Abstract

Current studies indicate that viruses of marine bacteria are biological carbon sinks, transforming bacterial carbon into dissolved organic matter, the majority of which is respired rather than incorporated back into biomass. In contrast, this dissertation focusses on viruses, not as a carbon sink but as a catalyst of nitrogen cycling, benefiting phytoplankton by liberating nitrogen from bacterial lysates that would otherwise be tied up in bacterial biomass. The results in this dissertation show that organic nitrogen released by viral lysis of heterotrophic marine bacteria is remineralised by uninfected bacteria, and the resulting ammonium taken up by phytoplankton.

In an initial laboratory experiment, only a portion of the amino acids derived from heterotrophic bacterial lysates could be taken up by other heterotrophic bacteria within the duration of the experiment. Both D- and L-amino acids were taken up in proportion to their initial concentrations, demonstrating a lack of preference for the generally more labile L-amino acids. In a subsequent field experiment, reduction of the viral fraction in a marine microbial community resulted in reduced ammonium remineralisation and phytoplankton abundance, suggesting that remineralised nitrogen from bacterial metabolism of viral lysates contributes to phytoplankton growth. Another experiment added a marine bacterium labeled with $^{15}$N and infected with a lytic virus to microbial communities. This experiment directly demonstrated that remineralised nitrogen from bacterial lysates released through the action of viruses was a significant source of nitrogen for phytoplankton. In a final series of experiments, viruses were reduced from seawater from 22 field stations using bacterial concentration techniques to explore correlations between environmental factors and ammonium remineralisation from viral lysis. Viral mediated
ammonium remineralisation changed with different chlorophyll a concentrations and salinities, suggesting potential predictive associations.

These results show that liberated nitrogen from viral lysis of bacteria is readily degraded by heterotrophic marine bacteria and remineralised into ammonium for uptake by autotrophic organisms. The results in this dissertation demonstrate that viruses are key players in the cycling of nitrogen in marine systems and stress the need to incorporate viral mediated nutrient release into models of global biogeochemical cycling.
Preface

Chapter 2 is based on work conducted at the Marine Biological Laboratory at the University of Copenhagen by Dr. M. Middelboe, Dr. N. Jørgensen, S. Rasmussen, and E. Shelford. E. Shelford developed the experimental plan with input from S. Rasmussen and guidance from M. Middelboe, conducted the experiment with S. Rasmussen, and analysed all the samples except for the amino acid analysis which was performed by N. Jørgensen. E. Shelford was responsible for the majority of the data analysis, with guidance from M. Middelboe, N. Jørgensen, and C. Suttle.

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incubations for GH and JP. SI and FRP are singleton measurements. Initial C concentrations in primary producers was calculated by adding together the contribution by larger primary producers (calculated by converting initial chlorophyll $a$ concentrations to C using a C:chlorophyll $a$ ratio of 50:1) and the contribution by picophytoplankton (calculated by multiplying the initial picophytoplankton concentrations by a cellular C quota for $Synechococcus$ of 51.9 fmol cell$^{-1}$ as measured in this experiment).

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List of Abbreviations

C = Carbon
D- AA = D-amino acid
DCAA = Dissolved combined amino acid
DFAA = Dissolved free amino acid
DON = Dissolved organic nitrogen
HMW = High molecular weight
L-AA = L-amino acid
LMW = Low molecular weight
N = Nitrogen
PCA = Principal components analysis
PON = Particulate organic nitrogen
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Dedication

This one’s for you, Steven.
Chapter 1: Introduction

1.1 The microbial world

Microbes in the ocean are invisible to the unaided eye, yet far outweigh the visible macroorganisms in abundance and total biomass. There are $\sim 10^{29}$ prokaryotic\(^{1}\) cells in the ocean (Whitman et al. 1998), constituting typically $\sim 10^6$ cells ml\(^{-1}\) in surface waters (Fuhrman et al. 1989, Cho & Azam 1990, DeLong et al. 1994) and an estimated 90% of total biomass in the ocean (Wilhelm & Suttle 1999, Suttle 2005). Typical abundances of viruses are an order of magnitude more than prokaryotes at $10^{30}$ viruses in the ocean (Suttle 2007) and an average of $10^7$ particles ml\(^{-1}\), and all the viruses in the ocean contain a total of 200 Mt of carbon (Suttle 2005). Microzooplankton such as heterotrophic nanoflagellates and ciliates are also relatively abundant at $10^3$ cells ml\(^{-1}\) (Fenchel 1982), although their abundances are approximately a thousand fold less than the prokaryotes. All of these microorganisms have a profound impact on the ocean ecosystem, through trophic interactions and subsequent nutrient cycling.

1.1.1 Viruses in the ocean

Viruses are very abundant in the ocean ranging from $10^4$-$10^5$ viruses ml\(^{-1}\) in deep waters (Weinbauer 2004) to $10^8$ viruses ml\(^{-1}\) in coastal surface waters (Suttle 2005). They infect everything from large animals (e.g. Arbelo et al. 2012) to zooplankton (e.g. Dunlap et al. 2013)

\(^{1}\) For simplicity throughout this dissertation, prokaryotes will be referred to as bacteria, although archaea also make up a substantial proportion of prokaryotic biomass (DeLong 1992). In addition, the term bacteria will be used exclusively to refer to heterotrophic bacteria, and when autotrophic prokaryotes are mentioned, they will be referred to as cyanobacteria.
to phytoplankton (Brussaard 2004a), but the vast majority of viruses in the ocean infect the highly abundant and diverse bacteria (Weinbauer 2004). The outcome of a viral infection of single celled organisms such as bacteria varies depending on the different viral life cycles or the environmental conditions. A common infection strategy is the lytic infection, whereby the virus infects the host and takes over the host’s metabolic processes to produce new virus particles. Lytic infection results in the bursting of the single-celled organism, which releases all contents of that cell to the seawater, contributing to the dissolved organic matter pool.

1.1.2 Bacterial mortality

Bacterial viruses and eukaryotic grazers of bacteria are responsible for the majority of bacterial mortality (Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995). Bacterial viruses have specific host ranges, often infecting a limited number of strains within a species or genus (Suttle 2007). The majority of the detectable viruses in the ocean infect bacteria (Fuhrman 1999, Wommack & Colwell 2000, Suttle 2005, Steward et al. 2013), which they encounter via passive diffusion (Fuhrman 1999). Marine viruses can be influential, with some phytoplankton viruses controlling and terminating phytoplankton blooms (Castberg et al. 2001, Brussaard et al. 2005), and bacterial viruses controlling bacterial community structure by increased infection rate of the most active strain (Thingstad & Lignell 1997, Schwalbach et al. 2004).

Grazers of bacteria are primarily flagellated and ciliated protists 2 to 5 µm in diameter, slightly larger than the bacteria they consume (Sherr & Sherr 2002). Because of high metabolic needs and reliance on passive contact grazing they require relatively high concentrations of bacteria to sustain them, often more than 2-10x10⁴ cells ml⁻¹ (Taylor 1978, Pace 1988, Eccleston-Parry & Leadbeater 1994). These protists are generally considered to be non-specific grazers of bacteria,
although larger and more active cells may be preferentially grazed (Gonzalez et al. 1990).

Grazing rates mostly depend on random contacts with their prey, although there are some reports of chemotaxis (Seymour et al. 2009).

There are numerous estimates of grazing rates on aquatic bacteria (McManus & Fuhrman 1988, Pace 1988, Jürgens & Massana 2008), and more recently estimates of viral mediated mortality (Suttle 1994, Fuhrman 1999, Weinbauer 2004). Fuhrman and Noble (1995) examined both grazing and viral lysis in coastal Californian waters, and concluded that viruses and grazers contributed equally to bacterial mortality. Although this result diverged from the idea that most bacterial mortality is due to protistan grazing (e.g. McManus & Fuhrman 1988), it was consistent with emerging ideas that viral lysis is a significant source of bacterial mortality. Since then, numerous studies have found evidence both contradicting and supporting Noble and Fuhrman’s work. Similar grazing and viral lysis rates were also found in the oligotrophic coastal waters of the Mediterranean (Boras et al. 2009). Significant viral lysis rates compared to grazing rates were documented in the northern Adriatic Sea (Weinbauer & Peduzzi 1995), in the bottom waters of an Arctic bay (Wells & Deming 2006a), and in coastal waters off Japan (Taira et al. 2009). However, higher grazing rates compared to viral lysis rates were found in an oligomesotrophic lake (Bettarel et al. 2003), coastal Malaysia (Lee & Bong 2012), the subtropical northeast Atlantic (Boras et al. 2010), and an oxic-anoxic interface in the Baltic Sea (Anderson et al. 2012). Temporal variations of both viral lysis and grazing have also been found. In a Korean eutrophic bay the ratio of viral mortality relative to grazing ranged from 0.03 to 2.0, mainly due to fluctuating flagellate populations (Choi et al. 2003). In eutrophic (Tijdens et al. 2008), mesotrophic (Jacquet et al. 2005), and oligotrophic (Ram 2005) lakes the viral contributions to bacterial mortality changed seasonally. Variation amongst these studies does not allow for any
unifying conclusions as to the importance of viral lysis versus grazing in different environmental conditions.

1.1.3 The microbial loop

Through the process of infecting and ingesting bacteria, viral lysis and microzooplankton grazing alter the composition of nutrient pools in the ocean, which can affect the growth of other organisms. Microbes have an integral role in the cycling of nutrients in the ocean. Carbon (C) is generally transferred to higher trophic levels from the base of the food web. Primary producers fix C via photosynthesis or chemoautotrophy, are subsequently consumed by herbivores which are in turn consumed themselves, and C is then passed up the food chain. Not all C follows this path, however. Excretion and sloppy feeding by higher trophic levels remove some C to the particulate and dissolved organic matter pools, and bacteria degrade this C for their own metabolism. Some of these bacteria are then ingested by grazers, and the C re-enters the traditional food chain this way. This process is known as the microbial loop (Azam et al. 1983; Figure 1.1). A major cause of C input to the dissolved organic matter pool is cell lysis by viral infection. Since viral lysis kills 10-20% of bacteria each day (Suttle 1994), there is a substantial amount of C removed from the system via this ‘viral shunt’ (Figure 1.1; Wilhelm & Suttle 1999). It is proposed that the viral shunt represents a loss of C from the system (Bratbak et al. 1994, Middelboe & Lyck 2002) due to bacterial respiration of dissolved organic C, which is only replenished by C fixation of primary producers.
Figure 1.1 Diagram of the microbial loop and the viral shunt. All components of the food web contribute to the pool of dissolved organics. Bacteria can use the nutrients from this pool for their own metabolism, but they also contribute to the pool via viral lysis.

1.2 Nutrient recycling

1.2.1 Nitrogen cycling

As a key component of proteins and nucleic acids, nitrogen (N) has a myriad of functions in cells. N is a ubiquitous nutrient that supports primary production. It was traditionally thought to be the major limiting nutrient in the ocean (e.g. Tyrrell & Law 1997, Tyrrell 1999). Although the discovery of huge areas of iron limitation and phosphorus limitation has challenged this notion, N is still often an important limiting or co-limiting nutrient (Zehr & Kudela 2011). With the exception of some cyanobacteria, N$_2$ is not accessible to primary producers and therefore N is most often acquired in another inorganic form, such as nitrate, nitrite, or ammonium. N from
outside the mixed layer such as from upwelling of nitrate, land runoff, and N$_2$ fixation is known as new production, and is often in the form of nitrate. N can also be recycled within a system by bacterial degradation of dissolved organic matter to produce ammonium. This recycled N is known as regenerated production (Dugdale & Goering 1967, Eppley & Peterson 1979).

N may take the form of eight different oxidation states in the ocean, and there are numerous processes that transform N from one compound to another (Figure 1.2). Ammonium is an available source of N to phytoplankton and bacteria, and is a key N source in low nutrient, oligotrophic regions where new N is scarce. It is important to understand the processes controlling ammonium concentrations to understand N cycling in the ocean.

Figure 1.2 The various compounds and transformations of nitrogen in the ocean. Modified from Hutchins et al. (2009).
The direct sources of ammonium are organic N remineralisation and N\(_2\) fixation, and the direct sinks are assimilation, nitrification, and anammox (anaerobic ammonium oxidation). Anammox is a low oxygen process that can be discounted in oxygenated surface waters (Zehr & Ward 2002). Ammonium oxidiation is an aerobic process accomplished by nitrifying bacteria that can be an important removal process of ammonium (Fernández et al. 2009). N\(_2\) fixation, the process by which N\(_2\) gas is reduced to reactive N, can be a significant source of organic ‘new’ N in some regions such as the North Atlantic where sufficient iron is available for cyanobacteria to fix N (Capone & Carpenter 1982, Zehr et al. 2001). Organic N from N\(_2\) fixation can eventually be remineralised as ammonium. However, the major controls of ammonium in oxygenated surface waters are remineralisation of organic matter by bacteria and grazers, and assimilation by phytoplankton and bacteria.

Dissolved organic nitrogen (DON) is another form of N in the ocean. DON is loosely defined as any organic material that contains N and can pass through a 0.2 \(\mu\)m pore-size filter (Benner et al. 1992). Material larger than 0.2 \(\mu\)m is defined as particulate organic nitrogen (PON). DON concentrations in coastal waters can range from 1-37 \(\mu\)M (Scudlark et al. 1998). DON is classified into two size classes, the high molecular weight compounds (HMW) and the low molecular weight compounds (LMW). HMW DON is mostly categorised as undefined ‘humus’ and is thought to be generally refractory, composed of substances such as heterocyclic compounds (Zehr & Ward 2002) and acetylated amino sugars (McCarthy et al. 1997). HMW DON is mostly in amide form, indicating that it derives from biological sources (McCarthy et al. 1997); as well, a portion of coastal DON is derived from anthropogenic sources (Seitzinger et al. 2010). LMW DON can include urea, amino acids, proteins, nucleic acids, nucleotides, and amino sugars (Berman & Bronk 2003). It was believed that the majority of DON is produced through
phytoplankton degradation, lysis, or excretion (Pujo-Pay et al. 1997); however, it is now known that a portion of DON is from bacterial cell walls (McCarthy et al. 1998), and therefore bacteria are both a source and a sink for DON (Zehr & Ward 2002).

1.2.2 Phytoplankton nitrogen uptake

Phytoplankton are ubiquitous and abundant in the ocean’s surface waters, and need sources of inorganic nutrients, including N, to support their primary production (Armbrust et al. 2004). Although some phytoplankton may prefer nitrate (Cochlan & Harrison 1991), many phytoplankton preferentially use ammonium as a source of N because it does not need to be further reduced in order to be incorporated into amino acids (Dugdale & Goering 1967, McCarthy et al. 1977, Middelburg & Nieuwenhuize 2000). Nitrate uptake may even sometimes be inhibited in the presence of ammonium (Dortch & Conway 1984, Dortch et al. 1991), possibly due to the high energy costs of nitrate reduction (Thompson et al. 1989). Ammonium is a key N source for primary production in oligotrophic regions where new N production is scarce, but is also periodically an important source of N in coastal environments. DON may also be a source of N for primary producers (Bronk et al. 1994), despite traditional views that bacteria are the main consumers of DON.

1.2.3 Nitrogen remineralisation by microzooplankton

Microzooplankton contribute to inorganic N remineralisation by metabolising ingested bacteria and excreting inorganic N (Dolan 1997, Sherr & Sherr 2002). Although microzooplankton contribute to the recycling of other nutrients such as phosphorus, iron, thorium, and others (Nagata & Kirchman 1992, Turk et al. 1992, Dolan 1997, Barbeau et al. 2001, Sherr & Sherr 2002), ammonium is an important excretory product (Paasche & Kristiansen 1982). Laboratory-
based studies have found microzooplankton N remineralisation efficiencies of 25 to 75% (Eccleston-Parry & Leadbeater 1995, Dolan 1997, Davidson et al. 2005). Remineralisation efficiencies vary depending on the N limitation of the microzooplankton and the nutritive quality of the bacteria being grazed (Goldman et al. 1985, Grover & Chrzanowski 2009), as well as on other factors such as temperature and prey species (Rose et al. 2008). The ammonium excreted by microzooplankton is available to other organisms such as bacteria (Selph et al. 2003) and phytoplankton (Nugraha et al. 2010).

1.3 Viral contribution to nitrogen cycling

Viral lysis of bacteria results in the release of dissolved organics (Scanlan & Wilson 1999) made up of bacterial cell constituents such as cell walls, membranes, proteins, and cytoplasm. The nitrogenous parts of the cell often consist largely of free and combined amino acids (Middelboe & Jørgensen 2006), as well as nucleic acids and inorganic N products (nitrate, ammonium, urea). C sources released by viral lysis include polysaccharides (Ieranò et al. 2010), lipids (Oliver & Colwell 1973), sugar components of peptidoglycan (Costerton 1974, Benner & Kaiser 2003), and other parts of the organelles, membrane, and cell wall structures. As well, the viral particles themselves can be a source of dissolved organic matter (Noble & Fuhrman 1999). These products are released to the dissolved organics pool, where they may be available for uptake by other microbes. However, DON is available only to organisms that can take it up directly such as heterotrophic bacteria, although some phytoplankton have heterotrophic capabilities (Antia et al. 1991, Mulholland et al. 2002, Purina et al. 2004). This is in contrast to ammonium excreted by microzooplankton, which is immediately available to all primary producers. This DON release is
suggested to be an important source of N in oligotrophic systems, where there are few new sources of N (Sandroni et al. 2007).

1.3.1 Bacterial incorporation of viral lysis products

Heterotrophic bacteria use dissolved organic matter for their metabolic requirements. Although N and other nutrients are needed for bacterial growth, C is needed for both growth and respiration. When these dissolved organics contain N, as in amino acids, part of the metabolic processing includes deamination of amine groups to liberate C from the molecules (Hollibaugh 1978, Goldman et al. 1987, Jardillier et al. 2005). If N is in excess of bacterial requirements, it is released as ammonium. This ammonium is available to be taken up by other organisms, such as primary producers (e.g. Dugdale & Goering 1967). Efficiency of DON use by bacteria is dependent on the nature of the DON (labile or refractory), and availability of other nutrients (Polimene et al. 2006). N remineralisation efficiency is affected by the C:N ratio of the nutrient source relative to the C:N ratio of the bacterial biomass and the amount of C consumed by respiration (Goldman et al. 1987). Since the microbial loop was introduced, other studies have described DON release from sources other than phytoplankton exudation, such as from viral lysis (Wilhelm & Suttle 1999).

Bacteria can assimilate DON produced from viral lysis. This was clearly demonstrated by a laboratory-based study in which a bacterial strain (Cellulophaga sp.) was able to incorporate the viral lysate products from another bacterial strain (Photobacterium sp.; Middelboe et al. 2003). The two bacteria were cultured together and fed a C source that was only available for uptake by Cellulophaga sp. The C-replete Cellulophaga sp. was infected and lysed by its specific virus, and an estimated 62% of the lysate was metabolised by the starved Photobacterium sp. An
increase in metabolism of uninfected bacteria after viral lysis was also found by Middelboe et al. (1996).

Bacteria can also use DON released by viral lysis of phytoplankton (Gobler et al. 1997, Bratbak et al. 1998). Gobler et al. (1997) infected the chrysophyte *Aureococcus anophagefferens* with a virus, and monitored the bacteria in the sample. They found significant growth of prokaryotes and attributed it to the availability of lysate. Haaber & Middelboe (2009) ran a similar experiment using the autotrophic flagellate *Phaeocystis pouchetii* and its isolated virus and monitored remineralisation of the lysates by bacteria. They found that 78% of N in the lysate was remineralised to ammonium. In addition, they found that 11% of the growth of a co-occurring flagellate could be attributed to the increase of ammonium from remineralisation of lysate.

Although lysis of eukaryotes can provide a large nutrient influx, especially in bloom situations (Brussaard et al. 1996), the continual turnover of bacteria by viruses contributes a large and steady input of lysate products. A study by Weinbauer et al. (2011) found that with removal of the viral fraction, primary production (predominantly from a *Synechococcus* bloom) was significantly reduced. They proposed three hypotheses. First, that *Synechococcus* growth could be directly stimulated by viral lysates of bacteria, as cyanobacteria may be able to take up DON released by viral lysis (Zubkov et al. 2003, Poorvin et al. 2004). Second, a reduction in bacterial abundance from viral lysis results in less competition for nutrients with *Synechococcus*. They favoured the third hypothesis, which was suggested that viral lysis of bacteria provides nutrients to stimulate bacterial growth and respiration, remineralising inorganic nutrients that *Synechococcus* can use.

Assuming that the C:N ratio of bacterial lysates is similar to that of bacteria (i.e. between 4:1 and
5:1, Goldman et al. 1987) and that growth efficiencies of uninfected bacteria are in the range of 0.2 to 0.3 (Middelboe et al. 1996) such that their C needs are greater than their N needs due to respiration, lysates contain N in excess of bacterial requirements. Consequently, bacterial metabolism of viral lysates should produce inorganic N that is available to primary producers (Davidson et al. 2007, Haaber & Middelboe 2009), dependent on the environmental conditions of the system and the other sources of N such as from grazing of bacteria. Therefore, viral lysis of bacteria may represent a significant pathway for the remineralisation of ammonium, a major N source that supports primary production in the ocean.

1.4 Objectives

This dissertation focused on studying DON released by viral lysis of bacteria, describing and quantifying the influence of viruses on N concentration and phytoplankton abundance, and comparing N remineralisation in different environments caused by both viral lysis and grazer ingestion. Because of high bacterial abundance and mortality rates, microbial N cycling has a considerable effect on larger-scale N availability in the ocean.

The overall goal of this dissertation was to investigate the influence of viruses on N cycling in the ocean by: 1) measuring the nitrogenous products of viral lysis of heterotrophic marine bacteria and the bacterial uptake of those products; 2) demonstrating a reduction of N remineralisation and phytoplankton growth in the absence of viruses; 3) quantifying the effect of bacterial viruses in providing N to support phytoplankton growth; and 4) comparing N remineralisation by viral lysis across environmental conditions and against grazing.
This dissertation is organised into four main chapters. The following describes each chapter by defining a specific scientific question and describing the hypothesis of the expected results.

Chapter 2:

Question: What parts of the DON from viral lysis of a marine bacterium (*Cellulophaga* sp.) can be used for the growth and abundance increase of another marine bacterium (*Photobacterium* sp.)?

Hypothesis: Released DON includes a high proportion of dissolved free and combined amino acids and ammonium. Ammonium remineralisation occurs as *Photobacterium* metabolises released DON for respiration and growth.

Chapter 3:

Question: Do viruses have an effect on ammonium remineralisation in the ocean?

Hypothesis: The absence of viruses reduces the amount of free ammonium in the system.

Chapter 4:

Question: Is viral lysate from heterotrophic marine bacteria available to support N-limited phytoplankton growth (*Synechococcus*)?

Hypothesis: A large proportion of lysate is available to support phytoplankton growth, but the majority of the released DON must be first metabolised by heterotrophic bacteria and remineralised as ammonium.
Chapter 5:

Question: How do N remineralisation rates (as supported by viral lysates) differ across environmental gradients? How do N remineralisation rates differ between viral lysis and grazer excretion?

Hypothesis: Regions with high nutrient and chlorophyll a concentrations and bacterial abundance have the highest viral mediated N remineralisation rates due to higher contact rates between viruses and bacteria.

1.5 Methodological considerations

Some of the studies examining grazer versus viral mortality rates use a Landry and Hassett-style dilution method (Landry & Hassett 1982) to determine viral lysis rates, in which seawater is diluted with virus-free water to reduce contact rates, and apparent bacterial growth rates for each dilution are measured (Jacquet et al. 2005, Wells & Deming 2006a, Tijdens et al. 2008, Taira et al. 2009). Apparent growth rate is plotted against the fraction of unfiltered seawater, and the predation rate can be calculated from the slope. This method works well for zooplankton grazers, but there are a number of objections to using it with viruses. When seawater is diluted to reduce the concentration of grazers, the decrease in grazing rate is immediate. The effects of dilution in the presence of viruses, however, take at least 12-24 hours to observe, as cell lysis happens only at the end of the lytic cycle, and lytic cycle length varies among bacterial strains. Another objection involves the dramatic increase in viral abundance compared to grazers. Viral replication results in burst sizes much greater than the doublings observed in grazers, which can
obscure dilution factors. Over the length of incubation grazers replicate very little, but every time a cell lyses from viral infection, many more viruses are produced. Also, bacteria already infected at the time of sample incubation will eventually burst, resulting in bacterial mortality that obscures manipulation effects.

The method used in this dissertation (Chapters 3 and 5) is known as the viral production method (Wilhelm et al. 2002). In this method, viruses are ‘washed out’ of a seawater sample by filtering the sample through a 0.2 µm filter and keeping the bacteria re-suspended above the filter through physical agitation and addition of virus-free seawater to keep the volume constant. This process significantly reduces viral abundance while maintaining ambient concentrations of bacteria. This method has been modified to reduce viral abundance via tangential flow filtration (Winget et al. 2005), which is the method used in this dissertation. There are some losses in bacterial abundance with this method, but viral abundance is reduced to a far greater extent, rendering this method suitable for determining the effect of viruses on the bacterial population. Manipulation effects might be expected, but can be measured by including a control treatment in which virus-replete filtrate is added back during the washing stage to maintain ambient viral concentrations.
Chapter 2: Released nitrogen from viral lysis supports bacterial production

2.1 Synopsis

Lysis of marine bacteria by viruses releases a range of organic compounds into the environment, including D- and L-amino acids; however, the uptake of these compounds by other bacteria is not well characterised. This study determined that *Photobacterium* sp. strain SKA34 (Gammaproteobacteria) increased in abundance following uptake of D- and L-amino acids from viral lysate from *Cellulophaga* sp. strain MM#3 (Flavobacteria). Ammonium and dissolved free amino acids (DFAAs) were taken up almost completely, suggesting the available C:N in the lysate was relatively high for *Photobacterium* sp. growth thus causing a net uptake of ammonium. In contrast, only 1.51 µmol l\(^{-1}\) of the 4.77 µmol l\(^{-1}\) total dissolved combined amino acids (DCAAs) were taken up, indicating that a fraction of lysate-derived DCAAs were semi-labile or refractory to bacteria. Both D- and L-amino acid uptake rates were approximately proportional to their concentrations, indicating similar availability for each enantiomer as well as unsaturated uptake rates. These results imply that under high C:N conditions both D-amino acids (mainly found in bacterial cell walls) and L-amino acids (found in proteins of the rest of the cell) are equally available for *Photobacterium* sp. growth, and they help us understand the fate of the products of viral lysis.
2.2 Introduction

Anywhere from 18-83% of the total nitrogen (N) in the ocean consists of dissolved organic nitrogen (DON), a fraction of which includes cellular components such as proteins, nucleic acids, metabolites, and cell wall substances, as well as transformed organic matter in humic substances (McCarthy et al. 1997, Berman & Bronk 2003). Among components in the DON pool, smaller nitrogenous molecules such as free and combined amino acids and urea are important in the cycling of N since they are relatively labile and can be assimilated by microorganisms (Antia et al. 1991).

Measurable concentrations of amino acids (AAs) in the surface ocean typically range from 0.3-1 μmol l⁻¹, but the total concentrations are likely higher due to inefficient methods for detection of all AAs and since many AAs are in the form of hydrolysis-resistant amide groups (Keil & Kirchman 1993, McCarthy et al. 1996) and most of the N in living organisms is contained in AAs (McCarthy et al. 1998), including both free AAs (DFAAs) and AAs combined into polypeptides (DCAAs; Antia et al. 1991). Although most AAs within organisms are homochiral with the L-AA enantiomer dominating (Bonner 1995), there is a significant pool of D-AAs in the ocean (McCarthy et al. 1998), mainly of bacterial origin. The majority of D-AA isomers in bacteria are components of the peptidoglycan in cell walls (McCarthy et al. 1998), but can also be found in other parts of bacteria such as in lipopeptides (Kaiser & Benner 2008). D-AA measurements in seawater indicate that a substantial portion of AAs in DON originates from bacteria (McCarthy et al. 1998), and D-AA enrichment in refractory DON pools is consistent with the idea that bacterial cell walls are more refractory than are proteins (Berman & Bronk 2003, Nagata et al. 2003, Kitayama et al. 2007).
In the ocean, other sources of DON in addition to cell wall material include release by phytoplankton (Sarmento et al. 2013) and bacteria (Kaiser & Benner 2008), as well as from viral lysis of phytoplankton (Gobler et al. 1997, Hewson et al. 2004, Haaber & Middelboe 2009) and bacteria (Middelboe et al. 2003, Middelboe & Jørgensen 2006). Lysis of infected bacteria releases DON including DCAAs, DFAAs, and other cellular components (Middelboe & Jørgensen 2006). This influx of organic matter enhances bacterial production and supports the microbial loop and the cycling of inorganic nutrients (Fuhrman 1999, Wilhelm & Suttle 1999, Middelboe & Lyck 2002). AAs are taken up via a variety of transport systems that vary in specificity (Razin et al. 1968, Crawford et al. 1974, Cheruel & Jullien 1979, Antia et al. 1991, Montuelle et al. 1992). In eukaryotes, the uptake is often stereospecific with different transport systems for D-AAs and L-AAs (Suzuki 1981, McDaniel et al. 1982). Similar uptake systems may occur in bacteria, since uptake and release of D-AAs appear involved in rearrangements of peptidoglycan during specific growth phases (Cava et al. 2011, Azúa et al. 2014), and since uptake of D-AAs is stimulated during low-nutrient conditions in marina bacteria (Pérez et al. 2003). Since bacteria constitute a large portion of the living biomass in the sea (Whitman et al. 1998), release and viral lysis of bacterial cells, including isomers of AAs in cell wall material, are assumed to contribute significantly to N cycling in the ocean, and possibly to the production of refractory organic matter (Jiao et al. 2010). Consequently, knowing the fate of labile N substances from bacteria, such as AAs, is relevant to understanding marine N cycling.

This study examined whether lysis products by one bacterium can support the growth of another. Previous studies have shown that compounds released by viral lysis of the Flavobacteria Cellulophaga sp. can be taken up by uninfected bacteria (Middelboe et al. 2003, Middelboe & Jørgensen 2006), but these studies did not examine uptake of the specific compounds of viral
lysis by other bacterial taxa. This study monitored the production of ammonium, DFAAs, and DCAAs during viral lysis of the marine heterotrophic bacterium *Cellulophaga* sp. strain MM#3, and their subsequent uptake, including D and L isoforms of AAs by another marine bacterium *Photobacterium* sp. strain SKA34. These experiments help us understand the uptake of viral lysis products by marine bacteria.

### 2.3 Methods

#### 2.3.1 Growth of *Cellulophaga* sp. and lysate preparation

*Cellulophaga* sp. strain MM#3 was grown in 70% 0.2 μm filtered seawater and 30% ultrapure water containing 0.25 mmol l\(^{-1}\) lactose as a carbon source, 0.07 mmol l\(^{-1}\) K\(_2\)HPO\(_4\), and 0.26 mmol l\(^{-1}\) NaNO\(_3\) (modified from Middelboe et al. 2003). The strain belongs to a group of *Bacteriodetes* strains that have been isolated from five different locations in Danish and Swedish coastal waters during a 10-year period (Holmfeldt et al. 2007), and thus are considered a prevalent group in this environment. Cultures were grown on the bench-top at room temperature. Lysates of *Cellulophaga* sp. previously infected with a *Cellulophaga*-specific virus (*Celluphaga* P1; Middelboe et al. 2003) was spun down at 37000xg for 1 h and re-suspended twice in buffer to remove cellular debris. At approximately 4.0 x 10\(^7\) cells ml\(^{-1}\), the virus was added at a multiplicity of infection of 5, i.e. 20 x 10\(^7\) viruses ml\(^{-1}\). Bacterial density was measured by flow cytometry as described below, and the concentration of infectious viruses was determined by plaque assay at 0 h, 21.25 h, and 28.25 h. Plaque assays were performed by combining a series of stock *Cellulophaga* sp. cultures with increasingly diluted experimental samples, adding these mixtures to agar plates, and counting the plaques (areas of plate that did not form a bacterial
lawn). When viral abundance had increased 200 fold, the culture was terminated by sequential filtration through glass-fibre filters (Whatman, United Kingdom) and 0.2 µm pore-size Cyclopore filters (Whatman, United Kingdom). The filtered sample thus represents the dissolved fraction of the virus-derived organic matter following cell lysis, as some of the lysate (e.g. the larger cell fragments) may have been retained on the 0.2 µm filter. Samples for bacterial abundance, ammonium, DFAAs, and DCAAs were taken at 0 h and 28.25 h.

2.3.2 Addition and uptake of lysate

With the exception of control cultures, 100 ml of filtered lysate was added to 400 ml triplicate cultures of the marine *Photobacterium* sp. strain SKA34 (initial cell density 16.0 ± 6.1 × 10^6 cells ml⁻¹) grown in a nutrient-rich medium, washed via centrifugation, and inoculated in artificial seawater at a salinity of 15 without added nitrate or phosphate (Harrison et al. 1980, modified as in Cottrell & Suttle 1993). Like *Cellulophaga* MM#3, this strain was also isolated from coastal Danish waters (Pinhassi et al. 2003). *Photobacterium* sp. is a member of the Gammaproteobacteria, a class that is often important in dissolved organic matter assimilation (Elifantz et al. 2007), and can dominate bacterial communities exposed to dissolved organic matter from diatoms (Landa et al. 2013). Because *Photobacterium* sp. SKA34 cannot use lactose as a carbon source (Middelboe et al. 2003), any growth or respiration with the addition of filtered lysate would not be from residual lactose in the *Cellulophaga* sp. growth medium. Cultures were kept in the dark at 10°C. Samples for bacterial abundance and ammonium, DFAA, and DCAA concentrations were taken approximately every 6 h for 69.5 h. Triplicate control cultures without lysate addition was established in parallel.
2.3.3 Treatment and analysis of samples

Samples for bacterial abundance were fixed with gluteraldehyde at a final concentration of 0.5%, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Bacterial abundance was measured using a flow cytometer (BD FACSCanto II, Beckon Dickinson, Franklin Lakes, NJ) following Brussaard (2004). Samples for ammonium, DFAA, and DCAA determination were filtered through glass-fibre filters (Whatman, United Kingdom) into 15 ml polycarbonate tubes, and frozen at -20°C until analysis. Ammonium concentrations were determined from the filtered samples with a fluorometer (Holmes et al. 1999).

Dissolved free and combined amino acids (DFAA and DCAA) in two of the replicate cultures (#1 and #2) were quantified by high performance liquid chromatography (HPLC) and fluorescence detection using two methods. Total amounts of DCAA (after hydrolysis, see below) and DFAA were detected as fluorescent primary amines after derivatization with o-phthaldialdehyde (OPA) according to Lindroth & Mopper (1979) and Jørgensen et al. (2003). For the analysis of DCAA, triplicate water samples were freeze-dried and subsequently hydrolysed by a microwave technique (Jørgensen & Jensen 1997). The hydrolysed samples were re-dissolved in 1.25 mol l⁻¹ borate buffer at pH 9.5 and analysed by HPLC. Concentrations of DFAA were subtracted from the concentrations of total dissolved hydrolysable amino acids to provide DCAA concentrations. The D and L isomers of Asp, Glu, Ser and Ala were detected according to Mopper & Furton (1999) with the exception that N-isobutyryl-L-cysteine (IBC) was used as a chiral agent (Brückner et al. 1994). Analysis of amino acid D isomers focused on Asp, Glu, Ser and Ala as these amino acids have been found to be dominant isomers in natural and biological material, including bacterial peptidoglycan (Brückner et al. 1994, Jørgensen et al. 2003). HPLC columns were 3.9 µm 150 mm steel columns, type Nova-Pak C18 (for the OPA
method) and XTerra RP18 (for the IBC method) (Waters Associates, USA). Analytical variation of DFAA concentrations between replicate analyses (injections) was below 5%.

2.3.4 Calculations

Uptake rates of DCAAs were calculated by subtracting the final concentration from the initial concentration, and dividing by the total hours of the experiment.

2.4 Results

2.4.1 Uptake of nitrogen from lysate by Photobacterium sp.

The abundance of Photobacterium sp. cells did not change significantly in the control culture but increased in response to lysate addition (Figure 2.1). Similarly, there were no detectable changes in the concentrations of nitrogenous compounds in the control; however, after 24 h, and coincident with the rapid disappearance of ammonium, Photobacterium sp. took up DFAAs and DCAAs released by viral lysis (Figure 2.2). The patterns of usage differed among compounds, with ammonium and DFAAs taken up rapidly (Figure 2.2A and Figure 2.2B), whereas only about a third of the initial 4.77 µmol l⁻¹ of DCAAs was removed by 69.5 h (Figure 2.2C). Moreover, with the exception of serine, there was uptake of most individual AAs for replicate #1 (Figure 2.3A), although uptake varied depending on the AA and the initial concentration (Figure 2.4). Replicate #2 also showed no uptake for L-Asp, D-Asp, and Thr (Figure 2.3B); however, replicate #1 showed better chromatograms and had better peak separation in the HPLC analysis, and so more weight is given to replicate #1 results.
Figure 2.1 (A) Changes in *Photobacterium* sp. abundance over time following addition of lysate and in the control culture. Error bars are standard deviation from triplicate incubations. (B) Natural logarithm of changes in *Photobacterium* sp. abundance over time in the treatment with added lysate. The growth rate of the exponential phase is 1.12 d$^{-1}$. 
Figure 2.2: Uptake of nitrogenous compounds from the addition of lysate by *Photobacterium* sp. over time: (A) ammonium, (B) dissolved free amino acids (DFAAs), and (C) dissolved combined amino acids (DCAAs). Error bars in (A) are standard deviation from triplicate incubations, in (B) are range from duplicate incubations, and in (C) replicates are shown separately.
Figure 2.3: Concentrations of amino acids at initial (T=0 h) and final (T=69.5 h) time points in the *Photobacterium* sp. experiment for replicate incubations (A) #1 and (B) #2. Data for both figures are only shown for amino acids with a difference between T=0 h and T=69.5 h that is greater than the average technical error, calculated from the average of the standard deviation of triplicate technical replicates for each amino acid. Error bars are standard error of triplicate technical replicates.
2.4.2 Uptake of D- and L-amino acid enantiomers

In order to determine the relationship between concentrations of DCAA and their uptake rates, the measured rates were plotted against concentration of each individual DCAA (Figure 2.4). The graph indicates that the uptake was concentration-dependent, at least for the most abundant DCAA such as L-Glu, L-Asp, D-Asp, L-Ala and Thr.

![Uptake rate vs Initial concentration graph](image)

Figure 2.4: Uptake of specific amino acids by *Photobacterium* sp. for each amino acid as a function of its initial concentration from replicate #1. The regression is significant (p <<0.001, $R^2 = 0.85$).

2.5 Discussion

This study demonstrates that the marine bacterium *Photobacterium* sp. strain BKA34 takes up D- and L-AAs released by viral lysis of *Cellulophaga* sp. strain MM#3 in proportion to the relative abundance of each amino acid; however, only about one third of the amino acid content of the
dissolved lysates is immediately available for bacterial uptake. These observations are important for understanding the fate of the products of viral lysis in the sea.

2.5.1 Bioavailability of amino acid-nitrogen in viral lysates

Bacterial lysates are generally assumed to contain excess N relative to bacterial N demand due to the low (4.5:1) C:N ratio of bacterial cells and bacterial respiratory loss of >70% of the carbon (C) uptake (Middelboe et al. 1996). Consequently, bacterial uptake of lysates is expected to result in net remineralisation of N. Instead of the expected ammonium remineralisation (Hollibaugh 1978, Shelford et al. 2012), ammonium and DFAAs were taken up rapidly (Figure 2.2A and Figure 2.2B), indicating that initial concentrations of organic C relative to N were in excess of the growth needs of Photobacterium sp. Assuming a cell C content of 20 fg cell$^{-1}$ (Carlsson et al. 1999) and a C:N ratio of 4.5 (Goldman et al. 1987) for Photobacterium sp., the estimated bacterial N uptake was $16 \pm 4 \mu$mol l$^{-1}$ N during the 69.5 h incubation. Of this N demand, on average $0.3 \mu$mol l$^{-1}$, $1.5 \mu$mol l$^{-1}$ and $2.3 \mu$mol l$^{-1}$ was supported by uptake of DFAAs, DCAAs, and ammonium, respectively, corresponding to 25% of the estimated total bacterial N demand. This suggests that available N sources other than AAs were released from viral lysis and emphasises that the initial C:N ratio of bioavailable organic matter in the lysate-enriched cultures was higher than required to allow mineralization of excess N.

The decrease in bacterial growth rate (i.e. stationary phase) after ~40 h (Figure 2.1) from 1.12 d$^{-1}$ to 1.06 d$^{-1}$ and eventually to 0.97 d$^{-1}$, along with no remineralisation of N, indicates a continuing shortage of N. Despite conditions where the DFAA pool was exhausted and bacterial growth was most likely substrate limited (presumably by N, although limitation by other nutrients such as phosphate cannot be discounted), 68% of the DCAA pool was still not used by the bacteria. This
suggests that a significant portion of the DCAAs were not immediately available for uptake and growth by *Photobacterium* sp. This may be because a portion of the DCAAs had yet to be degraded, possibly by enzymatic activity, into a form that bacteria could take up, thus resulting in a delay in the DCAA uptake (Nagata et al. 1998, Murray et al. 2007). However, DCAAs released by viral lysis may also potentially be a source of refractory DON, as DCAA derived from bacterial membranes have been shown to persist and accumulate in the ocean (Tanoue et al. 1995). More recently, the presence of viruses has been shown to increase the formation of humic-like dissolved organic matter by a factor of 2.8 compared to incubations without viruses (Lønborg et al. 2013), further supporting that viral lysis may contribute to the formation of refractory DON.

2.5.2 *Uptake of different amino acids by Photobacterium sp.*

The differences in uptake among AAs is likely the result of initial concentrations, as seen from the linear relationship between uptake rate and initial concentration (Figure 2.4), indicating that uptake rates were not saturated and maximum uptake rates were not reached. Uptake kinetics are dependent on the nutritional status of the bacteria (Jørgensen 1992), and different uptake systems can be present at different substrate concentrations (Unanue et al. 1999). In the current study, the AAs with the highest concentrations had the highest uptake rates, indicating that *Photobacterium* sp. took up AAs based on their relative availability.

Unlike the other AAs, there was production of L-Ser in both replicates and D-Ser in replicate #1 (Figure 2.3A and B). Serine is often a dominant DFAA in natural waters (e.g. Thurman 1985, Hubberten et al. 1995), and can sometimes be unavailable for uptake by bacteria and phytoplankton due to restricted uptake capacity (Palenik & Morel 1990, Ietswaart et al. 1994).
This may explain the presence of serine in natural waters. The present accumulation of L- and D-Ser at 69.5 h may speculatively reflect release of peptidoglycan components due to cell wall transformations in the stationary growth phase as observed for D-Ala in a *Vibrio* species by Azúa et al. (2014). Both isomers of Ser have previously been found in peptidoglycan (Pérez et al. 2003, Jørgensen et al. 2003). However, more studies are needed to document relations between specific extra- and intracellular D-isomers and cell growth phases.

2.5.3 Availability of both AA enantiomers

Both D- and L-AAs were taken up in proportion to their initial concentration over the course of the experiment from uptake by *Photobacterium* sp. (Figure 2.4). The main sources of D-AAs are bacterial cell wall components including peptidoglycans, lipopolysaccharides, and lipopeptides (Kaiser & Benner 2008). It is generally assumed that D-AAs and bacterial cell wall material in general are somewhat refractory (Grutters et al. 2002, Nagata et al. 2003, Kitayama et al. 2007) and accumulate in seawater (Dittmar et al. 2001), compared to more labile proteins originating from membranes and intracellular material. The D-enantiomer of AAs may be used as a prokaryotic biomarker, since prokaryotes are the main producers of D-AAs in the ocean (Kaiser & Benner 2008). Phytoplankton activity, on the other hand, supplies mainly L-AAs, which are taken up preferentially over D-AAs (Pérez et al. 2003). However, there is evidence that D-AA uptake increases in the low-nutrient deep ocean (Pérez et al. 2003), that it depends on whether the source is from Gram-negative or Gram-positive bacterial cell walls (Jørgensen et al. 2003), and that D-AA uptake rates increase with concentration (Azúa et al. 2014). Data from the current study suggests that D-AAs (from *Cellulophaga* sp. cell walls) and L-AAs (likely from intracellular and membrane sources) are taken up in proportion to their initial concentrations. For example, the sevenfold greater uptake of L-Glu relative to D-Glu is consistent with its higher
initial concentration, while the concentrations and uptake rates of L-Asp and D-Asp are similar (data not shown).

The results presented here indicate that AAs in viral lysates from *Cellulophaga* sp. are taken up by *Photobacterium* sp. Since both bacteria are Gram-negative bacteria, and since Gram-negative bacteria dominate the world’s oceans, this system is likely a reasonable model for the fate of AAs from the lysis of most marine bacteria. However, Gram-positive bacteria are diverse and potentially more abundant than previously considered (Gontang et al. 2007), as well as being biologically very different from Gram-negative bacteria (Gupta 2000). A previous study suggests that peptidoglycan of Gram-positive bacteria is more labile than that of Gram-negative bacteria (Jørgensen et al. 2003). Therefore, efforts should be undertaken to examine the fate of AAs released by viral lysis of Gram-positive bacteria in order to gather a more comprehensive picture of the role of viral lysis on nutrient cycling in the ocean.

### 2.5.4 Conclusions and ecological implications

There are a few surprising observations from the study presented here. First, the uptake of AAs was not associated with the remineralisation of ammonium that has been observed in many studies (e.g. Hollibaugh 1978, Goldman et al. 1987, Shelford et al. 2012). Second, only about a third of the DCAAs in the viral lysate was taken up over the five days of this study, indicating that many of the DCAAs released by viral lysis were not immediately available for uptake. Third, there was no evident discrimination in uptake of the D-AAs associated with bacterial cell walls, relative to L-AAs associated with bacterial proteins, in contrast to results from previous studies (Kawasaki & Benner 2006, Azúa et al. 2014). Because an estimated 10 to 20% of marine
bacteria are lysed by viruses every day (Suttle 1994), and bacteria represent most of the biomass in the oceans, it is crucial to determine the pathways and fate of nutrients released by viral lysis.
Chapter 3: Virus-induced ammonium remineralisation by heterotrophic bacteria contributing to primary production

3.1 Synopsis

Viruses have been implicated as major players in aquatic nutrient cycling, yet few data exist to quantify their significance. To determine the effect of viruses on ammonium remineralisation by bacteria, experiments were carried out in the oligotrophic Indian Ocean and eutrophic False Creek, Vancouver, Canada. Bacteria were concentrated, and then diluted with virus-free water to reduce virus abundance, or with virus-replete water to restore natural virus abundance. Virus-replete treatments showed increased ammonium concentrations compared to treatments with viruses removed (differences of 0.29 ± 0.14 and 1.44 ± 0.73 µmol l⁻¹ in the Indian Ocean and False Creek, respectively, mean ± standard deviation). Bacterial abundances were lower, while phytoplankton abundances and chlorophyll a concentrations were greater in the virus-replete treatments, consistent with the increased availability of ammonium in the presence of viruses. These data demonstrate that viral lysis leads to ammonium production, likely through the liberation of dissolved organic nitrogen that is remineralised by uninfected bacteria. In turn, the released ammonium supports primary production. These results show that viruses can play a critical role in marine nitrogen cycling, and suggest that viral lysis may contribute to the global nitrogen requirements of phytoplankton.
3.2 Introduction

Viruses are the most abundant biological entities in the ocean, the majority of which are thought to infect bacteria (Suttle 2007). Every day, viruses lyse an estimated 10 to 20% of the bacterial production in the ocean (Suttle 1994), releasing bacterial cell contents to the surrounding environment. This process has been termed the ‘viral shunt’ to describe the viral mediated transfer of nutrients from particulate to dissolved material (Wilhelm & Suttle 1999, Suttle 2005). The lysis products are used by uninfected bacteria (Middelboe et al. 1996, 2003); therefore, viruses play an important role in recycling carbon (C) and nutrients within the bacterial community. Viral loop models (e.g. Bratbak et al. 1994) incorporate virus-induced cycling of C within the bacterial size fraction into the pelagic food web model, and suggest that viral activity primarily acts as a sink of organic C, by removing whole cells from grazing and increasing bacterial respiration. This was verified experimentally by Middelboe & Lyck (2002) in a study in which viral activity stimulated C recycling and respiration by uninfected bacteria, and reduced the accumulation of microbial biomass. Further studies have confirmed efficient bacterial recycling of C from bacterial (Middelboe et al. 2003) or phytoplankton lysates (Haaber & Middelboe 2009).

There is also persuasive evidence that viruses are important agents of nitrogen (N) cycling in aquatic environments. Viral lysates are rich in free and combined amino acids (Middelboe & Jørgensen 2006) and are therefore a potentially important source of labile organic N. Moreover, bacteria assimilate dissolved organic N (DON) resulting from the lysis of infected microorganisms, especially when nutrient-limited (Gobler et al. 1997). During C-limited conditions, bacteria hydrolyse amino acids and other nitrogenous products to access the C, producing ammonium as a by-product (Goldman et al. 1987). Assuming that the C:N ratio of
viral lysates is similar to that of bacteria (i.e. between 4:1 and 5:1, Goldman et al. 1987) and that C growth efficiencies of uninfected bacteria are in the range of 0.2 to 0.3 (Middelboe et al. 1996), viral lysates contain N in excess of bacterial requirements. Consequently, bacterial metabolism of viral lysates should produce inorganic N that is available to phytoplankton (Haaber & Middelboe 2009). In addition to supporting bacterial metabolism, viral lysis therefore may also represent a significant pathway for the remineralisation of ammonium, a major N source that supports primary production in the ocean.

It is hypothesised that viral lysis of bacteria releases cellular debris to the dissolved organic matter pool, which is then accessible to uninfected bacteria. The subsequent turnover is potentially an important source of N for phytoplankton growth (e.g. Haaber & Middelboe 2009). Therefore, viral activity would not only be a C sink, but would also indirectly stimulate primary productivity, and thus C production. The strongest evidence that viral lysis supports the growth of phytoplankton was seen in several experiments conducted in the Gulf of Mexico and the Mediterranean in which the growth rates and the proportion of dividing Synechococcus cells were higher in treatments with viruses than in treatments in which viral concentration was either reduced by dilution with ultra-filtered seawater or heat-inactivated (Weinbauer et al. 2011).

To determine whether ammonium remineralisation associated with viral lysis influences phytoplankton growth, an initial experiment was conducted in the oligotrophic Indian Ocean and followed up with an experiment in False Creek, a small productive inlet in Vancouver, Canada. The experiments showed a decrease of ammonium remineralisation with removal of viruses, and a subsequent decrease in phytoplankton growth. These data are significant because they not only quantify and verify the significance of viruses in remineralising N, but also support the view that viruses are not simply parasites that disrupt food web flow (Azam & Worden 2004), but play a
significant role in nutrient recycling (Fuhrman 1999, Wilhelm & Suttle 1999) and ultimately provide N that supports primary production.

3.3 Methods

3.3.1 Description of sampling sites

Samples were collected from the surface waters of False Creek (FC), Vancouver, Canada (49°16’N, 123°7’W) on 3 Nov 2008, and from 10 m depth in the Indian Ocean (IO) (19°46’S, 114°52’E) on 12 Nov 2006.

The two study sites represented very different environments. The Indian Ocean station is open ocean and oligotrophic with a high degree of dissolved organic matter recycling (Visser et al. unpubl.), and was characterised by very low concentrations of chlorophyll $a$ (0.2 µg $l^{-1}$), inorganic nutrients (nitrate, 0.12 µmol $l^{-1}$; phosphate, 0.08 µmol $l^{-1}$; ammonium, 0.16 µmol $l^{-1}$), a sea surface temperature of 28°C, and a salinity of 35.0 (Table 3.1). In contrast, False Creek is a small productive inlet which is heavily influenced by neighbouring English Bay, the Fraser River, and freshwater runoff from the City of Vancouver. Tidal mixing results in a vigorous exchange of water between the sampling site at the mouth of False Creek and English Bay. Surface salinities in English Bay typically remain at ~25 to 26 during November and December (Frederickson et al. 2003), and temperatures range from ~7 to 10°C. These data are consistent with sea-surface temperatures for November 2008 of 10°C estimated from satellite data (http://las.pfeg.noaa.gov/oceanWatch/oceanwatch.php). In November, chlorophyll $a$ was low (0.47 µg $l^{-1}$), and nutrient concentrations high (nitrate, 14.8 µmol $l^{-1}$; phosphate, 1.43 µmol $l^{-1}$;
ammonium, 3.50 μmol l⁻¹). The low chlorophyll a concentrations despite high nutrient concentrations is consistent with light-limited conditions caused by seasonal mixing and turbidity.

Table 3.1 Location parameters, including temperature, salinity, chlorophyll a, nitrate, phosphate, and ammonium, for the Indian Ocean (IO) and False Creek (FC) stations.

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Salinity</th>
<th>Chlorophyll a (µg l⁻¹)</th>
<th>Nitrate (µmol l⁻¹)</th>
<th>Phosphate (µmol l⁻¹)</th>
<th>Ammonium (µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IO</td>
<td>28</td>
<td>35.0</td>
<td>0.2</td>
<td>0.12</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>FC</td>
<td>7-10</td>
<td>25-26</td>
<td>0.47</td>
<td>14.8</td>
<td>1.43</td>
<td>3.50</td>
</tr>
</tbody>
</table>

3.3.2 Experimental design

The IO and FC experiments were designed to examine the influence of viruses on ammonium production and subsequent effects on phytoplankton growth. In both experiments, triplicate treatments were set up with the objective of keeping bacterial abundances at near in situ levels, while reducing (Treatment -V) or maintaining (Treatment +V) at near in situ viral abundances.

In the FC experiment, water was filtered in series through 2.0 and 0.2 µm pore-size, 47 mm diameter polycarbonate filters (AMD Manufacturing, Canada), with the first filter removing larger phytoplankton and zooplankton, and the second concentrating bacteria (Wilhelm et al. 2002). A transfer pipette was used to keep the bacteria in suspension above the 0.2 µm filter. Viruses were removed using a Prep Scale-TFF Cartridge (Millipore, Billerica, MA) with a 30k Da molecular weight cut-off. The -V treatment was prepared by adding 50 ml of bacterial concentrate to 450 ml of virus-free water, and the +V treatment was prepared by adding 50 ml of bacterial concentrate to 450 ml of 0.2 µm filtered water. Incubations ran on a light/dark cycle of
14:10 h at in situ temperature, and subsamples were collected at time 0 h, 5.5 h, 10.25 h, 19 h, 24.5 h, 32.5 h, and 43 h.

The IO experiment was performed similarly; however, water was filtered through a 20 µm pore-size filter before bacteria were concentrated above a 0.2 µm pore-size Pellicon filter (Millipore, Billerica, MA) and viruses were removed using a 47 mm diameter 0.02 µm pore-size Anodisc filter (Whatman, United Kingdom). Incubations were run at in situ light and temperature conditions in flow-through on-deck incubators, and subsamples were collected at 0 h, 6 h, 10.5 h, 17.5 h, and 23.5 h. The effect of the concentration step on bacterial production in the IO experiment was monitored using tritiated thymidine (TdR) incorporation (Fuhrman & Azam 1980). TdR incorporation rate, normalised per cell, was 12 ± 5% higher in untreated water than after the concentration step, indicating a minor effect of the procedure (data not shown). Despite the differences in treatment set-up between the two experiments (filtration by 2.0 µm in FC versus by 20 µm in IO), the fact that the results from both treatments within each experiment are similar allows conclusions to be drawn separately for each experiment before making comparisons between experiments.

3.3.3 Cell and virus counts

Samples of 1 ml for bacteria and viruses and 5 ml for phytoplankton were fixed with gluteraldehyde at a final concentration of 0.5%. Samples were then flash frozen in liquid nitrogen and stored at -80°C. Bacteria and viruses were stained with SYBR Green I (Sigma-Aldrich, St. Louis, MO; Brussaard 2004b), and picophytoplankton were left unstained (Olson et al. 1993). All samples were counted using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).
3.3.4 Chlorophyll a and nutrient determination

Samples of 25 ml were filtered through acid washed syringes fitted with MilliQ-soaked 0.45 µm pore-size 25mm cellulose nitrate membrane filters (Whatman, United Kingdom) in a Swinnex filter holder (Millipore, Billerica, MA). The filters were used for chlorophyll $a$ determination, and the filtrate was used for ammonium determination. The first 5 ml were used to rinse the collection tubes, and 10 ml were collected into acid washed 15 ml polypropylene screw-cap tubes (Sarstedt, Germany) and frozen at -20°C. The filters were folded and placed in similar screw-cap tubes, kept in the dark and frozen at -20°C. Chlorophyll $a$ concentrations were determined from the frozen filters after preparation with 90% acetone and sonication (Parsons et al. 1984) using a 10AU fluorometer (Turner Designs, Sunnyvale, CA). Ammonium concentrations were determined from the filtered samples (Holmes et al. 1999) using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

3.3.5 Statistical methods

Differences of means were tested using two-tailed Student’s t-tests. Equality of variance was tested using an F-test.

3.4 Results

3.4.1 Viral abundances

Viral abundances in the initial whole water samples at IO and FC were $15.3 \pm 5.4 \times 10^6$ and $17.3 \pm 1.72 \times 10^6$ viruses ml$^{-1}$, respectively. As expected, viral abundances at the start of the IO
and FC experiments were significantly less in the -V than in the +V treatments (IO: decrease of 70%, p<0.001; FC: decrease of 39%, p<0.05). Increase in viral abundance over the FC experiment was significantly greater in +V than in -V (Figure 3.1B; p<0.001). Time-course data for the IO experiment indicates that viral production occurred in the +V treatment (Figure 3.1A), although there was a net decrease of viral abundance from the initial to final time point, possibly due to viral degradation from UV exposure (Noble & Fuhrman 1997).
3.4.2 Bacterial abundance and production

Bacterial abundances in the initial whole water samples at IO and FC were $8.61 \pm 0.45 \times 10^5$ and $19.1 \pm 1.58 \times 10^5$ cells ml$^{-1}$, respectively. Bacteria in IO increased to a maximum of $23.6 \pm 3.2 \times 10^5$ cells ml$^{-1}$ in -V, and only to a maximum of $17.3 \pm 3.3 \times 10^5$ cells ml$^{-1}$ in +V, indicating a treatment effect with removal of viruses (Figure 3.2A). In FC, bacterial abundance differed
between treatments initially, and bacteria in +V increased to a maximum of $33.0 \pm 3.0 \times 10^5$ cells ml$^{-1}$, before decreasing to a minimum of $0.41 \pm 0.03 \times 10^5$ cells ml$^{-1}$ (Figure 3.2B). Bacteria in -V increased steadily to a maximum of $36.3 \pm 4.6 \times 10^5$ cells ml$^{-1}$. The dramatic decrease in +V may be due to a near monoculture scenario, in which most of the bacteria were of a similar strain, and were therefore susceptible to a common virus in the treatment. The -V treatment contained a much smaller virus concentration, and so the bacteria were not subject to infection to the same degree.
Figure 3.2 Time series of bacterial abundance in seawater samples from the (A) Indian Ocean (IO) and (B) False Creek (FC) experiments to which viruses had been reduced (-V) or maintained at near in situ abundance (+V). Error bars represent the standard error of abundance estimates from triplicate incubations.
3.4.3 Phytoplankton growth

In the FC samples, small cyanobacteria that were not removed by 2 µm filtration (the majority were likely not removed; Morel et al. 1993) increased significantly (p<0.05) in +V compared to -V treatments consistent with the increase in chlorophyll a in +V (Figure 3.3). In the IO samples, chlorophyll a increased in the +V treatment and decreased in the -V treatment, with significant differences between treatments at the final time point (p<0.05).

![Figure 3.3 Increase in +V and –V from initial to final time point of chlorophyll a concentrations from the Indian Ocean (IO) and False Creek (FC) experiments, and abundances of picophytoplankton from the FC experiment, to which viruses had been reduced (-V) or maintained at near in situ abundance (+V). Error bars represent the standard error of abundance estimates from triplicate incubations. Chlorophyll a for FC has no replicates.](image-url)

3.4.4 Ammonium concentrations

Ammonium concentrations were initially indistinguishable in +V and -V for both experiments (Figure 3.4; IO: +V = 0.25 ± 0.02 µmol l⁻¹, -V = 0.22 ± 0.02 µmol l⁻¹; FC: +V = 3.38 ± 0.12 µmol l⁻¹, -V = 3.13 ± 0.18 µmol l⁻¹), but at the end of both experiments were significantly higher in +V than in -V (Figure 3.5; IO: p<0.005; FC: p<0.05).
Figure 3.4 Time series of ammonium concentrations in seawater samples from the (A) Indian Ocean (IO) and (B) False Creek (FC) experiments to which viruses had been reduced (-V) or maintained at near in situ abundance (+V). Error bars represent the standard error of abundance estimates from triplicate incubations.
3.5 Discussion

The most important finding of this study was that ammonium and chlorophyll \( a \) production decreased in temperate and tropical seawater samples in which viral abundances were reduced. These results imply that viruses are important but neglected agents of ammonium remineralisation in a range of marine environments.

Diluting the viral size fraction with virus-free seawater resulted in significantly less net ammonium production in both experiments. Ammonium production concurrent with a decrease in the abundance of bacteria supports the hypothesis that viral lysis of bacteria, followed by subsequent processing by uninfected cells, was the source of the observed ammonium increase. Viral lysates are rich in free and combined amino acids (Middelboe & Jørgensen 2006), and it is well known that bacterial mineralization of these compounds is associated with the production of
ammonium (Hollibaugh 1978, Haaber & Middelboe 2009, Boras et al. 2010). In turn, virus-induced ammonium remineralisation resulted in increased growth of phytoplankton, thus implying that viral infection contributes to N recycling in the ocean. The effect was observed under oligotrophic oceanic conditions as well as under coastal conditions, and is consistent with observations that the growth of *Synechococcus* in the ocean is associated with the lysis of heterotrophic bacteria (Weinbauer et al. 2011).

### 3.5.1 Nitrogen dynamics

The estimated N mass balance presented (Table 3.2) suggests that there is a tight coupling of organic N release from lysis and subsequent ammonium production. Of the estimated N released from lysed bacteria, 81-123% could be accounted for as free ammonium or as taken up by phytoplankton. The budget is based on assumptions of C and N cell quotas in bacteria and phytoplankton (see legend in Table 3.2), which may explain the excess N release compared to the calculated N in lysates in the IO experiment. However, the high estimated recovery indicates that these lysates are highly labile, and that a relatively small fraction of lysate remains as refractory DON. This is in agreement with previous studies in which an estimated 97% of lysate N (Haaber & Middelboe 2009) and 60-100% of lysate C (Middelboe et al. 2003) were metabolised by heterotrophic bacteria following viral lysis of a specific host population.
3.5.2 Increases in chlorophyll and picophytoplankton

Increases in chlorophyll $a$ in the presence of the ambient viral community is consistent with ammonium increases in the +V treatments supporting increased picophytoplankton growth (Figure 3.3 and Figure 3.4). Interestingly, the dilution of the viral size fraction in -V resulted in less picophytoplankton biomass and chlorophyll $a$ concentration in both experiments, indicating that picophytoplankton production varied along with changes in viral lysis of bacteria.

The increase in picophytoplankton biomass and chlorophyll $a$ concentration in FC was unexpected, as nutrient levels were high (Table 3.1), and an influx of ammonium from bacterial remineralisation appeared unlikely to stimulate primary production. FC was expected to be well-mixed and primary production to be therefore light limited. However, picophytoplankton biomass did not increase in the -V treatment. Therefore, there was a co-limiting factor that was present when viruses were present, and absent when they were removed. This might be ammonium (as ammonium is often a more bioavailable N source than nitrate), but since

Table 3.2 Calculated increase from initial to final time point between +V and -V of ammonium production, phytoplankton uptake, estimated DON consumed (ammonium + phytoplankton uptake), and N released from lysis (N from lysates).

<table>
<thead>
<tr>
<th>Location</th>
<th>Ammonium ($\mu$mol l$^{-1}$)</th>
<th>Phytoplankton uptake‡ ($\mu$mol l$^{-1}$)</th>
<th>Estimated DON consumed ($\mu$mol l$^{-1}$)</th>
<th>N from lysates* ($\mu$mol l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IO</td>
<td>0.300 ± 0.022</td>
<td>0.018 ± 0.004</td>
<td>0.318 ± 0.022</td>
<td>0.261 ± 0.13</td>
</tr>
<tr>
<td>FC</td>
<td>1.44 ± 0.76</td>
<td>0.136 ± 0.031</td>
<td>1.74 ± 0.76</td>
<td>2.07 ± 0.17</td>
</tr>
</tbody>
</table>

‡ Calculated assuming a C:chlorophyll ratio of 50 and an phytoplankton C:N ratio of 6.6 (Redfield ratio) for the IO experiment, and by multiplying phytoplankton abundance by an average nitrogen cell content of 2.50 fmol cell$^{-1}$ for *Synechococcus* (Bertilsson et al. 2003) for the FC experiments, as it was assumed the majority of phytoplankton cells <2 $\mu$m in False Creek would be *Synechococcus*.

* An average bacterial nitrogen quota of 0.37 fmol cell$^{-1}$ was assumed (assuming 20 fg C cell$^{-1}$ (Carlsson et al. 1999) and a C:N of 4.5 (Goldman et al. 1987)), and multiplied by the difference in bacterial abundance between -V and +V from initial to final time point.
ammonium was high initially (Table 3.1), it is unlikely to be the co-limiting nutrient. However, ammonium was decreased in the -V treatment, indicating uptake, perhaps by some portion of the picophytoplankton that were previously light-limited in the field. Therefore, there may be another factor released by cell lysis when viruses are present (such as another nutrient or vitamin). So, in eutrophic scenarios, ammonium remineralisation might not be the key factor causing increased primary production, but other released nutrients may also increase picophytoplankton abundance.

3.5.3 Impact of grazers

Contribution to the dissolved organic matter pool by grazers cannot be excluded from either experiment, although in the FC experiment grazers were not detected (<10^4 cells ml⁻¹). Infection of grazers by viruses might have occurred (e.g. Saura et al. 2011), but would not change the results, because infection would simply provide another source of virus-induced DOM to the treatment, intensifying the results. Grazing of viruses may also have occurred; however, removal rates are generally minimal relative to the large standing stock of viruses (Gonzalez & Suttle 1993, Bettarel et al. 2005).

3.5.4 Ecological implications

Significant rates of viral mediated ammonium remineralisation in both oligotrophic oceanic and productive coastal environments emphasises the importance of viruses in remineralising N and supporting phytoplankton production. Unlike C, for which viral lysis of bacteria has been considered a futile loop (Azam & Worden 2004), N remineralisation by viruses appears to be an important process in both a low productivity and a high productivity marine station. Our results show that the removal of viruses reduces ammonium production and decreases phytoplankton
growth. These results suggest a mechanism for the process described in another study (Weinbauer et al. 2011) in which the removal of viruses decreases growth rates of the cyanobacterium *Synechococcus*.

The dependence of phytoplankton growth on N remineralisation mediated by viral lysis is a feedback mechanism which is likely significant on a global scale. Instead of acting as a C sink, viral activity stimulates N recycling that in turn supports primary production and system productivity.
Chapter 4: Viral infection of a marine bacterium provides nitrogen for phytoplankton

4.1 Synopsis

Lytic infection of bacteria by viruses releases nutrients during cell lysis and stimulates the growth of primary producers, but to date no studies have directly traced the path of those nutrients from lysates to primary producers. The purpose of this study was to examine the ability of heterotrophic bacteria to remineralise nitrogen (N) from bacterial lysates to support primary production. In laboratory experiments, *Vibrio natriegens* strain PWH3a was infected with a lytic virus and added to cultured cyanobacteria *Synechococcus* WH7803 as well as a natural bacterial assemblage, and the abundance of *Synechococcus* WH7803 was monitored. In field experiments, *V. natriegens* (PWH3a) was labeled with $^{15}$N, infected with a lytic virus, filtered to remove any remaining whole bacteria, and added to seawater. The particulate fraction (>0.7 µm) was collected at the end of the incubation for stable isotope analysis. Of the 36.0 µmol l$^{-1}$ DON added to the laboratory cultures, 26.5 µmol l$^{-1}$ N was remineralised and taken up by *Synechococcus* WH7803, resulting in 74% of lysate N transferred from the lysed *V. natriegens* (PWH3a) to *Synechococcus* WH7803. In the field experiments, 0.50 to 4.16 µmol l$^{-1}$ N originating from *V. natriegens* (PWH3a) lysate was taken up by the >1 µm particulate fraction. The laboratory experiment demonstrated a significant increase in *Synechococcus* WH7803 abundance due to remineralisation of bacterial lysate, and the field experiment showed evidence of significant lysate uptake by primary producers. We can conclude that bacterial lysates released after viral infection are available for phytoplankton growth through bacterial remineralisation of N.
4.2 Introduction

Viruses are significant mortality agents of bacteria and phytoplankton in the ocean, consequently affecting pathways and rates of nutrient cycling (Fuhrman 1999, Wilhelm & Suttle 1999, Suttle 2005, 2007) as particulate and dissolved organic matter is released into the water as a result of cell lysis (Gobler et al. 1997, Middelboe & Jørgensen 2006). Major constituents of the released material are free and combined amino acids (Middelboe & Jørgensen 2006), which are taken up and metabolised by bacteria (Middelboe et al. 1996, 2003). Depending on the composition of the released material, and the relative availability of carbon (C) and N, ammonium may be released (Hollibaugh 1978, Goldman et al. 1987), which in turn can support phytoplankton growth (Haaber & Middelboe 2009, Weinbauer et al. 2011, Shelford et al. 2012) and support primary production.

The large bacterial biomass loss due to viral lysis implies a continuous and substantial flux of lysates into the dissolved organic matter pool. Weinbauer et al. (2011) provided evidence of the importance of this flux by showing that reducing viral abundance decreased the growth of *Synechococcus*, the dominant primary producer during these experiments in the Gulf of Mexico and Mediterranean Sea. It was postulated that *Synechococcus* growth may have been directly stimulated by uptake of dissolved organic nutrients released by lysis, or indirectly through the incorporation of these organics by uninfected bacteria and subsequent remineralisation of inorganic nutrients. Evidence that mineralization of dissolved organic matter and release of ammonium by uninfected bacteria stimulates phytoplankton growth was shown by Shelford et al. (2012).
The present contribution demonstrates, in the laboratory and field, that uninfected bacteria metabolise dissolved organic N (DON) from infected bacteria and release ammonium that supports the growth of phytoplankton.

4.3 Methods

4.3.1 Laboratory cultures

A semi-continuous culture of the *Synechococcus* strain WH7803 (DC2) was grown on artificial seawater (Berges et al. 2001), with 5 mol l\(^{-1}\) bicine (Healey & Hendzel 1979), 124 µmol l\(^{-1}\) NH\(_4\)Cl instead of nitrate, and 13 µmol l\(^{-1}\) K\(_2\)HPO\(_4\). Cultures were maintained at 19°C and continuous light levels (42 µmol quanta m\(^{-2}\) s\(^{-1}\)). Experiments were started when cultures were at the end of their exponential phase and nearing N-limitation, determined by epifluorescence microscopy counts.

PWH3a, a strain of the gram negative marine bacterium *Vibrio natriegens* (Suttle & Chen 1992, Weinbauer et al. 1997) was grown on artificial seawater with 5mmol l\(^{-1}\) bicine, 500 µmol l\(^{-1}\) NH\(_4\)Cl, 100 µmol l\(^{-1}\) K\(_2\)HPO\(_4\), and 1000 µmol l\(^{-1}\) glucose as a carbon source for a C:N:P ratio of 60:5:1. PWH3a cultures were grown at 25°C and continuously mixed at 100 rpm.

Bacteriophage PWH3a-P1 was added to the *V. natriegens* (PWH3a) culture at the end of exponential growth, determined from spectrophotometric absorbance (Ultrospec spectrophotometer, Biochrom, United Kingdom). The culture was incubated with the virus until absorbance measurements decreased to 20% of initial absorbance, after approximately 7 h. The culture was then filtered through a 0.22 µm Durapore filter (Millipore, Billerica, MA) and the
lysate (Lys) kept in a 4°C refrigerator overnight until treatment set-up (approximately 20 h). The number of *V. natriegens* (PWH3a) cells lysed by the start of the experiment was calculated by the decrease of cells from pre-infection with viruses to the time of filtration. Cell abundance was measured with flow cytometry (see section 4.3.4.1). This cell abundance decrease was multiplied by the measured cellular N quota for *V. natriegens* (PWH3a), 2.54 fmol cell⁻¹ (this measurement is outlined in section 4.3.4.4). The result is the amount of total N released by lysed *V. natriegens* (PWH3a).

Lysates for field experiments were prepared as above, except that *V. natriegens* (PWH3a) was grown on ¹⁵NH₄Cl instead of ¹⁴NH₄Cl (90+ atom % ¹⁵N, Isotec, Miamisburg, OH), and the filtered lysate was kept at 4°C for 2 - 5 d until treatment set-up.

### 4.3.2 Bacterial assemblage preparation

Water was collected from Queen Charlotte Sound (51° 02.37N, 127° 52.38W) on 2 Oct 2011. Temperature and salinity were 11°C and 32, respectively. Nitrate and phosphate concentrations were 21.3 and 1.9 µmol l⁻¹, respectively. Ultrafiltrate was made using a 30k Da molecular weight cut-off tangential flow filtration system (Prep/Scale, Millipore, Billerica, MA) after pre-filtration through a 0.45 µm membrane filter (Millipore, Billerica, MA). Ultrafiltrate was kept at 4°C until experimental set-up. Although ultrafiltrate is ostensibly free of all particles with a molecular weight greater than 30k Da, enough bacterial cells passed through the processing to multiply in the ultra-filtered seawater over time. Bacterial abundance in the sampled water at the time of the experiment was 4.18 x 10⁵ cells ml⁻¹, determined by flow cytometry (described in section 4.3.4.1).
4.3.3 Experiments

4.3.3.1 Laboratory experiments

Six treatments (Figure 4.1) were composed of three different components: *Synechococcus* strain WH7803 (DC2), lysate from *V. natriegens* strain PWH3a (Lys), and the marine bacterial assemblage from the Queen Charlotte Sound (Bac). Treatment DC2+Bac was a control for DC2 growth in the presence of Bac without a DON source from Lys. Treatment DC2+Lys was a control for remineralisation from bacteria in the non-axenic DC2 component. Treatment Bac+Lys was to measure the amount of ammonium remineralisation by Bac with the addition of Lys. The remaining three treatments were Bac only, DC2 only, and the experimental treatment DC2+Bac+Lys. All treatments were in triplicate, and volumes were kept equal between treatments by adding nitrate- and phosphate-free artificial seawater to control treatments.
Figure 4.1 Diagram of treatments in the laboratory experiment examining increase in *Synechococcus* WH7803 (DC2) abundance, in the presence of bacterial lysate from *V. natriegens* PWH3a (Lys) and an environmental bacterial assemblage (Bac). Lys, while a component of some of the treatments, was not a treatment by itself (indicated by parentheses). The * is an indication that DC2 is not axenic, and therefore contains some heterotrophic bacteria.

Treatments were incubated in the laboratory at 19°C and continuous light levels (42 µmol quanta m⁻² s⁻¹). Samples for cell abundance and ammonium concentration were collected daily for 5 d as described in section 4.3.4.2.

4.3.3.2 Field experiments

Water was collected from the surface waters at five stations in southern coastal British Columbia: Saanich Inlet (SI), Fraser River Plume (FRP), Gorge Harbour (GH), Semiahoo Bay
(SB), and Jericho Pier (JP; Table 4.1). SI, FRP, and GH were collected from shipboard Niskin bottles mounted on a rosette, and temperature and salinity were measured by a CTD mounted on the rosette (SBE 25 CTD, Sea-Bird, Bellevue, WA). SB and JP were collected using an acid-rinsed (rinsed once with 10% HCl and then three times with ultrapure water) 20 l carboy from the shore and pier, respectively, and temperature and salinity were measured by a hand-held thermometer and refractometer. Samples for phytoplankton identification were collected from the whole water and preserved with Lugol’s iodine solution. The water was filtered through a 118 µm nitex mesh to remove large particulates. The experimental treatment (SW+Lys) which consisted of this filtered seawater with the addition of filtered lysate from V. natriegens (PWH3a) prepared as described above (see section 4.3.1) was compared to a control treatment (SW) containing only filtered seawater. For SB and JP, there was a third treatment (SW+N) that included filtered seawater and $^{15}$NH$_4$Cl. This treatment was added to confirm that N was able to stimulate production in each system (i.e. that another factor was not limiting growth). $^{15}$NH$_4$Cl was added at 0.9 µmol l$^{-1}$ to mimic N levels of the estimated lysate addition in Treatment SW+Lys. All treatments were in triplicate. For stations SI, FRP, and GH, treatments were kept in an on-deck flow-through incubator with neutral density screening to reduce ambient light to approximate collected-depth conditions. For stations SB and JP, treatments were kept at 19°C and continuous light levels in a laboratory incubator (42 µmol quanta m$^{-2}$ s$^{-1}$). Samples for cell abundance and ammonium concentration were collected every 6 to 9 h for 1 to 2 days, samples for chlorophyll $a$ were collected at the initial and final time points, and samples for PO$^{15}$N were collected at the final time point, as described in section 4.3.4.3.
Table 4.1 Locations and environmental parameters for field sampling stations. Environmental and biological conditions measured include temperature (Temp), salinity (Sal), nitrate (NO$_3^-$), phosphate (PO$_4^{3-}$), ammonium (NH$_4^+$), and the dominant phytoplankton.

<table>
<thead>
<tr>
<th>Station</th>
<th>Location</th>
<th>Date</th>
<th>Temp (°C)</th>
<th>Sal (ppt)</th>
<th>NO$_3^-$ (µmol l$^{-1}$)</th>
<th>PO$_4^{3-}$ (µmol l$^{-1}$)</th>
<th>NH$_4^+$ (µmol l$^{-1}$)</th>
<th>Dominant phytoplankton</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI: Saanich Inlet</td>
<td>48.592, -123.505</td>
<td>13 Sep 2012</td>
<td>14</td>
<td>27.9</td>
<td>1.24</td>
<td>0.75</td>
<td>0.54</td>
<td>Mixed assemblage</td>
</tr>
<tr>
<td>FRP: Fraser River Plume</td>
<td>49.072, -123.402</td>
<td>13 Sep 2012</td>
<td>11</td>
<td>28.0</td>
<td>10.9</td>
<td>1.12</td>
<td>0.07</td>
<td><em>Phaeocystis, Skeletonema, Leptocylindrus</em></td>
</tr>
<tr>
<td>GH: Gorge Harbour</td>
<td>50.092, -125.012</td>
<td>16 Sep 2012</td>
<td>10.7</td>
<td>28.2</td>
<td>16.9</td>
<td>1.80</td>
<td>0.59</td>
<td><em>Rhizosolenia</em></td>
</tr>
<tr>
<td>SB: Semiahoo Bay</td>
<td>49.013, -123.037</td>
<td>26 Aug 2013</td>
<td>20.2</td>
<td>29</td>
<td>0.00</td>
<td>2.40</td>
<td>0.08</td>
<td>Cyanobacteria (Also an abundant unidentified ciliate)</td>
</tr>
<tr>
<td>JP: Jericho Pier</td>
<td>49.277, -123.202</td>
<td>27 Aug 2013</td>
<td>19.5</td>
<td>21</td>
<td>0.00</td>
<td>0.20</td>
<td>0.11</td>
<td>Diatoms (mixed assemblage)</td>
</tr>
</tbody>
</table>

4.3.4 Sample analysis

4.3.4.1 Cell and virus counts

Samples of 1 ml for bacteria and viruses were fixed with gluteraldehyde at a final concentration of 0.5%. Samples of 1 ml for phytoplankton were fixed with formaldehyde at a final concentration of 2%. Samples of 1 ml were fixed with gluteraldehyde at a final concentration of 0.5% for *V. natriegens* (PWH3a) counts. Samples were then flash frozen in liquid nitrogen and stored at -80°C. Bacteria and viruses were stained with SYBR Green I (Sigma-Aldrich, St. Louis, MO; Brussaard 2004b), and phytoplankton were left unstained (Olson et al. 1993). All cells were counted using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Initial phytoplankton samples for the field experiments were collected, preserved with
Lugol’s solution, and dominant taxa were identified using a settling chamber (Edler & Elbrächter 2010) and a light microscope (Axiovert 10, Zeiss, Canada).

4.3.4.2 Nutrient and chlorophyll \( a \) collection and analysis

Samples of 25 ml were filtered through acid-rinsed syringes fitted with MilliQ-soaked 0.45 \( \mu \)m 25 mm cellulose nitrate membrane filters (Whatman, United Kingdom) in a Swinnex filter holder (Millipore, Billerica, MA). The filters were used for chlorophyll \( a \) determination, and the filtrate was used for ammonium determination. The first 15 ml were used to rinse the collection tubes, and 10 ml were collected into acid-rinsed 15 ml polypropylene screw-cap tubes (Sarstedt, Germany) and frozen at -20°C. For the field experiments, the filters were folded, placed in aluminum foil packages, and frozen desiccated at -20°C for chlorophyll \( a \) determination.

Chlorophyll \( a \) concentrations were determined from the frozen filters after preparation with 90% acetone and sonication (Parsons et al. 1984) using a 10AU fluorometer (Turner Designs, Sunnyvale, CA). Ammonium concentrations were determined from the filtered samples (Holmes et al. 1999) using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

Nitrate+nitrite and phosphate concentrations of the ultrafiltrate for the laboratory experiment and 0.45 \( \mu \)m filtered seawater for the field experiments were analyzed on a Bran & Luebbe AutoAnalyzer 3 using air-segmented continuous-flow analysis.

4.3.4.3 \( PO^{15}N \) collection and determination

For the field experiments, samples for \( PO^{15}N \) were filtered through a 1 \( \mu \)m filter (AMD Manufacturing, Canada), adding ultrafiltrate to keep cells larger than 1 \( \mu \)m re-suspended in order to ‘wash out’ bacteria that may have taken up \( PO^{15}N \). Samples for cell counts were taken before
and after this washing process to determine the proportion of >1 µm phytoplankton cells lost. The ‘washed’ phytoplankton above the 1 µm filters were re-filtered through combusted (450°C for 4 h) glass-fibre filters (GF/F, Whatman, United Kingdom) and dried in a 50°C oven for 2 d. Samples were also filtered onto combusted glass-fibre filters without the washing step to compare the amount of ^15N uptake that was due to the >1 µm fraction (mainly phytoplankton) versus the whole fraction (including bacteria). For SB and JP, treatment SW+N (with ^15NH₄Cl addition) was filtered directly through the combusted glass-fibre filters and dried, without the washing step. δ^15N-PON and total particulate N on the filters was determined at the Stable Isotope Laboratory at Boston University on a GV Instruments IsoPrime isotope ratio mass spectrometer and a Eurovector elemental analyzer, calibrated against atmospheric N₂ and IAEA standards N-1, N-2, and N-3 (replicate analysis within ± 0.2‰). Due to instrument malfunction, only singletons of the stable isotope data for SI and FRP and duplicates for GH and JP were measured. Triplicates were measured for SB.

4.3.4.4 Particulate carbon and nitrogen analysis

N cell quotas of *Synechococcus* WH7803 and *V. natriegens* (PWH3a) were determined from cultures grown using the same media and conditions as described in section 4.3.1.

Once cultures had reached the desired density (pre-stationary for *Synechococcus* WH7803 as determined by epifluorescence microscopy counts, and mid-exponential for *V. natriegens* (PWH3a) as determined by spectrophotometric absorbance), they were filtered onto combusted glass-fibre filters, dried in a 50°C drying oven for two days, and placed in a desiccator until packaged into aluminum foil and sent for analysis on a CHN Elemental Analyser (Carlo Erba NA-1500).
Calculations and statistical analysis

Total particulate $^{15}$N on the filters was calculated from the total particulate N on the filters measured by elemental analyser (section 4.3.4.3) and the ratio of $^{15}$N:$^{14}$N measured by mass spectrometer (section 4.3.4.3), taking into account the natural ratio of $^{15}$N:$^{14}$N in seawater (0.36765%) and the ratio of the glycine standard (0.3716%). Total uptake rates of $^{15}$N in the field experiments were calculated by dividing the total particulate $^{15}$N on the filters by the volume filtered, as follows:

$$\text{Total } ^{15}\text{N uptake} = \frac{[N] \times \left( \left( \frac{\delta^{15}N}{1000} \right) + 1 \right) \times 0.0036765}{\text{Volume filtered}} - 0.003716$$

Normalised uptake rates were calculated by dividing the total uptake rate by the initial chlorophyll $a$ concentration for each incubation. The percent contribution of cells $>1$ µm to total $^{15}$N uptake was calculated by dividing the total particulate $^{15}$N on the filters of the ‘washed’ samples (adjusted for decrease in phytoplankton abundance) by the $^{15}$N on the unwashed samples.

The differences between initial and final time points for DC2 abundance and ammonium concentrations were both normally distributed and with equal variances according to Shapiro-Wilk tests and Levene’s tests, respectively. The significance of these data was analysed using one-way analyses of variance (ANOVA). The differences between initial and final time points for bacterial abundance in the laboratory experiment, while normally distributed, had unequal variances, and therefore a Kruskal-Wallis test was performed to test for significant differences.
The differences between initial and final time points for ammonium and chlorophyll $a$ concentrations and bacteria and picophytoplankton abundances were all normally distributed (except for the picophytoplankton data for treatment SW in SI, which had only two samples), and with equal variances (except the bacteria data for SI and JP). Significant differences between treatments were determined using two-tailed Student’s t tests. Data which did not meet the assumptions for the Student’s t test were analysed for significant differences using the non-parametric Mann-Whitney U test.

Because the final time point of the bacterial data for the SW+Lys treatment in SI did not adequately reflect the trends seen over the duration of the experiment, significance between the two treatments was calculated for the differences between the initial and fourth time points. The data were normally distributed and had equal variances (Shapiro-Wilk and Levene’s tests), and so a Student’s t test was run.

4.4 Results

4.4.1 Lysate contribution to Synechococcus proliferation in laboratory cultures

There was a greater increase in N-limited *Synechococcus* WH7803 (DC2) abundance when exposed to both lysate and a remineralising bacterial assemblage, than when grown with each component separately. DC2 abundance increased the most in the experimental treatment, DC2+Bac+Lys (Figure 4.2A). Final abundances were significantly different between all treatments ($p<0.005$). Ammonium concentrations in every treatment except Bac+Lys decreased to less than $0.2 \, \mu\text{mol} \, l^{-1}$ (Figure 4.2B). In Bac+Lys, free ammonium increased to $8.24 \pm 0.04$
µmol l$^{-1}$, significantly higher than the other treatments (p<0.001). Bacterial abundance increased in all treatments, but increased the most in treatments with Lys addition (Figure 4.2C).

Figure 4.2 Time series of (A) DC2 abundance, (B) ammonium concentration, and (C) bacterial abundance in the laboratory experiment. Error bars are standard error from triplicate incubations.
The proportion of the N in *V. natriegens* (PWH3a) that was taken up by *Synechococcus* WH7803 can be calculated. Lysate was prepared from viral infection of the cultured bacterium *V. natriegens* (PWH3a). The N quota as determined by elemental analyser (section 4.3.4.4) was 2.54 fmol cell\(^{-1}\) for *V. natriegens* (PWH3a), and the decrease in *V. natriegens* (PWH3a) from viral infection multiplied by the N cell quota corresponds to the amount of N released from lysis (Table 4.2). Lysate addition added approximately 36.0 \(\mu\)mol l\(^{-1}\) N to each incubation (Table 4.2). N cell content as determined by elemental analyser (section 4.3.4.4) was 7.83 fmol cell\(^{-1}\) for *Synechococcus* WH7803. The percent of N from lysate that was taken up by DC2 via remineralisation (by both the added bacterial assemblage and the contaminating bacteria) was 74%, calculated by the abundance decrease of *V. natriegens* (PWH3a), the subsequent abundance increase of *Synechococcus* WH7803, and their respective cellular N contents.

Table 4.2 PWH3a dynamics during the creation of lysates by viral infection (Laboratory = experiments with cultured *Synechococcus* WH7803, SI = Saanich Inlet, FRP = Fraser River Plume, GH = Gorge Harbour, SB = Semiahoo Bay, JP = Jericho Pier).

<table>
<thead>
<tr>
<th></th>
<th>Laboratory</th>
<th>SI and FRP</th>
<th>GH</th>
<th>SB and JP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. natriegens</em> (PWH3a) abundance pre-virus (cell l(^{-1}))</td>
<td>3.96 x 10(^{11})</td>
<td>7.87 x 10(^{11})</td>
<td>1.01 x 10(^{12})</td>
<td>7.89 x 10(^{10})</td>
</tr>
<tr>
<td><em>V. natriegens</em> (PWH3a) abundance post-virus incubation (cell l(^{-1}))</td>
<td>1.12 x 10(^{11})</td>
<td>9.43 x 10(^{10})</td>
<td>1.41 x 10(^{11})</td>
<td>6.38 x 10(^{10})</td>
</tr>
<tr>
<td>Multiplicity of infection (MOI)</td>
<td>1</td>
<td>0.41</td>
<td>0.24</td>
<td>5</td>
</tr>
<tr>
<td>Total N release ((\mu)mol l(^{-1}))</td>
<td>721</td>
<td>1760</td>
<td>2194</td>
<td>38.4</td>
</tr>
<tr>
<td>N addition to experimental incubations ((\mu)mol l(^{-1}))</td>
<td>36.0</td>
<td>67.7</td>
<td>84.4</td>
<td>0.44</td>
</tr>
</tbody>
</table>
4.4.2 *Uptake of N from bacterial lysate by primary producers in field studies*

Addition of lysate stimulated the microbial system in five different coastal marine stations. The field experiments were performed with 100% $^{15}$N-labelled lysate of *V. natriegens* (PWH3a) added to seawater from Saanich Inlet (SI), Fraser River Plume (FRP), Gorge Harbour (GH), Semiahoo Bay (SB), and Jericho Pier (JP; Table 4.1). Bacterial abundance in SW+Lys increased significantly over SW by the final time point for FRP (p<0.005), SB (p<0.0005), and JP (p<0.05; Figure 4.3). Bacterial abundances in the treatment with lysate added (SW+Lys) in SI increased almost tenfold before a large decrease to below initial values by the final time point, concurrent with an increase in viral abundance (data not shown). The SW+Lys treatment in GH had threefold-higher bacterial abundance than the SW treatment, possibly due to unintended carryover of PWH3a bacteria during the filtration of the lysate. In GH, SW+Lys had a significantly greater increase in picophytoplankton abundance (p<0.01) than the control, but chlorophyll *a* concentration changes were similar for both treatments (Figure 4.4). There were no significant differences between experimental and control treatments for picophytoplankton abundances or for chlorophyll *a* concentrations in the other stations. Ammonium concentrations in SI, SB, and JP decreased to less than 0.2 µmol l$^{-1}$. In FRP, the SW+Lys treatment decreased to less than 0.04 µmol l$^{-1}$ before climbing again to 0.25 µmol l$^{-1}$, correlated with a spike in bacterial abundance. Ammonium concentrations in GH SW+Lys were higher than in the other experiments, likely due to incomplete uptake of ammonium in the bacterial cultures before lysate creation. Ammonium concentrations in GH SW+Lys steadily decreased over the course of the experiment. There was significantly greater drawdown of ammonium in the SW+Lys treatment in every station except SB (SI p<0.001; FRP p<0.05; GH p<0.001; JP p<0.05). Increase of
bacteria and uptake of ammonium at most stations, and increase in cyanobacterial abundance in GH, indicate that these systems were stimulated by the addition of bacterial lysate.
Figure 4.3 Time-course data of ammonium and bacterial abundance for the five field stations. Solid lines indicate the treatment with lysate addition (SW+Lys), and dotted lines indicate the treatment without lysate (SW). Error bars are standard error of triplicate incubations. Asterisks indicate when the treatments are significantly different for where ammonium decrease is significantly different between treatments or where final bacterial abundances are different between treatments. Note: each plot has a different y-axis range.
Figure 4.4 Time-course data of chlorophyll $a$ concentration and picophytoplankton abundance for the five field stations. Solid lines indicate the treatment with lysate addition (SW+Lys), and dotted lines indicate the treatment without lysate (SW). Error bars are standard error of triplicate incubations. Asterisks indicate where the treatments are significantly different at the final time point. Note: each plot has a different y-axis range.
There was uptake of lysate-derived N by phytoplankton and cells >1 µm. Lysate was added to the SW+Lys treatment for each station, adding approximately 67.7 µmol l\(^{-1}\) N for SI and FRP, 84.4 µmol l\(^{-1}\) N for GH and 0.44 µmol l\(^{-1}\) N for SB and JP (Table 4.2). In order to add a more reasonable concentration of N to the systems, the concentration of lysate N added to SB and JP was lower than in the other three stations (which were run prior to SB and JP). Stable isotope data collected from particulate organic material collected on 0.7 µm glass-fibre filters indicate uptake of lysate by the particulate fraction (Table 4.3; Figure 4.5). From the \(^{15}\)N data, the calculated contribution to the total uptake of cells >1 µm was 46.3% (SI), 47.6% (FRP), and 100% (GH, SB, and JP). The lack of \(^{15}\)N signal in the bacterial size fraction in GH (Figure 4.5) corresponds to the lack of increase in bacterial abundance, and the significant increase in picophytoplankton abundance (Figure 4.3 and Figure 4.4). Similarly, the large (>50%) contribution of the bacterial fraction to \(^{15}\)N uptake in SI and FRP (Figure 4.5) corresponds to their bacterial increase and lack of picophytoplankton increase (Figure 4.3 and Figure 4.4).

Table 4.3 Uptake by the particulate fraction of \(^{15}\)N from bacterial lysate from five field stations, and the same uptake normalised by initial chlorophyll \(a\) concentrations. Error measurements are standard error of triplicate incubations for SB, and range of duplicate incubations for GH and JP. SI and FRP are singleton measurements.

<table>
<thead>
<tr>
<th>Station</th>
<th>Total uptake of (^{15})N (µmol l(^{-1}))</th>
<th>Uptake of (^{15})N normalised by chlorophyll (a) (µmol µg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saanich Inlet (SI)</td>
<td>1.67</td>
<td>0.27</td>
</tr>
<tr>
<td>Fraser River Plume (FRP)</td>
<td>1.76</td>
<td>0.70</td>
</tr>
<tr>
<td>Gorge Harbour (GH)</td>
<td>4.16 ± 0.36</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>Semiahoo Bay (SB)</td>
<td>0.60 ± 0.02</td>
<td>0.70 ± 0.16</td>
</tr>
<tr>
<td>Jericho Pier (JP)</td>
<td>0.50 ± 0.1</td>
<td>0.09 ± 0.00</td>
</tr>
</tbody>
</table>
Figure 4.5 Uptake of $^{15}$N normalised by initial C concentrations in primary producers during the experiment by the $>1 \mu m$ and $<1 \mu m$ fractions in treatments with lysate addition for Saanich Inlet (SI), Fraser River Plume (FRP), Gorge Harbour (GH), Semiahoo Bay (SB), and Jericho Pier (JP). Error bars are standard error of triplicate incubations for SB, and range of duplicate incubations for GH and JP. SI and FRP are singleton measurements. Initial C concentrations in primary producers was calculated by adding together the contribution by larger primary producers (calculated by converting initial chlorophyll $a$ concentrations to C using a C:chlorophyll $a$ ratio of 50:1) and the contribution by picophytoplankton (calculated by multiplying the initial picophytoplankton concentrations by a cellular C quota for Synechococcus of 51.9 fmol cell$^{-1}$ as measured in this experiment).

The treatment with addition of $^{15}$N-ammonium (SW+N; data not shown) indicated that the organisms at SB and JP were able to take up ammonium. Ammonium concentration in the SW+N treatment in both stations decreased, indicating ammonium uptake. There was also confirmed uptake of $^{15}$NH$_4$ in this treatment, with $0.55 \pm 0.18 \mu$mol l$^{-1}$ N (SB) and $0.40 \pm 0.23 \mu$mol l$^{-1}$ N (JP; data not shown) taken up into the particulate fraction. Any ammonium produced by remineralisation of lysate in the SW+Lys treatment, therefore, could be used by the microbes in these two stations.
4.5 Discussion

Experiments in the laboratory and with natural systems show that the N in viral lysates can be remineralised by bacterial communities to support primary production. Many studies have shown that lysate is a source of bioavailable dissolved organic matter (e.g. Middelboe et al. 2003, Poorvin et al. 2004), and that phytoplankton lysate can be remineralised (Gobler et al. 1997). Other studies have provided evidence that ammonium from remineralisation may stimulate primary production (Haaber & Middelboe 2009, Weinbauer et al. 2011, Sheldford et al. 2012), yet data are lacking on the specific mechanism involved in the transfer of N to primary producers from bacterial lysates produced by viral infection. This study determines that both cultured and environmental phytoplankton can take up N originating in bacterial lysates, and that ammonium produced through bacterial remineralisation of DON is one mechanism enabling that uptake.

4.5.1 Remineralisation of nitrogen in bacterial lysates

The increase in bacterial abundance in almost all experiments, along with the production of ammonium in the laboratory experiment, establishes that bacterial lysates produced through viral infection are available for bacterial growth and potential ammonium remineralisation. In the laboratory experiment, every treatment with lysate had greater bacterial increase than those without lysate, indicating the ability of bacteria to use the added lysate. For all field stations except GH, bacterial abundance in the treatment with lysate increased significantly over the control treatment (SI and FRP: p<0.01; SB: p<0.005; JP: p<0.05; Figure 4.3), demonstrating uptake of lysis products by the bacterial communities. Production of free ammonium increased eightfold in the laboratory treatment with lysate added to bacteria (Bac+Lys; Figure 4.2B) from remineralisation of N in the lysate by the bacterial community. In the other treatments, either
lysate was not added as a source of DON (Treatment Bac), or *Synechococcus* WH7803 was present and removing the ammonium being produced (the remainder of the treatments). There was no ammonium production in the field studies except in the Fraser River Plume (FRP; Figure 4.3). The increase in ammonium at the final time point in FRP may be due to the sharp increase in bacteria at the final time point, likely increasing ammonium remineralisation. The viral lysates can be used by the bacterial community for growth, and excess N remineralised to produce ammonium.

4.5.2 *Phytoplankton uptake of remineralised nitrogen*

The results from this study provide strong evidence that remineralised N from bacterial lysates can support the growth of primary producers. Evidence for the production of ammonium in the presence of viral lysis has been presented before (Haaber & Middelboe 2009, Shelford et al. 2012), but this study clearly demonstrates that bacteria can degrade the organic N in lysates and subsequently produce ammonium, which is then taken up by phytoplankton. The use of a cultivated bacterium for lysate production limits generalisation of quantitative data to environmental systems; however, there is evidence that N from lysates may provide a large portion of N for phytoplankton uptake, as seen from the uptake of 74% of the N in lysates by *Synechococcus* WH7803 in the current study.

DON from bacterial lysates was remineralised by the bacterial assemblage into ammonium and used to support primary production. In the laboratory experiment, the increase in free ammonium in treatment Bac+Lys did not occur in the experimental treatment with *Synechococcus* WH7803 (DC2+Bac+Lys; Figure 4.2B), indicating that there was uptake of remineralised ammonium by *Synechococcus* WH7803. This conclusion is in agreement with the concurrent increase in
*Synechococcus* WH7803 abundance in that treatment (Figure 4.2A). The uptake of $^{15}$N in the field experiments by primary producers $>1$ µm (Figure 4.5) also demonstrates the availability of bacterial lysate to support primary production (Figure 4.5). Extrapolating from the laboratory results and previous findings (Shelford et al. 2012), it is likely that the primary production in the field experiments was stimulated by ammonium remineralised by bacteria.

All five field stations had $^{15}$N in the particulate fraction, indicating that phytoplankton and/or bacteria were incorporating lysis products (Figure 4.5). The uptake, as measured by the presence of $^{15}$N in the particulate fraction at the end of the experiments, is a conservative estimate of the actual uptake occurring in the incubations. This method does not account for release of $^{15}$N through bacterial remineralisation or phytoplankton release (Bronk & Ward 2000). Although these field experiments do not provide a quantitative estimate of the amount of lysate able to be taken up, they do provide evidence to corroborate with the uptake of lysis products in the laboratory experiment. Uptake in SB (0.60 ± 0.02 µmol l$^{-1}$ N; Figure 4.5) was higher than the calculated lysate addition (0.44 µmol l$^{-1}$ N; Table 4.2), but the lysate contribution is an estimate, and some variation could occur. We can conclude that most of the lysate added in the SB and JP experiments was incorporated by primary producers and other large plankton, since both stations had 100% of $^{15}$N uptake occurring in the $>1$ µm fraction.

Not all N in lysates was taken up by primary producers in three of the five field studies, and only 74% of the N in the lysate contributed to an increase in cultured *Synechococcus* WH7803 abundance. *Synechococcus* WH7803 can use remineralised N from co-occurring bacteria (as seen in DC2+Lys; Figure 4.2A), and bacteria can remineralise a portion of the lysate (as seen in Bac+Lys; Figure 4.2B), but there was a portion unavailable to the cultured microbial community during the experimental timeframe. The gross amount of N that phytoplankton take up can be
almost 74% more than the net amount (Bronk et al. 1994), suggesting that more N may have been taken up (and then released as DON) than was measured in the current experiments. In that same study, total DON turnover times in field samples were found to be 4 to 18 days. The DON in that study included phytoplankton exudation and possibly refractory ambient DON in the environment and would therefore likely have a different composition compared to bacterial lysates from viral infection. However, the short incubation time in the current study could still have resulted in incomplete DON turnover, and the less labile (but still potentially useable) portions of the DON might have been incorporated with time (Middelboe & Jørgensen 2006).

For SI, FRP, and GH, the lysate was added at a very high concentration (Table 4.2), and was therefore unlikely to have been all taken up, especially within the experimental timeframe. The SB and JP experiments were subsequently run with a reduced concentration in order to see complete uptake of the N in lysate. The $^{15}$N from the lysate addition in SB and JP was all recovered in the particulate fraction, indicating that all of the lysate was labile in the field when added at an appropriate concentration. The incomplete transfer of N from V. natriegens (PWH3a) biomass to cultured Synechococcus WH7803 abundance, when compared to the complete uptake of $^{15}$N at SB and JP, may indicate that either remineralisation of the DON is dependent on the make-up of the bacterial assemblage, or that other phytoplankton species may be capable of taking up DON directly (Bronk et al. 2007).

Although chlorophyll a concentrations in the field studies showed no major differences between treatments, picophytoplankton abundances in Gorge Harbour (GH) were almost tenfold higher than in Saanich Inlet (SI) and Fraser River Plume (FRP; Figure 4.4), indicating a large cyanobacterial population (likely Synechococcus, a common primary producer in BC summer coastal waters; Ortmann et al. 2002, Frederickson et al. 2003). Because cyanobacteria contain
less chlorophyll $a$ per cell than other phytoplankton, due to both their small size and reliance on phycobilins as accessory pigments (McConnell et al. 2002), they do not contribute as much to the chlorophyll $a$ concentrations as do other taxa. This cyanobacterial population likely increased total primary production in GH overall, unaccounted for by chlorophyll $a$ measurements and light microscopy, and contributed to the greater uptake of $^{15}$N in GH compared to SI and FRP, even when normalised to chlorophyll $a$ (Figure 4.5). Semiahoo Bay (SB) and Jericho Pier (JP) also had substantial populations of picophytoplankton, corresponding to their maximal uptake of the available $^{15}$N despite the lack of chlorophyll $a$ increase.

The increase in *Synechococcus* WH7803 abundance in the laboratory experiment (Figure 4.2A) can be explained by the different sources of N present in the experimental treatment (DC2+Bac+Lys). A portion of the increase can be attributed to the 21.3 µmol l$^{-1}$ of nitrate present in the Bac ultrafiltrate, a portion to the remineralisation of ammonium by the added bacterial assemblage, and another portion to uptake of ammonium from remineralisation by contaminating bacteria in the non-axenic *Synechococcus* WH7803 culture. Despite evidence for uptake of DON directly by phytoplankton in field experiments (see Bronk et al. 2007 for a review), preliminary studies to the current one showed no evidence for uptake of *V. natriegens* (PWH3a) lysate uptake by axenic *Synechococcus* WH7803. If considered together, the increase in *Synechococcus* WH7803 in the experimental treatment (DC2+Bac+Lys) can be accounted for by the effects from each of these control treatments.

4.5.3 *Ecological implications and future directions*

Some studies have focussed on the influence of phytoplankton lysates on supporting bacterial production (Gobler et al. 1997, Haaber & Middelboe 2009). Phytoplankton lysates can be very
important in certain situations, such as during a bloom termination (Brussaard et al. 2005), but bacterial lysates are being produced constantly throughout the ocean.

The ability of primary producers to use the N from bacterial remineralisation of bacterial lysates indicates that viruses are not just C sinks that disrupt trophic levels (Azam & Worden 2004), but are important facilitators in N recycling pathways. Traditional food chain models state that C and nutrients flow from primary producers to higher trophic levels. The introduction of the microbial loop (Azam et al. 1983) included bacterial dynamics, whereby dissolved organic matter produced by the members of the traditional food chain is taken up by bacteria and reintroduced to the food web instead of being lost to the system. Including viruses in this model explains C cycling pathways more fully. The viral shunt (Wilhelm & Suttle 1999) introduced viruses as a ‘short-circuit,’ removing particulate C from primary producers, consumers, and bacteria to the dissolved organics pool. This model emphasises viruses as a loss mechanism of food web C. However, N and other nutrients are not included in these models. The loss of C from the particulate pool is clear, but there is evidence of increased productivity in the presence of viruses. In Fe-limited regions of the eastern Pacific Ocean viruses were shown to liberate Fe into the DOM pool, where it was available for uptake by primary producers (Poorvin et al. 2004). Weinbauer et al. (2011) provided evidence for this phenomenon when they removed the viral fraction from a cyanobacteria bloom and primary production ceased. Shelford et al. (2012) confirmed that observation by demonstrating a reduction in both ammonium remineralisation and phytoplankton abundance with removal of viruses. The current study indicates that there is a transfer of N from bacterial lysates to phytoplankton biomass, and that this process can be replicated in five separate samples from coastal BC. Weinbauer et al. (2011) hypothesised that primary production decreased (Hollibaugh 1978, Healey & Hendzel 1979) in their experiments
due to either the lysates providing a direct source of nutrients for the *Synechococcus* blooms, or bacterial remineralisation occurring to provide inorganic nutrients. Given the evidence from this study, bacterial remineralisation was likely occurring, and this phenomenon occurs in the waters of coastal BC. Future work measuring the magnitude of N use for different species or in the field would contribute to our knowledge of microbial processes in the ocean, and enable better ecosystem modelling.
Chapter 5: Ammonium remineralisation caused by viral lysis and grazing of bacteria in coastal and oceanic waters

5.1 Synopsis

Nitrogen (N) is a key nutrient that often limits or co-limits phytoplankton production in the ocean. The release of N from bacteria by viral lysis and microzooplankton grazing directly and indirectly supplies ammonium that supports this production; yet, there are few data examining the relative contribution of these processes under different conditions. Tied to this is the fact that bacterial mortality may be caused primarily by grazers or by viruses, but the reasons for these differences are unclear. Bacterial mortality by grazing versus viral infection results in very different pathways of N recycling; therefore, quantifying their relative contribution under different environmental conditions is essential for incorporating viruses into models of marine N cycling. In order to separate the effects of grazing and viral lysis on N cycling, ammonium production and uptake were measured in 24 to 48 h incubations at 12 locations in coastal British Columbia, Canada and in the mid-Atlantic Ocean. Overall, the relative contributions to ammonium remineralisation by viruses and microzooplankton were highly variable, although remineralisation from viral infection was greater at stations with higher chlorophyll $a$ concentrations and lower salinity. No correlations were found between ammonium remineralisation by grazers and environmental parameters. These results suggest that predicting the relative contributions of viral lysis and microzooplankton grazing to ammonium remineralisation is controlled by a set of factors more complex than the suite of measured environmental variables.
5.2 Introduction

Microzooplankton and bacterial viruses are the major sources of bacterial mortality in the ocean (Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995). Microzooplankton are considered to be relatively non-specific grazers of bacteria, although larger and more active cells may be preferentially grazed (Gonzalez et al. 1990). In contrast, viruses have very specific host ranges, often infecting a limited number of strains within a species or genus (Suttle 2007).

There have been numerous estimates of grazing rates on aquatic bacteria (e.g. Pearce et al. 2011, Vázquez-Domínguez et al. 2012, Unrein et al. 2014) and of viral-mediated mortality (e.g. Rowe et al. 2012, Maurice et al. 2013, Xu et al. 2013). In addition, studies have examined the relative rates of grazing and viral lysis in different systems. Relative rates differ considerably from study to study, with some reporting grazing as the main source of bacterial mortality (e.g. McManus & Fuhrman 1988, Garzio et al. 2013), and others finding significant lysis rates compared to grazing (Weinbauer & Peduzzi 1995, Wells & Deming 2006a, Taira et al. 2009, Boras et al. 2010). Pernthaler (2005) speculated that grazing is more important in oligotrophic waters due to fewer predators of grazers, while Zhang et al. (2007) suggested that viral lysis is more important in eutrophic waters where grazers are kept down by larger zooplankton. Yet, another study that compared mortality across seasons at one location did not find that trophic conditions were a good predictor of the source of bacterial mortality (Ortmann et al. 2011). Consequently, there is a poor predictive understanding on what controls the balance between bacterial mortality caused by viruses and grazers. In turn, it is difficult to develop predictive relationships describing the relative contributions of viruses and grazers of bacteria to N remineralisation.
This chapter compares ammonium remineralisation rates caused by viruses and grazers across a range of environments and environmental conditions. As abundance and production of viruses often co-vary with bacterial abundance which can correlate with chlorophyll $a$ and system productivity (Cochlan et al. 1993, Clasen et al. 2008, Magiopoulos & Pitta 2012, Paterson et al. 2012), regions with high nutrient and chlorophyll $a$ concentrations and bacterial abundance are hypothesised to have the highest viral mediated ammonium remineralisation rates. Therefore, viral mediated remineralisation rates are hypothesised to be relatively more important in regions with high nutrient and chlorophyll $a$ concentrations and high bacterial abundance.

5.3 Methods

5.3.1 Sample collection and processing

Samples were collected from 12 locations (Table 5.1 and Figure 5.1). The Jericho Pier (JP) location was sampled 7 times over a single year (2010-2011), collected from the pier using an acid-rinsed (rinsed with 10% HCl followed by three rinses with ultrapure water) 10L carboy. Samples were collected using Niskin bottles mounted on a rosette from the Strait of Georgia (SOG) during two week-long cruises in Aug 2010 and Sep-Oct 2011, from the Saanich Inlet (SI) on a day-cruise in Aug 2011, and from the Atlantic (CI) during a month-long cruise along a transect from the Canary Islands to Iceland in April 2011. The Saanich Inlet (SI) and Pendrell Sound (SOG11-6) locations were both sampled at 3 depths on the same cast. All this data collection resulted in 22 samples that were used for experiments.
Table 5.1 Environmental and biological data from each station, including bacteria, viruses, cyanobacteria, chlorophyll $a$ (Chla), temperature (Temp), salinity (Sal), nitrate (NO$_3^-$), phosphate (PO$_4^{3-}$), ammonium (NH$_4^+$), N remineralisation attributed to viral lysis (Viral remin), and total N remineralisation from the whole seawater treatment (Total remin).

<table>
<thead>
<tr>
<th>Station</th>
<th>Location</th>
<th>Date</th>
<th>Bacteria (x10$^5$ ml$^{-1}$)</th>
<th>Virus (x10$^6$ ml$^{-1}$)</th>
<th>Cyanobacteria (x10$^3$ ml$^{-1}$)</th>
<th>Chla (µg l$^{-1}$)</th>
<th>Temp (°C)</th>
<th>Sal</th>
<th>NO$_3^-$ (µmol l$^{-1}$)</th>
<th>PO$_4^{2-}$ (µmol l$^{-1}$)</th>
<th>NH$_4^+$ (µmol l$^{-1}$)</th>
<th>Viral remin (µmol l$^{-1}$ d$^{-1}$)</th>
<th>Total remin (µmol l$^{-1}$ d$^{-1}$)</th>
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<td>Virus</td>
<td>Cyanobacteria</td>
<td>Chla</td>
<td>Temp</td>
<td>Sal</td>
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<td>PO$_4^{2-}$</td>
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*UD = undetectable*
Figure 5.1 (A) Location of Strait of Georgia (SOG), Saanich Inlet (SI), and Jericho Pier (JP) sampling sites in BC. (B) Stations sampled in the Atlantic Ocean.
Locations for sample collection were chosen to encompass a wide variety of conditions. Samples from the Atlantic (CI) included both tropical and temperate open ocean waters, expected to have low nutrients and low productivity. Samples from the Strait of Georgia (SOG) and Saanich Inlet (SI) were all coastal temperate locations, but the amount of mixing, nutrient levels, and productivity varied. Samples from Jericho Pier (JP) were collected over the course of a year in order to see any changes from changing temperature and salinity from seasonal differences. The three depths sampled at SI and SOG11-6 from above and below the mixed layer were collected to determine if any changes were detectable in the same water column and at the same time, but in different nutrient conditions.

Virus-free water (ultrafiltrate) for the experiments was made by running 1.5 l of seawater through a 30k Da molecular weight cut-off tangential flow filtration system, either a Vivaflow 200 water purification unit (Thomas Scientific, Swedesboro, NJ) or a Prep Scale-TFF Cartridge (Millipore, Billerica, MA) after pre-filtration through a 1 or 2 µm pore-size polycarbonate filter (AMD Manufacturing, Canada).

Temperature and salinity were measured via CTD for SOG, CI, and SI locations, and with a thermometer and a handheld refractometer for the JP location. Approximately 10 l of seawater was collected from each location. Samples of 100 ml were used for environmental conditions analysis, and the rest was used for the experiment. Samples of 50 ml were filtered through acid-rinsed syringes fitted with MilliQ-soaked 0.45 µm 25mm cellulose nitrate membrane filters (Whatman, United Kingdom) in a Swinnex filter holder (Millipore, Billerica, MA). The filters were used for chlorophyll a determination, and the filtrate was used for ammonium determination. The first 15 ml were used to rinse the collection tubes, and 10 ml were collected into acid-rinsed 15 ml polypropylene screw-cap tubes (Sarstedt, Germany) and frozen at -20°C.
The filters were folded and placed in foil squares, kept in the dark and frozen at -20°C. Chlorophyll $a$ concentrations were determined from the frozen filters after preparation with 90% acetone and sonication (Parsons et al. 1984) using a 10AU fluorometer (Turner Designs, Sunnyvale, CA). Ammonium concentrations were determined from the filtered samples (Holmes et al. 1999) using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

Samples of 1 ml were collected, fixed with gluteraldehyde at a final concentration of 0.5% (Marie et al. 1999) and used for counting bacteria, viruses, and phytoplankton by flow cytometry, except at CI locations where an additional 3 ml sample was collected for phytoplankton counts by flow cytometry and fixed with formaldehyde at a final concentration of 2%. Samples were flash frozen in liquid nitrogen and stored at -80°C. Samples were counted using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), using SYBR Green I (Sigma-Aldrich, St. Louis, MO) to stain the bacteria and viruses (Brussaard 2004b); phytoplankton were enumerated unstained via flow cytometry (Olson et al. 1993).

5.3.2 Experimental design

The experiment consisted of three treatments (Figure 5.2), all of which consisted of 500-800 ml of sample in 1000 l Whirlpak bags (Nasco, Fort Atkinson, WI). Treatment SW was untreated seawater filtered through 118 µm Nitex mesh to remove large zooplankton grazers. The second and third treatments were prepared by filtering the Nitex-filtered seawater through either a 1 or 2 µm pore-size polycarbonate filter (AMD Manufacturing, Canada) to remove the majority of microzooplankton while retaining most bacteria and viruses in the filtrate. Although there may be differences between the 1 and 2 µm filtrates, the majority of microzooplankton are removed using a 2 µm pore-size filter (Shelford et al. 2012). Bacteria from the filtrate were concentrated
approximately tenfold by tangential flow filtration with a 0.22 µm pore-size membrane (Pellicon, Millipore, Billerica, MA). Viral particles were diluted by adding ultrafiltrate to the bacterial concentrate and reducing the volume again (Treatment B). For Treatment V, 0.22 µm pore-size filtrate was added back to the bacterial concentrate to create a treatment in which bacteria and viruses were at ambient levels. This treatment was included instead of a simple 1 or 2 µm filtrate in order to control for manipulation effects of bacterial concentration in Treatment B. All treatments were in triplicate.

Figure 5.2: Diagram of the experimental treatment set-up. The three treatments are B (bacteria only), V (bacteria and viruses), and SW (whole seawater).
For experiments, in situ light levels and temperatures were approximated using either flowing seawater incubators covered by neutral density screening (CI and SOG), or an incubator (JP and SI). Samples for cell and viral abundances and ammonium concentrations were taken at five equally spaced intervals over the 24 to 48 h incubations, while samples from chlorophyll a concentrations were taken at the initial and final time points.

At the final time point, $^{15}$N ammonium chloride (90+ atom % $^{15}$N, Isotec, Miamisburg, OH) was added at approximately 10% of ambient ammonium concentrations to each treatment to estimate ammonium uptake rates of larger phytoplankton present in the SW treatment that were removed in the V and B treatments. This measurement allowed gross ammonium remineralisation rates to be estimated for each treatment. After 1 to 2 h (2 to 4 h for CI locations), each treatment was filtered through 0.2 µm pore-size silver filters (Sterlitech, Kent, WA) in order to collect the particulate fraction. The filters were dried at 50°C for 2 d. $\delta^{15}$N-PON on the filters was determined at the Stable Isotope Laboratory at Boston University on a GV Instruments IsoPrime isotope ratio mass spectrometer and a Eurovector elemental analyzer, calibrated against atmospheric N$_2$ and IAEA standards N-1, N-2, and N-3 (replicate analysis within ± 0.2‰).

5.3.3 Calculations

The net ammonium remineralisation rate was calculated by taking the difference between the ammonium uptake rate (as measured by the $^{15}$N uptake) and the change in concentration of free ammonium over the duration of the experiments. In the SW treatment, this net remineralisation rate was due to both grazing and viral lysis. To obtain the amount of remineralisation that can be attributed solely to viral lysis, the ammonium uptake rate (as measured by the $^{15}$N uptake) was not needed, since treatments B and V both have similar cellular communities, and therefore
should have equivalent uptake rates. Therefore, the free ammonium increase or decrease over the duration of the experiment in the B treatment (in which any remineralisation that occurs is in the absence of both predators) was subtracted from that of the V treatment (in which only viral lysis contributes to remineralisation).

Total particulate $^{15}$N on the filters was calculated from the total particulate N on the filters measured by elemental analyser (section 4.3.4.3) and the ratio of $^{15}$N:$^{14}$N measured by mass spectrometer (section 4.3.4.3), taking into account the natural ratio of $^{15}$N:$^{14}$N in seawater (0.36765%) and the ratio of the glycine standard (0.3716%). Uptake rates were calculated without accounting for isotope dilution. The ammonium uptake rate ($\mu$mol l$^{-1}$ h$^{-1}$) was calculated as follows:

$$\text{Total } NH_4^+ \text{ uptake} = \left( \frac{[N] \times \left( \frac{\delta^{15}N}{1000} + 1 \right) \times 0.0036765}{\text{Volume filtered} \times \text{Proportion of added } ^{15}\text{N in seawater}} - 0.003716 \right)$$

5.3.4 Statistical analysis

5.3.4.1 Testing relationship between environmental variables and viral mediated ammonium remineralisation

Statistical tests were run to determine whether ammonium remineralisation by viral lysis changed with variations in environmental or biological variables. Figures were generated using ggplot2 in R (Wickham 2009). A variable was made, equivalent to the amount of ammonium remineralisation contributed by viral lysis, by subtracting the remineralisation that occurred with bacteria alone from the remineralisation that occurred with viruses present. The dataset was split
into two groups: stations with detectable viral remineralisation (JP6, JP7, SOG10-11, SOG11-6T, and SOG11-15), in which the mean of the new variable was at least one standard deviation above zero, and stations with undetectable viral remineralisation, in which the mean was below or within one standard deviation of zero. A non-parametric Mann-Whitney U test was used to test for differences between groups for salinity and for chlorophyll a.

5.3.4.2 Testing relationship between environmental variables and virus- vs. grazer-mediated ammonium remineralisation

To compare remineralisation rates among grazers, viruses, and bacteria, a principle components analysis (PCA) and a mixed-effects linear model (with treatment and PCA component as fixed effects and station as a random effect) were run in order to compare the gross remineralisation rates for selected stations. Each component in a PCA is an amalgamation of correlated variables that describe a portion of the variance of the data. Because N-depleted phytoplankton may rapidly take up ammonium in excess of growth requirements (Suttle and Harrison 1988), the only data analyzed were from stations at which the $^{15}$N ammonium addition was $<$30% of ambient concentration (JP0, JP1, JP3, SOG11-6T, SOG11-15, CI-3, CI-15, CI-18). A post hoc test on an ANOVA of the gross remineralisation rates was run in order to determine which treatments were significantly different.

5.3.4.3 Initial tests

5.3.4.3.1 Testing relationship between environmental variables and viral mediated ammonium remineralisation

Remineralisation rates caused by viruses were examined by running a PCA and a mixed-effects linear model on the data for all stations, with station as a random effect. However, treatment B
(bacteria only) had significantly greater remineralisation rates overall (p<0.05) than treatment V (bacteria and viruses). Because of this unexpected result, the data were split into two groups (stations with detectable viral remineralisation and stations without detectable viral remineralisation) and a Mann-Whitney U test was run, as outlined in section 5.3.4.1.

5.3.4.3.2 Testing relationship between environmental variables and virus- vs. grazer-mediated ammonium remineralisation

A PCA and a mixed-effects linear model were run to compare gross remineralisation rates among grazers, viruses, and bacteria at all stations (Table 5.2). However, a number of these stations had unacceptable amounts of $^{15}$N addition for the isotope incubations, and for this reason only certain stations were selected for analysis, as outlined in section 5.3.4.2.

Table 5.2 Results from mixed-effects linear models testing ammonium remineralisation rates attributed to grazers, viruses, and bacteria, with an ANOVA test for mixed-effects linear model with treatment and PCA component as fixed effects and station as random effect. Two models were run, one including all stations, and another only selected stations (JP0, JP1, JP3, SOG11-6T, SOG11-15, CI-3, CI-15, CI-18). Treatments were whole seawater including grazers (treatment SW), seawater with only viruses and bacteria (treatment V), and seawater with only bacteria (treatment B). Comp1 is a component from the principle components analysis (PCA) of environmental and biological variables from the stations, and is positively correlated with nitrate and phosphate and negatively correlated with temperature.

<table>
<thead>
<tr>
<th>Effect</th>
<th>All stations</th>
<th>Select stations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>p &lt; 0.005</td>
<td>p = 0.254</td>
</tr>
<tr>
<td>Comp1</td>
<td>p &lt; 0.01</td>
<td>p = 0.662</td>
</tr>
<tr>
<td>Treatment x Comp1</td>
<td>p = 0.196</td>
<td>p = 0.251</td>
</tr>
</tbody>
</table>

5.4 Results

5.4.1 Environmental and biological parameters

The stations chosen exhibited environmental and biological parameters characteristic of a wide range of conditions, from low nutrient tropical systems to highly productive coastal systems (
Stations JP0, JP1, JP3, JP4, JP8, and most of SOG11 were characterised by high nitrate and phosphate but low chlorophyll $a$ concentrations, likely indicating deeply mixed or recently upwelled waters. The stations from the Atlantic (CI) had mostly low nutrients, low chlorophyll $a$, and high salinity, indicative of oceanic regions. The Jericho Pier location (JP) was the only station sampled repeatedly throughout the year, and therefore ranged from highly productive bloom-like conditions (JP6, JP7) to high nutrient winter conditions (JP0, JP1, JP3, JP4), and had a wide range in salinity likely due to varying riverine and terrestrial inputs throughout the year.

Rates of total ammonium remineralisation in this study averaged $0.51 \mu\text{mol l}^{-1} \text{d}^{-1}$, ranging from undetectable to $3.08 \mu\text{mol l}^{-1} \text{d}^{-1}$. Although there was variation both within and among stations, most stations displayed ammonium remineralisation overall (Figure 5.4A). Detectable viral mediated ammonium remineralisation was limited to five stations, JP6, JP7, SOG10-11, SOG11-6T, and SOG11-15 (Figure 5.4B), and the magnitude was much less than that of the gross remineralisation rates, implying that much of the measured ammonium production came from grazers. Station JP3, while appearing to have significant viral mediated ammonium remineralisation from the standard error bars (Figure 5.4B), did not qualify for inclusion, as the mean was less than one standard deviation above zero.
Figure 5.3: Environmental data from all stations: (A) bacterial abundance, (B) viral abundance, (C) cyanobacterial abundance, (D) chlorophyll $a$ concentration, (E) temperature, (F) salinity, (G) nitrate concentration, (H) phosphate concentration, and (I) ammonium concentration.
Figure 5.4 Average ammonium remineralisation for each station of (A) unfiltered seawater in the SW treatment and (B) ammonium production in treatment with viruses, treatment V, subtracted by ammonium production in treatment without viruses, treatment B (i.e. viral-specific remineralisation rates). Error bars are standard error of triplicate incubations. The five stations with detectable viral mediated ammonium remineralisation (defined as the mean being at least one standard deviation above zero) are indicated with asterisks (*).
5.4.2 *Viral influence on ammonium remineralisation*

Five stations had detectable viral mediated ammonium remineralisation, stations JP6, JP7, SOG10-11, SOG11-6T, and SOG11-15 (Figure 5.4B). JP6 and JP7 had the highest chlorophyll $a$ concentrations of all the stations, SOG11-6T had one of the highest viral abundances, and JP6 had the lowest salinity, indicating a wide variety of conditions across all five stations. Relative to stations with no detectable viral remineralisation, stations with detectable viral remineralisation had chlorophyll $a$ concentrations that were significantly higher ($p<0.05$, Mann-Whitney U test; Figure 5.5A), salinity was significantly lower ($p<0.05$, Mann-Whitney U test; Figure 5.5B), and the range was much greater overall.

The $^{15}$N uptake data indicated that the stations with no detectable viral remineralisation had the lowest ammonium uptake rates. This result indicates that these stations did not have high ammonium turnover, and therefore it is not likely that high ammonium remineralisation rates were masked by high uptake rates.
Figure 5.5 Box plot of (A) chlorophyll $a$ concentrations ($p<0.05$) and (B) salinities ($p<0.05$) at stations with and without detectable viral remineralisation. The lower and upper limits of the boxes indicate the first and third quartiles, and the thick central line is the median. The whiskers extend to the minimum and maximum data points, and outliers are depicted as individual points.
5.4.3 Comparison of grazer and viral influence on ammonium remineralisation

There was no significant effect of environmental variables for the mixed-effects linear model comparing gross remineralisation rates against treatment (treatments SW, V, and B) and PCA component (Comp1, positively correlated with nitrate and phosphate and negatively correlated with temperature) with selected stations as the random effect (Table 5.2).

5.5 Discussion

Microzooplankton grazers and viruses are important players in marine N recycling. The goal of this study was to examine their relative contribution across a range of environmental and biological conditions. Overall, the results of this study found that the relative contributions of viruses and microzooplankton grazers to ammonium remineralisation were highly variable and not correlated with the measured environmental variables; however, there was a significant difference between stations with detectable viral mediated ammonium remineralisation and stations without, when comparing chlorophyll a concentrations and salinity. There were no significant correlations between environmental conditions and ammonium remineralisation by grazers. These results are discussed in detail below.

5.5.1 Increased ammonium remineralisation in the bacteria-only treatments

At some stations (JP0, JP1, JP4, SI 15m, SOG11-6M, SOG11-6B, CI-15), ammonium remineralisation in the treatment with only bacteria (B) was higher than in the treatment with viruses (V) or with both viruses and grazers (SW). This is counter-intuitive, as one would expect
more ammonium remineralisation to occur in the presence of viruses, or viruses and grazers. However, increased ammonium remineralisation in some B treatments may be an artefact of DON in the ultrafiltrate. Because bacteria were concentrated by tangential-flow filtration to control for the effect of concentration in both B and V treatments, the only difference between the two is the ultrafiltrate added to the B treatment. Ultrafiltration could result in the release of DON from cells that have passed through pre-filtration. The 30k Da molecular weight cut-off used in these experiments removes viral particles, but not most DON (Berman & Bronk 2003). Because of this potential effect, and because very few stations showed an effect of viruses on ammonium remineralisation rates, the results from the first mixed-effects linear model for the virus-only data were not used. Instead the data were analysed by a Mann-Whitney U test, sorting the data into groups of ‘stations with detectable viral remineralisation’ and ‘stations with undetectable viral remineralisation’.

5.5.2 Salinity and chlorophyll a as indicators of viral influence on remineralisation

Salinity was significantly lower at stations where there was an influence of viruses on ammonium remineralisation (Figure 5.5B). It is not clear why lower salinities would be associated with viral mediated ammonium remineralisation, although salinity can affect host-virus interactions, including adsorption. For example, many Gram negative bacteria have lipopolysaccharides (LPS) that act as binding sites for phage (Lüderitz et al. 1982), and even very small changes in salt concentration can alter LPS profiles (Soussi et al. 2001). In the case of Aeromonas hydrophila changes in the LPS with increased osmolarity also increased phage infectivity (Aguilar et al. 1997). Similarly, teichoic acid is a polysaccharide found in cell walls of Gram positive bacteria, and is an adsorption point for phage. Doyle et al. (1974) found that increased salt concentrations reversibly changed the conformation of teichoic acid, which
negatively affected phage adsorption rates. In another example, infectivity was affected by changes in salinity for a marine phage-host system isolated from the Arctic (Wells & Deming 2006b). Moreover, the effects of salinity can be phage dependent. Zachary (1976) examined two phages infecting the marine bacterium *Beneckea natriegens*, and found that one phage replicated optimally at higher salinities and the other at more estuarine salinities, although a certain salinity minimum was necessary for both phages. Williamson & Paul (2006) also found that an increase in salinity induced the lytic stage and increased phage titer in a pseudolysogenic bacterium *Listonella pelagia*. Although it is unknown how salinity might specifically have affected the bacterial and viral communities in the current study, it was lower in stations with detectable viral mediated ammonium remineralisation, just as chlorophyll *a* concentrations were higher in those stations.

It is not surprising that salinity and chlorophyll *a* concentration covaried, as these two variables are often correlated due to increased nutrients and stratification from freshwater runoff promoting phytoplankton bloom formation (Ingram et al. 1985, Zheng & Tang 2007). Therefore, it follows that ammonium remineralisation caused by viruses would vary with both parameters at once. Although one might expect other variables in this study such as viral or bacterial abundance to also increase with increasing ammonium remineralisation, it may be that a larger dataset would provide those correlations.

Chlorophyll *a* was significantly higher at stations with detectable viral remineralisation, corresponding to the initial hypothesis of this study. Higher chlorophyll *a* could mean that the site has more interactions of all types, more DON produced in the system, more bacterial production, and thus more opportunities for viral infection. A similar significant correlation might be found between viral remineralisation and bacterial and phytoplankton abundances in a
larger dataset. Viral abundances are generally positively correlated with bacterial abundances (Maranger & Bird 1995, Clasen et al. 2008), and often with chlorophyll \( a \) and system productivity (Boehme et al. 1993, Maranger & Bird 1995), although that is not always the case (Cochlan et al. 1993). In this study, higher chlorophyll \( a \) concentrations occurred along with detectable viral remineralisation, but the range of responses was large (Figure 5.5A).

5.5.3 Correlations with grazing

There was no statistical significance between environmental variables and remineralisation rates by grazers when examining the selected stations; however, there were correlations in the larger dataset, which suggest potential avenues for future research.

The model with all three treatments, and using data from selected stations where \(^{15}\text{N}\) ammonium was added at <30% of ambient concentration, did not show significant differences among treatments or a significant correlation with the PCA component (Table 5.2). The lack of a predictive variable to explain ammonium remineralisation is consistent with observations of Ortmann et al. (2011) and Berdjeb et al. (2011) who also did not find a predictive relationship between bacterial mortality and environmental conditions. Nonetheless, a larger dataset may yield significant correlations; when all the stations in the present study were included in the analysis (including those with high \(^{15}\text{N}\) additions), the PCA component was significant (Table 5.2). While conclusions cannot be made from correlations using the high \(^{15}\text{N}\) data, the results suggest that future experiments using larger datasets, more precise ammonium uptake measurements, or different environmental or biological variables might reveal the conditions affecting the relative contributions of viral lysis and microzooplankton grazing to ammonium remineralisation.
Environmental and biological conditions have been shown to affect grazing rates in some studies. Temperature range can influence nutrient cycling in cultured protists (Jürgens & Massana 2008), clearance rates of heterotrophic flagellates increase with temperature (Unrein et al. 2007) and decrease with prey biomass (Sherr & Sherr 2002), and numerous studies have shown a general seasonality of microzooplankton grazing rates of bacteria (Cleven & Weisse 2001, Domaizon et al. 2003, Jacquet et al. 2005), but the mechanism of these changes is unclear. One study found no significant relationship between temperature and mortality, but the temperature range was very narrow (Garzio et al. 2013). Although the current study did not show a significant relationship between the measured environmental and biological conditions and ammonium remineralisation by grazers, the correlation found in the larger dataset and evidence from other studies indicate that it would be worthwhile to examine this relationship further.

5.5.4 Methodological considerations

5.5.4.1 Comparison of ammonium uptake and remineralisation rates

Ammonium uptake rates measured in this study were within reported values in the literature. Mean uptake rates ($V_{\text{max}}$) vary from 0.0099 h$^{-1}$ for an oceanic phytoplankton assemblage (MacIsaac & Dugdale 1969) to 0.048 h$^{-1}$ for a coastal phytoplankton assemblage (Glibert et al. 1982), compared to this study’s maximum uptake rate of 0.021 h$^{-1}$ or mean uptake rate of 0.0025 h$^{-1}$.

Ammonium remineralisation rates in this study (mean of 0.51 µmol l$^{-1}$ d$^{-1}$, maximum of 3.08 µmol l$^{-1}$ d$^{-1}$) are consistent with ammonium remineralisation rates found by other researchers. Low remineralisation rates of 0.010-0.160 µmol l$^{-1}$ d$^{-1}$ were found in the oligotrophic Atlantic (Clark et al. 2008), and 0.5 µmol l$^{-1}$ d$^{-1}$ in the northeast Atlantic (Fernández & Raimbault 2007),
comparable to the rates found in this study of 0.05-0.07 µmol l\(^{-1}\) d\(^{-1}\) in the tropical Atlantic and 0.38 µmol l\(^{-1}\) d\(^{-1}\) in the northern Atlantic (Table 5.1). In coastal zones remineralisation rates can be higher, with 0.526 ± 0.584 µmol l\(^{-1}\) d\(^{-1}\) in spring and summer off Chile (Fernandez & Farías 2012) and 0.220 µmol l\(^{-1}\) d\(^{-1}\) in coastal northwest Mediterranean (Diaz & Raimbault 2000). In highly productive waters remineralisation rates range greatly; in the Mississippi during a drought remineralisation rates ranged from 0.432-2.976 µmol l\(^{-1}\) d\(^{-1}\) (Jochem et al. 2004), and in mangrove waters from 0.24-36 µmol l\(^{-1}\) d\(^{-1}\) (Dham et al. 2002). The results in this study are consistent with the literature.

5.5.4.2 Selecting stations for analysis

Because the initial ammonium concentrations were unknown in most experiments prior to the \(^{15}\)N incubations, \(^{15}\)NH\(_4\)Cl was not consistently added at 10\% or less of the initial NH\(_4\) concentration. As a result, stations JP4, JP6, JP7, JP8, SOG10-11, SOG10-15 and CI-7 had \(^{15}\)NH\(_4\)Cl added at high concentrations compared to ambient. In the analysis, only data from stations that had \(^{15}\)NH\(_4\)Cl additions < 30\% of ambient concentrations were included. Although higher than the <10\% additions that are typically used (e.g. Ward & Bronk 2001), the treatments which received additions of between 10 \% and 30 \% of ambient had ammonium concentrations of at least 0.3 µmol l\(^{-1}\) and therefore enhanced uptake is not as likely to be a factor (Conway & Harrison 1977).

5.5.4.3 Timing of the \(^{15}\)N incubations

Obtaining enough filtered water to run the experiments was a balance between having enough volume for analyses and experiments while minimizing manipulation times. Filtering, concentrating bacteria, and treatment preparation took approximately 2-3 h, and larger volumes
would have increased this time. Because volumes were minimised, $^{15}$NH$_4$Cl was added directly to the incubations after all other measurements had been taken at the end of the experiment. Bottle effects after long incubation times are inevitable (Bronk & Glibert 1994), and may have affected uptake rates. However, the purpose of the $^{15}$N incubations was not to estimate uptake in the environment, but to estimate uptake by phytoplankton and bacteria within the incubations.

5.5.4.4 Accounting for DO$^{15}$N production

The experiments run in the Atlantic (CI) were often in oligotrophic waters. For these experiments, the $^{15}$N incubations were run for 2 to 4 h instead of 1 to 2 h. Since productivity was very low, the longer incubations ensured that there was sufficient $^{15}$N taken up for analysis. However, longer incubation times can result in release of DO$^{15}$N. This can be a significant problem, especially in oligotrophic waters (Bronk & Glibert 1994), and can result in underestimation of ammonium uptake. This would most likely be a problem at stations CI-2, CI-3, and CI-7. Removing these stations did not have a significant effect on the results of the mixed-effects linear model. Bronk and Glibert (1994) discuss the release and uptake of DON by bacteria as a potential unmeasured sink of $^{15}$N in isotope tracer studies. However, silver filters with a 0.2 µm pore-size were used in the experiments discussed in this dissertation, which should capture all the bacteria on the filters. Hence, bacterial uptake was measured, thereby reducing the amount of unaccounted-for $^{15}$N in the incubation.

5.5.5 Conclusions

It was difficult to determine whether grazers or viruses had the larger net effect, because there were very few stations in which both occurred. However, most stations had significant ammonium remineralisation by grazers, but only five stations showed signs of ammonium
remineralisation by viral lysis. This is in contrast to other studies in this dissertation in which viral lysis was found to play an important role in marine N cycling; methodological limitations may be a factor in the current study, especially from the introduction of dissolved organic matter in experimental set-up (discussed in section 5.5.1). Ammonium remineralisation caused by viruses was high when chlorophyll $a$ concentrations were high and salinity was low, implying that these may be important factors for viral remineralisation. However, the large ranges in chlorophyll $a$ and salinity in stations with detectable viral remineralisation, as well as the lack of correlation found in the grazer-data model, indicate that the environmental conditions under which ammonium remineralisation occurs are highly variable.
Chapter 6: Conclusion

6.1 Conclusions

This dissertation examined the role viruses play in nutrient dynamics in the ocean. In particular, it focussed on the availability of nutrients released by viral lysis. This work was inspired by a study from Middelboe et al. (2003) in which lysates of Cellulophaga sp. increased the cell numbers of Photobacterium sp. This dissertation began by examining nitrogenous products of bacterial lysates of Cellulophaga sp. released from viral infection, and the ability of other organisms to incorporate these products. Amino acids in lysates from both the cell wall and intracellular proteins were taken up at similar rates by uninfected Photobacterium sp., although not all amino acids were readily available. Following this, remineralisation of nitrogenous components of lysate was measured in both the oligotrophic Indian Ocean and in eutrophic coastal British Columbia. Removal of viruses reduced ammonium remineralisation, as well as decreased the abundance of primary producers. The effect of virus removal on primary producers was also evaluated by measuring the amount of nitrogen (N) available to a cyanobacteria strain in the laboratory and observing uptake of N in lysate in the field. N from bacterial lysate was incorporated by both the cyanobacteria laboratory strain and in situ phytoplankton communities. Lastly, it was hypothesised that viral mediated N remineralisation was dependent on environmental conditions. Remineralisation caused by viral infection and bacterial lysis was measured in a wide variety of different marine environments. The contribution to N remineralisation by viruses and grazers was also compared, since both are major sources of bacterial mortality (e.g. Fuhrman & Noble 1995). There were few correlations between
environmental conditions and viral and grazer remineralisation, although there were some trends showing remineralisation caused by viruses changing with chlorophyll a and salinity.

This dissertation work has expanded our knowledge of the contribution of viral lysis of bacteria to N cycling in the ocean, and the ability of primary producers to benefit from remineralised N. It has also emphasised that the conditions under which remineralisation can occur are highly variable. This is the first time that the uptake of specific N products of viral lysis has been investigated, showing different availabilities for various N components of a bacterial cell, and to what extent lysates can provide nutrition for bacteria in the ocean. This is also the first time that the transfer of N has been traced and quantified from lysate to phytoplankton, in both a laboratory setting and in the environment. Although mortality rates have been examined before, this is the first time that remineralisation due to viral lysis or grazing has been compared between environments. These results are important to consider when integrating microbes into models of nutrient cycling in the ocean (Figure 6.1).
Suttle (1994) estimated that 10 to 20% of heterotrophic bacteria are lysed every day by viruses, a number which can vary widely dependent on location and conditions (e.g. Wilhelm et al. 1998, 2002). With an estimated 26-70 Pg C yr$^{-1}$ of bacterial production in the euphotic zone (Ducklow & Carlson 1992) a bacterial C:N of approximately 5 (Goldman et al. 1987), and loss rates of 10 to 20% from viral lysis, approximately 0.52-2.8 Pg N yr$^{-1}$ is released from viral lysis of bacteria in the photic zone (Figure 6.2). Primary production C demand is estimated to be 49.3 Pg C yr$^{-1}$ (Ducklow & Carlson 1992), corresponding to 7.4 Pg N yr$^{-1}$ according to the Redfield ratio (106C:16N). Therefore, an estimated 7-38% of global primary production can be supported from bacterial lysates from viral infection. This is a substantial source of recycled N available to support primary production, especially in regions that are N-limited.
Viruses release 0.52-2.8 Pg N yr\(^{-1}\) globally in the euphotic ocean from lysis of heterotrophic bacteria. That dissolved organic N (DON) is available for remineralisation by other bacteria into ammonium, which constitutes a large proportion of the total N demand of primary producers.

The role of microbes in the ocean is an important yet often underappreciated one, and it is only recently that microbes have been included in models of nutrient cycling. The importance of marine viruses especially has only been discovered in the past twenty-five years (Bergh et al. 1989, Jacquet et al. 2010), and even now there is not enough information on the role of viruses in nutrient cycling and mortality rates to be able to fully include their processes in biogeochemical models (e.g. Sarmiento et al. 1998, Brussaard et al. 2008, Hurwitz & Sullivan 2013). This dissertation explores the importance of viruses in N cycling, and provides evidence for the large effect of viral infection on N cycling and its importance to primary producers, which are the base of the marine food web. As well, the changes in ammonium remineralisation from viral lysis with chlorophyll \(a\) and salinity are the type of information needed to model viral mediated...
processes in marine ecosystems. Predictive models are crucial to our understanding of how the ocean will react in different scenarios such as climate change.

6.2 Method limitations and future directions

6.2.1 Improve methods for measuring phytoplankton uptake

Uptake of ammonium by phytoplankton during remineralisation experiments is a difficult variable to measure. In Chapter 5, phytoplankton ammonium uptake was measured to determine the gross remineralisation rate for grazers and viruses. There were some difficulties with this method such as stimulation of primary production by excess ammonium addition, bottle effects, and incubation timing, as discussed in detail in Chapter 5. Improved separation of total remineralisation by grazers from uptake by phytoplankton would be a very useful addition to this field. The effects of grazing and viruses can be segregated when examining mortality rates, but measuring remineralisation rates adds another layer of complexity. Other studies have attempted to reduce phytoplankton uptake by various means, such as keeping incubations in the dark (Kudela et al. 1997), and poisoning the phytoplankton to reduce uptake (Liu et al. 1995, Davidson et al. 2005). These methods have their flaws. Dark uptake of N by phytoplankton can be a significant contributor to total uptake (Cochlan & Harrison 1991, Maguer et al. 2011, Jauzein et al. 2011). It is often difficult to ensure that specific toxins do not affect any others besides the target, and sometimes inhibitors do not affect all members of the target population equally (Sanders & Porter 1986, Tremaine & Mills 1987, Devilla et al. 2005, Shimeta & Cook 2011). Filtration is not an acceptable option as the size range of primary producers overlaps with that of grazers of bacteria. Another way to target phytoplankton uptake would be ideal. A
radioisotope tracing method using tritium in the ammonium molecule may be beneficial. Very small amounts of radiolabelled ammonium would be needed to follow the ammonium uptake, and so there would be little stimulation of the system by adding nutrients. Similarly, incubations could be shorter, given the sensitivity associated with using radioisotopes.

6.2.2 Reduce viral abundance in experiments

The viral production method (Wilhelm et al. 2002) was chosen to determine viral mediated ammonium remineralisation. Other methods are available, but have caveats. The modified dilution approach has been used, but the presence of pre-infected cells is a difficult problem to address and makes manipulation results difficult to interpret (Jacquet et al. 2005), as discussed in Chapter 1. The viral production method tends to reduce bacterial abundance in the incubations and filtration may disrupt bacterial processes, but because similar manipulations were run for treatments with and without viruses, bacterial abundances and processes between treatments were comparable.

6.2.3 Determine the source of variation in viral mediated remineralisation

Viral mediated ammonium remineralisation varied between different locations in Chapter 5. Only a small number of stations had any measurable remineralisation, and when comparing those stations to the stations without remineralisation by viral infection, salinity and chlorophyll $a$ changed along with remineralisation. However, it is clear that further work needs to be done to elucidate the factors that control remineralisation. It is likely that there are different important factors in addition to the set of parameters that were measured here. In this dissertation, ammonium remineralisation was determined by measuring ammonium concentrations over 1 to
2 d incubations. Shorter incubations or ways to measure remineralisation with less sample manipulation would improve the precision of future results.

6.2.4 *Quantify lysate uptake by primary producers*

This is the first study to describe the substantial transfer of N from bacterial lysates through to primary producers. Calculating the amount transferred from cell quota data in the laboratory study and using stable isotopes to trace N in the field studies in Chapter 4 yielded clear results. However, the field of microbial N cycling would benefit from more information on the amount of N transferred using lysates from other species or environmental samples rather than from cultured bacterial lysates. Further work in this field, whether by measuring N transfer using different phytoplankton species, or by collecting more evidence from fieldwork, would greatly increase knowledge of the effect of viruses on the rest of the microbial ecosystem.

6.3 **Final thoughts**

This dissertation examined the N products from viral lysis of marine bacteria and the subsequent path of those released nutrients. The data suggest that viral lysates are highly labile sources of nutrients that can be used by both heterotrophic and autotrophic organisms in the marine microbial world. Given the importance of N in the ocean, and the high turnover of bacterial biomass due to viral infection, more research is needed to examine and quantify the effects of N in lysates. This dissertation contributes to the field of marine microbial nutrient cycling, and adds weight to the argument that viruses are not only removing C from the microbial food web, but are adding back vital N to the system for the benefit of other organisms.
Works Cited


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