Marine Viruses in Coral Reef Ecosystem

THESIS

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サンゴ礁生態系における海洋性ウイルス

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Chitra Ramphul

General Abstract

Five marine phages were successfully isolated from shallow coral reef seawater of Bise and Sesoko, Okinawa, Japan (26º42'N, 127º52'E; 26º39'N, 127º51'E). One of the phages denoted as Vibrio phage RYC (VCPH RYC) infected the pathogenic bacteria, *V. coralliilyticus* (AB 490821, Japan) and the rest infected *V. coralliilyticus* P1 (LMG 23696, Australia). Most of the phages isolated from *V. coralliilyticus* P1 regardless of their sampling sites displayed phages with head and tail structures. The size of the capsids and tails varied slightly from 57.65−58.33 nm and 95.38−105.88 nm, respectively. An elongated icosahedral symmetry capsid was observed denoting that the phages belong to Myoviridae family. However, the phage isolated with the host *V. coralliilyticus* (Japan) exhibited no tails and non-enveloped structures with a diameter of 84.45 nm, which could not be classified. All the phages were chloroform resistant and showed high host specificity. The latent phases for phages lyses of *V. coralliilyticus* P1 and *V. coralliilyticus* (Japan) were 45 and 60 min, respectively. The genetic material of the phages was extracted and the whole genome of the phages was sequenced through the next generation sequencing technique. It was found that the genome of Myoviridae-type viruses was 48 kb and 35 kb, whereas VCPH RYC had a genome size of 158 kb. Cluster analysis of the isolated Myoviridae-type viruses revealed that they were closely related to *Vibrio parahaemolyticus* phage VP16T and VP16C based on partial sequencing. However, analysis of VCPH RYC based on DNA polymerase showed that it was relatively close to Shewanella sp. Phage, though there was a lack of viral sequenced data. As a result, VCPH RYC could be considered a novel phage infecting *V. coralliilyticus*. Phages were also screened in healthy coral tissues (*Montipora digitata*) and lagoon sediments

at the two sampling sites. Nonetheless, only seawater exhibited the presence of phages.

Virus-like particles (VLPs) are very important for their ecological roles, particularly in biogeochemical cycles and for shaping marine communities in both oceans and coral ecosystem. Previous researches in enumeration and functions of VLPs were emphasized on deep sea/open ocean ecosystem instead of coral reef ecosystem. Hence, this study focused on coastal coral reef ecosystem, where Bise and Sesoko reef lagoons were opted as sampling sites. In order to determine the abundance of VLPs as well as prokaryotes, three sub-environments were chosen in the coral ecosystem: seawater, lagoon sediments and healthy coral tissues (*M. digitata*). Healthy *M. digitata* was chosen owing to its high coral cover at both sampling sites. Samplings were done during August 2013. Epifluorescence microscopy was used to determine the abundance of the VLPs and prokaryotes. Seawater was found to have the highest abundance of VLPs among lagoon sediments and coral tissues. However, the VLPs density differed only in lagoon sediments neither in seawater nor in coral tissues. The abundance of bacteria was highest in seawater followed by lagoon sediments and coral tissues. The prokaryotes abundance showed no variation in coral tissues in regards to its location. The only strong positive relationship was found between the abundance of VLPs and bacteria in Bise seawater. Nevertheless, some relationships were found in lagoon sediments and coral tissues at both sampling sites, but none were significant. Hence, the abundance of VLPs differed significantly in lagoon sediments but not in seawater and coral tissues, indicating that local locations showed the least influential effect on the VLPs abundance in seawater and coral tissues.

VLPs can infect all types of marine organisms, where they play important roles in regulating the marine food web, controlling microbial loop, particularly the dynamics of phytoplankton and prokaryote communities. Thus, the links between phytoplankton, the abundance of microorganisms (VLPs and prokaryotes) and different hydrographic factors (temperature, salinity, dissolved oxygen and pH), as well as nutrient availability (nitrates, nitrites, ammonium, total dissolved nitrogen, dissolved organic nitrogen, dissolved organic phosphorus and total dissolved phosphorus) were studied in coastal shallow reef seawater within 5 m depth at Bise (BE, Okinawa, Japan) and Tang Kheng Bay (TKB, Phuket, Thailand). The enumeration of VLPs was more abundant than prokaryotes. Phytoplankton carbon biomass was significantly higher in TKB than BE, but BE showed more planktonic diversity than TKB. VLPs abundance was strongly linked to phytoplankton, bacteria and inorganic nutrients. Nevertheless, the ratio of virus:plankton and virus:bacteria differed between TKB and BE. The abundance of VLPs was linked to nutrient availability rather than hydrographical parameters and ammonium was revealed to have greater influence on the abundance of VLPs.

Thus, it was shown that phages were most abundant in seawater and a possible novel phage VCPH RYC infecting *V. coralliilyticus* (Japan) was isolated. Besides, the abundance of VLPs was high in seawater at Bise, Sesoko and Tang Kheng Bay. Nonetheless, within a similar local environment the viral density of VLPs in seawater did not show any variation, but it differed between TKB and BE. This variation is attributed to the nutrients contents, particularly ammonium concentration in seawater. Thus, marine viruses in coastal coral areas fulfill their ecological roles mostly in accordance with the prevailing nutrient availability.

Keywords: *V. coralliilyticus*, Virbio phage, genomic size, nutrients, prokaryotes

CHAPTER 1

1. General Introduction

1.1 What is a virus?

A virus is a microscopic entity consisting of a single nucleic acid surrounded by a protein coat and is capable of replication only within the living cells of bacteria, animals, plants and Archea. Viruses are found in nearly every ecosystem on Earth. Yet, it is still debatable when it comes to its classification in the Tree of life, since viruses do not abide to all the properties of living things such as breathing, growing, metabolisation but abide only to the reproduction feature. Moreover, viruses do not possess the ribosomal RNA nucleotide sequence upon which the domains of cellular of life are founded; hence they cannot be integrated into the Tree of life (Breitbart et al., 2002; Rohwer and Edwards, 2002).

It is very important to distinguish between the terms "virus" and "virus-like particle". The term "virus" applied only when the microscopic obligate agent become a fully functional biological entity, which is capable of active infection and multiplication within a host. However, the virus-like particle (VLPs) is known to be physically similar to a virus but it is not infectious or it is assumed that it possesses similar genetic sequence to a virus but lack the infectious cycle. A complete structure of a virus particle is called a virion, whereby structures like lipid envelope, protein capsid, nucleic acid, spike projections and virion-associated polymerase can be observed. The term virion is often interchangeable with VLPs.

Basically, viruses range from 20-250 nm in size. They are mostly ~100 times smaller than an average bacterium (Kayser et al., 2005). The shapes of the virus vary widely from helical, icosahedral to more complex structures, with/without tails and

contractile and non-contractile tails. The genetic material is usually found inside a capsid for its protection. The nucleic acid can be single or double stranded DNA/RNA.

Viruses replicate through lytic, chronic and lysogenic processes. Viruses have no internal metabolism and therefore they rely on intracellular system of a susceptible living host cell for all its energy processes. Usually, the life cycles start with a diffusive passive fixation on specific receptors found at the surface of a host cell. This is followed by the injection of the viral genome into the host cell. Briefly, once the viral genetic material is inside the host cell, it starts the synthesis of the viral components together with the genetic material. This will result into various virions which will self-assembled and become the new viruses progeny. The latter will then be released into the surroundings by lysis/rupture of the cell or cell membrane to release the new-formed viruses (Fig. 1.1). This process is known as lytic life cycle. The viruses that undergo this lytic life cycle are known as virulent viruses. This lytic infection is a survival strategy, especially to nutrient-rich environments (Duckworth, 1987).

However in the chronic cycle, new viruses are constantly or sporadically released from the host cell by budding, secretion or extrusion, without any damage to the host cells (Fig. 1.2A; Weinbauer, 2004), but it is not common in aquatic environment. In the lysogenic or temperate cycle, the injected genetic material will embed its DNA/RNA into that of the host genome, known as prophage or provirus. The prophage thus hides into the host cell for long time and then replicate along with host cell. Usually, environmental stresses, physical and chemical factors (pH,

temperature, salinity, nutrients) will alter the life cycle from lysogenic to lytic cycle. Both the prohpage and the host cell benefit from each other as a means of persistence for viruses, when the host cells are low. Prophages may affect the metabolic properties of host cells, which can acquire immunity to other infections, new phenotypic features, virulence factors which result in a niche variety of viral hosts (Fig. 1.2B). A modification to the lysogenic cycle is the pseudolysogenic cycle. It is vastly distributed in the marine ecosystems, whereby the viral genome is not integrated with the host genome but rather remains in an "inactive state" within the host cell. There is no replication of the viral genome which is segregated irregularly into progeny cells, most likely for a few generations. Pseudolysogenic viruses probably occur in nutrients-restricted environment, in very poor nutrient conditions where host cells are enduring starvation and cannot offer enough energy for viral gene expression (Sime-Ngando, 2014).

The virus–host interaction are usually very specific. In other words, the viruses will generally infect a single species, however some viruses can infect related species or hosts belonging to the same genus. Viral infection, which can be triggered by one or more than one type of virus, can occur in all cells organisms (Fuhrman, 1999; Munn, 2006).

1.2 Introduction of marine viruses

Marine viruses were first discovered in 1946. After 30 years later, marine microbial loop was first hypothesized. Methods were developed in 1976 to enumerate marine microorganisms and soon afterwards, in 1979, when Torrella and Morita discovered that viral particles were mostly dominant at sea, and showed similar characteristics to a group of virus which killed certain bacteria known as

bacteriophages or phages. As a result, much emphasis was brought to microbial food web dynamics during that time (Berg et al., 1989). In the 1990s, much research was oriented towards genetic diversity of marine phages, their ecological roles to the marine plankton, and their importance in terms of biogeochemical cycles (Wommack et al., 1999; Bratbak et al., 1993; Fuhrman and Noble, 1995; Suttle, 1994; Gobler, et al., 1997). In the twentieth century, much focus was on viral genomes, genetic and metagenomics (Mannisto et al., 1999; Rohwer et al., 2000; Culley et al., 2006, 2007; Comeau et al., 2006; Jiang et al., 1998; Paul et al., 2005; Mann et al., 2003) as shown in the figure 1.3.

1.3 Abundance of marine virus

Viruses are the most abundant biological entities in the sea, ~10^6–10^{10}/ml (Bergh et al., 1989; Suttle, 2005), however their biomass is very small owing to its small size (Fig. 1.4.). It exceeds bacterial concentrations by 5–20 fold (Fuhrman, 1999). Since viruses are omnipresent across marine systems, a huge research interests from mangroves, estuaries, coastal bays, seas and ocean have been studied (Wommack et al., 1992; Paul et al., 1993; Weinbauer et al., 1993; Steward et al., 1996; Marchant et al., 2000; Hewson et al., 2001, 2006; Auguet et al., 2005; Pan et al., 2007; Seymour et al., 2007). In most cases, marine viruses showed extremely dynamic assemblages, changing from 2–100-fold over temporal scales from minutes to months (Bratbak et al., 1990; Suttle and Chan, 1994; Weinbauer et al., 1995; Bratbak et al., 1996; Winter et al., 2004a). Viral abundances are generally higher in more productive systems, with a general decrease in its abundance from coastal to offshore waters and surface to deep waters (Cochlan et al., 1993; Corinaldesi et al., 2003). Within any environment, the total viral abundance generally varies along with the prokaryotic abundance and

productivity (Cochlan et al., 1993; Jiang and Paul, 1994). Viruses have the highest genetic diversity in marine ecosystems, and the majority of them are bacteriophages, which is a key element controlling the mortality of microorganisms. Phage infection promotes horizontal gene transfer, and incites bacterial phage-resistant mutations. Marine virioplankton play important roles in controlling microbial population sizes, community structure and diversity, affecting microbial food web processes, and marine biogeochemical cycles, such as carbon and nitrogen cycles.

1.4 Marine virus – marine bacteriophage/phage

Bacteriophages are viruses that infect prokaryotes, including bacteria and Archaea. They were first discovered in 1915 and it was only in 1917 that it was named bacteriophage by d'Herelle (Duckworth, 1987). Since then, studies on bacteriophages became of great importance in terms of molecular biology (Duckworth, 1987), abundance of VLPs (Fuhrman, 1999), its ecological roles and biogeochemical cycles. It is estimated that the total amount of phages in the ocean is almost 10^{30} (Suttle, 2007). Phages exist ubiquitously in the ocean and the presence of microorganisms depends largely on the existence of phages. In surface seawater, there are about 10^{7} bacteriophages per mL, ~ 5–25 times higher than that of bacteria (Fuhrman, 1999). Hence, phages can be considered as the most abundant biological entities in the ocean.

1.5 Basic features and classification of marine phages

Marine phages have a high genetic diversity but are limited in phenotypic diversity. Some typical morphological characters include: a head, which encloses the genetic material, usually the double-stranded DNA (dsDNA) and a tail. Tailed phages

belong to Caudovirales, usually demonstrating 10-40% of the total abundance of viruses in aquatic systems (Wommack and Colwell, 2000; Sime-Ngando and Colombet, 2009). Through the tail characteristics, phages can be grouped into Siphoviridae, Podoviridae and Myoviridae (Fig. 1.5, Suttle, 2005). Generally, Myoviridae have a thick, strong and contractile tail, and are mostly virulent owing to its strong lytic ability. Moreover, they show a wide host range, and are mainly isolated from seawater. However, Podoviridae phages have a short and non-contractile tail, a relative strong lytic ability, and their host range is very small and shows strict host specificity. Thus, few of them can be isolated from seawater. However, Siphoviridae phages have long non-contractile and relatively weak lytic ability. They are considered to be temperate/lysogenic phages. In most studies, non-tailed phages or just capsids govern most of the viral abundances. Some authors proposed that this could be artifact due to some mechanical effects (Colombet et al., 2007) as 96% of the described phages are tailed (Ackermann, 2007). Nevertheless, recent studies suggested that non-tailed marine viruses consisted of 50-90% of the observed VLPs could characterize the most ecological importance in the environment (Brum et al., 2013).

1.6 Roles and importance of marine viruses

1.6.1 Microbial diversity

In the ocean, marine viruses are known to shape the microbial community and to regulate the carbon and nutrient availability. Marine viruses are closely linked to bacterial abundance over a broad range of trophic and temporal conditions (Cochlan et al., 1993; Weinbauer et al., 1993; Alonso et al., 2001; Culley and Welschmeyer, 2002; Corinaldesi et al., 2003; Seymour et al., 2006). This denotes that bacteria are among the major dominant hosts for marine viruses. Nonetheless, other microorganisms

including autotrophic prokaryotes (Suttle & Chan, 1993; Sullivan et al., 2003), eukaryotic microalgae (Nagasaki et al., 2004) and heterotrophic nanoflagellates (Nagasaki et al., 1993; Garza and Suttle 1995; Massana et al., 2007) are also considerable hosts for viral infection. For instance, the autotrophic prokaryotes – *Synechococcus* – is responsible for a substantial fraction of primary production in marine waters (Partensky et al., 1999) and 3% of cells are lysed on a daily basis (Suttle, 1994). Large-scaled mortality events, especially in bloom-forming microalgae, are caused by viral infection (Brussaard et al., 1996; Wilson et al., 2002). The effect of viruses on host microbial populations has been revealed through many studies involving changes in viral abundances by concentrating or diluting natural viral communities. The latter showed that viruses influence host cell abundances, growth and production rates and host species composition (Suttle, 1992; Larsen et al., 2001; Winter et al., 2004b; Bouvier and del Giorgio 2007; Zhang et al., 2007). In addition to their roles in microbial mortality, viruses are responsible for the exchange of genetic material between cells (Jiang and Paul, 1998), hence it has been proposed that viruses have played major roles in the evolution of eukaryotes (Villarreal and DeFilippis, 2000).

Furthermore, viral infections can increase the hosts' metabolism, immunity, distribution and evolution in various ways as illustrated in figure 1.6. For instance, the genes (psbA and psbD) which encode for phtotosynthesis in the genera *Synechococcus* and *Prochlorococcus*, contribute around 25% of the global photosynthsesis (Partensky et al., 1999) is in fact carried by a phage genome (Mann et al., 2005). By encoding psbA gene and other photosynthetic genes, phage acquired necessary energy for its production and the hosts benefit from initiating its photosynthetic mechanisms. This is known as horizontal gene transfer. Additionally, the occurrence of phage-encoded

photosynthesis increases the chances of recombination between phage and the host genes (Sullivan et al., 2005) and acts as gene reservoirs that alter the ecological niche of the host. Transformations prevalence can also trigger drastic effects on their hosts. They can change from symbiont/benign microorganism to pathogenic. A good example is in the case of *Vibrio cholera*, a common lagoon bacterium, normally harmless unless it encounters the phage cholera which integrate the toxin (CTX) genes (Waldor and Mekalanos, 1996). Many studies have shown that viruses have high numbers of virulence genes which ease antibiotic resistance, toxicity, host adhesion and host invasion. Hence, the bacteria that take up these genes tend to extend their ecological niches and some are involved in unanticipated metabolic and functional pathways. Another mechanism through which the transfer of genes from marine viruses/viral-like entities to the hosts occurs is through generalized tranducing agents (GTAs) agents (Stanton, 2007). GTA particles resemble to phage morphologically, but they are smaller (with a head diameter of 30–50 nm) and contain a smaller amount (about 4 kilobases, kb) of DNA. The importance of GTAs is that they carry only the host DNA which they then injected into the recipient (Lang and Beatty, 2007) being more effective in the transduction process. The study from Biers et al., (2008) showed that genes encoding GTAs were found in most marine systems and GTAs are produced by marine bacteria which moved genes between species (for example, α-proteobacteria). Hence, GTAs is a way to transfer related gene from one species to another which will finally contribute to nice portioning and microbial diversity. This kind of transduction is limited and restricted to certain hosts only. Moreover, viruses can effectively infect and replicate in marine microorganisms from unrelated environments (Sano et al., 2004). In other words, viruses can contribute to a global genetic pool. Viruses also infect protists and metazoans. In the case of protists, the

cocccolithophores, which are one of the major components of the eukaryotic phytoplankton, are taken as an example. Blooms of coccolithopores are often observed under the influence of increasing temperature and nutrients, for instance *Emiliania huxleyi*, which turn the sea into a milky blue from satellite view. These blooms suddenly disappear and at a fast rate. This mechanism can be referred to "kill the winner" effect. In other words, it is the lysis of a specific species owing to its high increase in population. This is due to viral infection of *E. huxleyi*. It is believed that VLPs co-occur with *E. huxleyi* and other phytoplankton in increasing-nutrient mesocosm experiments (Bratbak et al., 1993). This is further supported in areas where *E. huxleyi* were found in low concentrations but higher concentrations of viruses and free coccoliths were observed. These results suggested that viral lysis event had blown coccolithophores apart. The solar-powered sea slug is an example for metazoan viral infection (Mujer et al., 1996). Two surprising roles of viruses were found in this organism, where they dramatically change the slug's life history and secondly they maybe the vector for horizontal gene transfer between an animal and plant. As a result, marine viruses play essential roles in microbial diversity through horizontal gene transfer and transduction. It was also shown that viruses could shape and control marine organisms through many mechanisms, for instance "kill the winner" effect. Studies have also demonstrated that both the viruses and host organisms benefit from each other in a certain way, however there are certain environmental, physical and chemical factors that could disturb the virus-host interaction, survival strategies and the benefits as mentioned earlier.

1.6.2 Viruses in nutrient cycling

Irrespective of the type of microorganisms that marine viruses infected, the results of the lytic infection play very important roles in marine geochemical cycles. Viruses act as catalysts that will speed up the transmission of nutrients from particulate (living organisms) to dissolved state. The latter can be taken up by microbial communities. Some of the dissolved organic matter will sink and thus lowers the contents of cellular carbon, which means that the carbon is being respired in the surface waters. In the case of phytoplankton, the virus-mediated cells will sink very fast (Lawrence and Suttle, 2004) which will enrich the deeper waters (Lawrence et al., 2002). Others nutrients, including C, N, P, Fe and amino acids are released into surrounding waters through viral lysis (Gobler et al., 1997, Poorvin et al., 2004, Middelboe & Jørgensen, 2006). In such case, carbon is fundamentally averted away from higher trophic levels like grazing food chain. This mechanism is known as the 'viral shunt', through which almost more than one quarter of photosynthetically fixed carbon may be recycled back through the microbial loop (Wilhelm & Suttle, 1999). Viral lysis products and the effects of viruses have on marine microbial food web dynamics undergo vital roles in biogeochemical cycling processes on local through to global scales (Fuhrman, 1999, Suttle, 2005, Suttle, 2007).

1.7 Marine viruses in corals and coral reef ecosystem

The study of marine viruses in corals and coral reefs has just been emerged recently with less than 30 published publications in this field. Most of the studies were focused on the abundance of VLPs in open ocean water rather than shallow-water scleratinian (stony) corals. Corals consist of a variety of organisms, including

dinoflagellate algae (*Symbiodinium* spp.), bacteria, and archaea. The abundance of VLPs in oceanic coral reef seawater is analogous to or higher than 10^5 or 10^7/mL (Wilhelm et al., 2010). It is often reported that VLPs abundance in seawater is nearly an order of magnitude than bacterial abundance (Bergh et al., 1989; Proctor and Fuhrman, 1990). Some other studies demonstrated that increase in VLPs abundance is related to the concentration of local inorganic nutrients, proposing that human activity may impact the viral dynamics in coral reefs (Dinsdale et al., 2008). Depth, salinity, micro-scaled sampling, health state of corals, sampling location were reported to influence the distribution of VLPs in coral reef seawater (Dinsdale et al., 2008; Patten et al., 2006, 2008b; Paul et al., 1993; Seymour et al., 2005). VLPs were also found in corals tissues, coral surface microlayer (CSM) and mucus (Wilson et al., 2005; Davy and Patten, 2007; Lang et al., 2009; Wommack and Colwell, 2000; Davy et al., 2006). Viruses from Caudovirales, Myoviridae, Podoviridae and Siphoviridae were observed in CSM (Suttle, 2005). A few other VLPs were also identified such as small, twinned small, twinned Geminiviruses and long filamentous particles (Nawaz-ul-Rehman and Fauquet, 2009) and lemon-type or bottle-shaped (Prangishvili et al., 2006). These viruses are archaeal-like viruses. The roles and dynamics that these coral-associated archaea viruses remain unclear (Vega Thurber et al., 2009b) although they have formerly been documented (Beman et al., 2007; Kellogg, 2004; Siboni et al., 2008). A diversity of sizes, morphologies of VLPs were observed in white syndrome infected *Acropora muricata,* the most prominent one was from the family Phycodnaviridae and/or Iridoviridae (Patten et al., 2008). Molecular characterization of VLPs in corals showed that phage-like and eukaryotic virus-like particles were observed (Wegley et al., 2007). Sequencing with ss- and dsDNA showed that most of the virus-like sequences were from Microviridae and Myoviridae. However, much of

the difficulties to viromics analysis is due to the fact that viral genome are short (< 1kb - 1.2Mb). Despite, different methods used in amplification and sequencing of virus-associated with coral tissues, almost similar results were obtained whereby Herpesviruses, Phycodnaviruses and phages were the outcomes. The study carried out by Vega Thurber et al., (2008) showed that herpes-like VLPs target most or all cnidarians. The association of VLPs with *Symbiodinium* were projected by the study of Wilson et al., (2001); Lohr et al., (2007); Davy and Patten, (2007) and Fauquet et al., (2005). From all the studies, it was shown that VLPs were associated in many different components of the coral.

1.8 Marine viruses as coral pathogens and phage therapy

VLPs have been noticed in many stony corals – diseased, experimentally stressed and healthy ones too. Thus, the presence of VLPs in corals is barely linked to coral diseases (Patten et al., 2008b; Wegley et al., 2007; Wilson and Chapman, 2001; Vega Thurber et al., 2008). However, there are some studies which showed that coral diseases may be associated to the viral proliferation (Barash et al., 2005; Cervino et al., 2004; Fauquet et al., 2005; Denner et al., 2003; Richardson et al., 1998). It is hypothesized that pathologies of the yellow blotch and white band disease could be linked to viral infection (Cervino et al., 2004). Some coral diseases like White plague could be triggered by distinct agents, including various bacteria and viruses (van Oppen et al., 2009). It is further assumed that coral growth anomalies (GAs) could be associated to viral infection, however, studies in this direction is lacking. Although, it is proposed by Breitbart et al., (2005) that GAs is not generated by bacterial infection. Moreover, it is presumed that corals are vulnerable to viral infections. Since, many

reef-associated organisms, such as fish, invertebrates and macroalgae could possibly enhance the horizontal transfer of viral particles to corals. It is known that these reef organisms had played major roles in transmission of pathogens - bacterial vector (Mumby, 2009; Sheridan et al., 2008). For example, the feeding habit of corallivorous fish (e.g., parrotfishes and butterflyfishes) and invertebrates (Rotjan and Lewis, 2008; Williams and Miller, 2005) could be a possible method for introducing viruses to distinct coral colonies. Some researchers anticipated that macro-algae could be another possible vectors of coral disease, although the mechanisms involved are highly questioned (Jompa and McCook, 2002a,b; Nugues et al., 2004; Smith et al., 2006; Bruno et al., 2007; Rasher and Hay, 2010; Barott et al., 2011). Nevertheless, there is no proof yet to sustain or contest that macroalgae, fish or invertebrates could transmit coral viruses. Corals can also be considered as vectors of diseases. Griffin et al., (1999) were the first to show that corals can harbor human-borne viruses in their mucus and can be considered as an indicator for coastal pollution. Since then, many studies have been based on this concept (Futch et al., 2010; Lipp and Griffin, 2004; Wetz et al., 2004). These studies strongly support that coral mucus can accumulate potential pathogens thus turning into potential vectors. However, the mechanisms involved, the physiological, behavioral, and ecological factors related to this viral transmission frequency and efficiency of stony corals are virtually unexplored (Mao-Jones et al., 2010).

Certain viruses can be beneficial to corals, especially for certain types of coral diseases in terms of phage therapy. It consists of using a specific lytic virus, known as bacteriophage/phage, which will infect the pathogenic bacteria. The virus will replicate itself using the bacteria as its host and then lyse the bacteria cells to release more viruses and stop replication until there are no more host bacteria that are

available. This will result in decreasing the numbers of pathogenic bacteria finally help in decreasing the rate of infection or disease. The team of Effrony (2007) had successful results by using this concept in a closed system, where they used a specific phage, YB1, against *V. coralliilyticus* (LMG 21348) bacteria. Recently, in 2013, Cohen et al., has identified phage, YC, against *V. coralliilyticus* strain P1 (LMG 23696). He noted that the phage was able to hinder the bacterial-induced photosystem inhibition in pure cultures of *Symbiodinium*. Since *V. coralliilyticus* affect the zooxanthellae cells, using phage therapy to treat infected corals seemed favorable and effective. Housby and Mann (2009) also encouraged this promising therapy, since there is no known treatment for coral diseases up to now.

1.9 Aims and objectives

In order to bring more insights in this domain, the main aims of the study is to firstly determine the abundance of VLPs in shallow neritic coral areas from seawater, lagoon sediments and healthy corals tissues (*Montipora digitata*) and secondly to isolate and identify phages from seawater by using *Vibrio coralliilyticus* from Japan (AB 490821) and Australia (LMG 23696) as hosts bacteria. Emphasis were put on several points as mentioned below:

1. To determine and compare the abundance of VLPs, bacteria and cyanobacteria from lagoon sediments, coral tissues (*Montipora digitata*) and seawater from coastal coral areas of Sesoko and Bise, Okinawa, Japan
2. To understand the geographical distribution of the abundance of VLPs from seawater of neritic zone of Bise, Okinawa, Japan and Tang Kheng Bay, Phuket, Thailand.

3. To find out any possible links between the abundance of VLPs to water quality, in terms of nutrients and geographical data of coastal seawater
4. To isolate and identify phage/s using *Vibrio coralliilyticus* as hosts bacteria
5. To establish the geographical distribution of the isolated phages in Sesoko and Bise by comparing the phages in open ocean to that coastal waters
6. To identify the phage/s morphologically and genetically

Chapter 1: Figures

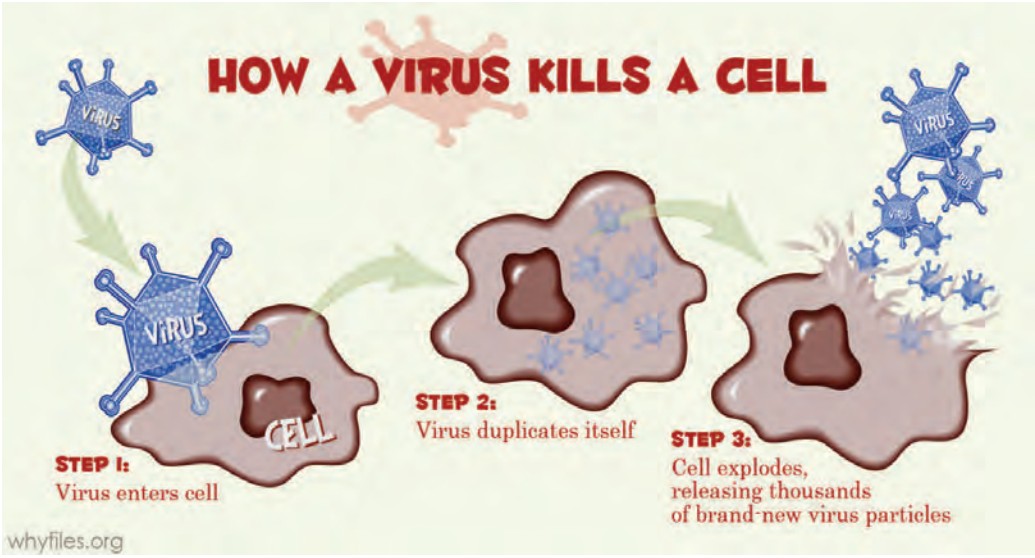
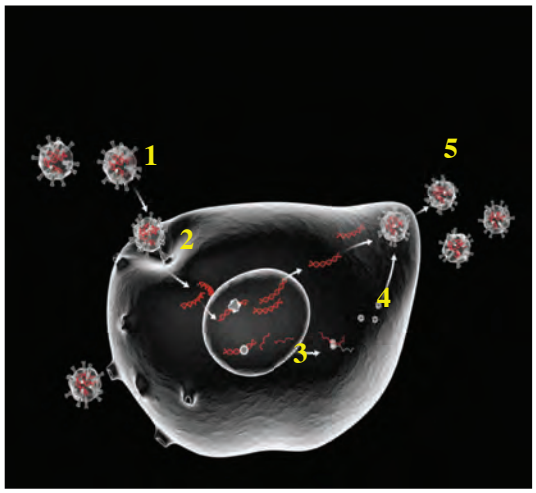
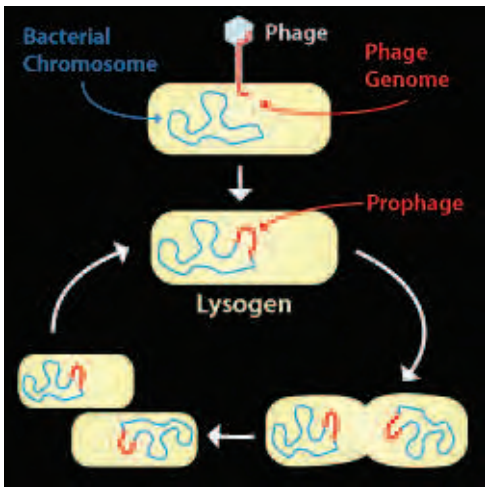


Fig. 1.1: Lytic cycle of a virus.

(http://whyfiles.org/2012/biobombs-blast-cancer/)



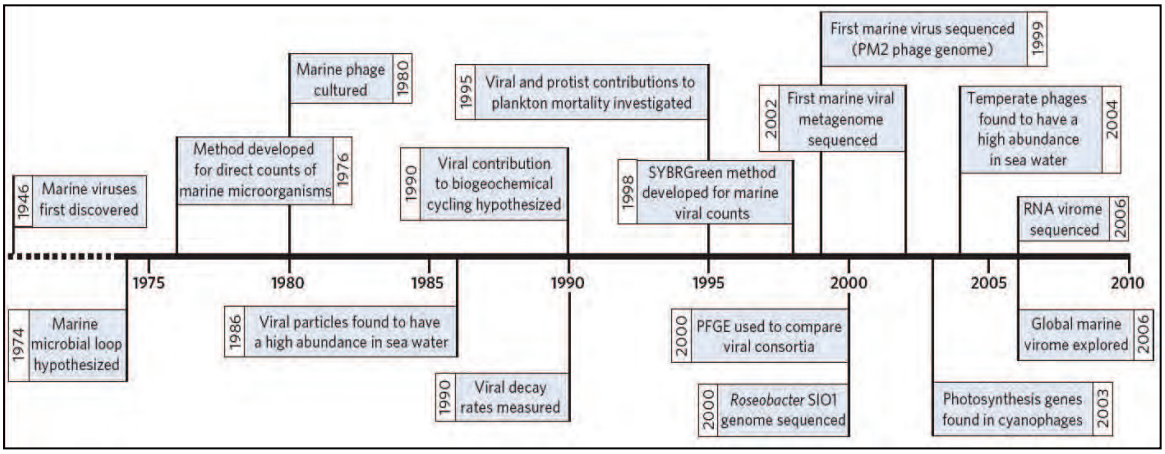


A B

(http://pixshark.com/lysogenic-cycle-labeled.htm)

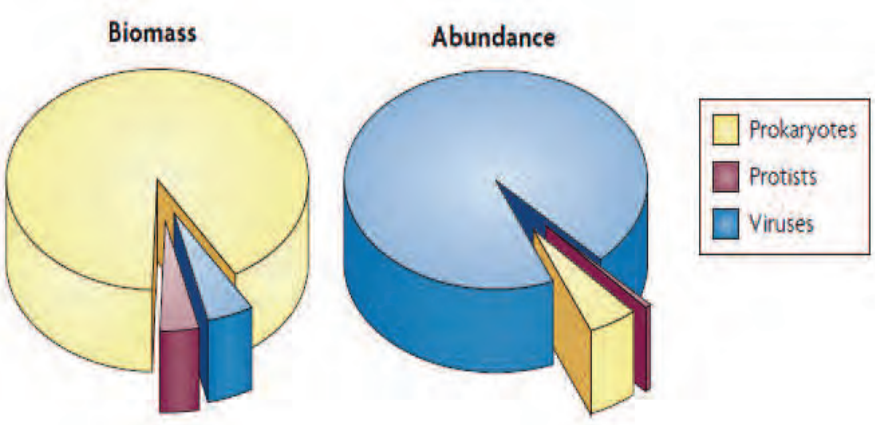
Fig 1.2A: Chronic life cycle of a virus. Steps involved are: 1: Adhere to the cell; 2. Release its genetic material; 3: Uses the host proteins and organelles to produce more virus; 4: The virus particles assemble itself and is released by the host cell; 5: The host cell does not undergo lysis it rather acts as a machinery for virus replication and usually leaving the host cell genetic material intact.

Fig 1.2B: Lysogenic life cycle of a virus. Steps involved are: Adhere to the cell; Release its genetic material; the injected genetic material will embed its DNA/RNA into that of the host genome, known as prophage/ provirus. The prophage thus hides into the host cell for long time and then replicate along with host cell



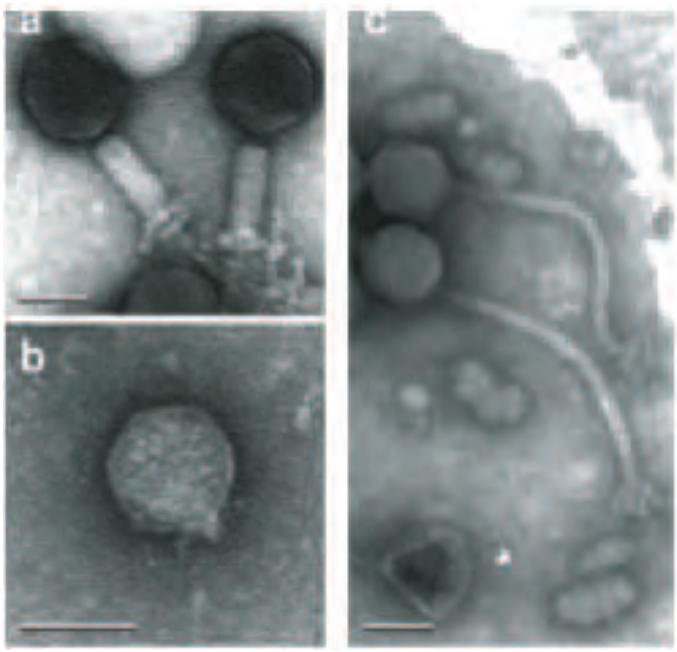
(Rohwer & Thurber, 2009)

Fig. 1.3: Major timeline events in marine virology research



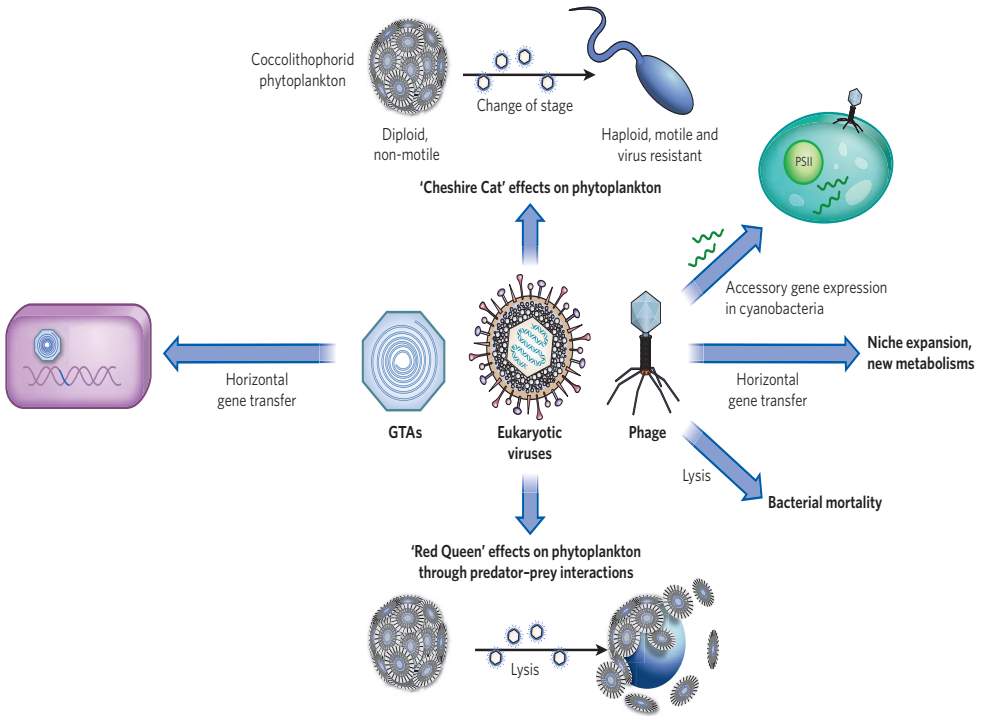
(Suttle, 2007)

Fig. 1.4. Abundance and biomass of viruses, prokaryotes and protists in seawater. The abundance of VLPs/viruses is greater than that of biomass because VLPs and viruses are very small in size.



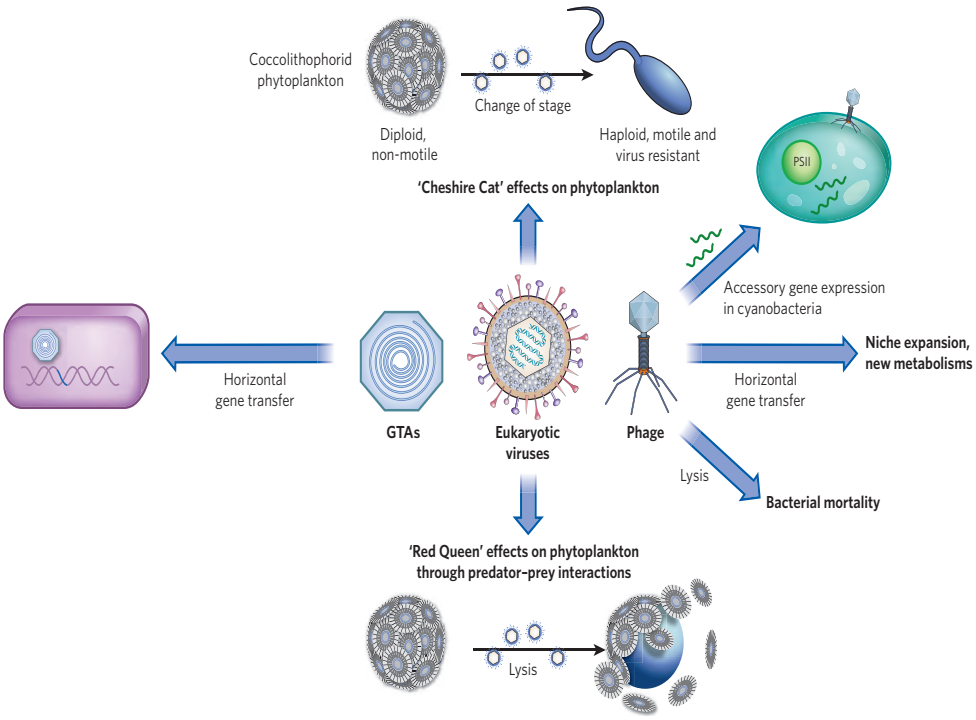
(Suttle, 2005)

Fig. 1.5. Morphological feature of marine phages (three families of tailed dsDNA) isolated from seawater: a – Myoviruses are mostly common isolated phage; b – Podoviruses possess a short non-contractile tail; c – Siphoviruses have long non-contractile tails. Scale bar, 50 nm.



(Rohwer and Vega Thurber, 2009)

Fig. 1.6. Effects of marine viruses on their hosts. Marine viruses, phages and generalized transducing agents (GTAs), can have numerous impacts on host cells. When a phage infects a bacterial cell, it can lyse or transfer the genetic material from previous host (horizontal gene transfer) or from its own genome (accessory genes expression). The transmitted genes can allow the host cell to develop into different niches (for example, through the activation of certain photosynthetic genes which are important to phytoplankton such as cyanobacteria and coccolithophorids). Similarly, small VLPs known as GTAs can transfer genes between marine organisms through 'Red Queen' effect whereby the virus and cell are locked in an evolutionary 'arms race', such that they continue to evolve mechanisms of resistance to each other until finally the virus causes the host cell (exemplified by a coccolithophorid in the figure) to die; in the 'Cheshire Cat' hypothesis, however, the coccolithophorid simply changes from its diploid, non-mobile stage to a motile, haploid stage, thereby evading the virus.



(Rohwer and Vega Thurber, 2009)

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CHAPTER 2

2. Abundance of virus-like particles and prokaryotes in healthy coral (*Montipora digitata*) tissues, lagoon sediments and seawater in Bise and Sesoko coral areas, Okinawa, Japan

Abstract

Virus-like particles (VLPs) are very important for its ecological roles not only in oceans but also in coral ecosystem. In order to determine the abundance of VLPs as well as prokaryotes, two sampling sites were opted: Bise and Sessoko (26º42'N, 127º52'E; 26º39'N, 127º51'E), Okinawa, Japan and three sub-environments samples were chosen in the coral ecosystem: seawater, lagoon sediments and healthy coral tissues (*Montipora digitata*). All the samplings were carried out during August 2013. Epifluorescence microscopy was used to determine the abundance of the VLPs and prokaryotes. It was found that seawater had the highest abundance of VLPs when compared to lagoon sediments and coral tissues. However, the VLPs density differed significantly in lagoon sediments, not in seawater and coral tissues. The abundance of bacteria was highest in seawater followed by lagoon sediments and coral tissues. Bacterial and coccoid cyanobacteria abundance showed no variation in coral tissues in regards to its location. A strong positive relationship was found between the abundance of VLPs and bacteria in seawater of Bise only. Nevertheless, some relationships were observed in lagoon sediments and coral tissues at both sampling sites, but none were significant. The abundance of VLPs and bacteria was highest in seawater. Healthy *Montipora digita* coral tissues from Bise and Sesoko showed no variation in VLPs and prokaryotes abundance. Generally, the abundance of VLPs differed significantly from the different studied components of the coral ecosystem. However, their locations showed the least influential effect in VLPs abundance in seawater and coral tissues but not in lagoon sediments.

Keywords: VLPs, epifluorescence microscopy, bacteria, coccoid cyanobacteria, healthy coral

2.1 Introduction

Virus-like particles (VLPs) is the most abundant biological entities in the marine environment by ~ 15-fold compared to bacteria and Archaea. Yet, VLPs represent only ~5% of the prokaryotic biomass as they are extremely small in size (Suttle, 2007). VLPs play key roles in the marine environment by controlling the population of various organisms and in global geochemical cycles (Suttle, 2005). Marine viral research had gained much more importance with developing tools and techniques both in direct and indirect methods such as viral counts. Most of the enumeration was done through Transmission Electron Microscope (TEM) (Bergh et al., 1989; Borsheim et al., 1990; Sime-Ngando et al., 1996) since the 1950s. However, this technique was very demanding, time consuming and expensive. Since then, new methods (less expensive, rapid and easy) were developed for indirect counts of VLPs, for instance using specific fluorescent dyes to stain the genetic material of VLPs such as SYBR Green I, SYBR Green II, SYBR Gold or YoPro I. Afterwards, the VLPs were counted using a specific light excitation wavelength by using fluorescence microscopy (EFM) (Hennes and Suttle, 1995; Xenopoulos and Bird, 1997; Noble and Fuhrman, 1998; Chen et al., 2001; Middelboe et al., 2003; Wen et al., 2004) or by the use of flow cytometry (Chisholm et al., 1988; Courties et al., 1994; Lindström et al., 2002; Rose et al., 2004). Since the invention of these methods in the late 1990s, much research had been conducted in this field. All these three methods are properly acceptable for enumerating viruses (Hara et al., 1991; Hennes and Suttle, 1995; Weinbauer and Suttle, 1997; Bettarel et al., 2000; Chen et al., 2001). The use of EFM became widely accepted owing to its fast and less expensive technology. Therefore, this study focused on using EFM for the viral counts as it provided a fast, easy, accurate and

inexpensive way of estimating VLPs compared to transmission electron microscope, plaque assays, most-probable-number assays and flow cytometry.

The distribution and abundance of VLPs within coral reef environment is mostly founded on studies done in tropical seawater and sediments. Those studies revealed that VLP abundances in coral reef environment are more or less to 10^5 to 10^7 VLPs/mL, which is a representative of VLP density in oceanic seawater (Wilhem et al., 2010). The VLP abundance is almost one magnitude higher than seawater bacteria (Bergh et al., 1989; Proctor and Fuhrman, 1990). A study carried out by Paul et al., in 1993, showed that the abundance of VLPs in surface water, sediments and several invertebrate taxa changes with distance in relation to the shore, season and salinity. They noted the highest VLP abundances in tropical seawater near mangrove forests ($2.2 \times 10^6 - 1.2 \times 10^7$ VLPs/mL) and during the rainy season, whereas abundances were lower above oceanic reefs (1.5 to 4.3×10^6 VLPs/mL). Overall, they noticed that VLP abundance was inversely proportional to seawater salinity. However, within sediments, VLP abundance ranged from 1.35 to 5.33×10^8 VLPs cm^{-3}, and similar densities were observed within deposit-feeding sea cucumber guts. Other studies estimated that the variation in VLP abundance across Pacific coral atolls were ~4.9 to 5.1×10^5 VLPs/mL of seawater, with no variation in VLP abundance across sampling depths of ~10 m, and 500 and 25 cm above reefs. The increase in VLP density was relational to the concentration of local inorganic nutrients, including human population sizes over time (Dinsdale et al., 2008). Other studies showed that VLP densities altered across several depths above a coral reef and within lagoon shoreward of the reef (4 to 8.5 x 10^5/mL) (Patten et al., 2006, 2008a; Seymour et al., 2005). This is to say that VLP density varies according to time, scale, sampling location and methods employed, however, it denotes that coral reefs contain important abundance of VLPs

(Dinsdale et al., 2008; Patten et al., 2006, 2008b; Paul et al., 1993; Seymour et al., 2005).

Some studies revealed the presence of VLPs in coral mucus and coral tissues through TEM technique. Wilson et al., (2005) were the first ones to characterize VLPs morphologically from stony corals, *Pavona danai*. They discovered that VLPs were present in coral tissues and *Symbiodinium* cells. They found that there was an important morphological diversity of VLPs in corals and *Symbiodinium* cells in both healthy and stressed coral specimens. Davy et al, in 2006, showed that VLPs were more abundant in *Acropora formosa* after heat shock through flow cytometry. Some coral-associated viruses were found in coral surface microlayer (CSM) of *Acropora muricata*, *Porites lobata*, *Porites lutea*, and *Porites australiensis* (Davy and Patten, 2007). The authors concentrated the mucus samples through filtration and ultracentrifugation and then seek for the VLPs through TEM. They found a diversity of VLPs of tailed phage, polyhedral/spherical, lemon-shaped, filamentous, and unique VLPs and 17 sub-groups based on morphological similarities to previously described viral Families. Nawaz-ul-Rehman and Fauquet, (2009) found small twinned Geminiviruses and long filamentous particles in the CSM. The first archaeal-like viruses were detected from stony corals (Beman et al., 2007; Kellogg, 2004; Siboni et al., 2008; Wegley et al., 2004), however their dynamics and roles in the coral microbial assemblage remain poorly understood (Vega Thurber et al., 2009b). Some studies showed that VLPs were mostly predominant in coral epidermis regardless to healthy or white syndrome-infected corals suggested that viruses entered the coral tissues mainly from seawater. The authors hypothesized that *A. muricata* colonies were persistently infected with members of the Phycodnaviridae and/or Iridoviridae (Patten et al., 2008a).

Overall, VLPs can be observed in coral reef seawater surface, sediments and coral tissues and coral mucus. This study focused on abundance of VLPs at two different lagoonal coral areas in Okinawa, Japan. The abundance of VLPs within the sub-environment: seawater, lagoon sediments and coral tissues were determined as well as prokaryotic abundance, including bacteria and coccoid cyanobacteria using epifluorescence microscopy. Moreover, possible relationships between VLPs abundance and prokaryotes were revealed.

2.2 Materials and methods

2.2.1 Sampling

To establish the abundance of VLPs at Bise and Sesoko (26º42'N, 127º52'E; 26º39'N, 127º51'E), Okinawa, Japan, regions were chosen as sampling sites (Map 2.1) owing to the high prevalence of *Montipora digitata* and due to their relative short distance for sampling processing. Healthy, *M. digitata,* corals (~ 5 cm x 3), seawater (5 L) and lagoon sediments (50 mL x 3) (Fig. 2.1 A-C) were collected within 5 m depth in triplicates during August 2013. All samples were treated within 4h to maximize extraction of most of the VLPs. The sites Bise was denoted by BE whereas Sesoko as SO.

2.2.2 Extraction of prokaryotes and VLPs from sub-environment samples

2.2.2.1. Seawater

Hundred milliliters of seawater was fixed with 38% formaldehyde (1% final concentration) onsite and stored in darkness at 4°C until further analysis.

2.2.2.2. Lagoon sediments

Fifty milliliters (x3) of sediments were collected within 5 cm depth from the benthic surface of sampling sites, BE and SO. Extraction of prokaryotes and VLPs were done according to modified protocol of Helton et al., (2006). Five milliliters of lagoon sediments was freshly weighed and sodium pyrophosphate (8 mL of 10 mM) and 8 µL of 5 mM EDTA were added to the sediments in a 50-mL sterile conical centrifuge tube (Millipore, Ireland). The tubes were subjected to sonication for 20 min and shake at the highest speed for another 20 min. The mixture was then centrifuged (5000g) for 25 min at 20°C. Part of the resultant supernatant was used for prokaryotes counts after filtration through 0.45 µm Sterivex (Advantec, USA) whereas the other part was filtered through 0.22 µm Sterivex (Advantec, USA) for viral analysis. All the solutions used were 0.02 µm prefiltered. The resultant samples were fixed with 38% formaldehyde (1% final concentration) for epifluorescence microscopy (EFM) analysis and stored in darkness at 4°C until analysis.

2.2.2.3. Coral tissues

Healthy *M. digitata* corals fragments (5 cm) were collected at both sites, BE and SO, in triplicates. The corals (~2 g) were rinsed thoroughly with 0.02 µm-filtered

autoclaved water. Coral tissues were extracted by using water-jet blasting using 0.02 µm-filtered autoclaved water. The high pressure of the water disrupted the tissues from the coral skeleton. The tissues were then homogenized, centrifuged (2000 × g, 20°C, for 10 min) and the supernatant was filtered through 0.8 µm nitrocellulose membrane (Millipore Co., Ireland) filters for prokaryotes analysis. For viral analysis the supernatant was filtered through 0.22 µm Sterivex (Advantec, USA). The resultant samples were fixed with 38% formaldehyde (1% final concentration) for EFM analysis and stored in darkness at 4°C until analysis.

2.2.3 Enumeration of virus-like particles and prokaryotes through epifluorescence microscopy analysis

The number of VLPs and bacteria was determined by epifluorescence microscopy according to Suttle and Fuhrman (2010). SYBR green I was used to stain the genetic material of microorganisms and in case of VLPs (DNA & RNA) showed low affinity to non-nucleic acids particles. All fixed formaldehyde samples (seawater, lagoon sediments and coral tissues) were filtered through a 0.02 µm Anodisc filter (25 mm Ø, Whatman, Germany) back up with 0.8 µm filter using vacuum filtration (15-20 kPa). The Anodisc filters were dried in darkness at room temperature for some minutes and 2.5 µL of 10% SYBR Green I (x10 000 in DMSO, Invitrogen) was added and left for staining for 15 min in the dark. The excess of stain was wiped away by barely touching the Anodisc filter with a damp filter paper. Slides were mounted using 12 µL of an anti-fade solution (50% of 0.05 M PBS/50% glycerol, pH 7.5, 10% *p*-phenylenediamine) before and after placing the filter on the slide.

Bacteria and coccoid cyanobacteria were stained using 6-diamidino-2-phenylindole (0.5 µg/mL, DAPI). DAPI was added to the fixed water samples (1:10)

and was kept in dark for 15 min. Simple vacuum filtration was done using the 0.2 μm black nitrocellulose filters. A drop of immersion oil (Olympus, Type-F, Japan) was added before and after putting the filter onto the glass slides. A coverslip was gently placed on the filter.

VLPs and prokaryotes cell abundance were determined by counting within 15 to 30 randomly selected fields using epifluorescence microscope (Nikon Eclipse E600, Japan). Bacteria were observed under light excitation of 400 nm whereas 450-490 nm was used to spot VLPs and coccoid cyanobacteria. VLPs appeared very small dim pin-pricks whereas prokaryotes were bigger and brighter stained. The abundance of the VLPs and prokaryotes per mL was estimated by the following equation of Suttle and Fuhrman (2010):

Abundance of VLPs mL^{-1} in the sample (Vt) $= [(Vc \div Fc) \times (At \div Af)] \div S$

Where Vc = total number of viruses counted,

Fc = total number of fields counted,

At = surface area of the filter (μm^2)

Af = area of each field (μm^2),

S = volume of sample filtered (mL).

2.2.4 Statistical analysis

All the data was tested for normality. The Friedman's ANOVA was used to screen for any discrepancies among the different samples groups: seawater; lagoon sediments and coral tissues. Kendall's W test was used to indicate the strength of variation among the samples groups. Two-tailed Independent Student's *t*-test was used to determine the significance difference in between sampling sites of a specific

sample group. Spearman rho's correlation was used to determine possible relationships between VLPs and prokaryotes.

2.3 Results

2.3.1 Abundance of VLPs

The abundance of VLPs showed significant difference among the studied samples: seawater; lagoon sediments and coral tissues according to Friedman ANOVA test (X^2 (2, N = 4) = 8, $p < 0.05$). Kendall's W test (0.99, p = 0.018) revealed significant strong differences among the samples. The density of VLPs was highest in seawater (1.32 x 10^6 particles/mL BE; 1.04 x 10^6 particles/mL SO) when compare to lagoon sediments and coral tissues (4.91 x 10^5 particles/mL BE; 5.07 x 10^5 particles/mL SO; 1.40 x 10^4 particles/mL BE; 1.80 x 10^4 particles/mL SO, respectively). However, no discrepancy was noted in VLPs enumeration between seawater of BE and SO (t = 0.024, df = 4, $p > 0.05$, 2-tailed). Similarly, *M. digitata* tissues bear almost same amount of VLPs regardless of their locations (t = -0.019, df = 4, $p > 0.05$, 2-tailed). Nevertheless, lagoon sediments showed variation in VLPs abundance according to their locations (t = 9.301, df = 4, $p < 0.05$, 2-tailed) as illustrated in the figure 2.2. The slides (Fig. 2.3) showed that VLPs appeared very dim tiny pin-pricks, whereas bacteria and other cells like protists are bigger in size and more brighter.

2.3.2 Abundance of bacteria

The Friedman ANOVA test revealed significant difference among the studied samples in terms of bacterial abundance (X^2 (2, N = 4) = 14.6, $p < 0.05$; Fig 2.4). A fairly strong difference was observed among the samples with a Kendall's W test of

0.528 ($p < 0.05$). No variation in bacterial abundance was noted from corals collected at BE and SO ($t = 3.552$, df = 4, $p > 0.05$, 2-tailed). However, seawater and lagoon sediments from BE and SO differed significantly in bacteria density, respectively ($t = 4.773$, df = 4, $p < 0.05$, 2-tailed; $t = -3.267$, df = 4, $p < 0.05$, 2-tailed). Bacteria appeared bright rod-like structures under the fluorescence microscope (Fig. 2.5).

2.3.3 Abundance of coccoid cyanobacteria

Coccoid cyanobacteria varied in seawater, lagoon sediments and coral tissues (X^2 (2, $N = 4$) = 9.333, $p < 0.05$; Fig 2.6). According to Kendall's W test (0.78, $p < 0.05$), a relatively strong variation was noted among the sample groups. Coccoid cyanobacteria enumeration differed from seawater of BE to that of SO ($t = -9.449$, df = 4, $p < 0.05$, 2-tailed). However, the abundance of coccoid cyanobacteria of lagoon sediments and coral tissues were almost similar at BE and SO ($t = -1.362$, df = 4, $p > 0.05$, 2-tailed; $t = 0.783$, df = 4, $p > 0.05$, 2-tailed). A few rounded reddish-orange cells were observed in lagoon sediments and coral tissues at both sampling sites (Fig. 2.7).

2.3.4 Relationship of VLPs to prokaryotes

A positive strong relationship was found between the abundance of VLPs and bacteria in BE seawater (Table 2.1). However, this trend was not observed in seawater of SO but a strong significant negative relation was noted with coccoid cyanobacteria. Nevertheless, some relationships were observed in lagoon sediments and coral tissues at both sampling sites, but were not significant.

2.4 Discussion

Quantification of VLPs abundance and prokaryotes is very important in order to understand the roles they play in coral ecosystem in terms of spatial and temporal dynamics. Hence, the abundance of VLPs in the studied neritic areas showed that VLPs density was highest in seawater at BE and SO. This VLPs density ranged within that of coral reef environment and oceanic seawater (Bouvy et al., 2012; Wilhem et al., 2010; Suttle, 2005). It was noted that the VLPs abundance in seawater at both sampling locations was one magnitude higher than that of seawater bacteria. This observation corresponded to Bergh et al., (1989) and Proctor and Fuhrman (1990). Some studies revealed that abundance of VLPs in seawater varied according to its location, seasonality and distance from the shore (Paul et al., 1993). However in this study, it was found that VLPs density in seawater showed no variation with respect to its location. This could be explained because healthy corals – *Montipora digitata* – dominated both sampling sites and sampling was done during the same season – summer. Though sampling depths could influence the VLPs abundance at both sites, it was demonstrated by a study carried out by Dinsdale et al., (2008) that sampling depths did not influence the VLPs density in seawater. Another study demonstrated that VLPs density was highest in tropical seawater in mangroves forests and during rainy season (Paul et al., 1993). This study was carried out during summer season where the rain effects was least influential and seawater was collected 50 cm above the corals colonies, which demonstrated no relation to mangrove forests.

The VLPs abundance in lagoon sediments was relatively low compared to other studies (Donovaro and Middelboe et al., 2010; Carreira et al., 2013). However, few studies in coral reef sediments are available (Paul et al., 1993; Patten et al., 2008). Most of benthic studies were from freshwater aquatic systems and deep-sea

ecosystems (Bettarel et al., 2006; Donovaro et al., 2008; Hewson et al., 2001; Middelboe et al., 2006; Middelboe et al., 2011). Sediments play essential roles in aquatic carbon cycle (Glud and Middelboe, 2004), which is mainly triggered by a high number of viruses, usually 10-100 times higher than in the water column. In principle, aquatic sediments could be regarded as an optimum platform for viral production, provided that the host organisms are present and active (Glud et al., 2005). In this study, the prokaryotes abundance was low compared to other studies (Helton et al., 2012; Carreira et al., 2013), which could explain the low VLPs density at both sites. Danovaro et al., in 2002, noted that the lowest VLPs densities were found when cell sizes were largest, bacterial growth and turnover rates were lowest. Other authors reported that benthic viral community could be very dynamic and is strongly associated with bacterial activity (Middelboe et al., 2003; Glud and Middelboe, 2004). It is possible that the low VLPs density could be due to methodological artifacts like vortexing, shaking and centrifugation (Danovaro et al, 2001; 2008 a, b). However, almost similar method as Helton et al., (2012) was used in this study. Nevertheless, the viral and bacterial abundance in this study was 5 and 3 magnitude lower than that reported by Helton et al., (2012). Yet, the abundance of VLPs in lagoon sediments differed significantly from BE and SO. This could be due to the presence of sea cucumbers and other marine organisms which were frequently present at BE compared to SO. There could be deposit-feeding from sea cucumbers' gut, which could explain the difference of VLPs in lagoon sediments at both sites (Paul et al., 1993).

Nonetheless, coral tissues at both sampling sites bear similar VLPs abundance. One of the reasons could be that same coral species were sampled at both sites. Another reason is that the sampled corals were in healthy state, as some studies found that VLPs abundance was higher (compare to controls) when the corals were under

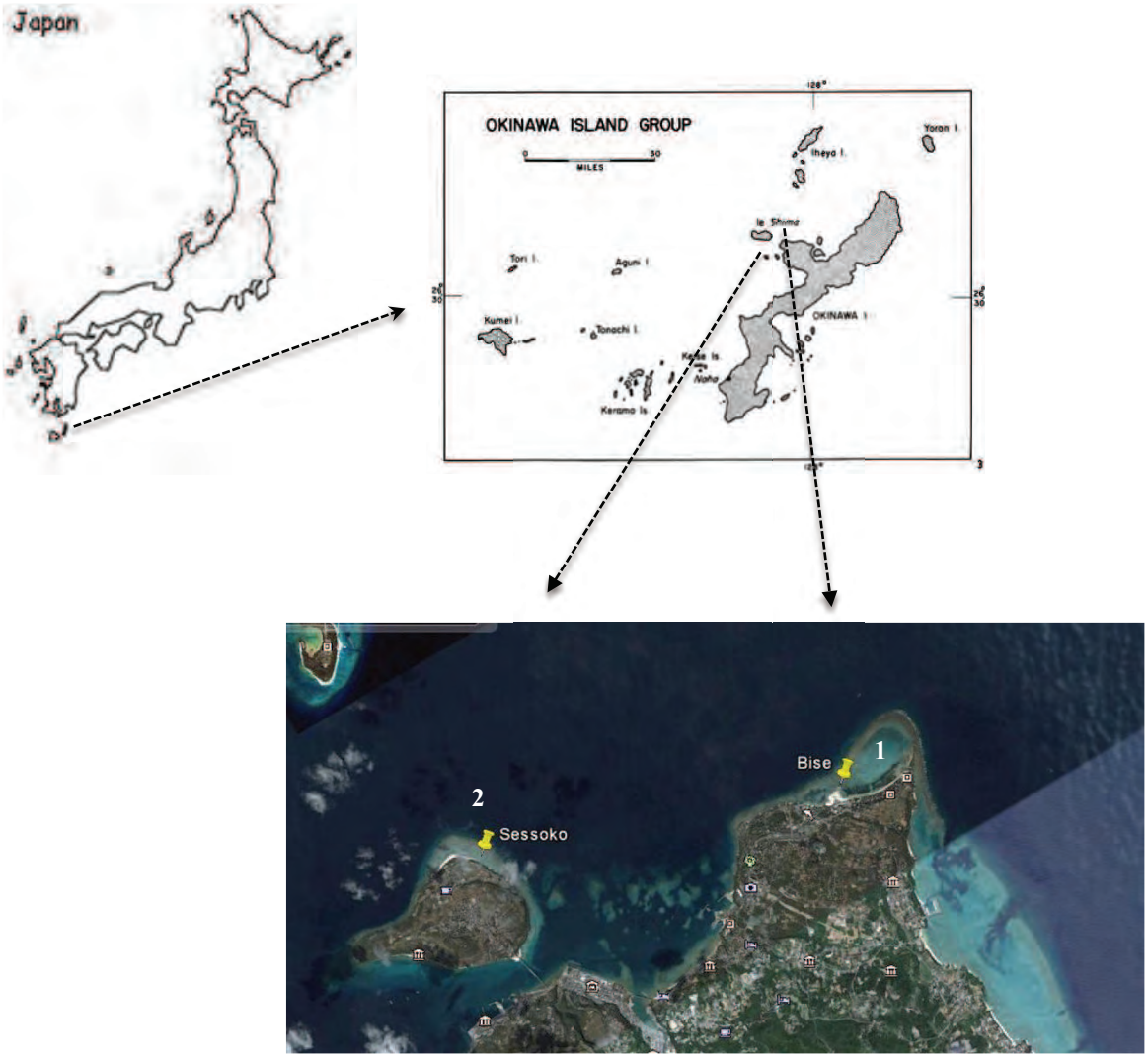
stress/shock (Davy et al., 2006). Other studies showed that a high diversity of VLPs existed in corals, especially coral surface microlayer (Davy and Patten, 2007; Nawa-ul-Rehman and Fauquet, 2009). Different morphology of viruses was also found in stony corals, for instance Archeal-like viruses (Kellog, 2004; Wegley et al., 2004). It is believed that VLPs form coral microbial assemblage whether the corals are healthy or not (Weil et al., 2006; Patten et al., 2008), however their dynamics and roles are still unclear (Vega Thurber et al., 2009b).

A positive relationship between VLPs abundance and bacteria were found in BE seawater only. This could be regarded as most of the VLPs present in seawater at BE are free VLPs and could be considered as bacteriophages (Paul et al., 1993; Wommack and Colwell, 2000; Suttle 2005). It is interesting to point out that this similar trend was observed in most marine systems from shore to offshore (Culley and Welschmeyer, 2002) and in oligotrophic waters (Alonso et al., 2001). They also found a positive significant relationship between VLPs and bacteria, which corroborated with this study. However, the VLPs density of seawater at SO did not divulge any relationship with bacteria. This lack of coupling between the two variables could be explained by the presence of bacterioplankton and vibrioplankton. However, this finding could be limited as a strong negative relationship was found between VLPs abundance and coccoid cyanobacteria. Moreover, in the other studied samples, no significant relationship was found. This could be due to the small plethora of studied microorganisms, which could also limit this finding.

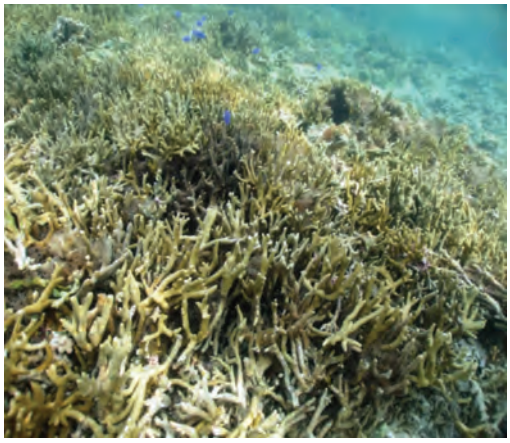
2.5 Conclusion

This study showed that VLPs abundance varied across seawater, lagoon sediments and coral tissues. The VLPs density did not differ in seawater and coral tissues from BE and SO, but location showed some influence on the VLPs abundance in lagoon sediments in this study. Moreover, the VLPs in seawater at BE could be regarded as bacteriophages, owing to its positive coupling with bacteria. The difference in VLPs density in lagoon sediments could be associated with the marine organisms that were mostly present at BE. Overall, VLPs abundance varied across the studied samples, however, location showed the least influential effect in VLPs abundance in seawater and coral tissues but not in lagoon sediments.

Chapter 2: Figures and Tables

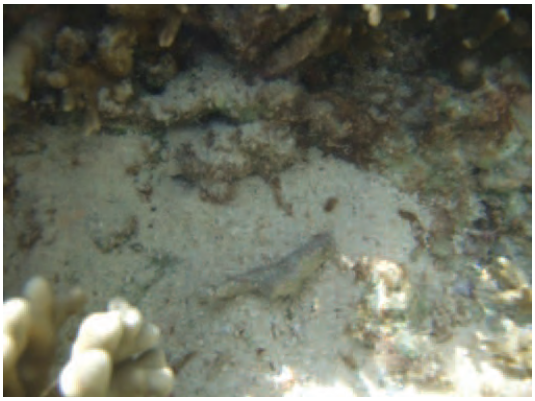


Map 2.1: Map showing sampling sites: (1) Bise (26º42'N; 127º52'E) & (2) Sesoko (26º39'N; 127º51'E).



A

B



C

Fig. 2.1: Samples in Bise and Sesoko, A: Healthy corals *Montipora digitata*; B: Seawater; C: lagoon sediments in corals areas.

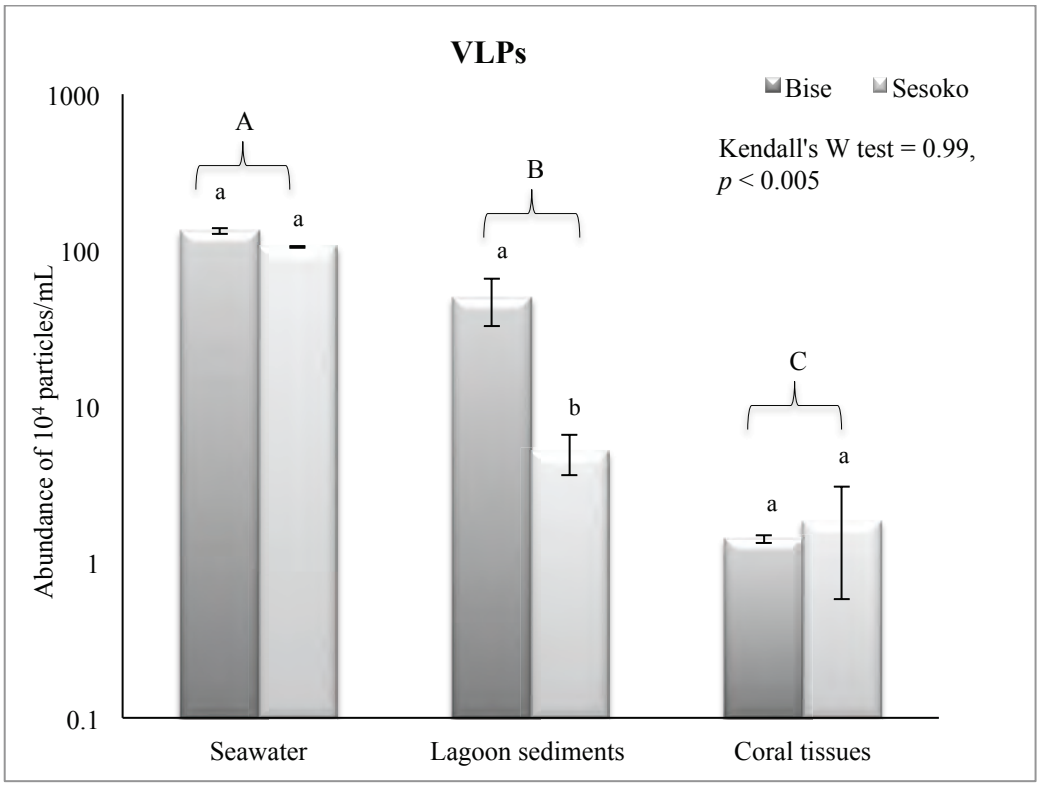
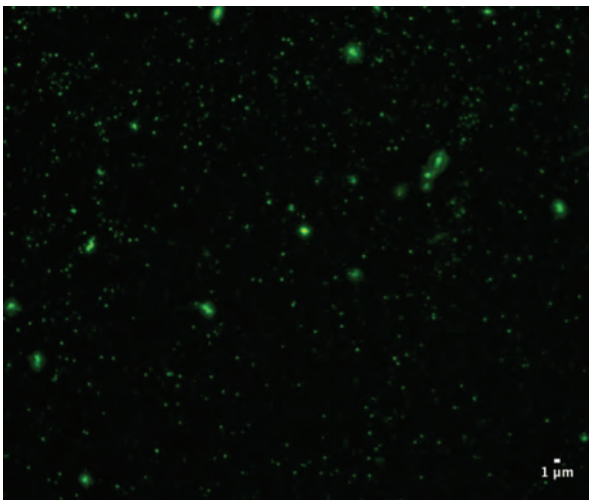


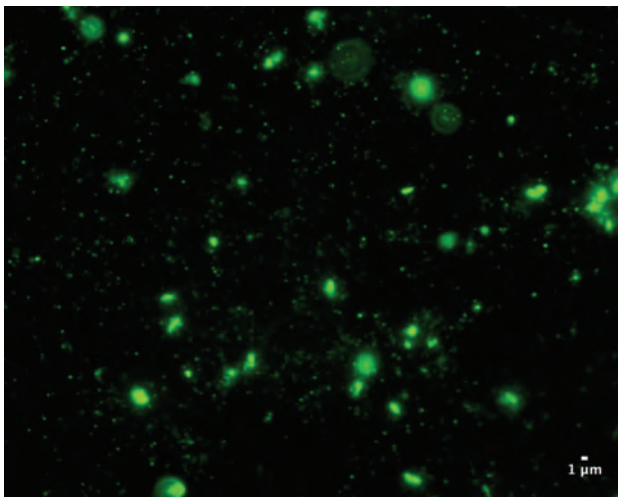
Fig. 2.2: Abundance of VLPs in seawater, lagoon sediments and coral tissues (*Montipora digitata*) of BE (Bise, Okinawa, Japan) and SO (Sesoko, Okinawa, Japan). Bars represent the Mean ± SD for n = 3; 15 readings per replicate. Uppercase letters represent comparison among the sample groups (Friedman ANOVA test, $\alpha = 0.05$). Kendall's W test = 0.99, $p < 0.005$. Lowercase letters represent comparison between the two sampling sites within a sample group (Independent Student's *t*-test). Bars not sharing similar letter are significantly different ($\alpha = 0.05$).

Virus-like particles (VLPs) in seawater, lagoon sediments and coral tissues

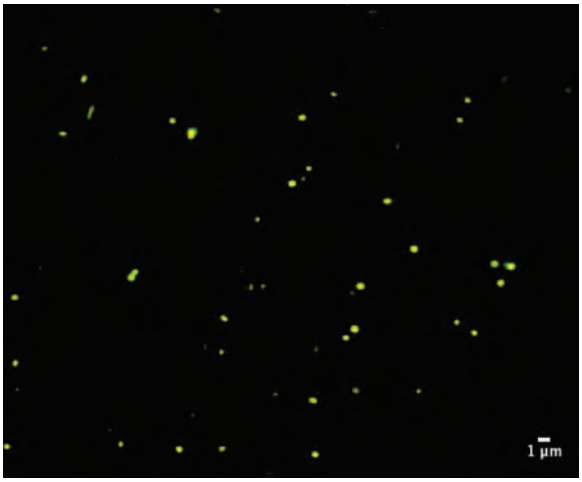
1. Seawater (Bise)



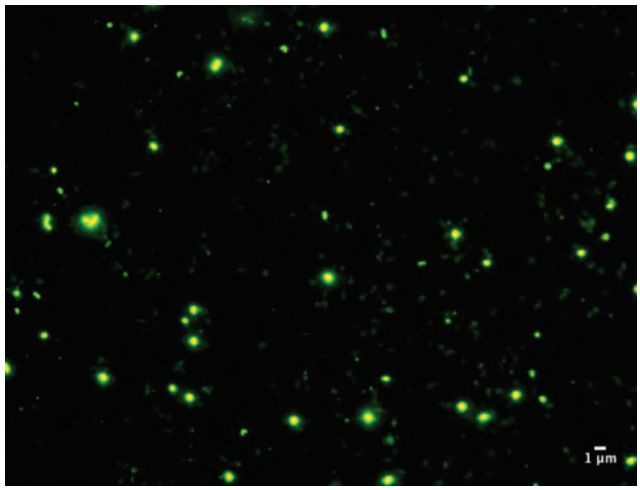
Seawater (Sesoko)



2. Lagoon sediments (Bise)



Lagoon sediments (Sesoko)



3. Coral tissues (Bise) Coral tissues (Sesoko)

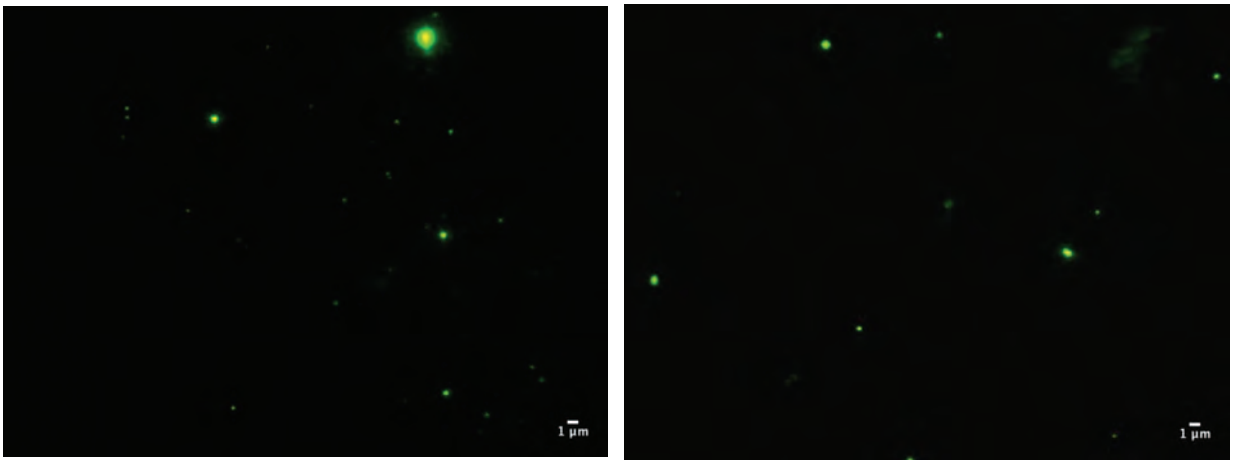


Fig. 2.3: VLPs in seawater, lagoon sediments and coral tissues (*Montipora digitata*). VLPs are represented by dim pin-pricks. Very bright and bigger structures could be bacteria and protists cells.

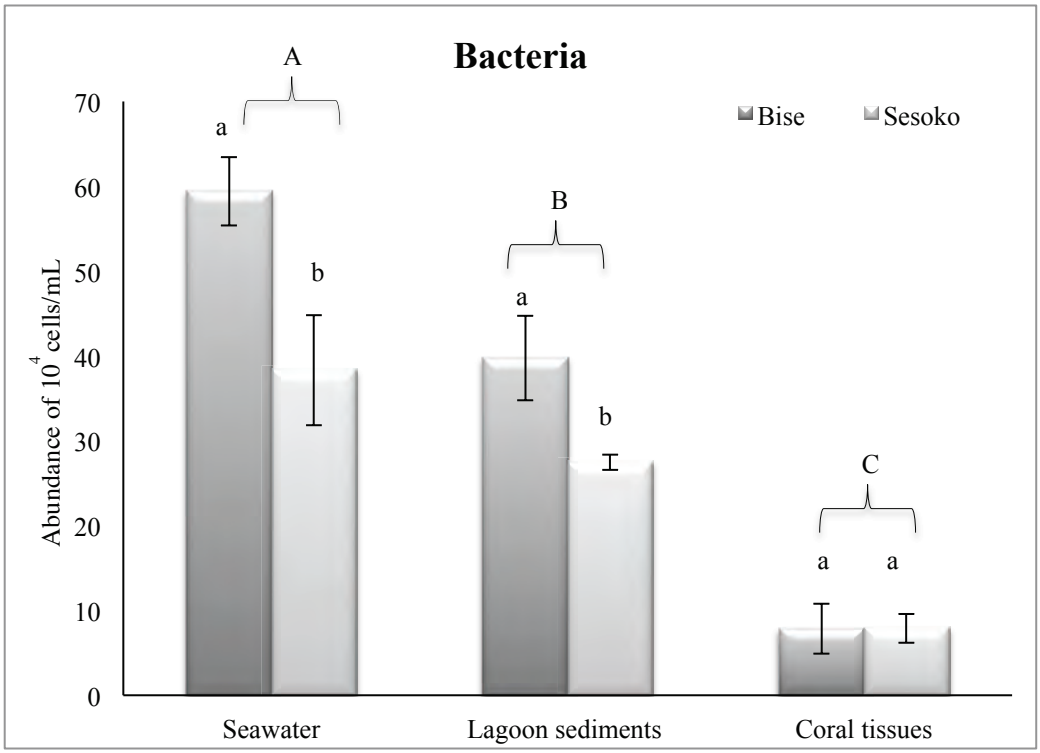
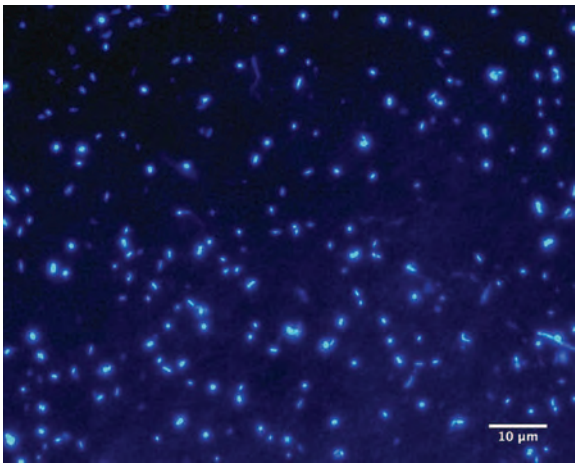


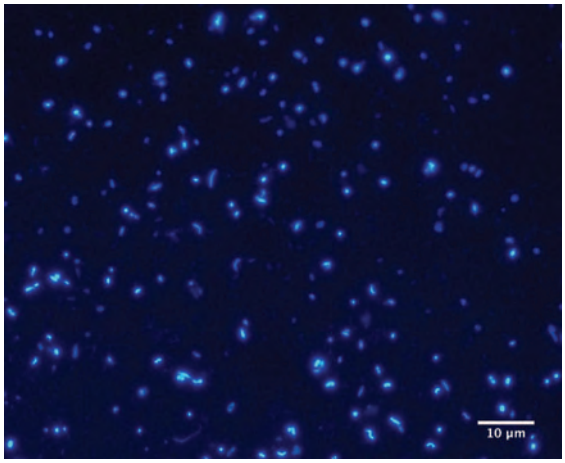
Fig. 2.4: Abundance of bacteria in seawater, lagoon sediments and coral tissues (*Montipora digitata*) of BE (Bise, Okinawa, Japan) and SO (Sesoko, Okinawa, Japan). Bars represent the Mean ± SD for n = 3; 15 readings per replicate. Uppercase letters represent comparison among the sample groups (Friedman ANOVA test, $\alpha = 0.05$). Kendall's W test = 0.58, $p < 0.005$. Lowercase letters represent comparison between the two sampling sites within a sample group (Independent Student's *t*-test). Bars not sharing similar letter are significantly different ($\alpha = 0.05$).

Bacteria

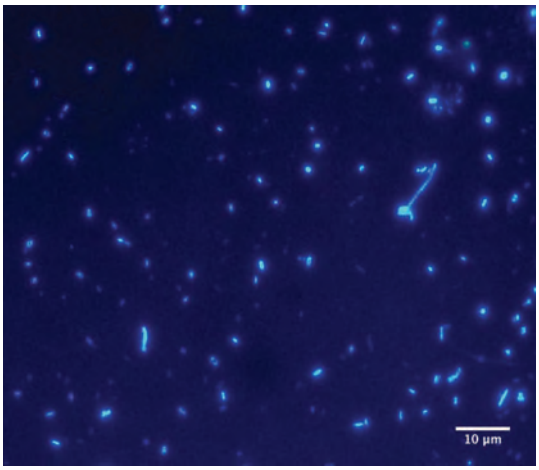
1. Seawater (Bise)



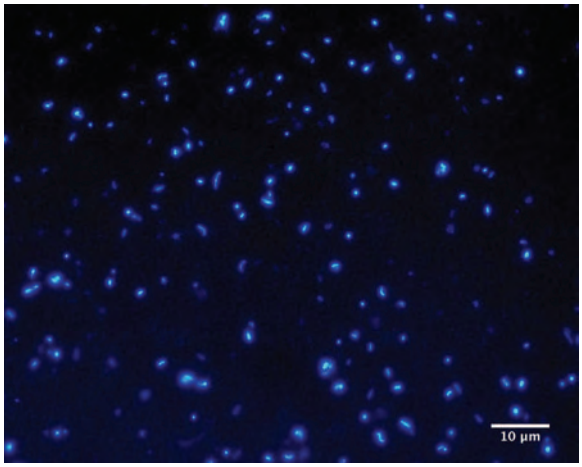
Seawater (Sesoko)



2. Lagoon sediments (Bise)



Lagoon sediments (Sesoko)



3. Coral tissues (Bise) Coral tissues (Sesoko)

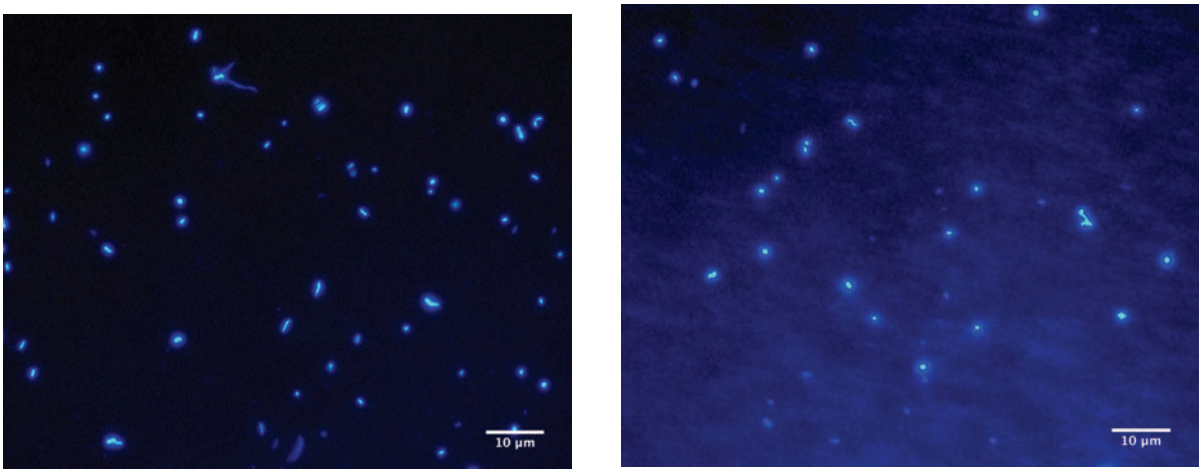


Fig. 2.5: Bacteria in seawater, lagoon sediments and coral tissues (*Montipora digitata*). Bacteria are represented by rod-like bright structures.

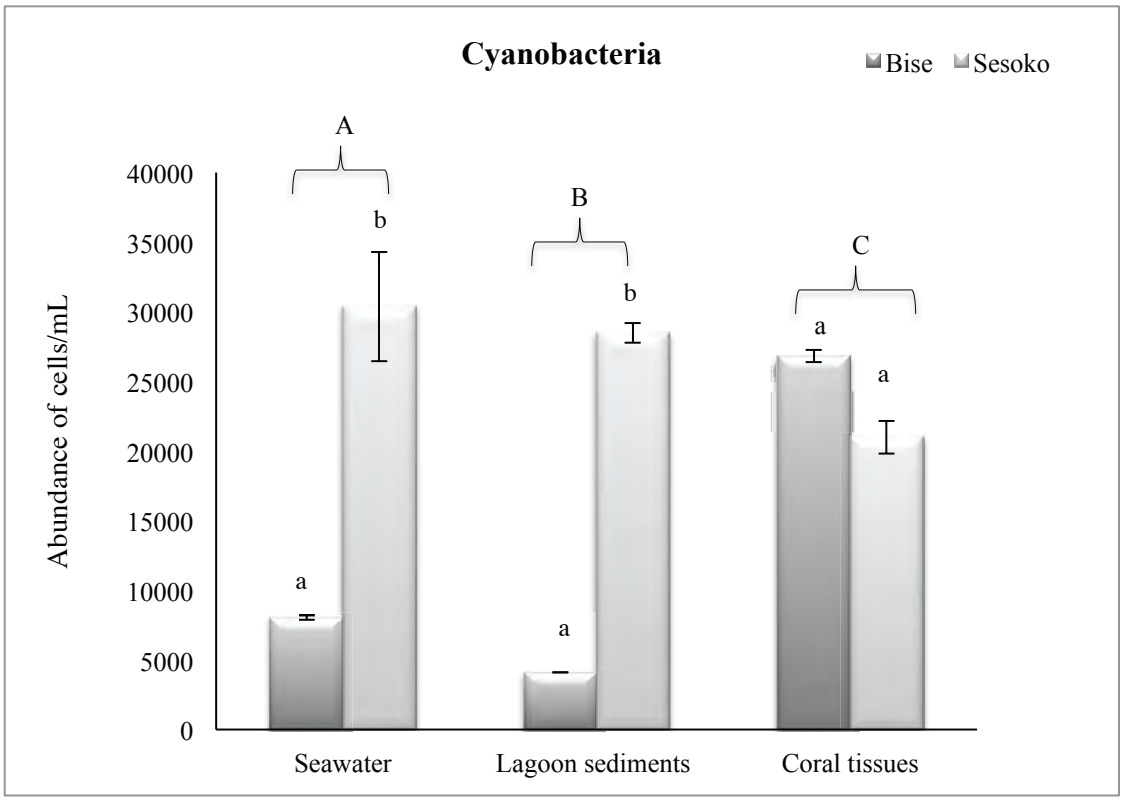
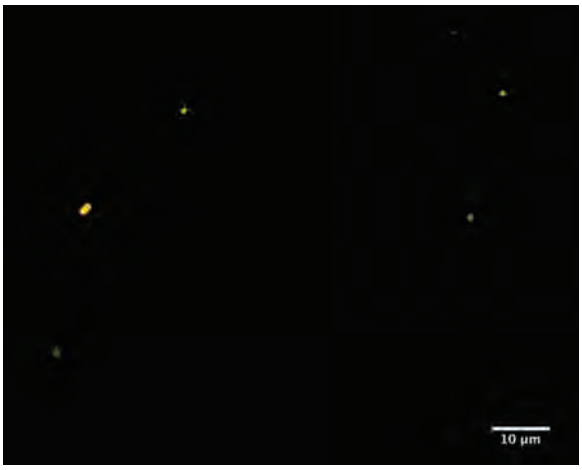


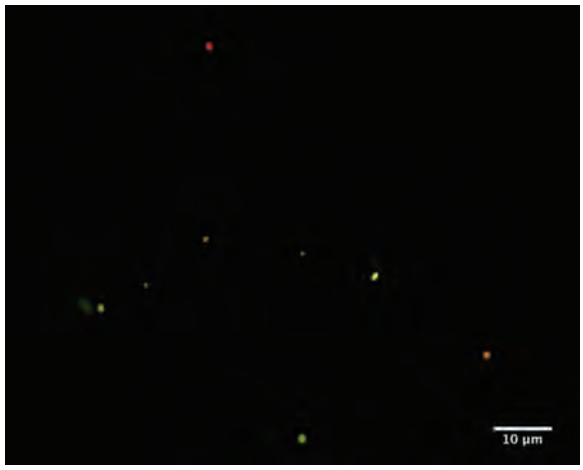
Fig. 2.6: Abundance of coccoid cyanobacteria in seawater, lagoon sediments and coral tissues (*Montipora digitata*) of BE (Bise, Okinawa, Japan) and SO (Sesoko, Okinawa, Japan). Bars represent the Mean ± SD for n = 3; 15 readings per replicate. Uppercase letters represent comparison among the sample groups (Friedman ANOVA test, α = 0.05). Kendall's W test = 0.78, $p < 0.005$. Lowercase letters represent comparison between the two sampling sites within a sample group (Independent Student's *t*-test). Bars not sharing similar letter are significantly different (α = 0.05).

Coccoid cyanobacteria

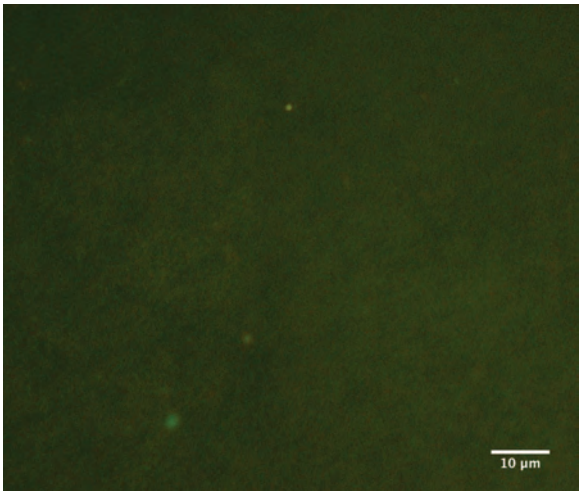
1. Seawater (Bise)



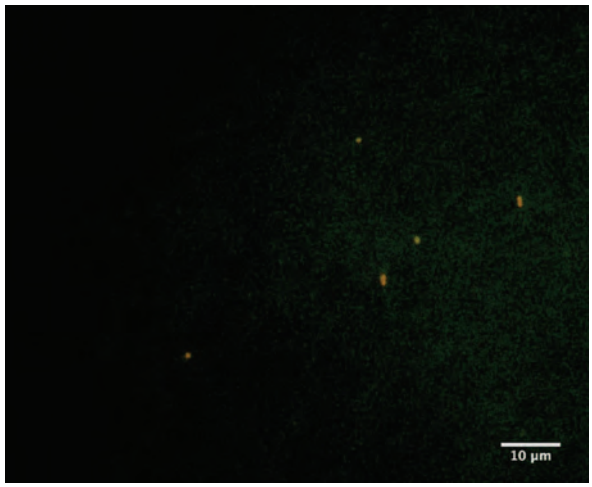
Seawater (Sesoko)



2. Lagoon sediments (Bise)



Lagoon sediments (Sesoko)



3. Coral tissues (Bise) Coral tissues (Sesoko)

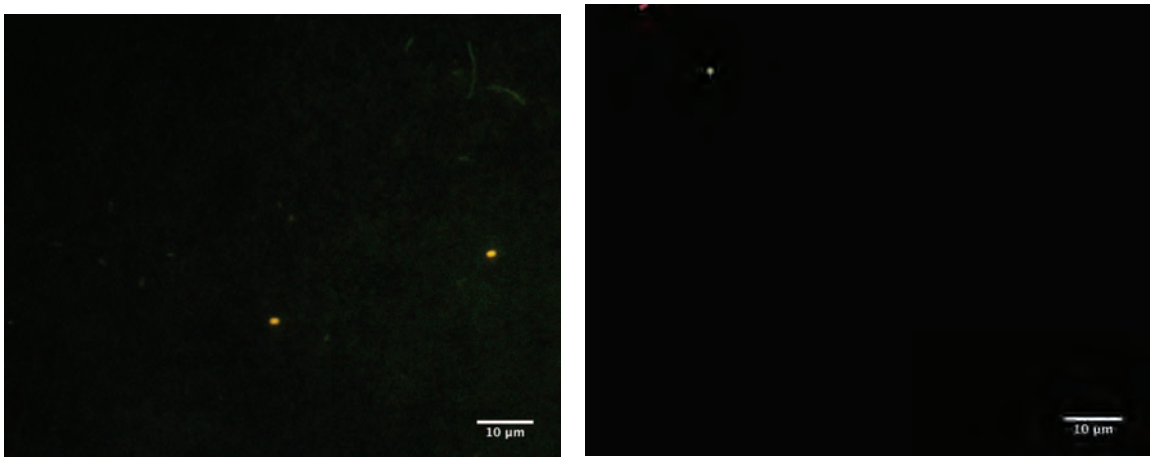


Fig. 2.7: Bacteria in seawater, lagoon sediments and coral tissues (*Montipora digitata*). Bacteria are represented by rod-like bright structures.

Table 2.1: Relationship between VLPs and prokaryotes in seawater, lagoon sediments and coral tissues at Bise and Sesoko, Okinawa, Japan.

Spearman's rho correlation				
Sampling sites	Microorganisms (cells/mL)	VLPs (particles/mL)		
		Seawater	Lagoon sediments	Coral tissues
Bise	Bacteria	0.98**	-0.5	0.5
	Coccoid cyanobacteria	0.87	-	-0.5
Sesoko	Bacteria	-0.85	0.5	0.5
	Coccoid cyanobacteria	-0.97**	-0.5	0.5

** Correlation is significant at the 0.01 level (2-tailed).

CHAPTER 3

3. Abundance of virus-like particles and its links to phytoplankton, bacteria and nutrients cycling in coastal coral ecosystem

This chapter was published in Eco-Engineering, 27 (3), 81-90, 2015

Abstract

Virus-like particles (VLPs) play key ecological roles in coral ecosystem. They can regulate the phytoplankton and prokaryote dynamics and communities. Yet, the factors that regulate the abundance of VLPs within coastal coral ecosystem are not clearly established. The link between phytoplankton, microorganisms (VLPs, bacteria, and coccoid cyanobacteria) abundance and hydrographic factors (temperature, salinity, dissolved oxygen and pH), as well as nutrient availability (nitrates, nitrites, ammonium, total dissolved nitrogen, dissolved organic nitrogen, dissolved organic phosphorus and total dissolved phosphorus) was studied in coral coastal seawater within 5 m depth at Bise (BE, Okinawa, Japan) and Tang Kheng Bay (TKB, Phuket, Thailand). Enumeration by epifluorecence microscopy revealed that VLPs were more abundant than bacteria and coccoid cyanobacteria. Phytoplankton biomass was significantly higher in TKB than BE, but BE showed more planktonic diversity than TKB. VLPs abundance was strongly linked to phytoplankton, bacteria and inorganic nutrients. However, the ratio of virus:plankton and virus:bacteria differed between TKB and BE. The abundance of VLPs is linked to nutrient availability rather than to the hydrographical parameters. Multiple regression analysis showed that ammonium had greater influence on the abundance of VLPs. Thus, marine viruses in coastal coral areas fulfill their ecological roles mostly in accordance to the prevailing nutrient availability.

Key words: coastal coral ecosystem, epifluorescence microscopy, phytoplankton, prokaryotes, VLPs

3 .1 Introduction

Coral reefs are very well known for its biological and economic importance not only for human beings, but also for other organisms. It sustains rich, diverse and active communities of microorganisms including bacteria and marine viruses (Paul et al., 1986; Rohwer et al., 2002; Vega Thurber and Correa, 2011). These microorganisms are important components in nutrient cycling processes (Ferrier-Pagès et al., 1998), thus contributing a major resource for coral species (Bak et al., 1998).

Viruses are the most dominant biological entities in the ocean. Marine viruses were first discovered in 1946 and since then, it became a new field to explore. In the 1990s, much emphasis was given on understanding the roles of marine viruses on biogeochemical cycles. Consequently, marine viruses was observed to shape and structure various communities through viral lysis of numerous microorganisms including autotrophic and heterotrophic organisms. Thus, marine viruses influence and regulate the carbon and nutrient availability (Proctor and Fuhrman, 1990; Fuhrman, 1999) in the ocean. However, the mechanisms on how marine viruses control host populations, structure microbial and plankton dynamics and communities and ocean ecosystem functions still remain unresolved. Various factors such as nutrients, hydrographical, environmental, abiotic factors can influence the distribution and abundance of virus like particles (VLPs). While many studies have focused on deep sea environment (Middelboe et al., 2006; Kellog, 2010), those dedicated for coral areas/reefs are recently emerging. Marine viruses in shallow-water scleractinian corals and coral reef ecosystem have demonstrated that viruses could infect corals and zooxanthellae (Wilson et al., 2005; Danovaro et al., 2008), and recently lytic phages

were isolated which could be used as phage therapy for some coral diseases (Cohen et al., 2013; Efrony et al., 2007).

To further understand the role of marine viruses on host population dynamics with respect to their environment, this study, therefore, focused on determining the abundance of VLPs and its links to phytoplankton, prokaryotes, hydrographical and nutrients availability.

3.2. Materials and methods

3.2.1 Sampling and collection of hydrographic data

Seawater samples (3L in triplicates) were collected into cleaned acid-washed opaque bottles (5L) within 5 m depth in the neritic coral areas of Bise (13:25; 26°42'N, 127°52'E; Okinawa, Japan) and Tang Kheng Bay (11:45; 07°48'N, 98°24'E; Phuket, Thailand) in August and November of 2013, respectively. Heavy rain was noticed at Tang Kheng Bay a few days before sampling and this could influence the sampling. Both sites were characterized by the presence of healthy corals. The dominant coral species at Bise was *Montipora digitata* whereas *Platygyra* sp. was the case at Tang Kheng Bay. Sampling was done during summer season at both locations and water was collected 50 cm above the corals colonies. Seawater temperature, salinity, dissolved oxygen concentration, pH and depth were measured at each sampling sites using a multisensor (Hydrolab MS5 MultiparameterSonde, HachHydromet, USA) within 5-minute intervals.

3.2.2 Analysis of seawater quality and nutrient contents

Nutrients such as ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), phosphates (PO_4^{3-}), total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were

analyzed according to Hansen and Koroleff (2007) using an auto-analyzer (TRAACS 2000, BL Tek, Germany). One hundred milliliters of seawater was prefiltered by GF/F (Whatman, UK) and stored in dark at −20 °C until analysis. Samples were analyzed in triplicates. TDN and TDP were determined by using the $K_2S_2O_8$ wet oxidation according to Grasshoff et al. (1999). Concentrations of dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) were acquired by subtracting the sum of NO_3^-, NO_2^- and NH_4^+ (DIN) and dissolved inorganic phosphorus (DIP, PO_4^{3-}) from TDN and TDP, respectively. The detection limits were 0.052 µM for $NO_3^- + NO_2^-$, 0.01 µM for NO_2^-, 0.020 µM for NH_4^+, and 0.020 µM for PO_4^{3-}. Reproducibility (margin of error) of nutrient analysis was ± 0.2 % for NO_3^-, ± 0.5% for NO_2^-, ± 1.2% for NH_4^+, and ± 0.8% for PO_4^{-3}. The results were expressed as µmol/L.

3.2.3 Enumeration of virus-like particles, bacteria and coccoid cyanobacteria

The abundance of VLPs and prokaryotes was measured by epifluorescence microscopy. The samples were fixed onsite using 38% formaldehyde (1% final concentration) and stored in the dark at 4 °C until microscopic analysis (processed within 15 d from collection). As described by Ortmann and Suttle (2009), 3 mL of fixed samples was filtered through a 0.02 µm Anodisc filter (Anodisc 25, Whatman, GmbH, Germany) using 0.8 µm as back up filter using vacuum filtration (15−20 kPa). The Anodisc filter was dried in dark, and was then stained with SYBR Green I (x10 000 in DMSO, Invitrogen, Oregon, USA). Slides were mounted using an anti-fade solution (50% of 0.05 M PBS/50% glycerol, pH 7.5, 10% *p*-phenylenediamine). Bacteria and coccoid cyanobacteria were stained using 6-diamidino-2-phenylindole (0.5ug/mL, DAPI). DAPI was added to the fixed water samples (1:10) and was kept in dark for 15 min. Simple vacuum filtration was done using the 0.2 µm black

nitrocellulose filters. A drop of immersion oil (Type-F, Olympus, Japan) was added before and after putting the filter on the slides. VLPs and prokaryotes cell abundance were determined by counting within 15 to 30 randomly selected fields using epifluorescence microscope (Nikon Eclipse E600, Japan). Prokaryotes were observed under light excitation of 400 nm whereas 450−490 nm was used to spot VLPs and coccoid cyanobacteria. VLPs appeared very small dim pin-pricks whereas prokaryotes were bigger and brighter stained. The abundance of the VLPs and prokaryotes per mL was estimated by the following equation of Suttle and Fuhrman (2010):

Abundance of VLPs mL^{-1} in the sample (Vt) $= [(Vc \div Fc) \times (At \div Af)] \div S$

Where Vc = total number of viruses counted,

Fc = total number of fields counted,

At = surface area of the filter (μm^2)

Af = area of each field (μm^2),

S = volume of sample filtered (mL).

3.2.4 Determination of phytoplankton diversity and abundance

One liter of water was collected into a cleaned plastic bottle at both sampling sites and buffered formalin at a final concentration of 5% was added. The samples were stored in darkness at room temperature until further analysis. An inverted microscope (200−400x magnification) was used to identify and count the plankton according to Chihara and Murano (1997). Phytoplankton was screened based on their composition and size and were categorized into taxonomic classes.

3.2.5 Determination of prokaryotes and phytoplankton carbon biomass

Phytoplankton biomass of different species was calculated using carbon-to-biovolume relationship. Average cell dimensions of phytoplankton were calculated from measurements of 30−50 cells for each species. The biovolume was estimated from the average cell dimensions assuming the closest approximation of geometric shape. Biomasses of diatoms and other micro- and nanoplankton were calculated using the relationships given in Menden-Deuer and Lessard (2000):

Diatoms: C $cell^{-1}$ in pg = biovolume $^{0.811}$ x 0.288

Other phytoplankton: C $cell^{-1}$ in pg = biovolume $^{0.819}$ x 0.76

Bacterial and coccoid cyanobacteria carbon biomass were calculated using biovolume-to-biomass conversion factor:

Bacterial and coccoid cyanobacteria carbon biomass were calculated using biovolume-to-biomass conversion factor given by Lee and Fuhrman (1987) as:

C $cell^{-1}$ in pg = biovolume x 0.38

Biovolume (in μm^3) of bacterioplankton was determined by measuring the size of more than 30 cells using a digital image analyzer attached to the fluorescence microscope, and assuming the closest approximation of geometric shape. The biomass was expressed in µg C/L.

3.2.6 Statistical analysis

The data were analysed for normality through the Shapiro-Wilk's test. Two-way analysis of variance (ANOVA) was performed to find out whether the variables varied according to geographic location as well as among microorganisms, and was validated

by the Tukey`s *post-hoc* test ($\alpha = 0.05$). Differences of means for phytoplankton were evaluated by using 2-tailed Independent Student's *t*-tests. However, Mann-Whitney-U test was performed for non-parametric data. All the data were transformed into $\log_{10}(x + 1)$ to abide to normality and then bivariate analysis was used to establish the possible relationships between variables, microorganisms, phytoplankton, nutrients and hydrographical data. Multiple regression analysis using the abundance of VLPs as dependent variable, and hydrographic data and concentration of nutrients as independent variables, was carried out to determine the parameters that influence on the abundance of VLPs in coral areas.

3.3.0 Results

3.3.1 Hydrographical and nutrients contents

The hydrographical features in the seawater of Bise (BE; Okinawa, Japan) and Tang Kheng Bay (TKB; Phuket, Thailand) differed significantly (Mann-Whitney-U test, $p < 0.05$; Fig. 3.1). Similarly, the nutrients contents of seawater of the two sampling sites revealed significant variation (Fig. 3.2), especially dissolved organic nitrogen (13.50 ± 1.46 µmol/L and 4.44 ± 0.18 µmol/L for BE and TKB, respectively), DIN (BE: 1.43 ± 0.02 µmol/L; TKB: 4.47 ± 0.58 µmol/L), ammonium (BE: 0.58 ± 0.04 µmol/L; TKB: 2.73 ± 0.21 µmol/L) and DIP (BE: 0.01 ± 0.00 µmol/L; TKB: 0.30 ± 0.07 µmol/L). No difference in the concentration of nitrates and dissolved organic phosphorus were obtained (Mann-Whitney-U test, $p > 0.05$, Fig. 3.2) between the two sites.

3.3.2 Abundance of VLPs, bacteria and coccoid cyanobacteria

The abundance of microorganisms (VLPs, bacteria, and coccoid cyanobacteria) differed between the two sampling sites (Fig. 3.3, Annex), where the seawater of TKB contained significantly higher ($F_{(2,59)}$ = 844.1; p < 0.001) concentration of microorganisms than BE. Similarly, there was a similar significant occurrence (main effect) among the microorganisms present in these two areas, although the concentration of VLPs was 4.39 and 335.64-times higher than that of bacteria and coccoid cyanobacteria, respectively ($F_{(2, 59)}$ = 1160; p < 0.001; Fig. 3.3). A significant interaction effect existed between the two sampling sites and the three microorganism categories, which indicates that the abundance of these microorganisms in seawater varied differently with respect to their coral area location ($F_{(2,59)}$ = 601.1; p < 0.001). Specifically, abundance of bacteria and coccoid cyanobacteria was not significantly different in BE and TKB. However, VLPs abundance at TKB was significantly higher than those at BE.

3.3.3 Phytoplankton diversity and abundance

It was observed that BE bear more diverse phytoplankton than TKB, however seawater samples in TKB showed higher abundance of plankton than BE, (Fig. 3.4, t = -2.860, df = 4, p < 0.001, 2-tailed). Bacillariophyceae (diatoms) was the most abundant phytoplankton at both sites (99% at TKB and 44% at BE, respectively). Dinophyceae varied at both sites being 8% at BE and almost 1% at TKB of the total screened phytoplankton. Besides, other classes of phytoplankton were found only at BE but not at TKB, where 46 % was filamentic Cyanophyceae (Oscillatoriaceae), 2 % was Cryptophyceae and Nanoflagellates were barely present (Table 3.1).

3.3.4 Prokaryotes and phytoplankton carbon biomass

The biomass of organisms varied significantly between the two studied sites, especially in terms of diatoms and bacteria. Diatoms were the dominant organisms at TKB (92%) whereas bacteria were most abundant with 76% at BE (Table 3.2). In addition, coccoid cyanobacteria biomass was higher in BE than TKB (0.58 µg C/L and 0.47 µg C/L).

3.3.5 Relationship of phytoplankton and microorganisms to VLPs

A strong significant relationship was obtained between VLPs and phytoplankton (Table 3.3). Correspondingly, bacteria was also significantly linked to VLPs ($r = 0.712$, $p < 0.01$, 2-tailed, Table 3.3). Hence, the ratio of VLPs to phytoplankton and bacteria denoted as VPR and VBR were determined at BE and TKB. The VPR at Bise was 44.5 times greater than TKB (0.089 and 0.002, respectively); whereas the VBR at TKB was almost 3 times greater than BE (1.99 and 5.81, respectively).

3.3.6 Influence of water quality (nutrients, hydrographical factors) on abundance of VLPs, VPR and VBR

The concentration of nitrite, ammonium, DIN and DIP had a strong positive influence on the abundance of VLPs, bacteria, phytoplankton and VBR (Table 3.3). However, negative significant associations were observed with VPR (Table 3.3). No

significant difference in concentration of nitrates and dissolved organic phosphorus were observed at both sites (Mann-Whitney-U test, $p > 0.05$), which did not contribute to the VLPs abundance as non-significant correlations were obtained (r = 0.735 and 0.461, N = 6, $p < 0.05$, 2-tailed, respectively). Significant negative relationships were also obtained with dissolved oxygen, temperature, pH, salinity and total phytoplankton (Table 1). In order to determine the most dominant factors influencing the abundance of VLPs multiple regression analysis was performed. As a result, it was found that ammonium was the main contributor to the abundance of VLPs among all the factors tested. Ammonium significantly predisposed the abundance of VLPs, unstandardized coefficient B = 2.017, $t_{(5)} = 13.05$, $p < 0.001$. Ammonium concentration might also contribute to the difference in VLPs abundance at both sites with a probability of 86% ($R^2 = 0.860$, $F_{(1, 5)} = 170.23$, $p < 0.001$).

3.4. Discussion

A high abundance of marine viruses can regulate the dynamics of their natural hosts as observed in phytoplankton and bacteria at the studied areas. The strong link between phytoplankton and VLPs abundance corroborate with other marine environments (Proctor & Fuhrman, 1990; Wilson et al., 1993; Bratbak et al., 1994; Suttle & Chan, 1995). Moreover, abundance of VLPs is associated with bacteria dynamics, at the two studied areas, implying that bacteria are potential hosts (Paul et al., 1993; Wommack and Colwell, 2000; Suttle, 2005). However, the difference of VPR at these two sites indicates that the marine viruses would have preferential host infection pattern. More phytoplankton would be infected at BE, whereas at TKB most of the free viruses would have a tendency to infect more bacteria than phytoplankton.

This difference in host-infection at BE and TKB is supported by the high bacterial and diatoms biomass composition, respectively. These marine viral infections of bacteria and phytoplankton would produce viral lysates, which is essential in the viral-shunt mechanism. Through this process the cells contents of the bacterial (~40%) and of primary producers (~78%) population are released to the surroundings on daily basis (Suttle et al., 1990). Consequently, the transfer of nutrients from particulate to dissolved materials is enhanced (Wilhelm and Suttle, 1999; Suttle, 2005) to be readily utilized by uninfected bacteria (Middelboe et al., 1996, 2003). Therefore, marine viruses actively participate in the recycling of nutrients and carbon. Because these lysates are rich in free or combined amino acids (Middelboe and Jørgensen, 2006), they represent an important pool of labile organic N, which is consumed by bacteria to produce ammonium as a by-product (Goldman et al., 1987), as observed by the high dissolved organic nitrogen concentration at TKB. In general, VLPs will infect the hosts to produce the required N source to drive primary production in the coral coastal areas.

In the case of BE, the concentration of dissolved organic nitrogen was higher, and the amount of ammonium and DIN was relatively lower in contrast to TKB. This condition could be explained because the dissolved organic nitrogen was in refractory form where bacteria could not degrade or convert it into inorganic form, which could result into the low concentration of inorganic N species. This similar observation was noted by Meekaew et al., (2014) at almost same sampling site, Bise, Okinawa, Japan. However, it was noticed that VPR was high at BE but low in VBR. This might indicate viral infection on phytoplankton, which would result into plankton lysates (Haaber & Middelboe, 2009). Another reason for this condition is that BE possessed a high diversity of plankton in contrast to TKB. Since a strong significant correlation

was noted between plankton and VLPs counts, this denoted that pico- and nanoplankton such as Cryptophycea, Cyanophycea and nanoflagellates could be potential viral hosts in BE water, in addition to Dinophyceae and Baccillariophyceae. Consequently, the viral lysis of phytoplankton and bacterioplankton population could deeply change the rates of nutrient regeneration and cycling processes in such condition (Seymour et al., 2005). Overall, it was observed that viral lysates was triggered both by bacteria and phytoplankton, which would stimulate the "viral shunt" mechanism, thereby helped in N recycling. The latter would then promote the phytoplankton growth at different rates upon the availability of nutrients.

Among the nutrients that could shape the abundance of VLPs at the two sampling sites, ammonium was the most influential. The availability is regulated by viral lysis and can influence phytoplankton growth as shown by a decrease of ammonium regeneration when viruses are removed, accompanying a decline in phytoplankton growth (Shelford et al., 2012). Moreover, ammonium production could be instigated through bacterial mineralization (Boras et al., 2010). Consequently, the viral infection independent of its host organisms will entail ammonium production through lysis. This is important in stimulating N recycling and primary production in seawater. Furthermore, phosphate availability might play a major role in marine viral production as significant positive correlation was obtained with DIP. This could be so, because virus particles constitute more P compared to C and N (C:N:P of 10:4.5:1, Hewson and Fuhrman, 2002) which is essential for viral production. In addition, Rohwer et al., (2000) revealed that phosphate had important roles to play in viruses since some phosphate metabolic genes were detected within a marine bacteriophage genome. However, phosphates could also act as limiting factor for virus abundance and production in the water column (Williamson et al., 2002; Hewson et al., 2001;

Wilson et al., 1993). Another reason is that increased in phosphate and ammonium will increase heterotrophic bacteria and phytoplankton, which will account for the viral production (Keil and Kirchman, 1991).

In this study, the abundance of VLPs is not linked to the hydrographical data irrespective of the difference in dissolved oxygen and salinity. Oxygen variation could affect the VLPs abundance whether in low or high oxygen waters as demonstrated by Taylor et al., (2003) and Weinbauer et al., (2003). Furthermore, Chiang and Quinones, in 2007, found that concentration of dissolved oxygen in their studied areas characterised the relationship between VLPs and bacteria. However, most of the studied areas were from coastal to oceanic areas (5-150 m) where changes in depth and dissolved oxygen were obvious. Nonetheless in this study, focus was brought on coastal coral areas in shallow areas and the depth was limited to nearly 5 m. Moreover, the studied areas were in the photic zone in contrast to hypoxic areas where an increase in both abundance of VLPs and prokaryotes was observed (Chiang and Quinones, 2007; Weinbauer et al., 2003) which might limit this finding. Salinity was found to have an indirect effect on the abundance of VLPs. This could be due to the fact that salinity was within the range of 30-35, because from 18-25 PSU, a very high VLPs abundance was demonstrated in brackish water and from 50-150 PSU an increase in both VLPs and bacteria was noted by Schapira et al., (2009). The salinity at both sites - BE and TKB - was above 25, which could limit this finding. Besides, it is challenging to link salinity to VLPs, as there are many other factors such as nutrients, solar radiation, organic matter and grazing processes (Schapira et al., 2007) that could influence the abundance of VLPs along with this parameter.

3.5. Conclusion

Abundance of VLPs at TKB and BE in coral areas was strongly linked to inorganic nitrogen availability and phytoplankton. Viral lysates triggered by bacterial and phytoplankton cells had great effect on availability of ammonium and helps in N recycle. Ammonium could be considered as a major by-product of viral infection. This nutrient was very essential in primary productivity as demonstrated at TKB.

Chapter 3: Figures and Tables

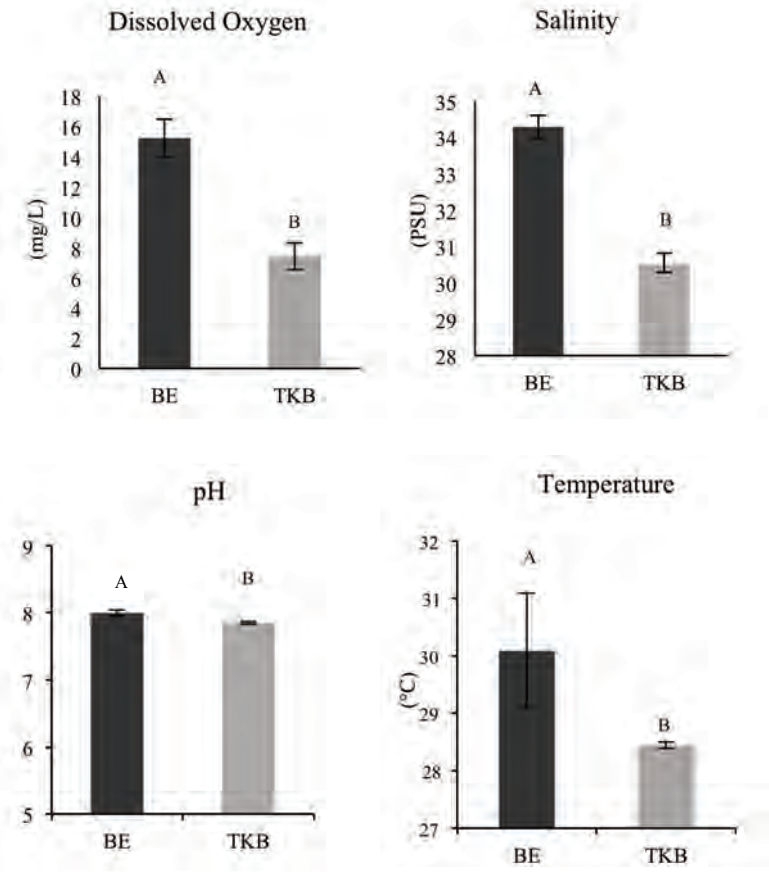


Fig. 3.1: Hydrographical characteristics of the seawater of BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Bars represent the Mean ± SD for n = 3. Letters A and B represent significant difference in the hydrographical parameters between the sites BE and TKB at the time sampling was performed (Mann-Whitney *U*-test, 1-tailed, $\alpha = 0.05$).

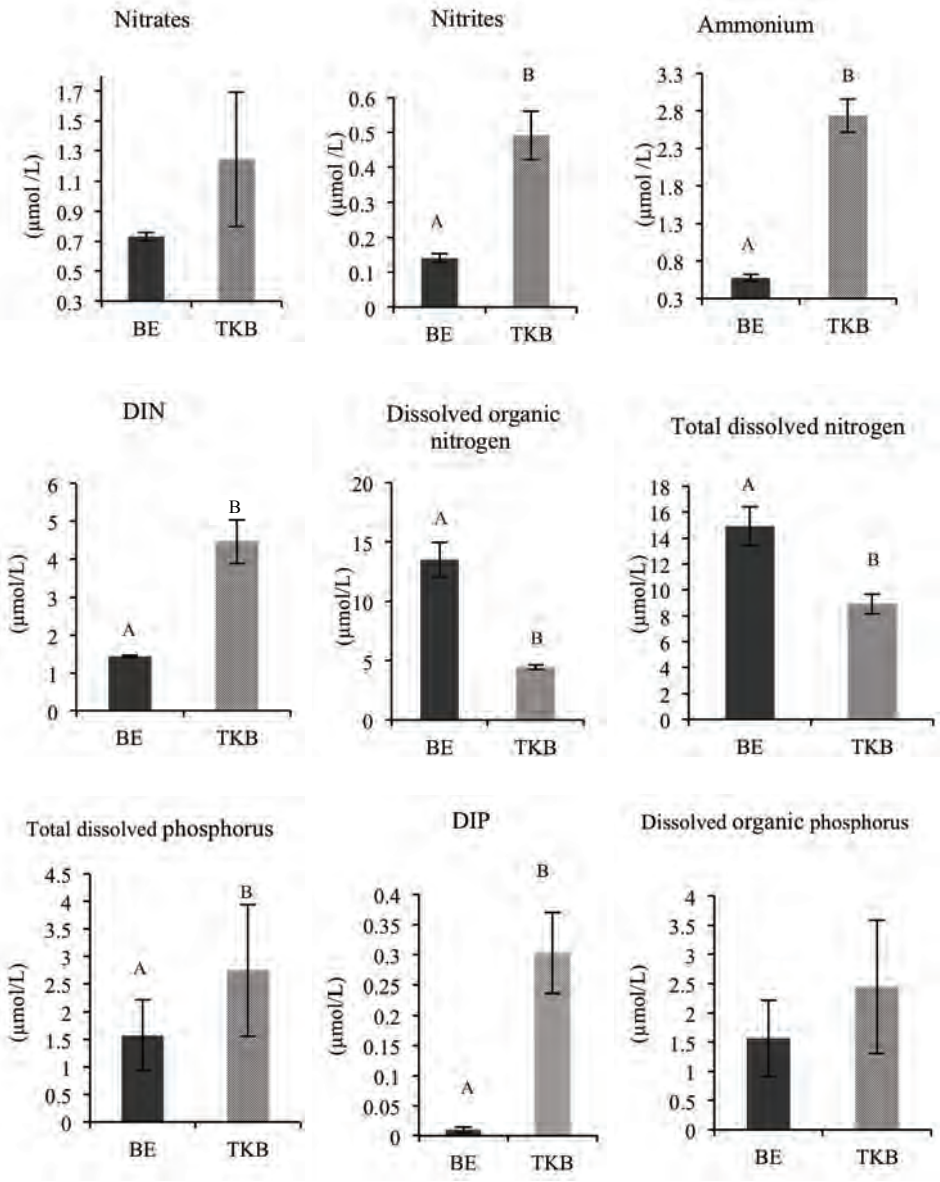


Fig. 3.2: Nutrient availability in the seawater of BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Bars represent the Mean ± SD for n = 3. Letters A and B represent significant difference in the nutrient concentration between the sampling sites BE and TKB (Mann-Whitney *U*-test, 1-tailed, $\alpha = 0.05$). DIN = dissolved inorganic nitrogen; DIP = dissolved inorganic phosphate.

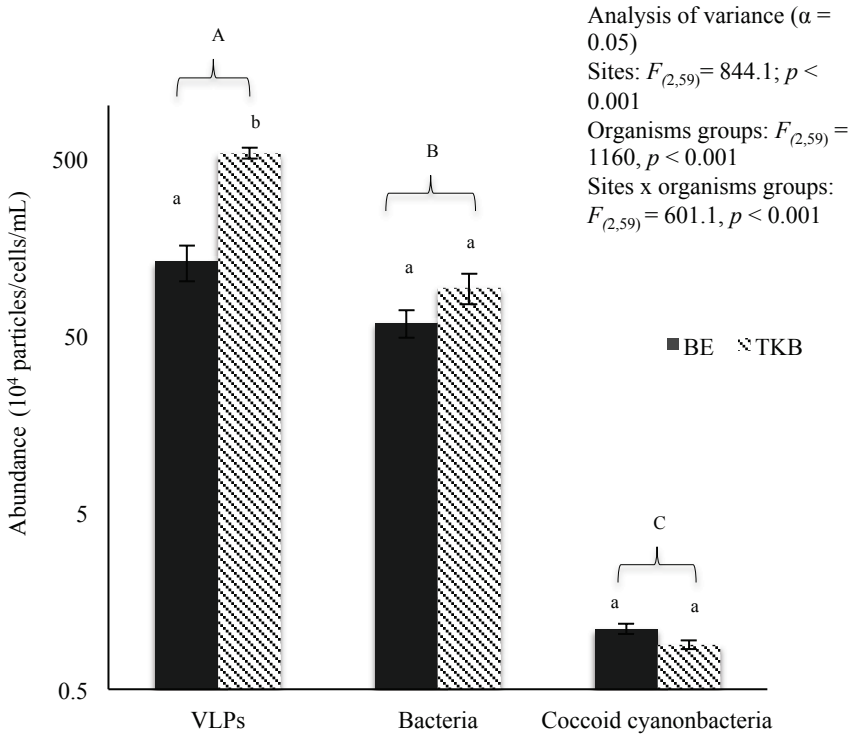


Fig. 3.3: Abundance of VLPs, bacteria, and coccoid cyanobacteria in the seawater of BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Bars represent the Mean ± SD for n = 3; 15 readings per replicate. Uppercase letters represent comparison among the microorganism groups. Lowercase letters represent comparison between the two sampling sites within a microorganism group. Bars not sharing similar letter are significantly different (α = 0.05).

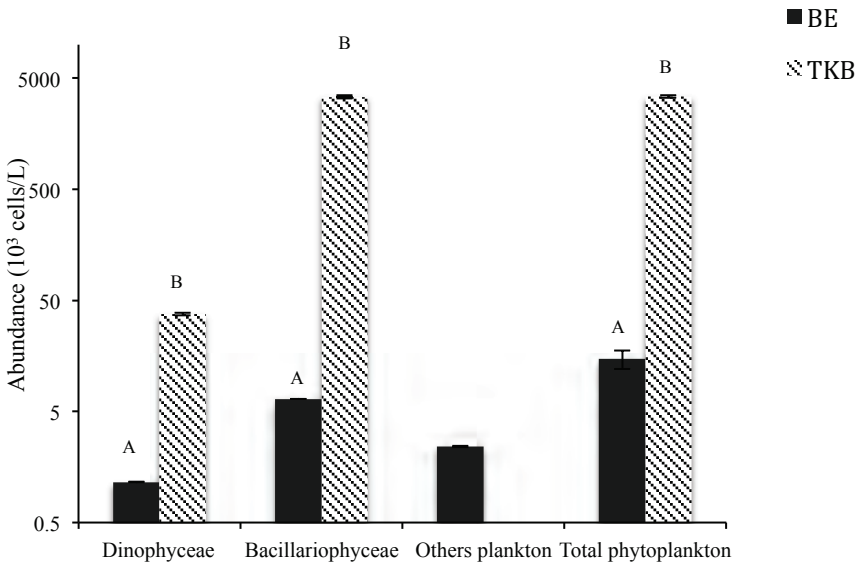


Fig. 3.4: Phytoplankton abundance at BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Bars represent the Mean ± SD for n = 3. Letters A and B represent significant difference in phytoplankton abundance between the sampling sites BE and TKB (Independent Student's *t*-tests, 2-tailed, $p < 0.001$).

Table 3.1: Phytoplankton diversity in seawater of BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Results expressed in percentage.

	Phytoplankton diversity (%)	
Phytoplankton family	BE	TKB
Cyanophyceae (filament type)	46.00	-
Cryptophyceae	2.00	-
Dinophyceae	8.00	0.10
Bacillariophyceae	44.00	99.90
Nanoflagellates	0.00	-

BE: Bise, Okinawa, Japan; TKB: Tang Kheng Bay, Phuket, Thailand, -: Cyanophyceae, Cryptophyceae and Nanoflagellates were not observed at TKB.

Table 3.2: Prokaryote and phytoplankton biomass at BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand). Results expressed in percentage.

	Biomass (%)	
Organisms	BE	TKB
Bacteria	76.01	2.84
Coccoid cyanobacteria	3.41	0.07
Cyanophyceae (filament type)	0.01	0.00
Dinophyceae	7.88	4.96
Bacillariophyceae	12.69	92.13

BE: Bise, Okinawa, Japan; TKB: Tang Kheng Bay, Phuket, Thailand.

Table 3.3: Relationships between microorganisms (virus-like particles, bacteria, and coccoid cyanobacteria), phytoplankton and the nutrient availability, as well as the hydrographical characteristics of the seawater of BE (Bise, Okinawa, Japan) and TKB (Tang Kheng Bay, Phuket, Thailand).

	Pearson's correlation coefficient				
	Abundance of VLPs (log_{10} particles/mL	Abundance of bacteria (log_{10} cells/mL)	Abundance of total phytoplankton (log_{10} cells/mL)	VBR	VPR
Abundance of VLPs (log_{10} particles/mL)		0.712**	0.990**		
Nutrients					
Nitrate (µmol/L)	0.735	0.417	0.729		
Nitrite (µmol/L)	0.962**	0.856*	0.979**	0.937**	-0.977**
Ammonium ions (µmol/L)	0.988**	0.902*	0.996**	0.956**	-0.987**
Dissolved inorganic nitrogen (µmol/L)	0.979**	0.868*	0.988**	0.955**	-0.979**
Dissolved inorganic phosphate (µmol/L)	0.966*	0.827*	0.973**	0.950**	-0.964**
Total dissolved nitrogen (µmol/L)	-0.881*	-0.961**	-0.931**		0.959**
Total dissolved phosphorus (µmol/L)	0.575	0.495	0.633		-0.671
Dissolved organic nitrogen (µmol/L)	-0.949**	-0.945**	-0.982**	-0.896*	0.994**
Dissolved organic phosphorus (µmol/L)	0.461	0.543	0.523		-0.565
Hydrographical parameters					
Dissolved oxygen	-0.959**	-0.919**	-0.974**	-0.916*	0.971**
Salinity (PSU)	-0.978**	-0.874*	-0.993**	-0.951**	0.990**
pH	-0.947**	-0.715	-0.933**		0.906*
Temperature (°C)	-0.895*	-0.623	-0.824*		0.751

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed); VBR: VLPs to bacteria ratio; VPR: VLPs to phytoplankton ratio

CHAPTER 4

4. Marine phages isolated against *Vibrio coralliilyticus* from Bise and Sesoko seawater, Okinawa, Japan

Abstract

Five marine phages were successfully isolated from coral reef seawater of Bise and Sesoko, Okinawa, Japan. Only one of the phages denoted as Vibrio phage RYC (VCPH RYC) infected the pathogenic bacteria, *V. coralliilyticus* (AB 490821, Japan) and the rest infected *V. coralliilyticus* P1 (LMG 23696, Australia). Most of the phages isolated infecting *V. coralliilyticus* P1, regardless of their sampling sites, displayed phages with head and tail structures. The size of the capsids and tails varies slightly from 57.65−58.33 nm and 95.38−105.88 nm, respectively. An elongated icosahedral symmetry capsid was observed denoting that the phages belong to Myoviridae family. However, the phages isolated with the host *V. coralliilyticus* (Japan) exhibited no tails and non-enveloped structures with a diameter of 84.45 nm, which yet remained unclassified. All the phages were chloroform resistant and showed high host specificity. The latent phases for phages lyses of *V. coralliilyticus* P1 and *V. coralliilyticus* (Japan) were 45 and 60 minutes. The genetic material of the phages was extracted and the whole genome of the phages was sequenced through next generation sequencing method. It was found that the four viruses belonging to the Myoviridae family had 48 kb and 35 kb as genomic size. However, the VCPH RYC had a genome size of 158 kb. Cluster analysis of the Myoviridae-type viruses revealed that they are closely related to *Vibrio parahaemolyticus* phage VP16T based on partial sequencing. However, VCPH RYC showed to be relatively close to Shewanella sp. Phage based on DNA polymerase. However, this finding could be limited owing to the lack of viral sequenced genome data. As a result, VCPH RYC could be considered as a novel phage infecting *V. coralliilyticus* (Japan).

Keywords: Vibrio phage, *V. coralliilyticus,* genome, Myoviridae, coral reef seawater

4.1 Introduction

One of the important worldwide factors that affect corals all over the globe is coral disease. Coral disease is a field that need to research on, so as to enhance our understanding and knowledge. Many studies have revealed that several colonies of corals throughout the world have been affected and some had even been completely devastated owing to certain type of coral diseases (Hoegh- Guldberg, 1999; Peters, 1997; Richardson, 1998). This led to huge global increase in the frequency, outbreaks and distribution of coral diseases. Subsequently, there is a universal decline of corals in terms of total abundance and species diversity (Loya et al., 2001). This effect does not only affect marine ecosystems and species in general, but also had a negative impact on humans, since many people depend on coral ecosystem for their food, services and living income (NMFS/NOAA, 2001).

The causes and causative agents of coral disease outbreaks are very complex and less understood (Rosenberg et al., 2007; Bourne et al., 2009). Little is known on the pathogens and up to now only five pathogens, mainly bacteria, followed the Koch's postulates, confirming the actual pathogens of some of the coral diseases such as: the White band II by *Vibrio carchariae*; sea fan disease by *Aspergillus sydowii* (Smith et al., 1996; Geiser et al., 1998); coral white plague II by *Aurantimonas coralicida* (Raymundo et al., 2008) and the bleaching of *Oculina patagonic*a by *V. shilonii* and *V. coralliilyticus* (Kushmaro et al., 1996, 1997).

The widely distributed and emergent coral pathogen can be referred to *V. coralliilyticus* and is also known as coral-dissolving bacteria. It belongs to the family Vibrionaceae, genus *Vibrio*, Gram-negative, non-spore-forming rods and are motile by a single polar and sheathed flagellum (Farmer & Hickman-Brenner, 1992). It was

first isolated in Indian Ocean (Ben-Haim et al. 2003a,b), and became famous in the Indo-Pacific for the White syndrome outbreaks (Sussman et al., 2008). Some of the strains of *V. coralliilyticus* which had been isolated are: YB1 (ATCC BAA450) from diseased coral (*Policipora damicornis*), Zanzibar, Tanzania, 1999; LMG 21348 from diseased coral (*P. damicornis*), Eilat, Israel, 2001; LMG P1 23696 from Magnetic Island, Australia from diseased *Montipora aequituberculata* (Sussman et al., 2008) and AB 490821 from Japanese seawater in 2007 (Sesoko, Motobu, Okinawa, Japan).

The strains of *V. coralliilyticus* affect both soft and hard corals all over the world by causing tissue lysis, bleaching and necrosis (Ben-Haim and Rosenberg, 2002). It was noted that in most hard corals, *V. coralliilyticus* triggers the lost of the endosymbiotic dinoflagellates, *Symbiodinium* or zooxanthellae cells, and eventually in tissue loss resulting into just a bare white skeleton (Sussman et al., 2008, 2009). The bacteria also show high proteolytic activities that disturb the cleavage of connective tissue and other cellular activities. These bacteria affect juvenile corals too by inhibition of their photosynthetic activity, loss of zooxanthellae cells, rapid onset of tissue lesions that ultimately result in the death of the juvenile colony (Sussman et al., 2009). With huge information on its virulence, *V. coralliilyticus* is a good candidate as coral bacterial pathogen to study on.

Though many studies have focused on coral diseases and on *V. coralliilyticus,* there is no known treatment for coral diseases. This is so, because it is difficult to implement, some diseases are still very complex and less understood, some are triggered by more than one pathogen, bacterial infection if treated with antibiotic can result into antibiotic-resistant strains (Parisien et al., 2007), corals cannot be

immuninized as they do not possess an acquired immune system (Nair et al., 2005). As a result, there is an urge to develop tools and strategies to control the progress and even treat certain coral diseases. Fortunately studies from Efrony et al., in 2007 and 2008, demonstrated an emerged possible therapy against coral diseases in the form of phage therapy. This promising therapy is also encouraged by Housby and Mann (2009). It consists of using a specific lytic virus, known as bacteriophage or phage, which will infect the pathogenic bacteria. The virus will replicate itself using the bacteria and then lyse the bacterial cells to release more viruses and stop replication until there are no more host bacteria that are available. This will result in a decrease in the numbers of pathogenic bacteria, which will finally help in decreasing/stopping rate of infection or disease. The team of Efrony had successful results by using this concept in a closed system, where they used a specific phage, YB1, against *V. coralliilyticus* (LMG 21348) bacteria. Recently, in 2013, Cohen et al., has identified phage, YC, against *V. coralliilyticus* strain P1 (LMG 23696). He noted that the phage was able to hinder the bacterial-induced photosystem inhibition in pure cultures of *Symbiodinium*. Since *V. coralliilyticus* affect the zooxanthellae cells, using phage therapy to treat infected corals seemed favorable and effective. In order to bring more understandings in this domain, the main aim of the study was to isolate and identify phages from seawater, lagoon sediments and healthy corals tissues (*Montipora digitata*) using *V. coralliilyticus* from Japan (AB 490821) and Australia (LMG 23696) as hosts bacteria. Morphological and molecular features were used to identify and classify the isolated phages.

4.2 Materials and Methods

4.2.1 Growth of bacterial strains

The bacterial strains used in this study were *V. coralliilyticus* (AB 490821) isolated from seawater of Sesoko, Okinawa, Japan and P1 (LMG 23696) isolated from diseased corals *Montipora aequituberculata* at Magnetic Island, Australia (Cohen et al., 2013; Sussman et al., 2008). Both strains were grown on marine agar (MA) plates containing: 1.8% marine broth (Difco™, Le Pont de Claix, France), 1.0% NaCl (Wako, Osaka, Japan), 0.5% Bacto tryptone (Difco™, Le Pont de Claix, France) and 1.5% agar (Wako, Osaka, Japan). A single colony of bacteria was transferred to 2 mL of liquid marine broth tryptone (MBT) medium containing 1.8% Difco marine broth; 1.0% NaCl, and 0.5% Difco Bacto tryptone. The bacteria was grown overnight at 28°C in a shaking incubator at 210 revolutions per minute (rpm).

4.2.2 Phage isolation

4.2.2.1 Sampling sites

Samples were collected at Sesoko (26º39'N; 127º51'E) denoted as site SO and at Bise (26°42'N, 127°52'E) called site BE, in Okinawa, Japan during May and late August to early September 2014. The estimated coral cover of healthy corals (*Montipora digitata*) was 50−60% at both sites per 10-m^2 areas. Seawater, lagoon sediments and healthy corals were sampled for extraction of phages.

4. 2.2.2 Samples 1: seawater

4.2.2.1.1 Tangential flow filtration

Three liters of seawater was collected in triplicates at site SO during May 2014 and during August 2014 at coral and outer reef areas. Similarly, seawater (3L x3) was collected at site BE during August 2014 at both coral and outer reef areas. During August sampling, water was collected at high and low tides at both sites

(Table 1). The water was pre-filtered with a series of filters: 5 µm (SIK, Osaka, Japan); 20 µm (SIK, Osaka, Japan); 500 µm (ITO Seisakusho Co. Ltd, Tokyo, Japan) and 0.20 µm (Vivaflow 50, membrane 0.2 µm PES, Satorius Stedium, Biotech, Germany). Then, the water was concentrated by passing through a membrane of 100,000 MWCO (Vivaflow 50, membrane 100,000 MWCO PES, Satorius Stedium, Biotech, Germany) through tangential flow filtration.

4.2.2.1.2 Enrichment method

Modified enrichment method (Cohen et al., 2013) was used to seek for phages in seawater. One milliliter of bacteria (10^5) cells was added to 10 mL of concentrated seawater in addition to 3 mL of marine broth. The mixture was incubated at 210 rpm at 28°C in a shaking incubator. This first enrichment was centrifuged at 2600g and then filtered through 0.22 µm disposable membrane filter Unit (Advantec®, Toyo Kaisha Ltd, Tokyo, Japan). The second enrichment was done by adding 3 mL of the first enrichment to 3 mL of concentrated seawater with 1 mL of MBT and 100 µL of bacteria (10^5cells). It was incubated at 28°C for 24h with shaking at 210 rpm. A-2 mL aliquot of the resulting culture was centrifuged at 2600g and filtered through a 0.22 µm sterile syringe filter (Advantec®, Toyo Kaisha Ltd, Tokyo, Japan). Both enrichments were plated according to the soft agar overlay technique to isolate phages.

4.2.2.3 Samples 2: lagoon sediments

Fifty milliliters (x3) of lagoon sediments were collected within 5 cm depth from the benthic surface of sampling sites, SO and BE. Five milliliters of lagoon sediments was freshly weighed and phages were extracted according to modified

Helton et al, (2006) protocol. Sodium pyrophosphate (8 mL of 10 mM) and 8 µLof 5 mM EDTA were added to the lagoon sediments in a 50-mL conical centrifuge tube (Milipore, Ireland). The tubes were then were subjected to sonication for 20 min and shake at the highest speed for another 20 min. The mixture was then centrifuged (5000g) for 25 min at 4°C. The resultant supernatant was 0.2 µm filtered, stored at 4°C and was used for plaque assay. All the solutions used were 0.02 µm prefiltered.

4.2.2.4 Samples 3: coral tissues

Healthy *M. digitata* corals fragments were collected at both sites, SO and BE, in triplicates. The corals (~2 g) were rinsed thoroughly with autoclaved 0.02 µm-filtered water. Coral tissues were extracted by using water-jet blasting with autoclaved 0.02 µm-filtered water. The tissues were homogenized and filtered through 0.2 µm filter to remove big particles and bacteria. The coral tissues were used for plaque assay.

4.2.3 Plaque assay

Plaque assay was done according to Sambrook et al., 1989, using the soft agar method. One hundred microliter of bacteria (10^5 cells) was added to 100 µL of samples in a 15-mL sterile corning tube. It was then incubated for 20 min at 28°C for absorption to occur. Afterwards, 3 mL of soft molten agar (0.6 % agar) at 47°C was added to the tube, mixed gently and poured on a MA plate. The plates were left to stand for 5 min to set the agar. The latter were incubated for 15 h at 28°C. The plates were then observed for clear zones known as plaques. A single plaque was collected by using a sterile Pasteur pipette in 1 mL of sterile phage buffer (0.06% NaCl; 0.02% $MgSO_4.7H_2O$; 1M Tris.Cl, pH 7.5; 2% gelatin) with addition of 10 µL of chloroform.

Several rounds of plaques purifications were prepared to ensure a pure phage stock. The isolated phage titer was determined by a series of dilution.

4.2.4 Production of high titer phage stocks

Phage lysates stock was prepared by mixing 100 µL of 10^4 pfu of phages to 100 µL of bacteria cells (10^5). The mixture was incubated for 20 min at 28°C. Three milliliters of soft agar was added, mixed gently and poured on the hardened bottom agar (MA). The plates were incubated at 28°C for 24 h. Honeybee hive-like plates were obtained as each plaque was touching each other. The viruses were collected by adding 5 mL of phage buffer to the plates and were gently shaken for 1h at 4°C. The top agar was delicately scraped to optimize the collection of viruses. Afterwards, 1 mL of phage buffer was added to the MA plate to collect the remaining viruses. Chloroform was added (200 µL) to the harvested phages. The mixture was vortexed and centrifuged at 15,000g for 10 min. The supernatant is recovered and filtered through 0.2 µm sterile disposable filters. A few drops of chloroform was added to the phage stock and it was stored at 4°C. The titer stock was estimated to 10^{10}–10^{11} pfu/mL.

4.2.5 Burst size experiment/one-step growth curve

One step growth curve or burst size experiment was used to determine the latent phase of phage lysis on its host bacteria as described by Adams (1959). This was done in triplicates by adding 10^7 bacterial cells to 10^5 phages (multiplicity of infection, MOI = 0.01). The mixture was allowed to incubate for 5 min at 28°C and then aliquots of the mixture was removed sequentially at 10 min intervals for 2 hr. The latter was centrifuged at 10,000g and the pellet was resuspended in phage buffer. Samples were plated according to plaque assay. The number of plaques was counted on the next day. The experiment was repeated three times, using the two hosts bacteria.

4.2.6 Transmission electron microscopy (TEM) analysis

Morphological characterization of phages (10^{10} pfu/mL) was performed by the use of transmission electron microscope (TEM). The phages (5 uL) were dropped on a hydrophilic TEM grid and were left to stand for 2 min in order to be absorbed by the TEM grids. A moist filter paper was used to wick the excess sample. Then, a first drop (5 uL) of EM stain (40%) was added to the grid and was followed by another drop of stain. It was left to stain for 2-3 min. A damp filter paper was used to remove the excess of stain. The grid was left to dry at room temperature for 30 min. The phages were examined by JOEL-1400 Plus (Tokyo, Japan) electron microscope at 80kV at Shizuoka University (Research Institute of Green Science and Technology Institute). The viral dimension was measured by using the software ImageJ 1.48v, USA.

4.2.7 Extraction of phage DNA

Phage lysates (10^{10} pfu/mL) was used for DNA extraction using the modified protocol of Henn et al., (2010). Briefly, the phage lysates were centrifuged at 16,000g to pellet any remaining cells and the pellet was discarded. For 50 mL of phage lysates, 2 mL of phage precipitant (33% polyethylene glycol, PEG-8000) was added and it was incubated on ice for 1 hr. The mixture was centrifuged at 10,000g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in phage buffer (150mM NaCl; 40mM Tris-HCl (pH 7.4); 10mM $MgSO_4$). The DNA was extracted by using the Wizard® PCR Preps DNA Purification System, (Promega, Madison, USA). Succinctly, purification resin was added (1 mL) and was gently mixed by inverting the tubes, it was then passed through the minicolumn, washed with 1 mL of 80% isopropanol. The tubes were briefly centrifuged to remove any remaining isopropanol. The DNA was eluted by adding 100 uL of TE (10mM Tris-HCl (pH 7.5); 1mM EDTA) buffer at 80°C to the minicolumn. The minicolumn was immediately centrifuge at 10,000g for 30 seconds. The purified DNA was stored at 4°C.

4.2.8 Extraction of bacterial DNA

Bacterial DNA was extracted by using DNeasy® Blood and Tissue Kit (50) (Qiagen, GmbH, Hilden, Germany). Single colonies of bacteria were grown in 2 mL of marine broth for 24hr. The bacterial cells (10^9) were harvested by centrifugation at 5000g for 10 min. The supernatant was discarded whereas the pellet was treated according to the manual's instructions.

4.2.9 Next generation sequencing

4.2.9.1 Genomic DNA Quantification

The DNA samples were quantified using Quant-iT PicoGreen dsDNA Reagent (Life Technologies, USA) according to the manufacturer's instructions. The structural integrity of DNA was verified and analyzed on 0.7% of agarose gel electrophoresis.

4.2.9.2 Library preparation and sequencing

Next generation sequencing technique was used to sequence the genome of the isolated phages and bacteria. Genomic libraries were constructed using TruSeq Nano DNA Sample Prep kit (Illumina, USA) following the manual's instructions. First, the DNA samples were quantified using Quant-iT PicoGreen dsDNA Reagent (Life Technologies, USA) according to the manufacturer's instructions. Then, the genomic DNA (200 ng) was fragmented by Covaris shearing (Hokkaido system science Co., Japan) and end-repaired was conducted. Second, library templates were size-selected by using AMPure XP beads to collect a DNA fraction of approx. 550 bp lengths. Third, the DNA fraction was subjected to 3' adenylation and each sample was labeled with a unique adaptor index. Each library sample was mixed with PCR Primer Cocktail and Enhanced PCR Mix to conduct library enrichment with initial denaturing step at 95 °C for 3 minutes, followed by 8 cycles at 98 °C for 20 seconds, 65 °C for 15 seconds, 72 °C for 30 seconds, and then at 72 °C for 5 minutes. Finally, the PCR products were purified with AMPure XP beads in 1:1 ratio. The libraries were analyzed using a Bioanalyzer 2100 and the DNA 7500 kit (Agilent technologies, USA). The adapter-ligated fragments were quantified by qPCR using the KAPA Library Quantification Kits (KAPA Biosystems, USA). Sequencing was performed by using the method of 300 bp of paired-end through Illumina MiSeq (Illumina, USA).

4.2.10 Cluster analysis

Phylogenetic tree was constructed using DNA polymerase sequence for the isolated phages and from gene bank using ClustalIW (DDBJ: http://www.ddbj.nig.ac.jp/) and the phylogenetic tree was created using TreeView software.

4.3 Results

4.3.1 Phage isolation

Plaques were observed from only seawater at both sampling sites, SO and BE. Healthy coral tissues and lagoon sediments did not show any plaques against the two hosts bacteria (Fig. 4.1) - *V. coralliilyticus* (AB 490821, Japan) and P1 (LMG 23696, Australia). A few putative plaques were obtained from Sesoko outer reef water (high tide, August 2014) with host bacteria P1 (LMG 23696, Australia) and from Bise coral areas water (low tide, August 2014) with *V. coralliilyticus* (AB 490821, Japan) as host bacteria. However, none of them could be confirmed by further reinfection of the host bacteria. Mostly, water that was collected during low tide showed the presence of plaques (Table 4.2). The plaques obtained using P1 as host bacteria was 1.0 mm in diameter and showed very clear small zones whereas that obtained from *V. coralliilyticus,* Japan, was bigger (3.0 mm in diameter), clear and bear a halo around them as shown in figure 4.2). All the phages obtained were chloroform resistant. It was noted that the titer of isolated phages obtained from outer reef water was higher than that of coral areas during low tides (Table 4.2).

4.3.2 Characterization of phages

4.3.2.1 Morphological features

Most of the phages isolated from P1, (LMG 23696, Australia) regardless of the sampling sites, SO and BE, displayed phages with head and tail structures. An elongated icosahedral symmetry capsid was observed denoting that the phages belong to Myoviridae family. The size of the capsid and tails varies slightly from 57.65−58.33 nm and 95.38−105.88 nm, respectively (Fig. 4.3 A-D). However, the

phages isolated with the host *V. coralliilyticus* (Japan) exhibited no tails (Fig. 4.3E) and non-enveloped structures with a diameter of 84.45 nm and could not yet be classified.

4.3.2.1 General genomic features of isolated phages

All the isolated phages had a linear, double stranded DNA. The genomic size of the Myoviridae-type viruses had 48 kb and 35 kb (Table 4.3). It is also noticed that phages isolated from coastal coral seawater contained analogous amount of genetic material (35 kb). Except for one of the samples revealed a genome size of 156 kb. This could be the result of the presence of more than one type of viruses. This sample was not taken into account for further genetic analysis. The genomic size of phage infecting *V. coralliilyticus* (Japan) was 158 kb.

4.3.3 One-step growth curve

The latent phases for phages lyses infecting P1 and *V. coralliilyticus* (Japan) were 45 and 60 min as shown by the figures 4.4 and 4.5. The experiment was conducted in phage buffer so as to determine burst size irrespective of the nutrients. The phages isolated using the two different strains of *V. coralliilyticus* showed different latent phases.

4.3.3 Cluster analysis

The cluster analysis of the phage infecting *V. coralliilyticus* (Japan), denoted as VCPH RYC, showed that it is relative close to Shewanella sp. phage using DNA polymerase as the marker (Fig. 4.6). The phages isolated using P1 strain was also analyzed for partial sequencing. They showed relatively close relationship to *Vibrio*

parahaemolyticus phage VP16T and VP16C. Three categories of similarity were observed. All the phages shared some common sequences to *Vibrio parahaemolyticus* phage VP16T and VP16C, denoted as "A". However, it was noted that phage with a genome of 35 kb (L8) shared another set of sequences to *Vibrio parahaemolyticus* phage VP16T and VP16C denoted as "B". Nevertheless, the phage with genome size 48 kb (L7) showed a coverage was of 4.38, which was too low and was denoted as "C" (Table 4.4).

4.3.4 Genomic features of VCPH RYC

The overall genome organization of the VCPH RYC was analyzed in terms of proteins with references to Vibrio phage helene 12B3, Vibrio phage eugene 12A10, Vibrio phage 11895-B1, Vibrio phage ICP1, Shewanella sp. phage 1/40, Shewanella sp. phage 1/4 and Vibrio phage PWH3a-P1 (Table 4.5). It was found that VCPH RYC had 105 proteins; most of them were hypothetical proteins. Nonetheless, VCPH RYC shared 70% similar proteins as Vibrio phage helene 12B3, 65% with Vibrio phage eugene, 57% Vibrio phage 11895-B1, 52% Vibrio phage ICP1, 40% Shewanella sp. phage 1/40, 37% Shewanella sp. phage 1/4 and 36% Vibrio phage PWH3a-P1.

Moreover, it was noted that VCPH RYC contained proteins like putative Gp5 baseplate hub subunit and tail lysozyme, putative major capsid protein and putative tail fiber protein, which summed up to 5.7% structural proteins. Functional proteins such as enzymes: DNA polymerase; putative phosphohydrolase; tRNA nulceotidyltransferase; AAA ATPase formed 19% of the total proteins and 76% of the proteins were hypothetical proteins as shown in the Table 4.5.

4.3.5 Bacterial (*V. coralliilyticus*) genome

The genome sequence of *V. coralliilyticus* revealed that *V. coralliilyticus* AB490821 and LMG23696 are different. This variation is mostly brought by the presence of plasmid. Two plasmids were found in *V. coralliilyticus* (Japan) and one plasmid in *V. coralliilyticus* (Australia).

4.4 Discussion

It is well known that phages and bacterial population sustain a balance in their natural environments. If however the host populations increase, this rise will be regularly control by viruses to decrease the numbers of the host cells, which is known as "kill the winner" theory (Thingsad, 2000). As a result, it is expected to find a specific phage where the host bacteria had been isolated in its natural surroundings (Chibani-Chennoufi et al., 2004; Stenholm et al., 2008). However, in this study *V. coralliilyticus* strain (Japan) phage was not observed from SO waters although *V. coralliilyticus* strain (Japan) was isolated at SO. Conversely, the phage (VCPH RYC) infecting *V. coralliilyticus* strain (Japan) was successfully isolated from BE waters. In other words, this study showed that phages could be isolated from coastal seawater regardless of the host bacteria isolation locations. For example, *V. coralliilyticus* strain P1 and its Phage YC was isolated from waters of Nelly Bay, Magnetic Island, Australia (Cohen et al., 2013). But, phages infecting the same bacteria strain could also be isolated from Okinawan waters at both sites, BE and SO. However, the species of the phages could differ from location to location.

Moreover, the titer of the isolated phages was higher in outer reef water compared to coral areas. This trend was seen at both locations. This could be so, because first of all, the coral areas had healthy corals at both sites and hence the host

(*V. coralliilyticus*) densities could be low as phage titer depended on bacterial host abundance. Secondly, the outer reef water is known to bear higher titer of viruses or VLPs owing to viral shunt mechanism in the water (Proctor and Fuhrman, 1990; Fuhrman, 1999; Gobler et al., 1997; Poorvin et al., 2004; Middelboe and Jørgensen, 2006). Thirdly, the natural titer of phages alters in seawater with various factors such as UV light exposure, depth, temperature, salinity, bacterial host abundance, nutrients, pH and season (Dinsdale et al., 2008; Patten et al., 2006, 2008b; Paul et al., 1993; Seymour et al., 2005). Nonetheless, seasonality did not play a main effect in this study as the sampling was done during the same season and nearly at the same sampling time within 1-2 days difference. Although certain studies noted that phage was isolated during summer when disease prevalence was high (Cohen et al., 2013; Haapkyla et al., 2010). Nevertheless, in this study phage could be successfully isolated during summer season although prevalence of the disease could be high. In addition, phage was successfully isolated at SO during May and August season. Hence, seasonality might not be a big factor in this particular case.

Morphological characteristics of the isolated phages suggested that all the viruses infecting *V. coralliilyticus* strain P1 showed head and tail features, which corresponded to Myoviridae family. Similarly, Cohen et al., (2013) isolated Phage YC with similar morphology belonging to Myoviridae family, using the same host bacterial strain P1. However, the phages isolated from Japan waters had a latent phase of 45 min compared to Phage YC with 60 min. This could indicate that Japanese isolated phages could be slightly different from Phage YC. Another possibility was Cohen et al., 2013 used nutrient-rich medium whereas in this study, phage buffer was used. This could limit this finding.

Phage VCPH RYC did not show the typical features of Caudovirales, as no tails were present through TEM analysis. This result could be related to that Efrony et al., (2007), where he isolated YB2 phage using *V. coralliilyticus* as host bacteria. Though, Efrony and his colleagues showed YB2 phage is chloroform sensitive, formed 1.5 mm clear plaques, possessed double stranded circular DNA, the burst size was at 80 min at 30°C and belong to Corticoviridae. Nonetheless, the characteristics of VCPH RYC did not correspond to the features of YB2 as VCPH RYC was chloroform resistant, the latent phase was 60 min, the plaques obtained was clear but possessed a halo at the center of the plaques and the DNA was double stranded linear but not circular. Additionally, in most studies, non-tailed phages or just capsids govern most of the viral abundances consisting of 51-92% of the observed VLPs could be characterized as non-tailed marine viruses (Brum et al., 2013). Some authors argued that tails could be lost through artifacts due to some mechanical and preparation effects (Colombet et al., 2007) as 96% of the described phages are tailed (Ackermann, 2007).

Nevertheless, the protein analysis of VCPH RYC revealed some structural proteins, which are associated with tails morphology were noted but no tails were observed through TEM analysis. One reason for this, although there could be mechanical artifacts, Brum et al., 2013, reported that marine viruses might lose their tails through natural decay just before sample collection. This could explain the absence of tails in TEM photographs of VCPH RYC. In addition, the cluster analysis of VCPH RYC based on DNA polymerase showed that VCPH RYC is relatively close to Shewanella sp. Phage, which could be a filamentous phage. However, the TEM photographs of VCPH RYC showed no filamentous structures at all. As a

result, it was not possible to classify the phage VCPH RYC on morphological characters.

Furthermore, the genome analysis divulged that VCPH RYC could be considered as novel phage as there was no correspondence to the gene banks. Genotypic classification was barely possible because there is a lack of taxonomic genetic marker and sequence database to identify phages (Paul and Sullivan, 2005; Angly et al., 2006; Hurwitz and Sullivan, 2013). Hence, questions about phage biogeography in terms of distribution, viral assemblages and comparisons remained unanswered, which led to VCPH RYC as unclassified phage. The four phages infecting *V. coralliilyticus* strain P1 demonstrated that they are closely related to *Vibrio parahaemolyticus* in terms of common shared sequences. However, three types of categories could be derived from this sequencing alignment.

Besides, it was found that *V. coralliilyticus* strain (Japan) contained two plasmids and strain P1 had one plasmid, respectively. One reason for this, phages infecting the bacteria could enter the bacteria cell and undergo lysogenic circumstance or pseudolysogenic condition within the natural environment and upon the optimum conditions the lysogenic phage turned into lytic state. This concept was demonstrated by (Moebus, 1996). In the study of Cohen et al., (2013), treated the samples (seawater) with chloroform first, in order to interrupt the host cell, which would free the phage. Plaques were obtained after this treatment compared to untreated chloroform samples. Nonetheless, samples in this study were not treated with chloroform but still phages were successfully isolated. Moreover, phages were neither isolated from lagoon sediments nor healthy *M. digitata* coral tissues, but from seawater only. It is estimated that the total amount of phages in the ocean is almost 10^{30} (Suttle, 2007). Phages exist ubiquitously in the ocean and the presence of

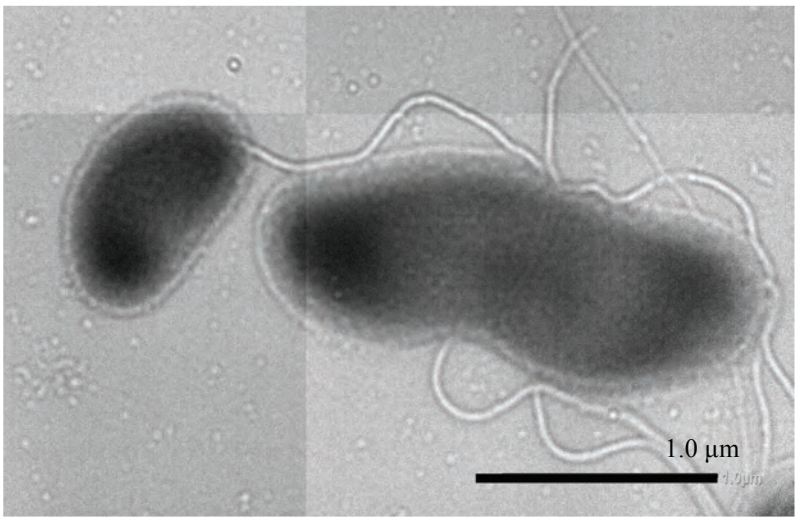
microorganisms depends largely on the existence of phages. In surface seawater, there are about 10^7 bacteriophages per mL, ~ 5–25 times higher than that of bacteria (Fuhrman, 1999). Hence, seawater provides a big pool for phages. Moreover, in other studies, it was found that phages were successfully isolated from seawater using *V. coralliilyticus* as host bacteria (Cohen et al., 2013; Efrony et al., 2007).

The explanation for the presence of the plasmids could be due to viral infections. These viral infections could increase the hosts' metabolism, immunity, distribution and evolution in various ways through horizontal gene transfer. Deeper analysis needs to be done in order to determine the mechanisms, roles and functions of the plasmids in the host cells.

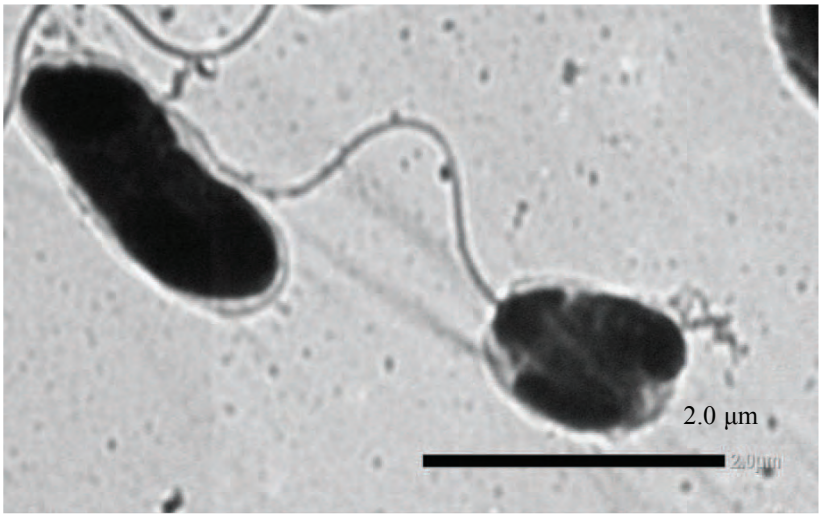
4.5 Conclusion

This study revealed a possible novel phage infecting *V. coralliilyticus* strain (Japan) named VCPH RYC. It was mainly concentrated at BE in outer reef water at low tide. Moreover, four phages infecting *V. coralliilyticus* strain (Australia) regardless of the sampling locations were isolated. Besides, it is argued that the location where the host bacteria was isolated their phages would be present too, however, this study showed that phages could be isolated irrespective to their host isolation location. Until now, there is a lack of molecular taxonomic database in terms of genotypic markers and sequences to classify phages, however morphological characterization showed that most of the phages isolated infecting *V. coralliilyticus* strain P1 (Australia) exhibited a head and a tail characteristic to Myoviridae family whereas that of VCPH RYC remained unclassified, so far.

Chapter 4: Figures and Tables



Vibrio coralliilyticus (LMG 23696) Australia



Vibrio coralliilyticus (AB 490821) Japan

Fig. 4.1: Host bacteria *Vibrio coralliilyticus* (LMG 23696, Australia and AB 490821, Japan).

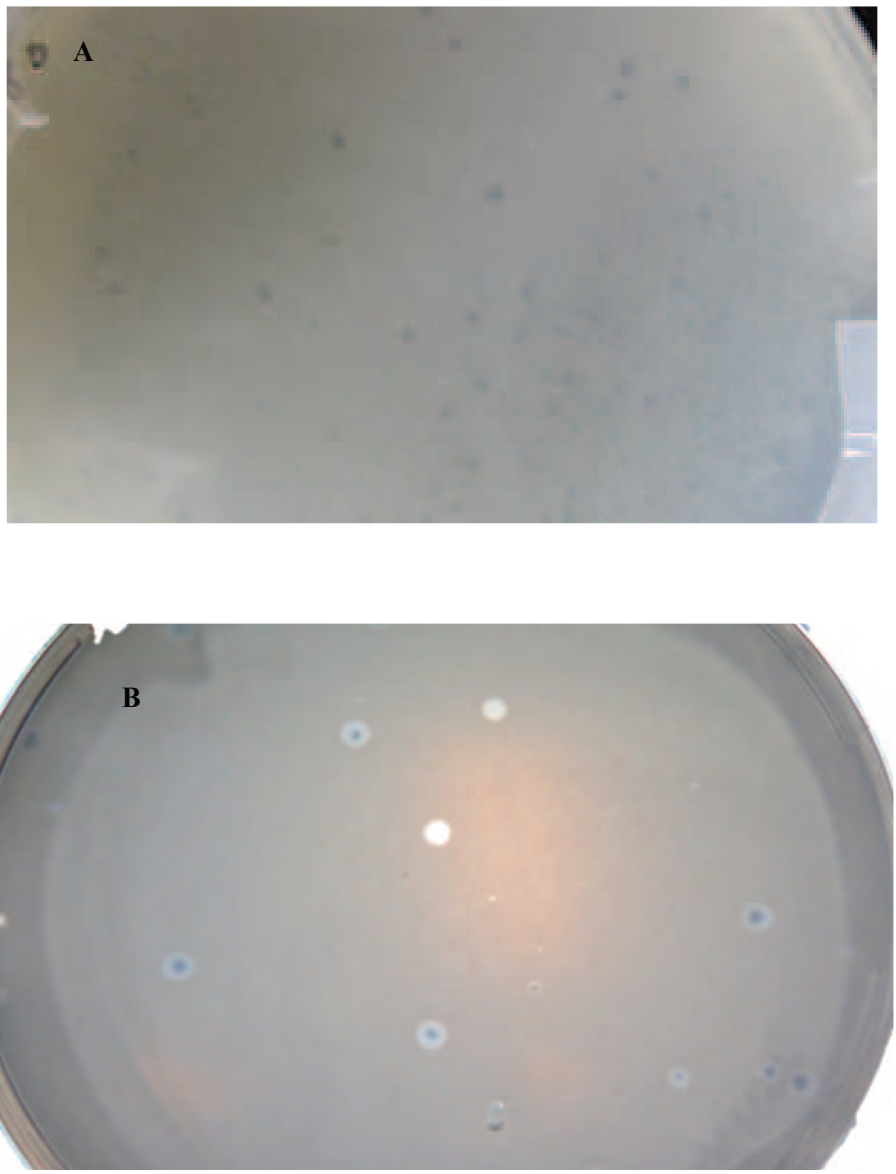


Fig. 4.2: Plaques obtained using host bacteria *Vibrio coralliilyticus* (LMG 23696, Australia) and *Vibrio coralliilyticus* (AB 490821, Japan). Plaques were 1.0 mm in diameter and showed very clear small zones (A) using host bacteria *Vibrio coralliilyticus* (LMG 23696, Australia). Those found from *Vibrio coralliilyticus* (AB 490821, Japan) were bigger (3.0 mm in diameter) and showed clear and bear a halo around them as shown above (B).

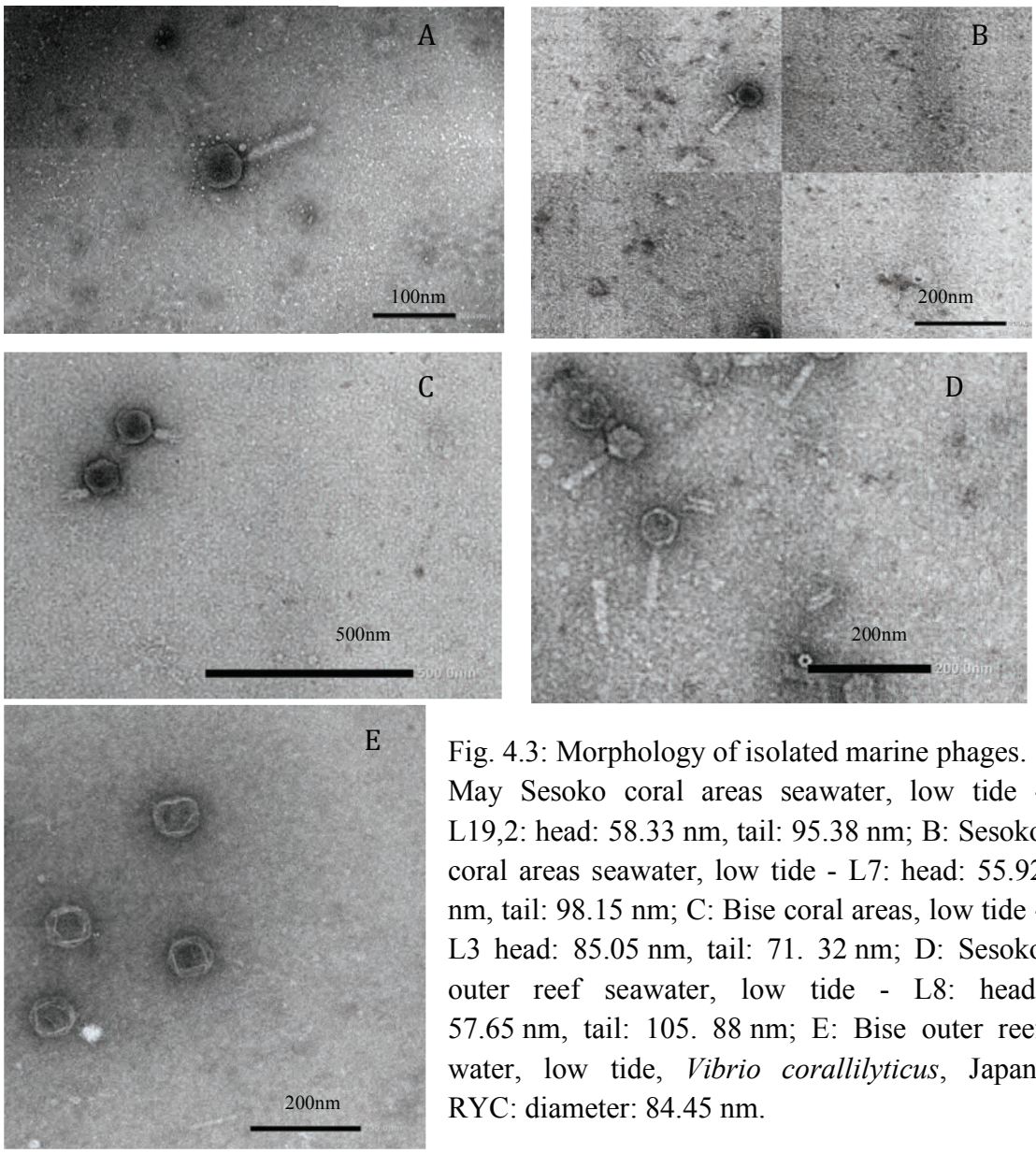


Fig. 4.3: Morphology of isolated marine phages. May Sesoko coral areas seawater, low tide - L19,2: head: 58.33 nm, tail: 95.38 nm; B: Sesoko coral areas seawater, low tide - L7: head: 55.92 nm, tail: 98.15 nm; C: Bise coral areas, low tide - L3 head: 85.05 nm, tail: 71. 32 nm; D: Sesoko outer reef seawater, low tide - L8: head: 57.65 nm, tail: 105. 88 nm; E: Bise outer reef water, low tide, *Vibrio corallilyticus*, Japan, RYC: diameter: 84.45 nm.

Phages (A-D) were isolated using *V. coralliilyticus* (LMG 236696, Australia) as host bacteria. Phage E infected *V. coralliilyticus* (AB 490821), Japan. Samples from B-E were collected during late August to early September 2014.

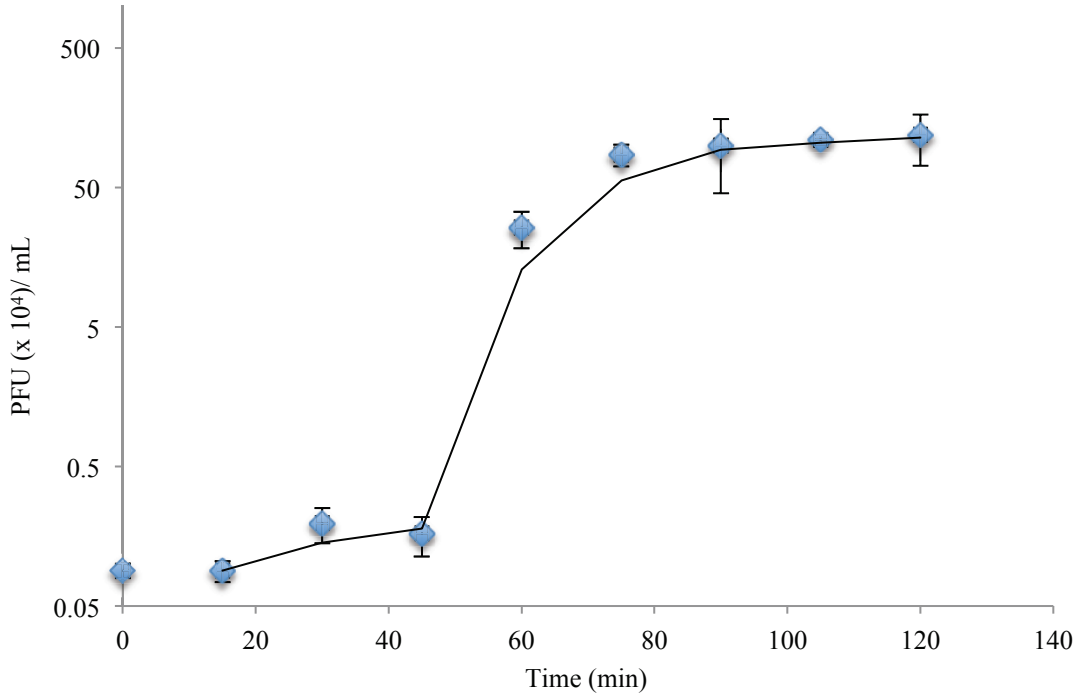


Fig. 4.4. One-step growth curve of phage infecting *V. coralliilyticus* (LMG 23696) Australia in phage buffer medium. Phage infecting *V. coralliilyticus* (Australia) was used at multiplicity of infection (M.O.I.) of 0.01.

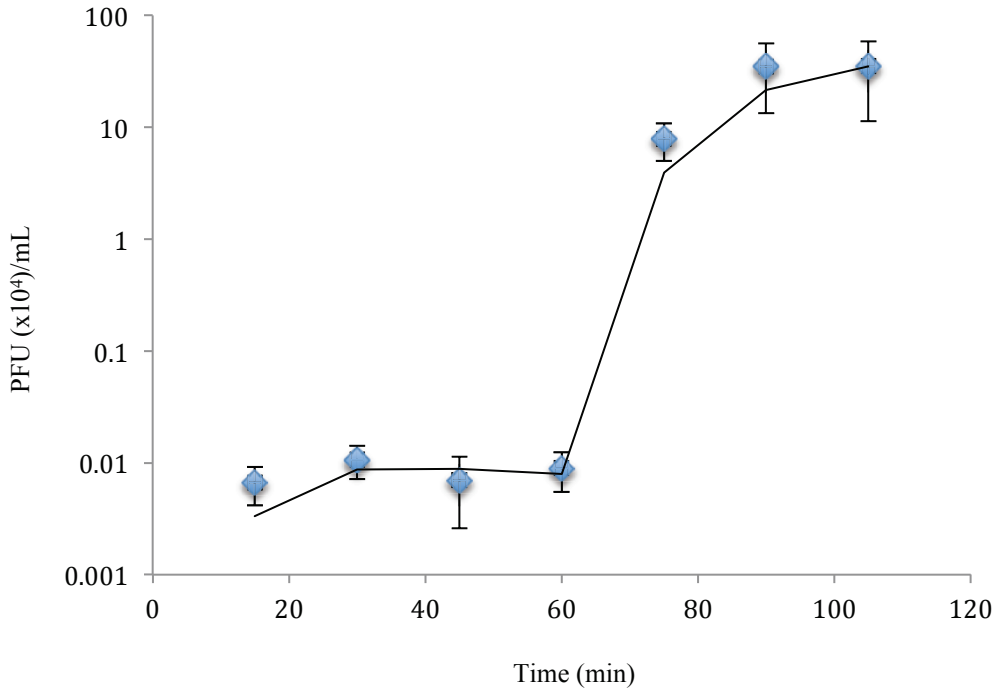


Fig. 4.5: One-step growth curve of phage infecting *V. coralliilyticus* (AB 490821) Japan in phage buffer medium. Phage *V. coralliilyticus* (Japan) was used at multiplicity of infection (M.O.I.) of 0.01.

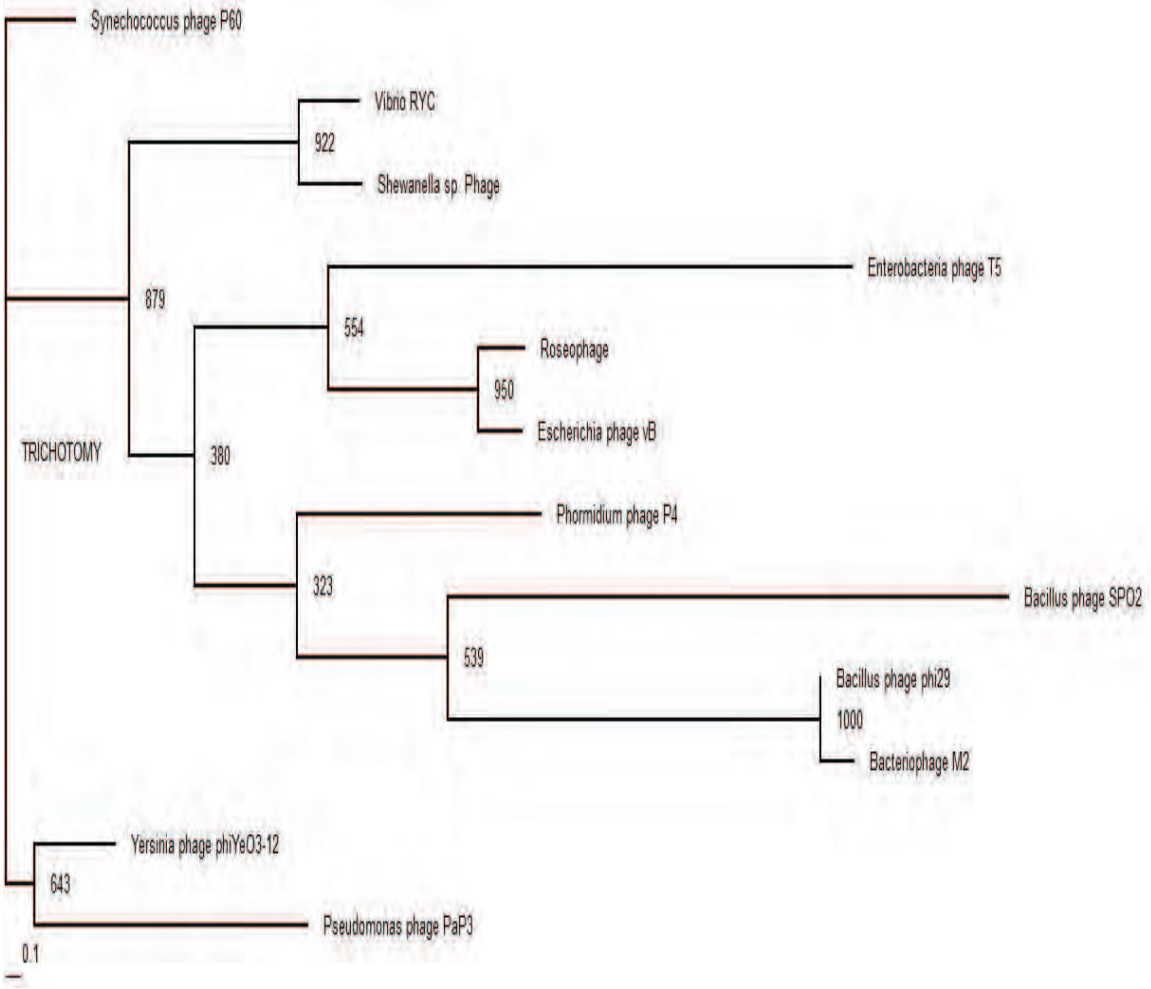


Fig. 4.6: Phylogenetic tree of VCPH RYC - *V. coralliilyticus* (Japan) phage.

Table 4.1: Seawater sampling at Sesoko and Bise.

	Sampling time				
Sampling sites	May 2014		August−September 2014		
	Coral areas (low tide)	Coral areas (low tide)	Coral areas (high tide)	Outer reef water (low tide)	Outer reef water (high tide)
Sesoko	9 L	9 L	9 L	9 L	9 L
Bise	9 L	9 L	9 L	9 L	9 L

Table 4. 2: Phage titer derived from seawater, Bise and Sesoko, Japan.

Sampling time (2014)	Seawater sampling sites	Tidal level	Concentration factor (TFF)	Host bacteria (*V. coralliilyticus*)	Isolated phage titer (pfu/mL)*
May	Sesoko – coral areas	Low tide	x 30	Australia	60
August	Bise – coral areas	Low tide	x 30	Australia	~2
September	Sesoko – Outer reef	Low tide	x 30	Australia	1.8×10^{8}
September	Sesoko – coral areas	Low tide	x 30	Australia	~2
August	Bise – Outer reef	Low tide	x 42	Japan	2.99×10^{5}

*The phage titer was determined by pfu/mL regardless to the concentrated water.

Table 4. 3: Estimated genome of phages isolated from seawater, Bise and Sesoko, Okinawa, Japan.

Sampling time (2014)	Seawater sampling sites	Phage labeling	Host bacteria (*V. coralliilyticus*)	Phage family	Estimated genome (kbp)
May	Sesoko – coral areas	L19,2	Australia	Myoviridae	48
August	Bise – coral areas	L3	Australia	Myoviridae	156
September	Sesoko – Outer reef	L8	Australia	Myoviridae	35
September	Sesoko – coral areas	L7	Australia	Myoviridae	48
August	Bise – Outer reef	RYC	Japan	Unclassified	158

Table 4. 4: Analysis of partial sequencing of the phages L19,2, L3, L8, L7 with reference to *Vibrio parahaemolyticus* phage VP16T & VP16C.

Phage labeling	Estimated genome (bp) length	GC	Coverage	Denotation
L8	48285	61.39	153.17	A
L7	48284	61.38	310.87	A
L19,2	48284	61.34	28.98	A
L8	34840	45.98	444.44	B
L7	46155	45.68	4.38	C

A: phages sharing common sequence in regards to genome length of about 48 kb and 35 kb; B: phages sharing common sequence in regards to genome length of about 35 kb; C: phages sharing common sequence in regards to genome length of about 46 kb but showed low coverage.

Table 4. 5: Analysis of proteins of VCPH RYC.

Protein_ID	Annotation	VCPH_RYC	Vibriophage helene12B3	Vibriophage eugene12A10	Vibriophage 1189 5-B1	Vibriophage ICP1	Shewanella sp. phage 1/40	Shewanella sp. phage 1/4	Vibriophage PWH3a-P1
VCPH_00034	hypothetical protein	1	0	0	0	0	1	0	0
VCPH_00041	nicotinate phosphoribosyltransferase	1	0	1	0	1	1	1	0
VCPH_00042	putative phosphatase	1	0	0	0	1	1	1	0
VCPH_00043	ribose-phosphate pyrophosphokinase	1	1	1	0	1	1	0	0
VCPH_00049	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00051	hypothetical protein	1	0	0	0	1	0	0	0
VCPH_00052	gp34	1	0	0	0	1	0	0	0
VCPH_00055	ATP-dependent DNA ligase	1	1	0	0	0	1	1	0
VCPH_00057	ClpP ATP-dependent protease subunit	1	1	1	1	1	0	0	1
VCPH_00059	PhoH family protein	1	0	0	0	0	0	0	1
VCPH_00061	putative Gp5 baseplate hub subunit and tail lysozyme	1	0	0	0	1	0	0	1
VCPH_00063	putative ribonucleotide reductase of class Ia (aerobic) beta subunit	1	1	1	1	1	1	1	0
VCPH_00064	putative ribonucleotide reductase of class Ia (aerobic) alpha subunit	1	1	1	1	1	1	1	1
VCPH_00065	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00066	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00067	putative adenine methyltransferase	1	1	1	1	1	0	0	0
VCPH_00068	dihydrofolate reductase	1	1	1	0	0	1	1	1
VCPH_00069	thymidylate synthase	1	1	1	0	0	1	1	1
VCPH_00072	hypothetical protein	1	1	1	0	1	0	0	1
VCPH_00073	gp05	1	0	0	0	0	1	1	0
VCPH_00076	hypothetical protein	1	1	1	0	0	0	0	0
VCPH_00077	putative cell wall hydrolase	1	1	1	1	0	0	0	1
VCPH_00078	phage protein	1	1	1	1	1	1	1	1
VCPH_00079	putative exodeoxyribonuclease	1	1	1	1	1	1	1	1
VCPH_00082	recombination associated protein	1	1	1	1	1	1	1	1
VCPH_00083	hypothetical protein	1	0	0	1	1	0	0	0
VCPH_00084	putative 5'(3')-deoxyribonucleotidase	1	1	1	1	1	0	0	1
VCPH_00085	ATP-binding protein	1	0	0	0	1	0	0	1
VCPH_00088	hypothetical protein	1	1	1	1	1	0	0	1
VCPH_00090	hypothetical protein	1	0	0	0	1	0	0	0
VCPH_00092	hypothetical protein	1	1	0	0	0	0	0	0
VCPH_00094	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00096	AAA ATPase	1	1	1	1	1	1	1	1

VCPH_00098	hypothetical protein	1	1	0	0	0	1	0	0
VCPH_00099	GTP cyclohydrolase II	1	0	0	0	0	0	0	1
VCPH_00100	hypothetical protein	1	0	0	0	1	0	0	0
VCPH_00109	hypothetical protein	1	1	1	0	1	0	0	0
VCPH_00111	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00117	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00118	hypothetical protein	1	0	0	1	1	0	0	0
VCPH_00119	putative terminase large subunit	1	1	1	1	1	1	1	1
VCPH_00123	hypothetical protein	1	0	0	1	0	0	0	0
VCPH_00126	hypothetical protein	1	0	0	0	0	0	0	1
VCPH_00127	tRNA nucleotidyltransferase	1	1	1	1	0	1	1	0
VCPH_00133	hyphothetical protein	1	1	1	1	1	1	1	1
VCPH_00134	hypothetical protein	1	0	0	0	0	1	1	1
VCPH_00135	hyphothetical protein	1	1	1	1	1	1	1	0
VCPH_00136	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00137	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00138	putative major capsid protein	1	1	1	1	1	1	1	1
VCPH_00139	hypothetical protein	1	1	1	1	1	1	1	1
VCPH_00140	hypothetical protein	1	0	0	0	1	1	1	0
VCPH_00141	hypothetical protein	1	1	1	0	0	1	0	0
VCPH_00143	hypothetical protein	1	0	0	0	0	1	1	0
VCPH_00144	hypothetical protein	1	0	0	0	0	1	1	0
VCPH_00148	putative UvrD-type helicase	1	0	0	1	0	0	1	1
VCPH_00154	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00155	hypothetical protein	1	1	1	1	1	1	1	1
VCPH_00157	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00159	hypothetical protein	1	1	0	0	0	0	0	0
VCPH_00160	hypothetical protein	1	1	0	0	0	0	0	0
VCPH_00161	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00162	hypothetical protein	1	1	1	1	1	0	1	1
VCPH_00164	hypothetical protein	1	1	1	1	1	1	1	1
VCPH_00165	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00166	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00168	hypothetical protein	1	1	1	1	1	1	1	1
VCPH_00169	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00170	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00171	hypothetical protein	1	1	1	1	1	1	1	1
VCPH_00172	putative baseplate assembly protein	1	1	1	1	1	1	1	1
VCPH_00173	hypothetical protein	1	1	1	1	1	1	1	0
VCPH_00174	putative baseplate component	1	1	1	1	1	1	1	1
VCPH_00175	hyphothetical protein	1	1	1	1	1	1	1	1
VCPH_00178	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00179	putative tail fiber protein	1	1	1	0	0	0	0	0
VCPH_00180	hypothetical protein	1	1	1	1	0	0	0	0
VCPH_00181	hypothetical protein	1	0	0	1	0	0	0	0
VCPH_00187	hypothetical protein	1	1	1	0	1	0	0	0
VCPH_00188	hypothetical protein	1	0	1	0	0	0	0	0
VCPH_00191	hypothetical protein	1	1	1	1	1	0	0	1
VCPH_00193	hypothetical protein	1	1	1	0	0	0	0	0
VCPH_00195	putative phosphohydrolase	1	1	0	0	0	0	0	0

VCPH_00200	hypothetical protein	1	1	0	0	0	0	0	0
VCPH_00207	DNA polymerase	1	0	0	0	0	1	0	0
VCPH_00208	hypothetical protein	1	1	1	0	0	0	0	0
VCPH_00209	putative helicase	1	0	0	1	0	0	1	0
VCPH_00210	hypothetical protein	1	1	0	0	0	0	0	0
VCPH_00214	hypothetical protein	1	1	1	0	0	0	0	0
VCPH_00215	hypothetical protein	1	1	1	1	0	0	0	1
VCPH_00222	anti-sigma factor	1	1	1	1	0	0	0	1
VCPH_00223	hypothetical protein	1	1	1	0	0	0	0	1
VCPH_00225	hypothetical protein	1	1	1	1	1	0	0	1
VCPH_00231	hypothetical protein	1	0	0	1	0	0	0	0
VCPH_00232	hypothetical protein	1	0	0	1	0	0	0	0
VCPH_00233	hypothetical protein	1	0	0	1	0	0	0	0
VCPH_00237	hypothetical protein	1	1	1	0	0	0	0	0
VCPH_00238	hypothetical protein	1	0	0	0	0	1	0	0
VCPH_00239	DenV endonuclease V	1	1	1	1	0	1	1	0
VCPH_00248	hypothetical protein	1	1	1	0	1	0	0	0
VCPH_00251	hypothetical protein	1	1	1	1	1	0	0	0
VCPH_00252	hypothetical protein	1	0	0	1	0	0	0	1
VCPH_00253	hypothetical protein	1	1	1	0	1	0	0	1
VCPH_00260	hypothetical protein	1	0	1	0	0	0	0	0
VCPH_00267	hypothetical protein	1	0	0	1	0	0	0	0
		105	73	68	60	55	42	39	38
	Percentage of Proteins (%)		69	65	57	52	40	37	36

Pink areas represent proteins which are absent whereas dark green areas are proteins which are present. Light green highlights are enzymes (functional proteins) whereas yellow highlights are proteins with structural functioning.

CHAPTER 5

General Conclusion and Further Research

5.1 General conclusion

Overall, this study focused on marine viruses, especially phages, and abundance of Virus-like particles (VLPs) in coral reef ecosystem. The study sites were mostly in Okinawa (local sites: Bise and Sesoko) and one overseas site – Tang Kheng Bay, Phuket, Thailand.

One of the big finding in this study is the isolation of a possible novel phage known as VCPH RYC infecting *V. coralliilyticus* strain (AB 490821, Japan). This phage could not be classified morphologically and genetically, although the whole genome was sequenced. Genotypic classification is still difficult as there is a lack of taxonomic marker in this field. Nonetheless, it shared certain proteins to other Vibrio phages such as Vibrio phage helene 12B3 (70%), Vibrio phage eugene (65%), Vibrio phage 11895-B1 (57%), Vibrio phage ICP1 (52%), Shewanella sp. phage 1/40 (40%), Shewanella sp. phage 1/4 (37%) and Vibrio phage PWH3a-P1 (36%). However, the phage remained unclassified and was registered in the Japanese database gene bank (DDJB). Other phages were also isolated infecting *V. coralliilyticus* strain P1 (LMG 23696, Australia). These phages could be characterized morphologically belonging to Myoviridae family, as they showed features of capsid and tail. Moreover, this study showed that phages could be isolated irrespective of their host/s isolation location.

One of the aims of the study was to seek if the distribution of VLPs in coral reef ecosystem followed the same trend at local and overseas coral ecosystem. It was demonstrated that viral density did not differ significantly between local sites but varied significantly over long geographical distances. This variation was triggered mostly by nutrients availability, especially the concentration of ammonium in seawater. Moreover, it was demonstrated that abundance of VLPs is strongly linked to phytoplankton and bacteria. Another purpose of this study was to find out the

distribution of VLPs in sub-environments (seawater, marine lagoon sediments and healthy *M. digitata* coral tissues) of coral reef ecosystem and to determine, if the same scenario would be found at the two studied sites: Bise and Sesoko. The findings showed that VLPs abundance varied across seawater, lagoon sediments and coral tissues. Nevertheless, the VLPs density did not differ in seawater and coral tissues at Bise and Sesoko, but significant variation was noted in lagoon sediments in this research. The difference in VLPs density in lagoon sediments could be associated with the marine organisms that were mostly present at Bise. Moreover, the VLPs in seawater at Bise could be regarded as bacteriophages, owing to its positive coupling with bacteria. Generally, VLPs abundance varied across the studied samples, however, location showed the least influential effect on VLPs abundance in seawater and coral tissues but not in lagoon sediments.

As a result, this study had answered to the following research questions: whether the abundance of VLPs, bacteria and coccoid cyanobacteria from lagoon sediments, coral tissues (*M. digitata*) and seawater differed in coastal coral areas of Sesoko and Bise, Okinawa, Japan; whether geographical distribution of the abundance of VLPs from seawater in neritic zone of Bise, Okinawa, Japan differed significantly to Tang Kheng Bay, Phuket, Thailand. More lights were shed on the links of the abundance of VLPs to water quality, in terms of nutrients and geographical data of coastal seawater. Furthermore, successful isolation of *Vibrio coralliilyticus* phages was achieved, whereby a novel phage was isolated infecting the Japanese *Vibrio coralliilyticus* strain (AB 490821), VCPH RYC. Four phages were isolated using the Australian *Vibrio coralliilyticus* strain P1 (LMG 23696). These isolated phages could be characterized morphologically belonging to Myoviridae family and showed close relationship to *Vibrio parahaemolyticus* phage VP16T and

VP16C. However, VCPH RYC remained unclassified at both morphological and at genotypic level.

5.2 Further research

5.2.1 Vibrio phages (Japan)

Since there is a lack of genotypic markers, further analysis of the sequenced genomes of the isolated phages - VCPH RYC and Myoviridae-type phages - will determine the conserved genes in those phages, which could be used as possible genotypic markers of Vibrio phages. Further analysis will determine the geographical distribution of the phages with respect to other sequenced phages, if possible.

5.2.2 *Vibrio coralliilyticus* genomic analysis

Since plasmids were found in the two host bacteria, further analysis in this direction will establish if the host bacteria were infected by the isolated phages or other phages. This finding will be very important in order to provide more knowledge and inputs in this domain: whether these plasmids will increase the bacterial resistance to viral infection or affect the bacteria in other ways; what are the functions of the plasmid to the bacteria, and whether environmental factors can turn the plasmid active (such as lytic) or not, remaining inactive.

5.2.3 Phage therapy

Phage therapy is a concept where lytic viruses are used to decrease the population of pathogenic bacteria by infecting and lyse (kill) the bacterial cells. Hence, phage therapy will decrease the rate of certain disease. First of all, *V. coralliilyticus* is a worldwide known pathogen that affects corals. The isolated phage VCPH RYC and other phages will be tested as phage therapy using the corals (*M. digitata* and *Acropora* sp.) in controlled incubation experiments. The outcomes will add more lights in the possible application of this treatment to certain coral diseases, since there is no known treatment, yet. The team of Rosenberg proposed phage therapy since

2007 and research in this field is still limited. Hence, further research in phage therapy using the isolated phages will bring more support to the only treatment of coral diseases, so far identified.

Moreover studying the bacterial genome, some questions to phage therapy could be answered:

Whether the bacteria will develop resistance to viral infection or not?

How long will the bacteria take to develop resistance to phages/after how many infections or generations, resistance to phages will occur?

Which type of horizontal gene transfer will occur?

Whether the phage genes/genetic material inside the host cell can develop into lytic or remain lysogenic?

What is the best time to infect the bacteria?

Whether it is possible to use a cocktail of phages, if the bacteria develop resistance to one specific phage.

5.2.4 Effect of nutrients on the abundance of VLPs

Nutrients have shown strong links to the abundance of VLPs in seawater, in particular ammonium. As a result, further research in this direction will confirm this hypothesis. An incubation experiment with different concentration of nutrients, ammonium, is proposed to study such effect on the abundance of VLPs. Nowadays, there is a great concern with nutrients pollution especially from land and agriculture run-off. Hence, an increase in ammonium could increase the abundance of VLPs and maybe its associated host cells. What will be the effects of the increased viral abundance in coral ecosystem is one of the questions that need to be addressed.

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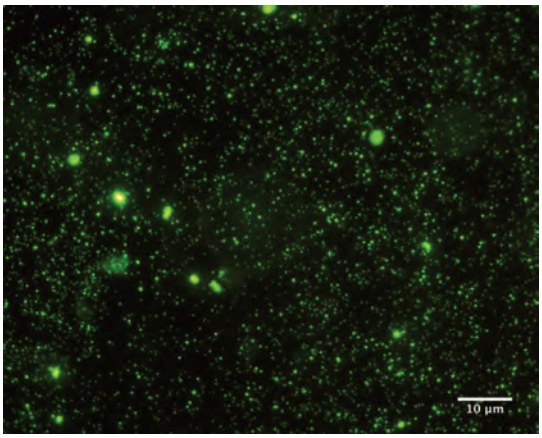
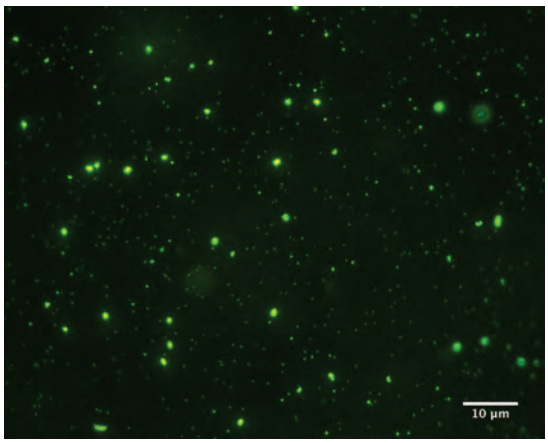
ANNEX

Slides photographs of VLPs, bacteria and coccoid cyanobacteria in seawater of Tang Kheng Bay, Phuket, Thailand and Bise, Okinawa, Japan.

1. VLPs

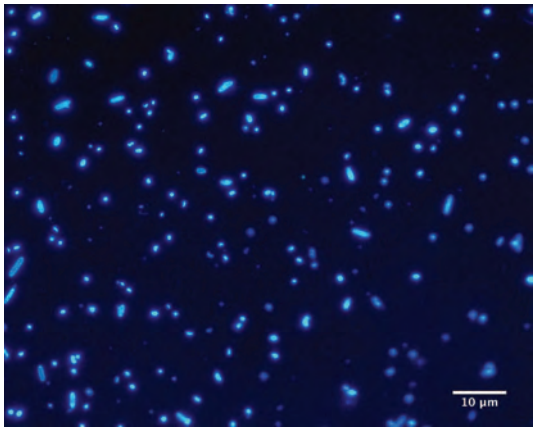


Tang Kheng Bay, Phuket (TKB)

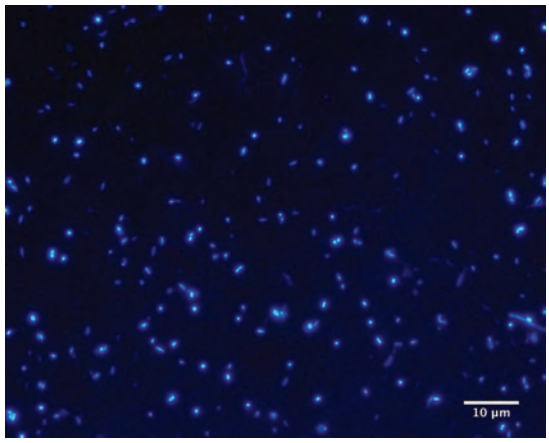


Bise, Okinawa (BE)

2. Bacteria

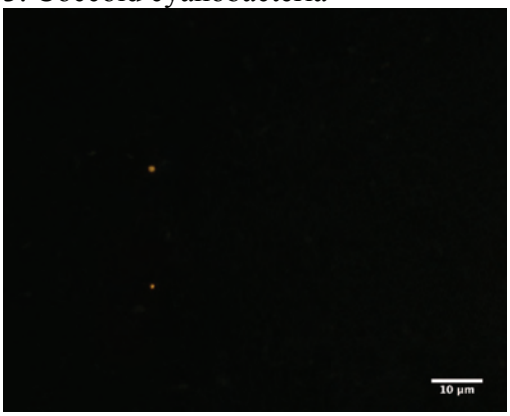


Tang Kheng Bay, Phuket (TKB)

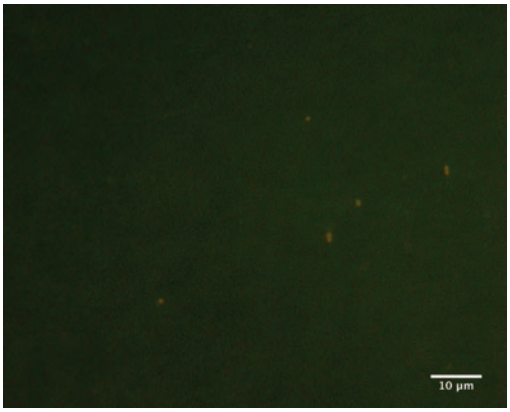


Bise, Okinawa (BE)

3. Coccoid cyanobacteria



Tang Kheng Bay, Phuket (TKB)



Bise, Okinawa (BE)