# Coevolutionary interactions between bacteria and phage in natural environments

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# Declaration of originality

I hereby declare that this thesis is a result of my own work under the supervision of Dr Thomas Bell of Imperial College London and Dr Andrew Singer of the Centre for Ecology and Hydrology.

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# Abstract

Bacteria and their viruses (bacteriophage, phage) are the most abundant and diverse taxonomic groups, but ecological and evolutionary research on bacteria-phage interactions has largely focused on studies of simplified communities using a few model organisms. The goal of the thesis is to understand how bacteria and phage interact within natural environments, and how these interactions impact the patterns of phage infectivity and bacterial resistance.

Here I investigate the effects of natural environments on the coevolutionary patterns of bacteria (*Pseudomonas fluorescens*) and phage (SBW25Φ2). In chapter 3 I investigate the effects of nineteen different communities on the coevolutionary interactions of SBW25 and phage, and the degree to which the infectivity of phage to its host, SBW25, changes depending on their local microbial community. Chapter 4 aimed to understand the effects of varying diversities of communities on coevolutionary interactions. In Chapter 5, I looked at how coevolutionary interactions were affected by different communities in different abiotic conditions (pH, temperature and nutrient concentration) and the effect communities had on the ability of SBW25 to adapt to the abiotic conditions.

Understanding how biological and physical factors affect coevolutionary interactions in natural environments allows predictions of how phage and bacteria coevolve in natural and unnatural settings.

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# Contents

# List of figures

## Chapter 1



## Chapter 2



## Chapter 3

## Chapter 4



## Chapter 5

# Appendix

# List of Tables

## Abbreviations

**Φ2** – SBW25 phage

**ANOSIM** – Analysis of Similarity

**ANOVA** – Analysis of Variance

**ARD** – Arms race dynamic

**bp** – Base pair

**CEH** – Centre for Ecology and Hydrology

**CFU** – Colony forming unit

**CRISPR** – Clustered Regularly Interspaced Short Palindromic Repeat system

**DNA** – Deoxyribonucleic acid

**dNTP** – Deoxyribose nucleotide triphosphates

**dsDNA** – Double stranded DNA

**FSD** – Fluctuating selection dynamics

**GFG** – Gene for Gene

**GI** – Genomic Island

**GLM** – Generalised Linear Model

**GMC** – Geographic mosaic of coevolution

**GPS** – Global positioning system

**KCl** – Potassium chloride

**LB** – Luria Broth

**LPS** – Lipopolysaccharide

**MA** – Matching allele

**MgCl** – Magnesium chloride

**NaCl** – Sodium chloride

**nMDS** – Nonmetric Multidimensional Scaling

**PBS** – Phosphate buffered saline

**RCF** – Relative centrifugal force

**RPM** – Revolutions per minute

**RM** – Restriction modification system

**OD** – Optical density

**PCR** – Polymerase Chain Reaction

**RNA** – Ribonucleic acid

**rRNA** – Ribosomal Ribonucleic acid

**SBW25** – *Pseudomonas fluorescens*

**SE** – Standard errors

**SS** – Sum of squares

**ssDNA** – Single stranded DNA

**TEM** – Transmission Electron Microscope

**TFF** – Tangential flow filter

**TRFLP** – Terminal restriction fragment length polymerisation

**Tris-HCl** – Tris Hydrochloride

**TukeyHSD** – Tukey's Honest significant difference

# Publications

Thompson, M. S. A., **Bankier, C**., Bell, T., Dumbrell, A. J., Gray, C., Ledger, M. E., Lehmann, K., McKew, B. A., Sayer, C. D., Shelley, F., Trimmer, M., Warren, S.L and Woodward, G (2015) **Gene-to-ecosystem impacts of a catastrophic pesticide spill: testing a novel multilevel approach in a natural system.** *Freshwater Biology.* Pp: n/a-n/a

Miller, M. A., **Bankier, C**., Al-Shaeri, M. A and Hartl, M. G, 2015. **Neutral red cytotoxicity assays for assessing *in vivo* carbon nanotube ecotoxicology in mussels – Comparing microscope and microplate methods.** *Marine Pollution Bulletin*. 101 (2), pp. 903 - 907

# Chapter 1 - Thesis Introduction

Bacteria underpin all ecosystems, and are typically the primary drivers of many nutrient cycles (Waring et al. 2013). Nonetheless, relatively little is known about how bacterial populations are regulated in natural environments. While viruses that infect bacteria (phage) are known to have the capacity to significantly reduce bacterial populations, the overwhelming abundance and diversity of both bacteria and phage have prevented a thorough understanding of community dynamics over ecological or evolutionary timescales.

In the natural environment, bacteria and phage are known to undergo coevolution, which is the reciprocal evolutionary change through interactions driven by natural selection (Thompson, 2005).

Several model bacteria and phage systems have been developed to understand interactions between individual bacterial strains and their phage. Some of the most well-defined model phage-bacteria systems are the "T-even" bacteriophages (T2, T4 and T6), the "T-odd" phage (T3 and T7) and lambda λ (T1 and T5) phage and their host *Escherichia coli* (Carter and Saunders, 2013). Their discovery and meticulous analysis of life history, physiology and genetics has paved the way for advances in molecular biology and defining the genetic code (Abedon 2000; Watson 1951). Other phage systems have also been studied extensively including the large dsDNA viruses *Phycodnaviridae* that infect marine and freshwater algae. It is known that the *Phycodnaviridae* infect coccolithophores (in particular *Emiliania huxleyi*) and have a major impact on effects of the algal bloom which in turn has significant biogeochemical (primary production) and ecological (ocean heat retention) impacts (Martínez et al. 2012). Here, I will focus on

*Pseudomonas fluorescens* SBW25 and podovirus φ2 as a model system that is particularly well understood and which I use for the experiments. SBW25 and φ2 are a well-known bacteria-phage model system first isolated from the phyllosphere of a sugar beet (*Beta vulgaris*) in 1989 to be used in a study that analysed the fate of a genetically modified organism in the environment (Bailey et al., 1995). *P. fluorescens* has also been used as a biocontrol to help protect plant roots against some fungal pathogens and nematodes (Haas & Défago 2005). Since then, SBW25 have been used extensively to study host-pathogen coevolutionary dynamics between bacteria and phage φ2 (Morgan et al. 2010; Hall, Scanlan, Morgan, et al. 2011; Gandon et al. 2008; Koskella & Brockhurst 2014). I was interested in understanding host-pathogen interactions in natural environments. I therefore selected the SBW25-phage system because of the prior extensive research on host-pathogen coevolution that could easily be extended to increase the degree of realism by incorporating biotic and abiotic complexity.

## Coevolutionary dynamics

Bacteria-phage interactions are characterised by antagonistic coevolution where reciprocal evolution occurs between a host defence and a pathogen counter-defence (Woolhouse et al. 2002), resulting in a constantly shifting adaptive landscape for both bacteria and phage (i.e. Red Queen Hypothesis) (Lively & Dybdahl 2000). In the SBW25 model system, the first few hundred generations of coevolution between SBW25 and φ2 are governed by an arms race dynamic (ARD) of host defence and pathogen counter defence. This directional coevolutionary interaction favours the reciprocal evolution of new bacterial resistance genes followed by new phage infectivity genes, and is an interaction

that escalates over time (Hall, Scanlan, Morgan, et al. 2011). This results in a pattern where the coevolved host can resist (and coevolved pathogen can infect) all of their ancestral genotypes. However, this dynamic cannot continue indefinitely due to two principal factors: firstly, costs associated with increasing resistance or infectivity and second the evolutionary potential of the bacteria and phage.

The costs involved in ARD are often due to bacteria becoming more broadly resistant and phage more broadly infective. These include impaired growth rates due to the impaired function of the cell surface lipopolysaccharide (LPS) molecules in which phage attach (Koskella & Brockhurst 2014). Scalan et al, (2015), showed in *Pseudomonas fluorescens* which evolved with their phage (φ2), a high proportion of mutations were observed in the phage target site on the LPS resulting in four different types of LPS structures (ranging from short to long banding, which is an important factor when it comes to phage resistance), however it was not clear how these bands affected resistance to phage.

The fitness costs for both bacteria and phage in the arms-race dynamic therefore results in a changing dynamic to frequency dependent selection. This occurs when bacteria and phage evolve different genotypes that have different resistance and infectivity specificities resulting in more specialised host-phage interactions. 'Negative frequency dependence' occurs where phage evolve to infect the most common bacterial genotype which gives the advantage to the rare bacterial resistance allele which then rises in frequency (Koskella & Brockhurst 2014). This changing mode of coevolution over time is due to the weakened ability of the bacteria to respond to directional selection and accumulating costs of bacterial resistance.

These different mechanisms result in distinct patterns of bacterial resistance and phage infectivity over space and time, shown in figure 1.

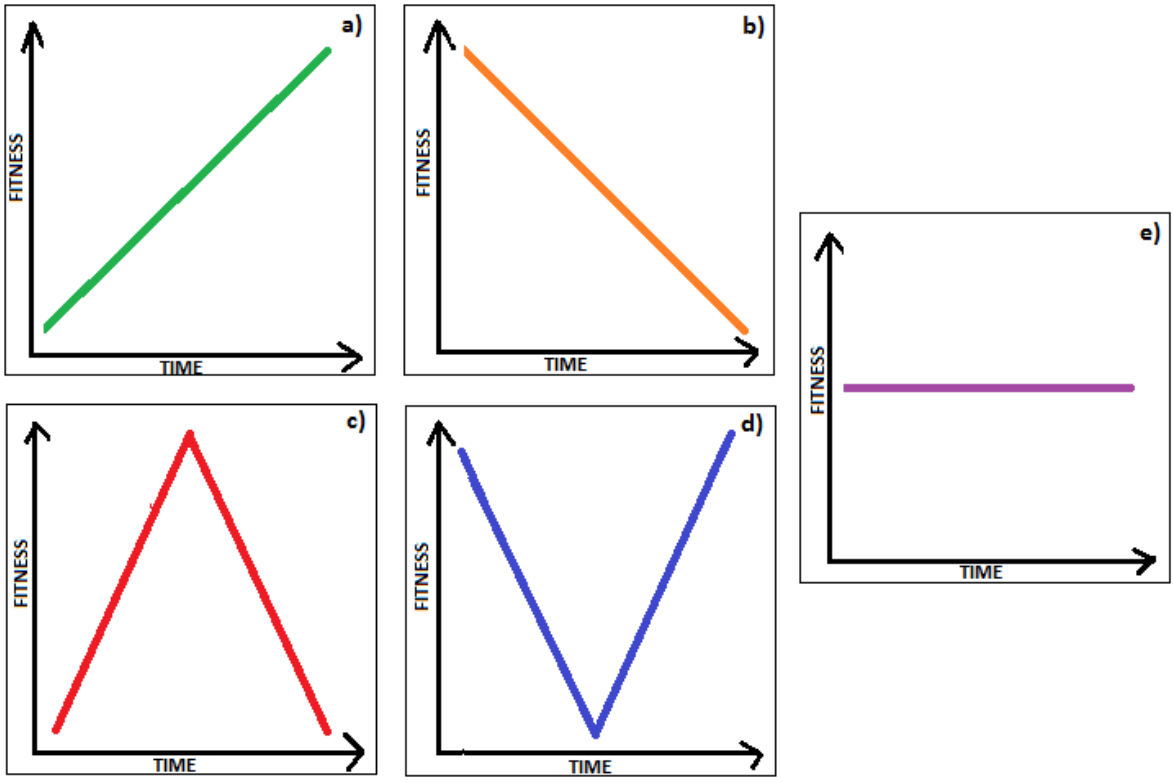


**Figure 1: Schematic diagram of different patterns observed when bacteria and phage coevolve. a and b) illustrate typical patterns observed in a time shift assay showing arms race dynamics. This is parasite defence followed by host counter defence where coevolution proceeds by mutations conferring bacterial resistance followed by new phage infectivity alleles. c and d) demonstrate typical patterns observed with fluctuating selection dynamics where phage infect the most common bacterial genotype which allows rare bacterial genotypes to rise in frequency and so on. e) shows no coevolution observed**

In an experiment that tracks coevolving bacteria and phage over time, ARD results in bacteria evolving increasing resistance to phage genotypes whilst phage

evolve broader host ranges. In ARD, the chances of beneficial mutations occurring simultaneously in the pathogen to counter-act the host defence, is small. However, a subset of these beneficial mutations might give an immediate advantage over the host which in turn, increases the chances that a full set of advantageous mutations will become fixed.

In contrast to ARD, fluctuating selection dynamics (FSD) does not require bacteria and phage to obtain new resistance and infectivity mutations with attack and defence alleles remaining constant at an individual level (Betts et al. 2014). FSD generally occurs when costs associated with ARD become too high and may cause impaired growth rates. This gives rise to oscillations that are sustained over time of different bacterial resistance and parasite infectivity genotypes. These oscillations are driven by negative frequency dependent selection, as phage evolve to infect the most common bacterial genotype, this allows for rare bacterial genotypes to rise in frequency and phage again evolve to infect this new, previously rare genotype which again, rises in frequency and so on (Koskella & Brockhurst 2014).

Two theoretical models exist that describe host-pathogen infection networks in plants and animals. The gene for gene (GFG) model, first described by Flor, 1956, results in a nested phage-bacteria infection network in which there is a compartmentalised/non-hierarchical network of host resistance and phage infectivity where host-pathogen interactions generally create generalist resistance and infectivity genotypes which predisposes this interaction towards ARD (Gómez et al. 2015). GFG is the ability of a host to resist infection with a resistance gene and the ability of a parasite to infect the host with an avirulence gene and follow ARD selection that can lead to generalist host and parasites.

Flores et al., (2011) showed through a metaanalysis of 38 laboratory studies, nested interaction structure occurred most often in nature when looking at the empirical patterns of infectivity.

At the other end of the continuum of host-pathogen infection networks, the matching allele (MA) model hypothesises that parasite genotypes must specifically match host genotypes at all interaction loci (Dennehy 2012; Poullain et al. 2008) and assumes positive epistatic interactions (Kouyos et al. 2009). The MA model is dependent on self/non self-recognition molecules where hosts have the ability to defend against a parasitic genome that is not recognised as their own (Quigley et al. 2012). The matching allele hypothesis can predict specialisation and local adaptation as any parasite can only infect a particular host whilst the GFG model does not. However, most evidence with phage and host systems supports the GFG model for antagonistic coevolution which results in generalist genotypes (Brockhurst, Morgan, et al. 2007). In contrast, Morgan et al. 2010 favoured the matching allele hypothesis by demonstrating that parasites were locally maladapted as phage could not rapidly adapt to the changes of the bacteria. Although most evidence lies with the GFG model overall, in the environment, systems that coevolve are likely to show traits of both models (Dennehy 2012). These interactions are of extreme importance due to the rapid rate of evolution which plays a key role in ecosystem functioning and the use of phage in pharmacological and agricultural contexts (Gómez & Buckling 2011a).

The pattern of infectivity and resistance across space or over time can therefore be used to gain insight into the coevolutionary dynamics of bacteria and phage, and the mechanisms that might be prevalent. An alternative method for assessing coevolutionary dynamics is to conduct time shift experiments. In time shift

experiments, hosts and pathogens from a given time point are compared with their host or pathogens isolated from the past or future. This allows investigation of adaptations to the host or pathogen (Koskella 2014).

As well as creating characteristic patterns over time, the different mechanisms result in patterns of adaptation over space. To detect spatial patterns, rather than comparing bacterial resistance and phage infectivity in time shift experiments, we conduct reciprocal transplants ('cross infection') of phage and bacteria over space and measure the degree to which bacteria are adapted to their local phage and vice versa.

The evolution of bacteria-phage interactions can also be impacted by their evolutionary potential. Evolutionary potential is defined as the capacity to produce new genetic variants (Schulte et al. 2013). The rate of new genotypes is influenced by a number of factors, such as population size, migration, mutation rates and recombination. In relation to host-parasite systems, each species is required to adapt in variable environments and this adaptation is dependent on the strength of the evolutionary potential with the ability to incorporate new genotypes that are able to overcome to adaptation of the counter-species. Phage are thought to have greater evolutionary potential as they have higher mutation rates, shorter generation times, and larger population sizes and have been shown to be locally adapted to their host (Gandon & Michalakis 2002). Dispersal is also thought to be influential in host-parasite evolution as it reflects gene flow between different habitats. However, dispersal is associated with fitness costs due to adapting to the new environment which may be unsuitable and movement between environments (Gandon et al., 1998).

## Mechanisms of bacteria defence and phage attack

Bacteria have evolved several ways to prevent phage infection through anti-phage barriers to help regulate bacterial populations during coevolution in various ecological niches (Labrie et al. 2010). Most bacteriophage are specific to the host bacteria in which they infect and attach to the host through receptors found on the cell surface and this defines which host the phage can infect. To prevent the phage from attaching in the first place, bacteria have evolved numerous ways to stop phage adsorbing on to the cell by modifying or concealing the surface receptors that the phage attach. Mechanisms for modifying the receptors are displayed by numerous bacteria. A plasmid isolated from *Lactococcus lactis* caused total inhibition of several phages from adsorbing to the host cell through production of a protein that conceals the surface cell receptors (Szczepankowska et al. 2013). *Staphylococcus aureus* has also been shown to conceal surface receptors with outer membrane protein immunoglobin G-binding Protein A and is known that when Protein A masked the cell surface receptors, phage adsorption is decreased (Drulis-Kawa et al. 2012; Nordström & Forsgren 1974). Another way in which bacteria prevent phage infection is through production of an exopolysaccharide in species such as *Pseudomonas* or *Campylobacter*, which form a protective coating around the bacterial capsule covering the receptors to prevent phage entry. However, some phage have evolved the ability to depolymerise the extracellular matrix (such as hydrolasaes and lyase) that degrade this matrix and as well as gaining access to the cell surface receptors, they can also make more bacteria available to infection by destabilising the biofilm (Nwodo et al. 2012; Drulis-Kawa et al. 2012).

One of the most common bacterial defence mechanisms is the use of a restriction modification system (R-M system) which has been characterised in many bacteria and seen as an innate immune response in bacterial cells by recognising non-self DNA. Mechanisms for recognising non-self DNA are performed by the enzymes REase and MTase. REase endonucleolytically recognises and cleaves foreign DNA at the phosphodiester bonds and highly conserved MTase transfers methyl groups to specific DNA found within the host's genome that helps protect the cell and discriminates against self and non self DNA as phage DNA is unmethylated and therefore recognised by the restriction enzyme (Vasu & Nagaraja 2013). There are four types of RM systems (type I – type IV) which are separated on their ability to recognise sequences, cleavage position, requirements for their cofactor and composition of subunits (Gormley et al. 2005).

One of the most recent discoveries on phage-resistance mechanisms are the Clustered, Regularly Interspaces Short Palindromic Repeat system, known as CRISPR (Sorek et al. 2008). First described in 1987, it was first thought the CRISPR/Cas system played a role in DNA repair with genome stability. However, it is now widely accepted that the system may play a role in protecting a host microbial cell from foreign DNA invasion from plasmids or viruses (Deveau et al. 2010). The CRISPR arrays are composed of ~2 – 250 repeats which are separated by similar sized non-repetitive spacers and the microbial response to avoid selective pressure by phage predations is the acquisition of these new spacer elements in the system. This system is subject to rapid and dynamic evolutionary changes that occur when a host is subjected to phage exposure and are involved in whether a phage can attack or a bacteria can resist an attack (Deveau et al. 2010). The system has numerous biotechnical

applications as a genome-editing tool used for modifying genes and cell engineering in bacterial and mammalian cells (Wang et al. 2013). CRISPR arrays can aid understanding of host-pathogen interactions evolved in a geographical mosaic and detect differences in phage in diverse populations. For example, Heidelberg et al. 2009 were able to distinguish between two thermophilic *Synechococcus* isolates from microbial mats in hot springs that were infected with different phage allowing them to evaluate the coevolution of host-pathogen genomes and their effect on the composition of microbial populations.

## Local adaptation

Diversification in communities occurs through mutation, speciation and dispersal. However, how to predict the fate of this diversity in natural communities is poorly understood. Bacteria are able to inhabit a wide range of niches and habitats and have great capacity for passive dispersal. It is thought that divergent selection of bacterial species will occur in different environments which will generate distinct genotypes in different environments leading to local adaptation. A population is locally adapted if its fitness is higher in its local environment than in other environments and this adaptation of the species to its native environment can lead to trade-offs in which adaptation to one environment will come at a cost of adaptation to another environment (Hereford 2009). Genetic differentiation in contrasting environments or along environmental gradients is also an indication of local adaptation (Savolainen et al. 2013). This theory is encompassed with Bass-Becking hypothesis that "Everything is everywhere, but the environment selects", where the distribution of microbes is selected by abiotic and biotic conditions of growth with dispersal having little influence of the structure and

composition of microbial communities (Fontaneto, 2011; Kraemer & Kassen 2015).

There is evidence of widespread local adaptation in several species of plants and animals with recent studies showing evidence of local adaptation in different macro and micro-organisms (Kniskern et al. 2012; Primmer 2011; Savolainen et al. 2007). Local adaptation in bacterial populations is poorly understood despite the fact that bacteria are one of the few groups where we can experimentally test local adaptation; numerous contrasting studies demonstrate evidence of bacterial local adaptation or maladaptation through results of reciprocal transplant experiments. Belotte et al. (2003) demonstrated that isolates from soil samples tended to grow better in their home site than isolates from elsewhere and showed clear evidence of local adaptation to spatial variation in the soil chemistry. In contrast to this, some studies have shown maladaptation of species, suggesting some populations are not more adapted to genotypes from foreign environments (Hereford, 2009). A recent study by Kraemer and Kassen (2015) investigated the extent of local adaptation in space and time of the soil bacterium *Pseudomonas* and found that divergent selection increases as sites become more distant from one another but that they are not locally adapted. Similar results of maladaptation were shown in Fox & Harder (2015) who also found that over space and time, aquatic bacteria were predominantly maladapted (i.e. higher fitness) to water chemistries different to their own, local site in which they were selected.

Local adaptation experiments determine whether a pathogen is more infective to a host from the same population and the average fitness of a population is higher relative to a host from another population and has been demonstrated in a range of host-pathogen systems (Koskella 2014; Dybdahl & Storfer 2003). Mutations

can arise in different populations of the same species resulting in differences in the genes that are under selection or the same gene under divergent selection pressures. Therefore genotype fitness of one species depends on the genotype fitness of the other species which could vary with abiotic conditions and/or community context (Thompson, 2005). Local maladaptation can occur when sympatric combinations of host and pathogens will be less compatible, i.e less infective/resistant than allopatric combinations (Woolhouse, 2008). This phenomenon could arise, for example in coevolving antagonists due to a time lag in response to adaptation where a new infective mutation has appeared in phage but the counter defence mutation has yet to appear in the bacterial population (or vice versa). When fluctuating selection occurs and new mutations do not appear, it can create mismatching of coevolving species and appear as local maladaptation. Therefore local adaptation must be analysed at multiple temporal and spatial scales (Schulte et al. 2011*;* Thompson, 2005). In general, when local adaptation occurs, pathogens are shown to be most fit against their host from the past and least infective to their hosts from the future. However, this is dependent on the timescale and model of coevolution as previously discussed. It is thought that pathogens will coevolve faster than their host due to their shorter generation times and can overcome new host counter defence strategies (Kaltz & Shykoff 1998). Koskella, (2014) demonstrated in Horse Chestnut trees (*Aesculus hippocastanum*) that phage are consistently adapted to bacteria isolated from the same populations. Similarly, Vos et al. (2009), tested if local phage that was isolated from soil samples were more infective to their local hosts than foreign phage from another soil sample. The study showed that local adaptation was occurring at a scale of less than a centimetre. Local adaptation in bacteria has

been identified as a key contributor to maintaining diversity at a global scale rather than dispersal (Whitaker 2009).

The pattern of local adaptation will be influenced by the environmental conditions. For example, factors that decrease the cost of adaptation at some locations but not others could create local populations with more- or less locally adapted hosts. Since environmental conditions vary enormously across a landscape, spatial and temporal heterogeneity in environmental conditions is predicted to create a patchwork of locally adapted or maladapted bacteria and phage populations, a phenomenon which has been termed the geographical mosaic of coevolution (GMC) (Thompson, 2005). Diverse mutations will arise in different populations due to differences in genes under natural selection in populations. Therefore, a host/pathogen pair could coevolve quite differently in two populations even if they have the same initial genes in different environments.

For example, Harrison et al. 2013 demonstrated that in rapidly deteriorating environments or fluctuating environments, nutrient concentration can impede the selective sweeps of resistance alleles and decelerate coevolution of bacteria and phage. Similarly, Zhang & Buckling 2011 showed that in a thermally deteriorating environment, phage populations decrease significantly when coevolved with bacteria suggesting that infectivity strategies by the phage were too costly as a result of coevolution.

The pattern of local adaptation can also depend on the biotic environment. Biotic environments can change rapidly as they are subject to evolution which results in adaptation of a species being matched and counteracted by an

interacting species, with average fitness of both species remaining constant, which is in accordance to the Red Queen Hypothesis as previously mentioned (Brockhurst et al. 2014). Competition between species in an ecological system is considered a significant factor in determining the dynamics and structure of a biological community and the outcome of these interactions is largely modulated by abiotic factors (Gomez-Mestre 2002).

GMC theory states that environmental heterogeneity conforms to local selection pressures (such as coevolutionary hot and cold spots) and the spatial distribution of genotypes is determined by gene flow (Drown & Wade 2014). A coevolutionary hot spot is a location where the fitness of a species is affected by the distribution of traits in another species. A coevolutionary cold spot is described as an area where reciprocal coevolution is absent. Local maladaptation can be a consequence of gene flow because alleles that are shaped by selection in one community context are introduced into another context. However, these alleles can also provide genetic variation for continued coadaptation and reciprocal selection to occur (Gomulkiewicz et al. 2007). One way to assess the GMC hypothesis is to investigate whether coevolved traits of a host-pathogen interaction differs among communities.

## Trade off

Local adaptation to one environment may cause lower fitness in other environments that can result in a fitness cost to the organism or a trade-off. Reciprocal transplant experiments can show evidence of these fitness costs due to adaptation or trade-offs. Trade-offs occur as cellular functions and energy within a bacterial cell are constrained and influenced by physical and chemical factors.

They also help maintain genetic variation in populations and ecological speciation. This can allow the coexistence of species as they can result in species utilizing and adapting to a specific energy source with each species having different growth requirements which can prevent competitive exclusion (Bohannan et al. 2002).

Resource use may be restricted in diverse communities due to competitive interactions from other species requiring the same resource use and a pre-adapted species population may increase at the expense of the less adapted species. This can cause trade-offs due to the cost of production of defence alleles and adaptations to the biotic and abiotic environment and there is evidence that species can evolve to utilize resources and waste products produced by other species (Lawrence et al. 2012). Species can diversify into unoccupied niches if there is strong competition from other colonizers within the habitat. Divergent selection is caused by the availability of multiple niches within a heterogeneous environment that leads to adaptive radiation which is thought to play an important role in evolution of diverse communities (Brockhurst, et al. 2007). Ecological specialisations are thought to be caused by adaptations that carry a pleiotrophic fitness cost that may have an undesirable or useful effect for the species such as antibiotic or pesticide resistance.

Fitness costs caused by trade-offs is the inability to perform biological cell functions as efficiently as they interfere with cellular functions. These costs can result in impaired motility, reduced growth rates, metabolic costs and a reduction in transmission (Zur Wiesch et al. 2010; Melnyk et al. 2015). However, they can also be beneficial to bacteria. One well known example of beneficial mutations is antibiotic resistance when bacteria are exposed to a lethal antibiotic in which only

a few cells can survive. However, these cells then become the surviving population and a rare bacterial mutation can then replicate and pass on the beneficial mutation through horizontal gene transfer. Genetic exchange is common within bacterial populations through ability to uptake foreign DNA from plasmids and bacteriophage and incorporate them into their genome (Salmond & Welch 2008). With the rise of antibiotic resistance, bacterial infections are becoming more difficult to treat in patients and the spread of these resistant bacteria is evident despite the bacteria carrying a fitness cost.

## Geographical Mosaic of Coevolution

If there is local adaptation of host and pathogen, we would expect the pattern of host and phage co-adaptation to vary over space due to local differences in environmental conditions or chance events. Across a landscape, we therefore expect a mosaic of coevolutionary patterns. The Geographic Mosaic Theory attempts to encapsulate this idea, and depends on 3 main components. These include 1. local adaptation/maladaptation which create conditions for selection mosaics in coevolving species among communities (Thompson, 1999), 2. coevolution hot and cold spots, and 3. trait remixing (Nash 2008). Selection mosaics occur geographically due to differences in how species fitness affects another species. These selection mosaics can be driven by biotic factors, such as the composition of species present within the community or abiotic factors such as nutrient concentration, temperature fluctuations or physical compositions of the habitat (Piculell et al. 2008) Local adaptation is the basis for coevolutionary dynamics as it creates a template for the GMC. Mosaics of local adaptation occur due to the presence or absence of host or parasite gene in some regions but not in

others. Through gene flow, local populations are connected and coevolutionary dynamics have the capacity to exhibit metapopulation dynamics. This allows for a geographic mosaic of traits to shift within any host-pathogen interaction (such as extinction, coevolution and recolonization) in a metapopulation structure (Thompson, 2005) and may obscure local adaptation processes or the level at which local adaptation occurs (Kaltz & Shykoff 1998).

The GMC model can help explain how coevolution occurs in natural environments with focus on local abiotic and biotic factors influencing community structure. Whilst GMC predicts local adaptation of coevolving species to their surrounding environment, understanding the biogeography of bacteria is also important for helping to explain the wider distribution and dispersal patterns of microbial species across different environments around the world.

Little is known about the biogeography of interacting species of bacteria and it has been shown that some bacteria do have biogeographical patterns however, exhaustive surveys are not yet possible. Baas Becking (1934) claimed "everything is everywhere – the environment selects", implying that different environments maintain microbial diversity and due to high dispersal, population size and rapid growth, bacteria are unlikely to go extinct (Lindström & Langenheder 2012). However, this claim has been widely disputed (Ramette & Tiedje 2007; O'Malley 2008; De Wit & Bouvier 2006). Since the advent of 16S rRNA based molecular techniques, measuring β diversity in microorganisms has shown that microbial communities do show biogeographical patterns. However, it still remains unclear as to whether these patterns are due to competitive exclusion or dispersal limitation (Lindström & Langenheder 2012). Coevolution

between individual species and the community occurs when a species is introduced and the community evolves as a composite of many species (Little et al., 2008). Simple model systems of host-pathogen interactions have driven a vast amount of knowledge about cellular processes and microbial interactions (Martiny et al. 2006).

## Host-pathogen coevolution in nature

To date, there have been numerous detailed empirical studies on the coevolution in the laboratory in several coevolutionary model systems but little is known about how these interactions occur in the context of natural systems. Viruses that infect bacteria (phage) are known to have the capacity to significantly reduce bacterial populations, but little is known about how communities of bacteria and phage interact in natural microbial communities due to the overwhelming abundance and diversity of both bacteria and phage.

Studying coevolution in nature is difficult as it requires evidence of change from specific species. Several abiotic and biotic conditions affect species interactions and therefore patterns of coevolution cannot be predicted. The Geographic Mosaic of Coevolution (mentioned above) is one theory that attempts to describe how coevolution might happen in the natural environment. However, more work needs to focus on the local context of coevolution to help understand what occurs in nature in terms of how communities and environmental fluctuations can alter and define coevolutionary patterns.

Nutrient availability within an environment is one example of an abiotic factor that can boost productivity and alter coevolutionary patterns as this can affect the evolutionary potential of the host. If conditions are more favourable to the host, resistance genes will become more dominant more quickly with the parasite less able to infect which can lead to extinction.

Biogeography of bacteria is also known to affect how species interact and therefore affect coevolutionary patterns. Bacterial communities were once thought to vary little over space, however, from the advent of DNA sequencing techniques, we now know that there are distinct biogeographical patterns of microbial communities that are constantly changing with migration rates, populations sizes and competition which all influence coevolution.

In this thesis, we use bacteria collected from Beech tree holes as a semi-natural community in the experiments. Tree holes are described as a semi-enclosed hollow which is formed naturally in the branch or trunk of a tree. They are an important habitat for many species and can contain unique communities of species.

By performing experiments under semi-natural conditions, i.e. using natural microbial communities, media and mimicking environmental fluctuations, we are able to make more accurate predictions of how bacteria and phage coevolution occurs in nature as competition from other species and external pressures may or may not be favourable to a host or parasite species within a semi-natural microcosm and will change how the host will react in response to increased competition and environmental limitations.

## Aims and objectives

My thesis will investigate how coevolution operates when it is embedded in semi-natural conditions. I do this by tracking coevolutionary changes in different diverse biotic environments (chapter 3 and 4) and in a variety of abiotic environments (chapter 5) using controlled laboratory experiments. The overall aim of the thesis is to begin to untangle the complex interactions between bacteria and phage isolated from natural environments. I aim to look at patterns of bacteria-phage infection networks, and the repercussions of these networks for understanding bacterial community dynamics.

The first chapter (chapter 3) investigates the effect natural microbial communities have on coevolutionary paths of pathogen-host interactions. We also tested whether phage were locally adapted to their host and if SBW25 fitness was also locally adapted to growing within the community in which it had coevolved with phage.

Chapter 4 uses community manipulations to create different orders of diversity between two communities and determine the effect phage has on SBW25 when coevolved within different diversities.

Chapter 5 follows the same experimental design as chapter 3 to investigate the impact of abiotic factors on coevolution between *P. fluorescens* and phage when in the presence of natural microbial communities and whether communities constrain adaptation of *P. fluorescens* to abiotic conditions.

# Chapter 2 - Materials and Systems Development

Phage have been successfully isolated from numerous environmental locations. As phage are valuable tools in microbiology, their ecology and what factors influence their multiplication, survival and the effect they have on microbial host cells are important questions, much of which remains unanswered (Seeley & Primrose, 1982). Phage isolation from environmental samples begins with growing cultures of potential host bacteria and exposing this to an inoculum that is expected to contain phage. Several environments have been successful in isolating phage. Raw sewage or effluent lagoons are classic examples of excellent places to isolate phage due to its high microbial diversity and abundance (McLaughlin et al., 2006). Flu & Flu, (1946) demonstrated positive isolation of bacteria and phage from canal water in the Netherlands due to high abundance of human and animal waste. Many methods rely on the viruses being present in a high enough abundance for detection; therefore obtaining a concentrate of virus from environmental samples is often vital to the success of an assay. Tangential flow filter (TFF) is frequently used when phage is difficult to isolate due to large

volumes of samples, such as marine or river systems. A water sample is filtered through the TFF collecting the phage and recirculating them so most of the water is filtered out leaving only a concentration of phage, however a TFF may damage phage due to the force from filtration. Successful phage isolation has also been performed from the phyllosphere of Horse Chestnut trees (*Aesculus hippocastanum*) (Koskella & Meaden, 2013).

Bacteriophage is commonly isolated using the plaque assay or double agar overlay technique (Abedon, 2008; Middelboe et al., 2010). Plaque assays are performed by preparing dilutions of phage with a compatible host bacterium which are both evenly dispensed onto solid agar medium to form lawns. Lysis of bacterial cells are shown when viral plaques form a zone of clearing where the infectious phage particles have inhibited the growth of cells (Kropinski, 2009), shown in figure 19.

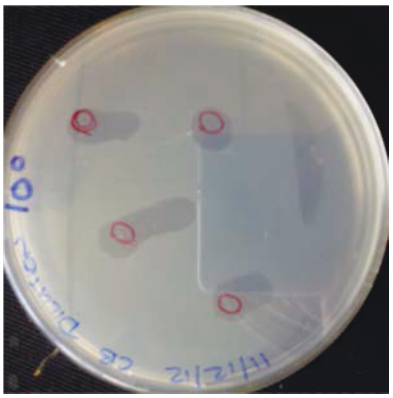

**Figure 2: Plaque assay. Zones of clearing are shown on the bacterial lawn of SBW25 with lytic phage where lysis has occurred**

Epifluorescent microscopy may be performed as a rapid and accurate method to determine the abundance of phage from environmental samples and cultures. It has been demonstrated to have higher accuracy than TEM

(transmission electron microscopy) and flow cytrometry for counting viral like particles (Ortmann & Suttle, 2009). These methods have been shown to work well for dsDNA viruses (~40% of all viruses); however, ssDNA and RNA viruses may be underestimated in abundance as current methods might not detect these viruses (Sandaa, 2008). The use of flow cytometry allows for identification of discrete groups of viruses in environmental samples that is based on their green fluorescence. A flow cytometer is able to detect different morphologies, genomes and size helping to identify different types of phage (Brussaard, 2009).

Sequencing of viruses has allowed phenotypic information to be connected to the genomic information to determine the capabilities and organisation of genes within a virus (Brum et al., 2013). A comprehensive metagenomic approach to studying a viral community can piece together and provide insight into the diversity within different environments (Edwards & Rohwer, 2005; Yoshida et al., 2013). Recent advances in high throughput sequencing has allowed for high resolution studies of ecology and evolution (Solonenko et al., 2013). With the development and advances of viral metagenomics, this will progress our understanding and knowledge of phage-host interactions.

## Bacteria and phage isolation from natural environments

Bacteria and phage are some of the most genetically diverse and abundant group of organisms known. With the advent of community genomics, this diversity has become increasingly apparent (Flores et al., 2011). Phage are ubiquitous and are found in every environment where bacteria are present. Therefore, understanding bacteria-phage dynamics in the environment is pivotal to understanding microbial ecology as phage continually regulate environmental processes such as nutrient and

carbon fluxes, food webs, microbial diversity and diversification (Shapiro & Kushmaro, 2011). To help understand this diversity and interactions between bacteria and phage, considerable time was spent developing methods to isolate and identify phage from natural environments. I have taken two approaches to method development. First, I have attempted to isolate phage from a wide variety of environmental samples (sediment, soil, river water, pond water). Second, I have tested a variety of methods on a known phage-bacteria pair to ensure that methods were working in principle.

Bacteria and phage were collected from various aquatic environments listed in table 2.

**Table 1: List of locations sampled throughout the year**

| Sample site | Location | GPS | Success Rate | Date sampled |
|---|---|---|---|---|
| Silwood soil and water | Ascot, Berkshire | 51.408410, -0.640697 | 0/96 | Nov – Dec 2012 |
| Forth & Clyde canal | Falkirk, Scotland | 56.001606,-3.806072 | 0 /96 | Mar 2013 |
| Aquatic Beech tree-holes | Burnham Beeches, Buckinghamshire | 51.5569157, -0.6318212 | 1/384 | Mar 2013 |
| River Seine | Paris, France | 48.85211, 2.353528 | 0/96 | Apr 2013 |
| Thames | Staines, | 51.430213,-0.510384 | 2/96 | Apr 2013 |
| Silwood Lake | Ascot, Berkshire | 51.413272, -0.647532 | 5/48 | Apr 2013 |
| Thames | Twickenham, London | 51.4464, -0.325014 | 5/216 | Apr 2013 |
| Thames | CEH, various | Various | 3/480 | May 2013 |
| Thames | CEH, various | Various | 6/528 | Jun-Jul 2013 |
| River Kennet | Wiltshire | 51.4229339 -1.134468 | 0/576 | Jul 2013 |
| Thames | Twickenham, London | 51.4464, -0.325014 | 42/1152 | Aug 2013 |

Numerous locations were sampled over the course of one year to isolate a phage and bacteria host pair to be used in interaction experiments. Several methods were employed to isolate bacteria and phage from the environment.

100µl of raw sample was pipetted onto R2A agar (Oxoid CM0906) which was then spread by glass beads (5mm) and incubated at 20°C for 24 hours to isolate single colonies. R2A was used due to the low nutrient content which mimics low nutrients found in rivers. The colonies were individually picked off using a sterile toothpick into a 96 deep well plate containing 500µl Luria Broth (LB Broth Miller Molecular Genetics. Granular. Fisher Scientific). Samples were placed on a shaker (Bibby Stuart Orbital Shaker SO1) at 150rpm for 12 – 24 hours. To isolate phage, several methods were employed. Samples were filtered through a 0.45µm and 0.2µm filter or treated with 100µl chloroform in 900µl of sample, centrifuged (Eppendorf Centrifuge 5430) at 20817rcf (14000rpm) for 2 minutes to isolate the phage from the supernatant. These methods have been described previously (Clokie and Kropinski, 2009; Lopez-Pascua & Buckling, 2008; Morgan et al., 2010; Summer et al., 2006). Bacterial lawns from the single colonies were prepared using 0.7% bacteriological agar at 60°c to maintain a liquid state, containing the log phase isolate. Once dried, spots of phage were placed onto the agar overlay described by Champagne & Gardner, (1995), where phage lysis can be detected and visualised by plaque formation (Middelboe et al., 2010).

## Qualitative assays of phage presence in bulk samples

Throughout the year, a high throughput method was developed to allow the maximum number of colonies to be paired with phage. Instead of petri dishes, 24 well plates were filled with 1ml of R2A to help increase the volume of samples. A master mix of all samples was prepared by taking 1ml from each of the original raw samples. Single log phase isolates were separately added to the master mix which was incubated at room temperature for 6 hours and phage were extracted with chloroform.

3µl of the solution was added to the corresponding well on the 24 well plates. These were incubated at room temperature for 48 hours. Figure 20 shows an example of the 24 well plate method with a positive phage result.

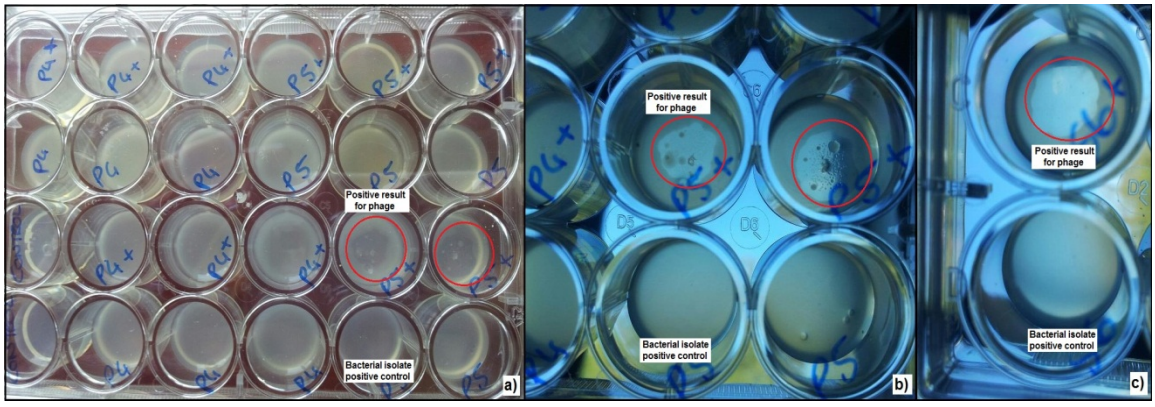


**Figure 3 a) 24 well plate filled with R2A agar containing bacterial isolates. Red circle indicates a positive presence of phage shown by a zone of clearing in the bacterial lawn. b) and c) Close up of zone of clearing compared with positive bacterial control below showing no clearing**

The high throughput method showed a higher success rate for phage isolation as more combinations of bacterial-phage were able to be performed compared with the petri dish method. Phage spots were spotted on the same day as bacteria were

lawned onto the R2A base in the wells and enriched samples were inoculated back into the original samples before being chloroformed.

The process was repeated with samples obtained by CEH along the Thames with minor changes. 30µl and 300µl of phage was spotted onto the lawns of bacteria to allow phage to flood the wells containing the lawns. This proved to be more efficient with several more phage isolated from samples.

Once a phage plaque was confirmed, attempts to isolate the phage from the lawn of the host bacteria was performed by using a sterile pipette tip to extract the phage from the outer area of the zone of clearing on the agar plate. This was then inoculated into broth media and grown for 12 – 24 hours. Plaque assays were repeated on the host bacteria to check that phage had been isolated correctly. This process proved to be unsuccessful numerous times and it was decided that use of an established host-phage pair would be suitable to perform semi-natural experiments.

## Results/Discussion on environmental isolation of bacteria and phage

Bacteriophage isolation techniques were developed in the first 9 months using several methods (Chaudhry et al., 2013; Clokie & Kropinski, 2009; Flores et al., 2011). Improvements to methods such as bacteriophage enrichment with single colony isolates and increasing the volume of the phage spot have proved to be successful developments. By using 24 well plates in replacement of petri dishes has allowed a larger number of samples to be processed using less consumables and more generating more results. Current methods for isolating the phage from the plaque through growing the plaque from the plug of agar into 10% LB broth then analysing the results of growth on the spectrophotometer have proven

unsuccessful. Therefore it was decided that using a known bacteria and phage in a natural community would be a way of looking at phage-bacteria interaction networks in the environment.

A method was developed with a known bacteria and phage to ensure the method described above was successful in producing viral plaques. Log phase isolates of a genetically modified strain of *Pseudomonas fluorescens* SBW25 that is resistant to gentamicin and phage were obtained from Dr Ville Friman, Oxford University that were originally isolated from a sugar beet lead in 1989 (Rainey and Travisano, 1998).

*P. fluorescens* SBW25 is a common bacterium often found in water, soil and surfaces of plants and animal tissue. It has been shown to enhance plant growth and protect crops against disease (Rainey & Bailey, 1996). SBW25 and its lytic phage are used in numerous studies to investigate the roles of coevolution on phage and bacteria due to their specificity to one another (Buckling & Rainey, 2002; Dennehy, 2012; Gomez & Buckling, 2011; Lopez-Pascua et al., 2010; Pal et al., 2007; Poullain et al., 2007), therefore it was used in this study to validate methods that were then used on environmental samples.

The same method for isolating and growing SBW25 colonies was used as mentioned above. SBW25φ2 was extracted from SBW25 isolates using 100µl chloroform in 900µl bacteria isolates. Plaque assays were performed using 0.7% bacteriological agar inoculated with SBW25 broth culture and spotting phage isolates onto the lawn of bacteria. This proved an effective method for phage isolation from SBW25.

**Spectrophotometry**

Growth assays were performed using spectrophotometry (Biotek) to show the effect of phage on bacterial growth. Phage was isolated into SBW25 broth cultures in a 96 well plate and a 48 hour kinetic read was implemented every 1 hour. Results are shown in figure 2.

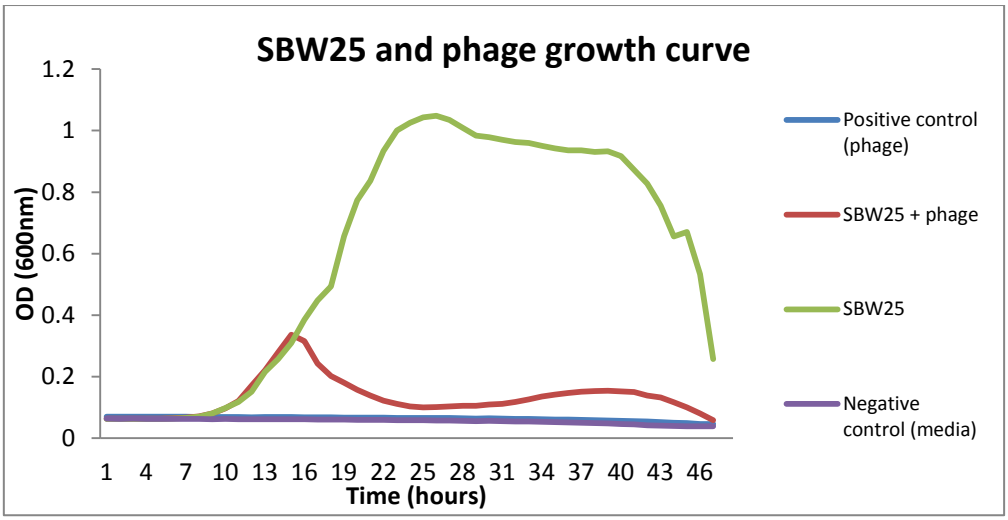


**Figure 4: Absorbance (OD600nm) readings from spectrophotometer data of SBW25 and phage growth curves. Six replicates of SBW25 and phage were inoculated into a 96 well microtitre plate and read on the spectrophotometer (Biotek) over 48 hours to ensure phage could sufficiently infect SBW25.**

The results from spectrophotometry data show that when SBW25 is inoculated with phage (red), the turbidity of the broth media and absorbance (OD) on the spectrophotometer decreases due to phage lysis of bacteria when compared with SBW25 (green) which shows a high growth and absorbance (OD) during the

exponential and stationary phase (9 – 41 hours) with a sharp decrease in growth during the death phase where bacterial cells are dying due to lack of nutrients and build-up of waste products. Comparison between the samples containing SBW25 only and SBW25 phage shows the ability of phage to prevent SBW25 growth reaching high densities due to cell lysis.

The results show that phage can lyse SBW25 sufficiently to be used in phage-bacteria experiments.

**Flow Cytometry Optimisation**

Flow cytometry was chosen as the method to use throughout the coevolutionary experiments to provide a quantitative way to determine phage lysis of SBW25 bacterial cells. This is advantageous over spectrophotometry as it gives absolute cell counts. Before experiments were performed, a dilution series of SBW25 in 0.2µm filtered beech tea media was analysed to determine the optimal dilution in which to analyse bacterial cell count on the flow cytometer. This enabled us to distinguish between background noise caused by media and high cell counts that might be caused by media and doublets (clumps of cells that cannot be determined as single SBW25 bacterial cells).

A 10-fold dilution was prepared by diluting 20µl of SBW25 stock solution ($10^{-5}$ cells per ml in LB) in 180µl of beech tea media in a 96 well microtitre plate. This was then serially diluted to $10^{-6}$ and analysed on the flow cytometer (10 seconds, fast fluidics). Figure 3 shows the logged cell counts for the 10-fold dilution series with standard error as a pink polygon around the mean.

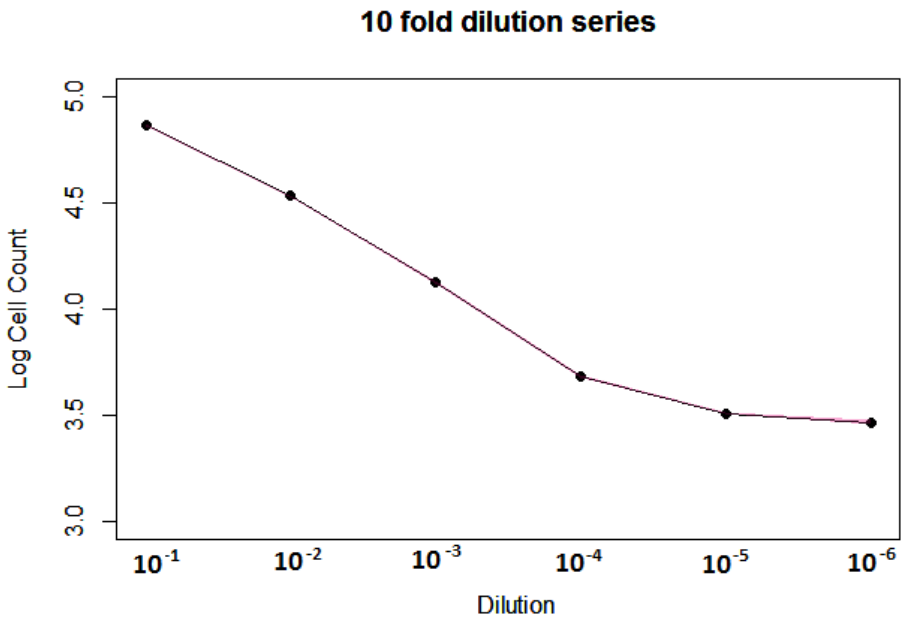


**Figure 5: 10-fold dilution series used to test the optimal dilution of SBW25 in 0.2µm filtered beech tea to read on the flow cytometer. 12 replicates of each dilution was prepared and standard error around the mean is shown by a pink polygon.**

The results show the optimal dilution for SBW25 on flow cytometry to be a 1:100 dilution when grown in beech tea media as this can easily be distinguished from background noise and single bacterial cells are shown when gating bacterial cells on the flow cytometer software. Therefore, throughout the experiments, a 1:100 dilution will be used.

**Flow cytometry phage optimisation**

To measure phage lysis of SBW25, flow cytometry was used as a culture-based method to detect lower counts of SBW25 cells when lysed by phage compared to SBW25 cell counts when phage was unable to lyse cells. Use of flow cytometry prevents bias or subjective results introduced using plate culture methods such as streak plate assays or plate culture that is common practice in coevolutionary

studies between bacteria and phage (Budzik et al. 2004; Gómez & Buckling 2011a; Koskella et al. 2011). Flow cytometry has also been used in bacteria-phage studies and can give quantitative as well as qualitative analysis of in-situ single cells and can be combined with fluorescent staining to distinguish between live and dead cells (Verthé & Verstraete 2006). To validate methods for use in my coevolutionary experiments, the flow cytometer was used to detect SBW25 bacterial cell counts in the presence and absence of phage (Michelsen et al. 2007; Garneau & Moineau 2011; Verthé & Verstraete 2006). SBW25 and phage were grown for 24 hours until log phase isolates were grown. SBW25 was inoculated with SBW25φ2 phage and grown for 24 hours in a 96 well microtitre plate. After 24 hours, these were placed on the flow cytometer at a 1:100 dilution (BD™ CSampler) and SBW25 cell densities counted when in the presence and absence of phage.

Figure 4 shows SBW25 flow cytometry counts in the presence and absence of phage.

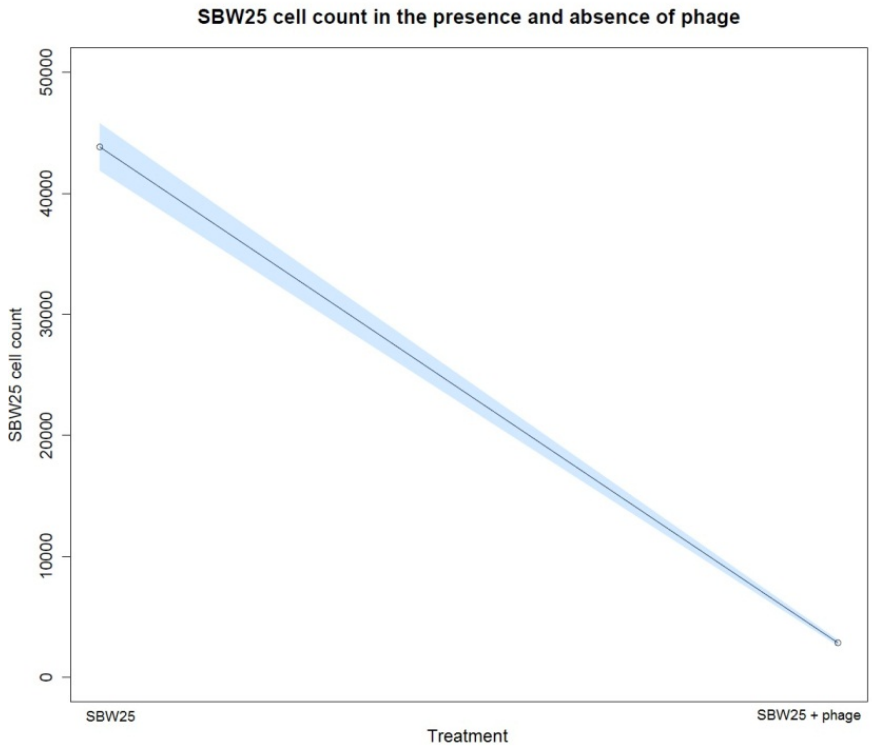


**Figure 6: SBW25 counts on the flow cytometer in the presence and absence of phage treatment. Twelve replicates of SBW25 and SBW25 + phage inoculations were performed. Standard error of the mean of cell densities was calculated (represented by the blue polygon).**

The results show a large decrease in cell counts detected when SBW25 is inoculated with phage showing that phage drastically decreases cell count that can be detected by flow cytometry one noise from the media was gated using BD Accuri™ C6 software (version 1.0.264.21, 2011). This result shows that the flow cytometry data is sensitive enough to detect high counts of live SBW25 cells and differentiates these from cells lysed by phage. As the results show some cells in the SBW25 + phage outcome, this shows that some lysed cells might be detected or that not all SBW25 have been lysed by phage. This method was determined suitable to detect SBW25 and phage coevolution assays.

Results from the plaque assay experiments, spectrophotometry and flow cytometry show that SBW25 and phage are suitable experimental isolates that will be used in the coevolutionary experiments. As the isolates have a natural origin, they were determined to be suitable for performing semi-natural experiments using natural bacterial communities isolated from beech tree holes.

**Antibiotic Resistance**

To ensure SBW25 isolates can be pulled out of the natural communities they were inoculated in during experiments, a genetically modified strain of SBW25 with gentamicin resistance was used. To use this strain, Beech treehole communities used in the experiments were screened by plating onto LB agar containing gentamicin (100µg/ml) to show gentamicin susceptibility. 70 communities that were isolated from Beech tree-holes at different locations were plated onto gentamicin agar plates and microbial colonies were observed. 19 communities were unable to grow on gentamicin agar plates and were chosen for the experiments. Methods for isolating and archiving the Beech tree-hole communities are detailed in chapter 3 methods. During the preliminary screening, it was shown that there was a pattern in gentamicin resistance within communities according to where they were sampled. This was analysed further by screening over 300 communities from different locations with 6 different antibiotics. The results are shown in appendix as the results were not used towards the thesis.

# Chapter 3 - Coevolution in natural microbial communities

## Abstract

Little is known about the coevolutionary interactions between bacteria and phage in natural microbial communities with much work focussing on coevolving in high nutrient sterile media.

Here we coevolved *P. fluorescens* and phage (SBW25φ2) in the presence of nineteen different semi - natural microbial communities isolated from beech tree holes to study if different coevolutionary trajectories (ARD or FSD) differed in the presence of natural communities. Results show that coevolutionary trajectories were consistent with either arms race dynamics or fluctuating selection dynamics.

The results show coevolutionary trajectories and adaptation are influenced by species present within the bacterial community. Predictions of how species will coevolve within different communities requires knowledge of species interactions within the communities.

## Introduction

Understanding how bacteria are regulated within the environment is pivotal to understanding the role of pathogens and how they interact with other microbes within natural ecosystems. Within natural environments, viruses and bacteria are in a continuous reciprocal coevolution in which the virus infects bacteria and the bacteria evolve to resist infectivity. Little is known on how bacterial communities affect these coevolutionary dynamics. Research in microcosms of rich media showed arms race dynamics of generalists (Buckling and Rainey, 2002, Koskella et al., 2011). However, when the experiments were conducted in soil, Gómez & Buckling (2011) showed when bacteria and phage were coevolved in the presence of a natural soil microbial community, coevolutionary dynamics changed from generalist arms race coevolution to fluctuating selection where phage and bacteria became specialist due to greater fitness costs associated with evolving resistance or infectivity alleles in a soil environment than when grown in culture media. The study also showed that coevolutionary outcomes were not different when bacteria and phage were grown within a microbial community and in the absence of a community which suggests that phage selection is not affected by interspecific competition from other organisms.

Empirical studies on phage-bacteria interactions within natural microbial communities are limited, with little known on how significant phage-bacteria

interactions are in shaping microbial communities in the environment. This is also true for how communities might alter coevolution between bacteria and phage and how this should vary between communities.

The surrounding community can influence phage-host dynamics in several ways. Microbial communities can slow down coevolution as bacterial population sizes are reduced overall due to competition. They can also constrain adaptation of bacteria if there is a trade-off between evolving to the phage and evolving to the surrounding community (de Mazancourt et al. 2008). The evolution of phage can also be affected if the phage can attach to the host cell but not attack non-target cells. Even if there is a small affinity for phage particles to misidentify SBW25 in amongst all the alien cells, this could affect the efficiency of phage infection. In other words, the surrounding community can limit the potential for evolution.

Interspecific interactions between microbial species within a community are often negative (Foster & Bell 2012) presumably because many microbial species compete for finite resources and predation can both lead to lower population sizes (Barraclough 2015). Competition is known to slow down the ability of organisms to adapt. The study shows decreased rates of adaptation caused by competition for resources between species and decreased morphological transformation. Adaptation to the new environment is slower due to these competitive interactions even if a species can adapt to the environment as they would have had they been in isolation. Evolutionary potential is known to increase when parasites and predators are generalist and can attain higher densities in diverse populations as these interactions are positive (Barraclough 2015). Therefore, microbial communities might alter coevolution between bacteria and phage as competition can lower the ability of bacteria to resist phage due to the higher

costs associated with phage defence alleles therefore leading to lower rates of coevolution due to lack of resources and phage parasitism which can ultimately lead to extinction.

The surrounding community can also influence host-phage coevolutionary trajectories because of shifting selection pressures exerted by the surrounding communities cause an altered response to selection. For example, co-occurring species can evolve to utilize resources and waste products from other species to reduce negative reactions within the community (Lawrence et al. 2012). Over time, the composition of the microbial community can change which will alter the direction and strength of selection upon the species within the community (Guimarães et al. 2011). Coevolution is the key driver in maintaining complex interaction networks between species; however, in communities that are highly diverse, more selective interactions are possible and multiple interactions between species might constrain coevolution due to the constantly changing community composition in which species interact. As resources and interactions are continuously changing, generalist species are more likely to evolve rather than specialist species as specialism is more likely to occur between two species without disturbance for an extended period of time (Howe 1984). The development of generalists influences phage/bacteria coevolution as host range can increase over time and can lead to an accumulation of infectivity mutations that can be effective against a range of host genotypes (Hall, Scanlan & Buckling 2011). Székely & Langenheder 2014 showed that the composition of an aquatic bacterial community was mainly habitat generalists that were able to tolerate a wider range of environmental conditions than specialist species that could only tolerate a small range of environmental factors.

Another way in which coevolutionary interactions can be different depending on different community compositions is through ecological sorting. In diverse communities, changing environmental conditions (biotic and/or abiotic) can filter out certain species of bacteria as some species that are already pre-adapted to the new conditions and can restrict the evolutionary opportunity for all species to respond; therefore, ecological sorting is a consequence of species abundance rather than the ability of a species to adapt to change (de Mazancourt et al. 2008; Lawrence et al. 2012; Johansson 2008). Ecological sorting can impact coevolutionary interactions between bacteria and phage as competitive abilities between microbial species will change in different environments which can increase or reduce the ability of an organism to adapt. Within a natural environment, a pairwise interaction with no other competitive forces is rare and it is exceedingly likely that organisms will have other interactions with other species within the community. Therefore, coevolution between a pairwise interaction between host and parasite is thought to change between and within different communities. This might be due to the composition of species within communities' can change between different habitats and therefore interactions between these species are likely to develop unique competitive interactions and mutualistic relationships which can alter coevolutionary trajectories.

The aim of this experiment is to understand how coevolution of *P.fluorescens* and phage is affected by bacteria present in different community assembleges. We predict that different species within a community of bacteria alter the coevolutionary trajectories of SBW25 and phage.

We investigated the effects of the surrounding community on the coevolutionary dynamics of a model phage-bacteria pair. There are very few experiments involving coevolution of bacteria and phage in the presence of other species. Therefore, this chapter aims to tease apart these interactions to help understand how coevolution proceeds in the natural environment. We used microbial communities isolated from pools of temporary rain water in *Fagus sylvatica* tree holes to test how they affected the coevolutionary trajectory of *P. fluorescens* (SBW25) and phage (SBW25φ2). We allowed SBW25 and phage to coevolve within microcosms containing 19 different beech tree communities in laboratory microcosms that were designed to mimic natural conditions and assessed how these different background communities affected SBW25 and phage coevolutionary dynamics.

The communities used are a biased sample of bacteria collected from beech tree holes due to processing, freezing, thawing and manipulation, detailed in the methods chapter. The communities were collected around well-known beech tree forests in Southern England. It is not currently known what species are within the natural communities collected, however initial testing suggests that the population is high in *Pseudomonads*.

# Methods

## Microcosm inoculation

### *Community selection*

Beech tree hole communities were sampled around southern England (figure 5).

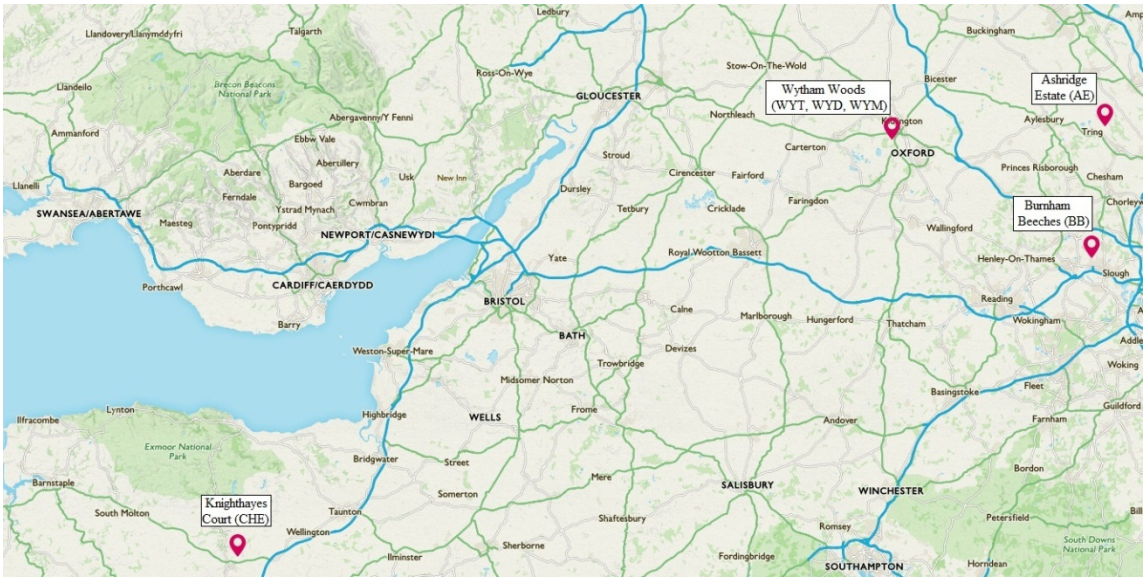


**Figure 7: Beech tree hole locations where tree hole water was sampled (Ordnance Survey, 2015).**

Using a sterile Pasteur pipette, sediment at the bottom of the tree hole was disturbed to homogenise the treehole water. Approximately 1.4ml of treehole water was pipetted into a 1.5ml microcentrifuge tube. The samples were placed into a container and the GPS coordinates were recorded (table 5, appendix). Once in the laboratory, 9 ml of PBS was aseptically transferred to a polypropylene centrifuge tube and was vortexed. 1 ml of the sample was added to the 9 ml of PBS using a widebore pipette top. A Whatman filter was placed

over an empty sterile polypropylene centrifuge tube and the diluted sample was filtered through. Freezing solution was prepared using 176 ml glycerol, 2.16 g NaCl and 74ml water and autoclaved before use. 800µl of the filtrate was added to a microcentrifuge tube and to each tube 650µl of freezing solution was added. This was vortexed and stored at -80°C.

We screened 96 communities for gentamicin resistance on LB again to ensure there were no gentamicin-resistant bacteria in the community. Of the 96 communnities, 19 were chosen on the basis of this susceptibility to gentamicin and are listed in appendix table 5.

**Experiment design and set up**

To investigate the effects a semi natural bacterial community has on the coevolutionary trajectories of SBW25 and phage, beech tea media was prepared as in Lawrence et al., (2012). Briefly, 50 g of dried beech tea leaves were suspended in 500 ml of water, autoclaved and then filtered through a 0.2 µm filter to make a concentrated stock. This was then diluted 1:32 to make the final concentration for the media.

250 µl of the archived beech tree community was grown up in 25 ml of beech tea media. From the archived beech tree communities, 250 µl was added to 10ml of beech tree media, 250 µl of SBW25 and phage stocks were also grown up in 25 ml beech tea media for 24 hours. Two-hundred and forty 50 ml polypropylene tubes were filled with 25 ml of beech tea media. The microcosms were then amended with the following treatments: one of the 19 communities or no community; SBW25 present or absent, and phage present or absent. Temperature was kept constant at room temperature and microcosms were subjected to natural

light conditions within the laboratory. The microcosm lids were loosened to allow an inflow of $O_2$. We conducted a fully factorial design of the 3 treatments, though we did not assay phage combinations in the absence of SBW25 following checks to ensure that phage did not survive in the absence of SBW25. Each treatment combination was replicated 4 times. From the grown up stocks, 250µl community, 250µl SBW25 and 100µl of phage were added to the amended microcosms. Before first sampling commenced, isolates were grown for 1 week before sampling.

**Community sampling**

Every week for 8 weeks, 1 ml of the microcosm was removed. We counted bacterial cells from a 20 µl sample which was diluted 10-fold in 0.2 µm filtered beech tea and placed in the flow cytometer (BD Accuri C6 flow cytometer, unstained cells, fast fluidics at 1 µl per second for 30 seconds). Filtered beech tea media was used to reduce background noise of beech tea particles counted as cells by the flow cytometer. We stored a 700 µl sample of each microcosm in 30% glycerol at -80°C for downstream assays. We isolated phage by placing a further 700 µl sample was added to a 1.5 ml microcentrifuge tube with 70 µl chloroform. The microcentrifuge tubes were vortexted and centrifuged at 13000 rpm for 3 minutes. The supernatant containing the phage was gently aspirated and stored at -20°C.

Microcosms were sampled weekly for 8 weeks. Every week the microcosms were replenished with 1420µl beech tea media (equal to the volumes removed every week with sampling).

**Coevolution experiments**

We conducted coevolution assays using samples taken from week 1, 4 and 8. We isolated individual SBW25 clones by plating 10 µl of the frozen samples onto LB agar containing 100 µg/ml of gentamicin. Plates were incubated at room temperature for 4 days, after which we picked 6 colonies with sterile toothpicks into vials containing 600 µl of LB media and grown for 2 days at room temperature. Isolates from broth cultures were stored in 30% glycerol at -80°C.

We tested the ability of SBW25 to resist past, contemporary and future phage by first inoculating 96 well plates with 180 ul of LB. 10 µl of each SBW25 clone from each week 4 were added to the LB. To each of the clones, 10µl of the corresponding phage from past (week 1), contemporary (week 4) and future (week 8) were added. After 24 hours incubation at 22°C, a 2µl subsample of the plate was added to 198 µl of LB and read in the flow cytometer for 10 seconds fast fluidics. Ancestral SBW25 was also grown in LB broth in the absence of phage and cell count was measured using flow cytometry. We assessed the ability of SBW25 to resist phage infections by taking the ratio of growth of the ancestor to the growth of the derived clones in the presence of phage.

We then tested the ability of phage to infect past, contemporary and future SBW25 by first filling 96 well plates with 180 µl of LB. 10 µl of phage from week 4 were added to the LB. To each of the phage, 10µl of the corresponding SBW25 from past (week 1), contemporary (week 4) and future (week 8) was added. After 24 hours incubation at 22°C, a 2 µl subsample of the plate was added to 198 µl of LB and read in the flow cytometer for 10 seconds fast fluidics.

Ancestral SBW25 was also grown in LB broth in the absence of phage and cell counts were measured using flow cytometry. We assessed the ability of SBW25 to resist phage infections by taking the ratio of growth of the ancestor to the growth of the derived clones.

**Local Adaptation Experiments**

We performed local adaptation assays by pairing SBW25 clones either with their native phage or with phage collected from a foreign microcosm. The clones used were another replicate of the same treatment (i.e. all clones chosen were from week 4 populations). We used a subset of the SBW25 clones collected for the coevolution experiments. SBW25 clones were paired with phage isolated from different communities to test whether local phages (isolated from the same sample and community) were more or less infected by phages from another community (foreign phages from other samples). Using a pipetting robot (Hamilton Starlet), 10 µl phage from one community was inoculated into a 96 well plate and paired with 10 µl SBW25 isolated from another community for the three timepoints (week 1 past, week 4 contemporary and week 8 future) in 180 µl LB broth. Plates were incubated at 22°C for 24 hours.

Foreign communities were picked and paired randomly using R. Two SBW25 clones used in the coevolution experiments were used for replicate pairwise comparisons and the experiment was replicated 4 times with two technical replicates.

After incubation, plates were inoculated with Flow Cytometer Microspheres (Cyto-Cal™ Thermo Scientific) and were placed on the HyperCyt® Autosampler

(IntelliCyt) and read through the flow cytometer (BD Accuri C6) where cell counts were recorded.

**TRFLP**

All DNA extractions were performed using Zymo ZR DNA Bacteria/Fungal extraction kits according to manufacturer's instructions.

We amplified part of the 16S rRNA gene region using forward primer 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′; Marchesi et al. 1998) and reverse primer 519R (5' -GT(N) TTA C(N)G CGG C(K)GC TG-3'; Handl et al. 2011).

PCR reactions consisted of 12.5µl RED*Taq* Readymix (Sigma-Aldrich 20 mM Tris-HCl, pH 8.3, with 100 mM KCl,3 mM MgCl2, 0.002 % gelatin, 0.4 mM dNTP mix (dATP, dCTP, dGTP, TTP), stabilizers, and 0.06 unit/mL of *Taq* DNA Polymerase), 11.3µl nuclease-free water and 0.1µl of each of the primers to make the final reaction volume to a total of 25µl. PCR Thermal Cycler conditions are described by *Liu et al., (1997)*. Initial denaturing occurred at 95°C, with 32 cycles at 94°C, 54°C and 72°C followed by 72°C. All PCRs were carried out using AB Applied Biosystems Thermal Cycler (Veriti).

Amplified PCR products were separated using 10% agarose gel electrophoresis at 120V for 30 minutes in TBE buffer. The DNA was visualised by staining the gel with GelRed™ and imaged using Gel Doc™ EZ imager (BioRad) and using Image Lab 5.0 software.

All PCR clean up were performed using ZR 96 DNA Clean Up Kit according to manufacturer's instructions.

After PCR clean up, samples were prepped for TRFLP analysis using FastDigest (Thermo Scientific) enzymes. A master mix of 1µl 10x FastDigest buffer, 0.3µl FastDigest MSP1 and 5.7µl of molecular grade water per sample was prepared. 7µl of the master mix was aliquoted into 96 well PCR plate and 3µl of PCR product was added to each well. The plate was sealed and placed in the thermalcycler at 37°C for 60 minutes, 65°C for 10 minutes.

After digestion, a master mix of 0.5µl of size standard (GeneScan™ 600LIZ®) and 9µl of HiDi Formamide (Applied Bioscience) was prepared for each of the samples. The master mix was prepared on ice into a 15ml falcon tube. The master mix was divided into aliquots of 1.5ml in Eppendorfs and placed in a hot plate at 94°C for 3 minutes. After heating, the Eppendorf contents were pipetted into a sterile trough and 9.5µl was pipetted into a PCR plate. 1µl of PCR digest sample was pipetted into the PCR plate containing the master mix and sealed with a plate seal. The plates were vortexed for 1 minute each and then centrifuged for 1 minute.

Fragment size detection was based on the TRFLP products using GeneMarker v2.6.3 (Biogene Ltd) sequencing software. The fragments were manually aligned using the software's binning analysis to remove size standards and determine fragment lengths. We converted absolute band intensity data to relative abundance by dividing each intensity measurement by the total fluorescence signal from the sample as a whole (the sum of the intensities from each microcosm.

**Trade off**

We used the viable Celltracker (Thermo) stain to estimate tradeoffs by competing clones isolated from the coevolution experiment. Celltracker is a cytoplasm stain that gets divided evenly between daughter cells and does not transferred to adjacent cells. The reduction in per-cell fluorescence can therefore be used to estimate growth rates. The effects on metabolism of the bacterial cell are not known.

The original microcosm from where the SBW25 was isolated for the coevolution experiments (chapter 3) were grown in beech tea media. A subsample of SBW25 LB colonies were inoculated in beech tea media in a 96 deep well plate and grown at 22°C for 24 hours.

After 24 hours of growth in LB, SBW25 colonies were stained with Cell Tracker™ (Life Technologies) at 1µl/200µl SBW25. After staining, colonies incubated in the dark on a shaker at 150rpm for 1 hour. The plate was centrifuged for 3 minutes at 4000rpm. The supernatant was aspirated from the bacterial pellet and the media was replaced. A shallow 96 well plate was filled with filtered beech tea media and using the reciprocal transplant experiment design, 50µl of the stained communities was inoculated into the plate with 50µl foreign community and its own community as shown in the plate configuration and grown at 22°C for 24 hours.

After 24 hours, a 10µl subsample of the microcosms was pipetted into 0.2µm filtered beech tea media and ran on the flow cytometer for 10 seconds fast fluidics.

**Statistical Analysis**

To analyse the changes in microcosms over time, cell densities of the microcosms were measured over time using flow cytometry. Coevolutionary interactions between treatments within communities were estimated using a general linear model (GLM) on log transformed ratios of SBW25 grown in the absence of phage vs the presence of phage for testing treatment (contemporary vs past and future) and differences of these treatments between communities with community fitted as a random effect. Flow cytometry cell counts were analysed using GLMs and one-way ANOVAs were performed to test the effects of community and treatment interactions. *Post hoc* Tukeys test was performed. Paired Student's T-Test was used to compare local adaptation of phage to SBW25 clones coevolved from different communities.

Paired T tests were performed to analyse the differences between SBW25 cell count, and thus fitness, when inoculated in sympatric vs allopatric microcosms. A significant difference is shown when $P < 0.05$.

TRFLP data was analysed using nonmetric multidimensional scaling (nMDS) as the data contained numerous 0 values to determine the dissimilarities and similarities between communities' composition between treatments. Analysis of Similarities (ANOSIM) was used to test the differences between community composition in week 1 and week 8. The R statistic tests whether the assemblage structure differs across the treatments. An R statistic of <0.25 typically shows that the groups are indistinguishable and R >0.75 shows that the groups are well separated (Lehmann et al., 2008). Distance measures for the multivariate analysis are based on Bray-Curtis dissimilarity matrix.

## Results

Microbial cell count data from each microcosm were monitored throughout the experiment.

Figure 6 shows log cell counts of bacterial cell densities of the communities containing SBW25 and/or phage or microbial community only with media as a negative control, community only and SBW25 and phage as positive controls. No significant difference in cell count density between treatments were shown in between community ($P > 0.08$) with the exception of community 2 and community 7 which showed a significant difference between treatments ($F_{2, 18} = 6.5$, $P = 0.007$ and $F_{2, 18} = 20.9$, $P > 0.001$, respectively) with post hoc Tukeys analysis. Community 2 showed a significant difference between community only with community and SBW25 and community 7 shows significant difference between community with SBW25 and community with SBW25 and phage. These results show that the majority of microcosm cell densities are very stable

throughout the week with cell counts not changing even with the addition of phage and/or SBW25 with the exception of community 2, which shows a decrease in cell density for communities inoculated with SBW25. Higher cell count was shown in community 7 for communities inoculated with SBW25 and phage than other microcosm treatments.

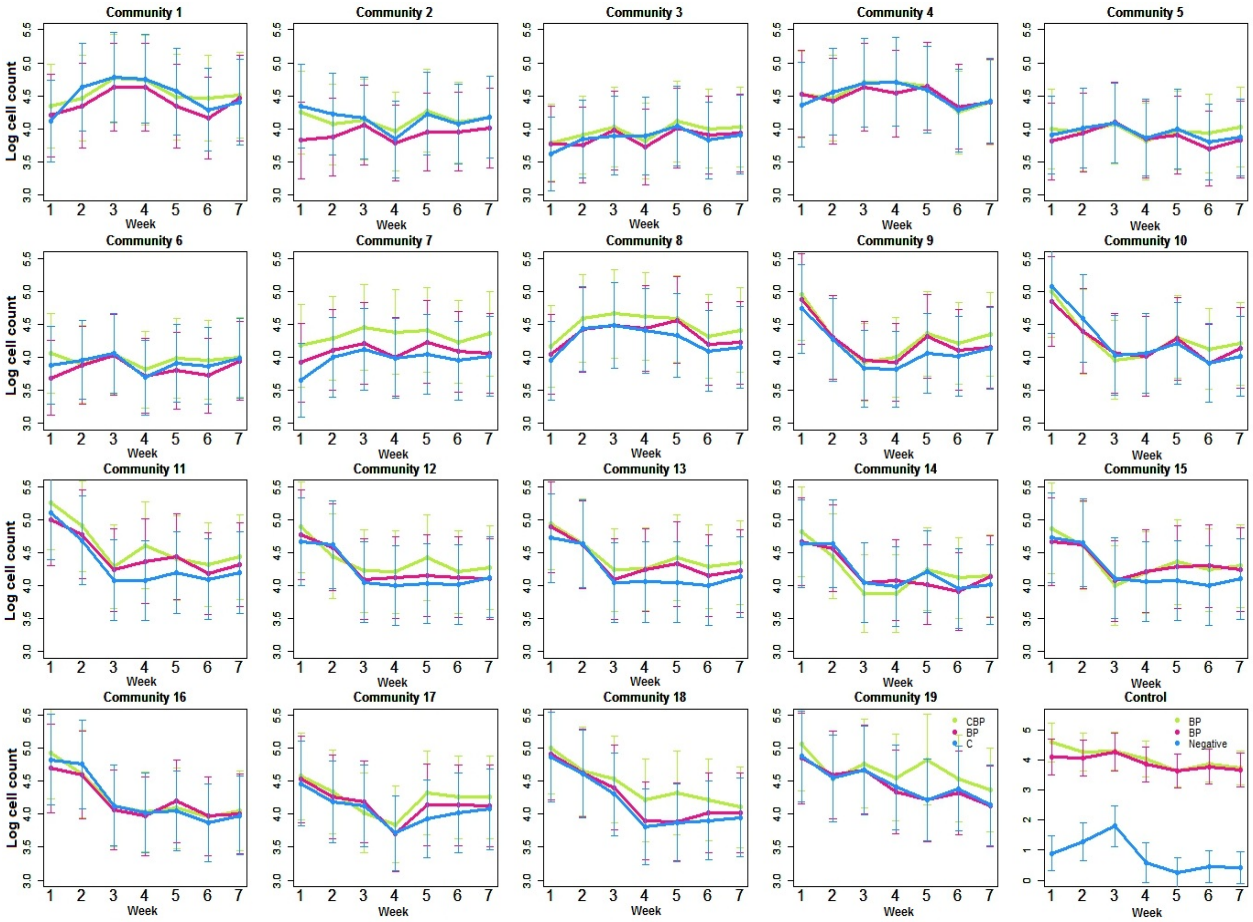


**Figure 8: Microcosm treatments with phage and/or SBW25 or community with no treatment. The results show stable microcosm growth between treatments. In communities 1 – 19, the green line shows communities inoculated with SBW25 and phage and were subject to the coevolutionary experiments below. Pink line shows microcosms with SBW25 and phage only and blue shows community with no SBW25 and phage. Error bars show standard error of the mean of all the microcosm and technical replicates. Most communities show high growth in week 1 with a decline in cell counts by week 3. Treatments in each community showed no significant difference (GLM P > 0.08).**

**Coevolutionary patterns of SBW25 inoculated with past, contemporary and future phage**

All SBW25 and phage isolates from week 1 to 8 were recovered and were used in the following experiment. Figure 7 shows the ability of phage populations from different time points (past (week 1), contemporary (week 4) and future (week 8)) (over an eight-week period) to infect SBW25 from contemporary populations and figure 8 shows the ability of contemporary phage to infect SBW25 past, contemporary and future populations.

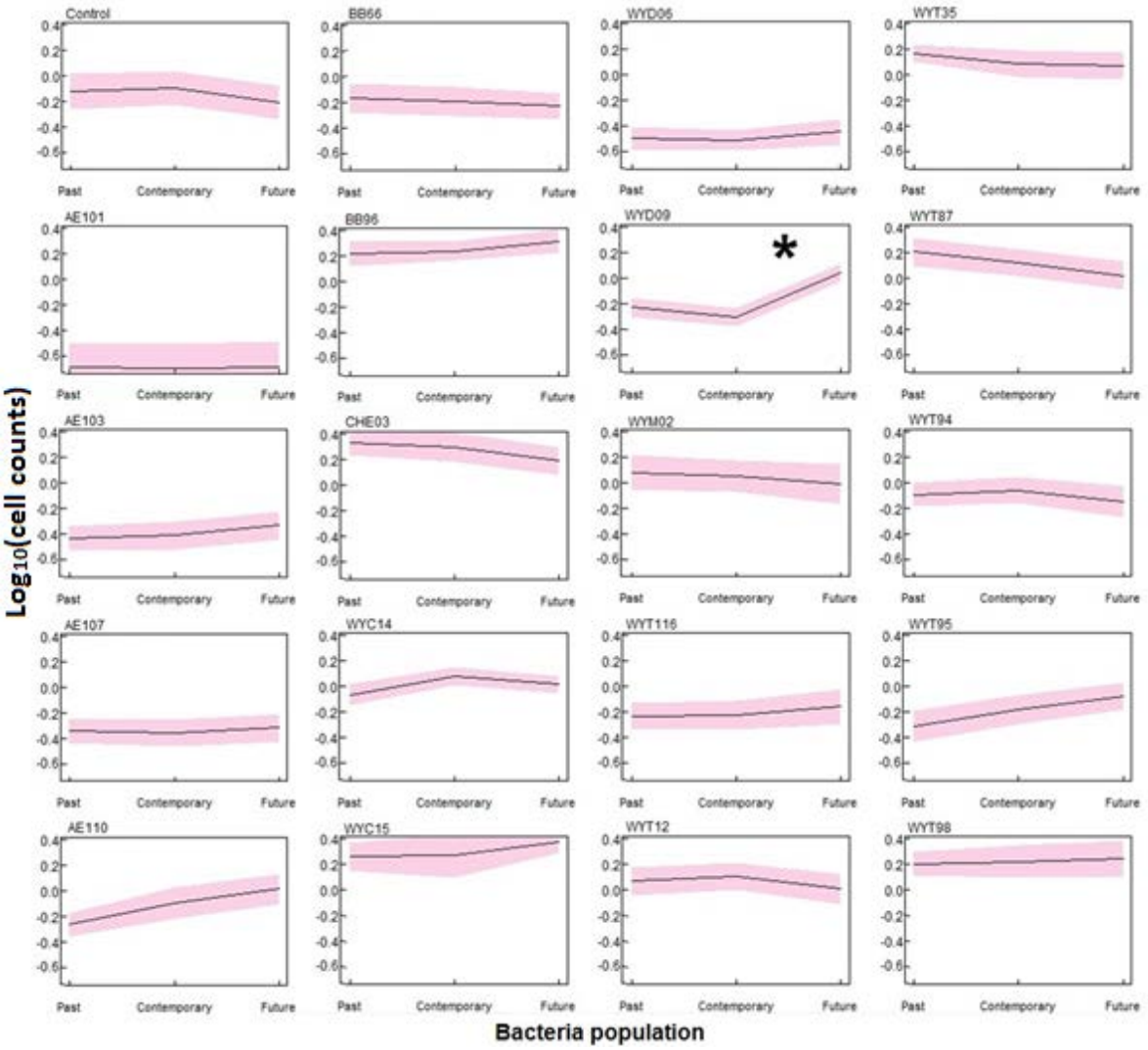


**Figure 9: Log transformed SBW25 mean cell counts (±SE) from flow cytometry data in different communities to test the infectivity of 'past', 'contemporary' and 'future' phage on 'contemporary' SBW25. (* denotes a significant difference between treatments). Cell ratio data from flow cytometry was standardised against the growth of SBW25 in the absence of phage. For each community, 6 SBW25 clones were used for each treatment (past, contemporary and future), this was replicated 4 times across the microtitre plate and 2 technical replicates were performed. Relative densities (y-axis) are calculated as the density of cells in the presence of phage (past, contemporary, future) divided by the density of cells grown in the absence of phage (control). Standard errors are depicted by the polygon colour (pink) around the mean (black line). GLM showed significant difference of phage infectivity between communities $F_{19, 1380} = 16.57$, $P < 0.001$.**

Over the eight-week period, SBW25 infected with past phage (isolated from week 1), contemporary (week 4) and future (week 8) showed a large variation in coevolutionary patterns when coevolved in different communities (Figure 7: one-way ANOVA revealed a significant difference between all communities GLM $F_{19, 1380} = 16.57$, $P < 0.001$, appendix table 6).

Treatment (past, contemporary and future) overall was non-significant (ANOVA GLM $F_{2, 1380} = 0.325$, $P > 0.7$, appendix table 6). However, Tukeys analysis showed community WYD09 was significant between treatments ($P < 0.02$, appendix table 7). Although patterns of coevolutionary trajectories differed between communities, treatment with the phage within the communities showed no significant different with ratio log cell count. The results show that although no significant difference between cell count of phage infection to bacteria in the majority of communities (with the exception of community WYD09), there is a difference in phage infection between the communities and show that coevolutionary trajectories of bacteria and phage cannot be predicted when evolved in the presence of different microbial communities with different species compositions.

**Coevolutionary patterns of phage inoculated with past, contemporary and future SBW25**

Figure 8 shows log ratio of contemporary phage on populations of past, contemporary and future populations. A one-way ANOVA revealed a significant difference between all communities (figure 8: GLM $F_{19, 1380} = 20.7$, $P < 0.001$, appendix table 8). Treatment (past, contemporary and future) overall was non-significant (GLM $F_{2, 1380} = 0.3$, $P > 0.8$, table 8). However, Tukeys analysis showed communities WYT116 and WYT12 were significantly different between treatments ($P < 0.05$ and $P < 0.03$, respectively, table 9 and 10). Figure 8 shows similarities to FSD patterns in several of the communities (AE101, AE107, WYT94 and WYT95) although none show a significant difference ($P < 0.05$). Community WYT116 resembles an arms race dynamic where phage fitness is highest in future populations of SBW25 and lowest with ancestral SBW25. WYT12 shows a significant FSD pattern where hosts are most resistant to their contemporary than past and future phage.

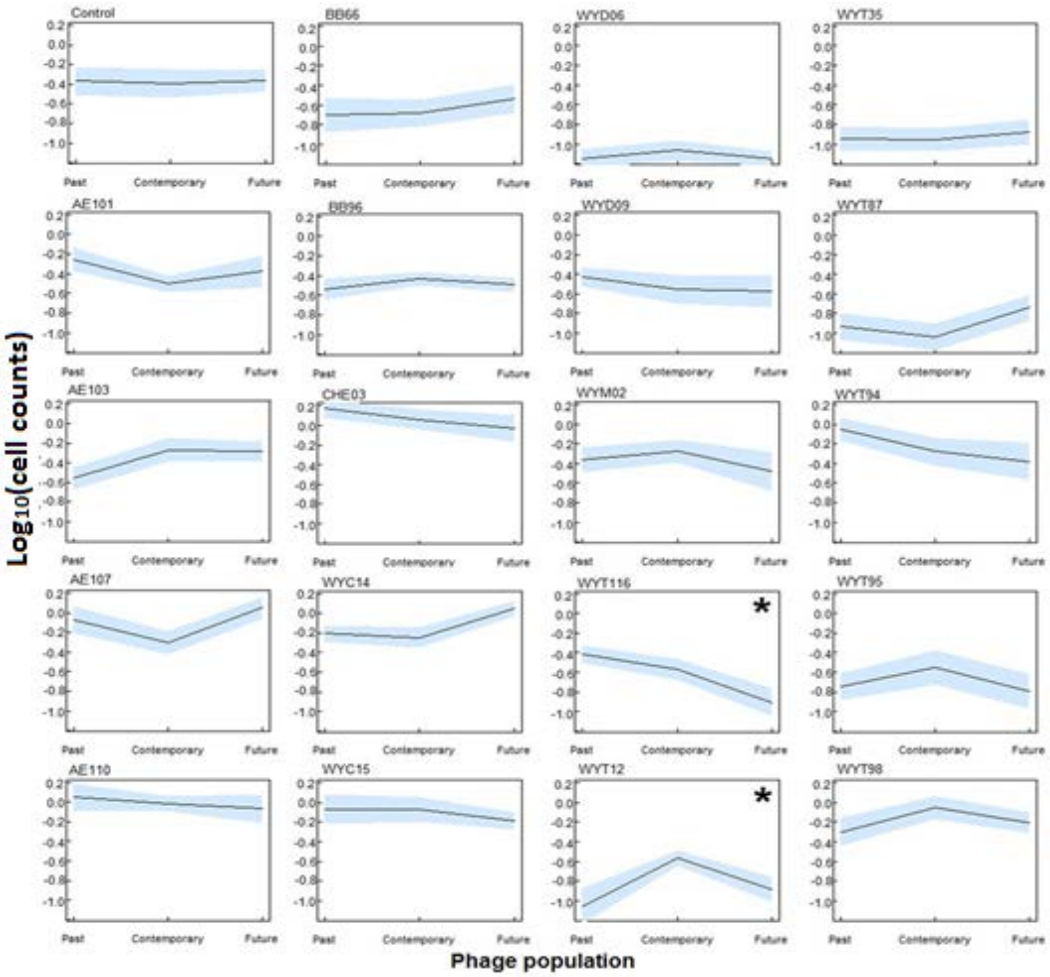


**Figure 10: Log transformed SBW25 mean cell counts (±SE) from flow cytometry data in different communities to test the infectivity of 'contemporary' phage on SBW25 isolated from the 'past', 'contemporary' and 'future'. (* denotes a significant difference between treatments). For each community, 6 SBW25 clones were used for each treatment (past, contemporary and future), this was replicated 4 times across the microtitre plate and 2 technical replicates were performed. Cell count data from flow cytometry was standardised against the growth of SBW25 in the absence of phage. Relative densities (y-axis) are calculated as the density of cells in the presence of phage (past, contemporary, future) divided by the density of cells grown in the absence of phage (control). Standard errors are depicted by the colours (blue) around the mean (black line). GLM showed significant difference of phage infectivity of SBW25 between communities.** $F_{19, 1380} = 20.738$, $P < 0.001$.

Similar results of coevolutionary patterns were shown in figure 8 were to figure 7, where coevolutionary patterns of SBW25 and phage are shown to be distinct from one another dependant on the community in which they evolved.

**Local Adaptation of phage with sympatric and allopatric SBW25**

To test local adaptation of phage to SBW25, the relationship of SBW25 when it was coevolved with its phage in the same community (sympatric) was compared with SBW25 coevolved with its phage from a different community (allopatric) was analysed using paired T-tests. This was done by isolating the phage from one community and inoculating it with an SBW25 isolated from another community. The t-tests compare SBW25 and phage in relation to which community they were isolated from. The results showed that all but one community from the sympatric SBW25 and phage pairs were significantly different from the allopatric SBW25 and phage pairs ($P < 0.05$). Community 15 (AE101) showed no significant difference between sympatric and allopatric coevolution ($t = 0.74$, $P = 0.4$). The results show that a majority of communities where SBW25 and phage were coevolved shows higher cells counts of SBW25 infected with phage from sympatric hosts than on allopatric hosts. This result suggests that the phage are locally maladapted to SBW25 as they perform better on hosts from foreign communities rather than their own communities as shown in figure 9

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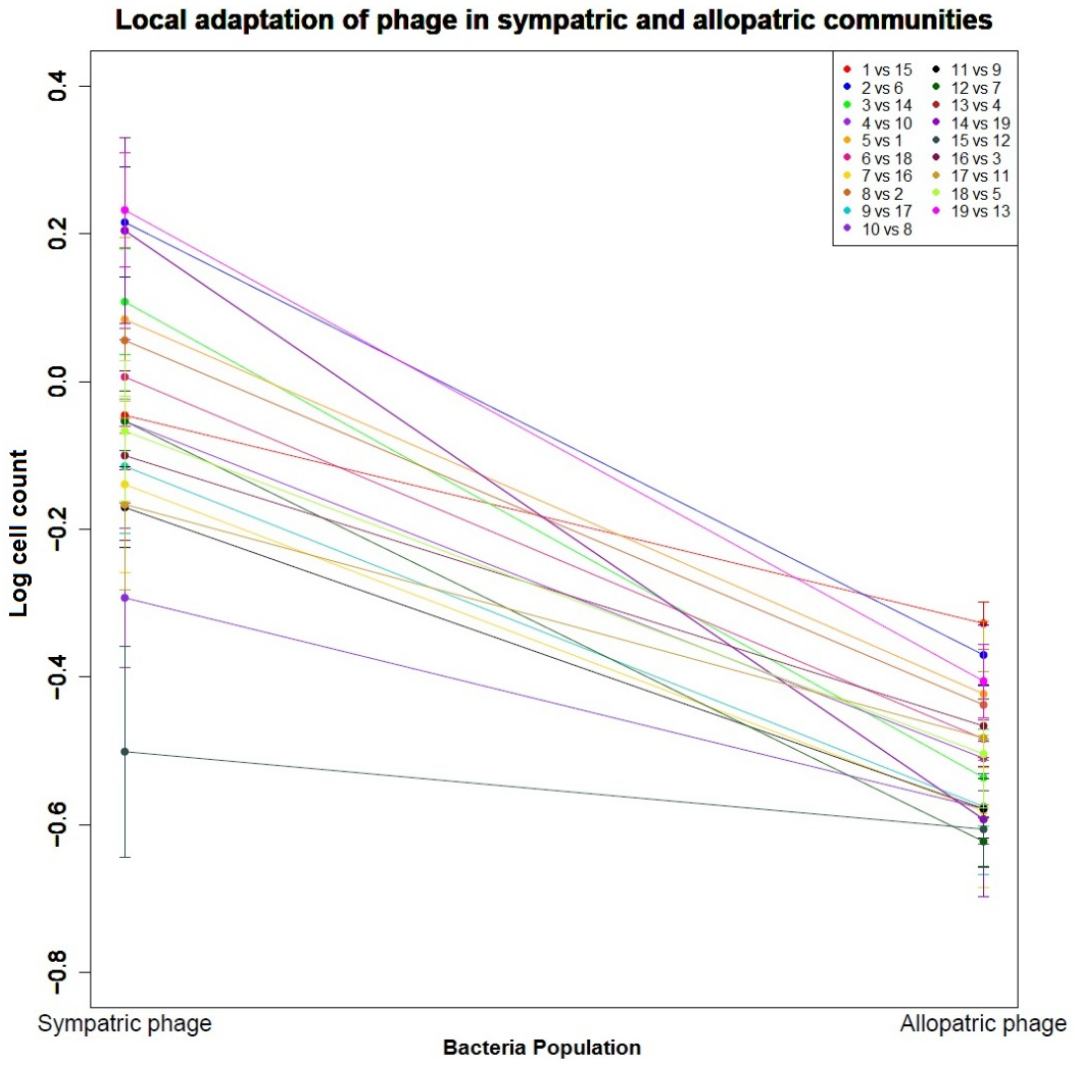


**Figure 11: Log transformed SBW25 mean cell counts (±SE) from flow cytometry data when coevolved with sympatric phage isolated from the same communities and allopatric phage isolated from different communities.**

**Each line represents a different community that isolated SBW25 was infected with its local phage (sympatric) or foreign phage (allopatric). T tests showed a highly significant difference in phage infectivity to foreign SBW25 compared to local SBW25 ($P < 0.05$).**

To test whether local adaptation increases if the foreign community is more similar to the local community, ANOSIM was performed on TRFLP community data. An R value >0.25 to 1 shows that there is a high separation of species composition between communities. An ANOSIM R value <0.25 suggests communities are more similar to one another. All community pairings (with the exception of one) were shown to be dissimilar to one another (ANOSIM R= >0.35, appendix table 12) suggesting that the local maladaptation observed in figure 9 is not because phage have evolved in similar local conditions when placed into a similar foreign community. However, one community (AE101) showed no significance and a very low R score (R = 0.0, P = 0.528). This result is interesting it was the only SBW25 and phage inoculated into a foreign community that did not show patterns of local maladaptation as the local and foreign community were similar to one another.

**Trade off**

Paired t tests were used to analyse the cell count data of SBW25 when inoculated into their sympatric community with local SBW25 compared to SBW25 inoculated into their allopatric community with foreign SBW25.

Communities 1, 2, 4 and 17 all showed significantly higher growth ($t_{31} = 3.17$, P = 0.003; $t_{31} = 2.27$, P = 0.03; $t_{31} = 3.8$, P = 0.0007; $t_{31} = 5.72$, P < 0.001 respectively) of SBW25 in their sympatric community with local SBW25. This shows that SBW25 grew better with the same SBW25 in which they were extracted and re-inoculated into. This is in contrast to communities 3, 10, 12, 13, 15 and 18 which all showed significantly higher growth ($t_{31} = 2.7$, P = 0.01; $t_{31} = 2.8$, P = 0.008; $t_{31} = 4.1$, P < 0.001; $t_{31} = 2.22$, P = 0.03; $t_{31} = 3.6$, P = 0.001; $t_{31} =$

4.14, $P < 0.001$, respectively) of SBW25 in their allopatric community with foreign SBW25 (blue bar), suggesting a maladaptation of SBW25 as they grew better in the presence of a diverged population of SBW25 as shown in figure 10. No significant difference in SBW25 growth between allopatric and sympatric populations are shown in communities 5, 6, 7, 8, 9, 11, 14, 16 and 19 with paired t test results of $P > 0.06$.

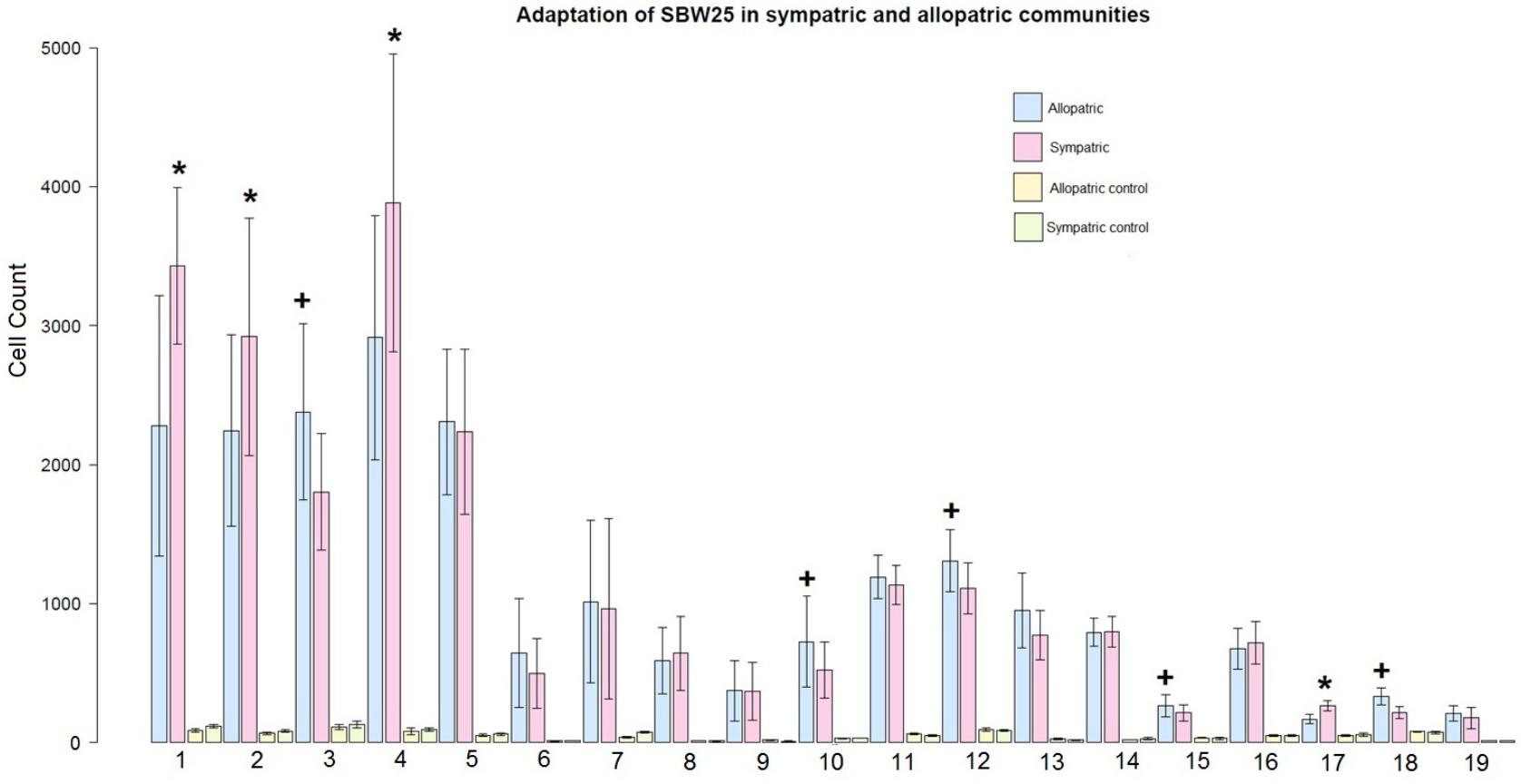


**Figure 12: Cell count data of stained SBW25 inoculated into local SBW25 and communities (red) and SBW25 growth inoculated into foreign SBW25 and communities (blue). * shows significantly higher growth of local SBW25 in its sympatric community ($P < 0.05$). Community native and foreign communities are listed in appendix. + shows significantly higher growth of foreign SBW25 in its allopatric community ($P < 0.05$). Community and SBW25 inoculated with SBW25 with no Cell Tracker™ controls (foreign – green, local – purple)**

**TRFLP**

Multivariate analysis of TRFLP data was used to determine differences of community composition between treatments (CBP, BP, CB) and weeks (week 1 and week 8 by measuring presence and absence of species). Week 1 results (figure 11, appendix table 11), show that communities overall were dissimilar (ANOSIM, P = 0.001, R = 0.333). Communities that were inoculated with SBW25 and phage (CBP) were compared to communities without inoculation (C) were shown to be similar in composition (P = 0.16, R = 0.05). This suggests that communities inoculated with SBW25 and phage were not distinct from communities alone meaning that SBW25 and phage are not having a significant effect with their abundance within the community in week 1. When comparing communities inoculated with SBW25 (CB) and communities alone (C) shows a significant difference between communities (ANOSIM, P = 0.004, R = 0.034).

**Week 1**

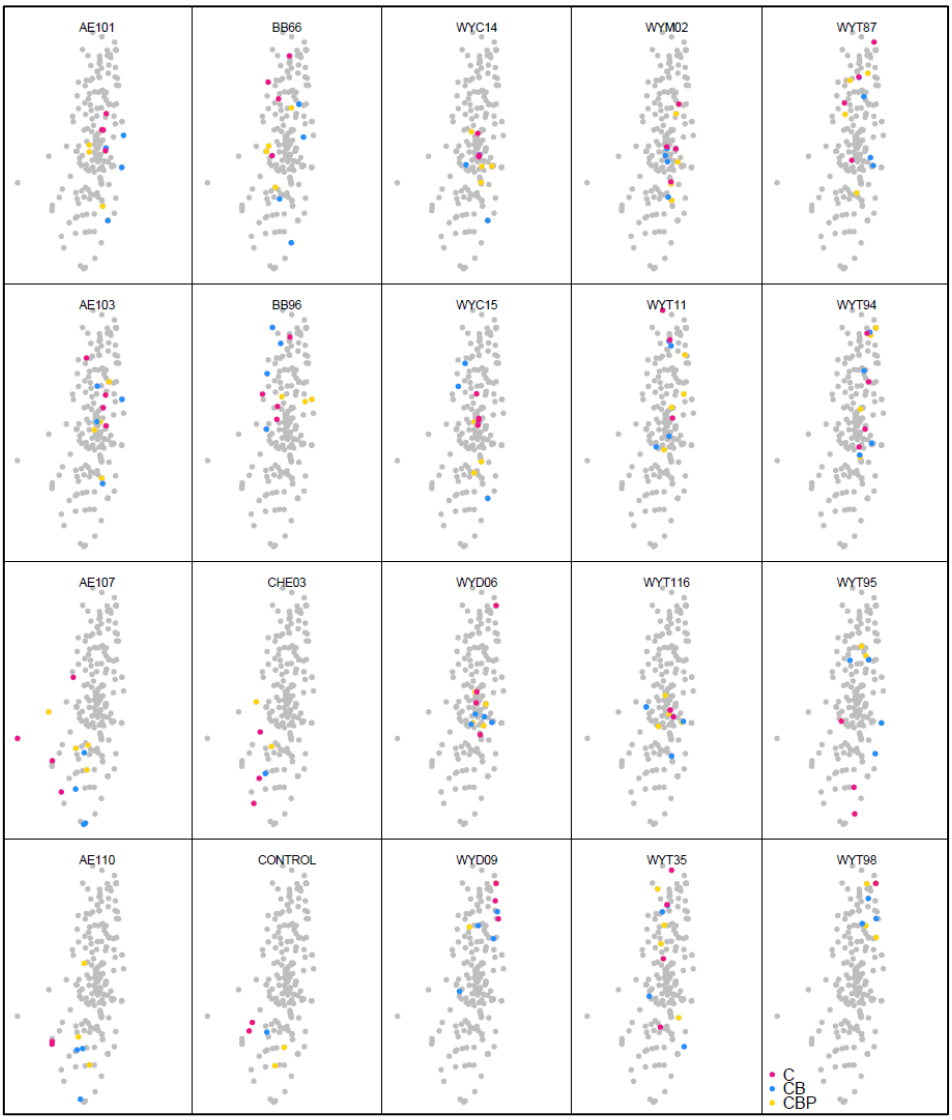


**Figure 13: nMDS ordination results for TRFLP of 19 communities and 1 control for week 1.**

**Each plot shows the similarities in species composition between each community with grey dots showing all microcosms and treatments in replicate for each community with colours showing specific community with treatments. Pink shows community with no SBW25 or phage, blue is community with SBW25 and yellow shows community inoculated with SBW25 and phage.**

Multivariate analysis of community composition 8 weeks (figure 12) after inoculation of SBW25 and phage show that there is still no distinction between communities alone (C) and inoculated communities (CBP) (ANOSIM, P = 0.01, R = 0.022), this is similar to the results in week 1 and suggests that communities have remained relatively stable with little change in composition. Again, this is reflected in comparisons of SBW25 inoculation (CB) compared with community alone (C) (ANOSIM, P = 0.001, R = 0.1512). However, the R score increased slightly from week 1 which shows some change in composition between species such as change in abundance of some but the results do not suggest a strong difference and compositions cannot be determined to be distinct.

Week 8 species composition of community alone show significant difference and a decrease in R score from week 1 (ANOSIM, P = 0.001, R = 0.26). This shows that species structure is converging after eight weeks and changes in abundances in species might be causing a change in composition.

**Week 8**


**Figure 14: nMDS ordination results for TRFLP of 19 communities and 1 control for week 8. Each plot shows the similarities in species composition between each community with grey dots showing all microcosms and treatments in replicate for each community with colours showing specific community with treatments. Pink shows community with no SBW25 or phage, blue is community with SBW25 and yellow shows community inoculated with SBW25 and phage.**

## Discussion

We show that the surrounding microbial community affects the patterns of coevolution of *P. fluorescens* and φ2 phage. Although we did not test for specific mechanisms, the patterns of coevolution between SBW25 and phage change when evolved in different communities with patterns resembling fluctuating selection and arms race dynamics (figures 7 and 8) in both the bacterial population with past, contemporary and future phage and with the phage population inoculated with past, contemporary and future SBW25. This is contradictory to numerous studies in which bacteria-phage coevolution patterns are predicted to follow reciprocal coevolution (Ashby & Buckling 2015; Lopez-Pascua & Buckling 2008; Buckling & Rainey 2002b) or fluctuating selection (Hall, Scanlan, Morgan, et al. 2011; Gómez & Buckling 2011a) with and without communities present. This study showed that host-parasite coevolutionary paths cannot be generalised as these trajectories are affected in different ways and dependent on the surrounding interspecific interactions of the community. Little work has been performed on coevolution of host-parasite interactions in the presence of a natural community. Overall, coevolution between SBW25 and past (week 1), contemporary (week 4) and future (week 8) phage showed resemblance of patterns of arms race dynamics or fluctuating selection dynamics and the trajectories of coevolution could not be predicted due to the effects of the surrounding community. Negative interactions from surrounding communities can impact pairwise interactions of host and parasites as competition can slow adaptation to resistant and infectivity alleles of the bacteria and phage, respectively. Adaptation is slowed because interspecific competition between microbes has fitness costs in that resources are finite and each microbe has to

adapt to utilize the resource within the changing environment as well as evolve to resist or infect another species for survival. Coevolution can be constrained when composition of species within a community is constantly changing. This experiment showed non-significant patterns of coevolutionwhich might show that in this instance coevolution was constrained within the community. . This suggests that the surrounding community in which SBW25 and phage had coevolved within had caused too high a fitness cost for either the phage or bacteria to evolve sufficient resistance or infectivity alleles. Several studies have shown strong coevolutionary dynamics of bacteria and phage without external competition from competing microbes for following an arms race dynamic (Dennehy 2012; Lopez-Pascua & Buckling 2008; Buckling & Rainey 2002a) in which the phage can continuously infect the bacteria through mutation of infectivity genes and the bacteria can resist infection by modifying its resistance mechanisms. However, in a natural environment, phage and bacteria will have external pressures upon them causing this constraint in coevolution observed.

Resemblance of fluctuating selection or an arms race dynamic pattern was shown in each of the communities. In sample WYD09 (figure 7), it is shown that the infectivity of phage from the past is most infectious to SBW25 in contemporary populations and least infective to SBW25 in future populations. From the other coevolved samples in figure 7, it is shown that different patterns emerge with some showing similarities to arms race dynamics where the phage from past populations is able to infect contemporary SBW25 best and is least infectivity with SBW25 from future populations (BB96, AE110 and WYT95). Patterns that resemble fluctuating selection dynamics also appears in several of the communities (control, WYC14, WYD09, WYT12 and WYT94, figure 8).

However, although both coevolutionary paths can be seen, these both represent two extremes in a continuum of more complex interactions (Gandon et al. 2008).

The differences in patterns of host and phage coevolution may be due to coevolutionary hot spots, where in one community (eg WYD09, figure 7 and WYT116, figure 8), there is clear evidence for reciprocal coevolution. Whereas in communities that do not show clear evidence for coevolution, might be a coevolutionary cold spot and reciprocal evolution does not appear to occur (*Thompson et al., 2002*). As coevolutionary trajectories appeared to be non-significant in the majority of communities, this could be caused by local maladaptation where the sympatric hosts are not coevolved to their parasites as there is evidence for poor phage infectivity to their hosts which can be caused by a mismatch of host defence and parasite counter defence genes created by evolutionary time lags that are caused by frequency and density dependent selection (Thompson et al., 2002).

There was a significant difference in the coevolution patterns exhibited between all communities. This suggests that there are considerable differences between the population dynamics of SBW25 and phage in the presence of each community and suggests that there might be an impact of the microbial community presence on coevolution and that interspecific competition may contribute to the coevolutionary trajectories exhibited by SBW25 and phage. However, these result also show that this might have occurred by chance as it is to be expected that distribution patterns might change if the experiment were to be run again, however this is true for every coevolutionary experiment. The experiment shows a snapshot of a possible evolutionary outcome at one moment in time In a study by Gomez and Buckling, (2011) demonstrated that in the presence of a natural

soil community, coevolutionary paths changed from arms race dynamics to fluctuating selection dynamics. However, this study shows that different coevolutionary patterns are exhibited when in the presence of different microbial communities and therefore one pattern cannot be used to describe the interactions that occur between phage and bacteria in a natural microbial community.

Figure 7 and 8, show the majority of SBW25 – phage coevolution showing patterns that reflect fluctuating selection dynamics (FSD). Gómez et al. 2015 showed that if populations of *P. fluorescens* and phage are physically mixed, this increases encounter rates and coevolution can shift from specialist FSD to generalist ARD. This increases the selective advantage of evolving broad resistance ranges that follow the Gene for Gene (GFG) model of infection which can result in ARD. As the microcosms were static in this experiment, this may have reduced the encounter rates of SBW25 and phage to coevolve rapidly and it is thought that as encounter rates will be low, coevolution is most likely to follow Matching Allele (MA) hypothesis (FSD) in which a specific infectivity allele is required to infect the host with a specific resistance allele (Quigley et al. 2012).

Recent work has shown that in nutrient rich media, host – phage interactions most commonly follow the GFG model as the costs associated with generalism are less with the high availability of nutrients (Brockhurst et al. 2007; Koskella & Brockhurst 2014) In contrast to this, Gomez and Buckling, (2011), again showed that in soil microcosms that are lower in nutrients, SBW25 and phage coevolution switches from ARD to FSD with neither phage nor host becoming more infective or resistant over time. The cost of resistance is therefore thought to be affected by a wide range of nutrient availability. Studies have suggested that increased nutrients result in elevated resistance of host and infectivity of phage (Lopez

Pascua et al. 2014). Beech tea media is very low in nutrients compared to nutrient rich laboratory media. However, it has not yet been determined if beech tea media is comparable to natural tree holes. The presence of other species in the community in which they are coevolving could alter the coevolutionary trajectory and extent of adaptation of host and parasite (Lawrence et al. 2012). Lopez Pascua et al. 2014 showed that higher nutrient availability shifted coevolution from FSD to ARD through elevated costs of resistance, which is thought to be a direct effect of increased nutrients. Therefore, as the beech tea media is low in nutrients, this would most likely result in FSD dynamics as shown in both figure 7 and 8.

In microbial communities, bacteria compete for resources and space with nutrient limitations being one of the most important reasons for microbial competition, Extinction can be high due to being outcompeted by more adapted microbes and nutrient limitation can occur (Hibbing et al. 2010). In this instance, small microcosms might have led to a depletion of nutrients and eventually the extinction of SBW25 and/or phage. Static liquid cultures offer numerous ecological niches and species that suit these particular niches are selected for by negative frequency dependent section. Static cultures also have lower nutrient availability as there is less mixing of the resources which causes a disadvantage to cell growth (Leiman et al. 2014). If microbial populations cannot grow to high densities, this creates less competition and therefore it is more likely that a population can coexist stably in the same microcosm with limited extinction rates. These competitive interactions with other species can affect coevolution through other species using much of the resources available, leading to fewer nutrients available to SBW25 which can result in FSD as this mode of coevolution is less

costly and beneficial mutations for resistance are reduced due to lower populations sizes of host and parasite and costs associated with resistance outweigh the benefits (Lopez Pascua et al. 2014). The microbial population may also mean that the SBW25 and phage encounter rates are lower which was also shown to lead to FSD dynamics where a specific resistance allele of the host is matched to a specific infection allele of the parasite.

Although no significant patterns were observed (with the exception of one community in figure 7 and two in figure 8), a significant difference was observed in patterns between communities showing that the way in which phage could infect bacteria differed depending on the community in which it evolved. This suggests that there are considerable differences between the population dynamics of SBW25 and phage in the presence of each community and suggests that there is an impact of the microbial community presence on coevolution and that interspecific competition may contribute to the coevolutionary trajectories exhibited by SBW25 and phage. This study shows that different coevolutionary patterns are exhibited when in the presence of different microbial communities and therefore one pattern cannot be used to describe the interactions that occur between phage and bacteria in a natural microbial community.

We also show that phage are locally maladapted to their foreign host with higher fitness of phage against SBW25 when evolved with bacteria from another community (figure 9). Local maladaptation can result due to numerous reasons and is often caused by an evolutionary time lag between host and parasite defence and counter defence genes (Thompson, 2005). Frequency dependent selection between parasite and host species can cause local maladaptation. As rare host genotypes are favoured by natural selection, to which the parasite is poorly

adapted, this creates a temporal mismatch between the host and phage within local communities. Density dependent selection is also known to generate these evolutionary time lags. As populations of host and parasite can rapidly fluctuate, this can create delays in response to selection. As host and phage are rapidly coevolving, mutations will lead to a diversity of hosts that the phage must adapt to. This can create compromise in selection that could make these pairwise interactions appear maladapted. These adaptations to different hosts could cause asymmetric effects on parasite infectivity to different hosts (Thompson et al., 2002). Trait mismatching can occur as traits and alleles are shaped in one community context and are then introduced into a different context. The mismatching of traits is important to the coevolutionary process as it can drive ongoing coevolutionary change in populations, this may have caused the phage to be less fit on the host in sympatric combinations. With negative frequency dependent selection, interactions between the parasite and host create continuous oscillation patterns, in line with arms race dynamics, which will give patterns of local adaptation and local maladaptation over time (Koskella & Brockhurst 2014). Therefore, they will appear to be locally maladapted due to a time lag, due to directional selection, until the parasite can track changes in the host species, local maladaptation only occurs due to the structure of the coevolutionary process (Thompson, 2005). This evolutionary lag between parasite genotype tracking may be the reason some parasites can exploit their allopatric hosts more efficiently than their sympatric hosts as the frequencies of the parasite and host cannot change their genotypes instantaneously within populations (Lajeunesse & Forbes 2002; Thompson et al., 2002). In species undergoing rapid coevolution, the parasite is always maladapted to their sympatric host by at least one

generation and greater parasite fitness in allopatric hosts can imply a greater adaptation to the hosts. A rare parasite genotype might also be very infective to an allopatric host and therefore as this parasite is more infective, its population rises rapidly which may produce a maladaptation pattern (Dybdahl and Strofer, 2003) as seen in figure 9. Another explanation for the local maladaptation could be due to the geographical structure of the communities in which the SBW25 and phage coevolved. As shown in figure 7 and 8, different coevolutionary trajectories are observed. Adaptation and coadaptation have been shown to be influenced by spatial structure. The strength of selection on host parasite interaction can vary across geographical range. This in turn, can result in coevolutionary hot and cold spots. In typical broth media, it has been shown that SBW25 and phage switch from rapid reciprocal evolution to fluctuating selection dynamics after 250 generations due to the costs involved with bacterial resistance (Koskella & Brockhurst 2014; Lopez Pascua et al. 2014). As beech tea media is a low nutrient broth, the reciprocal evolution (arms race dynamics) between SBW25 and φ2 would not occur as quickly as in high nutrient media such as LB. Therefore, as the coevolutionary experiments ran for eight weeks, this may not have been enough time for the parasites to develop narrow host infectivity and therefore may still be able to infect broad host ranges. Lajeunesse & Forbes 2002 showed that broad host range parasites are less likely to demonstrate local parasite adaptation and that this result may relate to the evolutionary time lags during broad host range coevolution shown with maladaptation. If the parasite was still able to infect a broad host range, this could explain why it was efficient at infecting allopatric hosts as well as sympatric hosts. Local maladaptation is not

itself evidence against coevolution. As seen in figure 7 and 8, coevolutionary process appears to show reciprocal change in SBW25 and phage interactions.

ANOSIM of community composition between communities in which local phage was cross infected with SBW25 evolved in a foreign community showed a large dissimilarity ($R>0.35$) between the local and foreign community comparisons. Community similarity between species was shown in community AE101 where no significant difference in phage fitness could be detected. ANOSIM revealed that both local and foreign communities in this instance were indistinguishable ($R=0$). My results indicate that local maladaptation did not occur because of community similarity in which phage and SBW25 grew as the species composition between communities the local adaptation experiment took place were distinct as determined by ANOSIM. This provides experimental evidence that communities can alter coevolution and that the composition of the community can vary the patterns of the coevolutionary paths. Furthermore, we also found that SBW25 was either local adapted or maladapted to its local or foreign community with no determined pattern (figure 10). TRFLP analysis showed that communities that were inoculated with phage and/or SBW25 did not alter community composition over the weeks and that the communities were dissimilar in species structure to enable analysis of the effects of bacterial communities on coevolutionary patterns.

Using a reciprocal transplant experimental design, fitness costs and adaptability of SBW25 in its sympatric and allopatric environments were assessed. Using paired t tests, the results show a higher fitness of SBW25 in their native environment in 4 out of 19 treatments with a significantly higher growth rate than SBW25 in a foreign environment ($P < 0.001$, figure 10, *) suggesting that

SBW25 are locally adapted to their local habitat and that this adaptation to their local environment might be at a cost of decreased fitness in another environment (Hereford, 2009). However, in other treatments, significantly higher growth ($P < 0.03$) of SBW25 was shown when placed in foreign environments in 6 out of 19 treatments (figure 10, +). This suggests that some SBW25 are maladapted to their environment. Almost half of all treatments showed high fitness of SBW25 in both sympatric and allopatric environments which implies that there is adaptation but without a fitness trade off.

How species interact and adapt to invasion of introduced species is important for understanding the dynamics of habitats and helps to predict the response to changes in natural environment (Lawrence et al., 2012).

TRFLP analysis of community composition from week 1 to week 8 (figure 11 and 12, respectively) show a slight decrease in R score ($P = 0.001$, $R = 0.333$ to $P = 0.001$, $R = 0.2601$). This suggests that species structure is converging and the communities are less distinct from one another. This can mean that species within microcosms are adapting to beech tea media and some species may not be able to tolerate laboratory conditions and have become extinct. They might also be affected from anthropogenic pressures from their surroundings, i.e, areas of high pollution from urban areas might affect the composition of species compared to undisturbed areas. Although many species will be known to inhabit these treeholes, many species present will present due to their surroundings causing differences in species between communities in which they are sampled from, shown in the results. As communities adapt and evolve in lab conditions over the eight weeks, this can cause rare or weaker species who are unable to tolerate the

new conditions to become extinct with similar species surviving between communities causing the decrease in dissimilarity between them.

The results also show that SBW25 (CBP) is not having an effect when compared with community alone (C) with no distinction between either detected ($P = 0.162$, $R = 0.005$ and $P = 0.1$, $R = 0.0224$, respectively). This shows that SBW25 abundance is non distinguishable from communities that do not contain SBW25. As SBW25 was originally isolated from a plant root, it might be adapted to coexist with some species present in the beech tree hole, however it has not been determined whether SBW25 occurs naturally in tree holes. This might create less competition between species as they are already adapted to specific niches in a semi-natural microcosm. This is also true for communities inoculated with SBW25 (CB) only compared with communities alone (C) (ANOSIM, $P = 0.004$, $R = 0.03$ and $P = 0.001$, $R = 0.1512$). The same reasons as above might be as to why no distinction between communities inoculated with SBW25 and not are not distinguishable from one another.

My results show evidence of interspecific microbial interactions affecting coevolutionary outcomes of bacteria and phage interactions; however, there are limitations to my experimental procedures and outcomes. Evidence for reciprocal coevolution might be absent due to methods used to measure host and phage infectivity. As the results rely on cell counts from flow cytometry, this might have picked up dead cells lysed by phage that may have been counted as a viable cell or noise created by particles within the media. This was accounted for by gating out larger particles and correcting the dilutions of bacteria so as there was a clear differentiation between bacteria cells and noise, however some noise of dead cells might have been counted. Numerous coevolutionary studies rely on

streak assays where a streak of bacteria is streaked across a plate and phage from past, contemporary or future populations are pipetted onto the streak. If a zone of clearing is visible, the phage is observed to have been infective (Buckling & Rainey 2002a; Budzik et al. 2004; Lopez-Pascua & Buckling 2008; Poullain et al. 2008). However, this method can be largely subjective due to faint clearing or methods in which the plaque assay was performed. Flow cytometry was used in this experiment due to the accuracy of bacterial cell counting when in the absence and presence of phage. As the results are not reliant on infective on non-infective, it would take a very high phage titre to lyse all the cells to create a significant decline in SBW25. If phage were not at a high enough titre to have a significant decline in SBW25, this may have caused some results to show as non-significant even though different coevolutionary trajectories can be observed.

Communities used in the microcosm study in which bacteria and phage were inoculated into were originally frozen after sampling from beech tree holes. This creates a somewhat unnatural or semi natural microcosm study in which to test coevolution within semi natural communities. To archive SBW25 and phage at -80°C to perform the coevolutionary studies, the phage were isolated from a subsample of the microcosm using chloroform and centrifuged. Chloroform that was used to isolate SBW25 phage from the communities might have damaged some of the phage making it unable to infect SBW25 in which it would be cross infected with and some chloroform might have remained within the supernatant after centrifugation. This can cause future issues and lysed bacteria if it was present during the cross infection studies, although every effort was made to prevent this from occurring as once centrifuged, a clear differentiation between the supernatant and chloroform was shown. When pipetting, the tip gently

aspirated the supernatant leaving a large gap at the chloroform-supernatant border therefore we were confident that no chloroform had entered the phage supernatant. To isolate SBW25, agar plates were used to pick isolates and grow them in broth culture for archiving. During this process, SBW25 genes that confer resistance or susceptibility to phage might have altered as it grew in media and competing for resources against other SBW25 cells. The alterations in the genotype might have caused it to become more or less resistant to phage infectivity and therefore affecting the experiments. To limit the effect this would have on the experiment, isolates were grown in microcosms containing a sufficient amount of media and grown until SBW25 were sufficiently dense to use in my experiments to isolates were not overgrown. Beech tea media was used as a laboratory substitute for water collected in beech tree holes. This does not mimic beech tree water entirely and it also semi-natural. The autoclaving process in which beech tea is made can also cause an increase in compounds such as tannins that may inhibit microbial growth, therefore affecting the microbes in a negative way which might also change interspecific interactions.

The study also focussed on studying single host-pathogen interactions in which only SBW25 and phage were analysed. This does not take into account the dozens of other interactions occurring within the microcosm and how SBW25 and phage affected the interactions of other species within the community. The SBW25 and phage were also removed from the microcosm in which they were grown in order to assess coevolutionary interactions. Assessing their coevolutionary interactions outside of the environment in which they coevolved might not give an accurate representation of their coevolutionary trajectories that occurred within the microcosm. This is also true for the local adaptation

experiments performed in order to assess how locally adapted each phage was to its local SBW25 had to be removed from the microcosm.

Although there were some limitations to the experiment, best efforts were made to keep the experiment as close to natural conditions as possible to infer coevolutionary patterns in natural communities.

## Conclusion

My results show that coevolutionary trajectories are influenced and affected by interspecific interactions within a natural community and these interactions can constrain or help promote coevolutionary paths between bacteria and phage. We found when a known bacteria-phage pair were inoculated into a natural community, phage infectivity and SBW25 resistance was affected by limited resource use and space by other competing bacteria within the community causing a large fitness cost to both phage and bacteria which restricted their ability to adapt and evolve resistance or infectivity alleles. It has been known that interactions of other species affect coevolutionary trajectories but most studies have focussed on single pairwise interactions in broth culture (Hall, Scanlan, Morgan, et al. 2011; Harrison, Laine, Hietala & Brockhurst 2013; Ashby & Buckling 2015) or interactions in the presence of one natural community (Gómez & Buckling 2011b). However, to date, no study has analysed coevolutionary interactions in the presence of multiple different communities. TRFLP analysis determined that community composition was not significantly altered when inoculated with SBW25 or phage suggesting that communities were stable and SBW25 did not have a significant benefit in competing with communities which allowed interaction amongst species. Local adaptation experiments showed

phage to be maladapted to their foreign host. This shows evidence of high costs associated with infection of hosts and host resistance.

# Chapter 4 - Coevolutionary interactions between SBW25 and phage in high and low diversity communities

## Abstract

Bacteria and phage are known to undergo rapid coevolution in laboratory conditions. However, little is known on how they coevolve with a diversity of natural communities. In Chapter 3 we showed that coevolutionary dynamics were influenced by the surrounding community. We build on this result here by looking at how community diversity influences coevolution.

Using communities from beech tree holes, *Pseudomonas fluorescens* SBW25 and its phage were coevolved in the presence of a diversity gradient of these natural communities. We predict coevolutionary trajectories (ARD or FSD) will be different depending on the community in which they coevolve. The results showed that in the presence of one community, densities of *P. fluorescens* increased and in the presence of another community, mean densities of *P. fluorescens* decreased

The results show that impact of phage on SBW25 can change depending on different diversities of the communities

## Introduction

Coevolution of bacteria and phage in the laboratory have been extensively studied (Hall, Scanlan, Morgan, et al. 2011; Lopez-Pascua & Buckling 2008). However, little is known about how bacteria and phage behave in a natural microbial environment as previously discussed. Similar habitats can contain different species compositions and abundances caused by biotic and abiotic interactions. This can lead to changes in species diversity within the community through positive and negative interactions between species that coexist and coevolve together.

If negative interactions between species persist, e.g. competition for resources, species present in a higher diversity community can reduce population sizes of certain species that cannot utilize resources as effectively. This makes it increasingly difficult for these species to increase their evolutionary potential as there is less genetic variation that might be transferred between these species via horizontal gene transfer. Higher species diversity can also cause some species to monopolise resources that can cause a decline in the abundance of some species within the community (Fiegna et al. 2014). However, the species that increase in abundance in higher diversity communities could have greater evolutionary potential due to positive interactions through generalist parasites or predators (Barraclough 2015). In the event of environmental perturbations, it is thought that a more diverse community will be able to exploit resources more effectively

as they are more likely to contain species that are tolerant to the changing conditions due to niche differences between species and are therefore able to maintain ecosystem functioning (Awasthi et al. 2014; Bell et al. 2005).

Community diversity often affects ecosystem functioning due to interactions between species and the presence of species that have specific capabilities and use certain resources such as competitors or producers (Bell et al. 2005). The impact of bacterial diversity on the ecosystem and other species within communities is highly dependent on abiotic conditions as well as the abundance and type of species present (Venail & Vives 2013). This suggests that higher diversity of species can lead to more stable populations.

It has been well documented that phage mediate populations of host bacterial species and can enter a coevolutionary arms race with their host for increasing resistance and infectivity. Numerous studies have shown arms race host-parasite dynamics in single monocultures in laboratory conditions (Brockhurst & Koskella 2013; Scanlan & Buckling 2012; Maclean et al. 2004; Paterson et al. 2010a). However, this might not reflect how they behave when in complex communities with varying degrees of species diversity as they will be affected by interactions from other species that cannot be studied in single host-parasite monocultures. Harcombe & Bull 2005 showed that in two-species bacterial communities, phage decrease the density of their target bacteria through reduced resistance capability of their host due to the cost of resistance in the presence of competitor bacteria. The Janzen-Connell hypothesis states that specific pathogens can target species of trees that keeps populations small and therefore this creates space for other tree species to grow within the same habitat. This might explain how pathogens mediate their host populations in the presence of microbial communities and

prevent the hosts from increasing to too high densities that prevents other species within the community from surviving. This negative frequency dependence created by predators or pathogens can maintain diversity and is known as "kill the winner" hypothesis (Jessup and Forde, 2008). However, as diversity of species increases, it is thought that phage populations might not be maintained due to the reduced host populations and increasing competition from other microbial species. If phage are too virulent, then they could kill off too much of the host populations.

The aim of this series of experiments was to understand how bacterial community dynamics affect a two-way interaction between *Pseudomonas fluorescens* SBW25 and its associated lytic phage SBW25φ2 in the presence of microbial communities of different diversities. Previous studies have shown that SBW25 phage, inoculated into a soil microbial community, reduced mean densities of SBW25. Conversely, while in the absence of a natural community, phage inoculation increased densities of the host SBW25. From this work it was apparent that SBW25 and φ2 follow different coevolutionary paths when placed with a natural community than in liquid broth with no community (Gómez & Buckling 2011a). Therefore, it is suggested that the presence of the natural community will reduce densities of SBW25 and its associated lytic phage SBW25φ2.

This interaction will be studied following the ecological and evolutionary dynamics of this two-way interaction which will be transferred into 2 separate bacterial communities isolated from beech tree holes (*Fagus sylvatica* in Silwood Park and Burnham Beeches) which are pools of rain water accumulated in a cavity of the beech tree. The aim of this experiment is to determine whether

communities of bacteria affect coevolutionary paths of SBW25 and phage when in different diversities (ranging from high to low). We know the diversity was high due to sequencing performed on the communities (data not included) and communities were diluted to near extinction. We predict that different diversities of species will affect coevolutionary trajectories of SBW25 and phage.

## Method

### Environmental sampling

Two beech tree bacterial communities were sampled from two different locations in beech tree holes (*Fagus sylvatica)*, Burham Beeches (51° 33' 34.20", -0° 38' 13.92) and Silwood Park (51° 24' 29.52", -0° 38' 42.72). Samples were collected using a 10 ml serological pipette to stir the beech tree hole and pipette up liquid and stored in a 50 ml polypropylene tube where it was transferred to a 4°C fridge. These samples are different to those used in the previous experiment.

### Microcosm inoculation

1 ml of each of the communities were inoculated into 9 ml of beech tea media (Lawrence et al. 2012) and placed on a shaker for 24 hours at 150 rpm. Gentamicin resistant SBW25 and its associate phage, SBW25φ2, were also inoculated into beech tea media and grown for 24 hours at 150rpm. Gentamicin resistant SBW25 was used as it could be plated onto agar containing gentamicin and survive whilst other bacteria from the communities would not, therefore isolating the SBW25 from the community. Phage was isolated from the beech tree hole communities using chloroform and using plaque assays, was spotted onto lawns of SBW25. It was established that no phage from the communities chosen could infect SBW25 by isolating phage from the community using

chloroform and spotting this onto a plate containing a lawn of SBW25. No zones of clearing on the lawn of SBW25 were observed.

100 µl of each community was pipetted into a 96 well plate containing 900 µl beech tea media. This was diluted two fold 6 times with beech tea media and inferred to create 6 diversity gradients with the rarest species being diluted out of the communities. To this, 10 µl of gentamicin-resistant SBW25 and SBW25 with SBW25φ2 was added and also diluted with the communities.

**Microcosm sampling**

Every week for 6 weeks, 50 µl of each microcosm was added to 50 µl of 0.2 µm filtered beech tea media ran on the flow cytometer (BD™ Accuri C6) for 30 seconds, fast fluidics. After cell counts were taken, each of the microcosms were transferred using a pin replicator calibrated to transfer 2 µl from the 96 well plate onto 200 µg/ml gentamicin plates to ensure the SBW25 was still present in the microcosms.

A further 50 µl of each microcosm from each community was plated neat onto 200 µg/ml gentamicin R2A plates, spread with 5 mm glass beads and incubated at room temperature for 5 days. After incubation, CFUs were recorded and picked using sterile toothpicks into sterile LB media, incubated for 48hours and stored at -80°C in 30% glycerol.

To isolate phage, 100 µl of the original microcosms were pipetted into separate 96 deep well plates with 800µl sterile beech tea. This was incubated at room temperature for 4 days and isolated by adding 100 µl chloroform to each well and

centrifuging at 3000 rpm for 8 minutes. The supernatant was pipetted into a 96 well plate and stored at -20°C.

Media in the original microcosms was refreshed using sterile beech tea and incubated at room temperature for another 7 days where the experiment was repeated for 6 further weeks.

Every week, 70 µl of the microcosms were stored with 30% glycerol at -80°C for use in the coevolutionary experiments.

**Cross infection coevolutionary experiments**

SBW25 CFUs collected from week 2 bacteria and phage treatments were collected from the microcosms stored at -80°C. Each SBW25 clone was grown in 1 ml LB overnight at room temperature. This was then transferred into a deep well plate where 8 replicates were set up and grown in 1 ml LB for a further 24 hours.

Phage from frozen stocks at -20°C and ancestral phage were obtained, grown in LB for 24 hours and isolated using 10% chloroform and centrifuged in deep wells at 2200rpm.

To each of the SBW25 clones, 10 µl of the corresponding phage from past (ancestral), contemporary (week 2) and future (week 6) were added. After 24 hours incubation at 22°C, a 2 µl subsample of the plate was added to 198 µl of LB and read in the flow cytometer for 30 seconds fast fluidics.

## Results

### Community dynamics

Due to extinction of SBW25 and phage isolates from the microcosms, the results for diversities 1, 4 and 5 were omitted from the experiment. The experiment could continue with specific isolates chosen from diversities 2, 3 and 6 across the weeks. These diversities represent the second most diluted diversity (diversity 2), third diluted (diversity 3) and the most dilute community (diversity 6). It is assumed that diversity 6 is this most diluted, however it is has not been tested whether diversity 6 contained less species than diversity 1 (least dilute).

Growth of bacterial community microcosms containing different treatments and microbial communities were monitored throughout the experiment. Log transformed cell count data from flow cytometry were read over 5 weeks for two communities (community 1, Burnham beeches (BB22) and community 2, Silwood Park (SP3)). Bacterial communities were maintained over the course of the experiment across all levels of diversity at approximately $10^5$ cells per ml. Figure 13 shows cell count data from flow cytometry measuring bacterial growth within different microcosm. Throughout all communities, cell count remained

relatively stable. However, a decrease in cell densities is shown in week 4 for all microcosms with the exception of community 2, SP3 diversity 6. To compare differences in growth between diversities a linear model was fitted to flow cytometry count data. One-way ANOVA showed no significant difference in community counts between diversities ($F_{2, 237} = 2.195$, $P = 0.114$, appendix table 16). This result shows that community growth within microcosms were relatively stable in cell count regardless of whether they were inoculated with SBW25 and phage.

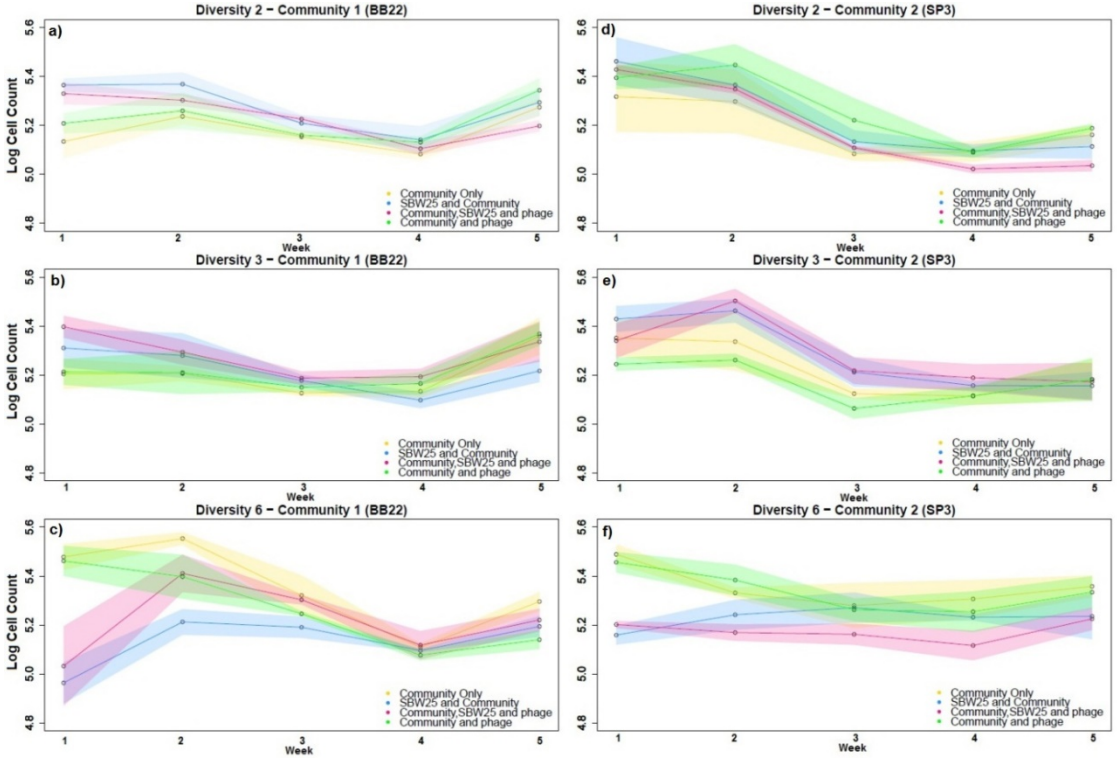


**Figure 15 Flow cytometry log cell counts measuring cell counts of different microcosms over 5 weeks. Coloured polygons show standard error around the mean with yellow lines showing community only, blue community with SBW25, pink polygons show community, SBW25 and phage and green represents community and phage. The graph shows flow cytometry counts of the microcosms over 5 weeks. Linear model ANOVA shows no significant difference between diversities (3 diversities) and treatments for flow cytometry count data over 5 weeks $F_{2, 237} = 2.195$, $P = 0.114$.**

The effect of each treatment on community growth for each diversity was measured using a linear model was fitted to flow cytometry cell count data. ANOVA and Tukeys Honest Significant Difference test were performed to identify significant differences between treatments. Community 1 (BB22), diversity 2 showed significant differences between treatments ($F_{3,76} = 2.801$, $P < 0.05$, table 17 appendix). *Post hoc* Tukeys analysis showed the significant differences to be between community and community inoculated with SBW25. A significantly higher cell count was recorded for communities inoculated with SBW25. No other significant differences between treatments were shown. No significant difference was shown between treatments for diversity 3, community 1 (figure 13, b) ($F_{3,76} = 1.473$, $P = 0.2$, table 18 appendix). This shows that despite different inoculations to the microcosms, cell densities remain relatively constant. Community 1, diversity 6 (figure 13, c) shows a significant difference between treatments ($F_{3,76} = 5.4$, $P = 0.001$, table 19 appendix). A *post hoc* Tukeys analysis shows the significant difference to be between community and community with SBW25.

Community 2 (SP3), diversities 2 and 3 both showed no significant difference between treatments ($F_{3,76} = 0.864$, $P = 0.46$ (table 20) and $F_{3,76} = 2.352$, $P = 0.078$ (table 21), respectively) showing cell densities remain stable over 5 weeks regardless of treatment. A significant difference between was shown in diversity 6 (figure 13, f) ($F_{3,76} = 10.65$, $P < 0.001$, table 22 appendix). *Post hoc* Tukeys test showed significant between community and community with SBW25, community and community with SBW25 and phage, community and phage with community and SBW25 and finally community and phage with community with SBW25 and phage.

## Coevolution

### Diversity

In order to assess SBW25 resistance when inoculated with past, contemporary and future phage within and between diversities as shown in figure 14. A GLM was used to determine whether a significant difference was shown. No significant different between past, contemporary and future phage was found for Silwood Park (SP3) community for diversity 2, 3 or 6 ($F_{2, 3}$ = 3.02, P = 0.191, $F_{2,12}$ = 0.054, P = 0.95, $F_{2, 3}$ = 0.16, P = 0.86, respectively). The same was true for Burnham Beeches (BB22) community for diversity 2, 3 or 6 ($F_{2,12}$ = 0.081, $F_{2, 12}$ = 0.22, P = 0.8 and $F_{2, 9}$ = 0.12, P = 0.89, respectively).

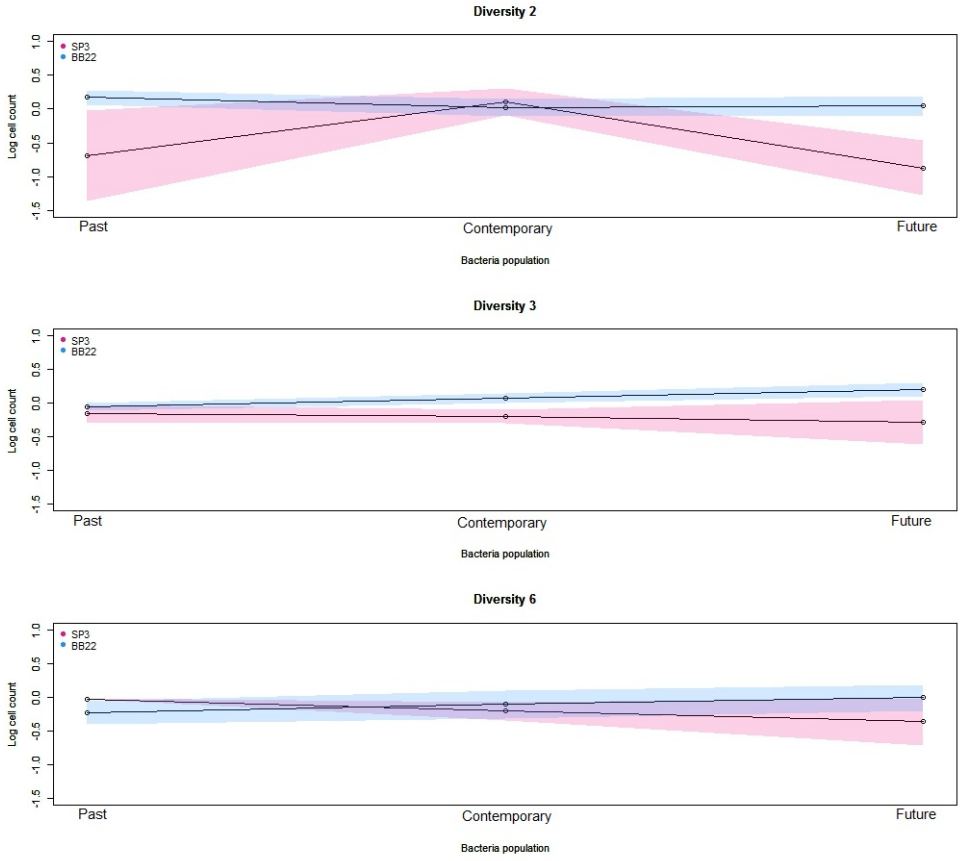


**Figure 16: Log10 relative frequency count data for two different communities for the different diversities.* Coloured polygons show SBW25 and phage standard error around the mean of Burnham Beech community (blue) and Silwood Park (pink). A GLM showed no significant difference between infectivity of past, contemporary and future phage on contemporary SBW25 (P<0.95). However, ANOVA fitted to a linear model shows a significant difference in SBW25 cell density in the presence of phage between diversity 2, 3 and 6 $F_{1,67} = 10.35$, *P=0.002*)**

A linear model was fitted to mean $log_{10}$ relative frequency flow cytometry count data to standardise the growth of each strain in absence of phage with standard error bars. $Log_{10}$ relative frequency counts were fitted as the response variable and treatment (past, contemporary and future) and community were fitted as the explanatory variables (figure 14). A one-way ANOVA revealed a significant difference between the two communities (Burnham beeches (BB22) and Silwood Park (SP3), $F_{1,67} = 10.35$, $P=0.002$) in the presence of phage. SBW25 and phage exposed to the microbial community isolated from Burnham Beeches revealed that in the presence of phage showed an increased in mean density of *P. fluorescens.* SBW25 and phage exposed to the microbial community isolated from Silwood Park (pink) reduced the mean density of *P. fluorescens*.

## Discussion

This chapter aimed to understand whether different diversities of two different communities had an effect on coevolution. From the results, we could not find any evidence of coevolution between SBW25 and phage. We did, however, establish that phage could significantly reduce SBW25 cell densities in the presence of one community, whilst in another community, SBW25 cell density increased.

It was found that in one community, mean densities of SBW25 increased when coevolved with phage in the presence of a community isolated from Burnham Beeches (BB22) and in the presence of a community isolated from Silwood Park (SP3), phage decreased mean densities of *P. fluorescens*. This suggests that phage can affect SBW25 in different ways and alter their abundances when competed together in a natural microbial community. However, coevolutionary interactions between SBW25 and phage was not observed in either of the communities or diversities.

Relative frequencies were used to standardise the growth of each strain by the growth in the absence of phage (control treatment). Data was log transformed to help understand how the growth was affected relative to what the growth would have been in the absence of phage. Figure 14 shows the log transformed relative frequencies for the flow cytometry counts in the presence of phage from the past, contemporary and future. From the graph we can see that community BB22 grew worse in the presence of phage as most values are <0 whereas SP3 appears to have grown better in the presence of phage >0. A one-way ANOVA was performed and revealed there was a significant difference between the

communities ($F_{1,94} = 11.08$, P=0.001). This result suggests that there is a significant overall impact of community. Gómez & Buckling 2011 observed a mean decrease in SBW25 in the presence of phage when placed into a single natural soil community. Here, as we have used two communities, shows that these results observed by Gomez & Buckling (2011), might not be generalizable. We have shown here that different communities might give different results as to how the phage impact SBW25 in the presence of a natural community.

No significant differences were observed when testing the infectivity of past, contemporary and future phage on ancestral SBW25. This result suggests that there is no measureable difference in phage infectivity and SBW25 resistance over the 6 weeks, showing that coevolution between the phage and bacteria was not shown. There are several reasons this might have occurred, the length of time (6 weeks) might not have been a long enough duration for an arms race to occur or phage might have become extinct throughout the experiment. As the communities in the microcosm were sampled every week and media was refreshed, this continuous removal of part of the microcosm community might have caused decay in the free-phage densities. As the rate of host cells (SBW25) lysed by the free phage is a function of free phage density, this causes a race between the rate at which the phage are removed from the system and the survival of phage sensitive bacteria (Calendar, 2006). If the rate at which the free phages are removed is higher than how fast they can infect their host, the phage will eventually become extinct.

Communities and treatments were monitored over the course of the experiment. Community 1, diversity 2 (figure 13, a) showed significant differences between community alone when compared with community with SBW25. This

significance might be caused by the addition of SBW25 to the community increasing the cell densities within the microcosm. Significantly higher cell density was shown in diversity 2 when SBW25 was inoculated into a community compared with community alone. As SBW25 is known to grow well in laboratory conditions, it might have introduced a bias and been better adapted to conditions within the microcosm than species in the community therefore, through ecological sorting, species within the natural community were unable to adapt as quickly as the SBW25 allowing it to grow to high densities, therefore giving a higher cell count when compared to community with SBW25. As the species within the community are pre-adapted to one another within the beech tree hole, this might have allowed the community to become more stable with community structure remaining relatively constant throughout the weeks.

Community 1 and diversity 6 (figure 13, c) showed significant difference between these two treatments. However, in diversity 6, microcosms containing the natural microbial community with SBW25 were significantly lower than microcosms containing only community. Diversity 6 was the most diluted community with rare species extinct. This might have allowed dominant remaining species within the community to increase in density and alter community structure. Within this diversity, community with SBW25 is lowest cell count when compared to other microcosms. The addition of SBW25 to species within this diversity might have allowed SBW25 to outcompete other species, resulting in an overall decline in abundance. However, at week 4, species abundances between communities converge to similar cell counts suggesting species adaptation to one another and microcosm conditions.

Community 2, diversity 6 (figure 13, f) also was significant between almost all treatments. As shown in (figure 13, c), microcosms appear less stable and competitive interactions are thought to occur as rarer species have been removed opening up previously unoccupied niches of other bacteria. Rare species are known to be important contributors to diversities between local habitats and across different habitats as many have important ecological roles and provide a pool of genetic and functional diversity (Lynch & Neufeld 2015).

Diversity was shown not have any significant impact on coevolutionary interactions. Competitors in ecological communities are shaped by mutual coevolution which allows species to coexist. Species that are not dependant on each other (SBW25 and bacteria isolated from beech tree holes) do not need to consistently coevolve together. Species that coexist in higher diversities are often associated with changes in species composition, this continually changing composition and high diversity further reduces the chances of consistent co-occurrence of a particular pair of species. Coevolution is most likely to occur in low species diversities where species composition changes at a lower rate (Connell, 1980). For phage and bacteria to coevolve, they need to be interacting over time. If they are in a diverse mixture of species, i.e. the phage is attaching to more hosts, the phage might have tried to infect other species and unable to track evolutionary changed in the host when compared to a situation where most of the cells the phage encounters are the host cells. This might be reason as to why SBW25 and phage became extinct. Even though SBW25 counts were high during the first few weeks in microcosms with low diversities, as beech tree species increased in population throughout the week, SBW25 cell counts declined rapidly when the beech tree hole species increased in density and outcompeted the

SBW25 and phage. It is also possible that even though diversity 6 had the lowest species composition, it is possible that this might have still been too high for coevolution to successfully occur between SBW25 and φ2.

Understanding how species co-exist and coevolve within different diversities is important as species diversity within communities is constantly changing. There is mounting evidence that phage contribute to increasing species diversity (Clokie et al. 2011) which is vital for ecosystem functioning as high species diversity utilises resources effectively during environmental perturbations and enhance community stability (Awasthi et al. 2014). It was thought that in high species diversity, coevolution between SBW25 and phage would be inhibited by strong inter-specific interactions by other competing species, whereas in low species diversity, the ability of phage and SBW25 to coevolve might have had a stronger if there was less interaction from other species. However, we did not find any evidence of coevolution regardless of species diversity. To create the diversity gradient used in the experiment, in the lower diversity of species (diversity 6), the rarer species were diluted out. This might have allowed the most dominant species in the community to become more dominant and more competitive thus inhibiting coevolution even though there were less species for SBW25 and phage to compete against.

Demonstrating coevolution empirically is notoriously difficult as is requires evidence of change (Brockhurst, Morgan, et al. 2007). *Pseudomonas fluorescens* SBW25 was originally isolated from a sugar beet leaf, Oxford in 1989 (Rainey and Travisano 1998) and genetically modified to be resistant to the antibiotic gentamicin. When this strain was introduced into a naturally occurring beech tree community, resources might be restricted by the competitors in the community

and SBW25 was potentially outcompeted by a community already adapted to the beech tea media as the isolated community was acclimatised to beech tea media for one week before the experiment commenced. This decline in SBW25 population (figure 13), might have reduced the rate of beneficial mutations leading to a reduced rate of adaptation. Pre- adapted species, the beech tree community, increase in abundance if variation in traits among species exceeds that within individual species, this increase in abundance is at the expense of less adapted species (SBW25). There might also have been trade-offs between the biotic and abiotic environment, including defences against phage which might have been costly allowing phage increase in abundance and causing a decline in SBW25. As SBW25 was not isolated from the beech tree and was instead introduced to the community, the natural community dynamics might have adapted to have positive interactions with one another, leading to increased productivity and population growth, as demonstrated by Lawrence et al. 2012. This might have led to competition in resource use between the naturally occurring communities and the introduced SBW25 and phage with the communities out competing SBW25.

The environment in beech tree holes differs drastically from the conditions in a laboratory as beech tree holes receive a wide variety of natural resources such as falling debris and are exposed to somewhat unpredictable biotic and abiotic factors, whereas laboratory conditions are uniform in terms of variation to the abiotic factors and to some extent the biotic factors. Understanding coevolution in different diversities of communities is important with the advent of climate change and the increasing threats from anthropogenic pressures, All aspects that affect species diversity (abundance, composition, interactions, spatial and

temporal variation) are expected to be impacted but effects remain unpredictable (Chapin et al. 2000).

**Conclusion**

Coevolutionary dynamics cannot be completely understood through a single interaction of bacteria and phage alone (Thrall & Burdon 2003). Therefore, following coevolutionary interactions in the presence of a natural community is important to help understand coevolution in nature. The importance of studying coevolution within naturally occurring communities in microcosms, such as soil or beech tree communities, is valuable as it reveals coevolutionary ecological constraints imposed by natural environments and might help guide studies of communities (Brockhurst & Koskella 2013). This importance is illustrated in the study by Gomez and Buckling, (2011) who were able to show coevolution of SBW25 and its phage followed fluctuating selection dynamics when placed with a naturally occurring soil community rather than arms race dynamics which has been demonstrated in liquid media and that phage reduced mean densities of SBW25 in the presence of phage and a natural community. However, in this experiment we showed that two communities from separate beech tree holes respond differently in the presence of phage. Community BB22 grew worse in the presence of phage and community SP3 grew better. This implies that although Gomez and Buckling, (2011), suggested that in a natural soil community, phage reduced mean densities of SBW25, this research shows that results depend on the nature of the background community.

# Chapter 5 - Coevolution in abiotic and biotic conditions

## Abstract

Understanding coevolution in abiotic conditions is vastly understudied. A wide range of abiotic and biotic environment pressures are constantly affecting the coevolution of bacteria and phage in the natural environment.

In this study, we paired *P. fluorescens* and phage (SBW25φ2) in the presence of two natural communities isolated from beech tree holes and with varying abiotic conditions (temperature, pH and nutrients). We also looked at the degree to which SBW25 was constrained by the community to adapt to the abiotic conditions.

The results show highly variable levels of phage infection to SBW25 with different abiotic and biotic pressures and that communities did constrain SBW25 adaptation to abiotic conditions. These results highlight that in this instance coevolution between SBW25 and phage in natural environments cannot be predicted and are dependent on the surrounding environment and species present in the communities.

## Introduction

Microbial communities are under constant abiotic and biotic selective pressures. These pressures can have positive or negative effects and can cause considerable impact on the composition, diversity and structuring of a microbial community (Singh et al., 2009). Beech tree holes contain a huge diversity of beneficial heterotrophic organisms that interact over many generations (Foster & Bell, 2012). A large proportion of the beech tree hole microbial communities are made up from decomposers where leaf litter is degraded and this then provides a source of carbon for growth. Although tree holes can contain similar species due to similar environmental conditions, different populations can have considerably different gene content. . Adaptation to specific niches within the tree hole is due to microbial strains and species containing unique genes in microbial populations and individual species that experience similar abiotic and biotic selection that pressures experience high levels of variation between genes. These pressures can cause species to diversify and can reflect adaptation by populations in certain microenvironments (Coleman & Chisholm, 2010).

Different abiotic conditions can cause stress within a community and reduce population sizes and interactions between species through lowered encounter rates and increased competition due to finite resources such as nutrients. When

encounter rates change, adaptation rates and selection within the community can change coevolutionary trajectories (Hiltunen et al., 2015).

As we saw in chapter 3, community composition can impact coevolutionary trajectories. If biotic factors can impact coevolution, then abiotic factors can also alter coevolution directly through alteration of species interactions and imposing costs as abiotic conditions can directly cause ecological sorting within a community.

The ability of a species to adapt to abiotic conditions can be affected by co-occurring species present within the community. These interactions can result in spatial variation between two distinct populations of the same species which impacts species interactions and creates a geographic selection mosaic (Thompson, 2005). If a species is already adapted to change in the environment, e.g, increasing temperature, this species can quickly multiply whereas species that are adapted to growing in a lower temperature can become outcompeted and go extinct regardless of whether they might have been able to adapt to the rising temperature when in isolation or a less competitive community. This competition by pre-adapted species reduces the likelihood of their survival (Barraclough, 2015). Higher nutrient concentrations can make it possible for a species to invest in more defence alleles against competitive or predatory species and can also result in higher growth rates for the species and alter the course of coevolution (Piculell et al., 2008).

The effects of competition from a microbial community on a species introduced into that community can constrain the adaptation to the abiotic conditions in which it is introduced. For example, costs might include investments in defence

alleles to resist phage that might make it more difficult for a species to adapt to unfavourable temperatures (Barraclough 2015). It is important to understand the consequences of extinction or introduction of a species into different ecosystems and the effect that has on ecosystem functioning. If there is a change in the environment, for example, increasing temperature caused by climate change, this could affect a particular species within that community and therefore have a knock-on effect on other species and indirectly affect the composition of the community and its ability to respond to changing conditions (Northfield & Ives 2013). It is important that a species can adapt locally to the changing environment. Gene flow can help a species to adapt rapidly or constrain its adaptation dependent on the community in which it is introduced due to competition for limited resources (Norberg et al. 2012). As well as adaptation to an environment, dispersal also can play an important role in allowing an organism to track environmental conditions and disperse to areas that have favourable conditions. It can allow populations to persist in the event of a changing environment (Lavergne et al. 2010).

Nutrient concentration has been shown to affect host-parasite interactions as productivity can drive bacterial community diversity and composition as resources are more widely available. Bell et al., (2010) showed that nutrients can alter predator - prey (protist - bacteria) dynamics when both are competed against one another in a nutrient gradient. It is well documented that when resource availability increases, bacteria can rapidly multiply and grow and if there are more bacterial cells available, predation is also thought to increase as predators such as protists or pathogens (phage) will ultimately have more resources in which to consume or infect.

pH is also known to strongly influence bacterial composition and growth with some species thriving in acidic or alkaline conditions. Adaptation of bacteria to pH can directly influence growth which can then have a direct effect on phage and alter coevolutionary paths. pH is also an important factor influencing phage stability in the environment as phage aggregation is influenced by hydrogen ion concentration which might influence their ability to infect bacteria (Langlet et al., 2007).

Temperature also strongly influences bacteria and phage growth. Under the correct conditions, bacteria can thrive and multiply rapidly, therefore allowing phage to also grow and multiply rapidly. However, both bacteria and phage are sensitive to temperature fluctuations and species dependant and can tolerate a narrow range of temperatures. Zhang & Buckling, (2011) showed that when SBW25 and phage were subjected to gradually increasing temperatures, the phage became rapidly extinct due to fitness costs associated with host infectivity but SBW25 were able to continue to grow.

There is good evidence that these abiotic factors (nutrient, temperature and pH) are significant in determining community composition in natural environments whether they affect bacteria and phage interactions directly or indirectly.

Coevolution between phage and bacteria is known to be affected by abiotic and biotic conditions and can be constrained if conditions are unfavourable. This can include competition between species which can result in nutrient depletion, lack of space and evolving resistance or infectivity alleles that are costly and can reduce the ability for the bacteria to evolve other traits. Although bacteria-phage dynamics have been extensively studied without consideration of abiotic and

biotic environments, this hascontributed massively to our understanding of host-phage interactions, it does not further our understanding of how relevant these interactions are within the wider microbial community and fluctuating environmental conditions or if the same coevolutionary paths are observed in nature as they are in the laboratory. Studying evolutionary interactions in natural environments is a major challenge due to massive diversity and fluctuating factors that can affect coevolutionary trajectories (Barraclough 2015).

Here I focus on observing host-phage interactions in the presence of different environmental conditions (pH, nutrient concentration and temperature). Two beech tree hole microbial communities used in this experiment were chosen on the basis of the results from previous experiments (chapter 3) where both communities showed strong coevolutionary patterns when coevolved with phage (1 community showing ARD and the other showing FSD).

Different nutrient concentrations of media were used in this experiment by preparing standard beech tree tea (Lawrence et al., 2012) and using half and double strength to mimic high and low nutrient availability.

Temperature was also chosen as a significant abiotic factor that could affect host-phage interactions. Three different temperatures were chosen (15°C, 22°C and 30°C) to coevolve bacteria and phage based on temperatures that might be experienced in the natural environment where the beech tree communities were isolated from (southern England).

Coevolving SBW25 and phage in different pH was also measured. Beech tea media was pH adjusted to three pH conditions that were known to be experienced

in different tree holes by previous measurements (T. Bell, personal communication).

To help understand whether community composition constrains adaptation of SBW25 to different abiotic conditions, SBW25 cell counts by flow cytometry were used to compare growth in the presence and absence of the community.

I follow the growth of the bacterial communities in the presence of the various environmental conditions and interactions of SBW25 and phage through cross infection evolutionary experiments throughout eight weeks using flow cytometry.

## Method

### Microcosm preparation

From previous coevolutionary experiments, two communities (WYD09 and WYT116) were chosen that demonstrated strong different coevolutionary trajectories (FSD and ARD, figure 7 and 8).

Beech tea media was prepared according to Lawrence et al., 2012. The media was used to simulate three different abiotic environmental conditions (nutrienavailability, pH and temperature). Incubators were set to 15°C, 22°C and 30°C to mimic realistic temperatures found in the environment throughout the seasons. Double and half strength beech tea was prepared to imitate different nutrient availability and the pH of the beech tea media was also altered to reflect the extreme and average conditions found in beech tree holes.

Beech tea media was shown to have an initial pH of 7.4 and was buffered to pH 8, 6.5 and 5 using 50mM phosphate buffer (0.5g/l, 5g/l and 7g/l monosodium

phosphate and 12.5g/l, 4g/l and 1.8g/l disodium phosphate, respectively) to adjust the beech tea media to the required pH.

Two hundred and eighty-eight 50 ml polypropylene tubes were each filled with 25 ml of the adjusted beech tea media consisting of different treatments and factors, shown in table 1

**Table 2: Shows the treatments with factors. All factors had three levels, high, medium and low nutrient, temperature and pH. Treatments and factors were replicated four times and two communities were used throughout the experiment. Controls looked at community alone (no SBW25 and phage), community with SBW25 only and a negative control of media**

| Treatment / Factor | Community | SBW25 | Phage |
|---|---|---|---|
| Nutrient | ✓ | ✓ | ✓ |
| Nutrient | ✓ | ✓ | |
| Nutrient | ✓ | | |
| Nutrient | | ✓ | ✓ |
| pH | ✓ | ✓ | ✓ |
| pH | ✓ | ✓ | |
| pH | ✓ | | |
| pH | | ✓ | ✓ |
| Temperature | ✓ | ✓ | ✓ |
| Temperature | ✓ | ✓ | |
| Temperature | ✓ | | |
| Temperature | | ✓ | ✓ |

Frozen (-80°C) beech tree communities were grown in beech tea media 24 hours before use in the experiments to help revive and adjust them to laboratory conditions. SBW25 stock was also grown in each of the adjusted beech tea media

(pH, temperature and nutrients) and were used to inoculate each of the microcosms.

250µl of beech tree communities was pipetted into the 50 ml polypropylene tubes containing the beech tea media. With this, 250 µl of gentamicin resistant SBW25 and 100µl of SBW25 phage were also pipetted into the appropriate beech tea microcosm. Isolates were allowed to grow for 1 week before sampling began.

Every week for 8 weeks 1 ml of the microcosm was removed. 20 µl of this was added to 180 µl of 0.2 µm filtered beech tea and placed in the flow cytometer (BD CSampler, fast fluidics, for 10 seconds). 700 µl of the microcosm was added to a deep well and 300µl of neat glycerol was added and stored at -80°C. A further 900µl of the microcosms containing phage was placed in a 1.5 ml microcentrifuge tube with 100µl of chloroform. The tubes were vortexed, and centrifuged at 13000rpm for 3 minutes. The supernatant was gently aspirated off and stored in a 96 deep well.

Microcosms were sampled for 8 weeks. Every week the media was refreshed with 1ml beech tea media (in accordance to their set environmental conditions). To ensure SBW25 and phage were still present in the microcosms, SBW25 was pin replicated (calibrated to 2 µl) onto 100µg/ml gentamicin plates and phage isolates were also pin replicated onto a soft agar plate containing ancestral SBW25 to check for phage plaques.

**Coevolution**

Week 1, 4 and 8 microcosms stored throughout the experiment at -80°C were plated at a 10-fold dilution onto 100µg/ml gentamicin LB agar plates. After 48

hours' growth at 22°C, six SBW25 clones were picked and grown in a 96 deep well plate in 700µl LB broth for 24 hours at 22°C. 300µl glycerol was added and the clones were stored at -80°C.

Week 4 SBW25 clones isolated from microcosms containing communities WYD09 and WYT116 were defrosted in the fridge overnight along with corresponding phage isolated from the same microcosms from weeks 1, 4 and 8. 180µl LB was pipetted into 96 well plates with 10µl of SBW25 from week 4 and 10µl of the corresponding phage from past (week 1), contemporary (week 4) and future (week 8) were added. Plates were grown for 24 hours at 22°C. After 24 hours, a 5µl subsample of the plates (2 technical replicates) was added to 195µl of 0.2µm filtered beech tea and ran on the flow cytometer (BD CSampler, fast fluidics, 10 seconds).

The coevolution experiment was repeated using contemporary (week 4) phage and SBW25 clones isolated from week 1, 4 and 8.

SBW25 growth in the absence of phage were also analysed on the flow cytometer to allow comparison of SBW25 before and after phage inoculation for week 1, 4 and 8.

**SBW25 fitness before and after experiment**

Before the experiment began, 10µl of gentamicin resistant SBW25 stock solution was inoculated into each of the altered beech tea microcosms (nutrient, pH, temperature) which was then used to inoculated the microcosms used in the experiments (as mentioned above).

A subsample was removed from the SBW25 adjusted beech tea media and a 10-fold dilution onto 100µg/ml gentamicin LB agar plates. After 48 hours' growth at 22°C, six SBW25 clones were picked and grown in a 96 deep well plate in 700µl LB broth for 24 hours at 22°C. After 24 hours, a 5µl subsample of the 96 well plates was added to 195µl of 0.2µm filtered beech tea and ran on the flow cytometer (BD CSampler, fast fluidics, 10 seconds). The six clones were replicated 3 times and 4 technical replicates were performed.

This enabled us to understand SBW25 fitness to the adjusted beech tea media before and after inoculation with communities and phage.

**Community constraint to adaptation**

To understand how adaptation of SBW25 to the abiotic conditions was constrained by the presence of the community, ancestral SBW25 counts were inoculated into adjusted beech tea media and grown for 24 hours. After eight weeks, SBW25 counts in the presence and absence of community were analysed on the flow cytometer. Coevolved counts were divided by ancestral cell counts to provide a ratio of growth. This enabled us to determine if communities constrain adaptation to abiotic conditions.

**Degree of community constraint**

To quantify the harshness of the environment of each abiotic treatment, the mean of the ancestral SBW25 growth was used to order the 3 treatments levels (eg high, standard and low nutrient) to show what treatment SBW25 grew best in.

The degree to which community constrains adaptation of SBW25 to each abiotic condition was calculated as the fitness of the evolved, divided by the fitness of the ancestor. Fitness without community was subtracted from fitness with community to give the difference 'community constraint'. A positive value indicates adaptation was better without the community. Fitness is defined as SBW25 growth.

**Statistical analysis**

Flow cytometry cell counts were log transformed to normalise the data.

I used a linear model fitted cell to count data to order to analyse the change in cell density when SBW25 was infected with phage from past, contemporary or future populations. To analyse SBW25 and phage coevolution, a ratio of flow cytometry cell counts of SBW25 counts with and without phage from week 1, 4 and 8 was analysed. Analysis of variance (ANOVA) and Tukeys Honest Significant Difference test were used to compare different abiotic and biotic treatments within the microcosm.

Paired T Test was used to compare SBW25 fitness from 6 clones before and after inoculation with phage and the communities in the adjusted beech tea media.

To test adaptation of SBW25 in the presence and absence of communities in abiotic conditions, cell counts from flow cytometry from ancestral and coevolved isolates were divided from one another. This was then averaged over 24 replicates for each treatment and level and a barchart was made with standard error of the mean. A generalised linear model (GLM) was fitted to an ANOVA to

determine the differences in fitness of SBW25 in the presence and absence of each community.

To measure the degree the communities constrained adaptation of SBW25, ancestral and coevolved SBW25 cell counts were averaged over the 24 replicates and coevolved SBW25 was divided by ancestral SBW25 counts for no community, community WYD09 and community WYT116. These counts for SBW25 without community were then subtracted from SBW25 counts from community WYD09 and the same for no community subtracted by community WYT116 to test the degree of community constraint in the presence of a community. If the results were positive, then this showed that SBW25 grew better in the absence of a community.

## Results

### Community dynamics

### Community 1- WYD09

The effect of the treatments on community composition for both communities (WYD09 and WYT116) were monitored by flow cytometry (log cell count) over the 8 weeks of the experiment. A one-way ANOVA was performed to analyse the differences between the cell count data between the different treatments. Figure 15 shows logged cell count flow cytometry data for community 1 (WYD09).

A significant difference ($F_{3,284}$ = 3.7, P<0.05) was shown between microcosms that contained SBW25 (BP, CB and CBP) and community (C) that did not have SBW25.

No significant difference ($F_{2,285}$ = 1.148, P > 0.05) was shown between cell counts between different factors (nutrient, pH and temperature) indicating that bacteria in the microcosms had similar growth. However, a significant difference ($F_{2,93}$ = 3.135, P < 0.05) was shown between cell counts recorded in low nutrient and high nutrient. No significant difference ($F_{2,93}$ = 0.6, P > 0.5) was shown between cell counts in different temperatures indicating temperatures did not have a significant effect in bacterial growth. Differing pH also did not significantly ($F_{2,93}$ = 1.4, P > 0.2) affect bacterial growth in microcosms.

Cell counts over the weeks were highly significant ($F_{7,280}$ = 139.5, P < 0.05), with the exception of week 1 and week 5, week 2 and week 4. Figure 15 shows a rapid decline in growth in all microcosms at week 2 and 3 but recovery in all microcosms is shown in week 4 with higher cell growths, similar to those shown in week 1 and increasing to week 5 where another decline around week 6 is shown and increasing again at week 8.

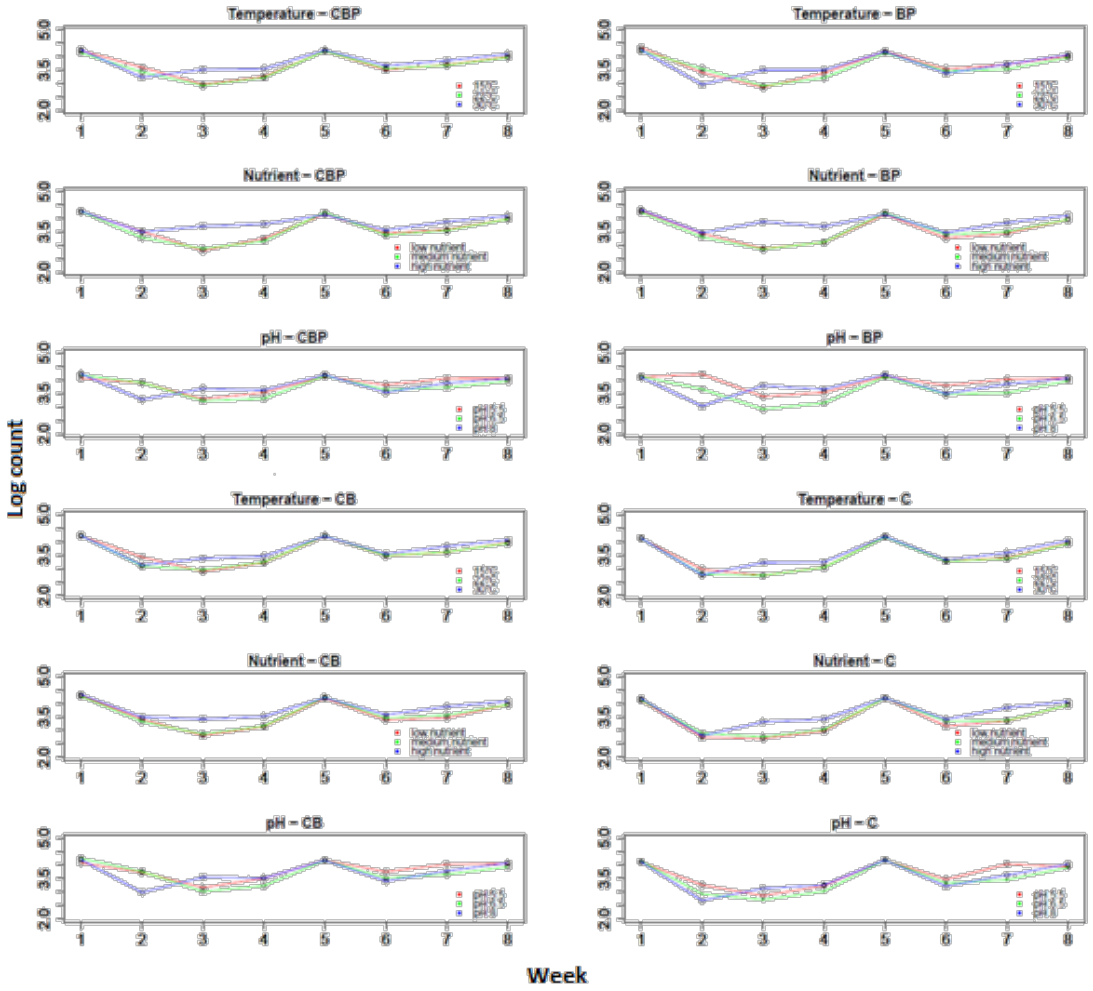


**Figure 17: Log transformed cell count data for community WYD09 from flow cytometry was measured every week for eight weeks to monitor cell density in each of the microcosms containing different treatments and inoculations (CBP – community, SBW25 and phage, BP – SBW25 and phage, CB – community and SBW25 and C – community only).**

The effect of treatment on community 2 (WYT116) was also monitored over the 8 weeks of the experiments, the results are shown in figure 16.

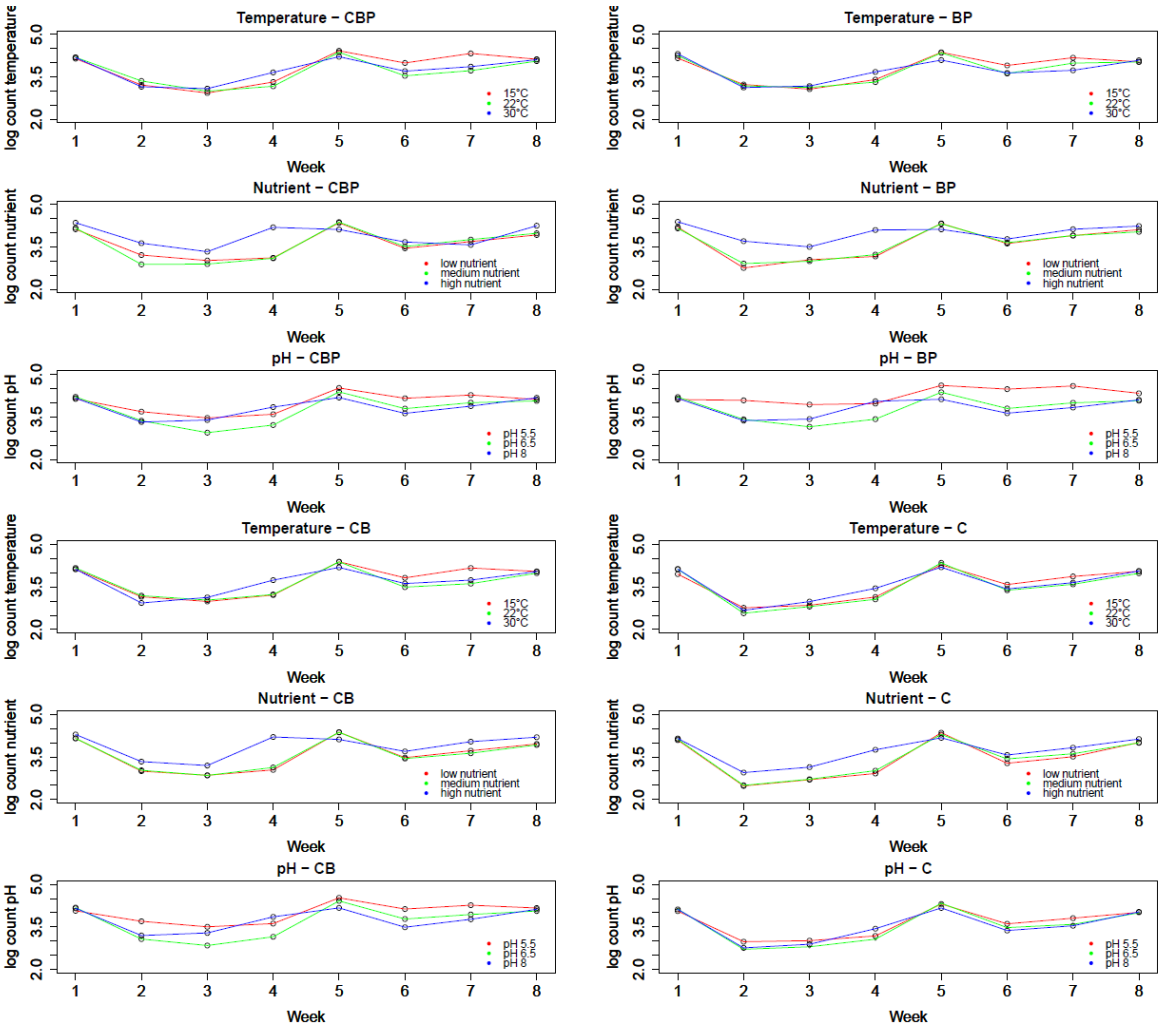


**Figure 18: Log transformed cell count data for community WYT116 from flow cytometry was measured every week for eight weeks to monitor cell density in each of the microcosms containing different treatments and inoculations (CBP – community, SBW25 and phage, BP – SBW25 and phage, CB – community and SBW25 and C – community only).**

A one-way ANOVA showed that there was a significant difference ($F_{3, 284} = 4.48$ $P < 0.004$) between microcosms that contained different microbial compositions (i.e those that contained SBW25 and phage and those that did not). *Post hoc*

Tukeys test revealed that the significant difference occurs between community only (C) with microcosms that have SBW25 and phage (CBP and BP). The microcosms containing SBW25 were shown to have on average, a higher cell count (figure 16). SBW25 is a strain that grows well in the lab, whereas the natural beech tree communities may have required more time to adapt to the media and laboratory conditions, allowing the SBW25 to grow faster and show higher cell densities than when the community is placed in beech tea media alone.

Different abiotic factors were not found to have a significant effect ($F_{2,285}$ = 2.036, P > 0.132) on growth of the microbes in the microcosms with cell counts remaining similar regardless of the abiotic pressure. However, different levels (eg high, medium and low temperatures) of abiotic factors were significant. *Post hoc* Tukeys showed a significant difference (P < 0.03) between half strength nutrient level and full strength nutrient, with an increase in growth of bacteria in microcosms in double strength media (figure 16). Although decreasing trends can be seen in week 2 and 3, exponential and stationary phase are achieved after week 4 where bacteria have been able to utilise the new media.

A significant difference ($F_{2,93}$ = 3.5, P = 0.04) was also shown between pH 5.5 and pH 6.5 with pH 5.5 showing a higher growth rate for bacteria in the microcosms. pH 5.5 – 8 were chosen as these represent the extreme pH's found in beech tree holes in the environment.

No significant difference in growth is shown in temperature controlled microcosms ($F_{2,93}$ = 0.279, P > 0.75) with growth rates remaining constant regardless of temperature. However, figures 15 and 16 show a slightly higher cell count for microcosms at 30°C. The results show little difference in growth rate

at the different temperatures (15 - 30°C), suggesting that the communities are able to adapt well to lower or higher temperatures without much effect on the cell integrity and functions. Cell counts over the length of the experiment also had a highly significant ($F_{7,280} = 127.5$, $P < 0.0001$) effect on bacteria growth over the eight weeks. *Post hoc* Tukeys showed high significance between all weeks with the exception of week 1 with weeks 7 and 8. Figure 17 shows cell counts decreasing over the weeks with cell counts in week 7 and 8 returning to similar counts seen in week 1. No significant difference was also shown between weeks 2 and 3 which showed lower but similar cell counts.

**Coevolution - SBW25 fitness across treatments**

ANOVA revealed a highly significant difference ($F_{2,645} = 38.98$, $P < 0.001$) between bacterial growth in the presence of phage in different abiotic factors (nutrient, temperature and pH) in community WYD09 (figure 17). *Post hoc* Tukeys analysis showed differences between microcosms affected by fluctuating temperatures with microcosms containing different nutrient concentrations ($P<0.001$) and temperature with pH ($P<0.001$). No significant difference was shown between pH and nutrient ($P = 0.143$).

Analysis of WYD09 of each of the abiotic factors showed no significant difference for any of the environmental perturbations ($F_{2,69,}$ $P > 0.4$). (appendix 1)

ANOVA was also used to analyse the patterns of coevolution when SBW25 and phage were coevolved in the presence of the varying environmental conditions and communities isolated from Beech tree hole WYT116 (blue). A significant difference was shown on SBW25 cell density in the presence of phage in each of

the different abiotic factors ($F_{2,645} = 79.07$, $P = 0.0001$). Tukeys revealed these differences to be between temperature and nutrients ($P < 0.001$) and temperature and pH ($P < 0.001$). However, no significance was shown for pH and nutrient ($P = 0.8$).

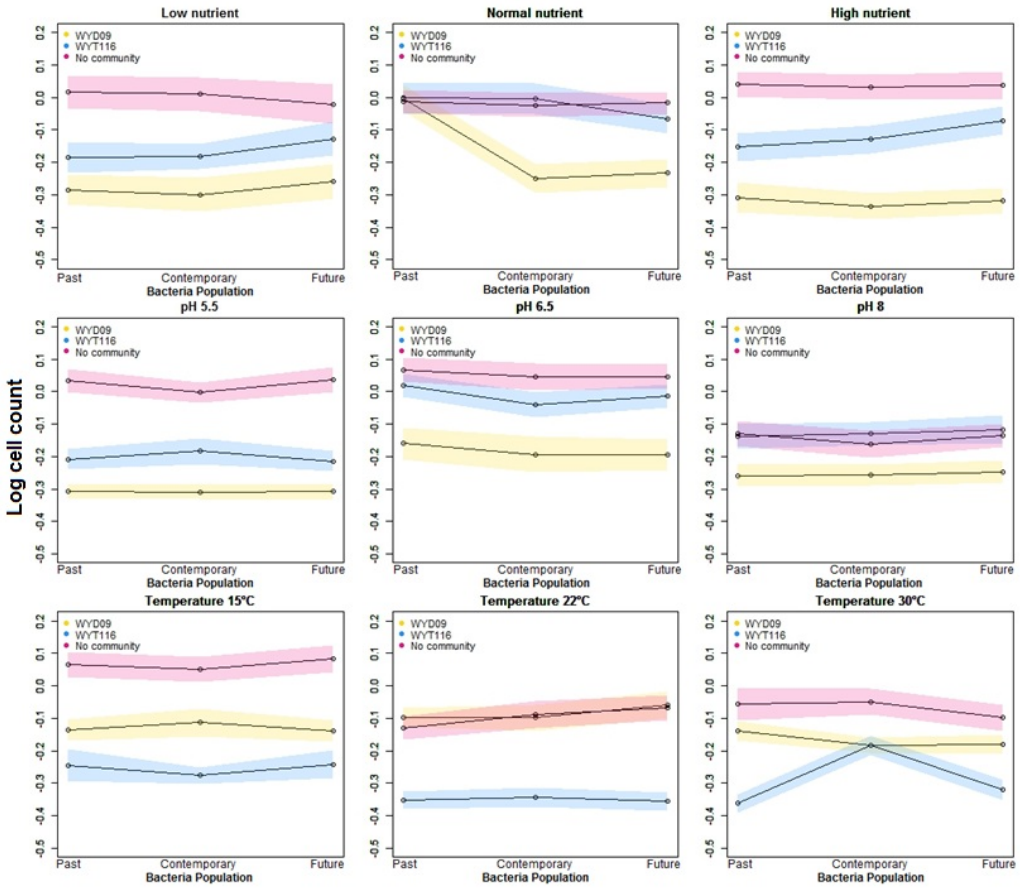


**Figure 19: Mean log cell count of SBW25 with past, contemporary and future phage showing coevolutionary dynamics in communities WYD09, WYT116 and no community. No significant difference was shown for coevolutionary patterns in either community or no community (P>0.05).**

**Coevolution**

The effects of different abiotic and biotic factors on the coevolution of SBW25 with past, contemporary and future phage (isolated from week 1, 4 and 8) were analysed over an eight-week period.

Each factor was individually analysed to measure whether phage significantly reduced SBW25 cell counts. A ratio cell count was calculated by measuring SBW25 in the presence of past, contemporary and future phage and SBW25 in the absence of phage.

One-way ANOVA found that there was no significant infectivity of SBW25 by past, contemporary and future phage ($F_{2,69,}$ $P > 0.4$), therefore no coevolution was observed.

SBW25 and phage coevolution was monitored when grown in the absence of community but in different abiotic factors (pink). No significant difference was shown ($F_{2, 645} = 2.085$, $P = 0.125$) in SBW25 cell growth between any abiotic factor.

**Coevolution of phage with past, contemporary and future SBW25**

Coevolution of bacteria and phage was monitored when coevolved in the presence of community WYD09, WYT116 and no community (figure 18) and different abiotic factors. Highly significant results were shown when comparing SBW25 cell growth in different abiotic factors ($F_{2, 645} = 68.49$, $P = 0.0001$). Post hoc Tukeys analysis showed that all abiotic factors were significant when compared to each other ($P < 0.0001$).

Analysing phage infection to SBW25 showed that in community WYD09 (yellow bar, figure 18) low nutrient (a), pH 5.5 (d), 22°C temperature (h) and 30°C temperature (i) all showed significant differences in SBW25 growth ($F_{2,69}$ = 5.771, $P < 0.005$, $F_{2,69}$ = 8.526, $P < 0.0001$, $F_{2,69}$ = 3.24, $P < 0.05$ and $F_{2,69}$ = 7.81, $P < 0.001$, respectively)

Similar results were also shown when SBW25 and phage coevolution was evolved in the presence of community WYT116 (blue) ($F_{2,645}$ = 51.76, $P < 0.0001$). Tukeys shows significant differences between all factors (P = 0.0001) with the exception of pH and nutrient (P = 0.17). Measuring SBW25 cell densities in the presence of phage showed no significant differences when phage was infected with past, contemporary and future SBW25 ($F_{2,69,}$ $P > 0.05$)

Coevolution of phage with past, contemporary and future SBW25 in the absence of a natural microbial community (pink) was measured and significant difference was shown when comparing factors ($F_{2,645}$ = 4.908, P = 0.007). A Tukeys analysis found that significance was only between pH and nutrient (P = 0.005) with temperature not showing significant interactions with nutrients or pH ($P > 0.1$ and $P > 0.3$, respectively). Analysis of past, contemporary and future SBW25 on phage populations in each of the microcosms was performed. No significant difference in cell counts of SBW25 was shown ($P > 0.07$).

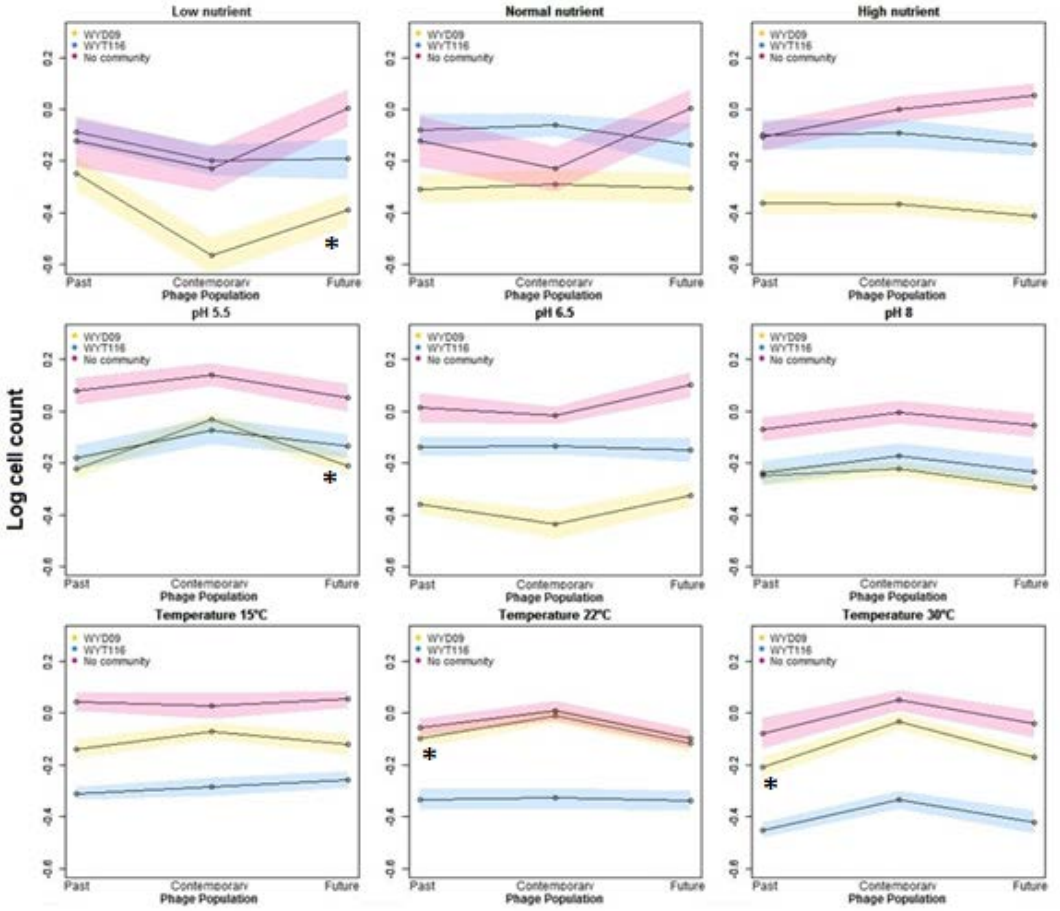


**Figure 20: Mean log cell count of phage with past, contemporary and future SBW25 showing coevolutionary dynamics in 2 communities (yellow and blue) and no community (pink). Coevolutionary patterns were shown in community WYD09 low nutrient, low pH, mid and high temperatures (P <0.05), as shown by *. No significant coevolutionary patterns were shown for community WYT116 (blue) or in no community (pink).**

**SBW25 fitness before and after experiment**

To test the effects of abiotic (pH, temperature and nutrient concentrations) and biotic (community and phage) stresses on SBW25, SBW25 (ancestral SBW25 clones) was analysed in the adjusted beech tea media before inoculation into the microcosms and after (week 8 SBW25 clones), the results are shown in figure 19. A decrease in SBW25 cell counts shows that SBW25 fitness has declined in that it cannot grow as efficiently in the adjusted beech tea media after exposure to abiotic and biotic conditions.

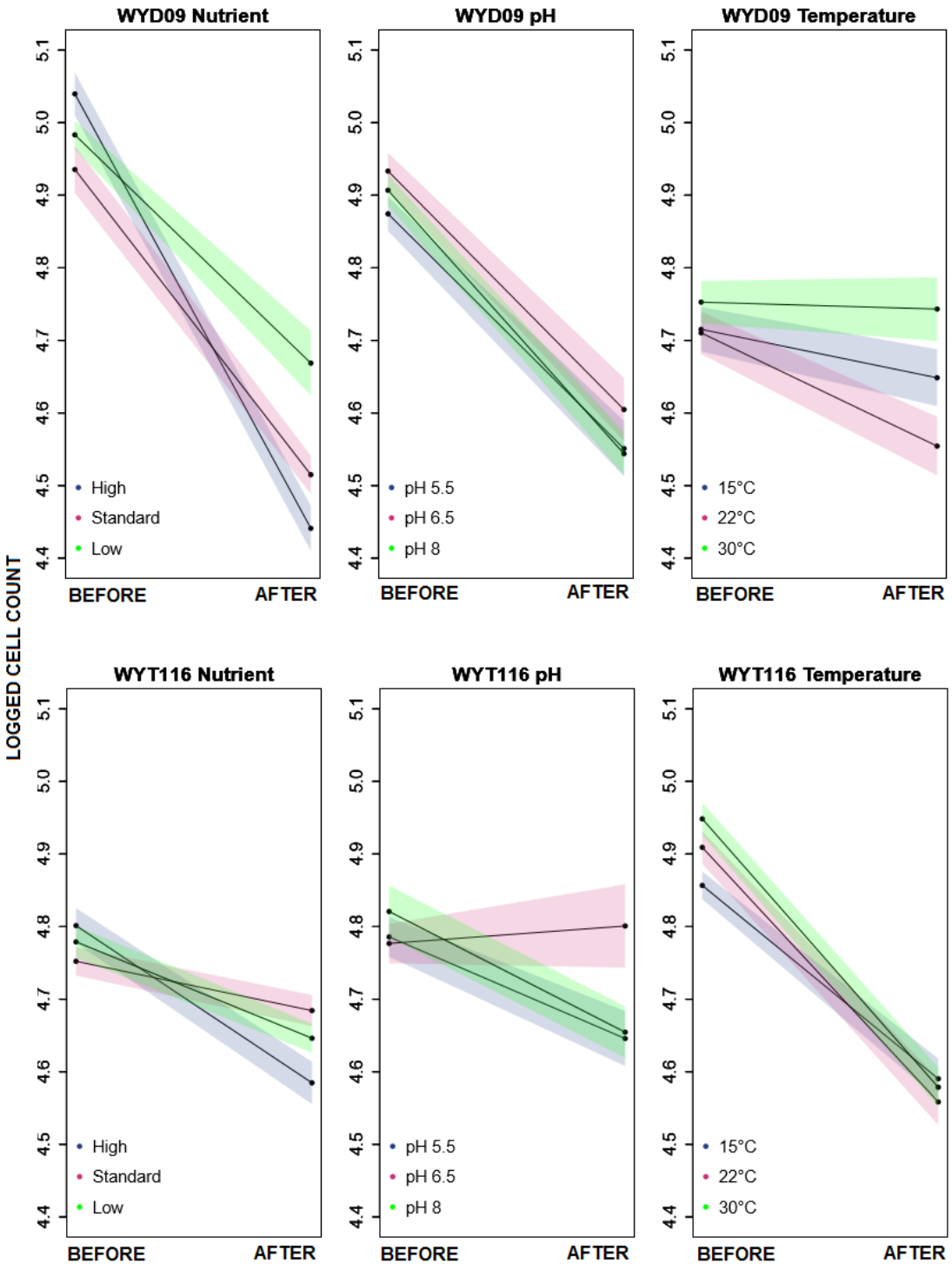


**Figure 21: SBW25 clones (six clones, 3 replicate clones) were analysed in adjusted beech tea media before (ancestral SBW25) and after (week 8 SBW25) exposure to abiotic (communities and phage) and abiotic (nutrients, pH and temperatures). Paired T tests were used to compare before and after SBW25 fitness according to cell count.**

Paired T tests were used to compare SBW25 fitness before and after treatments within the microcosms. The results show that most SBW25 clones had significantly higher cell counts (therefore higher fitness) before inoculation in the microcosms. The t-test shows significant differences before and after treatment for community WYD09 with high, standard and low nutrients ($T_{23}$ = 12.8, $P < 0.001$, $T_{23}$ = 13.1, $P < 0.001$, $T_{23}$ = 6.9, $P < 0.001$), pH 5.5, 6.5 and 8 ($T_{23}$ = 10.4, $P < 0.001$, $T_{23}$ = 6.7, $P < 0.001$, $T_{23}$ = 11.9, $P < 0.001$) and temperature at 22°C ($T_{23}$ = 4.6, $P < 0.001$), respectively. For community WYT116 T tests showed significant differences between high and low nutrients ($T_{23}$ = 5.3, $P < 0.001$, $T_{23}$ = 3.8, $P < 0.001$), pH 5.5 and 8 ($T_{23}$ = 3.1, $P < 0.005$, $T_{23}$ = 3.6, $P = 0.001$) and temperatures 15°C, 22°C and 30°C ($T_{23}$ = 9.5, $P < 0.001$, $T_{23}$ = 8.2, $P < 0.001$ and $T_{23}$ = 10.1, $P < 0.001$), respectively. All paired t-test results are shown in table 39 and 40, appendix.

**Adaptation to communities**

I aimed to test whether SBW25 adaptation to the abiotic factors (nutrient, temperature, pH) in the microcosms was slowed due to the presence of a community

Adaptation in the absence of the community (fitness of evolved in absence of community divided by fitness of ancestor) was compared to adaptation in the presence of community (evolved divided by fitness of ancestor). This gave the degree to which the community helps or hinders adaptation to the abiotic conditions. GLM was used to determine significant differences in adaptation to abiotic conditions in the absence of the community compared to adaptation in the presence of community. The results are shown in figure 20.

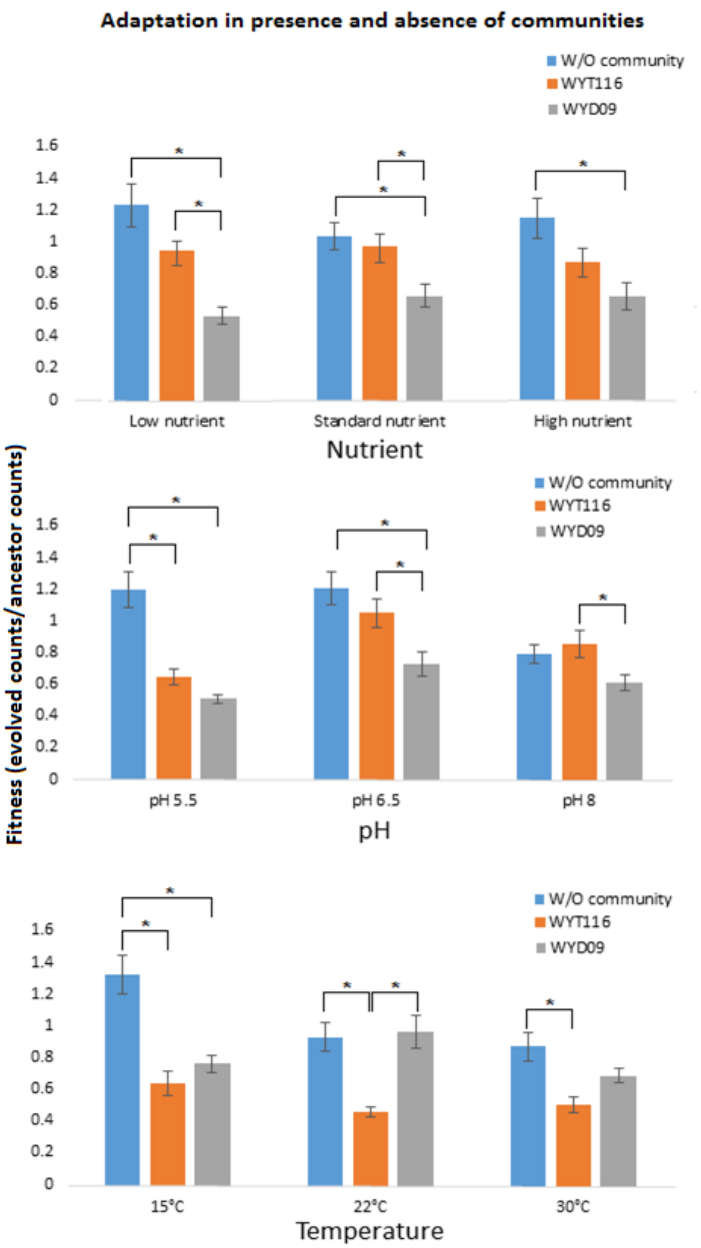


**Figure 22: Adaptation to abiotic conditions in the presence and absence of the communities. Comparison in fitness of SBW25 in the presence of community (orange – WYT116 or grey – WYD09) and absence of a community (blue bar). GLM ANOVA and Tukeys significance test showed significant differences by between fitness denoted by * between bars. Standard error is shown.**

Fitnesses of SBW25 in the presence and absence of communities were compared to determine whether the presence of a community constrained adaptation to abiotic conditions. GLM was fitted to a one-way ANOVA followed by Tukeys analysis which found significant differences between community WYD09 and no community ($F_{2,69} = 5.5$, $P = 0.006$) in high nutrient, WYD09 with no community and between WYD09 and WYT116 ($F_{2,69} = 5.0$, $P = 0.009$) in standard nutrient. For low nutrients, a significant difference was also shown in community WYD09 compared with no community ($F_{2,69} = 11.97$, $P < 0.001$).

For pH, significant differences were shown between fitness' of SBW25 in the presence of community WYD09, WYT116 and absence of community ($F_{2,69} = 24.95$, $P < 0.001$) of pH 5.5. pH 6.5 also showed significance between WYD09 and no community and between communities WYD09 and WYT116 ($F_{2,69} = 7.3$, $P < 0.002$). For pH 8, no significance was shown between presence and absence of communities, however, between communities WYD09 and WYT116 a significant difference was shown ($F_{2,69} = 3.4$, $P = 0.03$).

Fitness of SBW25 in microcosms at 15°C showed a significant difference between both presence of communities WYD09 and WYT116 when compared to the fitness of SBW25 in the absence of communities ($F_{2,69} = 17.11$, $P < 0.001$). No significant difference was shown between WYD09 and no community for 22°C ($P < 0.9$), however a significant difference was shown between WYT116 with no community and WYD09 and WYT116 ($F_{2,69} = 12.01$, $P < 0.001$). 30°C showed significant difference between WYT116 and no community only (($F_{2,69} = 8.1$, $P = 0.0007$).

**Degree to which community constrains adaptation**

The degree to which communities constrain adaptation to the abiotic environment was analysed. Mean ancestral counts of SBW25 fitness were used to determine the harshness of the environment (figure 21). Higher ancestral cell counts indicate that SBW25 grew well in that particular environment. It is shown that high nutrient levels in both WYD09 and WYT116 communities had the highest SBW25 growth. This is also true for temperature in that 30°C showed highest growth for SBW25. pH 6.5 showed the highest ancestral growth of SBW25 whilst WYT116 showed pH 8 to have the highest SBW25 cell count.

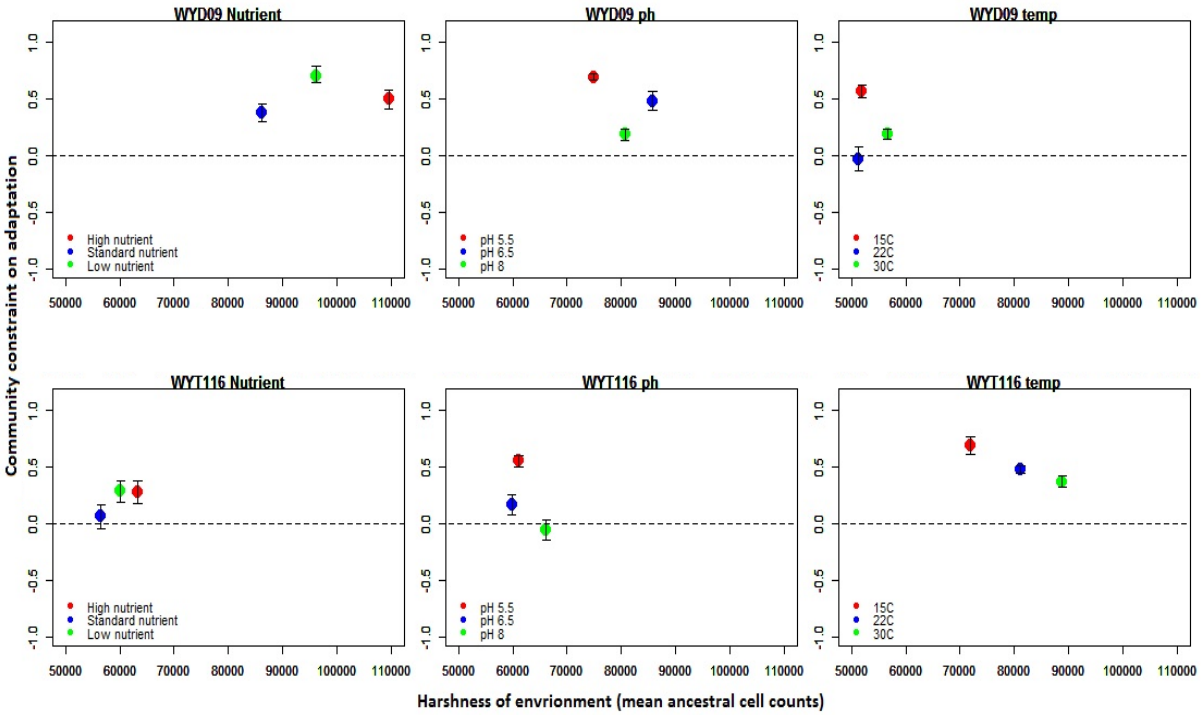


**Figure 23: Degree to which community constrains adaptation. X axis shows cell count by flow cytometry calculated as the average ancestral count of each factor. Y axis shows the degree of community constraint calculated as the coevolved cell counts, divided by the ancestral cell counts and subtracted from cell counts in the absence of a community. It is shown in the graph that as most results are positive (> 0 indicated by the dotted baseline) that SBW25 adaptation to abiotic conditions is constrained by the presence of a community with two exceptions (WYT116 pH 8 and WYD09 temperature 22°C). Standard error bars are shown.**

The difference between the evolved cell counts (SBW25 growth after eight-week experiment) and ancestral cell counts was calculated to give baseline of community adaptation in the absence of a community. These results were then used to subtract fitness in presence of community minus the fitness of SBW25 in the absence of community. Figure 21 shows that SBW25 could adapt to abiotic conditions most efficiently when in the absence of community as indicated by a positive result above the 0.0 baseline. WYT116 pH 8 and WYD09 temperature 22°C show the only two negative results showing that overall fitness was slightly better in the presence of a community. This is also mirrored in the bargraph (figure 20) in that both these factors (WYT116 pH 8 and WYD09 temperature) do not show a significant difference in fitness of SBW25 when comparing presence and absence of communities.

## Discussion

The results show different abiotic and biotic conditions affect coevolutionary dynamics between bacteria and phage. Strong coevolutionary patterns were observed in figure 18 (community WYD09) for low nutrient, low pH and temperature (22°C and 30°C) ($P < 0.05$ for contemporary phage coevolved with week 1, 4 and 8 SBW25). This shows that despite high competition from the surrounding community and stressors from the abiotic environment, phage was still able to effectively infect and coevolve with SBW25. Significant patterns observed in figure 18 resemble fluctuating selection dynamics (FSD).

Increasing temperatures and nutrient concentrations directly impact SBW25 growth rate and ability to adapt to external pressures of competing communities and fluctuating environmental conditions. These conditions are not known to impact phage directly, however, in unfavourable conditions if bacterial growth rates are slow, this will also affect phage populations as they cannot replicate without the host cell which can lead to extinction of both phage and bacteria populations.

Higher temperatures increase bacterial growth as this favours the internal enzyme kinetics which increase metabolism. If bacterial growth is higher, this allows the phage to infect more hosts and also increases populations. Coevolutionary patterns of SBW25 and phage were observed in higher temperatures showing that conditions were favourable.

FSD occurs when the cost of generalism is too high for SBW25 and phage to sustain an arms race dynamic and proceeds to specialism in that SBW25 and phage match infectivity and resistance alleles as described by the Matching Allele

Hypothesis (Sardany 2007). This pattern implies that because of the external pressures from the abiotic conditions and competition from the community, coevolution is slowed and FSD proceeds. Temperature is known to have a vital role in the length of the latent period of the bacterial life cycle and at low temperatures, fewer phage can penetrate the host cell and therefore cannot infect as efficiently as demonstrated by Zhang & Buckling, (2011) who showed that phage grew well in temperatures below 29°C but failed to reproduce above 30°C. This would not appear to be the case in this situation as it is apparent that phage can strongly infect bacterial cells at 30°C, (figure 18).

Figures 17 and 18 also show that in lower temperatures, there is no evidence for coevolution of SBW25 and phage for either community or on their own. This suggests that temperatures caused coevolution to slow down as bacteria cannot synthesise proteins as quickly and inhibition of DNA/RNA synthesis can occur (Beales, 2003).

Higher nutrient concentrations should lower the costs of adaptation of bacterial species so they can invest more in defence against parasites and competing species. Numerous studies have shown that phage and bacteria coevolve rapidly when placed in a high nutrient broth (Harrison, Laine, Hietala, Brockhurst, et al. 2013; Hall, Scanlan, Morgan, et al. 2011; Koskella & Brockhurst 2014; Pal et al. 2007). A significant difference ($F_{2,69} = 5.77$, $P = 0.005$) was shown for community WYD09 (figure 18) in low nutrient concentrations. Beech tea media is low in nutrients and is known to be high in tannins (Smith & Mackie 2004). With increasing concentrations of nutrients i.e. increased tannins, this might have inhibited bacterial growth and therefore indirectly affected phage ability to infect

and multiply, therefore, in this case, lower nutrients might have favoured bacterial growth as there were less inhibitory compounds within the media.

pH fluctuations represent a stress to both phage and bacteria. pH can interrupt the ability of bacteria to uptake nutrients as pH can change the ionization of nutrient molecules (Prescott et al., 2005). Phage are also affected by pH as they undergo a pH dependent surface change and are known to become slightly unstable in increasing acidic conditions. This is caused by an increase in hydrogen ions that can influence phage aggregation and is dependent on the phage isoelectric point that plays a large role in the sorption process and mobility (Michen & Graule 2010; Jończyk et al. 2011). Coevolution was shown to be significant in low pH 5.5 ($F_{2,69} = 8.5$, $P > 0.005$) (figure 18). The pH of rainwater tends to be slightly acidic at around pH 6, therefore, this would suggest that the beech tree community, SBW25 and phage are well adapted to growing in beech tree holes.

Figure 20 shows the fitness of SBW25 in the presence and absence of a community (fitness evolved/fitness of ancestral). It is shown that there are significant differences between SBW25 fitness in the absence compared to SBW25 fitness in the presence of a community with most cases showing a much higher fitness of SBW25 in the absence of a community. This indicates that SBW25 ability to adapt to the abiotic conditions is constrained by the presence of the community. If SBW25 is having to spend finite resources adapting to the surrounding community and if competition is too high, SBW25 will not be able to adapt and grow as quickly as if the competition from the community was absent. WYT116 pH 8 shows that fitness was higher when in the presence of the community. This might suggest that the community did not hinder adaptation of SBW25 to the abiotic environment. This is also true for SBW25 fitness in

community WYD09 at 22°C. The ability of an organism to adapt to the surrounding environment is largely dependent on the interspecific interactions of a surrounding community, particularly competition for finite resources. However, there can also be cooperation within the community for metabolic exchanges for example, cross feeding of by-products from other species (Zelezniak et al. 2015). This allows adaptation of a bacterial species when introduced into a community and therefore fitness is not affected.

The degree to which community constrained SBW25 adaptation was analysed and results shown in figure 21. Firstly, the harshness of the environment was quantified. The graphs show highest ancestral cell counts for high nutrient in both communities. This result is not surprising as higher nutrient concentration permits increased SBW25 growth as SBW25 can spend more on resources to increase growth rate. This is also true for high temperatures (30°C) as it is well documented that higher temperatures can cause increased growth rates of bacteria as metabolic activity is increased. Positive results (above > 0 baseline) showedthat fitness is higher in the absence of community. As SBW25 did not have to spend resources competing with other species present within the community, this allowed adaptation to the abiotic conditions as adaptation was not constrained by the surrounding community.

We also observed a decline in most microcosms in SBW25 fitness to the adjusted beech tea media by comparing SBW25 fitness before inoculation and after eight-week inoculation with biotic (community and phage) and exposure to different abiotic stressors (pH, nutrient and temperature). When a bacterial species is introduced into a new environment, there are certain metabolic and physiological costs associated with adapting to the new environment as it has evolved to grow

in a different niche than in which it has been introduced, which can result in poor growth. Figure 19 looks at how SBW25 were able to adjust to the new environment with t-tests showing in the majority of microcosms a significant decline in growth ($P < 0.005$) when SBW25 were exposed to communities, phage and abiotic stressors. Poor growth of SBW25 in the beech tea media after exposure might be due to the increased fitness costs associated with competing against numerous species for resources and defence against phage. Beech tea media might have also been responsible for poor growth of SBW25 as it might not have had the biochemical or physical properties required to adapt efficiently to such an environment (Hottes et al. 2013). Biotic interactions in the environment can often lead to species extinction if species cannot adapt to utilise the new environmental resources and species present (as described by the Red Queen Hypothesis). However, here we did observe extinction but a decline in growth. It would be expected that SBW25 in isolation in media would thrive as no other species are competing for resources and the build-up of waste products would be minimal. However, after the eight-week exposure and growth in isolation for 24 hours, SBW25 fitness had still declined.

Adaptation to different environments is a complex process in which mutational changes determine the ability of a species to adapt and subsequent fitness of a species will depend on the community and environment in which it had to adapt. Although most SBW25 isolates after treatment showed a decline in growth, in some there was no change in growth as shown in WYD09 temperature (in particular 30°C) and WYT116 pH 5.5 and 6.5 (figure 19). This suggests that SBW25 adapted to the media and could grow just as efficiently when in a community of species and in isolation. Consequently, these were both observed

to have significant coevolutionary patterns (figure 18) showing a strong adaptation of SBW25 and phage to the community and abiotic pressure.

Figures 15 and 16 show total cell count (logged flow cytometry cell counts) for the microcosms over the eight-week experiment. Microcosms in the higher nutrient level (blue), shows a higher cell count. As there are more nutrients available to bacteria, this enables higher growth as bacteria have more nutrients to utilise. Lower nutrients (red), showed a significantly lower cell count from bacteria from high nutrient microcosms. This further shows that bacteria in microcosms with less nutrients cannot grow as efficiently as when there are plenty of nutrients available.

As waste products build up in media, this can cause cell death in bacteria as nutrients are in decline. Media in the microcosms was refreshed every week to sustain bacteria in stationary phase, however, cell counts indicate a decline which could be due to bacteria death.

As bacterial counts are shown to increase again after week 4, this indicates that they might be adapting to the media and making use of the resources available to survive. Liebig's Law of the Minimum states that growth is controlled by the most limited resource, i.e. the nutrient present in the lowest concentration. An increase in essential nutrients will subsequently increase growth (shown in figure 15 and 16). However, if an essential nutrient remains low (e.g phosphate), changes in other nutrient concentrations will have no effect on growth. Multiple limiting abiotic factors such as pH, light or salinity can also influence microbial populations and the growth of a microbial community depends on environmental conditions as well as high nutrient supply to have optimal growth (Gorban et al.

2011). In response to low nutrient levels, competition between organisms occurs and morphological and physiological traits can be altered to exploit all available resources. Lawrence et al., (2012) demonstrated that competitive species in communities evolve to use waste products generated by other species and can diverge in their resource use. A slight difference (P = 0.051) is shown between normal strength media and double strength media in all nutrient microcosms. Although fluctuations in cell count growth are observed throughout the weeks, overall, cell count has plateaued and a stationary phase is seen.

The results show the coevolutionary trajectories are tending towards fluctuating selection dynamics in the majority of microcosms rather than an arms race dynamics, however, many of the microcosms, in particular figure 17, showed no obvious trends to either ARD or FSD patterns. FSD dynamics suggest that the phage and bacteria are specialists whereby a resistance gene is matched by an infectivity gene but resistance or infectivity to past resistance or infectivity genes are lost. ARD is driven by negative frequency dependent selection in which the rare alleles become the most common as phage infect and lyse the most common bacterial genotype which gives an advantage to rare bacterial resistance alleles which rise in frequency (Koskella and Brockhurst, 2014). Gomez and Buckling (2011) showed that in a natural soil community, coevolution between SBW25 and phage is more consistent with FSD which is in contrast to ARD which is commonly observed with high nutrient broth (Buckling & Rainey 2002a; Brockhurst, Morgan, et al. 2007) as neither SBW25 nor its phage become increasingly infective or resistant over time. This is apparent in this case where nearly all microcosms exhibit FSD, with the exception of microcosms in high nutrient microcosms where ARD is shown (figure 17).

Resistance of hosts to phage is most costly due to resistance mutations that are no longer beneficial so resistance to past phage is lost in preference to having specific genes that resist contemporary phage. Therefore, ARD often decelerates and gives way to FSD (Hall et al., 2011).

FSD was still consistently observed in the absence of a community but in the presence of abiotic pressures (figure 17, pink), with the exception of a pattern of ARD in high nutrient media (figure 17). The results shown in figure 17, show that in high nutrient media, whether in the presence or absence of communities, the typical pattern follows ARD which is typically observed in microcosms of high nutrient broth (Brockhurst et al. 2003; Buckling & Rainey 2002a). This suggests that there are sufficient nutrients and space for SBW25 and phage to coevolve whilst retaining their past infectivity and resistance mutations as costs of retaining these mutations are lower. As nutrients are higher than in other microcosms, growth rates of phage and bacteria are expected to be higher. This might lead to a higher encounter rate between SBW25 and phage which can lead to accelerated coevolution. It also suggests that interspecific interactions between SBW25, phage and other species in the community are not having a significant effect on the coevolution. Different species in microcosms can create competition for space and nutrients leading to external pressures to SBW25 and phage that might cause even more costly effects on fitness and may result in the loss of resistance and infectivity mutations, which would be observed as FSD. As ARD is observed in high nutrient media, this suggests that competition between other species on SBW25 is not having an effect on its ability to resist phage. Competition rather than cooperation is thought to dominate species interactions (Foster and Bell, 2012), however, many species can cooperate or facilitate

competitive strategies that require cooperation from individual species such as quorum sensing. Brockhurst et al. 2006 showed that phage can regulate competitive interactions between bacterial species and that abiotic factors can influence species coexistence.

Abiotic factors are known to affect species interactions and coevolution between phage and SBW25. It is known that coevolution between SBW25 and phage in higher temperatures can cause environmental stress to the virus and not the bacteria which is thought to be the result of costly infectivity strategies of the virus that contributed to the viral extinction (Zhang & Buckling 2012). No extinction was observed in this experiment, however in temperatures that were below optimal (15°C) or above optimal (30°C), a reduction in SBW25 cell count was observed. This suggests that SBW25 were unable to replicate as well or that phage were able to lyse the cells effectively.

The costs of adaptation for a bacterial species introduced into a new community can be high and dependent on the other species present within the community. There are often trade-offs with adapting to the biotic community whilst adapting to the abiotic environment. Here we showed that community does constrain adaptation of SBW25 with SBW25 fitness higher than when coevolved in a community in most cases (figure 20). Species have to contend with changing abiotic conditions through the seasons as well as disturbances caused by natural and anthropogenic pressures (Harmon et al. 2009). We have shown here that biotic pressures profoundly impact the ability of a species to adapt that can also affect their ability to coevolve. However, we also found SBW25 which had coevolved with communities in microcosms with altered pH (pH 8) and temperature (22°C) that SBW25 fitness was lower in isolation than when in

communities. It is possible that instead of negative interactions, the species within these communities were more cooperative by sharing of resources. However, this behaviour is more associated with the same species or similar species (Xavier & Foster 2007; Griffin et al. 2004). Diverse populations of species are also more likely to be productive as more resources can be exploited (Brockhurst et al. 2006), which might explain the increase in fitness of the SBW25 in the presence of the communities.

The fitness of one species within a community can depend on the distribution of certain genotypes of another species and these interspecific interactions can vary among different populations. It is thought that different environmental conditions (e.g. nutrient concentration) can affect how these species interact within that environment. This appears to be the case here in that coevolutionary interactions differ amongst populations of different communities and that abiotic interactions are affecting how well SBW25 can adapt to the environments. It may be the case, as described by the Geographic Mosaic Theory that coevolutionary coldspots have occurred in these communities because certain species with which they would reciprocally interact with are not present within that particular community (Thompson, 2005).

Competition between species occurs when one organism exploits and reduces a common resource which indirectly affects other organisms (competitive exploitation) or when one organisms directly interferes with another organisms ability to use a resource (interference competition) (Amarasekare, 2002). Inference competition can occur between and within species and is affected by the type of species and their life history within the population (Bourlot et al., 2014) whereas exploitative competition is much more common and understood.

In this experiment, we show that with fluctuating resources can affect coevolutionary interactions and growth of species, inferring competition between species for these resources. For example, figure 16 shows that in high nutrients, population growth is increased – therefore more resource for population growth and reduced exploitative competition. However, in low to medium nutrient levels there is less population growth and therefore competition between species is thought to be increased. Exploitative competition is proven here in figure 21 which shows that SBW25 adaptation to the abiotic environment (i.e its ability to grow) is constrained by competition from other species within the community.

The effects of changing abiotic conditions that can constrain adaptation to biotic interactions are well documented (Barraclough 2015; Northfield & Ives 2013). Environmental changes can lead to lowered biodiversity and extinction of keystone species which can cause changes in ecosystem functioning and the composition of communities. Here, we observed that these changes in abiotic conditions and competition from different communities have different effects on species ability to coexist and coevolve with one another.

**Conclusion**

These results show that abiotic and biotic factors are both important when determining the species that can coexist and coevolve together.

The results showed that overall, SBW25 could adapt to a beech tree hole community and abiotic stressors but at a cost as shown by the decline in fitness after an eight-week exposure to the microcosms (figure 19). This cost is thought to be caused by the adaptation to phage, communities and limiting resources within the environment. This was further analysed by SBW25 fitness in the presence and absence of community. It was shown that across the board (with two exceptions) adaptation of SBW25 was constrained by the presence of a community.

Coevolutionary patterns were observed in some microcosms that were known to simulate close conditions to beech tree holes (pH 5.5, low nutrient) and conditions that SBW25 grew well in (high temperature) where adaptation costs might have been lower than when SBW25 were placed in unfavourable conditions (high pH, high nutrient media potentially containing a higher level of tannins, but this has not been measured).

This research has given a semi-natural insight into how bacterial species adapt, coevolve and coexist with one another in different environments. It lends itself to support more studies to include more natural environments rather than focusing on pairwise interactions in unnatural settings.

# Chapter 6 – General discussion and conclusions

The overall aim of the thesis was to investigate how coevolutionary paths between a known phage and bacteria pair were affected by surrounding natural microbial communities.

Understanding how bacteria and phage interact in natural environments is of significant importance, in particular finding new treatments to fight bacterial infections with increased antibiotic resistance (Andersson & Hughes 2010). Recent research into phage therapy is now thought of as one way of targeting specific disease-causing bacteria by using viruses where antibiotics are no longer able to eradicate the disease, for example in the case of *P. aeruginosa* infection of the lungs in cystic fibrosis sufferers (Friman et al. 2013).

I researched bacteria and phage coevolution through a series of laboratory experiments using a well-studied bacteria and phage pair and natural microbial communities that were isolated from beech tree holes. Through experimental manipulations of abiotic and biotic conditions, my findings provided new insight into how coevolutionary trajectories of virus-bacteria interactions are affected by different external pressures of the natural environment.

To date, so far as we are aware, there have not been published studies of how natural microbial communities affect the coevolutionary dynamics of bacteria and phage; with most studies have focussed on single host-pathogen interactions (Hall, Scanlan, Morgan, et al. 2011), two-species communities (Harcombe & Bull 2005) or in the presence of one multi-species community (Gómez & Buckling

2011a). Therefore, this work gives a more realistic approach to how host-pathogen interactions occur in the environment.

Chapter 3 looked at how surrounding microbial communities affect phage-bacteria dynamics and how they coevolve. I found that coevolutionary trajectories of either arms race dynamics or fluctuating selection dynamics of SBW25 and φ2 phage could not be predicted and that through negative interactions with other species, coevolution was slowed down due to costs associated with evolving increased infectivity and resistance genes. The results showed different patterns of evolutionary responses of bacteria and phage when in the presence of different surrounding communities with some resembling classic patterns associated with fluctuating directional selection, arms race or no coevolution. This suggests that the composition and structure of the microbial communities is affecting how the phage and bacteria respond to one another depending on the competition of the other species present. Another interesting result was shown in the local adaptation experiments of phage and bacteria where phage isolated from one community was coevolved with SBW25 isolated from another community. The results showed that all phage (with one exception) were locally maladapted to SBW25, i.e. phage fitness was higher and could infect SBW25 evolved in foreign communities better than local SBW25 in which it had coevolved with. It was thought that this is due to a temporal mismatching of genotypes between the bacteria and phage caused by an evolutionary time lag of at least one generation due to natural selection. To ensure that this maladaptation did not occur because of similarities between communities, TRFLP data was used to compare species composition of the communities in which they coevolved in. All foreign and local communities from which the phage and SBW25 were

chosen from showed that they were very distinct from one another. Interestingly, the phage that showed no adaptation or maladaptation to foreign SBW25 was paired with an SBW25 that coevolved in a very similar community according to TRFLP data. If phage is paired with a SBW25 that evolved in very similar communities, then similar interactions with other species would have occurred which might have prevented SBW25 and phage from coevolving effectively. Therefore, we would not expect a similar phage to the local phage to have higher fitness as they might have similar genotype pairings.

Chapter 4 aimed to show how these bacteria-phage dynamics are affected in the presence of similar communities but with varying degrees of diversification with rarer species being diluted out. This enabled us to identify how varying diversities of communities affected coevolutionary dynamics and how much rare or dominant species affect these interactions. Due to extinctions of species, it could not be determined whether the changes in community diversity affected the coevolutionary paths of phage and bacteria. However, it was shown that in the presence of one community phage decreased SBW25 density and in the presence of another community, SBW25 density increased. This is contrary to previous findings by Gómez & Buckling 2011a which only looked at one community and found that phage decreased mean cell densities of SBW25. This outcome was consistent with findings in the previous chapter in that different communities can impact the interactions of bacteria and phage and that these interactions cannot be predicted. Together with the previous findings, this suggests that there is evidence for natural microbial communities to affect host-pathogen interactions and that the response of these interactions will differ in the presence of different competitive interactions of other species.

As well as understanding how biotic factors affect coevolutionary interactions, an experiment was performed by manipulating abiotic conditions (nutrient concentration, pH and temperature) and following coevolutionary paths of SBW25 and phage also in the presence of natural communities (chapter 5). Numerous studies have looked at how bacteria-phage are affected by abiotic conditions (Zhang & Buckling 2011; Harrison, Laine, Hietala & Brockhurst 2013) but none have shown these in the context of a natural community. The experiment aimed to show that as well as biotic factors affecting host-pathogen interactions, abiotic conditions will also affect them indirectly as abiotic conditions can affect species composition, in particular nutrients and temperature (Awasthi et al. 2014). As in the natural environment, phage and bacteria are not only in competition with other microbial species but also they must adapt to fluctuating environmental conditions. Similar results were shown to previous experiments (chapter 3) in that coevolutionary dynamics are unpredictable and are affected by abiotic conditions as well as biotic conditions.Comparing the coevolutionary patterns of bacteria and phage in only biotic with abiotic, shows a slowing down of coevolutionary patterns with many of them showing not to coevolve. This might suggest that although phage and bacteria can coevolve, this coevolutionary process might be slowed down by not only competitive interactions but by abiotic pressures that they might not be adapted to. This can also lead to reduced beneficial mutations and ecological sorting of the microbial community which might cause a species that is able to adapt to dominate and use resources further reducing the ability of phage and bacteria to coevolve.

To test how rates of adaptation of SBW25 that were affected by the surrounding community, coevolved SBW25 in the presence of a community was compared to

coevolved SBW25 in the absence of community. This allowed us to visualise the degree of constraint to adaptation of SBW25 was caused by the community. We found across the board, with 2 exceptions, that SBW25 fitness decreased when coevolving in the presence of a community rather than in isolation. This suggests that in the presence of a community, there is a cost to adapting defence alleles for competing species and potential predators, however this was not tested here.

All experiments (chapters 3 – 5) followed cell counts of the microcosms and consistently showed that SBW25 and phage addition to communities overall did not affect the cell populations of the microcosm with similar cell counts shown for community alone and communities inoculated with SBW25 and phage. This suggests that the communities reached an equilibrium where both SBW25 and phage could survive and be re-isolated from the community.

Although my results show weaker evidence for coevolution due to the nature of the study, it has given insight into how coevolution proceeds in the environment and that it is largely dependent on external factors. When compared to other studies that show strong evidence for coevolution (Paterson et al. 2010b; Pal et al. 2007; Poullain et al. 2008; Hall, Scanlan, Morgan, et al. 2011), this research gives a more realistic idea of how coevolution proceeds in natural environments in a large number of .. However, due to the large number of interactions within the microcosms, evidence for coevolution is weaker as the results show little evolutionary response in most microcosms.

The use of flow cytometry throughout this project gave quantitative results that allowed analysis of specific cell counts at given time points. I believe this was an excellent way to analyse cell counts when exposed to different factors (abiotic

and biotic – in particular phage) as it allowed us to analyse cell counts when in the presence and absence of phage that were not subjective when compared to other methods used to analyse phage infection to hosts (i.e. streak assay).

Using larger microcosms allowed SBW25 and phage to survive and coexist within a microbial community. Chapter 4 (diversity experiment) showed SBW25 and phage coevolved with a community in a 96 deep well plate. This created higher competition, lower nutrient availability and reduced space which led to the extinction of SBW25 and phage in some microcosms. However, when microcosms were changed to 50 ml polypropylene tubes, SBW25 and phage did not become extinct as there was less competition through higher nutrient availability and space. Other factors might have affected how well SBW25 and phage could coevolve. Performing the experiments for a longer period of time (longer than eight weeks) might have affected how well phage and SBW25 coevolved. The use of a manually assembled polyculture of bacteria species might have still given insight into how communities affect coevolutionary processes without affecting the response of coevolution, as shown here. To analyse abiotic conditions better and give a more realistic approach, fluctuating conditions could have been simulated by altering the incubator temperatures or fluctuating nutrient input and gradually increasing pH of the microcosms.

Future directions for this work include looking at how changing environments affect coevolutionary processes. This could include how coevolutionary processes are affected by climate change or new pollutants entering the environment, in particular antibiotics. Research into how these changes in coevolution affect higher trophic organisms such as protists and beyond (Friman

et al. 2013) as bacteria and phage are fundamental providers of nutrients to higher trophic organisms and their role in the microbial loop (Azam & Malfatti 2007).

This work might have fundamental applications in 'real world' situations as coevolution between bacteria and phage can have implications for informing strategies to control pathogens and wider repercussions for the food web as it can alter how available bacteria are to organisms in higher trophic levels such as protists (Friman et al. 2013; Friman & Buckling 2013; Råberg et al. 2014). Phage have been used to treat bacterial infections for several years in numerous countries, particularly the former Soviet state of Georgia. In recent years, phage therapy has come into focus in Western countries as a possiblity to treat bacterial infections due to increased antibiotic resistance (Meaden & Koskella 2013). If phage are used to treat an infection of a person with a compromised immune system, such as those with Cystic Fibrosis, the natural surrounding microbial community could affect how effective the phage treatment can target the infection due to trade-offs which can slow down evolutionary potential of bacteria and phage evolving beneficial mutations that can be used to resist the phage or infect the bacteria (Friman et al. 2013). An example of which is seen in these coevolutionary experiments as coevolutionary paths are not as well defined as those seen in other coevolutionary experiments in the laboratory, showing that phage-bacteria interactions cannot be predicted as these are largely influenced by the positive and negative interaction of the surrounding microbial community. Biogeography of microbial species can also affect coevolutionary dynamics with some microbial compositions allowing for high trait remixing which can lead to a hot spot for coevolutionary dynamics due to the availability of beneficial

mutations which is likely to lead to an accelerated coevolution of bacteria and phage.

Coevolution is a highly dynamic and complex ecological and evolutionary process occurring continuously across different landscapes. Through gene flow, horizontal gene transfer, mutations and migration, populations of microbial species are continually changing, for example, parasites driving their hosts extinct and species outcompeting others when environmental conditions change to their favour (Thompson, 2005). All of these processes create the ecological structure of communities and an explanation of how species can persist and coexist with one another.

To conclude, throughout this thesis I examined the ecological and evolutionary responses of SBW25 and phage (SBW25φ2) to one another when faced with naturally occurring biotic and abiotic external pressures. This research has provided novel insight into how SBW25 and phage coevolutionary patterns respond when faced with these stresses and how unpredictable these patterns are. These results are contrary to what has already been shown in that phage-bacteria interactions cannot be generalised and are largely dependent on the ecological interactions in the environment in which they are situated. It has also contributed to the understanding of local adaptation of bacteria and phage to their local environments and how interactions from other species can cause lowered fitness of coexisting species and how they adapt to respond in changing environments. I have shown that ecological sorting of bacterial species is fundamental in determining which species are present which also affects coevolutionary paths of bacteria and phage.

Overall I have shown that phage-bacteria dynamics are affected by surrounding biotic and abiotic factors by varying degrees depending on the microbial community and abiotic conditions and my findings contribute to the on-going research into coevolutionary studies of phage and bacteria dynamics. I hope that this work can contribute to further understanding of these processes and how they might affect predictions of phage infectivity of bacteria in the future.

# Appendix

## Antibiotic resistance

Communities were screened for antibiotic resistance/susceptibility on the basis of whether they could grow on gentamicin agar so as gentamicin resistant P. fluorescens could be pulled out from the microbial community within the experiment. During preliminary screening, it was shown to be a trend in gentamicin resistance and where the communities were isolated (chapter 2, method development). Therefore, 302 beech tree hole communities that were collected around the UK were obtained from - 80°C freezer and six different antibiotics were screened in order to assess antibiotic resistance to different antibiotics in relation to where they were sampled. The antibiotics chosen were based on the different characteristics of each antibiotic (table 4). 302 communities were inoculated into a 96 well plate containing 50% LB. Different antibiotic concentrations were chosen on the basis of minimum inhibitory concentrations given by the EUCAST MIC breakpoint shown in table 3.

**Table 3: EUCAST MIC breakpoints for microbial resistance which were used in the experiment.**

| Antibiotic | Concentration (µg/ml) |
|---|---|
| Tetracycline | 30 |
| Ampicillin | 30 |
| Gentamicin | 10 |
| Imipenen | 10 |
| Streptomycin | 10 |
| Kanamycin | 10 |

Once plates were inoculated with the antibiotic, community and media, they were placed on the plate stacker and read in a plate reader (BioTek) at $OD_{590}$ for 18 hours to monitor the growth curve. A positive control of the community with no antibiotic was used to determine if a community could or could not grow in the presence of the antibiotic. Using absorbance spectrophotometer readings, a community inoculated with antibiotic was determined as resistant or susceptible based on the positive control absorbance readings. After 18 hours, the mean of the absorbance readings were taken.

**Table 4: Antibiotic chosen based on the different characteristics.**

| Antibiotic | Conc (µg/ml) | Characteristics |
|---|---|---|
| Kanamycin | 10 | Aminoglycoside, narrow therapeutic use for serious infections, synergistic with beta lactams |
| Streptomycin | 10 | Aminoglycoside |
| Gentamicin | 10 | Aminoglycoside |
| Tetracycline | 30 | Once broad spectrum, resistance has now caused reduced use |
| Imipenen | 10 | Carbapenem. Broad spectrum. Same mechanism as Beta lactams. Often used as a last resort |
| Ampicillin | 30 | Aminopencillin, water soluble so pass through porin channel in cell wall of gram neg. |

A heatmap was used to help visualise antibiotic resistance and susceptibility to the 6 antibiotics. The results are shown in figure 21.

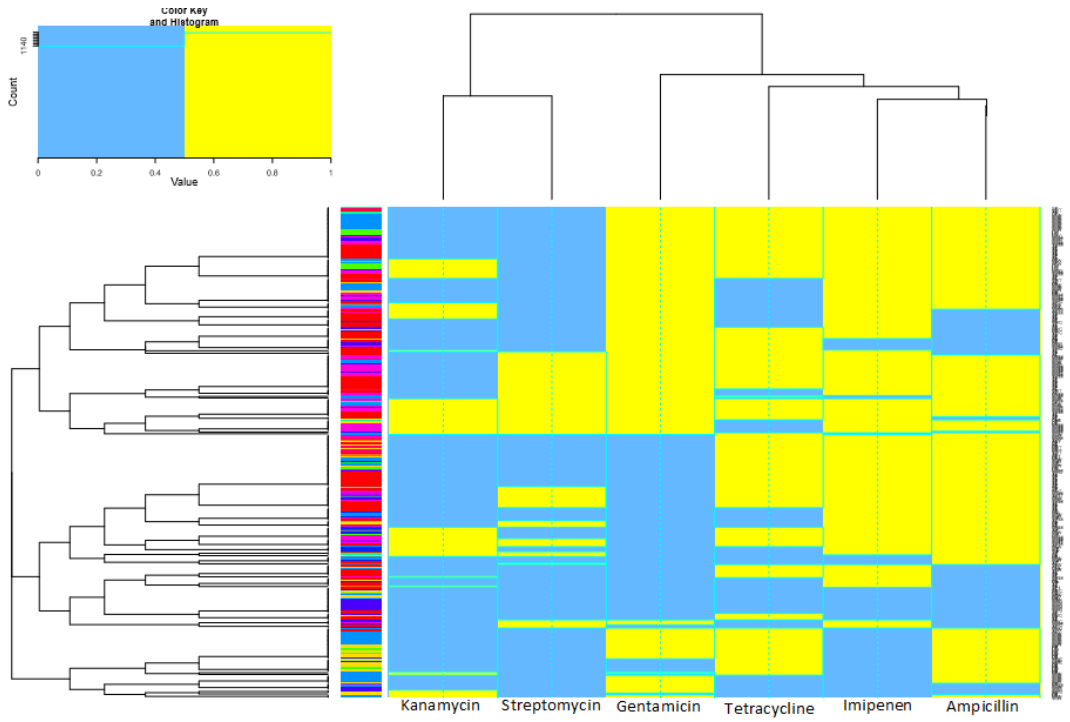


**Figure 24: Heatmap shows 6 antibiotics used to determine geographic patterns in communities that are susceptible/resistant to antibiotics. Blue shows that communities could grow on agar with the antibiotic and are resistant to the antibiotic. Yellow shows communities that were resistant to the antibiotic.**

Figure 21 shows results for different communities (right side of heatmap) grouped on the basis of similarity in resistance (blue)/susceptibility (yellow) to antibiotics. It is shown from the heatmap that Ampicillin and Imipenen to be the least effective antibiotic as most communities were able to grow when inoculated with both. Kanamycin showed to be the most effective antibiotic with gentamicin showing around 58% resistance/susceptibility.

These results are of interest as Imipenen is often used as a "last resort" antibiotic due to its broad spectrum potency to a wide range of gram positive and negative bacteria (Papp-Wallace et al., 2011). The results also show evidence of certain communities, dependant on where they were isolated from being grouped a

resistant or susceptible to types of antibiotics. This could indicate a possible association with the bacterial communities being isolated from environments that had previously been exposed to a particular antibiotic (e.g. urban pollution from agriculture).

**Table 5: Locations of tree hole samples with community ID and sample ID**

| Location | Sample ID | Community ID | Northings | Westings |
|---|---|---|---|---|
| Burnham Beeches | BB66 | 1 | 51.5596 | 0.6315 |
| Burnham Beeches | BB96 | 2 | 51.5564 | 0.6371 |
| Wytham Woods | WYT12 | 3 | 51.7673 | 1.3299 |
| Wytham Woods | WYT35 | 4 | 51.769 | 1.3291 |
| Wytham Woods | WYT87 | 5 | 51.7725 | 1.337 |
| Wytham Woods | WYT94 | 6 | 51.7728 | 1.337 |
| Wytham Woods | WYT95 | 7 | 51.7728 | 1.337 |
| Wytham Woods | WYT98 | 8 | 51.7728 | 1.337 |
| Wytham Woods | WYT116 | 9 | 51.772 | 1.3364 |
| Wytham Woods | WYD06 | 10 | 51.7688 | 1.329 |
| Wytham Woods | WYD09 | 11 | 51.769 | 1.3289 |
| Wytham Woods | WYC14 | 12 | 51.7693 | 1.3292 |
| Wytham Woods | WYC15 | 13 | 51.7688 | 1.3299 |
| Wytham Woods | WYM02 | 14 | 51.7697 | 1.3294 |
| Ashridge Estate | AE101 | 15 | 51.79484 | 0.56032 |
| Ashridge Estate | AE103 | 16 | 51.79487 | 0.56205 |
| Ashridge Estate | AE107 | 17 | 51.79507 | 0.56083 |
| Ashridge Estate | AE110 | 18 | 51.79537 | 0.56065 |
| Knighthayes Court | CHE03 | 19 | 50.9228 | 3.4745 |

**SBW25 PCF phage coevolution experiments**

**Table 6: Comparing communities and treatments**

| | DF | Sum Sq | Mean | F value | Pr(>F) |
|---|---|---|---|---|---|
| Community | 19 | 24.36 | 1.2822 | 16.569 | <2e-16*** |
| Treatment | 2 | 0.05 | 0.0251 | 0.325 | 0.723 |
| Community:treatment | 38 | 1.30 | 0.0343 | 0.444 | 0.999 |
| Residuals | 1380 | 106.79 | 0.0774 | | |

**Table 7: Tukeys for significant difference**

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| contemporary-future | -0.17467742 | -0.29575940 | -0.05359543 | 0.0026835 |
| past-future | -0.13681141 | -0.25789340 | -0.01572942 | 0.0230053 |
| past-contemporary | 0.03786601 | -0.08321598 | 0.15894800 | 0.7351941 |

**Phage PCF SBW25**

**Table 8: Comparing communities and treatments**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| community | 19 | 37.83 | 1.9909 | 20.738 | <2e-16 *** |
| treatment | 2 | 0.03 | 0.0174 | 0.181 | 0.834 |
| community: treatment | 38 | 4.28 | 0.1126 | 1.173 | 0.219 |
| Residuals | 1380 | 132.49 | 0.0960 | | |

**Table 9: Tukeys for significant difference. Community WYT116**

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| contemporary-future | 0.16793331 | -0.02440314 | 0.3602698 | 0.0990639 |
| past-future | 0.24468086 | 0.05234441 | 0.4370173 | 0.0090649 |
| past-contemporary | 0.07674755 | -0.11558891 | 0.2690840 | 0.6070997 |

**Table 10: Tukeys for significant difference. Community WYT12**

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| contemporary-future | 0.15908586 | -0.06886676 | 0.3870385 | 0.2233574 |
| past-future | -0.08722386 | -0.31517649 | 0.1407288 | 0.6317583 |
| past-contemporary | -0.24630973 | -0.47426235 | -0.0183571 | 0.0311712 |

**Table 11: Local adaptation experiment - community pairings**

| Community | |
|---|---|
| 1. BB66 | 15. AE101 |
| 2. BB96 | 6. WYT94 |
| 3. WYT11 | 14. WYM02 |
| 4. WYT35 | 10. WYD06 |
| 5. WYT87 | 1. BB66 |
| 6. WYT94 | 18. AE110 |
| 7. WYT95 | 16. AE103 |
| 8. WYT98 | 2. BB96 |
| 9. WYT116 | 17. AE107 |
| 10. WYD06 | 8. WYT98 |
| 11. WYD09 | 9. WYT116 |
| 12. WYC14 | 7. WYT95 |
| 13. WYC15 | 4. WYT35 |
| 14. WYM02 | 19. CHE03 |
| 15. AE101 | 12. WYC14 |

| | |
|---|---|
| 16. AE103 | 3. WYT11 |
| 17. AE107 | 11. WYD09 |
| 18. AE110 | 5. WYT87 |
| 19. CHE03 | 13. WYC15 |

**Table 12: Local adaptation ANOSIM results**

**Table 13: ANOSIM results for TRFLP community composition in week 1 compared with week 8**

| Community | ANOSIM sig | R |
|---|---|---|
| 1 vs 15 | 0.001 | 0.372 |
| 2 vs 6 | 0.001 | 0.3799 |
| 3 vs 14 | 0.005 | 0.2789 |
| 4 vs 10 | 0.001 | 0.4973 |
| 5 vs 1 | 0.001 | 0.4461 |
| 6 vs 18 | 0.001 | 0.4043 |
| 7 vs 16 | 0.001 | 0.3652 |
| 8 vs 2 | 0.001 | 0.2502 |
| 9 vs 17 | 0.001 | 0.4436 |
| 10 vs 8 | 0.001 | 0.6757 |
| 11 vs 9 | 0.001 | 0.8074 |
| 12 vs 7 | 0.001 | 0.4976 |
| 13 vs 4 | 0.001 | 0.3506 |
| 14 vs 19 | 0.001 | 0.618 |
| 15 vs 12 | 0.528 | -0.01381 |
| 16 vs 3 | 0.009 | 0.1913 |
| 17 vs 11 | 0.001 | 0.5364 |
| 18 vs 5 | 0.001 | 0.4337 |
| 19 vs 13 | 0.002 | 0.3986 |

| Treatment | Week 1 | Week 8 |
|---|---|---|
| Communities overall similarity | R = 0.3337<br>P = 0.001 | R = 0.2601<br>P = 0.001 |
| Communities inoculated with treatments vs control | R = 0.005676<br>P = 0.162 | R = 0.02243<br>P = 0.01 |
| Communities with SBW25 vs control | R = 0.03454<br>P = 0.004 | R = 0.1512<br>P = 0.001 |
| Communities inoculated with phage vs control | R = 0<br>P = 0.647 | R = 0.01841<br>P = 0.138 |
| Communities that contain SBW25 | R = 0<br>P = 0.441 | R = 0.08825<br>P = 0.001 |

**Chapter 4 - Diversity**

Coefficient t test

**Table 14 Paired T Test: Past and contemporary**

| | |
|---|---|
| data: past vs contemporary | |
| 95 percent confidence interval: | t = -0.1322, df = 31, p-value = 0.8957 |
| sample estimates: | -29517.63, 25923.34 |
| mean of the differences: | -1797.146 |

**Table 15 Paired T Test: Past and future**

| | |
|---|---|
| data: past vs contemporary | |
| 95 percent confidence interval: | t = -1.2179, df = 31, p-value = 0.2325 |
| sample estimates: | -50673.90 12781.44 |
| mean of the differences: | -18946.23 |

**Table 16: Between diversity significance (ANOVA and Tukeys)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity | 2 | 0.114 | 0.05723 | 2.195 | 0.114 |
| Residuals | 237 | 6.179 | 0.02607 | | |

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| 3-2 | 0.02101040 | -0.039205436 | 0.08122623 | 0.6891788 |
| 6-2 | 0.05310834 | -0.007107495 | 0.11332417 | 0.0961420 |
| 6-3 | 0.03209794 | -0.028117893 | 0.09231377 | 0.4208842 |

**Table 17: Between treatments significance (Community 1 Diversity 2 ANOVA and Tukeys)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 2 | 3 | 0.0995 | 0.03317 | 2.801 | 0.0455 * |
| Residuals | 76 | 0.8998 | 0.01184 | | |

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| C-B | -0.09904583 | -0.18943186 | -0.008659806 | 0.0261144 |

**Table 18: Between treatments significance (Community 1 Diversity 3 ANOVA)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 3 | 3 | 0.0699 | 0.02331 | 1.473 | 0.229 |
| Residuals | 76 | 1.2030 | 0.01583 | | |

**Table 19: Between treatments significance (Community 1 Diversity 6 ANOVA and Tukeys)**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 6 | 3 | 0.5064 | 0.16879 | 5.419 | 0.00197 ** |
| Residuals | 76 | 2.3672 | 0.03115 | | |

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| C-B | 0.21992454 | 0.07332490 | 0.36652418 | 0.0010104 |
| P-B | 0.13304521 | -0.01355442 | 0.27964485 | 0.0888698 |
| C-BP | 0.13452674 | -0.01207290 | 0.28112638 | 0.0836712 |

**Table 20: Between treatments significance (Community 2 Diversity 2 ANOVA)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 2 | 3 | 0.0872 | 0.02906 | 0.864 | 0.464 |
| Residuals | 76 | 2.5563 | 0.03364 | | |

**Table 21: Between treatments significance (Community 2 Diversity 3 ANOVA)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 3 | 3 | 0.1727 | 0.05758 | 2.352 | 0.0788 |
| Residuals | 76 | 1.8603 | 0.02448 | | |

**Table 22: Between treatments significance (community 2, diversity 6 ANOVA and Tukeys)**

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Diversity 6 | 3 | 0.4449 | 0.14830 | 10.65 | 6.33e-06 *** |
| Residuals | 76 | 1.0579 | 0.01392 | | |

| | diff | Lwr | upr | p adj |
|---|---|---|---|---|
| C-B | 0.12484964 | 0.02684375 | 0.22285552 | 0.0068484 |
| P-B | 0.11030676 | 0.01230087 | 0.20831264 | 0.0211298 |
| C-BP | 0.17773140 | 0.07972551 | 0.27573728 | 0.0000521 |
| P-BP | 0.16318852 | 0.06518264 | 0.26119440 | 0.0002208 |

**Table 23 Between diversities 2, 3 and 6**

| | DF | Sum Sq | Mean | F value | Pr(>F) |
|---|---|---|---|---|---|
| glm.diversity | 1 | 1.1430 | 1.14303 | 10.35 | 0.002 |
| Residuals | 67 | 9.261 | 0.1382 | | |

## Chapter 5 - Abiotic coevolution

### SBW25 PCF Phage

**Table 24 ANOVA and Tukeys WYD09 CBP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 2.892 | 1.4460 | 38.98 | <2e-16 *** |
| Residuals | 645 | 23.929 | 0.0371 | | |

| | Diff | Lwr | Upr | P adj |
|---|---|---|---|---|
| ph-nutrient | 0.03499728 | -0.008541953 | 0.07853651 | 0.1428744 |
| temperature-nutrient | 0.15593304 | 0.112393808 | 0.19947227 | 0.0000000 |
| temperature-ph | 0.12093576 | 0.077396529 | 0.16447499 | 0.0000000 |

**Table 25 ANOVA and Tukeys WYD09 BP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 0.181 | 0.09029 | 2.085 | 0.125 |
| Residuals | 645 | 27.931 | 0.04330 | | |

| | Diff | lwr | upr | p adj |
|---|---|---|---|---|
| ph-nutrient | -0.02902461 | -0.07606405 | 0.018014823 | 0.3162336 |
| Temperature-nutrient | -0.03945654 | -0.08649597 | 0.007582897 | 0.1203902 |
| temperature-ph | -0.01043193 | -0.05747136 | 0.036607510 | 0.8611266 |

**Table 26 ANOVA and Tukeys WYT116 CBP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 6.176 | 3.0880 | 79.07 | <2e-16*** |
| Residuals | 645 | 25.190 | 0.0391 | | |

| | diff | lwr | upr | P adj |
|---|---|---|---|---|
| ph-nutrient | -0.01205634 | -0.0567275 | 0.03261483 | 0.8014339 |
| temperature-nutrient | -0.21286192 | -0.2575331 | -0.16819075 | 0.0000000 |
| temperature-ph | -0.20080558 | -0.2454767 | -0.15613442 | 0.0000000 |

**Phage PCF SBW25**

**Table 27 ANOVA and Tukeys WYD09 CBP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 7.00 | 3.499 | 68.49 | <2e-16*** |
| Residuals | 645 | 32.95 | 0.051 | | |

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| ph-nutrient | 0.09885643 | 0.0477649 | 0.1499480 | 1.95e-05 |
| temperature-nutrient | 0.25256439 | 0.2014729 | 0.3036559 | 0.00e+00 |
| temperature-ph | 0.1537079 | 0.1026164 | 0.2047995 | 0.00e+00 |

**Table 28 ANOVA and Tukeys WYD09 BP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 0.72 | 0.3592 | 4.908 | 0.00766** |
| Residuals | 645 | 47.20 | 0.0732 | | |

| | diff | lwr | upr | p adj |
|---|---|---|---|---|
| ph-nutrient | 0.08142195 | 0.02027411 | 0.14256979 | 0.0052210 |
| temperature-nutrient | 0.04474893 | -0.01639891 | 0.10589677 | 0.1987765 |
| temperature-ph | -0.03667302 | -0.09782086 | 0.02447483 | 0.3368762 |

**Table 29 ANOVA and Tukeys WYT116 CBP**

| | DF | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Factor | 2 | 5.86 | 2.9294 | 51.76 | <2e-16*** |
| Residuals | 645 | 36.51 | 0.0566 | | |

| | Diff | Lwr | Upr | P adj |
|---|---|---|---|---|
| ph-nutrient | -0.04117774 | -0.09495502 | 0.01259953 | 0.1707919 |
| temperature-nutrient | -0.21911883 | -0.27289611 | -0.16534156 | 0.0000000 |
| temperature-ph | -0.17794109 | -0.23171837 | -0.12416382 | 0.0000000 |

## Community constraint bargraphs

### Table 30: Community constraint ANOVA and Tukeys – high nutrient

| Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|
| High nutrient | 2 | 2.933 | 1.4663 | 5.454 | 0.00632 ** |
| Residuals | 69 | 18.551 | 0.2689 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.4931943 | -0.8517254 | -0.1346631 | 0.0043849 |
| WYT116-none | -0.2759521 | -0.6344833 | 0.0825791 | 0.1631869 |
| WYT116-WYD09 | 0.2172422 | -0.1412890 | 0.5757734 | 0.3206510 |

### Table 31: Community constraint ANOVA and Tukeys – standard nutrient

| Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|
| Standard Nutrient | 2 | 1.952 | 0.9758 | 5.031 | 0.00913 ** |
| Residuals | 69 | 13.382 | 0.1939 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.37496804 | -0.67947673 | -0.07045936 | 0.0119358 |
| WYT116-none | -0.05896102 | -0.36346971 | 0.24554766 | 0.8883971 |
| WYT116-WYD09 | 0.31600702 | 0.01149833 | 0.62051570 | 0.0402078 |

### Table 32: Community constraint ANOVA and Tukeys – low nutrient

| | df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Low nutrient | 2 | 5.939 | 2.970 | 11.97 | 3.44e-05 *** |
| Residuals | 69 | 17.115 | 0.248 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.6992206 | -1.04359845 | -0.35484268 | 0.0000207 |
| WYT116-none | -0.2824814 | -0.62685930 | 0.06189647 | 0.1287094 |
| WYT116-WYD09 | 0.4167391 | 0.07236126 | 0.76111703 | 0.0137425 |

### Table 33: Community constraint ANOVA and Tukeys – pH 5.5

| Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|
| pH 5.5 | 2 | 6.375 | 3.188 | 24.95 | 7.04e-09 *** |
| Residuals | 69 | 8.817 | 0.128 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.6893663 | -0.9365372 | -0.4421954 | 0.0000000 |
| WYT116-none | -0.5497116 | -0.7968825 | -0.3025407 | 0.0000035 |
| WYT116-WYD09 | 0.1396547 | -0.1075162 | 0.3868257 | 0.3709706 |

### Table 34: Community constraint ANOVA and Tukeys – pH 6.5

| Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|
| pH 6.5 | 2 | 2.864 | 1.4320 | 7.304 | 0.00133 ** |
| Residuals | 69 | 13.528 | 0.1961 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.4798499 | -0.78601917 | -0.1736805 0.0010354 |
| WYT116-none | -0.1604880 | -0.46665737 | 0.1456813 0.4250242 |
| WYT116-WYD09 | 0.3193618 | 0.01319249 | 0.6255311 0.0389698 |

**Table 35: Community constraint ANOVA and Tukeys – pH 8**

| Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|
| pH 8 | 2 | 0.788 | 0.3940 | 3.435 | 0.0378 * |
| Residuals | 69 | 7.914 | 0.1147 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.18258687 | -0.41676177 | 0.05158804 0.1558498 |
| WYT116-none | 0.06442536 | -0.16974955 | 0.29860026 0.7879068 |
| WYT116-WYD09 | 0.24701223 | 0.01283732 | 0.48118713 0.0363609 |

**Table 36: Community constraint ANOVA and Tukeys – 15C**

| Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|
| Temperature 15C | 2 | 6.424 | 3.212 | 17.11 | 9.21e-07 *** |
| Residuals | 69 | 12.949 | 0.188 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.5639238 | -0.8634743 | -0.2643733 0.0000763 |
| WYT116-none | -0.6856709 | -0.9852214 | -0.3861203 0.0000019 |
| WYT116-WYD09 | -0.1217471 | -0.4212976 | 0.1778035 0.5959606 |

**Table 37: Community constraint ANOVA and Tukeys – 22C**

| Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|
| Temperature 22C | 2 | 3.855 | 1.9275 | 12.01 | 3.34e-05 *** |
| Residuals | 69 | 11.072 | 0.1605 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | 0.03214017 | -0.2448439 | 0.3091242 0.9583349 |
| WYT116-none | -0.47399311 | -0.7509772 | -0.1970091 0.0003251 |
| WYT116-WYD09 | -0.50613328 | -0.7831173 | -0.2291492 0.0001227 |

**Table 38: Community constraint ANOVA and Tukeys – 30C**

| Df | Sum Sq | Mean Sq | F value | Pr(>F) | |
|---|---|---|---|---|---|
| Temperature 30C | 2 | 1.616 | 0.8079 | 8.069 | 0.00071 *** |
| Residuals | 69 | 6.909 | 0.1001 | | |

| diff | lwr | upr | p adj |
|---|---|---|---|
| WYD09-none | -0.1851528 | -0.4039590 | 0.03365351 0.1133949 |

| | | | | |
|---|---|---|---|---|
| WYT116-none | -0.3669471 | -0.5857534 | -0.14814083 | 0.0004304 |
| WYT116-WYD09 | -0.1817943 | -0.4006006 | 0.03701193 | 0.1222751 |

**Table 39: T tests for SBW25 fitness WYD09**

| | |
|---|---|
| **data: WYD09 high nutrient** | t = 12.845, df = 23, p-value = 5.621e-12 |
| 95 percent confidence interval: | 0.5073187 0.7020990 |
| mean of the differences: | 0.6047089 |
| **data: WYD09 standard nutrient** | t = 13.053, df = 23, p-value = 4.05e-12 |
| 95 percent confidence interval: | 0.3468546 0.4774963 |
| mean of the differences: | 0.4121754 |
| **data: WYD09 low nutrient** | t = 6.9675, df = 23, p-value = 4.215e-07 |
| 95 percent confidence interval: | 0.2560355 0.4722714 |
| mean of the differences: | 0.3641534 |
| **data: WYD09 pH 5.5** | t = 10.42, df = 23, p-value = 3.496e-10 |
| 95 percent confidence interval: | 0.2809551 0.4201365 |
| mean of the differences: | 0.3505458 |
| **data: WYD09 pH 6.5** | t = 6.6728, df = 23, p-value = 8.301e-07 |
| 95 percent confidence interval: | 0.2562002 0.4864230 |
| mean of the differences: | 0.3713116 |
| **data: WYD09 pH 8** | t = 11.937, df = 23, p-value = 2.455e-11 |
| 95 percent confidence interval: | 0.3051994 0.4331545 |
| mean of the differences: | 0.3691769 |
| **data: WYD09 temperature 15°C** | t = 1.5237, df = 23, p-value = 0.1412 |
| 95 percent confidence interval: | -0.03010883 0.19849476 |
| mean of the differences: | 0.08419297 |
| **data: WYD09 temperature 22°C** | t = 4.6263, df = 23, p-value = 0.0001181 |
| 95 percent confidence interval: | 0.09346009 0.24464136 |
| mean of the differences: | 0.1690507 |
| **data: WYD09 temperature 30°C** | t = 0.74044, df = 23, p-value = 0.4665 |
| 95 percent confidence interval: | -0.05966684 0.12619114 |
| mean of the differences: | 0.03326215 |

**Table 40: T tests for SBW25 WYT116**

| | |
|---|---|
| **data: WYT116 high nutrient** | t = 5.3169, df = 23, p-value = 2.133e-05 |
| 95 percent confidence interval: | 0.1354346 0.3079385 |
| mean of the differences: | 0.2216865 |
| **data: WYT116 standard nutrient** | t = 2.5913, df = 23, p-value = 0.01632 |
| 95 percent confidence interval: | 0.0141658 0.1263049 |
| mean of the differences: | 0.07023534 |
| **data: WYT116 low nutrient** | t = 3.8474, df = 23, p-value = 0.0008211 |
| 95 percent confidence interval: | 0.06061936 0.20161820 |
| mean of the differences: | 0.1311188 |
| **data: WYT116 pH 5.5** | t = 3.1046, df = 23, p-value = 0.004993 |
| 95 percent confidence interval: | 0.05262218 0.26277835 |
| mean of the differences: | 0.1577003 |
| **data: WYT116 pH 6.5** | t = 0.58673, df = 23, p-value = 0.5631 |
| 95 percent confidence interval: | -0.09835069 0.17623013 |

| | |
|---|---|
| mean of the differences: | 0.03893972 |
| **data: WYT116 pH 8** | t = 3.5993, df = 23, p-value = 0.001513 |
| 95 percent confidence interval: | 0.07001751 0.25927072 |
| mean of the differences: | 0.1646441 |
| **data: WYT116 temperature 15°C** | t = 9.4686, df = 23, p-value = 2.12e-09 |
| 95 percent confidence interval: | 0.2160687 0.3368735 |
| mean of the differences: | 0.2764711 |
| **data: WYT116 temperature 22°C** | t = 8.1632, df = 23, p-value = 3.028e-08 |
| 95 percent confidence interval: | 0.2694809 0.4524180 |
| mean of the differences: | 0.3609494 |
| **data: WYT116 temperature 30°C** | t = 10.058, df = 23, p-value = 6.849e-10 |
| 95 percent confidence interval: | 0.2978734 0.4521208 |
| mean of the differences: | 0.3749971 |

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*"Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for*

*something and that this thing must be attained."*

— *Marie Curie*