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I am submitting herewith a thesis written by Julie Linda Higgins entitled "Virus Dynamics in High-Nutrient, Low-Chlorophyll Marine Surface Waters." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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VIRUS DYNAMICS IN HIGH-NUTRIENT, LOW-CHLOROPHYLL MARINE SURFACE WATERS

A Thesis
Presented for the
Master of Science Degree
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Julie Linda Higgins
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DEDICATION

This thesis is dedicated to my biggest fans: my parents, Andrew and Linda Higgins who always told me that I could be anything I wanted to be; and my sister, Jennifer Ansley Black and brother, Scott Higgins, my best friends and role models, for their continued love, support and inspiration.

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ABSTRACT

Iron (Fe) limitation of primary productivity in high-nutrient, low-chlorophyll (HNLC) regions is relatively well-studied. Iron fertilization experiments as well as bottle incubations have been used to study changes in phytoplankton community biomass and diversity, changes in bacterial growth rates, etc. However, viral activity has been largely ignored in these studies. Viral activity was monitored during an iron budget study (FeCycle) in the HNLC waters of the Southern Ocean southwest of New Zealand as well as during a mesoscacle iron fertilization in the subarctic Pacific (SEEDS II). The goal of these studies was to evaluate the role of viruses in the lysis of bacterial cells and the subsequent regeneration of iron and other key nutrients. Two methods, a transmission electron microscopy (TEM) approach and a dilution assay, were used to measure viral production in each study and comparisons were made as to the appropriateness of each. From these studies, it appears that the viral community indirectly responds to changes in trophic production as observed by changes in virus abundance and production, while burst size and frequency of infection remain constant. These results suggest that there is a decrease in the length of lytic cycle after productivity is stimulated. Virus-induced lysis was found to regenerate up to 70 pM Fe in the Southern Ocean, and nearly 200 pM Fe in the subarctic Pacific. While there is little doubt as to the usefulness of TEM and its importance in determining lytic burst sizes in natural populations, the observations in this study suggest that there are problems associated with inferences concerning community mortality from such observations, especially during periods of trophic change.

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LIST OF SYMBOLS

*a*g	atograms (10^{-18}grams)
C	carbon
CO_2	carbon dioxide
Fe	iron
fg	femtograms (10^{-15} grams)
kDa	kiloDaltons
KeV	kiloelectronvolts
kg	kilograms
L	liters
mL	milliliters
µatm	microatmospheres
µg	micrograms
µL	miroliters
µM	micromolar
µm	micrometer
nM	nanomolar
N	nitrogen
P	phosphorus
pM	picomolar
SF_6	sulfur hexafluoride
Si	silicate

ABBREVIATIONS

DOC	dissolved organic carbon
DOM	dissolved organic matter
EisenEx	eisen (iron) experiment
ESAW	enriched seawater/artificial water
FIC	frequency of infected cells
FVIC	frequency of visibly infected cells
GPS	global positioning system
HNLC	high-nutrient, low-chlorophyll
IronEx	iron fertilization experiment
NIWA	National Institute for Water and Atmosphere
POM	particulate organic matter
SEEDS	subarctic Pacific iron experiment for ecosystem dynamics study
SERIES	subarctic ecosystem response to iron enrichment study
SOFeX	Southern Ocean iron experiment
SOIREE	Southern Ocean iron release experiment
TEM	transmission electron microscopy
VIC	visibly infected cells
VIM	viral-induced mortality of bacteria
VMB	viral-mediated bacterial mortality

BACKGROUND

1. The Microbial Food Web

Traditionally, phytoplankton, which are responsible for nearly 50% of the photosynthesis on the planet (Field *et al.* 1998), are considered to be the lowest trophic level in marine food webs. These primary producers are grazed upon by zooplankton, providing carbon flow through the food web as well as leading to the liberation and recycling of both organic and inorganic nutrients. Recent attention, however, has also been focused on the interactions of viruses with members of the microbial community, including phytoplankton as well as protozoans and heterotrophic bacteria (Figure 1). Bacteriophage (from Greek *bakterion* "small staff", *-phagos*, "eater of", viruses infecting prokaryotic cells) and heterotrophic bacteria play a major role in the recycling of dissolved organic matter (DOM) as well as other nutrients (Fuhrman 1999). Referred to as the "microbial loop" (Azam *et al.* 1983), this model shows how bacteria feed on carbon released from phytoplankton, thus converting DOM into particulate organic matter (POM). Bacteria are then grazed upon by protozoans, again liberating nutrients for use by zooplankton. Viral infection contributes to this process by shunting organic nutrients into the dissolved phase where they are assimilated by heterotrophic bacteria (Fuhrman 1999; Wilhelm & Suttle 1999). Because the nutrients released by viral lysis of bacterial cells are organically complexed, and this supply is probably differentially bioavailable relative to that which is not bound to organics (Gobler *et al.* 1997; Wilhelm and Suttle 2000; Poorvin *et al.* 2004), much of this organic material is kept within the microbial loop and away from secondary consumers, who primarily use POM (Wilhelm and Suttle 1999).

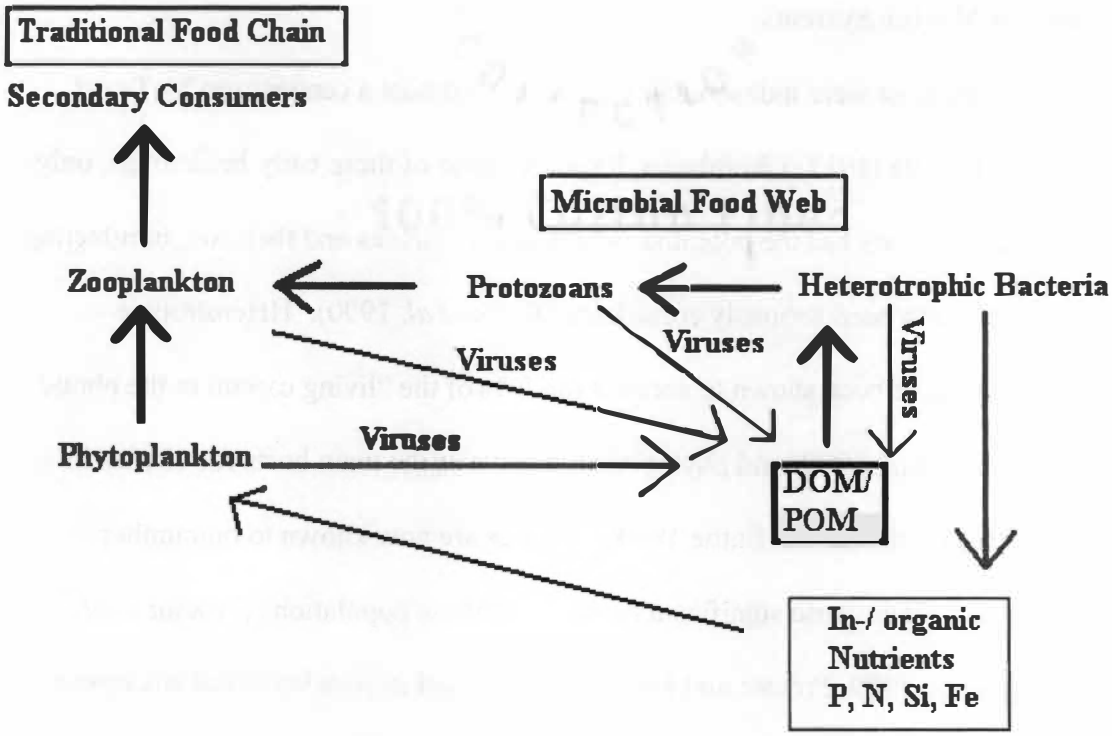

Figure 1. The Microbial Food Web
Illustration showing the importance of the microbial food web to nutrient cycling. Heterotrophic bacteria, protozoans and viruses contribute to the DOM pool. Viral lysis of microbial communities releases DOM, POM, and organic nutrients, which are then used by other members of the community (modified from DeBruyn *et al.* 2004).

2. Viruses in Marine Systems

Bacteriophage were independently discovered almost a century ago by Twort (1915) and d'Herelle (1917) (Weinbauer 2004). In spite of these early beginnings, only during the last 15 years has the potential importance of viruses and their role in infecting eukaryotic plankton been seriously considered (Suttle *et al.* 1990). Heterotrophic bacteria, which have been shown to account for 70% of the "living carbon in the photic zone" (Fuhrman *et al.* 1989) and phytoplankton serve as the main hosts for viruses in marine systems (Wilhelm and Suttle 1999). Viruses are now known to outnumber prokaryotes as well as cause significant mortality to these populations (Proctor *et al.* 1988; Bergh *et al.* 1989; Proctor and Fuhrman 1990), and to populations of eukaryotic algae (Sieburth *et al.* 1988; Suttle *et al.* 1990). Fuhrman and Noble (1995) reported that the extent to which viruses cause bacterial mortality is close to that caused by protists (*i.e.* grazing). Such findings have led to increased interest in the ecological role of viruses in marine systems.

Nutrients released as a result of viral lysis of bacteria are, in most cases, thought to be organically complexed, while grazing activity releases a mixture of organic and inorganic nutrients (Wilhelm and Suttle 2000; Poorvin *et al.* 2004). It is thought that marine plankton are better able to use some nutrients such as iron when they are organically bound (Rue and Bruland 1995; Poorvin *et al.* 2004), so particular interest has been placed on the potential impact that viral activity may have on nutrient cycling in natural systems. DOM (particles with < 0.2 μm diameter) is used by heterotrophic bacteria and phytoplankton, while POM (> 0.2 μm diameter) is taken up by zooplankton and other grazers. It has recently been shown that most of the organic matter released by

viral lysis of bacteria is into the dissolved phase (Poorvin *et al.* 2004). These materials include nucleic acids (Reisser *et al.* 1993; Weinbauer *et al.* 1995), carbohydrates (Weinbauer and Peduzzi 1995) and small proteins (Hirayama *et al.* 1993). Some dissolved nutrients are quickly assimilated by heterotrophic bacteria, while others require enzymatic and grazer activity to be broken down further. Larger proteins and cellular debris (*e.g.*, cell walls) are thought to operationally partition into the particulate form (Wilhelm and Suttle 1999).

Using radioactive tracers in laboratory studies, Gobler *et al.* (1997) demonstrated that the lysis of the marine algae *Aureococcus anophagefferens,* resulted in a nearly 150% increase in the concentration of dissolved organic carbon (DOC) relative to uninfected controls, and that this DOC resulted in a ten-fold increase in bacterial abundance. From this, Gobler *et al.* predicted a potential 40 μM increase in DOC if a natural bloom of *A. anophagefferens* were subjected to viral lysis. This study also demonstrated that the nutrients released were assimilated by (and therefore bioavailable to) other organisms. Similar increases in dissolved phosphorous (117%), selenium (150%), and iron (135%) were seen as well, followed by a decrease in dissolved forms of these nutrients and a rise in the particulate phase, indicating bacterial uptake. Similarly, Middelboe *et al.* (1996) reported a 72% increase in DOC uptake after viral lysis of heterotrophic bacteria. Although a two- to three-fold rise in bacterial production was reported in this study, a 66% decrease in the biomass production to substrate ratio was also seen, indicating that the bacteria required energy to assimilate the cellular products of viral lysis, and are converting more DOC toward energy than into biomass. Another important study (Poorvin *et al.* 2004) demonstrated that 250% more iron was in the

dissolved phase following viral lysis of both cyano- and heterotrophic bacteria. Additionally, this iron was assimilated more rapidly than iron leaked from unlysed control cells or iron added in inorganic forms.

Other studies, summarized in Table 1, have shown the estimated nutrients and carbon released each day as a result of viral lysis of bacterial cells. These estimates are determined by multiplying the number of cells lysed each day by the predetermined cellular quotas [iron (1.1 *a*g cell^{-1}; Tortell *et al.*1996), phosphorus (0.5 fg cell^{-1}; Heldal, *et al.* 1996), nitrogen (5.6 fg cell^{-1}; Lee and Fuhrman 1987), and carbon (10 fg cell^{-1}; Fukuda *et al.* 1998, or 23.3fg cell^{-1}; Simon and Azam 1989)]. The abundance of cells lysed each day is determined by dividing the experimentally determined viral production rate by the number of viruses released per lytic event (burst size). Burst sizes are either estimated (usually 25 to 50) or determined by transmission electron microscopy for each study.

Table 1. Viral Activity and Regeneration of Nutrients. Values in italics were not calculated in the reported study, but determined using viral production estimates given in each reported study.

Location	Burst Size	FVIC%	C released ($\mu M\ d^{-1}$)	N released ($\mu M\ d^{-1}$)	P released ($nM\ d^{-1}$)	Fe released ($pM\ d^{-1}$)
Lake Plußsee[a]	5-500	0.5-6.4%				
Lake Constance[b]	21-121					
Subtropical Pacific[c]			*1.89-7.46*	*0.39-1.54*	*15.74-62.05*	19.2-75.7
Gulf of Mexico (offshore)[d]	10-23		0.01-0.05	0.001-0.008	*0.08-0.38*	*0.01-0.46*
Gulf of Mexico (coastal)[d]	29-64		0.06-0.43	0.01-0.07	*0.5-3.6*	*0.61-4.4*
Gulf of Mexico (phytoplankton)[e]		VIM[1] 2-10%	0.12-0.35*			
Gulf of Mexico (phytoplankton)[f]		VIM[1] 5-14%	0.15**			
Bering and Chukchi Seas[g]		VIM[1] 9-23%	0.02-0.25	*0.005-0.051*	*0.21-2.8*	*0.25-2.54*
Strait of Georgia (stratified)[h]			0.09-0.13	0.02-0.03	0.65-0.97	0.9-1.3 x 10^3
Strait of Georgia (mixed)[h]			0.17-0.69	0.03-0.14	1.29-5.81	1.6-7.0 x 10^3

[1]VIM (viral-induced mortality of bacterial cells) measured according to Proctor and Fuhrman (1990). *Assuming 63-67 fg C $cell^{-1}$; **Assuming 125 fg C $cell^{-1}$
[a]Weilbauer & Höfle 1998; [b]Hennes & Simon 1995; [c]Poorvin *et al.* 2004; [d]Wilhelm *et al.* 1998; [e]Cottrell & Suttle 1991; [f]Suttle & Chan 1994; [g]Steward *et al.* 1996; [h]Wilhelm & Suttle 2000.

3. The Iron Hypothesis

Eppley *et al.* (1973) reported that primary production in the majority of the World's ocean is limited by nitrogen, while silica can limit growth in some taxa (Swift 1981). Bacterial growth is not only limited by carbon availability (Ducklow and Carlson 1992), but likely by phosphorus (Thingstad *et al.* 1998) and nitrogen (Kirchman 1994) as well. Although Leibig's Law of the Minimum suggests that aquatic organisms should be limited by whatever nutrient is in lowest concentration, Brandt and Raben (1992) suggested that a single limiting factor may not be possible to find. The difficulty in identifying a single limiting factor is evident when one considers the complex interactions of light, salinity, temperature and gas exchange, in addition to the relative nutrient concentrations in a given region.

The special cases in the World's ocean are those areas with high nutrient content, but relatively low levels of phytoplankton biomass (measured in terms of chlorophyll *a*). Such "high-nutrient, low-chlorophyll" (HNLC) regions include the Southern Ocean, the Equatorial Pacific, and the subarctic Pacific. There are several possible factors that may limit primary production in these waters, including zooplankton grazing, which may keep phytoplankton populations at a level that prevents them from utilizing the major nutrients in the area, trace metal limitation, and low light and temperature, especially in the Southern Ocean and sub-Arctic Pacific (Chisholm and Morel 1991; Hutchins 1995).

Trace metal limitation, especially iron, is likely in many pelagic regions, given that much of the supply of many trace metals enters the ocean via atmospheric dust, and dust entering these areas was found to be low by Duce and Tindale (1991). Prior to these findings, Hart (1941) suggested that iron was a limiting nutrient in several Antarctic

regions, including the Weddell Sea. Experimental design was a major limiting factor in this work and other early studies of trace metal limitation (Harvey 1933, 1947; Gran 1931; Menzel and Ryther 1961), as contamination (usually in the sub-picomolar range) was a constant problem, causing many of these studies to be discounted. The trace metal clean techniques developed by Bruland *et al.* (1979) served to circumvent many of these problems. Martin later applied these techniques to conduct on-deck bottle incubation experiments where iron and other nutrients such as zinc, sulfur, and copper were added. After conducting these experiments in the three HNLC regions mentioned above, Martin et al. (1991) concluded that iron was a limiting nutrient of phytoplankton biomass. Coale *et al.* (1991) found that adding 0.86 nM iron in bottle incubations in the subarctic Pacific increased phytoplankton production by 360 % in the > 0.7-μm size-class and by 1300 % in the > 25 - μm size-class. In addition, chlorophyll *a* concentrations increased by 924 % after the addition of iron. Similar experiments in the Weddell and Scotia Seas have shown that adding iron to shipboard incubations did not stimulate primary production in all areas of the Southern Ocean (de Baar *et al.* 1990; Buma *et al.*, 1991).

Many HNLC regions are dominated by picoplankton (planktonic organisms < 2 μm diameter). These organisms are more efficient nutrient scavengers under oligotrophic conditions possibly due to their greater surface area-volume ratio and are more efficient at assimilating iron (Sunda and Huntsman 1997). Microzooplankton (grazing organisms ranging from 2 to 20 μm diameter) are responsible for keeping the populations of picoplankton lower by grazing (Price *et al.* 1994). When iron is added to these waters, diatoms are thought to proliferate because of their ability to grow at faster rates than both the organisms that normally graze upon them and the smaller species that they compete

with for nutrients when in limited amounts (Geider and LaRoche 1994). These interactions are difficult to study using shipboard bottle experiments, as they seldom account for grazing activity or vertical export in the water column (sinking, Buma *et al.* 1991).

The "Iron Hypothesis" was first proposed by John Martin (1990), who hypothesized that adding iron to HNLC waters would stimulate primary production, thus sequestering CO_2 into the sea. A man of bold words, Martin stated in 1988 that "300,000 tons of iron in the Southern Ocean would cause a phytoplankton bloom and remove 2 billion tons of carbon dioxide." and that "with half a shipload of Fe… I could give you an ice age." This proposition was not received well in the scientific community, as many felt and still feel that, to reduce global warming, the United States (the top producer of greenhouse gases) should lower fossil fuel emissions (Chisholm and Morel 1991). The outspoken Martin responded more conservatively stating that *if the need arises*, fertilizing the ocean with iron may be the most feasible way to remove atmospheric CO_2 by stimulating phytoplankton production (Martin *et al.* 1990). Not until Roberts (1991) stated it more calmly, saying that small-scale iron fertilization experiments would not have global impacts, but simply test Martin's hypothesis (1991), did the idea seem practical.

4. Iron Fertilization Experiments

A number of mesoscale iron fertilizations have been performed in the equatorial Pacific Ocean (Martin *et al.* 1994; Coale *et al.* 1996), the Southern Ocean (Boyd *et al.* 2000; Gervais *et al.* 2002; Coale *et al.* 2004), and the subarctic Pacific Ocean (Tsuda *et al.* 2003; Boyd *et al.* 2004) to test Martin's hypothesis that adding iron to HNLC waters would increase phytoplankton productivity. The first iron fertilization experiment (IronEx I; Martin *et al.* 1994) took place in 1993 in the equatorial Pacific. This study involved a single addition of iron (4 nM) as ferrous sulfate over an 8 x 8 km area. A three-fold increase in chlorophyll *a* concentration was observed, primary production increased by a factor of four, and atmospheric CO_2 decreased by 10 μatm. However, the iron patch seemed to sink by day 4 of observation due to a marked decrease in salinity. At that time, primary production and chlorophyll *a* also decreased. It remains unclear if these observations were related, or if production in the area became limited by the availability of other micronutrients such as zinc or silicate.

In May 1995, the IronEx II study (Coal *et al.* 1996) addressed the problems encountered in IronEx I, discussed above and examined the activity of grazers and their potential effects on bloom formation. Before fertilization, the study area was surveyed to ensure that the patch was uniform and to eliminate the possibility of the patch sinking again. The study area received two further iron infusions (1 nM) on days 3 and 7 after the initial addition (2 nM) on day 1. At day 9, a 26-fold increase in chlorophyll a was reported, as well as a rise in diatom abundance (85x) and grazing rates. Bottle incubations indicated that the area was not limited by zinc or silicate. Due to these

findings, the authors from IronEx II claimed that the iron hypothesis should be termed the "iron theory" (Coale *et al.* 1996).

The Southern Ocean iron release experiment (SOIREE) took place in February 9-22, 1999 in the Australian sector of the Southern Ocean. Before iron fertilization, the area was found to have high concentrations of nitrate (25 μM) and phosphate (1.5 μM), medial concentrations of silicate (10 μM), and a low iron concentration (0.08 nM). After a 3 nM addition of iron on day 1 and three further 1 nM amendments on days 3, 5, and 7, a six-fold increase in chlorophyll *a* (primarily diatoms) and 75 % rise in primary production rates were reported. There was not a significant change in bacterial abundance, but a three-fold increase in bacterial production was observed. Increases in nitrate, phosphate, and silicate uptake were also seen, indicating that these nutrients were not limiting in the area at the time of the study (Boyd *et al.* 2000).

The SOIREE study prompted Gervais *et al.* (2002) to explore the effects of iron amendments in the Atlantic sector of the Southern Ocean. Eisen (eisen = iron in German) experiment (EisenEx) took place in the Austral spring (November, 2000), rather than summer, as in SOIREE (Boyd *et al.* 2000). Again, non-limiting concentrations of nitrate (> 20 μM), phosphate (>1.5 μM), and silicate (10 μM) were found in the area prior to fertilization, along with very low iron concentrations (< 0.1 nM). Three iron additions were made during this study, although the resulting iron concentrations were not reported. There was a five-fold increase in chlorophyll *a*. Primary production rates were also raised three and a half times. There was also a noteworthy shift in the dominant algal size class. Picophytoplankton (< 2 μM diameter) accounted for 40 % of the total chlorophyll *a* at the beginning of the study, but only made up 13% by the end of EisenEx.

Nanophytoplankton (2-20 μM diameter) decreased from 50 % to 44 %, while microphytoplankton (> 20μM) increased from 10 % to 43 % of the total chlorophyll *a* concentration in the area. These findings seem to confirm the ideas of Geider and LaRoche (1994) that diatoms (> 20 μM) would proliferate upon the addition of iron.

The most recent iron fertilization of the Southern Ocean, and perhaps the most unique to date, was the Southern Ocean iron experiment (SOFeX; Coale *et al.* 2004). In this study, low (< 3 μM)- and high (~ 60 μM)-silicate waters of the Southern Ocean were amended with three (1.2 nM) and four (0.7 nM) additions of iron respectively. Also unique to this experiment, the researchers left the experimental area and returned 4 weeks later to survey the patch dynamics. At this time, rates of photosynthesis had increased 21-fold in the low silicate waters, and 14-fold in the high-silicate waters, probably due to the increased amount of iron in the former, as well as higher temperatures. As expected, an increase in phytoplankton biomass was seen, but was dominated by diatoms only in the high-silicate patch, which increased by a factor of 20. In both patches, atmospheric CO_2 decreased by nearly 40 μatm. Wingenter *et al.* (2004) reported on changes in different gases over the course of SOFeX. They reported a 4-fold increase in isoprene gas, which is produced by phytoplankton and contributes to the longevity of atmospheric dimethyl sulfide thereby increasing cloud cover (Wingenter *et al.* 1996; 1999). This study also resulted in an increase in methyl bromide and methane gases, both known contributors to global warming and ozone depletion (Intergovernmental Panel on Climate Change 1995). Such findings are indications that, although iron fertilization may decrease CO_2 levels, the increase in planktonic activity may lead to the liberation of other harmful gases.

In July of 2001, the subarctic Pacific iron experiment for ecosystem dynamics study (SEEDS) took place in the western subarctic gyre of the North Pacific (Tsuda *et al.* 2003). This study involved a single (2.9 nM) addition of iron, resulting in a 20 μg L^{-1} (> 20-fold) rise in chlorophyll *a* and a 94 μatm decrease in atmospheric CO_2 over the study area. Interesting and unique to this study was the diatom bloom seen after fertilization. In this case, the dominant species was a centric, chain forming diatom, *Chaetoceros debilis*, whose growth rate in this study was higher than the pennate diatom (*Pseudonitzechia turgidula*) growth reported during IronEx II (Coal *et al.* 1996) and higher than all algal growth seen in SOIREE (Boyd *et al.* 2000). The centric diatom seen here is known to be fast growing, and is probably found in this area due to the intermittent natural iron amendments the area receives from dust from the Asian continent (Bishop *et al.* 2002). This observation is also evidence of the different responses likely from the complex ecosystems seen across HNLC regions. Finally, an increase in nitrate, silicate, and phosphate uptake rates indicated that these nutrients did not limit production at the time of the study (Tsuda *et al.* 2003).

The subarctic ecosystem response to iron enrichment study (SERIES) took place in the Gulf of Alaska in July of 2002 (Boyd *et al.* 2004). An initial (1nM) followed by a second (0.6nM) iron addition resulted in a five-fold increase in chlorophyll *a* and an increase in bacterial activity in the area. This bloom (primarily consisting of diatoms) lasted more than 30 days, indicating that the added iron was retained in the area.

Throughout each of these iron fertilization experiments, the influence of changes in system chemistry on virus activity has remained largely unstudied. Given the recent attention placed on viruses and their impact(s) on nutrient cycling, it is reasonable to

assume that the viral impact in high-nutrient, low-chlorophyll regions is potentially significant and worthy of investigation. Moreover, it is also reasonable to assume that changes in water column chemistry due to these mesoscale addition events will alter this relationship. The purpose of this thesis is to test the following hypotheses: 1.) that viral activity plays an important role in the cycling of nutrients in HNCL regions; and 2.) that a mesoscale iron fertilization experiment will result in heightened virus activity in these regions.

PART ONE: AN ESTIMATION OF VIRAL DYNAMICS IN AN SF_6-LABELED HNLC PATCH

1. Introduction

The Southern Ocean has been characterized as an HNLC region. Iron fertilization studies in this region have stimulated phytoplankton blooms, primarily caused by diatoms, as well as increased draw-down of CO_2 and nutrient uptake (Boyd *et al.* 2000; Gervais *et al.* 2002; Coale *et al.* 2004). This area has also been extensively studied without the addition of iron (Dugdale and Wilkerson 1998). However, during all of these studies, parameters associated with the activity of viruses were largely ignored. Due to the potential role of viruses in the recycling of nutrients (Poorvin *et al.* 2004; Wilhelm *et al.* 1998; Cottrell & Suttle 1991; Suttle & Chan 1994; others), it seems appropriate to determine the influence that virus-mediated bacterial cell lysis has on the regeneration of nutrients in an area that is iron-limited. During this study (denoted "FeCycle") in the low-iron waters of the Southern Ocean, the inert tracer SF_6 was used to mark and follow a patch of seawater and the entrained microbial community to construct a balanced Fe budget.

2. Materials and Methods

2.1 Study site and sampling

All samples for measurements taken in the Southern Ocean were obtained with surface water pumps on board the RV *Tangaroa* from February 3 to 11, 2003 (Figure 2). This study took take place near New Zealand's National Institute for Water and Atmosphere (NIWA)'s Southern mooring site (~ 46°30 S, 178°30 E). The area was examined (Boyd *et al.* submitted) and found to have high nitrate (> 25 μM), high phosphate (> 1 μM) high silicate (> 10 μM) and low chlorophyll *a* (< 1 μg L^{-1}). Extremely low iron concentrations (0.05 nM) in surface water suggested that phytoplankton growth in the area may be limited by iron. The center of the study site was marked with a GPS navigated drifter buoy, which transmitted its location to the ship every 10 minutes. Seawater was injected with SF_6 and released into the surface and mixed layers to form a 47 km^2 patch. This release was completed in a "lawnmower" pattern while the ship was navigated with a Lagrangian system (Law *et al.* 1998). The position and shape of the patch was mapped by an underway survey of SF_6 using gas chromatography.

2.2 Phytoplankton, bacterial, and viral abundance

Phytoplankton biomass at sample stations was inferred from measurements of chlorophyll *a*. Samples were collected on 0.2-μm nominal pore-size polycarbonate filters (47mm diameter; Millipore). Chlorophyll *a* was extracted in the dark in 90% acetone for 24 h at 4°C, and quantified using a Turner Designs TD 700 fluorometer according to the non-acidification approach (Welschmeyer 1994).

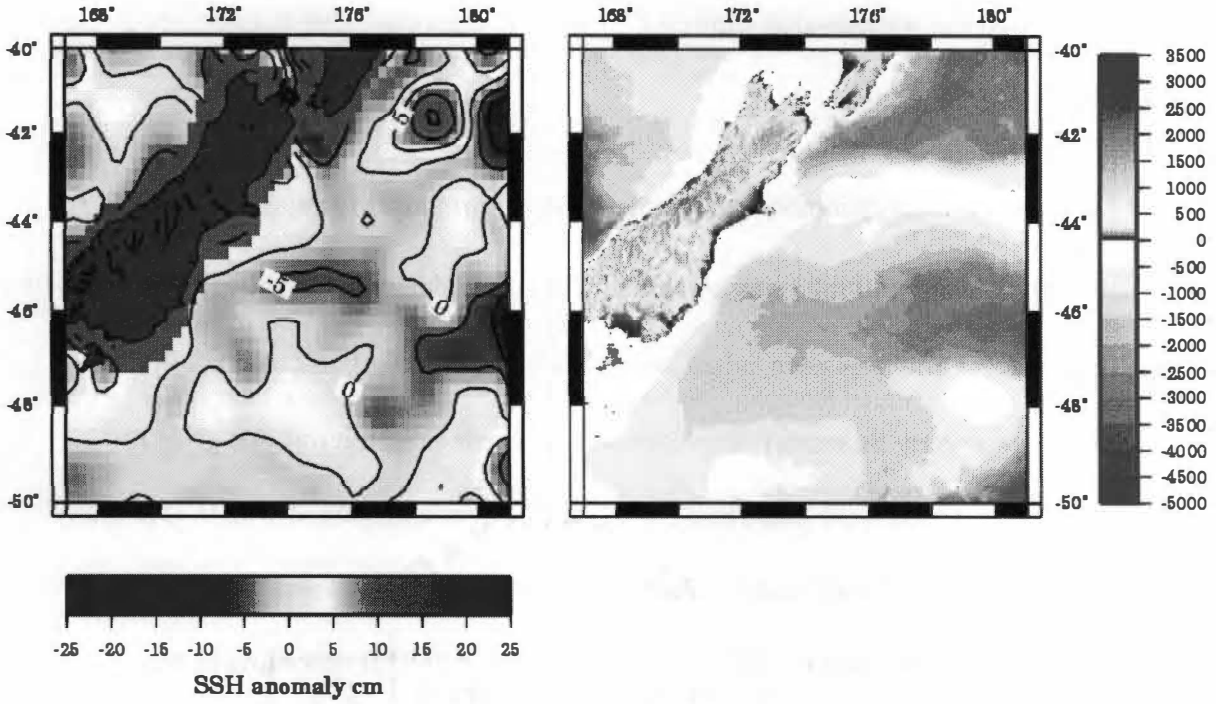

Figure 2. Study Area for FeCycle
Sea surface height anomaly (left) and bathymetry map for study area (right) prior to the beginning of the FeCycle experiment (Jan 26, 2003). Taken from Croot *et al.* 2005

Samples for bacteria and virus enumeration were preserved in glutaraldehyde (final concentration, 2.5%) and returned to the laboratory for analysis. Samples were stained with Acridine orange (Hobbie *et al.* 1977) for bacterial direct counts. Briefly, 2 mL of water was treated with Acridine orange and collected onto black polycarbonate 0.2 μm pore-size filters (Millipore GTBP). For determining viral abundance, sample water (0.8 mL) was collected onto 0.02 μm pore-size Anodisc filters (Whatman) and stained with SYBR Green I prior to enumeration of virus-like particles by epifluorescence microscopy (Noble and Fuhrman 1998). Samples with high virus abundance required dilutions: in this case 10 or 100 μL of the sample were respectively diluted with 790 or 700 μL of sterile marine media (ESAW). Samples were viewed with an epifluorescence

microscope (Leica DMRXA) with a standard Acridine orange filter set (ex_λ = 450-490 nm; em_λ = 510 nm; suppression $filter_\lambda$ = 510 nm). In each case, 200 particles or 20 fields of view were enumerated.

2.3 Determination of estimated burst size and the frequency of visibly infected cells

Whole water (40 mL) was preserved with glutaraldehyde (as above) and stored in the dark at 4°C. Samples were subsequently collected onto carbon-coated collodion (2%, Electron Microscopy Sciences) films atop 400-mesh electron microscope grids by centrifugation. Grids were then rinsed with sterile water and stained with 0.75% uranyl formate. The frequency of visibly infected bacterial cells (FVIC) and burst size were determined by use of transmission electron microscopy (TEM) according to the recommendations of Weinbauer and Suttle (1996). Samples were viewed with a Hitachi H-800 TEM with an accelerating voltage of 100 KeV. For each sample, two grids were prepared, and at least 1,000 bacteria cells were examined per grid for infection. Burst size was defined as the average number of viral particles in all visibly infected cells (VIC). This is likely the minimum burst size, as more viral particles may accumulate before the infected cell lyses.

2.4 Calculating the frequency of infected cells and viral-mediated bacterial mortality

Viral particles are only visible by TEM during the final ~10% of the lytic cycle (Vanentine and Chapman 1966), so the frequency of infected cells (FIC) was calculated from FVIC obtained by TEM using the empirically determined conversion factors of 3.7 to 7.17 (Proctor *et al.*1993). This process gave an estimated range for the percentage of infected cells in a sample. Viral-mediated mortality of bacteria (VMB) was found using

the factor-of-two rule of Proctor *et al.* (1993). FIC and VMB were also determined according to Binder (1999), where data were also given as percentages from the following equations:

$$\text{(a) } FIC = 7.1\ FVIC - 22.5\ FVIC^2$$

$$\text{(b) } VMB = (FIC + 0.6\ FIC^2) / (1 - 1.2\ FIC)$$

2.5 Bacterial and viral production and virus-induced mortality rate estimates

Bacterial production rates were measured using a ^{3}H-thymidine incorporation microcentrifuge method (Smith and Azam 1992). Briefly, triplicate samples were amended with 20 µL of a stock solution of ^{3}H-thymidine (final concentration, 40 nM) and incubated at *in situ* temperatures in the dark for one hour. Two controls were killed with the addition of 100% trichloroacetic acid (TCA) at T = 0. After incubation, the live samples received 100% TCA and all samples were treated with subsequent washes of 5% TCA and 80% EtOH, with centrifugation and liquid extraction between each treatment. After drying overnight, 1 mL of scintillation cocktail was added to each tube. A Wallac 1450 Microbeta Trilux scintillation counter was used to measure ^{3}H-thymidine incorporation by the bacteria to provide gross production estimates for all samples. Bacterial carbon production estimates were determined by converting mol thymidine to g C using the conversion factor of 2.4×10^{18} grams of carbon per mol of thymidine incorporated (Fuhrman and Azam 1982).

Viral production rates were independently estimated using the dilution approach of Wilhelm *et al.* (2002) and the TEM approach of Proctor *et al.* (1993) as modified by Binder (1999). For the dilution assay of virus production rates, the bacterial community

from 450 mL of seawater was gently collected on a 0.2-μm nominal pore-size polycarbonate filter (47-mm dia., Millipore) in order to wash viral particles from the sample. These cells were resuspended with a transfer pipette, while maintaining the initial volume with ultra-filtered seawater (< 30 KDa). Three 150 mL subsamples were transferred to individual 500 mL polycarbonate bottles and incubated at *in situ* temperatures in the dark. A 4 mL sample was collected from each bottle every 2.5 hours, and preserved with glutaraldehyde (final concentration, 2.5%). Each experiment was limited to 10 hours to reduce the amount of virus production observed from new infections. Mean production rates (viruses mL^{-1} h^{-1}) were calculated from the reoccurrence of viruses in each replicate over time. Virus-induced mortality (VIM) rates were inferred by dividing viral production by the burst size, giving the number of bacterial cells lysed mL^{-1} h^{-1} (Wilhelm *et al.* 2002). Using the TEM approach, viral production rates were determined by the following equation, where VMB is the viral mediated mortality of bacteria in a given population, at the time of sampling.

(c) Viral production= VMB x bacterial growth rate[a] x burst size

[a]assuming that bacterial growth equals bacterial mortality

As discussed above, dividing viral production by burst size gives the number of cells lysed each day by viral activity. Thus, the two methods for calculating viral-induced mortality rates can be summarized by the following two equations:

(d) VIM = viral production / burst size [dilution method (Wilhelm *et al.* 2002)]

(e) VIM = VMB x bacterial production [TEM method (Nobel and Steward 2001)]

These results were used to subsequently calculate the rates of nutrient regeneration by viral lysis of bacterial cells by multiplying the bacterial cell quota for iron (1.1 ag $cell^{-1}$, Tortell *et al.*1996), phosphorus (0.5 fg $cell^{-1}$, Heldal, *et al.* 1996), nitrogen (5.6 fg $cell^{-1}$, Lee and Fuhrman 1987), and carbon (10 fg $cell^{-1}$; Fukuda *et al.* 1998, or 23.3 fg $cell^{-1}$; Simon and Azam 1989), by the number of cells lysed each day by viral activity.

3. Results

3.1 Bacterial and viral abundance and production

Bacterial and viral abundance ranged from 5.75 - 21.8 x 10^5 (mean = 12.0 x 10^5) cells mL^{-1} and 4.1 - 84.1 x 10^7 (mean = 24.4 x 10^7) particles mL^{-1}, respectively throughout the study area in the Southern Ocean (Figure 3). Although there was no pattern in the distribution of these particles from station to station, the changes showed a weak correlation to the distributions of chlorophyll *a* (bacterial abundance, $r^2 = 0.45$; virus abundance, $r^2 = 0.35$) and dissolved iron (bacterial abundance, $r^2 = 0.41$; virus abundance, $r^2 = 0.41$) in the sample area. Bacterial and viral abundances were also positively correlated ($r = 0.71$; $p = 0.003$).

3.2 Chlorophyll a, *dissolved iron and iron regeneration*

During the survey of the area, an increase in chlorophyll *a* was observed, as well as an increase in dissolved iron and viral production. Chlorophyll *a* increased from 0.37 to 0.65 μg L^{-1} over a period of 9 days. During the same time frame, dissolved iron concentrations increased from ~ 30 to ~70 pM, while the flux of iron released by viral-mediated cell lysis (as estimated by the dilution method) increased from almost zero to nearly 75 pM d^{-1} (Figure 4). An apparent lack of agreement between the two viral production estimates is shown in Figure 5, where the dilution assay indicated that nearly one hundred times more bacteria are lysed each day, thus remobilizing more Fe, than was shown by the TEM method.

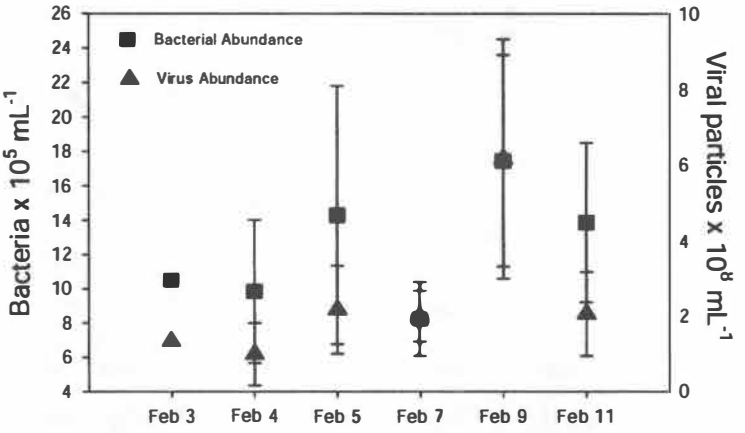

Figure 3. Bacterial and Viral Abundance for FeCycle Study.
These measurements were positively correlated (r = 0.71; p = 0.003) throughout the study

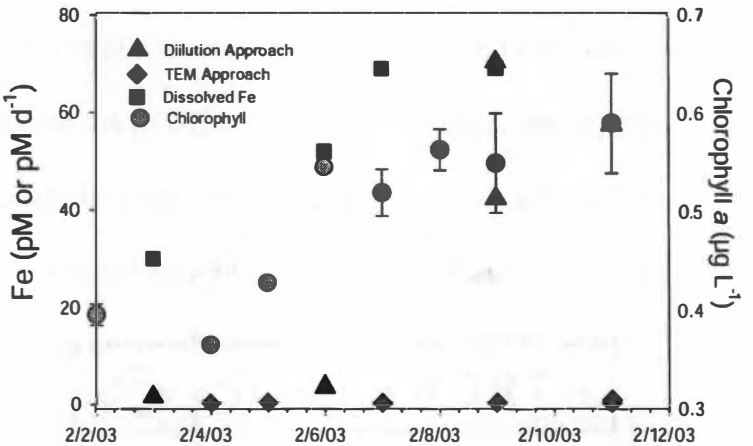

Figure 4. Viral Activity and Community Changes during FeCycle.
Viral activity (only when measured by the dilution method) increased as chlorophyll *a* and dFe rise during the study.

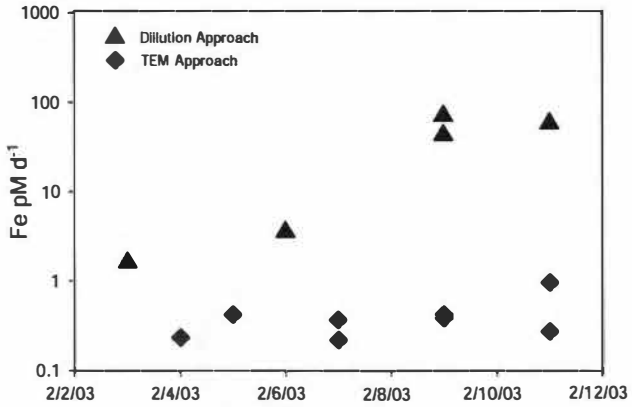

Figure 5. Two Methods Used to Measure Viral Production during FeCycle. When viewed on a logarithmic scale, it becomes apparent that the dilution method showed an increase in viral production by nearly 2 orders of magnitude, while the TEM method indicated little or no change in viral activity throughout the study.

3.3 VMB, burst size other nutrient regeneration

Values for burst size, % of bacterial mortality attributable to viral lysis (VMB), and nutrient regeneration (as measured by the dilution method) are presented in Table 2. Burst sizes ranged from 5 to 250 virus-like particles (VLP) per cell (mean = 29.75; n = 543) and did not vary over the course of the study. Virus- mediated bacterial cell lysis (VMB) ranged from 10.79 to 22.54% (mean = 15.66) and also did not change throughout the study. Viral lysis of bacteria was responsible for the regeneration of 0.16 – 120 μM C, 1.33 – 986 nM P, and 0.03 – 25 μM N each day. Nutrient remobilization estimates reported here were found using the dilution approach because they are consistent with nutrient remobilization reports of previous studies (Table 1). Because of this, they follow the same trends reported above for the remobilization of Fe in these waters (also determined according to the dilution method).

Table 2. Nutrient Remobilization and Viral Parameters during the FeCycle Study.
Mean bacterial abundance = 1.17×10^6 mL^{-1}
Water samples were taken using the ship's surface pump.

Date (Feb)	Mean Burst Size	n	% VMB	Elemental remobilization				
				*pM Fe d^{-1}	**pM Fe d^{-1}	**µM C d^{-1}	**nM P d^{-1}	**µM N d^{-1}
3	24.2	54	15	Nd	1.6	0.16	1.33	0.03
4	23.7	53	18.1	0.23	Nd	Nd	Nd	Nd
4	18.3	40	16.8	Nd	Nd	Nd	Nd	Nd
5	28.2	27	10.8	Nd	Nd	Nd	Nd	Nd
5	32.3	41	17.4	0.42	3.6	0.35	2.92	0.07
7	28.8	36	15	0.22	42.5	4.18	34.79	0.86
7	21.1	39	16.4	0.37	70.2	6.92	57.57	1.43
7	48.1	51	22.5	Nd	Nd	Nd	Nd	Nd
7	18.7	18	15.1	0.22	Nd	Nd	Nd	Nd
9	45	35	14.7	0.38	Nd	Nd	Nd	Nd
9	50.1	37	15.5	0.42	Nd	Nd	Nd	Nd
11	20.9	38	15.9	0.27	1203.0	118.5	986.1	24.41
11	34.7	21	11.5	Nd	Nd	Nd	Nd	Nd
11	22.6	53	14.6	0.96	Nd	Nd	Nd	Nd

VMB = Viral- mediated mortality of bacteria
Nd = No data
*Measured by TEM method. N = 5
**Measured according to the dilution method. n = 9

4. Discussion

Several interesting observations arise from this data set. The results demonstrate that a minor shift in phytoplankton biomass (as inferred from chlorophyll *a*) occurred during observations of the SF_6 labeled FeCycle patch of water. This shift was accompanied by changes in the dissolved Fe concentration in the upper mixed layer. As such, either an internal process released more Fe from the particulate to dissolved phase, or an intrusion / mixing event occurred, which introduced water with higher a Fe concentration. Secondly, the results demonstrate a lack of agreement between the two methods employed to estimate virus productivity. While the methods were in agreement during the early observations of the patch, concurrent to the increases in Fe and chlorophyll *a*, these methods produced markedly different results. Taken together, the results of this study suggest a potential role for viruses in the shift in production observed within this patch, and suggest that rapid changes in system trophic status may not be mirrored in the results of the TEM approach used to estimate viral–mediated bacterial cell lysis.

4.1 Increase in chlorophyll a, *dissolved iron, and viral activity (by dilution approach)*

As reported above, the dilution approach to estimate virus-mediated cell lysis suggested that viral activity resulted in the recycling of up to 75 pM Fe d^{-1} during the latter parts of the survey. This is a substantial amount, when one takes into account that the eukaryotic size class's daily demand for iron is 20-70 pM (McKay *et al.* submitted). This magnitude of Fe release is comparable to previous estimates in the subtropical southeastern Pacific Ocean (Poorvin *et al.* 2004). However, due to a limited data set

(samples were damaged during shipping) these findings need to be considered cautiously. Moreover, it is difficult to determine the cause of the increase in chlorophyll *a*, viral activity (according to the dilution method), and dissolved iron, although there are three possible explanations. The first is that an amount of iron entered the area. Originally, it was thought that this may have occurred due to the ship's activity in the study site, but, upon further analysis, it was found that the ship would have had to lose 111 kg of dissolved Fe to account for such an increase (KA Hunter, pers.com.). It is more likely that the change in iron concentration was due to the entrainment of another water mass, which in turn stimulated phytoplankton production. This is a logical assumption, as the SF_6-labeled patch appeared to shift during the experiments, becoming notably oblong, as opposed to the square-shaped area mapped at the onset of the sampling (Figure 6). While such movement in water masses made sampling a challenge, it could potentially bring in additional amounts of Fe to the area. Previous work has shown phytoplankton productivity in this region to be Fe-limited (Boyd *et al.* 2000). As such, shifts in cellular chlorophyll would be anticipated from such an event. Assuming this influenced cell abundance, the viral community would respond to the higher host abundance as contact rates (the frequency with which a virus contacts a host cell) are directly dependent on viral abundance (Murray and Jackson 1992; Wilhelm *et al.* 1998). Secondary effects, such as enhanced DOM release, leading to the stimulation of bacterial abundance and subsequent infection may have also occurred. This theory is difficult to verify however, as a higher amount of chlorophyll *a* does not always indicate a larger phytoplankton population, the sampling scheme may have missed any statistically significant shift in the abundance of a specific virus-sensitive group.

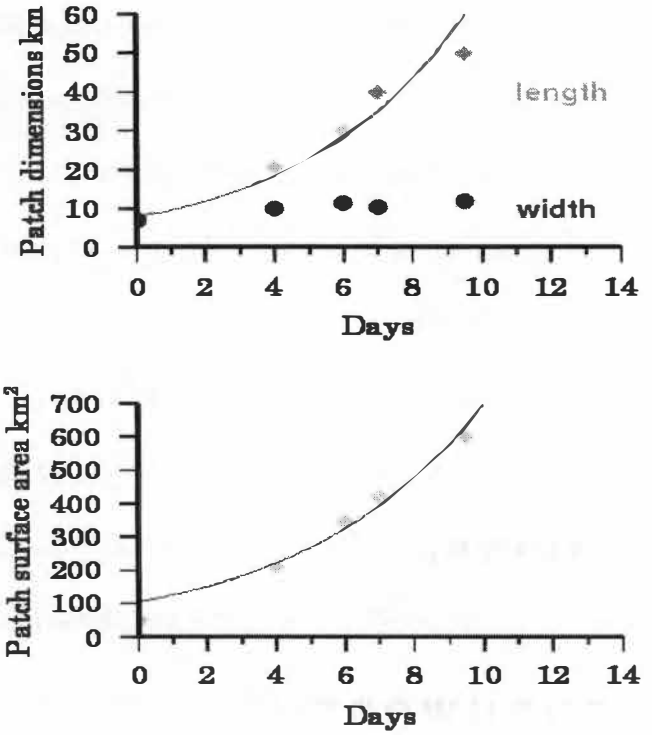

Figure 6. Movement of the SF_6 Patch during FeCycle from Croot *et al.* 2005.

A second possibility is that the viral community responded to an increase in heterotrophic bacteria (which occurred prior to the chlorophyll *a* increase), resulting in the release of iron into the area. Poorvin *et al.* (2004) demonstrated that iron released by viral-mediated cell lysis is bioavailable to other microorganisms, including marine phytoplankton. This may have led to the increase in chlorophyll *a*. The problem with this theory is that heterotrophic bacterial populations did not increase significantly throughout the study (Figure 3).

The final possibility is the increase in chlorophyll *a* occurred first, leading to heightened viral activity, thus liberating iron. This option is the least likely because in this high nutrient, low chlorophyll area, iron has been shown as the limiting nutrient (Boyd *et al.* 2000; Gervais *et al.* 2002; Coale *et al.* 2004), so it is difficult to say what

may have caused the increase chlorophyll *a* in the area. The first possibility is the most probable, when referring to Figure 4. Although it is difficult to accurately discern the sequence with which these parameters changed, according to this figure, the increase in dissolved iron occurred first, resulting in higher amounts of chlorophyll *a*, followed by heightened viral activity.

4.2 VMB, burst size and nutrient remobilization

Due to the time requirements and technical skills required to examine samples by transmission electron microscopy (TEM), very few studies include reports of burst size. Instead, an assumed burst size, ranging from 25 – 100 (depending on the author) is commonly used. In this study, TEM was used to empirically determine burst sizes. Of 543 infected cells examined during this study, the burst sizes ranged from 5 to 250, with a mean burst size of 29.75 virus-like particles (VLPs) produced per lytic event.
The significance of these TEM results is two fold: first, if an assumed (and in this case lower) estimate of burst size, such as 25 VLPs is used, the results will provide an overestimate of cells lysed each day (an average burst size of almost 30 was found in this study); and , second, using an assumed burst size of 25 across all samples does not account for the variation between samples, such as the 5 to 250 VLPs per lytic event observed in this study.

While the use of TEM is a useful method of determining burst size, the values found are likely minimum estimates due to the fact that these infected cells are not exactly viewed at the last moment before lysis. With more accurate estimates of burst sizes, it is becoming apparent that the use of 25 VLPs for a given sample is not only

possibly an underestimate, but fails to address the lack of uniformity within a study area, where burst sizes are concerned.

It is important to point out here that the average burst size did not vary over the study area, even when estimates of viral production seemed to increase (using the dilution method). Similarly, VMB did not change during the study. While the estimates of VMB are consistent with those reported in previous studies (Table 1), it was surprising to find that these values did not vary. It is possible that the TEM method is not useful for tracking the viral response to community changes, as it is expected that an increase in trophic production would lead to a rise in viral activity in the study area, and such an increase was not detected by this method. This is expected when one considers host contact kinetics as given by the following equation:

$$\text{(f)}\ C = (2S\pi \cdot \omega \cdot Dv)V \cdot B$$

Where C is the contact rate, S is the Sherwood number (1.06, Wilhelm *et al.*, 1998), ω is the diameter of a marine bacterium (*ca* 0.45×10^{-4} cm, Lee and Fuhrman, 1987), Dv is viral diffusivity (3.456×10^{-3} cm^2 d^{-1}, Murray and Jackson, 1992), and V and B are viral and bacterial abundance, respectively. Because S, ω, π, and Dv are constants, increases in bacterial abundance will increase the contact rate between bacteria and viruses. It follows that if 30 VLPs are liberated by one lytic event, the contact rate will increase a further 30- fold. This would lead to a rapid increase in the rate of host cell infection and ultimately to an increase in viral production. Therefore, the increase in viral production that is apparent when using the dilution method appears to label this method the more accurate of the two.

The nutrient remobilization due to viral lysis of bacterial cells (0.16 – 120 μM C, 1.33 – 986 nM P, and 0.03 – 25 μM N) each day found during this study is impressive. Although it is unclear if these nutrients are of use to the bacterial community, there is little doubt that viral activity plays an important role in their regeneration.

5. Part I Conclusions

The amounts of Fe, N, C, and P released due to viral lysis in this study provides strong evidence in support of the theory that marine bacteriophage play a major role in the cycling of nutrients in the Southern Ocean. Furthermore, because iron is released in an organically complexed form, it remains bioavailable to the microbial community in the area.

This study provides insight into the intricacies of aquatic microbial food webs, while it also offers insight into the utility of the two methods used to study viral production. The limited data set suggests that the dilution method is most useful for tracking changes in trophic production, as the TEM approach discerned little change in virus activity.

PART TWO: A MESOSCALE IRON FERTILIZATION IN THE SUBARCTIC PACIFIC OCEAN AND ITS EFFECTS ON VIRAL ACTIVITY

1. Introduction

Two iron fertilization experiments in the subarctic Pacific Ocean [SEEDS (Tsuda *et al.* 2003) and SERIES (Boyd *et al.* 2004)] have demonstrated that the availability of elemental iron (Fe) limits primary productivity of the phytoplankton community. Both studies reported significant increases in chlorophyll *a*, coupled with a rise in diatom abundance in waters to which Fe was added. The subarctic Pacific Ocean iron experiment for ecosystem dynamics study (SEEDS; Tsuda *et al.* 2003) resulted in a nearly 20-fold increase in chlorophyll *a*, and a bloom that lasted nearly 30 days as seen from satellite images. While it is known that this bloom consisted primarily of the centric diatom, *Chaetoceros deblis*, little else is said about other members of the microbial food web, and nothing is reported concerning viral activity following the addition of iron. In fact, during the six published iron fertilization experiments, the activity of the virus community has been left unstudied. The SOFeX experiment in the Southern Ocean (Coale *et al.* 2004) resulted in an increase in the release of other greenhouse gases, such as methane, isoprene, and methyl bromide (Wingenter *et al.* 2004). These gases are thought to have increased with a rise in planktonic activity. It stands to reason that virus-mediated lysis of these organisms could lead to the release of these gases.

During the FeCycle experiment, reported in Part One, an increase in virus activity was observed during a natural increase in both dissolved iron and chlorophyll *a* concentrations, which seemed to indicate that viruses were acting as feedback when primary production was stimulated. These results raise questions as to the response of viruses when changes occur in the trophic status of their host.

In July and August 2004, a second subarctic Pacific Ocean iron experiment for ecosystem dynamics study (SEEDS II) took place at the same location of SEEDS I (48.5ºN, 165ºE). As a component of this program, changes in the activity of the virus community following the mesoscale iron fertilization were monitored. Moreover, in order to follow up on observations from FeCycle concerning the discrepancies in the two common techniques used to examine virus activity, samples were processed by both the dilution method (Wilhelm *et al.* 2002) and the TEM method (Binder 1999) to estimate virus induced microbial mortality.

2. Materials and Methods

2.1 Study site and sampling

Samples from the subarctic Pacific Ocean were taken from July 16 to August 5, 2004 in the area shown in Figure 7 while on board the RV *Hakuho Maru* using acid washed Niskin-X bottles and Kevlar wire. To ensure that a uniform patch of water was monitored in the subarctic Pacific, the upper-mixed layer of the water column was labeled in a manner analogous to FeCycle with the inert tracer SF_6, which was combined with iron sulfate (6 to 10 metric tons) dissolved in seawater. Fe additions were made on days 1 and 3 of the study.

2.2 Phytoplankton, bacterial and viral abundance, and bacterial production

Phytoplankton biomass was inferred from estimates of chlorophyll *a* concentrations, as in Part One with two slight variations. Samples were collected on 0.2 μm nominal pore-size polycarbonate filters (47 mm diameter; Millipore) and stored in the dark at -20°C until processing. Samples were extracted in 90% acetone for 24 h at 4°C in the dark, and quantified using a Turner Designs TD700 fluorometer using the non-acidification approach (Welschmeyer, 1994).

Samples for the estimation of the abundance of viruses and bacteria were collected from stations both inside and outside the fertilized patch and direct counts were completed using the methods described in Part One. Slides for viral abundance measurements were, however, prepared while at sea and shipped back to the laboratory frozen (-20°C) where they were enumerated.

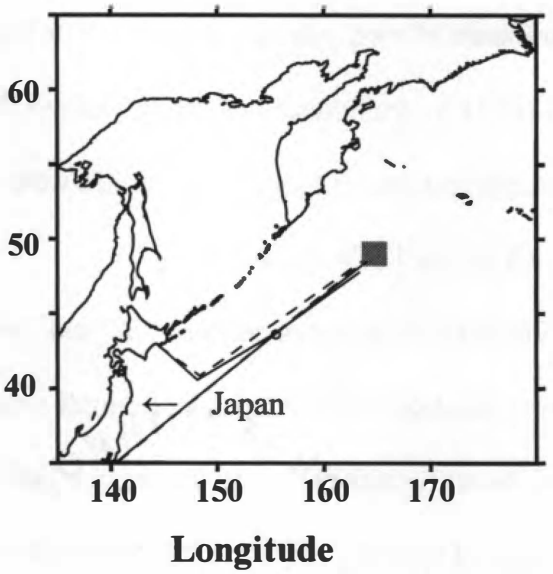

Figure 7. Study Area for SEEDS II Experiment

Bacterial production rates were measured using Kirchman's (2001) ^{3}H-leucine incorporation microcentrifuge method. Briefly, triplicate samples were amended with 20 μL of a stock solution of ^{3}H-Leucine (173 Ci mmol^{-1}, Perkin Elmer Life Sciences, Inc., final concentration of 40 nM) and incubated at *in situ* temperatures in the dark for one hour. Two controls were killed with 100% trichloroacetic acid (TCA) at T = 0. After incubation, the live samples received 100% TCA and all samples were treated with subsequent washes of 5% TCA and 80% EtOH, with centrifugation and liquid extraction between each treatment. After drying overnight, 1 mL of scintillation cocktail was added to each tube. A Wallac 1450 Microbeta Trilux scintillation counter was used to measure ^{3}H-Leu incorporation by the bacteria to provide gross production estimates for all samples. Where bacterial carbon production estimates are provided, the conversion factor (3.1 kg C mol^{-1} leucine) of Wetzel and Likens (2000) was employed.

2.3 Additional viral parameters and tests for significance

Other measurements of viral activity, including lytic burst size, frequency of visibly infected cells (FVIC), viral induced mortality rates (VIM) by TEM and dilution methods, viral production and nutrient regeneration rates were conducted using the methodology described for the FeCycle study.

All statistical tests for differences between "in" and "out" stations or before or after Fe addition were completed with independent sample t-tests at 95% confidence level. Statistical tests for differences between the two methods for measuring viral production were conducted by using paired sample t-tests using 95% confidence level. Finally, tests for the correlation between viral activity and chlorophyll *a* concentration were completed using Pearson correlation. All statistical tests were conducted using SPSS 12.0 software.

3. Results

3.1 Dissolved Fe and chlorophyll a

Inside the patch, dissolved iron (dFe) concentrations increased from 18 pM before fertilization to ~ 300 pM by day 4, and increased again after the second addition to 660 pM by day 8. This rise in dFe was significantly different from measurements taken prior to fertilization ($p = 0.033$). Dissolved Fe then dropped until the end of the experiment. The dissolved Fe outside of the patch averaged 89.62 pM and remained significantly lower ($p = 0.021$) than that inside the fertilization area (Figure 8 A).

Concurrent to the rise in Fe concentration, a substantial increase ($p < 0.001$) was observed in chlorophyll *a* concentrations from 0.36 $\mu g\ L^{-1}$ before Fe addition to as high as 2.25 $\mu g\ L^{-1}$ after Fe addition. As was observed for iron, chlorophyll *a* remained significantly lower ($p = 0.001$) outside the patch than inside (Figure 8 B). The rise in chlorophyll *a* seen in this study was primarily due to an increase in the picoplanktonic size class (Tsuda and Suzuki per. com), as opposed to the diatom bloom seen in SEEDS I (Tsuda *et al.* 2003).

3.2 Bacterial and viral abundance

The highest bacterial abundance was estimated prior to the addition of Fe in the study area [mean = 1.55 (± 0.3) x 10^6 cells mL^{-1}]. Following Fe fertilization, bacterial abundance significantly decreased ($p < 0.001$) and remained lower than the initial abundance [mean = 5.07 (± 1.8) x 10^5 cells mL^{-1}]. Although initial abundance estimates were high, the bacterial abundance found for the remainder of the study were not significantly different ($p = 0.59$) from those outside of the patch prior to day 12. There

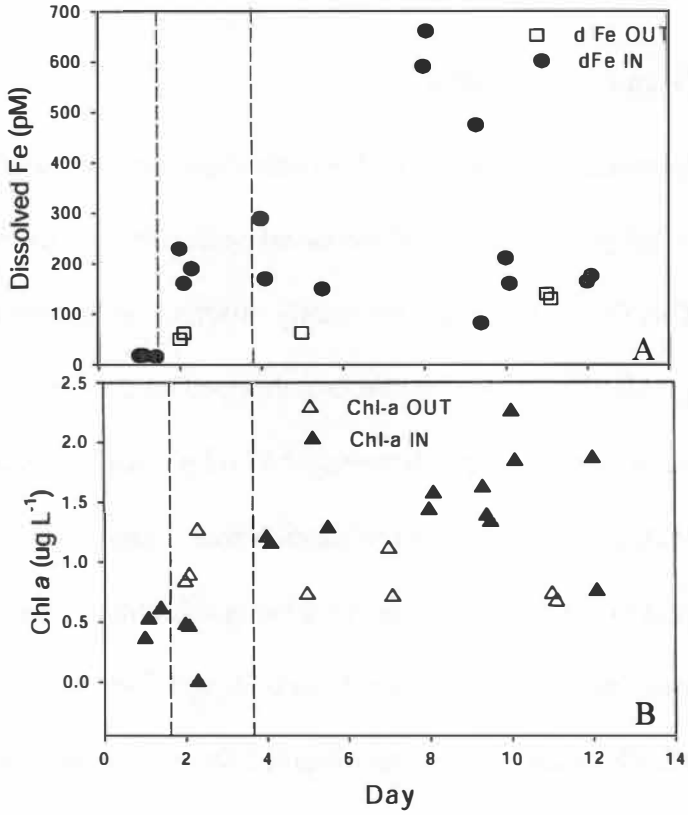

Figure 8. Dissolved Fe and Chlorophyll *a* Inside and Outside of the Patch during SEEDS II.

Significant increases can be seen in both dFe (A; $p = 0.033$) and chl *a* (B; $p < 0.001$) inside the patch following the addition of iron, but not outside. This indicates that the study area was in fact iron limited. Dotted lines stand for iron additions.

was, however a significant increase ($p < 0.001$) in bacterial abundance observed on day 12 inside the iron patch (Figure 9 A).

Viral abundance was also high [mean = 5.3 (± 0.03) x 10^7 particles mL^{-1}] prior to fertilization. After the Fe addition, a slight but insignificant ($p = 0.60$) decrease was seen, but abundance increased significantly ($p = 0.003$) at the end of sampling. As was observed for bacterial abundance, viral abundance inside the Fe patch was not substantially different ($p = 0.701$) from estimates from outside, (Figure 9 B).

3.3 Viral production measurements

Viral production, as measured by either method, appears to be weakly correlated with changes in chlorophyll *a* measurements (TEM r = 0.575; dilution r = 0.173; Figure 10). However, the TEM method gives values [mean = 1.85 (±0.57) x 10^7 bacterial cells lysed L^{-1} day^{-1}] that are much lower (~ 10^{-3}) than those found using the dilution method [mean = 6.31 (± 2.5) x 10^9 bacterial cells lysed L^{-1} day^{-1}]. Therefore, the rates estimated by these two methods for measuring viral production are significantly different ($p < 0.001$).

Although viral production was not significantly correlated with the increase in chlorophyll *a*, it was seen to significantly increase (dilution $p = 0.001$; TEM $p = 0.002$) from 2.58 (± 0.61) x 10^9 prior to Fe addition to 9.00 (± 2.5) x 10^9 cells lysed L^{-1} day^{-1} by the end of the study. Additionally, both methods showed that measurements taken inside the Fe patch (after day 5) were significantly different from those taken outside (dilution $p = 0.023$; TEM $p = 0.001$).

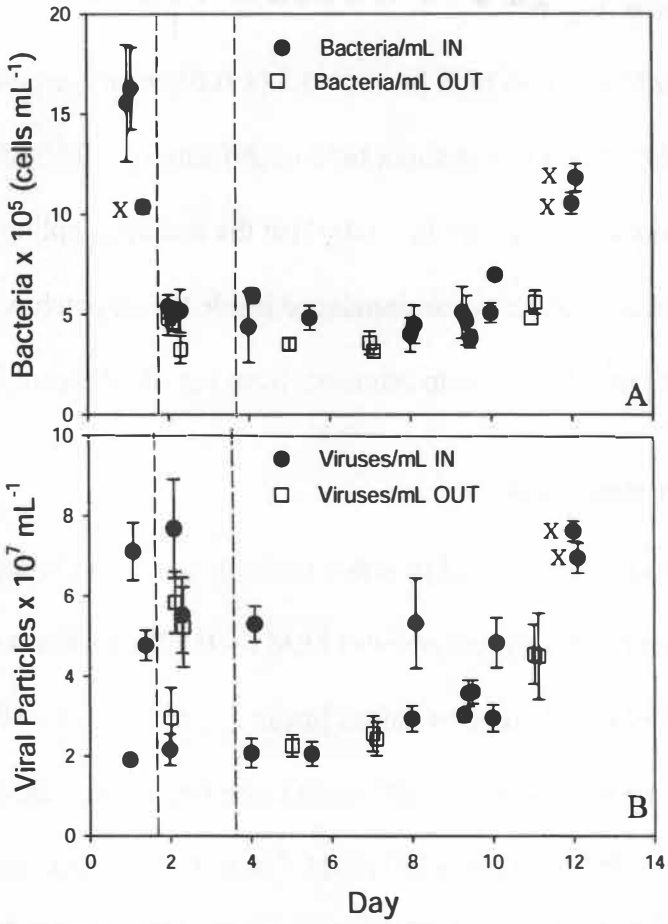

Figure 9. Bacterial and Viral Abundance Inside and Outside the Fe Patch during SEEDS II.

A. Bacterial abundance inside the Fe patch significantly dropped ($p < 0.001$) with the addition of Fe, remained statistically similar ($p = 0.59$) to measurements outside the patch, and significantly increased ($p < 0.001$) by D 12 in reference to all days prior to this following to the Fe addition. B. Virus abundance displays similar trends and begins to rise around D 9 with a significant change ($p = 0.003$) by D 12, compared to the days leading up to this after fertilization. $N = 3$ for all points shown, except where "x" indicates $n = 2$. Dashed lines represent Fe additions.

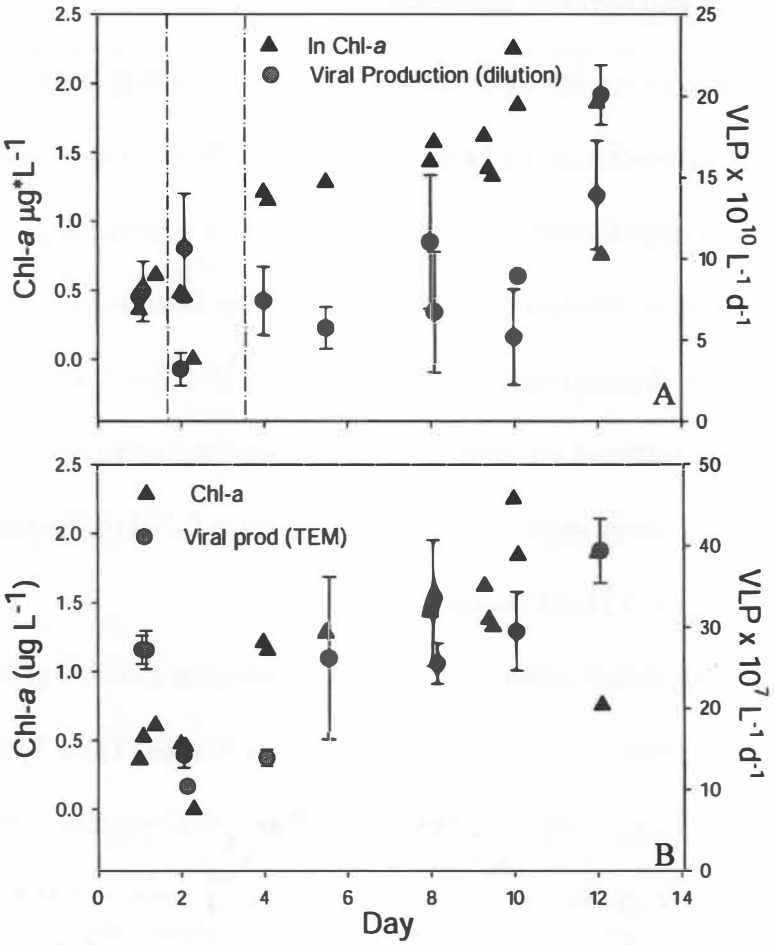

Figure 10. Chlorophyll *a* and Viral Production [measured by dilution (A) and TEM (B) methods] Inside the Fe Patch during SEEDS II.

Neither method indicates that viral activity is correlated with changes in primary production (TEM r = 0.433; dilution r = 0.173). The methods are statistically different ($p < 0.001$), with the dilution method showing values that are 10^3 higher. Dashed lines represent iron additions.

3.4 VMB, burst size and nutrient remobilization

Viral-mediated mortality of bacterial cells estimated using TEM (VMB) did not vary greatly inside or outside of the Fe patch ($p = 0.921$; Tables 3 & 4) and did not significantly change upon the addition of Fe ($p = 0.251$). The percentage of the heterotrophic population whose mortality was attributable to virus-mediated lysis was 7.32% (± 1.33) inside the patch and 7.16% (± 1.53) outside. Burst size followed similar trends, where values reflected no significant change after the addition of Fe ($p = 0.644$) and showed little difference inside or out of the patch ($p = 0.504$) during the study [mean = 12.9 (± 2.1) inside; 12.1 (± 0.8) outside].

The nutrient remobilization estimates found using the dilution assay, although high, are more consistent with values given in previous studies (Table 1) than estimates found using the TEM approach. In the waters outside of the Fe patch, viral lysis of bacterial cells was responsible for the remobilization of as much as 191.8 pM Fe, 9.73 μM C, 2.00 μM N, and 80.09 nM P (Table 3). In the area to which Fe was added, the viral release of 194.8 pM Fe, 11.47 μM C, 2.36 μM N, and 95.45 nM P was estimated on day 2, just after the addition of Fe (Table 4).

Table 3. Nutrient Remobilization and Virus Parameters during the SEEDS II Study Outside Fe Patch.
Water samples were taken using Niskin- X bottles.

Sampling Day Out of Patch	Mean Burst Size	n	% VMB	*Fe pM d^{-1} (+/-)	**Fe pM d^{-1} (+/-)	**µM C d^{-1}	**nM P d^{-1}	**µM N d^{-1}
D 02	11.5	20	5.66	0.22 (0.09)	152.5 (20.9)	5.40	44.2	1.11
D 05	12.8	22	6.74	0.29 (0.03)	98.7 (11.7)	2.30	19.1	0.47
D 11	12.9	25	6.97	0.19 (0.03)	191.8 (34.8)	9.73	80.9	2.00
D 11	11.4	14	9.27	0.21 (0.002)	339.8 (108.7)	20.12	167.4	4.15

VMB = viral- mediated bacterial cell lysis
*Measured by TEM method.
**Measured according to the dilution method.

Table 4. Nutrient Remobilization and Viral Parameters during the SEEDS II Study Inside Fe Patch.
Water samples were taken using Niskin- X bottles.

Sampling Day In Patch	Mean Burst Size	n	% VMB	Elemental remobilization				
				*pM Fe d^{-1} (+/-)	**pM Fe d^{-1} (+/-)	**µM C d^{-1}	**nM P d^{-1}	**µM N d^{-1}
PS	11.97	31	8.33	0.45 (0.03)	125.7 (2.88)	5.69	47.3	1.17
PS	15.24	17	7.25	0.34 (0.03)	103.2 (24.1)	5.16	42.9	1.06
D 02	12.35	23	6.44	0.23 (0.02)	87.6 (53.5)	2.49	20.7	0.51
D 02	10.7	13	5.53	0.19 (0.01)	194.8 (61.2)	11.5	95.5	2.36
D 04	10.3	23	6.61	0.27 (0.02)	141.5 (40.4)	7.97	66.3	1.64
D 05	14	11	10.05	0.37 (0.14)	80.7 (18.1)	4.45	37.6	0.92
D 08	12.13	31	8.90	0.53 (0.13)	179.1 (66.9)	8.55	71.2	1.76
D 08	12.54	28	7.67	0.40 (0.04)	105.5 (58.0)	4.34	36.1	0.89
D 10	16.6	22	6.08	0.30 (0.06)	61.5 (38.8)	2.01	16.7	0.41
D 12	15.59	17	7.09	0.50 (0.05)	175.8 (42.3)	7.93	66.6	1.63

VMB = viral- mediated bacterial cell lysis
*Measured by TEM method.
**Measured according to the dilution method.

4. Discussion

4.1 Dissolved Fe and chlorophyll a

After fertilization inside the patch, dissolved Fe predictably increased inside, but not outside of the patch. The timing of this increase coincides with the Fe additions on days 1 and 3 (Figure 8 A). An increase in particulate chlorophyll *a* concentration was also observed inside, but not outside of the patch (Figure 8 B), suggesting that the addition of Fe stimulated primary production. As mentioned, this increase was seen in the smallest size class of phytoplankton, the picophytoplankton. In SEEDS I, a 20- fold increase in chlorophyll a was reported along with a large bloom of the centric diatom, *Chaetoceros deblis* (Tsuda *et al.* 2003). However, in SEEDS II, little or no increase was observed in the microzooplanktonic size class, which would include this species. In comparison to SEEDS I, the magnitude of the chlorophyll *a* increase seen in SEEDS II was much lower (only 6- fold). The larger phytoplankton size class typically contains more chlorophyll *a* per cell than the picophytoplankton that was seen to increase in this study. Moreover, given that picoplankton typically have larger Fe:C demands (Wilhelm 1995) it is anticipated that the net amount of increased production in this group after Fe fertilization would be less.

4.2 Bacterial and viral abundance

It is difficult to determine the cause of the decrease in bacterial abundance following iron fertilization. It is possible that the addition of iron caused this decline. However, this argument is not a compelling one, as bacterial abundance outside of the

iron patch was not significantly different than that seen inside the patch following this addition. It is possible that sampling and/or microscopy errors resulted in these data, but this is doubtful as virus abundance also was seemingly high prior to fertilization. Natural fluxes in bacterial abundances are known to occur in natural systems (Ducklow and Carlson 1992). As previously discussed, when a bacterial population increases, a natural rise in viral activity will inevitably follow due to higher host-phage contact rates. This will serve to decrease the bacterial abundance, followed by a rise and subsequent drop in viral abundance when host populations are lowered. Figure 9 can possibly be explained by this phenomenon.

Perhaps of greater interest is the significant increase in both bacterial and viral abundances at day 12. While it is possible that these increases could be caused by the trends discussed above, it is also likely that the addition of iron caused the apparent heightened microbial activity seen in Figure 9. This is probable when considering the sequence of events when trophic activity is stimulated. Figure 8 shows that the concentration of chlorophyll *a* increased with the addition of Fe. The subsequent decay of these organisms releases a substantial supply of DOM on which heterotrophic bacteria will feed, leading to an increase in bacterial abundance, followed by the expected response by the viral community. Therefore, while the addition of iron does not directly lead to an increase in bacterial or viral abundance, the secondary effects are increases in both, as is apparent in Figure 9. It is important to mention that while these trends may be explained by the above, additional sampling after day 12 would be useful for constructing a clearer picture of the changes occurring here.

4.3 Viral production measurements

As seen in Figure 10, viral production measured by either the TEM or dilution methods is not significantly correlated with changes in phytoplankton biomass. It appears highly unlikely that viral activity would not follow community changes, given the above discussion of the close ties between communities. In fact, an increase in viral activity is seen with each method after the addition of iron, but it is not directly correlated with phytoplankton activity. Monitoring these populations for extended periods of time following iron addition might lead to results which offer further insight into population dynamics here. This is possible because viral activity measured here is of phage infecting heterotrophic bacterial populations, which seem to respond to the increased DOM released from phytoplankton decay Again, it is expected that viral activity would increase with somewhat of a delay after the addition of Fe. This is further verified by the fact that viral production was not seen to increase outside of the iron patch. While the FeCycle study seemed to indicate that viral activity (measured by the dilution method) follows primary production, it is likely that the limited data set was not sufficient to allow for such conclusions. It is also possible that since no amendments were made in the FeCycle study, viral activity would not take as long to respond to community fluxes.

This study again made the lack of agreement between the two methods used to measure rates of host cell lysis apparent (Figure 10). The dilution method gives results that are 10^3 higher than those estimated using the TEM method. In order to determine which of the methods is more accurate (or perhaps least prone to error), it is appropriate to reconsider the parameters of each. The dilution method, which gives results which are higher, relies on viral abundance estimates measured over time from a resuspension of

cells trapped on a 0.2 μm filter, therefore, the release of viral particles from any cell type larger than 0.2 μm diameter will be counted. In contrast, the TEM method only measures viral infection of heterotrophic bacteria cells. However, this difference is not substantial because the majority of cell types (other than heterotrophic bacteria) that would be resuspended from the filter are probably phototrophs. Since the incubations for this experiment are made in the dark, this would likely slow the production of these cells. Although it is probable that these phototrophic cells may still lyse at this point, it is also likely that incubating these organisms in the dark would hinder their lysis by viruses, as the lack of photosynthesis may inhibit the cellular energy exploited by viruses. Possibly accounting for a large difference in the results found using the two methods is the fact that, even though viral abundances in the resuspensions were greatly reduced due to filtering, they were still considerably high, and therefore, difficult to monitor for changes. The other measurement required for determining the number of cells lysed each day by viral activity using the dilution method is burst size. As mentioned earlier, burst sizes were low in this study [mean = 12.9 (± 2.1) inside; 12.1 (± 0.8) outside]. Dividing viral production rates, measured by the dilution method, by lower burst sizes, such as those reported in this study, yields higher estimates of cells lysed each day. The TEM method does not rely on burst size.

The TEM method, however, is not without its possible caveats. As mentioned above, this method only makes it possible to examine heterotrophic cells which are infected (other cell types require thin sectioning), reducing the number of infected cells that are visible in a sample. Furthermore, conversion factors are used in order to account for the following: the fact that viral particles are only able to be viewed with TEM

during the last approximately 10% of the lytic cycle; grazing; and natural mortality (that not imposed by viral activity) of bacteria cells. It is possible that the addition of Fe may lengthen the lytic cycle, making it more difficult to see changes in the frequency of infected cells after fertilization.

The lytic cycle is a function of viral production, burst size and virus-mediated mortality of bacteria, given by the following equation:

$$\text{(g)}\ V_{prod} \approx T_{LC}^{-1} \bullet (VMB \bullet BS)$$

Where V_{prod} is viral production, T_{LC} is the length of the lytic cycle, VMB is the percentage of bacterial mortality attributable to viral lysis, and BS is burst size (Dean *et al.* 2005). Utilizing this equation, it can be concluded that, because there was not a significant change in BS or VMB, but an increase was observed in V_{prod}, the lytic cycle must have been shortened. This would cause a problem with quantifying the frequency of visibly infected cells, while its effects would not be seen when using the dilution method, as measurements taken here would not allow for the observation of a lengthening in lytic cycles.

The TEM method is also dependent on bacterial production rates, as it is assumed that in a steady state, death equals growth. The problems with this are obvious. After the addition of iron, steady state was disrupted, as production (and therefore growth) was stimulated. This would lead to an imbalance in the production and loss processes, making this calculation unreliable. Also contributing to the discrepancy between the two methods is that the bacterial production rates only account for heterotrophic bacteria. Again, the dilution method does not discriminate here, leading to higher estimates of viral production.

As outlined here, the dilution method relies on far fewer possible "loopholes" than does the TEM method. The dilution assay is more reliable and less prone to error accrued during tedious measurements such as bacterial production and estimating VMB.

4.4 VMB, burst size and nutrient remobilization

The nutrient remobilization estimates reported in Tables 3 and 4, with the exception of iron, are given using the dilution method, as these values are more similar to those in previously reported studies. Estimates found using the TEM method were substantially lower. Although the nutrient remobilization approximations made using the dilution method are considerably higher than those previously reported they are more in support of previous results than those estimated using the TEM method. A possible explanation for the higher values reported here is that the burst sizes found during this study were very low (mean ~13 virus-like particles). This would result in the finding that more cells were lysed each day, as the virus production rate is divided by burst size in order to calculate cells lysed per day. As mentioned in Part One, previous studies have been conducted without estimating burst sizes by TEM, but assuming a burst size of 25 to 50. This is substantially higher than the mean of 13 reported here.

As was seen in the FeCycle study, there was not a significant change in burst size or VMB during the SEEDS II study. Notable here however, was the significantly lower ($p < 0.001$) mean burst sizes (~13) in this study compared to that of FeCycle (~30). This was also the case with VMB estimates, which were 15.7% in FeCycle and 7.32% in this study. There are several possible explanations for this trend, but unfortunately none are without much speculation. One thought is that increased day length typical of the

SEEDS II study area may have contributed to these differences. It was also thought that lower host (bacterial) abundance may lead to lower burst size; however, there was not a significant difference in bacterial abundance found in these studies. Finally, although unconfirmed by previous studies, adding Fe may change the viruses' ability to adhere to host cells, reducing rates of infection. Clearly, all that can be offered at this point is speculation as to the cause of the differences in viral activity between the two study sites.

5. Part II Conclusions

This study has again confirmed the hypothesis that iron limits primary productivity in the subarctic Pacific Ocean. However, there remains much to be understood about the intricacies of the microbial communities in HNLC areas and the world's oceans in general. Just as in FeCycle, the results of this study show that a disconnect exists between the two methods used to measure viral production, but unlike the FeCycle study, SEEDS II measurements failed to confirm any significant correlation between viral activity and chlorophyll *a* when production is stimulated. Furthermore, this study confirms that viral activity is responsible for the regeneration of substantial amounts of nutrients which are organically bound.

FINAL SUMMARY AND CONCLUSIONS

In this study, several objectives have been accomplished. The activity of viruses in HNCL regions was evaluated with respect to rates of host cell lysis, the regeneration of nutrients, parameters of infection, such as burst size, and the ability for these activities to be tracked through changes in trophic production using two methods by which to measure viral production. These parameters were monitored during an iron budget study in the Southern Ocean (FeCycle) and before, during, and after a mesoscale iron fertilization in the subarctic Pacific Ocean (SEEDS II).

From these findings, several conclusions can be drawn. The results from the Southern Ocean study established that viral lysis of bacterial cells is responsible for the regeneration of significant concentrations of nutrients. Of great importance is the fact that these nutrients are organically complexed, and, in the case of Fe (Poorvin *et al.* 2004, have been shown to be bioavailable to the nearby microbial community. The FeCycle study also revealed the need to further evaluate the two methods used to measure viral production, as the dilution method provided results that suggest that the virus community follows changes in trophic production, while the TEM method seems to report little or no change in virus activity when trophic production is stimulated.

The findings of FeCycle provided the framework for SEEDS II: the goal was to compare two methods used to measure virus production and to further examine the role that viruses play in the cycling of nutrients. From this work it was concluded that viruses do play a major role in nutrient recycling (especially Fe). This is of obvious importance, as this study confirmed Fe to be a limiting nutrient for phytoplankton growth in the subarctic Pacific. Additionally, it was concluded that, while virus activity does not directly follow changes in primary productivity, viral production does increase as a result

of the effects of community changes. With this study, more information was gathered to compare the methods used to measure virus production. As reported for FeCycle, a lack of agreement remains between the two methods. As a result, it was concluded the complexities of the TEM method in particular prevent it form being a useful tool by which to track viral activity through changes in system trophic status, leaving the dilution assay as the method of choice.

The conclusions drawn from this study serve several functions. First, they highlight the importance of viral activity in HNLC regions in the recycling of nutrients and indicate the appropriateness of studying such parameters. Secondly, it becomes apparent when examining these results that interpretation of these methods requires caution. Finally, the results of this study highlight the complexity of the microbial community within HNLC regions and the community's ability to regulate itself through the combined activity of each of its members.

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Vita

Julie Higgins was born in Nashville, Tennessee on May 18, 1979. She was raised in Brentwood and Nashville, Tennessee and attended Oak Hill Elementary School, junior high at Franklin Road Academy (Nashville), and graduated with honors from Brentwood High School in 1998. In the fall of 1998, she began her undergraduate studies at the University of Tennessee, Knoxville. During this time, she was involved in the Sea-Mester program in Long Key, Florida, where she discovered her love for research.

After receiving her B.S. in Ecology and Evolutionary Biology in May, 2002, she went on to work in Steven Wilhelm's microbial ecology laboratory at the University of Tennessee. In the fall of 2002, she took an internship opportunity at Mote Marine Laboratory's Center for Shark Research in Sarasota, Florida under the direction of Colin Simpfendorfer. After retuning to Knoxville, Julie continued work in Dr. Wilhelm's lab and became involved with the FeCycle project.

In the fall of 2003, Julie enrolled as a Masters candidate in Microbiology with Steve Wilhelm as her advisor. She will receive her Master of Science degree in May 2005. After this, Julie plans to remain with the Wilhelm lab for a brief period before seeking a technician position elsewhere, where she hopes to work with microbial ecology, harmful algal blooms, or elasmobranch research.

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