

Distribution of marine viruses in the Central and South Adriatic Sea

M. ORDULJ¹, N. KRSTULOVIĆ², D. ŠANTIĆ², S. JOZIĆ² and M. ŠOLIĆ²

¹ University of Split, University Department of Marine Science, Livanjska 5/III, 21000 Split, Croatia

² Institute of Oceanography and Fisheries, POB 500, 21000 Split, Croatia

Corresponding author: marin.ordulj@unist.hr

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Abstract

The seasonal distribution of marine viruses and their relationship with heterotrophic bacteria in the coastal and offshore area of the central and southern Adriatic was studied. Additionally, the percentage of high (HNA) and low (LNA) nucleic acid bacteria in the total number of bacteria and the distribution of heterotrophic nanoflagellates (HNF), as a major predator of bacteria, were studied as well. Viral abundance ranged from 3.55 to 27.32×10^6 virus-like particles mL^{-1} , and was on average 25-fold higher than bacterial abundances at all investigated stations. The highest viral abundances were found at coastal stations, especially in the area influenced by the rivers Krka and Jadro, whereas the lowest values were found in the open sea and in the coastal area of the southern Adriatic. No significant difference was established in the relationship of the viruses with HNA and LNA bacterial groups. The ratio between viruses and bacteria (VBR) was higher during the colder part of the year, which coincided with lower HNF abundance and vice versa during the warmer part of the year. This suggests that HNF grazing could be more important in controlling bacterial abundance during the warmer part of the year, and viral lysis during the colder part of the year.

Keywords: Viruses, Bacteria, Heterotrophic nanoflagellates, Distribution of viruses, Adriatic Sea.

Introduction

Viruses are the most abundant and ubiquitous component of marine microbial plankton (Thingstad *et al.*, 1993; Bratbak *et al.*, 1994; Fuhrman, 1999; Wommack & Colwell, 2000; Weinbauer, 2004; Suttle, 2005). Despite their small size, they represent a substantial biomass in the marine environment (Kirchman, 2008). Studies of marine viral abundance have been performed in various locations and habitats worldwide. Viral counts typically range from 10^4 to 10^8 per mL^{-1} depending on the trophic state of the study area. Viruses are in general an order of magnitude more abundant than marine bacteria. The important role of viruses in the microbial loop has been recognized and heavily studied in the last two decades. Marine viruses are significant agents in controlling the marine bacteria and phytoplankton, and represent a major force behind biogeochemical cycles (Fuhrman, 1999; Wommack & Colwell, 2000; Suttle, 2007). Today, it is generally accepted that viruses are responsible for about 10–70% of total bacterial mortality and can cause a 10–20% loss of bacterial production (Heldal & Bratbak, 1991; Fuhrman, 1999; Jacquet *et al.*, 2010). By lysing marine bacteria, viruses cause the release of particulated (POC) and dissolved (DOC) organic carbon, which can affect bacterial community structure and have an impact on the bacterial carbon cycle (Bongiorni *et al.*, 2005). Furthermore,

the virally induced mortality of bacteria in the marine environment can affect the flux of nutrients and organic matter by increasing their recycling through the microbial loop (Fuhrman, 1999).

In the Adriatic Sea, the studies of marine viral abundances were carried out mostly in the northern Adriatic Sea (Weinbauer *et al.*, 1993; Weinbauer & Peduzzi, 1994; Weinbauer *et al.*, 1995; Luna *et al.*, 2002; Corinaldesi *et al.*, 2003; Stopar *et al.*, 2003; Bongiorni *et al.*, 2005; Karuza *et al.*, 2010, 2012), whereas data from the eastern coastal Adriatic Sea are scarce. In a comprehensive study, Corinaldesi *et al.* (2003) determined the viral abundance throughout the Adriatic Sea, but did not collect data from Croatian territorial waters.

In this paper, complete distribution data for marine viruses along the eastern coast of the central and southern Adriatic Sea and in the open central Adriatic are presented. The main purpose of the study was to demonstrate the seasonal distribution of marine viruses and their relationship with marine bacteria and heterotrophic nanoflagellates in the coastal and open Adriatic Sea.

Materials and Methods

The abundance of viral, bacterial and heterotrophic nanoflagellates was determined at 23 stations across the coastal area of the central and southern Adriatic Sea, and

at two stations located in the open sea area of the central Adriatic (Fig. 1). Sampling was carried out monthly from September 2010 to September 2011. Samples were collected using 5-L Niskin bottles at standard oceanographic depths from the surface to the seabed in 5 to 10 m intervals. Temperature and salinity were recorded using the SeaBird 25 CTD profiler.

The abundance of marine viruses was determined as previously described in Noble and Fuhrman (1998), with slight adjustments. Collected samples were preserved in formaldehyde (2%, final concentration) and frozen at -80°C until analysis, which was performed in the laboratory within 24 hours after the end of the cruise. Preserved samples (2 mL) were filtered through 0.02-µm filters (Anodisc; diameter: 25 mm; Al₂O₃, Whatman) and stained with SYBR Green I (stock solution diluted 300×). Filters were incubated in the dark for 20 min and mounted on glass slides with a drop of 50% phosphate buffer (6.7 mM, pH 7.8) and 50% glycerol, containing 0.5% ascorbic acid. Slides were stored at -20°C until analysis. Viral counts were obtained by epifluorescence microscopy (Olympus BX 51, equipped with a blue excitation filter) under magnification of 1,250× (objective 100×, ocular 12.5×), and are expressed as virus-like particles (VLP) per mL.

The abundance of marine bacteria was determined by flow cytometry (Beckman Coulter Epics XL MCL) as described in Marie *et al.* (1997). Samples for heterotrophic bacteria were preserved in 2% formaldehyde and stored at 4 °C until analysis (5-10 days). 1 mL samples were stained with SybrGreen I and analysed on the Beckman Coulter EPICS XL-MCL (high flow rate from 1 to 1.2 µl s⁻¹). To standardise fluorescence intensity of cells,

1 µm yellow-green beads were added (Level-III Epics Division of Coulter Corporation Hialeah, Florida). Two groups of bacteria were distinguished according to their relative green fluorescence as a proxy for nucleic acid content (Jochem, 2001), referred to as the high nucleic acid (HNA) and the low nucleic acid bacteria (LNA), and light scattering.

Bacterial cell production was determined using the ³H-Thymidine incorporation techniques (Fuhrman & Azam, 1980). Conversion factors for bacterial production were calculated from bacterial cell number and ³H-thymidine incorporation during bacterial growth in 1 µm pre-filtered seawater (Riemann *et al.*, 1987) $CF = (N2 - N1) / ^3H$, where N1 and N2 represent the numbers of bacteria at the beginning and the end of the experiment, respectively, and ³H is the integrated ³H-thymidine incorporation rate during the experiment.

The number of heterotrophic nanoflagellates (HNF) was estimated using epifluorescence microscopy. Samples were stained with 4-6-diamidino-2-phenylindole (DAPI) for 10 min and filtered through 0.8-µm pore diameter polycarbonate filters (Millipore, Ireland). Microscope slides were observed with an Olympus microscope under UV light illumination at a magnification of 1000× (Porter & Feig, 1980).

The relationship among the investigated parameters was determined using Pearson's rank correlation index after testing the normal distribution of the data. Analysis of variance (ANOVA) and *t* tests were used to determine the differences in microbiological parameters throughout the water column and between investigated stations.

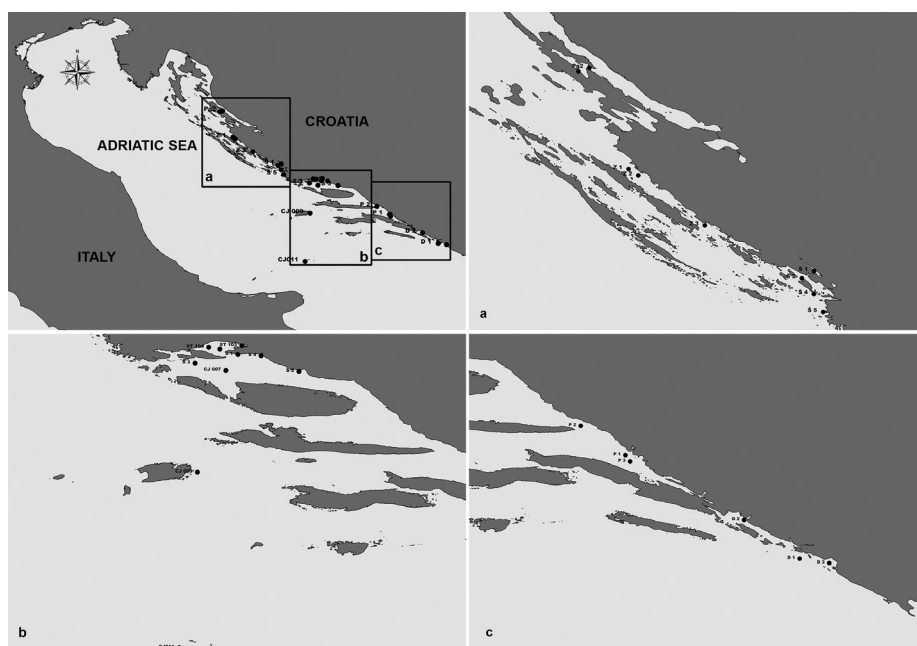


Fig. 1: Study area with sampling stations: a) Coastal areas: Pag (PG1, PG2), Zadar (Z1–Z3), Šibenik (Š1–Š3); b) Coastal areas: Kaštela Bay (ST101–ST104), Split (S1–S5, CA007), open sea stations (CA009, CA011); c) Coastal areas: Ploče (P1–P3), Dubrovnik (D1–D3).

Results and Discussion

Viral abundance and distribution

This study presents the first record of the seasonal distribution of marine viruses in the coastal and offshore area of the central and southern Adriatic Sea. The mean abundance of marine viruses, heterotrophic bacteria, heterotrophic nanoflagellates, the mean values of bacterial production and the virus-to-bacteria ratio (VBR), temperature and salinity in the investigated area are shown in Table 1.

Viral abundance in the mid-Adriatic coastal sea area ranged from 4.84×10^6 VLP mL⁻¹ to 27.32×10^6 VLP mL⁻¹, with a mean of $11.56 \pm 3.26 \times 10^6$ VLP mL⁻¹, and in the southern Adriatic coastal sea area, from 3.93 to 11.57×10^6 VLP mL⁻¹, with a mean of $7.49 \pm 2.24 \times 10^6$ VLP mL⁻¹. Notably, the fluctuation in viral abundance in the southern Adriatic was less pronounced compared to that in the central Adriatic. In the central Adriatic open sea area, viral abundance was similar to that in the southern Adriatic coastal sea area and ranged from 3.55 to 13.14×10^6 VLP mL⁻¹ with a mean of $7.51 \pm 1.85 \times 10^6$ VLP mL⁻¹. Results obtained in the sampling period fall within the range of previously published data for the coastal and estuarine environments of the north Adriatic Sea (Weinbauer *et al.*, 1993, 1995; Corinaldesi *et al.*, 2003; Stopar *et al.*, 2004; Bongiorno *et al.*, 2005; Karuza *et al.*, 2010, 2012) and the Mediterranean Sea (Alonso *et al.*, 2001; Weinbauer *et al.*, 2003; Magagnini *et al.*, 2007; Boras *et al.*, 2009; Magiopoulos *et al.*, 2012).

The viral abundance decreased from the more productive coastal sea to the open sea area along the trophic gradient, following the Kaštela bay to Palagruža Island transect

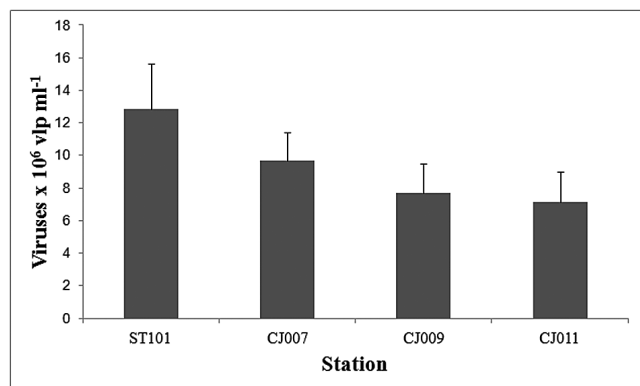


Fig. 2: Abundance of viruses along the trophic gradient following the Kaštela bay to Palagruža Island transect (mean value ± SD).

(ANOVA, $P < 0.01$) (Fig. 2). Weinbauer *et al.* (1993) also determined a decrease in viral number following a transect from the river Po to Rovinj in the north Adriatic, similar to Bongiorno *et al.* (2005), who observed a decrease in viral number following a transect from the river Po to the open sea area, with a 2.5-fold higher abundance in the eutrophic than in the oligotrophic area.

Significant differences ($P < 0.01$, ANOVA) in viral abundance were observed along the investigated coastal sea stations from Pag to Cavtat, as well as a difference in viral numbers along a trophic gradient from the coastal to the open sea area (Fig. 3). The lowest viral abundances were determined in the Dubrovnik area, with a mean of $7.49 \pm 2.24 \times 10^6$ VLP mL⁻¹, which is similar to the viral abundance for the oligotrophic part of the eastern Mediterranean Sea (Weinbauer *et al.*, 2003; Magiopoulos *et al.*, 2012). Hwang & Cho (2002) stated that the typical viral abundance in oligotrophic waters is 6×10^6 mL⁻¹, which agrees with our data for the open central and southern Adriatic Sea. Previous studies of various environmental parameters (inorganic salts, biomass of phytoplankton, heterotrophic bacteria, bacterial production and HNF) in the coastal area of the south Adriatic Sea also showed that this area can be classified as oligotrophic (Šantić, 2010).

Significant differences were observed in the vertical distribution of viruses between the surface and bottom layers at all investigated coastal (t -test, $p < 0.05$) and open sea (t -test, $p < 0.03$) stations. The extreme variations in viral numbers between surface and bottom layers were determined at station ST101 in Kaštela Bay with a maximum value of 20.20×10^6 VLP mL⁻¹ in the surface layer and in the Šibenik area in the surface layer of station Š1, where viral abundance reached 27.32×10^6 VLP mL⁻¹. These stations

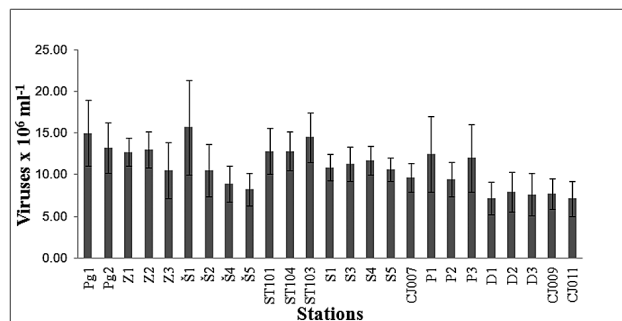


Fig. 3: Mean annual values of viral abundance at the sampling stations (mean ± SD).

Table 1. Temperature, salinity, viral abundance, bacterial abundance, virus-to-bacteria ratio (VBR), bacterial production (BP), percentage of LNA and HNA bacterial groups and abundance of HNF at the investigated coastal and open sea stations (mean value ± SD).

	Temperature (°C)	Salinity (‰)	Viruses (10 ⁶ mL ⁻¹)	Bacteria (10 ⁶ mL ⁻¹)	VBR	BP (10 ⁴ mL ⁻¹ h ⁻¹)	HNA (%)	LNA (%)	HNF (10 ³ mL ⁻¹)
Coastal Sea	16.71 ± 3.97	37.31 ± 1.89	11.24 ± 3.45	0.50 ± 0.21	24.59 ± 9.71	0.18 ± 0.10	42.37 ± 8.33	57.63 ± 8.33	0.94 ± 1.08
Open Sea	17.53 ± 3.74	38.43 ± 0.25	7.51 ± 1.85	0.31 ± 0.08	24.76 ± 6.12	0.11 ± 0.04	37.34 ± 5.01	62.66 ± 5.01	0.63 ± 0.51

are heavily influenced by the Jadro and Krka rivers, whose waters are rich in organic matter and nutrients. This is responsible for a higher abundance of heterotrophic bacteria, which are the main viral hosts, and ultimately probably for the higher abundance of marine viruses. According to previously published data, a negative correlation between viral abundance and salinity was observed in the estuarine area, which can be related to an input of viral particles with riverine water. Since fresh water spreads on top of the surface layer, this might result in an increased viral abundance in the surface layers of estuarine areas (Weinbauer *et al.*, 1993; Maranger & Bird, 1995).

The present study showed the highest viral abundance during spring and autumn at the investigated coastal stations in the central and southern Adriatic Sea. The lowest viral abundances were observed in early summer (June) at all sampling stations (Fig. 4a). Similar results was reported by Jacquet *et al.* (2010) who stated that a higher abundance of marine viruses was observed during early spring, summer and at the end of autumn, whereas a lower viral abundance was observed during the winter. In the northern Adriatic, a high viral abundance was observed during the summer and late autumn period (Weinbauer *et al.*, 1993; Weinbauer *et al.*, 1995). Although there are many potential causes behind the relative low viral numbers found in early summer, we were not able to determine the cause. Decay of viral particles in response to

various environmental parameters or lysogeny could be the most common reason but it was discarded in a parallel study we conducted (unpublished data). Another possible explanation for this unexpected summer minimum might be a substantial increase in the abundance of heterotrophic nanoflagellates at coastal and open sea sampling stations. The summer HNF abundance was three-fold and two-fold higher at coastal and open sea stations, respectively, than the abundance during the winter. We assumed that HNF significantly controlled the bacterial population during the warmer part of year, whereas viruses controlled the bacterial population during the colder part of year. We observed that viral abundance started to increase with a decrease in the abundance of HNF and bacteria in the investigated coastal area, which represents the beginning of viral domination over bacterial populations (Fig. 4c).

Viruses in relation to heterotrophic bacteria

We determined statistically significant correlations between marine viruses and heterotrophic bacteria at the investigated coastal sea stations ($r = 0.58$, $n = 490$, $p < 0.05$) and at the open sea stations of the central Adriatic ($r = 0.49$, $n = 130$, $p < 0.05$) (Table 2). The determined correlations suggest that bacteria were the main host for viral replication and are an important factor in the control of viral abundance. A similar correlation was found by

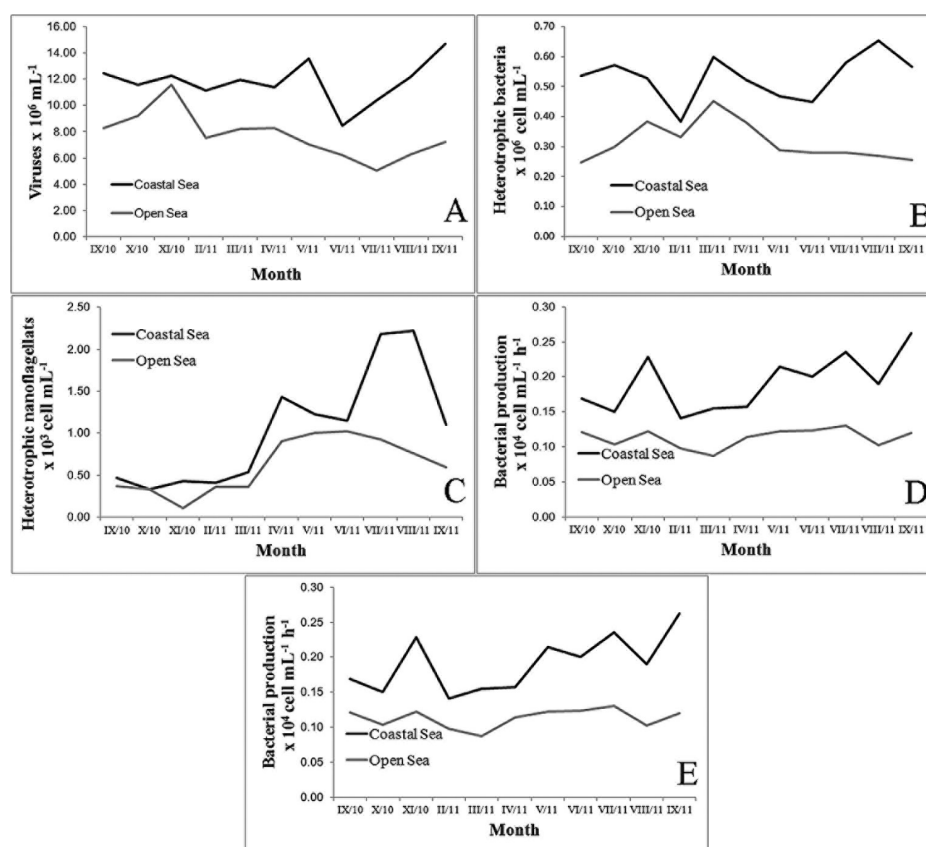


Fig. 4: Mean monthly abundance of viruses (a), heterotrophic bacteria (b), HNF (c) and mean monthly bacterial production (d) and virus/bacterium ratio (e).

Weinbauer *et al.* (1993) and Stopar *et al.* (2003) in the north Adriatic Sea, by Boehme *et al.* (1993) in the Mexican Gulf and by Cochlan *et al.* (1993) in the southern part of the Californian Bay and the Chucki Sea.

When the results from sampling stations in the Šibenik area and Kaštela Bay were analysed separately, the statistical correlation between marine viruses and heterotrophic bacteria was even stronger ($r = 0.74$, $n = 49$, $p < 0.05$). At the same sampling stations, a significant statistical correlation between bacterial production and viral abundance was also found. The area near Šibenik is greatly influenced by the Krka, Čikola and Gudača Rivers and is also under the influence of the waste waters of the cities of Šibenik, Drniš and Knin, which is reflected by low salinity in the surface marine layer (Šantić, 2010). Accordingly, a high concentration of organic and inorganic matter, which is transported by the riverine water stimulates the growth of bacterioplankton (Šantić *et al.*, 2012) and eventually virioplankton since viral abundance is closely correlated with the abundance of bacteria (Jacquet *et al.*, 2010).

The sampling station situated in the Vranjic Basin (Kaštela Bay) is under the strong influence of River Jadro, which enriches this area with nutrients that stimulate a high

abundance of both bacteria and viruses (Šolić *et al.*, 2010). Results in this paper also demonstrate a high negative statistical correlation between salinity and viral abundance at the previously mentioned sampling stations, where lower salinity was measured. The statistical correlation between salinity and viral abundance at coastal sampling stations was negative and lower ($r = -0.45$, $n = 490$, $p < 0.05$) than that at the open sea sampling stations ($r = -0.61$, $n = 130$, $p < 0.05$). Data for the correlation between viruses and other investigated parameters are shown in Table 2.

Viruses in relation to HNA and LNA bacterial groups

A low correlation between marine viruses and heterotrophic bacteria groups with a high (HNA) or low (LNA) nucleic acid content was determined at all investigated stations. Marine viruses and HNA bacteria at coastal sea stations were positively correlated, whereas at open sea stations they were negatively correlated (Table 2). This might demonstrate the dependence of marine viruses on the HNA bacterial group at coastal sea and the LNA bacterial group at open sea stations. This demonstrates that there is no dependence of marine viruses on any bacterial group regarding their nucleic acid content. At all stations,

Table 2. Pearson's correlation coefficients between viruses, heterotrophic bacteria (HB), heterotrophic nanoflagellates (HNF), low nucleic acid (LNA) and high nucleic acid (HNA) bacterial groups, bacterial production (BP), temperature and salinity.

Coastal Sea n=490, P<0.05	Viruses	HB	BP	HNF	HNA	LNA	Temperature	Salinity
Viruses	1.00	0.58	0.32	0.27	0.3	-0.3	-0.01	-0.45
HB	0.58	1.00	0.47	0.43	0.2	-0.2	0.24	-0.40
BP	0.32	0.47	1.00	0.31	0.2	-0.2	0.26	-0.25
HNF	0.27	0.43	0.31	1.00	0.2	-0.2	0.22	-0.40
HNA	0.25	0.24	0.24	0.20	1.0	-1.0	-0.24	-0.34
LNA	-0.25	-0.24	-0.24	-0.20	-1.0	1.0	0.24	0.34
Temperature	-0.01	0.24	0.26	0.22	-0.2	0.2	1.00	0.07
Salinity	-0.45	-0.40	-0.25	-0.40	-0.3	0.3	0.07	1.00
Š1 and ST103 n=49, P<0.05	Viruses	HB	BP	HNF	HNA	LNA	Temperature	Salinity
Viruses	1.00	0.74	0.59	0.63	0.4	-0.4	0.37	-0.60
HB	0.74	1.00	0.68	0.54	n.s.	n.s.	0.57	-0.51
BP	0.59	0.68	1.00	0.40	0.3	-0.3	0.49	-0.32
HNF	0.63	0.54	0.40	1.00	n.s.	n.s.	0.33	-0.56
HNA	0.43	n.s.	0.31	n.s.	1.0	-1.0	n.s.	-0.54
LNA	-0.43	n.s.	-0.31	n.s.	-1.0	1.0	n.s.	0.54
Temperature	0.37	0.57	0.49	0.33	n.s.	n.s.	1.00	n.s.
Salinity	-0.60	-0.51	-0.32	-0.56	-0.5	0.5	n.s.	1.00
Open Sea n=130, P<0.05	Viruses	HB	BP	HNF	HNA	LNA	Temperature	Salinity
Viruses	1.00	0.49	n.s.	-0.34	-0.34	0.34	n.s.	-0.61
HB	0.49	1.00	n.s.	n.s.	n.s.	n.s.	-0.44	-0.54
BP	n.s.	n.s.	1.00	n.s.	n.s.	n.s.	n.s.	n.s.
HNF	-0.34	n.s.	n.s.	1.00	0.18	-0.18	0.31	0.20
HNA	-0.34	n.s.	n.s.	0.18	1.00	-1.00	-0.17	0.25
LNA	0.34	n.s.	n.s.	-0.18	-1.00	1.00	0.17	-0.25
Temperature	n.s.	-0.44	n.s.	0.31	-0.17	0.17	1.00	n.s.
Salinity	-0.61	-0.54	n.s.	0.20	0.25	-0.25	n.s.	1.00

LNA bacteria slightly dominated over HNA bacteria, which has been previously described for the southern and central Adriatic Sea by Šantić (2012) (Fig. 5).

The prevalence of LNA bacteria can be explained by their morphological characteristics and better competition for food resources in an oligotrophic environment, compared to HNA bacteria (Button, 1998; Jochem *et al.*, 2004). Moreover, since LNA bacteria have smaller cells and a higher surface-to-volume ratio, Zubkov *et al.* (2001) observed that they have a higher specific growth rate in an oligotrophic environment than HNA bacteria, which is responsible for the better survival of this bacterial group.

Exceptions to our results were observed at stations in the Šibenik area (Š1) and Kaštela Bay (ST103), where a slight predominance of HNA bacteria was determined. This greater abundance of HNA bacteria is related to a higher trophic state and to higher bacterial production in the area, followed by high viral and bacterial abundance, which is the opposite to that in the other investigated areas. A significant statistical correlation between viruses and HNA bacteria was determined ($r = 0.4$, $n = 49$, $p < 0.05$) in this area, whereas at all other stations, the correlation between viruses and HNA or LNA bacteria was low as mentioned previously ($r = 0.1$ HNA, $r = -0.1$ LNA, $n = 441$, $p < 0.05$).

The abundance of LNA bacteria was slightly greater than that of HNA bacteria in the open sea area of the central Adriatic (Fig. 6) and statistical correlation showed that marine viruses were more dependent on this group of bacteria than on HNA bacteria ($r = 0.34$, $n = 130$, $p < 0.05$).

Therefore, in most cases, viral abundance showed better correlation with the most abundant bacterial group (HNA or LNA), suggesting that viruses generally did not show preference to any particular bacterial group according to their nucleic acid content.

Virus-to-bacterium ratio

The virus-to-bacterium ratio (VBR) is a good parameter that can be used to monitor the predominance of viral abundance over heterotrophic bacteria in the marine environment (Jacquet *et al.*, 2010). The VBR in different marine environments varies from 3 to 100 with a mean of 25. A higher VBR is characteristic of eutrophic and more productive sea areas. Nutrient-rich environments stimulate higher growth and production of bacteria, which influences viral production and thus increases the VBR (Jacquet *et al.*, 2010).

The VBR determined for the coastal stations of the southern and central Adriatic Sea varied from 8 to 76, with a mean of 25 ± 10 , whereas the VBR at the open sea stations of the central Adriatic Sea varied from 14 to 40, with a mean of 25 ± 6 . The VBR for coastal sea stations was similar to the values determined for the northern Adriatic Sea. The VBR at the open sea stations of the central Adriatic was within the range of data reported for the Mediterranean Sea. The highest VBR was determined in the northern part of the central Adriatic, suggesting a high dominance of viruses over bacteria within this area (Fig. 7).

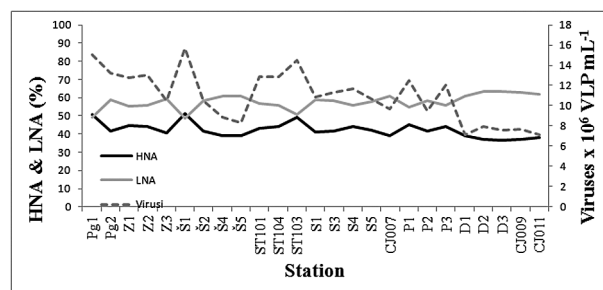


Fig. 5: Mean annual value of viral abundance compared to mean annual values of LNA and the HNA heterotrophic bacteria in the study area.

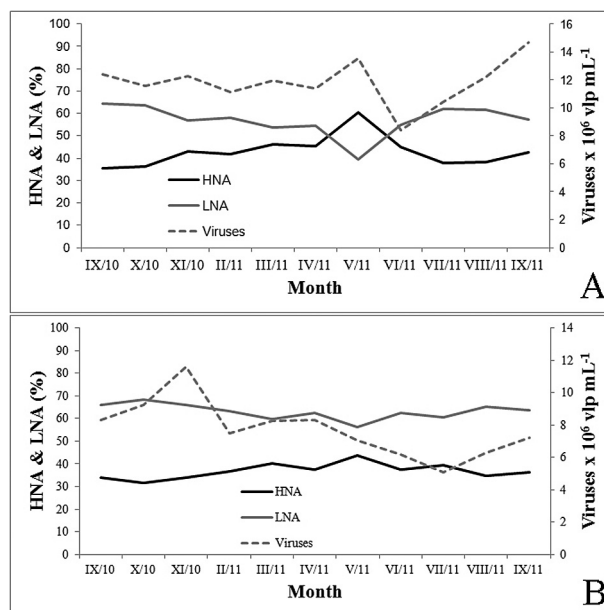


Fig. 6: Mean monthly viral abundance in relation to the HNA and LNA bacterial groups during the sampling period for coastal (a) and open (b) sea areas.

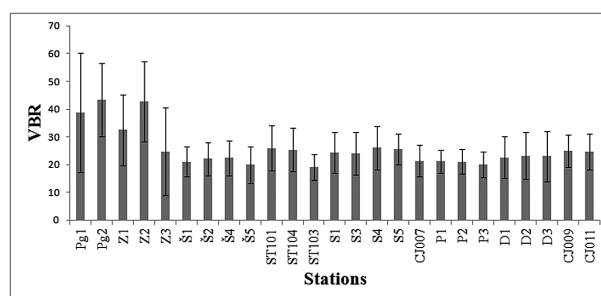


Fig. 7: Mean annual values of virus to bacteria ratio at the sampling stations (mean \pm SD).

In the coastal sea area, we observed a strong dominance of viruses over bacteria in February, May and June, when lower abundances of HNF were recorded. The abundance of HNF was high during the warmer part of the year at the coastal sea stations, causing a lower VBR, compared to the winter VBR. At the open sea stations, a lower VBR was determined during March, when bacterial abundance was high, and during July, when a high abundance of bacterial predators (HNF) was observed (Fig. 4e).

According to previously published data, similar ranges of the VBR were determined for the Mediterranean area and north Adriatic sea, varying from 4 to 44 (Alonso *et al.*, 2001; Weinbauer *et al.*, 2003; Magagnini *et al.*, 2007; Boras *et al.*, 2009; Magiopoulos *et al.*, 2012) and from 1 to 89 respectively (Weinbauer *et al.*, 1993, 1995; Corinaldesi *et al.*, 2003; Bongiorno *et al.*, 2005; Karuza *et al.*, 2010).

A high range of VBR, especially at the coastal sea stations of the central and southern Adriatic Sea, shows the variation in viral communities caused by different trophic states in the sampling areas. Variations might also be due to viruses infecting different types of host cells. Furthermore, since viral abundance changes in a short period of time, it is possible that sampling at different time scales results in viruses released from host cells at different phases of infection, which ultimately greatly affect the VBR (Siokou-Frangou *et al.*, 2010).

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