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Bactericidal efficacy of elevated pH on fish pathogenic and environmental bacteria ☆

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Abstract Ship ballast water is a recognized medium for transfer and introductions of nonindigenous species. There is a need for new ballast water treatment methods that effectively and safely eliminate or greatly minimize movements of these species. The present study employed laboratory methods to evaluate the bactericidal efficacy of increased pH (pH 10.0–12.0) for exposure durations of up to 72 h to kill a variety of Gram-negative and Gram-positive bacteria including fish pathogens (*Aeromonas* spp., *Yersinia ruckeri*, *Edwardsiella ictaluri*, *Serratia liquefaciens*, *Carnobacterium* sp.), other common aquatic-inhabitant bacteria (*Serratia marcescens*, *Pseudomonas fluorescens*, *Staphylococcus* sp., *Bacillus* sp.) and indicators listed in International Maritime Organization D2 Standards; namely, *Vibrio cholera* (an environmental isolate from fish), *Escherichia coli* and *Enterococcus faecalis*. Volumes of 5 N NaOH were added to tryptic soy broth to obtain desired pH adjustments. Viable cells were determined after 0, 4, 12, 24, 48, and 72 h. Initial (0 h) cell numbers ranged from 3.40×10^4 cfu/mL for *Bacillus* sp. to 2.44×10^7 cfu/mL for *E. faecalis*. The effective endpoints of pH and treatment duration necessary to realize 100% bactericidal effect varied; however, all bacteria tested were killed within 72 h at pH 12.0 or lower. The lowest parameters examined, 4 h at pH 10.0, were bactericidal to *V. cholera*, *E. ictaluri*, three of four isolates of *E. coli*, and (three of four) *Aeromonas salmonicida* subsp. *salmonicida*. Bactericidal effect was attained at pH 10.0 within 12 h for the other *A. salmonicida* subsp. *salmonicida*, and within 24 h for *P. fluorescens*, and the remaining *E. coli*.

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Introduction

Ship ballast (water) is a well-recognized conveyer of nonindigenous species [1–5]. In an effort to control movements and introductions of nonindigenous species via ballast, the Regulation D2 requirement to treat or decontaminate ballast water was developed from international legislation developed

by the International Maritime Organization (IMO), The International Convention for the Control and Management of Ships' Ballast Water and Sediments [6]. Regulation D2 specifies that ships constructed during and after 2009 with under 5000 m^3 ballast water capacity are required to have treatment capability to meet the D2 Standards. Ballast water treatment systems must be approved within relevant IMO guidelines and achieve treatment standards of: < 10 cells/m^3 of plankton > 50 μM; < 10 cells/mL of plankton 10–50 μM; < 1 colony forming unit (cfu)/100 mL of toxicogenic *Vibrio cholera*; < 250 cfu/100 mL of *Escherichia coli*; and < 100 cfu/100 mL of intestinal Enterococci. Ballast water exchanges, the replacement of freshwater with 35 ppt seawater during the voyage, is commonly used and is successful in controlling inadvertent introductions of nonindigenous organisms. This approach relies on the inability of organisms present in freshwater ballast to survive when abruptly placed in full salinity seawater without any progressive acclimation.

The use of ballast affords ships buoyancy, stability and maneuverability and when loaded or off loaded relative to cargo load, maintains proper trim. Loading and unloading of ballast water along with the travel between ports, including transoceanic voyages, presents the opportunity to move and introduce nonindigenous biota, including microorganisms. For example, the extent to which bacteria are dispersed among ports of call within untreated ballast water is largely unknown. McCarthy and Khambaty [4] confirmed the presence of fecal coliforms in ballast water samples (highest cell count was 5.80×10^2 cfu/mL) from 5 of 16 cargo ships that ballast water was sampled; also, toxigenic *V. cholera* was recovered from ballast water from five of the cargo ships docked at ports in the Gulf of Mexico, USA. Ruiz et al. [5] showed that ships arriving at the Chesapeake Bay, USA from foreign ports contained on average 8.30×10^8 cfu/L of bacteria in ballast, including *V. cholera*, and an average of 7.40×10^9 virus-like particles per liter. The harmful impacts of two relatively recent introductions of nonindigenous mollusks have been more widely recognized in North America; namely, zebra mussels *Dreissena polymorpha* and quagga mussels *Dreissena bugensis* [7–9]. Zebra mussels, for example, were first noted in the Laurentian Great Lakes in the 1980's [10,11] and are not only a major biological threat to native mussel species [12–14], but are also a significant biofouling problem to aquatic infrastructure, costing an estimated $1 billion (US) annually in the United States in damages and control measures [15].

Hydroxide alkalinity has been shown to be a very effective antimicrobial chemical in wastewater treatment and endodontics [16–18]. For example, in effluent from an activated-sludge plant that was adjusted with lime [Ca(OH)$_2$] to an average pH 11.1, Grabow et al. [17] showed reductions of 99.98% in total coliforms, 97.11% reduction in Enterococci, and 100% in enteric viruses with a retention time of approximately 50 min. Similarly, Grabow et al. [16] demonstrated greater than 99% reduction in Gram-negative bacteria in humus tank effluent that was lime-adjusted to pH 11.5 for 1 h.

We are exploring the use of hydroxide alkalinity (i.e. with chemical addition of sodium hydroxide) as a ballast decontaminant to meet or exceed the D2 Standards in decontaminating organisms, along with other important criteria associated with its use including cost effectiveness, mixing

characteristics, safety and ease of use for crew members, and neutralization. With the current study, we developed controlled laboratory procedures to evaluate the bactericidal efficacy of pH exposure in a range of pH 10.0–12.0 for exposure durations of up to 72 h to kill a variety of purified viable bacterial cultures including fish pathogenic bacteria that survive in and are transmitted via the water column, other common aquatic-inhabitant bacteria that may also be recovered from fish, and bacterial indicator organisms listed in D2 Standards; namely, *V. cholera* (an environmental isolate from fish), *E. coli* and intestinal Enterococci (i.e., *Enterococcus faecalis*).

Material and methods

A standard curve was developed using 0.2 μM filter sterilized 5 N sodium hydroxide (NaOH; Sigma–Aldrich, Co., St. Louis, MO, USA) in the test bacteriological medium used for growth of the cultures, which was steam-sterilized (standard parameters: 121 °C, 15 psi, 15 min) tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA). Three replicates were prepared with NaOH incorporated into the medium using a buret and the pH determined with a Denver Instruments Model 215 meter (Arvada, CO, USA). Volumes of NaOH were recorded at 9 pH intervals per replicate within pH ranges of approximately pH 7.3–12.49. Standard curves were developed using 500 mL volumes of TSB, and all subsequent preparations of TSB for controls and testing of cultures were completed using volumes of 500 mL. The data were analyzed using Tablecurve 2D 5.0 (AISN Software, Inc., Chicago, IL. USA) with $r^2 > 0.999$. Volumes of 5 N NaOH were determined from the standard curve to yield desired pH adjustments in TSB. The reproducibility of these pH curves was confirmed by adding specific volumes of 5 N NaOH and comparing the resultant pH values, measured with the Model 215 meter. Measured pH values were consistently within ± 0.04 pH units of each other.

In an effort to simplify the preparation and distribution of pH adjusted TSB and to ensure test cultures were challenged with the same media, the 500 mL volumes were distributed as 50 mL volumes into pre-sterilized 250 mL Erlenmeyer flasks for all tests. The effect of autoclaving the TSB after pH adjustment was assessed at pH 10.0, pH 11.0 and pH 12.0. After the media cooled, the pH was determined using the Model 215 meter; the values consistently were much lower than those prior to sterilization. In one of the tests for example, TSB adjusted to pH 10.0 was pH 9.37 after autoclaving. The pH adjusted media was significantly darkened after autoclaving, particularly so at higher adjusted pH values. Therefore, all subsequent testing with cultures was done using TSB that was pH adjusted after sterilization.

Thirty-one bacterial isolates of 15 different species/taxonomic groupings were used in this study (Table 1). When initially recovered, the purity of each isolate was ensured by streak-plating and transfer of single colonies to fresh media, typically TS agar or brain heart infusion agar (BHI; Becton, Dickinson and Company, Sparks, MD, USA). Isolates were archived at −70 °C in fresh broth supplemented with 20% glycerol that was used to wash log phase growth of bacteria on slanted TS or BHI agar culture media. Isolates were stored

Table 1 Origins of IMO (International Maritime Organization) D2 Standards and fish pathogenic bacteria used for evaluation of the bactericidal activity of pH 10.0, pH 11.0 and pH 12.0 tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) adjusted with 5 N sodium hydroxide (NaOH).

Bacterial isolate	Origin or source
IMO D2 Standards indicators	
Escherichia coli NM554	Provided by Dr. R.K. Cooper, II, Department of Veterinary Science, Louisiana State University, Baton Rouge, Louisiana, USA
E. coli JM109	Provided by Dr. R.K. Cooper, II, Department of Veterinary Science, Louisiana State University, Baton Rouge, Louisiana, USA
E. coli HB101	Provided by Dr. T. Aoki, Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan
E. coli 1932	Provided by Dr. R.E. Wooley, Department of Infectious Diseases, University of Georgia, Athens, Georgia, USA [47]
Enterococcus faecalis	Striped bass *Morone saxatilis*; Delaware Bay, USA; 2004
Vibrio cholera	Razorback sucker *Xyrauchen texanus*; New Mexico, USA; 1998
Gram-negative pathogenic bacteria	
Aeromonas salmonicida subsp. *salmonicida* 3.139	Atlantic salmon *Salmo salar*; West Virginia, USA; 1998
A. salmonicida subsp. *salmonicida* F1	Brown trout *Salmo trutta*; Maryland, USA; 2007
A. salmonicida subsp. *salmonicida* F2	Brown trout *Salmo trutta*; Maryland, USA; 2007
A. salmonicida subsp. *salmonicida* K1	Brown trout *Salmo trutta*; Maryland, USA; 2007
Aeromonas veronii bv. *sobria* T2	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. veronii bv. *sobria* T6a	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. veronii bv. *sobria* T13b	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
Aeromonas hydrophila F15b	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. hydrophila T21b	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. hydrophila F21a	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
Aeromonas caviae F4	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. caviae T13a	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
A. caviae T4	Ebonyshell *Fusconaia ebena*; Alabama, USA; 2008 [48]
Edwardsiella ictaluri 6051	Channel catfish *Ictalurus punctatus*; Mississippi, USA; 1984 [37]
E. ictaluri 6075	Channel catfish *Ictalurus punctatus*; Mississippi, USA; 1987 [37]
E. ictaluri Bio027 K	Channel catfish *Ictalurus punctatus*; Mississippi, USA; 1992 [37]
Yersinia ruckeri 11.34	Rainbow trout *Oncorhynchus mykiss*; Colorado, USA; 1977
Y. ruckeri 11.40	Rainbow trout *Oncorhynchus mykiss*; North Carolina, USA; 1978
Y. ruckeri 11.47	Rainbow trout *Oncorhynchus mykiss*; Colorado, USA; 1978
Serratia liquefaciens	Arctic char *Salvelinus alpinus*; West Virginia, USA; 2000 [35]
Serratia marcescens	National Fish Health Research Laboratory Collection; origin unknown
Pseudomonas fluorescens	Rainbow trout *Oncorhynchus mykiss*; Nevada, USA; 1991 [23]
Gram-positive pathogenic bacteria	
Staphylococcus sp.	White sucker *Catostomus commersonii*; West Virginia, USA; 2009
Carnobacterium sp.	Rainbow trout *Oncorhynchus mykiss*; Idaho, USA; 1989 [36]
Bacillus sp.	National Fish Health Research Laboratory Collection; origin unknown

in cryovials containing 0.5 mL of washed cells. The bacteria were characterized using standard biochemical–phenotypic methods and comparison of results with published phenotypic line data [19–37].

To maximize consistency in the number of viable colony forming units (cfu) in control and test flasks at the start (i.e. initial cfu/mL at time 0 h) of each trial, a standard method to recover the isolates from frozen storage was developed and used. The contents of one cryovial were used to inoculate 5 mL of TSB, which was incubated at room temperature (approximately 21–22 °C) for 48 h. A fresh 5 mL TSB was inoculated with 0.5 mL of the 48 h culture, which was also incubated at room temperature for 48 h. The inoculum for the control TSB (pH 7.3) and test pH flasks (pH 10.0, pH 11.0, pH 12.0) came from the second 48 h culture. Control and test flasks were inoculated with 1% (v/v; 0.5 mL + 50 mL) of the inoculum and incubated at optimum culture growth temperatures ranging from 21 to 35 °C (Table 2) on a rotary shaker (Innova 2050 Platform Shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 120 rpm. The number of viable cells in each flask was determined after 0, 4, 12, 24, and 48 h; if not bactericidal, 72 h cell counts were done. Viable

Table 2 Viable cell counts (cfu/mL) of various bacterial cultures in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) at neutral pH (control) and adjusted to pH 10.0, pH 11.0 and pH 12.0 using 5 N sodium hydroxide (NaOH). Sampling times after (0–72 h) hours of incubation and incubation temperatures (21–35 °C) for testing as indicated.

Bacterium and temperature	Hours	Control cfu/mL	pH 10.0 cfu/mL	pH 11.0 cfu/mL	pH 12.0 cfu/mL
Escherichia coli (n = 4)[a] 35 °C	0	4.93×10^6 (4.60×10^6–5.20×10^6)	4.93×10^6 (Same as Control)[b]	4.93×10^6	4.93×10^6
	4	2.49×10^8 (4.60×10^7–6.20×10^8)	0.00×10^0	0.00×10^0	0.00×10^0
	12	9.40×10^8 (3.87×10^8–1.60×10^9)	5.00×10^0 (0.00–2.00×10^1)	0.00×10^0	0.00×10^0
	24	1.42×10^9 (1.00×10^9–2.13×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
	48	1.53×10^9 (1.47×10^8–2.67×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
Vibrio cholera 21 °C	0	1.21×10^6	1.21×10^6	1.21×10^6	1.21×10^6
	4	1.80×10^7	0.00×10^0	0.00×10^0	0.00×10^0
	12	1.16×10^9	0.00×10^0	0.00×10^0	0.00×10^0
	24	1.28×10^9	0.00×10^0	0.00×10^0	0.00×10^0
	48	3.00×10^9	0.00×10^0	0.00×10^0	0.00×10^0
Enterococcus faecalis 30 °C	0	2.44×10^7	2.44×10^7	2.44×10^7	2.44×10^7
	4	4.60×10^8	2.22×10^7	4.60×10^5	5.20×10^4
	12	8.60×10^8	1.58×10^7	4.00×10^5	3.40×10^4
	24	6.40×10^8	9.40×10^6	1.52×10^5	1.38×10^4
	48	6.00×10^8	5.80×10^6	5.60×10^3	8.60×10^2
	72	3.80×10^8	8.60×10^5	4.80×10^2	0.00×10^0
Aeromonas salmonicida subsp. *salmonicida* (n = 4) 21 °C	0	2.40×10^6 (5.80×10^5–3.47×10^6)	2.40×10^6	2.40×10^6	2.40×10^6
	4	2.01×10^7 (9.40×10^5–2.70×10^7)	1.50×10^1 (0.00–6.00×10^1)	0.00×10^0	0.00×10^0
	12	1.30×10^9 (1.78×10^6–2.27×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
	24	1.38×10^9 (4.27×10^8–2.53×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
	48	1.40×10^9 (9.47×10^8–1.87×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
Motile *Aeromonas* spp.[c] (n = 9) 21 °C	0	2.08×10^7 (3.60×10^6–5.00×10^7)	2.08×10^7	2.08×10^7	2.08×10^7
	4	3.36×10^8 (4.80×10^7–9.20×10^8)	2.30×10^7 (7.20×10^5–6.80×10^7)	1.34×10^6 (0.00–1.20×10^7)	1.78×10^3 (0.00–1.60×10^4)
	12	3.56×10^9 (2.00×10^9–6.00×10^9)	3.23×10^8 (4.00×10^2–1.16×10^9)	1.11×10^5 (0.00–1.00×10^6)	4.00×10^1 (0.00–3.60×10^2)
	24	7.64×10^9 (3.20×10^9–1.48×10^{10})	3.29×10^9 (4.00×10^1–1.32×10^{10})	0.00×10^0	0.00×10^0
	48	1.21×10^{10} (4.80×10^9–2.40×10^{10})	5.45×10^9 (1.20×10^3–1.84×10^{10})	0.00×10^0	0.00×10^0
Edwardsiella ictaluri (n = 3) 30 °C	0	4.10×10^6 (3.00×10^5–6.40×10^6)	4.10×10^6	4.10×10^6	4.10×10^6
	4	1.03×10^7 (3.60×10^6–1.60×10^7)	0.00×10^0	0.00×10^0	0.00×10^0
	12	6.27×10^8 (2.80×10^8–9.60×10^8)	0.00×10^0	0.00×10^0	0.00×10^0
	24	1.60×10^9 (1.20×10^9–2.00×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
	48	3.37×10^9 (9.20×10^8–6.00×10^9)	0.00×10^0	0.00×10^0	0.00×10^0
Yersinia ruckeri (n = 3) 25 °C	0	5.78×10^6 (4.20×10^6–7.40×10^6)	5.78×10^6	5.78×10^6	5.78×10^6
	4	4.45×10^8 (8.40×10^7–7.20×10^8)	2.35×10^6 (6.60×10^5–3.60×10^6)	1.68×10^3 (2.80×10^2–4.00×10^3)	0.00×10^0
	12	2.07×10^9 (1.60×10^9–2.40×10^9)	1.33×10^6 (8.00×10^1–2.40×10^6)	3.07×10^2 (0.00–5.20×10^2)	0.00×10^0
	24	5.89×10^9 (2.60×10^9–7.60×10^9)	2.54×10^5 (1.36×10^3–6.00×10^5)	2.00×10^2 (0.00–6.00×10^2)	0.00×10^0
	48	9.07×10^9 (5.60×10^9–1.32×10^{10})	5.33×10^2 (0.00–8.00×10^2)	2.67×10^2 (0.00–8.00×10^2)	0.00×10^0
Serratia liquefaciens 21 °C	0	1.54×10^7	1.54×10^7	1.54×10^7	1.54×10^7
	4	2.64×10^7	1.40×10^5	0.00×10^0	0.00×10^0
	12	3.20×10^9	1.20×10^2	0.00×10^0	0.00×10^0
	24	8.40×10^9	1.60×10^2	0.00×10^0	0.00×10^0
	48	5.00×10^9	1.20×10^2	0.00×10^0	0.00×10^0

Table 2 (*Continued*)

Bacterium and temperature	Hours	Control cfu/mL	pH 10.0 cfu/mL	pH 11.0 cfu/mL	pH 12.0 cfu/mL
Serratia marcescens 30 °C	0	1.40×10^6	1.40×10^6	1.40×10^6	1.40×10^6
	4	1.04×10^8	3.20×10^2	0.00×10^0	0.00×10^0
	12	3.00×10^9	2.00×10^1	0.00×10^0	0.00×10^0
	24	6.00×10^9	6.00×10^1	0.00×10^0	0.00×10^0
	48	9.00×10^9	6.40×10^2	0.00×10^0	0.00×10^0
Pseudomonas fluorescens 21 °C	0	3.00×10^6	3.00×10^6	3.00×10^6	3.00×10^6
	4	7.20×10^6	8.80×10^4	0.00×10^0	0.00×10^0
	12	1.12×10^9	2.40×10^3	0.00×10^0	0.00×10^0
	24	2.00×10^9	0.00×10^0	0.00×10^0	0.00×10^0
	48	5.20×10^9	0.00×10^0	0.00×10^0	0.00×10^0
Staphylococcus sp. 30 °C	0	2.12×10^6	2.12×10^6	2.12×10^6	2.12×10^6
	4	6.20×10^6	9.40×10^4	4.00×10^1	4.00×10^1
	12	3.60×10^7	9.00×10^4	0.00×10^0	0.00×10^0
	24	4.20×10^8	0.00×10^0	0.00×10^0	0.00×10^0
	48	2.00×10^9	0.00×10^0	0.00×10^0	0.00×10^0
Carnobacterium sp. 30 °C	0	7.80×10^5	7.80×10^5	7.80×10^5	7.80×10^5
	4	1.60×10^7	2.60×10^5	0.00×10^0	0.00×10^0
	12	3.00×10^8	2.60×10^5	3.60×10^2	0.00×10^0
	24	4.40×10^8	1.16×10^4	0.00×10^0	0.00×10^0
	48	3.20×10^8	7.80×10^6	0.00×10^0	0.00×10^0
Bacillus sp. 30 °C	0	3.40×10^4	3.40×10^4	3.40×10^4	3.40×10^4
	4	4.20×10^6	1.60×10^2	8.00×10^1	1.40×10^2
	12	2.20×10^8	1.20×10^2	1.00×10^2	4.00×10^1
	24	4.00×10^8	4.00×10^1	8.00×10^1	2.00×10^1
	48	3.40×10^8	1.00×10^2	8.00×10^1	4.00×10^1
	72	3.00×10^8	6.00×10^1	0.00×10^0	0.00×10^0

[a] Bacterial cell counts are mean cfu/mL (with ranges in parentheses) for the number of isolates (*n*) tested. Those without an (*n*) are counts for single isolates.

[b] For all bacteria, the ranges in cell counts at 0 h for pH 10.0, pH 11.0, and pH 12.0 were the same as the controls.

[c] Includes three isolates each of three species: *Aeromonas veronii* bv. *sobria*, *A. hydrophila*, and *A. caviae*.

cell numbers were determined by preparing serial 10-fold dilutions in TSB and placing 25 µL volumes from each dilution on the surface of TS agar plates. Serial dilutions were made from each control and pH test flask by removing 0.5 mL at each sampling time. Following incubation of the plates at optimum temperatures (Table 2), typically for 24–48 h, resulting colonies were enumerated and the number converted to cfu/mL by multiplication by dilution factors. Minimum parameters of pH and duration of exposure necessary to attain 100% bactericidal (i.e. killing) effect for each bacterial isolate were noted. Bactericidal effect was represented as "no growth apparent" on the surface of the TS agar plates at any dilution. At the same sample times (0–72 h) that were examined for viable cell counting, 0.15 mL were transferred from the control and pH test cultures to pre-cleaned microscope slides. The slides were allowed to air dry and were heat-fixed, and either simple- or Gram-stained [26,27]. Each slide was examined qualitatively for the presence of intact cells using a Nikon Alphaphot-2 light microscope (1000×; Fryer Company, Inc., Cleveland, OH, USA). One representative isolate from each bacterial species was examined. The absence of intact cells along with no recovery of cfu from the serial dilutions was indicative of lethal effects of hydroxide ions to the bacterial cells.

Results

Viable bacterial cell counts for various bacteria from 0 to 48 h, or 72 h for *E. faecalis* and *Bacillus* sp., in TSB control and increased pH test media are presented in Table 2. A 100% bactericidal (killing) effect to all Gram-negative and Gram-positive bacterial cultures evaluated in this study was achieved within the maximum parameters tested of pH 12.0 for up to 72 h of exposure. However, the pH and exposure necessary for 100% bactericidal effect varied among the bacteria tested.

Initial (time 0 h) cell numbers in control and high pH test cultures ranged from a minimum of 3.40×10^4 cfu/mL for *Bacillus* sp. to the greatest of 2.44×10^7 cfu/mL for *E. faecalis*. It can be noted from the cell counts determined at the sequential sampling times that the cultures grew exponentially. The greatest cell numbers from control flasks for Gram-negative bacteria were attained from the motile *Aeromonas* spp. at 48 h, mean = 1.21×10^{10} cfu/mL, whereas the greatest number from a Gram-positive bacterium was 2.00×10^9 cfu/mL from *Staphylococcus* sp. also after 48 h of incubation. Because of the high initial cfu/mL of the cultures, there was no lag in growth of the cultures from 0 to 4 h, which indicated their vigorous growth status. However, four of the control cultures showed slight decreases in cfu/mL following selected incubation

durations. This was an anticipated outcome and is typical of bacterial cultures and indicative of depletion of nutrients in the media. For example, viable cell numbers in the control culture of *E. faecalis* were reduced at 24 h (6.40×10^8 cfu/mL) compared with 12 h (8.60×10^8 cfu/mL) and again following 48 h (6.00×10^8 cfu/mL) and 72 h (3.80×10^8 cfu/mL) incubations. Similarly, *Serratia liquefaciens*, *Carnobacterium* sp., and *Bacillus* sp. cell counts decreased, but the reduced cell numbers from these were first noted after 48 h of incubation.

On two occasions, viable cell counts were recorded from increased pH test cultures after 12 h of incubation and both followed 4 h counts that no viable cells were detected (Table 2). The two occasions were *E. coli* at pH 10.0 (5.00×10^0 cfu/mL) and *Carnobacterium* sp. at pH 11.0 (3.60×10^2 cfu/mL). In both instances, the cultures were no longer viable after 24 and 48 h. This was not an unexpected outcome for bacterial culture kinetics studies involving dilution series and viable cell counting techniques as the low viable cell numbers are near the threshold for sensitivity of the enumeration techniques. Viable cell counts contrast with other cell counting techniques, such as absorbance readings, which do not distinguish live from dead cells.

No growth was noted at pH 10.0, pH 11.0, or pH 12.0 from any of the sampling times from *V. cholera* and *Edwardsiella ictaluri*. At 0 h, there was 1.21×10^6 cfu/mL in the *V. cholera* control and pH test cultures with the cell count of the control TSB increasing to 3.00×10^9 cfu/mL after 48 h of incubation. Similarly, the initial mean cell numbers of the control TSB *E. ictaluri* cultures increased from 4.10×10^6 cfu/mL at 0 h to 3.37×10^9 cfu/mL after 48 h, which also showed excellent growth responses.

Bactericidal effect was attained within 12 h for *Aeromonas salmonicida* subsp. *salmonicida*, and within 24 h for *E. coli* and *Pseudomonas fluorescens*, all at pH 10.0. No bacterial growth was detected from these three bacterial species from pH 11.0 or pH 12.0 test media. The corresponding mean viable cell count for *A. salmonicida* subsp. *salmonicida* in control TSB at 12 h was 1.30×10^9 cfu/mL. Cell counts from TSB control cultures of *E. coli* and *P. fluorescens* at 24 h were 1.42×10^9 cfu/mL and 2.00×10^9 cfu/mL, respectively.

Both *Serratia* spp., *S. liquefaciens* and *Serratia marcescens* grew through 48 h in pH 10.0 TSB, but neither species grew at all in pH 11.0 or pH 12.0 adjusted TSB. Growth of both bacterial cultures after 48 h at pH 10.0 was reduced by greater than seven log(10) dilutions compared to growth in control TSB. The cell count for *S. liquefaciens* after 48 h at pH 10.0 was 1.20×10^2 cfu/mL while the count in control TSB was 5.00×10^9 cfu/mL, which were similar to the viable cell counts recorded from *S. marcescens* cultures at pH 10.0 and control, 6.40×10^2 cfu/mL and 9.00×10^9 cfu/mL, respectively.

Growth of *Carnobacterium* sp. at pH 10.0 was noted through 48 h with cell numbers approximately two to four log(10) dilutions less than from paired control pH cultures sampled at the same times. Higher pH media were bactericidal within 4 h at pH 12.0 and within 24 h at pH 11.0. The viable cell count in pH 11.0 was 3.60×10^2 cfu/mL at 12 h compared to 3.00×10^8 cfu/mL from the control.

In the pH 10.0 TSB, viable *Staphylococcus* sp. cell counts were recorded at 4 and 12 h, but not following 24 and 48 h of incubation. This bacterium also grew at pH 11.0 and pH 12.0, but only after the 4 h incubation sampling. The 4 h viable cell counts, 4.00×10^1 cfu/mL, were the same at pH 11.0 and

pH 12.0 compared with 6.20×10^6 cfu/mL from the TSB control after 4 h.

The cultures of *Yersinia ruckeri* and motile *Aeromonas* spp. grew comparatively well at pH 10.0, although producing lower viable cell counts than the paired controls at those times. One exception was *Y. ruckeri* at 48 h, which grew poorly (mean = 5.33×10^2 cfu/mL) compared to the control mean of 9.07×10^9 cfu/mL. *Y. ruckeri* also grew at all sampling times through 48 h at pH 11.0, but with mean cell counts of five to seven log(10) dilutions reduced from controls; however, did not grow at all at pH 12.0. Motile *Aeromonas* spp. cultures were viable after 4 and 12 h of incubation at pH 11.0 and pH 12.0, with mean cell counts at pH 12.0 of 1.78×10^3 cfu/mL and 4.00×10^1 cfu/mL at 4 and 12 h, respectively, relative to the mean counts of 3.36×10^8 cfu/mL and 3.56×10^9 cfu/mL in controls, respectively.

Incubation times greater than 48 h at pH 11.0 or pH 12.0 were necessary to be bactericidal for *E. faecalis* and *Bacillus* sp. Cell counts from *E. faecalis* cultures consistently decreased as the pH of the medium increased. For example, after 48 h, the cell count from the control was 6.00×10^8 cfu/mL whereas cell counts from pH 10.0, pH 11.0, and pH 12.0 were 5.80×10^6 cfu/mL, 5.60×10^3 cfu/mL, and 8.60×10^2 cfu/mL, respectively. The only test pH and sample time in which *E. faecalis* did not grow was after 72 h of incubation at pH 12.0. In contrast to the gradually reduced *E. faecalis* cell counts in higher pH media, all of the cell counts from *Bacillus* sp. were 1.60×10^2 cfu/mL or lower regardless of pH and duration of exposure. Growth was not noted at 72 h from *Bacillus* sp. cultures at pH 11.0 or pH 12.0.

Microscopy for intact bacterial cells was done for control and increased pH test cultures for ten bacteria. Intact cells were observed from all cultures (40 total) immediately following the inoculations (0 h). Intact cells were also observed from the pH control cultures at all sample collection times through 72 h. No cells were noted after 4 h or 12 h from increased pH cultures from *V. cholera*, *E. ictaluri* 6051, *A. salmonicida* 3.139, *E. coli* 1932 and *S. marcescens*. Similarly, no cells were detected from *Aeromonas hydrophila* F15b, *Y. ruckeri* 11.34, *E. faecalis*, *Bacillus* sp. or *Staphylococcus* sp. in pH 11.0 and pH 12.0 cultures typically at the next timed sampling following the last sampling that viable cells (i.e., cfu) were recovered on the TS agar plates.

Discussion

The minimum endpoints of pH and treatment duration necessary to achieve 100% bactericidal effect to the bacteria tested varied. However, all bacteria were affected within pH 12.0 and 72 h. The lowest test parameters of 4 h at pH 10.0 were bactericidal to many of the bacteria, including IMO D2 Standards isolates *E. coli* (three of four isolates were killed within 4 h) and *V. cholera*, as well as three of four isolates of *A. salmonicida* subsp. *salmonicida* and *E. ictaluri*. An assessment of the bactericidal effects to these bacterial cultures was completed with cultures ranging from 1.21×10^6 cfu/mL for *V. cholera* to a mean of 4.93×10^6 cfu/mL for *E. coli* at the initiation (time 0 h) of the trials (Table 2). The other IMO D2 Standards bacterium, *E. faecalis*, a Gram-positive and common fecal indicator organism, required 72 h at pH 12.0 to be bactericidal. Relative of all bacteria tested, the Gram-positive bacteria, one of the enteric bacteria (*Y. ruckeri*) and the motile

Aeromonas spp. were more tolerant to the conditions of increased pH adjusted growth media. Other enterics, for example *E. coli* and *Serratia* spp. were more sensitive.

Y. ruckeri, the cause of redmouth disease principally to rainbow trout *Oncorhynchus mykiss* [22], was specifically chosen for increased pH evaluations due to it's high-pH tolerance [38]. This provided a robust evaluation of increased pH as a potential effective bactericidal agent. Resistance of *Y. ruckeri* to high pH was highlighted with the differential primary isolation medium (SW) [39], which was described with a final pH 7.4. The recipe for this medium was published with the pH indicator bromthymol blue at 0.0003%, whereas the correct concentration should be 0.003% [40]. Quenching of the medium's color from the desired blue-green to yellow (i.e., decreased pH) masked the differentiation of the bacterial colonies based on carbohydrate utilization. In addition to use of the correct bromthymol blue concentration, the solution to the medium color problem was to adjust the pH of the medium "to color", which resulted in pH 9.0–9.5 [38]. This adjustment also aids the primary recovery of *Y. ruckeri* from fish because the high pH of SW is selective against some contaminating bacteria.

Gram-positive bacteria were included in the present study because of increased resistance to the lytic action of mild solutions of lye relative to Gram-negative bacteria [41]. The composition of the bacterial cell walls of Gram-positive and Gram-negative bacteria, which imparts the differential resistances to lye form the basis of the 3% KOH (i.e., potassium hydroxide) Gram reaction; a diagnostic test useful for characterizations of bacteria [42,43]. Results of the present study confirmed that Gram-positive bacteria are relatively tolerant to increased pH (Table 2), for example, *E. faecalis* requiring greater than 48 h at pH 12.0 to be bactericidal and *Bacillus* sp. also requiring greater than 48 h at pH 11.0 and pH 12.0 for bactericidal efficacy.

The pH comparative studies in the present study were conducted in TS broth medium. The bactericidal efficacy imparted by the alkaline pH concentrations were due to the action of the hydroxide ions instead of substantial alterations to the nutritional value of the medium components. One indicator of this was the survival or growth of those bacterial isolates which were anticipated to persist at the higher pH concentrations, including *Y. ruckeri* and certain Gram-positive isolates (Table 2). Another indication of the effects of hydroxyl ions was the inability to detect intact cells after staining samples taken from the high pH concentration test cultures after the times in which viable cells were recovered. Furthermore, plating the serial dilutions on pH 7.3 TS agar provided an opportunity for cells in the high pH concentration cultures to recover and grow if the cells were lacking essential nutrients in the high pH TS broth due to nutrient degradation. Hydroxide ions may impart several lethal effects to bacterial cells, including destruction of phospholipids, which are structural components of cell membranes, destruction of bonds of essential metabolic enzymes and loss of tertiary structure, and destruction of DNA [18].

Increased pH treated ballast water will not have an effect to the environment or to the aquatic ecosystem. Actual treatment of the ballast water with sodium hydroxide occurs within the ballast tanks and the treated water must be neutralized and returned to ambient pH prior to its deballasting. In the United States, for example, the pH of the deballasted water is regulated by the appropriate ruling regulatory authority. The present study was designed to determine the bactericidal efficacy of increased pH by use of sodium hydroxide against a variety of fish pathogenic and environmental bacteria. As a robust evaluation, the bacteria were purposely grown using optimal culture conditions, including the use of a high nutrient medium and laboratory-controlled temperatures. This study was one of a larger research project with the goal to establish a safe and effective ballast water treatment. Other studies in progress include the use of carbon dioxide as the pH neutralizing agent, thorough mixing dynamics of the chemicals in the water within ballast tanks, process and cost economics, and sodium hydroxide treatment efficacy of actual ballast water (fresh and saline), sediment and mixed bacterial populations.

It was imperative for this study to demonstrate viable and vigorous culture growth for each bacterium in the control, pH-neutral TSB, because it ensured that the inoculums for the increased pH testing were viable and, therefore, would quickly reach log phase growth. The high initial (0 h) cfu/mL selected for the cultures was done to eliminate or greatly minimize the lag in culture growth, which is often typical of broth cultures in their early stages of growth. This was particularly important to show for those cultures in which the lowest test parameters of pH (10.0) and duration (4 h) proved to be bactericidal. Also, for those bacterial cultures requiring higher pH or longer durations of exposure to be bactericidal, vigorous growth of the controls served as comparisons to show percent reductions in cfu/mL in the samples while leading up to 100% killing. Although the objective for this study was to demonstrate 100% eradication of each bacterial culture, the percent reductions in cfu/mL from high pH cultures were significant relative to the paired controls. For example, the mean cell count for *Y. ruckeri* from controls at 48 h was 9.07×10^9 cfu/mL (Table 2); however, at pH 10.0 the mean was 5.33×10^2 cfu/mL, which was greater than 7 log(10) dilutions less (>99.99% reduction).

The technologies to treat ballast water are typically derived from proven municipal and other industrial applications [6]. For example, increased pH from the incorporation of lime, has been used for many years at water treatment plants as a very effective agent for the elimination of coliform bacteria from effluent waters [17,44–46]. Van Arnum [45] provided a report on the use of lime at the Youngstown, OH (USA) water treatment facility to cleanse waters with a presumptive coliform bacteria index of 1.00×10^5 cfu/100 mL. Following treatment with lime dosages that yielded approximately 10 ppm causticity (i.e., excess lime treatment; pH not given), it was often shown that no gas-forming bacteria (e.g. coliforms) were detected after a 3.5 h detention. In another study, Wattie and Chambers [46] evaluated lime as a bactericidal agent to selected enteric bacterial pathogens common in untreated water, including *E. coli*. Pure cell suspensions of viable bacteria were added to pH-adjusted, sterilized water to initial (0 h) cell densities of approximately 1.50×10^3 cfu/mL. At pH 10.01–10.5, 8.67 h was necessary to obtain a 100% kill at 20–25 °C; whereas, 3.5 h was required at pH 11.01–11.5. This temperature range that the *E. coli* cultures were tested, which was lower than the temperature used in the present study, would be anticipated to increase the duration of high pH exposure to achieve complete bactericidal effect. In the present study, *E. coli* cultures were tested at 35 °C, with 100% bactericidal effect demonstrated in under 4 h with three of four isolates at pH 10.0, and for all isolates at pH 11.0.

Conclusion

A bacterial growth medium having the pH adjusted with sodium hydroxide to pH 10.0–12.0 proved to be an inhospitable environment for a variety of Gram-negative and Gram-positive bacteria. All of the bacteria tested were affected to some extent even at the lowest pH (10.0) evaluated as shown by the reduction in viable cell counts; pH 12.0 for 72 h was bactericidal for all isolates examined.

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