11-2004

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Original Publication Citation

Doblin, M.A., Popels, L.C., Coyne, K.J., Hutchins, D.A., Cary, S.C., & Dobbs, F.C. (2004). Transport of the harmful bloom alga Aureococcus anophagefferens by oceangoing ships and coastal boats. *Applied and Environmental Microbiology, 70*(11), 6495-6500. doi: 10.1128/aem.70.11.6495-6500.2004

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2004, p. 6495–6500 Vol. 70, No. 11
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.11.6495–6500.2004

Transport of the Harmful Bloom Alga *Aureococcus anophagefferens* by Oceangoing Ships and Coastal Boats

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Received 25 April 2004/Accepted 18 July 2004

It is well established that cyst-forming phytoplankton species are transported in ships' ballast tanks. However, there is increasing evidence that other phytoplankton species which do not encyst are also capable of surviving ballast transit. These species have alternative modes of nutrition (hetero- or mixotrophy) and/or are able to survive long-term darkness. In our studies of no-ballast-on-board vessels arriving in the Great Lakes, we tested for the presence of the harmful algal bloom species *Aureococcus anophagefferens* (brown tide) in residual (i.e., unpumpable) ballast water using methods based on the PCR. During 2001, the brown tide organism was detected in 7 of 18 ballast water tanks in commercial ships following transit from foreign ports. Furthermore, it was detected after 10 days of ballast tank confinement during a vessel transit in the Great Lakes, a significant result given the large disparity between the salinity tolerance for active growth of *Aureococcus* (>22 ppt) and the low salinity of the residual ballast water (~2 ppt). We also investigated the potential for smaller, recreational vessels to transport and distribute *Aureococcus*. During the summer of 2002, 11 trailered boats from the inland bays of Delaware and coastal bays of Maryland were sampled. Brown tide was detected in the bilge water in the bottoms of eight boats, as well as in one live-well sample. Commercial ships and small recreational boats are therefore implicated as potential vectors for long-distance transport and local-scale dispersal of *Aureococcus*.

It is well established that cyst-forming phytoplankton are transported in ballast tanks (21, 22, 24, 31, 33). Following introduction, harmful forms can become established (e.g., the toxic dinoflagellates *Gymnodinium catenatum* in southeast Tasmania, Australia, and *Alexandrium catenella* in Tau Lagoon, France) where they may have significant ecological and economic impact (23, 30). However, there is increasing evidence that at least some algal species which do not form cysts may survive ballast transit (12, 15). Furthermore, coast-wise vessels and recreational boats are increasingly being implicated in facilitating the spread of introduced species (27, 28, 37, 44).

If we consider that ballast and bilge tanks are dark, aerobic environments, the likely survivors of ballast transit (apart from cyst formers) are those phytoplankton species that have alternative modes of nutrition (hetero- or mixotrophy) and/or the ability to survive long-term darkness. The brown tide organism, *Aureococcus anophagefferens* (referred to below as *Aureococcus*), fits both of these criteria, since it is able to grow with organic enrichment (3, 9, 14, 18) and at low light (35) and it is able to survive at least 30 days in complete darkness (39). Brown tides have had significant detrimental impacts on the benthic communities in Narragansett Bay in Rhode Island, the bays of Long Island in New York, and New Jersey (5), causing eelgrass dieback (due to decreased light penetration) and starvation and recruitment failure of commercially important scallop and mussel populations (7, 43).

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New data suggest that there has been a dramatic extension of the known range of *Aureococcus* on the east coast of the United States (40). Previously, this organism was known to exist in shallow estuaries from New York to Maryland but not further south (1). The newly defined range extends from Florida north to New Hampshire (40), and blooms are now being recorded in Virginia's coastal bays (4). Blooms have also recently been observed in Saldanha Bay in South Africa (36, 38, 42). Thus, the distribution of brown tide seems to be rapidly increasing both within and outside the United States, suggesting that there is an anthropogenic dispersal vector, exploitation of a niche in previously uninhabited environments, or both.

In this study, we tested the hypothesis that brown tide is transported long distances via ships' ballast water and on the local scale via recreational boats. This species is small (diameter, approximately 2 to 4 μm) and difficult to detect directly by regular microscopic observation, particularly at low background cell levels. To confirm the presence of the organism, we used an *Aureococcus*-specific primer for detection in ballast water by PCR amplification of the 18S rRNA gene. *Aureococcus* concentrations in bilge water of recreational boats were determined by quantitative real-time PCR by using a species-specific molecular probe (40).

MATERIALS AND METHODS

Presence of *Aureococcus* in ships' ballast water. During 2001, we sampled residual water in no-ballast-on-board (NOBOB) bulk carrier ships arriving in the North American Great Lakes. These vessels were all foreign arrivals (mainly from ports in Western Europe, including one from the Baltic region and another from the Mediterranean, but also one ship from Asia). None of the ships had pumpable ballast on board. Although *Aureococcus* (a polyhaline organism that

inhabits waters with salinities of 18 to 30 ppt) poses little potential risk of establishment in the Great Lakes, we sampled commercial vessels destined for Great Lakes ports because their residual water should have reflected the integrated history of previous ballasting operations (i.e., multiple ballast loads and exchanges), including those at marine, brackish, and freshwater ports. Residual water was collected from ships at the ports of Hamilton, Thorold, and Windsor in Canada and the ports of Cleveland, Chicago, and Burns Harbor in the United States. Vessels were selected opportunistically without consideration of previous ports of call (i.e., probable ballast water sources). Residual water was collected by using either new (but not sterile) polyethylene siphon pumps or sterile plastic scoops from double-bottom, upper wing, or forepeak ballast tanks (most frequently double-bottom tanks on the port and starboard sides of a ship). Within a given tank, samples were taken at numerous locations and pooled. Five ships (a total of 10 tanks) were sampled during the summer (June to July 2001), and five ships (a total of eight tanks) were sampled during the fall (October to November 2001). The ships were between 1 and 18 years old, and the time between thorough tank cleaning and sampling ranged from 7.8 to 36 months.

Presence of *Aureococcus* during vessel transit through the Great Lakes. In October 2001, we sampled a commercial vessel during its transit through the Great Lakes. The ship was initially sampled in the Port of Hamilton as a NOBOB vessel (i.e., residual water was collected). The forepeak tank was then partially filled with harbor water (freshwater) and resampled approximately 1 m below the surface (zero time), and then it was sampled again in the Port of Windsor on day 6 and in the Port of Chicago on day 10.

Presence of *Aureococcus* in small boats. We sampled trailered small boats as they emerged from Delaware's inland bays and Maryland's coastal bays. Visits to Indian River Marina (in Delaware) and Ocean City (in Maryland) in the summer of 2002 yielded samples from 11 vessels. Permission was requested from recreational boaters before water was collected. Just after a boat was pulled up the ramp, the aft bung was unscrewed, and water from the bottom of the boat was collected directly in a clean sampling bottle. For one boat an onboard live well (approximately 0.25 m^2 of storage space filled with seawater and used for carrying fish or bait) was also sampled to determined whether it contained brown tide. A water sample was also collected adjacent to the boat ramp at each marina in order to determine whether *Aureococcus* was present in local water at the time.

Nucleic acid extraction and DNA amplification. Water samples were stored in coolers filled with water or ice to keep them at ambient temperature. Between 100 and 300 ml of each water sample was prefiltered through 20- and 5-μm-pore-size polycarbonate filters (Osmonics, Livermore, Calif.) to remove larger planktonic species and detritus. The 5-μm-pore-size filtrate was then filtered through 1-μm-pore-size polycarbonate filters to collect the plankton size fraction that included *Aureococcus* (diameter, 2 to 4 μm). The 1-μm-pore-size membranes were placed in 0.6 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 2% [wt/vol] cetyltrimethylammonium bromide, 0.4% [vol/vol] β-mercaptoethanol, 1% [wt/vol] polyvinylpyrrolidone) (11), heated to 50°C for 20 to 40 min, and stored at −80°C until extraction. All samples were heated in a 65°C water bath for 5 to 10 min immediately before extraction. Nucleic acids were extracted as described previously (10) and were diluted to a concentration of 25 ng/μl for amplification.

For ballast water samples DNA (50 ng) was amplified by PCR in 20-μl reaction mixtures consisting of 0.5 U of *Taq* polymerase (Promega, Madison, Wis.), 1× *Taq* polymerase buffer (Promega), 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 nM, 0.5 μM *Aureococcus*-specific primer Aa1685F (5′ ACCTCCGGACTGGGGTT 3′) (40), and 0.5 μM universal eukaryotic primer EukB (5′ GATCC[A/T]TCTGCAGGTTCACCTAC 3′) (34) as a reverse primer (representing a total of 122 bp). The PCR consisted of 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, followed by a 5-min extension at 72°C. PCR products were fractionated on a 2% agarose gel, stained with ethidium bromide, and visualized with a transilluminator. To verify the presence of *Aureococcus*, bands were sequenced and compared with previously published sequences (accession numbers AF117776 to AF117779, AF119119, AF118443, and AAU40257).

For recreational boat samples, DNA (62.5 ng) was amplified by quantitative PCR by using the same primers (Aa1685f and EukB) and a species-specific molecular probe (40). The concentration of *Aureococcus* was then estimated by the method described previously (40).

cDNA synthesis. Since RNA is more labile than DNA, the RNA pool in a nucleic acid sample is contributed primarily by those organisms that are metabolically active and transcribing RNA (29, 41). Therefore, to test for the presence of viable *Aureococcus*, samples determined to be positive by PCR were assayed by reverse transcription-PCR. Along with the ballast water samples, four samples from the small-boat survey were also subjected to reverse transcription-PCR as positive controls. About 100 ng of total nucleic acids was treated for 15 min at room temperature in 10-μl reaction mixtures containing 1 U of DNase (Invitrogen, Carlsbad, Calif.) and 1× DNase buffer. EDTA was added to a final concentration of 2.5 mM, and the DNase was inactivated by incubation at 65°C for 15 min. RNA was reverse transcribed in 20-μl reaction mixtures containing 200 U of Superscript III (Invitrogen), 1× buffer, each deoxynucleoside triphosphate at a concentration of 0.5 mM, 100 ng of random hexamers (Invitrogen), 10 mM dithiothreitol, and 2 U of RNaseOut (Invitrogen). The reaction was carried out at 50°C for 1 h, followed by 15 min at 70°C to inactivate the reverse transcriptase. RNA was removed by RNase H treatment at 37°C for 20 min. cDNA was amplified as described above.

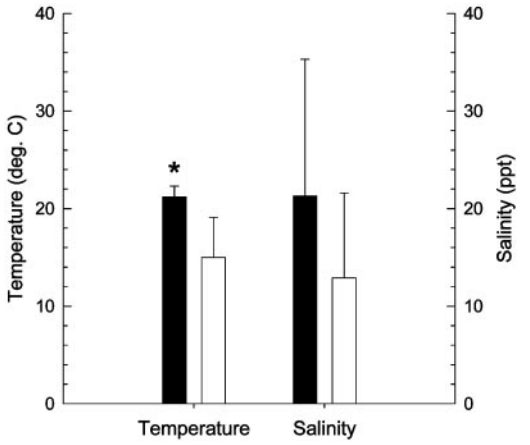

FIG. 1. Mean temperatures and salinities of ships' ballast tanks in which *Aureococcus* (brown tide) was detected (solid bars) and not detected (open bars). The error bars indicate standard deviations. *, differences were significant (T statistic = 3.89; $P < 0.01$; $n = 18$).

RESULTS

Presence of *Aureococcus* in ships' ballast water. The brown tide organism was detected only during summer months (7 of 10 tanks sampled), when the ballast water temperatures were above 19°C (Fig. 1). Cell abundance was generally low, as revealed by the relatively faint DNA bands on electrophoresis gels. Viable cells (i.e., cells transcribing DNA to RNA) were found in a vessel that had a history of freshwater ballast and had exchanged ballast water off Venezuela in May 2001, approximately 1 month before we sampled the tank (Table 1).

Generally, the longer ballast tanks are utilized and the more ballast tanks and higher ballast capacity a ship has, the greater the potential for sediment and organisms to accumulate. However, the ships in which *Aureococcus* was detected were similar ages and had similar ballast capacities and residual contents and the times between thorough tank cleanings were similar compared to the ships in which *Aureococcus* was not detected (Table 2). As far as we know, double-bottom tanks on the port and starboard sides of a ship had identical ballast histories, but in two of seven cases in which paired tanks were sampled, *Aureococcus* was detected in only one tank. In one other case, viable cells were found in the starboard tank but not in the corresponding port tank (Table 1).

Presence of *Aureococcus* during vessel transit through the Great Lakes. *Aureococcus* was also detected in a commercial vessel after 10 days of ballast tank confinement during transit through the Great Lakes (when the initial sampling temperature was 17.6°C). However, viable cells were detected in this

TABLE 1. Ballast activity of ships in which *Aureococcus* (brown tide) was detected and not detected

Ship code	Sample date (mo/day/yr)	Ballast load		Exchange[b]	Tank type[c]	Tank salinity (ppt)[d]
		Date (mo/yr)	Salinity[a]			
1004	6/25/01	6/01	FW	X	DB2 port	10 (+BT)
		5/01	FW		DB2 stbd	2 (−BT)
		5/01	BR			
		4/01	FW			
1005	6/27/01	5/01	BR	X	DB4 port	34 (+BT)
		3/01	BR	X	DB4 stbd	32 (+BT)
		2/01	BR	X		
		1/01	MW	X		
		12/00	FW			
1006	6/30/01	5/01	FW	X	DB6 port	29 (+BT)
		5/01	FW		DB6 stbd	34(+BT)[e]
		4/01	MW			
		4/01	FW			
1007	7/26/01	6/01	FW		UW6 stbd	8 (+BT)
		6/01	FW		FP	9 (−BT)
		5/01	FW			
		5/01	BR			
		3/01	MW	X		
1008	7/28/01	7/01	FW		DB4 port	5 (−BT)
		7/01	MW		DB4 stbd	2 (+BT)
1013	10/05/01	9/01	FW		DB3 stbd	7 (−BT)
		9/01	FW			
		8/01	FW			
		8/01	FW			
		7/01	FW			
1007	10/07/01	9/01	FW		DB2 port	3 (−BT)
		9/01	FW		DB2 stbd	23 (−BT)
1014	10/22/01	10/01	FW		DB3 port	20 (−BT)
		9/01	BR		DB3 stbd	22 (−BT)
		8/01	FW			
		8/01	FW/BR			
1015	10/25/01	10/01	MW	X	UW1 port	7 (−BT)
		9/01	FW			
		8/01	MW			
		7/01	FW			
		6/01	FW	X		
		5/01	MW			
1016	11/08/01	10/01	MW/BR		DB2 port	22 (−BT)
		10/01	MW		DB2 stbd	22 (−BT)
		9/01	FW			
		8/01	MW			
		8/01	FW			
1007[f]	10/01/01	9/01	FW		FP	1 (+BT)[e]
		8/01	FW	X		
		4/01	MW	X		
		1/01	MW	X		
		11/00	FW			

[a] FW, freshwater; BR, brackish water; MW, marine water.
[b] X, exchange took place after ballast load.
[c] DB, double bottom; UW, upper wing; FP, forepeak; stbd, starboard.
[d] +BT, *Aureococcus* was detected; −BT, *Aureococcus* was not detected.
[e] Viable cells were detected.
[f] Ship resampled after exit and reentry into the Great Lakes.

vessel directly only after the tank was filled with freshwater in the Port of Hamilton and were not detected at the Port of Windsor 6 days later. This vessel had exchanged its ballast water in the Atlantic after its second-to-last ballast load in Ghent, Belgium, about 2 months before it was sampled, but it had later taken on more ballast water in the Port of Amsterdam (Table 1). Upon entry into the Great Lakes, the residual ballast water in the tank had a salinity of 5 ppt.

Presence of *Aureococcus* in small boats. Sampling compliance during the small-boat survey was excellent. Of 20 people approached, only 1 refused to participate, and another 8 who were amenable to our sampling had boats either with no water or with not enough water for a sample. In the boats sampled, *Aureococcus* was detected in the bilge water in 8 of 10 cases, as well as in the one live-well sample which we collected. Two of the recreational boat samples had *Aureococcus* concentrations below the detection limit, five had concentrations of 1 to 25 cells ml^{-1}, and four had concentrations of 100 to 400 cells ml^{-1} (Fig. 2). Interestingly, the concentration of *Aureococcus* in the live-well sample (sample B4 from Indian River Marina) was

TABLE 2. Ship specifications for ballast water samples in which *Aureococcus* (brown tide) was detected and not detected

Parameter	+BT[a]	−BT
Ship age (yr)	10.2 ± 8.5	7.3 ± 6.9
Total ballast capacity (MT[b])	18,942 ± 5,237	17,698 ± 6,097
Time from sampling to last cleaning (months)	20.5 ± 10.1	31.0 ± 7.4
Total estimated residual water (MT)	72 ± 49	66 ± 58
Total estimated residual water (% of ballast capacity)	0.38 ± 0.19	0.35 ± 0.20

[a] +BT, samples in which *Aureococcus* was detected; −BT, samples in which *Aureococcus* was not detected.
[b] MT, metric tons.

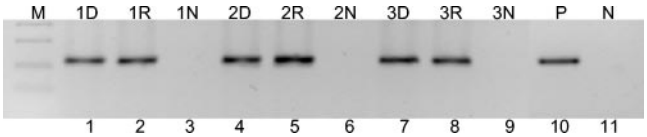

FIG. 3. Results obtained with molecular probes for *Aureococcus* (brown tide) in water samples from three small boats. D, DNA amplification; R, cDNA amplification; P, positive control; N, no-reverse-transcriptase control. Lane M, DNA ladder; lanes 1 to 3, bilge water collected at Indian River Marina in Delaware (sample B1 in Fig. 2); lanes 4 to 6, live-well water collected at Indian River Marina (sample B4 in Fig. 2); lanes 7 to 9, bilge water collected at West Ocean City Marina in Maryland (sample B8 in Fig. 2); lane 10, positive control; lane 11, negative control (no transcript).

higher than the concentration found in the water tested at the marina. Further investigation of four samples that were positive for brown tide (highest cell abundance) showed that cells had *Aureococcus*-specific RNA, which demonstrated that metabolically active cells were present (Fig. 3).

DISCUSSION

This study demonstrated that commercial ships and recreational boats allow delivery of viable brown tide cells at end points of transits. Of the ships sampled (Table 1) from June to November 2001 (a total of 19 tanks), two contained detectable levels of viable (actively transcribing) *Aureococcus* cells. This result is noteworthy given the ships' ballast histories, which indicated that both tanks were ballasted twice with freshwater before they were sampled and that the ships had exchanged their ballast in the open ocean either on their last or second-to-last voyage, as much as 1 month prior to sampling. For a polyhaline (salinity, 18 to 30 ppt) phytoplankton species to remain viable in the presence of water with a salinity of ≤5 ppt is remarkable and suggests that this information should be taken into consideration as new ballast regulations are developed (26).

For non-cyst-forming phytoplankton such as *Aureococcus*, the presence of a large initial population entrained in the ballast tank is likely key to successful transport and introduction, given the exponential decay of photosynthetic organisms during ballast tank confinement (13). Current ballast water management procedures recommend that vessel captains avoid algal blooms when they load ballast (25). However, for *Aureococcus*, it appears that temperature may also be an important physiological constraint on survival in ballast tanks (Fig. 1). Brown tide was not detected in ballast water having temperatures below 17.6°C, which is consistent with its temperature response in laboratory cultures (the optimal temperature for growth ranges from 20 to 25°C [8]). Other characteristics, such as the ability to survive in the dark and the ability to utilize organic substrates, may also facilitate ballast transport of *Aureococcus*. However, *Aureococcus* survives best in the dark at low temperatures (e.g., 6 and 12°C), presumably due to the decrease in metabolic activity, and addition of organic substrates has no effect on survival (39).

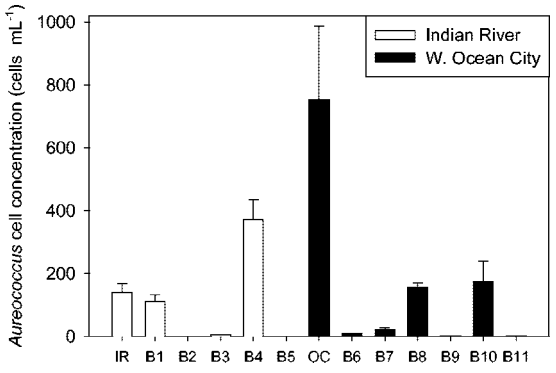

FIG. 2. Concentrations of *Aureococcus* found in marinas and recreational boats. IR, Indian River Marina; OC, West Ocean City Marina. Recreational boat samples B1 to B5 were collected at Indian River Marina, and samples B6 to B11 were collected at West Ocean City Marina. The error bars indicate standard deviations for duplicate samples.

One of the most intriguing aspects of this organism's survival in ballast water is its apparent tolerance of low salinities (Fig. 1), in contrast to its salinity preferences in the field (18 to 32 ppt) (1) and in culture (>22 ppt) (8). Brown tide was found in vessels with residual water salinities ranging from 2 to 34 ppt (Fig. 1), and in both cases when viable cells were detected, the tanks had taken on freshwater ballast during the last two ballast operations. In a previous study (40), *Aureococcus* was detected in locations that were periodically fresh (i.e., tidally influenced), but brown tide blooms have not occurred at these sites. Thus, it appears that *Aureococcus* is tolerant of low salinities in ballast tanks (perhaps due to buffering properties of the sediments) and can survive a 30-day exposure to 2-ppt ballast water. However, based on culture data (no growth at a salinity of 0 ppt) it is unlikely that cells would grow after delivery to the freshwater Great Lakes.

Of the six commercial ships containing *Aureococcus* (Table 1), only one had previously taken on ballast water in a port (Philadelphia) that overlaps the alga's currently recognized distribution (the east coast of the United States and Saldanha Bay in South Africa) (40). However, during the eastern seaboard survey of Popels et al. (40), relatively high cell densities were found in waters of the Delaware continental shelf, suggesting that brown tide may be an oceanic species rather than a coastal species. The implication is that ballast exchange (occurring in waters >200 nautical miles offshore), which is generally practiced as a barrier to future invasions by estuarine species, may actually increase the abundance of *Aureococcus* in

ships' ballast tanks rather than decrease it through displacement or osmotic shock.

With respect to its origin and dispersal, *Aureococcus* is estimated to have been present in Quantock Sound in New York for over 100 years (17), based on the presence of a unique sterol found in sediments (16). Furthermore, the small amount of sequence variation in 14 *Aureococcus* strains (18S ribosomal DNA gene) isolated from New York (2) caused Popels et al. (40) to design a species-specific molecular probe for a conserved region in the 18S ribosomal DNA sequence. However, the disjunct global distribution of *Aureococcus* may suggest that there was relatively recent introduction to South Africa, and the evidence provided here indicates that ships' ballast water could be a transport vector. Current investigations involving comparisons of DNA sequences of different geographic strains should help clarify whether *Aureococcus* is indeed an introduced species in South Africa (L. Botes, personal communication) rather than cryptogenic (a species which is neither clearly native nor clearly introduced [6]).

In this study, PCR techniques were used to detect *Aureococcus* and estimate its viability. Such molecular techniques provide an extremely powerful and efficient tool for the detection and quantification of microorganisms compared to traditional microscopic and cultural methods (10, 19, 20). Furthermore, these techniques are uniquely suited to analysis of ballast water and sediment samples in which viable target organism abundance may be below the level of microscopic detection, yet still pose a threat to human health or the environment. We detected the DNA of *Aureococcus* in eight ballast tanks of the commercial ships sampled (Table 1); however, we detected RNA (which is more labile than DNA [29, 41]) from only two tanks, suggesting that most *Aureococcus* cells present in our ballast water samples were inactive. Given the great disparity between the number of samples in which *Aureococcus* was viable and the number of samples in which it was detected, we suggest that studies to assess the metabolic activity or capacity for growth as a means of estimating invasive potential are warranted.

Aureococcus was present at both marinas where small boats were surveyed. Furthermore, boats were sampled immediately after they came out of the water, so it was not surprising that the recreational boat samples contained viable cells of *Aureococcus*, either in the bilge or in live-well water. Given the likelihood that recreational boaters will use their vessels in multiple locations on any given day or weekend or within the same month and the likelihood that that they will not fully drain or rinse their boats with freshwater, there is great potential for small boats to distribute the brown tide organism. A quantitative risk assessment could be achieved by sampling boats before they are launched at various marinas to test for the presence of viable cells, and boat owners could be interviewed and asked when and where they last used their boats.

The presence of a relatively high concentration of *Aureococcus* in the live-well sample also has implications for the alga's potential introduction with bait or its dispersal by commercial bait vendors. Other instances of such hitchhikers (e.g., macrophytes [27]) suggest that this is a real possibility. These findings add to those of previous studies which demonstrated the potential for recreational boaters to facilitate the spread of previously introduced species (e.g., zebra mussel into inland water bodies from Lake Michigan [37]) and highlight the importance of educating recreational boaters about their role in species dispersal.

Conclusions. To our knowledge, this is the only study that has demonstrated the presence of viable harmful microbes in ships' ballast water and small-boat bilge and live-well water. The variable presence of *Aureococcus* in paired ballast water tanks on the same ship, as well as in different ships, indicates the importance of tracking the history of individual tanks when workers attempt to determine outcomes of ballast management practices. With respect to risk assessment, current models incorporate the number of propagules at transit end points (32); however, this study shows that the number of viable propagules (2 of 19 tanks; 11%) is likely a better predictor of potential invaders.

ACKNOWLEDGMENTS

We thank members of the Great Lakes NOBOB team for their efforts in collecting samples, Leslie Kampschmidt for assistance in the laboratory, and Ric Ruof for assistance in collecting recreational boat samples.

This work was conducted under the multi-institutional Great Lakes NOBOB Project funded by the Great Lakes Protection Fund, the National Oceanic and Atmospheric Administration (NOAA), the U.S. Environmental Protection Agency, and the U.S. Coast Guard. The project was comanaged by the Cooperative Institute of Limnology and Ecosystems Research and the NOAA Great Lakes Environmental Research Laboratory and was sponsored under cooperative agreement NA17RJ1225 from NOAA's Office of Oceanic and Atmospheric Research. Additional funding for this project was provided by the Delaware Center for the Inland Bays and Delaware Sea Grant RB/41 to D.A.H. and S.C.C.

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