# Potential invasion of microorganisms and pathogens via 'interior hull fouling': biofilms inside ballast water tanks

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



## Abstract:

Surfaces submerged in an aquatic milieu are covered to some degree with biofilms – organic matrices that can contain bacteria, microalgae, and protozoans, sometimes including disease-causing forms. One unquantified risk of aquatic biological invasions is the potential for biofilms within ships' ballast water tanks to harbor pathogens, and, in turn, seed other waters. To begin to evaluate this vector, we collected biofilm samples from tanks' surfaces and deployed controlled- surface sampling units within tanks. We then measured a variety of microbial metrics within the biofilms to test the hypotheses that pathogens are present in biofilms and that biofilms have higher microbial densities compared to ballast water. Field experiments and sampling of coastwise and oceangoing ships arriving at ports in Chesapeake Bay and the North American Great Lakes showed the presence of abundant microorganisms, including pathogens, in biofilms. These results suggest that ballast-tank biofilms represent an additional risk of microbial invasion, provided they release cells into the water or they are sloughed off during normal ballasting operations.

**Key words:** bacteria, ballast water, Chesapeake Bay, Great Lakes, management, policy, virus Abstract

## Article:

### *Introduction*

Aquatic nuisance species have the potential to effect great ecological and economic change, and a primary vector for global transport of these species is ballast water discharged from ships. When ships load water in one port and discharge it at subsequent ports, their ballast water can contain a diverse mix of plankton, nekton, and benthic organisms (e.g., Carlton 1985). Research on ballast water introductions has emphasized the role of metazoans, with good reason (e.g., the zebra mussel's invasion of the North American Great Lakes), yet unicellular forms are indisputably the most abundant aquatic organisms, and, therefore, species most likely to be transported in ballast water (e.g., Ruiz et al. 2000; Drake et al. 2001).

In attempts to control aquatic nuisance species in ballast water, the only procedure currently in widespread use is open-ocean ballast water exchange (Federal Register 1991; Australian Quarantine and Inspection Service 2001). In this procedure, coastal water is removed from ballast water tanks and replaced with oceanic water. The threat of invasion is theoretically reduced by jettisoning coastal organisms into the open ocean. Further, when the organisms in the exchanged water are discharged at the next port of call, their survival may be reduced due to differences of temperature and salinity between the exchanged, oceanic water and coastal water (Smith et al. 1999).

The most intensively studied microorganisms in ballast water have been toxic dinoflagellates and the bacterium Vibrio cholerae. Dinoflagellates are an important, sometimes-dominant, component of estuarine and coastal phytoplankton communities (Taylor and Pollingher 1987); therefore, they are likely to be entrained in ballast

water pumped aboard ships. Of almost 1800 extant dinoflagellates, more than 80 species form resting cysts (Matsuoka and Fukuyo 1995) and thus constitute a vector for their potential global dispersal (Hallegraeff 1998). Indeed, dinoflagellate cysts have been recorded in ballast-tank sediments of ships arriving at ports around the globe (e.g., Hallegraeff and Bolch 1991, 1992; Kelly 1993; McCollin et al. 2000), and cyst abundances can be high. Hallegraeff and Bolch (1992) estimated a single ballast tank contained >300 million viable Alexandrium tamarense cysts, some of which germinated under laboratory conditions to form toxic cultures. The causative agent of human cholera, Vibrio cholerae, was found in ballast water of ships arriving at ports in Chesapeake Bay (Ruiz et al. 2000) and the North American Great Lakes (Knight et al. 1999; Zo et al. 1999), including V. cholerae O1 or O139, the two epidemic-causing serotypes (Ruiz et al. 2000).

Surfaces submerged in an aquatic milieu are quickly covered to some degree with biofilms, organic matrices that can contain bacteria, microalgae, and associated protozoans, sometimes including pathogenic forms (e.g., overviews by Decho 1990, 2000). These interfaces tend to have greater microbial abundance than surrounding water, whether the biofilms surround particles (e.g., ZoBell and Anderson 1936), occur on the surfaces of organisms (e.g., crustaceans, Carman and Dobbs 1997), or exist at the sediment–water interface (e.g., Novitsky and Karl 1986). Mature biofilms can contain microorganisms notoriously resistant to chemical disinfectants (Costerton et al. 1999) and predation by protozoans (Hülsmann et al. 2000); thus, biofilm matrices provide 'protective refugia' from chemical and biological stresses (Decho 2000).

Ballast water tanks, with their extensive structural architecture, have a high-surface area to volume ratio, and large areas of biofilms may accumulate in them to form 'interior hull fouling'. With respect to aquatic nuisance species, there are potential ecological ramifications of biofilms in ballast water tanks. First, communities within biofilms may sequester organisms during multiple fill and discharge cycles, with the result of concentrating organisms in a biofilm repository having a long-term 'microbial memory'. This environment may promote phenotypic modifications, as well as genetic exchanges among microorganisms. Second, biofilms may act as seed banks, releasing microorganisms, including pathogens, into water subsequently discharged at ports.

Field experiments have demonstrated that biofilm communities form on multiple types of artificial surfaces deployed in a ballast water hold during a trans-oceanic voyage (Meyer et al. 2000). When the substrata were removed from the hold and submerged in artificial seawater, they seeded secondary biofilms, which survived for years in the laboratory (Meyer et al. 2000). Thus, there is evidence that tank biofilms can indeed act as seed banks, but it is not known whether such is the case outside the laboratory.

The research described here was structured around two hypotheses. The first was that tank biofilms contain pathogenic microorganisms. The second was that the abundance of microorganisms contained in biofilms on tank surfaces exceeds that in the ballast water a ship carries. In testing these hypotheses, we quantified microorganisms, including pathogens, in tank biofilms, ballast water, and static samplers deployed in vessels arriving at ports in Chesapeake Bay and the Great Lakes.

## Materials and methods

### *Sample collection*

### Ballast water

A commercial steamship was sampled four times during June–July 2002 when it arrived in the lower Chesapeake Bay, Virginia, USA from Massachusetts, USA. Samples were collected into autoclaved bottles by carefully submerging bottles into the tank by hand or using a bleached Niskin bottle; separate casts of the Niskin bottle were made for each subsample. The containers were transported in a dark, insulated cooler to the laboratory and processed immediately.

Ballast water samples were collected to com-pare microbial metrics in a tank's water column to those values within the static biofilm sampler deployed in the same tank (see below). In each case, the ballast water was approximately 2 days old, having been sequestered during the voyages between Massachusetts and Virginia. Although water of that age is typical for coastwise vessels (e.g., Lavoie et al. 1999), it is 'young' for most

overseas arrivals (OSAs), which have longer ballast-containment periods. For comparison, data from water samples collected from OSAs to ports in Chesapeake Bay (Drake et al. 2001) are included in the results.

### *Biofilms in ballast tanks*

Biofilms were collected from a ballast water tank in each of three vessels arriving at the lower Chesapeake Bay (August 2002, December 2002, February 2003) and from two tanks in a ship arriving at the Great Lakes (October 2002) (Table 1). Biofilm collection began by surface sterilizing (with ethyl alcohol) a template made of polyvinyl chloride (PVC). The template resembled a rectangular picture frame (20 cm × 10 cm, inside dimensions) with a handle used to hold it securely against the ballast-tank wall. Inside the area defined by the template, the wall was scraped using a surface-sterilized polystyrene scraper (15 cm × 2.5 cm). After 1 to 3 1 0-cm transects were scraped (25–75 $cm^2$ area scraped), the scraper and attached material were placed in a sterile 50-ml centrifuge tube. The midsection of the scraper had been scored so it could be snapped neatly in two, leaving the sample end in the tube. Each centrifuge tube was considered a subsample of the tank. Tubes were capped, transported in a dark, insulated cooler to the laboratory, and processed immediately.

### *Biofilms on static sampling units*

Static sampling devices, named Ballast Organic Biofilm (BOB) units, were constructed from PVC sewer drain pipe. A section of pipe, oriented vertically, with a length of 35 cm and an inside diameter of 11 cm, was drilled with multiple holes 2 cm in diameter to allow flow of water through the unit during deployment. PVC caps were secured with nylon bolts or cable ties to both ends of the pipe. Each BOB contained two microscope slide trays, situated vertically and arranged back to back. Each slide tray held 44– 48 polystyrene coupons, in sets of two, oriented so that their treated surfaces were facing either toward the ballast water surface or toward the bottom of the ship (for a total of 88–96 coupons per BOB). The coupons were treated in 1 of 3 ways: untreated polystyrene (medium surface energy, hydrophobic), gas-plasma-treated (high-surface energy, hydrophilic), HumiSeal-coated (dimethylsiloxane polymer; low-surface energy, hydrophobic). Note that low-surface energy coatings yield the easiest release of biofilms (Forsberg et al. 2002).

*Table 1.* Vessel information.

| Sampling location sampling date | Vessel's last port of call | Source of ballast water | Salinity of ballast water (ppt) | Residence time of ballast water in tank (days) | Time tank was empty prior to sampling (days) |
|---|---|---|---|---|---|
| Chesapeake Bay Norfolk, Virginia, USA August 2002 | Houston, Texas, USA | Open ocean (32°22′ N 072°48′ W) | 36 | 3 | 1 |
| Chesapeake Bay Norfolk, Virginia, USA December 2002 | New York, New York, USA | New York, New York, USA | 29 | 5 | 1 |
| Chesapeake Bay Chesapeake, Virginia, USA February 2003 | Point Lisas, Trinidad and Tobago | Cartagena, Columbia | 15 | 7 | 21[a] |
| Detroit River Windsor, Ontario, Canada October 2002 | Korea | n/a | 21 | n/a | n/a |
| Detroit River Windsor, Ontario, Canada October 2002 | Korea | n/a | 33 | n/a | n/a |

[a] Tank walls and biofilm were still wet during sampling.

BOBs were deployed on two commercial vessels during summer 2002. One BOB was secured to a manhole ladder 2 m below the top of a steamship's topside ballast water tank (described above) for 38 days. During that time, it steamed 10 times between Massachusetts and lower Chesapeake Bay, Virginia (BOB Coastwise, BOB CW). The tank was filled and emptied during each 4-day trip (the vessel did not undergo open-ocean exchange), with the BOB remaining in the tank during the empty/refill process.

Two BOBs were deployed on a bulk carrier for 4 days, as it sailed from Rotterdam, Nether-lands to Sept Iles, Canada (BOB Oceanics, BOB OCs). BOBs were secured to manhole ladders 7 m below the top of two side

tanks, one designated as Exchange, the other, Control. The coastal water in the Exchange tank was replaced with oceanic water during the voyage (day 3). To accomplish exchange, the water height in the tank was reduced by 20% by gravity; then oceanic water was pumped into the tank in a flow-through manner such that a three-volume exchange was completed. During this process, the BOB from the Exchange tank was removed, submerged in a bucket of water from the tank, and placed in darkness at 4 °C overnight. The Control BOB was treated in the same manner. Both BOBs were returned to their respective tanks following exchange. All BOBs were transported to the laboratory in darkened containers, and samples were processed immediately (BOB CW) or 13 days after retrieval (BOB OCs; stored in the dark at 4 °C in ballast water until they were shipped to the laboratory and processed).

### *Removing bacteria and viruses from scrapers and coupons*

For all analyses except chlorophyll *a* (chl *a*) and a group of five bacteria (Halomonas aquamarina, Comamonas terrigena, Pseudomonas putrefaciens, Pseudomonas sp., and Vibrio alginolyticus), the following procedure was used to process samples: autoclaved, artificial seawater (Coralife, Energy Savers Unlimited) matching the salinity of the water in the tank (if there was enough water to measure salinity; otherwise, 35 ppt artificial seawater was used) was added to a centrifuge tube containing one scraper or coupon, and the tube was vortexed (Vortex-Genie 2, Fisher Scientific) on 'high' for 60 s (30 s for BOB OC samples). Subsamples of the biofilm suspended in the tube were used for analysis of microbial parameters. When applicable, the concentration of a parameter for a given area or volume was calculated.

The goal of this vortexing procedure was to approximate flushing of tanks, i.e., to dislodge the 'removable' portion of the biofilm community. To establish the appropriate vortexing time, four coupons from the Control BOB OC were tested (two with HumiSeal coating, two with no coating). Each coupon was placed in a centrifuge tube with sterile saline, vortexed for 30 s on the highest setting, then removed and placed into a second tube with sterile saline and vortexed for 30 s. This process was repeated a third time so that coupons were vortexed for a total of 90 s. One hundred microliters of saline from each tube was plated onto Luria-Bertani, Miller agar (LB, Difco), with 3–4 subsamples per tube. Plates were incubated at room temperature for 24 h, and then bacteria colonies were counted. Counts demonstrated that vortexing the coupons for 30 s removed >87% of 'removable' bacteria and 60 s of vortexing removed >93% (100% 'removable' bacteria was set at the sum of the colonies counted on plates from 30, 60, and 90 s for a given coupon; the plates with samples vortexed for 90 s had very low numbers of colonies) (data not shown). Most marine bacteria are not culturable under laboratory conditions (e.g., Amann et al. 1995). We assumed the bacteria grown on the agar plates were a representative subset of the bacteria attached to BOB coupons and in the scraping samples.

### *Bacteria enumeration*

Samples were fixed in a formaldehyde solution (final concentration 2.7%) and stored in the dark at 4 °C until they were enumerated via flow cytometry. Analyses were done using a Becton Dickinson FACScan flow cytometer equipped with a 15 mW, 488 nm, air-cooled Argon ion laser. Simultaneous measurements of forward light scatter, 90-degree light scatter, and green fluorescence were made on all samples. Pico-Green (Molecular Probes, Inc.), a DNA-specific probe, was used to detect and enumerate bacteria (Veldhuis et al. 1997). Detectors (photomultiplier tubes) were in log mode and signal peak heights from excitation wavelengths were measured. The volume of samples was determined gravimetrically using an A-160 electronic balance (Denver Instruments Co.) whereby each sample was weighed prior to analysis and immediately after analysis. All samples were run at a low flow rate setting (approximately 20 µl $min^{-1}$). For biofilm and BOB samples, values were converted from areal measurements to volumetric measurements by assuming a wet biofilm thickness of 250 µm, consistent with the architecture of primary, oligotrophic films (Baier 1984) typical of those found in ballast tanks (Meyer et al. 2000).

### Virus-like particle enumeration

Virus-like particles (VLPs) were counted using the fluorochrome SYBR® Green I (Noble and Fuhrman 1998). Upon return to the laboratory, samples that could not be prepared immediately were fixed in formalin (2.7% final concentration). Fixed and unfixed samples were diluted 1:8 with 0.02 µm-filtered distilled, deionized

water. Next, diluted samples were filtered onto 0.02 μm-pore size Anodisc filters (Whatman International Ltd.) and stained in the dark for 15 min at room temperature with a working solution of the nucleic acid stain SYBRO Green I (Molecular Probes, Inc.).

Filters were placed on microscope slides with a 25 μl drop of antifade mounting solution and counted immediately or stored in the dark at -85 °C until the VLPs were counted. Filters were randomly chosen (in groups of two), thawed in the dark at room temperature for about 5 min, and VLPs counted in 15–20 fields using an Olympus BX-50 System Microscope with a BX-FLA epifluorescent attachment. For each set of filters prepared, two control filters were prepared using only 0.02 μm-filtered distilled, deionized water, and their average VLP count was subtracted from values determined in field samples. For bio-film and BOB samples, values were converted to volumetric measurements as above.

### *Chlorophyll* a *and phaeopigment determination*

Chl *a* samples from ballast water were collected by filtering 500 ml of ballast water onto 47 mm-diameter glass fiber filters (GF/F, Whatman International Ltd.) at a vacuum pressure of 100 mm Hg. Filters were wrapped in foil and stored at -85 °C until the chl *a* and phaeopigment (phaeophytin and phaeophorbide) on the filters were extracted in acetone and measured fluorometrically (Parsons et al. 1992).

For biofilm samples from the field and the BOBs, scrapers or coupons were placed in 50-ml centrifuge tubes, stored at -85 °C until acetone was added to the centrifuge tubes, and the tubes were vortexed for 60 s. The chl *a* and degradation pigments were then analyzed fluorometrically as described above. For biofilm and BOB samples, values were converted to volumetric measurements as above.

### *Comparison of microbial abundance in ballast water vs biofilms*

Initial comparisons were made between 'bulk' microbial metrics (bacteria density, VLP abundance, and phytoplankton pigment concentration) in ballast water and those within tank biofilms. To more meaningfully estimate the separate contributions of microbes from each source during ballast discharge, metrics were normalized either to the volume (ballast water) or ballast-tank area (biofilms) of ships sampled. The ballast water capacity of nine bulk carriers boarded in this study was averaged. The potential maximum volume of ballast-tank biofilm was calculated by multiplying the internal, ballast-tank area of average bulk carriers (44,593 $m^2$ for vessels of 33,000 dead weight tons, John Kelly, International Paint, pers. comm.) by the estimated thickness of hydrated biofilms, 250 lm (Baier 1984; Meyer et al. 2000). Assuming that biofilms <50 μm thick would not have been collected using our techniques and, given the amount of biofilm on the scrapers, that the biofilms collected were <500 μm thick, 250 μm approximated the mid-point of the range. So estimated, the potential maximum volume assumes all surface areas within tanks are hydrated, which was not the case on the vessels boarded in this study. As a more conservative estimate, therefore, biofilm volume also was calculated assuming 10% of available ballast-tank surface area was covered with hydrated biofilm. Finally, to determine the average ship's capacity to discharge microorganisms, the mean concentration of each microbial metric was multiplied by either ballast water volume or estimated biofilm volume.

### **Vibrio cholerae** *detection*

### *Cultural and biochemical tests*

Samples were analyzed using a modification of Choopun et al. (2002). Briefly, suspended bio-films and ballast water samples (in aliquots of 1, 10, and 100 ml of each sample) were filtered onto 0.45 μm-pore size filters (Osmonics). Filters were placed directly onto Thiosulfate–Citrate–Bile– Sucrose agar (Difco) and incubated at 35 °C for 18–24 h. Up to four sucrose-positive (yellow) colony-forming units per sample were streaked for isolation onto LB agar (Difco). On colonies picked from the LB plate, the following biochemical analyses were performed: an arginine dihydrolase test, an esculine hydrolysis test, and an oxidase test. Isolates positive for oxidase, negative for esculin hydrolysis, and negative for arginine dihydrolase were scored as V. cholerae. A sample from a ship was considered positive if any of its subsamples contained V. cholerae.

### *Fluorescent antibody tests*

Vibrio cholerae serotypes O1 and O139 were identified using immunofluorescent antibodies. Ballast water samples (200 ml) were filtered onto 47 mm 0.22 μm-pore size filters (MSI, Inc.); the filters were scraped gently with a sterile loop to resuspend cells, and the samples were fixed in a final concentration of 2% formalin. For scrapings and BOB slides, scrapers and coupons were placed into centrifuge tubes, autoclaved saline solution was added, tubes were vortexed (described above), and then samples were fixed in a final concentration of 2% formalin. For ballast water, scraping, and BOB samples, a 10 μl subsample of the fixed mixture was removed and stained with fluorescent anti-body (New Horizons, Inc.), and cells were enumerated under epifluorescence microscopy using an Olympus BX50 System Microscope with epifluorescent attachment. Positive and negative reagent controls were run on each day of analysis.

### *Ferrographic tagging*

To test for very low abundance of V. cholerae, we used a ferrographic technique that combines the selectivity of immunomagnetic tagging with the high resolution of ferrography (Zhang et al. 1999a, b; Johnson et al. 2000). The quantification limit has been shown to be extremely low, 20 cells $ml^{-1}$, with analytical errors ranging between 10% and 20% (Johnson et al. 2000, 2001).

Following sample collection, 500 μl of water was removed from ballast water, biofilm, or BOB samples, and goat anti-rabbit antibody-coated super paramagnetic microbeads were tethered to cell surfaces selectively using monoclonal rabbit antibodies raised to whole cells of V. cholerae. Antibodies for V. cholerae O1 and O139 were produced at a commercial laboratory (New Horizons, Inc.). Immunomagnetically tagged cells were deposited onto an exceedingly small area on a glass coverslip using a device (Bio-Ferrograph, Guilfoyle, Inc.) that moved the sample through a closed chamber situated over a gap between two magnetic pole pieces. The magnetic gradient over the gap was sufficient to force deposition of only slightly magnetically susceptible particles onto a glass coverslip. Enumeration of deposited cells was performed using a Nikon Eclipse E400 microscope at 1000 × magnification. Standards were run with each analysis to ensure proper reaction conditions, not for numerical adjustments to counts. Standards were in the range of 100– 300 cells $ml^{-1}$. Standards counts within 20% of the expected count indicated analytical conditions were optimal. If standards counts exceeded 20% error, reaction conditions were modified and counts were performed again.

### *Detection of the 'Group of Five' bacteria*

Samples of ballast water and BOB coupons were analyzed for the presence of Halomonas aquamarina, Comamonas terrigena, Pseudomonas putrefaciens, Pseudomonas sp., and Vibrio alginolyticus, dominant organisms among culturable bacteria collected previously from a seawater environment (Zambon et al. 1984); some of these species can be pathogenic. It is not implied that they predominated in the present study, rather that their detection was useful in testing the hypotheses. Rabbit antisera were converted to immunofluorescent reagents for these species (Zambon et al. 1984) and immunofluorescence on a Leitz Wetzlar Ortholux II microscope system was used to detect and enumerate the bacteria. Biofilm scrapings were prepared as smears on gas-plasma-treated glass slides; samples on BOB coupons were characterized in situ. In both cases, each of the five fluorescently labeled antibodies was applied to a specific area of each slide or coupon. Prior results demonstrated the reagents can identify up to 39% of the attached bacteria in biofilms formed within a portable biofouling unit filled with freshly exchanged water from the Atlantic Ocean (Meyer et al. 1984; Zambon et al. 1984). A more recent study illustrated that the immunofluorescent staining techniques are valid for identifying these bacterial species in ballast-tank biofilms (Forsberg 2003). Tests with control microorganisms demonstrated that there was no cross-reactivity between the antibodies for the Pseudomonas putrefaciens and the unidentified Pseudomonas sp.

### *Determination of dinoflagellate cyst viability*

Isolation of cysts began with vortexing scrapers or coupons in artificial-seawater solutions matching the salinity of the sample, then filtering the sloughed biofilm through 90-μm and 20-μm mesh filters (following Bolch 1997). Material retained on the 20-μm mesh was examined under a dissecting microscope to ensure that vegetative dinoflagellate cells were absent, and then the material was backwashed into a beaker and subsampled

into six-well plates. To determine viability, isolated cyst-like objects (CLOs) were incubated under light conditions (12:12 L:D cycle) at 18 °C in f/10 (Guillard 1975) nutrient-enriched Chesapeake Bay water (salinity 0, 10, 20, 30 ppt) and scored for germination success by the presence of swimming vegetative cells. Since dinoflagellate cysts typically have endogenous dormancy (e.g., Blackburn et al. 1989; Anderson et al. 1995), incubations were conducted for a total of 60 days. A sample was considered positive for dinoflagellate presence or germination if any of its subsamples was positive.

### *Detection of Pfiesteria species*

Samples (100 ml for water samples, 40 ml for biofilm and BOB samples) for Pfiesteria piscicida and P. shumwayae were drawn onto glass fiber filters (GF/C or GF/F, Whatman International Ltd.), which were then immersed in cetyl-trimethyl-ammonium-bromide lysis buffer and stored at room temperature (Schaefer 1997). DNA was extracted and purified (Schaefer 1997), and aliquots of the purified DNA were assayed by real-time PCR using fluorescent probes (Bowers et al. 2000). Both positive (DNA extracted from cultures) and negative (no template) controls were run in every PCR reaction. A sample was considered positive if any of its subsamples was positive.

### *Data analysis*

All statistics were calculated using SPSS Base 9.0.0 (SPSS Inc.). Each tank within a ship was designated a sampling unit, with 2–4 subsamples collected per tank. Thus, within a given ballast tank, each Niskin-bottle cast to collect water and each scraping to collect biofilm was considered a subsample.

Data from the Control and Exchange oceanic BOBs were pooled because none of nine comparisons yielded significant differences between treatments (although bacteria samples did have significant interaction among factors). Preliminary analyses (ANOVAs) showed it was reason-able to pool BOB samples with respect to surface coating and orientation; among 18 possible comparisons, only five showed significant differences ($P < 0.05$). Four of the significant differences were in chl *a* and phaeopigment data, which tend to covary, from each of the two BOBs (CW and OC). The pigment differences were not consistent between BOBs – surface coatings yielded significantly different values in one BOB; coupon orientation yielded different values in the other BOB. Therefore, individual coupons within a BOB, regardless of surface coating or orientation, were considered replicates (for a total of 88–96 replicates per BOB). The specifics of the surface coatings will be discussed within the context of a larger dataset (Meyer et al. in prep).

Values for bulk microbial metrics (bacteria, VLPs, pigments) and for the group of five bacteria were analyzed as 1-factor or 2-factor ANOVAs on rank-transformed data (Conover and Inman 1981). Error variances were not equal across groups, therefore, post hoc comparisons of significant differences were performed with the Dunnett T3 test, which accounts for unequal error variance (n = 4–14 with 0–4 subsamples per replicate).

## Results

*Bulk microbial metrics – bacteria density, virus-like particle abundance, and algal pigment concentration*

Mean bacteria concentrations ranged 26-fold, from 1.73 to 44.9 × $10^9$ cells $l^{-1}$, with values lowest in overseas arrival (OSA) ballast water and highest on the BOB Coastwise (BOB CW) slides (Table 2). Bacteria abundances differed significantly among the types of samples collected. The biofilm mean was not significantly different from those of all other sample types, although each of them was significantly different from one another ($df = 4$, $P < 0.0001$, Table 2).

Densities of VLPs varied 88-fold, from 0.132 to 11.6 × $10^{11}$ $l^{-1}$ (Table 2). The mean VLP con-centration was lowest for samples of OSA water and highest for samples from the BOB CW slides. The only post hoc difference that emerged among sample types was that the mean VLP con-centration in OSA water samples was significantly lower than all others, save the biofilm mean ($df = 4$, $P < 0.0001$).

Chl *a* concentrations spanned 36,000-fold, from 0.02 to 715 μg $l^{-1}$ in OSA water and BOB CW samples, respectively (Table 2). The biofilm mean was not significantly different from those of all other sample types,

although each of them was significantly different from one another (df = 4, P < 0.0001, Table 2). Phaeopigment means also had a vast range, from 0.10 to 5109 μg $l^{-1}$, and, as with all other bulk microbial metrics, the mean was lowest in OSA water samples and highest in the BOB CW samples (Table 2). The biofilm mean was not statistically different from each of the other sample types, but each of them was significantly different from one another (df = 4, P < 0.0001, Table 2).

*Table 2.* Bulk microbial metrics in ships' ballast water, biofilm, and BOB samples.

| Metric | Coastwise water[a] | OSA water [b] | Biofilm | BOB (coastwise) | BOB (oceanic) |
|---|---|---|---|---|---|
| Bacteria mean ± SD (× $10^9$ $l^{-1}$) | 3.19 ± 1.17 $^{A}$ | 1.73 ± 3.06 $^{B}$ | 6.62 ± 8.83 $^{ABCD}$ | 44.9 ± 44.8 $^{C}$ | 12.1 ± 3.80 $^{D}$ |
| Bacteria *n* | 4 (3) | 18 | 3 (2–3) | 6 | 10 |
| VLP mean ± SD (× $10^{11}$ $l^{-1}$) | 2.96 ± 1.81 $^{A}$ | 0.132 ± 0.155 $^{B}$ | 6.33 ± 8.69 $^{AB}$ | 11.6 ± 12.6 $^{A}$ | 3.22 ± 2.33 $^{A}$ |
| VLP *n* | 4 (3) | 12 | 5 (2–3) | 6 | 10 |
| Chl *a* mean ± SD (μg $l^{-1}$) | 4.04 ± 2.21 $^{A}$ | 0.02 ± 0.01 $^{B}$ | 255 ± 393 $^{ABCD}$ | 715 ± 795 $^{C}$ | 36.7 ± 36.8 $^{D}$ |
| Chl *a n* | 4 (3) | 7 | 5 (2–3) | 8 | 14 |
| Phaeo mean ± SD (μg $l^{-1}$) | 1.81 ± 0.27 $^{A}$ | 0.10 ± 0.11 $^{B}$ | 2029 ± 1837 $^{ABCD}$ | 5109 ± 5858 $^{C}$ | 198 ± 158 $^{D}$ |
| Phaeo *n* | 4 (3) | 7 | 5 (2–3) | 8 | 14 |

Column labels: BOB = ballast organic biofilm sampling unit; bacteria = abundance of bacteria; VLP = abundance of virus-like particles; Chl *a* = concentration of chlorophyll *a*; Phaeo = concentration of phaeopigments; *n* = number of samples. Numbers in parentheses indicate number of subsamples. Different superscripted letters indicate significant differences among means in a row ($P < 0.05$). SD = standard deviation.

[a] This study.

[b] Data reported in Drake et al. (2001) recalculated to include surface samples only. Across all OSA samples, the age of ballast water ranged from 3 to 28 days (mean = 13 days, *n* = 20 ships), and samples were collected in all seasons and from exchanged and unexchanged tanks.

### *Comparison of microbial abundance in ballast water vs biofilms*

Estimates of microbial abundance in biofilms were much lower than or similar to estimates for ships' ballast water when it was assumed that 100% of the available tank surface was covered with biofilm (Table 3). The numbers of biofilm bacteria and VLPs were approximately 1000 times lower than those in CW water and OSA water. Biofilm chl *a* and phaeopigments estimates were similar to CW and OSA water values. When similar calculations were made assuming only 10% coverage of biofilms, the differences were magnified 10-fold (Table 3).

## Vibrio cholerae

### *Biochemical and cultural tests*

Half of the water samples were positive for the presence of V. cholerae (Figure 1). No V. cholerae were detected in the biofilm samples, but they were present in the BOBs (67% of the BOB CW samples and 33% of the BOB Oceanic [BOB OC] samples).

### *Fluorescent antibody tests*

No samples were positively scored for the O1 and O139 strains from analyses of water (n = 4, with 3 subsamples per sampling effort), biofilm (n = 4, with 2–3 subsamples per ship), BOB CW (n = 6 or 5, respectively), and BOB OC samples (n = 12 or 10, respectively) (data not shown).

### *Ferrographic tagging*

No Vibrio cholerae of either serotype (O1 or O139) were detected in water (n = 4, with 3 subsamples per sampling effort), biofilm (n = 2, with 2–3 subsamples per ship), BOB CW (n = 2), and BOB OC (n = 8) samples (data not shown).

## Group of five bacteria

Bacteria concentrations ranged from 4 × $10^3$ cells $cm^{-2}$ (Vibrio alginolyticus in biofilm samples) to 315 × $10^3$ cells $cm^{-2}$ (Pseudomonas putrefaciens in BOB OC samples) (Figure 2). The means varied such that BOB OC >BOB CW > Biofilm, but none of these comparisons within a given bacteria species was significant (1-way ANOVA on rank-transformed data, df = 2, P > 0.05 in all cases). When all data were analyzed together, there were no significant differences among bacteria species or type of sample collected, and no significant interaction among factors emerged (2-way ANOVA on rank-trans-formed data, df = 14, P = > 0.05).

### *Dinoflagellate cysts*

Samples were collected from ballast water (CW water), biofilm scrapings, and coupons deployed in BOB units (Figure 3). One of four (25%) water samples contained CLOs, but they did not germinate during 60 days' incubation. Biofilm scrapings had a higher incidence of CLOs (3 of 6 samples; 50%), but they did not germinate.

*Table 3.* Estimated values of bulk microbial metrics in ballast water and in biofilm.

| Metric | Ballast water | | Biofilm[c] | |
|---|---|---|---|---|
| | Coastwise water[a] | OSA water[b] | 100% | 10% |
| Bacteria ($\times 10^{16}$) | 5.6 | 3.2 | 0.0074 | 0.00074 |
| VLP ($\times 10^{18}$) | 5.2 | 0.24 | 0.0071 | 0.00071 |
| Chl *a* ($\times 10^{7}$ µg) | 7.1 | 0.028 | 0.28 | 0.028 |
| Phaeo ($\times 10^{7}$ µg) | 3.2 | 0.18 | 2.3 | 0.23 |

Column labels: Biofilm 100% = value of microbial metric in the total amount of biofilm within an average bulk carrier's ballast water tanks; Biofilm 10% = value of microbial metric in the total amount of biofilm within an average bulk carrier's ballast water tanks, assuming only 10% of the ballast-tank surface area was covered with biofilm; bacteria = number of bacteria; VLP = number of virus-like particles; Chl *a* = amount of chlorophyll *a*; Phaeo = amount of phaeopigments.

[a] This study.

[b] Using data reported in Drake et al. 2001, recalculated to include surface samples only. Across all overseas arrival (OSA) samples, the age of ballast water ranged from 3 to 28 days (mean = 13 days, $n$ = 20 ships), and samples were collected in all seasons and from exchanged and unexchanged tanks.

[c] Calculated biofilm metric assuming a biofilm thickness of 250 µm.

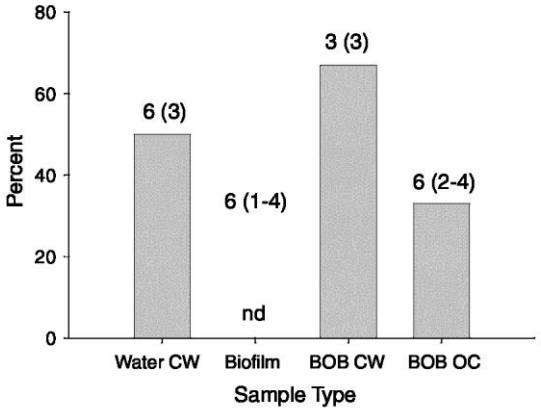


*Figure 1.* Percentage of samples testing positive for the presence of *Vibrio cholerae*. Water CW = ballast water from coastwise ship; BOB CW = Ballast Organic Biofilm sampling unit deployed in a coastwise ship; BOB OC = BOB sampling unit deployed in an oceanic ship; nd = none detected. Values above bars indicate the number of samples collected, with the number of subsamples per sample in parentheses.

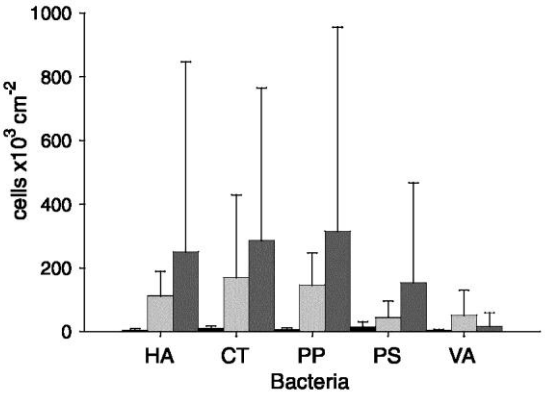


*Figure 2.* Group of five bacteria in ships' ballast water tanks. From left to right for each group, black bars represent biofilm samples ($n$ = 4, with 3–4 subsamples per ship); light-gray bars represent samples from the BOB sampling unit deployed in a coastwise ship (BOB CW) ($n$ = 6); dark-gray bars represent samples from the BOB deployed in an oceanic ship (BOB OC) ($n$ = 9). Error bars represent 1 SD. HA = *Halomonas aquamarina*; CT = *Comamonas terrigena*; PP = *Pseudomonas putrefaciens*; PS = *Pseudomonas* sp.; VA = *Vibrio alginolyticus*.

In contrast, most coupons recovered from BOBs deployed in ballast tanks contained CLOs, and of these, some germinated to actively swimming, vegetative dinoflagellates. The BOB CW contained 6 samples, all of which had CLOs. Of those, 4 germinated (67%). The BOB OC contained 12 samples, 9 of which had CLOs (75%), but none germinated.

Cysts from the BOB CW germinated in salinities of 0, 10, 20 and 30, indicating the biofilms contained species capable of germinating in fresh water, estuarine, or marine environments. Germinating cells were photographed when possible, but attempts to isolate them were unsuccessful.

### Pfiesteria species

Pfiesteria piscicida was present in 25% of ballast water samples (n = 4, with 2–3 subsamples per sampling effort) (data not shown). It was not detected in biofilm samples (n = 4, with 1–2 subsamples per ship) or BOB samples (n = 12; 6 from the BOB CW, 6 from the BOB OC). All samples were also analyzed for the presence of P. shumwayae, which was not detected.

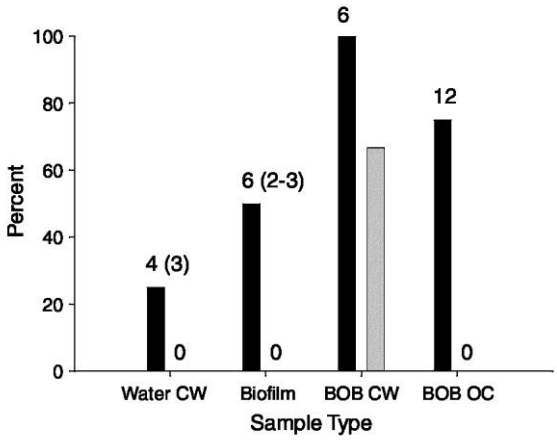


*Figure 3*. Cyst-like objects (CLOs) and dinoflagellates. Black bars represent the percent of samples containing CLOs; gray bars represent the percentage of CLOs that germinated into vegetative cells. Water CW = ballast water collected from the coastwise ship; BOB CW = Ballast Organic Biofilm sampling unit deployed in a coastwise ship; BOB OC = BOB sampling unit deployed in an oceanic ship. Values above bars indicate the number of samples collected, with the number of subsamples per sample in parentheses.

## Discussion

Ballast water tanks can be characterized as 'dark intertidal zones', environments with highly variable hydrologic conditions that nonetheless support organisms of various taxa. This study has demonstrated that ships arriving at ports in Chesapeake Bay and the Great Lakes contain biofilms comprised in part of bacteria, VLPs, and cysts of microalgae and are, in some cases, a reservoir for pathogens. We analyzed samples for only a very small suite of pathogens but speculate that a broader range of disease-causing microorganisms may be found in tank biofilms. In this discussion, we will first consider the particulars of the data, then comment on the managerial ramifications of this study.

Although we detected the bacterium Vibrio cholerae in ballast water, we found neither of the two epidemic-causing serotypes, O1 or O139, in ballast water, biofilms, or BOB samples. This result contrasts with that of Ruiz et al. (2000), who found both serotypes in ballast water of 93% of ships arriving at ports in Chesapeake Bay following a trans-Atlantic voyage. This inconsistency is puzzling, given that the methods in both studies employed the same antibody procedure for detecting bacteria. Fully resolving the disparity between studies will require more sampling to test for possible effects of seasonality, water quality, duration of voyages, and ports of origin. In addition, it is possible that detection of V. cholerae cells within biofilms was affected by rugose exopolysaccharide (rEXP) production. V. cholerae cells may, at low frequency, spontaneously enter a growth state in which they produce copious amounts of rEXP (Ali et al. 2002), which is most prevalent under nutrient-limiting conditions. Antibodies would probably inefficiently capture cells surrounded by a large rEXP layer.

The abundance of dinoflagellate cysts in ballast water and biofilm scrapings was relatively low compared to that on BOB slides. Although cysts have a similar density to silt and clay particles and therefore will tend to settle out of the water column onto horizontal, rather than vertical, surfaces, this result cannot be explained solely by settling dynamics: cysts were detected on BOB coupons in the 'down' position and were capable of germination (data not shown). Perhaps the BOB slides (with coatings) or BOB slides and their associated biofilm were sufficiently 'sticky' to capture cysts resuspended during ballasting operations or by sloshing ballast water while the ships were underway.

In contrast, Pfiesteria piscicida and P. shumwayae were not detected in either biofilm or BOB samples. We know our detection methods are appropriate for ballast water and ballast-tank sediments, since Pfiesteria has been detected in many such samples, including those from ships arriving at ports in Chesapeake Bay (Doblin et

al. in press). Therefore, either Pfiesteria species are not associated with tank biofilms (at least in these samples) or our methods were unable to detect them within biofilm matrices.

Three of four bulk microbial metrics were statistically higher in samples collected from the BOB CW than the BOB OC. This result was not surprising, given that ballast water aboard the CW ship was refreshed every 4 days with coastal water. Of interest, however, was that there was no difference in the distribution of species within BOBs with respect to the group of five bacteria. This result supports those of Forsberg (2003), who showed little difference in the abundance of the five groups of bacteria among samples collected from nine different surface-characterized substrata, deployed on five trans-oceanic voyages across all seasons, onboard three separate vessels having different ballasting histories. These results suggest the abundance of the group of five bacteria in the biofilms on the coupons were influenced most by initial conditions in the tanks.

Questions of marine public policy drive most ballast water studies, and from this work, we pose the question – what is the importance of ships' biofilms as an invasion vector? We acknowledge that our samplings of ballast-tank microorganisms represent only snapshots of aquatic microbial landscapes, which exhibit pro-found temporal changes (Drake et al. 2002; Doblin et al. 2003). Furthermore, biofilm samples collected by scraping relatively tiny areas surely underestimate the diversity inherent in a ballast tank's total microbial community. With these caveats, our data show tank biofilm samples, when extrapolated to an entire ship, contain – at a maximum – much lower abundances of bacteria and VLPs and subequal amounts of chl *a* and phaeopigments relative to the volume of ballast water typically carried by the ship (Table 3). Nonetheless, the total number of microorganisms contained within a single ship's ballast-tank bio-films is great. These results, coupled with our finding pathogens in biofilms (see also Forsberg 2003) suggest ballast-tank biofilms represent a risk of microbial invasion, should they release cells into the water or be sloughed off during ballasting operations.

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