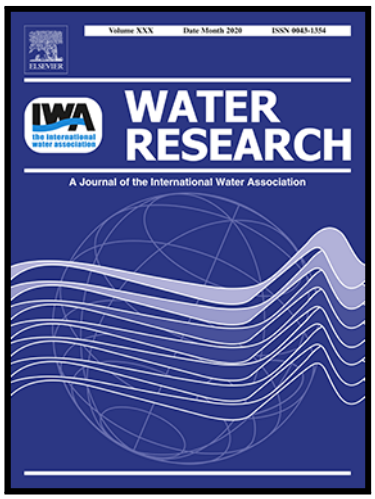

Effect of cetyltrimethylammonium chloride on various Escherichia coli strains and their inactivation kinetics by ozone and monochloramine

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PII: S0043-1354(22)00241-X
DOI: https://doi.org/10.1016/j.watres.2022.118278
Reference: WR 118278

To appear in: *Water Research*

Received date: 30 September 2021
Revised date: 23 February 2022
Accepted date: 7 March 2022

Please cite this article as: M. Voumard , F. Breider , U. von Gunten , Effect of cetyltrimethylammonium chloride on various Escherichia coli strains and their inactivation kinetics by ozone and monochloramine, *Water Research* (2022), doi: https://doi.org/10.1016/j.watres.2022.118278

Highlights

- Susceptibility of *E. coli* for CTMA is different in broth than in other waters
- A pre-exposure of *E. coli* to CTMA enhances the inactivation kinetics by ozone
- A pre-exposure of *E. coli* to CTMA reduces the inactivation kinetics by monochloramine
- The presence of QACs during the chemical disinfection enhances the inactivation of *E. coli*

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

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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. Currently, 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 the 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. The impacts of the pre-exposure and the presence of CTMA on inactivation by ozone and monochloramine were tested with three different *E. coli* strains: AG100 with the efflux pump *acrAB* intact, AG100A with it deleted and AG100tet with it overexpressed. Pre-exposure of *E. coli* AG100 to CTMA led to an increased susceptibility for ozone with second-order inactivation rate constants (~ 10^6 $M^{-1}s^{-1}$) increasing by a factor of about 1.5. An opposite trend was observed for monochloramine with second-order inactivation rate constants (~ 10^3 $M^{-1}s^{-1}$) decreasing by a factor of about 2. For *E. coli* AG100tet, the second-order inactivation rate constant decreased by a factor of almost 2 and increased by a factor of about 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 about 3. For monochloramine also an enhancement of the inactivation was

observed, which was at least additive but might also be synergistic. Enhancement by factors from about 2 to 4.5 were observed for CTMA concentrations > 2.5 mgL^{-1}.

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

1. Introduction

Quaternary ammonium compounds are widely used compounds for different purposes (Gilbert and Mcbain, 2003; Hegstad et al., 2010; Hora et al., 2020; Maillard et al., 2013; McDonnell and Russell, 1999; Morrison et al., 2019) such as disinfection, but also as surfactants, preservatives or biological stains, some of them are commonly used in consumers products (Hegstad et al., 2010; Maillard et al., 2013; McDonnell and Russell, 1999) QACs are used because of their broad-spectrum antimicrobial and surfactant-like properties. They contain an acyclic saturated hydrocarbon chain with a chain length 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 bactericidal effect with 12 – 14 carbons. This is hypothesized to correspond to the length required to penetrate the bacterial cell membrane (Jennings et al., 2016a, 2016b; Minbiole et al., 2016; Morrison et al., 2019). CTMA, for which the structure is provided in Figure 1, possesses an alkyl chain composed of 16 carbons, which is close to the ideal length to penetrate the bacterial membrane. The widespread use of CTMA in consumer products in addition to its presence in industrial products warrants further investigations on this compound (Hegstad et al., 2010). Furthermore, in contrast to other QACs, CTMA is an individual compound and not a mixture and therefore more suitable for experiments under controlled conditions.

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 (Jennings et al., 2016b). Low concentrations of QACs, in the sub-inhibitory range, have been shown to promote resistance in bacteria (Thomas et al., 2000; Voumard et al., 2020).

Additionally, promotion of resistance to antibiotics has been observed in some cases (Buffet-Bataillon et al., 2016, 2012; Hegstad et al., 2010) but not in others. The resistance mechanisms promoted below minimum inhibitory concentrations (MICs) of QACs are mostly achieved by modifications of the outer and inner cell membrane, the density and structure of porins and overexpression of efflux-pumps, including some that are specific for QACs (Buffet-Bataillon et al., 2016; Chapman, 2003; Fernandes et al., 2003; Jaglic and Cervinkova, 2012; Levy, 2002; Loughlin et al., 2002; Moen et al., 2012; Tezel and Pavlostathis, 2015, 2011; Walsh et al., 2003b).

The inclusion of QACs such as CTMA in industrial products and personal care products (PCPs) results in their inevitable direct release to the aquatic environment and the presence in sewage systems and wastewater (Hegstad et al., 2010; Hora et al., 2020; Kümmerer, 2001) and their removal during wastewater treatment is often incomplete (Boethling, 1984; Hora et al., 2020; Pati and Arnold, 2020; Tezel and Pavlostathis, 2015). Concentrations of QACs were detected in the mgL^{-1} range in hospital wastewater effluents (Kümmerer, 2001), in the μgL^{-1} to mgL^{-1} range in municipal wastewater treatment plant (WWTP) influents (Kreuzinger et al., 2007; Kümmerer, 2001) and in the μgL^{-1} to ngL^{-1} range in WWTP effluents (Pati and Arnold, 2020). Removal of QACs during wastewater treatment occurs mostly by sorption onto sludge (Hora et al., 2020; Östman et al., 2018, 2017). The sorptive properties of QACs also lead to an accumulation in sediments with patterns corresponding to the yearly use of QACs and the concentrations in sediments were found to be quite high from µg/kg to mg/kg (Li and Brownawell, 2010; Pati and Arnold, 2020; Zhang et al., 2015) and up to the g/kg range for sewage sludge (Li and Brownawell, 2010). 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 (Boethling, 1984; Buffet-Bataillon et al., 2012; Hegstad et al., 2010; Maillard, 2007).

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* (Mazzola et al., 2009; McDonnell and Russell, 1999; Walsh et al., 2003a), 0.5-75 mgL^{-1} for *S. aureus* (Mazzola et al., 2009; McDonnell and Russell, 1999), and 5-500 mgL^{-1} for *P. aeruginosa* (McDonnell and Russell, 1999; Voumard et al., 2020; Walsh et al., 2003b). 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 depend on the growth of bacteria (Wiegand et al., 2008). 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 lower than 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 (Sedlak and von Gunten, 2011; von Gunten, 2018). Among these disinfection methods, ozone has been shown to be most efficient (von Sonntag

and von Gunten, 2012) with second-order inactivation rate constants in the order of 10^6 M^{-1} s^{-1} for *E. coli* (Hunt and Mariñas, 1999, 1997). Inactivation by ozone occurs by direct oxidation with ozone (Hunt and Mariñas, 1997), leading to damages of membranes and internal cell components, and ultimately to cell death (Cho et al., 2010; von Sonntag and von Gunten, 2012; World Health Organization, 2004). 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 (von Sonntag and von Gunten, 2012). For other widely used disinfectants the inactivation kinetics are significantly lower (Heeb et al., 2017). One case in point is monochloramine, which was introduced to reduce the formation of chlorinated disinfection byproducts such as trihalomethanes (Sedlak and von Gunten, 2011). The mode of action of monochloramine on bacteria is mostly unknown, but some reactivity was found with cysteine and methionine (Dodd, 2012; Heeb et al., 2017), a slow reaction with DNA and RNA (Dodd, 2012) but no reaction is expected with fatty acids and polysaccharides of the cell membrane (Dodd, 2012). 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 (Cho et al., 2010; Dodd, 2012; Kouame and Haas, 1991; Ramseier et al., 2011; World Health Organization, 2004).

The presence of CTMA, before or during chemical disinfection of water and wastewater may be an additional factor influencing the inactivation efficiency 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, which CTMA is part of (Corless et al., 1989; Delanghe et al., 1991), because the quaternary ammonium group has no lone electron pair and aliphatic compounds have very low reactivity, but reactions with unsaturated QACs can occur (Corless et al., 1989; von Sonntag and von Gunten, 2012). QACs are also expected to have very low reactivity with monochloramine (Heeb et al., 2017). This absence of reactions between ozone or monochloramine and QACs and in particular CTMA 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.

Ozone and monochloramine disinfection kinetics have been well studied with several bacteria including *E. coli*, a gram-negative bacterium. Therefore, reference data on inactivation are available (Berry et al., 2010, 2009; Holder et al., 2013; Hunt and Mariñas, 1999, 1997; Jacangelo et al., 1991; Kouame and Haas, 1991; Lee et al., 2016; Zuma et al., 2009). Moreover, the effect of several QACs has been studied on this bacterium (Buffet-Bataillon et al., 2016; McDonnell and Russell, 1999; Walsh et al., 2003b, 2003a). The existence of reference data for the inactivation of *E. coli* by ozone and monochloramine as well as some information on the effect of QACs on it make this bacterium particularly interesting to investigate the combined effect of CTMA and ozone or monochloramine. Moreover, different strains of *E. coli* are available which allows to investigate different variations of the same bacteria. For instance, strains with different levels of activation in the efflux pump system (Martins et al., 2011; Viveiros et al., 2005) are particularly relevant to study, considering that the resistance to QACs is known to be linked to the efflux pump system (Buffet-Bataillon et al., 2016; Levy, 2002; Poole, 2005, 2002). Moreover, efflux

pumps have been shown to play a role in the inactivation by monochloramine of *E. coli* (Berry et al., 2010; Holder et al., 2013) . The impact of CTMA resistance and of mechanisms involved in the QAC resistance, such as efflux pumps on the inactivation by ozone or monochloramine has not been, or very poorly, investigated previously. As the bacterial resistance to biocides and to QACs such as CTMA is becoming a health concern, it is relevant to be investigated.

The aims of this study were to (i) investigate the susceptibility of *E. coli* to CTMA 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.

2. Material and Methods

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 (Viveiros et al., 2008). Briefly, AG100 has the efflux pump *acrAB* intact, AG100A has it deleted and AG100tet has it overexpressed (Martins et al., 2011). 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 2, cation adjusted; Sigma-Aldrich, Switzerland) and incubated overnight (12-16 hours, 37°C, 180 rpm). MHB was enriched with 100 mgL^{-1} kanamycin for AG100A and 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 re-suspended 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.

2.2. Quaternary Ammonium Compound

The disinfection agent used in this study was cetyltrimethylammonium chloride (CTMA, CAS 112-02-7). CTMA solutions (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.

2.3. Susceptibility to CTMA in different water matrices

The susceptibility of *E. coli* 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 1. Sterility of the water after filtration (Whatman® nitrocellulose membrane filters, 0.2 µm; Merck, Switzerland) 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 to 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.

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} (von Sonntag and von Gunten, 2012).

2.5. Ozone exposure

During the experiments, ozone exposures were controlled by a previously described method (Wolf et al., 2018). 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 (Wolf et al., 2018) 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 *t*-CA concentration, which differed from the original protocol where the produced benzaldehyde concentration was used (Wolf et al., 2018). 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 S1 (supporting information, SI).

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 hyxdroxyl radical (•OH) scavenger during ozonation. A 500 µL aliquot was taken before the experiment to determine the 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 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 S1 and Text S2, SI) and the second was diluted in PBS for bacteria quantification.

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 a previously published method (Schreiber and Mitch, 2005) by direct spectrophotometry at 245nm and 295nm. The equations and molar extinction coefficients used are provided in Text S3 (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 to 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 intervals (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. To determine the NH_2Cl exposure, 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) by the DPD method.

2.7. DPD method

The concentrations of monochloramine in the reactors were monitored at the beginning and at the end of the experiment using the DPD method (Rice et al., 2005). 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} sulfuric acid; 2.75 gL^{-1} *N,N*-Diethyl-p-phenylenediamine (DPD) sulfate 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.

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 S1 (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).

2.9. Data modelling and analysis

Ozone

Ozone inactivation followed pseudo first-order kinetics and was modelled using a modified version of the Chick-Watson model (Hunt and Mariñas, 1997). The model was modified to take into account the ozone exposure instead of the contact time (Table 2, equation 1) (Hunt and Mariñas, 1997).

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”.

Monochloramine

The non-linear inactivation curves for *E. coli* by monochloramine were modelled using a previously published model (Geeraerd et al., 2005). 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 was modified in this study 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” (Baty et al., 2015; Baty and Delignetter-Muller, 2013) 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.

3. Results and Discussion

3.1. Biocidal efficiency of CTMA in different water matrices.

An initial MIC of 25 mgL^{-1} CTMA was obtained for the *E. coli* strain AG100 in MHB with an increasing trend for multiple exposures to sub-inhibitory levels of CTMA (Table S1, SI). To assess the dependence of the susceptibility on the water matrices, the biocidal efficiency of CTMA was tested with four different waters for an experimental time of 60 minutes at room temperature. The corresponding results are presented in Figure 2. In absence of CTMA, no, or little, inactivation is visible. No inactivation was visible in MHB for concentrations of up to 10 mgL^{-1}. In the three other waters, 4-log inactivation was achieved at CTMA concentrations of about 5 mgL^{-1}.

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 including 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 (Giannakis et al., 2014; Scheuerman et al., 1988). 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 tested waters. 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 option. The possible remaining factors are a limitation of available nutrients and interaction between CTMA and MHB.

The first hypothesis is related to starvation of the cells. Indeed, all media have no or a limited nutrient level compared to MHB. Long-term starvation of *E. coli* has been shown to lead to an exponential decay of the viability of the cells (Schink et al., 2019). To maintain viability, the presence of nutrients is crucial and it has been demonstrated that adaptation was possible for *E. coli* in nutrient deprived environments, by e. g., the use of nutrients from dead cells (Schink et al., 2019). However, with the short time exposure in the experiments of this study (60 min), adaptation and survival in presence of CTMA was probably not possible in a nutrient-deprived medium. Additionally, the absence of starvation of the cells prior the experiment also reduced the probability of the nutrient limitation hypothesis without completely 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 can form micelles in aqueous media (Breider et al., 2018). If the interaction between CTMA and bacteria is based on electrostatic attraction with the positive-charged head of CTMA and the negatively charged membrane, 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 (Thomas et al., 2000). This could be an indication of the formation of micelles, in contrast to the other solutions, where no such effect was observed. 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 in the different waters.

The use of MIC to determine the susceptibility of bacteria to CTMA, and QACs in general is useful to determine the efficiency at the point of use, where the QACs may be in contact with solutions/agents promoting the growth of bacteria. However, based on the observation that the water matrix plays an important role, standard MIC tests are less representative of the efficiency QACs in natural and technical aquatic systems such as wastewaters.

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 S1, SI), consistent with previous findings that showed a decrease in the susceptibility to QACs after exposure to sub-inhibitory concentrations of CTMA for *P. aeruginosa* (Voumard et al., 2020). 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 Figures 3 and S2 (SI). Inactivation curves for ozone followed pseudo first-order kinetics and therefore, a modified Chick-Watson approach was applied (Table 2, eq. 1). The quality of the fit of the model was assessed with R^2 values,

which were in the range 0.72 - 0.95 (Table S2, SI). All the obtained second-order rate constants for ozone are in the order of 10^6 $M^{-1}s^{-1}$ and summarized in Figure 4A and Table S4 (SI).

For monochloramine, the inactivation curves presented a lag-phase and no tail (Figure 3B). Modelling was performed with a modified version of the empirical Geeraerd model (Geeraerd et al., 2005) (Table 2, eq. 3) and fitting of the curves was assessed using R^2 values, which were in the range 0.98 and 0.99 (Table S3, SI). For monochloramine, the second-order rate constant is calculated from the linear section of the inactivation plot. All the obtained second-order rate constants for monochloramine are in the order of 10^3 $M^{-1}s^{-1}$ and are summarized in Figure 4B and Table S3 (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 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 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 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 the 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 4). Tetracycline-resistant bacteria were reported to have an increased susceptibility to chlorine in a previous study (Macauley et al., 2006). 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 (Macauley et al., 2006). 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 (Alexander et al., 2016; Czekalski et al., 2016; Heß and Gallert, 2015; Oh et al., 2014; Rice et al., 2005). 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 is among the mechanisms of resistance to QACs (Buffet-Bataillon et al., 2016; Mc Cay et al., 2010; Sundheim et al., 1998). This was confirmed in the present study with the efflux of ethidium bromide, a fluorescent substrate (Figure S3, 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 S3, SI) (Martins et al., 2011; Viveiros et al., 2008), 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 (Berry et al., 2010). In another case, the response of *E. coli* to monochloramine showed an activation of genes belonging to the stress response mechanism, including the activation of multidrug efflux systems (Holder et al., 2013). These results show the complexity and the number of different mechanisms possible for the bacteria when responding to stress.

Apart from the mechanisms of resistance, membrane damages can also be the consequence of an exposure to CTMA (Voumard et al., 2020). Bacteria pre-exposed to CTMA would have an alteration of the membrane and efflux pumps overexpressed as it was shown with ethidium bromide (Figure S3, SI). In the case of inactivation by ozone, as the inactivation is very quick, a membrane alteration could be the dominant factor and thus enhance the inactivation. For monochloramine, the inactivation is much slower, with a difference of about 3 orders of magnitude for the second-order rate constants compared to ozone (Figure 4, Tables S2 and S3, SI). 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 trends are opposite for ozone and monochloramine, which indicates that this observation may be related to the different reactivities and properties of the applied chemical disinfectants.

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. QACs do not readily react with oxidants, because the nitrogen does not have a lone electron pair anymore. The aliphatic chain also has a low reactivity with both ozone and NH_2Cl. The use of a scavenger in the experiments prevent the reaction of hydroxyl radical, which are formed during ozonation, with the aliphatic chain of CTMA. Therefore, the CTMA concentration will remain constant in the oxidation experiments. This could be a possible situation in real water disinfection systems. Based on the data presented in Figure 2, different concentrations of CTMA in the range of 0.1 mgL^{-1} to ≤ 5 mgL^{-1} were selected for the combined experiments with CTMA and chemical oxidants. This range of QACs covers situations encountered in municipal, hospital and industrial wastewaters, with concentrations of up to 2.8 mgL^{-1} (Kreuzinger et al., 2007). The concentrations in this study above the range detected in aquatic systems are not unrealistic and they were selected to investigate the combined effect of CTMA and chemical oxidants over a wide range to better understand additive or synergistic effects. Inactivation curves for ozone and monochloramine are presented in Figures 5 and S4 (SI) and the corresponding second-order rate constants obtained by the modified Chick-Watson model and the modified Geeraerd model are presented Figure 6. The numerical values for the second-order rate constants and the corresponding fitting parameters for each curve are

provided in Tables S4 and S5 (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 constants could not be determined and are therefore not presented in Figure 6.

A similar pattern for the second-order inactivation rate constants of *E. coli* was observed for the simultaneous presence of CTMA and ozone or monochloramine (Figure 6). For low concentrations of CTMA, there is little influence on the inactivation kinetics. For higher CTMA concentrations, the inactivation kinetics are enhanced resulting in higher second-order inactivation rate constants.

An exponential-type increase in the rate constant is visible for the inactivation by ozone in presence of CTMA, for concentrations ≥ 1.5 mgL^{-1} (Figure 6). 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 and therefore, an antagonist effect 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.

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 5B could be modelled by the Geeraerd model provided in Table 2. Therefore, the individual contributions of CTMA and

monochloramine on the inactivation of *E. coli* could be elucidated. The inactivation curves representing the individual and combined contributions of monochloramine and CTMA to the inactivation of *E. coli* are shown in Figure 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 S5, SI).

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 similarly 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 CTMA and QACs in general, is mainly the disruption of the cell membrane and the modes of action of ozone and monochloramine are based on reactions with various cell components. A destabilization of the membrane enhances the penetration of ozone or monochloramine inside the cell allowing an enhanced 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 for CTMA (3 mgL^{-1}) and monochloramine were added and compared to the combined experimentally

observed inactivation. A CTMA concentration of 3 mgL^{-1} was chosen because of an apparent similar level of inactivation by individual treatment with 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 for the individual treatments with monochloramine and CTMA (Figure 8). The experimentally observed overall inactivation in the combined CTMA-monochloramine experiment is higher than calculated from the sum of the modelled curves for the individual inactivation experiments (Figure 8). This enhancement suggests a slight synergistic effect with 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 there is no antagonistic effect and that the inactivation is faster and enhanced in presence of both CTMA and monochloramine.

4. Conclusions

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 water quality and is higher in PBS, secondary wastewater effluent and lake water than under 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 an alternative 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 elucidated by the obtained results. The different results obtained for 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 and a potential synergistic effect needs to be confirmed by further investigations. The combination of ozone with CTMA resulted in non-linear pseudo first-order inactivation kinetics, which could not be quantified. 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 compounds during a chemical disinfection by ozone or monochloramine may enhance the inactivation of pathogenic bacteria.

5. 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, and Gabrielle Léger for helping with the ozone inactivation experiments. Caroline Gachet-Aquillon and Karim-Alexandre Essaïed are acknowledged for technical support during the experiments. Financial support by EPFL is acknowledged.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Characteristics of the different media used

Media	**MHB**	**PBS**	**Lake water**	**2nd wastewater effluent**
Origin	Sigma-Aldrich, CH	Sigma-Aldrich, CH	Geneva lake, Switzerland	Morges, Switzerland
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

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

Equation		**Parameters**
1.. Modified Chick-Watson model using the disinfectant exposure $\int [C]dt$ (Hunt and Mariñas, 1997)	$ln\left(\frac{N}{N_0}\right) = -k\int [C]dt$	N_0 = Initial number of organisms N = number of survivors k = second-order inactivation rate constant [C] = disinfectant concentrations
2. Time-based Geeraerd model (Geeraerd et al., 2005)	$\mathrm{N}(t) = (N(0) - N_{res})e^{-k_{max}t}\left(\frac{e^{k_{max}S_l}}{1+(e^{k_{max}S_l}-1)\cdot e^{-k_{max}t}}\right) + N_{res}$	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. Modified Geeraerd model to take into account the CT parameter instead of the time only (this study)	$\mathrm{N}(Ct) = (N(0) - N_{res})e^{-k_{max}Ct}\left(\frac{e^{k_{max}S_l}}{1+(e^{k_{max}S_l}-1)\cdot e^{-k_{max}Ct}}\right) + N_{res}$	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

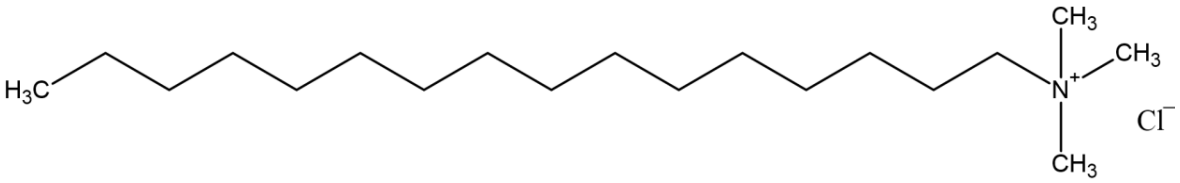


Figure 1: Chemical structure of cetyltrimethylammonium chloride (CTMA, CAS 57-09-0)

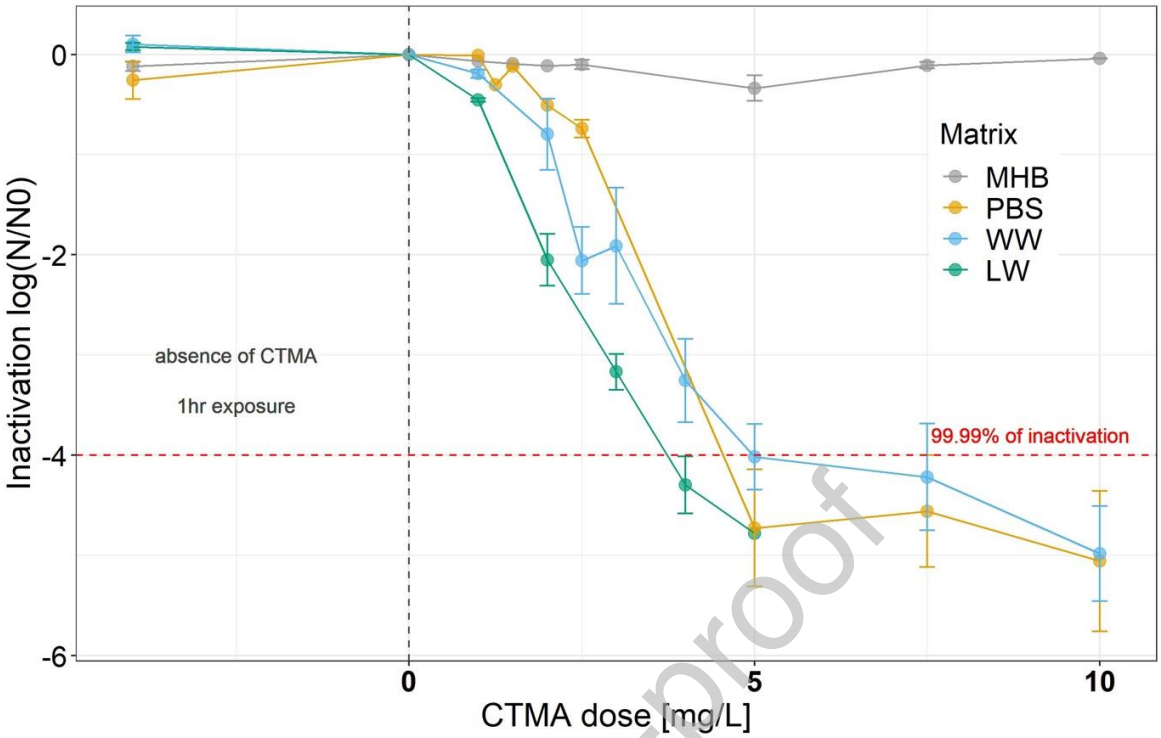


Figure 2. Inactivation levels of *E. coli* (AG100) as a function of the CTMA dose in MHB, PBS, secondary wastewater effluent (WW; wastewater effluent, wastewater treatment plant Morges, Switzerland) and lake water (LW; Lake Geneva, Switzerland). The left part (absence of CTMA) and the right part (presence of CTMA) show the results of two independent sets of experiments. For the left part, bacterial populations were exposed to the water matrix only, for one hour at room temperature (20°C). For the right part of the graph, in addition to the water matrix, the bacterial populations were exposed to increasing doses of CTMA. For each concentration of CTMA, the exposure time was one hour, and the experiments were performed at room temperature (20°C).

The pH-values were 7.5 (MHB), 7.4 (PBS), 8.2 (WW) and 8.5 (LW). Three independent experiments were performed for each condition, and at least 3 technical replicates

were obtained per data point. The error bars represent the standard deviations of the technical and experimental replicates.

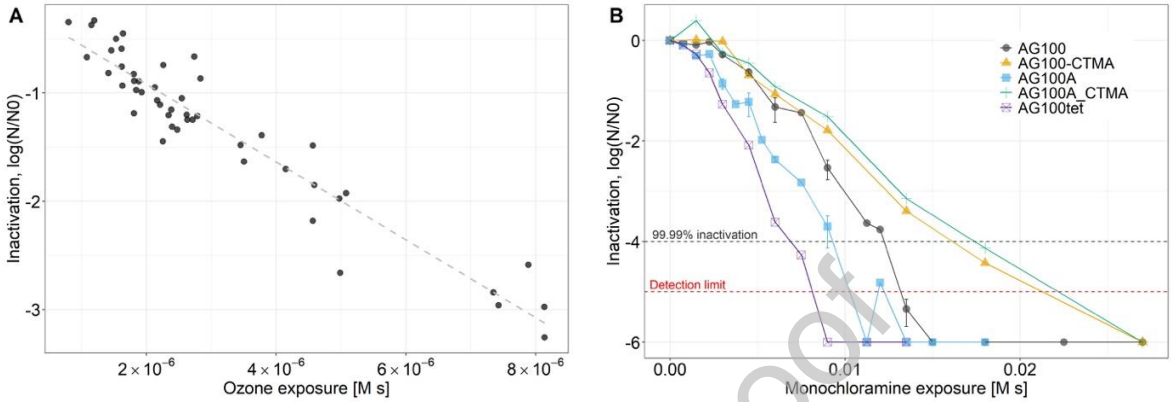


Figure 3. Inactivation kinetics of *E. coli* by ozone and monochloramine.

(A) AG100 inactivation by ozone (60 experiments), the other strains are available in Figure S4 (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 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. 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.

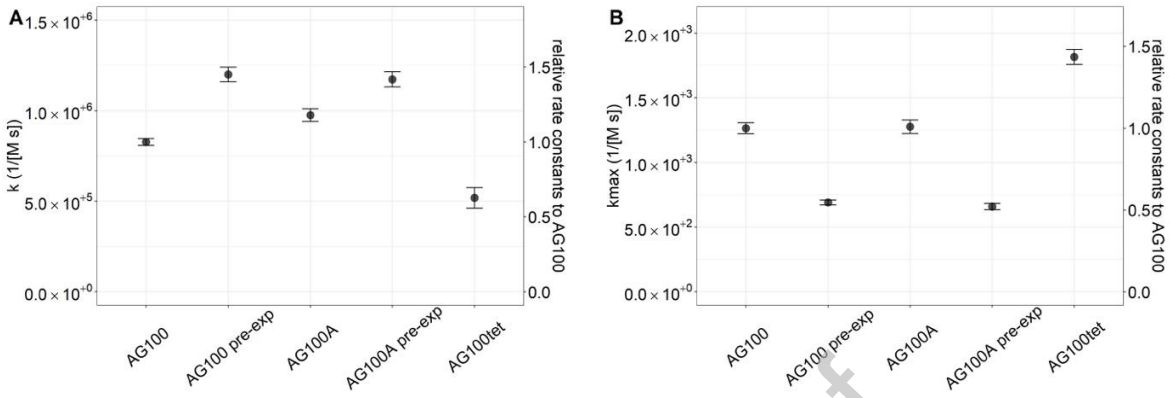


Figure 4. Second-order rate constants for the inactivation of *E. coli* AG100, AG100A not pre-exposed to CTMA, AG100 and AG100A pre-exposed to CTMA and AG100tet by (A) ozone and (B) monochloramine. All experiments were performed in PBS (10mM) at room temperature, pH 7.4. PBS was supplemented with *t*-BuOH (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 S2 and Table S3 (SI)

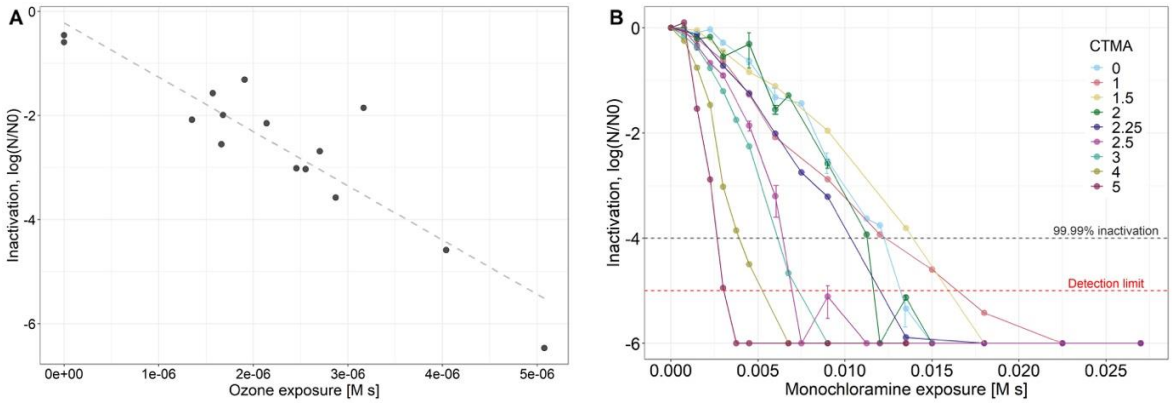


Figure 5. Inactivation kinetics of *E. coli* AG100 in absence and presence of CTMA.

(A) Ozonation in presence of 2.25 mgL^{-1} CTMA (15 experiments, see Figure S4 (SI) for the other CTMA concentrations). (B) Monochloramination with varying concentrations of CTMA. All experiments were performed in PBS (10mM) at room temperature (20 ^{o}C), 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.

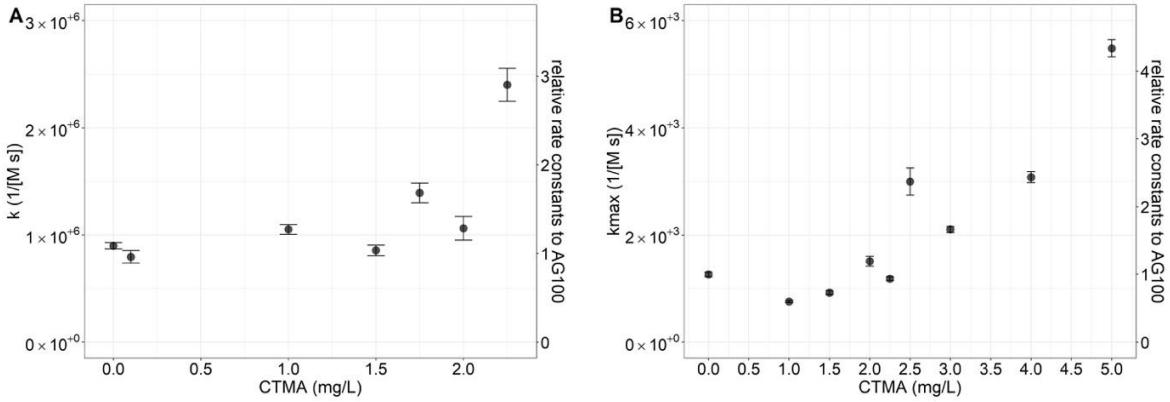


Figure 6. Effect of the simultaneous presence of CTMA and chemical disinfectants on the second-order rate constants for the inactivation of *E. coli* AG100. (A) ozone and (B) monochloramine.

All experiments were performed in PBS (10mM) at room temperature (20°C), 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 rate constants of at least three independent experiments. The error bars represent the standard deviations obtained from the modeling of the second-order rate constants. The rate constants are also available in Tables S4 and S5 (SI).

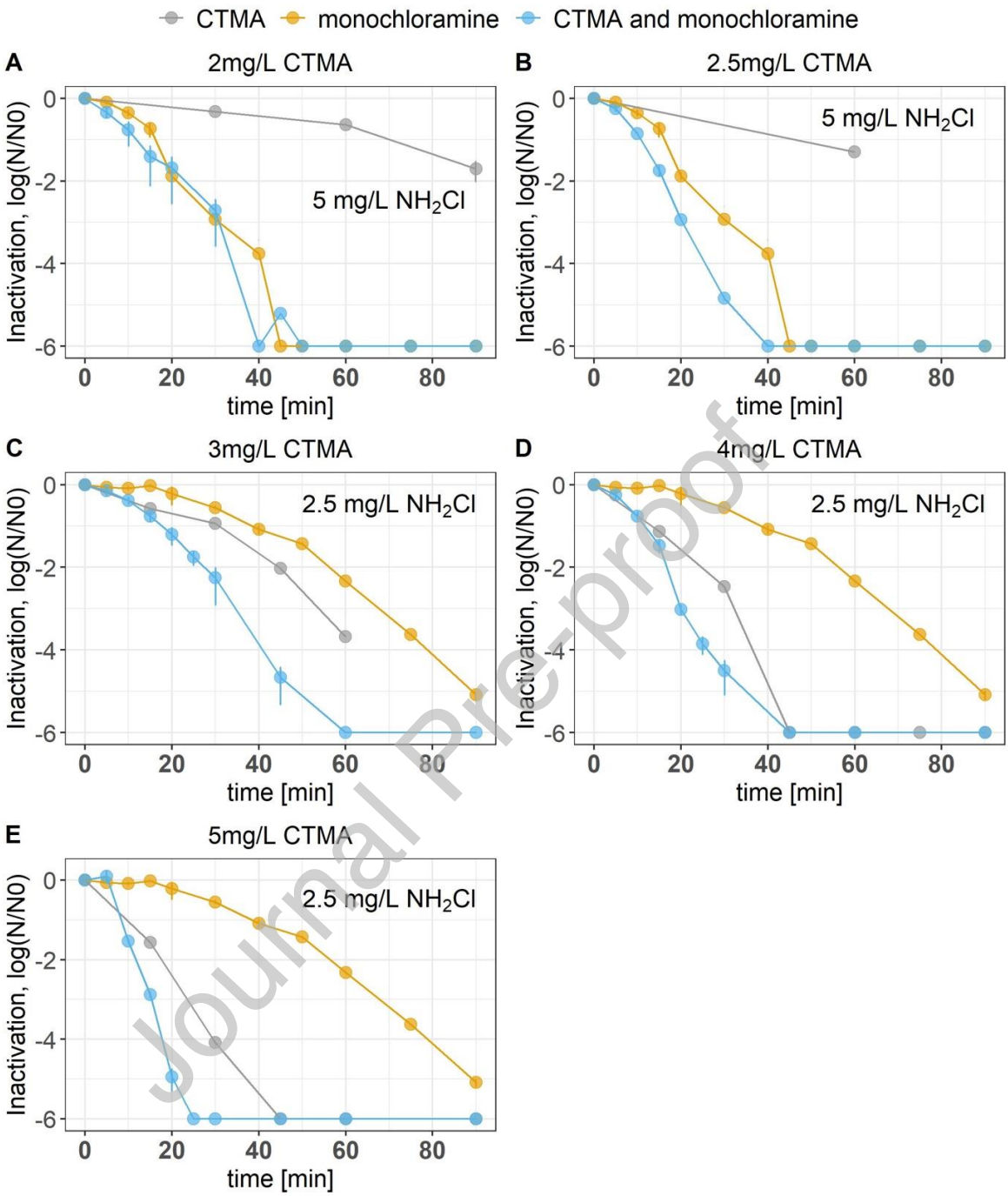


Figure 7. Inactivation of *E. coli* AG100 by CTMA, NH_2Cl and the combination of CTMA and NH_2Cl.

(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 (20 ^oC), pH 7.4. Each data point is the average of at least four replicates and the error bars represent the standard deviations.

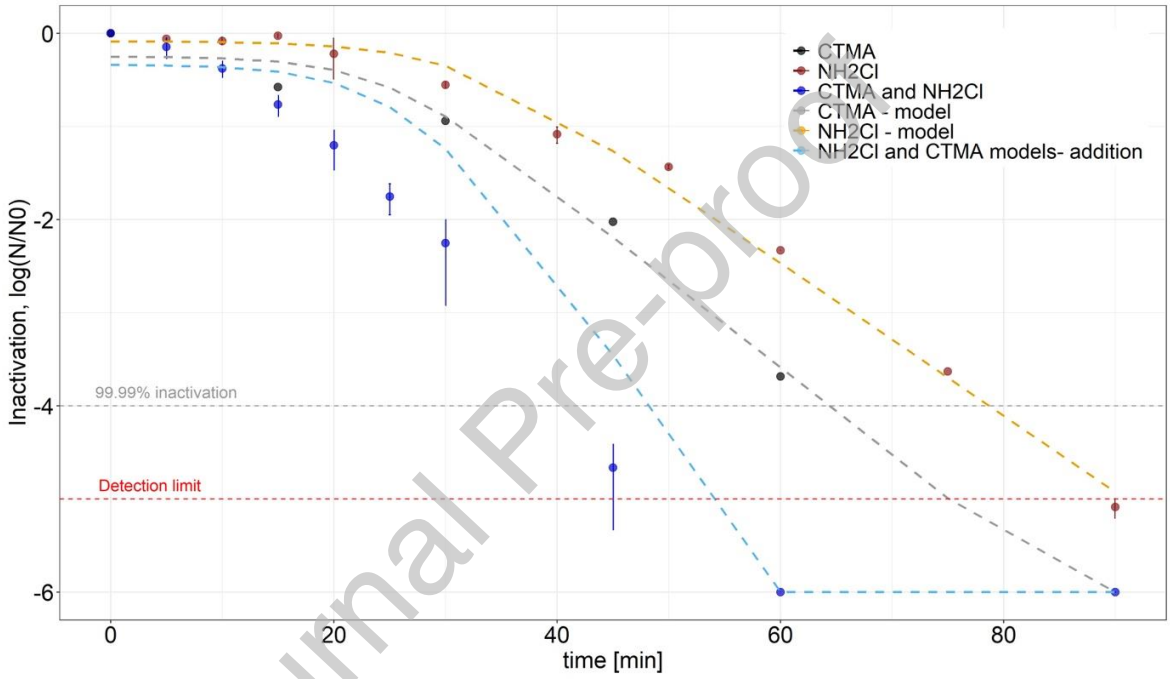
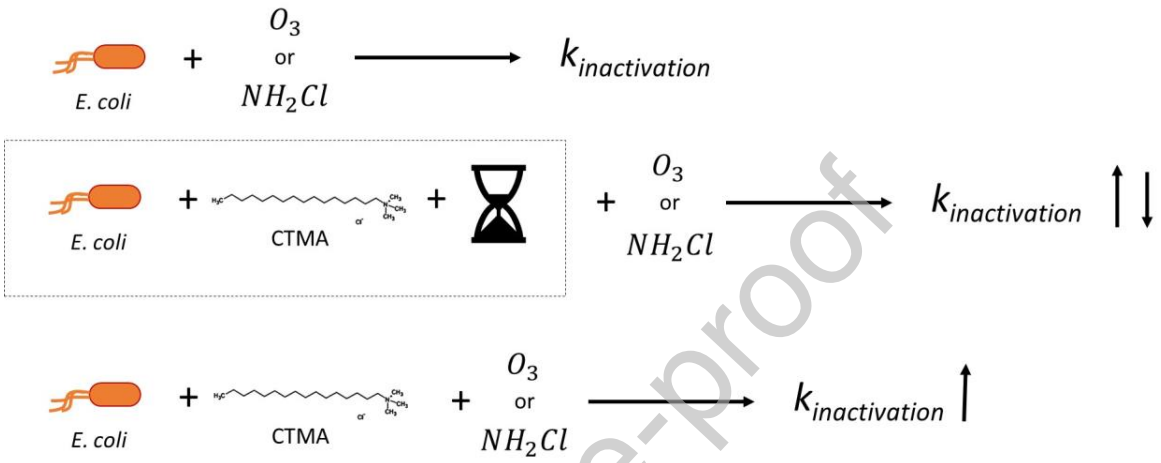


Figure 8. Separate and combined inactivation of *E. coli* AG100 by CTMA (3mgL^{-1}) and/or NH_2Cl (2.5 mgL^{-1}).

All experiments were performed in PBS (10mM) at room temperature (20 ^oC), pH 7.4. The calculated overall effect is the result of the sum 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 sum of the two other dashed lines. The error bars on the experimental

data represent the standard deviations of the experimental and technical replicates (3 independent experiments and at least four technical replicates)



Graphical