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Distribution of Virus-like Particles in an Oligotrophic Marine Environment (Alboran Sea, Western Mediterranean)

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ABSTRACT

Viruses are abundant in a variety of aquatic environments, often exceeding bacterial abundance by one order of magnitude. In the present study, the spatial distribution of viruses in offshore waters of the Alboran Sea (Western Mediterranean) have been studied to determine the relationships between viruses and host communities in this oligotrophic marine environment. Viral abundance was determined using two methods: (i) epifluorescence light microscopy using the dsDNA binding fluorochrome DAPI, and (ii) direct counts by transmission electron microscopy (TEM). The results obtained were significantly different; the highest viral counts were obtained by mean of TEM analyses. In all the samples tested the number of viruses was exceeded by the bacterial concentrations, with a ratio between viral and bacterial titers varying between 1.4 and 20. VLP (virus-like particle) counts were not significantly correlated ($p > 0.001$) with chlorophyll *a* concentration or the abundance of cyanobacteria. However, there was a positive and significant correlation with bacterial abundance ($p < 0.001$). The analysis of size and morphology of viral particles by TEM and the correlation obtained between the numbers of VLP and bacteria suggest that the majority of the viral particles in the Alboran Sea are bacteriophages. None of the indirect evidence suggested that eukaryotic algae or cyanobacteria were important host organisms in these waters.

Key words: Virus-like particles, Distribution of viruses, Marine bacteria, Chlorophyll-a, Oligotrophic marine environment

Introduction

Classically, it has been considered that the main organisms responsible for bacterial mortality in the marine environment are protists. However, several studies suggest that viruses play an important role in the mortality of host populations and as a mechanism influencing genetic and clonal diversity of host populations [2, 12, 51]. In addition, recent results show that viruses could be responsible for a reduction of up to 78% of primary production in the oceans [41], and

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for an important percentage (10–20%) of the mortality of heterotrophic bacterial populations [40].

Direct counts of virus-like particles (VLP) do not indicate if they are infectious particles, but they may be very useful tools in studies of viral ecology. The first study on the abundance of viral particles in seawater was carried out by Torrella and Morita [43], who calculated viral concentrations of up to 10^4 VLP ml^{-1} in coastal seawater from Oregon. Although at present it is well known that viruses can infect both marine bacteria and phytoplanktonic cells [51], only recently, with the development of new techniques applied to the viral count, has the existence of a large number of marine viruses in natural waters (10^5–10^8 ml^{-1}) been revealed [3, 8, 17, 30, 51, 52]. This viral number varies according to different factors, such as sampling depth, degree of stratification, season, and trophic conditions [11]. The improvement of the enumeration techniques has allowed elucidation of the ecology of viruses in aquatic environments, and their role as a factor controlling both prokaryotic and eukaryotic populations.

To assess the ecological role of marine viruses, it is necessary to establish the viral spatial distribution, and to determine the abundance of other microbial loop components, mainly bacteria, cyanobacteria, and phytoplankton. Based on the fact that algae, bacteria, or cyanobacteria may be the main viral hosts, a correlation with either of these parameters is expected [27]. Studies on spatial distribution of viruses and their specific hosts have been performed in estuarine and coastal waters and open oceans, as well as in freshwater ecosystems [5, 25, 29, 33, 51]. Since it has been suggested that viral impact may vary depending on the trophic characteristics of the water mass [5, 8], further studies in oligotrophic environments are needed.

In the present work, a study on the abundance and distribution of viral particles in an oligotrophic marine environment such as the Alboran Sea (Western Mediterranean) has been performed. The Alboran Sea is a well-characterized aquatic ecosystem, which can be considered as a scale model of the oceanic systems. In addition, the relationships between viral and bacterial or phytoplanktonic levels have also been analyzed.

Materials and Methods

Sampling Sites

A cruise on board the *Francisco de Paula Navarro* was performed in the Alboran Sea (Western Mediterranean) during the summer stratification period along several transects sited perpendicularly to the coastline. The cruise was carried out as part of a project in collaboration with the *Instituto Español de Oceanografía* (I.E.O.).

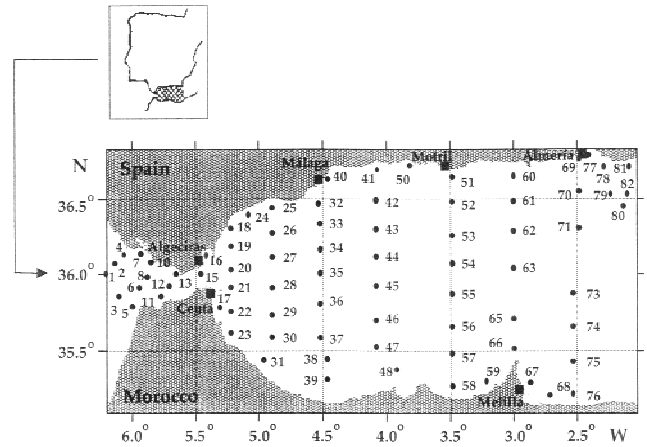

Fig. 1. Location of the stations sampled in the Alboran Sea.

Samples were collected from 82 stations at the depth of the subsurface chlorophyll maximum (from 40 to 55 m) (Fig. 1).

Chlorophyll a and Salinity Determination

Samples for chlorophyll *a* analysis (250 ml) were filtered through fiberglass GF/F Whatman filters. Chlorophyll *a* was extracted in a 90% acetone solution for 24 h in cold and dark, and determined fluorimetrically [53] with a Turner Designs fluorometer. Salinity was measured by conductivity using a Sea-Bird 25 CTD equipped with a Sea Tech fluorometer.

Bacterial and Cyanobacterial Enumeration

The samples for enumerating bacterial populations were immediately fixed with 2.5% glutaraldehyde (final concentration) and stored at 4°C in the dark until analyses were performed. Bacterial number was determined by epifluorescence microscopy of 2,4-diamidino-2-phenylindole (DAPI)-stained preparations filtered on 0.2-μm pore-size polycarbonate filters (Millipore, GTTP), as described by Porter and Feig [32]. At least 100 bacterial cells were counted on 10 randomly selected fields of each preparation. Filters were mounted onto slides with immersion oil (Nikon, type A) and inspected at 1000× magnification under UV light excitation, using a Nikon microscope with a 100 W mercury lamp for epifluorescence illumination.

The abundance of cyanobacteria was analyzed with a FACSCAN flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser providing a 488-nm light and the standard filter setup. Samples were run for 5 min at 60 μl min^{-1}, and the threshold was set on red fluorescence. Parameters were collected on logarithmic scales and analyzed with the Lysys II software. Cyanobacteria were distinguished by the orange phycoerithrin fluorescence.

Samples for flow cytometry were filtered through a net of 40-μm-pore size, immediately preserved with 1% glutaraldehyde [45], and stored in liquid nitrogen until they were processed.

Viral Enumeration

Viral abundance was determined by two methods: by transmission electron microscopy (TEM) direct counting, and by epifluorescence light microscopy using the fluorochrome DAPI (1 μg ml^{-1}, final concentration) [39]. DAPI-stained viral particles appear as tiny points of blue light. For epifluorescence microscopy, a modification of the method of Porter and Feig was developed [1]. Briefly, viral particles were collected under gentle vacuum suction (under 125 mm of Hg) on 0.02-μm pore-size aluminum oxide filters (Anodisc, 25). Filters were mounted onto slides following the methodology above described.

For TEM direct counting volumes of 500 ml of selected seawater samples were concentrated to 5–10 ml using a tangential flow filtration system with a membrane area of 50 cm^2 and an exclusion size of 50 kDa (Filtron, Mini-Ultrasette). Samples were pumped toward the filtration unit by means of a peristaltic pump (Masterflex) at a flow rate of 280 ml min^{-1}. Ultrafiltration centrifugal concentrators (Filtron, Microsep) containing OMEGA membranes with an exclusion size of 10 kDa were used for further concentration of the retentate volume [1].

Small volumes (5–10 μl) of concentrated samples as above described were placed on 400-mesh copper grids covered with a Formvar film. The grids were rinsed with deionized water, stained with 1% (wt/vol) uranyl acetate solution (pH 4.5), and observed under a transmission electron microscope (Philips EM100) at 80 kV accelerating voltage and magnification between 20,000× and 80,000×.

Different types of viruses were recognised on the basis of staining properties, size, and morphology. Head diameters of the viruses were determined from micrographs randomly taken using an Eastman Kodak film (400 ASA). Viral particles were classified into three size classes according to the head diameters: <30 nm, from 30 to 60 nm and >60 nm.

The number of viral particles was determined from micrographs. A minimum of 20 microscopic fields were randomly selected at 20,000×.

Statistical Tests

The Spearman nonparametric rank correlation coefficient (r_s) was calculated to study interactions among bacteria, VLP, chlorophyll *a,* cyanobacteria, temperature, and salinity. A positive correlation between viral count and the abundance of the main host organisms is expected, since higher host cell density increases the encounter rate.

Results and Discussion

Evaluation of Several Methods for Viral Count from Seawater Samples

VLP abundance was determined by several methodologies, and the viral titer varied (about 1 log) depending on the method used (Table 1).

The number of VLP (tiny fluorescent dots) obtained by using 0.02-μm pore-size aluminum oxide filters in conjunction with DAPI staining was significantly higher (paired *t*-test, $p = 0.007$) than that obtained on 0.2-μm pore-size polycarbonate filters, as is shown in Table 2. The recovery rate of VLP retained on polycarbonate filters in relation to aluminium oxide filters varied from <3% to 79%, with a mean of 26 ± 6%. However, significant differences in the number of bacteria (large fluorescent particles) on the two types of filters were not observed (paired *t*-test, $p = 0.437$) (Table 2). The low autofluorescence of aluminum oxide filters makes this technique appropriate for environmental studies. In addition, the flat and rigid surface of these filters results in easier focusing and produces a better definition of fluorochrome-stained viral particles. According to Hara et al. [18], this technique is rapid, easy, and suitable for processing a large number of samples, being especially useful for comparative spatial distribution studies among samples with similar characteristics. The successful enumeration of viruses generally requires intensification of the fluorescent signal by means of a photographic processing designed to increase film speed [17].

However, DAPI-stained viruses are close to the limit of visual detection and the overlap in size between small bacterioplankton cells and large VLP, in some environments, can be a source of error in direct counts of viruses [36]. For this reason, more recently two other nucleic acid binding fluorochrome stains, Yo-Pro-1 and SYBR Green I, have been used for virioplankton enumeration [10, 16, 19, 29]. Specific advantages of these stains are low background staining and brightness of fluorescence greater than that of DAPI. A notable disadvantage of Yo-Pro-1 is the 2-day incubation needed for adequate staining of virioplankton samples trapped on an aluminum oxide filter [47, 51]. In addition, the high concentrations of divalent ions present in the seawater samples may affect the binding between DNA and fluorochrome [19, 29].

The practical limitation of TEM direct counting is the equipment required. Thus, it is not suitable for most filed studies [51]. For this reason, only eight selected samples were determined by TEM analysis. The VLP abundance detected by TEM was statistically higher ($p < 0.01$) than that obtained by the DAPI method, coinciding with results obtained by other authors [15, 16, 30]. As shown in Table 1, the number of viruses calculated by TEM is only slightly higher than bacterial abundance, which is in agreement with Paul et al. [30] from oligotrophic environments. These authors suggest that virus abundance is only significantly higher than bacterial concentration in eutrophic environments, being quite similar in oligotrophic waters.

Although occasionally VLP counts by TEM lower than 10^5 ml^{-1} have been reported [3, 5, 8, 17, 18, 33, 35], they are

Table 1. Viral and bacterial abundance in seawater samples of an oligotrophic marine environment (Alboran Sea)

Viral abundance by DAPI (VLP[a] ml^{-1})	Viral abundance by TEM (VLP ml^{-1})	Bacterial abundance (bacteria ml^{-1})	Ratio VLP[b]/Bact	Ratio VLP[c]/Bact
(7.2 ± 2.6)[d] $\times 10^4$	$(2.6 \pm 0.5) \times 10^5$	$(1.0 \pm 0.7) \times 10^5$	0.7	2.6
$(3.3 \pm 1.6) \times 10^4$	$(1.4 \pm 0.3) \times 10^6$	$(1.3 \pm 0.5) \times 10^5$	0.2	10.8
$(1.4 \pm 0.9) \times 10^4$	$(2.2 \pm 1.1) \times 10^5$	$(1.6 \pm 0.2) \times 10^5$	0.1	1.4
$(1.9 \pm 0.9) \times 10^4$	$(6.2 \pm 0.9) \times 10^5$	$(1.4 \pm 0.3) \times 10^5$	0.1	4.4
$(2.3 \pm 1.1) \times 10^4$	$(7.3 \pm 1.9) \times 10^5$	$(6.8 \pm 2.5) \times 10^4$	0.3	10.7
$<10^3$	$(1.8 \pm 0.3) \times 10^6$	$(9.0 \pm 3.1) \times 10^4$	<0.01	20.0
$(3.4 \pm 2.0) \times 10^4$	$(3.0 \pm 0.8) \times 10^5$	$(1.1 \pm 0.2) \times 10^5$	0.3	2.7
$(2.3 \pm 1.2) \times 10^4$	$(4.5 \pm 1.7) \times 10^5$	$(2.0 \pm 0.4) \times 10^5$	0.1	2.2

[a] VLP: virus-like particles
[b] Viral abundance by DAPI
[c] Viral abundance by TEM
[d] Mean ± standard deviation

typically in the range of 10^5 to 10^6 ml^{-1} [44, 51], figures similar to the results obtained in the present study (Table 1). Some authors have obtained significantly higher VLP counts by epifluorescence microscopy than by TEM [17, 18, 19, 47]. All these studies have been carried out in eutrophic systems in which organic matter may impede VLP observation by TEM. The degree of underestimation depends on the amount of organic matter in the sample [19]. In addition, Weinbauer and Suttle [47] showed that TEM methods result in lower estimations when viral abundance exceeds 10^6 ml^{-1}.

Although viral abundance determined by epifluorescence microscopy using DAPI staining was significantly lower than that found with TEM, the former was used routinely to compare viral counts in all stations because it is faster, less complex, and useful when the number of samples that must be processed is high. However, other fluorochromes, such as SYBR Green I, stain only viruses and cells in seawater samples with a short (<15 min) incubation and are not affected by the presence of aldehyde fixatives [29]. In addition, SYBR Green I virus counts yielded a precision similar to that of TEM counts [51].

Abundance of VLP in Relation to Several Physical and Ecological Parameters

The spatial distribution of viruses in relation to bacterial abundance, chlorophyll *a* concentration, and number of cyanobacteria was analyzed in offshore waters of the Alboran Sea. Viral abundance ranged from 2.6×10^3 to 8.1×10^4 VLP ml^{-1} and follows the same general abundance pattern as bacteria (Fig. 2). This patterns includes an important increase in eastern area of the basin studied (from 4°W).

Bacterial number varied from 1.9×10^4 to 5.5×10^5 bac-

Table 2. Numbers of VLP and bacteria determined with the epifluorescence microscopy method using 0.02 and 0.2-μm pore-size filters

VLP ml^{-1}			Bacteria ml^{-1}		
Polycarbonate filters (0.2 μm pore size)	Aluminum oxide filters (0.02 μm pore size)	% Recovery[a]	Polycarbonate filters (0.2 μm pore size)	Aluminum oxide filters (0.02 μm pore size)	% Recovery[a]
$<10^3$	3.6×10^4	<3	1.5×10^5	1.1×10^5	136
3.5×10^3	1.1×10^4	32	1.1×10^5	1.4×10^5	79
$<10^3$	8.8×10^3	<11	2.9×10^5	4.3×10^5	67
2.1×10^4	1.5×10^5	14	8.0×10^4	1.9×10^5	42
1.3×10^4	1.3×10^5	10	2.4×10^5	3.0×10^5	80
1.4×10^4	7.9×10^4	18	3.4×10^5	3.0×10^5	113
4.1×10^3	3.1×10^4	13	2.1×10^5	2.8×10^5	75
2.6×10^3	5.3×10^3	49	1.3×10^5	1.3×10^5	100
1.1×10^4	6.5×10^4	17	2.7×10^5	2.1×10^5	129
1.3×10^4	8.7×10^4	15	1.6×10^5	1.3×10^5	123
8.8×10^3	6.2×10^4	14	1.3×10^5	7.0×10^4	186
3.4×10^4	4.3×10^4	79			
$<10^3$	3.5×10^3	<29			
$<10^3$	2.3×10^4	<4			

[a] The number of microorganisms detected using 0.02 μm pore-size aluminum oxide filters was considered as 100%.

teria ml^{-1}, exceeding viral concentration by one order of magnitude on average at all stations (Fig. 2). These bacterial levels are similar to those found from other marine environments with the same trophic conditions as the Alboran Sea in the summer stratification period [23, 34].

Viral abundance obtained is lower than numbers reported for most aquatic environments [3, 25, 33, 41], in which viruses were usually more abundant than bacterioplankton. The results vary significantly in relation to the method used to count viral particles [7, 16], but only in several studies performed in freshwater systems (Lake Superior and Lake Erie) was the bacterial number higher than the viral abundance [24, 42]. The widespread observations of high viral counts in bays and estuarine environments may be the result of induction of prophages by anthropogenic causes [9].

The ratio of mean viral count to mean bacterial count for each station was calculated to characterize the relationship between bacterial and viral communities. Values of the virus-to-bacteria ratio (VBR) varied from 0.02 to 0.75 (Fig. 3), although 33% of the stations presented a VBR value between 0.1 and 0.2 (Fig. 3). This variation among stations in the same marine environment was also observed in North Pacific [22], Tampa Bay [9], and Paradise Harbor (Antarctic Peninsula) [4]. These results suggest that viral and bacterial production does not take place at similar rates in all loca-

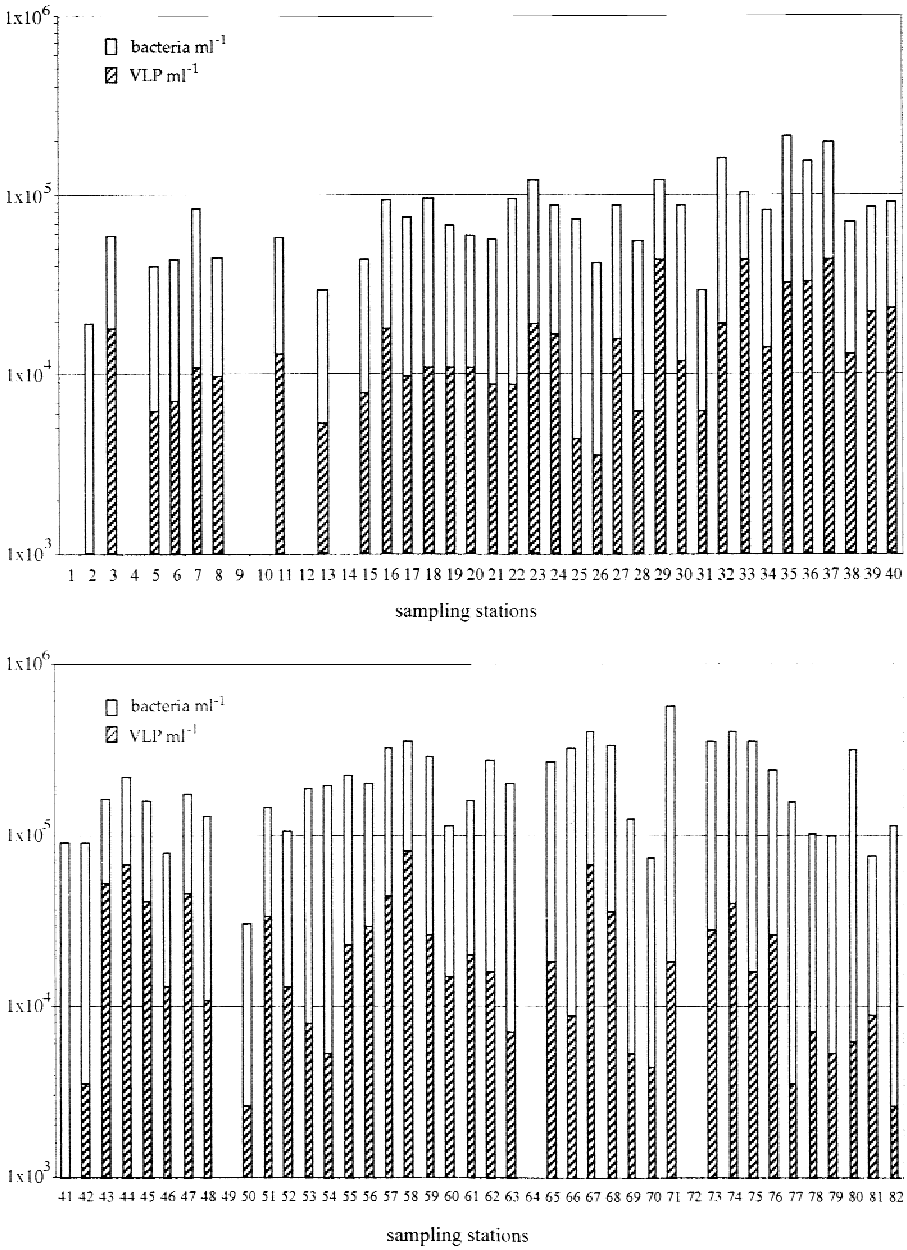

Fig. 2. Viral and bacterial abundance at stations sampled in the Alboran Sea.

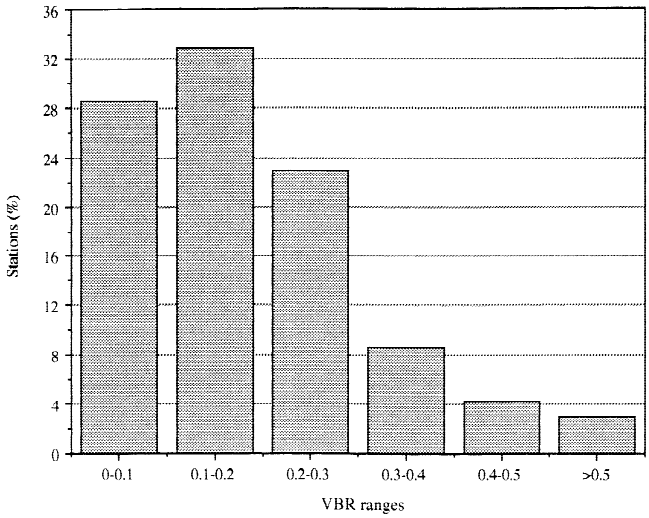

Fig. 3. Distribution of virus-to-bacteria ratio (VBR) ranges represented as percentage of the total stations.

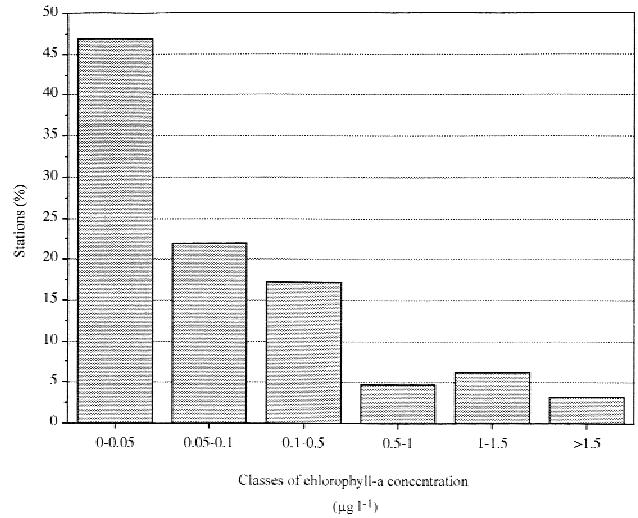

Fig. 4. Distribution of chlorophyll *a* concentration classes at the depth of subsurface maximum. Values are represented as percentage of the total stations.

tions. The values of VBR follow those of VLP concentration, with the highest VBR coinciding with the highest viral counts.

The low VLP concentrations obtained decrease the VBR, since the concentrations of bacteria were similar to those reported from other aquatic ecosystems. In the majority of the systems VBR is greater than 1, between 5 and 83 in seawater samples [48], and ranged from 5 to 20 in freshwater environments [26]. Low values of the VBR may indicate low infectious rates, low numbers of viral particles from each bacterial host, or high decay rates of viruses in this ecosystem. In spite of the low number of viruses detected in this study, they can control microbial community structure [21].

By means of direct observation of samples, it is only possible to count free viral particles. It has been suggested that an important proportion of marine bacteria are lysogenic [11], especially when bacterial concentration is low, as it is in the case of the Alboran Sea. In these conditions, at low rates of host cells and viruses, lysogeny may be a strategy for bacteriophage survival. For this reason, the percentage of viruses inside host bacteria may be high, and this may be an explanation for the low number of free viruses estimated in the studied ecosystem.

The concentration of chlorophyll *a* was between 0.004 and 1.8 μg L^{-1}. Of the samples, 47% yielded concentrations of chlorophyll *a* lower than 0.05 μg L^{-1}, and only 3.1% of the stations presented concentrations above 1.5 μg L^{-1} (Fig. 4). The low concentration of chlorophyll *a* indicates the oligotrophic nature of this environment, which can explain the low concentration of viruses detected. This observation is in concordance with several studies indicating that production and distribution of viruses in aquatic environments is determined by factors that affect the productivity and density of host populations, especially the nutrient availability, the viral abundance being higher in nutrient-rich waters [13, 30, 38, 48, 49, 50].

In oligotrophic environments such as the Alboran Sea, the phytoplanktonic community changes its size structure, the smallest cells (prochlorophytes and cyanobacteria) being the most abundant groups. The abundance of cyanobacteria observed in this study ranged from 1.1×10^3 to 1×10^5 cells ml^{-1}, similar to those observed in other marine environments. Although recent studies suggest that cyanophages can be a very numerous group of viruses in seawater [14], a spatial relation between the distribution of viruses and cyanobacteria was not established in the present work.

Several different morphological types of viral particles were observed by TEM. These viruses could be infective for prokaryotic or eukaryotic microorganisms. On the basis of morphological data, the lack of significant correlation between algal biomass (chlorophyll *a* concentration) and viral direct counts, and the greater abundance of bacteria over that of other planktonic hosts, bacteriophages make up the majority of viruses within the virioplankton.

Bacteriophage heads ranged from 30 to 80 nm in diameter; predominant viral particles were in the 30–60 nm size class (73%), and 27% of the bacteriophages possessed icosahedral heads above 60 nm. Measuring virioplankton diversity by capsid size is appropriate, since this feature varies

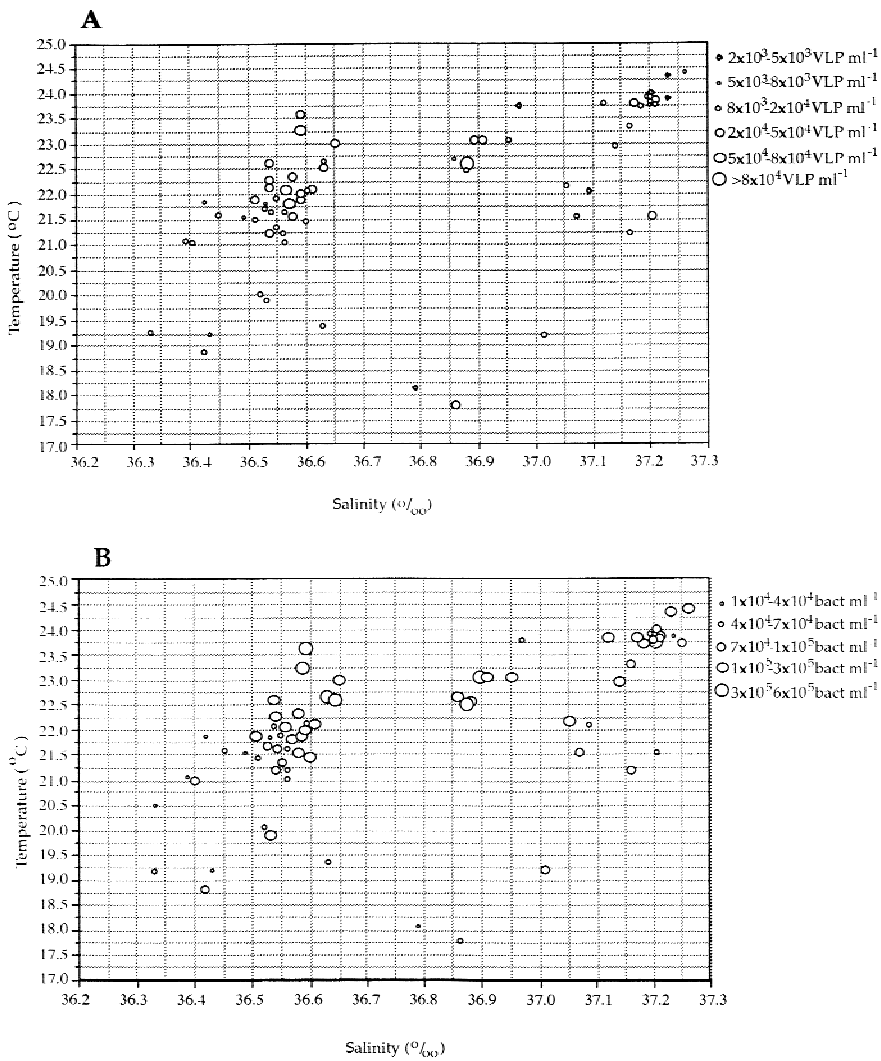

Fig. 5. Distribution of viral (A) and bacterial (B) abundance classes represented in a temperature–salinity diagram.

widely among the bacteriophages described to date (20 to 200 nm) and it is a consistent characteristic of bacteriophages [6, 44]. Data on the frequency distribution of capsid size within virioplankton populations have been reported for a variety of aquatic environments [51], with the dominant virioplankton capsid diameter being in the range of 30 to 70 nm [3, 8, 20, 25, 42, 48, 52]. The proportion of virioplankton observed to fall into the 30- to 60-nm size class was higher, 65% [51]. Several authors [20, 46] have suggested that the free bacteriophages most frequently observed and characterized are indeed produced *in situ.*

A correlation between the capsid size of the virioplankton and the composition of host populations has been proposed [46]. These authors showed that bacteria within different morphological groups carry viruses of a particular size class. Analysis of Adriatic Sea water samples revealed that the 30- to <60-nm capsid class made up 75 to 100% of intracellular viruses in rods and spirilla, respectively. Cocci, however, contained larger viruses (60 to <110 nm) more often than the smaller 30- to <60-nm viruses (65 and 35%, respectively).

The Alboran Sea exhibits strong horizontal physical gradients due to the input of surface less-saline Atlantic water into the Mediterranean basin through the Gibraltar Strait. For this reason, salinity among the different samples increases toward the eastern area of the basin. In Fig. 5 abundance ranges of bacteria and VLP are shown in Temperature-Salinity diagrams. In these diagrams the stations appear in two main groups according to their values of salinity and temperature. The first group presents salinity ranged from 36.5 to 36.7‰ and temperature from 20.5 to 23.5°C, and the second one is characterized by higher values of both temperature (between 22.5 and 25.4°C) and salinity (between 37.1 and 37.3‰).

In Fig. 5B is shown that the majority of the stations in both types of water present a bacterial abundance between 1×10^5 and 3×10^5 bacteria ml^{-1} (30% and 50% of the stations), whereas stations with the highest bacterial num-

bers are those with lower salinity. The distribution of VLP abundance represented in this kind of diagram (Fig. 5A) is very similar to that of bacteria, being the majority of the stations between 8×10^3 and 2×10^4 VLP ml^{-1}. Stations with VLP numbers above 2×10^4 VLP ml^{-1} constitute 52% of the stations with lower salinity and only 21% of the stations with higher temperature and salinity.

Although viral and bacterial numbers varied almost one order of magnitude in a narrow range of salinity, a clear and significant correlation with salinity has not been obtained. This lack of correlation indicates that there is not a considerable input of viral particles or bacteria into the Alboran Sea from the Atlantic waters.

Correlation of Parameters

Viral abundance was significantly correlated ($r_s = 0.447$; $n = 71$; $p < 0.001$) with bacterial concentration, but no correlation was found between viral counts and chlorophyll *a* concentration ($r_s = 0.031$; $n = 63$; $p = 0.8$) or the abundance of cyanobacteria ($r_s = -0.02$; $n = 71$; $p = 0.9$). A similar correlation between bacterial and viral abundance has been also reported by several authors [5, 8, 15, 30, 31, 47]. This result is consistent with the conclusion that bacteria are the main host organisms of marine viruses in Alboran Sea and suggests that bacterial abundance is the main factor governing the viral pattern in this oligotrophic marine environment. In fact, the rate of virus production increases with the increasing of bacterial density [28, 37].

Values of salinity and temperature varied within a range from 36.2 to 37.3‰ and from 17.7 to 24°C and were not correlated with viral abundance ($p > 0.1$).

A multiple regression analysis between VLP, bacteria, chlorophyll *a*, and cyanobacteria showed that virus numbers were positively correlated with the bacterial numbers ($p < 0.01$), whereas the chlorophyll *a* concentration and the abundance of cyanobacteria did not show a significant relationship ($p > 0.5$).

The correlation between viral and bacterial concentration would not be possible if there were a high proportion of temperate phages. In this case, virus production would not only depend on infection.

Acknowledgments

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