CORAL REEF VIRUSES IN KANE'OHE BAY, HAWAI'I

ABUNDANCE, DIVERSITY
& ENVIRONMENTAL DRIVERS

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When you make the finding yourself – even if you’re the last
person on Earth to see the light – you’ll never forget it.

(Sagan 1995)

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ABSTRACT

Viruses are a ubiquitous component of coral reef ecosystems, with several viral types, from at least seven prokaryotic and 20 eukaryotic virus families currently characterised from the surface mucopolysaccharide layer (SML), coral tissue and the water column. However, little is known about the ecology and function of these viruses. For example, what are the environmental drivers of viral abundance and diversity on coral reefs? In this study, the abundance and distribution of virus-like particles (VLPs) associated with the SML and reef water of the coral *Montipora capitata* were determined using epifluorescence microscopy, while transmission electron microscopy was employed to determine the morphological diversity of VLPs. Sampling was conducted across the Coconut Island Marine Reserve (CIMR) reef system, Kane'ohe Bay, O'ahu, Hawai'i. Viral abundance was correlated with select environmental drivers and prokaryote abundance, while non-metric multidimensional scaling was used to determine the key environmental drivers of the viral community assemblage. The water column contained high concentrations of VLPs ($5.98 \times 10^7 \text{ ml}^{-1}$) and prokaryotes ($3.11 \times 10^6 \text{ ml}^{-1}$), consistent with the considerable anthropogenic impacts at this location. In comparison, the SML contained lower concentrations of VLPs ($2.61 \times 10^7 \text{ ml}^{-1}$) and prokaryotes ($2.08 \times 10^6 \text{ ml}^{-1}$); of note, the densities of viruses and prokaryotes in the SML were strongly coupled while those in the reef water were not. VLP density in the water column varied spatially across the reef, with the most sheltered site and the only one not situated on the reef crest having a greater VLP density than the other sites. Temporal

variations in the density of microbes (i.e. viruses and prokaryotes) in the reef water were pronounced, while in the SML microbial densities remained constant. However, no specific environmental drivers of this variability could be identified. In contrast, temperature and water quality were correlated with shifts in the morphological diversity of VLPs across the reef. Small (< 50 nm) polyhedral/spherical VLPs were dominant, and were positively correlated to chlorophyll-*a* concentration when in the SML. In this same habitat, *Fuselloviridae*-like VLPs, filamentous VLPs and bead-shaped VLPs were positively correlated to temperature. In the reef water a different pattern was apparent: large (> 100 nm) *Podoviridae*-like VLPs and elongate *Myoviridae*-like VLPs, as well as lemon-shaped VLPs of both size classes showed positive associations with turbidity, while large filamentous VLPs, *Geminiviridae*-like VLPs and rod-shaped VLPs were positively associated with temperature. These results demonstrate that the viral community of Coconut Island's reef is highly diverse, and subject to spatial and temporal change, especially in the water column. However, while the environmental drivers of viral diversity were partly elucidated, we are still a long way from understanding the drivers of viral abundance. More detailed study, both spatially and temporally, of the CIMR environment is required, as is comprehensive molecular analysis of the viral community of Kane'ohe Bay. Only then can we begin to understand the importance of viruses to the health and function of this, and other reef sites.

DECLARATION

STATEMENT OF ORIGINALITY

The following work presented in this thesis, is to the best of my knowledge, original. It is a representation of my own work throughout my Masters of Science course, except as acknowledged throughout the text. All figures presented represent original work unless otherwise stated. The contained material has not been submitted for any degree at this or any other institution.

Candidate's signature

5 January 2012

Date

Supervisor's signature

5 January 2012

Date

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GLOSSARY OF TERMINOLOGY

Archaea: Domain (or subkingdom) of prokaryotic organisms containing the archaeobacteria. Distinct from eubacteria in certain aspects of their chemical structure, such as composition of their cell walls (Prangishvili 2011).

Bacteria: Single-celled organisms, spherical, spiral, or rod shaped, and appearing singularly or in chains (Karl 2007).

Bacteriophage (or *phage*): A virus that infects bacteria. A bacteriophage typically consists of a protein capsid that encloses genetic material. Phage are among the most diverse and widely distributed biological entities on the planet and are grouped within three viral Families: *Myoviridae*, *Siphoviridae* and *Podoviridae* (Ackermann 2007).

Capsid: A shell of proteins, which generally surrounds the viral genome (Carter and Saunders 2007).

Coral holobiont: The collective community of coral animal and associated zooxanthellae, endolithic algae, Protozoa, and microbiota (Rohwer et al. 2002).

Cnidaria: A phylum of exclusively aquatic, invertebrate animals characterised by radial symmetry, a sac-like internal cavity and nematocyst stinging cells encompassing the

Anthozoa which includes the stony corals, anemones, zoanthids, corallimorphs, soft corals, sea fans, sea pens and blue corals (van Oppen et al. 2009).

Eubacteria: A bacterium of a large group (kingdom Eubacteria) typically having simple cells with rigid cell walls and often flagella for movement. The group is comprised of the “true” bacteria and cyanobacteria, as distinct from the archaeobacteria (Ackermann 2007).

Eukaryote: An organism consisting of a cell or cells in which the genetic material is DNA in the form of chromosomes contained within a distinct nucleus. Eukaryotes include all living organisms other than the eubacteria and archaeobacteria (Fuhrman 1999).

Host cell: A living cell in which a virus reproduces (Hogan 2010).

Infectious virus: A viral particle that has the potential to: (1) attach to and enter its host, (2) decode its genomic information, (3) undergo replication and assembly, and (4) exit its host (Thurber and Correa 2011).

Lytic Virus: A virus that causes lysis of the host cell (Wilcox and Fuhrman 1994).

Lysogenic Virus: A virus that can adopt an inactive (lysogenic) state, in which it maintains its genome within a cell instead of entering the lytic cycle (Wilson and Mann 1997).

Microbe(s): A microorganism, here referring to viruses, bacteria and archaea.

Microbial community: An assemblage or population of microbes (Fuhrman 2009).

Microbial loop: The recycling of nutrients through microbes (Fuhrman 1999).

Picoplankton: The fraction of the plankton composed of cells of between 0.2 μm and 2 μm (Karl 2007).

Prokaryote: A unicellular organism having cells lacking membrane-bound nuclei; bacteria are the prime example but also included are archaea, blue-green algae, actinomycetes and mycoplasma (Weinbauer 2004).

Provirus (or *prophage*): The genetic material of a virus as incorporated into, and able to replicate with, the genome of a host cell (Prangishvili 2011).

Scleractinia: The taxonomic order Scleractinia (within the anthozoans), containing the hard/reef-building corals.

Symbiosis: A close interaction or association between evolutionarily distinct organisms (Baker 2003).

Virus: An infectious agent that can replicate only inside the living cells of organisms (Carter and Saunders 2007).

Virus-like particles (VLPs): Material that (1) physically resembles a virus but is non-infectious (e.g. self-assembled viral protein components), or (2) is hypothesized to be viral but for which an infectious cycle has not been observed (e.g. genetic sequence similar to or material physically resembling a known virus) (Thurber and Correa 2011).

Viral-shunt: The movement of material from heterotrophs and photoautotrophs into particulate organic matter (POM) and dissolved organic matter (DOM) (Suttle 2007).

Virion: The physical structure of a virus, which consists of a genome surrounded by a protein coat or capsid. This term is interchangeable with ‘viral particle’ (Thurber and Correa 2011).

Zooxanthellae: Symbiotic dinoflagellates, commonly of the genus *Symbiodinium*, that form endosymbiotic associations with a variety of marine invertebrates and protists including cnidarians, molluscs and foraminiferans (Rowan 1998).

ABBREVIATIONS

ATP	Adenosine triphosphate
DNA	Deoxyribose nucleic acid
dsDNA	Double-stranded deoxyribose nucleic acid
dsDNA-RT	Reverse transcribing double-stranded deoxyribose nucleic acid
dsRNA	Double-stranded ribose nucleic acid
EFM	Epifluorescence microscopy
FC	Flow cytometry
FVLPs	Filamentous virus-like particles
GAs	Growth anomalies
GBR	Great Barrier Reef
NLI	Northern Line Islands
PCR	Polymerase chain reaction
PGEF	Pulsed gel electrophoresis
Phage	Bacteriophage
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Scanning electron microscopy

SML	Surface mucopolysaccharide layer
ssDNA	Single-stranded deoxyribose nucleic acid
ssRNA	Single-stranded ribose nucleic acid
ssRNA-RT	Reverse transcribing single-stranded ribose nucleic acid
TEM	Transmission electron microscopy
VLP(s)	Virus-like particle(s)
VPR	Virus:prokaryote ratio (also called virus:bacteria ratio)

GENERAL INTRODUCTION

1.1 Defining the virus

Viruses are unique biological entities classified as non-cellular obligate intracellular parasites (Carter and Saunders 2007) that are known to infect all cellular life forms: eukaryotes - encompassing vertebrate and invertebrate animals, plants, algae and fungi; and prokaryotes - encompassing eubacteria ('true bacteria' and cyanobacteria) and archaea (Carter and Saunders 2007). While their presence can be seen in infected host organisms with disease, many organisms are host to non-pathogenic viral infections (Jiang and Paul 1994, 1998 Wilson and Mann 1997; McDaniel et al. 2002; McDaniel and Paul 2005).

Viruses are composed of a genome with either double- or single-stranded DNA (dsDNA, ssDNA) or RNA (dsRNA, ssRNA), though reverse-transcribing viruses can also contain double- or single-stranded (dsDNA-RT, ssRNA-RT) genomes (Thurber and Correa 2011). In all cases, these genetic components are encased in a protein capsid and many also incorporate a lipid component at the surface, forming an envelope containing proteins that aid entry into the host cell (Carter and Saunders 2007).

Viruses exist in two distinct states. The first, the virion, is defined as a virus outside a host cell, and functions as a gene-delivery system, allowing survival of the genetic material.

Virions are inert, with no internal biological activities occurring until they come into contact with an appropriate host, when they become active and are referred to as a virus, their second distinct state (Carter and Saunders 2007).

Viruses employ several successful reproductive strategies that have allowed them to become the most abundant biological entities currently known (Suttle 2007). By gaining an in-depth understanding of viral reproductive processes, knowledge of viral infection, disease and the other roles viruses play in the ecological processes of ecosystems, can be further elucidated. Virus replication is achieved by taking over the biosynthetic functions of a host cell (Munn 2006). Contact with the host is through passive diffusion, and the virus uses the host's exposed structures – often transporter proteins – to anchor and gain entry into the cell (Fuhrman 1999). Cell infection is then achieved through one of three basic types of viral reproduction: lytic infection, chronic infection and lysogeny (often termed latency; which includes pseudolysogeny) (Angly et al. 2006). Lytic viral infection involves the virus attaching to the host cell and injecting its genome inside. The host cell is then directed to produce numerous viral progeny, which are released by means of cell lysis. Chronic infection is similar to lytic infection, but the progeny viruses are released in a non-lethal manner (although the host cell is weakened, over several generations, by extrusion or budding from the host cell). Lysogeny involves the nucleic acid of the virus incorporating into the genome of the host cell; this genetic material is then reproduced, as a prophage or provirus, in the host cell. The switch from a lysogenic state to lytic infection can be triggered through a host cell stress event. Such stress events occur either in the form of an environmental trigger such as increased temperature (Edgar and Lielausis 1964) or nutrient availability (Scanlan and Wilson 1999), or host cell exposure to short wave ultra-violet (UV) light (Wilson et al. 2001) (*Figure 1.1*). Finally, pseudolysogeny involves a lysogenic infection in which the viral nucleic acid may remain dormant within the host cell for a number of generations before the switch to lytic infection and cell lysis occurs (Moebus 1996, 1997; Ripp and Miller 1997).

Figure 1.1 Virus reproductive cycles of chronic infection (columns one and three), lytic infection (columns two and three), and lysogenic infection (columns three and four). Here bacteriophage are seen infecting a *Vibrio* sp. host cell.

Host cell lysis can be initiated by the infecting virus through the production of enzymes (lysins, such as lysozymes) that break the bonds in the peptidoglycan of the host cell walls (Weinbauer 2004; Carter and Saunders 2007). Alternatively, phage can manufacture proteins that inhibit host cell enzymes involved in cell wall synthesis, weakening the walls, resulting in cell lysis (Carter and Saunders 2007).

Lytic infection is dependent on encounter frequency, which is determined by viral and host cell density (Wilcox and Fuhrman 1994), with higher host cell density being more conducive to lytic – rather than lysogenic – infections (Weinbauer 2004). Experimental evidence indicates that the majority of viral infections in the marine environment are lytic (Wilcox and Fuhrman 1994), although investigation into lysogenic, pseudolysogenic, and cryptic infection systems remains understudied and requires further investigation.

1.2 *Marine virus characterisation*

Viruses are posited to infect every species of living organism (Suttle 2005; Munn 2006). With such scope for potential infection, defining the host cell of a particular virus may seem like an illogical starting point. However, due to the majority of viruses having small host cell ranges, often being species or even strain specific (Suttle 2007), identifying host cells can be extremely informative.

1.2.1 *Prokaryotic viruses*

Bacteriophage (or ‘phage’) is a term previously used to describe viruses that infect bacteria, but recently broadened to mean “prokaryotic viruses” (Ackermann 2009). This revision follows the discovery of several genes in viruses infecting archaea that are homologous to head-and-tailed bacteriophage (Prangishvili et al. 2006a, b). In this study, the term ‘prokaryotic virus’ will be used, except when referring to viruses infecting bacteria or archaea specifically. Viruses infecting prokaryotic hosts cells have been documented in dominance in viral assemblages of phytoplankton (Bergh et al. 1989; Børsheim 1993; Cochlan et al. 1993; Wommack et al. 1999), with viral abundance

varying in accordance with prokaryotic abundance and productivity (Cochlan et al. 1993; Jiang and Paul 1994; Weinbauer 2004) indicating that the majority of marine viruses are phages (Rohwer 2003; Weinbauer 2004). Estimates of daily prokaryotic virus-induced mortality range from 4-50% (Heldal and Bratbak 1991; Steward et al. 1992a, b; Breitbart and Rohwer 2005), with the remaining prokaryotes being consumed by protist grazing (Fuhrman and Noble 1995). Through the expanding use of culturing techniques it has become apparent that multiple prokaryotic virus types can infect single microbial isolates (Rohwer 2003). This evidence, combined with the knowledge that prokaryotic viruses occupy narrow host cell ranges (Fuhrman 1999; Suttle 2007) implies that they are more diverse than the host cells they infect (Rohwer 2003).

Two viral classification systems are currently used. The first is the 'Baltimore classification' system, which categorises viruses into one of seven groups according to viral nucleic acid type, the negative strandedness of the monopartite genome (Fauquet et al. 2005), sense (comparison of nucleic acid molecule polarity – positive or negative) and method of replication (Baltimore 1971). The second is the 'International Committee on Taxonomy of Viruses (ICTV)' classification system, which imposes specific naming conventions and classification guidelines (Fauquet et al. 2005).

More than 520 sequenced bacteria and archaea viral genomes can be found within the GenBank prokaryotic virus database, of which 88% contain dsDNA, largely distributed within the order *Caudoviridae* (Maniloff and Ackermann 1998). The remaining 12% contain ssDNA or RNA and belong to the *Microviridae*, *Leviviridae* and *Inoviridae* families (Deschavanne et al. 2010). The order *Caudoviridae*, comprised of tailed prokaryotic viruses containing dsDNA, is a large group of viruses with diverse virion, genome and replication properties, corresponding to 84% of available genomes within GenBank (Ackermann 2001; Deschavanne et al. 2010). Three families are nested within the order, and are comprised of 57% *Siphoviridae*, 23% *Myoviridae* and 20% *Podoviridae* (Fauquet et al. 2005; Deschavanne et al. 2010).

Figure 1.2 Simplified prokaryotic virus morphologies categorised within families (for simplicity only those viral groups phenotypically or genetically linked to coral reef ecosystems are shown; see *Table 1.1* below for descriptions of each viral morphotype depicted. Basic morphology design is adapted from Ackermann et al. (2001, 2007). Sizes represented for each viral family are averages of the known size range. It should be noted that the tails of the three bacteriophage groups vary greatly in length (Fauquet et al. 2005). The scale bar represents 100 nm.

Table 1.1 Phenotypic characteristics and traits of prokaryotic viruses with significant similarities to sequences and/or visual evidence from coral reef ecosystem studies. *Figure 1.2* depicts the respective viral families.

SHAPE	NUCLEIC ACID	FAMILY	CITED IN	DESCRIPTION
Short-tailed isometric capsid	dsDNA	<i>Podoviridae</i>	Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008	Icosahedral head and short non-contractile tail, typically lytic. Includes many ecologically important marine vibriophages and cyanophages.
Non-contractile isometric capsid	dsDNA	<i>Siphoviridae</i>	Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008;	Icosahedral head and long flexible non-contractile tail. Many are capable of integrating into the host genome. Includes coliphages of enterobacteria, and cyanophages of <i>Synechococcus</i> - and <i>Phormidium</i> spp.
Contractile isometric capsid	dsDNA	<i>Myoviridae</i>	Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008;	Isometric (A) or elongate (B) polyhedral head and long contractile tail with short appendages. Typically lytic, with a relatively broad host range. Frequently isolated from seawater.
Icosahedral	ssDNA	<i>Microviridae</i>	Wegley et al. 2007; Marhaver et al. 2008	Icosahedral tail-less viruses. Capsids size varies with genus however they are typically very small often around 25 nm.
Icosahedral	dsDNA	<i>Corticoviridae</i>	Efrony et al. 2007	Icosahedral tail-less viruses with capsid diameters of approximately 56 nm. Six short spike proteins protrude from each of the six corners of the capsid.
Filamentous	ssDNA	<i>Inoviridae</i>	Wegley et al. 2007	Filamentous viruses known to infect marine bacteria including <i>Vibrio</i> spp. and coliphages of enterobacteria. Members of the <i>Inoviridae</i> have also been isolated from halophilic and thermophilic archaea.
Pleomorphic	dsDNA	<i>Fuselloviridae</i>	Davy and Patten 2007	Virions are lemon-shaped, ranging in size from 80-100 nm in length and 50-80 nm in diameter. Known to infect archaea these viruses have been observed associated with the coral holobiont, yet genetic evidence has yet to be obtained.

This ratio closely reflects Ackermann's (2007) review of TEM studies examining prokaryotic viruses, in which 5500 bacteriophages were categorised according to morphotype. The most abundant dsDNA viruses were tailed bacteriophages, totaling 96%. These were comprised largely of phage within the order *Caudoviridae* (61% *Siphoviridae*, 24.5% *Myoviridae*, and 14.5% *Podoviridae*). Conversely, non-tailed phage made up 3.7% of the total, consisting of polyhedral, filamentous, and pleomorphic morphologies (*Figure 1.2* and *Table 1.1*).

Wichels et al. (1998) provided sound evidence for the current system of categorisation, showing 85 prokaryotic viruses within the order *Caudoviridae* by utilising dot-blot DNA-DNA hybridisation. Using this method, the authors concluded that there is a lack of genetic homology between prokaryotic virus families, implying that current application of the nomenclature system, according to virion morphology, viral nucleic acid type, and host range characteristics, is consistent with genetic analysis of prokaryotic viral genomes.

1.2.2 Eukaryotic viruses

Eukaryotic viruses are, on average, distinctly larger than prokaryotic viruses and have icosahedral capsids rather than the polyhedral heads and tails that are characteristic of the majority of prokaryotic viruses (Weinbauer 2004). Filamentous, pleomorphic and rod-shaped morphologies have also been described in the eukaryotic viruses (Ackermann 2001). Eukaryotic viruses have been found to infect more than 50 species of algae in ten of the fourteen orders (Dodds 1979; Brussaard 2004; Ackermann 2007, 2009) and at least 20 different eukaryotic viral families have been associated with coral reef systems (*Figure 1.3* and *Table 1.2*).

The largest and most thoroughly studied family of eukaryotic viruses is the *Phycodnaviridae* (Ackermann 2007; Wilson et al. 2009; *Figure 1.3* and *Table 1.2*). Classified in the nucleotide group of viruses – the nucleocytoplasmic large DNA viruses (NCLDV) – members of the *Phycodnaviridae* are recognised as having a significant influence on critical ecological elements within the marine environment (Van Etten 2002).

Figure 1.3 Simplified eukaryotic virus morphologies categorised within families and genera (for simplicity only those viral groups phenotypically or genetically linked to coral reefs are shown; see *Table 1.2* below for descriptions of each viral morphotype depicted). Sizes represented for each family are averages of known size range. Simplified virus morphologies adapted from Fauquet et al. (2005). The scale bar represents 100 nm.

Table 1.2 Phenotypic characteristics and traits of eukaryotic viruses with significant similarities to sequences and/or visual evidence from coral reef ecosystem studies. *Figure 1.3* depicts the respective viral families.

SHAPE	NUCLEIC ACID	FAMILY	HOST	CITED IN	DESCRIPTION
Polyhedral	ssDNA	<i>Nanoviridae</i>	Plants	Wegley et al. 2007	Polyhedral tailless virions; non-enveloped capsids measure 17-20 nm in diameter.
Polyhedral	ssRNA	<i>Picornaviridae</i>	Mosquitoes, ticks, and some vertebrates	Culley and Steward 2007	Polyhedral/spherical tailless capsids; non-enveloped ranging in size from 22-30 nm. Small host-cell ranges.
Icosahedral	ssDNA	<i>Parvoviridae</i>	Vertebrates (sub family: <i>Parvovirinae</i>) and invertebrates	Vega Thurber et al. 2008	Icosahedral tailless virions; non-enveloped capsids measure 18-26 nm in diameter. Certain parvoviruses require helper viruses to replicate efficiently (e.g. adeno- and herpesviruses)
Icosahedral	ssDNA	<i>Circoviridae</i>	Birds, pigs	Wegley et al. 2007	Icosahedral tailless virions; non-enveloped capsids measure approximately 20-30 nm. Infection within this family is prevalent with widespread geographic distributions.
Icosahedral	dsDNA	<i>Papillomaviridae</i>	Vertebrates including humans	Vega Thurber et al. 2008	Icosahedral tailless virions; non-enveloped capsids measure 55 nm in diameter. Can cause benign tumors (warts and papillomas).
Icosahedral	dsDNA	<i>Adenoviridae</i>	Vertebrates	Vega Thurber et al. 2008	Icosahedral tailless virions; non-enveloped measuring 85 nm in diameter, 12 fibers protrude from the capsid surface ranging in size from 9-77.5 nm in length.
Isometric or bacilliform	dsDNA (RT)	<i>Caulimoviridae</i>	Plants	Wegley et al. 2007	Virions are either isometric or bacilliform, depending on genus. Isometric non-enveloped capsids measure 50 nm in diameter. Narrow host-cell ranges.

Table 1.2 continued.

SHAPE	NUCLEIC ACID	FAMILY	HOST	CITED IN	DESCRIPTION
Polyhedral	Nucleocytoplasmic large dsDNA viruses (NCLDV)	<i>Phycodnaviridae</i>	Eukaryotic algae	Wegley et al. 2007; Davy and Patten 2007; Marhaver et al. 2008	Non-enveloped virions are polyhedral with a multilaminar shell surrounding an electron dense core, ranging in size from 100-220 nm in diameter. Some virions have flexible hair-like appendages with swollen structures capping the ends.
Double polyhedral	ssDNA	Geminiviridae	Plants	Davy and Patten 2007; Vega Thurber et al. 2008	Virions are typically twinned "geminate" approximately 22 x 38 nm in size. Each twinned virion is thought to contain a single copy of ssDNA. Known to infect a wide range of plants.
Icosahedral	dsDNA	<i>Iridoviridae</i>	Nematodes, insects, poikilothermic vertebrates including marine animals	Wegley et al. 2007	Virions display icosahedral symmetry usually ranging in size from 100 - 170 nm. However, they can reach sizes of up to 350 nm (e.g. genus <i>Lymphocystivirus</i>).
Icosahedral	dsDNA	Mimiviridae	Protozoa	Wegley et al. 2007	<i>Acanthamoeba polyphaga mimivirus</i> (APMV) - 400 nm in diameter, spherical in shape and surrounded by an icosahedral capsid. Isolated from the amoeba <i>Acanthamoeba polyphaga</i> .
Icosahedral - enveloped	dsDNA	<i>Herpesviridae</i>	Molluscs, fish, turtles, mammals	Wegley et al. 2007; Marhaver et al. 2008; Patten et al. 2008	Polyhedral tail-less viruses. Specimens include members of viral families known to infect eukaryotic algae, protists and invertebrates. Notably Herpes-like viruses have been found within a number of scleractinian coral species.
Icosahedral - enveloped	dsRNA	<i>Asfarviridae</i>	Pigs, warthogs, ticks	Vega Thurber et al. 2008	Lipid envelopes encompass an icosahedral capsid 170-190 nm in diameter that contains an enveloped nucleoprotein core structure 70-100 nm in diameter.

Table 1.2 continued.

SHAPE	NUCLEIC ACID	FAMILY	HOST	CITED IN	DESCRIPTION
Prolate polyhedral	dsDNA	<i>Baculoviridae</i>	Decapods (shrimp), insects (Lepidoptera, Hymenoptera, Diptera)	Wegley et al. 2007; Patten et al. 2008; Vega Thurber et al. 2008	250-300 nm in length x 30-60 nm in diameter. Marginally sensitive to heat and pH between 8.0-1.2. Virions are embedded in occlusion bodies.
Spherical	ssRNA (RT)	<i>Retroviridae</i>	Vertebrates	Vega Thurber et al. 2008	Enveloped virions measure 80-100 nm in diameter. Glycoprotein surface proteins measure approximate 8 nm in length. Occur widely amongst vertebrates.
Ovoid or ellipsoid to bacilliform	dsDNA	<i>Nimaviridae</i>	Crustaceans	Wegley et al. 2007	Known to infect a wide range of crustaceans.
Pleomorphic	Multiple dsDNA	<i>Polydnaviridae</i>	Insects (Hymenoptera)	Wegley et al. 2007; Vega Thurber et al. 2008	Capsids range in size from 300-320 nm in length.
Pleomorphic	dsRNA	<i>Poxviridae</i>	Vertebrates, arthropods	Wegley et al. 2007; Vega Thurber et al. 2008	Virions are either brick-shaped (220-450nm L x 140-260nm W x 140-260nm H) with a lipoprotein surface membrane displaying tubular or globular units (10-40 nm L). They can also be ovoid (250-300nm L x 160-190nm W) with a surface membrane characterised with a regular spiral filament (10-20 nm W).
Filamentous	ssRNA (+)	<i>Closteroviridae</i>	Plants	Lohr et al. 2007	Virions range in size from 1.5-2.2 µm. Most virions are degraded by CsCl and are unstable in high saline concentrations.

This family encompasses the diverse group of icosahedral dsDNA viroplankton that are known to infect a broad range of primary producing organisms, including micro- and macro-algae (Van Etten et al. 1991; Wilson et al. 2009).

The *Phycodnaviridae* play a major role in the regulation of phytoplankton blooms and they assist in the conversion of marine material by way of geochemical cycling on a global scale (Wilson et al. 2009). Such influence is further illustrated through their sheer abundance within the marine environment; they are thought to contribute 0.01%-1% of the estimated $\times 10^{30}$ viruses within oceans (Suttle 2005, 2007). Therefore, members of the *Phycodnaviridae* represent a considerable proportion of the overall marine virus population.

Eukaryotic viruses are known to cause a number of fatal viruses in marine invertebrates, including bivalves (Renault and Novoa 2004). Several *Iridoviridae*-like viruses that have been found to be causative agents of mass mortality in the Portuguese oysters, *Crassostrea angulata* (Comps and Bonami 1977) and *Herpesviridae*-like viruses, associated with high-mortality rates of hatchery reared *Crassostrea gigas* in New Zealand (Hine et al. 1992). In crustaceans, white-spot syndrome, affecting shrimp, is caused by the dsDNA Whispovirus (Mayo 2002); this disease can cause 100% mortality of shrimp populations in just a few days.

1.3 *Determining the abundance and diversity of marine viruses*

The presence of marine viruses has been known for over sixty years (ZoBell 1946, Spencer 1955), but knowledge of their quantitative and diverse nature within the marine environment has only become clear more recently (Torrella and Morita 1979). The ubiquity of viruses within the marine environment is only now beginning to be understood, with the advent of technologies that allow for a higher level of precision when categorising, enumerating and sequencing viruses. These include: transmission electron microscopy (TEM) (Bergh et al. 1989; Bratbak et al. 1990; Proctor and Fuhrman 1990; Suttle et al. 1990; Hara et al. 1991; Hennes and Suttle 1995; Noble and Furham 1999);

flow cytometry (FC) (Olsen et al. 1991; Marie et al. 1999; Tomaru and Nagasaki 2007); epifluorescence microscopy (EFM) (Zimmerman and Meyer-Reil 1975; Hennes and Suttle 1995; Patel et al. 2007); pulsed field gel electrophoresis (PFGE) (Wommack et al. 1999); novel culturing techniques (Rehnstom et al. 1993); and genome sequencing and metagenomic approaches (Hölfe and Brettar 1995; Riesenfeld et al. 2004; Rodriguez-Brito et al. 2006). These have begun to reveal the presence of abundant, diverse and dynamic viral communities that influence many organisms and ecological processes.

Previously, estimates of viral abundance were based upon TEM analysis of virus particles that were removed from seawater samples (Bergh et al. 1989; Børsheim et al. 1990; Proctor and Fuhrman 1990). While TEM provided sound evidence of particles being viral, it resulted in widely variable counts and was both expensive and time consuming (Weinbauer and Suttle 1997; Brussaard 2004). At present, EFM is largely regarded as the superior choice for cost, efficiency, and accuracy when enumerating viral abundances in seawater, sediment and culture (Ackermann 2009). EFM allows for accurate and easy enumeration of viruses through the use of nucleic acid dyes (e.g. acridine orange, DAPI, YoPro, SYBR Green I and II, SYBR Gold) (Francisco et al. 1973; Zimmerman and Meyer-Reil 1975; Noble and Fuhrman 1998; Tuma et al. 1999). Moreover, EFM has been shown to have higher levels of accuracy and precision than both TEM and flow cytometry (Ferris et al. 2002; Ackermann 2009). In addition, EFM does not require access to large expensive equipment and can be conducted in the field with just a light microscope (Brussaard 2004).

Through these technique described, it is now known that viruses are the most abundant biological agents in the oceans (Fuhrman 1999; Suttle 2005, 2007), occurring in an order of magnitude superior to those of prokaryotes (Suttle 2007). These are estimated at 3.6×10^{29} (Sogin et al. 2006) and 1.3×10^{28} (Karner et al. 2001) for bacterial and archaeal cells, respectively (Webster and Hill 2006).

While EFM is the superior choice for viral enumeration, due to the minute size of viruses, TEM has become the standard method for providing visual evidence of particles being viral; these

particles are referred to as virus-like particles (VLPs), and not viruses, until an infectious cycle is observed (for a complete explanation of novel virus discovery terminology see Thurber and Correa 2011). Utilising TEM, Torrella and Morita (1979) provided the first evidence of the morphological diversity of marine viruses. ICTV viral classification is largely based around nucleic acid type and physiochemical properties of the virion (Ackermann and Haldal 2010), although any viral property can be used, including a range of viral morphological characteristics and host cell type, all of which are discernible using TEM.

The ability to measure viral diversity is essential in order to further understand the biogeography and ecology of both viruses and the marine microbes they infect (Cohan et al. 2007). However the genetic richness of marine viruses has become apparent with the use of rapidly developing genomic techniques such as restriction fragment length polymorphisms (RFLP) and hybridization techniques, and PCR methodologies such as denaturing-gradient gel electrophoresis and pulsed gel electrophoresis, and, most recently, metagenomic studies (Suttle 2007). With the use of the above techniques, viral genetic diversity has been found to be vast. Studies that sampled minute subsets of the viral population have found highly diverse viral populations over large (Wilson et al. 1999; Angly et al. 2006) and local (Marhaver et al. 2008) spatial scales. In the pioneering metagenomic study by Breitbart et al. (2002), partially sequenced shotgun libraries from uncultured viral communities isolated from seawater were utilised. The authors found an estimated 3318 and 7114 viral types in 200 L samples of surface seawater taken from Scripps Pier and Mission Bay in San Diego, USA, respectively. In these samples, approximately 65% of sequences were not significantly similar to those previously reported. Even higher viral diversity has been estimated within marine sediment communities, with estimates of at least 10^4 different viral genotypes per kilogram of marine sediment (Breitbart et al. 2004a).

1.4 Functions of marine viruses

Prokaryotes and phytoplankton constitute more than 90% of the living biomass in the world's oceans, with 20% of this biomass estimated to be killed daily by viruses (Suttle

dissolved inorganic carbon (i.e. carbon dioxide) (these compounds can affect virus-induced gene transfer, discussed later in this chapter) (Jiang and Paul 1998; Suttle 2007).

As well as mediating biogeochemical cycling, the viral infection process plays a significant role in the structuring of microbial host communities (Bench et al. 2007). As a large number of marine viruses have concise host cell ranges (Suttle 2007), the role that they play is not limited to simple population regulation through high mortality rates, but extends to complex host cell community structuring through selective mortality and gene exchange.

A number of Lotka-Volterra theoretical models known as 'killing-the-winner' have been used to describe the control of steady state bacterial communities through viral infection (Thingstad and Lignell 1997; Angly et al. 2006). Increases in the overall genetic diversity of the host cell populations occur through the regulation of the competitively dominant microbial strains that would otherwise occupy specific niches (Bratbak et al. 1990; Suttle et al. 1990; Wommack and Colwell 2000; Bench et al. 2007). Open niches can then be utilised by similar microbial strains resistant to the specific phage present in that niche (Angly et al. 2006).

Additional indirect effects of virus-mediated mortality help to further explain the dynamic nature of microbial communities. The most well known example is the bacterial gene homogenising effect through *in situ* horizontal gene transfer (HGT) via virus-mediated transduction (Jiang and Paul 1998). Such transduction can involve lytic or lysogenic phage and, to a lesser degree, eukaryotic viruses (Monier et al. 2007) transferring DNA from one host to the genome of another (Miller and Saylor 1992). This is a highly important process in the evolution and genetic diversification of microbial host cell populations (Weinbauer and Rassoulzadegan 2004). In the case of cryptic lysogenic infection, viruses incorporate their own nucleic acids into the host genome; this can lead to phage mediated phenotypic conversion of prokaryotic hosts (Bench et al. 2007). This conversion can confer the acquisition of new morphological and metabolic traits in the host cell, coded from the newly incorporated viral genome, bestowing increased evolutionary fitness to the host (Weinbauer et al. 2003). Lysogenic

phage conversion is thought to be highly prevalent (Boyd et al. 2001), especially throughout multifarious microbial communities (Munn 2006) such as those associated with scleractinian corals (Rohwer et al. 2001, 2002; Bourne and Munn 2005).

1.5 *Coral reefs*

Tropical coral reefs are among the most diverse and ecologically important ecosystems on the planet, with many human societies heavily reliant on them through fishing and tourism (Hoegh-Guldberg et al. 2007). However, there is a global trend of reef degradation. This decline in reef health stems from a number of factors, including the increase in seawater temperature (Hoegh-Guldberg and Salvat 1995; Hoegh-Guldberg 1999; Bruno et al. 2007), ocean acidification (Hoegh-Guldberg et al. 2007), anthropogenic pollution (Leão et al. 2005; Lipp et al. 2007), overfishing (Bellwood et al. 2005), marine tourism (Maragos and Cook 1995; Nontji 2000; Danovaro et al. 2008) and coral disease epizootics (Rosenberg 2009; Williams et al. 2010a). The detrimental effects these factors have on the homeostasis of reef-building corals, which are highly complex biotic structures, harbouring a plethora of microbial organisms within their skeleton, tissue and mucus (Marhaver et al. 2008) is of primary importance regarding the decline of global coral reef ecosystems.

1.5.1 *The coral holobiont*

Collectively, the coral animal and associated microbes comprise a ‘meta-organism’ (Thurber and Correa 2011) known as the coral holobiont (Rohwer et al. 2002). The holobiont is comprised of the coral animal, microbial eukaryotes including algae, fungi, and protists, prokaryotes including bacteria and archaea, and viruses (Rohwer et al. 2002; Wegley et al. 2007). Given the diversity of these various components, a coral colony represents a variety of potential target hosts for viruses (Thurber and Correa 2011). The majority of marine viruses have specific host cell ranges (Suttle 2007), indicating the potential for highly diverse viral communities associated with corals.

The most extensively investigated symbiotic relationship within the coral holobiont is between the single-celled autotrophic dinoflagellates of the genus *Symbiodinium* (commonly referred to as zooxanthellae) and the coral animal. Harboured within the gastrodermal (endoderm) cells of the coral tissue (Muller-Parker and D'Elia 1997; Smith et al. 2005) the *Symbiodinium* cells photosynthesise, providing photosynthate comprised of glycerol, glucose, amino acids and perhaps lipids to the coral (Falkowski and Raven 1997). In return, the coral host provides the dinoflagellate with nitrogenous compounds, phosphates, carbon dioxide and environmental protection (Davies 1984; Smith et al. 2005). Aside from these autotrophic protists, heterotrophic protists, which include the potentially pathogenic apicomplexans (Upton and Peters 1986; Toller et al. 2002), are also associated with scleractinian corals, though very little is known regarding the roles that such heterotrophic protists may play within the coral meta-organism.

Endolithic (i.e. within the skeleton) fungi are thought to form a parasitic relationship within the coral holobiont, attacking both the coral polyp and the endolithic algae, but it is unclear whether the endolithic fungi are completely parasitic or confer some benefit to the coral holobiont (Kendrick et al. 1982; Priess et al. 2000; Golubic et al. 2005; Wegley et al. 2007). It also remains to be seen what roles endolithic algae have within the coral holobiont (Shashar et al. 1997; Fine and Loya 2002) and in general little is known about the abundance, diversity, and metabolic contributions of any of the endolithic organisms (Wegley et al. 2007).

The interface between the coral holobiont and the environment is the surface mucopolysaccharide layer (SML) (Ritchie and Smith 2004; Brown and Bythell 2005). In contrast to the nutrient depleted waters surrounding the coral animal, the SML is a highly productive, carbon-rich mucus zone, extending several millimeters above the surface of the coral colony (Paul et al. 1986; Ferrier-Pagès et al. 1998; Gast et al. 1998; Davy and Patten 2007). The SML plays a variety of roles in the health and homeostasis of the coral animal and couples the coral holobiont to the overlying reef water environment (*Figure 1.6*). Bacteria are the dominant microbial component in

the SML and as a result of the carbon-rich environment provided by the coral mucus these bacterial communities have been demonstrated to have higher productivity in the SML than the overlying reef water (Paul et al. 1986; Coffroth 1990; Ritchie and Smith 2004). SML-associated bacteria are hypothesised to fulfil a number of roles: filling entry niches – thereby preventing the entry of pathogenic or opportunistic microbes (Rohwer et al. 2002); providing the coral with supplementary nutrients such as fixed nitrogen (Williams et al. 1987, Shashar et al. 1994); aiding in the uptake of phosphorus, iron and sulphur (Rohwer et al. 2002; Vega Thurber et al. 2009); producing antibiotic substances (Ritchie 2006; Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009); and acting as causal agents in specific cases of disease manifestation (Ben-Haim and Rosenberg 2002; Rosenberg 2009).

Figure 1.5 The coral holobiont model originally proposed after Rohwer et al. (2002). The model depicts a coral colony as a holobiont, consisting of coral animal, zooxanthellae, and associated microbiota. Microbiota includes endolithic algae, bacteria, archaea, fungi, Protozoa, viruses, and possibly unknown components yet to be discovered. 'Loop A' details the symbiosis between zooxanthellae and coral. 'Loop B' details the interaction between coral and associated microbiota. By-products of respective interactions are shown in *Table 1.3*.

Table 1.3 Organisms associated with the coral holobiont. Information sourced from: Fine and Loya (2002); Rohwer et al. (2002); Toller et al. (2002); Golubic et al. (2005); Smith et al. (2005); Ritchie (2006); Davy and Patten (2007); Wegley et al. (2007); Shnit-Orland and Kushmaro (2009).

ORGANISM	ASSOCIATED AREA OF THE HOLOBIONT	MUTUALISTIC OR PARASITIC	PREDICTED FUNCTIONAL ROLES	BY-PRODUCTS
Coral animal	Structure	Not applicable	Structure of holobiont, environmental protection to resident symbionts	Nitrogenous compounds, phosphates, CO ₂
Zooxanthellae (<i>Symbiodinium</i>)	Coral gastrodermal tissue	Mutualistic	Fixes carbon through photosynthesis, and lipids?	Glucose, glycerol, amino acids
Endolithic algae	Coral skeleton	Unknown	Unknown	Unknown
Protozoa	Unknown	Potentially parasitic	Unknown	Unknown
Bacteria	CSM, potentially harboured within the coral tissue	Mutualistic and parasitic	(1) utilise complex carbon compounds (proteins and polysaccharides) (2) carbon and nitrogen fixation (3) genes for stress response and virulence, in particular, DNA repair and antibiotic resistance (4) potential defence against infecting pathogenic bacteria/viruses	Potentially produce antibiotic substances
Archaea	CSM	Potentially mutualistic, and parasitic	Unknown, hypothetically similar to that of bacteria	Unknown
Cyanobacteria	CSM	Unknown	Unknown	Unknown
Endolithic fungi	Coral skeleton and polyps, endolithic algae	Parasitic	Nitrogen cycling, including ammonification. Thought to not live strictly parasitically as previously described	Enables fixed nitrogen to cycle within the coral holobiont
Viruses	Coral animal (CSM, Coral tissue), zooxanthellae, protozoa, bacteria, archaea, cyanobacteria, fungi	Mutualistic and parasitic	(1) Infect prokaryotic population associated with coral (2) cross-protection (3) niche opening	Unknown

The SML-associated microbial consortium is thought to be distinct from that of the surrounding reef water (Rohwer et al. 2001, 2002; Davy and Patten 2007), with bacterial communities being identified as being highly specific, even down to the individual coral colony level (Santavy 1995; Rohwer et al. 2001). Archaea communities in the SML have also been shown to be highly diverse (Kellogg et al. 2004), although unlike certain coral-associated bacteria they are not thought to be coral colony specific. Rather, SML-associated archaea communities may be associated with one species type throughout a reef system (Kellogg et al. 2004; Wegley et al. 2004, 2007; Beman et al. 2007).

Viruses are known members of the coral holobiont (Rohwer et al. 2002; Wilson et al. 2005; Davy et al. 2006; Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008; Patten et al. 2008a) and potentially play a lead role in coral health and breakdown (Marhaver et al. 2008). Viruses have been found within the gastrodermal, epidermal and, on rare occasions, mesogleal tissues of the coral animal (Patten et al. 2008a). They are also found in the SML (Davy and Patten 2007), can infect zooxanthellae (Wilson et al. 2001, 2005; Davy et al. 2006), and may potentially infect other, if not all, associated microbiota. This implies that corals harbour a highly diverse viral community, comprised of both prokaryotic and eukaryotic viruses of multiple types, although to date there are no known viral pathogens of corals (Bourne et al. 2009; but see Davy 2007).

Many of the organisms that comprise the coral holobiont are thought to be species-specific, leading to a unique form of co-evolution (Rohwer et al. 2002). In spite of the increasing effort to understand these interactions, and how they might affect coral health, it remains uncertain as to whether many of these associations are pathogenic, mutualistic, or opportunistic.

Figure 1.6 The coral surface mucopolysaccharide layer and coral tissue layers with associated microbial consortium. The microbes (viruses and prokaryotes) are not to scale in relation to the cell layer and endosymbiotic dinoflagellates. The surface mucopolysaccharide layer is depicted containing eukaryotic and prokaryotic viruses and bacteria. Viruses are seen further infiltrating the ectodermis and endodermis coral tissue layers with filamentous virus-like particles infecting the endosymbiotic dinoflagellates.

A number of studies have demonstrated that abiotic stressors alter the relationships between the members of the holobiont (Hoegh-Guldberg 1999; Ben-Haim et al. 2003a, b; Frias-Lopez et al. 2004; Bourne et al. 2008). For the coral *Porites compressa*, Vega Thurber et al. (2009) showed that exposure to selected abiotic stressors resulted in a shift of coral-associated microbiota from a 'healthy-associated' coral community (e.g. *Cyanobacteria*, *Proteobacteria* and the zooxanthellae) to a 'disease-associated' coral community (e.g. *Bacteroidetes*, *Fusobacteria* and fungi).

1.5.2 Viruses on coral reefs

Coral reef health is a serious concern, with the world currently losing approximately 2% of its reefs per year (Bruno and Selig 2007). Coral bleaching events, most notably caused by elevated seawater temperature (Brown 1997; Douglas 2003) and high solar irradiance (Le Tissier and Brown 1996), and coral disease outbreaks linked to pollution (Leão et al. 2005; Lipp et al. 2007), thermal stress (Bruno et al. 2007), over-fishing (Bellwood et al. 2005), nutrient increase (Bruno et al. 2003) and other anthropogenic influences (Dinsdale et al. 2008b), are strong contributors towards this global decline. However, despite the potential importance of viruses in this decline, to date there have been only 15 published studies that have considered the relationship of viruses to corals and coral reefs, and as such their role in coral reef function and health is poorly understood (*Table 1.5*; see review by Thurber and Correa 2011).

Table 1.4 Summary of published literature on the virus-like particles (VLPs) isolated from cnidarians to date, the location in which VLPs have been observed, and the approaches employed. RT-PCR = real time-polymerase chain reaction; TEM = transmission electron microscopy; FC = Flow cytometry (adapted from Thurber and Correa 2011).

STUDY	CNIDARIAN TAXA	SAMPLE TYPE	APPROACH
Wilson and Chapman (2001)	<i>Metridium senile</i> ¹	Anemone tissue	TEM
Wilson et al. (2001)	<i>Anemonia viridis</i> ¹	<i>Symbiodinium</i> spp.	TEM
Lipp et al. (2002)	Various stony coral spp. ²	CSM	RT-PCR
Lipp and Griffen (2004)	Various stony coral spp. ²	CSM	PCR
Cervino et al. (2004)	<i>Montastraea faveolata</i> ²	<i>Symbiodinium</i> spp.	TEM
Wilson et al. (2005)	<i>Pavona danai</i> ²	Coral tissue	TEM
Davy et al. (2006)	Various coral spp. ² , <i>Zoanthus</i> sp. ³	Coral tissue	TEM, FC
Davy and Patten (2007)	<i>Acropora</i> and <i>Porites</i> spp. ²	CSM	TEM
Lipp et al. (2007)	Various stony coral spp. ²	CSM	PCR
Lohr et al. (2007)	<i>Acropora formosa</i> ²	<i>Symbiodinium</i> spp.	TEM
Wegley et al. (2007)	<i>Porites astreoides</i> ²	Microbial community	Microbial metagenomics
Patten et al. (2008a)	<i>Acropora muricata</i> ²	Coral tissue	TEM
Marhaver et al. (2008)	<i>Diploria strigosa</i> ²	Coral tissue	Viral metagenomics
Danovaro et al. (2008)	<i>Acropora</i> spp. ²	Coral tissue	TEM
Vega Thurber et al. (2008)	<i>Porites compressa</i> ²	Coral tissue	Viral metagenomics

1. Anemone 2. Scleractinian coral 3. Zoanthid

The bulk of these studies have either directly investigated viral diversity of the coral animal through the use of metagenomics (Wegley et al. 2007; Marhaver et al. 2008; Vega Thurber et al. 2008) or through phenotypic or genomic techniques, focused on specific areas of the coral animal such as the coral SML (Lipp et al. 2002, 2007; Lipp and Griffen 2004; Davy and Patten 2007), or coral-associated microbiota such as *Symbiodinium* spp. (Cervino et al. 2004; Wilson et al. 2005; Davy et al. 2006; Lohr et al. 2007).

Table 1.5 Summary of published studies identifying virus-like particle (VLP) and prokaryote densities from coral-associated reef water and sediment sampled using direct counts. Values presented for VLP and prokaryote densities represent average VLP ($\times 10^6$) ml⁻¹ and prokaryotes ($\times 10^5$) ml⁻¹ in reef water, and $\times 10^8$ /cm³ for all sediment samples. VPR = virus to prokaryote ratio. ND = not determined. GBR = Great Barrier Reef.

STUDY	SAMPLED LOCATIONS	APPROACH (ES)	SAMPLED HABITAT TYPE	VLP ABUNDANCE	PROKARYOTIC ABUNDANCE	VPR
Moriarty (1979)	Lizard Island, GBR, Australia	Epifluorescence microscopy direct counts	Reef water	ND	10 - 25	ND
Moriarty et al.(1985)	Lizard Island and Yonge Reef, GBR, Australia	Epifluorescence microscopy direct counts	Reef water	ND	1.2 - 6	ND
Paul et al.(1986)	Dry Tortugas and Key West, FL, USA	Cell:polystyrene latex bead ratio	Reef water	ND	1.7 - 6.3	ND
Hoppe et al.(1988)	Danajon Bank, Central Visayas, Philippines	Epifluorescence microscopy direct counts	Reef water	ND	0.51 - 0.76	ND
Paul et al. (1993)	Key Largo, FL, USA	TEM direct counts	Reef water	1.7 - 12	ND	ND
Paul et al. (1993)	Key Largo, FL, USA	TEM direct counts	Sediment	1.35 - 5.3	ND	ND
Gast et al.(1998)	Curaçao	Epifluorescence microscopy direct counts	Reef water	ND	2 - 9	ND
Seymour et al. (2005)	Nelly Bay, Magnetic Island, Australia	Flow cytometry	Reef water	0.4 - 0.85	1.0 - 1.8	4.0 - 6.5
Patten et al. (2006)	Myricon Reef, GBR, Australia	Flow cytometry	Reef water	2.2 - 14	10 - 52	0.4 to 5.8
Dinsdale et al. (2008b)	Line Islands, Kingdom of Kirabati	Epifluorescence microscopy direct counts	Reef water	0.51 - 4.9	0.72 - 8.4	5.0 - 7.1
Patten et al. (2008b)	GBR, Australia	Flow cytometry	Reef water	1.55 - 3.27	4.5 - 8.6	1.2 - 4.8
Patten et al. (2008b)	GBR, Australia	Flow cytometry	Sediment	2.5 - 12	0.2 - 3.0	2 - 9.5

Viral diversity in coral reef water remains poorly characterised (Culley et al. 2006; Culley and Steward 2007) and the level of diversity within the coral holobiont is only just beginning to be understood (Thurber and Correa 2011). Understanding how microbial and viral abundances change across spatial scales in reef water and within the coral holobiont is also only now becoming elucidated (Gast et al. 1998; Rohwer et al. 2001, 2002; Seymour et al. 2005; Patten et al. 2006; Dinsdale et al. 2008a, b; Patten et al. 2008a, b).

To date, the small amount of research focused on viral abundance in coral reef ecosystems has concentrated on reef water and sediment environments rather than determining viral abundance directly associated with the coral holobiont (*Table 1.5*). Coral-associated VLP abundance and diversity will be discussed further in Chapters Two and Three, respectively.

1.5.3 *Viral roles in coral health and functioning*

The majority of research surrounding viral roles in coral health and functioning has focused towards implicating viruses as causative agents of coral disease; the obvious focal point, as the number of new coral diseases continues to rise, with over twenty currently described (Rosenberg 2009). However, the majority of these diseases have unknown etiologies (Rosenberg and Ben-Haim 2002; Rosenberg et al. 2007), with only eight pathogens of coral diseases known to conform to Koch's postulates (the four criteria designed to establish a casual relationship between an infecting microorganism and a disease) (for comprehensive reviews on these coral diseases see Rosenberg et al. 2007 and Rosenberg 2009).

Wilson and Chapman (2001) provided evidence of VLPs within the tissues of the non-symbiotic anemone *Metridium senile*, but viruses were first implicated as contributing to coral reef decline in the investigation by Wilson et al. (2001) of *Symbiodinium* spp. isolated from the temperate sea anemone *Anemonia viridis*. In this study, UV and temperature proposed to induce lytic cnidarian viruses, leading to bleaching. This study isolated a transferable infectious agent, believed to be a virus, following its induction after exposure of the isolated *Symbiodinium* spp. to elevated temperature. Changes in environmental conditions have also been shown to induce the lytic cycle of

viruses associated with scleractinian corals (Wilson et al. 2005). In this study, *Pavona danai* was subjected to heat-shock and VLPs were subsequently observed within both the coral tissue and dinoflagellate symbionts. Davy et al. (2006) built on this study, observing VLPs within the tissue and *Symbiodinium* spp. of heat-shocked corals *P. danai* and *Acropora formosa*, and the zoanthid *Zoanthus* sp. In both these studies, the authors concluded that VLPs were associated with either *Symbiodinium* spp. or the coral animal itself. Lohr et al. (2007) went on to observe the induction of a latent filamentous virus within *Symbiodinium* spp. cultures subjected to UV light stress, implicating both temperature and UV light stress in the induction of the lytic cycle. However, despite a mechanism for viruses being involved with coral bleaching being identified (Rosenberg 2009), evidence of any virus-mediated coral disease remains inconclusive (Thurber and Correa 2011). Marhaver et al. (2008) found a wide diversity of eukaryotic viruses associated with the scleractinian coral *Diploria strigosa*, with *Herpesviridae*-like viral sequences comprising 4-8% of the metagenome in both the healthy and partially bleached specimens sampled. Vega Thurber et al. (2008) also found high levels of *Herpesviridae*-like viral sequences associated with stressed specimens of the coral *P. compressa*, while *Herpesviridae*-like viral sequences were rarely found within the healthy specimens. The phenotypic identification of *Phycodnaviridae*-like VLPs in samples taken from the SML of a number of coral species has resulted in the hypothesis that *Phycodnaviridae* viruses target *Symbiodinium* spp. (Davy and Patten 2007). Viruses in the family *Iridoviridae* have also been associated with coral reef ecosystems (Marhaver et al. 2008; Vega Thurber et al. 2008), as viruses in this family are known pathogens of numerous aquatic organisms including reef fish, molluscs, and crustaceans. Reef organisms feeding on corals can potentially act as vectors for disease, transmitting infectious viruses between corals across a reef (Thurber and Correa 2011).

Some of the strongest evidence towards viruses being causal agents of coral disease can be found in the investigations of coral growth anomalies (GAs). Aeby et al. (2011) positively correlated GAs to human population size and host-density, in what is purportedly the first study to unambiguously link coral disease to increasing human numbers (Aeby et al. 2011). Kaczmarek and Richardson (2007) also found GAs to be transmissible,

suggesting viruses as causal agents. As viruses are known agents of cell proliferation disorders in a number of other marine organisms (Bourne et al. 2009) including turtles (Work et al. 2001, 2004) and fish (Anders and Yoshimizu 1994), the potential for viruses to be causing GAs in corals is likely, however, continued investigation is needed as the etiology of GAs remains largely unknown (Work et al. 2008; Aeby et al. 2011).

While the above studies have not directly implicated viruses in coral bleaching or disease, they do demonstrate that there is a close relationship between viruses, their coral host, associated symbiotic *Symbiodinium* spp. and other microbiota (Lohr et al. 2007). However, while viruses are highly influential within the coral holobiont, they are also the least studied constituent (Marhaver et al. 2008).

It should also be noted that coral-associated viruses need not be synonymous with disease; latent viruses within a coral host potentially play a vector role, transmitting infection to nearby colonies rather than succumbing to the infection themselves (Wilson and Chapman 2001). Viruses can confer beneficial traits to their host (e.g. lysogenic phage conversion, as previously described; Boyd et al. 2001; Weinbauer 2004), although examples are scarce in the marine environment. One well-described viral mutualism involves the relationship between the large *Chlorella* virus Marburg 1 (CVM-1) and the photosynthetic green alga, *Chlorella* (Seaton et al. 1996). In this alga, the virus initiates heat dissipation of excess light energy from photosystem II, preventing damage to the host's photosystem (Seaton et al. 1996). Lastly, virus-mediated structuring of SML-associated microbial communities through limiting overgrowth of opportunistic pathogens is another proposed benefit viruses can confer to the coral holobiont (Davy and Patten 2007). Whether such mutualistic relationships are occurring between the coral holobiont and affiliated viruses remains to be confirmed.

1.5.4 Study species and reef system

Kane'ohe Bay was chosen as the current studies reef system of investigation, characterised as an estuarine and coral reef ecosystem, the bay has had a long history of being adversely affected by a myriad of anthropogenic influences, offering insight into viral assemblages

in a heavily human impacted coral reef ecosystem. The antropogenic influences include increased soil erosion, sedimentation, dredging, channelling of streams in the bay, and over thirty years of primary sewage influx (Maragos and Chave 1973; Smith et al. 1981, Pastorak and Bilyard 1985, Hunter and Evans 1995).

Concomitant agricultural practices and urbanisation from 1940 - 1970 (Hunter and Evans 1995) have amplified these adverse impacts. The negative anthropogenic influences, coupled with naturally high levels of freshwater runoff, led to an algal (the green 'bubble' alga *Dictyosphaeria cavernosa*) and filter/deposit-feeder dominated community, and overall poor coral reef health (Hunter and Evans 1995).

At present, Kane'ohe Bay still has reduced water quality, a known stressor of corals. High nutrient and chlorophyll-*a* levels, indicative of poor water quality, have been recorded in the Coconut Island Marine Reserve (CIMR) (Jokiel et al. 1993; Williams et al. 2010a). Both these proxies for water quality have been linked to increased levels of certain coral diseases including yellow band disease, black band disease and *Montipora* white syndrome (MWS) (Williams et al. 2010a), with the latter found at levels four times higher in Kane'ohe Bay than in other areas of the main Hawai'ian Islands (Williams et al. 2010a).

Corals in Kane'ohe Bay are affected by high freshwater influx, with annual rainfall averaging 140 – 240 cm per year and significant freshwater discharge from the nine perennial streams entering the bay (Jokiel et al. 1993). During rare episodic rainfall events, freshwater influx can increase ten-fold, with salinity dropping from 34 to 15 ppt in surface waters (Jokiel et al. 1993). Such an event reportedly caused close to 100% mortality of coral to depths of 3 m in the western and southern portions of the bay in 1987 (Jokiel et al. 1993).

The reefs of Kane'ohe Bay harbour two competitively co-dominant space holders: *Montipora capitata* and *P. compressa*. *Pocillopora damicornis*, *Pocillopora meandrina* and *Fungia scutaria* are also space-holders in the reef system but are

seen at low densities (Williams et al. 2010a) (*Figure 1.7* gives a detailed explanation of *M. capitata* phenotypic traits and disease).

M. capitata colonies have encrusting bases, but are arborescent when fully developed. Colony level morphology is highly plastic, with colonies containing thick branches, thin branches, plates, whirls or a combination of these (Forsman et al. 2008, 2010).

M. capitata is present in two colour morphotypes within Kane'ohe Bay. The common 'brown' morphotype found at all depths, and the less common shallow dwelling 'orange' morphotype (LaJeunesse et al. 2004). Differences between the two colour morphologies are not only aesthetic, with LaJeunesse et al. (2004) finding clade C *Symbiodinium* within the 'brown' morphotypes and clade D *Symbiodinium* within the 'orange' morphotype.

Montipora white syndrome (MWS)- seen as a diffuse irregular lesion with indistinct edges. Tissue loss can occur gradually or in rapid bursts leaving behind intact white tissue skeleton colonized by algae (Williams et al. 2010). MWS exhibits high prevalence in the CIMR with 29.17% prevalence recorded by Williams et al. (2010). The etiology of MWS is currently unknown, disease prevalence has been positively correlated with chlorophyll-*a* concentration and negatively correlated with juvenile parrotfish abundance within the CIMR (Williams et al. 2010).

Figure 1.7 *Montipora capitata* in the Coconut Island Marine Reserve (CIMR). A: Colony morphology B: The two colour morphotypes of *Montipora capitata* found in the CIMR C: The tissue wasting disease *Montipora* White Syndrome (Image C courtesy of Gareth J. Williams).

1.6 *Aims and specific objectives of the study*

The current study employed EFM and TEM to enumerate and characterise the virus communities in the SML of *M. capitata* and the reef water directly above each colony. Sampling was undertaken on the reef surrounding Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i. Sites were chosen for their variable environmental characteristics, including turbidity, chlorophyll-*a* concentration, and temperature (Williams et al. 2010a). Specifically, the current study aimed to determine whether the abundance and composition of viral communities associated with *M. capitata* is influenced by the environmental regime and proximity to the SML.

The first section of the study (Chapter Two) aimed to explain the relationship between viral abundance and selected environmental factors. The specific aims were:

1. To determine the relative virus numbers associated with the SML of *M. capitata* and overlying reef water around Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i, and to establish whether the SML contains significantly more viruses.
2. To determine the relationship between key environmental variables and viral numbers in the SML and reef water.

The second section of the study (Chapter Three) aimed to characterise the viral community composition and establish the influence of key environmental factors on this community. The specific aims were:

1. To identify and classify the viruses associated with the SML of *M. capitata* and the reef water around Coconut Island, Kane'ohe Bay, Hawai'i, and thereby determine whether the SML has a distinct viral community.
2. To establish how key environmental factors influence viral community composition of both the SML and reef water, with respect to viral morphotype, size, and class.

This was the first attempt to use epifluorescence microscopy for enumeration of VLPs in the SML of scleractinian corals. It was an important undertaking, as no non-genetic quantification methodology exists for the microbial constituent of the coral SML. Moreover, there have been no previous attempts to establish the environmental drivers of viral community composition on coral reefs.

The two data chapters are structured as independent manuscripts; for the purposes of this thesis, each chapter is presented with more detail than will be submitted for publication. Therefore, there is some overlap between these two chapters, though every attempt has been made to keep the information and writing within these chapters distinctive, yet complementary.

ABUNDANCE OF VIRUS-LIKE PARTICLES WITHIN A CORAL REEF SYSTEM

Variation at a local spatial scale

2.1 *Introduction*

2.1.1 *Virus abundance in the marine environment*

Viruses are the most abundant biological agents in the ocean (Fuhrman 1999; Suttle 2005), with a total global figure estimated at 10^{30} within the marine environment (Suttle 2007). Due to their minute size, their abundance to biomass ratio is massively disparate, with viruses comprising a mere 5% of the total microbial biomass within the Earth's seas (Suttle 2007). Their small size does not infer low influence though, as high virus numbers, diversity and infection rates (10^{23} viral infections are predicted to occur every second within the ocean; Suttle 2007) affect major ecological processes including elemental cycling (Weinbauer and Höfle 1998; Wilhelm and Suttle 1999; Weinbauer 2004), regulation of microbial communities (Middelboe et al. 2001), and a wide range of diseases in organisms across all trophic levels (Munn 2006).

2.1.2 *Virus abundance on coral reefs*

Viral abundance studies on coral reefs have predominantly focused on viruses in the reef water (i.e. the sea water overlying a coral reef; Gast et al. 1998) and sediment (Paul et al. 1993; Patten et al. 2008b), rather than those directly associated with corals. This is largely because the coral holobiont – comprised of the coral animal, algae, fungi, protists, bacteria, archaea and viruses (Rohwer et al. 2002; Wegley et

al. 2007; Marhaver et al. 2008) – provides a hugely complex and difficult ecosystem to study.

Viral densities in reef water typically range from $2 - 14 \times 10^6 \text{ ml}^{-1}$ (Patten et al. 2006, 2008b) – which is also characteristic of open ocean densities (Wilhelm et al. 2010) – and $1.35 - 12 \times 10^8 \text{ viruses cm}^{-3}$ in reef sediment (Paul et al. 1993; Patten et al. 2008b). In seawater, viral counts are typically superior to those of prokaryotes by an order of magnitude (Proctor and Fuhrman 1990). Prokaryotes include eubacteria (‘true bacteria’ and cyanobacteria) and archaea (Carter and Saunders 2007), and their density normally ranges from $0.51 - 52 \times 10^5 \text{ ml}^{-1}$ in reef water. However, in reef sediment, prokaryote densities ($0.2 - 3 \times 10^8 \text{ cm}^{-3}$; Paul et al. 1993; Patten et al. 2008b) are of the same order of magnitude as viral densities (see Chapter One, *Table 1.5*). Viral and prokaryotic densities on coral reefs have been found to be positively correlated (Thurber and Correa 2011). The virus to prokaryote ratio (VPR; also known as the virus to bacteria ratio, VBR) typically ranges from 0.4 - 7.1 and 2 - 9.5 in the water column and sediment of coral reefs, respectively (see Chapter One, *Table 1.5*).

Paul et al. (1993) were the first to investigate viral abundance in coral reef water and sediment, and a number of invertebrate taxa, and correlated viral density with distance from shore, season and salinity. Viral density was inversely related to salinity, providing the first insight into environmental drivers of viral abundance in reef water and sediment. Following this investigation, several studies explored viral abundance in relation to various facets of the coral reef system, including spatial variation (Seymour et al. 2005; Dinsdale et al. 2008b), diseased and dead coral colonies (Patten et al. 2006), and mass coral spawning events (Patten et al. 2008b).

Spatial abundance patterns of viruses on coral reefs can be used to infer their influence on nutrient cycling and food-web structure (Seymour et al. 2005), as well as provide insight into coral reef health. For example, Dinsdale et al. (2008b) characterised the microbial ecology of four coral atolls in the Northern Line Islands (Central Pacific) against a gradient of anthropogenic impact, and positively correlated both viral and

prokaryote abundance with elevated nutrient levels and greater human population sizes. A strong positive correlation with nutrients has also been found in a number of studies investigating viral abundance in seawater (Cochlan et al. 1993; Maranger and Bird 1995; Jones et al. 2007; Clasen et al. 2008). However, the relationship between viral abundance on coral reefs and other potential environmental drivers such as turbidity and chlorophyll-*a* (both proxies for water quality), remains to be investigated.

2.1.3 *The surface mucopolysaccharide layer*

The interface between the coral holobiont and reef water is the surface mucopolysaccharide layer (SML), comprised of a carbon-rich polysaccharide-protein-lipid complex, secreted by the coral animal and enriched by the associated symbiotic dinoflagellates (Ferrier-Pagès et al. 1998; Gast et al. 1998, Ritchie and Smith 2004; Brown and Bythell 2005). The SML has a range of roles: (i) desiccation resistance (Krupp 1984); (ii) protection against UV radiation and oxygen radicals (Drollett et al. 1997); (iii) mucociliary transport of microbes for ingestion (Goldberg 2002; Ritchie 2006); (iv) a medium for secreted allelochemicals with antimicrobial properties (Ritchie 2006; Shnit-Orland and Kushmaro 2009); (v) assisting in the translocation of photosynthetic products that aid in coral reproduction (Rinkevich 1989); and (vi) the removal of invasive microbes and sediment through sloughing (Brown and Bythell 2005; Johnson and Rohwer 2007; Sharon and Rosenberg 2008). Across a coral reef system, mucus released to the seawater is a major contributor to the overall energy budget of the reef (Sharon and Rosenberg 2008), and plays a vital role in coupling the microbial and viral communities in the SML with the water column (Naumann et al. 2009).

To date, much more research has focused on bacterial rather than viral communities in the SML, with dense bacterial populations having been reported (Paul et al. 1986; Coffroth 1990; Ritchie and Smith 2004; Rohwer and Kelly 2004; Ritchie 2006; Mao-Jones et al. 2010). The microbial community (here referred to as viruses, bacteria and archaea) of the SML is thought to have an organised, stratified structure (Ritchie and Smith 2004; Rohwer and Kelly 2004), though it can shift between a 'healthy' community dominated by beneficial antimicrobial compound-producing bacteria to an unhealthy or 'disease-

associated' state in which pathogenic microbes dominate (Mao-Jones et al. 2010). The composition of viral morphological diversity in the SML significantly varies from that of reef water (Davy and Patten 2007). Metagenomics support this finding, identifying numerous viral strains within coral tissue samples dissimilar to those commonly found in the water column (Wegley et al. 2007; Marhaver et al. 2008; Vega Thurber et al. 2008). Distinct SML viral assemblages have been shown to change in their composition in relation to stresses exerted upon their associated coral holobiont (Vega Thurber et al. 2008, 2009). Whether such stress-induced changes in the SML viral composition have consequences for the rest of the coral-associated microbiota (Ritchie and Smith 2004; Rohwer and Kelly 2004; Mao-Jones et al. 2010) remains unknown.

Numerous studies have utilised epifluorescence microscopy (EFM) to determine viral and prokaryotic abundances in coral reef water and sediment (see Chapter One, *Table 1.5*), yet no study has attempted to quantify viral abundance in the SML by way of this non-genetic sampling technique. Rather, studies have focused on the morphological and genetic characterisation of viral diversity, both in and on corals and other cnidarians (Wilson and Chapman 2001; Wilson et al. 2001, 2005; Lipp et al. 2002, 2007; Lipp and Griffen 2004; Cervino et al. 2004; Davy et al. 2006; Davy and Patten 2007; Lohr et al. 2007; Danovaro et al. 2008; Marhaver et al. 2008; Patten et al. 2008a; Vega Thurber et al. 2008, 2009).

2.1.4 Abiotic effects on viruses

In all marine ecosystems, viral abundance varies with prokaryotic abundance and productivity (Suttle 2007), and hence also varies with chlorophyll-*a* concentration (Cochlan et al. 1993; Maranger and Bird 1995; Jones et al. 2007; Clasen et al. 2008). However, while abiotic factors can influence viral abundance – for example depth, oxygen concentration (Hara et al. 1996; Drake et al. 1998), temperature (Jiang and Paul 1994; Jiang et al. 2004; Ram et al. 2005), ultra-violet (UV) light (Wilhelm and Smith 2000) and salinity (Paul et al. 1993) – few studies (but see Paul et al. 1993) have attempted to correlate these drivers to viral abundance in a coral reef system.

Determining the influence that select environmental drivers have on coral reef virus

assemblages remains a key area of coral reef ecology that has yet to be properly investigated. Changes in environmental conditions are known to trigger lytic viral infection from a lysogenic viral state (Edgar and Lielausis 1964; Scanlan and Wilson 1999; Wilson et al. 2001). Several authors have conducted investigations into lysogenic viral infection in marine prokaryote communities (Weinbauer and Suttle 1996; Wilson and Mann 1997; Cochran et al. 1998; Jiang and Paul 1994, 1998; McDaniel et al. 2002; McDaniel and Paul 2005) and *Symbiodinium* spp. from scleractinian corals (Cervino et al. 2004; Wilson et al. 2005; Davy et al. 2006; Lohr et al. 2007). These studies indicate that a shift from lysogenic to lytic infection may occur in the coral holobiont. Thus, correlating select environmental drivers that are known to induce lysogenic-lytic infection shifts, such as temperature (Edgar and Lielausis 1964), nutrient availability (Scanlan and Wilson 1999), and UV light (Wilson et al. 2001) to viral densities can offer further insight into the microbial ecology of coral reefs.

2.1.5 Aims and specific objectives

This study aimed to quantify the abundance of viruses (using EFM) both in the SML of the coral *Montipora capitata* and the surrounding seawater, across the reef of Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i. Select environmental drivers were then correlated with viral abundance to assess their potential influence.

The specific objectives were to:

1. Measure and compare viral density in the SML of *M. capitata* and the overlying reef water, and so establish whether the SML or reef water contains more viruses.
2. Determine the relationship between key environmental variables and the viral density in the SML and reef water.

Because viral density correlates with prokaryotic density (Seymour et al. 2005; Patten et al. 2006, 2008b; Dinsdale et al. 2008b), and because higher bacterial numbers exist in the nutrient-rich SML than in the overlying reef water (Paul et al. 1986; Coffroth 1990;

Ritchie and Smith 2004), it was hypothesised that both viral and prokaryotic abundance would be highest in the SML. Furthermore, as high nutrient content (reflected by high levels of chlorophyll-*a* and turbidity) has been positively correlated to viral abundance on reefs across a large spatial scale (Dinsdale et al. 2008b), it was expected that sites of poorer water quality would have higher densities of both viruses and prokaryotes.

2.2 Methodology

2.2.1 Study site and sampled species

Two surveys were conducted: a pilot study from the 7th - 12th December 2008, and a full environmental study from the 26th May - 14th June 2009, both at Coconut Island Marine Reserve (CIMR) (21°26'N, 157°47'W), Kane'ohe Bay, O'ahu, Hawai'i. In the 2008 pilot study, four sampling sites (B, C, E and D; *Figure 2.1*) were chosen, running 50 m in length along the reef crest that surrounds Coconut Island. The pilot study primarily allowed for the development of survey and analytical methodologies. However, it also tested for any spatial variability of viruses and prokaryotes across the reef, and allowed for analysis of temporal variability when compared to the 2009 study.

The 2009 full environmental study was expanded to include six sites (Sites A-F; *Figure 2.1*), with the entire survey replicated one week after the first survey. Sites were chosen from previous surveys by Williams (2010) to provide the strongest gradients possible of a range of key environmental factors (see below).

All sites (excluding Site F) were on the reef crest, with the distance from shore ranging from 50 - 200 m. Site F was located in the north-eastern causeway channel, which is lined by mangrove forest and contains high coral cover throughout; it reaches the fringing reef at the end of the causeway (*Figure 2.1*).

2.2.1 Environmental data

Environmental data were only recorded during the 2009 full environmental study.

Depth, salinity, temperature, turbidity and chlorophyll-*a* concentration were measured using two RBRH XR-420 data loggers (www.rbr-global.com). Two data loggers were used to allow for sampling on consecutive days as the environmental recordings were taken once every minute over 48-hour periods at the depth from which the corals were sampled at each site. The chlorophyll-*a* value is a measure of how much of the suspended material present (turbidity) contains chlorophyll-*a* (Williams et. al. 2010), with both turbidity and chlorophyll-*a* used as proxies for water quality. The 48-hour time period was chosen to capture sufficient data to construct as reliable an environmental profile as possible, given time constraints in the field. Placement of the loggers was randomised among the chosen CIMR sampling regions throughout the designated sampling period (26th May – 14th June 2009).

2.2.2 Sample collection and preparation

Sample collection was conducted using snorkel, with mucus samples from the SML taken from the centre-top section of healthy *M. capitata* colonies (care was taken to avoid sampling any 'orange' colour morphs or any colonies with signs of MWS; see Chapter One, *Figure 1.7*), and samples from the reef water were taken 20 cm directly above each colony. In 2008, five SML and five reef water samples were taken at each site. In 2009, two sampling sessions were conducted, during each of which six SML and six reef water samples were collected at each of the six sites; this totalled 12 SML and 12 reef water samples per site across the field season.

SML samples were obtained using sterile, 25 ml, capped needleless syringes, with care taken to ensure that the syringe tip was in constant contact with the surface of the coral during sampling, so minimising contamination with seawater. Syringe caps were removed immediately before sampling and replaced immediately after to reduce contamination. Reef water samples were taken 20 cm directly above each *M. capitata* colony using sterile 50 ml Falcon centrifuge tubes, which were sealed *in situ*. Samples were processed within two hours of collection.

Figure 2.1 Satellite image of the Coconut Island study site depicting all six transect sites sampled in the 2009 summer sampling period. Four sites (B, C, E and F) were sampled in the 2008 winter sampling period. Image sourced from <http://www.flashearth.com>. Kaneohe Bay map adapted from Jokiel et. al. 1993.

Sample preparation was performed as described by Patel et al. (2007) and Ackermann (2009). Each sample was drawn through a 0.02 μm pore size, 19 mm diameter Anodisc A1203 filter (WhatmanTM) using a 25 mm glass microanalysis filter holder and a vacuum pump set to B20 kPa or 20 cm Hg VAC. The filter was removed and the underside blotted with tissue and left to dry in a dark container. It was then placed, sample side up, on a 10 μl drop of SYBR Gold solution (1:400 SYBR Gold and 0.02 μm filter-autoclaved MilliQ H₂O) in a plastic petri dish to stain for 18 minutes in the dark. The filter was then mounted onto a glass microscope slide with 30 ml of 0.1% (vol/vol) p-phenylenediamine anti-fade mounting medium, to ensure long-term sample integrity.

In the 2008 pilot study, sample filtration volumes were predominantly 5 ml ($n = 170$), however 2.5 ml ($n = 95$) and 10 ml ($n = 95$) samples were also filtered to determine the optimum sample volume for counting time versus accuracy. This optimum volume was in fact found to be 2.5 ml, as lower sample volumes reduced the chance of overcrowding of particles, so this volume was used throughout the 2009 study.

2.2.3 Enumeration of viruses

Samples were viewed with an Olympus Provis AX70 photomicroscope with an Olympus PlanApo 100 $\times$ fluorescence oil-immersion objective with DF grade immersion oil. A fluorescein isothiocyanate (FITC) filter (with excitation and emission spectra peak wavelengths of approximately 495 nm and 521 nm, respectively) optimised the observation of VLPs and prokaryotes. An Olympus DP70 digital camera was used to image the samples, with a field-of-view of 175 $\mu\text{m} \times 132 \mu\text{m}$ at 100 $\times$ magnification.

Each sample was imaged 10 times at random; to avoid potential imaging bias, the computer monitor used for visualising samples was 'blackened out' when moving between fields of view. Due to the high concentrations of VLPs observed, the images were then sub-sampled using a 20 $\mu\text{m} \times 20 \mu\text{m}$ one hundred square grid, which was superimposed onto each image using a random number generator assigned to the number of pixels across the x and y axes of each image (1 - 4080 and 1 - 3072, respectively). For each sample, all VLPs and prokaryotes were counted in each 20 $\mu\text{m} \times 20 \mu\text{m}$ grid on each of

the 10 images (*Figure 2.3*). This final protocol was based on a pilot study that compared the enumeration of VLP and prokaryote densities in SML and reef water samples when one, three, five, or ten grids per image were used (see Appendix B, *Figure 6.2*).

The following equation was used to calculate the abundance of VLPs and prokaryotes within samples (Suttle 1993; Ackermann 2009):

$$N_v = P_t \div F_t \times A_t \div A_f \div V_t \quad [\text{Equation 1}]$$

Where N_v = VLPs/prokaryotes ml^{-1} , P_t = total number of VLPs/prokaryotes counted, F_t = total number of fields, A_t = total area filtered (μm^2), A_f = area of each field (μm^2), and V_t = volume of sample filtered (ml).

An additional step was incorporated into the 2009 protocol, as in 2008 the mucus from the SML had produced an uneven spread of coral mucus across the filter. This unevenness resulted in the grid often chancing upon an area with no mucus cover, in which case additional time was spent calculating a new position for the grid until mucus was located. To avoid this, SML samples were lightly homogenised using a sterile hand-held tissue homogeniser before filtration and staining (see Appendix C, *Figure 6.3*).

2.2.4 Statistical analyses

In any cases of missing samples (as a result of an inability to count particles due to a lack of fluorescence, likely a result of an unknown error during sample preparation) the paired sample from the respective habitat was removed. For 2008, four samples were removed: two from the SML and two from the reef water. For 2009, nine samples were removed: seven from the SML and two from the reef water.

Figure 2.2 Examples of epifluorescence images. (A) Site B, surface mucopolysaccharide layer, 5 ml, 2008 (B) Site C, surface mucopolysaccharide, 2.5 ml, 2009 (C) Site B, reef water sample, 5 ml, 2008 (D) Site C, reef water sample, 2.5 ml, 2009 (E and F) Images of SML samples depicting the random placement of the 20 μm $\times$ 20 μm grid using a random number generator assigned to the number of pixels across the x and y axes of the image (1 - 4080 pixels and 1 - 3072 pixels, respectively); approximately 177 virus-like particles and eight prokaryotes are present in the grid displayed above.

Data was tested for normality, and a 2 (habitat: SML and reef water) $\times$ 4 (Site: B, C, E, F) factorial ANOVA design for VLP abundance, prokaryote abundance and the VPR was used for the 2008 pilot study, while a 2 (habitat: SML and reef water) $\times$ 6 (Site: A, B, C, D, E, F) factorial ANOVA design for VLP abundance, prokaryote abundance and the VPR was used for the 2009 full environmental study. In the case of any significant interactions between factors, simple effects tests were carried out using the denominator mean square from the interaction model to determine significant effects in each habitat type, with Bonferroni's post hoc test used to identify any inter-site differences.

For 2009, in order to determine which, if any, of the measured environmental parameters were driving abundance patterns, VLPs and prokaryotes in each habitat type were correlated to the environmental parameters across all six sites using independent linear regression. To determine any temporal differences, VLP and prokaryote densities, and the VPR value were compared between the two sampling periods using a 2 (sampling period: 2008 and 2009) $\times$ 2 (habitat: SML and reef water) repeated measures ANOVA. VLP density, prokaryote density and VPR values cited are means $\pm$ standard deviation. All statistical analyses were conducted using the statistical software program PASW® Statistics, Release Version 18.0 (SPSS Inc., 2009; Chicago, IL; www.spss.com).

2.3 Results

2.3.1 *Coral mucus thickness: impact on microbe enumeration*

To determine whether coral mucus thickness on the grids had an effect on the enumeration of VLP and prokaryote abundances in the SML (due to mucus autofluorescence), counts were compared across the four coral mucus thickness categories (see Appendix D, *Figure 6.4*) by univariate ANOVA. Following this analysis, all samples from Category One (little or no mucus cover; $n = 3$ from 2009, no samples from 2008) were removed, as these samples were not true representations of the SML environment; the other levels of mucus cover had no discernible impact on either VLP or prokaryote counts, or the VPR value, in either 2008 or 2009 (see Appendix D).

2.3.2 Across-reef microbe densities: habitat effects

In 2008, across all reef sites, VLP and prokaryote density, and the VPR were similar between the SML ($2.48 \pm 1.61 \times 10^7$ VLPs ml⁻¹; $1.6 \pm 0.94 \times 10^6$ prokaryotes ml⁻¹; 16.45 ± 6.65 VPR) and reef water ($1.95 \pm 0.55 \times 10^7$ VLPs ml⁻¹; $1.56 \pm 0.52 \times 10^6$ prokaryotes ml⁻¹; 13.94 ± 6.87 VPR) (univariate ANOVA, $p > 0.05$ for all comparisons; for clarity, the details of the statistical values are given in Appendix E, *Table 6.1*) (*Figure 2.3*).

In 2009, VLP and prokaryote density, and the VPR were significantly lower in the SML ($2.61 \pm 1.06 \times 10^7$ VLPs ml⁻¹; $2.08 \pm 1.02 \times 10^6$ prokaryotes ml⁻¹; 15.15 ± 7.76 VPR) than the reef water ($5.98 \pm 0.83 \times 10^7$ VLPs ml⁻¹; $3.11 \pm 0.76 \times 10^6$ prokaryotes ml⁻¹; 20.48 ± 6.19 VPR) (univariate ANOVA, $p < 0.001$ for all comparisons) (*Figure 2.4*).

In the 2008 study, linear regression indicated a strong positive relationship between VLP and prokaryote densities in the SML, whilst in the reef water no significant correlations were identified. Likewise, in 2009, VLP and prokaryote densities were strongly correlated in the SML but not in the reef water (*Figure 2.5*; for statistics see figure legend).

2.3.3 Spatial variability in microbe density

For the 2008 pilot study, VLP and prokaryote density were similar across all reef sites, for both the SML and seawater (univariate ANOVA, $p > 0.05$ for both comparisons), while the VPR differed in the water column, but only between Site B (21.25 ± 8.01) and Site F (9.92 ± 3.22) (univariate ANOVA simple effects test, $p = 0.016$) and not in the SML (univariate ANOVA simple effects test, $p > 0.05$).

The lack of a significant difference between VLP and prokaryote densities in the different habitats and sites is likely a result of lack of power due to the low samples size, exacerbated by the loss of four samples as a result of an inability to find a precise plane of focus for enumeration. This lack of power was remedied in 2009.

Figure 2.3 2008 sampling of virus-like particles (VLPs) and prokaryotes. (A) Density of VLPs; (B) prokaryote density; and (C) VLP to prokaryote ratio. Values are means $\pm$ standard deviation, and represent the means across all four sites in the Coconut Island Marine Reserve. White and grey bars represent samples taken from surface mucopolysaccharide layer and reef water, respectively. For location of sites see *Figure 2.1*.

Figure 2.4 2009 sampling of virus-like particles (VLPs) and prokaryotes. (A) Density of VLPs; (B) prokaryote density; and (C) VLP to prokaryote ratio. Values are means $\pm$ standard deviation, and represent the means across all six sites in the Coconut Island Marine Reserve. White and grey bars represent samples taken from surface mucopolysaccharide layer and reef water, respectively. For location of sites see *Figure 2.1*.

Figure 2.5 Relationships between virus-like particles and prokaryotes in 2008 (A) and 2009 (B). For 2008, surface mucopolysaccharide layer (SML) samples (solid black line) $r^2 = 0.726$, $n = 16$, $p < 0.001$ and reef water (dotted line) $r^2 = 0.063$, $n = 16$, $p > 0.05$. For 2009, SML samples $r^2 = 0.399$, $n = 60$, $p < 0.001$ and reef water (dotted line) $r^2 < 0.001$, $n = 60$, $p > 0.05$.

In 2009, in the SML, VLP density was the same across all reef sites (univariate ANOVA simple effects test, $p > 0.05$), while prokaryote density varied in the SML, but only

between Sites F ($1.48 \pm 0.94 \times 10^6 \text{ ml}^{-1}$) and A ($2.84 \pm 0.92 \times 10^6 \text{ ml}^{-1}$), with prokaryote density found to be significantly lower at Site F (univariate ANOVA simple effects test, $p < 0.001$). The VPR in the SML also varied across sites, with a greater value at Site F (21.73 ± 8.3) than anywhere else across the reef, and significantly higher than at Sites A (11.94 ± 3.2) and E (9.29 ± 3.51) (univariate ANOVA simple effects test, $p < 0.001$).

In 2009, the VLP density in the seawater was higher at Site F ($6.95 \pm 0.66 \times 10^7 \text{ ml}^{-1}$) than at all other sites [A ($5.37 \pm 0.74 \times 10^7 \text{ ml}^{-1}$), B ($5.6 \pm 0.52 \times 10^7 \text{ ml}^{-1}$), C ($5.85 \pm 0.77 \times 10^7 \text{ ml}^{-1}$), and E ($5.99 \pm 0.67 \times 10^7 \text{ ml}^{-1}$)] excluding Site D ($6.08 \pm 0.79 \times 10^7 \text{ ml}^{-1}$), with the largest difference in VLP density seen between Site F and Sites A and B (simple effects test univariate ANOVA, $p < 0.001$). Conversely, prokaryote density was lower at Site F ($2.58 \pm 0.67 \times 10^6 \text{ ml}^{-1}$) than at Sites A ($3.58 \pm 0.89 \times 10^6 \text{ ml}^{-1}$) and E ($3.61 \pm 0.68 \times 10^7 \text{ ml}^{-1}$) (simple effects test univariate ANOVA, $p = 0.005$). The VPR value followed the same trend as for VLP density, being significantly higher at Site F (28.24 ± 6.28) than at all other sites [A (16.16 ± 5.66), B (20.39 ± 4.09), C (3.08 ± 6.91), and E (16.96 ± 2.65)] excluding Site D (21.04 ± 6.96) (simple effects test univariate ANOVA, $p < 0.001$); this suggests a level of decoupling between the VLP and prokaryote densities, which was most pronounced at the most sheltered site (F); this is also the only sample site not on the fringing reef.

2.3.4 Temporal variability in microbe abundance

Sampling of Sites B, C, E and F in the 2008 pilot study allowed for an analysis of temporal variability between these four sites across the two years. Between 2008 and 2009, in the SML across all reef sites, VLP and prokaryote density, and the VPR were similar (repeated measures ANOVA, $p > 0.05$ for all comparisons; mean $\pm$ standard deviation values are given earlier in section 2.3.3). In contrast, in the reef water, VLP and prokaryote densities, and the VPR were significantly lower in 2008 ($1.95 \pm 0.55 \times 10^7 \text{ VLPs ml}^{-1}$; $1.56 \pm 0.52 \times 10^6 \text{ prokaryotes ml}^{-1}$; $13.94 \pm 6.87 \text{ VPR}$) than in 2009 ($6.12 \pm 0.53 \times 10^7 \text{ VLPs ml}^{-1}$; $3.02 \pm 0.65 \times 10^6 \text{ prokaryotes ml}^{-1}$; $21.7 \pm 5.75 \text{ VPR}$) (repeated measures ANOVA, $p < 0.001$; $p < 0.001$; $p = 0.007$, for VLP and prokaryote densities, and the VPR, respectively), with VLP densities in the reef water increasing more than 3-fold between 2008 and 2009, while seawater prokaryote densities increased 2-fold between the two sampling periods.

Comparing individual sites revealed a particularly interesting pattern at Site F, where VLP density significantly increased in the reef water from 2008 ($1.5 \pm 0.22 \times 10^7$ VLPs ml⁻¹) to 2009 ($6.85 \pm 0.25 \times 10^7$ VLPs ml⁻¹) (repeated measures ANOVA, $p = 0.012$), while no significant increase in prokaryote density was seen ($2.49 \pm 0.18 \times 10^6$ prokaryotes ml⁻¹ and $1.63 \pm 0.55 \times 10^6$ prokaryotes ml⁻¹ for 2008 and 2009, respectively) (repeated measures ANOVA, $p > 0.05$).

2.3.5 Environmental drivers

VLP and prokaryote abundances in the reef water and SML in 2009 were individually tested against the five environmental predictors (chlorophyll-*a*, depth, salinity, temperature, and turbidity) recorded (*Table 2.1*), using linear regression analyses. The VLP and prokaryote densities yielded no significant correlations with any of the measured environmental variables (*Tables 2.2* and *2.3*). Interestingly though, for both SML-associated VLPs and seawater prokaryotes, density was most strongly correlated (positively) with depth (single linear regression, $r^2 = 0.275$, $n = 5$, $p = 0.285$ and $r^2 = 0.249$, $n = 5$, $p = 0.314$, respectively).

Table 2.1 Environmental variables recorded during the 2009 sampling period. Sites with maximum and minimum values are in parentheses; for site locations see *Figure 2.1*.

26th May - 14th June 2009

VARIABLE	UNIT	MINIMUM	MAXIMUM	RANGE
Chlorophyll- <i>a</i>	µg/l	1.1 (B)	3.12 (C)	2.02
Depth	m	1.28 (C)	4.11 (A)	2.83
Salinity	ppt	34.19 (C)	35.15 (D)	0.96
Temperature	°C	26.02 (D)	27.62 (E)	1.6
Turbidity	STU	1.51 (B)	8.14 (D)	6.63

Table 2.2 Simple linear regression analysis correlating virus-like particle density in the surface mucopolysaccharide layer and reef water habitats with environmental parameters.

HABITAT TYPE	ENVIRONMENTAL VARIABLE	β	t	r^2	p
SML	Chlorophyll-a	0.35	0.749	0.123	0.495
	Depth	0.525	1.233	0.275	0.285
	Salinity	- 0.065	- 0.129	0.004	0.903
	Temperature	- 0.448	- 1.002	0.201	0.373
	Turbidity	- 0.323	- 0.682	0.104	0.533
Reef water	Chlorophyll-a	- 0.356	- 0.761	0.126	0.489
	Depth	- 0.397	- 0.864	0.156	0.436
	Salinity	0.02	0.04	< 0.001	0.97
	Temperature	- 0.052	- 0.105	0.003	0.921
	Turbidity	0.05	0.099	0.002	0.926

Degrees of freedom = 1,4; β = standardised beta coefficient; t = t -test statistic; p = p -level; r^2 = simple linear regression statistic.

Table 2.3 Simple linear regression analysis correlating prokaryote density in the SML and reef water habitats with environmental parameters.

HABITAT TYPE	ENVIRONMENTAL VARIABLE	β	t	r^2	p
SML	Chlorophyll-a	0.419	0.924	0.176	0.408
	Depth	0.431	0.955	0.186	0.394
	Salinity	0.018	0.036	< 0.001	0.973
	Temperature	- 0.111	- 0.223	0.012	0.835
	Turbidity	0.452	1.013	0.204	0.368
Reef water	Chlorophyll-a	0.252	0.521	0.063	0.63
	Depth	0.499	1.151	0.249	0.314
	Salinity	- 0.003	- 0.007	< 0.001	0.995
	Temperature	0.254	0.524	0.064	0.628
	Turbidity	0.373	0.804	0.139	0.467

Degrees of freedom = 1,4; β = standardised beta coefficient; t = t -test statistic; p = p -level; r^2 = simple linear regression statistic.

2.4 Discussion

A tight coupling of VLP and prokaryote abundances in the water column overlying coral reefs has been observed previously (Seymour et al. 2005; Dinsdale et al. 2008b), with VLP abundance positively correlated to increasing chlorophyll-*a* and nutrient concentrations (Cochlan et al. 1993; Dinsdale et al. 2008b). Therefore, given that the carbon-rich SML of scleractinian corals contains a diverse consortium of microbes (Rohwer et al. 2002; Mao-Jones et al. 2010) that is often at higher densities than in the reef water (Paul et al. 1986; Coffroth 1990; Ritchie and Smith 2004), it was expected that VLP abundance would be higher in the SML of *M. capitata* than in the water column. Furthermore, it was predicted that VLP density in both the SML of *M. capitata* and the reef water of Coconut Island would be explained, at least in part, by the environmental regime, with poor quality water (higher turbidity levels and chlorophyll-*a* concentrations) containing the highest densities. However, the results of the current study were not consistent with this hypothesis, as VLP density in the SML and seawater did not differ in 2008, while it was in fact greater in the seawater than the SML in 2009. Moreover, none of the water quality parameters (or indeed other environmental variables) were correlated with VLP abundance, suggesting that the spatial variability in VLP and prokaryote distribution was determined by a factor(s) other than those measured in the current study. It is also possible, though, that measurement of environmental variables over a longer timeframe may have resulted in different conclusions.

2.4.1 Virus-like particle abundance versus habitat type

Virus and prokaryote numbers in reef water typically range from 2 - 14 x 10⁶ VLPs ml⁻¹ and 0.51 - 52 x 10⁵ prokaryotes ml⁻¹ (Patten et al. 2006, 2008b). Counts at Coconut Island were within these ranges, except for the reef water VLP and prokaryote densities in 2009, which were greater.

The poor water quality resulting from Kane'ohe Bay's long history of anthropogenic impacts is, at least in part, likely to explain the high VLP and prokaryote densities observed. Frequent stressors in Kane'ohe Bay include high sediment loading (Jokiel

1991; De Carlo et al. 2007; Smith 2008), high nutrient levels (Jokiel 1991; Williams 2010), reduced salinity and increased nutrient levels as a result of high freshwater runoff (De Carlo et al. 2007; Ostrander et al. 2008), and sewage release or leakage (Maragos and Chave 1973; Pastorak and Bilyard 1985; Hunter and Evan 1995). Just one or a combination of several of these factors could result in an increase in host cell (i.e. prokaryote) density and viral abundance in the reef water. Furthermore, the microbial loading of bay water could potentially be increased as a direct result of transport in episodic freshwater runoff (De Carlo et al. 2007; Ostrander et al. 2008).

The nine perennial streams entering Kane'ohe Bay discharge approximately 214,000 m³ of water per day. These streams provide a direct means for the transport of anthropogenic and terrigenous materials from Kane'ohe Bay's high relief watershed to its coastal ocean environment (Ostrander et al. 2008). During episodic rainfall events, freshwater influx can increase to levels as high as 2,120,000 m³ day⁻¹ with salinity dropping from 34 to 15 ppt in surface waters (Jokiel et al. 1993). Turbidity and chlorophyll-*a* levels increase an order of magnitude directly following such large freshwater influxes (De Carlo et al. 2007). Investigation into the spatial variability of freshwater plumes in Kane'ohe Bay indicates that stream discharge and duration exert a primary control on plume persistence in the southern Kaneohe Bay system, where the majority of organic matter entering into the bay is transported (De Carlo et al. 2007; Ostrander et al. 2008). This results in sustained phytoplankton productivity for extended periods of time (De Carlo et al. 2007). The CIMR is located in the southern portion of Kane'ohe Bay, therefore the potential for a significant increase in VLP abundance in the reef water as a result of a large-scale freshwater influx event driving an increase in phytoplankton or prokaryote host cell abundance, or the direct transport of microbes from the terrestrial to the marine environment, is evident. It is interesting to note, however, that this increased density in the water column in 2009 did not elevate the VLP or prokaryote densities of the SML; this suggests limited coupling between these two habitats. The specificity of SML-associated microbiota likely limits the extent of coupling between the SML and seawater, with the composition of the SML microbial community being distinct from that of the water column (Rohwer et al. 2001, 2002; Kellogg 2004; Davy and Patten 2007).

While short-term episodic events could well have explained the temporal change in seawater microbial abundance, it is also possible that longer-term, seasonal effects also played a part. In 2008, sampling took place during winter, whilst sampling in 2009 took place during summer. Jiang and Paul (1994) found that viral populations showed a strong seasonal pattern in the water column in Tampa Bay, Florida, with the highest concentrations ($2.0 \pm 0.8 \times 10^7$) in the summer and lowest ($4.8 \pm 1.4 \times 10^6$) in the winter, consistent with the current study. This seasonal change was positively correlated with chlorophyll-*a* level and negatively correlated with salinity, and followed the summer increase in temperature (which stimulated prokaryote population growth).

The poor correlation between VLP and prokaryote abundance in the reef water was somewhat unexpected, as a positive relationship has been seen in a number of previous studies (Boehme et al. 1993, Cochlan et al. 1993, Drake et al. 1998, Middelboe et al. 2003). Nevertheless, both Seymour et al. (2005) and Dinsdale et al. (2008b) occasionally identified a similar lack of correlation in VLP and prokaryote densities in the water column of Magnetic Island, Great Barrier Reef (GBR), Australia and Palmyra Atoll (Northern Line Islands) respectively. These reasons for this poor correlation are unknown, though shifts in the host cell community composition may drive some of the variability in the VPR (Wommack and Colwell 2000), which in turn may be influenced by hydrodynamic regime (Dinsdale et al 2008b).

The relatively steady VLP density in the SML observed between 2008 and 2009 is consistent with the stable environment and microbial community typical of ‘healthy’ corals (Ritchie and Smith 1997, 2004; Rohwer and Kelly 2004; Ritchie 2006; Shnit-Orland and Kushmaro 2009). The alternative is a pathogen-dominated microbial community with an elevated number of microbes (Smith 2008; Mao-Jones et al. 2010). For example, Smith (2008) illustrated that *Montipora* white syndrome (MWS)-infected colonies of *M. capitata* around Coconut Island have approximately 25.6 times the density of culturable bacteria in their SML than do healthy colonies. She also found that bacterial communities sampled from the healthy colonies were predominantly comprised of *Alteromonas* spp. and *Streptomyces* spp., whereas MWS-associated

bacterial communities were comprised predominantly of *Vibrio* spp. Whether the microbial consortium quantified in the current study was also stable with respect to its community composition is unknown, but is worthy of future consideration, as is whether the microbial density remained constant over shorter periods.

2.4.2 *Virus-like particle abundance: spatial variability and the environment*

As in the current study, Seymour et al. (2005) identified spatial variation in both VLP and prokaryote abundance across a coral reef in Magnetic Island, GBR, Australia, and in particular between four reef water types (surface-water, coral-water, lagoon-water, sediment-water). The spatial variation in VLP density was most pronounced in the coral-water samples (taken 12 cm above coral colony), which had a significantly higher VLP density and virus to bacteria ratio (VBR) than did the other water types. The high VLP density of coral-associated water, as reported for Magnetic Island, can result from increased bacterial host cell density, stimulated by dissolved and particulate organic matter from the coral surface (Ducklow 1990; Ferrier-Pagès et al. 1998), while the increased VPR/VBR is typical of habitats that favour fast bacterial growth and high productivity (Wommack and Colwell 2000). However, this was not supported by the observations at Site F, where the relatively high VPR of the SML was in part caused by a low bacterial abundance; Seymour and colleagues likewise noted that the VBR value when close to the coral surface was related to a low bacterial abundance. The reasons for this low bacterial abundance are predominantly unknown, although removal of bacterial biomass by benthic filter-feeding organisms associated with the reef is likely to have contributed (Gast et al. 1998).

Seymour and colleagues also noted that VLP density was higher in lagoon water (mid-water at approximately 1 m depth) than surface-water (5 cm depth) and sediment-water (12 cm above the coral reef sediment floor). This was perhaps a result of higher UV radiation levels causing an inactivation of viruses in the surface-water samples (Suttle and Chen 1992; Wilhelm et al. 1998), while absorption of VLPs and suspended particles to the sediment (Sakoda et al. 1997) may have led to the low counts in this habitat. UV irradiance in particular would be a valuable addition to the study of viral ecology

in the CIMR and elsewhere, and could contribute to some of the unexplained spatial variability.

As in the current study (2009), VLP density in the seawater was highest at Site F. Site F was located in the middle of Coconut Island's eastern causeway channel, which is characterised by a gradual bathymetry, low flushing rate due to its sheltered position, and the second highest temperature and chlorophyll-*a* concentrations recorded; it was the only site not on the fringing reef and hence directly exposed to the hydrodynamic regime of Kane'ohe Bay. Nevertheless, the environmental reasons for the higher VLP counts in the seawater of Site F were not apparent from the environmental analyses. It therefore seems likely that biotic or abiotic parameters not measured here better explain this local spatial variability. For example, both reef topography and variable fluid dynamics can markedly influence the planktonic composition of reef water (Gast et al. 1998), with reefs situated in quiet waters or lagoon/bay areas characterised with higher particle accumulation through lack of hydrographical influence (Gast et al. 1998; Seymour et al. 2005); both of these factors could have contributed to the distinctiveness of Site F.

A further consideration is that the combined effect of certain environmental factors may cause them to exert a greater influence than when considered in isolation. For example, in 2009, Site F was characterised by the second highest chlorophyll-*a* concentrations and temperatures. Previously, both chlorophyll-*a* (Boehme et al. 1993; Cochlan et al. 1993; Jiang and Paul 1994; Maranger and Bird 1995; Clasen et al. 2008) and temperature (Jiang and Paul 1994; Jiang et al. 2004; Ram et al. 2005) have been found to be positively correlated with increased viral abundance, so moderate levels of both at Site F could have led to the high microbial counts.

The small sample size may in part explain why VLP and prokaryote abundances appeared similar in the SML and water column in 2008 across all sites. The number of *M. capitata* colonies sampled in 2008 was less than half of that sampled in 2009 ($n = 5$ versus 12). Kvennefors et al. (2010) suggested that at least six coral colonies are needed

for a robust examination of bacterial community composition, so a greater number of replicates may well have identified patterns not seen in the current study.

2.4.3 *Conclusions and future directions*

The results presented here indicate that viruses represent an abundant and dynamic component on the coral reef of Coconut Island, with significant spatial variation identified. However, the failure to correlate spatial distribution with environmental factors suggests that we still have a lot to learn about the ecology of viruses in this system (as we do on reefs elsewhere). Likewise, the lack of coupling between the seawater viral community and that of the SML warrants further investigation. Of particular note though, enumeration of VLPs and prokaryotes from the SML of *M. capitata* via EFM was successful, allowing for a cheaper alternative to genetic sequencing. However, as with any novel technique, refinements can be made, including comparisons between coral species. With the number of investigations of coral reef viruses steadily increasing, the focus over the coming years must shift from identifying the presence of viruses on corals and coral reefs, to incorporating viral abundance and distribution patterns into studies of the ecological and physiological processes linked to the health of the coral holobiont. By doing so, our knowledge of how to predict, prevent and mitigate detrimental effects occurring on coral reefs will be improved.

LOCAL ENVIRONMENTAL DRIVERS INFLUENCE THE COMPOSITION OF VIRUS-LIKE PARTICLE ASSEMBLAGES ASSOCIATED WITH THE CORAL *MONTIPORA CAPITATA*

3.1 *Introduction*

3.1.1 *Marine viruses*

Throughout marine environments, both prokaryotic and eukaryotic viruses are pervasive (Suttle 2007) and are phenomenally diverse (Breitbart et al. 2004a; Edwards and Rohwer 2005). Viruses cause disease and mortality in every known marine organism investigated for infection (Munn 2006), yet, while the diversity of marine viruses is known to be high, in the majority of marine systems this diversity remains poorly characterised (Culley and Steward 2007).

Viruses can be considered as ‘architects of the seas’, shaping and influencing major ecological processes through the regulation of microbial communities (Middelboe et al. 2001) and nutrient cycling through mass mortality and lysis of host cells (Weinbauer 1998; Wilhelm and Suttle 1999). In the water column, virioplankton are the most abundant planktonic component (Suttle 2007) and are likely to be the most diverse (Wommack and Colwell 2000). However, within the marine environment viral diversity is currently known to be highest in marine sediment communities, with estimates as high as one million viral genotypes per kilogram of sediment (Breitbart and Rohwer 2005).

3.1.2 *Coral reef viruses*

Determining the viral diversity associated with coral reefs, which are both structurally and environmentally complex in their array of habitats and species (Ainsworth et al. 2010), can provide a great deal of information regarding the potential roles that viruses may play in coral health and overall reef functioning.

Coral reefs are known to harbour a diverse community of microbes amongst the sediment (Danovaro et al. 2001) and the surrounding reef water (Seymour et al. 2005; Patten et al. 2006). For example, Culley and Stewart (2007) identified five new genera and 24 new species of ssRNA *Picornaviridae*-like viruses in the reef waters around O'ahu, Hawai'i. The high viral diversity in marine sediments is predominantly uncharacterised, with metagenomic analysis of Fiesta Island, Mission Bay, CA, USA indicating that three-quarters of the 104 different viral genotypes obtained, from just one kilogram of sediment, were previously unrecorded (Breitbart et al. 2004a). While this offers fascinating potential for the survey of coral reef sediment viral communities, investigation of their diversity has yet to be undertaken. Ultimately, the majority of virus diversity studies on coral reefs have focused on VLPs directly associated with the coral animal or one of the multitude of microbes associated with it.

Numerous microbes interact with scleractinian corals, making up a 'meta-organism' termed the coral holobiont (Rohwer et al. 2002; Thurber and Correa 2011). It is comprised of the coral animal itself, along with its resident dinoflagellate endosymbionts (Muller-Parker and D'Elia 1997; Smith et al. 2005), bacteria (Shashar et al. 1994; Ritchie and Smith 1995a, b, 1997; Rohwer et al. 2001, 2002; Bourne and Munn 2005; Ritchie 2006), archaea (Kellogg 2004; Beman et al. 2007), endolithic fungi (Kendrick et al. 1982; Priess et al. 2000; Gobulic et al. 2005) and algae (Shashar et al. 1997; Fine and Loya 2002), heterotrophic protists (Upton and Peters 1986; Toller et al. 2002) and viruses (Rohwer et al. 2002; Wilson et al. 2005; Davy et al. 2006; Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008; Patten et al. 2008a; Vega Thurber et al. 2008, 2009).

Viruses have been observed within the gastrodermal, epidermal, and mesogleal tissues

of scleractinian corals (Patten et al. 2008a), in the coral surface mucopolysaccharide layer (SML) (Davy and Patten 2007), and within the zooxanthellae (Wilson et al. 2005; Davy et al. 2006). Although direct evidence of viruses infecting microbes associated with scleractinian corals is still somewhat lacking, the ubiquitous and abundant nature of viruses throughout the oceans suggests that infection of all the holobiont components is likely (Marhaver et al. 2008).

The mucus layer secreted by all corals, referred to as the SML (Ritchie et al. 1994), is the interface between the coral and external environment (Brown and Bythell 2005). The sloughing of the SML into the surrounding water plays a vital role in coupling the microbial and viral communities in the SML with the water column. Despite this coupling, the SML is known to support a select and distinct resident bacterial community rather than a randomly assorted community as a result of passive settlement and entrapment of water-borne bacteria (Ritchie and Smith 1995b; Santavy 1995; Frias-Lopez et al. 2002; Guppy and Bythell 2006). Comparisons between the bacterial species diversity of the surrounding reef water and coral mucus show little to no overlap (Rohwer et al. 2001; Frias-Lopez et al. 2002), with similarly distinct archaeal communities between coral mucus and seawater samples (Kellogg et al. 2004; Wegley et al. 2004, 2007; Beman et al. 2007). SML-associated VLPs have also been found to be diverse when compared to those in the reef water, as illustrated in the study by Davy and Patten (2007), yet the drivers of this variability remain largely unknown.

3.1.3 The impact of the environmental regime on coral-associated virus-like particles

On coral reefs, microbial processes impact heavily on ecosystem functioning, and the effects of environmental change can be reflected and amplified in the microbial community over short time scales (Paerl et al. 2003; Manini et al. 2004; Guppy and Bythell 2006). With a growing body of evidence indicating the decline of coral reef health on a global scale (Hoegh-Guldberg 1999; Williams et al. 2010a), it is crucial that we advance our understanding of how the individual microbial components associated with the coral meta-organism may change in relation to environmental parameters.

Local and regional anthropogenic and environmental impacts have a major influence on coral reef health (Jordán-Dahlgren et al. 2005; Guppy and Bythell 2006), while environmental drivers such as salinity (Paul et al. 1993), temperature (Jiang and Paul 1994), and nutrient concentrations (Clasen et al. 2008) have been found to influence the abundance and virulence of viroplankton assemblages. No study has yet attempted to link coral reef-associated viral diversity with environmental regime. A greater understanding of viral diversity across coral reefs, in both the SML and seawater, is still needed, as is a more comprehensive appreciation of how environmental regime drives this diversity. With this information, we may better understand the mechanisms that affect homeostasis of the coral holobiont and the processes affecting coral reef health.

3.1.4 Aims and specific objectives

In this chapter, transmission electron microscopy (TEM) was used to characterise viral diversity of the SML of *Montipora capitata*, and in the overlying seawater, across the reef of Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i. Select environmental parameters were correlated with viral morphology to determine the influence exerted by these parameters.

TEM is currently the standard tool for visualising and characterising virus phenotypic diversity (Suttle 2007), providing evidence for particles being viral through characterisation of VLP morphology at the particle level. Moreover, morphological patterns of VLP assemblages can be accurately categorised according to virion size, shape, symmetry and finite compartmentalisation of small morphological features (Fauquet et al. 2005; Thurber and Correa 2011). Through the use of TEM, VLPs can also be classified according to the area of the host tissue in which they are found (e.g. coral gastrodermal tissue, SML, *Symbiodinium* cell).

The specific aims for this chapter were:

1. To identify and classify the viruses associated with the SML of *M. capitata* and the reef water around Coconut Island, Kane'ohe Bay, Hawai'i, and thereby determine whether the SML has a distinct viral community.

2. To establish how key environmental factors influence viral community composition of both the SML and reef water, with respect to viral morphotype, size, and class.

Prokaryotes dominate marine plankton, with the majority of virioplankton thought to be bacteriophage (Rohwer 2003; Weinbauer 2004). In contrast, the SML of corals, harbour a plethora of microbial eukaryotic and prokaryotic organisms which are often species- and, in the case of SML-associated bacteria, colony-specific. Due to the specific host range of the majority of marine viruses (Suttle 2007) it was hypothesised that viral morphologies in the SML of *M. capitata* would be distinct from those in the water column. Furthermore, given the links between water quality, microbial community composition, and the viral infection cycle, it was hypothesized that viral diversity would differ with environmental regime.

3.2 Methodology

Sampling was conducted between 26th May – 14th June 2009 at Coconut Island Marine Reserve (CIMR) (21°26'N, 157°47'W), Kane'ohe Bay, O'ahu, Hawai'i. The four sampling sites were located 50 – 200 m from the shore, on the reef crest (*Figure 3.1*), and selected on the basis of environmental data collected previously (October – November 2007; Williams 2010). This allowed the maximum variability between environmental predictors - turbidity, chlorophyll-*a*, salinity, temperature and depth – to be calculated.

3.2.1 Environmental Data

Temperature, turbidity, chlorophyll-*a* fluorescence, salinity and depth were recorded every minute using two RBRH XR-420 data loggers (www.rbr-global.com) over 48 hour periods across a depth range of 1.3-4.1 m (see Chapter Two, *Table 2.1*). The chlorophyll-*a* value is a measure of how much of the suspended material present (turbidity) contains chlorophyll-*a* (Williams et al. 2010a); both chlorophyll-*a* and turbidity are used as proxies for water quality. The 48 hour time period was believed

to give sufficient resolution of both viral and bacterial communities in response to short-term environmental changes over local spatial scales. Placement of the loggers was randomised among the chosen CIMR sampling regions throughout the designated sampling period (26th May - 14th June 2009; *Table 3.1*).

3.2.2 *Sample collection*

Snorkel was used to collect five mucus samples from the SML and five coral-associated reef water samples from *M. capitata* colonies. SML samples were taken from the centre-top area of each colony using sterile 25 ml capped, needleless syringes. Care was taken to ensure that the syringe tip was in constant contact with the surface of the coral during sampling, thereby minimising contamination with seawater.

Syringe caps were removed immediately before sampling and replaced immediately after to reduce contamination. Seawater samples were taken 20 cm above each *M. capitata* colony using sterile 50 ml centrifuge tubes. Both SML and coral-associated reef water samples (15 ml) were fixed with 300 µl EM-grade glutaraldehyde (2% final concentration) and stored in the dark at 4 °C for 10 min for fixation to be completed.

3.2.3 *Sample preparation and electron microscopy analysis*

Sample preparation followed the protocol outlined in Davy and Patten (2007). To remove particulate matter (including suspended coral tissue), the SML and reef water samples were centrifuged at 1500× g for 10 min at 4 °C in a Sigma 3K15 bench top centrifuge. Supernatant (13 ml) was then pipetted into polypropylene centrifuge tubes.

The VLPs within the supernatant were concentrated into a pellet by ultracentrifugation for two hours at 146,000× g and 4 °C, using a Beckman Optima L-XP ultracentrifuge with swinging bucket SW41Ti rotor. The viral pellet was carefully resuspended in 100 µl of supernatant and stored in the dark at 4 °C overnight (12 hours).

Formvar- and carbon-coated 200 µm mesh copper grids were glow-discharged immediately prior to floating on 20 µl sub-samples of the viral suspensions for one hour.

Figure 3.1 Kaneohe Bay (map adapted from Jokiel et. al. 1993) – the nine perennial streams that run into the bay are depicted. The accompanying satellite image of the Coconut Island study site illustrates the four 50 m transect sites sampled. Image sourced from <http://www.flashearth.com>.

Grids were then washed in 0.02 μm filtered water for two seconds; excess water was wicked away with filter paper (Whatman™) and the sample was negatively stained for 30 seconds with 3% uranyl acetate. Excess stain was wicked away and grids air-dried while covered. Grids were viewed on a Hitachi HT7700 transmission electron microscope (120 kV) fitted with a video camera for fast scanning and alignment, and an AMT XR-41 2048 $\times$ 2048 pixel bottom-mount camera for high-resolution images at 33,000-127,000 $\times$ magnification.

Scanning electron micrographs (SEM) were also imaged from an additional seawater sample taken above Colony One at Site D with the use of a Hitachi S-4800 Field Emission Scanning Electron Microscope with Oxford INCA X-Ray Microanalysis system. Categorisation counts from these SEM images were not included in the study, but rather SEM imaging was opportunistic and undertaken for a more detailed visual analysis of the morphology of VLPs and prokaryotes sampled.

Size and morphology of the first 100 VLPs observed on each grid were determined, and each VLP classified was assigned to one of 26 sub-groups within five major morphological classes: tailed bacteriophage-like VLPs; icosahedral/spherical tailless VLPs; lemon-shaped VLPs; filamentous VLPs; and miscellaneous/other VLPs - according to criteria outlined by the International Committee on Taxonomy of Viruses (ICTV) classification system (Murphy et al. 1995; Fauquet et al. 2005) and the species concept outlined in Ackermann et al. (1992, 2007).

3.2.4 *Data analysis*

Differences in VLP community structure between the SML and reef water, and between sites were tested using a two-factor multivariate regression model applied under permutation of dissimilarities using the FORTRAN computer program 'distance-based multivariate analysis for a linear model' (DISTLM v.5) (Anderson 2001, 2004; McArdle and Anderson 2001). This statistical method was chosen due to the lack of residual multivariate normality and variance-covariance homogeneity, missing data (due to the desiccation of three grids and loss of associated samples), and the large viral

morphotype-sample ratio, rendering the traditional MANOVA test statistics such as Pillai's Trace unreliable (McArdle and Anderson 2001). The permutation method does not rely on the above assumptions, as no theoretical population parameters are evoked (Manly 1997). Deviations from normality and constant variance resulted from zero counts of rare morphotypes and assumed aggregations (e.g. resulting from localized viral 'bursts'). In the case of significant interaction between factors, simple effects tests were carried out using the denominator mean square from the interaction model.

A dissimilarity matrix of the virus morphotype frequencies for each pair of replicate grids was constructed using the semi-metric Bray-Curtis measure. This was achieved using the *vegdist* function of the *vegan* package in *R* (*R* Development Core Team 2006; Oksanen et al. 2010). The Bray-Curtis measure was chosen as it is a suitable descriptor of multivariate ecological dissimilarities among assemblages of species. The measure is appropriate for ranking of VLP morphologies in relation to distance along the measured environmental gradients (temperature, turbidity, chlorophyll-*a* fluorescence, salinity and depth; Bray and Curtis 1957). The appropriately coded predictor (XSITE, XHABITAT and XSxH) and full (XFULL) matrices were constructed using the FORTRAN computer program XMATRIX (Anderson 2003). Orthogonal contrasts were used due to the unbalanced replication.

A non-metric multidimensional scaling (nMDS) ordination of site and species scores was carried out using the *metaMDS* function of the *MASS* package in *R* (*R* Development Core Team 2006; Oksanen et al. 2010). This was performed to illustrate the relative site differences in each habitat type, with points representing viral morphotypes and field sites.

To establish whether the patterns of VLP morphological composition correlated, and in which direction, with the environmental parameters across the four sample sites, a subset of environmental variables was chosen based on maximum correlation with community dissimilarities. For each habitat type (the SML and the reef water), environmental vectors were fitted and projected as vectors onto the ordination diagrams using the *envfit* procedure in the *vegan* package in *R* (*R* Development Core Team 2006, Oksanen et al.

2010). The goodness of fit for each vector was assessed using the squared correlation coefficient (r^2) test statistic based on 4999 permutations of the environmental variables and a 5% significance level.

Individual virus morphotype groups were compared between habitat type (SML and reef water) using paired samples *t*-tests. While univariate ANOVA was used to compare individual virus morphotypes across sites (A, B, C, D). These analyses were undertaken using the statistical software program PASW® Statistics, Release Version 18.0 (SPSS Inc., 2009; Chicago, IL; www.spss.com).

3.3 Results

3.3.1 Virus-like particle morphology

VLPs ($n = 3700$) representing 26 subgroups, and nested within five major morphological classes (see Appendix F, *Tables 6.2, 6.3*), were categorised in the current study, with many VLPs being morphologically similar to previously recognised families of viruses.

Throughout the 37 viable samples, both habitat types (SML and reef water) were dominated by tailless polyhedral/spherical VLPs, accounting for 55.9% of total VLPs. These VLPs were evenly distributed between both of the habitats, contributing 28.9% and 27% of total VLP counts from the SML and reef water, respectively. Tailless polyhedral/spherical VLPs in the < 50 nm size range dominated the sub-groups (see Appendix F, *Tables 6.2, 6.3*) with the highest counts at Site C (characterised as the shallowest site, and also the one with the lowest salinity and highest chlorophyll-*a* levels (see Chapter Two, *Table 2.1*); for both habitat types.

Bacteriophage-like VLPs from the *Myoviridae*, *Podoviridae* and *Siphoviridae* groups of tailed bacteriophages constituted 30.1% of total VLPs, with proportional abundance spread evenly across habitat type (14% and 16.2% in the SML and reef water samples, respectively).

Figure 3.2 Relative proportions of virus-like particle morphological sub-groups in the surface mucopolysaccharide layer (SML) and reef water at Sites A (deepest), B (warmest), C (highest chlorophyll-*a* concentration) and D (most turbid; see Chapter Two, *Table 2.1*). White and grey bars represent morphotype proportions in the SML and reef water, respectively. Values are mean $\pm$ standard deviation. Dimensions refer to capsid width (at narrowest point) or length for filamentous virus-like particles. For SML communities, $n = 5$ grids for Sites A, C and D and $n = 4$ for Site B. For reef water communities, $n = 5$ for Sites A and B and $n = 4$ for Sites C and D. 100 virus-like particles were counted per grid. For values, refer to Appendix F, *Table 6.2*, *6.3*.

Figure 3.2 continued.

Myoviridae-like VLPs comprised the majority of the bacteriophage-like VLPs (41.5%), followed by *Podoviridae*-like VLPs (37.2%) and *Siphoviridae*-like VLPs (21.3%), with a significantly greater proportion of *Siphoviridae*-like VLPs found in the seawater ($3.7\% \pm 3.3\%$) than in the respective SML ($2.0\% \pm 1.4\%$) (conditions; $t_{35} = -2.452$ $p = 0.042$).

The proportion of the total VLP assemblage comprised of polyhedral/spherical VLPs

within the two smaller capsid size ranges (< 50 and 50-100 nm) significantly varied across sites (univariate ANOVA; $F_{3,32} = 4.86$, $p = 0.007$ and $F_{3,32} = 3.213$, $p = 0.036$), respectively. Small (< 50 nm) polyhedral/spherical VLPs were observed in the highest proportional abundance across all morphotype categories; significantly higher proportions were observed at Site C ($54.2\% \pm 10.5\%$), which has the highest chlorophyll-*a* concentration (see Chapter Two, *Table 2.1*), than across the other three sites ($36.2\% \pm 12.1\%$) (conditions; $t_{35} = -3.913$ $p < 0.001$). Tailless polyhedral/spherical VLPs > 100 nm in size were observed in lower numbers, comprising 5.2% of the counts within their major group and 2.9% of the total VLPs (*Figure 3.4*; see Appendix F, *Tables 6.2, 6.3*).

Despite *Myoviridae*-like VLPs dominating the overall bacteriophage-like VLP counts, the most prevalent sub-group within the tailed bacteriophage-like VLPs was small (< 100 nm) *Podoviridae*-like VLPs, comprising 33.2% of the total tailed phage counts and 10% of the total VLPs. The second most abundant sub-group within the tailed phage was medium-sized (100-200 nm) isometric *Myoviridae*-like VLPs, making up 19.5% of the total tailed phage counts and 5.9% of the total VLPs (*Figure 3.3*).

Filamentous VLPs (FVLPs) ranged in length from < 50 nm to over 2 μm and had an average width of 25 nm. FVLPs were categorised into four sub-groups according to size (< 100 nm, 100 nm - 500 nm, 500nm - 1 μm , and > 1 μm). FVLPs comprised 7.2% of the total VLPs, with the largest proportion being in the 500 nm - 1 μm size class. Lemon-shaped viruses (categorised into two size groups, < 100 nm and ≥ 100 nm) made up 2.9% of the total counts, with other rare morphologies including *Geminiviridae*-like (1.2%), bead-shaped (0.9%), cuboid (0.7%) and hook-shaped VLPs (0.05%) (*Figures 3.5*). A number of bacteria that were heavily infected with bacteriophage-like VLPs were also found and, while the ratio of infected bacteria to 'healthy' bacteria was not quantified, electron micrographs were taken to illustrate the phenomenon (*Figures 3.6, 3.7*).

Figure 3.3 Transmission electron microscopy images of virus-like particles (VLPs) classified as bacteriophages in the reef water (A-G) and surface mucopolysaccharide layer (H-L) of *Montipora capitata* colonies, from Coconut Island Marine Reserve. Labels highlight distinguishing morphological features: icosahedral symmetry of capsid (IC); contractile tail (CT); helical symmetry of contractile tail sheath (HE); base plate (BP); tail fibres (TF); arm-like extensions (AE); prolate capsid (PC); collar (CO); non-contractile tail (NT); and short-tail (ST). Myovirus-like bacteriophages (A-E, H-K); siphovirus-like bacteriophage with filamentous non-contractile tail (F); podovirus-like VLPs with short non-contractile tails (G, L). Scale bars = 100 nm.

Figure 3.4 Transmission electron microscopy images of polyhedral/spherical virus-like particles (VLPs) in the reef water overlying *Montipora capitata* colonies from Coconut Island Marine Reserve. Labels highlight distinguishing morphological features: icosahedral symmetry of capsid (IC); capsomeres (CP); and electron-dense core (EC). *Phycodnaviridae*-like virus (A); enveloped isometric VLPs (B, C); and enveloped spherical VLPs (D-E). Scale bars = 100 nm.

The resolution of electron micrographs taken was sharp, resulting in clearly distinguishable ‘fine scale’ morphologies of VLPs. Within the bacteriophage-like VLPs, base plates with attached fibres, collars (present at the capsid-tail junction), helical symmetry along tails, 2-, 3- and 5-fold axis of rotational symmetry in a number of icosahedral capsids, and arm-like extensions extending from one unique bacteriophage were clearly visible (*Figure 3.3*). Within the tailless polyhedral/spherical VLPs, enveloped isometric capsids, electron dense cores, and protruding protein attachment points were clearly visible (*Figure 3.4*). FVLPs had clearly-visible helical symmetry and electron-dense central grooves (*Figure 3.5B*).

Figure 3.5 Transmission electron microscopy images of lemon shaped virus-like particles (VLPs), filamentous virus-like particles (FVLPs), and unique virus-like particles in the reef water (A-B, D, F-G) and SML (C, E) of *Montipora capitata* colonies, from Coconut Island Marine Reserve. Labels highlight distinguishing morphological features: tail fibres (TF); helical symmetry structure (HE); central groove (CG); and bead-shaped electron-dense core structures (BD). Lemon-shaped *Fuselliviridae*-like VLPs (A, D); FVLPs (B); unique filamentous VLP (C); unique cuboid VLP with central groove (E); and unique rod and filamentous-like VLPs (F, G). Scale bars = 100 nm.

Figure 3.6 Transmission electron microscopy images of bacteriophage virus-like particles (VLPs) and polyhedral/spherical VLPs infecting various bacteria species in the reef water (A, C-F) and surface mucopolysaccharide layer (SML) (B) of *Montipora capitata* colonies, from Coconut Island Marine Reserve. Both bacteriophage VLPs and polyhedral/spherical VLPs can be seen aggregating and/or infecting the bacterium. Although not quantified, infected bacteria were a common occurrence throughout both reef water and SML samples. Scale bars = 100 nm.

Figure 3.7 Scanning electron microscopy images of bacteriophage virus-like particles (VLPs) and polyhedral/spherical VLPs infecting various bacteria species in the seawater (A, C-G) and surface mucopolysaccharide layer (B and H) of *Montipora capitata* colonies, from Coconut Island Marine Reserve. Magnification varied from 30,000 $\times$ (image A, depicting a large portion of the seawater sample) to 350,000 $\times$ (image G). Scale bars = 100 nm.

The proportions of all VLP sub-groups for each habitat type and site are shown in *Figure 3.2*, with accompanying electron micrographs of: bacteriophage-like VLPs (*Figure 3.3*); tailless polyhedral/spherical VLPs (*Figure 3.4*); lemon-shaped VLPs, FVLPs, cuboid, rod and *Geminiviridae*-like VLPs (*Figure 3.5*); and virus-infected bacteria (*Figure 3.6*).

Figure 3.8 Ordination diagram of virus-like particle (VLP) assemblages in the surface mucopolysaccharide layer (A) and reef water (B), depicting the patterns of variation in VLP community composition explained by the environmental variables. Red labels are approximate relative centres of morphotype distributions; black labels indicate the approximate positions of each sampling unit (grid) from each site; and the blue arrows denote the relative size and direction of the fitted environmental gradients.

Figure 3.8 continued

The scanning electron micrographs (SEM) imaged from an additional seawater sample taken above Colony One at Site D (Figure 3.7), portrayed several bacteria species infected with VLPs, however, the resolution of the SEM images did not allow for morphological characterisation of these VLPs.

Non-parametric analysis of variance revealed a significant multivariate interaction between habitat and site in determining the compositional structure of the VLP assemblage (permutation, $p = 0.007$). For both habitat types, simple effects tests revealed a significant difference in VLP community composition between sites (permutation, $p = 0.004$ and < 0.001 for SML and reef water, respectively).

3.3.2 Environmental drivers of virus-like particle communities

The *envfit* variable selection analysis indicated that, of the five environmental drivers measured, temperature and turbidity had the highest significance in relation to VLP communities in the seawater (permutation $p = 0.013$ and 0.007 , respectively). In the SML, environmental factors that influenced the relative proportions of VLP morphotypes were temperature, turbidity and chlorophyll-*a* (permutation, $p = 0.004$, 0.001 and 0.012 for temperature, turbidity and chlorophyll-*a*, respectively).

nMDS ordinations were used to determine the distribution of VLP morphotypes along these selected environmental gradients. In the SML, a negative correlation between temperature and turbidity was evident by the near anti-parallel vectors (*Figure 3.8A*). The nMDS plots also indicated that subsets of the SML and reef water VLP communities were distributed along the temperature/turbidity gradient, with a number of individual morphotypes having their distribution mode centered in either a positive or negative direction along the axis.

In the reef water (*Figure 3.8B*), the negatively acute chlorophyll-*a* vector indicated that chlorophyll-*a* concentration was not correlated with either temperature or turbidity, though increased turbidity was associated with lower temperature. In both habitat types, simple effects tests revealed a significant difference in the composition of VLP morphologies between sites (permutation, $p = 0.004$ and < 0.001 for SML and reef water, respectively).

VLP diversity was similar between the SML of *M. capitata* and the reef water (permutation test, $p > 0.05$; Shannon-Wiener index $\pm S.D.$ = 2.00 ± 0.15 and 1.92 ± 0.08 for SML and reef water samples, respectively). Non-parametric analysis of variance showed a significant multivariate interaction between habitat type and site in determining the compositional structure of the VLP assemblage (permutation $p = 0.007$).

At both sites with good water quality (Site A and Site B; *Table 3.1*) (Appendix F; *Tables 6.2, 6.3*) there were no significant differences in VLP community composition

between the SML and the reef water (permutation, $p > 0.05$ for both sites). However, the composition of the VLP community differed between the two habitat types at the more degraded sites (Site C and Site D, respectively; permutation, $p = 0.021$ and 0.036 for the SML and seawater, respectively).

3.3.3 Individual viral morphologies: environmental drivers and habitat effects

The ordination diagrams (Figure 3.8) revealed a number of patterns in the association between the VLP communities, habitat type and the environmental parameters at the level of individual virus morphology. In the reef water, large (> 100 nm) *Podoviridae*-like VLPs and elongate *Myoviridae*-like VLPs, as well as lemon-shaped VLPs of both size classes, showed a positive association with turbidity. In contrast, the relative abundance of the three largest size classes of FVLPs, as well as *Geminiviridae*-like VLPs, and bead- and rod-shaped VLPs, showed a positive association with temperature. A different pattern was apparent in the SML, where the relative abundance of all four groups of small (< 100 nm) tailed phages, small lemon-shaped VLPs, FVLPs, and bead-shaped VLPs, were positively correlated with increased temperature. Lastly, the abundance of the numerically dominant, small (< 50 nm) polyhedral/spherical VLPs appeared to be positively correlated with chlorophyll-*a* concentration (Figure 3.8B).

3.4 Discussion

This study showed that the morphological diversity in both the SML and water column is diverse, with the majority of VLPs observed in both habitat types encompassing VLPs phenotypically similar to a range of previously observed eukaryotic and prokaryotic virus families (Davy and Patten 2007). Contrary to the habitat type hypothesis, the proportional abundance of VLP morphologies were similar between habitat type; small tailless/spherical VLPs dominated the morphologies in both the SML and reef water, while bacteriophage and larger eukaryotic algae infecting VLPs were also identified. Significant variation in VLP morphology in relation to the environmental regime was also hypothesised, with the findings from the current study conforming to this

hypothesis. A number of abiotic drivers were correlated to various VLP morphological sub-groups that differed between each habitat type. With strong relationships between temperature and turbidity and certain VLP morphologies in the reef water identified, while temperature, turbidity, and chlorophyll-*a* in SML-associated VLP populations were shown to have a significant effect.

3.4.1 *Virus-like particle assemblage versus habitat type*

The finding that the composition of VLP community was not significantly different between the SML and reef water was contrary to that of Davy and Patten (2007), who characterised the VLPs associated with the SML and reef water surrounding *Acropora muricata* and *Porites* spp. at Heron Island, on the Great Barrier Reef. The two studies used similar sampling and processing protocols, and analyses, and therefore it is possible that coupling between the SML and reef water is stronger at Kane'ohe Bay than at Heron Island, or that VLPs in Kane'ohe Bay's reef water are just more diverse than at Heron Island. Both of these scenarios are plausible when comparing the respective study sites, and subsequent anthropogenic and environmental pressures exerted on each. The water quality in the CIMR is currently classified as poor (Williams et al. 2010a), precipitated by a long history of anthropogenic impacts (Maragos and Chave 1973; Smith et al. 1981; Pastorak and Bilyard 1985) and high levels of environmental pressure (Jokiel et al. 1993; Hunter and Evans 1995). When compared to Heron Island's offshore (65 km off mainland Australia; Connell et al. 2004) coral reef system, which is subject to low anthropogenic impact and moderate environmental pressures (predominantly in the form of cyclones; Connell et al. 1997), it is likely that corals in the CIMR experience higher levels of stress. This might increase coral mucus production (Segel and Ducklow 1982), which in turn could strengthen the coupling between the SML and water column.

Temporal/seasonal differences between the current study and that of Davy and Patten (2007) are also potential explanations for the different patterns seen in the two studies. In particular, Davy and Patten (2007) undertook sampling in April 2006 (autumn) while the current study sampled throughout May/June (winter). However, it is not possible to determine whether any of these factors are truly driving the differences observed

between the two studies due to the considerable differences in sites and latitudes. Indeed, in an investigation of marine virome diversity across four distinct oceanic regions, VLP diversity was characterised by variable VLP morphologies within each region (Angly et al. 2006).

A further consideration is that stress can affect the composition of the microbial consortium in the mucus (Ritchie 1995a, b), with pathogenic microbes such as *Vibrio* spp. outcompeting bacteria associated with healthy coral colonies (Mao-Jones et al. 2010). Such a phase shift could result in a reduction of overall prokaryotic diversity and subsequently reduced viral diversity due to the high specificity of the majority of viruses to host cells (Suttle 2007). However, whether this played a part in the trends seen in the current study cannot be determined and requires further investigation.

3.4.2 *Environmental drivers of virus-like particle assemblage composition*

VLP diversity varied significantly between habitat types and between sites, suggesting that characteristics of the environment were driving certain aspects of the observed VLP diversity patterns in both the SML and seawater. This is supported by the nMDS ordinations, which indicate strong correlations between the proportional abundance of viral morphotypes and the environmental variables.

The positive correlation between SML-associated VLP diversity and chlorophyll-*a* concentration was greater for small (< 50 nm) polyhedral/spherical VLPs. Chlorophyll-*a* concentration is often used as a proxy for high nutrient levels, symptomatic of eutrophication, which has been widely documented as having detrimental effects on corals (Bruno et al. 2003; Voss and Richardson 2006; Baker and Romanski 2007). Eutrophication has been linked to increasing incidence of coral disease (Kim and Harvell 2002; Kuta and Richardson 2002; Bruno et al. 2003), and a positive correlation was found between chlorophyll-*a* and increasing prevalence of *Montipora* white syndrome (MWS) associated with *M. capitata* colonies in the CIMR (Williams et al. 2010a). High nutrient concentrations (indicated by chlorophyll-*a*) have also been linked to higher bacterial abundance and thus higher bacteriophage abundance on corals (Pastorok and

Bilyard 1985; Bruno et al. 2003; Marhaver et al. 2008). This suggests that there may be an indirect effect of chlorophyll-*a* concentration on the VLP diversity in the SML of *M. capitata*.

Salinity and depth were found to have less of an influence on VLP diversity than temperature, turbidity and chlorophyll-*a*. Paul et al. (1993) found that salinity had a significant negative correlation with VLP abundance, and that VLP abundance was decoupled from host cell density across varying salinity profiles. However, VLP diversity was not investigated in their study. The salinity of Kane'ohe Bay has been shown to fluctuate by as much as 19 ppt over a single 12 hour period, with the fluctuations being caused by rare episodic storm flood events that introduce high levels of freshwater influx into the bay (Jokiel et al. 1993). While these events are infrequent, they result in lasting effects on the corals and their associated communities in the bay (Jokiel et al. 1993; De Carlo et al. 2007; Ostrander et al. 2008). In contrast, salinity levels only fluctuated by 0.96 ppt during the sampling period of the current study, and therefore a longer period of sampling – encompassing a wider range of salinities – could yield discernible differences in VLP diversity in relation to salinity.

SML and reef water samples in the current study spanned just a 2.83 m range, and the single-point CTD sampling at each site would therefore have had insufficient resolution for determination of the finer-scale patterns of depth on VLP diversity. Determining the influence of depth on VLPs would require more fine-scale measurements, such as those achieved by micro-scale profiling of VLP abundance patterns in coral reef water by Seymour et al. (2005).

3.4.3 Individual virus-like particle morphotypes: comparisons between habitat types and possible environmental drivers

In both habitat types (i.e. SML and seawater), diverse ranges of VLP morphologies were seen, indicating a correspondingly wide range of host microbiota. VLP morphology in both habitat types was cosmopolitan, characterised by both prokaryotic- and eukaryotic-like VLPs, with several morphological types exhibiting trends across environmental gradients.

The finding that small (< 50 nm) tailless polyhedral/spherical VLPs comprised the majority of counts in the SML habitat type is consistent with observations of Davy and Patten (2007). Davy et al. (2006) made a similar finding in the tissues and *Symbiodinium* cells of heat-shocked colonies of the corals *Pavona danai* and *A. formosa*, and a *Zoanthus* sp., reporting that tailless polyhedral VLPs of 40-50 nm in diameter were in highest abundance. In the current study, the smallest particles encountered (< 30 nm) were difficult to characterize and, as they could not be clearly distinguished from similarly sized colloidal material, were not counted. Consequently, numbers of VLPs in the < 50 nm class range were likely to have been underestimated; further issues regarding phenotypic identification of this VLP sub-group will be discussed in detail in Chapter Four.

Larger tailless polyhedral/spherical VLPs with capsid diameters of > 100 nm were similar in size and morphology to the *Phycodnaviridae* (Van Etten et al. 1991; Wilson et al. 2009), *Herpesviridae* (Marhaver et al. 2008; Vega Thurber et al. 2008) and *Iridoviridae* families of eukaryotic viruses. All three of these virus families have previously been found to be associated with scleractinian corals (Wegley et al. 2007; Patten et al. 2008a; Vega Thurber et al. 2008, 2009). In the seawater samples, larger tailless polyhedral/spherical VLPs were positively associated with turbidity, especially at Sites C and D - the most turbid of the four sampled. This could reflect the impact of increased turbidity on the host organisms. For example, the presence of the green 'bubble' alga *Dictyosphaeria cavernosa* (Hunter and Evans 1995) and *Chlorella* spp. (Landry et al. 1984) in Kane'ohe Bay is related to the high turbidity levels (Hunter and Evans 1995), and these algae represent potential hosts for *Phycodnaviridae*-like VLPs. Moreover, *Symbiodinium* spp., filamentous algae and plankton have been observed in coral mucus aggregates (Wild et al. 2004a, b), representing a broad range of potential algal hosts for viruses of the *Phycodnaviridae* family in the SML. The potential for coral-associated *Herpesviridae*-like VLPs is illustrated by the identification of *Herpesviridae*-like sequences in the coral tissue metagenomic studies by Marhaver et al. (2008) and Vega Thurber et al. (2008), while Wegley et al. (2007) used metagenomic analysis to characterise the microbes associated with the coral *Porites astreoides*, of

which *Iridoviridae*-like viruses comprised the highest proportion of eukaryotic virus-like sequences (37%). The relationship between *Iridoviridae*-like viruses and corals is potentially of great interest, as the *Iridoviridae* are known infectors of a number of reef fish and other aquatic organisms (Walker and Winton 2010), which could act as vectors in the spread of viral infections between coral colonies.

The presence of bacteriophage-like VLPs, resembling all three known bacteriophage families, agrees with several studies (Breitbart et al. 2004b; Davy and Patten 2007; Wegley et al. 2007; Marhaver et al. 2008). Bacteriophage-like VLP counts were relatively low (14% and 16.2% of the total VLP counts from the SML and reef water samples, respectively). This supports the observations of Davy and Patten (2007), who found less than 6% of their total VLP counts to be bacteriophage-like VLPs. This small contribution of bacteriophage-like VLPs to the SML assemblage can potentially be explained by a decoupling of the archetypal open-ocean viral and bacterial abundance relationships (Seymour et al. 2005), though the almost equally low bacteriophage-like VLP counts in the seawater do not support this.

Lemon-shaped VLPs were present in both habitat types and showed a positive correlation with turbidity. Those with capsid sizes of > 100 nm were found exclusively at Site D, the most turbid site investigated. The lemon-shaped VLPs observed were morphologically similar to the archaea-infecting virus family *Fuselloviridae* (Ackermann 2001, 2007). Davy and Patten (2007) were the first to phenotypically link this family to the coral holobiont (Thurber and Correa 2011), and noted that the lemon-shaped VLPs were confined to the SML. The positive correlation with turbidity of lemon-shaped VLPs in both the seawater and SML may be explained, in part, by the distribution of archaeal host cells, whose distributions have also been found to correlate with increasing turbidity (Dang et al. 2008). The formation of abundant and diverse archaeal communities in the SML of scleractinian corals (Kellogg 2004; Wegley et al. 2004) and the plankton surrounding the colonies (DeLong 1994) may allow for abundant and diverse assemblages of archaeal viruses.

Several FVLP families have previously been found to be associated with scleractinian corals, including the *Inoviridae* associated with *P. astreoides* (Wegley et al. 2007) and *Closteroviridae*, suggested to be latent within certain *Symbiodinium* spp. (Lohr et al. 2007). The categorisation of observed FVLPs throughout the current study was difficult as a result of the considerable overlap between lengths. The *Inoviridae* range in length from 85 nm to 1.9 μm (Proctor 1997) and the *Closteroviridae* from 1.5 to 2.2 μm (Agranovsky et al. 1995), with the majority of FVLPs observed falling inside the overlapping range. However, certain phenotypic traits such as tail fibers, central grooves, and head structures were often visible. In accordance with Davy et al. (2006), who previously described FVLPs in the seawater immediately surrounding thermally stressed colonies of *A. formosa*, more FVLPs were found in the seawater samples taken from warmer sites. This could be indicative of latent FVLPs within the coral tissue being released due to thermal stress or as a result of light-mediated induction (Wilson et al. 2005; Davy et al. 2006). They may then have been introduced into the reef water either directly or through mucus sloughing. This hypothesis is supported by the findings of Lohr et al. (2007), who exposed cultured *Symbiodinium* cells to elevated levels of UV-irradiation, resulting in a marked increase in the number of *Closteroviridae*-like VLPs. Furthermore, investigations undertaken by Davy and Patten (2007) and Patten et al. (2008a) reported FVLPs in the SML, tissue layers, and surrounding seawater of *A. muricata* colonies, suggesting both strong coupling between these three environments, and the potential for latent coral- and *Symbiodinium*-associated FVLPs.

Unidentified FVLPs phenotypically similar to the bacteria-infecting virus family *Inoviridae* (Ackermann 2001, 2007), the ssRNA plant-infecting virus family *Closteroviridae* (Agranovsky et al. 1995), and the dsDNA archaea-infecting virus family *Rudiviridae* (Prangishvili et al. 1999, 2001; Prangishvili 2011), were observed in the current study (see Appendix F; *Figure 6.6*). The unidentified FVLP did not phenotypically conform to any of these three families (*Rudiviridae*, D. Prangishvili, Institut Pasteur, pers. comm; *Closteroviridae* and *Inoviridae*, Fauquet et al. 2005) and remains unclassified.

Geminiviridae-like, bead-shaped, bacilliform and cuboid VLPs were all observed, with all morphological groups previously detected by Davy and Patten (2007) in their investigation into VLP morphological diversity in the SML of *A. muricata* and *Porites* spp. and surrounding seawater from the Great Barrier Reef, Australia. Further evidence for *Geminiviridae*-like viruses in corals comes from the discovery of begomovirus-like sequences (a genus within the *Geminiviridae*) associated with the *P. compressa* holobiont (Vega Thurber et al. 2008). All unique VLPs in the SML (excluding cuboid VLPs) showed a positive relationship with temperature. Conversely, in the reef water, only bead-shaped VLPs showed a strong positive correlation with temperature. The potential for thermal induction of morphologically unique VLPs in the SML from lysogenic-lytic processes was evident, but hypotheses based on so little *a priori* knowledge are highly speculative and further investigation is warranted.

Polydnaviridae-like VLPs were observed in this study; they were found exclusively in the SML, and only at the highly turbid Site D. *Polydnaviridae* have a characteristic multi-segmented genome and to date have only been isolated from parasitoid wasps with which they form an obligate mutualistic symbiosis through virus-specific gene expression, replicating in the wasps, but only causing severe infection and disease in the wasps parasitised hosts (Webb et al. 2006). Moreover, coral-associated *Polydnaviridae*-like VLP sequences were extracted in both the Wegley et al. (2007) and Vega Thurber et al. (2008) metagenomic studies. The latter study observed a decrease in the proportional abundance of *Polydnaviridae*-like VLP sequences with elevated temperature, however, likely due to the low proportional percentage of *Polydnaviridae*-like VLPs in the current study, no correlation with temperature was identified. Despite this, the aversion to increasing temperature, as well as their symbiotic potential, suggest that coral-associated *Polydnaviridae* warrant further investigation.

3.4.4 Conclusions

The results presented here indicate that viruses are a highly dynamic component of coral reefs, and likely have many undiscovered roles (both positive and negative) in the functioning of the ecosystem. The presence of VLP morphotypes that are phenotypically

similar to viruses known to infect corals, *Symbiodinium* spp., bacteria, and archaea, and other coral associates suggests that environmentally-mediated induction of lysogenic viruses associated with corals and other members of the holobiont is potentially a common occurrence on coral reefs. Temperature and water quality were correlated with shifts in VLP morphological diversity over a local spatial scale, with this study offering the first insight into the effects that multiple environmental drivers have on the viral diversity of coral reefs. Characterisation of VLP morphology can offer a great deal of information regarding the diversity and distribution of coral-associated viruses, though the microscopy methods involved still have their limitations. Identification of viruses utilising both phenotypic and genetic methodologies, with samples collected across a wide range of locations and time-points, should be a focus of coral virology research in the coming years.

FINAL DISCUSSION

4.1 *Major findings of the study*

The current study measured and compared the abundance and morphological diversity of virus-like particles (VLPs) in the surface mucopolysaccharide layer (SML) of the coral *Montipora capitata* and the overlying seawater, at a range of sites around Coconut Island, Hawai'i, and related these measures to the environmental regime.

In general, VLP and prokaryote densities were high in the water column, while the SML contained comparatively low densities of both. However, the VLP density in the seawater varied spatially across the reef, with Site F, the most sheltered site and the only one not situated on the reef crest, having a greater VLP density than all the other sites (excluding Site D). Prokaryote densities differed spatially in both habitat types, with significantly lower density found at Site F than at the relatively exposed Site A in both the SML and reef water. Temporal variations in microbe densities (i.e. viruses, bacteria and archaea) in the reef water were pronounced, while in the SML microbial densities remained constant. Interestingly, VLP and prokaryote densities were strongly coupled in the SML but not the seawater. Despite these significant spatial and temporal differences, the specific environmental drivers of VLP and prokaryote abundance were not identified. In contrast, non-parametric multidimensional scaling (nMDS) plots indicated that VLP diversity in the reef water was driven predominantly by

temperature and turbidity while, in the SML, temperature, turbidity and chlorophyll-*a* were identified as the significant environmental drivers. Small (< 50 nm) polyhedral/spherical VLPs dominated the VLPs morphotype sub-groups, with a positive correlation to seawater chlorophyll-*a* levels when in the SML. In comparison, when in the SML, *Fuselloviridae*-like VLPs, filamentous virus-like particles (FVLPs), and bead-shaped VLPs were positively correlated to temperature. In the reef water, large (> 100 nm) *Podoviridae*-like VLPs and elongate *Myoviridae*-like VLPs, as well as lemon-shaped VLPs of both size classes, showed positive associations with turbidity, while large filamentous virus-like particles (FVLPs), *Geminiviridae*-like VLPs and rod-shaped VLPs exhibited a positive association with temperature.

These findings highlight some interesting patterns and methodological limitations that warrant further discussion here. In particular the simultaneous consideration of the abundance and diversity patterns has the potential to provide further information about the viruses of Coconut Island, while further development of the approaches used here and the additional use of molecular technologies will strengthen our ability to identify and elucidate their ecology.

4.2 *Virus-like particle abundance in relation to diversity*

The high proportion of tailless/spherical VLPs of < 100 nm diameter in the reef water, coupled with the significantly higher density of VLPs in the seawater than in the SML, indicates that VLPs conforming to this size range and morphotype likely dominated the water column. Of particular note, Sites C and D, which had the poorest water quality, had the second and third highest VLP densities in the water column, respectively, and so also had particularly high densities of small tailless/spherical VLPs.

There are eight different virus families that fall within this morphological/size category previously reported from coral reef ecosystems: two prokaryotic (*Microviridae* and *Corticoviridae*) and six eukaryotic virus families (*Caulomoviridae*, *Circoviridae*,

Nanoviridae, *Papillomaviridae*, *Parvoviridae* and *Picornaviridae*) (Culley and Steward 2007; Efrony et al. 2007; Wegley et al. 2007; Marhaver et al. 2008; Vega Thurber et al. 2008). Culley and Steward (2007) identified 24 new species of ssRNA *Picornaviridae*-like VLPs in the seawater of Kane'ohe Bay, indicating a high diversity in this particular virus family in the water column of the study site. The genetic analysis undertaken by Culley and Steward indicates that *Picornaviridae*-like VLPs likely infect a wide range of marine protists (Culley and Steward 2007), indicating the potential for these VLPs to be found at high densities in the plankton rich waters of Kane'ohe Bay. Moreover, the potential for human enteric viruses in the water column in the bay is high, as a result of the sewage pipes running through Kane'ohe Bay, in which periodic breakdowns in the sewer system continue to dump thousands of liters of raw sewage into the water column (Jokiel 1991). Viruses of the *Papillomaviridae* family are known infectors of humans (Fauquet et al. 2005), and hence might occur at high densities in parts of the bay exposed to effluent waste water; Sites C and D are located on the northern side of Coconut Island, positioned closest to any sewage that would flow into the southern portions of the bay.

Despite some tentative conclusions arising from the simultaneous consideration of the abundance and diversity data, a more thorough ecological survey is necessary to better understand viral ecology of the CIMR. One suggestion for future study is Site F, which differed the most from the other sites in terms of location and reef topography, and had the highest density of VLPs and lowest density of prokaryotes in the seawater. Unfortunately, however, as VLP morphological diversity was not measured at Site F, further analysis was not possible.

4.3 *The modelling of ecological interactions of microbial communities*

The modelling of coral-associated viral and microbial communities is notoriously difficult (Dinsdale et al. 2008b; Kvennefors et al. 2010), requiring a large sample size in order to properly infer spatial changes in microbial community composition (Kvennefors et al. 2010). This is due to the highly complex intra- and inter-specific

interactions between the microbes on a coral reef. The lack of correlation identified between VLP and prokaryote densities in the reef water could potential be a result of a lack of replication, however a more comprehensive sampling design, encompassing a higher number of coral colonies and seawater samples, over more sites, is the only way to determine this for certain. This may likewise explain why no difference was detected between the SML and seawater densities (of both VLPs and prokaryotes) in 2008, when replication was less than half of that in 2009 ($n = 5$ versus 12). Indeed, Kvennefors et al. (2010) suggested that bacterial diversity of corals cannot be accurately quantified with sample sizes of less than six colonies.

In addition to the problems that arise from these complex microbial interactions, modelling is only truly successful if it incorporates all potential abiotic and biotic drivers; this is unlikely the case in the present study. For example, the photosynthetic activity of the symbiotic dinoflagellates in corals results in a circadian cycle of oxygen availability within the mucus, which is highly oxygen-rich during the day and anaerobic during the night (Shashar et al. 1993). Also, viral density in the water column is influenced by the hydrodynamic regime and reef structure, with flow rate, current and eddy dynamics, and reef topography all previously shown to alter virioplankton abundance (Gast et al. 1998; van Duyl and Gast 2001). Environmental variables such as these may greatly affect the viral assemblages in Kane'ohe Bay, and lead to de-coupling of the microbial consortia associated with the SML and seawater. It should be borne in mind, however, that the VLP and prokaryote densities measured here were based upon 'snap-shot' measurements, and so may be atypical of longer-term trends. A more temporally comprehensive survey of VLP densities of the Coconut Island reef system is therefore warranted, which includes a greater variety of environmental variables.

Scale is an additional factor that makes modelling of microbial communities inherently difficult, as suggested by the fact that bacterial communities even change along the length of a coral branch (Rohwer et al. 2002). Moreover, the specificity of coral-microbe associations is thought to be considerable (Rohwer et al. 2001, 2002; Ainsworth et al. 2010). Microbial communities are therefore likely to change rapidly in response to host

and environmental variability at the colony scale (Ainsworth et al. 2010), with abiotic drivers measured on scales dissimilar from the study (i.e. coral) species unlikely to elucidate the majority of interactions and patterns occurring in the associated microbial community (Azam and Worden 2004). The measurements in the current study were over a scale of tens of metres, and so may well have lacked sufficient resolution to truly elucidate viral ecology of the SML and the overlying seawater. Clearly there is a need to modify studies such as that conducted here to include environmental sampling at the colony, and even intra-colony scales.

4.4 Limitations of surface mucopolysaccharide layer sampling and future directions

The study undertaken in Chapter Two was the first attempt to determine VLP abundance in the SML of scleractinian corals through the use of epifluorescence microscopy (EFM). SML samples that were fixed, filtered and stained for EFM produced reliable counts. This method therefore has potential as a non-genetic alternative to other more costly techniques such as shotgun sequencing, though caution has to be exercised with respect to the quantity of mucus on the slides, as this generated a degree of auto-fluorescence.

The properties and location of the SML mean that obtaining ‘pure’ mucus, uncontaminated by the surrounding reef water is extremely difficult. The procedure used in the current study (collection via a sterile syringe) has been widely employed elsewhere (e.g. Coles and Strathmann 1973; Ducklow and Mitchell 1979; Coffroth 1990; Davy and Patten 2007). However, when sampling the coral SML *in situ*, samples immediately become contaminated with sediment, particles released by the coral, microbes from the reef water, and the reef water itself (Brown and Bythell 2005). Several studies have trialed the collection of ‘pure’ mucus using a variety of different methods. These include artificial stressors such as sediment (Mitchell and Chet 1975), air exposure (Daumas et al. 1982; Wild et al. 2004a, b) and water-jet stimulation (Benson and Muscatine 1974; Richman et al. 1975; Rublee et al. 1980). However, Segel

and Ducklow (1982) and Ritchie and Smith (1995a, b) demonstrated that stress alters the chemical nature and quantities of mucus produced, inducing shifts in populations of SML-associated microbes. Unnatural representations of the SML will therefore likely result from studies employing such techniques. Alternatively, the SML can be sampled *in vitro*, thereby removing any potential for reef water contamination (e.g. by sampling out of water or in filtered seawater). Significant stress can again result, however, from the removal of the coral colony from its environment (Crossland et al. 1980; Means and Sigleo 1986). Perhaps the best approach was that used by Crossland (1987), who performed *in situ* sampling of coral mucus from *Acropora variabilis* and *Stylophora pistillata* via clear perspex chambers fitted with a pre-filter, pump system and collection system. This allowed for acclimatisation of the corals before collection of mucus, with no reef water in the mucus samples upon removal.

A further consideration is that, in addition to stress, coral mucus production and composition can vary with depth and/or irradiance, between coral species, and temporally (Brown and Bythell 2005 and references therein). This highly dynamic nature therefore has the potential to introduce inconsistencies in sampling and errors in enumeration by EFM.

Given these methodological limitations, refinements of the EFM technique described here are recommended for future studies. In particular, sampling at more regular time intervals is suggested, with more coral species. Furthermore, *in situ* sampling of mucus within an enclosed environment is recommended to reduce potential contamination with overlying reef water.

4.5 Limitations of phenotypic identification of virus-like particles and future directions

Microscopy-based characterisation of VLPs – like genomic approaches – is an imperfect means of assessing the total diversity within a population (Thurber and Correa 2011). Indeed, there is continual debate about phenotypically classifying viruses without the use of genetic identification (Proctor 1997; and reviewed by Thurber and Correa

2011). Electron microscopy analyses can result in false-positive detection of viruses as a result of inert particles appearing morphologically similar to infectious viruses, and the improper classification of VLP morphology either resulting from a lack of detail or human error (e.g. misinterpretation of capsid size; Thurber and Correa 2011). Furthermore, genomic studies have demonstrated extremely high genetic diversity of viruses in the sea, far exceeding their morphological diversity (Wichels et al. 1998); this indicates a realm of categorisation beyond the reach of phenotypic identification. The power of genomics is demonstrated by Marhaver et al. (2008), who estimated that 28,600 viral types were associated with a single colony of the Caribbean coral *Diplora strigosa*, based on shotgun sequencing and metagenomic extrapolation of the VLP assemblage.

One particular limitation of VLP classification using microscopy techniques is the significant overlap between capsid sizes of VLP groups. For example, the average capsid diameter of viruses infecting microalgae is approximately 150 nm whereas bacteriophages are generally smaller, with a capsid diameter of approximately 70 nm (Ackermann and DuBow 1987a, b), yet more than a 25% of microalgal viruses have a capsid diameter smaller than 60 nm (Van Etten et al. 1991). Finer scale morphological structures such as capsomers or 2-, 3- and 5-fold axial symmetry are often required to discern differences within morphological sub-groups, especially where capsid size and shape are similar (e.g. the morphologically-similar *Microviridae* and *Leviviridae*; Fauquet et al. 2005). Further division of sub-groups (e.g. based on number of tail-fibers or capsomer appearance) could have revealed distributional patterns not readily apparent with the broader categories used here. Finer-scale groupings are recommended in future.

The majority of tailless polyhedral/spherical VLPs are morphologically similar, with few identifying features. This made definitive morphological categorisation of these VLP families especially problematic in the current study, where small (< 50 nm) VLPs of this type were the most abundant sub-group, with the *Microviridae*, *Corticoviridae*, *Parvoviridae*, *Nanoviridae*, *Circoviridae*, *Picronaviridae*, and *Caulomoviridae* viral families all falling within this morphological/size category (Fauquet et al. 2005), highlighting the limited resolution of the microscopical techniques used here.

A further problem can result from sample preparation, as a proportion of the tailed bacteriophage-like VLPs may be damaged during the ultracentrifugation process and their tails subsequently detaching from their capsids (Proctor 1997); this can then lead to misidentification (Davy and Patten 2007). However, the high quality of tailed bacteriophages imaged in the TEM micrographs in the current study, with intact fine-scale structures such as tail filaments and arm-like projections, suggests that sample preparation was suitably careful and unlikely to have led to major misinterpretation.

Another limitation of phenotypic identification is that it excludes viruses in the prophage phase of the lysogenic cycle (Thurber and Correa 2011). However, genetic techniques such as shotgun sequencing have limitations also, as while they can produce large quantities of sequence data, the fraction of viruses in an incomplete virion form – dormant within a host cell (or nucleus) – during sampling is omitted, resulting in significant underestimates (Thurber and Correa 2011). Furthermore, shotgun sequence samples are often comprised of both coral tissue and mucus, and so the specific site of origin (i.e. SML, coral tissue, *Symbiodinium* spp., and other microbial associates) is impossible to determine (Thurber and Correa 2011). Future studies investigating viral diversity should therefore employ both phenotypic and genomic diversity sampling techniques, that are spatially and temporally comprehensive (Thurber and Correa 2011), to properly determine the diversity of viral assemblages associated with coral reefs and their dynamics.

4.6 *Concluding remarks: Viruses and coral reef health*

Studies of coral reef microbes have increased in response to the global decline in coral reef health (Hoegh-Guldberg et al. 2007; Thurber and Correa 2011). However, while viruses are now known to be abundant and diverse on coral reefs, we still know virtually nothing about their role in coral reef health. Elucidating patterns of viral abundance and diversity, and in particular the abiotic and biotic drivers of these patterns on coral reefs should therefore be a research focus in the years to come, as should the potential role of viruses in coral bleaching and disease susceptibility/resistance.

The decline in coral reef health can, in part, be explained by increasing incidences of coral bleaching (Hoegh-Guldberg 1999) and coral disease (Nugues 2002; Richardson and Voss 2005; Bruckner and Hill 2009). Of note, several studies have linked disease signs with viruses (Cervino et al. 2004; Barash et al. 2005; Davy 2007; Aeby et al. 2011), while the link between viruses and coral bleaching has also been made in several studies (Wilson et al. 2001, 2005; Davy et al. 2006; Lohr et al. 2007; Danovaro et al. 2008). Of particular note is the thermal induction of viral lysis in the symbiotic dinoflagellates of corals and other anthozoans (Wilson et al. 2001, 2005; Davy et al. 2006; Lohr et al. 2007). However, to date, no viral causative agents have been definitively linked to coral disease etiologies (Rosenberg 2009; Thurber and Correa 2011), indicating that a greater understanding of disease pathology and viral ecology is needed. By focusing the investigation of coral diseases toward those that display viral-like characteristics (i.e. rapid progression and high transmission; Thurber and Correa 2011) the characterisation of the first unambiguously viral-induced coral disease might be just over the horizon.

The role of physical stressors on coral reef viruses is an obvious starting point to further establish whether they play a leading role in coral disease and bleaching. This is because physical stressors have been shown to significantly alter coral-associated microbial (including viral) constituents in coral tissue (Vega Thurber et al. 2009) and the SML (Segel and Ducklow 1982; Ritchie and Smith 1995a, b; Mao-Jones et al. 2010). Such alterations involve transitions from a healthy microbial community to a pathogenic one (Smith 2008; Vega Thurber et al. 2008, 2009; Bourne et al. 2009; Mao-Jones et al. 2010). Knowledge of how viruses are involved in these SML-associated microbial phase shifts is not well known, yet as the SML plays a critical role in coral disease transmission and resistance (Mao-Jones et al. 2010), this region of the coral holobiont and the interactions with viruses should be a research focus in future.

Continued investigation into how environmental stressors influence coral reef virus assemblages, within and surrounding the coral holobiont, should be a priority for future research. As our understanding grows of how viruses are involved in the homeostasis of the coral holobiont and the overall functioning of coral reef systems, so too will our ability to predict, slow and even mitigate detrimental drivers of coral reef health.

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6 APPENDICES

APPENDIX A ENVIRONMENTAL DATA

Figure 6.1 Chlorophyll-*a* (1), depth (2) salinity (3), temperature (4), and turbidity (5) measurements taken during the main sampling period (26th May - 14th June 2009) from all six sites in the Coconut Island Marine Reserve. Error bars represent standard deviation.

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Figure 6.1 continued

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Figure 6.1 continued

APPENDIX B
PILOT STUDY OF VIRUS-LIKE PARTICLE DENSITY MEASUREMENTS
USING SUB-SAMPLING GRIDS

Background and Methods

Epifluorescence microscopy (EFM) images were taken at 100 $\times$ magnification and each showed an area measuring 175 μm $\times$ 132 μm of the Anodisc filter onto which samples were collected. Very high numbers of virus-like particles (VLPs) and prokaryotes were visible, more than it was possible to count. Therefore, the images were sub-sampled using 20 μm $\times$ 20 μm gridded squares (representative of a grid reticle used in a photomicroscope).

To determine the optimum balance between accuracy and counting effort, a comparison of grid number per image was undertaken. Site A was chosen as being representative, and 100 images (ten per sample for each of five coral surface mucopolysaccharide layer (SML) samples and five reef water samples) were counted using one, three, five and ten grids each per image; the VLP and prokaryote densities were then averaged. Counts of both VLPs and prokaryotes were compared using univariate ANOVA.

Results

VLP and prokaryote counts did not significantly vary whether one, three, five or ten grids were counted (univariate ANOVA; $F_{3,36} = 0.086$, $p = 0.967$ and $F_{3,36} = 0.084$, $p =$

0.969, for VLPs and prokaryotes, respectively; *Figure 6.2*). Naturally, counting effort was increased substantially in relation to increased grid number per image. Thus, it was decided that a single grid per image (totaling ten per sample) could be used.

Figure 6.2 Average virus-like particle (A) and prokaryote (B) abundances in samples collected during 2009 at Site A. Counts were made using one, three, five, or ten grids superimposed on each image. White bars represent samples from the coral surface mucopolysaccharide layer; grey bars represent samples from the reef water. Error bars represent standard deviation.

APPENDIX C

CORAL SURFACE MUCOPOLYSACCHARIDE LAYER SAMPLE PREPARATION

Background and Methods

To test whether gentle homogenising had an affect on the efficiency of counting VLPs and prokaryotes a pilot study comparing homogenised SML samples versus non-homogenised control samples was undertaken. The homogenizing step was hypothesised to produce a more even spread of coral mucus (and thus VLPs and prokaryotes) across the 0.02 μm Anodisc filters. Samples were taken from four *Montipora capitata* colonies at Site A and transferred to a sterile tissue homogeniser, whereby three up and down movements with the homogeniser's piston were made. The samples were then further processed as outlined in Chapter Two. Four additional control SML samples were taken from the same coral colonies at Site A and processed for comparison, without the homogenizing step. Samples were analysed using the Hawai'i Institutes of Marine Biology's photomicroscope with a 100 $\times$ fluorescence oil-immersion objective with DF grade immersion oil.

Results

VLP and prokaryote densities did not significantly differ between the homogenised samples ($1.36 \pm 0.81 \times 10^7 \text{ ml}^{-1}$) and the controls ($1.56 \pm 0.24 \times 10^7 \text{ ml}^{-1}$) (paired sample t-test; $t_6, p = 0.652$). However, homogenising resulted in a more even spread of mucus

across the Anodisc with fewer 'mucus free' patches, making counting easier, and it was therefore decided that this homogenising step would be carried out on the samples collected in the main study in 2009.

Figure 6.3 Epifluorescence sample preparation pilot study. Surface mucopolysaccharide layer (SML) control sample (no homogenising step) (A); Homogenised SML sample (B). Images displayed were taken at 10× magnification.

APPENDIX D

MUCUS THICKNESS

Background and Methods

Following analysis of the 2009 SML samples, an assessment was undertaken to determine whether coral mucus in the SML samples was acting as a confounding variable, masking VLPs and prokaryotes in the epifluorescence images. The SYBR Gold stain caused the coral mucus to appear bright green in the EFM samples; thicker levels of mucus appeared to have a brighter green background than samples with a thinner layer of mucus, while reef water samples had an even darker green/black background. As images in the current study were taken with standardised settings on the photomicroscope with only minor brightness adjustments made, a comparison of the green background colour levels in images taken from the SML samples was undertaken, thus determining the relative mucus thickness in each image. The background colour level was quantified by using the Adobe Photoshop CS4 imaging software photo image option, which gives point sample statistics on red, blue, and green colour spectrum levels of an image. Four mucus thicknesses were determined, encompassing green colour values from 0 – 181+, and all SML images taken were placed into one of the categories along this mucus thickness spectrum (*Figure 6.4*; see Chapter Two, Section 2.2.4).

Grade one represented values 0–60 (similar to those of reef water samples), grade two represented 61–120, grade three represented 121–180, and grade four represented

181+. It should be noted that VLP and prokaryote particle fluorescence values typically range above 220 on the green colour scale, peaking at around 260 for large prokaryote particles.

Univariate ANOVAs were conducted with the mucus thickness spectrum category as the independent variable and VLPs and prokaryote densities as the dependent variables for both the 2008 and 2009 VLP prokaryote densities, and the virus to prokaryote ratio (VPR).

Results and Discussion

Following analysis, all samples from category one (background green levels 0–80; $n = 3$ from 2009, no samples from 2008) were removed, as these samples were primarily comprised of seawater with very little mucus and thus were not true representations of the SML environment.

Across mucus grades two, three and four there was no significant difference in VLP and prokaryote densities for 2008 (univariate ANOVA; $F_{2,13} = 1.12$ $p = 0.356$ for VLPs and $F_{2,13} = 1.071$ $p = 0.371$ and prokaryotes) or 2009 (univariate ANOVA; $F_{2,57} = 0.706$ $p = 0.498$; $F_{2,57} = 2.089$ $p = 0.133$ for VLP and prokaryote densities, respectively).

For the VPR value, a similar trend was identified for 2008 (univariate ANOVA; $F_{2,13} = 1.473$ $p = 0.265$) or 2009 (univariate ANOVA; $F_{2,57} = 1.21$ $p = 0.306$) (*Table 6.5*). These results indicate that higher levels of coral mucus were not acting as a confounding variable, causing the lower VLP and prokaryote densities seen in the 2009 SML samples.

Figure 6.4 Coral mucus cover spectrum. 0- 60 represents low levels of green background fluorescence, comparable to reef water samples (1); 61 - 120 represents medium green background fluorescence (2); 121 - 180 represents high green background fluorescence (3); 181+ represents a very high green background fluorescence (4). All sample images were graded from one-four in terms of the coral mucus thickness (level of green background fluorescence). Grade one was later removed from the analysis.

Figure 6.5 Virus-like particle (VLP) abundances, prokaryote abundances, and virus to prokaryote ratio (VPR) values plotted against the mucus cover and thickness spectrum grades. Grade two represents the lowest mucus cover and thickness, increasing through to grade four. All values from grade one were omitted. (A) 2008 pilot study: VLPs ($\times 10^7$) ml^{-1} (white bars) and prokaryotes ($\times 10^6$) ml^{-1} (grey bars); (B) 2009 full environmental study: VLPs ($\times 10^7$) ml^{-1} (white bars) and prokaryotes ($\times 10^6$) ml^{-1} (grey bars); (C) VPR values from the 2008 pilot study (white bars) and the 2009 full environmental study (grey bars). Error bars represent standard deviation.

APPENDIX E
STATISTICAL ANALYSES OF MICROBE DENSITIES

Table 6.1 Univariate ANOVA results testing virus-like particle and prokaryote densities, and virus to prokaryote ratio values against habitat type (surface mucopolysaccharide layer and reef water), site (2008: B, C, E, F; 2009: A, B, C, D, E, F) and temporal comparisons.

Sampling period	Statistical analysis	Fixed factor	Independent factor	df	F	p
2008	Two-way univariate ANOVA	Habitat type	Virus-like particles	1,24	2.642	0.117
			Prokaryotes	1,24	0.167	0.687
			Virus to prokaryote ratio	1,24	1.473	0.237
2008	Two-way univariate ANOVA	Site	Virus-like particles	3,24	2.766	0.117
			Prokaryotes	3,24	2.133	0.122
			Virus to prokaryote ratio	3,24	4.813	0.009 *
2008	Simple effects test for site	Surface mucopolysaccharide layer	Virus to prokaryote ratio	3,12	1.099	0.387
		Reef water	Virus to prokaryote ratio	3,12	4.813	0.009 *
2009	Two-way univariate ANOVA	Habitat type	Virus-like particles	1,108	386.539	< 0.001 *
			Prokaryotes	1,108	39.852	< 0.001 *
			Virus to prokaryote ratio	1,108	26.631	< 0.001 *
2009	Two-way univariate ANOVA	Site	Virus-like particles	5,108	2.392	0.042 *
			Prokaryotes	5,108	6.954	< 0.001 *
			Virus to prokaryote ratio	5,108	12.431	< 0.001 *
2009	Simple effects test for site	Surface mucopolysaccharide layer	Virus-like particles	5,54	1.325	0.627
		Reef water	Virus-like particles	5,54	6.65	< 0.001 *
2009	Simple effects test for site	Surface mucopolysaccharide layer	Prokaryotes	5,54	3.584	0.007 *
		Reef water	Prokaryotes	5,54	3.781	0.005 *
2009	Simple effects test for site	Surface mucopolysaccharide layer	Virus to prokaryote ratio	5,54	6.112	< 0.001 *
		Reef water	Virus to prokaryote ratio	5,54	8.201	< 0.001 *
2008 and 2009	Repeated measures ANOVA for sites B, C, D, E	Surface mucopolysaccharide layer	Virus-like particles	1,15	22.99	0.131
			Prokaryotes	1,15	5.65	0.464
			Virus to prokaryote ratio	1,15	0.035	0.854
2008 and 2009	Repeated measures ANOVA for sites B, C, D, E	Reef water	Virus-like particles	1,15	86.811	< 0.001 *
			Prokaryotes	1,15	86.811	< 0.001 *
			Virus to prokaryote ratio	1,15	9.894	0.007 *

df = degrees of freedom; F = F-statistic; p = p-value; * = p-value is significant at the 95% confidence interval.

APPENDIX F

SUPPLEMENTARY VIRUS-LIKE PARTICLE MORPHOTYPE DATA

Figure 6.6 Transmission electron microscopy images of the unclassified filamentous virus-like particles observed in samples from both surface mucopolysaccharide layer and reef water samples. All images pictured here were sampled from reef water with images A, B, and D sampled from Site D, and Image C sampled from Site C. TF = tail-fiber. Scale bars represent 100 nm.

Table 6.2 Relative proportions of virus-like particle (VLP) morphotypes in the surface mucopolysaccharide layer of *Montipora capitata* colonies at each of the four sites sampled (A - D) for VLP morphology. Site A - the greatest depth, Site B - the highest temperature, Site C - the highest chlorophyll-*a* concentration, and Site D - the highest turbidity. Values are mean proportion $\pm$ standard deviation. ($n = 5$ grids for Sites A, C and D, and 4 for Site B; 100 VLPs were counted per grid).

Morphotype	Site A	Site B	Site C	Site D
Podovirus < 100 nm	0.14 $\pm$ 0.032	0.113 $\pm$ 0.028	0.062 $\pm$ 0.037	0.058 $\pm$ 0.032
Podovirus > 100 nm	0.02 $\pm$ 0.023	0.003 $\pm$ 0.005	0.014 $\pm$ 0.013	0.004 $\pm$ 0.005
Elongate myovirus < 100 nm	0.004 $\pm$ 0.009	0.008 $\pm$ 0.01	—	—
Elongate myovirus 100 - 200 nm	0.008 $\pm$ 0.008	0.013 $\pm$ 0.015	0.004 $\pm$ 0.005	0.012 $\pm$ 0.011
Elongate myovirus > 200 nm	0.008 $\pm$ 0.005	0.013 $\pm$ 0.005	—	0.008 $\pm$ 0.008
Isometric myovirus < 100 nm	0.058 $\pm$ 0.029	0.098 $\pm$ 0.083	0.028 $\pm$ 0.024	0.020 $\pm$ 0.012
Isometric myovirus 100 - 200 nm	0.076 $\pm$ 0.06	0.07 $\pm$ 0.026	0.038 $\pm$ 0.024	0.05 $\pm$ 0.035
Isometric myovirus > 200 nm	0.01 $\pm$ 0.012	—	0.002 $\pm$ 0.004	0.002 $\pm$ 0.004
Siphovirus < 100 nm	0.03 $\pm$ 0.029	0.028 $\pm$ 0.031	0.006 $\pm$ 0.005	0.008 $\pm$ 0.008
Siphovirus 100 - 200 nm	0.024 $\pm$ 0.026	0.023 $\pm$ 0.01	0.018 $\pm$ 0.016	0.026 $\pm$ 0.013
Siphovirus > 200 nm	0.002 $\pm$ 0.004	0.005 $\pm$ 0.006	0.006 $\pm$ 0.005	0.004 $\pm$ 0.005
Polyhedral/Spherical < 50 nm	0.32 $\pm$ 0.074	0.319 $\pm$ 0.123	0.564 $\pm$ 0.134	0.385 $\pm$ 0.209
Polyhedral/Spherical 50 - 100 nm	0.123 $\pm$ 0.057	0.142 $\pm$ 0.069	0.118 $\pm$ 0.031	0.149 $\pm$ 0.082
Polyhedral/Spherical > 100 nm	0.01 $\pm$ 0.007	0.015 $\pm$ 0.017	0.020 $\pm$ 0.007	0.084 $\pm$ 0.084
Lemon-shaped < 100 nm	0.06 $\pm$ 0.09	0.02 $\pm$ 0.008	0.028 $\pm$ 0.02	0.012 $\pm$ 0.013
Lemon-shaped > 100 nm	0.012 $\pm$ 0.018	0.018 $\pm$ 0.029	0.01 $\pm$ 0.007	0.006 $\pm$ 0.009
Filamentous < 100 nm	0.014 $\pm$ 0.022	0.033 $\pm$ 0.01	0.002 $\pm$ 0.004	—
Filamentous 100 - 500 nm	0.03 $\pm$ 0.039	0.03 $\pm$ 0.014	0.016 $\pm$ 0.013	0.034 $\pm$ 0.015
Filamentous 500 nm - 1 μ m	0.006 $\pm$ 0.005	0.018 $\pm$ 0.013	0.016 $\pm$ 0.017	0.04 $\pm$ 0.019
Filamentous > 1 μ m	0.018 $\pm$ 0.011	0.008 $\pm$ 0.01	0.004 $\pm$ 0.005	0.032 $\pm$ 0.019
Geminiviridae-like	0.008 $\pm$ 0.004	0.01 $\pm$ 0.000	0.02 $\pm$ 0.012	0.026 $\pm$ 0.026
Bead-shaped	0.01 $\pm$ 0.014	0.015 $\pm$ 0.013	0.016 $\pm$ 0.015	0.002 $\pm$ 0.004
Rod-shaped	0.008 $\pm$ 0.004	0.005 $\pm$ 0.006	0.008 $\pm$ 0.008	0.026 $\pm$ 0.021
Cuboid	0.004 $\pm$ 0.005	0.01 $\pm$ 0.008	—	0.006 $\pm$ 0.009
Hook-shaped	0.002 $\pm$ 0.004	—	—	0.002 $\pm$ 0.004
Other	0.004 $\pm$ 0.009	—	—	0.012 $\pm$ 0.027

Table 6.3 Relative proportions of virus-like particle (VLP) morphotypes in the reef water overlying *Montipora capitata* colonies at each of the four sites sampled (A - D) for VLP morphology. Site A - the greatest depth, Site B - the highest temperature, Site C - the highest chlorophyll-*a* concentration, and Site D - the highest turbidity. Values are mean proportion $\pm$ standard deviation. ($n = 5$ grids for Sites A and B and 4 for Sites C and D; 100 VLPs were counted per grid).

Morphotype	Site A		Site B		Site C		Site D	
Podovirus < 100 nm	0.13 $\pm$	0.046	0.134 $\pm$	0.076	0.075 $\pm$	0.017	0.073 $\pm$	0.035
Podovirus > 100 nm	0.004 $\pm$	0.005	0.012 $\pm$	0.013	0.003 $\pm$	0.005	0.05 $\pm$	0.02
Elongate myovirus < 100 nm	—		0.008 $\pm$	0.013	—		—	
Elongate myovirus 100 - 200 nm	0.002 $\pm$	0.004	0.01 $\pm$	0.007	0.025 $\pm$	0.044	0.023 $\pm$	0.04
Elongate myovirus > 200 nm	0.004 $\pm$	0.005	—		0.008 $\pm$	0.05	—	
Isometric myovirus < 100 nm	0.068 $\pm$	0.058	0.036 $\pm$	0.029	0.02 $\pm$	0.008	0.03 $\pm$	0.026
Isometric myovirus 100-200 nm	0.05 $\pm$	0.019	0.048 $\pm$	0.029	0.08 $\pm$	0.036	0.073 $\pm$	0.035
Isometric myovirus > 200 nm	0.006 $\pm$	0.009	—		0.02 $\pm$	0.014	0.003 $\pm$	0.006
Siphovirus < 100 nm	0.04 $\pm$	0.032	0.08 $\pm$	0.118	0.005 $\pm$	0.006	0.043 $\pm$	0.012
Siphovirus 100-200 nm	0.032 $\pm$	0.018	0.038 $\pm$	0.022	0.023 $\pm$	0.013	0.033 $\pm$	0.04
Siphovirus > 200 nm	0.006 $\pm$	0.005	0.004 $\pm$	0.005	0.025 $\pm$	0.013	0.007 $\pm$	0.006
Polyhedral/Spherical < 50 nm	0.422 $\pm$	0.044	0.404 $\pm$	0.143	0.515 $\pm$	0.061	0.3 $\pm$	0.066
Polyhedral/Spherical 50-100 nm	0.066 $\pm$	0.051	0.064 $\pm$	0.038	0.08 $\pm$	0.018	0.28 $\pm$	0.199
Polyhedral/Spherical > 100 nm	0.004 $\pm$	0.005	0.064 $\pm$	0.115	0.008 $\pm$	0.01	0.03 $\pm$	0.017
Lemon-shaped < 100 nm	0.002 $\pm$	0.004	0.002 $\pm$	0.004	0.005 $\pm$	0.01	0.01 $\pm$	0.01
Lemon-shaped > 100 nm	—		—		—		—	
Filamentous < 100 nm	0.012 $\pm$	0.008	0.018 $\pm$	0.02	—		0.013 $\pm$	0.012
Filamentous 100-500 nm	0.026 $\pm$	0.013	0.028 $\pm$	0.027	0.018 $\pm$	0.01	0.017 $\pm$	0.015
Filamentous 500 nm -1 μ m	0.03 $\pm$	0.026	0.014 $\pm$	0.013	0.038 $\pm$	0.022	—	
Filamentous > 1 μ m	0.024 $\pm$	0.038	0.016 $\pm$	0.018	0.023 $\pm$	0.015	—	
Geminiviridae-like	0.006 $\pm$	0.009	0.01 $\pm$	0.01	0.01 $\pm$	0.008	0.003 $\pm$	0.006
Bead-shaped	0.024 $\pm$	0.015	0.002 $\pm$	0.004	—		0.003 $\pm$	0.006
Rod-shaped	0.022 $\pm$	0.011	0.002 $\pm$	0.004	0.023 $\pm$	0.01	—	
Cuboid	0.02 $\pm$	0.007	0.006 $\pm$	0.009	—		0.007 $\pm$	0.006
Hook-shaped	—		—		—		—	
Other	—		—		—		—	

APPENDIX G

PALMYRA ATOLL

Background and Methods

Palmyra Atoll study site

Sampling was conducted between 15th November – 5th December 2008 at an additional study site, Palmyra Atoll National Wildlife Refuge (05°52' N, 162°06' W). The atoll is one of the Line Islands, situated in the Central Pacific Ocean, and one of the most remote coral reefs on the planet (Williams et al. 2010b). It is a near pristine coral reef environment and was chosen as a study site to allow comparison with the highly degraded Coconut Island Marine Reserve (CIMR) in Hawai'i.

Sampling design and collection

Samples were collected from five sites around the atoll (*Figure 6.7*), with SML samples and reef water samples taken from five colonies of the coral *Pocillopora meandrina* at each site. In addition, SML and reef waters samples were collected from the Western Terrace (WT) for a species type comparison of VLP morphology across ten coral species (*Acropora acuminata*, *Pavona duerdeni*, *Lobophyllia* sp., *Astreopora gracilis*, *Stylophora pistillata*, *Favia stelligera*, *Pocillopra meandrina*, *Montastrea curta*, *Hydnophora microcouos*, *Porites* sp.). Samples were prepared for transmission electron microscopy (TEM) analyses following the protocol outlined in Chapter Three, with samples stored at 4 °C prior to transportation to the University of Hawai'i.

Unfortunately, as a glow discharge machine was not available during grid preparation, VLP did not appear to stick to grids, resulting in extremely low VLP numbers (often zero VLPs on the majority of grids). It was therefore not possible to collect data for this particular part of the study.

Figure 6.7 Satellite image of the Palmyra Atoll National Wildlife Refuge, showing the location of the five sample sites. Back reef sites: Penguin Spit Inner Buoy (PSi); North Barren (NB). Fore-reef sites: PSi, Penguin Spit; HB, Holei and Burn (HB); Western Terrace (WT); Strawn (St). Ikonos 2000 image courtesy of Space Imaging and Gareth J. Williams.

APPENDIX H

PORTABLE VIRUS COUNTER FIELD TRIALS

Introduction

Developing and applying novel technologies to the expanding field of coral virology is an important step to help determine the numerous potential roles of coral reef viruses (Thurber and Correa 2011). A collaboration with the nanotechnology company iZON (Christchurch, New Zealand) was forged in which I took their newly developed qViro nano-particle counting instrument for the first field trials to Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i and Palmyra atoll of the Line Islands, in an attempt to further develop the instrument's ability to count and characterise individual virus particles in real-time.

The qViro uses scanning ion occlusion sensing (SIOS), a technique that incorporates a novel resizable elastic nanopore to detect the passage of individual nano-scale objects (Roberts et al. 2010; Roberts et al. in press). Acting as a miniature coulter counter (an apparatus for counting and sizing particles suspended in electrolytes; Coulter 1953), the qViro runs an electrical current across the top of the nanopore and particles are added to one side of the pore in an electron buffer. The nanopore is opened mechanically by stretching the plastic cruciform; as a result the electrical current also increases. As the pore opens, particles pass through and as they do, the change in the electrical current trace is recorded in what is called a blockade event. The size measurement of the blockade is representative of the volume of the particle (Roberts et al. 2010; Vogel et al. 2011).

Figure 6.8 The qViro portable field counter prototype. Trials were undertaken with samples from Coconut Island Marine Reserve and Palmyra Atoll National Wildlife Refuge.

As the qViro instrument allows for manual opening, closing and changing of size of the nanopores in real time, individual particles can be counted and sized, and the instrument thus effectively operates as a small, portable flow cytometer. During the field trials (15th November – 5th December 2008 at Palmyra Atoll, and 7th – 12th December 2008 at Coconut Island), reef water samples were tested using the qViro instrument (*Figure 6.8*). As the instrument is still in the early development stage and collection of viable data on particle density was not possible. However, important information on the ability of the instrument to record the abundance of particles of approximately 100 nm in diameter from sea water samples, the effect of the fixative agent gluteraldehyde on the functioning of the instrument, and sampling seawater without the use of an electron changed buffer was reported, thus assisting with future development.

The iZON qViro instrument has been used to determine accurate, direct, rapid, in-sample measurements of quantitative nanoparticle concentrations, for particles of approximately 100 nm to 1 μ m from both synthetic and biological samples (Vogel et al. 2011; Roberts et al. in press). Many marine viruses fall below this size range (Ackermann 2001, 2007; Fauquet et al. 2005), but the system is rapidly developing and thus in the future cheaper, accurate, in-sample characterisation of seawater samples (without the use of fixatives) may be possible.