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VIRIOPLANKTON ABUNDANCE IN TROPHIC GRADIENTS OF AN UPWELLING FIELD

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ABSTRACT

This work correlates time series of biological and physical variables to the marine viruses across trophic gradients within Arraial do Cabo upwelling system, Southeast of Brazil. The objective is to investigate the major controlling factors of virioplankton dynamics among different water masses. It was used an *in situ* and *ex situ* flow cytometry for accessing the plankton community. Viruses were highly correlated to bacteria and phytoplankton, but although the lack of direct correlation with physicals, upwelling turned out to be the main contributing factor to the highest values of viral abundance and virus:bacterial ratio. Our data suggest that the lowest temperature of upwelled South Atlantic Central Waters would help to maintain a high viral abundance and higher temperatures of Coastal and Tropical Waters might be another ecological niche allowing the co-existence.

Keywords: Marine virus, coastal waters, trophic gradients, upwelling system

INTRODUCTION

Nowadays, viruses are considered ubiquitous and ecologically important members of aquatic communities influencing biogeochemical cycles, community composition and horizontal gene transfer (18, 24, 38, 42). The use of transmission electron microscopy (TEM) for investigating aquatic viruses were followed by the epifluorescence microscopy (EFM) combined with the development of a variety of highly fluorescent nucleic acid dyes. More recently, flow cytometry (FCM) emerged as a new method, because it is a faster technology for direct counts. There have only been a few studies comparing the efficiency of these different techniques (4, 11), even though FCM has been reported to be 1 and 2 times more efficient (11, 26). It has been successfully used to analyze and count microbial communities such as protist, small algae, bacteria and viruses (3, 14, 25, 27, 35).

Currently, our best estimates range from ~3 X 10^6 viruses/ml in deep sea hydrothermal vent system (30) to ~10^8/ml viruses in the more productive coastal waters. It is also known high viral concentration (10^6–10^{10}) in marine surface sediments (16, 20). Most of them are phages that infect bacteria but there is a diverse community infecting all other organisms (28, 38).

There are two major pathways of phage replication (43).

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In the lytic cycle, the phage genome replicates immediately after infection and release progeny during lysis of the host cell. In the lysogenic cycle, a temperate phage genome is integrated into the host chromosome where it is carried in a dormant form (prophage) for generations until induction of lytic cycle by environmental factors. Thus, lytic phage production depends on encouter rates between virus and host, whereas, lysogenic phage production depends on the number of lysogens in the community and the presence of an induction factor. Comparisons of metagenomic analysis between near shore sediment samples and previously sequenced seawater viral communities showed that certain phage phylogenetic groups were abundant in both marine communities, while others were under represented or absent (7).

However, the factors influencing the dynamics of viral abundance in aquatic environments might be dependent upon complex interactions of both abiotic factors such as pressure, redox conditions, temperature, light effects, and biotic like host abundance, host metabolic rate, viral supply (2, 23, 46). The interaction between these factors and virus remains poorly understood. Many works have reported viruses along several environments and trophic conditions (44), from oceanic (12) to neritic and estuary (22), from euphotic and oxic surface to deep or anoxic waters since the tropics to the arctic (31). To date, and to our knowledge, no work has ever been done in upwelling areas and this is why we present this preliminary investigation. So, the aim of this paper is to present the temporal abundance of viral community and its relationship with some biotic and abiotic variables at the Arraial do Cabo upwelling system.

MATERIALS AND METHODS

Studied areas

The Southwest Atlantic Ocean off Brazil is known by its oligotrophy due to the prevailing Brazil Current (BC) that runs southwards, carrying the Tropical Water (TW) from the vicinity of the Equator (19). Moving in the bottom on the opposite direction, there is the cold South Atlantic Central Water (SACW) mass. In Arraial do Cabo, Northeast of Rio de Janeiro state (Figure 1), the positioning of the Cabo Frio island (23^0S, 42^0W) related to the coast line forms the small ($45Km^2$) and narrow (~10m depth) Anjos inlet. This site can be considered yet a pristine area and the hydrologic conditions are strongly influenced by the winds that determine the distribution of water masses. The action of E-NE winds results in a shunting of the nutrient-depleted (<1 µM l^{-1} NO_3-N) surface TW of Brazil Current to offshore followed by the up-flow of the deeper (~300 meters) and nutrient-rich (~12 µM l^{-1} NO_3-N) of SACW (33). The inverse pattern comes with the S-SW winds when cold fronts bring the oligotrophic TW back to the coast. According (41) these processes have a direct impact on the quantity and composition of the phytoplankton communities, shifting the trophic structure.

Figure 1. The studied area and sampling site location.

Sampling procedure

A total of 38 seawater samples were weekly collected at the surface (0,5 m depth) with a Nansen bottle with reverse thermometer outside, and in the bottom (water/sediment interface), by scuba diving using a 2 liters polyethylene bottle (three samples). The salinity, oxygen and nutrients (PO_4,

NO_2, NO_3, NH_4) were determined ashore as described in (37). Subsamples of 200 ml were immediately fixed with 4% of paraformaldehyde at final concentration (filtered in 0.02 m Anodisc, Whatman Inc., Piscataway, NJ) for further cytometric (~3h) analyzes. Merozooplankton larvae were collected by means of 100 mesh plankton net. The water mass identification were made according to the interval of temperature and salinity data provided by the Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM) as shown in Table 1.

Flow Cytometry

Phytoplankton enumerations were performed *in situ* with the CytoBuoy bench top (17) flow cytometer (Cytobuoy b.v. Nieuwerbrug, The Netherlands). It is equipped with a solid blue laser providing 20 mW at 488 nm, one side scatter (SWS, 446/500 nm) detector and three others to red (chlorophyll-a) fluorescence (FL-1, 669/725 nm); orange/yellow (FL-2, 601/651) and green/yellow (FL-3, 515/585 nm) fluorescence respectively. It was left to run for 3 min at a fixed flow rate of 2 m/s and the discriminator was set on red fluorescence. Parameters were collected on log scale using the CytoSift software and analyzed in the CytoClus software both provided by the manufacturer (Figure 2a).

The enumeration of bacteria and virus were performed with a FACScan flow cytometer (Becton Dickson, San Jose, Calif.) equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter setup. A yellow-green 0,92-µm beads (Fluoresbrite Microparticles, Polysciences Inc.-Warrington, PA) were added in all samples as an internal standard (Figure 2b). All samples were passed through a MF 0,45µm filter (Millipore). Bacterial samples were stained with SYBR-Green-I at a final concentration of 0.5 X 10^{-4} of the commercial stock solution (27). The samples were incubated at room temperature for 15 min in the dark, the discriminator was set on green fluorescence, and the samples were analyzed for 1 min at a rate of 50 µl/min.

For virus enumeration, we performed dilutions from 1:10 to 1:200 in previously filtered (0.02□m Anodisc, Whatman Inc., Piscataway, NJ) TE buffer (10mM Tris, 1 mM EDTA [pH 8.0] to avoid coincidence on the flow cytometer and to minimize the error due to low-volume pipeting. These dilutions were heated at 80ºC for 10 min in the dark in presence of SYBR-Green-I at a final concentration of 0.5 X 10^{-4} and left to cool for 5 min according (8). The samples were analyzed in the FACScan flow cytometry for 1 min at a delivery rate of 50 µl/min. The cytometer was triggered to green fluorescence and the detection threshold was progressively decreased until viruses could be detected. The parameters for bacteria and viral counts were collected on log scale and analyzed in the CellQuest™ Pro software provided by the manufacturer.

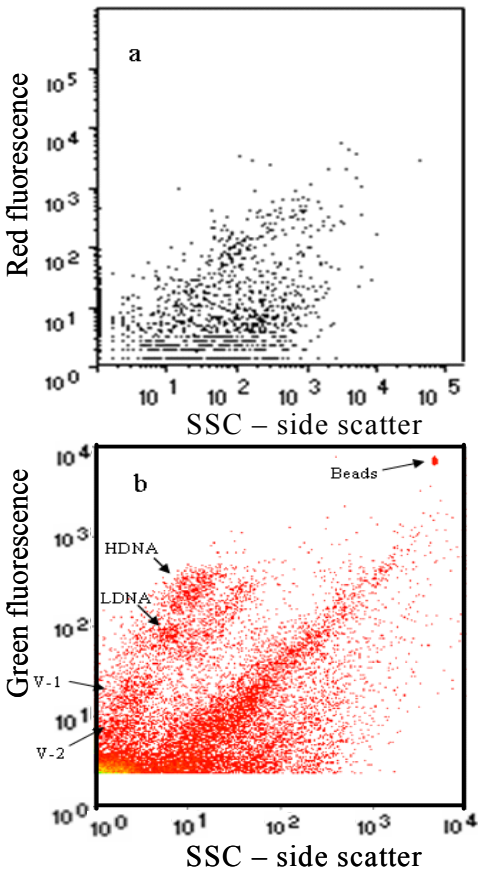

Figure 2. Dot plots of citometric data. In a, the red fluorescence *vs* side scatter signals of *in situ* real time phytoplankton monitoring. b, green fluorescence of stained nucleic acids of bacteria (HDNA and LDNA) and virus (V-1 and V-2).

Table 1. Temperature and salinity characteristics of Arraial do Cabo Water masses

Water Mass	T°C	S
SACW	T<18	S<36
SACW/COASTAL	18<T<20	35,4<S<36
COASTAL	T>20	S<35,4
SACW/TROPICAL	18<T<20	S>36
COASTAL/TROPICAL	T>20	35,4<S<36
TROPICAL	T>20	S>36

Source – Brazilian navy oceanographic department

Statistical Analyses

A Correlation matrix (Table 2) describes correlation among M variables. It is a matrix of n random variables X_1, ..., X_n is the n x n matrix whose i,j entry is corr(X_i,X_j). If the measures of correlation used are product-moment coefficients, the correlation matrix is the same as the covariance matrix of the standardized random variables X_i /SD(X_i) for i = 1,..., n. Consequently it is necessarily a positive-semidefinite matrix. The correlation matrix is symmetric due to the correlation between X_i and X_j is the same as the correlation between X_j and X_i. The diagonal elements are correlations of variables with themselves and are always equal to 1.

There are different coefficients for many situations. The correlation coefficient denotes any relationship, not necessarily a linear relationship between two variables. It always lies between -1 and +1. -1 indicates a perfect and negative relationship between two variables, +1 indicates a perfect positive relationship while 0 indicates lack of any relationship. We used the well known Pearson product-moment correlation coefficient, which is obtained by dividing the covariance of the two variables by the product of their standard deviations. All data analyses were performed in the Statistica 6.0 software.

Table 2. Studied variables: V1 e V2 (viral sub-groups of low and high fluorescence), VT, total virus (sum of V1 and V2), HET BAC, heterotrophic bacteria, PHYTO, phytoplankton, Larvae, total of meroplankton larvae, TEMP, temperature, SAL, salinity, O2, oxygen, PO4, phosphate, NO2, nitrite, NO3, nitrate, NH4, ammonium, VBR, virus bacteria ratio and BAC PHYTO, bacteria phytoplankton ratio . Significance in bold numbers. Level of significance $p < 0.05$.

Variables	V1	V2	VT	HET BAC	PHYTO	LARVAE	TEMP	SAL	O2	PO4	NO2	NO3	NH4	VBR	BAC PHYTO
V1	1	**0,88**	**0,64**	**0,91**	**-0,91**	-0,12	-0,01	-0,07	0,27	0,23	-0,03	0,10	0,14	0,26	-0,12
V2		1	**0,73**	**0,97**	**0,97**	**-0,37**	-0,31	0,06	**-0,42**	-0,01	-0,08	0,02	0,00	-0,05	-0,19
VT			1	**0,76**	**0,76**	-0,22	-0,26	-0,04	**-0,36**	0,17	-0,02	0,09	0,07	0,18	-0,22
HET BAC				1	-0,05	**0,36**	0,27	0,26	-0,07	-0,14	-0,08	-0,23	-0,15	**-0,33**	**0,83**
PHYTO					1	-0,17	-0,12	0,00	0,01	-0,27	-0,10	-0,01	0,17	-0,18	-0,18
LARVAE						1	0,16	0,18	-0,18	-0,27	-0,06	-0,15	-0,16	-0,06	0,23
TEMP							1	**-0,44**	**-0,36**	0,07	0,01	-0,08	-0,19	**-0,36**	**0,35**
SAL								1	-0,16	-0,02	0,01	0,04	0,05	-0,16	0,19
O2									1	0,03	-0,01	0,01	0,23	0,29	0,00
PO4										1	0,18	0,20	-0,10	0,24	0,02
NO2											1	0,20	0,02	-0,03	-0,05
NO3												1	0,11	-0,10	-0,20
NH4													1	**0,35**	-0,28
VBR														1	-0,27
BAC PHYTO															1

RESULTS AND DISCUSSION

Each dot depicted in Figure 2 is a suspended particle read by the two cytometers. In the case of Figure 2a, it presents the spread of real time data of phytoplankton cells accquired (radio transmited) by the CytoBuoy flow cytometer once the red fluorescence signals are the results of chlorophyll-a response to laser excitation. The side scatter (SSC) is usually considered a measure of complexity. During the studied period the total phytoplankton concentration varied from 8,66 X 10^2 cells/ml in the summer to only 3,30 X 10^2 cells/ml in the winter. The Figure 2b shows the picoplankton particle distribution related to 1:100 dilution stained with SYBR-Green I accessed by the FACScan flow cytometer. It seens to be a general distribution found in many places and authors (1, 22, 31). It is easily recognized two clusters of bacterial populations (LDNA and HDNA) with different green fluorescence intensities, consequently different nucleic acid contents. Although the HDNA are always the most abundant during the studied period and considered more active (36), in this work the two bacterial groups were quantified together and the total bacterioplankton varied from 1,24 X 10^6 cells/ml in the summer to 1,51x 10^3 cells/ml in the winter. It is also verified two viruses population. V-2 with smallest fluorescence is usually considered as bacteriophages according (11, 34, 43) while for (23) V-1 is a diverse group that infects eukaryotes. The total virioplankton community varied from 2,86 X 10^6 particles/ml in summer (January 2007) to 6,21x 10^5 particles/ml in the same time of year (February 2007). The average and the standard deviation values of virus community among the three samples from the water/sediment interface were 5,62 X 10^7 particles/ml and 9.40 respectively. The highest (1,09 organism/ml) and smallest (0,01 organism/ml) values of meroplankton larvae were found in summer.

The correlations among biotic and abiotic variables are demonstrated in Table 2. The highest and most significant correlation is observed between heterotrophic bacteria (Het Bac) and the virus group V-2 (r^2 = 0.97, n = 38, p< 0.05), coincidently phytoplankton (Phyto) shows the same correlation to V-2. Although (11, 34, 43) have stated that V-2 are bacteriophages these results suggest that it is a diverse group and their statements can not be supported in our case. In the same way, V-1 presents a bit small but still high correlation to heterotrophic bacteria (r^2 = 0.91, n = 38, p< 0.05) and a negative correlation (r^2 = -0.91, n = 38, p< 0.05) with phytoplankton. This is a clear indication of the V-l sub population controlling phytoplankton. The total virioplankton community (VT), the sum of V-1 and V-2, also shown significance to bacteria and microalgae communities (r^2 = 0.76, n = 38, p< 0.05). Thus, our results suggest that both viral groups are equally active in bacteria and phytoplankton however; to date we do not have any indication about phage infecting eukaryotic cells. A more deep investigation would be necessary by means of sorting, concentration, bioassays and TEM. Table 2 still presents that V-2 is highly correlated to V-1 (r^2 = 0.88, n = 38, p< 0.05) and this is more correalted to VT than V-1 (r^2 = 0.73, n = 38, p< 0.05).

The meroplankton larvae (Larvae) present a negative significance (r^2 = -0.37, n = 38, p< 0.05) to V-2 and positive correlation to heterotrophic bacteria (r^2 = 0.36, n = 38, p< 0.05) indicating that virus infection may be a more effective factor controlling bacteria. It is also verified a negative correlation between temperature and salinity (r^2 = -0.44, n = 38, p< 0.05), an indication of upwelling process. Significances were also found with oxygen. It is negative correlated to V-2 (r^2 = -0.42, n = 38, p< 0.05), VT (r^2 = -0.36, n = 38, p< 0.05) and temperature (r^2 = -0.36, n = 38, p< 0.05). Although oxygen is quite stable in the studied area (around 5 mg/l) it is verified some increased values related to lower temperatures (data not shown) probably due to the turbulence of upwelling. Consequently, the up flow brings some viruses into the water column. The nutrients did not present any correlation to the other variables; notwithstanding the effect of phosphate on cyanophage infection has been demonstrated by (47). However, the ratio between VT and heterotrophic bacteria (VBR) shows an obviously and negative correlation with heterotrophic bacteria (r^2 = -0.33, n = 38, p< 0.05) and

interesting and negative significance with temperature (r^2 = -0.36, n = 38, p< 0.05) indicating higher viral amounts in deep waters and at the same time positively correlation to NH4 (r^2 = 0.35, n = 38, p< 0.05). It is well known that ammonia is a by-product of anaerobic digestion (21) and could enter the water column by the upwelling process. By the other hand, since 1983 (10) and others have shown that ammonia may cause the inactivation of bacteriophages nucleic acids as the temperature increase indicating a complex interaction between these factors and virus. The heterotrophic bacteria and phytoplankton ratio also show an obviously correlation with heterotrophic bacteria (r^2 = 0,83, n = 38, p< 0.05) and temperature (r^2 = 0.35, n = 38, p< 0.05). The last one indicates that the primary production takes place in warm waters (32).

Despite this, the Figure 3 shows a seazonal behavior of virioplankton with highest values occuring during the spring-summer period and the smallest in the autumn-winter period. Seazonality in virioplankton has been previously reported by (2, 43) but the present results demontrates a close relationship with upwelling events. In spite of the absence of statistical correlation it reveals an interesting pattern between temperature (Figure 3a), salinity (Figure 3b) and the virus community. It is not difficult to see that decreased temperatures and encreased salinities (see Table 1 for water mass identification) are slightly coupled to viruses and should have some influence in the total viral community. While many factors are involved in the survival of marine viruses, temperature probably plays the most important environmental parameter, and viable phages can be found from freezing such as sea ice and hot springs. (45) reported that a bacteriophage, which was isolated from a marine sediment sample, produced only plages below 23^{o}C, whereas the host could grow at temperatures up to 33^{o}C.

Salinity as dissolved salt content is another important environmental factor. In this way, (48) have shown that the differences in synthetic abilities of nt-1 and nt-6 phage-infected cells at various NaCl levels raises the possibility of natural selection based on salinity, since these phage compete for the same host cells in estuarine water. It is also known that the concentration of salts may affect the thermal resistance of viruses. Ecological niche conditions depending on chemical parameters are known as well (6). The ionic environment can affect marine phages at all stages of the life cycle such as the adsorption, replication, lytic activity and survival. For example, divalent cations such as Mg^{+2} or Mg^{+2} + Ca^{+2} frequently seem to have a positive effect on phages, whereas the effect of Na^{+} is quite variable. Such data suggest a strong effect of the ionic environment on the definition of the ecological niche of phages.

Clustering the samples according Table 1 allowed us to verify the average and standard variation values of the studied variables in each water mass. These data are shown in Table 3. It is evident that V-1 is numerically dominant over V-2. The virus community (VT) is more abundant than bacteria in at least one order of magnitud and bacteria are three decades higher than phytoplankton. The highest average values of virioplankton occurs in the SACW (n=4) while the highest mean values of bacteria, phytoplankton and meroplankton larvae occurs in the mixing of coastal and tropical waters (Cost/Trop, n=21). The cold water of SACW shows the smallest bacterial/phytoplankton ratio (Bac/Phyto) and the highest virus/bacterial ratio (VBR) indicating a great viral activity. It could explain why the more nutrient rich waters are not so productive.

Ecological Interpretation

Both (13) in lakes and (44) in seawater suggested that the quantity of viruses may reflect the trophic status of the ecosystem. Our inverse occurrence of Bac/Phyto ratio and VBR corroborate with their suggestions. It has been proposed on several occasions that sediments can constitute a potentially important reservoir of infectious bacteriophages from the water column (7), cyanophages (43) or algal viruses (29)**.** However, no processes involving the recruitment of viruses into or their release from sediments had so far been clearly demonstrated. Previous studies have shown that viruses can be absorbed onto sinking particles, and thus

carried down to the sea-floor (20) so that sediments receiving a high influx of particles might also receive large inputs of viruses from the water column. However, our data indicates the upwelling process can likely to play a key role by producing the resuspension of the virus and organic mater through the water column. Based in the literature and our results we speculate that during the up flow, environmental factors such as nutrients (15), temperature, light and host biochemical features (5) may act as viral activation factor resulting in high rates of infectivity and mortality of bacteria and phytoplankton nevertheless, the heating and the decreased host availability can induce virus to lysogenic life cycle as demonstrated by (46). In this way, the higher temperature and oligotrophic conditions of the coastal and tropical waters in the Arraial do Cabo upwelling system could be considered as ecological niches resulting in a steady state that allows the co-existence of all populations. One predicts impact of viral activity is increased net respiration, bur our small Bac/Phyto ratio in SACW has shown against. By shunting biotic carbon toward the release of dissolved organic matter, viruses also accelerate the recycling of potentially growth-limiting nutrient elements in the photic zone. Thus, a critical question is whether viruses hinder or stimulate biological production?

Figure 3. Temporal distribution of total virioplankton, temperature in a and salinity in b.

Table 3. Average and Standard deviation values of studied variables in three different water masses of Arraial do Cabo upwelling system.

Water mass	V1/ml	V2/ml	VT/ml	Het Bac/ml	Phyto/ml	Bac/Phyto	VBR	Larvae/m3	Temp °C	Sal ‰
SACW	**1,45E+06**	**8,02E+03**	**2,38E+06**	8,06E+04	1,14E+02	7	**28**	94,33	18,91	35,55
n = 4	± 2,55	± 6,22	± 3,12	± 8,05	± 9,75	± 20,80	± 36,64	± 80,33	± 0,64	± 0,36
COAST/TROP	8,19E+05	4,60E+03	1,40E+06	**2,63E+05**	**1,29E+02**	20	5	**328,21**	**23,12**	35,59
n = 21	± 4,13	± 2,40	± 6,57	± 2,84	± 2,16	± 75,49	± 32,72	± 189,14	± 2,02	± 0,20
TROPICAL	9,32E+05	5,89E+03	1,47E+06	1,40E+05	6,41E+01	**21**	11	231,27	22,84	**36,14**
n = 13	± 27,88	± 15,92	± 31,78	± 26,42	± 75,26	± 50,05	± 66,48	± 320,05	± 1,15	± 0,09

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