EPFL

Thèse n° 8072

Presence of sub-inhibitory concentrations of quaternary ammonium compounds in water: effects on bacteria and their inactivation by ozone and monochloramine

Présentée le 5 novembre 2021

Faculté de l'environnement naturel, architectural et construit
Laboratoire pour le traitement et la qualité de l'eau
Programme doctoral en génie civil et environnement

pour l'obtention du grade de Docteur ès Sciences

par

Margaux Océane VOUMARD

Acceptée sur proposition du jury

Prof. S. Takahama, président du jury
Prof. U. von Gunten, Dr F. F. V. Breider, directeurs de thèse
Prof. M. Prévost, rapporteuse
Prof. M. Dodd, rapporteur
Prof. T. Kohn, rapporteuse

École polytechnique fédérale de Lausanne

2021

Acknowledgements

This work and thesis would not have happened without the support of many persons.

First, to Urs von Gunten and Florian Breider, my supervisors, you both supported me during these five challenging years. It has not been an easy road, but finally the result is here, in this manuscript. You supported me when everything was going well and when it was not. Thank you for giving me this opportunity and welcoming me in your team.

To my lab-mates Jaedon, Tianqi, Yishuai and to my former lab-mates, Sandro, Minju, Eva, Lucy, Juan, Andrea… Thanks for the lunch and coffee breaks, the afterwork beers or barbecues by the lake and all the nice discussions. Un merci spécial à Caroline et Karim, pour les pauses cafés hebdomadaires, les discussions et le support pendant toutes ces années de thèse. Et merci à Alina et Magda, qui ont aidé et contribué à réduire le stress administratif.

To my adoption lab, the LCE and especially Tamar Kohn, who adopted me when I was alone. To Odile, Margot, Marie, Aline, Camille, Simon, Qingxia, Virginie, Jason, Suzanne, Xavi. Thanks for letting me be a part of your group.

To the people who joined for a little while my project, to Leonardo, Myriam, Vincent and Gabrielle; your help was really valuable.

To my thesis external experts, Prof. Michèle Prévost and Prof. Mike Dodd, your feedback was valuable and very constructive. Thank you for joining me for this last part of my thesis.

To my fellows and former PhD friends, to Annie, Nick, Nikunj, Adrian, Arnaud, Gaelle, Niclas, thanks for the many afterwork beers, the coffee breaks, the discussions, the lunch breaks, the yoga sessions. Merci à Léo pour les repas, les pièces de théâtre partagées, le séjour chez toi à Montréal, les discussions, merci pour tout ! And a big thanks and hug to Annie, meeting you and knowing you made my PhD life better, the endless discussions, the lunches at EPFL or at the lake, the suppers, the hikes, the ski weekend, the Aar, and so more… You are a true friend!

To PolyDoc, the Photography Club and to the PhD representatives. Being a part of you helped me a lot when I was struggling with loneliness during my PhD; it helped me to stay motivated and to keep my well-being. To Myriam, Hoang, Nico, Curdin, Francesco, and so many more…

To my former lab, the GPAO, and more specifically Cesar and Stefanos. Thank you for welcoming me in your lab, for introducing me in the wonderful topic of water treatment and bacterial inactivation, for pushing me to start a thesis.

A l'aumônerie, et plus spécialement à Christian, Alex et Anouk. Vous avez permis de créer une petite bulle de bien-être et de bienveillance sur le campus. Un endroit où l'on peut se retrouver et se ressourcer. Merci pour les discussions et toute l'aide et le support que vous m'avez apportés. Merci aux prières de Taizé, à la Géode ou à Saint-Laurent, elles m'ont permis de survivre à toutes ces années sur le campus.

Au groupe Biol(for)ever, plus de 10 ans que l''on se connait et s'apprécie. A nos soupers, nos cafés, nos balades, nos discussions, nos Skypes et tout ce que l'on partage depuis des années. Merci pour tout, merci à vous. A Rosanne, Émilie, Muriel, Jitka, Romain, Aless, Heinz, Thaïs, et leurs familles !

A Jérémie, tu m'as accompagné et supporté cette dernière année de thèse, tu as découvert et accepté mon monde, merci pour tout ce que tu m'apportes et à nos moments à deux, bienvenus pendant cette fin de thèse.

Merci à ma famille pour leur soutien sans faille, à mes grands-parents, à Papi qui me voit finir cette thèse depuis le ciel.

Et finalement, les plus importants, merci à mes parents, Papa, Maman, vous m'avez soutenue dans mon choix d'études et depuis toujours, vous m'avez accueillie les week-ends, nourrie, permis de souffler, de me reposer, de me ressourcer. Sans vous, cette thèse n'aurait jamais eu lieu. Á Stéphanie et Thibaut, vous êtes les meilleurs frères et sœurs possibles, merci d'avoir été et d'être là pour moi.

Lausanne, le 5 juillet 2021

Abstract

Disinfection agents have been present in our daily lives for several decades in detergents or in personal care products, where their role is to prevent the spread of pathogenic microorganisms. One of the largest class of disinfection agents is quaternary ammonium compounds (QACs). Within this class, cetyltrimethylammonium chloride (CTMA) and benzalkonium chloride (BAC) are widely used. QACs have a broad-spectrum effect on bacteria, where they primarily target the membrane. After use, QACs are partially discharged to municipal wastewaters, where they are diluted to concentrations in the sub-inhibitory range. These low concentrations of QACs can trigger resistance mechanisms in bacteria, including membrane modifications and the expression of efflux pumps.

The aim of this thesis was to investigate the consequences and the impact of the presence of low concentrations of QACs in wastewater and surface water on the survival of bacteria. It could be demonstrated that strains of *P. aeruginosa* long-term exposed to constant sub-inhibitory concentrations of either BAC or CTMA showed decreased susceptibility to both QACs, regardless of which QAC the bacteria had been exposed to. However, no resistance to selected antibiotics was found after this exposure. Investigations of the membrane using atomic force microscopy showed an increase in surface roughness following exposure to both BAC and CTMA, demonstrating that QACs affect bacterial membranes.

QACs are poorly biodegradable and their presence, even at constant sub-inhibitory concentrations, affect bacteria. QACs are poorly reactive with chemical oxidants commonly used for water disinfection, which aims to inactivate microorganisms. Therefore, QACs remain present during water and wastewater treatment. Several methods of disinfection exist, where ozonation and monochloramination are widely applied worldwide. The effect of a pre-exposure of *E. coli* to CTMA and of the presence of CTMA during disinfection with ozone (O_3) and monochloramine (NH_2Cl) were investigated. The pre-exposure to CTMA led to contrasting results: inactivation of *E. coli* was enhanced when using ozone but decreased when using monochloramine. This result demonstrates the different modes of inactivation of bacteria for ozone and monochloramine. Moreover, the role of efflux pumps during inactivation was tested with two strains of *E. coli*, expressing different efflux pump systems. These two strains showed contrasting behaviors when treated with either ozone or monochloramine. Nevertheless, we were unable to resolve the role of efflux pumps in this system. The presence of CTMA enhanced the inactivation when concentrations were higher than 2 mgL^{-1} for ozone and 2.25 mgL^{-1} for monochloramine.

Standard methods to determine the minimum inhibitory concentration use ideal conditions for bacterial growth, which do not occur in natural water. Therefore, the susceptibility of *E. coli* to CTMA in PBS, lake water (Lake Geneva, Switzerland) and a wastewater effluent was investigated and compared to nutrient broth. It could be shown that *E. coli* was significantly more susceptible to CTMA in dilute aqueous solutions compared to broth. These results are relevant to assess the effect of CTMA and potentially other biocides on bacteria under realistic conditions, as it is probably overestimated using the current standard methods for MICs determination.

Keywords

Quaternary ammonium compounds; *Escherichia coli*; *Pseudomonas aeruginosa*; disinfection; ozone; monochloramine; drinking water; wastewater; kinetics; resistance

Résumé

Les substances désinfectantes font partie de notre quotidien sous la forme de détergents ou de produits d'hygiène personnelle, dans le but de prévenir la transmission d'agents pathogènes. Les ammonium quaternaires (QACs) forment une classe largement représentée. Le benzalkonium chloride (BAC) et le cetyltrimethylammonium chloride (CTMA) font partie des QACs les plus utilisés. Les QACs ont un mode d'action à large spectre sur les bactéries et ciblent la membrane. Après utilisation, les QACs sont en partie éliminés par les eaux usées, où ils sont dilués à des concentration sous-inhibitrices. Cependant, ces faibles concentrations peuvent promouvoir l'expression de mécanismes de résistance comme par exemple, dans le cas des QACs, une modification de la membrane ou l'expression de pompes à efflux.

Le but de cette thèse était d'étudier les conséquences de la présence de QACs à de faibles concentrations dans les eaux usées et les eaux de surface sur la survie des bactéries. Il a été démontré qu'une exposition de souches de *P. aeruginosa* avec des concentrations constantes sous-inhibitrices de CTMA et BAC ont promu une résistance à ces mêmes substances, indépendamment du QAC auquel elles ont été exposées. Cependant, aucun développement de résistance aux antibiotiques n'a été observé. Une analyse par microscopie à force atomique a permis de montrer une augmentation de la rugosité de la membrane suivant l'exposition, ce qui a montré que les QACs affectent la membrane

Les QACs ne sont que faiblement biodégradable et leur présence, même à faible concentrations, impacte les bactéries. Les QACs sont peu réactifs avec les oxydants chimiques utilisés lors de la désinfection de l'eau. Différentes méthodes existent, parmi elles, l'ozone et la monochloramine sont largement répandues. L'effet d'une préexposition et la présence de CTMA pendant la désinfection à l'ozone ainsi qu'à la monochloramine ont été étudiées pour la bactérie *E. coli*. Il a été montré qu'une préexposition au CTMA a conduit à une amélioration de la désinfection par l'ozone mais une diminution pour la monochloramine. Ce résultat démontre des modes d'action différents entre l'ozone et la monochloramine. De plus, l'activation des pompes à efflux a été testée avec l'aide de deux souches d'*E. coli* expriment deux systèmes différents de pompes à efflux. Ces deux souches ont montré des résultats opposés entre elles, que ce soit avec l'ozone

ou la monochloramine. Cela n'a pas permis d'élucider le rôle des pompes à efflux lors de la désinfection. La présence de CTMA a permis d'améliorer l'inactivation pour des concentrations de CTMA supérieures à 2 mgL^{-1} pour l'ozone et 2.25 mgL^{-1} pour la monochloramine.

Finalement, la susceptibilité d'*E. coli* dans différentes matrices a été étudiée. Les méthodes standards d'évaluation de la concentration minimale inhibitrice impliquent des conditions idéales pour la croissance bactérienne, qui n'ont pas lieu dans les eaux naturelles. La susceptibilité d'*E. coli* au CTMA a été évaluée et comparée dans une solution tamponnée, de l'eau de lac et une eau de sortie de STEP à un bouillon de culture. Il a été démontré que la susceptibilité au CTMA d'*E. coli* était supérieure dans les trois solutions aqueuses en comparaison au bouillon de culture. Ces résultats permettent de donner une indication sur les effets du CTMA, et potentiellement des autres biocides sur les bactéries en milieux naturels, les méthodes d'évaluation de la susceptibilité utilisant la MIC surestimant probablement celle-ci.

Mots-clés

Ammonium quaternaire ; désinfection ; *Escherichia coli*; *Pseudomonas aeruginosa* ; ozone ; monochloramine ; cinétique ; eau potable; eau usée ; résistance.

Table of contents

List of Figures

List of Figures – Supporting Information

List of Tables

List of Tables – Supporting Information

List of Equations

List of abbreviations

ABC – ATP-binding cassette

AFM – atomic force microscopy

ARB – antibiotic resistance bacteria

ARG – antibiotic resistance gene

BAC – benzalkonium chloride

CFU – colony-forming unit

CTMA – cetyltrimethylammonium chloride

DOM – dissolved organic matter

DBP – disinfection by-product

EUCAST – European Committee for Antibiotic Susceptibility Testing

FAC – free available chlorine

HAA – haloacetic acid

IZD – inhibition zone diameter

LB – Luria-Bertani broth

MATE – multidrug and toxic extrusion family

MFS – major facilitator superfamily

MHB – Mueller-Hinton broth

MIC – minimum inhibitory concentration

NDMA - *N*-nitrosodimethylamine

PBS – phosphate buffered saline

PCA – plate count agar

PCP – personal care product

QAC – quaternary ammonium compound

RND – resistance nodulation and cell division family

SMR – small multidrug resistance family

TC – total chlorine

THM – trihalomethane

UV – ultraviolet

VBNC – viable but not culturable

WHO – world health organization

WWTP – wastewater treatment plant

1 Introduction

1.1 Historical context and general background of water and wastewater treatment

1.1.1 Water supply and treatment – The origins

Water is essential for life, and even more, life evolved around water. A human being can only survive 3 days without drinking. This limitation of the human body forced the location of early villages near water sources. With growing populations and the beginning of large civilization, villages became cities, and the proximity of water sources was no longer an available option. To cope with this, water supplies had to be engineered. Examples of early water supplies systems are found in the antiquity: in Greece, Persia or the Roman Empire [1,2]. The ancient Rome is considered as the first metropolis, and the transition from a village to a metropolis lead to changes in the architecture of the city and the way to bring water to the population [1]. Roman engineers conceived aqueducts, networks of pipes, mostly underground to bring water from up to 50 km away to the numerous fountains of the city, to the public baths and to some private houses [1]. They created a developed system with separation of the water from the reservoir with different pipe systems, to allow the water to go in priority to the public fountains, that were the main water source for the population, in case of scarcity [1]. Interestingly, having fountains as an emergency system for water supply still exists today. The city of Zürich (Switzerland) has 400 fountains out of 1,200 fountains (Figure 1-1) fed by spring water which is independent from the tap water system. This system ensures the access to clean drinking water in case of problems with the main drinking water system [3].

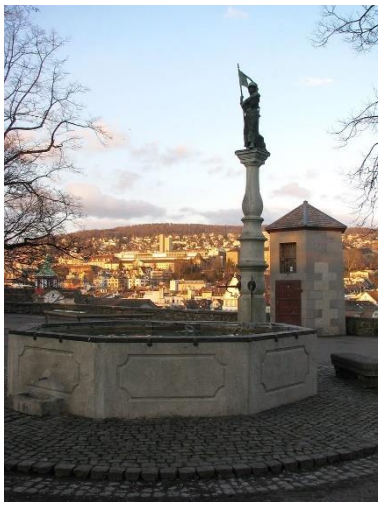


Figure 1-1: ©Roland zh. *Hedwig fountain* in the city of Zürich.

If the problem of supplying water to the city was solved with the aqueducts and underground plumbing systems, they also needed systems to discharge the used water without flooding the city. For this, they used and improved the Cloaca Maxima in Rome, also considered as the first sewage system in history, that was present in the city. The creation of this sewage system, also led to the creation of the first public toilets, with holes in the upper protection of the cloacae [1,4]. This was done without any knowledge of infectious diseases and its level was close to what can be found today. The use of an underground system prevented contamination of the water sources. Unfortunately, the end of the Roman Empire, led to a dark era for water supply and treatment. The Middle-Age period, was a period of poor sanitation, water was mostly abstracted from wells, surface water and rivers and people were avoiding to drink it, preferring other beverages such as beer [1]. Moreover, with increasing population, the level of fecal contamination of waters increased and in the fourteenth century a new pandemic arrived, the bubonic plague. The cause of bubonic plaque was hypothesized as the contamination of water by rotting wastes, this led to some effort to remove human wastes before they could contaminate water [1]. Several cities achieved quite efficient systems to remove wastes. With the beginning of the modern era and the emergence of bigger and urbanized cities, centralized water supplies were implemented to fulfil the water needs of the growing population. Unfortunately, poor sanitation conditions led to contamination of the water supply and the emergence of waterborne

diseases [5]. In 1831, a cholera epidemic spread in the city of London. It appears that the cause of the disease was the water of a well contaminated by feces of sick people. John Snow, at the origin of this discovery, was able to propose measures that prevented the spread of the disease [1,5]. Later, in the 1880s, Robert Koch was able to isolate the cause of the disease, the *Vibrio cholerae* bacteria. The discoveries of John Snow and Robert Koch are the very beginning of the epidemiology and drinking water microbiology. The outbreak of typhoid fever, another water-borne disease, in the USA led to the first research and implementation of water treatment. The first treatment applied were slow sand filters that were able to remove a considerable part of the pathogens [1]. However, in case of strongly contaminated water, filtration was not enough, and diseases continued to spread. To solve this issue, new treatments were looked for, treatments that would inactivate pathogens instead of removing them.

Even though, disinfection methods have been applied far before the modern era, by boiling water or adding silver or copper by antique civilizations, these treatments were not applicable at large scale. The first large-scale disinfectant for water applied was chlorine. It was first introduced as a continuous treatment in the early 1900s in a Belgian city [1,5]. The use of chlorine spread in Europe and in the USA. The presence of particles in water was recognized as a negative influence on the efficiency of chlorination by potentially protecting bacteria from the disinfectant. Therefore, water filtration was added prior to chlorination to enhance the efficiency of the treatment.

In the 1970s, it was discovered that trihalomethanes (THMs), are produced during the chlorination of waters when dissolved organic matter is present [6,7]. THMs were suspected and then identified as potentially carcinogenic as health threats and led to changes in water treatment processes [7,8] Other disinfectants were applied to try to solve the disinfection by-products (DBPs) issues, such as chloramine or chlorine dioxide [8]. Even though, this decreased the level of THMs, other DBPs are generated such as *N*-nitrosodimethylamine (NDMA) or chlorite/chlorate which are also potentially carcinogenic [8]. In Europe, ozone, another disinfectant has been applied since the beginning of the 20th century [1,9]. However, it was discovered that this disinfectant also has drawbacks, one of its DBPs is bromate, a possibly carcinogenic compound [1,9]. Therefore, no disinfectant is a silver bullet and the choice of the disinfectant must depend on other parameters, such as the raw water quality, costs, existing facilities, etc. Today, all three

chemical disinfection methods are commonly used but others are also applied alone or in combination such as UV or membrane filtration [10].

1.1.2 Wastewater treatment

In the antiquity, wastewaters were collected and discharged in rivers or other surface water. Knowledge existed that wastewater was not drinkable, mostly due to odor and color, but not more. Collecting and discharging wastewater in rivers or in canals was common until the middle of the 19th century [11]. This led to severe contaminations of rivers, such as the Thames River in London (England) or the Spree river in Berlin (Germany) (Figure 1-2) [11]. Pollution came from the growing population but also from industries. The high level of pollution led to the creation of institutions monitoring and assessing the quality of such rivers and canals [11]. Some scientists believed that "self-purification" of the water was possible during its transport to rivers and could reduce the level of pollution [11]. "Self-purification" consisted in the oxidation of pollutants by vigorously mixing the water, thus incorporating some oxygen from the air [1,11]. However, some laboratory experiments revealed that only a very small fraction of the pollutants were removed by this approach [11]. Other experiments, supervised by Robert Koch, revealed that microbiological processes produce CO_2 and NO_3^- by oxidizing organic compounds and NH_4^+ [11,12].

Figure 1-2. The 'Silent Highway' - Man.
'Your money or your life'. Cartoon published in Punch magazine, 10 July 1858, No. 35. The high level of pollution of the Thames River associated with a hot summer caused bad smell. In this cartoon, death is associated with pollution and disease. (Source: Museum of London)

With the high level of pollution of canals and rivers, the discharge of wastewater was becoming a serious problem and other solutions were looked for. In Germany and in Poland, at the end the 19th century, irrigation fields were used for wastewater treatment, and this system allowed to remove around 65% of dissolved organics, with a nearly complete nitrification [11]. Irrigation fields were used to avoid the discharge of wastewater in rivers and canals and acted as a biological treatment [13]. This biological treatment was then successfully applied in other countries with the creation of contact-beds [11]. Approximately, during the same period, cholera epidemics started in Europe, with water as the source of the epidemics. Treating wastewater and avoiding contamination of fresh water sources was efficient in Germany, two treatments were found to be beneficial: sand filtration followed by discharge in river water (Hamburg, Germany) and groundwater abstraction as riverbank filtrate and wastewater treatment by irrigating fields (Berlin. Germany) [11]. Later, in the early 20th century, the modern activated sludge process was slowly introduced [11,12]. A German engineer, Karl Imhoff developed a solution to separate the solids from the water and promote the growth of anaerobic microorganisms that would consume solids rich in organic matter releasing methane [14]. The Imhoff tank and settling basins became what is known the primary treatment today [1].

From the 1960s, a new problem arose, the eutrophication of water bodies due to the large discharge of nutrients to receiving water bodies. Phosphate was mainly responsible for this problem [15] and advanced wastewater treatment methods were developed. Phosphate removal became mandatory in 1976 in Switzerland and this was mainly achieved by precipitation with iron(III). Prevention of eutrophication was also achieved by the introduction of a anoxic zone in an activated sludge process [12]. In the 1960s, membranes for wastewater treatment were developed for biomass separation at laboratory- and full-scale aerobic membrane reactors were applied for the first time in the 1970s in the US and they became standard in some water treatment in the 1990s [12].

The disinfection of wastewater started at the end of the 19th century after the link between pathogenic microorganisms and diseases was discovered. Chlorine was first applied in Hamburg (Germany) in 1893 and ozone in France in 1906 [12]. Chlorine gas used in wastewater treatment plants was installed in the US at the beginning of the 20th century [12]. Ultraviolet light was used in France in the 1910s [12]. Nowadays, the US mostly relies on chlorine and chloramine for disinfection, as Europe uses other methods, such as ozone, UV light, membranes.

Moreover, the source of drinking water is also important. Indeed, wastewater disinfection is crucial when river water or the receiving water body is taken as the drinking water source directly, whereas, when riverbank filtration is used, because of an efficient pathogen removal, no disinfection is needed.

Towards the end of the 20th century, a new kind of pollution in municipal wastewaters became a concern: micropollutants. The increased use of chemicals, increasing population and a continuous and increasing urbanization increased the micropollutant problems in wastewaters [15]. Switzerland acted as a pioneer on the micropollutant issue with the introduction of the Water Protection Act in March 2014 [15]. This law has the purpose to reduce the load of micropollutants and the toxicity of wastewater to receiving water bodies and targets densely populated urban zones by enhancing and optimizing existing wastewater treatment plants (WWTPs) for micropollutant abatement [15]. The global plan for the improvement of WWTPs has several advantages, by being supported by experts in the field, having received a political and societal acceptance, being manageable, adaptable in time, financially possible but most importantly technically feasible [15]. Optimizing and improving the WWTPs will rely on mainly two technologies: ozonation followed by sand filtration and activated carbon (powdered and granular activated carbon, PAC and GAC respectively) [15,16]. Trials in Switzerland and Germany allowed to abate the overall load of micropollutants in WWTPs by 80% [15].

1.2 Disinfection methods in water treatment

The disinfection step in water treatment has the purpose to inactivate pathogenic microorganisms. Sterilizing water, in the frame of water treatment is impossible. Therefore, disinfection aims at reducing microbial pathogens to levels defined by the authorities, should not increase the toxicity of the water, be reliable and cost-effective and the handling of disinfection chemicals should be safe [17]. Water disinfection is inactivating bacteria to levels that are below the problematic range but can also promote the viable but not culturable (VBNC) state in bacteria [18–20]. The VBNC state is important, because bacteria are still viable but not detected when methods relying on culturability are used to detect pathogens. It was recently found that VBNC bacteria are present after disinfection, especially after chlorine and chloramine treatment, at significant levels, but conditions for resuscitation from this state remain unclear [18,20].

Disinfectants are chosen according to several parameters. First, their efficiency against waterborne pathogens is crucial. Other important considerations are the monitoring and control of the disinfectant added, the residual, the potential of the disinfectant to affect the aesthetic qualities of the water, the formation and control of disinfection by-products. Finally the feasibility of the disinfectant for large-scale applications and economic considerations are also important [21].

The efficacy of a disinfectant to inactivate waterborne pathogens depends on multiple parameters such as the nature of the disinfectant and the microorganism but also on the water quality. The water matrix itself can consume disinfectants by e.g. reactions with some constituents of dissolved organic matter (DOM) [8]. Treatments prior to disinfection and removal of potentially highly disinfectant-consuming components are important to achieve the required disinfection level. The disinfectant should also be distributed uniformly in the water to ensure an optimal disinfection [21].

Moreover, the concentration and the contact time, which defines the exposure (or CT) and physico-chemical parameters such as the temperature and the pH are essential for the inactivation of microorganisms [21,22]. The temperature affects the kinetics according to the Arrhenius law [23] and the pH will influence it by affecting the speciation of some chemical disinfectants, such as chlorine [23]. Furthermore, reactor hydraulics is an essential parameter for disinfection [24]. A summary of some of the main disinfection methods used for municipal wastewater treatment or for drinking water is provided in Table 1-1.

Table 1-1: List of chemical and physical disinfection methods commonly used in water treatment and their main properties, applications and advantages/disadvantages

Disinfection method	Formula	Application	Mode of action	Advantages	Disadvantages	Reference
Chlorine	Cl_2, mostly present as $HOCl/OCl^-$	Drinking water Wastewater	Free chlorine ($HOCl/OCl^-$) is the most reactive form. Reaction with different components of the cell.	Cheap Residual for protection of the distribution system	Formation of potentially toxic DBPs Storage on-site can be hazardous Regrowth of microorganisms if no residual	[25–27]
Chloramine	NH_2Cl	Drinking water Wastewater	Formed when ammonia is present in water Similar to chlorine	More stable compared to chlorine Avoid some DBPs from chlorine Efficient against microorganisms	Formation of toxic by-products Ca. 100x less efficient than chlorine Regrowth of microorganisms in absence of residual	[28,29]
Ozone	O_3	Drinking water Wastewater	Targets the DNA and the membrane of microorganisms causing lethal damages Formation of hydroxyl radicals	Strong oxidant Highly efficient No residual disinfectant Production on-site Less DBPs formation then chlorine	More costly than chlorine Bromate formation No residual disinfectant Potential regrowth of microorganisms	[9,30–33]
UV-radiation 254 nm	NA	Drinking water Wastewater	Direct action through attacks on the DNA and RNA of the microorganism	No known toxic by-products	Low turbidity of the water required No residual disinfectant Potential regrowth of microorganisms	[34–36]
Membranes	NA	Drinking water Wastewater	Retention of particles and microorganisms bigger than the pore size Removal of ions possible by reverse osmosis	Wide range of substances removed Works for water with different qualities	Fouling of the membrane No residual disinfectant Potential regrowth of microorganisms	[37,38]
SODIS	NA	Drinking water	Synergy between a rise of the temperature of the water and the solar UV radiation	Good for areas where water treatment is not available Efficient to inactivate bacteria and viruses No or little maintenance required	Potential regrowth of microorganisms Enough solar radiation time needed Small volumes of water Low turbidity of water required	[39–42]

At the beginning of the 20th century, it has been discovered that inactivation of microorganisms by disinfectants follows second-order kinetics [22]. Two main hypotheses were developed to explain the inactivation of bacteria. Firstly, the *mechanistic hypothesis* based on the assumption that all bacteria in a population have the same resistance to the applied disinfectant resulting in first-order kinetics if the concentration of the disinfectant is kept constant [22,43]. Secondly, the *vitalist hypothesis* is based on the assumption that inside a population, individuals are unique, leading to different degrees of resistance to the disinfectant in the same population [22,43]. The *vitalist hypothesis* has the advantage to consider the tailing of the log-survival curves, whereas the mechanistic hypothesis does not include this option. Repair processes, visible by a shoulder in the inactivation curve are also often not considered by the *mechanistic hypothesis*. However, both hypotheses are still under debate and none has been entirely falsified [43]. The inactivation kinetics are usually fitted to mathematical models such as the Chick-Watson model (Table 2), based on the mechanistic hypothesis and the Hom model (Table 1-2), an empirical model that can be fitted to non-linear log-survival data [43]. Many other models exist including combinations of these models to take into account the tailing and repair processes influencing the inactivation kinetics [9,43,44]. However, such more advanced models are often complicated and too difficult to use, and the Chick-Watson and Hom models are preferentially applied [43].

Most models include the CT values, which is the disinfectant concentration multiplied by the contact time. This parameter is often used to evaluate the efficiency of a chemical disinfectant and tables are available from regulatory agencies or from the World Health Organization (WHO) [45]. However, those models are only valid when a constant concentration of disinfectant is used. For other cases, the disinfectant exposure can be used. This parameter allows taking into account differences in water treatment conditions that can affect the stability of disinfectants. This parameter was introduced by von Gunten & Hoigné [46] for experiments with ozone and is represented by the integral under the disinfectant (in this case, ozone) curve [46]. Therefore, the Chick-Watson equation can be modified to a new form where the term $C^n t$ is replaced by the integral of the disinfectant curve ($\int [O_3] dt$) (Table 1-2).

Table 1-2: Chick-Watson and Hom equation to model the inactivation of microorganisms by disinfectants.

Equation		Parameters
1-1 Chick-Watson equation	$ln\left(\frac{N}{N_0}\right) = -kC^n t$	N_0 = Initial number of organisms N = number of survivors k = inactivation rate constant C = disinfectant concentrations n = coefficient of dilution, frequently assumed to be 1 t = contact time
1-2 Modified Chick-Watson model using the disinfectant exposure	$ln\left(\frac{N}{N_0}\right) = -k\int [C]dt$	N_0 = Initial number of organisms N = number of survivors k = second-order inactivation rate constant [C] = disinfectant concentrations
1-3 Hom equation	$log\left(\frac{N}{N_0}\right) = -kC^m t^h$	N_0 = Initial number of organisms N = number of survivors k = inactivation rate constant C = disinfectant concentrations m = Hom dilution constant t = contact time h = Hom time exponent

1.2.1 Chlorine and Chloramine

1.2.1.1 Chlorine chemistry in water

Chlorine was the first disinfectant for drinking water, which was applied in large scale and is still commonly used to date [1,24]. Chlorine can be used either in pre-treatment processes, for primary disinfection or as a post-treatment to prevent the re-growth of microorganisms with a residual concentration in the distribution system [47]. Chlorine can be added to water either in gaseous (Cl_2) or in liquid form (NaOCl, sodium hypochlorite solution) or as a solid (CaCl(OCl)). Once in the water, the following reactions take place to form hypochlorous acid (HOCl) and hypochlorite (OCl^-):

$$Cl_2\ (g) + H_2O \rightarrow HOCl + H^+ + Cl^- \quad (1)$$

$$NaOCl\ (l) \rightarrow OCl^- + H^+ \quad (2)$$

$$CaCl(ClO)\ (s) \rightarrow OCl^- + Cl^- + Ca^{2+} \quad (3)$$

$$HOCl \leftrightarrow OCl^- + H^+ \qquad pKa = 7.5 \text{ at } 25°C \quad (4)$$

Hypochlorous acid (HOCl) is a weak acid and its dissociation is pH- and temperature-dependent [10]. At pH 7.5 and 25°C, the HOCl and OCl^- concentrations are equivalent [10,47,48]. The sum of HOCl and OCl^- is also known as free available chlorine (FAC) (Figure 1-3).

The pH of the water is an important parameter of chlorine chemistry. Depending on the pH, one or the other species will dominate and can lead to different reaction kinetics, as the second-order rate constants for the reactions of the two species differ significantly. Mostly, HOCl has a higher reactivity than OCl^- (13). HOCl and OCl^- are the main species present, but chlorine monoxide may also be formed by dehydration of HOCl with an equilibrium constant $K = 8.74\ 10^{-3}\ M^{-1}$ at 25°C [49]. Under typical drinking water treatment conditions, chlorine monoxide concentrations are very small compared to HOCl. Therefore, its contribution for disinfection can be neglected [49].

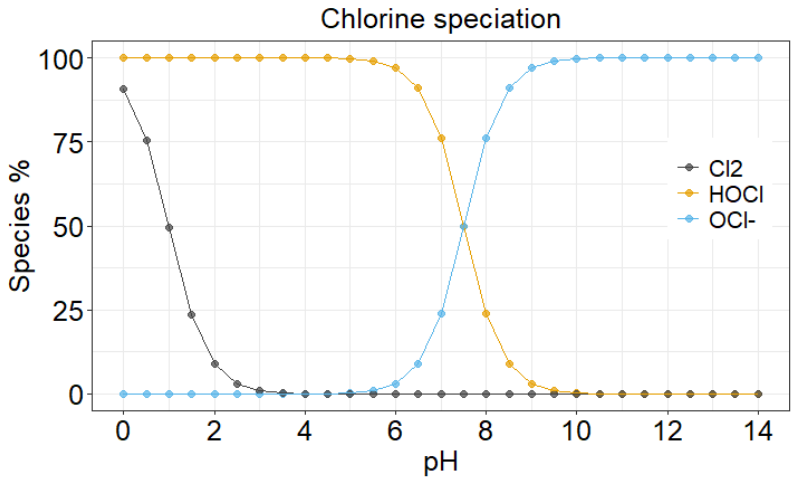


Figure 1-3. Speciation of chlorine as a function of the pH.
Relative distribution of the main chlorine species at 25°C. Chloride concentration was set at $5\ 10^{-3}$ M (a typical concentration of Cl^- in fresh water) and chlorine monoxide was ignored due to its small concentrations relative to the other chlorine species.

Chlorine is relatively stable in clean water but reacts with DOM present in real water systems. The reaction of chlorine DOM leads to the formation of (potentially) toxic DBPs such as THMs and haloacetic acids (HAAs) [47]. These toxic DBPs are regulated in the EU (THMs) and in the USA (THMs and HAAs) [47] and have to be monitored to comply with drinking water regulations. However, they only represent a small portion of the DBPs. To date, more than 700 DBPs have been identified [50]. Finally, if the water contains bromide (Br^-), bromine can be formed by the oxidation of Br^- with chlorine and as a consequence brominated by-products can be formed which are typically more toxic than the chlorinated analogous [32,47].

1.2.1.2 Chloramine chemistry in water

When ammonia is present in the water, chlorine reacts with it to chloramines. Chlorine will react with ammonia to form three species: monochloramine (NH_2Cl), dichloramine ($NHCl_2$) and trichloramine (NCl_3) according to the following reactions [10,32]:

$$H^+ + NH_3 \rightarrow NH_4^+ \quad (5)$$

$$HOCl + NH_3 \rightarrow NH_2Cl + H_2O \quad (6)$$

$$HOCl + NH_2Cl \rightarrow NHCl_2 + H_2O \quad (7)$$

$$HOCl + NHCl_2 \rightarrow NCl_3 + H_2O \quad (8)$$

The sum of the three chloramine species is also known as combined chlorine. The fraction of each species in the water is pH-dependent but also depends on the molar ratio of Cl_2/NH_4^+ [10]. In presence of ammonia, monochloramine is first formed with the addition of chlorine. Dichloramine formation follows; however, dichloramine is not stable and quickly decomposes to nitrogen gas. This decomposition occurs at a molar ratio of $HOCl:NH_4^+$ = 3:2 and leads to a complete depletion of the residual oxidant. This point is called the breakpoint. After this point, the free chlorine residual increases proportionally to the added chlorine (Figure 1-4).

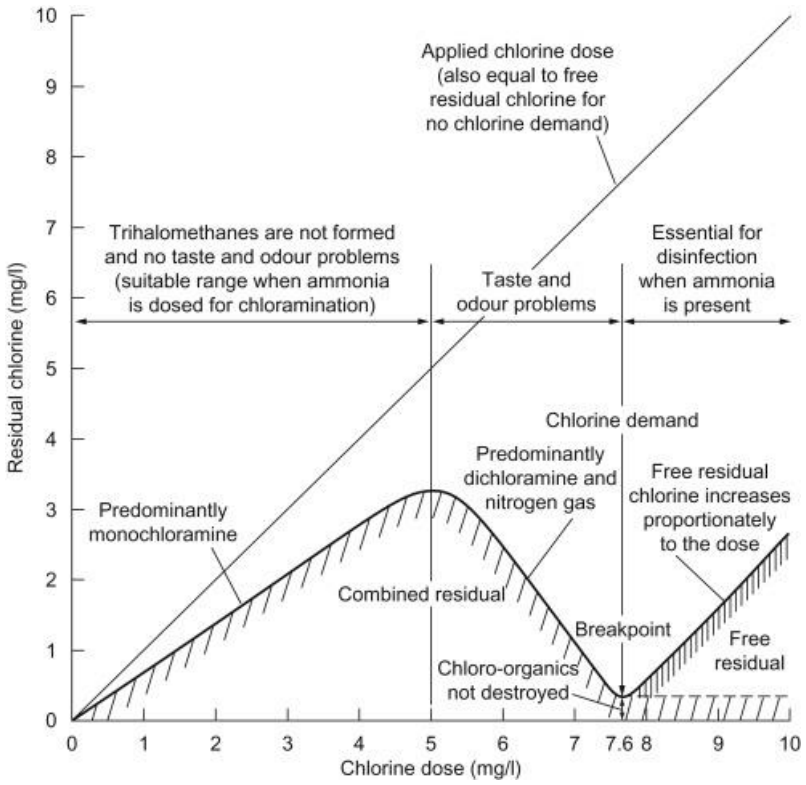


Figure 1-4. Breakpoint chlorination.

The curve corresponds to a concentration of ammonium of 1.0 mg/L (as N). (From Malcolm J. Brandt, K. Michael Johnson, Andrew J. Elphinston, Don D. Ratnayaka, Chapter 11 - Disinfection of Water, Editor(s): Malcolm J. Brandt, K. Michael Johnson, Andrew J. Elphinston, Don D. Ratnayaka, Twort's Water Supply (Seventh Edition), Butterworth-Heinemann, 2017, Pages 475-511, License 5041340481650)

Breakpoint chlorination has been used in the past for removal of ammonium from water. Today, it is usually not desirable because of the high required chlorine doses [24]. The breakpoint also highlights the importance of monitoring the water quality before and during treatment to avoid a depletion of disinfectant. If the formation of chloramine is not desired, ammonium has to be removed prior to chlorine addition. This can be achieved by a nitrification process prior to chlorination. Usually, a chlorine residual is desired to avoid a regrowth of microorganisms in the water distribution systems.

1.2.1.3 Inactivation of bacteria

As mentioned before, in aqueous environments, free chlorine exists mainly in two forms as hypochlorous acid (HOCl) and hypochlorite (ClO^-), with a pH- and temperature-dependent speciation [47,51]. HOCl is known to be approximately 80-fold more germicidal compared to OCl^- [52].

The mode of action of HOCl is summarized in Figure 1-5. HOCl is neutral, and, therefore, it can easily penetrate the bacterial cell membrane by passive diffusion (Figure 1-5(i)). HOCl will first rapidly react with nucleophilic structures, such as purine and pyrimidine bases, iron-sulfur proteins, heme and porphyrins, sulfhydryl groups [52]. HOCl also reacts with membrane components and proteins, including some involved in transport and energy production, leading to ATP hydrolysis (Figure 1-5(ii)) [52]. The action of HOCl on the membrane leads to a loss of membrane stability and potential leakage of some intracellular components to the outside of the cell (Figure 1-5 (iii)) [52]. The action of HOCl on proteins and amino acids leads to fragmentation and protein unfolding and ultimately aggregation of essential cellular components that can cause cellular death [23,47,52].

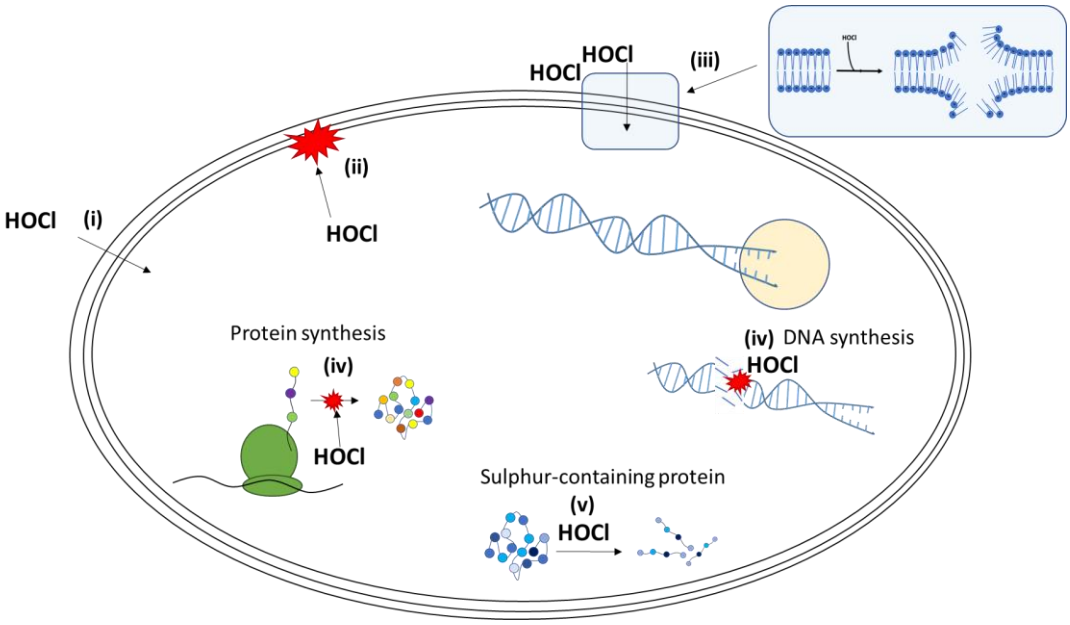


Figure 1-5. HOCl action on Gram-negative bacteria. (adapted from Nizer *et al.* [52])
(i) HOCl penetrates the bacterial cell by passive diffusion due to its neutral nature. (ii) HOCl attacks several membrane components, including transport proteins and ATP-ase, disrupting the ATP production. (iii) HOCl attacks the lipids of the membrane, leading to membrane permeation. (iv) HOCl attacks proteins involved in protein synthesis. (v) HOCl attacks proteins, especially sulfur-containing ones by

cleaving peptide bonds and affecting the folding of proteins. (vi) HOCl attacks DNA and its synthesis by affecting proteins involved in translation and transcription.

HOCl leads to loss of cultivability, substrate responsiveness, membrane potential, respiratory activity and membrane integrity [53]. Several studies confirmed the effects of HOCl, by showing differences in the Zeta potential of the membrane, increased permeability and decrease of the oxygen uptake [23,54].

The damages induced by chlorine are often irreversible, especially at high doses. Nevertheless, the susceptibility to chlorine remains microorganism-dependent and an adaptive response to the chlorine stress is possible. Spore-forming bacteria are quite resistant to chlorine as well as some Gram-negative bacteria due to their membrane composition [23]. Another parameter that can affect the susceptibility to chlorine is the presence of resistance genes. While being a major concern for human health, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG), as well as multiresistant bacteria, can influence the resistance patterns. Chlorine is reducing the overall number of bacteria but is less efficient against ARB, leading to an increased proportion of ARBs after treatment [55–59]. Regrowth of bacteria after treatment is also a major concern, with reactivation of bacteria, including ARBs, after treatment [59–62]. Reactivation is especially problematic when low doses of chlorine are applied, or when the exposure of bacteria and microorganisms is not sufficient for a total inactivation, and repair mechanisms can take place [62]. Moreover, chlorination is known to promote the VBNC state and recovery from this state is possible under the right conditions [18–20] and recovery from biofilms or from aggregation with flocs is also possible and would explain the reactivation. ARGs are particularly resistant to chlorine and it has been suggested that chlorination was not damaging plasmids, allowing transfer processes [58,59]. A recent study even demonstrated that chlorine-injured bacteria were more prone for receiving plasmids compared to non-injured ones [63]. Therefore, chlorine may promote horizontal gene transfer, and thus transmission of antibiotic resistance genes from resistant to non-resistant bacteria.

Additionally, when the exposure to chlorine is not enough, an adaptive response in Gram-negative bacteria has been observed with the expression of several enzymes, including catalases, peroxidase, superoxide dismutase [52]. Chaperones are also activated to repair unfolded and

damaged proteins and DNA and other protein repair systems are also activated [52]. Membrane changes were also observed with decreased permeability, decreased number of porins and increased hydrophobicity [52].

Monochloramine is orders of magnitude less reactive compared to free chlorine [33], with higher CT required compared to FAC for similar inactivation levels of microorganisms [23,53,64,65]. The mode of action of monochloramine is less known and few studies are available on this topic. Monochloramine is not affecting the cell membrane to levels that are leading to lysis but monochloramine seems to affect glucose and methionine transport systems and the bacterial respiration (Figure 1-6) [66]. Monochloramine has been hypothesized to act on the cell by a "multi-hit" model, where the inactivation is the result of several reactions in the bacterial cell [66]. As the mode of action of monochloramine does not rely on the cell lysis, monochloramine may accumulate in the cell, where it can slowly react with several components such as amino acids, peptides, phenolic compounds (Figure 1-6) [33].

When ARB and ARG are present in water, it is hypothesized that similar problems as with chlorine will occur, reduction of the total number of bacteria but increase in the proportion of resistant ones after treatment. Sampling before and after full-scale chloramination revealed higher proportions of resistant bacteria after treatment [67].

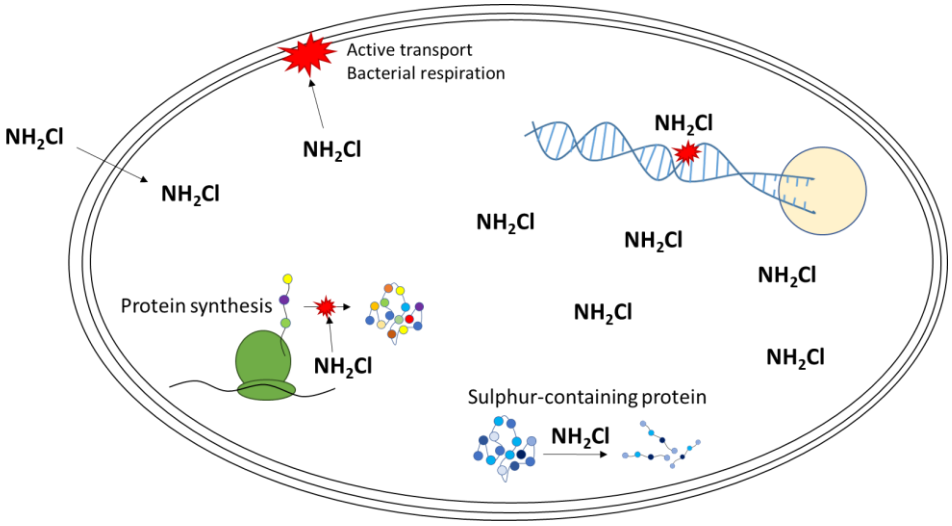


Figure 1-6. NH_2Cl action on Gram-negative bacteria

NH_2Cl affects some active transport systems and the bacterial respiration. It also accumulates in the cell, where it will slowly react with nucleic acids, amino acids, protein synthesis and sulphur-containing proteins.

1.2.2 Ozone

1.2.2.1 Ozone chemistry in water

Ozone is among the strongest chemical disinfectants used in water treatment. It is added as a gas to the water, where it dissolves. Aqueous ozone will then decay leading to the formation of hydroxyl radicals (·OH) [9,10,68] and disinfection processes and micropollutant abatement are the result of direct oxidation by ozone and/or by the secondary oxidant •OH [9].

Ozone is unstable in water with many factors affecting its stability such as temperature, pH, carbonate alkalinity and presence of DOM [9]. Decreasing the pH increases the ozone stability, and low temperature also increases its stability [9]. In drinking water and wastewater, the main parameter affecting ozone stability is the concentration and nature of DOM. DOM affects ozone stability by directly reacting with it but also by scavenging ·OH, whereby a radical chain reaction occurs [69]. Ozone reacts with some electron-rich aromatic DOM moieties, such as activated aromatic groups, by electron transfer reactions, which leads to the formation of ·OH [9]. Hydroxyl radicals then react with C-H bonds in the DOM by H-abstraction leading to carbon-centered radicals, which upon reaction with O_2 may lead to the formation of superoxide radicals. This, in turn reacts very fast with ozone by electron transfer, leading to another ·OH, as a carrier of the chain reaction [9]. Ozonation reduces the formation of several DPBs during post-chlorination, such as THMs and HAAs. However, other DBPs can be formed such as chloropicrin (during post-chlorination) [70,71] and bromate in bromide-containing waters [46].

1.2.2.2 Inactivation of bacteria

Ozone is among the most effective treatments for the inactivation of bacteria [9,23,64]. Ozone itself is the main oxidant for the inactivation of bacteria, as some studies revealed that similar inactivation rates were obtained in presence and in absence of •OH scavengers [72,73]. Nucleic acids, amino acids, proteins and electron-rich functional groups of proteins are particularly reactive with ozone, in contrast to carbohydrates and fatty acids which have low reactivity [23]. It is not clear yet if nucleic acids are the main reason for the inactivation of the cells or if another inactivation mechanism, such as the membrane damages are the reason. Membrane damages are occurring as ozone will react with several functional groups of membrane proteins, however, it is unclear to which extent those are contributing for the total inactivation of bacterial cells [9,23]. It seems that membrane damages are first occurring without being lethal to the cell,

but allowing the penetration of ozone into the cell, where it will react with internal cell components, leading to cell inactivation (Figure 1-7) [9,64]. Ozone reactions with nucleic acids are also the source of cellular mutations that can, ultimately, become lethal for cells [9] (Figure 1-7).

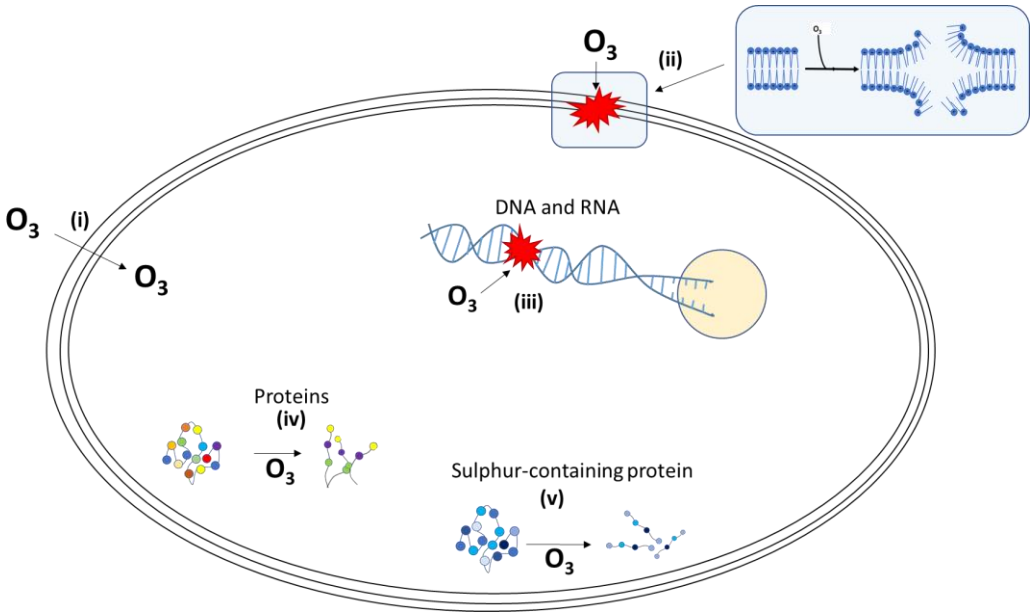


Figure 1-7. Mode of action of ozone on the bacterial cell.
(i) Ozone diffuses into the cell; (ii) destabilization of the membrane; (iii) ozone attacks the nucleic acids of DNA and RNA; (iv) ozone attacks proteins including (v) sulfur-containing proteins.

Several studies investigated the fate of ARBs and ARGs during ozonation [59,74–76]. As with chlorine, reduction in the number of bacteria, including resistant and multi-resistant ones is observed. ARGs alone were quite resistant to ozone, however no comparison was made with non-antibiotic resistant genes which could mean that the genetic material is simply resistant to ozone [74]. Full-scale ozonation of wastewater led to a limited inactivation of ARB, only 1-2 log removal was observed and ARG were not significantly affected by the ozone treatment [74]. In addition, ARBs regrew after the biological post-filtration, which reversed the benefits of ozone almost entirely.

1.3 Micropollutants

The emergence of new analytical techniques such as LC-HRMS, led to the detection of anthropogenic organic contaminants, micropollutants in the μg l^{-1} range and below [77,78]. Classification of micropollutants is possible based on the application of the compounds, including pharmaceuticals, disinfectants, X-ray contrast media, personal care products, surfactants, pesticides, dyes, paints, preservatives, food additives but also their metabolites and transformation products have to be considered [77]. Within these groups, some classifications exist either by chemical structures or by their mode of action. Disinfection agents, for example, are classified according to their chemical structures, with alcohols, aldehydes, halogen-releasing compounds or quaternary ammonium compounds to only cite a few. Micropollutants are either compounds that were not affected by the use or treatment but can also be transformation products and DBPs.

The presence of micropollutants was detected worldwide in the majority of rivers, surface waters and groundwaters [77,79,80]. The number of micropollutants and the low concentrations at which they exist in different aquatic environments made the assessment of their impacts quite complex [81]. Moreover, micropollutants are present as a complex mixture and many studies have only investigated the effect of single compounds [82]. For example, pesticides exert toxic effects on the fauna and flora, they led to e. g., changes in the composition of macroinvertebrate communities [82] and disinfection by-products are problematic for human health [81].

1.3.1 Disinfection agents

Disinfection agents are a class of micropollutants. Disinfection agents should not be confounded with chemical disinfectants, the similar name indicates a similar purpose, and both classes have the purpose to inactivate microorganisms and, therefore, act as disinfectants. What differs is their chemistry, their application and in which system they are used. Chemical disinfectants are used for municipal water treatment and disinfection agents are used in hospitals, public spaces and households to prevent the transmission of disease from one individual to another through direct and indirect contact. For the prevention through direct contact, disinfection agents exist in the form of soaps, hand sanitizers and other consumer products. For the prevention through indirect contact (via surfaces for example), they exist in the form of detergents. Disinfection agents have also been included in many other products such as paints to prevent the growth of

mold and fungi on walls. After use, they will partially end up in wastewaters and ultimately in the aquatic environment if they are persistent during the applied treatments (Figure 1-8).

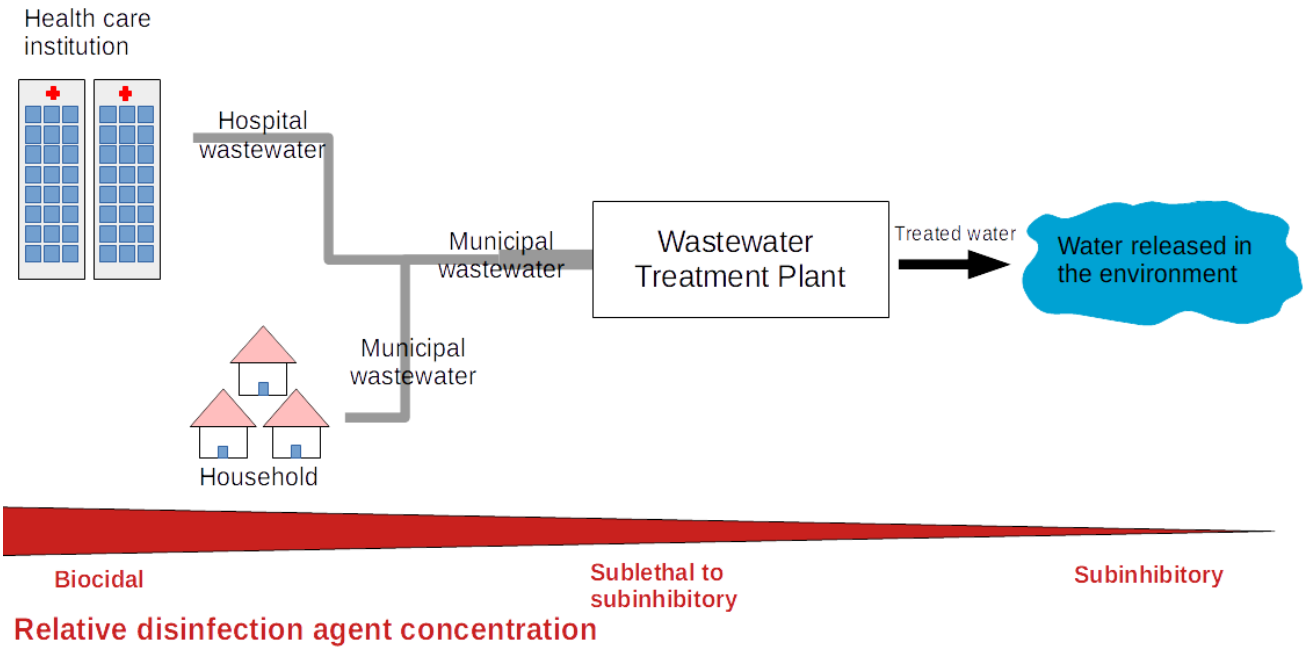


Figure 1-8. Schematic representation of the possible fate of disinfection agents from usage to release to the environment and the changes in the concentrations.

Beyond hand sanitation, one of the main applications of disinfection agents is to prevent the transmission of pathogens from indirect contact, by inanimate surfaces for example (Figure 1-9). Contamination of surfaces occurs through contact with fluids: blood, urine, feces, or droplets from sneezing or coughing. When coughing or sneezing, droplets can travel up to 8 meters from the individual and can even go further with ventilation systems [83]. Therefore, the pathogens can end up on diverse surfaces, where they can survive several hours to several months [84–87]. Disinfection can act as a barrier to the contamination from diverse environmental sources and is used in addition to other methods, such as gloves or masks (Figure 1-9). Hand disinfection with either hydro alcoholic solutions or with soap is one of the main barriers to contamination as the hands are one of principal vectors for transmission of infections [88,89]. The contamination of inanimate surfaces is a well-known problem for health care environments where the use of antimicrobial disinfection agents has been proven to reduce the rate of

pathogen transmission [90]. This problem reaches households, not only when it hosts someone sick, but also as some parts of homes are particularly prone to contamination. For instance, kitchens and bathrooms, however, microorganisms can also be found all around the house [91]. Even though most microorganisms are harmless, others can be pathogenic, especially from food spoilage, fecal sources, pets or when a house inhabitant is sick [92]. This led to the development of household disinfectants and the habits to not only clean but also disinfect homes. Public fear from infections may further increase the use of such products, especially in periods of a pandemic [93]. Additionally, disinfection agents are used in the food industry as preservatives and included in consumer and daily use products. This led to a discharge of disinfection agents in wastewater where some are poorly eliminated and are released to the environment. It is unknown what effects they have in the aquatic environment. It is even more problematic, because of the unknown implications of some disinfection agents for the multidrug resistance problem that we are currently facing.

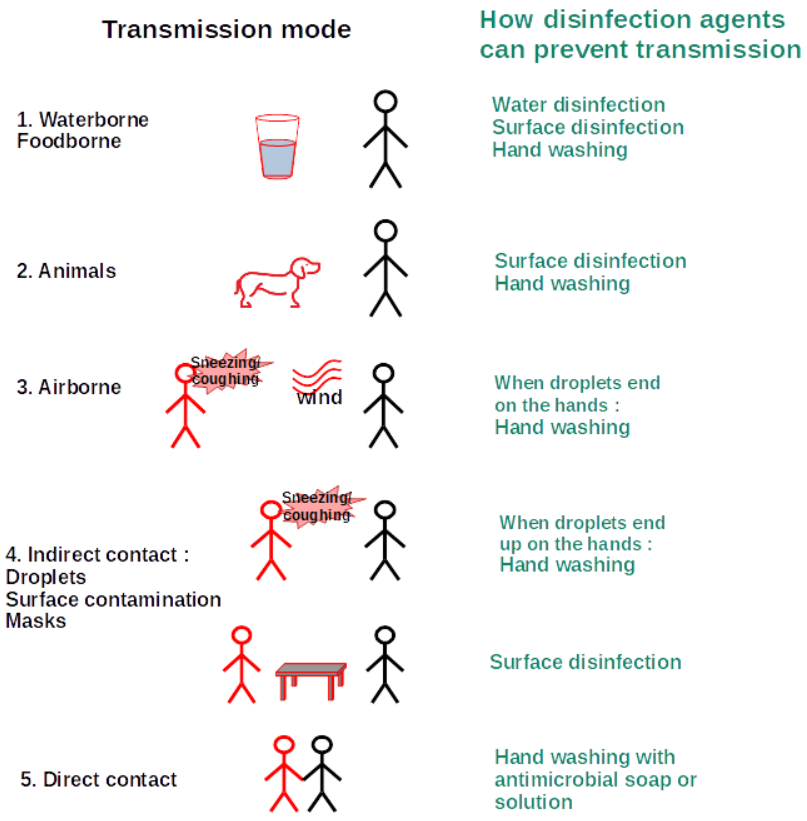


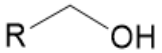
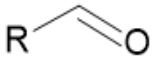
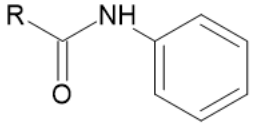
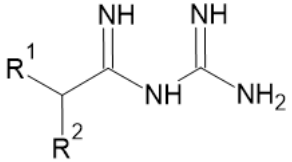
Figure 1-9. Transmission mode of pathogens and how disinfection agents can prevent the spread of pathogens.

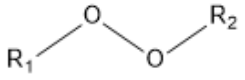
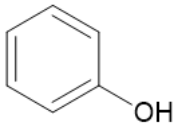
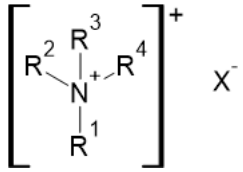
1.3.2 Classes of disinfection agents

Many different types of chemical substances are used because of their biocidal properties. A summary of the applied principal classes of disinfection agents, their structures, mode of action and fate in municipal wastewater is provided in Table 1-3.

Some disinfection agents are easily biodegradable such as alcohols or peroxides and will not persist in the environments [77]. Others have some stable transformation products, such as triclosan, a phenol, which transforms to methyl-triclosan, under aerobic conditions [77]. Finally, some are not easily biodegradable, such as quaternary ammonium compounds (QACs) and will end up in the aquatic environment [77,94,95]. QACs are of special interest because they have a high sorption capacity, due to their positively charged head and they are particularly prone to end in various parts of the environment but mostly in sediments [96–98].

Table 1-3. Main disinfection agents: Classes, structures, mode of action, use and fate in WWTP and the environment.

Class	Structure	Mode of action	Main use	Fate	Minimum inhibitory concentration (MIC) for *E. coli* (mg L^{-1})	Reference
Alcohols		Membrane damage, denaturation of protein	- Surface disinfection - Skin antisepsis	Easily biodegradable	Ethanol: ~65000	[95,99–102]
Aldehydes		- Cross-linking of proteins, RNA, DNA - Membrane alteration	- Sterilization of surgical instruments	Biodegradable Found in surface water at concentrations up to 4 mg L^{-1} in wastewater	Glutaraldehyde: ~3000 Formaldehyde: ~150	[95,99,102,103]
Anilids		Cytoplasmic membrane permeability alteration	- Antiseptics used in deodorants and soaps.	Not fully removed in WWTP	Triclocarban: > 5	[99,104–107]
Biguanides		Membrane permeability alteration, leakage of intracellular components	- Handwashing solutions - Mouthwash solutions - Pool disinfectants - Used in the food industry	Removal in the sludge in WWTP	Chlorhexidine: 1-100	[99,102,108–111]
Halogen-releasing agents	chlorine-, bromine- and iodine-based	Destruction of cell membranes Reaction with DNA Oxidation of proteins	Chlorine-based: - Hard-surface disinfections - Antiseptics Iodine-based: - Antiseptics Br-based: - Pool disinfection	Biodegradable Depending on the water quality, toxic by-products are possible	Chlorine-releasing agents: 1% pH>9: ~1000 1% pH 7: ~150 Iodine-releasing agents: Ethanol + 1% iodine: 43750	[99,102,112,113]
Silver compounds	Ag-based	Interaction with the thiol groups	- Eye infections - Prevent burns' infections	Retained in the sludge	$AgNO_3$: ~0.5	[99,114–116]

Class	Structure	Mode of action	Main use	Fate in WWTP	Minimum inhibitory concentration (MIC) for *E. coli* (mg L^{-1})	Reference
Peroxides		Generation of hydroxyl radicals that will attack different components of the cell in presence of activators such as iron or by degradation of the peroxide	- Hard surface disinfection - Sterilization - Antiseptics	Easily biodegradable	H_2O_2: ~2500 (*E. coli)*	[99,102,117]
Phenols		Membrane permeability alteration, leakage of intracellular material	- Disinfection - Sterilization - Antiseptics	Not fully removed. Concentrations of e.g., triclosan in the ng L^{-1} found in surface waters	Triclosan: ~5 Phenol: ~2000 *o*-Phenylphenol: ~500	[93,99,118–120]
Quaternary ammonium compounds		Membrane alteration, leakage of intracellular material, lysis of the cell	- Surface disinfection - Used in cosmetics products. - Used in paints	Poorly biodegradable Found in surface waters in the ng L^{-1} range. Sorbed on sediments	5-100	[95,99,102,107]
Natural compounds	Vinegar Citric acid Essential oils	Vinegar: inhibiting protein expression Citric acid: alone not bactericidal Essential oils: Membrane alteration	- Surface disinfection	No data available	Cinnamon oil: 0.4 µL mL^{-1} Thyme oil: 0.8 µL mL^{-1} Clove oil: 0.8 µL mL^{-1}	[107,121–127]

1.3.2.1 Quaternary Ammonium Compounds (QACs)

QACs are among the most used disinfection agents for surface cleaning and aside from their antimicrobial properties, they are also used as cationic surfactants in laundry detergents and in personal care products (PCPs) and finally, they are also used as biological stains such as crystal violet or propidium iodide [128,129].

They are cationic surfactants, composed of a polar head, a positively charged quaternary ammonium and a hydrophobic tail composed of aliphatic hydrocarbons or aromatic moieties [128]. Benzalkonium chloride (BAC) and cetyltrimethylammonium chloride (CTMA) are among the most used QACs (Figure 1-10A and B).

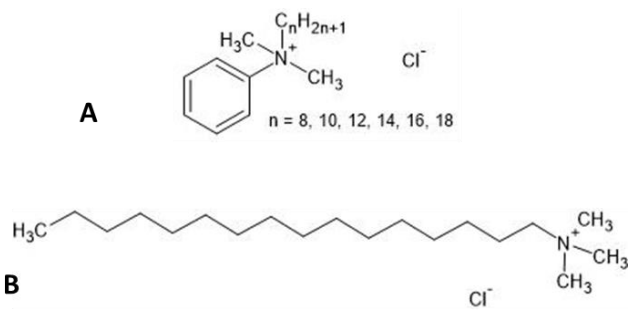


Figure 1-10. Quaternary ammonium-type disinfection agents.
(A) Benzalkonium chloride (BAC); (B) cetyltrimethylammonium chloride (CTMA).

The antimicrobial effect of QACs is linked to the length of their hydrophobic hydrocarbon chain, with the more bactericidal QACs having 12 to 14 carbons. This number seems to be related with the length required to penetrate and disrupt cell membranes of bacteria [129–132]. With a primary target being the bacterial cell membrane, they can be considered as broad-spectrum biocidal agents [129]. The positively charged nitrogen head associates with the negatively charged head of acidic phospholipids. The hydrophobic tails then interact with the hydrophobic part of the membrane. This leads to permeation and ultimately lysis of the bacterial cell (Figure 1-11) [129,133]. Aromatic moieties, present in some QACs, such as BAC, are likely to have been incorporated to mitigate the toxicity of the compound, leading to a lower antibacterial power [129].

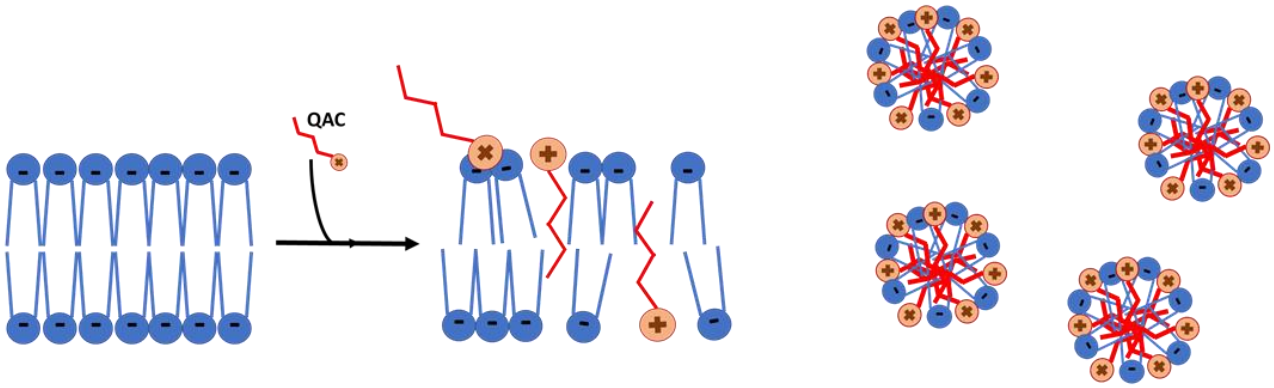


Figure 1-11. Interaction of the QAC with the membrane of a bacterial cell and the formation of micelles

At concentrations used in disinfection agents formulations, QACs and cell membrane components aggregate in the form of micelles, leading to membrane solubilization and cell lysis [133].

The structure of the membrane is a key component in the susceptibility of the bacteria to QACs. For instance, the outer membrane of *Pseudomonas spp* makes these bacteria particularly resistant to QACs and other disinfection agents.

Due to the introduction of QACs in many industrial and household products, they will inevitably reach sewage systems. Benzalkonium chloride was found in wastewater treatment plants influents and effluents (Table 1-4). In a survey in Sweden, BAC was detected in 100% of influents of wastewater treatment plants [133]. The potential constant presence of QACs at low concentrations and their incomplete elimination during conventional wastewater treatment can affect microorganisms present in wastewater and their release to the aquatic environment but also the natural bacterial population, especially close to the treated wastewater discharge points [99,134–136]. Moreover, the adsorption of QACs on sludge and the discharge of QAC-containing sludge is also a potential source of QACs released to the environment [94]. Additionally, the recent pandemic of SARS-COV-2 led to an increase of the used volumes of disinfection agents and of quaternary ammonium compounds and the current numbers are probably underestimating the real situation [137].

Table 1-4. Concentrations of benzalkonium chloride found in different aquatic environments.

Molecule	Water type	Concentration	Reference
Benzalkonium chloride	Hospital wastewater effluents	0.05 - 6.03 mg L^{-1}	[130,138]
	Wastewater treatment plant influent	55 - 170 µg L^{-1}	[130]
	Wastewater treatment plant effluent	175 – 630 ng L^{-1}	[130]

1.3.2.2 Reactions of QACs with chlorine, chloramine or ozone

Very few studies are available regarding the reactions of QACs with chlorine, chloramine or ozone. It has been suggested that the difficulty to analyze them in water bodies and the low amount produced compared to other surfactants is the cause of these lack in the literature [139]. No data was found about oxidation of QACs with chlorine and chloramine, however based on their structural features, no reaction is expected. For ozone, the aliphatic QACs were reported to be non-reactive with ozone. This is also expected from their chemical structure, as there are no electron-rich moieties. However, aromatic QACs can react with ozone, however, with a low reactivity [139–142]. Nevertheless, during ozonation a certain abatement is expected due to reactions with $^{\bullet}OH$.

More generally, some studies showed the presence of QACs in wastewater effluents, suggesting limited effects of the water treatment processes on the QACs [143–145].

1.4 Resistant bacteria

One of the biggest health threats nowadays is an increase of antibiotic resistance and the lack of new antibiotics or treatments to inactivate resistant bacteria. The problem of resistance to antibiotics is considered a consequence of an intense use of antibiotics. ARBs and ARGs have been detected in the environment, in municipal wastewater but also in surface water [146,147]. Wastewater treatment plants have been identified as hotspots for the dissemination of resistance as mentioned previously [148,149]. Therefore, it is crucial to understand how resistance is developed, which mechanisms are involved and how disinfection agents are contributing to the problem.

Resistances mechanism can be either intrinsic or acquired. Intrinsic resistance is due to the natural properties of the bacteria, for example, Gram-negative bacteria are more resistant towards certain biocides compared to Gram-positive ones because of their particular membrane structure. Acquired resistance is the result of either adaptation, mutation or horizontal gene transfer. A third way of resistance can build up in biofilms. Biofilms act as a barrier by lowering the diffusion rates of disinfection agents. Inside biofilms are high concentrations of organic molecule which will also react with the disinfectant agents, therefore, lowering the concentration of disinfection agents reacting with the bacteria themselves, leading to a "protective environment" [99,136]. Biofilms are also considered as "population-based" resistance mechanisms [150].

Intrinsic properties of the cells are a permanent resistance mechanism. In contrast, acquired mechanisms can also be transient effects. Indeed, genetic mutations will be responsible for more permanent resistances mechanisms but phenotypical changes, such as increased protein expressions are only transient and bacteria will regain their susceptibility to the biocide once the selective pressure is gone [151].

If antibiotics are the main cause of antibiotic resistance, disinfection agents can also promote the development of resistance to antibiotics but also to disinfection agents and multidrug resistance [107,152–155]. Resistance is possible when bacteria are exposed to low concentrations of disinfection agents, in the sub-inhibitory range [99,117]. Multiple resistance arise either when the target, way of action or death mechanisms are shared between two or more drugs/disinfection agents, which is defined as cross-resistance [151] (Figure 1-12B) but can also result from resistance genes located on the same plasmid, transposon or integron and activated together, which is called co-resistance [151] (Figure 1-12B).

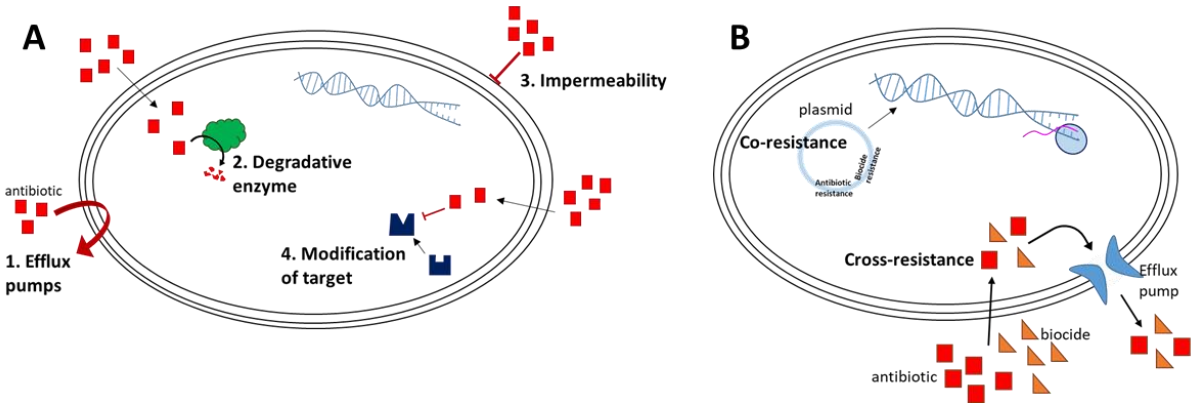


Figure 1-12. Resistance of bacteria.

(A) Mechanisms of resistance; (B) mechanisms of co- and cross-resistance

1.4.1 Implications of disinfection agents on the resistance problem

Disinfection agents are known to be involved in resistance to disinfection agents, but also to antibiotics and multi-resistance. Even though there is a large body of information in literature on antibiotic resistance mechanisms, only few studies have been performed regarding the nature of resistance to disinfection agents and the studies are biased towards certain compounds. For instance, triclosan has been widely studied, QACs and peroxides are also well documented but other compounds, and especially the more recently developed substances such as glucoprotamine, or mixtures of disinfection agents have been poorly investigated [151,154].

However, despite the biased reporting of resistance towards disinfection agents, some mechanisms have been revealed to be systematically involved in resistance but also in multiresistance issues.

1.4.1.1 Outer membrane structure

For many disinfection agents, the effectiveness of the compound is linked to its ability to penetrate the cell and reach its target site at sufficiently high concentrations. As mentioned before, Gram-negative bacteria are intrinsically more resistant to disinfection agents compared to Gram-positive ones due to their membrane structure. The structure of the outer membrane is therefore a key parameter in the susceptibility of the bacteria.

[99,156]. The outer membrane structure of bacteria can also be modified following exposure to a disinfection agent. For instance, downregulation of membrane proteins such as porins can prevent the diffusion of some disinfection agents [156]. *P. aeruginosa* has been shown to modify its outer membrane structure in response to the antibiotics imipenem and isothiolazone and the bactericide sodium dimethyldithiocarbamate [156].

1.4.1.2 Efflux pumps

Efflux pumps are transmembrane, proton-motive force-dependent and cation export proteins [151,157]. They are also part of the membrane structure and are ubiquitous in Gram-negative bacteria and mycobacteria and are responsible for the excretion of toxins from the cytoplasm to the extracellular matrix [157,158]. Efflux pumps can be either substrate-specific or non-specific and the latter class is the most problematic. Non-substrate specific efflux pumps can pump out many different toxins, such as different classes of antibiotics or disinfection agents. It has been identified that most classes of antibiotics, except glycopeptides are sensitive to efflux pumps [158]. Efflux pumps are divided into two main classes, the primary ones are ATP-driven, ATP-binding cassette (ABC) transporters, the secondary transporters are proton-driven ones [158]. In bacteria, secondary transporters are dominant [158]. ABC transporters are mainly drug-specific transporters but a few of them are involved in multiresistant bacteria [158]. Secondary transporters are classified into four families and are less drug-specific than primary ones. The four classes are the major facilitator superfamily (MFS), the resistance nodulation and cell division family (RND), the small multidrug resistance family (SMR) and the multidrug and toxic compound extrusion family (MATE) [157,158]. MFS pumps are ubiquitous for transporting sugars, intermediate metabolites and drugs [158]. RND pumps are transporting lipophilic and amphiphilic molecules and toxic divalent cations [158]. SMR pumps are transporting lipophilic cationic drugs [158]. MATE pumps are antiporters for Na^+ and drugs [158].

Efflux pumps are known to be involved in resistance to biocides and to antibiotics. They are ubiquitous but can be upregulated in response to environmental signals such as low concentrations of drugs or by mutation of genes [134]. Multidrug resistant efflux pumps

have been identified as a resistance mechanism expressed when bacteria acquired resistance to pine oils, triclosan, QACs or chlorhexidine [134,157,159]. The expression of this efflux pump (AcrAB/TolC complex) led to multiresistant bacteria, resistant to the cited biocides but also to antibiotics [134]. Other well-studied efflux pump systems are the ones regulated by the *qac* genes. The *qac* genes are regulating secondary efflux pumps of the SMR family [160]. The main substrates of *qac* genes are obviously QACs but they also target other lipophilic cationic compounds such as intercalating dyes, biguanides, diamidines, and the majority of QACs [160]. *qac* genes have been identified to be involved in adaptation to various cationic compounds and to confer resistance to antibiotics at the same time [160]. However, the exact role of *qac* mediated efflux pumps in the resistance to QACs and antibiotics is still to be determined, as several studies also showed adaptation and resistance in *qac*-negative bacteria [160].

In summary, efflux pumps have been identified as resistance mechanisms to several disinfection agents but also conferring multiresistance to either disinfection agents and/or antibiotics.

1.4.1.3 Enzymatic degradation

Some bacteria are able to produce enzymes that will transform the disinfection agent to a non-toxic form (Figure 1-12A) [154,156]. Catalase and superoxide dismutase, for example, are involved in the resistance to oxidizing agents. Resistance to heavy metals and formaldehyde is also enzyme mediated. Resistance to heavy metals occurs by an enzymatic reduction of the cation to the elemental metal, and activation of such an enzyme gives resistance to several heavy metals such as mercury, nickel, arsenal, copper or silver [156]. Resistance to formaldehyde is acquired through the reduction of it by NAD+-glutathione-dependent dehydrogenase. Activation of this enzyme gives resistance to most formaldehyde-releasing condensates [156].

1.4.1.4 Stress response in bacteria

The presence of sub-lethal concentrations of a biocide in the environment will trigger a stress response in bacteria. The stress response is the activation of several bacterial mechanisms that will confer transient resistance to the biocide in a short time following the exposure.

Activation of enzymes such as in the previous paragraph are part of the response but other mechanisms such as repair mechanisms are also contributing to the stress response. Stress response can give resistance to several biocides, as some global regulators such as *marA* or *soxS* can be activated. The activation of those global regulators can also contribute to antibiotic resistance as their resistance mechanisms are controlled by the same regulators [154,156].

1.5 Research Objectives

The implication of disinfection agents and more precisely of quaternary ammonium compounds (QACs) on the global resistance and multi-resistance problematic is still under debate. The increased use of disinfection agents in our daily lives has the consequence of a constant discharge of disinfection agents to municipal wastewaters but also to the aquatic environment, as some compounds are poorly biodegradable. Three main objectives are covered in this thesis and they are briefly described below.

Objective 1: Adaptation of *Pseudomonas aeruginosa* to constant sub-inhibitory concentrations of quaternary ammonium compounds (Chapter 2)

QACs are widely used in consumer products and have been detected at sub-inhibitory concentrations in the aquatic environment. Limited data is available on the effect of these low and constant concentrations of QACs in technical and natural aquatic environments on the development of bacterial resistance. The objectives of this study were to (i) investigate the effect of a long-term exposure of *P. aeruginosa* to constant sub-inhibitory concentrations of two QACs and two commercial disinfection agents; (ii) the stability of the adaptation; (iii) the cross-resistance between the two used QACs and (iv) with selected antibiotics and (v) morphological changes using atomic force microscopy at the end of the exposure period.

Objective 2: Effect of cetyltrimethylammonium chloride on various *Escherichia coli* strains and their inactivation by ozone and monochloramine (Chapter 3)

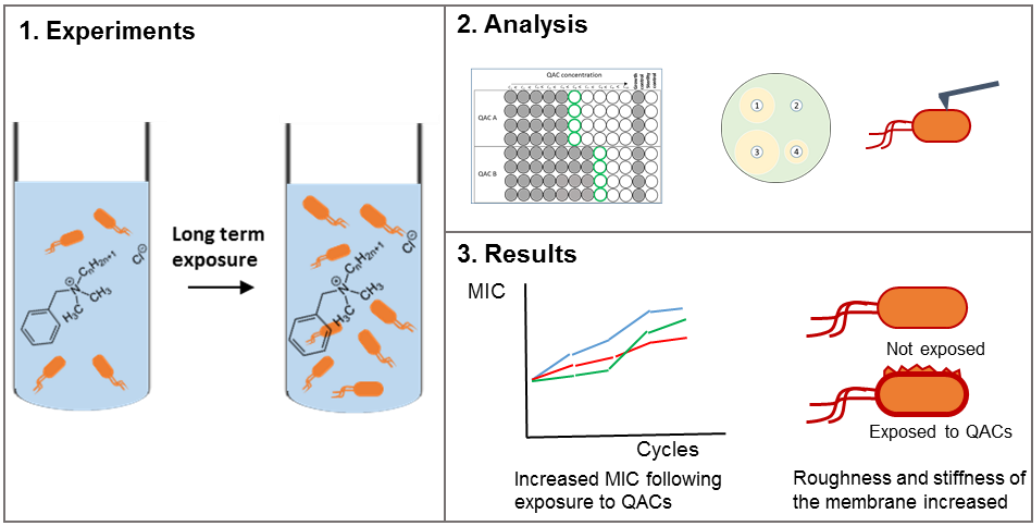
QACs have been detected at low concentrations in municipal wastewaters but also in surface waters. QACs are only partially eliminated in municipal wastewater treatment due to a poor biodegradability. Therefore, they can interact with bacteria in biological processes. So far, there is only limited information on the antimicrobial efficiency of CTMA in matrices other than standard growth media and if and how CTMA influences conventional chemical disinfection. The objectives of this study were to (i) investigate the susceptibility of *E. coli* to CTMA in different matrices: broth, PBS, lake water and municipal wastewater effluent; (ii) investigate the effects of a pre-exposure to CTMA and (iii) of the presence of

CTMA on the inactivation kinetics of various *E. coli* strains (AG100, AG100A, AG100tet) by ozone and monochloramine.

Objective 3: Development of CTMA resistance in *E. coli* using the microbial evolution and growth arena (MEGAPLATE) approach (Chapter 4)

The development of resistance in laboratory conditions mainly occurs in liquid media enriched with the compound of interest, with increasing concentrations over time. Even though, this method has been proven successful, it can sometimes be different from realistic conditions. One aspect missing in the experiments with liquid media is the space constraint. Indeed, once a species or a mutant has invaded a location, no space or nutrient is remaining for the other species or mutants, blocking access to a specific area. The MEGAPLATE device was developed previously to solve this issue and provide a giant spatial evolution arena for bacteria to compete [161]. This device has been successfully applied for antibiotics and its potential with other antimicrobial substances is unknown. The aim of this objective was to investigate the use of the MEGAPLATE for the development of resistance to QACs.

2 Adaptation of *Pseudomonas aeruginosa* to constant sub-inhibitory concentrations of quaternary ammonium compounds



This chapter was published as:

M. Voumard, L. Venturelli, M. Borgatta, A. Croxatto, S. Kasas, G. Dietler, F. Breider, U. Von Gunten, Adaptation of Pseudomonas aeruginosa to constant sub-inhibitory concentrations of quaternary ammonium compounds, Environ. Sci. Water Res. Technol. 6 (2020) 1139–1152. https://doi.org/10.1039/c9ew01056d.

Margaux Voumard performed the experimental design, method development, experimental part, data analysis and writing of the MIC determinations, repeated exposure, stability experiments and cross-resistance experiments as well as the global analysis with the scientific advising of U. von Gunten, F. Breider, A. Croxatto, M. Borgatta. Leonardo Venturelli performed the AFM part, experimental part, statistical analysis and writing with the scientific advising of S. Kasas and G. Dietler.

Abstract

Quaternary ammonium compounds (QACs) are widely used in consumer products for disinfection purposes. QACs are frequently detected in aquatic systems at sub-inhibitory concentrations and were found to affect the development of antimicrobial resistance if bacteria are exposed to increasing concentrations. However, the effect of a constant sub-inhibitory concentration on the development of bacterial resistance is unknown. A constant exposure to 88% of the minimum inhibitory concentration (MIC) of benzalkonium chloride (BAC) led to an increase of the MIC of *P. aeruginosa*. It increased from 80 mg l^{-1} to 150 mg l^{-1} after 10 cycles of exposure and remained stable after removal of BAC. When exposed to cetyltrimethylammonium chloride (CTMA), *P. aeruginosa* MIC increased from 110 mg l^{-1} to 160 mg l^{-1} after 10 cycles of exposure and decreased to 120 mg l^{-1} after removal of CTMA. Additionally, cross-resistance between the QACs was observed. When exposed to BAC, the MIC for CTMA increased from 110 mg l^{-1} to 200 mg l^{-1}, and when exposed to CTMA, the MIC for BAC increased from 80 mg l^{-1} to 160 mg l^{-1}. In contrast, the susceptibility to 16 antibiotics was not significantly affected by exposure to QACs. Finally, analyses of the membranes' nanomechanical properties of *P. aeruginosa* with atomic force microscopy (AFM) showed increases in cell roughness, adhesion and stiffness after treatment with CTMA. Since sub-inhibitory concentrations of QACs can be detected in (technical) aquatic systems including sediments, this may lead to a dissemination of bacteria with higher QAC resistance in the environment.

2.1 Introduction

Quaternary ammonium compounds (QACs) are widely used disinfection agents in industrial, medical and household environments [152,162]. Benzalkonium chloride (BAC, Figure 2-1A), a mixture of alkyl dimethyl benzylammonium chlorides, is among the most common QACs found in many consumer, industrial and medical products [129,153,163]. Another widely used QAC is cetyltrimethylammonium chloride (CTMA; also known as cetrimonium chloride, Figure 2-1B). In the clinical context, high loads of QACs are used for the disinfection and cleaning of surfaces and medical instruments [94]. The majority of the QACs is discharged to wastewaters [144,164–167], where they are further diluted and become micropollutants [168]. QACs are known to have a poor biodegradability and to persist in waters [95,146,169–172]. In hospital wastewaters, their concentrations have been measured in the mg l^{-1} range [173–175], which is in the sub-inhibitory range for several bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) [173,174] In municipal wastewaters, where the dilution effect is bigger, concentrations were measured in the µg l^{-1} range [95,175]. Concentrations of QACs in the ng l^{-1} to the µg l^{-1} have been detected in surface water [95,173]. In soils and sediments, relatively high concentrations of QACs were also found, the sorption of QAC being high and also highly dependent on their structure [176]. As desorption was also observed [176,177], local increases of concentrations of QACs are also possible in surface waters.

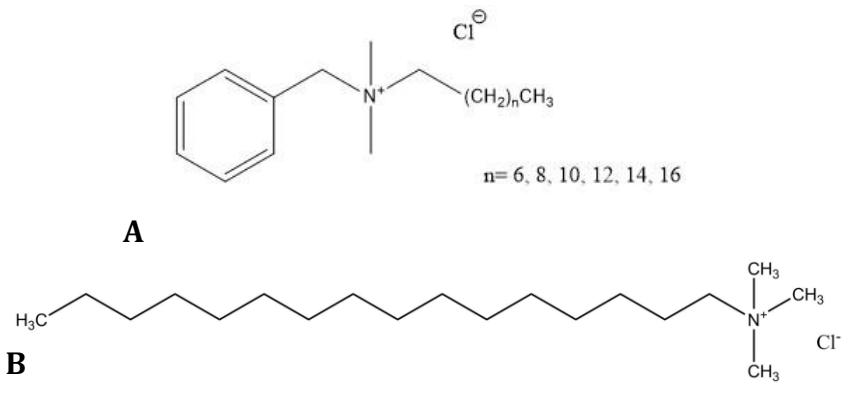


Figure 2-1. Chemical structures of quaternary ammonium compounds.

A. Benzalkonium chloride (BAC); B. Cetyltrimethylammonium chloride (CTMA)

Low concentrations of QACs can lead to a selection pressure on microorganisms' populations, that may yield to more QAC-resistant populations [129,178]. Bacterial populations have been known to adapt to sub-inhibitory concentrations by mechanisms such as modification of cell membrane structure, efflux pump expression and enhancement of biofilm formation [166,179]. The mode of action of QACs at low concentrations may differ from the one at higher concentrations and may involve multiple processes similar to antibiotics [166,180]. Because of these similar mechanisms, cross-resistance towards other antibacterial agents can occur [150,174,181,182]. Previous studies reported different results, either cross-resistance of *P. aeruginosa* to other disinfection agents and antibiotics or an absence of cross-resistance [110,181,183–187]. The different results obtained in these previous studies highlight the need for more information on a potential development of cross-resistance.

The level of resistance of bacteria or the potential for resistance development is linked with intrinsic properties of bacterial species [99]. Among the different bacterial species, *P. aeruginosa* is known to be particularly resistant to QACs and to adapt easily to the presence of antibacterial agents [183]. Moreover, *P. aeruginosa* is an important nosocomial bacterium, which is present in tap, recreational and surface water and has been involved in many infectious outbreaks [188–191]. These characteristics make this species particularly suitable for investigating the effect of constant sub-inhibitory concentrations of QACs and other disinfection agents.

QACs are part of commercial disinfection agents because of their antibacterial properties. QACs have been used for decades as active substances in commercial disinfection agents. Deconex® 53 PLUS, is a commercial product containing various disinfectants and a QAC (see below). Deconex® 53 PLUS is used for the pre-cleaning and disinfection of medical instruments. Other commercial disinfection agents include recently developed substances other than QACs. Incidin® PLUS, for example contains glucoprotamin as an active substance, which is included in several commonly used surface and instruments disinfection agents in the health care environment [192,193]. A tetracycline-resistant strain of *P. aeruginosa* (PAO-LAC ATCC 47085) was found to be more resistant to this product than a non-antibiotic-resistant strain [193]. However, no studies were found on the potential of this product to promote resistance at sub-inhibitory concentrations.

To investigate the effects of QACs or other disinfection agents on bacteria, classical methods such as cultivation are commonly used. In addition to classical microbiological methods, atomic force microscopy (AFM) has been successfully applied to investigate and characterize the bacterial morphology related to resistance to antimicrobial compounds at a single-cell level [194–197]. Measurements of alive bacteria in physiological conditions is possible with AFM, preventing any changes in the structure of the membrane when imaging the cells. The primary target of QACs is the bacterial membrane and changes in the membranes are suspected to be involved in the resistance mechanisms of *P. aeruginosa* to QACs. Therefore, AFM is likely to reveal potential changes in the membrane morphology as well as changes in the properties of the membrane in real-time [198]. The aforementioned methods (classical microbiology and AFM) have been used to determine the effect of BAC exposure of bacteria. Other QACs, including CTMA, have been scarcely investigated. Studies on BAC focused on the development of resistance when exposed to increasing concentrations of the QAC, but the effect of a constant sub-inhibitory concentration of a QAC or glucoprotamin has had little attention [131].

In this study, the consequences of an exposure of *P. aeruginosa* to BAC, CTMA, Deconex® 53 PLUS and to glucoprotamin were investigated. More specifically, the evolution of the MIC, cross-resistance with QACs and antibiotic susceptibilities following exposure were studied. Culture-based methods combined with AFM investigations were applied to characterize the properties of *P. aeruginosa* and highlight any potential change in the bacterial membrane.

2.2 Materials and Methods

2.2.1 Bacterial strains

Pseudomonas aeruginosa (ATCC® 27853™) was obtained from the ATCC collection. The strain was stored at -80°C using cryoinstant vials (VWR, Switzerland). Stock cultures were recovered from beads prior to each exposure experiment, by plating the beads on Lysogeny Broth Agar (LBA; Sigma-Aldrich, Switzerland) and on Columbia Blood Agar (Sigma-Aldrich, Switzerland). Colonies from these stock cultures were picked and dissolved in Mueller-Hinton broth (MHB; Sigma-Aldrich, Switzerland) to prepare overnight cultures.

Overnight cultures were prepared by incubating the tubes at 37°C and 220 rpm for 12 to 18 hours.

2.2.2 Disinfection agents

The disinfection agents used in this study were two QACs, benzalkonium chloride (BAC, CAS 63449-2) and cetyltrimethylammonium chloride (CTMA, CAS 112-02-7) and two ~~one~~ commercial products, Deconex® 53 PLUS and Incidin® PLUS. BAC (Sigma-Aldrich, Switzerland) was composed of 70% benzyldimethyldodecyl ammonium chloride and 30% benzyldimethyltetradecyl ammonium chloride. The concentration of the product was ≥ 95% and was of the highest purity available. CTMA (Sigma-Aldrich, Switzerland) had a concentration of 25 wt. % in H_2O with the highest purity available. Deconex® 53 PLUS (Ecolab Healthcare, Switzerland) contains 9.4% of the active substances (3.8 g alkyl propylene diamine guanidinium diacetate and 5.6 g *N,N*-didecyl-*N*-methyl-poly(oxyethyl)ammonium propionate per 100g of product). Incidin® PLUS (Ecolab Healthcare, Switzerland) contained 26% of glucoprotamin as the active substance. Stock solutions of 10,000 mg l^{-1} of the different antibacterial agents were prepared in Mueller Hinton Broth (MHB). The MHB was sterilized by autoclaving before the addition of the QACs or the disinfection agents and the stock solution (MHB and disinfection agent) was filtered by 0.2 µm filters (Filtropur S 0.2S; Sarstedt, Switzerland). The filter-sterilized solutions were then further diluted with autoclaved MHB to the concentrations of interest for the experiments (MIC determination and repeated exposure). The stock solutions were kept at room temperature and used within a week.

2.2.3 Determination of MICs

MICs were determined with broth microdilutions following the protocol by Wiegand *et al.* (2008) [199]. Bacterial suspensions were prepared by overnight culture, two to three colonies were diluted in 10 ml of autoclaved MHB and incubated during 12 to 18 hours at 37°C and 220 rpm. The overnight cultures were diluted to achieve an optical density of 0.12 at 600 nm (OD600) with an optical path length of 1 cm, which was determined to

correspond to a concentration of (1-2) x 10^8 CFU ml^{-1} (colony forming units per ml). This solution was then diluted 1:100 to reach a final concentration of (1-2) x 10^6 CFU ml^{-1}.

The 10 first rows of a sterile 96-well microplate (Nunclon™ Delta Surface, Thermo Scientific, Switzerland) were filled with 50 µl of increasing concentrations of antibacterial solutions. The eleventh column was filled with 50 µl of MHB and 50 µl of bacterial suspension (growth control) and the twelfth with 100 µl of a sterile control. Each well of the antibacterial testing and the growth control were inoculated with 50 µl of the bacterial suspension. The microplate was incubated at 37°C for 16-20 hours. The concentrations used for the MIC determination are available in the Supporting Information (Table S 2-1, ESI). After incubation, growth was assessed by turbidity or sediments in the wells. The MIC is defined as the lowest concentration of the antibacterial agent that inhibits visible growth [199,200].

2.2.4 Repeated exposure to a sub-inhibitory concentration of a disinfection agent

Figure 2-2 summarizes the exposure experiments carried out with *P. aeruginosa*. Tubes containing 10 ml of sub-inhibitory concentrations of BAC, CTMA, Deconex® 53 PLUS or Incidin® PLUS were inoculated with 100 µl of an overnight culture of *P. aeruginosa* diluted to obtain a final concentration of 10^6 CFU ml^{-1} in the tube at the beginning of the cycle. Concentrations of CTMA and BAC were set at 86 and 88% of the MIC, at 87% of the MIC for Deconex® 53 PLUS and at 88% of MIC for Incidin® PLUS, to remain in the sub-inhibitory range while still having a high selective pressure. For BAC, an additional exposure concentration of 50% of the MIC was tested. For the QACs, one exposure cycle consisted of a 48 hours' incubation at 37°C and 220 rpm. After 48 hours, the MIC was determined and the bacterial suspension was used to inoculate a new series of tubes with the same sub-inhibitory concentration. A purity check was added by plating 50 µl of the bacterial suspension on plate count agar (PCA; Sigma-Aldrich, Switzerland). This was repeated to reach 10 cycles. Samples were cryopreserved using cryoinstant vials after 5 and 10 cycles of exposure. Two controls were added, one by cycling the bacteria in absence of the disinfection agent in the growth medium (MHB) and a negative control with the growth medium only. A slightly different protocol was used for the exposure to Deconex® 53 PLUS

and to Incidin® PLUS, one exposure cycle was defined as 24 hours and the total experiment lasted 10 cycles of exposure for Deconex® 53 PLUS and 15 for Incidin® PLUS followed by 5 cycles of stability. The MICs were determined every five cycles of exposure and the samples were cryopreserved at the same time.

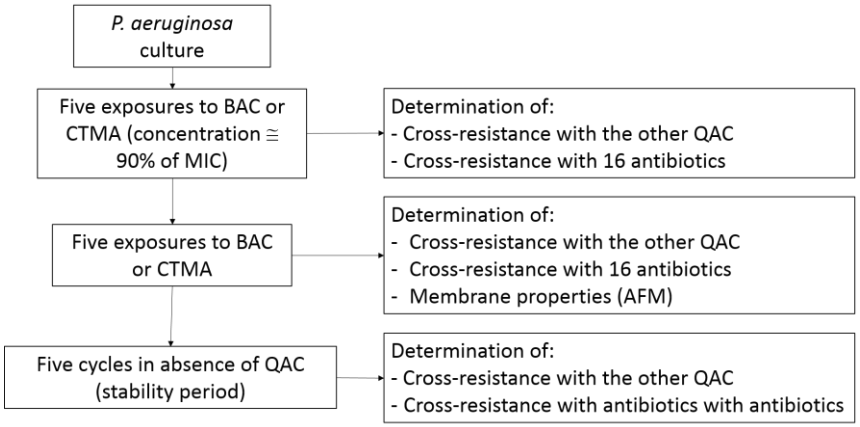


Figure 2-2. Summary of the experiments performed with *P. aeruginosa* and the two QACs.
Tubes of MHB with a sub-inhibitory concentration of QAC (either BAC or CTMA) were inoculated with a pure *P. aeruginosa* culture. After five and ten exposure cycles, cross-resistance to the other QAC and to antibiotics was determined. Additionally, after 10 cycles, populations were analyzed by AFM. After exposure to QACs, populations were sub-cultured for five additional cycles in QAC-free MHB and cross-resistance to the other QAC and antibiotics determined.

2.2.5 Stability

To investigate if the effects of the exposure to disinfection agents remains stable in absence of the disinfection agent from the medium, five cycles in disinfection agent-free MHB were added at the end of the total exposure (10 cycles). As in the exposure experiments, cycles of 48 h were carried out and a purity check was added after each cycle. The MIC was determined after each cycle, the antibiotic susceptibility profile and the cross-resistance was assessed at the end of the three cycles. For Deconex® 53 PLUS and Incidin® PLUS, cycles of 24 h were added for the stability experiments.

2.2.6 Determination of the antibiotic susceptibility profiles

10 antibiotics, which are known to be effective against *P. aeruginosa*, were selected according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [201]. The selected antibiotics belong to the classes of aminoglycosides (tobramycin 10 µg; amikacin 30 µg; gentamycin 10 µg), cephalosporin (cefepime 30 µg; ceftazidime 10 µg), fluoroquinolones (ciprofloxacin 5 µg; levofloxacin 5 µg); penicillin (piperacillin-tazobactam 36 µg) and carbapenem (imipenem 10 µg; meropenem 10 µg). In addition, six antibiotics, to which *P. aeruginosa* is naturally resistant, were also tested. These antibiotics belong to the classes of cephalosporin (cefoxitine 30 µg; cefuroxime 30 µg), penicillin (ampicillin 10 µg), sulfonamide (co-trimoxazole 25 µg), nitrofuran (nitrofurantoin 100 µg) and tetracycline (minocycline 10 µg). They were used in the form of antimicrobial susceptibility disks (Thermo Scientific™ Oxoid™ Gentamycin Antimicrobial Susceptibility Disks, Thermo Scientific, Switzerland). The disks were stored at -20°C, according to the manufacturer's instructions.

The antibiotic susceptibility profiles were determined using the disc diffusion method after 5 and 10 cycles of exposure, following the EUCAST methodology [201]. The bacterial growth was diluted in MHB to reach an OD600 of 0.12, as in the determination of the MIC. This solution was swabbed on Mueller-Hinton agar plates (MHA plates). Antibiotic discs were then applied to the agar surface using a disc dispenser (Oxoid™ Antimicrobial Susceptibility Disk Dispenser) and the plates were incubated for 24 hours at 37°C. The inhibition zone diameters (IZD) were recorded using calipers after the incubation period. The IZDs were then compared to the EUCAST database for *P. aeruginosa* [201].

2.2.7 Cross-resistances among QACs

Susceptibility to the QAC to which the bacterial populations were not exposed was determined to investigate if exposure to one QAC leads to a higher resistance to the other one. The MIC for CTMA was determined after 5 and 10 cycles of exposure to BAC and after the stability period using the broth microdilutions method. The MIC for BAC was determined after 5 and 10 cycles of exposure to CTMA and after the stability period using the broth microdilutions method.

2.2.8 AFM

2.2.8.1 Imaging

AFM measurement were performed on the treated cells with approximately 90% of the MIC during 10 cycles (for CTMA and BAC only). The measurements were performed before the stability period but in absence of the QACs. The bio-mechanical properties of *P. aeruginosa* strains were measured using a JPK NanoWizard 3 AFM (Bruker Nano GmbH, Germany), equipped with a Zeiss TE-100 inverted microscope (Bruker, Germany) in PBS buffer at room temperature (25 °C). The so called "quantitative imaging" mode was used to image the cells to gather both high quality 3D topology and determine mechanical properties, adhesion and stiffness. The scan size of the AFM images was adapted to fit an area with at least 20 cells and 10 areas per sample have been imaged. Typically, a square ranging from 15x15 to 25x25 μm was chosen. Every image consisted of either a 64×64 or a 128×128 force-distance (F/d) curve recorded with an indentation dwell-time of 80 milliseconds and a set-point force of 1200 pN. Specifically, each pixel in the AFM adhesion image has a value of force that is basically the registered minimum in the retrace function of the F/d curve. The adhesion force is a minimum value because it is considered as a pulling force from the cell towards the cantilever, and thus generated from the cell. For these experiments, we used 200 μm -long triangular silicon nitride probes, supplied by pyramidal tips with a nominal radius of 20 nm (DNP-10 Bruker, Bruker Nano Inc., CA, USA) and a nominal spring constant of 0.06 N/m. The scanned height was set to 1 μm, to be able to completely cover the cell height. The bottom glass-modified petri dishes, employed for the AFM experiments, allowed us to strongly improve the stiffness image contrast, due to few orders of magnitude differences between cells (hundreds of kPa) and glass (hundreds of MPa) Young's modulus.

2.2.8.2 Data Processing

The AFM data were processed using the "JPK Data Processing" software to obtain the values for the adhesion parameters and by applying the Hertz-Sneddon fit, it calculates the Young's modulus. A Matlab (MathWorks Inc., CA, USA) script, developed in our laboratory allowed to sharply discriminate and extract the points belonging to the bacteria cells from the background and then use them to plot the final graphs. Briefly, the script analyses each stiffness, adhesion and height image files and it creates a new matrix text file (64×64 or

128×128 points, based on the original image size) made of 0 and 1, where 0 means background and 1 means cell. The selection process consists of applying a threshold on the height AFM image. The so generated matrix files are then plotted as black and white images to check whether the script worked correctly. Afterwards the matrix files are used as a mask to extract the positive matching points from the stiffness, adhesion and height files. The selected points are finally plotted and averaged using the software OriginPro 2018 (OriginLab Corporation, MA, USA).

The average roughness was calculated by the "JPK Data Processing" software, which can calculate the average roughness (here named also Ra) of a specifically selected cross section from the raw files.

2.2.9 Statistical analyses

2.2.9.1 MIC evolution upon exposure to disinfection agents

To determine the statistical significance of the results for the evolution of the MIC during exposure to sub-inhibitory QAC concentrations, linear regression analysis was used. The analyses were performed in R using the R package lme4 [202]. To test if the MIC significantly changes over the cycles when exposed to a disinfection agent, measurements taken during the stability period were excluded and a linear regression was performed. The variables of the mixed model were selected using a backward approach; a reduced model missing the variable of interest was compared to the full model by an ANOVA. The p-value was extracted and the variable was considered as having a significant effect on the MIC for p-values < 0.01.

2.2.9.2 Stability period and cross-resistance

To determine the statistical significance of the stability period, dependent t-tests were performed. MIC at the end of the exposure period (after 10 cycles) were compared to values after the three cycles of stability. If significant differences were observed (p-value < 0.01), the MIC after stabilization was compared to the MIC before exposure to assess if the MIC was reversible.

The significance of the cross-resistance values was investigated using dependent and independent t-tests. MICs for one disinfection agent were compared at similar time points after exposure to both disinfection agents and to the control. Differences were considered significant for p-values < 0.01.

2.2.9.3 Antibiotic susceptibilities

The effects of the exposure to disinfection agents on antibiotic susceptibilities were analyzed using ANOVA on three populations (exposed to CTMA, exposed to BAC and not exposed) after 5 and 10 cycles of exposure and after the stability period. False discovery rate adjustment (Bonferroni) [203] was applied to correct the p-values. For the antibiotics for which the difference was still significant after correction (p-value < 0.01), Tukey's HSD was applied to determine for which pairs the difference was significant. The results were then compared to the EUCAST threshold values for resistance [201].

2.2.9.4 AFM data

The effect of the exposure to disinfection agents on the bacterial mechanical properties was analyzed using a one-tail ANOVA on the three populations (exposed to CTMA, exposed to BAC and not exposed). The p-value was considered significant if < 0.05. To assess the roughness property difference, a non-parametric Mann-Whitney test was applied with a p-value < 0.05.

2.3 Results

2.3.1 Evolution of the MIC of *P. aeruginosa* for CTMA and BAC with exposure cycles and stability after removal of the disinfection agent

2.3.1.1 CTMA

The MIC of *P. aeruginosa* for CTMA was determined by broth microdilutions to be 110 mg l^{-1}. Based on this result, the sub-inhibitory concentration to which the bacterial populations were exposed was set at 95 mg l^{-1} (86% of MIC). This concentration is in the sub-inhibitory range but will also exert a mild selective pressure [178]. For *P. aeruginosa* exposed to this sub-inhibitory concentration of CTMA a significant increase of the MIC was

observed. Each cycle contributed significantly ($p<0.001$) to an average increase of the MIC between 5.0 ± 0.8 mg l^{-1} (replicate 1), 3.6 ± 0.9 mg l^{-1} (replicate 3) and 2.7 ± 0.7 mg l^{-1} (replicate 2). After 10 cycles, the MIC reached a value of 150 ± 10 mg l^{-1} (Figure 2-3A). Overall, the MIC increased by a factor of 1.3 - 1.45. In the control experiment (cycling without exposure), no significant changes in the MICs were found (Table S 2-1, ESI).

The stability of the adaptation to CTMA was tested by five additional cycles in absence of CTMA. The MIC was again tested at the end of these cycles and a significantly lower value was found. However, the MIC after this procedure was still significantly higher (127.5 ± 9.6 mg l^{-1}) than the initial value. The values were at an intermediate level between samples that were non-exposed and exposed to CTMA for 10 cycles (Figure 2-3A, Table S 2-2, ESI).

2.3.1.2 BAC

The MIC of *P. aeruginosa* for BAC was determined to be 80 mg l^{-1}. *P. aeruginosa* was exposed to 40 mg l^{-1} BAC, which corresponds to 50% of the MIC (Figure S 2-1, ESI). The effect of exposure on the MIC of the strains was significant but small for replicate 1, where an average increase of the MIC of 1.36 ± 0.45 mg l^{-1} per cycle was observed. The effect of the cycles was not significant and no increase was observed for replicates 2 and 3. After removal of the BAC from the medium, the small increase observed after 10 cycles is no more present and the MIC is back towards the initial value.

To induce a potentially larger effect, *P. aeruginosa* was exposed to a concentration equal to 88% of the MIC (70 mg l^{-1}). When exposed to this concentration of BAC, the MIC increased first to 125 mg l^{-1} and then to 150 mg l^{-1} (replicates 1 and 2). For replicate 3, the MIC increased to 100 mg l^{-1}. Thus, the final observed increase factors were 1.9 (replicates 1 and 2) and 1.25 times the initial MIC (replicate 3, Figure 2-3B, Table S 2-2, ESI).

The stability of the adaptation to BAC was tested by five additional cycles in absence of BAC. The MIC was again measured after these cycles and the value obtained was similar to the one obtained at the end of the exposure period. Therefore, the MIC values remained at 150 mg l^{-1} (replicates 1 and 2) and a small decrease was observed for replicate 3, with a MIC at 90 mg l^{-1} after the stability period.

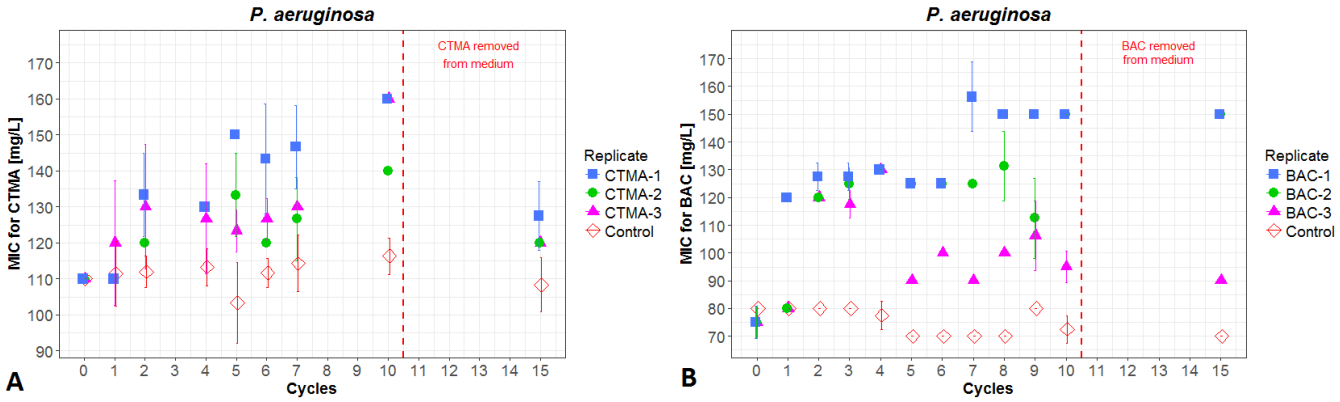


Figure 2-3. Evolution of the MICs of *P. aeruginosa* when exposed to sub-inhibitory concentrations of (A) CTMA (95 mg l^{-1}) or (B) BAC (70 mg l^{-1}).

The effect of absence of the QACs after 10 cycles on the MICs is also shown. Each exposure cycle lasted 48 hours. Each point is the average of at least 4 technical measurements, error bars represent the standard deviations. A. MIC evolution of three *P. aeruginosa* populations exposed to CTMA (CTMA-1/2/3) and one control (not exposed to CTMA) as a function of the exposure cycles. B. MIC evolution of three *P. aeruginosa* populations exposed to BAC (BAC-1/2/3) and one control (not exposed to BAC) as a function of the exposure cycles.

2.3.2 Cross-resistance between the QACs

Cross-resistance between the QACs was assessed by testing the populations for 5 and 10 cycles of exposure to BAC followed by 5 cycles of stability against CTMA and the CTMA-exposed population against BAC. The results are provided in Table S 2-3 (SI) and in Figure 2-4A and B. A weak positive correlation was also found between the increase of the MIC for BAC and the increase of the MIC for CTMA when exposed to BAC (Spearman correlation factor of 0.6) (Figure 2-4A). A stronger positive correlation between the increase of the MIC for exposure to CTMA and the increase of the MIC for BAC when pre-exposed to CTMA was found (Spearman correlation factor of 0.8) (Figure 2-4B).

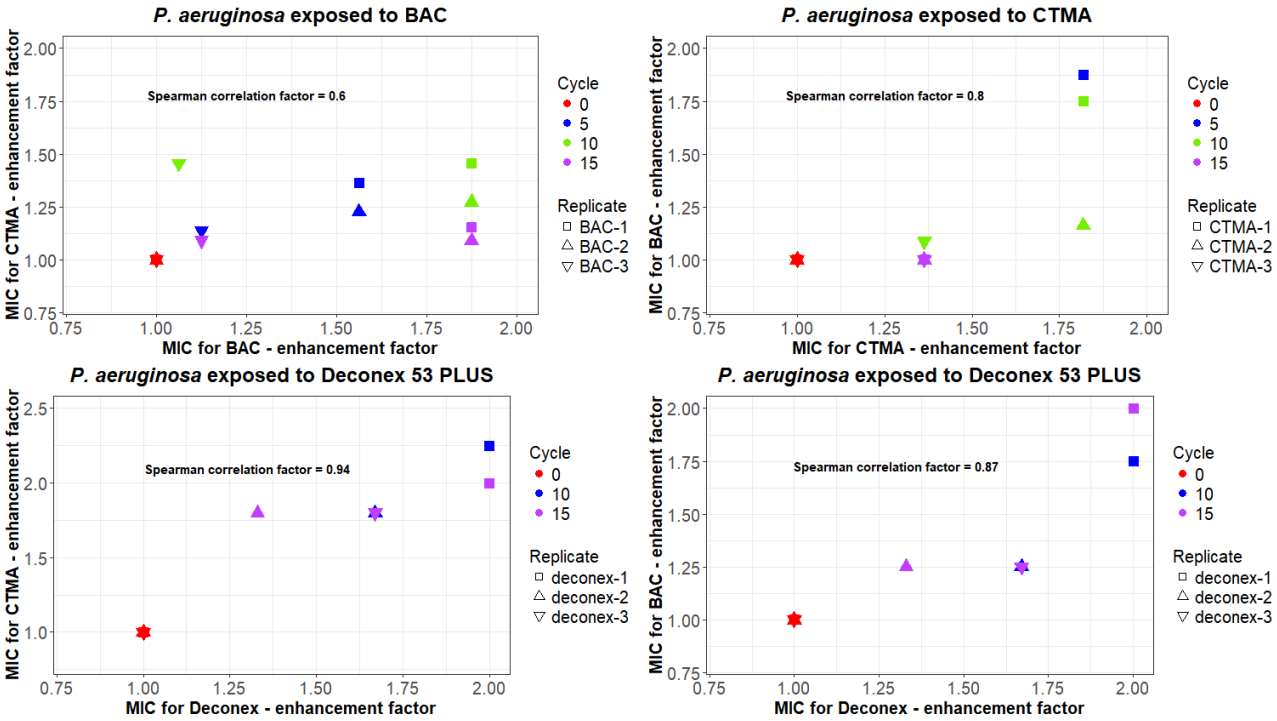


Figure 2-4. Correlation factors for the cross-resistance experiment.

A. Correlation between the enhancement factor of the MIC for CTMA and the MIC for BAC when exposed to CTMA. B. Correlation between the enhancement factor of the MIC for BAC and the MIC for CTMA when exposed to CTMA. C. Correlation between the enhancement factor of the MIC for Deconex® 53 PLUS and the MIC for BAC when exposed to Deconex® 53 PLUS. D. Correlation between the enhancement factor of the MIC for Deconex® 53 PLUS and the MIC for CTMA when exposed to Deconex® 53 PLUS.

2.3.3 Antibiotic susceptibilities after exposure to QACs

The effects of the exposure of *P. aeruginosa* to sub-inhibitory QAC concentrations after 5 and 10 cycles on the antibiotic susceptibilities was tested using an analysis of variance between the control and the exposed populations followed by a Dunnett post-hoc test (Table S 2-4, ESI). From the results of the statistical analyses, after 10 cycles of exposure, significant differences ($p<0.01$) were observed for amikacin, tobramycin, gentamycin (higher resistance of *P. aeruginosa*), and piperacillin-tazobactam (lower resistance of *P. aeruginosa*) for the strain exposed to CTMA versus the control. Bacteria exposed to BAC exhibited statistical differences for levofloxacin, imipenem and minocycline (lower re-

sistance of *P. aeruginosa*) after 10 cycles of exposure. However, the difference in resistance observed with these antibiotics was insufficient to modify the categorical interpretation (Susceptible /Intermediate /Resistant) according to EUCAST breakpoints. Among the 16 antibiotics tested, *P. aeruginosa* is intrinsically resistant to six of them and being exposed to either BAC or CTMA did not affect this intrinsic resistance.

2.3.4 Evolution of the MIC of *P. aeruginosa* for Deconex® 53 PLUS with exposure cycles and stability after removal of the disinfection agent

Figure 2-5 shows the evolution of the MIC of *P. aeruginosa* for exposure to 140 mg l^{-1} of Deconex® 53 PLUS (13 mg l^{-1} active substance), which corresponds to 87% of the MIC (160 mg l^{-1}, 15 mg l^{-1} of active substance). The MIC increased to 300 mg l^{-1} (28 mg l^{-1} of active substance) for one replicate (replicate 1) and to 266 mg l^{-1} (25 mg l^{-1}) for two replicates (replicates 2 and 3). Each cycle contributed to an increase of the MIC by an average of 10 mg l^{-1}, independently of the replicate. The control remained at 168±20 mg l^{-1} (16±1.8 mg l^{-1} active substances). After a stability period of 5 cycles in absence of the disinfection agents, the MIC either stayed at the higher value (replicates 1 and 3) or decreased to 212 mg l^{-1} (replicate 2).

An exposure to Deconex® 53 PLUS also led to a decreased susceptibility to both CTMA and BAC (Figure 2-4C and D). When exposed to Deconex® 53 PLUS, the MIC for CTMA increased to 250 mg l^{-1}, 2.25 times the initial MIC, for replicate 1, and to 200 mg l^{-1} , 1.8 times the initial MIC, for replicates 2 and 3 (Figure 2-4C). The MIC for BAC increased to 140 mg l^{-1}, 1.75 times the initial MIC for replicate 1 and to 100 mg l^{-1}, 1.25 times the initial MIC, for replicates 2 and 3 (Figure 2-4D).

The increase in the MIC was strongly correlated with increases in the MIC for BAC and CTMA with Spearman correlation factors of 0.94 (for CTMA) and 0.84 (for BAC).

2.3.5 Evolution of the MIC of *P. aeruginosa* for Incidin® PLUS with exposure cycles and stability after removal of the disinfection agent

Exposure to Incidin® PLUS during 15 cycles of 24 hours led to a significant increase of the MIC of *P. aeruginosa* The MIC was determined to be 40 mg l^{-1} (10 mg l^{-1} of glucoprotamin) prior to exposure. The concentration of exposure was set at 35 mg l^{-1} (9 mg l^{-1} glucoprotamin; 88% of the MIC). The MIC reached a maximum of 100 mg l^{-1} (26 mg l^{-1} glucoprotamin) after 15 cycles of exposure (Figure 2-5B, replicate 3), which correspond to an increase of the initial MIC by a factor of 2.5 and each cycle increased the MIC by an average of 3.2 ± 1.2 mg l^{-1}. An increase to 70 mg l^{-1} (18 mg l^{-1} glucoprotamin) was observed for replicate 3, which corresponds to an increase of the initial MIC by a factor 1.75 and an increase per cycle of 1.73 ± 0.5 mg l^{-1}. The control (not exposed to Incidin® PLUS) remained at 40 mg l^{-1} (10 mg l^{-1} glucoprotamin) during the cycling period (Figure 2-5B).

The stability of the adaptation to Incidin® PLUS was investigated by removing the disinfection agent from the growth media during 5 cycles (in this case, of 24 hours). The increases in the MICs were stable and irreversible, remaining at the values obtained at the end of the exposure period.

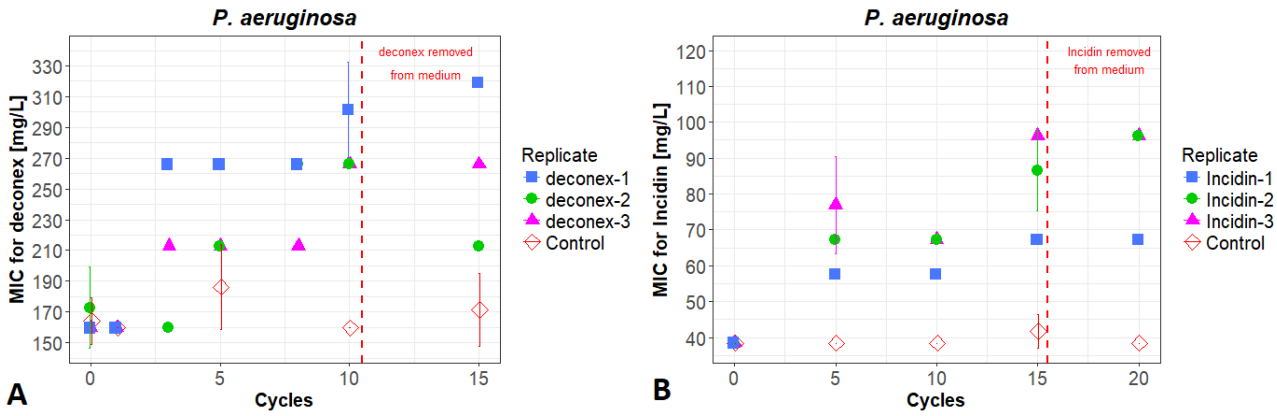


Figure 2-5. Evolution of the *P. aeruginosa's* MIC when exposed to (A) Deconex® 53 PLUS or (B) Incidin® PLUS.

Three *P. aeruginosa* populations (exposed to Deconex® 53 PLUS (A) or Incidin® PLUS (B)) and one control (not exposed to Deconex® 53 PLUS or Incidin® PLUS) as a function of the exposure cycles. Each cycle lasted 24 hours. Each point is the average of at least 4 technical measurements and error bars represent the standard deviations.

2.3.6 Antibiotic susceptibilities following exposure to Deconex® 53 PLUS and Incidin® PLUS

The effects of exposure to sub-inhibitory concentrations of Deconex® 53 PLUS and of Incidin® PLUS concentrations after 5, 10 and 15 cycles on the antibiotic susceptibilities were tested using an analysis of variance between the control and the exposed populations followed by a Dunnett post-hoc test, as with QACs. No differences were observed between the Incidin® PLUS exposed strains and the controls (data not shown).

2.3.7 AFM

The AFM measurements allowed investigating the mechanical properties of single *P. aeruginosa* cells at the nanometric scale. The main properties that were elucidated by this method are: (1) The roughness of the bacterial outer membrane, (2) the stiffness of the whole bacteria cell and (3) its adhesion properties. The AFM probe (also named cantilever tip) having a diameter of roughly 40 nm, is able to detect and scan very small details of the bacterial surface. The *P. aeruginosa* cells showed an average roughness (Ra) of 30nm (Figure 2-6A - left) when no QAC exposure occurred. CTMA treatment strongly affected the outer membrane characteristics of *P. aeruginosa*. The measured Ra after exposure to CTMA was around 95nm, on average, with peaks of 200 nm (Figure 2-6A – right). When BAC was administered, the average measured Ra was around 75 nm (Figure 2-6A – middle). Hence, for both tested QACs the outer membrane roughness significantly changed as a consequence of the positive charge interactions with the membrane components.

Furthermore, the probe indentation on the bacterial cell allowed to obtain a precise measurement of cell stiffness. In addition, the recording of any occurring interaction event, of either weak or strong value in between the tip and the protein/lipid matrix of the outer membrane, allowed to obtain roughness and adhesion parameters. Even though, the cantilever tip was not functionalized with any biological molecule (for instance: fibronectin, collagen, concanavalin-A, antibodies, etc.) and the indentation contact time was less than 100ms, a measurable interaction still remained with adhesion peaks plotted in Figure

2-6B. In Figure 2-6B and C, each point corresponds to a pixel belonging to the whole bacteria population that has been imaged with the AFM (almost 100 cells per treatment in at least 5 different images of 20 μm × 20 μm, with a resolution of 128x128 pixels). For *P. aeruginosa,* the registered average value of adhesion was almost 1.0 nN, whereas after the treatment with BAC it dropped to less than the half, specifically 0.4 nN (Figure 2-6B). Interestingly, the treatment of *P. aeruginosa* with CTMA increased the adhesion properties between the outer membrane and the cantilever tip by almost 50% compared to the original strain, yielding an average value of 1.5 nN. When the adhesion properties after BAC treatment is compared with the CTMA treatment a statistically significant difference (one-tail ANOVA, p-value < 0.05) of more than 3-fold was found, 0.4 versus 1.5 nN, respectively.

In Figure 2-6C the Young's modulus values distribution is plotted as a parameter to represent the bacterial stiffness. The measured stiffness from the three bacteria populations differed significantly only for the CTMA treated case. The determined value for the investigate P. aeruginosa strain (ATCC 27853) is around 210 kPa, which is the same as for the BAC-treated case (almost 210 kPa). Remarkably, CTMA acted differently again from the BAC counterpart by significantly increasing the bacteria stiffness value by almost a factor of three, up to 610 kPa (one-tail ANOVA, p-value < 0.01).

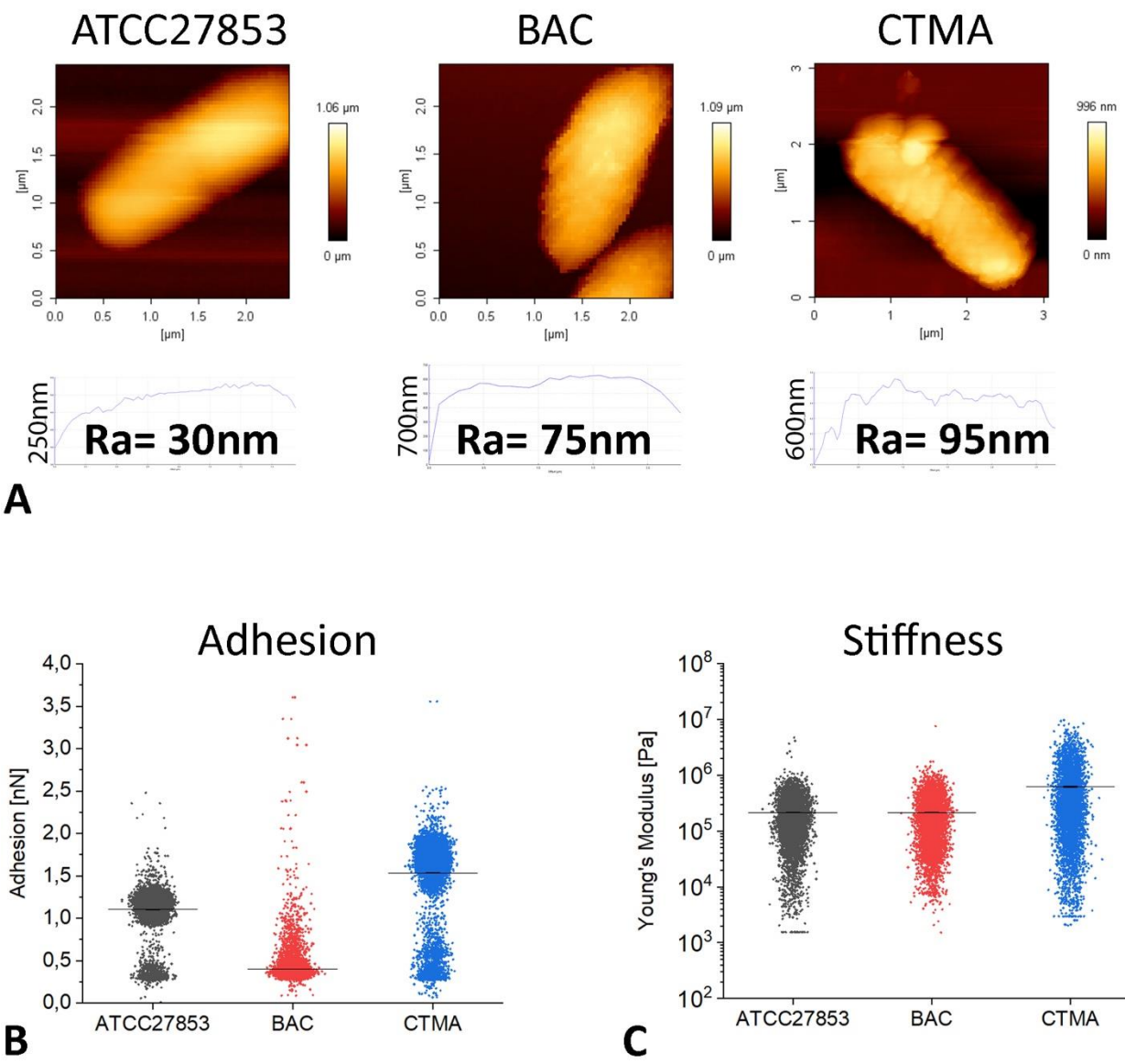


Figure 2-6. AFM investigation of the mechanical properties of *P. aeruginosa*.

A: Two representative images are shown for each tested QAC (BAC, middle and CTMA, right) and one without treatment (ATCC 27583). Below, a typical profile for the outer membrane roughness (Ra) is plotted as its average value. The Y-axes scales differ significantly and from left to right are as follows: 250nm, 700nm and 600nm, respectively. Both treatments with QACs significantly changed the membrane average roughness as reported in numbers in each plot: 30, 75 and 95 nm, respectively. Statistical significance was assessed by the Mann-Whitney test and a p-value < 0.05 was considered significant (n=15). B: Adhesion plot distribution. Each dot represents a single measured pixel from a bacteria cell. C: Stiffness plot distribution. Each dot represents a single measured pixel from a bacteria cell.

2.4 Discussion

2.4.1 Evolution of the MIC of *P. aeruginosa* for CTMA and BAC with exposure cycles, stability after removal of the disinfection agent and cross-resistances between the QACs and with antibiotics

Populations of *P.* aeruginosa were exposed to constant sub-inhibitory concentrations of CTMA and BAC, and adaptation occurred (increase in MIC) when the bacteria were exposed to a concentration of the selected QACs corresponding to approximately 90% of the MIC. A small increase in the MIC was observed for one replicate (out of three) when *P. aeruginosa* was exposed to 50% of the MIC. The strength of the selection pressure was important, as in the case of BAC, the adaptation was much stronger with an exposure concentration at 90% of the MIC compared to an exposure concentration of 50% of the MIC. However, it cannot be excluded that stronger adaptation may occur for longer exposure periods with < 90% of the MIC. When exposed to approximately 90% of the MIC of the selected QACs, the general trend was an increase of the MIC, however, the individual replicates showed different patterns. Previous studies revealed that a long-term exposure to BAC led to a reduction in the community diversity and an increase in the resistant bacteria [179,204]. Variability in the adaptation was also observed here, resulting from a low selective pressure. This variability can be the result of either different mechanisms or differences in the speed of adaptation. Exposure of *E. coli* strains to sub-inhibitory concentrations of BAC led to different phenotypical variants [179] and it is possible that the resulting exposed populations in this study are also comprised of phenotypic variants.

The results showed that an increase was still present at the end of the 10 cycles investigated (Figure 2-3 and Figure 2-5). In previous studies it has been shown that an increasing concentration of QACs ultimately reaches a plateau of the MIC [187]. It can be hypothesized that an exposure to a constant sub-inhibitory concentration of QACs also ultimately leads to a plateau of the MIC.

After removal of the QAC from the growth medium, the MIC for CTMA decreased to similar values prior to the exposure. However, when exposed to BAC, the MIC reached during the exposure period remained stable at the higher level. If the mechanisms are of similar nature, as shown with the experiments on cross-resistance, exposure to CTMA seems to trig-

ger a transient mechanism of adaptation, which is different from BAC. Mechanisms of adaptation or resistance to QACs includes modification of the membrane or expression of efflux pumps [182,185,204]. These mechanisms are consistent with a transient change either in the structure of the membrane or in the level of expression of other resistance mechanisms. The differences observed between the two selected QACs might be linked to the composition of the QACs, BAC being a mixture of several quaternary ammonium compounds with different aliphatic chains ranging from 8 to 16 carbon atoms with an aromatic ring (Figure 2-1A), while CTMA is a single quaternary ammonium compound with one aliphatic chain length (Figure 2-1B). However, this would need further investigations.

When exposed to CTMA first, *P. aeruginosa* became more resistant to BAC and when exposed to BAC, it became more resistant to CTMA (Figure 2-4A and B,). This result was demonstrated by a positive correlation score obtained in both cases. This might mean that the mechanisms involved are shared or similar for the two QACs. The nature of the mechanism might rather be the result of phenotypical changes for CTMA, because the increase of the MIC after removal of the QAC was reversible. For the other tested compounds, genotypic changes cannot be excluded. The increased resistance might be linked to morphological changes of the membrane observed by AFM or to the expression of other mechanisms, such as efflux pumps as shown in previous studies [182]. When exposed to a commercial disinfection agent containing QACs, cross-resistance was also observed with BAC and CTMA. Strains exposed to Deconex® 53 PLUS demonstrated a higher tolerance for CTMA and BAC compared to their control. Similarly, to BAC and CTMA only, the degree of tolerance was strongly correlated with the level of adaptation. The decrease in the susceptibility, when exposed to Deconex® 53 PLUS, was stronger for CTMA, for which a maximum increase in the MIC of up to a factor 1.25 was observed (Figure 2-4C). For BAC, it reached a maximum increase of the MIC by a factor 1.8 (Figure 2-4D).

Cross-resistance with antibiotics was less evident than for QACs. Statistical differences were observed with either increased or decreased resistance to antibiotics. However, the categorical interpretation (S/I/R) according to EUCAST breakpoints were not modified. These results are in accordance with previous studies, in which exposure to BAC or CTMA did not lead to any significant difference in the resistance profile to antibiotics [110,183,184].

2.4.2 Morphological evolution of the bacteria

AFM investigations revealed some differences in the treated compared the non-treated bacterial cells. The roughness of the cells increased with the treatment, like the results of other studies investigating antibiotics or antimicrobial agents [197,205,206]. As QACs are agents targeting the outer membrane of bacteria, this result indicates that even at subinhibitory concentrations, bacterial membranes are affected by QACs. The mechanism of action of QACs is known to be an association of the positively charged quaternary nitrogen with the head groups of acidic-phospholipids of the membrane [207]. This interaction decreases the fluidity of the membrane at concentrations close to the MIC [207]. Adaptation of *P. aeruginosa* to one QAC was observed to result in changes in the cell surface hydrophobicity and biofilm formation, but also to be the cause of changes in the outer membrane proteins and the permeability [183,185]. When exposed to CTMA, the stiffness of the cells increased by a factor of 3 (Figure 2-6C), leading to more rigid cells compared to the control, which is consistent with the expected mode of action of QACs. This was not the case for the cells exposed to BAC, as such an increase in the Young modulus was not observed.

Interestingly, the bacterial surface adhesion properties, measured as the pulling force exerted by the membrane matrix towards an uncoated-silicon probe, revealed a strong decrease when the bacteria were treated with BAC, whereas in the case of CTMA an increase in the unspecific adhesion properties was registered (Figure 2-6B). Adhesion properties of a bacterial cell are also linked with their ability to create biofilms [208]. *P. aeruginosa* is known to form biofilms and to use biofilms as a defense against different chemicals. It was previously observed that BAC inhibits the biofilm formation of *P. aeruginosa*, which could explain the observed decrease in the adhesion properties when exposed to BAC [209]. In contrast, in this study, the adaptation of *P. aeruginosa* to CTMA was associated with modifications of the bacterial membrane and an increase in adhesion properties, which could enhance the biofilm formation. These two mechanisms are consistent with previous observations when increasing concentrations of QACs were used, either with biofilm as a resistance mechanism [133] or with an exposure to BAC preventing the biofilm formation [209].

Even though CTMA and BAC modified the biomechanical properties of *P. aeruginosa* in a significantly different way, they both strongly affect the cells by modifying their morphological properties and hence the bacterial interaction with the external environment. Besides, the outer membrane roughness significantly changed by increasing the numbers and the height of "hills" and "valleys" of the external lipid bilayer and the lipo-poly-saccharides matrix. Potentially, the measured roughness could be a consequence of a deeper and stronger modification at a lower membrane level, mainly affecting the inner membrane.

2.4.3 Practical implications: exposure to commercial disinfection agents

Two commercial disinfection agents, currently used in the health care context for medical instrument pre-cleaning/disinfection and surface cleaning detergent were tested to compare their effects with single compounds (BAC and CTMA). Exposure to both disinfection agents, in their commercial forms, led to an adaptation of the exposed populations. The increase of the MIC, after 10 cycles, was similar for both products, an average factor of 1.7 for Deconex® 53 PLUS and 1.75 for Incidin® PLUS. Both commercial products led to adapted populations of *P. aeruginosa* like with QACs. The adaptation is stable during the 5 cycles in absence of the product, which is similar to BAC but different to CTMA. Exposure to QAC-containing commercial products also yielded an increased tolerance to the two pure QACs tested, but no changes in the antibiotic resistances.

This result is relevant for cases where bacterial populations are in contact with residual concentrations of the product. Due to a widespread application of QACs in hospitals, some uses in cleaning and discharge of wastewaters or other point sources may contain relatively high sub-inhibitory concentrations of disinfection agents, which may lead to development of increased MICs in the bacterial communities. Depending on the applied QAC-containing disinfection agents, it may also lead to morphological changes and adhesion and biofilm formation potentials. In contrast to these high sub-inhibitory concentration scenarios, the exposure to the µg/L levels of QACs present in municipal wastewater treatment plants may not lead to changes in the MICs.

2.5 Conclusion

This study showed for two selected QACs and two commercial disinfection agents that *P. aeruginosa* cells could adapt to these treatments, when exposed to approximately 90% of the MIC. This was exemplified by an increase of the MICs by a maximum factor of 1.45 for CTMA, 1.9 for BAC, 1.7 for Deconex® 53 PLUS and 1.75 for Incidin® PLUS. When exposed to a lower concentration (approximately 50% of MIC), strains exposed to BAC showed a slight adaptation to the product in one replicate. The slight increase observed upon exposure to 50% of the MIC, may suggest that adaptation is also possible at smaller concentrations, but longer exposure times are necessary. Moreover, not only, the MIC for the QAC to which the bacteria were exposed was increased but also the MIC for the other selected QAC was increased, which means that there is cross-resistance between the two selected QACs. The increase in the resistance to the compounds can be a result of an increase of the stiffness as shown by the AFM results for CTMA. However, the increase in resistance observed when exposed to BAC, is likely caused by a different mechanism, as no increase in the stiffness was observed. Differences were also observed in the adhesion properties following exposure and would require further investigation especially on the biofilm formation potential after exposure to sub-inhibitory concentrations of QACs. The roughness, was increased when exposed to both QACs, confirming that QACs are affecting the membrane and the observed increase might be the consequence of changes at the inner membrane level. The differences observed in the nanomechanical properties of the membrane are also consistent with the differences observed in the resistance evolution following exposure. This may indicate that different or slightly different mechanisms are involved with the different QACs. In contrast, no significant change of the QAC pre-treated bacteria was observed regarding the resistance to antibiotics in this experimental setup based on the EUCAST interpretational scheme. This result seems to exclude the spread of antibiotic resistant *P. aeruginosa* in the environment following exposure to constant sub-inhibitory concentrations of QACs. The effects obtained with two commercial disinfections agents (containing a QAC or glucoprotamin) were similar to results obtained with QACs.

Acknowledgments

Funding of this project by the Swiss Confederation represented by the Federal Office for the Environment FOEN 3003 Bern (15.0023.KP/P214-1092), EPFL are acknowledged for M.V., M.B., F.B., U.v.G. Fundings by the Swiss National Grants 200021-144321, 407240_167137, CRSII5_173863, the Gebert Rüf Stiftung GRS-024/14 and NASA NNH16ZDA001N-CLDTCH are acknowledged for L.V, G.D and S.K. We also would like to thank Myriam K.S. Ballo (EPFL), Dessislava Savova Bianchi (university center of legal medicine (CUMRL)), Virginie Bachmann (EPFL) and Caroline Gachet Aquillon (EPFL) for their technical assistance during this project.

Supporting Information for Adaptation of *Pseudomonas aeruginosa* to constant sub-inhibitory concentrations of quaternary ammonium compounds

Table S 2-1. MIC determination – concentration used

To define the initial MIC (i.e., the MIC of the bacterial strain prior to any exposure), two different sets of concentrations were used. The first set of concentrations used was from 0.06 to 32 mg/l and from 1 to 512 mg/l. Once the MIC for the selected was determined, the concentration interval was refined to have a more precise data.

Table S2. Initial MICs determination

CTMA – concentration interval [c_{min} – c_{max}] mg/l	MIC [mg/l]	BAC – concentration interval [c_{min} – c_{max}] mg/l	MIC [mg/l]
[0.06 – 32]	>32	[0.06 – 32]	>32
[1 – 512]	128	[1 – 512]	128
[10 – 150]	110	[50 – 150]	80

Deconex 53 PLUS– concentration interval [c_{min} – c_{max}] mg/l	MIC [mg/l] (active substance)	Incidin® PLUS – concentration interval [c_{min} – c_{max}] mg/l	MIC [mg/l]
[0.06 – 32]	16	[0.06 – 32]	>32
[1 – 512]	16	[1 – 512]	64
[10 – 150]	15	[10 – 100]	40

Table S 2-2. Average and standard deviations (4 data points) for the MIC before the exposure (cycle 0) during the exposure (cycles 1 to 10) to the QACs and after the stability period (QAC removed from the media for cycles 11 to 15; cycle 15)

QAC	Cycle (48 h)	Repli-cate	MIC [mg l^{-1}]		Repli-cate	MIC [mg l^{-1}]		Repli-cate	MIC [mg l^{-1}]		Replicate	MIC [mg l^{-1}]	
			Average	Standard deviation		Aver-age	Standard deviation		Average	Standard de-viation		Average	Standard devia-tion
CTMA	0	1	110.0	0.0	2	110.0	0.0	3	110.0	0.0	Control	110.0	0.0
CTMA	1	1	110.0	0.0	2	110.0	0.0	3	120.0	17.3	Control	111.4	9.0
CTMA	2	1	133.3	11.5	2	120.0	0.0	3	130.0	17.3	Control	112.0	4.5
CTMA	4	1	130.0	0.0	2	130.0	0.0	3	126.7	15.3	Control	113.3	5.2
CTMA	5	1	150.0	0.0	2	133.3	11.5	3	123.3	5.8	Control	103.3	11.2
CTMA	6	1	143.3	15.3	2	120.0	0.0	3	126.7	5.8	Control	111.7	4.1
CTMA	7	1	146.7	11.5	2	126.7	11.5	3	130.0	0.0	Control	114.3	7.9
CTMA	10	1	160.0	0.0	2	140.0	0.0	3	160.0	0.0	Control	116.4	5.0
CTMA	15	1	127.5	9.6	2	120.0	0.0	3	120.0	0.0	Control	108.3	7.5
BAC	0	1	80.0	0.0	2	80.0	0.0	3	80.0	0.0	Control	80.0	0.0
BAC	1	1	120.0	0.0	2	80.0	0.0	3	80.0	0.0	Control	80.0	0.0
BAC	2	1	127.5	5.0	2	120.0	0.0	3	120.0	0.0	Control	80.0	0.0
BAC	3	1	127.5	5.0	2	125.0	5.8	3	117.5	5.0	Control	80.0	0.0
BAC	4	1	130.0	0.0	2	130.0	0.0	3	130.0	0.0	Control	77.5	5.0
BAC	5	1	125.0	0.0	2	125.0	0.0	3	90.0	0.0	Control	70.0	0.0
BAC	6	1	125.0	0.0	2	125.0	0.0	3	100.0	0.0	Control	70.0	0.0
BAC	7	1	156.3	12.5	2	125.0	0.0	3	90.0	0.0	Control	70.0	0.0
BAC	8	1	150.0	0.0	2	131.3	12.5	3	100.0	0.0	Control	70.0	0.0
BAC	9	1	150.0	0.0	2	112.5	14.4	3	106.3	12.5	Control	80.0	0.0
BAC	10	1	150.0	0.0	2	150.0	0.0	3	95.0	5.8	Control	72.5	5.0
BAC	15	1	150.0	0.0	2	150.0	0.0	3	90.0	0.0	Control	70.0	0.0

Table S 2-3. Cross-range results for sequential exposures of P. aeruginosa to BAC followed by CTMA or CTMA followed by BAC.

Populations exposed to CTMA were tested against BAC and exposed to BAC were tested against CTMA to investigate cross-resistance. This table summarizes the MIC obtained for the cross-resistance experiments.

Exposed to CTMA			**Exposed to BAC**			**Exposed to Deconex® 53 PLUS**		
Cycle (48 h)	MIC	Cycle (48 h)	MIC	MIC [mg l^{-1}]	MIC [mg l^{-1}]	Cycle (48 h)	MIC	MIC [mg l^{-1}]
0	CTMA[a]	0	CTMA[a]	110	110	0	CTMA[a]	200
	BAC[a]		BAC[a]	80	80		BAC[a]	80
5	CTMA	5	CTMA	150[b] / 200[c]	130[b] / 150[c]	5	CTMA	NA
	BAC		BAC	80[b] / 150[c]	90[b] / 125[c]		BAC	NA
10	CTMA	10	CTMA	150[b] / 200[c]	140[b] / 160[c]	10	CTMA	200[c] / 250[b]
	BAC		BAC	90[b] / 150[c]	90[b] / 140[c]		BAC	100[c] / 140[b]
15[d]	CTMA	15[d]	CTMA	150	90[b] / 150[c]	15[d]	CTMA	200[c]/250[b]
	BAC		BAC	80	150		BAC	100[c]/160[b]

[a] control without QAC, [b] replicate 1; [c] replicates 2 and 3; [d] MIC obtained after the stability period

Table S 2-4. Average and standard deviation data of the Inhibition Zone Diameters (IZD) obtained with the disc diffusion method.

cycle	Treatment	Amikacin IZD [mm]		Ceftazidime IZD [mm]		Ciprofloxacin IZD [mm]		Gentamycin IZD [mm]		Cefepime IZD [mm]		Imipenem IZD [mm]	
		Average	Std deviation	Average	Std deviation	Average	Std deviation	Average	Std deviation	Average	Std deviation	Average	Std deviation
0	NA	24	1	25	0.9	35.1	0.8	21	2.2	31.2	1.2	24.1	1.1
5	CTMA	26.6	0.9	29.8	1.2	36.9	1.5	20.7	1.1	35.6	1.4	27.7	0.7
10	CTMA	22.8	2	26.6	1.1	34.8	1	18.7	2	32.6	1	27.2	0.8
15	CTMA	24	1.7	25.8	0.8	33.8	1.2	20	0.9	30.2	1.8	24.8	1.5
5	BAC	28.9	2.3	30	2.5	38.1	2	24.4	1.2	35.4	1.8	27.8	2.6
10	BAC	26.6	1.2	30.7	1.9	37	1	22.8	0.7	33.2	1.3	28	1.6
15	BAC	27.2	0.7	27.3	1.2	30.8	1.5	23.6	1.2	28.8	2.4	26.6	1.6
5	Control	27.1	2.6	27.9	5.7	33.7	3	19.9	1.6	33.3	3.6	26.1	1
10	Control	25.4	2.4	25.8	1.4	31.7	1.6	21.1	2.1	27.1	1.4	25.2	0.8

cycle	Treatment	Levofloxacin IZD [mm]		Meropenem IZD [mm]		Minocycline IZD [mm]		Tobramycin IZD [mm]		Piperacillin-tazobactam IZD [mm]	
		Average	Std deviation	Average	Std deviation	Average	Std deviation	Average	Std deviation	Average	Std deviation
0	NA	26.7	1.1	36.9	1.5	18.4	1.4	25.6	1.1	30.2	1.1
5	CTMA	28.6	1.7	38.1	1.9	20.4	1.7	27.6	1.5	31.2	1.2
10	CTMA	27.7	1	35	1	13.2	1.6	26.6	1	27.9	2
15	CTMA	26	0.7	35.6	0.9	15.4	1.1	22.6	1.3	28	1.3
5	BAC	32	1.1	35.6	2	14	1.3	24.3	0.7	28.9	1.5
10	BAC	30.7	0.9	35.5	1.2	15.8	0.8	24.3	0.8	27.8	1
15	BAC	27	1.8	34	1.1	11.2	2	23.8	2.3	26.6	1.2
5	Control	28	2.3	35.8	4.4	10	2.1	24.3	0.9	30.4	2.7
10	Control	27	1.1	33.3	1	14.7	0.7	23.2	1	26.4	0.9

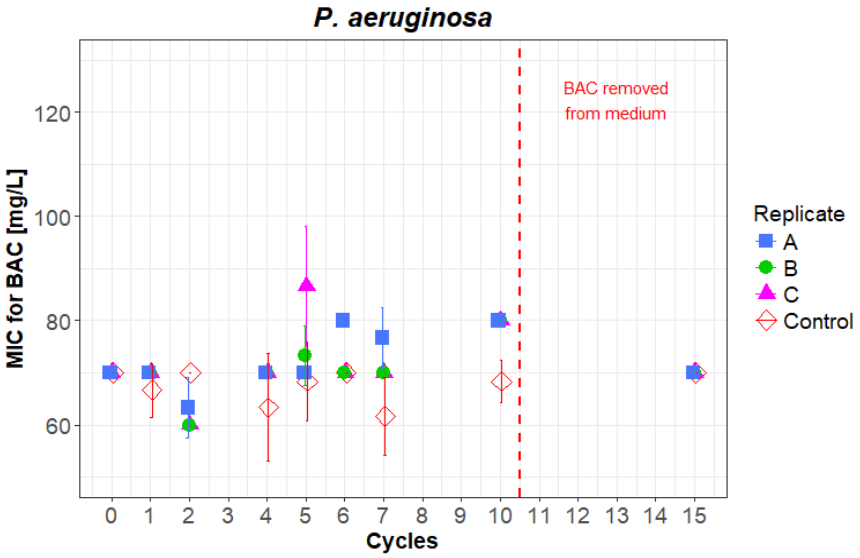


Figure S 2-1. Evolution of the MICs of *P. aeruginosa* when exposed to sub-inhibitory concentrations of BAC (40 mg l^{-1}).

The effect of absence of the QACs after 10 cycles on the MICs is also shown. Each exposure cycle lasted 48 hours. Each point is the average of at least 4 technical measurements, error bars represent the standard deviations.

3 Effect of cetyltrimethylammonium chloride on various *Escherichia coli* strains and their inactivation by ozone and monochloramine

M. Voumard, F. Breider, U. von Gunten

This chapter is in preparation for publication in *Water Research.*

Experimental design, protocol development, experimental part, data modelling and statistical analysis, writing of the paper were performed by Margaux Voumard under the scientific advising of U. von Gunten and F. Breider.

Pre-exposure to CTMA was performed by Vincent Berweiler and the ozone inactivation experiments were done with the help of Gabrielle Léger.

Abstract

Cethyltrimethylammonium chloride (CTMA) is one of the most used quaternary ammonium compounds (QACs) in consumer products. CTMA and other QACs are only partially eliminated in municipal wastewater treatment and they can interact with bacteria in biological processes. So far, there is only limited information on the antimicrobial efficiency of CTMA in matrices other than standard growth media and if and how CTMA influences conventional chemical disinfection. The results obtained in this study showed that susceptibility of *E. coli* to CTMA was significantly enhanced in phosphate-buffered saline, lake water and wastewater compared to broth. In broth, a minimum inhibitory concentration (MIC) of CTMA of 20mgL^{-1} was observed for *E. coli*, whereas a 4-log inactivation occurred for CTMA concentrations of about 4 mgL^{-1} in buffered ultra-purified water, a lake water and wastewater effluent. Pre-exposure of *E. coli* AG100 to CTMA led to an increased susceptibility for ozone with second-order inactivation rate constants increasing by a factor of 1.5. An opposite trend was observed for monochloramine with second-order inactivation rate constants decreasing by a factor of 2. For the tetracycline-resistant *E. coli* AG100tet, the second-order inactivation rate constant decreased by a factor of almost 2 and increased by a factor of 1.5 for ozone and monochloramine, respectively, relative to the strain AG100. The simultaneous presence of CTMA and ozone enhanced the second-order inactivation rate constants for CTMA concentrations of 2.5 mgL^{-1} by a factor of 3. For monochloramine also an enhancement was observed, which was at least additive but might also be synergistic. Enhancement by factors from 2 to 4.5 were observed for CTMA concentrations > 2.5 mgL^{-1}.

Keywords: cetyltrimethylammonium chloride; ozone; monochloramine; *E. coli;* susceptibility, disinfection

3.1 Introduction

Some disinfection agents have become widely used consumer products in the past decades [99,152,210]. Moreover, the recent COVID-19 pandemic has increased the use of such disinfection agents [137]. They are used to disinfect surfaces, surgical instruments and objects, such as toys, phones, or anything that can be contaminated by pathogenic microorganisms and they are also included in soaps, laundry washing detergents and personal care products (PCPs) [132,211]. Finally, they are used as biological stains such as crystal violet or propidium iodide [128,129]. Among such disinfection agents, quaternary ammonium compounds (QACs) represent a large part of the active ingredients present in commercial formulations [137]. QACs are used because of their broad-spectrum antimicrobial and surfactant-like properties. QACs contain an acyclic saturated hydrocarbon chain with a chain length mainly between 12 and 18 carbons, and a quaternary ammonium group. The antimicrobial effect of QACs is linked to the hydrocarbon chain length, with an optimum of the bactericidal effect with a chain length between 12 – 14 carbons. This is hypothesized to correspond to the length required to penetrate the bacterial cell membrane [129–132]. The most common commercial compounds are benzalkonium chloride (BAC; Figure 3-1A), cetyltrimethylammonium chloride (CTMA; Figure 3-1B), dimethyldodecyl ammonium chloride (DDAC; Figure 3-1C) and cetylpyridinium chloride (CPC; Figure 3-1D) [129].

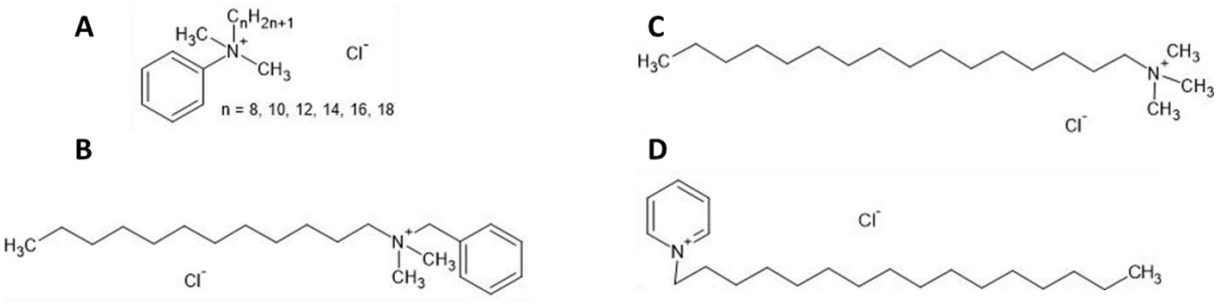


Figure 3-1. Chemical structures of selected quaternary ammonium compounds (QACs).

(A) benzalkonium chloride (BAC, CAS 63449-41-2); (B) cetyltrimethylammonium chloride (CTMA, CAS 57-09-0); (C) dimethyldodecylammonium chloride (DDAC, CAS 139-07-1) and (D) cetylpyridinium chloride (CPC, CAS 123-03-5)

Among the QACs, CTMA is the most commonly used in personal care products such as shampoos and conditioners, cosmetics and in household products [152]. The widespread inclusion of CTMA in consumer products in addition to its presence in industrial products warrants further investigations on this compound.

QACs act on bacteria by destabilization of the cell membrane, formation of micelles of QACs and cell membrane components, ultimately leading to membrane solubilization and cell lysis [129]. Low concentrations of QACs, in the sub-inhibitory range, have been shown to promote resistance in bacteria [110,212]. Additionally, promotion of resistance to antibiotics has been observed in some cases [152,153,182]. The resistance mechanisms promoted below minimum inhibitory concentrations (MICs) of QACs are mostly achieved by modifications of the membrane: modification of the outer and inner cell membrane, modification of the density and structure of porins and overexpression of efflux-pumps, including some that are specific for QACs [107,134,135,150,157,160,166,182,183,213].

The inclusion of QACs in industrial products and PCPs results in their inevitable direct release to the aquatic environment and the presence in sewage systems and wastewater [95,137,152] and their removal during wastewater treatment is often incomplete [137,145,166,214]. Concentrations of QACs were detected in the mgL^{-1} range in hospital wastewater effluents [95], in the μgL^{-1} range in municipal wastewater treatment plant (WWTP) influents and in the μgL^{-1} to ngL^{-1} range in WWTP effluents [145]. Removal of QACs during wastewater treatment occurs mostly by sorption onto sludge [111,137,215]. The sorptive properties of QACs also lead to an accumulation in sediments with patterns corresponding to the yearly use of QACs and the quantities in sediments were found to be quite high from µg/kg to mg/kg [96,145,216] and up to the g/kg range for sewage sludge [96]. This creates environmental reservoirs for these contaminants. The accumulation and possible local release of QACs in water from these reservoirs is of concern because of potential resistance promotion among bacteria in these aquatic environments [152,153,214,217].

Typically, QAC concentrations in wastewater effluents are below the calculated MICs and therefore below the toxicity level of bacteria, which were determined to be in the range of 5-60 mgL^{-1} for *E. coli* [99,102,174], 0.5-75 mgL^{-1} for *S. aureus* [99,102], and 5-500 mgL^{-1} for *P. aeruginosa* [99,107,212]. Even though, the MICs determined under standard conditions are useful to assess

the efficiency of biocides, they do not necessarily indicate the potential toxicity of QACs in natural waters. MICs are typically determined in broth or in agar, and usually include incubation at the ideal temperature for the growth of the bacteria of interest [199]. Even though, these conditions are ideal for bacteria, they are not representative of natural and technical aquatic systems, where nutrients can be limited, temperatures are far from the optimum for bacterial growth and other parameters such as pH can influence the susceptibility of bacteria. Therefore, broth can represent the worst-case scenario in terms of the efficiency of QACs and the required dose but may not be a good model for other matrices. Moreover, the determination of MICs could lead to an overestimation of the susceptibility of bacteria to a biocide in a natural environment.

Chemical disinfectants such as chlorine, ozone or chloramine are often used to inactivate microorganisms during water treatment [7,8]. Among these disinfection methods, ozone has been shown to be most efficient [9] with second-order inactivation rate constants in the order of 10^6 M^{-1} s^{-1} for *E. coli* [72,218]. Inactivation by ozone occurs by direct oxidation with ozone [218], leading to damages of membranes and internal cell components, and ultimately to cell death [9,23,64]. Additionally, ozone also reacts with nucleic acids, leading to mutations in the cell and an inhibition of DNA replication and ultimately division of the cell [9]. For other widely used disinfectants the inactivation kinetics are significantly lower [33]. One case in point is monochloramine, which was introduced to reduce the formation of chlorinated disinfection byproducts such as trihalomethanes [7]. The mode of action of monochloramine on bacteria is mostly unknown, but some reactivity was found with cysteine and methionine [33,219], a slow reaction with DNA and RNA [219] but no reaction is expected with fatty acids and polysaccharides of the cell membrane [219]. The required oxidant exposures (CT values) for the same extent of inactivation of bacteria for monochloramine are several orders of magnitude higher compared to ozone or free chlorine [23,53,64,65,219].

The presence of QACs before or during chemical disinfection of water and wastewater treatment may be an additional factor influencing the inactivation of microorganisms. To this end, it is currently unknown if additive and/or synergistic effects could occur for systems in which chemical disinfectants and QACs are both present.

Ozone has very low reactivity with saturated QACs [9,141,220], but reactions with unsaturated QACs can occur [9,220]. QACs are also expected to have very low reactivity with monochloramine [33]. This absence of reactions between ozone or monochloramine and QACs enables a simultaneous presence and allows an evaluation of combinations of QACs with ozone or with monochloramine in comparison to scenarios with pre-exposure of bacteria to QACs before a chemical disinfection.

The aims of this study were to (i) investigate the susceptibility of *E. coli* to CTMA, a widely used QAC, in broth, buffered ultra-purified water, secondary municipal wastewater effluent and a surface water and (ii) to investigate the impacts of pre-exposure or simultaneous presence of CTMA on the inactivation kinetics of different strains of *E. coli* by ozone and monochloramine.

3.2 Material and Methods

3.2.1 Bacterial strains

E. coli AG100, AG100A and AG100tet were kindly provided by Miguel Viveiros (Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal). Characteristics of the strains are described elsewhere [221,222]. Briefly, AG100 has the efflux pump *acrAB* intact, AG100A has it deleted and AG100tet has it overexpressed [222]. Bacteria were stored at -20°C in glycerol (Sigma-Aldrich, Switzerland) and broth (20% glycerol) for short-term storage and at -80°C for long-term storage.

Frozen stocks were streaked on Luria Bertani (LB; Sigma-Aldrich, Switzerland) agar and stored at 4°C weekly for short-term storage. Bacterial stocks were prepared by inoculating 2-3 colonies into LB or Mueller-Hinton broth (MHB; Sigma-Aldrich, Switzerland) and incubated overnight (12-16 hours, 37°C, 180 rpm). MHB was enriched with 100 mgL^{-1} kanamycin for AG100A; 10 mgL^{-1} tetracycline for AG100tet. To maintain the CTMA adaptation, MHB was enriched with it for the pre-exposed strains. The CTMA concentrations used were 35 mgL^{-1} for AG100 and 25 mgL^{-1} AG100A.

Overnight cultures were washed three times by centrifugation (5000rpm, 4°C, 15 min) and resuspended in phosphate-buffered saline (PBS, 10mM, pH 7.4). Concentrations of the bacteria were adjusted with PBS using the optical density at 600nm (OD600) to reach an absorbance of

1.1-1.3 in a 1 cm cuvette, which was determined to correspond to a concentration of 10^8 CFUmL^{-1} (colony forming units).

Bacteria were enumerated by serial dilution in PBS and spreading 100µL on agar media (plate count agar, PCA; Sigma-Aldrich). The limit of quantification of bacteria with this method is 1 CFUmL^{-1} and only plates with colonies between 3 and 300 CFUmL^{-1} were considered. For experiments with monochloramine, thiosulfate (final concentration 1mM) was added to the PBS for the first dilution to quench monochloramine.

3.2.2 Quaternary Ammonium Compound

The disinfection agent used in this study was cetyltrimethylammonium chloride (CTMA, CAS 112-02-7). CTMA (Sigma-Aldrich, Switzerland) had a concentration of 25 weight % in H_2O with the highest purity available. Stock solutions of 10,000 mgL^{-1} CTMA were prepared in PBS. PBS solutions were sterilized by autoclaving them prior to the addition of CTMA and the stock solutions were filtered by 0.2 µm filters (Filtropur S 0.2S; Sarstedt, Switzerland). The filter-sterilized solutions were then further diluted with PBS to reach the concentrations of interest. The stock solutions were kept at room temperature and used within a week.

3.2.3 Susceptibility to CTMA in different water matrices

The susceptibility was tested with four different media, MHB, PBS, water from Lake Geneva (Switzerland) and a secondary wastewater effluent from the wastewater treatment plant in Morges, Switzerland. The characteristics of the different media are presented in Table 3-1. Sterility of the water was checked by plating them on agar plates.

The different water matrices were spiked with bacteria to reach an initial concentration of 10^6 CFUmL^{-1}. Different doses of CTMA were added in the reactors and the solutions were mixed for 1 hour at 600rpm, room temperature. After one hour, an aliquot was taken and serial diluted in PBS to be plated on PCA for colony enumeration.

Table 3-1. Characteristics of the different media used

	MHB	**PBS**	**Lake water**	**2nd wastewater effluent**
Origin	Sigma-Aldrich, CH	Sigma-Aldrich, CH	Geneva lake	Morges
Sterilization	Autoclave, 15min, 121°C	Autoclave, 15min, 121°C	Filtered at 0.2µm	Filtered at 0.2µm
pH	7.5	7.4	8.5	8.2
Temperature for inactivation experiments	20±2°C	20±2°C	20±2°C	20±2°C
DOC	NA	NA	1.5 mgL^{-1}	22 mgL^{-1}
Alkalinity	NA	NA	1.75 $mmolL^{-1}$	7.26 $mmolL^{-1}$
Composition	Casein acid hydrolysate: 17.5 gL^{-1} Beef extract: 3 gL^{-1} Starch: 1.5 gL^{-1}	NaCl: 8 gL^{-1} KCl: 0.2 gL^{-1} Na_2HPO_4: 1.44 gL^{-1} KH_2PO_4: 0.24 gL^{-1}	NA	NA

3.2.4 Ozone generation

Ozone was produced with an ozone generator (model CMG 3-3 or CMG 3-5, Innovatec, Rheinbach, Germany) from pure oxygen. The resulting ozone/oxygen mixture was bubbled in ultra-purified water (Milli-Q) at 20°C. The temperature was controlled using a recirculating chiller (model F-108, Büchi Labortechnik AG, Switzerland) and a cylindrical reaction vessel with thermostatic jacket when the room temperature exceeded 25°C. The ozone stock solutions reached concentrations ranging from 0.45 to 0.55 mM. The ozone concentration in the stock solution was measured for each experiment by direct spectrophotometry in a 1 cm quartz cuvette at 260 nm with a molar absorption coefficient of 3200 $M^{-1}\,cm^{-1}$ [9].

3.2.5 Ozone exposure

During the experiments, ozone exposures were controlled by a method described in Wolf *et al*. (2018) [223]. Briefly, the ozone stability and hence the ozone exposure was controlled by its reaction with trans-cinnamic acid (*t*-CA) in the presence of *tert*-butanol (*t*-BuOH) to avoid interferences by hydroxyl radicals. *t*-CA is highly reactive with ozone, with a second-order rate constant $k = 7.6\times10^5\ M^{-1}s^{-1}$ at 20°C [223] and it does not affect the viability of bacteria (data not shown). In the protocol used for this study, the ozone dose was determined by the difference

between the initial and the final trans-cinnamic acid concentration, which differed from the original protocol were the benzaldehyde concentration was used [223]. This choice was based on the finding that there was an interaction between the bacteria and benzaldehyde (data not shown). A detailed description of the method is provided in Text S 3-1.

50 mL reactors were prepared each day of experiment with a range of t-CA concentrations (100-400 µM) in PBS. *t*-BuOH (20 mM) was added as •OH scavenger during ozonation. A 500 µL aliquot was taken before the experiment to determine the exact initial *t*-CA concentration. Bacteria were added to the reactors to reach an initial concentration of 10^6 CFU mL^{-1}. A 100 µL aliquot was taken to obtain the exact initial bacterial concentration. The reactors were then sealed and an aliquot of the ozone stock solution was injected by a syringe to reach a range of concentrations of 50-375 µM and mixed at 600 rpm for 2-3 min. During the ozone injection, a second syringe connected to a 0.2 µm filter was inserted to avoid overpressure in the reactors and contamination by microorganisms. The syringe was removed immediately after the ozone injection. After 5-10 min, when ozone was fully depleted, two aliquots were withdrawn from the reactors. The first was subjected to HPLC analysis for *t*-CA quantification (Text S 3-1 and Text S 3-2, SI) and the second was diluted in PBS for bacteria quantification.

3.2.6 Monochloramine

Stock solutions of NH_2Cl (2mM) were produced on each experimental day by mixing solutions of HOCl (4 mM) and NH_4Cl (6 mM) in sterile PBS (10mM, pH 7.4) in a 1:1.5 Cl:N molar ratio. The solutions were mixed for one hour to ensure completion of monochloramine formation. The concentration of monochloramine was determined using the method described in Schreiber and Mitch [224] by direct spectrophotometry at 245nm and 295nm. The equations and molar extinction coefficients used are provided in Text S 3-3 (SI).

The determined monochloramine concentrations were between 1.7 and 2 mM and in the ≤ µM range for dichloramine. As the solutions were further diluted to reach µM concentrations for monochloramine, dichloramine became negligible.

As with ozone, 50mL reactors were prepared on each experimental day in parallel to the monochloramine solution. The reactors were filled with PBS and bacteria to reach a concentration of 10^6 CFU mL^{-1}. A sample was taken prior the addition of monochloramine to determine the

exact initial concentration of bacteria. Monochloramine was added and the reactors were closed and mixed at 600 rpm for the duration of the experiment. 100µL aliquots were withdrawn from the reactors at different time interval (between 0-120 min) and mixed with PBS and thiosulfate for 2-3 minutes to quench monochloramine. The samples were then further diluted and plated on PCA for counting. Monochloramine concentrations in the reactors were measured at the beginning (2.5-5 µM) and at the end of the experiments (2.4-5 µM) using the DPD method to determine the NH_2Cl exposure.

3.2.7 DPD method

The concentration of monochloramine in the reactors was monitored at the beginning and at the end of the experiment using the DPD method [225]. 1.5 mL of the solution was added to a cuvette containing 75 µL of a DPD solution (200 mgL^{-1} EDTA; 2 mLL^{-1} Sulphuric acid; 2.75 gL^{-1} *N,N*-Diethyl-p-phenylenediamine sulphate salt) and 75 µL of a buffer solution (46 g L^{-1} KH_2PO_4; 24 gL^{-1} NaH_2PO_4; 800 mgL^{-1} EDTA; 20 mgL^{-1} $HgCl_2$). The absorbance at 510 nm was read immediately for free chlorine measurements, as a control for the theoretical absence of free chlorine, and a few crystals of KI were added for the chloramine measurements.

3.2.8 Pre-exposure to CTMA and determination of MIC

Tubes containing 10 mL of MHB with a range of CTMA concentrations were inoculated with 100 µL of an overnight culture of *E. coli* AG100 or AG100A diluted to obtain a final concentration of 10^6 CFU mL^{-1} in the tube at the beginning of the cycle. The tubes were incubated at 37°C and 180 rpm for 48 hours and growth was examined. The lowest concentration not presenting growth was determined as the MIC.

According to the determined MIC, a new series of 10 mL MHB tubes was prepared and inoculated with 10µL of the bacterial solution from the tube with the highest concentration presenting growth. A summary of the concentrations and MICs obtained is presented in Table S 3-1 (SI). This step was repeated 10 times. A purity check was added after each cycle by striking the inoculate on non-specific agar (plate-count agar, PCA; Sigma-Aldrich, Switzerland).

3.2.9 Data modelling and analysis

3.2.9.1 Ozone

Ozone inactivation followed pseudo first-order kinetics and was modelled using a modified version of the Chick-Watson model [226]. The model was modified to take into account the ozone exposure instead of the contact time (Table 2, equation 1) [226].

The modelling and analysis were performed using the software R. The following packages were used: “dplyr”, “scales”, “lsmeans”. The visualization of the data and the creation of the graphs were performed with the packages “ggplot2” and “gridExtra”.

3.2.9.2 Monochloramine

The non-linear inactivation curves for *E. coli* by monochloramine were modelled using a model introduced by Geeraerd et al. (2005) [227]. The model is empirical and takes into account a lag-phase and a tailing. The model was designed for inactivation curves as a function of time (Table 2, equation 2) and in a modified version to take into account the oxidant exposure instead of the contact time only (Table 2, equation 3).

The modelling and analysis were also performed using R. The package “nlsMicrobio” [228,229] containing the Geeraerd model was used in addition to the models for ozone. The data visualization and the plotting of the figures were done by the same packages as for ozone.

Table 3-2. Chick-Watson and Geeraerd equation to model the inactivation of microorganisms.

Equation		**Parameters**
1-2. Modified Chick-Watson model using the disinfectant exposure $\int [C]dt$ [226]	$ln\left(\frac{N}{N_0}\right) = -k \int [C]dt$	N_0 = Initial number of organisms N = number of survivors k = second-order inactivation rate constant [C] = disinfectant concentrations
3-1. Time-based Geeraerd model [227]	$N(t) = (N(0) - N_{res})e^{-k_{max}t}\left(\frac{e^{k_{max}S_l}}{1 + (e^{k_{max}S_l} - 1) \cdot e^{-k_{max}t}}\right) + N_{res}$	N(0): initial number of colonies N(t): number of colonies at a particular t N_{res} : residual population density k_{max} : specific inactivation rate constant Sl = Parameter for the lag-phase t = time of exposure
3-2. Modified Geeraerd model to take into account the CT parameter instead of the time only (this study)	$N(Ct) = (N(0) - N_{res})e^{-k_{max}Ct}\left(\frac{e^{k_{max}S_l}}{1 + (e^{k_{max}S_l} - 1) \cdot e^{-k_{max}Ct}}\right) + N_{res}$	N(0): initial number of colonies N(Ct): number of colonies at a particular Ct N_{res} : residual population density k_{max} : specific inactivation rate constant Sl = Parameter for the lag-phase Ct = Ct value= oxidant exposure

3.3 Results and Discussion

3.3.1 Susceptibility of *E. coli* to CTMA: Effect of water matrix

An initial MIC of 25 mgL^{-1} CTMA was obtained for *E. coli* strain AG100 in MHB with an increasing trend for multiple exposures to sub-inhibitory levels of CTMA (Table S1, SI). The susceptibility of the *E. coli* strain AG100 to CTMA was further tested in different water matrices and the corresponding results are presented in Figure 3-2. In absence of CTMA (left part of Figure 3-2), the concentration of the *E. coli* strain AG100 remained constant over one hour. In presence of ≤ 10 mg/L CTMA, the bacteria concentration remained constant in MHB. However, 4-log inactivation of *E. coli* was observed at about 4 mgL^{-1} CTMA in PBS, lake water, and secondary municipal wastewater effluent.

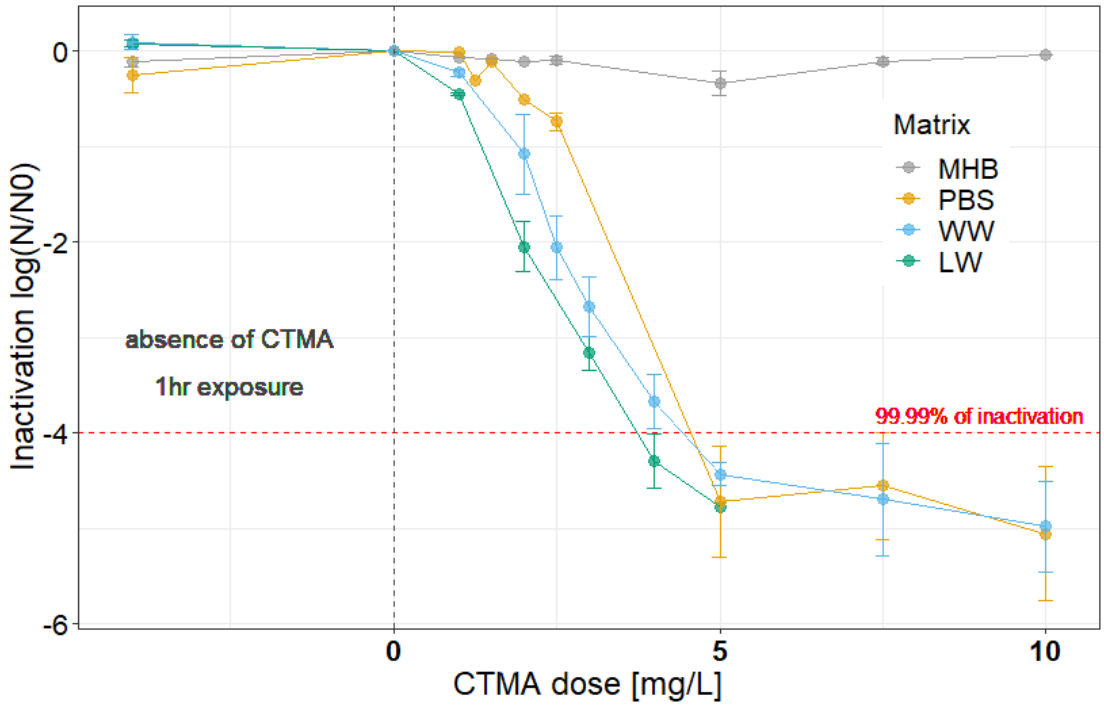


Figure 3-2. Dose-response curve for the exposure of *E. coli* (AG100) to CTMA in MHB, PBS, secondary wastewater effluent (WW; wastewater effluent, wastewater treatment plant Morges, Switzerland) and lake water (LW; Lake Geneva, Switzerland).

The exposure time was one hour and room temperature for all matrices and the left part of the figure represents the control experiment with no CTMA exposure for one hour. The pH-values were 7.5 (MHB), 7.4 (PBS), 8.2 (WW) and 8.5 (LW).

The significant differences between the experiments with MHB and the other water matrices show that the water matrix composition seems to be an important factor. Several factors have been identified to influence the survival and replication of bacteria in natural waters. They include light, temperature, pH, grazing of zooplankton or other predators such as bacteriophages, osmotic pressure, resistance to starvation and the presence of low molecular weight toxins [230,231]. As the control experiments showed no differences in the number of colonies after one hour, parameters such as light, pH, temperature and osmotic pressure can be ruled out since they were similar for all matrices. No inactivation was observed in the blank lake water and WW experiments, indicating no effect of zooplankton or bacteriophages. The presence of low molecular weight toxins would only be possible in lake water and wastewater. However, due to the similar results in PBS, it seems an unlikely effect. The possible remaining factors are a limitation in the nutrients available and interaction between CTMA and MHB.

The first hypothesis is starvation of the cells. Indeed, all media have no or a limited nutrient levels compared to MHB. Long-term starvation of *E. coli* has been shown to lead to an exponential decay of the viability of the cells [232]. To maintain viability, the presence of nutrients is crucial and it has been demonstrated that adaptation was possible for *E. coli* in nutrient deprived environment, by e. g., the use of nutrients from dead cells [232]. However, with the short time exposure in the experiments of this study, adaptation and survival in presence of CTMA was probably not possible in a nutrient-deprived medium.

However, the short duration of the experiments (60 min) and the fact that the bacteria were not starved prior the experiments reduces the probability of the nutrient limitation hypothesis without excluding it. The second possibility is the interaction of CTMA with some components of the MHB. CTMA, and QACs in general, are cationic surfactants which form micelles in aqueous media [233]. The formation of micelles due to the composition of MHB would prevent an inactivation. If the interaction between CTMA and bacteria is electrostatic attraction with the positive-charged head of CTMA and the negatively charged membrane, the lysis of the cell is a result of the penetration of the CTMA tail in the membrane. The formation of micelles would prevent the penetration step, by blocking the tail inside the micelle, thus inhibiting the action of CTMA. Turbidity was observed in MHB at high concentrations of CTMA in this study and was also reported previously [110]. The formation of larger and more stable micelles in MHB and not in

PBS, lake water or wastewater effluent would explain the differences observed in the susceptibility of *E. coli* to CTMA.

The differences observed between MHB and the other media, including lake water and wastewater are significant with respect to the determination MICs and minimum bactericidal concentrations (MBCs). MICs are determined in broth, usually MHB, and include incubation at 37°C. This represents ideal conditions for bacteria but is not representative of natural or technical aquatic systems. The values obtained for conditions other than MHB are smaller compared to the values available in literature (5-60 mgL^{-1} for QACs in general and *E. coli*) [99,102,234] and the value obtained in this study for CTMA, using a standard method [199] (20-25 mgL^{-1}, Table S 3-1, SI). Lake water, wastewater effluent and PBS showed a 4-log inactivation of *E. coli* at CTMA concentrations of approximately 4 mgL^{-1}, which is significantly below the MIC calculated at 20-25 mgL^{-1}(Table S 3-1, SI) with a standard method. The standard MIC method relies on the growth of bacteria, and more precisely, on the inhibition of visible growth [199]. As it relies on growth, the composition of the media and the presence of nutrients is crucial, and therefore, it is not possible to determine the MICs in media (e.g., PBS, lake water, wastewater) not allowing the growth of the bacteria of interest. However, MICs are used as an indication for the susceptibility of bacteria to a QAC and to other biocides are therefore over-estimating the susceptibility compared to dose-response curves. Therefore, the standard method to determine MICs of surfactants is useful for a worst-case scenario, with conditions that are ideal for bacteria but it is not representative of antimicrobial effects of QACs in the aquatic environment.

3.3.2 Effect of a pre-exposure to CTMA on the inactivation kinetics of AG100 and AG100A by ozone and monochloramine

Strains of *E. coli* AG100 and AG100A were pre-exposed to CTMA using a stepwise protocol. The susceptibility to CTMA decreased after this exposure (Figure S 3-1, SI), consistent with previous findings that showed a decrease in the susceptibility to QACs following exposure to sub-inhibitory concentrations of CTMA for *P. aeruginosa* [212]. After pre-exposure to CTMA, the inactivation kinetics of the *E. coli* strains by ozone and monochloramine were investigated. The corresponding results for ozone and monochloramine are provided in Figure 3-3A and Figure S 3-2 (SI). Inactivation curves for ozone followed pseudo first-order kinetics and therefore, a modified Chick-Watson approach was applied (Table 3-2, 1). The quality of the fit of the model was

assessed with the R^2 values, which were in the range 0.72 - 0.95 (Table S 3-2, SI). All the obtained second-order rate constants for ozone are also summarized in Figure 3-4A.

For monochloramine, the inactivation curves presented a lag-phase and no tail (Figure 3-3B). Modelling was performed with a modified version of the empirical Geeraerd model [227] (Table 3-2, 2) and fitting of the curves was assessed using the R^2 values, which were in the range 0.98 and 0.99 (Table S 3-3, SI). All the obtained second-order rate constants for ozone are also summarized in Figure 3-4B.

The modelling described in Table 3-2 allowed to obtain second-order inactivation rate constants. For monochloramine, the second-order rate constant is calculated from the linear section of the inactivation plot. All the obtained second-order inactivation rate constants are presented in Figure 3-4, Figure S 3-2, Table S 3-2 and Table S 3-3 (SI).

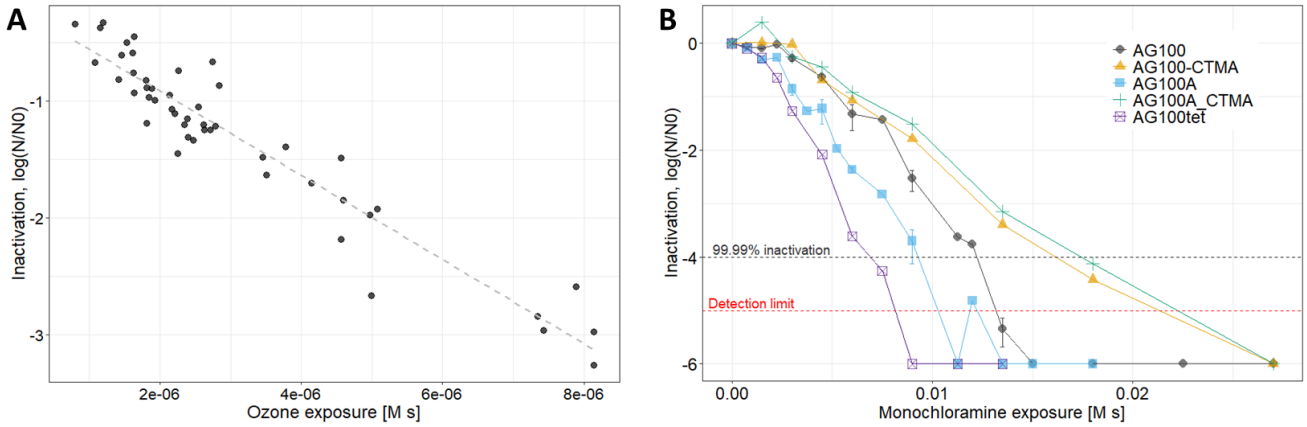


Figure 3-3. Inactivation kinetics of *E. coli* by ozone and monochloramine.
(A) AG100 inactivation by ozone (60 experiments), the other strains are available in Figure S 3-2 (SI); (B) inactivation of AG100 and AG100A not pre-exposed to CTMA, AG100 and AG100A pre-exposed to CTMA and AG100tet by monochloramine. All experiments were done in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (20mM) for the experiments with ozone to exclude hydroxyl radical reactions. The data points are the average values of at least 4 technical measurements of each replicate and the dashed line represents the linear regression of the points for the ozone experiments. For monochloramine, each point is the average of at least four independent experiments and the error bars are the standard deviations. For ozone experiments, all the bacterial counts were above the detection limit. For monochloramine experiments, the lines show 99.99% inactivation and the detection limit, respectively.

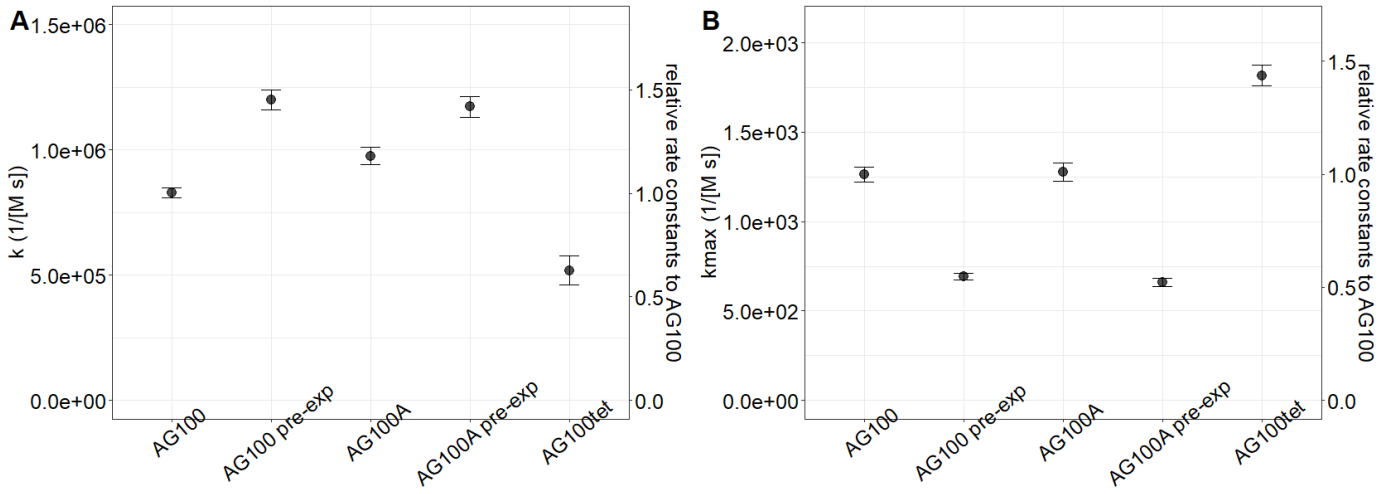


Figure 3-4**.** Second-order rate constants for the inactivation of *E. coli* AG100, AG100A not pre-exposed to CTMA, AG100 and AG100A pre-exposed to CTMA and AG100tet by (A) ozone and (B) monochloramine.

All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (20mM) for the experiments with ozone to exclude hydroxyl radical reactions. Left and right y-axes represent the second-order rate constant in M^{-1} s^{-1} and the relative second-order rate constant normalized to $k_{ox,AG100}$ in absence of CTMA, respectively. The data points are the average of at least three independent experiments with at least five replicates per experiment. The error bars represent the standard deviations obtained from the modelling for the second-order rate constants. The rate constants are also available in Table S 3-2 and Table S 3-3 (SI).

Overall, the obtained results differed between ozone and monochloramine. Pre-exposed to CTMA, *E. coli* (AG100 and AG100A) had a greater susceptibility to ozone with higher second-order inactivation rate constants, whereas the opposite trend was observed for monochloramine (Figure 3-4). Moreover, the overexpression of the AcrAB-TolC efflux pump system in AG100tet led to a decreased and increased susceptibility to ozone and monochloramine, respectively (Figure 3-4). Overall, the observed patterns are exactly opposite, which indicates different modes of action for ozone and monochloramine.

AG100, AG100A and AG100tet differ by their expression of efflux pumps and antibiotic resistance (kanamycin for AG100A and tetracycline for AG100tet). The inactivation of the AcrAB-TolC efflux pump system in AG100A had no effect on the inactivation kinetics by either ozone or monochloramine (Figure 3-4). This result indicates that a normal efflux is not involved in

any resistance to ozone or monochloramine with no change in the susceptibility to both disinfectants. In addition, the kanamycin resistance of this strain is also not influencing the inactivation kinetics. However, the over-expression of that same efflux pump system as in AG100tet has an impact on the kinetics of inactivation, decreasing the second-order rate constant by a factor of almost 2 for ozone and increasing it by a factor of 1.5 for monochloramine (Figure 3-4). Tetracycline-resistant bacteria were reported to have an increased susceptibility to chlorine in a previous study [235]. It was suggested that the over-expression of efflux pumps allowed more chlorine to interact with the bacterial cell membrane, however it was not clear how this may happen [235]. The results obtained here with monochloramine are consistent with this finding. In the case of ozone, the tetracycline resistance led to bacteria that are slightly more resistant. Previous studies on the ozonation of antibiotic-resistant bacteria showed various results with either no differences in the susceptibility or a reduction in the susceptibility [74,75,225,236,237]. However, there is no evidence in the literature of a reduction in the susceptibility to ozone caused by efflux pumps.

The activation of efflux pumps are among the mechanisms of resistance to QACs [181,182,238]. This was confirmed in the present study with the efflux of ethidium bromide, a fluorescent substrate (Figure S 3-3, SI). The decrease in the fluorescence of the colonies exposed to CTMA confirmed the activation of multidrug efflux pumps following the exposure to CTMA (Figure S 3-3, SI) [222,239], however, the exact type of efflux pump is unknown. Furthermore, the results differed between AG100tet and the pre-exposed strains which indicates either that efflux pumps play no role in the susceptibility to ozone and monochloramine or that the type of efflux pump is crucial. For monochloramine, it has been shown that *E. coli* with modifications of the expression of membrane proteins, including a down-regulation of the *acrE*, a multi-drug efflux pump system led to a reduction in the susceptibility [240]. In another case, the response to monochloramine of *E. coli* showed an activation of genes belonging to the stress response mechanism, including the activation of multidrug efflux systems [241]. These results show the complexity and the number of different mechanisms possible for the bacteria when responding to a stress.

Apart from the mechanisms of resistance, membrane damages can also be the consequence of an exposure to CTMA [212]. Bacteria pre-exposed to CTMA would have an alteration of the membrane and efflux pumps overexpressed as it was shown with ethidium bromide (Figure S 3-3). In the case of inactivation by ozone, as the inactivation is very quick, the membrane alteration could be the dominant factor and thus enhance the inactivation. For monochloramine, the

inactivation is much slower, with a difference of 3 orders of magnitude for the second-order rate constants compared to ozone (Figure 3-4). This lower reaction rate could allow the activated efflux pump system to pump out monochloramine, thus reducing the inactivation.

Overall, the differences observed in the pre-exposed strains compared to the other strains are caused by CTMA but there might be multiple reasons of the observed changes. It is interesting to note that the differences are opposite for ozone and monochloramine, which indicates that this observation may be related to the reactivity and the properties of the applied chemical disinfectant.

3.3.3 Effect of the presence of CTMA on the inactivation kinetics of AG100 by ozone and monochloramine

Since CTMA has very low or no reactivity with ozone and monochloramine, disinfection experiments can be performed in presence of CTMA simultaneously with the selected chemical disinfectants. This could be a situation encountered in real water disinfection systems. Different concentrations of CTMA in the range of 0.1 mgL^{-1} to ≤ 5 mgL^{-1} were chosen according to Figure 3-2 for the combined CTMA-chemical oxidants experiments. Inactivation curves for ozone and monochloramine are presented in Figure 3-5 and Figure S 3-4 (SI) and the corresponding second-order rate constants obtained by the modified Chick-Watson model and the modified Geeraerd model are presented Figure 3-6. The fitting parameters for each curve are presented in Table S 3-4 and Table S 3-5 (SI). Linearity was observed for ozone inactivation up to a concentration of 2.25 mgL^{-1}. For higher concentrations, the inactivation was too fast for the method used here to exhibit first-order kinetics and the corresponding second-order inactivation rate constant are therefore not presented in Figure 3-6.

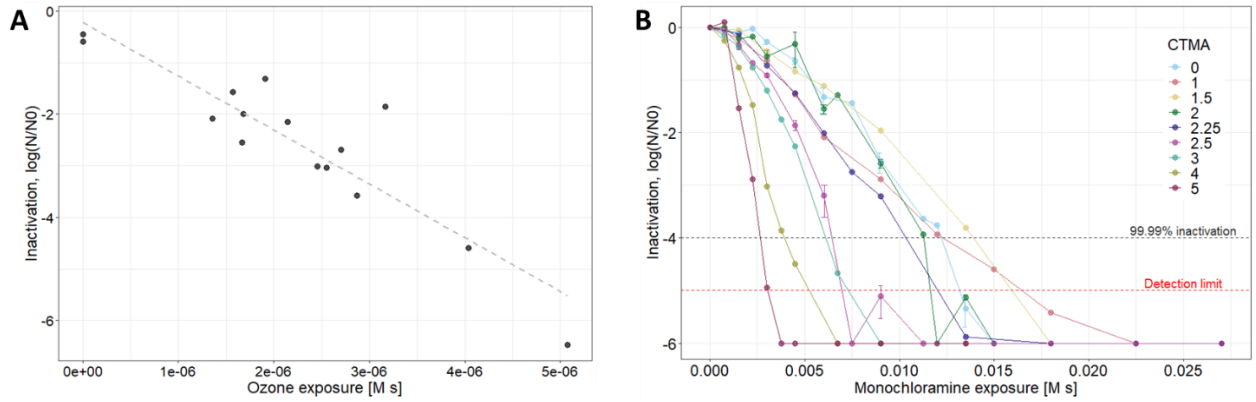


Figure 3-5. Inactivation kinetics of *E. coli* AG100 in absence and presence of CTMA.
(A) Ozonation in presence of 2.25 mgL^{-1} CTMA (15 experiments, see Figure S 3-4(SI) for the other CTMA concentrations). (B) Monochloramination for varying concentrations of CTMA. All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (20mM) for the experiments with ozone to exclude hydroxyl radical reactions. For ozone, the data points are the mean values of at least four technical measurements of each replicate and the dashed line represents a linear regression of the points. For monochloramine, each point is the average of at least three independent experiments and the error bars are the standard deviations.

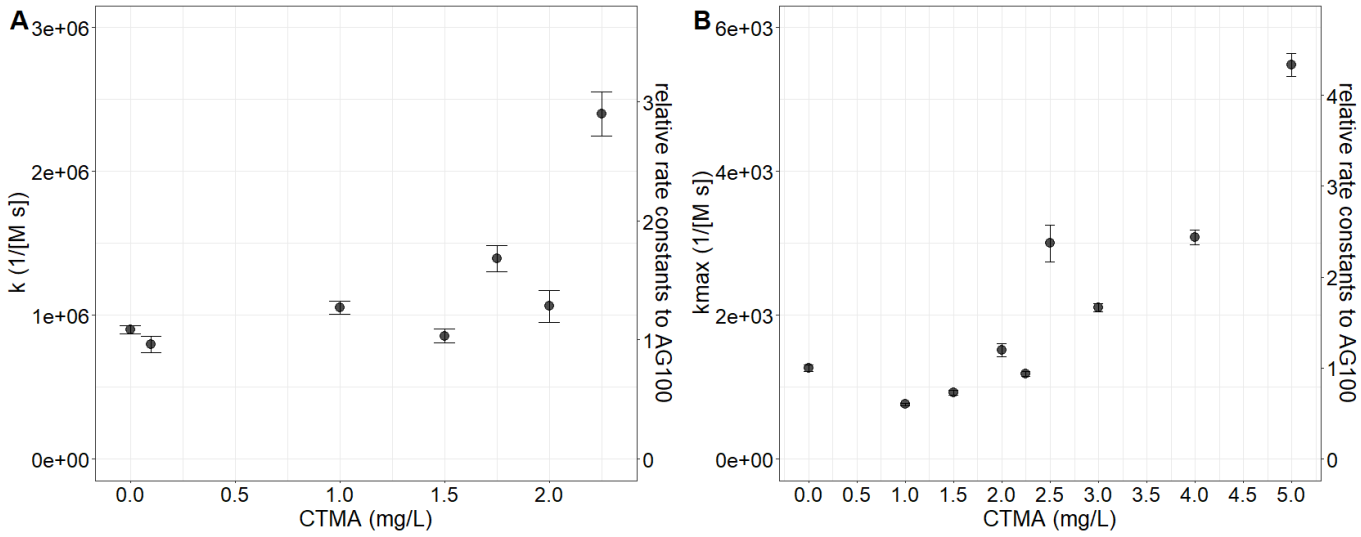


Figure 3-6**.** Effect of the simultaneous presence of CTMA and chemical disinfectant on the second-order rate constants for the inactivation of *E. coli* AG100. (A) ozone and (B) monochloramine.
All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (20mM) for the experiments with ozone. The left y-axis represents the second-order rate constant in $M^{-1}s^{-1}$ and the right y-axis represents the relative second-order rate constant normalized to

the absence of CTMA. Each point is the average of the second-order constant of at least three independent experiments. The error bars represent the standard deviations obtained from the modelling of the second-order rate constants. The rate constants are also available in Table S 3-4 and Table S 3-5.

A similar pattern for the inactivation of *E. coli* was observed for the simultaneous presence of CTMA and ozone or monochloramine (Figure 3-6). For low concentrations of CTMA, there is little influence on the inactivation kinetics. For higher CTMA concentrations, the inactivation kinetics are enhanced with higher second-order inactivation rate constants. For ozone, for CTMA concentrations > 2.25 mgL^{-1}, the applied method did not allow to determine second-order inactivation rate constants.

In absence of ozone, CTMA concentrations ≥ 1.5 mgL^{-1} already led to 0.5-2-log inactivation for the applied experimental conditions (Figure S 3-4, SI). In presence of CTMA, the same level of inactivation was achieved with smaller ozone doses (Figure S 3-4, SI). This increase of inactivation efficiency is consistent with an additive or synergistic effect resulting from the combination of ozone with CTMA. There is only limited reactivity between ozone and CTMA; therefore, an antagonist effect it is unlikely. Moreover, CTMA and ozone have different modes of action on the cell, targeting different constituents. While targeting different cell components, they both act on the membrane, leading to destabilization and permeation of it, allowing an enhanced penetration of ozone inside the bacterial cell.

Ozone reaction took place in seconds, and a Chick-Watson model was applied. The Chick-Watson model relies on (pseudo-)first order kinetics, which was not the case for concentrations of CTMA > 2.25 mgL^{-1}. Despite not being able to determine second-order rate constants, it was still possible to observe an enhanced inactivation trend from the inactivation curves themselves (Figure S 3-4, SI).

For monochloramine, a gradual increase of the second-order inactivation rate constants was observed for CTMA concentrations between 2.5 and 5 mgL^{-1}. In this case, the curves showed similar patterns and the data shown in Figure 3-5B could be modelled by the Geeraerd model provided in Table 3-2. Therefore, the individual contributions of CTMA and monochloramine on the inactivation of *E. coli* could be elucidated. The inactivation curves were plotted as a function of time with a comparison of the individual contributions of monochloramine and CTMA

to the overall inactivation of *E. coli* (Figure 3-7). To obtain similar levels of inactivation for the different CTMA-monochloramine combinations for the same time, two different monochloramine concentrations were chosen: 5 mgL^{-1} NH_2Cl for 2 and 2.5 mgL^{-1} of CTMA and 2.5 mgL^{-1} NH_2Cl for 3, 4 and 5 mgL^{-1} of CTMA. Both concentrations of monochloramine led to similar levels of inactivation based on the monochloramine exposure (Figure S 3-5).

At CTMA concentrations $\leq$ 2.5 mgL^{-1}, the inactivation curves are similar for monochloramine only and the combination of monochloramine with CTMA, demonstrating that monochloramine is the main contributor to inactivation. At 3 mgL^{-1} CTMA, both monochloramine and CTMA seem to contribute equally to the inactivation. For CTMA concentrations > 4mgL^{-1}, the trend changes and inactivation seemed to be mostly controlled by CTMA with only a minor effect of monochloramine. These results indicate that there is at least an additive effect for the two disinfectants.

The mode of action of QACs, which includes mainly the disruption of the cell membrane and the modes of action of ozone and monochloramine, which include reactions with cell components is consistent with the results obtained here. A destabilization of the membrane enhances the penetration of ozone or monochloramine inside the cell allowing a more efficient inactivation. Moreover, because ozone and monochloramine do not react with CTMA, a consumption of this QAC is not expected during the reaction time of the experiment.

To investigate the combined effect, the individual inactivation curves of AG100 by CTMA (3 mgL^{-1}) and monochloramine were added and compared to their combination. A CTMA concentration of 3 mgL^{-1} was chosen because of an apparent similar level of inactivation by monochloramine and CTMA. Because the sampling times were different, the curves were modelled using the Geeraerd model and a good agreement between model and experiment was obtained (Figure 3-8). A comparison of the overall inactivation in the combined CTMA-monochloramine experiment shows that the overall inactivation is higher than calculated from the sum of the modelled curves (Figure 3-8). This enhancement suggests a slightly synergistic effect and not completely independent damages. However, the approximations of the modelling approach and the variation of the experimental data, leave some uncertainty regarding synergism. Nevertheless, it is clear from the data that no antagonistic effect is taking place and that the inactivation is faster and enhanced in presence of both CTMA and monochloramine.

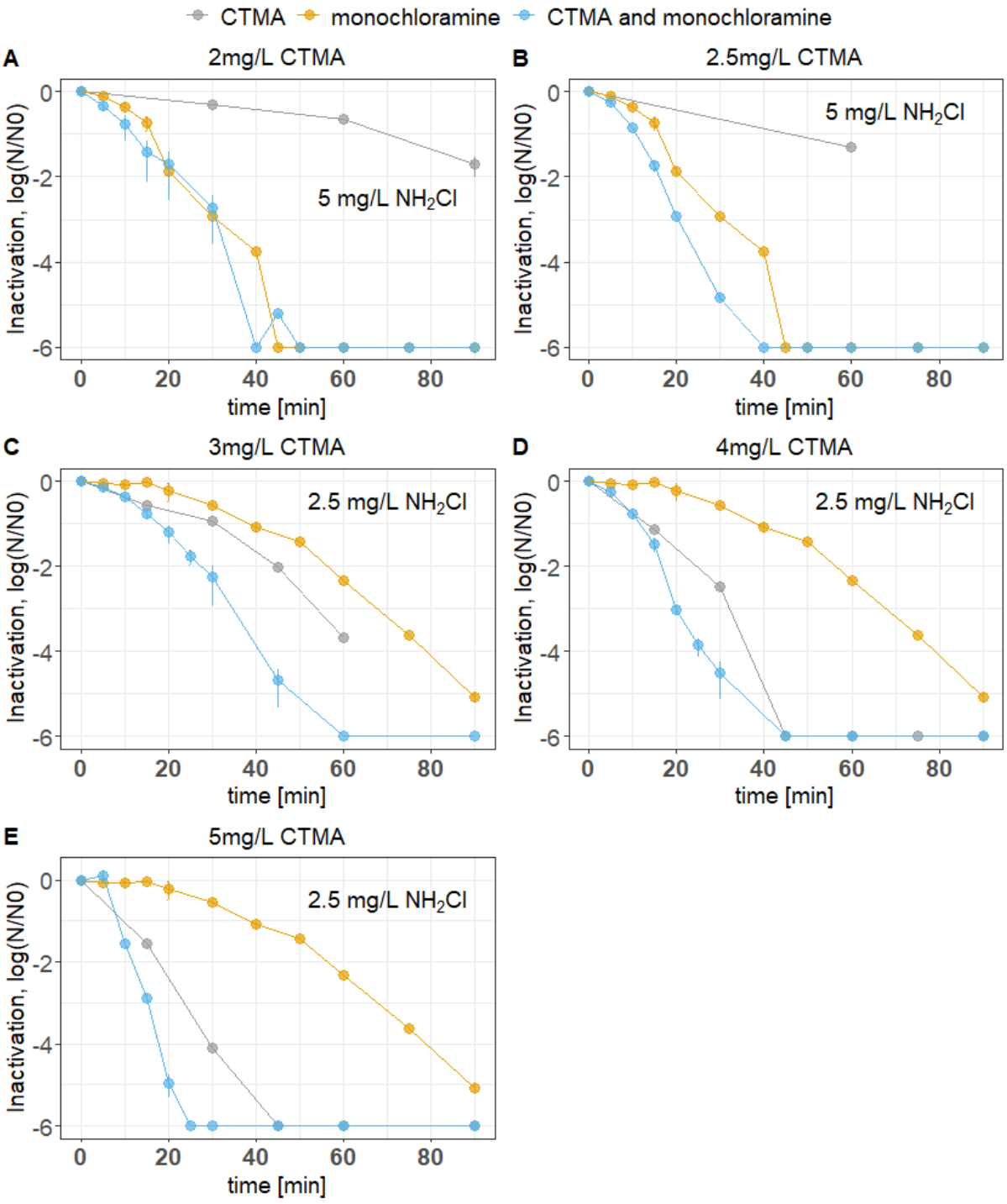


Figure 3-7. Inactivation of *E. coli* AG100 by CTMA, NH_2Cl and the combination of both for different concentrations of CTMA.

(A) 2 mgL^{-1} CTMA, (B) 2.5 mgL^{-1} CTMA (C) 3 mgL^{-1} CTMA, (D) 4 mgL^{-1} CTMA and (E) 5 mgL^{-1} CTMA. The NH_2Cl concentrations were 5 mgL^{-1} for (A) and (B) and 2.5 mgL^{-1} for (C), (D) and (E). All experiments were performed in PBS (10mM) at room temperature, pH 7.4. Each data point is the average of at least four replicates and the error bars represent the standard deviations.

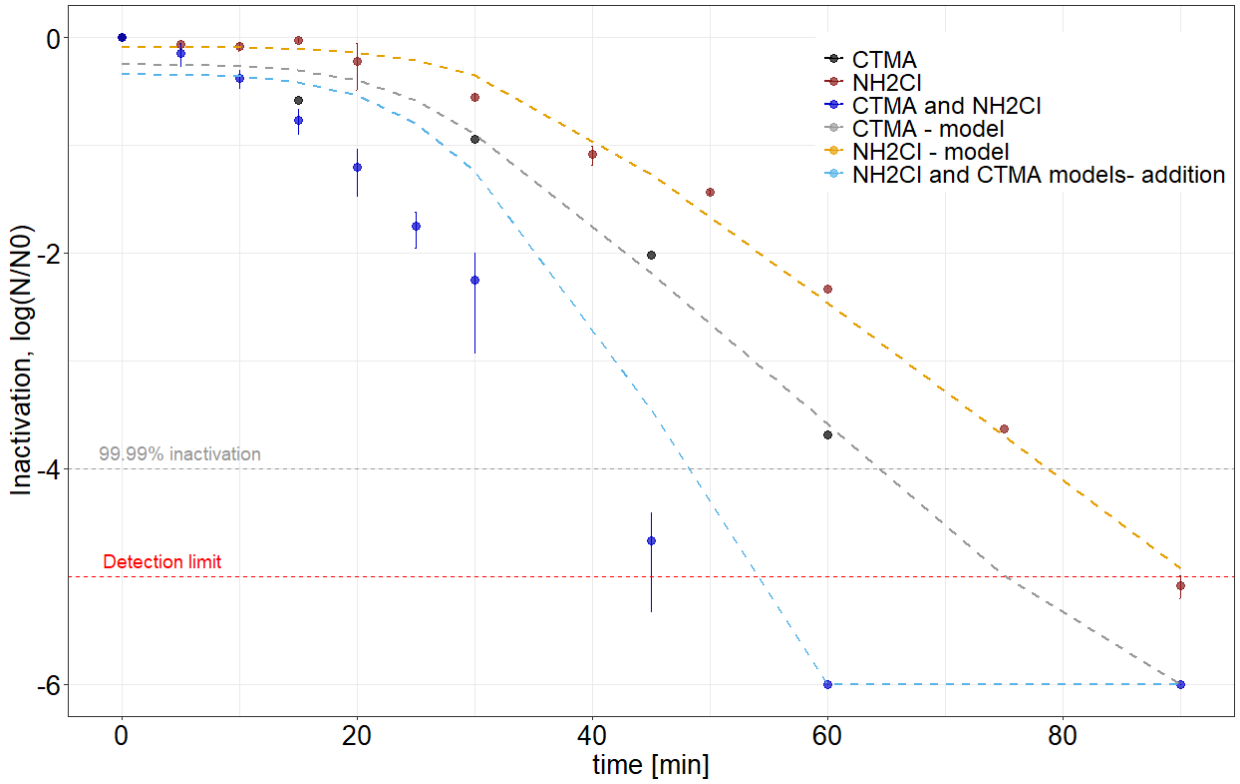


Figure 3-8. Separate and combined inactivation of *E. coli* AG100 by CTMA (3mgL^{-1}), NH_2Cl (2.5 mgL^{-1}). All experiments were performed in PBS (10mM) at room temperature, pH 7.4. The calculated overall effect is the result of the addition of data fitted for CTMA only and NH_2Cl only with the Geeraerd model. The dashed lines are the fitted data (NH_2Cl model fit: $R^2 = 0.99$; CTMA model fit: $R^2 = 0.99$), and the filled circles are the experimental data. The blue dashed line represents the addition of the two other dashed lines.

3.4 Conclusion

The susceptibility of *E. coli* to cetyltrimethylammonium chloride (CTMA) was investigated in different media. MHB, a common media used to determine the minimum inhibitory concentrations (MICs) was compared to phosphate-buffer saline (PBS), a secondary wastewater effluent and a lake water. Furthermore, the impact of CTMA on the inactivation kinetics of *E. coli* by ozone and monochloramine was investigated for various strains of *E. coli* (AG100, AG100A, AG100tet). The main findings of this study are:

- The susceptibility of *E. coli* for CTMA depends on the aquatic environment and is higher in PBS, secondary wastewater effluent and lake water compared to the standard conditions in MHB. The standard determination of MIC, in broth, is therefore not representative of natural and engineered aquatic systems and the assessment of the presence of QACs in the environment requires a new approach.
- A pre-exposure to CTMA and the resulting reduction of susceptibility of *E. coli* to CTMA influences the inactivation kinetics by ozone and monochloramine. The inactivation was enhanced for ozone and reduced for monochloramine. This difference is due to different modes of action for ozone and monochloramine.
- The role of the efflux pumps for the susceptibility to ozone or monochloramine could not be determined with the obtained results. The different results obtained between strains expressing different types of efflux pumps (AG100tet vs pre-exposed strains) indicates complex mechanisms, which require further investigations.
- The presence of mgL^{-1} of QACs during the inactivation by either ozone or monochloramine enhanced the inactivation of *E. coli*. The inactivation kinetics were at least additive for monochloramine, a potential synergistic effect needs to be confirmed by further investigations. For ozone, the combination of ozone with CTMA resulted in non-linear inactivation curves, which could not be compared. Nonetheless, the presence of CTMA led to enhanced inactivation compared to ozone alone.
- Natural waters and especially wastewater effluents contain a mixture of different QACs and other biocides. The presence of these substances during a chemical disinfection by ozone or monochloramine may enhance the inactivation of pathogenic bacteria.

Acknowledgements

We would like to thank the Prof. Miguel Viveiros for kindly providing the *E. coli* strains. Vincent Berweiler for having performed the pre-exposure part of the experiments. Gabrielle Léger for helping with the ozone inactivation experiments. Caroline Gachet-Aquillon and Karim Essaied are acknowledged for the technical support during the experiments. Finally, the financial support by EPFL is acknowledged.

Supporting Information for Effects of cetyltrimethylammonium chloride on various *E. coli strains* and their inactivation by ozone and monochloramine

Text S 3-1. Ozone exposure

The applied experimental approach used was adapted from Wolf et al. (2018) [223].

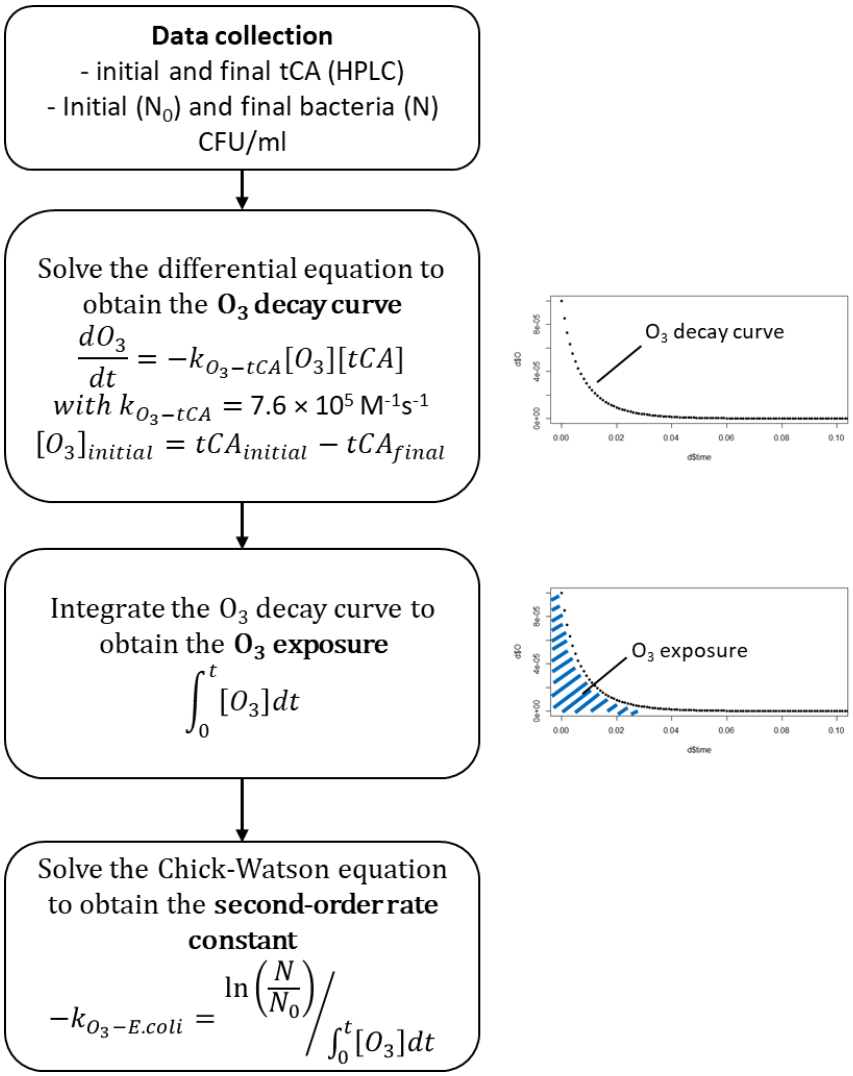
Ozone exposure was controlled using *trans*-cinnamic acid (*t*-CA), which has a known high reactivity with ozone (see main text). *t*-CA was added in excess of the ozone dose in the solution. *t*-CA reacts with ozone according to the following reaction:

$$tCA + O_3 \xrightarrow{k_{O_3-tCA}} Benzaldehyde + Glyoxylic\ acid + H_2O_2 \quad (3\text{-}1)$$

Benzaldehyde is produced in a stoichiometric ratio 1:1 from the reaction of ozone with *t*-CA. In a previous study, benzaldehyde was used to determine the exact applied ozone dose [223]. However, in contrast to this previous study in which the concentration of benzaldehyde was not affected the experimental conditions, in the current study, the concentration was affected by the presence of bacteria (data not shown). Therefore, the ozone dose was determined by the difference between the initial and the final *t*-CA concentration.

t-CA was quantified by a HPLC method before and after adding ozone to the solution (Text S 3-2). The second-order rate constant $k_{O3\text{-}tCA}$ was obtained from Wolf et al. (2018) [223], as 7.6 $\times 10^5$ M^{-1} s^{-1} at 22°C. To prevent any interference with hydroxyl radicals (•OH), tert-butanol (*t*-BuOH) was added as a scavenger (20mM).

The ozone exposure and the inactivation rate constants were calculated based on scheme S 3-1.



Scheme S 3.1. Flow-Chart for the determination of the second-order rate constant for the inactivation of *E. coli* by ozone.

Text S 3-2. HPLC method for quantification of benzaldehyde and trans-cinnamic acid

Benzaldehyde and *t*-CA) were quantified using an EC 125/3 Nucleosil 100-5-C18 column (Marcherey-Nagel) on an Ultimate 3000 HPLC system (Thermoscientific, Sunnyvale, CA, USA) with a multiple wavelength diode array detector recording the absorbance at 254 nm (benzaldehyde) and 266 nm (*t*-CA).

50 µL of the sample were injected in an isocratic flow (40% H_3PO_4 10mM, pH 2.3; 60% methanol) with a flow rate of 1mLmin^{-1}. The total run time of the sequence was 9 min, and the retention times were 3 min for benzaldehyde and 6 min for t-CA. Calibration curves were obtained for each experiment. The calibrations were carried out with concentrations in the range 5-400 µM for both compounds and the measured concentrations were in the range 10-350 µM for both compounds.

Text S 3-3. Spectrophotometric method for the determination of the concentrations of monochloramine and dichloramine.

The concentrations were determined from the measurement of the direct absorbance of the solution at the wavelengths 245nm and 295 nm. The concentrations were determined with the following equations:

$$A_{245} = \epsilon_{NH_2Cl,245} C_{NH_2Cl} l + \epsilon_{NHCl_2,245} C_{NHCl_2} l \quad (1)$$

$$A_{295} = \epsilon_{NH_2Cl,295} C_{NH_2Cl} l + \epsilon_{NHCl_2,295} C_{NHCl_2} l \quad (2)$$

The following molar absorption coefficients were used:

Molar absorption coefficients (ϵ, M cm^{-1}) of monochloramine and dichloramine:

	λ (nm)		**Reference**
Species	**245**	**295**	
NH_2Cl	445	14	[224,242]
$NHCl_2$	208	267	[224,242]

Table S 3-1. Evolution of the MIC for *E. coli* AG100 and AG100A during a stepwise exposure to CTMA in MHB at pH 7.5 and room temperature.
Each step was 48hours. The value at the step 1 corresponds to the initial MIC of the bacteria for CTMA.

	AG100				**AG100A**			
	Replicate 1		**Replicate 2**		**Replicate 1**		**Replicate 2**	
Step	**Conc MHB**	**MIC**	**Conc MHB**	**MIC**	**Conc MHB**	**MIC**	**Conc MHB**	**MIC**
1	15	25	15	20	5	7.5	5	7.5
2	20	25	15	20	5	7.5	5	7.5
3	20	25	20	25	5	7.5	5	7.5
4	20	25	20	25	5	7.5	5	7.5
5	20	25	20	25	5	7.5	5	15
6	25	30	25	30	7.5	10	7.5	15
7	25	30	25	30	15	10	15	20
8	30	35	25	30	15	20	20	25
9	30	40	25	30	25	30	25	30
10	NA	40	NA	40	NA	40	NA	40

Table S 3-2. Second-order rate constants for the inactivation of different *E. coli* strains by ozone, including CTMA pre-exposed strains in MHB at pH 7.5 and room temperature.
The strains were pre-exposed using a stepwise method as described in section 3.2.8.
The second-order rate constants were computed with a modified Chick-Watson model (Table 2, main text). The standard deviation of the second-order rate constant and the fitting quality (R^2) of the data to the model are also provided.

Strain	**k [M^{-1} s^{-1}]**	**stdev**	**R^2**
AG100	8.3E+05	4.0E+04	0.91
AG100A	9.8E+05	7.0E+04	0.91
AG100tet	5.2E+05	1.1E+05	0.72
AG100 pre-exp	1.2E+06	8.0E+04	0.95
AG100A pre-exp	1.2E+06	8.3E+04	0.95

Table S 3-3. Second-order rate constants for the inactivation of different *E. coli* strains by monochloramine, including CTMA pre-exposed strains in MHB at pH 7.5 and room temperature. The strains were pre-exposed using a stepwise method as described in section 3.2.8.
The second-order rate constants were computed with a modified Geeraerd model (Table 3-2, main text) and the inactivation curves are presented in Figure 3-3 B (main text). The model also provides a parameter for the lag-phase. The standard deviations and the fitting quality (R^2) of the data to the model are also provided.

Strains	k_{max} [M^{-1} s^{-1}]	Stdev k_{max}	Lag-phase parameter [M s]	Stdev lag-phase	R^2
AG100	1.3E+03	8.4E+01	4.5E-03	5.1E-04	0.99
AG100 pre-exp	6.9E+02	3.7E+01	2.5E-03	7.8E-04	1.00
AG100A	1.3E+03	1.0E+02	2.0E-03	7.2E-04	0.98
AG100A pre-exp	6.6E+02	4.8E+01	2.5E-03	1.1E-03	0.99
AG100tet	1.8E+03	1.1E+02	1.6E-03	3.9E-04	0.99

Table S 3-4. Second-order rate constants for the inactivation of the *E. coli* strain AG100 by ozone in presence of different CTMA concentrations in MHB at pH 7.5 and room temperature.
The second-order rate constants were computed with a modified Chick-Watson model (Table 3-2). The standard deviation of the rate constants and the fitting quality (R^2) of the data are also provided.

CTMA concentration [mg L^{-1}]	k [M^{-1} s^{-1}]	stdev	R^2
0.0	9.0E+05	5.7E+04	0.91
0.1	8.0E+05	1.2E+05	0.82
1.0	1.1E+06	9.2E+04	0.92
1.5	8.6E+05	9.8E+04	0.76
1.75	1.4E+06	1.9E+05	0.84
2.0	1.1E+06	2.2E+05	0.48
2.25	2.4E+06	3.1E+05	0.84
2.5	7.4E+05	2.9E+05	0.39

Table S 3-5. Second-order rate constants for the inactivation of *E. coli* AG100 by monochloramine in presence of different CTMA concentrations in MHB at pH 7.5 and room temperature.

The second-order rate constants were computed with a modified Geeraerd model (Table 3-2, main text). The model also provides a parameter for the lag-phase. The standard deviations and the fitting quality (R^2) of the data are also provided.

CTMA concentration [mg L^{-1}]	**k_{max} [M^{-1} s^{-1}]**	**Stdev k_{max}**	**Lag-phase parameter [M s]**	**Stdev lag-phase**	**R^2**
0	1.3E+03	8.4E+01	4.5E-03	5.1E-04	0.99
1	7.6E+02	3.5E+01	2.9E-04	7.6E-04	1.00
1.5	9.2E+02	7.3E+01	3.7E-03	8.5E-04	0.99
2	1.5E+03	1.8E+02	4.7E-03	7.4E-04	0.98
2.25	1.2E+03	6.7E+01	2.2E-03	4.6E-04	1.00
2.5	3.0E+03	5.1E+02	3.4E-03	4.6E-04	0.98
3	2.1E+03	1.1E+02	1.9E-03	2.2E-04	1.00
4	3.1E+03	2.0E+02	9.9E-04	2.2E-04	1.00
5	5.5E+03	3.2E+02	9.0E-04	1.1E-04	1.00

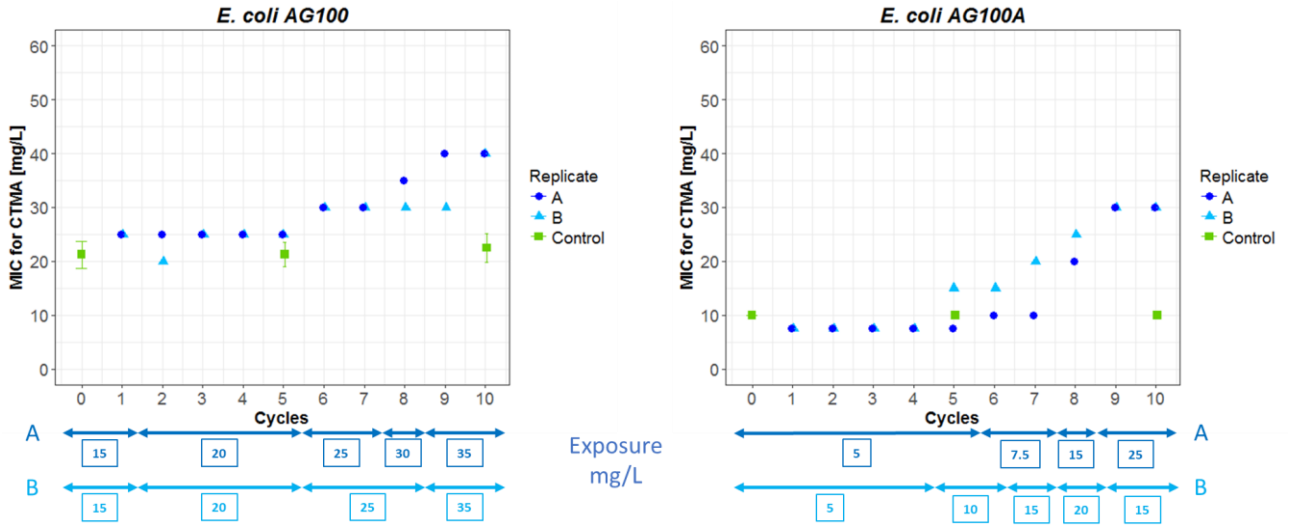


Figure S 3-1. Evolution of the MIC of the *E. coli* strains AG100 and AG100A during a stepwise training against CTMA.

The corresponding data are available in Table S 3-1. The pattern of exposure, with the concentrations used is available below the x-axis.

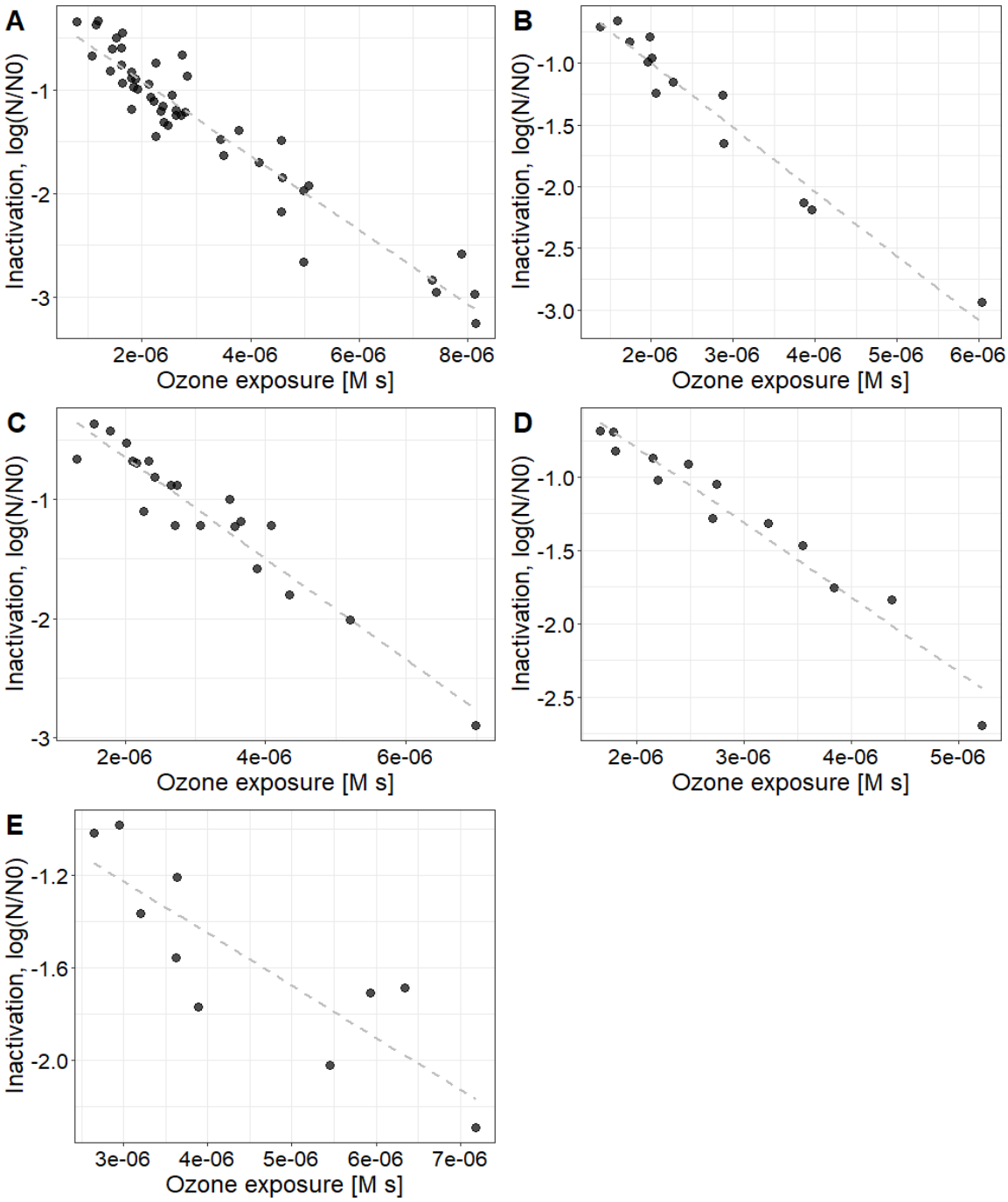
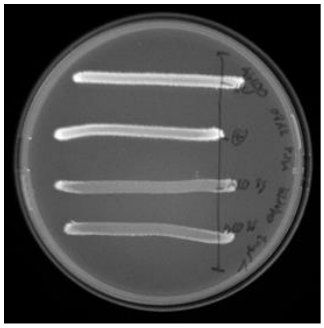


Figure S 3-2. Disinfection by ozone.

Inactivation curves of (A) *E. coli* AG100; (B) AG100 pre-exposed to CTMA; (C) AG100A; (D) AG100A pre-exposed to CTMA; (E) AG100tet. A summary of these data is provided in Figure 3-4, main text. All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with t-BuOH (20mM). The points are the mean values of at least four technical measurements of each replicate and the dashed line represents the linear regression of the points. The calculated second-order rate constants are also provided in Table S 3-2.

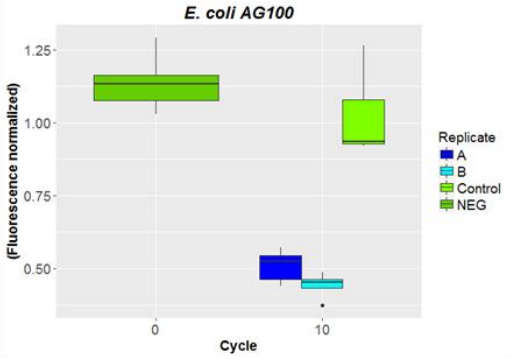


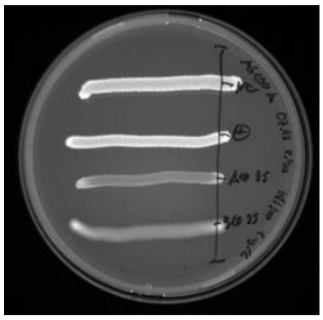
AG100 not cycled

AG100 cycled but not exposed

Replicate A

Replicate B





AG100A not cycled

AG100A cycled but not exposed

Replicate A

Replicate B

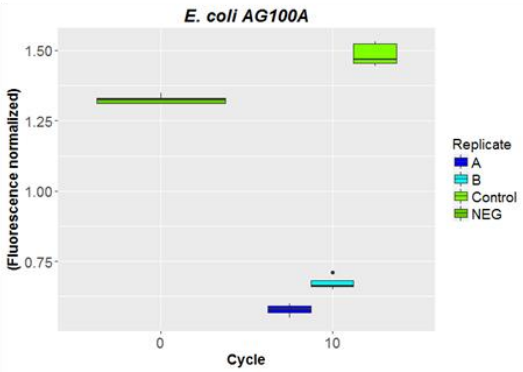


Figure S 3-3. Fluorescence of the trained *E. coli* strains exposed to CTMA with ethidium bromide and the relative fluorescence to the control strain.

The strains were grown on LB agar enriched with ethidium bromide. After incubation, the strains were imaged using a UV-light imaging system. The fluorescence of the strains is the result of the accumulation of ethidium bromide inside the cell. The more fluorescent the line, the more ethidium bromide are in the bacteria. The level of ethidium bromide inside the cell is correlated with the level of expression of multidrug efflux pumps. To semi-quantify the level of fluorescence, the results were compared by averaging and normalizing the brightness of the pixels of different areas of the strike by the fluorescence of the AG100 strain not cycled and by subtracting the noise.

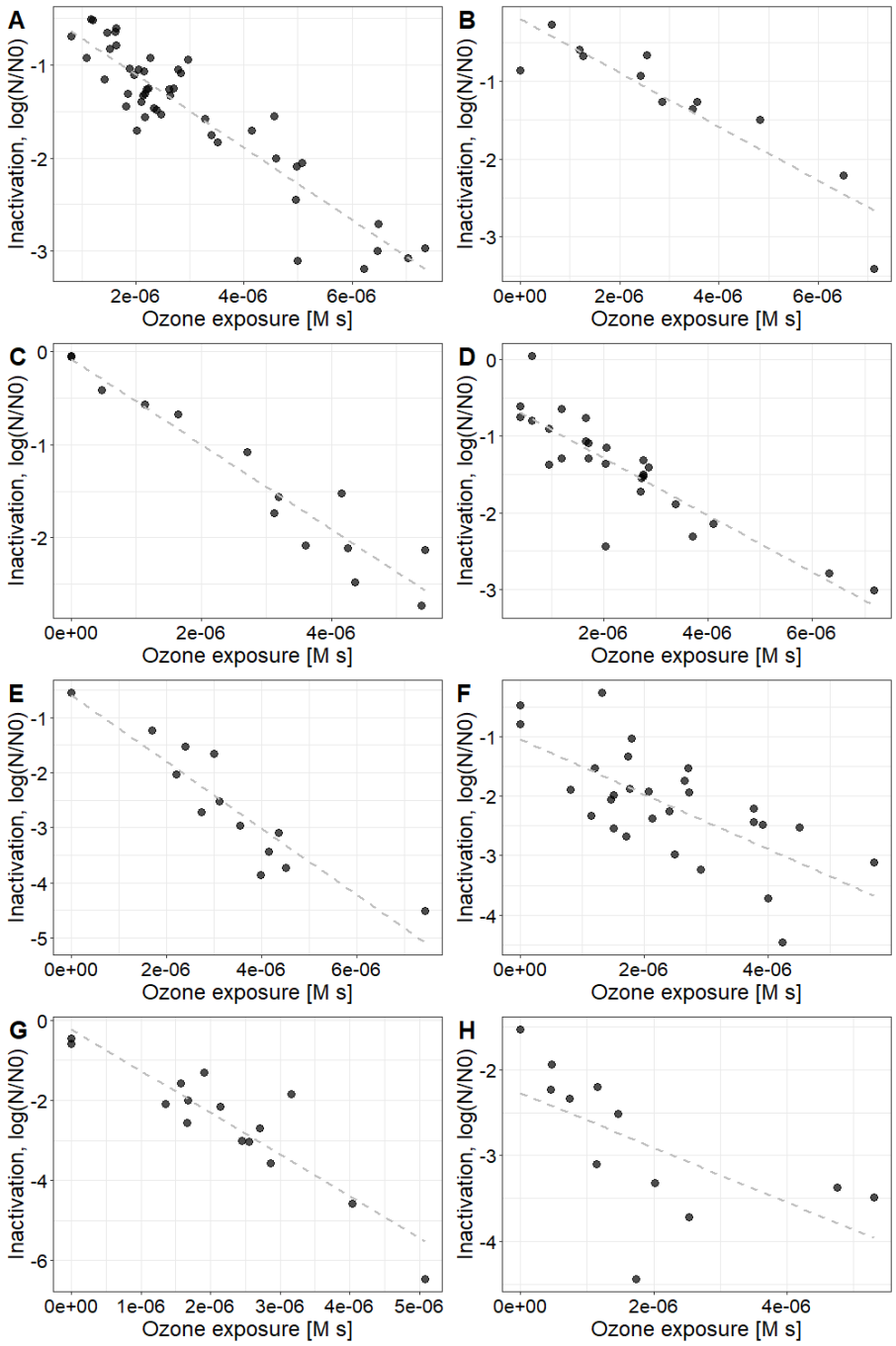


Figure S 3-4. Inactivation of the *E. coli* AG100 strain in presence of CTMA by ozone in presence of CTMA.

(A) no CTMA; (B) 0.1mgL^{-1} CTMA; (C) 1mgL^{-1} CTMA; (D) 1.5mgL^{-1} CTMA; (E) 1.75mgL^{-1} CTMA; (F) 2 mgL^{-1} CTMA; (G) 2.25 mgL^{-1} CTMA; (H) 2.5 mgL^{-1} CTMA. All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (20mM) to exclude hydroxyl radical reactions. The points are the mean values of at least four technical measurements of each replicate and the dashed line represents the linear regression of the data points. The calculated second-order rate constants are also provided in Table S 3-4.

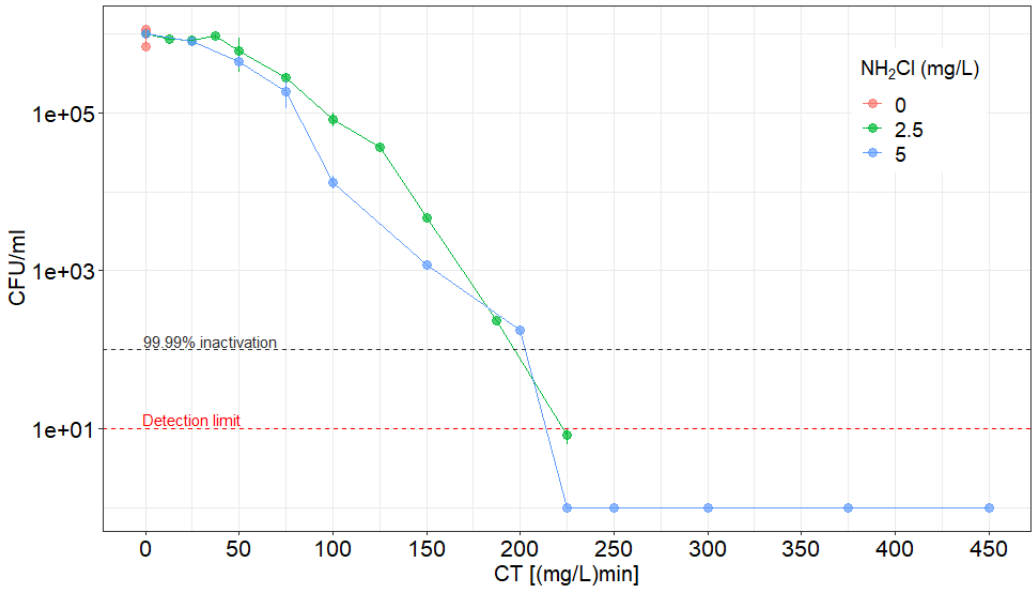


Figure S 3-5. Comparison of the inactivation level of *E. coli* AG100 by two different concentrations of monochloramine as a function of the NH_2Cl exposure.

The experiments were performed in PBS (10mM) at room temperature, pH 7.4. The data points are the mean values of at least four technical measurements of each replicate and the error bars represent the standard deviation.

4 Development of CTMA resistance in *E. coli* using the microbial evolution and growth arena (MEGAPLATE) approach

M. Voumard, F. Breider, U. Von Gunten

Experimental design, protocol development, experimental part, data analysis, writing of the chapter were performed by Margaux Voumard under the scientific advising of U. von Gunten and F. Breider.

The experimental part was done at 60% by Margaux Voumard and at 40% by Vincent Berweiler under the supervision of Margaux Voumard.

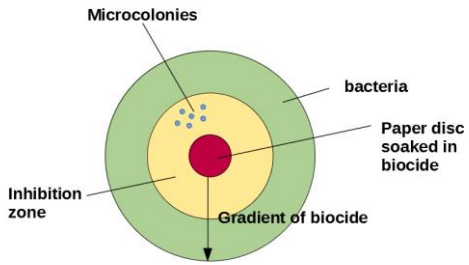
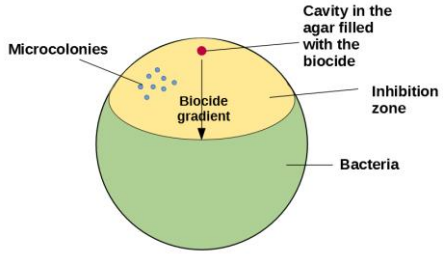
Abstract

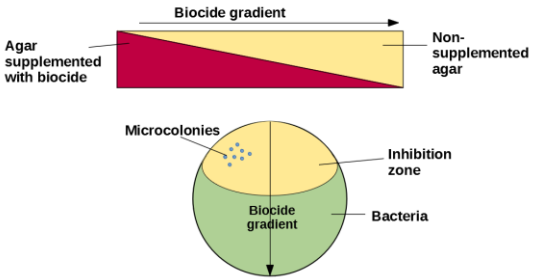
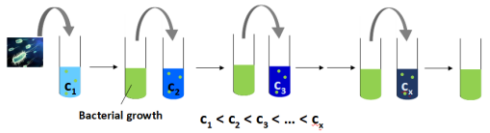
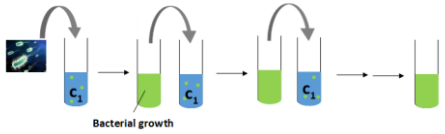
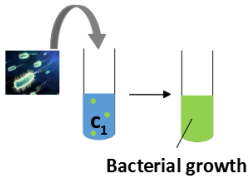
Bacteria resistant to antibiotics is a worldwide concern and resistance to other biocidal compounds such as disinfection agents is of growing concerns. Different methods have been proposed and used to investigate the development of resistance in bacteria under laboratory conditions. However, these methods are not necessarily reflecting the environments in which resistance are appearing. Moreover, these methods were developed for antibiotics and their suitability for other drugs is unknown. To overcome this limitation, a new method was developed and successfully used for antibiotics, the MEGAPLATE device. This device includes a spatial component, often missing in other laboratory setups. The potential of this device with other biocides such as quaternary ammonium compounds (QACs) is unknown. The objective of this chapter was to test the MEGAPLATE approach with CTMA, a widely used QAC and *Escherichia coli*. If the design of the MEGAPLATE and the application of CTMA was a success, the inoculation with the bacteria led to contaminations and failure to avoid them. Due to the lack of suitable structure to incubate the MEGAPLATE in sterile conditions, it was not possible to achieve any complete experiment.

4.1 Introduction

Development of resistance to antibiotics or other drugs in bacteria is a major well-known worldwide problem. The high use of antibiotics but also other antibacterial products is known as the main cause of for the emergence of resistant and multiresistant bacteria [243,244]. Field investigations for the development of resistance are crucial, but the need to elucidate the most important parameters requires the development of simple and robust laboratory-scale methods. Therefore, protocols to promote resistance under defined and selected conditions have been developed. The protocols need to be reproducible and to allow testing different parameters. Multiple methods exist that are commonly used by laboratories worldwide. A study from Walsh et al. (2003) [107] compared 6 different methods and their potential to create stable mutants. A summary of these methods is provided in Table 4-1. In this study, two out of the six methods led to stable mutants, the paper disc diffusion and the gradient agar plate. All other tested methods led to increased resistance but no stable mutants [107].

Table 4-1. Description of several laboratory methods to promote the development of antibiotic resistance

Method	Description	Scheme	Advantages and disadvantages
Agar diffusion methods			
Paper disk diffusion	A paper disk is soaked with the biocide of interest and placed on an agar plate swabbed with bacteria. The biocide diffuses in the agar creating a gradient of concentration. After incubation, microcolonies growing in the inhibition zone are tested		Easy to setup, reproducible. Need to make sure that the microcolonies are not contaminated. Limited space for the gradient.
Cup plate diffusion	Principle similar to the paper disc diffusion. Instead, a cavity is created in the agar and the biocide poured in the cavity. It will diffuse creating a gradient. After incubation, microcolonies growing in the inhibition zone are tested		Easy to setup, reproducible. Need to make sure that the microcolonies are not contaminated. Limited space for the gradient.

Biocide gradient plate	Agar plates are prepared with a two-layer system. A first layer of agar supplemented with biocide is let to solidify at a defined angle. A second, non-supplemented layer is added on top. The biocide will diffuse creating a gradient. Bacteria are swabbed and the ones growing at the highest concentration are re-inoculated on novel plates until a stable MIC is obtained.		Reproducibility can be difficult as the inclination needs to be similar in all experiments. Need to make sure that the microcolonies are not contaminated.
Liquid methods			
Stepwise training in broth	Bacteria are sub-cultured in broth-containing increasing concentrations of biocides. The MIC is tested after each sub-culture until stabilization.		Easy to set up, reproducible Time and material consuming.
Repeated exposure to sub-lethal concentrations	Bacteria are sub-cultured in broth containing a constant sub-lethal concentration of biocide. The MIC is tested after each sub-culture.		Easy to set up, reproducible Time and material consuming.
Basal medium supplemented with biocide	Bacteria are grown in basal medium (basic medium for non-specific growth) supplemented with a defined concentration of biocide. The MIC is checked after incubation.		Easy to set up, reproducible. Can be extended to either stepwise or repeated exposure to constant concentrations. In this case, time and material consuming.

Even though laboratory-scale methods can provide some useful insights about development of antibiotic resistance, they do not necessarily reflect natural or clinical environments. Under realistic conditions, microorganisms are facing spatially separated areas with different conditions. For instance, surfaces regularly cleaned might carry a heavy load of detergents or biocides, but some hidden spots may experience lower doses. Bacteria are then alternatively exposed to ideal, not stressful environments and more dangerous locations related to their survival. The first bacteria that will develop a mutation that allows it to invade the new hostile environment will be able to replicate and live there. Adaptation to new environments via mutation or phenotypical changes has been known for centuries as a pillar of evolution [245]. Therefore, including a spatial component in a laboratory protocol is of importance. Baym et al. (2016) [161] developed a novel method to investigate the development of resistance in bacteria. The set-up consists of a giant plate (MEGAPLATE), filled with bands of agar with increasing antibiotic concentrations (Figure 4-1). The agar is put in two layers, the first one consists of bands of hard agar (increased quantities of agar-agar), and with different concentrations of an antibiotic and the top layer is soft agar, with a lower amount of agar, which will allow the motility of bacteria. Bacteria were inoculated on the band with the lower concentration where they replicate and deplete nutrients. Once there is no more space and no more nutrients on the area with the lowest concentration, the first bacteria that will be able to pass to the next concentration will be able to spread and to conquer this area. The experiment is recorded in order to follow the evolution pathways and the lineages. At the end of the experiments, bacteria can be sampled from the plate at the different locations and their evolution pathway, in terms of mutations, can be sequenced. The large size of the MEGAPLATE (120 x 60 cm) prevents an equilibrium of the antibiotic concentration and provides enough space and nutrients for a large bacterial population to survive and mutate [161].

This method has been set up and successfully used with antibiotics. However, the potential with other antibacterial substances, such as quaternary ammonium compounds is unknown. Therefore, the objectives of this study were to (i) re-create the device, (ii) adapt the method to QACs, (iii) follow the adaptation of bacteria live with an imaging system and (iv) investigate the time necessary for bacteria to adapt to each concentration or to each concentration gap. This was performed using one QAC, cetyltrimethylammonium (CTMA) and a wild-type strain of *E. coli.*

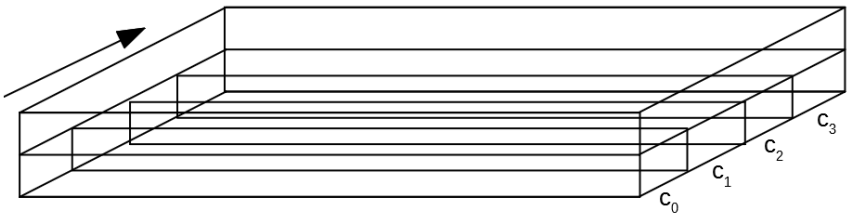


Figure 4-1. Schematic representation of the MEGAPLATE.
The arrow represents the migration direction.

4.2 Material and methods

4.2.1 Construction of the MEGAPLATE

The MEGAPLATE was constructed using Plexiglas of a thickness of 4 mm and glued together with dichloromethane. Waterproofing tests were performed prior the first filling of the plate. The pieces were built following the plan in Figure 4-2.

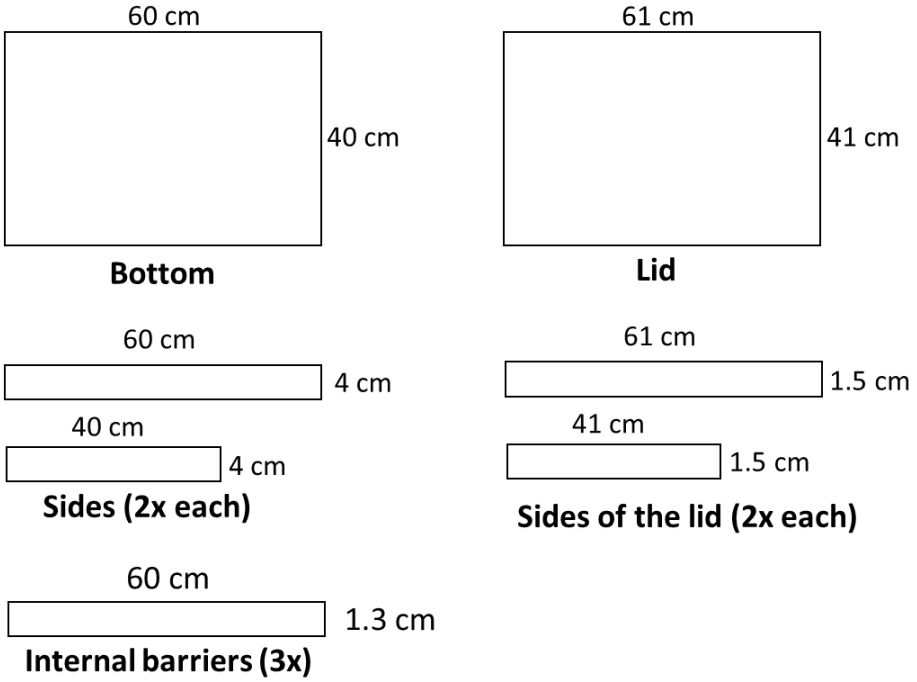


Figure 4-2. Plan for the construction of the different pieces of the MEGAPLATE device.

4.2.2 Disinfection and cleaning procedure of the MEGAPLATE

Prior and between all tests, the plate had to be cleaned and disinfected properly.

4.2.2.1 Disinfection procedure

The plate was placed in a ventilated chemical hood. A bleach solution of a final concentration of 1.3-1.5% (10-fold dilution of a 13-14.7% solution) was poured in the agar plate. The total volume of bleach solution was approximately 8-9 liters. The bleach was kept in the plate overnight for at least 12 hours at room temperature. After 12 hours, the bleach solution was removed using a pump. Ethanol at 70% was sprayed on the plate for the transport to avoid contamination between the disinfection and the pouring place.

If contamination was still observed, the plate was treated by UV irradiation.

4.2.2.2 Cleaning procedure

The agar was removed and placed in autoclavable containers and eliminated according to the safety rules. The plate was wiped down to remove the entire agar. All garbage was discarded in appropriate containers in order to be autoclaved and correctly discarded.

4.2.3 MEGAPLATE setup

4.2.3.1 Agar layers

All the pouring of the agar was done inside a biosafety cabinet to avoid contamination of the media.

4.2.3.1.1 Hard agar

Five liters of hard agar (20g of agar, 20g of Luria-Bertani broth, LB, per L of MilliQ water) were prepared, one liter for each band and one for an intermediate layer. The solutions were sterilized (121°C for 40 minutes) and cooled down to 56°C.

CTMA solutions in LB) were prepared and filter sterilized. India ink solutions were prepared and sterilized by autoclaving (india ink darkens the agar without affecting bacteria and helps visualization). The risk of contamination was prevented by adding cycloheximide, an anti-fungus solution to the agar. In addition, CTMA was added to each liter of agar to reach the wanted concentrations of CTMA. Agar was then poured one band after the other and let to solidify before pouring the next one, band per band (Figure 4-3A).

Once the agar has solidified (a few hours after pouring), a small layer of hard agar (0.8 L of agar) without CTMA is poured on top to equalize the level of each band and let to solidify (1 hour). (Figure 4-3B)

4.2.3.1.2 Soft agar

One liter of soft agar (3g agar-agar, 20g LB broth per L MilliQ water) was prepared. It was sterilized by autoclaving at 121°C for 40 minutes. The soft agar layer was then poured over the intermediate layer (Figure 4-3B). The lid was closed and the plate was let to solidify at room temperature in the biosafety cabinet.

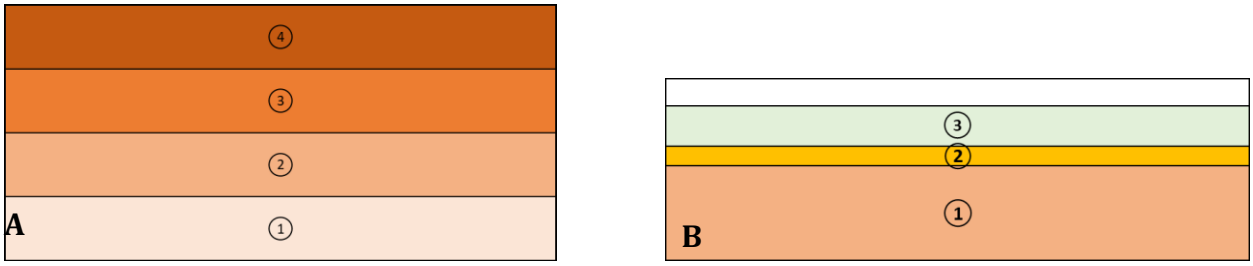


Figure 4-3. Agar Setup.

(A) Top view of the MEGAPLATE. Each band (1 to 4) was poured one after the other. (B) Side view of the MEGAPLATE. Layer 1 is the hard agar layer, layer 2 is the intermediate layer to equalize the level of each band and layer 3 is the soft agar layer to allow the motility of bacteria.

4.2.3.2 Concentrations of CTMA

Concentrations of CTMA used for each band were defined according to the MIC and with a large enough interval to create a gradient of concentrations. They were also taken in accordance with the set of concentrations used by Baym et al. (2016) [161]. Therefore, the following set of concentration was tested: 0-200-1000-2000 mg L^{-1}.

4.2.4 Inoculation of the plate

Inoculation was performed at four different points on the first agar band (without CTMA) with 10 µL of an overnight *E. coli* solution (Figure 4-4).

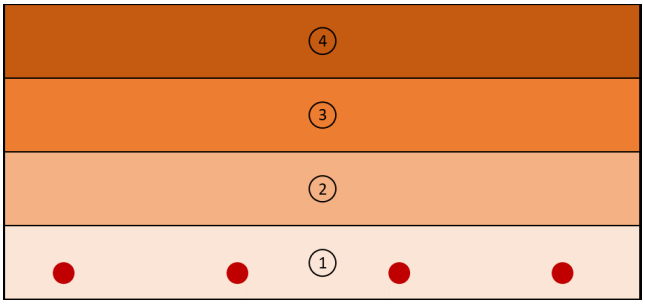


Figure 4-4. Top view of the MEGAPLATE.

Each red dot represents an inoculation point on the first agar band.

4.2.5 Imaging system

In order to record the evolution of the adaptation, an imaging system was created. It consisted of two webcams linked to a Raspberry Pie computer. Led lights were stuck in the incubator to light it when the pictures were taken. A python code was developed to define at which interval the pictures should be taken, to switch on the lights and to store the images on an external server.

4.3 Results

4.3.1 Preliminary test:

4.3.1.1 Migration of the agar from the hard to the soft agar layer

The method is highly dependent on the diffusion of the molecule of interest, in this case, CTMA, from the hard agar layer to the soft agar layer. As QACs have different chemical properties compared to antibiotics, it was necessary to ensure the diffusion of CTMA.

For this purpose, small scale experiments were designed with a colorimetric indicator reacting with QACs, bromophenol blue [246]. In absence of QAC, bromophenol blue in broth has a purple color, whereas in presence of QAC, it turns to blue-green (Figure 4-5A).

Petri dished were filled with the three same layers of agar as in the MEGAPLATE. The third layer, the soft agar one, was supplemented with bromophenol blue. Plates were let to equilibrate for 24 hours at room temperature and a change in the color of the soft agar layer was

checked. Different concentrations of CTMA in the hard agar layer were tested. In all cases, a change in the color of the soft agar layer was observed, except for the control, where no CTMA was present in the hard agar layer. An example of the obtained results is shown in Figure 4-5B and C.

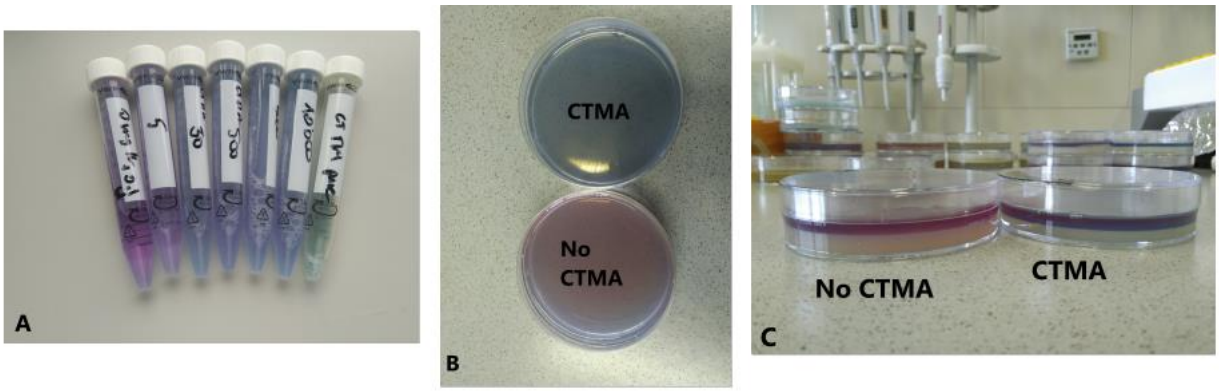


Figure 4-5. Migration experiments of CTMA.
(A) CTMA in LB broth and in presence of bromophenol blue, concentrations are (from left to right: 0, 5, 50, 500, 5000, 10'000, 2.5 10^5 mg/L; (B) agar plates with a double agar layer in presence and in absence of CTMA after 24 hours period; (C) same as B, but profile picture of the plates.

4.3.2 Trial 1: incubation temperature 37°C

The first tests were performed with a constant incubation temperature of 37°C. However, the water from the soft agar layer was evaporating quickly, preventing the migration of bacteria (Figure 4-6). Based on this problem, the temperature was reduced to 30°C and the border of the plate were sealed with parafilm to decrease evaporation and drying of the agar.

Figure 4-6. Drying of the agar because of a too high incubation temperature.

4.3.3 Trials 2 and further:

After the evaporation problems were solved, the experiments restarted. Contamination problems quickly arrived, with the whole plate covered in 24 hours by a bacteria film (independent on the CTMA concentration, Figure 4-7). Based on this issue a disinfection step consisting of UV irradiation of the plate was added and solved the problem.

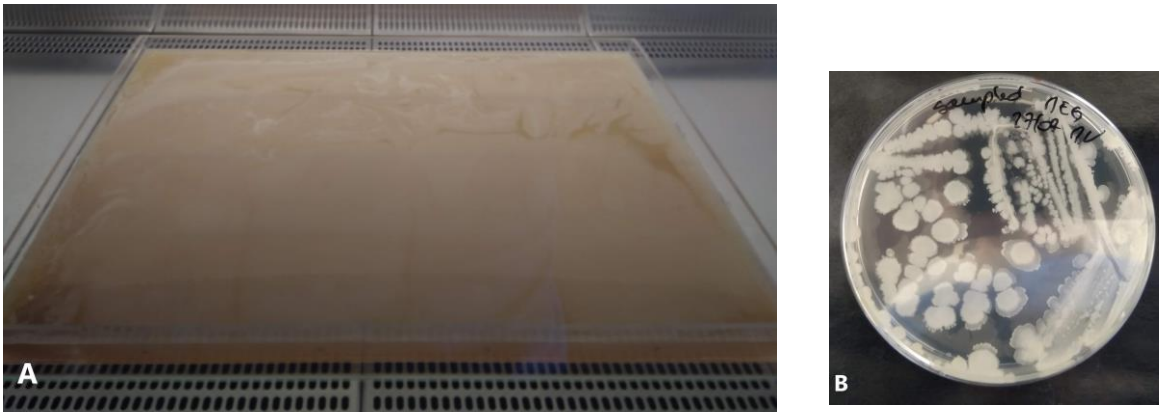


Figure 4-7. Contamination of the MEGAPLATE.
(A) The whole plate covered by a film of an unknown bacteria after 24 hours of incubation (uniform white color all over the plate); (B) result of the sampling from the MEGAPLATE, the type of microorganism is unknown.

Experiments restarted, however, if this contamination problem was solved, other appeared. In the original study, contamination was also present, but not critical for the results, as they were able to discriminate their initial bacteria from the one of interest [161]. In our case, the contamination took too much place and discrimination was not possible. Samples taken from presumably "clean areas" appeared to be also contaminated with unknown microorganisms (Figure 4-7B).

4.4 Discussion and Conclusion

Despite the fact, that bacteria seemed to evolve and adapt to the increasing concentrations of CTMA, contaminations and unknown microorganisms (bacteria and/or fungi) quickly invaded

the plate and inhibited the bacteria of interest. After several trials leading to the same problems and no solutions, these experiments were stopped. Other than contaminations, the system seemed to be working. Overall, we faced two main limitations, linked to the infrastructure and logistics of the experiment and the linked to microbiological aspects of the experiment.

4.4.1 Infrastructure

Having access to the right infrastructure, such as a room dedicated for the experiment with limited access to prevent contamination is crucial. Moreover, space must be enough to set in place an imaging system, to keep the temperature and humidity at the right conditions to avoid evaporation of the soft agar layer.

In this case, the cleaning and disinfection were in the laboratory A, the pouring and solidification of the agar in the laboratory B and the incubation in the laboratory C. The different locations and moving of the plate have increased the risks of contamination. Therefore, the infrastructure was part of the failure of the experiment.

4.4.2 Contamination

Contaminations were unavoidable, and it should have been theoretically possible to discriminate between them and the bacteria of interest in a similar manner as the Baym study [161]. However, in our case, the contaminations were much bigger, taking all the plate in a few days and prevented the bacteria of interest to grow. The absence of access to method of bacteria identification such as 16S rRNA sequencing or kits to identify the bacteria, led to an impossibility to identify the contamination. Moreover, identifying the contamination could have helped to investigate the source of contamination and to prevent it.

Another advantage of having access to sequencing method is the identification of the bacteria lineage and potential mutations that are involved in the adaptation and resistance mechanisms.

4.4.3 Outcome of the experiment

Despite being promising on the paper and have been proven to work with antibiotics, the realization of such experiments has some limitations. Not considering the bacterial part, the experiment works, the QAC is diffusing from the hard to the soft agar layer, making a gradient field for the bacteria. However, the impossibility to control the contaminations forced us to a stop of this project.

The project went back to another method for the development of the resistance that has been proven to work and to avoid contamination risk with past experiment. The method chosen is stepwise training in liquid broth (Chapter 3). This method has the advantage to have all steps performed in the same laboratory and to include a purity check at each step. However, it takes longer time and material, but considering the limited risks of contaminations, the time and material limitation is a small sacrifice.

Acknowledgements

We would like to thank the “Atelier des maquettes” at EPFL for their help in building the MEGAPLATES, Antoine Wiedmer for building the imaging system and the Python code and the Central lab (EPFL) for the large enough incubator. We also would like to thank Vincent Berweiler who has performed a big part of the experiments.

5 Conclusion

5.1 Achieved results

The use of disinfection agents increased in the past decades. They are not only used in health care facilities and industries but also applied in consumer products to clean textiles, surfaces and sanitary facilities. They are part of many detergent formulations for surface cleaning or laundry and commonly used in personal care products such as conditioners, toothpastes, or cosmetics. The aim of the addition of disinfection agents in such products is to prevent the spread of pathogenic microorganisms. For instance, during the recent COVID-19 pandemic, the disinfection procedures of surfaces were promoted as well as the hand disinfection with soap or hydro alcoholic gels. The fear of spreading an infection triggered a massive use of disinfection agents.

Several compounds such as alcohols, phenols or peroxides are used as disinfection agents. Quaternary ammonium compounds (QACs) are among the most widely applied disinfection agents. QACs are used because of their broad-spectrum mode of action and in the class of QACs, benzalkonium chloride (BAC) and cetyltrimethylammonium chloride (CTMA) are among the most used, e.g., included in many surface cleaning and personal care products. The main target of QACs is the cell membrane of microorganisms. The positively charged ammonium group of QACs interacts with the negatively charged membrane. This leads to a destabilization of the membrane, leakage of the intracellular components and finally cellular death. Resistance to QACs is linked to membrane modifications and the expression of efflux pumps. After use in hospitals, industries or households, QACs are often discharged to municipal wastewaters. QACs are poorly biodegradable and will mostly persist during municipal wastewater treatment. QACs have been detected in the range of up to mgL^{-1} in hospital and municipal wastewater. Such concentrations are significantly below the application levels because of dilution effects. Nevertheless, such low concentrations can trigger some resistance mechanisms in bacteria.

In Chapter 2 the effect of constant sub-inhibitory concentrations of BAC and CTMA on *P. aeruginosa* were evaluated. It was observed that long-term exposure to concentrations close to the minimum inhibitory concentrations (MICs) led to a decrease in the susceptibility to the compound to which *P. aeruginosa* was exposed and to cross-resistance to the other QAC. However, no resistance to antibiotics was observed after exposure to constant sub-inhibitory concentra-

tions of BAC or CTMA. Morphological changes and membrane properties were investigated after exposure to BAC or CTMA. The roughness of the cellular membranes increased after exposure to both compounds, confirming QAC-induced modifications of the cell membrane. The stiffness and the adhesion increased after exposure to CTMA but not for BAC. This indicates that decreased susceptibility is possible after exposure to constant sub-inhibitory concentrations of QACs and that the bacterial response might be specific to each compound.

QACs are poorly biodegradable and persistent compounds. During municipal wastewater treatment, they are mostly removed by sorption on activated sludge. However, a small fraction escapes sorption and is released to the aquatic environment. Consequently, this fraction of QACs might be present during chemical disinfection.

Chapter 3 focuses on the effect of CTMA on the inactivation kinetics of *E. coli* by ozone or monochloramine, which are commonly used chemical disinfectants for water treatment. The effects of a pre-exposure to CTMA, in this case, a stepwise exposure to create the maximum selection pressure, and of the presence of CTMA during the inactivation by ozone or monochloramine were investigated. It was observed that pre-exposure to CTMA led to different results. The inactivation by ozone was enhanced, whereas it was diminished for monochloramine. This is most likely due to the differences in the mode of action of ozone and monochloramine. In presence of CTMA, the inactivation was enhanced for both monochloramine and ozone. The inactivation was at least additive, if not synergetic for monochloramine and for ozone, it led to non-linear behavior because of too fast kinetics for the method used. The role of efflux pumps during inactivation could not be resolved. The inactivation of AG100tet, overexpressing an efflux pump system and the inactivation of AG100 strains pre-exposed to CTMA, overexpressing another efflux pumps system led to opposite results for ozone and monochloramine.

Another important outcome of Chapter 3 is the susceptibility of *E. coli* to CTMA in different matrices. One of the standard methods to determine susceptibility of bacteria to an antimicrobial agent is the measurement of the MIC. Its determination is carried out in nutrient broth (e.g., MHB) and relies on the growth of bacteria in presence of different concentrations of a disinfection agent. However, in natural aquatic environments, bacteria will not be exposed to CTMA under those ideal conditions. Therefore, the susceptibility of *E. coli* to CTMA in MHB, PBS, a lake water and a wastewater effluent was investigated with dose-response curves. It was observed that the susceptibility to CTMA is higher in PBS, lake water and wastewater effluent compared to the same experiments in broth. Furthermore, the inactivation curves were similar for PBS,

lake water and wastewater effluent. It is hypothesized that the formation of micelles in MHB may explain the differences to the other waters. This result is important for the aquatic environment where the susceptibility to CTMA, and potentially to other biocidal compounds can be under-estimated with the standard MIC determination method.

Chapter 4 of this thesis deals with an alternative method to test the development of resistance to QACs. Previously, this method was successfully applied to test the susceptibility of bacteria to antibiotics by a large agar plate (MEGAPLATE). However, in this thesis, the method could not successfully be implemented due to several limitations and shortcomings. The large size of the device and the volume of agar required with the lack of a dedicated sterile space to create the plate and incubate it at the same time increased the problem of contamination. In addition, the plate was incubated for several days, also increasing the risk of contamination. The contamination that occurred multiple times took over the whole plate, did not allow discriminating it with the original bacteria and occurred during the first days, inhibiting the growth of the bacteria of interest. The source of contamination could not identified, wherefore this project was stopped. The MEGAPLATE approach remains an interesting approach to follow the adaptation and the development of resistance to a biocide. However, this method requires a strict disinfection protocol and a strict control of the incubation period (time, temperature, humidity, airflow).

5.2 Future development

QACs are increasingly present in our daily lives and their massive use is not without consequence. In Europe, the amount of QACs imported or manufactured is in the range ≥ 1000 and <10,000 tons per year, representing a market of several billions of US dollars. It led to an accumulation of these chemicals in the environment, mainly in sediments, which may affect the surface water quality. This creates local hot spots with low but potentially sufficiently high concentrations of QACs that may promote resistance in microorganisms. The presence of QACs in the environment should be monitored and the impact of these concentrations of antimicrobial agents should be assessed for different kinds of microorganisms. Moreover, the difference of susceptibility found between MHB and other matrices such as lake water and wastewater effluent, needs to be further evaluated to understand the reasons of these differences and support or falsify the nutrients' shortage or the micelle hypothesis. The method for the determination of the susceptibility should be adapted for the presence of biocides in the environment and not only for their applications under standard conditions.

The promotion of resistance mechanisms also calls for more investigations. The results of Chapter 2 show that an adaptation of bacteria to QAC concentrations higher than the MIC is possible when they are pre-exposed to constant sub-inhibitory concentrations. The experiments were performed in broth, and it would be important to understand if such a promotion is possible in other matrices such as wastewater or surface water.

Pre-exposure of bacteria to CTMA before water treatment led to different results for the inactivation efficiency by ozone and monochloramine. The reasons of this difference, apart from different modes of action of the two chemical oxidants, remains unclear and requires more investigation. Moreover, the role of efflux pumps during inactivation should be further evaluated. Different results were observed for a strain with one efflux pump system overexpressed (AG100tet) and strains pre-exposed to CTMA with another, unknown efflux pump system expressed. Moreover, different disinfectants such as chlorine dioxide, permanganate or hydroxyl radical should be investigated, because they have different modes of action with possibly different outcomes. Additionally, UV-radiation at 254 nm should also be investigated, as it is physical and not chemical method.

The development of resistance in liquids, as applied in this thesis, can be related to the development of resistance in water or in wastewater. In the liquid form, regular mixing is often involved, ensuring the same access to nutrients and to the biocide to all bacteria. However, in natural environment, ideal mixing does not always occur, and local or microenvironments are occurring such as in biofilms or when QACs are released from sediments. The MEGAPLATE approach included this spatial parameter. Bacteria where in competition and the first ones to adapt had access to nutrients and could spread blocking the other ones. Moreover, this approach allowed to follow and to sequence strains to obtain genetic information of the resistance development. The plates also contained a gradient of concentrations, which allowed investigating the size of the steps required to prevent the development of resistance. Apart from the water and wastewater problematic, this kind of approach is also useful to investigate the development of resistance on surfaces.

Finally, this study focused on two QACs, CTMA and BAC. However, there are plenty of other disinfection agents used and some of them have been identified as poorly biodegradable and/or detected in surface waters. Among them are aldehydes, anilids, biguanides, phenols or halogens-releasing agents. The literature focused on some compounds, such as triclosan, a phenolic compound found in numerous PCPs until the regulations became stricter in the past years.

However, concentrations in the ngL^{-1} are still found in surface water. Chlorhexidine, a biguanide and triclocarban, an anilid are also well studied for resistance development. However, the fate of these disinfection agents in the environment is not known yet. In addition, there are many more compounds, such as the glucoprotamin tested in Chapter 2, that are used in commercial formulations of detergents that would require more investigations.

References

[1] D. Sedlak, Water 4.0: The past, present, and future of the world's most vital resource, Yale University Press, 2014.

[2] A.P. Fraise, P.A. Lambert, J.-Y. Maillard, eds., Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization, Blackwell Publishing Ltd, Oxford, UK, 2004. https://doi.org/10.1002/9780470755884.

[3] ZURICH , THE WATER CITY, (2015). https://www.zuerich.com/sites/default/files/media_text_water_city_2015_05.pdf.

[4] J.N.N. Hopkins, the Cloaca Maxima and the Monumental Manipulation of Water in Archaic Rome, Waters Rome. 4 (2007) 1–15.

[5] M.J. McGuire, Eight revolutions in the history of US drinking water disinfection, J. / Am. Water Work. Assoc. 98 (2006) 123–149. https://doi.org/10.1002/j.1551-8833.2006.tb07612.x.

[6] J.J. Rook, Formation of haloforms during chlorination od natural waters, Water Treat. Exam. 23 (1974) 234–243.

[7] D.L. Sedlak, U. von Gunten, The Chlorine Dilemma, Science (80-.). 331 (2011) 42–43. https://doi.org/10.1126/science.1196397.

[8] U. Von Gunten, Oxidation Processes in Water Treatment: Are We on Track?, Environ. Sci. Technol. 52 (2018) 5062–5075. https://doi.org/10.1021/acs.est.8b00586.

[9] C. von Sonntag, U. von Gunten, Chemistry of Ozone in Water and Wastewater Treatment: From Basic Principles to Applications, 2012. https://doi.org/10.2166/9781780400839.

[10] J.C. Crittenden, R.R. Trussell, D.W. Hand, K.J. Howe, G. Tchobanoglous, MWH's Water Treatment, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2012. https://doi.org/10.1002/9781118131473.

[11] U. Wiesmann, S. Choi, E.-M. Dombrowski, Historical Development of Wastewater Collection and Treatment Water Supply and Wastewater Management in Antiquity, in: Fundam. Biol. Wastewater Treat., 2007: pp. 1–25.

[12] G. Lofrano, J. Brown, Wastewater management through the ages: A history of mankind, Sci. Total Environ. 408 (2010) 5254–5264. https://doi.org/10.1016/j.scitotenv.2010.07.062.

[13] Y. Zhang, Y. Shen, Wastewater irrigation: past, present, and future, Wiley Interdiscip. Rev. Water. 6 (2019) e1234. https://doi.org/10.1002/wat2.1234.

[14] M. Hotchkiss, T.J. Murray, the Relative Prevalence of Bacteria in an Imhoff Tank, Am. J. Public Health. 13 (1923) 562–567. https://doi.org/10.2105/ajph.13.7.562.

[15] C. Stamm, R.I.L. Eggen, J.G. Hering, J. Hollender, A. Joss, M. Schärer, Micropollutant removal from wastewater: Facts and decision-making despite uncertainty, Environ. Sci. Technol. 49 (2015) 6374–6375. https://doi.org/10.1021/acs.est.5b02242.

[16] J. Margot, C. Kienle, A. Magnet, M. Weil, L. Rossi, L.F. de Alencastro, C. Abegglen, D. Thonney, N. Chèvre, M. Schärer, D.A. Barry, Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon?, Sci. Total Environ. 461–462 (2013) 480–498. https://doi.org/10.1016/j.scitotenv.2013.05.034.

[17] EPA Victoria, Guidelines for environmental management: Disinfection of treated wastewater, 2002.

[18] L. Guo, K. Wan, J. Zhu, C. Ye, K. Chabi, X. Yu, Detection and distribution of vbnc/viable pathogenic bacteria in full-scale drinking water treatment plants, J. Hazard. Mater. 406 (2021) 124335. https://doi.org/10.1016/j.jhazmat.2020.124335.

[19] J.D. Oliver, Recent findings on the viable but nonculturable state in pathogenic bacteria, FEMS Microbiol. Rev. 34 (2010) 415–425. https://doi.org/10.1111/j.1574-6976.2009.00200.x.

[20] S. Chen, X. Li, Y. Wang, J. Zeng, C. Ye, X. Li, L. Guo, S. Zhang, X. Yu, Induction of Escherichia coli into a VBNC state through chlorination/chloramination and differences in characteristics of the bacterium between states, Water Res. 142 (2018) 279–288. https://doi.org/10.1016/j.watres.2018.05.055.

[21] National Research Council (US) Safe Drinking Water Committtee, Drinking Water and Health, Volume 2, National Academic Press (US), Washington (DC), 1980. https://doi.org/10.17226/1904.

[22] O. CERF, A REVIEW Tailing of Survival Curves of Bacterial Spores, J. Appl. Bacteriol. 42 (1977) 1–19. https://doi.org/10.1111/j.1365-2672.1977.tb00665.x.

[23] World Health Organization, 3 Inactivation (disinfection) processes, in: M.W. LeChevallier, K.-K. Au (Eds.), WHO Water Treat. Pathog. Control Process Effic. Achiev. Safe Drink. Water, IWA Publishing, London, UK, 2004: pp. 41–65.

[24] J.C. Crittenden, R.R. Trussell, D.W. Hand, K.J. Howe, G. Tchobanoglous, MWH's Water Treatment, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2012. https://doi.org/10.1002/9781118131473.

[25] A.D. Nikolaou, S.K. Golfinopoulos, T.D. Lekkas, Formation of organic by-products during chlorination of natural waters, J. Environ. Monit. 4 (2002) 910–916. https://doi.org/10.1039/b202965k.

[26] C. Shang, E.R. Blatchle, Chlorination of pure bacterial cultures in aqueous solution, Water Res. 35 (2001) 244–254. https://doi.org/10.1016/S0043-1354(00)00248-7.

[27] X. Hu, S. Geng, X. Wang, C. Hu, Inactivation and Photorepair of Enteric Pathogenic Microorganisms with Ultraviolet Irradiation, Environ. Eng. Sci. 29 (2012) 549–553. https://doi.org/10.1089/ees.2010.0379.

[28] D.J. Jurgens, S.A. Sattar, T.F. Mah, Chloraminated drinking water does not generate bacterial resistance to antibiotics in Pseudomonas aeruginosa biofilms, Lett. Appl. Microbiol. 46 (2008) 562–567. https://doi.org/10.1111/j.1472-765X.2008.02354.x.

[29] R.L. Wolfe, N.R. Ward, B.H. Olson, Inorganic chloramines as drinking water disinfectants: A review, J. / Am. Water Work. Assoc. 76 (1984) 74–88. https://doi.org/10.1002/j.1551-

8833.1984.tb05337.x.

[30] U. Von Gunten, Ozonation of drinking water: Part I. Oxidation kinetics and product formation, Water Res. 37 (2003) 1443–1467. https://doi.org/10.1016/S0043-1354(02)00457-8.

[31] Y. Lee, S. Imminger, N. Czekalski, U. von Gunten, F. Hammes, Inactivation efficiency of Escherichia coli and autochthonous bacteria during ozonation of municipal wastewater effluents quantified with flow cytometry and adenosine tri-phosphate analyses, Water Res. 101 (2016) 617–627. https://doi.org/10.1016/j.watres.2016.05.089.

[32] M.B. Heeb, J. Criquet, S.G. Zimmermann-Steffens, U. Von Gunten, Oxidative treatment of bromide-containing waters: Formation of bromine and its reactions with inorganic and organic compounds - A critical review, Water Res. 48 (2014) 15–42. https://doi.org/10.1016/j.watres.2013.08.030.

[33] M.B. Heeb, I. Kristiana, D. Trogolo, J.S. Arey, U. von Gunten, Formation and reactivity of inorganic and organic chloramines and bromamines during oxidative water treatment, Water Res. 110 (2017) 91–101. https://doi.org/10.1016/j.watres.2016.11.065.

[34] EPA, Wastewater Technology Fact Sheet - Disinfection for Small Systems, Water Technol. Fact Sheet. (2002).

[35] J.J. Huang, H.Y. Hu, Y.H. Wu, B. Wei, Y. Lu, Effect of chlorination and ultraviolet disinfection on tetA-mediated tetracycline resistance of Escherichia coli, Chemosphere. 90 (2013) 2247–2253. https://doi.org/10.1016/j.chemosphere.2012.10.008.

[36] M.R. Templeton, R.C. Andrews, R. Hofmann, Inactivation of particle-associated viral surrogates by ultraviolet light, Water Res. 39 (2005) 3487–3500. https://doi.org/10.1016/j.watres.2005.06.010.

[37] S.S. Madaeni, The application of membrane technology for water disinfection, Water Res. 33 (1999) 301–308. https://doi.org/10.1016/S0043-1354(98)00212-7.

[38] M. Bodzek, K. Konieczny, M. Rajca, Membranes in water and wastewater disinfection – review, Arch. Environ. Prot. 45 (2019) 3–18. https://doi.org/10.24425/aep.2019.126419.

[39] M. Boyle, C. Sichel, P. Fernández-Ibáñez, G.B. Arias-Quiroz, M. Iriarte-Puña, a. Mercado, E. Ubomba-Jaswa, K.G. McGuigan, Bactericidal effect of solar water disinfection under real sunlight conditions, Appl. Environ. Microbiol. 74 (2008) 2997–3001. https://doi.org/10.1128/AEM.02415-07.

[40] K.G. McGuigan, R.M. Conroy, H.J. Mosler, M. du Preez, E. Ubomba-Jaswa, P. Fernandez-Ibañez, Solar water disinfection (SODIS): A review from bench-top to roof-top, J. Hazard. Mater. 235–236 (2012) 29–46. https://doi.org/10.1016/j.jhazmat.2012.07.053.

[41] K.G. McGuigan, T.M. Joyce, R.M. Conroy, J.B. Gillespie, M. Elmore-Meegan, Solar disinfection of drinking water contained in transparent plastic bottles: Characterizing the bacterial inactivation process, J. Appl. Microbiol. 84 (1998) 1138–1148. https://doi.org/10.1046/j.1365-2672.1998.00455.x.

[42] J. Lonnen, S. Kilvington, S.C. Kehoe, F. Al-Touati, K.G. McGuigan, Solar and photocatalytic

disinfection of protozoan, fungal and bacterial microbes in drinking water, Water Res. 39 (2005) 877–883. https://doi.org/10.1016/j.watres.2004.11.023.

[43] R.J.W. Lambert, M.D. Johnston, Disinfection kinetics: A new hypothesis and model for the tailing of log-survivor/time curves, J. Appl. Microbiol. 88 (2000) 907–913. https://doi.org/10.1046/j.1365-2672.2000.01060.x.

[44] L.L. Gyürek, G.R. Frinch, MODELING WATER TREATMENT CHEMICAL DISINFECTION KINETICS By Lyndon L. Gyiirek 1 and Gordon R. Finch/ Member, ASCE, J. Environ. Eng. 124 (1998) 783–793.

[45] A.T. Rachmadi, M. Kitajima, T. Kato, H. Kato, S. Okabe, D. Sano, Required Chlorination Doses to Fulfill the Credit Value for Disinfection of Enteric Viruses in Water: A Critical Review, Environ. Sci. Technol. 54 (2020) 2068–2077. https://doi.org/10.1021/acs.est.9b01685.

[46] U. von Gunten, J. Hoigné, Bromate Formation during Ozonization of Bromide-Containing Waters: Interaction of Ozone and Hydroxyl Radical Reactions, Environ. Sci. Technol. 28 (1994) 1234–1242. https://doi.org/10.1021/es00056a009.

[47] M. Deborde, U. von Gunten, Reactions of chlorine with inorganic and organic compounds during water treatment-Kinetics and mechanisms: A critical review, Water Res. 42 (2008) 13–51. https://doi.org/10.1016/j.watres.2007.07.025.

[48] A.K. Kivaisi, The potential for constructed wetlands for wastewater treatment and reuse in developing countries: A review, Ecol. Eng. 16 (2001) 545–560. https://doi.org/10.1016/S0925-8574(00)00113-0.

[49] J.D. Sivey, C.E. McCullough, A.L. Roberts, Chlorine monoxide (Cl2O) and molecular chlorine (Cl2) as active chlorinating agents in reaction of dimethenamid with aqueous free chlorine, Environ. Sci. Technol. 44 (2010) 3357–3362. https://doi.org/10.1021/es9038903.

[50] S.D. Richardson, M.J. Plewa, To regulate or not to regulate? What to do with more toxic disinfection by-products?, J. Environ. Chem. Eng. 8 (2020) 103939. https://doi.org/10.1016/j.jece.2020.103939.

[51] S. Dukan, D. Touati, Hypochlorous acid stress in Escherichia coli: Resistance, DNA damage, and comparison with hydrogen peroxide stress, J. Bacteriol. 178 (1996) 6145–6150. https://doi.org/10.1128/jb.178.21.6145-6150.1996.

[52] W.S. da C. Nizer, V. Inkovskiy, J. Overhage, Surviving reactive chlorine stress: Responses of gram-negative bacteria to hypochlorous acid, Microorganisms. 8 (2020) 1–27. https://doi.org/10.3390/microorganisms8081220.

[53] M.K. Ramseier, U. von Gunten, P. Freihofer, F. Hammes, Kinetics of membrane damage to high (HNA) and low (LNA) nucleic acid bacterial clusters in drinking water by ozone, chlorine, chlorine dioxide, monochloramine, ferrate(VI), and permanganate, Water Res. 45 (2011) 1490–1500. https://doi.org/10.1016/j.watres.2010.11.016.

[54] C. Venkobachar, L. Iyengar, A.V.S. Prabhakara Rao, Mechanism of disinfection: Effect of chlorine on cell membrane functions, Water Res. 11 (1977) 727–729. https://doi.org/10.1016/0043-1354(77)90114-2.

[55] V.K. Sharma, N. Johnson, L. Cizmas, T.J. McDonald, H. Kim, A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes, Chemosphere. 150 (2016) 702–714. https://doi.org/10.1016/j.chemosphere.2015.12.084.

[56] C. Bouki, D. Venieri, E. Diamadopoulos, Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review, Ecotoxicol. Environ. Saf. 91 (2013) 1–9. https://doi.org/10.1016/j.ecoenv.2013.01.016.

[57] G.E. Murray, R.S. Robin, B. Junkins, D.J. Kushner, Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria, Appl. Environ. Microbiol. 48 (1984) 73–77. https://doi.org/10.1128/aem.48.1.73-77.1984.

[58] Q. Bin Yuan, M.T. Guo, J. Yang, Fate of antibiotic resistant bacteria and genes during wastewater chlorination: Implication for antibiotic resistance control, PLoS One. 10 (2015) 1–11. https://doi.org/10.1371/journal.pone.0119403.

[59] C.X. Hiller, U. Hübner, S. Fajnorova, T. Schwartz, J.E. Drewes, Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review, Sci. Total Environ. 685 (2019) 596–608. https://doi.org/10.1016/j.scitotenv.2019.05.315.

[60] X. Bai, X. Ma, F. Xu, J. Li, H. Zhang, X. Xiao, The drinking water treatment process as a potential source of affecting the bacterial antibiotic resistance, Sci. Total Environ. 533 (2015) 24–31. https://doi.org/10.1016/j.scitotenv.2015.06.082.

[61] S. Jia, P. Shi, Q. Hu, B. Li, T. Zhang, X.X. Zhang, Bacterial Community Shift Drives Antibiotic Resistance Promotion during Drinking Water Chlorination, Environ. Sci. Technol. 49 (2015) 12271–12279. https://doi.org/10.1021/acs.est.5b03521.

[62] J.J. Huang, H.Y. Hu, F. Tang, Y. Li, S.Q. Lu, Y. Lu, Inactivation and reactivation of antibiotic-resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant, Water Res. 45 (2011) 2775–2781. https://doi.org/10.1016/j.watres.2011.02.026.

[63] M. Jin, L. Liu, D. ning Wang, D. Yang, W. li Liu, J. Yin, Z. wei Yang, H. ran Wang, Z. gang Qiu, Z. qiang Shen, D. yang Shi, H. bei Li, J. hua Guo, J. wen Li, Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation, ISME J. 14 (2020) 1847–1856. https://doi.org/10.1038/s41396-020-0656-9.

[64] M. Cho, J. Kim, J.Y. Kim, J. Yoon, J.H. Kim, Mechanisms of Escherichia coli inactivation by several disinfectants, Water Res. 44 (2010) 3410–3418. https://doi.org/10.1016/j.watres.2010.03.017.

[65] Y. Kouame, C.N. Haas, Inactivation of E. coli by combined action of free chlorine and monochloramine, Water Res. 25 (1991) 1027–1032. https://doi.org/10.1016/0043-1354(91)90195-V.

[66] J.G. Jacangelo, V.P. Olivieri, K. Kawata, Investigating the mechanism of inactivation of Escherichia coli B by monochloramine, J. / Am. Water Work. Assoc. 83 (1991) 80–87. https://doi.org/10.1002/j.1551-8833.1991.tb07152.x.

[67] C. Xi, Y. Zhang, C.F. Marrs, W. Ye, C. Simon, B. Foxman, J. Nriagu, Prevalence of antibiotic resistance in drinking water treatment and distribution systems, Appl. Environ. Microbiol. 75 (2009) 5714–5718. https://doi.org/10.1128/AEM.00382-09.

[68] J. Hoigné, H. Bader, Ozonation of Water : Role of Hydroxyl Radicals as Oxidizing Intermediates Author (s): J . Hoigné and H . Bader Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1741602 Ozonation of Water : Role, Science (80-.). 190 (1975) 782–784.

[69] J. Staehelin, J. Hoigne, Decomposition of Ozone in Water in the Presence of Organic Solutes Acting as Promoters and Inhibitors of Radical Chain Reactions, Environ. Sci. Technol. 19 (1985) 1206–1213. https://doi.org/10.1021/es00142a012.

[70] H. Bader, V. Sturzenegger, J. Hoigné, Photometric method for the determination of low concentrations of hydrogen peroxide by the peroxidase catalyzed oxidation of N,N-diethyl-p-phenylenediamine (DPD), Water Res. 22 (1988) 1109–1115. https://doi.org/10.1016/0043-1354(88)90005-X.

[71] D.L. McCurry, A.N. Quay, W.A. Mitch, Ozone Promotes Chloropicrin Formation by Oxidizing Amines to Nitro Compounds, Environ. Sci. Technol. 50 (2016) 1209–1217. https://doi.org/10.1021/acs.est.5b04282.

[72] N.K. Hunt, B.J. Mariñas, Inactivation of Escherichia coli with ozone: Chemical and inactivation kinetics, Water Res. 33 (1999) 2633–2641. https://doi.org/10.1016/S0043-1354(99)00115-3.

[73] F. Zuma, J. Lin, S.B. Jonnalagadda, Ozone-initiated disinfection kinetics of Escherichia coli in water, J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 44 (2009) 48–56. https://doi.org/10.1080/10934520802515335.

[74] N. Czekalski, S. Imminger, E. Salhi, M. Veljkovic, K. Kleffel, D. Drissner, F. Hammes, H. Bürgmann, U. Von Gunten, Inactivation of Antibiotic Resistant Bacteria and Resistance Genes by Ozone: From Laboratory Experiments to Full-Scale Wastewater Treatment, Environ. Sci. Technol. 50 (2016) 11862–11871. https://doi.org/10.1021/acs.est.6b02640.

[75] J. Oh, D.E. Salcedo, C.A. Medriano, S. Kim, Comparison of different disinfection processes in the effective removal of antibiotic-resistant bacteria and genes, J. Environ. Sci. (China). 26 (2014) 1238–1242. https://doi.org/10.1016/S1001-0742(13)60594-X.

[76] I.C. Iakovides, I. Michael-Kordatou, N.F.F. Moreira, A.R. Ribeiro, T. Fernandes, M.F.R. Pereira, O.C. Nunes, C.M. Manaia, A.M.T. Silva, D. Fatta-Kassinos, Continuous ozonation of urban wastewater: Removal of antibiotics, antibiotic-resistant Escherichia coli and antibiotic resistance genes and phytotoxicity, Water Res. (2019). https://doi.org/10.1016/j.watres.2019.05.025.

[77] K. Kümmerer, Emerging Contaminants, Treatise Water Sci. 3 (2011) 69–87. https://doi.org/10.1016/B978-0-444-53199-5.00052-X.

[78] R.P. Schwarzenbach, B.I. Escher, K. Fenner, T.B. Hofstetter, A.C. Johnson, U. von Gunten, B. Wehrli, The Challenge of Micropollutants in Aquatic Systems, Science (80-.). 313 (2006) 1072–1077. https://doi.org/10.1126/science.1127291.

[79] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance, Environ. Sci. Technol. 36 (2002) 1202–1211. https://doi.org/10.1021/es011055j.

[80] F.H.M. Tang, M. Lenzen, A. McBratney, F. Maggi, Risk of pesticide pollution at the global scale, Nat. Geosci. 14 (2021) 206–210. https://doi.org/10.1038/s41561-021-00712-5.

[81] R.P. Schwarzenbach, B.I. Escher, K. Fenner, T.B. Hofstetter, A.C. Johnson, U. von Gunten, B. Wehrli, The Challenge of Micropollutants in Aquatic Systems, Science (80-.). 313 (2006) 1072–1077. https://doi.org/10.1126/science.1127291.

[82] C. Kienle, E.L.M. Vermeirssen, A. Schifferli, H. Singer, C. Stamm, I. Werner, Effects of treated wastewater on the ecotoxicity of small streams - Unravelling the contribution of chemicals causing effects, PLoS One. 14 (2019) 1–30. https://doi.org/10.1371/journal.pone.0226278.

[83] C. Lok, The snot-spattered experiments that show how far sneezes really spread, Nature. 534 (2016) 24–26. https://doi.org/10.1038/534024a.

[84] A. Kramer, I. Schwebke, G. Kampf, How long do nosocomial pathogens persist on inanimate surfaces? A systematic review, BMC Infect. Dis. 6 (2006) 1–8. https://doi.org/10.1186/1471-2334-6-130.

[85] N.-O. Hübner, C. Hübner, A. Kramer, O. Assadian, Original Research: Survival of Bacterial Pathogens on Paper and Bacterial Retrieval from Paper to Hands: Preliminary Results, AJN, Am. J. Nurs. 111 (2011) 30–34. https://doi.org/10.1097/01.NAJ.0000408181.37017.82.

[86] C. Webster, K.J. Towner, H. Humphreys, Survival of Acinetobacter on Three Clinically Related Inanimate Surfaces, Infect. Control Hosp. Epidemiol. 21 (2000) 246–246. https://doi.org/10.1086/503214.

[87] V. Russotto, A. Cortegiani, S.M. Raineri, A. Giarratano, Bacterial contamination of inanimate surfaces and equipment in the intensive care unit, J. Intensive Care. 3 (2015) 54. https://doi.org/10.1186/s40560-015-0120-5.

[88] M.M. Comer, M. Ibrahim, V.J. McMillan, G.G. Baker, S.G. Patterson, Reducing the spread of infectious disease through hand washing, J. Ext. 47 (2009) 1–8.

[89] L. Clack, M. Scotoni, A. Wolfensberger, H. Sax, First-person view of pathogen transmission and hand hygiene - use of a new head-mounted video capture and coding tool, Antimicrob. Resist. Infect. Control. 6 (2017) 1–9. https://doi.org/10.1186/s13756-017-0267-z.

[90] A. Kramer, P. Guggenbichler, P. Heldta, M. Jünger, A. Ladwig, H. Thierbach, U. Weber, G. Daeschlein, Hygienic Relevance and Risk Assessment of Antimicrobial-Impregnated Textiles, in: U.-C. Hipler, P. Elsner (Eds.), Biofunctional Text. Ski., Karger, 2006: pp. 78–109. https://doi.org/10.1017/CBO9781107415324.004.

[91] E. Scott, S.F. Bloomfield, C.G. Barlow, An investigation of microbial contamination in the home, J. Hyg. (Lond). 89 (1982) 279–293. https://doi.org/10.1017/S0022172400070819.

[92] D.H. Kusumaningrum, Behaviour and cross-contamination of pathogenic bacteria in household kitchens – relevance to exposure assessment, Wageninen University, 2003.

[93] H.P. Schweizer, Triclosan: a widely used biocide and its link to antibiotics, FEMS Microbiol. Lett. 202 (2001) 1–7. https://doi.org/10.1111/j.1574-6968.2001.tb10772.x.

[94] K. Kümmerer, A. Eitel, U. Braun, P. Hubner, F. Daschner, G. Mascart, M. Milandri, F. Reinthaler, J. Verhoef, Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection, J. Chromatogr. A. 774 (1997) 281–286. https://doi.org/10.1016/S0021-9673(97)00242-2.

[95] K. Kümmerer, Drugs in the environment: Emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - A review, Chemosphere. 45 (2001) 957–969. https://doi.org/10.1016/S0045-6535(01)00144-8.

[96] X. Li, B.J. Brownawell, Quaternary ammonium compounds in urban estuarine sediment environments - A class of contaminants in need of increased attention?, Environ. Sci. Technol. 44 (2010) 7561–7568. https://doi.org/10.1021/es1011669.

[97] W. Ding, Y. Liao, Determination of Alkylbenzyldimethylammonium Chlorides in River Water and Sewage Effluent by Solid-Phase Extraction and Gas Chromatography / Mass Spectrometry, Anal. Chem. 73 (2001) 36–40.

[98] M. Clara, S. Scharf, C. Scheffknecht, O. Gans, Occurrence of selected surfactants in untreated and treated sewage, Water Res. 41 (2007) 4339–4348. https://doi.org/10.1016/j.watres.2007.06.027.

[99] G. McDonnell, A.D. Russell, Antiseptics and Disinfectants : Activity , Action , and Resistance, Clin. Microbiol. Rev. 12 (1999) 147–179. https://doi.org/0893-8512/99/$04.00+0.

[100] E.A. Bryce, D. Spence, F.J. Roberts, An In-Use Evaluation of an Alcohol-Based Pre-Surgical Hand Disinfectant, Infect. Control Hosp. Epidemiol. 22 (2001) 635–639. https://doi.org/10.1086/501835.

[101] C.R. Smith, Alcohol as a Disinfectant against the Tubercle Bacillus, Public Heal. Reports. 62 (1947) 1285. https://doi.org/10.2307/4586265.

[102] P.G. Mazzola, A.F. Jozala, L.C.D.L. Novaes, P. Moriel, T.C.V. Penna, Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents, Brazilian J. Pharm. Sci. 45 (2009) 241–248. https://doi.org/10.1590/S1984-82502009000200008.

[103] N. Henoun Loukili, H. Becker, J. Harno, M. Bientz, O. Meunier, Effect of peracetic acid and aldehyde disinfectants on biofilm, J. Hosp. Infect. 58 (2004) 151–154. https://doi.org/10.1016/j.jhin.2004.06.022.

[104] H.B. Drugeon, B. Rouveix, A. Michaud-Nerard, Activité antibactérienne du triclocarban sur les staphylocoques, entérocoques et streptocoques résistant, Med. Mal. Infect. 42 (2012) 276–279. https://doi.org/10.1016/j.medmal.2012.04.004.

[105] D.L. Breneman, J.M. Hanifin, C.A. Berge, B.H. Keswick, P.B. Neumann, The effect of antibacterial soap with 1.5% triclocarban on Staphylococcus aureus in patients with

atopic dermatitis, Cutis. 66 (2000) 296–300.

[106] N. Lozano, C.P. Rice, M. Ramirez, A. Torrents, Fate of Triclocarban, Triclosan and Methyltriclosan during wastewater and biosolids treatment processes, Water Res. 47 (2013) 4519–4527. https://doi.org/10.1016/j.watres.2013.05.015.

[107] S.E. Walsh, J.Y. Maillard, A.D. Russell, C.E. Catrenich, D.L. Charbonneau, R.G. Bartolo, Development of bacterial resistance to several biocides and effects on antibiotic susceptibility, J. Hosp. Infect. 55 (2003) 98–107. https://doi.org/10.1016/S0195-6701(03)00240-8.

[108] N. Rohrer, A.F. Widmer, T. Waltimo, E.M. Kulik, R. Weiger, E. Filipuzzi-Jenny, C. Walter, Antimicrobial Efficacy of 3 Oral Antiseptics Containing Octenidine, Polyhexamethylene Biguanide, or Citroxx: Can Chlorhexidine Be Replaced?, Infect. Control Hosp. Epidemiol. 31 (2010) 733–739. https://doi.org/10.1086/653822.

[109] M. Addy, Chlorhexidine compared with other locally delivered antimicrobials. A short review, J. Clin. Periodontol. 13 (1986) 957–964. https://doi.org/10.1111/j.1600-051X.1986.tb01434.x.

[110] L. Thomas, J.-Y. Maillard, R.J.W. Lambert, A.D. Russell, Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a “residual” concentration, J. Hosp. Infect. 46 (2000) 297–303. https://doi.org/10.1053/jhin.2000.0851.

[111] M. Östman, J. Fick, M. Tysklind, Detailed mass flows and removal efficiencies for biocides and antibiotics in Swedish sewage treatment plants, Sci. Total Environ. 640–641 (2018) 327–336. https://doi.org/10.1016/j.scitotenv.2018.05.304.

[112] C.A. Parnes, Efficacy of sodium hypochlorite bleach and “alternative” products in preventing transfer of bacteria to and from inanimate surfaces, J. Environ. Health. 59 (1997) 14–20.

[113] T.E. Odlaug, Antimicrobial Activity of Halogens, J. Food Prot. 44 (1981) 608–613. https://doi.org/10.4315/0362-028X-44.8.608.

[114] S. Silver, L.T. Phung, G. Silver, Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds, J. Ind. Microbiol. Biotechnol. 33 (2006) 627–634. https://doi.org/10.1007/s10295-006-0139-7.

[115] R. Kaegi, A. Voegelin, C. Ort, B. Sinnet, B. Thalmann, J. Krismer, H. Hagendorfer, M. Elumelu, E. Mueller, Fate and transformation of silver nanoparticles in urban wastewater systems, Water Res. 47 (2013) 3866–3877. https://doi.org/10.1016/j.watres.2012.11.060.

[116] G. Mulley, A.T.A. Jenkins, N.R. Waterfield, Inactivation of the antibacterial and cytotoxic properties of silver ions by biologically relevant compounds, PLoS One. 9 (2014) 2–10. https://doi.org/10.1371/journal.pone.0094409.

[117] G. McDonnell, Peroxygens and other forms of oxygen: Their use for effective cleaning, disinfection, and sterilization, ACS Symp. Ser. 967 (2007) 292–308. https://doi.org/10.1021/bk-2007-0967.ch013.

[118] C.A. Bartzokas, J.E. Corkill, T. Makin, Evaluation of the Skin Disinfecting Activity and Cumulative Effect of Chlorhexidine and Triclosan Handwash Preparations on Hands Artificially Contaminated with Serratia marcescens, Infect. Control. 8 (1987) 163–167. https://doi.org/10.1017/S0195941700065838.

[119] D.J. Jeffrey, Chemicals used as disinfectants: active ingredients and enhancing additives., Rev. Sci. Tech. 14 (1995) 57–74. https://doi.org/10.20506/rst.14.1.828.

[120] H. Singer, S. Müller, C. Tixier, L. Pillonel, S. Muller, C. Tixier, L. Pillonel, 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. 36 (2002) 4998–5004. https://doi.org/10.1021/es025750i.

[121] S. Mahmoud Sadjjadi, J. Rostami, M. Azadbakht, Giardiacidal activity of lemon juice, vinifer and vinegar on Giardia intestinalis cysts., Southeast Asian J. Trop. Med. Public Health. 37 Suppl 3 (2006) 24–27.

[122] D. Fong, C. Gaulin, M. Lê, M. Shum, Effectiveness of Alternative Antimicrobial Agents for Disinfection of Hard Surfaces, (2011) 1–19.

[123] B. Baldas, E.M. Altuner, The antimicrobial activity of apple cider vinegar and grape vinegar, which are used as a traditional surface disinfectant for fruits and vegetables, Commun. Fac. Sci. Univ. Ankara Ser. C Biol. Geol. Eng. Geophys. Eng. 27 (2018) 1–10. https://doi.org/10.1501/Commuc_0000000187.

[124] I.Y. Sengun, M. Karapinar, Effectiveness of household natural sanitizers in the elimination of Salmonella typhimurium on rocket (Eruca sativa Miller) and spring onion (Allium cepa L.), Int. J. Food Microbiol. 98 (2005) 319–323. https://doi.org/10.1016/j.ijfoodmicro.2004.07.011.

[125] Y.S. Malik, S.M. Goyal, Virucidal efficacy of sodium bicarbonate on a food contact surface against feline calicivirus, a norovirus surrogate, Int. J. Food Microbiol. 109 (2006) 160–163. https://doi.org/10.1016/j.ijfoodmicro.2005.08.033.

[126] M. Bouaziz, T. Yangui, S. Sayadi, A. Dhouib, Disinfectant properties of essential oils from Salvia officinalis L. cultivated in Tunisia, Food Chem. Toxicol. 47 (2009) 2755–2760. https://doi.org/10.1016/j.fct.2009.08.005.

[127] F. Lu, Y.C. Ding, X.Q. Ye, Y.T. Ding, Antibacterial effect of cinnamon oil combined with thyme or clove oil, Agric. Sci. China. 10 (2011) 1482–1487. https://doi.org/10.1016/S1671-2927(11)60142-9.

[128] W.A. Rutala, S.L. Barbee, N.C. Aguiar, M.D. Sobsey, D.J. Weber, Antimicrobial Activity of Home Disinfectants and Natural Products Against Potential Human Pathogens, Infect. Control Hosp. Epidemiol. 21 (2000) 33–38. https://doi.org/10.1086/501694.

[129] M.C. Jennings, K.P.C. Minbiole, W.M. Wuest, Quaternary Ammonium Compounds: An Antimicrobial Mainstay and Platform for Innovation to Address Bacterial Resistance, ACS Infect. Dis. 1 (2016) 288–303. https://doi.org/10.1021/acsinfecdis.5b00047.

[130] K.P.C. Minbiole, M.C. Jennings, L.E. Ator, J.W. Black, M.C. Grenier, J.E. LaDow, K.L. Caran, K. Seifert, W.M. Wuest, From antimicrobial activity to mechanism of resistance: the multifaceted role of simple quaternary ammonium compounds in bacterial eradication,

Tetrahedron. 72 (2016) 3559–3566. https://doi.org/10.1016/j.tet.2016.01.014.

[131] M.C. Jennings, B.A. Buttaro, K.P.C. Minbiole, W.M. Wuest, Bioorganic Investigation of Multicationic Antimicrobials to Combat QAC-Resistant Staphylococcus aureus, ACS Infect. Dis. 1 (2016) 304–309. https://doi.org/10.1021/acsinfecdis.5b00032.

[132] K.R. Morrison, R.A. Allen, K.P.C. Minbiole, W.M. Wuest, More QACs, more questions: Recent advances in structure activity relationships and hurdles in understanding resistance mechanisms, Tetrahedron Lett. 60 (2019) 150935. https://doi.org/10.1016/j.tetlet.2019.07.026.

[133] A.J. McBain, R.G. Ledder, L.E. Moore, C.E. Catrenich, P. Gilbert, Effects of Quaternary-Ammonium-Based Formulations on Bacterial Community Dynamics and Antimicrobial Susceptibility, Appl. Environ. Microbiol. 70 (2004) 3449–3456. https://doi.org/10.1128/AEM.70.6.3449-3456.2004.

[134] S.B. Levy, Active efflux, a common mechanism for biocide and antibiotic resistance., Symp. Ser. Soc. Appl. Microbiol. (2002) 65S-71S. https://doi.org/10.1046/j.1365-2672.92.5s1.4.x.

[135] B. Moen, K. Rudi, E. Bore, S. Langsrud, Subminimal inhibitory concentrations of the disinfectant benzalkonium chloride select for a tolerant subpopulation of Escherichia coli with inheritable characteristics, Int. J. Mol. Sci. 13 (2012) 4101–4123. https://doi.org/10.3390/ijms13044101.

[136] D.G. White, P.F. McDermott, Biocides, drug resistance and microbial evolution, Curr. Opin. Microbiol. 4 (2001) 313–317. https://doi.org/10.1016/S1369-5274(00)00209-5.

[137] P. Hora, S.G. Pati, P.J. McNamara, W.A. Arnold, Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications, Environ. Sci. Technol. Lett. (2020). https://doi.org/10.1021/acs.estlett.0c00437.

[138] G.G. Ying, Fate, behavior and effects of surfactants and their degradation products in the environment, Environ. Int. 32 (2006) 417–431. https://doi.org/10.1016/j.envint.2005.07.004.

[139] K. Ikehata, M.G. El-Din, Degradation of recalcitrant surfactants in wastewater by ozonation and advanced oxidation processes: A review, Ozone Sci. Eng. 26 (2004) 327–343. https://doi.org/10.1080/01919510490482160.

[140] K. Ikehata, M.G. El-Din, S.A. Snyder, Ozonation and advanced oxidation treatment of emerging organic pollutants in water and wastewater, Ozone Sci. Eng. 30 (2008) 21–26. https://doi.org/10.1080/01919510701728970.

[141] B. Delanghe, C.I. Mekras, N.J.D. Graham, Aqueous Ozonation Of Surfactants: A Review, Ozone Sci. Eng. 13 (1991) 639–673. https://doi.org/10.1080/01919512.1991.10555707.

[142] R.F. Dantas, M. Darcissac, C. Lesueur, S. Contreras, C. Sans, M. Fuerhacker, S. Esplugas, Assessment of Cationic Surfactants Mineralization by Ozonation and Photo-Fenton Process, Water Environ. Res. 81 (2009) 201–205. https://doi.org/10.2175/106143008x274338.

[143] C. Zhang, F. Cui, G. ming Zeng, M. Jiang, Z. zhu Yang, Z. gang Yu, M. ying Zhu, L. qing Shen, Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment, Sci. Total Environ. 518–519 (2015) 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007.

[144] D. Fatta-Kassinos, K. Kümmerer, Pharmaceuticals in the environment: sources, fate, effects and risks, Environ. Sci. Pollut. Res. 17 (2010) 519–521. https://doi.org/10.1007/s11356-009-0276-4.

[145] S.G. Pati, W.A. Arnold, Comprehensive screening of quaternary ammonium surfactants and ionic liquids in wastewater effluents and lake sediments, Environ. Sci. Process. Impacts. 22 (2020) 430–441. https://doi.org/10.1039/c9em00554d.

[146] K. Kummerer, Resistance in the environment, J. Antimicrob. Chemother. 54 (2004) 311–320. https://doi.org/10.1093/jac/dkh325.

[147] L. Nuñez, J. Moretton, Disinfectant-resistant bacteria in Buenos Aires city hospital wastewater, Brazilian J. Microbiol. 38 (2007) 644–648. https://doi.org/10.1590/S1517-83822007000400012.

[148] I. Michael, L. Rizzo, C.S. McArdell, C.M. Manaia, C. Merlin, T. Schwartz, C. Dagot, D. Fatta-Kassinos, Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review, Water Res. 47 (2013) 957–995. https://doi.org/10.1016/j.watres.2012.11.027.

[149] L. Rizzo, Inactivation and injury of total coliform bacteria after primary disinfection of drinking water by TiO2 photocatalysis, J. Hazard. Mater. 165 (2009) 48–51. https://doi.org/10.1016/j.jhazmat.2008.09.068.

[150] J.S. Chapman, Biocide resistance mechanisms, Int. Biodeterior. Biodegrad. 51 (2003) 133–138. https://doi.org/10.1016/S0964-8305(02)00097-5.

[151] J.S. Chapman, Disinfectant resistance mechanisms, cross-resistance, and co-resistance, Int. Biodeterior. Biodegrad. 51 (2003) 271–276. https://doi.org/10.1016/S0964-8305(03)00044-1.

[152] K. Hegstad, S. Langsrud, B.T. Lunestad, A.A. Scheie, M. Sunde, S.P. Yazdankhah, Does the Wide Use of Quaternary Ammonium Compounds Enhance the Selection and Spread of Antimicrobial Resistance and Thus Threaten Our Health?, Microb. Drug Resist. 16 (2010) 91–104. https://doi.org/10.1089/mdr.2009.0120.

[153] S. Buffet-Bataillon, P. Tattevin, M. Bonnaure-Mallet, A. Jolivet-Gougeon, Emergence of resistance to antibacterial agents: The role of quaternary ammonium compounds - A critical review, Int. J. Antimicrob. Agents. 39 (2012) 381–389. https://doi.org/10.1016/j.ijantimicag.2012.01.011.

[154] J.-Y. Maillard, Resistance of Bacteria to Biocides, Microbiol. Spectr. 6 (2018) 183–188. https://doi.org/10.1128/microbiolspec.ARBA-0006-2017.

[155] J.Y. Maillard, Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern?, J. Hosp. Infect. 65 (2007) 60–72. https://doi.org/10.1016/S0195-6701(07)60018-8.

[156] T.E. Cloete, Resistance mechanisms of bacteria to antimicrobial compounds, Int. Biodeterior. Biodegrad. 51 (2003) 277–282. https://doi.org/10.1016/S0964-8305(03)00042-8.

[157] P. Fernandes, B. Sommer Ferreira, J.M. Sampaio Cabral, Solvent tolerance in bacteria: Role of efflux pumps and cross-resistance with antibiotics, Int. J. Antimicrob. Agents. 22 (2003) 211–216. https://doi.org/10.1016/S0924-8579(03)00209-7.

[158] B. Marquez, Bacterial efflux systems and efflux pumps inhibitors, Biochimie. 87 (2005) 1137–1147. https://doi.org/10.1016/j.biochi.2005.04.012.

[159] A.J. McBain, A.H. Rickard, P. Gilbert, Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance, J. Ind. Microbiol. Biotechnol. 29 (2002) 326–330. https://doi.org/10.1038/sj.jim.7000324.

[160] Z. Jaglic, D. Cervinkova, Genetic basis of resistance to quaternary ammonium compounds - The qac genes and their role: A review, Vet. Med. (Praha). 57 (2012) 275–281.

[161] M. Baym, T.D. Lieberman, E.D. Kelsic, R. Chait, R. Gross, I. Yelin, R. Kishony, Spatiotemporal microbial evolution on antibiotic landscapes, Science (80-.). 353 (2016) 1147–1151. https://doi.org/10.1126/science.aag0822.

[162] K.Z. Sanidad, H. Yang, W. Wang, E.I. Ozay, J. Yang, M. Gu, E. Karner, J. Zhang, D. Kim, L.M. Minter, H. Xiao, G. Zhang, Effects of consumer antimicrobials benzalkonium chloride, benzethonium chloride, and chloroxylenol on colonic inflammation and colitis-associated colon tumorigenesis in mice, Toxicol. Sci. 163 (2018) 490–499. https://doi.org/10.1093/toxsci/kfy045.

[163] C.P. Gerba, Quaternary Ammonium Biocides: Efficacy in Application, Appl. Environ. Microbiol. 81 (2015) 464–469. https://doi.org/10.1128/AEM.02633-14.

[164] G.R. Boyd, J.M. Palmeri, S. Zhang, D.A. Grimm, Pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) in stormwater canals and Bayou St. John in New Orleans, Louisiana, USA, Sci. Total Environ. 333 (2004) 137–148. https://doi.org/10.1016/j.scitotenv.2004.03.018.

[165] P. Verlicchi, A. Galletti, M. Petrovic, D. BarcelÓ, Hospital effluents as a source of emerging pollutants: An overview of micropollutants and sustainable treatment options, J. Hydrol. 389 (2010) 416–428. https://doi.org/10.1016/j.jhydrol.2010.06.005.

[166] U. Tezel, S.G. Pavlostathis, Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology, Curr. Opin. Biotechnol. 33 (2015) 296–304. https://doi.org/10.1016/j.copbio.2015.03.018.

[167] C. Zhang, F. Cui, G. ming Zeng, M. Jiang, Z. zhu Yang, Z. gang Yu, M. ying Zhu, L. qing Shen, Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment, Sci. Total Environ. 518–519 (2015) 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007.

[168] J. Margot, L. Rossi, D.A. Barry, C. Holliger, A review of the fate of micropollutants in wastewater treatment plants, Wiley Interdiscip. Rev. Water. 2 (2015) 457–487. https://doi.org/10.1002/wat2.1090.

[169] A. Lindström, I.J. Buerge, T. Poiger, P.A. Bergqvist, M.D. Müller, H.R. Buser, Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater, Environ. Sci. Technol. 36 (2002) 2322–2329. https://doi.org/10.1021/es0114254.

[170] R.J. Ash, J.L. Iverson, Antibiotic and disinfectant resistant bacteria in rivers of the United States, Proc. 4th Int. Conf. Pharm. Endocr. Disrupting Chem. Water. (2004) 1–7.

[171] T.A. Ternes, A. Joss, H. Siegrist, Peer Reviewed: Scrutinizing Pharmaceuticals and Personal Care Products in Wastewater Treatment, Environ. Sci. Technol. 38 (2004) 392A-399A. https://doi.org/10.1021/es040639t.

[172] F. Orias, Y. Perrodin, Characterisation of the ecotoxicity of hospital effluents: A review, Sci. Total Environ. 454–455 (2013) 250–276. https://doi.org/10.1016/j.scitotenv.2013.02.064.

[173] A.D. Russell, Mechanisms of bacterial resistance to biocides, Int. Biodeterior. Biodegradation. 36 (1995) 247–265. https://doi.org/10.1016/0964-8305(95)00056-9.

[174] S.E. Walsh, J.Y. Maillard, A.D. Russel, C.E. Catrenich, A.L. Charbonneau, R.G. Bartolo, Activity and Mechanism of Action of Selected Biocidal Agents on Gram -positive and -negative Bacteria, J. Appl. Microbiol. 94 (2003) 240–247.

[175] E. Martínez-Carballo, A. Sitka, C. González-Barreiro, N. Kreuzinger, M. Fürhacker, S. Scharf, O. Gans, Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria, Environ. Pollut. 145 (2007) 489–496. https://doi.org/10.1016/j.envpol.2006.04.033.

[176] Z.Z. Ismail, U. Tezel, S.G. Pavlostathis, Sorption of quaternary ammonium compounds to municipal sludge, Water Res. 44 (2010) 2303–2313. https://doi.org/10.1016/j.watres.2009.12.029.

[177] B. Sarkar, M. Megharaj, Y. Xi, G.S.R. Krishnamurti, R. Naidu, Sorption of quaternary ammonium compounds in soils: Implications to the soil microbial activities, J. Hazard. Mater. 184 (2010) 448–456. https://doi.org/10.1016/j.jhazmat.2010.08.055.

[178] T. Oz, A. Guvenek, S. Yildiz, E. Karaboga, Y.T. Tamer, N. Mumcuyan, V.B. Ozan, G.H. Senturk, M. Cokol, P. Yeh, E. Toprak, Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution, Mol. Biol. Evol. 31 (2014) 2387–2401. https://doi.org/10.1093/molbev/msu191.

[179] B. Moen, K. Rudi, E. Bore, S. Langsrud, Subminimal inhibitory concentrations of the disinfectant benzalkonium chloride select for a tolerant subpopulation of Escherichia coli with inheritable characteristics, Int. J. Mol. Sci. 13 (2012) 4101–4123. https://doi.org/10.3390/ijms13044101.

[180] L. Sandegren, Selection of antibiotic resistance at very low antibiotic concentrations, Ups. J. Med. Sci. 119 (2014) 103–107. https://doi.org/10.3109/03009734.2014.904457.

[181] P.H. Mc Cay, A.A. Ocampo-Sosa, G.T.A. Fleming, Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture, Microbiology. 156 (2010) 30–38.

https://doi.org/10.1099/mic.0.029751-0.

[182] S. Buffet-Bataillon, P. Tattevin, J.-Y. Maillard, M. Bonnaure-Mallet, A. Jolivet-Gougeon, Efflux pump induction by quaternary ammonium compounds and fluoroquinolone resistance in bacteria, Future Microbiol. 11 (2016) 81–92. https://doi.org/10.2217/fmb.15.131.

[183] M.F. Loughlin, M. V. Jones, P.A. Lambert, Pseudomonas aeruginosa cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics., J. Antimicrob. Chemother. 49 (2002) 631–639. https://doi.org/10.1093/jac/49.4.631.

[184] J.A. Joynson, B. Forbes, R.J.W. Lambert, Adaptive resistance to benzalkonium chloride, amikacin and tobramycin: The effect on susceptibility to other antimicrobials, J. Appl. Microbiol. 93 (2002) 96–107. https://doi.org/10.1046/j.1365-2672.2002.01667.x.

[185] A. Tabata, T. Maeda, H. Nagamune, H. Kourai, Characterization of Pseudomonas aeruginosa Resistant to a Quaternary Ammonium Compound., Biocontrol Sci. 7 (2002) 147–153. https://doi.org/10.4265/bio.7.147.

[186] L. Thomas, A.D. Russell, J.Y. Maillard, Antimicrobial activity of chlorhexidine diacetate and benzalkonium chloride against Pseudomonas aeruginosa and its response to biocide residues, J. Appl. Microbiol. 98 (2005) 533–543. https://doi.org/10.1111/j.1365-2672.2004.02402.x.

[187] M. Kim, M.R. Weigand, S. Oh, J.K. Hatt, R. Krishnan, U. Tezel, S.G. Pavlostathis, K.T. Konstantinidis, Widely Used Benzalkonium Chloride Disinfectants Can Promote Antibiotic Resistance, Appl. Environ. Microbiol. 84 (2018) 1–14. https://doi.org/10.1128/AEM.01201-18.

[188] F. Bert, E. Maubec, B. Bruneau, P. Berry, N. Lambert-Zechovsky, Multi-resistant Pseudomonas aeruginosa outbreak associated with contaminated tap water in a neurosurgery intensive care unit, J. Hosp. Infect. 39 (1998) 53–62. https://doi.org/10.1016/S0195-6701(98)90243-2.

[189] K.G. Kerr, A.M. Snelling, Pseudomonas aeruginosa: a formidable and ever-present adversary, J. Hosp. Infect. 73 (2009) 338–344. https://doi.org/10.1016/j.jhin.2009.04.020.

[190] K.D. Mena, C.P. Gerba, Risk Assessment of Pseudomonas aeruginosa in Water, in: Rev. Environ. Contam. Toxicol., 2009: pp. 71–115. https://doi.org/10.1007/978-1-4419-0032-6_3.

[191] H.P. Loveday, J.A. Wilson, K. Kerr, R. Pitchers, J.T. Walker, J. Browne, Association between healthcare water systems and Pseudomonas aeruginosa infections: A rapid systematic review, J. Hosp. Infect. 86 (2014) 7–15. https://doi.org/10.1016/j.jhin.2013.09.010.

[192] S. Tyski, W. Grzybowska, S. Grzeszczuk, P. Leszczynski, M. Staniszewska, E. Röhm-Rodowald, B. Jakimiak, Antimicrobial Activity of Glucoprotamin-Containing Disinfectants, Polish J. Microbiol. 58 (2009) 347–353.

[193] A. Chojecka, O. Wiercińska, E. Röhm-rodowald, Glucoprotamin antimicrobial activity against selected standard antibiotic-resistant bacteria and reference strains used in the

assessment of disinfection efficacy, Natl. Inst. Public Heal. - Natl. Inst. Hyg. 66 (2015) 281–288.

[194] P.C. Braga, D. Ricci, P.C. Braga, D. Ricci, Atomic Force Microscopy : Application to Investigation of Escherichia coli Morphology before and after Exposure to Cefodizime, Antimicrob. Agents Chemother. 42 (1998) 1–6.

[195] Y.F. Dufrêne, Application of atomic force microscopy to microbial surfaces : from reconstituted cell surface layers to living cells, Micron. 32 (2001) 153–165.

[196] G. Longo, S. Kasas, Effects of antibacterial agents and drugs monitored by atomic force microscopy, Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 6 (2014) 230–244. https://doi.org/10.1002/wnan.1258.

[197] C. Formosa, M. Grare, E. Jauvert, A. Coutable, J.B. Regnouf-de-Vains, M. Mourer, R.E. Duval, E. Dague, Nanoscale analysis of the effects of antibiotics and CX1 on a Pseudomonas aeruginosa multidrug-resistant strain, Sci. Rep. 2 (2012) 575. https://doi.org/10.1038/srep00575.

[198] H.A. Eskandarian, P.D. Odermatt, J.X.Y. Ven, M.T.M. Hannebelle, A.P. Nievergelt, N. Dhar, J.D. McKinney, G.E. Fantner, Division site selection linked to inherited cell surface wave troughs in mycobacteria, Nat. Microbiol. 2 (2017) 17094. https://doi.org/10.1038/nmicrobiol.2017.94.

[199] I. Wiegand, K. Hilpert, R.E.W. Hancock, Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances., Nat. Protoc. 3 (2008) 163–175. https://doi.org/10.1038/nprot.2007.521.

[200] J.M. Andrews, Determination of minimum inhibitory concentrations, J. Antimicrob. Chemother. 48 Suppl 1 (2001) 5–16. https://doi.org/10.1093/jac/48.suppl_1.5.

[201] EUCAST, ESCMID, Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution, Clin. Microbiol. Infect. 6 (2000) 509–515. https://doi.org/10.1046/j.1469-0691.2000.00142.x.

[202] D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting Linear Mixed-Effects Models Using lme4, J. Stat. Softw. 67 (2015). https://doi.org/10.18637/jss.v067.i01.

[203] J. Shaffer, Multiple Hypothesis Testing, Annu. Rev. Psychol. 46 (1995) 561–584. https://doi.org/10.1146/annurev.psych.46.1.561.

[204] M. Tandukar, S. Oh, U. Tezel, K.T. Konstantinidis, S.G. Pavlostathis, Long-Term Exposure to Benzalkonium Chloride Disinfectants Results in Change of Microbial Community Structure and Increased Antimicrobial Resistance, Environ. Sci. Technol. 47 (2013) 9730–9738. https://doi.org/10.1021/es401507k.

[205] C. Formosa, M. Grare, R.E. Duval, E. Dague, Nanoscale effects of antibiotics on P. aeruginosa, Nanomedicine Nanotechnology, Biol. Med. 8 (2012) 12–16. https://doi.org/10.1016/j.nano.2011.09.009.

[206] N.P. Mortensen, J.D. Fowlkes, C.J. Sullivan, D.P. Allison, N.B. Larsen, S. Molin, M.J. Doktycz, Effects of Colistin on Surface Ultrastructure and Nanomechanics of Pseudomonas aeruginosa Cells, Langmuir. 25 (2009) 3728–3733. https://doi.org/10.1021/la803898g.

[207] P. Gilbert, L.E. Moore, Cationic antiseptics: Diversity of action under a common epithet, J. Appl. Microbiol. 99 (2005) 703–715. https://doi.org/10.1111/j.1365-2672.2005.02664.x.

[208] J. Zabielska, A. Kunicka-Styczyńska, A. Otlewska, Adhesive and hydrophobic properties of Pseudomonas aeruginosa and Pseudomonas cedrina associated with cosmetics, Ecol. Quest. 28 (2018) 41. https://doi.org/10.12775/EQ.2017.037.

[209] A. Houari, P. Di Martino, Effect of chlorhexidine and benzalkonium chloride on bacterial biofilm formation, Lett. Appl. Microbiol. 45 (2007) 652–656. https://doi.org/10.1111/j.1472-765X.2007.02249.x.

[210] J.-Y. Maillard, S. Bloomfield, J.R. Coelho, P. Collier, B. Cookson, S. Fanning, A. Hill, P. Hartemann, A.J. Mcbain, M. Oggioni, S. Sattar, H.P. Schweizer, J. Threlfall, Does Microbicide Use in Consumer Products Promote Antimicrobial Resistance? A Critical Review and Recommendations for a Cohesive Approach to Risk Assessment, Microb. Drug Resist. 19 (2013) 344–354. https://doi.org/10.1089/mdr.2013.0039.

[211] P. Gilbert, A.J. Mcbain, Potential Impact of Increased Use of Biocides in Consumer Products on Prevalence of Antibiotic Resistance, Clin. Microbiol. Rev. 16 (2003) 189–208. https://doi.org/10.1128/CMR.16.2.189.

[212] M. Voumard, L. Venturelli, M. Borgatta, A. Croxatto, S. Kasas, G. Dietler, F. Breider, U. Von Gunten, Adaptation of Pseudomonas aeruginosa to constant sub-inhibitory concentrations of quaternary ammonium compounds, Environ. Sci. Water Res. Technol. 6 (2020) 1139–1152. https://doi.org/10.1039/c9ew01056d.

[213] U. Tezel, S.G. Pavlostathis, Role of Quaternary Ammonium Compounds on Antimicrobial Resistance in the Environment, in: Antimicrob. Resist. Environ., First edit, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2011: pp. 349–387. https://doi.org/10.1002/9781118156247.ch20.

[214] R.S. Boethling, Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants, Water Res. 18 (1984) 1061–1076. https://doi.org/10.1016/0043-1354(84)90220-3.

[215] M. Östman, R.H. Lindberg, J. Fick, E. Björn, M. Tysklind, Screening of biocides, metals and antibiotics in Swedish sewage sludge and wastewater, Water Res. 115 (2017) 318–328. https://doi.org/10.1016/j.watres.2017.03.011.

[216] C. Zhang, F. Cui, G. ming Zeng, M. Jiang, Z. zhu Yang, Z. gang Yu, M. ying Zhu, L. qing Shen, Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment, Sci. Total Environ. 518–519 (2015) 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007.

[217] J.Y. Maillard, Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern?, J. Hosp. Infect. (2007). https://doi.org/10.1016/S0195-6701(07)60018-8.

[218] N.K. Hunt, B.J. Mariñas, Kinetics of Escherichia coli inactivation with ozone, Science (80-.). 31 (1997) 1355–1362.

[219] M.C. Dodd, Potential impacts of disinfection processes on elimination and deactivation of

antibiotic resistance genes during water and wastewater treatment, J. Environ. Monit. 14 (2012) 1754–1771. https://doi.org/10.1039/c2em00006g.

[220] C. Corless, G. Reynolds, N. Graham, R. Perry, T.M. Gibson, J. Haley, Aqueous ozonation of a quaternary ammonium surfactant, Water Res. 23 (1989) 1367–1371. https://doi.org/10.1016/0043-1354(89)90075-4.

[221] M. Viveiros, A. Jesus, M. Brito, C. Leandro, M. Martins, D. Ordway, A.M. Molnar, J. Molnar, L. Amaral, Inducement and Reversal of Tetracycline Resistance in, Society. 49 (2005) 3578–3582. https://doi.org/10.1128/AAC.49.8.3578.

[222] M. Martins, M. Viveiros, I. Couto, S.S. Costa, T. Pacheco, S. Fanning, J.M. Pagès, L. Amaral, Identification of efflux pump-mediated multidrug-resistant bacteria by the ethidium bromide-agar cartwheel method, In Vivo (Brooklyn). 25 (2011) 171–178.

[223] C. Wolf, U. Von Gunten, T. Kohn, Kinetics of Inactivation of Waterborne Enteric Viruses by Ozone, Environ. Sci. Technol. 52 (2018) 2170–2177. https://doi.org/10.1021/acs.est.7b05111.

[224] I.M. Schreiber, W.A. Mitch, Influence of the Order of Reagent Addition on NDMA Formation during Chloramination, Environ. Sci. Technol. 39 (2005) 3811–3818. https://doi.org/10.1021/es0483286.

[225] E.W. Rice, R.B. Baird, A.D. Eaton, eds., Standard methods for the examination of water and wastewater, American Public Health Association, American Water Works Association, Water Environment Federation, 2005.

[226] N.K. Hunt, B.J. Mariñas, Kinetics of Escherichia coli inactivation with ozone, Water Res. 31 (1997) 1355–1362.

[227] A.H. Geeraerd, V.P. Valdramidis, J.F. Van Impe, GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves, Int. J. Food Microbiol. 102 (2005) 95–105. https://doi.org/10.1016/j.ijfoodmicro.2004.11.038.

[228] F. Baty, M. Delignetter-Muller, nlsMicrobio: data sets and nonlinear regression models dedicated to predictive microbiology, (2013) 1–16.

[229] F. Baty, C. Ritz, S. Charles, M. Brutsche, J.P. Flandrois, M.L. Delignette-Muller, A toolbox for nonlinear regression in R: The package nlstools, J. Stat. Softw. 66 (2015) 1–21. https://doi.org/10.18637/jss.v066.i05.

[230] P.R. Scheuerman, J.P. Schmidt, M. Alexander, Factors affecting the survival and growth of bacteria introduced into lake water, Arch. Microbiol. 150 (1988) 320–325. https://doi.org/10.1007/BF00408301.

[231] S. Giannakis, A.I. Merino Gamo, E. Darakas, A. Escalas-Cañellas, C. Pulgarin, Monitoring the post-irradiation E. coli survival patterns in environmental water matrices: Implications in handling solar disinfected wastewater, Chem. Eng. J. 253 (2014) 366–376. https://doi.org/10.1016/j.cej.2014.05.092.

[232] S.J. Schink, E. Biselli, C. Ammar, U. Gerland, Death Rate of E. coli during Starvation Is Set by Maintenance Cost and Biomass Recycling, Cell Syst. 9 (2019) 64-73.e3. https://doi.org/10.1016/j.cels.2019.06.003.

[233] F. Breider, I. Salihu, U. Von Gunten, Formation of N-nitrosamines by micelle-catalysed nitrosation of aliphatic secondary amines, Environ. Sci. Process. Impacts. 20 (2018) 1479–1487. https://doi.org/10.1039/c8em00335a.

[234] K. Nakata, T. Tsuchido, Y. Matsumura, Antimicrobial cationic surfactant, cetyltrimethylammonium bromide, induces superoxide stress in Escherichia coli cells, J. Appl. Microbiol. 110 (2011) 568–579. https://doi.org/10.1111/j.1365-2672.2010.04912.x.

[235] J.J. Macauley, Z. Qiang, C.D. Adams, R. Surampalli, M.R. Mormile, Disinfection of swine wastewater using chlorine, ultraviolet light and ozone, Water Res. 40 (2006) 2017–2026. https://doi.org/10.1016/j.watres.2006.03.021.

[236] J. Alexander, G. Knopp, A. Dötsch, A. Wieland, T. Schwartz, Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts, Sci. Total Environ. 559 (2016) 103–112. https://doi.org/10.1016/j.scitotenv.2016.03.154.

[237] S. Heß, C. Gallert, Sensitivity of antibiotic resistant and antibiotic susceptible Escherichia coli, Enterococcus and Staphylococcus strains against ozone, J. Water Health. 13 (2015) 1020–1028. https://doi.org/10.2166/wh.2015.291.

[238] G. Sundheim, S. Langsrud, E. Heir, A.L. Holck, Bacterial resistance to disinfectants containing quaternary ammonium compounds, in: Int. Biodeterior. Biodegrad., 1998: pp. 235–239. https://doi.org/10.1016/S0964-8305(98)00027-4.

[239] M. Viveiros, A. Martins, L. Paixão, L. Rodrigues, M. Martins, I. Couto, E. Fähnrich, W. V. Kern, L. Amaral, Demonstration of intrinsic efflux activity of Escherichia coli K-12 AG100 by an automated ethidium bromide method, Int. J. Antimicrob. Agents. 31 (2008) 458–462. https://doi.org/10.1016/j.ijantimicag.2007.12.015.

[240] D. Berry, D. Holder, C. Xi, L. Raskin, Comparative transcriptomics of the response of Escherichia coli to the disinfectant monochloramine and to growth conditions inducing monochloramine resistance, Water Res. 44 (2010) 4924–4931. https://doi.org/10.1016/j.watres.2010.07.026.

[241] D. Holder, D. Berry, D. Dai, L. Raskin, C. Xi, A dynamic and complex monochloramine stress response in Escherichia coli revealed by transcriptome analysis, Water Res. 47 (2013) 4978–4985. https://doi.org/10.1016/j.watres.2013.05.041.

[242] R.L. Valentine, K.I. Brandt, C.T. Jafvert, A spectrophotometric study of the formation of an unidentified monochloramine decomposition product, Water Res. 20 (1986) 1067–1074. https://doi.org/10.1016/0043-1354(86)90051-5.

[243] E. Larson, Community Factors in the Development of Antibiotic Resistance, Annu. Rev. Public Health. 28 (2007) 435–447. https://doi.org/10.1146/annurev.publhealth.28.021406.144020.

[244] A.E. Aiello, B. Marshall, S.B. Levy, P. Della-Latta, S.X. Lin, E. Larson, Antibacterial cleaning products and drug resistance, Emerg. Infect. Dis. (2005). https://doi.org/10.3201/eid1110.041276.

[245] O. Carjaa, U. Liberman, M.W. Feldman, Evolution in changing environments: Modifiers of

mutation, recombination, and migration, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 17935–17940. https://doi.org/10.1073/pnas.1417664111.

[246] K. Yamamoto, Sensitive determination of quaternary ammonium salts by extraction-spectrophotometry of ion associates with bromophenol blue anion and coextraction, Anal. Chim. Acta. 302 (1995) 75–79. https://doi.org/10.1016/0003-2670(94)00428-O.

Curriculum Vitae

Margaux VOUMARD

Avenue d'Yverdon 5 - 1004 Lausanne
0041 78 917 97 13
margauxvoumard@hotmail.com
www.linkedin.com/in/margaux-voumard-98345695

Highlights

- 5 years' experience in research in biosafety level 2 laboratories with bacteria and oxidants.
- 6 years of work on water and wastewater disinfection problematics
- Master of Science in Bioengineering

Professional experience

2016 –2021 **Doctoral Assistant – EPFL, Switzerland**
Laboratory for Water Quality and Treatment

- Design, development and experimental work linked to adaptation of bacteria to quaternary ammonium compounds, research on resistance profiles and inactivation. kinetics of bacteria by ozone and monochloramine.
- Data analysis, including advanced statistical analysis.
- Presentation of results in the form of bi-annual internal talks; posters, scientific reports and articles.
- Teaching experience for bachelor and master students.

2015 –2016 **Master Thesis and Internship – EPFL, Switzerland**
Group of Advanced Oxidation Processes

- Design, development and experimental work on the inactivation of bacteria and viruses by solar and UV-C irradiation, Fenton and photo-Fenton method.
- Data analysis and presentation of results in the form of a talk, scientific reports and articles.

February-2014 **Internship - EssentialMed Foundation, Switzerland and Cameroon**
Radiology and Radioprotection in Cameroon

- Design and development of a work protocol to collect data on-field regarding the protection measures in X-ray departments of health care facilities in Cameroon.
- Data collection on the field in 31 different health care facilities in Cameroon.
- Organization of a workshop to present the results to members of the EssentialMed Foundation and members of the Cameroonian Health Ministry, Professors and Radiology specialists.

Technical Skills

- Laboratory work in aseptic conditions, including in biosafety level 2
- Bacterial cultures, including P2 organisms
- Inactivation of bacteria by oxidants and UV-methods
- HPLC; spectrophotometry
- Susceptibility testing, including to antibiotics and disinfection agents
- Programming in R and Matlab
- Data analysis and advanced statistical modelling

- French as mother tongue
- Fluent in English (C1 level)

- B2 level in German for comprehension and reading, B1-B2 for oral and written expression

Education

2021 **PhD in Civil and Environmental Engineering, EPFL, Suisse**
Thesis: "Presence of quaternary ammonium compounds in water: effects on bacteria and their inactivation by ozone or monochloramine"

2015 **Master of Science MSc in Bioengineering, EPFL, Suisse**
Specialization in Bio-imaging and bio-photonics
Minor in Biotechnology

2013 **Bachelor in Life Sciences and Technologies, EPFL, Suisse**
Exchange at Polytechnique Montréal, Quebec, Canada during the 3rd year

2009 **Certificat de Maturité, Morges, Suisse**
Option : Biology and Chemistry

2008 **Goethe Zertifikat B2, Lausanne, Suisse**

Publications

Voumard, M., Venturelli, L., Borgatta, M., Croxatto, A., Kasas, S., Dietler, G., Breider, F. & von Gunten, U. (2020). Adaptation of Pseudomonas aeruginosa to constant sub-inhibitory concentrations of quaternary ammonium compounds. *Environmental Science: Water Research & Technology*, *6*(4), 1139-1152.

Voumard, M. O., Venturelli, L., Breider, F., & von Gunten, U. (2019, September). Resistance to disinfection agents: Adaptation of P. aeruginosa to sub inhibitory concentrations of quaternary ammonium compounds (QACs). In *20th Symposium on Health-Related Water Microbiology (HRWM)* (P198).

Voumard, M., Giannakis, S., Carratalà, A., & Pulgarin, C. (2019). E. coli–MS2 bacteriophage interactions during solar disinfection of wastewater and the subsequent post-irradiation period. *Chemical Engineering Journal*, *359*, 1224-1233.

Giannakis, S., **Voumard, M**., Rtimi, S., & Pulgarin, C. (2018). Bacterial disinfection by the photo-Fenton process: extracellular oxidation or intracellular photo-catalysis?. *Applied Catalysis B: Environmental*, *227*, 285-295.

Giannakis, S., **Voumard, M.,** Grandjean, D., Magnet, A., De Alencastro, L. F., & Pulgarin, C. (2016). Micropollutant degradation, bacterial inactivation and regrowth risk in wastewater effluents: Influence of the secondary (pre) treatment on the efficiency of Advanced Oxidation Processes. *Water research*, *102*, 505-515.

Extra-Professional activities

- Photography skills (Event photography: theatre, concert, festivals)
- Running (Participation in diverse 10km races)
- Sailing

Associative work:

- PhD student representative at EPFL (2017-2020)
- Vice-President of the EPFL PhD student association PolyDoc (2020) and committee member (2019)
- President of the EPFL photography club (2014-2016)

Personal situation

DoB: 16.10.1991; single; Swiss nationality