"...it takes all the running you can do to stay in the same place."

- Through the Looking Glass, Lewis Carroll
VIRUS INDUCED CELL DEATH, EVASION AND RESISTANCE IN THE 
HARMFUL BLOOM-FORMING ALGA, *Heterosigma akashiwo*

by

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ABSTRACT

Phytoplankton make up a minute proportion of the Earth’s total biomass yet they contribute almost half of global primary productivity, the discrepancy being a result of the rapid turnover of phytoplankton due to grazing and environmentally-induced mortality. Marine viruses also contribute to this high mortality and often outnumber their hosts by at least an order of magnitude. With an estimated $10^{23}$ infections per second, viruses can be responsible for the death of 20% of the ocean’s biomass per day. The magnitude of the threat implies that phytoplankton employ strategies to avoid and limit the extent of viral lysis. However, for most phytoplankton groups, there is little knowledge regarding specific defensive strategies. This thesis investigated specific responses activated in the bloom-forming alga *Heterosigma akashiwo* in response to heat-stress and three viruses (HaV, HaNIV and HaRNAV) and provides evidence that this alga is capable of an active form of cell death known as programmed cell death (PCD). This cell death has classic hallmarks consistent with apoptosis-like cell death in response to heat-stress, HaV and HaNIV infection, and paraptosis during HaRNAV infection. In addition to PCD, *Heterosigma* is capable of forming resting cells in response to high-heat (40 and 50°C) and to viral infection with HaV, a DNA virus (Phycodnaviridae), which represents a successful but short-term evasion mechanism against cell death in this alga. The various responses, multiple PCD programs and the formation of resting cells, represent cell and population level survival strategies under stress conditions. The resting-cell phase of *Heterosigma*’s life cycle is essential for population survival during viral infection, in addition to the previously reported overwintering survival strategies. This avoidance strategy would contribute to bloom
termination by allowing a proportion of the cells to settle out on the ocean floor and would presumably contribute to a “seed stock” for future blooms when the viral load has decreased. Understanding the triggers and mechanisms underlying the virus induced resting-cell formation could also lead to an approach for the control of toxic algae, like *Heterosigma*, that are known to kill fish and harm coastal environments.
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Run, hide or evolve
Driven to suicide, poor
Heterosigma
NOTES ON AUTHORSHIP AND PUBLICATION STATUS OF CHAPTERS

Chapters 2, 3 and 4 of this thesis were prepared as manuscripts.

Chapter 2: "Heat-stress-induced programmed cell death in Heterosigma akashiwo (Raphidophyceae)," was published in Harmful Algae and permission to reproduce it for this thesis has been confirmed from the journal’s copyright agreement under its personal use policy. This work was formulated, carried out and written by myself under the supervision of Dr. Janice Lawrence.

The data contained in Chapter 3: "Cell death programs of the harmful bloom-forming raphidophyte, Heterosigma akashiwo, during viral infection with HaRNAV, HaNIV, and HaV," was collected, in part, at the National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Hiroshima, Japan in collaboration with Dr. Keizo Nagasaki. HaV viral stocks were provided to me for future experiments in Canada by Dr. Nagasaki. With my guidance, James K. Roherty, an undergraduate honors student at the University of New Brunswick, collected the data for the HaRNAV infection experiments, however, statistical analysis and the conclusions are solely mine. The manuscript contained in Chapter 3 was prepared for Harmful Algae and is currently in the review process. The development of this project was formulated by myself and Dr. Janice Lawrence. Written work was primarily guided by Dr. Dion Durnford, although final approval was received from all scientist named as authors (Dr. Dion Durnford, Dr. Janice Lawrence, Dr. Keizo Nagasaki, James K. Roherty)
Chapter 4: "Three cell death fates during viral infection: apoptosis-like cell death, survival and resting-cell formation in *Heterosigma akashiwo* - a marine phytoplankton" has not yet been submitted for review at any journal. This work was formulated, carried out and written by myself under the guidance of Dr. Dion Durnford.
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Chapter 1 - INTRODUCTION

Phytoplankton are important in the marine environment because of the role they play in nutrient cycling in the ocean. To understand the significance of phytoplankton in biomass cycling, the ecology of marine food webs must be explored. Phytoplankton are unicellular photoautotrophs that form the base of most marine food webs. Some of the nutrients derived from photosynthesis are drawn up through the trophic levels by zooplankton grazing while the dissolved organic carbon/nitrogen released by phytoplankton through secretion or cell lysis is metabolized by bacteria, a process referred to as the microbial loop (Azam et al. 1983). In this thesis, I explore population cycling, cell death and viral infection in _Heterosigma akashiwo_— a toxic bloom-forming alga. I will describe experiments that will address three main questions. Is _Heterosigma_ capable of undergoing programmed cell death (PCD) when exposed to a classic trigger for PCD, namely heat-stress? Do different lytic viruses induce differences in cell death morphology in _Heterosigma_? What are the dynamic changes in population structure following viral infection?

**Overview of Phytoplankton**

Phytoplankton are photosynthetic microorganisms that live in the euphotic zone of a water body. They are planktonic in that they float and drift with ocean currents and wind action. Phytoplankton is a term that has no taxonomic significance as it includes an enormous diversity of organisms spanning multiple kingdoms. Phytoplankton have many different life history strategies and can be
sessile or motile, autotrophic or mixotrophic. Phytoplankton can be found in fresh and salt water as well as in moderate and extreme climates (i.e. polar ice and hot springs). Given the vast diversity of this group, the physiological plasticity among phytoplankton (including the eukaryotic "true algae" and prokaryotic cyanobacteria and prochlorophytes) is exceptional in the biological world and makes the group fascinatingly complex.

Cyanobacteria are responsible for the oxygenation of the atmosphere 2.7 billion years ago (Buick 2008). Eukaryotic phytoplankton evolved later (approximately 1.5 billion years ago) and became dominant in the world's oceans in the Mesozoic Era (251 to 65 million years ago) (Falkowski et al. 2004). The evolutionary history of phytoplankton is complex and is complicated by the spread of photosynthesis by both primary and secondary endosymbiotic events. Currently, the Eukaryotes are divided into five super-groups, four of which (Plantae, Chromalveolates, Excavates and Rhizaria) contain modern algae (Figure 1-1; Keeling et al. 2005). The kingdom Plantae includes the red and green algae, the glaucophytes and the land plants. In the Plantae, plastids were derived by a single primary endosymbiotic event with a cyanobacterium, giving rise to the first photosynthetic eukaryotes. Chromalveolates (e.g. coccolithophores, cryptophytes, diatoms, dinoflagellates, and raphidophytes) make up most of the eukaryotic algal diversity (Keeling et al. 2005). In this group, plastids arose through secondary endsymbiosis with a red algal-like organism. In the euglenoids (Excavate) and the chlorarachniophytes (Rhizaria), plastids arose via separate secondary endosymbiotic events, but unlike the Chromalveolates, these endosymbiosis involved green alga-like organisms.
Ecological Importance and Cycling of Phytoplankton

Phytoplankton are ecologically important because they contribute almost half of global primary production, yet they make up less than 1% of the Earth’s standing biomass (Field et al. 1998, Geider et al. 2001). This apparent discrepancy is due to the phytoplankton turnover rate and it is estimated that all phytoplankton on Earth are replaced weekly (Valiela 1995, Field et al. 1998, Bidle and Falkowski 2004). This high turnover rate leads to nutrient and carbon-flow through three fates: 1) transfer to higher trophic levels through grazing/predation, 2) transport to the deep ocean through sinking and 3) metabolism of nutrients following cell death by bacteria, which is called the microbial loop. Carbon, phosphorus, nitrogen, iron, silica and vitamin B$_{12}$ are the primary limiting nutrients and vitamins in aquatic systems (Wilhelm and Suttle 1999) and are often metabolized into organic forms and/or sequestered in phytoplankton (Capone et al. 1997). Thus the fate phytoplankton and the nutrients they contain can regulate productivity at all trophic levels.

There are two pools of organic carbon that have drastically different bioavailability: particulate organic carbon (POC) and dissolved organic carbon (DOC). Much of the POC is utilized by higher trophic levels (microzooplankton to macrozooplankton), the rest is either shunted into the deep ocean or broken down by heterotrophic bacteria. Sedimentation of large or heavy phytoplankton, such as diatoms or coccolithophores, creates a carbon sink where nutrients (including organic carbon) are taken from the pelagic zone and deposited in deep ocean sediments. This “biological pump” has been touted as a method for reducing
atmospheric carbon (CO$_2$) and thus a solution to global warming (Siegentharler and Sarmiento 1993). The hypothesis is that a transient increase in phytoplankton populations, such as coccolithophores, will cause a reduction of atmospheric CO$_2$ through fixation during photosynthesis and deposited in appropriate sinks, such as the production of large calcium-carbonate coccoliths. Many open-ocean fertilization experiments have been conducted to examine the possibility of reducing atmospheric CO$_2$ levels through phytoplankton blooms (Buesseler and Boyd 2003, Boyd et al. 2000, Raven and Falkowski 1999). Unfortunately, it is now known that the majority of phytoplankton biomass is not exported to the deep ocean, and phytoplankton are consumed more often by heterotrophic zooplankton (Kintisch 2007), allowing nutrients and carbon to move through the trophic levels of the marine ecosystem. For the most part, DOC can only be used by bacteria and other microbes because of the small size (<0.2µm; Fuhrman 1999, Azam et al 1993, Azam 1998). DOC is produced by cellular excretions, sloppy feeding, and egestion (Furham 1999), but also by microbial death (Wilhelm and Suttle 1999) via necrosis, lysis or PCD. Therefore, biological stressors, such as viruses, plus abiotic stressors, like UV irradiation, induce necrosis or PCD causing nutrients to flow into the microbial loop rather than through higher trophic levels.

Many species of phytoplankton are bloom-forming. During a bloom, phytoplankton can accumulate to hundreds of millions of cells per litre (Hallegraeff 1993). Some blooms, known as red- or brown-tides, can reach such extreme densities over vast areas that they can be seen from space through enhanced satellite images or remote sensing. Bloom forming species are opportunistic, utilizing nutrients quickly allowing for high growth rates, and can often outcompete...
non-bloom-forming algae. Blooms are a result of increased resource availability often caused by an influx of iron, nitrogen or phosphorus. Localized nutrient-rich water can stem from upwellings, sewage contamination or pollution (i.e. industrial waste or agriculture runoff). Algal blooms can be detrimental to other organisms and the surrounding environment by outcompeting non-bloom forming organisms, releasing noxious toxins, reducing light infiltration or creating dead-zones due to the depletion of oxygen in the water column (Heisler et al. 2008). As algal blooms peak, transparency of the water is reduced which impedes light penetration and decreases survivorship of other natural flora. Toxic chemicals can be released from algal cells and can accumulate in filter feeders such as shellfish. Several dinoflagellates, including species from the genus *Alexandrium* release the paralytic shellfish toxin, saxitoxin (Anderson 1998), which is a potent neurotoxin. Other harmful blooms cause finfish mortality by clogging the gills, resulting in suffocation (Onoue 1990).

There are numerous biotic and abiotic factors that trigger crashes of algal blooms. Heavy grazing (Boyd et al. 2004) by macro- and microzooplankton, a variety of benthic or water column filter feeders, or virus-induced cell lysis can quickly reduce algae populations (Jacquet et al. 2002). Unfavorable environmental conditions such as nutrient depletion, light limitation or temperature changes may also cause cyst formation or cell death (Hecky 1988), leading to sedimentation and subsequent bloom reduction or termination. Wind action or ocean currents can cause dilution or disruption (Vargo 2009) that can result in bloom crashes. Abrupt bloom termination can cause high levels of localized DOC leading to a sharp and sudden increase in bacteria concentration. This can reduced dissolved oxygen (DO),
which leads to a hypoxic or anoxic environment (Heisler et al. 2008). Reduced DO results in massive fish kills and death of invertebrates, areas that are known as dead-zones. Algal bloom formation and termination have substantial effects on ecology, industry and human health, thus predicting the onset, severity and duration of blooms is of great interest. Although the cause of blooms has been well established, mechanisms that cause trimming –reduction in population density– or termination of blooms are less understood.

Overview of Viruses

Viruses are small, ubiquitous agents that require a host for replication and progeny production. Dmitri Iwanowski described the first infectious agent in 1896 when he discovered that disease could be transmitted from extracts from contaminated tobacco plants that had been passed through filters fine enough to retain bacteria. Two years later, Friedrich Loeffler (1898) discovered that the causative agent for foot-and-mouth disease was a virus. Thus, viruses were known to infect both plants and animals. Viruses that infect bacteria (known as phages) were first identified almost 20 years later by Twort in 1915 and d’Hérelle in 1917 (Duckworth 1976). We now know that viruses infect organisms from all three domains of life: Archaea, Bacteria and Eukaryota. Although viruses are relatively host-specific, many hosts are susceptible to infection by multiple viruses (Breitbart et al. 2007). Therefore, estimates of virus diversity are many times the estimated number of species in existence. Although there is vast diversity within viruses and hosts, all viruses are obligate parasites that are composed of a protein capsid and a genome composed of RNA or DNA. Viruses may contain DNA, RNA or both, depending on the virus and stage of reproduction. The genomic material can be
single-, double-, or sections of single- and double-stranded DNA or RNA. The genome is organised in linear, circular or segmented strands. Adding to the complexity of replication, viral genomes can be positive (+), negative (-) or ambisense (+/-) (ambisense is a combination of complementary and negative sections along the genome). The combination of all of these genetic features makes viral replication the most unique and diverse mechanisms for genome replication amongst any other group of organisms on Earth.

The International Committee on Taxonomy of Viruses (ICTV) use four main characteristics to group and name viruses: nature of the nucleic acid, symmetry of the capsid, presence/absence of an envelope and size of the virion and particle size (Figure 1-2). To date, there are three families of viruses that infect Eukaryotic phytoplankton: the Phycodnaviridae (Van Etten 1983, not shown in Figure 1-2), the Picornaviridae (Lang et al. 2004) and the Bacillariornaviridae (Tomaru et al. 2009). However, new evidence is emerging that a member of Megaviridae, a nucleocytoplasmic large DNA viruses (NCLDV), infects Aureococcus anophagefferens, a brown tide alga (Moniruzzaman et al. 2014).

Viruses have immense genetic variation and there is no single genetic element shared by all viruses (Rohwer and Edwards 2002). There are, however, key replication "checkpoints" or elements that are common among viruses: attachment (adsorption), genome transport (penetration) and viral-progeny replication, assembly and release. Examples of functionally conserved genes within groups of viruses are the RNA-dependent RNA polymerase (RdRp) protein found in Picornaviridae (small, icosahedral, RNA-containing viruses) (Culley et al. 2003) and the DNA polymerase gene shared by many Phycodnaviridae (DNA-containing
algal viruses) (Chen et al. 1996, Chen and Suttle 1996). However, mutation and evolution in viral strains is relatively fast because of the high rates of replication, especially in RNA-viruses (Drake and Holland 1999) due to the lack of proof-reading capacity during RNA replication.

The struggle between successful viral replication and host immunity has lead to a vast array of host-virus interactions. Successful viral infection is dependent on evading host-immune responses at each replication checkpoint. A compounding factor that makes microbial virology difficult is that the majority of microbial hosts belong to uncultured groups and therefore the isolation of the associated viruses is currently impossible.

**Marine Viruses**

Marine viruses are ubiquitous and abundant. Some estimates suggest there are $10^{31}$ total viruses globally (Weitz and Wilhelm 2012) and the majority of these viruses are specific to microbial hosts in the marine environment. Here, I focus on aquatic viruses because most of the studied protist-viral systems to date are aquatic, primarily marine (Hyman and Abedon 2012). Marine viruses can reach densities as high as $10^4 - 10^8$ per milliliter of seawater (Bergh et al. 1989, Proctor and Fuhrman 1990, Fuhrman 1999). With such extreme abundances, viruses often outnumber their hosts by an order of magnitude. It is estimated that $10^{23}$ viral infections per second occur in the ocean and responsible for the death of 20% of the ocean’s biomass per day (Suttle 2007). These estimates are based on enumeration of visibly infected microbes and calculated from viral production rates. Thus, viral infections of phytoplankton are drivers for a significant portion of global nutrient cycling.
Algal Viruses

The Phycodnaviridae is named because of their infectivity of algal hosts ("phyco-") and because they contain large dsDNA (160 to 560 kb) genomes ("-dna-") (Wilson et al. 2005). They do not yet belong to an Order and are not depicted in Figure 1-2. However, there is a substantial scientific community contributing to the advancement of knowledge of these algal viruses and to date, Phycodnaviridae contains 6 Genera (Chlorella virus, Coccolithovirus, Phaeovirus, Pryminesiovirus and Raphidovirus) and 33 species; yet, they have only been identified in about 0.1% of the ~40,000 known algal species (Guiry and Rindi 2005).

There are a number of algal virus systems that have been isolated and cultured in laboratories around the world, but they represent a tiny fraction of the algal-virus systems in natural environments. However, our understanding of algal viruses has increased substantially since the first characterization.

Virus Driven Nutrient Cycling

Viruses are important in the marine food web because they can control plankton blooms. Wilhelm and Suttle (1999) estimate that viral infection leading to lysis of phytoplankton cells disrupts the biological pump and reduces deep-ocean carbon sequestering and instead contributes between 6% and 26% of dissolved organic matter (DOM) which remains at the surface ocean. DOM fuels the microbial food web and therefore viruses play a key role in shunting carbon and other nutrients otherwise destined for higher trophic levels or the ocean floor, into the microbial food web. Bloom trimming or termination by lytic viruses has been well discussed (Bratbak et al. 1993, Bratbak et al. 1996). In fact, viruses are often a
more substantial driver for microbial mortality (both phytoplankton and bacteria) than grazers (Winget and Wommack 2009, Vargo 2009). In addition to the high rate of DOM production via viral-induced lysis, a substantial component of the ocean's particulate organic matter (POM) is produced through lysis. Release of cellular components through viral lysis may stimulate population growth of other heterotrophic protists and microbes, especially in nutrient limited environments (Gobler et al. 1997).

_Virus Mediated Interactions_

Suttle (2007) suggests that viruses are the largest reservoir of genetic diversity in the ocean. In addition to virus-mediate nutrient cycling, viruses are also involved in horizontal gene transfer (HGT) (Jiang and Paul 1998, Chiura 2002), which some argue is their most important ecological role (Weynberg et al. 2011). Host-derived, viral sequences have frequently been identified through global ocean sampling (GOS), indicating that gene transduction is a ubiquitous mechanism across the world's oceans. Such virus-host horizontal gene transfer can theoretically lead to an increase in genetic diversity and ongoing evolution of microbial lineages in the ocean. For example, the genomes of two common cyanophages are known to contain genetic elements that encode for components of the photosynthetic machinery of their hosts, _Prochlorococcus_ and _Synechococcus_ (Williamson et al. 2008). Additionally, there is emerging evidence of horizontal gene transfer between algal viruses and their eukaryotic hosts. For example, an entire metabolic pathway of the coccolithophore _E. huxleyi_ was found in EhV, a large DNA virus specific to _E. huxleyi_ (Monier et al. 2009). Because the gene sequence is more similar to the _E._
huxleyi host than other eukaryotic algae, it is likely the transfer occurred after the divergence of the E. huxleyi lineage (Monier et al. 2009).

Viruses can also modulate the metabolism of their hosts. Emiliania huxleyi and its associated virus (EHV-86) have been extensively studied (Llewellyn et al. 2007, Evans et al. 2009, Wilson et al. 2005). During infection and subsequent culture decline, E. huxleyi exhibits a reduction and shift in proportion of polyunsaturated and monounsaturated fatty acids plus a change in carotenoid and chlorophyll composition. The reduction in these metabolites was speculated to be due to a shift from normal cell processes to upregulation of the production of cellular components for viral replication. On a population level, reduction in fatty acid synthesis caused by viral infection decreases the amount of some essential nutrients utilized by higher trophic levels. Thus, viral infection of E. huxleyi, could cause a decrease in productivity of marine ecosystems (Evans et al. 2009). Net effect of differential metabolic activities on a population or ecosystem level is not yet fully known. However it is speculated that viral-driven metabolic effects could alter growth efficiency, respiration, photosynthesis and life cycle of their hosts.

Cell Death

In marine systems, viral infection can ultimately lead to cell death either directly through viral infection and cell rupture or by the activation of intrinsic cellular pathways that lead to a programmed cell death during the infection process. With the exception of immediate and drastic cell injury, death is most often the result of genetically controlled cell destruction (Edinger and Thompson 2004). These controlled pathways include well-described, cell-death programs such as
apoptosis and autophagy plus the lesser-known programs such as caspase-independent cell death, non-lysosomal cell death, paraptosis and aponecrosis. All cell death programs, including necrosis, are categorized by morphological and biochemical features of the cell exhibited after exposure to a death-inducing stimulus.

Necrotic cells caused by catastrophic stress, usually acute abiotic stress (Berges and Falkowski 1998, Goldstein and Kroemer 2007), exhibit organelle swelling, cytoplasmic disintegration, unorganized DNA fragmentation, and increased plasma membrane permeability. No specific enzyme activity is required for progression and ultimately leads to irreversible, complete cell lysis (Table 1-1). This type of cell death is different from the recently described "programmed necrosis" that is induced by ligands that bind to specific plasma membrane receptors and can therefore be regulated (Galluzzi and Kroemer 2008).

The hallmarks of active forms of death, known as programmed cell death (PCD), can be grouped as following: (1) DNA degradation, as detected by either in situ labeling or laddering of nucleic acid during electrophoresis; (2) alterations of the plasma membrane, including the externalization of phosphatidylserine residues and increased membrane permeability during late stages of PCD; (3) caspase activation, inhibition, or the presence of a caspase gene; (4) organelle degradation; and (5) nuclear and cytoplasmic blebbing. There are multiple methods to identify each of these markers, and not all of them occur in every type of PCD. For this reason, no individual hallmark is accepted as definitive proof of PCD, and there is no standard group of hallmarks used to confirm the occurrence of PCD. However, the presence of as little as one hallmark and as many seven hallmarks have been
used to confirm PCD in different phytoplankton studies thus far (reviewed in Chapter 2). Therefore, when attempting to detect PCD in an unknown system, it is most informative to assay for a number of PCD hallmarks that are as distinct as possible.

The most well-described form of programmed cell death (PCD) is apoptosis, which is characterized as cell death preceded by cytoplasmic condensation, cytoskeletal degradation, nuclear blebbing – the formation of extensions that eventually bud off – and condensation, DNA fragmentation and maintenance of intact organelles, and plasma membrane integrity – all orchestrated by a caspase-mediated cascade (Table 1-1). The final step of apoptosis is the formation of apoptotic bodies – discrete fragmentation of cellular components. Apoptotic bodies have yet to be reported in single celled organisms and thus apoptosis is a term strictly reserved for multicellular organisms. Another form of PCD, paraptosis, shares morphological features with apoptosis but has other significant benchmarks including cytoplasmic vacuolization that is not present during apoptosis and cells do not exhibit DNA fragmentation nor are caspase enzymes involved (Table 1-1).

Recently, apoptosis-like cell death pathways have been described in unicellular organisms (see Nedelcu et al. 2011 for a review) and marine phytoplankton (see Bidle 2015 for a review). In addition to apoptosis-like death, other forms of cell death such as autophagic cell death and programmed necrosis have also been described in unicells (Deponte 2008). Forms of PCD have now been documented in yeast, bacteria and protists (Berges and Falkowski 1998, Chaloupka and Vinter 1996, Engelberg-Kulka et al. 2006, Gordeeva et al. 2003, Martin 2008)
and it has been speculated that the origin of a self-destruction pathway may be as ancient as the origin of the first cell (Ameisen 2002).

*Biochemistry of the Classic Apoptosis Pathway*

The apoptotic pathway is tightly choreographed and has critical control points (Danial and Korsmeyer 2004) that manifest in biochemical and morphological hallmarks. Apoptosis is initiated by extrinsic (cytoplasmic) or intrinsic (mitochondrial) pathways. During extrinsic activation in animals, Fas ligands bind to Fas receptors, found on the plasma membrane, to form the death-inducing signal complex (DISC) (Holler et al. 2003). After formation of the DISC, a cascade of biochemical events occur mediated primarily by caspases that are cysteine-requiring, aspartate-directed proteases. This pathway in metazoans begins with the activation of the initiator caspase (Caspase-8 and -9) (Chowdhury et al. 2006). Initiator Caspases are responsible for activation and/or amplification of the effector caspases, namely Caspase-3, -6, -7. Caspase-3 cleaves Bid, a member of the anti-apoptotic protein Bcl-2 family that is responsible for reduction in the mitochondrial transmembrane potential (MTP). Due to reduced MTP, cytochrome-c leaks out of the mitochondria (Desagher and Martinou 2000). In the intrinsic pathway, PCD is initiated by cytochrome-c leakage from the mitochondria, thus the extrinsic and intrinsic PCD pathways converge at this step. Cytochrome-c in the cytoplasm interacts with dATP, apoptotic protease activating facor-1 (Apaf-1) and pro-caspase-9 to form a complex known as the apoptosome (Zou et al. 1999). Within the apoptosome, the initiator caspase-9 is activated thus completing the loop.
Effector caspases are responsible for degradation of structural and regulatory constituents of the cell (Thornberry and Lazebnik 1998). During PCD, the cytoskeleton is degraded resulting in cell shrinkage and blebbing. Externalization of phosphatidylserine residue, a hallmark of PCD, is caused by caspase-mediated deactivation of translocase, an enzyme that binds phosphatidylserine to the interior of the plasma membrane, as well as activation of flipases, enzymes that transfer the negatively charged phospholipids from the inner to outer leaflet of the plasma membrane (Brantton et al. 1997). Effector caspases also degrade nuclear components of the cell. Poly ADP ribose polymerase (PARP), a component of the DNA repair mechanism, is cleaved during PCD (Yu et al. 2002). Internucleosomal DNA fragmentation occurs, often leaving DNA fragments with predictable sizes that appear as a ladder on agarose gels. The nuclear material may also undergo ultrastructural and biochemical changes including irreversible condensation and margination of the heterochromatin to the inner periphery of the nuclear envelope. In some cases, there is fragmentation of the nucleus itself, resulting in complete degradation of the nucleus and causing cell death (Liu et al. 1998).

In the last ~15 years, programmed cell death (or forms of PCD such as parapoptosis, PCD-like death and apoptosis-like death) has been documented in a wide range of phytoplankton groups (Bidle 2015) including the chlorophytes, (Segovia et al. 2003, Moharikar et al. 2006, Zuppini et al. 2007, Affenzeller et al. 2008), coccolithophores (Bidle et al. 2007, Schatz et al. 2014), cyanobacteria (Ross et al. 2006, Berman-Frank et al. 2004), diatoms (Bidle and Bender 2008), dinoflagellates (Zhang et al. 2006) and raphidophytes (Dingman and Lawrence
2012). In these cases, PCD was triggered by a variety of physiological stressors including heat stress (Nedelcu 2006, Dingman and Lawrence 2012), light stress (Berges and Falkowski 1998, Segovia et al. 2003, Moharikar et al. 2006) nutrient deprivation (Berges and Falkowski 1998, Berman-Frank et al. 2004, Segovia and Berges 2005), oxidative stress (Vardi et al. 1999) and viral infection (Bidle et al. 2007, Vardi et al. 2009).

Heterosigma akashiwo

*Heterosigma akashiwo* is a harmful, bloom-forming raphidophyte that inhabits temperate coastal waters globally (Honjo, 1993). The species is of economic and ecological interest because it is responsible for fish, oyster and urchin mortality due to dense blooms known as “red tides” resulting in millions of dollars of lost revenue to global fisheries (Nagasaki et al. 1999). The impacts of these blooms on non-economically important species are not well documented, but likely substantial. Accordingly, understanding the life cycle and physiology and predicting the onset, duration and severity of *H. akashiwo* blooms is of great interest.

Members of class *Raphidophyceae* are either nuisance or harmful algae containing three marine genera: *Heterosigma* (Imai 1989, Imai and Iakura 1991, 1999), *Chattonella* (Imai and Itoh 1987, Imai 1989), and *Fibrocapsa* (Yoshimatsu 1987). All three have two life history phases: vegetative cells and resting cells (or cysts). Cells in the vegetative phase are free swimming and metabolically active and are often visible in the water columns as red or brown blooms. Conversely, resting-phase cells are metabolically static and non-motile, thus sink and
accumulate in the sediment. This phase provides a selective advantage for these genera because resting cells overwinter and can survive nutrient and light stress. Additionally, the resting cell life phase represents a “seed” population for future vegetative populations and secondary transport (through natural ocean currents or vessel ballast transport) can spread seed populations vast distances (Connell 2000).

Unlike the cysts in the other two genera, *Heterosigma* is unique in that resting cells do not require a prolonged mandatory dormancy period before germination into vegetative cells (Han et al. 2002, Itakura et al. 1996), allowing them to quickly switch life phases as nutrient, temperature and light availability change. However, no single environmental cue promotes resting-cell formation and germination. Light and temperature cues must be concomitantly “perceived” (Jacobs et al. 1999) by *Heterosigma* to induce resting-cell formation (Han et al. 2002) and germination (Shikata et al. 2007). Temperature is tightly correlated with proportion and abundance of vegetative and resting cells (Imai and Itakura 1991, Taylor and Haigh 1993). In the laboratory, temperatures below 10°C and darkness trigger resting-cell formation while resting-cell activation is triggered by 20°C and light (Han et al. 2002). Similarly, in Hiroshima Bay, Japan, a site with extensive summer *Heterosigma* blooms, oscillates between 10.5 and 24.6°C (Imai and Itakura 1991). During resting-cell formation, cells initially stop dividing but maintain their ability to swim. Subsequently, their mobility is reduced until they become completely immobile. Resting cells retain their two flagella, become more rounded in shape, do not develop a cell wall or protective scales; however, they are protected by a polysaccharide matrix (Han et al. 2002). Another important environmental cue that triggers resting-cell formation is nitrate starvation (Han et
al. 2002). While *Heterosigma* can actively uptake nitrogen and phosphorus from deep within the water column at night (Watanabe et al. 1983), the strategy to form resting cells when nutrient condition are lean, provides an additional long-term survival advantage.

*Heterosigma akashiwo Viruses*

This thesis includes work on three *Heterosigma* viruses - HaV, HaRNAV, and HaNIV. HaV (*Heterosigma akashiwo virus*) is a member of the *Phycodnaviridae* because it has a large dsDNA genome (294 kb) contained within an icosahedral capsid (Nagasaki et al. 1999). Although the mechanism for attachment is unknown, HaV is known to replicate within the cytoplasm with a latent period of 30 to 33 h and a burst size of approximately 770 progeny per host cell. The first virus isolate was taken from Hiroshima Bay, Japan (Nagasaki and Yamaguchi 1997), and since then many more strain-isolates have been cultured in the laboratory having various degrees of infectivity on cultured strains of *H. akashiwo* (Tarutani et al. 2000, Tomaru et al. 2004). HaRNAV (*Heterosigma akashiwo RNA virus*) is a member of *Picornaviridae* because it is a tiny, ssRNA-containing virus (25 nm, 9.1 kb, respectively) (Tai et al. 2000). Like HaV, HaRNAV replicates in the cytoplasm but has a much greater burst size (2.1x10⁴ virus like particles (VLP) per cell) and a longer lytic length (up to 8 days) (Tai et al. 2000). HaNIV (*Heterosigma akashiwo nuclear inclusion virus*) has not yet been assigned a genera because its genetic characteristics are yet to be described, however, given its particle size (10 to 20 nm), it is likely not a member of *Phycodnaviridae*. HaNIV progeny form a crystalline array of virus inclusions in the
nucleus (Lawrence et al. 2001). HaNIV has a lytic length of approximately 60 h and produces up to $3 \times 10^5$ VLP/cell (Lawrence et al. 2001).

HaRNAV and HaNIV were isolated off the pacific coast of Canada by testing infectivity with three North American, Pacific-coast strains of *H. akashiwo*, (NEPCC 102, 522, and 764) (Tai et al. 2003, Lawrence et al. 2001). Neither of these viruses can effectively infect Atlantic-strains of *H. akashiwo* and only HaNIV was shown to also infect Japanese Pacific-coast strains. Unlike the other viruses, HaV was isolated in Japanese waters but not all HaV clones infect all Japanese Pacific-coast *H. akashiwo* strains with equal virulence (Nagasaki et al. 1999, Tarutani et al. 2000). No studies have examined the infectivity of HaV with North American, Pacific-coast or Atlantic strains. Although the outcome of infection, cell lysis, is well documented in *Heterosigma*, the finite mechanisms that trigger death, or what type of cell death occurs, is not well understood.

**Goals**

The following chapters address the questions; 1) Is PCD an outcome of stress response in *Heterosigma*, and if so, what hallmarks are exhibited prior to cell death? 2) Does viral infection trigger PCD prior to lysis? Do different viruses elicit different morphological and biochemical death-related hallmarks? 3) What is the role of virus infection in *Heterosigma* population cycling?

In Chapter 2, I investigated *Heterosigma akashiwo*’s ability to undergo programmed cell death (PCD) after acute stress. In Chapter 3, I examined the effect of three, unrelated viral-infections on the induction of different PCD-hallmarks during infection in *Heterosigma*. In Chapter 4, I provided evidence for the
population-level effects of the HaV-induced cycling of *Heterosigma* and explored an alternate response – resting-cell formation – that is likely a viral-evasion strategy. Overall, I argue for the significance of PCD, viral-infection and resting cell formation in the cycling and long-term survival of *Heterosigma* populations.
Chapter References


Megaviridae, elucidates NCLDV genome expansion and host-virus coevolution.

Virology. 466-467: 60-70.

Nagasaki, K., Tarutani, K., and Yamaguchi, M. 1999. Growth characteristics of 


Characterization of Viruses within Aquatic Microbial Samples. PLoS ONE 3(1): e1456. doi:10.1371/journal.pone.0001456


Table 1-1. Characteristics of the most well defined types of cell death. The classification of programmed cell death (PCD) pathways ranges from the most genetic involvement (apoptosis) to the least (paraptosis), followed by catastrophic necrosis, which has no known-genetic programming. Most of the features listed here are common to plants, animals and single celled organisms.

<table>
<thead>
<tr>
<th>Cytosolic/Organelle</th>
<th>Apoptosis</th>
<th>Autophagy</th>
<th>Caspase-independent cell death</th>
<th>Non-lysosomal cell death</th>
<th>Paraptosis</th>
<th>Necrosis</th>
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<tbody>
<tr>
<td></td>
<td>· Intact organelles(^{1,7})</td>
<td>· Intact organelles(^{1})</td>
<td>· Intact organelles(^{1})</td>
<td>· Formation of empty spaces in cytoplasm(^{4})</td>
<td>· ER swelling</td>
<td>· Organelles swell &amp; lyse(^{1,2,7})</td>
</tr>
<tr>
<td></td>
<td>· Intact mitochondria(^{1})</td>
<td>· Degradation of cellular components by lysosomes(^{3,4})</td>
<td>· Mitochondria outer membrane premeabilization(^{5})</td>
<td>· Mitochondrial swelling (late)(^{2})</td>
<td>· Mitochondrial swelling</td>
<td>· Cytoplasmic vacuolation(^{2})</td>
</tr>
<tr>
<td></td>
<td>· Mitochondrial swelling (sometimes)(^{2})</td>
<td>· Vacuole formation(^{7})</td>
<td>· Autophagosome formation(^{3,7}) or rupture of lysosome(^{3})</td>
<td>· Organelle swelling(^{4})</td>
<td>· Cytoplasmic vacuolization(^{2,8})</td>
<td>· Ribosomes disassociate from ER</td>
</tr>
<tr>
<td></td>
<td>· Disintegration of cytoskeletal elements(^{4})</td>
<td>· Autophagosome formation(^{3,7})</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>· Condensation of cytoplasm(^{1,4})</td>
<td></td>
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<tr>
<td></td>
<td>· No cytoplasmic vacuolation(^{2})</td>
<td></td>
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<tr>
<td></td>
<td>· Apoptotic bodies(^{2}) (metazoans only)</td>
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Table 1-1. Continued.

<table>
<thead>
<tr>
<th>Nuclear</th>
<th>Plasma Membrane</th>
<th>Enzymes</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>Nuclear blebbing &amp; fragmentation(^{(2,3,7)})</td>
<td>Maintenance of PM integrity(^{(1)})</td>
<td>Caspases(^{(1,3,7)})</td>
<td>z-VAD-FMK(^{(2,6)})</td>
</tr>
<tr>
<td>Nuclear condensation</td>
<td>Maintenance of PM integrity(^{(1)})</td>
<td>DEVD-cleaving activity(^{(2)})</td>
<td>BAF(^{(2)})</td>
</tr>
<tr>
<td>Chromatin condensation(^{(1)})</td>
<td>Maintenance of PM integrity(^{(1)})</td>
<td>Caspases(^{(3)})</td>
<td>Xiap(^{(2,6)})</td>
</tr>
<tr>
<td>DNA fragmentation(^{(1)})</td>
<td>Externalized PS residues(^{(1)})</td>
<td>BcL-2/Xi(^{(7)})</td>
<td>Bcl(^{(2)})</td>
</tr>
<tr>
<td>Internucleosomal cleavage of DNA (DNA laddering)(^{(2,3)})</td>
<td>Lipid reassembly(^{(1)})</td>
<td>Beclin 1(^{(1,7)})</td>
<td>Actinomycin D (sometimes)(^{(2)})</td>
</tr>
<tr>
<td>Nuclear condensation</td>
<td>Maintenance of PM integrity(^{(1)})</td>
<td>Caplains(^{(1)})</td>
<td>VPS(^{(8)})</td>
</tr>
<tr>
<td>Chromatin condensation(^{(7)})</td>
<td>Externalized PS residues(^{(1)})</td>
<td>Bcl-2 family (Bax and Bak)(^{(5)})</td>
<td>Unknown</td>
</tr>
<tr>
<td>DNA fragmentation(^{(1)})</td>
<td>No DNA fragmentation(^{(5)})</td>
<td>Ras(^{(6)})</td>
<td>Unknown</td>
</tr>
<tr>
<td>Chromatin degradation (5bp)(^{(5)})</td>
<td>Membrane destabilization(^{(3)})</td>
<td>Unknown</td>
<td>No response to caspase-inhibitors(^{(2,6)})</td>
</tr>
<tr>
<td>DNA fragmentation(^{(1)})</td>
<td>Unknown</td>
<td>MEK-2(^{(6)})</td>
<td>None(^{(1)})</td>
</tr>
<tr>
<td>No DNA fragmentation(^{(5)})</td>
<td></td>
<td>JNK-1(^{(6)})</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td>No enzyme activity(^{(1)})</td>
<td></td>
</tr>
<tr>
<td>PARP Cleavage (50-60 KDa fragments(^{(2)}))</td>
<td></td>
<td>Calpains and cathepsins(^{(7)})</td>
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<tr>
<td>DNA degradation (late)</td>
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<tr>
<td>No chromatin condensation(^{(2,4)})</td>
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<tr>
<td>Increased membrane permeability(^{(1,7)})</td>
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<td>Lysis (late)(^{(1)})</td>
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Table 1-1. Continued.

<table>
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<th>Abbreviations</th>
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<tbody>
<tr>
<td>A1P-1/Alix - atrophin-1-interacting protein</td>
</tr>
<tr>
<td>BAF - Bafilomycin A1</td>
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<tr>
<td>Bak - Benzalkonium chloride</td>
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<tr>
<td>Bax - Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2 - B-cell lymphoma-2</td>
</tr>
<tr>
<td>Bcl-xl - B-cell lymphoma-extra large</td>
</tr>
<tr>
<td>DEVD – Asp-Glu-Val-Asp</td>
</tr>
<tr>
<td>JNK-1 – Jun N-terminal kinase-1</td>
</tr>
<tr>
<td>MEK-2 - Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PARP - poly-ADP-ribose polymerase</td>
</tr>
<tr>
<td>PM – Plasma membrane</td>
</tr>
<tr>
<td>PS – phosphatidylinerine</td>
</tr>
<tr>
<td>XIAP - x-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>z-VAD-FMK - Benzylxoycarbonyl-Val-Ala-Asp-fluoromethylketon</td>
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</tbody>
</table>

Figure 1-1. A tree of eukaryotes (Keeling et al. 2005). The tree is a hypothesis build from various types of data (i.e. molecular phylogenies and characters, morphological and biochemical evidence). Of the five ‘supergroups’ shown, four contain algae: Plantae, Chromalveolates, Excavata and Rhizaria. *Heterosigma akashiwo* (Raphidophyceae), the organism explored in this thesis is contained in the Chromalveolates. (Permission to reproduce figure confirmed through RightsLink® Copyright Clearance Centre).
Figure 1-2. The virosphere (Abrescia et al. 2012). The International Committee of Taxonomy of Viruses (ICTV) scheme (adapted from the Universal Virus Database 2005) is shown with the currently defined orders colored, and the virus families within the order defined by letters, colored to correspond to the Virus Order. The key defines the coloring for each order. Those families that in 2005 were not assigned to an order are drawn in black, among them the *Phycodnaviridae*. (Permission to reproduce figure confirmed through RightsLink® Copyright Clearance Centre).
Chapter 2 - HEAT-STRESS-INDUCED PROGRAMMED CELL DEATH IN Heterosigma akashiwo (Raphidophyceae)

Abstract

Programmed cell death (PCD) was previously thought to only occur in metazoans. Recent studies have revealed PCD in yeast, prokaryotes, and some species of phytoplankton. However, the need for PCD assays with reliable controls currently limits the study of PCD in natural environments. We examined heat-stressed cultures of Heterosigma akashiwo (Y. Hada) Y. Hada ex Y. Hara & M. Chihara for DNA fragmentation using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, externalization of phosphatidylserine residues using Annexin-V and caspase-like activity using CaspACE and Caspase-Glo 8 assays, all of which are all diagnostic of cells undergoing PCD but not necrosis. Additionally, we treated cultures with the broad-spectrum caspase inhibitor z-VAD-FMK to rescue cells otherwise destined for death. Our results demonstrate the range of inducible responses of H. akashiwo to heat-stress: recovery from 35°C heat-stress, PCD-induction due to 37°C and 40°C heat-stress, and necrotic death or cyst formation due to 50°C heat-stress. The identification of a reliable inducer and markers for PCD provides key tools for further studies into the physiology and ecology of this harmful bloom-former.
Introduction

Roughly half of the planet’s primary production is attributed to oceanic phytoplankton (Field et al. 1998). While phytoplankton blooms drive nutrient cycling in the ocean, toxic blooms cause millions of dollars of lost revenue to international fisheries (Nagasaki et al. 1999). Despite their importance, the general physiology and ecology of marine phytoplankton are poorly understood. Over the past century it was thought that, because of ongoing cell division, single-celled phytoplankton do not die unless affected by external factors such as predator grazing or sedimentation. More recently, however, internally mediated cell mortality has been described with increasing detail by Brussaard et al. (1995) who address phytoplankton cell lysis following large blooms, Berman-Frank et al. (2004) who discuss cyanobacteria undergoing autocatalytic cell death due to nutrient deprivation, and Bidle et al. (2007) who link viral infection to enhanced expression of metacaspases and activation of caspase activity leading to programmed cell death. These and other works give evidence to the multifaceted life strategies and death processes involved in the cycling of phytoplankton.

In multicellular organisms cell death can occur via necrosis or programmed cell death (PCD). Necrosis is unorganized degradation resulting in death, and cells undergoing necrosis characteristically show loss of membrane integrity, swelling and lysis as a response to injury or infection. After lysis, cellular contents leak into the surrounding environment. In contrast, PCD describes a range of active, choreographed processes that result in the organized disassembly of the cell. The most well-known form of PCD is apoptosis, however, this term is strictly reserved
for multicellular organisms because the morphological end-point has not yet been seen in unicellular: apoptotic bodies. Recently apoptosis-like cell death pathways have been described in unicellular organisms (see Nedelcu et al. 2011 for a review) and marine phytoplankton (see Bidle 2015 for a review). In addition to apoptosis-like death, other forms of cell death such as autophagic cell death, paraptosis, Ferroptosis and programmed necrosis have also been described in unicells (Deponte 2008) and unicellular marine phytoplankton (Bidle 2015). The processes of PCD are internally mediated by the production and activation of enzymes generally known as caspases (enzymes in the cysteine protease family), with paracaspases specifically found in slime moulds and metazoans, and metacaspases found in plants, fungi and protists (Uren et al. 2000) and diverse types of bacteria (Bidle and Falkowski 2004).

Although PCD was originally thought to occur only in multicellular organisms, it has now been documented in yeast, bacteria and protists that span multiple kingdoms (Berges and Falkowski 1998, Chaloupka and Vinter 1996, Engelberg-Kulka et al. 2006, Gordeeva et al. 2003, Martin 2008) and it has been speculated that the origin of a self-destruction pathway may be as ancient as the origin of the first cell (Ameisen 2002). In the last 15 years, programmed cell death (or forms of PCD such as parapoptosis, PCD-like death and apoptosis-like death) has been documented in a wide range of phytoplankton groups under various physiological stressors including heat, light, salt and oxidative stress, nutrient limitation, viral infection and culture senescence (Table 2-1).
The single-celled Raphidophyte *Heterosigma akashiwo* is a harmful bloom-forming phytoplankton that is found in temperate coastal water around the globe (Honjo 1993). Some phytoplankton species, including *H. akashiwo*, form cysts which play important roles in seed-reservoirs for overwintering and future bloom formation (Imai and Itakura 1999). Dense blooms of *H. akashiwo* often result in large-scale fish kills of cultured and wild fish populations resulting in millions of dollars of lost revenue to global fisheries (Nagasaki et al. 1999). Therefore, understanding and predicting the onset, duration, severity and termination of such blooms are of great interest. The first step toward understanding the dynamics of a population is to understand the growth and death of the individuals that comprise the population. While numerous studies have investigated cyst germination, growth characteristics and bloom termination (often associated with viral infection (Nagasaki et al. 1994a,b)), little is known about process of encystment or death in the species.

Heat stress is a common inducer of PCD in other organisms (Nedelcu 2006, Zuppini et al. 2007). In our study, we used heat stress to induce PCD in *H. akashiwo*, which enabled us to develop methods for detecting a range of PCD hallmarks in single-celled algae, and determine the hallmarks present during PCD in this species. Although the temperatures used in this study exceed those found in the natural environment, the goal was to identify a reproducible, positive control for future PCD studies, and to develop methods that can be applied to other ecologically relevant stressors that may cause PCD, such as viral infection, light limitation/over exposure or nutrient deprivation. The hallmarks we examined for
were DNA fragmentation and laddering, externalization of phosphatidylserine (PS) residues to the external leaflet of the plasma membrane, caspase-like activity, and caspase inhibition leading to cell survival. These markers, which identify genetically encoded or “programmed” cell death, were used to evaluate death as either PCD or not without specifically determining the type of PCD.

Methods

Culturing Heterosigma akashiwo

*Heterosigma akashiwo* (NEPCC strain 522, nonaxenic) was grown in f/2 seawater media (30 psu) enriched with 10nM sodium selenite (Guillard 1975) at 20°C under a 14:10 h light-dark cycle. Cultures were grown in 50 mL borosilicate tubes and were monitored for growth by *in vivo* chlorophyll a fluorescence (Turner Designs TD-700 fluorometer, Sunnyvale, CA, USA). Cell morphology and autofluorescence were also monitored with bright field and epifluorescence microscopy using a Leica DM2500 microscope (Richmond Hill, ON, Canada).

Heat Stress

An exponential-phase batch culture was split into sub-cultures that were heat stressed, in triplicate, for 1 h at 35, 37, 40 or 50°C. Triplicate cultures maintained at 20°C served as controls. Cultures were monitored for growth by *in vivo* fluorescence as described above, and examined for the presence of PCD hallmarks at 0, 24, 48 and 72 h post-heat stress. DNA damage, externalized phosphatidylserine residues, quantitative caspase-8-like activity and *in situ* pan-
caspase-like activity were examined at 0, 24, 48 and 72 h post-heat stress using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) (Fisher), Annexin-V FITC (Invitrogen, Carlsbad, CA, USA), Caspase-Glo 8 (Fisher Canada, Nepean, ON, Canada), and CaspACE (Fisher), respectively.

*Phosphatidylserine Residue Detection and Cell Abundance Determination*

For each time-point a 200 μL- aliquot of each culture was incubated with 10 μL of Annexin-V FITC (Invitrogen), which is an impermeable fluorescent phosphatidylserine marker, in the dark at 20°C for 20 min. The cells were then immobilized with 2 μL of 1.0% formaldehyde and immediately loaded into a 0.099 mL nannoplankton counting chamber (PhycoTech Inc., St. Joseph, MI, USA) for examination under epifluorescence microscopy. At this concentration, the formaldehyde treatment was confirmed to have no effect on the proportion of cells with permeable membranes or externalized phosphatidylserine residues using SYTOX Green and Annexin-V, respectively. Total cell abundance was determined by enumerating autofluorescent cells under green excitation (Ex. 515nm-560nm, Em. 590nm+) with a minimum of 200 cells being scored for +/- Annexin-V FITC staining by switching to blue excitation (Ex. 450nm-490nm, Em. 515nm+). During cell death increased plasma membrane permeability can lead to false-positive Annexin-V staining. To correct for this plasma membrane permeability was assessed by staining a 200 μL-aliquot of cells from each culture with SYTOX Green, final concentration 0.5 μM (methods adapted from Lawrence et al. 2006).
Cells that were positively stained with SYTOX Green, as viewed with blue excitation, were subtracted from the proportion of Annexin-V stained cells.

**In situ TUNEL Assay**

A 200 μL-aliquot of cells from each culture was permeabilized with 50 μL of ice-cold 70% ethanol and incubated for 5 min, then placed on a poly-L-lysine-coated slide (Sigma-Aldrich Co., St Louis, MO, USA) for an additional 5 min. Affixed cells were rinsed with 1x PBS. A 100 μL-aliquot of equilibrium buffer (supplied with the Promega Dead End fluorometric TUNEL system, Fisher) was added and incubated at room temperature for 10 min. Slides were blotted before 50 μL of rTdT incubation buffer (prepared as specified by Fisher) and plastic cover slips were added. Slides were incubated at 37°C in a humidifying chamber and protected from light for 60 min. Slides were then soaked in 2x SSC for 15 min. Slides were washed with 1x PBS and cells examined immediately using epifluorescence microscopy with green excitation. A minimum of 200 cells were scored to determine the proportion of TUNEL-stained cells by switching to blue excitation to detect TUNEL staining.

**DNA Laddering**

Cells were lysed with 0.5% SDS and 20 μg·mL⁻¹ proteinase K. DNA was phenol-chloroform extracted, treated with 25 μg·mL⁻¹ RNase H (New England BioLabs, Pickering, ON, Canada) and separated on a 1.8% agarose gel (Nedelcu 2006) visualized by SYBR gold.
In situ CaspACE VAD-FMK-FITC Assay

For each time-point, a 200 µL- aliquot of cells from each culture was permeabilized by adding 50 µL of ice-cold 70% ethanol and incubating for 5 min. The aliquot was then placed on a 0.01% poly-L-lysine-coated slide (Sigma-Aldrich) covered with a plastic cover slip and incubated for an additional 5 min. The cover slip was removed and cells attached to the slide were rinsed twice with 1x PBS. A 100 µL- aliquot of PBS and 10µL of CaspACE VAD-FMK-FITC (Fisher, final concentration 10 µM) were added to the slides and another cover slip placed on top. Slides were incubated in the dark for 20 min, the cover slip was removed, and slides rinsed twice with 1x PBS. Slides were viewed immediately under epifluorescence microscopy using blue and green excitation as described above, with +/- CaspACE-staining identified under the blue excitation.

Caspase-Glo®8 Assay

Whole-culture caspase-8-like activity was measured for each culture at each time point. For each sample, a 100 µL- aliquot of cell culture and 100 µL of Caspase-Glo 8 reagent (Fisher; contains a cell-lysing agent, caspase-8-specific substrate and luciferase) were added to a 96-well plate and incubated at 20°C in the dark for 1 h. Luminescence was quantified using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). Cell lysis due to addition of Caspase-Glo 8 reagent was confirmed by examination under epifluorescence microscopy.
**Caspase Inhibition Assay**

The pan-caspase inhibitor z-VAD-FMK (Fisher) was added at a final concentration of 20 μM to triplicate, exponentially growing cultures and incubated at room temperature for 20 min before heat stress. A DMSO-only treatment (0.1%) was used to account for the final concentration of DMSO in the inhibitor treatment, in addition to a no-reagent control. Cultures were heat stressed for 1h and then placed in a 96-well plate and returned to initial culture conditions. Cultures were sampled at 0, 24 and 48 h post-heat stress, fixed with formaldehyde, and cell abundance was determined using epifluorescence microscopy as described above.

**Photography and Image Processing**

Cells were photographed under bright field or epifluorescence using a Leica DMRXAZ microscope with a DC500 camera. Images were processed using Thumbs plus 4.0. Filter cubes N2.1 and GFP (excitation 470/40nm, suppression 525/50nm) were used for viewing red autofluorescence and green fluorescence staining, respectively.

**Data Analysis**

All statistical analyses were performed using R version 2.11.1 (R Development Core Team). We assessed the assumptions of homogeneity of variance and normal distribution, using Cochran’s and Shapiro-Wilk’s tests. We log transformed Annexin-V data, and square-root transformed abundance, TUNEL and CaspACE data. For the hallmarks of PCD study, we conducted a repeated measures analysis of variance with cell abundance, proportion of cells stained (with TUNEL,
Annexin-V or CaspACE), and relative caspase activity (Caspase-Glo 8) as the responses with time, temperature and their interaction as predictors. Preliminary analyses revealed that less than 1% of variation in the data was explained by resampling tubes. Therefore, we did not explicitly account for the repeated measures. Post-hoc contrasts were performed using Tukey’s HSD. For the inhibitor study, we used analysis of variance to assess the affect of inhibitor and DMSO on cell abundance. We used data from cultures 72 h after no-heat stress (control), 35 and 37°C heat stress, as our previous results suggested this was the time when cells were most likely to undergo PCD. Post-hoc contrasts were performed using Tukey’s HSD.

**Results**

*Cell Abundance and Morphology*

There was no significant difference in cell abundance between control and 35°C heat-stressed cultures from 0-72 h (p>0.9618, inclusive) (Figure 2-1A). Cells in control and 35°C cultures maintained membrane integrity and were motile, oblong with a smooth surface (Figure 2-2A) and had strong red autofluorescence when excited by blue light (Figure 2-3). There was no significant difference between cell abundance for control and heat-stressed cultures at 35, 37, 40 and 50°C immediately following heat stress (t=0 h, p=1.0000, 1.0000, 0.1244 and 1.0000, respectively) (Figure 2-1A). Cells in these cultures were non-motile but exhibited strong autofluorescence, were oblong with a smooth surface immediately after heat-stress. In cultures heat-stressed at 37°C, cells maintained membrane
integrity at 0 h post-stress. However, cell abundance decreased by 16% by 24 h and decreased by another 50% by 48 h, at which point the abundance became significantly lower than controls (p<0.0001). Immediately after heat-stress, the majority of cells in cultures heat-stressed at 40 and 50°C were positively stained with SYTOX Green (data not shown) and therefore had lost membrane integrity. These cultures contained no motile, healthy cells by 24 h post-stress; rather, cells were non-motile, spherical with a rough cell surface (Figure 2-2B), and photo-bleached under green excitation. These observations are consistent with Itakura et al. (1996)’s description of *H. akashiwo* cysts. However, cells from cultures heat-stressed at 40°C were clumped at immediately after heat-stress and cysts were clumped at 24 h while cells heat-stressed at 50°C were not. Approximately 30% of 40°C and 85% of 50°C heat-stressed cells encysted and these cysts persisted throughout the rest of the experiment (Figure 2-1A). Cysts abundance in 40°C heat-stressed cultures increased slightly by 72 h, which may be an artifact of the reduction in clumping by 48 h in these cultures. Cysts persisted in 40 and 50°C heat-stressed cultures for weeks after stress (data not shown). There were no cysts in control, 35 or 37°C cultures.

**DNA Fragmentation**

We did not detect characteristic DNA laddering when genomic DNA of heat-stressed cultures was isolated and fractionated on an agarose gel (Figure 2-5). However, the absence of DNA laddering during PCD is not unusual because DNA smearing from necrotic cells can overshadow the laddering signal. Many other
organisms have been conclusively shown to undergo PCD without visible DNA laddering, including yeast (Madeo et al. 1999), the green alga *Chlorella saccharophila* (Zuppini et al. 2007), and the dinoflagellate *Peridinium gatunense* (Vardi et al. 1999).

In cultures heat-stressed at 35°C, 13% of cells were TUNEL-positive at 48 h post-heat stress, which was significantly higher than control cultures (p=0.0017) (Figure 2-1B). At 0, 24 and 72 h post-stress, the proportion of TUNEL-positive cells in control and 35°C heat-stressed cultures was not significantly different (p>0.6101, inclusive). In cultures heat-stressed at 37°C, 15% to 47% of cells stained positive for TUNEL throughout the experiment, which was significantly higher than control cultures (p<0.0016, inclusive). The highest proportion of TUNEL-positive cells occurred 24 h post-stress. In cultures heat-stressed at 40°C, 51% of cells were TUNEL-positive at 0 h post-heat stress, which was significantly higher than control cultures (p<0.0001). In cultures heat-stressed at 50°C, no cells were TUNEL-positive at any time point post-heat stress.

*Externalization of Phosphatidylserine Residues*

Control cultures and those heat-stressed at 35°C were not significantly different at any time with respect to Annexin-V staining (p>1.0000, inclusive) (Figure 2-1C). In cultures heat-stressed at 37°C, less than 2% of cells had permeable membranes at any point in time. However, at 0, 24 and 48 h post-heat stress, 4, 9 and 8.5% of viable cells stained positively with Annexin-V, respectively, which was significantly more than controls (p<0.0001, inclusive).
However, at 72 h post-heat stress, there was no significant difference between the number of positively stained cells in control and 37°C-stressed cultures (p=1.0000). At 0 h post-stress, 72 and 98% of cells from 40 and 50°C heat-stressed cultures had permeabilized membranes. Therefore, 6% of 40°C-heat-stressed cells stained positively with Annexin-V, which was significantly more than controls (p<0.0001, inclusive), while none of 50°C-heat-stressed cells stained positively with Annexin-V.

In situ Caspase-like Activity

In cultures heat-stressed at 35°C, 31, 12 and 7% of cells tested positive with the CaspACE assay 24, 48 and 72 h post-stress, respectively. This was significantly higher than controls at the same time-points (p<0.0001, inclusive) (Figure 2-1D). However, cultures heat-stressed at 35°C were not significantly different from controls at 0 h post-stress (p=1.0000). Caspase-like activity was present in 18, 46, 35 and 11% of cells heat-stressed at 37°C at 0, 24, 48 and 72 h post-stress (p<0.0002, inclusive). Heat stress at 40°C resulted in 70% of cells expressing active caspase-like enzymes 0 h post-stress, which was significantly higher than controls (p<0.0001). At 0 h post-stress, none of the cells in cultures heat-stressed at 50°C expressed active caspase-like enzymes and after 24 h only cysts remained in these cultures.

Whole Culture Caspase-8-like Activity

Caspase-8-like activity in cultures heat-stressed at 35°C and 37°C was not significantly different than in control cultures at any time-point (p>0.4118,
inclusive) (data not shown). Activity in cultures heat-stressed at 40°C was not significantly different from control cultures at 0 and 24 h post-heat stress (p>0.6422, inclusive). However, even though only cysts were present from 24 h post-heat stress onward, caspase-8-like activity increased and was significantly higher in 40°C heat-stressed cultures compared to control cultures at 48 and 72 h (p<0.0001), with values 3.8x and 2.2x greater than controls, respectively. Heat stress at 50°C did not result in caspase-8-like activity at any time-point.

**Caspase Inhibition**

Addition of the pan-caspase inhibitor z-VAD-FMK had no effect on cell abundance at 72 h in cultures kept at 20°C (control temperature) (p=0.9665) or heat-stressed at 35°C (p=0.3323), nor was there any difference in cell decline between inhibited or DMSO-only cultures heat-stressed at 40 or 50°C. Additionally, there was no difference between DMSO-only treated cultures compared to untreated cultures at the same temperatures (p=1.0000, inclusive) (Figure 2-4). However, the abundance of cells in cultures heat-stressed at 37°C was 1.5x greater in cultures treated with caspase inhibitor compared to non-inhibitor controls at 72 h post-stress (p=0.0239).

**Discussion**

The goal of this study was to use heat stress to induce PCD in *H. akashiwo*, thus permitting us to develop assays to detect PCD and determine the type and temporal sequence of hallmarks displayed during death. We examined a range of
heat-stress temperatures to determine the best inducer of PCD, thus identifying a positive control for future PCD studies.

Cultures heat-stressed at 37°C contained the highest proportion of cells exhibiting PCD hallmarks 24 h after stress that died by 48 h. Cultures heat-stressed at 40°C exhibited the same PCD hallmarks immediately after heat stress but died more quickly by 24 h. These results indicate the temperature range required for induction of PCD, below which (35°C) no effect is observed and above which (40°C) cells either undergo cyst formation or necrosis.

When cultures were heat-stressed at 35°C there was no evidence of cell stress by visual inspection, and the increase in cell abundance throughout the experiment was tantamount to that of the control (20°C) cultures. However, beginning at 24 h, 35°C heat-stressed cultures contained cells that tested positive for caspase-like activity, and there were also a small proportion of cells that exhibited DNA fragmentation visualized via TUNEL. It is possible that a small proportion of the initial population stressed at 35°C underwent PCD, but these cells represented a smaller and smaller proportion of the population as healthy cells continued to replicate. Thus, cell death was undetectable according to cell abundance in this treatment.

Cultures stressed at 37 and 40°C exhibited a number of biochemical markers of PCD immediately after heat-stress. These were: the externalization of PS residues, caspase-like activation, and DNA fragmentation. In the 37°C treatment, approximately half of the cells contained fragmented DNA and active caspase-like enzymes at 24 h. Over the next 24 h, cell abundance declined by 50%.
This indicates that DNA fragmentation and in situ caspase-like enzyme activity are good predictors of impending cell death. At 24 h only 9% of cells were positive for externalized PS residues even though 50% of them were destined to die, suggesting that this hallmark is short-lived, and therefore not a good hallmark for detection of PCD. At 24 h after heat-stress at 40°C, 33% of cells had encysted. Given that 24 h previously 6%, 70% and 51% of cells tested positive for external PS residues, active caspase-like enzymes, and DNA fragmentation, respectively, it is reasonable to conclude that up to 70% of cells heat-stressed at 40°C rapidly underwent PCD, while the remaining ~30% formed cysts. The presence of multiple PCD hallmarks in a significant proportion of cells and a decline in cell abundance relative to controls indicates that 37 - 40°C heat stress is a good inducer of PCD, and that the process of death observed in this treatment is most likely PCD.

When exposed to 50°C, a significant proportion (98%) of cells had compromised membrane integrity at 0 h, and exhibited none of the PCD hallmarks assayed for at this or other time-points. Between 0 and 24 h post-heat stress, there was only a 15% reduction in cell abundance due to necrosis while the rest of the population formed cysts. Therefore, increased membrane permeability followed by encystment is the major stress-response to 50°C heat stress. This is the first study to report on laboratory induction of cyst formation, however, the cellular physiology involved and natural causes of cyst formation in *H. akashiwo* are still unknown (Itakura et al. 1996). In natural environments the ability to encyst is a selective advantage that allows the species to overcome negative environmental conditions (lack of nutrients, light availability, unfavourable temperature) (Imai and Itakura...
Our findings suggest that some cells will undergo necrosis while others will form cysts.

**DNA Fragmentation**

DNA degradation is a key hallmark of PCD. During PCD, endonuclease activation leads to double stranded DNA breaks. Cells that contain damaged DNA can be identified by a number of *in situ* markers. We used the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and detected DNA fragmentation in cells from cultures heat-stressed at 35, 37 and 40°C. In cultures heat-stressed at 35°C, a small proportion of cells contained fragmented DNA and yet there was no significant decline in cell abundance. It is likely that a small proportion of dying cells was overshadowed by the survival and subsequent growth of the majority. In cultures heat-stressed at 37 and 40°C, the proportion of cells containing fragmented DNA was an accurate indicator of the magnitude of impending population decline. Cultures heat-stressed at 50°C did not contain cells with fragmented DNA, which indicated the cells from these cultures are not undergoing PCD. Rather, the decline in motile cells was caused by a combination of cyst formation and necrosis. Our study indicates the utility of the TUNEL assay and underlines the importance of assessing *in situ* DNA fragmentation when classifying cell death.

**Caspase-like Activity**

Caspases and related caspase-like enzymes are essential to the organized disassembly of a cell undergoing the PCD pathway. All metazoans share this highly
conserved group of enzymes whose primary functions are initiation and regulation of the PCD pathway. There are three families of proteins in the caspase superfamily: true caspases, paracaspases are found in slime moulds and metazoans, and metacaspases are found in plants, fungi and protists (Uren et al. 2000). Caspase-like activity has been reported during PCD in higher plants (Korthout et al. 2000, Lam and del Pozo 2000, Zuppini 2006), phytoplankton (see Table 2-1), and other unicellular organisms (Madeo et al. 1997 and 1999). In our study heat-stressed cultures of *H. akashiwo* contained a significantly higher proportion of cells expressing active caspase-like enzymes than controls, which indicates that caspase-like enzymes are active during PCD, but it is still possible these enzymes may have additional or alternative functions in *H. akashiwo* (see Chapter 4).

There are a number of PCD-initiator caspases in metazoans, including caspase-8, which represent the beginning of a cascade and lead to the ordered disassembly of the cell. Using the quantitative Caspase-Glo 8 assay, which was developed for the detection of the mammalian caspase-8, we detected an increase in caspase-8-like activity in 35, 37 and 40°C heat-stressed cultures of *H. akashiwo* but not in 50°C heat-stressed cultures. However, Caspase-8-like activity was the latest hallmark detected in cultures and neither preceded nor correlated with the intensity of other PCD markers. This suggests the activity does not represent PCD initiation in *H. akashiwo*. The increase in caspase-8-like activity between 24 and 72 h in 40°C heat-stressed cultures despite the there being no change in cell abundance further suggests this assay is not detecting PCD-relevant caspase activity; during this time period only cysts were present in the cultures. In contrast, cultures
exposed to 37°C did not exhibit caspase-8-like activity relative to controls. Thus, there is a disparity between the other PCD markers, which provide similar results for 37 and 40°C heat-stress treatments, and this marker, which provides very different results. These findings suggest that the Caspase-Glo 8 assay is an unreliable indicator of PCD in our system.

Caspase inhibition using the pan-caspase inhibitor z-VAD-FMK saved cells otherwise destined to die in cultures heat-stressed at 37°C suggesting the inhibitor limited temperature-induced PCD in Heterosigma. Our finding, along with that of Bidle and Bender (2008), Zuppini et al. (2007), and Vardi et al. (1999), gives evidence to the integral role of caspase-like enzymes in the execution of the PCD pathway.

**Externalization of Phosphatidylserine Residues**

Another key feature of PCD is externalization of phosphatidylserine (PS) residues (Bidle and Falkowski 2004). PS residues are specific phospholipids found on the interior layer of the lipid bilayer of a healthy cell membrane. During PCD, PS residues are actively translocated to the outer leaflet of the membrane by the enzyme flippase. In our study, cultures heat-stressed at 37 and 40°C contained significantly more cells with externalized PS than controls at 0 h post-stress. The diatom *Thalassiosira pseudonana* also exhibits this PCD hallmark (Bidle and Bender, 2008). The proportion of cells with everted PS residues was much lower than the number of cells destined to die. Flipping phospholipids from the inner leaflet of the plasma membrane to the outer leaflet when the lipid bilayer is in the
normal gel-phase is an energetic process that requires enzymatic activity by scramblase and flippase (Fraser 1999). However, phospholipids in liquid phase, which occur at high temperatures, move more freely between the inner and outer leaflet of the plasma membrane. It is possible that heat stress causes a change in phase of the lipid bilayer of H. akashiwo. Therefore, externalized PS residues are freely moving between the inner and outer leaflet and therefore PS residue externalization is not a reliable indicator for PCD induced by heat stress, but may be a useful indicator for PCD induced by other stressors.

This study demonstrates that H. akashiwo cells survive and exhibit normal growth after heat-stress for 1h at 35°C, while they undergo PCD when exposed to 37 - 40°C. Exposure to 50°C mostly causes encystment, but also a small proportion of the population undergoes necrosis. The proportion of cells that test positive for in situ DNA fragmentation, as identified by the TUNEL assay, at any given time is the most representative estimate of impending death via PCD. Externalization of PS residues is an early indication of PCD, although the phenomenon is too ephemeral to provide a good indication of the proportion of impacted cells, and may lead to false-negatives. This study underlines the complexity of H. akashiwo physiology. In natural environments, a combination of PCD, necrosis and cyst formation may be involved in bloom termination.
Chapter References


Nagasaki, K., Tarutani, K. and Yamaguchi, M. 1999. Growth characteristics of

*Heterosigma akashiwo* virus and its possible use as a microbiological agent for


Ross, C., Santiago-Vazquez, L. and Paul, V. 2006. Toxin release in response to
oxidative stress and programmed cell death in the cyanobacterium *Microcystis
aeruginosa*. Aquat. Toxicol. 78: 66-73.

unicellular chlorophyte *Dunaliella tertiolecta*. A hypothesis on the apoptosis in

Uren, A.G., O’Rourke, K., Aravind, L., Pisabarra, M.T., Seshaqiri, S., Koonie, E.V. and
families of caspase-like proteins, one of which plays a key role in MALT

Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated

Figure 2-1. *Heterosigma akashiwo* cell and cyst abundance (A) 1 h prior to and 0, 24, 48 and 72 h following 1 h stress at 35, 37, 40 and 50°C (grey bar indicates the duration of the 1 h heat stress). PCD hallmarks exhibited by *Heterosigma akashiwo* cells when heat-stressed for 1h at 35, 37, 40 and 50°C from 0 - 72 h post-heat stress: (B) proportion of TUNEL-positive cells, (C) proportion of Annexin-V positive cells, and (D) proportion of cells testing positive with the CaspACE assay. Control cultures were kept at 20°C. Error bars represent ±1 st.dev.
Figure 2-2. Light micrographs of *Heterosigma akashiwo* (A) vegetative cell and (B) resting cyst. Cells were grown in control conditions at 20°C and cysts were formed by 40 or 50°C heat stress for 1 h.
Figure 2-3. Representative micrographs of *Heterosigma akashiwo* cells from control and heat-stressed (for 1 h) cultures causing no effect (control), PCD and necrosis at 0 h post-stress. Autofluorescent cells appear red using green excitation, and positively stained cells for A) TUNEL, B) CaspACE and C) Annexin-V FITC appear green using blue excitation.
Figure 2-4. Cell abundance (cells mL⁻¹) of *Heterosigma akashiwo* cultures after heat stress for 1 h at 35, 37, 40 and 50°C. Cultures were untreated or treated with 0.1% DMSO or 20μM pan-caspase inhibitor (z-VAD-FMK) prior to heat stress. Control cultures were kept at 20°C. Error bars represent ±1 st.dev.
Figure 2-5. DNA fragmentation in *Heterosigma akashiwo* cultures at control conditions (20°C) and after a 1 h heat-stress at 35, 36 and 37°C at multiple timepoints: (0) immediately after heat-stress, (4) 4 h post heat-stress and (24) 24 h post heat-stress. First well: 100 bp ladder.
Table 2-1. The active death processes (including programmed cell death, PCD-like, apoptosis-like and paraptosis) found in various phytoplankton species, including the features of death and corresponding methods for detection.

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<td>DNA fragmentation, chromatin condensation, loss of nuclei, caspase 1, 3, 6, 8, 9 activity, caspase activity inhibited, caspase-like activity</td>
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<td>TUNEL staining, TEM, TEM, Western blot, TEM, TUNEL staining, Nucleic acid electrophoresis, H2DCFDA staining, DEVD-R110 fluorometric &amp; colorimetric assay, Western blot</td>
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<td><em>Micrasterias denticulate</em></td>
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<td>cytoplasmic vacuolization, mitochondrial degradation, ultrastructural changes of the Golgi and ER, DNA fragmentation - ionic but not osmotic stress, DNA laddering, ROS production, no caspase-3-like activity, no cytochrome-c</td>
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**Abbreviations**

Ac-DEVD-CHO - acetyl Asp-Glu-Val-Asp aldehyde (caspase 3 inhibitor)
Ac-DEVD-pNA - acetyl Asp-Glu-Val-Asp p-nitroaniline (colorimetric caspase-3/7 substrate)
Ac-VD-FMK - acetyl Val-Ala-Asp (OMe)-fluoromethylketone (caspase inhibitor)
AC-YEVD-CMK - acetyl Tyr-Glu-Val-Asp-chloromethylketone
Boc-D-FMK - Butyloxycarbonyl-Asp-fluoromethylketone (pancaspase inhibitor)
DAPI - 4',6-diamidino-2-phenylindol (fluorescent DNA stain)
DEVD-AFC - Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 3 substrate)
E-64 - L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (cysteine proteinase inhibitor)
FITC-VD-FMK - fluorescein isothiocyanate-Val-Ala-Asp-fluoromethylketone (in situ caspase marker)
H2DCFDA - 2',7'-dichlorodihydrofluorescein diacetate (fluorescent ROS detection reagent)
IETD-AFC - benzoxycarbonyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 8 substrate)
ISEL - in situ end labelling (DNA fragmentation labelling)
LEHD-AFC - Lue-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 9 substrate)
PM - plasma membrane
PS - phosphatidylserine
qRT-PCR - quantitative reverse transcription polymerase chain reaction
ROS - reactive oxygen species
TEM - transmission electron microscopy
TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labelling
VEID-AFC - Val-Glu-Ile-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 6 substrate)
WEHD-AFC - Trp-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 1 substrate)
z-DEVD-AFC - benzoxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (fluorogenic caspase 3 substrate)
z-VD-FMK - benzoxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (caspase inhibitor)
Chapter 3 - CELL DEATH PROGRAMMES IN THE HARMFUL BLOOM-FORMING RAPHIDOPHYTE, *Heterosigma akashiwo*, DURING VIRUS INFECTION WITH HaRNAV, HaNIV or HaV

Abstract

*Heterosigma akashiwo* (*Raphidophyceae*) is a harmful bloom forming, unicellular alga that can be abundant in temperate coastal waters worldwide. Viruses that infect *Heterosigma* are numerous, diverse and are one of the factors for natural bloom-termination. We compared the progression of viral infection and viral-induction of cell death in *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara & M. Chihara, during infections with three unrelated lytic viruses (HaRNAV, HaNIV and HaV). We examined the prevalence of three, classic cell-death hallmarks in *Heterosigma* to assess the importance of PCD-mechanisms during viral infection: DNA fragmentation, phosphatidylserine externalization and caspase-like activity. Caspase-like enzyme activity was detected in *H. akashiwo* during infection with all three viruses. However, DNA fragmentation was observed with only HaNIV and HaV infections, and phosphatidylserine externalization was only observed during HaV infection. This suggests the same algal host utilizes different cell-death programmes during HaRNAV, HaNIV and HaV infections, outlining the complexity of virus-induced mortality in *H. akashiwo*. 

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Introduction

Freshwater and marine phytoplankton contribute roughly half of the planet’s primary production (Geider et al. 2001) and yet accounts for less than 1% of global biomass (Field et al. 1998). These estimates are calculated using on satellite-sensing chlorophyll $a$ concentrations and net primary production algorithms (Geider et al. 2001). This low-standing biomass under-represents global production because of high turnover rates. On average, phytoplankton growth and mortality rates equate to their total global biomass being replaced weekly (Field et al. 1998, Bidle and Falkowski 2004, Bidle 2015) and thus are major contributors to carbon cycling and sedimentation. This rapid turnover is due to high growth rates coupled with high rates of sedimentation, grazing, and cell death (Bidle and Falkowski 2004). While the significance of cell death in phytoplankton attributed to unfavourable environmental conditions such as light or nutrient stress (Bidle and Falkowski 2004), seasonal changes in temperature, or ocean mixing events is generally recognized, there is also a significant impact of viral infection-induced mortality within phytoplankton populations. Virus-induced death of phytoplankton is associated with high rates of cell lysis in phytoplankton blooms (Brussaard et al. 1995, Brussaard 2004) and the reduction in global primary productivity during infection (Suttle et al. 1990). In the oceans, viruses are ubiquitous and can reach densities of $10^4 - 10^8$ per milliliter of seawater (Bergh et al. 1989, Proctor and Fuhrman 1990, Fuhrman 1999). Marine viruses influence abundance, species composition and diversity of the microbial community. Viral infection can alter the flow of nutrients and energy within the microbial loop (Fuhrman 1999). Lytic viral
infections trim phytoplankton blooms and alter the species and strain composition within a phytoplankton bloom (Bratbak et al. 1993, Bratbak et al. 1996). Many studies have described the interactions between marine viruses and their photosynthetic hosts on the population level (Brussaard 2004, Suttle 2007); however, the role of a programmed cell death and the cellular mechanisms involved in such a process during viral infection in phytoplankton is now beginning to become more clear (Bidle et al. 2007, Vardi et al. 2012, Ray et al. 2014).

Genetically programmed, self-destruction mechanisms are widely accepted to exist in multicellular organisms but a number of studies have shown that single-celled organisms, including bacteria (Bayles 2014), protists (reviewed in Deponte 1998) and yeast (Madeo et al. 1999), are also capable of self-destruction through the process of programmed cell death (PCD). PCD is a collection of cell death programs that are active, ordered, and genetically programmed (Galluzzi et al. 2012). In phytoplankton, PCD is defined as an autocatalytic, cell-suicide mechanism mediated by endogenous biochemical pathways. These pathways lead to a series of morphological and biochemical changes, many of which are similar to the hallmarks of the better-understood metazoan, apoptotic pathways (Bidle and Falkowski 2004, Bidle 2015) including caspase-like activity, externalized PS residues and DNA fragmentation.

The mechanisms that drive PCD and necrotic responses are not well known in unicellular organisms. A number of unicellular algae have been identified as PCD-capable in response to a variety of stressors including heat stress (Nedelcu 2006, Dingman and Lawrence 2012), light stress (Berges and Falkowski 1998,
Segovia et al. 2003, Moharikar et al. 2006) nutrient deprivation (Berges and Falkowski 1998, Berman-Frank et al. 2004, Segovia and Berges 2005), oxidative stress (Vardi et al. 1999) and viral infection (Bidle et al. 2007, Vardi et al. 2009). Although the adaptive function of genetically programmed and self-promoted death in unicells is a source of ongoing debate, many roles of active death pathways have been proposed, including population level adaptive advantages (reviewed in Nedelcu et al. 2011).

Cell death has been characterized along a continuum, from unregulated necrosis to highly choreographed, genetically-programmed cell death, each pathway with its own unique biochemical hallmarks. While necrotic cell death is accidental, chaotic and energy-independent, a genetically programmed death requires de novo protein synthesis and is enzymatically regulated. There are several well-characterized, genetically programmed cell death pathways in multicellular organisms, which include apoptosis, autophagy, caspase-independent cell death, paraptosis, and non-lysosomal cell death (Galluzzi et al. 2012). Each of these identified programs have varying degrees of genetic involvement - some more energy dependent than others (Galluzzi et al. 2012). These cell death programs are distinguishable from one another by the biochemical and morphological characteristics, or so called hallmarks of PCD. These hallmarks are diverse in nature and reflect differences in membrane permeability, activation of specific enzymes and transcription factors and so forth. In this study, we observed markers from the each of the 5 major components of a cell: (1) cytosol or organelles (by examining previously published TEM images), (2) nucleus (through assessment of
DNA fragmentation by TUNEL and DNA laddering), (3) plasma membrane 
(externalization of phosphatidylserine residues using Annexin-V), (4) associated 
enzymes (Caspase-like enzyme activity through CaspACE Staining) and (5) known 
inhibitors of these death pathways (using the caspase inhibitor z-VAD-FMK).

PCD as a result of viral infection is a common strategy in diverse 
multicellular organisms, which presumably limits transmission of the virus 
may initiate an autocatalytic cell death pathway to prevent viral release or to reduce 
the number of viruses released during lysis (Teodoro and Branton 1997). As a 
counter-measure, some viruses induce the production of caspase inhibitors and 
other proteins to inhibit PCD (Roulston et al. 1999). However, the interaction with 
PCD pathways can be complex since several viruses actively promote and/or 
require PCD during late stages of infection to complete their replication cycle 
(Wilson et al. 2005, Bidle et al. 2007 and reviewed by Teodoro and Branton 1997). 
Thus, depending on the viral-host system, it is possible that PCD is a host strategy 
to reduce viral replication or a viral strategy to liberate host resources by 
synchronizing cell lysis with viral progeny maturation (Bidle et al. 2007). Because 
the physiology of host and virus is intimately intertwined, it is likely that host-
mediated or virus-induced PCD are not mutually exclusive and gradients of each 
likely occur within the cell concomitantly.

Few studies have examined the cellular mechanisms involved in cell death 
during viral infections in phytoplankton blooms. To date only one host-virus system 
has linked PCD to viral infection of phytoplankton. Bidle et al. (2007) and Ray et
al. (2014) determined that PCD-related metacaspases are activated during viral infection of the coccolithophore *Emiliania huxleyi* and two other haptophytes, *Haptolina ericina* and *Phaeocystis pouchetii*, are likely required for successful viral infection in this host-virus system. In *E. huxleyi*, EhVs not only trigger PCD markers like caspase activity, they also actively recruit it. When caspases are inhibited, lysis was significantly reduced and evidence shows that the EhV proteome contains proteins that possess caspase cleavage sites (Bidle et al 2007). Our study examined the potential role of the PCD pathway in the same host (*Heterosigma akashiwo*) when infected with three unrelated viruses.

*H. akashiwo*, a red-tide forming alga, is responsible for large scale fish kills of wild and farmed fish populations in temperate coastal waters (Honjo 1993). These “red tide” blooms can reach densities of $5 \times 10^8$ cells•L$^{-1}$ (Taylor and Haigh 1993) and are often made up of multiple isolates of *H. akashiwo* (Tarutani et al. 2000). A single-species phytoplankton bloom often fluctuate in genotype composition because host-strains have varying sensitivity to environmental conditions and different HaVs (*Heterosigma akashiwo*-specific viruses) (Tarutani et al. 2000, Tomaru et al. 2004). Therefore, blooms can persist in the water column but the host-strain can be constantly changing as a result of varying sensitivity to viral infection (Tomaru et al. 2004).

Recently, a number of different PCD hallmarks were observed in *H. akashiwo* during heat stress (Dingman and Lawrence 2012) including DNA fragmentation, externalization of phosphatidylserine residues and caspase-like enzyme activation. In this study, we are testing the hypothesis that PCD is involved
in viral infection in a marine Raphidophyte. *H. akashiwo* provides a unique opportunity for examining the effects of viral infection leading to cell death because different viruses that infect the same species have been isolated from around the world and can be maintained in the laboratory. The three viruses used in this study—HaRNAV, HaNIV and HaV — are in different viral groups yet infect the same host species (*Heterosigma akashiwo*) (Table 3-1). In order to identify some of the characteristics of cell death during infection with HaRNAV, HaNIV and HaV, we describe PCD-related morphological and biochemical features of the cytosol, organelles, nucleus and plasma membrane and assay for death-related enzyme activity during viral infection.

**Methods**

**Culturing**

For this study we used *H. akashiwo* strain NEPCC522, a single clonal algal-population that was originally isolated from English Bay, BC. The culture was grown non-axenically in modified SWM3 media (Chen et al. 1969, Itoh and Imai 1987) enriched with 2 nM Na₂SeO₃ or modified f/2 media (Guillard 1975) enriched with 10 nM Na₂SeO₃ under 14:10 light-dark cycle (~45 μmol photons m⁻²s⁻¹ of cool white fluorescent lights). While infection of NEPCC522 by HaRNAV and HaNIV are well established (Tai et al. 2003, Lawrence et al. 2001), we had to determine the optimal HaV strain for NEPCC522 infection. To do this, cells were challenged with various strains of HaV, ultimately settling on viral strain HaV53.
Viral propagation was achieved by inoculating an exponentially growing culture of *H. akashiwo* with 10% v/v lysate from a previously viral-lysed culture. Lysates were stored in the dark at 4°C and used within ~1 week.

**Cell Counting and Viability**

Cells were fixed with 70% ice-cold ethanol and cells counted in a nanoplankton counting chamber (PhycoTech Inc., St. Joseph, MI, USA) using epifluorescence microscopy. Total cell abundance was determined by enumerating autofluorescing cells in the counting chamber using green excitation light (Ex 515nm-560nm, Em >590nm; Leica DM250, Richmond Hill, ON, Canada). Cell viability was determined by assessing plasma membrane permeability using 0.5 μM SYTOX Green (Invitrogen, Carlsbad, CA, USA) (methods adapted from Lawrence et al. 2006). The ethanol fixation was compared to unfixed cells and was confirmed to have no effect on membrane permeability using SYTOX Green.

**Virus Inoculation and PCD Assays**

HaRNAV and HaNIV viral lysates were clarified to remove cell debris by means of centrifugation (3220 rcf, 30 min) (Eppendorf 5810R, Mississauga, ON, Canada). HaV lysate was passed through a 0.8 μm track-etch membrane filter (Whatman Nucleopore, Tokyo, Japan) to clarify. Exponentially growing cultures (50 ml) were inoculated, in triplicate, with 10% v/v lysate of HaRNAV, HaNIV or HaV. Additional seawater media was added to control cultures in place of viral lysate. At 24 h intervals, post-inoculation cultures were subsampled to determine cell abundance and examined for the presence of the following PCD hallmarks: in
situ DNA fragmentation, DNA laddering, externalized phosphatidylserine residues, in situ caspase-family activity and whole-culture caspase-8-like activity as described in Dingman and Lawrence (2012).

Data Analysis

Statistical analysis was performed using R version 2.11.1 (R Development Core Team). The assumptions of homogeneity of variance and normal distribution were assessed using Cochran’s and Shapiro-Wilk’s tests. TUNEL data were log transformed. We conducted a repeated measures analysis of variance with cell abundance, proportion of cells stained (with TUNEL, AnnexinV-FTIC or Caspase) as responses with time, viral inoculation (HaRNAV or HaNIV) and their interaction as predictors. Preliminary analyses revealed that less than 1% of variation in the data was explained by resampling tubes. Therefore, we did not explicitly account for the repeated measures. Post-hoc contrasts were performed using Tukey’s honest significant difference (HSD) to compare all means against each other.

Results and Discussion

The purpose of our study was to examine virus-mediated death in Heterosigma akashiwo for hallmarks of programmed cell death during the infection cycles of three genetically unrelated lytic viruses (HaRNAV, HaNIV, HaV). These viruses are morphologically and biochemically unique and have different life-history strategies within the same host (Table 3-1). Our research shows that infection with each virus results in the manifestation of a unique set of cellular morphological, and biochemical changes within host cells, demonstrating the
complexity of cell death during infection and suggesting that H. akashiwo is capable of multiple cell death programmes.

**Growth Rate and Population Decline**

Upon inoculation of Heterosigma cultures with viruses, there was little change in growth rate until after 24 h [doubling time (DT): 20 h]. At 48 h, the net growth in HaRNAV- and HaNIV-infected cultures effectively stopped as cell abundance plateaued shortly after 48 h p.i. (Figure 3-1). This low rate of growth indicates a cessation of cell division as the infection proceeded or a balance between cell division and cell lysis. Of the cells remaining in the culture at 72 h p.i., 32 ±13 and 20 ±11% (±st.dev) were non-viable in HaRNAV and HaNIV infected cultures, respectively (Figure 3-2A). Either way, exponential growth typical of these bloom-formers (20 h DT) was halted during infection. During HaV infection, however, rapid cell lysis occurred where 75% lysed within 48h p.i. (Figure 3-1). Of the remaining cells visible in the culture at 48 h, 8 ±1% were nonviable as assessed by SYTOX staining).

**PCD Hallmarks: DNA Fragmentation, DNA Laddering, Caspase-like Enzymes, PS Residues**

DNA fragmentation has long been associated with active death processes. In metazoans, a consistent size pattern, or laddering, is identifiable from DNA isolated from cells undergoing apoptosis. The pattern is due to the activation of nucleases that non-specifically cleave DNA between nucleosomes, leaving behind DNA fragments of equal size (~180 bp) (Matassov et al. 2004). Heterosigma, however,
did not exhibit DNA laddering during cell death induced by either HaRNAV or HaNIV infection (HaV infected cells were not examined) (this study) or heat-stress (Dingman and Lawrence 2012) presumably because histones are irregularly spaced rather than because DNA fragmentation did not occur. In other studies involving phytoplankton, *in situ* detection of DNA fragmentation is more common than DNA laddering during electrophoresis (reviewed in Dingman and Lawrence 2012); therefore, in this study, we examined the presence of DNA nicks *in situ*, which would be expected if intact cells contained fragmented DNA. Cells from HaRNAV infected cultures did not exhibit DNA fragmentation as assessed by TUNEL (Figure 3-2B), indicating that death due to HaRNAV infection is not apoptosis-like cell death. However, in HaNIV and HaV infected cultures, cellular DNA degradation was significantly greater than that observed in control cultures (p<0.0001 and p=0.0341, respectively) although it was detected much later and in fewer cells in HaNIV infected cultures (72h, 18 ±1.8%) compared to HaV-infected cultures (48 h, 79 ±12%). This indicates that either (1) the trigger for PCD is detected sooner during HaV infection or that (2) the cell death program involved in HaV-induced lysis is shorter than that of HaNIV.

In metazoans, caspases are important enzymes in execution and inhibition of PCD, and are particularly well characterized for the mammalian apoptotic pathways (Carmona-Gutierrez et al. 2010). Caspases are cysteine proteases that cleave substrates after an aspartate residue (Uren et al. 2000) and can be detected *in situ* using a fluorogenic compound containing aspartic acid (FITC-VAD-FMK). However, this staining method has also been used to detect caspase-like enzymes in
organisms that are known to only have metacaspases and not caspases (eg. plants, protists and fungi) but it is still a diagnostic hallmark of the specific activity regardless of whether true caspases gene sequences are found in the genome. In fact, caspase-like activity have been detected during cell death in members of the Chlorophytes, (Moharikar et al. 2006, Zuppini et al. 2007, Segovia et al. 2003, Affenzeller et al. 2008), Haptophytes (Bidle et al. 2007, Vardi et al. 2013, Ray et al. 2014), Cyanobacteria (Ross et al. 2006, Berman-Frank et al. 2004), Diatoms (Bidle and Bender 2008, Thamatrakoln et al. 2012), Dinoflagellates (Zhang et al. 2006) and Raphidophytes (Dingman and Lawrence 2012). In Heterosigma, the percentage of cells with caspase-like enzyme activity during viral infection was negligible until 48 h p.i. At this time point, infections with all three viruses induced an increase in the proportion of cells with caspase-like activity, but it was particularly significant for HaV where over 55 ±5 % (p<0.0001) of the cells had detectable caspase-like activity. HaRNAV and HaNIV also had detectable caspase-like activity, but the proportion of cells was considerably smaller, though significant (HaRNAV: 14 ±10%, p=0.0033, 48 h p.i.) (HaNIV: 17 ±3%, p=0.0037, 72 h p.i.) (Figure 3-2D). These results suggest that, like other unicellular organisms, Heterosigma activate caspase-like proteases after stress. These proteases are detectable in cells, although at different proportions, in cultures exposed to all three viruses and also after a 1 h heat-stress event (Dingman and Lawrence 2012), indicating the activation of caspase-like enzymes is a general stress-response for this organism. Part of the increase in caspase-like activity may be related to the formation of resting cells that occurs during response to HaV-infection (Chapter 4).
In multicellular organisms, membrane alterations during apoptosis include externalized phosphatidyleserine (PS) residues, a well-studied process (Witasp et al. 2008, Savill 1997, Williamson and Schlegel 1994). Cells undergoing apoptosis use signaling molecules that are recognized and quickly engulfed by phagocytic cells to prevent inflammation of surrounding tissue (Fadeel et al. 2010, Elmore 2007). Many unicellular organisms exhibit externalization of PS residues during apoptosis-like cell death however, the function remains unclear (Nyoman and Lüder 2013, Dingman and Lawrence 2012, Bidle and Bender 2008). During viral infection of Heterosigma, cells from HaRNAV-infected cultures did not exhibit PS residue externalization at any time. At 48 h p.i., very few (8.2 ±3.0%) HaNIV-infected cells had external PS residues although this was significantly more than then control (p=0.0037), however, at that time, 20 ±11% of the culture had compromised cell membranes (assessed by SYTOX) which may have allowed Annexin V to permeate into the cells and stain internal PS residues. Nearly half (49.2 ±5.3%) of the cells in HaV-infected cultures had external PS residues at 48 h p.i. (p<0.0001). These results suggest that Heterosigma is capable of PS residue externalization during cell death but does not occur during all viral infections. The variability in the PS-externalization response provides evidence for more than one programmed cell death pathway during viral-mediated cell death in Heterosigma.

Cell Death Pathways

Although caspase-like enzyme activity was observed in H. akashiwo cells during HaRNAV infection, the lack of DNA fragmentation and externalized PS
residues combined with the previously described ultrastructural changes
[endoplasmic reticulum swelling and cytoplasmic vacuolization (Tai et al. 2003)]
suggests infected cells employ a death strategy that resembles paraptotic-like death
(Jimenez et al. 2009) (Table 3-2). This type of cell death has been seen in the
unicellular dinoflagellate Amphidinium carterae during light limitation and culture
senescence (Franklin and Berges 2004), which caused vacuolization, degradation of
cellular structure and maintenance of membranes yet lacked DNA fragmentation.

Death during HaNIV infection resembled apoptosis-like cell death. We
confirmed the presence of DNA fragmentation and caspase-like activity. However,
Annexin-V staining in a small proportion of the HaNIV-infected cells could be due
to leaky membranes in these dying cells and not necessarily PS-externalization.
Similar to HaRNAV-infected cultures, cell abundance in HaNIV-infected cultures
did not change after 24 h p.i. likely due to equal proportions of cells remaining in
culture (dividing or stationary) and cells that have lysed. These findings, combined
with the ultrastructural changes previously described (Lawrence et al. 2001) (Table
3-2), indicates that H. akashiwo cells exhibit most of the morphological and
biochemical features consistent with cells undergoing apoptosis-like cell death
during HaNIV infection.

HaV infected cultures exhibited all the PCD hallmarks we examined.
Additionally, the temporal sequence and the proportion of cells exhibiting these
hallmarks were different than those seen in HaRNAV and HaNIV infections. HaV
was previously described to have a lytic cycle length between 30-33 h post-
inoculation with mature particles appearing in the cytoplasm by 24 h (Nagasaki et
al. 1999). In HaV infected cultures, DNA fragmentation was observed in the majority of cells at 24 h p.i. while more than half of cells exhibited externalization of PS residues and caspase-like activity, which occurred concomitantly with abundance decline (48 h p.i.). These results suggest that DNA fragmentation in *H. akashiwo* cells occurs early in the HaV lytic cycle and is detectable for the length of the pathway while externalization of PS residues and caspase-like activity represents a later stage of the pathway and may be short-lived features. Our findings are consistent with the progression of PCD observed following heat stress in this species (Dingman and Lawrence 2012) and similar to results reported for viral infection of haptophytes (Ray et al. 2014). All the hallmarks we tested for were seen in a high proportion (>50%) of cells during the lytic cycle of HaV (Table 3-2), indicating that HaV infection will lead to a high proportion of cells undergo apoptosis-like cell death (Table 3-2).

The adaptive role of PCD pathways in single-celled organisms is a source of ongoing debate. However, a number of proposed functions have been discussed (Bidle and Falkowski 2004, Nedelcu et al. 2011, and Bidle 2015) and may be common among many single-celled organisms. There are a number of proposed roles for PCD such as limiting the spread of viral infection, regulation of population size during resource limitation, bloom termination and removal of mutated or damaged cells. Nevertheless, the specific selective pressure(s) maintaining active cell death in single celled organisms is still unknown. It is plausible that the induction of PCD during viral infection in *H. akashiwo* has evolved in response to kin selection (as individuals within a bloom are related) and/or reflects elements
developed through the host-virus co-evolution. Often referred to as the Red Queen hypothesis, the co-evolution “arms race” proposes that host and virus must constantly adapt for competing species to survive (Stenseth and Smith 1984).

As more single-celled organisms are being identified as PCD-capable, questions about the activation and regulation of the pathway are beginning to surface. It is not yet clear if the host or virus is initiating the pathway, or whether it is beneficial for either host or virus. Interestingly, by comparing the PCD pathways executed during HaRNAV, HaNIV and HaV infection, it is also likely that the *H. akashiwo* is capable of multiple cell death programmes; different stressors elicit different, yet choreographed, cellular disassembly.
References


Table 3-1. Comparison of three, unrelated *Heterosigma akashiwo* -infecting viruses, HaV, HaRNAV, and HaNIV.

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Isolated</th>
<th>Genome type, size</th>
<th>Burst size, particle size</th>
<th>Viroplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaV&lt;sup&gt;1,2&lt;/sup&gt; <em>Phycodnaviridae</em></td>
<td>Hiroshima Bay, Japan</td>
<td>dsDNA, 294kb</td>
<td>770, 185-220 nm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>HaRNAV&lt;sup&gt;3&lt;/sup&gt; <em>Picornaviridae</em></td>
<td>Strait of Georgia, BC. Canada</td>
<td>ssRNA, 9.1kb</td>
<td>2.1x10&lt;sup&gt;4&lt;/sup&gt;, 25 nm</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>HaNIV&lt;sup&gt;4&lt;/sup&gt; <em>(unknown)</em></td>
<td>Discovery Passage, BC. Canada.</td>
<td>Unknown</td>
<td>3x10&lt;sup&gt;5&lt;/sup&gt;, 10-20 nm</td>
<td>Nuclear inclusions</td>
</tr>
</tbody>
</table>

<sup>1</sup>Nagasaki et al. 1999  
<sup>2</sup>Tarutani et al. 2000  
<sup>3</sup>Tai et al. 2003  
<sup>4</sup>Lawrence et al. 2001
Table 3-2. Summary of cellular morphological and biochemical characteristics exhibited by *Heterosigma akashiwo* during infection with three, unrelated viruses: HaRNAV, HaNIV and HaV. Data for hallmarks exhibited during HaV infection are from literature as footnoted below.

<table>
<thead>
<tr>
<th></th>
<th>HaRNAV</th>
<th>HaNIV</th>
<th>HaV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytosolic/Organelle</strong></td>
<td>· Swelling of the ER&lt;sup&gt;1&lt;/sup&gt;</td>
<td>· Intact organelles&lt;sup&gt;2&lt;/sup&gt;</td>
<td>· Intact organelles&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>· Cytoplasmic vacuolization&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nuclear</strong></td>
<td>· No DNA fragmentation*</td>
<td>· DNA fragmentation*</td>
<td>· DNA fragmentation*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>· Chromatin condensation&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Membrane</strong></td>
<td>· Permeable PM*</td>
<td>· Maintenance of PM integrity*</td>
<td>· Maintenance of PM integrity*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>· Externalized PS residues*</td>
<td>· Externalized PS residues*</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td>· Caspase-like activity*</td>
<td>· Caspase-like activity*</td>
<td>· Caspase-like activity*</td>
</tr>
<tr>
<td><strong>Cell death program</strong></td>
<td>Paraptosis-like</td>
<td>Apoptosis-like</td>
<td>Apoptosis-like</td>
</tr>
</tbody>
</table>

*this study

<sup>1</sup>Tai et al. 2003

<sup>2</sup>Lawrence et al. 2001

<sup>3</sup>Nagaski et al. 1999
Figure 3-1. Cell abundance of *Heterosigma akashiwo* cultures during normal culture growth (diamonds) and during lytic viral infection of HaRNAV (solid boxes), HaNIV (open boxes) and HaV (solid circles) between 0 and 72 h post infection. Error bars represent ±1 st.dev. n=3.
Figure 3-2. PCD hallmarks exhibited by *Heterosigma akashiwo* cells across the 72 h post-infection (h.p.i) of the lytic infection cycle with HaRNAV, HaNIV, and HaV. Proportion of cells (%): that are non-viable assessed by SYTOX staining (A), containing DNA fragmentation as assessed by TUNEL (B); externalized phosphatidylserine (PS) residues assessed by Annexin V-FITC staining (C) and active caspase-like enzymes assessed by CaspACE staining (D). In control cultures, less than 1% of cells exhibited any of the PCD-related hallmarks at any time point.

Error bars represent ±1 st.dev. n=3
Chapter 4 - THREE CELL FATES DURING VIRAL INFECTION of *Heterosigma akashiwo*: APOPTOSIS-LIKE CELL DEATH, SURVIVAL AND RESTING-CELL FORMATION

Abstract

In the oceans, phytoplankton can form blooms quickly and subsequently turnover in rapid timescales compared to terrestrial primary producers. Bloom dynamics can be complex, but are primarily regulated by seasonal light availability, temperature, and nutrient cycles. Viruses can also contribute to the dynamic changes in phytoplankton abundance but the interactions between host and virus can be thought of as an arms race and thus algae likely have strategies to minimize or defend against viral attacks. We show that the marine microalga *Heterosigma akashiwo*, a toxic bloom former, has three fates when exposed to the Phycodnaviridae HaV: death, evasion and resistance. Apoptosis-like cell death is induced, but resting cells can form and some cells survive infection and remain vegetative. Here we show resting cells are a short-term mechanism for evading viral infection. I propose that infected or lysed cells may release a signal that is detected by uninfected members of the population, triggering the formation of resting cells. Alternatively, a viral replication error could cause formation of an incomplete or incompetent progeny that triggers a stress response leading to resting cell formation. These population level dynamics during viral infection leading to death, evasion and resistance are consistent with the Red Queen Hypothesis of host-virus co-evolution—"It takes all the running you can do to keep in the same place."
Introduction

Phytoplankton make up less than 1% of the Earth’s total biomass yet they contribute almost half of global primary productivity (Falkowski 1994). The discrepancy is a result of the rapid turnover of phytoplankton due to grazing and environment-induced mortality. Marine viruses also contribute to this high mortality and often outnumber their hosts by at least an order of magnitude. With an estimated $10^{23}$ infections per second, viruses can be responsible for the death of 20% of the ocean’s biomass per day (Suttle 2007). The severity of the threat implies that phytoplankton employ strategies to avoid and limit the extent of viral lysis. While morphotype (cell size, cell membrane structures) and cell-cycle phase may affect infection or viral burst size in phytoplankton (Bratbak et al. 2007, Brussaard et al. 1996, Brussaard 2004), a more active defense strategy includes the initiation of programmed cell death, presumably to limit viral replication, as evidenced by the activation of caspase-like enzymes (Bidle et al. 2007, Vardi et al. 2012, Ray et al. 2014, Bidle 2015) and the production of reactive oxygen species (Vardi et al. 2012). However, for most phytoplankton groups, there is little known about defensive strategies.

*Heterosigma akashiwo*, a harmful-bloom forming raphidophyte, is the dominant marine genera in a class that includes *Chattonella*, and *Fibrocapsa*. All have a two-phase, life-history consisting of an active vegetative phase and quiescent resting-cell or cyst phase (Imai and Itoh 1987, Yoshimatsu 1987, Imai and Itakura 1991, Taylor and Haigh 1993, Imai and Itakura 1999). Vegetative, free swimming cells are visible in the water column while resting cells (Han et al. 2002)
or cysts (Imai and Itakura 1999, Shikata et al. 2007) are non-motile and are a known survival strategy induced during seasonal changes, nutrient stress and other environmental stressors (Anderson et al. 1995). Unlike the cyst phase of other Raphidophytes, *Heterosigma*'s resting-cell phase does not require a mandatory dormancy period before germination (Han et al. 2002, Itakura et al. 1996), allowing it to quickly switch phases as nutrient, temperature and light conditions change. In *Heterosigma*, both light and temperature cues must be concomitantly perceived (Jacobs et al. 1999) to induce resting-cell formation (Han et al. 2002) and subsequently, germination (Shikata et al. 2007). The association between temperature and resting-cell formation is tightly correlated in laboratory and field studies (Imai and Itakura 1991, Taylor and Haigh 1993, Han et al. 2002) where the formation of resting cells is triggered by cold temperatures (<10°C) and germination and vegetative growth stimulated in warm temperatures (>16°C). The specific cellular mechanisms that promote resting-cell formation are not known in *Heterosigma* (Tobin et al. 2011). However, in *Scrippsiella trochoidea*, a cyst-forming dinoflagellate, allelopathic chemicals induce encystment (Fistarol et al. 2004) suggesting receptor involvement.

Here we examined the role viral infection in *Heterosigma* population cycling. Previous work (Chapter 3) shows that after inoculation with HaV, the vast majority of cells are lost due to virus-induced PCD (Chapter 3) and lysis yet little is known about the minute proportion of the population that does not undergo lysis. Our aim was quantify the proportion of cells that undergo PCD during infection and describe the fate of the remaining cells (those that did not die via the PCD
We propose that *Heterosigma* has adapted strategies to avoid total population loss during a viral infection. Concomitantly, HaV has likely adapted strategies to successfully infect *Heterosigma*. The interaction of a host and parasite is described by Leigh Van Valen's (1973) Red Queen hypothesis, where host and virus are constantly adapting to survive a co-evolutionary race yet neither ever win. Just as the Red Queen and Alice, in *Through the Looking-Glass* by Lewis Carroll, are continuously running yet remain in the same spot. This continual process services to stimulate diversity of genotypes.

**Methods**

**Culturing**

*Heterosigma akashiwo* (NEPCC 522, nonaxenic) were grown as previously described (Dingman and Lawrence 2012) and were monitored for population growth by in vivo chlorophyll a fluorescence (Turner Designs TD-700 fluorometer, Sunnyvale, CA, USA). Control cultures were grown at standard conditions in light at 20°C. These standard control cultures were run in parallel to those incubated under experimental conditions.

**Resting Cell Induction (cold-dark induction)**

Resting cell induction experiments were done by removing cultures from their normal 20°C, 14:10 light:dark cycle and placing them at 4°C in total darkness for different time periods. Cultures were monitored by *in vivo* relative fluorescence
Virus Inoculation

*Heterosigma akashiwo* virus isolate HaV53 was originally isolated from Itsukaichi Fishing Port (northern Hiroshima Bay, Japan) (Tarutani et al. 2000) and provided by K. Nagasaki from the National Fisheries Research Agency, Hatsukaichi, Japan. Initial confirmation that HaV53 specifically lysed algal strain NEPCC 522 was done in the lab of K. Nagasaki. Viral propagation was achieved by inoculating a fresh, exponentially growing culture of *H. akashiwo* with 10% v/v lysate. Lysate was clarified to remove cell debris before use in future infections by filtration. HaV lysate was passed through a 0.8 μm track-etch membrane filter (Whatman Nucleopore, Tokyo, Japan) to clarify. Exponential growing 5- or 50 mL cultures were inoculated at 20°C, in triplicate, with 10% v/v lysate of HaV. Additional seawater media was added to control cultures in place of viral lysate. At 24 h intervals post-inoculation cultures were monitored by *in vivo* relative fluorescence and/or subsampled to determine cell abundance.

Cell Abundance and Viability

Total cell abundance was determined by loading cultures treated with 1% Lugol's Iodine Solution onto a 0.099 mL nannoplankton counting chamber (PhycoTech Inc., St. Joseph, MI, USA) for examination under light microscopy. A minimum of 200 cells was counted in each sample. The proportion of resting cells was determined by first counting immobile, round cells that had settled onto the
bottom of the chamber, then Lugol's was added and the total number of cells and resting cells were counted.

Cell viability was tested by assessing plasma membrane permeability using 0.5 μM SYTOX Green (Invitrogen) (Lawrence et al. 2006). First, total cell abundance was determined by enumerating autofluorescing cells in the counting chamber using green excitation (Leica; Ex 515nm-560nm, Em 590nm+). Then a minimum of 200 cells was then scored for +/- SYTOX Green staining by switching to blue excitation (Leica; Ex 450nm-490nm, Em 515nm+).

*Casparse Inhibition*

The pan-caspase inhibitor z-VAD-FMK (Fisher) was added at a final concentration of 20 μM to triplicate, exponentially growing cultures and incubated at room temperature for 20 min before inoculation with the virus or introduction to cold-dark conditions. A previous study (Dingman and Lawrence 2012) demonstrated that a DMSO-only treatment (0.1%), which represents the final concentration of this chemical in the inhibitor treatment, had no effect on cell abundance in *Heterosigma akashiwo*. Control cultures for caspase-inhibition experiments experienced the same conditions experimental cultures did but were free of the caspase-inhibitor z-VAD-FMK. Previous experiments (Chapter 2) show that the DMSO (delivered with the inhibitor) was below the threshold to affect cultures and thus was not added to control cultures.
Photography and Image Processing

Cells were photographed under bright field using a Leica DMRXAZ microscope with a DC500 camera. Images were processed using Thumbs plus 4.0. Epifluorescence images were captured using Leica TCS-SP2 confocal laser scanning microscope with a Leica DM IRE2 inverted stand and the Leica Confocal software. A 488 nm laser was used to excite samples and the emission wavelength collected from the samples was 680 nm to 700 nm using an Acousto-Optic-Beam-Splitter (AOBS) to select the emission fluorescence.

Results and Discussion

HAV Infection Leading to Three Fates: death, resting and vegetative cells

Infection of an exponential-phase Heterosigma culture with HaV53 causes a population crash after 48 h that leads to clarification of the culture. However, close examination of the culture medium six days post-infection indicates that while 92.3 ± 1.5% of cells were lost due to viral-induced apoptosis-like cell death (Chapter 3), 7.2 ± 0.1% remained as resting cells (Figure 4-1). Resting cells were obvious as non-motile, spherical cells with intact plastids (Figure 4-2) that settled on the bottom of the culture flask. These resting cells were morphologically identical to those triggered by the classic treatments such as low temperature and darkness (Figure 4-2). No resting cells were observed in the uninfected control culture. In addition to lysis and resting cells, 0.49 ± 0.03% of cells survived the infection as vegetative cells (Figure 4-1, 4-2) that would eventually repopulate the culture in approximately two weeks. These survivors grew identically to the virgin cell...
culture line but these cells remained resistant to further infection (Figure 4-3). Thus, cells were destined for three fates when exposed to HaV: apoptosis-like cell death, formation of resting cell or survival as resistant vegetative cells.

Abiotic factors (e.g. light, temperature or nutrients) can contribute to a productive viral infection or reduced viral proliferation (Brown et al. 2007, Gobler et al. 2007, Brown and Bidle 2014) but biotic parameters can also set the stage for viral success or host survival. Genetic diversity among the host or the virus, multiplicity of infection (M.O.I.) (the ratio of viruses to hosts), host physiological state or life stage can all influence the success of the host or the virus (Murray and Jackson 1992, Bratback et al. 1998, Thyrrhaung et al. 2002, Frada et al. 2008, Brown and Bidle 2014). In this host-virus system, some of these biotic conditions, such as viral titre and cell cycle, are extremely difficult to control for. Our work focused on altering the abiotic factors light and temperature. However, biotic parameters may also dictate which of these three observed cell fates are dominant among HaV-exposed Heterosigma cultures.

Vegetative Cells Survive Re-infection

The vegetative cells that survived HaV infection had similar growth rates to the virgin-culture line but the survivors were immune to re-infection and have retained their immunity for at least over 12 months (Figure 4-3). While the Heterosigma akashiwo culture was derived from a single-cell line (NEPCC strain 522), this resistance may be due to a mutation in the population that confers resistance. It is also possible that this represents an inducible immunity, though the
persistence for several months without any viral exposure would seem to eliminate that possibility. Alternatively, the vegetative survivors could be experiencing a chronic infection where HaV is replicating within the host at a low rate and virus particles are either not released or released without host-cell lysis. As a next step, examining the vegetative survivors, visually using TEM or biochemically using PCR, may help identify a chronic infection.

Resting Cells: a short-term viral evasion strategy

A relatively small proportion of the cultured cells were capable of inducing resting-cell formation, though this is under viral titers that are likely higher than those in the environment. In the oceans, viral titer would likely increase more gradually because biotic and abiotic factors may cause temporal variability of infection leading to a multi-step infection (i.e., viral replication and lysis may occur multiple times before population termination). Therefore, evasion by resting cell formation could be a more significant strategy for long-term survival in populations with a viral outbreak. In the natural environment, these resting cells would presumably germinate when conditions were appropriate to allow the rebounding of the algal population, including a reduced viral load. In laboratory cultures, the resting cells that formed following HaV exposure germinated within 8 days after dilution with fresh media. Significantly, the resulting vegetative cells remained susceptible to HaV infection when exposed to new virus lysate (data not shown). For the strategy of resting cell formation to be effective, the cells would have to have either immunity to infection or reduce the probability of viral interaction,
perhaps by sinking to the bottom. To test if resting cells were resistant to virus infection, we transferred an actively-growing culture of *Heterosigma* to 4°C in the dark for 7 days, which are conditions known to induce resting-cell formation (Imai and Itakura 1991, Taylor and Haigh 1993). Resting cells were then divided into HaV exposed and unexposed (control) and kept at 4°C in the dark, transferred to 20°C in the dark, or returned to the normal growing conditions (20°C in the light). Resting cells transferred to the normal conditions germinated and resumed growth, but in the presence of HaV the culture had evidence of viral lysis starting after the second day and the culture eventually crashed (Figure 4-4). Resting cells exposed to the virus under conditions not favourable for germination (4°C/dark) remained in the culture after 8 days (Figure 4-4). However, when these cultures were returned to germination-permissible conditions, the resting cells germinated and then were lysed by the virus (Figure 4-4C). Under 20°C in the dark, conditions that are also unfavourable to germination, neither culture survived the eight-day incubation, with or without the addition of virus (Figure 4-4B) likely because these cells had insufficient energy to fuel essential metabolic activity. Resting cells triggered by cold-dark conditions were successful at delaying viral-induced lysis (Figure 4-5).

When an actively growing *Heterosigma* culture exposed to HaV under normal growth conditions for 24 h was triggered to form resting cells by transferring into the dark at 4°C, the progression of the infection was halted as vegetative cells transitioned into the resting form over the subsequent 5 days (Figure 4-5). The control infection, however, was almost entirely lysed by the second day (Figure 4-5). Transfer to 4°C likely delayed viral burst from infected cells, allowing more
time for resting-cell formation. When control cultures were returned to conditions that allowed germination, the resting cells were activated within a day (Figure 4-5). Resting cells from HaV-infected cultures also reactivated, but then stalled (Figure 4-5). Virus lysed newly germinated vegetative cells while the remaining resting cells failed to germinate and remained at the bottom of the culture tubes. The remaining resting cells may abort germination when components of lysed cells are perceived, possibly via cell-to-cell communication.

We propose that resting-cell formation blocks new infections, likely through changes in the plasma membrane and specific protein structure such that the appropriate receptors are not present or inactive. It also seems that resting-cell formation halts the infection cycle, likely by changes in the general metabolic rate and or the activity of any number of proteins involved in translation, replication and the cell cycle that may be required for the completion of the HaV life cycle.

Caspase-like enzymes: involved in resting-cell formation

While there are well-known environmental cues such as temperature, light, nutrient status, and now virus exposure that are triggers for resting-cell formation, the cellular mechanisms that regulate the transition between vegetative and resting stages are not known in Heterosigma. We have evidence to suggest that the pathway, at some stage, requires caspase-like activity. Thus we speculate the caspase-like enzymes are involved in cyst formation and also programmed cell death in this alga. Cultures treated with the caspase-inhibitor z-VAD-FMK prior to viral infection showed a consistently lower capacity to form resting cells (3.9 ±
0.08 %) compared to the non-treated controls (7.2 ± 0.1 %, Figure 4-1), though the differences were not significant (t-test, p=0.0539). However, when resting cell formation was induced at 4°C in the dark, cultures treated with the caspase-inhibitor, showed a delayed transition to the resting-cell stage (Figure 4-6). This transient delay was unusual, stalling at a resting cell proportion of 40%, but achieving equivalent proportion of resting cells as the control by day 4 (Figure 4-6). This suggests that in cultures treated with caspase-inhibitor, there was a bottleneck during the transition to resting cells that was alleviated by changes in the inhibitor effectiveness or stability. We hypothesize that the caspase inhibitor is blocking a stage in the cell cycle that prevents the majority of cells forming resting cells but allows others to proceed, perhaps because these cells have already transitioned through that stage. This transient delay could have increased the susceptibility to virus infection, explaining the reduction in the amount resting cells induced by HaV presence observed in Figure 4-1.

Conclusions

Here we observed, for the first time, that Heterosigma cells are destined for three different cell fates after exposure to HaV. Infection that leads to lysis via an apoptosis-like cell death pathway (Chapter 3), an evasion strategy as a long-term survival mechanism in response to viral exposure that involves the formation of non-motile, resting cells, and cell survival by an unknown mechanism. Resting cells are resistant to viral infection, though they remain sensitive to the virus in the subsequent vegetative phase. The nature of the triggering mechanism in response to
infection is unknown, however, it is plausible that the cells may either be sensing molecules released from the lysed cells or by possible structural variability in viral-proteins or defective viral-genes that might cause attachment but not successful infection. In *E. huxleyi*, only the bloom-forming, diploid phase is susceptible EhV (*Phycodnaviridae*) and virus exposure can trigger transition into the resistant, haploid, resting phase (Frada et al. 2008). As in *H. akashiwo*, the specific mechanism that trigger the transition are not known in *E. huxleyi*. Understanding these pathways will be an important advance in the deciphering the population dynamics of natural phytoplankton populations and perhaps leading to a strategy for limiting toxic algal bloom formation.
Chapter References


Dingman, J.E. and Lawrence, J.E. 2012. Heat-stress-induced programmed cell death in


Falkowski, P.G. 1994. The role of phytoplankton photosynthesis on global

formation in phytoplankton: a response to allelopathic competitors? Environ.
Microbiol. 6: 791–798.

"Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in
response to viral infection. PNAS. 105: 15944-15949.

aspects of viral infection and lysis in the harmful brown tide algae *Aureococcus

Han, M., Kim, Y. and Cattolico, R.A. 2002. *Heterosigma akashiwo* (Raphidophyceae)
resting cell formation in batch culture: Strain identity versus physiological

Imai, I. and Itakura, S. 1991. Densities of dormant cells of the red tide flagellate

*Heterosigma akashiwo* (Raphidophyceae) in bottom sediments of northern

Imai, I. 1989. Cyst formation of the noxious red tide flagellate *Chattonella marina*

Imai, I. and Itakura, S.1999. Importance of cysts in the population dynamics of the red


Figure 4-1. Relative abundance of *Heterosigma akashiwo* vegetative, resting and dead cells remaining six days post-HaV infection (d.p.i). The cultures included the uninfected, control culture (black bars), the HaV-infected cultures (grey bars) and the HaV-infected cultures treated with caspase inhibitor (z-VAD-FMK; white bars). Cultures were kept in the light at 20°C for 6 days post-infection and the proportion of vegetative, resting and dead cells determined. The total number of dead cells for each replicate was calculated as the difference between the cell abundance before and after culture decline, minus the number of resting cells. Error bars ±1 std.dev.
Figure 4-2. Cell and resting-cell morphology of *Heterosigma akashiwo* cultures A) under control culture conditions (20°C, 14:10 light:dark), B) cold treated (4°C, dark) and C) after viral infection with HaV under bright field (A, B, and C) and epifluorescence (D - control, E - cold treated, and F - viral infection).
Figure 4-3. Surviving *Heterosigma akashiwo* cells from cultures previously exposed to HaV exhibit resistance to subsequent HaV exposure. A) Cell lysis occurs in cultures not previously exposed to HaV 3 days post-infection (d.p.i) (open boxes) while cells in cultures that were previously exposed to HaV (open circles) do not (closed boxes). Resistant cultures were challenged with HaV approximately 1 month after initial infection. B) Resistance in the survivor cultures is retained for at least 12 months. n=3 for control and infection control, n=12 for survivors and re-infection of survivors. Error bars ±1 st. dev.
Figure 4-4. The effects of light and temperature on *Heterosigma akashiwo* resting-cell germination, culture growth and susceptibility to HaV. Mother cultures (n=3) were subjected to 4°C (dark) conditions for 7 days to produce resting cells and then split into treatment groups. (A) Cultures were exposed to normal culture conditions (20°C, 14:10 light:dark cycle) where culture growth occurred immediately. (B) Cultures were placed in the dark at 20°C, resulting in population decline. (C) Cultures remained in cold-dark conditions (4°C) and then transferred to normal culture conditions. Cultures were also challenged with HaV lysate and monitored (using relative fluorescence) for at least 8 days. Successful viral infection was observed in the cultures at 20°C in a 14:10 light:dark cycle. Cultures kept in the dark at 20°C (with or without viral infection) died by the 8th day. There was no change in the culture kept in the dark at 4°C until returned to normal culture conditions (after dashes line), where resting cells germinated and viral infection proceeded. Error bars ±1 st.dev.
Figure 4-5. HaV infection leading to lysis in *H. akashiwo* during normal culture conditions (A) and during a 5-day cold-dark phase (B), beginning at 1 day post inoculation, ending at 6 days post inoculation. Error bars represent ± 1 st. dev.
Figure 4-6. Rate of resting-cell formation in *H. akashiwo* in 4°C, dark conditions in control cultures (closed squares) and cultures treated (open circles) with the caspase-inhibitor z-VAD-FMK. Error bars represent ±1 st. dev.
Chapter 5 – GENERAL DISCUSSION

The objective of this work was to better understand the host-virus dynamics between three *Heterosigma* viruses (HaRNAV, HANIV and HaV) and their host, *Heterosigma akashiwo*. The main goals were to determine 1) if cell death by viral infection in *Heterosigma* triggered classic PCD characteritics, 2) if there are differences in cell responses to each virus, and 3) to assess the potential role of virus infection on population cycling including PCD and lysis, resting-cell formation and resistance. Because PCD has not previously been described in *Heterosigma*, I first wanted to examine the potential of inducing PCD in response to a classic, abiotic stress - high temperature. I used heat-stress to optimize standard PCD assays before assessing PCD hallmarks during viral infection. In this model organism, no previous studies existed to determine heat-stress thresholds nor had any of the methods used to identify hallmarks of PCD been modified for *Heterosigma*. After optimizing a 1 h heat-stress and adapting the protocols for a few available PCD-hallmark assays, I assessed *Heterosigma* cultures for PCD hallmarks during viral infection. Among the findings of this work, I explored the unexpected but intriguing results of resting-cell formation during heat-stress and viral infection.

*Heat Stress - A Positive Control for PCD*

This study demonstrates that *Heterosigma* cells survive and exhibit normal growth after heat-stress for 1h at 35°C, and undergo PCD when exposed to 37 - 40°C. Exposure to 50°C predominantly causes resting-cell formation, but also
approximately 1/8th of the population undergoes necrosis. On a population level, exposure to extreme temperature (50°C) may be a more survivable stress than the 37 - 40°C because the majority of the population (85%) form resting cells. Although these high temperature ranges are not ecologically realistic, they may indicate that *Heterosigma* is better suited to survive a short, severe stress rather than a short, mild stress.

*Heterosigma* undergoing cell death exhibited hallmarks consistent with apoptosis-like cell death including DNA fragmentation, PS externalization and caspase-like enzyme activation. The classic PCD hallmark of DNA laddering, visualized by DNA electrophoresis, was not detected. Lack of DNA laddering could indicate that histones are irregularly spaced, that cells are not synchronously undergoing PCD or DNA is not specifically cleaved between nucleosomes. Other organisms have been conclusively shown to undergo PCD without visible DNA laddering, including yeast (Madeo et al. 1999), the green alga *Chlorella saccharophila* (Zuppini et al. 2007), and the dinoflagellate *Peridinium gatunense* (Vardi et al. 1999). After 1 h heat shock in *Heterosigma* populations, *in situ* DNA fragmentation is the most representative estimate of impending death via PCD.

In *Heterosigma*, externalization of PS is an early indication of PCD after 1 h heat stress, although the phenomenon is too ephemeral or the assessment regime (every 24 h) was too long to provide a good indication of the proportion of impacted cells, and therefore may lead to false-negatives. In multicellular organisms, PS externalization can allow cell-to-cell communication. In multicellular organisms, membrane alterations during apoptosis include
externalized phosphatidyleserine (PS), a well-studied process (Witasp et al. 2008, Savill 1993, Williamson and Schlegel 1994, Fadok et al. 1992). Cells undergoing apoptosis use signaling molecules, including PS, which are recognized and quickly engulfed by phagocytic cells to prevent inflammation of surrounding tissue (Fadeel et al. 2010, Elmore 2007). Many unicellular organisms exhibit externalization of PS residues during apoptosis-like cell death; however, the function remains unclear (Nyoman and Lüder 2013, Bidle and Bender 2008). However, there is evidence that infochemicals, including aldehydes and Nitric Oxide, can influence cell demise in marine diatoms (Vardi et al. 2006, Bidle 2015), although the understanding of relative infochemical signals in phytoplankton is in it infancy. Another confounding factor reducing the reliability of PS testing is that phospholipids in liquid phase, which occur at high temperatures, move more freely between the inner and outer leaflet of the plasma membrane (Contreras et al. 2010). It is possible that heat stress causes a change in phase of the lipid bilayer of *Heterosigma* allowing passive movement.

Caspase-like enzymes were also detected, using the fluorescence assay CaspACE designed to detect characteristic caspase biochemical activity. These caspases are cysteine proteases that cleave substrates immediately after an aspartate residue (Uren et al. 2000, Carmona-Gutierrez et al. 2010). However, it is now known that there are two other families of caspase-like enzymes: Paracaspases (found in metazoans and *Dictyostelium*) and Metacaspases (found in plants fungi and protozoa) (Uren et al. 2000). Metacaspases, the caspase-like enzyme likely produced by *Heterosigma*, cleave proteins following arginine or lysine rather than
aspartate (Carmona-Gutierrez et al. 2010). Although caspases and metacaspases are phylogenetically distant and have different proteolytic specificities, there is emerging evidence to support the homology between these enzymes. For example, caspase-3, a major execution caspase, has similar cleavage affinity to metacaspases found in yeast (Carmona-Gutierrez et al. 2010). Thus the CaspACE assay, can reliably detect caspase-like enzymes and provide evidence for PCD, although there is still a missing link between metacaspase genes and the genes or proteins likely responsible for the observed caspase activity in Heterosigma. Additionally, positive caspase detection is not definitive evidence for PCD as these enzymes can often be components of other cell signalling cascades that do not lead to death (Los et al. 2001).

Host-Virus interactions

a) Susceptibility - Viral Induced PCD

When Heterosigma cultures are exposed to HaRNAV, HaNIV or HaV lysates, the culture crashes because the majority of cells undergo cell lysis. The lytic length is longer during HaRNAV and HaNIV infections compared to HaV (72 h and 48 h, respectively). During this shorter timeframe, HaV kills a larger proportion of the culture than HaRNAV and HaNIV. However, we are just beginning to understand the interactions between Heterosigma and the viruses that infect it. For example, no conclusive evidence shows the optimal viral load to produce a successful one-step infection. Also, the number of nonviable virus particles produced during infection is unknown. All three viruses do not reliably developed
into a successful infection at each inoculation. It is possible that the variability in viral titre was too low to consistently produce a successful infection.

During productive viral infection, caspase-like enzyme activity was detected in *Heterosigma* with all three viruses. However, *in situ* DNA fragmentation was observed with only HaNIV and HaV infections, and phosphatidylserine externalization was only observed during HaV infection. This suggests that this algal host utilizes different cell-death programmes during HaRNAV, HaNIV and HaV infections, outlining the complexity of virus-induced mortality in *Heterosigma*.

The programmed cell death pathway may have evolved through a host-virus arms race. PCD during viral infection is speculated to limit viral production and reduce or abolish viral progeny spread throughout the population (Teodoro and Branton 1997). Therefore, viruses have evolved strategies to evade progeny attenuation caused by PCD. There are many examples of animal viruses containing genes responsible for inhibition or promotion of the PCD pathway. African swine fever, Human adenovirus, Epstein-Barr virus and Herpes simplex virus have all been shown to have evolved similar PCD inhibiting strategies which block specific PCD-regulatory proteins and promote cell survival (Teodoro and Branton 1997). Alternatively, PCD late in lytic viral infection represents an efficient mechanism for viral progeny escape (Bidle and Falkowski 2004, Bidle 2015). Viruses can be contained and transported within apoptotic bodies (small compartmentalized components of the original cell), which can then be phagocytosed by surrounding cells whereby the virus evades extracellular antibodies or environmental
degradation. Chicken Anemia virus, Human Adenovirus and Human Influenza virus-1 have been shown to contain PCD promoting genes (Teodoro and Branton 1997). Other viruses such as *Emiliania huxleyi* virus (Bidle et al. 2007, Wilson et al. 2005) and Hepatitis C virus (Best and Bloom 2004) require PCD related, caspase-like enzymes to cleave host proteins and complete viral maturation and therefore virus infection actively triggers PCD in their host (Schatz et al. 2014, Ray et al. 2014). Although the PCD plays a role in the relationship between host and virus in many different systems, the complexity of this relationship remains elusive.

*b) Viral Evasion- Formation of Resting cells*

In addition to cell death during viral infection with HaV, a small proportion of the *Heterosigma* population forms non-motile, resting cells. This phenomenon was not observed during HaRNAV or HaNIV infections, although more study is needed to draw conclusions. Resting-cell formation during HaV infection represents a major defensive mechanism and evasion strategy that prevents total population loss during wide-spread infection. These cells are resistant to viral infection, though they remain sensitive to the virus following transition to the vegetative phase. Thus this stage represents a short-term evasion strategy for *Heterosigma*. Resting-cell formation is somehow triggered, whether by cell-to-cell communication or by cells perceiving the virus particles themselves, which acts as a twofold protection mechanism for further infection: 1) cells sink out of the photic zone where active viral infection is occurring and 2) full-formed resting cells are not susceptible to viral infection - whether because of alterations in plasma
membrane receptors which prevent attachment or by a reduction in cellular metabolism that prevents the completion of infection.

c) Viral Resistance - Natural or Acquired Resistance

A small proportion of cell (<1%) of cells survived infection with HaV and were subsequently resistant to addition challenges with HaV lysate for many months. There are a varities of possible explainations for this phenomenon. The strategy may be intrinsic to the host-virus system where genetic variability within the culture allows for a small proportion to be unreceptive to the virus or the resistance is gained, perhaps by down-regulation in virus-associated receptors or some other cellular characteristic required by HaVs (Bidle and Kwityn 2012, Fulton et al. 2014). Another plausable explaination is that a small proportion of the population is undergoing a non-lethal, chronic infection where infection proceeds without causing lysis, rather a slow "shedding" of virus progeny over several generations (Fuhrman 1999). Survivors from HaRNAV and HaNIV infection were not tested for resistance. In other systems, resistance is physiologically expensive because resistance cells often lack or have lost important receptors, so even though they may avoid viral infection, they cannot become dominant among a virus-susceptible population (Furhman 1999).

Future Work

To understand the significance of PCD on nutrient cycling driven by Heterosigma, we must first estimate the standing stock of phytoplankton undergoing PCD. I predict the proportion of nutrients that are lost through
sedimentation in blooms where PCD is the predominate termination mechanism would be higher than that of blooms where necrosis is predominant. As cells undergo PCD, they become non-motile and, therefore, are likely to sink before they rupture while necrotic cells can rupture almost immediately after a catastrophic stressor. The evidence that cells sink before they burst is that viruses, which are so tiny they rarely sink, are found in high abundance sediments. Likely they are transported to the ocean floor through infection of already sinking cells or that cells begin to sink as an artifact of infection (Lawrence and Chan 2002). Therefore, cells undergoing PCD may contribute more heavily to nutrient deposition to the deep ocean while nutrients contained in quickly dying cells, those undergoing necrosis, are more likely to stay in the photic zone and be utilized by other organisms.

Another key to understanding the interactions between host and virus is to understand the intricacies of the checkpoints of infection. Specifically, how do *Heterosigma* viruses attach to the host's plasma membrane? Is attachment sufficient to elicit a cell response (i.e. PCD and/or resting-cell formation)? Experiments including inactivated viruses (where attachment is possible but genome injection is not) could show if the PCD response occurs after the attachment checkpoint during viral infection.

*Concluding remarks*

In natural environments, a combination of PCD, necrosis and cyst formation are involved in bloom termination. The triggers of these cell responses are both biotic (viral infection) and abiotic (temperature and light cues) and thus
*Heterosigma* blooms likely are in constant flux because of changing seasons, tidal patterns, and viral infection. Making prediction of onset and duration of blooms is difficult and multifaceted. Thus reducing or eradicating these harmful and costly blooms has proven to be very difficult.
Chapter References


Nyoman, A.D.N. and Lüder, C.G.K. 2013. Apoptosis-like cell death pathways in the unicellular parasite *Toxoplasma gondii* following treatment with
apoptosis inducers and chemotherapeutic agent: a proof-of-concept study.

Bidle, K.D. and Sandaa, R. 2014. Virus infection of *Haptolina ericina* and
*Phaeocystis pouchetii* implicates evolutionary conservation of programmed cell
943-955.


Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S.G., Ben-Dor, S. and
Vardi, A. 2014. Hijacking of an autophagy-like process is critical for the life
cycle of a DNA virus infecting oceanic algal blooms. New Phytologist. 204(4):
854-863.

Teodoro, J.G. and Branton, P.E. 1997. Regulation of apoptosis by viral gene

Uren, A.G., O’Rourke, K., Aravind, L., Pisabarra, M.T., Seshaqiri, S., Koonie, E.V.
and Dixit, V.M. 2000. Identification of paracaspases and metacaspases:
two ancient families of caspase-like proteins, one of which plays a key role


Vardi, A., Berman-Frank, I., Rosenberg, T., Hadas, O., Kaplan, A. and Levine, A.
1999. Programmed cell death of the dinoflagellate *Peridinium gatunenseis*


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Dingman, J.E., Roherty, J.K., Nagasaki, K., Lawrence, J.E. 2012. Cell death programs of the harmful bloom-forming Raphidophyte, Heterosigma akashiwo, during viral infection with HaRNAV, HaNIV or HaV. North East Algal Symposium. Schoodic Point, ME, USA. (Presentation)
Havegna, M., Dingman, J.E. and Lawrence, J.E. 2011. Examination of programmed cell death hallmarks in Micromonas pusilla by viral infection with MpV

