FACULTY OF SCIENCE AND TECHNOLOGY

BACHELOR THESIS

Study programme / specialisation:

Chemistry and Environmental Engineering / Chemistry

The spring semester, 2022

Open access

Author: Natalija Jakovljevic

Supervisor: Krista Michelle Kaster

Thesis title: Review of the effect of disinfectants on antibiotic resistance in wastewater treatment plants

Credits (ECTS): 20

Keywords: disinfectant, antibiotic, antibiotic resistance, wastewater treatment plant

Pages: 50

Stavanger, 15.05.2022

Abstract

Antibiotic resistance (AR) has been identified by the World Health Organization (WHO) as one of the biggest threats to human health, due to the increased and unrestricted antibiotic use in humans and animals. In addition, there is growing concern that substances with antimicrobial properties, such as disinfectants coming from industry and housings, functions as selective agents in the proliferation of AR mainly due to co- and cross-resistance. Wastewater treatment plants (WWTPs) provide several different environmental conditions potentially favoring the selection of ARGs and thereby their spread in the environment. Although WWTPs show removal effectiveness of antibiotics and disinfectants from the wastewater influents, they fail to eliminate antibiotic resistance genes (ARGs). For that reason, WWTPs are among the main sources of the release of antibiotic resistant bacteria (ARB) and ARGs into the environment. Even though the distribution of different ARGs in WWTPs has been deeply investigated, the ecology and the molecular mechanisms underlying the selection of specific ARGs, especially those associated with disinfectants, are still poorly understood.

The review aims at investigating the effect of disinfectants in WWTPs on AR. First the mechanisms of action of different types of disinfectants on bacteria, as well as some of the resistance mechanisms bacteria possess against these chemicals, are described. The impact antimicrobial agents have on ARGs have been studied and linked to potential spread of these genes between bacterial genomes. WWTPs act as a final barrier to prevent the release of ARGs into the environment. Therefore, it is important to understand the different treatment processes and the effect of disinfectants in the treatment facilities on the spread and release of ARGs. The results of several studies support the hypothesis that the use of disinfectants can contribute to AR. Additionally, they show that present day WWTPs need some further improvements and induction of some more advanced treatment methods to increase their removal efficiency of antimicrobial agents and ARGs. This review concludes that more research is required to fully determine the effect of disinfectant use on the fate of ARGs in WWTPs.

Acknowledgements

First and foremost, I would like to express my most sincere gratitude and appreciation to Associate Professor Krista Michelle Kaster for her professional guidance and continuous support throughout this thesis, for her patience, enthusiasm, and immense knowledge. Krista was always available to answer my questions. Her guidance helped me a lot in writing of this thesis and made this work possible.

Finally, I would like to thank family and friends for their relentless support during my time as a bachelor's student at the University of Stavanger and for always encouraging me to do better. I would also like to thank my fellow students for many helpful discussions and good ideas along the way. During these special times in regard to the global pandemic, these people have given me the necessary motivation to always keep going.

Selected abbreviations and acronyms

AAS – anaerobic-aerobic sequence

ABC – ATP-binding cassette

ALX – alexidine

AR – antibiotic resistance

ARB – antibiotic resistant bacteria

ARG – antibiotic resistance gene

ATP – adenosine triphosphate

BAC – benzalkonium chloride

BOD – biochemical oxygen demand

CA-MRSA – community-associated methicillin-resistant *Staphylococcus aureus*

CHX - chlorohexidine

CRA – chlorine-releasing agent

CTR – cetrimide

CW – constructed wetland

DDAC – didecylmethylammonium

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

EPA – Environmental Protection Agency

FA - formaldehyde

GTA – glutaraldehyde

HGT – horizontal gene transfer

HMRG – heavy metal resistance gene

HPO – hydrogen peroxide

LPS – lipopolysaccharide

LTA - lipoteichoic acid

MACH – monoaromatic hydrocarbon

MATE – multidrug and toxic compound extrusion

MBR – membrane bioreactor

MDR – multiple-drug resistance

MFS – major facilitator superfamily

MGE – mobile genetic elements

MRSA – methicillin-resistant *Staphylococcus aureus*

OPA - *o*-Phthalaldehyde

PAA – paracetic acid

PAO – phosphorus accumulating organism

PFA – performic acid

PMF – proton motive force

QAC – quaternary ammonium compound

rDNA – ribosomal DNA

RNA – ribonucleic acid

RND – resistance-nodulation-division

SMR – small multidrug resistance

SRT – solid retention time

TCC – triclocarban

TLR – Toll-like receptor

TMSH - trimethylsulfonium hydroxide

UASB – upflow anaerobic sludge blanket

UV - ultraviolet

VGT – vertical gene transfer

WHO – World Health Organization

WWTP – wastewater treatment plant

Contents

List of figures and tables

Tables

Figures

1 Introduction

During the ongoing COVID-19 pandemic the use of disinfectants has increased in public facilities, healthcare systems, and even households worldwide.[1] Active ingredients in these disinfectants used for fighting against the spread of SARS coronavirus 2 (SARS-CoV-2), which causes the disease COVID-19, include chemicals where alcohols, formaldehyde, glutaraldehyde, hydrogen peroxide and quaternary ammonium compounds (QACs) are the most commonly used.[1-3] Ethanol, formaldehyde, isopropanol, sodium hypochlorite, hydrogen peroxide and benzalkonium chloride have been used to inactivate SARS-CoV-2 at following concentrations, 75%, 0.7%, 70%, 0.21%, 0.5%, and 0.1%,respectively.[4, 5] Although, disinfecting chemicals work together to achieve the same goal, they differ in their structures, properties, modes of action, environmental behaviors, and effects on human health upon exposure.[1] After use, these disinfectants end up in wastewater treatment plants (WWTPs) or directly enter the aquatic environment.[6]

In addition to disinfectants, it is also important to consider the affect antibiotic compounds have on the global health as they have been used for fighting bacterial infections for almost a century.[7] For instance, over 250 million antibiotic prescriptions are written in the United States annually, while the overall use of antibiotics in China in 2007 was 96 million kg.[2] It is believed that these numbers as well as those reported for the disinfectant use have increased even more during the COVID-19 pandemic due to an overuse of both disinfectants and antibiotics.[2] Even though antibiotics cannot be used for the treatment of the COVID-19 directly, they have been utilized to resist the COVID-19 induced inflammation and other diseases.[2] However, the number of effective antibiotics is decreasing due to the rising numbers of multi-drug-resistant pathogens.[8] Since human body cannot completely metabolize these compounds, 30-90 % of them are excreted uncharged into the waste system.[2]

The scientific reports collected during this study indicate that the overuse of antibiotics and disinfectants, characterized by high concentrations and high doses, can lead to antimicrobial resistance to these compounds, which has long been considered one of the major risks to global public health.[2, 9] The resistance can be achieved through horizontal gene transfer (HGT), vertical gene transfer (VGT), and co-selection.[2, 10] In addition, the scientific experiments show

that disinfectants have a significant effect on the fate of antibiotic resistance genes (ARGs) under favorable conditions.

Water is one of the most essential substances on Earth.[11] Although it covers 71% of the Earth's surface, only 2.5% is the fresh water.[11] Rapid urbanization and industrialization, especially in developing countries, discharge huge volumes of wastewater.[11] WWTPs collect sewage containing disinfectants, antibiotics, and resistant bacteria, thereby favoring the spread of resistance genes.[10] Even though different types of treatment methods are applied, the inlet and outlet concentrations reported in different experimental studies show that these plants are far from 100% effective at removal of these micropollutants. Consequently, ARGs and antibiotic resistant bacteria (ARB) present in the effluent water are released into the environment, thereby leading to more stress on ecological safety.[2] Therefore, optimizing the treatment efficiency of WWTPs is important for global health.[2]

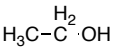
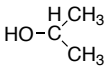
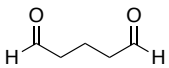
Most of the research done so far focus mainly on the relationship between antibiotics and ARGs. However, as it has been proven that disinfectants also have a huge impact on the spread of antibiotic resistance (AR), more research need to be done in that field. This literature review collects relevant data on the impact of disinfectant use on the spread of ARGs to obtain more comprehension on the work done this far on the link between disinfectants and ARGs. This review found that the link between disinfectants and ARGs is still poorly understood. In addition, this article also highlights that the removal of disinfectants from wastewater using different treatment methods needs more consideration, as traditional WWTPs are not designed for the removal of emerging pollutants, particularly antibiotic and disinfectant removal.

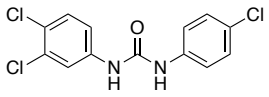
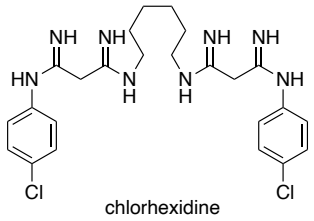
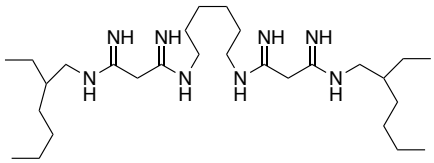
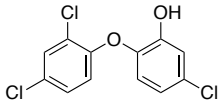
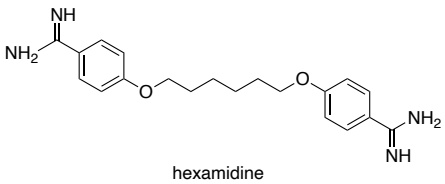
2 Disinfectants and biocides

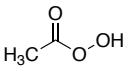
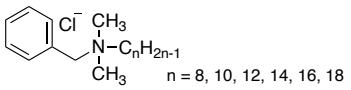
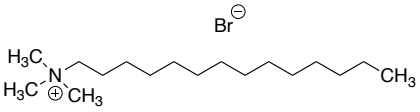
Biocides are chemical agents, more precisely inorganic or synthetic organic molecules,[12] that inactivates microorganisms.[13] Based on the range in antimicrobial activity, biocides can be divided into two groups.[13] The suffix "-static" refers to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic), while "-cidal" refers to agent which kill the target organism (e.g., bactericidal, virucidal and sporicidal).[13] Biocides are widely used in antiseptics, disinfectants, preservatives, and other biostatic and biocidal products.[14]

Although, both antiseptics and disinfectants are biocides or products that destroy or inhibit growth of microorganisms, the key to distinguishing the two groups is the area of use.[13] Antiseptics are applied on living tissues, such as human skin, while disinfectants are applied on non-living surfaces.[13] Since disinfectants are not necessarily sporicidal, the process of disinfection removes pathogens but leaves endospores.[15] However, sometimes it is necessary to destroy both endospores and pathogens in a process known as sterilization.[13, 15] In food and pharmaceutical industry biocides are used as preservatives for both longevity of preparations and to maintain sterility.[14]

Table 1. *Chemical structure and uses of biocides.* Adapted from McDonnell and Russell 1999[13]

Type of biocide	Chemical structure	Concentration	Area of use	Reference
Alcohols	 ethanol  isopropanol	60-80%	Antiseptic Disinfection Sterilization Preservation	[13, 16]
Aldehydes	 glutaraldehyde	0.1 - 2%	Disinfection Sterilization Preservation	[13, 17]

Anilides	 triclocarban	1.5%	Antisepsis	13, 18
Biguanides	 chlorhexidine  alexidine	0.05 – 4% 2%	Antisepsis Antiplaque agents Preservation Disinfection	13, 19, 20
Bis-phenols and structurally similar compounds	 triclosan	0.1-0.3 %	Antisepsis Antiplaque agents Deodorants Preservation	13, 21
Diamidines	 hexamidine	0.1 %	Antisepsis Preservation	13, 22
Halogen-releasing agents	 sodium hypochlorite	10% 50% industry	Disinfection Antisepsis Cleaning	13, 23

Heavy metals	Ag silver compounds Cu copper compounds		Preservation Antisepsis Disinfection	[13]
Peroxygens	HO−OH hydrogen peroxide  peracetic acid  performic acid	3-90 %	Disinfection Sterilization	[13]
Phenols	 phenol	5 %	Disinfection Preservation	[13, 16]
Quaternary ammonium compunds	 benzalkonium chlorides  cetrimide	0.1 %	Disinfection Antisepsis Preservation Cleaning	[13, 24]

2.1 Antimicrobial agents

One possible classification of disinfectants can be based on their ability to oxidize other substances, in other words to take their electrons, thereby acting as oxidizing agents.[15] The opposite action is the process of reduction and is carried out by non-oxidizing agents.[15] Oxidizing agents are also known as destroyers, while non-oxidizing agents are seen as coagulators.[15]

2.1.1 Aldehydes

Glutaraldehyde (GTA) ($C_5H_8O_2$; 1,5-pentanedial) has gained wide acceptance as a high-level disinfectant and chemical sterilant, typically used in health-care institutions.[25] This aldehyde is found to be active against bacteria and their spores, fungi, and viruses.[13] GTA is composed of glutaric acid and two aldehydes, and in the presence of water a hydroxyl group (•OH) attaches

to it forming a hydrate.[15] GTA is more active at alkaline than at acidic pHs.[13] The main reason for this is the formation of more reactive sites at the bacterial cell surface and thus a more rapid bactericidal effect as the external pH increases.[13] As the surrounding environment becomes alkaline, it releases more hydrogen and the solution becomes a strong acid.[15] It is believed that acidic GTA interacts with and remains at the cell surface where it forms cross-linkage as it reacts with proteins, thereby disturbing normal enzymatic activity crucial for the cell survival.[14] Alkaline GTA penetrates more deeply into the spore, acting sporicidal.[25] Another important aspect to take into account when considering the activity of GTA is its concentration. At low concentrations (0.1 %), GTA inhibits germination, whereas at higher concentrations (2%) it gains greater sporicidal activity.[13]

o-Phthalaldehyde (OPA) ($C_8H_6O_2$; benzene-1,2-dicarboxaaldehyde) is a high-level disinfectant used in dental and medical purposes as an alternative to GTA.[13] It is been proposed that the antimicrobial action mechanism of OPA is similar to that of GTA, both effecting the bacterial surface.[13] OPA is formed of two aldehydes attached to benzene, in other words GTA is made into a ring shape.[15] This ring shape is the reason why steric hindrance is less likely to occur than with GTA, making it easier for OPA to penetrate into the cells.[15] Although OPA induces less cross-linkage with the bacterial cells, the resulting increase in penetration might account for its increase in antimicrobial efficacy.[14]

Formaldehyde (FA) (CH_2O; methanal) is a disinfectant and sterilant used in numerous bactericidal, sporicidal, and virucidal properties.[13] FA is a monoaldehyde and it works more slowly compared to dialdehydes, such as GTA and *o*-phthalaldehyde.[13] However, FA is highly reactive and its interaction with proteins is characterized by a combination with the primary amide and the amino groups.[13] FA acts also as a mutagenic agent and as an alkylating agent by reaction with carboxyl, sulfhydryl, and hydroxyl groups.[13] In some bacteria and viruses, FA also reacts with deoxyribonucleic acid (DNA), more precisely with nucleic acids, thereby inhibiting DNA synthesis.[13] Finally, these interactive and cross-linking properties of FA result in microbial inactivation.[13]

2.1.2 Alcohols

Alcohols are known to be effective antimicrobials against vegetative bacteria, some viruses and fungi, but their ability to inhibit sporulation and spore germination is reversible.[13] Alcohols are not recommended for sterilization, but they have been shown to be effective hard-surface

disinfectants and skin antiseptics.[13] Ethanol (C_2H_5OH) and isopropanol (C_3H_8O) are the most widely used alcohols for these purposes.[13]

Ethanol is used an antiseptic at 60-80 % concentration.[15] At higher concentrations, the coagulation of the bacterial cell wall prevents the disinfectant from enter the cell.[15] While isopropanol is more suitable against bacteria, ethanol is more effective against viruses.[13] In order to increase product efficiency, biocides and excipients are being added to alcohol products.[13] For example, chlorohexidine is a biocide known to remain on skin following evaporation of the alcohol.[13] The main function of excipients is to decrease the evaporation time of the alcohol.[13]

The antimicrobial activity of alcohols increases with their increasing concentration.[13] In addition, they are even more effective in the presence of water.[13] Based on this, it is believed that ethanol and isopropanol act against microbes by causing membrane damage as a result of rapid denaturation and coagulation of proteins, mainly in the hydrocarbon part of the phospholipid bilayer.[13, 14] It is the hydroxyl group (•OH) of the alcohol that binds to microbial proteins causing enzyme inhibition and protein deposition.[15] Once inside the cell, alcohols interference with cell metabolism, inhibiting DNA, ribonucleic acid (RNA), protein and peptidoglycan synthesis (e.g. in *Escherichia coli*) until cell lysis occurs.[13]

2.1.3 Anilides

The anilides have antiseptic properties, but they are rarely used as disinfectants in hospital setting.[13] Instead, anilides are widely used as herbicides for inhibition of shoot growth.[26] Triclocarban (TCC) ($C_{13}H_9Cl_3N_2O$; 3,4,4'-triclocarbanilide) is an important anilid that has found usage in soaps and deodorants.[13] TCC is found to be less active against gram-negative bacteria and fungi as compared to gram-positive bacteria.[13] The anilides attack the cell by adsorbing and destroying the cell membrane causing cell death.[13]

2.1.4 Biguanides

Chlorhexidine (CHX) ($C_{22}H_{30}Cl_2N_{10}$) is an effective biocide widely used as a topical antiseptic in oral products and hand washing, but also as a disinfectant and preservative.[27] CHX is formed by the addition of chlorine to two bonded biguanides.[15] The activity of CHX is pH and concentration dependent, and its activity is reduced in the presence of organic matter.[13] Similar, to many other biocides, CHX first destabilizes the cell wall.[15] The positively charged CHX

binds to the cell wall of negatively charged bacteria, and remains in place for a long time.[15] In low concentrations, this binding results in cracks in the bacterial cell membrane and causes leakage of cellular components resulting in cell death.[13] In the case of high concentrations, the cytoplasm becomes congealed or solid.[13] CHX is not sporicidal and it has little effect on the germination of bacterial spores.[13] When it comes to antiviral activity, it is found to be restricted to the lipid-enveloped viruses[13]

Alexidine (ALX) ($C_{26}H_{56}N_{10}$) is a bisbiguanide disinfectant similar to CHX and has been previously used as a mouthwash and contact lens solution.[28] It has been showed that ALX also can be used in endodontics for eradication of *Enterococcus faecalis* because of its higher virulence factors for bacteria and better bacterial penetrability in comparison to CHX.[29] In addition, ALX is less toxic to corneal tissues in vivo when applied topically.[28] Both compounds are amphipathic bisbiguanides and can neutralize bacterial membrane components by stimulating Toll-like receptors (TLRs).[30] Recent studies have shown that CHX and ALX bind not only to lipopolysaccharide (LPS) from gram-negative bacteria but also to lipoteichoic acid (LTA) from gram-positive bacteria.[30] Both CHX and ALX prevent cell activation of TLR4 and TLR2 by LPS and LTA, respectively.[30]

2.1.5 Bis-phenols and structurally similar compounds

Triclosan ($C_{12}H_7Cl_3O_2$; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is one of the most widely used biocides in personal care products, such as hand soaps and cosmetics.[13] Triclosan is a broad-spectrum antibacterial and antifungal agent especially effective against gram-positive bacteria, whereas its efficiency against gram-negative bacteria and fungi can be significantly enhanced by formulation effects such as in combination with ethylenediaminetetraacetic acid (EDTA).[13] At low concentrations triclosan is predominantly bacteriostatic or fungistatic, while higher concentrations are bactericidal.[31] Some bacteria, including *Pseudomonas aeruginosa (P. aeruginosa)* are highly resistant to triclosan.[31] In addition, reports have suggested that triclosan may also have anti-inflammatory activity.[13] Further studies have shown that triclosan primary attacks the cytoplasmic membrane.[13] At low, bacteriostatic concentration triclosan inhibits the uptake of essential nutrients, while higher, bactericidal concentrations result in the rapid release of cellular components and cell death.[13]

2.1.6 Diamides

The two diamides, propamidine ($C_{17}H_{20}N_4O_2$; 4-[3-(4-carbamimidoylphenoxy) propoxy] benzenecarboximidamide) and dibromopropamidine ($C_{17}H_{18}Br_2N_4O_2$; 3-bromo-4-[3-(2-bromo-4-carbamimidoylphenoxy)propoxy]benzecarboximidamide) form isethionate salts which have been used as antibacterial agents for the topical treatment of wounds.[13] Another widely used diamide in skin disinfection is hexamidine ($C_{20}H_{26}N_4O_2$).[22, 32] This biocide is bacteriostatic against both gram-positive and gram-negative strains.[32] It has been shown that diamides act by inhibiting oxygen uptake and inducing leakage of amino acids as would be expected if they are considered as cationic surface-active agents.[13]

2.1.7 Halogen-releasing agents

Chlorine-releasing agents (CRAs) are considered to be both antiseptics and disinfectants.[13] Sodium hypochlorite (NaOCl), commonly known as bleach, is one of the most important CRAs.[13] It is a broad-spectrum disinfectant that is virucidal, bactericidal, sporicidal, and fungicidal, and is widely used for hard-surface disinfection, public water treatment, and for disinfecting spillages of blood containing human immunodeficiency virus (HIV) or hepatitis B virus (HBV).[13] When sodium hypochlorite is released in water, it decomposes into Na^+ and the hypochlorite ion, OCl^-, which establishes and equilibrium with hypochlorous acid, HOCl.[13] This acid is also the most effective moiety responsible for microbes' inactivation.[13] DNA synthesis has been considered as a target as it is almost fully inhibited even at low concentrations of hypochlorous acid.[13] Hypochlorites have deleterious effects on bacterial DNA synthesis that results from the formation of chlorinated derivatives of nucleotide bases.[31] In addition, they are also known as highly active oxidizing agents which destroy the cellular activity of proteins.[31]

2.1.8 Heavy metals (Silver compounds as disinfectants)

Copper and silver ions have been used in disinfection due to their interaction with cell protein surface and nucleic acids.[33] It is believed that these metal ions alter enzyme structure and function, facilitate hydrolysis and nucleophilic displacement.[33]

Silver compounds are well known as antimicrobial agents.[13] Silver nitrate ($AgNO_3$) is a common form of silver used as a disinfectant.[13] Silver ions acts as antibacterial agents, but their activity can be neutralized by interacting with thiol (sulfydryl, -SH) groups present in some amino acids and other compounds.[13] In some yeasts, such as *Cryptococcus neoformans (C.*

neoformans) silver nitrate inhibits growth, while in *P. aeruginosa* it inhibits cell division and damages the cell envelope and contents.[13] Silver sulfadiazine (AgSD) ($C_{10}H_9AgN_4O_2S$) is one of the most important silver compounds. Bacterial inhibition by AgSD and its antiphage properties are due to binding to the DNA, thus inhibiting transcription.[13] AgSD is also known to produce surface blebs in susceptible bacteria.[13]

2.1.9 Peroxygens

Hydrogen peroxide (HPO) (H_2O_2) is a widely used biocide typically at concentrations 3-90%.[13] It is used for disinfection, sterilization, and antisepsis.[13] The fact that HPO rapidly can degrade into water and oxygen makes this biocide environmentally friendly and is also the main reason why stabilizers are being added to pure solutions.[13] HPO is effective against viruses, yeast, bacterial spores and bacteria, especially gram-positive bacteria.[13] This oxidant performs sterilization by forming hydroxyl free radicals (•OH) which attack lipids, proteins, and DNA within the cell.[13] It is also believed that exposed sulfhydryl groups and double bonds are particularly targeted.[13] Radicals are known to act rapidly by stealing electrons from the target element.[13, 15] In case of contact with a microorganism, disinfection and sterilization occur immediately.[15]

Peracetic acid (PAA) (CH_3COOOH) is a highly biocidal oxidizer, being sporicidal (0.3%), bactericidal (0.001%), virucidal, and fungicidal (0.003%) at low concentrations,[15, 34] which is also the reason why PAA is considered more potent than HPO.[35] PAA decomposes to acetic acid, oxygen and hydrogen peroxide and remains active in the presence of organic soil.[36] It is mainly used as a low temperature sterilant for medical, surgical, and dental instruments, but it is also used as an environmental surface sterilant.[13] It is believed that the disinfectant activity of PAA is based on destruction of sulfhydryl (-SH) and sulfur (S-S) bonds in the cell wall that cause denaturation of proteins and enzymes leaving the cell membrane more permeable.[15, 34]

Performic acid (PFA) (CH_2O_3) is a disinfectant used in the medical field, food industry, against bacteria and viruses, but also in WWTPs for removing fecal coliforms.[36, 37] PFA is considered environmentally friendly due to its degradation products formic acid and water.[36]

2.1.10 Phenols

Phenols are known for their antiseptic, disinfectant, and preservative properties, as they are antibacterial, antifungal and antiviral.[13] Their antimicrobial activity is based on membrane-

active properties, more precisely denaturation and coagulation of proteins within the membrane, which trigger the leakage of cytoplasmic constituents, including the release of K^+.[13] At bactericidal concentration, the chlorinated bis-phenol fenticlor triggers the leakage of 260-nm-absorbing material, more precisely nucleic acids,[38] and in some bacteria, it even causes a selective increase in permeability to protons with the consequence of dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation.[13] Coagulation of cellular components occurs at higher phenol concentrations and causes irreversible cellular damage.[13] Chlorocresol is another important phenol which is known to have a similar mode of action.[13] It is believed that the antifungal action of phenols is similar to the mode of action for bacteria.[13] Phenols have no effect on phage DNA within the capsid, and their antiviral properties are limited and treatment times of 20 min or longer is a require for antiviral activity.[13]

2.1.11 Quaternary ammonium compounds

QACs are cationic surface-active agents used for disinfection, cleaning, and deodorization.[13] The structure of QACs is characterized by a nitrogen atom covalently bonded to four residues, making the nitrogen positively charged.[35] One of these four residues is typically an alkyl chain between 5 and 18 carbons in length; another one either one more alkyl chain or a benzyl moiety; and two methyl residues.[35] The biological activity of QACs is based on forming an electrostatic bond between their cationic charge and the negatively charged cell walls of microorganisms.[39] The rate of leakage might be higher for Gram-positive than for Gram-negative bacteria, because of the presence of multilayered cell wall in Gram-negative bacteria.[14] QACs act against bacteria and yeast by disrupting the cytoplasmic and the plasma membrane, respectively,[13] resulting in distortion of cell wall permeability.[14, 39] This interaction has a huge impact on the flow of nutrients into the cell, the discharge of wastes leaving the cell, and protein denaturation.[39] QAC are also sporostatic and have an effect on enveloped viruses.[13]

Benzalkonium chloride (BAC) ($C_{22}H_{42}ClNO$) is a quaternary ammonium antiseptic and disinfectant with alkyl chain ranging from 8 to 18 carbons in length, and widespread applications due to their broad-spectrum antimicrobial properties against bacteria, fungi, and viruses. [40, 41] It is commonly used in pharmaceutical formulations as an antimicrobial preservative, but also in domestic, agricultural and industrial applications.[40, 41] BAC is more active against Gram-positive than Gram-negative bacteria, showing minimal activity against bacterial endospores.[40] The antimicrobial activity of BAC is dependent upon the alkyl

composition of the homolog mixture.[40] It has been recognized that the release of BAC into lakes and other waters is toxic to the aquatic environment and its inhabitants.[41]

Cetrimide (CTR) ($C_{17}H_{38}BrN$), a quaternary ammonium derivative, is active against bacteria, both gram-positive and gram-negative, and fungi.[28] CTR is found to act bacteriostatic against *Staphylococcus aureus (S. aureus)* by causing the discharge of the pH component of thePMF.[13] In addition to its bacteriostatic activity CTR can also be used to reduce surface tension so that liquids easily can entry the places of difficult access, such as dentin tubules.[28]

Didecylmethylammonium chloride (DDAC) ($C_{22}H_{48}ClN$) is a dialkyl-quaternary ammonium biocide used for various purposes due to its effectiveness against bacteria, viruses and fungi.[42, 43] This potent disinfectant is used in hospitals, food industries, environmental sanitation, water treatment etc.[39]

3 Mechanisms of action of biocides

Biocides vary greatly in their chemical structure, and thus interact with different molecules.[13,14] Understanding the mechanisms of action of biocides requires a strong knowledge of the microbial cell's structure and the chemical interactions biocides trigger at their target sites.[13,14] Most biocides are known to have several target sites within the bacterial cell and based on the biocide concentration they can lead to either bacteriostatic or bactericidal effect.[14]

The initial reaction of a biocide with a microbial cell involves an interaction with the cell surface, which is also the primary target site for many biocides.[14] In order to penetrate the cell and reach its target site, these biocides have to change the outer cell layer. The key to understanding why some types of bacteria are more resistant to biocides than others is to study the diverseness in their cell envelopes. As shown in *Figure 1*, mycobacteria and Gram-negative bacteria possess multilayered cell envelop unlike Gram-positive bacteria.[14] Some bacteria can also be surrounded by a capsule and slime.[14] Consequently, mycobacteria appears to be the most resistant to disinfection, followed by Gram-negative bacteria, with Gram-positive being the most sensitive.[14]

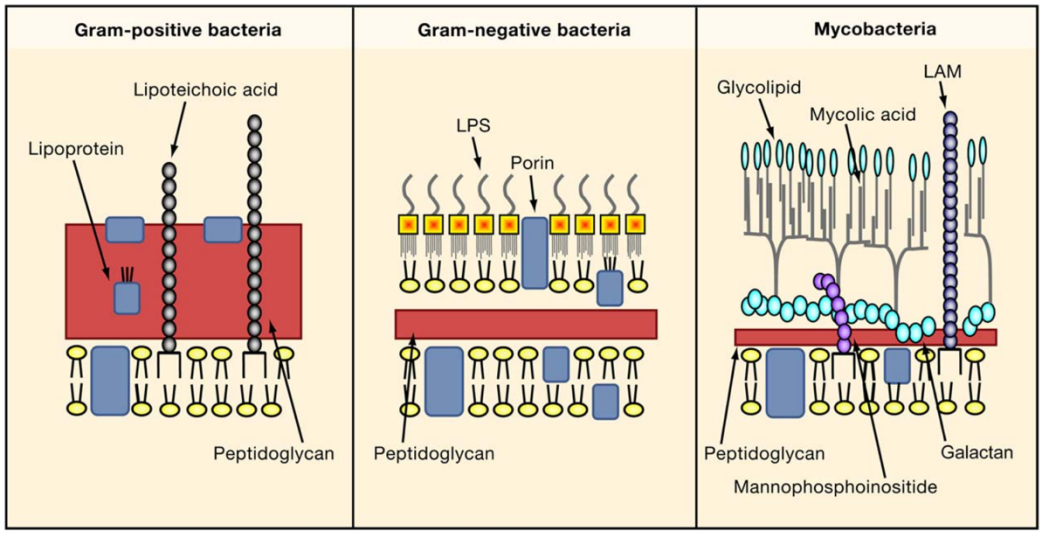


Figure 1. *Schematic representation of bacterial cell walls of (a) Gram-positive bacteria, (b) Gram-negative bacteria, and (c) mycobacteria.* Adopted from Akira, Uematsu and Takeuchi 2006.[44]

3.1 Interactions with outer cell components

The cell wall built up of peptidoglycan, with an additional lipopolysaccharide overlayer in Gram-negative bacteria, gives the cell strength and structure.[45] In addition, it also serves as an excellent target for antibiotics, even though it rarely proves a suitable site for direct biocide action.[45]

3.1.1 Hydrophobicity

Cationic compounds, such as CHX and BAC, are membrane active agents known to affect the hydrophobicity of Gram-negative bacteria.[14] The positively charged CHX cations binds to the negatively charged cell wall and remains in place for a long time due to this strong binding.[15] It has been proposed that they damage the cell wall and outer membrane of Gram-negative bacteria promoting their own uptake which results in their penetrating through these layers until their target sites are reached, either at the cell cytoplasmic membrane or within the cell.[14]

QACs are another group of cationic compounds that bind to the cell wall in a similar way as CHX.[15] This bonding is known to cause leakage of bacterial cells components, thereby disrupting the membrane potential and pH gradient of the cell.[15] Further studies with smooth, rough and deep rough strains of *Escherichia coli (E. coli)* and *Salmonella typhimurium (S. typhimurium)* have shown that deep rough strains are more sensitive to QACs than wild-type strains, also the ones that produce smooth LPS, but of equal sensitivity to CHX.[31]

On the other hand, oxidizing agents, such as PAA destroys the surface of a bacterium by a process known as oxidation.[15] PAA produces hydroxyl radicals (•OH) by breaking sulfhydryl (-SH) and sulfur (S-S) bonds, thereby breaking down the proteins.[15] Hypochlorites are another group of oxidizing agents that act on the cell wall and the amino groups in the proteins.[31]

3.1.2 Cross-linking

Aldehydes are known for cross-linking all amino acids or proteins when reaching the cell surface, resulting in destroyed protein structure, while the nucleic acids and lipid structures are swept together.[15] Amino acids containing an amino group ($-NH_2$), such as lysine, asparagine, glutamine, and arginine, are favored.[15]

GTA is an aldehyde known for its cross-linking properties,[14] and in Gram-negative bacteria it mainly interacts with lipoproteins.[14, 15] It has been found that cross-linking actions of the 5 carbon long stretch of GTA effects amino groups in bacterial proteins, where GTA agglutinates bacterial cells, increasing their settling rate.[15, 31, 46] Consequently, the steric hindrance disrupts penetration into the cell wall.[15, 46] Low concentrations of GTA might protect the cell from other harmful compounds, while high concentrations cause high degree of cross-linking that disrupt most, if not all, of the bacterial cell's essential functions, leading to cell death.[14]

OPA is another aldehyde, more precisely an aromatic dialdehyde,[31] that possess cross-linking properties and affects the bacterial cell surface.[14, 15] It acts in a similar way as GTA, but induces less cross-linkage with the bacterial cells because of its ring shape.[14, 15] Thence, steric hindrance is less favored to occur with OPA than with GTA and the compounds can more easily penetrate into cells.[15] The resulting increase in penetration might be a good explanation for the increase in antimicrobial efficiency of OPA.[14]

3.2 Interaction at the cell cytoplasmic membrane level

The cytoplasmic membrane is the key for interactions between phospholipid, enzymic and structural proteins, which controls impermeability and topological organization, thereby maintaining intracellular homeostasis and vectorial transport.[45]

3.2.1 Disruption of the cytoplasmic membrane

Because of their double bonds, unsaturated fatty acids within the cytoplasmic membrane are a preferred target for oxidizing agents which are known for breaking down lipids into smaller fatty acids.[15] Disruption of the cytoplasmic membrane is generally associated with the leakage of intracellular components, such as potassium ions (K^+), inorganic phosphates, amino acids and materials absorbing at 260 nm, more precisely nucleic acids and proteins.[14] Leakage can be defined as a measure of the disruption of the cell permeability barrier, and this action causes rather a bacteriostatic than bacteriocidic effect.[14]

Phenols and cresols are examples of membrane active agents well known for inducing leakage of intracellular materials from bacterial cell.[14] It has been proposed that low concentrations lyse growing cultures of *E. coli*, staphylococci and streptococci.[31] However, at high concentrations, these biocides cause intracellular coagulation.[31]

The cationic agent CHX also appears to have the same properties as phenols and cresols when applied in correct confrontation, otherwise it causes coagulation of the cytosol, as mentioned earlier.[14] ALX is another biguanide and although its structure differs from that of CHX only by the nature of the end-group substituent, it possesses additional binding targets at the cell envelope or at the cytoplasmic membrane.[14] It is believed that both QACs and CHX cause disruption of the cytoplasm membrane by interaction with membrane phospholipids.[14, 31] Further studies have shown that CHX binds well to the cell wall and membrane and cause cracks in the bacterial cells which further lead to leakage of the intracellular contents, and even bacterial burst.[15] This is due to a strong bonding between negatively charged bacteria and positively charged CHX cations which keep in place for a long time.[15]

Ethanol and isopropanol are membrane-active biocides which penetrate into the hydrocarbon part of the phospholipid bilayer.[14, 31] Ethanol-induced disruption of membrane structure further causes inhibition of the enzymes involved in glycolysis, fatty acid and phospholipid uptake and change in cell permeability.[31]

Triclosan is a membrane-disrupter which induces K^+ leakage at lethal concentrations.[31] Z-patter adsorption of triclosan is believed to break down the cytoplasmic membrane and generate new adsorbing sites.[31]

3.2.2 Dissipation of the proton motive force

Proton motive force (PMF) is the force that promotes movement of protons across the cytoplasmic membrane from the interior of the cell to the outside.[14] It is critical for the formation of energy in cell. PMF is essential for the cell survival, and the most crucial cellular metabolic functions, such as active transport, phosphorylation and make of adenosine triphosphate (ATP), relies on a proper functioning PMF.[14]

As mentioned previously, PMF is responsible for proton movement across the cytoplasmic membrane, and that is the main reason why interactions of some compounds with the PMF causes a change in pH.[14] One such example is acetic acid which neutralizes the PMF, lowers the pHi, thereby denaturing proteins.[14] Some other acids, such as dinitrophenol, inhibits ATP synthesis, thereby uncoupling oxidative phosphorylation.[14]

3.2.3 Interactions with other enzymatic systems

Membrane enzyme complexes act as a starting point for many chain reactions that proceed inside the cell.[14] One such example is ethanol which inhibits the enzymes involved in glycolysis, fatty acid and phospholipid synthesis by disrupting the structure of the cytoplasmic membrane.[14] Ethanol is known for denaturation and coagulating of proteins with the help of the hydroxyl group which binds to proteins via hydrogen bonding and damages protein structure and function, thereby resulting in enzyme inhibition and protein deposition.[15]

Other biocides, such as copper and silver, react with the thiol groups which are vital for the activity of many enzymes.[14] It has been demonstrated that amino acids and other compound containing thiol groups neutralized the activity of silver against *P. aeruginosa*.[13]

3.3 Interactions with cytoplasmic constituents

Many catabolic and anabolic processes, in addition to the replicative machinery, take place in the cytoplasm.[45] This region is also the last region for biocide accumulation.[45] A number of biocides are able to block DNA synthesis and inhibit RNA synthesis.[14] Here, the target sites are the nucleic acids.[14] Alkylating agents cross-link the base structures of DNA or RNA to adjacent nucleotide bases.[15] This disordered state of nucleotide bases inhibits proper DNA separation, thereby blocking both replication and transcription.[15]

Ribosomes, responsible for the process of DNA translation, can be damaged by biocides, such as hydrogen peroxide.[14] Hydroxyl radicals, formed when hydrogen peroxide takes one electron, break DNA or RNA strands directly or attack the phosphate backbone of purines or pyrimidines and ribose or deoxyribose.[15] When a hydroxyl radical attacks thymine, the thymine becomes a thymine glycol.[15] This broken thymine is unable to perform its tasks as a nucleic acid in replication, transcription and translation.[15] Hydroxyl radicals are also known to attack the sugar deoxyribose of the DNA, thereby forming deoxyribonolactone and breaking the base.[15] In addition, hypochlorites are also known for their deleterious effects on DNA synthesis as a result from the formation of chlorinated derivatives of nucleotide bases.[31]

Table 2. *Summary of mechanisms of antibacterial action of various biocides.* Adapted from McDonnel and Russell 1999.[13]

Target	Antiseptic or disinfectant	Mechanism of action	Reference
Cell wall	GTA, OPA	Cross-linking of proteins	14, 15, 31, 46
	CHX, BAC	Hydrophobicity	14, 15, 31
Cytoplasmic membrane	CHX, QACs, ethanol, isopropanol	Membrane damage involving phospholipid bilayers	14, 15, 31
	CHX, phenols	Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm	14, 15, 31
	Ethanol, isopropanol	Enzyme inhibition and change in cell permeability	14, 31
	Acetic acid	Dissipation of the proton motive force	14
	TCS	Breaking down of the cytoplasmic membrane	31
Cross-linking of macromolecules	FA	Cross-linking of proteins, RNA, and DNA	14
	GTA, OPA	Cross-linking of proteins in cell envelope and elsewhere in the cell	14, 15, 31, 46
Proteins and enzymes	Silver and copper compounds	Membrane-bound enzymes (interaction with thiol groups)	14
	Ethanol	Enzyme inhibition and protein deposition	15
	Peroxygens	Hydrogen peroxide: oxidization of thiol groups in enzymes and proteins with the help of free hydroxy radicals (•OH); PAA: disruption of thiol groups in proteins and enzymes	13
Effects on DNA	Halogens	Inhibition of DNA synthesis	13
	HPO, silver ions	DNA strand breakage	13, 14, 15

4 Resistance mechanisms

While antibiotic resistance has been a well-known global problem for ages, resistance to biocides has not gained as much attention, although bacteria can evolve resistant to both groups of antibacterial chemicals.[47] Antibiotics are known to have one specific target site, while biocides have multiple target sites within a bacterial cell.[7] However, there are still many similarities in the actions of some antibiotics and biocides.[7] Resistance to biocides might be induced by the same mechanisms as antibiotic resistance.[47] Biocides can also make bacteria more resistant to antibiotics.[7, 47] Changes in cell surface and permeability, enzymatic inactivation, efflux pumps, inhibition of a metabolic pathway, and biofilm formation are some of the most known resistance mechanisms against antibiotics and biocides.[7]

4.1 The spread of antibiotic resistance through genetic material

4.1.1 Horizontal gene transfer

Horizontal gene transfer (HGT) is considered the fastest and the most effective transfer mechanism of ARGs between bacteria, compared to antibiotic resistance obtained by vertical gene transfer (VGT) or mutation.[48, 49] Horizontal, or lateral, gene transfer, means that genes are exchanged between cells of the same generation, not by inheritance.[50] The genetic information is transferred from chromosomes to and between mobile genetic elements (MGEs), such as plasmids and transposons, and this type of gene transfer is mostly facilitated by integrons.[48]

The three methods of HGT for the spread of MGEs are conjugation, transformation, and transduction.[48] Conjugation requires direct cell-to-cell contact, where a donor bacterium transfers genetic material to a recipient bacterium via a plasmid. [48] Transformation is characterized by uptake of extracellular DNA followed by its incorporation into the recipient's chromosome or into a plasmid of a component bacterium through the process of recombination.[48] Transduction occurs when a bacteriophage, a type of virus that infects bacteria, transport genetic elements from their host to the receiver, leading to their incorporation into the genome of the new host by recombination.[48] This mode of transfer can either be general or specialized.[48] Generalized transduction occurs most commonly in lytic cycles of virulent phages where bacteriophage randomly package host cell DNA into the phage due to packaging errors.[51] In specialized transduction temperate bacteriophages, as part of their life cycle, integrate into the bacterial host cell DNA where it is replicated along with the host cell DNA.[51]

At a certain point of the temperate phage's lifecycle, the temperate bacteriophage will enter the lytic stage where its DNA then detaches from bacteria cell chromosome.[51] The viral DNA can then be replicated, packaged into new phage particles, and released.[51] In the case of specialized transduction, the temperate phage's DNA is excised imprecisely along with some of the host bacterial DNA.[51] This excised DNA contains some of host bacterial cell's genes and is packaged along with the phage into new bacteriophage particles able to carry the DNA to new bacterial host cells.[51]

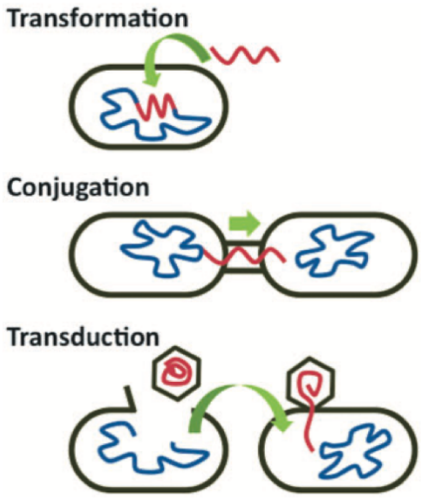


Figure 2. *Mechanisms of bacterial horizontal gene transfer.* Adopted from Burmeister 2015.[49]

One of the many examples of HGT is associated with methicillin-resistant strain CC398 of *S. aureus*.[49] The presence of antibiotics, such as tetracycline and ß-lactams, used in veterinary purpose provide a favorable environment for HGT of ARGs into a sensitive human-associated *S. aureus* strain.[49] Once a strain gains resistance, the genes and pathogens continue to evolve, often resulting in bacteria with greater resistance as they move among patients and hospitals.[49]

4.1.2 Co-selection

The presence of heavy metals, antibiotics, resistant bacteria, and resistance genes in WWTP effluent may favor the selection of multi-resistant bacteria and the spread of resistance into the environment through co-selection.[10] Metal contamination has been considered as a selective agent in the proliferation of antibiotic resistance,[52] due to experimental evidences showing a relation between the acquisition of heavy metal resistance genes (HMRGs) and ARGs, particularly on plasmids.[10] The co-selection of resistance genes is generally caused by stress, even without a direct impact, and is characterized by the selection of the corresponding

resistance gene promoting the persistence of other resistance genes.[10] Mechanism of co-selection is highly favored when the diverse resistance genes are located on the same mobile genetic element, such as an integron, a plasmid, or a transposon.[10] Documented associations between the types and levels of metal contamination and specific patterns of antibiotic resistance suggest that several mechanisms underlie the co-selection process.[52] These co-selection mechanisms include co-resistance, with different resistance determinants present on the same genetic element, and cross-resistance, where the same genetic determinant is responsible for resistance to both antibiotic and metals.[52]

In addition to earlier mentioned stress, some antibiotic and metal resistance mechanisms, such as biofilm induction, also represent potential co-selection mechanisms in prokaryotes.[52] For that reason, metal contamination has been considered as a long-standing, widespread and intractable selection pressure potentially contributing to the maintenance and spread of AR.[52]

4.1.2.1 Co-resistance

Co-resistance occurs when the genes specifying resistant phenotypes are physically linked together on the same genetic element such as a plasmid, transposon or integron, thereby resulting in the co-selection for other genes located on the same element.[52] Experimental evidences support the fact that metal- and antibiotic- resistance genes are linked, mostly on plasmids.[52] Antibiotic-metal co-selection came from studies that used transformation, plasmid curing and plasmid sequencing approaches.[52] Experiments showing that mercury resistance was co-transferred with antibiotic resistances in a subset of matings between *Enterobacteriaceae* and recipients confirmed further the genetic linkage of metal- and antibiotic-resistance traits on plasmids.[52] Furthermore, another experiment on *Salmonella abortus (S. abortus)* showed that upon removal of plasmids from strains resistant to ampicillin, arsenic, chromium, cadmium and mercury, the strains became sensitive to these toxicants.[52] A study by Yazdankhan et al. done on zinc and cooper, both used in disinfection, has shown that HMRGs against these metals increase the rate of AR dissemination by co-resistance.[48, 53] In addition, Jiao et al. showed that co-resistance is also present in *qac* genes encoding for efflux pumps against QACs and monoaromatic hydrocarbons (MACHs), typically located on MGEs together with ARGs.[48, 54]

4.1.2.2 Cross-resistance

Cross-resistance occurs when bacteria have developed survival methods against different antimicrobial agents that share the same strategy to attack the cell.[48, 52] The end result will be

the development of resistance to one antibacterial agent that is accompanied by resistance to another agent.[52] Mechanism of cross-resistance mainly manifest itself through efflux of structurally dissimilar compounds using the same mechanism.[52] An example supporting this is the multiple-drug resistance (MDR) pump in *Listeria monocytogenes (L. monocytogenes)* which can export both metals and antibiotics.[52]

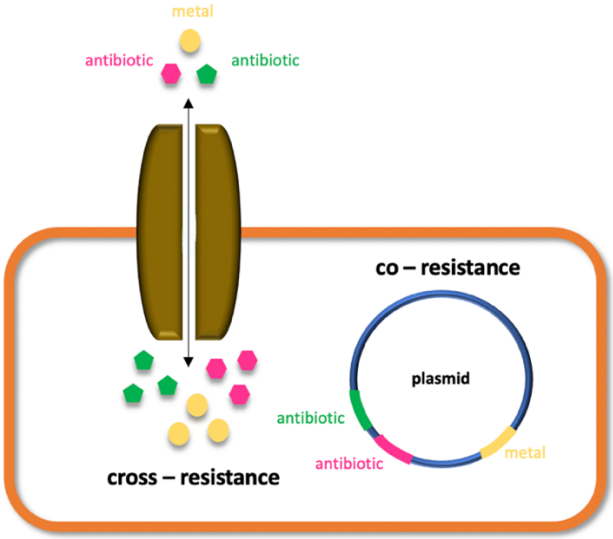


Figure 3. *Mechanisms of cross – resistance and co – resistance.*

4.2 Mechanisms of bacterial resistance to antibiotics and biocides

4.2.1 Efflux pumps

Efflux pumps are transport proteins found in the cytoplasmic membrane of both Gram-positive and -negative bacteria[55] that work as channels and pump solutes out of the cell.[56] These proteins allow bacteria to regulate their internal environment by removing toxic substances, including antimicrobial agents.[56] Some pumps are specified for transport of one substrate while other can transport multiple substrates.[55] Furthermore, efflux pumps are formed either by a single component or by multiple components, such as in Gram-negative bacteria.[56]

Based on the characteristics mentioned above, efflux pumps have been classified into five major families in the prokaryotes: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE), the small multidrug resistance (SMR) family, and the resistance-nodulation-division (RND) superfamily.[55] Common to all these families, apart from the ABC family, is that they utilize the PMF for

energy, while the ABC family utilizes ATP hydrolysis as an energy source to drive the export of substrates.[55]

Efflux pump genes are generally part of an operon, with a regulatory gene controlling expression.[55] Mutations within local repressor genes can lead to over-expression of these genes which again lead to resistance to the substrate (e.g. antibiotic and biocide) by transporting it out of the bacterium through the efflux pumps, thereby allowing cell survival.[55] Over-expression of a pump will often result in resistance to more than one type of antibiotics, as well as some biocides.[55] Another problem is cross-resistance; exposure to any one agent that belongs to the substrate profile of a pump would favor over-expression of that pump and consequent cross-resistance to all other substrates of the pump.[55] Over-expression of efflux pumps can also result from activation of a regulon regulated by a global transcriptional regulator such as MarA or SoxS of *E. coli.*[55] This has also been seen in *P. aeruginosa* mutants that over-produce MexAB and cause resistant to some antibiotics and triclosan.[55] Over-expression of a multidrug resistance efflux pump alone often does not confer high-level, clinically significant resistance to antibiotics.[55] However, such bacteria are better equipped to survive antibiotic pressure and develop further mutations in genes encoding the antibiotic target sites.[55]

It has been shown that expression of the Mex systems of *P. aeruginosa* and the *acrAB* efflux system of *E. coli* is most effective when the bacteria are exposed to inhospitable conditions, such as growth in a nutrient-poor medium, they are in stationary phase or osmotic shock.[55] For example in nutrient-poor medium, not only will toxic substances will be pumped out of the cell, but also nutrients and metabolic intermediates may be exported due to unregulated over expression of efflux pumps which is also detrimental to the cell.[55]

The efflux pumps systems have been seen as the resistance mechanisms for QACs, where QACs are being removed from the cell interior by the efflux pumps.[12] The reason why QACs first need to enter the cell could be that QACs only can access the membrane from the interior, or that they first need to interact with the cytosolic compounds responsible for growth inhibition.[12] There are several QACs efflux systems in Gram-positive (e.g. *qacA, qacB*, and *smr*) and in Gram-negative bacteria (e.g. *emrE, qacE,* and *qacEΔ1*).[12] For example, the *qacA* pump is a membrane-bound transport protein which cation export is dependent on PMF.[12] Besides QACs, other antimicrobial organic cations, such as biguanides and diamidines, are some of the well-known substrates for the *qacA* pump.[12]

4.2.2 Enzymatic inactivation

Enzymatic inactivation is associated with changes in antibiotic structure either by a hydrolysis or a modification by adding of acetyl-, adenyl-, or phosphorous groups.[57] This is mainly achieved by specialized enzymes with narrow substrate spectrum limited to the respective antibiotics, and such inactivating enzymes do not play a role in physiological cell metabolism.[57] The genes for these enzymes are found on mobile genetic elements.[57]

Besides directly attacking the antibiotic, enzymatic modification can also alter the target site and make it inaccessible to antibiotics.[57] Specific ribosomes protective proteins have been shown to inhibit binding of antibiotics to ribosomes active in protein synthesis, and these genes have been found on plasmids and conjugative transposons.[57] Another way to avoid the inhibitory action of antimicrobials is to replace sensitive target structures by new targets with reduced sensitivity to the antimicrobials.[57] This can be achieved by overproducing the native target, by acquiring new resistant targets or by undergoing mutational changes of the gene which code for the target structures.[57]

4.2.3 Changes in cell surface and permeability

The cytoplasmic membrane controls the entry of cytoplasmatically targeted compounds.[58] Alterations in the composition of the membrane such as loss of non-essential transporters, lack of porins or mutations in channels in the outer membrane of Gram-negative bacteria can all lead to decreased influx.[58] For example, resistance to QACs in *P. aeruginosa* has been associated with decreased amount of palmitic acid and increased levels of hydroxylated fatty acids and lauric acid.[12] *Serratia marcesens (S.marcesens)* and *Providencii stuartiis (P. stuartiis)* show both resistant to CHX as a result of changes in the composition of the cytoplasmic membrane.[12] Furthermore, many bacteria are naturally resistant to some antimicrobial molecules that are too large to enter the cell.[58]

Decreased susceptibility to BACs has long been associated with changes in the membrane composition. It has been shown that resistant strains of *P. aeruginosa* have different phospholipid and fatty acid compositions compared to the susceptible strain.[41] Similar, the lipopolysaccharide composition of an *E. coli* strain with reduced susceptibility to BACs differ from that of the susceptible strain.[41] Furthermore, recent studies have suggested that *Pseudomonas* strains can partially adapt to BACs by stabilizing the membrane charge through the increase in polyamine synthesis gene expression and mutations in *pmrB*.[41]

4.2.4 Bypass metabolic pathway

Some of the most known target by-pass strategies are: creating new pathways so that originally targeted enzyme remains unchanged, overproduction of the target compound, changes in the cell wall structure, and prevention of the antibiotic from binding to its target.[48] Modification of target molecule is a necessary action to prevent the antibiotic from binding to its target site.[59] These target site changes are generally a result of spontaneous mutations in a bacterial gene on the chromosome.[59] Even a minor alteration in target molecule structure can inhibit antibiotic interaction, because of their highly specific binding sites.[59]

4.2.5 Biofilm

Biofilms are surface-attached complex microbial associations enclosed in a matrix of extracellular polymeric substances that they have produced, and can be found in clinical, natural and industrial settings.[56, 60] The biofilm matrix provides structural stability to the biofilm and it is composed of polysaccharides, proteins and DNA from the microorganisms.[56, 60] It also makes it easier for microorganisms to communicate with other microorganisms either within or outside of the biofilm and to transfer genetic trait via HGT in the biofilm.[56] Nutrients circulate between cells through interstitial voids which enclose the extracellular matrix.[60] The biofilm structure protects the cells against host-defense mechanisms, phagocytosis, biocides, hydrodynamic shear forces and antibiotic treatment.[56]

Biofilms are known to be more resistant to antimicrobial agents than planktonic cells, and are responsible for 65% of all bacterial infections.[56] In addition, antimicrobial resistance increases as the biofilm ages until the maximum resistance is reached at the mature stage.[56] Consequently, infections caused by biofilm forming bacteria can lead to a serious clinical problem.[56] There are a couple of mechanisms known to be responsible for the antimicrobial resistance in biofilm structures. One of them is poor diffusion of antibiotics through the biofilm polysaccharide matrix, although some antibiotics penetrate the matrix.[56] The second one is induced due to physiological changes due to slow growth rate and starvation responses.[56] Phenotypic change of the cells forming the biofilm and quorum-sensing are also known to result in antimicrobial resistance.[56] The expression of efflux pumps, as earlier mentioned, possess the same antimicrobial resistance mechanism in biofilms, and finally, the persistent cells that resist killing when exposed to antimicrobials.[56]

One of the additional AR responses of bacteria observed in biofilm is developed self-production of antibiotics to inhibit the increase of other bacteria populations in order to have an evolutionary advantage.[9] On the other hand, some other bacteria generate antibiotic resistance to compete against these antibiotic producing bacteria. For example, this adaptation was observed in an experiment done on *S. aureus* that initially were not resistant to antibiotics.[8] The bacteria left under typical biofilm conditions in a confined space with limited nutrient supply for 5 days resulted in three different bacteria groups, the susceptible bacteria, antibiotic producing bacteria and antibiotic resistant bacteria.[8,9] The experiment focused on the community-associated methicillin-resistant *S. aureus* (CA-MRSA) where the original wild-type (WT) strains of *S. aureus* formed an orange center origin (O).[8] Over time, a second unpigmented white (W) antibiotic and phenol soluble producing strain emerged and rapidly surrounded the origin.[8] Finally, a third yellow strain (Y) which was resistant to the products of the white unpigmented strain evolved from the origin through the white sector.[8]

4.3 The effect of disinfectant on antibiotic resistance

4.3.1 Quaternary ammonium compounds

4.3.1.1 Benzalkonium chloride

There are many studies describing cross-resistance between BACs and antibiotics.[41] One of them shows that the presence of BACs increases the MICs for multiple antibiotics, such as oxacillin, cefazolin, and ofloxacin, in methicillin-resistant *S. aureus* (MRSA) strains.[41] MRSA strains nonadapted to BACs were already resistant to ofloxacin, while the MICs of the antibiotic increased up to 4-fold for the adapted strains.[41] Similar results were observed with *E. coli*, where the laboratory strain *E. coli* K-12 exposed to increasing concentrations of BACs resulted in higher MICs for several antibiotics, such as amipicillin, ciprofloxacin, and nalidixic acid, on such a strain.[41] Increased MICs for multiple antibiotics were observed for the pathogenic strain *E. coli* 0157, but also for *E. coli* ATCC 11775 and DSM 682.[41] Finally, some *E. coli* strains became resistant to antibiotics, such as chloramphenicol and ampicillin, after adaptation to BACs.[41] In addition, strains of *Salmonella* serovar Virchow showed resistance to amoxicillin under similar exposure.[41] Strains of some other bacteria adapted to BACs, namely *Listeria monocytogenes (L. monocytogenes)*, became more sensitive to ciprofloxacin and gentamicin, while those of *P. aeruginosa*, became less sensitive to ciprofloxacin but more sensitive to minocycline.[41]

Although the exposure and adaptation to BACs can result in either decreased or increased susceptibility to several antibiotics, an increase in MIC demonstrates the existence of cross-resistance.[41] Further evidences have showed that bacteria that are merely tolerant to antibiotics can develop resistance to them faster, because their ability to survive the presence of the antibiotics unit the MIC has reached clinical standards helps them accumulate mutations that later can result in the emergence of antibiotic resistant strains.[41]

4.3.1.2 The *qac* genes

The *gac* genes encode a large family of bacterial efflux pumps, and are named after one of their main substrates, namely quaternary ammonium compounds.[54, 61] In addition to QACs, *qac* encoded efflux pumps are capable of eliminating many other cationic compounds from cell interior, such as intercalating dyes, diamidines and bigianides, thereby leading to decreasing susceptibility to these compounds.[54, 61] The *qac* genes are generally horizontally transferred to other bacteria via MGEs, often in combination with other ARGs because of their linkage with each other typically on plasmids.[48, 54, 61] This linkage explains also the *qac*-mediated resistance to both antiseptics and antibiotics.[61]

In a study by Jaglic Z. and Cervinkova D., the experiments done on staphylococci reported linkage between genes resistant to trimethoprim (*dfr*A), β-lactams (*bla*Z), aminoglycosides (*aac*A-*aph*D) and antiseptics (*qac*C).[61] This resistance was due to a multi-resistance plasmid.[61] In *S. aureus*, the *qacA* and *qacB* genes are typically located on the pSK1 and β-lactamase/heavy metal-resistance plasmids which also give resistance to a number of antibiotics.[61] One such example is the incidence of *qac* and ß-lactamase *bla* genes in the same plasmid resulting in a linkage between selection pressure for resistance to disinfectants, such as BACs, and antibiotics, such as penicillin.[41] In addition, the BAC-resistant isolates harboring plasmids with *qacA* and *qacB* genes were less sensitive to multiple antibiotics compared to BAC-sensitive ones.[41]

In addition to *S. aureus*, there are many other bacteria that develop antibiotic resistance in a similar way. For instance, the *qac* genes in Gram-negative bacteria are most frequently linked with plasmid-mediated class 1 integrons which harbour a variety of ARGs.[61] A relationship between the *qac* genes and a couple of ARGs, such as those coding for resistance to aminoglycosides, chloramphenicol, suphonamides, trimethoprim and β-lactams, was also

reported in Enterobacteriaceae.[61] Similar results were also reported in *P. aeruginosa*. In *Aeromonas hydrophila (A. hydrophila)* and microflora from a WWTP, the *qac* genes were found in combination with macrolide inactivation genes.[61] All these results therefore suggest that the use of cationic biocides may result in the selection of antibiotic resistant bacteria.[61]

4.3.2 Heavy metals

In addition to industrial WWTPs, there are dissolved heavy metals in influents of WWTPs in urban areas.[10] These contaminants, which include cadmium, zinc and the metalloid arsenic, are efficiently removed in industrial WWTs, while their removal was not planned in urban WWTPs; as a result, there can be high heavy metal concentrations in urban WWTPs effluxes can be large, thereby favoring the selection of multi-resistant bacteria and the spread of resistances into the environment, when antibiotics, ARB and ARGs also are present.[10]

Experimental evidence, mostly on plasmids, demonstrated a relationship between the acquisition of heavy metal resistance genes (HMRGs) and ARGs through co-selection.[10] In addition, the comparison of the abundances of ARGs, HMRGs and of the class 1 integron within the resident bacterial communities showed specific co-occurrence of ARGs, HMRGs, and the class 1 integron in the different treatment steps in three urban WWTPs. [10]

Bacterial communities in WWTPs are often exposed to a chemical stress, such as heavy metals and antibiotics, which create favorable conditions for the potential co-selection of resistant genes.[10] Different studies demonstrated the concomitant presence of genes encoding for resistance against different metals and of ARGs, in plasmids and integrons, that originate from contaminated soils and WWTPs.[10] The concentrations of heavy metals detected in WWTPs are typically two to four orders of magnitude greater than the levels of antibiotics.[10] This supports the fact that in contrast to antibiotics, heavy metals are not subjected to rapid degradation.[10] Consequently, the high levels of heavy metals can represent a long-term selection pressure.[52] Experimental results between different ARGs, more precisely sulII, HMRGs and int1 indicated a potential co-selection of at least some of the ARGs and HMRGs in the same bacterial strains.[10] The strict relations between them hinted to indirect selection of antibiotic resistances within the WWTPs.[10]

5 Wastewater treatment plants

The main purpose of WWTPs is the removal of pollutants, including antibiotics, biocides, and other chemicals, as well as the reduction of biological oxygen demand (BOD) from the wastewater they receive, thereby returning good quality water to the environment.[11, 48, 62-64] However, the final WWTPs effluents are far from being sterile.[64] The ineffectiveness of the existing WWTPs in removing micropollutants, ARB and ARGs is the main reason why WWTPs have been considered one of the main sources of antibiotics' release into the environment.[48, 63]

WWTPs contain a remarkable diversity of microorganisms and provide highly favorable conditions for the spread and reproduction of AR because during wastewater treatment bacteria are continuously mixed with antibiotics, heavy metals, and other pharmaceutical residues.[48, 63] The wastewater microbiome brings together bacteria of environmental, human and animal origin.[64] Many of these bacteria may contain ARGs and present a potential threat to human and animal health, as ARGs may lead to infections that are resistant to antibiotics and difficult to treat.[64] The selective pressure for ARGs can be increased by heavy metals, anti-fouling agents, detergents, QACs, and some other organic compounds through the process of co-selection.[48] In order to develop a comprehensive strategy to contain resistance and protect human health, a better understanding of the behavior of ARG and the various pathways by which they are spread is needed.[65]

5.1 Treatment methods in WWTPs

WWTPs are among the most common forms of pollution control.[66] Sewers collect the wastewater from residential, mechanical, business or farming exercises, as well as from surface overflow or storm water,[11] and deliver it to WWTPs for treatment.[66] WWTPs are built to clean wastewater by removing impurities, so that the final effluent does not present a new source of pollution for receiving water.[66, 67] However, the WWTPs are far from 100 percent effective. Even though sewage passes through multiple stages of both physical and biological/chemical treatment methods, the final effluent appears to still contain significant number of micropollutants, including ARB with their ARGs.[68, 48]

5.1.1 Pre-treatment

Pre-treatment of the raw wastewater is based on removal of gross solids.[69] This process, also known as screening, is the first unit operation in WWTPs.[70]

5.1.1.1 Screening

A screen is a device composed of parallel bars, rods, or wires, grating, wire mesh, or perforated plate, with openings used to retain solids found in the influent.[70] As a sewage flows through a screen, large objects, such as rags, sticks, and grit, are removed.[66] If not removed properly, these solids may cause damage to subsequent process equipment, clog pipes,[66] or even contaminate waterways.[70] Two types of screens are generally used in preliminary treatment: coarse screens with screen openings between 6 and 150mm, and fine screens with openings less than 6 mm.[70] When greater solid removal is required, fine screens are used either in place of or following coarse screens.[70] Microscreens with screen openings less than 50 μm are used in removing fine solids from effluents.[70]

5.1.2 Primary treatment

The purpose of primary treatment is to remove the floating and settleable materials through physical operations.[71] Gravity and sedimentation have traditionally been used to remove suspended solids.[71]

5.1.2.1 Grit removal

After sewage has been screened, and large solid objects have been removed, grit, consisting of sand, cinders, and small stones has to be settled out of the water.[66, 70] This can be accomplished in grit chambers or by the centrifugal and gravitational separation of solids.[70] Grit chambers are especially important in cities with combined sewer systems where sand or gravel may wash into sewers along with storm water.[66]

Grit chambers are most commonly located between screens and primary sedimentation tank.[70] Grit chambers are designed to remove grit and other heavy inorganic solid materials that have subsiding velocities or specific gravities greater than those of the organic putrescible solids in wastewater.[70] This is accomplished by reducing the flow velocity in a long narrow or a circular tank, so that grit materials can easily settle to the bottom by the force of gravity.[66, 70] Generally, grit chambers are based on the removal of grit particles having a specific gravity of 2.65 and a

wastewater temperature of 15.5 °C.[70]

There are three types of grit chambers based on the flow type of sewage. In the horizontal-flow type, the flow passes through the chamber in a horizontal direction with a straight-line velocity, allowing the sand particles to be deposited to the bottom.[70] The aerated type is known for a spiral-flow aeration tank where the spiral velocity is induced and controlled by the tank dimensions and quantity of air pumped into it.[70] The air causes a spiral of water to flow through the tank and heavier particles are removed.[70] The vortex type consists of a cylindrical tank in which the flow enters tangentially creating a vortex flow pattern.[70] Grit settles by gravity into the bottom of the tank while effluent exits at the top of the tank.[70]

5.1.2.2 Sedimentation

Organic and inorganic matter along with other suspended solids that were able to pass through screens and grit chambers can be removed from the sewage in sedimentation tanks.[66, 70] In sedimentation, the separation of suspended particles that are heavier than water occurs by gravitational settling.[70] As the speed of the flow through one of these tanks is reduced, the solids gradually sink and settle on the bottom.[66] The settled solids, also known as raw or primary sludge, are usually removed from the tank bottom by periodically pumping.[66] To increase purification efficiencies, primary sedimentation must be followed by secondary treatment that includes activated sludge or tricking filter.

5.1.3 Secondary treatment

In secondary treatment, a huge amount of the biodegradable organic matter and suspended solids are removed by making use of the bacteria in it.[66, 71] The main purpose of biological WWT facilities is to stimulate the purification process that occurs naturally in rivers, lakes and streams.[72] The are a number of factors which determine whether the treatment process is aerobic or anaerobic, including the composition of the wastewater, the degree of stabilization required for environmental compliance and economic viability.[72]

5.1.3.1 Aerobic treatment process

5.1.3.1.1 Trickling filter

A trickling filter is a tank loaded up with a bearer material, such as volcanic shake, rock or engineered material, ranging from one to two meters in depth.[11, 66] Sewage is delivered from above and as it trickles through filter media, microorganisms gather and multiply on the stones, forming a slime layer or biological film.[11, 66] The steady flow of sewage over these growths allows the microbes to absorb the dissolved organic material, thereby lowering the BOD of the sewage.[73] Sufficient oxygen for the metabolic processes is provided by air circulating upward through the spaces among the stones.[73] The cleaner water trickles to the bottom, and then further out through pipes to another sedimentation tank to remove excess microbes.[66, 73] In order to increase treatment efficiencies, two or more trickling filters may be connected in series.[73] The trickling filter method is less complex compared to activated sludge method, but it is much more expensive because of the increased electrical power usage.[11]

5.1.3.1.2 Activated sludge

Activated sludge process is a biological treatment method whose main purpose is to eliminate organic matter from wastewater.[11] This method involves an aeration tank followed by a secondary clarifier.[73] To continue the attentiveness of active bacteria in the tank, about 30 percent of the activated sludge is recirculated from the secondary clarifier and introduced into the aeration tank, where it is mixed with air and the new sewage.[11, 73]

In the aeration tank, compressed air is generally injected into the mixture through porous diffusers located at the bottom of the tank.[73] As the diffused air bubbles to the surface, it provides oxygen and favorable conditions for rapid mixing action.[73] As a result of the high oxygen concentration, microorganisms form an active, healthy suspension of biological solids, mostly bacteria, called activated sludge.[73] The microbes are given enough time in the aeration tank to break down the organic matter into harmless by-products.[11, 66, 73] The higher concentration of oxygen, the less aeration time is required, thereby reducing the required tank volume.[73] After the mixture leaves the aeration tank, it flows into the secondary clarifier with main purpose is settling out activated sludge by gravity force.[73] The sludge is pumped out from a hopper at the bottom of the tank, while clear water from the surface of the clarifier is skimmed, disinfected, and discharged as secondary effluent.[73] Part of the sludge is recirculated back into

the aeration tank since the recycled microbes are well acclimated to the sewage environment and readily metabolize the organic materials in the primary effluent.[73]

In the recent years, more WWTPs have replaced trickling filter with activate sludge process.[66] The main reason for this is that the activated sludge method occupies a smaller place compared to trickling filter method, and in addition the discharged effluent is of better quality.[11]

5.1.3.2 Anaerobic treatment process

Anaerobic treatment processes include anaerobic suspended growth, upflow and downflow anaerobic attached growth, fluidized-bed attached growth, upflow anaerobic sludge blanket (UASB), anaerobic lagoons, and membrane separation anaerobic processes.[74]

Anaerobic microbes play an important role in natural environments for the biological degradation of organic matter in the absence of oxygen. [75] In case no other inorganic electron acceptors than carbon dioxide is present in anaerobic environments, the final degradation of organic compounds result in their conversion to gaseous methane and carbon dioxide.[75] Although both aerobic and anaerobic treatment processes are still being used for removing of organic pollutants in WWTPs, the anaerobic digestion process is known for its many advantages over aerobic treatment.[75] Some of the most important advantages of anaerobic treatment processes are low energy required, lower biomass yield, fewer nutrients required, and higher volumetric loadings.[74]

5.1.3.2.1 Suspended growth

The complete-mix anaerobic growth digester without sludge recycling is suitable for wastes containing high concentration of solids or organic matter, where thickening the effluent solids otherwise is difficult.[74] The anaerobic contact process overcomes the disadvantages of a complete-mix process without recycle by separating biomass and returning it to the complete-mix or contact reactor.[74] Although gravity separation is the most common approach for solids separation and thickening prior to sludge recycle, it possesses some disadvantages, including a sludge with poor settling properties.[74] Therefore, gas flotation is often used instead.[74] However, solids-liquid separation can be inefficient in anaerobic process because gas is produced under the anaerobic conditions and can even continue in the separation process.[74] The anaerobic batch reactor (ASBR) process combines a suspended growth process with reaction and solids-liquid

separation in the same vessel, thereby reducing organic removal and gas production rates, and providing better conditions for solids settling before decanting the effluent.[74]

5.1.3.2.2 Sludge blanket

The UASB process is the most used sludge blanket process.[74] In the UASB process the influent wastewater enters the reactor from the bottom and flows up through the sludge blanket.[74] The main characteristic of this process is the development of a dense granulated sludge with particle size ranging from 1.0 to 3.0 mm.[74] There are many factors affecting this development.[74] For example, treating of wastewater rich on carbohydrate or sugar results in successful granulation, while more fluffy floc particles result from wastewater high in protein.[74] Other factors favoring the formation of dense granulated sludge floc particles are a pH around 7.0, a plug-flow hydraulic regime, a zone of high hydrogen partial pressure, a nonlimiting supply of NH_4-N, and a limited amount of the amino acid cysteine.[74]

5.1.3.2.3 Membrane bioreactors

Membrane Bio-Reactor (MBR) treatment techniques combine biological treatment, specifically suspended growth bioreactors, with membrane filtration, such as microfiltration or ultrafiltration.[11, 76] The degradation of biomass occurs inside the bioreactor tank, while the membrane serves as a filter for separation of treated wastewater from microorganisms, resulting in a clarified and disinfected product effluent.[76] The major challenge in the applications of MBRs is membrane fouling which significantly reduces membrane performance and lifespan, leading to a significant increase in maintenance and operating costs.[76, 77]

5.1.4 Tertiary treatment

Tertiary treatment is the third and the final stage in the wastewater treatment process. Its main purpose is to further remove oxygen-demanding substances, to remove nitrogen and/or phosphorus and to eliminate microorganisms and pathogens.[78, 79, 80, 81, 82, 83, 84] Removing of these harmful substances makes the effluent water safe to reuse, recycle or release into the environment.

5.1.4.2 Disinfection

Disinfection, also known as the last step of the wastewater treatment process, is responsible for destroying most pathogens in WWTP effluents before they are discharged into the environment.[78] Some of the most common treatments applied are chlorination, ultraviolet (UV),

and ozonation disinfection.[78] These disinfection processes are highly effective at decreasing ARBs.[78] The main purpose of the disinfection of water after wastewater treatment is to kill or inactivate bacterial cells.[79] More precisely, removal or destruction of DNA is highly important because of ARGs are typically carried on plasmids and integrons.[79] If not removed properly, these highly transmissible DNA elements can remain functional and confer ARGs to downstream bacteria by transformation and/or transduction.[78, 79] Once released, ARG can persist in the environment, and have even been observed to establish and proliferate in drinking water biofilms.[79]

5.1.4.3 Biological phosphorus-removal process

Biological suspended growth process configurations used for biological phosphorus removal all include the same basic steps, an anaerobic zone followed by an aerobic zone.[80] In some applications, aerobic zone is replaced by an anoxic zone.[81] In addition, different types of further modifications are possible.[80] The phosphorus from the influent wastewater is taken up by specialized bacterial cells known as phosphorus accumulating organisms (PAOs), and as the biomass is wasted, stored phosphorous is removed from the biotreatment reactor.[80, 81]

5.1.4.4 Biological nitrogen-removal processes

Biological nitrogen-removal processes use an aerobic zone where nitrification takes place.[82] Furthermore, an anoxic zone is also required for biological denitrification for complete nitrogen removal by both NH_4-N oxidation and NO_3-N and NO_2-N reduction to nitrogen gas.[82] At the very end, an electron donor is required to complete the nitrogen reduction.[82] An electron can be supplied in the form of influent wastewater BOD, by endogenous respiration, or an external carbon source.[81, 82]

5.1.4.5 Removal of heavy metals using different methods

Due to their potential accumulation and toxicity in municipal sewer system, it is important to remove heavy metals from wastewater effluents in order to avoid their discharge into the environment.[83] Heavy metals can be removed from wastewater by chemical precipitation, carbon adsorption, reverse osmosis, and ion exchange.[84]

5.2 Occurrence of disinfectants in WWTPs

As mentioned earlier, use of disinfectants has increased drastically during the last two years with the emergence of the COVID-19 pandemic. QACs, more precisely BACs, are the most common ingredients in the disinfectants used to inactivate the SARS-CoV-2 virus.[85, 86] In addition, while high income countries have stabilized their use of antibiotics, low to upper-middle income countries are increasing their consumption of antibiotics drugs.[85] Huge amounts of both disinfectants and antibiotics present in wastewater represent a real danger to aquatic organisms and AR once they enters the environment.[79]

5.2.1 QACs

BACs is a QAC reported in wastewater effluents, with highest concentration coming from hospitals (up to 6.03 mg/liter).[41] The occurrence of 19 QACs has been detected in over 90% of residential dust samples during the COVID-19 pandemic, at concentrations ranging from 1.95 to 531 μg/g (median of 58.9 μg/g).[3] The QACs concentrations were significantly higher compared to samples collected before the COVID-19 pandemic, where the median concentration was 36.3 μg/g.[3] As mentioned earlier, WWTPs are not designed to remove QAC from wastewater, resulting in the release of a portion of them into the environment. In 2006 Environmental Protection Agency (EPA) found BACs to be toxic to the aquatic environment and its inhabitants, thereby advising against their release into rivers, lakes and other water.[41] QACs are known for their low vapor pressures, and are therefore not expected to volatilize from soil or water. [86] In addition, the high log organic carbon-water partitioning coefficient (K_{OC}) and partition coefficient (K_d) of BAC indicate that BAC tends to accumulate once it enters soil.[85] Another important characteristic of BAC supporting this retention in soil is its positive charge.[85] Efflux pump genes – acrA, acrB, qacG, and qacH – have been found in BAC resistant bacteria. Cross-resistance can be induced after exposure to other disinfectants.[85]

5.2.2 TCC and TCS

TCC and TCS have been found at high in the influents in Bangkok, Thailand. TCC was detected at the highest concentrations in sludge and sediment.[6] It is believed that this is due to its strong adsorption onto particles.[6] Therefore, adsorption plays a key role in the removal of TCC in WWTPs. In addition, high concentrations of TCS have been reported in fish samples from receiving rivers and canals.[6]

Table 3. *Occurrence of biocides in wastewater and their fate in the WWTPs (A/O: anaerobic/oxic); CAST: cyclic activated system technology; AS: activated sludge; TF: trickling filter).*

Disinfectant	Source	Location	Reported concentrations	Removal percent	Reference
Benzalkonium chlorides	Hospital effluent	Europe	0.05 – 6.03 g/L		[41]
	Municipal sewage sludge	China	0.09 – 191 mg/kg		[41]
	BAC-C_{12} in wastewater treatment influents	Unknown	170 µg/L		[41]
	WWTP	Unknown	Up to 0.17 mg/L		[41]
	WWTP influent	China	0.641 µg/L		[87]
	WWTP effluent		0.076 µg/L	88% A/O	[87]
	WWTP influent	China	0.870 µg/L		[87]
	WWTP effluent		0.010 µg/L	99% CAST	[87]
Triclosan	WWTP influent	Gossau, Switzerland	700 µg/L (?)		[88]
	Primary clarified effluent		520 µg/L		[88]
	Secondary effluent		45 µg/L		[88]
	WWTP effluent		42-213 µg/L	70-94%	[88]
	WWTP influent	USA	3.8-16.6 µg/L		[89]
	WWTP effluent		0.2-2.7 µg/L	96 % AS 58-86 % TF	[89]
	WWTP effluent	U.K.	340-1100 ng/L		[21]
	WWTP influent	Australia	573-845 ng/L		[21]
	WWTP effluent		60.5-159 ng/L	85 % (mean)	[21]
	WWTP influent	Brazil	17.8-67.1 µg/L		[90]
	WWTP effluent		0.9-3.6 µg/L	95 % (mean)	[90]
	WWTP influent	Gauteng Province, South Africa	2.01-17.6 µg/L		[91]
	WWTP effluent		0.990-13.0 µg/L	29 % (mean)	[91]
Chlorhexidine	WWTP influent	Sweden	18-164 kg/year		[92]
	WWTP effluent		0.2-3.3 kg/year	Up to 98 %	[92]
Copper	WWTP influent	Thessaloniki, Greece	79 µg/L		[93]
	WWTP effluent		33 µg/L	58 %	[93]

	WWTP influent WWTP effluent	Venice, Italy	5-430 µg/L 3-90 µg/L	 79 % (mean)	[94] [94]
Silver	WWTP influent WWTP effluent	Venice, Italy	1-13.7 µg/L 0.6-12.2 µg/L	 13 % (mean)	[94] [94]
Triclocarban	WWTP influent WWTP effluent	Gauteng Province, South Africa	0.086-2.84 µg/L <LOD-1.89 µg/L		[91] [91]

5.2 Removing of ARGs from WWTPs

Removal or destruction of DNA is highly important because of ARGs typically carried on plasmids and integrons.[79] If not removed properly, these highly transmissible DNA elements can remain functional and be assimilated by down-stream bacteria.[79] One released, ARG can persist in the environment, and have even been observed to establish and proliferate in drinking water biofilms.[79]

5.3.1 Secondary treatment

5.3.1.1 Aerobic/anaerobic treatment

In order to determine the effect of biological treatment on the levels of ARGs water from a dairy in northern Colorado was incubated anaerobically or aerobically at 20 °C or 4 °C. The effect of both aerobic/anaerobic and temperature treatment on the levels of tetracycline – *tet*(W) and *tet*(O), sulfonamide – *sul*(I) and *sul*(II), and macrolide – *ere*(A) and *msr*(A) was explored. The results showed that higher temperatures result in the degradation of antibiotics at a faster rate compared to treatment at lower temperature of 4 °C. In addition, aerobic treatment showed higher removal of ARGs than anaerobic at the same temperature.[65]

Another study on ARGs – *tet*(G), *tet*(W), *tet*(X), *sul*(I), and *intl*(I) – in a full-scale municipal WWTP with A ^{2}O-MBR system showed that membrane bioreactors (MBRs) are possibly more effective at removing ARB and ARGs than traditional activated sludge.[48, 95] This due to the extra filtration of the effluent through the membrane.[48] The concentration of ARGs in wastewater decreased in the anaerobic effluent and anoxic effluent, but increased in the aerobic effluent, before it declined in the MBR effluent. The following concentration trends both in influent and MBR effluent were observed *sul*(I) > *intl*(I) > *tet*(X) > *tet*(G) > *tet*(W).[95]

The results indicate that *sul*(I) is one of the most prevalent genes in the environment.[95] High concentrations of *intI(I)* can be explained by subsequent HGT event that could occur in the WWTP and result in the spread of ARGs among microorganisms.[95] The concentration of ARGs in sludge samples increased during along the treatment process, while the ratio of ARGs to 16S ribosomal DNA (rDNA) changed little from anaerobic to anoxic to aerobic basins, but increased in MBR. The results showed also that the reduction of *tet*(W), *intl*(I), and *sul(I)* was positively correlated with the reduction of 16S rDNA. It is believed that the anaerobic treatment is much better at removing ARGs in wastewater, because the bioactivity of the microorganism is lower under anaerobic conditions and the spread of resistance genes is inhibited.[95]

Reducing the amount of energy consumed in treatment processes has become the main challenge for the future.[96] Three different wastewater treatment strategies – anaerobic, aerobic, and anaerobic-aerobic sequence (AAS) bioreactors – have been compared regarding energy use, treatment performance, and ARG abundance.[48] Contrarily to the previous study mentioned, the results showed higher ARG effectiveness of aerobic bioreactors and AAS bioreactors compared to anaerobic bioreactors alone.[96] However, the AAS bioreactors showed both higher removal of ARGs and lower energy consumption than the two other strategies.[96]

5.3.1.2 Membrane filtration

Sequential filtration across decreasing membrane pore sizes showed significant effect on the removal of ARGs ($p < 0.001$).[79] In addition, the presence of colloidal material in the aqueous matrix was found to have the same effect on the removal of bla_{TEM} (p ¼ 0.004) and vanA ARGs (p ¼ 0.002), genes present on the spiked plasmids.[79] Further analysis showed that as membrane pore size decreased, the influence of colloids and the removal of ARGs became more apparent.[79] For example, microfiltration through 0.45 and 0.1 mm pore-size membranes resulted in less than 1-log removal of ARGs either in the presence or absence of colloidal material, while filtration through the 100, 10, and 1 kDa membranes resulted in 0.9, 3.6, and 4.3 e-log reduction respectively across no-colloidal controls and ARGs, and 1.7, 4.9, and 5.9 e-log reductions of ARGs across all WWTP effluents.[79] The conclusion that could be made from these results was that the membrane removal of ARGs is actually enhanced in the presence of wastewater rich in colloidal material.[79] A significant effect on the removal of bla_{TEM} and vanA observed at 1.2 mm-filtered wastewater effluent matrix is probably a result of interactions with colloidal material present in the effluent.[79] This assumption was further supported by the

correlations observed with the TOC, proteins, and polysaccharide concentrations present in the filtrates.[79]

5.3.2 Tertiary treatment

5.3.2.1 UV disinfection and chlorination

The effects of tertiary treatment methods, such as ultraviolet (UV) disinfection and chlorination, on the frequency of ARGs transfer have been studied based on the conjugative transfer model between Gram-negative strains of *E.coli*.[68] Low UV doses, up to 8 mJ/cm^2, had little influence on the frequency of conjugative transfer, while low chlorine doses, up to 40 mg Cl min/L, considerably promoted the frequency by 2-5 fold.[68] Furthermore, it was observed that low UV doses decreased bacterial number without changing the cell permeability.[68] After the treatment with low chlorine doses the generated chloramine stimulated the bacteria and improved the cell permeability, as a result of more pilus induced on the surface of conjugative cells, which acted as pathways for ARGs transfer.[68] High doses of both UV and chlorine, >10 mJ/cm^2 and >80 mg Cl min/L, respectively, resulted in suppressed frequency of ARG transfers.[68] The effect of high UV doses (>10 mJ/cm^2) on bacteria led to the complete inhibition of conjugative transfer.[68] Although causing the reduction of ARB or ARGs concentrations, the general observation is that UV or chlorine cannot completely eliminate antibiotic resistance, so that the risk of ARGs transfer in the final effluent still exists.[68]

One of the recent studies on the inactivation of two ARGs – sul1 and tetG, and the integrase gene of class 1 integrons – intI1, shows that chlorination achieved more inactivation of selected genes than UV irradiation and ozonation.[78] Furthermore, for the inactivation of 16S rDNA, UV irradiation was the least effective method.[78] The reason why chlorine exhibited the best removal of ARGs may be its ability to react moderately with cell envelope, thereby penetrating the cytoplasm to deactivate the ARGs.[78] Contrarily, ozone reacts too rapidly with the cell envelope leading to an inability of the dose to penetrate into the cell and reach the ARGs.[78]

Experiments conducted using bacteria resistant to antibiotics ampicillin, cephalothin, tetracycline and trimethoprim demonstrate a potential with chlorination disinfection. Although it previously has been shown that different doses of chlorination reduced the percentage of ampicillin-resistant bacteria in sewage, opposite results have also been reported in other studies. In a study done by Murray et al. an increase in proportion of bacteria resistant to ampicillin and cephalothin were observed after treating urban wastewater influent with chlorine.[97] Although

the chlorination process was found to initially reduce the total number of bacteria in wastewater, it may substantially increase the proportions of ARB.[97] Further evidence supporting this assumption relies on experiments done by Huang et al. using highly tetracycline-resistant *E. coli* strains, where inactivation was significantly lower for the tetracycline resistant strain when compared the antibiotic-sensitive *E. coli* at high chlorine doses (>1.0 mg/L, 10 min contact time).[48, 63, 98] However, opposite results were reported for ampicillin- and trimethoprim-resistant *E. coli* strains, and the conclusion made from these results suggests that the chlorination process is unlikely to select for ampicillin- or trimethoprim-resistant survivors during wastewater treatment.[99] Templeton et al. compared the effect of free chlorine and UV (UV intensity 0.247 mW/cm^2) disinfection on *E. coli* strains resistant to ampicillin and trimethoprim with an antibiotic-susceptible *E. coli* strain.[99] The results found the trimethoprim-resistant *E.coli* strains to be slightly more resistant to chlorine than the antibiotic-susceptible strain and the ampicillin-resistant *E. coli*.[99] Moreover, no significant differences between the antibiotic-resistant and antibiotic-susceptible *E. coli* strains were observed over the UV dose range tested. [48, 63, 99]

5.4 Constructed wetlands (main characteristics)

Constructed wetlands (CWs) are artificial systems that use natural processes involving wetland vegetation, soils, and the associated microbial populations to improve water quality.[100] CWs are an substitute for secondary and tertiary treatment of industrial and municipal wastewater as their main purpose is removing bacteria, enteric viruses, suspended solids, BOD, metals and phosphorus, from wastewater.[11, 101] Treatment methods present in CWs replicates those happening in natural wetlands consisting of substrate, macrophytes, and microbial assemblage.[11] CWs can be classified either according to the life form of the dominating macrophyte (free-floating, floating leaved, rooted emergent, or submerged) or according to the wetland hydrology (free water surface and subsurface) and surface flow (horizontal and vertical).[100] CWs have been proven to have several benefits over the conventional techniques, where the most important ones are lower costs linked to construction, operation and maintenance, and lower energy absorption.[11]

6 Discussion

Both heavy metals and QACs have been shown to lead to an increase in ARGs through co-resistance. For heavy metals, such as zinc and copper, the study by Yazdankhan et al. showed that heavy metal resistance genes increased the rate of spreading of antibiotic resistance.[48, 53] Similar results have been seen for QACs in Jiao et al. study, which showed the co-resistance of *gac* genes, typically located on MGEs together with ARGs.[48, 54] Another example is seen on plasmids where *qac* and ß-lactamase *bla* genes are linked, resulting in resistance to both disinfectants, such as BACs, and antibiotics, such as penicillin.[41] Similar co-resistance is also reported in a subset of matings between *Enterobacteriaceae* and recipients, where mercury resistance was co-transferred with antibiotic resistances.[61]

Cross-resistance has been found in *L. monocytogenes* where the multi-drug efflux pump exports both metals and antibiotics.[52] While over-expression of a pump alone results in resistance to some antibiotics and biocides, when exposed to these agents frequently it favors cross-resistance to all other substrates of the pump.[55] Such mechanisms are seen in *P. aeruginosa* mutants known for overproduction of MexAB, thereby resulting in resistance to some antibiotics, and triclosan.[55] The over-expression of the gene encoding efflux pumps happens to be most effective at leading to resistance when the bacteria are exposed to inhospitable condition.[55]

Many studies have described cross-resistance between BACs and antibiotics. One such study showed that the presence of BACs increases the MICs for multiple antibiotics, such as oxacillin, cefazolin, and ofloxacin, in MRSA strains.[41] In addition, similar results have been observed with *E. coli*, where increasing concentrations of BACs resulted in increasing the MIC of ampicillin, ciprofloxacin, and nalidixic acid on such a strain.[41] Even though these bacterial strains did not become resistant to antibiotics, the increase in the MICs for antibiotics gives the bacteria more time to accumulate mutations so that emerge in antibiotic resistant strains may emerge.[41]

The process of co-selection is favored in the presence of heavy metals, biocides, antibiotics, resistant bacteria, and resistance genes, WWTPs provide conditions which are suitable for the selection of multi-resistant bacteria.[10] However, highly specialized methods are required for the removal of ARB and ARGs. The different types of treatment methods used to treat wastewater

vary significantly in their removal efficiencies of ARB and ARGs. For example, looking at secondary treatment methods, aerobic biological treatment shows higher removal of ARGs when compared to anaerobic treatment at the same temperature. Higher temperatures result in the degradation of antibiotics at faster rates than at lower temperatures.[65] A study using membrane bioreactors showed that they are probably more effective at removing ARB and ARGs than the traditional activated sludge method.[48, 95] Within tertiary treatment methods, chlorination achieved more inactivation of ARGs than UV radiation and ozonation.[78]

7 Concluding remarks

Wastewater treatment plants concentrate wastewater containing residual antibiotics, disinfectants and antibiotic resistant bacteria from diverse origins, and are therefore considered a hotspot for the spread of antibiotic resistance genes into the environment. Even though the results show that exposure to disinfectants might increase antibiotic-susceptibility of bacteria, their mechanisms of action are still poorly understood. Additionally, WWTPs are not designed for the removal of antibiotics and disinfectants and there are no specific technologies for the reduction of antibiotic resistant bacteria and of antibiotic resistant genes in wastewater currently. Although some secondary and tertiary treatment methods probably lead to the reduction in ARB, ARGs, and disinfectants, they are still unlikely to completely eliminate antibiotic resistance, so that the risk of ARGs transfer in the final effluent still exists.[68] Additionally, high concentration and high dose of disinfectants and antibiotics used during the COVID-19 pandemic, which entered into environment, are accelerating the target selection of antimicrobial resistance in environements.[2]

8 Future perspectives

Future population growth and water scarcity pose significant risks to global food security and drive the need to reuse wastewater especially in arid and semiarid regions.[102] Global antibiotic consumption is also likely to increase.[9] Although high income countries have stabilized their use of antibiotics, low to upper-middle income countries are increasing their consumption of antibiotics drugs.[85] Additionally, new global pandemics, as seen with COVID-19, will also possibly lead to an increased use of disinfectants in daily life. With increasing water reuse, antibiotic consumption, and disinfectants use even more ARGs and ARB will be released into the environment if currently applied wastewater treatment methods remain the same. Some of the improvements that should be made involve plans for implementation of more advanced treatment processes focusing on removal of ARGs and ARB, as well as setting limits for release from point sources of compounds driving co-selection of resistance such as antibiotics and disinfectants.[48] Lastly, more research needs to be done on disinfectants' mode of action, the resistant mechanisms bacterial cells possess against them, as well as their occurrence and removal in WWTPs, and most importantly on how their presence leads to the development of antibiotic resistance.

References

1. Dewey, H. M.; Jones, J. M.; Keating, M. R.; Budhathoki-Uprety, J., Increased Use of Disinfectants During the COVID-19 Pandemic and Its Potential Impacts on Health and Safety. *ACS Chem. Health Saf. 2022* **2021,** *29*, 27-38.
2. Chen, Z.; Guo, J.; Jiang, Y.; Shao, Y., High concentration and high dose of disinfectants and antibiotics used during the COVID-19 pandemic threaten human health. *Environtal Sciences Europe* **2021,** *33*.
3. Zheng, G.; Filippelli, G. M.; Salamova, A., Increased Indoor Exposure to Commonly Used Disinfectants during the COVID-19 Pandemic. *Environmental Science and Technology Letters* **2020,** *7*, 760-765.
4. Dhama, K.; Patel, S. K.; Kumar, R.; Masand, R.; Rana, J.; Yatoo, M. I.; Tiwari, R.; Sharun, K.; Mohapatra, R. K.; Natesan, S.; Dhawan, M.; Ahmad, T.; Emran, T. B.; Malik, Y. S.; Harapan, H., The role of disinfectants and sanitizers during COVID-19 pandemic: advantages and deleterious effects on humans and the environment. *Environmental Science and Pollution Research* **2021,** *28*, 34211-34228.
5. Pedreira, A.; Taskin, Y.; García, M. R., A Critical Review of Disinfection Processes to Control SARS-CoV-2 Transmission in the Food Industry. *Foods* **2021,** *10*.
6. Juksu, K.; Zhao, J.-L.; Liu, Y.-S.; Yao, L.; Sarin, C.; Sreesai, S.; Klomjek, P.; Jiang, Y.-X.; Ying, G.-G., Occurence, fate and risk assessment of biocides in wastewater treatment plants and aquatic environments in Thailand. *Science of the Total Environment* **2019,** *690*, 1110-1119.
7. Russell, A. D., Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *Journal of Applied Microbiology Symposium Supplement* **2002,** *92*, 121-135.
8. Koch, G.; Yepes, A.; Förstner, K. U.; Wermser, C.; Stengel, S. T.; Modamio, J.; Ohlsen, K.; Foster, K. R.; Lopez, D., Evolution of Resistance to a Last-Resort Antibiotic in *Staphylococcus aureus* via Bacterial Competition. *Cell* **2014,** *158*, 1060-1071.
9. Hiller, C. X.; Hübner, U.; Fajnorova, S.; Schwartz, T.; Drewes, J. E., Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. *Science of the Total Environment* **2019,** *685*, 596-608.
10. Cesare, A. D.; Eckert, E. M.; D'Urso, S.; Bertoni, R.; Gillan, D. C.; Wattiez, R.; Corno, G., Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Research* **2016,** *94*, 208-214.
11. Fahad, A.; Mohamed, R. M. S.; Radhi, B.; Al-Sahari, M., Wastewater and its Treatment Techniques: An Ample Review. *Indian Journal of Science and Technology* **2019,** *12(25)*.
12. Chapman, J. S., Biocide resistance mechanisms. *International Biodeterioration & Biodegradation* **2003,** *51*, 133-138.
13. McDonnel, G.; Russell, D., Antiseptics and Disinfectants: Activity, Action, and Resistance. *Clinical Microbiology Reviews* **1999**, 147-179.
14. Maillard, J.-Y., Bacterial target sites for biocide action. *Journal of Applied Microbiology Symposium Supplement* **2002,** *92*, 16-27.
15. Yoo, J.-H., Review of Disinfection and Sterilization - Back to the Basics. *Infect Chemother 2018* **2018,** *50(2)*, 101-109.

16. Department, O. S. a. H. B. o. t. L., Guidance Notes on Safe Use of Chemical Disinfectants. *Chemical Safety in the Workplace* **2007**.
17. Herruzo-Cabrera, R.; Uriarte, M. C.; Rey-Calero, J., Antimicrobial effectiveness of 2% glutaraldehyde versus other disinfectnats for hospital equipment, in an in vitro test based on germ-carriers with a high microbial contamination. *Rev. Stomatol. Chir. Maxillofac.* **1999,** *100(6)*, 299-305.
18. Health, M. D. o., Triclocarban and Drinking Water. *Clean Water Land & Legacy Amendment* **2013**.
19. Safety, N. S. C. f. F., Chlorhexidine compounds in cosmetic products. Risk assessment of antimicrobial and antibiotic resistance development in microorganisms. **2010**.
20. Kermeoglu, F.; Aksoy, U.; Kalender, A.; Oztan, M. D.; Oguz, E. I.; Kiyan, M., Determination of the Minimum Inhibitory Concentrations of Alexidine and Chlorhexidine Against Enterococcus feacalis and Candida albicans: An In Vitro Study. *Cureus* **2018,** *10(2)*.
21. Ying, G.-G.; Kookana, R. S., Triclosan in wastewater and biosolids from Australian wastewater treatment plants. *Environmental International* **2007,** *33*, 199-205.
22. Geller, C.; Fontanay, S.; Finance, C.; Duval, R. E., A new Sephadex^TM-based method for removing microbicidal and cytotoxic residues when testing antiseptics against viruses: Experiments with a human coronavirus as a model. *Journal of Virological Methods* **2009,** *159*, 217-226.
23. England, P. H., Soldium hypochlorite. General Information. *Compendium of Chemical Hazards* **2016**.
24. Liebert, M. A.; Inc.; Publishers, Final Report on the Safety Assessment of Benzalkonium Chloride. *Journal of the American College of Toxicology* **1989,** *8*.
25. Biazik, J.; Vihinen, H.; Jokitalo, E.; Eskelinen, E.-L., Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging. *Role of Autophagy in Therapeutic Applications* **2015**, 57-68.
26. Sartori, S. K.; Alvarenga, E. S.; Franco, C. A.; Ramos, D. S.; Oliveria, D. F., One-pot synthesis of anilides, herbicidal activity and molecular docking study. *Pest Manag Sci* **2018**.
27. Przybysz, T. M.; Heffner, A. C., 42 - Central Venous Access Procedures. *Cardiac Intensive Care (Third Edition)* **2019**, 447-455.
28. Ruiz-Linares, M.; Ferrer-Luque, C. M.; Arias-Moliz, T.; de Castro, P.; Aguado, B.; Baca, P., Antimicrobial activity of alexidine, clorhexidine and cetrimide against *Streptococcus mutans* biofilm. *Annals of Clinical Microbiology and Antimicrobials* **2014,** *13:41*.
29. Varadan, P.; Ganesh, A.; Konindala, R.; Nagendrababu, V.; Ashok, R.; Deivanayagan, K., Comparison of the Antibacterial Efficacy of Alexidine and Chlorhexidine Against Enterococcus Faecalis: An in Vitro Study. *Cureus* **2017,** *9(10)*.
30. Zorko, M.; Jerala, R., Alexidine and chlorhexidine bind to lipopolysaccharide and lipoteichoic acid and prevent cell activation by antibiotics. *Journal of Antimicrobial Chemotherapy* **2008,** *62*, 730-737.
31. Russell, A. D., Similarities and differences in the responses of microorganisms to biocides. *Journal of Antimicrobial Chemotherapy* **2003,** *53*, 750-763.
32. Grare, M.; Mourer, M.; Fontanay, S.; Regnouf-de-Vains, J.-B.; Finance, C.; Duval, R. E., *In vitro* activity of *para*-quanidinoethylcalix[4]arene against susceptible and antibiotic-resistand Gram-negative and Gram-positive bacteria. *Journal of Antimicrobial Chemotherapy* **2007,** *60*, 575-581.

33. Thurman, R. B.; Gebra, C. P.; Bitton, G., The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. *Critical Reviews in Environmental Control* **2009,** *18*, 295-315.
34. Kitis, M., Disinfection of wastewater with paracetic acid: a review. *Issue 1* **2004,** *30*, 47-55.
35. Wessels, S.; Ingmer, H., Modes of action of three disinfectant active substances: A review. *Regulatory Toxicology and Pharmacology* **2013,** *67*, 456-467.
36. Chhetri, R. K.; Thornberg, D.; Berner, J.; Gramstad, R.; Öjstedt, U.; Sharma, A. K.; Andersen, H. R., Chemical Disinfection of Combined Sewer Overflow Waters Using Performic Acid or Paracetic Acids. *Science of the Total Environment* **2014,** *490*, 1065-1072.
37. Maffettone, R.; Manoli, K.; Santoro, D.; Passalacqua, K. D.; Wobus, C. E.; Sarathy, S., Performic Acid Disinfection of Municipal Secondary Effluent Wastewater: Inactivation of Murine Norovirus, Fecal Coliforms, and Enterococci. *Environ. Sci. Technol.* **2020,** *54*, 12761-12770.
38. Miksusanti; Jenie, B. S. L.; Priosoeryanto, B. P.; Syarief, R.; Rekso, G. T., Mode of Action Temu Kunci (*Kaempferia pandurata*) Essential Oil on *E. coli* K1.1 Cell Determined by Leakage of Material Cell and Salt Tolerance Assays. *HAYATI Journal of Biosciences* **2008,** *15*, 56-60.
39. Yoshimatsu, T.; Hiyama, K.-I., Mechanism of the Action of Didecyldimethylammonium chloride (DDAC) against *Escherichia coli* and Morphological Changes of the Cells. *Biocontrol Science* **2007,** *12*, 93-99.
40. Rowe, R. C.; Sheskey, P. J.; Cook, W. G.; Fenton, M. E., Handbook of pharmaceutical excipients *Pharmaceutical Press and American Pharmacists Association* **2012,** *eds.*, 60-61.
41. Pereira, B. M. P.; Tagkopoulos, I., Benzalkonium Chlorides: Uses, Regulatory Status, and Microbial Resistance. *Issue 1* **2019,** *85*.
42. Lim, C.-H.; Chung, Y.-H., Effects of Didecyldimethylammonium Chloride and Sprague-Dawley Rats after Two Weeks of Inhalation Exposure. *Toxical. Res.* **2014,** *30*, 205-210.
43. Anderson, S. E.; Shane, H.; Long, C.; Lukomska, E.; Meade, B. J.; Marshall, N. B., Evaluation of the irritancy and hypersensitivity potential following topical application of didecyldimethylammonium chloride. *Journal of Immunotoxicology* **2016,** *13*, 557-566.
44. Akira, S.; Uematsu, S.; Takeuchi, O., Pathogen Recognition and Innate Immunity. *Cell* **2006,** *124*, 783-801.
45. Denyer, S. P.; Stewart, G. S. A. B., Mechanisms of action of disinfectants. *International Biodeterioration & Biodegradation* **1998,** *41*, 261-268.
46. Migneault, I.; Dartiguenave, C.; Bertrand, M. J.; Waldron, K. C., Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques* **2004,** *37*, 790-802.
47. Bischofberger, A. M.; Baumgartner, M.; Pfrunder-Cardozo, K. R.; Allen, R. C.; Hall, A. R., Associations between sensitivity to antibiotics, disinfectants and heavy metals in natural, clinical and laboratory isolates of *Escherichia coli*. *Environmental Microbiology* **2020,** *22(7)*, 2664-2679.
48. Uluseker, C.; Kaster, K. M.; Thorsen, K.; Basiry, D.; Shobana, S.; Jain, M.; Kumar, G.; Kommedal, R.; Pala-Ozkok, I., A Review on Occurrence and Spread of Antibiotics Resistance in Wastewaters and in Wastewater Treatment Plants: Mechanisms and Perspectives. *Frontiers in Microbiology* **2021,** *12*.
49. Burmeister, A. R., Horizontal Gene Transfer. *Evolution, Medicine, and Public Health* **2015,** (193-194).

50. Sevillya, G.; Adato, O.; Snir, S., Detecting horizontal gene transfer: a probabilistic approach. *BMC Genomics* **2020,** *21*, 106.
51. Touchon, M.; de Sousa, J. A. M.; Rocha, E. P. C., Embracing the enemy: the diversification of microbial gene repertoires by phage-mediated horizontal gene transfer. *Current Opinion in Microbiology* **2017,** *38*, 66-73.
52. Baker-Austin, C.; Wright, M. S.; Stepanauskas, R.; McArthur, J. V., Co-selection of antibiotic and metal resistance. *TRENDS in Microbiology* **2006,** *14*.
53. Yazdankhan, S.; Rudi, K.; Bernhoft, A., Zinc and copper in animal feel - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microbial Ecology in Health and Disease* **2014,** *25*, 258-262.
54. Jiao, Y.-N.; Chen, H.; Gao, R.-X.; Zhu, Y.-G.; Rensing, C., Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems. *Chemosphere* **2017,** *184*, 53-61.
55. Webber, M. A.; Piddock, L. J., The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **2003,** *51*, 9-11.
56. Soto, S. M., Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. *Virulence* **2013,** *4:3*, 223-229.
57. Schwarz, S.; Noble, W. C., Aspects of bacterial resistance to antimicrobials used in veterinary dermatological practice. *Veterinary Dermatology* **1999,** *10*, 163-176.
58. Bockstael, K.; Van Aerschot, A., Antimicrobial resistance in bacteria. *Cent. Eur. J. Med.* **2009,** *4(2)*, 141-155.
59. Kapoor, G.; Saigal, S.; Elongavan, A., Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology Clinical Pharmacology* **2017,** *33*.
60. Patel, R., Biofilms and Antimicrobial Resistance. *Clinical Orthopedics and Related Research* **2005,** *437*, 41-47.
61. Jaglic, Z.; Cervinkova, D., Genetic basis of resistance to quaternaty ammonium compounds - the *qac* genes and their role: a review. *Veterinarni Medicina* **2012,** *57*, 275-281.
62. Hospido, A.; Moreira, M. T.; Fernández-Couto, M.; Feijoo, G., Environmental Performance of a Municipal Wastewater Treatment Plant. *Int J CLA* **2004,** *9 (4)*, 261-271.
63. Rizzo, L.; Manaia, C.; Merlin, C.; Schwartz, T.; Dagot, C.; Ploy, M. C.; Michael, I.; Fatta-Kassinos, D., Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of the Total Environment* **2013,** *447*, 345-360.
64. Manaia, C. M.; Rocha, J.; Scaccia, N.; Marano, R.; Radu, E.; Biancullo, F.; Cerqueira, F.; Fortunato, G.; Iakovides, I. C.; Zammit, I.; Kampouris, I.; Vaz-Moreira, I.; Nunes, O. C., Antibiotic resistance in wastewater treatment plants: Tackling the black box. *Environment International* **2018,** *115*, 312-324.
65. Pei, R.; Cha, J.; Carlson, K. H.; Priden, A., Response of Antibiotic Resistance Genes (ARG) to Biological Treatment in Dairy Lagoon Water. *Environ. Sci. Technol.* **2007,** *41*, 5108-5113.
66. Agenct, U. S. E. P., How Wastewater Treatment Works… The Basics. **1998**.
67. Ratola, N.; Cincinelli, A.; Alves, A.; Katsoyiannis, A., Occurence of organic microcontaminants in the wastewater treatment process. A mini review. *Journal of Hazardous Materials* **2012,** *239-240*, 1-18.
68. Guo, M. T.; Yuan, Q.-B.; Yang, J., Distinguishing Effects of Ultraviolet Exposure and Chlorination on the Horizontal Transfer of Antibiotic Resistance Genes in Municipal Wastewater. *Environ. Sci. Technol.* **2015,** *49*, 5771-5778.

69. Schier, W.; Frechen, F.-B.; Fischer, S., Efficiency of mechanical pre-treatment on European MBR plants. *Desalination* **2007,** *236*, 85-93.
70. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 5 Physical Unit Operations. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 311-474.
71. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 1 Wastewater Engineering: An Overview. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 1-26.
72. Robinson, D. G.; White, J. E.; Callier, A. J., Aerobic versus anaerobic wastewater treatment. *Chamical Engineering* **1997**.
73. Britannica *Wastewater treatment and disposal,* Available from https://www.britannica.com/technology/wastewater-treatment/Primary-treatment. (accessed 20 March 2022).
74. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 10 Anaerobic Suspended and Attached Growth Biological Treatment Processes. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 983-1035.
75. Kleerebezem, R.; Macarie, H., Treating industrial wastewater: anaerobic digestion comes of age: anaerobic treatment systems offer important advantages over conventionally applied aerobic processes for removing organic pollutants from water-based streams. . *Chemical Engineering* **2003,** *110*.
76. Al-Asheh, S.; Bagheri, M.; Aidan, A., Membrane bioreactor for wastewater treatment: A review. *Case Studies in Chemical and Environmental Engineering* **2021,** *4*.
77. Iorhemen, O. T.; Hamza, R. A.; Tay, J. H., Membrane Bioreactor (MBR) Technology for Wastewater Treatment and Reclamation: Membrane Fouling. *Membranes* **2016,** *6*.
78. Zhuang, Y.; Ren, H.; Geng, J.; Zhang, Y.; Zhang, Y.; Ding, L.; Xu, K., Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection. *Environ. Sci. Pollut. Res.* **2015,** *22*, 7037-7044.
79. Breazeal, M. V. R.; Novak, J. T.; VIkesland, P. J.; Pruden, A., Effect of wastewater colloids on membrane removal of antibiotic resistance genes. *Water Research* **2013,** *47*, 130-140.
80. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 8 Suspended Growth Biological Treatment Processes. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 659-886.
81. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 7 Fundamentals of Biological Treatment. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819) ed.; McGraw-Hill: 2003; pp 545-659.
82. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 8 Suspended Growth Biological Treatment Processes. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819) ed.; McGraw-Hill: 2003; pp 749-799.
83. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 11 Advanced Wastewater Treatment. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George

Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 1035-1217.
84. Tchobanoglous, G.; Burton, F. L.; Stensel, H. D.; Eddy, M., 6 Chemical Unit Processes. In *Wastewater engineering: treatment and reuse*, 4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel., pp.XXVIII, 1819 ed.; McGraw-Hill: 2003; pp 475-545.
85. Barber, O. W.; Hartmann, E. M., Benzalkonium chloride: A systematic review of its environmental entry through wastewater treatment, potential impact, and mitigation strategies. *Critical Reviews in Environmental Science and Technology* **2021**.
86. DeLeo, P. C.; Huynh, C.; Pattanayek, M.; Schmid, K. C.; Pechacek, N., Assesment of ecological hazards and environmental fate of disinfectant quaternary ammonium compounds. *Ecotoxicology and Environmental Safety* **2020,** *206*, 111-116.
87. Zhu, F.-J.; Ma, W.-L.; Xu, T.-F.; Ding, Y.; Zhao, X.; Li, W.-L.; Liu, L.-Y.; Song, W.-W.; Li, Y.-F.; Zhang, Z.-F., Removal characteristics of surfacants in typical industrial and domestic wastewater treatment plants in Northeast China. *Ecotoxicology and Environmental Safety* **2018,** *153*, 84-90.
88. Singer, H.; Müller, S.; Tixier, C.; Pillonel, L., Triclosan: Occurrence and Fate of a Widely Used Biocide in the Aquatic Environment: Field Measurements in Wastewater Treatment Plants, Surface Waters, and Lake Sediments. *Environ. Sci. Technol.* **2002,** *36*, 4998-5004.
89. McAvoy, D. C.; Schatowitz, B.; Jacob, M.; Hauk, A.; Eckhoff, W. S., Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology and Chemistry* **2002,** *21*, 1323-1329.
90. Komolafe, O.; Mrozik, W.; Dolfing, J.; Acharya, K.; L., V.; Mota, C. R.; Davenport, R., Occurence and removal of micropollutants in full-scale aerobic, anaerobic and facultative wastewater treatment plants in Brazil. *Journal of Environmental Management* **2021,** *287*, 112-286.
91. Lehutso, R. F.; Daso, A. P.; Okonkwo, J. O., Occurence and environmental levels of triclosan and triclocarban in selected wastewater treatment plants in Gauteng Province, South Africa. *Emerging Contaminants* **2017,** *3*, 107-114.
92. Östman, M.; Fick, J.; Tysklind, M., Detailed mass flows and removal efficiencies for biocides and antibiotics in Swedish sewage treatment plants. *Science of the Total Environment* **2018,** *640-641*, 327-336.
93. Karvelas, M.; Katsoyiannis, A.; Samara, C., Occurence and fate of heavy metals in the wastewater treatment process. *Chemosphere* **2003,** *53*, 1201-1210.
94. Busetti, F.; Badoer, S.; Cuomo, M.; Rubino, B.; Traverso, P., Occurence and Removal of Potentially Toxic Metals and Heavy Metals in the Wastewater Treatment Plant of Fusina (Venice, Italy). *Ind. Eng. Chem. Res.* **2005,** *44*, 9264-9272.
95. Du, J.; Geng, J.; Ren, H.; Ding, L.; Xu, K.; Zhang, Y., Variation of antibiotic resistance genes in municipal wastewater treatment plant with A 2O-MBR system. *Environ. Sci. Pollut. Res.* **2015,** *22*, 3715-3726.
96. Christgen, B.; Yang, Y.; Ahammad, S. Z.; Li, B.; Rodriquez, D. C.; Zhang, T.; Graham, D. W., Metagenomics Shows That Low-Energy Anaerobic-Aerobic Treatment Reactors Reduce Antibiotic Resistance Gene Levels from Domestic Wastewater. *Environ. Sci. Technol.* **2015,** *49*, 2577-2584.
97. Murray, G. E.; Tobin, R. S.; Junkins, B.; Kushner, D. J., Effect of chlorination on antibiotic resistance profiles of sewage related bacteria. *Appl Environ Microbiol* **1984,** *48*.

98. Huang, J.-J.; Hu, H.-Y.; Wu, Y.-H.; Wei, B.; Lu, Y., Effect of chlorination and ultraviolet disinfection on tetA-mediated tetracycline resistance of *Escherichia coli*. *Chemosphere* **2013,** *90*, 2247-2253.
99. Templeton, M. R.; Szczepanowski, R.; Braun, S., Chlorine and UV disinfection of ampicillin-resistant *Escherichia coli*. *Can J Civ Eng* **2009,** *36*, 889-894.
100. Vymazal, J., Constructed Wetlands for Wastewater Treatment. *Water* **2010,** *2*, 530-549.
101. Scholz, M.; Lee, B.-H., Constructed wetlands: a review. *International Journal of Environmental Studies* **2005,** *62*, 421-447.
102. Aburizaiza, O. S.; Mahar, G. A., Degree of wastewater treatment versus types of reuses: Case study, Saudi Arabia. *Global NEST Journal* **2016,** *18*.