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Phytoplankton and bacterial assemblages in ballast water of U.S. military ships as a function of port of origin, voyage time, and ocean exchange practices

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Abstract

We characterized the physical/chemical conditions and the algal and bacterial assemblages in ballast water from 62 ballast tanks aboard 28 ships operated by the U.S. Military Sealift Command and the Maritime Administration, sampled at 9 ports on the U.S. West Coast and 4 ports on the U.S. East Coast. The ballast tank waters had been held for 2–176 days, and 90% of the tanks had undergone ballast exchange with open ocean waters. Phytoplankton abundance was highly variable (grand mean for all tanks, 3.21×10^4 viable cells m^{-3}; median, 7.9×10^3 cells m^{-3}) and was unrelated to physical/chemical parameters, except for a positive relationship between centric diatom abundance and nitrate concentration. A total of 100 phytoplankton species were identified from the ballast tanks, including 23 potentially harmful taxa (e.g. *Chaetoceros concavicornis*, *Dinophysis acuminata*, *Gambierdiscus toxicus*, *Heterosigma akashiwo*, *Karlodinium veneficum*, *Prorocentrum minimum*, *Pseudo-nitzschia multiseries*). Assemblages were dominated by chain-forming diatoms and dinoflagellates, and viable organisms comprised about half of the total cells. Species richness was higher in ballast tanks with coastal water, and in tanks containing Atlantic or Pacific Ocean source waters rather than Indian Ocean water. Total and viable phytoplankton numbers decreased with age of water in the tanks. Diversity also generally decreased with water age, and tanks with ballast water age >33 days did not produce culturable phytoplankton. Abundance was significantly higher in tanks with recently added coastal water than in tanks without coastal sources, but highly variable in waters held less than 30 days. Bacterial abundance was significantly lower in ballast tanks with Atlantic than Pacific Ocean source water, but otherwise was surprisingly consistent among ballast tanks (overall mean across all tanks, $3.13 \pm 1.27 \times 10^{11}$ cells m^{-3}; median, 2.79×10^{11} cells m^{-3}) and was unrelated to vessel type, exchange status, age of water, environmental conditions measured, or phytoplankton abundance. At least one of four pathogenic eubacteria (*Listeria monocytogenes*, *Escherichia coli*,

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Mycobacterium spp., *Pseudomonas aeruginosa*) was detected in 48% of the ballast tanks, but toxigenic strains of *Vibrio cholerae* were not detected. For ships with tanks of similar ballasting history, the largest source of variation in phytoplankton and bacteria abundance was among ships; for ships with tanks of differing ballasting histories, and for all ships/tanks considered collectively, the largest source of variation was within ships. Significant differences in phytoplankton abundance, but not bacterial abundance, sometimes occurred between paired tanks with similar ballasting history; hence, for regulatory purposes phytoplankton abundance cannot be estimated from single tanks only. Most tanks (94%) had adequate records to determine the source locations and age of the ballast water and, as mentioned, 90% had had ballast exchange with open-ocean waters. Although additional data are needed from sediments that can accumulate at the bottom of ballast tanks, the data from this water-column study indicate that in general, U.S. Department of Defense (DoD) ships are well managed to minimize the risk for introduction of harmful microbiota. Nevertheless, abundances of viable phytoplankton with maximum dimension >50 μm exceeded proposed International Maritime Organization standards in 47% of the ballast tanks sampled. The data suggest that further treatment technologies and/or alternative management strategies will be necessary to enable DoD vessels to comply with proposed standards.

Keywords: Bacteria; Ballast water exchange; Harmful algae; Phytoplankton; Ship; Transport

1. Introduction

Non-indigenous species transport by ships has been ongoing since exploration and trading activities by early civilizations, and transport of biota in ballast water of modern steel ships was first suggested a century ago (Ostenfeld, 1908). Although it is often expressed that only a small percentage of introduced species have become invasive and caused a significant detrimental impact in their receiving environment, in estuaries where the problem has begun to be well studied it has generally been difficult to separate, with certainty, native from non-native taxa (Ruiz et al., 1997). Long-distance dispersal of marine organisms in ship ballast waters may be increasing and remains an environmental problem because, although the oceans are continuous, coastal marine life is often geographically discontinuous (Ruiz et al., 1997, 2000). The fact that many microflora and microfauna species presently have widespread distribution thus may reflect a long history of global transport by ships, migratory waterfowl and animals, winds, water currents, and other mechanisms. Yet, the continuing effects of human activities in non-indigenous species introductions and resulting economic and ecological impacts can be so major that entire ecosystems have been completely changed (Cohen and Carlton, 1995, 1998; Ruiz et al., 1997, 1999a).

Undesirable introduced aquatic species have the potential to become dominant when environmental conditions are conducive and natural predators are lacking. They can dramatically alter ecosystem structure and diversity, and some invasive species have greatly reduced or eliminated desirable species; they can also adversely affect public health, energy and food supplies, and local economies (e.g. Rayl, 1999; Pimentel et al., 2000). Of the introductions that have occurred in the last 20–30 years, many are believed to have occurred via ballast water (Carlton, 1985; Carlton and Geller, 1993; Ruiz et al., 1997; Cohen, 1998; Cohen and Carlton, 1998). Thus, the movement of ballast water and ballast tank sediments is regarded as the most important mechanism at present for transfer of aquatic non-indigenous species (Ruiz et al., 1997, 2000). Ballast water exchange practices have been linked to the proliferation of previously rare or undetected, sometimes harmful organisms in discharge locations, including certain potentially toxic dinoflagellates (Hallegraeff, 1998).

Ballast water exchange is defined as the replacement of ballast water taken up in coastal areas with water from the open ocean, either by completely deballasting and reballasting, or by continuously flushing the ballast tanks (NRC, 1996). The goal is to discard from the ballast tanks coastal organisms that were taken up in or near the port of departure. Oceanic organisms generally do not survive when released into the coastal or fresh waters of the destination port (NRC, 1996). The exchange process can be highly variable, however, in affecting abundances of microflora and microfauna in the ballast water (e.g. Smith et al., 1996; Wonham et al., 2001; Drake et al., 2002).

Ballast water exchange is, at present, the only widely used measure for mitigating ballast water transport (e.g. International Maritime Organization IMO, 1998, 2001, 2004; National Invasive Species Act [NISA]—U.S. Congress, 1996; U.S. Coast Guard, 2002; U.S. Senate, 2005). There are known shortcomings associated with ballast water exchange on commercial as well as military vessels, however, including lack of applicability to intra-coastal voyages; difficulty in assessing trans-vessel efficacy; lengthy time required for exchange; hull stress and other issues compromising

ship safety; and the potential for large amounts of inocula to remain in tanks (e.g. Carlton et al., 1995; Hallegraeff, 1998; Zhang and Dickman, 1999). These issues likely will prevent use of ballast water exchange as the primary means of controlling ballast water-related species invasions in the future.

Scientific understanding about non-indigenous species introductions via ballast water mostly has been based upon studies of commercial cargo vessels. The U.S. Department of Defense (DoD) operates a large fleet, requiring unrestricted access to national and international waters to facilitate domestic commerce, and to protect and promote national interests (U.S. DoD, 2000). Some DoD ships, for example, warships and naval auxiliary ships, are exempted from compliance with IMO standards; such ships are encouraged to follow the standards insofar as reasonable and practicable (IMO, 2004, Article 3.2(e)). DoD vessels have been documented to transport non-indigenous aquatic species (Coles et al., 1999). Previous information on ballast water transport of microflora by DoD ships is lacking, however, except for a study that reported minor transport of larger phytoplankton (diatoms and dinoflagellates retained by a plankton net with mesh size 80 μm, ~1 to ~410 cells m^{-3}) by naval vessels in Chesapeake Bay (35 voyages; October 1994–September 1996; Ruiz et al., 1999b). The same study also reported concentrations of 0.2–1.0 × 10^6 bacteria ml^{-1} in the ballast water of five voyages sampled, similar to bacterial densities found in ballast water of commercial ships entering the Bay.

The Uniform National Discharge Standards program (UNDS, 1999) estimated that 131 vessels of the U.S. Navy and U.S. Military Sealift Command carried ballast water suitable for discharge, with a total capacity of ~6.1 × 10^8 liters of water. These numbers do not include vessels of the Maritime Administration (MARAD—civilian contract operators) fleet, which also are commonly used by DoD. This project was designed to increase understanding about the extent to which DoD vessels may be introducing non-indigenous, harmful microflora species to U.S. coastal waters. The data are contributed toward reducing environmental risks of transport of non-indigenous organisms by DoD vessels, thereby enhancing public safety and health protection. Our primary objective was to characterize the phytoplankton and bacterial assemblages in ballast water from a selection of DoD vessels, including assessment of between-ship versus within-ship sources of variation. Harmful taxa were defined as photosynthetic or heterotrophic algae and bacteria capable of directly or indirectly causing disease or death of humans or beneficial aquatic life (Burkholder, 1998). The data were interpreted considering vessel travel routes to determine the types and concentrations of organisms arriving from ports within and outside the U.S., for use in assessing risks associated with various ballast water management variables.

2. Methods and materials

2.1. Sampling design and methods

2.1.1. Ballast tank sampling

The intent of our sampling strategy, jointly developed in collaboration with Holm et al. (2005), was to encompass the tank variability resulting from ballast age, season, region, vessel characteristics, and exchange status. Ballast tanks on various ships were screened for suitability of sampling using a decision tree developed in collaboration with Holm et al. (2005) (Fig. 1). A total of 28 vessels were sampled from September 2002 through July 2004, including 16 vessels from 9 ports on the U.S. West Coast and 12 vessels from 4 ports on the U.S. East Coast (Table 1). The study primarily included oilers (similar to commercial oil tankers) and cargo vessels operated by or for the U.S. Military Sealift Command (MSC). One U.S. Army lighter (similar to a commercial barge) was also sampled. There were two main vessel operator or service groupings, the Military Sealift Command (i.e., vessels operated directly by MSC) and the MARAD. Four different vessel classes were sampled from among the MSC vessels, and five were sampled from the MARAD grouping. Vessels designated in the exchange field as M fell under the mandatory (required) exchange requirements of the U.S. Navy (2003); those designated as V were subject to the voluntary (recommended) requirements of the NISA (U.S. Congress, 1996) and the Nonindigenous Aquatic Nuisance Prevention and Control Act (NANPC; U.S. Congress, 1990). At least two ballast tanks usually were sampled from each ship, accessed by manholes. Paired tanks with parallel history mostly were sampled (designated *P*); however, the number of tanks sampled varied from 1 to 8 tanks $vessel^{-1}$ $voyage^{-1}$. Multiple tanks within a ship were included for several ships to assess intra-ship variability. Vessels and tanks were represented numerically. A suffix was used to designate the voyage as opposed to the actual ship, because one vessel was sampled twice during the study. Voyage numbers were established chronologically by sampling date; for example, 1-*P*1a,1b represents Voyage 1, 1st pair (*P*, in that voyage, the only pair) of tanks sampled, tanks 1a and 1b. As another example, 6-*P*2a,2b

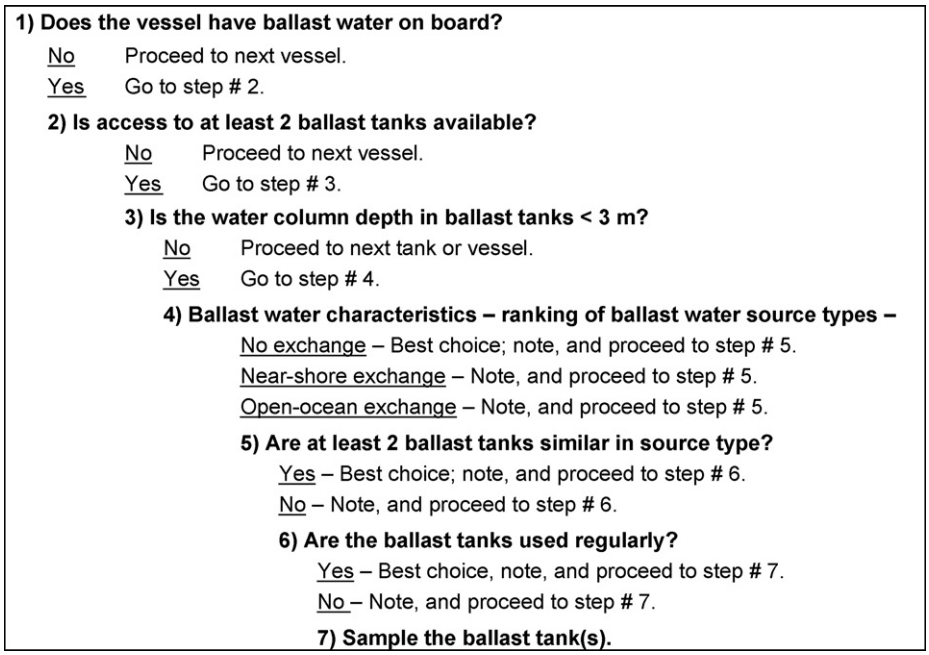

Fig. 1. Ballast tank sampling decision tree.

represents Voyage 6, 2nd pair of tanks sampled (in that voyage, from a total of four pairs sampled), designated as tanks 2a and 2b.

In each ballast tank, water depth was measured, and background environmental conditions in the ballast water (temperature, salinity, pH, dissolved oxygen [DO] concentration, turbidity) were profiled using a hand-held meter (YSI model 85, Yellow Springs Instruments, Inc.), or a Hydrolab datasonde sensor bundle (Hach, Inc., Loveland, CO, USA). Measurements were taken below the surface (0.5 m) and at depths of 1, 2, 5 and 10 m depending on the depth of the water column and the tank architecture. These data were taken to assess for relationships between environmental variables and species abundance and diversity. Three major categories of samples were also collected: chemical water quality samples, and both net-concentrated samples and whole-water samples for phytoplankton analyses.

Whole-water samples were collected in duplicate using an air-displacement composite sampler (Lab-Line, Inc., Melrose Park, IL, USA). This sampling canister was capable of collecting equal aliquots of water at set depth intervals, combined for a 2-l vertical composite sample. The composite samples were used for chemical analyses, bacteria quantification and molecular identifications, and culturing of phytoplankton taxa of interest. This sampling device was selected for two reasons: first, the air displacement enabled dependable water collection over multiple depths, and collection could be triggered by simply removing plugs (fill and displacement) from the surface through a spring-loaded line, independent of the weight- bearing line. Thus, the device could be triggered on the bottom of the tank and retrieved with periodic stops at 1-m intervals. Secondly, the smooth polypropylene surface comprising the interior canister wall could be thoroughly cleaned between sampling events, important to ensure sample integrity for bacterial identifications and chemical analyses. The hardened, smooth interior surfaces of the air displacement canister were cleaned with bleach and then with 10% sulfuric acid, followed by thorough rinsing with deionized water prior to each scheduled boarding. Canisters were also rinsed prior to sampling with water from the subject tank.

Quantitative net tows were taken for phytoplankton in duplicate using a 20-μm-mesh net; the volume sampled was estimated from the net circumference and the length (depth) of each tow. Initially, 5-μm-mesh nets were tested for use with phytoplankton and were found to be problematic because of clogging and resulting poor reproducibility and overall performance. These samples were used for determination of phytoplankton abundance (maximum dimension $\geq$20 μm); thus, smaller taxa were not included in the counts. For species richness determinations, we augmented the 20-μm-mesh net samples with a qualitative 5-μm-mesh net tow, following the approach used by Gollasch et al. (2003). Smaller phytoplankton that would have been missed by the

Table 1
Ballast water management summary for the voyages and tanks examined

Voyage; vessel type[a]	Operator (exchange rule); sampling port[b]	Tank(s)/ identification[c]	Exchange date, activity[d]	Coastal[e]	Distance (km/n.m.)[f]	Age when sampled (days)	Exchange location[g] and comments
1 UR-Oiler	MSC (M) San Diego, CA	*P*1a,1b	30 August '02, X3	Y	222/120	6	ECP, ~185–371 km (100–200 n.m.) off coast of southern Mexico
2 UR-Oiler	MSC (M) San Diego, CA	*P*1a,1b	12 November '02, XU	N*	NA	10	ECP, between the Hawaiian Islands, USA, and Midway
3 UR-Oiler	MSC (M) Norfolk, VA	*P*1a,1b	?, ?	?	NA	2?	?
4 UR-Oiler	MSC (M) San Diego, CA	*P*1a,1b	8 May '03, XU	N*	NA	10	ECP, >371 km (>200 n.m.) north of Wake Island
5 RORO	MARAD (V) Portsmith, VA	1	5 May '03, X1	Y	196/106	14	WCA, ~185–371 km (100–200 n.m.) off LA coast, USA
6 UR-Oiler	MSC (M) Norfolk, VA	*P*1a,1b	18 May '03, XU	N*	NA	9	NWA (Mediterranean[h] highly diluted with Gulf Stream waters)
		*P*2a,2b	10 May '03, XU	N*	NA	17	NEA (Mediterranean[h] highly diluted with eastern Atlantic waters)
		*P*3a,3b	22 May '03, XU	Y	44	5	WCA (Mediterranean[h] with Gulf Stream dilution and some coastal Carolina water, USA)
		*P*4a,4b	22 May '03, XU	Y	44	5	NWA (Mediterranean[h] with significant coastal Carolina water, USA)
7 RORO	MARAD (V) Alameda Pt., CA	*P*1a,1b	17 February '03, XU	Y	217/117	99	WCA, ~185–371 km (100–200 n.m.) off LA coast, USA
8 RORO	MARAD (V) Alameda Pt., CA	1	11 May '03, X1	N	NA	15	ECP—open Pacific, ~741 km (400 n.m.) east of Mariana Islands
9 Container	MARAD (V) Pt. Townsend, WA	*P*1a,1b	17 December '02, XU	Y	215/116	173	ECP, ~185–371 km (100–200 n.m.) off coast of southern Mexico
10 UR-Oiler	MSC (M) Norfolk, VA	*P*1a,1b	26 June '03, X1	N	NA	5	WCA, ~741 km (400 n.m.) west of Africa on northwest track to open Atlantic
11 RORO	MSC (V) Norfolk, VA	1	22 July '03, XU	N*	NA	22	NWA (with small portion of Indian Ocean water[h])
		2	29 May '03, X1	N	NA	76	IO (mid-Indian Ocean water[h])
12 RORO	MSC (V) San Francisco, CA	*P*1a,1b	8 August '03, X1	N	NA	12	ECP, open ocean >556 km (>300 n.m.) north of Midway Island
13 UR-Oiler	MSC (M) Norfolk, VA	1	25 August '03, X2	N	NA	9	NWA
14 UR-Oiler	MSC (M) San Diego, CA	*P*1a,1b	15 August '03, XU	N*	NA	32	ECP, ~93-185 km (50–100 n.m.) west of Hawaii, USA
15 RORO	MSC (V) Long Beach, CA	1, 2	2 October '03, X1	N	NA	46	IO (central Indian Ocean)
16 RORO	MSC (V) Everett, WA	1	29 October '03, X1	N	NA	23	ECP, ~463 km (250 n.m.) off northern coast of CA, USA
		2	16 November '03, X1	Y	2.8/1.5	5	ECP, ~463 km (250 n.m.) off northern coast of CA, also with WA state port water
17 UR-Oiler	MSC (M) Norfolk, VA	*P*1a,1b	7 February '04, X3	N	NA	5	WCA, ~649 km (350 n.m.) south of Bermuda
		*P*2a,2b	1 February '04, X3	N	NA	10	ECA, ~834 km (450 n.m.) west of Canaries
18 UR-Oiler	MSC (M) San Diego, CA	*P*1a,1b	16 February '04, X1	Y	<2/<1	17	WCP (Fuel Pier, Guam)
19 RORO	MARAD (V) Tacoma, WA	1	7 April '04, XU	N	NA	26	IO—had previously held fresh water, was loaded in open ocean, then more fresh water was added → 9 psu salinity
		2	20 January '04, XU	N	NA	104	ECP—had previously held fresh water; was loaded in open ocean

20 RORO	MARAD (V) Pt. Hueneme, CA	1	5 May '04, XU	Y	26/14	2	ECP (with Persian Gulf waters[h])
		2	2 May '04, XU	Y	48/26	5	ECP (with Persian Gulf waters[h])
21 RORO	MSC (V) Philadelphia, PA	1	9 February '04, XU	N*	NA	101	ECA—open Atlantic ~741 km (400 n.m.) west of Gibralter
22 RORO	MARAD (V) Baltimore, MD	*P*1a,1b	20 May '04, X1	N	NA	13	Mid-Atlantic
23 RORO	MARAD (V) San Francisco, CA	1	5 May '04, X1	Y	85/46	30	WCA—Gulf of Mexico ~148 km (80 n.m.) off Corpus Christi, TX, USA
24 RORO	MARAD (V) Olympia, WA	*P*1a,1b	26 April '04, X1	N	NA	43	IO (open ocean), ~1483 km (800 n.m.) west of Sri Lanka
		2	24 March '04, X1	N	NA	76	IO (open ocean)
25 RORO	MSC (V) Norfolk, VA	1, 2	?, ?	?	?	?	?
26 RORO	MARAD (V) Tacoma, WA	1	25 June '04, X1	Y	4.3/2.3	4	ECP—Puget Sound, WA, USA
		2	31 May '04, X3	N	NA	29	NWA
27 UR-Oiler	MSC (M) Norfolk, VA	*P*1a,1b	23 June'04, XU	Y	41	15	NEA, ~148 km (80 n.m.) off Portugal, ~111 km (60 n.m.) off Sicily
		*P*2a,2b	1 July '04, XU	N	NA	8	NWA, ~371 km (200 n.m.) off Norfolk, VA, USA
28 Lighter	U.S. Army Fort Eustis, VA	*P*1a,1b	10 July '04, X1	N	NA	11	Atlantic (region unknown)

[a] Vessel type—underway replenishment (UR-) oiler, roll-on-roll-off (RORO) carrier, container, or U.S. Army lighter.

[b] Operator—Military Sealift Command (MSC), Maritime Administration (MARAD; civilian-operated), or U.S. Army. Exchange rule—indicates whether the vessel operated under the mandatory (M) or voluntary (V) ocean exchange rule. Abbreviations of states with in the USA—CA, California; LA, Louisiana; PA, Pennsylvania; TX, Texas; VA, Virginia; WA, Washington.

[c] *P* designates paired tanks, followed by the pair number sampled on a given voyage; the two tanks of a pair are indicated as "a" and "b".

[d] Exchange activity—U, could not determine whether any exchange or open-ocean dilution was applied; XU, exchange or open-ocean dilution occurred, but to an undetermined extent; X1, one tank volume exchange; X2, two tank volume exchanges; X3, three tank volume exchanges; ?, no records or logs available.

[e] Coastal status was designated as Y (yes; $n = 20$ tanks in total); non-coastal (open-ocean) status was designated as N or N*, (N*, coastal water considered negligible at <5%; $n = 38$ tanks in total). The four tanks from vessels 3 and 25 were excluded from analysis by source area because no ballasting records were available.

[f] Distance in nautical miles (n.m.) between the nearest coastline and where exchange took place.

[g] Exchange locations, i.e. source regions for ballast water, are indicated by abbreviations of the oceans designated by the United Nations Food and Agriculture Organization: ECA, eastern central Atlantic; ECP, eastern central Pacific; IO, Indian Ocean; MBS, Mediterranean and Black Sea; NEA, northeast Atlantic; NEP, northeast Pacific; NWA, northwest Atlantic; NWP, northwest Pacific; SEP, southeast Pacific; SWA, southwest Atlantic; SWP, southwest Pacific; WCA, western central Atlantic; WCP, western central Pacific.

[h] Previous source region.

20-μm-mesh net were analyzed from whole-water samples and, for the potentially toxic cyanobacterium, *Microcystis aeruginosa* Kützing, emend. Elenkin using molecular techniques. Plankton nets were cleaned between sampling events by rinsing thoroughly with deionized water. Six tanks (from vessels-tanks 9-*P*1a,1b; 19-2; 20-2; 25-1; 26-1) had to be sampled with a manual diaphragm pump because tank architecture precluded use of nets.

Samples for phytoplankton enumeration were preserved with acidic Lugol's solution (Vollenweider, 1974), and samples designated for bacterial enumeration via flow cytometry were preserved with 50% glutaraldehyde (final concentration 4%; Sournia, 1978). During warm months, ice packs were included in the kit to avoid heat stress for organisms in samples designated for culture.

2.1.2. Water quality analyses

Samples for analysis of total suspended solids were maintained at ≤4 °C, filtered within 48 h, and measured gravimetrically (method 2540D, American Public Health Association [APHA] et al., 1998; practical quantitation limit, 2 mg l^{-1}). Measurement of total suspended solids was terminated halfway through the study because samples consistently were at or below the detection limit.

Nutrients were analyzed using a Technicon Traacs 800 autoanalyzer (Technicon, Pulse Instrumentation, Ltd., Saskatoon, Saskatchewan, Canada) or Quickchem 8000 autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). Variances from the U.S. Environmental Protection Agency (EPA) and the North Carolina Department of Environment, Health and Natural Resources—Division of Water Quality were obtained to enable use of procedures for nutrient sample storage and analysis (substitution of freezing at −20 °C for acidification; 2-month limit), that accommodated low-level analysis of estuarine matrices (U.S. EPA, 1992, 1997a). Water samples for total phosphorus (TP) analysis were frozen at −20 °C until analysis, using a variance of EPA method 365.1 (U.S. EPA, 1992, 1993; practical quantitation limit (APHA et al., 1998), 10 μg l^{-1}). Samples for NO_3^- + NO_2^- analysis (henceforth referred to as NO_3^-) were frozen and analyzed within 2 months, using a variance of EPA method 353.4 (U.S. EPA, 1992; practical quantitation limit, 6 μg NO_3^- l^{-1}). Samples for total Kjeldahl nitrogen (TKN = free ammonia + organic nitrogen) were assayed using a modification of EPA method 351.2 (U.S. EPA, 1993; samples held at −20 °C and not preserved with sulfuric acid; practical quantitation limit, 140 μg N l^{-1}). Total organic carbon (TOC) concentrations were determined using high-temperature non-dispersive infrared-combustion techniques (NDIR; APHA et al., 1998; practical quantitation limit, 2 mg l^{-1}).

Chlorophyll *a* (Chl*a*) was assessed as an indicator of total phytoplankton biomass (Wetzel and Likens, 2001). Chl*a* samples were filtered under low vacuum (Whatman GF/C filters, 55–69 kPa) and low light (20 μmol photons m^{-2} s^{-1}) within 12 h of collection, and were stored frozen (−20 °C) with desiccant in darkness until analysis within 2 months. Chl*a* was extracted in 90% basic acetone (practical quantitation limit, 1 μg l^{-1};U.S. Environmental Protection Agency [EPA], 1997b; Wetzel and Likens, 2001), and fluorescence was determined using a Turner 10-AU fluorometer.

2.1.3. Phytoplankton assemblages

Each 250-ml preserved phytoplankton sample (from 20-μm-mesh net tows) was settled undisturbed for ≥48 h; the upper 200 ml were then gently siphoned and removed; the remaining 50 ml were remixed, and 25 ml were settled in an Utermöhl chamber (Lund et al., 1958; Wetzel and Likens, 2001) for 24 h. Total phytoplankton, and phytoplankton cells that appeared viable when collected (in preserved material, with intact membranes and cytoplasm, and with no signs of damage or decay; Burkholder and Wetzel, 1989) were enumerated and identified to lowest possible taxa using phase contrast light microscopy (600×) following Wetzel and Likens (2001), with taxonomic phyla designated as in Graham and Wilcox (2000). Viable cells with maximum dimension ≥10 μm were divided into two size classes designated as small and large (maximum cell dimension 10–50 and >50 μm, respectively), to assess compliance of these ballast tanks with proposed IMO (2004) and U.S. Senate (2005) standards for ballast water discharge.

Assemblages from each ballast tank (from both net and whole-water samples) were cultured to assist in identification of some taxa. Fresh (unpreserved) samples were express-mailed (in coolers over icepacks as needed) by overnight shipment after sampling. Sub-samples were incubated in six-well plates (six wells per tank) for up to 8 weeks in *f*/2 growth media, ±hydrated silica (made using ultra-filtered tank water or natural seawater, filter pore size 0.22 μm; Guillard, 1975) at 23 °C, using a 14-h light:10-h dark photoperiod and light intensity (source, fluorescent tubes) of 100 μmol photons m^{-2} s^{-1}. At weekly intervals, sub-samples were examined under light microscopy (phase contrast, 600×) for actively growing algal taxa. Phytoplankton species from these initial assemblage cultures were isolated and maintained in monoculture for further identification.

Scanning electron microscopy was used to identify some cultured taxa, following methods of Parrow et al.

(2006). To discern the plates of armored dinoflagellates, cells first were treated with a 40% reduction in salinity for 30 min (i.e. 40% reduction in osmolality, monitored using a Vapor Pressure Osmometer—Wescor, Inc., Logan, UT, USA), then combined with an equal volume of fixative cocktail (1% OsO_4, 2% glutaraldehyde, and 0.1 M sodium cacodylate final concentration) at 4 °C for 20 min. Fixed cells of dinoflagellates and other taxa were filtered onto polycarbonate filters (3-μm porosity), rinsed in 0.1 M sodium cacodylate, dehydrated through an ethanol series, CO_2 critical-point-dried, sputter-coated with 25 nm Au/Pd, and viewed at 15 kV on a JEOL 5900LV scanning electron microscope.

Species identifications of potentially harmful algal taxa were also checked with species-specific molecular probes (18S rDNA; 16S rDNA for cyanobacteria), where available (Scholin et al., 2003). For DNA extraction, 50 ml of acidic Lugol's-preserved sample were centrifuged and total DNA extracted from the pellet using an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA). Extracted DNA was stored at −20 °C until PCR was performed. For real-time PCR assays (Wittwer et al., 1997), each reaction contained the following: 0.1 U *Taq* Pro (Denville Scientific, South Plainfield, NJ, USA), 1× PCR buffer, 4 mM $MgCl_2$, 0.2 μM forward primer, 0.2 μM reverse primer, 0.3 mM each deoxynucleotide triphophosphate, 0.25 mg ml^{-1} bovine serum albumin, 0.3 μM species-specific Taqman probe, molecular grade water to 10 μL and 1 μl DNA template. Assays were performed on the Lightcycler (Idaho Technology, Idaho Falls, ID, USA). The following cycling parameters were used: initial denaturing step for 2 min at 95 °C, followed by 50 cycles at 94 °C (touchdown), and annealing temperature (varied for each species) for 20 s. Fluorescence acquisition occurred after each cycle. Various real-time PCR assays were performed (Table 2), specific for harmful algal species and the cyanobacterium *M. aeruginosa*. Lower limits of detection were determined by serial dilution of purified DNA from each organism. The last dilution detected in the series was then spiked into background DNA extracted from various ballast samples. This allowed assessment of potential inhibitory materials in the ballast water samples that were co-extracted during DNA extraction. Although the cycle number at which the dilution was detected by real-time PCR varied with different background samples used, the dilution was still detectable, thus validating the assays as a qualitative tool.

Conventional PCR methodology was applied to assess diversity of potentially harmful dinoflagellate clones, since dinoflagellates include a large number of potentially toxic taxa in comparison to other estuarine/marine algal groups (Burkholder, 1998). A dinoflagellate group-specific primer (Oldach et al., 2000) was used in conjunction with a eukaryotic general primer (4618; Medlin et al., 1988) to amplify a 149 base pair segment at the 3′ end of the 18S ribosomal RNA locus. Each 50 μl PCR reaction contained 1.5 U of Mega-Frag™ Taq polymerase (Denville Scientific, Metuchen, NJ, USA); 10× PCR buffer and 4 mM $MgCl_2$; 2 mM each deoxynucleotide triphosphate (dNTP; Invitrogen, Alameda, CA, USA), 0.25 mg ml^{-1} bovine serum albumin (Idaho Technology), 0.8 μM of each primer (Qiagen/Operon, Alameda, CA, USA), 1 μl DNA template and molecular biology grade water to a final volume of 50 μl. Cycling was performed on a DNA Engine Dyad Peltier Thermocycler (Bio-Rad Laboratories, Inc., Waltham, MA, USA) as follows: initial denaturation at 94 °C for 2 min, followed by 45 cycles at 94 °C for 10 s, 55 °C for 30 s, 68 °C for 10 s, and a final extension at 68 °C for 6 min 20 s.

PCR products were examined on a 1.4% ethidium bromide-stained agarose gel, and bands were extracted from the gel following manufacturer's instructions supplied with the MinElute kit (Qiagen, Alameda, CA, USA). The purified products were immediately ligated

Table 2
Real-time PCR assays used to screen for potentially harmful microalgae and cyanobacteria in ballast water, including target species, target loci, loci descriptions, PCR techniques used, and lower limits of detection

Target species	Target	Loci	Lower limit of detection	Reference
Fibrocapsa japonica Toriumi et Takano	LSU	Large subunit	<10 copies μl^{-1}	Bowers et al. (2006)
Heterosigma akashiwo (Hada) Hada ex Hara et Chihara	LSU	Large subunit	<10 copies μl^{-1}	Bowers et al. (2006)
Karlodinium veneficum (Ballentine) J. Larsen[a]	16S	Chloroplast	<1 cell ml^{-1}	Tengs et al. (2001)
Microcystis aeruginosa	myc	Encodes enzyme that makes microcystin; codes for DNA binding protein	<10 copies μl^{-1}	Foulds et al. (2002)
Pfiesteria piscicida Steidinger et Burkholder	18S	18S rRNA	<1 cell ml^{-1}	Bowers et al. (2000)
Pfiesteria shumwayae Glasgow et Burkholder[a]	18S	18S rRNA	<1 cell ml^{-1}	Bowers et al. (2000)

[a] See Bergholtz et al. (2006—*K. veneficum*), Marshall et al. (2006—*P. shumwayae*).

into pCR® 2.1 vector and transformed into One Shot® Top 10F' chemically competent cells following the manufacturer's protocol (Invitrogen Technologies, Carlsbad, CA, USA). Colonies were screened for inserts using 1 μl of template in PCR, as described above, using primers specific for the pCR® 2.1 vector. DNA from colonies with an insert yielded an amplicon of 465 base pairs (149 bp insert + 316 bp vector sequence) while DNA derived from colonies without an insert resulted in a 316 bp amplicon.

PCR products containing DNA with inserts were sequenced. The sequencing reactions contained 0.5 μl of template DNA, 2 μl dye (DYEnamic™ ET Terminator Cycle Sequencing kit, Amersham Biosciences, Piscataway, NJ, USA), 1 μl of vector primer (0.4 μM final concentration), and sterile H_2O to 5 μl. Cycling parameters were as follows: 25 cycles at 95 °C for 20 s, 55 °C for 15 s and 60 °C for 1 min. After cycling, sequencing reactions were centrifuged through Sephadex G50 to remove unincorporated dye (Amersham Biosciences, Uppsala, Sweden). Sequencing was performed on the 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Dinoflagellate sequences were aligned and inspected for nucleotide ambiguities using Sequencher (version 4.1.2, Gene Codes Corporation, Ann Arbor, MI, USA) and then aligned to each other utilizing the software ClustalX (version 1.83.1; Thompson et al., 1997). The Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997), available on the National Institute for Biotechnology Information website maintained by the National Institutes of Health (http://www.ncbi.nlm.nih.gov/), was used to determine the closest genetic matches available in GenBank. GenBank sequences with a sequence similarity of 90% or greater were included in the tree. A total of 29 representative sequences from the following dinoflagellate genera were also included in the analysis: *Alexandrium*, *Amphidinium*, *Amyloodinium*, *Dinophysis*, *Gonyaulax*, *Gymnodinium/Gyrodinium*, *Heterocapsa*, *Oxyrrhis*, *Oxytricha*, *Peridinium*, *Prorocentrum*, *Scrippsiella*, *Strombiodinium*, *Takayama*, and *Noctiluca* (the latter used as the outgroup). The Phylogenetic Analysis Using Parsimony program (PAUP*, version 4.0, Swofford, 1999) was used to perform neighbor-joining analysis with the Jukes-Cantor evolutionary model.

2.1.4. Bacterial assemblages

Total bacterial abundance was quantified from the same tanks as phytoplankton using flow cytometry after DNA staining with SYBR® Green I nucleic acid stain (Molecular Probes, Eugene, OR, USA) (Gasol and Del Giorgio, 2000; Button and Robertson, 2001). Bacterial populations were identified and individual cells counted on flow cytometric histograms of right-angle light scatter versus green DNA fluorescence. Samples were analyzed in duplicate, with periodic quality control matrix spikes. Spikes were made by injecting 10 μl of a *Vibrio* spp. bacterial culture into 1 ml of sample and comparing the total cell counts with and without spiking solution. Nominal spike value was determined by running 10 μl *Vibrio* prepared with filtered sheath fluid (0.2 μm pore size) following the above protocol.

Molecular techniques were used to screen samples for potentially harmful bacterial taxa. For DNA extraction, 50 ml of acidic Lugol's-preserved sample was centrifuged and total DNA extracted from the pellet using an UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA). Extracted DNA was stored at −20 °C until PCR was performed.

Various types of PCR assays were performed for detection of selected bacterial pathogens (Table 3). Lower limits of detection were determined by serial dilution of purified bacterial DNA. The last dilution detected in the series was then regenerated by spiking into background DNA extracted from various ballast samples, to assess potential inhibitory effects from other materials in the ballast water samples that were co-extracted. Although the cycle number at which the dilution was detected by real-time PCR varied with different background samples used, the dilution was still detectable, thus validating the assays as a qualitative tool.

Several of the real-time PCR assays incorporated SyBr Green as the detection platform, analyzed using a Lightcycler (Idaho Technology, Inc., Salt Lake City, UT, USA) (Wittwer et al., 1997). Assays included a step of 94 °C for 2 min to release the antibody from the hot start Taq polymerase, followed by 50 cycles at 94 °C for 0.5 s, annealing temperature (varied for each assay) for 0.5 s, and 72 °C for 10 s (fluorescence acquisition occurred after this step in each cycle). The following cycling was used to generate the melting curve: 97 °C for 20 s, 50 °C for 20 s, and then reactions were slowly ramped back up to 97 °C at 0.2 °C s^{-1}. Additional assays incorporated Taqman probes as the detection platform (Holland et al., 1991): the probes, specific for target loci, were fluorescently labeled on the 5′ end with a reporter dye (e.g. FAM [carboxy-fluorescein]) and at the 3′ end with a quencher dye (e.g. TAMRA [carboxytetramethylrhodamin] or BHQ [black hole quencher]). The following cycling parameters were used to run Taqman-based assays: 50 cycles at 94 °C for 0.5 s, and annealing temperature for 20 s. Fluorescence acquisition occurred

Table 3
Real-time PCR assays used to screen for potentially harmful eubacteria, including target species, target loci, loci descriptions, PCR techniques used, and lower limits of detection

Target species	Target	Loci	PCR	Lower limit of detection	Reference
Aeromonas spp.	16S	16S rRNA	Conven.	n.d.	Figueras et al. (2000)
Escherichia coli	16S	16S rRNA	Conven.	n.d.	Sabat et al. (2000)
Leptospira spp.	16S	16S rRNA	Taqman	n.d.	Smythe et al. (2002)[a]
Listeria monocytogenes	*hly*	listeriolysin	Taqman	n.d.	Rodriquez-Lázaro et al. (2004)
Morganella morganii	16S	16S rRNA	Conven.	<10 fg μl^{-1}	Kim et al. (2003)
Mycobacterium spp.	16S	16S rRNA invasion plasmid antigen	Taqman	~ 58 fg μl^{-1}	Oldach and Bowers[b]
Pseudomonas aeruginosa	23S	23S rRNA	SyBr	74 fg μl^{-1}	Ludwig et al. (1994)
Salmonella spp.	*himA*	Protein—codes for hemolysin	SyBr	60 fg μl^{-1}	Bej et al. (1994)
Shigella spp.	*lpaH*	H	Conven.	<45 fg μl^{-1}	Kong et al. (2002)
Vibrio alginolyticus	*hsp*	Heat shock protein	SyBr	178 fg μl^{-1}	Oldach and Bowers[c]
Vibrio cholerae	*ctxA*	Toxin gene	SyBr	2 fg μl^{-1}	Nandi et al. (2000)[d]
Vibrio vulnificus	*vvhA*	Hemolysin/cytolysin gene	Taqman	250 fg μl^{-1}	Campbell and Wright (2003)

[a] Probe is specific for pathogenic *Leptospira* spp.
[b] Probe detects *Mycobacterium marinum*, *M. ulcerans, M. chesapeakii, M. pseudoshottsii*, and *M. liflandii*.
[c] Probe detects *Vibrio* spp.
[d] Probe detects toxic strains of *Vibrio cholerae*.

after each cycle. For real-time assays using either SyBr Green or Taqman probes, we used a hot-start Taq polymerase suitable for bacterial sequences that might be difficult to amplify (Takara Taq; Takara Bio Inc., Shiga, Japan). Each reaction contained the following: 0.05 U Taq polymerase, 1× PCR buffer, 5 mM $MgCl_2$, 0.2 μM forward primer, 0.2 μM reverse primer, 0.3 mM each deoxynucleotide triphophosphate, 0.25 mg ml^{-1} bovine serum albumin, molecular grade water to 10 μl, 1 μl of DNA template, and either 1× SyBr Green or 0.3 μM Taqman probe.

Conventional PCR (i.e. non-real-time) was used for detecting several bacterial species (Table 3). Each 50 μl PCR reaction contained 1.5 U of MegaFrag™ Taq polymerase (Denville Scientific, Metuchen, NJ, USA) or TaKaRa *Ex* Taq™ (Takara Bio Inc.); 10× PCR buffer and 4 mM $MgCl_2$; 2 mM each dNTP (Invitrogen), 0.25 mg ml^{-1} bovine serum albumin (Idaho Technology), 0.8 μM each primer (Qiagen/Operon), 1 μl DNA template, and molecular biology grade water to a final volume of 50 μl. Cycling was performed on the DNA Engine Dyad Peltier thermocycler (Bio-Rad Laboratories, Inc.) as follows: initial denaturation at 94 °C for 2 min, followed by 45 cycles of 94 °C for 10 s, annealing temperature (varied for each species) for 30 s, and 68 °C for 10 s, and a final extension at 68 °C for 6 min 20 s. PCR products were analyzed on an ethidium bromide-stained gel.

For generating bacterial clones from samples via molecular methods, two bacterial SSU rRNA group-specific primers, BAL-F (5′-AGACTCCTACGGGAGGCAGCAGT-3′; modified from Wilmotte et al., 1993) and BAL-R (5′-TCGAATTAAACCACATRCTCCACCG-3′) were used to amplify a fragment of approximately 600 bp. PCR was performed as described above with either MegaFrag™ Taq polymerase (Denville Scientific) or TaKaRa *Ex* Taq™ (Takara Bio Inc.). Cycling was performed as described above, with an annealing temperature of 60 °C and an extension time of 40 s. PCR products were ligated, cloned, and screened with PCR using vector primers as outlined above for dinoflagellate clones. DNA from colonies with an insert yielded an amplicon of approximately 916 base pairs (approximately 600 bp insert + 316 bp vector sequence). PCR products containing inserts were used as template in sequencing reactions as described above, with BAL-F and BAL-R.

Sequences from other eubacteria and other detected prokaryotes were aligned and inspected for nucleotide ambiguities using Sequencher (version 4.1.2). The presence of chimeric sequences was assessed using the Bellerophon server (Huber et al., 2004; http://foo.maths.uq.edu.au/~huber/bellerophon.pl). Sequences were uploaded to the Ribosomal Database Project II Release 9.40 (Cole et al., 2005; http://rdp.cme.msu.edu/) and a Seqmatch search was performed to determine the closest 16S rRNA sequence match (243,909 sequences available in the Ribosomal Database Project [RDP] database when the analysis was completed in July 2006; see http://rdp.cme.msu.edu/).

2.1.5. Data analysis

Tanks were divided into two major source groups based on whether they were known to contain some

Table 4
Age distribution of water (days) in ballast tanks that contained at least a portion of coastal source(s) water[a]

Metric	Coastal (n = 20 tanks)	Open-ocean (n = 38 tanks)
Mean ± 1S.D.	35.0 ± 54.9	24.7 ± 25.8
Median	10.0	12.5
Range	2–173	5–104
Coefficient of variation (%)	157	105

[a] Note that of the 62 tanks assessed, 4 (from voyages 3 and 25) could not be classified as coastal or open-ocean because there were no ballast records.

portion of coastal water, as defined by the regulatory Exclusive Economic Zone (EEZ) of ~370 km (200 nautical miles, n.m.) (United Nations, 1982). These sources were designated as coastal versus open-ocean (i.e. non-coastal source water, or with negligible coastal source water defined as <5% of the total). The ballast water age, defined as the time water was held within ballast tanks, was highly variable and averaged ~35 (median 10) days and ~25 (median 12.5) days for coastal versus open-ocean ballast water sources, respectively (Table 4). Voyages 7 and 9, considered within the coastal source group, strongly influenced the ballast water age data because the paired tanks that were sampled from those two voyages contained "old" water (voyage 7, 173 days old; voyage 9, 99 days old). Removal of these four tanks from the coastal group reduced the mean water age to ~10 days (median 6 days); thus, most tanks in the coastal group (16 of 20) contained water that was, on average, approximately half the age of the water in other tanks.

Phytoplankton taxa abundance and species richness data, and bacterial abundance data were subjected to exploratory statistical analyses such as scatter plots and box plots of various combinations of tank criteria and sampling information, including water quality and taxonomic data. Patterns in phytoplankton assemblage structure were compared considering all tanks collectively, and tanks with versus without coastal water sources. PROC VARCOMP or PROC MIXED procedures (SAS Institute Inc., 1999) was used to determine whether the more appropriate sampling unit in studies such as this is the tank or the vessel (i.e., is as much variability captured by sampling eight tanks on one ship as by sampling one tank on each of eight ships?). In addition, variance related to abundance data for phytoplankton and bacteria was examined in isolation from the environmental- and age-related variables and compared within and across ships using the SAS MIXED procedure (SAS Institute Inc., 1999). Given that many tanks were sampled as pairs with similar ballasting histories, the data were analyzed in total as well as in subsets in an attempt to characterize the relative influence of this sampling aspect on the apportioning of variance among the components. This approach resulted in four datasets comparing phytoplankton and bacterial abundances as: (1) the entire dataset; (2) a subset of data including voyages wherein a single set of paired tanks with similar ballast exchange history was sampled; and (3) a subset of data including voyages in which two or more unpaired tanks were sampled. The objective was to determine which components demonstrated higher variance to inform future sampling designs.

Appropriate determinative analyses were applied to further characterize relationships among the measured variables. These included correlation analyses and a standard set of general linear models (GLM) analyses (linear regressions, ANOVAs) to examine relationships among response variables (phytoplankton abundance, phytoplankton species richness, bacterial abundance) and explanatory variables (physical/chemical data, ballast management), including log and other transformations. It should be noted that four tanks (Voyage 3, tank pair; Voyage 25, both tanks) were eliminated from statistical analyses involving coastal versus non-coastal source waters because there were no ballast records available. Various transformations and different data

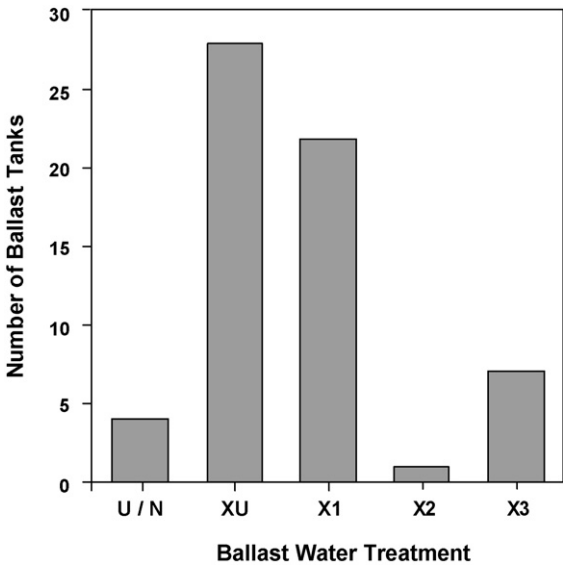

Fig. 2. Distribution of tanks based on ballast water management status. U = "unknown" tanks with insufficient records to determine whether exchange had occurred; N = no exchange was conducted; XU = exchange or open-ocean dilution occurred, but to an undetermined extent of tank volume exchange; X1 = 1 tank-volume exchange; X2 = 2 tank-volume exchanges; X3 = 3 tank-volume exchanges.

Table 5
Salinity, temperature, pH, and dissolved oxygen (DO) in the ballast water of sampled tanks

Variable	Atlantic ocean	Indian ocean	Pacific ocean
Temp. (°C)	21.4 ± 4.7 (CV 22%, n = 32)	17.4 ± 3.8 (CV 21%, n = 7)	17.0 ± 3.8 (CV 22%, n = 21)
Salinity (ppt)	36.7 ± 1.6 (CV 4%, n = 29)	31.3 ± 9.8 (CV 31%, n = 7)	34.1 ± 3.0 (CV 9%, n = 19)
pH	8.1 ± 0.3 (CV 4%, n = 12)	8.0 ± 0.02 (CV 0.2%, n = 2)	8.0 ± 0.27 (CV 3%, n = 15)
DO (mg l^{-1})	6.0 ± 1.1 (CV 18%, n = 30)	6.0 ± 1.7 (CV 28%, n = 7)	5.5 ± 0.7 (CV 13%, n = 19)

Each parameter was averaged over all depths for each sampling event, and then an overall mean was calculated for the ballast water from each ocean. Data are given as means ± 1S.D. (n = number of replicates); coefficients of variation (CVs) are also indicated.

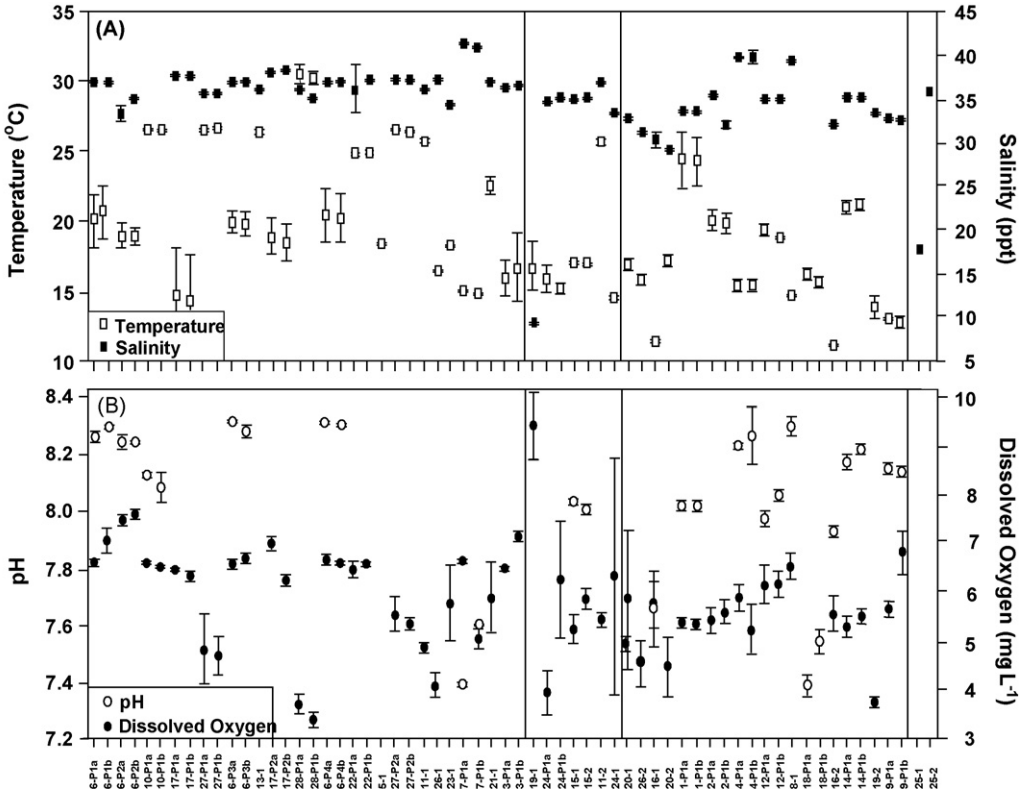

Fig. 3. (A) Temperature and salinity and (B) pH and dissolved oxygen in each ballast water tank, considered by major source region (note that U = unidentified major source region). Within each major source region, tanks are arranged by age in ascending order from left to right. Data are given as means ± 1 standard deviation (S.D.) for each tank.

Table 6
Nutrient concentrations and TN:TP ratios (molar basis) in the ballast water of sampled tanks (means ± 1S.E., with S.D.s given in parentheses; data available for 56 of 62 tanks)[a]

TN (μg l^{-1})	TKN (μg l^{-1})	$NO_3^- + NO_2^-$ (μg l^{-1})	TP (μg l^{-1})	TN:TP	TOC (mg l^{-1})
Tanks with coastal water sources (n = 20)					
164 ± 20 (S.D. 88)	107 ± 16 (S.D. 71)	56 ± 17 (S.D. 76)	33 ± 4 (S.D. 18)	7.7 ± 2.1 (S.D. 9.3)	14 ± 3 (S.D. 11)
Tanks with ocean water sources (n = 36)					
156 ± 28 (S.D. 169)	136 ± 27 (S.D. 164)	20 ± 7 (S.D. 42)	82 ± 41 (S.D. 248)	5.9 ± 1.5 (S.D. 9.1)	9 ± 2 (S.D. 11)
All tanks (n = 56)					
156 ± 19 (S.D. 144)	124 ± 18 (S.D. 138)	33 ± 8 (S.D. 58)	64 ± 26 (S.D. 198)	6.4 ± 1.2 (S.D. 9.1)	11 ± 1 (S.D. 11)

[a] TKN values estimated (below detection limit).

filters were also applied, including independent analysis of tanks that lacked a coastal water source. Data presentations were organized in graphics by source region and by ascending age of the tank water, moving from left to right within each major ocean source (Atlantic, Indian, and Pacific). Within this organizational scheme, ballast tanks were also differentiated by whether they contained a proportion of coastal water. The age of water within a given ballast tank was based upon the last documented ballasting event prior to sampling.

The variance in phytoplankton and bacterial abundances for paired and unpaired tanks was also characterized using principle components analysis (PCA), in an attempt to assess linear variable combinations that explain the variance in the data set. PCA considered physical/chemical variables, organism abundances, and primary biodiversity metrics. Ballast water age, temperature, salinity, dissolved oxygen (DO) concentrations, TN, TP, NO_3^-, and TOC concentrations were included in each PCA analysis, after log-transforming all data except ballast water age.

The experimental design (duplicate samples taken from each of two or more ballast tanks per ship for most vessels) additionally allowed us to examine variability in phytoplankton and bacteria abundances within and among ships, for ships that used different ballasting practices. We compared all tanks collectively and two subsets of data: paired tanks (with apparently similar ballasting history: 13 ships as 1–4, 7, 9, 10, 12, 14, 15, 18, 22, 28; $n = 26$) and unpaired tanks (vessels with at least two ballast tanks of differing management histories: 10 ships as 6 (with 8 tanks), 11 (with 2 tanks), 16 (with 2 tanks), 17 (with 4 tanks), 19 (with 2 tanks), 20 (with 2 tanks), 24 (with 3 tanks), 25 (with 2 tanks), 26 (with 2

Table 7
Phytoplankton taxa found in ballast water samples, also indicating potential harmful traits

Taxon	Distribution	Cultured (tank[s])
Diatoms (Phylum Ochrophyta)		
Actinocyclus curvatulus Janisch in A. Schmidt	Arctic	+(16-1, 26-1)
Actinoptychus senarius (Ehrenberg) Ehrenberg	Widespread	–
Amphora spp. (4)	Widespread	+(14-*P*1a, 27-*P*1b, 28-*P*1b)
Asterionella formosa Hassall	Widespread frw	–
Asterionellopsis glacialis (Castracane) Round	Widespread	–
Asterolampra marylandica Ehrenberg	Widespread (temp, trop)	–
Asteromphalus hookerii Ehrenberg	Widespread (cold)	–
Asteromphalus roperianus (Grevelle) Ralfs in Pritchard	Widespread (temp, trop)	–
Attheya septentrionalis (Østrup) Crawford	Widespread N. hemisphere (cold)	–
Aulacoseira sp.	Widespread	+(27-*P*1b)
Bacteriastrum comosum Pavillard	Widespread (trop, subtrop)	–
Bacteriastrum elongatum Cleve	Widespread (temp, trop)	–
Bacteriastrum furcatum Shadbolt	Widespread (?) (temp-trop)	–
Bacteriastrum hyalinum Lauder	Widespread (temp)	–
Bellerochea horologicalis von Stosch	Widespread (temp, trop)	–
Chaetoceros coarctatus Lauder	Widespread (trop, subtrop)	–
Chaetoceros concavicornis Mangin	Widespread (except Antarctic)	–
Chaetoceros criophilus Castracane	Widespread S. hemisphere (cold)	–
Chaetoceros curvisetus Cleve	Widespread (temp-subtrop)	+(13-1)
Chaetoceros decipiens Cleve	Widespread (polar to temp)	+(27-*P*2a)
Chaetoceros didymus Ehrenberg	Widespread (temp-subtrop)	+(18-*P*1a)
Chaetoceros lorenzianus Grunow	Widespread (temp-subtrop)	–
Chaetoceros similis Cleve	Widespread N. hemisphere (Arctic to temp)	–
Chaetoceros socialis Lauder	Widespread (polar–subtrop)	+(18-*P*1a)
Chaetoceros tetrastichon Cleve	Widespread (trop-temp)	–
Corethron criophilum Castracane	Widespread except Arctic	–
Cylindrotheca closterium (Ehrenberg) Reimann et Lewin	Widespread	+(20-1)
Delphineis sp.	–	+(28-*P*1b)
Ditylum brightwellii (West) Grunow in Van Huerck	Widespread except polar regions	+(16-1)
Eucampia zodiacus Ehrenberg	Widespread except polar regions	–
Helicotheca tamensis (Shrubsole) Ricard	Widespread (temp-trop)	–
Hemiaulus hauckii Grunow in Van Huerck	Widespread (temp-trop)	–
Hemiaulus indicus Karsten	Indian Ocean, Sea of Java	–
Hemiaulus membranaceus Cleve	Widespread (temp-trop)	–

Table 7 (*Continued*)

Taxon	Distribution	Cultured (tank[s])
Hemiaulus sinensis Greville	Widespread (temp-trop)	–
Lauderia annulata Cleve	Widespread (temp-trop)	–
Leptocylindrus danicus Cleve	Widespread except Antarctic	–
Leptocylindrus minimus Gran	Widespread except Antarctic	–
Nanofrustulum sp.	–	+(28-*P*1b)
Nitzschia acicularis (Kützing) W. Smith	Widespread estuarine/frw (temp-subtrop)	–
Nitzschia americana Hasle	Widespread (temp-trop)	+(26-1)
Nitzschia kolaczeckii Grunow	Widespread(?) (trop-subtrop)	+(20-1)
Nitzschia laevis Hustedt	Widespread	+(20-1)
Nitzschia longissima Brébisson in Kützing Ralfs in Prichard	Widespread (temp)	–
Nitzschia sinuata (Thwaites) Grunow	Widespread frw	–
Odontella aurita (Lyngbye) C.A. Agardh	Widespread (polar-temp)	–
Odontella mobilensis (Bailey) Grunow	Widespread	–
Odontella sinensis (Greville) Grunow	Widespread (temp)	–
Proboscia alata (Brightwell) Sundström	Widespread(?) (temp)	–
Pseudo-nitzschia lineola (Cleve) Hasle	Widespread	–
Pseudo-nitzschia fraudulenta (Cleve) Hasle	Widespread (temp-trop)	+(18-*P*1a)
Pseudo-nitzschia pungens (Grunow ex Cleve) Hasle	Widespread (polar-temp)	+(26-1)
Pseudo-nitzschia seriata (Cleve) ex Peragallo in H. et M. Peragallo	Widespread (polar-temp)	–
Pseudo-nitzschia spp. (2)	Widespread (polar-temp)	+(18-*P*1a, 20-1)
Rhizosolenia hebetata Bailey	Widespread	–
Rhizosolenia imbricata Brightwell	Widespread except polar regions	–
Rhizosolenia pungens Cleve-Euler	Widespread (temp-subtrop)	–
Rhizosolenia setigera Brightwell	Widespread (temp-subtrop)	–
Skeletonema costatum (Greville) Cleve	Widespread except polar regions	+(16-1)
Skeletonema potamos (Cl. Webber) Hasle	Widespread estuarine (frw)	–
Thalassionema bacillaris (Heiden in Heiden et Kolbe) Kolbe	Widespread	+(27-*P*2a)
Thalassiosira eccentrica (Ehrenberg) Cleve	Widespread except polar regions	+(6-*P*4b)
Thalassionema nitzschioides (Grunow) Mereschowsky	Widespread except Arctic	+(16-1)
Thalassiosira nordenskioeldii Cleve	Widespread N. hemisphere (Arctic-temp)	+(26-2)
Dinoflagellates (Phylum Dinophyta)		
Akashiwo sanguineum (Hirasaka) G. Hansen et Moestrup	Widespread (temp-trop)	–
Balechina coerulea (Dogiel) F.J.R. Taylor	Widespread (trop-temp)	–
Ceratium candelabrum (Ehrenberg) Stein	Widespread (warm temp-trop)	–
Ceratium furca (Ehrenberg) Claparède et Lachmann	Widespread (cold temp-trop)	–
Ceratium fusus (Ehrenberg) Dujardin	Widespread (cold temp-trop)	–
Ceratium lunula (Schimper) Jørgensen	Widespread (warm temp-trop)	–
Ceratium macroceros (Ehrenberg) Vanhöffen	Widespread (cold temp-trop)	–
Ceratium tripos (O.F. Müller) Nitzsch	Widespread (cold temp-trop)	+(18-*P*1a)
Dinophysis acuminata Claparède et Lachmann	Widespread (polar-temp)	–
Dinophysis caudata Savelle-Kent	Widespread (temp-trop)	–
Gambierdiscus toxicus Adachi et Fukuyo	Widespread (trop-subtrop)	–
Gonyaulax spinifera (Claparède et Lachmann) Diesing	Widespread (temp-subtrop)	–
Heterocapsa rotundata (Lohmann) Hansen	Widespread (temp)	+(20-1)
Heterocapsa triquetra (Ehrenberg) Stein	Widespread (temp)	+(3-*P*1a)
Karlodinium australe de Salas, Bolch et Hallegraeff	Tasmania, SE Australia	+(20-1)
Karlodinium veneficum	Widespread	–
Lingulodinium polyedrum (Stein) Dodge	Widespread (temp-trop)	+(18-*P*1a)
Ornithocercus magnificus Stein	Widespread (warm temp-trop)	–
Oxyrrhis marina Dujardin	Widespread	+(27-*P*2b)
Peridiniella danica (Paulsen) Okolodkov et Dodge	Widespread (polar-temp)	–
Peridinium aciculiferum (Lemmerman) Lindeman	Widespread frw/estuarine (temp)	–
Phalacroma rotundatum (Claparède et Lachmann) Kofoid et Michener	Widespread	–
Podolampas palmipes Stein	Widespread (warm temp-trop)	–

Table 7 (*Continued*)

Taxon	Distribution	Cultured (tank[s])
Prorocentrum micans Ehrenberg	Widespread (cold temp-trop)	+(6-*P*4b, 27-*P*2b)
Prorocentrum minimum (Pavillard) Schiller	Widespread estuarine/marine	+(16-1, 20-1, 27-*P*2a, 27-*P*2b)
Protoperidinium brevipes (Paulsen) Balech	Widespread (cold)	–
Protoperidinium conicum (Gran) Balech	Widespread (temp-trop)	–
Protoperidinium depressum (Bailey) Balech	Widespread (temp-trop)	–
Protoperidinium pallidum (Ostenfeld) Balech	Widespread (cold-warm tem)	–
Protoperidinium pellucidum Bergh	Widespread (temp-trop)	–
Protoperidinium pentagonum (Gran) Balech	Widespread (temp-trop)	–
Scrippsiella trochoidea (Stein) Loeblich III	Widespread	+(20-1, 26-1)
Cryptophyte Flagellates (Phylum Cryptophyta)		
Chroomonas minuta (Skuja) Santore	Widespread estuarine/marine	–
Cryptomonas erosa Ehrenberg	Widespread estuarine/marine	–
Cyanobacteria		
Jaaginema geminatum (Meneghini ex Gomont) Anagnostidis et Komárek	Widespread estuarine (frw)	–
Golden Flagellates (Phylum Ochrophyta)		
Apedinella spinifera (Throndsen) Throndsen	Widespread	–
Dictyocha fibula Ehrenberg	Widespread	–
Dictyocha speculum Ehrenberg	Widespread (polar-temp)	–
Pseudopedinella pyriforme N. Carter	Widespread	–
Flagellates—Other Ochrophytes (Class Raphidophyceae)		
Heterosigma akashiwo (Hada) Hada	Widespread	+(18-*P*1a)
Green Algae (Phylum Chlorophyta)		
Scenedesmus quadricauda de Brébisson in de Brébisson et Godey	Widespread frw	–
Westella botryoides (W. West) de Wildeman	Widespread frw	–

Taxa are marine/estuarine unless otherwise indicated (note: temp—temperate, trop—tropical, subtrop—subtropical, frw—freshwater, ?—distribution uncertain). References consulted for harmful traits include Burkholder (1998), Rhodes et al. (1998), Rengefors and Legrand (2001), Landsberg (2002), Hallegraeff et al. (2003) and Salas et al. (2005). Potentially harmful taxa are indicated in bold.
Harmful taxa were defined as photosynthetic and heterotrophic algae capable of directly or indirectly causing disease or death of humans or beneficial aquatic life.

tanks), and 27 (with 4 tanks); $n = 31$). Restricted maximum likelihood estimates of variance in biota abundances among ships, between or among ballast tanks within ships, and between duplicate samples per tank were calculated using PROC VARCOMP and SAS MIXED (SAS Institute Inc., 1999).

3. Results

3.1. Vessels sampled and ballast water management

Of the 62 tanks analyzed, 94% (all but 4, from voyages 3 and 25) had ballasting records either in the form of ballast logs, U.S. Coast Guard ballasting forms, or watch logs/communiqués that provided sufficient information to identify the recent source location(s) and age of the related water (Table 1). Of the remaining 58 tanks, 20 were classified as coastal and 38 were classified as open-ocean (non-coastal). Most tanks analyzed (90%, or 56 of 62) were subjected to some form of ballast management practice designed to ensure that upon arrival at a U.S. port, at least a portion of the ballast water was from an open ocean source (Table 1; Fig. 2). About 48% (30 of 62) of the tanks were subject to complete exchanges (1–3 tank volumes exchanged, X1, X2, and X3), and an additional 45% (28 of 62) were exchanged in uncertain proportions (XU in Table 1). The uncertainty was due to inadequate specificity in records and/or combining multiple tank pairs in log entries without maintaining tank-specific tonnages. Of the remaining four tanks, the exchange status was unknown for two tanks (U), and two tanks received no exchange (N). Overall, records were adequate to determine the source locales and the age of the water in most ballast tanks.

Based on the ballast records and utilizing the ~371 km (200 n.m.) exclusive economic zone (EEZ) for coastal boundaries, 32% of the tanks contained some coastal water, from either full exchanges in coastal areas or partial ballasting of coastal sources prior to making

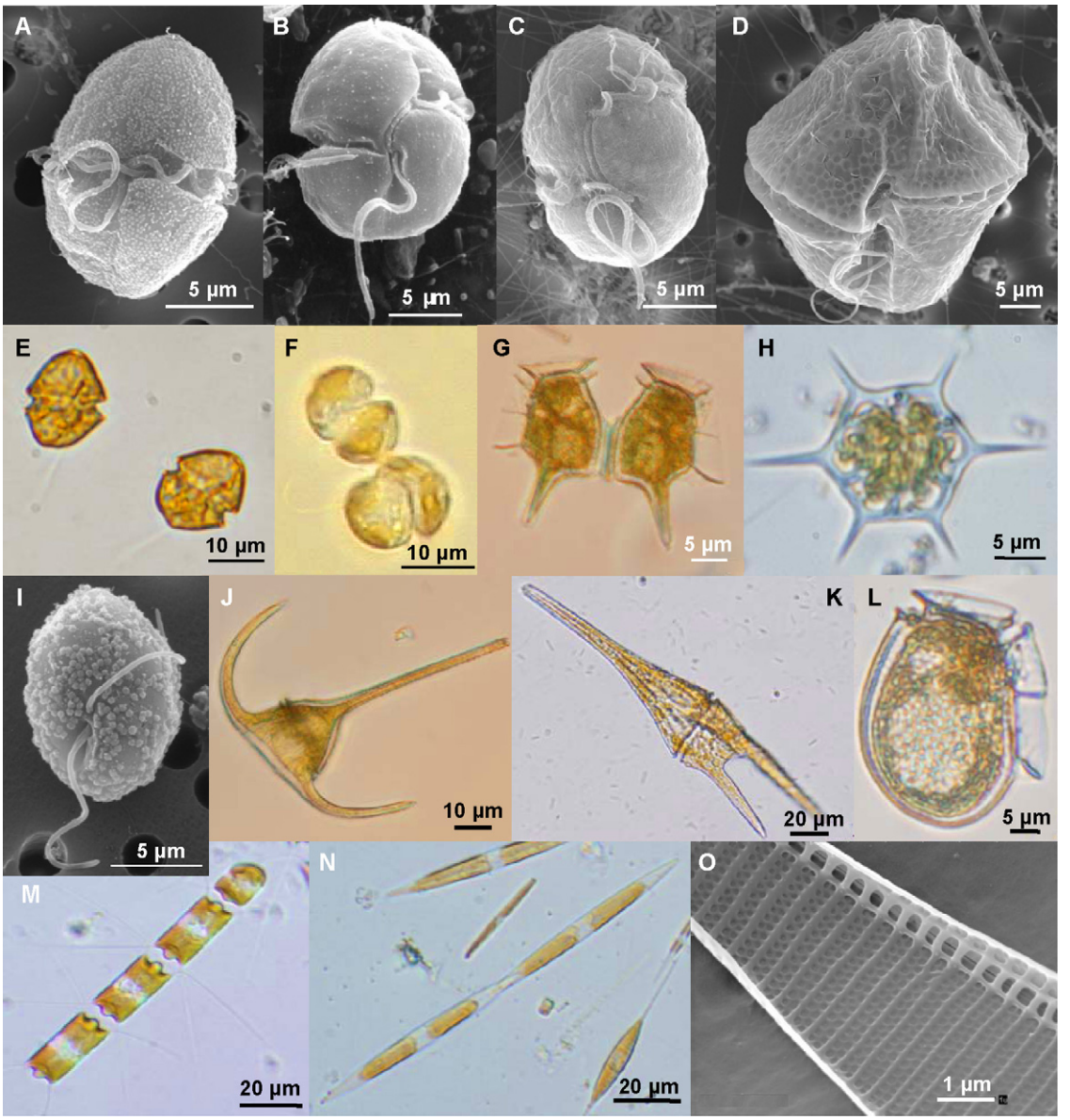

Fig. 4. Examples of phytoplankton taxa in the ballast tanks, as scanning electron micrographs (SEMs) or light micrographs (LMs), and indicating whether the species was successfully cultured from ballast tank waters: (A and E) cultured *Heterocapsa rotundata*—SEM and LM, respectively; (B and F) cultured harmful species *Karlodinium veneficum*—SEM and LM, respectively; (C) cultured harmful species *Karlodinium australe*—SEM; (D) cultured *Lingulodinium polyedrum*—SEM; (G) *Dinophysis caudata*—LM of two flagellate cells; (H) *Dictyocha speculum*—LM; (I) cultured *Heterosigma akashiwo*—SEM; (J) *Ceratium tripos*—LM; (K) *Ceratium furca*—LM; (L) *Dinophysis acuminata*—LM; *Chaetoceros didymus*—LM; and (N, O) *Pseudo-nitzschia fraudulenta*—LM (two cells in center) and SEM, respectively.

port. Distance to the adjacent coastline was calculated for all coastal-source tanks using geographic information systems (GIS) analysis (Table 1). Of the 20 tanks containing some coastal water, 10 were ballasted within the bounds of the primary continental shelf of the adjacent land mass. Four of these tanks (6.5%) were ballasted over coastal shelf zones from foreign locales (Voyages 18, 20—Table 1); the remaining 6 were ballasted over shelf zones in U.S. waters. There was no indication that any coastal foreign waters were discharged into the U.S. receiving ports.

3.2. Physical and chemical data

The mean salinities of ballast water in tanks with Atlantic, Indian, and Pacific Ocean water sources were

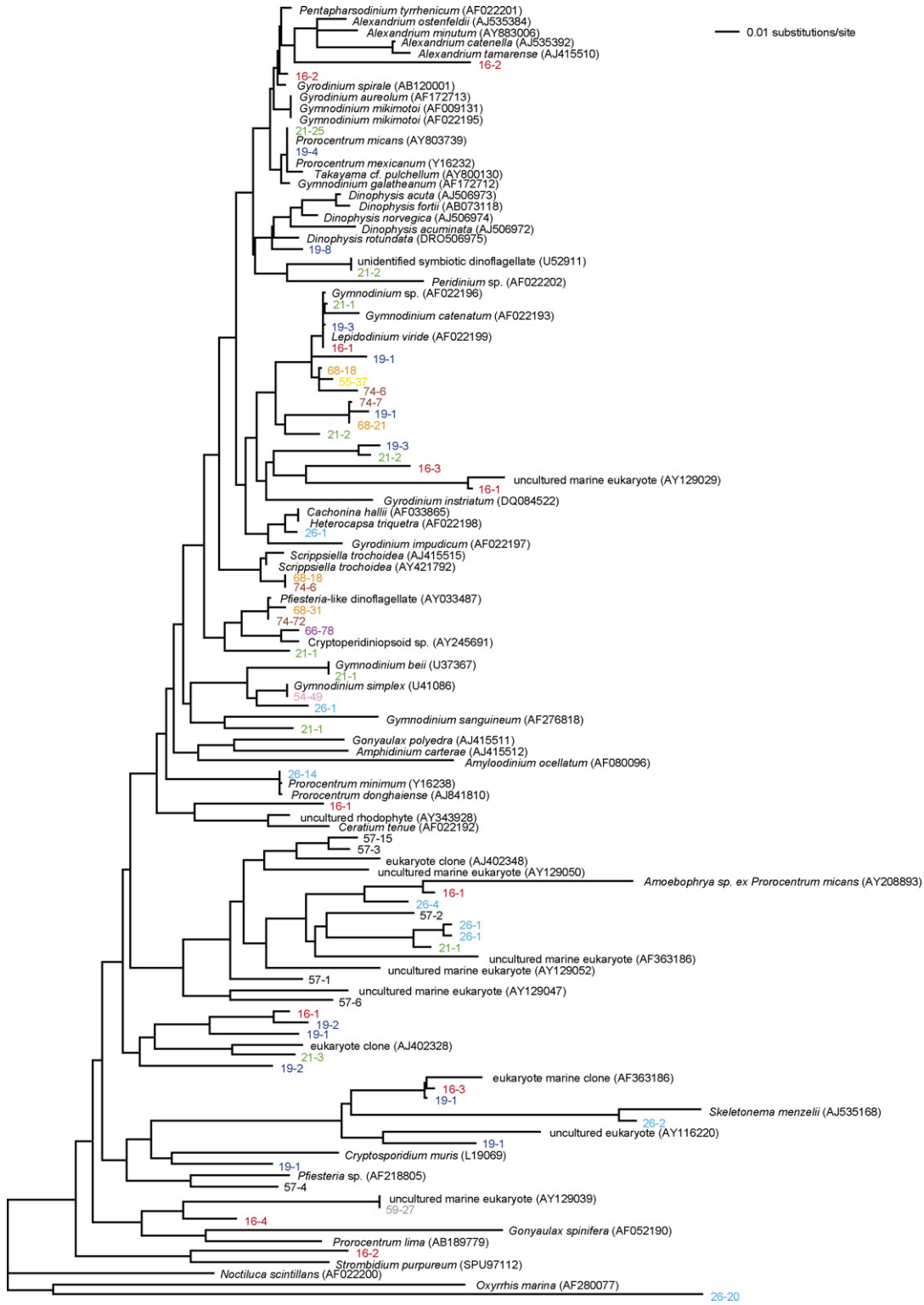

Fig. 5. Neighbor-joining analysis using the Jukes-Cantor evolutionary model. Analysis is based on a 149-bp region of the 3′-end of the 18S rRNA dinoflagellate locus. Sequences available in GenBank with >90% similarity to ballast clones were included. In addition, 29 representative sequences from other dinoflagellate genera were included: *Alexandrium*, *Amyloodinium*, *Amphidinium*, *Dinophysis*, *Gonyaulax*, *Gymnodinium/Gyrodinium*, *Heterocapsa*, *Oxyrrhis*, *Oxytricha*, *Peridinium*, *Prorocentrum*, *Scrippsiella*, *Strombiodinium*, *Takayama* and *Noctiluca* (the latter, as the outgroup).

comparable, but tanks with Atlantic Ocean water sources were significantly warmer ($p < 0.05$; Table 5; Fig. 3A). The pH was within expected ranges (alkaline, 7.2–8.4), and DO was sufficient to maintain the biota (regional means from data averaged over all depths, 5.52–6.02 mg DO l^{-1}), except for several tanks that were at somewhat lower levels of 3–4 mg DO l^{-1} (Table 5; Fig. 3B). Most tanks showed little depth stratification, indicating that they were well mixed at the time of sampling (Fig. 3). Turbidity was negligible in all tanks throughout the study (data not shown). Nutrient concentrations in all tanks were low to moderate relative to typical concentrations in coastal waters (e.g. Day et al., 1989) (Table 6). Using log-transformed data, no significant differences were found between TN, NO_3^-, TP, or TOC and water age, water source, dissolved oxygen, or other factors (PROC GLM).

3.3. Phytoplankton assemblages

A total of 100 phytoplankton species were identified from the ballast tanks examined, including 23 potentially harmful taxa (Table 7; Fig. 4). Phytoplankton species included 59 diatoms, 32 dinoflagellates, 2 cryptophyte flagellates, 1 cyanobacterium, 1 raphidophyte flagellate, 4 other ochrophyte (golden) flagellates, and 2 colonial green algae. Nearly all of the species found have been reported from various regions and are presumed to be widespread. Of the 62 tanks sampled, 14 tanks (23%) yielded a total of 36 species of culturable phytoplankton that were useful in species identifications. About half of the tanks with culturable phytoplankton had a coastal water source, including the four that yielded most cultures (tank 16-2, coastal Washington state; tank 18-*P*1a, Guam Fuel Pier; tank 20-1, Persian Gulf; tank 26-1, Puget Sound). The latter two tanks also contained the "newest" water (held <5 days). Tanks with ballast water age more than 33 days did not produce culturable phytoplankton. Six tanks yielded viable cultures of potentially harmful species: *Prorocentrum minimum* was cultured from 4 tanks (16-1, 20-2, 27-*P*2a,2b); *Karlodinium veneficum* was cultured from tank 20-1; *Scrippsiella trochoidea* was cultured from tank 26-1; *Ceratium tripos*, *Chaetoceros socialis*, *Heterosigma akashiwo*, *Lingulodinium polyedrum*, and *Pseudo-nitzschia fraudulenta* were cultured from tank 18-*P*1a.

Other potentially harmful phytoplankton species were tested for using molecular methods. The six tanks tested with the real-time PCR assays (Table 2) were negative for all six harmful algal species (*Heterosigma akashiwo*, *Fibrocapsa japonica*, *Karlodinium veneficum*, *Microcystis aeruginosa*, *Pfiesteria piscicida*, *Pfiesteria shumwayae*). A total of 513 dinoflagellate clones from 11 different tanks were analyzed and BLASTed to determine the closest genetic match in GenBank. A total of 85 clones (17%) were 100% identical to a sequence in GenBank (Fig. 5): 49 clones were 100% identical to *Gymnodinium simplex* (U41086); 27 clones were identical to an uncultured marine eukaryote (AY129039); 4 clones were 100% identical to *Lepidodinium viride* (AF022199); 2 clones were 100% identical to *Pentapharsodinium tyrrhenicum*; 2 clones were100% identical to an unidentified symbiotic dinoflagellate (U52911); and 1 clone was 100% identical to *Gymnodinium beii* (U37367). The remaining 405 clones (80%) were either novel (novel sequences with ≥2 identical clones were included) or shared a sequence similarity between 89% and 99% with dinoflagellate sequences on GenBank, and were included in Fig. 5.

Viable phytoplankton in ballast tank waters were dominated by chain-forming diatoms and dinoflagellates (Fig. 6). From all tanks, flagellated green algae and flagellated ochrophytes (golden-brown algae) in the size categories emphasized in this study were negligible in abundance, and cyanobacteria contributed <10% of the viable cells, expected considering the 20 μm net mesh size used. Comparable relative abundances of centric and pennate diatoms, dinoflagellates, and cyanobacteria occurred in tanks with open-ocean versus coastal water sources (Fig. 6A). However, tanks with known extent of exchange (X1, X2, X3) contained higher relative abundances of centric diatoms and lower relative abundances of dinoflagellates than tanks with unknown extent of exchange, unknown or no exchange (XU, U, N) (Fig. 6B).

Phytoplankton abundance was highly variable (Fig. 7): the grand mean (±1S.D.) for total phytoplankton was $3.73 \pm 2.68 \times 10^4$ cells m^{-3} (range ~0–5.95×10^5 cells m^{-3}; median 7.91×10^3 cells m^{-3}). The grand mean (±1S.D.) for viable phytoplankton was $3.28 \pm 2.26 \times 10^4$ cells m^{-3} (range ~0–4.743×10^5 cells m^{-3}; median 3.8×10^3 cells m^{-3}). Highest cell densities were from tanks containing coastal water that had recently been added. The proportion of viable phytoplankton was also highly variable (Fig. 7); considering median values, viable phytoplankton comprised ~50% of the

Note that *Gyrodinium galatheanum* has been renamed as *Karlodinium veneficum*; *Gymnodinium mikimotoi* has been renamed as *Akashiwo mikimotoi*; and *Gymnodinium sanguineum* has been renamed as *Akashiwo sanguineum*. Clone groups are designated by the tank number and the number of clones in that group (e.g. 16-2 represents 2 identical clones from tank 16).

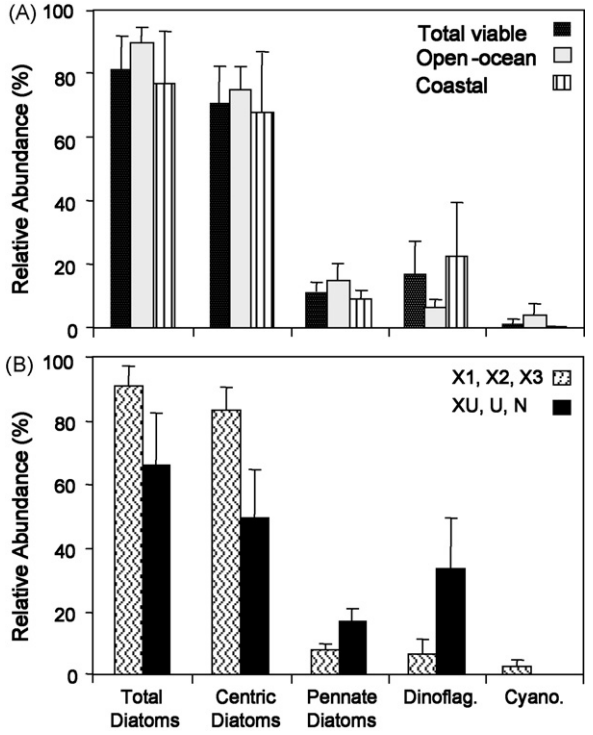

Fig. 6. Relative abundance of viable phytoplankton as major groups, for: (A) All ballast water tanks collectively, all tanks with negligible (<5%) coastal water sources, and tanks with coastal sources (dinoflag., dinoflagellates; cyano., cyanobacteria); and (B) ballast tanks that had 1–3 complete exchanges (X1, X2, X3) vs. tanks that received no exchange (N), tanks that were exchanged but to an unknown extent (XU = 22 of 62 tanks), and ''unknown'' tanks with insufficient records to determine whether exchange had occurred (U). Data are given as means ± 1S.E.

total cells. Median phytoplankton abundance in ballast waters from the Atlantic and Pacific Oceans was comparable (8.2×10^3 and 15.0×10^3 cells m^{-3}, respectively), whereas median density in ballast waters from the Indian Ocean was lower (0.6×10^3 cells m^{-3}). Tanks with Indian Ocean water were fewer in number and did not include coastal sources. In addition, the greater distance between the Indian Ocean and U.S. sampling ports resulted in a longer average time interval between the last ballast water additions and sampling efforts. Of the 62 tanks sampled, 58 tanks (94%) contained $<1 \times 10^4$ viable cells m^{-3} in the small size class (maximum dimension 10–50 μm), whereas 33 tanks contained >10 viable cells m^{-3} in the large size class (maximum dimension >50 μm; Fig. 8).

Pair-wise Student's *t* tests were performed to compare phytoplankton abundances from the three source regions, excluding tanks with coastal source waters, to assess whether there were regional differences independent of age and coastal variables. Phytoplankton abundances in tanks with Atlantic or Pacific versus Indian Ocean source waters were significantly higher ($p = 0.02$) and abundances were comparable in tanks with Atlantic versus Pacific waters. ANOVA indicated that phytoplankton densities between tanks with coastal water sources versus the other tanks were significantly higher ($p = 0.016$). Phytoplankton abundance (both viable and total cell number) was regressed against several potential explanatory variables including vessel, ballast water age, and 11 physical/chemical factors. Several different data filtering scenarios were applied across variable pairings, such as removal of tanks based upon sampling method, season, coastal status, or exchange status. Lower phytoplankton abundance occurred in older ballast water, but there was high variability in phytoplankton abundance within water held less than 30 days (Fig. 9). The only other statistically significant relationships discerned were between the abundance of phytoplankton (both viable and total), total diatoms, or centric diatoms (both viable and total) and nitrate concentrations ($r^2 = 0.44$ to 0.72, $P < 0.001$).

As expected, mean total phytoplankton abundance was ~4-fold higher in ballast water with coastal sources (7.74×10^4 cells m^{-3}) than in ballast water from the open-ocean (1.97×10^4 cells m^{-3}; $p = 0.026$). Median values approximated the same ratio (3.5-fold difference). Mean viable phytoplankton were ~5.5-fold higher (median, 3-fold higher) in ballast water with coastal sources ($p = 0.022$). Ballast tanks containing coastal source waters had significantly higher dinoflagellates, both as total cells and as viable cells, than tanks with open-ocean sources ($p = 0.05$). Ballast tanks within the coastal source group were considered as two major age classes (recent, 1–14 days; older, ≥15 days) to further explore the influence of age on phytoplankton abundance, and similar results were obtained. Overall, the data indicate that coastal status influenced phytoplankton abundance in the ballast tanks regardless of water age.

Species richness among the ballast tanks ranged from 5 to 47 and was highest in tank 18-*P*1b (Fig. 10), which had received water from the Guam fuel pier area 16 days before the tank was sampled. It should be noted that comparable species richness was obtained from samples collected with both net mesh sizes (data not shown). Although ballast records indicated that the tanks were managed as pairs with similar histories, species richness for paired tanks sometimes differed markedly. In general (except for voyages/tanks 4, 12, 24-1, and 18-*P*1b), within each source region, phytoplankton species richness decreased with ballast water age.

Principal components analysis of phytoplankton abundance showed that the paired tank ordinations were

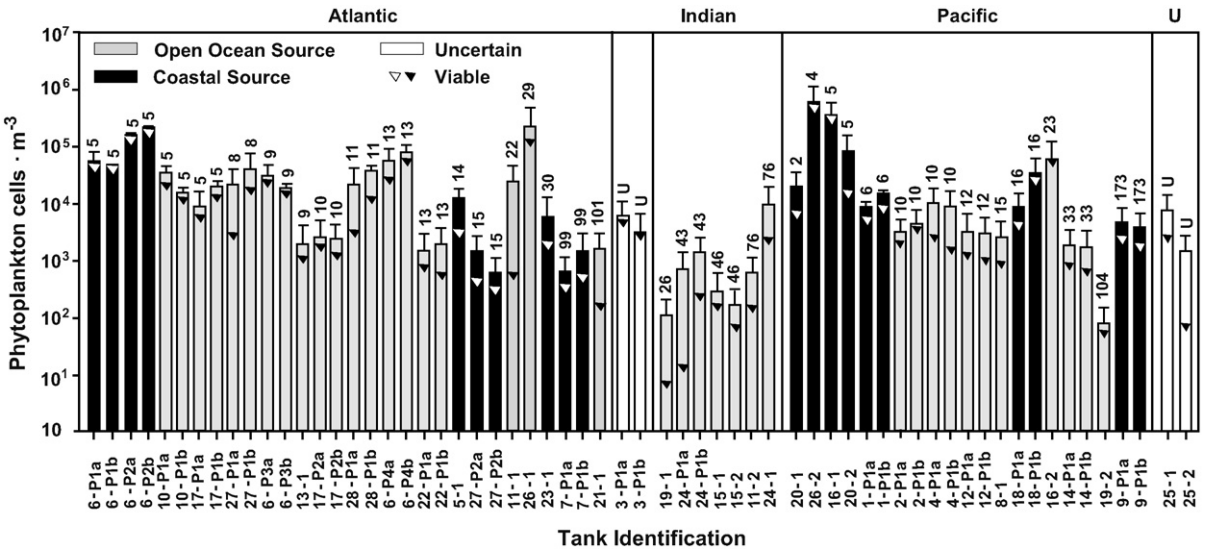

Fig. 7. Total and viable (▼) phytoplankton abundance for each ballast tank, quantitatively collected from plankton net tows (20-μm mesh) (means ± 1S.D., $n = 2$). The data are grouped by major source region (U = unidentified major source) and arranged by ascending age proceeding from left to right within each major ocean source. The age (in days) of the ballast water (time held in tanks), based upon the most recent documented ballasting event prior to sampling, is shown over each bar.

tightly clustered in comparison to the unpaired tank ordinations (Fig. 11). The percentage of the variance in the dataset that was explained by PC1 and PC2 was moderate, with means of 58% and 62% for the paired tank and unpaired tank datasets, respectively. For the paired tanks dataset, the dominant variables for PC1 were salinity, DO, and TOC, and the dominant variables for PC2 were ballast water age and temperature. For the

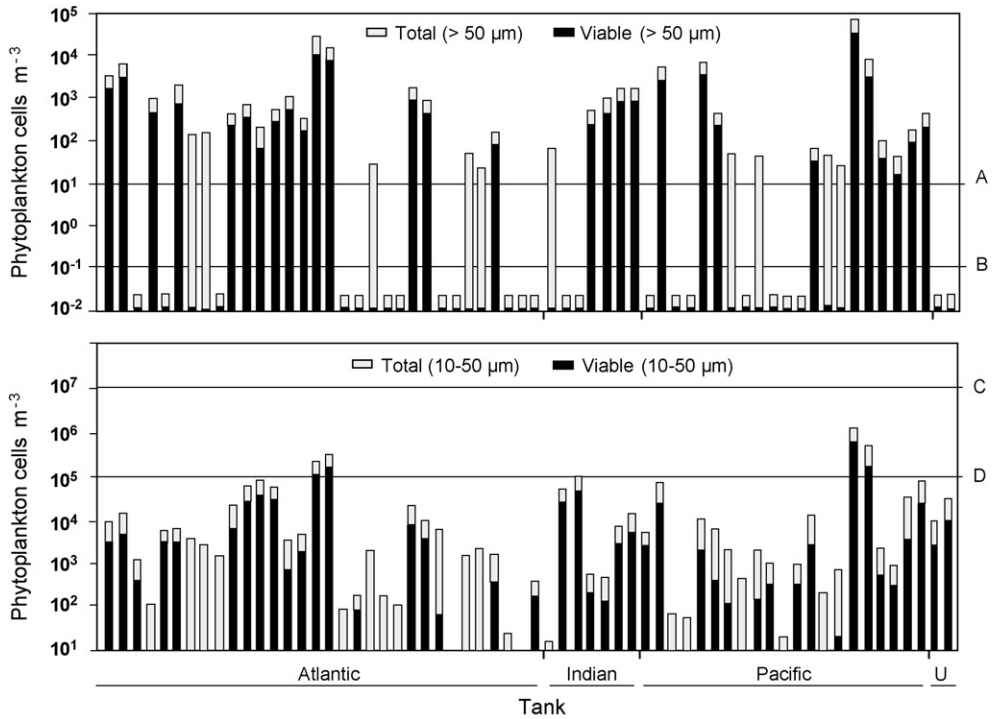

Fig. 8. Total and viable phytoplankton abundances by size class (maximum cell dimension), arranged in order of water age from youngest to oldest within each ocean source, in comparison to proposed regulatory discharge limits (lines) as (A) IMO (2004), 10 viable cells m^{-3}; (B) U.S. Senate (2005), 0.1 viable cell m^{-3}; (C) IMO (2004), 10^7 viable cell m^{-3}; (D) U.S. Senate (2005), 10^5 viable cells m^{-3}.

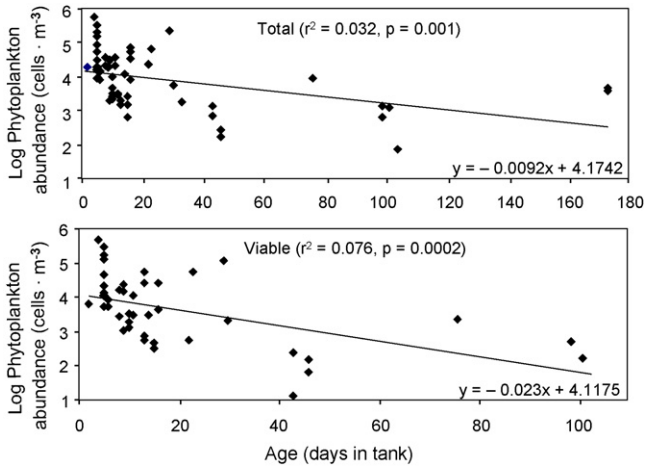

Fig. 9. Relationship between phytoplankton abundance (log-transformed data) and ballast water age, showing total cells and viable cells (upper and lower panels, respectively).

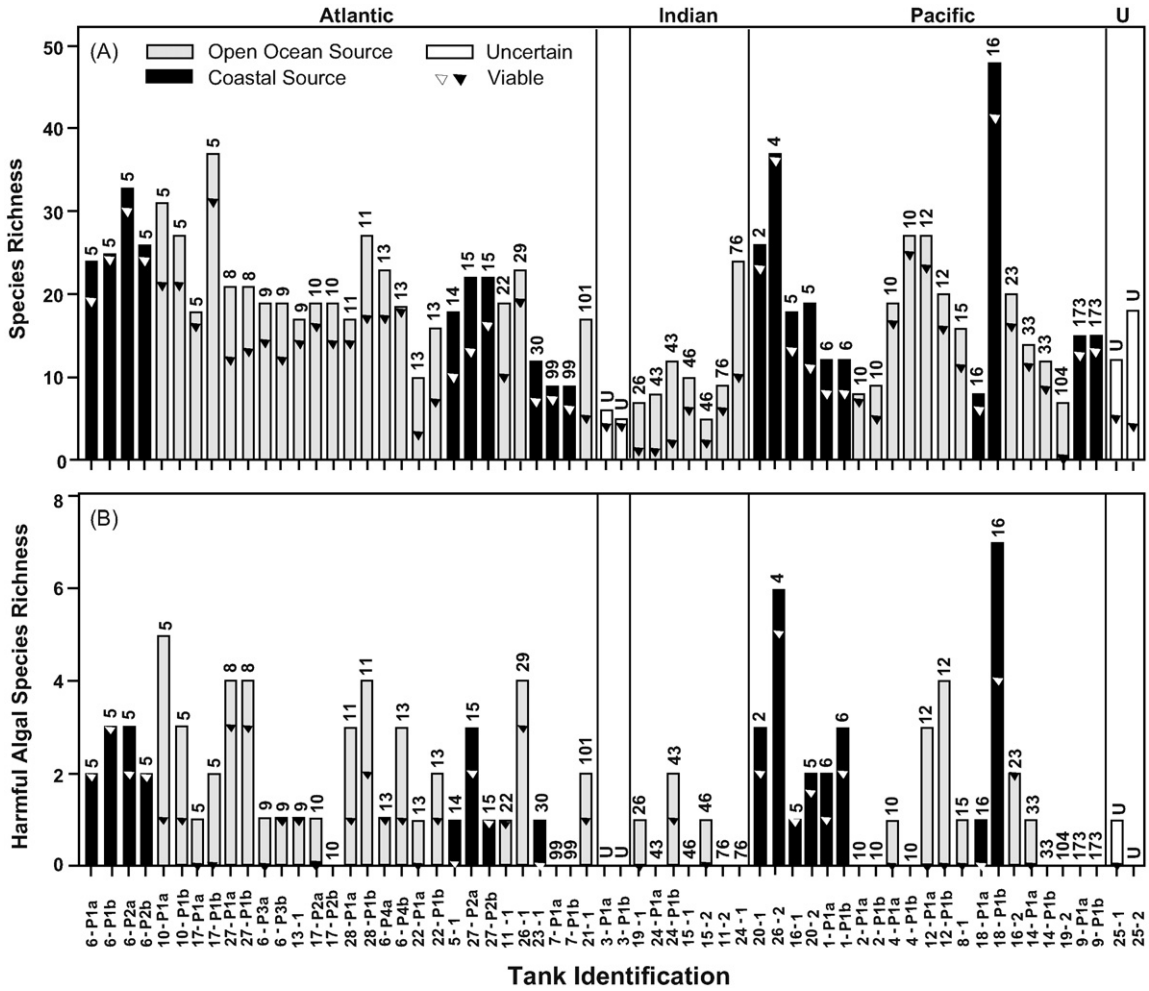

Fig. 10. Phytoplankton species richness and harmful algal species richness in each ballast tank (upper and lower panels, respectively), considering total and viable cells from quantitative 20-μm-mesh net tows. The data are presented as in Fig. 7.

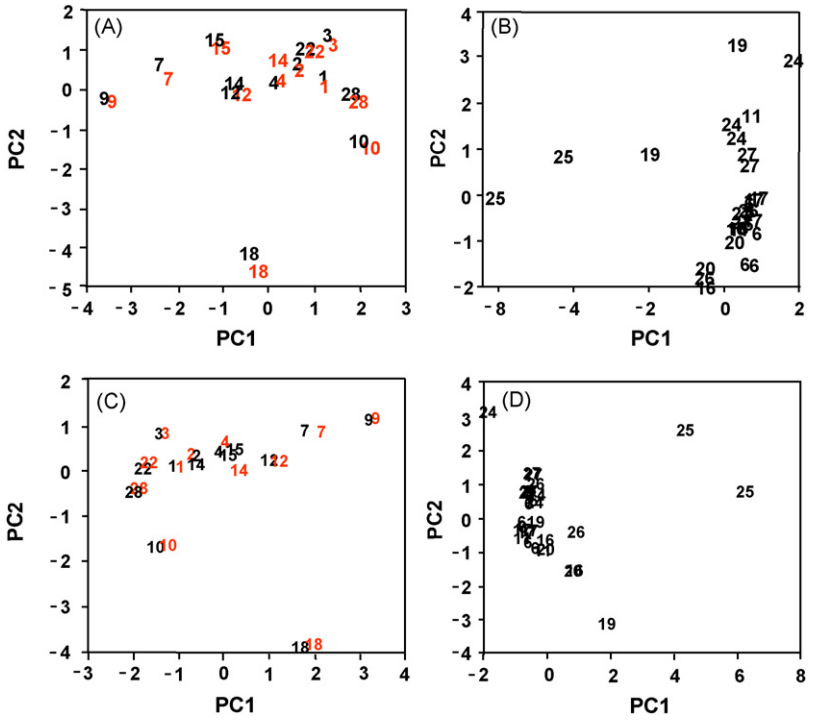

Fig. 11. Principal component plots of biota abundance considering paired tanks with similar ballasting history (A, phytoplankton; C, bacteria) vs. ''unpaired'' tanks with different ballasting history (B, phytoplankton; D, bacteria). Paired tanks were from voyages 1, 2, 3, 4, 7, 9, 10, 12, 14, 15, 18, 22, and 28; voyage numbers are shown, with the second member of each pair in red. For (B and D), the following tanks were included: voyages 11, 16, 19, 20, 25, and 26, each with two tanks; voyage 24 with three tanks; voyages 17 and 27 each with 4 tanks; and voyage 6 with 8 tanks. In (B), the lower right cluster includes six tanks from voyage 6, four tanks from voyage 17, two tanks from voyage 27, and one tank each from voyages 16, 20, and 26. In (D), the upper left cluster includes four tanks from voyage 6, two from voyage 24, one from voyage 26, and four from voyage 27; the lower left cluster includes four tanks from voyage 6, two from voyage 11, four from voyage 17, and one each from voyages 18, 19, and 20.

unpaired tanks dataset, the dominant variables for PC1 were temperature and DO, and the dominant variables for PC2 were TOC and NO_3^-.

3.4. Bacterial assemblages

Bacterial abundance in ballast tank water was surprisingly consistent across this study among ballast tanks and seasons, varying within one order of magnitude (Fig. 12a). The overall mean (±1S.D.) across all tanks examined was $3.05 \pm 0.52 \times 10^{11}$ cells m^{-3} (median, 2.79×10^{11} cells m^{-3}). Means (±1S.D.) by region were $2.70 \pm 0.37 \times 10^{11}$, $3.21 \pm 0.74 \times 10^{11}$, and $3.49 \pm 0.62 \times 10^{11}$ cells m^{-3} for the Atlantic, Indian, and Pacific Oceans, respectively. Means (±1S.D.) for ballast tanks with coastal and non-coastal waters were $3.16 \pm 0.39 \times 10^{11}$ cells m^{-3} (median, 3.2×10^{11} cells m^{-3}) and $2.97 \pm 0.58 \times 10^{11}$ cells m^{-3} (median, 2.66×10^{11} cells m^{-3}), respectively.

Vibrio spp. were detected in 16 (26%) of the 62 tanks sampled (Fig. 12b), and densities ranged from ~200 CFU 100 ml^{-1} to TNTC (too numerous to count; practical estimate of minimum, $\sim 3.0 \times 10^4$ CFU 100 ml^{-1}). *Vibrio* spp. comprised ~0–10% of the total bacterial abundances in the ballast tanks where they occurred, but toxigenic *Vibrio cholerae* strains were not

Table 8
Data summary from molecular screening for pathogenic eubacteria detected in ballast tanks[a]

Taxon	Occurrence
Aeromonas sp. (2 tanks)	9-1, 19-1
Escherichia coli (23 tanks)	2-*P*1b, 4-*P*1a, 6-*P*1a, 6-*P*1b, 6-*P*2b, 7-*P*1a, 7-*P*1b, 9-*P*1a, 9-*P*1b,11-1, 13-1, 15-1, 16-1, 17-*P*1b, 19-1, 22-*P*1a, 22-*P*1b, 24-*P*1a, 24-*P*1b, 24-2, 25-1, 27-*P*1a, 28-*P*1a
Listeria monocytogenes (1 tank)	3-*P*1b
Mycobacterium spp. (2 tanks)	5-1, 16-2
Pseudomonas aeruginosa (4 tanks)	19-1, 20-1, 21-1, 22-*P*1a

[a] All blanks were negative for these taxa; data not available for water from ballast tanks 12-*P*1a,b and 26-1. Note that *Pseudomonas putida* was also detected from tank 10-*P*1b using other techniques (see Table 9).

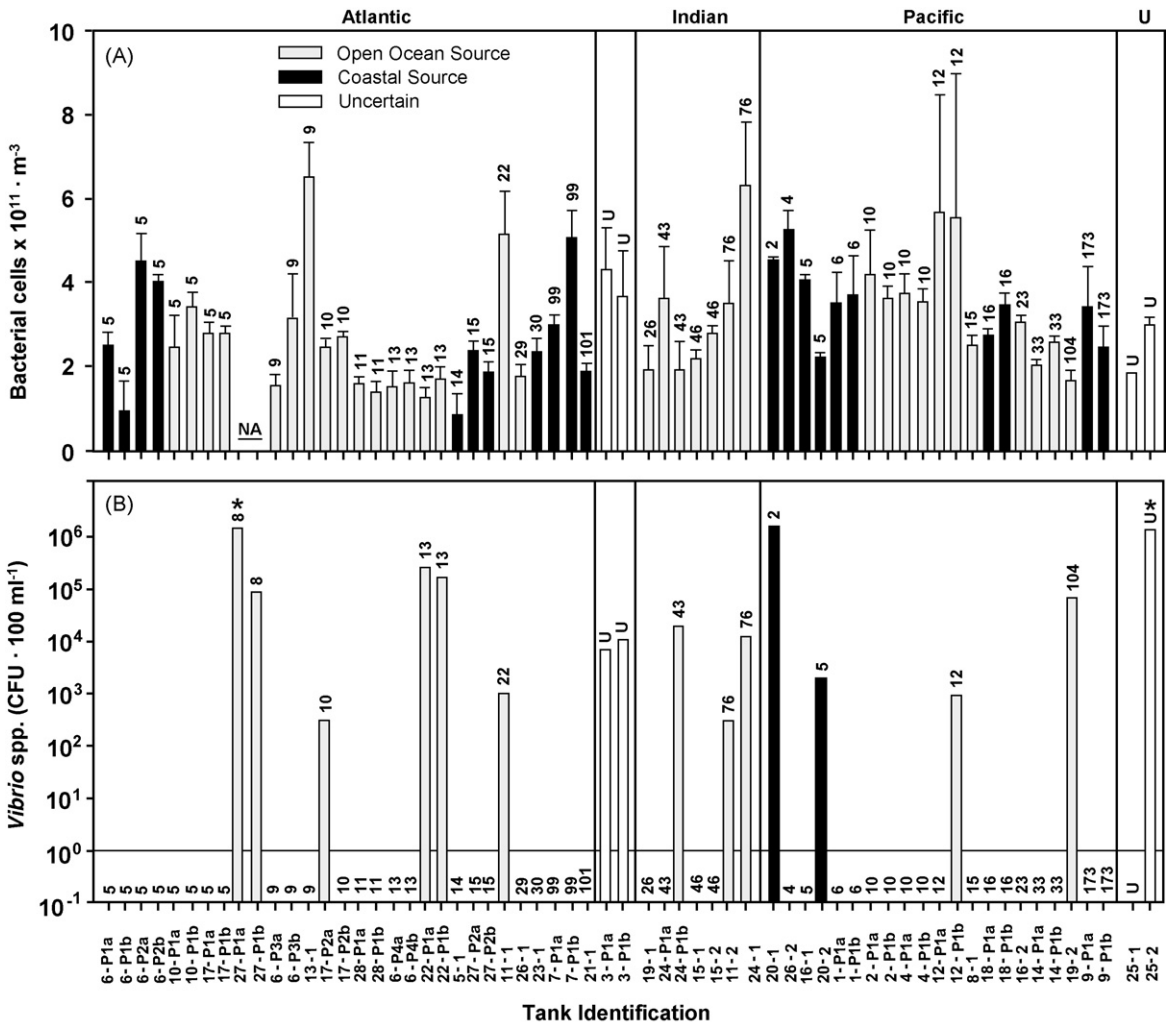

Fig. 12. Bacterial abundance, with data given as in Fig. 7. (A) Total bacterial abundance by major source region, age, and status (coastal, open-ocean, uncertain). The presence of copious flocculent materials prevented analysis of the water from tanks 27-*P*1a,1b. (B) Abundance of *Vibrio* spp. in each ballast tank by source region, age, and status (coastal, open-ocean, uncertain). The data were obtained using TCBS plating, which is selective for *Vibrio* but does not permit speciation. Numbers indicate the age of the water based upon the most recent ballasting activity; the asterisk (*) indicates a sample for which *Vibrio* spp. were too numerous to count. The line (at 1 CFU 100 ml^{-1}) indicates the regulatory treatment standards threshold of the IMO Convention (2004) and the U.S. Ballast Water Management Act (2005; see United States Senate 2005) for *Vibrio cholerae*.

detected in any tanks. Of the 10 other pathogenic eubacterial taxa screened for using species-specific assays, *Escherichia coli* (found in 23 tanks, including tanks with both coastal and open-ocean source waters) and *Pseudomonas aeruginosa* (found in 4 tanks) were most commonly detected (Table 8); 2 tanks also were positive for *Aeromonas* sp. or *Mycobacterium* sp., and 1 tank was positive for *Listeria monocytogenes*.

A total of 387 prokaryote clones representing 36 eubacteria, 6 cyanobacteria, and 5 other taxa were generated from 9 different ballast water tanks (Table 9). Approximately, 30% (118) of those clones had a sequence similarity score of ≥0.900 with a sequence in the Ribosomal Database Project (RDP) database. Forty-seven different RDP sequences were represented by ≥1 ballast clone (Table 9). Twenty-four clones had a sequence similarity of 1.000 with a sequence available on RDP (1 with *Pseudomonas putida* [AB029257], which is common in putrefaction and can cause septicemia and septic arthritis in immunocompromised patients—Anaissie et al., 1987; Macfarlane et al., 1991); 1 with an uncultured bacterium [AY186077] associated with ferromanganese deposits—Northup et al., 2003; 3 from an unidentified bacterial isolate [AY227855, characterized by BioMerieux, France—Drancourt et al., 2000]; and 19 (13 from vessel 24, 6

Table 9
Prokaryote clones isolated from the ballast water tanks, indicating the taxon, GenBank accession number, vessel and tank number, and the sequence similarity score with a sequence in the RDP database

Taxon	GenBank Accession No.	2-*P*1b	4-*P*1a	4-*P*1b	6-*P*3a	5-1	10-*P*1a	10-*P*1b	16-2	27-1
Eubacteria										
Acinetobacter schindleri	AJ275041	–	–	–	–	–	–	0.970	–	–
Acinetobacter schindleri	AJ275040	–	–	–	–	–	–	0.989	–	–
Afipia genosp.	U87782	0.937 (3)	–	–	–	–	–	–	–	–
Alpha proteobacterium	AB106120	–	–	–	–	0.903	–	–	–	–
Bacterium	AY345413	–	0.910	0.920/0.932	0.914	–	–	–	–	–
Bacterium	AF227855	–	–	–	–	–	1.000 (3)	–	–	–
Bacterium	AY345457	–	–	–	–	–	–	0.988	–	–
Dibenzofuran-degrading bacterium	AB086228	–	–	–	–	–	0.982–0.998 (13)/1.000 (13)	0.979–0.993 (6)/1.000 (6)	–	–
Erythrobacter sp.	AJ391206	–	–	–	–	–	–	–	0.974	–
Granulicatella elegans	AY15413	–	–	–	–	–	–	0.985	–	–
Halomonas meridiana	AJ306891	–	–	–	–	–	–	0.997	–	–
Halomonas venusta	AJ306894	–	–	–	–	–	0.975/0.985 (2)	–	–	–
Iodide-oxidizing bacterium	AB159201	–	–	–	–	0.913	–	–	–	–
Methylocella tundrae	AJ555244	–	0.957	–	–	–	–	–	–	–
Pseudomonas putida	AB029257	–	–	–	–	–	–	1.000	–	–
Psychrobacter marincola	AJ309941	0.998 (2)	–	–	–	–	–	–	–	–
Roseobacter sp.	DQ120728	–	–	–	–	–	0.945–0.948 (3)	0.919	–	–
Streptococcus sanguinis	AY691542	0.980	–	–	–	–	–	–	–	–
Uncultured alpha proteobacterium	AF473921	–	0.901–0.902 (3)	0.909–0.912 (5)	0.900 (2)	–	–	–	–	–
Uncultured alpha proteobacterium	DQ432334	–	–	0.978	–	–	–	–	–	–
Uncultured alpha proteobacterium	DQ351770	–	–	–	–	0.909	–	–	–	–
Uncultured alpha proteobacterium	AF432337	–	–	–	–	0.981	–	–	–	–
Uncultured bacterium	AY186077	1.000	–	–	–	–	–	–	–	–
Uncultured bacterium	DQ015800	–	0.942	0.942 (2)	–	–	–	–	–	–
Uncultured bacterium	AM176849	–	–	–	–	–	0.903/0.913	–	–	–
Uncultured bacterium	AF382128	–	–	–	–	–	0.990	–	–	–
Uncultured bacterium	DQ015815	–	–	–	–	–	0.929 (2)	–	–	–
Uncultured bacterium	AF382123	–	–	–	–	–	–	0.938	–	–
Uncultured bacterium	AY945873	–	–	–	–	–	–	0.829	–	–
Uncultured gamma proteobacterium	AJ40921	–	–	–	–	–	0.972 (2)	–	–	–
Uncultured gamma proteobacterium	AB002655	–	–	–	–	–	–	0.988/0.998	–	–
Uncultured gamma proteobacterium	DQ234113	–	–	–	–	–	–	0.941 (4)	–	–
Uncultured *Halomonas* sp.	AY687476	–	–	–	–	–	–	–	0.997	0.997
Uncultured marine bacterium	AJ400342	–	0.979 (2)	–	–	–	–	–	–	–
Uncultured marine bacterium	DQ071157	–	–	–	–	–	0.981 (2)/0.988 (4)	–	–	–
Uncultured proteobacterium	DQ330951	–	–	0.909	–	–	–	–	–	–

Table 9 (*Continued*)

Taxon	GenBank Accession No.	2-*P*1b	4-*P*1a	4-*P*1b	6-*P*3a	5-1	10-*P*1a	10-*P*1b	16-2	27-1
Cyanobacteria										
Synechococcus sp.	AY172801	–	0.990	–	0.990	–	–	–	–	–
Synechococcus sp.	AY172804	–	–	0.978/0.981	–	–	–	–	–	–
Synechococcus sp.	AF539812	–	–	0.990	–	–	0.969	–	–	–
Synechococcus sp.	AY172834	–	–	0.945	–	–	–	–	–	–
Uncultured *Synechococcus* sp.	AF245618	–	–	0.964	–	–	–	–	–	–
Uncultured *Synechococcus* sp.	Y664232	–	–	–	–	–	–	0.966	–	–
Other										
"Formosa alga"	AY771766	–	–	–	–	–	–	–	–	0.906
Uncultured organism	DQ396137	–	–	–	–	–	–	–	0.930 (2)	0.930
Uncultured organism	DQ395499	–	–	–	–	–	–	0.947	–	–
Uncultured organism	DQ396344	–	–	–	–	–	–	0.990	–	–
Uncultured organism	DQ395758	–	–	–	–	–	–	–	0.990	–

from vessel 25) with a dibenzofuran-degrading bacterium [AB086228; Fuse et al., 2003]).

ANOVA indicated that bacterial density differed by source region (significantly lower in tanks containing Atlantic than Pacific Ocean waters; $p = 0.01$), but was unrelated to vessel type, service group (MSC or MARAD), exchange status, location, or season. There also were no statistically significant correlations between log-transformed bacterial densities or *Vibrio* spp. densities and minimum ballast water age or physical/chemical variables. In PCA, the first two principal components explained only 41% of the total variability; the dominant Eigen vectors were TN for PC1 and bacterial density and minimum ballast water age for PC2.

Similarity matrix analyses (rank-sum procedures, Kendall, Spearman, and weighted Spearman) revealed no comparable patterns among the phytoplankton and bacterial assemblages. The low correlations ($r \leq 0.197$) confirmed the lack of pattern match considering all tanks, tank subgroupings, seasonality, and other factors.

3.5. Variance in phytoplankton and bacterial abundances within versus among ships

Considering all ships and tanks, variance across tanks within ships was the dominant component (67–85% of the total) (Table 10). For ships with paired tanks of similar ballasting history, the largest source of variation was among ships. Variation in bacterial abundance between paired tanks was much lower than variation in abundance among ships; variation in phytoplankton abundance between paired tanks was about two-thirds of that among ships. Duplicate samples contributed little to the variation in the data. For ships with unpaired tanks of differing histories (both data subsets 1 and 2), the largest source of variation in biota abundances was between or among ballast tanks within ships.

4. Discussion

In this study, no obvious relationships were detected between measured environmental variables and phytoplankton or bacterial abundances in ballast waters of U.S. military vessels, except for positive relationships between nitrate and phytoplankton (both viable and total), diatom (total), or centric diatoms (both viable and total). Abundance of centric diatoms. Moreover, there were no similarities in abundance patterns among phytoplankton and bacterial assemblages. A companion study by Holm et al. (2005) reported sparse macro-

Table 10
Restricted maximum likelihood variance components for phytoplankton and bacterial abundance, expressed as proportions of the overall variance within and across ships, for all tanks (complete data set) and for tank subsets 1–3

Variance component	Complete data set	Paired subset 1[a]	Unpaired subset 1[b]	Unpaired subset 2[c]
Phytoplankton				
Across ships	0.33	0.57	0.53	0.28
Within ships	0.67	0.43	0.47	0.72
Bacteria				
Across ships	0.15	0.90	0.08	0.04
Within ships	0.85	0.10	0.92	0.96

Note that the error term for each of the four data set analyses was a very small proportion of the overall variance (<0.01).
[a] Paired subset—subset of ships for which only paired tanks (same ballasting history) were sampled (voyages 1, 2, 3, 4, 10, 12, 14, 15, 18, 22, 28).
[b] Unpaired subset 1—subset of ships for which more than a single set of paired tanks was sampled (voyages 6, 11, 17, 24, 27).
[c] Unpaired subset 2—subset of ships for which multiple pairs or a single set of unpaired tanks was sampled (voyages 6, 11, 16, 17, 20, 24, 26, 27).

zooplankton in the ballast tanks, suggesting that zooplankton grazing pressure was low. Environmental conditions in the ballast tanks were comparable: tanks were well mixed, with little evidence of depth stratification in most, adequate dissolved oxygen for animal biota, negligible turbidity, and low to moderate nutrient concentrations. The phytoplankton assemblages were both highly variable and generally low in abundance, with centric diatoms as the dominant group. These data support findings from the earlier study by Ruiz et al. (1999a,b) of ballast water from U.S. military ships, and of studies of commercial vessels. As more general observations, Forbes and Hallegraeff (1998) noted that viable diatom cells are common in ballast waters of commercial cargo ships, and various diatom species can survive extended periods in low light or darkness (e.g. Smayda and Mitchell-Innes, 1974; Chan, 1980; Lewis et al., 1999). Bacterial abundance, in contrast, was remarkably similar among the ballast tanks samples, and considerably higher ($\sim 10^{11}$ cells m^{-3}) than in the tanks studied by Ruiz et al. (1999a,b; $\sim 10^{6}$ cells m^{-3}).

Potentially harmful phytoplankton taxa were well represented in this study; 23 potentially harmful species were detected from the ballast tanks overall (1 cyanobacterium—*Jaaginema geminatum*; 5 diatoms—*Chaetoceros concavicornis*, *Chaetoceros socialis*, *Leptocylindrus minimus*, *Pseudo-nitzschia fraudulenta*, *Pseudo-nitzschia seriata*; 15 dinoflagellates—*Akashiwo sanguineum*, *Ceratium tripos*, *Dinophysis acuminata*, *Dinophysis caudata*, *Gambierdiscus toxicus*, *Heterocapsa triquetra*, *Karlodinium australe*, *Karlodinium veneficum*, *Lingulodinium polyedrum*, *Peridinium aciculiferum*, *Phalacroma rotundatum*, *Prorocentrum minimum*, *Protoperidinium depressum*, *Protoperidinium pellucidum*, *Scrippsiella trochoidea*; 1 silicoflagellate—*Dictyocha speculum*; 1 raphidophyte—*Heterosigma akashiwo*); and about one-third (8) of these harmful taxa were culturable. Several taxa of potentially pathogenic bacteria were also detected. Notably, toxic strains of *Vibrio cholerae* were not detected in any tanks of these DoD vessels. In a more general analysis that included non toxic as well as toxic strains, Ruiz et al. (1999a) reported various densities of *V. cholerae* serotypes in the ballast waters of commercial vessels. Several other potentially pathogenic bacteria (*Listeria monocytogenes*, *Escherichia coli*, *Mycobacterium* spp., *Pseudomonas aeruginosa*, *Pseudomonas putida*) were also detected. While most were found in only a few tanks (1–4, ≤6%), *E. coli* occurred in 23 (37%) tanks.

In numerous studies molecular methods (e.g. cloning and sequencing) have been used to assess genetic diversity of bacterial species, since it has been recognized that <1% of bacterial species are culturable (Amann et al., 1995). Only recently has this approach been applied for exploring the diversity of eukaryotic species in various environments (e.g. Diez et al., 2001; Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001). Additional studies have begun to identify novel sequences belonging to novel lineages (Dawson and Pace, 2002; Stoeck and Epstein, 2003). Although this study targets a small segment of the ribosomal 18S from only one class of dinoflagellates (Dinophyceae), to our knowledge it is the first report using cloning and sequencing methods to assess eukaryotic diversity in ballast water samples. Previous work on diversity in ballast samples was based upon identification of cyst assemblages or phytoplankton counts via microscopy (e.g. Hallegraeff and Bolch, 1992; Hamer et al., 2000)

Several caveats should be mentioned, however, regarding data analysis from cloning and sequencing efforts on environmental samples (Polz and Cavanaugh, 1998; Amann, 2000). Mis-incorporations of nucleotides can occur during PCR and lead to incorrect sequences, but proofreading enzymes, which were used in this

study, reduce this possibility. Formation of chimeric molecules during PCR can also affect the outcome of data interpretation (Wang and Wang, 1997; Wintzingerode et al., 1997). Another bias is the inherent nature of environmental samples to contain a heterogeneous pool of organisms, which results in an undetermined number of different DNA templates, all of which can compete for primers and other reagents during PCR (Suzuki and Giovannoni, 1996). It has also been demonstrated that DNA targets that are abundant can be preferentially amplified, consequently suppressing amplification of less abundant targets (e.g. Suzuki and Giovannoni, 1996; Chandler et al., 1997; Becker et al., 2000). Differences in copy number of the target in the genome (Farrelly et al., 1995; review in Wintzingerode et al., 1997), genetic heterogeneity demonstrated in bacteria species (Wang et al., 1997), and even the composition of the DNA flanking the target region (Hansen et al., 1998) can all affect results, thereby skewing conclusions of species abundance. Finally, limited data are available in the GenBank and RDP databases, which must be taken into consideration for data interpretation. As more sequence data become available, repeating BLAST and RDP SeqMatch searches may link ''novel'' sequences to characterized organisms. Regardless of these pitfalls and biases, molecular methods for determining population diversity in environmental samples have increasingly become attractive to overcome the limitations of identifications based on microscopy and culture methods (in the case of bacteria).

Although comparative data are not available for the number of DoD ships versus commercial vessels arriving in U.S. ports, a previous estimate for Chesapeake Bay suggests that DoD ships are comparatively few: Ruiz et al. (1999b) estimated that naval vessels transported ~750,000 tonnes of ballast water to the Bay, representing less than 1.2% of the volume of ballast water discharged by commercial vessels. This research provides further insights about how present ballasting practices of DoD ships may influence transfer of phytoplankton species. Abundance and diversity can indicate risk for undesirable species introductions (McCarthy and Crowder, 2000). Phytoplankton species richness in the ballast tanks examined here was moderate, with 100 species identified and harmful taxa well represented, and species diversity was low to moderate. The generally low abundance and low/moderate diversity of the phytoplankton assemblages from ballast waters of these DoD ships suggest that present DoD ballasting practices do not present a high risk for undesirable phytoplankton species introductions. Additional insights would be gained from analysis of sediments that can accumulate at the bottom of ballast tanks (Hamer et al., 2000, 2001). Previous research on ballast waters of U.S. military ships indicated that DoD ships were well managed in comparison to commercial ships in minimizing the risk for introduction of harmful microbiota (Ruiz et al., 1999a,b). We found that a high percentage (94%) of the tanks examined in this study had adequate records to determine the source locales and age of the ballast water. Moreover, ~90% of the tanks had undergone some form of ballast exchange, and contained at least a portion of ballast water from an open-ocean source by the time ships entered a U.S. port. For ~35% of the tanks, however, the extent of exchange could not be determined from available records; thus, DoD ships should be encouraged to maintain detailed records of ballasting activities as standard operating procedure. Phytoplankton abundance was higher in tanks with coastal water sources than in tanks with open-ocean water, as expected (e.g. Locke et al., 1991; McCarthy and Crowder, 2000), likely reflecting the generally higher phytoplankton abundance in coastal waters (Raymont, 1980). Thus, ballast water exchange by DoD ships should be conducted as far from shore as possible, supporting previous recommendations by Ruiz et al. (1999a) and Holm et al. (2005).

Despite a general pattern of reduction in phytoplankton abundance with ballast water age, regression analyses indicated only a weak (although significant) negative relationship between tank water age and abundance. Previous researchers (Drake et al., 2002) have reported a more striking, exponential decline in phytoplankton biomass as chlorophyll *a* over time in darkened ballast tanks. Other important influences on phytoplankton abundance may include spatial/temporal variation during ballasting, ballast water management practices, and environmental stress factors among ballast tanks and ships. The DoD ships included in this study used different ballast tanks depending on operational requirements, resulting in ballast tanks with different histories. The data suggest that metrics such as phytoplankton abundance in ballast water at the scale of a ship cannot be estimated from single tanks; the number of tanks sampled should encompass the magnitude of variation in tank histories. Although PCA indicated strong similarity between paired tanks in phytoplankton abundance and controlling factors, species richness sometimes differed markedly between paired tanks. In a companion study of macrozooplankton from the same ballast tanks, Holm et al. (2005) reported that mean abundances were not significantly

correlated between paired tanks. Thus, significant differences in biota abundances even in paired tanks may arise, likely related to environmental factors that can diverge in one tank versus another over time. Evaluation of phytoplankton abundance within versus among ships showed that for ships with tanks of similar ballasting history, the largest source of variation was *among ships*. In contrast, for ships with tanks of differing ballasting histories, and for all ships/tanks considered collectively, the largest source of variation in biota abundances was between/among ballast tanks *within ships*.

Comparison of size class data for viable phytoplankton with proposed IMO (2004, Annex, Regulation D2) ballast discharge standards indicated that all of the ballast tanks sampled on the DoD vessels would have been in compliance for the small size category (organism maximum dimension, 10–50 μm; Table 11). However, in the larger (>50 μm) size category, viable phytoplankton in 47% of the ballast tanks exceeded the proposed standards. As Doblin and Dobbs (2006) noted, ballast tanks containing mostly small cysts (<50 μm) would have been in compliance with the IMO performance standard, whereas tanks with larger cysts could require sometimes-multiple log reductions in abundance to meet the performance standard. Both Senate bill S363 (U.S. Senate, 2005) and the IMO (2004) convention would exempt military vessels, but would require these vessels to manage ballast water so as to be consistent with the proposed requirements. Thus, development, evaluation, and adoption of treatment technologies or alternative management strategies, along with open-ocean ballast water exchange, will be necessary to enable DoD vessels to comply with proposed standards, if adopted, for ensuring safe and environmentally sound operations.

For example, previous research (Hallegraeff, 1998) suggests that a temperature of 34 °C can be attained in ballast tanks, at least for some ship designs, by using waste engine heat. Disadvantages of heat treatment may include the elevated corrosivity of warm seawater, the hull stress related to multiple heating and cooling cycles, and the potential limitation of this management practice to warmer geographic regions. However, if an effective stand-alone or combination treatment system could be made feasible that includes waste heat, its environmental and economic benefits may outweigh added vessel maintenance costs (Rigby et al., 1999). Other techniques such as UV treatment, ozonation or filtration (50 μm pore size) could also substantially decrease phytoplankton numbers and viability, if feasible at the scale of ballast tank volumes (NRC, 1996).

Table 11
Proposed regulatory discharge limits for viable phytoplankton, in comparison to the data from this study

Size range	Regulation*	Viable phytoplankton exceedences
IMO (2004)		
10–50 μm	10^7 organisms m^{-3}	0 tanks
>50 μm	10^1 organisms m^{-3}	29 tanks (47%)
U.S. Senate (2005)		
10–50 μm	10^5 organisms m^{-3}	4 tanks (6%)
>50 μm	10^{-1} organism m^{-3}	29 tanks (47%)

Data are given as number (percentage) of ballast tanks.

Although many of the observed aquatic microorganisms are considered to have widespread distribution, ballast water discharge can alter the abundances of harmful species and set up conditions where previously rare populations proliferate (e.g. Rigby and Hallegraeff, 1996; Forbes and Hallegraeff, 1998; Hallegraeff, 1998). Once introduced, many site-specific factors acting in concert—for example, climatic conditions, season, light regime, the available suite of nutrient supplies, the presence of potential predators, mixing characteristics, water column depth, bottom sediment characteristics, and the presence and abundance of potential competitor microbiota—will control whether a given harmful species can successfully establish and thrive in an area where it is introduced (e.g. Smith et al., 1999). The dogma of phytoplankton cosmopolitanism has led to a false complacency about the potential for continued risks from ballast water introductions of these microorganisms. The new strains, if more toxic or physiologically robust (e.g. Burkholder and Glibert, 2006), could be more harmful or could hybridize with native populations, leading to unexpected consequences such as increased hybrid vigor.

Additional research to characterize the role of DoD ships in ballast water transport of phytoplankton species would be instructive in several areas. Amphibious vessels of the U.S. Navy were not available for this study. These vessels carry a substantial proportion of the ballast water transported by DoD vessels that are regulated under the Uniform National Discharge Standards program (see U.S. EPA, 1999). Different types of vessels and their ballast tanks can strongly influence the transport of viable harmful algae and other microbiota (e.g. Dickman and Zhang, 1999); thus, characterization of the phytoplankton assemblages in ballast tanks on naval amphibious vessels is recommended when operations allow. This study indicates that operations of DoD vessels may present a low risk

for transfer of pathogenic bacteria such as toxic strains of *V. cholerae*. Additional research encompassing a broader suite of microbial pathogens (bacteria, viruses, protozoans) would strengthen insights about the potential for transport of pathogenic microbes. Research is also needed to assess the importance of DoD ship-fouling for introducing harmful microorganisms, and to examine the effects of ballast water exchange by DoD ships on coastal phytoplankton, when ships holding coastal water that has not been exchanged become available for comparison with tanks containing exchanged water. As previously mentioned, future work should include assessment, as well, of the role of ballast tank sediments, known to include encysted and other resistant stages of phytoplankton species such as certain harmful dinoflagellates (e.g. Hallegraeff and Bolch, 1992; Hamer et al., 2000, 2001), and exterior ship fouling (e.g. Holm et al., 2000, 2003) for transport of undesirable phytoplankton species.

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