SOIL BACTERIAL AND VIRAL DYNAMICS

by

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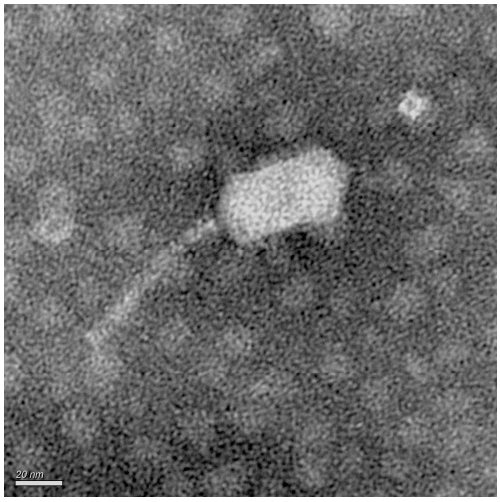

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF FORMULA

LIST OF PHOTOGRAPHS

ABSTRACT

Viruses have been shown to be responsible for considerable bacterial mortality and nutrient cycling in aquatic systems. As yet no detailed studies have been published on the role of viruses in natural soil bacterial communities despite common knowledge that viruses exist in the soil. This thesis sought to address some key questions on the ecology of soil bacterial viruses and their hosts. Disturbance through soil desiccation, nutrient inputs, rhizosphere effects and protozoan predation pressure were investigated. The first study of lysogeny in natural soil systems was also undertaken.

The work presented here utilised tools and techniques commonplace in aquatic systems research and applied them to soil. A novel protocol was developed based on physical extraction of bacteria and viruses from soil and direct counting with epifluorescence microscopy. Physical extraction was achieved using shaking, ultrasound sonication and low speed centrifugation. The fluorochrome SYBR Gold was used to stain nucleic acid of extracted bacteria and viruses, and image analysis software used to determine bacterial cell volumes.

Bacterial and viral abundances were in the region of 10^7-10^9 per gram of soil over a range of soil types. Significant fluctuations in viral and bacterial abundances were recorded at timescales of less than 24 h. Glucose and nitrogen addition led to substantial increases in bacterial and viral abundance. Loss of soil moisture resulted in peaks of viral abundance in sandy soils but not in a clay soil. A six-week microcosm study demonstrated that phage were not a significant regulator of bacterial abundance. Low levels of lysogeny were recorded over a range of soils when measured explicitly with Mitomycin C. The implication from that study was that viruses in soil behave differently to those in aquatic systems.

Bacterial and viral abundances were highly coupled in most instances, irrespective of the potential activity of bacteria. Further fundamental studies are recommended.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1 BACKGROUND TO BACTERIOPHAGE

1.1.1. What is a Virus?

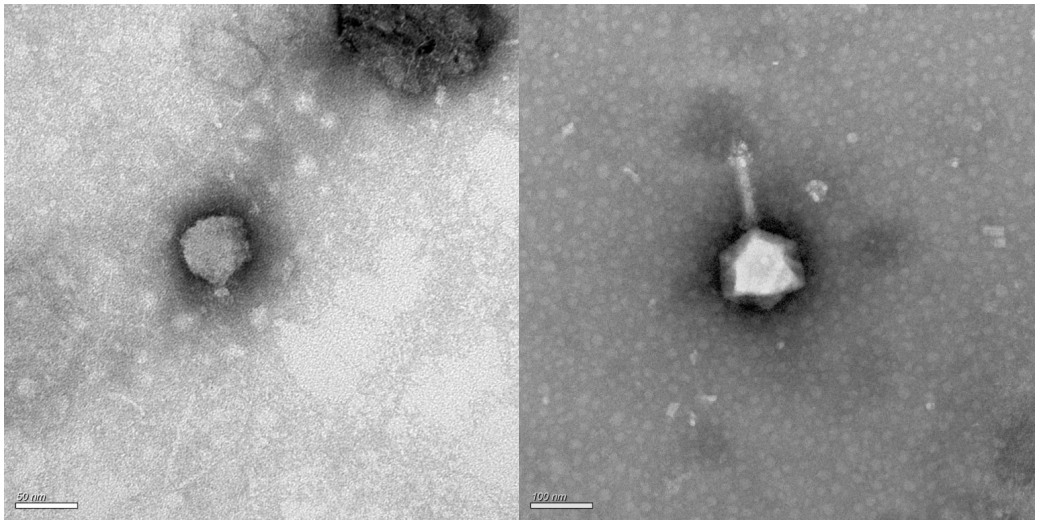
"any number of numerous kinds of very simple organisms smaller than bacteria, mainly of nucleic acid in protein coat, multiplying only in living cells and often able to cause disease"

(Concise Oxford dictionary)

There is debate as to whether viruses are living or non-living. They contain nucleic acids, an essential component of living organisms, as single or double stranded forms of RNA or DNA. The genome is surrounded by a protein coat, which may be covered by a lipid layer. They have no self-replicating mechanism and so rely on parasitising living host cells to replicate. Consequently they lack the ability of all living organisms in possessing the ability to reproduce and instead must make their host reproduce more viruses. Viruses may have a broad host range (Jensen *et al.* 1998) so any bacterium may be potentially infected by a number of different viruses. For example, data from one culture study on 50 *Bacillus subtilis* strains showed that nearly 50% were susceptible to more than 5 phage types (Tan and Reanney 1976). Resistance to viral infection can be developed through changes in cell membrane structure or internally within the organism's genome or cellular composition (Wagner and Hewlett 1999).

The smallest viruses at 17 nm are satellite plant viruses, whilst the tobacco mosaic virus at 300 nm is larger than some bacteria (McKenna and Faulkner 2001). Viruses that infect bacteria are technically referred

to as bacteriophage, ‘eaters of bacteria’, although the term ‘virus’ is common in general literature. Bacteriophage (‘phage’) can be classified into two groups based on their morphology; tailed and un-tailed (Photograph 1.1). Both groups typically have a head of about 100 nm in diameter, and tails can be up to 800 nm or more (Maniloff, Ackermann, and Jarvis 1999). Both groups display a variety of replication systems.



Photograph 1.1: Phage from Rothamsted Highfield Grassland soil. TEM image using the negative stain phosphotungstic acid. EM grids and pictures made by staff at the Centre for Bioimaging, Rothamsted. Copyright Rothamsted Research. Detail on sample preparation is outlined in Chapter 2.

1.1.2. Replication of Bacteriophage

There are six stages to successful replication of a phage when it encounters a host: adsorption to the bacterium cell surface, penetration and injection of the DNA, transcription of phage DNA, replication of phage DNA, production of new phage, and release of mature phage (Freifelder 1978). Phage do not all replicate in the same manner as some do not transcribe their DNA, but instead form a prophage associated with the bacterial genome, in effect becoming part of the bacterium. The prophage is reproduced in the same manner as the bacterial DNA when the bacterium divides, and phage may not be

released for several bacterial generations. The replication cycles are known as the lytic and lysogenic pathways.

1.1.2.1. Lytic pathway

The lytic pathway is initiated after a few hours denoted as the latent period. The latent period is determined in part by the phage itself, and in part by the generation time of the bacterium (Abedon, Herschler, and Stopar 2001), which differs between species and in relation to environmental factors. When mature, the new phage lyse the cell and are released, along with cell lysate, into the surroundings. The number of phage produced within the cell is referred to as the burst size, which can vary from less than 10 to over 300 (Granoff and Webster 1999). Bacteriophage infecting the same host may yield different burst sizes, for instance a wild type phage for *Escherichia coli* had a burst size 30% greater than a variant phage (Abedon, Hyman, and Thomas 2003).

Burst size as a component of the phage-host system is also influenced by the metabolic state of the host cell. The direct effect of resource limitation on host activity has been shown to significantly reduce the burst sizes of lysing phage. In one study starved *Pseudomonas aeruginosa* cells had a mean burst size of 11 compared with 211 during exponential growth (Schrader *et al.* 1997). An indirect effect on host activity has been shown by varying resource type; *E. coli* cells grown on tryptone agar had burst sizes three times greater than cells grown with glycerol, and nearly twice that when glucose was the carbon source (Howes 1965). An increase in incubation temperature from 12°C to 27°C was shown to increase burst sizes by up to 200 times

(Williams, Mortimer, and Manchester 1987). Thus genetic and environmental factors influence burst size in a number of ways.

1.1.2.2. Lysogeny

The second main cycle is the lysogenic pathway, where the viral genome integrates to a greater or lesser extent with the bacterial genome as a prophage. In this way phage DNA will replicate only once each time the bacterium divides; a much slower process than the lytic pathway, but one which has survival value, particularly in nutrient-poor conditions (Stewart and Levin 1984). These bacteria are referred to as lysogens, and they may have different attributes to bacteria not similarly infected, such as increased fitness (Bossi *et al.* 2003; Brüssow, Canchaya, and Hardt 2004). In extreme cases phage tail genes have been utilised for production of adhesion proteins by the bacterium (Brüssow, Canchaya, and Hardt 2004), or encode for toxin production in *Corynebacterium diphtheriae* (Fuchs 1998) and *Escherichia coli* (Teel *et al.* 2002). Prophage also may have negative effects, such as increasing autolysis incidence in *Streptococcus thermophilus* strains (Husson-Kao *et al.* 2000). Trigger events may cause the prophage to disassociate and enter the lytic cycle (Echols 1972). This is termed induction. Natural induction results from exposure to ultra-violet light, chemicals in the environment, or other stress events. Lysogens can be artificially induced, with the result that phage are released. Artificial induction is commonly carried out using mitomycin C or UV light.

1.1.2.3. Intermediates

The distinction between the lytic and lysogenic pathways is not clear-cut, and various states between the two conditions have been documented (Wagner and Hewlett 1999). One modification is chronic infection, where phage bud from the bacterium over time, this is a delayed and non-lethal lytic system. The other is pseudolysogeny. Pseudolysogeny is a scenario whereby phage enter into an unstable equilibrium within the bacterial cell, and does not form prophage (true lysogeny) nor lyses the cell. It was first described in soil bacteria in 1961 (Romig and Brodetsky 1961). The condition has been described as being a strategy for low host activity, where the host's metabolic processes are not adequate for phage replication. Phage using this strategy can survive within a starving bacterial cell without compromising their own survival (Miller 2001).

1.1.2.4. Multiple infection

Cells can be infected simultaneously with different phage, resulting in either lysis releasing both, releasing just one or no lysis (Adams 1959). Given that phage nucleic acid sequences can occupy different sites on bacterial DNA this increases the likelihood of lysogeny. Lysogeny can arise from infection by an active phage; one fraction of the bacterial population will lyse, and release more phage, the other fraction will survive and give rise to more lysogens. The benefit of lysogeny is that bacteria can acquire immunity to homologous infective phage.

1.2 EFFECTS OF BACTERIOPHAGE ACTIVITY ON BACTERIAL COMMUNITIES

The dominance of individual bacteria is likely to be held in check by phage (Thingstad and Lignell 1997; Fuhrman 1999). Release of large numbers of phage into an ecosystem may lyse all susceptible cells, but equilibrium must exist between phage and bacteria, given that host populations are not universally driven to extinction. Contributory factors against extinction include spatial refuge, development of host resistance, population densities of both host and phage and broad host range on the part of the phage (Alexander 1981). Resistant bacteria that arise by mutation may occur independently of phage action (Luria and Delbrück 1943) or mutations may be driven by interaction with phage (Lenski 1984). In either case, defence mechanisms continually evolve, driving genetic diversity and resilience in the environment.

Errors in phage replication will transfer parts of bacterial genomes to other bacteria on subsequent infection. This horizontal gene transfer mediated by phage, known as transduction, is believed to be commonplace (Paul and Jiang 2001; Canchaya *et al.* 2003; Brüssow, Canchaya, and Hardt 2004) and may in part explain prokaryotic diversity (Curtis, Sloan, and Scannell 2002; Papke and Doolittle 2003). An experiment on marine phage demonstrated the capability of transferring antibiotic-resistant plasmids between bacteria (Jiang and Paul 1998a). Lysogenic bacteria can be more resilient under nutrient limitations than their uninfected counterparts (Brüssow, Canchaya, and Hardt 2004), so this would increase the propensity of phage to occur as prophage in the natural environment.

Single strain studies with *Pseudomonas fluorescens* have shown a clear link between viral parasitism and host evolution (Buckling and Rainey 2002a). These authors demonstrated increases in host resistance to phage and increases in phage infectivity over 400 bacterial generations. Phage were always able to infect the ancestral bacterial strain whilst 'evolved' bacteria were always resistant to the ancestral phage. In a separate study populations of bacteria were propagated with and without virulent phage (Buckling and Rainey 2002b). Diversity indices (based on colony morphology) indicated that sympatric diversity decreased and allopatric diversity increased when bacteria were incubated with phage: niche specialism was possible in the absence of phage as host density was not restricted, whilst in the presence of phage host resistance dictated divergence from the original community.

Introduced *Pseudomonas fluorescens* populations can be decimated by natural virulent phage in the soil (Keel *et al.* 2002). This is presumably as laboratory-propagated strains have not evolved suitable defence mechanisms. Reviews detailing reduced survival of bacteria introduced into soil have not included viral activity as a source of mortality (Mawdsley *et al.* 1995; van Veen, van Overbeek, and van Elsas 1997) despite the finding that many soil phage have a broad host range (Hegazi and Jensen 1973; Reanney and Teh 1976). Host range may be interspecific, for example phage capable of lysing both *Pseudomonas aeruginosa* and *Escherichia coli*, or *E. coli* and *Sphareotilus natans* have been isolated from ponds and sewage treatment plants (Jensen *et al.* 1998).

1.2.1. Viruses in the Aquatic Environment

Viruses can be a significant component in aquatic systems, where they can impose a significant impact. For example addition of virus-enriched water to sea water reduced primary productivity by up to 78% (Suttle, Chan, and Cottrell 1990), and decreased bacterial abundance by up to 60% (Hewson *et al.* 2001b). In high nutrient systems the significance of viral lysis is increased (Weinbauer, Fuks, and Peduzzi 1993). Abundances of viruses in excess of 10^8 per millilitre have been recorded from freshwater (Bergh *et al.* 1989). They are also present in marine (Danovaro and Serresi 2000) and freshwater (Ricciardi-Rigault, Bird, and Prairie 2000) sediments. Viruses infecting eukaryotic algae are abundant (van Etten, Lane, and Meints 1991) and cyanophage have been enumerated and isolated in oligotrophic (Sullivan, Waterbury, and Chisholm 2003) coastal (McDaniel and Paul 2005) and paddy soil (Singh 1973) environments. Viruses are present in the Arctic (Borriss *et al.* 2003) and abundances of 10^6-10^8 per millilitre have been found in Antarctic lakes (Laybourn-Parry, Hofer, and Sommaruga 2001; Madan, Marshall, and Laybourn-Parry 2005).

Virus to bacteria ratios (VBR) across a range of aquatic environments show that viral abundance is generally higher than bacterial abundance (Weinbauer 2004). VBR in eutrophic environments are at the higher end of the range 3–10 found over numerous studies (Wommack and Colwell 2000), however VBR as low as 0.1 have been recorded in marine sediments (Danovaro, Manini, and Dell'Anno 2002) and as high as 50 in an Antarctic saline lake (Laybourn-Parry, Hofer, and Sommaruga 2001). VBR may not be sufficient to indicate productivity.

The presence of bacteriophage was first established in the marine environment using transmission electron microscopy in 1979 (Torrella and Morita 1979), since then other studies have captured images of marine and freshwater bacteria infected with phage (Proctor and Fuhrman 1990; Steward, Smith, and Azam 1996; Weinbauer and Höfle 1998b).

Using the frequency of visibly infected bacterial cells through electron microscopy, the percentage of virus-mediated/induced mortality ranges from 2-40% over a range of geographical locations (Wommack and Colwell 2000). In the northern Baffin Bay 6-28% of bacterial production was assumed to be lost due to viral lysis (Middelboe, Nielsen, and Bjørnsen 2002), and in samples from a eutrophic freshwater lake between 8% and 97% of bacterial production was removed by viral lysis (Weinbauer and Höfle 1998a). Batch cultures of Danish coastal water samples showed significantly greater bacterial abundance in reduced-virus water (0.02 μm filtered) than the control (Middelboe and Lyck 2002).

Non-infected bacterioplankton can benefit from the action of viral lysis because of the release of carbon providing greater substrate availability and uptake (Middelboe, Jørgensen, and Kroer 1996). Studies with fluorescently labelled viruses showed the abundance of an individual host strain bacterium was markedly reduced although overall bacterial abundance remained unchanged (Hennes, Suttle, and Chan 1995). Hence viral action appears to modify bacterial community structure.

When bacteria are lysed by phage they are no longer available for consumption by Protozoa; thus viral activity has implications on food-web structure (Wilhelm and Suttle 1999). The scenario is further complicated by the findings that nanoflagellates may consume viruses (González and Suttle 1993). One study using fluorescently labelled viruses indicated that viruses may contribute from 0.2-9% of the carbon and 0.6-28% of the phosphorus requirements that bacteria supply (González and Suttle 1993). This equated to an ingestion rate of approximately 3 viruses per flagellate per hour, supported in findings elsewhere, where based on viral decay rates through filtered water nanoflagellates were assumed to each consume on average 3.3 viruses (Suttle and Chen 1992). The applicability of these culture studies to the natural environment however is uncertain.

Viral lysis and protozoan grazing are two significant causes of bacterial mortality in aquatic environments. A seawater mesocosm study demonstrated that viral lysis and grazing contributed equally to bacterial death (Fuhrman and Noble 1995), whilst bacterial mortality due to viral lysis was greater than that by protozoan grazing in three out of four marine stations sampled from several locations off the Alaskan coast (Steward, Smith, and Azam 1996). The extent of viral lysis also varies within stratified lakes. In the epilimnion of lake Plußsee, Germany, protozoan grazing was dominant whereas in the hypolimnion viral lysis was dominant (Weinbauer and Höfle 1998a). Moreover viral activity influenced cell size in that when viral activity was highest there were significantly greater numbers of bacteria <0.3 μm (Weinbauer and Höfle 1998b). Thus in aquatic environments there is clear evidence that

viruses play a role in influencing bacterial production and biogeochemical cycling (Fuhrman 1999; Wilhelm and Suttle 1999; Wommack and Colwell 2000).

If viruses do not find a host cell they decay over time. Viral decay in marine systems calculated by dividing viral loss by the production rate gives ranges from 10% to over 80% per day (Wommack and Colwell 2000), with sunlight, particulate and dissolved matter being the main factors (Suttle and Chen 1992; Noble and Fuhrman 1997). The proportion of bacterial mortality due to viruses will be great in environments where viral abundance is maintained despite rapid decay (Jiang and Paul 1994).

1.2.2. Viruses in Soil Systems

"Bacterial viruses are produced by indigenous soil bacteria and may have a role in the ecology of soil micro-organisms".

(Farrah and Bitton 1990)

Given the significance of bacteriophage in aquatic systems, it is likely that they may play a role in soil, where bacteria are abundant. Bacteriophage are expected to be the most abundant members of the soil viral community, and are perhaps "the most numerous genetic object in the soil habitat" (Williams, Mortimer, and Parry 1999). The soil as a 'bank' for animal, plant, protozoan and fungal viruses should not be overlooked, but it is not clear how large an impact they will have. Most protozoan viruses are entirely persistent within their hosts, only replicating when the host undergoes cell division or reproduces (Bruenn 2000).

Soil is a highly complex three-dimensional environment that poses considerable problems when attempting to quantify its microorganisms. Soil structure is heterogeneous, comprising microaggregates less than 20 μm up to macroaggregates greater than 250 μm (Tisdall and Oades 1982). Soil organisms are typically adsorbed to the surface of particles, and the greater proportion of bacteria are located within aggregates and not on outer surfaces (Hattori 1973). At the millimetre scale numerous environments may be present (Figure 1.1). Soil aggregates are clumped together and structured in such a way as to leave voids (pores) between and within them. Pores will retain water to different degrees depending on their size: as water naturally drains down from the topsoil by gravity, pores with large apertures will drain quicker than pores with small ones (Childs 1969). Bacteria reside within thin water films surrounding particles and also stick to clay and other particles. This adhesion may be via polysaccharide exudates or by electrostatic forces. Different bacteria will adhere in different ways depending on the nature of their cell wall, exudates and appendages (Theng and Orchard 1995). The majority of bacteria are adsorbed onto particle surfaces and are unable to move any great distance (Stotzky 1986).

Channels within the greater soil structure facilitate the downward movement of water away from the topsoil. These channels are caused by diverse influences such as the climate, soil use and management practice, plant roots or earthworms (Juma 1993). The two factors of aggregation and channels determine the mass flow of water through the soil and hence the flow of free microbes and viruses. Gas diffusion is

another contributory factor in microbial activity: saturated pores can quickly become anaerobic (Sierra, Renault, and Valles 1995).

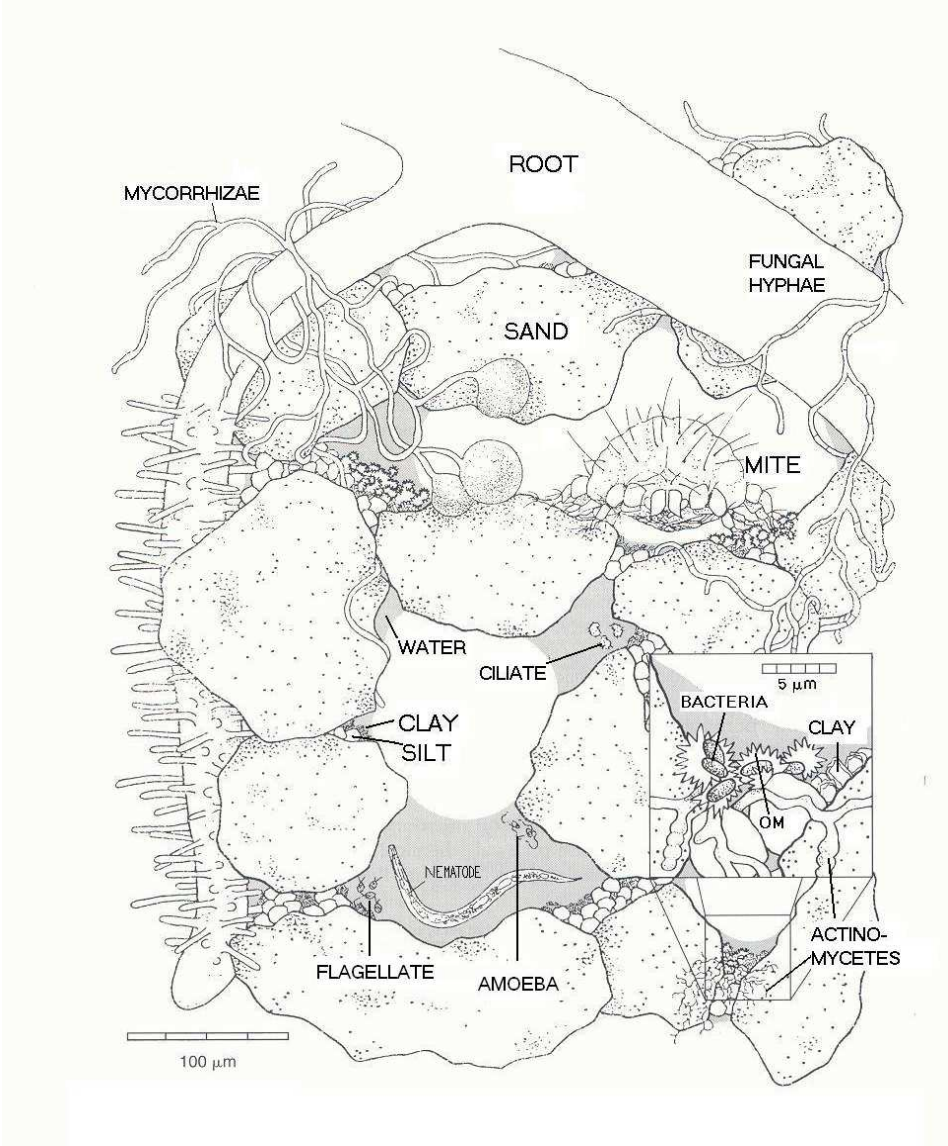


Figure 1.1: Typical constituents of soil habitat at the millimetre scale, modified from Sylvia *et al.* (1999), based on a drawing by K. Luoma. OM-organic matter. Viruses would be dot-sized objects in the 5 µm section.

Soil might be described as a fluctuating habitat, (Lenski 1984), whereby viruses and their hosts are confined in a comparatively unchanging habitat until a water (or nutrient) influx brings renewed activity. In conditions where bacterial activity is stimulated both lytic and temperate phage will benefit, as reproduction and activity rates will

support growth of their host. As bacterial activity reduces there may be less production of lytic phage and of lysogens.

Soil is an oligotrophic environment, and bacteria exist only because they can survive starvation phases (Morita 1997). In a stable soil system it is not moisture that limits bacterial activity but available nutrients (Morita 1997; Strong, Sale, and Helyar 1998). Starvation of bacteria will delay any phage development within them (Lwoff 1953; Schrader *et al.* 1997), whilst without water bacteria will not function at all.

As soil rewets, bacterial activity will be renewed if nutrients are available. Phage replication will be initiated and virulent phage released under pseudolysogenic conditions. This has been demonstrated in cultures (Ripp and Miller 1998). In this way lytic phage are not solely dependent on external adsorption to bacteria or soil matrix for survival. They have been shown to adsorb to dead bacteria; Krueger (1931), found that phage adsorbed equally well to heat-treated bacteria as they did to living bacteria. In one study *Staphyloccocus aureus* were vigorously shaken with glass beads in order to burst the cells, and were then differentially centrifuged to separate cell membranes. These membranes were then incubated with phage for 30 min, after which, based on plaque assays, 90% adsorption of phage was recorded (Hotchin, Dawson, and Elford 1952). Hence phage may attach to both living and dead bacteria.

1.2.2.1. Survival of phage in soil

Survival studies of viruses have generally been restricted to enteric and rotaviruses that have significance for human health (Sobsey

et al. 1980; Moore *et al.* 1981; Fong and Lipp 2005). Influencing factors on virus survival in soil include adsorption, pH, temperature osmotic shock, humic materials and the ionic composition of the soil water (Gerba 1984; Farrah and Bitton 1990; DeFlaun and Gerba 1993; England, Holmes, and Trevors 1998; Jin and Flury 2002). In addition, horizontal movements and loss may occur through topography, rainfall and agricultural practice (Mawdsley *et al.* 1995; Dighton *et al.* 1997). Adsorption is considered to be the dominant factor in phage survival (Farrah and Bitton 1990). Studies with *B. subtilis* phage and two clays; kaolinite and montmorillonite (Vettori *et al.* 1999) have shown that adsorption is rapid, resists wash-out, and maintains phage infectivity.

Soil pH may influence adsorption. Low pH appeared to aid adsorption by a variety of viruses (Goyal and Gerba 1979), however actinophages were not found in soils with pH values below 6.0 (Sykes, Lanning, and Williams 1981). Adsorption of phage MS2 was not significantly affected at pH 4-9, although it decreased slightly at higher pH (You *et al.* 2003). Differences in adsorption are in part due to different iso-electric points – the charge on their coat – which are particular to individual phage and do not follow taxonomic divisions (Dowd *et al.* 1998). As the surrounding pH changes the net charge of the phage also changes, so affecting its adsorptive capability. Higher concentrations of electrolytes may lead to greater adsorption (Taylor, Moore, and Sturman 1981). Cations can reduce the net negative charges on the outer surfaces of clay particles and so allow negatively charged viruses to go closer to the particles, while anions will compete with viruses for adsorption sites (Gerba 1984; Zhuang and Jin 2003).

Humic substances can also be charged, and will affect viral adsorption, with some humic acid particles as large as viruses or larger (Osterberg, Lindqvist, and Mortensen 1993). Soils with higher organic matter had a lower adsorption of poliovirus than substances with less organic matter (Moore *et al.* 1981). There may be relatively few sites available for adsorption; only 1% of the surface of a sandy soil in that study was covered at maximum adsorption of virus. Adsorption might in addition be affected by whether the protein coats contain lipids or not (Kinoshita *et al.* 1993). Thus conditions that suit the adsorption of one phage type might release others from the soil environment, hampering effective extraction.

Degradation of viruses can occur by enzyme action, with degrees of sensitivity between viruses. In one study protease enzymes inactivated over 90% of a seeded Cox-A9 virus but did not affect other viruses (Nasser, Glozman, and Nitzan 2002). Not all viruses may be at risk from naturally occurring enzymes and some viral particles persist for years, although they may be no longer infective (Farrah and Bitton 1990).

1.2.2.2. Transport

The sensitivity of viruses to soil perturbation depends in part on their location within the soil. Viruses freely suspended in the liquid phase of the soil solution and adsorbed on the air-liquid interface are more likely to be influenced by fluctuations in water content than those adsorbed on clay, other soil particles or organisms (Gerba 1984; Sim and Chrysikopoulos 1999). Free virus movement will be determined by

water flow, although it is likely that most viruses will be trapped in the pore in which they were released, and so concentrated in microsites (Williams, Mortimer, and Parry 1999). If there is sufficient water movement, viruses may be transported through soil (Jin and Flury 2002). A proportion may be carried within bacterial cells ingested by Protozoa, in a similar way to that reported with virus-like particles within zoochlorellae endosymbionts of *Hydra viridis* and *Paramecium burgaria* (van Etten, Lane, and Meints 1991). The net result of abiotic and biotic factors is that the spatial variability of viruses in soil is likely to be lower than that of their hosts.

1.2.2.3. Soil architecture determining phage activity

Bacteria are attacked by various groups of nematodes and Protozoa. Protozoan grazing is most likely to occur at higher water contents, when Protozoa are able to move about on or feed within a deeper water film (Vargas and Hattori 1986). For example, Darbyshire (1976) found a positive relationship between soil moisture and populations of an introduced ciliate. Viral particles contained within bacteria may be egested by Protozoa during feeding, rather than be digested (Clarke 1998). Bacteria can escape predation by residing in areas too small for predators to enter (Postma and van Veen 1990; Heijnen and van Veen 1991). In pores < 6 μm, a cut off for protozoan predation, the impact of phage on bacterial mortality could be significant, as movement of bacteria out of these pores would be impeded. The potential outcomes of this viral activity could be an increase in bacterial

production and activity as lysis releases cellular contents and makes them potentially available for other, resistant bacteria.

1.2.2.4. Effects of phage on bacterial population dynamics

Models attempting to describe the relationship between bacteriophage and bacteria have incorporated both purely theoretical and laboratory studies (Rodin and Ratner 1983; Ratner and Rodin 1984; Lenski and Levin 1985). Several chemostat experiments have shown that phage limit bacteria to a level much lower than that of resource-limited controls (Lenski 1988).

Phage can reduce the population of *Bacillus subtilis* (Pantastico-Caldas, Duncan, and Istock 1992) and *Streptomyces* (Cresswell *et al.* 1992) in soil. In other studies populations of *Streptomyces* have remained high and the heterogeneity of the soil environment is proposed as the means by which bacteria escape from viral lysis such that growth is not restricted by phage presence (Burroughs, Marsh, and Wellington 2000).

Phage-controlled bacterial populations occur only when the host is already present at a sufficient density, recorded at 10^4 colony forming units ml^{-1} (Wiggins and Alexander 1985). Although total counts of bacteria in soil may be high, susceptible host strains may be present at densities below the phage-controlling limit. In one study plaque formation showed that pseudomonad bacteria were parasitized by more than one phage species and phage dominance was temporal, implying switches in replication strategies even though the host strains were quite similar (Ashelford *et al.* 1999). In another study there were clear

differences in population numbers of two phage and their bacterial host, with correlation between the bacterial populations and phage populations (Ashelford *et al.* 2000).

1.2.2.5. Finding phage in soil

It follows that an analysis of soil should find very few phage, as they would be mostly located within their hosts. Enrichment is often necessary either by addition of more host or nutrients to boost activity of existing populations prior to analysis (Reanney and Teh 1976; Williams, Mortimer, and Manchester 1987). Earlier work on soil phage used agar plating methods and 'plaque forming units' (PFUs) to count phage. The inherent flaw in this is that very few soil bacteria are cultivable, so it would be inevitable that, irrespective of the number of plaque forming units recorded, the value would be unrepresentative for the soil as a whole and would constitute "PFUs of cultivable bacteria". More recently specialised media have been prepared and used successfully to cultivate specific groups of soil bacteria (Alef and Nannipieri 1995; Janssen *et al.* 2002).

1.2.3. Models

Detailed models have been constructed which quantify the relationship between protozoa and bacteria (Coûteaux *et al.* 1988; Juma 1993). Relationships thus derived feed into the construction of soil food webs (Hunt *et al.* 1984) and subsequently into the measurement of carbon dynamics in soils (Stapleton *et al.* 2005). A suite of models needs to be developed for phage-host bacteria to better quantify the dynamic nature of their interactions.

Simulation models on soil nutrient fluxes typically contain parameters such as respiration, predation, dissolved organic carbon pools, estimated carbon to nitrogen ratios, net assimilation and production efficiencies and specific natural death rate per unit time (Hunt *et al.* 1984; De Ruiter *et al.* 1993). If abundance or biomass data are known of the most significant participating organism groups, mineralisation can be modelled and compared with experimental data. None of the current models for soil nutrient dynamics have a phage component although phage will have an impact. Phage lysis will contribute to bacterial mortality and bacterially-derived dissolved organic carbon values. Lysate bacterial carbon would be unavailable to protozoan grazers and may not be wholly taken up by surrounding bacteria. Other effects might be an increase or decrease in bacterial, fungal or protozoan diversity and growth/death rates.

1.3 OBJECTIVES

The aim of this thesis is to assess relationships between viral and bacterial counts in soil, and their implications for nutrient cycling and soil food webs. The thesis will explore whether bacterial viruses do have a measurable impact on bacterial population size and to what extent perturbations of the soil environment influence this. In order to address these aims some key objectives are set out:

To identify an effective technique for extraction of bacteria and virus-sized particles from soil for direct counting by epifluorescence microscopy.

To determine the extent to which nutrient addition and other factors influence viral populations in soil.

To determine the extent of lysogeny of soil bacteriophage in contrasting soils.

1.4 THESIS OVERVIEW

Chapter 2 reviews current practice in extracting and enumerating bacteria and viruses from aquatic and terrestrial samples. A protocol is described based on shaking, ultrasound sonication, centrifugation for extraction, and filtering, fluorochrome staining and microscopic counting for enumeration.

Chapter 3 explores the effects of addition of simple nutrient and carbon sources on soil bacteria and virus populations over a four-day period.

Chapter 4 explores the effects of changing soil moisture. As one of the key environmental disturbances that affect topsoil is moisture stress, some short-term studies on drying clay-rich and clay-poor soils were carried out.

Chapter 5 examines how stable the interactions between virus and bacteria are in soil by determining to what extent phage are lysogenic. Lysogeny was measured using the antibiotic Mitomycin C.

Chapter 6 explores rhizosphere, predation and nutrient addition as factors that may influence viral abundance in soil. A multi-factorial microcosm study was run for 35 days with bacteria and virus counts made at near-weekly intervals.

Chapter 7 gives some general conclusions on the studies carried out, with recommendations for future work.

CHAPTER 2: METHODOLOGY

2.1 INTRODUCTION

Bacteria and viruses are often tightly bound to soil particles and will therefore be variable in their distribution (Chapter 1). A procedure is required to extract as many bacterial cells and viruses as possible so that counts may be compared within and between soils under a variety of treatments. There are numerous recorded methods for extracting bacteria from soil, sediment and aquatic samples, but far fewer for viruses. The aim was to develop a technique suitable for enumerating bacteria and viruses from single samples rather than using separate protocols for each. In this way more samples could be processed, and a more accurate representation of abundance gained.

2.1.1. Extraction Methodologies

2.1.1.1. Soil processing

Prior to use soil must be processed and stored appropriately in order for continuity between experiments and time. Standard practice involves drying and sieving soil, which may have no significant effect on soil respiration and amino acid mineralization (Jenkinson and Powlson 1980; Jones and Shannon 1999), but may affect carbon mineralization (Franzluebbers 1999). There are no reports on the effects soil processing might have on virus infectivity or survival, although natural soil perturbations have been studied (Chapter 1) which would suggest decreased virus survival in very dry soil (Chu *et al.* 2003).

Fixation kills and preserves cells and viruses and strengthens cells, thus reducing losses during processing. Only samples processed immediately do not require fixing. Most studies use aldehyde fixation (Kepner and Pratt 1994), but viral abundance in aquatic samples decreases even with fixation by as much as 50% within 24 hours (Wen, Ortmann, and Suttle 2004).

2.1.1.2. Soil solution sampling

The abundance of small bacteria and free viruses in the soil solution can be determined with the use of a Rhizon soil moisture sampler (Eijkelkamp Agrisearch Equipment, The Netherlands). These samplers are made of an inert plastic micro-porous material with a given pore size of 0.2 μm. They are inserted into the soil and remove soil water under suction, thus keeping the structure of the soil reasonably intact (Spangenberg, Cecchini, and Lamersdorf 1997). Suction can be applied and soil water is drawn through them over several hours into a collection syringe. These samplers are routinely used for chemical analysis of the soil solution (Knight *et al.* 1998) rather than biotic components. One study in which Rhizons were used for nitrate, ammonium and phosphate quantification reported values that were lower and more variable than those from a standard soil solution method (Farley and Fitter 1999) so they may therefore not be suitable for the purposes of cell and virus extraction. Furthermore, viruses have been shown to aggregate in solution over time (Floyd 1979), possibly reducing the efficacy of the sampler.

2.1.2. Chemical Disruption of Soil

The interaction of bacteria with soil particles is complex (Huang and Schnitzer 1986): no procedure will adequately disrupt all of these associations. Detergents and other chemicals interact with particle surfaces and can remove adsorbed objects (Taylor, Moore, and Sturman 1981), with enhanced extraction of bacteria reported (Yu *et al.* 1995). However other authors have found no improvement in extraction with or without detergent (Lindahl and Bakken 1995). Sodium pyrophosphate has been demonstrated to increase extraction of bacteria in soil (Weinbauer, Beckmann, and Höfle 1998) and sediment (Danovaro *et al.* 2001), so this chemical was explored in the current work.

Soil Family	Clay (%)	Organic matter (%)	pH	Phage Adsorption (%)			
				T2	T4	ØX174	f2
Fine, mixed, thermic	39	0.30	4.5	99	99	99	99
Fine, mixed, thermic	28	1.4	8.0	86	99	0	16
Sandy, hyperthermic	3	3.64	7.1	8	0	18	0
Sandy loam	3	0.88	7.8	36	72	0	0

Table 2.1: Showing percentage adsorption of added phage to selected soils. Selected data from Goyal and Gerba (1979).

Viruses have an electric charge (iso-electric point) that arises through the structure of the protein capsid. This charge is a key factor in the adsorption of the virus to surfaces (Gerba 1984). As iso-electric points differ between viruses (Dowd *et al.* 1998) adsorption is not uniform across soil or virus type (Table 2.1). Extraction of viruses from soil has used a variety of eluents (Table 2.2). including organic substances such as egg albumin (Williams, Mortimer, and Parry 1999).

Extraction of Bacteria					
Centrifuge speed	**Time (min)**	**Substrate**	**Eluent**	**Sonication details**	**Reference**
–		Marine sediment	0.0005M $Na_4P_2O_7$	Branson sonifier 2200 60 W 1 min x 3	(Danovaro, Manini, & Dell'Anno 2002)
500 rpm	5	Tidal sand	0.001M $Na_4P_2O_7$ or 0.0001% Tween	Branson S0125 100W 20kHz 109 μm	(Epstein & Rossel 1995)
-	-	Freshwater sediment	-	Sonic bath 15 min	(Gough & Stahl 2003)
-	-	Freshwater sediment	-	Artek sonic 300 dismembrator 30 sec	(Maranger & Bird 1996)
-	-	Soil Sediment	0.01M $Na_4P_2O_7$	Labsonic 4mm tip U 2000 50 W 0.5 second pulses for 1 min.	(Weinbauer, Beckmann, & Höfle 1998)
-	-	Sediment	Tween 1mg/litre	Biomic GE-100 100W 3mm tip 40% output	(Kuwae & Hosokawa 1999)
-	-	Sediment	-	Branson sonifier 2200 60 W 3 x 1min	(Luna, Manini, & Danovaro 2002b)
750 rpm 7000 rpm	10	Sediment	0.01M $Na_4P_2O_7$	Branson 2210E-DTH bath 0-5 min	(dos Santos Furtado & Casper 2000)
-	-	Sediment	0.005-0.5M $Na_4P_2O_7$	Sonicate 100W 30-60 seconds	(Velji & Albright 1993)
1000 rpm	5	Sediment	0.1M $Na_4P_2O_7$ (not if many cations)	Sonicator 8 x 5 second pulses 160W	(Griebler, Mindl, & Slezak 2001)
-	-	Soil	0.1% $Na_4P_2O_7$ and 0.8% NaCl (best)	-	(Yu, *et al*., 1995)
-	-	Freshwater sediment	0.1% $Na_4P_2O_7$ buffered formalin	Branson sonifier 250 80 W 76 μm, 11.4mm 0.5 – 7 min	(Buesing & Gessner 2002)
630-1060 rpm	15	Soil	Various – water chosen	-	(Bakken 1985)
750 rpm	10	Soil	Water	-	(Bååth 1996)

1-600 rpm	5-30	Soil	0.2% $Na_4P_2O_7$	-	(Riis, Lorbeer, & Babel 1998a)
500 rpm	1-2	Soil	Various	Various	(Hopkins, Macnaughton, & O'Donnell 1991)
-		Soil	Saline solution	2 min at 100w	(Toyota, Ritz, & Young 1996)
10000 rpm	10	Soil	0.05M $Na_4P_2O_7$ and Water	-	(Lindahl 1996)
7000 rpm	60	Soil	Water	-	(Priemé *et al*., 2003)
Extraction of Viruses					
Centrifuge speed	**Time (min)**	**Substrate**	**Eluent**	**Sonication details**	**Reference**
1200 rpm	14	Soil	Various Including water	-	(Lanning & Williams 1982)
5000-10000 rpm	30	Soil	Various	-	(Sobsey, Dean, Knuckles, & Wagner 1980)
480 *g*	5	Saline sediment	None		(Roper & Marshall 1974)
800 *g*	1	Marine sediment	0.001M $Na_4P_2O_7$	Branson sonifier 2200 47kHz 100W 3 min	(Danovaro, *et al*., 2001)
-	-	Marine sediment	0.001M $Na_4P_2O_7$	Branson sonifier 2200 60 W 1 min x 3	(Danovaro & Serresi 2000)
1000 rpm	10	Marine sediment	-	-	(Hewson *et al*., 2001a)
800 *g*	1	Marine sediment	0.0005M $Na_4P_2O_7$	Branson sonifier 2200 60 W 1 min x 3	(Danovaro, Manini, & Dell'Anno 2002)
3000 rpm	30	Freshwater sediment	0.001-0.1M $Na_4P_2O_7$	Artek sonic 300 dismembrator 45 sec	(Maranger & Bird 1996)

Table 2.2: Summary of extraction procedures for bacteria and viruses from the environment

2.1.3. Physical Disruption of Soil

In seminal work Hattori (1973) showed that bacterial abundance was higher in inner aggregates than on the outer layer of aggregates, and that the composition of the bacterial and fungal communities differed between these two sites. Disruption of these aggregates increases extraction of bacteria, and has been achieved using a variety of physical methods. Approaches commonly use two or more techniques (Table 2.2) and as sonication combined with centrifugation has been shown to be successful in extracting both bacteria and viruses from sediments and soil these methods were adopted in the present study.

The use of sonic probes or baths is widespread, as bacteria released are not destroyed and thus can be enumerated or used in culture studies. Work has shown that after 2 minutes of sonication at 100W, extracted bacteria can still grow on agar plates (Toyota, Ritz, and Young 1996). Sonic probes dislodge bacteria and viruses from soil particles by bombardment with high intensity ultrasound waves. Sonication periods can be regulated in intensity, wattage, and length of sonication, as well as having optional pulses, pulse duration and different diameter probes. This complicates comparisons of sonication studies, as the actual energy delivered per unit volume or mass of sample may have varied considerably (Lindahl and Bakken 1995; De Cesare *et al.* 2000).

2.1.4. Centrifugation

2.1.4.1. Centrifuge speed

High-speed centrifugation is effective in creating a clear supernatant for subsequent enumeration of viruses, but will sediment bacteria into the pellet and thus could not be used in a protocol designed to extract both. A compromise speed is required for appropriate retention of bacteria and effective clearing of the supernatant for viruses. The optimum centrifugation will vary depending on the nature of the soil: very sandy soils with low organic matter content will require lower speeds than clay-rich, high organic matter soils due to different sedimentation rates of particles. As the current study involved several different soils over its duration, it was deemed inappropriate to determine an optimum centrifugation speed for each soil, but rather to apply a single compromise speed suitable for all.

2.1.4.2. Repeat centrifugation

Repeated centrifugation should lead to higher extraction of organisms from soil, as there will be greater disruption to aggregates than in a single centrifuge step. In work aimed at optimising extraction, many cycles of centrifugation and resuspension have been proposed (Hopkins, Macnaughton, and O'Donnell 1991; Epstein and Rossel 1995; Riis, Lorbeer, and Babel 1998). There is a trade-off between the amount of time spent on sample processing and ever diminishing returns.

2.1.5. Losses During Processing

Losses of viruses and bacteria will occur after successful elution from the soil due to sorption onto the surface of sample tubes during processing (Thompson *et al.* 1998). Glass tubes are preferable to polypropylene as it interacts less with bacteriophage (Thompson and Yates 1999), but as samples were being centrifuged and sonicated, glass could not be used due to safety considerations. If samples are not to be processed immediately, then storing at –20°C leads to fewer losses of virus than at +4°C (Brussaard 2004).

2.1.6. Enumeration Methodologies

2.1.6.1. Agar plating

Plating on agar with some nutrient addition is a highly effective technique when specific bacteria and phage are of interest. A substantial body of work on *Streptomyces* bacteria and associated phage has been collected in this way (Cresswell *et al.* 1992; Herron and Wellington 1994), resulting in advances in understanding of transduction, transconjugation and plasmid transfer of this group. The double-layer plaque assay developed over fifty years ago (Adams 1959) can be used when studying phage on specific bacteria (Mocé-Llivina, Lucena, and Jofre 2004). Selective plating of different functional bacterial groups (Alef and Nannipieri 1995) and looking for their associated phage could be considered, but the drawback for plating is that very few bacteria are culturable so even with modified plates (Davis, Joseph, and Janssen 2005) only a small proportion are represented.

2.1.6.2. Molecular techniques

The *Zeitgeist* in microbial ecology is molecular characterisation of communities. 'Polymerase chain reaction (PCR) methods to 'fingerprint' communities in such forms as denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (TFLP) are in widespread use (Kent and Triplett 2002). Alternatives to PCR include phospholipid fatty acid analysis (PLFA), where cellular constituents are of interest. Microscopic investigation of target groups can be carried out using fluorescence *in situ* hybridisation (FISH) or pulsed-field gel electrophoresis (PFGE), where, for instance several *Campylobacter* phage were classified (Atterbury *et al.* 2003). Ribosomal intergenic space analysis (RISA) was used to show that microbial diversity increased with decreasing soil particle size (Sessitsch *et al.* 2001). To date these methods have not been utilised in ecological studies of soil viruses (Weinbauer 2004).

2.1.6.3. Flow cytometry

This technique incorporates detectors and counters to enumerate and classify small objects by size. It has been used in aquatic samples (Brussaard, Marie, and Bratbak 2000), and counts were comparable to those from epifluorescence microscopy (Marie *et al.* 1999). However, other workers have found that a good viral signal is not obtainable in all aquatic environments (Laybourn-Parry J. personal communication). It is unlikely that a good viral signal will be obvious in soil samples against a background rich in organic matter and humic acids. One comparative

study of soil virus enumeration using flow cytometry and direct counting found counts were lower by one order of magnitude with flow cytometry (Page and Burns 1991).

2.1.6.4. Electron microscopy

The ability to look at the structure of bacterial viruses has been exploited for many decades. Papers published from the 1960's onward have demonstrated that electromicrographs can be used to classify viruses by their morphology (Bradley and Kay 1960; Hegazi and Jensen 1973; Casida and Liu 1974; Hemphill and Whiteley 1975). The method is commonly used in culture studies, for example on *Bacillus subtilis* phage (Hemphill and Whiteley 1975). Electron microscopy has been used to count viruses in samples from marine (Hara, Terauchi, and Koike 1991) and soil environments (Ashelford, Day, and Fry 2003) but it underestimates populations (Chen *et al.* 2001). It is useful as a confirmation that virus-sized particles seen under epifluorescence microscopy are viruses. It has also been used for spatial location of bacteria in soil (Bae and Casida 1973) and is particularly informative for identifying infected bacteria and burst sizes. Transmission electron microscopy (TEM) was used to obtain electron micrographs of phage from soil samples in the work presented here.

2.1.6.5. Epifluorescence microscopy

For this project, epifluorescence microscopy has several advantages over other techniques. The technique is quantitative without the need for dilution series, and cell dimensions can be determined on a large number of cells. It does not require the culturing of bacteria and

provides virus counts that are usually higher than electron microscopy (Bettarel *et al.* 2000). Various fluorochromes are available, but the most commonly used are acridine orange, DAPI, calcifluor and the SYBR family. DAPI is easy to use and rapid in sample preparation, but can overestimate bacteria as it also stains ghost cells – which are no longer viable (Zweifel and Hagström 1995). The SYBR stains (Molecular Probes 2001) have been shown to give very satisfactory results for virus and bacteria counts in aquatic samples (Marie *et al.* 1997; Noble and Fuhrman 1998) and have been used successfully to enumerate bacteria in soils (Weinbauer, Beckmann, and Höfle 1998). Initial work here was carried out using SYBR Green 1, but it had unstable fluorescence and faded rapidly, as reported elsewhere (Chen *et al.* 2001) so a switch was made to SYBR Gold. Most phage contain double stranded DNA (Maniloff, Ackermann, and Jarvis 1999) so will be detected by the SYBR stains, which primarily detect dsDNA (Molecular Probes 2001). An exception is the Cystoviridae that contain dsRNA and infect *Pseudomonas* (Maniloff, Ackermann, and Jarvis 1999). These phage will be detected at a lower sensitivity.

2.2 METHODS

A variety of soils were used to reflect differences in soil management history and soil texture (Table 2.3). All soils, with the exception of the Highfield soils, were taken from the Silsoe Research Institute at Wrest Park. The sites are all within one mile of each other, yet a wide range of textural types are present. All have been under conventional or modified rotations as part of the experimental farm. The Highfield soil samples are from the long-term “Broadbalk” site at

Rothamsted, where some management practices have been maintained for close to 150 years.

Soil name	Location	Particle size			Organic C (%)	Cation Exchange Capacity	pH (H_2O)
		Sand	Silt	Clay			
*Boot Field	TL 0758 3423	9	18	73	4.1	32	6.9
*Bypass Field	TL 0840 3479	26	19	56	3.8	7	5.4
*Garden Gate	TL 0872 3570	80	15	5	0.8	2	4.5
*Cashmore	TL 0853 3545	49	15	36	3.2	4	6.7
*Cashmore Clay	TL 0856 3551	35	20	45	4.3	5	6.4
**Highfield Fallow	TL 1190 1390	9	66	25	1.1	nd	7.1
**Highfield Grassland	TL 1190 1390	11	67	23	3.2	nd	7.3

Table 2.3: Selected soil properties used in this work. *data from Barton, A. – personal communication, and **Watts, C. – personal communication.

2.2.1. Soil Processing

Standard practice of soil processing involved bulking samples taken from several locations in the chosen site (Rowell 1994). Any litter layer was removed and soil was taken either by spade or core to a depth of 10 cm. These soil samples were thoroughly mixed on clean polythene sheeting to make one bulked sample. Soil was then dried by evaporation in air for sieving through a 4 mm sieve, and stored at 5°C until needed. Samples were stored for a maximum of 3 months before use. When required the soil was equilibrated by rewetting to about 40% water holding capacity and placing in a loosely secured plastic bag. Bags were placed in a metal drum with jars of de-ionised water and soda lime (to remove evolving CO_2) and left for one week to equilibrate before use (Grace, Hart, and Brookes 2004). This rigorous stepwise method allows for comparative analysis of stored soil, and can stabilise microbial activity, reducing the effect of storage.

Small samples were used for centrifugation. Other researchers have used 2 g (Lindahl 1996; Weinbauer, Beckmann, and Höfle 1998) or 5 g (Hopkins, Macnaughton, and O'Donnell 1991; Bååth 1996). Sediment samples used have been as small as 1 ml or less (dos Santos Furtado and Casper 2000; Griebler, Mindl, and Slezak 2001 ; Luna, Manini, and Danovaro 2002). In this work samples were commonly between 1.5 - 2 g wet weights, with typically three or four replicates per treatment.

2.2.1.1. Fixation

Phosphate-buffered glutaraldehyde was used at 4% v/v as it is a common preservative in such studies (Kepner and Pratt 1994). Trials of processing samples without fixation gave supernatants in which bacteria and viruses could not be enumerated.

2.2.2. Fluorochrome Staining

A 1 g sub-sample was added to 10 ml of 4% phosphate buffered, 0.2 μm filtered glutaraldehyde in a 15 ml polycarbonate centrifuge tube. Samples were sonicated for 30 s with a Bandelin Sonopuls set at 70% intensity. They were then centrifuged at 2000 rpm (600 *g*) for 10 min, removed from the centrifuge and shaken, then centrifuged for a further 10 min. Supernatants were then filtered, dried, stained and mounted based on trials of several options resulting in the use of the procedure by Noble and Fuhrman (1998). Samples were filtered onto a 0.02 μm Anodisc 25 mm membrane filter [Whatman] resting on a 0.8 μm Millipore AA filter to assist in uniform filtration. Between 20 and 50 μl of sample

was added to 1 ml of virus free water (0.02 μm filtered). This was followed by a further 1 ml of virus free water to further disperse the sample. The vacuum was set at 4 kPa (Kepner and Pratt 1994). The underside was dried using a tissue before placing in a desiccator for 10 min. The dried filter was then placed on a 100 μl drop of SYBR Gold stain (at x500 dilution of the x10000 concentrate supplied) incubated in the dark for 15 min and dried in a desiccator for a further 10 min. It was then placed on an ethanol-cleaned microscope slide with a 40 μl drop of anti-fade solution. The anti-fade solution consisted of 50% glycerol, 50% phosphate-buffered saline and 0.1% *p*-phenylenediamine and was made up daily. The method was adapted from Noble and Fuhrman (1998).

Filters were either viewed immediately or stored at –18°C for less than 5 days before viewing at x1600 magnification with a Ziess Axioskop using a blue exciter filter (BP 450-490 nm – set 48 79 09). At least 20 randomly selected Whipple grids were counted on each filter for viruses and bacteria, depending on the number of objects in each field. To determine an appropriate number of grids to count, work by Cassell (1965) and Kirchman (1993) were used as guidelines. The conversion factor used to multiply counts per grid to counts per 1 ml of filtrate was 55850, based on the radius of the filtration tower and filter and the area of the grid. A further calculation, based on the mass and original water content of the soil sample used, converted that figure into a bacterial or viral abundance per gram of dry soil.

2.2.2.1. Filter volume and replicates

The optimum filtrate volume was generally determined on the clarity of supernatant: aliquots of up to 100 μl could be used from clear supernatants, but with other supernatants 40-50 μl was more suitable. This volume is comparable to previous work (Weinbauer, Beckmann, and Höfle 1998). Replicate filters from single samples were counted to assess the variability of the supernatant. The pipette was always inserted to 10 mm below the meniscus of the supernatant.

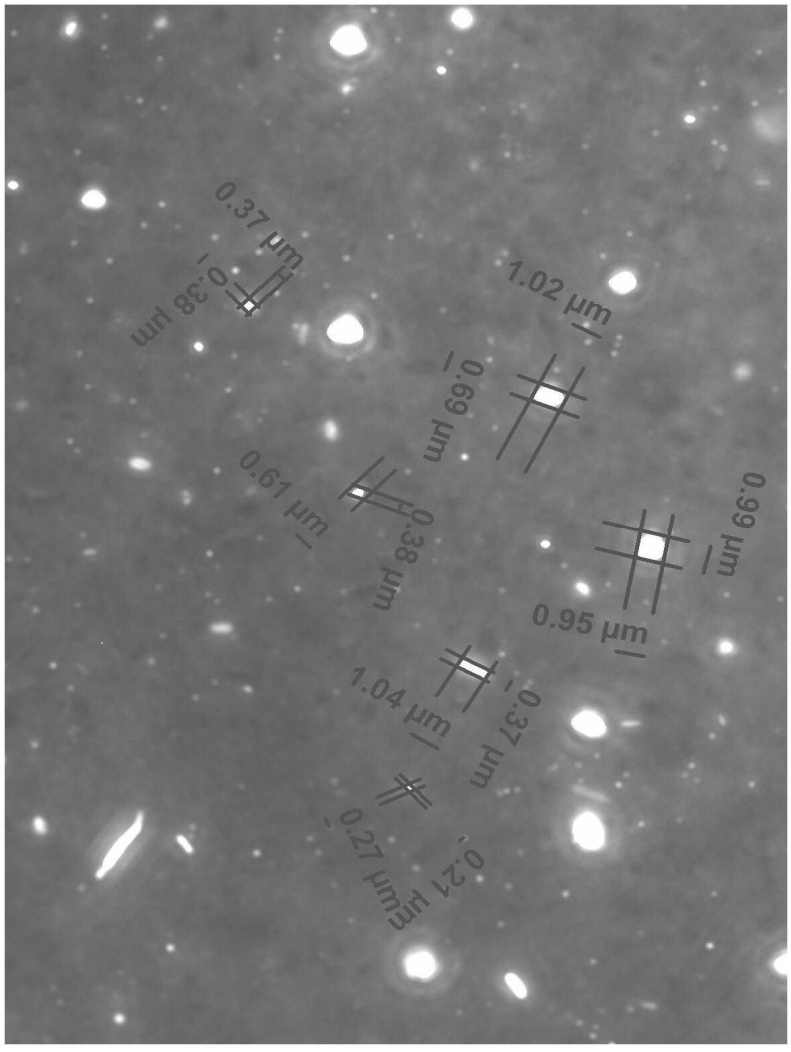
2.2.2.2. Bacterial biovolume and biomass

Two or three photographs were captured for each sample with an Axiovision camera mounted on top of the microscope and at least twenty randomly selected bacteria per photograph were measured using Axioplan software. The focus was set to reduce the artefacts of fluorescence enlarging the bacteria, and the lengths (L) and widths (W) measured (Photograph 2.1). A micrometer slide was photographed and used to calibrate the measurements. These measurements were converted into a cell volume according to the equation used by Bratbak (1985): $(\pi/4)\bullet W^2\bullet(L-W/3)$, suitable for the rod and cocci shapes seen in the photographs.

Bacterial biomass carbon was determined by applying a conversion factor of 0.56 pg carbon per μm^3 (Bratbak 1985) as calculated with the above formula.

2.2.3. Detergent

Sodium pyrophosphate ($Na_4P_2O_7$) was tried as an elution agent at 0.001M concentration (Danovaro *et al.* 2001). Equilibrated soil samples of 1.5 g wet weight from Boot field and Garden Gate (Table 2.3, p34) were incubated in 10 ml 0.001M $Na_4P_2O_7$ for 5 min with gentle shaking on a horizontal shaker before centrifugation at 600 *g* for 10 min and processed as above (section 2.2.2). Three sub-samples were processed and triplicate slides prepared from each soil.



Photograph 2.1: Example photograph captured with the Zeiss Axioskop microscope and image analysis software, showing measurement of length (L) and width (W) of selected bacteria. The smaller bright specks are viruses.

2.2.4. Sonication

Equilibrated clay Cashmore soil (Table 2.3, p34) samples of 2 g wet weight were either treated with ultrasound or left untreated. A Bandelin Sonopuls H2200 (230V, 70W, frequency 20kHz) was used at 70% intensity. The sonication time was set at 0, 30, 60 and 90 s. A maximum of 90 s was chosen as it has been shown to have a limited effect after a long period (Stemmer, Gerzabek, and Kandeler 1998). The longer duration timings were carried out in 30 s intervals with a 30 s resting period in between to allow the sample to cool slightly. This practice is commonly adopted if samples are not set in ice during sonication. Three sub-samples were processed and triplicate slides prepared at each sonication period. Biovolume data were collated by recording cell dimensions of at least twenty bacteria from each photograph of the grids.

2.2.5. Centrifugation

2.2.5.1. Centrifuge speed

A nominal high speed of 4000 rpm (2000 *g*) was set near the operating maximum of a bench-top centrifuge. A lower speed of 2000 rpm (600 *g*) allowed minimum centrifugation which gave a clear enough supernatant. At lower speeds there was too much particulate matter remaining for reasonable filtering of the supernatant. Centrifugation periods were set at around 10 min (Bååth, Pettersson, and Söderberg 2001). Three sub-samples were processed and triplicate slides prepared from each sub-sample at each centrifuge speed.

2.2.5.2. Repeated centrifugation

Repeated centrifugation allowed comparison of relative proportions of bacteria and viruses extracted after each step. Soil samples from Garden Gate and Boot field (Table 2.3, p34) were selected as they contrasted in clay content. Samples of 1.5 g wet weight soil were centrifuged for 10 min at 600 *g*. The supernatant was decanted for enumeration and the pellet re-suspended in 0.2 μm filtered water. After 1 min shaking, the sample was again sonicated for 30 s and centrifuged again (Riis, Lorbeer, and Babel 1998). This was repeated for a total of six centrifuge steps. Three sub-samples were processed and triplicate slides prepared from each sub-sample at each centrifugation step.

2.2.6. Soil Solution Samplers

Three soils were used: Garden Gate, Bypass and Boot fields. As Rhizons extract the soil solution they require wet samples so 10 g sub-samples were diluted 1:10 to form slurries. Samples were preserved with 10 ml 20% buffered glutaraldehyde (c. 2% final concentration). The mixtures were made up in 150 ml plastic bottles with screw-cap lids. The samples were shaken on a Rotatest shaker table (at speed 5) for 1 h, lying longitudinally along the main direction of movement. They were sonicated for 30 s. Rhizons were inserted into the slurry, with a vacuum applied by connecting a closed 10 ml syringe, then fully extending and securing the stopper to ensure equal suction. They were left for 18 h, during which time an average of 8 ml soil extract liquid had been collected from each sample. Aliquots of <100 μl were then filtered as

detailed above (section 2.2.2). Three syringes were processed for each soil and triplicate slides prepared.

2.2.7. Electron Microscopy

Aliquots of 5 μl supernatant were spotted directly onto copper formvar-coated grids and dried in a petri dish. They were then stained with a 5 μl drop of 2% methylamine tungstate placed on top of the grid. Grids were dried once again in a petri-dish. The method was taken directly from Ashelford *et al.* (2003) in which TEM images of soil bacteriophage were shown. Despite the use of a variety of soils this yielded no useable grids on several occasions.

Modifications of the method and a standard protocol using phospho-tungstic acid and uranyl acetate as stains were carried out by staff at the Centre for Bioimaging at Rothamsted Research. Three aspects of grid preparation were examined: sample droplet placement, stain droplet placement and drying technique (Hoppert and Holzenburg 1998). Sample droplets may be placed on top of the grid or the grid may be placed on top of a sample droplet. The assumption is that phage will preferentially adsorb to the solid surface of the grid rather than remain in suspension. In the first instance, debris may also deposit onto the grid, obscuring any phage present. Another contributory factor is that phage may be drawn toward the air, water and solid interface (Thompson *et al.* 1998) and aggregate around the edge of the grid rather than spread evenly on it. The placement of the stain droplet may affect staining ability – the droplet may cause phage to leave the grid and re-suspend.

If grids are blotted dry any phage in suspension will be carried off with the liquid and poor grids may result.

2.3 RESULTS

2.3.1. Soil Equilibration

There were differences in counts between soil samples processed straight from cold storage (4°C) and samples that had equilibrated for three days at 20°C. In a clay soil (Boot field) bacteria counts were similar at 5 $x10^7$ cells g^{-1} dry soil (Figure 2.1A), but viruses increased significantly ($p<0.001$) from under 10 $x10^7$ particles g^{-1} dry soil to over 20 $x10^7$ particles g^{-1} dry soil. A sandy soil (Garden Gate) showed an insignificant increase in bacterial counts from 2 to 4.5 $x10^7$ cells g^{-1} dry soil, yet virus counts decreased slightly (Figure 2.1B). Hence bacteria responded to elevated temperatures in the sandy soil more strongly than in the clay soil, yet viral production was only stimulated in the clay soil.

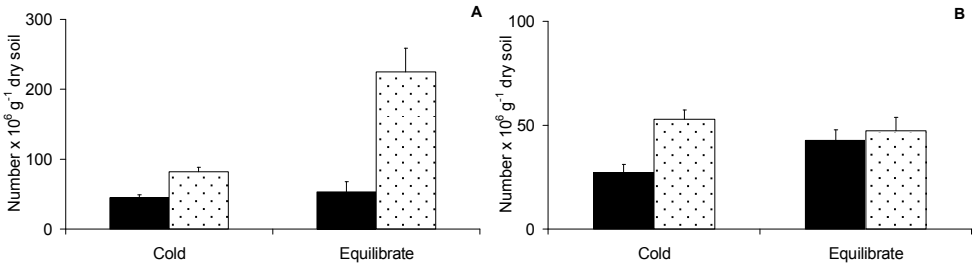


Figure 2.1: Effect of equilibration to room temperature over 3 days on abundances of bacteria (solid columns) and viruses (dotted columns) in A – clay soil (Boot field) and B – sandy soil (Garden Gate). Standard error shown (n=3).

2.3.2. Filter Volume and Replicates

The size of the aliquot filtered had a negligible effect on filter counts, but some were easier to count. The optimum aliquot depended on how opaque the supernatant was, and it ranged from 20 to 100 μl. The sample supernatants were not homogenous as the difference in

counts of bacteria and viruses within single samples were occasionally as high as 40% (Figure 2.2) although this was an exceptional case.

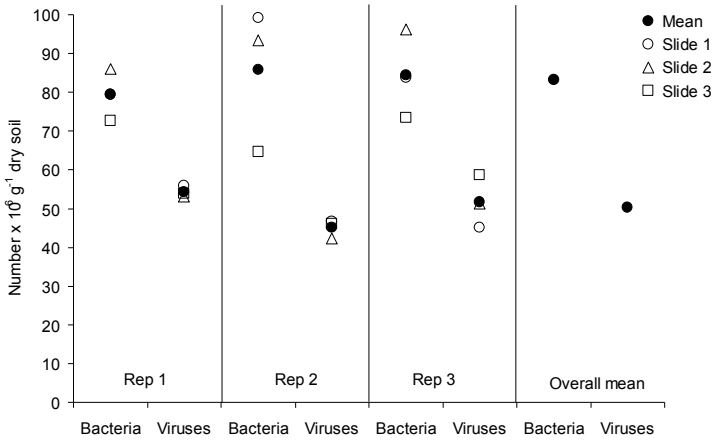


Figure 2.2: Variability of counts within 1 g sub-sample tubes from Highfield Grassland soil. Single slides (open), mean (solid).

2.3.3. Detergent

Bacterial counts in both soils, at 4 $x10^7$ cells g^{-1} dry soil, were comparable when extracted with sodium pyrophosphate ($Na_4P_2O_7$) or water (Figure 2.3). Virus counts in the clay soil were slightly reduced when extracted with pyrophosphate, whereas in the sandy soil counts increased from 2 $x10^7$ cells g^{-1} dry soil to 3 $x10^7$ cells g^{-1} dry soil. Although not significant, the increase recorded in the sandy soil may indicate that the detergent is effective in sandy soils. Enumeration was made more difficult in pyrophosphate samples due to high background fluorescence, thus although slightly higher virus counts were recorded in the sandy soil using pyrophosphate it was decided to extract without it in further work.

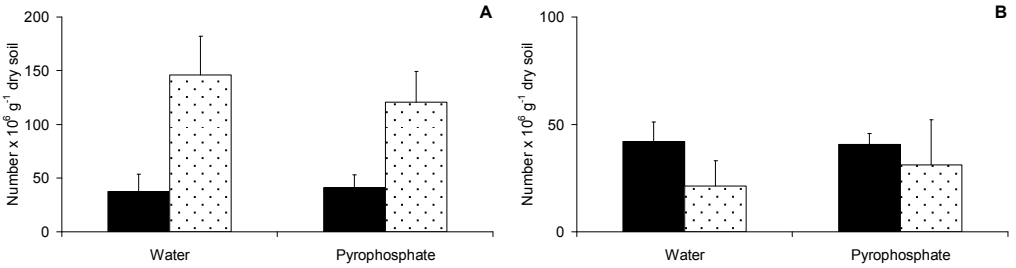


Figure 2.3: Effect of tetrasodium pyrophosphate ($Na_4P_2O_7$) as an eluting agent compared with water in: A – clay soil (Boot field) and B – sandy soil (Garden Gate). Bacteria (solid columns), viruses (dotted columns). Standard error shown (n=3).

2.3.4. Sonication

The use of a sonic probe significantly ($p<0.001$) increased both bacteria and virus counts (Figure 2.4A). The highest counts of bacteria and viruses were at 30 s. Increasing sonication time beyond 30 s had no significant effect on extracted bacterial counts and decreased virus counts, although these were still slightly higher than with no sonication. Mean cell volume of extracted cells decreased as the period of sonication increased (Figure 2.4B). There was a significant decrease in mean cell size when samples were sonicated for 30 s compared to no sonication, indicating the possibility of smaller cells in more protected aggregates.

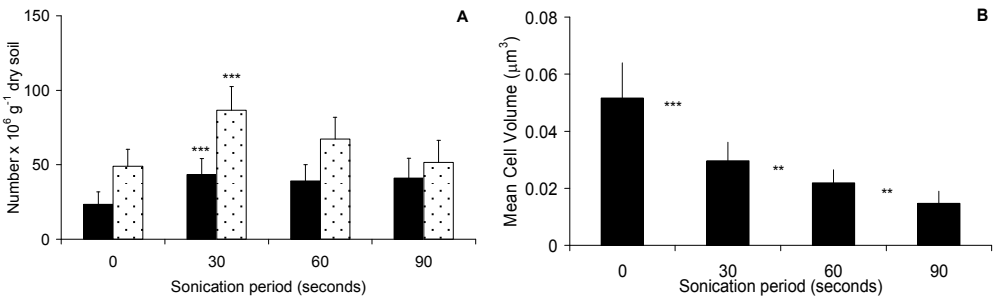


Figure 2.4: Effect of sonication on clay Cashmore soil: A – extraction of bacteria (solid columns), and viruses (dotted columns); and B – mean bacterial cell volume. Significance between two columns: *** p<0.001, ** p<0.01. Standard error shown (n=3).

2.3.5. Centrifugation

2.3.5.1. Centrifuge speed

Centrifugation at high speed (2000 *g*) decreased virus counts significantly ($p<0.001$) in the clay soil, but bacteria counts were unchanged (Figure 2.5A). High speed centrifugation of the sandy soil resulted in significantly lower bacteria counts and slightly higher virus counts when compared to lower speed centrifugation (Figure 2.5B). The low values extracted in both cases reflect the lack of sonication prior to supernatant processing.

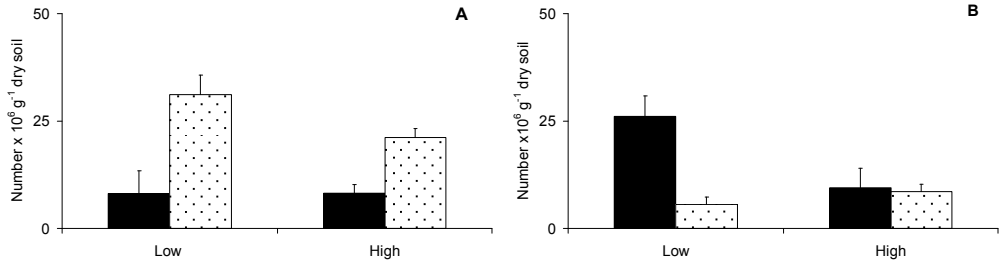


Figure 2.5: Effect of increased centrifuge speed on extraction of bacteria (solid columns) and viruses (dotted columns) in: A – clay soil (Boot field) and B – sandy soil (Garden Gate). Low – 600 *g*, High – 2000 *g*. Standard error shown (n=3).

2.3.5.2. Repeated centrifugation

One centrifugation step was not sufficient to release the majority of bacteria from soil samples. In the clay soil (Boot field) less than 20% were extracted initially, with significantly higher counts recorded on centrifuge step 2 and 3 (Figure 2.6A). In the sandy soil counts from the first two steps were of similar magnitude, thereafter they decreased (Figure 2.6B). The pattern for virus extraction was similar to that of bacteria; increased extraction in the second and third centrifugation for the clay soil and sequential decrease in the sandy soil (Figure 2.6A,B).

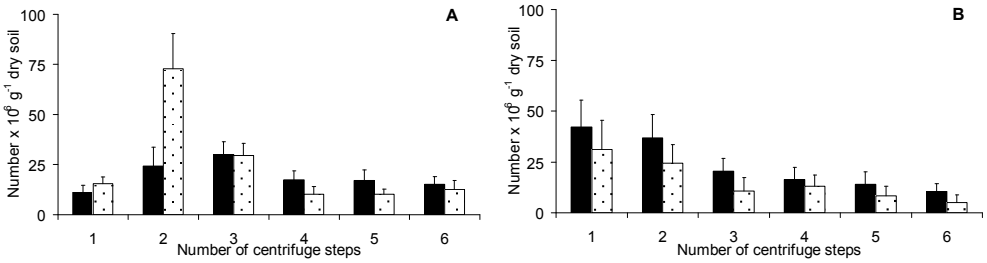


Figure 2.6: Extraction of bacteria (solid columns) and viruses (dotted columns) with sequential centrifuge steps in: A – clay soil (Boot field), and B – sandy soil (Garden Gate). Standard error shown (n=3).

2.3.6. Soil Solution Samplers

Bacteria counts were similar in all three soils at about 1 x10^7 cells g^{-1} dry soil. It must be noted, however, that the samplers are sold as "impermeable to bacteria" (Rhizon instructions for use). The counts then are of the smallest bacteria, of an estimated cell length of <0.2 μm, which can pass through the porous material of the sampler. Virus counts were considerably lower in Boot field (clay soil) than the other two soils, so it is possible that the suction from the sampler was not sufficient to remove viruses from adsorbed sites. Virus counts in Boot field samples were 2 x10^7 particles g^{-1} dry soil, compared with 10 x10^7 particles g^{-1} dry soil in the other two soils. There was very high variability in counts of both bacteria and viruses in all three soils (Figure 2.7).

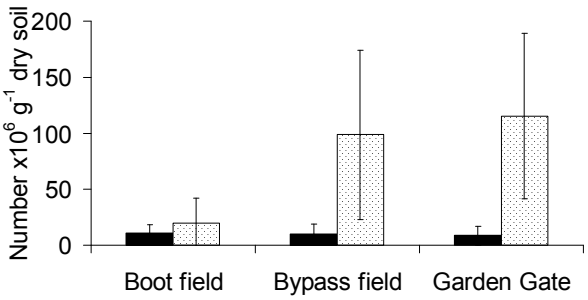


Figure 2.7: Extraction of bacteria (solid columns) and viruses (dotted columns) from three contrasting soils using Rhizon samplers. Standard error shown (n=3).

2.3.7. Electron Microscopy

Several soil samples were processed over the course of the programme for electron microscope viewing. No useable images were obtained when the method outlined by Ashelford *et al.* (2003) was adopted.

Initial views of grids showed that a high virus concentration was required in order to reduce operator fatigue. Two concentration procedures were used; the first used high-speed centrifugation at 12000 rpm for one hour, resulting in virus-enriched aliquots. The second utilised high-speed centrifugation directly on to grids. Neither method enhanced grid clarity or increased virus sightings.

The method attempted by the author in Nottingham, taken from Ashelford *et al.* (2003), was tried by staff at the Centre for Bioimaging at Rothamsted Research. It compared unfavorably with standard protocols using phosphotungstic acid (Carzaniga, R and Devonshire, B.J. unpublished data). An example of micrographs obtained is illustrated in Chapter 1, p2. Despite further high-speed centrifugation of soil supernatants the density of viruses on the TEM grids was too low for quantitative work and resulted in highly inefficient scanning for possible phage. This work demonstrated that concentration of viruses from soil samples considerably greater than 2 g is needed in order to achieve useable grids.

2.4 DISCUSSION

This chapter has reviewed a range of techniques that have been adopted for extracting and enumerating bacteria and viruses from soils. Its aim was to find an adequate protocol suitable for a range of soils. Optimising a joint extraction of bacteria and viruses from different soils would be a Herculean task and one that would not yield useful data on its own as bacteria and virus adsorption is soil moisture dependent (Gerba 1984; Jin and Flury 2002). Counts using the protocol are of similar magnitude (although smaller) to bacteria counts made with epifluorescence elsewhere (Weinbauer, Beckmann, and Höfle 1998).

Sonication was shown to significantly increase extraction, which is in agreement with other findings (Kuwae and Hosokawa 1999). The optimum sonication speed of 30 s was shorter than 2 min reported elsewhere (De Cesare *et al.* 2000). Sonication may not disrupt all the aggregates however, as it has been shown that 2-20 μm diameter aggregates can resist sonication (Tisdall and Oades 1982). As bacteria are known to exist in these small microaggregates (Hattori 1973), and viruses are likely to exist there also, there may be some underestimation inherent in the method.

Sodium pyrophosphate had a negative effect on counts. This is in contrast to work carried out in sediments which showed that $Na_4P_2O_7$ had a significant effect on elution of bacteria and viruses (Danovaro *et al.* 2001). The difference in electrolytes between sediments and soil may diminish the effect of pyrophosphate. An increased quantity and nature of humic matter in soil as compared to sediments may contribute,

as $Na_4P_2O_7$ extracted these materials rendering the supernatant quite dark (personal observation), presumably an explanation for the high background fluorescence on filters in other work (Williamson, Wommack, and Radosevich 2003). Phosphate addition has been shown to increase release of viruses from soil (Hurst, Gerba, and Cech 1980) and promotes virus stability (Jin, Pratt, and Yates 2000) so it is probable that the glutaraldehyde phosphate buffer solution may have acted as an eluting agent alone. Detergents may be more effective in sediment (Danovaro *et al.* 2001) as sediments are saturated and hence viral adsorption may differ from that in unsaturated soil.

Considerably slower or faster centrifuge speeds could be used to extract more bacteria or viruses respectively, but the speed of 600 *g*, in line with those used in other studies (Table 2.2), was taken as a suitable compromise for simultaneous extraction of bacteria and viruses. Increasing centrifuge speed may underestimate larger bacteria if they sediment into the pellet instead of remaining in the supernatant (Balkwill, Labeda, and Casida 1975).

Multiple centrifugation and re-suspension steps were not viable, as they would have been too time-consuming for practical use. A compromise of one centrifugation for 10 minutes, stopping to resuspend the pellet, followed by a further centrifugation for 10 minutes was considered the most appropriate way to increase counts without adding considerable time to sample preparation.

There are no clear recommendations in the literature for adequate sampling of supernatants, but Kirchman (1993) suggested having more

slides rather than counting more fields in an effort to reduce variation. If time permitted, then four filters could be made up from four sub-samples for every sampling point throughout an experiment. Filter preparation and enumeration alone at this replication would take over 5 hours for a single datum point. If one or two filters were prepared from four sub-samples, then this dropped to 1½ to 2½ hours per datum point – a more manageable time frame. Filtering too large an aliquot of supernatant resulted in obscuring of viruses and bacteria on the filter surface. A consistent supernatant volume from centrifuged samples was used in each experiment to reduce potential errors that may have arisen from converting counts on the slide to abundance per gram of soil (multiplication factors were 250 for 40 μl supernatant, but 200 for 50 μl supernatant). The aliquot volume used at the start of an experiment may not be appropriate if populations were to rise or fall significantly during the course of it.

The Rhizon samplers gave unsatisfactory results, and cannot be compared with other data as nothing has been published where they have been used in a similar way. The samplers did demonstrate the presence of bacteria smaller than 0.2 μm in soil. A high clay content, and thus increased adsorption, may have been responsible for the reduced viral count in Boot field.

2.5 PROTOCOL SUMMARY

A suitable protocol was established for processing soil for direct enumeration of bacteria and viruses. Whilst there are inevitable limitations and it is labour-intensive, it is preferable to plating as all bacteria extracted are counted, rather than the cultivable few. Here follows the protocol in detail.

Topsoil samples were air-dried to allow 4 mm sieving. Plant material and stones were removed, and the sieved soil rewetted up to 40% water holding capacity. The soil was then stored at 4°C until use (within 3 months). One week prior to use, soil was placed in a loosely tied plastic bag and stored with a jar of soda-lime (to remove CO_2) in an air-tight drum at 20°C. Soil was then ready for use in incubations. Fresh soil samples could also be used, but for consistency the author carried out soil processing as outlined.

Soil sub-samples of 1.5-2 g wet-weight were placed in 15 ml polycarbonate centrifuge tubes. Fixative was with 10 ml of 0.02 μm filtered, phosphate-buffered glutaraldehyde (4% v/v, adjusted to pH 7 with NaOH). Manual shaking the slurry for 30 s was sufficient to disperse larger soil clumps. A 'high power' sonication period of 30 s disrupted small aggregates and dispersed the sample further. Centrifugation was by two 10 min periods at low speed (<600 *g*). Between the two periods, samples were removed from the centrifuge and manually shaken for 30 s.

Concentration of was done by filtering first 1 ml 0.02 μm filtered water, then an aliquot of supernatant (typically 50 μl), which was then

dispersed with a further 1 ml 0.02 μm filtered water onto a 0.02 μm aluminium oxide filter. Aliquots were small to prevent particulate matter from obscuring bacteria or viruses.

Filters were dried in a desiccator after filtration for at least 10 min and then stained by resting on a 100 μl drop of SYBR Gold at 5000x dilution for 15 min in the dark. Filters were blotted dry on clean lint-free tissue and dried in a desiccator. Filters were placed on a clean slide and a 40 μl drop of fresh 'anti-fade' solution applied onto a coverslip and inverted over the filter. A commercial anti-fade solution could be used or, as was used here, 0.1% p-Phenylenediamine (in sterile 50:50 glycerol: phosphate-buffered saline mix). Slides were viewed immediately or were stored at -18°C for less than 24 hours for optimum enumeration.

Enumeration was by epifluorescence microscopy using a Ziess Axioskop with an exciter filter (BP 450-490 nm – set 48 79 09) under x1600 magnification. At least 20 Whipple grids, or fields of view, or 200 bacteria were counted on each filter, depending on their abundance. The counting procedure allowed reasonable validity of counts with respect to the variability expected on filters. For estimates of biovolume, and hence biomass, two or more photographs were taken from each filter.

CHAPTER 3: EFFECTS OF NUTRIENT AMENDMENT ON VIRAL AND BACTERIAL DYNAMICS IN SOIL

3.1 INTRODUCTION

Soil is an environment where moisture and crucial nutrients that support plant and bacterial growth (nitrogen, phosphorus and a source of DOC for bacteria) are often limiting. Small molecular weight dissolved organic and inorganic nutrients will swiftly become incorporated into microbial biomass, with corresponding growth curves occurring typically within 2 or 3 days of a nutrient input (Rutherford and Juma 1992). The addition of water often results in similar responses (Clarholm and Rosswall 1980), but production may not be as marked as with nutrients (Shaver *et al.* 1998). What results after such activity is usually an increased abundance of microbes (Robertson *et al.* 1988), and a build up of recalcitrant compounds that can not swiftly be broken down (Morita 1997).

Bacterial activity is a key factor in determining the rate of phage production and the final number of mature phage released (Lenski 1988). As a general rule a stimulation of bacterial production results in increased bacteriophage production. Substantial work has been carried out on nutrient utilisation by soil microbial populations (Coleman 1994) but this has not extended to investigating bacteriophage. Most research on soil phage has focused on the classification of phage on particular host species (Chapter 1). A few investigations have been carried out on the deleterious effect of phage on susceptible host strains, and

demonstrated differences between strains. For example one fluorescent *Pseudomonas* strain population was reduced nearly 100-fold when inoculated into sterile soil with bacteriophage, whilst abundance of another strain was hardly affected (Stephens, O'Sullivan, and O'Gara 1987).

Nutrient addition studies carried out in aquatic systems show that amendment may not always stimulate phage production. Variable results were found when coastal water was amended with nitrate, ammonium and phosphate, with significant increases in counts occurring only when data were collected on individual hosts rather than at a community level (McDaniel and Paul 2005). Elsewhere viral abundance was significantly increased with the addition of phosphate or ammonium but not with nitrate (Williamson and Paul 2004). The nature of the carbon source may have an effect on virus production; in a microcosm experiment Hewson *et al.* (2001a) showed highest viral abundance with adenosine as a substrate – although glucose had the greatest effect on bacterial abundance. On individual phage-host systems isolated from the North Sea an increase in yeast extract and peptone concentration increased lytic phage production and production of pseudolysogens (Moebus 1997), whilst in the alga *Phaeocyctis pouchetii* combined nitrate and phosphate addition doubled phage production (Bratbak *et al.* 1998).

3.1.1. Hypothesis

Addition of simple sources of nutrients and DOC to soil microcosms will stimulate bacterial activity and hence viral production. One hypothesis is that bacterial abundance will increase in the short term in response to nutrient and glucose addition. The other is that viral abundance will also increase. Viral production is expected to be at the maximum in treatments where nutrients and glucose are present in combination rather than as separate additions. If inorganic nutrients are not limiting within the soil then increases in abundances will only be observed on the addition of a carbon source. If bacteriophage in lytic cycles are responsible for significant bacterial mortality a decrease in bacterial abundance may coincide with increased viral abundance.

3.1.2. Aims

A microcosm study was set up with a nonsterile soil at optimal moisture content for microbial activity with different amendments: glucose as the carbon source, ammonium-nitrate for inorganic N and sodium dihydrogen phosphate + potassium dihydrogen phosphate for inorganic P. Abundances both of bacteria and viruses made over the short term (less than four days) illustrated the effects that amendments have on bacterial activity, in a similar fashion to the evolution of CO_2 for respiration studies. Recording of bacterial mean cell volume (MCV) allowed comparisons between potential virus production and mean community cell size.

3.2 METHODS

3.2.1. Soil Processing

Bypass field topsoil (Table 2.3, p34) was collected, bulked up and kept in a refrigerated room (4°C) for three months before processing as outlined previously (section 2.5).

3.2.2. Microcosm Set Up

Microcosms were set up with 25 g wet-weight soil in sterile 50 ml plastic centrifuge tubes. Gravimetric water content was calculated by drying triplicate 30 g portions at 105ºC for 24 hours and the mean moisture content at the start of the experiment was 29% w/w. Mean bulk density of the microcosms was approximately 1 g cm^{-3}. The amendment levels selected (Table 3.1) were similar to those as used in a study in which Protozoa and soil respiration were investigated (Christensen *et al.* 1996).

Treatment		Volume added (µl)			
		Glucose $C_6H_{12}O_6$	Phosphate PO_4^-	Ammonium Nitrate[1] NH_4NO_3	Sterile deionised H_2O
1	Control				1500
2	+C	278			1222
3	+N			75	1425
4	+P		48		1452
5	+CN	278		75	1147
6	+CP	278	48		1174
7	+NP		48	75	1377
8	+CNP	278	48	75	1174

[1]Glucose and **PO^{4-} were 1M solutions, NH_4NO_3 was 2M

Table 3.1: Nutrient amendments g^{-1} soil applied: 800 µg C (as $C_6H_{12}O_6$), 149 µg P (NaH_2PO_4 and KH_2PO_4 were combined 50:50) and 168 µg N (as NH_4NO_3).

Tubes were kept in an incubator at 18°C with their lids resting at an angle on their tops to allow gas exchange with minimum evaporation. Nitrogen was added as ammonium nitrate as it has been shown that virus production may be enhanced when ammonium is present (Williamson and Paul 2004), and soil bacterial production may be increased with ammonium rather than nitrate (Stapleton *et al.* 2005), whilst nitrate has been shown to increase respiration of rhizosphere bacteria (Söderberg and Bååth 2004).

3.2.3. Bacteria and Virus Counts

For analysis 1 g soil was extracted using a clean metal spatula from near the centre of each tube. Sample times were 20, 35, 50, 65 and 80 hours after amendment. The 1 g sub-samples were put into 15 ml centrifuge tubes and processed as outlined in section 2.5.

3.2.4. Bacterial Biovolumes

Bacterial cell volumes were determined with a Zeiss Axioscope Microscope with an attached image analysis system. Cell dimensions of 60 random, in focus, bacteria per sample per treatment at each sampling time were recorded using the Axiovision image software as described in section 2.2.2.2.

3.2.5. Statistical Analyses

Bacteria and virus counts were log transformed for normality before analysis with Genstat (version 7.0 VSN International). Standard errors of the mean were generated through general analysis of variance.

3.3 RESULTS

3.3.1. Control

There were marked increases in bacterial abundance by 20 hours in the control in which water only was added (Figure 3.1a, open symbols). Bacterial and viral abundances were both 2.3 $x10^7$ particles g^{-1} dry soil at the start of the incubation, and by 20 hours bacteria had increased two-fold to 5.0 $x10^7$ cells g^{-1} dry soil. Bacteria counts decreased at 35 and 50 hours until a low count of 2.8 $x10^7$ cells g^{-1} dry soil at 65 hours. By 85 hours bacteria counts had increased to 3.9 $x10^7$ cells g^{-1} dry soil by 85 hours. Bacterial mean cell volume (MCV) increased from an initial 0.25 μm^3 to 0.29 μm^3 at 20 hours, and as with the abundance data decreased until a low of 0.14 μm^3 recorded at 65 hours (Figure 3.2a, open symbols). The final MCV was 0.29 μm^3. The increased bacterial abundance and cell size indicated a response to incubation set-up, presumably through physical disturbance of the soil and temperature changes.

Viral counts increased five-fold from 2.3 $x10^7$ particles g^{-1} dry soil to 13 $x10^7$ particles g^{-1} dry soil at 20 hours (Figure 3.1b, open symbols). Viral abundance then decreased at 35 hours to 11 $x10^7$ particles g^{-1} dry soil but unlike bacterial counts then steadily increased to a maximum of 23 $x10^7$ particles g^{-1} dry soil at 85 hours.

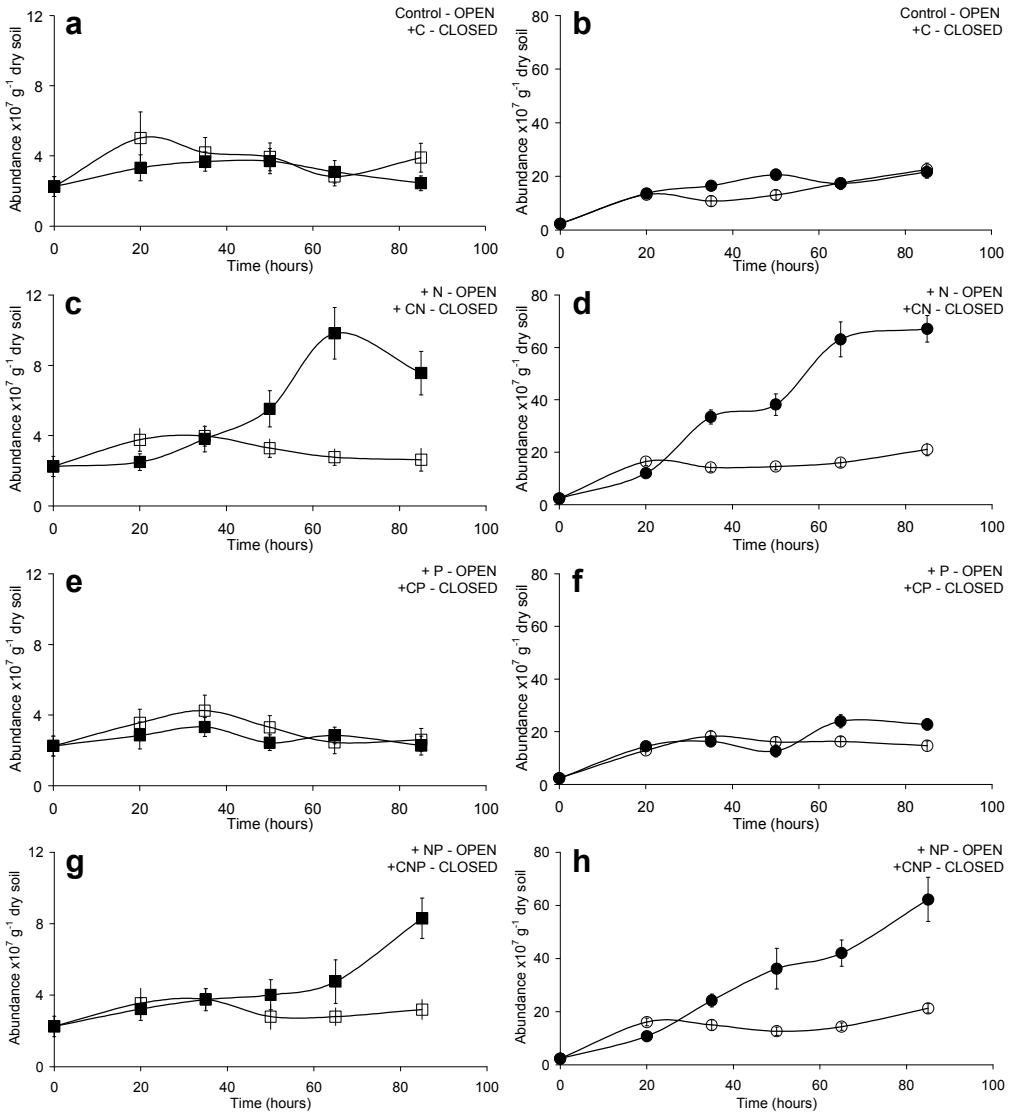


Figure 3.1: Bacterial (a,c,e,g) and viral (b,d,f,h) abundance over time in unsterile soil with nutrient amendments, with (■●) and without (□○) Carbon. P and N were added as $*PO_4^-$ and NH_4NO_3, C added as $C_6H_{12}O_6$. Standard error shown (n=3). Note differences in scale between bacteria and virus charts.

3.3.2. Carbon Addition

Bacteria counts in +C increased to 3.3 x10^7 cells g^{-1} dry soil by 20 hours (Figure 3.1a, closed symbols). This value was 66% of the control. Bacterial counts in +C were smaller than the control at all time points, except at 65 hours, where they were insignificantly greater. MCV by contrast was generally higher (101-186% of control) in +C than in control, apart from at 20 hours when at 0.29 μm^3 it was 74% of the control (Figure 3.2a, closed symbols). The addition of carbon

presumably stimulated bacterial activity (possibly reflected in larger MCV values in +C over control), but inhibited population growth.

Virus counts were marginally higher in +C than in the control at 20, 35 and 50 hours, but then decreased relative to the control at 65 and 85 hours (Figure 3.1b, closed symbols). The increase by 20 hours was not significantly greater than the control, at 14 x10^7 particles g^{-1} dry soil. Differences between +C and control increased to 50 hours, where virus counts were 21 x10^7 and 13 x10^7 particles g^{-1} dry soil for +C and control respectively. Hence carbon addition significantly stimulated viral production up to 50 hours, although net bacterial abundance was unaltered or decreased compared with the control.

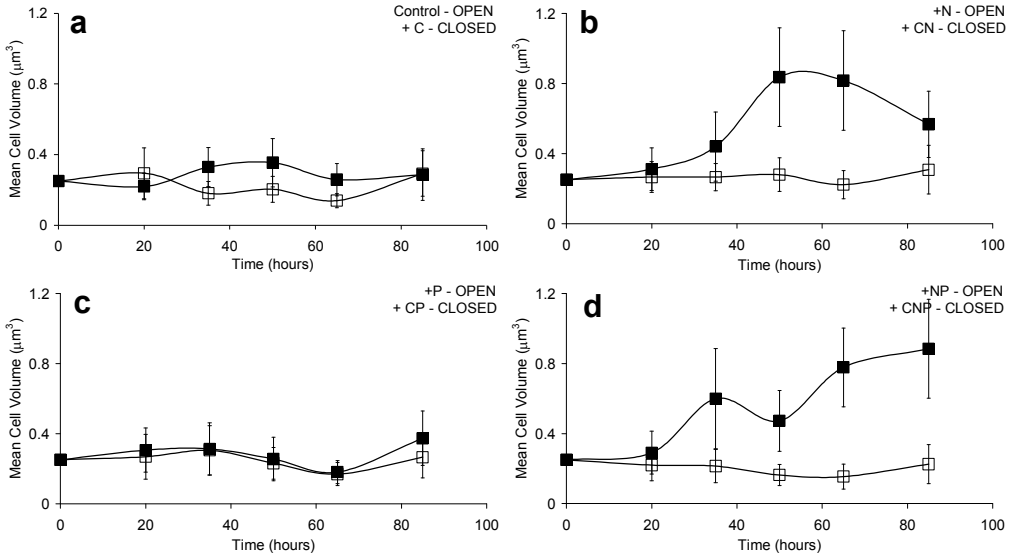


Figure 3.2: Bacterial mean cell volume over time under different nutrient amendments with (■) and without (□) carbon as glucose. Standard error shown (n=6)

3.3.3. Nitrogen and Phosphorus Addition

In the absence of C (Figure 3.1, open symbols) bacterial counts increased within the first 20 hours: in +N to 3.8 x10^7 cells g^{-1} dry soil (Figure 3.1c, open symbols) in +P to 3.6 x10^7 cells g^{-1} dry soil (Figure 3.1e, open symbols) and in +NP to 3.6 x10^7 cells g^{-1} dry soil (Figure

3.1g, open symbols). These counts were all lower than the control count of 5.0 $x10^7$ cells g^{-1} dry soil (Figure 3.1a, open symbols). In these treatments most bacterial counts were lower than the control: in +N were 68-98%, in +P were 67-102% and in +NP were 71-99% of control counts. Bacterial counts in +N, +P and +NP at 50 and 85 hours were significantly ($p<0.05$) smaller than the control. Addition of nitrogen and phosphorus singly or combined therefore had a negative or no effect on bacteria counts.

There were differences in bacterial MCV between the treatments +N, +P and +NP (Figure 3.2b.c.d, open symbols) when compared with the control (Figure 3.2a, open symbols). MCV ranged in +N from 0.22-0.31 μm^3, in +P from 0.17-0.30 μm^3 and +NP from 0.15-0.23 μm^3. These values were in the range of 90-170% of the control, at 0.14-0.29 μm^3.

The initial increase in virus counts in +N, +P and +NP was slightly greater than the control. At 20 hours virus counts were 16 $x10^7$ particles g^{-1} dry soil for +N (Figure 3.1d, open symbols), 13 $x10^7$ particles g^{-1} dry soil for +P (Figure 3.1f, open symbols), and 16 $x10^7$ particles g^{-1} dry soil for +NP (Figure 3.1h, open symbols). At 35 hours the difference between amendments and the control was at its highest and most significant ($p<0.005$), when virus counts in +N, +P and +NP were 130-170% of the control count of 11 $x10^7$ particles g^{-1} dry soil. The positive effect of nutrient amendment on viral production lasted less than 50 hours as all virus counts in +N, +P and +NP were lower than the control by 65 and 85 hours. Combined amendment of N and P had no additive effect on virus counts over single additions.

3.3.4. Carbon with Nitrogen or Phosphorus

Addition of carbon in nutrient combinations initially reduced bacterial counts relative to the control or corresponding treatment without carbon (Figure 3.1 c-h, closed symbols). Only after 35 hours were increased bacteria counts recorded. At 20 hours bacterial counts in +CN were 66% of +N counts (Figure 3.1c), in +CP were 80% of +P counts (Figure 3.1e) and +CNP were 91% of +NP counts (Figure 3.1g).

By 35 hours counts in +CN, +CP and +CNP were still smaller than the equivalent treatment without carbon, but the difference had reduced (Figure 3.1c,e,g). By 50 hours bacterial counts in +CN and +CNP exceeded those of +N and +NP by 140% and 170% (Figure 3.1c,g), and continued to exceed them for the remainder of the incubation. By 85 hours bacterial counts in +CNP were 8.3 $x10^7$ cells g^{-1} dry soil, respectively 210% and 260% of control and +NP counts. In contrast, counts in +CP were smaller than in +P throughout, except at 65 hours where +CP counts were 120% of +P, at 2.9 $x10^7$ cells g^{-1} dry soil (Figure 3.1e).

MCV in +CN was already larger than +N at 20 hours (Figure 3.2b), and the trend continued throughout the incubation. At 50 hours MCV in +CN was 0.84 μm^3 compared to 0.28 in +N (Figure 3.2b). A similar difference occurred at 65 hours, but by 85 hours MCV had decreased to 0.57 μm^3 in +CN. MCV were also slightly greater in +CP than in +P until 85 hours, but the difference was less than 0.04 μm^3 (Figure 3.2c). The only marked difference was at the end where MCV was 0.37 μm^3 for +CP against 0.27 μm^3 for +P. MCV in +CNP were

larger than +NP throughout the incubation, and reached a maximum of 0.88 μm^3 by 85 hours (Figure 3.2d), the largest MCV recorded.

Comparisons of virus counts in +CN and +N showed that at 20 hours +CN counts were 73% of +N, but subsequently were 240-390% of +N counts (Figure 3.1d). They reached their maximum (and the maximum recorded for the study) of 67 $x10^7$ particles g^{-1} dry soil at 85 hours. This significant ($p<0.001$) increase indicates that combined, carbon and nitrogen stimulated phage production, but each applied singly did not. Virus counts in +CP were similar to +P at 20, 35 and 50 hours and only increased to 150% of +P toward the end of the incubation (Figure 3.1f). Counts with +CP were therefore only slightly greater than the control. Virus counts in +CNP were lower than +NP and control at 20 hours but then increased at each sampling point until a maximum at 85 hours of 62 $x10^7$ particles g^{-1} dry soil, 275 and 290% of control and +NP counts (Figure 3.1h).

Therefore the combination of carbon and phosphorus had a negative effect on bacterial abundance, and only moderately increased MCV and viral abundance. This was in contrast to combinations with carbon and nitrogen. Of note was the delayed positive effect on abundances when carbon was added, compared to the equivalent amendment without carbon, thus the addition of a suitable carbon source with N enhanced growth.

3.3.5. Virus to Bacteria Ratio

Virus-to-bacteria ratios increased slightly over the incubation period and C addition resulted in higher VBR than corresponding

treatments without C (Figure 3.3a, closed symbols). VBR in treatments without C peaked at 6:1, with the exception of +N, which rose to >8:1 at 85 hours (Figure 3.3b, open symbols). In treatments with C, VBR were similar irrespective of amendment treatments but VBR in the control (Figure 3.3 a) was considerably lower than in all treatments.

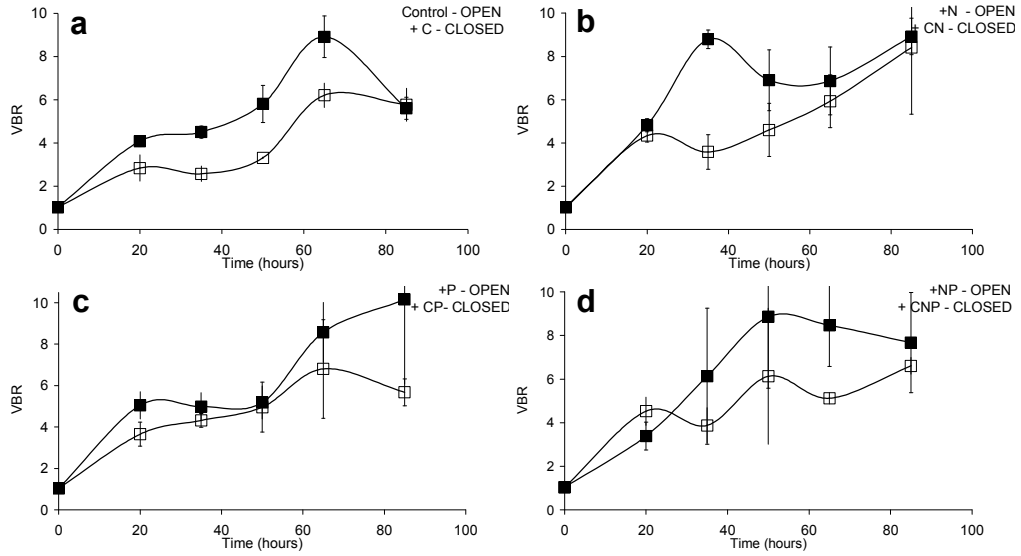


Figure 3.3: Virus-to-bacteria ratios over time under different nutrient amendments with (■) and without (□) carbon as glucose. Standard error shown (n=3)

3.4 DISCUSSION

The results from this amendment study demonstrated that nutrient addition to unsterile soil increased bacterial and viral abundance. The initial hypotheses were upheld, although there were marked differences in the extent of increases in bacteria and viruses dependent on the combination supplied. Carbon and nitrogen were limiting to bacterial growth and viral production as increased bacterial and viral populations were only recorded in +CN and +CNP treatments. The phosphorus status of the soil was not determined but was unlikely to be limiting, as the field has been in agricultural use with standard 'NPK' fertiliser application for some time.

The reduction in bacterial counts with a variety of amendments may be attributable to several causes. If protozoan populations were stimulated by increases in bacterial production then grazing would have increased. The low value for bacteria counts would in this case represent a net count in which grazing removed bacterial production. Protozoa counts were not performed, but it has been shown that increased C and N combined increase Protozoa in soil over single additions (Christensen *et al.* 1996). Given the decrease in bacterial populations, substantial grazing must have occurred within 20 hours. Excystment of protozoa on water addition is rapid, often taking place within 24 hours (Clarholm 1981) and can be as short as one hour (Ekelund and Rønn 1994), although only fast-growing flagellates are likely to respond so quickly, so grazing may have accounted for some loss of bacterial production.

Phage can reduce populations of susceptible bacteria in soil. In one microcosm study with *Bacillus subtilis*, bacterial abundance was significantly reduced within one day of incubation with inoculated temperate phage but thereafter (up to 200 days) abundance was similar with or without the phage present (Pantastico-Caldas, Duncan, and Istock 1992). Separate incubation for 45 days with a virulent phage decreased the bacterial population for a whole period, with phage rapidly decreasing in abundance when incubated alone or with a resistant strain of *Bacillus subtilis*. Applying findings from that study here, it may be inferred that bacteria were susceptible to phage attack, and phage populations were sustained by interactions with the bacteria. Given the large populations of bacteria normally resident within soil, phage

probably do not cause continued permanent losses at the community level (Marsh and Wellington 1994).

In one batch-culture experiment with 1 μm filtered seawater (thus Protozoa-removed), a similar rise of bacterial abundance from 0-50 hours was recorded, followed by a fall at 65 hours. Viral abundance throughout the same period continued to increase, hence viruses were likely responsible for the decline in bacterial abundance (Middelboe, Nielsen, and Bjørnsen 2002). In that study, viral doubling times ranged from 1.2 – 15 days, so viral mortality of natural bacterial assemblages can be rapid. Bacterial production and viral abundance correlated postively (r=0.57, n=58), so more active bacteria produced more viruses. Bacterial production was not estimated, but viral and bacterial abundance over the whole dataset correlated positively (r=0.69, n=120) (Figure 3.4).

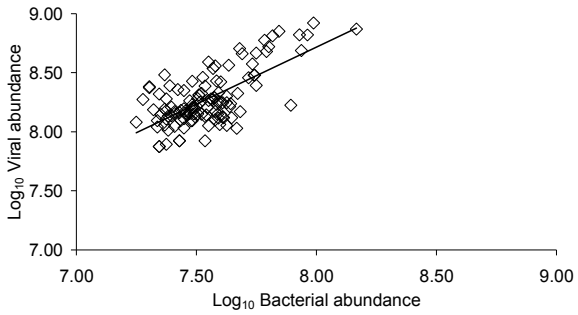


Figure 3.4: Log-log scattergraph of bacterial and viral abundance. All data are plotted.

Attributing bacterial mortality to phage in this study is difficult due to unknown gross bacterial abundance, predator populations and frequency of infected cells. Because MCV was generally larger in the amended tubes, it may be assumed that bacterial activity was also greater. Glucose addition increased bacterial production in seawater, but N and P did not (Middelboe, Nielsen, and Bjørnsen 2002). Marked

differences in MCV between glucose and N and P treatments in this study suggest a similar finding here. Another study of freshwater bacterial assemblages found that single additions of glucose or N or P did not significantly increase bacterial production, only in combination (+CNP) of all three (Anesio *et al.* 2004). Despite increased bacterial production and abundance in +CNP between 10 and 72 hours incubation, viral abundance was much reduced when compared with +C, +NP or control at the same time points. Significant increases in viral abundance in +CNP were only recorded at 360 hours. That study indicated high viral adsorption to host cells and a long latent period as an explanation for the delayed release of viruses. As evidenced by the continued increase in virus and bacteria counts in +CNP treatment in this study it is unlikely that similar mechanisms are at work here.

Glucose has been used as a carbon source in a variety of studies with differing results. Glucose decreased bacterial abundance and increased virus abundance when compared to counts from incubations with fulvic acids as the carbon source in one study (Anesio *et al.* 2004). In another study glucose increased bacterial abundance as compared against incubations with glutamate, ATP and other C sources, but virus abundance was similar (Williamson and Paul 2004). Hence there are variable responses to the addition of glucose to natural bacterial communities. Reduced N or P from the growth medium with the autotrophic *Phaeocystis pouchetti* resulted in smaller virus counts and smaller burst sizes than from full medium (Bratbak *et al.* 1998). Algae were significantly reduced within 3 days by viruses, with concomitant increases in virus numbers.

The magnitude of difference in mean cell volume between treatments also implies that protozoan grazing was not disproportionately removing the larger cells, yet the soil was sufficiently wet for optimum grazing pressure to occur. As the soil had quite high clay content, it is likely that many protected sites occurred within it, and thus reduced grazing pressure existed (Juma 1993). Hence whilst it is highly probable that grazing accounted for some decreases in bacterial abundance, other factors were also at play.

Virus production can thus be very rapid in soil at ambient temperatures on addition of simple nutrient and carbon sources. The combination of carbon with nitrogen is a stimulant to viral production, with concomitant increases in bacteria numbers. Phage were not responsible for significant bacterial mortality in this soil over the 3.5 day period investigated. Based on these findings it is suggested that bacteria and virus abundance would be increased in (high fertility) soils where C and N are not so limiting.

3.5 SUMMARY

Nutrient limitation of bacterioplankton has been shown in aquatic systems to result in decreased phage production. The effect of adding glucose, ammonium nitrate and phosphate on phage production in soil was examined, with the hypothesis that increased carbon source and inorganic nutrients would increase virus production and hence abundance. Addition of a combination of carbon and nitrogen resulted in significant increases in bacterial and viral abundance, but nitrogen and phosphate addtions alone or combined had negligible effects on virus abundance. Mean bacterial cell volume doubled from control values when carbon and nitrogen were present in combination. Phosphorus addition appeared to have no impact on bacteria abundance, mean cell volume or lytic phage production. These data indicate that soil bacteria respond to nitrogen and carbon addition by increasing phage production, which is similar to findings in aquatic systems.

CHAPTER 4: EFFECTS OF VARYING SOIL MOISTURE CONTENT ON VIRAL AND BACTERIAL DYNAMICS

4.1 INTRODUCTION

Moisture availability is critical to bacterial survival and activity. Too much or too little available water creates saturated or dry conditions, which decreases bacterial activity as oxygen or water become limiting (Leffelaar 1993). Anaerobic bacteria typically comprise a small (<5%) fraction of the total number of bacteria in soil (Linn and Doran 1984). The system is dominated by aerobic metabolism, consequently total respiration and bacterial populations are typically largest at intermediate soil water contents (Williams *et al.* 1972).

Successive drying may result in different stresses for bacteria in a sandy soil and in a clay soil (Theng and Orchard 1995). In the former, water will leave the soil completely, whereas in the latter more will be retained, but through its association with clay, will have high negative potential and may not be available for microbial use (Stotzky 1986).

Bacteria that are actively growing are more likely to be suitable hosts for phage in the lytic cycle and may trigger replication of lysogenic phage, producing more phage (Lenski 1988). In addition, studies with inoculated viruses have shown that wet conditions allow greater movement of viruses within soil columns (Chu *et al.* 2003), so if this holds true for natural systems then there is a greater probability of finding hosts and replicating when the soil is wet. It is expected that viral abundance will be correlated to bacterial abundance, with greatest

numbers at intermediate soil moisture levels. The relationship between viruses and bacteria in soil is not simple, however, as numerous factors influence the susceptibility of the bacterial population to viral attack such as dormancy, long or short generation times, spore formation, and population size (Farrah and Bitton 1990; Williams, Mortimer, and Parry 1999). Phage on *Azospirillum brasilense* were shown to require host populations exceeding 10^3 cells g^{-1} soil, yet were continually present at low levels throughout the study (Germida 1986).

4.1.1. Spatial Location of Microbes within Soil

Bacteria are not uniformly distributed within the soil. There is a greater abundance in the topsoil and rhizosphere than in bulk or subsoil (Sørensen 1997). *Azospirillum* population densities vary, comprising up to 10% of total bacteria in rhizosphere but insignificant (<1%) in bulk soil communities (Bashan 1999) – thus host location will also contribute to phage dynamics. Micro-architecture gives rise to spatial variability; sequential analysis of soil aggregates showed a higher proportion of bacteria (colony forming units) in aggregates <0.1 mm than in larger aggregates (Gupta and Germida 1988). Aggregation may affect the distribution of bacterial groups in soil. For instance in one study *Actinobacteria* predominated inside aggregates and *Proteobacteria* predominated on the outer surfaces of aggregates (Mummey and Stahl 2004). Moreover, Gram negative bacteria are found more within aggregates (Hattori 1973). The spatial difference in communities might be a reflection of pressure from selective grazing on different bacteria

groups by grazers such as protozoans and nematodes as well as differences in environment.

Soils that are habitually wet are probably largely anoxic and likely to have reduced microbial activity compared with drier soils (Torbert and Wood 1992). They may support different bacterial communities compared to drier soils; novel members of *Verrucomicrobiales* were found in anoxic paddy soil (Janssen *et al.* 1997). These were small (volume $<0.1\mu m^3$) bacteria that have also been found inside small aggregates (Mummey and Stahl 2004). The implication is that other bacteria within aggregates may also be small. It may be a microhabitat that specifically favours small cells. Clayey soils that undergo periodic flooding may be anaerobic (Smith 1977), particularly in larger aggregates (Sierra and Renault 1996). Sandier soils by contrast are unlikely to remain anoxic for long provided there is sufficient drainage (Stepniewski, Glinski, and Ball 1994). Management practices that compact soil also tend to reduce microbial biomass (Beare, Hendrix, and Coleman 1994; Beare *et al.* 1994).

Thin sectioning and fluorochrome staining techniques have been developed since the 1970's enabling the photography of bacteria *in situ* (Balkwill, Labeda, and Casida 1975). Recent work employs sophisticated processing on photographs of thin soil sections to enhance resolution and remove background noise. After several image transformation steps, automatic determination of bacteria stained with a fluorochrome has been achieved (Nunan *et al.* 2001; 2002; 2003). In this way the vertical distribution of soil bacteria was assessed, with abundances rapidly declining below the plough layer of the sampled field

sections, and confirmed spatial aggregation and clustering of bacteria within soil profiles.

4.1.2. Soil Moisture Affects Predation

The influence of Protozoa on bacterial activity is documented in Chapter 5. It is recognised that protozoan grazing stimulates bacterial turnover (Elliott *et al.* 1980; Görres *et al.* 1999). In aquatic systems bacteria are more vulnerable to predation as there are few exclusion zones which the Protozoa cannot enter. The soil system allows three main opportunities for bacteria to escape from predation: pore openings, aggregate formation and moisture stress.

Bacteria will be protected in pores that have openings that are too small to allow Protozoa to enter. Intuitively, the size of protozoa can be used as an approximate guide to cut-off points for their movement. Some protozoa are able to change their cell shape in order to squeeze through these small cavities, but in general large ciliates and testate amoebae are restricted to large cavities, whilst naked amoebae and flagellates can access much smaller spaces. As such, the spatial variability of protozoa in soil is likely to be considerably greater than that of bacteria. In one study it was shown that over 10% of aggregates harboured no protozoa at all (Vargas and Hattori 1986) and it is not unexpected that flagellates have been found to be most widespread, and amoebae most abundant, in soil (Vargas and Hattori 1990). Inoculation studies in which identifiable bacteria were placed within soil pores of differing sizes indicate that openings of less than 6 μm offer protection. *Pseudomonas fluorescens* survived predation from *Colpoda steinii* more

in pores of <6 μm than in pores of 6–30 μm (Wright *et al.* 1993). Introduced rhizobia had higher survival rates when there were more pores of <6 μm than in controls (Heijnen and van Veen 1991) as seen in other studies (Postma and van Veen 1990).

A formula (Formula 4.1) can be used to describe the maximum pore size that would remain full of water as suction is increased on a soil. S is the suction, γ is the surface tension of water, ρ the density of water, *g* the acceleration due to gravity and *r* the radius of the pore (Marshall and Holmes 1979).

$$S = 2\gamma\rho gr$$

Formula 4.1: The relationship between pore size and suction

Soil under suctions of over 500 mbar (potential of –50 kPa) will have most pores of 6 μm drained of water (Table 4.1) so it can be assumed that grazing will be restricted at or beyond this point. Recording bacterial abundance at soil moisture contents beyond this may give some indication of how viral lysis influences the population size.

Potential (-kPa)[a]	0.1	1	3	10	50	100	500	1500
Drained pore diameter (μm)	3000	300	100	30	6	3	0.6	0.2

a. Conversion of suction to potential: 1mbar = 0.1kPa.

Table 4.1: Showing the relationship between suction applied to a soil and the size of pores remaining full of water, based on Formula 4.1.

Due to the complexity of its structure soil contains protected sites for bacteria (Hattori 1994; Hassink and Whitmore 1997). High concentrations of organic nitrogen can be located in pores <0.6 μm (Strong, Sale, and Helyar 1998), presumably as bacteria are protected from grazing. This is illustrated by the study of bacterial and protozoan

populations in the inner and outer zones of aggregates. The bacterial population was stable at 10^8 g^{-1} soil when the ciliate *Colpoda* were not able to enter the aggregate, but decreased to 10^4 cells g^{-1} soil when aggregates were disrupted by sonication – allowing the Protozoa access (Vargas and Hattori 1986).

At high water contents, Protozoa can move between aggregates and through the smallest pore openings their size allows. At low moisture contents, continuous water pathway length decreases and the thickness of the water film decreases, reducing protozoan movement. As moisture becomes limiting, Protozoa may change their motility mechanisms, prey targets or feeding method, or encyst (Vickerman and Cox 1967; Foissner 1987).

If the majority of soil bacteria are in a starvation phase most of the time (van Elsas and van Overbeek 1993; Morita 1997), altering the soil moisture might not have much effect, in which case virus production will be low and unchanging throughout a drying period. There is evidence from batch experiments that virus production is reduced in starvation conditions (Schrader *et al.* 1997), but moisture stress may not result in a similar outcome. A detectable proportion of bacteria are active in soil as populations fluctuate over short time scales (Clarholm and Rosswall 1980), so some virus production is likely. The effects of drying on this production might be two-fold: many bacteria might release lysogenic phage, or ordinarily virulent phage might switch replication strategies and enter pseudolysogeny. In addition, increased adsorption and decreased movement of viruses may offer bacteria protection from viral lysis (Williams, Mortimer, and Parry 1999).

Evidence suggests that in drier conditions there will be stronger adsorption to solid interfaces in the soil (Stotzky 1986), which may increase survival (England, Lee, and Trevors 1993). Natural soil microbes survive periods of desiccation and can rapidly 'resurrect' once conditions are more favourable: bacteria counts were reduced to <1 $x10^4$ cells g^{-1} in soil dried to 1.4%, but on incubation with 1% sterile glucose solution increased to 10^7 cells g^{-1} soil (Sparling and Cheshire 1979).

Changes in soil moisture content will affect virus adsorption (Chapter 1). Viruses in soil can be adsorbed to bacteria or particles, suspended in water or on air-water interfaces (Sim and Chrysikopoulos 1999). As they are non-motile they will move if the water around them moves so the likelihood of encountering new host cells is increased. When soil dries fewer viruses will be suspended in the water and will adsorb to surfaces, thus reducing the chance of infecting new bacteria.

Strongly adsorbed bacteria may be more active than those found in the free soil water (Bååth 1996), although this may be short-lived if fresh nutrient input is lacking (Stotzky 1986). In aquatic systems increased bacterial activity leads to higher viral production and greater bacterial mortality (Middelboe, Glud, and Finster 2003). Adsorbed viruses can remain active outside bacteria for several days in aquatic systems (Middelboe, Jørgensen, and Kroer 1996), so they may do likewise in soils.

4.1.3. Site Variability

Ideal samples for analysis of microbial communities in soil are intact cores, as the gross architecture of the soil is also taken into

account. It is likely that macro-channels in soil make a significant contribution to water flow and this is not captured in repacked soil cores. Furthermore, the use of intact soil cores allows for undisturbed communities to be placed under moisture stress. Highest microbial biomass carbon was recorded in moist intact cores (Franzluebbers 1999), followed by dried intact and then sieved - soil drying was more significant than sieving overall. Natural variability in soil structure is a drawback against intact soil cores: analysis of 25 intact cores had coefficients of variation of over 300% for nitrogen mineralisation (Sierra 1997). A preliminary study was set up to determine whether the use of intact cores was feasible for the subsequent drying experiments.

4.1.4. Hypotheses

Bacterial activity will decrease as soil moisture becomes limiting. Within this study three hypotheses may be tested. First, that reduced bacterial activity will lead to a concurrent decrease in viral abundance as soil moisture decreases. Second, that decreased viral abundance is likely to be recorded at a lower suction in sandy than in clay soil, given the potential buffering capacity of clay. Hence the third, that there will be no difference between counts of bacteria and viruses over several suctions in clay.

4.1.5. Aims

To enumerate bacterial and viral abundance and bacterial mean cell volume over a series of suctions in contrasting soils. As soil dries the effect of protozoan grazing is expected to diminish, so it is of interest whether this results in changes in virus production. Comparison of

abundances of intact and re-packed soil cores give indication of spatial variability of bacteria and viruses within the soil. The hypothesis is that bacteria will be distributed more heterogeneously in soil than viruses.

4.2 METHODS

4.2.1. Moisture Tension

The water content of soils can be regulated in soil cores. Suction is applied by gravitational flow or by vacuum pump, creating a pressure potential within the system. Sand and clay tables are capable of drying to maxima of around –8 kPa and clay up to –40 kPa (Soil Survey 1974). Further drying can be carried out using a pressure membrane apparatus, which can exert potentials of up to –1.5 MPa. Soil samples need to equilibrate for from a few days to more than a fortnight at each potential, so the duration of one of these experiments can last several months.

Moisture release curves relate pressure potential to water content for each soil. As the sampling for bacteria and virus counts disrupts the cores, the resultant curve for each soil is made up from several cores, all of which were sampled for counts. The curve allows estimations of the abundance of different pore size classes within the soil, which may assist in explaining any differences observed in bacterial or viral abundance as the soil dries.

4.2.2. Soils Selected

Soils of contrasting organic matter and clay content were chosen to determine if fluctuations of bacterial populations might be influenced

by soil properties. The soils were taken from various sites around Wrest Park, the site of Silsoe Research Institute (Table 2.3, page 34).

4.2.3. Spatial Variability

Bypass field was selected to look at spatial variability of bacterial abundance. The site was appropriate for road building nearby had disturbed the field when earth was excavated and relocated. The topsoil had been replaced and had been subsequently cropped, but a consideration was that soil further away from the road would be less disturbed than that closer to it.

Four cores were removed from a metre-wide circle from each of five sites located approximately 10, 30, 50, 70, 90 metres away from the roadside. These cores were allowed to equilibrate on a sand tension table at –5 kPa for 3 days to allow standard suction on each core. The average moisture content was 26% (range 25-27%).

4.2.3.1. Bacteria and virus counts

Slides were prepared as outlined in section 2.5.

4.2.4. Soil Drying Effects on Bacteria and Viruses

Plots were chosen at random from within each field away from driveways, field edges, tramlines and other experimental plot areas. After removing the top 10–20 mm surface layer soil was collected by spade and emptied into clean polythene bags for sieving through a 4 mm sieve. Three cores (40 mm height, 50 mm diameter) were placed grid-wise within a 2 square metre plot. The cores were pushed into the soil until the uppermost surface was flush with the top of the soil.

Sieved soil was repacked into fresh cores at a bulk density equivalent to the average bulk density of three intact cores, sampled from the same site. Repacked cores were placed on sand and kaolin tension tables in a constant temperature room set at 20°C. The cores were saturated over a period of 24 hours with de-ionised water and left to stabilise for a further 48 hours prior to sampling at the first stage with longer equilibration times as the soil dried. Three sampling stages were taken on the sand tables and one or two from the kaolin table, covering a range of soil water potentials from –1 to –30 kPa.

Soil samples, equivalent to half a core, were placed onto pressure plates to reach drier states. Five or six sampling stages were taken, covering potentials from –100 kPa to –1.5 MPa. At each stage three or four replicate cores were removed and a 7 mm diameter cork borer used to extract 1 g of soil vertically from the centre of the core. Garden Gate and Cashmore soils had triplicate slides made for three replicates and for the clay Cashmore soil duplicate slides of four replicates were made.

4.2.4.1. Bacterial and viral counts

Slides were prepared as outlined in section 2.5.

4.2.4.2. Mean cell volume and biomass

Mean cell volume and biomass were determined as outlined in section 2.2.2.2.

4.3 RESULTS

4.3.1. Site Variability

Bacteria and virus counts were generally similar between the five sites of Bypass field (Figure 4.1). Across the five sites bacteria and virus counts were 0.9 $x10^8$ cells and 4.2 $x10^8$ particles g^{-1} dry soil respectively, with coefficients of variance of 77% and 43%. Counts were variable within the two replicates in each core, but when means from several cores were collated there was little variability between sites (Figure 4.2). After checking for normality of distribution, a general analysis of variance was carried out. Most variability within bacteria counts came from cores ($p=0.03$) or the two replicates within each core ($p<0.001$), rather than between sites ($p=0.846$). Variability in virus counts were core ($p<0.001$), within core ($p=0.011$) and site ($p=0.016$). Differences in habitats and bacterial abundance at the centimetre scale are masked when data are collated from several samples as variability of counts within cores was higher than that over tens of metres.

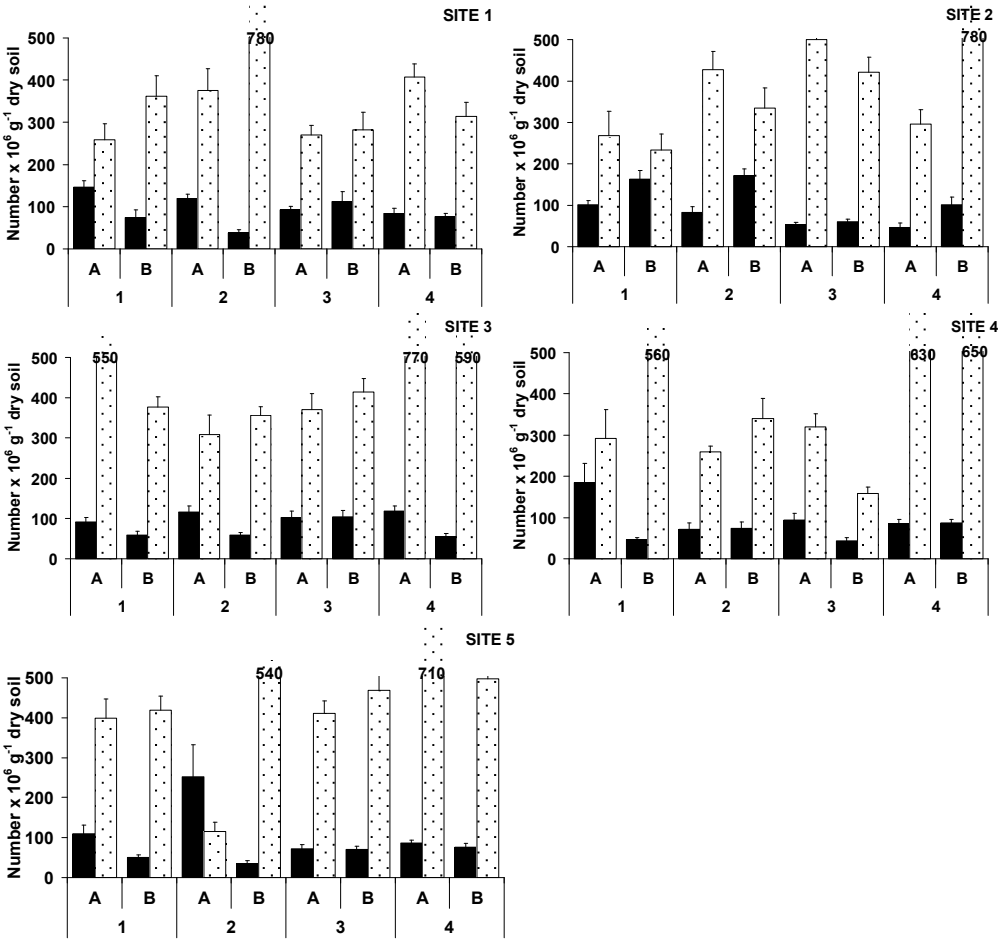


Figure 4.1: Abundances of bacteria (solid columns) and viruses (dotted columns) within (A+B) and between four cores from sites on Bypass field. From each core two duplicate samples, A + B, were made. Site 1 closest to and site 5 furthest from road excavation disturbance. Standard error shown (n=2).

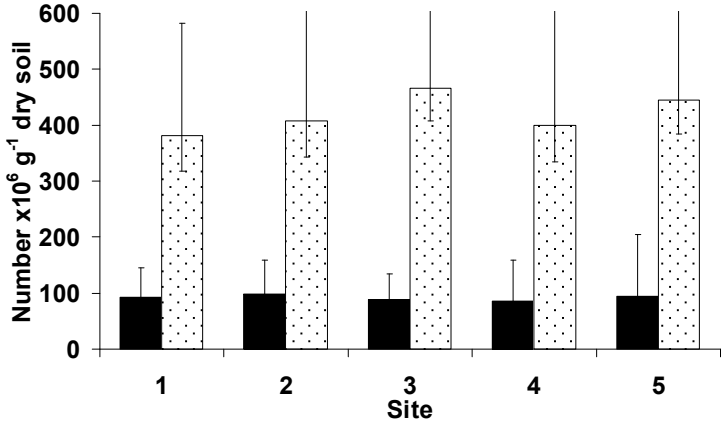


Figure 4.2: Mean abundances from 4 cores of bacteria (solid columns) and viruses (dotted columns) at each of five sites. Site 1 closest to and site 5 furthest from road excavation disturbance. Standard error shown (n=16).

As the field had undergone disturbance in the past, some differences in microbial abundance were anticipated but were not obvious. Analysis of the variance of the counts was carried out to verify this. F-test analysis at a 95% confidence level for variance did show that sites 4 and 5 (furthest away from the road) had significantly greater variances ($p<0.001$) in bacteria counts than sites 1-3. There was no significant difference in variances ($p>0.1$) of virus counts between sites. Disturbance of the soil appears to have decreased the spatial variability of bacteria slightly but not viruses. This is expected if the majority of bacteria are adsorbed onto soil particle surfaces. Newly released viruses however will move passively within soil water films before adsorbing either to bacteria or particles.These data prompted the use of repacked cores for the future studies involving drying soils down to –1.5 MPa, as the replication required for reasonable variability of data would be lower.

4.3.2. Soil Moisture Effects on Bacteria and Viruses

4.3.2.1. Moisture release curves

The two sandy soils exhibited characteristic drying patterns, whilst the drying regime had little impact on the clay soil (Figure 4.3). From these curves and Formula 4.1 it can be inferred that the sandy soils contained a significant number of pores greater than 6 μm, but that most water in the clay Cashmore soil was within pores in the region of 0.2 μm.

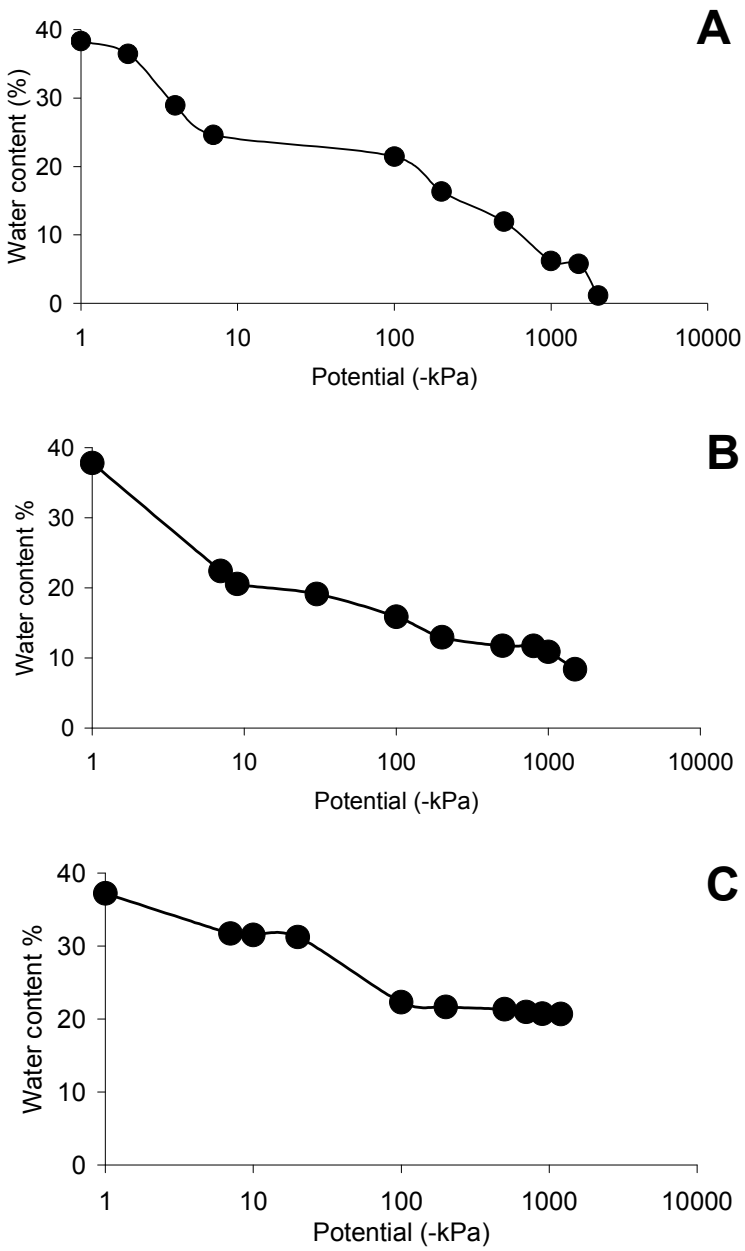


Figure 4.3: Moisture release curve for soils used in the drying study. Points are the mean of 3 or 4 cores. A – Garden Gate, B – sandy Cashmore, C – clay Cashmore.

Using the conservative cut-off for protozoan exclusion of 3 μm, pressure potentials between –50 and –100 kPa is where grazing is likely to be minimal. Approximate moisture contents for each soil at this potential are; Garden Gate, 21%; sandy Cashmore, 16%, clay Cashmore 22%. Virus-induced mortality of bacteria would be more significant than predation at these and lower moisture contents.

Variability in water contents between repacked cores over the drying period was small (standard deviations in Figure 4.3 were typically <5%), which indicated that the cores had been dried to a similar extent at each suction, and that repacking was reasonably consistent. The kaolin suction table was difficult to maintain suction on, so although water content in samples did decrease, the measured suction may have been any value between –10 and –40 kPa. Data collected from the table have been removed if anomalous.

4.3.2.2. Bacterial and viral counts

Bacterial and viral counts in all three soils were at or close to their maximum at –1 to –10 kPa potential, with the exception of virus counts in the clay Cashmore soil, which fluctuated throughout. Viral counts tended to mirror bacterial counts over the drying period, but significant peaks in viral abundance were seen in Garden Gate and sandy Cashmore, the two sandy soils. The drying periods differed between soils; as expected the sandier soil required less time for equilibration of sample moisture content at each pressure potential. Sampling intervals were recorded, however these were approximate sample equilibrium as not all samples were weighed and were not monitored on a daily basis.

Correlation coefficients for viral and bacterial abundances increased with increasing clay content: Garden Gate ($r=0.31$, $n=81$), sandy Cashmore ($r=0.44$, $n=80$) and clay Cashmore ($r=0.62$, $n=81$). These values are an indication that bacteria and viruses are more closely associated when interacting with clay than if little clay is present.

4.3.2.3. Garden Gate soil

In soil samples from Garden Gate bacterial counts decreased from an initial 67 $x10^7$ cells g^{-1} dry soil at saturation to 31 $x10^7$ cells g^{-1} dry soil by –10 kPa (Figure 4.4A,B). Viral counts decreased from 140 $x10^7$ to 61 $x10^7$ particles g^{-1} dry soil, over the same period (Figure 4.4A,B). The decrease may be in part due to a movement of free bacteria and viruses out of soil water, either to relocation within the core or transport out of it. Bacterial and viral counts were stable between –10 kPa and –100 kPa, and soil moisture was maintained above 20%. Protozoan grazing may have removed any increases in production of both bacteria and viruses. After –100 kPa bacterial counts increased significantly ($p<0.001$) from 31 $x10^7$ cells g^{-1} dry soil to 55 $x10^7$ cells g^{-1} dry soil, before declining.

At –200 kPa there was a significant increase in viral counts from 47 $x10^7$ particles g^{-1} dry soil to 129 $x10^7$ particles g^{-1} dry soil. Viral counts were still above 100 $x10^7$ particles g^{-1} dry soil at -500 kPa, but subsequently decreased three-fold. Counts in 'air-dry' soil were very low, at 13 $x10^7$ particles g^{-1} dry soil. The increase in counts after –200 kPa supports the suggestion that protozoan grazing did remove bacteria and viruses when the soil was wetter. The crash in viral counts at very low moisture contents at the highest suction may be a reflection of decreased survival in dry soil.

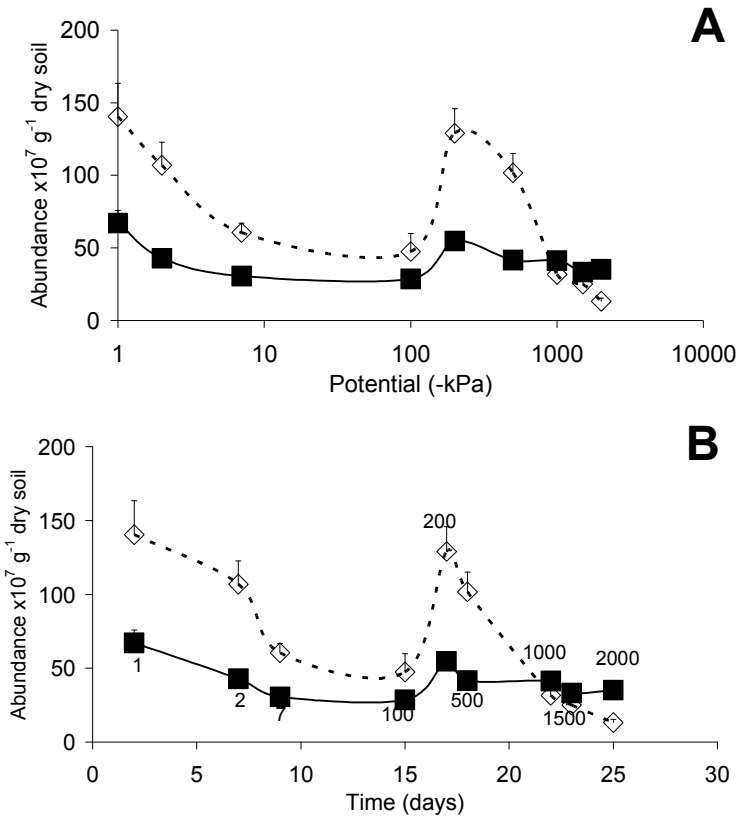


Figure 4.4: Bacterial and viral abundance in Garden Gate soil. A: at decreasing pressure potentials (-kPa), B: at time of sampling (potential indicated adjacent to data point). Bacteria (–■–), viruses (- ◊ -). Standard error shown (n=9).

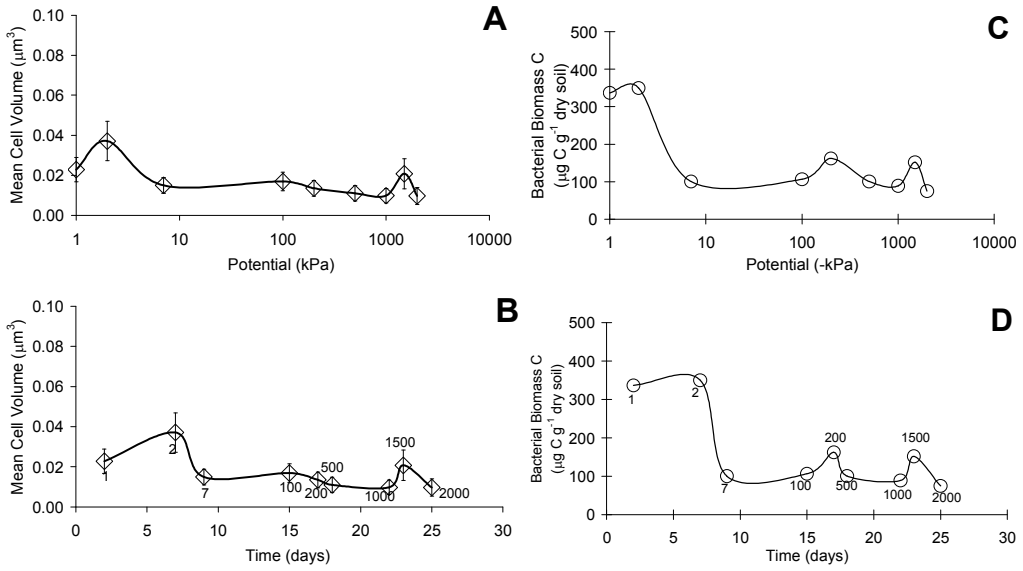


Figure 4.5: Bacterial mean cell volume (A,B) and bacterial biomass C (C,D) in Garden Gate soil. A and C: at decreasing pressure potentials (-kPa), B and D: at time of sampling (potential indicated adjacent to data point). Bacterial biomass was calculated using abundance data, MCV and a conversion figure of 0.56pg C µm^{-3}. Standard error shown (n=9).

Mean cell volume (MCV) decreased from a maximum of 0.037 μm^3 to 0.015 μm^3 by –10 kPa and continued to decrease (Figure 4.5A,B). Bacterial abundance was also highest when soil was wettest (Figure 4.5 C,D), so overall bacterial biomass C was maximum at –1 and –10 kPa potential. MCV decreased markedly initially, in spite of the soil being still very wet (–10 kPa). An increase in MCV at –1500 kPa led to increased bacterial biomass carbon (Figure 4.5C,D), despite a reduction in abundance. Hence both abundance data and biovolume were necessary for adequate assessment of biomass. Samples reached equilibrium at each pressure potential reasonably quickly, and the time intervals indicated (Figure 4.5B,D) may have been an over-estimation.

4.3.2.4. Sandy Cashmore soil

Bacterial counts fluctuated little around a mean of 110 $x10^7$ cells g^{-1} dry soil as soil dried until –500 kPa, when they decreased (Figure 4.6). This was despite the soil moisture content dropping from 37% to 12%. Viral counts peaked to 380 $x10^7$ particles g^{-1} dry soil between –10 kPa and –100 kPa, suggesting a possible switch to the lytic cycle. Viral counts then decreased with bacteria counts until –500 kPa, when there was an increase in bacterial counts and another significant peak ($p<0.001$) in viral counts before both decreased.

Mean cell volume increased from 0.015 μm^3 to a maximum of 0.04 μm^3 at –400 kPa. MCV decreased for two sampling points at –500 to –800 kPa and then reached 0.05 μm^3 when the soil was at its driest (Figure 4.7).

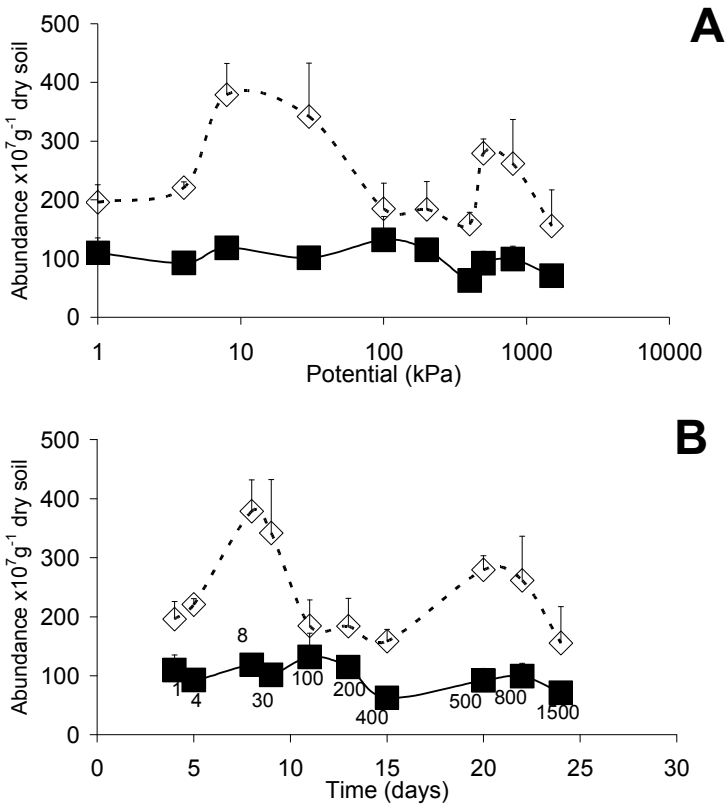


Figure 4.6: Bacterial and viral abundance in sandy Cashmore soil. A: at decreasing pressure potentials (-kPa), B: at time of sampling (potential indicated adjacent to data point). Bacteria (–■–), viruses (- ◊ -). Standard error shown (n=8).

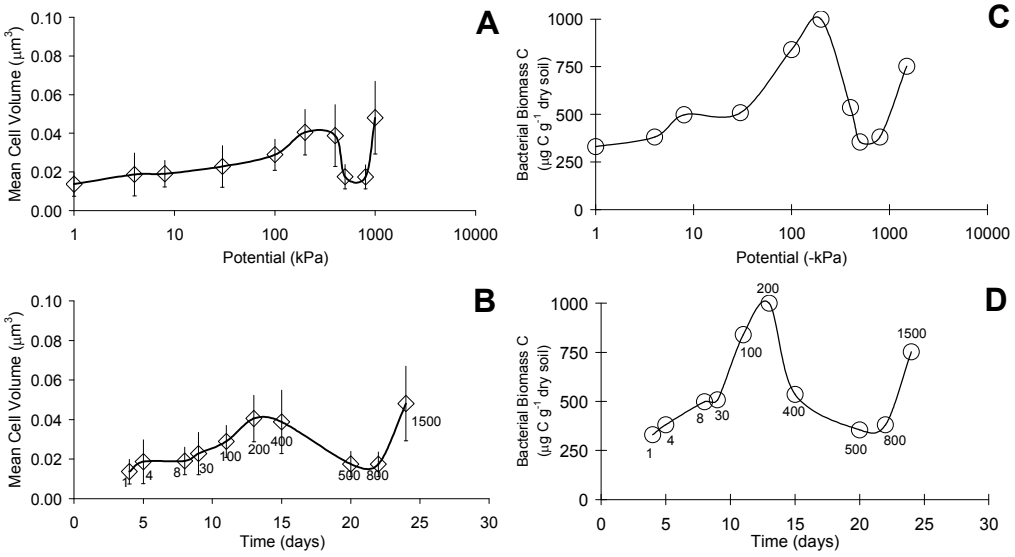


Figure 4.7: Bacterial mean cell volume (A,B) and bacterial biomass C (C,D) in sandy Cashmore soil. A and C: at decreasing pressure potentials (-kPa), B and D: at time of sampling (potential indicated adjacent to data point). Bacterial biomass was calculated using abundance data, MCV and a conversion figure of 0.56pg C μm^{-3}. Standard error shown (n=8).

4.3.2.5. Clay Cashmore soil

Bacterial and viral counts fluctuated together throughout the drying period (Figure 4.8). The initial counts were 211 x10^7 cells g^{-1} dry soil and 290 x10^7 particles g^{-1} dry soil for bacteria and viruses respectively. Counts decreased slightly at –30 kPa and increased again at –100 kPa. This fluctuating trend continued; counts fell and rose again at –800 kPa, with bacterial counts of 174 x10^7 cells g^{-1} dry soil and 213 x10^7 particles g^{-1} dry soil for viruses.

Mean cell volume was significantly ($p<0.001$) greater than both sandy soils (Figure 4.9A,B), being 0.06 μm^3 at the start, increasing to 0.08 μm^3 by –100 kPa and then falling and fluctuating at the later stages of drying. Samples required at least one week longer than the sandier soil samples for equilibration at the drier stages (Figure 4.9C,D), highlighting the dominance of very small pores.

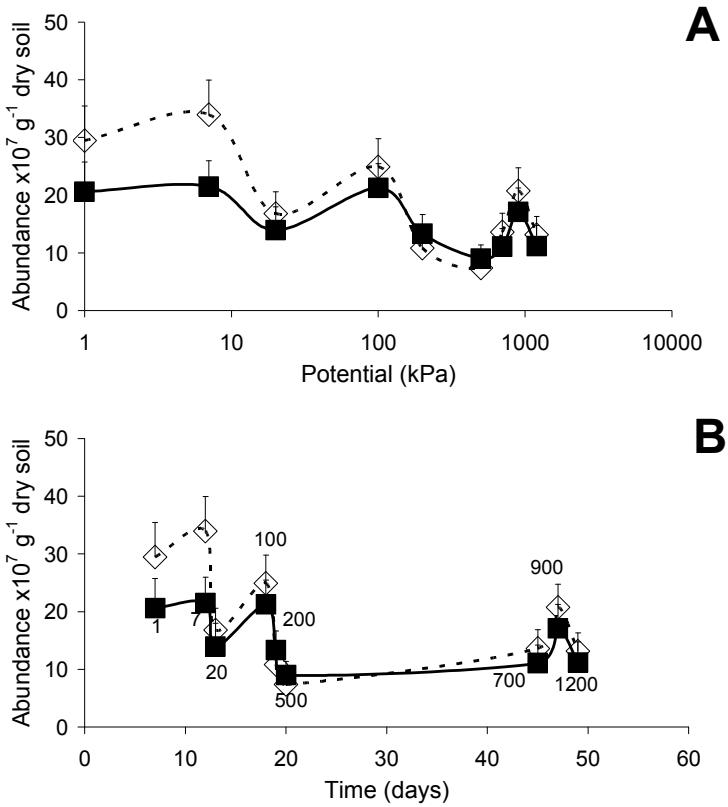


Figure 4.8: Bacterial and viral abundance in clay Cashmore soil. A: at decreasing pressure potentials (-kPa), B: at time of sampling (potential indicated adjacent to data point). Bacteria (–■–), viruses (- ◊ -). Standard error shown (n=9).

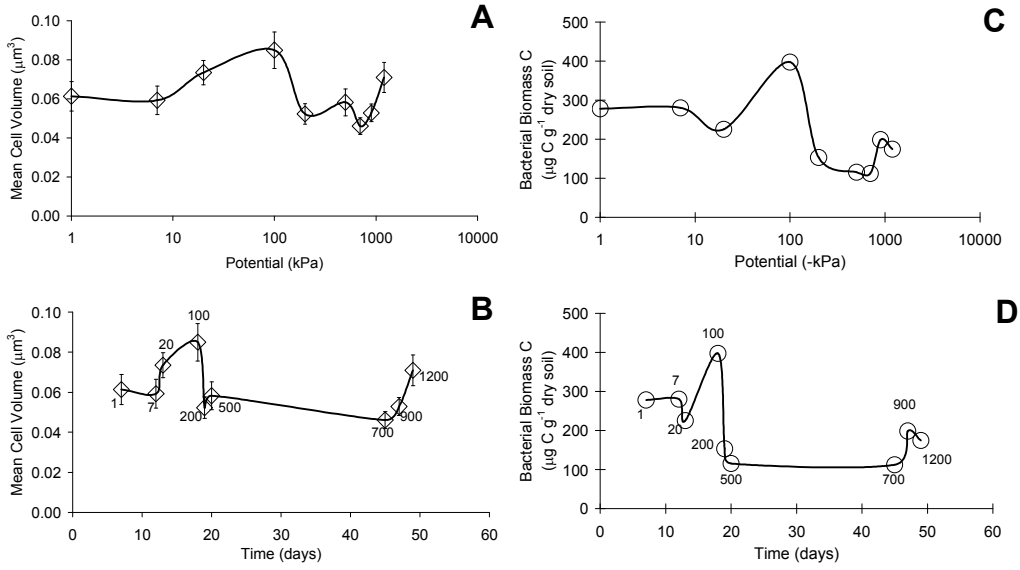


Figure 4.9: Bacterial mean cell volume (A,B) and bacterial biomass C (C,D) in clay Cashmore soil. A and C: at decreasing pressure potentials (-kPa), B and D: at time of sampling (potential indicated adjacent to data point). Bacterial biomass was calculated using abundance data, MCV and a conversion figure of 0.56pg C μm^{-3}. Standard error shown (n=9).

4.4 DISCUSSION

These data have demonstrated the dynamic nature of bacterial and viral populations in drying soils. Wetter conditions were more favourable for larger numbers of bacteria and phage in the soil system, irrespective of clay content. However, the first hypothesis that viral abundance would decrease as soils dried, was not conclusive due to peaks of high abundance in the sandier soils (Garden Gate and sandy Cashmore) at low potentials and fluctuations in abundances throughout in the clay Cashmore soil. The second hypothesis, that effects of soil drying would be apparent at higher potentials in the sandier soils, was accepted. The sandiest soil, Garden Gate, showed significant decreases in viral abundance when the soil was still fairly wet, in sandy Cashmore the same trend occurred, but at lower potentials, whilst in clay Cashmore significant decreases in abundance occurred later still. The third hypothesis, that abundances over several potentials would be stable in a clay soil needed further investigation as the fluctuating pattern of abundance in clay Cashmore soil dominated.

Mean cell volumes of extracted bacteria from wet soil were larger than in drying soil, which may have been an indication of increased metabolism: small cells result as the bacteria enter the starvation phase (Bae and Casida 1973; Morita 1997). Based on MCV, some bacteria in the sandy soils could be classified as ultra-microbacteria (Bae, Cota-Robles, and Casida 1972), as they had volumes of less than 0.02 μm^3.

Bacteria and virus abundance was stable over the range of suctions –10 kPa to –100 kPa in Garden Gate, which suggested that

protozoan grazing removed production. A study in which inoculated protozoan and bacterial populations were recorded showed they stabilised at pF of 3.08 (≈ -100 kPa) (Vargas and Hattori 1986). A similar result was not shown in the sandy Cashmore soil, as peaks in viral abundance occurred at this point. The sandy Cashmore soil contains more organic matter and thus may have a higher number of smaller pores than the Garden Gate soil as organic matter can lead to the formation of micro-aggregates (Tisdall and Oades 1982; Oades 1993). The peaks in viral abundance may have been a direct response to changes in soil moisture.

Bacterial and viral abundance in the clay Cashmore soil decreased over time but fluctuated widely. Other studies have linked similar 'harmonic' fluctuations of bacteria over time to nutrient inputs (Zelenev, van Brugen, and Semenov 2005), but it is unclear what mechanism associated with soil drying might drive such fluctuations.

Each drying study took over 2 months to complete; therefore the peaks in viral production may be associated solely with a time-mediated factor. A study elsewhere in which soil was kept continually wet or underwent a drying-wetting cycle showed that bacteria population and the frequency of dividing cells (a measure of activity) changed over time irrespective of soil moisture content (Bloem *et al.* 1992). In that study, when soil water potential had dropped to –100 kPa, equivalent to pore sizes of 3 μm being drained, 10% of the cells were still undergoing division, although N mineralisation had decreased significantly from wet soil values.

The virus production factors governed by time (latent and rise periods) extend to hours rather than days (Williams, Mortimer, and Manchester 1987; Abedon, Hyman, and Thomas 2003) so fluctuations of virus numbers would be expected over time. Soil moisture in soils is greater for longer on a drying cycle than on the return wetting cycle due to hysteresis (Whitmore and Heinen 1999). More marked differences might be expected were counts to be made whilst the soils were systematically dried and then re-wetted to saturation.

If adsorption of bacteria and viruses increase in dry soil then the numbers presented here would be an underestimation. Using radioactively labelled enteroviruses, Yeager and O'Brien (1979a; 1979b) showed that viruses in dry soils become inactivated and degraded rather than irreversibly bound. In the sand and sandy loam they used there were no differences in adsorption, even with the difference in clay contents. If bacteriophage behave similarly they are unlikely to have been underestimated as the soils dried. Other studies (Farrah and Bitton 1990) have shown fewer phage in dry soil, but they did not quantify the proportion that were inactivated. As resources become limited more phage may remain in host cells rather than be released; studies in chemostats have shown a significant rise in pseudolysogens at successive nutrient dilution (Ripp and Miller 1998), with lower resultant virus-to-bacteria ratios.

An explanation for the peaks in viral abundance maybe attributed to bacterial diversity. Different bacterial groups may have different susceptibility to virus infection and release; thus factors influencing the metabolism of different groups may result in changes in overall viral

production. The assumption made was that all active bacteria were infected and could release phage, so as activity decreased, so too would viral production. Applying that assertion to these data would lead to the inference that in the sandy soils dominant bacterial groups occupying particular zones exhibited variable phage production characteristics, whilst there was uniform production in the clay soil, possibly because insufficient drying did not distinguish between bacteria in different pore size classes. One study with a sandy loam soil (1.8% organic matter and 15% clay, physical characteristics in between those of Garden Gate and sandy Cashmore soil), maximum plasmid transfer frequency occurred at 8% moisture, a potential of –500 kPa to –1 MPa, with decreases in transfer frequency at 5% and 2% moisture (Richaume, Angle, and Sadowsky 1989). Hence bacteria can be active at quite low moisture contents.

Other studies have shown that neither bacterial community structure and diversity (Fierer, Schimel, and Holden 2003) nor bacterial abundance (Bloem *et al.* 1992) are significantly affected by stresses of drying and wetting, although in the latter study mineralisation increased upon rewetting, indicating an expected increase in activity. Increases of mineralisation on rewetting may be in part due to the disrupted aggregates releasing organic matter, which is then mineralised (Denef *et al.* 2001), and in part due to microbes actively releasing extra-cellular solutes in response to the osmotic shock. These solutes are then mineralised resulting in a pulse of carbon dioxide (Fierer and Schimel 2003).

Overall mean cell volumes were small. MCV in the sandy soils were typically around 0.02 μm^3, whilst those in the clay soil were 0.06 μm^3. These were nearly half the size of biovolumes recorded elsewhere (Jenkinson, Powlson, and Wedderburn 1976; Lin and Brookes 1999). Part of this may be due to overestimations through the use of eyepiece graticules, but the likelihood also is that the centrifugation process utilised in the current study sedimented larger bacteria thus reducing mean cell volume appreciably.

A valid inference from these findings is that bacteria and virus dynamics are comparatively stable in clay soil, but are more variable in sandy soils. Virus abundance appears to be strongly coupled to bacterial abundance at the time of sampling. Prior releases of large numbers of viruses are not detected after a period of a week or more, indicating that they either degrade or strongly adsorb.

4.5 SUMMARY

Soil bacteria and viral abundances were investigated over a range of moisture contents, from saturation to air dry. Soils with contrasting clay and organic matter content were sampled, the premise being that adsorption to clay and organic matter by bacteria and viruses buffers the effect of decreasing moisture. Mean cell volumes were calculated at each sampling point, as this is a factor that is frequently overlooked but is crucial in estimating biomass. Bacterial abundance fluctuated to a similar extent in both the sandy and clay soils, while viral abundance fluctuated more strongly in the clay than in the sandy soil. The data indicate that viral production is enhanced when sandier soils are dried, but due to fluctuating abundances in bacteria and viruses it is unclear whether clay offers protection against soil drying. Mean cell volumes were half the size in the sandy soils compared with the clay soil. Cell volumes in one sandy soil decreased as the soil dried, but increased in the other sandy soil and the clay soil.

CHAPTER 5: THE EXTENT OF LYSOGENY IN SOIL BACTERIA.

5.1 INTRODUCTION

5.1.1. Effects of Phage Replication on Bacterial Abundance

Phage and bacteria exist in a dynamic equilibrium in soil, as other chapters in this volume demonstrate. Viral and bacterial abundance is one component of this dynamic; another is the degree of stability of these populations within the soil system. The 'choice' of replication strategy employed by phage will have a significant impact on this stability.

If soil phage are predominantly temperate and many bacteria are therefore lysogens, then triggers that induce prophage will cause mass lysis of bacteria (Lwoff 1953) decreasing bacterial populations with a concomitant rise in viral abundance. Conversely in *status quo* conditions low viral replication and production would be expected (Echols 1972).

If, however, the majority of phage are virulent (undergo solely lytic cycles), then bacterial metabolism will be sufficient to initiate phage replication (Lenski 1988). In this case bacterial and phage abundance may be in continual flux when resources are present and external perturbations will not have such a dramatic effect: phage will be a stronger regulator of bacterial abundance.

5.1.2. Effects of Phage Replication on Bacterial Communities

Understanding of the extent of lysogeny in soil bacteria may also assist in ascribing the relative importance of phage in genetic exchange between bacteria.

Bacteria may become recipients or donors of genetic exchange in three ways: transduction, conjugation and transformation. The first is phage-mediated, and takes place when DNA segments of an infected bacterium are mis-packed within developing phage particles. On infection of a new host cell, the donor DNA can integrate with the recipient cell to form a transductant or form a plasmid. Many bacterial genomes also contain large sections of prophage DNA (Canchaya *et al.* 2003), suggesting past interactions with a multitude of different phage, and *Escherichia coli* has been transduced by phage in soil (Germida and Khachatourians 1988; Zeph, Onaga, and Stotzky 1988). Whilst *E. coli* is not a natural soil inhabitant, the studies illustrate that the environment is suitable for transduction to occur.

Transduction can be either generalised or specialised. In the former, any part of the bacterial or plasmid DNA may be incorporated into phage. In specialised transduction, only a number of genes either side of the site of integrated prophage are transferred (Paul and Jiang 2001). Thus the degree of lysogeny in a system may have implications on the nature of horizontal gene transfer between bacteria.

5.1.3. Studies of Lysogeny in Aquatic Systems

The extent of lysogeny has been examined in bacteria from aquatic environments. Studies with whole bacterial communities (Williamson *et al.* 2002) showed that most induction (release of phage from lysogens) took place in winter months, when bacterial abundance and activity was low. Individual host studies using *Synechococcus* (McDaniel *et al.* 2002) exhibited the same trend, suggesting that phage infecting these cyanobacteria can switch replication dependent on conditions. Highest percentage of induced phage occurred when primary production in *Synechococcus* was low and counts of phage and host were small. Lysogenic production did not appear to be responsible for a significant proportion of total cyanophage counts in this case.

Examination of natural communities has demonstrated that there is a greater incidence of lysogeny in oligotrophic sites than less nutrient-limiting sites (Jiang and Paul 1998b; Weinbauer, Brettar, and Höfle 2003). As polar lakes also show similar trends (Lisle and Priscu 2004; Laybourn-Parry, Marshall, and Madan 2006), it implies that lysogeny is favoured under certain nutrient limiting conditions.

Other studies however showed that less than 5% of bacterial populations were lysogenic, with no difference between coastal and more oligotrophic waters (Weinbauer and Suttle 1996). This finding has been supported by amendment studies on bacterial communities; the addition of phosphate and ammonium increased lytic phage production in bacteria from oligotrophic and mesotrophic sites, but did not affect lysogenic production in either case (Williamson and Paul 2004). Thus

lysogeny may or may not be influenced by nutrient supply, host activity or abundance. The uncertainty in the significance of lysogeny has been noted by at least one author, who concluded that although lysogenic phage production is a widespread phenomenon, it is not a significant factor in regulating bacterial populations in aquatic systems (Weinbauer 2004).

5.1.4. Lysogeny in Soil

Characterisation of soil phages initially showed the majority to be lytic. Examples of lytic phage were found in *Bacillus subtilis* (Hemphill and Whiteley 1975), *Azotobacter* (Hegazi and Ciampor 1976) *Nitrobacter* (Bock, Düvel, and Peters 1974) and *Arthrobacter globiformis* (Casida and Liu 1974). Temperate phage however have been isolated for *Streptomyces lividans* (Marsh *et al.* 1993). If the methodologies employed in the first instance did not induce prophage, then it is little wonder that the majority seen were lytic.

Reviews on soil phage dynamics support the idea that lysogeny dominates in soil (Marsh and Wellington 1994), but the basis for this has been from work on individual species: no studies have been conducted on natural communities. Investigation of the lytic and lysogenic pathways at the molecular level indicate that repression of the lytic cycle is associated with the rate of protein synthesis in the host and the number of phage infecting the cell (Echols 1972). In soil both strategies are adopted, as was found when both lysogenic and lytic phage of *Serratia liquefaciens* were characterised (Ashelford *et al.* 2000). The extent of lysogeny has also been examined in other systems: lytic phage

dominated in sewage samples, but lysogens dominated in faecal samples (Dhillon *et al.* 1976).

In addition to conferring immunity to other particular phage, lysogeny may give bacteria a competitive advantage (Bossi *et al.* 2003; Weinbauer and Rassoulzadegan 2004; Brüssow, Canchaya, and Hardt 2004). Populations of lysogenic *Streptomyces*, however, declined more rapidly in a soil microcosm than non-lysogens (Herron and Wellington 1994), suggesting that they were less fit.

5.1.5. Hypotheses

It is expected that lysogeny will be significant in soil due to the oligotrophic nature of this environment: most bacteria are dormant for much of the time, so resources for replication would be minimal. Despite large total abundance of bacterial–host populations in soil, the spatial heterogeneity of the environment may lead to localised shortages of host. The two factors of nutrient limitation and low host abundance are associated with increased lysogeny in the aquatic environment, so the presence of lysogenic populations in soil is expected if soil bacteriophage behave in a similar way to aquatic phage.

Hypothesis 1: A higher incidence of lysogeny will be present in drier soil than wetter soil.

Water addition to a dry soil stimulates bacterial activity and overall phage production (Chapter 3). Water also facilitates movement of liberated phage to sites where there are new hosts. Host availability is therefore increased in a wetter soil, even if total abundance remains unchanged. Given that increased host abundance is associated with

phage favouring the lytic cycle, the assumption is that there will be less lysogeny recorded in the wetter soil.

Hypothesis 2: Energy-rich soils will exhibit lower lysogeny than energy-poor soils.

A greater abundance would be expected both of bacteria and phage in an energy-rich soil than in an energy-deficient soil. Increased incidence of lysogeny has been found in aquatic systems with nutrient limitations so a similar finding would be expected from very carbon-poor soil. Nutrient limitation in soil is commonplace and results in starvation states for bacteria (Morita 1997), favouring (pseudo)lysogeny (Ripp and Miller 1998). Pseudolysogeny is discussed in Chapter 1, but in brief; after injection, a phage may delay either straightforward lysis or integration into prophage until conditions within the bacterial cell change. If conditions are perpetually bleak, then pseudolysogenic phage are unlikely to initiate lysis. Bacterial turnover in very oligotrophic soil will be protracted as against that in a richer soil. A greater incidence of lysogeny is therefore expected in the poor soil as a means of survival for the phage, as there would be insufficient resources for much phage replication.

5.1.6. Aims

To test the hypotheses, several aims had to be met. In the first instance, a suitable method of detecting lysogeny had to be established. In studies where the proportion of lysogens is of interest, chemicals or UV can be employed to induce lysogenic phage to replicate and lyse from bacteria. Mitomycin C, a mutagen, has been used for many years

as a chemical inducing agent, (e.g Auling, Mayer and Schlegel (1977). Whilst it may not necessarily induce all lysogenic bacteria (Cochran, Kellog, and Paul 1998), it is the standard currently in use, with incubation periods typically less than 24 hours at a concentration of 1-5 $\mu g\ ml^{-1}$ sample (Paul and Jiang 2001). Mitomycin C has been widely used to determine the extent of lysogeny in aquatic systems, but to the author's knowledge has not been used before in soil. Therefore it was necessary to determine effective concentrations of mitomycin C during this study. The effect of adding sodium chloride with the mutagen (mitomycin C is supplied as 4% w/w in NaCl) needs consideration lest it stimulates or inhibits phage production on its own.

Mitomycin C incubations in aquatic science commonly employ a '*t=0*' and '*t=x*' sampling procedure, with 'x' being around 16 to 24 hours (although some have extended to 72 hours (Anesio *et al.* 2004)). It was unclear whether the normal 'beginning and end' points would be sufficient to capture the effects of the antibiotic in soil. In aqueous samples there would be few losses onto particulate matter, but adsorption of liberated phage might be significant in soil. Given the different particulate nature of wet soil samples to aqueous samples, it was deemed necessary to test for lysogeny over several time-points.

Adding an aliquot of mitomycin C to a sample from aquatic habitats clearly will not alter its water status, but the added water will affect bacterial activity in soil samples. The positive effect of water on bacteria will diminish over time as bacterial metabolism slows down once more, or it may increase as host cells are lysed by phage detecting the decrease in activity.

Several calculations have been utilised in the literature in induction studies on aquatic systems. They address slightly different aspects of lysogeny and associated phenomena so are reviewed and incorporated into the results here for comparison where appropriate.

5.2 METHODS

5.2.1. Effective Mitomycin C Concentration

A series of different concentrations of mitomycin C were applied to soil samples, with enumeration of bacteria and viruses after 24 hours to determine an appropriate level of mitomycin C to be used. Typical concentrations applied in aquatic studies have been 1 μg mitomycin C ml^{-1} sample. A higher concentration of mitomycin C might be needed when incubating with solid matter given the impediments to free movement. In addition, mitomycin C may interact with particulate matter. A concentration range from 1 μg g^{-1} soil to 50 μg g^{-1} soil was selected.

Highfield grassland soil (Table 2.3, p34) was used. Triplicate samples of thoroughly sieved, mixed and equilibrated 1.5g soil were placed in polypropylene centrifuge tubes and were incubated with 400 μl of either sterile de-ionised water or an aliquot of mitomycin C for 24 hours. The volume of 400 μl was chosen so as not to fully saturate the soil, but to achieve 'field capacity' for optimum bacterial activity. Final water content was 28% (g/g). At the end of the incubation viral and bacterial abundance was recorded.

Mitomycin C (Sigma chemicals) is supplied at 4% by weight as a crystalline powder in NaCl, so at the highest level of addition (50 μg

mitomycin C g^{-1} soil) a significant input (1200 μg g^{-1} soil) of NaCl was also being added. Separate samples were incubated with equivalent levels of NaCl without mitomycin C to check the effect of the salt on bacterial or viral abundance. Viral and bacterial abundance were recorded after 24 hours incubation by the protocol outlined in section 2.5. Each tube in its entirety was processed as a single sample, hence there were three samples duplicated.

5.2.2. Soil Moisture as a Variable

To test the first hypothesis, two separate incubations were run with the same soil at different moisture contents to determine whether it altered the extent of lysogeny. Clay Cashmore soil (Table 2.3, p34) was selected as its moisture release curve had already been established (Figure 4.3, p84). Topsoil was stored and processed as outlined in section 2.2.1. Triplicate 1.5 g samples were incubated for 90 hours with 300 μl aliquots – the drier incubation and 170 hours with 400 μl aliquots – the wetter incubation. Aliquots were either mitomycin C (10 μg g^{-1} soil – see section 5.3.1) or sterile de-ionised water.

Final soil moistures were 26% and 32% (g/g) for the 90 and 170 hours incubations respectively: moisture content had to be sufficiently high to allow adequate movement of mitomycin solution within the sample for effective action. The incubations were run at different times due to logistical reasons, with the drier incubation run first – hence the difference in incubation periods. All other experimental variables, apart from moisture, were identical between the two incubations.

Viral and bacterial abundance were recorded at 15 – 20 hour intervals throughout the incubation periods by the protocol outlined in section 2.5. Several time points over the incubation period were selected to determine if apparent levels of lysogeny differed over time, as it was uncertain whether 24 hours would be appropriate in soil. Each tube in its entirety was processed as a single sample, hence there were three samples duplicated.

5.2.3. Organic Carbon as a Variable

To assess the hypothesis that carbon-poor soil would exhibit greater lysogeny than a carbon-rich soil, suitable samples were required that reduced the large inherent variability between soils. Contrasting soils were selected that have been under the same, continuous management at Rothamsted Research Institute for more than 45 years. One is a high input grassland plot at Highfield -hereafter referred to as soil A. Soil A has been in grass for more than 100 years. Another plot has been continuously fallowed four times per year since 1959 (low input) – hereafter referred to as soil B. Organic carbon values (loss on ignition) were 3.2% for soil A and 1.2% for soil B. The soils contain on average 25% clay, 66% silt and 9% sand. Topsoil was used within one month of collection. Soil was air dried so it could be sieved through a 2 mm sieve, hand picked for removal of root matter and equilibrated for one week at 20°C. The day before use the soil was left to air-dry to reduce moisture to allow aliquots of mitomycin C or water to be added.

Samples were concomitantly wetted with either 400 μl of a solution containing mitomycin C (10 μg g^{-1} soil) or with 400 μl of sterile

de-ionised water. Initial soil moisture content was 35% (g/g) and maintained for up to 170 hours. In soil B it was necessary to water at 60 hours because the soil was losing moisture and portions of the sample had dried. Soil A retained its moisture level throughout. As with the study for hypothesis 1, viral and bacterial abundance were recorded at nine 15 – 20 hour intervals up to 170 hours incubation by the protocol outlined in section 2.5. Each tube in its entirety was processed as a single sample, hence there were three samples duplicated.

5.2.4. Calculation of Lysogeny

Different formulae have been adopted in the literature to express levels of lysogeny. As the extent of lysogeny calculated will differ depending on the calculation used, it was felt prudent to apply those most common to the data here. The following pages highlight the derivation of the formulae.

5.2.4.1. Standard burst size method

The standard burst size method utilises counts of viruses in control and induced samples and counts of bacteria at the start, and is widely used (Paul and Jiang 2001; Weinbauer, Brettar, and Höfle 2003; Anesio *et al.* 2004). Burst sizes would ideally be determined through counting of comparative TEM grids of samples, but in most reported work a burst size standard of 30 or so is applied, based on other studies (Jiang and Paul 1996; Wommack and Colwell 2000). In the work reported here TEM showed no definitive cases of phage assembled in bacteria (section 2.2.7) and, as no data are available on burst sizes from soil communities, the average of 30 from aquatic studies was used.

Work on particular soil bacteria has shown wide variation; a burst size of 100 was recorded in *Rhizobium* (Dhar *et al.* 1978), whilst in a review on *Bacillus subtilis* phage burst size ranged from 23 to over 500 (Hemphill and Whiteley 1975).

Percentage lysogeny was calculated at each sampling time point using Formula 5.1.

$$\text{Lysogeny} = [(V_I - V_C)/Z]/C_0 \times 100 \qquad [1]$$

Formula 5.1: Standard burst size calculation of percentage lysogeny

Where V_I is the viral count in induced samples, V_C is the control, Z the burst size and C_0 is the bacterial count at the start of incubation.

5.2.4.2. Modified burst size method

If at the start of incubations only a proportion of the total bacterial community is counted, growth of the uncounted fraction may overestimate the final percentage lysogeny if *t=0* bacteria counts are used. Taking the bacteria count at each time point (Formula 5.2) may compensate for this.

$$\text{Lysogeny} = [(V_{it} - V_{ct})/Z]/C_t \times 100 \qquad [2]$$

Formula 5.2: Compensatory burst size calculation of percentage lysogeny

Where V_{it} is the viral count in induced samples at time t, V_{ct} is the viral count in the control at time t, Z is the burst size, and C_t is the bacterial count at time t. This formula has been used less widely (Williamson *et al.* 2002).

5.2.4.3. Lysogenic fraction

Rather than comparing viral counts, bacteria counts may be considered. The lysogenic fraction can be denoted by the proportion of bacteria in the population lost in mitomycin C treatments. Bacterial mortality is based on differences in bacterial abundance between mitomycin C and control (Formula 5.3), and higher percentage lysogeny may be recorded than if the standard 'burst size' method is used (Lisle and Priscu 2004).

$$\text{Lysogenic fraction} = (B_c - B_i)/B_c \; x \; 100 \qquad [3]$$

Formula 5.3: Calculation of percentage lysogenic fraction

Where B_c is the bacterial count in control tubes and B_i is bacterial count in mitomycin C tubes. The calculation may be 'corrected' by subtracting the average lysogenic fraction calculated in treatments in which viral abundance did not increase significantly. If there were significant increases in viral abundance in all treatments, or the variability in bacterial abundance was too high for reliable calculation of abundance the correction will serve little purpose and may detract from the calculation's worth.

5.2.4.4. Induced burst size

An estimate of the number of phage released from each bacterium can be determined using Formula 5.4.

$$\text{Burst size} = (V_{it} - V_{ct})/(B_{it} - B_{ct}) \; x \; 100 \qquad [4]$$

Formula 5.4: Calculation of percentage induced burst size

Where V_{it} is the viral count in induced samples at time t, V_{ct} is the viral count in control samples at time t, B_{it} is the bacterial count in

induced samples at time *t* and B_{ct} is the bacterial count in induced samples at time *t*.

The calculation of induced burst size has been seen by the author in one other report only (Williamson *et al.* 2002), where induced burst size ranged from 1.4 to 103 viral particles liberated from each host. These data reflects the variability common in studies of lysogeny.

5.2.4.5. Percentage induced phage

Some studies express viral and bacterial abundance data from induced samples as a percentage of the control. This is a simple means of eliminating the necessity of plotting both control and mitomycin C data points on figures. [5]

5.2.4.6. Incidence of lysogeny in samples

The indication of lysogeny may be skewed by a few samples liberating many induced phage. One measure to reduce this likelihood is to record the proportion of samples in which significant increase in virus counts occurred, irrespective of the magnitude of this increase. Using this measure the actual incidence of lysogeny may be high even though "percentage lysogeny", as determined by the standard or compensatory burst size formulae is low. [6]

5.3 RESULTS

5.3.1. Effective Mitomycin C Concentration

After 24 hours bacteria and virus counts in the control treatments were 20 $x10^7$ cells g^{-1} dry soil and 104 $x10^7$ particles g^{-1} dry soil respectively (Figure 5.1). At 1 μg mitomycin C g^{-1} dry soil, bacteria counts were 17 $x10^7$ cells g^{-1} dry soil (90% of the control) and virus counts were 160 $x10^7$ particles g^{-1} dry soil (154% of the control) (Figure 5.1). Across all concentrations of mitomycin C, bacteria counts in mitomycin C tubes ranged from 90-98% of control tubes, whilst virus counts were 154-174% of control. Hence mitomycin C had been effective in inducing lysis, and mitomycin C at the lowest concentration (1 μg mitomycin C g^{-1} dry soil) resulted in significantly higher virus ($p<0.001$) and lower bacteria ($p=0.007$) counts between control and mitomycin C amended tubes.

Increasing mitomycin C concentration to 5 μg mitomycin C g^{-1} dry soil gave significantly higher ($p=0.002$) virus counts over 1 μg mitomycin C g^{-1} dry soil. Bacteria counts at 19 $x10^7$ cells g^{-1} dry soil were again similar to the control. Increases above 5 μg mitomycin C g^{-1} dry soil did not increase virus counts above 170 $x10^7$ particles g^{-1} dry soil, nor did it markedly change bacteria counts. At 50 μg mitomycin C g^{-1} dry soil the highest count of viruses was recorded, at 180 $x10^7$ particles g^{-1} dry soil although this was not significantly ($p=0.12$) greater than at 10 μg mitomycin C g^{-1} dry soil. Based on these findings the concentration of 10 μg mitomycin C g^{-1} dry soil was applied to remaining incubations.

An increase in the concentration of NaCl to 1200 μg NaCl g^{-1} soil decreased viral counts but did not alter bacteria counts significantly. After 24 hours incubation, bacteria counts with NaCl were 22 $x10^7$ cells g^{-1} dry soil, compared to 20 $x10^7$ cells g^{-1} dry soil in the control. Virus counts were decreased to 80 $x10^7$ particles g^{-1} dry soil from >100 $x10^7$ particles g^{-1} dry soil in the control. Given that alone, NaCl had a negative effect on virus production, it demonstrates that the differences observed in counts between mitomycin C and control tubes was most likely due to induction of prophage and not an artefact of incubation.

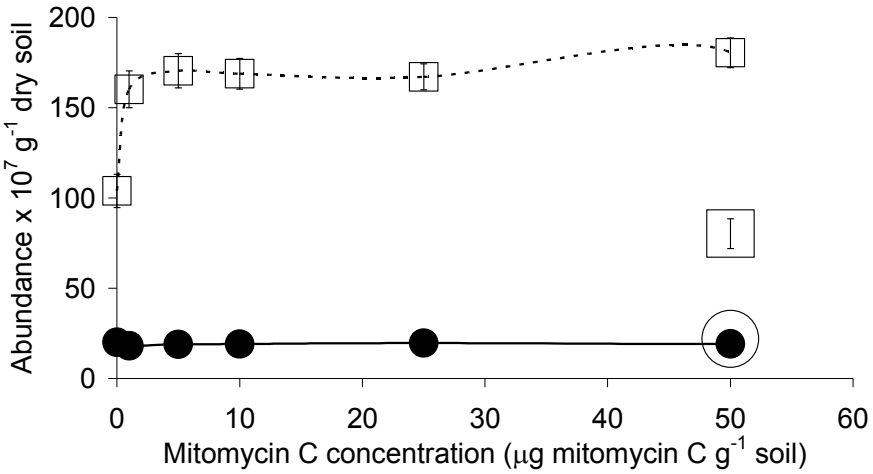


Figure 5.1: Effect of increasing concentration of mitomycin C on bacterial (circles) and viral (squares) abundance after 24 hours incubation with Highfield grassland soil. Standard error shown (n=3) or they do not exceed the size of the symbol. Large single points represent counts with maximum NaCl added without mitomycin C.

Determination of lysogeny using various methods is summarised in Table 5.1. Percentage lysogeny did not differ from 10% or so and the greatest differences in bacterial abundance between control and mitomycin C treatments (the lysogenic fraction), occurred at 1 μg mitomycin C g^{-1}dry soil. The low value for the lysogenic fraction implies that only a small proportion of bacteria were killed as a result of

incubation with mitomycin C. The calculated induced burst size ranged from 3 to 27; within the ranges reported in aquatic studies previously.

Mitomycin C Concentration ($\mu g\ g^{-1}$ soil)	Lysogeny (%) [2]	Lysogenic Fraction [3]	Induced Burst size [4]	Induced virus (% of control) (5)
1	9.3	10.3	27.3	154.1
5	11.0	6.2	-12.6[1]	164.0
10	10.7	5.2	8.2	162.4
25	10.5	2.4	3.1	160.7
50	12.7	5.0	25.5	173.6

1. A negative burst size either arises when induced virus counts are lower than the control, or when bacteria counts with mitomycin C are higher than the control, as in this case.

Table 5.1: Indicators of lysogeny at different concentrations of mitomycin C.

5.3.2. Soil Moisture as a Variable

Initial bacteria counts for all four incubations were similar, at approximately 45 $x10^7$ cells g^{-1} dry soil for the drier incubation (26%) and 40 $x10^7$ cells g^{-1} dry soil for the wetter incubation (32%) (Figure 5.2a,b,c,d). By contrast, virus counts were some three-fold lower in the drier incubation, at 5 $x10^7$ particles g^{-1} dry soil when compared to 17 $x10^7$ particles g^{-1} dry soil in the wetter incubation (Figure 5.2a,b,c,d). Mitomycin C treatments typically had lower bacterial counts than control due to viral lysis of induced host cells. The difference in bacterial counts between mitomycin C treatments and control was more marked in the wetter incubation, at 56-118% of control counts, compared to an average 72-110% of control counts in the drier incubation.

Mitomycin-induced bacteria released significant numbers of viruses in both incubations: in the drier incubation (26% moisture) virus counts were 84-181% of control counts (Figure 5.2a,b), whilst in the wetter incubation (32% moisture) they were 73-410% (Figure 5.2c,d). In

the drier incubation this was most apparent after 70 hours incubation (Figure 5.2a,b). In the wetter incubation there was a siginificant difference ($p<0.001$) between virus counts in mitomycin C and control tubes at 30 hours incubation (Figure 5.2c,d). The greatest difference occurred at 90 hours incubation, where a second addition of water appeared to stimulate further mitomycin C action (Figure 5.2d).

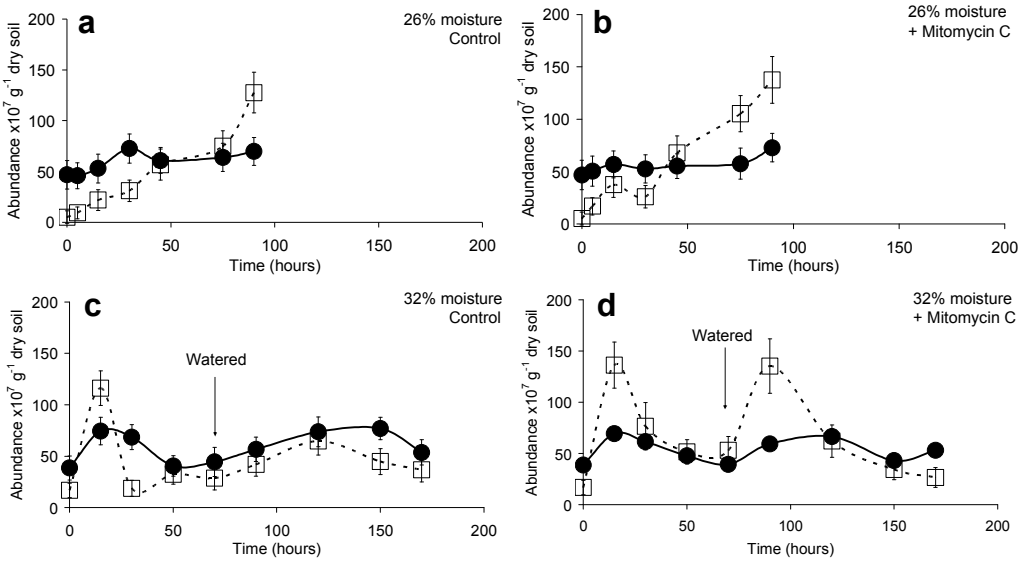


Figure 5.2: Bacterial (-●-) and viral (.□.) abundances in clay Cashmore soil incubated at 26% (a,b) or at 32% moisture content (c,d), with water (a,c) or with 10μg g^{-1} mitomycin (b,d). The drier incubation (26%) ran for 90 hours, the wetter incubation (32%) for 170 hours. Standard error shown (n=3).

Increased soil moisture markedly increased the proportion of viruses produced, and bacteria in the wetter incubation may have been more exposed to mitomycin C based on comparative decreases in counts. The sequential rise in viral abundance observed in the drier incubation is an indication that few viruses were adsorbing (e.g. to new hosts) so as to render them unextractable over the incubation. In the wetter soil viral abundance did not show a cumulative increase, but fluctuated with peaks corresponding to increased bacterial activity and abundance directly after the two watering events.

5.3.2.1. Percentage lysogeny

Lysogeny was detected in both incubations, but it was most significant in the wetter incubation. In the drier incubation lysogeny was undetectable at most points, with the maximum of 1.8% (calculation [1]) only after 70 hours incubation (Figure 5.3A). In contrast, lysogeny (calculation [1]) in the wetter incubation was significantly greater than in the dry incubation being 2.8% at 30 hours and 5.5% at 90 hours. As net bacterial abundance increased over time, application of calculation [2] raised those percentages to 5 and 8% respectively (Figure 5.3B). There was no detectable lysogeny beyond 90 hours incubation in the wetter soil.

With reference to optimum incubation duration, there was therefore no common time at which both incubations exhibited significant lysogeny.

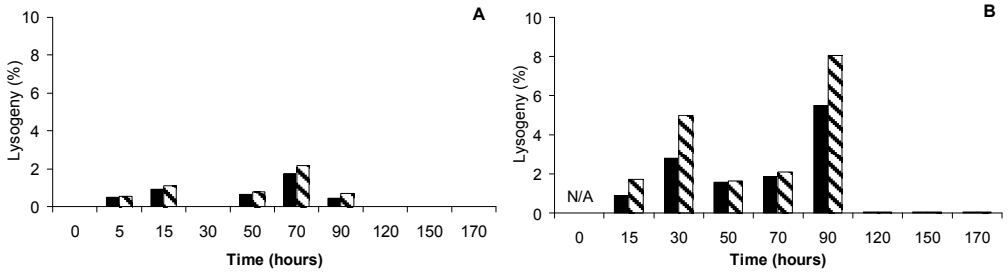


Figure 5.3: Percentage lysogeny as calculated by the standard burst size [1] (hatched columns) or compensatory burst size [2] (solid columns). A –Cashmore clay soil at 26% moisture, incubated for 90 hours, and B –Cashmore clay soil at 32% moisture incubated for 170 hours.

5.3.2.2. Other indicators of lysogeny

Considerably more phage were induced in the wetter soil than the drier soil (Table 5.2), even though net bacterial abundance was comparable at 60 x10^7 cells g^{-1} soil.

Time (hours)	Lysogenic Fraction (%)[1] [3]		Induced Burst Size [4] (virus/bacterium)[2]		Induced virus (% of control) (5)	
	A	B	A	B	A	B
5	-7.6	Nd	-1.6	nd	181	nd
15	-8.4	9.0	-3.1	2.7	171	117
30	25.3	11.4	-0.2	6.9	84	410
50	8.6	-17.2	2.0	-2.9	119	159
70	10.1	6.7	4.3	8.9	141	185
90	-3.3	-4.5	-3.9	-36.6	108	322
120	na	10.4	na	-0.9	na	96
150	na	45.8	na	-0.3	na	77
170	na	1.6	na	-9.1	na	73

1. Negative values arise when bacteria counts with mitomycin C are larger than in the control. 2. A negative burst size either arises when induced virus counts are smaller than the control, or when bacteria counts with mitomycin C are larger than the control, as in this case.

Table 5.2: Indicators of lysogeny in Cashmore soil at A - 26%, and B - 32% moisture.

Maximum induced percentage virus was over 400% in the wetter soil, compared with less than 200% for the drier soil. Either many more bacteria were producing phage in the wetter incubation, or burst sizes were double that of the drier incubation. Maximum lysogenic fraction – that is the proportion of bacteria lost in mitomycin C treatments over the control – was greater in the wetter incubation (46%) than in the drier incubation (25%) (Table 5.2).

It may be inferred that more bacteria in the wetter soil were indeed lysogenic. Furthermore, maximum positive induced burst size for the wetter soil was 9 viruses per bacterium, more than double that of 4.3 in the drier soil, lending support to the explanation offered above. These burst sizes are at the lower end of reported burst sizes however.

5.3.2.3. Incidence of lysogeny

Incidence of lysogeny (calculation [6]) was similar for both incubations. Significantly ($p<0.05$) higher viral abundance in mitomycin C treatments were found in 75% and 77% of tubes for the wetter and drier soil respectively. Decreases in bacterial abundance brought about by mitomycin C were less pronounced, with significantly smaller counts in mitomycin C treatments occurring in only 46% and 39% of samples for wetter and drier soils respectively. Concomitant significant decreased bacteria and increased virus counts occurred in 29% and 27% of samples for wetter and drier soils. Of note was that at 30 hours incubation in the drier soil all mitomycin C replicates had significantly ($p<0.05$) lower bacteria counts than controls – in which counts had increased from those at 15 hours. Hence bacterial production had increased over that time period, and mitomycin C appeared to check this increased growth of bacteria without leading to increased phage production.

5.3.3. Organic Carbon as a Variable

In both soils, bacteria counts increased in response to the initial water and mitomycin C addition and in the fallow soil (soil B) to subsequent watering (Figure 5.4). Mitomycin C was not added at the second watering stage. Bacterial abundance increased within 15 hours from 21 $x10^7$ cells g^{-1} dry soil to 34 $x10^7$ cells g^{-1} dry soil in soil A and from 3 $x10^7$ cells g^{-1} dry soil to 4 $x10^7$ cells g^{-1} dry soil in soil B. Bacterial counts were 10-fold higher in soil A. Mean bacteria counts over the incubation period were 52 $x10^7$ cells g^{-1} dry soil and 3 $x10^7$ cells g^{-1} dry

soil for soil A and soil B respectively. Bacteria and virus counts increased after the second addition of water on soil B and then decreased once more. The trend over time was that abundances increased in soil A and decreased in soil B.

In soil A, virus counts increased from an initial 16 $x10^7$ particles g^{-1} dry soil at the incubation start to 175 $x10^7$ particles g^{-1} dry soil at 170 hours, whilst in soil B virus counts decreased slightly from 0.8 $x10^7$ particles g^{-1} dry soil at the start to 0.5 $x10^7$ particles g^{-1} dry soil by 170 hours. Bacteria counts in soil A rose from 21 $x10^7$ cells g^{-1} dry soil to 68 $x10^7$ cells g^{-1} dry soil, whilst in soil B they fell from 2.9 $x10^7$ cells g^{-1} dry soil to 18 $x10^7$ cells g^{-1} dry soil (Figure 5.4). Viral abundance was up to 300-fold greater in soil A, and an overall mean of 101 $x10^7$ particles g^{-1} dry soil compared with 0.8 $x10^7$ particles g^{-1} dry soil for soil B. The correlation between bacterial counts and virus counts was significant for soil A ($r=0.77$), but not for soil B ($r=0.27$). Mean virus to bacteria ratios (VBR) during incubations were 2.0 and 0.3 for soil A and soil B respectively.

Bacterial mean cell volume (MCV) was ten times greater in soil A (0.11 μm^3) than in soil B (0.01 μm^3). Mean cell volume in soil A increased from an initial 0.05 μm^3 to nearly 0.2 μm^3, whilst in soil B it stayed at around 0.01 μm^3 throughout. In soil B bacterial MCV tracked bacterial abundance, while in soil A MCV increased relative to abundance (Figure 5.4).

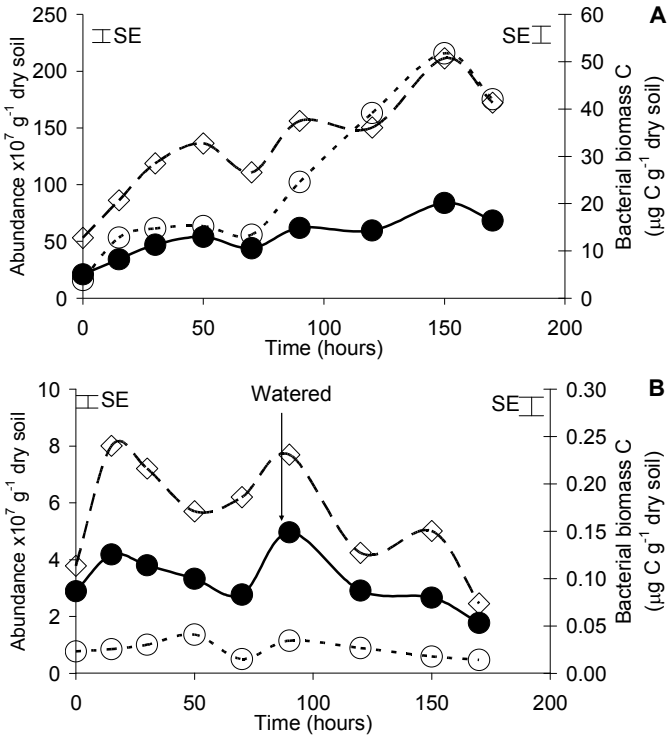


Figure 5.4: Bacterial and viral abundance and bacterial biomass C over time in incubations with mitomycin C. A - Grassland soil – high input, B - Fallowed soil – low input. SE bars refer to the data associated with the axis adjacent to them (n=4). Bacteria (●), viruses (○), and biomass (◊). Note differences in scale.

5.3.3.1. Percentage lysogeny

In soil A, the maximum calculated lysogeny of 3-9% was at 90 hours incubation and lysogeny was not detectable at all at most time points in soil B (Figure 5.5). The highest value of percentage lysogeny, recorded at 15 hours, did not exceed 1%. Using the standard burst size formula [1] (section 5.2.4.1), percentage lysogeny in soil A was three-fold higher than using the modified formula [2] (section 5.2.4.2) due to increased bacterial abundance over the incubation period. Percentage lysogeny was virtually unchanged in soil B using either formula [1] or [2] as bacterial abundance was constant throughout.

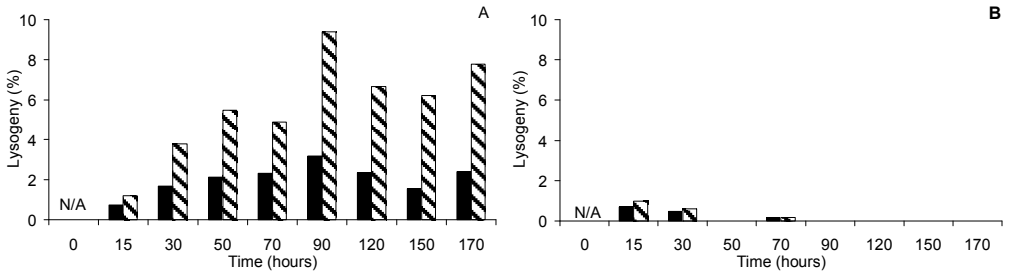


Figure 5.5: Percentage lysogeny as calculated by standard burst size [5.2.4.1] (hatched columns) or modified burst size [5.2.4.2] (solid columns). A - Grassland – high input soil, and B - Fallowed – low input soil.

5.3.3.2. Other indicators of lysogeny

Soil A and soil B showed similar magnitudes of percentage of induced virus over the control. The pattern in soil A fluctuated, whilst in soil B there were two maxima, one at 15 and another at 70 hours incubation (Table 5.3). After 70 hours there was evidence that mitomycin C had a negative effect on virus production in soil B, as fewer viruses were being counted in the mitomycin C treatments. In soil A mitomycin C continued to be effective to the end. The second watering of soil B after 70 hours therefore had a negative or no effect on induction of prophage.

The maximum positive induced burst size was 7.6 in soil A and 1.2 in soil B (Table 5.3). If the absolute value of induced burst size is considered, then the difference in magnitude is reflected by bacterial mean cell volume. Larger bacteria in soil A would be able to support a realistic burst size of 20 phage per bacterium, whilst the tiny bacteria extracted from soil B are barely larger than phage in the first instance.

Of interest is the contrast between the magnitude of the lysogenic fraction. Soil B bacterial populations were reduced by mitomycin C (hence the large 'lysogenic fraction'), although they did not necessarily

release phage, whereas in soil A, bacterial populations actually increased with mitomycin C in many samples.

Time (hours)	Lysogenic fraction (%)[1] [3]		Induced burst size (virus/bacterium)[2] [4]		Induced virus (% of control) [5]	
	A	B	A	B	A	B
15	-6.4	16.3	-3.6	1.2	114	201
30	-4.3	19.8	-11.5	0.7	139	152
50	-8.4	16.8	-7.1	-0.1	154	96
70	-16.0	36.4	-4.1	0.1	155	129
90	-2.3	22.8	-42.3	-0.1	158	96
120	-2.8	-10.4	-28.2	0.6	126	77
150	6.3	30.2	7.6	-0.2	118	62
170	-2.7	10.2	-31.6	-0.5	128	80

1. Negative values arise when bacteria counts with mitomycin C are larger than in the control. 2. A negative burst size either arises when induced virus counts are smaller than the control (as is the case in soil B), or when bacteria counts with mitomycin C are larger than the control (as is the case in soil A).

Table 5.3: Indicators of lysogeny in A - High input grassland soil, and B - low input fallowed soil.

5.3.3.3. Incidence of lysogeny

Incidence of lysogeny [6] differed markedly between the soils. In soil A, 81% of samples had significantly ($p<0.05$) higher virus counts with mitomycin C, compared with only 21% of samples in soil B. Bacteria counts were significantly ($p<0.05$) lower in equal proportions of soil A and B tubes, 53% and 50% respectively. Thus although mitomycin C addition resulted in decreases of bacterial counts in the same proportion of samples in soil A and B, prophage induction was only widespread in soil A.

The percentage of samples in which both bacterial counts decreased and virus counts increased significantly ($p<0.05$) was 44% and 8% for soil A and soil B respectively. From these data it can be inferred that lysogens are widespread in the high input soil (soil A) and

considerably less common in the low input soil (soil B). The low abundance of bacteria and viruses in soil B might lead to an implication that lysogeny should be widespread, but these data appear to refute this idea, and the hypothesis set out in the introduction is not supported.

5.4 DISCUSSION

5.4.1. Effective Mitomycin C Concentration

Mitomycin C was effective at the lowest dose applied. Given the complexity of soil structure and the fact that bacteria do not occupy most of the exposed surface area, the finding that a chemical can be effective at so low a concentration is remarkable. It demonstrated that mitomycin C was equally able to reach bacteria within the soil as resources such as water or substrate.

The negative effect on virus counts observed with the application of a high concentration of NaCl may be due to several factors. Increased adsorption of viruses to soil particles may have occurred as a result of increases in soil water electrolytes. In addition phage may have adsorbed more strongly to bacteria with increased NaCl – in a plating study 0.2M NaCl increased phage adsorption to bacteria by a factor of 10^4 (Hershey (p452 in (Adams 1959)). Fewer phage may have been released initially from infected cells, or taken longer to be produced, if bacteria were osmotically stressed by the NaCl.

The temperature of incubation was set at 18°C, as this would not ordinarily be an extreme for the soil biota. Temperature has been shown to have a marked effect on phage production. The time taken for viral lysis plaques to appear on plates of *Bacillus stearothermophilus* reduced

from 12 hours to just 2 when plates were incubated at 37°C instead of 30°C (Reanney and Marsh 1973). The author has not seen any work that carried out plate counts of phage at 18°C, but the assumption is that the time scale will be increased several fold. All the incubations carried here were done in constant temperature rooms or incubators, but the margin of error of 2°C or so may be sufficient for significant effects.

5.4.2. Soil Moisture as a Variable

Contrary to expectation, increased lysogeny was not observed in a drier soil environment. Increasing the soil moisture content by just 6% appeared to change the dynamic of phage-bacterium relationships. One explanation suggested for the differences in viral and bacterial abundance between the two incubations is based on phage transport in the soil. The soil is a clay loam, and thus water within is likely to be as water films surrounding clay domains and particles.

With reference to the moisture release curve for Cashmore clay soil (Chaper 3), a moisture content of 26% may occur at the range of potentials from –20 kPa to –100 kPa, whilst 32% would be associated with a potential below -10kPa. The wetter soil would be equivalent to pores of 30 μm draining, but in the drier soil pores of about 6 μm will be drained. Water film thickness differences between the two potentials would be great. At 26% moisture, released phage may have remained close to the site of release, as there would be insufficient water to move them rapidly elsewhere. In contrast, 32% moisture would have been sufficient to effect mass movement of viruses free in the soil water allowing far greater possibilities to adsorb to host cells. As a result

increased phage production was possible. Phage production was also more rapid in the wetter soil. Phage released when mitomycin C acted on bacteria within smaller pores may have moved into more accessible (for extraction) zones in the wetter soil, whilst in the drier soil they may have remained closer to their release site. Increased movement of phage is the most likely explanation for the differences observed in lysogeny. The soil was incubated at relatively high moisture content to allow effective action of mitomycin C. Investigations with a wider range of soil moistures may yield other differences, and will help answer questions as to the spatial variability of lysogeny within soil. How would the outer surfaces of aggregates, consisting of bacteria subject to many perturbations and protozoan grazing pressure but with potentially more substrate, compare with the relatively stable inner aggregate with perhaps less productive bacteria?

In environments where host abundance is high, virulence may be a better survival strategy than lysogeny but if they are spatially isolated in unconnecting pores then the proportion available to phage may be considerably lower.

5.4.3. Organic Carbon as a Variable

Contrary to expectation, levels of lysogeny in the energy-rich soil A were low and were virtually non-existent in the energy-deficient soil (soil B). Estimates of lysogeny in aquatic environments are typically higher, although 1% is within recorded range (Weinbauer 2004). The maximum percentage lysogeny calculated for soil B was at 15 hours, an incubation period consistent with aquatic studies. For soil A, the

maximum was recorded after 90 hours incubation, far longer than in most incubations of aquatic systems (Paul and Jiang 2001).

An increase in the abundance of larger cells during incubation has been shown before (Bae and Casida 1973). Mean cell volume of bacteria from soil B was very small, although similarly sized bacteria have been reported in lake water (Loferer-Krössbacher, Klima, and Psenner 1998). The difference that management practice has on bacterial MCV has been demonstrated elsewhere: MCV was twice as great (0.8 μm^3) in a carbon-rich soil compared to 0.4 μm^3 in a carbon-deficient soil (Schnürer, Clarholm, and Rosswall 1985).

It is suggested that the majority of the bacteria in the fallow soil might be ultramicrobacteria. They are abundant in other environments, and their significance in soil nutrient cycling has been postulated (van Elsas and van Overbeek 1993). Dwarf cells were categorised as having a diameter of <0.3 μm (Bae, Cota-Robles, and Casida 1972). This equates to a cell volume in the order of 0.015 μm^3, yet 80% of cells in soil B were smaller than this. The higher proportion of ultramicrobacteria in soil B and the much lower viral counts suggests that these small bacteria with their low DNA content may be too small to support prophage. A study of lake bacterioplankton showed very little viral infection of bacteria of diameter <0.3 μm (Weinbauer and Höfle 1998b). Larger cells have been shown to have higher burst sizes in aquatic systems (Weinbauer and Höfle 1998b). Thus in soil B only a relatively small proportion of the bacteria (the larger cells) might support viruses, hence the very low overall levels of lysogeny in this community. Spatial

variability of host bacteria within the soil (Marsh and Wellington 1994), or low bacterial abundance (Wiggins and Alexander 1985) might also contribute to the low incidence of viruses in soil B.

Surviving bacteria in soil A may have benefited from cell lysates produced during viral lysis, thus increasing abundance. This supports the theory that viruses can increase nutrient turnover and bacterial abundance (Wilhelm and Suttle 1999), however other studies have found that bacterial abundance is increased in the absence of phage (Middelboe, Jørgensen, and Kroer 1996).

In contrast to aquatic systems the extent of the lysogenic cycle appears to differ in that the percent of lysogenic hosts was low, and it was greater in the energy rich environment. In the low carbon soil (B) the lytic cycle predominated in a bacterial community dominated by small bacterial cells. In aquatic ecosystems lysogeny is typical of conditions that are not conducive to strong bacterial growth, when finding suitable new hosts might be problematic (Williamson *et al.* 2002).

Virus and bacterial populations in soils fluctuate significantly over a matter of hours, so single point estimates of abundance do not reflect the true state of bacterial/viral dynamics. Moreover, carbon status is a significant factor in determining the abundance and biomass of bacteria and viral activity, as it is in aquatic ecosystems.

5.5 SUMMARY

Although there is a growing body of work on the extent of lysogeny in aquatic ecosystems, and in a few species of soil bacteria, there is a paucity of information on lysogeny in natural soil communities. The mutagen mitomycin C is commonly employed in aquatic systems as an inducing agent to test for lysogenic phage but has not been reported in use in soil. This work aimed to set out some baseline data on the action of mitomycin C in several soils.

From the data presented here mitomycin C is effective at determining lysogeny in natural soil bacteria. It is recommended that incubation periods of soil samples should be longer than those reported from aquatic research, as maximum lysogeny in more than one study did not arise until 90 hours incubation. Concentrations as low as 1 μg mitomycin C g^{-1} soil were effective, but 5-10 μg mitomycin C g^{-1} soil obtained higher viral counts.

Two hypotheses were tested based on findings from aquatic systems. The first was an expectation that a wetter soil would exhibit lower lysogeny than a drier soil due to increased host availability in the wetter soil. The second was the expectation that a carbon-rich soil would exhibit lower lysogeny than a carbon-poor soil due to increased resources for phage replication. Neither hypothesis was supported by the data, indicating that viral dynamics in soils differ from patterns seen in aquatic ecosystems.

CHAPTER 6: EFFECTS OF PLANTS AND PROTOZOA ON VIRAL AND BACTERIAL DYNAMICS IN SOIL

6.1 INTRODUCTION

Previous chapters have looked at viral and bacterial dynamics in isolation of other soil biota or nutrient status. Predation and availability of nutrient sources influence bacterial abundance and turnover and so should have an effect on viral production in the soil.

6.1.1. Bacterial Nutrient Sources

Heterotrophic soil bacteria obtain their nutrient and energy requirements from constituents of their surroundings. Bacteria are capable of utilising many forms of organic carbon and nitrogen, but simple compounds are more rapidly taken up as complex polymers must first be (typically) hydrolysed into sub-units by extra-cellular enzymes, before being transported into the cell (Gottschalk 1979). Soluble forms of organic matter such as amino acids, organic acids and sugars are utilised quickly, whereas cellulose, lignin and fibrous proteins are more recalcitrant (Paul and Clark 1996). The decomposition of this organic matter by bacteria and fungi is the basis of soil food webs (De Ruiter *et al.* 1993), and a measure of this activity is mineralisation – the formation of inorganic carbon and nitrogen when organic compounds are degraded. Bacteria that are more active are likely to release more phage (Chapter 1). Three processes that increase the availability of nutrient and energy sources for soil bacteria will be explored here: external amendment, protozoan grazing, and presence of plants.

6.1.2. Protozoan Grazing Pressure

Heterotrophic Protozoa found in the soil belong to four main taxonomic groups: flagellates, naked amoebae, testate amoebae and ciliates. All feed on bacteria, and for many it is their main food source (Nisbet 1984). They feed mainly by engulfing prey or by filter feeding and in ciliates this depends on the oral apparatus, while in most flagellates and amoebae there is no cell mouth or cytostome and food items enter the cell through the cell membrane. Amoebae typically require a surface on which to move and feed, whilst ciliates and flagellates can filter water for food while in suspension themselves (Laybourn-Parry 1984). Protozoa have similar carbon-to-nitrogen ratios as their prey (C:N are about 5) (Griffiths 1994) and they are relatively inefficient (40% efficiency) at assimilating carbon (Heal 1971). When they metabolise bacterial biomass they excrete otherwise immobilised nitrogen and phosphorus into the environment (Eccleston-Parry and Leadbeater 1995) mainly as ammonium and orthophosphates, but also in more complex organic forms (Pussard, Alabouvette, and Levrat 1994). This nutrient source is readily assimilated by surviving bacteria, and increases net mineralisation (Griffiths 1989) and respiration (Elliott *et al.* 1980). The clearest illustration comes from increased nitrogen fixation by *Azotobacter chroococcum* when *Colpoda steinii* ciliates were present, as the ciliate can not fix nitrogen (Darbyshire 1972). Further findings are summarised in various reviews (Bamforth 1988; Ekelund and Rønn 1994; Hattori 1994; Clarholm 1994; Bonkowski 2003).

Protozoan grazing also has an effect on the composition of the bacterial community. A mixed protozoan inoculum decreased the

proportion of Gram-negative bacteria and increased Gram-positive bacteria in one study (Rønn *et al.* 2002), slightly decreasing the diversity of the community overall. Numbers of nitrifying bacteria decreased more than heterotrophic bacteria when *Adriamonas* flagellates were present (Verhagen and Laanbroek 1992), showing selective grazing.

Effects of Protozoa on bacterial cell size have also been reported: an increase in cell size of a bacterial strain from 1.5 μm to as much as 20 μm occurred when incubated with a ciliate predator (Shikano, Luckinbill, and Kurihara 1990). The effect of grazing on the community as a whole appears to be reduced bacterial mean cell volume, as has been demonstrated by studies in chemostats (Hahn and Höfle 1999), aquifer sediment (Kinner *et al.* 1998), and in soil (Clarholm 1985). In the last study bacterial cell size was increased in microcosms with plants compared to those in unplanted tubes, suggesting that the increased activity associated with the rhizosphere led to larger bacterial cells.

6.1.3. Plants as a Source of Carbon for Bacterial Growth

Plants are responsible for the greatest proportion of organic matter that enters the soil (Sørensen 1997). They produce muscilage polysaccharides, slough off dead cells and actively exude substances, with the release of low molecular weight organic acids, amino acids and sugars to the surrounding soil (Jones, Hodge, and Kuzyakov 2004). Concentrations of carbon and nitrogen contained within these exudates can be in the region of 30 mg g^{-1} of root (Zwart, Kuikman, and van Veen 1994), so this can be a significant addition to the dissolved organic matter pool in the soil. These contributions are not simply by-products of

plant growth, but some are actively exuded as chemo-attractants to microbes (Dakora and Phillips 2002). Bacterial death due to exudates has also been demonstrated; lysozyme released from potato roots significantly decreased survival of adsorbed *B. subtilis* (Ahrenholtz *et al.* 2000).

Plants mediate other changes in soil through the physical actions of root growth and water uptake (Six *et al.* 2004). Root penetration decreases soil porosity immediately adjacent to it; water uptake will dry soil but water films will occur on roots, providing habitats for microbes (Sørensen 1997); and aggregate formation is increased (Oades 1984; Oades 1993) through root exudates and entanglement. These factors lead to increases in: the abundance of Protozoa (Griffiths 1990), bacterial activity (Söderberg and Bååth 2004), C mineralisation (Christensen *et al.* 1995), and N mineralisation (Klemedtsson *et al.* 1987).

The rhizosphere therefore supports much higher bacterial concentrations than the bulk soil and this in turn attracts higher concentrations of their protozoan predators. Where plants are present their role in turning over carbon together with protozoan predation might be expected to have a combined impact on bacterial community dynamics.

6.1.4. Implications of Rhizosphere and Predation on Phage

If bacteria respond to grazing pressures and changes in their microenvironment by adapting their morphology and increasing growth rates, or if the diversity of the community is affected, then this will have

implications on their relationship with phage. In one aquatic system studied the removal of protozoan grazers increased bacterioplankton production rates and increased viral infection but did not alter burst sizes (Simek *et al.* 2001), so the effects of Protozoa were not that significant in viral production overall. Viral infection may be affected by the development of filamentous cells (Hahn, Moore, and Höfle 1999) or shifts in mean cell volume (Hahn and Höfle 1999) as a result of grazing. Soil phage have been shown to have a wide host range (Tan and Reanney 1976), so these changes may not be a deterrent to infection. The conclusion from one study on aquatic bacterial strains was that grazing increased viral production (Middelboe *et al.* 2001). However, if grazing pressure leads to an overall reduction of mean cell size, it is probable that these smaller bacteria will liberate fewer phage than larger bacteria, as has been shown in bacterioplankton (Weinbauer and Höfle 1998b) (Chapter 4). In other words they have lower burst sizes. Thus it is uncertain whether virus production will be increased or not in the presence of Protozoa.

The effect of rhizosphere activity on bacteria is likely to increase virus production as the metabolic activity of their host is enhanced. Community shifts to an increased proportion of nitrifying bacteria in the rhizosphere will be unlikely to change virus production significantly, as nitrifying bacteria such as *Nitrobacter* (Bock, Düvel, and Peters 1974) have been shown to harbour phage. Increasing the supply of carbon and inorganic nitrogen through amendments also stimulates bacterial activity and reproduction, and will lead to an increase in virus production (Tuomi *et al.* 1995).

6.1.5. Microcosm Studies

Microcosm studies can be used to explore the interactions described above as they can be manipulated more easily than field studies and reflect at least some of the variability and conditions of natural soil (Prosser 1997). They can be broadly classified into two categories: sterilisation and specific inoculation. Sterilisation studies remove existing soil communities before adding selected organisms. The addition of Protozoa and bacteria at known population densities and recording total abundance during or at the end of an incubation has been widely practised (Darbyshire and Greaves 1967; Elliott, Coleman, and Cole 1979; Clarholm 1985; Vargas and Hattori 1986; Kuikman and van Veen 1989; Wright *et al.* 1995). These studies have led to greater understanding of the significance of Protozoa on the population density of their prey; susceptible accessible bacteria are consumed and the population diminishes to a stable level relative to that in predator-free microcosms.

Specific inoculation studies have the advantage that the existing community does not need to be removed from the soil. Common practice has been to add antibiotic-resistant bacteria to communities that can be subsequently identified through plating. For example introduced streptomycin-resistant *Rhizobium* populations decreased concurrent with an increase in Protozoa (Danso, Keya, and Alexander 1975; Habte and Alexander 1977). Organisms possessing an engineered *lux* gene can be enumerated through fluorescence; spatial protection of *Pseudomonas fluorescens* from ciliate attack was demonstrated using this technique (Wright *et al.* 1993). Molecular characterisation analyses, as outlined in

Chapter 2, have a role to play in determining diversity of communities (Kent and Triplett 2002). DGGE was used sucessfully to show that bacterial communities were changed by grazing, with bacteria similar to *Arthrobacter* being favoured by the Protozoa for a food source (Rønn *et al.* 2002).

Sterilisation studies have the advantage that any number of organisms can be added to the system, creating complex communities, albeit artificially generated. The disadvantage is that the system is no longer 'natural'. Specific-inoculation studies can track the fate of one or few organisms in a mostly untouched system, but this may not be representative of organisms in their natural environment. A sterilisation study was considered to be most appropriate for this work, as there have been previous group- or species-specific inoculation studies on soil phage: on *Streptomycetes* (Cresswell *et al.* 1992; Herron and Wellington 1994) and *Serratia* spp. and *Pseudomonas* spp (Ashelford *et al.* 1999; Ashelford *et al.* 2000). A bacterial community may in addition highlight a more general pattern of phage dynamics than a few closely related strains.

6.1.5.1. Microcosm case study

One study, cited 200 times (Thomson Scientific ISI Web of Knowledge, Web of Science – Science Citation Index, accessed 4/1/2006), explored the effect of Protozoa on bacterial abundance and nitrogen uptake by plants (Clarholm 1985). Microcosms of increasing complexity were created by the addition of bacteria to planted and unplanted autoclaved loam soil with or without Protozoa added. Bacteria

were obtained by mixing soil with water and then filtering the liquid phase through 3 μm filters. Protozoa from the same soil were used after incubation with bacterial suspension. Amoebae and flagellates were added at a density of 3 $x10^3$ cells g^{-1} soil, with a low number of ciliates added (<50 cells g^{-1} soil). Additional nutrient amendments of carbon (as glucose), nitrogen (as ammonium nitrate), and carbon plus nitrogen combined were added. Three germinated wheat seeds were added for the planted tubes.

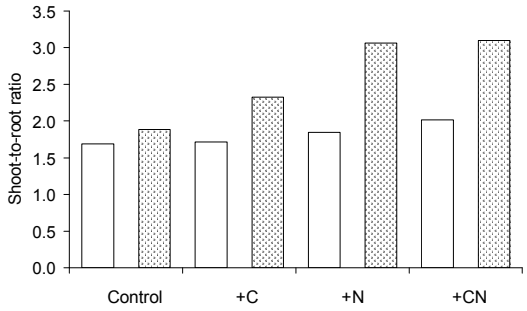


Figure 6.1: Shoot to root ratios of wheat seedlings after 6 weeks grown in microcosms with added nutrients. Protozoa present (dotted columns), Protozoa absent (white columns). Calculated from data in Clarholm 1985.

The experiment ran for six weeks, at the end of which protozoan abundance, bacterial abundance, soil N and plant dry weight and N were recorded. The results in brief were as follows: plants grown with Protozoa present were heavier (266 mg) than those without (181 mg), but with lower shoot N concentrations (3.0% against 3.7%). Soil ammonium N was 3 times lower with Protozoa present, but nitrate N was unchanged, a possible indication that bacterial uptake of NH_4 was increased. All Protozoa increased in abundance, but amoebae were significantly more numerous in unplanted soil. Nutrients had a negative effect overall on protozoan abundance.

6.1.6. Hypothesis

Soil that contains protozoan predators and active plant roots will enhance bacterial activity. Increased bacterial and viral abundance is expected in planted microcosms with Protozoa than in unplanted microcosms without Protozoa. Nutrient addition will increase abundances of both bacteria and viruses over the controls.

6.1.7. Aim

The experiment outlined here is a modified version of Clarholm's study reported in 1985, but with counts of virus numbers made concurrent with bacteria, and more frequent analyses over time. Fluctuations and magnitude of viral abundance over time in different microcosms might allow an assessment of the relative importance of Protozoa, nutrients and/or carbon, and plant roots on bacterial production of phage.

6.2 METHODS

6.2.1. Soil Preparation

Soil was collected from Bypass field using the method in Chapter 2. Soil was air-dried, passed through a 2 mm sieve, followed by sterilisation by autoclaving for 20 minutes at 15 *p.s.i.* pressure at 135°C. After heating, the soil was thoroughly mixed and turned to allow gases to escape, then dried at 105°C for 24 hours. Slides prepared from the soil using SYBR Gold showed that no organisms remained in the soil after processing.

6.2.1.1. Soil extract media

Soil extract medium was prepared according to guidelines from the Culture Collection of Algae and Protozoa handbook (2005). A soil sample (stored sieved <2 mm moist at 4°C) of 50 g was mixed in 330 ml de-ionised water. After thorough mixing, it was autoclaved at 15 *p.s.i.* pressure for 15 minutes, left to stand for 24 hours and autoclaved again. The bottles were allowed to sediment for a few days before filtering the liquid through a GF/C filter and stored at 4°C.

6.2.2. Bacteria Inoculation

A bacterial community was prepared by shaking 10 g of fresh soil with distilled water for 5 minutes. The supernatant was left to settle for 5 minutes to allow particulate matter to sediment before 100 μl aliquots were plated onto nutrient agar. Plates were left for 2 days at room temperature to initiate growth before storing at 12°C. After lawns had developed, bacteria were washed off plate surfaces with sterile Chalkley's solution (Culture Collection of Algae and Protozoa 2005) and transferred into plastic tissue culture flasks.

6.2.2.1. Lysogeny

As viral abundance was one of the key parameters of this study the cultured bacterial community was tested for virus production. Adding mitomycin C and comparing counts with and without tested the presence of phage and the extent of lysogeny (Chapter 4) in the bacterial suspension. An aliquot of 1 ml bulked from several culture flasks was diluted fifty times in $^{1}/_{10}$ nutrient broth and incubated at room temperature

until the suspension had an absorbance of 0.4-0.6 at 600 nm (Paul and Jiang 2001). A sub-sample of 1 ml was then removed and processed for initial bacteria and virus counts. The remainder was then divided equally into two flasks. A 200 μl aliquot of sterile de-ionised water was added to the control, and to the experimental flask a 200 μl aliquot of water containing 10 μg of mitomycin C. After 18 hours sub-samples were fixed in glutaraldehyde and processed for enumeration of bacteria and viruses with SYBR Gold.

6.2.3. Protozoa Inoculation

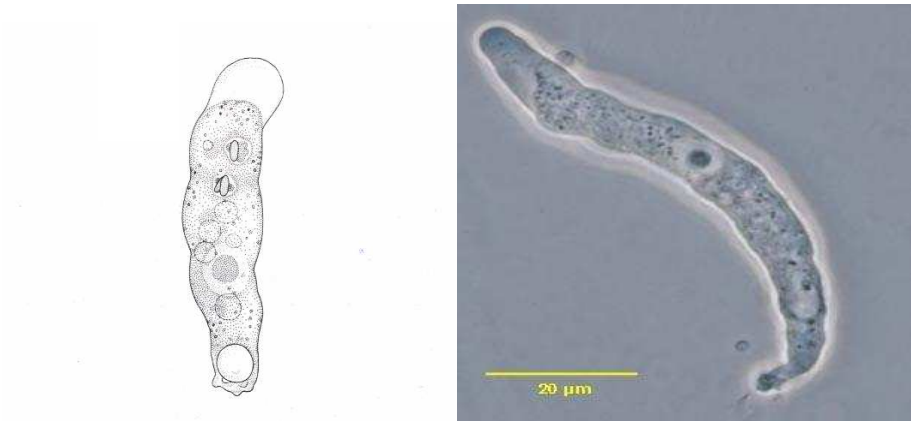
Protozoa selected for inoculation were: *Bodo saltans* (flagellate), *Colpoda steinii* (ciliate), and *Hartmannella* sp (naked amoeba) (Photograph 6.1). *Bodo saltans* and *Colpoda steinii* were obtained from the Culture Collection of Algae and Protozoa, and *Hartmannella* was kindly provided by Dr J. Parry, Department of Biological Sciences, Lancaster University. All are organisms that are commonly found in the soils. The flagellates and ciliates were maintained in culture flasks containing Chalkey's medium (Culture Collection of Algae and Protozoa 2005) with a few drops of bacteria concentrate. The concentrate was made using sterilised soil water and bacteria washed off plates. Amoebae were cultured on amoeba agar plates using *Klebsiella* sp. as a food source. All Protozoa were cultured in an incubator at 12°C in the dark.

6.2.4. Experimental Set Up

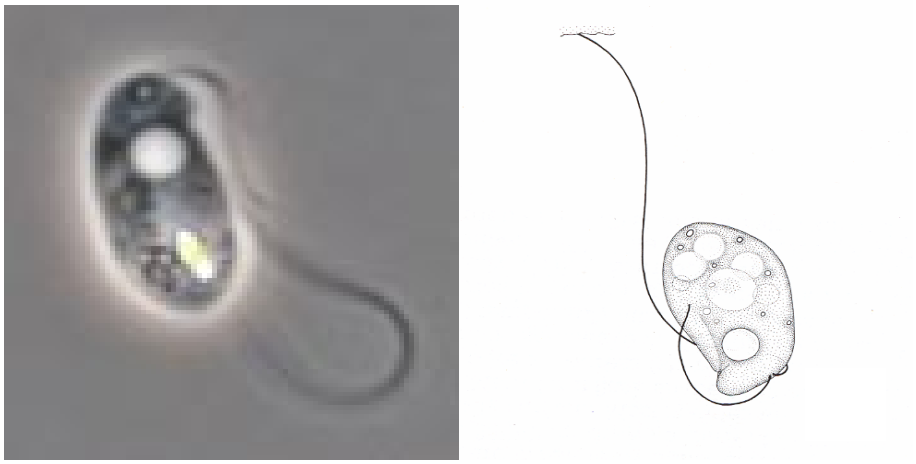
Sterile straight-sided glass boiling tubes (diameter 22 mm, height 150 mm) were used as experimental vessels. Sterilised soil samples (35 g dry weight) were added to 240 tubes and packed to an approximate density of 1.0 g cm^{-3} by tapping the tube gently whilst filling, and pressing a clean flat-bottomed sample jar gently on the surface. Half the tubes were seeded with 7 seeds of *Lolium multifolium* (Italian ryegrass) surface sterilised with alcohol. The other half was not planted. All tubes were wetted with 5 ml of sterile de-ionised water, sealed with cotton wool plugs and placed in a laboratory environment lit by both natural and fluorescent light. After germination extra seedlings were thinned out so that each planted tube contained 3-5 seedlings. The tubes were left for one week to allow the plants to develop some root structure independent of any subsequent nutrient additions (Photograph 6.2).

Initial bacterial inoculations on day 0 were in the order of 1 $x10^7$ cells g^{-1} dry soil. On day 1 Protozoa were added: flagellates and amoebae were in approximate concentrations of 5 $x10^3$ cells g^{-1} dry soil, and ciliates were added at very low levels (<1 $x10^2$ cells g^{-1} soil). Inoculation concentrations were similar to those used by Clarholm (Clarholm 1985). Inocula were added in 1 ml sterile water. Nutrient treatments were 500 μg of carbon (glucose) g^{-1} dry soil and 125 μg ammonium nitrate g^{-1} dry soil given as 4 ml aliquots at the start of the experiment as carbon only (+C), nitrogen only (+N) or carbon and nitrogen combined (+C+N). The control tubes acted as a baseline of

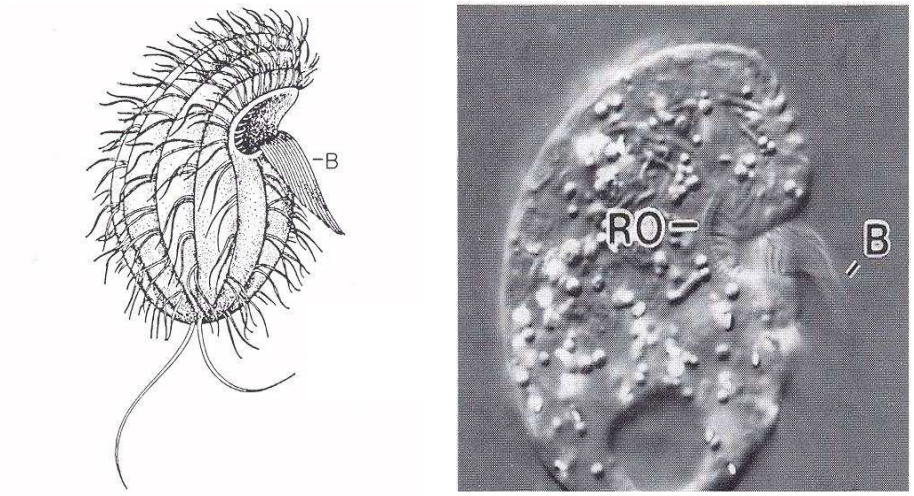
available nutrients, and the amended tubes provided additional carbohydrate and inorganic nitrogen for rapid bacterial production.



Hartmannella sp. 10-50 µm (Patterson 1996)



Bodo saltans 5-10 µm (Patterson 1996)



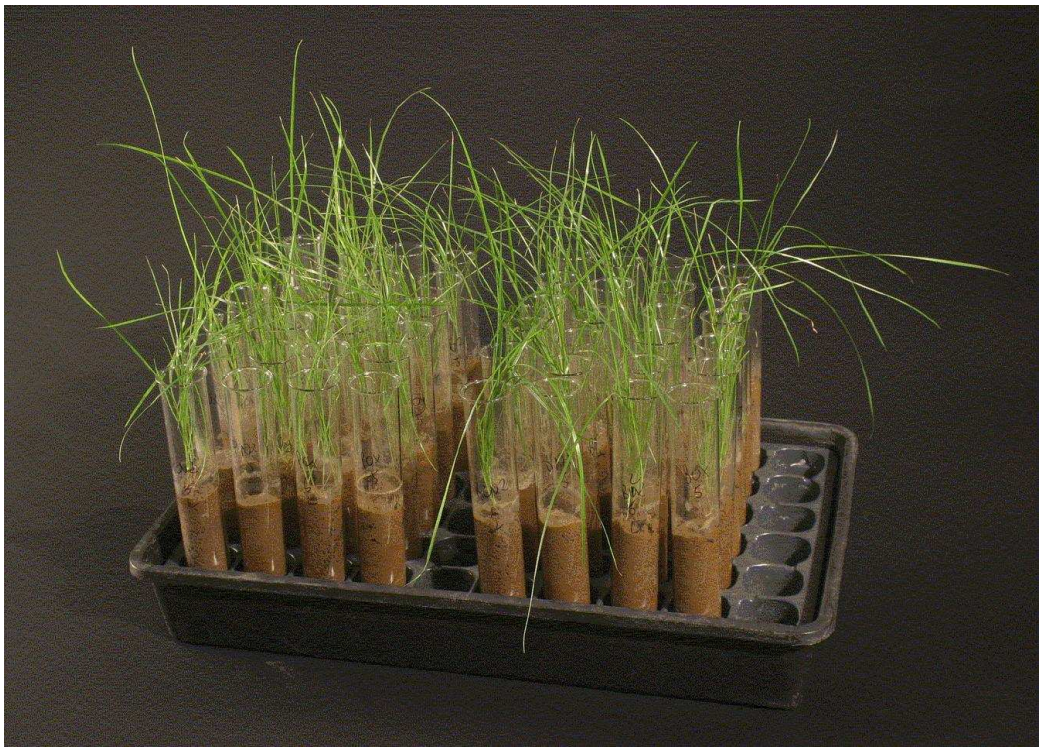
Colpoda steinii 15-40 µm (Foissner, Berger, and Schaumburg 1999)

Photograph 6.1 Images of the three Protozoa species used in this study.

Microcosms were set up as a multiple factorial design. Factors were nutrients, plants and Protozoa. Levels of nutrients were control, carbon only, nitrogen only and carbon and nitrogen combined. Plants were present or absent, Protozoa were present or absent (Table 6.1). There were three replicate tubes for each treatment thus 48 tubes were destructively sampled for each treatment at each sampling time. The tubes were incubated in a glass-house and arranged so that each treatment was randomly placed on trays for each harvest period, one tray per harvest. The trays were moved and rotated around the bench to minimise any location effect. The microcosms were well watered initially with water or nutrient solution. Sampling times were on days 2, 7, 14, 23 and 35 days after inoculation. Timings were similar to those used in other microcosm work (Elliott *et al.* 1980; Kuikman, van Vuuren, and van Veen 1989).

WITHOUT PLANTS								
TREATMENT	1	2	3	4	P1	P2	P3	P4
BACTERIA	√	√	√	√	√	√	√	√
PROTOZOA					√	√	√	√
AMENDMENT	CONTROL	C	N	C+N	CONTROL	C	N	C+N
WITH PLANTS								
TREATMENT	L1	L2	L3	L4	LP1	LP2	LP3	LP4
BACTERIA	√	√	√	√	√	√	√	√
PROTOZOA					√	√	√	√
AMENDMENT	CONTROL	+C	+N	C+N	CONTROL	+C	+N	+C+N

Table 6.1: Treatments established in the microcosm study in 35 g soil. Bacteria were added at 1 x10^7 bacteria g^{-1} dry soil. Flagellates and amoebae at 5 x10^3 g^{-1} dry soil, and ciliates at <1 x10^2 g^{-1} soil. Nutrient treatments were (C) 500 μg of carbon (glucose) g^{-1} dry soil, (N) 125 μg ammonium nitrate g^{-1} dry soil and (C+N), a combination of 500 μg glucose and 125 μg ammonium nitrate g^{-1} dry soil given as 4 ml aliquots at the start of the experiment. There were 3 replicate tubes for each treatment. Treatment codes are referred to in the text. L-Lolium (planted), P-Protozoa. 1 = control, 2 = +C, 3 = +N and 4 = +C+N.



Photograph 6.2: Microcosm set up. Planted tubes only are shown from day 35, after sorting for sampling.

6.2.4.1. Statistical analysis

Abundance data were log transformed for normality and treated in separate multiple analysis of variances for each time point. Statistical analysis was by Genstat (Version 7, VSN International). The significance of differences of means is expressed at 95% unless otherwise stated. Abundance data are expressed as means ± one standard error of the mean.

6.2.5. Plant Dry Weight

At sampling the soil was removed from the tube using a spatula or knife. In the planted tubes, plants were teased away from the soil and washed clean with de-ionised water. Plants were placed in paper bags, oven-dried at 85°C, and subsequently weighed to determine dry weights.

6.2.6. Soil Moisture Content

A portion of approximately 20 g from each tube was oven-dried at 105°C for 24 hours to determine gravimetric moisture content.

6.2.7. Dissolved Organic Carbon and Nitrogen

A 10 g portion from each tube was added to 30 ml of sterile de-ionised water (Chen, Xu, and Mathers 2004), shaken on a bench-top shaker for 14 hours and centrifuged at 4000 rpm (c. 3000 *g*) for 20 min. Samples of 20 ml were filtered through GF/F filters (treated beforehand by heating at 400°C for 4 hr in a furnace) for the measurement of organic carbon (DOC) and dissolved organic nitrogen (DON). Filtrates were stored at -18°C until frozen in acid washed plastic bottles. After freezing at –18°C for 24 hr, they were transferred to a –80°C freezer. A Shimadzu TOC/TON analyser was used for the analysis of DOC and DON.

6.2.8. Bacterial and Viral Counts

Soil from each tube was emptied onto a foil square and well mixed. A sub-sample of 1.5 g was put into a 15 ml centrifuge tube with 10 ml of buffered 4% glutaraldehyde. Samples were shaken on a bench top shaker for 10 min and allowed to settle for a few minutes before a 3 ml portion was removed from all tubes: those portions from treatments with Protozoa (P1-4 & LP1-4) were processed for flagellates as detailed below. The remaining slurry was then processed as outlined in section 2.5.

6.2.9. Protozoa

6.2.9.1. Flagellates

The 3 ml portions from P1-4 and LP1-4 were carefully added to the top of a 15 ml Eppendorf centrifuge tube containing a Percoll layer (created by adding 5 ml of sterile Sorensen's phosphate buffer to the tube, then slowly adding 5 ml of Percoll) (Griffiths and Ritz 1988). This stood for 30 minutes, and then was centrifuged for 1 hour at 3500 rpm (c. 2500 *g*). After centrifugation the supernatant was decanted into a clean fresh tube and 4 drops of 1% DAPI added for staining. The tubes were stored at 4°C for a maximum of 12 hours before processing. For slide preparation 10 ml aliquots were filtered under low pressure onto 2 µm polycarbonate filters that were subsequently mounted on microscopes slides. Slides were stored in the dark at 4°C for up to 3 days prior to microscopic analysis under epifluorescence using an UV filter at x1600 magnification.

6.2.9.2. Amoebae

A modified most probable number method (Darbyshire *et al.* 1974) was employed as follows. A sample of 1.5 g wet weight soil from each P* treatment was mixed with 10 ml diluted soil extract. To each tube, 5 ml of sterile soil extract and 5 ml of sterile Page's amoeba saline solution (Culture Collection of Algae and Protozoa 2005) was added. After gentle shaking aliquots of 100 µl were pipetted from each sample into four rows of the first column of a microtitre well plate, to which had been added 100 µl of bacterial soil extract. The extract was prepared by adding 5 ml of thick bacterial broth to 100 ml of sterile soil extract. Ten-

fold dilution was then carried out in the next four columns. Applying full suction on the pipette, evacuating it and repeating the process several times mixed the cell contents before a 10 μl aliquot was removed. Four samples were pipetted on one plate, with empty cells filled with sterile water to reduce evaporation. Plates were left in covered boxes in the dark at around 20°C in which beakers of sterile de-ionised water were placed to reduce evaporation.

6.2.9.3. Ciliates

Ciliates were counted according to the method outlined by Bamforth (Bamforth 1991). A sub-sample of 3 ml of the sample described above was taken after shaking and examined drop by drop for active ciliates. Droplets of 50 μl were pipetted onto a microscope slide and viewing under an inverted microscope at x400 magnification under phase contrast.

6.3 RESULTS

6.3.1. Lysogeny

During the course of incubation with mitomycin C and control incubations bacteria counts stayed at about 115 $x10^5$ cells ml^{-1} (Table 6.2). However, virus counts were 20% higher in the control relative to the mitomycin C treatment which suggests that viruses were predominantly in lytic cycles and the lysogenic cycle was not operating. Applying the lysogenic fraction calculation (section 5.2.4.3), 3.5% of the bacteria were lysogenic. Using the burst size calculation, lysogeny was negative at -0.3%.

	T=0	T=18 hours	
		Control	Mitomycin-C
Bacteria	120 (±6.6)	114	110
		(± 5.8)	(± 7.1)
Viruses	7.54 (±1.3)	44.8	34.8
		(± 4.8)	(± 4.5)

Table 6.2: Bacteria and virus counts (x10^5 ml^{-1} suspension) after 18 hours with and without mitomycin C ± standard error of the mean (n=2).

6.3.2. Soil Moisture

From the initial 20% (g/g) water content, moisture decreased to 15% in unplanted tubes (1-4 & P1-4) and below 10% in planted tubes (Figure 6.2). There was no significant difference in moisture content between nutrient additions or between treatments with or without Protozoa. After sampling on day 23, the planted tubes were re-watered up to 20% water content (g/g). By day 35, moisture had again dropped to 12-15%. The water status of the planted treatments (L1-4 & LP1-4) underwent significant wet-dry cycles during the incubation, whereas the unplanted soil did not. Consequently there was likely to be reduced protozoan activity during the dry periods.

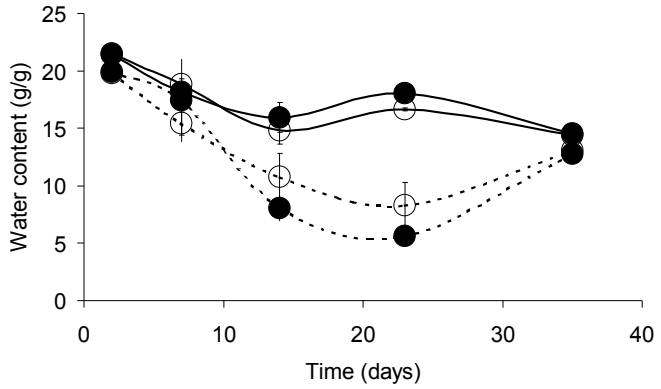


Figure 6.2: Soil moisture over time in microcosms of Bypass soil. Amendments and control (*1-*4) are aggregated into single data points. Protozoa present (solid), Protozoa absent (open). Planted (dotted lines), Unplanted (solid lines). Bars are one standard deviation (n=12).

6.3.3. General Trend in Abundance

The general trend was that bacterial and virus numbers increased significantly in the first two days of incubation and then decreased to <1 $x10^7$ cells or particles g^{-1} soil for three weeks and subsequently rose again after day 23. Thus there was a rapid response to the availability of nutrients followed by either death or strong adsorption to soil particles. Virus numbers mirrored fluctuations in bacterial numbers throughout the incubation. Protozoa numbers increased from initial inoculation and then decreased probably as a result of grazing pressure on the bacterial community.

6.3.4. Week 1 – High Activity Period

6.3.4.1. Bacterial and viral abundance

An increase in bacterial counts indicated a rapid response to nutrients. These rose from the initial 1 $x10^7$ cells g^{-1} soil to a mean of 1.9 $x10^7$ cells g^{-1} dry soil in the controls (1,P1,L1,LP1), 2.3 $x10^7$ cells g^{-1} dry soil in +C treatments (2,P2,L2,LP2), 3.5 $x10^7$ cells g^{-1} dry soil in +N treatments (3,P3,L3,LP3), and 3.6 $x10^7$ g^{-1} dry soil in +C+N treatments (4,P4,L4,LP4) (Figure 6.4): bacteria increased more with combined addition of carbon and nitrogen than with single additions. The increase in control tubes was due to utilisation of liberated organic matter from the sterilisation procedure. Bacteria counts with +C were no different from control counts in three treatments, but in unplanted tubes with Protozoa (P2), they were over five-fold higher.

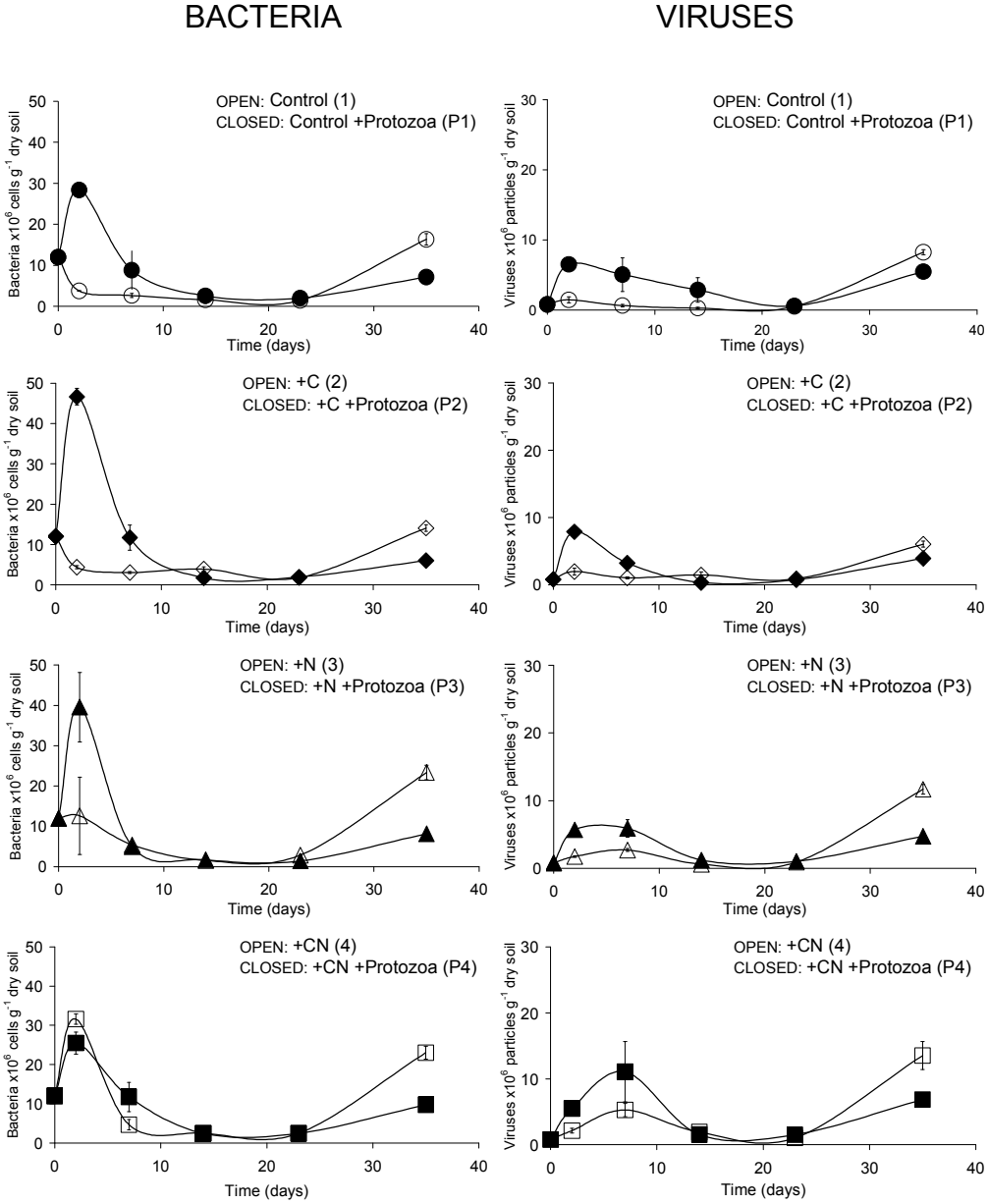


Figure 6.3: Bacterial and viral abundance x10^6 g^{-1} dry soil over time in unplanted tubes (1-4 & P1-4). Protozoa present (solid), Protozoa absent (open). Control (o), Carbon (◊), Nitrogen (Δ), Carbon+Nitrogen (□). Bars are one standard error of the mean (n=3).

Bacterial counts were significantly higher ($p<0.001$) in all +N (3,P3,L3,LP3) and +C+N (4,P4,L4,LP4) treatments compared with +C (2,P2,L2,LP2) or controls (1,P1,L1,LP1), so autoclaving may have released an excess of readily-assimilated carbon, but not of nitrogen so bacteria benefited from the amendments.

As was expected, bacterial counts were generally higher in planted than unplanted tubes. Amendment-aggregated means were 1.3

$x10^7$ cells g^{-1} dry soil for unplanted (1-4) and 3.8 $x10^7$ cells g^{-1} dry soil for planted tubes (L1-4), a three-fold increase. Plants therefore directly increased bacterial abundance. When Protozoa were added to the system, bacterial counts were increased in unplanted tubes but decreased in planted tubes, with overall means of 3.5 $x10^7$ cells g^{-1} dry soil for unplanted with protozoa (P1-4) and 2.8 $x10^7$ cells g^{-1} dry soil for planted with protozoa (LP1-4). Bacteria may have been under increased vulnerability to grazing in the rhizosphere. The small abundance of bacteria in +C+N (P4) on day 2 coincided with the highest abundance of flagellates on day 7 (Figure 6.5).

Viral counts increased more than bacteria over the first two days: they increased from an initial 0.1 $x10^7$ particles g^{-1} dry soil to 0.75 $x10^7$ particles g^{-1} dry soil in the controls (1,L1,P1,LP1), 0.83 $x10^7$ particles g^{-1} dry soil in +C treatments (2,P2,L2,LP2), 0.95 $x10^7$ particles g^{-1} dry soil in +N treatments (3,P3,L3,LP3) and 1.14 $x10^7$ particles g^{-1} dry soil in +C+N treatments (4,P4,L4,LP4) (Figure 6.3 and Figure 6.4). Although nutrient amendments increased virus counts, the effect was less pronounced than on bacteria counts. The largest increase of more than ten times the original inoculum, was in +C+N planted without Protozoa (L4), with a maximum of 2.5 x 10^7 particles g^{-1} dry soil (Figure 6.4,L4).

Planted tubes had more viruses than unplanted: aggregated means were 0.18 $x10^7$ particles g^{-1} dry soil in unplanted (1-4) and 1.9 $x10^7$ particles g^{-1} dry soil in planted (L1-4) tubes. Viruses reflected increased bacteria concentration. When Protozoa were present, viral counts increased to 0.64 $x10^7$ particles g^{-1} dry soil in unplanted tubes (Figure 6.3, P1-4) but decreased to 1.0 $x10^7$ particles g^{-1} dry soil in

planted tubes (Figure 6.4, LP1-4). Thus viruses showed the same pattern as bacteria, suggesting that Protozoa either grazed on viruses as well as bacteria or grazed on bacteria in which phage were present.

There was only a slight indication that viruses may be regulators of bacterial abundance. In +C+N unplanted tubes lacking Protozoa (4), virus abundance more than doubled from day 2 to day 7, concurrent with a marked decrease in bacteria from 3.2 $x10^7$ cells g^{-1} dry soil to 0.47 $x10^7$ cells g^{-1} dry soil over the same period (Figure 6.3, 4). A similar, but less pronounced, pattern was seen in the +N treatment. There was a positive correlation between bacteria and virus counts: r=0.67 for day 2 and r=0.87 for day 7 (n=48, $p<0.001$).

6.3.4.2. Protozoa abundance

Flagellate abundance increased in all tubes from the initial 5 $x10^3$ cells g^{-1} dry soil to amendment-aggregated means of 7.2 $x10^3$ cells g^{-1} dry soil in unplanted (P1-4) tubes and 6.4 $x10^3$ cells g^{-1} dry soil in planted (LP1-4) tubes on day 2 (Figure 6.5, A,B). Counts were marginally greater for +N and +C+N tubes than the control. By day 7, abundance had doubled to 16.4 $x10^3$ cells g^{-1} dry soil in planted (LP1-4), and 13.4 $x10^3$ cells g^{-1} dry soil in unplanted (P1-4) tubes respectively, indicating they fed first and divided later.

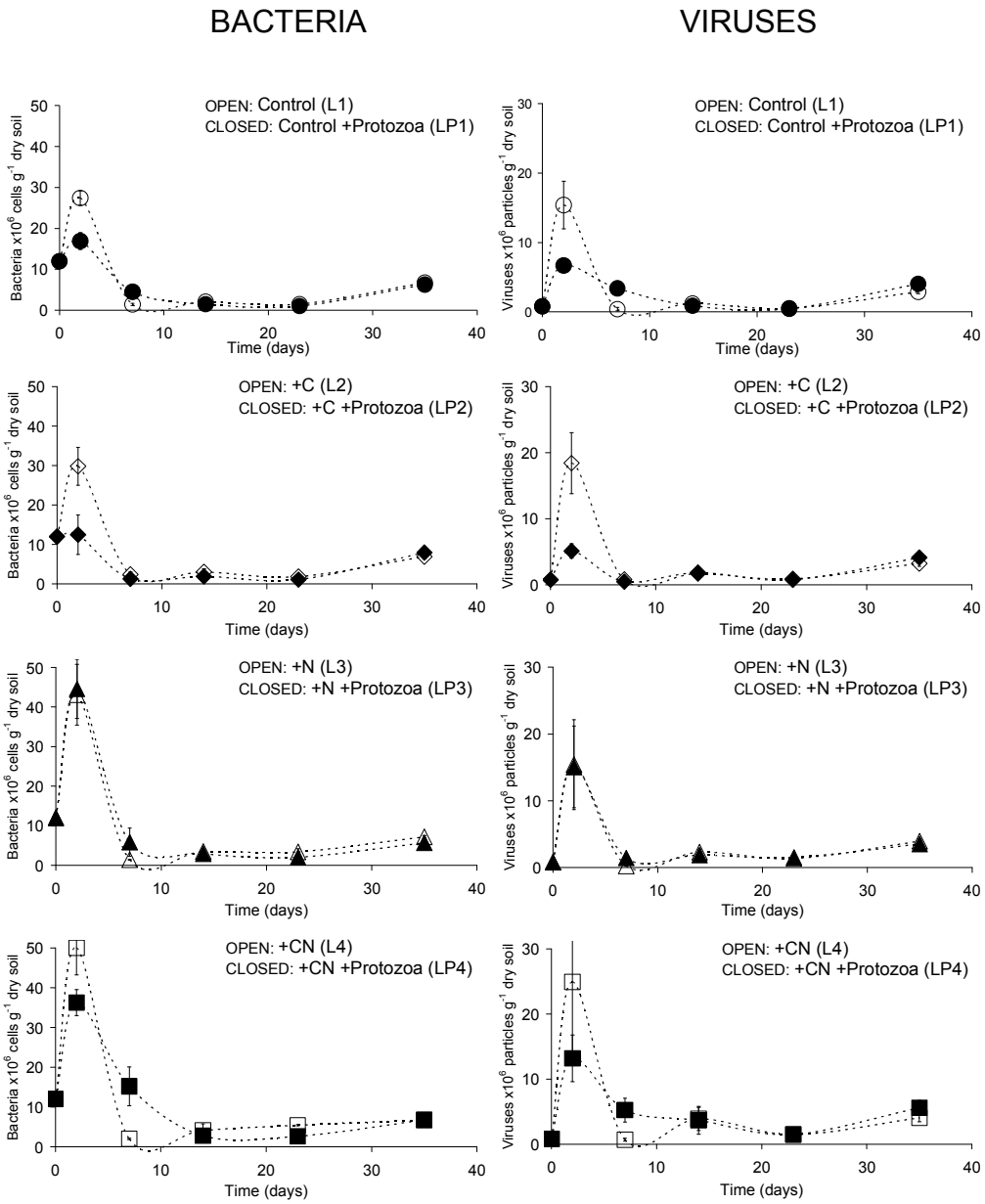


Figure 6.4: Bacterial and viral abundance over time in planted (L1-4&LP1-4) tubes. Protozoa present (solid), Protozoa absent (open). Control (o), Carbon (◊), Nitrogen (Δ), Carbon+Nitrogen (□). Bars are one standard error of the mean (n=3).

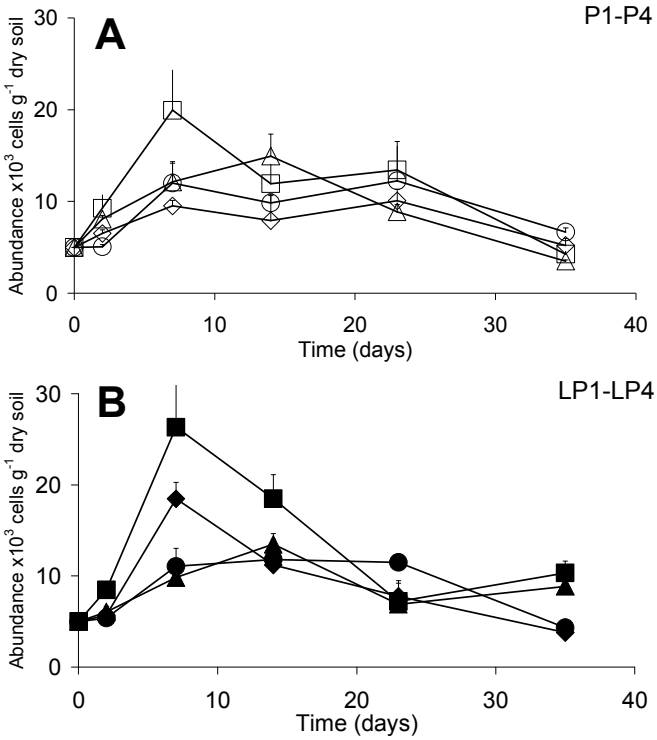


Figure 6.5: Flagellate abundance at selected points over six weeks. A. Unplanted (P1-4), B. Planted (LP1-4). Control (o), Carbon (◊), Nitrogen (Δ), Carbon+Nitrogen (□). Bars are one standard error of the mean (n=3).

Contrary to expectation, there was no clear increase in flagellate counts in planted tubes compared with unplanted tubes apart from one instance: there was a 100% increase in +C planted (LP2) tubes from 9.5 $x10^3$ cells g^{-1} dry soil to 18.5 $x10^3$ cells g^{-1} dry soil. The regulating influence of flagellates on bacterial abundance is illustrated by a low abundance of both bacteria and flagellates on day 2 in +C-planted (LP2) tubes, and a subsequent rapid rise in flagellate counts in this treatment by day 7. In unplanted tubes, flagellates appear not to have checked bacterial production in the control and C tubes as bacterial abundance was high with minimal increases in flagellate abundance.

6.3.4.3. Virus to bacteria ratio

Virus-to-bacteria ratios (VBR) were generally lower when Protozoa were present and higher in microcosms with plants. Amendment-aggregated mean VBR were 0.26 (1-4) and 0.19 (P1-4) for unplanted tubes without and with Protozoa. In planted tubes, amendment-aggregated VBR were 0.56 (L1-4) and 0.38 (LP1-4) without and with Protozoa respectively (Figure 6.6). VBR was particularly low for C+N unplanted without Protozoa tubes on day 2, due to low virus abundance. It is unclear why this occurred. VBR was lower with Protozoa on day 2, yet higher in the majority by day 7 (Figure 6.6).

VBR in unplanted tubes were 0.55 (1-4) and 0.74 (P1-4) without and with Protozoa, and in planted tubes were 0.28 (L1-4) and 0.43 (LP1-4) accordingly. When Protozoa were present, virus production may have been reduced in the initial stages of rapid grazing activity, followed by an increase some days later. Surviving bacteria may have benefited from the grazing and increases in their metabolism led to increased viral production. By contrast, VBR were higher in planted tubes than unplanted on day 2 but lower on day 7. Bacterial metabolism may have been reduced by day 7 as the pool of readily available organic matter from roots became depleted.

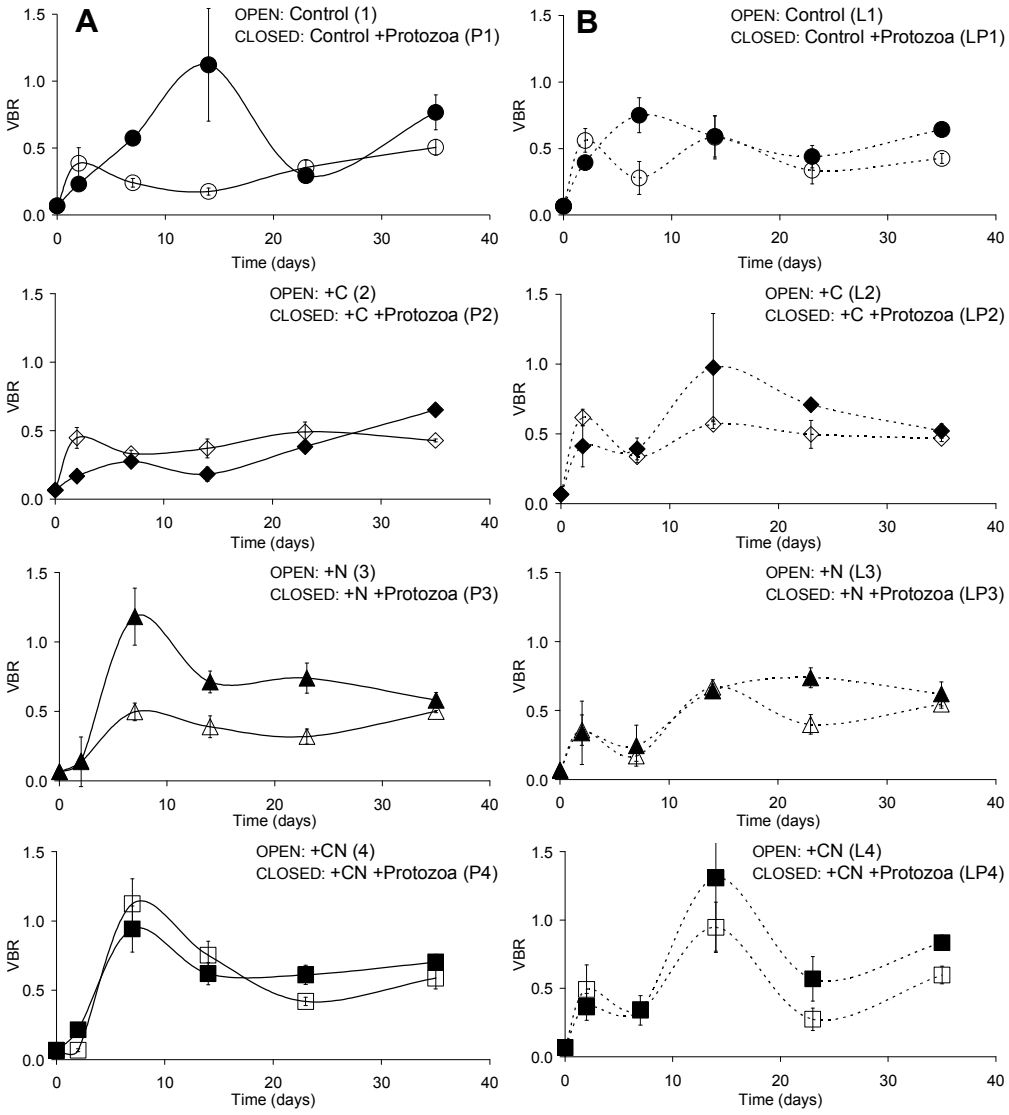


Figure 6.6: Virus-to-bacteria ratios at selected points over six weeks. A. Unplanted (1-4&P1-4) and B. Planted (L1-4&LP1-4). Protozoa present (solid), Protozoa absent (open). Control (o), Carbon (◊), Nitrogen (Δ), Carbon+Nitrogen (□). Bars are one standard error of the mean (n=3).

6.3.4.4. Ciliates

Ciliate abundance increased from the initial 50 cells g^{-1} to a maximum of 570 cells g^{-1} in C+N planted tubes at day 7 (Figure 6.7B). All counts were greater in planted tubes than unplanted on day 7, and nutrient amendments were greater than control, thus ciliates benefited from the increased bacterial production.

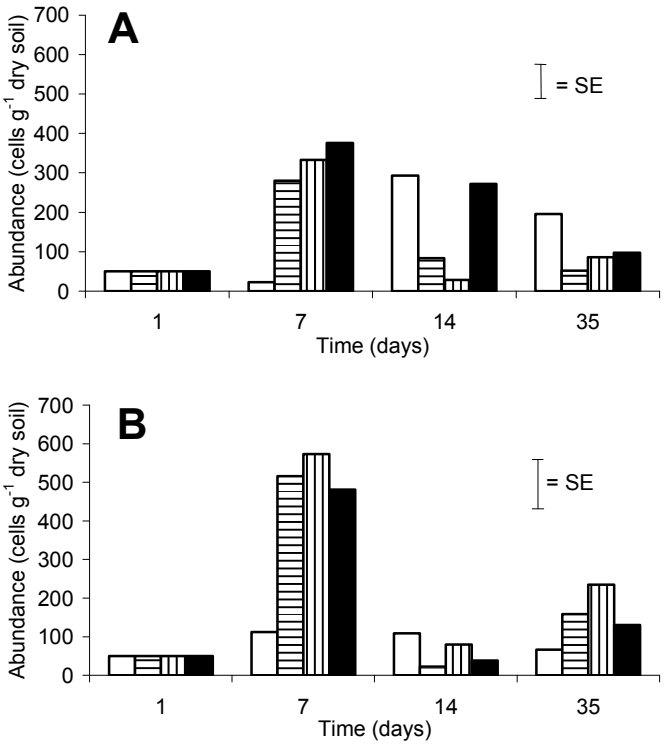


Figure 6.7: Ciliate abundance at selected points over six weeks. A. Unplanted. B. Planted. Columns: Control (clear), Carbon (horizontal), Nitrogen (vertical), Carbon+Nitrogen (solid). Mean standard error shown for day 7 (n=12)

6.3.4.5. Dissolved organic carbon and nitrogen

The added glucose was largely assimilated by day 2, as there were little differences in DOC between amendments. Mean values in unplanted tubes were 274 and 283 μg C g^{-1} dry soil without (1-4) and with (P1-4) Protozoa, and in planted tubes were 291 and 322 μg C g^{-1} dry soil without (P1-4) and with (LP1-4) Protozoa (Figure 6.8). Thus both Protozoa and plants marginally increased DOC singly and in combination. DON values were significantly higher in the N amended treatments. Overall means were 30 and 80 μg N g^{-1} dry soil for –N and +N treatments respectively (Figure 6.9). Hence the inorganic N addition resulted in increased dissolved organic nitrogen, presumably as bacterial biomass. There were no significant differences in DON values between the other treatments.

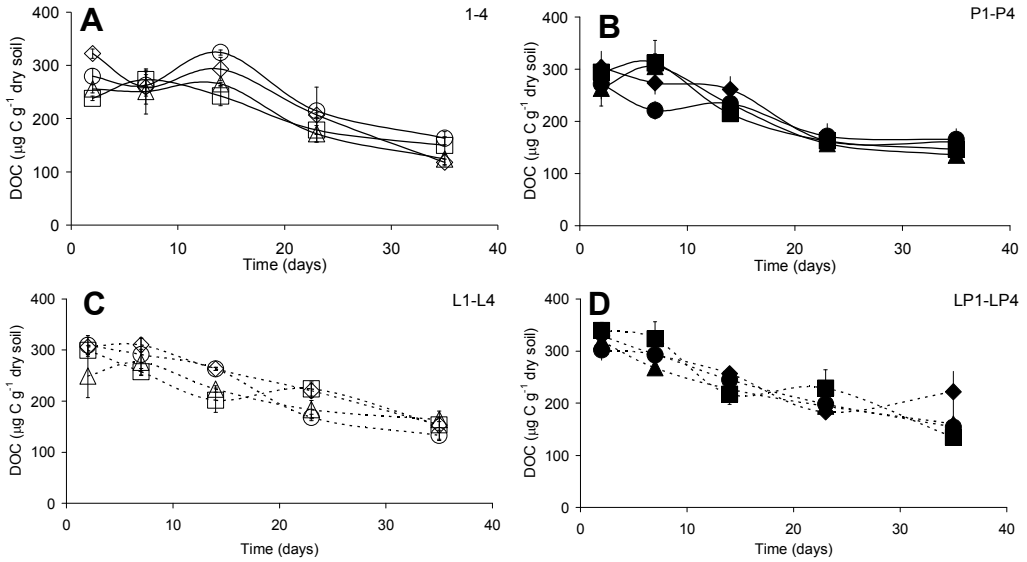


Figure 6.8: Dissolved Organic Carbon at selected points over six weeks. A: Unplanted without Protozoa (1-4), B: Unplanted with Protozoa (P1-4), C: Planted without Protozoa (L1-4), and D: Planted with Protozoa (LP1-4). Symbols: Control (o), Carbon (◊), Nitrogen (Δ), Carbon + Nitrogen (□). Bars are one standard error of the mean (n=3).

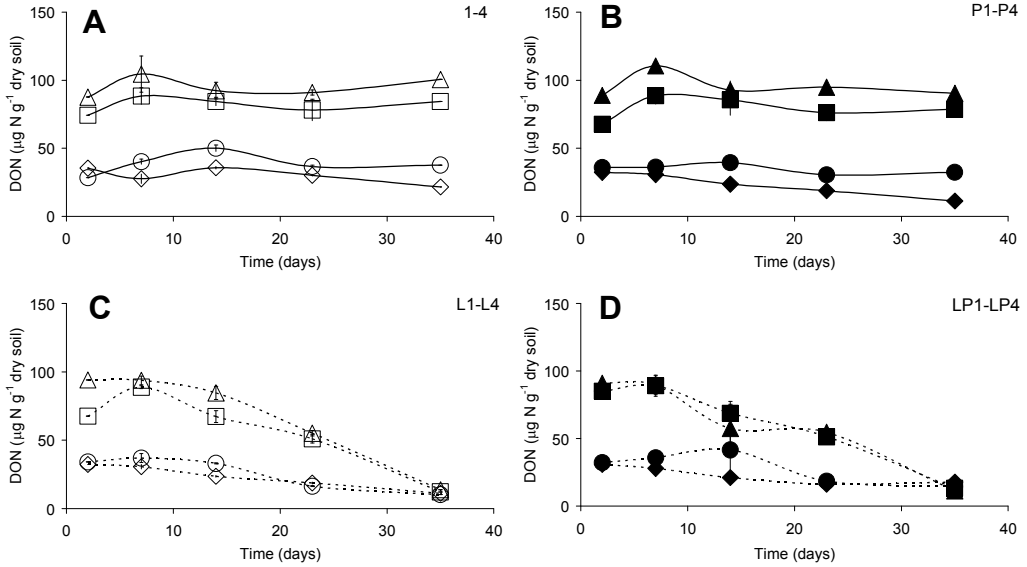


Figure 6.9: Dissolved Organic Nitrogen at selected points over six weeks. A: Unplanted without Protozoa (1-4), B: Unplanted with Protozoa (P1-4), C: Planted without Protozoa (L1-4), and D: Planted with Protozoa (LP1-4). Symbols: Control (o), Carbon (◊), Nitrogen (Δ), Carbon + Nitrogen (□). Bars are one standard error of the mean (n=3).

6.3.5. Weeks 2-4 – Low Activity Period

6.3.5.1. Bacterial and viral abundance

Bacterial counts were much reduced from the first week, ranging from a minimum of 0.11 $x10^7$ cells g^{-1} dry soil to a maximum of only 0.54 $x10^7$ cells g^{-1} dry soil (Figure 6.3 and Figure 6.4). On day 14 amendment-aggregated counts in unplanted tubes (1-4, P1-4) were 2.4 $x10^7$ cells g^{-1} dry soil and in planted tubes (L1-4, LP1-4) were 3.2 $x10^7$ cells g^{-1} dry soil. Counts of bacteria were only greater than control in planted tubes. On day 23 amendments were still having an effect as counts were generally higher relative to the control. As soil moisture decreased, production of root exudates may have reduced, so the plant effect would have been less pronounced. The lasting effect of nutrients suggests that the initial dose was adequate to sustain considerable cycles of bacterial reproduction.

Virus counts also decreased over this period, ranging from a minimum of 0.03 $x10^7$ particles g^{-1} dry soil, to a maximum of 0.39 $x10^7$ particles g^{-1} dry soil (Figure 6.3 and Figure 6.4). Amendment counts were greater than controls in all cases with the exception of an anomalous count of 0.29 $x10^7$ particles g^{-1} dry soil in control unplanted with Protozoa (P1), which was more than double the count in any of the amendments. As with bacteria, the most significant factor in increasing counts was plants ($p=0.001$) on day 14 and then nutrients ($p=0.001$) on day 23. There was a positive correlation between virus and bacteria counts: $r=0.76$ for day 14 and $r=0.67$ for day 23 ($n=48$, $p<0.001$).

6.3.5.2. Protozoa abundance

Flagellate abundance generally decreased over this period. Amendment-aggregated means were 11.2 $x10^3$ cells g^{-1} dry soil and 13.7 $x10^3$ cells g^{-1} dry soil in unplanted (P1-4) and planted (LP1-4) tubes respectively on day 14 (Figure 6.5). By day 23 these had decreased in the planted tubes to 8.3 $x10^3$ cells g^{-1} dry soil. By contrast, flagellate abundance increased slightly in unplanted tubes (apart from +N, P3), so they were still reproducing. The pattern in P3 may have been due to an already very low bacteria count below 5 x 10^7 cells g^{-1} dry soil, on day 7, so food availability was a limiting factor earlier.

Ciliate abundance on day 23 had decreased to values close to the initial inoculation. All ciliate counts were much reduced with the exception of a rise in the unplanted control (P1) to 300 cells g^{-1} dry soil, and in C+N (P4) of 270 cells g^{-1} dry soil (Figure 6.7). The probability is that higher soil moisture in the unplanted tubes sustained a greater proportion of active ciliates. They may have encysted in the planted soils as the soils dried out. There was wide variability in ciliate counts between replicate sub-samples indicating spatial factors influenced location.

6.3.5.3. Virus to bacteria ratio

On day 14 VBR were generally greater in tubes with Protozoa: unplanted means rose from 0.42 to 0.69, planted rose from 0.66 to 0.88 (Figure 6.6). These data support the idea that grazing pressure and plants enhanced virus production even at presumed low activity levels due to moisture stress.

6.3.5.4. Dissolved organic carbon and nitrogen

By day 23, DOC had decreased by 30% compared with day 7 values (Figure 6.9). Values in planted tubes decreased gradually, whilst in unplanted tubes they decreased between day 14 and 23. Treatments with Protozoa had higher decreases than without, suggesting possible immobilisation into cells or cysts. Decreases in DON values on day 23 compared to day 7 were 10% and 44% for unplanted and planted tubes respectively, -N tubes decreasing slightly more than +N values. Mean DON values were 32 μg N g^{-1} dry soil and 85 μg N g^{-1} dry soil for unplanted –N (1,2,P1,P2) and +N (3,4,P3,P4) treatments. DON values in planted tubes were 18 μg N g^{-1} dry soil for –N (L1,L2,LP1,LP2) and 53 μg N g^{-1} dry soil for +N (L3,L4,LP3,LP4) treatments. Protozoa had no effect on DON values at this period. In this period planted tubes were still supporting sufficiently high microbial activity to mineralise substantial amounts of organic nitrogen.

6.3.6. Week 5 - Recovery

6.3.6.1. Abundance

Re-watering had a positive effect on bacterial counts, but the increase was only marked in the unplanted microcosms that lacked Protozoa. Bacterial abundance in unplanted without Protozoa (1-4) was 1.9 $x10^7$ cells g^{-1} dry soil, and with Protozoa (P1-4) 0.8 $x10^7$ cells g^{-1} dry soil. In in planted tubes (L1-4, LP1-4) bacterial counts were similar irrespective of Protozoa presence at 0.7 $x10^7$ cells g^{-1} dry soil. Bacteria counts in planted tubes with Protozoa increased in response to the

second watering, but the increase was minimal in +N and +C+N treatments, coinciding with increases in flagellate abundance (Figure 6.5) and very high amoebae abundance (Figure 6.11). Reactivation of the Protozoa removed much of the increase in bacterial production.

Viral abundance in unplanted tubes without Protozoa (1-4) were 0.9 x10^7 particles g^{-1} dry soil, with protozoa (P1-4) 0.4 x10^7 particles g^{-1} dry soil, and in planted tubes without Protozoa (L1-4) were 0.5 x10^7 particles g^{-1} dry soil and 0.4 x10^7 particles g^{-1} dry soil for planted with Protozoa (LP1-4). Abundance again reflected bacterial abundance with greater increases in unplanted than planted tubes, greater abundance in +N and +C+N, and reduced abundance in the presence of Protozoa.

Correlation between virus and bacteria counts at the last sampling point was significant ($r=88$, $n=48$, $p<0.001$). Across all time points virus and bacteria counts were very highly correlated ($r=0.859$, $n=227$, $p<0.001$) (Figure 6.10).

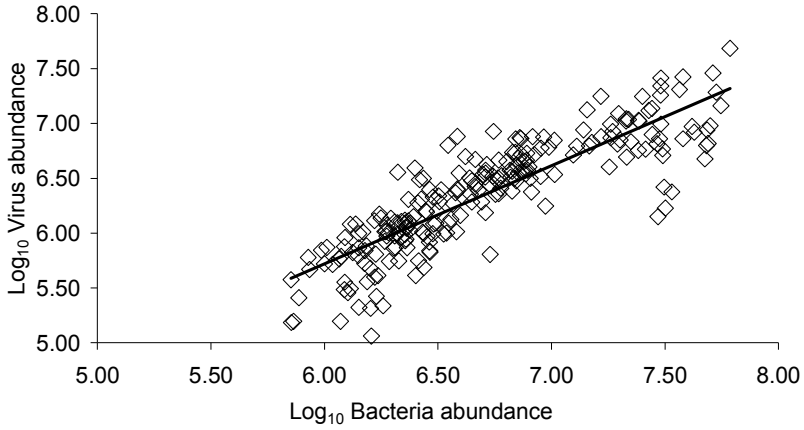


Figure 6.10: Log-log scatter graph of bacteria and virus abundance. All data are plotted.

6.3.6.2. Amoebae

Amoebae counts were recorded on day 35 but no other sampling point. Flat-bottomed micro-titre well plates were used for day 35 samples but all previous samples were on older round-bottomed plates, which for unknown reasons yielded no data. At the highest dilution debris might have obscured the cells, but at lower dilution one might have expected some counts. Counts from unplanted soil were 45 $x10^3$ cells g^{-1} dry soil in control (P1), <10 $x10^3$ cells g^{-1} dry soil for both +C and +N (P2,P3), and 25 $x10^3$ cells g^{-1} dry soil for +C+N (P4) (Figure 6.11). From planted soil samples counts were very low in control and +C (LP1,LP2), but highly abundant at <80 $x10^3$ cells g^{-1} dry soil in +N and +C+N (LP3,LP4). Means were highly variable, with standard errors of between 9 and 99%.

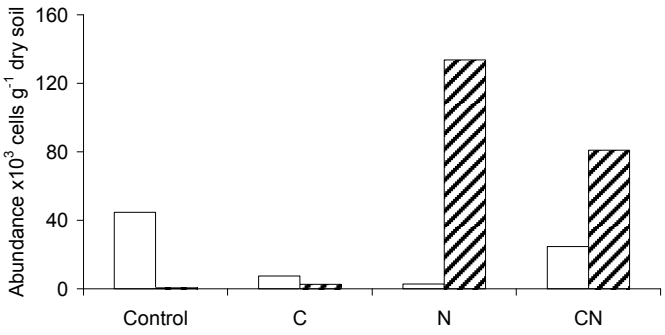


Figure 6.11: Amoebae abundance based on MPN at day 35. Unplanted (P1-P4)(clear columns), planted (LP1-LP4) (hatched columns).

6.3.6.3. Virus to bacteria ratio

VBR were larger with Protozoa present than absent. In unplanted tubes VBR increased from 0.51 (1-4) to 0.68 (P1-4). In planted tubes VBR increased from 0.51 (L1-4) to 0.65 (LP1-4) in planted tubes (Figure 6.6). Protozoa were therefore still influencing virus production.

6.3.6.4. Dissolved organic carbon and nitrogen

By day 35, DOC values had decreased further. In unplanted tubes, final values were 139 μg C g^{-1} dry soil without Protozoa (1-4) and 152 μg C g^{-1} dry soil with Protozoa (P1-4), and in planted tubes values were 151 μg C g^{-1} dry soil (L1-4) and 168 μg C g^{-1} dry soil (LP1-4) accordingly (Figure 6.8). Thus the increase observed in bacteria and protozoan abundance was not reflected in DOC.

DON values in unplanted tubes at day 35 were 30 μg N g^{-1} dry soil in –N treatments (1,2,P1,P2) and 89 μg N g^{-1} dry soil in +N treatments (3,4,P3,P4). In planted tubes there were minimal differences between –N (L1,L2,LP1,LP2) and +N (L3,L4,LP3,LP4) treatments – both were 13 μg N g^{-1} dry soil (Figure 6.9).

6.3.7. Plants

6.3.7.1. Plant dry weight

Plant dry weight was small at the end of six weeks, reflecting the moisture stress. Dry weights for plants with Protozoa absent were 0.11g (control) 0.11g (+C), 0.17g (+N) and 0.16g (+C+N) so amendments with nitrogen resulted in higher plant dry weight (Figure 6.12). Plant dry weights without Protozoa were 83-93% of those when Protozoa were present, confirming that Protozoa do aid plant uptake and growth.

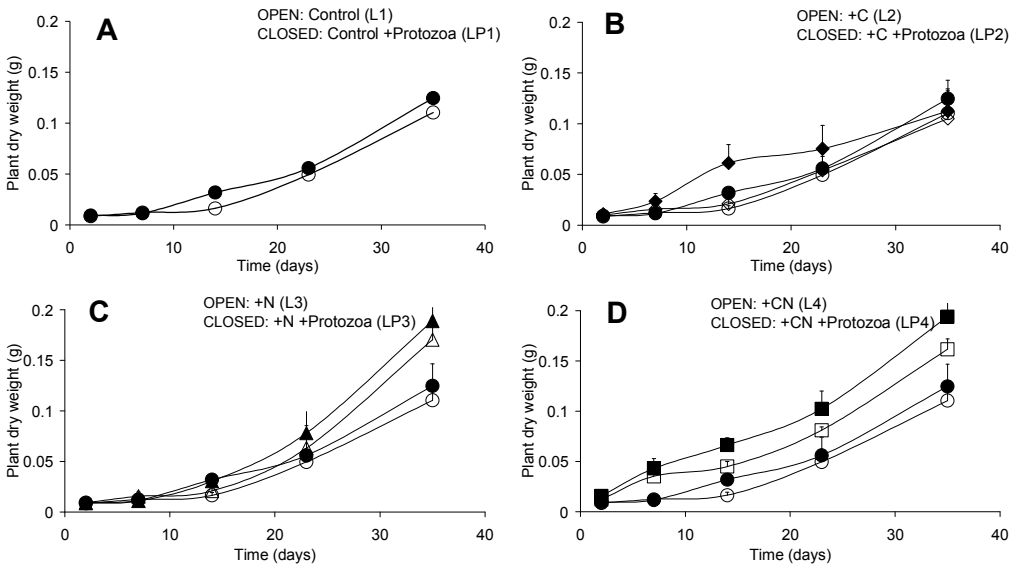


Figure 6.12: Plant dry weight at the end of the incubation period. A: Control (L1,LP1), B: Carbon (L2,LP2), C: Nitrogen (L3,LP3), D: Carbon + Nitrogen (L4,LP4). Open symbols: Protozoa absent (L1-L4)(open), closed symbols: Protozoa present (LP1-LP4). Bars are one standard error of the mean (n=3).

6.3.7.2. Shoot to root ratio

Mean shoot-to-root ratios were 0.9 (–N treatments) and 2.2 (+N treatments) (Figure 6.13). Nitrogen alone or in combination increased ratios compared to +C and the control by the end of the incubation, so plants benefited from the addition of N. As ratios were greater when Protozoa were present (Figure 6.13), plants may have benefited from increased availablility of nutrients as a result of grazing. Ratio differences were similar to those found by Clarholm (1985) (Figure 6.1).

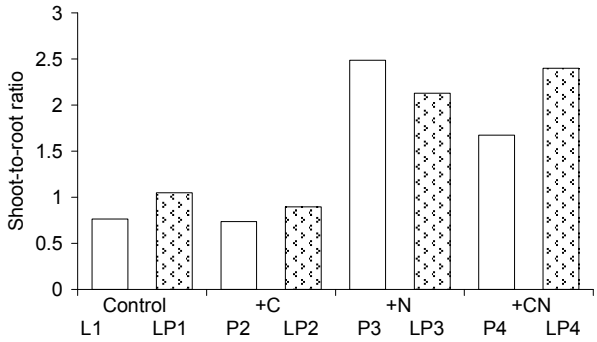


Figure 6.13: Shoot-to-root ratios of Italian ryegrass seedlings after 6 weeks grown in microcosms with added nutrients. Protozoa present (hatched), Protozoa absent (plain).

6.4 DISCUSSION

The results from this complex microcosm study showed that bacteria and their associated phage are influenced by a range of biological and chemical parameters.

Amendment with nitrogen led to greater bacterial abundance and activity, as recorded in microcosm studies elsewhere (Mamilov and Dilly 2002). However, nitrogen amendments to natural soil do not always result in increased bacterial number but may increase bacterial production (Stapleton *et al.* 2005). That is, there maybe an increase in the production to biomass ratio. The lack of a response to additions of glucose (carbon source) and glucose combined with inorganic nitrogen over nitrogen alone suggest that the system was N limited for bacterial growth. In this study nitrogen was added as both ammonium and nitrate as ammonium was shown to be the preferred form of N in a study of a tundra soil (Stapleton *et al.* 2005), and has also been shown to be the preferred source of N by marine bacteria (Kirchman, Keil, and Wheeler 1989; Keil and Kirchman 1991). In the current study the carbon source was a carbohydrate requiring the bacteria to take up inorganic N to effect bacterial production. Where amino acids are available the bacteria can use organic N and organic carbon from the amino acid substrate. Plants may also utilise organic N, although their capacity to do so may be compromised by bacterial activity (Miller and Cramer 2004).

Bacteria increased in abundance on initial inoculation into the soil, as expected and supported by other data. Bacterial counts (colony forming units) of eight inoculated species were at their highest at 2-4

days incubation before declining (Acea, Moore, and Alexander 1988). Thus soil bacteria respond quickly to changed conditions that provide increased sources of N or C or both. As in this study the addition of nutrients increased overall population size, but did not change the rise-fall pattern. It has been postulated (Clarholm 2002) that within 5 days of a stimulus, most bacterial production has either been lost as CO_2 or formed dead organic matter or protozoan cysts. The current study supports this supposition, as differences between counts were minimal by day 14 irrespective of previous abundance. However, Clarholm's study missed the first few days of the incubations, when most activity occurred, as she made her analysis after six weeks rather than following a time series.

Apart from viral attack and lysis one of the major causes of bacterial mortality in the natural environment is predation by Protozoa. In aquatic environments viral induced mortality has been shown to equal protozoan grazing on occasions (Fuhrman 1999). In the current study the greatest reduction of bacterial production through protozoan grazing was within the first week, as shown in other studies (Elliott and Coleman 1977; Rutherford and Juma 1992). The rapid decline in bacteria counts followed by an increase in Protozoa, particularly flagellates, suggests significant grazing pressure.

Where grazing rates for soil flagellates have been measured using modern epifluorescence techniques, clearance rates of 0.37–0.49 nl $cell^{-1}$ $hour^{-1}$ have been recorded, equating to 1.59 bacterial cells ingested per hour (Stapleton *et al.* 2005). Applying that value to this study leads to 1-3% bacterial death by flagellate grazing on the first day;

by day 7 this had increased to 47-54%, and by day 14 grazing may have been faster than production as 92-230% bacterial death could be calculated. Based on decreases of a prey population when *Colpoda* were present, Vargas and Hattori (1986) estimated that each ciliate consumed 1 $x10^4$ bacteria per division. Assuming this value for this study, by day 7 ciliates may have been responsible for 10% of bacterial mortality. Thus protozoan predation was probably one of the major factors in bacterial mortality.

This study showed a close coupling of virus abundance to bacteria abundance, as has been reported in some sediments (Middelboe, Glud, and Finster 2003). Other work showed increases in sediment bacteria coincided with decreases in virus counts (Maranger and Bird 1996). The overall mean VBR in this study, an indicator of the relationship between bacteria and viruses was 0.52 $\pm$ 0.29, so despite considerable differences between microcosms the ratio did not alter greatly. The two other studies in soil where VBR were calculated gave values of 0.04 (Ashelford, Day, and Fry 2003) and 4-12 (Williamson, Radosevich, and Wommack 2005). [Note: an extreme VBR of >3000 was reported in the latter study, but exceptionally low bacteria counts were responsible for this, so is not likely to be typical]. All but one calculated VBR over the course of this incubation were between 0.14 and 1.3, with slight increases in VBR in planted tubes. This suggests that although plants may have had some influence on virus and bacteria interactions, this was not significant: i.e. viruses were not switching replication cycles dependent on resource availability or predation pressure. The data indicate that the lytic cycle predominated throughout

the study and that lysogeny was not occurring to any significant extent. Bacterial and viral numbers were closely correlated throughout the incubation, thus decreases in bacteria were reflected in virus numbers and fairly consistent VBR. VBR were similar to those found in marine sediments (VBR<1) (Danovaro, Manini, and Dell'Anno 2002), so some comparison between soil and sediment bacteria-phage dynamic may be valid.

During the first few days of the incubation in the absence of plants in both controls and C+N amended microcosms it appears as if protozoan predation and subsequent excretion of N and P enhanced bacterial production. That Protozoa enhance bacterial activity is well established (Griffiths 1989; Darbyshire 1994; Ekelund and Rønn 1994). The pattern of viral abundance and VBR suggests that viruses were not the major cause of bacterial mortality, but rather protozoan grazing was exerting the greater effect. In planted microcosms the picture was more complex because there would have been competition for recycled N between plants and bacteria. Only where N was in excess, because of amendments, was there an increase in bacteria when protozoa were present.

Following the initial high response both bacterial and viral numbers declined in tandem as did flagellates, the major grazers of bacteria in this study. Protozoa are known to respond quickly to increased bacteria in soil and then to encyst and wait for the next phase of high activity (Clarholm 1994). The small increase in bacteria and viruses toward the end of the experiment in response to watering did not elicit a corresponding increase in flagellate numbers as the experiment

was terminated before they had an opportunity to reproduce. They typically lag behind bacteria in a characteristic predator/prey oscillation (Clarholm 1981; Laybourn-Parry 1984; Wright *et al.* 1995).

Plant shoot-root ratio differences are a measure of the plant's ability to apportion resources to either photosynthetic growth or nutrient and water uptake. If nitrogen is plentiful then more resources will be put into shoot growth (Davidson 1969). It is clear from these data that the addition of inorganic N had that effect, as did the presence of Protozoa, so Protozoa increased the amount of available nitrogen for the plants. Clarholm (1985) reported the same findings, however the effect of nutrients was only apparent when Protozoa were present.

Artificial microcosms offer both advantages and disadvantages. The soil was sterilised, and the diversity of the bacterial community much reduced from that which originally existed. The process of autoclaving soil has several effects on organic matter. It kills all organisms within it, releasing their cell contents. Bonds of more resistant compounds will be broken, allowing more rapid assimilation and soil structure is disrupted releasing previously bound substances (Paul and Clark 1996). These actions result in increased O_2 consumption, N mineralisation and CO_2 evolution compared with untreated soil (Powlson and Jenkinson 1976).

The bacterial suspension for inoculation was prepared using nutrient agar, so the bacteria that formed the microcosm community had been pre-selected to grow on high levels of simple nutrient sources. The substantial decreases in DON, and slight decreases in DOC irrespective of treatment are an indication that a significant proportion of dissolved

organic matter was accessible to bacteria, suggesting only a small recalcitrant component. The marked decline in DON in N amended microcosms where Protozoa were both present and absent suggests significant recycling of N between the inorganic and organic forms as a result of biological activity. Plants can access both inorganic and organic N via their roots; amino acid transporters have been described in roots (Jones, Hodge, and Kuzyakov 2004) and uptake of glycine has been reported (Hawkins, Wolf, and Stock 2005): through uptake of amino acids there is competition between plants and bacteria for N (Owen and Jones 2001).

Abundance is just one of several factors to be measured for a complete picture on the extent of bacterial mortality due to viruses. Bacterial production, frequency of infected cells, burst size, decay rate for viruses, duration of latent period and burst size need to be recorded (Noble and Steward 2001). This study took a multi-factorial approach that considered the presence of bacterial predators, plants and levels of N. Viruses may be a contributory factor to bacterial mortality in soil systems, but that it is less significant than grazing pressure.

In aquatic systems bacterial mortality due to viral lysis is highly variable (see chapter 1); in one study it was up to ten fold higher in anoxic than oxic water, although total abundances of both bacteria and viruses were similar (Weinbauer, Brettar, and Höfle 2003). In instances where protozoan grazing is limited there is evidence of increased significance of viral lysis (Weinbauer and Höfle 1998a). The converse scenario may be proposed that where protozoan grazing dominates,

viral lysis is of lesser importance. The present study may fall into that category.

6.5 SUMMARY

Biotic and abiotic influences on bacterial abundance and activity were examined in relation to viral abundance over 35 days. Protozoan grazing, planting and amendment of carbon and/or nitrogen were considered. Bacteria and virus numbers increased significantly within the first two days after inoculation and watering, and then decreased over time. Amendment of carbon and nitrogen combined resulted in increased bacteria and virus numbers when compared to individual or no amendment addition. Plants had the effect of increasing bacterial and viral abundances when Protozoa were absent, but these decreased when Protozoa were present. Thus Protozoa were actively grazing bacteria, and possibly phage, which resulted in reduced population sizes. Protozoa and nitrogen addition increased the shoot to root ratio of plants by day 35. Overall it was evident that viruses had a negligible effect on bacterial population size.

CHAPTER 7. GENERAL DISCUSSION

7.1 MAIN FINDINGS

The key objectives of this thesis were achieved, namely to investigate the magnitude and fluctuation of bacteriophage populations over a range of soil conditions. Viral abundance is substantial; exceeding that of bacteria, in some soils and fluctuates widely under changing soil conditions yet was closely coupled to bacterial abundance in most instances. Although viral abundance in soil was often considerable, the data presented in this thesis indicate that phage do not appear to regulate bacterial population density to any significant extent.

7.1.1. Method

A procedure for enumerating viruses and bacteria within a single sample was established and utilised throughout the study. It was adapted from procedures in use for aquatic samples and refined with several modifications appropriate to soil. The protocol was shown to be suitable for a range of soils differing in clay, cation exchange capacity and organic matter content. Calibrated image analysis software was used to determine bacterial cell volumes, a considerable improvement over sizing cells using an eyepiece graticule.

It is considered a useful, straightforward method to determine bacteria and virus abundance in soils with readily extractable bacteria of approximate abundances greater than 1 $x10^8$ cells g^{-1} soil. In order to reduce variability of within-filter counts it is preferable to count 300-400 bacteria or viruses per filter. In some samples (notably the carbon-poor

permanent fallow – chapter 5) abundance was too low for ease of counting and so although the method is sufficiently sensitive, it is not considered appropriate for abundances less than 1 $x10^7$ cells g^{-1} soil.

The method gives certainty, as 'organisms' stained by the fluorochromes definitely possess nucleic acid, and are therefore distinguished from the multitude of other particles in soil. Furthermore bacteria stained in this manner are not ghost cells so whilst they may be dormant are not incapable of activity. Size class distinction of viruses at the lowest viable bacterial cell size (cell length and width <0.2 μm), combined with visual cues gives reasonable assurance that particles defined as viruses are indeed viruses.

Any procedure that incorporates reasonable sampling handling time will inevitably lead to partial extraction. Comparisons with other studies (Weinbauer, Beckmann, and Höfle 1998) indicate that bacteria counts in this thesis may be underestimated by up to one order of magnitude. In the protocol outlined here, some of the larger bacteria were likely sedimented and not counted; the small biovolumes recorded are an indication of this. The compromise of centrifuging, shaking and recentrifuging rather than several repeated centrifuge and resuspension steps indubitably led to losses left within the sediment pellet. Any future improvements of the method would in the first instance deal with this so more total extraction might be performed. However centrifugation at any speed will lead to sedimentation. Extraction with detergents may have increased counts further, but was deemed unsuitable for soil with high organic matter. Water and buffer as extraction media were not tested in the other the studies in which fluorochrome staining was used on soil

bacteria or viruses (Weinbauer, Beckmann, and Höfle 1998; Williamson, Wommack, and Radosevich 2003; 2005) so comparisions cannot be drawn.

Advances in automated image processing allow rapid enumeration of soil bacteria with accuracies comparable to human counts. The use of image analysis software in this study was limited to the (manual) determination of bacteria cell sizes. Computer programmes can be devised comparatively easily which would determine bacterial area based on the number of pixels occupied in the image by each bacterium. However when this was investigated by the author two factors prevented its further use. The first was that two dimensions are required for calculation of cell volume, and secondly the image processing required to reduce background noise eliminated the majority of stained viruses.

Abundance data have limitations, as counts at any given time are net of production and loss. Thus relatively inactive populations that remain stable over time may yield similar values to a population with significant production and associated losses through death and decay. One indirect method used was changes in the virus-to-bacteria ratio over time or between treatments. When VBR remain constant the inference is that there are no large changes in phage production or loss and there is a coupling between phage and bacteria abundances. Marked changes in VBR would be indicative of greater fluctuations in phage production and hence dynamism in the system. Given the small range of VBR recorded in this work, comparisons of VBR may not be appropriate in determining potential bacterial activity.

One method employed at the Centre for Bioimaging at Rothamsted Research gave some clear TEM images of phage. Phage density on grids was never high enough to allow quantification of phage by TEM, and search for phage was laborious. Some form of phage and bacteria concentration was therefore required but two methods investigated gave unsatisfactory results.

A microscope adapted for fluorescence is the most expensive equipment required for this method. Investigators with no prior expertise in this area can readily carry out the procedure, as acclimatisation of the eyes for microscope use is the greatest hurdle to clear. Thus its simplicity and adaptability commends it for further use.

7.1.2. Experimental Data

Viral and bacterial abundance were closely coupled in a variety of soils and conditions. Direct counts of bacteria and virus ranged from less than 10^7 to more than 10^9 particles or cells per gram of soil. Calculated virus to bacteria ratios ranged from less than 0.1 to over 10: these encompass the published ranges in aquatic systems (Wommack and Colwell 2000). Bacteriophage do not appear to be significant contributors to bacterial mortality; unlike many aquatic systems where they equal or exceed protist grazing (Wommack and Colwell 2000; Weinbauer 2004).

A useful addition to the microcosm study would have included unsterilised soil, as sterilised soil is an unnatural environment for plants and microorganisms to inhabit. The activity of other microfauna and flora may however have masked any fluctuations in bacterial and viral

abundances: each new factor also has associated time implications, and it would not have been feasible for the study as conducted.

The inference from mean cell volume data is that more active bacteria are bigger, as would be expected, but also that viral abundance data concurrent with the presence of larger cells is greater. This may be self-evident, but it illustrates on a community level that which has hitherto only been examined on individual bacterial strains.

Analysis of marine virus communities has been carried out based on genome size, subsequently separated using pulsed-field gel electrophoresis. In this way spatial and temporal differences in viral communities have been established (Steward and Azam 2000). Future investigation of soil viral communities is required, and will also complement the growing body of data on bacterial community diversity and structure.

The most significant finding from this study was the extent of lysogeny in soils. This novel work demonstrated that lysogeny is prevalent in soil, but at a much reduced level than that hypothesised previously. Assumptions made based on models of phage-host interactions have consistently stated that lysogeny would be widespread and significant in soil. Data presented here illustrate that lysogeny is neither a significant cycle for viral production nor a major contributor to bacterial mortality.

To better understand the dynamics of phage in soil, abundance data should be supported by a measure of phage production. This thesis has demonstrated a procedure in which reliable and reproducible

abundance data may be obtained. Viral production, however, was only demonstrated unequivocally in the sterile soil microcosms in Chapter 6. In natural soil, viral production was inferred through changes in virus-to-bacteria ratios.

In aquatic systems viral production has been successfully determined by staining a proportion of the viral community with a fluorochrome. Changes in the relative proportion of stained versus unstained viruses can be used to calculate production rates (Noble and Fuhrman 2000). This method is unlikely to be transferable to soils as adsorption will reduce recovery rates; in samples from aquatic systems the proportion of viruses potentially lost through irreversible adsorption is very low when compared to soils. It is unclear what techniques could be used in successful measurement of phage production in natural soil systems.

7.1.3. Further Studies

Abundance data do not give any indication as to community composition or diversity. Given that known bacterial diversity in soil is bound to be a considerable under-estimation of the actual diversity present, the question is: which bacterial groups are involved in phage-host interactions in soil? A useful study could investigate that by enriching a soil with complex nutrient solutions to stimulate bacterial activity and population growth. A 0.2 μm filtered soil extract could be removed and divided into two. Irradiation of one portion would inactivate phage, whilst the other should contain viable phage. These extracts could then be added to new soil samples and bacterial community

diversity profiles made before and after application. In this way the effect of an increased phage presence on community composition could be measured. Fractionation of soil into different aggregate size classes would yield even more useful information, as to how prevalent phage-host interactions are.

Further electron microscope work on the range of burst sizes in soil might give valuable insights into how dynamic the interaction between phage and hosts are. Are more phage released per bacterium in nutrient-rich soil than in poorer soil?

Is protozoan grazing a significant factor in reducing phage populations in soil? Data from the soil drying incubations (Chapter 4) did not answer that question, and the reduction in viral abundance in Chapter 6 may have been solely due to reductions in the bacterial populations. Protozoa do ingest virus-sized inorganic particles (Laybourn-Parry, J. and Parry, J. personal communication), but it is unlikely they would actively seek out phage. Inoculation studies using natural soil and capturing TEM images of Protozoa might give further insights.

Data in Chapter 5 indicated low levels of lysogeny in soil, but the samples were incubated after soil drying, and water or mitomycin C only were added. The data may have been very different had bacterial activity been stimulated with the addition of nutrients, i.e. percent lysogeny may have been low because bacterial activity was too low for induction of phage (Dodd, C. personal observation).

What is the temporal component of phage-host interactions? Is it linked to nutrient, moisture or temperature fluxes? Longer-term studies, in conjunction with field trials, would give insights into rhizosphere bacteriophage dynamics through the growing season of different crop species. Data from the Highfield grassland and fallow soils (Chapter 5) emphasised how different environments lead to significantly different microflora populations. A comparative study on soils from other locations (e.g. tundra, peatland, forest) could provide useful insights as to the significance of soil bacteriophage in the natural environment. Shifts in bacterial community composition may occur in polluted soil. How bacteriophage exist in these altered systems is also of interest, particularly if they reduce the biodegradation capacity of the soil.

The time-limiting step in the work presented here was in enumeration of bacteria and viruses. It would be profitable to develop an automatic image analysis programme sufficiently sensitive so as to discriminate between background, viral and bacterial particles on slides. If that were to be achieved, the whole procedure would be much more rapid and would have more widespread application.

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