Microbial ecology of ballast water during a transoceanic voyage and the effects of open-ocean exchange

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ABSTRACT: The only procedure used frequently to reduce the risk of invasion by ballast-mediated biota is open-ocean exchange of ballast water, a procedure in which vessels release coastal water and replace it with oceanic water. Limited information exists concerning the effects of transport upon the aquatic microbial community throughout transit and following open-ocean exchange. A transoceanic voyage aboard a commercial bulk carrier afforded us the opportunity to sample the microbial community in exchanged and unexchanged ballast-water holds during the journey from Hadera, Israel to Baltimore, USA. Five days following the exchange process, all microbial metrics tested (i.e. bacteria concentration, virus-like particle density, chl a and phaeopigment concentration, and microbial biomass) had decreased 1.6- to 34-fold from initial values. With respect to microbial measures, no significant differences existed between exchanged and unexchanged holds on Day 15, the final day of sampling. We stress that we quantified differences in total microorganism abundance and biomass, not species composition, and more research is necessary to determine the changes that nonindigenous microorganisms, including potential pathogens, may effect in receiving waters.

KEY WORDS: Bacteria · Invasive species · Management · Non-indigenous species · Non-native species · Policy · Virus

INTRODUCTION

Ships carry ballast water for vessel stability and trim, and it is well established that ballast water can contain a diversity of metazoan (e.g. Medcof 1975, Carlton 1985, Williams et al. 1988, Carlton & Geller 1993, Chu et al. 1997, Gollasch et al. 1998, Lavoie et al. 1999) and protozoan taxa (e.g. Galil & Hülsmann 1997, Pierce et al. 1997), as well as other microorganisms (e.g. Subba Rao et al. 1994, Gollasch et al. 1998, McCarthy & Crowder 2000, Ruiz et al. 2000, Drake et al. 2001) and their resting stages (e.g. Hallegraeff & Bolch 1991, 1992, Macdonald 1998, Hamer et al. 2000). Some of these forms are pathogenic (e.g. McCarthy et al. 1992, McCarthy & Khambaty 1994, Gosselin et al. 1995, Hallegraeff 1998, Ruiz et al. 2000). Several highly visible (and oft-cited) instances of likely ballast-water introductions, including the zebra mussel's arrival to the Great Lakes (e.g. Hebert et al. 1989, 1991, Griffiths et al. 1991) and the introduction of toxic dinoflagellates to Australian waters (Hallegraeff et al. 1988, Hallegraeff & Bolch 1991) have brought to bear international

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attention on ballast-mediated introductions (International Maritime Organization 1991).

Microorganisms possess a great capacity to invade new environments. Bacteria and viruses, for example, are numerically dominant in seawater (e.g. Ducklow & Shiah 1993) and occur typically in densities of 10^6 to 10^{11} l^{-1} (Ducklow & Shiah 1993, Wommack & Colwell 2000), many times greater than the abundance of macroorganisms. Further, many microorganisms employ survival strategies, such as the formation of cysts (e.g. Hallegraeff & Bolch 1991, 1992) and the ability to undergo morphological changes (Wai et al. 1999), that enable them to withstand prolonged periods of inhospitable conditions, such as confinement in darkened ballast-water tanks. The sheer number of microorganisms in coastal waters, combined with the great volumes of ballast water delivered annually to ports—an estimated 79 million tonnes to the USA alone (Carlton et al. 1995)—suggest that inoculation of coastal waters by microorganisms proceeds globally on a staggering scale. In support of that hypothesis, Ruiz et al. (2000) quantified microorganisms in ballast water of vessels arriving to Chesapeake Bay from foreign ports and reported mean abundances of 8.3×10^8 bacteria and 7.4×10^9 virus-like particles l^{-1}.

The only method now in widespread use to reduce the spread of nonindigenous species via ballast-water discharge is open-ocean ballast-water exchange (Federal Register 1999, Australian Quarantine and Inspection Service 2001). In this procedure, coastal water is removed from ballast-water tanks and replaced with oceanic water. Open-ocean exchange theoretically reduces the threat of invasion by jettisoning nonindigenous propagules into the open ocean, where they will presumably not survive. Subsequently, the survival of organisms in exchanged, oceanic ballast water may be low due to temperature or salinity mismatch between the oceanic ballast water and the coastal water into which it is discharged (Smith et al. 1999).

The purpose of this work was 2-fold. First, we quantified changes in the marine microbial community during sequestration in ballast-water tanks throughout a transoceanic voyage. Second, we evaluated differences in the microbial communities in exchanged and unexchanged ballast water.

MATERIALS AND METHODS

Motor Bulk Carrier 'Hadera'. MBC 'Hadera' is 290 m long, with a gross tonnage of 93 052. In July/August 1999, we rode the vessel from Hadera, Israel to Baltimore USA on a 19 d voyage. Of the 9 cargo holds on 'Hadera', 4 are configured to carry ballast water, each with a capacity of 21 560 to 21 780 t.
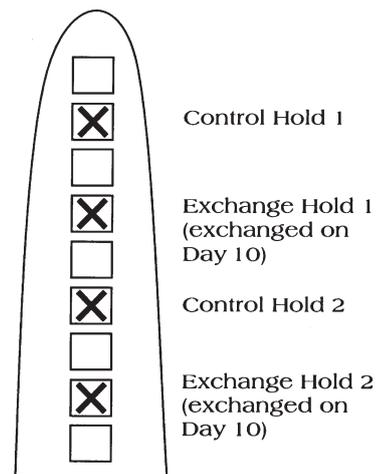

Fig. 1. Summary of the experimental design expressed in a plan view of the cargo holds on MBC 'Hadera'

Two were designated as 'exchange' and were subsequently exchanged in the open ocean; 2 were designated as 'control' and were not exchanged in the open ocean during the course of sampling (Fig. 1). The 4 holds were filled with ballast water at the berth in Israel: the 2 control holds were filled 1 to 2 d before sailing; the 2 exchange holds were filled 2 to 3 d before sailing. Three days prior to sailing, we added flourescent red FWT50 dye (Forestry Suppliers, Jackson, MS) to Exchange Hold 1 to measure the efficacy of exchange (dye data are not presented in this paper). Although holds were filled on different days and dye was added to Exchange Hold 1, there were no significant differences between control and exchange holds in any of the microbial measures on Day 0 of sampling (Table 1). On Day 10 of the voyage, the ballast water in both exchange holds was replaced in the North Atlantic Ocean by overflowing the holds with oceanic water for 13 h (theoretically, a 149% exchange), ending at 35° 57' N, 030° 30' W.

Because it took an entire day to collect and process samples from 2 holds, we sampled Control Hold 1 and Exchange Hold 1 at each of the following time points throughout the voyage: Days 0, 6, 12, 15 (for all metrics, with additional sampling on Days 3 and 9 for pigment analyses and hydrographic properties). Samples for Control Hold 2 and Exchange Hold 2 were collected 1 d after the samples from Control Hold 1 and Exchange Hold 1 were collected. For clarity of presentation in this paper, data from replicate holds were considered as if they were collected on the same day.

Sample collection. A Niskin bottle was cleaned in bleach and rinsed with tap water, then lowered through a vent on top of the hold cover to collect water samples. Two or 3 samples were taken from the surface

and 10 m below the water surface from each hold at each time point. Samples were transferred to bottles that had been surface sterilized with ethyl alcohol, rinsed with tap and deionized water, then rinsed twice with ballast water immediately before collection. Sample bottles were protected from light and transported to the shipboard laboratory.

Bacteria enumeration. Samples were fixed in formaldehyde solution (final concentration 2.7%) and stored in the dark at 4°C until they were enumerated via flow cytometry. Analyses were done using a Becton Dickinson FACScan flow cytometer equipped with a 15 mW, 488 nm, air-cooled Argon ion laser. Simultaneous measurements of forward light scatter, 90 degree light scatter, and green fluorescence were made on all samples. PicoGreen (Molecular Probes, Eugene, OR), a DNA-specific probe that emits in the green wavelengths when excited with 488 nm light, was used to detect and enumerate bacteria. Detectors (photomultiplier tubes) were in log mode and signal peak integrals were measured. The volume of sample analyzed by the FACScan was determined gravimetrically using an A-160 electronic balance (Denver Instrument, Arvada, CO) whereby each sample was weighed prior to analysis and immediately after the analysis was terminated. All samples were run at a low flow rate setting (approximately 20 μl min^{-1}).

Virus-like particle enumeration. Virus-like particles (VLPs) were counted using the method of Hennes & Suttle (1995). Upon return to the ship's laboratory, unfixed, duplicate samples were diluted with 0.1 μm filtered distilled, deionized water. Next, diluted samples were filtered onto 0.02 μm pore size Anodisc filters (Whatman International, Maidstone, UK) and stained in the dark for 2 d at room temperature with a cyanide-based working solution of the nucleic acid stain Yo-Pro^{TM}-1 iodide (491/509) (Quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-,diiodide) (Molecular Probes). Filters were rinsed twice with 0.02 μm filtered distilled, deionized water, placed on microscope slides, and stored in the dark at –20°C on board the ship. Upon return to the laboratory, slides were stored in the dark at –85°C until the VLPs were counted. Filters were randomly chosen (in groups of 2), thawed in the dark at room temperature for ca. 5 min, and VLPs were counted using an Olympus BX50 System Microscope with an Olympus BX-FLA epifluorescence attachment. For each set of filters prepared, 2 control filters were prepared using only 0.02 μm filtered distilled, deionized water and their average VLP count was subtracted from values determined in field samples.

Microbial biomass. Under a vacuum pressure of 100 mm Hg, 900 ml of seawater was filtered onto 47 mm diameter glass fiber filters (GF/F Whatman). Filters were wrapped in foil and stored in liquid nitrogen on the ship; upon return to the laboratory, filters were stored at –85°C until they were placed into a modified (White et al. 1979) Bligh & Dyer (1959) solution (methanol-chloroform-buffer) to extract lipids. From an aliquot of the extracted bulk lipid, microbial biomass was determined by oxidizing the phosphorus-containing cell-membrane lipids, thus releasing inorganic phosphate, then performing an inorganic phosphate determination (Dobbs & Findlay 1993). Finally, phosphate concentrations were converted to carbon equivalents assuming 100 μmol P gC^{-1} (Dobbs & Findlay 1993).

Chlorophyll *a* and phaeopigment determination. Chl *a* samples were collected by filtering 500 ml of seawater onto 47 mm diameter glass fiber filters (GF/F Whatman) at a vacuum pressure of 100 mm Hg. Filters were wrapped in foil and stored in liquid nitrogen for the duration of the voyage; upon return to the laboratory, filters were stored at –85°C until the chl *a* on the filters was extracted in acetone and measured fluorometrically (Parsons et al. 1992). Phaeopigment concentration (phaeophytin and phaeophorbide) was quantified by acidifying the chl *a* sam-

Table 1. Changes in microbial metrics throughout the voyage. For each treatment, n = 2 replicates with 3 to 6 subsamples per replicate. All comparisons are 2-level, mixed model, nested ANOVAs on ranked data. Significant differences (p-values < 0.05) are indicated in bold type, and the relationship between the groups compared is presented in subscripted text. Column labels: Bacteria = abundance of bacteria; VLP = abundance of virus-like particles; Chl *a* = concentration of chl *a*; Phaeo = concentration of phaeopigments; Biomass = microbial biomass. Comparisons between values on Days 9 and 12 were possible only for pigment data, as samples for other metrics were not collected on Day 9

	Bacteria	VLP	p-values Chl *a*	Phaeo	Biomass
Day 0					
Control vs exchange holds	0.669	0.705	0.370	0.830	0.356
Day 15					
Control vs exchange holds	0.264	0.676	0.114	0.161	0.665
Control holds					
Day 0 vs 15	**0.013**$_{0>15}$	**0.046**$_{0>15}$	**<0.001**$_{0>15}$	**0.002**$_{0>15}$	0.062$_{0>15}$
Exchange holds					
Day 0 vs 15	0.355	**0.011**$_{0>15}$	**0.008**$_{0>15}$	**0.009**$_{0>15}$	**0.037**$_{0>15}$
Day 6 vs 12	0.355	**0.033**$_{6>12}$	0.135	0.066$_{6>12}$	0.706
Day 9 vs 12	–	–	0.424	0.222	–

ples with 5% hydrochloric acid and again determining the sample's fluorescence.

Hydrographic data. Temperature and dissolved oxygen (percent saturation) were measured using a YSI Model 58 Dissolved Oxygen Meter (YSI, Yellow Springs, OH) at the following depths within each hold: 0, 1, 2, 5, 10 and 23 m (bottom). Salinity of samples collected from 0 and 10 m depth was measured using a hand-held refractometer (ATAGO Model S-28E).

Data analysis. All statistics were calculated using SPSS Base 9.0.0 (SPSS, Chicago, IL). Each hold was designated a sampling unit. From each hold at each sampling time point, 4 to 6 subsamples were collected, i.e. 2 or 3 from the surface, and 2 or 3 from bottom. Preliminary analyses showed it was reasonable to pool surface and bottom samples collected within holds; among 16 comparisons of surface versus bottom samples of bacteria and VLP abundances, no significant differences emerged.

Data were analyzed as 2-level, mixed-model nested ANOVAs on rank-transformed data, with n = 2 (i.e. 2 control holds and 2 exchange holds) and 2 to 6 subsamples per replicate hold. We did not treat the subsamples as independent samples, that is, as deliberate pseudoreplicates, but we instead treated them as subsamples to estimate the mean of a given metric within a specific hold.

RESULTS

Hydrographic characteristics

Temperature in the ballast water ranged from 26.2 to 29.4°C (Fig. 2). In control holds, mean temperature decreased from 29.3°C on Day 0 to 28.1°C on Day 15. Following exchange on Day 10, mean temperature in the exchange holds decreased on Day 12 to 26.4°C, then increased on Day 15 to 26.8°C. On Day 15, the mean temperature in the control holds was significantly greater than in the exchange holds (n = 2 with 6 subsamples per replicate; p = 0.048). Within the water columns, temperature decreased with depth in 21 of 24 profiles; in the remaining 3 profiles, temperature remained constant with depth. Among all profiles, the average temperature difference between surface and bottom was 0.4°C.

Salinity ranged from 38.0 to 42.0 ppt (data not shown). In the control holds, mean salinity increased from 40.2 ppt on Day 0 to 40.4 ppt on Day 15. Following exchange, mean salinity in the exchange holds decreased to 38.5 ppt on Day 12, then increased to 39.0 ppt on Day 15. Salinity was not significantly different between control and exchange holds on Day 15 (n = 2 with 2 subsamples per replicate; p = 0.057).
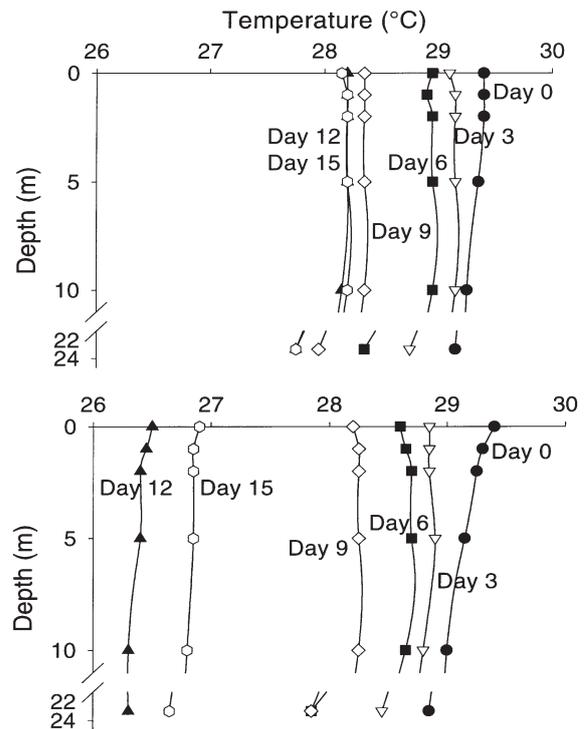

Fig. 2. Temperature profiles in control holds (upper panel) and exchange holds (lower panel) on Day 0 (●), Day 3 (▽), Day 6 (■), Day 9 (◇), Day 12 (▲) and Day 15 (○). Data are mean values (n = 2)

Within holds, salinity remained constant with depth in 16 of 24 profiles; in 2 profiles, salinity increased with depth; in the remaining 6 profiles, salinity decreased with depth. The mean difference between salinity measurements taken at 0 and 10 m was 0.2 ppt.

Dissolved oxygen (DO, measured as percent saturation) in the ballast water ranged from 77.6 to 99.7% (data not shown). In the control holds, mean DO decreased during the voyage, from 92.2% on Day 0 to 85.2% on Day 15. Following exchange, mean DO in the exchange holds increased to 91.7% on Day 12 and to 96.3% on Day 15. On Day 15, the mean DO value was not significantly different between the control and exchange holds (n = 2 with 6 subsamples per replicate; p = 0.106). In depth profiles, DO decreased with depth in 21 of 24 profiles; in the remaining 3 profiles, it increased with depth. The average difference in DO between surface and bottom was 2.6%.

Bacterial density

Mean bacteria concentrations ranged from 9.2 to 22×10^7 l^{-1} and decreased by a factor of 2.3 (control holds) and 1.6 (exchange holds) throughout the voyage (Fig. 3A). Bacteria concentrations were signifi-
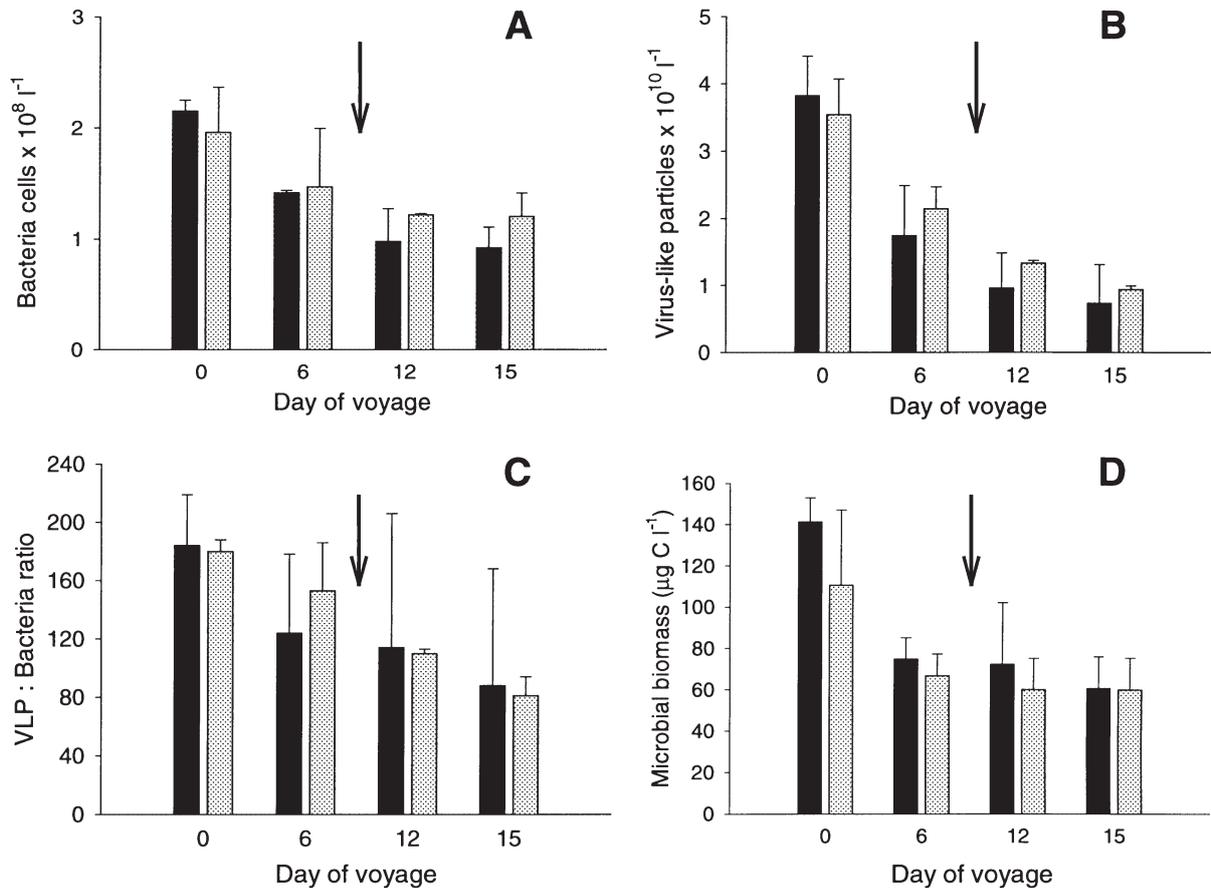
Fig. 3. Microbial metrics in ballast water. Black bars represent samples from control holds; gray bars represent samples from exchange holds. (A) Bacterial abundance; (B) virus-like particle abundance; (C) VLP-to-bacteria ratio; (D) microbial biomass. In all plots, the arrow indicates Day 10, when exchange holds were exchanged in the open ocean. Data are mean values (n = 2, with 3 to 6 subsamples per replicate); error bars represent +1 SD

cantly higher on Day 0 than on Day 15 in the control holds (p = 0.013), but not in the exchange holds (Table 1). In the exchange holds, there was no significant difference in bacteria abundance before (Day 6) and after (Day 12) the exchange on Day 10 (Table 1). On Day 15, the mean bacteria concentration did not differ significantly between control and exchange holds (Table 1).

Virus-like particle abundance

Average VLP densities varied from 0.7 to 3.8×10^{10} l^{-1} and decreased by a factor of 5.2 (control holds) and 3.8 (exchange holds) throughout the voyage (Fig. 3B). VLP densities were significantly higher on Day 0 than on Day 15 in both control holds (p = 0.046) and exchange holds (p = 0.011; Table 1). Within exchange holds, the mean VLP density was significantly greater before exchange than after exchange (Day 6 vs 12; p = 0.033; Table 1). On Day 15, the mean VLP concentration did

not differ significantly between the exchange and control holds (Table 1).

VLP-to-bacteria ratio

The VLP-to-bacteria ratio ranged from 81 to 184 and decreased during the voyage by 2.1- and 2.2-fold (control and exchange holds, respectively; Fig. 3C). Within exchange holds, the VLP-to-bacteria ratio showed a 1.4-fold decrease from measurements taken before (Day 6) and after (Day 12) exchange.

Microbial biomass

The mean microbial biomass fluctuated from 60 to 141 µg C l^{-1} and decreased 2.3- and 1.8-fold (control and exchange holds, respectively) over the course of sampling (Fig. 3D). Biomass on Day 0 was not significantly higher than on Day 15 in control holds (although

p = 0.062), but it was in exchange holds (p = 0.037). There was no significant difference in the mean microbial biomass in samples collected from exchange holds before and after the exchange (Day 6 vs 12; Table 1). Finally, there was no significant difference between the mean microbial biomass in samples collected from control and exchange holds 5 d following exchange (Day 15).

Phytoplankton pigments

Mean chl *a* values ranged from 0.01 to 0.34 µg l^{-1}, with decreases of 34- and 21-fold (control and exchange holds, respectively) during the crossing (Fig. 4). Chl *a* concentrations were significantly higher on Day 0 than on Day 15 in both types of holds (control p < 0.001; exchange p = 0.008; Table 1). Samples collected from exchange holds on Days 6 and 9, prior to exchange, did not have significantly different chl *a* concentrations from samples collected after exchange on Day 12 (Table 1). On Day 15, the mean chl *a* concentration did not differ statistically between the control and exchange holds (Table 1).

Average phaeopigment concentrations varied between 0.02 to 0.14 µg l^{-1} (Fig. 4). Phaeopigment values decreased by a factor of 4.7- and 7.0-fold (control and exchange holds, respectively) throughout the voyage. Phaeopigment values were significantly greater on Day 0 than on Day 15 in both control (p = 0.002) and exchange holds (p = 0.009; Table 1). Data collected on Days 6 and 9 show large variability, presumably related to particle-associated phaeopigment, given the

notable amount of particles in some of those samples (Fig. 4). The mean phaeopigment concentration in the exchange holds was not significantly different before open-ocean exchange (Days 6 and 9) than after the procedure (Day 12; although p = 0.066 for the Day 6 vs 12 comparison; Table 1). On Day 15, as with the chl *a* samples, the mean phaeopigment concentration did not differ statistically between hold types (Table 1).

The ratio of chl *a* to phaeopigment ranged from 0.45 to 2.3 and decreased during most of the trip, as the phaeopigments became an increasingly large part of the pigment pool through Day 9 (data not shown). There was an increase in the ratio in both control and exchange holds on Day 12 relative to Day 9, then a decrease on Day 15. The overall decrease from Day 0 to 15 was 5.2- and 2.9-fold (control and exchange holds, respectively).

DISCUSSION

Although ballast-water research has grown greatly in the past decades, most studies—with the exception of those on dinoflagellates—have centered on metazoans. Furthermore, sample collection has largely occurred at the termination of voyages, with little research on the processes leading to end-point conditions (although see Rigby & Hallegraeff 1994, Yoshida et al. 1996, Gollasch et al. 2000). Bacterio- and virioplankton are becoming a focus of ballast-water studies (Ruiz et al. 2000, Drake et al. 2001), and here we present the first data tracking microbial communities throughout a transoceanic voyage and a comparison of microorganismal abundance in paired exchanged and unexchanged ballast-water samples.

What happens to a parcel of water when it is sequestered in a darkened ballast-water tank? One hypothesis is that ballast-water tanks and holds act as incubators for microorganisms: in the darkness, photosynthesis ceases, phytoplankton die, and, in turn, zooplankton starve and die. Concentrations of dissolved organic matter (DOM) increase as these planktonic constituents decompose. The augmented DOM pool subsequently fuels bacteria production, and viral production proceeds via viral infection and lysis of plentiful bacteria hosts. The outcome of such a scenario is that concentrations of microorganisms in ballast water could exceed those in coastal waters prior to their uptake by ships.

Data gathered from sampling ballast water at the end points of voyages, however, do not support the 'incubator' hypothesis, because microbial abundances are generally lower in ballast-water samples than in coastal-water samples (Ruiz et al. 2000, Drake et al. 2001). An alternative scenario for microbes transported
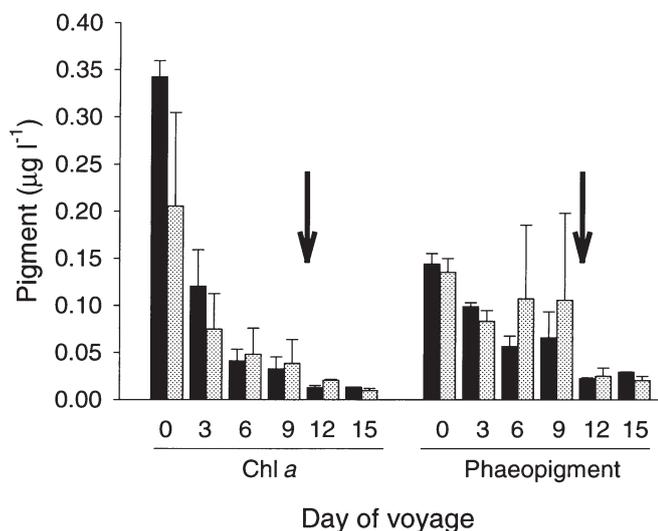

Fig. 4. Chl *a* and phaeopigment concentrations. Format is the same as in Fig. 3. Data are mean values (n = 2, with 3 to 4 subsamples per replicate). Error bars represent +1 SD

via ballast water, therefore, is that their abundance decays over time. The decrease is possibly due to a number of reasons, which are not necessarily mutually exclusive: (1) an uncoupling of the microbial loop occurs, specifically, there is a lower-than-expected utilization of DOM resulting from phyto- and zooplankton die-offs; (2) bacteria are removed by microzooplankton and there is a subsequent accumulation of microzooplankton biomass; or (3) the pulse of DOM from phyto- and zooplankton die-offs is respired by bacteria in the beginning of the voyage, and the remaining, small amount of DOM in the holds leads to a steady state of low microbial biomass throughout the voyage. Although we did not collect samples of ship-side water in Israel before the voyage, our data lend support to the 'decay' hypothesis because: (1) microbial metrics showed decay, not stimulation, throughout the voyage; (2) VLP abundance, bacteria concentration, and the VLP-to-bacteria ratio decreased over time (Fig. 3A,B,C), suggesting that VLP abundance declined faster than bacteria, rather than being increased by viral lysis of abundant bacterial hosts; and (3) microbial metrics on Day 15 were low, especially for the algal pigments (Figs. 3 & 4).

In this study, all microbial variables decreased over time in the control holds. Likewise, Williams et al. (1988) documented a decrease in planktonic species and taxa with increasing voyage length between Japan and Australia; Yoshida et al. (1996) noted a decrease in phytoplankton species on a voyage from Japan to the USA; and Lavoie et al. (1999) showed a decrease in taxa richness in plankton tows and ballast-water samples before and after intracoastal voyages along the US East Coast. Gollasch et al. (2000) documented a similar pattern of decreasing biomass and diversity of zooplankton and phytoplankton during a 23 d voyage from Singapore to Germany, with the notable exception of the harpacticoid copepod *Tisbe graciloides*, which increased in abundance 100-fold during the voyage. The present study showed that ballast-water holds did not act as incubators to stimulate general microorganism growth during the course of a journey from Israel to the USA, and, in fact, microbial constituents decreased throughout the voyage.

The efficacy of exchanging ballast water in the open ocean to decrease abundances and biomass of microorganisms can be evaluated by comparing values in control and exchange samples collected on Day 15 (Table 1). In all 5 metrics tested, there was no significant difference between treatment means. We stress, however, that the efficacy of open-ocean exchange to reduce invasion by non-indigenous microorganisms cannot be based solely on microbial abundances and biomass. First, it is likely that species composition shifted following exchange (Hülsmann et al. 2000), resulting in a decline in coastal constituents and an increase in oceanic species. Second, the likelihood of microorganisms surviving in a new environment following their discharge with ballast water, in part a function of their species composition, is an element critical to evaluating the risk of microbial invasion. Composition and survivorship, therefore, represent important yet uninvestigated facets of ballast-water microbiology and will provide future direction for the field.

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