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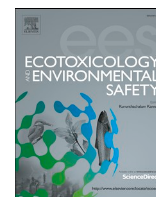
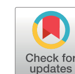


Evaluation of residual toxicity of hypochlorite-treated water using bioluminescent microbes and microalgae: Implications for ballast water management



Jung-Suk Lee[a,1], Seongjin Hong[b,1], Junghyun Lee[c], Tae Seob Choi[a], Kitae Rhie[d], Jong Seong Khim[c,*]

[a] Neo Environmental Business Co. (NeoEnBiz), Bucheon 14523, Republic of Korea
[b] Department of Ocean Environmental Sciences, Chungnam National University, Daejeon 34134, Republic of Korea
[c] School of Earth and Environmental Sciences & Research Institute of Oceanography, Seoul National University, Seoul 08826, Republic of Korea
[d] Department of Biology, Kyung Hee University, Seoul 02447, Republic of Korea

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ABSTRACT

Total residual oxidants (TRO) in treated ballast water can produce various disinfection by-products (DBPs) depending on local conditions, such as salinity and organic matter content in water. Because TRO and DBPs are known to be harmful to aquatic organisms and humans, ecotoxicity tests have been proposed for screening the residual toxicity before discharging treated ballast water. In the present study, we aimed to address the decay rates and toxicity changes of TRO under various conditions in salinity, initial TRO concentrations, and residence time of TRO. In addition, the toxicological sensitivities of bioluminescent bacteria *Vibrio fischeri* and a commonly-used microalgae *Skeletonema costatum* relative to the residual toxicity of TRO and six selected DBPs were determined. Decay rate of TRO concentration increased as a function of salinity and was affected by the initial concentrations of TRO. Unexpectedly, significant bioluminescence inhibition was observed for hypochlorite-treated water at $< 0.1 \text{ mg L}^{-1}$ TRO (expressed as Cl_2), which is a lower concentration than the maximum allowable discharge concentration (MADC) to marine waters established by the International Maritime Organization (IMO). The ecotoxicological thresholds of no observed effective concentration and median effect concentration for all tested DBPs were about 3–10 times lower for *V. fischeri* than for *S. costatum*. The results indicate that bioluminescent microbes possess an ecologically-relevant sensitivity to both TRO and DBPs in ballast water. In general, bioassay using *V. fischeri* was potentially more effective than microalgae for screening the total toxicity of TRO and DBPs in treated ballast water, especially given that ballast water usually contains a highly variable and complex mixture of toxicants.

1. Introduction

The discharge of ballast water has been a concern since it was known to be a major route by which invasive aquatic species occur and spread into new habitats (Ruiz et al., 1997). Alien invasive marine species often has threatened native ecosystems, adversely affecting local aquatic-based economic activities (such as fisheries) and human fatalities. To minimize such problems, the International Maritime Organization (IMO), in 2004, adopted to propose the international convention for the control and management of ship ballast water and sediment, which requires that discharged ballast water should meet specific water quality standards with respect to the concentration of living organisms (IMO (International Maritime Organization), 2004). The standards also make it mandatory for ships to install a ballast water management system (BWMS) on board.

Electrolysis, ultraviolet irradiation, ozonation, and thermal treatment are typically used for treating ballast waters (Delacroix et al., 2013; Duan et al., 2016; Tsolaki and Diamadopoulos, 2010; Werschkun et al., 2014). Although electrolysis which most technically efficient and cost-effective treatment approach is widely-used, it creates both free chlorine (Cl_2) and chlorine compounds in ballast water (Duan et al., 2016). These active substances (ASs) are defined as concentration of total residual oxidants (TRO), quantified as free chlorine (IMO, 2007). The highly reactive TRO are adversely impact physiological and

* Corresponding author.
E-mail address: jskocean@snu.ac.kr (J.S. Khim).
[1] These authors contributed equally to this work.

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metabolic processes of phytoplankton by damaging cell membranes, leading to leakage of intracellular materials, reduction in photosynthetic efficiency, and denaturation of proteins and nucleic acids (Virto et al., 2005). This is why the IMO requires that TRO be neutralized before being discharged into aquatic environments. In addition, approval for the use of ASs, including chlorine, must be granted by the Marine Environment Protection Committee (MEPC) of the IMO in accordance with mandated procedures established for BWMS (G9 protocol) (IMO, 2007, 2008).

The formation of disinfectant by-products (DBPs) which impact aquatic organisms and marine ecosystems have been a problem for most BWMS using ASs to treat ballast water. The most-frequently occurring DBPs in chlorinated ballast water include bromoform (BF), monobromoacetic acid (MBAA), bromodichloromethane, and bromoacetonitrile (Fabbricino and Korshin, 2005). The formation of DBPs has been observed variously depending on environmental parameters such as salinity, residence time, bromide content, and initial properties of the ballast water (Delacroix et al., 2013; Gregg et al., 2009; Richardson et al., 2007; Zhang et al., 2013). Previous studies have examined how DBPs are formed during the treatment freshwater used for drinking (Delacroix et al., 2013; Werschkun et al., 2014), but there is still limited information on the formation, fate, and effects of DBPs in treated seawater (Čulin and Mustać, 2015; Fisher et al., 2014). DBPs could potentially cause cytotoxicity, carcinogenicity, and mutagenicity in organisms (Richardson et al., 2007; Werschkun et al., 2014). However, these toxicities cannot be neutralized and some DBPs can persist for long periods of time in marine environments, and could bioaccumulate in organisms (Gregg et al., 2009; Lee et al., 2015). Thus, the IMO requires that ecotoxicological tests and chemical analyses of potential DBPs in seawater be tested prior to discharge. In fact, the IMO mandates that at least two different types of water tests be conducted (e.g., seawater, brackish water, and/or freshwater) before the Marine Environment Protection Committee provides approval for a BWMS to operate.

For many approved BWMS, it is difficult to assess treated ballast water within a practical time frame. This is because most DBP analyses and ecotoxicity tests take days to weeks to perform, even by trained staff in well-managed laboratories. Thus, there is a need for compliance monitoring and ecotoxicity tests for ballast water that are both simpler to use and can be more rapidly performed. Currently, under the G9 protocol, testing of microalgae, invertebrate, and vertebrate (e.g., fish) taxa must be examined in whole-effluent toxicity (WET) tests for ballast water after being treated. Of these three taxa, microalgae are the most frequently utilized test organisms because they are highly sensitive to treated ballast water (IMO, 2007).

Even though bacteria are a very important component of aquatic ecosystems and they have been already established as standard test organisms by the International Organization for Standardization (ISO, 1134-2008), toxic effects of ASs and/or DBPs on bacteria have not been applied (ISO, 2007). It would be a practical, rapid, and sensitive assessment tool to use a microbial assay with a bioluminescent bacterium for residual toxicity evaluation of treated ballast water. The specific aims of this study were to: (1) measure decay rates of TRO (total residual oxidants) under various salinity conditions and initial TRO concentrations; (2) evaluate changes in ecotoxicity after injecting hypochlorite (as TRO) relative to water salinity and residence time of the TRO; and (3) compare toxicological sensitivities of the bioluminescent bacteria (*Vibrio fischeri*) and the commonly-used microalgae (*Skeletonema costatum*) by determination of residual toxicity of TRO and six selected DBPs.

2. Materials and methods

2.1. Experimental settings

We created a stock solution by adding sodium hypochlorite (NaOCl) (Sigma-Aldrich, St. Louis, MO) to millipore water filtered through a 0.22 μm filter. This stock solution was injected into artificial seawater (for use in our TRO decay tests) in an amount sufficient to produce initial residual oxidant concentrations of 0.31, 0.63, 1.3, 2.5, 5.0, and 10 mg L^{-1} (expressed in TRO as Cl_2) for use in our TRO decay tests (Dataset I) (Table 1). A TRO nominal concentration gradient from 0.15 to 5 mg L^{-1} was established for Dataset II, whereas a gradient from 0.08 to 10 mg L^{-1} was established for Dataset III. TRO decay was determined periodically: Dataset I provided a long-term (120 h) evaluation of TRO (sampled at 0, 24, 48, 72, 96, and 120 h), whereas Dataset II provided a short-term (24 h) evaluation of TRO (sampled at 0, 1, 2, 3,

Table 1
Experimental design of the three Datasets (I, II, and III) examined in this study.

	Dataset I	Dataset II	Dataset III	
Specific purpose	Determination of TRO decay rates at varying conditions of salinity and initial concentrations	Evaluation of toxicity changes after injecting TRO at (1) three salinity conditions and (2) over time	Comparison of toxicological sensitivity between assays of *V. fischeri* and *S. costatum* on TRO and 6 DBPs	
Test species		*V. fischeri*	*V. fischeri*	*S. costatum*
Experimental conditions				
Exposure concentrations of TRO (mg L^{-1})	0.31–10 (6 levels)	(1) 5 (2) 0.15–5 (6 levels)	0.08–10 (9 levels)	0.08–10 (9 levels)
Exposure concentrations of DBPs (mg L^{-1})	–	–	0.78–100	[a]7.81–1000 (8 levels) [b]3.91–500 (8 levels)
Salinity treatments (psu)	2.7, 21.3, and 33.2 ± 0.1	(1) 2.7, 21.3, and 33.2 (± 0.1) (2) 33.2 ± 0.1	33.2 ± 0.1	33.2 ± 0.1
Temperature (°C)	25 ± 1	25 ± 1	15	20
Test duration (hours)	120	120 and 24	0.5	72
Initial cell concentrations (cells mL^{-1})	–	–	–	1–2 × 10^4
Replicates	3	3	3	3
Measurement	TRO concentration	Luminescence inhibition (EC_{50})	Luminescence inhibition (NOEC, LOEC, & EC_{50})	Growth inhibition (NOEC, LOEC, & EC_{50})
Data presented in	Fig. 1	Fig. 2	Figs. 3–4 and Table 2	

[a] Concentrations for bromate and chloroform.
[b] Concentrations for bromoform, monochloroacetic acid, dichloroacetic acid, and monobromoacetic acid.

4, 8, 12, and 24 h). We used a DR/2000 spectrophotometer (Hach Company, Loveland, CO) to measure TRO concentrations using the *N*, *N*-diethyl-*p*-phenylenediamine/ferrous ammonium sulfate (DPD/FAS) titration method, based on US EPA (U. S. Environmental Protection Agency) method 330.4 (Greenberg et al., 1998).

Batches of water were prepared at three salinity concentrations to investigate the effect of salinity on toxicity: 2.7 psu (representing freshwater conditions), 21.3 psu (representing brackish water), and 33.2 psu (representing seawater). All three conditions were produced using artificial sea salt (Sigma-Aldrich). The six TRO treatment concentrations (0.31–10 mg L^{-1}) were tested for each salinity concentration. Salinity and temperature of water were monitored over time with a YSI 6000 multi-parameter Sonde monitor (YSI Inc., Yellow Springs, OH).

We selected six target DBPs for our toxicity tests based on the frequency at which particular DBPs occur in BWMS-treated or neutralized ballast water and/or their concentrations (references for IMO reports reviewed for the selection of DBPs are listed in Table S1 of the Supplementary material). The six targeted chemicals included bromate (BR), chloroform (CF), BF, monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), and MBAA. All chemicals were purchased from Sigma-Aldrich with guaranteed reagents. The six DBP stock solutions were injected into bacterial and microalgal media to achieve initial concentrations of 500 mg L^{-1} for BF, MCAA, DCAA, and MBAA and 1000 mg L^{-1} for BR and CF (Table 1). In order to determine the appropriate and relevant ecotoxicological effects between the two bioassays, we conducted range-finding toxicity tests before conducting the definitive toxicity tests (Table 1).

2.2. Luminescent inhibition assay using a microbe N-Tox test

A bioluminescence test with *V. fischeri* (NRRL B-11177) was conducted using a luminescent bacteria toxicity measurement apparatus (N-TOX model 100; NeoEnBiz Inc., Bucheon, Korea) following the standard method specified by the Ministry of Maritime Affairs and Fisheries of South Korea (MOMAF Ministry of Maritime Affairs and Fisheries of South Korea, 2005). Lyophilized *V. fischeri* were prepared by adding 1 mL of a reconstituted solution of VF100® (NeoEnBiz Inc.). A bacterial solution was prepared with 50 mL of diluent (at 4 °C) by adding 1 mL of bacterial reagent to it (Lee et al., 2008). Serially-diluted test water (with TRO or DBPs) were prepared and introduced into a 96-well plate (100 µL to each well). Then 100 µL of bacterial solution was added to each well. Luminescence was measured at 10 min intervals for a total of 30 min to track changes in light intensity. A zinc sulfate solution was used as a reference chemical; this standard was compared with every fresh vial of bacteria to ensure the validity cross all tests (ISO The International Organization for Standardization, 1998).

2.3. Growth inhibition assay using microalgae

Ecotoxicity tests using microalgae were performed following the ISO standard protocols 10253 and ASTM E1218 (ASTM American Society for Testing and Materials, 1997; ISO, 2006). Microalgae were pre-cultured and then inoculated with the control and test solutions (detailed descriptions of culture conditions for microalgae provided in Table S2). Validation criteria for the algal culture control group were met by the protocol (ASTM American Society for Testing and Materials, 1997; ISO, 2006). We measured inhibition of population growth of microalgae after 72 h of incubation.

2.4. Toxicity data analysis

Two bioassays (of microbes and microalgae) were used to compare sensitivities to TRO and the six DBPs. Toxicological parameters were subjected to toxicological classification following EU legislation guidelines (Directive 93/67/EEC; EC (European Commission), 1993). No observed effective concentration (NOEC), lowest observed effective concentration (LOEC), and median effect concentration (EC_{50}) values were calculated based on reductions in bioluminescence (for bacteria) and population growth rates (for microalgae), respectively, associated with the concentration gradients of the samples. Toxicity parameters were calculated using ToxCalc™ Ver. 5.0 (Tidepool Scientific Software, McKinleyville, CA), which is specifically designed for testing environmental toxicity, being compatible with US EPA statistical guidelines. A log-linear model was used to calculate the 50% effective concentration (EC_{50}) with a 95% confidence limit. The *t*-test was used for comparison of replicates and was performed with SPSS 23.0 (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Decay rates of TRO under three salinity conditions

Freshwater (2.7 psu), brackish water (21.3 psu), and seawater (33.2 psu) were evaluated to compare the impact of salinity on the decay rate of TRO (Fig. 1a–c). Concentrations of measured TRO were compared to the injected nominal concentrations of TRO at initial dosages of 0.31–10 mg L^{-1} (expressed as Cl_2) at the three tested seawater salinities (Fig. 1d–e). A suitable agreement between the injected and measured values was observed in all tested salinities at 0 h (Fig. 1a–c). Under all treatment conditions, the concentrations of TRO in the tested water rapidly declined with residence time (24–120 h), particularly during the initial stage (within the first 24 h). Reduction of TRO over time is related to a number of factors including oxidation, addition-, or substitution-reactions, decomposition by light, concentration of dissolved organic carbon, and formation of DBP due to the reactions (Lee et al., 2017; Nanayakkara et al., 2011). Although the concentrations of TRO were reduced by such reactions in our treatments, DBPs seem to have been formed in the initial stage of treatment (within 24 h), when toxic effects of DBPs were likely greatest.

The decay rates of TRO increased with salinity. The decay rate of TRO was highest in brackish water and seawater than in freshwater. At mid-concentration of TRO (5 mg L^{-1}, nominal concentration, expressed as Cl_2), 27% of TRO was degraded at salinity 2.7 psu during the experimental period (120 h), while 56% and 77% of TRO were degraded at salinities of 21.3 and 33.2 psu, respectively (Fig. 1d). These results agreed with a previous finding by Wang et al. (2008), where the rate of TRO decay in water was influenced by salinity. One possible explanation for the association of decay rate of TRO with salinity is that the chemical reactions in chlorinated seawater and brackish water are very different than in chlorinated freshwater, mainly due the presence of bromide ions in saline waters (Oemcke and Leeuwen, 2004). Natural seawater possesses bromide concentrations of 65–68 mg L^{-1} (Magazinovic et al., 2004), which introduces the much greater complexity of DBP following chlorination, due to the rapid formation of reactive bromine. This complexity can produce abundant brominated DBPs, such as haloacetic acids, halogenated phenols, halogenated acetonitriles, and halogenated hydrocarbons (Gonsior et al., 2015). However, it is not clear what mechanism(s) specifically caused the decay of the TRO observed in artificial seawater. Our results could have

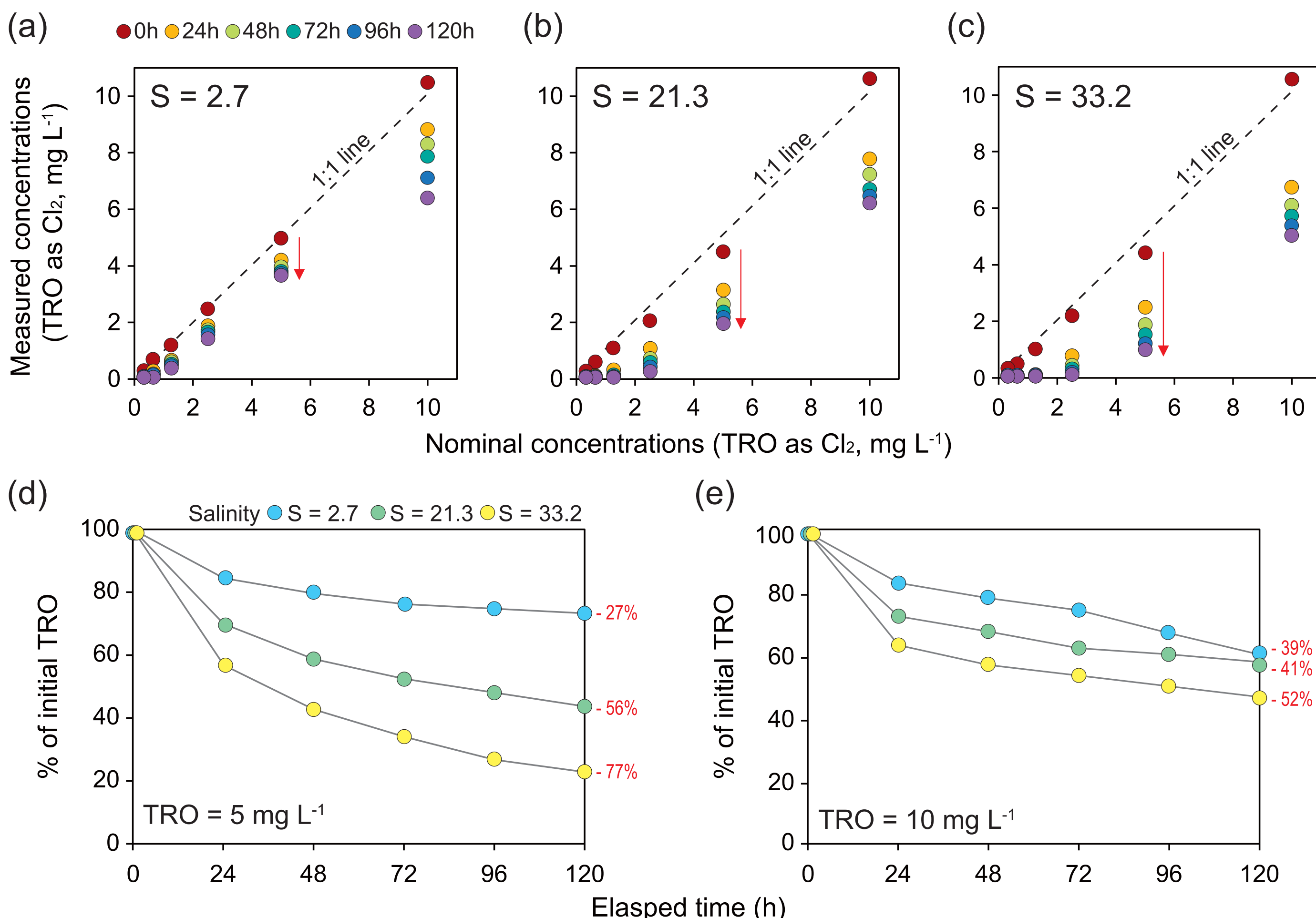

Fig. 1. Decay of total residual oxidant (TRO) in synthetic seawater prepared at three salinity conditions: (a) 2.7 psu, (b) 21.3 psu, and (c) 33.2 psu. Six TRO concentrations were prepared via serial dilution (from 0.31 m to 10 mg L^{-1}, expressed as Cl_2). TRO concentrations were daily measured during 120 h period. Decay of TRO in water at three different salinities, based on the initial concentrations of TRO: (d) 5.0 mg L^{-1} and (e) 10.0 mg L^{-1}.

underestimated the decay rate of TRO in natural seawater because artificial seawater contains a lower concentration of dissolved organic carbon.

Rates of TRO decay were also affected by the initial dose concentrations of TRO. During the initial stage of degradation (within 24 h), we found a significant difference (*t*-test; $p < 0.05$) in the rate of TRO decay between the TRO concentrations of 5.0 and 10.0 mg L^{-1} (Fig. 1d–e). The mid-concentration (5.0 mg L^{-1}) of initial TRO decayed more rapidly than the higher initial concentration of TRO (10.0 mg L^{-1}), especially at the highest salinity treatment (33 psu). Our results indicate that high concentrations of DBPs can be produced with TRO concentrations of approximately 5 mg L^{-1}. Although TRO concentrations declined to a distinct threshold after 120 h, toxicity associated with hypochlorite treated water could have persisted due to an increased concentration of DBPs produced during the disinfection process, most of which cannot be neutralized.

3.2. Toxicity changes of TRO with aging time

At mid-concentrations of TRO (5 mg L^{-1}, nominal concentrations expressed as Cl_2), bioluminescence of *V. fischeri* was inhibited at all salinities (i.e., 2.7, 21.3, and 33.2 psu) (Fig. 2a). However, the inhibition did not show any clear directional trend among salinity conditions over time, similar to a previous study by Cook et al. (2000). Over the time period from 0 to 120 h, EC_{50} values declined to 56%, 63%, and 34% at salinities of 2.7, 21.3, and 33.2 psu, respectively. This indicates that inhibition of bioluminescence by *V. fischeri* increased over time, at least until the end of the 120 h time frame (i.e., 5 d decay time for TRO). At the earliest stage of degradation (within 24 h), the EC_{50} values were the lowest (i.e., toxicities were their highest) under all salinity conditions. In particular, under the ocean condition (33.2 psu), the EC_{50} was 0.09 mg L^{-1}, which was lower than the 0.1 mg L^{-1} of maximum allowable discharge concentration (MADC) mandated for treated ballast water by the IMO (IMO, 2015).

Because the EC_{50} values at 24 h were the lowest (in 33 psu water), we further examined TRO concentration responses during the prior 24 h (Fig. 2b). Bioluminescence inhibition of *V. fischeri* was maximized at 24 h (elapsed time). Bioluminescence inhibition of *V. fischeri* has been shown to increase even as TRO decay, causing them to lose their ability to emit light in the presence of a toxicant at even very low concentrations of TRO (i.e., below the detection limit of 0.01 mg L^{-1}). This response might be explained by the fact that TRO reacts with reducing agents to form complicated DPBs over time (Fabbricino and Korshin, 2005). Thus, short-term survival of organisms is likely detrimentally affected by TRO in treated water, while long-term survival is more likely influenced by the DBPs remained as the TRO are converted to DBPs in response to their reactions with organic carbon typically occurring in natural seawater (Park et al., 2017).

The EC_{50} value for inhibition of bioluminescence due to TRO was 0.48 mg L^{-1} (expressed as Cl_2), which was the concentration at which the bacteria test was conducted immediately following the spiking of TRO (Fig. 2c). After spiking, EC_{50} generally declined in concentration over time (with an exception of EC_{50} at 1 h), and then finally declined to 0.05 mg L^{-1} after 24 h. The highest EC_{50} values that occurred at 1 h

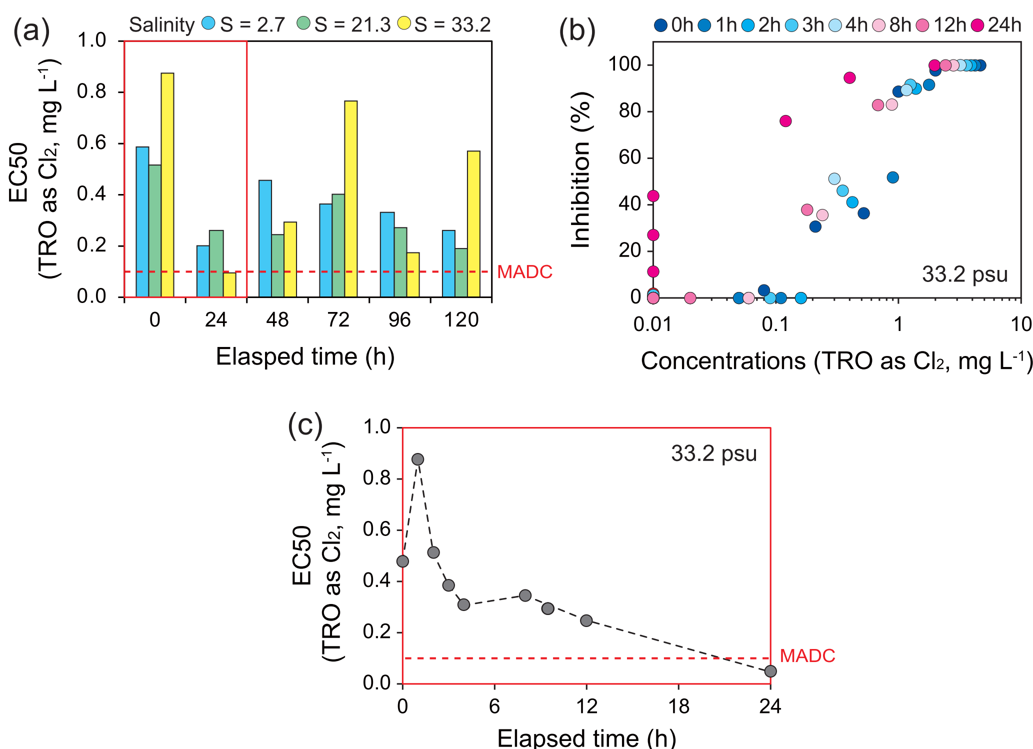

Fig. 2. (a) Variation of relative bioluminescence inhibition at the median effect concentration (EC_{50}) by the microbe *V. fischeri* under various salinity conditions over a period of 120 h, (b) bioluminescence inhibition of *V. fischeri* at 33.2 psu salinity during a 24 h period (at various injected TRO concentrations), and (c) EC_{50} values of *V. fischeri* at 33.2 psu salinity during a 24 h period. Dotted line denotes the maximum allowable discharge concentration (MADC) mandated by the International Maritime Organization.

may have been due to a reduction in the toxicity effect by TRO in response to the various chemical reactions that occurred immediately after spiking and the rapidity at which DBPs formed. EC_{50} values indicated that toxicity of TRO drastically increased over time to about 10 fold at any given TRO concentration level (Fig. 2). The MADC mandated by the IMO is 0.1 mg L^{-1} TRO by five days after treatment, i.e., if the TRO concentration of ballast water is below this MADC threshold after five days, ballast water can be discharged without being neutralized (IMO, 2015). However, our results suggested that a lower concentration of TRO, at 10 times below MADC regulation limit mandated by the IMO, was still significantly toxic to the bioluminescent microbe *V. fischeri*.

3.3. Comparison of ecotoxicological sensitivities for TRO and DBPs between bacteria and microalgae

Measured toxicological parameters (NOEC, LOEC, and EC_{50}) for TRO and six selected DBPs are provided in Table 2 and Figs. 3 and 4. The EC_{50} of TRO for *V. fischeri* was nine times lower than that for *S. costatum*. Except for MBAA and BR, DBPs were similarly toxic to both *V. fischeri* and *S. costatum*. Our EC_{50} values indicated that MBAA exerted a greater toxic effect on *V. fischeri* than did any of the other DBPs studied (Table 2 and Fig. 4). This finding is consistent with our other results of algal toxicity, which generally reflected that MBAA had the lowest EC_{50} value among the tested toxicants. In contrast, BR exhibited the least toxic effect on both *V. fischeri* and *S. costatum*. However, *V. fischeri* had about 3- to 10-times lower values of NOEC and EC_{50} for all tested DBPs than did *S. costatum* (Fig. S1 and Table 2). According to the toxicity classification established by Directive 93/67/EEC (EC (European Commission), 1993), BR, CF, BF, and DCAA could be classified as exerting “harmful effects” on *V. fischeri* after 30 min of exposure, whereas a “no harmful effect” designation could be established for *S. costatum* at the same concentrations. Our study shows that *V. fischeri* is sensitive to chemicals produced during the disinfection process (in response to reactions of TRO with various substances in seawater).

The toxicity of TRO can be negated with a neutralizer, but the neutralization only affects compounds with oxidizing abilities. Hence, non-oxidizing DBPs in treated water might still detrimentally affect aquatic organisms (Lee et al., 2015). In several cases, DBP concentrations reported for BWMS exceeded the toxicologically-derived WHO drinking water guideline values for BF (0.1 mg L^{-1} threshold) and BR (0.01 mg L^{-1} threshold) (Table S1). Although the concentrations of DBPs detected in treated ballast water were generally lower than the LOEC values determined for *V. fischeri* and *S. costatum* (Table S1), potential adverse effects could be observed, possibly due to the synergistic effects and/or existence of unknown toxic DBPs (Delacroix et al., 2013). Furthermore, concentrations of DBPs in natural ballast water are much higher than those in synthetic ballast water, presumably because natural freshwater and saltwater generally have a higher potential for forming brominated DBPs.

Because biological conditions in ballast water are complex and vary widely among ships, current algal toxicity methodologies are sometimes inadequate for evaluating the toxicity of discharged water. *V. fischeri* has been shown to be more sensitive to TRO and DBPs than *S. costatum*, even though microalgae are generally considered to be more sensitive than crustaceans and fish in BWMS reported by the IMO. *S. costatum* is likely less sensitive because it has siliceous cell walls with slender spines around the cell that provide it with a compact and protective cell structure (Li et al., 2007); therefore, it is predisposed to having fewer outside interferences and is more difficult to kill. A recent study reported that other standard microalgae used to test toxicity, *Isochrysis galbana*, was found to be more sensitive to TRO and DBP contamination than the microbe *V. fischeri* because *I. galbana* does not have a thick cell wall (Park et al., 2017). Thus, toxicological sensitivities on TRO and DBPs in treated ballast water vary among species of microalgae due to differences in their morphologies (Smit et al., 2008). Meanwhile, algal toxicity tests take a relatively long time to produce results (at least 3 days), whereas bacterial bioluminescence assays can rapidly screen for toxic conditions in treated ballast water (within 30 min). In addition, bacterial bioluminescence assays can detect toxic

Table 2
Comparison of toxicological parameters (NOEC, LOEC, and EC_{50} values) for TRO and six DBPs using the bioluminescent microbe *Vibrio fischeri* and the microalgae *Skeletonema costatum*. Exposure time frames provided in footnotes.[a,b].

TRO and DBPs	Abb.	CAS number	Luminescence inhibition[a] (*V. fischeri*)				Population growth inhibition[b] (*S. costatum*)			
			NOEC[c]	LOEC[d]	EC_{50}[e]	Toxicity classification[f]	NOEC	LOEC	EC_{50}	Toxicity classification[f]
Sodium hypochlorite solution (TRO as Cl_2)	TRO	7691-52-9	0.11	0.22	0.13	Very toxic	1.0	2.2	1.3	Toxic
Sodium bromate	BR	7789-38-0	30	60	48	Harmful	50	100	180	Not harmful
Chloroform (trichloromethane)	CF	67-66-3	5.0	10	33	Harmful	100	200	170	Not harmful
Bromoform (tribromomethane)	BF	75-25-2	0.63	13	37	Harmful	100	200	110	Not harmful
Monochloroacetic acid	MCAA	79-11-8	0.63	13	15	Harmful	50	100	77	Harmful
Dichloroacetic acid	DCAA	79-43-6	0.63	1.3	20	Harmful	100	200	160	Not harmful
Monobromoacetic acid	MBAA	79-08-3	0.31	0.63	10	Very toxic	50	100	72	Harmful

[a] ISO 11348, Luminescence inhibition (30 min).
[b] ASTM E1218, Population growth inhibition (72 h).
[c] NOEC: No observed effective concentration ($mg\ L^{-1}$).
[d] LOEC: Lowest observed effective concentration ($mg\ L^{-1}$).
[e] EC_{50}: Concentration of a target chemical that gives a half-maximal response ($mg\ L^{-1}$).
[f] Toxicity categories based on the EC_{50} toxicity endpoint: $\leq 1\ mg\ L^{-1}$ indicates "*very toxic*"; 1–10 $mg\ L^{-1}$ indicates "*toxic*"; and, 10–100 $mg\ L^{-1}$ indicates "*harmful.*" The $EC_{50} > 100\ mg\ L^{-1}$ category is considered to be "*not harmful*" to aquatic organisms.

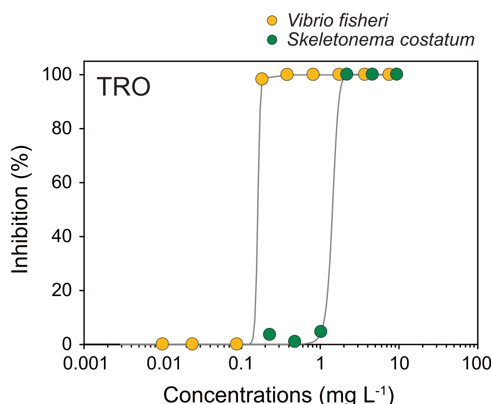

Fig. 3. Initial (after spiking) dose-response relationship between TRO concentration and ecotoxicity responses by the bioluminescent microbe *V. fischeri* and the microalgae *S. costatum*.

effects of TRO and DBP contamination at low concentrations. Therefore, we conclude that bacterial bioluminescence assays can produce more sensitive and rapid test results than other test species typically used (e.g., microalgae) in evaluating toxic effects on TRO and DBPs remaining in discharged ballast water.

3.4. Implications for ballast water management

The WET testing used to assess the toxicity of effluents in ballast water is used by port authorities to monitor and assess the environmental risk of ballast water discharges. However, the WET test could be simplified and be designed to more-rapidly assess discharged ballast water. To do this, quick-identification guides could be created for organisms typically living in ballast water. For example, the bioluminescent microbe bioassay test is a well-established tool that is very simple and rapid to conduct under both laboratory and field conditions. The bacterial bioluminescence assay has been used to measure baseline

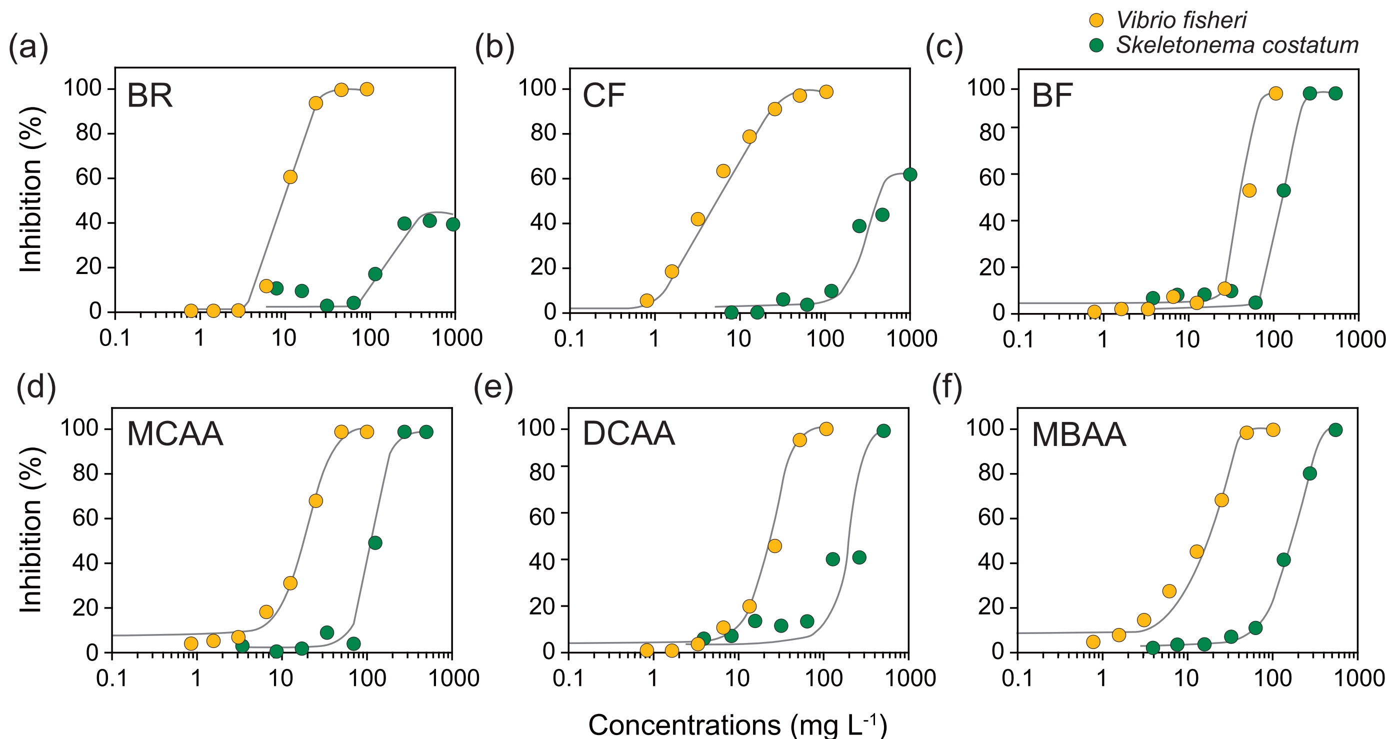

Fig. 4. Dose-response relationship between concentrations of six selected DBPs and ecotoxicity responses of the bioluminescent microbe *V. fischeri* and the microalgae *S. costatum*.

toxicity and so it encompasses a bacterium's entire cytotoxic response, regardless of whether the underlying toxic mechanism is non-specific, specific, and/or reactive (Escher and Leusch, 2011). However, although microbes are one of the important components of marine ecosystems, they are not yet widely utilized to test for ASs in ballast water during the approval process in most BWMS.

Our study results indicated that a considerable amount of hypochlorite-treated water remained toxic (within 24 h) after microbes have been exposed to seawater with concentrations of 0.1 mg L^{-1} TRO (as Cl_2), which is lower than the MADC criteria established by the IMO. EC_{50} values of *V. fischeri* were greater than the MADC in all salinity conditions after 48 h of aging time, thus we suggest that hypochlorite-treated ballast water has sufficient aging time before discharge. Bioluminescent microbes are even more sensitive to toxic conditions than *S. costatum*, which has been reported in the literature as being the most-sensitive microalgae test organism for evaluating the residual toxicity of treated ballast water. NOEC and EC_{50} of ecotoxicity testing thresholds (for all tested DBPs) are about three to ten times lower when using bioluminescent microbes than when using microalgae. Bioluminescent microbes also show an ecologically-meaningful sensitivity to chemicals produced by the reaction of TRO with various substances in seawater during the disinfection process. Therefore, bioluminescent microbes could be used to efficiently and accurately test the ecotoxicity of residual DBPs, hypochlorite ions, or TRO in treated ballast water following the G9 IMO protocol guidelines established for BWMS using ASs.

4. Conclusions

The present study provides an approach for assessing the residual toxicity of treated ballast water using the bioluminescent microbe *V. fischeri* and the microalgae *S. costatum*. We provide several key findings on the toxicity of TRO and DBPs in hypochlorite treated water:

- TRO decay rates and formation of the DBPs are affected by water salinity and the initial concentrations of TRO in water.
- Toxicity to microbes after injecting hypochlorite in salt water increased most rapidly within the first 24 h, which seems to be due to the production of DBPs.
- The bioluminescent microbe *V. fischeri* could elicit significant ecotoxicological effects at a 10-fold lower concentration than guidelines established by the MADC regulations established by the IMO.
- *V. fischeri* was found to be more sensitive to residual ASs and/or DBPs in hypochlorite treated water than is *S. costatum.*

Overall, the present study found that a bacterial bioluminescence assay test would produce more sensitive and rapid results than tests with other species when evaluating toxic effects of DBPs remaining in discharged ballast water. A comprehensive study for risk assessment is necessary to determine potential deleterious impacts on marine ecosystems and human populations arising from the discharge of ballast water associated with the implementation of ballast water decontamination approaches.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.10.002.

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<Supplementary Materials>

Evaluation of residual toxicity of hypochlorite-treated water using bioluminescent microbes and microalgae: Implications for ballast water management

Jung-Suk Lee [1], Seongjin Hong [1], Junghyun Lee, Tae Seob Choi, Kitae Rhie, Jong Seong Khim [*]

Table of Contents

Table S1. Summary of reported concentrations of DBPs from ballast water samples treated for five days. S2

Table S2. Culture conditions for the maintenance of algal stock cultures of microalgae used in this study. S3

[1] These authors contributed equally to this work.

[*] Corresponding author. *E-mail address*: jskocean@snu.ac.kr (J.S. Khim).

Table S1. Summary of reported concentrations of DBPs from ballast water samples for treated for five days.

Chemical ($\mu g\ L^{-1}$)	Control		Experimental			
	Seawater or Brackish water		Seawater		Brackish water	
	Range	Mean	Range	Mean	Range	Mean
Bromate Ion (Postassium)	0.08-0.5	0.19	2.22-327	69.5	0.15-9.46	3.6
Chloroform	0.34-1.05	0.63	0.01-0.7	0.3	0.1-10.6	4.3
Bromoform	0.01-1.17	0.41	69.1-1,100	380	49-300	144
Monochloroacetic acid	0.24-27.1	13.7	0.24-217	38.1	1-517	170
Dichloroacetic acid	0.02-1.64	0.83	1-2.42	1.7	1-3.8	2.5
Monobromoacetic acid	0.04	0.04	1-30.4	15.2	1-204	73.0

Data from IMO, 2011, 2012a-e, ISO 10253, 2006.

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Table S2. Culture conditions for the maintenance of algal stock cultures of microalgae used in this study.

Test parameter / Species	*Skeletonema costatum*
Media	Erdschreiber's medium
Temperature	20°C
Light : Dark (h)	16 : 8
Photoperiod	45 μM photons$\cdot$m$^{-2}\cdot$sec^{-1}
Test volume (mL)	50 mL / 250 mL flasks
Inoculation Concentration (cells$\cdot$mL^{-1})	1.0 ~ 2.0×10^{4}
Replication	3
Test duration (h)	144 h
Aeration	Flask shake 100 rpm$\cdot$min^{-1}
Reference	ISO 10253