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Effect of Sub-Inhibitory Wastewater Stressors on Mutation Frequency in Clinically Relevant Aeromonas Species

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EFFECT OF SUB-INHIBITORY WASTEWATER STRESSORS ON MUTATION FREQUENCY BETWEEN CLINICALLY RELEVANT AEROMONAS SPECIES

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ABSTRACT

EFFECT OF SUB-INHIBITORY STRESSORS ON MUTATION FREQUENCY BETWEEN CLINICALLY RELEVANT AEROMONAS SPECIES

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Antimicrobial resistance among pathogens is steadily increasing resulting in untreatable infections and elevated mortality rates, with an estimation of claiming 10 million lives worldwide by 2050. A common factor accelerating resistance is the presence of sub-inhibitory microbial stressors (e.g. antimicrobials, heavy metals, and disinfectants), which can drive horizontal gene transfer and mutagenesis in various environments. Improper disposal of pharmaceuticals, excretion of antimicrobial byproducts from humans and livestock, and excess storm runoff are common sources of these pollutants. An environmental reservoir rich in stressors and bacterial populations, including the emerging pathogen *Aeromonas*, is wastewater. Our objective in this study was to determine the mutagenic impact of sub-lethal concentrations of wastewater, common antimicrobial contaminants, and wastewater disinfectants amongst residential wastewater bacterial populations. Fluctuation assays were performed to quantify mutation frequencies in environments with these pollutants. Briefly, clinically relevant bacterial cultures of *A. hydrophila* and *A. caviae* were incubated with various stressors for 24 hours with subsequent plating on tryptic soy agar containing eight times the MIC value of rifampin. For each treatment group evaluated, sixteen or more independent experiments were included. Mutation frequencies were determined by dividing resistant colonies by total colonies on tryptic soy agar without antibiotic. In all, sub-lethal concentrations of wastewater influent, four antibiotics (i.e., ciprofloxacin, tetracycline,

trimethoprim, and cefotaxime) each with different molecular targets, and common wastewater disinfectants (i.e. ultraviolet light and calcium hypochlorite) were assessed for their role on accelerating mutagenesis. Our findings showed that filtered influent wastewater increased mutagenic evolution by 3-fold within *A. caviae*. When looking at the impact of sub-inhibitory concentrations of antibiotics, the strongest impact on mutagenesis was tetracycline, trimethoprim, and cefotaxime among *A. hydrophila*, whereas ciprofloxacin and trimethoprim were most influential among *A. caviae* populations. Although majority of the antimicrobials appear to lose their effect at 0.25X of the MIC value, trimethoprim impacted mutation frequencies as low as 0.0078X the MIC (0.156 µg/mL). Common wastewater disinfectants also accelerated the presence of specific mutations in *A. caviae*. Together, although wastewater treatment is instrumental in reducing microbial populations, as well as resistant populations, our findings demonstrate the potential impact wastewater and its disinfectants have on the evolution of antimicrobial resistance and virulence.

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LIST OF ABBREVIATIONS

ADAVI: ampicillin dextrin infused agar with vancomycin and irgasan
AMR: antimicrobial resistance
ARGs: antibiotic-resistant genes
CIP: ciprofloxacin
CFU: colony forming unit
CFU/mL: CFUs per milliliter
CTX: cefotaxime
DNA: deoxyribonucleic acids
dsDNA: double-stranded DNA
EFF: effluent wastewater
FRE: frequency of recombination
HGT: horizontal gene transfer
IWW: influent wastewater
LB: Luria broth
mCFU: mutant CFUs
mCFU/mL: mutant CFUs per milliliter
MIC: minimum inhibitory concentration
MID: minimal inhibitory dose
rRNA: ribosomal ribonucleic acid
UVL: ultraviolet light
ssDNA: single-stranded DNA
TET: tetracycline
TMP: trimethoprim
TSA: trypticase soy agar
WW: wastewater
WHO: World Health Organization

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CHAPTER 1: BACKGROUND

I: Introduction

1. Antimicrobial resistance

The prevalence of antimicrobial-resistant microorganisms are accelerating at an alarming rate, contributing to devastating medical complications and causing over 700,000 deaths worldwide annually [1]. Antimicrobial resistance (AMR) plays a substantial role in untreatable bacterial infections among hospitalized patients, leading to increased mortality rates [2]. Individuals with immune deficiencies are more susceptible to acquiring bacterial infections [2]. Causes of AMR include the introduction of mutagenic events in DNA, as well as the uptake of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT) [3]. The continual presence of sub-lethal bacterial stressors has contributed to AMR populations, including *Streptococcus, Pseudomonas, Staphylococcus, Lactobacillus,* and *Escherichia coli* [4-8]. Antimicrobial compounds to disinfecting agents have been identified as bacterial stressors [4-8]. For example, long-term exposure to sub-lethal concentrations of penicillin and virginiamycin resulted in increased viability to otherwise lethal concentrations of penicillin in *Lactobacillus* [8]. The World Health Organization has predicted that mortality rates associated with AMR populations will reach over 10 million annually within the next 30 years [2], highlighting the urgency to discover a solution for these resistant populations. According to the WHO [9], some factors in reducing AMR microorganisms are having access to clean drinking water, stricter regulations on antibiotics, and the reduction of waste being expelled into our sewage facilities [2]. Despite these recommendations, AMR is still on the rise in both the clinic and environmental

bacterial populations. A reason for the continual rise of AMR includes overusing and disposing of bacterial stressing agents from users, medical facilities, agriculture, and factory pollution [3].

2. Bacterial stressors in the environment

A diversity of environments are home to various microorganisms that have evolved to adapt to their surroundings. For example, Gram-negative microorganisms like *Aeromonas* thrive best in aquatic environments, such as riverbanks, lakes, and wastewater (WW) [10]. These environments can exhibit an array of contaminants that exert a continual pressure on bacteria [11]. The presence of these compounds can progress the mutagenic evolution of resident microorganisms by undergoing adaptive alterations, including recombinational events and mutagenesis [12]. Pathogenicity and AMR are vital outcomes of bacterial evolution that aid in microbial survival. Variation of surface antigens can contribute to pathogenicity by aiding in the adhesion to various host receptors [12]. Microorganisms have developed other strategies to combat cellular damage that occurred in the environment by maintaining membrane integrity [13]. Regulation of the bacterial membrane controls a stressor's anti-microbial effects, allowing mutations to occur in the genome before bacterial lysis [13]. These adaptive mechanisms initiate due to changing environmental pressures, increasing genomic transformation, and potentially accelerating microbial evolution [13, 14]. The continuous release of these stressing agents into the environment indicates the importance of further understanding their effect on mutagenesis among environmental microorganisms [3, 11]. An assortment of microbes and antibiotic compounds, including beta-lactams, fluoroquinolones, sulfonamides, cephalosporins, and trimethoprim, have been observed in aquatic environments, with heightened levels in WW [11, 15, 16]. Considering

the impact of bacterial stressors on mutagenesis [3], the effect of WW on mutation frequencies among emerging pathogenic inhabitants is essential to investigate.

3. Wastewater treatment

WW treatment facilities receive mixtures of untreated water, containing a high frequency of contaminants, from a diversity of sources [11, 15]. These contaminants, including sewage, storm runoff, medical waste disposal, and industrial pollution can be found at varying concentrations, acting as stressors on residential WW microorganisms [11, 15, 16]. A diversity of microorganisms reside in WW, including commensals and potential pathogens such as *Escherichia coli, Aeromonas, Bacillus, Citrobacter, Klebsiella,* and *Pseudomonas* [16, 17]. Treatment begins with untreated water entering the treatment facility by removing large debris via preliminary screening. Materials, including sand and grease, not removed in preliminary screening are separated using centripetal force in a process termed primary screening [18]. Buoyant substances are then removed by a skimming unit in clarification tanks, whereas the smaller debris sinks to the bottom. The smaller particles get sent to anaerobic digestor tanks, where gases are collected and can be converted into energy to power the plant [18]. After preliminary and primary screening processes have finished, biological treatment can begin. Microscopic organisms are used during this stage to remove a surplus of organic materials [18]. After a substantial amount of organic material is broken down, the final but optional stage of WW treatment, known as disinfection, can begin. The principle of the disinfection stage is to remove additional microorganisms and other contaminants present in the treated WW before being released into the environment [18]. Numerous elements

play a role in the effectiveness of the disinfection stage, including time of exposure, as well as the concentration and type of bacteria present, attributing to potential treatment limitations [19, 20].

i. Differences among wastewater samples

WW can be classified into two categories during the purification processes, identified as pre- and post- treated waters. Influent wastewater (IWW) is the pre-treated raw water containing numerous contaminants that comes directly into the treatment facility [19]. Effluent wastewater (EWW) is the post-treated water that has endured preliminary, primary, biological, and, potentially, disinfecting stages, resulting in a reduced number of contaminants [19]. This WW classification meets the agricultural standards to be "safely" released back into the environment [19]. One study examined the presence of over 140 *E. coli* strains in IWW and EWW samples and observed a 200-fold reduction of this population in post-treated water, though antimicrobial resistant strains survived past treatment [21]. This poses a compelling health concern, as AMR populations may enter the environment and potentially infect humans and wildlife [21, 22]. One potential explanation for survival past disinfection is the increased adaptive alteration of microorganisms [23-25]. Microbes under selective pressure can readily take up ARGs from previously lysed bacteria throughout stages of disinfection [23, 25]. IWW being a rich source of bacterial stressors, intracellular and extracellular ARGs, identify an ideal environment for bacterial mutagenesis and acquisition of resistance through HGT [23-25]. The benefits and potential drawbacks between methods of disinfection in WW should be further analyzed from a mutational perspective.

ii. Wastewater disinfection methods

WW disinfection is commonly performed via two methods, chlorination, or ultraviolet light (UVL) exposure. Disinfection through chlorination produces hypochlorous acid during contact between hypochlorite and water, increasing reactive oxygen species, which can cause damage to cellular membranes and DNA [25]. Chlorination significantly reduces microorganisms; however, it only contributes up to a 3.6 log reduction, thus allowing potentially pathogenic microorganisms to be released into the environment [26, 27]. Chlorination treatment increased transformational HGT in *Acinetobacter baylyi* in sub-lethal doses, aiding in AMR to tetracycline and ampicillin [25]. After exposure to sub-lethal doses of hypochlorite, resistance to lethal concentrations of hypochlorite and antibiotics increased in *Pseudomonas* through oxidative stress-induced mutagenesis [5]. The potential of hypochlorite to induce AMR through mutagenesis and HGT suggests alternative disinfecting methods are warranted. After chlorination, the chemicals must be removed to be released back into the environment. De-chlorinating processes use chemical compounds like sodium bisulfite to remove significant levels of hypochlorite residues to meet agricultural standards [28]. It has been shown that improperly de-chlorinated effluent water increases health risks to humans, including colon cancer in men and adverse developmental effects in infants [29].

Due to a lack of toxigenic compounds, the use of UVL presents a reduced health risk to humans and wildlife compared to chlorination [20]. Although, microorganisms can have replicational and transcriptional functions inhibited under UVL exposure, resulting in their eventual death [20]. This may occur from the creation of pyrimidine dimers causing subsequent nucleic acid damage or activation of the error-prone SOS response [20]. Pyrimidine dimers are

two pyrimidine bases located on the same strand of DNA binding together and stalling the polymerase. Excision repair is triggered once a stall is identified, removing the dimer [30]. Mutations within the dimer sites may also arise due to translesion DNA polymerases, which aid in error-prone DNA repair. Bacterial DNA repair mechanisms, such as the SOS response, can also be activated during DNA damage, which introduces mutations in the genome due to the "sloppy" repair by DNA polymerase V [14]. For example, UVL exposure increased spontaneous mutagenic events by 40-times among *E. coli* populations, resulting in resistance to nalidixic acid [31]. In bacterial species like *Legionella pneumophila*, which lacks genes responsible for the SOS response, transformational HGT was more common in response to UV exposure [32]. Some resistant bacterial populations have a decreased susceptibility to UVL, raising the concern of these microbes entering the environment post-treatment [33]. Irradiation with UVL significantly reduces the viability of microorganisms post-exposure; however, the greatest inactivation is similar to that of chlorination, 3.4-log in WW, allowing potential AMR populations to enter the environment [20]. In contrast, up to a 6-log inactivation of microorganisms occurred after UV exposure in saline solution [20]. The difference between log inactivations can be explained due to the sensitivity of UVL towards initial steps of WW treatment. For example, debris not adequately removed during treatment may block or absorb the UV light from fully penetrating the microorganisms [20]. UVL exposure is still a preferred method of WW disinfection due to the lack of toxicity compared to chlorination [20].

4. *Aeromonas* species

Aeromonas is a genus of over 35 bacterial species that are Gram-negative, with several species considered as clinically relevant [9]. This genus is associated with diseases ranging from sub-clinical to severe in wildlife and mammals such as fish, rabbits, dogs, cats, and humans [10, 34]. The most prevalent *Aeromonas spp*. associated with human infections include *A. hydrophila*, *A. caviae*, *A. veronii*, and *A. dhakensis* [10]. These species contributed to 95.4% of *Aeromonas*-acquired human infections, with *A. caviae* being the most prevalent of the four [10]. One of the most common illnesses that individuals infected with *Aeromonas* experience is gastroenteritis, including symptoms like fever, diarrhea, and nausea [10, 35]. Examination of 863 individuals that were experiencing travelers' diarrhea, indicated that 2% of those infections were induced by *Aeromonas* spp., specifically *A. caviae, A. veronii, and A. hydrophila* [35]. This illness results in chronic diarrheal episodes, which may last up to a year, as shown by infections caused by *A. caviae* [35]. Severe diseases like necrotizing fasciitis have been observed from bacterial infections induced by *A. caviae* and *A. hydrophila*, with the latter causing the majority of cases [10, 36]. Necrotizing fasciitis can affect immunocompetent populations; however, this infection is typically associated with immunocompromised individuals [10, 36]. A symptom of this infection is myonecrosis, which is the death of soft tissues [36, 37]. This illness is associated with a high mortality rate of almost 100% for untreated cases, and up to 36% if treated within 24 hours, reflecting the fatal capabilities of *Aeromonas* spp. [10, 37].

i. Relevance of *Aeromonas* species

Aeromonas resides among diverse environments ranging from food products to clinical infections, and most notably aquatic ecosystems, including WW [10]. Among cultured bacteria

identified in WW samples, *Aeromonas* spp. were of highest abundance in IWW at around 49% of the total population [16]. This prevalence was reduced to 19.8% of the population in EWW, being the second most abundant genera, with *Bacillus* species being predominant [16]. The prevalence of the genus *Bacillus* post-treatment may be due to this species capacity for forming endospores. Accumulation of ARGs encoding AMR to fluoroquinolones, carbapenems, and tetracyclines were evident in *Aeromonas* spp. residing in WW [38]. For example, an elevated resistance to tetracycline arose in *A. hydrophila* due to the acquisition of the *tetE* gene, which was also present post-treatment [38]. Due to intrinsic AMR to penicillins, treatments for *Aeromonas*-induced infections include the use of fluoroquinolones and third-generation cephalosporins [38]; However, recent studies have indicated emerging resistance to ciprofloxacin (CIP) among Lake Erie isolates, identifying the urgent risks from recreational exposure [34]. The clinical and environmental presence of *A. hydrophila* and *A. caviae* [36, 37] demonstrate the importance of determining a potential cause of AMR.

ii. Identifying *Aeromonas* species

At a molecular level, the genus of *Aeromonas* can be classified by the 16S rRNA [10]. However, due to close homology of the 16S rRNA gene between species, others are preferable, like the house keeping genes *gyrB* and *rpoD* [10]. *gyrB* encodes the beta subunit of the DNA gyrase, which contributes to negative supercoiling of dsDNA in the replication process [10]. Supercoiling is defined as tension between DNA strands, which can be positive or negative, impacting the regulation of genetic material [10]. The *rpoD* gene encodes sigma factor S70, which plays a role in synthesizing RNA from DNA by binding to the RNA polymerase. These genes are

known to play a significant role in the survival of the bacterial genus, labeled as house-keeping genes, which are used to aid in differentiating species [10].

5. Bacterial mechanisms of survival

Microorganisms residing among bacterial stressing agents, including hypochlorite, UVL, or antibiotic compounds, have the potential to undergo adaptive alterations to survive in the environment [12, 25, 31]. A universal way that a bacterium may undergo these beneficial alterations, such as developing antibiotic resistance, is through mutagenesis. We can analyze the frequency at which a microorganism mutates, along with its overall rate of mutagenesis. The difference between mutation frequency and mutation rate is the respective mutated population that is analyzed [39]. For example, mutation frequency examines a single mutated locus, whereas mutation rate estimates the overall mutagenesis throughout the genome, per bacterial generation [39]. Therefore, mutation frequency is considered as a fraction of the estimated mutation rate [39].

i. SOS response

One bacterial mechanism that triggers DNA repair, also contributing to elevated mutagenesis, is the SOS response, which becomes activated after nucleic acid damage [14]. This mechanism is often a last resort for a bacterium to maintain viability in harmful environments. In microorganisms associated with WW, including *E. coli* and *Aeromonas*, a functional *recA* has been noted, potentially leading to activation of the SOS response [40, 41]. A major outcome of the bacterial SOS response is increased survival of the microorganism; however, it also results in a 'sloppy' repair process leading to increased mutagenesis [14, 42]. This latter effect can potentially

impact the evolution of AMR, especially among WW residents like *Aeromonas* species. Over twenty genes play a role in the functionality of the SOS response, which includes the essential repair gene, *recA* [14]. The LexA repressor cleaves itself under DNA damage, termed autoproteolysis, resulting in the SOS response genes, including *recA,* being de-repressed [14]. Once de-repressed, the RecA protein can be produced, which has two functions during the SOS response [14]. Firstly, it acts as a co-protease which aids in the self-cleavage that occurs with LexA [14]. Secondly, it acts as a ssDNA nucleoprotein filament, which aids in the recombinational repair of ssDNA [14]. As the damaged DNA is repaired, the amount of ssDNA needed to trigger the RecA nucleoprotein filaments decreases [14]. After DNA damage has halted, the LexA repressor can again bind to the SOS pathway and repress the process [14].

SOS expression is one mechanism that can play a role in viability among bacterial populations during WW treatment. It was determined that specific residues of the *recA* nucleotide sequence may play a significant role in the level of SOS regulation [43]. In a previous comparison, *Pseudomonas aeruginosa* underwent frequency of recombination (FRE) over 6.5 times that of *E. coli*, though *recA* homology was around 86% [43]. Homology differed primarily in the C-terminal end of RecA, specifically the Mg^{2+} binding site, located between amino acids 329-352, regulating *recA* expression and DNA binding affinity. Residual variation and similarities in the C-terminal end exist between *Aeromonas*, *E. coli*, and *Pseudomonas* (Table 13). For example, compared to *A. hydrophila*, the presence of glutamic acids and alanine are elevated in *A. caviae*, which is also observed in *P. aeruginosa* (Table 13). Alanine aids in the construction and stabilization of proteins, whereas glutamic acids form salt bridges with positively charged amino acids aiding in stabilizing proteins. The specific amino acid residues, having a potential role in SOS-induced mutagenesis [43], reflect the importance of observing mutational variation between these two microorganisms.

ii. Horizontal gene transfer

Another approach that microorganisms can use to adapt in stressful environments is HGT. This method involves the uptake of genetic material by the host organism, including virulence genes and ARGs [24]. HGT can occur in three forms: conjugation, transduction, or transformation [24]. Conjugation is the exchange of plasmid DNA between microorganisms via a sex pilus, also known as the F-pilus, connecting the organisms [24]. Transduction is the exchange of DNA via a bacteriophage, which can integrate its chromosome into the host through the lysogenic cycle [24]. This viral vector can also enter the lytic cycle forming new phages within the host [24]. Lastly, transformation is the acquisition of exogenous DNA by the microorganism due to membrane competency of the cell wall [24]. The presence of hypochlorite, a common disinfecting agent in WW, increased membrane permeability in *Acinetobacter baylyi* and *E. coli*, accelerating transformational HGT of ARGs [23]. *Aeromonas spp*. have also been noted to naturally uptake genetic material through transformation among aquatic populations, specifically *A. hydrophila* and *A. caviae* [23]. The potential influence of HGT on AMR between *A. caviae* and *A. hydrophila* in the presence of common WW stressors has not been thoroughly examined. Understanding the link between WW treatment and mutational variation between emerging pathogens, such as *A. caviae*, and *A. hydrophila* will aid in determining the potential progressive effects of these stressing agents on mutagenic evolution.

6. Conclusion

Aeromonas species are Gram-negative microorganisms commonly found in aquatic environments, agricultural products, and clinical environments [10]. This genus is typically associated with diseases ranging from gastroenteritis to wound infections, such as necrotizing fasciitis [10]. The most prevalent *Aeromonas spp*. associated with human disease are *A. hydrophila*, *A. caviae*, *A. veronii*, and *A. dhakensis* [10, 36]. Antibiotic treatment is a common medical practice to subdue and treat bacterial infections, including those induced by *Aeromonas spp.* [38]. AMR populations pose substantial complications to human health worldwide due to increased mortality rates from untreatable infections. Improper usage and disposal of antimicrobial agents can induce adaptive mechanisms among microbial populations including *Streptococcus, Pseudomonas, Staphylococcus,* and *Escherichia coli* [4-7]. These bacterial stressors are commonly found in aquatic environments, specifically WW, co-existing with emerging pathogens like *Aeromonas* [11, 15, 16]. Microbial evolution is primarily due to microorganisms' survival mechanisms, including HGT and the SOS response. There is a lack of knowledge on the effects that WW and specific stressors encountered throughout treatment have on mutagenesis between species of *Aeromonas.* The purpose of this study is to analyze the impact of WW and its treatment on mutagenesis among clinically relevant *A. hydrophila* and *A. caviae*.

II: Hypothesis & Specific Aims

1. Hypothesis and Specific Aims

The hypothesis of this proposal is: *IWW, common antibiotics associated with this environment, and treatment disinfectants will increase mutagenesis in clinically relevant* Aeromonas *species.* To test this hypothesis, the following specific aims will be addressed:

1. **Specific Aim 1: Determine the impact of filtered IWW on mutation frequency among *A. hydrophila* and *A. caviae.*** The working hypothesis of this specific aim is: *Culturing* Aeromonas *species with sub-inhibitory dilutions of filtered influent WW will increase the mutation frequency in these microbes.*

2. **Specific Aim 2: Determine the impact of sub-inhibitory concentrations of common antibiotics in WW on mutation frequency among *A. hydrophila* and *A. caviae*.** The working hypothesis of this specific aim is: *Culturing* Aeromonas *species with sub-inhibitory concentrations of commonly found antibiotics in WW induces increased mutagenesis in these microorganisms.*

3. **Specific Aim 3: Determine the impact of sub-inhibitory exposure to WW treatment disinfectants on mutation frequency among *A. hydrophila* and *A. caviae.*** The working hypothesis of this specific aim is: *Culturing* Aeromonas *species with sub-inhibitory concentrations of calcium hypochlorite or sub-inhibitory irradiance doses of UVL contribute to an increased frequency of mutational events within these microbes.*

CHAPTER 2: MATERIAL AND METHODS

I: MIC determination

1. Microorganisms used in study

In this study we used the *A. hydrophila* strain ATCC7966 and the *A. caviae* strain ATCC15468, obtained from the American Type Culture Collection. When working with these microorganisms, proper personal protective equipment was used throughout the experiments, since *A. hydrophila* is a biosafety level 2 organism, whereas *A. caviae* is biosafety level 1. Pure cultures of the bacteria were stored in an –80° Celsius freezer until further use in our experiments. Overnight cultures were initiated by inoculating separately labeled Luria broth [44], a nutrient media, with a fresh scrape of each individual bacterium. Test tubes containing the newly inoculated culture were shaken in aerobic conditions at 150 RPM and 30°C, allowing for optimal overnight growth into stationary phase. To reduce the occurrence of mutations based on temperature, we incubated our species at 30°C, which is the ideal climate for this genus [34]. Identification of *Aeromonas* was performed by streaking the bacteria onto ampicillin dextrin infused agar with vancomycin (2µg/ml) and irgasan (5µg/ml) (ADA-VI). Confirmation of *Aeromonas* was indicated by colony growth surrounded by a yellow halo [34]. ATCC strains of *A. hydrophila* and *A. caviae* mentioned above, were used for each experiment performed throughout this study.

2. Determining MICs of filtered IWW

Using fresh cultures of *A. caviae* and *A. hydrophila*, MICs of filtered IWW were determined [45]. The definition of a bacterial MIC is the lowest concentration of a specific stressor that inhibits the growth of a specific bacterium, allowing sub-inhibitory concentrations to be determined for use in our experiments [1, 46]. Fresh IWW samples were obtained from Jones

Island Water Reclamation Facility in Milwaukee, Wisconsin on the day of each experiment. Samples were kept on ice until further use, to reduce degradation of any contaminants present. To prepare the IWW used in our experiments, we filtered each sample twice. The first filtration was performed using a 0.45µm filter, followed by a 0.22µm syringe filter, thus minimizing the presence of other smaller microbes.

A modification of the standard MIC protocol was performed to identify these values in response to filtered IWW [46]. Specifically, this involved using a 96 deep-well plate with triplicates of decreasing dilutions of filtered IWW, beginning at a ½ dilution. In total 8 different dilutions of filtered IWW were included to ensure a dilution that did not inhibit bacteria growth. Each bacterium was diluted 1:20 in LB (50 µL into 950 µL LB), followed by a 1:100 dilution (100 µL into 9,900 µL LB) in sterile 15mL conical tubes containing various dilutions of filtered IWW, resulting in a final dilution of 1:2000. Mixed samples were then transferred to a 96 deep-well plate in triplicates at a volume of 500 µL per well. Positive controls contained culture only, and negative controls had LB with no bacteria. Control groups were also performed in triplicate. Incubation of the 96 deep-well MIC plates took place at 30°C, while shaking at 250RPM, for 24 hours in aerobic conditions [1, 46]. After the incubation period, turbidity was visually observed by examining the bottom of the 96-well plate. If visually similar, each sample was examined further using a spectrophotometer at a wavelength of six hundred nanometers (OD_{600nm}), a standard bacterial turbidity measurement. If similar turbidities were not observed, the MIC assay was repeated with the varied groups. The absorbances of each well were compared with the positive controls, which had optimal growth. These comparisons were used to determine the dilutions that shared similar optical densities, and therefore were considered sub-inhibitory. Due to the daily

fluctuation and variation of contaminants in WW, MICs were performed each time a new IWW sample was obtained.

3. Determining MICs of antibiotic compounds

A. caviae ATCC 15468 and *A. hydrophila* ATCC 7966 were used to determine the MICs of various antibiotics. The antibiotic compounds being tested in this specific aim include ciprofloxacin (CIP), tetracycline (TET), trimethoprim (TMP), and cefotaxime (CTX). Additionally, the MIC of rifampin (RIF) was identified to be used in our selective media. To determine the MICs of these antibiotics to each bacterium, the following protocol was performed [46]. We began by creating overnight cultures of both bacterial species from the -80°C frozen stock, followed by incubation at 30°C with shaking at 150RPM. Next, the initial wells of each sample group were filled with 200 μL of antibiotic at double the concentration than what was being analyzed. This is required due to the addition of bacterial samples, which results in the concentration being diluted 1:2. The starting concentrations of antibiotics were as follows: 64μg/mL of RIF, 2μg/mL of CIP, 2μg/mL of TET, 32μg/mL of TMP, and 1μg/mL of CTX [47]. The remaining wells were then filled with 100 μL of LB. Once each individual well was filled with appropriate antibiotics and LB, a 1:2 dilution was performed using a multichannel pipet until the final row. After final wells were properly mixed, the same volume that was added was removed to keep the concentration at a 1:2 difference. Positive and negative controls were similar to the MIC experiment testing IWW.

After 24 hours of incubation, bacterial optical densities were measured between experimental and control groups. Bacterial absorbances were read at OD_{600nm} and were performed using a 96-well plate reader. Optical densities were compared to the positive controls, as a guide

to determine the optimal sub-inhibitory concentrations to be used in further experiments, specifically the fluctuation assay. To be confident in the MIC values analyzed, antibiotic groups were performed in triplicates for each microorganism.

4. Determining MICs of calcium hypochlorite

Steps taken to determine the MICs of calcium hypochlorite were identical to that in the antibiotic MIC experiments. The initial concentration of hypochlorite for each microorganism was 1000μg/mL, followed by a series of 1:2 serial dilutions. McFarland standards of each bacterium were further diluted in LB (1:100), which were then aliquoted to the corresponding wells at a volume of 100 μL. Positive and negative controls were included as mentioned previously. In order to be confident in the MIC values analyzed, calcium hypochlorite groups were performed in triplicates for each microorganism.

5. Determining minimal inhibitory irradiance doses of ultraviolet light

The minimal inhibitory doses (MIDs) of UVL was measured by comparing the number of bacterial colonies from cultures exposed to UVL and those without exposure, to determine similar bacterial counts. This was achieved by plating each bacterium on TSA after the emittance of UVL, or those not exposed. Overnight cultures, as performed previously, were centrifuged and washed, removing any growth media from the bacteria, and diluted in buffered demand free (BDF) water to obtain an absorbance of 0.03 at OD_{600nm} for light exposure preparation [48]. BDF water was used for UVL exposure instead of LB, since it has been shown that UVL is sensitive to factors including media coloration [20]. Acknowledging previous work conducted with Dr. Brooke Mayer, Dr. Patrick McNamara, and Nicole Heyniger at Maquette University, the effects of UVL

beginning at a frequency of 1 mJ/cm^2 were examined at wavelengths of 255nm and 285nm [48]. UV-C is in the range of 100-280nm and UV-B is in the range of 280-315nm [9]. Throughout varying exposures, small magnetic stir bars were continuously mixing samples, making sure the bacteria were evenly treated [48]. Serial dilutions were performed post-light exposure followed by plating on non-selective TSA to determine bacterial viability. Once plated, the TSA plates were incubated at 30ºC for 24 hours in aerobic conditions. After the incubation period, we counted bacterial CFUs to determine the viability of the samples compared to the positive control [48]. Positive controls for the UVL MIDs were the bacterial organisms not exposed to light, and our negative controls were BDF alone, both plated on TSA. Determination of MIDs was conducted in triplicates to have greater confidence in our results. The UV-LED source was provided by the laboratory of Dr. Patrick McNamara at Marquette University.

II: Fluctuation analysis

1. Fluctuation analysis with IWW

To determine the effect of filtered IWW on microbial mutagenesis, we briefly exposed *A. hydrophila* and *A. caviae* to varying sub-inhibitory concentrations of filtered IWW. Our fluctuation assay followed previously published procedures with minor adjustments [49]. We began by culturing fresh bacterial samples overnight, following the procedure above. After overnight incubation, each microorganism was sub-cultured at a 1:2000 dilution in 15mL conical tubes containing varying volumes of LB and filtered IWW, with a total volume of 10mL each [49]. Performing a high initial dilution increases the number of bacterial replications, allowing for a greater chance of mutational events to occur. After each bacterial sample was vortexed with sub-inhibitory volumes of filtered IWW, we aliquoted 0.5mL of each separate culture into 19

independent wells of a 96 deep-well plate [49]. Once every well was filled with control or treatment samples, each 96 deep-well plate was covered with a sealing membrane to minimize evaporation and incubated in a 30ºC incubator for 24 hours, while shaking at 250 RPM.

Independent wells were plated accordingly onto either selective or non-selective TSA, a standard growth media, after the 24-hour incubation [34]. Three wells were designated for plating on non-selective agar, and 16 wells were used for the selective agar [49]. Non-selective TSA is used to determine the total bacteria present in each sample being tested, which can be compared to the control group. To achieve this, serial dilutions were performed, up to a 10^{-8} dilution, onto the non-selective agar at a volume of 50μL. Selective TSA was used to determine the number of mCFU in our sample, due to the presence of a lethal concentration of RIF. To make the media selective, a concentration eight-times the MIC of RIF was used specific to the species [49, 50]. The selective agar is referred to as 8X RIF throughout this study. Unlike the non-selective agar, serial dilutions were not performed to determine the total mCFUs present. Instead, the entirety of each 0.5mL independent sample was aliquoted onto individual 8X RIF plates and set out in aseptic conditions to allow the culture to dry [49].

After each independent sample was spread, the various plates were inverted and incubated for 48 hours at 30ºC in aerobic conditions. The individual CFUs from each non-selective plate were counted and used to determine CFU/mL. To calculate CFU/mL, the total CFUs on our non-selective media are multiplied by the dilution factor and then divided by the volume plated. When determining mCFU/mL, the number of mutant colonies was divided by 0.5, since we originally plated half a milliliter. Once CFU/mL and mCFU/mL were determined, mutation rates were calculated by dividing the mCFU/mL by the corresponding CFU/mL [39, 49]. Final mutation rates calculated from this specific aim were compared between bacterial species and the control groups.

Positive controls were bacterial colonies on non-selective media, whereas negative controls consisted of TSA plates with LB, used for identification of contamination. Experimental groups in these experiments were the culturing of each microorganism with different concentrations of filtered IWW [4, 7], plated on the selective media.

2. Fluctuation analysis with antibiotic compounds

To determine the impact of sublethal doses of antibiotics on mutation rates between *A. caviae* and *A. hydrophila*, these microorganisms were cultured with sub-inhibitory concentrations of CIP, TET, CTX, or TMP. Overnight cultures of each bacterium were prepared following the same methods as performed above. The fluctuation assay used for examining mutation variation in the presence of different antibiotics followed the steps performed in the IWW fluctuation assay, including temperatures, incubation periods, dilutions, number of independent wells, controls, and medias used [49]. We calculated and aliquoted the appropriate sub-inhibitory concentrations of CIP, TET, CTX, and TMP into individual 15mL conical tubes containing separate bacterial samples. Antibiotic concentrations of 0.5X and 0.25X respectively [4, 7] were tested for each species of bacteria and antibiotic to analyze mutagenic differences (Table 1). We used the same calculations used previously to calculate CFU/mL, mCFU/mL, and the mutation frequencies between the two microorganisms.

Microorganism	CIP	TET	TMP	CTX	$Ca(OCl)_2$
A. hydrophila (0.5X)	0.0078µg/mL	0.125µg/mL	1µg/mL	1µg/mL	125µg/mL
A. hydrophila (0.25X)	0.0039µg/mL	0.0625µg/mL	0.5µg/mL	0.5µg/mL	62.5µg/mL
A. caviae (0.5X)	0.0156µg/mL	0.125µg/mL	2µg/mL	0.25µg/mL	125µg/mL
A. caviae (0.25X)	0.0078µg/mL	0.0625µg/mL	1µg/mL	0.125µg/mL	62.5µg/mL

Table 1: Antibiotic and disinfectant concentrations corresponding to respective 0.5X and 0.25X values of each sub-inhibitory stressor used in the fluctuation experiments.

3. Fluctuation analysis with calcium hypochlorite

To determine the effect of chlorination on inducing mutations in microbes, *A. hydrophila* and *A. caviae* were exposed to sub-inhibitory concentrations of calcium hypochlorite. The fluctuation assay and overnight procedure followed the same protocol and variables as stated in previous sections. Since de-chlorination was not conducted, the only variable that was altered was the stressing agent used. Experimental groups in this section were the different sub-inhibitory concentrations of calcium hypochlorite, respectively at 0.5X and 0.25X the MIC (Table 1).

4. Fluctuation analysis with UVL

Exposure to sub-inhibitory UVL was performed using *A. hydrophila* and *A. caviae* to determine its effect on bacterial mutagenesis. The mutation analysis and overnight setup followed the same procedure and variables as stated in previous sections, with additional steps of UVL exposure [49]. Overnight cultures used in the UVL experiment were centrifuged and washed, followed by obtaining an OD_{600nm} absorbance of 0.03, roughly a 1:40 dilution, for each bacterium [48]. During UV exposure, small magnetic stir bars were continuously mixing samples, exposing

the bacteria equally in each petri dish [48]. After exposure to irradiances of 0.5mJ/cm^2 and 0.25mJ/cm^2 of UVL, an additional 1:50 dilution of the cells was performed to obtain a final 1:2000 dilution [49]. The final dilution allowed for an increased number of bacterial replications, which was followed by aliquoting 0.5mL from the 10mL cultures of each independent sample into the 96-deep well plates. The fluctuation assay continued as mentioned previously.

III: Analysis of mutation frequencies

1. Statistical analysis

The data obtained from our experiments were analyzed using GraphPad Prism 9. Significance between test groups of our mutation frequency experiments used the analysis of variance test [51]. Results between treatment groups were analyzed using a one-way ANOVA followed by Dunnett's multiple comparison test to determine significant differences between treatment groups. Welch's t-test was also used in our control experiment since one-way ANOVA requires more than two experimental groupings to be included. Data collected in our experiments were identified as significant ($P<0.05$) and indicated by asterisks above the bar graphs in the results and discussion section of this thesis.

CHAPTER 3: RESULTS

Specific Aim I:

To test for mutation frequencies, we needed to determine the MICs in response to filtered IWW for both *A. hydrophila* and *A. caviae.* In total, two samples of filtered IWW were analyzed, with the MICs consistent between samples in *A. hydrophila;* however, *A. caviae* showed varying MICs between samples. Specifically, the MIC of *A. caviae* was a 1/8 dilution for sample one, and a 1/16 dilution for sample two (Table 2.). In our fluctuation assay, an additional 1:2 dilution was performed from each of the indicated MICs mentioned above, which is respective of a 0.5X concentration.

Bacterium	**96-deep well plate (Sample #1)**	**96-deep well plate (Sample #2)**
A. hydrophila	1/4 dilution	1/4 dilution
A. caviae	1/8 dilution	1/16 dilution

Table 2. MICs of filtered IWW to *A. hydrophila* and *A. caviae*.

IWW contains a diverse array of contaminants ranging from chemical compounds to detergents. Therefore, we wanted to determine the impact of IWW on mutation frequency. In Table 3, the mean mutation frequencies of each *A. hydrophila* treatment group in response to diluted filtered IWW is displayed, with minimal differences among mutation frequencies. To further analyze our IWW data with *A. hydrophila*, we performed a one-way ANOVA of the 16-independent samples from each treatment group (Fig. 1). These data represent the mutagenic progression of *A. hydrophila* in the presence of 0.5X and 0.25X dilutions of filtered IWW (Fig. 1).

Here we analyzed two samples of filtered IWW from different time periods. Since WW varies daily regarding its contaminants [52], it was essential to perform the fluctuation assay with more than one sample. After performing statistical analyses for *A. hydrophila* there was no significant difference between the control groups and each experimental group (Fig. 1).

Average mutation frequency		
Experimental groups	**Sample #1**	**Sample #2**
Control AH	2.20E-09	5.79E-09
1/8 Inf. AH	7.00E-09	1.12E-08
1/16 Inf. AH	2.53E-09	1.10E-08

Table 3. Average mutation frequencies of *A. hydrophila* with filtered IWW. Means represent 16-independent experiments.

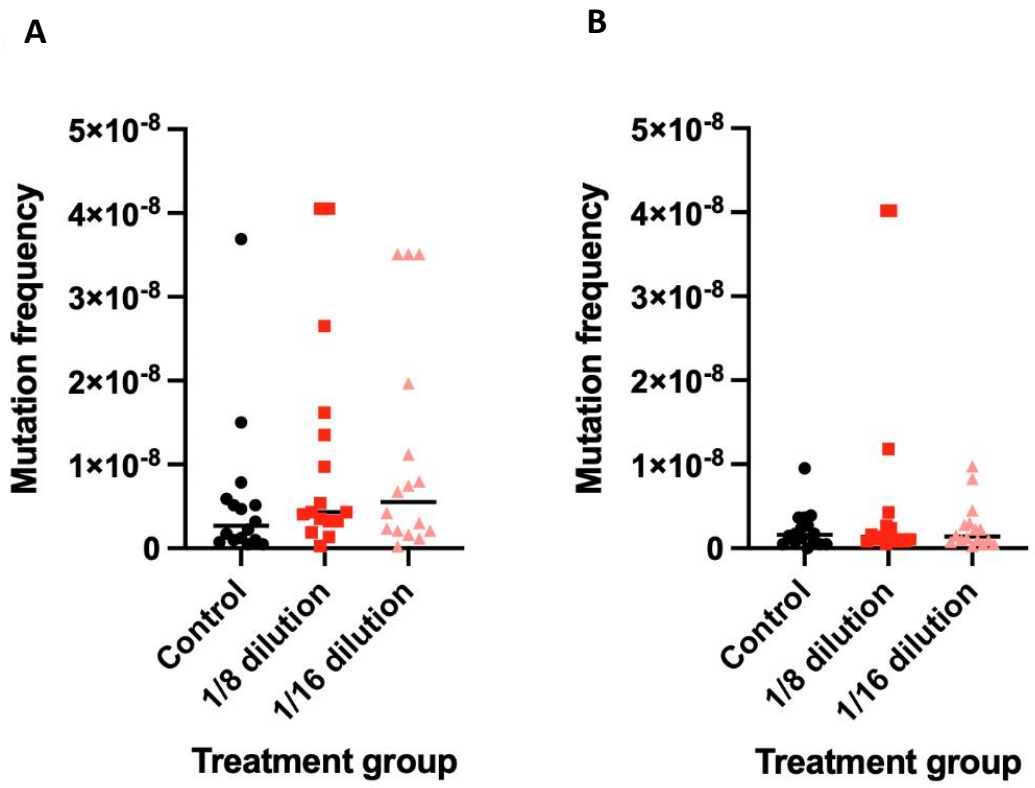

Figure 1: Impact of filtered IWW on mutation frequencies in *A. hydrophila*. Briefly, *A. hydrophila* was cultured with sub-lethal dilutions of filtered IWW. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment. Experiments were performed with IWW from two different dates (A and B).

Similar to the previous data examining the mutagenic effects of filtered IWW on *A. hydrophila* (Fig. 1), these data represent the mutational effect of filtered IWW on *A. caviae*. In Table 4, the means of each *A. caviae* treatment group in response to filtered IWW is displayed. It is important to note that the sub-inhibitory concentrations examined varied between each sample of IWW. Sample one contributed to an increased mutation frequency over sample two; however,

this cannot be strongly correlated since MIC values varied between samples. Bacterial growth in the sample with a 1/16 dilution of filtered IWW was slightly below that of the control; however, to maintain consistency throughout our experiments, regarding bacterial growth, we chose to use the 1/32 dilution (absorbance readings not shown). We further analyzed the 16-independent samples from each treatment group of *A. caviae* (Fig. 2). Incubation with filtered IWW caused significant changes in mutation frequency amongst *A. caviae* (Fig. 2 A, P = 0.002); however, this was only present in IWW from one date.

Average mutation frequency			
Experimental groups	**Sample #1**	**Experimental groups**	**Sample #2**
Control AC	7.25E-09	Control AC	7.14E-09
1/16 Inf. AC	2.19E-08	1/32 Inf. AC	1.26E-08
1/32 Inf. AC	1.92E-08	1/64 Inf. AC	1.29E-08

Table 4. Average mutation frequencies of *A. caviae* with filtered IWW exposure. Means represent 16-independent experiments.

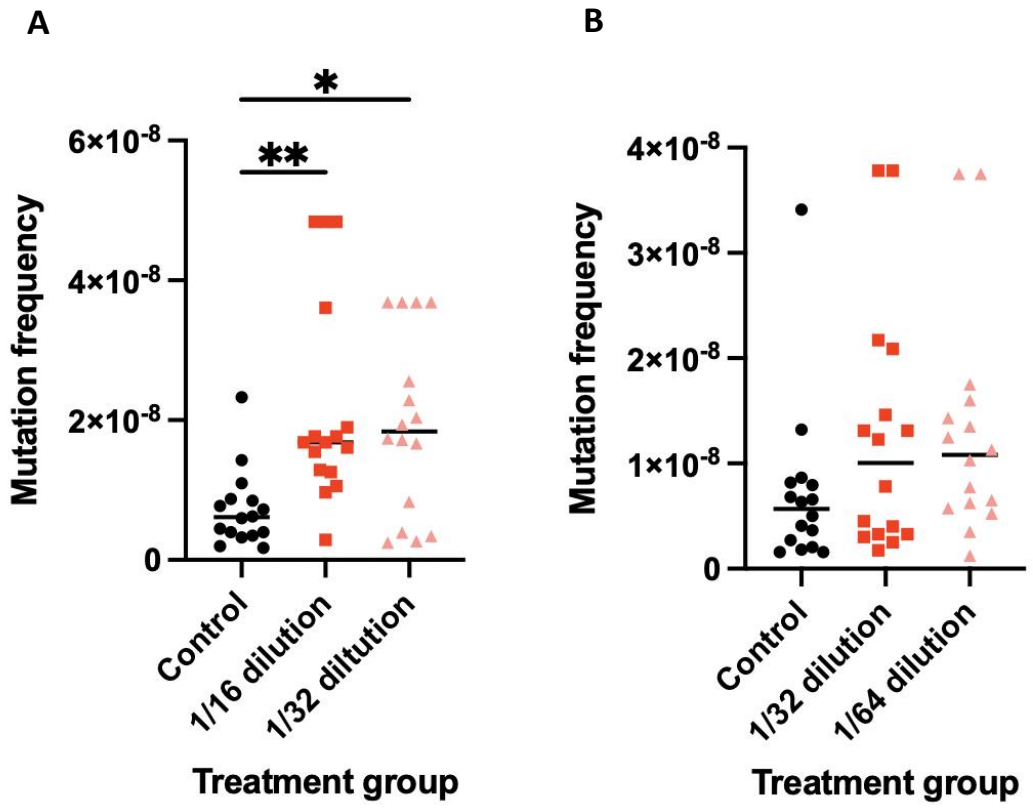

Figure 2: Impact of filtered IWW on mutation frequencies in *A. caviae*. Briefly, *A. caviae* was cultured with sub-lethal dilutions of filtered IWW. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 16 independent experiments for each species and individual dots represent one experiment. Experiments were performed with IWW from two different dates (A and B).

* $P = 0.0112$, ** $P = 0.002$

Specific Aim II:

Due to the presence of antibiotics within WW, we analyzed the individual effects that specific compounds had on mutagenesis, specifically TMP, CIP, TET, and CTX [45].In order to assess sub-inhibitory concentrations of each antibiotic to be used in the fluctuation assay, MICs were performed on both *A. hydrophila* and *A. caviae* (Table 5). We also performed an additional MIC procedure, which involved using a 96-deep well plate. Between the standard 96-well plate and 96-deep well plate, TMP and CIP MICs varied for *A. hydrophila*, whereas the MICs of TMP and CTX varied for *A. caviae* (Table 5). Sub-inhibitory values used in our fluctuation assays (Fig. 3 and Fig. 4) were performed using the concentrations from the 96-deep well MICs.

Bacterium	Antibiotic	96-well plate	96-deep well plate
A. hydrophila	Trimethoprim	64 µg/mL	2µg/mL
A. hydrophila	Cefotaxime	2µg/mL	2µg/mL
A. hydrophila	Ciprofloxacin	0.03125µg/mL	0.0156µg/mL
A. hydrophila	Tetracycline	0.25µg/mL	0.25µg/mL
A. caviae	Trimethoprim	32µg/mL	4µg/mL
A. caviae	Cefotaxime	2µg/mL	0.5µg/mL
A. caviae	Ciprofloxacin	0.03125µg/mL	0.03125µg/mL
A. caviae	Tetracycline	0.25µg/mL	0.25µg/mL

Table 5. MICs of each antibiotic to *A. hydrophila* and *A. caviae*. Data provided are the MICs in a standard 96-well plate and 96-deep well plate.

We aimed to determine the mutagenic effects of these antibiotics, at sub-inhibitory concentrations, on *A. hydrophila*. In Table 6 , the mean mutation frequencies of 16-independent experiments are displayed. In total, two separate fluctuation analyses were performed, due to the high sample size of each (Table 6, Fig. 3). Average mutation frequencies varied between specific

antibiotics in *A. hydrophila*; however, analysis of significance was not conducted. We performed 16-independent samples of each experimental group to measure any significance between them (Fig. 3). Significance was determined in *A. hydrophila* for sub-inhibitory concentrations of TMP (Fig. 3 A: P = 0.0005*)*, CTX (Fig. 3 B: P = 0.0010*)*, and TET (Fig. 3 D: P = 0.0028*)*. No mutagenic significance was evident in *A. hydrophila* after exposure to subinhibitory concentrations of CIP (Fig. 3 C: P > 0.5).

Average mutation frequency	
Experimental group	Experiments #1
Control	2.16E-09
0.5X TMP	7.87E-09
0.25X TMP	1.14E-08
0.5X CTX	1.10E-08
0.25X CTX	5.00E-09
Experimental group	Experiments #2
Control	1.32E-09
0.5X CIP	3.51E-09
0.25X CIP	2.77E-09
0.5X TET	1.03E-08
0.25X TET	4.34E-09

Table 6. Average mutation frequencies of *A. hydrophila* to antibiotics. Data represents the mean values of 16-independent samples. Two separate fluctuation analyses were performed.

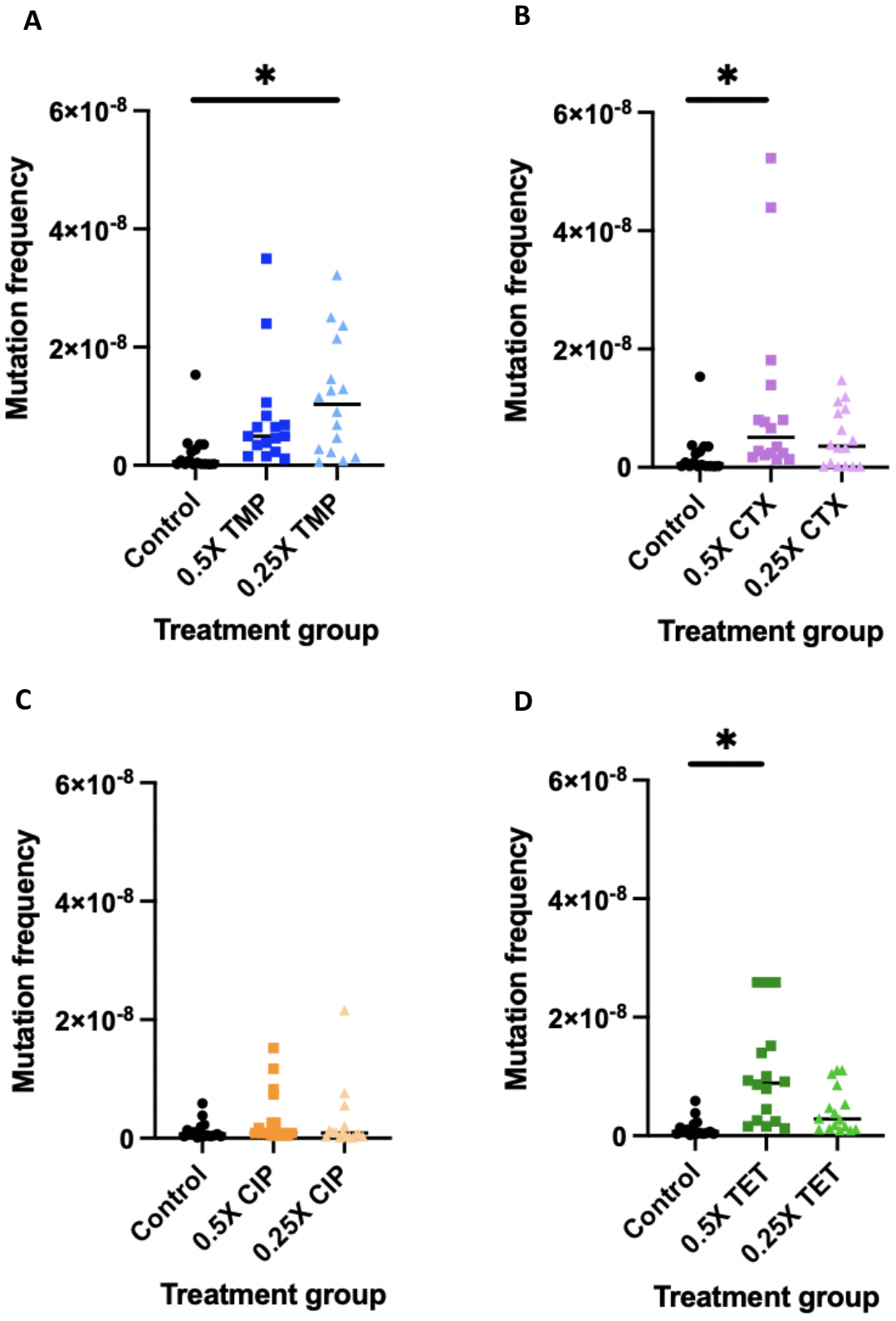
A
*
6×10-8
4×10-8
2×10-8
0
Mutation frequency
Control
0.5X TMP
0.25X TMP
Treatment group
B
*
6×10-8
4×10-8
2×10-8
0
Mutation frequency
Control
0.5X CTX
0.25X CTX
Treatment group
C
6×10-8
4×10-8
2×10-8
0
Mutation frequency
Control
0.5X CIP
0.25X CIP
Treatment group
D
*
6×10-8
4×10-8
2×10-8
0
Mutation frequency
Control
0.5X TET
0.25X TET
Treatment group

Figure 3: Antibiotic impact of on mutation frequencies of *A. hydrophila*. Briefly, *A. hydrophila* was incubated with sub-inhibitory concentrations of TMP (A), CTX (B), CIP (C), and TET (D) then mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment. * $P < 0.005$

We aimed to determine the mutagenic effects of certain antibiotics, at sub-inhibitory concentrations, on *A. caviae*. Table 7 identifies the mean mutation frequencies of *A. caviae* in response to sub-inhibitory antibiotic exposure. We performed 16-independent samples of each experimental group to measure any significance between them (Fig. 4). In *A. caviae,* we observed a significant increase of mutagenic populations after exposure to TMP at both 0.5X (Fig. 4 A: $P < 0.0001$*)* and 0.25X (Fig. 4 A: $P < 0.0001$*)*, as well as 0.5X CIP (Fig. 4 C: $P = 0.0007$*)*. No mutagenic significance was evident in *A. caviae* after exposure to subinhibitory concentrations of CTX (Fig. 4 B: $P > 0.05$) and TET (Fig. 4 D: $P > 0.05$)

Average mutation frequency	
Experimental group	**Experiment #1**
Control	4.17E-09
0.5X CIP	1.28E-08
0.25X CIP	6.95E-09
0.5X TET	5.57E-09
0.25X TET	6.14E-09
Experimental group	**Experiment #2**
Control	6.97E-09
0.5X TMP	3.02E-08
0.25X TMP	2.88E-08
0.5X CTX	7.85E-09
0.25X CTX	3.72E-09

Table 7. Average mutation frequencies of *A. caviae* to antibiotics. Data represents the mean values of 16-independent samples. Two separate fluctuation analyses were performed.

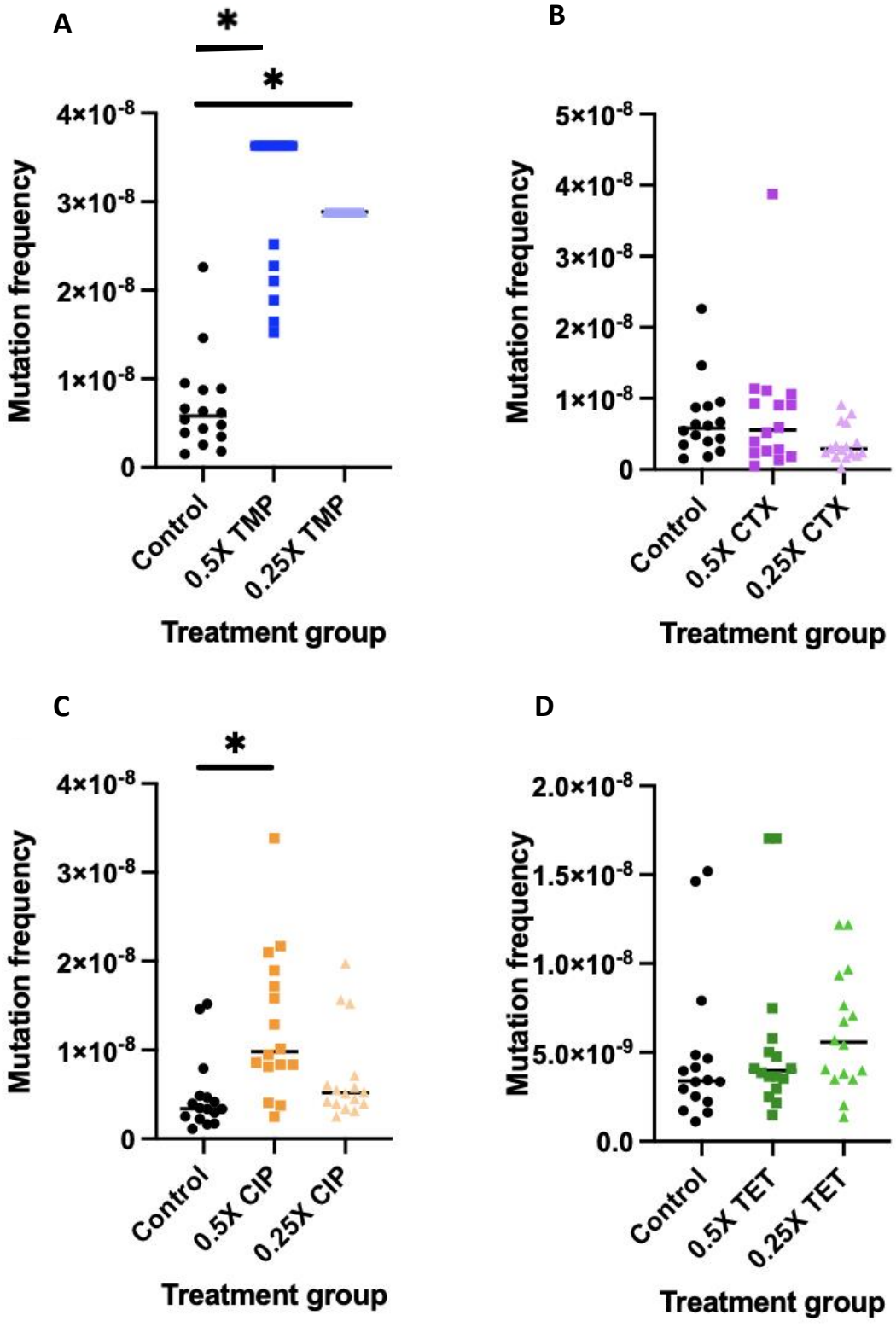
A
*
*
4×10-8
3×10-8
2×10-8
1×10-8
0
Mutation frequency
Control
0.5X TMP
0.25X TMP
Treatment group
B
5×10-8
4×10-8
3×10-8
2×10-8
1×10-8
0
Mutation frequency
Control
0.5X CTX
0.25X CTX
Treatment group
C
*
4×10-8
3×10-8
2×10-8
1×10-8
0
Mutation frequency
Control
0.5X CIP
0.25X CIP
Treatment group
D
2.0×10-8
1.5×10-8
1.0×10-8
5.0×10-9
0.0
Mutation frequency
Control
0.5X TET
0.25X TET
Treatment group

Figure 4: Antibiotic impact of on mutation frequencies of *A. caviae*. Briefly, *A. caviae* was incubated with sub-inhibitory concentrations of TMP (A), CTX (B), CIP (C), and TET (D) then mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment. * $P < 0.005$

Due to the effect that sub-inhibitory exposure of TMP had on elevating mutagenesis within *A. caviae*, we wanted to determine at what concentration this effect was lost. Since we performed this analysis over three different dates, we normalized the data from our experiments. This was performed by taking the mutation frequency of each independent experiment, divided by the average control mutation frequency. A normalized value of 1 for the control was then compared between groups to compare mutagenic variation from each TMP treatment. Table 8 displays the normalized mean mutation frequencies of 16-independent experiments. A steady decline of mutagenesis is seen for the varying concentrations of TMP starting at 0.03125X and ending at 0.00195X (Table 8 and Fig. 5). TMP maintained a significant effect on mutations compared to the control group until the 0.0078X concentration (Table 8 and Fig. 5: $P < 0.0005$). A significance was lost at a TMP concentration less than 0.0039X (Table 8 and Fig. 5: $P = 0.1320$).

Average normalized mutation frequencies (48-hr)	
Control	1.00
0.5X TMP	4.33
0.25X TMP	4.14
0.125X TMP	4.10
0.0625X TMP	4.31
0.03125X TMP	3.09
0.0156X TMP	2.74
0.0078X TMP	2.12
0.0039X TMP	1.56
0.00195X TMP	1.38

Table 8. Average mutation frequencies of *A. caviae* to TMP. Data represents the mean values of normalized mutation frequencies in 16-independent samples.

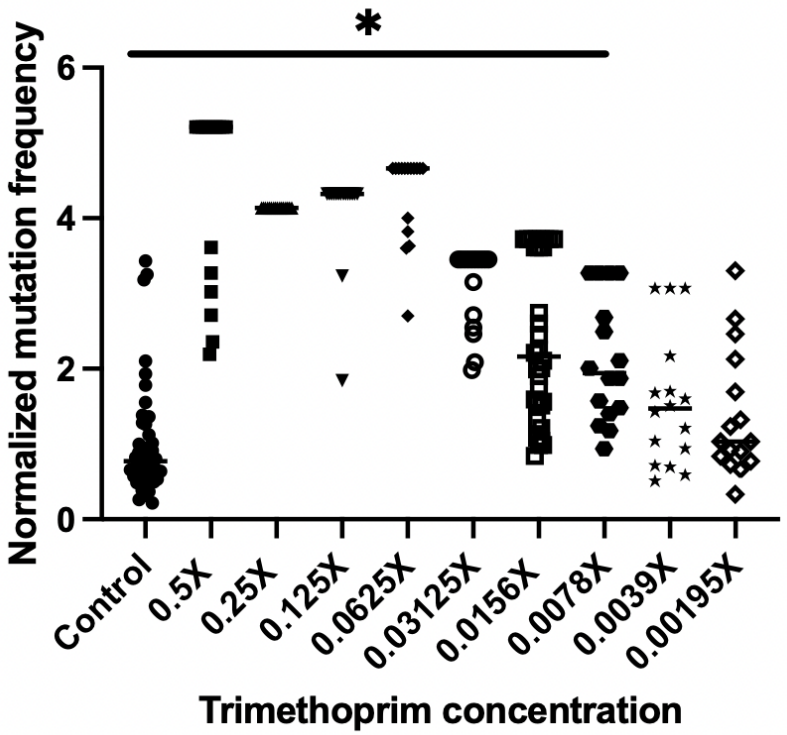

Figure 5: Impact of TMP on mutation frequencies in *A. caviae*. Briefly, A. caviae was incubated with various sub-inhibitory concentrations of TMP. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment.

* $P < 0.005$

Specific Aim III:

We next examined the mutagenic effects of the disinfecting agent calcium hypochlorite. During the WW treatment process, calcium hypochlorite is a common disinfecting agent used to remove microorganisms. Firstly, we performed an MIC analysis to determine sub-inhibitory values

for our fluctuation assay (Table 9). As previously mentioned, determination of MIC values for both *A. hydrophila* or *A. caviae* were performed in distinct 96-well plates. In the MIC experiments using calcium hypochlorite, values were similar in *A. hydrophila*, but differed in *A. caviae.* Specifically, the MIC decreased for *A. caviae* when performed in a 96-deep well plate (Table 9).

Bacterium	**96-well plate**	**96-deep well plate**
A. hydrophila	250ug/mL	250ug/mL
A. caviae	500ug/mL	250ug/mL

Table 9. MICs of calcium hypochlorite to *A. hydrophila* and *A. caviae*. MICs were performed in a standard 96-well plate and 96-deep well plate.

Table 10 represents the mean mutation frequency values for *A. hydrophila* and *A. caviae*, in response to sub-lethal concentrations of calcium hypochlorite. For *A. caviae*, mean values indicated a strong increase of mutation frequencies, specifically in response to the 0.5X concentration of calcium hypochlorite (Table 10 and Fig. 6 B: $P < 0.0005$). Once the concentration was lowered to 0.25X, significance was no longer observed for *A. caviae*. These results identify that *A. caviae* (Fig. 6 B) has a greater mutagenic potential over *A. hydrophila* (Fig. 6 A) when exposed to calcium hypochlorite and further support the risk that may be associated with certain bacterial populations during the disinfection process of WW treatment.

Average mutation frequency		
Experimental groups	***A. hydrophila***	***A. caviae***
Control AH	2.72E-09	4.91E-09
0.5X CaClO2 AH	7.12E-09	1.53E-08
0.25X CaClO2 AH	5.16E-09	8.94E-09

Table 10. Average mutation frequencies of *A. hydrophila* and *A. caviae* with calcium hypochlorite. Data represents the mean values of 16-independent samples.

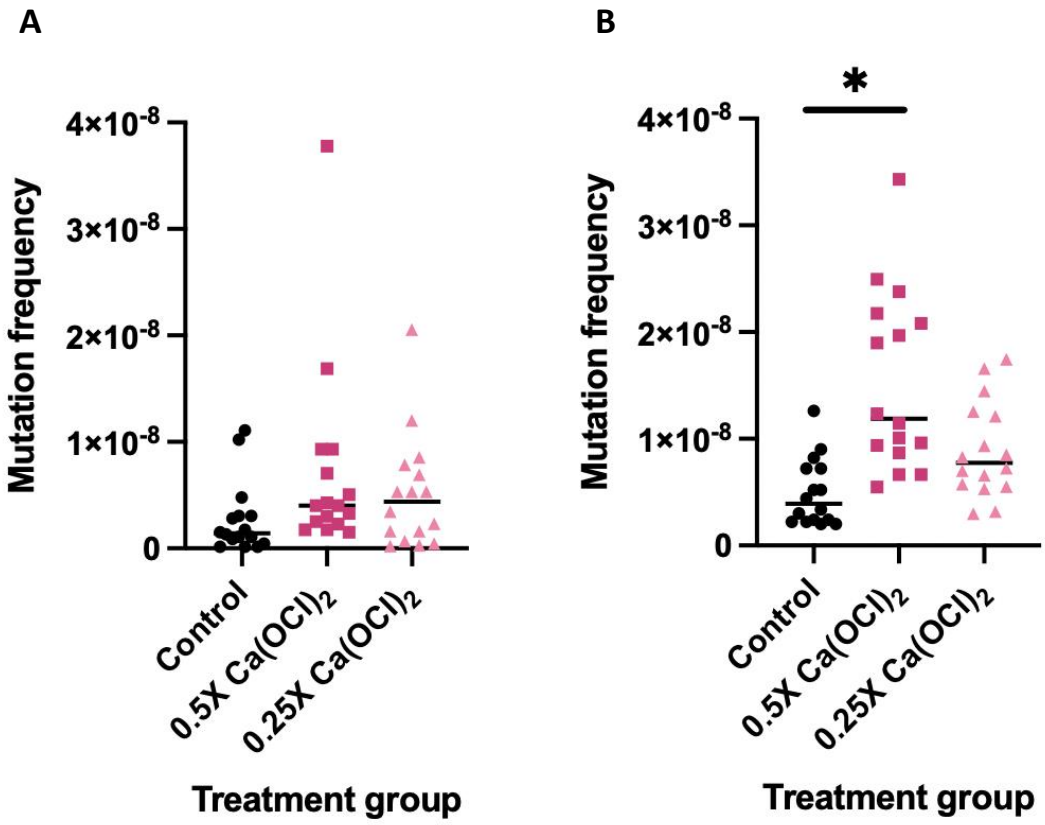

Figure 6: Impact of calcium hypochlorite on mutation frequencies in *A. hydrophila* and *A. caviae*. Briefly, *A. hydrophila* (A) and *A. caviae* (B) were incubated with sub-inhibitory concentrations of calcium hypochlorite. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment. * $P < 0.005$

Another form of disinfection that is commonly used in WW treatment is the use of UVL. In Table 11, respective MIDs of each bacterium in response to UVL, based on bacterial inactivation, are provided. Intensities used in the UVL experiments were similar between both species of *Aeromonas*, in which a reduction was observed for bacterial colonies at 1mJ/cm^2.

Bacterial inactivation experiments were performed with the help of Dr. Brooke Mayer, Dr. Patrick McNamara, and Nicole Heyniger at Marquette University.

Bacterium	Bacterial inactivation	Intensity used (255nm/285nm)
A. hydrophila	1 mJ/cm^2	0.5 mJ/cm^2 and 0.25 mJ/cm^2
A. caviae	1 mJ/cm^2	0.5 mJ/cm^2 and 0.25 mJ/cm^2

Table 11. MID values of UVL to *A. hydrophila* and *A. caviae*.

Table 12 displays the mean mutation frequency values of *A. hydrophila* and *A. caviae* after UVL exposure. Mean values of *A. hydrophila and A. caviae* shared minor variation post-UVL exposure when compared to the control group (Table 12). For each treatment, 16-independent experiments were performed to determine any significant mutagenic activity (Fig. 7). We show that *A. hydrophila* (Fig. 7 A) had minimal differences on mutagenic activity for the treatment groups over the control; however significant differences of mutation frequencies was evident in *A. caviae* (Fig. 7 B), specifically for the 255nm treatment groups at both 0.5mJ/cm^2 (Fig. 7 B: $P < 0.0005$) and 0.25mJ/cm^2 (Fig. 7 B: $P < 0.0005$). The effect of UVL at a wavelength of 285nm had no significant effect on mutagenesis for *A. caviae*. These data further support the potential of WW treatment to increase mutagenesis within bacterial species, specifically those that are considered emerging pathogens.

Average mutation frequency		
Experimental groups	***A. hydrophila***	***A. caviae***
Control	2.64E-09	8.05E-09
UV 255 0.25mJ	6.44E-09	2.28E-08
UV 285 0.25mJ	6.01E-09	1.25E-08
UV 255 0.5mJ	7.51E-09	2.43E-08
UV 285 0.5mJ	4.22E-09	1.07E-08

Table 12. Average mutation frequencies of *A. hydrophila* and *A. caviae* to UVL. Data represents the mean values of 16-independent samples. Two separate fluctuation analyses were performed.

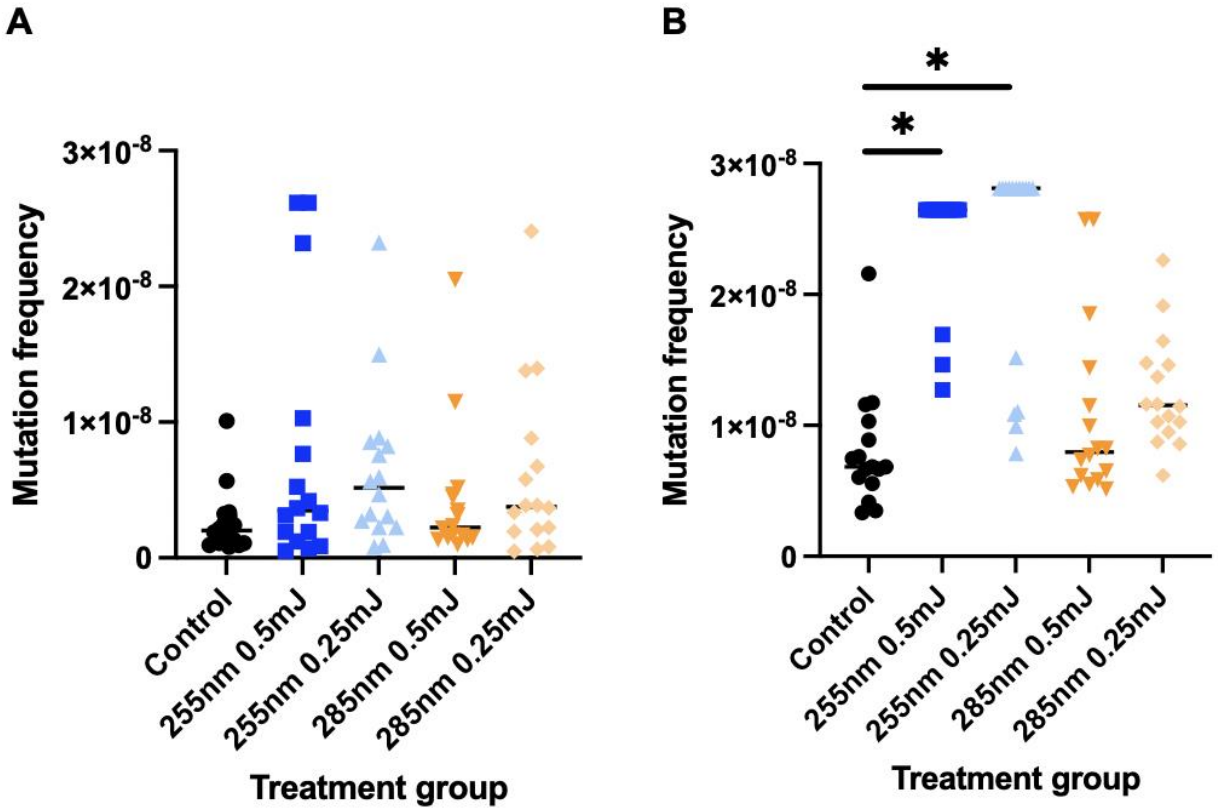

Figure 7: Impact of UVL on mutation frequencies in *A. hydrophila* and *A. caviae*. Briefly, *A. hydrophila* (Fig. 6 A) and *A. caviae* (Fig. 6 B) were exposed to sub-lethal irradiances of UVL. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Mutation frequencies were analyzed after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 16 independent experiments for each species with individual dots representing one experiment. * $P < 0.0005$

Due to the increased mutagenesis of *A. caviae* throughout our study, we wanted to identify the mutagenic variation between *A. hydrophila* and *A. caviae* under normal conditions (Fig. 8). A clear mutagenic distinction between the microorganisms was observed, with *A. caviae* exhibiting

increased mutation frequencies under normal growth conditions, compared to *A. hydrophila* (Fig. 8: 6.451e-009 vs. 2.064e-009 respectively, $P < 0.0005$).

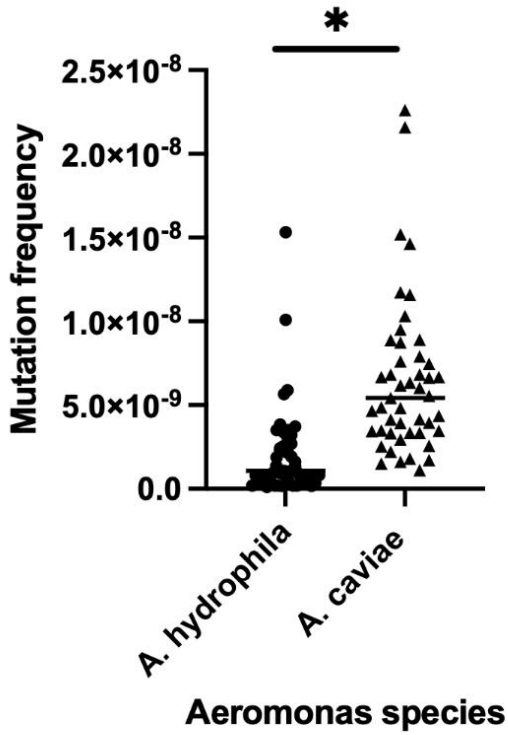

Figure 8: Comparison of mutation frequencies between control groups of *A. hydrophila* and *A. caviae*. Briefly, bacteria were cultured in media alone for 24 hours. Mutant CFUs were counted and compared to total CFUs to determine mutation frequencies. Briefly, mutation frequencies were determined after 48 hours on RIF agar at eight-times the MIC. Results represent the mean (line) of 32 independent experiments for each species (individual dots represent one experiment).

* $P < 0.0005$

CHAPTER 4: DISCUSSION

Exposure to sub-inhibitory stressing agents has been shown to accelerate bacterial mutagenesis in numerous microorganisms, contributing to increased pathogenicity and antimicrobial resistance [4-8]. Expanding on these previous data, we examined filtered IWW, common antibiotics found in WW, as well as disinfectants in this environment, on their mutagenic effects in *A. hydrophila* and *A. caviae*. Our findings indicate that certain stressors contributed to increased mutagenesis in either *A. hydrophila* or *A. caviae.* After exposure to filtered IWW, a significant mutagenic effect was noted in one of two samples obtained when cultured with *A. caviae*. When analyzing the four antibiotics CIP, TET, TMP, and CTX, which are commonly found in WW [44, 45, 53], mutational frequencies varied. In *A. hydrophila*, a significant increase in mutation frequencies was shown post-exposure to TET, TMP, and CTX, whereas in *A. caviae* significance was only shown for CIP and TMP. While a decreased significance of mutagenesis to the number of antibiotics was shown in *A. caviae,* it is important to note that trimethoprim impacted mutation frequencies as low as 0.156 µg/mL (0.0078X MIC). A significant increase of mutation frequencies was also shown after exposure to the common WW disinfectants calcium hypochlorite and UVL at a wavelength of 255nm in *A. caviae*. The findings from this current study support the idea that WW treatment may accelerate the manifestation of mutations within microbes.

IWW is immensely populated with a variety of contaminants making this a likely environment for mutagenic events to be induced among microorganisms [11, 15]. A challenge of examining WW at individual time points is the variation of the contaminants in each sample [52]. A likely factor contributing to this variation is seasonal trends, with a lower presence of stressing

agents in the winter months [52]. The samples analyzed in our experiments were obtained during the month of February, which can be linked to having a lower presence of contaminants than months such as June and July [52]. Therefore, WW from summer months may contribute to increased mutagenic effects among microbes. Since we did not analyze the contaminants in each sample, we are unable to further link specific stressors to any induction of mutations evident among our bacterial species. When interpreting our findings, it is important to note the dilutions of our filtered IWW samples (i.e., 1/16 dilution for *A. caviae*), since this further lowers the concentrations of any stressors present. Although undiluted IWW had minor growth, these dilutions were essential in this experiment in order to achieve similar growth of our microbial species, ensuring the number of replications was similar between control and treated groups.

Previous work examining the mutational effects of 38 compounds found in conventional hospital WW on *Salmonella enterica*, found an induced resistance to CIP through the Ames test [54]. Since AMR populations have been observed in both IWW and EWW [19], it was imperative to determine the impact the treatment process has on the acquisition of mutations in the resident WW genus, *Aeromonas*. Similar to the study that identified increased mutagenesis, causing AMR, in *S. enterica* post-IWW exposure [54], we also determined an increase of mutations among *A. caviae* when exposed to our first filtered IWW sample. While the mutagenic effects of WW have not been studied among *Aeromonas* species, the results of this study further support our hypothesis that this environment may act as a stressor that accelerates bacterial mutagenesis.

Antimicrobials are common stressors found in wastewater [44, 45, 53]. Among urban and hospital WW samples, the highest concentrated antibiotics found are CIP [45] and TMP [45],

although TET [44] and CTX [53] also reside in this environment. These common contaminants have been found to induce increased mutations among various bacteria [4, 6, 7]. Exposure to sub-inhibitory concentrations of CIP and streptomycin have increased resistance to RIF between 2- to 5-fold in the Gram-positive bacterium *Streptococcus pneumoniae* [4]. Similar mutagenic activity is also seen in the Gram-negative bacterium *P. aeruginosa*, in which resistance to imipenem was identified after sub-inhibitory exposure to CIP [7]. Our findings identified increases in mutations among *A. hydrophila* that was exposed to sub-inhibitory concentrations of 0.5X TET (0.125µg/mL), 0.5X CTX (1µg/mL), and 0.25X TMP (0.5µg/mL). Significant changes in mutation frequency was also evident with *A. caviae* post-exposure to sub-inhibitory concentrations of CIP (0.0156µg/mL) and TMP (>0.0078µg/mL). Previously, concentrations of CIP (0.0409µg/mL) [45], TMP (0.07285µg/mL) [45], TET (0.05µg/mL) [44], and CTX (0.00727µg/mL) [53] were identified in IWW. While the concentrations of TET and CTX were greater in our experiments than what is found within WW, CIP and TMP were lower. Due to the two latter antibiotics having a significant effect on mutation frequencies in *A. caviae* at concentrations lower than those found in WW, this may signify the potential of this environment to accelerate mutagenic evolution.

The disinfecting agents in WW treatment, whether calcium hypochlorite or UVL, are required to remove high frequencies of microorganisms [20, 26]. However, previous studies have identified their inductive effect on antimicrobial resistance [5, 31, 32]. In *E. coli*, calcium hypochlorite accelerated HGT of ARGs up to 550-times that of the untreated group [23]. It has also been shown that stress responses corresponding to DNA damage are induced post-calcium hypochlorite exposure, specifically in *Pseudomonas* [5]. In particular, the mutagenic effects of the SOS response were upregulated through increased oxidative stress in this bacterium [5]. Similar

to previous studies analyzing the mutagenic effects of calcium hypochlorite exposure in *Pseudomonas* and *E. coli* [5, 23], our data also identified an increased mutagenesis in *A. caviae* when exposed to sub-inhibitory concentrations of this stressor.

An alternative method of disinfection is UVL, which has also been shown to accelerate resistance among microorganisms [31, 32]. Exposure to UVL results in DNA damage, inducing mutagenesis with a strong influence from the SOS response [14, 40]. A unique comparison, which displays the activity of this survival mechanism during UVL exposure, is between *E. coli* and *Legionella pneumophila*. In *E. coli*, the occurrence of spontaneous mutagenic events increased by 40-times, whereas in *L. pneumophila*, only a 7-fold increase was shown post-UVL exposure [31, 32]. This mutable variation may be due to the absence of the functional components of the SOS response in *L. pneumophila* [32]. Comparable results to the *E. coli* study of an increased mutagenesis also occurred in *A. caviae* at both millijoules of UVL at 255nm. While the use of UVL in WW treatment has been identified as having decreased health risks to humans over chlorination [20], the mutagenic effects of UVL identified in this study demonstrate the potential risks as well. The influence of both disinfection agents on increasing mutagenesis in *A. caviae* reflect the need for alternative methods to be identified, such as the potential use of photodynamic inactivation, due to its strong anti-microbial effects [55].

Similar to the studies that indicated a variation of mutational frequencies between species with and without a functional *recA* [31, 32], the increased mutagenesis in *A. caviae* after UVL exposure may be justified from the sequence variation of this gene, resulting in an accelerated SOS response [43]. Variation in the C-terminal end of RecA, specifically the Mg^{2+} binding site,

regulates *recA* expression and DNA binding affinity [43]. This area differs between *P. aeruginosa* and *E. coli,* resulting in an 8-fold increase of mutagenesis over *E. coli* [43]. Some of these specific amino acid variations can be seen between *A. hydrophila, A. caviae,* and *P. aeruginosa* in Table 13. It is important to note the presence of alanine and glutamic acids in *A. caviae* and *P. aeruginosa* since the latter species expressed an increased mutagenesis over *E. coli.* Since alanine promotes construction of proteins and glutamic acid aids in maintaining the structure of proteins, this may be an explanation for the increased mutation frequencies [51]. In our current study, similar comparisons can be seen between our bacterial species. If the variation in the C-terminal end of the RecA contributes to an accelerated mutagenesis of *Aeromonas,* potential remedies that disrupt the *recA* gene may be used to prevent global health complications, such as AMR, via inhibitors like phthalocyanine terasulfonate [56].

Concentrations of selective media used may play a major role in quantifying mutation frequencies among microbes. Similar to our experiments, a previous study cultured *Staphylococcus aureus* and *P. aeruginosa* with concentrations ½ and ¼ the MICs of CIP. This was followed by plating on media containing 4 or 8-times the MIC of antibiotics, including imipenem and TET [7]. When analyzing mutant colonies on plates containing 4-times the MIC of a given antibiotic, the spontaneous mutation frequencies were increased by over a log-difference compared to plates containing 8-times the MIC [7]. Initially we were examining mutation frequencies using plates containing 4-times the MIC of RIF; however, an uncountable number of mutant colonies were seen for the majority, if not all, of the experimental groups. Once we increased our concentration to 8-times the MIC of RIF, we observed fewer mutants, allowing us to quantify mutation frequencies more readily. An alternative approach that may have been taken is to use

selective media containing 4-times the MIC of RIF, while reducing the amount of culture added to each selective plate. According to one source, the entire volume of each independent sample must be plated on selective media to reliably identify mutation frequency values (42). To achieve a lower volume plated on this media, the initial volumes aliquoted in the 96-deep well plate would be reduced. For example, in this study we initially aliquoted 0.5mL of our samples into individual wells, although we could reduce this volume to 0.25mL or lower.

It is indicative that the antibiotic used in selective media plays a role in quantifying mutation frequencies. In our study, *A. caviae* only had a mutagenic increase of 3-fold, compared to the 40-time increase in *E. coli* after UVL exposure [31]. Specifically, this study measured mutagenic populations using the antibiotic nalidixic acid [31], whereas we used RIF. Resistance to the latter antibiotic is conferred when the beta subunit of the RNA polymerase is altered through mutagenic events, specifically in the *rpoB* gene [57]. As few as one amino acid change can confer resistance, which reduces the binding affinity of RIF to the polymerase [57]. Mutations occurring between amino acids 507-687 of the *rpoB* gene are the most common [57]. Resistance to nalidixic acid is acquired through point mutations of multiple genes (e.g. *gyrB, gyrA,* and *parC*), as well as other numerous sites [59]. The elevated mutation frequency of *E. coli*, by using nalidixic acid [31], may due to numerous genes conferring resistance to this antibiotic. Initially, we began our fluctuation assays using nalidixic acid as an indicator for mutagenic populations; however, plates at both 4X and 8X the MIC displayed lawn growth, inhibiting our ability to determine mutation frequencies. Previously it was indicated that 95% of *Aeromonas* were resistant to nalidixic acid in post-chlorinated WW samples suggesting mutations are occurring in WW, considering there was

0% resistance to this antibiotic in recipient WW [58]. This signifies that an increased mutation frequency of our bacterial species may be identified if spiking our media with nalidixic acid.

This study focused on the quantification of mutation frequencies through RIF resistance in *A. hydrophila* and *A. caviae*; however, there is a high probability that mutations outside this gene occurred. For instance, while AMR is one of the main factors contributing to increased mortality rates in infected individuals, factors increasing pathogenicity may also be affected due to mutational events affecting virulence factors, including adherence sites, biofilm production, and capsule formation [6]. For instance, mutations may play a significant role on the functionality of the RpoS regulon, which aids in biofilm expression [6]. Whole genome analysis in future studies could identify new hypermutable regions in the *Aeromonas* genome that are undetected only by looking at mutation frequencies.

CHAPTER 5: FUTURE DIRECTIONS AND CONCLUSIONS

Future studies may involve performing whole genome sequencing of the wildtypes and mutants, conducting a series of gene knockouts/knock-ins for *recA*, and examining additional microbes or WW stressors. Whole genome sequencing of both the wild-type and the mutated species can identify each locus where mutations occurred. We can speculate that the mutated loci within the bacterial species examined in this study, were within the *rpoB* gene, due to the mutated colonies having conferred resistance to RIF. However, another phenotype potentially affecting the mutagenic evolution of a bacterium is identified as hypermutable, which occurs due to the lack of a functional mismatch repair system [61]. A hypermutable phenotype means that a microbe has acquired mutational alterations in their genome, without being able to excise them, contributing to the evasion of otherwise lethal conditions [61]. There is also a probability that the fitness of a bacterium may be negatively impacted by this phenotype, through microbial competition or ultimate death [61]. It is important to identify all mutations that occurred within the genome, to assess the potential evolutional effects that these sub-inhibitory stressors contribute to.

It has been demonstrated that the C-terminal sequence of RecA, including the Mg^{2+} binding site, plays a significant role in the mutagenesis between *E. coli* and *P. aeruginosa* [43]. This domain is the area that determines its affinity for binding dsDNA [43]. The variation that has been noted in the C-terminal domain of RecA in *A. hydrophila* and *A. caviae* (Table 13), as well as the varied mutagenesis between species (Fig. 6 and Fig. 7), justify a need for further analysis of this mechanism. To further test our hypothesis of the SOS response playing a role in the mutagenesis of *Aeromonas*, gene knockouts and knock-ins can be performed with *recA*. Previously, Sarah Duhr

M.S. (University of Wisconsin-Milwaukee, Department of Biomedical Sciences) collaborated with Dr. Sonia Bardy (University of Wisconsin-Milwaukee, Department of Biological Sciences) to develop a suicide vector, which allowed for the removal of *recA* from *A. hydrophila* [62]. Sarah took the *recA* knock-out of this bacterium and performed the fluctuation assay, post-CIP exposure, which established that this gene significantly contributed to mutagenesis either directly or inderectly [62]. However, the *recA* still needs to be re-introduced into *A. hydrophila* to confirm that these mutagenic effects return. We can further analyze the effects of the SOS response by cloning in the *recA* from *A. caviae* into *A. hydrophila* and performing another fluctuation assay, assessing the mutagenic capabilities driven by this gene. We would hypothesize that *A. hydrophila* containing the *A. caviae recA* would stimulate an increased mutagenic effect after exposure to sub-inhibitory stressors, particularly that of TMP. However, the SOS response is not the only method by which microbes may undergo adaptive alterations, further supporting the need to analyze other mechanisms of survival, such as HGT and repair systems [24, 61].

Table 13. MegaX alignment of amino acid residues of RecA from *E.coli*, *P. aeruginosa*, *A. hydrophila*, and *A. caviae*. Solidly colored boxes indicate residual variation, whereas non-colored background indicates residual homology. (Table from Sarah Duhr, Skwor Lab [62])

We can also test additional stressing agents and clinically relevant microorganisms to understand their mutagenic responses. Specifically, we can test more WW samples, including post-

treated EWW, to compare pre- and post- treated WW on mutagenesis. Since we only observed a significant increase of mutagenesis in *A. caviae* to one sample of IWW, additional specimens within this classification should be examined. It is also important to assess WW samples from summer months (i.e., June and July), due to the elevated influx of contaminants compared to winter months (i.e., January and February). To further aid in understanding the effect of WW on accelerating bacterial mutagenesis, chemical analyses can be performed to identify the specific stressing compounds found within each sample. In this study, we only examined four antibiotic compounds found within WW; however, there are numerous other antimicrobials and chemicals found in this environment that should be analyzed in future work.

In conclusion, our study is the first to show that exposures to sub-inhibitory concentrations of filtered IWW, antimicrobial compounds, calcium hypochlorite, and UVL can increase mutagenesis in the emerging human pathogens *A. caviae* and *A. hydrophila*. Our findings correlate with previous research examining the effects that individual stressors have on accelerating mutational events among various microorganisms. While there are a variety of bacterial mechanisms that may be contributing to the increased mutagenic evolution among *A. caviae* and *A. hydrophila*, we hypothesize that the SOS response is playing a significant role due to previous work conducted by Sarah Duhr. Gene knockouts and knock-ins should be performed with the *recA* gene to further analyze if the SOS response is a driving force of bacterial mutagenesis. While WW treatment plants provide "clean water" to re-enter the environment and our homes, the continual presence of AMR populations, specifically of emerging pathogens, raises a major concern for public health. Additionally, identifying wastewater as a cause of mutations suggests that more research is needed to assess its role in accelerating the evolution of AMR and pathogenicity.

REFERENCES

1. Khan, Z.A., M.F. Siddiqui, and S. Park, *Current and Emerging Methods of Antibiotic Susceptibility Testing.* Diagnostics (Basel), 2019. **9**(2).
2. Organization, W.H. *Antimicrobial Resistance*. 2021 November 17th; Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
3. Larsson, D.G.J. and C.F. Flach, *Antibiotic resistance in the environment.* Nat Rev Microbiol, 2021.
4. Henderson-Begg, S.K., D.M. Livermore, and L.M. Hall, *Effect of subinhibitory concentrations of antibiotics on mutation frequency in Streptococcus pneumoniae.* J Antimicrob Chemother, 2006. **57**(5): p. 849-54.
5. Tong, C., et al., *Chlorine disinfectants promote microbial resistance in Pseudomonas sp.* Environ Res, 2021. **199**: p. 111296.
6. Laureti, L., I. Matic, and A. Gutierrez, *Bacterial Responses and Genome Instability Induced by Subinhibitory Concentrations of Antibiotics.* Antibiotics (Basel), 2013. **2**(1): p. 100-14.
7. Fung-Tomc, J., *Ciprofloxacin-Induced, Low-Level Resistance to Structurally Unrelated Antibiotics in Pseudomonas aeruginosa and Methicillin-Resistant Staphylococcus aureus.* Antimicrobial agents and Chemotherapy, 1993.
8. Walter, A.L., et al., *Assessment of antibiotic resistance from long-term bacterial exposure to antibiotics commonly used in fuel ethanol production.* World J Microbiol Biotechnol, 2019. **35**(4): p. 66.
9. (WHO), W.H.O. *Radiation: Ultraviolet (UV) radiation*. 2016; Available from: https://www.who.int/news-room/questions-and-answers/item/radiation-ultraviolet-(uv).

10. Fernandez-Bravo, A. and M.J. Figueras, *An Update on the Genus Aeromonas: Taxonomy, Epidemiology, and Pathogenicity.* Microorganisms, 2020. **8**(1).

11. Felis, E., et al., *Antimicrobial pharmaceuticals in the aquatic environment - occurrence and environmental implications.* Eur J Pharmacol, 2020. **866**: p. 172813.

12. Denamur, E. and I. Matic, *Evolution of mutation rates in bacteria.* Mol Microbiol, 2006. **60**(4): p. 820-7.

13. Guan, N., et al., *Microbial response to environmental stresses: from fundamental mechanisms to practical applications.* Appl Microbiol Biotechnol, 2017. **101**(10): p. 3991-4008.

14. Maslowska, K.H., K. Makiela-Dzbenska, and I.J. Fijalkowska, *The SOS system: A complex and tightly regulated response to DNA damage.* Environ Mol Mutagen, 2019. **60**(4): p. 368-384.

15. Ribeiro, A.R., B. Sures, and T.C. Schmidt, *Cephalosporin antibiotics in the aquatic environment: A critical review of occurrence, fate, ecotoxicity and removal technologies.* Environ Pollut, 2018. **241**: p. 1153-1166.

16. Zhang, S., et al., *Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes.* Chemosphere, 2015. **135**: p. 138-45.

17. Beattie, R.E., T. Skwor, and K.R. Hristova, *Survivor microbial populations in post-chlorinated wastewater are strongly associated with untreated hospital sewage and include ceftazidime and meropenem resistant populations.* Sci Total Environ, 2020. **740**: p. 140186.

18. District, M.M.S. *The four stages of cleaning wastewater*. 2022 [cited 2022.

19. Lin, X., et al., *Occurrence and risk assessment of emerging contaminants in a water reclamation and ecological reuse project.* Sci Total Environ, 2020. **744**: p. 140977.

20. Collivignarelli, M.C., et al., *Disinfection of Wastewater by UV-Based Treatment for Reuse in a Circular Economy Perspective. Where Are We at?* Int J Environ Res Public Health, 2020. **18**(1).

21. Reinthaler, F.F., et al., *Antibiotic resistance of E. coli in sewage and sludge.* Water Res, 2003. **37**(8): p. 1685-90.

22. Liedhegner, E., et al., *Similarities in Virulence and Extended Spectrum Beta-Lactamase Gene Profiles among Cefotaxime-Resistant Escherichia coli Wastewater and Clinical Isolates.* Antibiotics (Basel), 2022. **11**(2).

23. Jin, M., et al., *Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation.* ISME J, 2020. **14**(7): p. 1847-1856.

24. Bello-Lopez, J.M., et al., *Horizontal Gene Transfer and Its Association with Antibiotic Resistance in the Genus Aeromonas spp.* Microorganisms, 2019. **7**(9).

25. Zhang, S., et al., *Chlorine disinfection facilitates natural transformation through ROS-mediated oxidative stress.* ISME J, 2021. **15**(10): p. 2969-2985.

26. Macauley, J.J., et al., *Disinfection of swine wastewater using chlorine, ultraviolet light and ozone.* Water Res, 2006. **40**(10): p. 2017-26.

27. Bojar, B., et al., *Antibiotic resistance patterns of Escherichia coli isolates from the clinic through the wastewater pathway.* Int J Hyg Environ Health, 2021. **238**: p. 113863.

28. Resources, W.D.o.N., *Basic Disinfection Study Guide*. 2016: Madison, WI. p. 43.

29. Hamidin, N., Q.J. Yu, and D.W. Connell, *Human health risk assessment of chlorinated disinfection by-products in drinking water using a probabilistic approach.* Water Res, 2008. **42**(13): p. 3263-74.

30. Cordeiro-Stone, M., et al., *Analysis of DNA replication forks encountering a pyrimidine dimer in the template to the leading strand.* J Mol Biol, 1999. **289**(5): p. 1207-18.

31. Shibai, A., et al., *Mutation accumulation under UV radiation in Escherichia coli.* Sci Rep, 2017. **7**(1): p. 14531.

32. Charpentier, X., et al., *Antibiotics and UV radiation induce competence for natural transformation in Legionella pneumophila.* J Bacteriol, 2011. **193**(5): p. 1114-21.

33. Papajova, I., et al., *Effect of Wastewater Treatment on Bacterial Community, Antibiotic-Resistant Bacteria and Endoparasites.* Int J Environ Res Public Health, 2022. **19**(5).

34. Skwor, T., et al., *Aeromonas hydrophila and Aeromonas veronii predominate among potentially pathogenic ciprofloxacin- and tetracycline-resistant aeromonas isolates from Lake Erie.* Appl Environ Microbiol, 2014. **80**(3): p. 841-8.

35. Vila, J., et al., *Aeromonas spp. and traveler's diarrhea: clinical features and antimicrobial resistance.* Emerg Infect Dis, 2003. **9**(5): p. 552-5.

36. Kumar, S., et al., *Necrotizing fasciitis caused by Aeromonas caviae.* Avicenna J Med, 2012. **2**(4): p. 94-6.

37. Tsujimoto, Y., et al., *Necrotizing fasciitis and sepsis caused by Aeromonas hydrophila.* Infez Med, 2019. **27**(4): p. 429-435.

38. Piotrowska, M., Popowska, M., *The prevalence of antibiotic resistance genes among Aeromonas species in aquatic environments.* Ann Microbiol, 2014.

39. Foster, P.L., *Methods for determining spontaneous mutation rates.* Methods Enzymol, 2006. **409**: p. 195-213.

40. Renzette, N., et al., *Localization of RecA in Escherichia coli K-12 using RecA-GFP*. Mol Microbiol, 2005. **57**(4): p. 1074-85.

41. Resnick, D. and D.R. Nelson, *Cloning and characterization of the Aeromonas caviae recA gene and construction of an A. caviae recA mutant.* J Bacteriol, 1988. **170**(1): p. 48-55.

42. Cirz, R.T., et al., *Inhibition of mutation and combating the evolution of antibiotic resistance.* PLoS Biol, 2005. **3**(6): p. e176.

43. Del Val, E., et al., *RecA and DNA recombination: a review of molecular mechanisms.* Biochem Soc Trans, 2019. **47**(5): p. 1511-1531.

44. Islam, G.M. and K.A. Gilbride, *The effect of tetracycline on the structure of the bacterial community in a wastewater treatment system and its effects on nitrogen removal.* J Hazard Mater, 2019. **371**: p. 130-137.

45. Langbehn, R.K., C. Michels, and H.M. Soares, *Antibiotics in wastewater: From its occurrence to the biological removal by environmentally conscious technologies.* Environ Pollut, 2021. **275**: p. 116603.

46. Andrews, J.M., *Determination of minimum inhibitory concentrations.* J Antimicrob Chemother, 2001. **48 Suppl 1**: p. 5-16.

47. Ko, W.C., et al., *Increasing antibiotic resistance in clinical isolates of Aeromonas strains in Taiwan.* Antimicrob Agents Chemother, 1996. **40**(5): p. 1260-2.

48. Heyniger, N., *UV light emitting diodes (LED) for degradation of antibiotic resistant bacteria and genes*. 2022, Marquette University.

49. Krasovec, R., et al., *Measuring Microbial Mutation Rates with the Fluctuation Assay.* J Vis Exp, 2019(153).

50. B. baker, P.M., *Evaluation of resistance development of the novel antimicrobial MBN-101 against gram-positive and gram-negative pathogens.* ASM Microbes, 2019.

51. Bakhlanova, I.V., T. Ogawa, and V.A. Lanzov, *Recombinogenic activity of chimeric recA genes (Pseudomonas aeruginosa/Escherichia coli): a search for RecA protein regions responsible for this activity.* Genetics, 2001. **159**(1): p. 7-15.

52. Comber, S.D.W., M.J. Gardner, and B. Ellor, *Seasonal variation of contaminant concentrations in wastewater treatment works effluents and river waters.* Environ Technol, 2020. **41**(21): p. 2716-2730.

53. Yao, S., et al., *Occurrence and removal of antibiotics, antibiotic resistance genes, and bacterial communities in hospital wastewater.* Environ Sci Pollut Res Int, 2021. **28**(40): p. 57321-57333.

54. Birosova, L.B., Lucia) ; Lepesova, K (Lepesova, Kristina) ; Grabic, R (Grabic, Roman) ; Mackulak, T (Mackulak, Tomas), *Non-antimicrobial pharmaceuticals can affect the development of antibiotic resistance in hospital wastewater.* Web of Science.

55. Skwor, T.A., et al., *Photodynamic inactivation of methicillin-resistant Staphylococcus aureus and Escherichia coli: A metalloporphyrin comparison.* J Photochem Photobiol B, 2016. **165**: p. 51-57.

56. Alam, M.K., et al., *RecA Inhibitors Potentiate Antibiotic Activity and Block Evolution of Antibiotic Resistance.* Cell Chem Biol, 2016. **23**(3): p. 381-91.

57. Goldstein, B.P., *Resistance to rifampicin* Journal of antibiotics, 2014. **67**.

58. Skwor, T., et al., *Prevalence of Potentially Pathogenic Antibiotic-Resistant Aeromonas spp. in Treated Urban Wastewater Effluents versus Recipient Riverine Populations: a 3-Year Comparative Study.* Appl Environ Microbiol, 2020. **86**(3).

59. Bhatnagar, K. and A. Wong, *The mutational landscape of quinolone resistance in Escherichia coli.* PLoS One, 2019. **14**(11): p. e0224650.

60. Bakkeren, E., et al., *Impact of horizontal gene transfer on emergence and stability of cooperative virulence in Salmonella Typhimurium.* Nat Commun, 2022. **13**(1): p. 1939.

61. Jolivet-Gougeon, A., et al., *Bacterial hypermutation: clinical implications.* J Med Microbiol, 2011. **60**(Pt 5): p. 563-573.

62. Duhr, S., The effect of subinhibitory concentrations of ciprofloxacin on *recA* expression and mutation rates of *Aeromonas hydrophila*, in *College of Health Sciences*. 2021, University of Wisconsin - Milwaukee. p. 87.