Rapid examination of microorganisms in ballast waters

Examen rápido de microorganismos en aguas de lastre

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Resumen.- Se examinaron las comunidades microbianas del agua de lastre mediante microscopía de epifluorescencia. Se realizaron análisis cualitativos y cuantitativos con el fin de probar si los perfiles microbianos encontrados en diferentes barcos estaban relacionados con sus itinerarios de viaje y/o el manejo de sus tanques de agua de lastre. Las concentraciones de VLPs ("virus-like particles") y bacterias presentaron diferencias evidentes en barcos que arribaron a un mismo puerto. Estas diferencias son coherentes con los respectivos itinerarios de viaje.
Además, la extensión del tiempo de confinamiento del agua de lastre en un tanque determina que algunas formas bacterianas predominen progresivamente sobre otras. Lo anterior permitiría, en principio, determinar si el agua de lastre ha sido renovada en mar abierto antes de arribar a puerto.
Los resultados presentados se restringen al examen del agua de lastre de unos pocos barcos. Sin embargo, pensamos que una mayor investigación en esta área determinará que la microscopía de epifluorescencia será cada vez más útil para caracterizar microbiológicamente las aguas de lastre, contribuyendo así a develar su historia reciente.

Palabras clave: Agua de lastre, microscopía de epifluorescencia , microorganismos.

Introduction

Global movement of ballast water by ships represents a long-distance dispersal mechanism of harmful species. In order to reduce the dispersal risks, ballast water exchange practices are currently performed in oligotrophic oceanic waters as a practical methodology for ballast water management (Gollasch 1998). Therefore, it is important to determine if ballast water exchange in the open ocean has been done before the arrival of the ship to its destiny port. A problem to be solved is precisely, the design of a technology that allows controlling whether such procedures have been performed in ships.

Marine waters contain variable amounts and species of microorganisms depending if samples have been taken from littoral or oceanic waters as well as other factors such as latitude or pollution. Virus-like particles (VLPs) and bacteria are an order of magnitude more abundant in coastal waters than in open ocean and big-sized bacteria are typical of coastal waters (Ducklow 2000, Wommack & Colwell 2000). So, a microbiological profile should reflect the origin of the ballast water.

The abundance of VLPs, total bacteria and the bacteria *Vibrio cholerae* has been measured in samples taken from ballast water from vessels (Ruiz *et al*. 2000). Those results demonstrated the great potential significance of ship-mediated transfer of microorganisms. In our case, we aim to find out, also by microbiological techniques, if the history of a ship's ballast water can be traced back by a microbiological profile analysis. Theoretically, it should be possible to check, according to the International Maritime Organisation (IMO) guidelines, if a given ship has undertaken measures to minimize the impact of discharging its ballast water (Gollasch 1998).

The viability of different control options depends not only on their biological effectiveness but also on their technical affordability in terms of speed, costs and safety in order to preserve a given environment from harmful species. The microscopic examination of water samples stained with fluorescent dyes seems to fulfil the appropriate requirements (Bettarel *et al.* 2000). Several protocols have recently been compiled (Paul 2001). Microorganisms, including viruses, are visualized as fluorescent dots that can be easily enumerated with the proper software. Moreover, shapes and sizes of bacteria can also be used to further characterize the water being analyzed.

The aim of this work is to show that a microbial epifluorescence profiling can be useful to characterize ballast waters of ships and that this information allows

the tracing of the history of a ballast water in a given ship tank.

Materials and methods

Sampling

Four vessels arrived to different ports of Chile were studied in this research. Seawater samples were collected with sterile propylene bottles of 250 ml capacity at 50 cm depth and 100 m from the coast. One ballast water tank was monitored before and after its water was changed. Triplicate samples from ballast tank waters were taken through the manhole immersing the propylene bottles 50 cm below water surface. From each of them, three filters containing the microorganisms were prepared for the staining procedure.

All samples were filtered by a 35 µm pore-size net and maintained in the dark at 4ºC until staining. The samples for bacteria examination, were kept in 1 ml aliquots containing 5% formaldehyde. Those aliquots for virus examination were maintained in NaCN 2 mM.

Staining

The SYBR Green I method was used (Noble 2001). Briefly, seawater samples were filtered with an ultra-fine pore size filter (Whatman Anodisc, 0.02 µm Membrane Filters); the filter was placed over a drop of SYBR Green I standard solution (Molecular Probes Inc.)1:1000 in Milli Q water. After 15 min in the dark, at room temperature, the filter was washed three times with Milli Q water, removed from the vacuum equipment, placed on a glass slide and covered with a 25 mm square glass containing a drop of anti-fade mounting solution (50% PBS / 50% glycerol with 0.1% p-phenylenediamine).

The stained samples were examined in an Olympus BX60 epifluorescence microscope. Randomly selected fields were counted in order to enumerate about 250 VLPs or 250 bacteria per filter. Standard deviations <5% were obtained between the replicated filters.

The particles counting was done using the Image Pro® Plus 4.5 (Media Cybernetics) software.

In order to analyze the ballast water we sampled the tanks of ships upon arrival to ports at two different latitudes. Puerto Montt (41°29'S, 72°57'W) and Antofagasta (23º38'S, 70º24'W). At Puerto Montt, samples were obtained from a ship (A) ending a transoceanic trip and a ship (B) coming from a shorter trip from the southern part of the country. A third ship (C), arrived to the port of Antofagasta (northern Chile), was sampled as well.

Results

As shown in Fig. 1, bacteria were more abundant in ship A than in B, by at least a factor of ten. Besides, there were differences in shape and size. Bacteria from ship A were rod-shaped and had a rather big cellular volume. Bacteria from ship B, in contrast, were spherical in shape and smaller in size compared with those of ship A (Fig. 1). Both samples of ballast waters contained similar concentrations of VLPs, 1.8 (s = 0.12; n =3) x 10^7 and 2.0 (s = 0.16; n=3) x 10^7 VLPs/ml.

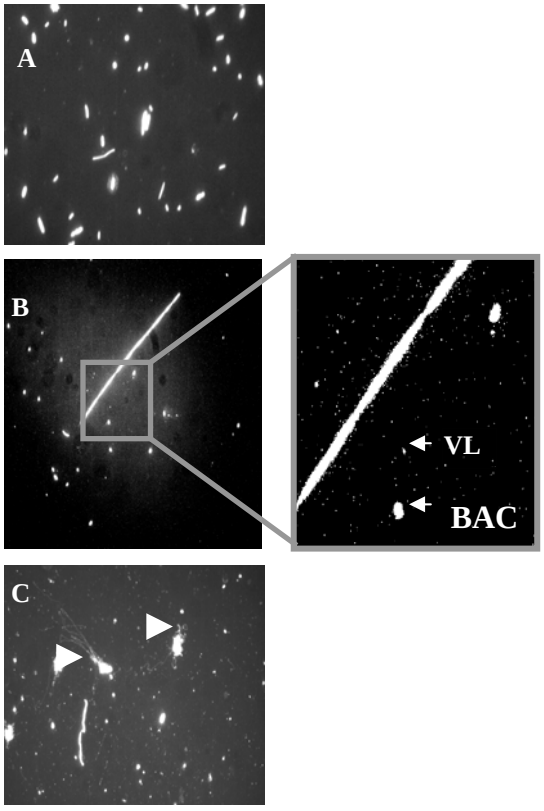

Figure 1

Bacteria and VLPs in ballast waters from three ships. Ships A and B arrived to Puerto Montt, (A) and (B) respectively. Ship C arrived to Antofagasta (C). Arrow shows microorganisms; arrow heads show cell debris and / or lysed cells

Bacterias y VLPs en agua de lastre de tres barcos. Los barcos A y B arribaron a Puerto Montt, (A) y (B) respectivamente. El barco C arribó a Antofagasta (C). La flecha muestra microorganismos; las cabezas de flecha muestran restos celulares y/o células lisadas

The ballast waters from ship C contained fewer bacteria - one order of magnitude - than those detected in vessel B.

Different kinds of microbiological profiles can be seen in samples from ballast waters taken from different vessels. Aiming to know how sensitive to confinement are the microbial communities present in ballast waters, we analyzed the microbiological profile of one tank of another ship (D) during five days since it was filled with sea water.

Bacteria and VLPs were very abundant in the ballast water sample taken from the vessel upon arrival (Fig. 2, left columns, "before change"). Levels like those found in eutrophic marine coastal environments were found, several times higher when compared with the bay water in which the ship was anchored (Fig. 2, right columns). When compared with seawater of the surrounding environment, the concentration of bacteria and VLPs in the ballast water did not seem to change during five days of confinement in the tank. However, a closer examination of the bacterial communities showed interesting differences.

As shown in Fig. 3, the spherical shapes were the predominant bacterial forms at the first day after having changed the tank water. During the following days, the rod-shaped bacteria became increasingly predominant (B and C). Notably, the ballast water maintained during a long time inside the tank contained only rod-shaped bacteria (D).

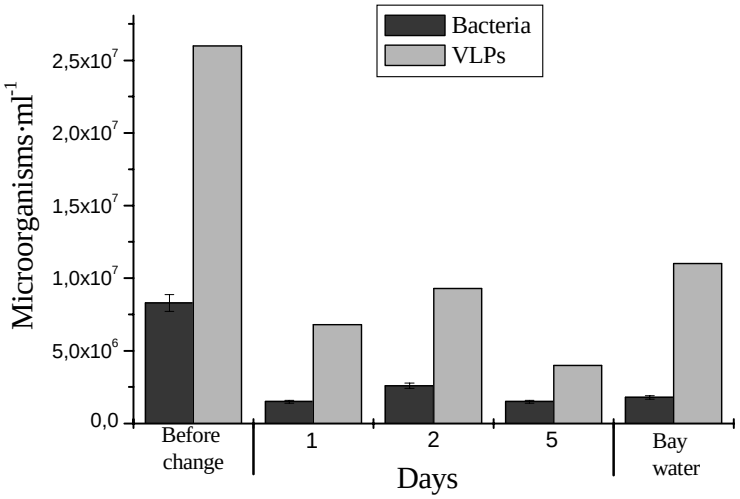

Figure 2

Bacteria and VLPs in ballast water from ship D before and after the exchange in bay waters. Samples were taken from: ballast water upon arrival (before change), bay water from the place where the interchange was done (bay water) and days 1, 2 and 5 from the tank. Standard deviations never exceeded 6-8% between replicate samples. As an example, *s* bars corresponding to bacteria counting data are shown

Bacterias y VLPs en agua de lastre del barco D antes y después del intercambio en aguas de la bahía. Muestras de agua de lastre se obtuvieron al arribo (antes de cambiar), de agua de la bahía en el sitio que se produjo el intercambio (agua de la bahía) y del tanque 1, 2 y 5 días después del intercambio. La desviación estándar nunca excedió el 6-8% entre réplicas. Como ejemplo se muestran las barras de *s* correspondientes al conteo de bacterias

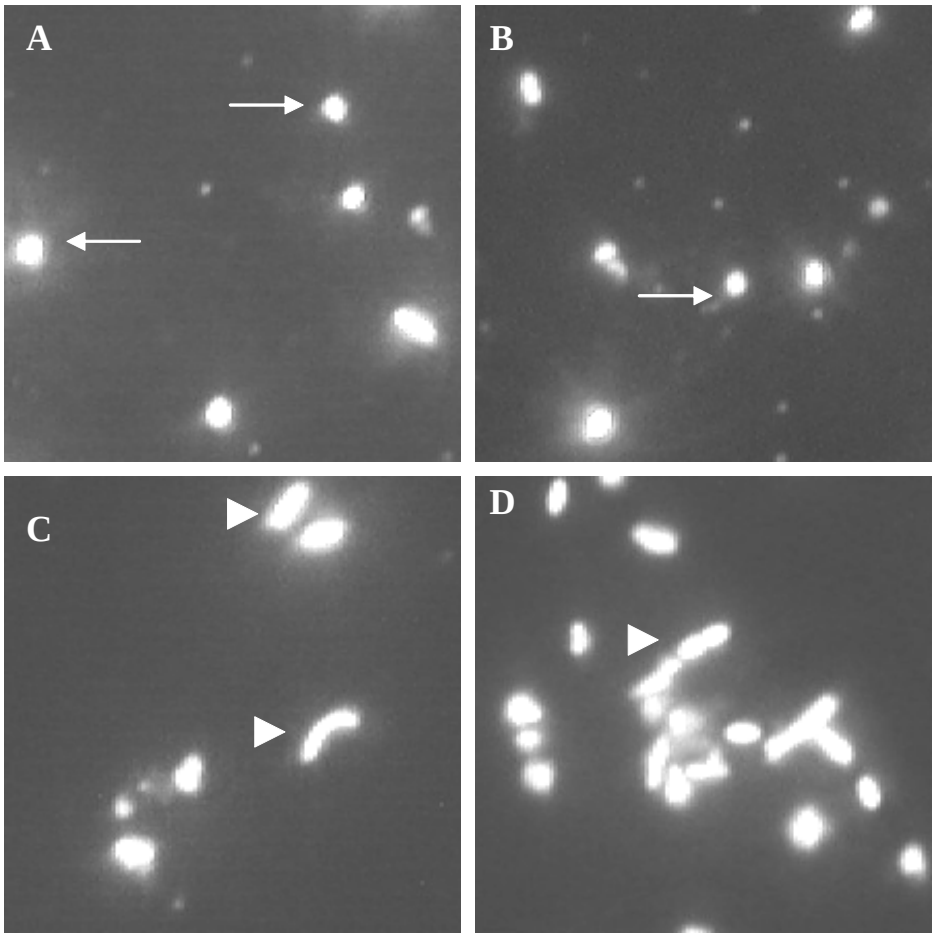

Figure 3

Bacteria in ballast water of ship D after the exchange in bay water. (A), (B) and (C): after day 1, 2 and 5 respectively. (D): control sample of ballast water before the interchange. Arrows show spherical bacteria; Arrow heads show rod-shaped bacteria

Bacterias en el agua de lastre del barco D después del intercambio en aguas de la bahía. (A), (B) y (C): después de los días 1, 2 y 5 respectivamente. (D): Muestra control de agua del tanque de lastre antes del intercambio. Las flechas muestran bacterias esféricas; Las cabezas de flecha muestran bacterias bacilares.

Discussion

We predict that a microbiological profile can be useful to gain information about the history of a ship's ballast water. Due to their short life cycles and adaptability, microorganisms communities can change in numbers and in composition in short periods as a consequence of modifications in their environments.

The differences observed in the ballast waters of ships A and B can be attributable to, among others, the environment of the ports of origin, the duration of the trip and whether or not they exchange ballast water prior to a port arrival (Fig. 1). Our results are coherent with this assumption because they are in accordance with the traveling schedule of both vessels.

Ship B arrived to Puerto Montt ending a short trip through the inner channels of southern Chile. Therefore, it can be expected that the microbiological profile reflects the fact that the ballast water tank was filled with water similar to that found at the arrival port and that the travel lasted at most three days. As showed in Fig. 2, only minor changes in the microbiological abundance profile can be detected when the ballast water is maintained inside the ballast tank during short periods.

Ship A begun its three weeks trip in Japan. The size of the bacilar forms of the bacteria found in its ballast water does not correspond to those characteristic of coastal waters (Ducklow 2000, Rheinheimer 1987). In the later, as well as in the case of ballast waters of ship B, small sized bacteria are the predominant ones. In addition, the microbiological profile of ship C differs completely with the ones of ships A and B. Bacteria and VLPs were present in levels even lower than those found in the oligotrophic waters of open ocean and, cell debris and lysed bacteria were observed in the sample. This last situation can be frequent in the ballast waters of tanks with environmental conditions too harsh for supporting microbial life.

In order to test if a microbiological profile can be used as a tool to verify mid-ocean exchange of ballast waters, we analyzed the microbiological profile of ballast waters immediately after exchange. The rationale behind this experiment is that the composition and quantity of major microbial communities should not change a dramatically between the moment of the water exchange and the moment of verification sampling. Otherwise, it will become difficult to discriminate between having exchanged ballast waters few days before verification and without accomplishing the norms at all.

As seen in Fig. 2, the concentration of bacteria and VLPs in the replaced ballast water did not change significantly during five days. However, the bacterial community composition changed progressively, judged by shape changes, during the sampling period (Fig. 3A, B and C). The change should be interpreted as a consequence of the new environmental conditions prevailing in the ballast tank. The rod-shaped bacteria could be heterotrophic bacteria best adapted to the new conditions. This speculation is in accordance to the fact that only rod shaped bacteria were visualized in the sample taken before the ballast water change (Fig. 3D).

To our knowledge, there is only one reference dealing with the analysis of microbiological communities as a tool for testing if ballast water has been changed before arrival to a port (Thomson *et al.* 2001). The methodology uses the metabolic properties of heterotrophic bacteria to distinguish among aquatic microbial communities. The authors suggested that the proposed methodology has potential for applications involving ballast water characterization. This method and the one described in this paper are based in the same principle; the bacterial community reflects the history of ballast water in a tank. The main difference between both methodologies relies in the fact that one requires the cultivation of the ballast water bacterial community and the one proposed by us, instead, implies the direct visualization of the bacterial community in only few hours.

As the quantity and composition of microbes at any moment is the consequence of the environmental factors acting on a rapidly adaptable community, the microbiological profile of ballast water is expected to be the result of many interacting factors, as confinement time in a tank and the absence of light.

Although the results presented here are restricted to the examination of the ballast waters of few ships, we believe that the epifluorescence methodology can be increasingly useful because the technology is rapid, reliable and low cost.

Acknowledgments

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