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Distribution and activity of virioplankton in the Toliara Large Reef (Madagascar)

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ABSTRACT

Until then, the study of aquatic viruses concerned almost exclusively temperate and polar environments while the tropics were rarely explored. In this study, we examined the spatial and temporal variability of viral and bacterial communities in two different tide periods (spring/ neap tide), on 22 stations covering the Toliara Large Reef (TLR) located in Madagascar. The coupling of these two communities was also analyzed in several contrasted stations of the reef by physiological and ecological descriptors means. The epifluorescence microscopy technique allowed to achieve the bacterial and viral count support of this work. The results obtained show that the bacterial and viral concentrations for the whole TLR were significantly higher during spring than neap tide, probably because of the hydrodynamics very marked in this area. In addition, the *Mitomycin C* used permitted to establish that the cells fraction in phase of lysogenic infection was very low (< 3%) despite the oligotrophic status of TLR waters. This suggests that the lysogeny was the viral reproduction dominant strategy, which was diverted spontaneously and in a continuous way towards the lytic pathway, under the solar conditions' influence. Finally, we show that the viruses form a dynamic and ubiquitous component of reef ecosystems, whose activity appears once again, closely related to the physico-chemical and biological environment nature.

Keywords: Virus, lysogeny, lysis, tropical latitude.

INTRODUCTION

Viruses constitute the most abundant biological entity in the water ecosystems. They are obligatory parasites potentially capable of infecting all living cells. Recent knowledge revealed that, these viruses do not represent only agents of the bacterioplankton control but also intervene in many ecological and biogeochemical processes such as nutrients recycling (Emma et al., 2012; Tsai et al., 2013), genetic materials transfer (McDaniel et al., 2010; Anderson et al. (2013) are suspected of affecting the diversity of the whole microbial communities (Paul, 2008). However, the majority of studies relating to

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virioplankton's ecology concern the temperate and polar areas, whereas this viral compartment is beginning to be examined in fresh water from tropical latitudes (Bettarel et al., 2009; Bettarel et al., 2010; Bettarel et al., 2011; Sime-Ngando et al., 2011). At the low latitudes (ecological niches) like coral reef zones, little informations are available regarding viral and microbial point of view (Marc Bouvy et al., 2012). To this end, the aim of this work is to study the environmental constraints that impact on the functioning of a coral reef ecosystem - the one of Toliara (Madagascar).

The Toliara Large Reef (TLR) is a medium, placed at the same time under continental and oceanic influence. Subjected to the consequences of the current climatic change and threatened by the growing anthropogenic effects, this site is fairly degraded today. These forcings are characterized by an increase in the allochtons contributions (nutritive, particulate and polluting) which could be at the origin of the reef medium peroration quality and losses of biodiversity, resources, and patrimony. This study is specifically aimed at testing this assumption by examining the distribution, activity, persistence and reproduction strategies of planktonic viruses in relation to that their microbial hosts, by covering a broad surface of the TLR. For that, two studies were undertaken: the first aimed at studying space and temporal variability of viral and bacterial communities at two different periods of tide [spring tide (ST)/ neap tide (NT)], the second attempted to examine more exhaustively, using physiological and ecological descriptors, the coupling between these two communities in several contrasted stations of the reef.

MATERIALS AND METHODS

This study was carried out in the TLR located at the edge of the Mozambique Channel. It's a zone which belongs to the Madagascar South-west reef complex (Figure 1). The climate is characterised and largely dominated by a South-west trade winds mode. The entropic developmental activities (mining, touristic, aquaculture, etc.) considerably contributes to increase the polluting load of the TLR. The water samplings at TLR took place from the 7 to 21 of September, 2007. This sampling campaign took place under typical dry season conditions, with relatively high temperatures (24.3 °C in average) and an important lighting (pyranometric values not presented here). The study zone sampling stations gathered in two main networks: "estuary" and "TLR". Figure 2, Tables 1 and 2 respectively, present the station names and sampling depths.

This was realized at each station using a bottle "Niskin" with a 5 liters sampling volume sufficient for various incubations and analyses. The TLR is largely subjected to the effects of the tides, therefore the water samplings were made twice: from the 11th to September 14, in spring tide and from the 18th to September 21 in NT.

In each station, 5 ml of water were placed in cryotubes (freezable tubes until -80 °C), were fixed in formol (final concentration 2% v/v) then were deposited in a cryogenic bottle (liquid nitrogen) to be conveyed at the IRD's laboratory in Dakar Bel air. To limit skew related to the degradation of fixed particles, the samples treatment were made in the two months which followed their collection. For all the samples, viral and bacterial abundance was observed with epifluorescence microscope (ME) after colouring of particles using a fluorochrome, the SYBR Gold (Molecular Probes) according to the method described by Bettarel et al. (2000).

A water subsample (1 ml) was filtered through glass fibre membranes of porosity 0.02 µm and of diameter 25 mm (Anodisque 25, Whatman) placed at the subfilters top (cellulose nitrate, Whatman) of porosity 0,2 µm. This superposition filter/subfilter allows to ensure a better distribution of the viral

particles on the membrane. After filtration, each membrane was deposited on a SYBR Gold girl solution drop (~50 µl) prepared beforehand starting from a stock solution of this fluorochrome (20 µl diluted in 2 ml of distilled water filtered on 0.02 µm). The preparation obtained, was afterwards incubated for 15 minutes in the dark, and then rinsed by filtration using distilled water. Lastly, the membranes were assembled between microscopes slides after deposit one drop of oil immersion. From each cryotube, two slides (duplicate) were produced with the aim to obtaining a statistically reliable enumeration. The enumeration of virus-like particles (VLPs) and bacteria were undertaken using an epifluorescence microscope (Olympus BX60), at 100x, under blue light. The viral and bacterial particles were counted on 15 fields selected randomly in order to minimize the errors due to a possible heterogeneity in the distribution of the micro-organisms on the membrane. The microscope objective being equipped with a squaring made up of 100 squares, the bacteria were reckoned on 10 squares and the viruses, of smaller size on 4 squares. The viral and bacteria's number computed on average by field was converted into viral concentration (particles number per ml of water) using the following formula: $\mathbf{N = (X/n) \times \Delta \times (1/V)}$

N = Particle number per millilitre; X = Average of the particles counted on a field,

N = Squares number which compose this fields; Δ = Correction factor defined according to the surface of the counting field as well as the membrane filter surface (Δ = 3 810 200).

V = filtered sample volume (ml).

The study of the lysogenic strategy and the viruses persistence in the TLR took place through 7 pilot stations: *Rade, large* (*Exterieure surface*, *Exterieure fond*), *Vasque, Mangrove, Resurgence, Estuaire.* These 7 stations were chosen because they were showing contrasted physicochemical and geographical characteristics. The viral reproduction strategy was related to bacteria in lytic and lysogenic infection phase. To this end, the proportion of bacteria in infection lytic phase was determined by the Clermont-Ferrand University (Protistes Biology Laboratory) in France.

To determine the bacterioplankton fraction in lysogenic infection phase, 50 mL of water from each pilot station were incubated in darkness at ambient temperature (25-27 °C), in presence of an antibiotic: the *mitomycine C* [(Sigma, final concentration 5 µg l^{-1}) (Jiang et al., 1996)]. This antibiotic allows the starting of a lytic cycle among the lysogenic cells and thus the prophage activation, up to that point encoded inside the genome of its host. The *mytomycin C* inoculation is then translated, a few hours later by the massive release of virus in the ambient medium. It is this liberation that we measured by comparing with the viral abundance of a sample in which nothing was added (control). After 24 hours of incubation, 5 ml from each sample (mitomycine & controls) were thus fixed using formol (2% final concentration) then deposited in cryotubes and freezed until their analysis (enumeration of the viruses and bacteria, as presented previously).

The bacteria lysogenic fraction (FBL) was obtained by using the viral concentration (VM) in the water incubated in the presence of mitomycine C, after 24 hours. It was also employed to determine this FBL: the viral concentration (Vc) in control, after 24 hours; the bacterial concentration (Ab) in control at the beginning of incubation T0 and bacterial Burst size (BS) (virus by bacteria) which corresponds to the number of mature viruses produced by infected cells and thus released in the ambient medium after cellular lysis. The following formula was applied:

FBL = 100 * [(VM - Vc)/(bs * Ab)].

The virus survival rate expresses the virus proportion which disappeared after 24 hours in absence of their bacterial hosts. To determine it, 100 ml of water were filtered

beforehand on 0.2 µm (cellulose nitrate membrane) which corresponds to the size of the smallest bacteria, in order to recover only the particles of viral size. This filtrate was then incubated in darkness (to limit the degrading effect of UV) for 24 hours. The percentage of viral losses could be estimated by measuring the difference between the viral concentrations [VIR] at the beginning (T0) and the end (T24) of the experimentation according to the following formula:

% of viral losses = [([VIR] $_{To}$ - [VIR] $_{T24}$)/[VIR] $_{To}$)*100].

The water chemical analyses (concentrations of NO_3, NO_2, NH_4, PO_4 and *chlorophyll a*) were supplied by the biochemistry laboratory of Ur CyRoCo.

Data analysis

Viral and bacterial abundances were compared statistically using the software Sigmastat 2.0. The correlations analysis (Pearson correlation coefficient R, Excel) between viruses and bacteria and of their distribution constituted the other statistical analyses carried out.

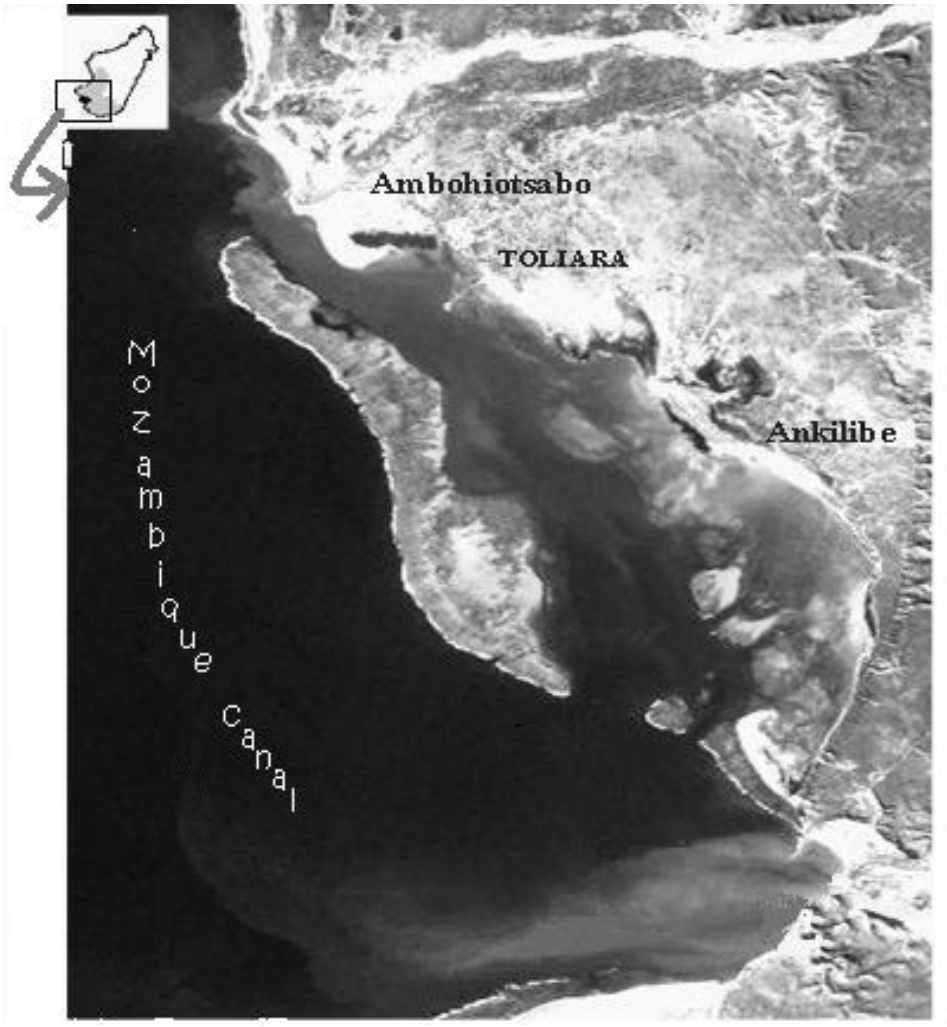

Figure 1: General presentation of the TLR (Arfi et al., 2007).

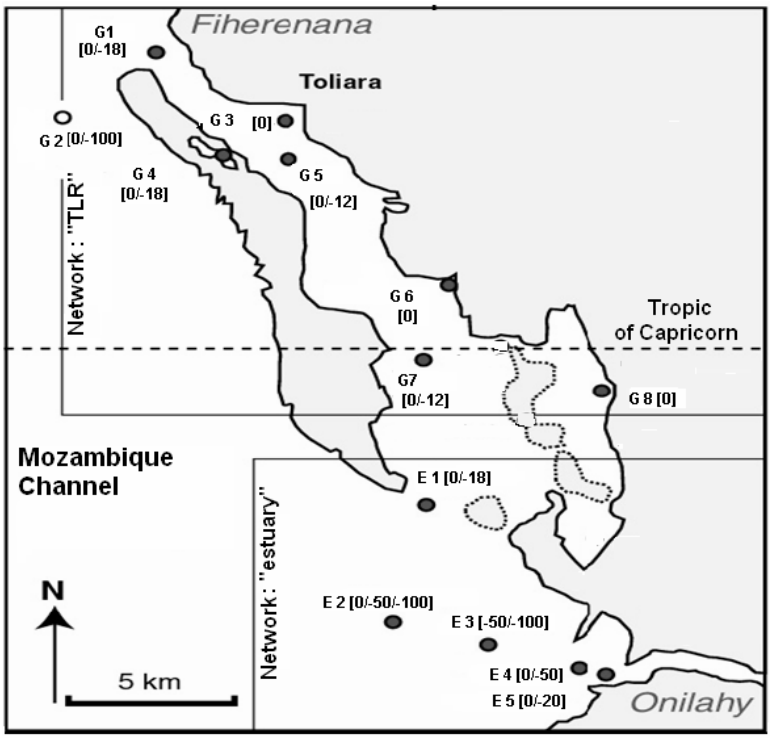

Figure 2: Distribution of the stations and sampled depths (Arfi et al., 2007).

Table 1 : Sampled stations.

Stations Network	Stations Name	Stations notation on the map
TLR	*Observatoire 1*	G 1
	*Exterieure**	G 2
	*Rade**	G 3
	*Vasque**	G 4
	Observatoire 2	G 5
	*Mangrove**	G 6
	Observatoire 3	G 7
	*Resurgence**	G 8
ESTUARY	*Estuaire 1*	E 1
	Estuaire 2	E 2
	Estuaire 3	E 3
	Estuaire 4	E 4
	*Estuaire 5**	E 5

The asterisk (*) indicates the considered pilot stations

Table 2: Sampled depths by station.

Stations	Sampled depths (m)	Stations
Observatoire1	0	1
	-18	2
Rade	0*	3
Vasque	0 *	4
	-18	5
Observatoire 2	0	6
	-12	7
Mangrove	0*	8
Observatoire 3	0	9
	-12	10
Resurgence	0*	11
Estuaire 1	0	12
	-18	13
	0	14
Estuaire 2	-50	15
	-100	16
Estuaire 3	-50	17
	-100	18
Estuaire 4	0	19
	-50	20
Estuaire 5	0*	21
	- 20	22

The asterisk (*) indicates the considered depths in the experimental stations. The *Exterieure* station was sampled on the surface (0 m) and in depth (-100 m).

RESULTS AND DISCUSSION

Distribution of viruses and bacteria in the TLR

For the whole study zone, the viral abundances were situated between 1.3 and 14.3 X 10^6 viruses ml^{-1} (average: 4.9 X 10^6 viruses ml^{-1}) in spring tide, while it was included between 0.8 and 6.1 X 10^6 viruses ml^{-1} (average:2.5 X 10^6 viruses ml^{-1}) in NT (Figure 3). The bacterial abundances were located in the interval 3.4 and 45.9 X 10^5 viruses ml^{-1} (Figure 4). The recorded densities were similar to those generally noticed in the literature for the low productivity zones (oligotrophic) (Weinbauer, 2004 ; Bouvy et al., 2004 ; Marc Bouvy et al., 2012). The natural concentrations in nutrients as well as the contents in chlorophyll measured in the various stations which cover the reef zone testify to the clearly oligotrophic status of the TLR (Table 3). Viruses are always parasites which need a host for their proliferation and development. If all the living cells are sensitive to the viral infection, the highly significant correlations between the dynamic of viruses and bacteria at the two maregraphic periods (rve = 0.8, rme = 0.9, N = 20) clearly indicates that the majority of TLR virioplankton is constituted of bacteriophages. The great coupling seems to attest to this host/parasite relation. Given that reef zones prokaryotes are generally represented by strong proportion of picocyanobacteria like *Synechococcus* (Charpy, 2005), we thus suspect that the virioplankton was mainly made up by cyanophages.

Temporal variability: spring and neap tide

The strongest viral and bacterial densities recorded in ST, seem to indicate a marked effect of the hydrodynamism in the TLR community distribution. The effect of

maregraphic context and water masses dynamics were already recorded, respectively by Bongiorni et al. (2007) in Adriatic Sea and Weinbauer et al (2010) in SW lagoon of New Caledonia. The strong tides result observed in the TLR has probably explained a bank fuller washing, and de facto, by a strong handing-over in suspension of the nutrients trapped in the surface sedimentary layers of the seashores.

In spite of the significant difference which exists between the average abundance of viruses in ST and NT, these however followed same spatial distribution at the two periods of study ($R = 0.47$, $p < 0.05$, $N = 22$).

This seems to indicate that the enrichment of TLR water (in nutrients and cells) in ST was very homogeneous on the space level and therefore didn't benefit any station in particular. It thus appears that the hydrodynamism's described effects on the functioning of the microbial trophic networks could extend not only to the TLR but to the whole marine littoral zones.

*Space variability***.** The strongest viral and bacterial abundances were always recorded in the stations of *Mangrove* and *Résurgence*. This could be explained by the fact that these stations are ecosystems with an important productivity and sheltering many ecological niches (Blanchot et al., 1989; Charpy et al., 1998).

Viral reproduction strategy

The viruses reproduce primarily according to two infectious modes: lytic and lysogenic which depends in particular on the physiological state of the host (Weinbauer, 2004). Thus, the lysogenic type infections are generally met in the mediums poor in nutrients (Paul, 2008), therefore unfavourable with the growth of the bacteria hosts. In the TLR, considered as oligotrophic, a strong proportion of lysogenic cells (i.e. infected by a temporarily inactive virus, also called prophage) were thus awaited. However, the mitomycine C used, recognized like the most powerful inductor (causing the swinging of a lysogenic cycle towards the lytic voice) (Jiang et al., 1996), indicates a very small proportion of lysogenic bacteria with values ranging between 0 and 14.5% (average = 3.2%) (Table 4).

If the lysogeny can concern up to the totality of infected cells occasionally, as it's the case in polar zone (Sawstrom et al., 2007; Laybourn-parry et al., 2007), it is known on other hand that the values recorded in the coastal zones of the globe are generally low (< 15%) (Paul et al., 2005). The very low levels of lysogeny observed in this study seem nevertheless to characterize tropical surface waters since Bettarel et al. (2008) and Marc Bouvy et al. (2012) had already revealed such values (average = 0.6% ; 2.5-8%) respectively in Bay of Hann (Senegal) and Tuomotu Archipelago (French Polynesia). The strong radiance at low latitudes could explain these observations partly. Indeed, it is known that the ultraviolet wavelengths represent, as well as the mitomycin C or pollutants (Danovaro et al., 2005) prophages' powerful activators and thus the implementation process of the intracellular replication. We therefore suggest that in tropical zone, UV radiations play the role of natural inductor on the lysogeny potential stock, giving back the mitomycin use quasi obsolete, the recorded lysogeny proportion after treatment corresponding to those having resisted UV. If this theory is correct, the lysogenic cycles could finally represent the dominating strategy selected by the TLR viruses, but diverted spontaneously and continuously towards the lytic pathway, under the influence of solar conditions. A nychthemeral study is now necessary to confirm this hypothesis in order to determine the night replication methods. Among the 7 pilot stations, the strongest lysogenical values were recorded in *Mangrove* station (m:

14.5%) and *Exterieure surface* (m: 5.1%); In all the others stations, values were lower than 3% (Table 4). Based on the hypothesis stated earlier, the relation between the proportion of bacteria in lytic infection phase and the *chlorophyll a* contents (r = 0, 7; p < 1‰; n=22) (Table 5) seems to suggest that the lysogenic viral production is primarily ensured by photosynthetic organisms. However, for a long time authors Odum (1975) and Odum et al. (1982) showed that in lagoon and reef environments, the primary production is essentially ensured by picocyanobacteria (*Synechococcus* kind).

Then, we suspect that the TLR virioplankton and probably more widely reef environments, are largely dominated by cyanophages. Another hypothesis to explain this correlation could hold in the photosynthetic activity of the eukaryotic microalgae whose secreted organic matter could rather favour the lytic infection cycles than lysogenic cycles. This relation remains however dubious and other studies will be necessary to identify the circumstances which determine the release of one or the other viral reproduction modes.

Virus persistence in water

The prokaryotes withdrawal from the samples by filtration permit to establish, before the contact of the viruses with their bacterial hosts, the viral losses horary rate related only to virucidal environmental factors. These rates of losses vary from one station to another ranging from 22.7 to 61.2% (average: 39.5%). These differences could be related to the nature of the viruses and also to the intrinsic physicochemical conditions at these strongly contrasted niches, as it has been reported by Bettarel (2009).

Here, no significant correlation could be observed between the losses rates and the nutrient contents. Nevertheless, it is interesting to note that another positive correlation exists between the fraction of lytic bacteria (R = 0.77) (Table 5) and *chlorophyll a*. This relation between virus coming from lytic bacteria and *Synechococcus* have been reported by Baudoux et al. (2007) in ologotrophic system.

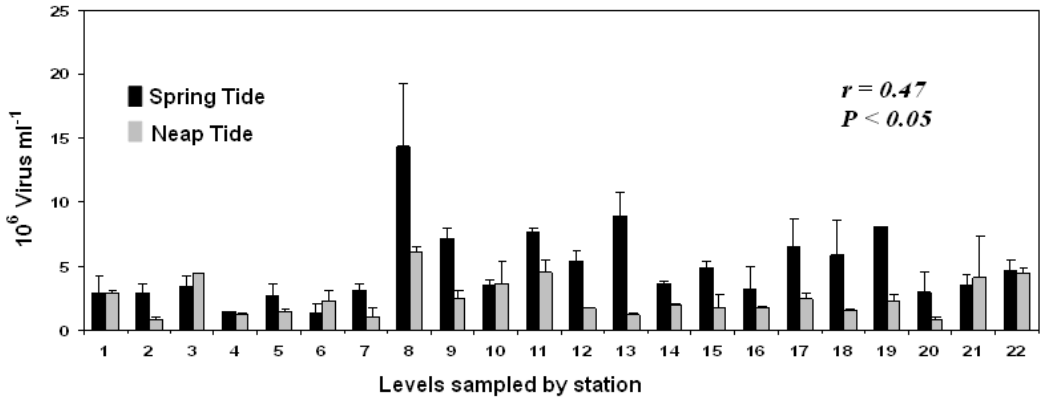

Figure 3: Comparison of the viral concentrations in spring and neap tide.

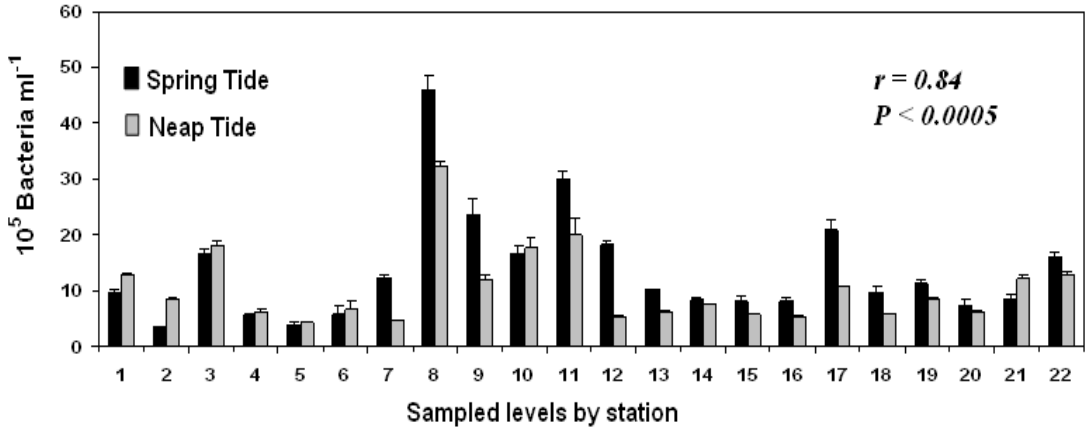

Figure 4: Comparison of the bacterial concentrations in spring and neap tide.

Table 3: Physicochemical characteristics of the pilot stations.

Stations	NO_2 (nM)	NO_3 (nM	H_4 (µM)	O_4 (µM)	l *a* (µg L^{-1})
Estuaire	358.0	1501.0	1.7	0.5	1.0
Rade	6.0	226.0	0.0	0.4	1.5
Exterieure Fond	24.0	403.0	0.1	0.3	0.3
Vasque	56.0	1096.0	0.1	0.3	0.2
Resurgence	73.0	12327.0	0.3	0.3	2.1
Exterieure Surface	21.0	249.0	0.2	0.2	0.1
Mangrove	14.0	329.0	1.2	0.2	1.5

Table 4: Viral parameters in seven experimental stations of the TLR.

	[VIR] (10^6 ml^{-1})	[BAC] (10^6 ml^{-1})	VBR	LYT(%)	LYS (%)	Viral losses (%)
Estuaire	4.1 ± 0.7	1.2 ± 0.1	3.4	0.0	0.2 ± 0	22.7 ± 2.7
Rade	3.3 ± 0.9	1.7 ± 0.1	1.9	10.9	0.0	43.0 ± 7.2
Exterieure Fond	2.6 ± 0.5	1.3 ± 0.3	1.9	2.1	0.0	42.9 ± 12.4
Vasque	1.4 ± 0.1	0.6± 0.1	2.3	1.3	0.8 ± 0	29.1± 2.9
Resurgence	4.5 ± 0.3	2.0 ± 0.3	2.3	15.1	1.8 ± 0	61.2 ± 12
Exterieure Surface	1.6 ± 0.8	0.5 ± 0.1	3.2	0.8	5.1± 0	30.7 ± 3.3
Mangrove	6.1 ± 0.4	3.2 ± 0.1	1.9	0.0	14.5 ± 5.8	44.7 ± 5.7

Table 5: Correlation between the parameters of study.

	[VIR]	**[BAC]**	**VBR**	**NO_2**	**NO_3**	**NH_4**	**PO_4**	**Chl *a***	**LYT (%)**	**LYS (%)**	**Viral losses (%)**
[VIR]	**1**										
[BAC]	**0.93*** **	**1**									
VBR	-0.26	-0.57	**1**								
NO_2	0.17	-0.18	**0.7***	**1**							
NO_3	0.29	0.2	-0.07	0.07	**1**						
NH_4	0.64	0.37	0.43	**0.76****	-0.06	**1**					
PO_4	0.16	-0.06	0.3	**0.72***	-0.04	0.43	**1**				
Chl *a*	**0.79****	**0.75****	-0.35	0.02	0.62	0.23	0.23	**1**			
LYT (%)	0.17	0.21	-0.37	-0.23	**0.74***	-0.41	0.13	**0.7***	**1**		
LYS (%)	0.58	**0.66***	-0.19	-0.30	-0.15	0.35	-0.55	0.20	-0.33	**1**	
Viral losses (%)	0.46	0.6	**-0.67***	-0.50	**0.69***	-0.31	-0.26	**0.71***	**0.77****	0.16	**1**

In fact, this correlation seems to indicate that the cyanophages are, at the Free State, relatively more sensitive than the bacteriophages to the environment destroying factors. In order to confirm this hypothesis, some new study should be carried out.

In conclusion, this study examined the viral and bacterial component in tropical zone [the Toliara Large Reef (TLR) in Madagascar], In seeking to determine the space and temporal variability of these two communities in 22 stations covering the TLR and the environmental and biotic conditions which support one or the other viral reproduction strategies (lytic vs lysogenic) and those which enable them to remain in a free state in aquatic environment.

By the use of epifluorescence microscopy, it is shown that the bacterial and viral concentrations for the whole of TLR were significantly higher in ST period than NT, because of the very marked hydrodynamism in this zone. The mitomycin C utilization permitted to establish that the cells fraction in lysogenic infection phase was very weak (< 3%) in spite of TLR water's oligotrophic status. This would be explained by the lysogenic cycles' theory (dominating strategy of viral reproduction in TLR), diverted spontaneously and continuously towards the lytic way, under the influence of the solar conditions. The virus-bacterium strong coupling and the positive correlation between the bacteria in lytic infection phase and *chlorophyll a* at the various stations suggest that prokaryotes' viruses in TLR are phages dominated by cyanophages. Finally, our results demonstrate that the viruses that were persistence at the Free State was more related to the virus nature. As it happens, we suspect that TLR cyanophages are probably more sensitive to the environment virucidal factors (UV radiations and temperature) than phages targeting none pigmented cells. Finally, we show that the viruses form a dynamic and ubiquity component of reef ecosystems.

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