Available online at www.sciencedirect.com
ScienceDirect
Marine Pollution Bulletin 55 (2007) 333–341
MARINE POLLUTION BULLETIN
www.elsevier.com/locate/marpolbul

Potential microbial bioinvasions via ships' ballast water, sediment, and biofilm

Lisa A. Drake [a,*], Martina A. Doblin [b], Fred C. Dobbs [c]

[a] Department of Science, US Coast Guard Academy, 27 Mohegan Avenue, New London, CT 06320, USA
[b] University of Technology, Sydney, P.O. Box 123, Broadway New South Wales 2007, Australia
[c] Department of Ocean, Earth and Atmospheric Sciences, Old Dominion University, 4600 Elkhorn Avenue, Norfolk, VA 23529, USA

Abstract

A prominent vector of aquatic invasive species to coastal regions is the discharge of water, sediments, and biofilm from ships' ballast-water tanks. During eight years of studying ships arriving to the lower Chesapeake Bay, we developed an understanding of the mechanisms by which invasive microorganisms might arrive to the region via ships. Within a given ship, habitats included ballast water, unpumpable water and sediment (collectively known as residuals), and biofilms formed on internal surfaces of ballast-water tanks. We sampled 69 vessels arriving from foreign and domestic ports, largely from Western Europe, the Mediterranean region, and the US East and Gulf coasts. All habitats contained bacteria and viruses. By extrapolating the measured concentration of a microbial metric to the estimated volume of ballast water, biofilm, or residual sediment and water within an average vessel, we calculated the potential total number of microorganisms contained by each habitat, thus creating a hierarchy of risk of delivery. The estimated concentration of microorganisms was greatest in ballast water $\gg$ sediment and water residuals $\gg$ biofilms. From these results, it is clear microorganisms may be transported within ships in a variety of ways. Using temperature tolerance as a measure of survivability and the temperature difference between ballast-water samples and the water into which the ballast water was discharged, we estimated 56% of microorganisms could survive in the lower Bay. Extrapolated delivery and survival of microorganisms to the Port of Hampton Roads in lower Chesapeake Bay shows on the order of 10^{20} microorganisms (6.8×10^{19} viruses and 3.9×10^{18} bacteria cells) are discharged annually to the region.

Keywords: Aquatic nuisance species; Bacteria; Invasive species; Management; Viruses

1. Why study microorganisms?

In the context of invasive species, the reasons for investigating the transfer of aquatic microorganisms, including viruses, bacteria, protists, and microalgae are threefold: their high densities in natural waters, ability to form resting stages, and potential toxicity or pathogenicity. Aquatic microorganisms are orders of magnitude more abundant than macroorganisms such as copepods and fish: naturally occurring bacteria and viruses are found in concentrations on the order of 10^6–10^{11} l^{-1} (e.g., Ducklow and Shiah, 1993; Proctor, 1997; Fuhrman, 1999; Wommack and Colwell, 2000). Given such high densities, microorganisms are transferred and introduced globally via ships in greater numbers than any other size class of organisms. Nearly all such microorganisms, incidentally, are innocuous to humans. Instead, the viruses infect naturally occurring bacterial and phytoplankton hosts, in which they can cause significant mortality (Fuhrman and Noble, 1996; Suttle, 2005).

Once released, microorganisms are well poised to be invasive species. They are small, a size that facilitates their passive dispersal. They appear to have simpler requirements for survival than do metazoans, based upon their ubiquity in the biosphere, including extreme environments (Deming, 1997). They predominantly reproduce asexually and grow

* Corresponding author. Tel.: +1 401 789 1461.
E-mail address: lisa.drake1@verizon.net (L.A. Drake).

0025-326X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2006.11.007

rapidly, factors also contributing to their widespread distribution. Finally, life cycles of many invertebrate metazoans and unicellular organisms such as bacteria, eukaryotic phytoplankton (including toxic dinoflagellates), and other protist species, include resting stages (variously called cysts, spores, auxospores, ephippia, or resting eggs according to taxon) capable of surviving prolonged periods of unfavorable conditions (e.g., Bailey et al., 2003).

These resting stages are typically produced at very low frequency, if at all, under favorable conditions, and at high frequency when environmental conditions deteriorate (e.g., declining nutrient concentrations, shortened photoperiod, or reduced food quality; Blackburn and Parker, 2005). Production of resting stages ensures long-term viability of the population because they are extremely resistant to adverse conditions, including anoxia, exposure to noxious chemicals, freezing, and passage through digestive tracts of fish and waterfowl. Resting eggs of invertebrates and cysts of dinoflagellates are usually negatively buoyant and sink when released or formed. Resting stages may remain viable in sediments in a virtual suspended metabolic state for decades or even centuries (Hairston et al., 1995) and can germinate under a combination of favorable light, temperature, and other environmental conditions (e.g., Kremp and Anderson, 2000; Itakura and Yamaguchi, 2001; Figueroa et al., 2006).

Pathogenic or toxic aquatic bacteria, viruses, protists, and microalgae can have devastating effects on ecosystems and economic resources. There are well-studied pathogen–host systems among many aquatic phyla; for example, viruses terminating algal blooms (e.g., Milligan and Cosper, 1994; Nagasaki et al., 1994; Van Etten et al., 1991; Short and Suttle, 2002), viruses infecting seals (e.g., Osterhaus et al., 1985; Grachev et al., 1989), and protists decimating seagrass beds (e.g., Muehlstein, 1992; Ralph and Short, 2002). Furthermore, the apparent increasing frequency and distribution of toxic microalgal blooms has received much attention in the past two decades (see reviews by Hallegraeff, 1993; CENR, 2000). Given marine pathogens can spread locally much more quickly than terrestrial pathogens (even when instances of obvious human intervention are excluded, McCallum et al., 2003), and considering the relatively fast transport by ships, the threat of global dispersal of aquatic pathogens appears more immediate than the threat of invasion by other groups of organisms.

Thus, by virtue of their abundance, life-history characteristics, and potential pathogenicity or toxicity, microorganisms possess a great capacity to invade and cause detrimental effects in new environments. This paper will explore the extent and potential consequences of bacteria and virus transport within ships.

2. Types of habitats within ships

Microorganisms can be found in several locations within a ship—ballast water, residual sediment and water, and biofilms formed on interior tanks surfaces—each of which will be considered separately. Transfer among these habitats has not been fully explored (although see Meyer et al., 2000). We do not consider microorganisms within hull fouling communities in this review.

2.1. Ballast water

Ships' ballast waters are the best investigated of these habitats. Although water has been used regularly as ballast since the 1880s (Carlton, 1985), the transfer of organisms by ballast-water discharge was investigated only sporadically until the late 1980s. The interest in ballast water at that time stemmed largely from the dramatic ecological and economic impacts of introduced species, such as comb jellies (*Mnemiopsis leidyi*) in the Black Sea and zebra mussels (*Dreissena polymorpha*) in the North American Great Lakes (International Maritime Organization, 1999). Much of the work on ballast invaders has been dedicated to studying metazoans (Fofonoff et al., 2003), despite the high densities of naturally occurring microorganisms in aquatic environments. To test the hypothesis that vast quantities of bacteria and viruses are carried in ships' ballast tanks, Ruiz et al. (2000) quantified their abundance in ballast water of vessels arriving to Chesapeake Bay from foreign ports. Indeed, the numbers were high: mean abundances of 8.3×10^8 bacteria l^{-1} and 7.4×10^9 virus-like particles (VLPs) l^{-1} were documented.

2.2. Sediment and water residuals

We now know ships declaring no (pumpable) ballast on board may also serve as vectors. Concerns about no ballast on board (NOBOB) invasions have risen from a position of relative obscurity a few years ago to one of the chief environmental concerns in the Great Lakes basin today (e.g., Grigorovich et al., 2003). The potential for NOBOB-mediated invasions lies within tanks' muddy puddles; residuals of sediment and water can contain an assortment of metazoans and microorganisms, including resting stages (e.g., Hallegraeff and Bolch, 1992; Galil and Hülsmann, 1997; Gollasch et al., 1998; Hamer et al., 2000). When NOBOB tanks are later filled with ballast water, the accumulated sediment (and associated biota) may be resuspended and discharged immediately or at subsequent ports of call.

Sediment accumulation can be appreciable, depending on elapsed time since the ship was last dry-docked. For example, double-bottom ballast tanks of a cargo vessel contained up to 30 cm of sediment after only two years of use (Hamer et al., 2000). While circumstances vary from ship to ship, the unpumpable water that remains in most vessels, together with any residual sediment, potentially harbors nonindigenous organisms. A metastudy of 13 European studies recorded 990 species in a combination of ballast water and sediment samples (Gollasch et al., 2002). Furthermore, Kelly (1993) reported Japanese ships visiting the USA carried viable cysts and spores of nonindigenous species after 11–15 days' voyage. Finally, Mac-

Isaac et al. (2002) modeled NOBOB vessel-mediated inoculation of the Great Lakes using available plankton densities in coastal European waters, ship transit times, species' survival curves, and residual ballast volumes; individual NOBOB vessels potentially discharge $>10^7$ individuals of rotifers, cladocerans, and copepods, and 10^{11} bacteria, depending on conditions inside ballast tanks.

In this regard, tank sediments serve as a repository for particles, living or otherwise, that settle from water within the tank. With respect to issues of invasions, therefore, sediment and water residuals in a NOBOB tank will contain a temporally integrated assortment of organisms found in the water columns that overlay it days and weeks earlier, and possibly months and years earlier, in the case of resting stages of organisms.

2.3. Biofilms on ballast-water tank surfaces

Aquatic surfaces are colonized to some degree with biofilms, organic matrices that can contain bacteria, microalgae, and associated protists, sometimes including pathogenic forms (e.g., Decho, 1990, 2000). Microorganisms in mature biofilms are notoriously resistant to chemical disinfectants for reasons that have yet to be satisfactorily explained (Costerton et al., 1999). The production of exopolymer secretions, "slimes", by surface-bound bacteria is a well-recognized—but insufficient—mechanism to explain such resistance to chemical treatment. Confocal microscopy and other tools have demonstrated the complicated, heterogeneous architecture of thick biofilms (many as thick as hundreds of micrometers, Baier, 1984; Cook et al., 2000). In addition to providing a chemical barrier to potentially lethal agents, biofilms might also provide refuge for bacteria from predatory protists (Hülsmann et al., 2000) or promote interactions among pathogenic bacteria and protist grazers such that evolutionarily successful pathogens survive digestion by protists or become their endosymbionts (Barker and Brown, 1994).

For these reasons, it is worthwhile to consider ships' ballast-water biofilms, but there are only two such reports. First, biofilm communities formed on multiple types of artificial surfaces deployed in a ballast tank during a transoceanic voyage (Meyer et al., 2000). When the substrata and associated biofilms were removed and submerged in artificial seawater, they seeded secondary biofilms, which survived for years (Meyer et al., 2000). Second, bacteria and VLPs have been enumerated in biofilms collected from ships arriving to Chesapeake Bay and the Great Lakes. Microbial concentrations were up to ten times greater than those in ballast water, and some samples contained potentially harmful bacteria and microalgae (Drake et al., 2005).

3. Ship-borne microbial transport: case studies

Here we consider examples of ship-borne microbial transport, discharge, and subsequent deleterious effect. First in most such litanies is the introduction of toxic dinoflagellates to Australia (Hallegraeff et al., 1988; Hallegraeff, 1998). Upon commencement of woodchip export from southern Tasmania to Japan and South Korea, the previously undetected toxic dinoflagellate *Gymnodinium catenatum* was likely introduced to Tasmania (McMinn et al., 1997). It is impossible to say ships' ballast water was responsible for the translocation, but the evidence to that effect is compelling. First, the large, chain-forming dinoflagellate and associated human poisonings were previously unknown in this area prior to the opening of the woodchip mill. Second, radioisotope dating of sediment cores shows the appearance of the dinoflagellate's cysts coincident with the beginning of woodchip export (McMinn et al., 1997). This species is now well established in the Derwent and Huon estuaries, and high concentrations of paralytic shellfish toxins in shellfish have caused regular closure of aquaculture farms there.

A second example of probable microbial transport by ships is the oft-cited discovery of the bacterium *Vibrio cholerae*, agent of human cholera, in ships arriving to ports in the Gulf of Mexico (McCarthy and Khambaty, 1994). During routine monitoring of shellfish and fish in the Gulf, an epidemic-causing strain of *V. cholerae*, serotype O139, previously unreported in the Gulf, was detected (DePaola et al., 1992). At the time, an epidemic caused by *V. cholerae* O139 was underway in South America. When ships with last ports of call in South America were sampled in Mobile Bay, Alabama, their ballast water contained the epidemic strain of cholera. Subsequent testing showed it to be indistinguishable from the strain found in Gulf fish and shellfish (McCarthy et al., 1992). Although no illnesses were reported in the US from this strain, the incidents illustrate the potential for ships to transport—and, importantly, deliver—viable, toxic bacteria.

Lastly, there are a growing number of data sets illustrating the *transport* of potentially toxic microorganisms by ships. Organized by broad taxonomic groups, they include: bacteria, such as *V. cholerae* (e.g., Knight et al., 1999; Zo et al., 1999; Ruiz et al., 2000) and *Vibrio* spp. (Mimura et al., 2005); autotrophic protists (e.g., Hallegraeff and Bolch, 1992; McCollin et al., 2000; Doblin et al., in press); and heterotrophic protists (e.g., Galil and Hülsmann, 1997).

4. Can free-living microorganisms be invaders? Biogeographical considerations

Except for spectacular examples provided by highly infectious pathogens (see review in McCallum et al., 2004), the invasion biology of aquatic microorganisms is not as well understood as those of vertebrates, macroinvertebrates, and macroalgae. In general, many members of the latter groups have biogeography, i.e., their distribution is specific to certain geographical areas. If we assume, however, free-living microorganisms have no biogeography and are global in their distribution, as some researchers

contend (e.g., Finlay, 2002; Finlay and Fenchel, 2004), then they cannot be considered to "invade" environments. This topic is explored with respect to ship-borne microorganisms by Dobbs and Rogerson (2005). We will not reiterate their arguments here, but in short, they contend resolution of the microbial-ubiquity hypothesis is highly relevant to considerations of ballast management.

5. Methods to determine numbers of microorganisms, estimate their survival, and calculate the total number of microorganisms delivered

5.1. Relative propagule pressure among habitats

Propagule pressure (i.e., the number of individuals introduced into a given environment) is understood as a prime factor explaining the success of an invasion (see Occhipinti–Ambrogi in this volume). Furthermore, the concentration of microorganisms in ballast waters has been considered a proxy for propagule pressure in most studies and for devising microbiological standards for ballast water treatment. We know, however, there are microorganisms in ballast-tank sediments and biofilms. How might these other microbiological repositories figure into treatment strategies and regulatory policy? To address the question, we calculated the number of microorganisms contained within each ballast-tank habitat (ballast water, biofilm, and residual sediment and water) to create a numerical hierarchy based on potential delivery of microorganisms. We extrapolated the average concentrations of microorganisms within ships sampled to their estimated concentrations within a so-called "average" vessel. We considered our average vessel to be a bulk carrier, because most ballast-tank microbiology has been gleaned from such ships, they contain the largest volume of ballast water compared to other ship types, and they are highly abundant in the world fleet and therefore representative.

The dynamics of biofilm detachment from ballast-tank walls remain uninvestigated, as do the dynamics of residuals' resuspension and entrainment into ballast water. Therefore, the calculations below relate to the number of microorganisms within each ballast-tank habitat prior to ballast discharge at a receiving port.

Between 1996 and 2003, we sampled bulk carriers and colliers (coal-carrying ships) arriving to the lower and upper Chesapeake Bay from foreign and domestic ports, largely from Western Europe, the Mediterranean region, and the US East and Gulf coasts. Data sources for estimates of microbial concentrations are described in Table 1. Despite the large number of ballast-water samples collected for microbial enumeration ($n = 31$ for VLPs, 53 for bacteria), there was little if any replication for a given port/season combination, so data were not analyzed by combination. Likewise, the number of vessels sampled for

Table 1
Data used to estimate the total number of microorganisms contained within ballast-tank habitats in an "average" bulk carrier

Parameter	Value (SD)	Sample size and data source
Ballast water		
Volume	18,484 MT[a] (11,988)	$n = 10$; category 1
Bacteria concentration	$0.803 \times 10^9\ l^{-1}$ (1.88)	$n = 53$, 2 subsamples; category 2
VLP concentration	$13.9 \times 10^9\ l^{-1}$ (15.7)	$n = 31$, 2 subsamples; category 2
Residuals: sediment pore water		
Volume	25 MT	$n = 8$; category 3
Bacteria concentration	$26.3 \times 10^9\ l^{-1}$ (36.2)	$n = 12$, 0–2 subsamples; category 4
VLP concentration	$1170 \times 10^9\ l^{-1}$ (1124)	$n = 12$, 0 subsamples; category 4
Residuals: overlying water		
Volume	17 MT	$n = 8$; category 3
Bacteria concentration	$0.439 \times 10^9\ l^{-1}$ (0.342)	$n = 13$, 0–2 subsamples; category 4
VLP concentration	$62.4 \times 10^9\ l^{-1}$ (48.7)	$n = 13$, 0–2 subsamples; category 4
Biofilm		
Area covered by biofilm[b]	4,459 m^2	Pers. com[c]
Thickness of biofilm	250 μm	Baier (1984); Meyer et al. (2000)
Volume	1.1 MT	Calculated using above two variables
Bacteria concentration	$6.62 \times 10^9\ l^{-1}$ (8.83)	$n = 3$, 2–3 subsamples; category 5
VLP concentration	$633 \times 10^9\ l^{-1}$ (869)	$n = 5$, 2–3 subsamples; category 6

HR—Hampton Roads, which encompasses ship terminals in the lower Chesapeake Bay cities of Norfolk, Newport News, Portsmouth, and Chesapeake, Virginia, USA; category 1—colliers and bulk carriers arriving to HR from foreign and domestic ports in 2001–2003; category 2—colliers arriving from foreign ports to upper and lower Chesapeake Bay in 1996–2001; category 3—bulk carriers arriving to HR from foreign and domestic ports in 2003–2004; category 4—bulk carriers arriving from foreign and domestic ports to HR in 2003; category 5—colliers and bulk carriers arriving from foreign and domestic ports to HR and the Great Lakes in 2002 (Drake et al., 2005); category 6—colliers and bulk carriers arriving from foreign and domestic ports to HR and the Great Lakes in 2002–2003 (Drake et al., 2005). VLP—virus-like particle.

[a] 1 MT = 1000 kg = 1000 l of freshwater.

[b] Assumes 10% of the total tank surface area covered by biofilm.

[c] J. Kelly, International Paint Inc.

residuals or biofilms precluded analysis by port/season combination.

For a complete description of field and laboratory methods, see Drake et al. (2001, 2005). Briefly, field samples were collected as follows: ballast-water samples were collected by hand or using a bleached Niskin bottle and dispensed into sterile containers. Because residual samples consisted of sediment and a layer of overlaying water, both fractions were collected independently and aseptically into sterile containers. Finally, biofilm samples on ballast tank surfaces were scraped from an area of known dimensions with a sterile polystyrene scraper, and the scraper with attached material was placed in a sterile container. In all cases, samples were transported from vessel to laboratory in a cooler.

In the laboratory, bacteria were enumerated using the nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole; Sigma Chemical Company, St. Louis, Missouri; Porter and Feig, 1980) or by flow cytometery using PicoGreen® (Molecular Probes, Inc., Eugene, Oregon; Veldhuis et al., 1997). VLPs were visualized and enumerated with the nucleic acid stains YO-PRO™-1 (Hennes and Suttle, 1995) or SYBR® Green I (Molecular Probes, Inc., Eugene, Oregon; Noble and Fuhrman, 1998). When different methods were used, an intercalibration was conducted to ensure data from both methods could be pooled for analysis. Statistical analyses were conducted using SPSS for Windows Release 11.0.1.

Most of the vessels sampled for ballast water had undergone open ocean exchange prior to sampling (70% of vessels sampled for bacteria analysis, 77% of vessels sampled for VLP analysis). Because there were no significant differences between mean bacteria or VLP concentrations in exchanged vs. unexchanged tanks (Mann–Whitney *U*-test, $p = 0.69$ for bacteria and 0.345 for VLPs), samples from both types of vessels were pooled to calculate average microbial concentrations. Biofilm and residual samples inherently represent an amalgamation of previous ballasting operations, so their prior condition as exchanged or unexchanged was not considered in these analyses.

To determine the average quantity of ballast water and residual volume present in a bulk carrier, vessel officers were interviewed. In the case of residual volume, vessel captains did not partition unpumpable ballast between sediment and overlying water. Based on personal observations, the majority of residual volume was sediment, so residual volume was apportioned as 80% sediment and 20% overlying water. The microbial abundance of each fraction was calculated, and the two were added to determine the total microbial abundance in the residual volume. The final number underestimates the total residual abundance because microorganisms attached to sediment grains or meiofauna were not removed and counted. Instead, the microbial component of sediment residuals was measured by enumerating microorganisms in sediment pore water. Pore water was analyzed after it was expressed from sediment samples by centrifugation at 1000*g* at 4 °C for 10 min in a Marathon 2100R centrifuge (Fisher Scientific, Hampton, New Hampshire). The volume of pore water contained in an average sediment residual volume was 38% (SD = 14%, $n = 10$ with up to four subsamples per sample).

The amount of biofilm on ballast tank walls was determined by multiplying the average surface area of the tanks and the thickness of aquatic biofilms (Table 1). Field sampling showed only a small portion of the tanks' surfaces were covered by biofilm, so the calculations include a tank area coverage of 10%.

5.2. Survival success of discharged microorganisms

Once organisms are delivered to a new location, their invasion success is a function of their ability to survive and reproduce (Carlton, 1985). With respect to microorganisms, little is known in this regard. One proxy of survival used for invertebrates determines the hydrographic match between the ballast water and receiving (pier side) waters (e.g., Smith et al., 1999). We used this model and estimated the percentage of vessels arriving to lower Chesapeake Bay that encounter optimum temperature conditions for microorganisms upon ballast-water discharge. Using empirical data from ships arriving to the lower Bay, we calculated temperature differences between ballast water and receiving waters and then applied assumptions about bacteria temperature tolerances. Our discussion is limited to ballast water because the dynamics of sediment and biofilm discharge are more complex; that is, the amount of sediment and biofilm discharged by a vessel will depend on vessel type, operations, tank history, and characteristics of the tank habitats themselves.

Thirty-two commercial ships were boarded in the Port of Hampton Roads, Virginia, which encompasses ship terminals in the lower Chesapeake Bay cities of Norfolk, Newport News, Portsmouth, and Chesapeake. The vessels arrived from foreign and domestic ports, largely from Western Europe, the Mediterranean region, and the US East and Gulf coasts between 1999 and 2003. Most vessels (72%, 23 of 32) had undergone open ocean exchange. Temperature was measured in surface water samples of ballast tanks and surface water collected adjacent to the vessel pier.

First, we considered the *tolerance* of microorganisms. Assuming bacteria have a temperature tolerance range of 30 °C (Madigan et al., 2003) and they inhabit ballast water at the midpoint of that range, then they can tolerate discharge into water ±15 °C that of the ballast water. Second, we considered the *optimum* temperature conditions. If bacteria have a 10 °C temperature range for optimum growth (Madigan et al., 2003), and if they inhabit ballast water at the midpoint of their optimal range, then they will grow best when discharged into receiving water ±5 °C that of the ballast water. We have simply and conservatively assumed virus tolerances and optima reflect those of bacteria. Furthermore, our use of the term "surviving viruses" is

Table 2
Values used to estimate bacteria and VLPs surviving discharge in ballast water from commercial vessels arriving to the Port of Hampton Roads, Virginia, USA each year

Parameter	Mean (SD)	Sample size and data source
Ballast water volume discharged per container ship	321 MT (575)	$n = 4$ vessels arriving to HR in 1999
Ballast water volume discharged per collier	30,788 MT (18,391)	$n = 80$ vessels arriving to HR in 1998–2003
Ballast water volume discharged per bulk carrier	12,070 MT (7828)	$n = 10$ vessels arriving to HR in 2001–2003
Total number of vessels arriving weekly to HR	39 (5.0)	$n = 77$ weeks in 2002–2004; arrival data from the Hampton Roads Maritime Association (Norfolk, Virginia)
Container ships arriving weekly to HR	26.1 (3.6)	$n = 77$ weeks in 2002–2004
Colliers arriving weekly to HR	3.8 (2.3)	$n = 77$ weeks in 2002–2004
Bulk carriers arriving weekly to HR	3.5 (1.8)	$n = 77$ weeks in 2002–2004
Bacteria concentration in ballast water	8.03×10^8 l^{-1} (18.8)	$n = 53$, see category 2 in Table 1
VLP concentration in ballast water	13.9×10^9 l^{-1} (15.7)	$n = 31$, see category 2 in Table 1

HR—Hampton Roads; VLP—virus-like particle.

merely for ease of reading; we appreciate the fundamentally different nature of viruses compared to bacteria.

5.3. Total number of surviving bacteria and VLPs delivered to the Port of Hampton Roads

Because the number of microorganisms contained in ballast water is far greater than in residuals or biofilms (see Section 6), these calculations used ballast water only. To determine the amount of ballast water released annually in the port, we multiplied the volume of ballast-water discharged per vessel type by the frequency of vessel arrivals (Table 2). By interviewing ships' officers onboard colliers and container ships, we determined the average volume of ballast-water discharged in port by those vessel types. However, for bulk carriers, we obtained data solely describing ballast water capacity. Assuming their ballasting operations are similar to colliers (a type of bulk carrier), we calculated bulk carriers' ballast-water discharge using the same percentage (65% of total ballast water capacity; SD = 28%; $n = 85$ colliers boarded in Hampton Roads between 1998 and 2003). Thus, we estimated the volume of ballast-water discharged annually from colliers, bulk carriers, and container ships. Together, these three types of vessels represented 85% of the vessel arrivals to the port over the 77-week tracking period.

Next, combining (1) the volume of ballast-water discharged in port, (2) the density of microorganisms in ballast water, and (3) the estimated survival of discharged microorganisms, we calculated the total number of microorganisms annually discharged from ships' ballast water and surviving in the Port of Hampton Roads (Table 2).

6. Results

6.1. Differences in propagule density among habitats within an average bulk carrier

In every ship sampled, all habitats contained viruses and bacteria, but the concentrations of microorganisms varied over 1000-fold among habitats. Per ship, the total number of bacteria was 7.4×10^{12} (within the biofilm habitat), 6.8×10^{14} (residuals = sum of bacteria in sediment pore water and overlying water), and 1.5×10^{16} (ballast water); corresponding VLP numbers were 7.1×10^{14} (biofilm), 3.1×10^{16} (residuals), and 2.6×10^{17} (ballast water) (data not shown). In sum, the potential delivery of microorganisms was greatest in ballast water $\gg$ sediment and water residuals $\gg$ biofilms.

6.2. Survival success of discharged microorganisms

The difference between the temperature of ballast water and the pier side water ranged from 13 to −2.5 °C. Vessels with exchanged ballast water (23 vessels) had temperature differences that spanned the entire range; for vessels with unexchanged water, the range was 6 to −2 °C (nine vessels). We tested whether a significant difference existed between vessels with exchanged and unexchanged ballast water. If there were a difference, it would indicate—using this analysis—that one group of microorganisms (exchanged or unexchanged) would have a greater survivability, and therefore, a greater chance of invading the waters where discharged. There was no significant difference, however, in the average temperature difference between exchanged or unexchanged ballast water and the water sampled pier side (5.7 °C, exchanged; 2.8 °C, unexchanged, T-test, $p > 0.05$).

If microorganisms tolerate water ±15 °C that of the ballast water, then these data show 100% (32 of 32) of ships sampled contained microorganisms that could tolerate the new environment upon delivery (Fig. 1). If microorganisms grow optimally at temperatures ±5 °C of the ballast water, then 56% (18 of 32) of ships sampled contained microorganisms that encountered optimum temperatures in the receiving waters (Fig. 1).

6.3. Total number of bacteria and VLPs surviving ballast-water discharge in the Port of Hampton Roads

Assuming 56% survival (as above) and applying estimates of ship traffic (Table 2), then the total number of

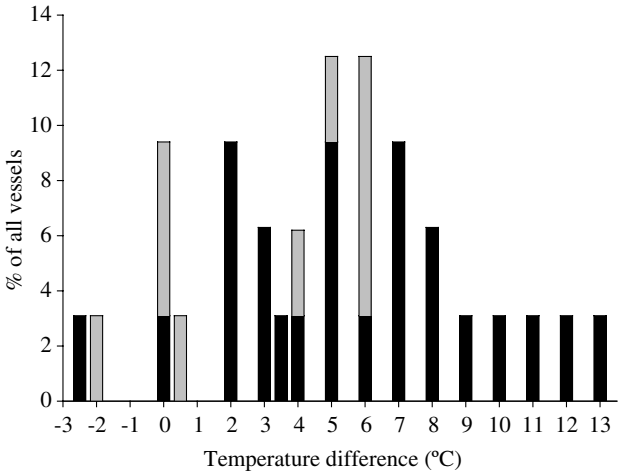

Fig. 1. Distribution of temperature differences between undischarged ballast water and pier side water for 32 vessels arriving to the Port of Hampton Roads. Values greater than zero indicate the ballast water was warmer than the pier side water. Black bars represent vessels with exchanged ballast water; gray bars represent unexchanged water. When both exchanged and unexchanged vessels have the same temperature difference, they are stacked. The sum of all bars' values is 100%.

bacteria discharged from vessels and surviving in the Port is 3.9×10^{18} cells per year. The number of surviving VLPs is 6.8×10^{19} per year. Taken together, 7.2×10^{19} bacteria and viruses annually survive delivery via ships' ballast water to the Port.

7. Conclusions

In the past century, global shipping has increased with respect to the number and size of ships; thus, the volume of ballast water transported has increased as well. We hypothesize this increased inoculation of microorganisms has had ecological or economic impact, and case studies of *V. cholerae* and toxic dinoflagellates demonstrate some potential consequences of microorganisms' release by ships. As economies expand and the number of ships in the world fleet increases, dissemination of microorganisms will in all likelihood grow. Also, as global sea surface temperature is expected to rise, tropical diseases—including water-borne ones—may enlarge their geographic distribution (Pascual et al., 2000, although see Rogers and Randolph, 2000). Thus, not only do we expect delivery of microorganisms to the world's ports to escalate, but release of potential pathogens may increase as well.

From a numerical standpoint, these results show far more bacteria and viruses were contained in ballast water compared to residuals and biofilms. Thus, treatment options should first target the ballast-water fraction of ship-borne microorganisms. We acknowledge our residual and biofilm calculations are likely *under*estimates of the microbial community, because attached bacteria and viruses were not enumerated. However, we must assume only a fraction of residual and biofilm volume (and their associated microorganisms) is resuspended and discharged in a given port. Thus, a calculation of the microbial community contained within the entire volume of residual or biofilm in a ship is most likely an *over*estimate of the microorganisms to be discharged. Even so, residuals and biofilms contain high densities of microorganisms, including pathogenic forms, and, at a minimum, should be addressed using best management practices. The ongoing 'invasional meltdown' (Simberloff and Von Holle, 1999) in the Great Lakes illustrates the risk of biological invasions from vessels carrying "only" sediment and water residuals.

The temperature mismatch between ballast water and pier side water in the Port of Hampton Roads is not great and suggests most bacteria (and presumably viruses) survive following ballast-water discharge; however, other variables must be considered with respect to their subsequent survival and growth. Among them are the concentrations of dissolved organic material (DOM) and nutrients, pH, dissolved oxygen, density and feeding behavior of potential predators, interactions with other members of the foodweb in competition for resources, and salinity. The concentrations of DOM and predators would need to be addressed according to discharge site as they vary spatially on scales that range from μm to km, and temporally from tides to seasons. With respect to salinity, we are unaware of useful, broad assumptions regarding microorganisms' salinity tolerances and suggest evaluation on a case-by-case basis using model microorganisms from freshwater, estuarine, and oceanic environments. Additional variables to take into account include microorganisms' tolerances to abrupt shifts in temperature and salinity, annual temperature and salinity cycles in temperate ports, shipping patterns, tank history, and concentrations of DOM in receiving waters.

An estimated 10^{20} bacteria and viruses in ballast water are annually delivered to and survive in the lower Chesapeake Bay. We recognize this calculation contains a number of assumptions, and the biotic community will vary temporally and with changes in vessel routes, vessel history, vessel type, season, length of voyage, and other factors (e.g., Verling et al., 2005). Nonetheless, to our knowledge, this number is the first estimate of annual microbial delivery to a port.

Acknowledgements

We are thankful for the courtesy of the many ship Captains, Chief Officers, and crewmembers who helped us collect these data. The Hampton Roads Maritime Association and local shipping agents provided updates of vessels' arrivals, departures, and ports of call, which allowed us to track ships arriving to the lower Bay. Many students and colleagues helped us collect and analyze samples—we are grateful to all. Finally, we appreciate the input from two anonymous reviewers. This paper includes data collected with the support of the following research awards: Maryland Sea Grant College Program Grant Number MSG-SC3527704-C, National Sea Grant College Program grant

number MSG-SA7528006-B, and NOAA Office of Sea Grant award numbers NA16RG1697 and NA16RG2271 to the Virginia Graduate Marine Science Consortium and Virginia Sea Grant College Program. The US Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright notation that may appear hereon.

References

Baier, R.E., 1984. Initial events in microbial film formation. In: Costlow, J.D., Tipper, R.C. (Eds.), Marine Biodeterioration: An Interdisciplinary Study. Naval Institute Press, Annapolis, pp. 57–62.

Bailey, S.A., van Overdijk, C.D.A., Jenkins, P., MacIsaac, H.J., 2003. Viability of invertebrate resting stages collected from residual ballast sediment of transoceanic vessels. Limnology and Oceanography 48, 1701–1710.

Barker, J., Brown, M.R.W., 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140, 1253–1259.

Blackburn, S.I., Parker, N., 2005. Microalgal life cycles: encystment and excystment. In: Andersen, R.A. (Ed.), Algal Culturing Techniques. Elsevier/Academic Press, Amsterdam, pp. 399–417.

Carlton, J.T., 1985. Transoceanic and interoceanic dispersal of coastal marine organisms: the biology of ballast water. Oceanography and Marine Biology Annual Review 23, 313–371.

CENR, 2000. National Assessment of Harmful Algal Blooms in US waters. National Science and Technology Council Committee on Environment and Natural Resources, Washington, DC.

Cook, G., Costerton, J.W., Darouiche, R.O., 2000. Direct confocal microscopy studies of the bacterial colonization in vitro of a silver-coated heart valve sewing cuff. International Journal of Antimicrobial Agents 13, 169–173.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322.

Decho, A.W., 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. Oceanography and Marine Biology Annual Review 28, 73–153.

Decho, A.W., 2000. Microbial biofilms in intertidal systems: an overview. Continental Shelf Research 20, 1257–1273.

Deming, J.W., 1997. Unusual or extreme high-pressure marine environments. In: Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., Walter, M.V. (Eds.), ASM Manual of Environmental Microbiology. ASM Press, Washington, DC, pp. 366–376.

DePaola, A., Capers, G.M., Motes, M.L., Olsvik, O., Fields, P.I., Wells, J., Wachsmuth, I.K., Cebula, T.A., Koch, W.H., Khambaty, F., Payne, W.L., Wentz, B.A., 1992. Isolation of Latin American epidemic strain of *Vibrio cholerae* O1 from US Gulf Coast. Lancet 339, 624.

Dobbs, F.C., Rogerson, A., 2005. Ridding ships' ballast water of microorganisms. Environmental Science and Technology 39, 259–264.

Doblin, M.A., Coyne, K.C., Rinta-Kanto, J.M., Wilhelm, S.W., Dobbs, F.C., in press. Dynamics and short-term survival of toxic cyanobacteria species in ballast water from NOBOB vessels transiting the Great Lakes—implications for HAB invasions. Harmful Algae.

Drake, L.A., Choi, K.-H., Ruiz, G.M., Dobbs, F.C., 2001. Global redistribution of bacterioplankton and virioplankton communities. Biological Invasions 3, 193–199.

Drake, L.A., Meyer, A.E., Forsberg, R.L., Baier, R.E., Doblin, M.A., Heinemann, S., Johnson, W.P., Koch, M., Rublee, P.A., Dobbs, F.C., 2005. Potential invasion of microorganisms and pathogens via 'interior hull fouling': biofilms inside ballast-water tanks. Biological Invasions 7, 969–982.

Ducklow, H.W., Shiah, F.-K., 1993. Estuarine bacterial production. In: Ford, T.E. (Ed.), Aquatic Microbiology: An Ecological Approach. Blackwell, London, pp. 261–284.

Figueroa, R.I., Bravo, I., Garces, E., Ramilo, I., 2006. Nuclear features and effect of nutrients on *Gymnodinium catenatum* (Dinophyceae) sexual stages. Journal of Phycology 42, 67–77.

Finlay, B.J., 2002. Global dispersal of free-living microbial eukaryote species. Science 296, 1061–1063.

Finlay, B.J., Fenchel, T., 2004. Cosmopolitan metapopulations of free-living microbial eukaryotes. Protist 155, 237–244.

Fofonoff, P.W., Ruiz, G.M., Steves, B., Hines, A.H., Carlton, J.T. 2003. National Exotic Marine and Estuarine Species Information System. <http://invasions.si.edu/nemesis/>.

Fuhrman, J.A., 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399, 541–548.

Fuhrman, J.A., Noble, R.T., 1996. Viruses and protists cause similar bacterial mortality in coastal seawater. Limnology and Oceanography 40, 1236–1242.

Galil, B.S., Hülsmann, N., 1997. Protist transport via ballast water—biological classification of ballast tanks by food web interactions. European Journal of Protistology 33, 244–253.

Gollasch, S., Dammer, M., Lenz, J., Andres, H.G., 1998. Non-indigenous organisms introduced via ships into German waters. ICES Cooperative Research Report 224, 50–64.

Gollasch, S., MacDonald, E., Belson, S., Botnen, H., Christensen, J.T., Hamer, J.P., Houvenaghel, G., Jelmert, A., Lucas, I., Masson, D., McCollin, T., Olenin, S., Persson, A., Wallentinus, I., Wetsteyn, L.P.M.J., Wittling, T., 2002. Life in ballast tanks. In: Leppäkoski, E., Gollasch, S., Olenin, S. (Eds.), Invasive aquatic species of Europe: distribution, impacts and management. Kluwer Academic Publishers, Dordrecht, pp. 217–231.

Grachev, M.A., Kumarev, V.P., Mamaev, L.V., Zorin, V.L., Baranova, L.V., Denikina, N.N., Belikov, S.I., Petrov, E.A., Kolesnik, V.S., Kolesnik, R.S., Dorofeev, V.M., Beim, A.M., Kudelin, V.N., Nagieva, F.G., Sidorov, V.N., 1989. Distemper virus in Baikal seals. Nature 338, 209.

Grigorovich, I.A., Coulatti, R.I., Mills, E.L., Holeck, K., Ballert, A.G., MacIsaac, H.J., 2003. Ballast-mediated animal introductions in the Laurentian Great Lakes: retrospective and prospective analyses. Canadian Journal of Fisheries and Aquatic Sciences 60, 740–756.

Hairston Jr., N.G., Van Brunt, R.A., Kearns, C.M., Engstrom, D.R., 1995. Age and survivorship of diapausing eggs in a sediment egg bank. Ecology 76, 1706–1711.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79–99.

Hallegraeff, G.M., 1998. Transport of toxic dinoflagellates via ships' ballast water: bioeconomic risk assessment and efficacy of possible ballast water management strategies. Marine Ecology Progress Series 168, 297–309.

Hallegraeff, G.M., Bolch, C.J., 1992. Transport of diatom and dinoflagellate resting spores in ships' ballast water: implications for plankton biogeography and aquaculture. Journal of Plankton Research 14, 1067–1084.

Hallegraeff, G.M., Steffensen, D.A., Wetherbee, R., 1988. Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. Journal of Plankton Research 10, 533–541.

Hamer, J.P., McCollin, T.A., Lucas, I.A.N., 2000. Dinoflagellate cysts in ballast tank sediments: between tank variability. Marine Pollution Bulletin 40, 731–733.

Hennes, K.P., Suttle, C.A., 1995. Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnology and Oceanography 40, 1050–1055.

Hülsmann, N., Galil, B., Baier, R., 2000. Spatio-temporal distribution of viable heterotrophic protists in ballast water and sediments during a transatlantic voyage. In: Aquatic Sciences Meeting hosted by the American Society of Limnology and Oceanography, Copenhagen, Denmark.

International Maritime Organization, 1999. Alien invaders – putting a stop to the ballast water hitch-hikers. <http://www.imo.org/includes/blastDataOnly.asp/data_id%3D7991/BALLASTAlieninvaders1999.pdf>.

Itakura, S., Yamaguchi, M., 2001. Germination characteristics of naturally occurring cysts of *Alexandrium tamarense* (Dinophyceae) in Hiroshima Bay, Inland Sea of Japan. Phycologia 40, 263–267.

Kelly, J.M., 1993. Ballast water and sediments as mechanisms for unwanted species introductions into Washington state. Journal of Shellfish Research 12, 405–410.

Knight, I., Wells, C.S., Wiggins, B., Russell, H., Reynolds, K.A., Huq, A., 1999. Detection and enumeration of fecal indicators and pathogens in the ballast water of transoceanic vessels entering the Great Lakes. In: General Meeting of the American Society for Microbiology. American Society for Microbiology, Chicago, IL, p. 594.

Kremp, A., Anderson, D.M., 2000. Factors regulating germination of resting cysts of the spring bloom dinoflagellate *Scrippsiella hangoei* from the northern Baltic Sea. Journal of Plankton Research 22, 1311–1327.

MacIsaac, H.J., Robbins, T., Lewis, M.A., 2002. Modeling ships' ballast water as invasion threats to the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 59, 1245–1256.

Madigan, M.T., Martinko, J.M., Parker, J., 2003. Brock Biology of Microorganisms, 10th ed. Prentice Hall/Pearson Higher Education Group, Upper Saddle River, NJ.

McCallum, H., Harvell, D., Dobson, A., 2003. Rates of spread of marine pathogens. Ecology Letters 6, 1062–1067.

McCallum, H.I., Kuris, A., Harvell, C.D., Lafferty, K.D., Smith, G.W., Porter, J., 2004. Does terrestrial epidemiology apply to marine systems? TRENDS in Ecology and Evolution 19, 585–591.

McCarthy, S.A., Khambaty, F.M., 1994. International dissemination of epidemic *Vibrio cholerae* by cargo ship ballast and other nonpotable waters. Applied and Environmental Microbiology 60, 2597–2601.

McCarthy, S.A., McPhearson, R.M., Guarino, A.M., Gaines, J.L., 1992. Toxigenic *Vibrio cholerae* O1 and cargo ships entering Gulf of Mexico. Lancet 339, 624–625.

McCollin, T.A., Hamer, J.P., Lucas, I.A.N. 2000. Transport of phytoplankton via ship's ballast into ports around England and Wales. In: J. Pederson (Ed.) Marine bioinvasions: Proceedings of the First National Conference, Cambridge, MA: Massachusetts Institute of Technology, MIT Sea Grant College Program, pp. 282–288.

McMinn, A., Hallegraeff, G.M., Thomson, P., Jenkinson, A.B., Heijnis, H., 1997. Cyst and radionucleotide evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters. Marine Ecology Progress Series 161, 165–172.

Meyer, A.E., Baier, R., Hülsmann, N., Galil, B., Friedmann, D., Forsberg, R. 2000. Risk assessment, prediction, and limitation of transport of bioinvaders in biofilms. Abstracts Book. In: American Society of Limnology and Oceanography, Aquatic Sciences Meeting, June 5–9, 2000, Copenhagen, Denmark.

Milligan, K.L.D., Cosper, EM., 1994. Isolation of virus capable of lysing the brown tide microalga, *Aureococcus anophagefferens*. Science 266, 805–807.

Mimura, H., Katakura, R., Ishida, H., 2005. Changes of microbial populations in a ship's ballast water and sediments on a voyage from Japan to Qatar. Marine Pollution Bulletin 50, 751–757.

Muehlstein, L.K., 1992. The host–pathogen interaction in the wasting disease of eelgrass, *Zostera marina*. Canadian Journal of Botany 70, 2081–2088.

Nagasaki, K., Ando, M., Itakura, S., Imai, I., Ishida, Y., 1994. Viral mortality in the final stage of *Heterosigma akashiwo* (Raphidophyceae) red tide. Journal of Plankton Research 16, 1595–1599.

Noble, R.T., Fuhrman, J.A., 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquatic Microbial Ecology 14, 113–118.

Osterhaus, A.D.M.E., Yang, H., Spijkers, H.E.M., Groen, J., Teppema, J.S., van Steenis, G., 1985. The isolation and partial characterization of a highly pathogenic herpesvirus from the harbor seal (*Phoca vitulina*). Archives of Virology 86, 239–251.

Pascual, M., Rodó, X., Ellner, S.P., Colwell, R., Bouma, M.J., 2000. Cholera dynamics and El Niño-Southern oscillation. Science 289, 1766–1769.

Porter, K.G., Feig, Y., 1980. The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25, 943–948.

Proctor, L.M., 1997. Advances in the study of marine viruses. Microscopy Research and Technique 37, 136–161.

Ralph, P.J., Short, F.T., 2002. Impact of the wasting disease pathogen, *Labyrinthula zosterae*, on the photobiology of eelgrass *Zostera marina*. Marine Ecology Progress Series 226, 265–271.

Rogers, D.J., Randolph, S.E., 2000. The global spread of malaria in a future, warmer world. Science 289, 1763–1766.

Ruiz, G.M., Rawlings, T.K., Dobbs, F.C., Drake, L.A., Mullady, T., Huq, A., Colwell, R.R., 2000. Global spread of microorganisms by ships. Nature 408, 49–50.

Short, S.M., Suttle, C.A., 2002. Sequence analysis of marine virus communities reveals that groups of related algal viruses are widely distributed in nature. Applied and Environmental Microbiology 68, 1290–1296.

Simberloff, D., Von Holle, B., 1999. Positive interactions of nonindigenous species: invasional meltdown? Biological Invasions 1, 21–32.

Smith, D.L., Wonham, M.J., McCann, L.D., Ruiz, G.M., Hines, A.H., Carlton, J.T., 1999. Invasion pressure to a ballast-flooded estuary and an assessment of inoculant survival. Biological Invasions 1, 67–89.

Suttle, C.A., 2005. Viruses in the sea. Nature 437, 356–361.

Van Etten, J.L., Lane, L.C., Meints, R.H., 1991. Viruses and viruslike particles of eukaryotic algae. Microbiology and Molecular Biology Reviews 55, 586–620.

Veldhuis, M.J.W., Cucci, T.L., Sieracki, M.E., 1997. Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. Journal of Phycology 33, 527–541.

Verling, E., Ruiz, G.M., Smith, L.D., Galil, B., Miller, A.W., Murphy, K.R., 2005. Supply-side invasion ecology: characterizing propagule pressure in coastal ecosystems. Proceedings of the Royal Society 272, 1249–1257.

Wommack, K.E., Colwell, R.R., 2000. Virioplankton: viruses in aquatic ecosystems. Microbiology and Molecular Biology Reviews 64, 69–114.

Zo, Y., Grimm, C., Matte, M., Matte, G., Knight, I., Huq, A., Colwell, R.R., 1999. Detection and enumeration of pathogenic bacteria in ballast water of transoceanic vessels entering the Great Lakes and resistance to common antibiotics. In: General Meeting of the American Society for Microbiology, Chicago, IL. American Society for Microbiology, Washington, DC, p. 594.