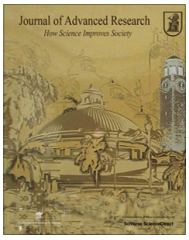
Journal of Advanced Research (2015) 6, 501–509

Cairo University

Journal of Advanced Research



ORIGINAL ARTICLE

Efficacy of pH elevation as a bactericidal strategy for treating ballast water of freight carriers

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ARTICLE INFO

Article history:
Received 17 December 2014
Received in revised form 13 February 2015
Accepted 23 February 2015
Available online 6 March 2015

Keywords:
Ballast water
Nonindigenous
Bacteria
pH
Treatment

ABSTRACT

Treatment of ship ballast water with sodium hydroxide (NaOH) is one method currently being developed to minimize the risk to introduce aquatic invasive species. The bactericidal capability of sodium hydroxide was determined for 148 bacterial strains from ballast water collected in 2009 and 2010 from the M/V Indiana Harbor, a bulk-freight carrier plying the Laurentian Great Lakes, USA. Primary culture of bacteria was done using brain heart infusion agar and a developmental medium. Strains were characterized based on PCR amplification and sequencing of a portion of the 16S rRNA gene. Sequence similarities (99+ %) were determined by comparison with the National Center for Biotechnology Information (NCBI) GenBank catalog. *Flavobacterium* spp. were the most prevalent bacteria characterized in 2009, comprising 51.1% (24/47) of the total, and *Pseudomonas* spp. (62/101; 61.4%) and *Brevundimonas* spp. (22/101; 21.8%) were the predominate bacteria recovered in 2010; together, comprising 83.2% (84/101) of the total. Testing was done in tryptic soy broth (TSB) medium adjusted with 5 N NaOH. Growth of each strain was evaluated at pH 10.0, pH 11.0 and pH 12.0, and 4 h up to 72 h. The median cell count at 0 h for 148 cultures was 5.20×10^6 cfu/mL with a range 1.02×10^5–1.60×10^8 cfu/mL. The TSB adjusted to pH 10.0 and incubation for less than 24 h was bactericidal to 52 (35.1%) strains. Growth in pH 11.0 TSB for less than 4 h was bactericidal to 131 (88.5%) strains and pH 11.0 within 12 h was bactericidal to 141 (95.3%). One strain, *Bacillus horikoshii*, survived the harshest treatment, pH 12.0 for 72 h.

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Peer review under responsibility of Cairo University.

Introduction

Due to their small size and high densities, microbes have a relatively high potential to be translocated with ballast water compared to other larger aquatic-borne species [1]. Bacterial asexual reproduction, ability to adapt, possible alternative resting stages (e.g., spores), and survival outside of a host

http://dx.doi.org/10.1016/j.jare.2015.02.005
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are a partial list of factors that may contribute to their dispersal or transmission [1], including via ships' ballast water [2–6]. An example of the volume of bacterial cells dispersed was provided in a study by Ruiz et al. [4], in which they showed that samples of ballast water from ships arriving at Chesapeake Bay, USA contained an average of 8.30×10^5 bacteria per mL. They provided an estimate that 1.20×10^{10} L of ballast water was received in the Bay in 1991; therefore, there is a real threat that a bacterium could survive and multiply. McCarthy and Khambaty [2] conducted a study of nonpotable water from ships docked at various ports in the Gulf of Mexico, USA. *Vibrio cholerae* was recovered from ballast water collected from several of the ships. Analyses of these isolates showed that they were indistinguishable from a Latin America *V. cholerae* epidemic strain, thus showing that ships can facilitate the international dissemination of pathogenic bacteria.

Elevated pH is one solution being developed at the U.S. Geological Survey to decontaminate ship ballast water to eliminate or greatly reduce the risk of transporting and introducing nonindigenous organisms. Under this scenario, the pH of the ballast water will be elevated on-board-ship through the introduction of hydroxide alkalinity, such as sodium hydroxide, in which the appropriate amount of hydroxide (i.e., hydroxyl – OH) may be added during ballasting such that an effective dose or pH is achieved and the water and hydroxide are uniformly mixed. A contact time of hydroxide with the targeted waterborne biota will be necessary to produce the desired decontamination. In a previous study by Starliper and Watten [7], minimum parameters of pH and contact duration to produce 100% bactericidal effects were determined for a suite of fish pathogenic and environmental bacteria and Regulation D2 standards indicator bacteria [8]. Controlled laboratory studies were developed and employed with pure bacterial cultures to determine bactericidal parameters. A variety of Gram-negative and Gram-positive bacteria were tested to create a robust evaluation of the efficacy of sodium hydroxide. High initial bacterial loads or colony forming units (cfu/mL) were also a part of the study design to minimize typical lag-phase culture growth. Initial time 0 h viable cell counts ranged from 3.40×10^4 cfu/mL to 2.44×10^7 cfu/mL and strains were grown in optimal growth conditions. At pH 12.0 for 72 h or less, which were the harshest parameters tested, sodium hydroxide was 100% bactericidal to all of the bacteria tested. However, a lower sodium hydroxide concentration was bactericidal to many bacteria. For example, pH 10.0 was 100% bactericidal to fish pathogenic *Aeromonas salmonicida* subsp. *salmonicida*, *Edwardsiella ictaluri*, *Pseudomonas fluorescens* and *Staphylococcus* sp., and to two Regulation D2 indicators, *Escherichia coli* and *V. cholerae*.

In the present study, the bactericidal capacity of sodium hydroxide was further evaluated by testing bacteria that were recovered from ballast tank water from the American Steamship Company's (Williamsville, NY, USA) M/V Indiana Harbor, a bulk-freight hauling vessel that operates on the Laurentian Great Lakes, USA.

Material and methods

Water samples

The M/V Indiana Harbor is a bulk material carrying vessel with a capacity of 72,575 metric tons. This ship is 310 m long and 30 m wide, and has seventeen separate ballast tanks, which are connected by a series of pipes and valves. Two water samples were collected in April 2009 from the "No. 3" (3-P) ballast tank on the port side. Ballast tank 3-P has a capacity of 4765.8 m^3 and was filled with water from southern Lake Michigan near Gary, IN, USA. The water samples were collected immediately after deballasting and when the vessel was loaded with cargo. The samples were collected by dipping sterile 125 mL bottles into pools of water that remained in the ballast tank, which is typical after deballasting.

In May 2010, sixteen ballast water samples were collected from two different ballast tanks on the M/V Indiana Harbor (Table 1). Eight water samples were collected from the no. 4 port (4-P) ballast tank; water to fill this tank was from Lake Michigan and taken on board near Gary, IN, USA. Eight water samples were also from the no. 4 starboard (4-S) ballast tank; water for this ballast tank was a mixture (proportions unknown) from Lakes Michigan, Huron and Superior. Both ballast tanks were full when the water samples were collected. Sampling points were set up throughout the water columns within the ballast tanks, which were plumbed with tygon tubing to a manifold for ease of sampling. To collect the water samples, each valve was opened and water was allowed to run for 2–3 min, then the sample was collected using a clean-catch method in a sterile 125 mL bottle. Water samples were kept on ice until bacterial sampling was done within 4 h.

Bacterial cultures

A series of ten-fold dilutions was prepared for each water sample in 0.1% tryptone-0.05% yeast extract (pH 7.2; Becton, Dickinson and Company, Sparks, MD, USA). Volumes (0.025 or 0.15 mL) of each sample dilution, including the undiluted water sample, were used to inoculate the surfaces of two media prepared in petri plates, brain heart infusion agar (BHIA; Becton, Dickinson and Company, Sparks, MD, USA) and a developmental medium consisting of 0.5% tryptone, 0.05% yeast extract, 0.05% beef extract, 1.5% agar (all sourced from Becton, Dickinson and Company, Sparks, MD, USA), 0.028% sodium acetate trihydrate, 0.02% calcium chloride dihydrate, and 0.074% magnesium sulfate heptahydrate (pH 7.2; all sourced from Sigma–Aldrich, Company, St. Louis, MO, USA). Both BHIA and the developmental medium are general growth media and neither was expected to culture certain bacteria that the other would not. The inoculated plates were incubated aerobically at 21–22 °C until the resulting bacterial colonies were distinguishable; within 3 d. Bacterial colonies were enumerated from those plates having the lowest water sample dilutions with isolated colonies. Bacterial counts were reported as colony forming units per mL (cfu/mL) of water after multiplication of all sample dilution factors. Single bacterial colonies representative of all colony morphologies recovered were transferred to fresh homologous media for growth. Each strain was transferred to a 5 mL homologous medium agar slant in a 16×125 mm tube for growth. The bacterial growth from each slant was loosened by pipetting and suspended in 5 mL of a freezing medium. Strains were archived at −70 °C in sterile cryovials containing 0.5 mL of suspended cells per vial. The freezing medium consisted of the developmental medium previously described minus the agar and supplemented with 20% glycerol (Becton, Dickinson and Company, Sparks, MD, USA).

Table 1 Bacterial cell counts (cfu/mL) from M/V Indiana Harbor ballast water samples in 2010 from developmental medium incubated aerobically at 21 °C.

Ballast water sample number	cfu/mL	Ballast water sample number	cfu/mL
4P-B2	9.00×10^3	4S-B2	3.50×10^4
4P-B3	1.55×10^4	4S-B3	1.10×10^4
4P-B4	1.30×10^4	4S-B4	3.00×10^4
4P-B5	1.15×10^5	4S-B5	2.00×10^4
4P-D1	3.50×10^3	4S-D1	2.50×10^4
4P-D2	7.00×10^3	4S-D3	2.50×10^4
4P-D3	3.50×10^4	4S-E1	7.50×10^3
4P-E1	9.50×10^4	4S-E2	6.50×10^3
Mean ($n = 16$)	2.83×10^4		
Standard deviation	3.08×10^4		

Strains were archived until they were recovered for identifications and sodium hydroxide testing.

Bacterial characterizations

Bacteria were characterized using a polymerase chain reaction (PCR) that targeted a portion of the 16S rRNA gene. Bacterial DNA was extracted using the DNA blood and tissue kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's methods. DNA was stored at 4 °C prior to amplification. For PCR amplification, a PCR cocktail consisting of 1 μM of each of the following primers, F63 (5′ – CAG GCC TAA CAC ATG CAA GTC – 3′) and R1389 (5′ – AGC GGC GGT GTG TAC AAG – 3′) [9,10] was added to GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA). The universal bacterial primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). The PCR cycling profile consisted of a 2 min denaturation step at 94 °C, 35 cycles of 45 s at 94 °C, 30 s at 58 °C, 2 min at 72 °C, and a 7 min extension at 74 °C. PCR success was verified by subjecting 5 μL of each PCR product to electrophoresis at 90 V for 2 h on a gel containing 1.2% I.D.NA® agarose (FMC Bioproducts, Rockland, ME, USA).

The PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Sequencing was done using Applied Biosystems Big Dye Cycle Sequencing Kit (Foster City, CA, USA) according to the manufacturer's instructions for both the forward and reverse primers. The samples were then subjected to a PCR cycling profile: 25 cycles of 30 s at 96 °C, 15 s at 58 °C, and 4 min at 60 °C, and a 10 min extension at 72 °C. The PCR sequencing reactions were cleaned with Agencourt CleanSEQ (Beckman Coulter Genomics, Beckman Coulter Inc., Brea, CA, USA) and loaded onto an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA, USA). Amplicons were sequenced in both directions, aligned, and analyzed with BioEdit software. Amplified PCR fragments were cropped to yield sequences of approximately 910 base pairs in length. Sequences were compared to the National Center for Biotechnology Information (NCBI) GenBank catalog for taxonomic identifications. Similarities of 99% or greater of ballast water strain sequences to GenBank sequences led to the identifications.

Sodium hydroxide testing

Bactericidal testing of sodium hydroxide (NaOH) to the bacterial strains was done in tryptic soy broth medium (TSB; Becton, Dickinson and Company, Sparks, MD, USA); TSB was used in the development of the standard curve with 5 N NaOH as previously described [7]. For consistency, the TSB was always prepared in volumes of 500 mL. The pH of unadjusted TSB for growth of controls was pH 7.3 ± 0.2; whereas, the pH-test media were adjusted using volumes of 5 N NaOH (Sigma–Aldrich, Company, St. Louis, MO, USA) that were previously determined from the standard curve. The TSB was autoclave-sterilized and allowed to cool to room temperature, then appropriate volumes of 0.2 μm filter sterilized 5 N NaOH were added to yield pH 10.0, pH 11.0 and pH 12.0 batches of TSB. For example, 1.007 mL of 5 N NaOH in 50 mL TSB yielded pH 12.0; this change in volume was considered insignificant. Reproducibility of accurate pH-adjusted TSB was confirmed in a previous study [7]. Fifty-mL volumes of control and pH-adjusted TSB were aseptically distributed into pre-sterilized 250-mL Erlenmeyer flasks. Each bacterial strain was recovered from low temperature storage using the standard method described by Starliper and Watten [7]. There was a 100% recovery rate of strains from frozen archive. Four flasks were inoculated with 1% inoculum (0.5 mL + 50 mL) prepared from each strain, one control and one each of the three pH-adjusted TSB's. Strains were incubated by placing the flasks on a rotary shaker (Innova 2050, New Brunswick Scientific Co., Inc., Edison, NJ, USA) set at 120 rpm and 21–22 °C. The cfu/mL in the culture flasks were determined using counting techniques similar to that previously described. Bacteria were diluted ten-fold in TSB and 0.025 mL volumes of all dilutions were placed on the surfaces of TS agar medium (pH 7.3 ± 0.2; Becton, Dickinson and Company, Sparks, MD, USA). Plates were incubated at 21–22 °C and resulting colonies were enumerated as described previously. The cfu/mL were determined at 0 h (initial) and after 4, 12, 24 and 48 h incubation; additionally, in 2010, cfu/mL were enumerated after 72 h. Minimum pH and duration of exposure (h) were recorded after 100% bactericidal effect was noted from each culture flask as indicated by the absence of bacterial colonies on TS agar inoculated with the dilution series. Durations were reported as less than (<) the hours of

Table 2 Identification of bacteria recovered from M/V Indiana Harbor ballast water in 2009, time 0 h cell counts of controls and pH test cultures, highest cell counts from controls during 48 h, and minimum (100%) bactericidal parameters of pH 10, pH 11 or pH 12 and exposure duration (h).

Identification (*n*)[a]	Accession(s)[b]	Time 0 h cfu/mL median; range	Control highest cfu/mL median; range	Bactericidal pH/h (number of cultures)
Flavobacterium xinjiangense (9)	200, 201, 203, 204, 207, 210, 212, 218, 219	1.49×10^6; 2.82×10^4–1.90×10^7	1.35×10^9; 4.00×10^8–7.60×10^9	10/ < 4 (3) 10/ < 24 (3) 11/ < 4 (3)
Flavobacterium psychrolimnae (2)	205, 211	1.61×10^6; 1.58×10^6–1.63×10^6	2.20×10^9; 2.00×10^9–2.40×10^9	10/ < 4 (1) 11/ < 4 (1)
Flavobacterium sinopsychrotolerans (2)	213, 216	2.10×10^7; 2.38×10^6–3.96×10^7	5.00×10^9; 2.80×10^9–7.20×10^9	11/ < 4 (1) 11/ < 12 (1)
Flavobacterium frigidimaris (1)	185	2.38×10^6	2.80×10^9	10/ < 4
Flavobacterium limicola (1)	220	2.04×10^6	2.80×10^9	10/ < 4
Flavobacterium soli (1)	206	9.39×10^6	1.88×10^{10}	10/ < 24
Flavobacterium pectinovorum (1)	208	5.54×10^4	5.20×10^7	10/ < 4
Flavobacterium sp. (7)	183, 192, 196–198, 214, 215	3.96×10^6; 9.90×10^5–2.38×10^7	4.80×10^9; 1.20×10^9–1.04×10^{10}	10/ < 4 (6) 11/ < 4 (1)
Pseudomonas putida (1)	182	9.11×10^5	3.20×10^9	10/ < 4
Pseudomonas fluorescens (1)	186	3.09×10^6	4.80×10^9	11/ < 24
Pseudomonas migulae (1)	188	9.50×10^6	2.00×10^9	10/ < 4
Pseudomonas gessardii (1)	191	6.34×10^6	1.04×10^{10}	11/ < 4
Pedobacter koreensis (1)	209	3.96×10^6	4.40×10^8	10/ < 24
Pedobacter sp. (3)	199, 217, 221	2.06×10^7; 1.02×10^b–2.38×10^7	5.20×10^9; 7.60×10^3–8.00×10^9	10/ < 4 (1) 11/ < 4 (2)
Janthinobacterium lividum (2)	181, 193	3.35×10^7; 3.56×10^6–6.34×10^7	5.60×10^9; 4.40×10^9–6.80×10^9	10/ < 4 (2)
Psychrobacter psychrophilus (1)	195	6.34×10^5	2.80×10^8	10/ < 24
Psychrobacter sp. (1)	184	1.98×10^5	2.80×10^9	10/ < 12
Arthrobacter sulfureus (1)	187	1.19×10^7	6.00×10^9	11/ < 24
Arthrobacter siccitolerans (1)	190	9.50×10^6	7.20×10^9	11/ < 4
Brevundimonas diminuta (1)	189	7.13×10^7	6.80×10^9	11/ < 4
Agreia pratensis (1)	194	2.35×10^6	5.60×10^9	11/ < 12
Sphingobacteriaceae bacterium (1)	202	7.05×10^6	7.20×10^9	10/ < 12
Unknown (6)	ID's not attempted	4.38×10^6; 3.84×10^5–1.60×10^8	1.14×10^7; 2.80×10^5–5.60×10^9	10/ < 4 (6)

[a] *n* = number of strains.

[b] Accessions were assigned by NCBI GenBank; all accessions begin with the prefix KP762-; for example, KP762200.

Table 3 Identification of bacteria recovered from M/V Indiana Harbor ballast water in 2010, time 0 h cell counts of controls and pH test cultures, highest cell counts from controls during 72 h, and minimum (100%) bactericidal parameters of pH 10, pH 11 or pH 12 and exposure duration (h).

Identification (*n*)[a]	Accession(s)[b]	Time 0 h cfu/mL median; range	Control highest cfu/mL median; range	Bactericidal pH/h (number of cultures)
Pseudomonas veronii (15)	240–243, 247, 260, 262, 263, 265, 273, 278, 286, 290, 291, 297	9.60×10^6; 3.20×10^6–2.00×10^7	4.80×10^9; 2.40×10^8–1.52×10^{10}	10/ < 4 (1) 10/ < 12 (1) 10/ < 24 (3) 11/ < 4 (10)
Pseudomonas grimontii (10)	239, 266, 274, 275, 277, 279, 280–282, 293	6.40×10^6; 7.20×10^5–1.48×10^7	5.20×10^9; 2.80×10^9–1.24×10^{10}	11/ < 4 (10)
Pseudomonas fluorescens (9)	244–246, 255–257, 287–289	1.40×10^7; 4.80×10^6–2.80×10^7	8.80×10^9; 3.60×10^9–2.08×10^{10}	11/ < 4 (9)
Pseudomonas brenneri (6)	250, 253, 258, 267, 268, 276	2.36×10^7; 8.00×10^6–6.00×10^7	8.60×10^9; 6.40×10^9–3.00×10^{10}	11/ < 4 (4) 11/ < 12 (2)
Pseudomonas frederiksbergensis (3)	231, 261, 317	3.20×10^6; 1.56×10^6–2.40×10^7	6.80×10^9; 5.60×10^9–1.40×10^{10}	10/ < 12 (2) 11/ < 48 (1)
Pseudomonas gessardii (3)	252, 271, 272	1.40×10^7; 1.32×10^7–2.28×10^7	1.00×10^{10}; 9.20×10^9–1.64×10^{10}	11/ < 12 (2) 11/ < 4 (1)
Pseudomonas anguilliseptica (2)	310, 311	3.60×10^6; 2.00×10^6–5.20×10^6	1.10×10^{10}; 9.60×10^9–1.24×10^{10}	11/ < 4 (2)
Pseudomonas mandelii (2)	259, 318	2.54×10^7; 6.80×10^6–4.40×10^7	6.60×10^9; 6.00×10^9–7.20×10^9	10/ < 4 (1) 11/ < 4 (1)
Pseudomonas antarctica (1)	292	3.60×10^6	4.40×10^9	10/ < 24
Pseudomonas fragi (1)	285	4.00×10^6	6.80×10^9	11/ < 4
Pseudomonas salomonii (1)	283	7.20×10^6	8.40×10^9	11/ < 4
Pseudomonas simiae (1)	269	8.00×10^6	5.20×10^9	11/ < 4
Pseudomonas umsongensis (1)	302	2.80×10^6	5.60×10^9	11/ < 4
Pseudomonas sp. (2)	233, 305	1.01×10^7; 2.40×10^5–2.00×10^7	4.80×10^9; 1.20×10^9–8.40×10^9	10/ < 4 11/ < 12
Pseudomonas spp. (5)	223, 251, 254, 270, 284	8.00×10^6; 5.60×10^6–2.00×10^7	5.20×10^9; 2.40×10^9–7.60×10^9	11/ < 4 (4) 11/ < 24 (1)
Brevundimonas mediterranea (10)	249, 299–301, 307, 309, 312–315	3.20×10^6; 5.60×10^5–2.80×10^7	2.00×10^{10}; 4.80×10^9–2.88×10^{10}	10/ < 24 (2) 11/ < 4 (8)
Brevundimonas sp. (12)	225–227, 229, 230, 235–238, 248, 294, 306	2.40×10^6; 6.40×10^5–6.40×10^6	1.56×10^{10}; 5.20×10^9–2.24×10^{10}	10/ < 4 (1) 10/ < 12 (2) 11/ < 4 (9)
Janthinobacterium lividum (2)	222, 228	1.92×10^5; 1.04×10^5–2.80×10^5	2.80×10^9; 2.80×10^9–2.80×10^9	10/ < 4 (2)
Janthinobacterium sp. (1)	224	4.40×10^4	5.20×10^9	11/ < 4
Arthrobacter scleromae (1)	295	4.40×10^6	4.80×10^9	11/ < 12
Arthrobacter sp. (1)	234	2.48×10^6	8.80×10^9	12/ < 24
Bacillus horikoshii (1)	316	2.32×10^4	1.24×10^9	12/ > 72
Bacillus sp. (1)	264	8.80×10^6	1.12×10^{10}	11/ < 4
Flavobacterium sp. (2)	304, 308	6.78×10^6; 7.60×10^5–1.28×10^7	2.16×10^9; 1.52×10^9–2.80×10^9	10/ < 4 (2)
Cryobacterium sp. (1)	298	1.60×10^6	1.48×10^{10}	11/ < 12
Psychrobacter psychrophilus (1)	296	4.00×10^5	8.40×10^9	11/ < 4
Sphingomonadaceae bacterium (1)	232	1.44×10^2	1.08×10^4	10/ < 4
Vogesella perlucida (1)	303	2.40×10^6	6.40×10^9	11/ < 72
Unknown (4)	ID's not attempted	2.40×10^7; 1.28×10^5–3.20×10^7	6.80×10^9; 4.00×10^9–1.36×10^{10}	11/ < 4 (3) 11/ < 12 (1)

[a] *n* = number of strains.

[b] Accessions were assigned by NCBI GenBank; all accessions begin with the prefix KP762-; for example, KP762240.

Table 4 Cell counts (cfu/mL) from bacteria recovered from M/V Indiana Harbor ballast water from 2009. Strains were grown in pH-adjusted tryptic soy broth (TSB) at 21 °C. Cell counting was performed at the indicated times.

TSB pH	Time 0 h	4 h	12 h	24 h	48 h
Control (*n*)[a]	(47)	(47)	(47)	(47)	(47)
Median	3.56×10^6	4.80×10^6	7.60×10^7	1.32×10^9	2.80×10^9
Mean	1.28×10^7	2.20×10^7	4.12×10^8	2.14×10^9	3.58×10^9
SD	2.70×10^7	3.45×10^7	6.71×10^8	2.47×10^9	3.77×10^9
pH 10.0 (*n*)	(47)	(20)	(19)	(14)	(11)
Median	3.56×10^6	1.80×10^5	1.00×10^4	9.80×10[b]	7.20×10[b]
Mean	1.28×10^7	2.74×10^6	9.14×10^5	5.88×10^5	2.21×10^5
SD	2.70×10^7	9.53×10^6	3.37×10^6	2.06×10^6	6.89×10^5
pH 11.0 (*n*)	(47)	(6)	(4)	(0)	(0)
Median	3.56×10^6	3.20×10^3	2.40×10[b]	NG[b]	NG
Mean	1.28×10^7	3.08×10^3	5.40×10[b]	NG	NG
SD	2.70×10^7	1.68×10^3	6.21×10[b]	NG	NG
pH 12.0 (*n*)	(47)	(1)	(1)	(0)	(0)
Median	3.56×10^6	4.80×10^3	2.40×10[b]	NG	NG
Mean	1.28×10^7	4.80×10^3	2.40×10[b]	NG	NG
SD	2.70×10^7	0	0	NG	NG

[a] *n* = number of strains included in individual data summaries; median, mean and standard deviation (SD) cfu/mL's. The range of cells per culture at Time 0 h was 1.02×10^2–1.60×10^8 cfu/mL.
[b] NG = no bacterial growth.

Table 5 Cell counts (cfu/mL) from bacteria recovered from M/V Indiana Harbor ballast water from 2010. Strains were grown in pH-adjusted tryptic soy broth (TSB) at 21 °C. Cell counting was performed at the indicated times.

TSB pH	Time 0 h	4 h	12 h	24 h	48 h	72 h
Control (*n*)[a]	(101)	(101)	(101)	(101)	(101)	(101)
Median	6.40×10^6	2.80×10^7	4.80×10^8	2.80×10^9	6.00×10^9	6.40×10^9
Mean	9.56×10^6	4.22×10^7	6.80×10^8	3.10×10^9	7.52×10^9	8.39×10^9
SD	1.04×10^7	5.33×10^7	7.59×10^8	1.96×10^9	5.61×10^9	6.20×10^9
pH 10.0 (*n*)	(101)	(92)	(83)	(76)	(69)	(69)
Median	6.40×10^6	1.48×10^5	4.00×10^4	5.40×10^3	1.20×10^4	1.60×10^4
Mean	9.56×10^6	2.66×10^6	1.24×10^6	2.17×10^7	4.45×10^7	7.80×10^7
SD	1.04×10^7	7.84×10^6	4.00×10^6	1.82×10^8	2.09×10^8	4.89×10^8
pH 11.0 (*n*)	(101)	(10)	(5)	(6)	(3)	(2)
Median	6.40×10^6	1.00×10^2	4.00×10[a]	2.80×10^2	4.80×10^2	2.20×10^4
Mean	9.56×10^6	1.16×10^4	3.60×10^2	9.13×10^2	5.07×10^2	2.20×10^4
SD	1.04×10^7	3.35×10^4	5.46×10^2	1.27×10^3	6.80×10[a]	2.20×10^4
pH 12.0 (*n*)	(101)	(1)	(2)	(1)	(1)	(1)
Median	6.40×10^6	1.60×10^2	6.00×10[a]	4.00×10[a]	2.00×10^2	4.00×10[a]
Mean	9.56×10^6	1.60×10^2	6.00×10[a]	4.00×10[a]	2.00×10^2	4.00×10[a]
SD	1.04×10^7	0	2.00×10[a]	0	0	0

[a] *n* = number of strains included in individual data summaries; median, mean and standard deviation (SD) cfu/mL's. The range of cells at Time 0 h was 1.44×10^2–6.00×10^7 cfu/mL.

exposure indicated (Tables 2, 3 and 6). This was because cells were present at previous sampling times, but not at the indicated times. The treatment was bactericidal at some time between the two sample times. Data were managed and analyzed using Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

Results

The concentration of bacteria from the developmental medium from the two ballast water samples collected in 2009 was 7.20×10^3 cfu/mL and 2.00×10^4 cfu/mL. The 2010 median was 1.78×10^4 cfu/mL; the mean was 2.83×10^4 cfu/mL (SD = 3.08×10^4 cfu/mL) and counts ranged from 3.50×10^3 cfu/mL to 1.15×10^5 cfu/mL (Table 1). Minimum bactericidal parameters of pH and duration of exposure were determined for 148 bacterial strains from ballast water primary isolation plates. Forty-seven strains were from 2009 (Table 2) and 101 were from 2010 (Table 3).

In 2009, the median cell count of the 47 control and pH test cultures at time 0 h, the starting inoculum, was

Table 6 Minimum bactericidal pH and exposure durations to 148 bacteria recovered from M/V Indiana Harbor ballast water in 2009 and 2010.

Treatment duration	pH 10.0 2009–2010[a]	pH 11.0 2009–2010	pH 12.0 2009–2010	Cumulative totals
< 4 h	24–9	11–68	0–0	35 + 77
< 12 h	2–5	2–8	0–0	39 + 90
< 24 h	6–6	2–1	0–1	47 + 98
< 48 h	0–0	0–1	0–0	47 + 99
< 72 h	0–0	0–1	0–1 (> [b])	47 + 101
Cumulative totals	32–20	47–99	47–101	148

[a] Number of strains in 2009 and (–) 2010 that the pH and treatment duration were bactericidal.
[b] *Bacillus horikoshii* survived pH 12.0 for 72 h; therefore, an exposure greater than (>) 72 h will be necessary to be bactericidal.

3.56×10^6 cfu/mL and the mean was 1.28×10^7 cfu/mL (SD = 2.70×10^7 cfu/mL; Table 4). The range in cfu/mL at 0 h was 1.02×10^2–1.60×10^8 cfu/mL (Table 2). Subsequent sample counts from controls showed that all remained viable throughout the experiment (Table 4). The mean starting inoculum (0 h) in control and pH test flasks for bacteria from ballast water collected in 2010 was 9.56×10^6 cfu/mL (SD = 1.04×10^7 cfu/mL; Table 5). The median was 6.40×10^6 cfu/mL and the range was 1.44×10^2–6.00×10^7 cfu/mL (Table 3). In the controls, 94/101 (93.1%) strains had greater cfu/mL after 4 h of incubation compared to their respective inoculum cfu/mL and 100% of the controls had greater cfu/mL after 12 h. In all but two controls from both 2009 and 2010 (148 bacteria), subsequent cfu/mL were greater compared to their respective cfu/mL at 0 h, which indicated no apparent lags in growth of cultures (Tables 2–5).

Flavobacterium spp. were the most prevalent bacteria characterized from ballast water sampled in 2009, comprising 51.1% (24/47) of the total. The most common species was *F. xinjiangense* (9/24; 37.5%); other species recovered included *F. psychrolimnae*, *F. sinopsychrotolerans*, *F. frigidimaris*, *F. limicola*, *F. soli* and *F. pectinovorum*. Seven additional Flavobacteria represented by seven different accessions did not match at a species confidence level. Four *Pseudomonas* spp. were recovered, including the opportunistic fish pathogen *P. fluorescens* [11]. Four strains of *Pedobacter*, including *P. koreensis*, also comprised bacteria from 2009.

At pH 10.0, the effect of increasing the duration of exposure was shown with fewer numbers of viable strains as well as reduced numbers of cells (Table 4). For example, after 48 h in pH 10.0 TSB, 11/47 strains remained viable with a median of 7.20×10^2 cfu/mL, compared to 20/47 viable strains at 4 h with a median of 1.80×10^5 cfu/mL. Additionally, as pH concentrations are increased within the same sampling time, a similar treatment effect was shown with reduced viable strains and fewer cfu/mL. For example, at 4 h, 20/47 pH 10.0 treated strains were viable while six (*F. xinjiangense*, *F. sinopsychrotolerans*, *P. fluorescens*, *Pedobacter koreensis*, *Arthrobacter sulfureus*, and *Agreia pratensis*) were viable at pH 11.0 and only one (A7; *Arthrobacter sulfureus*) remained viable at pH 12.0 (Table 4). The minimum parameters tested, which were 4 h exposure at pH 10.0, were bactericidal to 24/47 (51.1%) of the strains (Table 6) and this included thirteen *Flavobacterium* spp. (Table 2). After 24 h exposure to pH 10.0, no cells were recovered from 32 (68.1%) of the cultures. At pH 11.0, 43 (91.5%) of the cultures succumbed at less than 4 h of exposure and less than 24 h was bactericidal to all 47 strains from 2009.

Pseudomonas spp. (62/101; 61.4%) and *Brevundimonas* spp. (22/101; 21.8%) were the predominate bacteria recovered from 2010 water samples, together comprising 83.2% (84/101) of the total. The most prevalent species was *P. veronii* (15/63; 23.8%), followed by *P. grimontii* (15.9%) and *P. fluorescens* (14.3%). The highest mean cfu/mL, regardless of sampling time, recorded from the controls was 9.46×10^9 cfu/mL (SD = 6.66×10^9 cfu/mL), which was nearly a three log-ten increase compared to the mean inoculum (9.56×10^6 cfu/mL; Table 5). The highest median was 7.20×10^9 cfu/mL and the range was 1.08×10^4–3.00×10^{10} cfu/mL (Table 3). All strains from 2009 and 2010 were assigned accessions by NCBI GenBank (Tables 2 and 3).

Similar to the responses shown with cultures from 2009, as the medium pH was increased and the duration of exposure to the higher pH was increased, the number of viable strains decreased as did the cfu/mL (Tables 3, 5 and 6). Less than 24 h at pH 10.0 was bactericidal for 20/101 (19.8%) of the bacteria; whereas, at pH 11.0, 4 h of exposure was bactericidal to 88 (87.1%) strains and 96 (95.1%) succumbed at this pH within 12 h (Table 6). *Arthrobacter* sp. was viable in pH 12.0 at 12 h, but not at 24 h; at 12 h there were 4.00×10^1 cfu/mL while the control had 8.80×10^9 cfu/mL (Tables 3 and 6). *Bacillus horikoshii* remained viable after 72 h in pH 12.0 medium (4.00×10^1 cfu/mL) and was the only bacterium to do so (Table 3), compared to 2.32×10^4 cfu/mL at 0 h and 1.24×10^9 cfu/mL after 72 h in the control.

When the data from 2009 to 2010 were combined, inoculation into pH 10.0 growth medium and less than 24 h was bactericidal to 35.1% (52/148; Table 6). Growth in pH 11.0 TSB for 4 h was bactericidal to 131 (88.5%) strains and 12 h was bactericidal to 141 (95.3%). Two *Pseudomonas* strains, *P. fluorescens* and *P. gessardii*, were recovered in both 2009 and 2010. No additional strains were recovered in both sampling years.

Discussion

In laboratory studies, bacterial growth conditions were predictable and controlled whereas in the ballast tank environment, varying conditions of the water can be anticipated to affect bacterial abundance and community composition [12]. The initial (Time 0 h) inocula developed for the control and

pH test strains were intentionally high for two reasons. First, as a part of the design of the study, the high numbers of cells created a rigorous evaluation of the effectiveness of sodium hydroxide as a bactericide. Second, the high numbers of cells at 0 h would eliminate or greatly reduce the anticipated lag phases typical of culture growths. If the inocula cfu/mL were too low, it is possible that during the lag phase there would be too few cells at 4 h and perhaps 12 h to recover using viable cell counting techniques. The lack of recovery of cells from controls after the short growth times (e.g. 4 h and 12 h) would have confounded in determining whether the lack of cells in pH test flasks at the short growth times was due to a lag in growth or the bactericidal effect of sodium hydroxide. There were only three strains (unknown; Table 2) in which the 0 h cell counts were greater than all subsequent timed cell counts, yet cells were recovered from the controls at all subsequent sampling times. Thus, sustained viability of all strains was clearly shown for the durations of the trials.

It might appear that there are discrepancies in the cumulative totals provided in Table 6 when compared with the results in Tables 4 and 5. For example, in Table 4 (2009 cultures) cells were recovered from twenty (of 47) strains at pH 10.0 and 4 h. Accordingly, the data summary provided in that table cell was on the cfu/mL from those strains. Therefore, it could be assumed that 27 strains would be killed at pH 10.0 at <4 h. However, in Table 6 only 24 are shown to succumb at pH 10.0 within 4 h. The reason for this difference was there were three strains (two strains of *F. xinjiangense*, *Brevundimonas diminuta*) from which cells were not recovered at the 4 h sampling time, but cells were recovered at subsequent sampling times, for example at 12 h, 24 h and/or 48 h. Thus, the minimum bactericidal parameters for these three strains were reported as pH 11.0 at <4 h for *B. diminuta* and one strain of *F. xinjiangense* and pH 10.0 at <24 h for the other *F. xinjiangense*, which changed their positions in Table 6. A likely reason for not recovering cells at 4 h, but doing so from subsequent sampling times could be at 4 h the cell numbers might have been too low and near a threshold for detection (i.e. bacterial colonies formed on primary plates) using viable culture techniques. Presumably, if larger volumes from the TSB dilution series had been used to inoculate the TSA plates, it is probable that colonies would have formed. Similarly, in Table 5, there were seven similar instances. It was assumed that no cfu were present when no colonies were recovered; however, the ability to kill bacteria that are capable of entering a viable, but nonculturable state was not determined [13]. In a previous study, Starliper and Watten [7] showed that sodium hydroxide apparently destroyed bacterial cell walls because microscopically, intact cells were not detected after treatments that were the same as administered in the present study.

Bactericidal parameters were determined for all but one strain (147 of 148); *B. horikoshii*, which was viable at pH 12.0 and after the longest duration of pH exposure, 72 h. For the current study, exposure durations of greater than 72 h were considered unreasonable for actual ship ballast applications because on short voyages the ballast would not be on board for this length of time. Although *B. horikoshii* cells were recovered at pH 12.0 at 72 h, the cell count (4.00×10^1 cfu/mL) was much lower compared with 1.24×10^9 cfu/mL from the control. *B. horikoshii* is Gram-positive rod and its survival in higher pH media, albeit few cfu/mL, was not too surprising. Previous studies have determined that *B. horikoshii* is alkaliphilic with a pH tolerance range of pH 7.0–12.0 and an optimal pH 8.0–9.0 [14–15]. Previous results by Starliper and Watten [7] also showed that Gram-positive bacteria *Enterococcus faecalis* and *Bacillus* sp. survived pH 12.0 for greater than 48 h. Likewise, *Arthrobacter* spp. are Gram-positive [16–17] and also display varying degrees of high pH tolerance. In the present study, *Arthrobacter* sp. required 12 h at pH 12.0 to be bactericidal efficacy and pH 11.0 within 12 h was required for *A. scleromae* (Table 3). In another study, *A. mysorens* was recovered on a pH 10.0 bacteriological medium from an alkaline Lonar Lake, India where the pH of the water was pH 10.5 [15].

Data from the present study illustrated that multiple durations of treatment and pH concentrations were bactericidal to bacteria recovered from ballast water. An example of this was with three of the strains of *P. veronii* in which pH 10.0 within 24 h was bactericidal (Table 3). However, pH 11.0 within 4 h was also bactericidal (data not reported). Similarly, *P. frederiksbergensis* was killed within 48 h at pH 11.0 and *Vogesella perlucida* succumbed to pH 11.0 within 72 h (Table 3); whereas, pH 12.0 within 4 h was lethal to both. This indicates that a choice in the bactericidal parameters could be made to decontaminate the ballast water. This offers flexibility in choosing the treatment parameters of how high to raise the pH and the duration available to conduct the exposure. The treatment parameters chosen could best be adapted to the needs of the shipping company depending on the length of a voyage or for how long a ship may be docked in waiting. Clearly, there is a favorable cost benefit with the investment of lesser product (sodium hydroxide) and therefore, a resulting lower pH treatment. This may be possible if the logistics provides a sufficient contact time for an efficacious treatment. The amount of sodium hydroxide required is also dependent on additional factors such as water temperature and alkalinity [18].

The mean initial inocula in the present study, 1.28×10^7 cfu/mL in 2009 and 9.56×10^6 cfu/mL in 2010 (Tables 4 and 5), were much greater than the mean bacteria enumerated from the ballast water samples, which were 1.36×10^4 cfu/mL and 2.83×10^4 cfu/mL from 2009 to 2010, respectively. However, these bacterial counts from ballast water were within a range of bacterial counts from previous studies of ballast water [4,19] and source/destination port waters [13]. Maranda et al. [19] reported a range in marine heterotrophic bacterial counts of 2.00×10^2 cfu/mL to greater than 2.00×10^4 cfu/mL from ballast water taken on board in Newark Bay, Newark, New Jersey, USA. Ruiz et al. [4] recovered an average of 8.30×10^5 cfu/mL from ballast water from ships arriving to Chesapeake Bay, USA. Seiden et al. [13] recovered an average 1.27×10^6 cfu/mL from port source and destination waters from two trans-Pacific voyages between Japan and the west coast of Canada. It is reasonable to anticipate that if sodium hydroxide was effective for high cfu/mL as was shown in the present study, it would be more effective at lower bacterial concentrations. A successful product that will thoroughly decontaminate ballast water will be effective for a wide range in fauna and flora including bacteria, algae and mollusks. Additionally, the agent must meet or exceed expectations for cost effectiveness for expendables as well as infrastructure to apply and mix the agent. The agent and process must also be safe for crew members and the agent readily inactivated or neutralized prior to release of treated ballast into the

environment. Fortunately, although often quite large, a ballast tank is a defined space that can be individualized and holds a measurable amount of water, thus providing an opportunity to be completely disinfected.

Conclusions

The present study of bacteria recovered from ballast water showed that raising the pH using sodium hydroxide was an very effective bactericide. Future studies will evaluate sodium hydroxide as a decontaminant for bacteria in ballast tank conditions and ballast tank sediment. If as effective as in this study, sodium hydroxide could prove to be a cost-effective agent when scaled up to actual ballast tank applications. Additionally, sodium hydroxide may be readily neutralized prior to deballasting, and provides anti-corrosion to steel that ballast tanks are constructed from.

Conflict of interest and animal welfare statement

Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government. Animals were not used in this study; therefore, institutional and national guidelines for care and use of animals were not relevant.

Acknowledgments

This research was funded by the U.S. Geological Survey and the National Park Service.

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