공학석사 학위논문

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해수에서 염소를 활용한 에드워드시엘라 타르다와

비브리오 하베이의 불활성화

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조지윤의 석사 학위논문을 인준함

2021 년 1 월

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Abstract

Inactivation of *Edwardsiella tarda* and *Vibrio harveyi* in seawater by chlorination

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Edwardsiella tarda (*E. tarda*) and *Vibrio harveyi* (*V. harveyi*) are known as representative fish pathogenic bacteria that cause serious mortality among fish. Chlorination, which has been widely applied in the conventional disinfection process, can be employed to effectively control these bacteria. This study demonstrated the microbial inactivation kinetics of *E. tarda* and *V. harveyi* by free chlorine in seawater and deionized water. Experiments were performed by varying the initial concentration of free chlorine, the concentration of natural organic matter (NOM), temperature, and pH in order to quantify the disinfection efficacy of free chlorine. Both microorganisms showed similar inactivation tendencies, and disinfection efficacy decreased as NOM concentration increased or temperature decreased. In seawater, *Ct*

(Concentration-time product) values of free chlorine for achieving 1 log inactivation of *E. tarda* and *V. harveyi* at pH 7.1 were 0.0489 and 0.0551 mg·min/L, respectively. However, both *Ct* values increased nearly 3.5 times at pH 8.2 (0.1822 and 0.1984 mg·min/L, respectively). The difference in *Ct* values was due to the speciation of free chlorine which in turn, was affected by pH as well as the presence of bromide and ammonium in seawater.

Keywords: Chlorination, Disinfection, *Edwardsiella tarda*, *Vibrio harveyi*, Seawater, Inactivation kinetics, Chlorine speciation

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Chapter 1.Introduction

In recent years, research on pathogenic microorganisms that cause fish infectious diseases has been actively conducted with the rapid development of the global aquaculture industry (Toranzo et al. 2005; Austin and Zhang, 2006). Among these microorganisms, *Edwardsiella tarda* (*E. tarda*) and *Vibrio harveyi* (*V. harveyi*) cause a variety of diseases in fish, including discoloration of the body, eye lesions, gastroenteritis, and hemorrhage in fin and skin (Egusa, 1976; Miyazaki and Kaige, 1985; Lee et al., 2002; Park et al., 2012; Mohamad et al., 2019). As such, they have been previously reported as representative fish infectious bacteria in many studies.

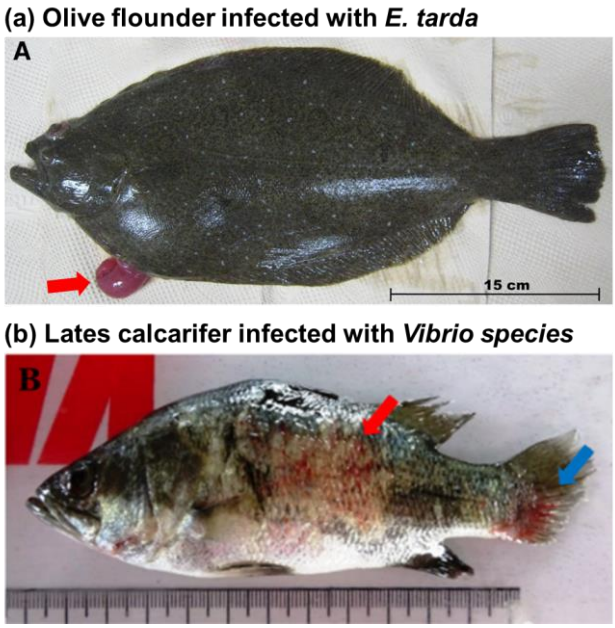

Figure. 1.1. Fishes infected with (a) *E. tarda* and (b) *Vibrio species*. (Park et al., 2012; Mohamad et al., 2019)

Fish infections generally lead to mass mortality and can result in economic losses to the fisheries industry. Every year, several million USD worth of economic losses occur in fish and shrimp farms (Zorriehzahra and Banaederakhshan, 2015). Especially in Korea, aquaculture of olive flounder, which accounts for 56.5% of the total fisheries production, suffers enormous losses due to infection by *E. tarda* (Park et al., 2012). In addition, predators can also be infected and damaged by infected fish, and even cases of human infection have been reported (Jordan and Hadley, 1969; Toranzo et al. 2005; Mohamad et al., 2019). To mitigate the damage caused by fish infection, disinfection techniques to control the pathogenic bacteria must be applied.

Chemical disinfection is one of the most effective methods for microbial control, and chlorination is widely used for microbial disinfection. Effective microbial control can be expected by using chlorine to attack components of the inner cell (Kouame and Haas, 1991; Shin and Sobsey, 2008; Cho et al., 2010). Previous studies have already reported that free chlorine is an effective disinfectant for the inactivation of *E. tarda* and *V. harveyi* (Kim et al., 2008; Mainous and Smith, 2010; Tanaka et al., 2013). However, the haphazard use of disinfectants can lead to higher operating costs, as well as the formation of harmful disinfection by-products (DBPs), especially in seawater (e.g., trihalomethanes, haloacetic acids, etc; Kim et al., 2015; Deborde and von Gunten, 2008). For this reason, it is important to optimize both disinfection time and initial concentration of disinfectant.

Generally, the microbial inactivation kinetics of disinfectants are reported as *Ct* (Concentration-time product) values (Thurston-Enriquez et al., 2003; Shin and Sobsey, 2008; Cho et al., 2010; Kim et al., 2019). However, no studies have previously reported the *Ct* values of free chlorine for *E. tarda* and *V. harveyi* inactivation in seawater. Inactivation information in seawater is important because these microorganisms can have a huge impact on aquaculture systems. Thus, it is necessary to derive *Ct* values of free chlorine for both microorganisms in seawater.

Additionally, in seawater, the inactivation efficacy of free chlorine can be affected by various water conditions such as natural organic matter (NOM), temperature, and pH (Lavonen et al., 2013; Butterfield et al., 1943; Fair et al., 1948). In particular, the speciation of free chlorine, which significantly affects disinfection efficacy, can change depending on water conditions. (Bousher et al., 1986; Sugam and Helz, 1981; Abarnou and Miossec, 1992; Heeb et al., 2014). Therefore, the influencing factors in chlorination should also be assessed.

This study investigated the microbial inactivation kinetics of *E. tarda* and *V. harveyi* by free chlorine and assessed the influencing factors. The inactivation efficacy of each microorganism and total residual oxidant (TRO) decomposition were examined by varying the concentration of initial free chlorine, the concentration of NOM, temperature, and pH in seawater and deionized (DI) water conditions. To identify the dominant disinfectant species in a certain pH, a chemical kinetic model was developed. The microbial inactivation efficacy of the identified dominant disinfectant species at each condition was compared to evaluate the difference in inactivation efficacy between oxidant species. The Chick-Watson model was applied to derive *Ct* values of free chlorine for *E. tarda* and *V. harveyi* inactivation.

Chapter 2. Materials and Methods

2.1. Reagents

All chemicals were of reagent grade and were used without further purification. Tryptic soy broth (TSB), tryptic soy agar (TSA) (Becton Dickinson), sodium hypochlorite (NaOCl), sodium chloride (NaCl), sodium bromide (NaBr), ammonium chloride (NH_4Cl) sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), methanesulfonic acid (MSA), perchloric acid ($HClO_4$), humic acid and sodium sulfite (Na_2SO_3) (Sigma-Aldrich) were used in this study. All solutions were prepared using DI water (>18.2 MΩ cm, Milli-Q Integral Water Purification System, Millipore), and stored at 4˚C. All glassware was washed with DI water, and sterilized prior to use by autoclaving at 121˚C for 15 minutes.

2.2. Seawater samples and water quality parameters

The seawater samples were collected from the coast of Busan, Korea. The seawater was filtered with a 0.45 μm PTFE membrane filter (Advantec) after sampling and stored at 4°C. The water quality parameters of the seawater samples can be found in Table 2.1. pH of samples was measured using a pH meter (Orion Star A326, Thermo Scientific). Conductivity was measured using a conductivity meter (LAQUA F-74BW, Horiba) and turbidity was measured using a turbidity meter (Turb 430 IR, WTW). Alkalinity was analyzed using APHA Method 2320. Total organic carbon (TOC) analyzer (Sievers InnovOx, Suez) was employed for TOC measurement and UV absorbance at 254 nm was detected by UV/Vis spectrophotometer (LAMBDA 465, PerkinElmer). The concentrations of anion and cation excluding ammonium were quantified by ion chromatography (Dionex Aquion, Thermo Scientific). The chromatographic separation was performed on an anionic column (4 mm × 250 mm, Dionex IonPac AS22, Thermo Scientific) using a mixed carbonate solution as eluent (4.5 mM Na_2CO_3 and 1.4 mM $NaHCO_3$), and a cationic column (4 mm × 250 mm, Dionex IonPac CS12A, Thermo Scientific) using 20 mM MSA solution as eluent. Indophenol method was applied to detect the low concentration of ammonium ion (Ma et al., 2018). Artificial seawater was prepared by adding 38,000 mg/L NaCl, 61 mg/L Br^- (NaBr), and 1mg/L NH_4^+ (NH_4Cl) to DI water.

Table 2.1. Water quality parameters of Busan coastal water.

Parameter		Value
pH		8.2
Conductivity (mS/cm)		48.10
Turbidity (NTU)		0.04
Alkalinity (mg/L as $CaCO_3$)		107.1
TOC (mg/L)		3.81
A_{254} (cm^{-1})		0.009
Anion (mg/L)	Cl	18,302.0
	SO_4^{2-}	1,918.0
	Br^-	61.1
Cation (mg/L)	Na^+	11,110.0
	K^+	449.2
	Mg^{2+}	1,333.1
	Ca^{2+}	361.8
	NH_4^+	1.0

2.3. Culture and analysis of microorganisms

The *E. tarda* (ATCC 23712) and *V. harveyi* (ATCC BAA-1116) were inoculated in 30 mL of medium (TSB + 2% NaCl) and grown at 25˚C for 18-24 hours. The cells were harvested by centrifugation at 3000 g (rcf) for 15 minutes and were washed at least 3 times with 30 mL phosphate buffer solution (PBS, 10 mM, pH 7.2). The bacteria stock suspensions (~10^9 CFU/mL) were prepared by resuspending the cells in 20 mL PBS and were stored at 4˚C until use. The spread plate method was used to determine the cell population in the samples (Figure 2.1). After spreading, the plates were grown in an incubator at 25˚C for 18-24 hours, and then the numbers of cell colonies were counted. The average number of colonies of three plates were used to measure the microorganism population and analysis was carried out in duplicate.

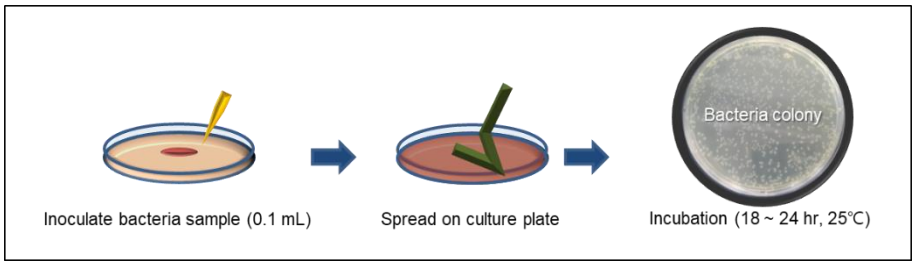

Figure 2.1. Spread plate method for analysis of microorganisms.

2.4. Inactivation experiment

All experiments were performed using sterilized 60 mL Pyrex flasks with 50 mL of reaction solution open to the atmosphere under vigorous stirring (600 rpm). To control the temperature, either a probe-type chiller (TC45E-F, Huber Co.) or water bath heater was used. The pH of seawater was adjusted with $HClO_4$ and the pH of artificial seawater and DI water was adjusted with phosphate buffer. Humic acid was used as a NOM. Microbial inactivation was initiated by adding free chlorine into the reactor. Samples (1 mL) were withdrawn at a pre-determined time, immediately mixed with sodium sulfate, and serially diluted with PBS to quench the reaction. At least duplicate experiments were conducted, and the average values with the standard deviations were presented.

2.5. Chlorine/bromine species and total residual oxidant

To determine the dominant oxidants in seawater, stock solutions of chlorine/bromine species (hypochlorous acid (HOCl), hypochlorite (OCl^-), monochloramine (NH_2Cl), hypobromous acid (HOBr), and monobromamine (NH_2Br)) were prepared in DI water. The conditions for preparing stock solutions for each oxidant species can be found in Table 2.2.

Table 2.2. Conditions for preparing stock solutions of chlorine/bromine species.

Species	pH	Reagent
HOCl	7.1	1.4 mM NaOCl
OCl^-	8.2	1.4 mM NaOCl
NH_2Cl	8.2	1.4 mM NaOCl + 6 mM NH_4Cl
HOBr	7.1	1.4 mM NaOCl + 76.5 mM NaBr
NH_2Br	7.1	1.4 mM HOBr + 6 mM NH_4Cl

* pH was controlled by phosphate buffer

A UV/Vis spectrophotometer was used to identify these chlorine/bromine species. TRO concentration was determined by the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (Figure 2.2). The UV absorbance of the TRO sample was detected at 515 nm.

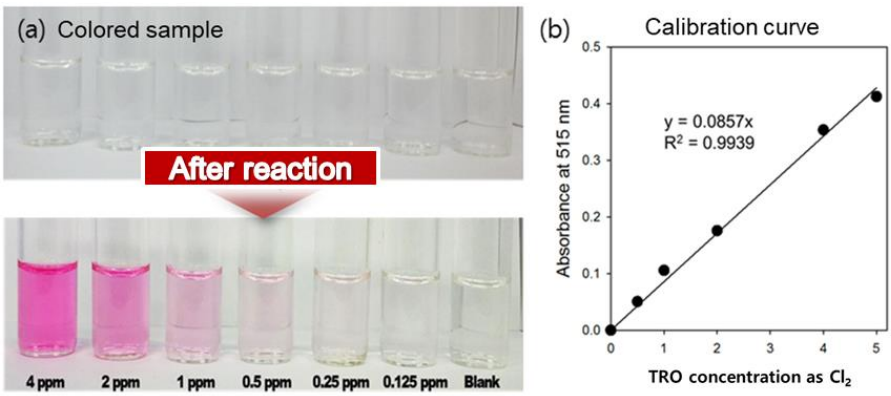

Figure 2.2. (a) Colored sample and (b) calibration curve of DPD colorimetric method.

2.6. Kinetic modeling

2.6.1 Chemical reaction model

To predict the dominant oxidant in seawater, the chemical reaction model was calculated by using COMSOL Multiphysics software (version 5.5). The initial concentration of the compounds, pKa values, and the reaction rate constants of each reaction were entered into COMSOL Multiphysics to create a chemical reaction model.

2.6.2. Chick-Watson model

The Chick-Watson model (Equation 1) was applied to determine the microbial inactivation kinetics.

$$\mathrm{Log}(\mathrm{N}/\mathrm{N}_0) = kCt \quad (1)$$

where N and N_0 represent the numbers of viable cells (CFU/mL) at times *t* and 0, respectively, *k* represents the inactivation rate constant (L/mg·min), *C* represents the concentration of disinfectant (mg/L), and *t* represents the reaction time (min).

Chapter 3. Results and Discussion

3.1. Influencing factors

3.1.1. Effects of free chlorine dose, NOM concentration, and temperature

Effect of free chlorine dose. The effect of free chlorine dose on *E. tarda* and *V. harveyi* inactivation was examined at different initial free chlorine concentrations of 1.0-2.0 mg/L in seawater (Figure 3.1). As the initial concentration of free chlorine increased, the inactivation efficacy also increased for both microorganisms, which showed similar inactivation tendencies. When 1.0 mg/L free chlorine was injected, *E. tarda* and *V. harveyi* were inactivated about 2.5 log in 20 seconds; both showed almost 4 log inactivation when injecting 2.0 mg/L free chlorine.

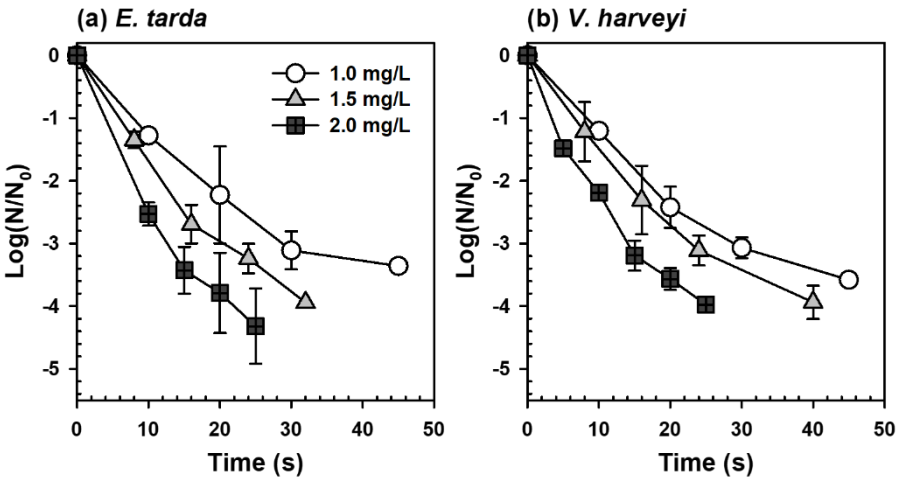

Figure 3.1. Effect of free chlorine dose on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in seawater ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, Temperature = 25°C).

Figure 3.2 shows the effect of free chlorine dose on *E. tarda* and *V. harveyi* efficacy in DI water. The conditions of initial free chlorine dose are 0.1-0.3 mg/L. Since DI water does not contain ions or organic matter, a similar degree of inactivation was possible even when the free chlorine dose was 10 times lower than seawater conditions. Both microorganisms showed less than 1 log inactivation in 20 seconds at 0.1 mg/L, and 4 log inactivation at 0.3 mg/L. As in seawater, both microorganisms showed a similar inactivation tendency, and the inactivation efficiency increased as the injected chlorine concentration also increased.

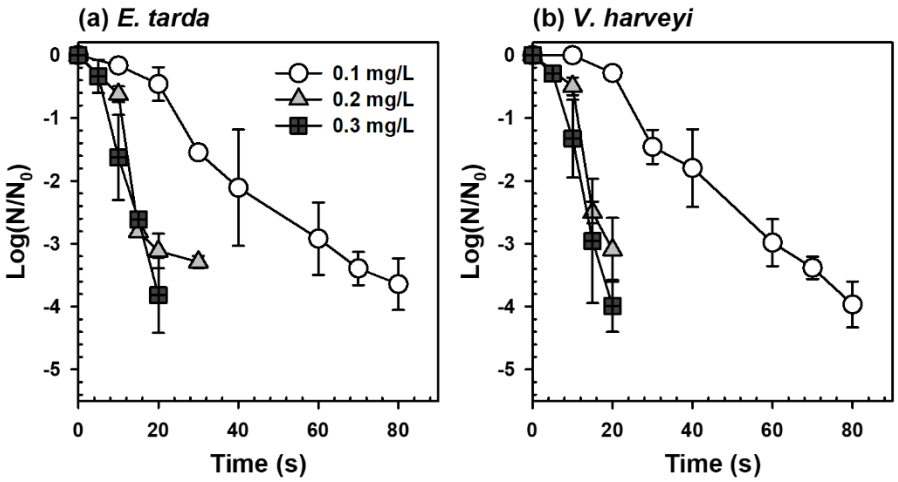

Figure 3.2. Effect of free chlorine dose on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Phosphate buffer] = 10 mM, pH = 8.2, Temperature = 25°C).

At each free chlorine dose, TRO decomposition was measured (Figure 3.3). In seawater, the reaction time was short due to a high concentration of initial free chlorine, so TRO decomposition hardly occurred. While TRO decomposition seems to occur in DI water. It should be pointed out that the free chlorine concentration in DI water is much lower than that of seawater conditions. The decay constant of free chlorine becomes large as the initial dose decreases (Hua et al., 1999). Also, as the chlorine concentration is lower, a longer reaction time is required for microbial inactivation. Therefore, in DI water, TRO decomposition occurs due to low initial free chlorine concentration and long reaction time.

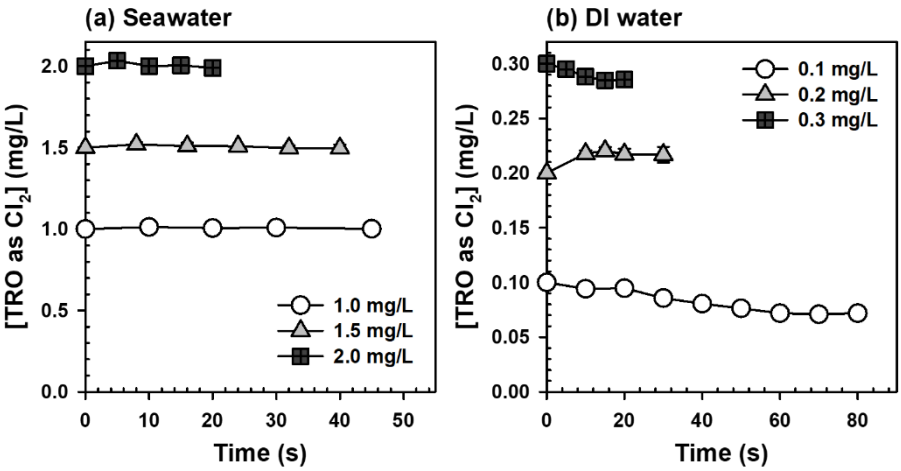

Figure 3.3. Effect of free chlorine dose on TRO decomposition in (a) seawater and (b) DI water ([*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, Temperature = 25°C, [Phosphate buffer] = 10 mM for (b)).

Effect of NOM concentration. Figure 3.4 presents the effect of NOM on microbial inactivation in seawater. In all conditions, 1 mg/L of free chlorine was injected, but the disinfection efficacy decreased as NOM concentration increased. In the absence of NOM, both microorganisms showed higher than 3 log inactivation in 25 seconds, but only about 1 log of microbial inactivation was observed when 1.0-2.0 mg/L NOM was added.

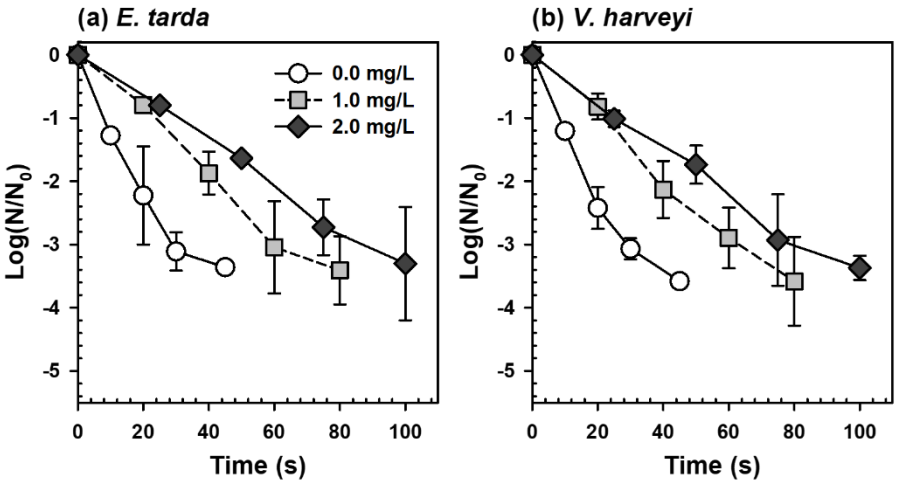

Figure 3.4. Effect of NOM on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in seawater ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, [Free chlorine]$_0$ = 1.0 mg/L, Temperature = 25°C).

Disinfection tendency in DI water was similar with seawater (Figure 3.5). Inactivation efficacy of *E. tarda* and *V. harveyi* decreased with increasing NOM concentration. About 4 log and 2 log microbial inactivation were exhibited at 20 seconds in the presence of 0.0 mg/L and 1.0 mg/L of NOM, respectively. However, unlike seawater, the tail phase was observed after 20 seconds when NOM was added.

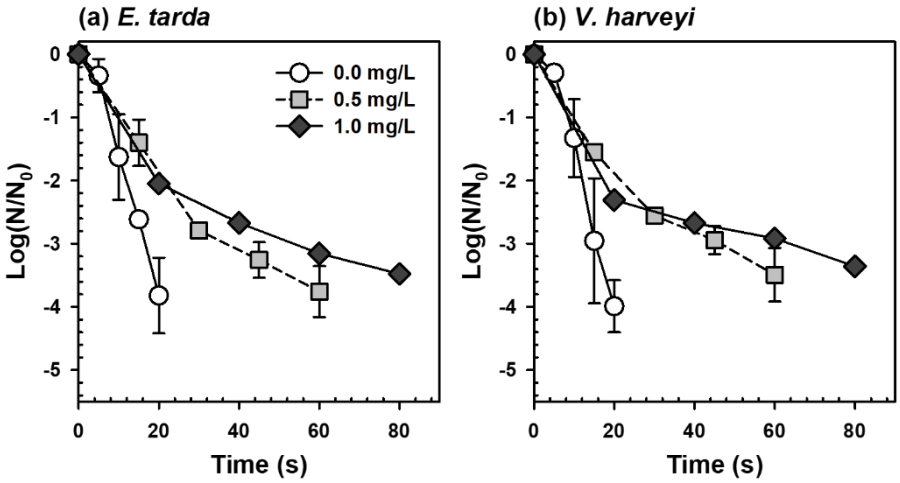

Figure 3.5. Effect of NOM on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Phosphate buffer] = 10 mM, pH = 8.2, [Free chlorine]$_0$ = 0.3 mg/L, Temperature = 25°C).

The effect of NOM on TRO decomposition is shown in Figure 3.6. In both seawater and DI water, TRO degradation was accelerated as NOM concentration increased. In the presence of 1.0 mg/L NOM, 3% of TRO decomposed in seawater, and 20% of TRO decomposed in DI water at 40 seconds. The tail phase of the DI water condition can be explained by the deterioration of disinfection efficacy due to the acceleration of TRO decomposition. NOM tends to react with disinfectants to form DBPs and inhibit disinfection efficacy (Lavonen et al., 2013; Lee et al., 2015). Thus, a higher NOM concentration leads to an increase in TRO decomposition, lowing disinfection efficacy.

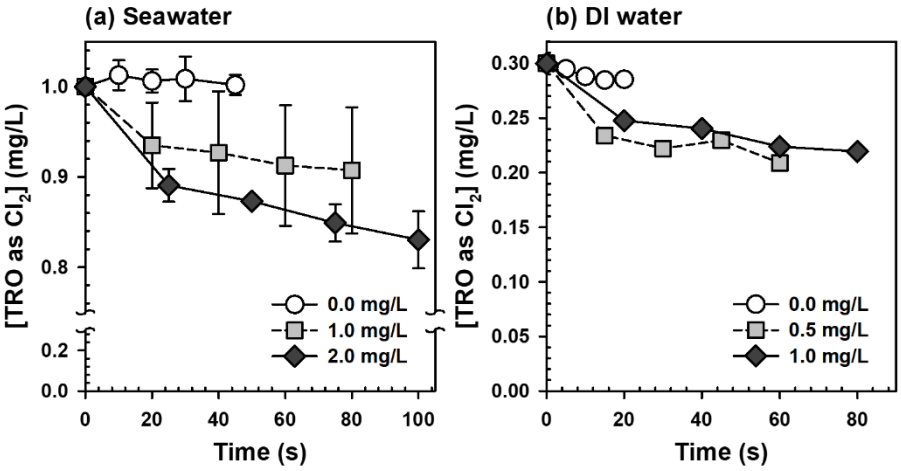

Figure 3.6. Effect of NOM on TRO decomposition in (a) seawater and (b) DI water ([*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, Temperature = 25°C, [Free chlorine]$_0$ = 1.0 mg/L for (a), 0.3 mg/L for (b), [Phosphate buffer] = 10 mM for (b)).

Effect of temperature. To investigate the temperature dependency in seawater, microbial inactivation was examined with varying temperatures from 4°C to 33°C (Figure 3.7). There was no significant difference at 4°C and 25°C but slightly higher disinfection efficacy was observed at 33°C. Both microorganisms exhibited about 3 log inactivation at 4°C and 25°C, but about 4 log inactivation at 33°C at 30 seconds.

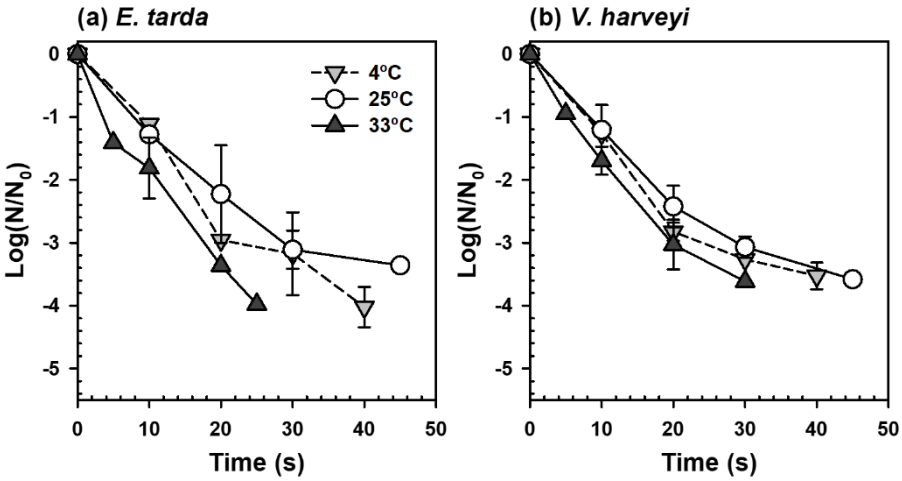

Figure 3.7. Effect of temperature on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in seawater ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, [Free chlorine]$_0$ = 1.0 mg/L).

The effect of temperature was examined under the same temperature range in DI water. As shown in Figure 3.8, two microorganisms showed very similar inactivation trends, and the disinfection efficiency at 25°C and 33°C was also not that different. The data exhibited an initial lag phase (shoulder) at 4°C. It seems that the reaction rate between the microorganisms and the disinfectant decreases due to the low temperature, resulting in a lag phase at the beginning. As mentioned earlier, DI water has no ions or organic matter, so the effect of temperature appears to have a higher impact than in seawater.

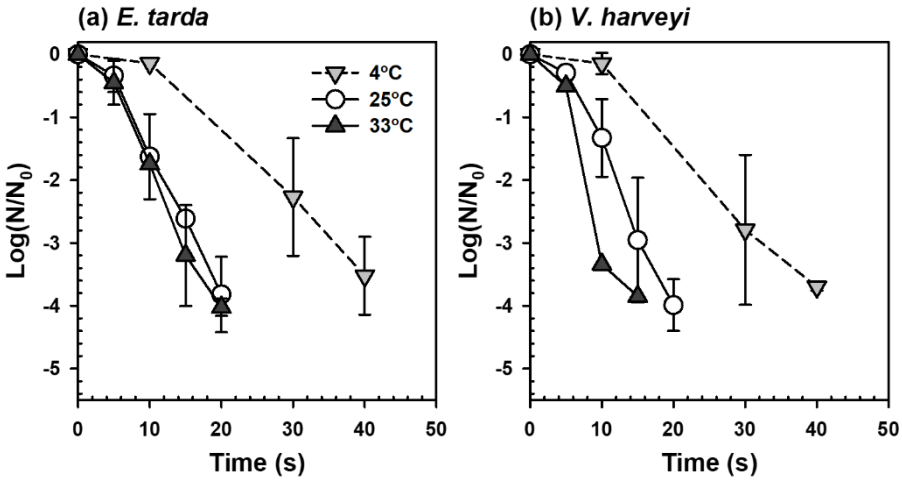

Figure 3.8. Effect of temperature on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Phosphate buffer] = 10 mM, pH = 8.2, [Free chlorine]$_0$ = 0.3 mg/L).

Figure 3.9 shows the effect of temperature on TRO decomposition. There is no significant change at 4°C and 25°C, but it can be seen that TRO decomposition accelerates at 33°C in both seawater and DI water. As the temperature increases, the decomposition of TRO is accelerated (Hua et al., 1999). However, the rate of microbial inactivation is also accelerated (Butterfield et al., 1943), so even if the TRO concentration decreases, the inactivation efficacy still increases at a higher temperature.

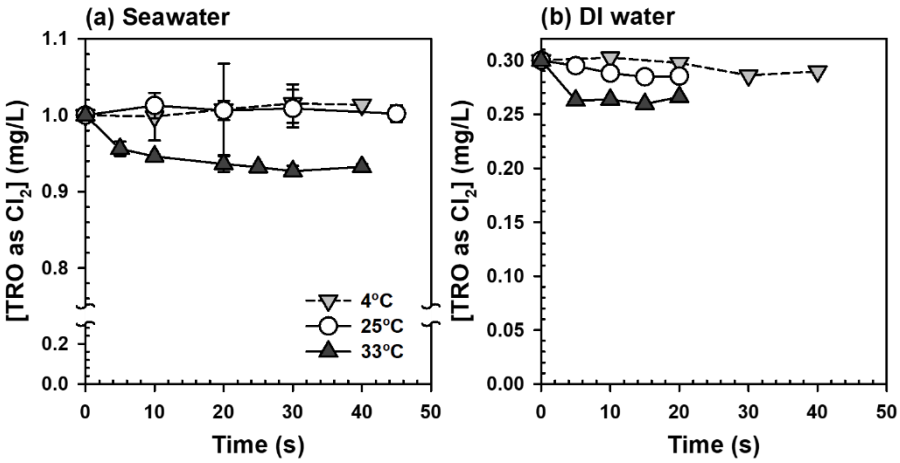

Figure 3.9. Effect of temperature on TRO decomposition in (a) seawater and (b) DI water ([*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 8.2, [Free chlorine]$_0$ = 1.0 mg/L for (a), 0.3 mg/L for (b), [Phosphate buffer] = 10 mM for (b)).

3.1.2. Effect of pH

The effect of pH on *E. tarda* and *V. harveyi* inactivation efficacy was examined in seawater (Figure 3.10). The pH range of seawater is generally between 7.5 and 8.4, but experiments in this study were performed at pH 7.1 and 8.2 to see a clear inactivation efficacy difference. For the same initial free chlorine dose (0.5 g/L), the inactivation efficacy was much higher at pH 7.1 than 8.2. At pH 7.1, 4 log inactivation occurred within 30 seconds, but at pH 8.2, the inactivation efficacy was less than 2 log even after 120 seconds.

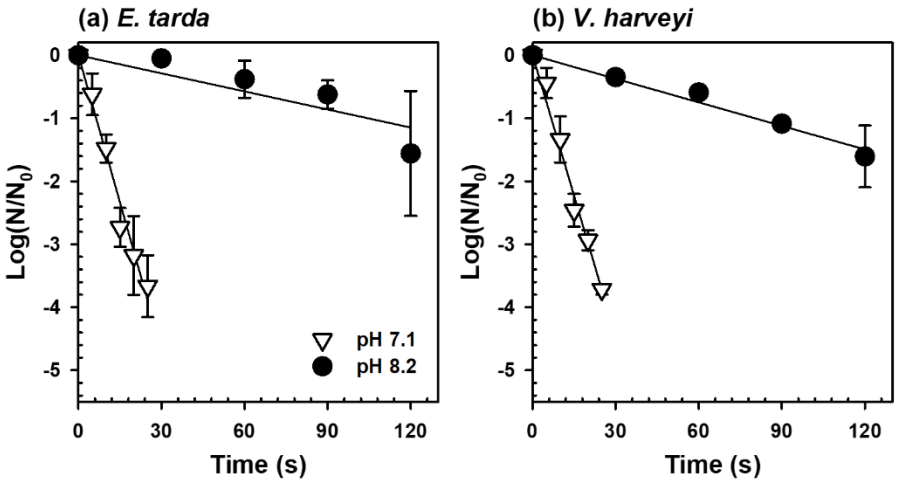

Figure. 3.10. Effect of pH on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in seawater ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Free chlorine]$_0$ = 0.5 mg/L, Temperature = 25°C).

Figure 3.11 shows the effect of pH in DI water. The initial free chlorine dose was 0.1 mg/L. In DI water, speciation of free chlorine changes based on the pKa value (pH 7.54; Morris, 1966). Thus, at pH 7.1, the dominant species of TRO is most likely to be HOCl (73 %) due to the protonation of OCl^-. In the case of pH 8.2, the dominant species of TRO becomes OCl^- (82 %). The difference in disinfection efficacy shows that HOCl is a stronger disinfectant compared to OCl^-, as Fair et al. (1948) demonstrated.

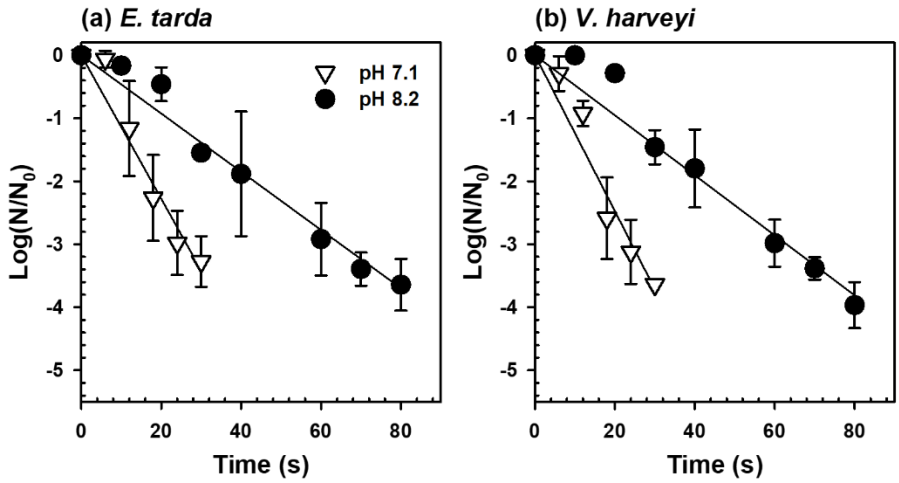

Figure. 3.11. Effect of pH on (a) *E. tarda* and (b) *V. harveyi* inactivation efficacy in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Free chlorine]$_0$ = 0.1 mg/L, [Phosphate buffer] = 10 mM, Temperature = 25°C).

Figure 3.12 presents the effect of pH on TRO decomposition. In seawater, TRO was decomposed up to 10% at both pH 7.1 and 8.2. Although TRO did not decompose sharply, its inactivation efficacy was much lower at pH 8.2. It can be inferred that the dominant TRO species at pH 8.2 has a weaker oxidation power.

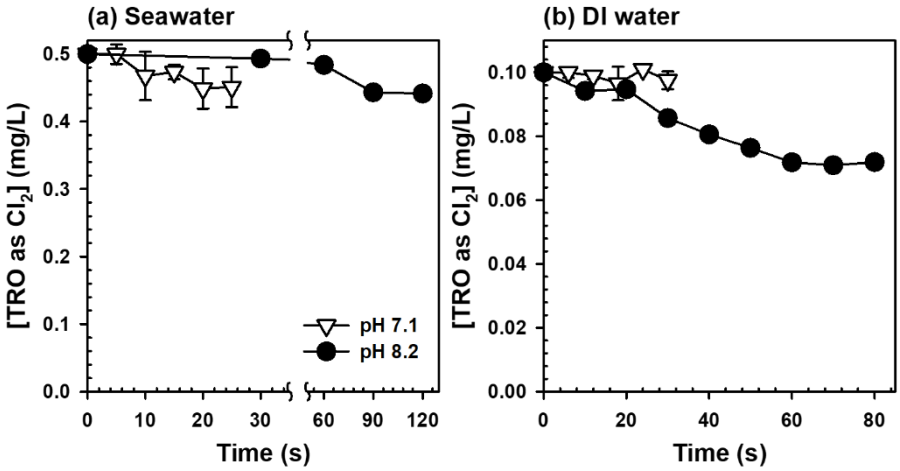

Figure. 3.12. Effect of pH on TRO decomposition in seawater and DI water ([*V. harveyi*]$_0$ = 10^7 CFU/mL, [Free chlorine]$_0$ = 0.5 mg/L for (a), 0.1 mg/L for (b), [Phosphate buffer] = 10 mM for (b), Temperature = 25°C).

3.2. Determination of dominant disinfectant at each pH

3.2.1. Chemical reaction modeling

In seawater, chlorine species other than HOCl/OCl^- may be the dominant disinfectant due to the influence of ions or other matters. It is well known that in seawater, free chlorine mainly reacts with bromide (Br^-) and ammonium (NH_3/NH_4^+) to generate HOBr, NH_2Br, NH_2Cl, and other species (Bousher et al., 1986; Sugam and Helz, 1981; Abarnou and Miossec, 1992). Within a few seconds, the free chlorine in seawater will transform into various chlorine/bromine species (HOCl, OCl^-, HOBr, NH_2Cl, NH_2Br, hypobromite (OBr^-), and bromochloramine (NHBrCl)). Therefore, in this study, the chemical reaction model between chlorine, bromine, and ammonium species was created to determine the major oxidant species at pH 7.1 and 8.2 in seawater. In this model, the initial doses of Br^-, NH_4^+ and, free chlorine were set to 61 mg/L, 1 mg/L, and 1 mg/L (≈14uM), respectively. The initial concentrations of other chlorine/bromine species were set to zero. The pKa values used in the model were summarized in Table 3.1.

Table 3.1. Dissociation constants of hypochlorous acid, hypobromous acid, and ammonium ion.

Compounds	pKa (25°C)	Ionic strength (M)	Reference
$HOCl/OCl^-$	7.54	–	Morris, 1966
$HOBr/OBr^-$	8.80	0.50	Troy and Margerum, 1991
NH_4^+/NH_3	9.25	–	Emerson et al., 1975

The reaction rate constants of chlorine/bromine species that can be generated were summarized in Table 3.2. To calculate the reaction constant of the reactions between $HOBr/OBr^-$ and NH_3 at 25°C, the Arrhenius equation (Equation 2) was applied.

$$k = A \cdot exp(-E_a/RT) \quad (2)$$

where k represents the rate constant, A represents the pre-exponential factor, E_a represents the activation energy, R represents the universal gas constant and T represents the temperature. The pre-exponential factors for these reactions are 4.7×10^{10} (HOBr) and 3.2×10^{13} $M^{-1} \cdot s^{-1}$ (OBr^-), and the activation energies are 15.7 (HOBr) and 48.4 kJ/mol (OBr^-) (Wajon and Morris, 1982). For the activity coefficient of all reactions, the Davies equation (Equation 3) was employed to compensate for the ionic strength in seawater.

$$\mathrm{Log}\,\gamma = -0.5z^2 \cdot (\sqrt{I}/(1+\sqrt{I}) - 0.2I) \quad (3)$$

where γ represents the activity coefficient, I represents the ionic strength, and z represents the charge of ion (Stumm and Morgan, 1995). It should be noted that the Davies equation does not fit perfectly in seawater with 0.72 M ionic strength

since the Davies equation is recommended for waters with less than 0.5 M ionic strength. However, the equation was applied because it does not deviate significantly from the recommended value and other methods are quite complicated and beyond the scope of this work. Thus, all pKa values and reaction rate constants were corrected by the Davies equation, and the model was developed using COMSOL Multiphysics software.

The kinetic modeling results are shown in Figure. 3.13. The results show distinct speciation at each pH condition. At pH 7.1, disinfectants containing bromide (e.g., HOBr, NH_2Br and NHBrCl) were predominantly produced while NH_2Cl was mainly generated at pH 8.2. At 10 seconds, the species with the highest ratio at pH 7.1 and 8.2 were HOBr (39%) and NH_2Cl (82%), respectively. Theoretically, at pH 7.1, HOCl is the major disinfectant, and since most of the ammonium is present as NH_4^+ (99%) which has low reactivity with HOCl (Qiang and Adams, 2004), HOCl reacts rapidly with Br^- to produce HOBr. However, at pH 8.2, OCl^- is the dominant oxidant, and NH_2Cl is produced predominantly since OCl^- has a much higher reactivity with NH_4^+ compare to Br^-. This is clearly demonstrated through the kinetic model.

Table 3.2. Rate constants for the reaction of chlorine/bromine species.

Reactions	k_f (M^{-1} s^{-1})	k_r (s^{-1})	Ionic strength (M)	Temperature (°C)	Reference
$HOCl + NH_3 \leftrightarrow NH_2Cl + H_2O$	3.07×10^{6}	2×10^{-5} †	0.075	25	Qiang and Adams, 2004
$OCl^- + NH_4^+ \rightarrow NH_2Cl + H_2O$	5.93×10^{4}	–	0.075	25	Qiang and Adams, 2004
$HOCl + Br^- \rightarrow HOBr + Cl^-$	1.55×10^{3}	–	0.50	25	Kumar and Margerum, 1987
$OCl^- + Br^- \rightarrow OBr^- + Cl^-$	0.90×10^{-3}	–	0.50	25	Kumar and Margerum, 1987
$HOCl + NH_2Cl \leftrightarrow NHCl_2 + H_2O$	3.5×10^{2}	5×10^{-9} †	–	–	Morris and Isaac, 1983
$HOBr + NH_3 \leftrightarrow NH_2Br + H_2O$	7.5×10^{7}	4×10^{-2} †	–	20	Wajon and Morris, 1982
$OBr^- + NH_3 \rightarrow NH_2Br + OH^-$	7.6×10^{4}	–	–	20	Wajon and Morris, 1982
$HOBr + NH_2Cl \leftrightarrow NHBrCl + H_2O$	2.86×10^{5}	5×10^{-5} †	0.50	25	Gazda and Margerum, 1994
$OBr^- + NH_2Cl \rightarrow NHBrCl + OH^-$	2.20×10^{4}	–	0.50	25	Gazda and Margerum, 1994

† Taken from Trogolo and Arey, 2017

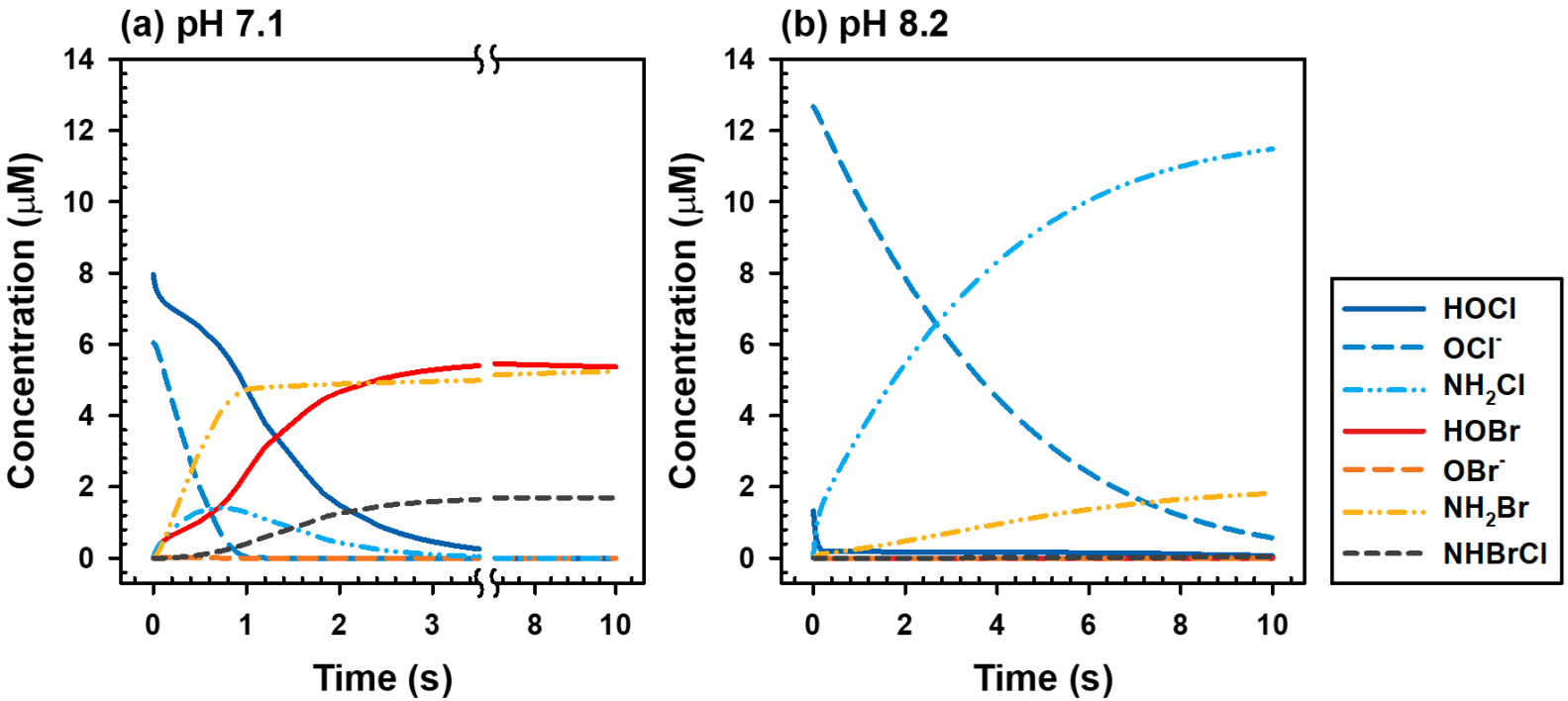

Figure. 3.13. Kinetic modeling results at (a) pH 7.1 and (b) pH 8.2 ([Free chlorine]$_0$ = 1 mg/L, [NH_4^+]$_0$ = 1 mg/L, [Br^-]$_0$ = 61 mg/L, Temperature = 25°C, Reaction time = 10 sec).

3.2.2. UV/Vis absorption spectra

To validate previous kinetic modeling results, several experiments were conducted to confirm the dominant disinfectant at each pH. UV/Vis absorption spectra of chlorine/bromine species were measured using stock solution (Table 3.3 and Figure 3.14). The peaks of chlorine/bromine species were found to coincide with the literature values of Soulard et al. (1981).

Table 3.3. Characteristics of absorption spectra of chlorine/bromine species.

Species	λ_{max} (nm)	ε (M^{-1} cm^{-1})
HOCl	233	97
HOBr	260	160
OCl^-	292	350
OBr^-	329	345
NH_2Cl	244	457
NH_2Br	278	380

* Taken from Soulard et al., 1981

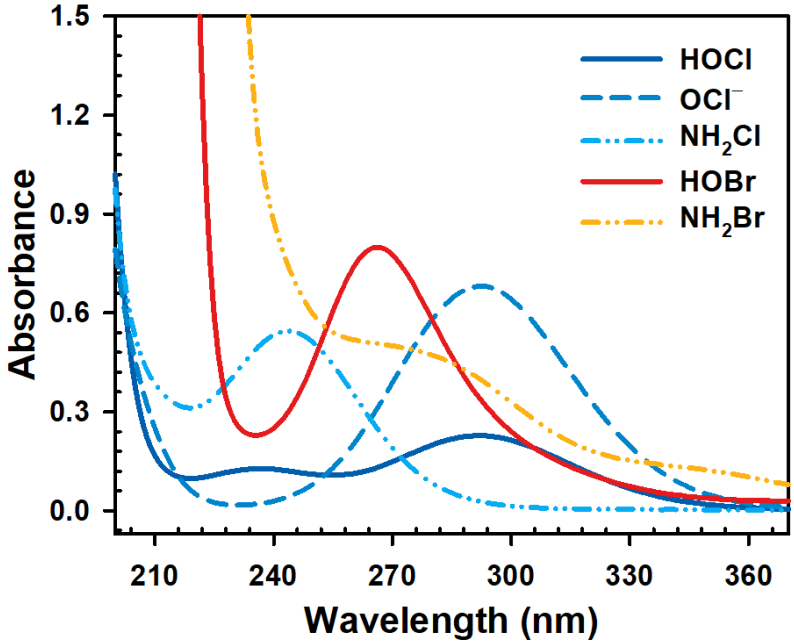

Figure. 3.14. UV/Vis absorption spectra of chlorine/bromine species in DI water ([Oxidant]$_0$ = 100 mg/L, [Phosphate buffer] = 10 mM, Cell path length = 1 cm, Temperature = 25°C (4 times dilution for HOBr, 200 mg/L concentration and 10 cm cell path length for NH_2Br)).

Based on the spectra, absorption spectra were analyzed to find the major disinfectant after injecting free chlorine into seawater. However, it was difficult to observe a significant peak for each species due to a low initial concentration of free chlorine (1 mg/L) and severe absorption interference from seawater. Thus, artificial seawater was used to overcome the aforementioned problems. Nevertheless, even in artificial seawater, it was difficult to observe the peak of 1 mg/L free chlorine, so the absorbance was confirmed 10 seconds after an injection of 5 mg/L free chlorine.

The results are shown in Figure 3.15. A peak was observed around 260 nm at pH 7.1, indicating that HOBr was a major oxidant. At pH 8.2, a peak around 244 nm confirmed the formation of NH_2Cl. Therefore, that the dominant disinfectants at pH 7.1 and 8.2 were HOBr and NH_2Cl, respectively, and this is consistent with the kinetic modeling results.

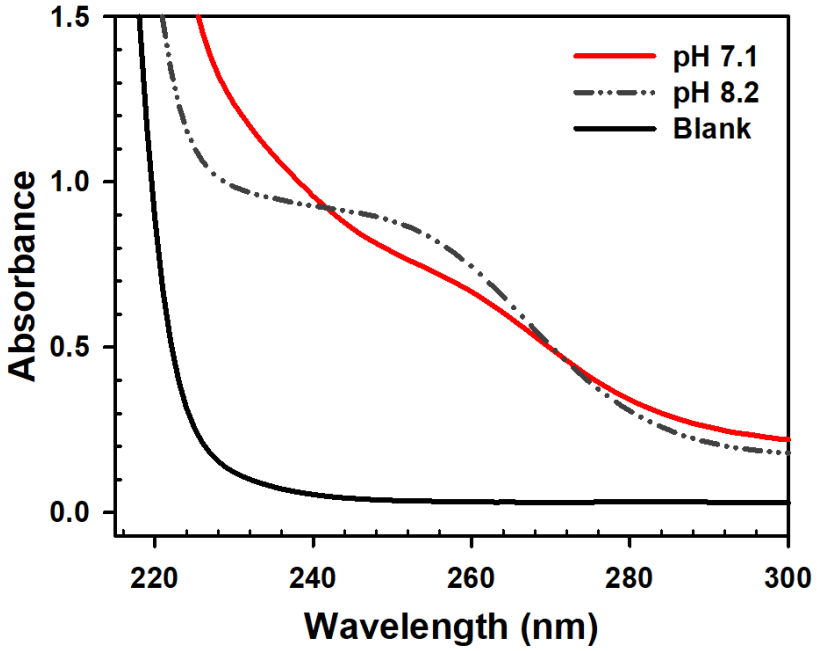

Figure. 3.15. UV/Vis absorption spectra of artificial seawater after chlorination at pH 7.1 and pH 8.2 ([Free chlorine]$_0$ = 5 mg/L, [NaCl]$_0$ = 38,000 mg/L, [NH_4^+]$_0$ = 1 mg/L, [Br^-]$_0$ = 61 mg/L, [Phosphate buffer] = 10 mM, Cell path length = 10 cm, Temperature = 25°C, Reaction time = 10 sec).

3.3. Inactivation efficacy of chlorine/bromine species

In order to compare whether there is an actual difference in inactivation efficacy between the disinfectants identified as major species, inactivation experiments with chlorine/bromine species in DI water were performed (Figure. 3.16). *E. tarda* and *V. harveyi* showed similar levels of inactivation when each disinfectant was injected at 0.1 mg/L. In *V. harveyi* disinfection, the inactivation efficacy of HOBr was slightly lower than HOCl, but overall, the two disinfectants showed similar tendencies. Both disinfectants inactivated 3 log of microorganisms within 30 seconds. OCl^- showed 3 log inactivation in 1 minute, showing lower disinfection efficacy than HOCl and HOBr. NH_2Cl and NH_2Br, produced by ammonium reacting with HOCl and HOBr, respectively, showed the lowest efficacy as they were not able to inactivate the microorganisms even after 8 minutes. This is because they have 20-50 times less oxidation power compare to HOCl or HOBr (Berman et al., 1988; Heeb et al., 2014). Thus, in seawater, the lower disinfection efficacy at pH 8.2 than 7.1 is attributed to the significantly lower disinfection efficacy of NH_2Cl compared to HOBr.

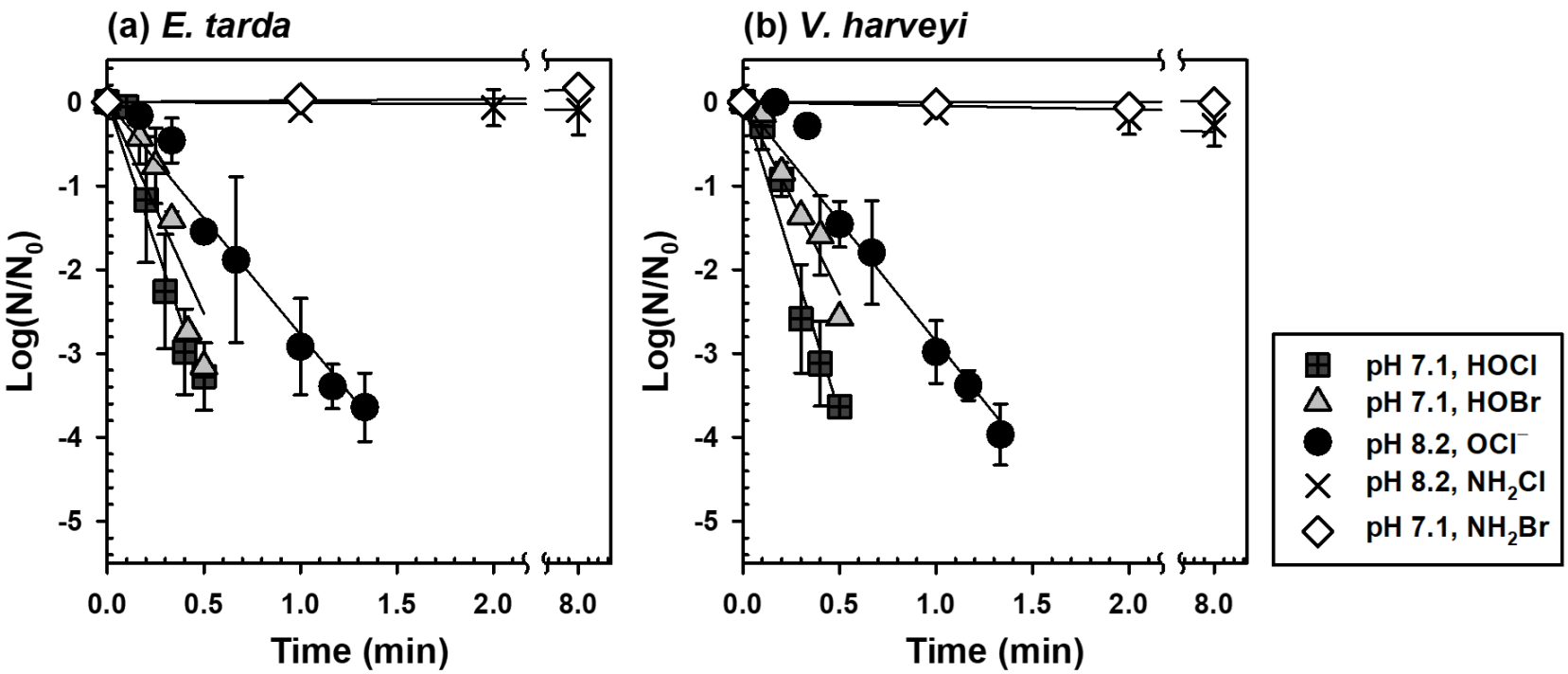

Figure. 3.16. Inactivation of (a) *E.tarda* and (b) *V.harveyi* by chlorine/bromine species in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Oxidant]$_0$ = 0.1 mg/L, [Phosphate buffer] = 10 mM, Temperature = 25°C).

3.4. *Ct* values

At pH 7.1 and 8.2, the inactivation efficiency showed a large gap in both seawater and DI water due to the speciation of disinfectant. For this reason, the *Ct* values should be derived separately at each pH, thus additional microbial inactivation experiments were carried out at pH 7.1 (Figure 3.17). Because a strong disinfectant (i.e., HOCl and HOBr) was produced at pH 7.1, a relatively small amount of free chlorine was injected compared to pH 8.2 experiments. In both water conditions, the disinfection efficacy correlated with the gradient of initial free chlorine concentration, and the decomposition of TRO is almost negligible.

Finally, the *Ct* values for *E. tarda* and *V. harveyi* at 25°C were determined using microbial inactivation and TRO decomposition data previously shown in Figures 3.1-3.3 and 3.17. Only the effect of initial free chlorine concentration was considered because including the effects of NOM and temperature makes it difficult to observe the correlation between the inactivation rate and the TRO concentration. The log inactivation of *E. tarda* and *V. harveyi* in seawater and DI water were plotted as a function of *Ct* in Figures 3.18 and 3.19. The microorganism inactivation rate was linearly correlated with the *Ct* values. In both seawater and DI water, a steeper slope appeared at pH 7.1.

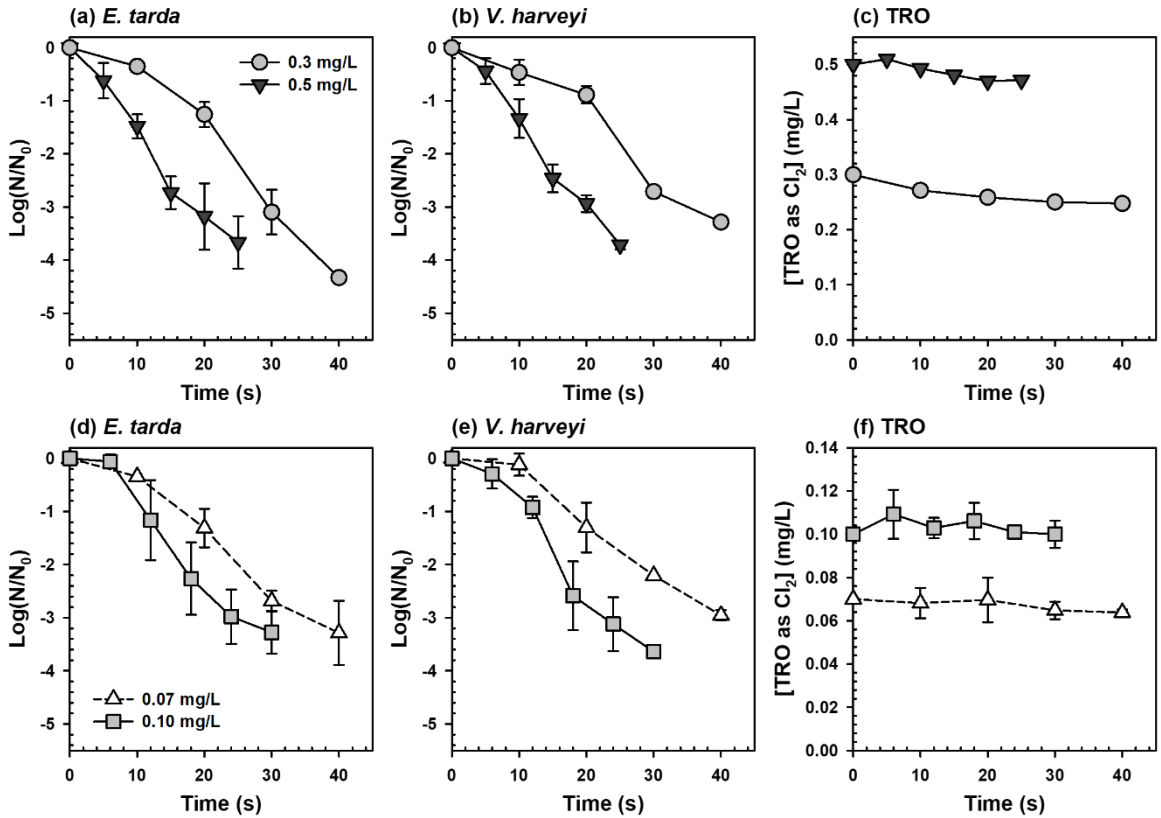

Figure. 3.17. Effect of free chlorine dose on *E. tarda* and *V. harveyi* inactivation efficacy and TRO decomposition in (a), (b), (c) seawater and (d), (e), (f) DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, pH = 7.1, Temperature = 25°C, [Phosphate buffer] = 10 mM for (d), (e), (f)).

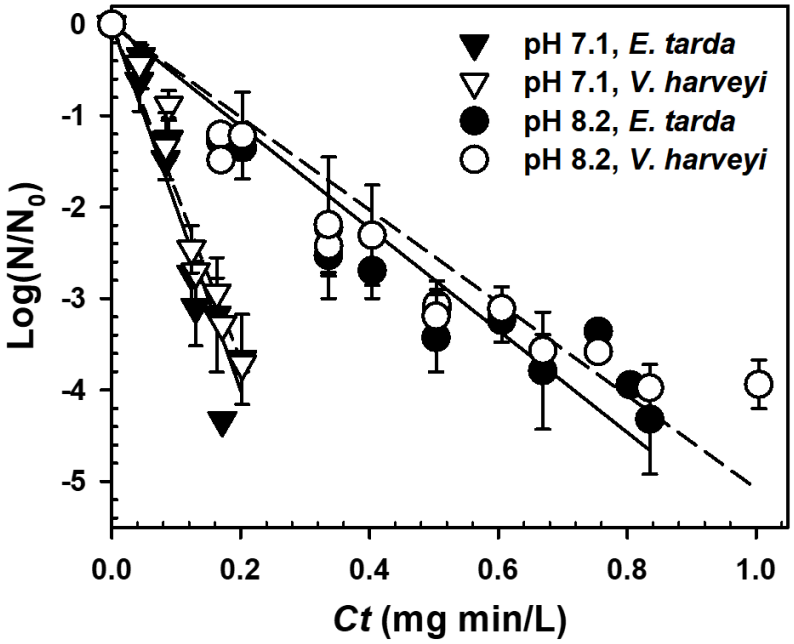

Figure 3.18. *Ct* values for *E. tarda* and *V. harveyi* inactivation in seawater ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, Temperature = 25°C).

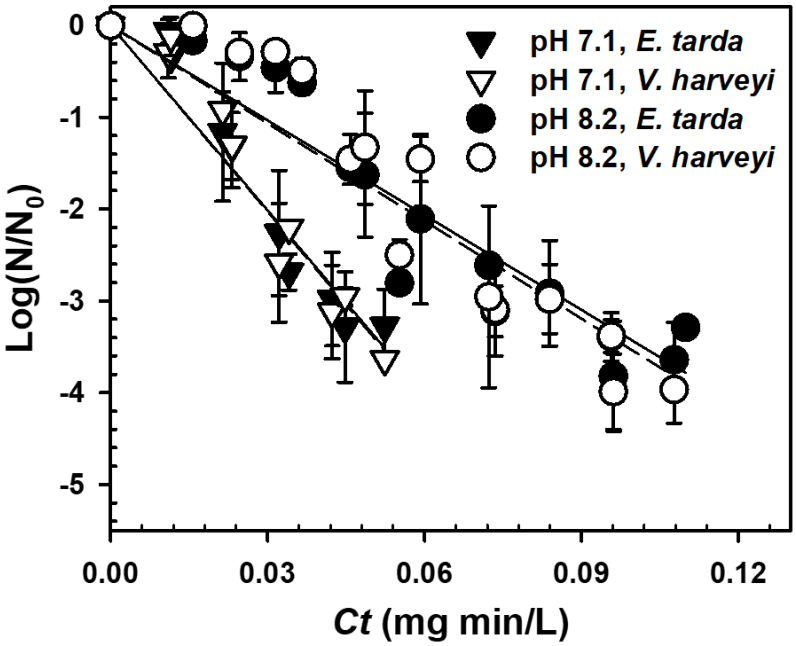

Figure 3.19. *Ct* values for *E. tarda* and *V. harveyi* inactivation in DI water ([*E. tarda*]$_0$ = [*V. harveyi*]$_0$ = 10^7 CFU/mL, [Phosphate buffer] = 10 mM, Temperature = 25°C).

Table 3.4 summarizes the required *Ct* values to achieve 1 log (90%) inactivation of *E. tarda* and *V. harveyi*. Both microorganisms represented very similar *Ct* values. In seawater, the *Ct* values at pH 7.1 are 0.0489 and 0.0551 mg·min/L for each microorganism. These values are about 3.5 times higher compare to the DI water condition (0.0149 and 0.0150 mg·min/L). Previously, in Figure 3.16, HOCl and HOBr, the major species at pH 7.1, showed similar disinfection efficacy. However, HOCl accounts for the highest portion (73%) in DI water, whereas in seawater, HOBr only accounts for 39%. Instead, around 37% of total oxidants is composed of NH_2Br, which has a weaker disinfection efficacy, so the overall inactivation efficiency is lower in seawater. In addition, disinfection efficacy in seawater was inhibited by organic/inorganic substances, increasing the *Ct* values. At pH 8.2, the dominant disinfectant in seawater is NH_2Cl and the *Ct* values of NH_2Cl for *E. tarda* and *V. harveyi* are 0.1822 and 0.1984 mg·min/L, respectively. Since NH_2Cl has lower efficacy than HOCl, it has *Ct* values about 3.5 times higher. High *Ct* values at pH 8.2 and a gentle slope that can be seen in Figure. 3.18 indicates that it requires a longer reaction time or higher initial oxidant concentration to achieve a similar degree of microbial inactivation at pH 7.1 condition.

In DI water, HOCl and OCl^- are the dominant oxidants at pH 7.1 and 8.2, respectively. Therefore, the *Ct* values at each pH can be said to be the *Ct* value of the dominant oxidants. The *Ct* values of HOCl for *E. tarda* and *V. harveyi* inactivation are 0.0149 and 0.0150 mg·min/L, respectively. For OCl^-, *Ct* values were about 2 times higher (0.0286 and 0.0279 mg·min/L) than that of HOCl. In conclusion, the *Ct* value varies greatly depending on the speciation of free chlorine.

Table 3.4. Required *Ct* values to achieve 1 log (90%) inactivation of *E. tarda* and *V. harveyi*.

	***Ct* (mg·min/L)**			
	Seawater		**DI water**	
	E. tarda	*V. harveyi*	*E. tarda*	*V. harveyi*
pH 7.1	0.0489 (R^2 = 0.909)	0.0551 (R^2 = 0.940)	0.0149 (R^2 = 0.930)	0.0150 (R^2 = 0.925)
pH 8.2	0.1822 (R^2 = 0.862)	0.1984 (R^2 = 0.809)	0.0286 (R^2 = 0.895)	0.0279 (R^2 = 0.880)

Chapter 4. Conclusions

This study investigated the microbial inactivation kinetics of *E. tarda* and *V. harveyi* by chlorination in seawater and DI water. As NOM concentration increased, disinfection efficacy decreased due to the degradation of injected free chlorine by NOM. The inactivation efficacy and TRO decomposition indicated similar temperature dependencies. Slightly higher disinfection efficacy and TRO decomposition were observed as the temperature was increased from 4 to 33°C. Depending on the pH value and water condition, the major disinfectant species may change, and the dominant disinfectant and their inactivation efficacy have been identified at each condition. In DI water, the oxidants differentiated into either HOCl and OCl^- depending on the pKa value of free chlorine. Chemical reaction model results and UV/Vis absorbance spectra in artificial seawater revealed that HOBr and NH_2Cl are the main species in seawater that contained bromide and ammonium. The inactivation efficacy of disinfectant identified as the major species was confirmed in order of HOCl ≈ HOBr > OCl^- >> NH_2Cl in DI water. *E. tarda* and *V. harveyi* showed similar inactivation tendency in all conditions. Finally, based on the previous experiments, the *Ct* values of free chlorine for each microorganism were determined. Depending on the speciation of free chlorine, *Ct* values changed. *Ct* values derived from each condition can be applied to prevent the indiscriminate use of disinfectants in aquaculture systems.

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요약

에드워드시엘라 타르다와 비브리오 하베이는 어류 대량폐사를 유발하는 대표적인 어류 병원성 박테리아로 알려져 있으며, 해당 미생물에 의한 피해를 줄이기 위해서는 양식 체계에 적절한 소독 기술을 적용할 필요가 있다. 기존의 소독 공정에 널리 적용되고 있는 염소 처리는 이러한 어류 병원성 박테리아를 효과적으로 제어 가능한 방법이다. 그러나 해수에서 염소의 무분별한 사용은 유해한 소독 부산물을 생성할 수 있으며 이는 소독 부산물의 독성에 의한 어류 폐사 등의 문제로 이어져 양식 체계에 경제적 손실을 유발한다. 이에 따라 병원성 미생물에 대한 소독제의 소독능을 정량적으로 평가하여 소독제의 사용 농도 및 시간을 최적화하는 연구가 우선되어야 한다. 이전의 어류 병원성 미생물 제어에 관한 연구는 염소를 활용한 에드워드시엘라 타르다와 비브리오 하베이의 불활성화 가능성을 보여주었지만, 실제 소독공정이 적용되어야 할 해수 상에서 염소의 소독능에 대한 정량적 평가는 보고된 바가 없다. 따라서 본 연구에서는 해수에서 염소에 의한 에드워드시엘라 타르다와 비브리오 하베이의 소독능을 정량적으로 평가하였다. 염소의 살균 효과를 정량 평가하기 위해 증류수와 해수에서 염소의 농도, 자연 유기물질의 농도, 온도, pH 조건을 변화시켜 실험을 진행하였다. 실험 결과, 두 미생물은 유사한 불활성화 경향을 보였으며, 유기물 농도가 증가하거나 온도가 감소함에

따라 소독능이 감소하였다. pH 7.1 조건의 해수에서 에드워드시엘라 타르다와 비브리오 하베이의 90% 불활성화를 달성하기 위한 염소의 *Ct* (농도-시간의 곱)값은 각각 0.0489 및 0.0551 mg min/L으로 나타났다. 그러나 pH 8.2 조건의 해수에서 *Ct*값은 각각 0.1822 및 0.1984 mg min/L으로, pH 71 조건보다 약 3.5배 높은 값을 나타내었다. 이러한 *Ct*값의 차이는 pH 및 해수에 존재하는 브롬 이온과 암모늄의 영향으로 인한 염소 종 변화 때문임을 확인하였다.

주요어: 염소 소독, 미생물 소독, 에드워드시엘라 타르다, 비브리오 하베이, 해수, 불활성화 정량 평가, 염소 종 변화

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