# Virus mediated transfer of nitrogen from heterotrophic bacteria to phytoplankton

Emma J. Shelford[1] and Curtis A. Suttle[1,2,3,4]

[1]Department of Earth, Ocean and Atmospheric Sciences, University of British Columbia, Vancouver, Canada

[2]Department of Botany, University of British Columbia, Vancouver, Canada

[3]Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada

[4]Institute of Oceans and Fisheries, University of British Columbia, Vancouver, Canada

Correspondence to: C. A. Suttle (suttle@science.ubc.ca)

# Abstract

Lytic infection of bacteria by viruses releases nutrients during cell lysis and stimulates the growth of primary producers, but the path by which these nutrients flow from lysates to primary producers has not been traced. This study examines the remineralisation of nitrogen (N) from *Vibrio* lysates by heterotrophic bacterioplankton and its transfer to primary producers. In laboratory trials, *Vibrio* sp. strain PWH3a was infected with a lytic virus (PWH3a-P1) and the resulting 36.0 µmol $l^{-1}$ of dissolved organic N (DON) in the lysate was added to cultures containing cyanobacteria (*Synechococcus* sp. strain DC2) and a natural bacterial assemblage. Based on the increase in cyanobacteria, 74% (26.5 µmol $l^{-1}$ N) of the DON in the lysate was remineralised and taken up. Lysate from *Vibrio* sp. strain PWH3a labeled with $^{15}NH_4^+$ was also added to seawater containing natural microbial communities, and in four field experiments, stable isotope analysis indicated that the uptake of $^{15}N$ was 0.09 to 0.70 µmol N per µg of chlorophyll *a*. The results from these experiments demonstrate that DON from lysate can be efficiently remineralised and transferred to phytoplankton, and provides further evidence that the viral shunt is an important link in nitrogen recycling in aquatic systems.


## 1 Introduction

Nutrient recycling is an important link between phytoplankton and heterotrophic bacterioplankton (henceforth, referred to as bacteria) in the ocean. Cell death of phytoplankton and bacteria release dissolved organic material (DOM), which is rich in free and combined amino acids (Middelboe and Jørgensen, 2006) that are taken up and metabolised by bacteria

(Middelboe et al., 1996, 2003). When the C:N of DOM is low relative to bacterial nutritional requirements, bacteria deaminate DOM and release ammonium (Goldman et al., 1987; Hollibaugh, 1978) to acquire carbon for energy and growth (e.g. Brussaard et al., 1996, Fouilland et al. 2014); the release of ammonium can support phytoplankton growth (Haaber and Middelboe, 2009; Shelford et al., 2012; Weinbauer et al., 2011).

Viruses are significant mortality agents of bacteria and phytoplankton in the ocean, and consequently of DOM release (Gobler et al., 1997; Middelboe and Jørgensen, 2006; Noble and Fuhrman, 1999), thereby affecting pathways and rates of nutrient cycling (Fuhrman, 1999; Suttle, 2005, 2007; Wilhelm and Suttle, 1999). Although many nutrients are released during cell lysis, nitrogen typically limits phytoplankton growth in coastal BC waters (e.g. Yin et al., 2017), the location of the current study. High rates of bacterial mortality from viral lysis imply a continuous and substantial flux of DOM from cells into seawater. 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 their 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 mineralisation of DOM 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) released as the result of viral lysis of bacteria, and produce ammonium that supports the growth of phytoplankton.

# 2 Methods

## 2.1 Laboratory cultures

A non-axenic semi-continuous culture of *Synechococcus* sp. strain DC2 (Bigelow, CCMP #1334; WH7803); henceforth, referred to as *Synechococcus*, was grown on artificial seawater (Berges et al., 2001), modified by adding 5 mM bicine (Healey and Hendzel, 1979), 124 µM $NH_4Cl$ instead of nitrate, and 13 µM $K_2HPO_4$, to ensure a low N:P ratio and N-limited growth. Cultures were maintained at 19°C and continuous light (42 µmol quanta $m^{-2}$ $s^{-1}$ photosynthetically active radiation). Experiments were started when cultures entered N limitation near the end of exponential growth, as determined by epifluorescence microscopy counts.

The gram-negative marine bacterium *Vibrio* sp. strain PWH3a (henceforth referred to as *Vibrio* PWH3a), also known as *Vibrio natriegens* strain PWH3a (Suttle and Chen, 1992; Weinbauer et al., 1997) and *Vibrio alginolyticus* strain PWH3a (Poorvin et al. 2011), was grown on artificial seawater with 5 mM bicine, 500 µM $NH_4Cl$, 100 µM $K_2HPO_4$, and 1 mM glucose as a carbon source for a C:N:P ratio of 60:5:1. The cultures were grown at 25°C and continuously mixed at 100 rpm. This bacterium was chosen as a model to produce lysates for the current study because it originated from a coastal marine source, and because it has an isolated lytic virus (PWH3a-P1). It is assumed that lysate from *Vibrio* PWH3a is a reasonable proxy for DOM produced by viral lysis of marine heterotrophic bacteria, and throughout the manuscript is referred to as bacterial lysate.

Bacteriophage PWH3a-P1 was added in eight-fold excess abundance (multiplicity of infection of 8:1) to cultures of *Vibrio* PWH3a at the end of exponential growth, as determined by absorbance at 660 nm (Ultrospec spectrophotometer, Biochrom, United Kingdom). The culture was incubated with the virus until absorbance decreased to 20% of the initial value (~7 h). The lysate was filtered through a 0.22-µm pore-size Durapore membrane (Millipore, Billerica, MA) and kept at 4°C for approximately 20 h. The number of cells lysed prior to filtration was determined by flow cytometry as described below (section 2.4.1). The amount of DON released was determined by the number of cells lysed multiplied by the measured cellular N quota for *Vibrio* PWH3a, 2.54 fmol cell$^{-1}$ as described below (section 2.4.4). The result is the amount of total N released by lysis of *Vibrio* PWH3a.

Lysates for field experiments were prepared as above, except that *Vibrio* PWH3a was grown on $^{15}NH_4Cl$ instead of $^{14}NH_4Cl$ (90+ atom % $^{15}N$, Isotec, Miamisburg, OH), and the filtered lysate was kept at 4°C for 2 to 5 d until the experiments were initiated. Excess ammonium in the lysate was not removed before adding to the experiments; however, the added ammonium was less than the increase in particulate organic N (PON) in every field experiment.

## 2.2 Growth of *Synechococcus* on lysate from *Vibrio* PWH3a

Water was collected from Queen Charlotte Sound (51$^o$ 02.37N, 127$^o$ 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 µM, respectively. The water was ultrafiltered using a 30 kDa molecular weight cut-off tangential flow cartridge (Prep/Scale, Millipore, Billerica, MA) after pre-filtration through a 0.45 µm pore-size polyvinylidene fluoride (PVDF) Durapore membrane (Millipore, Billerica, MA) as outlined in Suttle et al. (1991). The ultrafiltrate was stored in the dark at 4°C

for a year until used in experiments. Bacteria in the ultrafiltrate grew to a density of $4.18 \times 10^5$ cells $ml^{-1}$, as determined by flow cytometry (described in section 2.4.1), and were used for the remineralisation experiment described below. The bacteria in the ultrafiltrate were derived from the environment and persisted at low nutrient concentrations for an extended period, and hence were more representative of in situ communities than a monoculture. They were an essential component of the laboratory study, where they served as remineralisers.

The experiment combined *Synechococcus* (Syn), lysate from *Vibrio* PWH3a, and the bacterial assemblage in ultrafiltrate from Queen Charlotte Sound (Bac) in the following six combinations (Table 1): 1) Syn+Bac+lysate was the experimental treatment with DON from lysate; 2) Syn+Bac was a control for *Synechococcus* growth in the presence of the bacterial assemblage without a DON source from lysate; 3) Syn+lysate was a control for bacterial remineralisation in the non-axenic *Synechococcus* culture; 4) Bac+lysate was a control to quantify ammonium remineralisation by the bacterial assemblage with the addition of lysate; 5) Bac only was a control to determine the ammonium concentration of the bacterial assemblage by itself; 6) Syn only was a control to determine the ammonium concentration and increase in cell number of *Synechococcus* by itself. All treatments were in triplicate in 1 l polycarbonate Erlenmeyer flasks (Corning, New York). To each appropriate treatment was added 10 ml of *Synechococcus* culture, 100 ml of bacterial assemblage, and/or 10 ml of lysate. The experimental treatment volume was 200 ml, and volumes of control treatments were topped up to 200 ml by adding nitrate- and phosphate-free artificial seawater to control treatments.

Treatments were incubated in the laboratory at 19°C under continuous light (42 µmol quanta $m^{-2}$ $s^{-1}$ of photosynthetically active radiation) and sampled daily for cell abundance and ammonium concentration.

## 2.3 Uptake of $^{15}N$ from lysates in the field

Water was collected from the surface at Saanich Inlet (SI), the Fraser River Plume (FRP), Semiahoo Bay (SB) and Jericho Pier (JP) in southern coastal British Columbia (Table 1 Treatments in the laboratory experiment to examine increase in *Synechococcus* abundance (Syn), in the presence of bacterial lysate from *Vibrio* PWH3a (lysate) and an environmental bacterial assemblage (Bac).

| Treatment | Syn (ml) | Bac (ml) | Lysate (ml) | Media (ml) | Aim |
|---|---|---|---|---|---|
| Syn+Bac+lysate | 10 | 100 | 10 | 80 | Experimental treatment |
| Syn+Bac | 10 | 100 | - | 90 | Control for Syn growth with bacterial assemblage but not lysate |
| Syn+lysate | 10 | - | 10 | 180 | Control for remineralisation in non-axenic Syn culture |
| Bac+lysate | - | 100 | 10 | 90 | Control for remineralisation by bacterial assemblage |
| Bac only | - | 100 | - | 100 | Control for remineralisation in bacterial assemblage |
| Syn only | 10 | - | - | 190 | Control for Syn growth and remineralisation |

Table 2). SI and FRP samples were collected using Go-Flo bottles mounted on a rosette, which held a SBE 25 CTD (Sea-Bird, Bellevue, WA) for measuring temperature and salinity. SB and JP samples were collected by hand using a 20 l carboy rinsed with 10% HCl and ultrapure water, and temperature and salinity were measured with a hand-held thermometer and refractometer. Samples for phytoplankton identification were collected from the whole water and preserved with Lugol's acidic iodine solution (Edler and Elbrächter, 2010). The water was filtered through 118-µm mesh-size Nitex screening to remove large particulates.

For each experiment, 0.22-µm filtered lysate from *Vibrio* PWH3a was added to Nitex-filtered seawater (SW+lysate) and compared to a control containing 0.22-µm filtered seawater (SW). For SB and JP, a third treatment included 0.22-µm filtered seawater and 0.9 µM $^{15}NH_4Cl$ (SW+N), to confirm that N stimulated production and that another factor was not limiting phytoplankton growth, and to mirror the estimated N contributed by the lysate in the SW+lysate treatment. Treatments were in triplicate. SI and FRP experiments were done in an on-deck flow-through incubator with neutral density screening to reduce sunlight to approximate in situ irradiance. For SB and JP, experiments were done at 19°C and 42 µmol quanta $m^{-2}$ $s^{-1}$ continuous irradiance. Samples for cell abundance and ammonium concentration were collected every 6 to 9 h for 1 to 2 d; 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.

## 2.4 Sample Analysis

### 2.4.1 Cell and virus counts

One ml samples were fixed with a final concentration of 0.5% gluteraldehyde for bacteria and viruses, and 2% formaldehyde for phytoplankton, and flash frozen in liquid nitrogen and stored

at -80°C. Bacteria and viruses were stained with SYBR Green I (Sigma-Aldrich, St. Louis, MO), while picophytoplankton were left unstained, immediately before counting by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ) following the procedures of Brussaard (2004), Gasol and Del Giorgio (2000), and Olson et al. (1993). Larger phytoplankton were preserved with Lugol's solution, and the dominant taxa identified using a settling chamber (Edler and Elbrächter, 2010) and light microscope (Axiovert 10, Zeiss, Canada).

### 2.4.2 Nutrient and chlorophyll *a* collection and analysis

Samples of 25 ml were filtered through acid-rinsed syringes fitted with MilliQ-soaked 0.45-µm pore size, 25-mm diameter, cellulose-nitrate filters (Whatman, United Kingdom) in a Swinnex filter holder (Millipore, Billerica, MA). The first 15 ml were used to rinse the 15-ml acid-rinsed polypropylene screw-cap collection tubes (Sarstedt, Germany), before collecting and freezing the final 10 ml at -20°C for subsequent nutrient analysis. The filters were folded, placed in aluminum foil packages, and frozen desiccated at -20°C until chlorophyll *a* was extracted using 90% acetone and sonication, and analysed fluorometrically (Turner Designs, 10AU fluorometer, Sunnyvale, CA) following Parsons et al. (1984).

Nitrate+nitrite and phosphate concentrations were analyzed on a Bran & Luebbe AutoAnalyzer 3 using air-segmented continuous-flow analysis (Technicon, Oakland, CA), while ammonium concentrations were determined fluorometrically (Holmes et al., 1999) using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA).

### 2.4.3 $PO^{15}N$ size-fractionation, collection and determination of $^{15}N$ enrichment

For the field experiments, the $^{15}N$ incorporated was determined by collecting the particulate material onto combusted (450°C for 4 h) glass-fibre (GF) filters (nominal pore size of 0.7 µm, Whatman, United Kingdom), which were subsequently dried at 50°C for 2 d. Prior to collection onto the GF filters, the SI and FRP samples were poured into a Sterifil (Millipore, Germany) filtration tower fitted with a 1-µm pore-size polycarbonate filter (AMD Manufacturing, Canada). While the sample was being gently filtered, the phytoplankton were kept in suspension and rinsed, while the volume was maintained by adding 200 ml of ultrafiltrate. In this way, cells captured by the 1-µm filter were retained for analysis of isotopic enrichment, while smaller cells passed through. Samples for cell counts were taken before and after washing to determine the proportion of cells lost by this process. Samples that were not rinsed with ultrafiltrate were also filtered onto combusted GF filters to estimate the amount of $^{15}N$ uptake that was due to bacteria that passed through the 1-µm pore size membrane. These GF filters were not rinsed with ultrafiltrate after collecting the sample; hence, if there was noncellular $PO^{15}N$ in the <1-µm size fraction, uptake by the bacterial size fraction would be overestimated. However, there is no reason to expect significant noncellular $PO^{15}N$ in the <1-µm size fraction, and it is uptake by the >1-µm size fraction, which was washed, that is the focus of this study. The SW+N treatment (SB and JP) was filtered directly onto GF filters without rinsing. The $\delta$ $^{15}N$-PON and total 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‰). Due to instrument error, the stable-isotope data for SI and FRP are not replicated; whereas, duplicates for JP and triplicates for SB were measured.

### 2.4.4 Particulate C and N analysis

N cell quotas of *Synechococcus* and *Vibrio* PWH3a were determined from cultures grown using the same media and conditions as described in section 2.1. Cultures at the end of exponential phase for *Synechococcus* as determined by epifluorescence microscopy counts, and mid-exponential phase for *Vibrio* PWH3a as determined by absorbance, were filtered onto combusted GF filters, dried at 50°C for two days, and placed in a desiccator until packaged into aluminum foil and analyzed on a CHN Elemental Analyser (Carlo Erba NA-1500).

### 2.4.5 Calculations and statistical analysis

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. 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 *Synechococcus* abundances and ammonium concentrations were normally distributed and with equal variances according to Shapiro-Wilk and Levene's tests, respectively; hence, the significance of the results was analysed using one-way analysis of variance (ANOVA). The differences between initial and final time points for bacterial abundances 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 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+lysate treatment in SI was approximately six-fold lower than the previous time point, concurrent with a significant increase in viral abundance (data not shown), significance between the two treatments was calculated for the differences between the initial and the 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.

# 3 RESULTS

## 3.1 Response of N-limited *Synechococcus* to the addition of lysate

There was a greater increase in the abundance of N-limited *Synechococcus* cells exposed to lysate and a remineralising bacterial assemblage than when grown with each component separately; the greatest increase occurred in the treatment Syn+Bac+lysate (Figure 1A), with final abundances significantly different among all treatments ($p<0.05$). Ammonium decreased from ~1.0 µM to less than 0.2 µM except in the Bac+lysate treatment, in which it increased to

8.24 ± 0.04 µM (Figure 1B), significantly higher than in the other treatments ($p<0.05$). Bacterial abundance increased in all treatments, but the greatest increases occurred in treatments with lysate addition (Figure 1C). Bacterial abundance increased in the Syn+lysate treatment, because bacteria were present in the non-axenic *Synechococcus* and were remineralising (see Figure 1C Syn+lysate). The bacteria added with the ultrafiltrate (Bac) simply added additional "environmental" bacteria to the treatment and were associated with higher rates of ammonium regeneration.

The proportion of N released by lysis of *Vibrio* PWH3a that was taken up by *Synechococcus* cells was calculated using the N cell quotas for *Vibrio* PWH3a of 2.54 fmol $cell^{-1}$, and for *Synechococcus* of 7.83 fmol $cell^{-1}$. Based on the decrease in *Vibrio* PWH3a cells from viral infection, multiplied by the N cell quota of *Vibrio* PWH3a, approximately 36.0 µmol $l^{-1}$ N was added to each incubation from bacterial lysis (Table 3). The mean percent of N from lysate that was taken up by *Synechococcus* cells via remineralisation by bacteria was 74%.

## 3.2 Uptake of N from bacterial lysate by primary producers in field studies

Lysate from $^{15}$N-labeled *Vibrio* PWH3a cultures was added to seawater from Saanich Inlet (SI), the Fraser River Plume (FRP), Semiahoo Bay (SB), and Jericho Pier (JP; Table 1 Treatments in the laboratory experiment to examine increase in *Synechococcus* abundance (Syn), in the presence of bacterial lysate from *Vibrio* PWH3a (lysate) and an environmental bacterial assemblage (Bac).

| Treatment | Syn (ml) | Bac (ml) | Lysate (ml) | Media (ml) | Aim |
|---|---|---|---|---|---|
| Syn+Bac+lysate | 10 | 100 | 10 | 80 | Experimental treatment |

| | | | | | |
|---|---|---|---|---|---|
| Syn+Bac | 10 | 100 | - | 90 | Control for Syn growth with bacterial assemblage but not lysate |
| Syn+lysate | 10 | - | 10 | 180 | Control for remineralisation in non-axenic Syn culture |
| Bac+lysate | - | 100 | 10 | 90 | Control for remineralisation by bacterial assemblage |
| Bac only | - | 100 | - | 100 | Control for remineralisation in bacterial assemblage |
| Syn only | 10 | - | - | 190 | Control for Syn growth and remineralisation |

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Table 2). Changes were followed in ammonium concentrations and in abundances of bacteria (Figure 2). Bacterial abundances in SW+lysate treatments were significantly higher than in samples without lysate (SW) by the final time point for FRP, SB, and JP ($p<0.05$). In the SI sample, bacterial abundances in the treatment with lysate added (SW+lysate) increased almost tenfold before decreasing to below initial values by the final time point, concurrent with an increase in viral abundance (data not shown). There were no significant differences between experimental and control treatments for picophytoplankton abundances, or for chlorophyll *a* concentrations at the other stations (data not shown). Ammonium concentrations in SI, SB, and JP decreased to less than 0.2 µM. In FRP, ammonium concentrations in the SW+lysate treatment decreased to less than 0.04 µM by 19.75 h before both ammonium and bacterial abundance increased concurrently. There was significantly greater drawdown of ammonium in the SW+lysate treatment at every station except SB ($p<0.05$). The increase of bacteria and greater ammonium uptake at most stations indicates that bacterial growth and overall uptake of ammonium were enhanced in these samples by the addition of bacterial lysate.

There was uptake of lysate-derived N by cells >1 µm, which would be comprised primarily of phytoplankton. The addition of lysate to the SW+lysate treatment for each station contributed approximately 67.7 µmol $l^{-1}$ N for SI and FRP, and 0.44 µmol $l^{-1}$ N for SB and JP (Table 3). To more closely reflect ambient concentrations, the amount of lysate N added to SB and JP was greatly reduced compared to the two previous experiments. Stable isotope data from PON collected on 0.7-µm pore-size glass-fibre filters indicated uptake of lysate by the particulate fraction (Table 4; Figure 3). From the $^{15}N$ data, the calculated contribution to the total uptake by cells in the >1 µm size fraction was 46.3% (SI), 47.6% (FRP), and 100% (SB and JP). The large

(>50%) contribution of the bacterial size fraction to $^{15}N$ uptake in SI and FRP (Figure 3) corresponded to an increase in bacterial abundances (Figure 2).

There was uptake of $^{15}NH_4$ in the SW+N treatment, with 0.61 ± 0.20 µmol $l^{-1}$ N (SB) and 0.44 ± 0.26 µmol $l^{-1}$ N (JP) taken up into the particulate fraction (corrected for 90 atom % $^{15}N$, data not shown); therefore, ammonium produced by remineralisation of lysate in the SW+lysate treatment could be used by the microbes at these two stations.

# 4 DISCUSSION

Experiments in the laboratory and with natural systems demonstrate that N in viral lysates can be remineralised by bacterial communities to fuel primary production. Studies have shown that lysis by viruses produces bioavailable DOM (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 and Middelboe, 2009; Shelford et al., 2012; Weinbauer et al., 2011); yet, data are lacking on the mechanism involved in the transfer of N to primary producers from bacterial lysates produced by viral infection. This study shows that phytoplankton in culture and in environmental samples take up N from bacterial lysates, and that ammonium produced through bacterial remineralisation of DON enables that uptake.

## 4.1 Remineralisation of nitrogen in bacterial lysates

The increase in bacterial abundance in 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 ammonium remineralisation. In the laboratory, every treatment with added lysate had a greater increase of bacteria than treatments without lysate, indicating that the bacteria used the added lysate for growth. For all field studies, bacterial abundances increased significantly in the treatments with lysate over the controls ($p<0.05$; Figure 2), demonstrating uptake of lysis products by the bacterial communities.

Ammonium concentration increased eightfold in laboratory treatments with lysate added to bacteria (Bac+lysate; Figure 1B) 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* was present and consumed the ammonium that was produced (the remainder of the treatments). There was no measured ammonium production in the field studies except in the Fraser River Plume (FRP; Figure 2). The increase in ammonium at the final time point in FRP may be due to the concurrent rapid increase in bacteria, which likely resulted in increased ammonium remineralisation. There was some ammonium in the added lysate (see Figure 1B, T=0), which was present because the *Vibrio* PWH3a was in exponential growth to facilitate maximum lysis by PWH3a-P1, and so not all the ammonium in the medium was taken up by the bacteria prior to lysis. However, the amount that was added was minor relative to the ammonium that was produced by remineralisation. The data clearly show that lysates were used by the bacterial community for growth, and excess N remineralised to produce ammonium.

Although viruses (PWH3a-P1) were present in the bacterial lysate, it is unlikely that they would infect members of the bacterial community. *Vibrio* phages are typically species specific, and often strain specific (Comeau et al. 2005), and experiments have shown undetectable binding of PWH3a-P1 to natural populations of bacteria, even in the environment from which it was isolated (Hennes et al. 1995). Finally, even if PWH3a-P1 was able to bind to *Vibrio* spp., the estimated abundance of *Vibrio* spp. in BC coastal waters ranges from ~1 $ml^{-1}$ to several hundred $ml^{-1}$ (Comeau and Suttle 2007), and is not enough to significantly affect N release. Moreover, this would not affect the observation that N released by viral lysis is incorporated into cells.

## 4.2 Phytoplankton uptake of remineralised nitrogen

This study shows that remineralised N from viral lysis of bacteria can fuel the growth of primary producers. Observations of increased ammonium production in the presence of viral lysis (Haaber and Middelboe, 2009; Shelford et al., 2012), are extended by this study, which demonstrates that bacteria remineralise the organic N in lysates and produce ammonium, which is then taken up by primary producers. The use of a cultivated bacterium for lysate production limits generalisation of quantitative data to environmental systems; however, the observation that 74% of the N in bacterial lysates was taken up by cultures of *Synechococcus* provides strong evidence that N from lysates is available to phytoplankton.

DON from bacterial lysates was remineralised by the bacterial assemblage into ammonium and used to fuel primary production. In the laboratory experiment, the increase in ammonium in the Bac+lysate treatment did not occur in the treatment with *Synechococcus* (Syn+Bac+lysate; Figure 1B), indicating that the remineralised ammonium was taken up and led to the concurrent increase in *Synechococcus* cells (Figure 1A). The increase in *Synechococcus* in treatment

Syn+lysate may have resulted from remineralisation by contaminating bacteria (bacteria in the non-axenic *Synechococcus* culture, or from uninfected *Vibrio* PWH3a in the lysate), or possibly from direct DON uptake, although preliminary experiments indicated that direct uptake of DON was not significant.

All four field studies showed that $^{15}N$ from labeled bacterial lysate was taken up by the >1-µm fraction (Figure 3), which demonstrates that remineralised N in lysate supports primary production, as suggested previously (Shelford et al., 2012; Weinbauer et al., 2011). The reason that increases in phytoplankton or chlorophyll were not observed is likely because there are often significant lags between incorporation of ammonium and growth in N-limited phytoplankton (Collos, 1986, Davidson et al., 1992). As well, these rates represent minimum estimates, as $^{15}N$ released through rapid recycling of lysis products (Noble and Fuhrman, 1999) or phytoplankton exudation (Bronk and Ward, 2000) is not included. Uptake of $DO^{15}N$ concurrent with $^{15}NH_4$ could also have resulted in an underestimation of N uptake from remineralisation. The ability of phytoplankton to take up DON directly varies greatly, and is dependent on factors such as trophic status of the environment and the species of phytoplankton present (Berman and Bronk, 2003), but is generally less important than inorganic sources of N. Although the field experiments do not provide quantitative estimates of the amount of N in lysate that was taken up, the results corroborate those from the laboratory experiment and indicate that N uptake from lysates occurs. The anomalous result in the SB experiment that the estimated uptake (0.67 ± 0.02 µmol $l^{-1}$ N; corrected for 90 atom % $^{15}N$; Figure 3) was more than the calculated N added (0.44 µmol $l^{-1}$ N; Table 3) suggests that the N in the lysate was underestimated. This is plausible, as lysate N was calculated from the cellular N quota multiplied by the decline in abundance of *Vibrio* PWH3a, and cell count data were not replicated. Nonetheless, the data are convincing that

most of the lysate added in the SB and JP experiments was incorporated by primary producers, since at both stations 100% of the estimated $^{15}N$ uptake was into the >1 µm fraction. Interestingly, we did not find a relationship between ambient concentrations of nitrate, phosphate, or ammonium, and ammonium uptake despite evidence of an increase in uptake of lysis products with lower trophic status in previous studies (e.g. Noble and Fuhrman, 1999), although this could be due to all the stations in these studies being relatively productive.

Nonetheless, not all the N in lysates was taken up by primary producers. Although bacteria can remineralise lysate (Bac+lysate; Figure 1B), and *Synechococcus* can use remineralised N from co-occurring bacteria (Syn+lysate; Figure 1A), in the laboratory only 74% of the N in the lysate contributed to an increase in *Synechococcus* abundance. As well, in the FRP and SI samples, only about half of the $^{15}N$ uptake was into the >1 µm fraction. Discrepancies between N uptake and phytoplankton growth is likely because of the lag between ammonium uptake and growth that is observed in many N-limited phytoplankton (Collos, 1986), and because phytoplankton can take up substantially more N than they incorporate because of DON release (Bronk et al., 1994). As well, longer incubation times may have allowed the in situ bacterial communities to break down more complex DON in the lysate and regenerate additional ammonium that could be incorporated (Middelboe and Jørgensen, 2006), but would add the additional complication of secondary release and uptake of labelled DON. For SI and FRP, lysate N was added at relatively high concentrations (Table 3), which may have led to incomplete incorporation. In contrast, lysate N was added at much lower concentrations in the SB and JP experiments, and the $^{15}N$ in the lysate was completely recovered in the particulate fraction. Finally, the incomplete transfer of N from lysate to *Synechococcus* in the laboratory, relative to the complete uptake of $^{15}N$ at SB

and JP, may indicate that complete remineralisation of the DON depends on the make-up of the bacterial assemblage, or that N uptake of *Synechococcus* was saturated.

Laboratory experiments were conducted on *Synechococcus* cultures grown under a low N:P ratio and which were transitioning from exponential growth, to ensure N limitation but not starvation and potential cell death with release of DOM. The bacterial community (Bac) in these experiments was unlikely N-limited, and most likely C-limited, given nitrate in the medium and the production of ammonium in the Bac+lysate treatment. The experiment was designed to show ammonium production by the bacterial community (Bac+lysate), and its subsequent use by *Synechococcus* (Syn+Bac+lysate).

The increase in *Synechococcus* abundance in the laboratory experiment (Figure 1A) can be explained by the different sources of N present in the experimental treatment (Syn+Bac+lysate). The increase can be attributed as follows (calculated from the increase in cell number multiplied by cell quota): 1) 16.7 µmol $l^{-1}$ N from the 21.3 µM of nitrate in the Bac ultrafiltrate, 2) 9.2 µmol $l^{-1}$ N from remineralisation of ammonium by the added bacterial assemblage, and 3) 9.8 µmol $l^{-1}$ N from the uptake of ammonium from remineralisation by heterotrophic bacteria in the *Synechococcus* culture. Despite evidence for direct uptake of DON by phytoplankton (see Bronk et al. 2007 for a review), preliminary experiments showed no evidence for uptake of N from *Vibrio* PWH3a lysate by axenic *Synechococcus*. Despite the addition of N from other sources, the results clearly show that remineralization of N from the lysate contributed to the increase of *Synechococcus* in the Syn+Bac+lysate treatment.

## 4.3 Ecological implications and future directions

Some studies have focused on the influence of phytoplankton lysates on fueling bacterial production (Gobler et al., 1997; Haaber and Middelboe, 2009), which can be especially important in certain situations, such as bloom termination (Brussaard et al., 2005). In contrast, 10 to 20% of heterotrophic bacteria are estimated to be lysed every day by viruses (Suttle 1994), although it can vary widely (e.g. Wilhelm et al. 1998, 2002). With an estimated $3.6 \times 10^{28}$ prokaryotic cells in the upper 200 m of the ocean and an average carbon quota of $2.0 \times 10^{-14}$ g cell$^{-1}$ (Whitman et al., 1998), a bacterial C:N of approximately 5 (Goldman et al., 1987), and estimated loss rates of 10% d$^{-1}$ from viral lysis, approximately $1.44 \times 10^{13}$ g N d$^{-1}$ is released from viral lysis of bacteria in the photic zone. Primary production is estimated to be ~$1.35 \times 10^{14}$ g C d$^{-1}$ (Ducklow and Carlson, 1992), corresponding to ~$2.04 \times 10^{13}$ g N d$^{-1}$ assuming the Redfield ratio (106C:16N). Therefore, an estimated 71% of global primary production could be supported by N released by viral lysis of bacteria. While these estimates need to be verified, evidence suggests that viral lysis supplies a substantial portion of the N required for primary production, and emphasizes the importance of the viral shunt, especially in N-limited regions.

The use of N by phytoplankton from bacterial remineralisation of DOM originating from viral lysis of bacteria indicates that viruses are not simply C sinks that disrupt trophic levels (Azam and Worden, 2004), but are important facilitators in N recycling pathways. Traditional food chain models state that C and other nutrients flow from primary producers to higher trophic levels. The introduction of the microbial loop (Azam et al., 1983) included bacterial dynamics, whereby DOM 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. The viral shunt (Wilhelm and

Suttle, 1999) introduced viruses as a 'short-circuit,' removing particulate C from primary producers, consumers, and bacteria to the pool of organic matter. This model emphasises viruses as a loss mechanism of food web C; however, implicit in these models is that nutrients, as well as C, must also be released by viral lysis, and that N and P are likely recycled with greater efficiency than C (Suttle, 2007). 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 demonstrated in four separate field experiments that N is transferred from bacterial lysates to phytoplankton. Weinbauer et al. (2011) hypothesised that lysates either directly provided nutrients supporting *Synechococcus* growth, or that bacteria provided inorganic nutrients by remineralising the products of viral lysis. This study demonstrates that remobilization of nutrients by viral lysis of bacteria and subsequent remineralisation by uninfected bacteria can fuel the growth of primary producers.

## Author contributions

E.J.S. and C.A.S. designed the experiments. E.J.S. performed the experiments and analysed the samples except for the stable isotope data which was analysed by the Stable Isotope Facility at UC Davis, and the nitrogen cell content which was analysed by Maureen Soon at UBC. E.J.S. prepared the manuscript and analyzed the data with contributions and guidance from C.A.S.

## Competing Interests

The authors declare that they have no conflict of interest.

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Table 1 Treatments in the laboratory experiment to examine increase in *Synechococcus* abundance (Syn), in the presence of bacterial lysate from *Vibrio* PWH3a (lysate) and an environmental bacterial assemblage (Bac).

| Treatment | Syn (ml) | Bac (ml) | Lysate (ml) | Media (ml) | Aim |
|---|---|---|---|---|---|
| Syn+Bac+lysate | 10 | 100 | 10 | 80 | Experimental treatment |
| Syn+Bac | 10 | 100 | - | 90 | Control for Syn growth with bacterial assemblage but not lysate |
| Syn+lysate | 10 | - | 10 | 180 | Control for remineralisation in non-axenic Syn culture |
| Bac+lysate | - | 100 | 10 | 90 | Control for remineralisation by bacterial assemblage |
| Bac only | - | 100 | - | 100 | Control for remineralisation in bacterial assemblage |
| Syn only | 10 | - | - | 190 | Control for Syn growth and remineralisation |

Table 2 Locations and environmental parameters for field sampling stations: Saanich Inlet (SI), Fraser River Plume (FRP), Semiahoo Bay (SB), and Jericho Pier (JP). Environmental and biological conditions measured include temperature (Temp), salinity (Sal), nitrate ($NO_3^-$), phosphate ($PO_4^{3-}$), ammonium ($NH_4^+$), and the dominant phytoplankton.

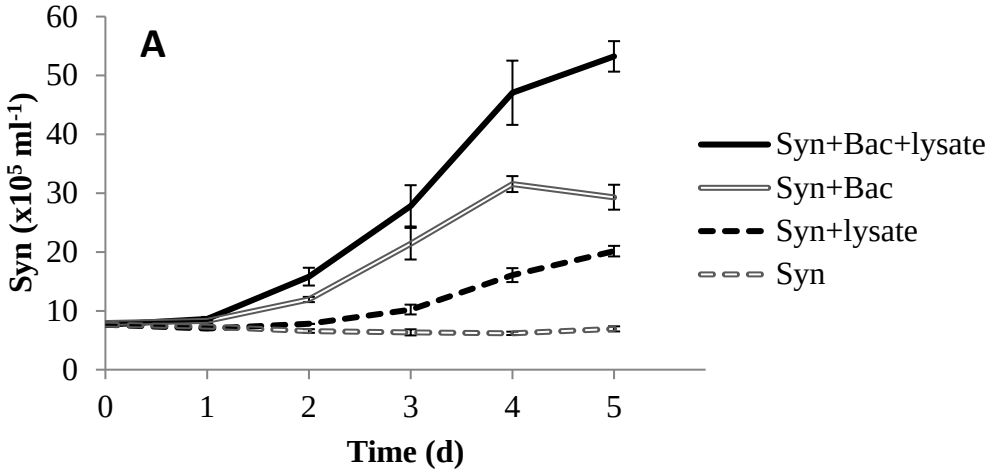
| Station | Location | Date | Temp (°C) | Sal | $NO_3^-$ (µM) | $PO_4^{2-}$ (µM) | $NH_4^+$ (µM) | Dominant phytoplankton |
|---|---|---|---|---|---|---|---|---|
| SI | 48.592°N, -123.505°W | 13 Sep 2012 | 14 | 27.9 | 1.24 | 0.75 | 0.54 | Mixed assemblage |
| FRP | 49.072°N, -123.402°W | 13 Sep 2012 | 11 | 28.0 | 10.9 | 1.12 | 0.07 | *Phaeocystis*, *Skeletonema*, *Leptocylindrus* |
| SB | 49.013°N, -123.037°W | 26 Aug 2013 | 20.2 | 29 | 0.00 | 2.40 | 0.08 | Cyanobacteria Unknown ciliate |
| JP | 49.277°N, -123.202°W | 27 Aug 2013 | 19.5 | 21 | 0.00 | 0.20 | 0.11 | Diatoms (mixed assemblage) |

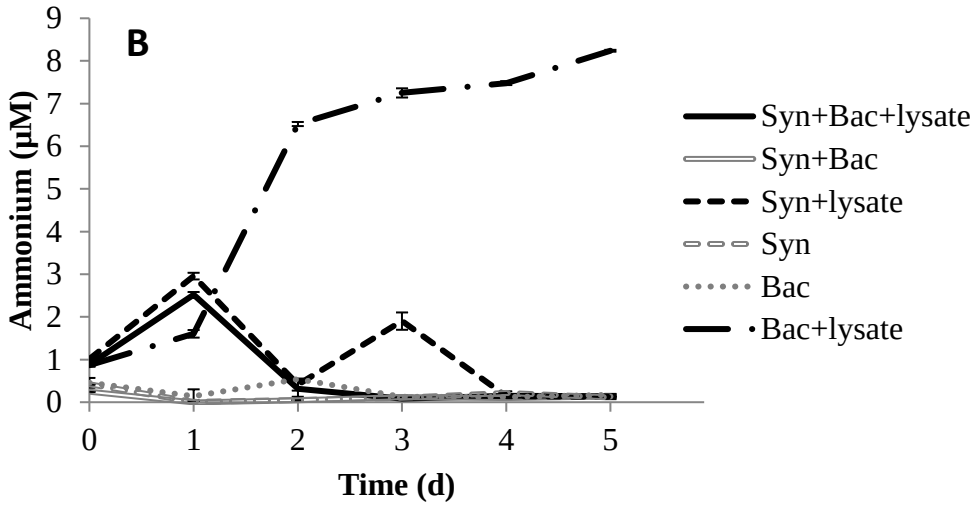
Table 3 *Vibrio* sp. strain PWH3a dynamics during the creation of lysates by viral infection (Laboratory = experiments with cultured *Synechococcus*, SI = Saanich Inlet, FRP = Fraser River Plume, SB = Semiahoo Bay, JP = Jericho Pier). N was added at lower concentrations for stations SB and JP to approximate in situ concentrations.

| | Laboratory | SI and FRP | SB and JP |
|---|---|---|---|
| *Vibrio* PWH3a abundance pre-virus (cell $l^{-1}$) | $3.96 \times 10^{11}$ | $7.87 \times 10^{11}$ | $7.89 \times 10^{10}$ |
| *Vibrio* PWH3a abundance post-virus incubation (cell $l^{-1}$) | $1.12 \times 10^{11}$ | $9.43 \times 10^{10}$ | $6.38 \times 10^{10}$ |
| Total N release (µmol $l^{-1}$) | 721 | 1760 | 38.4 |
| N addition to experimental incubations (µmol $l^{-1}$) | 36.0 | 67.7 | 0.44 |
| N addition to experimental incubations (µmol) | 7.21 | 33.8 | 0.31 |

Table 4 Uptake by the particulate fraction of $^{15}N$ from bacterial lysate from four 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 JP. SI and FRP are singleton measurements.

| | Total uptake of $^{15}N$ (µmol) | Total uptake of $^{15}N$ (µmol $l^{-1}$) | Uptake of $^{15}N$ normalised by chlorophyll *a* (µmol $µg^{-1}$) |
|---|---|---|---|
| Saanich Inlet (SI) | 0.83 | 1.67 | 0.27 |
| Fraser River Plume (FRP) | 0.88 | 1.76 | 0.70 |
| Semiahoo Bay (SB) | 0.42 ± 0.01 | 0.60 ± 0.02 | 0.70 ± 0.16 |
| Jericho Pier (JP) | 0.35 ± 0.04 | 0.50 ± 0.1 | 0.09 ± 0.00 |



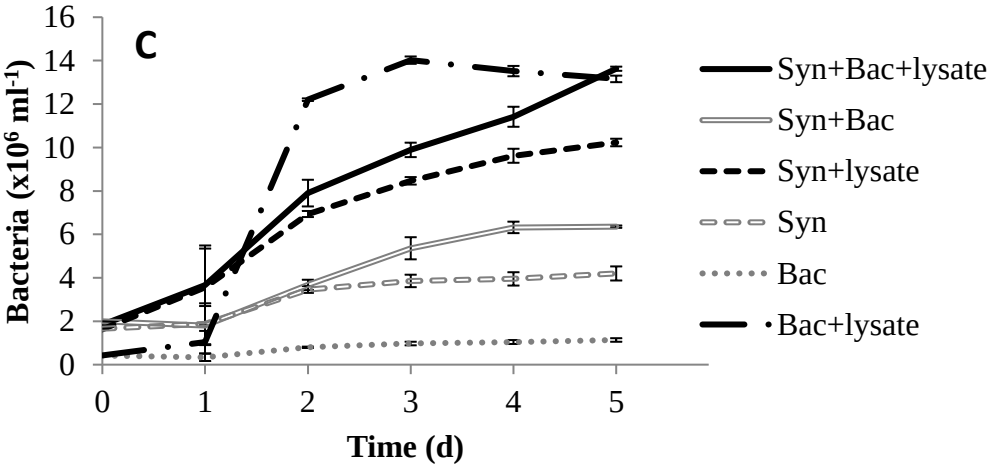




Figure 1 Time series of (A) *Synechococcus* abundance, (B) ammonium concentration, and (C) bacterial abundance in the laboratory experiment. Error bars are standard error from triplicate incubations.

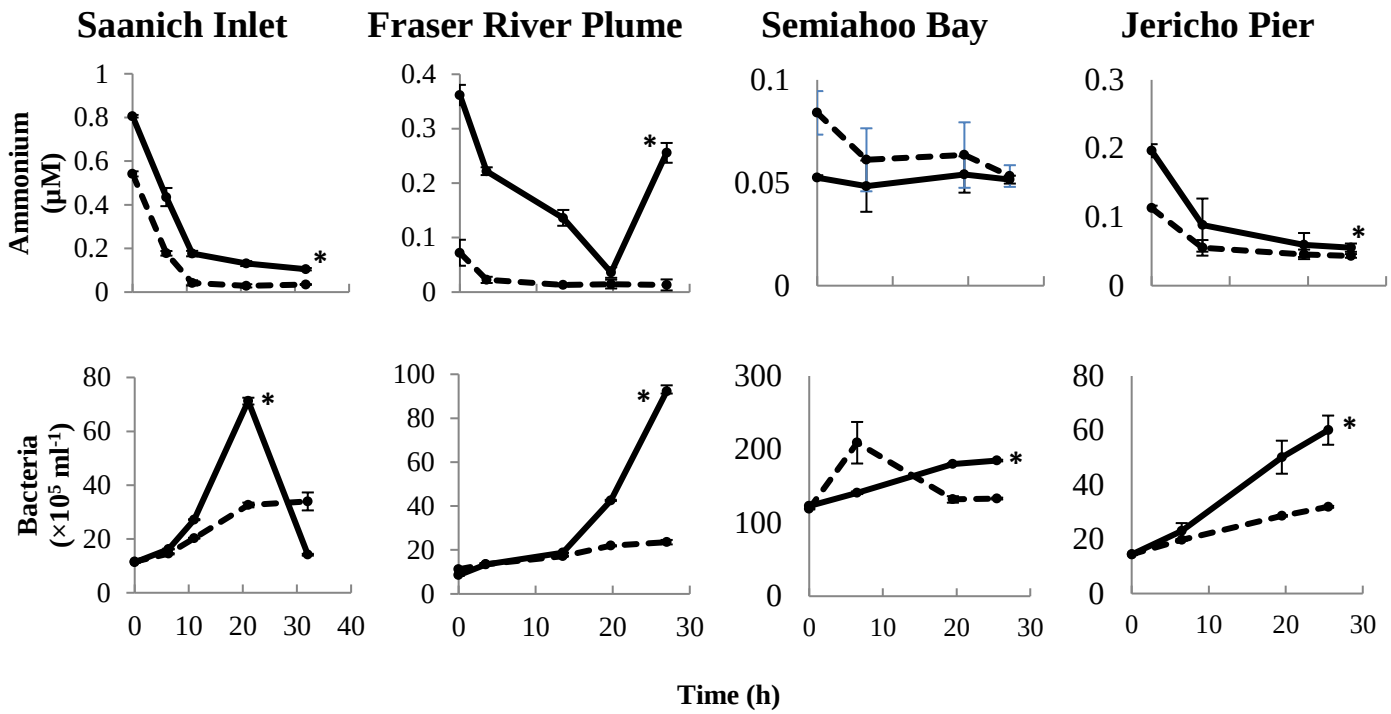



Figure 2 Time-course of ammonium concentrations and bacterial abundances in the field experiments. Solid and dashed lines indicate treatments with lysate addition (SW+lysate), or without (SW), respectively. Error bars are standard errors of triplicate incubations. Asterisks indicate significant differences between treatments. Note that the scales are different among plots.

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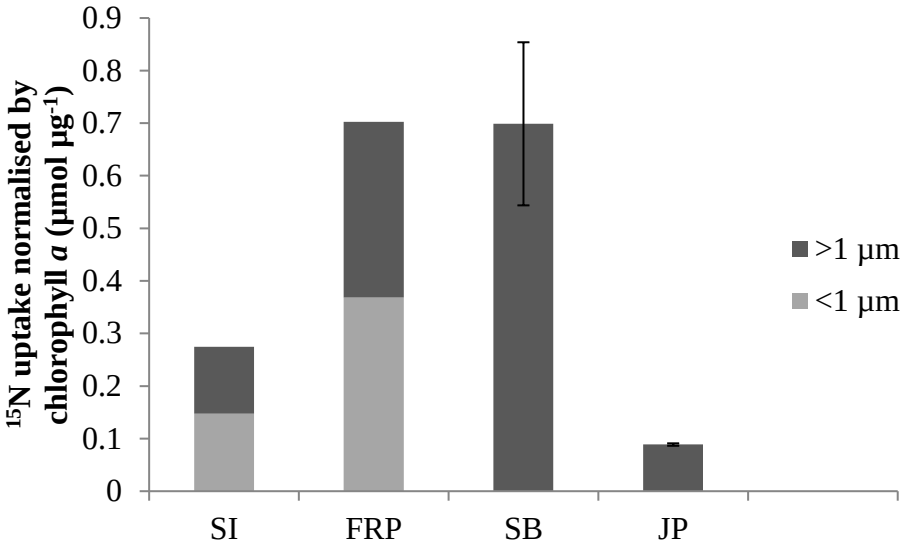


Figure 3 Uptake of $^{15}N$ normalised by initial chlorophyll *a* concentrations by the >1 µm and <1 µm fractions in treatments with lysate addition for Saanich Inlet (SI), Fraser River Plume (FRP), Semiahoo Bay (SB), and Jericho Pier (JP). Error bars are standard error of triplicate incubations for SB, and range of duplicate incubations for JP. SI and FRP are singleton measurements.