Characterization of Microalgal Viruses from Aquatic Systems

A thesis submitted to Goa University

For the award of the degree of

Doctor of Philosophy

in

Biotechnology

by

Judith Miriam Noronha

Department of Biotechnology

Goa University

Taleigao Plateau, Goa

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2021

CERTIFICATE

This is to certify that the thesis entitled **"Characterization of microalgal viruses from aquatic systems"** submitted by Ms. Judith Miriam Noronha, for the award of the degree of Doctor of Philosophy in Biotechnology, is based on original studies carried out by her under our supervision.

The thesis or any part thereof has not been submitted for any other degree or diploma in any university or institution.

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Date: 08.01.2021

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STATEMENT

As required under the Goa University Ordinance OB-9A, I state that the present thesis entitled **"Characterization of microalgal viruses from aquatic systems"** is my original contribution, and that the same has not been submitted on any previous occasion for any degree. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made, wherever facilities and suggestions have been availed of.

Place: Goa University

Date: 08.01.2021 Judith Miriam Noronha

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"Alone we can do so little; together we can do so much." – Helen Keller

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CONTENTS

LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CTAB	Cetyl trimethyl ammonium bromide
DAPI	4′,6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EFM	Epifluorescence microscopy
FCM	Flow cytometry
ITS	Internal transcribed spacer
MEGA	Molecular evolutionary genetics analysis
MPN	Most probable number
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque-forming units
PSU	Practical salinity unit
RNA	Ribonucleic acid
SC	Santana Creek
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV	Ultraviolet
VL	Verna Lake
VLP	Virus-like particles

LIST OF FIGURES

Chapter 5

Chapter 6

LIST OF TABLES

CHAPTER ONE

INTRODUCTION AND RESEARCH OBJECTIVES

Aquatic Viruses

Thirty years ago, it was a novel idea that the smallest biological entity of any ecosystem – the nanoscopic virus – could well be one of the most important (Bergh et al. 1989; Proctor and Fuhrman 1990). This fact is no surprise now, as it is universally acknowledged that the virus component of any ecosystem is by far the most dominant, numerically. Viruses have been estimated to number 10^6 to 10^7 per ml, on average, in aquatic environments (Wommack and Colwell 2000), and 10^8 to 10^9 in sediments (Danovaro et al. 2008). Nevertheless, the importance of viruses is in the fact that they directly affect each and every trophic level, by infection and subsequent lysis or disease. Specific viruses exist, which infect every form, ranging from bacteria and protists, to phyto-and zoo-plankton as well as larger plant and animal forms (Fuhrman 1999; Suttle 2005).

Until the discovery of a large number of viruses in seawater, viruses were considered important only in their capacity to cause diseases to humans as well as animals and plants of economic value (Grafe 1991). Bacteriophages were thought to be only as abundant as the cultivable forms, similar to the earlier concept of bacteria (Clokie et al. 2011). This created gross underestimates in terms of numbers as well as importance of these phages. It is now estimated that viruses, totally across aquatic and terrestrial biomes, number at least 4.8×10^{31} (Cobián Güemes et al. 2016; Mokili, Rohwer, and Dutilh 2012; Mushegian 2020). Concurrently, the number of virus genotypes on earth has been estimated at somewhere between 3.9×10^6 and 2×10^9 (Cesar Ignacio-Espinoza, Solonenko, and Sullivan 2013; Rohwer 2003).

The sheer numbers of viruses and their attendant activities have far-reaching consequences on the ecosystem. Thus has arisen the concept of a ‘virocentric ecology’ (Hurst and Lindquist 2000; O’Malley 2016; Rohwer and Thurber 2009), wherein ecology, biogeochemical cycling and even evolution should be understood from a viral perspective.

With compelling evidence supporting the importance of aquatic viruses, researchers worldwide have focused their attention on several aspects of these viruses. Most of the current knowledge in this field has originated from studies on marine systems, with limited focus on freshwater systems and extreme environments. Advancements in experimental techniques have facilitated important discoveries on the roles of viruses. For example, direct counts of viruses, through microscopy or flow cytometry (Brussaard, Marie, and Bratbak 2000; Noble and Fuhrman 1998), made clear the actual numbers of viruses in various

ecosystems. Needless to say, these were far higher than any previous estimates. Moreover, these techniques enabled the measurement of viral production rate – and further inference of viral activity on ecosystems as a whole (Danovaro et al. 2008; Wommack et al. 2015). More recently, the advent of next-generation sequencing and high-throughput metagenomics analysis, has facilitated the discovery of virus diversity and community composition in a wide variety of biomes (Dávila-Ramos et al. 2019; Gregory et al. 2019; Hayes et al. 2017; Paez-Espino et al. 2016).

In all this, traditional laboratory-based isolation of viruses and cultivation and characterization of virus-host systems, has not lost its value (Brum and Sullivan 2015; Weitz et al. 2013; Wommack and Colwell 2000). On the contrary, robust laboratory cultures of individual viruses are necessary to complement counts and sequences of total virus populations. Moreover, sequencing of individual virus genomes is required to update genomic databases, and improve the annotation of metagenomic sequences obtained from various environments – which, to date, comprise a large proportion of unknown sequences or viral dark matter.

1.1 Microalgae and Cyanobacteria

Microalgae – microscopic, photosynthesising organisms – are one of the most abundant and widely distributed groups of organisms on earth. They are found mostly in aquatic systems, but also in soil surfaces, either free-living or as part of symbiotic associations (Khan, Shin, and Kim 2018; Richmond 2004; Singh and Saxena 2015). The term ‘microalgae’ includes the prokaryotic cyanobacteria and the eukaryotic unicellular algae (Masojídek and Torzillo 2014; Richmond 2004). These together constitute the base of any food chain – in other words, they are the primary producers in any aquatic ecosystem, accounting for nearly half the global net primary production annually (Falkowski et al. 2004; Field et al. 1998). Microalgae have been used as food for humans for thousands of years, and continue to be utilized for a wide range of commercial and pharmaceutical applications (Sathasivam et al. 2019; Spolaore et al. 2006).

Although cyanobacteria are prokaryotes, and often considered along with bacteria in terms of cellular structure and genetic features (Garrity 2012), their ecological role is more closely linked to eukaryotic algae than to bacteria. Hence, it is appropriate to consider cyanobacteria along with microalgae in ecological studies (Brussaard 2004; Suttle 2000).

Cyanobacteria, in particular, are present everywhere that light penetrates (Moss et al. 2018). This includes oceans and lakes, as well as thermal springs, snow-covered areas and even deserts (Chapman 2013). Cyanobacteria display a high level of adaptability, thriving in environments that cover a range of temperature, light, salinity and nutrient conditions (Waterbury 2006). This group of organisms has probably been in existence for over 3.5 billion years (Knoll 2008). Oxygenic photosynthesis by cyanobacteria was largely responsible for creating the first oxygen-rich environment and kick-starting evolution (Flores, López-lozano, and Herrero 2015). Unicellular cyanobacteria are responsible for a quarter of global oxygen production (Field et al. 1998; Zhang, Jiao, and Hong 2008). Another important function is nitrogen fixation – converting inert nitrogen gas into nitrates and nitrites usable by other organisms (Whitton 2012). Secondary metabolites of cyanobacteria have been a source of many compounds of pharmaceutical value (Newman and Cragg 2016).

Among the numerous branches of research on microalgae and cyanobacteria, the discovery and characterization of viruses that infect these organisms, is an important aspect, as these viruses are ubiquitous and significant agents of population control in any natural system.

1.2 Cyanophages – Aquatic Viruses which Infect Cyanobacteria

Virus abundance in a given ecosystem is directly correlated with the presence of respective hosts (Jacquet et al. 2010; Wigington et al. 2016). In aquatic systems, cyanobacteria are the second most abundant class of planktonic micro-organisms, after bacteria. It follows, therefore, that cyanophages – viruses which infect cyanobacteria – are a major component of virioplankton (Mann and Clokie 2012; Suttle 2001) and one of the most significant groups of aquatic viruses (Hargreaves, Anderson, and Clokie 2013; Jaskulska and Mankiewicz-Boczek 2020). Evidence supporting this has come from numerous studies. In studies on lake systems, for instance, virus and cyanobacterial abundance (Dorigo, Jacquet, and Humbert 2004) and virus abundance and chlorophyll a (Clasen et al. 2008; Maranger and Bird 1995) displayed strong positive correlations. Metagenomic investigations have been carried out in varied environments, ranging from oceanic sites (DeLong et al. 2006), to freshwater lakes (Mohiuddin and Schellhorn 2015; Skvortsov et al. 2016; Tseng et al. 2013) to desert ponds (Fancello et al. 2013). In all these cases, cyanophage-like sequences

represented the majority of identified viral genomes, indicating the dominance of cyanophages in diverse viral communities.

Viruses impact prokaryotic communities in either of two ways – directly, through lysis-induced mortality (also referred to as top-down control) or indirectly, by altering nutrient pools (Liu et al. 2015; Pradeep Ram, Keshri, and Sime-Ngando 2020). Lytic cyanophages account for 5 to 25% mortality of unicellular cyanobacteria on a daily basis (Brussaard 2004; Suttle 2000). Significant cyanophage-induced mortality of cyanobacteria has been reported in lake systems (Personnic et al. 2009). Infection of cyanobacteria by cyanophages reduces their photosynthetic efficiency, as cellular energy and reducing power are diverted towards viral particle production (Padan and Shilo 1973; Zimmerman et al. 2020). As in the case of bacteriophages, cyanophages also control to a great extent the diversity and community composition of their cyanobacterial hosts (Brussaard 2004; Deng and Hayes 2008; Thingstad and Lignell 1997; Weinbauer and Rassoulzadegan 2004).

The 'indirect' effect of viral activity on prokaryotic communities, also known as the 'viral shunt' component of aquatic nutrient cycles (Wilhelm and Suttle 1999), increases the volume and efficiency of nutrient cycling. For example, around 55% of bacterioplankton production in marine systems has been ascribed to carbon released through viral lysis (Winget et al. 2011). Moreover, nutrients released through viral lysis are rapidly converted to bioavailable ions which actually stimulate growth and productivity of micro-organisms (Haaber and Middelboe 2009). On a larger scale, the lytic activity of bacteriophages and cyanophages, has an overwhelming effect on the cycling of major nutrients, including carbon, nitrogen and phosphorous, and thereby on the dynamics of the entire food web (Bonetti et al. 2019; Fuchsman et al. 2019; Fuhrman 1999; Sime-Ngando 2014). "Host–virus interactions at nanoscale eventually shape ecosystem processes at geographical scales (Moniruzzaman, Gann, and Wilhelm 2018)."

A further significant contribution of cyanophages is in genetic diversity and evolution of cyanobacterial hosts – through horizontal gene transfer (Lindell et al. 2004; Mann et al. 2003). The existence of cyanophage-derived genes within cyanobacterial hosts is now well-documented. These genes, known as auxiliary metabolic genes, have wide-ranging functionalities, coding for proteins involved in photosynthesis, carbon metabolism and pigment degradation (Hurwitz, Hallam, and Sullivan 2013; Thompson et al. 2011; Warwick-Dugdale, Buchholz, et al. 2019). Although the acquisition and expression of these

genes is to the phage's advantage, rather than the host's, there is a net benefit to the entire ecosystem, in terms of increased efficiency of photosynthesis and nutrient cycling (O'Malley 2016; Weitz and Wilhelm 2012).

1.3 Cyanophages as Biocontrol Agents of Harmful Cyanobacteria

The general role of aquatic viruses in regulating host populations assumes great significance in the case of viruses that infect bloom-forming cyanobacteria. As in the case of other algal viruses, cyanophages have been associated with sudden decline (crash) of cyanobacterial blooms (Gerphagnon et al. 2015; Hewson, O'Neil, and Dennison 2001; Suttle 2000). Moreover, the ratio of phage-resistant to sensitive strains present in a given community may be a significant factor in influencing the progress of blooms (Coloma et al. 2019).

Cyanobacterial blooms are on the rise globally (Davis and Gobler 2016). All of the evidence so far points to the fact that climate change, rising global temperatures, and increasing eutrophication of water bodies will favour the proliferation of cyanobacterial blooms (Glibert 2020; O'Neil et al. 2012; Paerl and Paul 2012). Such blooms, which come under the category of harmful algal blooms (HABs) cause harm to aquatic animals and plants, due to blockage of light, reduction in oxygen levels and production of toxins (Chorus, Ingrid and Bartram 1999; Paerl et al. 2001). Some toxins can kill animals and even cause severe health hazards to humans (Carmichael 1997; Lawton, Linda A, Codd 1991).

Various methods have been utilized for the control of cyanobacterial HABs (Lürling, Waajen, and de Senerpont Domis 2016; Paerl 2017). Most of these employ nutrient manipulations, as well as input of high concentrations of lethal chemicals. Among these, an ecologically acceptable method is biological control using lytic cyanophages against the harmful cyanobacterial species. First proposed in 1964, by the same research group which isolated the first cyanophage in culture (Safferman and Morris 1963), the use of cyanophages as a cyanobacterial control agent was endorsed by several researchers over subsequent decades (Deng and Hayes 2008; Jassim and Limoges 2017; Sigee et al. 1999; Yoshida et al. 2006a). With more extensive research and isolation of cyanophages which specifically target bloom-forming cyanobacteria, this method could potentially be used in combination with nutrient control, to effectively bring down the populations of these cyanobacteria (Aligata, Zhang, and Waechter 2019).

1.4 Focus on Freshwater Aquatic Systems

Freshwater systems occupy only about 1% of the earth's surface, yet harbour immense biological diversity, upto 10% of all described species and store almost three times the organic matter of all the oceans combined (Dudgeon et al. 2006). Moreover, these systems cycle significant quantities of carbon (Tranvik et al. 2009). They have a direct impact on human health (P. A. Green et al. 2015) and are directly impacted by human activities on short as well as long time-scales (Okazaki et al. 2019; Posch et al. 2012). Freshwater aquatic systems are highly diverse, ranging from ponds and lakes, to rivers, to polar ice-cap freshwaters. They are the link between terrestrial and marine systems and convey dissolved and particulate matter from land into the sea (Eiler and Bertilsson 2004).

Although initial work on aquatic virus ecology was carried out in freshwater systems (Miller et al. 1992) marine viruses, including cyanophages, have subsequently been studied in far greater detail than freshwater ones (Dreher et al. 2011; Ghai et al. 2014).

In the area of freshwater aquatic virology, research at the international level has, thus far, focused on certain key areas, notably ecosystem-level studies on virus populations and the isolation of specific viruses that have an ecological relevance. At the ecosystem level, virus populations from large lakes and rice field ecosystems have been characterized, in terms of their abundance, morphological variation, and temporal and spatial distribution (Clasen et al. 2008; Filippini, Buesing, and Gessner 2008; Nakayama et al. 2007b, 2007c; Sime-Ngando et al. 2016). Further, the viral genetic diversity has been elucidated, through surveys of marker genes, such as *g20* and *psbA* (Adriaenssens and Cowan 2014; Dorigo et al. 2004; Wang et al. 2010; Zhong and Jacquet 2013), or characterization of entire virus metagenomes (Cai et al. 2016; Chopyk et al. 2018; Skvortsov et al. 2016; Taboada et al. 2018). Where the isolation and characterization of ecologically relevant viruses is concerned, a lot of research has been carried out on cyanophages. For example, phages of the ubiquitous unicellular cyanobacterium *Synechococcus* sp. have been isolated from a variety of aquatic niches and characterized (Dillon and Parry 2008; Wang and Chen 2008). Another area that has received attention from the point of view of its direct application, is the characterization of cyanophages infecting bloom-forming cyanobacteria, such as *Planktothrix* sp. (Gao et al. 2009), *Cylindrospermopsis* sp. (Pollard and Young 2010) and *Microcystis* sp. (Watkins et al. 2014).

From the perspective of viral discovery, there is a vast availability of unexplored freshwater systems (Mohiuddin and Schellhorn 2015; Palermo et al. 2019; Roux, Enault, et al. 2012). Our attention in this study, therefore, has been focused on the freshwater microalgal viruses, specifically cyanophages.

1.5 Aquatic Virology Research in India

There are minimal published reports in the field of aquatic virology from India. A few previous studies (carried out majorly by a single research group) have focused on the abundance (Mitbavkar, Rajaneesh, and Sathish Kumar 2011; Parvathi et al. 2011) and ecological effects (Jasna et al. 2017, 2019; Jasna, Ram, et al. 2018; Parvathi et al. 2013) of viruses in selected niches. Studies on microalgae/cyanobacteria-infecting viruses, in particular, have also been limited, with a few reports of cyanophage isolation (Singh 1973, 1974, 1975) and characterization of externally sourced cyanophage isolates (Amla 1981; Kashyap, Rai, and Singh 1988; Singh and Kashyap 1977).

Objectives of the Present Study

The present study is based in a geographical region of the West-Coast of India (the state of Goa). Microalgae and cyanobacteria have been isolated from freshwater and estuarine aquatic niches, and cultured with the intention of utilizing them as hosts for virus isolation. Subsequently, lytic viruses infecting some of these cultured forms have been isolated – from the same or proximate aquatic niches. Thus, we have established specific virus-host systems in laboratory culture. Further, in order to broaden the scope of the study to include whole viral communities from aquatic systems of interest, we have carried out several kinds of studies on total virus populations. Firstly, virus particle abundance in representative ecosystems has been compared, by the techniques of flow cytometry and epifluorescence microscopic counts. Secondly, at the molecular level, specific virus families have been detected by PCR-amplification of marker genes. Thirdly, the viromes (virus component of the metagenomes) of two representative niches, have been characterized.

The objectives of the study were as follows:

1. Culturing of microalgae from aquatic systems of Goa.
2. Isolation and characterization of a selected microalgal virus.
3. Molecular and microscopic studies of microalgal viruses from various niches.

CHAPTER TWO

REVIEW OF LITERATURE

This chapter elaborates on the literature related to isolation, culturing and identification of microalgae and cyanobacteria from aquatic systems. This is followed by a review of the existing literature pertaining to isolation of microalgal viruses, and finally the molecular and microscopic studies of aquatic viruses as a whole.

2.1.1 Microalgae – an Introduction

Microalgae are organisms possessing chlorophyll a and a thallus not differentiated into roots, stems and leaves (Lee 1989). Thus, they include both the eukaryotic microscopic algae and the prokaryotic photosynthetic cyanobacteria (Richmond 2004).

The microalgal cell organization may take any of several different forms – unicellular (flagellate / non-flagellate), colonial (flagellate / non-flagellate) or filamentous (branched / unbranched). In addition, cells may undergo various morphological adaptations to perform specialized functions. These include

i) Spores and akinetes, thick-walled non-dividing cells formed under unfavourable conditions, which divide once favourable conditions return
ii) Heterocysts, involved in nitrogen fixation in certain cyanobacterial genera
iii) Pili and flagella for locomotion in cyanobacteria and microalgae respectively.

Microalgae further possess a wide variety of pigments, and these are characteristic of the algal class.

The current molecular system of classification, as reported by (Sigee 2004) divides algae (including micro- and macro-algae) into ten divisions, namely:

1. *Cyanophyta*: blue-green algae
2. *Chlorophyta*: green algae
3. *Euglenophyta*: euglenoids
4. *Xanthophyta*: yellow-green algae
5. *Dinophyta*: dinoflagellates
6. *Cryptophyta*: cryptomonads
7. *Chrysophyta*: chrysophytes
8. *Bacillariophyta*: diatoms
9. *Rhodophyta*: red algae
10. *Phaeophyta*: brown algae

Since the present study deals specifically with members of the Cyanophyta, Chlorophyta and Bacillariophyta, the following sections will elaborate on certain aspects of these divisions, referred to by the general terms 'Cyanobacteria', 'Green microalgae' and 'Diatoms' respectively.

2.1.2 Culturing of Microalgae and Cyanobacteria: The Beginnings

The beginnings of algal culturing date back to the late 1800s. Ferdinand Cohn in 1850 demonstrated the culturing of *Hematococcus* sp, a chlorophyte (Cohn 1850). However, the culture was not pure (devoid of other organisms), nor could it be maintained over a long period of time; moreover, the culture medium was undefined. Later, Famintzin grew green algae in a defined medium made of a few salts (Famintzin 1871). The first report of pure algal cultures came from the Dutch microbiologist Beijerinck (Beijerinck 1890). Beginning with *Chlorella* sp. and *Scenedesmus* sp., he successfully applied the established bacterial purification techniques to cultivate various green algae and cyanobacteria in subsequent years. Miquel (during 1890-1900) was the first to isolate and establish axenic cultures of diatoms, and pioneered the method of isolating single cells under the microscope, using a micropipette (Miquel 1893). Numerous other researchers made valuable contributions to this field during the nineteenth and early twentieth century, including Naegli, Klebs, Chodat, Allen, Warburg and many others. An exhaustive review of the history of algal culturing may be found in (Andersen 2005).

Provasoli and associates were in the forefront, in the development of artificial culture media for micro-algae, bearing as close a resemblance as possible to natural nutrient conditions. They were also the first to use antibiotics to eliminate bacterial contaminants associated with microalgae (Provasoli 1960). Major culture collections of algae were established by Provasoli and Guillard – National Center for Culture of Marine Phytoplankton (CCMP) at the Bigelow Laboratory for Ocean Sciences in Maine, and by Starr and Zeikus at the University of Texas at Austin (UTEX Culture Collection of Algae).

Pringsheim was the first to succeed in establishing and maintaining cyanobacterial cultures which were free from associated bacteria. He introduced several other refinements to culturing technique, summarized in the book *Pure Cultures of Algae* (Pringsheim 1946). He established a total of more than 2000 algal cultures during his lifetime, which were used to

set up several prominent culture collections – the Culture Centre of Algae and Protozoa (CCAP) in Cambridge and the Sammlung von Algenkulturen Göttingen (SAG) in Germany.

Warburg (Warburg 1921) pioneered the application of fast growing microalgal cultures like *Chlorella* sp. in physiological studies such of photosynthesis. The growth of such cultures at high density in the laboratory, led to the beginnings of microalgal mass culturing for commercial application (Burlew 1953).

2.1.3 Important Steps in the Culturing of Microalgae and Cyanobacteria

The isolation and culturing of algae from the environment and establishment of unialgal or axenic laboratory cultures, is a complicated process, influenced by numerous factors, and subject to repeated failure. Important stages of this process include:

i. Sample collection: Collection of microscopic planktonic forms differs from that of visible or surface-attached ones. Precautions during sample collection include avoidance of contamination. Microscopic observation and possible separation of collected organisms is required (Waterbury 2006).
ii. Choice of appropriate culture media: A variety of culture media have been used for cyanobacteria and microalgae, with a few of the most common being BG-11 (Rippka et al. 1979), f/2 (Guillard 1975) and Walne's medium (Walne 1970). Important constituents of culture media include nitrogen, phosphorous and trace elements, along with specific vitamins required by specific target organisms.
iii. Purification by serial dilution, micropipette isolation or agar streaking, with or without antibiotics: Purification of microalgal and cyanobacterial cultures involves separating them from contaminating heterotrophic bacteria, through a combination of streaking, microscopic observation and appropriate use of antibiotics (Andersen 2005; Waterbury 2006)

Of paramount importance is understanding and simulating the natural conditions under which the alga thrives, for example, temperature, salinity, specific nutrients and light. Only a careful combination of all such factors ensures success in isolation and purification of algal cultures (Andersen 2005).

2.1.4 Molecular Identification of Cyanobacteria and Green Microalgae

Cyanobacteria

Cyanobacteria being prokaryotic in nature, the universally used method for bacterial identification – amplification of 16S rRNA sequences – was applied to cyanobacteria as well (Neilan 1995; Nelissen et al. 1996). However, suitable primers had to be developed which would specifically amplify cyanobacterial, and exclude bacterial, sequences. Garcia-pichel et al., (1997) pioneered the development of a now widely-used primer pair CYA106F (or CYA359F) and CYA781R, specifically designed to detect and identify cyanobacteria in non-axenic samples. This primer pair has been successfully used for cyanobacterial detection and identification in marine, freshwater and culture samples (Casamatta, et al., 2005; Faldu et al., 2014; Keshari et al., 2015; Kumar et al. 2018; Moss et al. 2018). Identification of cyanobacteria at the molecular level is not merely of taxonomic value, but can provide information on toxin-producing species (Casero et al. 2019; Patel et al. 2019; Ramos et al. 2017) or species producing metabolites of medicinal and economic value (Luo 2015).

Green Microalgae

Taxonomic identification of algae has traditionally been carried out by morphological methods. In the case of microalgae, this involves careful microscopic observation and a certain level of taxonomic expertise, as the morphological differences between species are very minor. Hence the rising popularity of molecular methods of identification, based on conserved gene sequences known as barcodes (Krienitz and Bock 2012).

In the case of "coccoid green algae", taxonomic classification presents a further challenge due to the lack of sexual reproduction in most of the members. Hence, identification solely on the basis of phenotypic features, have led to major errors in taxonomic assignment (Friedl and Rybalka 2012; Krienitz and Bock 2012). The coccoid green algae belong to the classes Chlorophyceae, Trebouxiophyceae and Prasinophyceae, in the division Chlorophyta. These are unicellular green algae possessing a clockwise orientation of flagella basal apparatus (Krienitz and Bock 2012).

However, no single DNA barcode can be used across algal species, due to high diversity in nuclear as well as organelle genome sequence and organization. Various candidate genes include the nuclear-encoded 18S rRNA gene and the internal transcribed spacer sequences

(ITS) as well as the plastid-encoded genes such as *matK* and *rbcL* (Hall et al., 2010). The ITS2 sequence which lies between the genes encoding the large and small ribosomal subunits has displayed wide applicability (Caisová et al., 2013). Its only drawback is that it is insufficiently conserved, and this has been partially overcome by complementing ITS2 sequence with secondary structure information to provide a far more accurate phylogenetic picture (Buchheim et al. 2011; Coleman 2003). Identification of green algae upto species level in natural samples and cultures has been carried out, based on ITS2 sequence and secondary structure information (D'Elia et al. 2018; Ferro, Gentili, and Funk 2018; Hoda 2016). Further, this marker has been used for species delimitation among Chlorophyceae members (Hegewald et al. 2010; Leliaert et al. 2014).

2.1.5 Microalgae Previously Reported from Aquatic Ecosystems of Goa

Bacillariophyta (Diatoms)

The phytoplankton community existing in the coastal waters of Goa has been widely studied in different seasons (Devassy and Goes 1988; Kumari and John 2003; Parab et al. 2006, 2013; Pednekar et al. 2011; Redekar and Wagh 2000). The focus has been on the impact of the annual monsoon on these communities. Several researchers have reported an increase in blooming diatom species during the monsoon (Parab et al. 2006; Patil and Anil 2011)

Chlorophyta (Green microalgae)

Green microalgae inhabiting various freshwater niches across Goa have been documented by (Kanolkar and Kerkar 2009; Kerkar and Madkaiker 2003; Shetiya and Kerkar 2004). The inventories have been carried out with samples from rice fields and various types of temporary and permanent ponds, indicating the various genera and species indigenous to the area. The Goa State Biodiversity Strategy and Action Plan has also documented numerous algal forms found within aquatic niches in the state (Desai 2002).

A new microalgal species *Tetraselmis indica* was isolated from salt pans, cultured and characterized (Arora et al., 2013). To the best of my knowledge, there are no other reports of laboratory culturing of microalgae for research purposes in Goa.

Cyanophyta (Cyanobacteria)

A comprehensive survey of cyanobacterial species present in rice fields of Goa was carried out by Gomes and co-workers (Gomes, Veeresh, and Rodrigues 2011). From an agricultural

point of view, the density and diversity of cyanobacteria in rice fields under various soil and microclimatic conditions was compared, revealing greater density and diversity in undisturbed fields compared to those subject to influence of mining and other activities. In total, 84 species belonging to 16 genera were identified. Of these, 13 were unicellular forms, 30 non-heterocystous filamentous and 41 heterocystous.

A preliminary survey of most common marine cyanobacteria found at various coastal sites may be found in (Pereira and Almeida 2012). Various marine forms were isolated and cultured (Nagle et al., 2010).

The effects of UV radiation and high light treatment on two filamentous cyanobacteria in culture were studied – freshwater *Nostoc spongiaeforme* and marine *Phormidium corium* (Bhandari and Sharma 2006, 2007). UV-B treatment led to an increase in photosynthetic pigments. However, high light reduced photosynthetic efficiency, led to bleaching of pigments and degradation of DNA.

The above-mentioned studies are some of the few published reports in the field of microalgal / cyanobacterial culturing and identification from the region of Goa.

2.2.1 Introduction to Cyanophages

Ever since the first quantitative estimate of virus abundance in aquatic environments (Bergh et al. 1989), the isolation and characterization of viruses from aquatic (mainly marine) systems, became a vast area of biological and ecological study. Fairly soon (Proctor and Fuhrman 1990) it was understood that, after bacteriophages, cyanophages numerically dominate the virus population of freshwater as well as marine environments. In fact, cyanobacteria as a branch evolutionarily distinct from bacteria, were established around 3.5 billion years ago (Schopf and Packer 1987). Therefore, the origin of cyanophages is probably older. Moreover, this implies that cyanophages were the earliest predators of cyanobacteria, as eukaryotes evolved much later (Suttle 2000).

Like all viruses, cyanophages are obligate intracellular parasites. The majority of cyanophages characterized to date have been tailed double-stranded DNA viruses (Kaletta et al. 2020; Mann and Clokie 2012), assigned to the order Caudovirales. They belong to just three of the 13 families within the Caudovirales. On the basis of tail structure, these are designated the *Myoviridae* (long, contractile tail), *Siphoviridae* (long, non-contractile tail) and *Podoviridae* (short, non-contractile tail). The *Myoviridae* have much larger genome sizes than the other two families (Puxty et al. 2015). The nomenclature of cyanophages has been arbitrary, mostly based on the hosts they infect. However, Suttle (Suttle 2000) suggested a standardized system of nomenclature which has been largely followed. The first letter indicates the host genus, the next two, the geographical location of isolation, the fourth, the phage morphology and the final numeral indicates the serial number of the phage of the specific family isolated from the specific location.

Cyanophages follow any of the basic modes of infection adopted by bacteriophages. Following adsorption on a host cell surface, lytic phages divert host cellular machinery towards their own multiplication, assembly and release of numerous progeny viruses which perpetuate the infection, while temperate phages integrate their genome with that of the host and do not cause any external effect until environmental conditions favour the induction of a regular lytic cycle. Other modes of infection such as pseudolysogeny (Abedon 2009) are less common and chronic infection is not known among cyanophages (Mann and Clokie 2012). Temperate phages follow a ‘lysogenic’ mode of infection. Lysogeny protects the viral DNA from environmental conditions as well as confers on the host, immunity to further phage infections. Lysogeny is common in freshwater filamentous cyanobacteria, and

less common among unicellular forms (Suttle 2000). The significance of lysogeny is that if a large proportion of cyanobacterial communities is lysogenized, a single environmental factor such as ultraviolet light or additional availability of nutrients could trigger large scale induction, which would lead to mass mortality and ripple effects on the food chain (Weinbauer, Brettar, and Höfle 2003).

2.2.2 Host Range of Cyanophages

Unlike most bacteriophages, cyanophages typically have a broad host range. This implies that the same cyanophage has the ability to infect different strains, species or even genera of cyanobacteria. Broad host ranges may be an evolutionary advantage in low-nutrient waters where host abundances are low (Watkins et al. 2014).

Several of the earliest isolated cyanophages, including the very first one (Safferman and Morris 1963) were consigned to the 'LPP' group for their ability to infect several genera of filamentous cyanobacteria, namely *Lyngbya*, *Plectonema* and *Phormidium*. It was hypothesized that this putative broad host range could in fact be a consequence of discrepancies in cyanobacterial taxonomy (Suttle 2000) However, a number of researchers later isolated broad host range phages thereby confirming the phenomenon (Dekel-Bird et al. 2015; Deng and Hayes 2008; Watkins et al. 2014).

Here again the distinction between the families of cyanophages seems to be that myoviruses have a broader host range – 'generalists' , while podo- and sipho- viruses have a narrower host range – 'specialists'. It has been suggested that tRNAs encoded by certain phages could play an important role in their adaptation to infecting different genera of hosts, as these enable the phages to modify codon usage according to that of the host (Dekel-Bird et al. 2015; Enav, Béjà, and Mandel-Gutfreund 2012).

2.2.3 Isolation and Characterization of Cyanophages

To an extent, the methods conventionally used for bacteriophage isolation from the environment, may be applied to cyanophages as well (Clokie and Kropinski 2009). The plaque assay is the most widely used of these. Host cultures which are capable of growing in agar medium, can be mixed into a small volume of soft agar along with putative phage-containing samples, and plated on top of a base layer of higher percentage agar. A

permissive host such as *Synechococcus* WH7803 is helpful for isolating phages from new environments. The plaque assay, however, is not suitable for detecting cyanophages where the host is motile or cannot grow on solid media (Deng and Hayes 2008; Wilhelm et al. 2006). In such cases, liquid propagation is utilized.

In oligotrophic waters, concentration of natural samples for virus isolation may not be required (Clokie et al. 2006). However in, for instance, open ocean samples, prior concentration may facilitate better detection of lytic viruses. Concentration is commonly carried out by using filters (Jing et al. 2014), chemical flocculants (John et al. 2011) or polyethylene glycol (Colombet et al. 2007).

2.2.4 *Synechococcus* Cyanophages

Synechococcus sp., along with *Prochlorococcus* sp., both unicellular cyanobacteria, together account for upto a quarter of primary productivity in the oceans (Partensky, Blanchot, and Vaulot 1999). It is no surprise, therefore, that the first marine cyanophages to be isolated and characterized were those of *Synechococcus*. Waterbury and Valois (Waterbury and Valois 1993) isolated diverse phages (representatives of all three families of tailed phages) against several strains of *Synechococcus*. However, the lytic effect was negligible and they reasoned that this could be due to high resistance in natural *Synechococcus* populations. Subsequent studies have confirmed his hypothesis (Marston and Sallee 2003).

At around the same time, Suttle and Chan (Suttle and Chan 1993) isolated phages infectious to both marine and freshwater strains. Abundances were estimated by TEM as well as plaque assays and could reach a maximum of 10^5 particles per ml. Nearly a decade later, the first genome sequence of lytic phage P60, infecting a marine *Synechococcus* strain, was reported (Chen and Lu 2002). Among *Synechococcus* phages isolated from Rhode Island coastal waters over a 3-year period (Marston and Sallee 2003), phylogenetic analysis based on the *g20* marker revealed 36 distinct cyanomyovirus genotypes. The first report of a marine 'synechophage' (term coined by (Wang and Chen 2008)) from the Eastern world was from the South China Sea (Zhang et al. 2013).

Gradually synechophages from freshwater, estuarine and polar environments were isolated. Wang and Chen used the conventional plaque assay method to isolate seven synechophages on four estuarine host strains. These phages showed a high host-specificity and some isolates possessed the photosynthetic *psbA* gene marker which, nevertheless was distinct

from the marine counterpart (Wang and Chen 2008). In another study however, the genome of a freshwater synechophage indicated phylogenetic relationships with marine isolates (Dreher et al. 2011).

Wang and co-workers (Wang, Asakawa, and Kimura 2011) tracked the abundances of *Synechococcus* strains and their co-occurring phages in an estuarine environment – Chesapeake Bay, over a period of 5 years. The respective titres of phage and host were found to covary and to be seasonally dependent.

Chénard and co-workers (Chenard et al. 2015) reported an isolate from Arctic freshwaters, whose genome sequence indicated little similarly to previously sequenced synechophages, but shared features with metagenomic data from diverse environments, indicating the possibility of widespread dispersal of such phages.

In general, the vast majority of isolated synechophages have been myoviruses, with a few reports of podoviruses and siphoviruses (Zhong et al. 2018). Further, these phages generally infect phycocyanin-rich strains in freshwater and phycoerythrin-rich strains in marine systems (Mann 2003; Suttle 2000).

Studies on synechophages have laid the foundation for diverse cyanophage research and the story of these phages continues till date. While the vast majority of isolated synechophages have been myoviruses, and the few siphovirus isolates have been exclusively marine, Zhong and co-workers report several interesting features of a freshwater siphovirus isolate (Zhong et al. 2018). Similar to the virus isolated by Chénard and associates (Chenard et al. 2015), the genome of this isolate shares similarity with metagenomic sequences from diverse aquatic environments, but is highly divergent from previous siphovirus isolates.

2.2.5 Cyanophages of Filamentous Cyanobacteria

A disproportionate number of cyanophages studies so far, whether individual or metagenomic, have come from marine environments. Freshwater cyanophages are under-represented in metagenomic datasets and 94% of all sequenced genomes belong to *Synechococcus* and *Prochlorococcus* (Šulčius et al. 2019). This fact is surprising, given that the earliest cyanophages to be isolated were from freshwater niches. One possible reason could be that, during the initial years post-discovery of cyanophages, the major interest was in their potential to control harmful cyanobacterial blooms (Safferman and Morris 1963),

The failure of efforts in this direction may have shifted the focus to marine systems. During the last couple of decades, freshwater cyanophages research has picked up. Efforts are still on to isolate highly virulent phages against bloom-forming cyanobacteria, which could in future be used as a biological control agent. Since many of the bloom-formers are filamentous forms, some of the recent work that has focused on cyanophages infecting freshwater filamentous cyanobacteria is summarized below.

Lyngbya majuscula forms toxic summer blooms over Moreton Bay, Australia. The blooms were observed to decay rapidly, suggesting the presence and action of a lytic cyanophage, which was isolated and characterized (Hewson et al. 2001).

A lytic cyanophage against another bloom-forming species, *Planktothrix agardhii*, was isolated from Lake Donghu, China (Gao et al. 2009). Lake water was tested against 24 strains of filamentous cyanobacteria. The susceptible *P. agardhii* strain showed lysis after 8 days of inoculation. Shortening of host filaments was observed, and infected filaments lost mobility. Further, regrowth of certain resistant host filaments occurred. Virus particles were observed through TEM to have an icosahedral structure with a mean diameter of 76 nm. Genomic analysis of this phage (Gao, Gui, and Zhang 2012) confirmed the tailless structure, by the absence of genes coding for typical tail-associated proteins

In a study by (Pollard and Young 2010), natural virus-containing samples from lake water were tested for infectivity against the filamentous *Cylindrospermopsis raciborskii*. Lysis was confirmed by a decrease in host cell abundance, and a corresponding increase in virus-like particles. Further, the host filaments fragmented post-viral infection. Interestingly, the authors hypothesize that fragmentation would facilitate dispersal and hence, could be a survival strategy. The process of virus release from the cell was captured by epifluorescence microscopy. The virus had a burst size of 64, similar to other aquatic cyanophages.

A lytic cyanophage infecting *Phormidium orientale* were isolated from three different freshwater locations in Egypt – a rice field, reservoir and river (Ali et al. 2012). It was host-specific and caused visible lysis in liquid medium as well as formed plaques on lawns of host. TEM analysis confirmed the virus belongs to *Siphoviridae* and has a head of diameter 85 nm and tail of length 182 nm.

As reported by many workers in the field, cyanophages typically have a broad host-range. One such ‘generalist’ was found to infect two unrelated genera – *Planktothrix* and *Microcystis* (Watkins et al. 2014). The study used non-axenic cultures of host cyanobacteria.

Members of the genus *Planktothrix* are motile, which make the plaque assay difficult, hence liquid assays were carried out. The virus was structurally characterized by TEM and Atomic Force Microscopy, and found to belong to *Podoviridae*, with an unusually large capsid of 100-120 nm. Another cyanophage infecting *Plectonema* and *Phormidium* caused complete lysis of host within 24 hours (Zhou et al. 2013).

Anabaena phages were isolated from a tropical freshwater reservoir (Yeo and Gin 2013). Host species used were *A. circinalis* and *A. cylindrica*, and isolation was carried out using standard liquid and plaque assays. The phages showed potential as biocontrol agents in preventing bloom formation, as they inhibited the formation of dense mats.

Sulcius and co-workers characterized a phage of the harmful filamentous cyanobacterium *Aphanizomenon flos-aquae* (Šulčius et al. 2015, 2019). vb-AphaS-CL131 is the second largest cyanosiphovirus discovered to date, with a genome size of around 120 kb. In the case of the host, *A. flos-aquae*, the existence of a lytic phage in bloom conditions assumes greater significance due to the fact that toxins produced by this alga, as well as dense filament structure prevent zooplankton grazing, and the resultant population control. Hence viruses are virtually the only control mechanism. Phage CL131 demonstrated a very long latent period of about 108 hours in laboratory assays. Further, CL131 was highly host –specific, infecting only two out of a total of 60 strains of *Aphanizomenon* and the related *Dolichospermum*. The genome of CL131 included a CRISPR-cas system, rarely found in phages.

The only known cyanophage to infect the genus *Limnothrix* was isolated from Lake Donghu, China (Xiangling et al. 2015). Lysis was observed by yellowing of host culture (degradation of filaments) followed by clarification of the culture. Purification of the phage followed by TEM analysis revealed a *Siphoviridae* structure with a unique ‘collar’ between the head and tail, previously unknown among phages.

2.2.6 Microalgal Viruses: A Brief Introduction

Although cyanobacteria are phylogenetically closer to bacteria than to eukaryotic algae, their ecological role is more closely linked to that of algae, particularly microalgae. These two groups of organisms together are the major primary producers in aquatic ecosystems. Hence it is also meaningful to consider viruses of cyanobacteria and microalgae together (Suttle 2000).

Viruses infecting eukaryotic algae are genetically diverse, encompassing single- as well as double-stranded DNA and RNA genomes in the size range of 4.4 to 638 kb (Short et al. 2020). The activity of these viruses assumes global significance due to their well-established effects on controlling algal blooms (Brussaard and Martínez 2008; Suttle 2007).

The very first such virus (Gibbs et al. 1975) was isolated fairly soon after the initial discovery of aquatic viruses. However, not much attention was devoted to algal viruses until the 1980s, when James van Etten initiated a dedicated quest to characterize viruses infecting *Chlorella* sp. (Van Etten et al. 1983; Van Etten and Dunigan 2012).

Today, viruses infecting approximately 60 host species exist in culture collections worldwide (Coy et al. 2018). The vast majority of these belong to two families of large, ds DNA viruses – the *Phycodnaviridae* and the *Mimiviridae*, collectively known as the nucleo-cytoplasmic large DNA viruses or NCLDVs (Wilson, Van Etten, and Allen 2009).

The *Phycodnaviridae* are a family of morphologically similar (icosahedral) but genetically diverse viruses (Van Etten et al. 2002; Wilson et al. 2009). Presently phycodnaviruses comprise six genera, based on the hosts they infect, and on genomic characteristics: *Chlorovirus, Coccolithovirus, Raphidovirus, Prasinovirus, Prymnesiovirus* and *Pheovirus* (Brussaard et al. 2012). Among these, the Chloroviruses target freshwater algae while all the rest infect marine algae (Chen et al. 2018). Diverse viral life cycles are represented by chlorovirus PBCV-1 (lytic), pheovirus EsV-1 (lysogenic) and coccolithovirus EhV-86 (chronic) (Wilson et al. 2009).

Chloroviruses are among the most interesting groups of viruses ever characterized. Physiologically, they are unique as they do not infect free-living hosts but only chlorella-like unicellular green algae in a symbiotic association with zooplankton, called zoochlorellae (Van Etten et al. 1983; Van Etten and Dunigan 2012, 2016). Chlorovirus genomes encode a huge variety of proteins, including many unusual ones such as sugar metabolism enzymes and DNA restriction endonucleases. Many proteins and enzymes encoded by the prototype PBCV-1 are the smallest in their family (Van Etten and Dunigan 2012; Sandaa and Bratbak 2018). The most astonishing discovery has been the presence of chlorovirus genes in the human brain (Yolken et al. 2014).

2.3.1 Virus Enumeration

Until the latter part of the twentieth century, the only methods used, to estimate virus counts in natural waters, were the plaque assay and the most probable number (MPN) method, both of which relied on the availability of culturable hosts. These methods were useful for detecting only the bacteriophage component in environmental samples. Unsurprisingly, phage populations were thought to be as low as, for instance, 10^3 per millilitre in seawater samples (Frank and Moebus 1987).

Then came a landmark study in 1989, wherein Bergh and co-workers concentrated aliquots of natural water by ultracentrifugation, stained virus particles with uranyl acetate and observed them under an electron microscope. They came up with an unprecendented estimate of 2.5×10^8 virus particles per ml. As of December 2020, this study (Bergh et al. 1989) has been cited more than 1750 times (Google Scholar data).

Transmission Electron Microscopy for Virus Enumeration

Electron microscopy as a technique to study the structure of viruses was developed since the 1930s (early work reviewed by Ackermann 2011 and Almeida, Leppänen, Maasilta, & Sundberg, 2018). However, quantification of viruses by TEM began with the work of Bergh and associates (Bergh et al. 1989). Numerous important conclusions on the ecological contributions of viruses were made possible based on TEM counts of viruses, both free and within infected host cells (Cochlan et al. 1993; Fuhrman 1999; Maranger and Bird 1995; Proctor and Fuhrman 1990; Suttle and Chen 1992). However, TEM was later superseded by more accurate enumeration techniques.

Epifluorescence Microscopy for Virus Enumeration

In a departure from the trend of major innovations in virus research coming from the Western World, a team of Japanese scientists (Hara, Terauchi, and Koike 1991) were the first to report a different method for enumeration of viruses in natural waters. Virus particles were stained with DAPI (4′,6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to double-stranded DNA. Larger DNA-containing particles – bacteria and plankton were also stained for comparison. This was done by collecting whole seawater samples on 0.015 µm polycarbonate filters, followed by staining and immediate viewing under an

epifluorescence microscope (EFM). Viral counts thus obtained were well correlated with those obtained through TEM.

Hennes and Suttle (1995) rigorously standardized the EFM technique, by estimating virus abundances in a range of environments, from marine to freshwater and oligotrophic to hypereutrophic. They used Yo-Pro, a cyanine-based dye, which stains nucleic acids. This method was found to be far more precise than TEM. Virus abundance values ranged from 10^7 to 10^8 in surface seawater to 10^9 in water surrounding a cyanobacterial mat.

As EFM does not reveal virus structure, but only a quantitative estimate of virus particles, the term virus-like particles (VLPs) was used to denote those satisfying the size criteria of viruses in a given sample analysed through EFM.

In the first study investigating virus and bacterial population abundance in sediment pore water, VLPs were found to be 10 times more abundant in pore water than in the water column, indicating their significance in the sedimentary microbial community (Drake et al., 1999). Further, the abundance of VLPs was correlated with bacterial abundance and not with *Chl* a, suggesting the predominance of bacteriophages.

With refinement of the EFM technique, important conclusions could be drawn concerning the impact of viruses on the ecology of aquatic systems. In a mesocosm experiment, the populations of viruses, bacteria and nanoflagellates were measured using EFM (Guixa-Boixereu et al., 1999). Virus counts increased by an order of magnitude during the experiment. Viral lysis was found to be a significant factor in bacterial lysis, at stages more so than bacterivory by flagellates. However, virus counts were found to differ, depending on whether DAPI or Yo-Pro was used as stain, with Yo-Pro giving higher counts. This difference was consistent.

The relative importance of lysis and lysogeny on bacterial and cyanobacterial hosts, respectively, during bloom conditions, was studied (Ortmann, Lawrence, and Suttle 2002). Seawater samples were incubated with and without Mitomycin C, and direct counts of viruses using Yo-Pro-1, followed by EFM, over a 24-hour period. A very high level of bacterial lysogeny was observed, along with the possibility of induction in the cyanobacteria *Synechococcus* sp.

The contribution of viral lysis vis-à-vis flagellate grazing in bacterial mortality was estimated in the freshwater of Lake Pavin, and compared between the metalimnion and epilimnion of the lake (Bettarel et al. 2003).

Most of the mentioned studies used DAPI or Yo-Pro as stain. (Noble and Fuhrman 1998) reported great success with the use of SYBR Green in EFM experiments. SYBR Green I and Later SYBR Gold became the 'gold' standard for EFM staining. The reasons for this were far greater brightness and longevity of the stain, clarity of imaging and reduced staining time required, compared to other stains. Detailed protocols for this have been published (Patel et al. 2007; Suttle and Fuhrman 2010). Since then, EFM has become the routine method for virus enumeration in a variety of samples.

Flow Cytometry for Virus Enumeration

The drawbacks of TEM were the time, expense and effort associated with sample preparation as well as analysis. Further, TEM is strictly a laboratory method, and could not be applied to field investigations. EFM, while scoring over TEM in most of the above aspects, still involved tedious sample preparation and strenuous microscopic counting.

The technique of flow cytometry (FCM), involving rapid and specific staining of all cells, with nucleic acid-specific stains such as SYBR Green, had been used for detection and enumeration of phytoplankton and bacteria. Marie and co-workers (Marie et al. 1999) extended this technique to viruses, thus establishing a simple, elegant and speedy method to enumerate viruses in a variety of water samples, especially applicable to on-board studies. With the high throughput capacity of FCM, a large number of samples could be rapidly analysed, generating statistically significant information. The method was first optimized with an algal virus culture, after which it was successfully used with seawater samples from several locations.

FCM was further applied to several families of viruses – *Baculoviridae, Podoviridae, Herpesviridae, Myoviridae, Siphoviridae, Phycodnaviridae,* and others (Brussaard et al. 2000). They found that the output cytogram could differentiate various classes of viruses, with fluorescence signal emitted being proportional to the genome size of the virus. At the lowest limit of detection were small genome-sized RNA viruses. Many others (Ma et al. 2013; Zhong and Jacquet 2013) reported a clear-cut distinction in virus populations

estimable through FCM. Upto four different groups of viruses (differing according to size and genetic material) could be distinguished by their position in the cytogram.

Brussaard (Brussaard et al. 2010) provides a comprehensive description of the methodology used to obtain accurate virus counts and eliminate background signals – a major challenge with this method. Numerous researchers have subsequently applied this method to marine samples. An in-depth explanation of the principles behind the FCM method may be found in (Gasol and Moran 2016).

Ma and co-workers studied the variation in virioplankton populations in the Haihe River, China, over a one-year period and correlated this data with environmental parameters. Viral counts peaked in the spring season and were highest in estuarine sites (Ma et al. 2013).

Duhamel and Jacquet (Duhamel and Jacquet 2006) counted viruses in lake water as well as sediments by modifying the established protocol, including fixation time (optimum – 1 Hour) and the use of SYBR Green II. The study was carried out with samples from Lakes Geneva and Bourget. Samples from Lakes Geneva, Bourget and Annecy, three of the largest lakes in France, were analysed by FCM for seasonal variations in virus populations (Personnic et al. 2009). Two seasonal peaks were observed – in summer and autumn.

While the above studies focused on surface water samples, another interesting (and, quite literally, ‘in-depth’) study carried out on Priest Pot Lake in the English Lake District, documented the changes in viral abundances with depth and across seasons (Goddard et al. 2005). This was done in conjunction with changes in other microbial abundances for a broader perspective. Total virus abundances were highest in the summer and lowest in the winter. However, while counts were relatively constant with changing depth during the spring and winter, in the summer, a distinct increase in virus concentration in the deepest anoxic layers was observed, correlated with a peak population of green sulphur bacteria.

High virus counts, apparently exist not just in the deepest water layers but in the permanently anoxic sediments of lakes as well (Borrel et al. 2012).

Jacquet and co-workers (Jacquet, Dorigo, and Personnic 2013) fine-tuned many of the steps prior to FCM analysis, including type of dilution solution and stain used, type and concentration of fixative, and incubation temperature, with the caveat that a similar fine-tuning process be carried out by researchers working on different kinds of samples.

Nakayama and co-workers (Nakayama et al. 2007a) studied viral abundances in a previously unexplored ecosystem – the rice field floodwaters. While viral abundance was found to vary almost 50-fold across the rice cultivation period, a mean abundance of 1.5 x 10^8 particles per ml was found and this figure correlated significantly with counts obtained by EFM for the same samples. They reported for the first time that virus counts in paddy field floodwaters exceeded typical counts in other freshwater and marine aquatic systems.

The only study in this field from any aquatic system in India was carried out by (Mitbavkar et al. 2011) where viruses were enumerated from the Zuari estuary, Goa, through FCM, and two different populations of viruses were distinguished.

2.3.2 Virus Diversity Studies Based on Signature Genes

Studies of viral abundances in various environments helped in elucidating the ecological roles of viruses. In parallel with this information, uncovering the viral diversity in a particular environment provided new insights into the structure and function of microbial communities in that environment. The experimental use of signature genes to reveal viral diversity began in the late 1990s. The challenge here is that viruses do not possess a universal signature gene similar to the 16S gene in bacteria. Hence a variety of potential marker genes were used, most of which are family-specific. Broadly these consisted of structural genes, auxiliary metabolism genes and DNA polymerase genes (Adriaenssens and Cowan 2014). The application of each of these candidate markers towards studying viral (predominantly cyanophage) diversity in freshwater environments is briefly covered below.

Structural genes

The *g20* gene was the first marker that was applied to the study of environmental cyanophage diversity (Fuller et al. 1998). It encodes the portal protein in T4-like phages (*Myoviridae*). A variety of primer sets were designed to amplify different portions of the gene. Initial studies using DGGE of amplified g20 fragments in various marine systems revealed interesting facts such as depth variation of viral diversity, corresponding with host diversity and nutrient levels (Wilson et al. 2000). In freshwater environments, viruses belonging to the same clades as marine were identified in Lake Bourget France (Dorigo et al. 2004) and Lake Erie Canada (Matteson et al. 2011). Further, seasonal variations were found to occur. *g20* analysis of paddy field floodwater and soil (Wang, Murase, Asakawa, &

Kimura, 2010; Wang, Asakawa, & Kimura, 2011) revealed distinct viral communities, with water communities showing some degree of similarly to other aquatic environments. Surprisingly, in a large study covering samples from diverse environments from marine (including tropical, polar and deep sea) to freshwater (lakes and ponds), highly similar sequences were found in widely separated environments, a finding which could point to either of two possibilities – movement of phage across ecosystems (Breitbart and Rohwer 2005) or horizontal gene transfer (Hatfull and Hendrix 2011). The only drawback of the *g20* gene is that is not represented in the entire *Myoviridae* family of cyanophages (McDaniel, DelaRosa, and Paul 2006).

In comparison to *g20,* the *g23* gene coding for the major capsid protein of T4 like phages, was found to be more widely distributed in the *Myoviridae* (Filée et al. 2005). *g23* sequence analysis uncovered previously unknown groups of viruses in marine systems. The sequence has also been extensively used in samples from paddy field niches, i.e. floodwater, soil at different levels and straw, to discover virus community structure therein (Fujii et al. 2008; Jia et al. 2007). Overall, the *g23* marker was found to represent a greater diversity of myoviruses than *g20*. However, it could amplify sequences from heterotrophic T4-like phages as well, hence was not totally specific to cyanomyoviruses (Mann and Clokie 2012).

Auxiliary metabolism genes

The discovery that cyanophages carry photosynthetic genes acquired from host cyanobacteria (Mann et al. 2003; Millard et al. 2004) was a game-changer in the field of aquatic virology. Homologues of the genes *psbA* and *psbD,* coding for core proteins D1 and D2 of photosystem II are found in the genomes of numerous *Myoviridae* and *Podoviridae* members. In fact, approximately 88% of marine cyanophages (Sullivan et al. 2006) and 90% of all cyanophage isolates (Puxty et al. 2015) carry the *psbA* gene. Because D1 has a short lifetime, yet is critical for photosynthesis to take place, it is hypothesized that viral expression of *psbA* is also required to maintain photosynthesis, as viral infection shuts down mRNA production by the host (Sieradzki et al. 2019). The expression of photosynthetic genes thus confers a fitness advantage on phages, by allowing photosynthesis to continue after host machinery shuts down, thereby supporting phage release from host cells (Lindell et al. 2004; Mann et al. 2003; Puxty et al. 2016).

psbA analysis can distinguish between viruses from freshwater and marine environments, and even between *Synechococcus* and *Prochlorococcus* viruses (Chénard and Suttle 2008).

DGGE analysis of *psbA* sequences present in paddy field floodwater (Wang et al. 2010; Wang, Jing, et al. 2016) too indicated differences from marine sequences but a narrow distribution within the specific niche. Further, the gene was found in estuarine podoviruses infecting *Synechococcus* (Wang and Chen 2008) and in lake freshwaters (Zhong and Jacquet 2013). Recently, *psbA* gene expression was measured in cultures of lytic cyanophages isolated from Lake Erie (Jiang et al. 2019).

Various other host-derived metabolic genes have been detected in cyanophage genomes as well as metagenomic samples (Adriaenssens and Cowan 2014; Gao, Huang, and Ning 2016). These include genes involved in the phosphate acquisition pathway, carbon metabolism and pigment degradation. The function of such horizontally transferred genes is clear – they facilitate the ecological success of cyanophages (Gao et al. 2016).

DNA polymerase genes

While the *g20* and *psbA* markers majorly target cyanomyoviruses, the *pol* gene encoding Family A DNA polymerase has been used to detect cyanopodoviruses in estuarine (Chen et al. 2009), marine (Labonté, Reid, and Suttle 2009), lake (Wang et al. 2015), paddy field (Wang, Liu, et al. 2016) and wetland (Li et al. 2019) water samples.

Another DNA polymerase gene, *polB*, has been widely used in the detection of viruses of eukaryotic algae, belonging to the family *Phycodnaviridae*. In a seminal work by Chen and Suttle, the gene was successfully amplified from cultures of viruses infecting *Chlorella* and *Microcystis* sp. as well as natural virus assemblages (Chen and Suttle 1995). Short and Suttle (Short and Suttle 2002) demonstrated that closely related algal viruses could be found in marine systems across the world. DGGE and phylogenetic analysis of amplified *polB* fragments from lake samples in Canada (Clasen et al. 2008), revealed subtle differences at the inferred amino acid level, which could potentially be used to predict virus hosts. In a yearlong survey of two French lakes (Jacquet, Zhong, and Parvathi 2013), phycodnavirus signatures were found to be unique and distinct from other freshwater as well as marine niches. *polB* sequencing has provided useful information on algal virus populations in various other niches such as a bay (Rozon and Short 2013), an estuarine transition zone (Labbé et al. 2018) and a freshwater pond (Long and Short 2016).

2.3.3 Virus Metagenomics

Metagenomics is defined as the direct sequencing and analysis of all genetic material recovered from an environmental sample (Thomas, Gilbert, and Meyer 2012). Over the past two decades, metagenomics has been the method of choice for studying total microbial communities in a culture-independent manner. Initially, the approach followed was cloning and Sanger sequencing of microbial genetic material (Angly et al. 2006; Venter et al. 2004). Later, the use of universal marker genes which are conserved within phyla, increased in popularity. Generally ribosomal genes, such as 16S for bacteria, 18S for eukaryotes and the internal transcribed sequence (ITS) for fungi and algae, the PCR-amplification followed by sequencing of fragments of these genes could reveal information on the taxonomic diversity of microbes in a given niche. Since viruses do not possess a universal marker, family-specific markers were used, as described in detail above, such as *g20*, *g23* and *polB*.

Next-generation-sequencing (NGS) took metagenomic studies to a different level, free from limitations of gene marker analysis, wherein whole microbial communities could be studied. Advances in NGS instrumentation and techniques have made the impossible possible and also accessible to the common researcher. Novel bacterial and archaeal species have been discovered in a wide variety of ecosystems and ecological niches (Yarza et al. 2014). Where the virus component is concerned, the lack of a universally conserved genetic marker makes metagenomic studies more complicated than in the case of bacteria, where the 16S rRNA sequence is routinely used to uncover novel taxa. In the case of viruses, a whole-genome sequencing or shotgun approach must be employed, wherein data analysis is far more time-consuming. Challenges in analyzing viral metagenomes include the lack of sufficient viral sequences in databases, the vast diversity of viral types in any given environmental sample, and inevitable contamination with bacterial DNA, necessitating additional purification steps as well as filtering out bacterial sequence data (Bruder et al. 2016; Hayes et al. 2017).

Nevertheless, this technique has been widely used, beginning with marine environments (Duhaime and Sullivan 2012). The pioneering study was by (Breitbart et al. 2002), at a time when NGS did not exist and a complex approach of preparing linker-amplified shotgun libraries in the laboratory was followed. Later, with advancements in sequencing technology and bioinformatics tools, marine viromics has been at the cutting edge of environmental virology research, with important contributions by many researchers (Coutinho et al. 2017;

Culley, Lang, and Suttle 2006; Hurwitz and Sullivan 2013; Mizuno et al. 2013; Winter et al. 2014).

Since the focus of our study is on various freshwater environments, I review in greater detail the studies that have explored the viromes of freshwater systems, including lakes, ponds and groundwater reservoirs. Understanding the virus populations that exist in freshwater systems is of great importance as these systems have a direct impact in human health, being in close proximity to human habitation. Further, from the point of view of discovery of novel viruses, freshwater bodies present a huge variety of unexplored yet accessible aquatic niches.

Broadly the workflow of any virome analysis project comprises the following steps:
1) Sample collection 2) Removal of higher organisms 3) Concentration of viral particles
4) Viral nucleic acid extraction 5) Library preparation and sequencing
6) Read error correction 7) Assembly 8) Comparison to reference databases 9) Annotation

Roux and co-workers (Roux, Enault, et al. 2012) demonstrated for the first time, a high degree of specificity in freshwater virus communities, compared to those from marine and other ecosystems. Two temperate freshwater lakes were compared, the mesotrophic Lake Bourget, and the oligotrophic Lake Pavin. The species richness of Lake Bourget was found to be far greater than that of Lake Pavin, a trend observed with microorganisms as well. However, overall virus communities of the two lakes were found, through phylogenetic analysis, to be very similar.

The community composition at several sites of the temperate eutrophic Lake Matoaka revealed that high anthropogenic activity adversely affects viral diversity (J. C. Green et al. 2015). The results of this study also supported the conclusion that freshwater virus assemblages are genetically distinct from those in other environments.

Another hypertrophic Lake, however – Lough Neagh, the largest freshwater lake in Ireland – exhibited a highly diverse viral community (Skvortsov et al. 2016). Further, compared with earlier studies on lake viromes, a very low proportion of ssDNA viruses was found. This could be attributed to biases in sample preparation techniques, highlighting the importance of sample preparation in the characterization of metagenomes.

In one of the few studies conducted on river ecosystems, (Labbé et al. 2018) followed an amplicon-based approach to characterize the diversity of two viral families in the St

Lawrence estuary. The *Picornavirales* (RNA viruses) and *Phycodnaviridae* (ds DNA viruses of eukaryotic algae) demonstrated high diversity and uniqueness.

Unlike large bodies like oceans, rivers and to some extent lakes, microbial and viral communities in smaller ponds and wetlands are directly exposed to fluctuations in climatic conditions as well as anthropogenic activity. Changes in virus community structure in an agricultural pond were monitored over a three-month span during the autumn-winter change of season (Chopyk et al. 2018). A parallel bacterial community profiling revealed a broader picture of virus-host dynamics in such systems. The viral population was dominated by *Siphoviridae*.

A similar investigation of the virus community (with emphasis on bacteriophages) in conjunction with that of bacterial hosts, was carried out in a groundwater site contaminated with hydrocarbon-containing effluent (Costeira et al. 2019). Phages infecting hydrocarbon-degraders were prominent members of the community, and included generalists (those with a broad host range). Studying the phage and bacterial populations in combination would contribute to current research on the bioremediation of polluted groundwater aquifers.

Anthropogenic activity influences the composition of freshwater viromes, particularly at sites that are in close proximity to human habitation. The viromes of three freshwater reservoirs in the Ile-Balkash region of Kazakhstan (Alexyuk et al. 2017) showed the presence of not only typical autochthonous viruses, but also allochthonous viruses of families *Coronaviridae*, *Reoviridae* and *Herpesviridae*, whose presence is indicative of sewage contamination in water.

In a unique study on the Cuatro Ciénegas, a water basin in a Mexican desert, the viral metagenome at several locations was examined (Taboada et al. 2018). A very high diversity of endemic species of prokaryotes, plants, invertebrates and vertebrates has previously been reported from the region. A corresponding high viral diversity, with a large proportion of unique (possibly endemic) taxa was found. Unsurprisingly, bacteriophages predominated; surprisingly, algal viruses too were numerous, in spite of algal populations not being very high in the specific niche.

Fancello and co-workers (Fancello et al. 2013) compared the viral metagenomes of several perennial ponds in the central Sahara desert. As in the study by Green et al., the ponds exposed to higher anthropogenic effects had a lower viral diversity. In general, tailed

bacteriophages predominated, both in pristine and relatively polluted aquifers. These were mostly lytic cyanophages, whereas, lysogenic phages dominated in the pond experiencing extreme conditions of dryness at certain times.

The existence of high viral diversity in extreme environments too, is no longer a novel fact, as pointed out by (de Cárcer et al. 2016; Das et al. 2020) and many others.

While most of the above studies have dealt with the viral metagenomes of specific aquatic niches, or comparisons between niches of the same environmental type, a comparison of the viromes of nine different biomes was carried out by Dinsdale and co-workers (Dinsdale et al. 2008). The biomes included marine, freshwater, subterranean, hypersaline, microbialites and animal-associated. Significant findings of the study included distinct functional (metabolic) profiles in each biome, and a viral metagenome in each, that was predictive of the biogeochemical conditions therein.

Among various published protocols for preparation of samples for viral metagenomics analysis, the protocol described by (Thurber et al. 2009), has been successfully used by a number of subsequent researchers. A detailed and immensely helpful overview of the entire workflow of viral metagenomic analysis, with emphasis on freshwater samples, has been provided (Putonti, Diener, and Watkins 2018). A number of authors have reviewed the bioinformatic tools available for meaningful analysis of vast amount of metagenomic data (Bruder et al. 2016; Hayes et al. 2017; Nooij et al. 2018). Some of the possible biases inherent at different stages of methodology have been elucidated (Kim and Bae 2011; Rastrojo and Alcamí 2017).

Thanks to virus metagenomics, more and more viral ‘dark matter’ is being brought to light (Krishnamurthy and Wang 2017; Roux, Matthijnssens, and Dutilh 2019). New bioinformatic tools to analyse the vast quantities of sequence data, are constantly being developed and modified for greater utility. However, metagenomics and laboratory isolation and characterization of viruses have to go hand in hand, because these studies complement each other. While metagenomics studies can uncover a vastly greater diversity of virus sequences present in the environment (far more than can ever be isolated or propagated in the lab), the up-to-date annotation of metagenomics sequences depends on the constant addition of virus whole genome sequences to reference databases. Metagenomics without sufficient resources for annotation is of little utility.

CHAPTER THREE

CULTURING OF MICROALGAE AND CYANOBACTERIA FROM AQUATIC SYSTEMS OF GOA

Microscopic algae, generally referred to as microalgae, are widely found in freshwater as well as marine ecosystems. They are typically unicellular, existing in solitary form, or as chains or colonies. In the present chapter, 'microalgae' refers to diatoms, cyanobacteria and green microalgae that have been isolated from aquatic niches within the state of Goa. Goa is endowed with a variety of aquatic systems. This chapter reports the isolation and culturing of microalgae from lakes, ponds, rice field floodwaters, estuarine and coastal sites. The broad morphological identification of these cultures has been corroborated by molecular phylogenetic analysis. The objective of this study was to isolate and identify the microalgae, and subsequently use them as hosts for isolating viruses from similar aquatic niches.

Materials

Media: BG-11, f/2 (composition in Appendix), nutrient broth, Luria-Bertani broth

Chemicals: Sodium nitrate, sodium hydrogen phosphate, sodium silicate, agar, Tris chloride, sodium dodecyl sulphate, sodium chloride, CTAB, EDTA, phenol, chloroform, isoamyl alcohol, isopropanol. All chemicals were obtained from HiMedia Laboratories and were of analytical grade.

Antibiotics: Penicillin, streptomycin, gentamycin, tienam

Kit: PCR purification kit (GeNei)

PCR reagents (GeNei): *Taq* polymerase, dNTP mix (10mM), 10X PCR assay buffer

Methods

3.1 Water Sample Collection

Water samples were collected from various marine, freshwater and estuarine sites situated within Goa, India. In all cases, surface water samples (between a depth of 0 to 2 m) were collected using a sterile container. Samples were collected in various seasons, as the primary aim of collection at this stage was establishment of microalgal cultures.

3.2 Isolation of Marine Diatoms

Water samples were collected from various coastal locations (**Table 3.1A; Figure 3.1**). Samples were first filtered through a 200 μm nylon mesh to remove zooplankton and other large particles. The filtered water was then passed through a 20 μm nylon mesh and the retentate containing phytoplankton was transferred to a Petri plate. Phytoplankton were viewed under an Olympus inverted microscope at 100 X magnification.

The following techniques were used to establish diatom cultures:

a) Enrichment culture: Diatoms were enriched as follows. Nutrients (0.8 mM sodium nitrate, 0.03 mM sodium hydrogen phosphate, 0.1 mM sodium silicate) were added to the concentrated volume of collected water sample and further incubated at 25°C, under a 16:8 light:dark cycle at irradiance between 10 and 20 µmol m^{-2} s^{-1}.

b) Serial dilution: Mixed cultures of diatoms were serially diluted in stages. The culture was diluted upto 10^{-4}, incubated for one week, under the conditions mentioned above, and then diluted again.

c) Single cell isolation: Using an inverted microscope to observe the sample at 100X or 200X magnification, an individual diatom cell was carefully aspirated using a micropipette tip attached to a sterile tubing and placed in a drop of filtered seawater. After several washes in filtered seawater (under microscopic observation), the cell was transferred to a 24-well plate containing f/2 medium (Guillard 1975) and incubated under standard conditions.

d) Size-selective filtration: Mixed cultures, containing a larger diatom form and a smaller one, were separated by means of a nylon mesh of varying pore size, from 5 µm to 50 µm).

Table 3.1: Sample collection sites, for isolation of

A: Diatoms; B: Cyanobacteria and green microalgae

A	
Location	**Coordinates**
Anjuna beach	15° 34.583′ N, 73° 44.398′ E
Bogmalo beach	15° 22.188′ N, 73° 50.018′ E
Zuari estuary	15° 30.496′ N, 73° 54.765′ E
Dona Paula bay	15° 27.24′ N, 73° 48.12′ E
Miramar beach	15° 29.233′ N, 73° 48.47′ E
Mormugao harbour	15° 24.907′ N, 73° 47.867′ E
Old Goa jetty	15° 30.365′ N, 73° 54.722′ E
Reis Magos	15° 29.69′ N, 73° 48.7′ E
St Jacinto island	15° 24.786′ N, 73° 51.755′ E
Vagator beach	15° 36.179′ N, 73° 44.017′ E
Vainguinim beach	15° 27.335′ N, 73° 48.805′ E

B	
Location	**Coordinates**
Chicalim rice field	15°23'52.92’N, 73°50'39.37’E
Chicalim pond	15°23'58.4’N, 73°50'29.7’E
Curca pond	15°27’30.30’N, 73°52’22.01’E
Santana creek	15° 27' 8.28’ N, 73° 52' 30.36’ E
Mandovi estuary	15°30'19.16’N, 73°50'43.68’E
Verna lake	15°20'51.68’N, 73°56'43.72’E
Verna rice field	15°20'51.68’N, 73°56'43.72’E
Verna spring	15°20'52.3"N, 73°56'46.3"E
Zuari estuary	15° 25.732′ N, 73° 50.379′ E

3.3 Isolation of Cyanobacteria and Green Microalgae from Freshwater and Estuarine Sites

Water samples were collected from rice fields, ponds, lakes, creeks and estuarine regions of Goa (**Table 3.1B; Figure 3.1**). After filtration through a 20 µm nylon mesh to eliminate larger organisms, the water was passed through a 0.22 µm nitrocellulose filter. The concentrated retentate on the filter was inoculated into BG-11 medium (Rippka et al. 1979) and incubated at 25°C, under a 16:8 light:dark cycle and exposed to an irradiance of 10 to 20 µmol m^{-2} s^{-1}.

Mixed cultures of cyanobacteria and green microalgae obtained were repeatedly purified by the serial dilution method used for diatoms.

3.4 Purification of Microalgal Cultures

Algal cultures generally harbour heterotrophic bacteria. A bacteria-free unialgal culture is referred to as an 'axenic' culture. Establishment of axenic cultures was attempted by:

a) Streaking: Liquid culture was streaked across agar plates, to isolate colonies, which were re-streaked to obtain a bacteria-free culture.
b) Use of antibiotics: For diatoms, an antibiotic cocktail consisting of penicillin, streptomycin and gentamycin (final concentrations 10, 2.5 and 2.5 mg/ml, respectively) was added to the growth medium in varying concentrations, i.e. 0.5%, 1%, 2%, 4%. A drop of sterile nutrient broth was added to stimulate bacterial growth. The culture was incubated for 24 or 48 hours, after which a small volume was withdrawn and inoculated in fresh, antibiotic-free medium (Andersen 2005).
c) In the case of cyanobacteria, Tienam, a combination of 500 mg imipenem and 500 mg cilastatin, was used (Sarchizian and Ardelean 2010). Briefly a 25 mg/ml stock of Tienam was prepared in sterile distilled water. To 20 ml of exponentially growing cyanobacterial culture, 10 ml of fresh BG-11 media was added along with 5 ml of Luria Bertani broth. This was kept on a shaker in the dark, at 30°C for one hour, to stimulate growth of contaminating bacteria, following which, 80 µl of tienam stock was added and kept on a shaker in the dark, at 30°C, for 24 hours. After two washes with sterile BG-11, the culture was inoculated in fresh media and incubated in light for 24 hours, following which it was maintained under a normal light: dark cycle of 16:8 hours.

3.5 Maintenance of Microalgal Cultures

Cultures of diatoms, cyanobacteria and green microalgae, isolated as described, were maintained under standard conditions (25°C, 16:8 h light:dark cycle, irradiance 10-20 µmol m^{-2} $s^{-}1$) and subcultured every three weeks in fresh medium – f/2 for diatoms, BG-11 for cyanobacteria and green microalgae.

3.6 Identification of Microalgal Cultures

3.6.1 Morphological identification:

For the purpose of identification, cultures were observed microscopically. Diatoms were observed under an inverted optical microscope at 200X magnification, while cyanobacteria and green microalgae were mounted on slides and observed under oil immersion at 1000X. Identification was carried out using standard keys (Tomas 1997).

Diatom morphology was studied by scanning electron microscopy. 500 µl of diatom culture at appropriate dilution was passed through a 0.2 micron polycarbonate filter under vacuum. The filter was dehydrated in an ethanol series (30%, 60%, 90% ethanol) and air dried. The filter was mounted on an aluminium stub, sputter-coated with gold particles and observed under a Zeiss Evo 18 scanning electron microscope.

3.6.2 Molecular level identification of cyanobacteria and green microalgae:

Genomic DNA isolation

i) Genomic DNA was extracted from five cyanobacterial cultures using a modified bacterial genomic DNA extraction protocol (Keshari et al. 2015). 5 ml of overgrown culture was pelleted and 567 µl lysis buffer (10 mM Tris Cl, pH 8; 1mM trisodium citrate, 1.5% SDS) added to the cell pellet. 30 µl of 10% SDS, along with 3 µl of Proteinase K (20 mg/ml) was added and incubated for 1 hour at 37°C. 100 µl of 5M NaCl and 80 µl of CTAB/NaCl solution (10% CTAB, 0.7M NaCl) was added and incubated at 65°C for 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the tube centrifuged at 11,000 x g for 5 minutes. The aqueous layer was transferred to a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) added. After centrifugation and recovery of the aqueous layer, DNA was precipitated by addition of 0.6 volumes of isopropanol, incubation at room temperature for 20 minutes, followed by centrifugation at 11,000 x g for 20 minutes. The DNA pellet was resuspended in 10 mM Tris chloride and stored at –20°C until further use.

ii) In the case of green microalgae, DNA isolation was carried out according to previously published protocols (Keshari et al. 2015; Newman et al. 1990; Radha et al. 2013), with modifications. 15 ml of microalgal culture was pelleted. The pellet was resuspended in 500 µl of TEN buffer (10 mM Tris-Cl, 10 mM EDTA, 150 mM NaCl, pH 8), transferred to a microfuge tube and centrifuged at 6,000 x g for 5 minutes. The resulting cell pellet was crushed with glass powder and 600 µl lysis buffer (2% SDS, 400 mM NaCl, 40 mM EDTA, 100 mM Tris-Cl, pH 8) added and kept at room temperature for 5 minutes. After centrifugation at 6,000 x g for 5 min, the pellet was resuspended in 10 mM Tris chloride. An equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) was added, and the tube centrifuged at 11,000 x g for 5 minutes. The aqueous layer was transferred to a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) added. After centrifugation and recovery of the aqueous layer, DNA was precipitated by addition of 0.6 volumes of isopropanol, incubation at room temperature for 20 minutes, followed by centrifugation at 11,000 x g for 20 minutes. The DNA pellet was resuspended in 10 mM Tris chloride and stored at –20°C until further use.

PCR Amplification of Marker Genes

Cyanobacterial 16S rDNA was PCR-amplified using universal primers (Nubel et al. 1997), while for green microalgae, the internal transcribed spacer 2 (*ITS-2*) sequence, was amplified using primers ITS_f and ITS_r (Liu, Gerken, and Li 2014).

The reaction components were as follows:

	16S	***ITS-2***
Component	Volume in µl	
10X PCR assay buffer containing $MgCl_2$	5.0	5.0
dNTP mix (2.5 mM each)	5.0	5.0
Forward primer (5 µM)	3.2	2.5
Reverse primer (5 µM)	2.4	2.5
Algal DNA (template)	4.0	4.3
Taq polymerase	0.7	0.7
Nuclease free water	29.7	30.0

The PCR reaction conditions were as follows:

PCR parameters:	***16S***		***ITS-2***	
(30 cycles of the following)	Temperature	Time	Temperature	Time
Denaturation	94°C	1 min	95°C	30 s
Annealing	60°C	30 s	50°C	30 s
Extension	72°C	1 min	72°C	1 min

Amplification was performed using a Bio-rad MJ Mini thermal cycler. After addition of PCR reaction components (excluding *Taq* polymerase), initial denaturation was carried out at 95°C for 5 minutes, after which *Taq* polymerase was added and the amplification reaction cycle was initiated.

In the case of both cyanobacteria and green microalgae, the amplified fragments of between 700-800 bp (**Figure 3.2**) were purified using a GeneiPure™ Gel Extraction Kit, according to the manufacturer's protocol. Briefly, PCR products were loaded on a 1% low-melting agarose gel. The band containing the desired fragment was excised, the appropriate volume (approximately 3 x weight of gel slice) of gel solubiliser added, and the tube incubated at 50°C till complete dissolution. 10 µl of 3M sodium acetate (pH 5.2) was added to adjust the pH, followed by addition of 100 µl isopropanol per 100 mg gel slice. The sample was then loaded on a GeneiPure™ DNA spin column, washed with the provided wash buffer and finally eluted with 20 µl of preheated elution buffer and stored at –20°C.

Purified fragments were sequenced by automated Sanger sequencing. Forward and reverse sequences obtained were combined to generate a contig, which was analysed by BLAST (Altschul et al., 1990) against existing sequences in the NCBI-GenBank database, to find the closest match. The sequences showing >97% identity to the query sequence, and belonging to related genera, were selected and aligned using CLUSTALW. A phylogenetic tree was generated in MEGA software (Tamura et al. 2011), using the Neighbour-Joining heuristic, with the Maximum Composite Likelihood model and 1000 bootstrap replications.

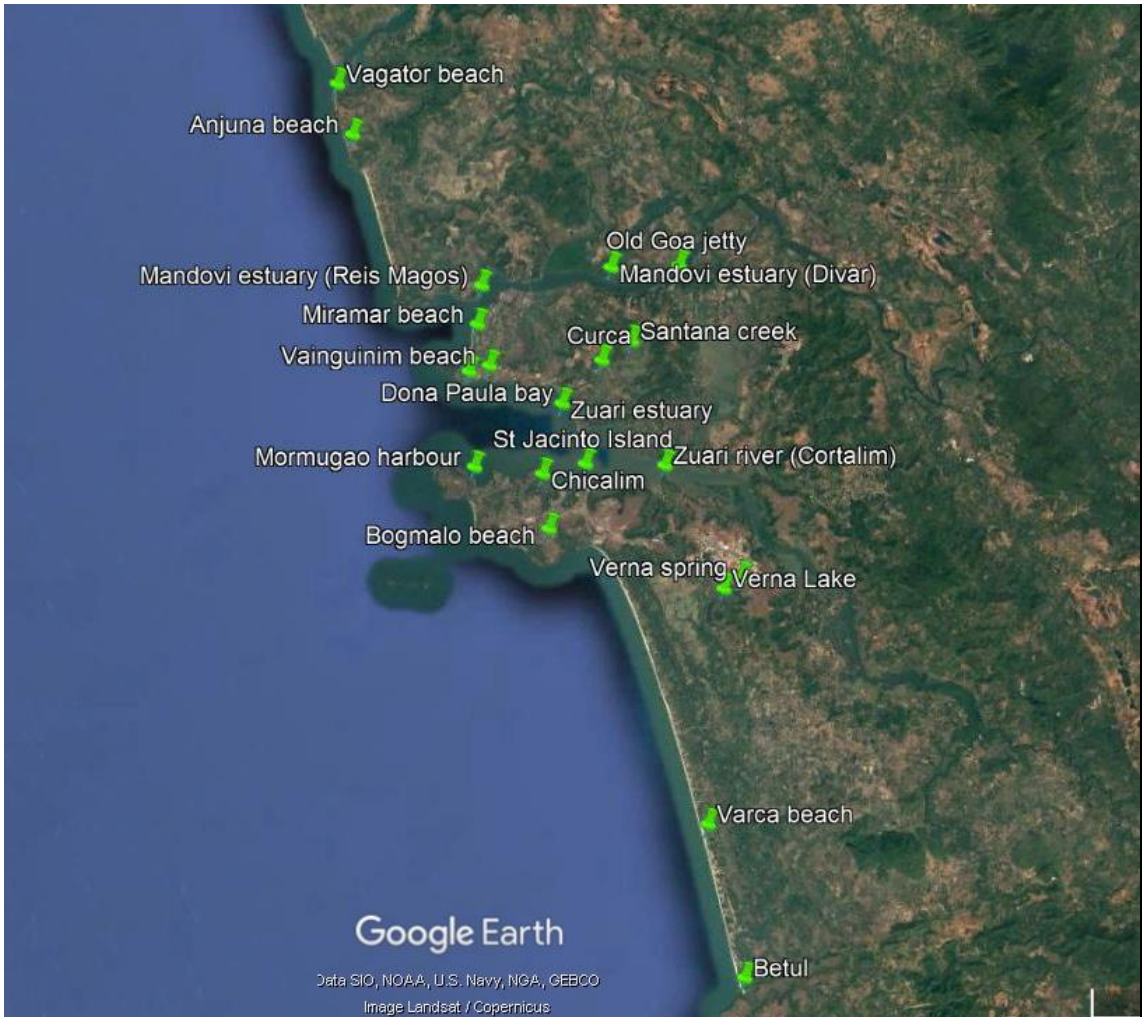


Figure 3.1: Map displaying locations of sample collection sites

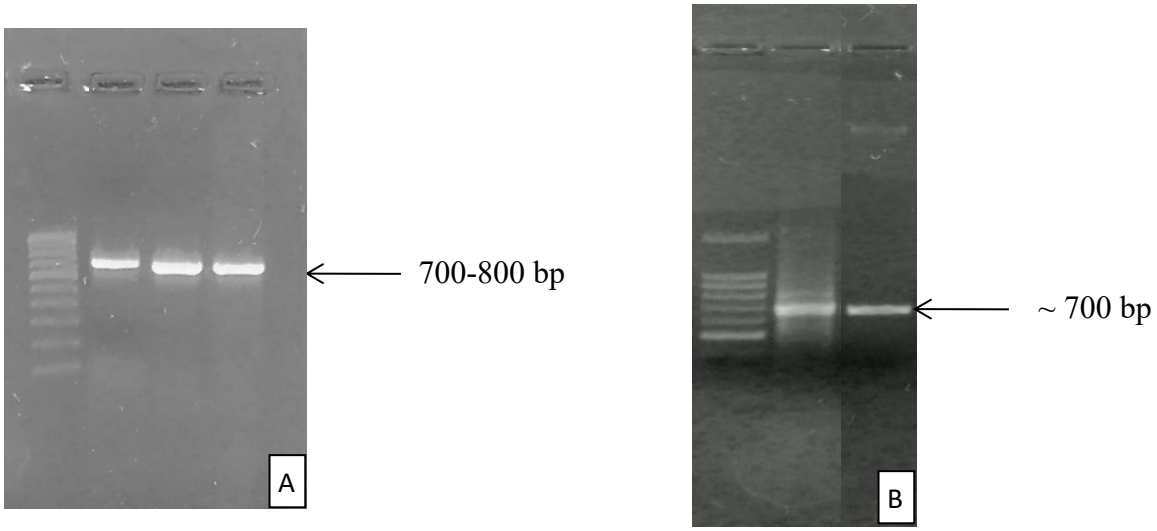


Figure 3.2: PCR-amplification of

A: ITS fragments from microalgal cultures; B: 16S fragments from cyanobacterial cultures

Lane 1: 100 bp DNA ladder
Lane 2, 3 and 4: Respective PCR products of cultures **CF01**, **ME01** and **VL02**

Lane 1: 500 bp DNA ladder
Lane 2 and 3: Respective PCR products of cultures **DP01** and **MZ01**

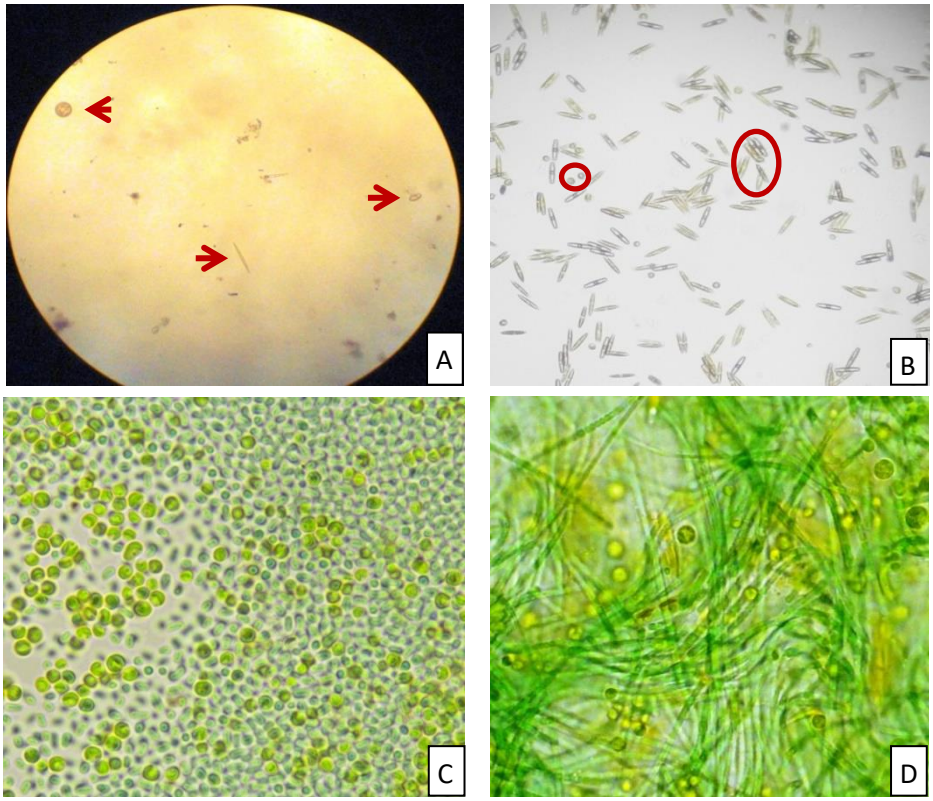


Figure 3.3: **Mixed cultures of microalgae**
A, B: Diatoms; C,D: Green microalgae and cyanobacteria

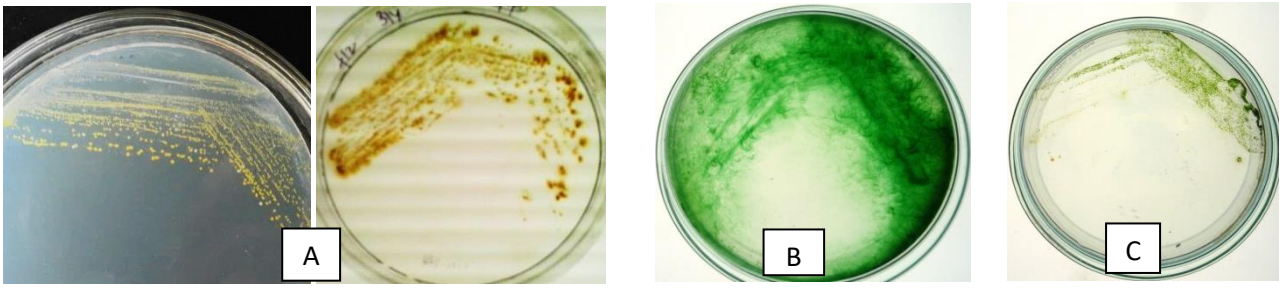


Figure 3.4: Microalgal cultures on agar plates
A: Diatoms; B: Cyanobacteria; C: Green microalgae

Results and Discussion

Water samples were collected from various aquatic sites, spanning a major part of the state of Goa, India (**Figure 3.1**), as described in the 'Methods' section. Samples were used to isolate native microalgal species. After preliminary purification and inoculation in respective growth media, mixed cultures of microalgae were obtained (**Figure 3.3**). These were subsequently purified by various techniques detailed in the 'Methods' section.

3.7 Isolation of Marine Diatoms

The marine diatom cultures were purified. Certain species of diatoms demonstrated poor growth on agar plates. This is a common observation: In an attempt to establish an axenic culture of a *Synedra* sp., it was observed that cells plated on agar medium did not divide and propagate (Shishlyannikov et al. 2011). Centric diatoms are considered particularly difficult to grow on solid media (Iwasaki 1979; Kimura & Tomaru, 2013). In general, there are few reports of successful cultivation of diatoms on solid media (Kourtchenko, Rajala, and Godhe 2018).

However, some of the isolates in the present study grew (**Figure 3.4 A**) and formed individual colonies which could be inoculated in a liquid medium, to obtain a pure culture. Single cell isolation was found to be the most effective method of purification, resulting in isolation of several unialgal populations of cells, which were subcultured in larger volumes of medium. Diatom species exhibiting varied morphologies, i.e. centric, pennate and chain forms were cultured (**Figure 3.5**). However, after several subcultures, other isolates were lost or contaminated, including those depicted in **Figure 3.6**. Moreover, diatoms did not survive the antibiotic treatments attempted. Other researchers have previously reported difficulty or inability of diatom species to grow in the presence of antibiotics. This could be the result of direct effects on cultivated diatoms, such as reduction in their light utilization efficiency (Guo, Selby, and Boxall 2016) or rate of division (Hagenbuch and Pinckney 2012; Kline and Pinckney 2016). It could also be a consequence of indirect effects – elimination or suppression of bacteria associated with diatoms, which are necessary for their survival (D'Costa and Anil 2011).

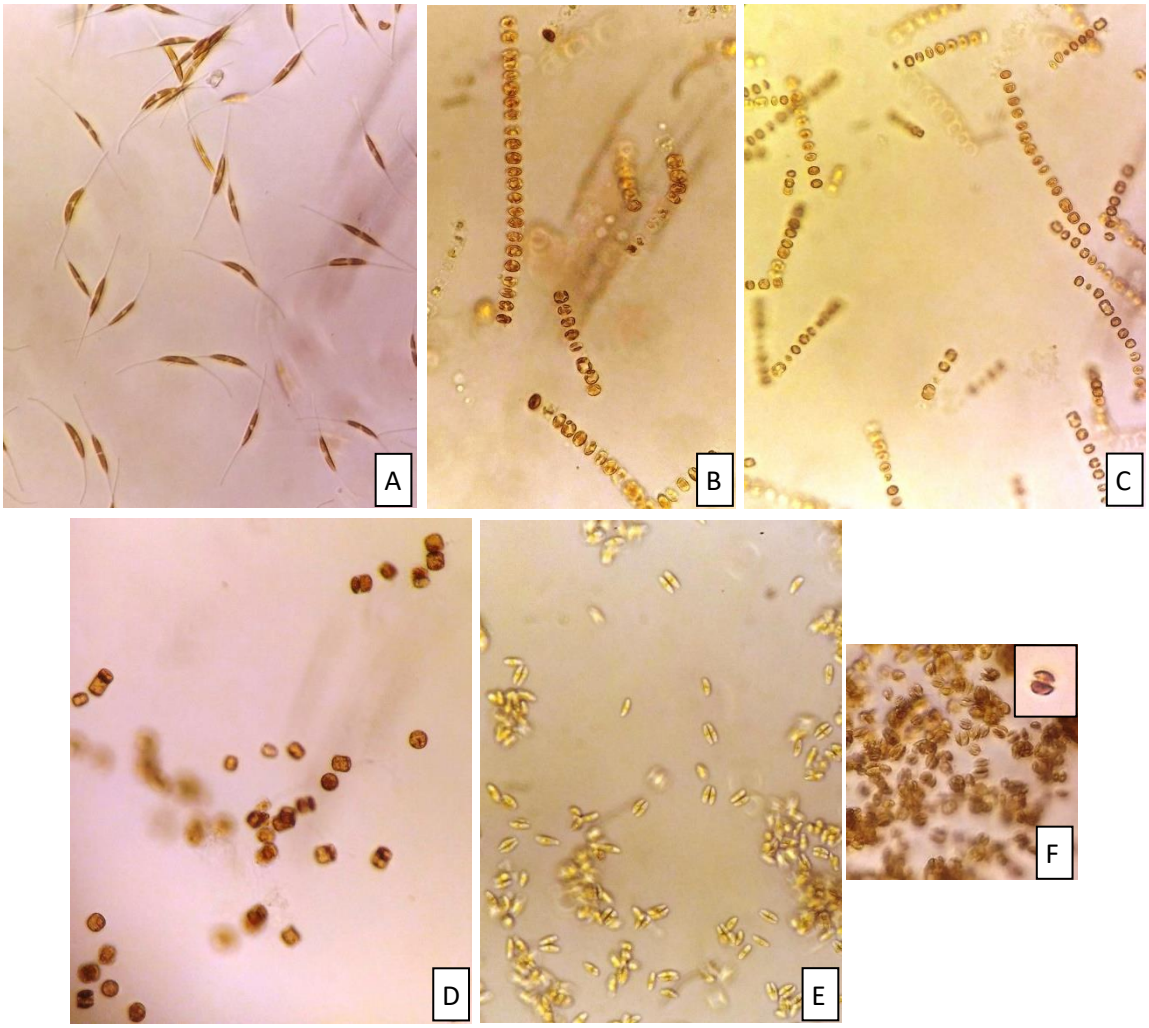


Figure 3.5: Diatom isolates, as seen under light microscope (40X):
A: *Cylindrotheca closterium* ; B: *Skeletonema* sp. 1; C: *Skeletonema* sp. 2;
D: *Thalassiosira* sp.; E: *Amphora* sp. 1; F: *Amphora* sp. 2 (inset: Two attached cells – enlarged view)

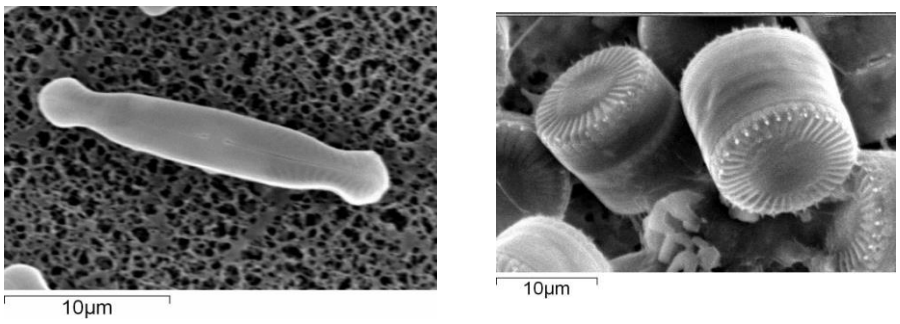


Figure 3.6: SEM images of diatom unicells: A: *Navicula* sp.; B: *Cyclotella* sp.

Single cell isolation primarily aims at isolating a particular target species. Often, the target species dies after a few subcultures, either due to lack of trace essential components in artificial media, or due to accumulation of toxic wastes. Both these factors are artifacts of the *in vitro* system, as in nature, wastes are metabolized by lower organisms, and trace components are available in the complex aqueous system (Andersen 2005).

Six diatom isolates, belonging to four genera, were ultimately obtained and cultured in the laboratory. These were identified morphologically on the basis of typical features (Pal and Choudhury 2014; Verlecar and Desai 2004), by optical microscopy (**Table 3.2**; **Figure 3.5)**

Table 3.2: Details of diatom isolates

Location	**Isolate**	**Identifying features**
Dona Paula	*Cylindrotheca closterium*	Spindle-shaped cells, thinly silicified; frustules relatively long and narrow with attenuated apices; one raphe on each valve.
	Skeletonema sp. 1 and 2	Cells short or elongated–cylindrical, bound into long, slender chains by delicate siliceous spines or gelatinous threads.
	Thalassiosira sp.	Cells disc-shaped, in gelatinous masses – solitary or in flexible chains; valves with delicate radial grooves.
Old Goa	*Amphora* sp. 1 and 2	Pennate, biraphid; frustules lanceolate to elliptic; raphe located towards ventral margin of valve.

All four of the above genera have previously been isolated from coastal and estuarine waters of Goa, specifically the sampling sites mentioned.

In the present study, sampling was carried out during the monsoon season (June-September). The annual South-West Monsoon strongly influences the dynamics of marine and estuarine systems in Goa, physicochemical characteristics such as nutrients and salinity, and biological communities. Abundance and community structure of diatom populations at various depths of the water column are influenced by heavy rainfall, increased runoff from

rivers, as well as turbulence characteristic of the monsoonal period (Qasim & Sen Gupta, 1981; Shetye, Shankar, Neetu, & Suprit, 2007; Vijith, Sundar & Shetye, 2009).

While earlier researchers reported a drop in phytoplankton and diatom abundances during this season (Devassy and Goes 1988; Kumari and John 2003; Redekar and Wagh 2000), more recent studies have identified blooms that occur specifically during this time, and an overall high phytoplankton abundance (Parab et al. 2006; Patil and Anil 2011).

The Dona Paula Bay has been the subject of a number of studies. *Navicula* sp. and *Nitzschia* sp. were reported to be most abundant at this site in all seasons (Patil and Anil 2015). At another coastal site investigated over a yearlong period (Parab et al. 2006), *Navicula* sp., *Nitzschia* sp. and *Thalassiosira* sp. were reported to be dominant in different times of the year. *Skeletonema* sp. blooms have been observed in Goan coastal waters as well as other sites along the west coast of India, during the onset and restart of the monsoon (Patil & Anil, 2008). *Skeletonema* sp. and *Thalassiosira* sp. reportedly dominate the diatom population during post-monsoon (Pednekar et al. 2011) and in low-light conditions (Ramakrishnan et al. 2018). These two genera, along with other diatoms and at times dinoflagellates, dominate the community at the Dona Paula site (Patil & Anil, 2015; Patil & Anil, 2019). However, at this site, as at other points along the Zuari estuary, sampled by earlier researchers, shifts in blooming species occur, with changes in nutrient and salinity conditions consequent to the rise and ebb of the monsoon (Patil & Anil, 2011; Redekar & Wagh, 2000).

The site at Old Goa (from where we have isolated some of the diatoms) is located towards the upper section of the Mandovi estuary, hence with a lower salinity. However, silicate concentrations are high in this niche (Pednekar, Kerkar, and Matondkar 2014), promoting abundant growth of diatoms.

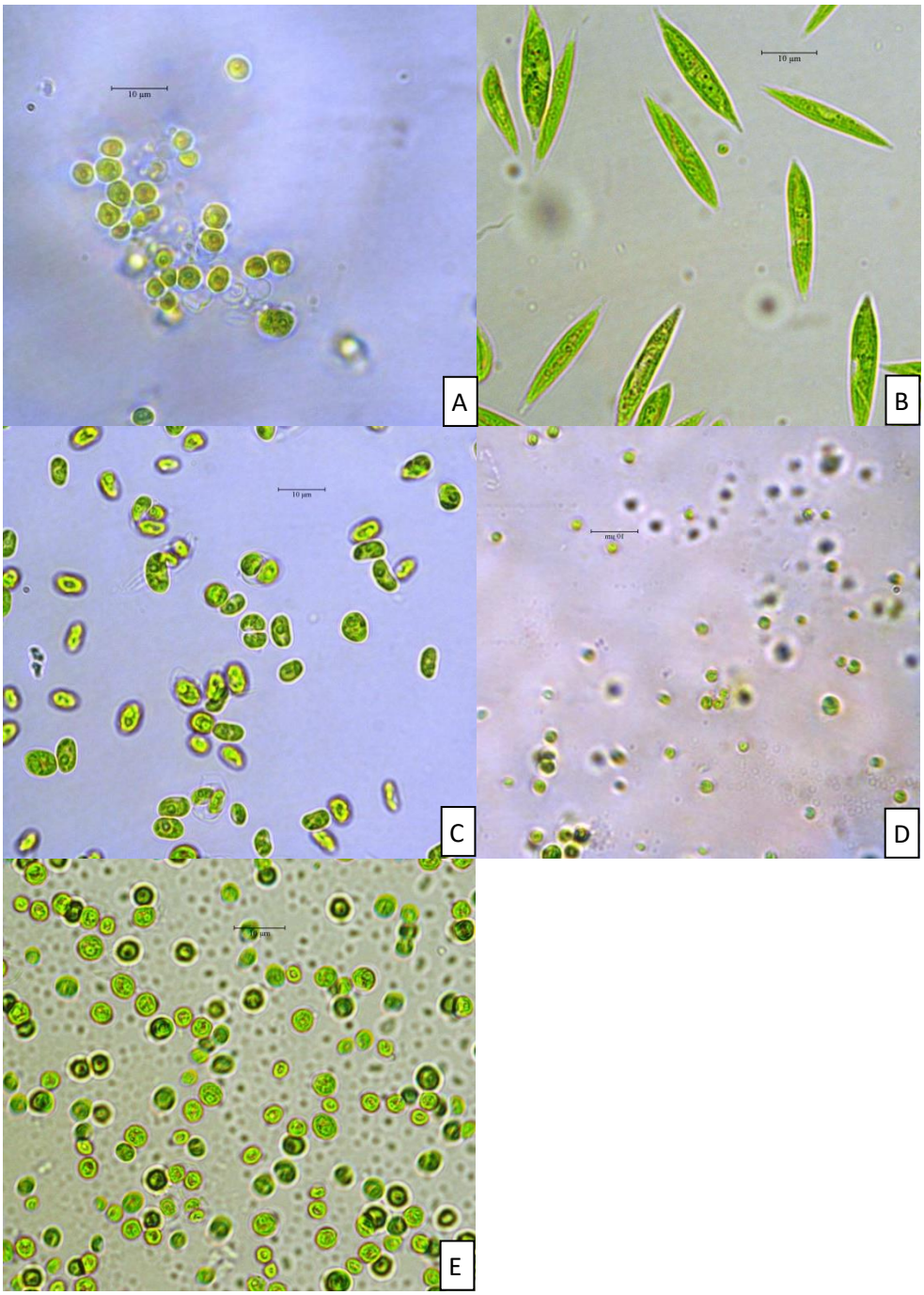


Figure 3.7: Green microalgal isolates as seen under light microscope (40X).
A: *Chlorella / Micractinium* sp. CF01; B: *Scenedesmus* sp. VL01; C: *Asterarcys* sp. ME03; D: *Chlorella* sp.; E: *Chlorella* sp.

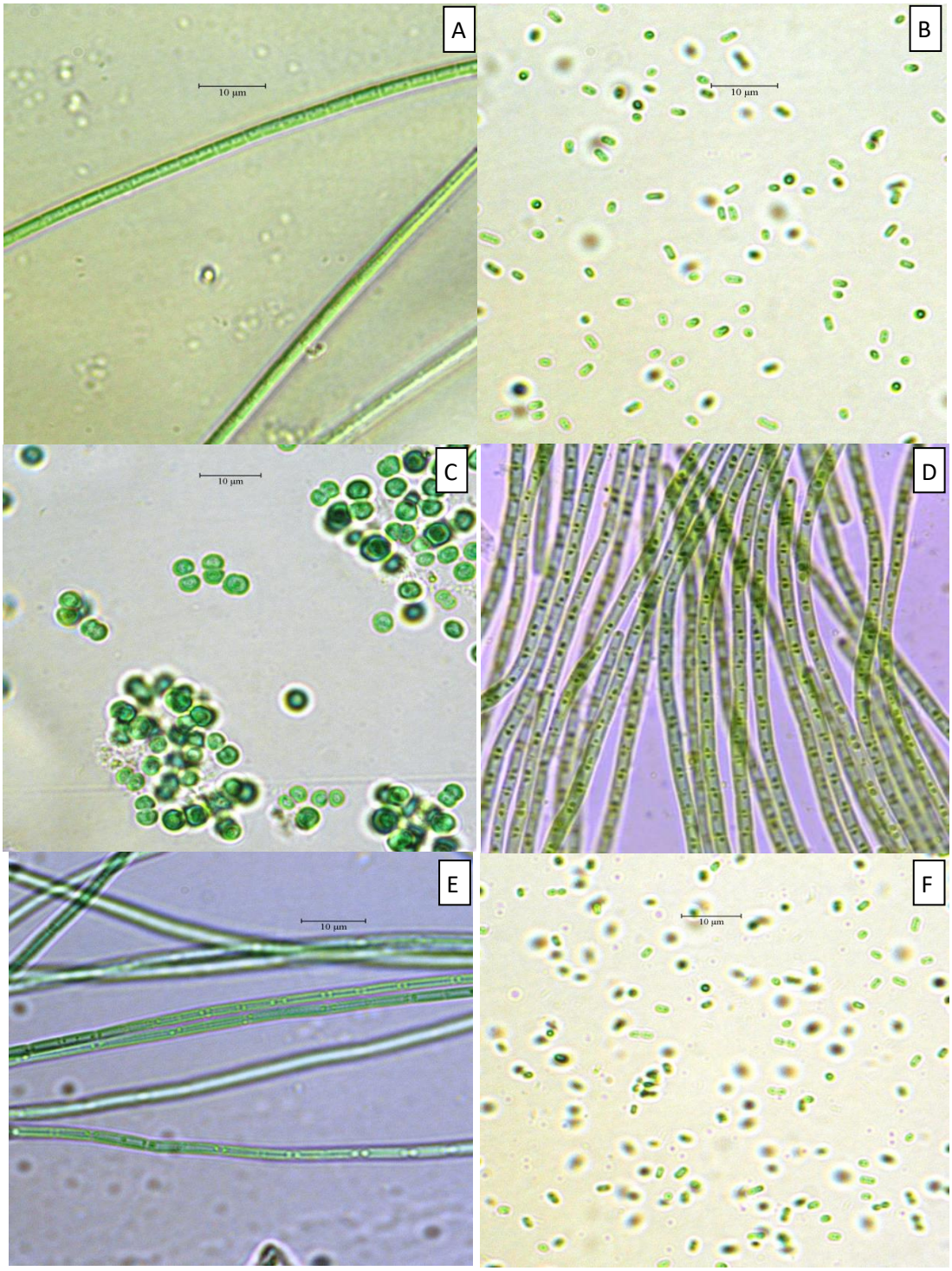


Figure 3.8: Cyanobacterial isolates as seen under light microscope (40X)
A: *Limnothrix* sp. CP01; B: *Synechococcus* sp. DP01; C: *Synechocystis* sp. ME01; D: *Limnothrix* sp. VL01; E: *Limnothrix* sp. TP02; F: *Synechococcus* sp. TP01

3.8 Isolation of Cyanobacteria and Green Microalgae from Freshwater and Estuarine Locations

Mixed cultures of cyanobacteria and green microalgae were obtained from various freshwater and estuarine locations (**Figure 3.3**).

Serial dilution of mixed cultures was carried out in stages, over a period of several months, as these cultures were relatively slow-growing. Unialgal cultures emerged from the consortium. The final cyanobacterial / microalgal form constituting the unialgal culture thereby gave an indication of the most highly populated or robust taxa in the respective aquatic niches (Allen and Nelson 1910).

Thus, a total of five green microalgal (**Figure 3.7**) and six cyanobacterial (**Figure 3.8**) cultures were established and maintained in the laboratory, with BG-11 as growth medium. BG-11 is widely used as a culture medium both for microalgae and cyanobacteria (Ferris and Hirsch 1991; Sharma et al. 2018; Waterbury 2006).

As in the case of diatoms, several of the green microalgal and cyanobacterial cultures too, depicted susceptibility to the antibiotic formulations used during preparation of axenic cultures. A few isolates, however (VL01, DP01), grew in the presence of imipenem, thereby establishing axenic cultures (indicated by * in **Table 3.3 B**).

3.9 Identification of Green Microalgal / Cyanobacterial cultures

Most of the cultures have been identified by morphological and / or molecular methods, as shown in **Table 3.3 A and 3.3 B**.

Table 3.3 (A): Green microalgal cultures isolated from various aquatic niches

GenBank Accession number	Identification	Niche	Location	Geographical coordinates
MN954686.1	*Chlorella/ Micractinium sp.* CF01	Rice field	Chicalim	15°23'45.4"N 73°50'42.5"E
MN954687.1	*Asterarcys* sp. ME03	Estuary	Mandovi	15°30'19.16'N, 73°50'43.68'E
MN954688.1	*Scenedesmus* sp. VL02	Lake	Verna	15°20'51.68'N, 73°56'43.72'E
n/a	*Chlorella* sp.	Spring	Verna	15°20'52.3"N 73°56'46.3"E
n/a	*Chlorella* sp.	Pond	Curca	15°27'30.30'N, 73°52'22.01'E

n/a: The cultures were identified on the basis of morphological features

Table 3.3 (B): Cyanobacterial cultures isolated from various aquatic niches

GenBank Accession number	Identification	Location	Niche	Geographical coordinates
MN808642.1	*Synechococcus* sp. DP01*	Dona Paula	Bay	15° 27.24′ N, 73° 48.12′ E
MN808644.1	*Synechocystis* sp. ME01	Mandovi	Estuary	15°30'19.16'N, 73°50'43.68'E
MN808643.1	*Limnothrix sp.*VL01*	Verna	Lake	15°20'51.68'N, 73°56'43.72'E
MN808645.1	*Synechococcus* sp. TP01	Curca (Talaulim)	Pond	15°27'30.30'N, 73°52'22.01'E
MN786794.1	*Limnothrix* sp. CP01	Chicalim	Pond	15°23'58.4'N 73°50'29.7'E
MT649653.1	*Limnothrix sp.* TP02	Curca / Talaulim	Rice field	15° 27' 8.28' N, 73° 52' 30.36' E

* = Axenic

Some of the cyanobacterial genera isolated cultured in the present study, have earlier been reported from freshwater sites and paddy fields in Goa, include *Synechococcus* sp. (Gomes et al. 2011; S. Kamat 2004; Kanolkar and Kerkar 2009; Shetiya and Kerkar 2004); *Limnothrix* sp. has not been reported thus far.

Among green microalgae, *Chlorella* sp. and *Scenedesmus* sp. have been reported and their occurrence is widespread, for examples, in freshwater wetlands (S. Kamat 2004); in various small freshwater bodies viz, ponds, puddles, paddy fields (Desai 2002; Kanolkar and Kerkar 2009).

Unlike the well-characterized marine and estuarine diatom community in Goan waters, very limited studies have been carried out on freshwater algal populations in this region.

3.10 Phylogenetic Analysis of Green Microalgae and Cyanobacteria

3.10.1 Green Microalgae

Nucleotide BLAST analysis of the ITS sequences obtained from each of the isolates, was carried out. The respective hit tables obtained are depicted below. A combined phylogenetic tree was constructed, using sequences of all the isolates of the present study, along with comparative sequences obtained in BLAST analysis of respective sequences (**Figure 3.9**).

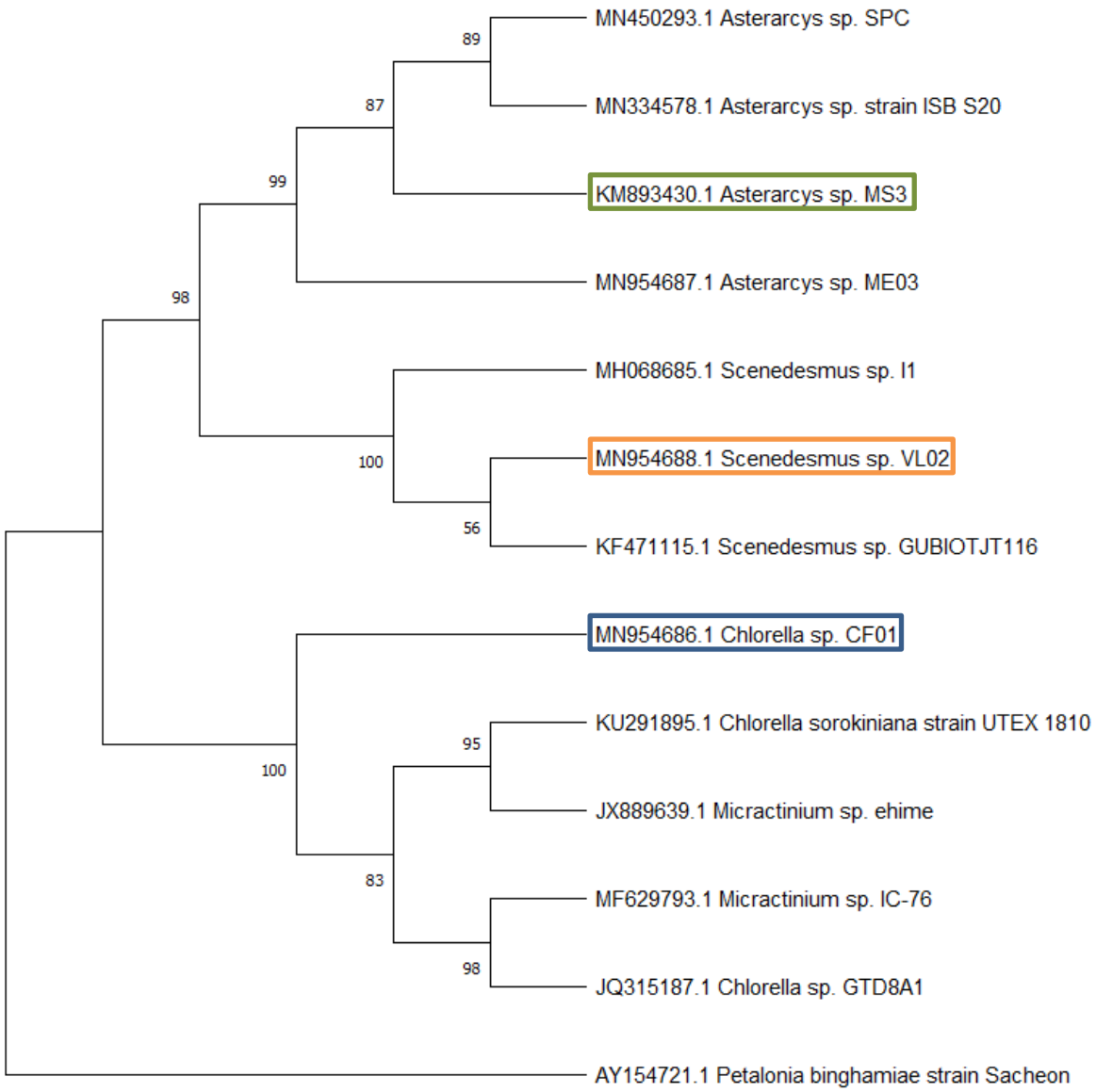
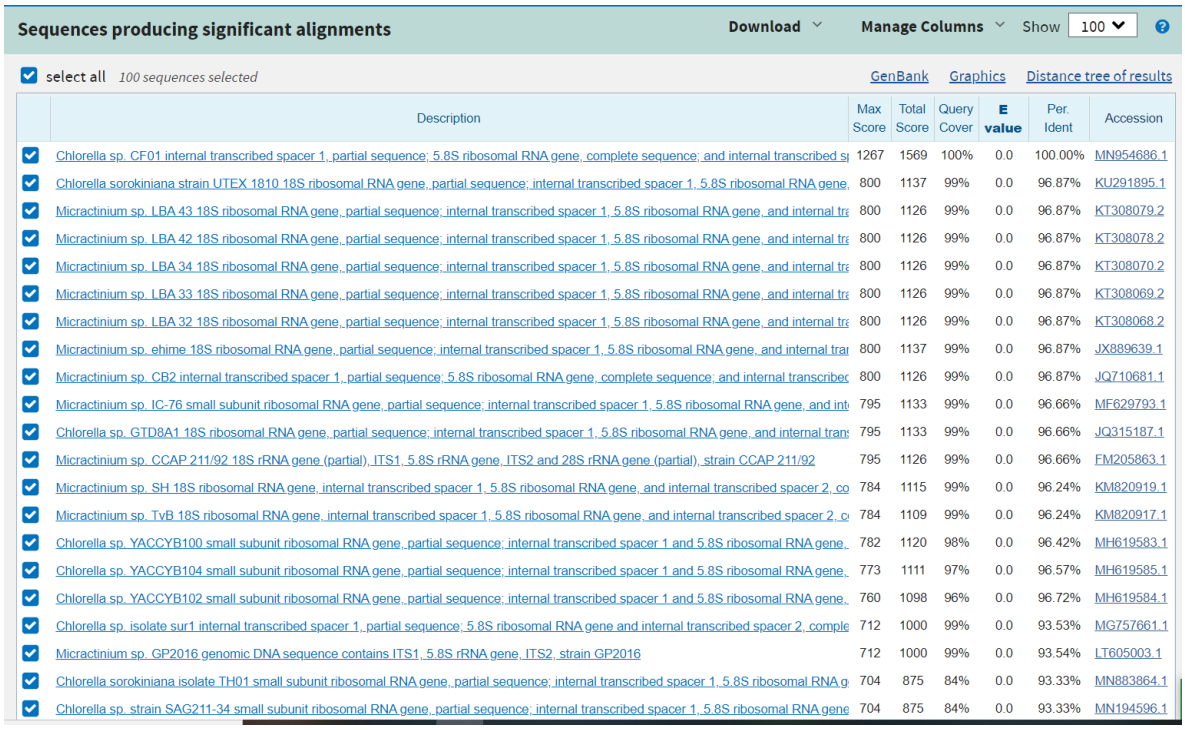


Figure 3.9: Phylogenetic tree constructed using ITS sequences of all green microalgal isolates used in the present study (highlighted), with *Petalonia binghamiae* as the outgroup.

Chlorella/Micractinium sp. CF01



Sequences producing significant alignments Download Manage Columns Show 100

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Chlorella sp. CF01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed sp	1267	1569	100%	0.0	100.00%	MN954686.1
Chlorella sorokiniana strain UTEX 1810 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,	800	1137	99%	0.0	96.87%	KU291895.1
Micractinium sp. LBA 43 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	800	1126	99%	0.0	96.87%	KT308079.2
Micractinium sp. LBA 42 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	800	1126	99%	0.0	96.87%	KT308078.2
Micractinium sp. LBA 34 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	800	1126	99%	0.0	96.87%	KT308070.2
Micractinium sp. LBA 33 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	800	1126	99%	0.0	96.87%	KT308069.2
Micractinium sp. LBA 32 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	800	1126	99%	0.0	96.87%	KT308068.2
Micractinium sp. ehime 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trar	800	1137	99%	0.0	96.87%	JX889639.1
Micractinium sp. CB2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed	800	1126	99%	0.0	96.87%	JQ710681.1
Micractinium sp. IC-76 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	795	1133	99%	0.0	96.66%	MF629793.1
Chlorella sp. GTD8A1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	795	1133	99%	0.0	96.66%	JQ315187.1
Micractinium sp. CCAP 211/92 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene (partial), strain CCAP 211/92	795	1126	99%	0.0	96.66%	FM205863.1
Micractinium sp. SH 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, co	784	1115	99%	0.0	96.24%	KM820919.1
Micractinium sp. TvB 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, c	784	1109	99%	0.0	96.24%	KM820917.1
Chlorella sp. YACCYB100 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene,	782	1120	98%	0.0	96.42%	MH619583.1
Chlorella sp. YACCYB104 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene,	773	1111	97%	0.0	96.57%	MH619585.1
Chlorella sp. YACCYB102 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene,	760	1098	96%	0.0	96.72%	MH619584.1
Chlorella sp. isolate sur1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, comple	712	1000	99%	0.0	93.53%	MG757661.1
Micractinium sp. GP2016 genomic DNA sequence contains ITS1, 5.8S rRNA gene, ITS2, strain GP2016	712	1000	99%	0.0	93.54%	LT605003.1
Chlorella sorokiniana isolate TH01 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA g	704	875	84%	0.0	93.33%	MN883864.1
Chlorella sp. strain SAG211-34 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	704	875	84%	0.0	93.33%	MN194596.1

Culture CF01 showed 96.87% identity with *Chlorella sorokiniana* strain UTEX1810, and 96.87% identity with several strains of *Micractinium* sp. These were considered in the phylogenetic analysis. CF01 was found to cluster with *Chlorella* sp. and *Micractinium* sp., with a bootstrap value of 100. Hence, this isolate was identified as *Chlorella* / *Micractinium* sp. CF01.

Classification:

Kingdom: Plantae

Phylum: Chlorophyta

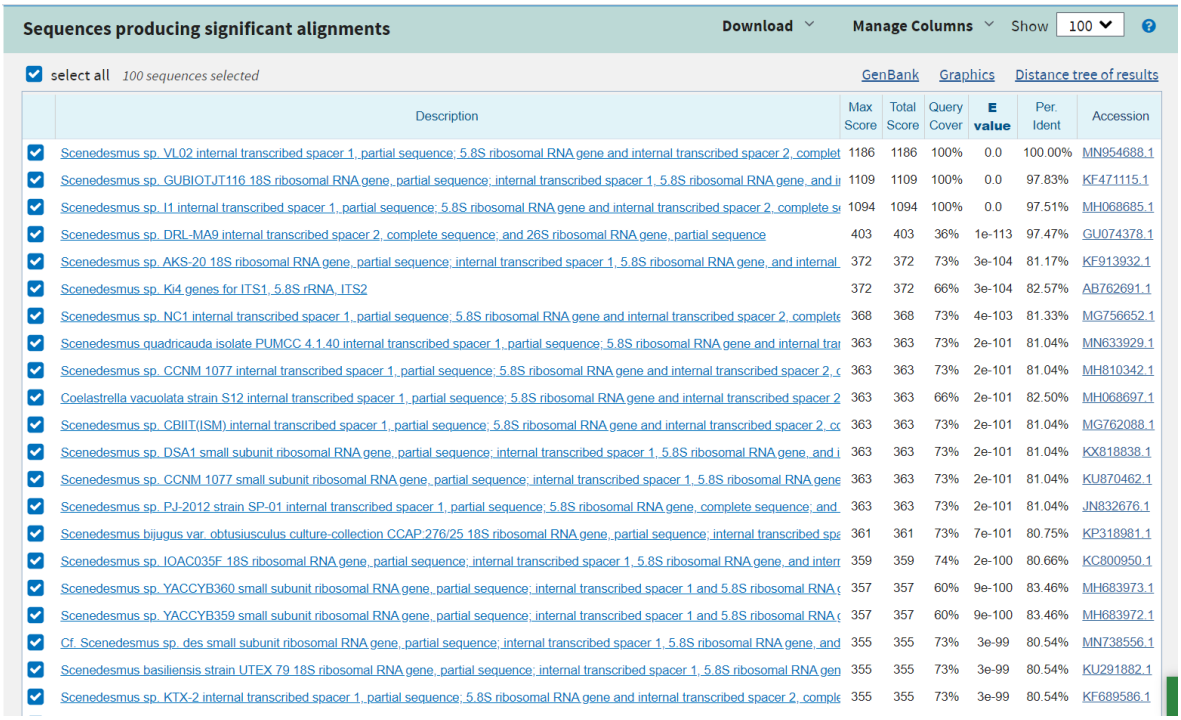
Class: Trebouxiophyceae

Order: Chlorellales

Family: Chlorellaceae

The morphological features of isolate CF01 (**Figure 3.7A**) were in consonance with the description of *Chlorella* / *Micractinium* sp., i.e. cells spherical to ellipsoid, cell diameter 3-5µm, with a predominant cup-shaped parietal chloroplast; cells arranged in coenobia (Bellinger and Sigee 2010). While the presence of spines generally differentiates *Micractinium* from *Chlorella* (Luo et al. 2005), *Micractinium* lacking spines has been reported, both in natural samples (Hong et al. 2015) and as a result of dense laboratory culture (Luo et al. 2006), thereby making the two genera morphologically indistinguishable.

Scenedesmus sp. VL02



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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Scenedesmus sp. VL02 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complet	1186	1186	100%	0.0	100.00%	MN954688.1
Scenedesmus sp. GUBIOTJT116 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and i	1109	1109	100%	0.0	97.83%	KF471115.1
Scenedesmus sp. I1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete s	1094	1094	100%	0.0	97.51%	MH068685.1
Scenedesmus sp. DRL-MA9 internal transcribed spacer 2, complete sequence; and 26S ribosomal RNA gene, partial sequence	403	403	36%	1e-113	97.47%	GU074378.1
Scenedesmus sp. AKS-20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	372	372	73%	3e-104	81.17%	KF913932.1
Scenedesmus sp. Ki4 genes for ITS1, 5.8S rRNA, ITS2	372	372	66%	3e-104	82.57%	AB762691.1
Scenedesmus sp. NC1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete	368	368	73%	4e-103	81.33%	MG756652.1
Scenedesmus quadricauda isolate PUMCC 4.1.40 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal tra	363	363	73%	2e-101	81.04%	MN633929.1
Scenedesmus sp. CCNM 1077 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, c	363	363	73%	2e-101	81.04%	MH810342.1
Coelastrella vacuolata strain S12 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2	363	363	66%	2e-101	82.50%	MH068697.1
Scenedesmus sp. CBIIT(ISM) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, cc	363	363	73%	2e-101	81.04%	MG762088.1
Scenedesmus sp. DSA1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and i	363	363	73%	2e-101	81.04%	KX818838.1
Scenedesmus sp. CCNM 1077 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	363	363	73%	2e-101	81.04%	KU870462.1
Scenedesmus sp. PJ-2012 strain SP-01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and	363	363	73%	2e-101	81.04%	JN832676.1
Scenedesmus bijugus var. obtusiusculus culture-collection CCAP:276/25 18S ribosomal RNA gene, partial sequence; internal transcribed spa	361	361	73%	7e-101	80.75%	KP318981.1
Scenedesmus sp. IOAC035F 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and intern	359	359	74%	2e-100	80.66%	KC800950.1
Scenedesmus sp. YACCYB360 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA g	357	357	60%	9e-100	83.46%	MH683973.1
Scenedesmus sp. YACCYB359 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA g	357	357	60%	9e-100	83.46%	MH683972.1
Cf. Scenedesmus sp. des small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	355	355	73%	3e-99	80.54%	MN738556.1
Scenedesmus basiliensis strain UTEX 79 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	355	355	73%	3e-99	80.54%	KU291882.1
Scenedesmus sp. KTX-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, compl	355	355	73%	3e-99	80.54%	KF689586.1

Culture VL02 showed 97.83% identity with *Scenedesmus* sp. GUBIOTJT116, and 97.51% with *Scenedesmus* sp. I1. Its identity with other sequences in the hit table was below 97%, hence these were not considered in the phylogenetic analysis. VL02 was found to cluster with *Scenedesmus* sp. GUBIOTJT116, with a bootstrap value of 56. Hence, this isolate was assigned to the genus *Scenedesmus*.

Classification:

Kingdom: Plantae

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Spaeropleales

Family: Scenedesmaceae

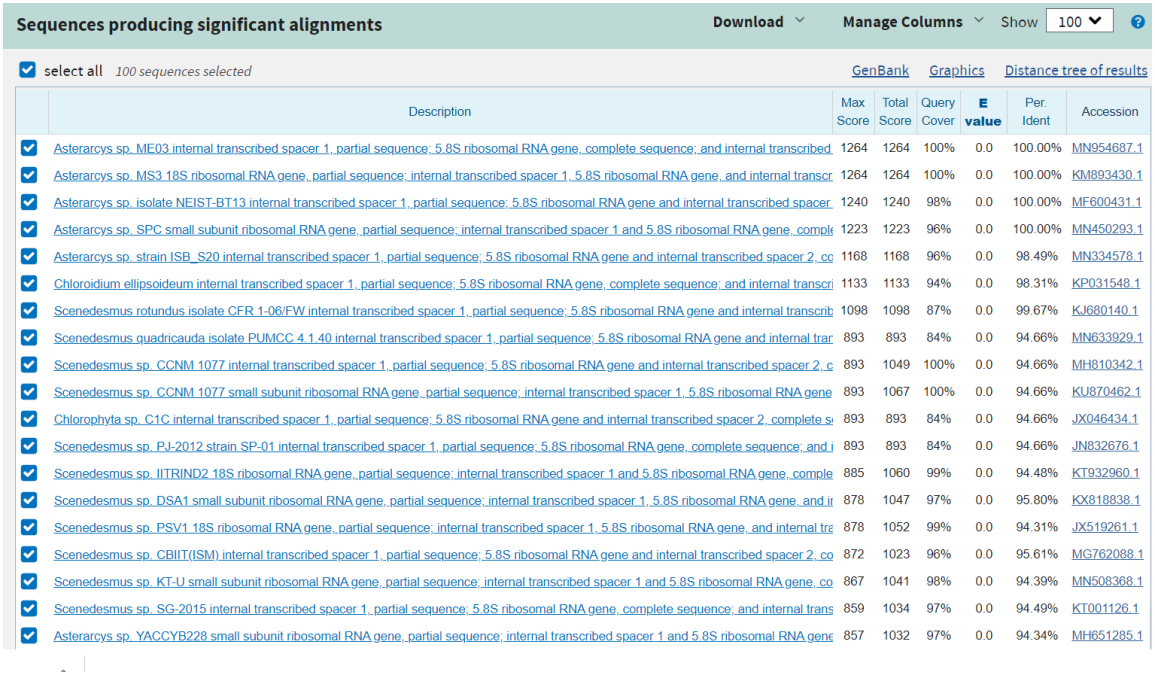
The morphological features of isolate VL02 (**Figure 3.7B**) were in consonance with the description of *Scenedesmus* sp., i.e. cells solitary, fusiform, with slightly curved apices; single parietal chloroplast (Akgül et al. 2017).

Like Chlorella, *Scenedesmus* sp. is another widely distributed green microalga, often dominant in freshwater niches worldwide (Trainor 1998). The cosmopolitan nature of this

genus points to its phenotypic flexibility and ability to withstand a range of environmental conditions (Lürling 2003).

In India, *Scenedesmus* has been reported in high abundance from Kalyana Lake Jodhpur (Barupal and Narayan 2016); the Ganges River at Kanpur (Rishi, Tripathi, and Awasthi 2016), Komati Cheruvu Lake Telangana – dominant genus (Srinivas and Aruna 2018);

Asterarcys sp. ME03



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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Asterarcys sp. ME03 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed	1264	1264	100%	0.0	100.00%	MN954687.1
Asterarcys sp. MS3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcr	1264	1264	100%	0.0	100.00%	KM893430.1
Asterarcys sp. isolate NEIST-BT13 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer	1240	1240	98%	0.0	100.00%	MF600431.1
Asterarcys sp. SPC small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, comple	1223	1223	96%	0.0	100.00%	MN450293.1
Asterarcys sp. strain ISB_S20 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, co	1168	1168	96%	0.0	98.49%	MN334578.1
Chloroidium ellipsoideum internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcri	1133	1133	94%	0.0	98.31%	KP031548.1
Scenedesmus rotundus isolate CFR 1-06/FW internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcrib	1098	1098	87%	0.0	99.67%	KJ680140.1
Scenedesmus quadricauda isolate PUMCC 4.1.40 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal trar	893	893	84%	0.0	94.66%	MN633929.1
Scenedesmus sp. CCNM 1077 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, c	893	1049	100%	0.0	94.66%	MH810342.1
Scenedesmus sp. CCNM 1077 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	893	1067	100%	0.0	94.66%	KU870462.1
Chlorophyta sp. C1C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete s	893	893	84%	0.0	94.66%	JX046434.1
Scenedesmus sp. PJ-2012 strain SP-01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and i	893	893	84%	0.0	94.66%	JN832676.1
Scenedesmus sp. IITRIND2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, comple	885	1060	99%	0.0	94.48%	KT932960.1
Scenedesmus sp. DSA1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and in	878	1047	97%	0.0	95.80%	KX818838.1
Scenedesmus sp. PSV1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	878	1052	99%	0.0	94.31%	JX519261.1
Scenedesmus sp. CBIIT(ISM) internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, co	872	1023	96%	0.0	95.61%	MG762088.1
Scenedesmus sp. KT-U small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, co	867	1041	98%	0.0	94.39%	MN508368.1
Scenedesmus sp. SG-2015 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal trans	859	1034	97%	0.0	94.49%	KT001126.1
Asterarcys sp. YACCYB228 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene	857	1032	97%	0.0	94.34%	MH651285.1

Culture ME03 showed 100% identity with *Asterarcys* sp. MS3, *Asterarcys* sp. NEIST BT-13 and *Asterarcys* sp. SPC respectively. These were considered in the phylogenetic analysis. ME03 was found to cluster with *Asterarcys* sp., with a bootstrap value of 99. Hence, this isolate was assigned to the genus *Asterarcys*.

Classification:

Kingdom: Plantae

Phylum: Chlorophyta

Class: Chlorophyceae

Order: Spaeropleales

Family: Scenedesmaceae

The morphological features of isolate ME03 (**Figure 3.7C**) were in consonance with the description of *Asterarcys* sp., i.e. cells irregularly oval in shape, mean length 4-5 µm and width 2-3 µm. Cells arranged in 2 or 3-celled coenobia, surrounded by mucilaginous envelope; single cup-shaped chloroplast present (Hong et al., 2012; Singh, Khattar, Rajput, Chaudhary, & Singh, 2019).

Unlike *Chlorella* and *Scenedesmus*, which have a worldwide distribution, *Asterarcys* sp. has so far been reported mainly from freshwater locations in Asia, including India (Ghosh et al. 2017; Karthikeyan and Thirumarimurugan 2017; Sehgal et al. 2019; Varshney et al. 2018), Pakistan (Alam et al. 2019) and Korea (Hong et al., 2012), with a few reports from Africa (Odjadjare et al. 2018; Saber et al. 2018).

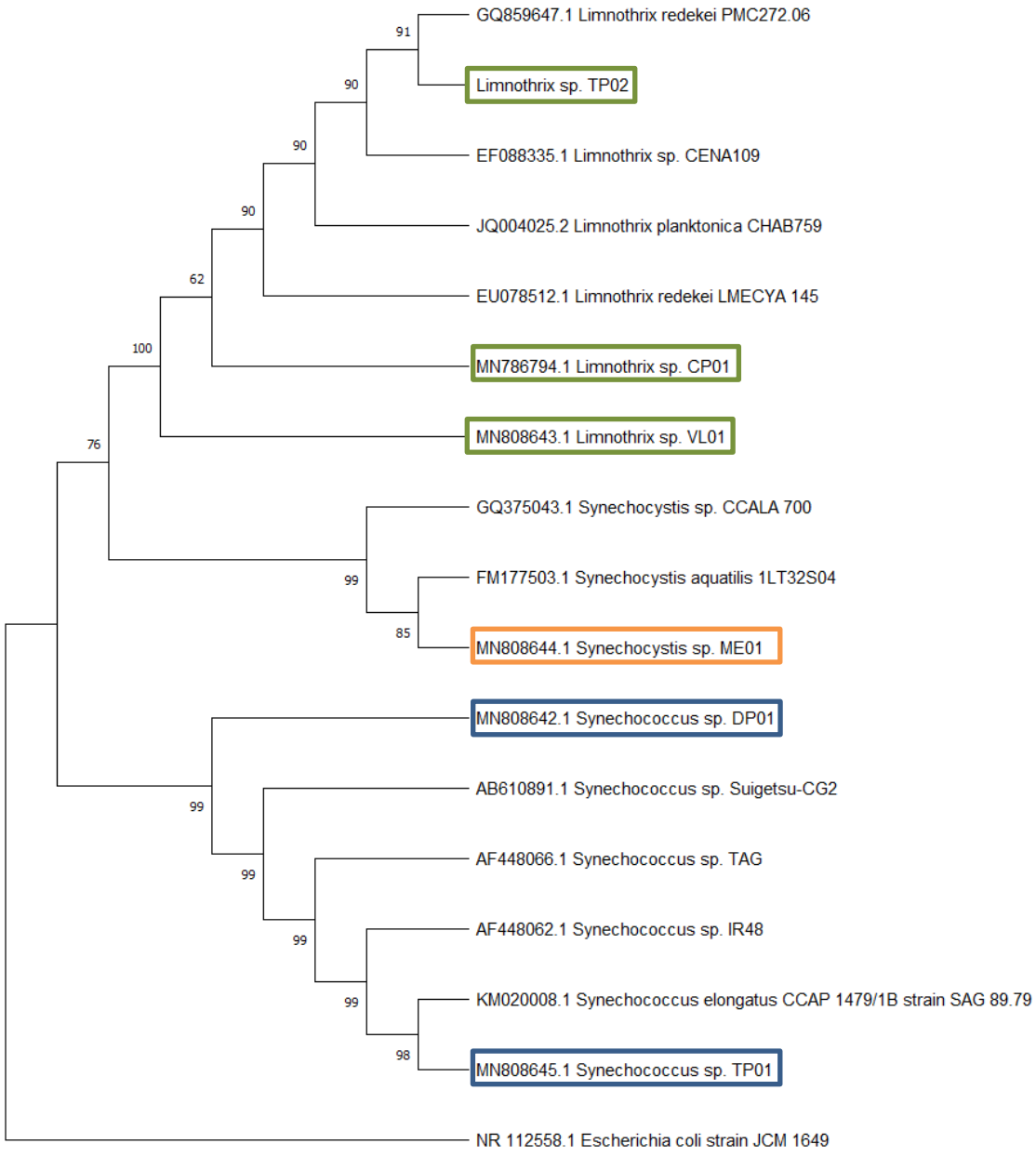


Figure 3.10: Phylogenetic tree constructed using 16S rDNA sequences of all isolates used in the present study (highlighted), with *Escherichia coli* as the outgroup.

3.10.2 Cyanobacteria

Nucleotide BLAST analysis of the 16S rDNA sequences obtained from each of the isolates, was carried out. The respective hit tables obtained are depicted below. A combined phylogenetic tree was constructed, using sequences of all the isolates of the present study, along with comparative sequences obtained in BLAST analysis of respective sequences (**Figure 3.10**).

Synechococcus sp. DP01

Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results

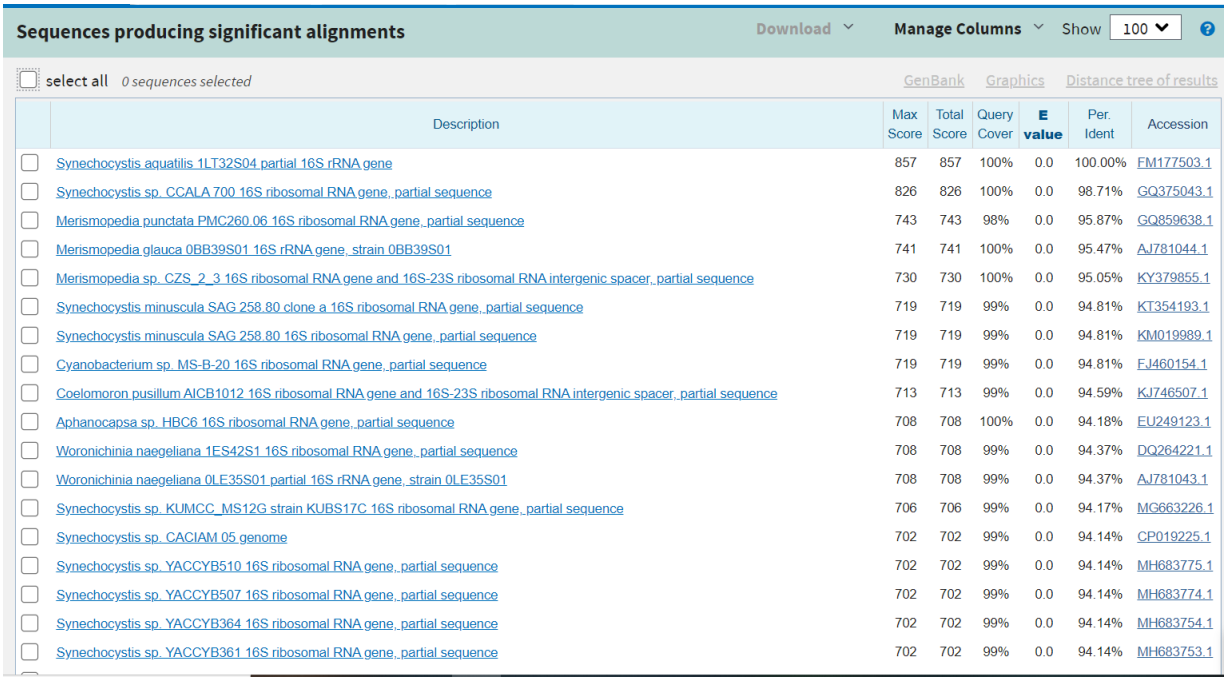
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Synechococcus sp. DP01 16S ribosomal RNA gene, partial sequence	830	830	100%	0.0	100.00%	MN808642.1
Synechococcus sp. Suigetsu-CG2 genes for 16S rRNA, ITS1, tRNA-Ile, tRNA-Ala and 23S rRNA, partial and complete sequence	830	830	100%	0.0	100.00%	AB610891.1
Synechococcus sp. TAG 16S ribosomal RNA gene, partial sequence	830	830	100%	0.0	100.00%	AF448066.1
Synechococcus sp. IR48 16S ribosomal RNA gene, partial sequence	830	830	100%	0.0	100.00%	AF448062.1
Cyanobacterium CBS1105 16S ribosomal RNA gene, partial sequence	824	824	100%	0.0	99.78%	KC513896.1
Cyanobacterium CBS1104 16S ribosomal RNA gene, partial sequence	824	824	100%	0.0	99.78%	KC513895.1
Cyanodictyon sp. JJCD partial 16S rRNA gene, strain JJCD	819	819	100%	0.0	99.55%	AM710382.1
Cyanobium sp. JJ22K partial 16S rRNA gene, strain JJ22K	813	813	100%	0.0	99.33%	AM710364.1
Synechococcus elongatus CCAP 1479/1B strain SAG 89.79 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	KM020008.1
Synechococcus elongatus CCAP 1479/1B partial 16S rRNA gene	808	808	100%	0.0	99.11%	HF678480.1
Synechococcus elongatus CCAP 1479/1A partial 16S rRNA gene	808	808	100%	0.0	99.11%	HF678478.1
Chroococcales cyanobacterium PELL partial 16S rRNA gene, isolate PELL	808	808	100%	0.0	99.11%	HE805930.1
Synechococcus sp. CCAP 1479/10 partial 16S rRNA gene, strain CCAP 1479/10	808	808	100%	0.0	99.11%	HE975006.1
Cyanobacterium IHB-347 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	JF966680.1
Synechococcus sp. PCC 7009 partial ribosomal RNA operon, strain PCC 7009	808	808	100%	0.0	99.11%	AM709628.1
Synechococcus sp. EW15 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	DQ275602.1
Synechococcus sp. BAC 106-1 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	DQ407518.1
Synechococcus sp. NAN 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	AF448065.1
Synechococcus sp. BO8806 16S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; tRNA-Ile and tRI	808	808	100%	0.0	99.11%	AF317072.1
Synechococcus PCC7009 16S ribosomal RNA gene, partial sequence	808	808	100%	0.0	99.11%	AF216945.1
Synechococcales cyanobacterium GJ_2B_I3 16S ribosomal RNA gene, partial sequence	802	802	100%	0.0	98.89%	MH918157.1

Culture DP01 showed 100% identity with *Synechococcus* sp. strains Suigetsu-CG2, TAG and IR48 respectively. In the phylogenetic analysis, DP01 was found to cluster with *Synechococcus* sp., with a bootstrap value of 99. Hence, this isolate was assigned to the genus *Synechococcus*.

The morphological features of isolate DP01 (**Figure 3.8B**) were in consonance with the description of *Synechococcus*, i.e. rod-shaped unicells, 2-3 µm in diameter, cells longer than wide, and lacking a mucilaginous sheath; cells found solitary or in loose colonies (Cabello-Yeves et al. 2017). Recent 16S rRNA-based analyses have confirmed that several widely

varying clades of this genus display no corresponding morphological or ecological signature (Robertson, Tezuka, and Watanabe 2001). Hence, confirmation of identification by molecular phylogenetic analysis is necessary.

Synechocystis sp. ME01



Sequences producing significant alignments Download Manage Columns Show 100

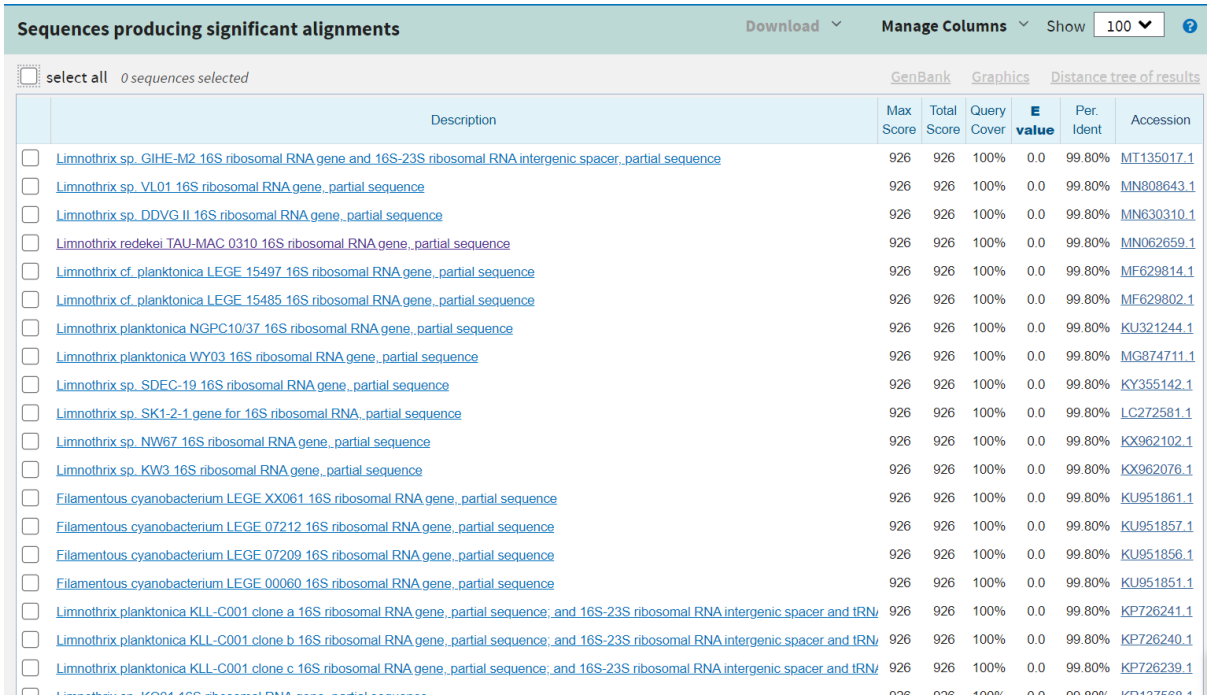
select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Synechocystis aquatilis 1LT32S04 partial 16S rRNA gene	857	857	100%	0.0	100.00%	FM177503.1
Synechocystis sp. CCALA 700 16S ribosomal RNA gene, partial sequence	826	826	100%	0.0	98.71%	GQ375043.1
Merismopedia punctata PMC260.06 16S ribosomal RNA gene, partial sequence	743	743	98%	0.0	95.87%	GQ859638.1
Merismopedia glauca 0BB39S01 16S rRNA gene, strain 0BB39S01	741	741	100%	0.0	95.47%	AJ781044.1
Merismopedia sp. CZS_2_3 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	730	730	100%	0.0	95.05%	KY379855.1
Synechocystis minuscula SAG 258.80 clone a 16S ribosomal RNA gene, partial sequence	719	719	99%	0.0	94.81%	KT354193.1
Synechocystis minuscula SAG 258.80 16S ribosomal RNA gene, partial sequence	719	719	99%	0.0	94.81%	KM019989.1
Cyanobacterium sp. MS-B-20 16S ribosomal RNA gene, partial sequence	719	719	99%	0.0	94.81%	FJ460154.1
Coelomoron pusillum AICB1012 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	713	713	99%	0.0	94.59%	KJ746507.1
Aphanocapsa sp. HBC6 16S ribosomal RNA gene, partial sequence	708	708	100%	0.0	94.18%	EU249123.1
Woronichinia naegeliana 1ES42S1 16S ribosomal RNA gene, partial sequence	708	708	99%	0.0	94.37%	DQ264221.1
Woronichinia naegeliana 0LE35S01 partial 16S rRNA gene, strain 0LE35S01	708	708	99%	0.0	94.37%	AJ781043.1
Synechocystis sp. KUMCC_MS12G strain KUBS17C 16S ribosomal RNA gene, partial sequence	706	706	99%	0.0	94.17%	MG663226.1
Synechocystis sp. CACIAM 05 genome	702	702	99%	0.0	94.14%	CP019225.1
Synechocystis sp. YACCYB510 16S ribosomal RNA gene, partial sequence	702	702	99%	0.0	94.14%	MH683775.1
Synechocystis sp. YACCYB507 16S ribosomal RNA gene, partial sequence	702	702	99%	0.0	94.14%	MH683774.1
Synechocystis sp. YACCYB364 16S ribosomal RNA gene, partial sequence	702	702	99%	0.0	94.14%	MH683754.1
Synechocystis sp. YACCYB361 16S ribosomal RNA gene, partial sequence	702	702	99%	0.0	94.14%	MH683753.1

Culture ME01 showed 100% identity with *Synechocystis aquatilis* 1LT32S04, and 98.71% with *Synechocystis* sp. CCALA 700. Its identity with other sequences in the hit table was below 97%, hence these were not considered in the phylogenetic analysis. ME01 was found to cluster with *Synechocystis aquatilis*, with a bootstrap value of 85. Hence, this isolate was assigned to the genus *Synechocystis*.

The morphological features of isolate ME01 (**Figure 3.8C**) were in consonance with the description of *Synechocystis*, i.e. unicellular; 1.5-2 µm in length; cells spherical or slightly widely oval in shape, solitary or agglomerated, but without common mucilage (Komarek 2003).

Limnothrix sp. VL01



Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Limnothrix sp. GIHE-M2 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	926	926	100%	0.0	99.80%	MT135017.1
Limnothrix sp. VL01 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MN808643.1
Limnothrix sp. DDVG II 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MN630310.1
Limnothrix redekei TAU-MAC 0310 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MN062659.1
Limnothrix cf. planktonica LEGE 15497 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MF629814.1
Limnothrix cf. planktonica LEGE 15485 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MF629802.1
Limnothrix planktonica NGPC10/37 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KU321244.1
Limnothrix planktonica WY03 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	MG874711.1
Limnothrix sp. SDEC-19 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KY355142.1
Limnothrix sp. SK1-2-1 gene for 16S ribosomal RNA, partial sequence	926	926	100%	0.0	99.80%	LC272581.1
Limnothrix sp. NW67 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KX962102.1
Limnothrix sp. KW3 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KX962076.1
Filamentous cyanobacterium LEGE XX061 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KU951861.1
Filamentous cyanobacterium LEGE 07212 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KU951857.1
Filamentous cyanobacterium LEGE 07209 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KU951856.1
Filamentous cyanobacterium LEGE 00060 16S ribosomal RNA gene, partial sequence	926	926	100%	0.0	99.80%	KU951851.1
Limnothrix planktonica KLL-C001 clone a 16S ribosomal RNA gene, partial sequence; and 16S-23S ribosomal RNA intergenic spacer and tRN	926	926	100%	0.0	99.80%	KP726241.1
Limnothrix planktonica KLL-C001 clone b 16S ribosomal RNA gene, partial sequence; and 16S-23S ribosomal RNA intergenic spacer and tRN	926	926	100%	0.0	99.80%	KP726240.1
Limnothrix planktonica KLL-C001 clone c 16S ribosomal RNA gene, partial sequence; and 16S-23S ribosomal RNA intergenic spacer and tRN	926	926	100%	0.0	99.80%	KP726239.1

Culture VL01 showed 99.8% identity with several strains of *Limnothrix* sp. Comparative sequences for phylogenetic analysis were selected, based on previously published material on the respective strain, validating the identification as *Limnothrix* sp. In the phylogenetic analysis, VL01 was found to cluster with *Limnothrix* sp., with a bootstrap value of 100. Hence, this isolate was assigned to the genus *Limnothrix*.

The morphological features of isolate VL01 (**Figure 3.8D**) were in consonance with the description of the genus *Limnothrix*, i.e. non-heterocystous, solitary, unsheathed filaments consisting of narrow, long, cylindrical cells with gas vacuoles (Meffert 1988).

Limnothrix sp. is largely a freshwater form, which at times dominates the freshwater phytoplankton (Gkelis et al. 2005) and can form blooms (Köhler et al. 2005; Kormas et al. 2011; Rucker, Wiedner, and Zippel 1997; Zwart et al. 2005). In India, this genus has been reported from lakes in Manipur and Himachal Pradesh (Singh, Khattar, Singh, Rahi, & Gulati, 2014), rice fields in Uttar Pradesh and Punjab (Khattar et al. 2010; Tiwari et al. 2001), thermal springs of Maharashtra and Odissa (Bhakta, Das, and Adhikary 2016).

CHAPTER FOUR

ISOLATION AND CHARACTERIZATION OF CYANOPHAGES FROM AQUATIC SYSTEMS

This chapter describes the isolation, maintenance and finally characterization of several cyanophages. After the establishment of several stable microalgal and cyanobacterial cultures (Chapter 3), the objective of the current chapter was to isolate specific viruses that would infect any of the said cultures. In order to evaluate the presence of viral particles in the collected water sample, concentration of viral particles would be a critical step. After detection of viral lytic activity, the selected virus would be propagated, and its infectivity characteristics studied.

In aquatic ecosystems, microalgae and cyanobacteria constitute the base of the food chain (Falkowski et al. 2004; Field et al. 1998). The populations of these organisms are, to a great extent, regulated by the top-down control exerted by viruses (Brussaard 2004; Suttle 2000). Thus, viruses that infect and kill microalgae and cyanobacteria are responsible for ripple effects on the entire food chain. Moreover, cyanobacteria are the second most abundant planktonic group after bacteria; hence, cyanophages too are a numerically significant component of the virus community (Mann and Clokie 2012; Suttle 2001). The lysis of cyanobacteria contributes significantly to the nutrient pool, ultimately influencing nutrient cycling as well (Bonetti et al. 2019; Fuhrman 1999). Further, viruses that infect microalgae / cyanobacteria influence their diversity and community composition (Deng and Hayes 2008; Thingstad and Lignell 1997). They reduce the photosynthetic efficiency of their hosts (Padan and Shilo 1973; Suttle, Chan, and Cottrell 1990) and contribute to their genetic diversity through horizontal gene transfer (Lindell et al. 2004; Mann et al. 2003).

With this background cyanophages from marine systems have been well characterized (Chow et al. 2015; Huang et al. 2014; Sullivan, Waterbury, and Chisholm 2003; Suttle and Chan 1993; Xiao et al. 2018). Cyanophages from freshwater and estuarine systems however, have been less studied, thus presenting an immense opportunity for the discovery of novel viruses (Mohiuddin and Schellhorn 2015; Roux, Enault, et al. 2012).

Materials

Media: BG-11, f/2 (composition in Appendix)

Chemicals: Tris base, sodium-EDTA, magnesium chloride, oxalic acid, agar, PEG 8000, cesium chloride, sodium chloride, Tris chloride, methanol, ethanol, formaldehyde, proteinase K, sodium dodecyl sulphate, phenol, chloroform, isoamyl alcohol, isopropanol. All chemicals were obtained from HiMedia Laboratories and were of analytical grade.

Stain: SYBR^{TM} Green I (Thermo Fisher Scientific)

PCR reagents (GeNei): *Taq* polymerase, dNTP mix (10 mM), 10X PCR assay buffer

Methods

4.1 Preparation of Virus Concentrates from Water Samples

Water samples were collected from marine, estuarine and freshwater sites. In addition to the sampling sites detailed in Chapter 3 (p. 34), repeated sampling at selected sites was also carried out. These included Dona Paula Bay (15°27.24′ N, 73°48.12′ E) and Verna Lake (15°20'51.68'N, 73°56'43.72'E). Several microalgal cultures had been isolated from both these sites during the present study, namely, *Cylindrotheca closterium*, *Skeletonema* sp., *Thalassiosira* sp. and *Synechococcus* sp. from Dona Paula Bay (p. 43); *Limnothrix* sp. and *Scenedesmus* sp. from Verna Lake (p. 47-48).

Water samples were filtered through sterile 0.22 µm nitrocellulose membranes and the filtrate was subsequently treated by one of the following methods to concentrate the viruses:

i) Retention on a 0.02 µm membrane (Jing et al. 2014): Prefiltered water was filtered through a 0.02 µm Whatman Anodisc membrane (Sigma-Aldrich Co.). The membrane was flushed with 5 ml of sterile BG-11 medium and the resulting retentate was designated as virus concentrate.

ii) Precipitation with ferric chloride (John et al. 2011): 1 ml of a 1% $FeCl_3$ solution was added per 10 litres of prefiltered water. After an hour's incubation, the ferric-virus flocculate was collected by passing the entire sample through a 0.8 µm polycarbonate filter. The flocculate was resuspended in ascorbate buffer (pH 6.5). This was used as virus concentrate.

4.2 Infection of Microalgal Cultures with Viral Concentrates

Viral concentrates, prepared as above, were tested against all existing microalgal cultures by the following methods:

i) For diatoms: Testing was done in liquid culture, using 24-well plates. To 1 ml of f/2 medium in each well, 10 µl of exponentially growing host culture and 100 µl of virus-concentrate were added. Control wells had just 10 µl host culture added to 1 ml medium. The plates were incubated under the following culture conditions (hereafter referred to as 'standard conditions') 25°C, 16:8 h light:dark cycle and irradiance between 10 and 20 µmol m^{-2} s^{-1}. Test wells were observed for decreased growth compared to controls (Nagasaki and Bratbak 2010).

ii) For cyanobacteria and green microalgae: The above method for detecting viral infection in liquid culture was followed, with the growth medium being BG-11. Since these cultures are amenable to growth in solid medium, the double agar overlay method was carried out after two weeks of growth in liquid culture. For this, the contents of each test well were centrifuged to pellet algal and bacterial cells. 100 µl of supernatant and 300 µl of the relevant exponentially growing host culture cells were added to 3 ml of molten 0.5% BG-11 agar, mixed well and poured on the surface of a 1% BG-11 bottom agar plate. Plates were incubated under standard conditions for 2-4 weeks and observed for viral plaques (Bubeck and Pfitzner 2005).

4.3 Exposure of Microalgal Cultures to UV Radiation to Isolate Possible Lysogenic Viruses

The previous methods of infection of microalgal cultures using virus concentrates were specifically aimed at isolating lytic viruses.

To determine the presence of inducible lysogenic viruses, the unialgal cultures isolated during the present study were exposed to UV light. Aliquots of each culture were transferred to Petri plates, placed at a distance of 40 cm from a 240V, 50 Hz UV lamp, and exposed to UV radiation for 30, 60, 90 and 120 seconds respectively. The plates were incubated overnight in light. The 0.22 µm-filtered culture supernatant was used to infect fresh host cultures in liquid and solid medium (Bratbak, Wilson, and Heldal 1996).

4.4 Amplification / Enrichment of Natural Virus Populations

Viruses infecting specific host microalgae / cyanobacteria are generally present in low titre in natural water samples. In addition to the concentration methods described earlier, to increase the likelihood of detecting viruses of interest, the following method was used.

Water samples were prefiltered after collection, as described, followed by addition of major nutrients – nitrate, phosphate (and silicate for diatoms) in concentrations comparable to culture media. The relevant host culture was inoculated at 10% (v/v) and incubated under standard conditions. The inoculated flasks were observed for signs of decreased growth compared to controls (controls had host culture inoculated in the same volume of autoclaved water sample). Supernatants were further checked for the presence of lytic viruses by solid / liquid assays described above (Brussaard 2004).

Studies on Cyanophages S-BE01 and S-SC01

4.5 Propagation of Isolated Cyanophages

Cyanobacterial culture plates which demonstrated the presence of viral plaques were selected. The following methods for propagation of viral plaques were carried out.

i) Plaque harvesting and propagation (Abedon 2018) – A single plaque was scooped out, suspended in 200 µl of BG-11 medium and incubated at 25°C for 2 hours. After centrifugation at 8000 x g for 10 minutes, the pellet containing debris was discarded. 100 µl of the supernatant was added to 300 µl of exponentially growing host algal culture, mixed into molten BG-11 agar, and poured onto the surface of BG-11 agar plates. This was also used as the method for carrying out plaque assays.

ii) Preparation of plate lysates – Plaque plates which showed confluent lysis (near-complete clearance of host lawn) were used. The plate was flooded with BG-11 medium, with light scraping of the surface, and incubated at 25°C for 2 hours. The supernatant was harvested, centrifuged at 8000 x g for 10 minutes and used as plate lysate for further plaque assays.

iii) Plate lysate-Liquid: The plate lysate was added to various concentrations of exponentially growing host culture in liquid medium.

iv) Plate lysate-Solid: The supernatant of liquid infection with plate lysate (After incubation at standard conditions for 2 weeks) was used as a source of viral inoculum for plaque assay in solid medium.

v) Plate lysate-PEG: The supernatant of liquid infection with plate lysate (After incubation at standard conditions for 2 weeks) was concentrated by PEG precipitation, after which the obtained viral concentrate was used as a source of viral inoculum for plaque assay.

4.6 TEM analysis of Isolated Cyanophages

The phage-host infection was carried out in a large volume for the purpose of obtaining a concentrated phage suspension. For this, 1 ml of exponentially growing host culture and 50 ml phage lysate was mixed and incubated for one hour in light (irradiance between 10 and 20 μmol m^{-2} s^{-1}), for adsorption to take place. This was then inoculated in one litre of BG-11 medium and incubated under the conditions suitable for host growth, described earlier. After two weeks of growth, the phage was concentrated by PEG precipitation (Baker et al. 2006) as follows: The culture suspension was centrifuged at 13,000 x g for 10 minutes to pellet algal cells and debris. To the supernatant, 1M NaCl was added and incubated in ice for one hour. After a second centrifugation, PEG 8000 (10% w/v) was added to the supernatant, mixed gently and incubated at 4°C overnight. The suspension was centrifuged at 13,000 x g for 10 minutes, the supernatant discarded and the pellet containing precipitated phage particles was resuspended in 5 ml of 10 mM Tris-chloride.

The sample was loaded on a cesium chloride gradient (densities 1.45, 1.50 and 1.70 gml^{-1} respectively) and centrifuged at 1,60,000 x g for 24 hours using a Beckman Optima Max ultracentrifuge. A fine band was observed at the region corresponding to a density of 1.6 g ml^{-1}. 0.5 ml-fractions were taken in separate tubes and the fraction containing the band of purified viral particles was dialyzed overnight against SM buffer. A portion of this purified sample was adsorbed onto a formvar-coated copper grid and stained with 2% (w/v) uranyl acetate for 30 seconds, followed by analysis with a Jeol JEM 2100 200kV transmission electron microscope.

Studies on Cyanophage L-VL01

4.7 Solid Propagation and Optimization of Plaque Formation for L-VL01

Cyanophage L-VL01, which infects the filamentous *Limnothrix* sp. VL01, was propagated in solid as well as liquid medium. For propagation in solid medium, 100 µl of virus lysate and 300 µl of overgrown host culture were added to 3 ml of molten 1% BG-11 agar, mixed well and poured on the surface of a 1% BG-11 agar plate. Plates were incubated under standard conditions for two weeks and observed for viral plaques.

To optimize the conditions for plaque formation, the following parameters were varied.

i) The host: phage ratio was varied in the liquid infection preceding the plaque assay. This was achieved by varying the volume of host inoculum (10, 50, 100 µl) added to a fixed volume (500 µl) of phage lysate.

ii) Adsorption time after mixing of host and phage lysate was varied (1 hour, 3 hours and overnight).

iii) For plaque assay, the age of host culture (early middle or late stationary phase) and volume of inoculum of host (300, 350, 400 µl, for each stage) were varied.

4.8 Liquid Propagation and Host Specificity of Cyanophage L-VL01

Phage L-VL01 was propagated in liquid medium as follows. Virus lysate was centrifuged at 13,000 x g for 10 minutes, to pellet host cells and debris. To 500 µl of supernatant, 5 µl of host culture was added and incubated at 25°C for 2 hours for adsorption to take place. This mixture was then added to 50 ml of BG-11 broth in a conical flask and incubated under the conditions for optimal host growth mentioned above. After 2 weeks of incubation, the resultant virus lysate was propagated further, in a similar manner. The virus lysate was also used to characterize the infectivity, morphological and genetic features of the virus, as described in the following sections.

The infectivity of L-VL01 against two other strains of freshwater filamentous cyanobacteria cultured in our laboratory was tested. The strains had been identified as *Limnothrix* sp. CP01 and *Limnothrix* sp. TP02 on the basis of 16S rDNA amplification and phylogenetic analysis (Chapter 3, p. 48).

4.9 Comparison of Growth of Virus-Infected and Uninfected Host Culture

The growth rate of the host cyanobacterium was compared in virus-infected and uninfected (control) cultures. Growth rate was measured in terms of change in concentration of chlorophyll a, over 16 days. For extraction of chlorophyll a, 1 ml of culture (infected or uninfected) was centrifuged at 7000 x g for 5 minutes to pellet cyanobacterial cells. To the cell pellet, 1 ml of ice-cold methanol was added, vortexed and incubated at 4°C for 20 minutes. After a centrifugation step at 7000 x g for 10 minutes, the extracted pigment in the supernatant was estimated at 665 nm and 720 nm on a Shimadzu UV-Mini-1240 Spectrophotometer (Zavřel, Červený, and Sinetova 2015).

Chlorophyll a concentration was calculated by the following formula:

$$Chl_a\ [\mu g/ml] = 12.9447\ (A_{665} - A_{720})$$

4.10 Comparison of Virus-infected and Non-infected Cyanobacterial Filaments

By Optical Microscopy

20 µl of culture medium containing cyanobacterial filaments was withdrawn from control and virus-infected flasks, and mounted on a glass slide. This was observed at 100X and 1000X magnification, using a Lynx Light Microscope.

By scanning electron microscopy (SEM):

The filament morphology of exponentially growing uninoculated host cyanobacteria *Limnothrix* sp. VL01 and inoculated (virus-added) host were compared. 500 µl of each suspension was passed through a 0.2 micron polycarbonate filter under vacuum. The filter was dehydrated in an ethanol series (30%, 60%, 90% ethanol) and air dried. The filter was mounted on an aluminium stub, sputter-coated with gold particles and observed under a Zeiss Evo 18 scanning electron microscope.

4.11 Virus Particle Count by Epifluorescence Microscopy (EFM)

Virus particles present in in the phage lysate were counted by the established SYBR-Green staining method (Patel et al. 2007).

Briefly, the lysate was fixed with 2% (v/v) 0.22 µm-filtered formaldehyde and kept at 4°C for 30 minutes. 5 ml of this sample was pre-filtered through a 0.22 µm polyethersulfone syringe filter. The pre-filtered sample was vacuum-filtered through a 0.02 µm anodisc filter mounted on a pre-wetted 0.8 µm polycarbonate backing filter. The anodisc was filtered to dryness.

SYBR Green commercial stock was diluted 1:10 with sterile, 0.02 µm-filtered MilliQ water to get a working stock. A drop containing 97.5 µl 0.02 µm-filtered water and 2.5 µl SYBR working stock, was placed on a sterile Petri plate in the dark. The anodisc filter, after complete drying on a tissue paper, was placed, sample side up, on the drop of diluted stain, in the dark, for 15 minutes. After wicking away moisture, the filter was placed, sample side up, on a clean glass slide and a coverslip bearing a drop of antifade reagent (Invitrogen SlowFade Kit, Reagent A) was inverted onto it. The slide was mounted with a drop of non-fluorescent immersion oil, and viewed at 1000 X magnification, under the blue excitation filter of an epifluorescence microscope.

Virus particles visualized in 10 distinct fields were photographed and the image analysed using TCapture v. 5.1.1 software. Virus particles present in 10 distinct squares, each of measurement 100 µm x 100 µm were counted. The obtained mean count was extrapolated to the entire filterable area of the Anodisc filter (42 mm), to arrive at the virus particle count per ml.

4.12 TEM Analysis

TEM analysis was carried out as described in section 4.6.

4.13 Isolation of the Phage Genomic DNA

One litre of phage lysate was concentrated to approximately 5 ml by PEG precipitation, as described in the preparation for TEM analysis. 2 ml of this concentrated lysate was loaded on a cesium chloride density gradient and subjected to ultracentrifugation as described earlier. The aspirated virus-containing fraction was dialysed against SM buffer and then used for genomic DNA isolation by a standard phenol-chloroform protocol (Sambrook and Russell 2006). Briefly, proteinase K (final concentration 50 µg/ml) and SDS (final concentration 0.5%) were added to the purified phage lysate, mixed gently and incubated at 37°C for one hour. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the digestion mixture, mixed gently and centrifuged at 8000 x g for 5 minutes to separate the aqueous and organic phases. The aqueous phase was aspirated and transferred to a clean tube and an equal volume of chloroform/isoamyl alcohol (24:1) added, mixed and centrifuged. Finally the aqueous phase was transferred to a clean tube, 0.7 volumes of isopropanol added, mixed gently and incubated at room temperature for 20 minutes to precipitate DNA. The DNA was collected by centrifugation at 13,000 x g, 4°C, for 15

minutes. The supernatant was discarded and the pellet washed twice with 70% ethanol, air dried and re-suspended in 30 µl of RNase-free water.

4.14 Amplification of Virus Family-Specific Genes

PCR-amplification of virus family specific genes, with a view to better characterization of Φ L-VL01, was attempted for the following genes: The *g20* gene, a structural gene, encoding the capsid assembly protein, and the *psbA* gene, a functional gene, encoding the D1 protein involved in photosynthesis.

Reaction mixture components and parameters for the respective genes were as follows:

Component	Volume in µl	
	g20	***psbA***
10X PCR assay buffer containing $MgCl_2$	5.0	5.0
dNTP mix (2.5 mM each)	5.0	5.0
Forward primer (50 pmol)	0.5	2.0
Reverse primer (50 pmol)	0.5	2.0
Virus lysate (template)	4.0	4.0
Taq polymerase	0.5	0.5
Nuclease free water	34.5	31.5

PCR parameters:	***g20***		***psbA***	
(30 cycles of the following)	Temperature	Time	Temperature	Time
Denaturation	94°C	45 s	92°C	1 min
Annealing	40°C	45 s	50°C	1 min
Extension	72°C	1 min	68°C	1 min

Primers used:
g20: CPS1 and CPS 8 (Wang et al., 2010)
psbA: psbA-F and psbA R (Zeidner et al., 2003)

In addition, the viral genomic DNA was subjected to PCR-amplification using universal 16S rDNA primers (28F and 1492R), to check for the presence of contaminating bacterial DNA, if any. The parameters used for the same were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, extension at 72°C for 1 min.

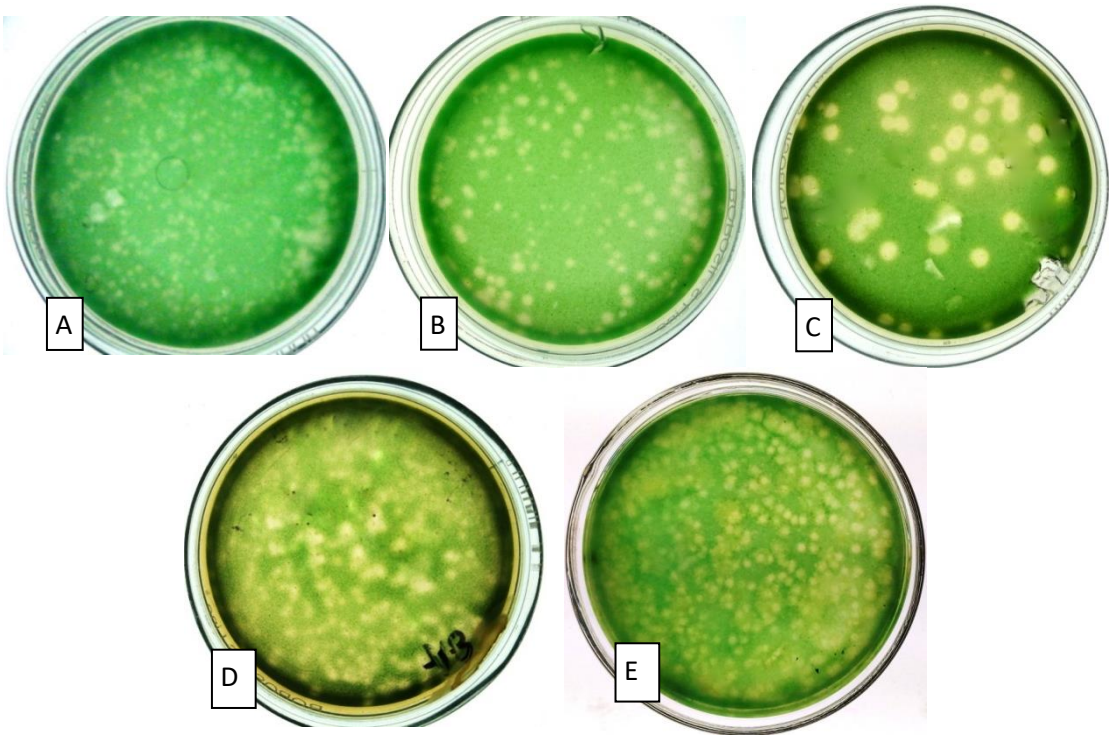


Figure 4.1: Cyanophages isolated in the present study, evidenced by plaque assays:
A: Φ S-VL01; B: Φ S-BE01; C: Φ S-SC01 D: Φ S-CF01 E: Φ L-VL01

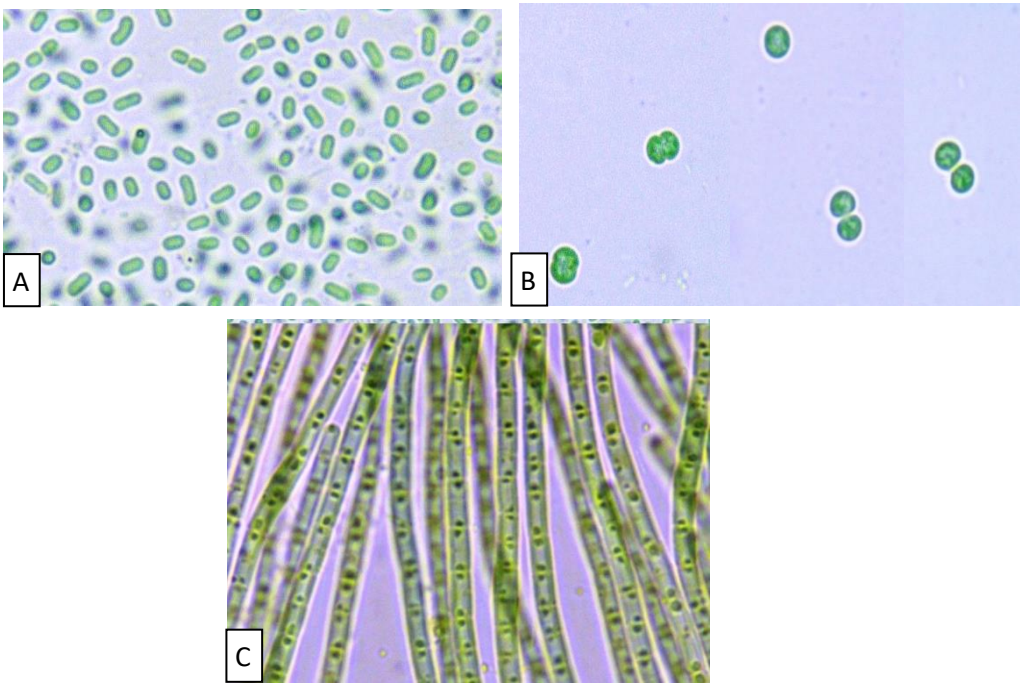


Figure 4.2: Respective host cyanobacteria of isolated cyanophages
A: *Synechococcus* sp. DP01; B: *Synechocystis* sp. ME01; C: *Limnothrix* sp. VL01

Results and Discussion

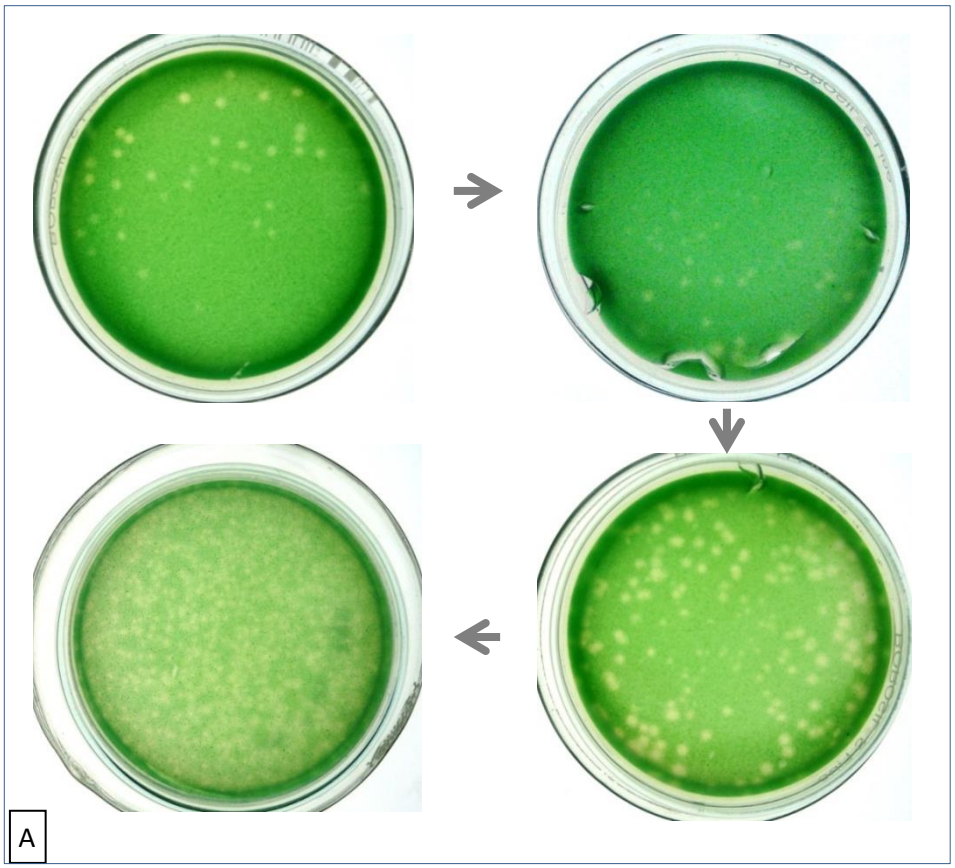
In aquatic ecosystems, virus abundance is generally correlated with that of the respective hosts (Jacquet et al. 2010; Wigington et al. 2016). Bacteriophages comprise more than 90% of the aquatic viral community (Duhamel and Jacquet 2006; Li and Dickie 2001; Marie et al. 1999). This group has been well studied and characterized in diverse environments. Cyanophages, the second most abundant category, comprise about 7% of the viral population (Mann and Clokie 2012; Personnic et al. 2009; Suttle 2001) and play significant ecological roles, impacting the entire system (Hargreaves et al. 2013; Jaskulska and Mankiewicz-Boczek 2020). As mentioned in the introduction to this chapter, cyanophages from non-marine systems have not been extensively studied. Within India, cyanophage-related studies have been limited to a few descriptions of cyanophage isolation (Singh 1973, 1974, 1975) and characterization of externally sourced isolates (Amla 1981; Kashyap et al. 1988; Singh and Kashyap 1977). The present study is the first to focus on isolating and characterizing native cyanophages from freshwater / estuarine systems in the geographical region of Goa, India.

4.15 Isolation of Five Cyanophages

After screening close to 40 water samples against 17 cultures (including diatoms, green microalgae and cyanobacteria), we isolated a total of five novel cyanophages. All the cyanophages were detected by the formation of plaques on lawns of the respective host cultures (**Figure 4.1**).

The three host cyanobacteria that showed susceptibility to infection were identified as *Synechococcus* sp. DP01, *Synechocystis* sp. ME01 and *Limnothrix* sp. VL01, respectively, on the basis of their morphological features (**Figure 4.2**) and phylogenetic analysis (Chapter 3). The alphabets DP, MZ and VL represent the niches from where host cyanobacteria were isolated (**Table 4.1**)

The two cyanophages infecting *Synechococcus* sp. DP01 were designated Φ S-VL01 and Φ S-BE01 respectively, while the two infectious to *Synechocystis* sp. MZ01 were designated Φ S-SC01 and Φ S-CF01 respectively and the one infectious to *Limnothrix* sp. VL01 was designated Φ L-VL01. In this nomenclature, the first alphabet represents the first letter of the host genus, while the next two represent the niche from where the respective cyanophage was isolated (**Table 4.1**).



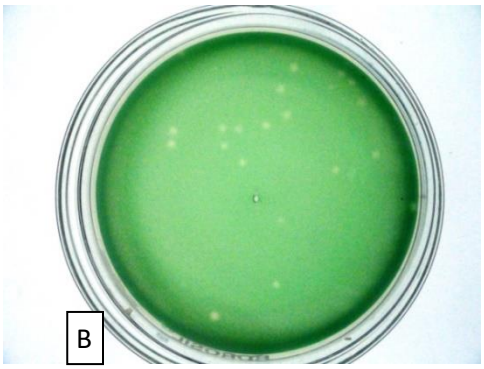


Figure 4.3: Φ S-BE01
A: Increase in phage titre over four rounds of propagation
B: Plaque formation after re-inoculation of plate lysate with host in solid media

Table 4.1 Details of cyanophages isolated in the present study

Host cyanobacterium	**Virus**	**Host location of isolation**	**Virus location of isolation**
Synechococcus sp. DP01	Φ S-VL01	**D**ona **P**aula Bay	**V**erna **L**ake
	Φ S-BE01		**B**etul **E**stuary
Synechocystis sp. ME01	**Φ S-SC01**	**M**andovi **E**stuary	**S**antana **C**reek
	Φ S-CF01		**C**ortalim Rice **F**ields
Limnothrix sp. VL01	**Φ L-VL01**	**V**erna **L**ake	**V**erna **L**ake

Two of the cyanophages could not be propagated beyond two generations in the laboratory. Cyanophages **S-BE01, S-SC01 and L-VL01** were propagated for 8 or more generations; however only ΦL-VL01 could be cultured for an extended period of time.

4.16 Propagation of Cyanophages S-BE01 and S-SC01

Cyanophages S-BE01 and S-SC01 were lytic phages infecting *Synechococcus* sp. DP01 and *Synechocystis* sp. ME01 respectively. During isolation of these phages, initial plating of the filtered water sample with host culture on solid medium did not indicate the presence of phage (plaques). Plaque formation was obtained by preliminary infection in liquid medium, followed by plaque assays in solid medium. Subsequently, several methods for propagation of the virus were carried out, as detailed in the Methods section. In the case of both these phages, the phage titre increased over several rounds of propagation (**Figure 4.3[A]; Fig 4.4[A]**). As indicated in **Table 4.2**, phage infection did not cause complete lysis of host in liquid infection, even when a high phage titre (plate lysate) was inoculated.

Table 4.2 Qualitative characteristics of infection by Φ S-BE01 and Φ S-SC01

Phage	**S-BE01**	**S-SC01**
Generation time	2 weeks	2 weeks
Titre	1.5 x 10^4 PFU / ml	8.0 x 10^4 PFU / ml
Plate lysate-Liquid	No visible lysis	Decrease in culture turbidity
Plate lysate -Solid	Plaques obtained (**Figure 4.3 B**)	Plaques obtained **(Figure 4.4 B)**
Plate lysate -PEG	Infectivity lost	Infectivity retained **(Figure 4.4 C)**

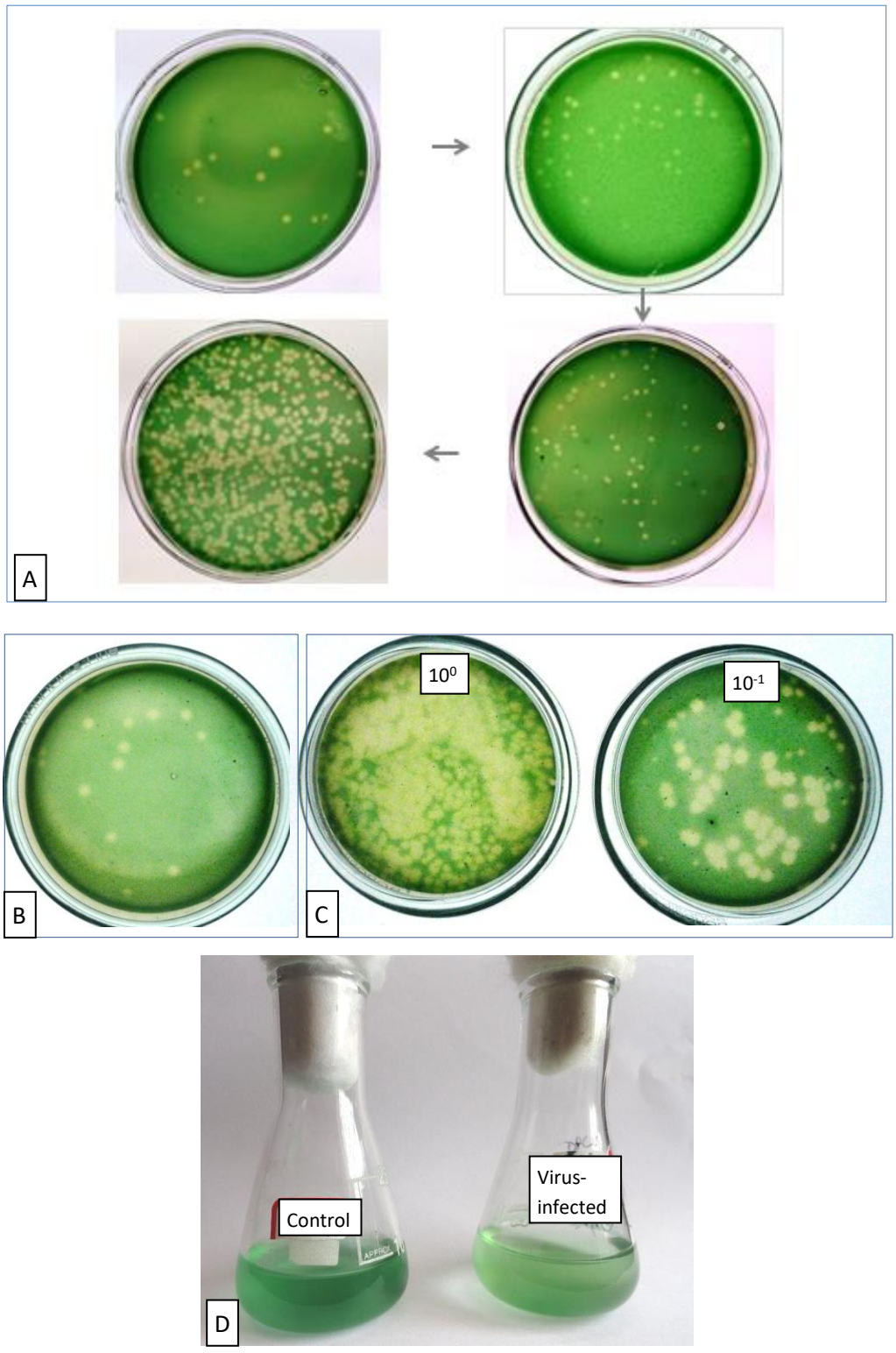


Figure 4.4: Φ S-SC01

A: Increase in phage titre over four rounds of propagation

B: Plaque formation after re-inoculation of plate lysate with host in solid media

C: Plaque formation after concentrating virus lysate with PEG (dilutions 0 and 10^{-1})

D: Inoculation in liquid medium

Synechococcus Phages

Synechococcus is one of the most abundant cyanobacteria in the oceans, and together with *Prochlorococcus*, accounts for nearly 25% of oceanic primary productivity (Liu et al. 1998; Partensky et al. 1999; Scanlan and West 2002). However, though most extensively studied as a component of marine systems, this is a cosmopolitan genus, found in freshwater, terrestrial and subaerial habitats as well, and possesses great adaptability, facilitating its successful establishment in the entire range of tropical to polar regions (Cabello-Yeves et al. 2017; Dvořák et al. 2014). In freshwater systems, *Synechococcus* sp. constitutes a major component of the picocyanobacteria, along with *Cyanobium* sp., both of which are non-bloom formers (Callieri 2008).

Synechococcus has been described as "one of the most successful and influential organisms in Earth's history" (Dvořák et al. 2014). Consequently, the significance of the phages that regulate the populations of this organism has been recognized. *Synechococcus* phages have previously been isolated from a variety of aquatic ecosystems, including marine (Wilson et al. 1993; Zhang et al. 2013), estuarine (Wang and Chen 2008) and freshwater (Dillon and Parry 2008; Dreher et al. 2011) systems. In the current study, the isolation of Synechophages has been reported for the first time, from water systems within Goa – an estuarine and a lake system respectively. Water at the estuarine site (Betul) had a salinity of 25 p.s.u., indicating the influence of seawater, which would in turn influence the species composition of the specific niche. Salinity has been found to be a chief determinant of cyanophage distribution in river estuaries (Lu, Chen, and Hodson 2001). In contrast, the other site (Verna Lake) had a salinity of 0 p.s.u., indicating its freshwater characteristic. The occurrence of related cyanophages in freshwater and marine environments has previously been reported (Chénard and Suttle 2008; Dreher et al. 2011).

Natural *Synechococcus* communities are often dominated by cells resistant to viral infection. This may be attributed to the phenomenon of "antagonistic co-evolution" – a process wherein the host evolves resistance to infection by a particular virus, the virus then evolves in a manner to infect the resistant host, followed by further development of resistance in the host (Buckling and Rainey 2002; Lenski and Levin 1985). In marine *Synechococcus*, such evolutionary dynamics give rise to new resistant strains at a rapid rate (Marston et al. 2012). Synechophages commonly found in aquatic systems might well be maintained only by infecting the rare sensitive cells present in the community (Marston and Sallee 2003;

Waterbury and Valois 1993). From this perspective, the detection of viral lysis and the isolation of lytic phages in the laboratory is significant.

Synechocystis Phages

Synechocystis is a widely distributed, unicellular, coccoid, freshwater cyanobacterium (Ikeuchi and Tabata 2001). However, the vast majority of research on this genus (and a significant proportion of recent research on cultured cyanobacteria) has been based on one specific cultured strain. *Synechocystis* sp. PCC 6803 is a phenotypically versatile strain, that has been used as a model organism for laboratory studies on photosynthesis, signal transduction and stress responses (Hernández-Prieto et al. 2016; Zavřel, Sinetova, et al. 2015) and been referred to as the 'green *E. coli*' (dos Santos, Du, and Hellingwerf 2014).

To the best of our knowledge, this study is the first report of isolation of *Synechocystis* sp. phages. We isolated two lytic phages against *Synechocystis* sp., from an estuarine site and a rice field respectively. The isolation of a cyanophage from a rice field is significant to our study, as in Chapter 5 (p. 97), we report the highest concentration of total virioplankton from rice field water, when compared with lake and estuarine water samples. Laboratory studies of virus-host systems from rice fields would advance the understanding of this ecosystem, which is relatively unexplored from an ecological point of view.

Sustained propagation and maintenance of freshwater cyanophages is reported to be challenging (Watkins et al. 2014). Limited propagation of phages isolated in the laboratory may be attributed to either of the equally dynamic components – the virus and the host – of the virus-host system. In natural systems, resistance to viral infection typically comes at a 'fitness cost'. This implies that resistant strains are typically less efficient at utilizing limiting resources than sensitive ones, and thereby at a disadvantage for survival (Buckling and Rainey 2002; Lennon et al. 2007). However, in the laboratory, where growth media are replete with nutrients, this fitness cost may be irrelevant, thereby promoting the development of resistant strains within a natural population of cultured cyanobacterial cells. The production of defective interfering particles (DIPs) in a virus population – progeny viruses that do not further infect host cells (Brown and Bidle 2014; Kirkwood and Bangham 1994) – may also lead to eventual resistance of the host population.

Moreover, the dynamics of viral infection strongly depend on the physiological state of the host (Fulton et al. 2014; Thyrhaug et al. 2002). Light and nutrient conditions *in vitro* are often vastly different from field conditions (Brown and Bidle 2014). The presence of certain ions is critical for phage infection and lysis (Mole, Meredith, and Adams 1997; Yeo and Gin 2013).

Characterization of Cyanophage L-VL01

Cyanophage L-VL01, which infects *Limnothrix* sp. VL01, is the only cyanophage infectious to a filamentous cyanobacterium, to be isolated in the present study. Its host cyanobacterium had been isolated from a shallow lake in Verna village, Salcete, Goa, in the month of January (post-monsoon season). The virus was isolated in the month of April (pre-monsoon) from a water sample collected from the same location. The phages isolated previously were detected by plaque formation in solid medium, but depicted limited lysis when used to infect hosts in liquid medium (**Figure 4.4 D**). However, L-VL01 demonstrated visible lysis both in liquid and solid media (**Figure 4.5)**.

4.17 Solid Propagation and Optimization of Plaque Formation

Unlike bacterial hosts, cyanobacteria do not always depict plaque formation upon phage infection (Wilhelm et al. 2006). The following conditions were found optimal for the formation of plaques by Φ L-VL01 (**Figure 4.5 A**).

i) Before solid propagation, the host-phage infection was first carried out in liquid BG-11 medium, as described previously, and incubated for a period of two weeks. The supernatant of this infection was used as inoculum for solid propagation.
ii) Prior to solid propagation, an adsorption time of one hour was found optimal, wherein the appropriate aliquots of host culture and phage lysate respectively, were directly mixed and incubated, followed by addition of growth medium.
iii) Plaque formation was optimal when a 35-40 days old host culture was used. The volume of host added to the mixture for plating had to be adjusted according to cell density.

The above-mentioned parameters are indicative of phage infectivity characteristics. The multiplicity of infection (the ratio of infective viral particles to host cells) is a vital parameter in determining the success of a phage-host infection. Stage of growth of the host culture represents the density of host cells, wherein a higher density would lead to a far less effective phage infection (Watkins et al. 2014).

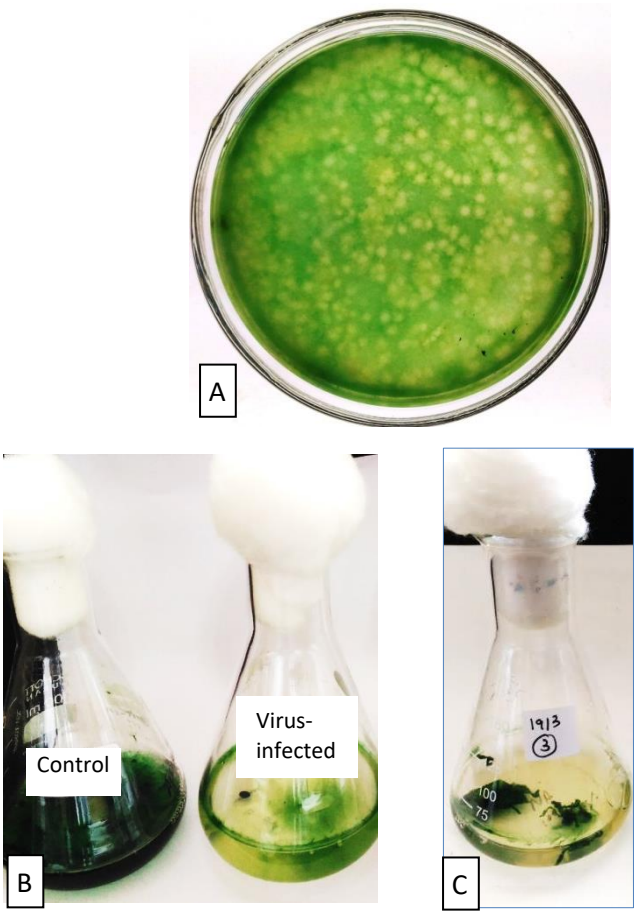


Figure 4.5: Φ L-VL01

A: Plaque formation under optimum conditions
B: Liquid propagation – near-complete lysis of cyanobacterial host filaments
C: Poor regrowth of resistant filaments

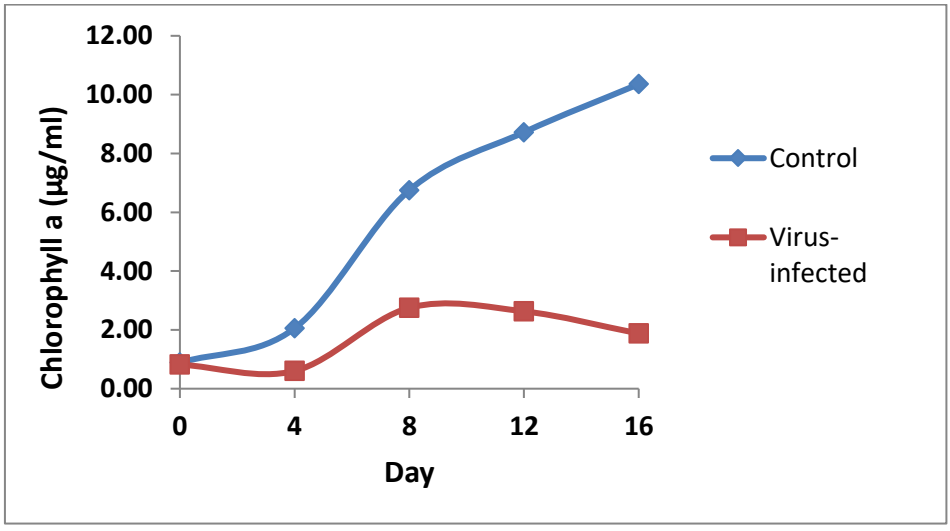


Figure 4.6: Growth of phage-infected and uninfected *Limnothrix* sp. VL01 culture

4.18 Liquid Propagation and Host Specificity

When propagated in liquid medium, Φ L-VL01 demonstrated a pattern of infection similar to phages of other filamentous cyanobacteria (Gao et al. 2009; Pollard and Young 2010; Šulčius et al. 2015). Host filaments gradually turned from dark green to yellow, an indication of filament degradation (Xiangling et al. 2015).

Lysis of host was not complete (**Figure 4.5 B**), but there was a residual growth of host filaments. The growth of these 'resistant' filaments increased till about 2 weeks post-infection, after which there was no further growth. When washed and reinoculated in fresh media, the resistant filaments showed poor growth (**Figure 4.5 C**) compared to control host filaments. Incomplete lysis of filamentous host has been reported in phage-infection of *Planktothrix agardhii* (Gao et al. 2009) and *Aphanizomenon flos-aquae* (Šulčius et al. 2015).

Φ L-VL01 did not lyse either of the alternative strains used to test its infectivity. Host specificity varies among cyanophages. While some are highly host-specific (Ali et al. 2012; Šulčius et al. 2015), others are 'generalists', infecting more than one host strain, sometimes across species and even across genera (Liao et al. 2010; Watkins et al. 2014).

Liquid propagation of Φ L-VL01 was preferred over solid, due to the filamentous nature of the host, which at times interferes with plaque formation (Wilhelm et al. 2006) Thus, the phage lysate prepared through liquid infection was used for further experiments.

4.19 Growth of Phage-Infected and Uninfected VL01 Culture

The growth of virus-infected versus uninfected host cultures was compared in terms of total chlorophyll a concentration. There was an 82% reduction in growth of virus-infected cultures over 16 days (**Figure 4.6**). Host lysis was evident from the second day post-infection.

A cyanophage infecting *Lyngbya* sp. reduced chlorophyll concentrations in host cultures by 95% over 14 days, while a phage infecting *Anabaena* sp. reduced host chlorophyll concentrations by 80% over 30 days (Phlips, Monegue, and Aldridge 1990). Another report (Yeo and Gin 2013) states that chlorophyll concentrations in phage-infected *Anabaena* sp. remained unchanged for 3 days post-inoculation, followed by a sudden drop on the 4th day.

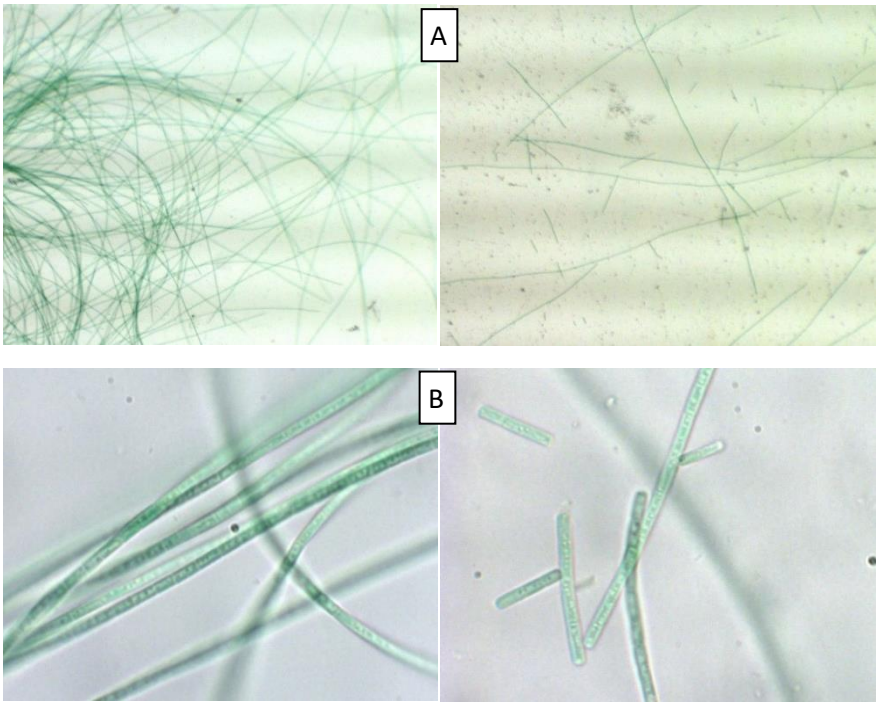


Figure 4.7: Filaments of control / uninfected (left panel) **and phage-infected** (right panel) ***Limnothrix* sp. VL01, viewed under optical microscope:**
A: 100 X magnification; B: 1000 X magnification

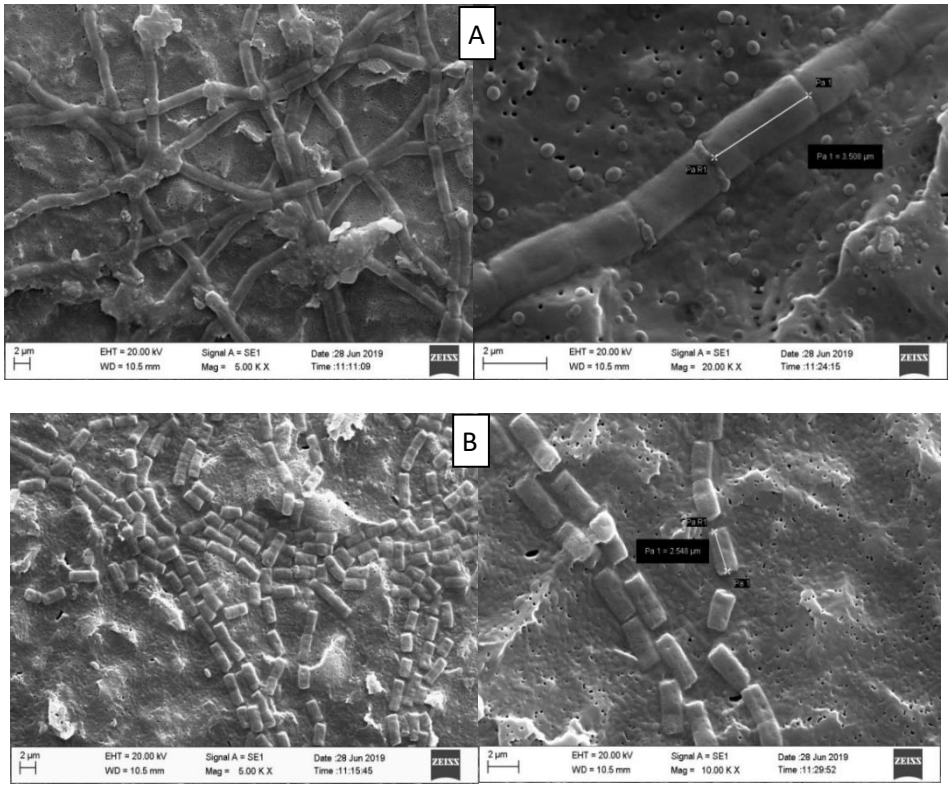


Figure 4.8: **Comparison of virus-infected and non-infected filaments of *Limnothrix* sp. VL01 by scanning electron microscopy**

A: Intact control filaments at 5,000 X and 20,000 X magnification

B: Fragmented virus-infected filaments at 5,000 X and 10,000 X magnification

4.20 Comparison of Virus-Infected and Control Filaments by Microscopy

Cyanobacterial filaments of a control culture of *Limnothrix* sp. VL01 were compared with filaments of virus-infected culture, by optical and scanning electron microscopy. Control filaments appeared intact, while in virus-infected filaments, cells were detached from each other, giving a fragmented appearance to the filament. **Figure 4.7 A** represents control and virus-infected filaments viewed under optical microscope (100X magnification); **Figure 4.7 B** represents the same, viewed under 1000X magnification. The SEM images of control and infected filaments are depicted in **Figure 4.8 A** and **B** respectively.

Fragmentation of cyanobacterial filaments following phage infection has been reported in *Anabaena* sp. (Mole et al. 1997) *Planktothrix* sp. (Gao et al. 2009), *Cylindrospermopsis* sp. (Pollard and Young 2010) and *Anabaena flos-aquae* (Šulčius et al. 2015). This phenomenon is hypothesized to be a strategy for host survival by increased chances of dispersal and propagation.

4.21 Virus Particle Count by EFM

Virus particles viewed under EFM are depicted in **Figure 4.9**. A mean count of 3.43 x 10^4 virus-like particles per ml was obtained. This count was correlated to the count obtained through plaque assay, which was 1.0 x 10^4 plaque forming units per ml.

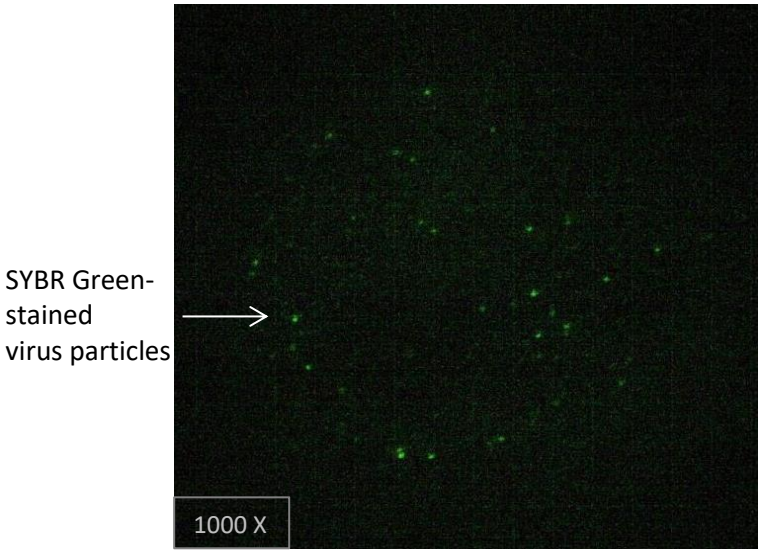


Figure 4.9 Virus particle count by epifluorescence microscopy

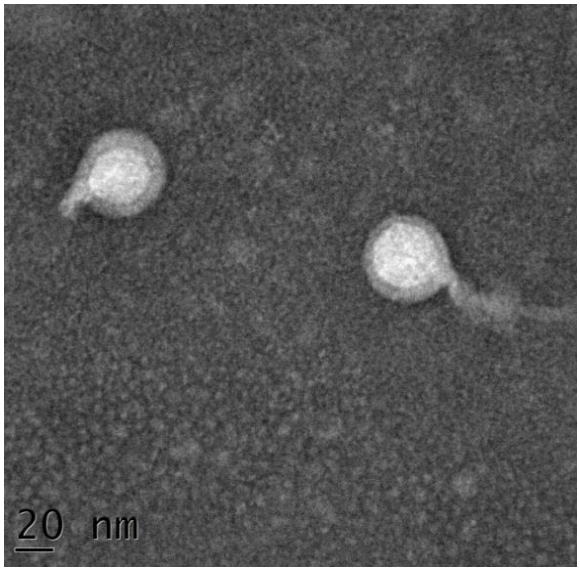
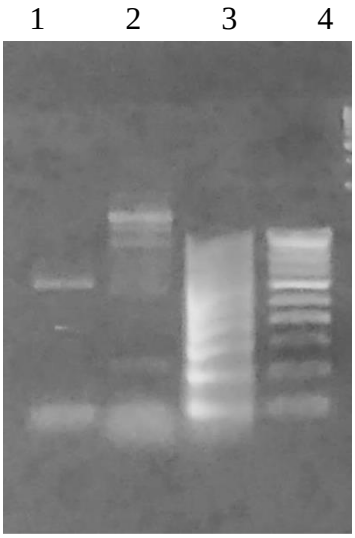
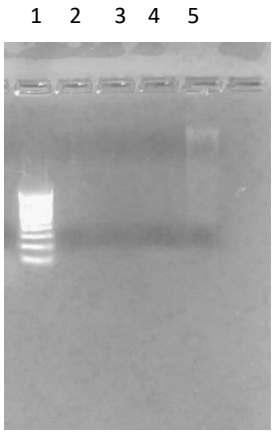


Figure 4.10: **TEM image of Φ L-VL01**

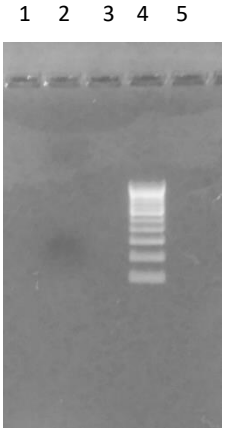


Lane 1: Amplified *g20* fragment from L-VL01 culture sample
Lane 4: 100 bp DNA ladder

Figure 4.11: **Amplification of *g20* gene fragment from genomic DNA of Φ L-VL01**



Lane 1: 100 bp DNA ladder
Lane 5: Genomic DNA of Φ L-VL01



Lane 2: 16S rDNA PCR product (no amplification)
Lane 4: 100 bp DNA ladder

Figure 4.12: A: Genomic DNA preparation of Φ L-VL01;
B: No amplification of 16S rDNA from virus DNA preparation

4.22 TEM

TEM analysis revealed the following morphological features of Φ L-VL01 (**Figure 4.10**): an icosahedral head of diameter 49 nm and a short tail. Among cyanophages of filamentous cyanobacteria reported in literature (**Table 4.3**), several have been short-tailed (*Podoviridae*) or tailless.

Table 4.3: Comparative dimensions of phages of filamentous cyanobacteria reported in literature

Cyanophage	**Host**	**Head diameter (nm)**	**Tail type/ length (nm)**	**Reference**
Various	*Nodularia* sp.	53-137	Long/ 126-888	Jenkins 2006
Pf-WMP4	*Phormidium foveolarum*	55	Short/ NA	Liu 2007
Various	*Anabaena* sp.	Mostly Podoviridae		Deng 2008
PaV-LD	*Planktothrix agardhii*	76	Tailless	Gao 2009
Cr-LS	*Cylindrospermopsis raciborskii*	70	Long	Pollard 2010
Various	*Anabaena circinalis, A. cylindrica*	123-170	Mostly Podoviridae	Yeo 2013
L-DHS1	*Limnothrix* sp.	72	Long / 660	Xiangling 2015
CL131	*Aphanizomenon flos-aquae*	97	361	Sulcius 2015
CrV	*Cylindrospermopsis raciborskii*	65	612	Steenhauer 2016
PA-SR01	*Pseudoanabaena*	91	Tailless	Zhang 2020
L-VL01	*Limnothrix* sp.	49	Short	Present study

NA: data not available

4.23 Amplification of Virus Family-Specific Genes

The *g20* gene coding for the capsid assembly protein of myoviruses, was successfully amplified from the lysate of cyanophage L-VL01. **Figure (4.11)** shows the PCR product of expected size around 600 bp. The presence of this gene confirms that L-VL01 belongs to the family *Myoviridae*.

g20 was the first marker that was applied to the study of environmental cyanophage diversity (Fuller et al. 1998). It has been used in metagenomic studies (Matteson et al. 2011;

Wang et al. 2011) as well as studies of cultured cyanophages from freshwater environments (Wang et al. 2010; Wilhelm et al. 2006).

Φ L-VL01, however, did not show amplification of the *psbA* gene. *psbA* comes under the category of 'auxiliary metabolism genes' present in cyanophage genomes. It is a host-derived metabolic gene, which codes for a homologue of the D1 protein of photosystem II found in cyanobacteria. Approximately 90% of all cyanophage isolates carry the *psbA* gene (Puxty et al., 2014).

There was no amplification of 16S rDNA (**Figure 4.12 B**), confirming the absence of bacterial DNA in the phage genomic DNA preparation (**Figure 4.12 A)**.

The first cyanophages ever isolated were from freshwater niches (Safferman and Morris 1963). During the initial years post-discovery of cyanophages, the major interest was in their potential to control harmful cyanobacterial blooms, a concept which was endorsed by several researchers over the decades (Deng and Hayes 2008; Jassim and Limoges 2017; Sigee et al. 1999; Yoshida et al. 2006b). However, the failure of efforts in this direction may have shifted the focus to marine systems. Today, a disproportionate number of cyanophage studies, whether individual or metagenomic, have come from marine environments (Šulčius et al. 2019). During the last couple of decades, freshwater cyanophage research has picked up. Efforts are still on to isolate highly virulent phages against bloom-forming (many of them filamentous) cyanobacteria, which could in future be used as a biological control agent. Several of these studies, discussed above, have isolated phages from lake systems in various geographical regions. The phages display patterns of infection and host morphological changes similar to our observations with L-VL01.

The *Limnothrix* isolate used in the present study was purified from a mixed consortium of cyanobacteria isolated from lake water. The serial dilution method used for establishing a unialgal culture, intuitively selects for the fastest-growing / most robust form within the consortium (Allen and Nelson 1910). The final constituent of the culture thereby gives an idea of the most highly populated / robust taxa in the specific aquatic niche. Therefore, we consider *Limnothrix sp.* VL01 to be a potential bloom-former. With respect to the isolates we similarly obtained from two other freshwater bodies within geographical proximity, the

dominant forms that emerged also belonged to *Limnothrix* sp., indicating the possibility of widespread dominance of this genus.

Among filamentous cyanobacteria, *Limnothrix* sp. depicts less physiological plasticity than related bloom-formers such as *Planktothrix* sp. (Yang et al. 2020). This implies a low potential for coexistence with other species, and high likelihood of blooms where *Limnothrix* sp. is the dominant form. From this perspective, the isolation of a lytic cyanophage against a member of this genus assumes significance. It is worthy of note that Φ L-VL01 is only the second *Limnothrix* sp. phage to be reported.

Moreover, from a general virological perspective, the isolation and characterization of novel viruses is a critical area in aquatic virus research. Laboratory studies on individual virus-host systems enable a better understanding of the contribution of viruses to the aquatic ecology (Weitz et al. 2013; Wommack and Colwell 2000). Such studies are also required, to complement metagenomic investigations of various ecosystems (Ackermann 2012; Brum and Sullivan 2015).

CHAPTER FIVE

MOLECULAR AND MICROSCOPIC STUDIES OF AQUATIC VIRUS COMMUNITIES

The studies reported in the previous two chapters demonstrate virus-host systems isolated from freshwater and estuarine ecosystems. The current chapter elaborates on virus abundance and community studies from the respective ecosystems.

Water samples were collected from aquatic sites representing ecosystems such as rice field floodwater, lakes and estuaries. Total viral counts in the water samples were determined by flow cytometry (Brussaard et al. 2010). Virus communities from two of the sites which depicted high viral counts were further studied by epifluorescence microscopy (Patel et al. 2007) and PCR-based detection of family-specific marker genes (Chen and Suttle 1995; Wang et al. 2010).

Flow cytometry and epifluorescence microscopy are commonly used to enumerate viruses in natural and culture samples (Jacquet, Dorigo, et al. 2013; Sulcius, Sigitas, Staniulis, and Paskauskas 2011). These techniques are based on the use of fluorescent stains that bind tightly and specifically to nucleic acids, thus facilitating the visualization of cells or virus particles. First reported in 1999 for enumerating viruses in seawater samples (Marie et al. 1999), flow cytometric enumeration has subsequently been used to quantify viral populations and sub-populations in, for example, marine (Brussaard et al. 2010), lake (Personnic et al. 2009) and rice field (Nakayama et al. 2007c) systems. One of the few published reports in the field of aquatic virology from Goa, India, describes virus enumeration from an estuarine system (Mitbavkar et al. 2011). Compared to previously used techniques for virus enumeration, epifluorescence microscopy is convenient, rapid and cost-effective. It has routinely been used to quantify viruses in natural systems and laboratory experiments (Patel et al. 2007; Suttle and Fuhrman 2010). Over the past few decades, epifluorescence enumeration has facilitated important conclusions on the factors contributing to viral abundance in marine and freshwater systems (Clasen et al. 2008) and the ecological roles of viruses (Bettarel et al. 2003; Guixa-Boixereu et al. 1999).

Enumeration techniques provide an idea of total viral populations. To measure the presence and abundance of specific virus sub-populations, molecular methods based on signature genes are used. There is no universal marker gene for viruses as for bacteria; hence, family-specific markers are used (Adriaenssens and Cowan 2014). The AVS (algal virus-specific) primers were designed to amplify a portion of the DNA *polB* gene from the family of phytoplankton viruses called *Phycodnaviridae* (Chen and Suttle 1995). Phycoviruses from

diverse niches have been detected by this method (Clasen et al. 2008; Long et al. 2018; Short and Suttle 2002).

Cyanophages fall into any of the major bacteriophage families based on their structure, viz. *Myoviridae*, *Podoviridae* and *Siphoviridae*. Cyanomyoviruses constitute a large majority of aquatic cyanophage communities (Marston and Sallee 2003; Suttle and Chan 1993). The *g20* gene, encoding the capsid assembly protein of cyanomyoviruses, has been developed as a biomarker for this family. Characterization of this gene from viral concentrates has unearthed a large diversity of *g20* sequences from marine (Short and Suttle 2005; Wang and Chen 2004), estuarine (Zhong et al. 2002), freshwater (Dorigo et al. 2004; Wilhelm et al. 2006), wetland (Sun et al. 2015; Yeo and Gin 2015) and paddy field floodwater (Jing et al. 2014; Wang et al. 2010) environments.

Cyanophages from the myovirus as well as podovirus families carry a number of photosynthetic genes, acquired from host cyanobacteria during evolution (Lindell et al. 2004; Mann et al. 2003). Homologues of the *psbA* gene, which codes for the D1 protein of cyanobacterial photosystem II, have been found in a wide variety of cyanophage genomes – both cultured and environmental samples, sourced from marine, freshwater and terrestrial aquatic systems (Alperovitch-lavy et al. 2011; Gao et al. 2016).

Materials

Chemicals: SYBR Green, Tris-EDTA buffer, Tris chloride, PEG 8000, sodium chloride, ferric chloride, ascorbate-EDTA buffer, SM buffer, formaldehyde, formamide, EDTA, ethanol, phenol, chloroform, isoamyl alcohol, proteinase K, CTAB. All chemicals were obtained from HiMedia Laboratories and were of analytical grade.

Kit: Viral Nucleic Acid Isolation Kit (APS Labs)

Methods

5.1 Flow Cytometric Enumeration of Viruses

5.1.1 *Standardization of the flow cytometric enumeration protocol*:

Water samples from five sites representing different ecosystems were collected. A standard protocol (Brussaard et al. 2010) was used for flow cytometric enumeration of virus (virioplankton) particles; however, parameters were optimized for the instrument and conditions. Briefly, 1 ml of 0.22 µm-filtered water sample (*per se* or diluted 1:1 with Tris-EDTA buffer) was stained with SYBR Green at a final concentration of 10^{-4} of the commercial stock. Tris-EDTA buffer was used as a negative control. An aliquot of purified Φ MC-1 – a bacteriophage isolated in our laboratory (Poduval et al. 2018) – was used as a positive control.

Staining was carried out for 10 minutes in the dark at 80°C. After a cooling period of 5 minutes, the samples were 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. Flow cytometry acquisition was set on the FL1 channel (threshold value-500), and analysis was carried out for 30 seconds (Flow rate: 60 µL min^{-1}). A combination of FSC (forward scatter) and SSC (side scatter) was used to distinguish virus particles from bacteria, if present in the sample. The data was analysed with the BD CellQuest™ Pro software.

The established methodology for bacterial and picoplankton enumeration was adapted for viral particle size. The following formula was used to calculate the number of virus particles in a sample:

$$\text{Number of virus particles (per ml)} = \frac{\text{Total events}}{\text{Time x flow rate calibration factor}} \times 1000$$

5.1.2 *Enumeration of Virioplankton from Lakes, Estuaries and Rice Field Floodwaters*:

Several types of aquatic ecosystems were selected for estimating the overall virioplankton populations present. These included estuarine systems, shallow lakes / reservoirs (hereafter referred to as 'lakes') and rice field floodwaters. Estuaries represent a completely natural system, lakes, a natural system under some human control – for example, drainage of water during the monsoon season through floodgates – and rice fields, a predominantly artificial ecosystem. Surface water samples from five sites for each type of ecosystem (**Table 5.1**) were collected during the months of August-September (monsoon season). Samples were subjected to flow cytometric analysis as described above.

Table 5.1: Sampling sites for flow cytometric enumeration of virioplankton

Type of ecosystem	**Location**	**Geographical coordinates**
	Nuvem	15°18'47.56"N, 73°57'7.45"E
	Utorda	15°19'4.75"N, 73°53'59.81"E
Rice fields	Verna	15°20'46.43"N, 73°56'2.28"E
	Cortalim	15°23'51.85"N, 73°55'3.09"E
	Agassaim	15°25'40.96"N, 73°54'13.14"E
	Verna	15°20'26.42"N, 73°56'21.42"E
	Sarzora	15°13'14.90"N, 74° 0'14.34"E
Lakes	Ralloi Tollem	15°16'14.67"N, 74° 1'7.06"E
	Curtorim	15°17'16.17"N, 74° 1'9.71"E
	Muxiwado	15°16'42.47"N, 74° 0'38.60"E
	Chorao	15°31'30.27"N, 73°52'29.55"E
	Dona Paula	15°27'10.68"N, 73°48'8.28"E
Estuaries	Divar	15°30'59.44"N, 73°54'31.36"E
	Santana	15°28'21.42"N, 73°53'30.80"E
	Betul	15° 8'37.43"N, 73°56'53.49"E

5.2 Detection of Virus Families in Metagenomic Samples from Various Niches

5.2.1 *Water Sample Collection*:

2 to 4-litre water samples were collected from sites mentioned in **Table 5.2**. Water samples were transported to the laboratory within one hour of collection, stored at 4°C and processed within 24 hours. Samples were centrifuged at 22,000 x g and the supernatant filtered through a 0.45 µm polyvinylidene fluoride membrane.

Table 5.2: Sample collection sites for preparation of metagenomic DNA

Ecosystem	Location	Geographical coordinates
Estuarine creek	Santana Manos	15°28'04.2"N 73°53'19.4"E
Rice field	Cortalim	15°23'25.6"N 73°54'53.0"E
Bay	Dona Paula	15°27'04.1"N 73°48'12.1"E
Lake	Verna	15°20'51.68'N, 73°56'43.72'E
River	Assolna	15°11'06.7"N 73°58'17.0"E

5.2.2 *Concentration of Virus Particles*:

Virus particles were concentrated by combinations of the following methods:

i. Precipitation with polyethylene glycol (Cai et al. 2016; Colombet et al. 2007): A final concentration of 0.5 M sodium chloride and 10% (w/v) PEG 8000 were added to 1 litre of filtered water sample, dissolved slowly and kept at 4°C overnight. Precipitated viral particles were collected by centrifugation at 13,000 x g for 30 minutes; the pellet was resuspended in 15 ml of SM buffer.

ii. Flocculation with ferric chloride (John et al. 2011): 400 µl of ferric chloride solution (0.48 g $FeCl_3$ dissolved in 10 ml ultrapure water) was added to 1 litre of filtered water sample, mixed thoroughly and kept undisturbed at room temperature for one hour. The flocculated particles were collected on a 0.8 µm polycarbonate filter. Filters were resuspended overnight in an ascorbate-EDTA buffer, to recover viruses.

iii. Concentration on 0.02 µm Whatman Anodisc filters (Wang et al. 2010): Water samples were filtered onto a 0.02 µm Whatman Anodisc filter (Sigma-Aldrich Co.). Viral particles retained on the filter were eluted by flushing with sterile 3% beef extract.

iv. Direct pelleting: Virus particles in water samples were concentrated by three rounds of ultracentrifugation at 1,60,000 x g for 3 hours.

5.2.3 *Isolation of Viral DNA*:

A modification of the standard protocol for isolation of viral metagenomic DNA was used (Thurber et al. 2009). The eluate was passed through a 0.2 µm polyethersulfone syringe filter and treated with 3-5 U of pancreatic DNase I for 2 hours. Virions were extracted by adding to each 1 ml of sample, the following: 100 µl of 2M Tris-HCl (pH 8.5)/0.2 M EDTA, 10 µl of 0.5 M EDTA (pH 8.0), 1 ml of formamide (molecular biology grade) and 4 µl of glycogen (20 mg/ml). After incubation at room temperature for 30 min, the DNA was precipitated by adding 2 volumes of absolute ethanol and centrifuging for 20 min at 8000 x g at 4°C. The pellet was washed with 70% ethanol and resuspended in a minimum volume of 10 mM Tris chloride. DNA was further extracted by one of two methods: by means of a virus DNA isolation kit or by the standard phenol-chloroform method.

i) Kit method: Viral DNA was extracted using The APS Labs Viral Nucleic Acid Isolation Kit, according to the manufacturer's protocol. Briefly, 150 µl of sample was mixed with 570 µl VNE buffer, vortexed and kept at room temperature for 10 min. After addition of 570 µl absolute ethanol, the sample was passed through the provided column by centrifuging at 8000 g for 1 min, and the flow-through was discarded. The column was washed once with 500 µl Wash Buffer 1, followed by two washes with 750 µl Wash Buffer 2. Finally, the column was placed in a fresh elution tube and the DNA eluted with 20 µl of preheated RNase-free water.

ii) Phenol-chloroform isolation: To 567 µl of extract obtained in the previous step, 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase K was added, and incubated at 37°C for 1 hour. 100 µl of 5M NaCl and 80 µl of CTAB/NaCl solution (10% CTAB, 0.7M NaCl) was added and incubated at 65°C for 10 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added, and the tube centrifuged at 11,000 x g for 5 minutes. The aqueous layer was transferred to a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) added. After centrifugation and recovery of the aqueous layer, DNA was precipitated by the addition of 0.6 volumes of isopropanol, incubation at room temperature for 20 minutes, followed by centrifugation at 11,000 x g for 20 minutes. The DNA pellet was resuspended in 30 µl of 10 mM Tris chloride and stored at –20°C until further use.

5.2.4 Detection of virus families by targeted PCR amplification of marker genes:

Viral DNA, extracted as above, was used as a template for PCR amplification of target genes, representing three virus families – *Myoviridae*, *Podoviridae* and *Phycodnaviridae*.

For *Phycodnaviridae*, the target gene was the *polB* encoding DNA polymerase B. For *Myoviridae*, the *g20* gene, encoding the capsid assembly protein, and for *Myoviridae* / *Podoviridae*, the *psbA* gene encoding the photosynthetic D1 protein were used.

Reaction mixture components and parameters for the respective genes were as follows:

	g20	***polB***	***psbA***
Component	Volume in µl		
10X PCR assay buffer containing $MgCl_2$	5.0	5.0	5.0
dNTP mix (2.5 mM each)	5.0	5.0	5.0
Forward primer (10 µM)	0.5	1.0	2.0
Reverse primer (10 µM)	0.5	0.8	2.0
Viral DNA (template)	4.0	5.0	4.0
Taq polymerase	0.7	0.7	0.5
Nuclease free water	34.3	32.5	31.5

PCR parameters:	***g20***		***polB***		***psbA***	
(30 cycles of the following)	Temperature	Time	Temperature	Time	Temperature	Time
Denaturation	94°C	45 s	94°C	30 s	92°C	1 min
Annealing	40°C	45 s	48°C	30 s	50°C	1 min
Extension	72°C	1 min	72°C	30 s	68°C	1 min

Primers used:
g20: CPS1 and CPS 8 (Wang et al. 2010)
polB: AVS 1 and AVS 2 (Chen and Suttle 1995)
psbA: psbA-F and psbA R (Zeidner et al. 2003)

5.3 Enumeration of Viruses in Natural Samples by Epifluorescence Microscopy

Virus particles present in freshly collected samples were counted by the established SYBR-Green staining method (Patel et al., 2007), described in Chapter 4 (p. 65). Briefly, formaldehyde-fixed and pre-filtered samples were filtered onto a 0.02 µm Anodisc membrane and stained with SYBR Green (final concentration 10^{-4}). The filters were mounted on glass slides and viewed at 1000 X magnification, under the blue excitation filter of an epifluorescence microscope. Virus particles in 10 different fields were counted to arrive at an estimate of total virus particles present in the sample.

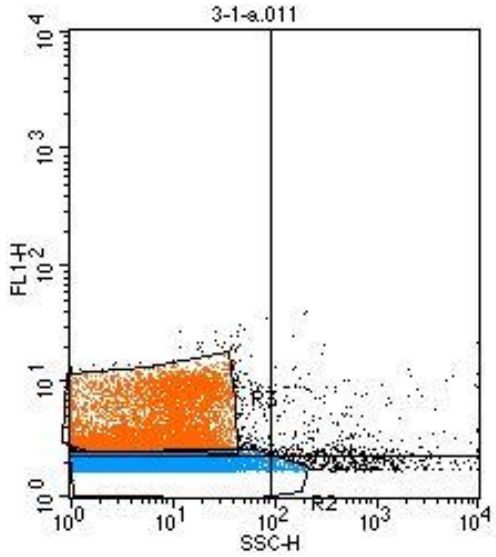


Figure 5.1: Typical flow cytometry dot plot for virus enumeration from water samples

R3: Fluorescence signal from virus population

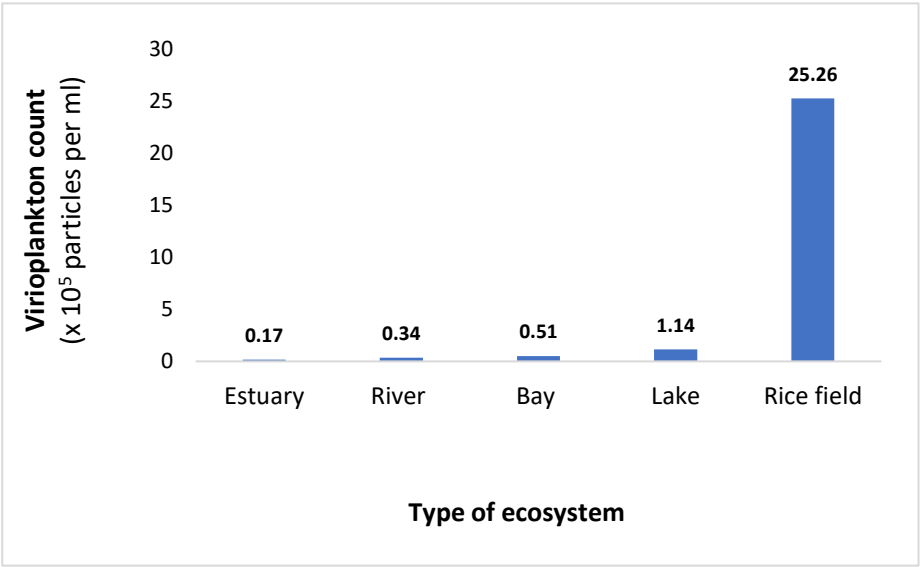


Figure 5.2: **Preliminary enumeration of virioplankton from selected aquatic sites**

A) Virioplankton count

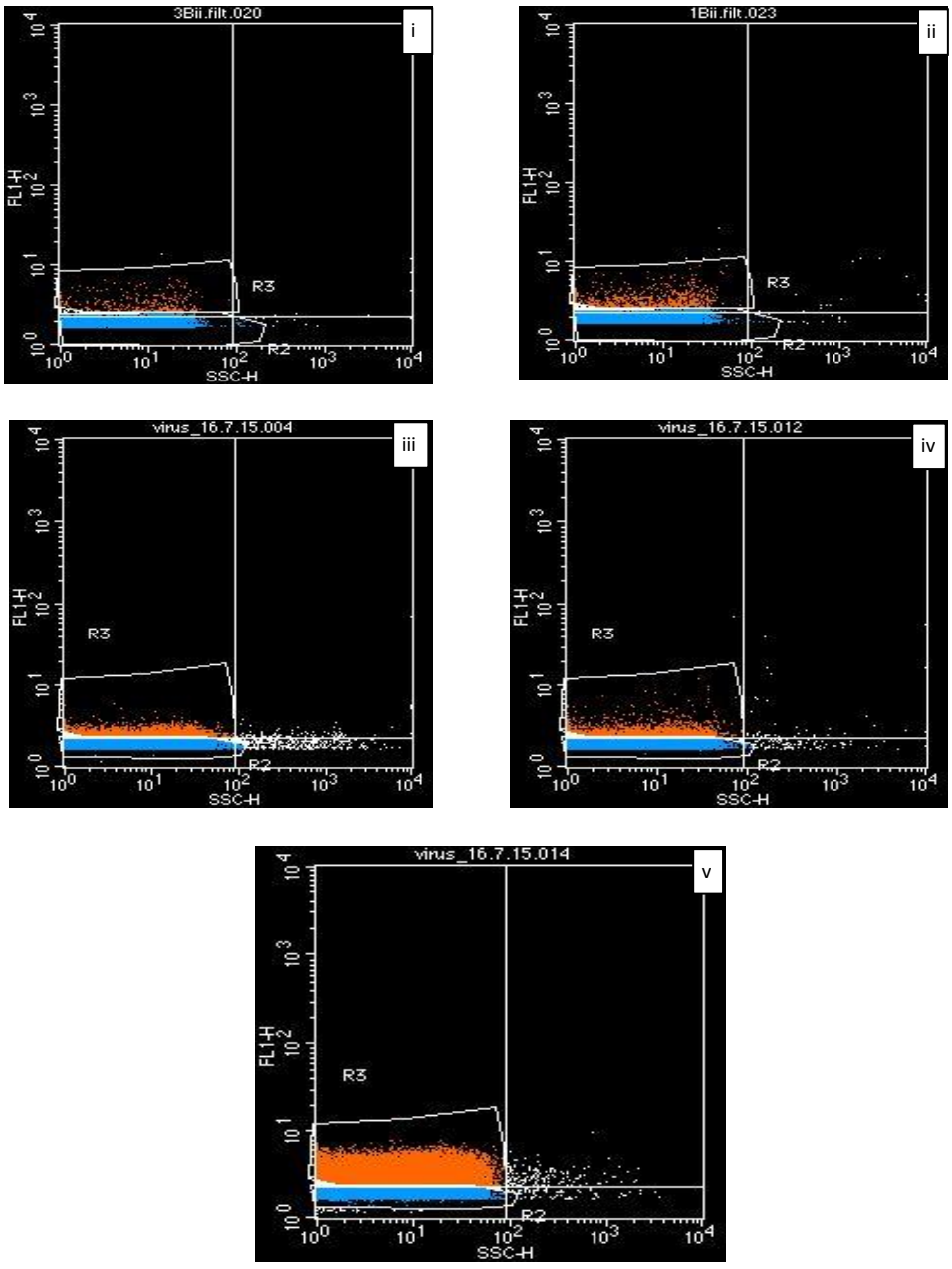


Figure 5.2 cont.: **Preliminary enumeration of virioplankton from selected aquatic sites**

B) Flow cytometric dot plot: i. Estuary; ii. River iii. Bay iv: Lake v: Rice Field

Results and Discussion

5.4 Flow Cytometric Enumeration of Virioplankton from Selected Aquatic Ecosystems

A typical plot obtained in the flow cytometric measurement is depicted in **Figure 5.1**. This is a plot of fluorescence (proportional to the DNA content of the particle) against side scatter (proportional to particle size). The region designated R3 represents fluorescence and particle size values typical of the viral population, as per the existing literature (Brussaard et al. 2000; Mitbavkar et al. 2011). The signal obtained in this region is quantified to arrive at a count of virioplankton particles in the sample.

Preliminary measurements carried out from five representative samples during protocol standardization, yielded the following result (**Table 5.3**; **Figure 5.2**)

Table 5.3: Virioplankton count in water samples representing various ecosystems

Type of ecosystem	**Virioplankton count** (x 10^5 particles per ml)
Estuary	0.170
River	0.340
Bay	0.51
Lake	1.14
Rice field	25.26

Evidently, closed freshwater ecosystems, i.e. rice field and lake, showed higher overall viral populations than riverine and estuarine systems. To further explore and statistically confirm these results, the next study focused on three types of ecosystems, with multiple sites of collection for each.

5.5 Flow Cytometric Enumeration of Virioplankton from Lakes, Estuaries and Rice Field Floodwaters

Figure 5.3 illustrates representative sampling sites from each category of ecosystem. Virioplankton counts obtained from each site by flow cytometry are depicted in **Figure 5.4** (A-C). The mean values for each type of ecosystem are depicted in **Figure 5.4** D. The highest mean virioplankton count (average particles per ml x 10^6) was obtained from rice field floodwaters (12.1), followed by lake (3.9) and estuarine (2.1) samples.

Figure 5.3: Representative sampling sites from

A) Rice field (Verna); B) Lake (Sarzora); C) Estuary (Betul)

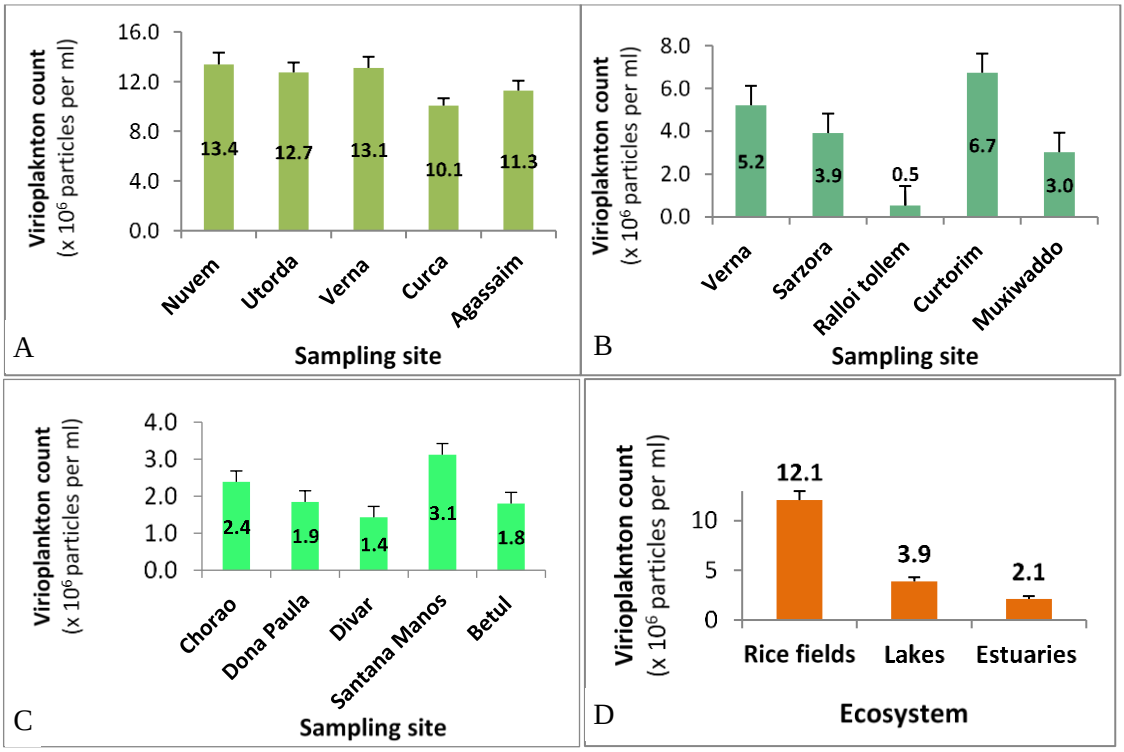


Figure 5.4: Virioplankton counts from rice fields, lakes and estuarine ecosystems

A) Rice fields B) Lakes C) Estuaries D) Mean values from each type of ecosystem

Table 5.4: Comparison of virioplankton counts in studies on various aquatic systems

	Virioplankton counts (average particles per ml)	**Reference**
Rice Fields	12.1 x 10^6	**This study**
	2.2 x 10^6 to 8.0 x 10^7	Nakayama et al. 2007
	5.6 x 10^6 to 1.2 x 10^9	Kimura et al. 2010
Lakes	3.9 x 10^6	**This study**
	4.38 x 10^7 (reservoirs)	Steenhauer 2013
	5.19 x 10^7 (lakes)	
Estuaries	2.1 x 10^6	**This study**
	1.4 x 10^7	Auguet et al. 2005
	2.0 x 10^7	Peduzzi and Schiemer 2004

Table 5.4 compares virioplankton counts obtained in the present study with those obtained in other published studies on the same ecosystem types.

Among the few studies which have previously enumerated virioplankton or virus-like-particles (VLPs) in rice field floodwater, Nakayama and co-workers (Nakayama et al. 2007c) reported an abundance of between 2.2 x 10^6 and 8.0 x 10^7 per ml for these particles. This was the first evidence of virus counts in the rice field ecosystem exceeding typical counts in other freshwater and marine systems. Kimura and co-workers (Kimura et al. 2010) found that VLP abundance fluctuated between 5.6 x 10^6 and 1.2 x 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 (Maranger and Bird 1995; Peduzzi and Schiemer 2004; Wommack and Colwell 2000). Moreover, the temporal and seasonal rise and fall in viral populations in a given ecosystem is closely tied to the variation in host populations, particularly bacterioplankton and algal hosts (Filippini et al. 2006; Jackson and Jackson 2008; Wommack and Colwell 2000). The rice field is a closed, anthropogenically influenced aquatic ecosystem, constituted for the specific purpose of cultivating the rice crop. The submerged conditions favour the accumulation of organic matter (Sahrawat 2003). 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 between this soil layer and the overlying shallow floodwater (Roger 1996; Watanabe and Furusaka 1980) ensure high bacterial and algal populations in the water layer, supporting high viral populations. Another study found higher virus populations in agricultural aquatic systems versus natural reservoirs, possibly attributed to some of the reasons elaborated above (Yang 2019).

Rice fields have been scientifically defined as temporary, seasonal, wetland ecosystems (Bambaradeniya and Amarasinghe 2003). The dynamics of virus-host infections in wetland ecosystems have not been extensively studied (Bonetti et al. 2019; Jackson and Jackson 2008); however authors of a recent collation on freshwater virus studies hypothesize that in periodically flooded wetlands, the transition from dry to wet state triggers a wave of viral infections. This is the situation with rainwater-irrigated rice fields in our study, which are dry during the summer months but suddenly flooded with the onset of the monsoon. These

environmental factors could be responsible for release of large numbers of lytic viruses, as breaking up of microbial aggregates leads to increased virus-host encounters (Bonetti et al. 2019). This hypothesis is supported by a study wherein large numbers of cyanophages (evidenced by plaque assays) were isolated shortly after transplantation of seedlings in rice fields (Singh 1973).

In contrast to rice fields, lakes, though also 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. Moreover, several studies of viral populations in lake ecosystems have found higher viral content in the sediment layer, compared to the water column (Bettarel et al. 2006; Filippini et al. 2008). One possible explanation is that sediments provide viral particles protection against decay due to UV radiation (Danovaro et al. 2008). Conversely, in lakes / wetlands with varying water levels, periods of low water, combined with high ambient temperatures have been shown to be accompanied by higher concentrations of viruses (Farnell-Jackson and Ward 2003; Peduzzi and Schiemer 2004).

Steenhauer's review (Steenhauer 2013) 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 the present study may 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 (Cochlan et al. 1993; Paul et al. 1997; Weinbauer et al. 1995)

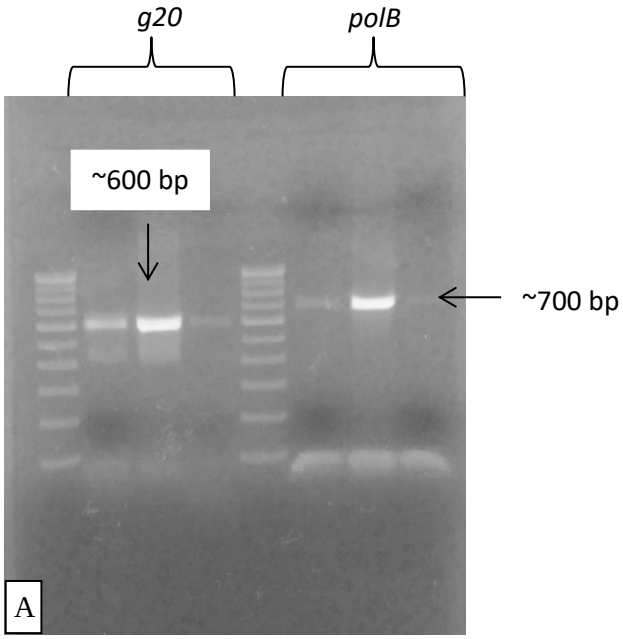
5.6 Detection of Virus Families in Aquatic Niches by PCR

Metagenomic DNA obtained from several of the sampling sites depicted amplification of viral family marker genes (**Figure 5.5**).

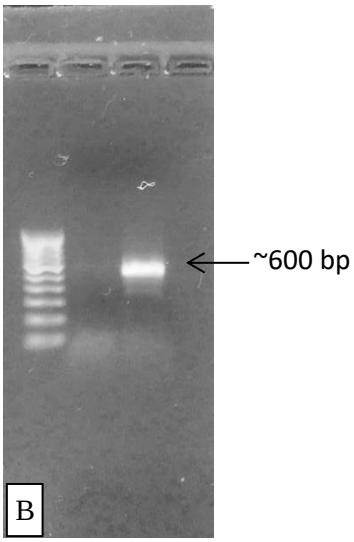
Viruses, unlike bacteria, lack a universal marker gene (Rohwer 2002; Adriaenssens 2014); hence molecular detection and quantification of viruses has utilized family-specific markers. These genes broadly fall into three categories, depending on their function – structural genes, auxiliary metabolism genes and DNA polymerase genes.

The ***g20*** gene, indicating the presence of *Myoviridae*-family viruses, was amplified from the Santana Creek and Verna Lake samples (**Figure 5.5** A, B; fragment size ~ 600 bp). *g20* is a structural gene, encoding the capsid assembly protein of myoviruses. Its original application was in the detection of viruses infecting marine *Synechococcus* (Fuller et al. 1998). The gene has subsequently been widely used to detect cyanomyoviruses in varied aquatic environments (Adriaenssens and Cowan 2014; Jing et al. 2014). *g20* is an ideal marker, being a slow-evolving gene, whose protein product functions in the highly complex and precise process of capsid assembly (Sullivan et al. 2008). The ubiquity of this gene has enabled its use as a proxy for cyanophage abundance in various environments, such as seawater (Jameson et al. 2011; Short and Suttle 2005; Wang and Chen 2004), lakes (Dorigo et al. 2004; Matteson et al. 2011; Zhong and Jacquet 2013), and even paddy fields (Jing et al. 2014; Wang et al. 2010, 2011), despite differences in freshwater and marine viral communities. While certain primers targeting this gene, amplified *g20* sequences from *Myoviridae* bacteriophages as well (Short and Suttle 2005), the CPS1/CPS8 primer – used in the present study – has previously demonstrated specificity towards the *Myoviridae* cyanophages, to the exclusion of bacteriophages as well as podo- and sipho- viruses (Wang et al. 2010; Zhong et al. 2002).

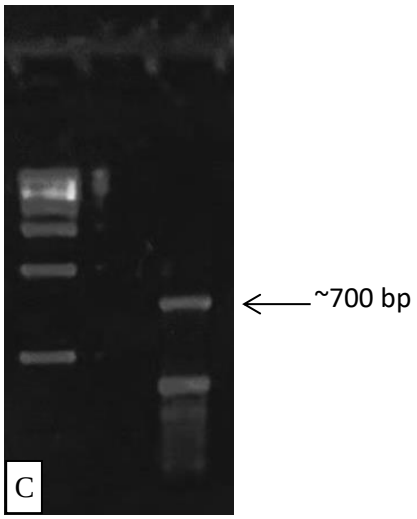
The ***polB*** gene, representing *Phycodnaviridae*, was amplified from Santana Creek and Dona Paula Bay samples (**Figure 5.5** A, C; fragment size ~700 bp). *polB*, coding for DNA polymerase B, was the first marker to be used for detection of algal viruses in environmental samples (Chen and Suttle 1995). Algal virus-specific (AVS) primers amplified a portion of this gene specifically from members of the *Phycodnaviridae*. This is a family of viruses infecting eukaryotic microalgae belonging to the Chlorophyta, Dinophyta and other groups, and structurally consisting of non-tailed, polyhedral forms with large (upto 560 kb) dsDNA genomes (Van Etten et al. 2002; Wilson et al. 2009).



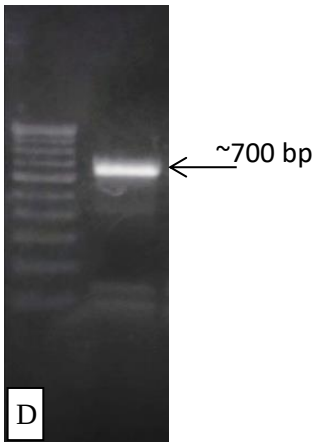
Lane 1, 5: 100 bp DNA ladder
Lane 2,3,4: Amplification of *g20* gene from SC sample, different sample preparations
Lane 6,7,8: Amplification of *polB* gene from SC sample, different sample preparations



Lane 1: 100 bp DNA ladder
Lane 3: Amplification of *g20* gene from VL sample



Lane 1: 500 bp DNA ladder
Lane 3: Amplification of *polB* gene from DP sample



Lane 1: 100 bp DNA ladder
Lane 3: Amplification of *psbA* gene from VL sample

Figure 5.5: **PCR-amplification of virus family-specific genes from metagenomic samples**

A) Santana Creek (SC): *g20* and *polB* B) Verna Lake (VL): *g20*

C) Dona Paula (DP): *polB* D) Verna Lake: *psbA*

The cited study (Chen and Suttle 1995) reported amplification of the *polB* gene from natural virus communities as well as cultures of specific microalgal viruses. The *polB* marker has provided useful information on algal virus populations in most of the major aquatic systems such as marine (Short and Suttle 2002), riverine (Short and Short 2008), estuarine (Labbé et al. 2018) and freshwater lakes (Clasen et al. 2008; Zhong and Jacquet 2013) and ponds (Long and Short 2016).

Verna Lake demonstrated the amplification of the ***psbA*** gene (**Figure 5.5** D; fragment size ~700 bp). Numerous cyanophages belonging to the *Myoviridae* and *Podoviridae* families carry photosynthetic genes *psbA* and *psbD*, which code for proteins D1 and D2 respectively, of photosystem II (Mann et al. 2003; Millard et al. 2004; Sullivan et al. 2006). Post-cyanophage infection, the photosynthetic machinery of the cyanobacterial host shuts down; Phage-expressed photosynthetic proteins maintain continued photosynthesis, thus aiding their own propagation (Lindell et al. 2004; Mann et al. 2003; Puxty et al. 2016). Approximately 90% of all cyanophage isolates carry the *psbA* gene (Puxty et al. 2015). Previous studies have demonstrated the presence of *psbA* in varied aquatic environments – marine (Chénard and Suttle 2008; Sandaa, Clokie, and Mann 2008); lake freshwaters (Chénard and Suttle 2008; Zhong and Jacquet 2013); paddy field floodwater (Wang et al. 2009; Wang, Jing, et al. 2016) as well as in cultured cyanophages (Puxty et al. 2015; Sullivan et al. 2006).

Changes in virus abundance and community composition are known to occur on a seasonal and even on a daily basis (Dorigo et al. 2004; Jasna et al. 2019; Long et al. 2018; Weinbauer et al. 1995). This could explain the absence of viral marker genes from certain sites at the time of sampling.

5.7 Enumeration of Viruses by Epifluorescence Microscopy

Virus particles in samples collected from Santana Creek and Verna Lake sites were enumerated by epifluorescence microscopy (**Figure 5.6**). The count obtained for Santana Creek was 2.4 x 10^6 particles per ml, which was consistent with the flow cytometric viral count from the site, i.e. 3.1 x 10^6 particles per ml. The count obtained for Verna Lake through epifluorescence microscopy was 3.8 x 10^6 particles per ml, which corresponded to the flow cytometric count of 5.2 x 10^6 particles per ml.

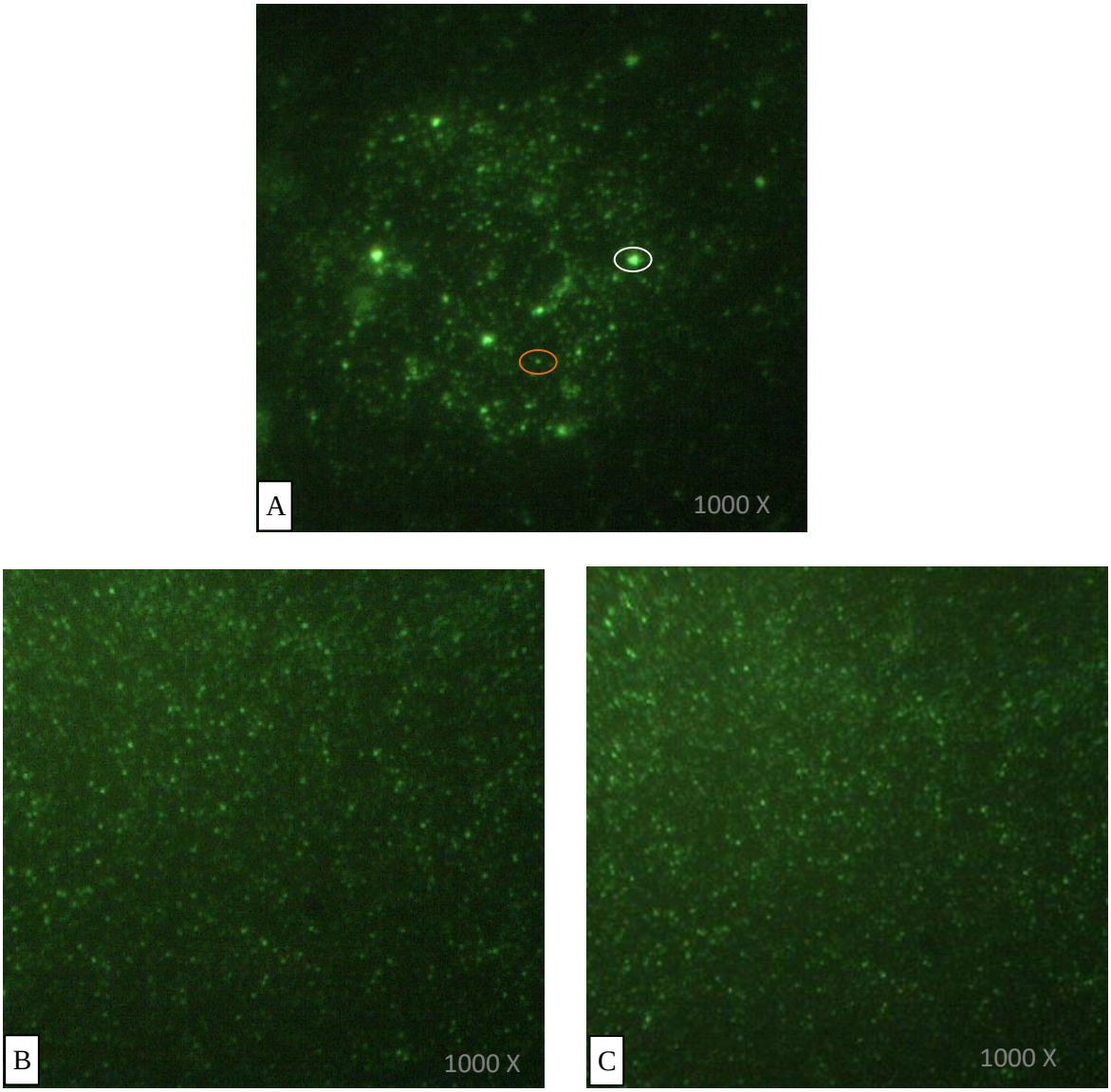


Figure 5.6: Virus enumeration by epifluorescence microscopy

A) Viruses and bacteria in an unfiltered water sample, viewed under epifluorescence; white oval – bacterial cell; orange oval – virus particle

B) Santana Creek sample C) Verna Lake sample

The direct visualization of viruses by epifluorescence microscopy was an important innovation in aquatic virology (Hara et al. 1991; Noble and Fuhrman 1998). The technique is independent of sample type, equally applicable to natural samples from varying aquatic chemistries and to culture samples (Suttle and Fuhrman 2010; Sulcius et al. 2011). Significantly, many of the features of aquatic viral populations which are known today, were discovered through direct counts of viruses obtained by this method (Wommack et al. 2015). In the present study, viral counts obtained by epifluorescence microscopy corresponded to those obtained by flow cytometry. Previous studies have reported a similar correlation (Chen et al. 2001; Marie et al. 1999).

The obtained estimates of viral abundance as well as the detection of specific families through their marker genes, provided the basis for a metagenomic study of two aquatic viral communities, described in Chapter 6.

CHAPTER SIX

CHARACTERIZATION OF TWO AQUATIC VIROMES

The present chapter describes a metagenomic analysis of virus communities (viromes) from two selected aquatic systems. Although several representative sites were sampled for this purpose, metagenomic DNA was successfully obtained from *Verna Lake* and *Santana Manos Creek*. These two sites provided a case study for representative freshwater and estuarine systems respectively. Verna Lake is a purely freshwater system, with a salinity of 0 p.s.u. throughout the year. The salinity level at Santana Manos Creek (hereafter referred to as Santana Creek) changes according to the season, with a value of 0 p.s.u. during the monsoon, consequent to high levels of precipitation, and upto 20 p.s.u during other seasons. As described in previous chapters, high virus counts have been obtained from both these sites through flow cytometry (p. 96) and confirmed by epifluorescence microscopy (p. 101). Further, we have isolated cyanophages from both these sites (p. 71). The uniqueness from ecosystem point of view of each of these (described in section 6.7) further supports their selection for a comparison of respective viromes.

Over the past two decades, metagenomics has been the method of choice for studying total microbial communities in a culture-independent manner (Bruder et al. 2016). In the case of virus metagenomics, a whole-genome sequencing or shotgun approach is generally followed (Putonti et al. 2018; Rastrojo and Alcamí 2017). Metagenomics has been used to study virus communities in a wide variety of aquatic systems, including marine (Duhaime and Sullivan 2012), freshwater lakes (J. C. Green et al. 2015; Skvortsov et al. 2016), ponds (Chopyk et al. 2018), desert ponds (Taboada et al. 2018), arctic ponds (de Cárcer et al. 2016) and groundwater reservoirs (Costeira et al. 2019). Understanding the virus communities that exist in freshwater systems is of great importance as these systems have a direct impact on human health, being in close proximity to human habitation. Further, from the point of view of discovery of novel viruses, freshwater and estuarine systems present a huge variety of unexplored yet accessible aquatic niches.

Methods:

6.1 Water Sample Collection

Water samples were collected from five sites (**Table 6.1**) which had demonstrated a high presence of viruses in the previously described flow cytometric measurements (p. 96). Surface water samples (between a depth of 0 to 5 m) were collected using a sterile vessel, stored at 4°C and processed within 24 hours for DNA extraction. Physicochemical parameters of the water samples were measured.

Table 6.1: Sites selected for viral metagenomic studies

Ecosystem	Location	Geographical coordinates
Estuarine creek	Santana Manos	15°28'04.2"N 73°53'19.4"E
Rice field	Cortalim	15°23'25.6"N 73°54'53.0"E
Bay	Dona Paula	15°27'04.1"N 73°48'12.1"E
Lake	Verna	15°20'51.68'N, 73°56'43.72'E
River	Assolna	15°11'06.7"N 73°58'17.0"E

6.2 Isolation of Metagenomic DNA

Metagenomic DNA was extracted from water samples following the protocol described in Chapter 5 (p. 90). Briefly, samples were pre-filtered to remove bacterial and other microbial cells and virus particles were concentrated by a combination of physical and chemical methods. DNA was extracted by an established protocol (Thurber et al. 2009).

6.3 Library Preparation and Sequencing

The concentration of DNA obtained was measured using a Qubit spectrofluorometer.

A whole genome sequencing library was prepared with the NEXTflex Rapid DNA sequencing bundle (BIOO Scientific, Inc. U.S.A.) according to the manufacturer's protocol. Briefly, DNA was sheared to generate specific fragments in the size range of 200-300 bp.

Purified fragments were end-repaired, adenylated and ligated to Illumina multiplex barcode adaptors. Adapter-ligated DNA was amplified for 4 cycles of PCR using Illumina-compatible primers. Final PCR products (sequencing libraries) were cleaned up and quantified by a Qubit fluorometer. The fragment size distributions were analyzed on Agilent 2200 TapeStation.

Samples were sequenced on an Illumina HiSeq X Ten sequencer (Illumina, San Diego, USA) using 150 bp paired-end chemistry, following the manufacturer's protocol. After completion of the sequencing run, the data was de-multiplexed using bcl2fastq software ver. 2.20 and FastQ files were generated based on the unique dual barcode sequences. The sequencing quality was assessed using Fast QC ver. 0.11.8. Adapter sequences were trimmed and low-quality bases filtered out during read pre-processing, while reads above Q30 were retained for downstream analysis.

6.4 Virome Quality Trimming and Assembly

Paired ended reads from each virome were quality-checked using FastQC ver. 0.11.8 (Andrews 2010) and trimmed using Trimmomatic ver. 0.39 (Bolger, Lohse, and Usadel 2014) using the following parameters: Leading 3, sliding window 4:20, minimum length 50. Reads were then assembled *de novo* using metaSPAdes (ver. 3.14.0) as well as MEGAHIT (ver. 1.2.9), with the intention of selecting the better assembly of the two.

Commands and parameters used for each software are detailed below:

Trimmomatic
Version 0.39
Parameters: Leading 3, sliding window 4:20, minimum length 50
Command:

```
java -jar trimmomatic-0.39.jar PE -phred33 SM_F.fastq SM_R.fastq
output_F_paired.fastq.gz output_F_unpaired.fastq.gz
output_R_paired.fastq.gz output_R_unpaired.fastq.gz ILLUMINACLIP:TruSeq3-
PE.fa:2:30:10 LEADING:3 SLIDINGWINDOW:4:20 MINLEN:50
```

MetaSPAdes
Version 3.14.0
Parameters: default
Command:

```
metaspades.py --pe1-1 ../R1_pe.fq.gz --pe1-2 ../R2_pe.fq.gz --pe1-s
../R1_unpe.fq.gz --pe1-s ../R2_unpe.fq.gz  -t 16 -o ../metaspades/
```

MEGAHIT
Version 1.2.9
Parameters: k-mers used for assembly 21,29,39,59,79,99,119,141
Command:

```
megahit -1 SM_F_TRIM.fastq  -2 SM_R_TRIM.fastq -r SM_F_UNPE.fastq,
SM_R_UNPE.fastq -m 0.5  -t 4  -o megahit_result
```

6.5 Taxonomic Annotation of Viromes

The respective contig files from each virome were analysed for taxonomic composition, by comparison with three databases, namely, NCBI RefSeq, Kraken Viruses and Minikraken. The alignment against sequences in NCBI RefSeq was carried out using Kaiju ver. 1.7.3 (Menzel, Ng, and Krogh 2016) with the following parameters:
Minimum match length 11; Minimum match score 75; Allowed mismatches 5.

The alignment against Kraken Viruses and Minikraken was carried out using Kraken ver. 1.1.1 (Wood and Salzberg 2014) with default parameters.

The Shannon-Weiner diversity index was calculated to measure species diversity in each virome.

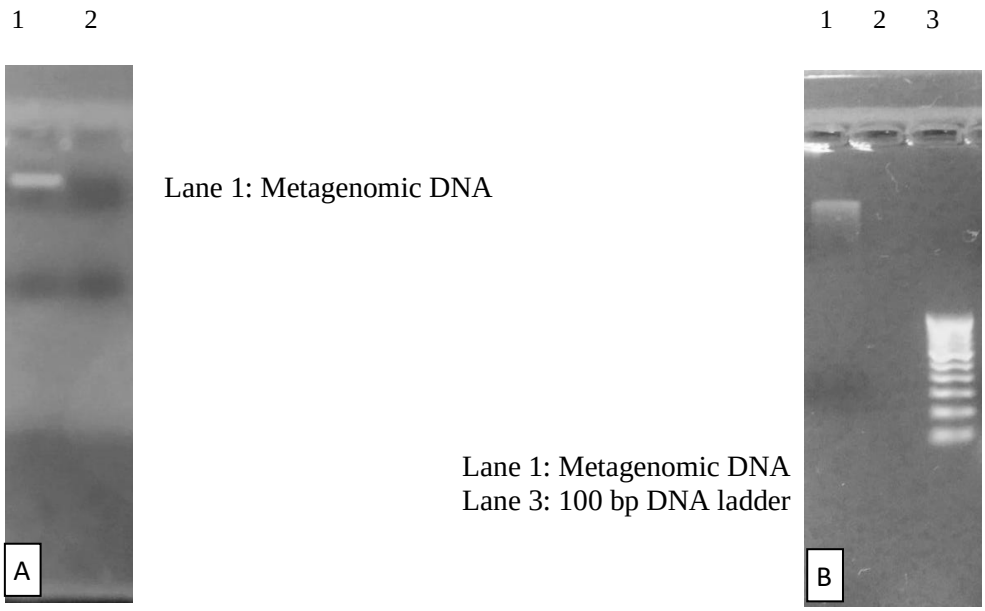
6.6 Functional Annotation of Viromes

The respective contig files were run through VirSorter ver. 1.0.3 (Roux et al. 2015) which selects for viral sequences based on viral gene content or characteristic viral genomic features. The output files enriched in viral sequences were functionally annotated through the MG-RAST server ver. 4.0.3 (Keegan, Glass, and Meyer 2016).

Figure 6.1: Sampling sites

A) Santana Creek

B) Verna Lake



Lane 1: Metagenomic DNA

Lane 1: Metagenomic DNA
Lane 3: 100 bp DNA ladder

Figure 6.2: Metagenomic DNA isolated from A) Santana Creek and B) Verna Lake

Results and Discussion

6.7 Description of the Sampling Sites

Metagenomic DNA was successfully isolated from two of the five selected sites, i.e. Santana Creek and Verna Lake. The sampling sites are depicted in **Figure 6.1** and the profile of isolated DNA in **Figure 6.2**.

Santana Creek and Verna Lake are located in the Northern and Southern district respectively, of Goa, India. Both these sites may be categorized as lentic ecosystems of Goa (Gokhale, Reddy, and Gad 2015).

Santana Creek is connected to the estuarine system of the River Zuari on the south-western side, and to agricultural fields towards the northern side. It belongs to a type of ecosystem unique to Goa – known as the *khazan* lands. This is a form of coastal zone management practised for centuries, with a view to maintaining the agricultural productivity of the land. *Khazan* lands are basically reclaimed lands from estuary or sea, where the tidal water flow is regulated by sluice gates (N. Kamat 2004; Sonak 2014).

Verna Lake, locally known as Ambulor Tollem, is the origin of the river Sal, one of the seven major rivers of Goa. This river flows along the South Goa Coast, before meeting the Arabian Sea at Betul (Kerkar, Pradhan, and Dandekar 2016). Known as a site favoured by migratory birds, more than 150 species of birds have been reported here (Baidya 2018).

Thus, both the selected sites play an important role in the ecology of Goa. Similarities between the two sites include their utilization in agriculture and their regulation by sluice gates. Both these water bodies are agriculturally utilized reservoirs, connected to the rivers Zuari and Sal respectively, which ultimately join the Arabian Sea.

The climate of Goa is strongly influenced by the annual monsoon which brings heavy rainfall over the months June-September. The monsoon and accompanying conditions are the single largest defining factor in the climate and aquatic systems within Goa and the surrounding geographical region, which receive a substantial rainfall during this period. The months October-January are therefore referred to as post-monsoon period, and February-May as pre-monsoon. As regards the two sampling sites, the structure of these water bodies is altered by the opening and closing of sluice gates, depending on whether the water needs to be drained out (monsoon) or retained (other periods). In the present study, sampling at both sites was carried out during pre-monsoon season, when water is retained.

The salinity of the Santana Creek sample was measured to be 20 p.s.u. and that of Verna Lake was 0 p.s.u. pH values were 6.7 and 6.5 respectively, concentrations of nitrate were 0.75 µM and 1.58 µM respectively and of phosphate, 2.18 µM and 1.59 µM respectively, for the Santana Creek and Verna Lake samples.

6.8 Isolation and Sequencing of Metagenomic DNA

The highest concentrations of DNA were obtained by the following protocol: Concentration of virus particles by a combination of three methods (direct pelleting, precipitation with PEG 8000 and physical concentration on a 0.02 µm Anodisc membrane), followed by manual extraction of DNA by the standard phenol-chloroform method.

Table 6.2 lists the statistics of respective libraries prepared from each sample.

Table 6.2: Statistics of libraries prepared from metagenomic DNA

Parameter	**Santana Creek**	**Verna Lake**
DNA concentration on Qubit (ng/µl)	10.3	6.8
DNA concentration after library preparation	14.4	66.6
Average fragment size of library (bp)	400	372

A total of 15.04 and 18.85 million raw reads were generated for the samples from Santana Creek and Verna Lake respectively (**Table 6.3**). All the reads were of good quality. As seen from Table 6.4, the mean GC content for both samples was 48% and mean sequence length was 150 bp. The raw reads were submitted to the NCBI sequence read archive (BioProject Accession: **PRJNA687641**).

Table 6.3: Basic statistics of reads obtained from Illumina sequencing

	Santana Creek	**Verna Lake**
Total raw reads (million)	15.04	18.85
Reads of poor quality	0	0
Mean sequence length (bp)	150	150
Mean GC content (%)	48	48

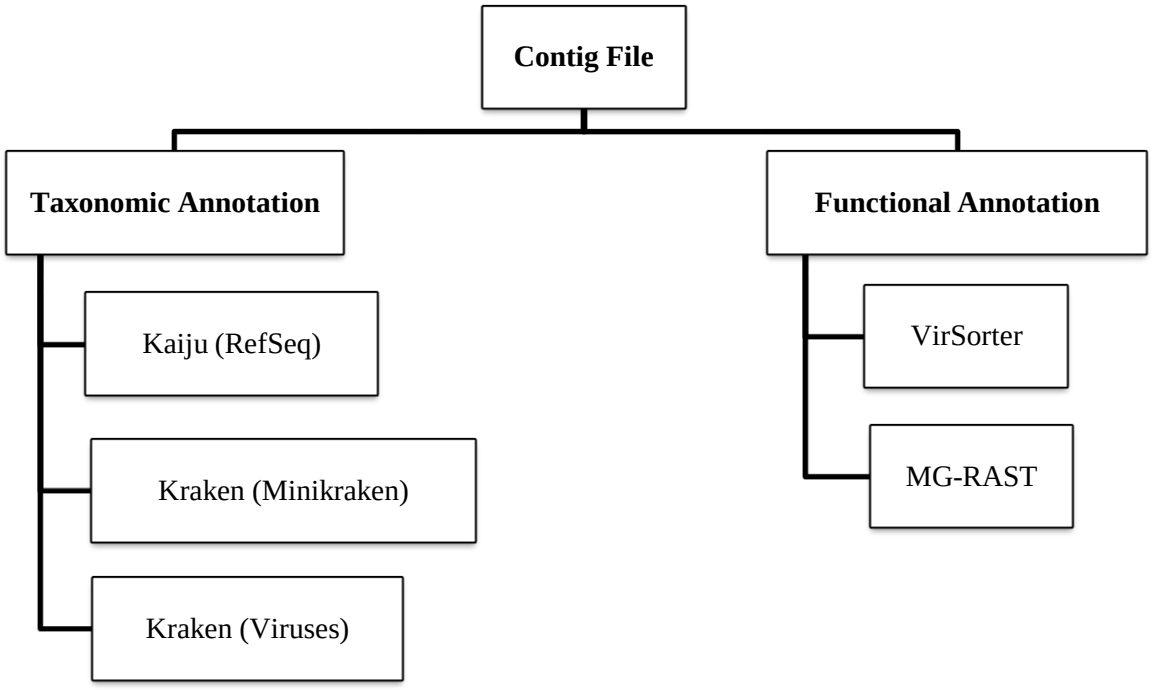
Following trimming, a total of 14.09 and 16.98 million paired ended reads were obtained from Santana Creek and Verna Lake respectively (**Table 6.4**). The paired reads were assembled using MEGAHIT, as a higher N50 value was obtained, compared to assembly with MetaSPAdes. Table 6.4 depicts the statistics regarding N50 value and lengths of the longest contigs from respective samples. The mean contig length for Santana Creek and Verna Lake was 644 and 908 respectively.

Table 6.4: Assembly statistics for respective viromes

Parameter	Santana Creek	Verna Lake
Trimmomatic Output		
Number of reads in each paired ended read file	1,40,94,682	1,69,88,934
Total number of reads in unpaired files	8,10,197	16,15,167
MEGAHIT Output		
GC content	47.5	47.4
N50 value	666	1271
Length of longest contig	74,173	2,88,804
Length of shortest contig	200	200
Mean contig length	644	908

6.9 Annotation of Santana Creek and Verna Lake Viromes

Following assembly with MEGAHIT, the respective contig files were subjected to the analyses described in the Methods Section.



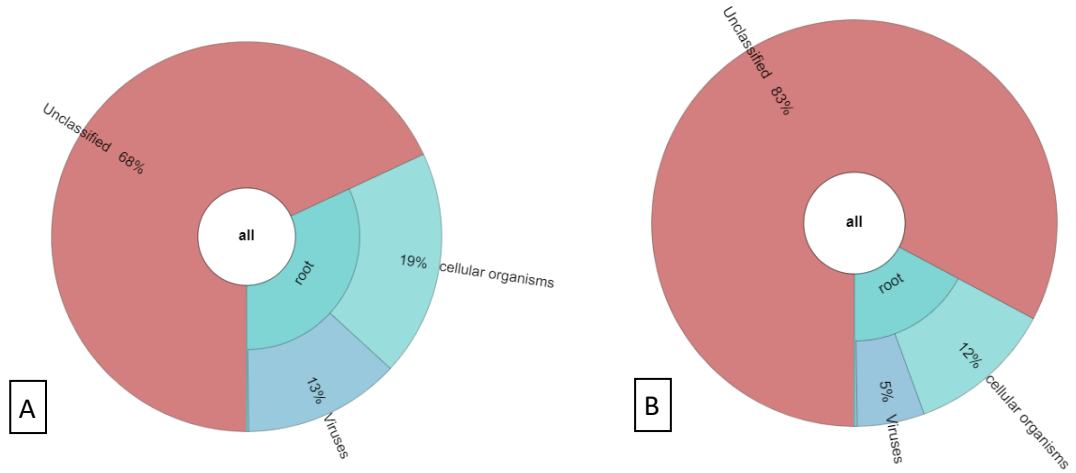


Figure 6.3: Overview of the viromes A) SC; B) VL

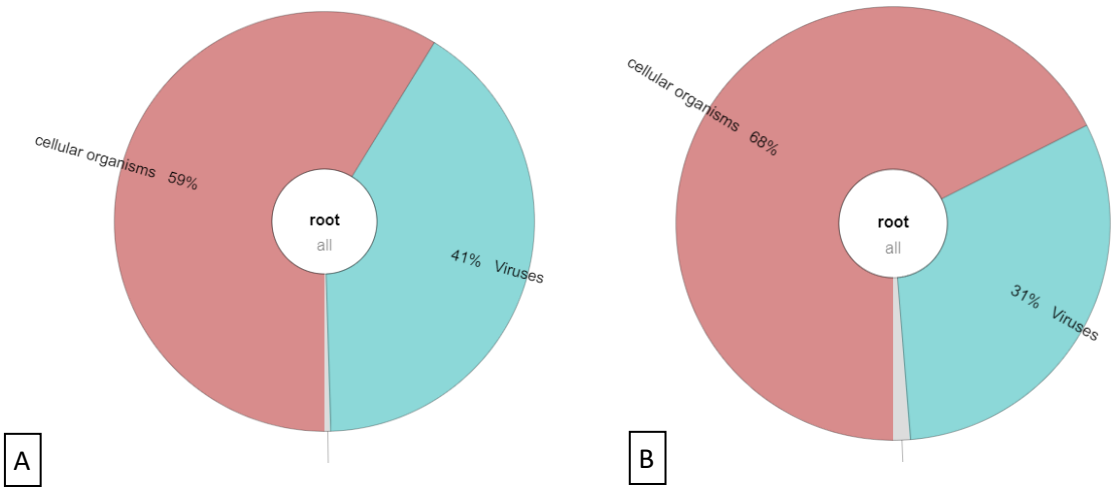


Figure 6.4: Distribution of classified sequences from each virome

A) SC; B) VL

6.10 Overview of the Viromes

The majority of raw as well as assembled sequences showed no homology to sequences in existing databases (**Table 6.5**), testifying to the novelty of both the selected viromes. **Figure 6.3** depicts the overall distribution of assembled sequences in the respective viromes, annotated with NCBI RefSeq (Kraken does not output unclassified sequences). 13% of contigs from the Santana Creek ('**SC**') virome belonged to viruses, 19% to cellular organisms, specifically bacteria, and 68% were unclassified. With respect to the Verna Lake ('**VL**') virome, 5% of contigs were of viral origin, 12% of bacterial and 83% were unclassified. Over numerous viral metagenomic studies, 40-90% of reads have been found unclassified, an indication of the vast reservoirs of viral dark matter present in the biosphere (Bruder et al. 2016; Krishnamurthy and Wang 2017; Roux, Adriaenssens, et al. 2019).

Figure 6.4 depicts the distribution of classified sequences only. Viral contigs constituted 41% and 31% of all classified sequences in the SC and VL viromes respectively. However, the total number of reads, both raw and assembled in VL was far greater than SC (**Table 6.5**). Consequently, the percentage of unclassified reads was far greater in VL, pointing to a much higher proportion of novel sequences in the VL virome.

Table 6.5: Summary of annotation of SC and VL viromes by various modalities

	Total number of sequences	**Number of viral sequences**
Santana Creek		
Raw (NCBI nr)	1,27,88,735 (72% unclassified)	12,58,482
Assembled		
1) Kaiju (RefSeq)	2,85,800 (68% unclassified)	37,192
2) Kraken (Minikraken)	n/a	5,717
3) Kraken (Viruses)	n/a	7,473
Verna Lake		
Raw (NCBI nr)	1,88,54,018 (85% unclassified)	7,74,625
Assembled		
1) Kaiju (RefSeq)	1,72,806 (83% unclassified)	9,275
2) Kraken (Minikraken)	n/a	309
3) Kraken (Viruses)	n/a	462

n/a: Kraken does not report unclassified sequences. Kraken Viruses only outputs sequences identified as viral.

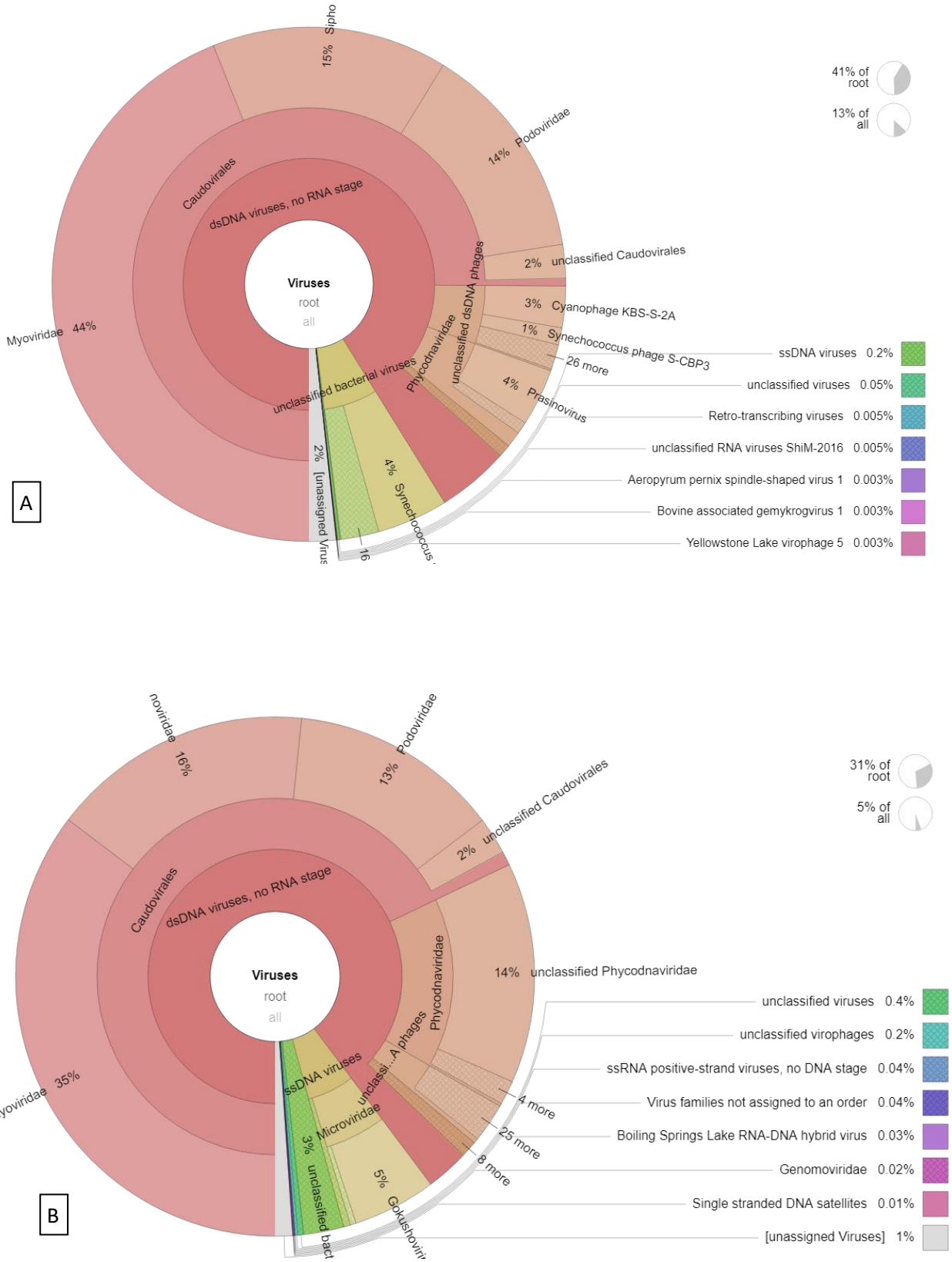


Figure 6.5: Order / family level classification of the viromes based on NCBI RefSeq

A) SC; B) VL

The major difference between the two sites may lie in their varying salinity levels, a consequence of location and proximity to the sea. Verna Lake is purely freshwater, with a salinity of 0 p.s.u. throughout the year. Santana Creek is quasi-freshwater, as salinity levels vary depending on the season – with a higher salinity during pre-and post-monsoon, and near zero salinity during the monsoon season. Due to the close proximity of Santana Creek to the sea, the SC virome is likely to have some proportion of sequences which are of marine origin. A similar finding was reported in the virome characterization of the Jiulong River Estuary in China (Cai et al. 2016). Marine viromes have been better characterized the world over (Mohiuddin and Schellhorn 2015; Putonti et al. 2018), possibly resulting in a greater proportion of marine-origin sequences in databases, than those derived from inland or freshwater systems. Freshwater viromes have also been shown to diverge from marine (Potapov et al. 2019; Roux, Enault, et al. 2012). Studies on other estuarine locations have demonstrated that salinity is a significant determinant of viral community composition (Parvathi et al. 2013; C. Zhang et al. 2020).

The presence of bacterial-origin sequences in virome-enriched samples is a common observation. Most researchers follow the strategy of separating bacterial-origin sequences during downstream bioinformatics analysis, to circumvent the limitations of currently available sample preparation protocols (Pinto et al. 2020). Further, prophage sequences in databases are commonly classified as bacterial-origin, thereby increasing the percentage of sequences annotated as ‘bacterial’ (Potapov et al. 2019).

6.11 Order / Family Level Classification of the Viromes based on NCBI RefSeq

The NCBI nonredundant (nr) protein database is a collection of translations of all nonredundant coding sequences from GenBank, RefSeq, PDB, SwissProt and PRF databases. BLAST analyses use this database by default (Sayers et al. 2018). The NCBI Reference Sequences (RefSeq) protein database contains translated sequences from all the whole genomic sequences of bacteria, archaea and viruses deposited in NCBI (Pruitt et al. 2012). The comparison of virome contigs against the RefSeq database in the present study, was carried out using the Kaiju programme (Menzel et al. 2016).

As depicted in **Table 6.6** and **Figure 6.5**, sequences identified as belonging to Order *Caudovirales* (tailed bacteriophages) predominated in both viromes. This result was expected, given the similar predominance of *Caudovirales* in viromes ranging from marine

(Flaviani et al. 2018; Garin-Fernandez et al. 2018) to freshwater lakes (Mohiuddin and Schellhorn 2015; Potapov et al. 2019), agricultural ponds and creeks (Chopyk et al. 2018, 2020) and extreme environments (Dávila-Ramos et al. 2019). Within the *Caudovirales*, the highest proportion of annotated sequences belonged to Family *Myoviridae*. The other families within Order *Caudovirales,* i.e. *Siphoviridae* and *Podoviridae* had similar distributions in both viral communities.

Table 6.6: Overview of virus community structure in each virome (based on RefSeq)

	Santana Creek	**Verna Lake**
Order *Caudovirales* (total)	75	68
Family *Myoviridae*	44	35
Family *Podoviridae*	14	13
Family *Siphoviridae*	15	16
Family *Phycodnaviridae*	5	15
Family *Microviridae*	-	6
Unclassified bacterial viruses	22	15

Note: All figures expressed as % of total viral sequences

The abundant presence of *Myoviridae* members (44% of SC virome and 35% of VL virome) was supported by amplification of the *g20* marker gene from metagenomic DNA of both sites (p. 99). Previous studies based on the *g20* marker have demonstrated the dominance of *Myoviridae* in diverse aquatic systems (Matteson et al. 2011; Wilhelm et al. 2006). The relative distribution of families within *Caudovirales* elucidated through metagenomic sequencing however, varies. While certain freshwater ecosystems, notably other lakes – Lake Baikal (Potapov et al. 2019), Lakes Ontario and Erie (Mohiuddin and Schellhorn 2015) – displayed a predominance of *Myoviridae*, other niches such as an agricultural freshwater pond (Chopyk et al. 2018) and a groundwater reservoir (Costeira et al. 2019) had a majority of *Siphoviridae* sequences.

The second most abundant category of viruses was Family *Phycodnaviridae*, constituting 5% annotated viral contigs in SC virome and 15% in VL. A substantial presence of *Phycodnaviridae* was also supported by amplification of the *polB* gene described earlier in Chapter 5 (p. 99).

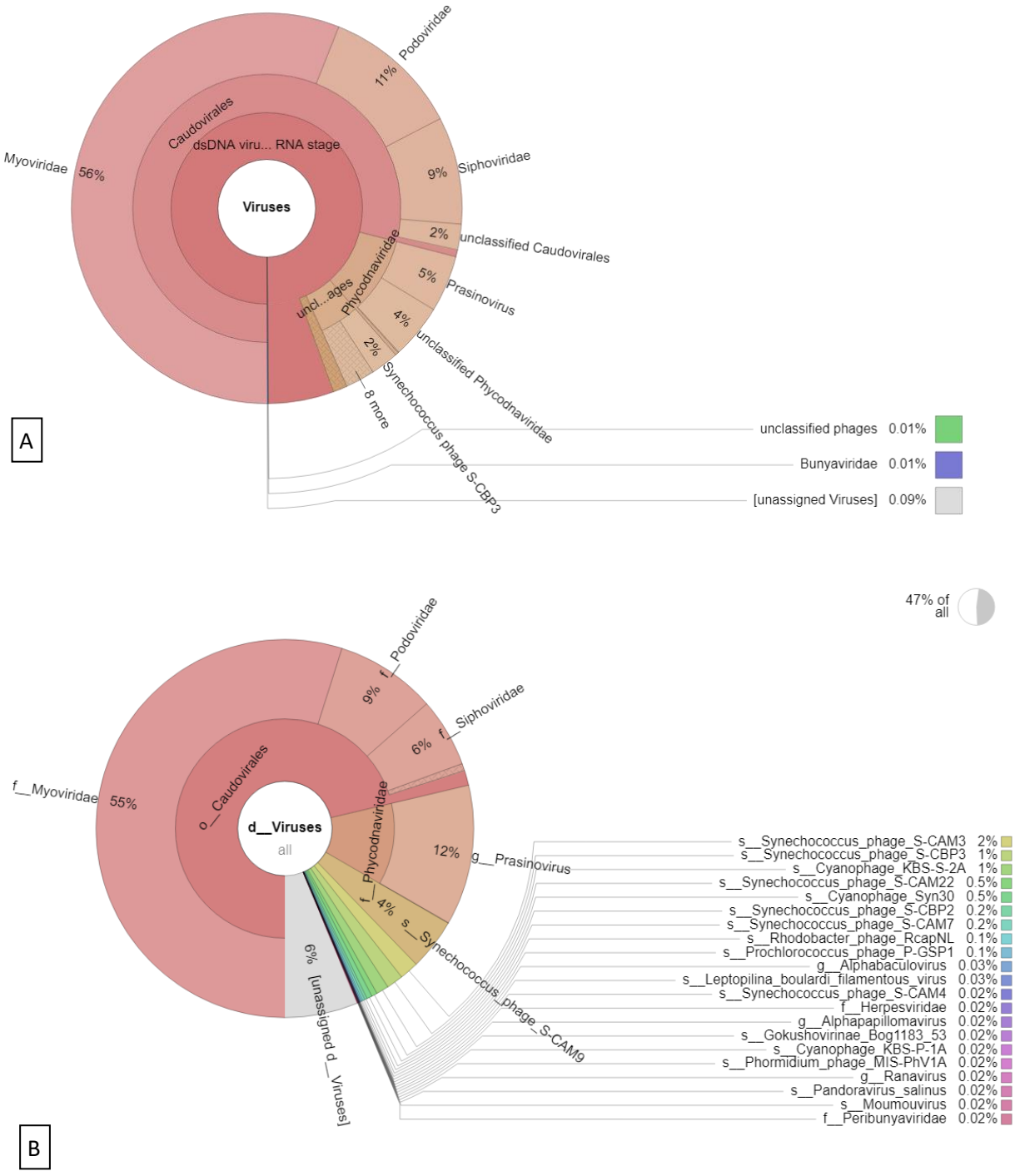


Figure 6.6: Order / family level classification of the SC virome, based on

A) Kraken Viruses B) Minikraken

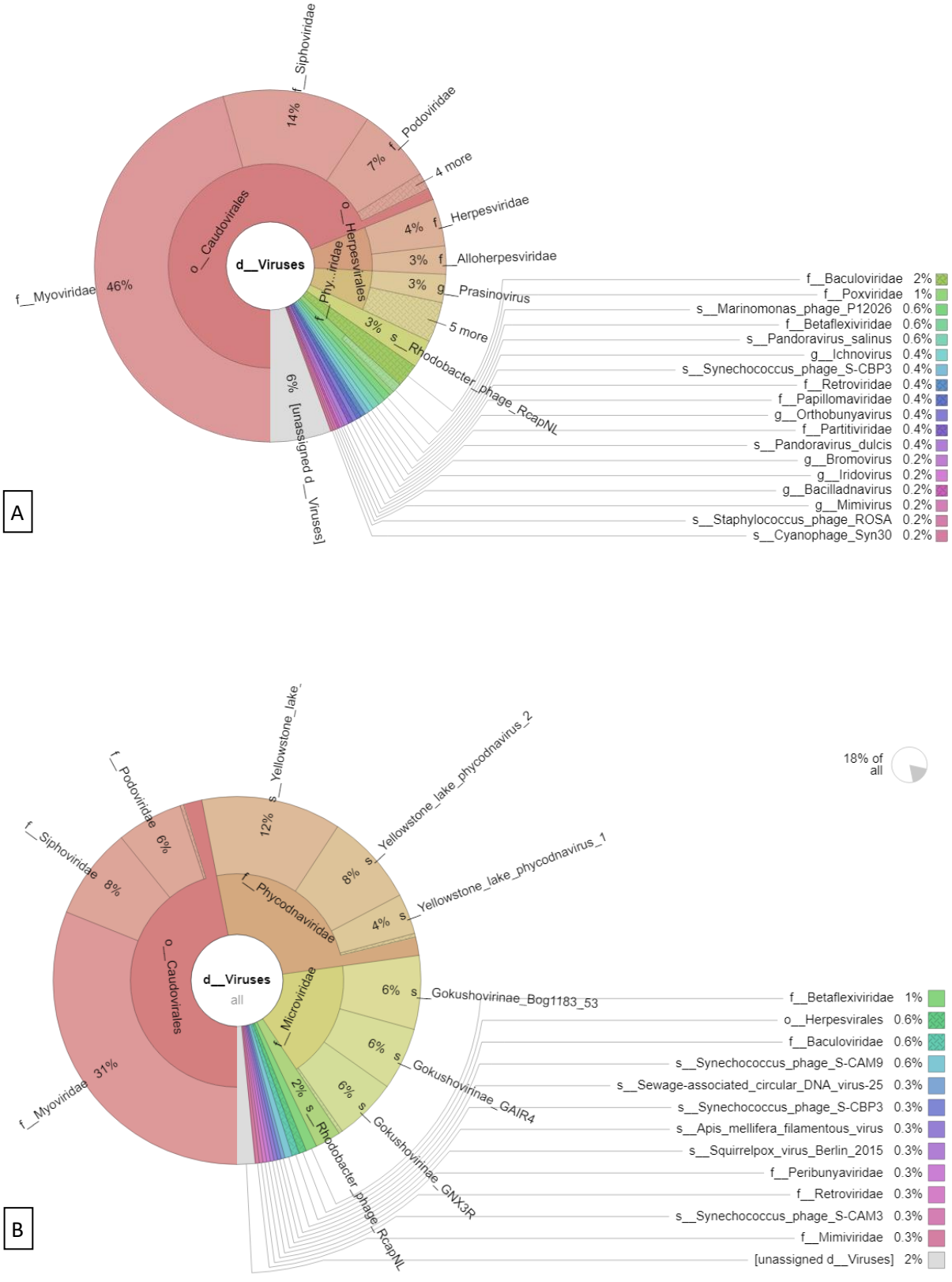


Figure 6.7: Order / Family Level Classification of the VL virome based on

A) Kraken Viruses B) Minikraken

One family which constituted 6% of viruses of the VL virome but was conspicuously absent from the SC virome, was Family *Microviridae*. ssDNA viruses consistently represent a very small proportion of viromes, compared to dsDNA; This has, at times, been attributed to biases induced by amplification methods used in library preparation (Kim and Bae 2011; Roux et al. 2016). Among ssDNA viruses, the *Microviridae* have been detected in a variety of soil, aquatic and gut-associated metagenomes (Roux et al. 2016; Roux, Krupovic, et al. 2012; Székely and Breitbart 2016). In the present study, members of subfamily *Gokushovirinae* constituted 94% of *Microviridae* sequences. The bacterial hosts of this subfamily are mostly intracellular parasites such as *Chlamydia* and *Bdellovibrio* (Roux, Krupovic, et al. 2012). Marine viromics studies have reported a restricted distribution of gokushoviruses, likely tied to the distribution of their hosts (Labonté and Suttle 2013). The presence of gokushoviruses exclusively in the VL virome, could indicate sewage contamination containing host parasitic bacteria.

6.12 Order / Family Level Classification of the Viromes based on Kraken

The contigs obtained from both viromes were also annotated against two Kraken databases, namely, 'Minikraken' and 'Viruses' (**Table 6.7, Figures 6.6 and 6.7**). While Kraken annotation maintained an overall similar pattern to the RefSeq annotation, a few notable differences emerged, both between Kraken and RefSeq and between the two Kraken databases. The similarities included overall dominance of families *Myoviridae and Phycodnaviridae*; however, percentages were widely different (**Table 6.7, Figures 6.6 and 6.7**). Further differences included additional virus sequences revealed by Kraken annotation which were absent in the RefSeq annotation. Notable were the *Herpesvirales*, comprising 7% of total viruses in the VL virome. Within *Herpesvirales*, the only identified human pathogenic virus was Human Herpesvirus 7 constituting 0.1% of all viruses; the remaining were animal herpesviruses.

Table 6.7: Overview of virus community structure in each virome (based on Kraken)

	Santana Creek		***Verna Lake***	
	Viruses	**Minikraken**	**Viruses**	**Minikraken**
Order *Caudovirales* (total)	79	71	69	47
Family *Myoviridae*	46	55	56	31
Family *Podoviridae*	7	9	11	6
Family *Siphoviridae*	14	6	9	8
Family *Phycodnaviridae*	10	12	6	26
Family *Microviridae*	-	-	-	18
Order *Herpesvirales*	-	-	7	-

Note: All figures expressed as % of total viral sequences

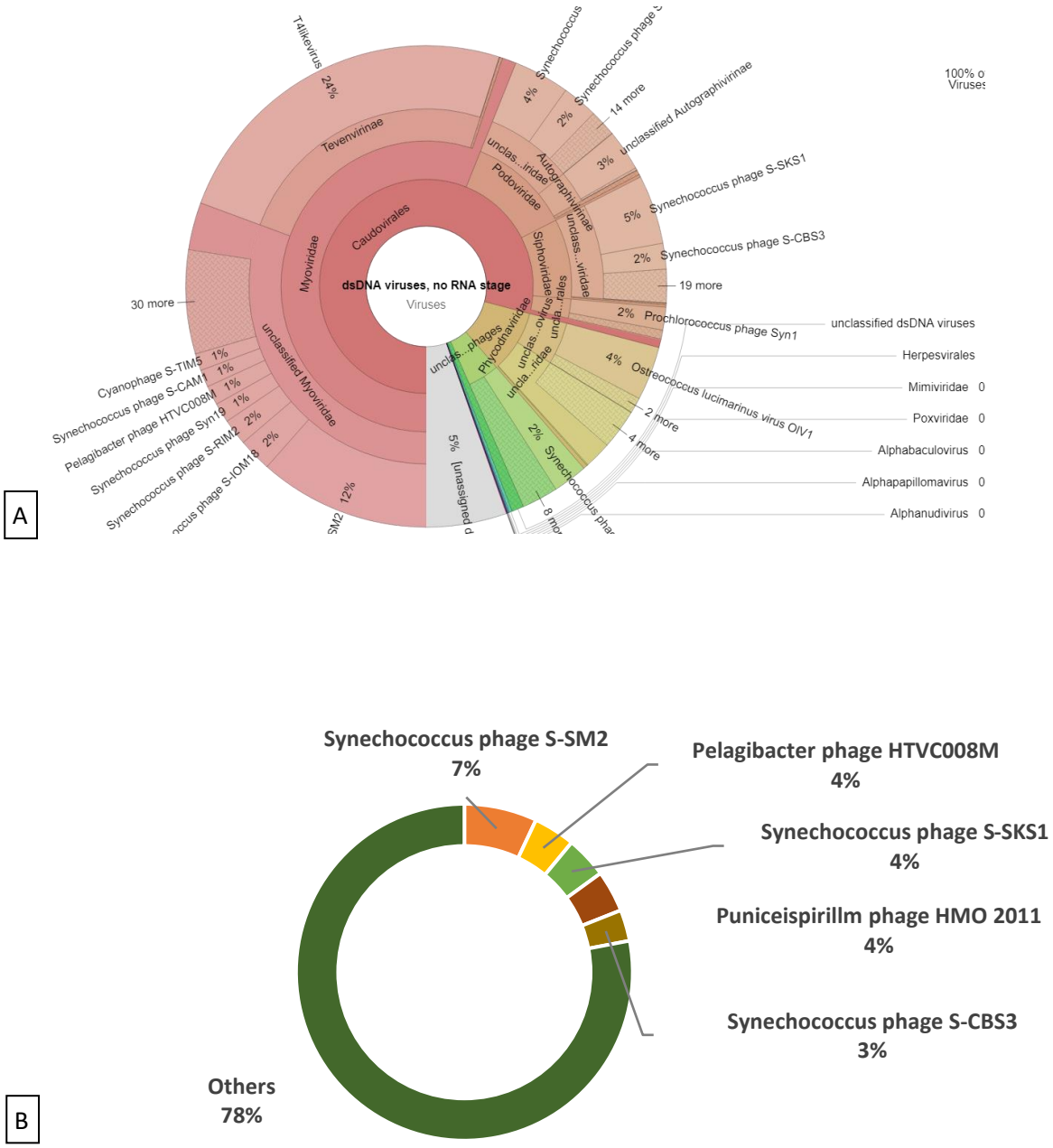


Figure 6.8: **Genus / species level classification of the SC virome based on RefSeq**

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

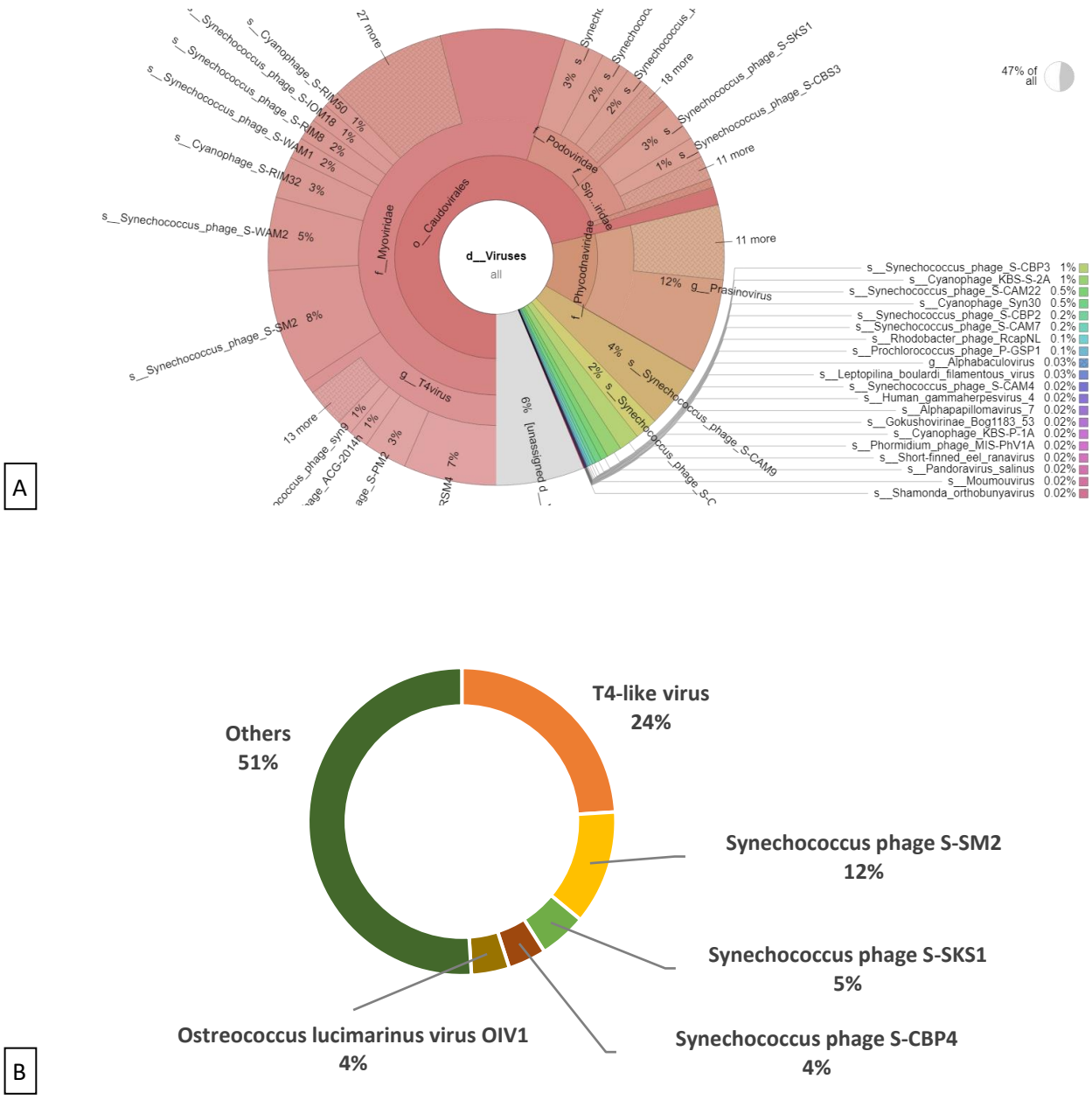


Figure 6.9: **Genus / species level classification of the SC virome based on Kraken Viruses**

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

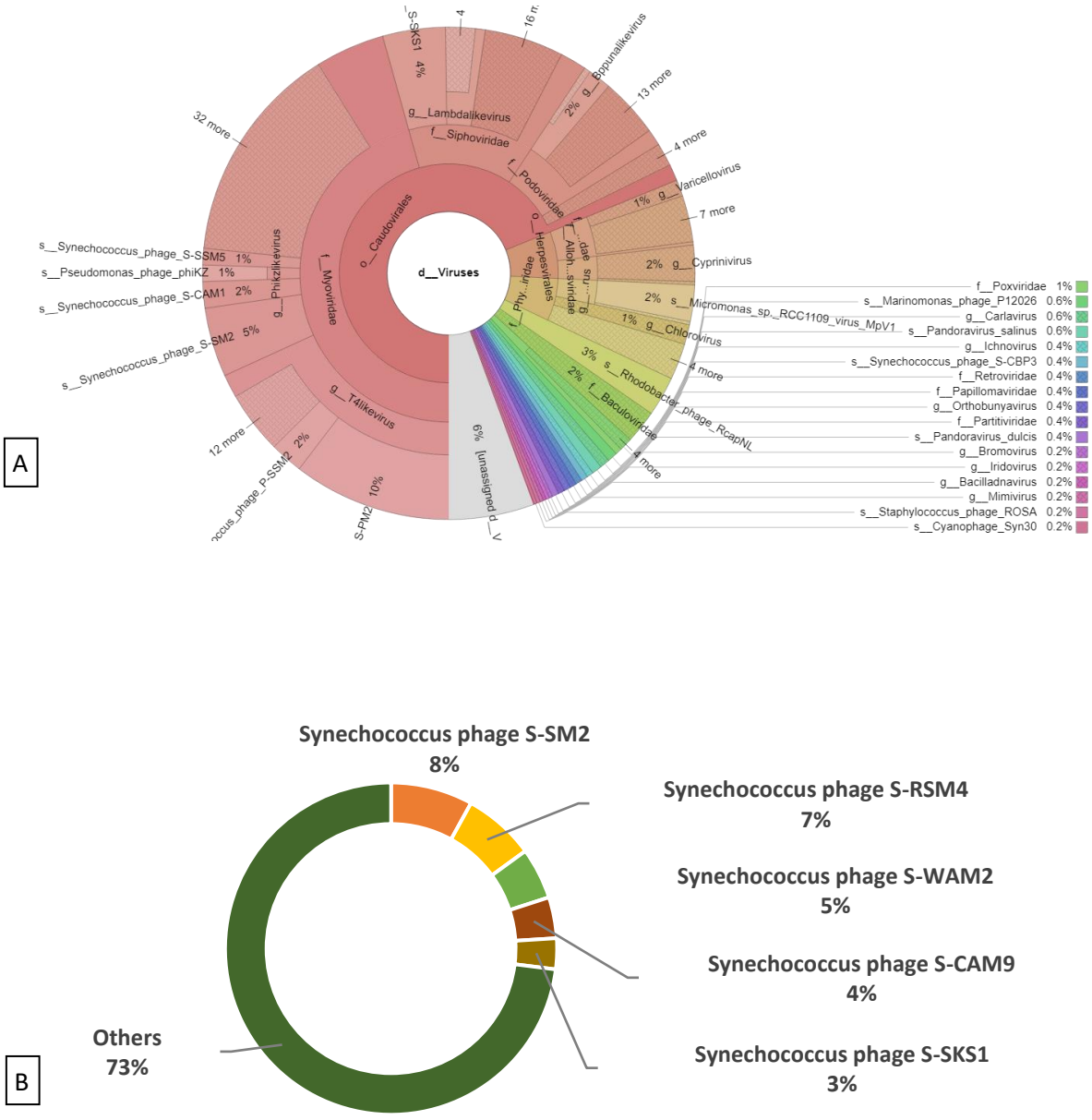


Figure 6.10: **Genus / species level classification of the SC virome based on Minikraken**

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

6.13 Genus / Species Level Classification of the Viromes

The top five predominant species in each virome based on the three different classifications are presented in **Table 6.8** and **Figure 6.8 to 6.13.**

Table 6.8: Five predominant viral species in each virome, based on separate annotations

	Virus species	**% of total viruses in virome**
SC (RefSeq)	*Synechococcus* phage S-SM2	7
	Pelagibacter phage HTVC008M	4
	Synechococcus phage S-SKS1	4
	Puniceispirillum phage HMO 2011	4
	Synechococcus phage S-CBS3	3
SC (Viruses)	T4-like virus	24
	Synechococcus phage S-SM2	12
	Synechococcus phage S-SKS1	5
	Synechococcus phage S-CBP4	4
	Ostreococcus lucimarinus virus OIV1	4
SC (Minikraken)	*Synechococcus* phage S-SM2	8
	Synechococcus phage S-RSM4	7
	Synechococcus phage S-WAM2	5
	Synechococcus phage S-CAM9	4
	Synechococcus phage S-SKS1	3
VL (RefSeq)	Yellowstone lake phycodnavirus 3	6
	Yellowstone lake phycodnavirus 2	4
	Yellowstone lake phycodnavirus 1	3
	Synechococcus phage S-SM2	3
	Pelagibacter phage HTVC008M	2
VL (Viruses)	*Synechococcus* phage S-PM2	10
	Synechococcus phage S-SM2	5
	Synechococcus phage S-SKS1	4
	Rhodobacter phage RCapNL	3
	Micromonas sp. RCC 1109 virus MpV1	2
VL (Minikraken)	Yellowstone lake phycodnavirus 3	12
	Synechococcus phage S-PM2	9
	Yellowstone lake phycodnavirus 2	8
	Gokushovirinae Bog1183 53	6
	Gokushovirinae GAIR4	6

Synechococcus phages consistently appear to be dominant members of virus communities, as discussed in Chapter 4 (p.73). This is a natural fallout of the ubiquity of the host *Synechococcus*. This is also aided by the ease of culturing phage-host systems and subsequent genome sequencing leading to them constituting a significant proportion of sequences in all viral databases (Bruder et al. 2016).

The VL virome was dominated by species of Yellowstone Lake Phycodnavirus. These are *Prasinovirus*-related large phycodnaviruses, whose hosts are currently unknown, but whose genomes have been assembled from a previous Lake location (Zhang et al. 2015).
The presence of significant proportions of these viruses raises the interesting possibility of studying them in conjunction with their hosts which have not been previously isolated from aquatic ecosystems in Goa.

The Kraken and Kaiju programmes use alternative algorithms to classify metagenomic sequences. Kraken uses a k-mer based approach where nucleotide sequences or contigs are directly classified based on characteristic k-mers found therein. Kraken utilizes databases built by selecting individual k-mer values and the lowest common ancestor (LCA) of all organisms whose genome contains that k-mer. The set of LCAs corresponding to all k-mers in a read are co-ordinately analysed to assign a single label to the read. Minikraken is a minimal version of the complete Kraken database, intended for fast classification based on first hit rather than exact matches, while Kraken Viruses contains virus sequences only (Wood and Salzberg 2014).

Kaiju on the other hand, compares sequences against protein databases. Here the algorithms have been improved compared to standard BLAST, in terms of speed and sensitivity. The rationale of Kaiju against k-mer based approaches is that microbial genomes have a high density of protein coding sequences, therefore comparison of whole sequences may achieve a more sensitive classification. In other words, sequences which might be missed by the k-mer approach are identified here (Menzel et al. 2016).

The annotation results based on **NCBI Refseq** were selected for functional annotation of the viromes, as the maximum viral contigs in both viromes were annotated via this database.

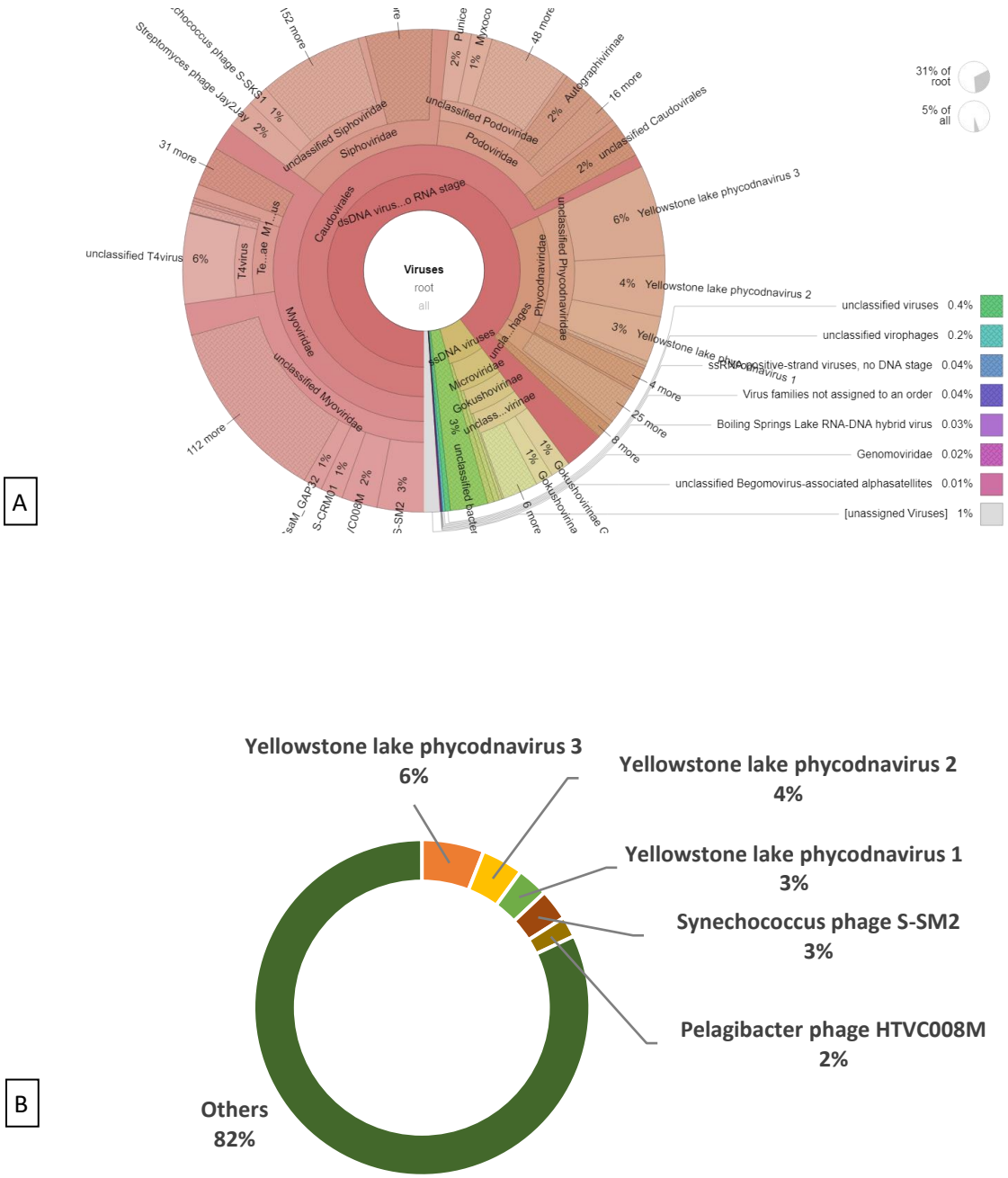


Figure 6.11 Genus / species level classification of the VL virome based on RefSeq

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

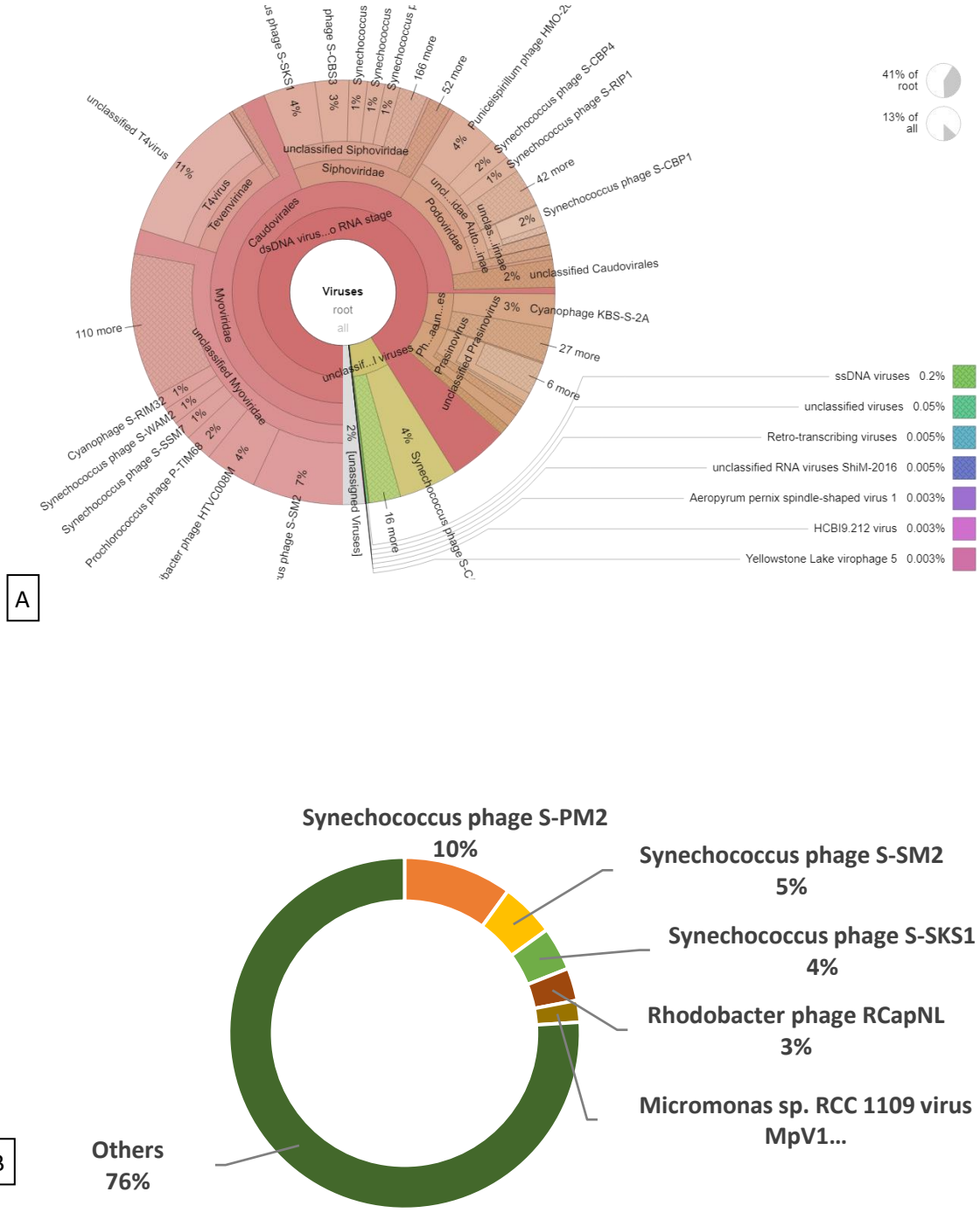


Figure 6.12: **Genus / species level classification of the VL virome based on Kraken Viruses**

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

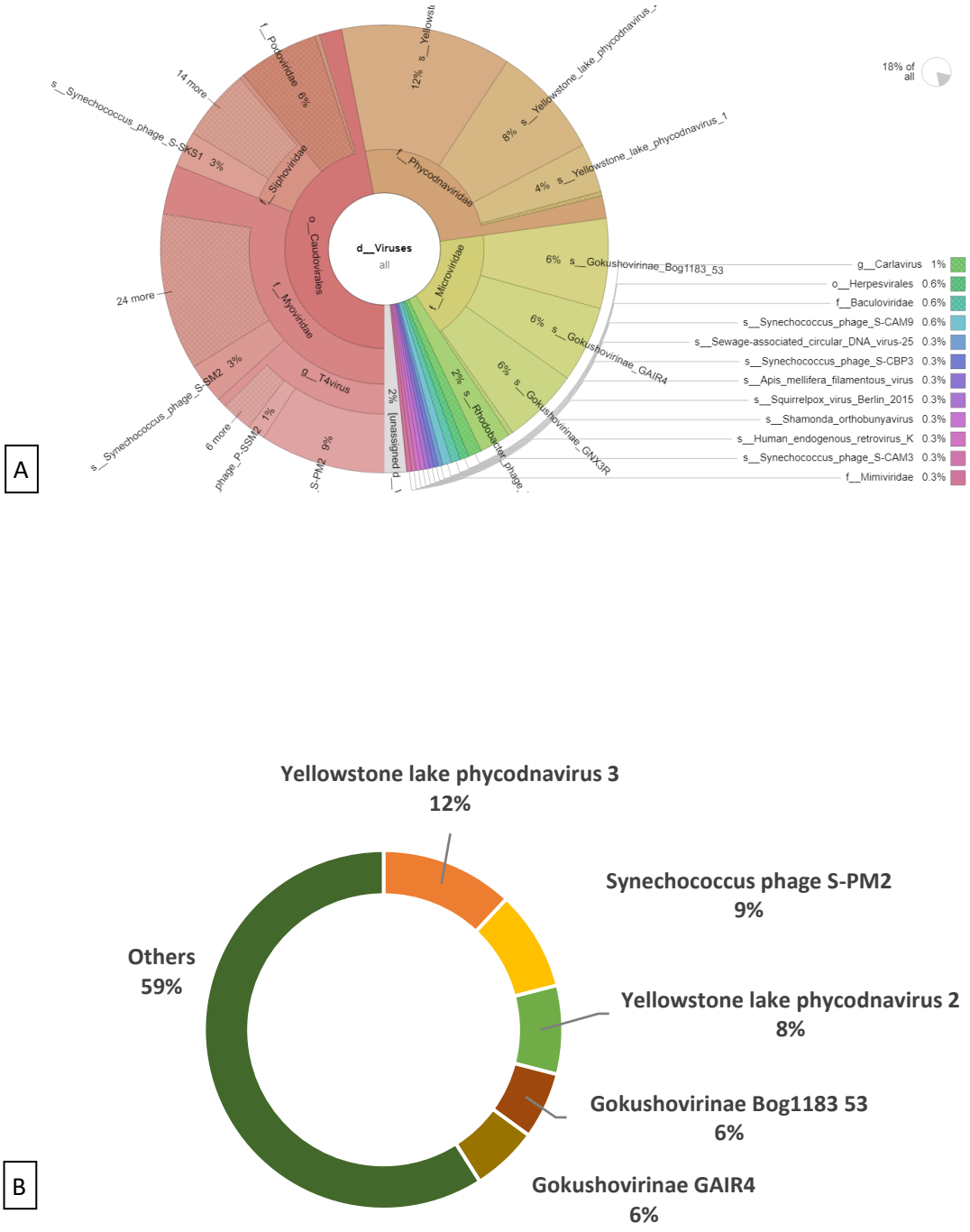


Figure 6.13: **Genus / species level classification of the VL virome based on Minikraken**

A) Overview; B) The 5 predominant genera along with the proportion of total viruses constituted by them

6.14 Functional Annotation of Viromes with MG-RAST

When the raw reads / assembled contigs of the entire sample were input into MG-RAST, the output was complicated by the presence of bacterial sequences in the raw as well as assembled files. For this reason, the contig files were first run through VirSorter, which outputs those contigs likely to be of viral origin in separate FASTA files – one file with highly likely and one with less likely (designated Category 1 "***Cat1***" and Category 2 "***Cat2***" respectively). VirSorter identifies viruses based either on enrichment in viral genes or the presence of at least one genomic feature characteristic of viruses. The utility of VirSorter is represented in **Figure 6.14**. The Cat1 and Cat2 files were separately input into MG-RAST, to obtain functional annotation of largely viral origin sequences. MG-RAST uses a subsystems-based approach to annotation, wherein protein families are grouped according to their functional roles across genomes. The annotation is not specific to an organism, but rather, to a functional category (Keegan et al. 2016).

MG-RAST provides several levels of annotation (**Figure 6.15**). To arrive at a relevant set of protein functions annotated from ORFs within the contigs of the respective viromes, an e value cut-off of 1e-5 was applied, along with an identity of 70% and length of 15. Further, to avoid annotation of bacterial-origin proteins, a filter of RefSeq Viruses was applied.

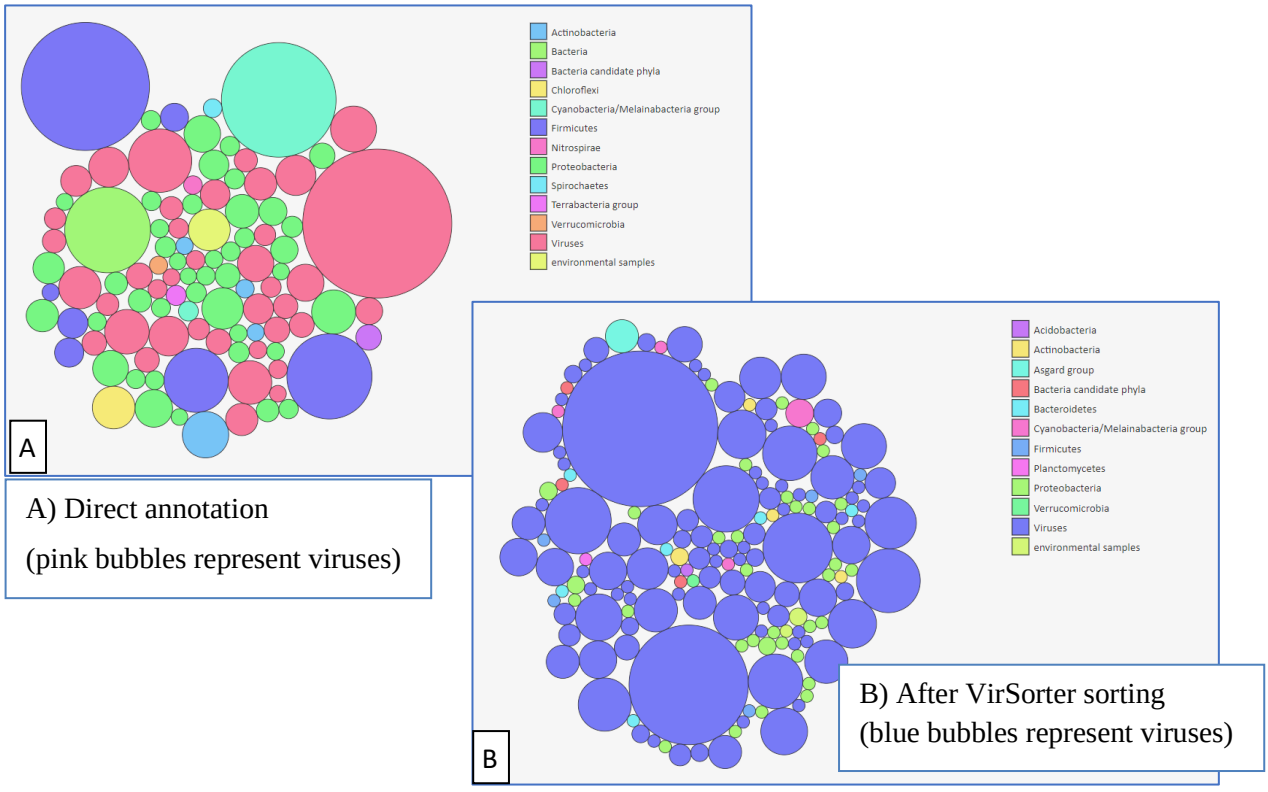


Figure 6.14: Utility of VirSorter, represented as proportion of virus sequences annotated with RefSeq in two situations

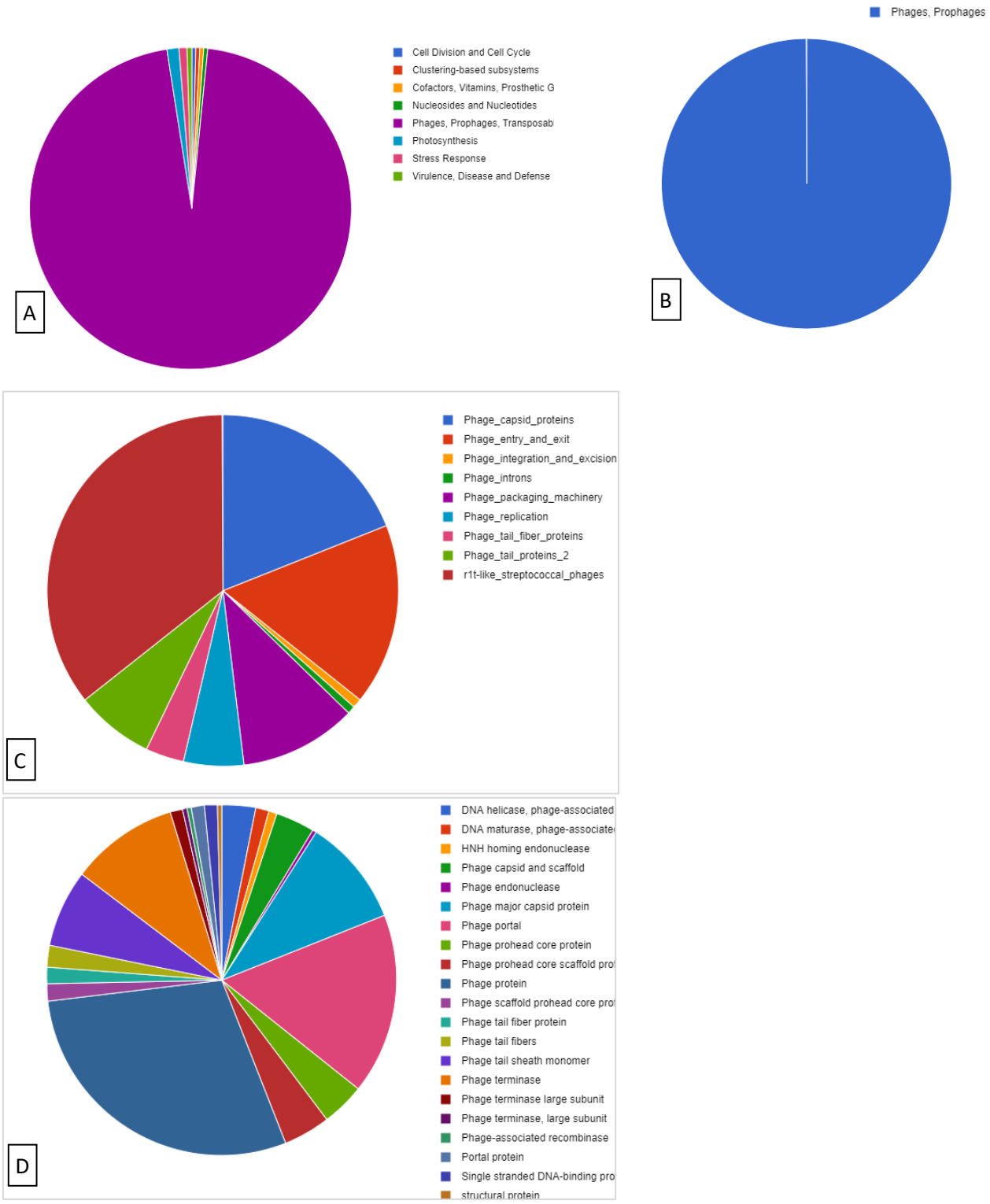


Figure 6.15: Levels of annotation of SC Cat 1 proteins through MG-RAST

A) Level 1: Broad categories of proteins B) Level 2: Phages and Prophages category

C) Level 3: Functional categories (broad) D) Level 4: Functional categories (specific)

Table 6.9 lists the respective functional annotations for each category of proteins in SC (Cat 1, Cat 2) and VL (Cat 1, Cat 2).

Table 6.9 : Annotated proteins in SC and VL

Category	**Protein function**	**Number of hits**			
		SC Cat 1	SC Cat 2	VL Cat 1	VL Cat 2
Phage baseplate proteins	Phage baseplate	9	0	0	9
	Phage baseplate hub	4	0	0	3
	Phage baseplate wedge	4	0	0	1
Phage capsid proteins	Phage head completion protein	4	0	0	3
	Phage prohead core protein	3	10	10	1
	Phage prohead core scaffold protein and protease	1	11	13	0
	Phage scaffold prohead core protein	3	4	9	1
	T7-like phage head-to-tail joining protein	10	0	0	3
Phage DNA synthesis	3'-phosphatase, 5'-polynucleotide kinase, phage-associated	2	0	0	0
	Adenine DNA methyltransferase, phage-associated	2	0	0	0
	DNA adenine methyltransferase, phage-associated	1	0	0	0
Phage entry and exit	Phage lysin	0	0	0	5
	Phage portal	3	42	29	0
Phage integration and excision	Phage endonuclease	3	1	0	1
	Phage exonuclease	0	0	0	2
	Phage-associated recombinase	0	1	0	0
Phage neck proteins	Phage neck	2	0	0	2
	Phage neck protein	4	0	0	4
Phage packaging machinery	Phage DNA packaging	1	0	0	0
	Phage terminase	9	24	24	3
Phage regulation of gene expression	RNA polymerase sigma factor	2	0	0	10
Phage replication	DNA helicase	1	0	0	4
	DNA helicase, phage-associated	21	8	5	20
	DNA maturase, phage-associated	1	3	1	0
	DNA polymerase sliding clamp, phage-associated	2	0	0	0
	RNA polymerase, phage-associated	7	0	0	9
	Single stranded DNA-binding protein, phage-associated	1	3	0	3
Phage tail fiber proteins	Phage tail fiber protein	4	1	0	1
	Phage tail fibers	7	4	3	5
Phage tail proteins	Phage tail assembly	1	0	0	2
	Phage tail sheath monomer	0	18	16	0
r1t-like streptococcal phages	Phage capsid and scaffold	12	8	3	1
	Phage major capsid protein	3	23	10	1
	Phage protein	693	72	60	501
	Structural protein	3	0	0	3
T7-like phage core proteins	T7-like phage DNA Polymerase	1	0	0	0

Figure 6.16: Heatmap of functional annotations from each virome

(Cat 1 and 2 respectively)

The heatmap in **Figure 6.16** indicates functional categories of each virome. The Cat 1 annotations from respective viromes depicted similarities, with enrichment in several categories of phage structural proteins, in comparison with Cat 2 annotations.

The respective annotations in Cat 1 and 2 were combined for each virome to obtain an overall picture of viral functions. A functional comparison of SC and VL viromes is depicted in **Table 6.10**.

Table 6.10: Comparison of viral functions in SC and VL viromes

Category	**Total number of hits**	
	SC	**VL**
Phage DNA synthesis	5	0
Phage baseplate proteins	17	13
Phage capsid proteins	73	48
Phage entry and exit	45	34
Phage integration and excision	5	3
Phage neck proteins	6	6
Phage packaging machinery	30	23
Phage regulation of gene expression	2	10
Phage replication	47	42
Phage tail fiber proteins	16	9
Phage tail proteins	19	18
T7-like phage core proteins	2	0

The category “r1t-like streptococcal phages” was omitted for this comparison. Most of the proteins in this category were labelled non-specifically as “phage protein”, indicating that they could not be placed in any recognizable category of the Subsystems annotation.

Figure 6.17 depicts a graphical comparison of viral functions of both the viromes.

A greater number of viral proteins was annotated from the SC virome than from the VL virome, consistent with the respective numbers of viral sequences that were taxonomically annotated. In both the viromes, phage capsid proteins formed the most abundant category of functional elements, followed by proteins involved in phage entry and exit, and phage replication. The functions of viruses in a specific ecosystem depend on the environmental conditions prevalent (Jasna, Parvathi, and Dash 2018). Thus, the predominance of proteins involved in normal phage functions such as structure, replication and interaction with hosts,

indicates favourable conditions for replication and propagation of lytic phages in both the Santana Creek and Verna Lake ecosystems during the pre-monsoon season (the time of sampling). Lytic phages also formed the largest taxonomic category in both viromes, supporting the presence of corresponding functional elements.

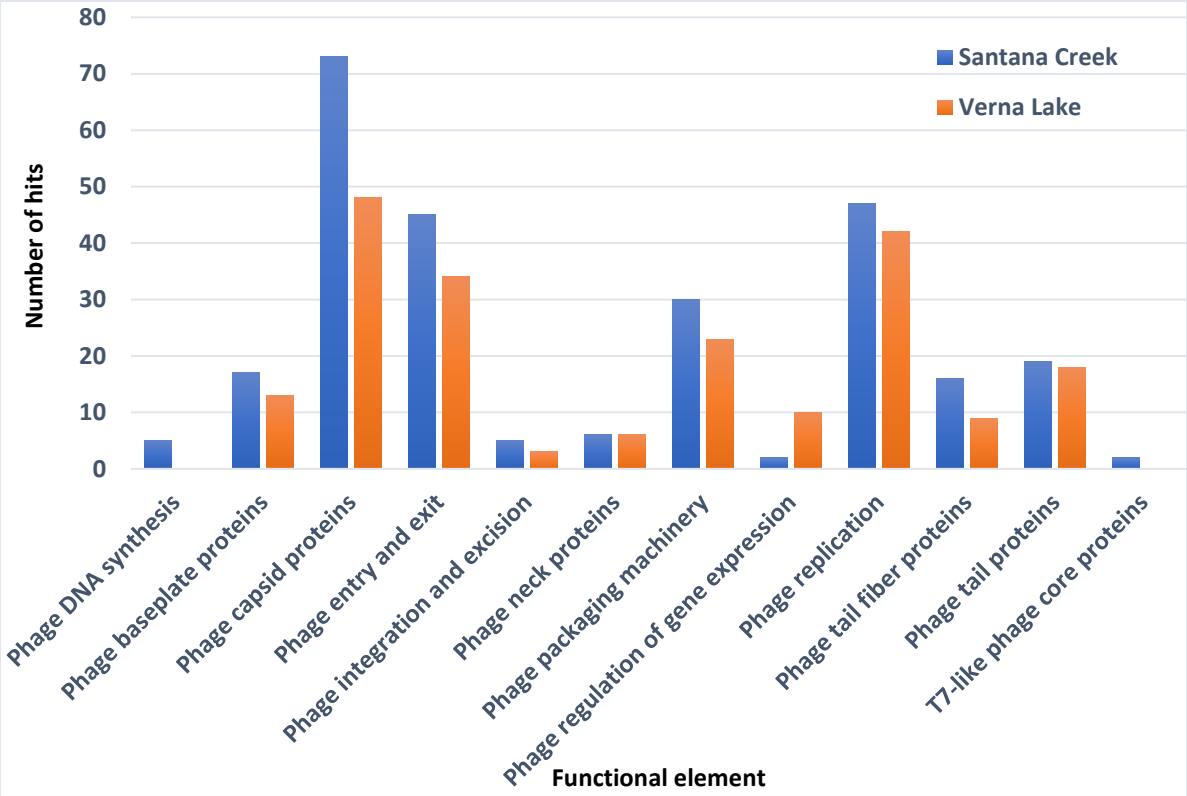


Figure 6.17: Overview of functional comparison between SC and VL viromes

6.15 Diversity indices

The Shannon Weiner Diversity index (*H'*) of SC virome was 3.548 and of VL 3.812. **Figure 6.18** depicts the same, along with confidence intervals.

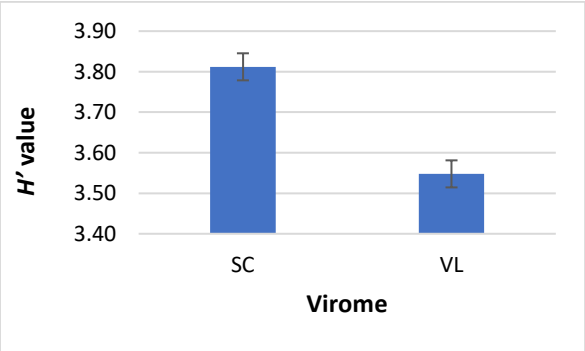


Figure 6.18: Shannon-Weiner diversity indices of SC and VL viromes

There is a general scarcity of metagenomic studies on viruses from freshwater and estuarine ecosystems. Moreover, most freshwater studies have focused on large lakes (Green et al. 2015; Mohiuddin and Schellhorn 2015; Potapov et al. 2019; Roux et al. 2012; Skvortsov et al. 2016) and it would not be strictly accurate to compare results of the present study with those. The dynamics of small lakes / ponds are considerably different from those of large lakes. Some of the reasons for this are greater terrestrial-aquatic interchange, a more insular nature – protected from wind as well as isolated from other water bodies – and relatively stagnant water (Søndergaard et al. 2005). One study which characterized bacterial and viral metagenomes from an agricultural freshwater pond, reported a similar dominance (nearly 99%) of *Caudovirales*; however within *Caudovirales*, *Siphoviridae* was the dominant family (Chopyk et al. 2018). The study also reported a majority of functional elements in the category of phage structural proteins.

The only published report on virome characterization of an estuarine ecosystem in India (Jasna, Parvathi, et al. 2018) reports dominance of *Caudovirales*, specifically *Myoviridae*, in all the four sites studied. Here, functional elements were dominated by DNA-binding and ATP-binding proteins.

As with other areas of aquatic virology, metagenomic studies thus far have focused on marine ecosystems. Marine viromics has been at the cutting edge of environmental virology research, from the very first studies (Angly et al. 2006; Culley et al. 2006) till today (Pérez-Losada et al. 2020; Sunagawa et al. 2020; Warwick-Dugdale, Solonenko, et al. 2019)

In spite of greater abundances of viruses in inland waters than in the open ocean, the viromes of such waters have been comparatively less explored (Chopyk et al. 2018; Putonti et al. 2018; R. Zhang, Weinbauer, and Peduzzi 2020), thus presenting a vast reservoir of novel viruses yet to be discovered. Freshwater aquatic systems have a direct bearing on human health (P. A. Green et al. 2015) and are directly impacted by human activities (Okazaki et al. 2019; Posch et al. 2012). Viromics studies could contribute to a better understanding of both these aspects.

With respect to the present study, an interesting angle would be the study the relation of the Verna Lake viromes to the migratory birds that flock there. Such studies could prove useful, given the substantial possibility of viral pandemics occurring in the future (Jester, Uyeki, and Jernigan 2018; Neumann and Kawaoka 2019). Migratory birds as common reservoirs of

influenza viruses could transmit the viruses through the water bodies where they halt (Lickfett et al. 2018).

From a global perspective, the wide-ranging effects of viruses on aquatic ecosystems are well-known. The coming decades would bring with them the challenges of climate change, increasing aquatic temperatures, increased runoff from inland waters into oceans and other factors. While the interactions of viruses with all these aspects has not been studied in great detail, several studies have indicated a link between temperature and virus abundance (R. Zhang et al. 2020). Moreover, virus communities have been found to be robust, capable of adjusting to changes brought about by salinity, temperature and precipitation, which cellular microbial communities are generally impacted by (Bonilla-Findji et al. 2009; Tseng et al. 2013; Wei et al. 2019).

Metagenomics is currently the method of choice for microbiome studies. Perhaps the greatest contribution of viral metagenomic studies to science would be to annotate long reads of unknown viruses, and to use this information to obtain a broader view of the ecological roles of those viruses (Chow et al. 2015; Okazaki et al. 2019; Weitz et al. 2015; Wommack et al. 2015). As metagenomic studies become routine and less prohibitively expensive to carry out, monitoring virus communities over shorter time scales would be possible.

Over the years, several researchers have proposed that viruses are major regulators of global ecosystems, a concept aptly termed “virocentric ecology” (Hurst and Lindquist 2000; O’Malley 2016; Rohwer and Thurber 2009). The numerical dominance of viruses in aquatic systems is now indisputable. What remains is identifying, classifying and elucidating the roles of those viruses. As metagenomic studies become more routine, combined with better annotation of reference databases, the true contribution of viruses to changing ecosystems would become clearer. It would be no surprise to the scientific community if, through such studies, the virocentric ecology concept was substantiated in the future.

SUMMARY

AND

CONCLUSION

Summary

Isolation and Culture of a Microalgal Virus-Host System

1. Microalgae, specifically cyanobacteria, green microalgae and diatoms, were isolated from freshwater, estuarine and marine ecosystems. Six isolates each of cyanobacteria and diatoms, and five green microalgal isolates were established in culture.
2. Five lytic cyanophages were isolated. The respective host cyanobacteria were *Synechococcus* sp. DP01 (isolated from an estuarine ecosystem), *Synechocystis* sp. ME01 (also isolated from an estuary) and *Limnothrix* sp. VL01 (isolated from a freshwater lake). Phage infection of host cultures was detected by plaque formation.
3. *Limnothrix* sp. VL01 is a bloom-forming, filamentous cyanobacterium. Φ L-VL01, which infects *Limnothrix* sp. VL01, was selected for further characterization. The phage caused lysis of its host in liquid as well as solid medium. Lysis was quantitatively indicated by an 82% reduction in chlorophyll content of host culture over 16 days. Fragmentation of host filaments upon phage infection was observed during SEM studies.
4. Structurally, Φ L-VL01 had an icosahedral head of diameter 49 nm and a short tail. Amplification of the *g20* marker from the phage genomic DNA supported its affiliation to the *Myoviridae* family.

A Study of Selected Aquatic Virus Communities

1. In the present study, among various aquatic ecosystems (rice field floodwaters, lakes, estuaries), flow cytometry studies indicated the highest abundance of virus particles, i.e. 12.1 x 10^6 particles per ml, in rice field floodwater.
2. Virus-family-specific marker genes, such as *g20* representing the *Myoviridae*, and *polB*, representing the *Phycodnaviridae*, were amplified from metagenomic DNA isolated from selected aquatic niches.
3. A metagenomic comparison of two representative ecosystems – Verna Lake (freshwater) and Santana Creek (estuarine) revealed the dominance of the order *Caudovirales*, specifically Family *Myoviridae*, in the respective viral communities. Other families present were the *Siphoviridae*, *Podoviridae* and *Phycodnaviridae*. Family *Microviridae* was exclusively present in the Verna Lake virome.

4. Phages infecting the cyanobacterium *Synechococcus* were abundant in both communities, while the Verna Lake virome also possessed a high percentage of Yellowstone Lake Phycodnavirus.
5. From a functional perspective, viral structural proteins, and proteins involved in activities such as host entry and viral replication, predominated in both viromes.

Conclusion

The present study contributes to research in the field of aquatic virology, with a focus on viruses from previously unexplored freshwater and estuarine ecosystems. The isolation of Φ L-VL01, a lytic cyanophage infecting the filamentous *Limnothrix* sp. VL01, is significant from the perspective of control of cyanobacterial blooms. Studies of virus abundance in various aquatic ecosystems have indicated that rice field floodwaters possess very high viral populations. The presence of large numbers of lytic bacteriophages and cyanophages could have significant repercussions on an ecosystem in which the host organisms play important roles. The predominance of *Myoviridae*-family viruses across diverse aquatic systems has been corroborated in the present study, comparing a freshwater and estuarine virome respectively. A high proportion of unidentified sequences testifies to the presence of novel viruses in both viromes, and provides an incentive for further metagenomic studies of unexplored aquatic systems.

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APPENDIX

BG-11 Medium

A) Stock solutions for BG-11:

	Concentration (grams per litre)
Stock 1:	
Na_2-EDTA	0.1
Ferric ammonium citrate	0.6
Citric acid . $1H_2O$	0.6
$CaCl_2$. $2H_2O$	3.6
Stock 2:	
$MgSO_4$. $7H_2O$	7.5
Stock 3:	
K_2HPO_4 . $3H_2O$	4.0
Stock 5 (Microelements):	
H_3BO_3	2.86
$MnCl_2$. $4H_2O$	1.81
$ZnSO_4$. $7H_2O$	0.222
$CuSO_4$. $5H_2O$	0.079
$CoCl_2$. $6H_2O$	0.050
$NaMoO_4$. $2H_2O$	0.391

All stock solutions may be filter-sterilized and stored at 4°C.

B) For basic BG-11 medium combine the following stock solutions:

Stock Solution	Per Litre of medium
Stock 1	10 ml
Stock 2	10 ml
Stock 3	10 ml
Na_2CO_3	0.02 g
Stock 5	1.0 ml
$NaNO_3$	1.5 g

Adjust the pH to 7.2. Filter-sterilize and store at 4°C.

f/2 Medium

A) f/2 Trace Metal Solution:

$FeCl_3 \cdot 6H_2O$	3.15 g
$Na_2EDTA \cdot 2H_2O$	4.36 g
$CuSO_4 \cdot 5H_2O$ (9.8 g/L dH_2O)	1.0 ml
$Na_2MoO_4 \cdot 2H_2O$ (6.3 g/L dH_2O)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (22.0 g/L dH_2O)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (10.0 g/L dH_2O)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (180.0 g/L dH_2O)	1.0 ml
Distilled water to	1.0 L

B) f/2 Vitamin Solution:

Vitamin B_{12} (1.0 g/L dH_2O)	1.0 ml
Biotin (0.1 g/L dH_2O)	10.0 ml
Thiamine HCl	200.0 mg
Distilled water to	1.0 L

C) For basic f/2 medium combine the following:

$NaNO_3$ (75.0 g/L dH_2O)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ (5.0 g/L dH_2O)	1.0 ml
$Na_2SiO_3 \cdot 9H_2O$ (30.0 g/L dH_2O)	1.0 ml
f/2 Trace Metal Solution	1.0 ml
f/2 Vitamin Solution	0.5 ml
Filtered seawater to	1.0 L

Filter-sterilize and store at 4°C.

Tris-EDTA (TE) Buffer

1. 1 M Tris-Cl: Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.
2. 0.5 M EDTA: Add 186.1 g of disodium EDTA•$2H_2O$ to 800 ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by autoclaving

To prepare 100 ml of TE buffer:

Reagent	*Amount to add*	*Final concentration*
Tris-Cl (1 M, pH 8.0)	1 ml	10 mM
EDTA (0.5 M, pH 8.0)	200 µl	1 mM
H_2O	98.8 ml	

Store at room temperature.

Tris Borate EDTA (TBE) Buffer

For a 5X stock solution:

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
H_2O	to 1 L

Adjust the pH to 8.3. Pass the stock solution through a 0.22 µm-filter and store at room temperature.

Sodium Magnesium (SM) Buffer

Reagent	*Amount to add*	*Final concentration*
Tris-Cl (1 M, pH 7.5)	50 ml	50 mM
NaCl	5.8 g	100 mM
$MgSO_4.7H_2O$	2 g	8 mM
H_2O	to 1 L	

Sterilize by autoclaving. Dispense into aliquots.

Ascorbate Buffer

Prepare the following reagents, each in 10 ml of H_2O

Reagent	*Amount to add*	*Final concentration*
Tris base	0.151 g	0.125 M
Na_2-EDTA.2 H_2O	0.372 g	0.1 M
$MgCl_2.6H_2O$	0.407	0.2 M
Ascorbic acid	0.352 g	0.2 M

Combine equal parts of all the four prepared reagents, adjust pH to 6-7, with 10 N NaOH. Prepare fresh, within 48 hours of use.

PUBLICATIONS

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1. **Noronha, J. M**., Mulla, A. B., Gauns, M. U., & Ghadi, S. C. (2018). Enumeration of total virioplankton and isolation of specific cyanophages from selected aquatic ecosystems in Goa, India. *Current Science, 115*(11), 2147-2150.
2. Poduval, P. B., **Noronha, J. M**., Bansal, S. K., & Ghadi, S. C. (2018). Characterization of a new virulent phage ϕMC1 specific to *Microbulbifer* strain CMC-5. *Virus Research, 257*, 7-13.

CONFERENCE PRESENTATIONS

1. Paper entitled "Flow cytometric and PCR-based detection of algal viruses from aquatic ecosystems of Goa" presented at conference on "New Frontiers in Microbiology and Applied Biology", Goa: January 7-8, 2016.
2. Paper entitled "Flow cytometric enumeration of viruses from selected aquatic ecosystems of Goa" presented at the International Conference "VIROCON", Bengaluru: December 8-10, 2016. Awarded "Best Oral Presentation (First Place)".