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Research Article

Current status and recommendations toward a virus standard for ballast water

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Abstract

Ballast water contains and transfers a variety of biological and non-biological materials between biomes. This study aimed to summarize published literature on the viruses found in ballast water, to evaluate ultrafiltration and plaque assay as methods for concentrating and enumerating viruses, and to assess the efficacy of UV irradiation in the inactivation of infectious viruses in ballast water. Previous studies reported high virus concentrations in ballast tanks, ranging from 10^9 to 10^{14} viral particles/L. The ultrafiltration and plaque assay were efficient in concentrating and enumerating four types of phages: MS2, PhiX174, P22, and PP7. The UV dose (radiant fluence) necessary to achieve a 4-log reduction of the four phages ranged from 6.1 to 77.7 mJ/cm^2 with MS2 being the most UV-resistant phage. The results illustrated the applicability of ultrafiltration and plaque assay techniques for quantifying viruses in ballast waters and pointed to the need for additional test microorganisms for assessing ballast water quality.

Key words: ballast water management; ballast water treatment; phage; plaque assay; UV irradiation

Introduction

Ships draw and discharge ballast water to maintain trim, stability, and structural integrity, yet this water contains a variety of biological materials. This practice leads to a potential introduction of non-native micro- and macro-organisms to the receiving body of water during ballast water discharge. Discharged water can include invasive species, which present ecological, economic, and public health threats to major ports and waterways worldwide (Carlton 1985; Ruiz et al. 2000; Ibrahim and El-Naggar 2012). In the U.S., ballast water management is regulated by the federal agencies, U.S. Environmental Protection Agency (USEPA) and U.S. Coast Guard (USCG), and at the state level (NOAA 2018). In 2012, because of the limited ecological protection afforded by ballast water exchange

practice, USCG issued a Final Rule, which requires ballast water discharges to meet the International Maritime Organization ballast water management Convention D-2 standard (Department of Homeland Security 2012). Under the D-2 standard, three indicator bacteria must not exceed the specified concentrations in discharged ballast water: < 250 colony-forming units (CFU) of *Escherichia coli* (*E. coli*), < 100 CFU of intestinal enterococci, and < 1 CFU of toxicogenic *Vibrio cholerae* per 100 mL. At the state level, several states have developed regulations for numeric ballast water discharge standards with California having the most stringent requirements (David and Gollasch 2015). California has "interim" and "final" ballast water discharge performance standards, which are proposed by the California State Lands Commission (CSLC) to go into effect January 1, 2020 and January 1, 2030, respectively (Brown et al. 2017). For viruses in particular, the interim standard requires less than 10,000 viruses per 100 mL and the final discharge standard requires zero detectable viable organisms for all organism size classes, including viruses.

The final discharge standard for ballast water is more stringent, thus regulation D-3 establishes approval requirements for ballast water management systems to treat the water in order to comply with the D-2 as a performance standard. However, many technologies for ballast water treatment are still in the research and development phase (David and Gollasch 2015) with 21 ballast water treatment systems certified as of December 2018 (USCG 2018); yet their ability to meet the California's interim standards have not been established (Brown et al. 2017). Two key steps in ballast water treatment are 1) physical removal of species via filtration and 2) disinfection. Disinfection technologies include UV irradiation, gamma radiation, microwave, and ultrasonication (Ibrahim and El-Naggar 2012). While UV irradiation has been the primary means of disinfection, the application of gamma radiation, microwave, and ultrasonication is relatively new and demonstrated only in laboratory tests (Faez and Sarkar 2006; Collings et al. 2007; Boldor et al. 2008; Gregg et al. 2009). In addition, species in ballast water can be inactivated using chemical disinfectants via chlorination, ozonation, and oxidation by hydrogen peroxide (Ibrahim and El-Naggar 2012). Among the various disinfection approaches, UV irradiation is currently the most broadly used method applied to ballast water. While UV disinfection of viruses has a long history in water treatment, to the best of our knowledge, there is no study on efficacy of UV irradiation in the inactivation of the various types of viruses in ballast water.

Viruses are the most abundant biological entities on earth, with typical concentrations ranging from 10^9 to 10^{10} viral particles/L of seawater (Bergh et al. 1989; Fuhrman 1999). The identification and quantification of viruses in aquatic environments are inherently challenging due to their small size and the vast variety of virus species. Quantifying viruses in disinfected

water is especially challenging as virus counts are often below the detection limit of current counting techniques. Thus, effective techniques for concentrating viruses from large volumes of water are required in order to reach the detection limits necessary to comply with the California ballast water discharge standards. In fact, since the development of a standard method under the Information Collection Rule coordinated by USEPA (1996), there have been many advances in both the detection as well as concentration of viruses from water. For ballast water, however, no viral standard method currently exists and the development of virus concentration and detection methods is needed to address the new performance standard. Critically, any method used for concentrating viruses must include an analysis of viral recovery. This effort will help control the emergence of deadly viruses, such as viral hemorrhagic septicemia virus in the Great Lakes (Bain et al. 2010).

In this paper, we evaluated ultrafiltration because it can concentrate diverse microorganisms in water samples based on size exclusion (Morales-Morales et al. 2003), and therefore has been widely used in aquatic virus research. Four types of phages (viruses that infect bacteria) were used in the disinfection study, including those found in ballast waters as model systems where enumeration was performed by a plaque assay to determine infectivity. Phages were used as virus surrogates for evaluating an ultrafiltration system and efficacy of UV irradiation because it is now known that they are much more numerous in aquatic systems, especially in oceans, than bacteria and other organisms (Bergh et al. 1989; Mokili et al. 2012). Moreover, plaque assay as a method of quantifying phage concentration is safe and straightforward, and directly indicates viral infectivity. UV dose requirements for the most UV resistant of the viruses, MS2, were further characterized in filtered, natural samples of fresh, brackish, and saline waters representative of realistic conditions in ballast tanks. The specific objectives of this study were to:

i) Conduct a literature review on viral concentrations in ballast tanks.
ii) Evaluate the recovery efficiencies of an ultrafiltration system and detection capability of plaque assay for addressing their technical feasibility.
iii) Evaluate the efficacy of UV irradiation in the inactivation of infectious viruses in ballast water to undertake a preliminary determination on how ship operators might attain the CSLC standards.

Materials and methods

Ultrafiltration setup and procedure

The effectiveness of a low-cost ultrafiltration system using disposable hollow fiber ultrafilters (USD 22) was evaluated for concentrating four types of phages (MS2, PhiX174, P22, and PP7) from marine water. Before

each experiment, artificial marine water was prepared by mixing MilliQ water (resistivity > 18.2 MΩ·cm) with sea salt (Instant Ocean, Spectrum Brands, Inc., VA, USA) to obtain a final salinity of 3% and mixed for 30 min at room temperature. To evaluate phage recovery efficiency, five samples of artificial marine water, 10 L each, were seeded with all four phages at levels of 10^5, 10^6, 10^7, 10^8, and 10^9 plaque-forming units (PFU)/mL (final concentrations of each phage in each 10 L of water at approximately 10^1, 10^2, 10^3, 10^4, and 10^5 PFU/mL) and mixed for 30 min at room temperature. Experiments were run in triplicate.

The ultrafiltration system was set up as described previously with a few modifications (Hill et al. 2005). Briefly, a peristaltic pump (model 7554-90; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and a pump head (model 77800-52; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) were used with L/S 36 and L/S 24 silicone tubing (Masterflex; Cole-Parmer Instrument Co., Vernon Hills, IL, USA). Immediately before filtration, ultrafilters were primed by recirculating 500 mL of sterile 0.01% NaPP solution for 15 min with the filtrate port closed. Water samples seeded with phages were passed through single-use Asahi Kasei REXEED 25S ultrafilters (2.5 m^2 surface area, 30,000 Dalton MWCO; Asahi Kasei Medical Co., Ltd., Tokyo, Japan). Filtration was performed at a rate of approximately 1,000 mL/min until approximately 250 mL of concentrated sample remained in the system. Elution was performed by the recirculation of 300 mL of sterile surfactant solution (0.001% Antifoam A, 0.01% NaPP, and 0.5% Tween 80) through the system for 5 min. The eluent was then added to the retentate and produced the final volume of approximately 500 mL.

The four phages were enumerated using single agar overlay plaque assay (USEPA method 1602) before and after filtration to calculate their recovery efficiencies. Briefly, 2 mL of sample and 0.5 mL of log phase host culture were added to 2.5 mL of 1.5% agar overlays. This solution was mixed and poured over a solidified 1.5% tryptic soy agar. The plates were incubated at 37°C for 16 to 24 hours and plaques were then counted. For each sample, three replicated counts were conducted.

Sample collection and characterization

Natural water samples were collected from three different locations: Mississippi River (MR: 30.412572; −91.198142), Lake Pontchartrain (LP: 30.1575671; −89.8571398), and Grand Isle (GI: 29.202685; −90.037211; Figure 1) in the southeast United States. All the samples were filtered through 0.45 μm polypropylene membrane filter prior to analysis. Total Organic Carbon (TOC) concentrations were measured using a Shimadzu TOC-VCSH analyzer (Shimadzu Scientific Instruments, MD, USA). The light absorbance of samples at 254 nm was measured in a 1 cm quartz cuvette with a StellaRad spectrometer (StellarNet Inc., Tampa, FL, USA).

Figure 1. Map of the sample locations in southeastern United States including GPS co-ordinates. Abbreviations refer to locations MR: Mississippi River; LP: Lake Pontchartrain; and GI: Grand Isle.

Table 1. Characteristics of natural water samples used in this study for seeded experiments. Sample name abbreviations refer to MR: Mississippi River; LP: Lake Pontchartrain; and GI: Grand Isle.

Sample name	TOC (mgC/L)	Absorbance @ 254nm (cm^{-1})	$SUVA_{254}$ (L/mg/m)	pH	Salinity (ppt)	Electroconductivity (mS/cm)	Turbidity (NTU)	$[NO_3^-]$ (mg/l NO_3^-N)	$[NO_2^-]$ (mg/l NO_2^-N)
MR	4.73	0.19	4.04	7.75	0.3	0.4	13	0.9	0.000
LP	4.25	0.21	4.96	7.51	5.4	6.36	3.0	0.2	0.000
GI	29.5	0.14	0.46	8.16	24.2	27.4	7.0	0.9	0.005

The specific UV absorbance ($SUVA_{254}$) was then calculated as the ratio of UV_{254} absorbance and TOC value (Weishaar et al. 2003). pH and electroconductivity were measured using a benchtop multiparameter meter (VWR International, Radnor, PA, USA). Turbidity, nitrate, and nitrite were measured with a colorimeter (HACH Co., Loveland, CO, USA). These data are recorded in Table 1.

Virus inactivation by bench-scale UV irradiation

Experimental solutions were prepared using 150 mL of MilliQ water with and without addition of sea salt (Instant Ocean, Spectrum Brands Inc., VA, USA) to represent artificial freshwater and marine water with salinity of 3%, respectively. Based on turbidity range (Min; 0, Mean; 2.5, Max; 11 NTU) of 21 ballast waters originating from freshwater and marine water (Kim et al. 2015, 2016), two 150 mL-samples of artificial marine water with turbidity levels of 5 NTU and 10 NTU were also prepared by adding ISO 12103-1, A2 Fine Test Dust (Powder Technology Inc., MN, USA). The

turbidity of the experimental solutions was further adjusted using a 2020we portable turbidity meter (LaMotte Company, Chestertown, MD, USA). Each 150 mL of the test waters was inoculated with 1.5×10^8 PFU (final concentrations of approximately 1×10^6 PFU/mL) of the phages MS2, PhiX174, P22, and PP7. Experiments containing MS2 phages only in natural water samples collected from GI, MR, and NP were performed in an identical manner using filtered, natural samples spiked with MS2 phages.

UV disinfection was performed using a bench-scale collimated-beam UV reactor equipped with UV exposure box, UVC lamp (16 W), stir plate, and a beaker (diameter, 10 cm). The UV lamp emitted within the germicidal range, with 95% of emitted energy at the 254 nm wavelength. Prior to the experiment, the UV lamp was turned on for 10 min. The UV irradiance ($\mu W/cm^2$) at 254 nm at surface of the test waters was measured using a radiometer (Model UVX-25, UVP Co., USA). The UV absorbance of the test waters was measured using an UV/Vis spectrophotometer (Model MultiSpec 1501 spectrophotometer, Shimadzu Scientific Instruments, MD, USA). Sample aliquots were withdrawn from the reactor at various reaction times (0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 min). Experimental solutions were stirred very gently via magnetic stirrer to ensure well mixed conditions without perturbing the solution surface, so as not to increase reflection of incident UV light. Experiments were run in duplicate. The UV dose was calculated following Bolton and Linden (2003) and was defined as the average irradiance throughout the experimental solution in the reactor multiplied by the UV exposure time. In addition, the effects of reflection from the water surface (reflection factor) and divergence of the beam (divergence factor) were taken into account for the calculation.

To assess the viability of viruses after UV irradiation, infectious phages MS2, PhiX174, P22, and PP7 were quantified using single agar overlay plaque assay (USEPA method 1602) as described previously. Since the initial viral concentration of each phage was 1×10^6 PFU/mL and CSLC proposes less than 1×10^2 viruses/mL, a reduction of 10^4 viruses/mL was targeted. In order to predict the UV fluences necessary to achieve a 4-log reduction, the results of viral inactivation tests were fitted by linear regression analysis.

Results and discussion

High concentration of viruses found in ballast tanks

A limited number of studies have examined viruses present in ballast tanks and most of the reported viral concentrations came from one research group (Fred C. Dobbs). A review of published studies of viruses in ballast tanks - including ballast tank water, ballast tank residuals (after discharge), ballast tank biofilms, and sediment pore water from ballast tank sediments – was performed; the results of 10 reports are summarized in Table 2. Overall,

Table 2. Viral particle concentrations found in ballast tanks.

Sample type/origin	Virus concentration (10^{10}/L)	N[a]	Enumeration method	Reference
Ballast water				
Chesapeake Bay, USA	0.74	11	NA[b]	Ruiz et al. 2000
Chesapeake Bay, USA	1.4	18	Epifluorescence microscope	Drake et al. 2001
Baltimore, USA	0.7–3.8	8	Epifluorescence microscope	Drake et al. 2002
Chesapeake Bay, USA	1.3	18	Epifluorescence microscope	Drake et al. 2005
Different ports, Chile	1.8	3	Epifluorescence microscope	Soto et al. 2005
Great Lakes, USA	33	5	Epifluorescence microscope	Wilhelm et al. 2006
Chesapeake Bay, USA	1.39	53	Epifluorescence microscope	Drake et al. 2007
Vancouver, CA	1.1	50	Epifluorescence microscope	Leichsenring and Lawrence 2011
Japan	0.1–1.0	10	Epifluorescence microscope	Tomaru et al. 2014
Residual ballast tank water				
Great Lakes, USA	1–100	75	Epifluorescence microscope	Johengen et al. 2005
Chesapeake Bay, USA	6.2	13	Epifluorescence microscope	Drake et al. 2007
Pore water of ballast tank sediments				
Great Lakes, USA	1–10,000	73	Epifluorescence microscope	Johengen et al. 2005
Chesapeake Bay, USA	120	12	Epifluorescence microscope	Drake et al. 2007
Ballast tank biofilms				
Chesapeake Bay, USA	63	3	Epifluorescence microscope	Drake et al. 2005

[a] Number of samples.
[b] Information not applicable.

these studies reported the number of viral particles measured by epifluorescence microscopy and revealed a high concentration of viruses in ballast water, ranging from 1.0×10^9 to 3.3×10^{11} viral particles/L (see Table 2 for references). When ballast tanks are emptied after deballasting, the residual water associated with sediment particles and biofilms on the sides of ballast tanks are left behind. As such, many of the viruses may remain in the ballast tanks even after the ballast water is discharged (Drake et al. 2007), resulting in a build up in virus concentrations. Commercial ships have to undergo dry dock on a regular basis (twice within five years) for inspection and maintenance of the components of ships (IMO 2015). Prior to dry dock, ballast tanks are emptied, such that sediments and biofilms accumulated at the bottom of ballast tanks can be sampled, allowing a brief window for sample collection. Thus, sample collection for enumerating viral particles, particularly those present in sediments and biofilms of ballast tanks is challenging, as ballast tank sediments are not accessible during routine operation of ships. Because of these difficulties, the data are sparse regarding viral concentrations in residual water, sediments, and biofilms of ballast tanks. Viral concentrations in residual ballast tank water range from 10^{10} to 10^{12} viral particles/L (Johengen et al. 2005; Drake et al. 2007). Pore water of ballast tank sediments contains 10^{10} to 10^{14} viral particles/L (Johengen et al. 2005; Drake et al. 2007). Drake et al. (2005) examined three samples of ballast tank biofilms and reported viral concentrations of 6.3×10^{11} viral particles/L. Taken together, viral concentrations found in residual water, sediments, and biofilms of ballast tanks are higher than those found in ballast water, suggesting that viruses

accumulate in the bottom waters associated with the sediments and biofilms in the ballast tank. In summary, these studies reported average concentrations of viral particles in ballast water, ranging from 1.0×10^9 to 3.3×10^{11} viral particles/L. This finding suggests that over 4 to 6.5 log removal of viral particles would be needed to achieve the CSLC standard (less than 10,000 particles/100 mL).

The efficacy of ballast water exchange was also evaluated by direct measurement of viral particles in ballast water. Studies reported no significant differences in viral concentrations when comparing samples directly before and after ballast water exchange (Drake et al. 2002) and comparing un-exchanged (control) and exchanged ballast tanks (Drake et al. 2002; Leichsenring and Lawrence 2011). On the contrary, Tomaru et al. (2014) reported that both viral and bacterial concentrations were steady at approximately 1.0×10^7 viral particles and 1.0×10^6 cells per milliliter, respectively and sharply decreased by approximately one order of magnitude each after ballast water exchange. In most cases, including the studies by Drake et al. (2002) and Leichsenring and Lawrence (2011), however, viral and bacterial concentrations showed no significant changes following ballast water exchange. Molina and Drake (2016) suggested that a decrease in concentrations may not be related to removal of viruses and bacteria following ballast water exchange. Instead, this may be due to lower concentrations of microorganisms in mid-oceanic water (1.5 to 1.7×10^6 viral particles/mL) compared to coastal water (15.0 to 21.6×10^6 viral particles/mL (Culley and Welschmeyer 2002; He et al. 2009). Moreover, as suggested by Leichsenring and Lawrence (2011), viral concentrations in ballast tanks are highly variable, which hinders observing statistically significant differences between viral concentrations in exchanged and un-exchanged tanks. This variability is mainly due to challenges associated with obtaining biologically representative samples from ballast tanks: (i) uneven distribution of organisms in ballast tanks (Murphy et al. 2002; Gollasch and David 2010; Costa et al. 2015); (ii) tanks containing ballast water from different sources in a vessel, (iii) non-representative sampling, (iv) presence of biofilm and sediment in ballast tanks, and (v) irregular shapes of ballast tanks (Murphy et al. 2002). Molina and Drake (2016) also suggested that challenges of obtaining representative samples from ballast tanks as well as water depth and salinity differences between ballast water exchange sites could contribute conflicting results of viral and bacterial concentrations.

Overall, the differences in virus reporting imply that viral concentrations may not always serve as a reliable indicator of the efficacy of ballast water exchange in reducing number of viral particles in ballast tanks. In addition to number of viral particles directly counted, viability, composition and diversity of viral communities, or dynamics of specific viruses in ballast water need to be better understood to evaluate the risk of viral invasion

(Drake et al. 2002; Leichsenring and Lawrence 2011; Molina and Drake 2016; Darling and Frederick 2018). Two recent studies characterized composition and diversity of viral communities in freshwater- (Great Lakes, Kim et al. 2015) and ocean-captured ballast water (Ports of Los Angeles/Long Beach and Singapore (Kim et al. 2016), with the use of next-generation sequencing (NGS) technologies. Kim et al. (2016) showed that ballast water contains a diverse group of viruses including phages, which were the most abundant but also a variety of animal and plant viruses. Kim et al. (2016), in particular, demonstrated the important role of ballast water in initiating long-distance distribution of viruses including viral pathogens by revealing viral metagenomes (viromes) unique to geographic locations. Moreover, virus richness (total number of distinct viral species) of ballast water viromes was found to be correlated with local environmental conditions, such as pH, salinity, and temperature of ballast water, which was consistent with the suggestion by Molina and Drake (2016). Lastly, Kim and her colleagues (2016) stressed that efficacy of current ballast water exchange practice should be assessed by examining the reduction of different types of viruses rather than virus abundance estimates as proposed regulatory parameter. Although the subject of bacteria in ballast water is beyond the scope of this paper and we limit our discussion to viruses, the importance of better understanding of bacterial composition and diversity is also being recognized with increasing concerns about transport of non-indigenous bacteria during ballasting operations and the wide application of NGS technologies (Ng et al. 2015; Johansson et al. 2017; Lymperopoulou and Dobbs 2017).

Efficient concentration of viruses from artificial marine water by ultrafiltration

Large volumes of water need to be concentrated for enumerating viruses present in ballast water. Ultrafiltration is widely used as the primary concentration technique capable of recovering viruses with high efficiency. In the present study, effectiveness of a low-cost ultrafiltration system using disposable hollow fiber ultrafilters was evaluated for concentrating four types of phages from 10 L of artificial marine water. MS2 and PhiX174 were chosen for the evaluation of ultrafiltration efficacy, as they are used extensively as surrogates for human enteric viruses for water quality assessment. MS2, a member of the family *Leviviridae*, is an icosahedral single-stranded RNA (ssRNA) virus of *E. coli*, with a diameter of 27 nm (Fiers et al. 1976). PhiX174 is a single-stranded DNA (ssDNA) virus that also infects *E. coli*, which belongs to the family *Microviridae* with an icosahedral particle of 25 nm in diameter (Yazaki 1981). Since both phages are of similar size, we included a double-stranded DNA (dsDNA) virus of *Salmonella typhimurium*, P22, based on its larger size of approximately 60 nm

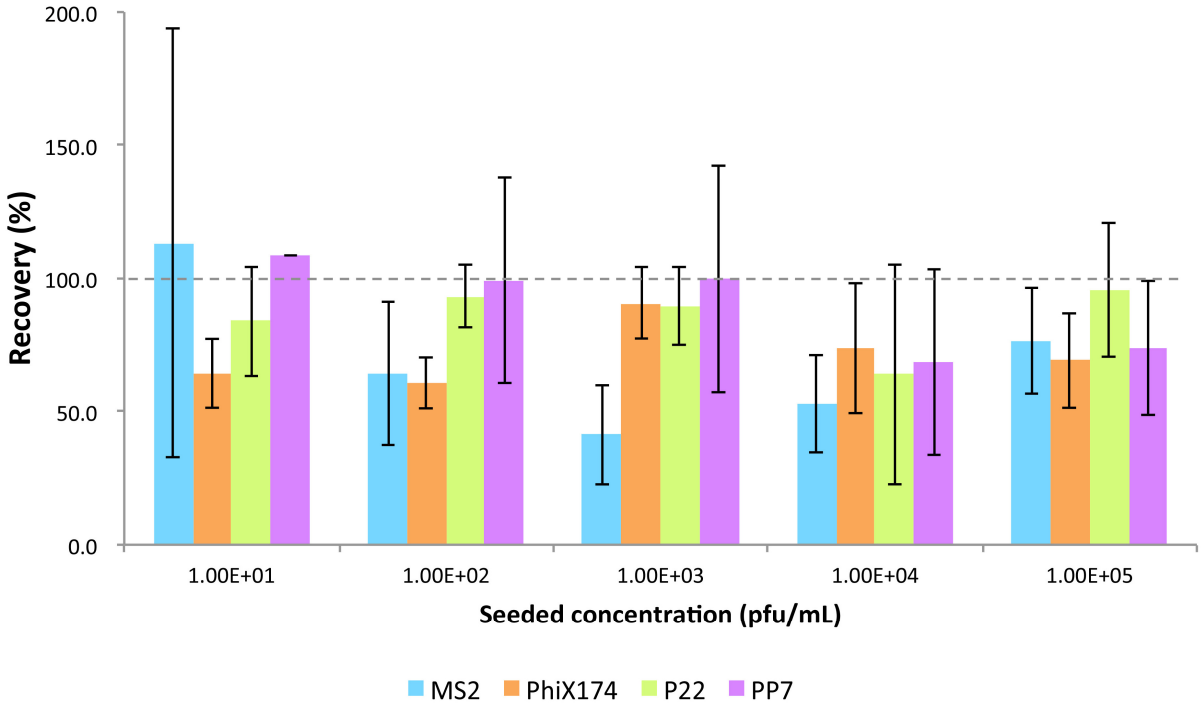

Figure 2. Percent recovery efficiencies of four phages seeded with five different concentrations in 10 L of artificial marine water. The error bars represent standard deviation of the recovery efficiency values calculated from three replicated runs. Abbreviation: PFU, plaque-forming units.

(Vander Byl and Kropinski 2000). These three viruses are well characterized, can be grown to high titers, and are safe to work with. We also included PP7 in this study. PP7, another ssRNA virus, is a distant relative of MS2, in the family *Leviviridae*, infecting *Pseudomonas aeroginosa*. PP7 was selected based on the previous metagenomic study of marine ballast water (Kim et al. 2016) for the following reasons: i) it was present in all 24 ballast and harbor waters, ii) it had high relative abundance, and iii) there is a widely used plaque assay method for it. Although phages infecting *Synechococcus* spp. were found to be present with high abundances across all the ballast and harbor water samples, its plaque assay method requires several weeks while the results are available within 24 hours for the four phages used in this study. The ultrafiltration system achieved high recovery for all four phage types and for each of five feed concentrations evaluated. The average recovery efficiencies for MS2, PhiX174, P22, and PP7 were 69.6 ± 27.6, 71.7 ± 11.6, 85.2 ± 12.8, and 90.0 ± 17.6%, respectively for the five feed concentrations (Figure 2). There was no significant difference in recovery efficiencies between different viruses or between different feed concentrations ($p < 0.05$, n = 60). The recoveries of phages using the Asahi Kasei REXEED 25S ultrafilters were comparable with results reported in previous studies that relied on the same type of ultrafilter: MS2 (57%) from 100 L of tap water (Smith and Hill 2009); MS2 (73%) from 100 L of tap water amended with surface water (Smith and Hill 2009); MS2 (66%) from 100 L of surface water (Mull and Hill 2012); and MS2 (62%) and PhiX174 (95%) from 100 L of drinking water (Holowecky et al. 2009).

Table 3. Comparison between calculated concentrations based on phage stock concentration and dilution and measured concentration of the four phages enumerated by plaque assay (PFU/mL) before (A) and after (B) ultrafiltration procedure. Concentration is expressed as mean ± standard deviation of three replicated runs. Efficiency is measured concentrations of the phages divided by calculated concentrations and dilution and expressed as a percentage.

A. Pre-ultrafiltration

MS2			PhiX174		
Calculated conc.[a]	Measured conc.[b]	Efficiency (%)	Calculated conc.	Measured conc.	Efficiency (%)
10	3 ± 2	33.0	10	22 ± 0	15.0
100	51 ± 6	51.1	100	20 ± 2	19.7
1,000	445 ± 170	44.5	1,000	145 ± 50	14.5
10,000	4,856 ± 952	48.6	10,000	1,622 ± 210	16.2
100,000	60,056 ± 7,782	60.1	100,000	19,556 ± 4,138	19.6
P22			PP7		
Calculated conc.	Measured conc.	Efficiency (%)	Calculated conc.	Measured conc.	Efficiency (%)
10	7 ± 2	67.0	10	1 ± 1	12.0
100	62 ± 4	61.6	100	39 ± 9	39.2
1,000	588 ± 58	58.8	1,000	396 ± 98	39.6
10,000	6,028 ± 982	60.3	10,000	5,361 ± 2,269	53.6
100,000	65,222 ± 4,171	65.2	100,000	77,444 ± 3,372	77.4

B. Post-ultrafiltration

MS2			PhiX174		
Calculated conc.[c]	Measured conc.[d]	Efficiency (%)	Calculated conc.	Measured conc.	Efficiency (%)
95	77 ± 29	81.2	42	27 ± 6	64.0
1,664	1,024 ± 372	61.6	637	380 ± 12	59.6
11,641	5,139 ± 4,118	44.1	3,726	3,222 ± 1,028	86.5
160,012	83,000 ± 23,605	51.9	54,992	38,111 ± 2,983	69.3
1,399,607	1,048,333 ± 307,531	74.9	442,054	306,111 ± 98,606	69.2
P22			PP7		
Calculated conc.	Measured conc.	Efficiency (%)	Calculated conc.	Measured conc.	Efficiency (%)
189	165 ± 24	87.2	31	86 ± 69	275.1
2,013	1,856 ± 343	92.2	1,345	1,261 ± 549	93.8
14,603	13,183 ± 3,642	90.3	10,179	10,244 ± 7,028	100.6
204,557	114,556 ± 47,732	56.0	170,840	105,556 ± 21,495	61.8
1,506,507	1,420,000 ± 376,928	94.3	1,773,266	1,341,667 ± 624,222	75.7

[a] Calculated expected concentration of the phages in the 10 L of artificial marine water.
[b] Measured concentration of the phages in the 10 L of artificial marine water.
[c] Calculated concentration of the phages in the recovered 10 L of artificial marine water.
[d] Measured concentration of the phages in the ultrafiltration concentrates.
Abbreviation: PFU, plaque-forming units.

Utility of plaque assays for the enumeration of infectious viruses in artificial marine water

As the ultrafiltration system was found to be effective for concentrating different types of phages, we next evaluated the detection capability of plaque assays by seeding four types of phages at five different concentrations in 10 L of artificial marine water. As mentioned earlier, we chose plaque assay for virus quantification because it is the current "gold standard" being straightforward, cost-effective, reliable, and most importantly, yielding infectious virus titer.

In general, positive correlations were observed between the calculated (based on seeded levels) and measured concentrations with R^2 values ranging from 0.999 to 1 for all four phages in the 10 L of artificial marine water (Table 3A, Supplementary material Figure S1). Across the different

phages and seeding concentrations, the concentrations measured by the plaque assays were lower than the expected concentrations of the phages. This difference represents the recovery, which compares the calculation of the starting concentration and the volume and to what was actually observed. The losses may be due to adsorption of the phages to surfaces of the containers and tubing during the experimentation. In the test waters, the highest difference between the two concentrations was observed with PhiX174 as indicated by an average efficiency of 17.0% and the lowest with P22 by an average efficiency of 55.0%. The efficiency of the plaque assay, (measured concentrations of the phages divided by calculated concentrations based on phage stock concentrations and dilution and expressed as a percentage) for MS2 (ranged from 33.0 to 60.1%) and PP7 (ranged from 12.0 to 77.4%), increased overall with increasing concentrations of phages seeded in the 10 L of artificial marine water. On the other hand, PhiX174 showed low efficiency (ranged from 14.5 to 19.7%) while P22 showed relatively high efficiency (ranged from 58.8 to 67.0%) regardless of seeded concentrations. The relationships between calculated concentrations and measured concentrations of the four phages were again examined after ultrafiltration procedure to address recovery. Positive correlations were also observed between the two concentrations with R^2 values ranging from 0.996 to 1 for all four phages after ultrafiltration (Table 3B, Figure S1). The overall efficiency of plaque assay was higher in the post-concentrated water samples (84.5%) compared to the pre-concentrated water samples (40.9%) by ultrafiltration. In the post-concentrated water samples, the highest difference between the two concentrations was observed with MS2 as indicated by an average efficiency of 62.7% and the lowest with PP7 by an average efficiency of 121.4%. An increase in the efficiency was not observed with increasing concentration of phages seeded in the 10 L of artificial marine water.

Although plaque assays can be used to efficiently quantify infectious viruses, several limitations restrict their use for various types of viruses. First, the hosts must be able to grow on agar media (which may not be always possible) and thus the host species must be known *a priori*. Second, multitudes of bacterial host cultures are needed to measure the array of viruses found in ballast water. Based on the recent study (Kim et al. 2015, 2016) using metagenomic techniques, which are capable of detecting and characterizing a diverse array of viruses, freshwater- and ocean-captured ballast and harbor waters contained various viruses, particularly phages with high abundance and diversity (e.g., *Microviridae*, *Myoviridae*, *Podoviridae*, and *Siphoviridae*). Thus, plaque assays are limited in detecting the variety of viruses, which could be used as indicators for all viruses. Finally, another limitation is the possible presence of toxic substances in the environmental samples, which can lead to virus die-off and potential false-negative results (Julian and Schwab 2012).

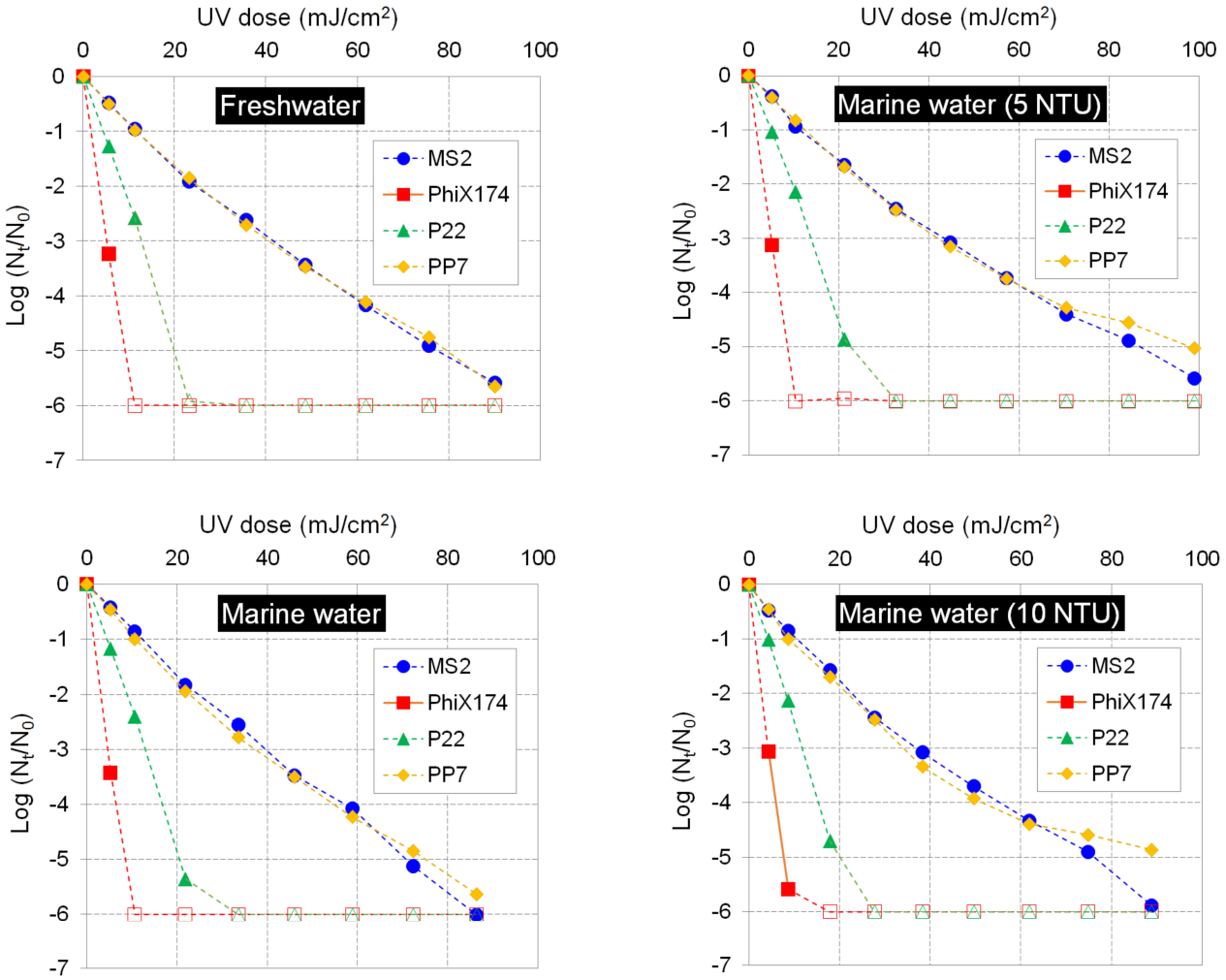

Figure 3. Inactivation kinetics of DNA (PhiX174 and P22) and RNA phages (MS2 and PP7) in artificial freshwater, marine water, marine water with a turbidity of 5 NTU, and marine water with a turbidity of 10 NTU as measured by plaque assay. X-axis and Y-axis represent UV dose and log inactivation, respectively. Dotted lines indicate no plaque detection.

Efficacy of UV irradiation in the inactivation of infectious viruses in freshwater and marine water

UV dosing experiments were performed to quantify the susceptibility of the four phages to UV irradiation under various water conditions. The inactivation kinetics obtained for the phages in different artificial water conditions as a function of UV dose are presented in Figure 3. Further, the kinetics of MS2 inactivation by UV in filtered, natural water samples are shown in Figure 4. First order inactivation rates were evident for all four phages in the different artificial water types without showing an initial lag phage (a linear response to UV dose). In general, similar patterns of UV resistance were observed for the four phages regardless of type or turbidity of test waters. Specifically, the UV resistance found for the two RNA phages, MS2 and PP7, was higher compared to the two DNA phages, PhiX174 and P22. MS2 is known to be remarkably resistant to UV inactivation as compared to other ssRNA phages and thus has been used as a model virus to measure UV light dose in UV reactors (Calgua et al. 2014;

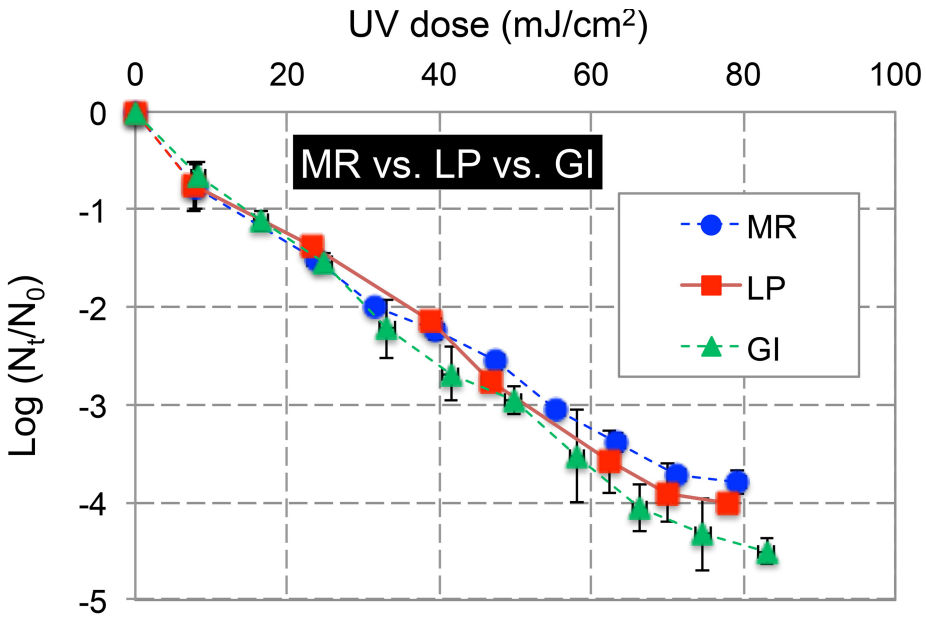

Figure 4. Inactivation kinetics of MS2 in natural water samples as measured by plaque assay. X-axis and Y-axis represent UV dose and log inactivation, respectively. Abbreviations for sampling locations refer to MR: Mississippi River; LP: Lake Pontchartrain; and GI: Grand Isle.

Table 4. UV dose (mJ/cm^2) required for an inactivation of 4-log of each phage in the different water types. Values in parentheses represent slope, rate constants for dN/d [UV dose], when a linear trendline was added in the chart. Sample name abbreviations refer to MR: Mississippi River; LP: Lake Pontchartrain; and GI: Grand Isle.

Phage (genome type)	Artificial freshwater	Artificial marine water	Artificial marine water (5 NTU)	Artificial marine water (10 NTU)	MR (13 NTU)	LP (3 NTU)	GI (7 NTU)
MS2 (ssRNA)	60.6 (−0.06)	56.0 (−0.07)	65.6 (−0.06)	57.1 (−0.06)	77.7 (−0.05)	76.8 (−0.05)	65.5 (−0.06)
PhiX174 (ssDNA)	6.9 (−0.58)	6.1 (−0.65)	6.6 (−0.60)	6.1 (−0.64)	N/A	N/A	N/A
P22 (dsDNA)	16.1 (−0.26)	16.6 (−0.25)	17.9 (−0.23)	15.4 (−0.26)	N/A	N/A	N/A
PP7 (ssRNA)	69.5 (−0.06)	57.2 (−0.06)	69.3 (−0.05)	60.7 (−0.06)	N/A	N/A	N/A

World Health Organization 2014). Interestingly, this study showed that PP7 was very similar to MS2 in its UV resistance. Between the two DNA viruses, UV resistance of P22 was consistently higher than that of PhiX174. For the two DNA phages, no infective viruses were observed at a UV dose equal to or greater than 35.6 and 11.4 mJ/cm^2 in artificial freshwater, 33.6 and 21.8 mJ/cm^2 in artificial marine water, 32.7 and 21.3 mJ/cm^2 in artificial marine water (5 NTU), and 38.3 and 27.7 mJ/cm^2 in artificial marine water (10 NTU) for P22 and PhiX174, respectively.

Values of UV fluence necessary to achieve a 4-log reduction are summarized in Table 4. As stated earlier, UV resistance found in the two RNA phages, MS2 (average 59.8 mJ/cm^2) and PP7 (average 64.2 mJ/cm^2), was high followed by P22 (average 16.5 mJ/cm^2) and PhiX174 (average 6.4 mJ/cm^2). An increase in turbidity in the test waters (artificial marine water (10 NTU) > artificial marine water (5 NTU) > artificial marine water > artificial freshwater) did not result in an increase in UV fluences necessary to achieve a 4-log reduction in the study, as expected, since the obstruction of

UV light was accounted for when calculating the fluence values. Notably, there was no observable difference between artificial freshwater and marine waters, implying that photochemical production of radicals, a known phenomenon in marine water (Zafiriou 1974), was not a significant inactivation route.

Experiments on natural water samples showed that despite accounting for variation in fluence, and thereby light attenuation by natural organic matter (NOM), natural water samples required a larger dose to achieve a 4 log reduction in MS2 viability. In these cases, required doses for 4 log inactivation of MS2 for the MR, LP, and GI samples were 77.7, 76.8, and 65.5 mJ/cm^2, respectively. This is 9.5% (GI) to 29.9% (MR) greater than the average UV dose (59.8 mJ/cm^2) required for artificial water samples. The difference between artificial and natural water samples may be explained in part by the differences in turbidity (13 NTU in the MR sample), but other factors likely played a role. If reactive radical intermediates do play a role in inactivation mechanisms, NOM present in natural water samples could act as a protective agent (Phong and Hur 2016), resulting in slightly diminished inactivation kinetics. Based on the dose required for 4 log inactivation and to reach the 100 virus/mL standard for marine water, it is recommended that UV applications be designed to deliver a dose of at least 66 mJ/cm^2 (the highest UV dose for inactivation of phages in the marine water was 65.5 mJ/cm^2).

Conclusions and recommendations

A review of the current literature showed that viruses exist in ballast tanks in high concentrations and can be found in the bulk ballast water as well as in residual ballast tank water, pore water of ballast tank sediments, and ballast tank biofilms. The data suggest the urgent need for developing effective treatment strategies to achieve the CSLC standard. The ultrafiltration system evaluated in the present work was efficient in concentrating four different types of phages and takes 1–2 hours to process one sample depending on turbidity. Plaque assay methods were assessed for detection of infective viruses in ballast water and the results are available within 24 hours. Both methods are shown to be a practicable technique, suitable for testing the ability of treatment technologies to remove viruses from ballast water. However, considering several limitations of the plaque assay, including the need for *a priori* knowledge about the host bacteria and the use of a multitude of hosts to measure the array of viruses found in ballast tanks, nucleic acid-based tools, such as qPCR and NGS technologies are recommended to be used as supplementary tools for a comprehensive assessment of risk of viral invasions to marine fauna and flora. Lastly, similar patterns of UV resistance of phages PP7 to MS2 and high abundance of PP7 found in ballast water viromes (Kim et al. 2015,

2016) as well as a negative signal of *E. coli* measured by quantitative PCR (Lymperopoulou and Dobbs 2017) suggest that additional model organisms are needed to serve as potential indicators.

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Contributions

Conceived and designed the experiments: YK, JR, VT, SS. Performed the experiments: YK, VR, SS, MM, GL. Wrote the paper: YK, JR. Discussed the results and commented on the manuscript: YK, JR, VT, SS, VR, MM.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Relationships between calculated concentrations and measured concentrations of the four phages enumerated by plaque assay (PFU/mL) before and after ultrafiltration procedure.

This material is available as part of online article from:
http://www.reabic.net/journals/mbi/2019/Supplements/MBI_2019_Kim_etal_SupplementaryMaterial.pdf