Enumeration of total virioplankton and isolation of specific cyanophages from selected aquatic ecosystems in Goa, India

Judith Miriam Noronha[1], Amara Begum Mulla[2], Manguesh U. Gauns[2] and Sanjeev C. Ghadi[1,*]

[1]Department of Biotechnology, Goa University, Taleigao Plateau, Goa 403 206, India
[2]CSIR-National Institute of Oceanography, Dona Paula, Goa 403 004, India

Viruses are known to be highly abundant and, therefore, ecologically significant entities of all aquatic ecosystems. However, very few studies from marine and freshwater ecosystems in India have dealt with enumeration, isolation or characterization of their virus (virioplankton) populations. In the present study, we have estimated total virioplankton populations from several kinds of aquatic niches, viz. rice fields, lakes and estuaries, using flow cytometry. Rice field floodwaters displayed the highest virioplankton count of 1.21×10^7 particles per ml. As cyanophages form the second most abundant class of virioplankton (after bacteriophages), we also isolated four cyanophages from the same aquatic niches.

Keywords: Aquatic ecosystem, cyanophage, flow cytometry, virus enumeration.

VIRUSES in aquatic ecosystems numerically vary between 10^6 and 10^7 per ml on an average, and are generally an order of magnitude more abundant than bacteria[1]. Due to such a vast and ubiquitous presence, they affect the ecosystem in profound ways – both at the micro and macro level. Thus, any comprehensive ecological study is incomplete without an assessment of the impact of viruses on individual groups of organisms as well as on the ecological balance of the system as a whole.

Microalgae, including cyanobacteria, are the most important primary producers in any aquatic ecosystem; they are responsible for producing almost half of the atmospheric oxygen. Viruses that infect microalgae affect their photosynthetic physiology[2] and community composition[3]. Moreover, viruses have been associated with the decline of algal blooms[4,5].

Among aquatic viruses, cyanophages that infect cyanobacteria, constitute a major group. Cyanophage research preceded that of other microalgal viruses[6,7]. Subsequently, cyanophages infecting various host cyanobacterial species have been isolated from a variety of locations and characterized. Examples are cyanophages of *Synechococcus* sp.[7–9], *Prochlorococcus* sp.[10], *Microcystis* sp.[11,12], *Nodularia* sp.[13] and phages that infect more than one host[14,15]. However, there is lack of published research on cyanophages isolated and characterized from aquatic niches within the Indian subcontinent, barring a few studies that reported viral enumeration only[16–18].

The climate of India is strongly influenced by monsoonal winds, which bring sizeable rainfall, particularly during June–October. Precipitation in the form of rain and snowfall provides over 4000 km^3 of freshwater to India. While most of this water is returned to the oceans, a small percentage is stored in inland water bodies (and groundwater aquifers) that are classified as rivers and canals, reservoirs, ponds, lakes, etc.

The varied marine and freshwater ecosystems of Goa, India have been studied with respect to their physicochemical characteristics, monsoonal influence and populations of bacteria, plankton and higher organisms, but not much explored from the virological perspective[18]. To the best of our knowledge, there are no reports on the isolation of microalgal viruses from aquatic ecosystems of Goa. Here we present a comparison of virus (virioplankton) populations enumerated from various aquatic niches of Goa by flow cytometry. Further, we describe the isolation of four cyanophages from these niches.

Several types of aquatic ecosystems were selected for estimating the overall virioplankton populations present therein. These included estuarine systems, lakes/natural reservoirs (hereafter referred to as 'lakes') and rice-field floodwaters. Estuaries represent a completely natural system; lakes represent a natural system under some human control – for example, drainage of excess water during monsoon season through the use of floodgates, and rice fields represent a predominantly artificial ecosystem. Surface water samples from various sites, at least five for each type of ecosystem (Supplementary Table 1), were collected during August–September 2016 (monsoon season). Samples were pre-filtered through 0.22 μm nitrocellulose membranes and stained with SYBR Green I at a final concentration of 10^{-4} of the commercial stock. Staining was carried out for 10 min in the dark at 80°C, followed by a cooling period of 5 min. Samples were then analysed on a BD FACS Calibur flow cytometer equipped with a 15 mW, 488 nm air-cooled argon-ion laser and a standard filter set-up. The trigger was set to green fluorescence[19].

In addition to a broad viral enumeration study, we specifically screened the water samples from different niches for the presence of cyanophages infectious to some of the cyanobacterial cultures maintained in our laboratory. These cultures had been isolated during the period July–September 2014 (monsoon season), from the same or similar locations to those selected for viral enumeration as described above. All cultures were maintained at 25°C in BG-11 medium[20], under a 16 : 8 h light : dark cycle at irradiance between 10 and 20 μmol m^{-2} s^{-1}. To detect viral infection of these cyanobacterial cultures, water samples were collected from the various aquatic niches as described above. One of the best methods for the

*For correspondence. (e-mail: saga@unigoa.ac.in)

screening of algal viruses is direct addition of natural water samples to unialgal cultures[21]. Hence, water samples were pre-filtered through 0.22 µm nitrocellulose membranes, and 100 µl of each filtrate was added to 1 ml of exponentially growing cyanobacterial host culture. After two weeks of incubation under standard conditions (mentioned earlier), an aliquot of the culture broth was taken, the algal cells pelleted by centrifugation and the supernatant used to re-infect the relevant host culture as follows: 100 µl of supernatant and 300 µl of exponentially growing host culture were added to 3 ml of molten 0.5% BG-11 agar, mixed well and poured on the surface of a 1% BG-11 agar plate. Plates were incubated under standard conditions for a few weeks and observed for viral plaques[22]. Cyanobacterial culture plates which demonstrated the presence of viral plaques were selected. A single plaque was excised and used to infect fresh, exponentially growing host culture by means of the plaque assay described above. This procedure was repeated multiple times to purify the virus. Cyanobacterial cultures which showed susceptibility to viral infection were identified by microscopic observation at 1000×, on the basis of standard keys.

Among the different aquatic niches, the highest mean virioplankton count (average particles per ml $\times 10^6$) was obtained from rice-field floodwaters (12.1), followed by lake (3.9) and estuarine (2.1) samples. Among the few studies which have previously enumerated virioplankton or virus-like-particles (VLPs) in rice-field floodwater, Nakayama *et al.*[23] reported an abundance of between 2.2×10^6 and 8.0×10^7 per ml for these particles. Kimura *et al.*[24] found that VLP abundance fluctuated between 5.6×10^6 and 1.2×10^9 particles per ml during the entire cultivation period.

Virus enumeration studies in aquatic systems spanning a wide variety of marine, estuarine and freshwater systems have unequivocally demonstrated that nutrient-rich environments support larger populations of viruses[1]. Moreover, the temporal and seasonal rise and fall in viral populations in a given ecosystem are closely associated with the variation in host populations, particularly bacterioplankton and algal hosts[1]. The rice field is a closed, anthropogenically influenced aquatic ecosystem, constituted for the specific purpose of cultivating the rice crop. The submerged conditions favour accumulation of organic matter[25]. Further, nutrients are artificially pumped into the system in the form of fertilizers. Thus, the nutrient-rich soil layer supports luxurious growth of bacteria and algae. Continuous exchanges occurring between this soil layer and the overlying shallow floodwater ensure high bacterial and algal populations in the water layer as well, which support high viral populations[26,27].

In contrast, lakes, though closed ecosystems, possess greater dynamics as well as greater depth. Hence the surface water layer is not in close association with the soil layer of the lake bed. Viral particles in the surface layer may constitute only a proportion of the total viral population in the water column. Moreover, several studies of viral populations in lake ecosystems have found higher viral content in the sediment layer compared to the water column[28,29]. One possible explanation is that sediments provide viral particles protection against decay due to UV radiation[30]. Steenhauer's[31] review of viral counts obtained from lakes and reservoirs in various parts of the world found an average count of 4.38×10^7 per ml in reservoirs and 5.19×10^7 per ml in lakes (averaging the counts obtained from eutrophic, mesotrophic and oligotrophic lakes). However, oligotrophic lakes alone showed a comparatively low average count of 1.84×10^7 per ml. The low viral counts obtained from lakes selected in our study may be a consequence of their oligotrophic nature.

The lowest viral counts from estuarine sites obtained in our study can be explained by the fact that estuarine systems are highly dynamic, subject to constant mixing of water layers. Hence unlike in lakes, reservoirs or wetlands, where viral counts are influenced by the depth of the water column and proximity of sediments, in estuarine regions viral counts have been shown to be relatively constant throughout the water column[32–34].

In total counts of viruses, bacteriophages comprise more than 90% (refs 35–37). This group has been well studied and characterized in diverse aquatic environments. The second most abundant group of cyanophages, comprising about 7% of the total viral population is comparatively less studied[38,39]; hence, we chose to study this community in more detail. All the water samples from which viral particles were enumerated were therefore used to detect the presence of cyanophages infectious

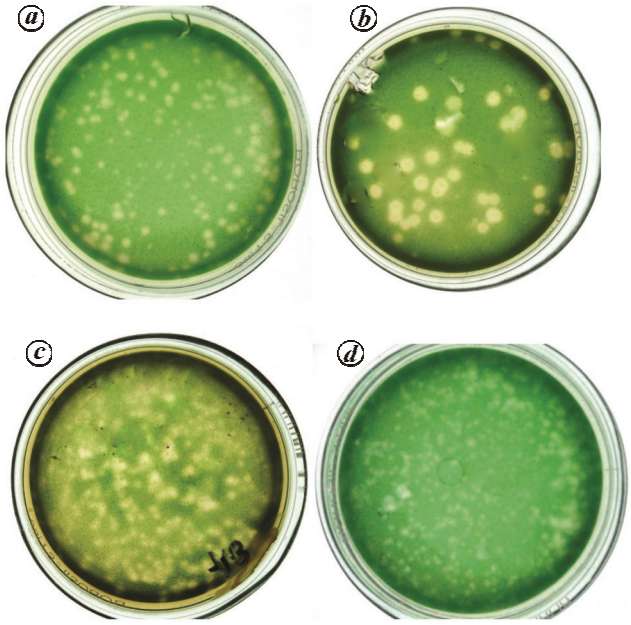

Figure 1. Cyanophages isolated from different ecosystems, evidenced by plaque assays: ***a***, ***b***, estuaries, ***c***, lakes and ***d***, rice fields.

to cyanobacterial cultures maintained in the laboratory. In total, four cyanophages were isolated. The two host cyanobacteria which showed susceptibility to infection were identified as *Synechococcus* sp. DP01 and *Synechocystis* sp. MZ01 respectively, on the basis of their morphological features (Supplementary Figure 1). The two cyanophages infecting *Synechococcus* sp. DP01 were designated S-VL01 and S-BE01 respectively, whereas the two infectious to *Synechocystis* sp. MZ01 were designated S-SE01 and S-CF01 respectively. All the cyanophages were detected by the formation of plaques on lawns of host culture (Figure 1). Plaques were excised and propagated as described earlier.

Synechococcus phages have been previously isolated from a variety of aquatic ecosystems, including marine[7,40,41], estuarine[42] and freshwater[43,44] systems. To the best of our knowledge, there are no previous reports on the isolation of *Synechocystis* phages.

In conclusion, we have described a preliminary study on virus enumeration and isolation from aquatic niches of Goa. A comparison of total viral counts from surface waters of three representative ecosystems, namely rice-fields, lakes and estuaries, revealed highest counts from rice-field floodwaters. Similar studies from a large number of sites could throw light on the viral ecology of aquatic systems in the region. Water samples from various sites were also checked for the presence of infectious viruses specific to cyanobacterial cultures maintained in our laboratory. Four cyanophages were isolated and propagated, two against *Synechococcus* sp. DP01 and two against *Synechocystis* sp. MZ01. The latter are potentially novel phages and await further characterization.

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Responses of short-nosed fruit bat, *Cynopterus sphinx* (Vahl 1797) towards distress calls of their conspecifics from related and unrelated sites: implications for building a social relationship

A. F. P. Allwin Mabes Raj[1,2], K. Emmanuvel Rajan[3] and H. Raghuram[1,*]

[1]Department of Zoology, The American College, Madurai 625 002, India
[2]Department of Genetics, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India
[3]Department of Animal Sciences, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, India

Distress calls emitted by bats signal their conspecifics either to warn them or inform them about the situations. Conspecifics may also get attracted towards distress calls as a behaviour of cooperative mobbing or just selfishly assessing the potential source of danger. The exact functions of distress calls in bats therefore vary to a great degree and are very hard to pinpoint. We conducted playback experiments to test the response of short-nosed fruit bat, *Cynopterus sphinx* towards the distress calls of their conspecifics from related and unrelated sites. Bats were attracted to their conspecifics from both related and unrelated sites and in one occasion towards fruit bat (*Rousettus leschenaulti*) of another genus within the same family. The response towards the opposite sex was significant in most of the playback trials and the reasons remain unclear. This symmetric response towards conspecifics from related and unrelated sites suggests the possibility of fruit bats building social relationships among unrelated individuals and probably between species.

Keywords: Chiroptera, *Cynopterus sphinx*, conspecifics, distress calls, social relationship.

SHORT-NOSED fruit bat (*Cynopterus sphinx*) is a non-echolocating, frugivorous bat that is very common in India and South East Asia[1]. They usually roost in small groups of 3 to 6 individuals in so-called tents constructed typically by the harem males within the fronds of palm leaves or behind creeping vines[2]. Morphologically they exhibit sexual dimorphism where males are larger than females and also characteristically orange tinted on the shoulders, sides of chest and thighs[1]. *C. sphinx* emits characteristic audible distress vocalizations when they are entangled in mist nets to warn or inform their counterpart or conspecifics[3]. During such distress call emissions, the

*For correspondence. (e-mail: hraghuram@gmail.com)