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# High frequency of virus-infected bacterial cells in a sheep grazed pasture soil in New Zealand

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ABSTRACT

Viruses cause significant mortality of microbes in aquatic environments and thus play a major role in biogeochemical cycling. While viruses are known to be present in soil, the rate of infection of bacteria, and therefore the potential for mortality and lysis, are unknown. In this study we measured the frequency of viral infected of bacterial cells in soils from a New Zealand sheep grazed pasture under ambient or elevated atmospheric carbon dioxide levels at the New Zealand Free Air Carbon Dioxide Enrichment facility (NZ-FACE). Averaged across the $CO_2$ treatments, the frequency of visibly infected cells (FVIC) was 23%. This is far greater than the reported FVIC values for marine or fresh water environments. The average FVIC of soils under ambient and elevated atmospheric $CO_2$ conditions was 28% and 18% respectively. These results are discussed in relation to nutrient cycling in grazed pastures.



## 1. Introduction

Viruses are the most abundant biological entities on earth, with genomes consisting of either single- or double-stranded DNA or RNA encapsulated in a protein coat (capsid) (Kimura et al., 2008). Viruses can only reproduce inside metabolically active host cells. Those that have bacteria as their hosts are known as bacteriophages (usually called phages). Viral ecology is the study of the interactions of viruses with host organisms and the environment. Studies on viral ecology in marine systems (Weinbauer, 2004; Suttle, 2005; Rohwer and Thurber, 2009) have shown that viruses have "the ability to manipulate the life histories and evolution of their hosts in remarkable ways" (Rohwer and Thurber, 2009). They are a major cause of microbial mortality, a driver of global biogeochemical cycles and a reservoir of enormous genetic diversity (Suttle, 2005).

At deeper sea layers nearly all of the heterotrophic production by prokaryotes is transformed into organic detritus by phages leading to a readily bio-available organic source for future growth by uninfected prokaryotes (Danovaro et al., 2008). This is the so-called 'viral shunt' which can promote the recycling of key elements such as nitrogen (N) and phosphorus (P) associated with prokaryotic biomass. Although the accuracy of various estimates of virus-mediated microbial mortality is a source of discussion (Binder, 1999; Weinbauer et al., 2002; Suttle, 2005), these estimates consistently indicate that viruses cause significant microbial mortality in a wide range of aquatic environments (see Table 6 in Weinbauer, 2004). In contrast, terrestrial environments remain inadequately explored with regard to this phenomenon and surprisingly this includes soil ecosystems (Kimura et al., 2008) where diverse and abundant prokaryotic organisms coexist. Yet the "soil and the rhizosphere's virosphere are essentially uncharted" (Buée et al., 2009).

Nutrient cycling in terrestrial ecosystems is strongly influenced by the 'microbial loop' (Coleman, 1994; Bonkowski, 2004) where nutrients that are temporarily held in microbial biomass are released through grazing or also natural life cycle growth stages by microfauna thus becoming available to plants. It is plausible that viruses could also act to release nutrients from soil bacteria just as they do in aquatic environments. In addition, viral infection could potentially modify bacterial abundance and diversity and facilitate horizontal gene transfer.

Grasslands grazed by animals generate numerous niches for soil microorganisms. There is a constant recycling of organic and inorganic matter through animal dung, urine, plant litter and decomposing organic matter (Whitehead, 1995). These conditions favour many heterotrophic and autotrophic prokaryotic species and therefore potentially high soil viral abundance.

A few data have been collected on viral presence in soils (e.g. Ashelfold et al., 2003; Williamson et al., 2005) but we are not aware

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of any data on phage infection rates in soils. In this study we investigated the frequency of virally infected bacterial cells obtained from a sheep grazed pasture under elevated and ambient $CO_2$ in a long running (12 year) Free Air Carbon Dioxide Enrichment (FACE) experiment.

## 2. Materials and methods

### 2.1. Site of soil sampling and soil properties

The NZ-FACE experiment is situated in the North Island of New Zealand (40°14′S, 175°16′E). Enrichment with $CO_2$ is achieved by releasing $CO_2$ mixed in air from a circular ring of pipes and allowing the enriched air to be carried across the ring by the wind. Our experiment consists of six rings (diameter 12 m); three enriched with $CO_2$ to a target concentration of 475 ppm and three control rings with ambient $CO_2$ concentrations. At the start of the experiment, rings were blocked based on initial botanical composition and soil characteristics. Enrichment is during the photoperiod year-round and has been in progress since October 1997. Unique among grassland FACE experiments, our pastures are grazed. Adult sheep (3–5) are penned within each ring (so nutrients are returned to the site of their removal) for periods of 3–5 d under a protocol described in Newton et al. (2006). The pasture is a permanent sward that has not been resown for at least 50 years. The pasture is botanically diverse with legumes, forbs, C3 and C4 grasses (see Newton et al., 2006 for details).

The soil at the research site is a Pukepuke black sand (Mollic Psammaquent) with a 0.25-m black loamy fine-sand top horizon underlain by grayish-brown, fine sand-textured horizons (Cowie and Hall, 1965). At the time of sampling (see below), the soil of the ambient and elevated $CO_2$ plots had a pH of 5.9 and 6.0, a total C of 45.2 and 50.2 g $kg^{-1}$ soil and a total N of 3.7 and 4.1 g $kg^{-1}$ soil for, respectively. No nitrogen fertilizer has been added since the start of the experiment; de novo additions of N coming from N fixation by legumes (Newton et al., 2006).

Soil samples were collected during an active plant growth period (average pasture growth rates of 20 kg DM $ha^{-1}$ $d^{-1}$) between two grazing events in autumn (March) 2009. Soil was sampled for several experiments and thus about 280 soil cores (7 cm diameter; 0–10 cm depth) were sampled from each ring. The soil cores from within each ring were mixed thoroughly, sieved (4 mm) and stored at 4 °C. For this study we took two 200 g from the mixed soil of each ring giving 12 samples in total.

### 2.2. Observation of viral-infected bacterial cells

The most common approach for inferring virus-mediated bacterial mortality is extrapolating the frequency of visibly infected cells (FVIC). In this study we modified the protocol of Nakayama et al. (2009) who estimated FVIC by transmission electron microscope (TEM) for floodwater samples from Japanese rice paddies.

Two 0.5 g sample of soil from each $CO_2$ ring were weighed into polypropylene 40 ml centrifuge tubes and 100 μl HF (50% v/v) and 20 ml 3 N HCl were added. The tubes were slowly shaken by hand to mix the soil with the solution and kept at room temperature for 2 d. The tubes were then vortexed for 10 s and centrifuged at 14 000 g at 4 °C. The supernatant was decanted and 20 ml of TE buffer (10 mmol $l^{-1}$ Tris, 1 mmol $l^{-1}$ EDTA pH 7.5) was added before further vortexing and centrifuging. This step was repeated twice and supernatant decanted. Then 5 ml of TE buffer was added, vortexed and then filtered through a 5 μm Millex syringe driven filter unit. The filtrate was then stored at 4 °C. The filtrate was used to make a serial dilution with sterilized milliQ water up to $10^3$. Bacterial cells in 1 ml of $10^3$ dilution solution were collected onto formvar-coated 400 mesh Cu grids by ultracentrifugation at 53 000 g for 15 min (Beckman TL-100 tabletop ultracentrifuge, Beckman Coulter K.K, Tokyo). Each grid was stained for 3 min with 1% uranyl acetate and washed for 10 s with milliQ water. Air dried Cu grids were examined for viral infection with a TEM (H-7500AMT Advantage HR, Hitachi High Technologies Corp., Tokyo, Japan) by randomly taking 50 photographs of individual bacterial cells from each sample. The TEM was operated at an accelerating voltage of 100 kV at magnifications between ×40 000 and ×80 000. Virus-infected bacterial cells were identified as containing virus-like particles. They were darkly stained, had a regular shape (e.g. circular) and were approximately 50–100 nm in size (Nakayama et al., 2009).

To facilitate our identification of virus-like particles in bacterial cells, we conducted a separate study inoculating a specific phage isolated from the floodwater of a Japanese paddy field into *Bacillus pseudomycoides* cells and observing them by TEM with the same procedures described above. We used these observations as our reference for identification of virus-like particles in bacterial cells extracted from the soil.

## 3. Results

In total, 600 photographs of bacterial cells were taken. We observed rod shaped (Fig. 1A), spherical shaped (Fig. 1B) and spiral shaped (Fig. 1C) bacterial cells. The majority of the cells were rod shaped. The filtration of soil suspension by a 5 μm filter might have removed most spherical-shaped bacterial cells. Most of the rod shaped cells observed had a flagellum (Fig. 1A).

Most of the virus-infected cells observed in this study carried several virus-like particles of different sizes (Fig. 3A, D and E), indicating simultaneous infection by phages that were either variable in their size or were different types. To distinguish between these two possibilities we measured the size of a *Bacillus* phage in its *Bacillus* host and found the size of the dark particles had a standard deviation of less than 15% (data not shown); whereas the size ranges we found in Fig. 3 were 55–66 nm (Fig. 3A), 87–130 nm (Fig. 3 D) and 37.5–50 nm (Fig. 3E). Consequently we expect that we are observing different types of phage in our bacterial cells.

The cells inoculated with a specific phage of *B. pseudomycoides* had a high frequency of cells with darkly stained spherical particles (Fig. 2). These had a similar appearance and size to previously described virus-like particles within bacterial cells (Steward et al., 1996; Säwström et al., 2007).

Examples of bacterial cells without virus-like particles and with virus-like particles are illustrated in Figs. 1 and 3, respectively. The dark phage-like particles ranged from approximately 50–100 nm in diameter. There were 140 virus-infected cells from the total of 600 bacterial cells observed giving a value of 23% FVIC. The range of FVIC of the twelve soil samples was from 12% to 48%. The average FVIC of soils under ambient atmospheric $CO_2$ and elevated $CO_2$ was 28% and 18% (Fig. 4). This difference was statistically significant at the 90% level ($p = 0.1$) when compared by $t$-test.

The number of virus-like particles in each bacterial cell ranged from 2 to 13 with most of the infected cells having 4–7 particles (Fig. 5).

## 4. Discussion

Dark particles with a range in size from 50 to 100 nm in bacterial cells were regarded as phage-particles in this study, because most phages with isometric capsids have a size in the 50–70 nm range (Ackermann and DuBow, 1987). Some bacteria are known to have particles of other origins in the cells such as polyhydroxyalkanoate (PHA) particles and glycogen granules in anammox bacteria. However, PHA particles are stained white with uranyl acetate (Maehara et al., 1999), and the size of glycogen granules is less than