Diversity and Competitive Interactions in Experimentally Evolved Bacterial Populations

Quan-Guo Zhang

Jesus College, University of Oxford

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Supervisors: Prof. H Charles J Godfray

Dr. Angus Buckling

Dr. Richard J Ellis

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Abstract

Laboratory bacterial populations provide ideal opportunities to experimentally test theories in ecology and evolutionary biology. I used a model laboratory microbial system, *Pseudomonas fluorescens* SBW25, to address an array of questions on the origin, maintenance, and functional role of biodiversity, and the evolution of biotic interactions. My thesis reports experiments with the following conclusions. (1) The extent of diversification in *P. fluorescens* populations is not affected by the presence of an interspecific competitor *P. putida*, although the early stage of the diversification in one environment (spatially homogeneous environment) could be speeded up by the competitor. (2) Niche and neutral mechanisms simultaneously contribute to the maintenance of phenotypic diversity in *P. fluorescens* populations; but the operation of niche processes does not lead to a positive effect of biodiversity on ecosystem functioning. (3) The competitive interactions among bacterial phenotypes are generally transitive, and competitive hierarchies inferred from pair-wise competition are fairly consistent to those from multi-species competition. (4) The niche complementarity and selection effects evaluated by random assembly biodiversity experiments can be used to predict the functional consequences of particular non-random species extinction scenarios. (5) *P. fluorescens* does not show an evolutionary trade-off in using several carbon substrates (glucose, galactose and trehalose), and evolution in environments containing these resources results in imperfect generalists; migration among populations may speed up fitness evolution of some generalists. (6) Biofilm formation at the air-broth interface by wrinkly spreader phenotypes in *P. fluorescens* is a cooperative behaviour which is costly to individuals but benefits the group; this behaviour could be exploited by smooth morph phenotypes. The cooperators and cheats in this system show reciprocal antagonistic coevolution in resistance and cheating performance.

Statement

I carried out all experimental work and statistical analyses in this thesis. Charles Godfray, Angus Buckling and Richard Ellis provided guidance in experimental design and data analyses, and helped me with writing.

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Contents

Chapter 1: Introduction

1.1 Laboratory microbial populations for ecological and evolutionary studies

Ecology and evolutionary biology stem from natural history studies; however, without rigorous experimental tests of their main principles, they are unlikely to become 'hard sciences' with high predictive power and repeatability. The complexity of environmental context in which ecological processes and evolutionary changes occur in nature often makes it very difficult, if not impossible, to detect the responsible mechanisms. Ecological and evolutionary studies based on field observations are often unrepeatable either spatially or temporally. Experiments in well-controlled environments reduce complexity to the points where ecological consequences and evolutionary outcomes can be directly attributable to particular mechanisms (Lenski 1992; Rainey *et al.* 2000).

Laboratory microbial populations are well suited for experimental ecological and evolutionary studies: environmental factors can be carefully manipulated in the laboratory; reproduction by binary fission means that replicate isogenic populations can be established; they have short generation time and large population sizes; evolved and ancestral genotypes can be viably stored at -80ºC. Therefore, laboratory microbial populations provide experimental systems with high simplicity and repeatability, in which genetic drift can be very weak, and fitness change can be attributed to given selection regimes. Long-term studies are tractable with such systems, and evolved genotypes can be compared with their ancestors directly (Travisano & Rainey 2000).

1.2 The origin and maintenance of diversity in bacterial populations

The origin and maintenance of biodiversity are among the central topics in ecology and evolutionary biology. Biodiversity has intraspecific and interspecific components. Intraspecific diversity is converted to species diversity by the process of speciation. In sympatric speciation, within-population diversity becomes interspecific diversity, while in allopatric speciation between-population diversity does so. Therefore intraspecific variation is the starting point for understanding the origin and maintenance of biodiversity at higher levels. Between-population (allopatric) diversity can be attributed to different selection regimes over habitats or genetic drift. Within-population (sympatric) diversity is a more challenging and interesting topic.

1.2.1 The origin of diversity in bacterial populations

Bacteria are haploids and asexually reproduce themselves. In natural bacterial populations diversity can arise from both mutation and sexual processes, conjugation, transformation and transduction (Maynard Smith 1990; Cohan 1994); the relative importance of mutation and sexual processes is unclear. In benign laboratory environments, however, sexual processes are very limited; microbial populations can be founded from single genotype, and various genotypes are entirely produced by mutation.

1.2.2 The maintenance of diversity in bacterial populations

Bacteria reproduce themselves asexually in most situations, therefore each genotypic lineage is analogous to a species in communities of sexual organisms (Kassen *et al.* 2000). This makes some essential differences between bacteria and animals or plants; for instance, a mechanism to maintain within-population (genotypic) diversity in sexual populations is overdominance in which heterozygotes are selected for and

continually produce homozygotes (Lewontin 1974), but it is irrelevant to bacteria. Since genotypes in a diversified bacterial population are analogous to species, community ecology can immediately provide a framework for understanding the maintenance of genetic diversity within bacterial populations. Maintenance of species diversity can be achieved in two ways: (1) species coexistence resulting in constant species diversity and composition, or (2) continuous replacement of species resulting in constant species diversity but dynamical species composition. The former process received much more attention from ecologists than did the latter one (Chesson 2000). Species coexistence mechanisms can function in two major ways: stabilizing (increasing negative intraspecific interactions relative to interspecific interactions; see below 'stable coexistence'), or equalizing (minimizing fitness differences between species to reduce the speed of competitive exclusion). Stabilizing and equalizing mechanisms can operate simultaneously; equalizing mechanisms by themselves can slow the process of competitive exclusion but do not ensure long-term stable species coexistence (Chesson 2000; Hubbell 2001).

1.2.2.1 Mechanisms of species diversity maintenance in community ecology

Stable coexistence

Stable coexistence requires mechanisms to favour rare species in population growth rate, i.e. negative frequency-dependent selection. The stable coexistence mechanisms can be further classified into fluctuation-independent and fluctuation-dependent mechanisms (Chesson 1994, 2000). In fluctuation-independent mechanisms, spatial or temporal variation in environment is unnecessary for species coexistence. Examples of fluctuation-independent mechanisms are resource or habitat partitioning

(Tilman 1982; Grover 1997), frequency-dependent predation (Gendron 1987), and population size-dependent sex ratio adjustment by which local mate competition and inbreeding lead to female-biased sex ratio in rare species and hence promote stable coexistence of species (Zhang & Hanski 1998).

Fluctuation-dependent mechanisms involve temporal or spatial change in environment. One example is the storage effect, in which different species use the same resources, but differ from each other in the time when they are most actively consuming the resources (Armstrong 1976; Abrams 1984). Spatial variation can promote species coexistence in a way analogous to temporal fluctuation, i.e. spatial storage effect (Chesson 2000). In discrete and patchy habitats, species with a trade-off in competitive ability and dispersal abilities can coexist (Armstrong 1989b; Tilman 1994); some species show intraspecific aggregation in ephemeral patchy habitats, more than one such species can coexist even when they have the same resource requirements (Atkinson & Shorrocks 1981; Hanski 1981).

Furthermore, some interspecific interactions *per se* can promote coexistence between species without niche differentiation in respect of environment requirements. Non-intransitive competitive interactions, like rock-paper-scissors games, occur among some species; in such cases, species can coexist because, although there is a winner in any pair-wise competitive interaction, each species outcompetes and is outcompeted by an equal number of competitors (Gilpin 1975; Feldgarden 1999; Huisman & Weissing 1999; Czaran *et al.* 2002). Recently, such a rock-paper-scissors interaction was even found among two genotypes within one species and a second species (Lankau & Strauss 2007). Intraguild predation may also promote species coexistence, if the species capable

of intraguild predation are less effective in exploring shared resource than their competitors (Polis *et al.* 1989; Holt 1997).

Neutral coexistence

Species that are similar enough to each other can coexist over a very long period of time because their difference in fitness is very small and the process of competitive exclusion is very slow. Hubbell (1979; 2001) and Bell (2001) developed a neutral theory of biodiversity to explain species abundance distribution, in which species compete for space and individuals from all species are ecologically identical. In their models there is no fitness difference between species or stabilizing mechanisms for species coexistence; species in such systems undergo a very slow random walk to extinction. In some respects, a very slow loss of species is equivalent to indefinite coexistence. Furthermore, Hubbell (2005; 2006) proposed that ecological equivalence for resource use between species is likely to evolve easily and often, especially in species-rich communities that are dispersal and recruitment limited. The neutral theory of biodiversity enjoys great success in fitting species abundance distribution in many communities (Hubbell 2001; Chave 2004). However, niche processes may also produce species abundance patterns fitting neutral predictions (Purves & Pacala 2005; Leibold & McPeek 2006). It is unclear whether neutral coexistence is common in the natural world.

Unstable coexistence

Continuous immigration may ensure the persistence of species in habitats where otherwise they are purged by competitive exclusions (Shmida & Wilson 1985; Holt 1993; Loreau & Mouquet 1999). This type of coexistence is unstable and needs no biological explanation at the local scale, but may have some ecological consequences. For instance,

it is one mechanism to produce functionally redundant species in local communities (Loreau 2004).

Maintenance of diversity with dynamical species composition

Species diversity is often consistent over time in a habitat but species composition is dynamical. For instance, in the classic 'island biogeography theory' (MacArthur & Wilson 1967), an equilibrium of species diversity in islands can be achieved, where species introduction and extinction balance each other; but the species composition may be varying over time.

1.2.2.2 Mechanisms relevant to genotypic diversity of bacterial populations

Theoretically, all mechanisms of species diversity maintenance mentioned above, except for those requiring sexual processes, should be applicable to genotypic diversity in bacterial population. Many of the mechanisms have been well documented in microbial studies. For example, in static microcosms divergent genotypes of the bacterium *Pseudomonas fluorescence* use different habitats (liquid phase, air-broth interface and vial bottom) to stably coexist (Rainey & Travisano 1998). In the presence of bacteriophage, spontaneous evolution of phage-resistant bacteria from populations of sensitive types have been detected in several studies, where a trade-off between bacterial growth rate and susceptibility to predation by bacteriophage ensure coexistence of sensitive and resistant genotypes (Lenski & Levin 1985; Bohannan & Lenski 2000). Temporal variation in resource may promote coexistence of genotypes if the competitive superiors in different temporal seasons are distinct genotypes (Turner 1996; Spencer *et al.* 2007). Cross-feeding (analogous to intraguild predation), in which some genotypes consume metabolites excreted by the others, can lead to stable coexistence of genotypes

in bacterial populations (Helling *et al.* 1987; Turner 1996; Rozen 2000). Nearly stable coexistence is possible when mutations of small fitness benefit are responsible for the emergence of new genotypes and purifying periodic selection is not strong enough to eliminate the diversity quickly (Maharjan *et al.* 2006; Fukami *et al.* 2007).

On the other hand, methodological limitations have frustrated microbial studies on some questions. For example, we do not know whether there is fugitive coexistence or coexistence through intra-genotypic aggregation in bacterial communities. It is difficult to artificially produce a patchy environment on a spatial scale fine enough for bacteria; it is also difficult to measure the mobility and aggregation behaviours of bacteria.

1.2.3 Adaptive radiation in experimental bacterial populations

Research on the origin and maintenance of biodiversity is well integrated in the studies of radiation, which is the evolution of ecological and phenotypic diversity in a rapidly multiplying lineage. A radiation involves the differentiation of a single ancestor into an array of species/phenotypes that can coexist over a period of time. Schluter (2000b) classified radiation into adaptive radiation and non-adaptive radiation. In the former, a single ancestor differentiates into an array of species/phenotypes that inhabit a variety of environments and that differ in the morphological and physiological traits used to exploit those environments. In the latter, rapid proliferation of species from an ancestor occurs with negligible or infrequent ecological differentiation (Gittenberger 1991) or with morphological and physiological differentiation unrelated to resource use or environmental adaptation (Brooks et al. 1985). Adaptive radiation has received much more attention than non-adaptive radiation, possibly because it is more likely to occur in nature, or there is a study or publication bias. Divergent natural selection between

environments is the key factor permitting adaptive radiation, and divergence of lineages from a single ancestor can happen via resource partitioning, apparent competition, or antagonistic interference (Schluter 2000b). Examples of adaptive radiation in microbes are evolution of stable polymorphism of cross-feeding in *Escherichia coli* (Helling et al. 1987) where one genotype consume metabolites excreted by the other, and spatial niche differentiation in diversified *P. fluorescens* populations (Rainey & Travisano 1998). The latter system is very promising for evolutionary studies because the divergent genotypes have obvious spatial preferences and distinct colony morphologies, and this adaptive radiation has been repeatedly observed in different laboratories.

In spatially heterogeneous environment (a static bottle containing nutrient broth medium), *P. fluorescens* (SBW25) populations rapidly diversify, generating by mutation a range of phenotypes distinguishable by their heritable colony morphologies on agar plates. These variants fall into three categories: smooth (SM) morphs which resemble the ancestor and inhabit the liquid phase, wrinkly-spreader (WS) morphs which colonize the air-broth interface and form a mat, and fuzzy spreader (FS) morphs which colonize the bottom of the vial. FS are much rarer than SM and WS. Within each of the three main categories multiple variants exist (Rainey & Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2000). Population evolving in spatially homogeneous environment (continuously shaken tubes) are dominated by the SM types. Diversity in spatially heterogeneous environment is maintained by negative frequency-dependent selection. When the three categories of genotypes are competing with each other, the rare categories generally enjoy an advantage in growth rate over the abundant ones (Rainey & Travisano 1998). However, it remains unclear how variants within each category coexist, although

studies have reported both stable (Brockhurst *et al.* 2006) and neutral (Fukami *et al.* 2007) coexistence among several WS phenotypes.

Several environmental factors were found to shape the diversification of *P. fluorescens* populations. First, the diversification can occur in spatially heterogeneous environment but not in homogeneous environment (Rainey & Travisano 1998). Second, in spatially heterogeneous environments, phenotypic diversity of *P. fluorescens* populations shows a unimodal relationship with habitat productivity (Kassen *et al.* 2000); such a relationship is also found for phenotypic diversity and disturbance frequency (Buckling *et al.* 2000). When nutrient and disturbance frequency are manipulated simultaneously, diversity peaked at intermediate nutrient concentration and disturbance frequency (Kassen *et al.* 2004). Third, in the presence of a virulent phage SBW25Φ2, the within-population phenotypic diversity of *P. fluorescens* in spatially heterogeneous environment is reduced, while between-population diversity is increased (Buckling & Rainey 2002b; Brockhurst *et al.* 2004); furthermore, the presence of this phage can eliminate the unimodal relationship between diversity and disturbance frequency (Morgan & Buckling 2004), or that between diversity and productivity and disturbance (Benmayor *et al.* 2008), in spatially heterogeneous environment. In the presence of a protist predator (*Tetrahymena thermophila*), the diversification of *P. fluorescens* can be delayed, probably due to the reduction in prey density caused by predation, but the extent of diversification is not affected (Meyer & Kassen 2007).

1.3 The functional consequences of diversity in bacterial populations

The relationship between biodiversity and ecosystem functioning (BEF) has been a long-standing question in ecology since Charles Darwin and motivated considerable research in the past two decades (Schulze & Mooney 1993; Kinzig *et al.* 2001; Loreau *et al.* 2002). The majority of studies in this area have been concerned with the effect of plant species richness on ecosystem productivity; they often manipulated species richness by using randomly chosen species from a pool to assemble communities containing different numbers of species (Tilman *et al.* 1996; Hooper & Vitousek 1997; McGrady-Steed *et al.* 1997; Symstad *et al.* 1998; Hector *et al.* 1999; Naeem *et al.* 2000; Mulder *et al.* 2001; Tilman *et al.* 2001; Cardinale *et al.* 2002; Fridley 2002; Pfisterer & Schmid 2002; van Ruijven & Berendse 2005). Ecologists often, although not always, found a positive effect of biodiversity on productivity, which could be explained by two non-mutually exclusive mechanisms: (1) niche complementarity, differences in resource/habitat use among species or interspecific facilitation (Tilman *et al.* 1997; Loreau 2000), and (2) the selection effect, which means that species-rich communities have a greater chance to contain a high-yielding species (Aarssen 1997; Huston 1997).

Laboratory microbes are ideal systems for testing the theoretical hypotheses on BEF relationships. Several studies have employed *P. fluorescens* for this purpose. A study found that that the selection effect is the main diver of a positive effect of phenotypic diversity on productivity or stability in *P. fluorescens* populations (Hodgson *et al.* 2002); another study with co-evolved biofilm-forming WS phenotypes found that character displacement in resource utilization among phenotypes increased the biofilm's productivity and resistance to the invasion of SM (Brockhurst *et al.* 2007a); a more recent study with meta-populations of this bacterium found that intermediate dispersal promotes

adaptation of populations to their local environments, which results in high levels of diversity and productivity on the meta-population level (Venail *et al.* 2008).

Laboratory microbial systems may be especially useful for linking species coexistence mechanisms and BEF relationships. The mechanisms responsible for the maintenance of species diversity are often believed to influence the BEF relationships (Kinzig & Pacala 2001; Mouquet *et al.* 2002; Fox 2003; Loreau 2004). For example, models based on niche processes often predict a positive relationship between diversity and ecosystem properties such as biomass production, as a result of more efficient total resource use (Tilman 1999; Loreau 2000); neutral theory assumes that all species are functionally equivalent and hence diversity has no effect on ecosystem properties (but see Loreau 2004; Pueyo *et al.* 2007; Zhou & Zhang 2008). Both stable and neutral coexistence may contribute to phenotypic diversity in bacterial populations (as mentioned above); in particular, a single *P. fluorescens* system may see both stable (Rainey & Travisano 1998) and neutral (Fukami *et al.* 2007) coexistence of bacterial phenotypes. Therefore, *P. fluorescens* may be an ideal system to address the link between BEF relationship and species coexistence mechanisms.

1.4 Evolution of ecological interactions in microbes

Not only physical but biotic environments are crucial to fitness of organisms. Meanwhile, the ecological interactions among organisms can evolve through time. There are two basic classes of ecological interactions, mutualistic ([+,+]) and antagonistic ([-,-] or [+,-]). Mutualistic interactions, especially highly specialized ones, should not change much over time, because once a mutualistic partnership has formed, the genotypes

responsible will be fixed in the populations. Most of the change experienced by organisms is contributed by antagonistic relationships (Bell 1997); the mutual antagonistic organisms live in the country of the Red Queen where it is necessary to run as one can to stay the same place (van Valen 1973).

There have been some theoretical insights into the evolution of ecological interactions, for example, it has long been suggested that sympatric congeneric species should exhibit greater morphological divergence than allopatric ones (i.e. character displacement), resulting from competition in the past (Brown & Willson 1956); predators evolve to be more and more aggressive and preys more and more efficient in defence, i.e. arms race (Abrams 1986). However, direct empirical evidence is scarce because evolution in most organisms is very slow and undetectable during the period of an investigator's life. Laboratory microbes provide ideal systems for such studies.

1.4.1 Resource competition

It is a simple logic that species (or genotypes within species) sharing resources should become more and more different because the types that are most different from the bulk of the combined community will suffer less competition (Bell 1997; Schluter 2000b). In this process, the competitors should evolved specialization on particular niches. Organisms can escape competition by exploring new resources, in some cases they even use the metabolites excreted by their competitors (Helling *et al.* 1987; Turner 1996; Rozen 2000).

However, competitors can also become more and more similar because of diffuse coevolution in species-rich communities (Connell 1980), which results in niche convergence towards generalist strategies rather than divergence to specialist strategies.

Hubbell and colleagues (Hubbell & Foster 1986; Hubbell 2005; Hubbell 2006) elaborated this idea. In species-poor communities with dispersal and recruitment limitation the direction of selection on progeny of individuals of each species is strong and consistent because the individuals of a species always encounter the same competitors. However, in species-rich communities, there is no consistency in the direction of selection within and between species because different individuals within a species would meet different competitors and experience different selection regimes; therefore the species evolves to be generalist with broad niche overlap, although individuals can be habitat-specialized. At the species level, no character displacement can be observed. In this case the competitive species becomes more and more similar to each other, and ecological equivalence is manifested (Hubbell 2005; Hubbell 2006).

Some authors have experimentally addressed the coevolution of competitors, mostly using paired crop species. They observed inconsistent results (see Bell 1997). A more promising approach is to examine the evolution of close related competitors, to detect character displacement (Schluter 2000a). A study with monomorphic *P. pseudomonas* populations (MacLean *et al.* 2005) investigated whether competition for shared resources cause adaptation to alternative resources; all populations were founded by two isogenic clones, and grown in spatially homogeneous environment for 160 generations. Differentiation in metabolic traits evolved among within-population (sympatric) genotypes, but not between-population (allopatric) genotypes. These metabolic traits are related to resource acquisition and frequency-dependent trade-offs in competitive ability. In their study the bacteria derived from one ancestral isolate evolved narrower catabolic function (i.e. the evolved bacteria consume smaller number of carbon

substrates than did the ancestor) and those from the other ancestral isolate showed no obvious change. In another study with the same bacterium (Barrett *et al.* 2005), environments with substitutive carbon sources (up to 8 carbon substrates) favoured imperfect generalists but not narrow specialists or a complete generalist.

There is yet no empirical evidence for diffuse coevolution. Many factors may shape competitors' coevolution. For example, the presence of predators might drive the competitors to differentiate in defence behaviours rather than in resource acquisition traits; migration among communities might dampen the local adaptation between competitors. These questions have been largely unexplored.

1.4.2 Prey-exploiter interactions: bacteria and phages

Exploiters (grazers, predators, parasites and parasitoids) may have arms race-like interactions with their victims. A common prey-exploiter interaction in microbial world is bacteria-bacteriophage interaction. The genetics underlying bacteria-bacteriophage interactions are frequently 'gene-for-gene' (Thompson 1992), which allows the parasites to have 'universal virulence' (infecting all genotypes of the hosts), and allows the hosts to be resistant to a wide range of parasite genotypes. The coevolution between bacteria and phages is like an 'arms race'. Ancestral sensitive bacteria evolve resistance to phages (this change most often occurs through mutations that change or eliminate the cell surface receptor molecule), after which the phages evolve expanded host range (e.g. by mutations that change the tail fibres). Coevolution occurs in which host bacteria become resistant to a wider range of phage genotypes and the phages infect a wider range of host genotypes. In some cases this arms race is asymmetrical, e.g. in *E. coli* B some resistant types can arise that no mutant phage of T7 can infect (Lenski & Levin 1985). Coevolution between

bacteria and phages can follow divergent trajectories in replicate communities and result in local adaptation of bacteria to phages (Buckling & Rainey 2002a).

The evolution of bacteria-phage system may be shaped by several factors. (a) Higher habitat productivity can cause faster evolution and invasion of resistant bacteria (Bohannan & Lenski 1997; Lopez-Pascua & Buckling 2008). (b) Community homogenization (mixing) can accelerate the coevolution rate (Brockhurst *et al.* 2003). (c) Migration can drive dynamics of local adaptation. In one study dispersal of both bacteria and phage from a nutrient-rich community to a nutrient-poor community accelerated adaptation of phages in the nutrient-poor community (Forde *et al.* 2004). In another study migration of bacteria and phages were independently manipulated; phage migration promoted phage local adaptation, but bacteria migration had little effect (Morgan *et al.* 2005).

1.4.3 Cooperation and cheating

Cooperators and cheats compete for shared resources, meanwhile the cheats benefit from the cooperators; therefore there are essential differences between resource competition and cooperation/cheating interactions. In resource competition, organisms are under negative density-dependent selection and negative-frequency dependent selection; in cooperation/cheating interactions, the cheats are under both negative density and negative frequency-dependent selection, but for the cooperators, selection can be negative density-dependent, because of competition among cooperators, and positive frequency-dependent, because of the presence of the cheats.

There are abundant modelling studies on the question of how a population consisting of cooperators can resist invasion by cheaters who selfishly use common

resources to maximize their individual reproduction at the expense of the group, and there have been some experimental tests. The followings are several examples. In an experiment with two strains of yeast with alternative pathways of glucose metabolism (MacLean & Gudelj 2006), the cheats (respiro-fermenter) excluded the cooperators (respirer) when they competed for a global pool of resources in a homogeneous environment; cooperators resisted invasion by cheats when they competed for local resource patches in spatially structured environment; temporal variability in resource availability also promoted coexistence of cooperators and cheats. In another experiment with mercury resistant (cooperator) and sensitive (cheat) strains of *P. fluorescence* (the former carries a plasmid that can remove mercury from environment; while the latter benefits from the former and has no burden of the plasmid), researchers found that the cheats and cooperators could coexist in both spatially homogeneous and structured environments, probably due to the temporal dynamics of bacterial growth and mercury removal (Ellis *et al.* 2007). In an experimental metacommunity of *E. coli* and T4 phages, the phages evolved different strategies under different migration regimes. When migration is spatially restricted, prudent phages (cooperators, which consume host slowly and maintain high productivity of host and thus that of themselves) dominate; under unrestricted migration, rapacious phages (cheats, which consume host rapidly and lead to 'tragedy of the commons') exclude the prudent ones (Kerr *et al.* 2006).

Many questions in this field want for experimental tests. For example, is there a minimum threshold population size for the invasion of the cheats; how does this antagonistic interaction respond to habitat productivity; is there local adaptation in

cooperators or cheats to each other; do cooperators and cheat show arms race-like coevolution?

1.5 Aims

My thesis aims to investigate an array of questions on the origin, maintenance and consequence of biodiversity, and the dynamics of cooperator-cheat coevolution, using *P. fluorescens* system. Specifically, I address the following questions:

(1) How interspecific competition shapes the adaptive radiation within *P. fluorescens* populations (Chapter 2).

(2) What is the relative importance of stabilizing and equalizing mechanisms (niches and neutrality, respectively) for the coexistence of phenotypes in divergent *P. fluorescens* populations (Chapter 3)?

(3) Whether the competitive interactions among phenotypes are hierarchy-like or non-transitive (Chapter 4)?

(4) How the random assembly biodiversity experiments can predict the functional consequence of non-random species (phenotypes) loss for ecosystem productivity (Chapter 5)?

(5) How bacteria evolve in habitats along a continuous environmental gradient, and how migration affects the evolution (Chapter 6)?

(6) Whether cooperators and cheats can show arms race-like coevolution (Chapter 7)?

Chapter 2: The effect of interspecific competition on diversification in experimental *Pseudomonas fluorescens* populations

Summary

To reveal the ecological forces shaping adaptive radiations is important for understanding the evolution of biodiversity. A radiating lineage may experience competition from other taxa; such competition has long been suggested as a constraint on adaptive radiation of the radiating lineage. I experimentally addressed how competition from the bacterium *Pseudomonas putida* affected the diversification of a bacterium *Pseudomonas fluorescens*. The extent of diversification (the number of co-occurring phenotypes) in *P. fluorescens* populations was not affected by the presence of the interspecific competitor in either spatially heterogeneous or homogeneous environment. In spatially homogeneous environment, however, the rate of diversification was increased by the competitor in the early stage of the radiation. The results suggest that there may not be a general pattern in the effect of competitors on diversification in radiating lineages.

2.1 Introduction

Adaptive radiation, the generation of ecological and phenotypic diversity in a lineage to allow its colonization of new niches, plays an important role in the evolution of biodiversity. Understanding the forces causing and shaping adaptive radiation is an important and challenging task in evolutionary biology. Adaptive radiation could result from divergent selection for specialization on alternative habitats or different resources within habitats. For adaptive radiations occurring in sympatry, competition for common resources among forms within a radiating lineage is thought to generate divergent selection for specialization on underutilized or novel resources (Schluter 2000b). The ecological opportunity (vacant niches or unutilized resources) in a given region determines the extent of adaptive radiations (Simpson 1953; Schluter 2000b; Kassen *et al.* 2004), while both abiotic (e.g. food abundance) and biotic factors (e.g. predation) may influence the ecological opportunity for a radiation event (Rainey & Travisano 1998; Buckling & Rainey 2002b; Kassen *et al.* 2004; Nosil & Crespi 2006; Brockhurst *et al.* 2007b; Meyer & Kassen 2007; Benmayor *et al.* 2008).

Resource competition has been considered as the most common selection agent to shape adaptive radiations (Schluter 2000b). The effect of competition on the breadth of adaptive radiation could be dependent on the source of the competition. In a traditional view, competition among forms within a radiating lineage can promote diversification, but competition from species of other taxa may inhibit it by reducing the width of potential niches for the radiating lineage (Mayr 1942; Lack 1947; Simpson 1953). The hypothesis that competitors impede adaptive radiation received evidence mainly from comparative studies (Schluter 1988; DeSalle 1995) on morphological divergence and

speciation rates of sister taxa on islands (where unrelated competitors are absent) and continents (where the competitors are present). More recently, several authors suggested that the presence of competitors may create novel niches by increasing the complexity of biotic interactions (Jones *et al.* 1997; Vellend & Geber 2005), and hence promote diversification (Emerson & Kolm 2005). The latter 'diversity drives diversification' hypothesis is supported by a positive correlation between species diversity and the rate of endemic diversification across some islands (Emerson & Kolm 2005). Direct tests of these competing hypotheses have been very rare, although there do have been several such studies (Davidson 1978; Brockhurst *et al.* 2007b; Fukami *et al.* 2007).

Here I experimentally investigate whether interspecific competition affects the extent of diversification in a bacterial species. In spatially heterogeneous environment (static tubes containing growth media) populations of *Pseudomonas fluorescens* rapidly diversify by mutation into numerous phenotypes that are distinguishable by heritable colony morphology and spatial niche occupancy. The phenotypes fall into three categories: smooth morphs (SM) that resemble the ancestral type and inhabit the liquid phase, wrinkly spreader (WS) types that form a biofilm at the air-broth interface, and fuzzy spreader (FS) types that colonize the bottom of tubes; each category consisted of several variants (Rainey & Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2000). Phenotypic diversity in this system is maintained through both negative frequency-dependent selection (Rainey & Travisano 1998; MacLean *et al.* 2005) and neutral coexistence (Fukami *et al.* 2007). Populations in spatially homogeneous environment (continuously shaken microcosms) are dominated by SM phenotypes (Rainey & Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2000). Two previous studies have

used this system to address the effect of niche occupation on the magnitude of diversification (Brockhurst *et al.* 2007b; Fukami *et al.* 2007). They made a priori predictions that the diversification of the ancestral type should be suppressed when some niche-specialist types have been introduced into the system before the process of diversification; their predictions were perfectly supported. The present study extends their considerations and examines the effect of an interspecific competitor, *P. putida*, on the diversification of *P. fluorescens* populations in both spatially heterogeneous and homogeneous environments.

2.2 Methods

I grew *P. fluorescens* SBW25EeZY6KX (Bailey et al. 1995) and *P. putida* UWC1::GFP (van Overbeek *et al.* 2002) in monocultures and mixtures, in both unshaken (spatially heterogeneous) and shaken (spatially homogeneous) environments. Six replicate microcosms of each species composition were destructively sampled every two days until day 14. Such an experimental design yielded 252 microcosms (3 species compositions × 2 environments × 7 points of sampling time × 6 replicates). During the experiment, I did not observe diversification in *P. putida* populations in terms of colony morphology or niche occupancy (specifically, *P. putida* had never formed a biofilm at the air-broth interface).

The bacteria were grown in 30 mL universal bottles with loose lids, each containing 6 mL of King's Medium B (KB). The monocultures were initialized with 10^7 cells of *P. fluorescens* or *P. putida*, and the mixtures, 10^7 cells of each species. The unshaken microcosms were grown in a static incubator at 28 °C, and the shaken

microcosms were grown in a shaking incubator (150 rpm, shaking radius 25 mm) at 28 °C. Bacterial densities were estimated by plating diluted cultures onto KB agar plates and counting the number of colony forming units (CFUs) after 72 h of incubation at 28 °C. The KB agar contained 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal; 0.004%, w:v), on which the colonies of *P. fluorescens* SBW25EeZY6KX showed blue colour and were easily distinguishable from those of *P. putida* UWC1::GFP.

I measured the total number of morphologically distinct phenotypes (see appendix of this chapter for description of colony morphologies of the different types) in *P. fluorescens*, as determined from ~100 random colonies (Buckling *et al.* 2000; Kassen *et al.* 2000). Phenotypic diversity was expressed as the complement of Simpson's index (Simpson 1949), $1-\lambda=\left(1-\sum_i p_i^2\right)$ where p_i is the frequency of the *i*th phenotype.

To look at whether the competitor *P. putida* showed differential impacts on the three major categories of phenotypes (SM, WS and FS) in static microcosms, I performed a 'competition experiment'. Twelve colonies of each of the three types (SM, WS and FS) were isolated randomly from the six *P. fluorescens* monoculture populations at day 6. These isolates were grown in monocultures and mixtures with *P. putida* for three days in static microcosms (culturing conditions as above). Bacterial densities were determined by counting the number of CFUs on KB agar plates. The intensity of competition of *P. putida* on each *P. fluorescens* isolate was measured by a log response ratio index lnRR developed by plant ecologists (Goldberg *et al.* 1999), calculated as a natural logarithmic transform of the ratio of *P. fluorescens* density in the absence of the competitor to that in the presence. A zero lnRR value means null competition effect of *P. putida* on *P. fluorescens* isolates, with greater values indicating stronger competition.

The differences in phenotypic richness, diversity and proportion of SM or WS cells in *P. fluorescens* populations (see below) between microcosms with and without *P. putida* was analyzed using ANCOVA, with the presence of *P. putida* as a categorical explanatory variable and time as a continuous variable. The richness data were log-transformed and the proportional data were arcsine-transformed before analyses.

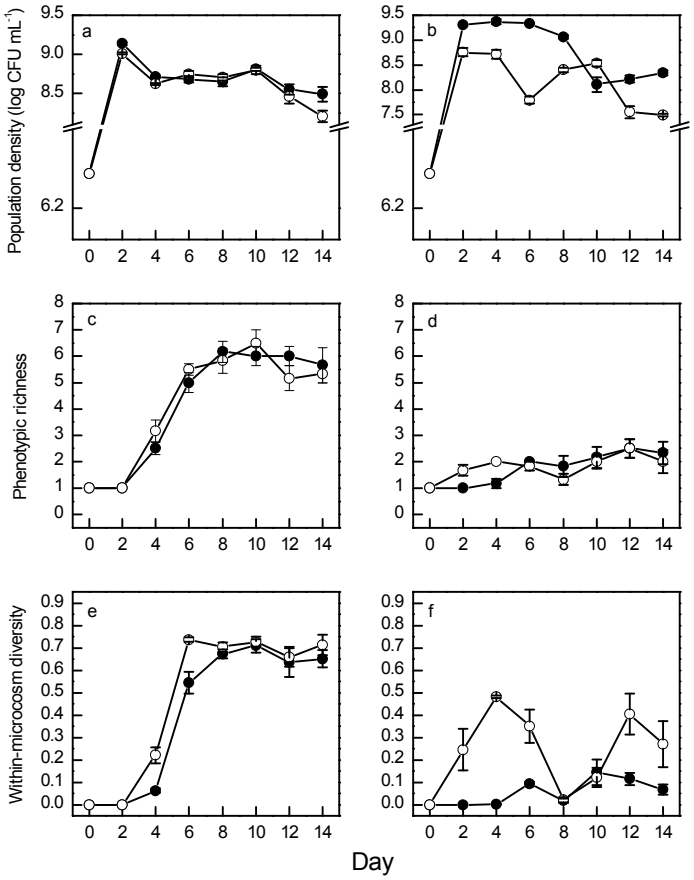

Figure 2.1 Density, phenotypic richness and diversity of *P. fluorescens* populations in unshaken (a, c, e) or shaken (b, d, f) microcosms, with *P. putida* absent (filled circles) or present (open circles). Data show mean ± SE.

2.3 Results and discussion

In spatially heterogeneous microcosms, population density of *P. fluorescens* was overall unaffected by the presence of *P. putida* ($F_{1, 81} = 2.98$, $P = 0.088$), and deceased

with time ($F_{1, 81} = 74.1$, $P < 0.001$); more detailed analysis shows that population density of *P. fluorescens* was decreased by the competitor at day 2, 4 and 14 (two-sample *t* test, $P < 0.05$). The phenotypic richness in *P. fluorescens* did not differ between microcosms with and without *P. putida* ($F_{1, 81} = 0.043$, $P = 0.836$), and increased though time ($F_{1, 81} = 93.00$, $P < 0.001$); at any time point the presence of the competitor did not affect the *P. fluorescens* phenotypic richness ($P > 0.10$; Fig. 2.1c). Diversity (1-λ) was overall unaffected by the competitor ($F_{1, 81} = 2.94$, $P = 0.090$) and increased with time ($F_{1, 81} = 112.35$, $P < 0.001$); specifically, diversity was increased by the competitor only at day 4 and 6 ($P < 0.01$; Fig. 2.1e).

In homogeneous microcosms, *P. fluorescens* density was decreased by the presence of *P. putida* ($F_{1, 81} = 69.08$, $P < 0.001$) and decreased with time ($F_{1, 81} = 119.74$, $P < 0.001$); the competitor decreased *P. fluorescens* density at any time ($P < 0.001$) except for day 10, when the density of *P. fluorescens* was higher in microcosms with *P. putida* present than without ($P = 0.024$; Fig. 2.1b). Phenotypic richness in *P. fluorescens* was increased by the presence of *P. putida* ($F_{1, 80} = 7.00$, $P = 0.010$), and increased over time ($F_{1, 80} = 15.24$, $P < 0.001$); the competitor × time interaction effect was also significant ($F_{1, 80} = 6.50$, $P = 0.013$): the competitor increased *P. fluorescens* phenotypic richness at day 2 and 4 ($P < 0.050$) but not at the remaining days ($P > 0.300$; Fig. 2.1d). Diversity (1-λ) was increased by the presence of *P. putida* ($F_{1, 81} = 33.00$, $P < 0.001$) and did not change over time ($F_{1, 81} = 0.11$, $P = 0.737$); detailed analysis shows that diversity was increased by *P. putida* at day 2, 4, 6 and 12 ($P < 0.05$; Fig. 2.1f).

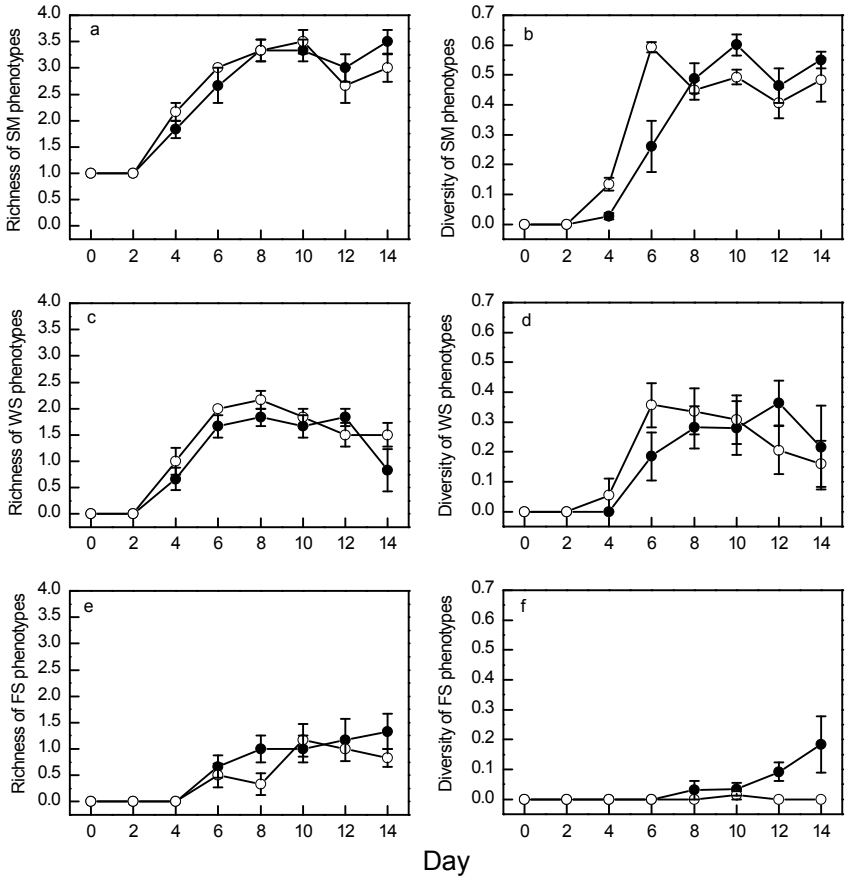

Figure 2.2 Phenotypic richness and diversity within each category of niche specialist (SM [panels a and b], WS [c and d] and FS [e and f]) in unshaken microcosms, in the absence of *P. putida* (filled circles), and in the presence (open circles). Data show mean ± SE.

The extent of diversification of *P. fluorescens*, measured as richness of phenotypes, was largely unaffected by the presence of an interspecific competitor *P. putida*, in either spatially heterogeneous or homogeneous environment. However, the early stage of diversification in homogeneous microcosms (where several SM and very few WS variants evolved from the ancestral SM type) was speeded up by the competitor. These results are inconsistent with the traditional view that competition from other taxa should limit the extent of diversification in a radiating lineage (Mayr 1942; Lack 1947; Simpson 1953). The hypothesis that competitors can drive adaptive radiation (Emerson &

Kolm 2005) is to some extent supported: early diversification of *P. fluorescens* was accelerated only in the homogeneous environment.

In spatially homogeneous microcosms (where *P. fluorescens* populations were dominated by SM) phenotypic richness of *P. fluorescens* was increased by the competitor in the early stage (Fig. 2.1d, f). There are two possible explanations. First, *P. putida* competes only with the abundant phenotype and prevents it from dominating the other rare variants. Second, *P. putida* is a generalist and competes with all *P. fluorescens* phenotypes; consequently, its effect on *P. fluorescens* is the same as decreased habitat productivity, which here shows either positive or null effect on coexistence of phenotypes in *P. fluorescens*.

In spatially heterogeneous microcosms phenotypic richness in *P. fluorescens* was unaffected by the competitor, and phenotypic diversity was increased at day 4 and 6 (Fig. 2.1c, e). The positive effect of the presence of competitor on diversity at day 4 and 6 was due to the response of SM diversity to the competitor. Phenotypic richness within each of the three major categories of variants (SM, WS or FS) did not differ between microcosms with and without *P. putida* ($P > 0.05$ at any point of time; Fig. 2.2). Phenotypic diversity within SM was increased by the competitor at day 4 and 6, decreased at day 10 ($P < 0.05$), and unaffected at the remaining days; while phenotypic diversity within WS or FS had no response to the presence of *P. putida* ($P > 0.100$; Fig. 2.2). The relative ratio among the three types of niche specialists (SM, WS and FS) was unaffected by the competitor except at day 8 when the proportion of SM cells in *P. fluorescens* populations was decreased by the competitor ($P = 0.048$) and the proportion of WS in non-SM types was increased ($P = 0.039$; Fig. 2.3).

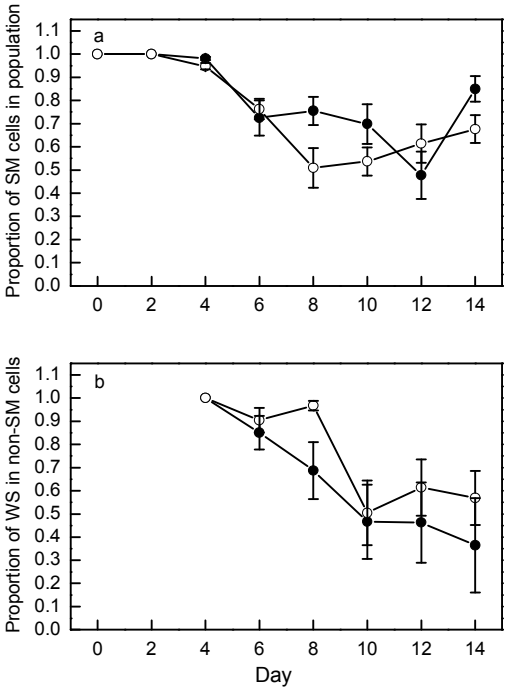

Figure 2.3 Proportion of SM cells in *P. fluorescens* populations (a) and proportion of WS cells in non-SM types (b), in the unshaken microcosms. Filled circles, in the absence of competitor; open circles, in the presence. Data show mean ± SE.

One possible explanation for the absence of competitor's effect on diversification in heterogeneous environment is that *P. fluorescens* experienced very weak or even undetectable competition from *P. putida*. The population density of *P. fluorescens* was significantly influenced by *P. putida* only at day 2, 4 and 14 in heterogeneous microcosms (Fig. 2.1a); meanwhile, the competition of *P. putida* on *P. fluorescens* was much stronger in homogeneous microcosms (Fig. 2.1a, b). *P. fluorescens* suffered weaker competition from *P. putida* in heterogeneous environment possibly because *P. putida* is less tolerant to oxygen-poor environments than *P. fluorescens*. In shaken microcosms where oxygen is not limiting, the two competing species, *P. fluorescens* and *P. putida*, negatively influenced each other; however, in static microcosms where oxygen is probably the limiting resource (Kassen *et al.* 2004), population size of *P. putida* was always reduced by the presence of *P. fluorescens* (data not shown) but the latter was impacted by the former only at the beginning (day 2 and 4) and the end (day 14) of the

experiment, when oxygen might not be limiting. At the beginning of the experiment the biofilms of WS phenotypes had not formed and oxygen may not have been exhausted to very low level. In old microcosms, the WS biofilms often become very thick and sink under their own weight (Kassen *et al.* 2004); the collapse and sinking of the biofilms might cause movement of liquid and increase the concentration of oxygen in broth cultures. It is also possible that some resources other than oxygen became limiting at the end of the experiment. Another explanation for the lack of effect of *P. putida* on diversification of *P. fluorescens* is that *P. putida* is a generalist and competes with all phenotypes of *P. fluorescens*. In a 'competition experiment' between *P. putida* and the three main categories of phenotypes in *P. fluorescens* (SM, WS and FS) in static microcosms, *P. putida* exhibited similar competitive effect on the different niche specialists (Fig. 2.4).

In summary, the present study suggests a lack of a clear-cut answer to the question of how competitors affect a radiating lineage's diversification. The competitors can be either generalists that colonize all potential niches and compete with all forms within a radiating lineage, or specialists that only use part of the potential niches and compete with particular forms. The effect of a generalist competitor on diversification might be the same as that of decreased habitat productivity on species diversity; the effect can be positive (if dominance among the divergent forms within the radiating lineage is prevented), negative (if the probability of the rare forms going extinct is raised), or neutral (if the competitive effect is weak and different forms showed proportionate reduction; see discussion on species diversity-productivity relationship (Abrams 1995)). A specialist competitor can decrease the size of an adaptive radiation if it drives some

forms in the radiating lineage extinct, or promote the radiation if it only competes with the abundant forms and prevent their dominance. Therefore, the effect of competitors on adaptive radiation of a lineage might depend on the competition intensity and the nature of the competitors. Finally I would notify the limitation of this study with using morphological variation as a measure of diversity: different morph-types may be ecologically equivalent (Fukami *et al.* 2007) and on the other hand within-morph diversity may be ecological relevant (MacLean *et al.* 2005). Both morphological and genotypic variation can be measured for study systems like mine, but we do not know which measure is of more ecological importance. Functional variation might be more ecological relevant, but measurement of such variation is sensitive to the choice of functions to estimate (e.g. a same population may show high variation in carbon metabolism but low variation in antibiotics resistance). Given that the data on morphological variation are very easy to collect in this system, it is worth using morphological data to address general ecological questions but caution is needed when the results are to be generalized.

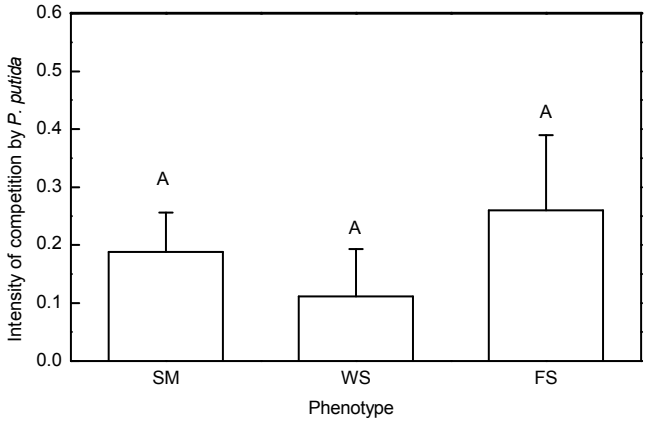

Figure 2.4 Intensity of competition of *P. putida* on the three niche specialists (SM, WS, and FS) in a competition experiment, where 12 isolates of each of the three types were competed by *P. putida*. The same letter 'A' above the bars indicates non-significant difference in the competition intensity between any two types ($P > 0.300$). Data show mean ± SE.

Appendix of Chapter 2

Appendix 1: Colony morphologies of the different phenotypes

I identified a total of nine phenotypes based on colony morphologies in this experiment: SM1 (ancestor-like SM), SM2 (SM with bright-yellow edge), SM3 (small SM), SM4 (tiny SM), WS1 (large WS), WS2 (wheel-like WS), WS3 (small WS), FS1 (large FS), and FS2 (small FS).

Chapter 3: An experimental test of the relative importance of niches and neutrality for coexistence

Summary

Ecologists have identified two types of processes promoting species coexistence: stabilizing mechanisms (niche differentiation and related processes) that increase negative intraspecific interactions relative to negative interspecific interactions, and equalizing mechanisms (neutrality) that minimize the differences in species' demographic parameters to reduce the speed of competitive exclusion. I here address the relative importance of stabilizing and equalizing mechanisms in explaining phenotypic diversity in experimental communities of the bacterium *Pseudomonas fluorescens*. Initially isogenic bacteria in microcosms diversify into a series of major and minor classes of phenotypes that can be treated as analogues of species. I estimated the relative growth rates when rare of 32 phenotypes from six lines of microcosms. Approximately two thirds of the phenotypes showed neutral or near-neutral dynamics while the remaining one third had a growth rate advantage when rare. Though there was some variation amongst different lines of microcosms there was overall little evidence that productivity increased with phenotypic diversity, nor that niche complementarity had a major effect on productivity. The results suggest that both niche and neutral mechanisms contribute to the maintenance of phenotypic diversity in this system, and the operation of niche processes do not necessarily lead to a positive biodiversity effect on ecosystem properties.

3.1 Introduction

One of the oldest problems in ecology is how to identify the mechanisms that allow species to coexist in nature (Hutchinson 1961). Traditional explanations based solely on niche differences face difficulties in explaining persistent communities containing numerous similar species in seemingly homogeneous environments with a small number of niche dimensions (Hutchinson 1961; Hubbell 2001). Ecologists have identified two classes of processes that promote species coexistence: (a) stabilizing mechanisms that increase negative intraspecific interactions relative to negative interspecific interactions (niche differentiation), and (b) equalizing mechanisms (neutrality) that minimize differences amongst species in population growth rate (Chesson 2000). Stabilizing mechanisms result in negative frequency-dependent selection: each species enjoys an advantage in population growth rate when rare. They are essential for long-term stable coexistence among species, and include processes such as resource partitioning (Schoener 1974; Tilman 1982; Tilman & Pacala 1993), frequency-dependent predation (Janzen 1970; Armstrong 1989a; Wills *et al.* 1997; Chase *et al.* 2002), and those dependent on fluctuations in population density and environmental factors in space and time (Hutchinson 1961; Grubb 1977; Chesson & Warner 1981; Chesson 2000). Equalizing mechanisms contribute to species coexistence by reducing the speed of competitive exclusion but do not lead to increased population growth rates when rare. The neutral model of biodiversity (Hubbell 2001) is a special case of an equalizing mechanism where all species have identical ecologies and hence population growth rates, and niche-based stabilizing processes are completely absent. It has been demonstrated theoretically that either perfect neutrality (Hubbell 2001) or near neutrality (Zhou &

Zhang 2008) can allow species to co-occur for long periods of time in the absence of any stabilizing processes.

Stabilizing and equalizing mechanisms can operate simultaneously to structure communities (Chesson 2000); and a synthesis of neutral theory with classical niche theory may provide a better understanding of the maintenance and patterns of biodiversity (Bell 2001; Hubbell 2001; Tilman 2004; Chase 2005; Purves & Pacala 2005; Gravel *et al.* 2006; Holt 2006; Leibold & McPeek 2006; Scheffer & van Nes 2006; Adler *et al.* 2007; Cadotte 2007). To create such a synthesis it is important to understand the relative importance of niche and neutrality mechanisms in natural communities (Bell *et al.* 2006; Gravel *et al.* 2006; Adler *et al.* 2007). Ecologists have realized that this goal cannot be achieved simply by analyzing species abundance patterns (Adler *et al.* 2007) because frequently the same data can be explained by either neutral or niche-based processes (Chave *et al.* 2002; McGill 2003; Sugihara *et al.* 2003; Tilman 2004; Purves & Pacala 2005). Direct examination of the underlying stabilizing and equalizing processes seems essential to resolve this issue (Adler *et al.* 2007).

One testable difference between stabilizing and equalizing mechanisms is that the former assumes negative frequency-dependency in population growth rate of species while the latter does not. Here I experimentally estimate the relative importance of the two types of mechanisms by examining how many "species" in a stable community tend to increase in relative abundance when rare. I used communities made up of diverse phenotypes of the bacterium *Pseudomonas fluorescens,* a system that has become an important model in experimental ecology (Rainey & Travisano 1998; Rainey *et al.* 2000; Travisano & Rainey 2000; Hodgson *et al.* 2002; MacLean 2005; Brockhurst *et al.* 2006;

Brockhurst *et al.* 2007b). When propagated in spatially heterogeneous environments (static tubes of growth media), initially isogenic *P. fluorescens* populations rapidly diversify, generating by mutation various phenotypes that are distinguishable by their heritable colony morphologies on agar plates and whose genetic bases have been partially identified (Spiers & Rainey 2005; Goymer *et al.* 2006; Bantinaki *et al.* 2007). These phenotypes fall into three main categories based on colony morphology and niche occupancy, and there are multiple variants within each category. Smooth morphs (SM) resemble the ancestral phenotype and inhabit the liquid phase; wrinkly spreaders (WS) form a biofilm at the air-liquid interface; and fuzzy spreaders (FS) colonize the bottoms of the tubes (Rainey & Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2000). The bacterial populations reproduce asexually, so these phenotypes can be considered as analogous to species. Previous studies have reported the operation of negative frequency-dependent selection among the three major categories of phenotypes (Rainey & Travisano 1998), as well as both neutral (Fukami *et al.* 2007) and stable coexistence (Brockhurst *et al.* 2006) of several WS phenotypes. It thus seems likely that both niche mechanisms and neutrality contribute to the maintenance of phenotypic diversity in this system, but their relative importance has not been systematically examined. I address this question by determining how many phenotypes can increase in relative abundance from rare.

The mechanisms responsible for the maintenance of species diversity are also believed to influence the relationship between biodiversity and ecosystem functioning (Kinzig & Pacala 2001; Mouquet *et al.* 2002; Fox 2003; Loreau 2004). Models based on niche processes often predict a positive relationship between diversity and ecosystem

properties such as biomass production as a result of more efficient total resource use, i.e. niche complementarity (Tilman 1999; Loreau 2000). In contrast, neutral theory assumes that all species are functionally equivalent and hence diversity has no effect on ecosystem properties (but see Loreau 2004; Pueyo *et al.* 2007; Zhou & Zhang 2008). I look at whether the relationship between phenotypic diversity and productivity in this system could be predicted by the estimated importance of niche processes for the maintenance of phenotypic diversity.

3.2 Methods

Bacterial phenotypes

Six replicate lines of *Pseudomonas fluorescens* SBW25 (Rainey & Bailey 1996) were propagated in static 30 mL glass universal bottles (which will be referred to as microcosms) containing 6 mL of M9KB media (glycerol, 10 g L^{-1}; proteose peptone no. 3, 20 g L^{-1}; Na_2HPO_4, 6 g L^{-1}; KH_2PO_4, 3 g L^{-1} NH_4Cl, 1 g L^{-1}; NaCl, 0.5 g L^{-1}) at 28°C, each of which was initially inoculated with approximately 10^8 bacterial cells. 60 μL (1%) of each culture was transferred to fresh media every two days. At day 6 the diversified bacterial populations (referred to as 'base communities' hereafter) were vortexed and an aliquot from each microcosm was diluted and plated onto M9KB agar. I counted ~ 400 random colonies for each base community, and isolated a colony for every distinguishable phenotype with a proportional abundance ≥ 0.02. Of the different phenotypes identified two fell within the broader SM category while five were members of the WS category. A total of 32 isolates from the six base communities were obtained,

propagated and stored at ˜80°C in 50% glycerol. No FS phenotype was observed in the present experiment.

Invasion experiment

For each phenotype from each of the six base communities, I performed an invasion experiment to determine whether it could increase in relative abundance from rare within its own community. To test whether a phenotype, *A*, can invade its community, I grew all the component phenotypes of that community in a new microcosm at initial densities 100-fold lower than the final densities observed in the base community with the exception of phenotype *A* which was introduced at a 1000-fold lower density. The new microcosm was grown in a static incubator for 2 days at 28°C. Each invasion experiment was replicated three times.

I calculated relative growth rate when rare (W) for each phenotype. For a phenotype, *A*, in a given community, let m_A be the estimated Malthusian parameter of *A* and $m_{A'}$ the equivalent compound growth rate for the rest of the community excluding *A*. The relative population growth rate of *A* was $W_A = m_A / m_{A'}$; and $m = \ln (N_f / N_0)$ where N_0 and N_f were the initial and final densities, respectively (Lenski *et al.* 1991). $W > 1$ suggests that the focal phenotype can invade from rare (and hence persist stably in the community), with $W = 1$ indicating neutrality, and $W < 1$ suggesting transient persistence (that is, the phenotype will be competitively excluded).

I used two methods to assess the numbers of neutral and non-neutral phenotypes. First, the difference of every phenotype's W values from one was determined by one-sample *t* test, with the phenotypes with W values non-significantly different from one considered as neutral; but type II statistical error may lead to an overestimation of the

number of neutral phenotypes. Second, I compared the observed frequency distribution of W values with that expected under the assumption of neutrality. I obtained the latter by assuming all 32 phenotypes had no low-frequency advantage (mean $W = 1$) and that the sampling variance of expected W values was equal to the observed among-replicate (within-phenotype) variance, $(\sum_i^M \sum_j^N (W_{ij} - \overline{W_i})^2)/(M \bullet N)$, where W_{ij} means the observed W value of the jth replicate of the ith phenotype ($i = 1, 2, \ldots, 32$; $j = 1, 2, 3$) and $\overline{W}_i$ is the mean of the observed W values of the ith phenotype.

Ecosystem function experiment

I performed a 'phenotype removal experiment' to investigate the relationship between diversity and biomass (cell density). Phenotypes isolated from each base community were used to initiate new assemblages with one or more component phenotype(s) omitted. The initial density of each phenotype in the new assemblage was 100-fold lower than that in the base community. All possible phenotype removal combinations were carried out for each community. The new assemblages were incubated in a static incubator for 2 days at 28°C, and the final density of each phenotype was measured by plating on M9KB agar. Each assemblage was replicated three times, and the average across the three replicates was used in analyses. Biomass data (colony forming units, CFU, per mL) were log-transformed before analysis of ANCOVA in which phenotypic richness was included as a continuous explanatory variable, and base community a random factor.

Biodiversity can influence ecosystem processes through two types of mechanisms: niche complementarity and the selection effect (Aarssen 1997; Huston 1997; Tilman 1999; Loreau *et al.* 2001). Complementarity relies on species occupying different niches

or interacting together positively so that diverse communities are more efficient in carrying out a specific process such as biomass production. The selection effect explains the high efficiency of diverse communities by the higher chance that more diverse assemblages include particularly efficient species that come to govern the community's properties. For example, diverse communities are more likely to include the most productive species and hence produce more biomass.

I estimated the complementarity effect by relative yield (RY) analysis (Trenbath 1974; Harper 1977; Vandermeer 1989; Hector 1998; Hooper 1998). The relative yield (RY) of a phenotype in a mixed assemblage is the ratio of its biomass in that mixture to its yield in monoculture. The sum of the RYs of all the phenotypes in a mixture is relative yield total (RYT). RYT = 1 indicates perfect niche overlap (no niche differentiation among phenotypes). RYT > 1 indicates some degree of niche partitioning, with the size of the effect determined by the amount of niche overlap and the size of the different niches. Finally, RYT < 1 suggests interference among phenotypes. RYT was analyzed using mixed-model ANOVA with phenotype morphology as a categorical explanatory variable and base community as a random factor.

Evidence for a selection effect can be found by looking for a positive correlation between the (normalized) RY of phenotypes in diverse communities and their monoculture yields (see Zhang & Zhang 2007 for details). I used this method rather than 'additive partitioning' (Loreau & Hector 2001) or 'tri-partitioning' (Fox 2005; Fox 2006) because the latter apply to experiments with substitutive design (i.e. mixtures at different diversity levels have the same initial total abundance) while my experiment had an

additive design (diverse mixtures have higher initial total abundance than species-poor ones).

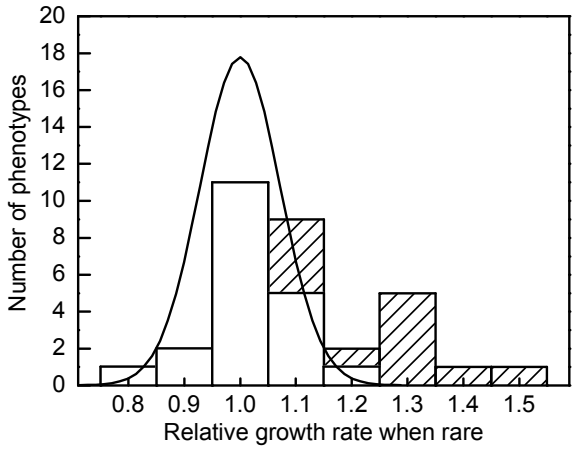

Figure 3.1 Frequency distribution of estimated relative population growth rates when rare (*W*) of the 32 phenotypes identified in the study. The histograms show the observed frequency distribution (1.11 ± 0.028 as mean ± SE.), with the shaded parts of the bars representing the 12 *W* values that were significantly greater than one. The curve shows the expected distribution of *W* values for neutral phenotypes (*W* = 1) with the observed sampling variance (0.005162)

3.3 Results

Across all six base communities two SM and five WS phenotypes could be distinguished based on consistent heritable differences in colony size and morphology. They are referred to as SM1, SM2, WS1, WS2, WS3, WS4, and WS5. I observed SM1, SM2, and WS1 in every community while WS2, WS3, WS4, and WS5 occurred in five, two, four, and three communities, respectively. All communities contained either five or six phenotypes, and in total 32 phenotypes isolated from the six communities were analyzed.

Stable and neutral persistence

The relative population growth rate when rare (*W*) was calculated for 32 phenotypes derived from six base communities. Eleven *W* values were significantly

greater than one (one-sample t test, $P < 0.05$) while a twelfth approached significance ($0.05 < P < 0.10$; Fig. 3.1, 3.2). No case of relative growth rates significantly less than one was found. The remaining 20 phenotypes formed a near-symmetrical distribution centred on $W = 1$ that had a variance similar to that predicted by the null hypothesis of neutrality with the observed among-replicate sampling variance (Fig. 3.1). In the absence of a fixed hypothesis for the distribution of non-neutral W values one cannot formally estimate the proportion of the two classes of phenotypes. Nevertheless the results suggest that ~1/3 the phenotypes are non-neutral and 2/3 are neutral or nearly neutral.

W values did not differ among the two major categories of phenotypes (SM versus WS; $F_{1, 25} = 1.058$, $P = 0.31$; base community, $F_{5, 25} = 1.09$, $P = 0.39$), nor across all the seven phenotypes ($F_{6, 20} = 1.14$, $P = 0.38$; base community, $F_{5, 20} = 1.08$, $P = 0.40$).

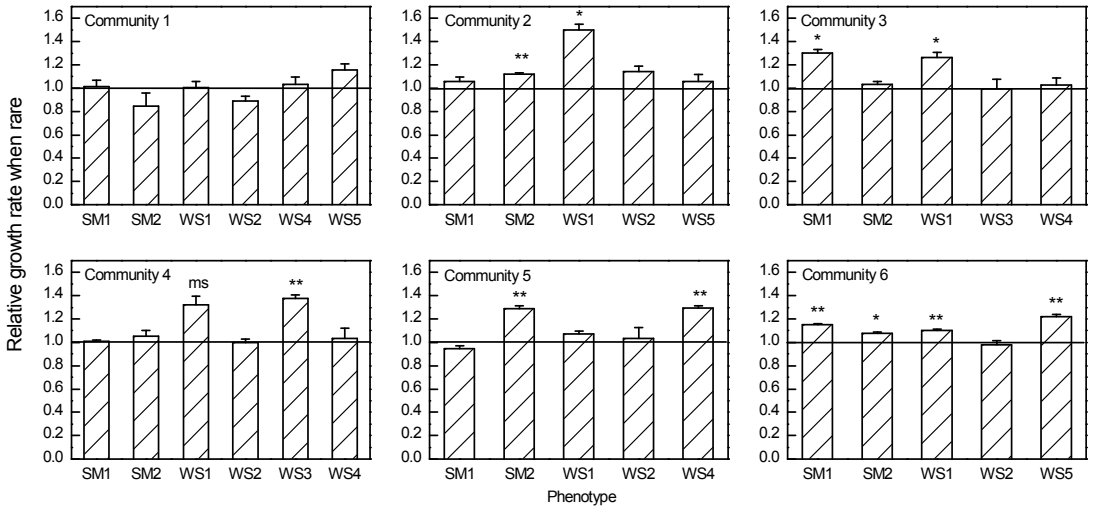

Figure 3.2 Relative population growth rate when rare (W) of each phenotype in each community. In each panel a horizontal line through one shows the null hypothesis that a phenotype, when rare, has the same growth rate as the other members of its community in total. Data show mean ± SE. ($N = 3$). Asterisks above the bars indicate significant difference from one (based on one-sample t test), single asterisk, $P < 0.050$; double, $P < 0.010$, ms (marginally significant), $P < 0.100$.

Diversity-biomass relationship

Biomass production was measured in a total of 250 assemblages made up of one to six phenotypes. Overall, there was no significant relationship between biomass production and phenotypic richness ($F_{1, 238} = 0.078$, $P = 0.78$) but biomass differed among assemblages constructed from different base communities ($F_{5, 238} = 9.98$, $P < 0.001$) and there was a significant richness × base community interaction effect ($F_{5, 238} = 3.75$, $P = 0.003$). Examination of the assemblages derived from individual base communities revealed a positive relationship for base community 1, a negative relationship for community 4, and non-significant relationship for the remaining four (Fig. 3.3).

A relative yield total (RYT) equal to one indicates perfect niche overlap while RYT > 1 indicates a degree of niche complementarity. The grand mean RYT across all non-monoculture assemblages was just higher than one (1.025 ± 0.012, mean ± SE; $P = 0.035$). Comparing assemblages constructed from different base communities I found a significant richness □ base community interaction ($F_{5, 206} = 4.33$, $P = 0.001$) a significant main effect of base community ($F_{5, 206} = 3.85$, $P = 0.002$), but the effect of phenotypic richness was not significant ($F_{1, 206} = 0.039$, $P = 0.88$). RYT was significantly higher than one in assemblages from base communities 2 and 3 but significantly less than one for base community 4 (and marginally significantly lower than one for base community 5; Fig 3.4).

A negative selection effect, measured as the correlation between normalized RYs and monoculture yields, was found when all the data was analyzed together (Pearson's, $r = -0.19$, $P < 0.001$). When the analyses were performed separately for assemblages derived from different base communities, a negative selection effect was found for

assemblages from base communities 3, 4 and 6 ($P < 0.001$), and assemblages from the remaining three base communities had no significant selection effect ($P > 0.05$).

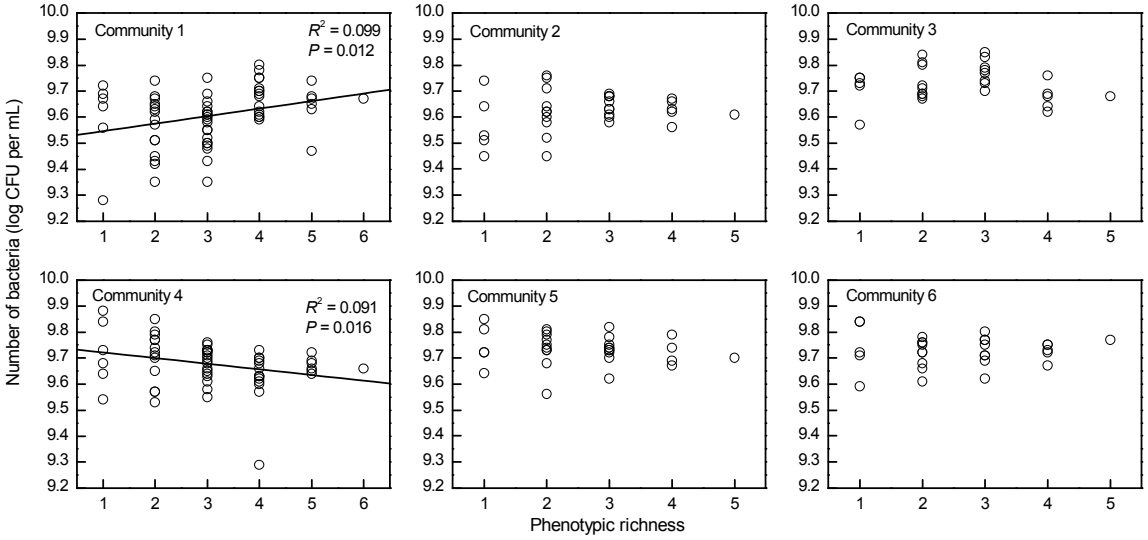

Figure 3.3 The biomass of assemblages created from the six different base communities as a function of the number of phenotypes each includes. Fitted regression lines are drawn for the two significant relationships.

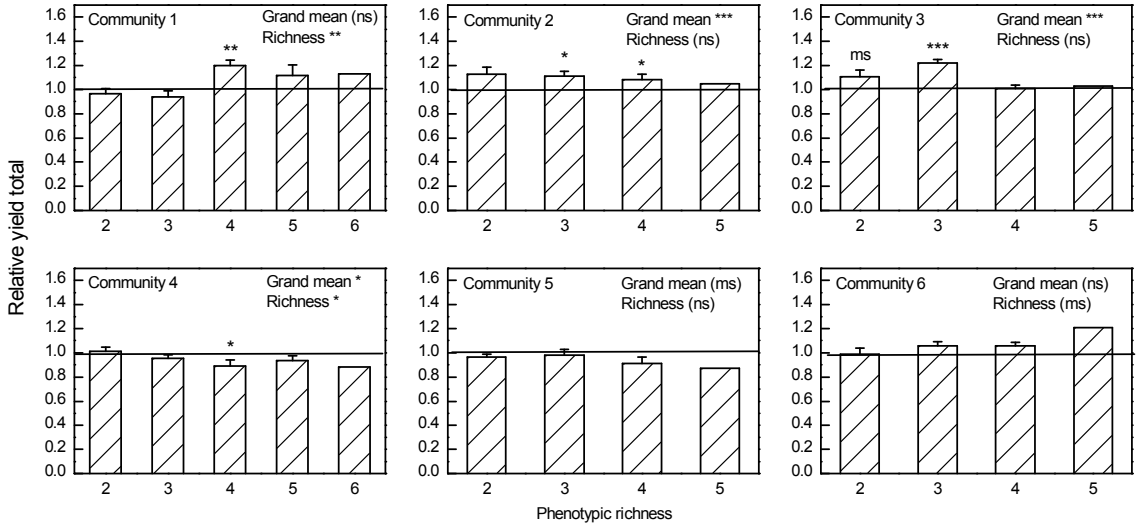

Figure 3.4 Relative yield total (RYT) of mixtures as a function of the number of phenotypes they include. A horizontal line through one in each panel indicates the prediction of the null hypothesis that no niche complementarity occurs. In each panel 'Grand mean *' indicates a grand mean significantly different from one while 'Richness *' indicates a significant linear relationship between RYT and phenotypic richness. Bars show mean ± SE. with significance indicated by: single asterisk, $P < 0.050$; double asterisk, $P < 0.010$; triple asterisk, $P < 0.001$; ms (marginally significant, $0.50 < P < 0.10$).

3.4 Discussion

Niches and neutrality

Of the phenotypes scored I estimated that approximately 2/3 were neutral and 1/3 had the ability to increase in frequency from rare. It is important to state exactly what I mean by neutral here because these figures depend to a large extent on sampling intensity (W is a continuous trait and given a sufficiently large sample it will always be possible to distinguish $W \neq 0$). The estimate of sampling variance indicates that I can distinguish from neutral most phenotypes with $W > \sim 1.1$ (Fig. 3.1).

The results suggest that both stabilizing and equalizing mechanisms may have played significant roles in the maintenance of phenotypic diversity in this experimental microbial system. The coexisting phenotypes in the microcosms have diversified from one common ancestral form, so explanations are required for not only their maintenance but also how they initially increased in frequency. The emergence and maintenance of a phenotype which has a selective advantage when rare can both be explained by adaptation to a distinct niche. Niche differentiation in this system may reflect spatial specialization (Rainey & Travisano 1998) or nutrient partitioning (MacLean *et al.* 2005; Brockhurst *et al.* 2006). It is harder to explain the emergence of neutral phenotypes: every new phenotype that arises through mutation has a very low initial abundance and no advantage in growth rate when rare. My experimental microcosms contained large bacterial populations ($\sim 10^8$ cells at bottleneck and $\sim 10^{10}$ cells at stationary phase) and neutral mutants were unlikely to spread by drift. Two other explanations are plausible. First, recurrent weak beneficial mutations may lead to the coexistence of several phenotypes. The mutants will ultimately exclude the ancestor, but very slowly. My

experimental assays may not be able to detect the advantage when rare for such mutants. The possibility that weak beneficial mutations allow near-neutral coexistence has been shown in a previous study with a chemostat *Escherichia coli* population (Maharjan *et al.* 2006): an ancestral isolate diversified into more than five phenotypic clusters within 26 days, but none of the evolved phenotypes showed negative frequency-dependent growth rate. Second, more than one ecologically equivalent mutant may appear at or around the same time and compete for fixation (Gerrish & Lenski 1998; de Visser *et al.* 1999; Rozen *et al.* 2002; Brockhurst *et al.* 2007b). The competing mutants drive their ancestor extinct, but then persist with nearly neutral dynamics for a long period of time. This type of interaction may be especially common in large bacterial populations (Cooper 2007; Perfeito *et al.* 2007) and I believe it is the most likely explanation for the establishment of neutral phenotypes in my experimental system.

Extrapolating the results here to communities of 'real' species must be done with great caution. My experimental microcosms differ from natural communities in many ways, for example their large community sizes, the absence of immigration, restriction to a single trophic level, and the generation of new 'species' through mutation. As mechanisms underlying neutral coexistence are more sensitive to stochastic processes than those responsible for stable coexistence (Adler *et al.* 2007), small communities may allow fewer neutral or nearly neutral species to co-occur (Hubbell 2001; Zhou & Zhang 2008). Immigration, however, may introduce more (nearly) neutral species into a local community (Zhou & Zhang 2008). Complex abiotic and biotic interactions involving multiple trophic levels may increase the number of niche dimensions, which in turn, allow more opportunities for stable coexistence (Harpole & Tilman 2007). Note also that

the influence of ecological context on neutral or near-neutral species diversity could be scale-dependent. For instance, small local communities may each contain few neutral or near-neutral species, but they may experience greater turnover in species composition at the regional scale (β-diversity) because the outcome of competition among nearly neutral species in small communities are unpredictable and different local communities are likely to be dominated by different species. Thus niche and neutral mechanisms may contribute to species coexistence in nature, but their relative importance may vary dramatically across habitats. The results of the present study and those by other groups (Bell 2001; Hubbell 2001; Tilman 2004; Chase 2005; Purves & Pacala 2005; Gravel *et al.* 2006; Holt 2006; Leibold & McPeek 2006; Scheffer & van Nes 2006; Adler *et al.* 2007; Cadotte 2007) support the current efforts to integrate niche and neutral theory in a same theoretical framework.

Diversity and ecosystem function

In my experimental bacterial communities the overall effect of phenotypic diversity on biomass production was negligible. I also found little evidence for complementarity: though the relative yield total (RYT) was significantly greater than one the biological significance of a RYT value of 1.025 is likely to be minor. The results do not support the prediction that the operation of niche processes in maintaining biodiversity leads to a positive relationship between biodiversity and ecosystem functioning (Kinzig & Pacala 2001; Mouquet *et al.* 2002; Fox 2003; Loreau 2004).

The absence of a stronger complementarity effect is surprising as it is known that the different major phenotypic categories inhabit distinct spatial niches in static microcosms. SM phenotypes are confined to the liquid medium, WS are specialized to

the air-liquid interface, and FS colonize the bottom of vials. A priori I expected mixed communities to be more efficient at using resources and hence have higher total biomass. This prediction is rejected. Phenotypes may interact in other ways than through resource competition. For example, FS phenotypes can invade a community containing both SM and WS, but not a pure WS community (Rainey & Travisano 1998). This suggests that WS phenotypes may have an allelopathic effect on FS, and that SM may rescue FS by countering the allelopathic agent(s) or in some way having a facilitative effect on FS (but see Day & Young 2004). Though I did not observe FS phenotypes here it is likely that interference effects occur among the different SM and WS phenotypes in my experiments, and these may explain the lack of complementarity. A previous study (Hodgson *et al.* 2002) including all three major categories of phenotype (SM, WS, and FS) found a positive diversity effect on biomass production mainly driven by a selection effect. Significant niche complementarity was also detected in certain combinations of phenotype (WS & FS and WS & FS & SM). The present study found consistently very weak complementarity and selection effects in different phenotype combinations (SM assemblages, WS assemblages or SM & WS assemblages). It is unclear whether the differences between the two studies were due to the absence in my experiments of the FS phenotype or the presence of greater number of WS phenotype classes.

The absence of effect of phenotypic diversity on biomass production may also be explained by resource-independent population regulations. For example, quorum sensing processes may terminate the growth of bacteria once their density is beyond a threshold (Lazazzera 2000; Vulic & Kolter 2001), even when resources are abundant; in this case, the low- and high-diversity communities produce the same amount of biomass, although

the diverse communities may potentially be able to consume more resources. To my knowledge, no evidence for quorum sensing behaviours has been reported for my experimental system; but this possibility cannot be ruled out.

Biodiversity and ecosystem function studies may involve artificially constructed assemblages of species or naturally self-organized communities. With the caveat that my experimental community developed in an artificial microcosm from an isogenic ancestor, the present study was of the second type. I found little effect of biodiversity on productivity and it is interesting that this is in accord with most other studies of naturally assembled communities (e.g. Wardle *et al.* 1997; Grime 1998; Loreau *et al.* 2001; Wardle 2001; Mokany *et al.* 2008) but contrasts with experiments that create new communities where positive effects are much more common (reviewed by Hooper *et al.* 2005; Spehn *et al.* 2005; Balvanera *et al.* 2006; Cardinale *et al.* 2006; Cardinale *et al.* 2007). This suggests that the two different experimental approaches may be answering subtly different questions; for example 'species removal' experiments may be a better approach to understand the consequences of biodiversity declines in nature (Diaz *et al.* 2003) while the creation of new assemblages may be more appropriate in studies of the consequences of invasions.

Chapter 4: Competitive hierarchies inferred from pair-wise and multi-species competition experiments

Summary

A 'competitive hierarchies' framework has been developed to predict the structure of multi-species communities by ranking species in competitive ability based on pair-wise competition experiments. Here I give some suggestions for the future development of this framework. Most importantly, the competitive hierarchies hypothesis should relax its definition of 'asymmetric competition', by allowing for species coexistence due to niche or (nearly) neutral processes; thus, an asymmetric competitive interaction, $a > b$, should indicate that species *a* reduces population size of species *b* more than vice versa, rather than that species *a* is capable of excluding species *b*. I then construct competitive hierarchies for species in two biodiversity experiments (one with algae and the other with bacteria). Transitive competitive interactions (which means that for competing species *a*, *b* and *c*, if $a > b$, $b > c$, then $a > c$) are common; and competitive hierarchies inferred from multi-species competitions are consistent with those from pair-wise competitions. The results imply that the competitive hierarchies framework could be a working approach to understanding the relative abundance of species in multi-species communities.

4.1 Introduction

Understanding the patterns and underlying mechanisms of the relative abundance of species is a long-standing challenge to ecologists. As competition has been believed to be one of the most important interspecific interactions due to its potential for shaping distribution, abundance and traits of co-occurring species, it has received much attention from researchers. A school of ecologists (mainly plant ecologists) have developed a 'competitive hierarchies' framework to predict structure and composition of communities by ranking species according to their competitive abilities. They measure the relative competitive abilities of species in pair-wise competition experiments, and construct a ranking order of species in competitive ability, which is then extrapolated to explain the structure of natural communities (Roush & Radosevich 1985; Mitchley & Grubb 1986; Miller & Werner 1987; Keddy & Shipley 1989; Goldberg & Barton 1992; Shipley & Keddy 1994; Keddy 2001; Perkins *et al.* 2007). This competitive hierarchies hypothesis, although having received some attention in the past three decades, is still out of the mainstream ecological theory. Here I give some suggestions for the future development of this framework, and used the data from two biodiversity experiments to address two issues with this hypothesis.

4.1.1 Competitive hierarchies: the conceptual hypothesis

The competitive hierarchies hypothesis assumes two properties of interspecific competition, asymmetry and transitivity (Keddy & Shipley 1989); and also assumes that multi-species interactions are additive, that is, high-order interactions in multi-species communities can be simply predicted by pair-wise interactions (Perkins *et al.* 2007). An asymmetric competitive interaction, $a > b$, occurs when species a is capable of excluding

species *b*. A competitive hierarchy can be constructed when every pair-wise interaction is asymmetric and competitive interactions among multiple species are transitive (e.g. for competing species *a*, *b* and *c*, if $a > b$, $b > c$, then $a > c$).

Ideally, the competitive ability should be measured using replacement series competition experiments (most appropriate for plants). Performance of species can be measured by an index, relative yield per plant (RYP). The competitive ability of species *i* relative to species *j* is its relative yield per plant in a community of species *i* and *j*, $RYP_{ij} = Y_{ij}/Y_{ii}$ where Y_{ii} is the yield of an average individual of species *i* grown in monoculture, and Y_{ij}, the yield of an average individual of species *i* when grown in the two-species community (averaged across all replacement series). An asymmetric pair-wise competition, $a > b$, occurs when the individuals of species *a* grow better in mixture with species *b* than they do in monoculture ($RYP_{ab} > 1$) but individuals of species *b* grow worse in mixture with species *a* than they do in monoculture ($RYP_{ba} < 1$). In this case species *a* is predicted to be capable of excluding species *b*. Thus, the competitive hierarchies hypothesis does not allow for stable coexistence among species (Keddy & Shipley 1989; Silvertown & Dale 1991).

4.1.2 Competitive hierarchies: empirical evidence

Empirical tests of this hypothesis have usually been done in plant communities. First, pair-wise competition experiments in green house, often with replacement series design, measured species competitive ability by the RYP index, and suggested that asymmetric transitive competitive interactions were common, although not universal (reviewed by Keddy & Shipley 1989; Keddy 2001). Second, many experiments conducted in the field also supported this hypothesis (Goldberg 1987; Miller & Werner

1987; Panetta & Randall 1993; Zamfir & Goldberg 2000; Perkins *et al.* 2007), although they often used a different measure of competitive ability, relative yield of species (RY). This index measures species competitive ability based on the performance of populations rather than individuals. RY of a species in a mixture is calculated as the ratio of its biomass in the mixture to that in its monoculture, with higher RY values indicating greater competitive ability (e.g. in a mixture of species *i* and *j*, if species *i* has higher RY than *j*, then species *i* is considered more competitive). Third, some authors addressed the consistency between pair-wise and multi-species competitive interactions, by either linking species abundance in natural communities to their competitive ranks in pair-wise competition experiments (Mitchley & Grubb 1986; Miller & Werner 1987; Aarssen 1988; Howard & Goldberg 2001; Fraser & Keddy 2005), or constructing competitive hierarchies from both pair-wise and multi-species competitions (Perkins *et al.* 2007). These studies found evidence both for and against the consistency between pair-wise and multi-species interactions.

In RYP or RY analyses, a species is given a competitive ability value by each pair-wise competition experiment. To predict multi-species community structure based on pair-wise competition experiments, one needs to normalize the competitive ability values of the species on a standard scale. Two indices, 'competitive effect' (CE) and 'competitive response' (CR), have been employed for this purpose, each of which gives a single competitive ability value to each species based on the species' performance in all pair-wise combinations (see below 'Techniques for construction of competitive hierarchies'). Such indices may construct competitive hierarchies based on pair-wise interactions that include symmetric or non-transitive components (Perkins *et al.* 2007).

4.1.3 Competitive hierarchies and species coexistence

The competitive hierarchies hypothesis lacks a sound explanation for species coexistence (Silvertown & Dale 1991). The conceptual model of this hypothesis does not allow for stable coexistence of species. Keddy and Shipley (1989) also rejected neutral coexistence as an explanation for the co-occurrence of species within a competitive hierarchy; they pointed out that very similar species may coexist indefinitely or show competitive outcome dependent on stochastic factors. They supposed that the outcome of competition among similar species was unpredictable, and such neutral coexistence would disrupt competitive hierarchies. They proposed that species generally show enough difference with each other (so that competitive exclusion can be common) but species coexistence in nature is maintained by external forces. This explanation is unconvincing to many ecologists (Silvertown & Dale 1991).

Surprisingly, the empirical studies of competitive hierarchies showed little reliable evidence for competitive exclusion; in effect, they ignored this question. An asymmetric interaction, $a > b$, will be given if species *a* has higher RYP or RY than species *b* in pair-wise competition experiments. In this situation, however, the possibility of negative frequency-dependency in population growth (which leads to stable coexistence of species) could not be ruled out. Species *b* may enjoy an advantage in population growth rate when rare, which could not be detected by RY data. The replacement series competition experiments can estimate RYP of species when rare, potentially being capable of detecting negative frequency-dependent competition. However, these experimental studies used the average RYP of individuals within a species across different replacement series to estimate species competitive ability. A

species may have a RYP value greater than one when rare but an average RYP value lower than one. Furthermore, the rarest species in replacement series experiments often have proportional abundance greater than 0.25, which is higher than the abundance of most species in natural communities; a species will not necessarily be excluded in natural communities even it has a RYP value lower than one when rare in such competition experiments. Therefore, an asymmetric interaction, $a > b$, obtained in the empirical studies only means that the presence of species *a* decreases the population size of species *b* more than vice versa; but cannot suggest that species *a* can competitively exclude species *b*.

Stable or neutral coexistence are compatible with competitive hierarchies

I suggest that acceptance of the compelling explanations for species coexistence, niche or neutrality processes, does not undermine, but solidifies, the competitive hierarchies hypothesis. The significance of this hypothesis is to understand the structure of communities comprising of naturally co-occurring species, rather than to understand which species may exclude the others. Niche processes lead to negative frequency-dependency in species population growth rate, promoting stable coexistence; while neutrality (ecological equivalence among species) reduces the speed of competitive exclusion. The two types of mechanisms may operate simultaneously to maintain species diversity in the real world (Chesson 2000; Adler *et al.* 2007).

When the competitive hierarchies framework permit stable coexistence among species, a competitive interaction, $a > b$, does not indicate that species *a* can exclude species *b* but that the presence of species *a* reduces population size of species *b* more than

vice versa. Stable coexistence means that each species tends to recover its abundance when getting rare.

Keddy and Shipley (1989) pointed out that 'nearly equivalent' species could coexist for long periods of time, but the outcome of competition among such species may be unpredictable (or say, such species could not form a competitive hierarchy). It is right that the outcome of competition among perfectly equivalent species could not be predictable; but perfect neutrality may not be common. A recent nearly neutral model of biodiversity shows that species with slight differences in fitness can coexist for a fairly long period of time ($> 10^3$ turnovers), and the slightly stronger competitors tend to have higher abundance in both local and meta- communities (Zhou & Zhang 2008), suggesting that nearly neutral species can form a competitive hierarchy.

In this chapter I use data from two biodiversity experiments, one with algae (Zhang & Zhang 2006) and the other with bacteria (Chapter 3 of this thesis), to address two questions: (a) how common transitive competitive interactions are, and (b) whether competitive hierarchies inferred from pair-wise competitions are consistent with those from multi-species competitions. In each of the two experiments, all possible species combinations from a species pool were constructed (i.e. all possible pair-wise and multi-species competitive interactions could be examined).

4.2 Methods

4.2.1 The algal experiment

Five algal species (*Ankistrodesmus falcatus*, *Chlamydomonas reinhardtii*, *Euglena gracilis, Haematococcus* sp. and *Navicula incerta*) were grown in monocultures

and all possible species combinations. The biomass of each species in each community was measured at 7-d interval from day 28 to 98. All communities received a cold perturbation during day 85-91, and the temperature was restored after day 91 (see details in Zhang & Zhang 2006). A niche complementarity effect among species (by which more diverse communities had greater biomass production) was detected (Zhang & Zhang 2006), implying that the niche processes may have contributed to species coexistence.

4.2.2 The bacterial experiment

I obtained six laboratory bacterial populations, each of which consisted of five or six phenotypes. In total, seven phenotypes were observed, which were referred as SM1, SM2, WS1, WS2, WS3, WS4, and WS5. Phenotypes from each population were grown in all possible monoculture and mixtures for two days, and the yield of every phenotype in every mixture was measured (Chapter 3 of this thesis). I here consider the phenotypes as species and the phenotypic mixtures as communities since bacteria reproduce asexually. In this system both niches and neutrality contribute to species coexistence (Chapter 3).

4.2.3 Techniques for construction of competitive hierarchies

Pair-wise comparison of relative yields

Competitive hierarchies were constructed using the relative yield of species. The relative yield of species *a* in pair-wise competition with species *b* is calculated as ${}_{b}r_{a} = Y_{ab}/Y_{a}$ where Y_{ab} is the yield of species *a* in the presence of species *b*, and Y_a is the yield of species *a* in its monoculture (Keddy 2001). I consider species *a* competitively superior to species *b* if ${}_{b}r_{a} > {}_{a}r_{b}$. Species can be ranked by comparing the relative yields of every pair of competing species. There are three possible outcomes of ranking: (1)

hierarchical ranking (e.g. $a > b > c$), in which competitive relationship among species is transitive and competitive relation within each pair is asymmetric (Keddy & Shipley 1989), (2) hierarchical ranking including symmetric competition (e.g. $a > b = c$), and (3) non-transitive ranking (e.g. $a > b > c > a$).

Competitive effect and competitive response

The construction of competitive hierarchies based on pair-wise comparison of relative yields can be disrupted by symmetric competition or non-transitive interactions (Perkins *et al.* 2007). This problem can be solved by constructing a relative yield matrix in which ${}_jr_i$ is the entry in row *i*, column *j*. The sum of entries in the *j*th column is the neighbour score for species *j*, measuring the ability of species *j* to suppress the other species; the sum of entries in the *i*th row is the target score for species *i*, measuring the ability of species *i* to survive the competition from the other species (Wilson & Keddy 1986). These two scores were competitive effect (CE) and competitive response (CR), respectively (Goldberg & Fleetwood 1987; Miller & Werner 1987). Either low CE or high CR values indicate high competitive ability. CE and CR are normalized competitive ability values of more than two species on a standard scale, making it possible to construct a competitive hierarchy even when non-transitive interactions occurs (Perkins *et al.* 2007).

Competitive hierarchies based on multi-species competitions

I adopt the methodology by Perkins *et al.* (2007) to construct competitive hierarchies for multi-species competition experiments. For tri-wise competitions, ${}_{bc}r_a$ is defined as the relative yield of species *a* in competition with species *b* and *c*. To measure the competitive effect of species *b* on species *a*, another parameter 'effective relative

yield of species *a*' is defined as the sum of all relative yields of *a* in the presence of species *b* and any other species *x*, calculated as ${}_{b}R_{a} = \sum_{x\in\{c,d,\ldots\}} {}_{bx}r_{a}$. I consider species *a* competitively superior to species *b* if ${}_{b}R_{a} > {}_{a}R_{b}$. CE and CR can be calculated from an effective relative yield matrix. This method can be applied to even higher-order (e.g. quadruple-wise) competition experiments.

4.2.4 Competitive hierarchies in my experiments

The commonness of transitive interactions

For each time point of the algal experiment, and each community in the bacterial experiment, I looked at whether or not the competition interactions among species was transitive by pair-wise comparison of relative yields values in two-species communities. I did not use effective relative yield values in multi-species communities to address this question, because non-transitive interactions could be masked in multi-species competition. For instance, for three species with a rock-paper-scissors interaction (i.e. $a > b > c > a$), the relative yields of *a* and *b* in the three-species community are mediated by the presence of species *c*, and thus cannot reliably infer the competitive ability of *a* and *b*.

Consistency of competitive hierarchies from pair-wise and multi-species interactions

I calculated competitive effect (CE) and competitive response (CR) for each species in pair-wise interactions using the relative yield matrix, and those in higher-order interactions using the effective relative yield matrix. For simplicity, I gave a zero value to the relative yield of a species in the presence of itself (i.e. ${}_{i}r_{i}$, or ${}_{i}R_{i}$). The CE and CR values were used to rank the species in competitive ability. I calculated a consistency index for hierarchies from high-order competitions and those from pair-wise competition experiments. Every hierarchy could be written as a series of two-species interactions, for

example, a hierarchy $a > b > c$ can be written as $a > b$, $a > c$, and $b > c$. I decomposed every hierarchy into the basic elements (two-species interactions), base on which the consistency index was calculated. For example, to measure the consistency between two hierarchies, $a > b > c$ and $a > c > b$, the former was decomposed into $a > b$, $a > c$, and $b > c$, and the latter, $a > b$, $a > c$, and $c > b$. The two hierarchies shared two basic elements ($a > b$ and $a > c$), but differed from each other in the third element (the former had $b > c$ and the latter, $c > b$). The consistency between these two hierarchies was defined as 2/3. This consistency index ranges from zero to one.

Table 4.1 Ranking of species in competitive ability produced by pair-wise comparison of relative yield values in two-species communities. In parentheses are rankings of non-transitive interactions. Species in the algal experiment are referred to by the first letters of their genus names (A, *Ankistrodesmus falcatus*; C, *Chlamydomonas reinhardtii*; E, *Euglena gracilis*; H, *Haematococcus* sp.; N, *Navicula incerta*).

	Ranking of species
Algal experiment	
day 28	E>C>N>A>H
day 35	E>C>H>A>N
day 42	E>C>H>A>N
day 49	(N>A>E>C>H>N)
day 56	E>(N>H>C>N)>A
day 63	E>H>C>A>N
day 70	E>H>C>A>N
day 77	E>H>C>A>N
day 84	H>E>C>A>N
day 91 (under-perturbation)	E>H>C>A>N
day 98 (post-perturbation)	H>E>C>A>N
Bacterial experiment	
Community 1	WS5>WS4>WS1>SM1>SM2>WS2
Community 2	WS1>SM1>SM2>WS2>WS5
Community 3	WS1>WS3>WS4>SM1>SM2
Community 4	SM1>WS1>WS2>SM2>WS3>WS4
Community 5	(SM1>WS4>SM2>WS1>SM1)>WS2
Community 6	WS1>SM1>WS2>SM2>WS5

4.3 Results

The algal experiment

Non-transitive rankings of species in competitive ability were observed at two points of time: at day 49 the totally five species formed a circular ranking, and at day 56 three of the five species formed a circular ranking. Transitive rankings were detected at the other nine time points (Table 4.1).

The consistency between multi-species and pair-wise interactions was fairly high (Table 4.2). For hierarchies based on competitive effect (CE), the mean consistency value across all points of time and all levels of species interactions was 0.86 (± 0.020, S.E.); for hierarchies based on competitive response (CR), 0.89 (± 0.014). The consistency between hierarchies based on CE and those based on CR was also high (0.93 ± 0.014).

The bacterial experiment

Non-transitive ranking of species occurred in one out of six communities: in community 5, four species formed a circular ranking. The other five communities had only transitive competitive interactions (Table 4.1).

The consistency between multi-species and pair-wise interactions was fairly high (Table 4.2). For hierarchies based on competitive effect (CE), the mean consistency value across all communities and all level of species interactions was 0.84 (± 0.034); for hierarchies based on competitive response (CR), 0.84 (± 0.020). The consistency between hierarchies based on CE and those based on CR was 0.93 (± 0.019).

4.4 Discussion

I suggest that the competitive hierarchies hypothesis should allow for stable or neutral species coexistence, thus an asymmetric competitive interaction, $a > b$, requires

that species *a* reduces population size of species *b* more than vice versa, not that species *a* excludes species *b*. In the algal experiment most communities saw species coexistence over a long period of time (98 days); extinction occurred very rarely (Zhang & Zhang 2006); in the bacterial system both niche and neutral processes are involved in the maintenance of diversity (Chapter 3). My analyses here show that the competitive hierarchies approach can be applied to communities with stable or neutral species coexistence.

In both experiments transitive competitive interactions were much more common than non-transitive ones. In the algal experiments, the competitive relations among five species were estimated at eleven time points, and non-transitive interactions were observed only at two points of time. In the bacterial experiments, non-transitive ranking of species competitive ability was detected only in one out of six communities. Both experiments saw fairly high consistency between hierarchies based on pair-wise competitions and those based on multi-species competitions.

Transitive competitive relations among species have been observed in many types of communities (Mitchley & Grubb 1986; Miller & Werner 1987; Keddy & Shipley 1989; Keddy *et al.* 2002). Meanwhile, non-transitive interaction also received some attention, and has been suggested as a mechanism for stable coexistence of species (e.g. Buss & Jackson 1979; Sinervo & Lively 1996; Huisman & Weissing 1999; Kirkup & Riley 2004; Karolyi *et al.* 2005; Lankau & Strauss 2007). It is unclear how general transitive or non-transitive competitive interactions are. As suggested by Brooker *et al*. (2008), interference competition is often involved in non-transitive interactions, e.g. cheating by 'sneaker males' in lizards (Sinervo & Lively 1996), or toxin production in bacteria

(Kirkup & Riley 2004); exploitative resource competition may generally lead to transitivity of competitive ability, and thus transitive interactions might be common in systems where interference is unusual, such as plant communities (Harper 1975, 1977).

The indirect interactions among species make it impossible to predict multi-species community structure by pair-wise competition experiments. Ecologists have tried to estimate the consistency between pair-wise and high-order competitive interactions by examining the relations between species abundance in natural communities and their competitive ranking orders in pair-wise competition experiments (Mitchley & Grubb 1986; Miller & Werner 1987; Aarssen 1988). Rarely has this problem been addressed directly in an experimental approach, except for Perkins *et al.* (2007) and the present study. There is yet insufficient evidence either for or against this consistency.

The most serious problem confronted by the competitive hierarchies hypothesis might be the reversals of ranking orders in species competitive ability over space or time; Reversals occur when, e.g., species *a* is competitively superior to species *b* in high-resource habitats but inferior in low-resource habitats. Such reversals make pair-wise competition experiments uninformative to predicting natural community structure (Herben & Krahulec 1990; Silvertown & Dale 1991). There is no doubt that shift in ranking orders of competitive ability over environments can occur. But the key question is how common such reversals are and how sensitive the ranking orders of competitive ability are to environmental change (Keddy & Shipley 1989; Shipley & Keddy 1994). If such reversals occur over environments with small differences, pair-wise competition experiments are not useful for predicting natural community structure; however, if such

reversals only happen when environments change dramatically, the competitive interactions among species could be invariant across a range of environments.

There has been some evidence for the consistency of competitive rankings of species across environments (Keddy *et al.* 1994; Keddy *et al.* 2000). But evidence for the reversals of competitive rankings (i.e. trade-off among species in colonizing different environments) seems more abundant. Ecological studies on interspecific competition find such reversals (Sharitz & McCormick 1973; Sousa *et al.* 1981; Rice & Menke 1985; Aerts *et al.* 1990; Keddy *et al.* 1994; Keddy *et al.* 2000); population genetic research offers even more evidence, although in this case trade-offs among variants within species rather than those among species are concerned: many studies (usually of crops or grass species) found that the variation in performance of genotypes across environments may partially be attributable to genotype-by-environment interactions (Bell 1997). However, I tend to believe that the rarity of evidence against such reversals of competitive rankings (trade-offs) may be due to a publication bias, as 'positive' results demonstrating trade-offs may have greater chance to be published, or to receive more attention. For instance, a green house experiment with six plant species found that experimentally increased temperature changed the competitive interaction between two species (Niu & Wan 2008). In effect, the competitive hierarchies of the totally six species was quite consistent between the two temperature environments, but the authors rather emphasized in their paper the reversal of ranking order of the two species. Therefore, I think it is still too early to make a conclusion on whether the reversals of competitive rankings are common or not.

In my algal experiment, shifts in ranking orders of competitive ability occurred over time. It is not surprising that the species ranking orders changed in the early stages when the communities were at young ages. Reversals also occurred during the late stage (day 84-98): a cold perturbation (day 91) changed the ranking order of the two most competitive species (Table 4.1). However, the ranking of the five species is still fairly consistent across over time: the consistency in the 5-species ranking between day 84 and 91, or day 91 and 98, is 0.9. The bacterial experiment is not appropriate for addressing this question, because the different communities have emerged through independent radiation and a 'same' species may have different characters in different communities, although with the same morphology.

The present study showed that the transitive competitive interactions among species were quite common in two biodiversity-ecosystem functioning experiments; the competitive hierarchies inferred from multi-species competitions are highly consistent with those from pair-wise competitions. But great caution is needed when attempting to generalize the findings that are reported here to natural communities or populations due to the increased complexity of natural settings.. For instance, an experimental study with *Daphnia*, together with observational research in field, found that a larger-bodied species dominates over a smaller-bodied species in many habitats (consistent to the prediction of competitive hierarchies hypothesis), but the presence of a fish species decreases the abundance of both *Daphnia* species which then coexist and do not show competitive effect on each other (Gliwicz & Wrzosek 2008). This implies that co-occurring species in nature that belong to a same guild may not compete with each other at all and the 'competitive' hierarchies hypothesis is definitely irrelevant in such situations.

Nevertheless, this framework may be a working approach to understanding the structure of communities that are likely to be regulated by bottom-up processes (e.g. grasslands).

Table 4.2 Competitive hierarchies based on CE and CR in pair-wise and multi-species competitions. In square brackets are consistency between hierarchies from multi-species competition and those from pair-wise competitions. Species abbreviations as in Table 4.1.

	CE	CR	Consistency: CE and CR
Algal experiment			
Day 28			
Pair-wise	E>N>C>H>A	E>C>A>N>H	7/10
Triple-wise	E>C>A>H>N [6/10]	E>C>H>A>N [8/10]	9/10
Quadruple-wise	E>C>N>H>A [9/10]	E>C>N>A>H [9/10]	9/10
Quintuple-wise	N>C>E>A>H [7/10]	N>C>E>A>H [6/10]	10/10
Day 35			
Pair-wise	E>C>H>A>N	E>C>H>A>N	10/10
Triple-wise	E>C>H>A>N [10/10]	E>C>H>A>N [10/10]	10/10
Quadruple-wise	E>C>H>A>N [10/10]	E>C>A>H>N [9/10]	9/10
Quintuple-wise	E>C>A>H>N [9/10]	E>C>A>H>N [9/10]	10/10
Day 42			
Pair-wise	E>C>H>A>N	E>C>H>A>N	10/10
Triple-wise	E>C>H>A>N [10/10]	E>C>H>A>N [10/10]	10/10
Quadruple-wise	E>C>H>A>N [10/10]	E>C>H>A>N [10/10]	10/10
Quintuple-wise	E>C>H>A>N [10/10]	E>C>H>A>N [10/10]	10/10
Day 49			
Pair-wise	H>E>C>A>N	E>C>A>H>N	7/10
Triple-wise	E>C>A>H>N [7/10]	E>C>H>A>N [9/10]	9/10
Quadruple-wise	E>C>H>A>N [8/10]	E>C>H>A>N [9/10]	10/10
Quintuple-wise	E>C>H>A>N [8/10]	E>C>H>A>N [9/10]	10/10
Day 56			
Pair-wise	E>H>C>N>A	E>C>H>N>A	9/10
Triple-wise	E>H>C>N>A [10/10]	E>C>H>N>A [10/10]	9/10
Quadruple-wise	E>C>H>A>N [8/10]	E>C>H>A>N [9/10]	10/10
Quintuple-wise	E>C>A>H>N [7/10]	E>C>A>H>N [8/10]	10/10
Day 63			
Pair-wise	E>C>A>H>N	E>H>C>A>N	8/10
Triple-wise	E>C>H>A>N [9/10]	E>C>H>A>N [9/10]	10/10
Quadruple-wise	H>A>E>C>N [5/10]	H>E>C>A>N [9/10]	8/10
Quintuple-wise	H>E>C>A>N [7/10]	H>E>C>A>N [9/10]	10/10
Day 70			
Pair-wise	E>H>C>A>N	E>C>H>A>N	9/10
Triple-wise	E>C>H>A>N [9/10]	E>C>H>A>N [10/10]	10/10
Quadruple-wise	E>C>H>A>N [9/10]	E>C>H>A>N [10/10]	10/10
Quintuple-wise	E>A>C>H>N [7/10]	E>A>C>H>N [8/10]	10/10
Day 77			
Pair-wise	E>H>C>A>N	E>C>H>A>N	9/10
Triple-wise	E>C>H>A>N [9/10]	E>H>C>A>N [9/10]	9/10
Quadruple-wise	E>H>C>A>N [10/10]	E>H>C>A>N [9/10]	10/10
Quintuple-wise	E>H>C>A>N [10/10]	E>H>C>A>N [9/10]	10/10
Day 84			
Pair-wise	H>E>A>C>N	H>E>C>A>N	9/10
Triple-wise	E>H>N>A>C [7/10]	E>H>C>A>N [9/10]	7/10

Quadruple-wise	E>H>C>A>N [8/10]	E>H>C>A>N [9/10]	10/10
Quintuple-wise	E>H>C>A>N [8/10]	E>H>C>A>N [9/10]	10/10
Day 91			
Pair-wise	E>H>A>C>N	H>E>C>A>N	8/10
Triple-wise	E>H>C>N>A [8/10]	E>H>C>A>N [9/10]	9/10
Quadruple-wise	E>H>C>A>N [9/10]	E>H>C>A>N [9/10]	10/10
Quintuple-wise	E>H>C>A>N [9/10]	E>H>C>A>N [9/10]	10/10
Day 98			
Pair-wise	E>H>A>C>N	H>E>C>N>A	7/10
Triple-wise	E>H>C>N>A [8/10]	E>H>C>A>N [8/10]	9/10
Quadruple-wise	E>H>C>A>N [9/10]	E>H>C>A>N [8/10]	10/10
Quintuple-wise	E>H>C>A>N [9/10]	E>H>C>A>N [8/10]	10/10
Bacterial experiment			
Community 1			
Pair-wise	WS5>WS4>WS1>SM1>SM2>WS2	WS4>WS5>SM1>WS1>SM2>WS2	13/15
Triple-wise	WS5>SM1>WS4>WS1>SM2>WS2 [13/15]	WS4>WS5>SM1>WS1>SM2>WS2 [15/15]	13/15
Quadruple-wise	SM1>WS5>WS4>WS1>WS2>SM2 [11/15]	WS5>SM1>WS4>WS1>SM2>WS2 [13/15]	13/15
Quintuple-wise	SM1>WS5>WS4>WS1>SM2>WS2 [12/15]	WS5>WS4>SM1>WS1>SM2>WS2 [14/15]	13/15
Sextuple-wise	SM1>WS5>WS4>WS1>SM2>WS2 [12/15]	SM1>WS5>WS4>WS1>SM2>WS2 [12/15]	15/15
Community 2			
Pair-wise	SM1>WS1>WS2>WS5>SM2	SM1>WS1>WS2>SM2>WS5	9/10
Triple-wise	SM1>WS1>SM2>WS5>WS2 [7/10]	SM1>WS1>SM2>WS5>WS2 [8/10]	10/10
Quadruple-wise	SM1>WS1>SM2>WS5>WS2 [7/10]	SM1>WS1>WS5>SM2>WS2 [7/10]	9/10
Quintuple-wise	SM1>WS1>WS5>SM2>WS2 [8/10]	SM1>WS1>WS5>SM2>WS2 [7/10]	10/10
Community 3			
Pair-wise	WS1>SM1>WS3>WS4>SM2	WS1>WS3>WS4>SM1>SM2	8/10
Triple-wise	WS1>WS3>SM1>WS4>SM2 [9/10]	WS1>WS3>SM1>WS4>SM2 [8/10]	10/10
Quadruple-wise	WS1>SM1>WS3>WS4>SM2 [10/10]	WS1>SM1>WS3>WS4>SM2 [8/10]	10/10
Quintuple-wise	WS1>SM1>WS3>WS4>SM2 [10/10]	WS1>SM1>WS3>WS4>SM2 [8/10]	10/10
Community 4			
Pair-wise	WS1>SM1>WS2>SM2>WS3>WS4	WS1>WS2>SM1>SM2>WS3>WS4	14/15
Triple-wise	WS1>SM1>WS2>SM2>WS3>WS4 [15/15]	WS2>WS1>SM1>SM2>WS3>WS4 [14/15]	13/15
Quadruple-wise	WS1>SM1>WS2>SM2>WS3>WS4 [15/15]	WS1>SM1>WS2>SM2>WS3>WS4 [14/15]	15/15
Quintuple-wise	WS1>SM1>WS2>SM2>WS3>WS4 [15/15]	WS1>SM1>WS2>SM2>WS3>WS4 [14/15]	15/15
Sextuple-wise	SM1>WS1>WS2>SM2>WS3>WS4 [14/15]	SM1>WS1>WS2>SM2>WS3>WS4 [13/15]	15/15
Community 5			
Pair-wise	WS1>WS4>SM2>SM1>WS2	SM1>WS4>WS1>SM2>WS2	6/10
Triple-wise	WS1>WS4>SM1>SM2>WS2 [9/10]	WS1>WS4>SM1>SM2>WS2 [7/10]	10/10
Quadruple-wise	SM1>WS4>SM2>WS1>WS2 [5/10]	WS4>SM1>WS1>SM2>WS2 [9/10]	8/10
Quintuple-wise	SM1>SM2>WS1>WS4>WS2 [5/10]	SM1>SM2>WS1>WS4>WS2 [7/10]	10/10
Community 6			
Pair-wise	WS1>SM1>WS2>SM2>WS5	WS1>SM1>WS2>SM2>WS5	10/10
Triple-wise	WS1>SM1>WS2>WS5>SM2 [9/10]	WS1>SM1>WS2>WS5>SM2 [9/10]	10/10
Quadruple-wise	SM1>WS1>WS2>WS5>SM2 [8/10]	SM1>WS1>WS2>SM2>WS5 [9/10]	9/10
Quintuple-wise	SM1>WS1>WS2>SM2>WS5 [9/10]	SM1>WS1>WS2>SM2>WS5 [9/10]	10/10

Chapter 5: Predicting the functional consequences of non-random species loss by random assembly biodiversity experiments

Summary

Recent studies have highlighted the importance of understanding the functional consequences of non-random biodiversity declines. Here I show that the existing random assembly biodiversity experiments may provide valuable information to answer this question. The random assembly experiments generally estimate niche complementarity and selection effects that jointly explain biodiversity effect on productivity. The magnitude of the two effects may predict the intensity of functional recovery due to species compensatory growth following species loss under particular extinction scenarios. Greater complementarity effect suggests smaller niche overlap among species (i.e. weaker interspecific competition) which, in turn, predicts weaker compensatory growth following species loss. The selection effect values indicate the relation between species' abundance in multi-species communities and their functional performance, which can be used to predict the magnitude of functional recovery due to species compensatory growth under specific extinction scenarios. I re-analyzed the data of two biodiversity experiments, and obtained the diversity-biomass relationships under two extinction scenarios, ascending-ordered (where rare species go extinct before the abundant) and descending-ordered (where abundant species are lost first) species loss. The resultant diversity-biomass relationships were in general consistent with those predicted by an analysis of niche complementarity and selection effects.

5.1 Introduction

The global declines in biodiversity have motivated considerable research on biodiversity-ecosystem functioning relationships in the past two decades (Schulze & Mooney 1993; Kinzig *et al.* 2001; Loreau *et al.* 2002). The majority of studies in this area have focused on the effect of species richness on ecosystem productivity; they often manipulated species richness by using randomly chosen species from a species pool to assemble communities containing different numbers of species (Tilman *et al.* 1996; Hooper & Vitousek 1997; McGrady-Steed *et al.* 1997; Symstad *et al.* 1998; Hector *et al.* 1999; Naeem *et al.* 2000; Mulder *et al.* 2001; Tilman *et al.* 2001; Cardinale *et al.* 2002; Fridley 2002; Pfisterer & Schmid 2002; van Ruijven & Berendse 2005). Ecologists often, although not always, found a positive effect of biodiversity on productivity; this effect could be explained by two non-mutually exclusive mechanisms: (a) niche complementarity, resulting from differences in resource/habitat use among species or interspecific facilitation (Tilman *et al.* 1997; Loreau 2000), and (b) selection effect, which occurs because the species-rich communities have a greater chance to include high-yielding species (Aarssen 1997; Huston 1997). These random assembly experiments offer a general understanding of biodiversity effects on ecosystem functioning. However, they do not immediately predict the consequences of realistic species extinctions that are often non-random (Pimm *et al.* 1988; Tracy & George 1992; McKinney 1997; Petchey *et al.* 1999; Thomas *et al.* 2004). The functional consequences of non-random species loss have attracted some attention in recent years (Sala *et al.* 1996; Lyons & Schwartz 2001; Adler & Bradford 2002; Ostfeld & LoGiudice 2003; Smith & Knapp 2003; Ives & Cardinale 2004; Gross & Cardinale 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005; Fox

2006; Suding *et al.* 2006; Ball *et al.* 2008); in particular, several authors examined the effect of realistic species loss in several types of natural communities, where the realistic species loss orders were determined by existing knowledge of species extinction risk (Jonsson *et al.* 2002; Zavaleta & Hulvey 2004; Cross & Harte 2007; Bracken *et al.* 2008). I here address whether the random assembly experiments can be useful for understanding the functional consequences of non-random species loss.

To predict the consequences of realistic species loss, ecologists must know (a) the extinction risk of species, and (b) the functional contributions of the lost species and the responses of the surviving species (Adler & Bradford 2002; Gross & Cardinale 2005; Fox & Harpole 2008). The random assembly experiments do not tell much about the former, but can help understand the latter. Such experiments often estimate the strength of niche complementarity effect (namely niche partitioning), which can indicate the possibility of compensatory growth following species loss. One extreme situation is perfect niche partitioning (i.e. no competition among species), where every species in a multi-species community is as productive as in monoculture, ecosystem productivity shows a proportionately linear increase with species richness. In this case, the surviving species show negligible compensatory response to species loss, and the current functional contribution of a species to a community directly predicts the functional loss caused by the extinction of that species. The consequences of non-random extinction for biodiversity-ecosystem functioning relationships depend on the relation between species' functional importance and extinction risk; for example, when functionally important species have higher extinction risk, the initial species loss causes abrupt functional loss

and further species extinctions have smaller functional consequence (Sala *et al.* 1996; Adler & Bradford 2002; Larsen *et al.* 2005), as shown by the dash lines in Figure 5.1.

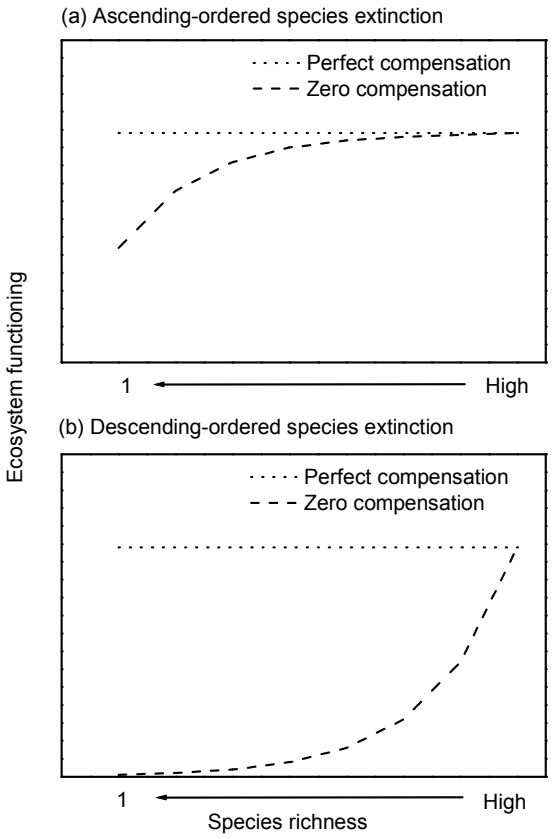

Figure 5.1 Hypothetical relationships between species richness and ecosystem functioning under two species extinction scenarios, ascending-ordered (in which the rarest species is lost first, followed by the secondly rarest, and so on) and descending-ordered extinction (in which the most abundant species goes extinct first). The dashed lines show the expected relationships with an assumption of zero compensatory growth among species, and the dotted lines, perfect compensation.

When niche complementarity is not perfect (which is often the case), the surviving species should have compensatory growth following species loss. The smaller the complementarity effect, the stronger the compensation. Meanwhile, the magnitude of compensatory growth also depends on the functional traits of the surviving species. Selection effect values estimated by random assembly experiments can predict the magnitude of functional compensation under specific conditions. For example, if rare species are more likely to be lost, a positive selection effect (which means that productive species are more competitive and have higher abundance in multi-species communities) suggests that low-yielding species go extinct first, where the remaining productive

species may well compensate the functional loss. In contrast, a negative selection effect suggests that productive species be lost first, where the remaining low-yielding species may only poorly compensate the functional loss (Adler & Bradford 2002; Gross & Cardinale 2005). A special scenario is perfect compensation (dotted lines in Fig. 5.1) which can occur, for example, when niche complementarity is zero and every species have the same functional performance.

5.2 Methods

I here consider two extreme and simplistic scenarios of species extinction, ascending- and descending-ordered extinction, and re-analyze the data of two biodiversity experiments to look at whether random assembly studies can be used to predict the consequences of the two extinction scenarios. Ascending-ordered species extinction occurs when the rarest species is lost first, followed by the second rarest, and so on. In the descending-ordered extinction scenario, species extinction begins with the most abundant (Sala *et al.* 1996). I examine the diversity-biomass relations produced by the two extinction scenarios in two biodiversity experiments. In each of the experiment, all possible monocultures and all possible combinations of species from a species pool were grown. Such an experimental design allows me to directly assess the consequences of extinction of particular species by comparing the 'full species mixture' (which contains all species from a pool) to a mixture that consists of all but the focal species. Then I can look at whether the niche complementarity and selection effects can predict the consequences of non-random species loss.

In the first experiment, five algal species were grown in all possible monocultures and mixtures; biomass of every species in every community was measured once a week, for eleven weeks (Zhang & Zhang 2005; Zhang & Zhang 2006). Biomass production increased with species richness but further increase in species richness when beyond 3-4 did not lead to further increase in biomass. Both complementarity and selection effects were significantly positive (Zhang & Zhang 2005; Zhang & Zhang 2006); but the niche complementarity among species was far from perfect, indicated by the saturating increase in biomass with species richness (Fig. 5.A1). In the second experiment (Chapter 3 of this thesis), I obtained six laboratory bacterial communities each of that contained five or six phenotypes. I consider the phenotypes as analogues of species as they reproduce asexually. From each community, the species were harvested, and then grown in all possible monocultures and mixtures. Either complementarity or selection was zero or very weak, and species richness showed little effect on biomass production (Chapter 3).

An analysis of complementarity and selection effect can make some predictions of the consequences of non-random species loss. In the algal experiment, species showed imperfect niche partitioning, so compensatory growth should occur after species extinction. Under the ascending extinction scenario, the rare species (also low-yielding species, as suggested by a positive selection effect) go extinct first, the functional loss may be well compensated by the remaining productive species; the diversity-biomass curve should differ from that expected with zero compensation (dash line in Fig. 5.1a), but close to expected with perfect compensation (dotted line in Fig. 5.1a). Under the descending extinction scenario, the abundant (also high-yielding) species go extinct first, the remaining rare species may not be able to recover the functional loss very well; the

diversity-biomass relation might be close to the expected with zero compensation (dash line in Fig. 5.1b). In the bacterial experiment, niche complementarity was zero or very weak, predicting strong compensatory growth following species extinction. The selection effect was zero or very weak because there was no large difference in monoculture yields among species, and the dominance of species in multi-species communities were not correlated with their performance in monocultures. Therefore, the functional role of either abundant or rare species should be replaceable, and the diversity-biomass relationship under either extinction scenario (ascending or descending-ordered) would be near the expected with perfect compensation (dotted lines in Fig. 5.1).

To test the predictions above, I obtained the 'observed' diversity-productivity relationship under the two extinction scenarios. I considered the two experiments as species removal studies, and estimated the consequences of extinction of particular species by comparing a full mixture (which contains all species from a species pool) with less diverse communities (Adler & Bradford 2002). With ascending-ordered species extinction, the observed community biomass with one species lost was defined as the biomass of the mixture consisting of all but the rarest species in the full mixture; community biomass with subsequent species extinction was defined in the same way. The expected community biomass with an assumption of zero compensation was obtained based on the full mixture; the expected community biomass with one species lost was defined as the biomass of the full mixture minus the biomass of the rarest species in that mixture, and so on. The expected community biomass with an assumption of perfect compensation was the biomass of the full mixture. The diversity-biomass relationships

under the descending extinction scenario were derived in the same way, with the most abundant species 'removed' first.

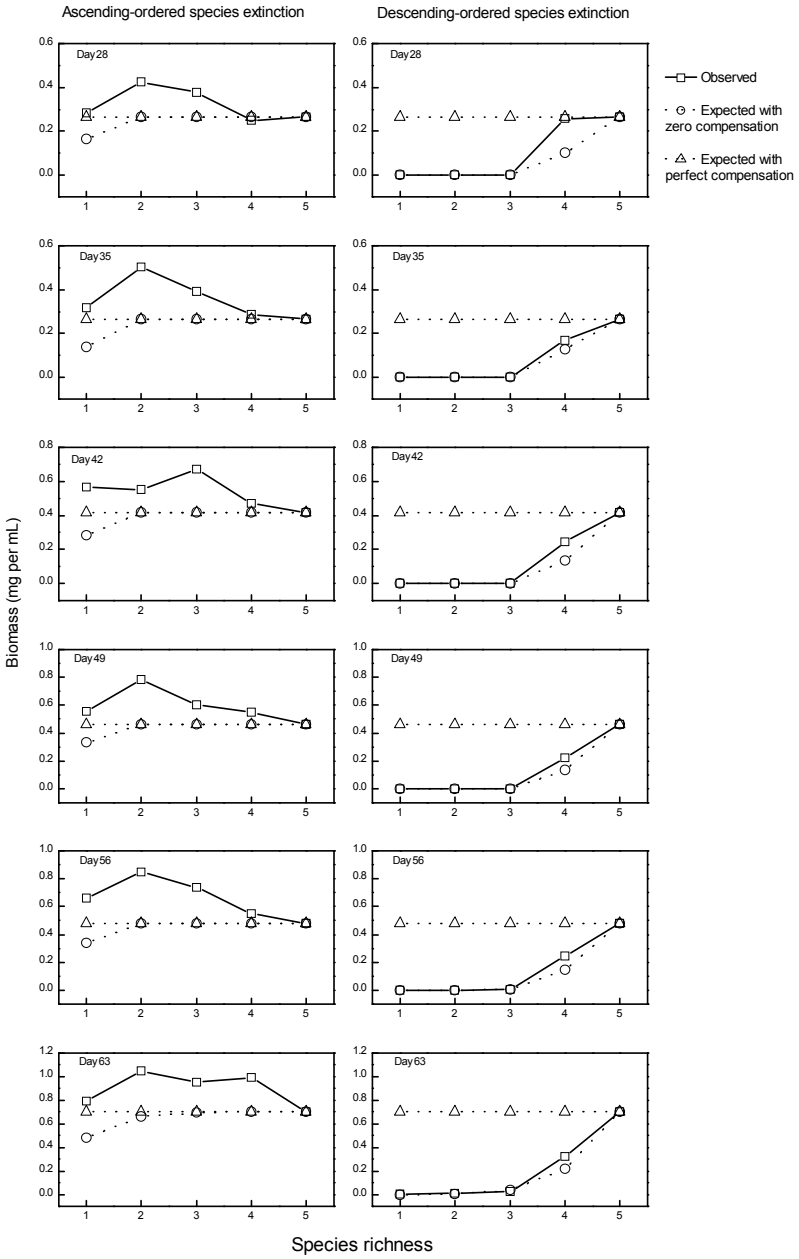

Figure 5.2 The observed diversity-biomass relationships, compared with the expected with an assumption of zero or perfect compensation, in the algal experiment.

The relation between species richness and community biomass under each extinction scenario was estimated by Pearson's correlation test. As each diversity-biomass relation was described by five or six data points, with one data point at each

level of species richness, I determined the difference between any two diversity-biomass relations (e.g. the observed diversity-biomass relation produced by ascending extinction and that by descending extinction) with paired-sample *t* tests. Biomass data were log transformed before analyses (log 10 (mg per L +1) for the algal experiment, and log 10 (colony formation units per mL +1) for the bacterial experiment).

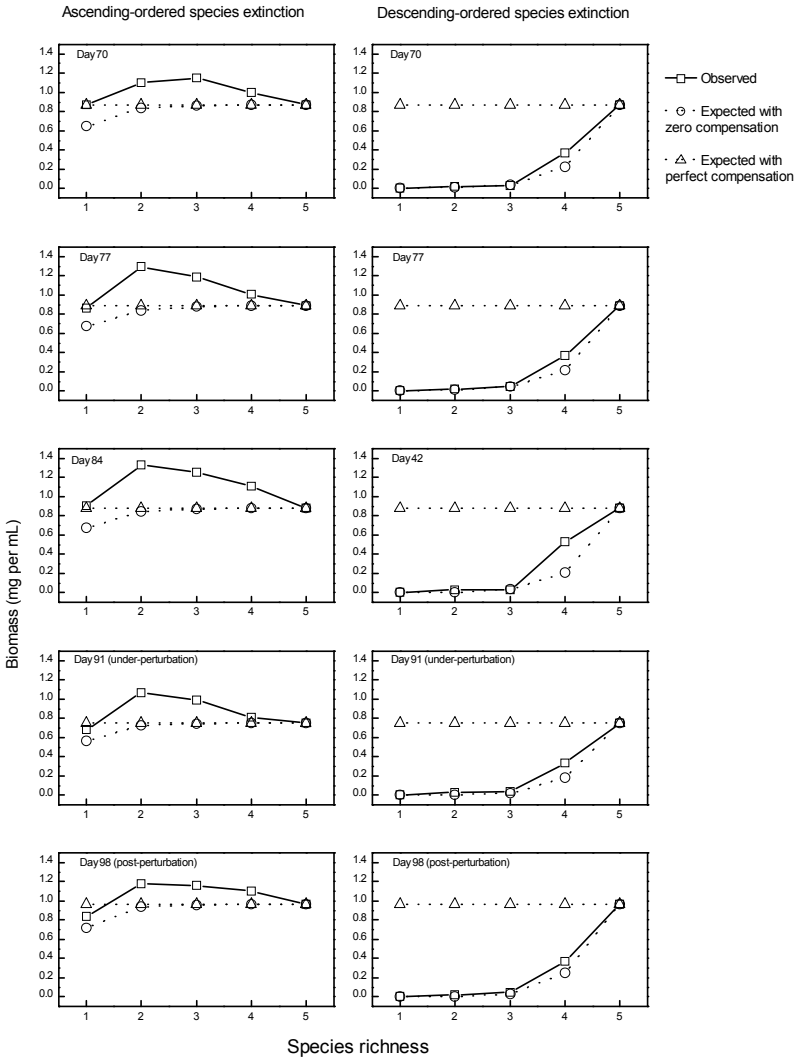

Figure 5.2 Extended.

5.3 Results

The algal experiment

I observed different diversity-biomass relationships under different species extinction scenarios. When species extinction occurs in an ascending fashion, no significant correlation was observed between community biomass and specie diversity (Pearson's, $P > 0.10$ at any day; Fig. 5.2). Under the descending extinction scenario, the diversity-biomass relation was always positive ($P < 0.02$ at any day; Fig. 5.2).

Under the ascending extinction scenario, the observed diversity-biomass relation was different from expected with zero compensation throughout the experiment except for day 28, and different from expected with perfect compensation at some points of time (Fig. 5.2; Table 5.A2). Whenever the observed diversity-biomass relation was different from expected with perfect compensation, initial species extinction (loss of rare species) did not decrease but increased community biomass; but the loss of the second most abundant species from the two-species mixture (which was composed of the most and the second most abundant species) decreased biomass production (Fig. 5.2). This suggests that the functional loss due to rare species extinction could be fully or over compensated by the remaining abundant species, but the functional role of the second most abundant species could not be fully replaced by the most abundant species. Under the descending extinction scenario, the observed diversity-biomass relation was or was not distinguishable from expected with zero compensation, and always different from expected with perfect compensation (Fig. 5.2; Table 5.A2), implying that the functional loss caused by abundant species extinction can only partially be compensated by the remaining rare species at some time points.

The Bacterial experiment

In general, species extinction order did not influence the diversity-biomass relationships. Under the ascending extinction scenario, non-significant correlations ($P > 0.20$) between species richness and biomass were observed for all but one community (community 5 had a negative correlation, Pearson's, $r = -0.921$, $P = 0.020$). Under the descending extinction scenario, non-significant diversity-biomass correlations ($P > 0.20$) were found for all but one community (community 4 showed a negative correlation, $r = -0.862$, $P = 0.027$; Fig. 5.3).

With ascending species extinction, the functional loss caused by species loss was fully compensated (slightly overcompensated for community 5), except for community 6 (Fig. 5.3; Table 5.A2). Similar results were found for the descending extinction scenario: the observed diversity-biomass relation was indistinguishable from expected with perfect compensation in every community (Fig. 5.3; Table 5.A2).

5.4 Discussion

The random assembly experiments may be useful for predicting the functional consequences of non-random species loss (Gross & Cardinale 2005; Fox & Harpole 2008). In the present study, I made some predictions of the diversity-biomass relations under two particular extinction scenarios, based on an analysis of the niche complementarity and selection effects. I then considered my experiments as species removal studies, and obtained the observed diversity-biomass relationship under the two extinction scenarios by comparing a 'full' species mixture that contains all species with a series of mixtures that comprise of subsets of these species. The predictions are generally supported.

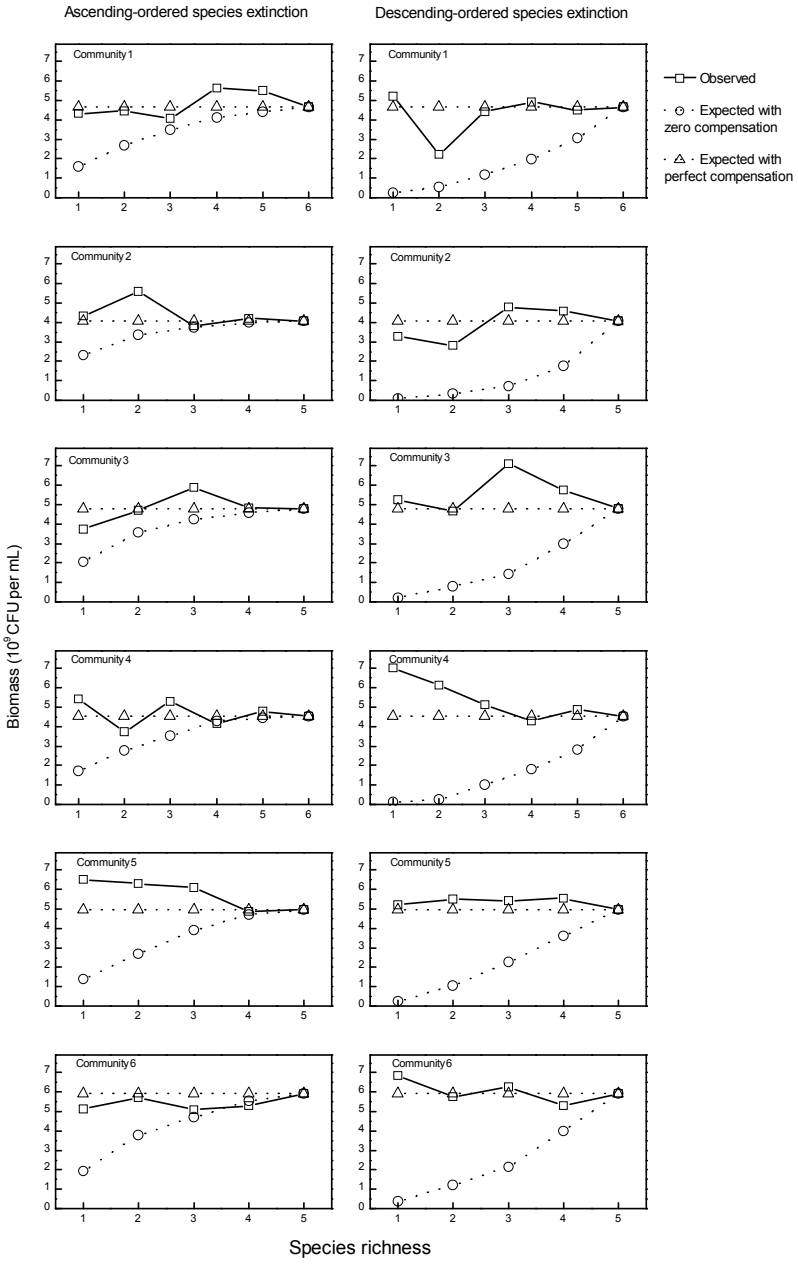

Figure 5.3 The observed diversity-biomass relationships, compared with the expected with an assumption of zero or perfect compensation, in the bacterial experiment.

In the algal experiment, the positive but imperfect niche complementarity effect suggests partial compensatory growth following species loss; and the positive selection effect suggests that the compensatory growth should be strong under the ascending extinction scenario (in which the rare species go extinct first) but weak under the

descending extinction scenario (in which the abundant species are to be lost first). The predictions are supported. The observed diversity-biomass relations under the ascending extinction scenario suggest that functional loss caused by the extinction of the three rare species could be fully or over compensated by the two remaining abundant species, although the most abundant species may not fully replace the functional role of the second most abundant one. When species were lost in a descending fashion, however, the remaining rare species could only partially, or could not at all, replace the functional role of the abundant ones (Fig. 5.2). In a multi-species community, a species may occupy some niche space that can only used by itself, and also occupy some common niche space shared by more than one species. A species' extinction would leave its unique niche space vacated, compromising the functional performance of the entire community; meanwhile, the shared niche space could be fully occupied by the remaining species. When a low-yielding species is lost, its unique niche space is empty, leading to functional loss; but the overlapped niche space previously occupied by this species would be used by more productive species. The increase in the performance of productive species may overcompensate the functional loss (Gross & Cardinale 2005). Another possible explanation for overcompensation is that the lost species had interference effect on the other species and extinction of such species removed the interference effect (Adler & Bradford 2002).

The bacterial experiment saw zero or very weak niche complementarity effects, suggesting strong compensatory growth among species. Selection effect was zero or very weak; there was no large difference in monoculture yields among species (see the data points for species richness of one in Fig. 5.3 for the monoculture yields of the most

abundant and the rarest species in the full communities), and the dominance of species in diverse communities were not correlated with their monoculture performance. This suggests that the functional role of any species could be well replaced by the others. Under either the ascending or descending extinction scenario, the observed diversity-biomass relations indicated perfect compensation (Fig. 5.3).

The diversity-productivity relationship observed in my algal experiment, that is, a saturating increase in productivity with species richness resulting from positive complementarity and positive selection effect, may be quite general, especially in plant biodiversity experiments (Tilman *et al.* 1996; Loreau & Hector 2001; Schmid *et al.* 2001; Spaekova & Leps 2001; Tilman *et al.* 2001; Hooper *et al.* 2005; Spehn *et al.* 2005; Cardinale *et al.* 2007). The functional loss caused by extinction of rare species in such systems may be much smaller than predicted by the diversity-productivity relationships inferred from random assemblages. Since ascending-ordered species extinction is supposed more general than the other extinction scenarios in natural communities as rare species are more prone to stochastic extinction and suffer from Allee effect (Pimm *et al.* 1988; Tracy & George 1992), the functional loss caused by realistic biodiversity declines may be smaller than previously thought. However, great caution is needed to generalize this finding, as most studies have only examined one to a few functions of ecosystems (e.g. primary productivity, nutrient cycling, or decomposition) but to conserve multiple functions may require greater biodiversity (Reich *et al.* 2004; Lyons *et al.* 2005; Spehn *et al.* 2005; Hector & Bagchi 2007).

Appendix of Chapter 5

Appendix 1

Table 5.A1 Summary of paired-samples *t* tests for the difference in diversity-biomass relationships between different extinction scenarios.

	Difference between the ascending and descending extinction scenarios	Difference between the ascending extinction and the average of all possible extinction scenarios	Difference between the descending extinction and the average of all possible extinction scenarios
Algal experiment			
Day 28	t = 2.439, P = 0.071	t = -1.826, P = 0.142	t = 2.468, P = 0.069
Day 35	t = 2.625, P = 0.058	t = -1.818, P = 0.143	t = 2.681, P = 0.055
Day 42	t = 2.664, P = 0.056	t = -2.226, P = 0.090	t = 2.640, P = 0.058
Day 49	t = 2.750, P = 0.051	t = -2.287, P = 0.084	t = 2.738, P = 0.052
Day 56	t = 2.678, P = 0.055	t = -1.922, P = 0.127	t = 2.755, P = 0.051
Day 63	t = 2.763, P = 0.051	t = -2.357, P = 0.078	t = 2.814, P = 0.048
Day 70	t = 2.798, P = 0.049	t = -2.345, P = 0.079	t = 2.870, P = 0.045
Day 77	t = 2.776, P = 0.050	t = -2.414, P = 0.073	t = 2.849, P = 0.046
Day 84	t = 2.702, P = 0.054	t = -2.518, P = 0.065	t = 2.709, P = 0.054
Day 91	t = 2.762, P = 0.051	t = -2.612, P = 0.059	t = 2.776, P = 0.050
Day 98	t = 2.814, P = 0.048	t = -2.639, P = 0.058	t = 2.845, P = 0.047
Bacterial experiment			
Community 1	t = 1.028, P = 0.351	t = -3.413, P = 0.019	t = 0.183, P = 0.862
Community 2	t = 0.814, P = 0.461	t = -0.661, P = 0.545	t = 0.905, P = 0.417
Community 3	t = -2.152, P = 0.098	t = 1.452, P = 0.220	t = -0.807, P = 0.465
Community 4	t = -1.494, P = 0.196	t = -0.729, P = 0.499	t = -2.009, P = 0.101
Community 5	t = 1.006, P = 0.371	t = -1.539, P = 0.199	t = 0.698, P = 0.524
Community 6	t = -1.635, P = 0.177	t = 0.583, P = 0.591	t = -2.201, P = 0.092

Appendix 2

Table 5.A2 Summary of paired-samples *t* tests for the difference between the observed diversity-biomass relationships and the expected.

	Ascending extinction scenario		Descending extinction scenario	
	Difference between the observed and the expected with zero compensation	Difference between the observed and the expected with perfect compensation	Difference between the observed and the expected with zero compensation	Difference between the observed and the expected with perfect compensation
Algal experiment				
Day 28	t = 2.122, P = 0.101	t = 1.593, P = 0.186	t = 1.085, P = 0.339	t = -2.462, P = 0.070
Day 35	t = 2.597, P = 0.060	t = 2.410, P = 0.074	t = 1.886, P = 0.132	t = -2.592, P = 0.061
Day 42	t = 2.498, P = 0.067	t = 2.856, P = 0.046	t = 1.934, P = 0.125	t = -2.630, P = 0.058
Day 49	t = 2.601, P = 0.060	t = 2.339, P = 0.079	t = 0.803, P = 0.467	t = -2.730, P = 0.052
Day 56	t = 2.832, P = 0.047	t = 2.864, P = 0.046	t = 2.458, P = 0.070	t = -2.635, P = 0.058
Day 63	t = 3.683, P = 0.021	t = 3.150, P = 0.035	t = 1.440, P = 0.223	t = -2.595, P = 0.060
Day 70	t = 3.392, P = 0.027	t = 2.203, P = 0.092	t = 1.684, P = 0.167	t = -2.697, P = 0.054
Day 77	t = 2.983, P = 0.041	t = 1.928, P = 0.126	t = 2.164, P = 0.096	t = -2.658, P = 0.056
Day 84	t = 3.511, P = 0.025	t = 2.450, P = 0.070	t = 1.969, P = 0.120	t = -2.545, P = 0.064
Day 91	t = 2.732, P = 0.052	t = 1.475, P = 0.214	t = 2.526, P = 0.065	t = -2.650, P = 0.057
Day 98	t = 3.658, P = 0.022	t = 1.178, P = 0.304	t = 2.600, P = 0.060	t = -2.695, P = 0.054
Bacterial experiment				
Community 1	t = 2.538, P = 0.052	t = 0.337, P = 0.750	t = 2.728, P = 0.041	t = -0.872, P = 0.423
Community 2	t = 1.824, P = 0.142	t = 1.064, P = 0.347	t = 2.898, P = 0.044	t = -0.610, P = 0.575
Community 3	t = 2.335, P = 0.080	t = -0.199, P = 0.852	t = 2.677, P = 0.055	t = 1.679, P = 0.168
Community 4	t = 1.757, P = 0.139	t = 0.241, P = 0.819	t = 2.642, P = 0.046	t = 1.866, P = 0.121
Community 5	t = 2.000, P = 0.116	t = 2.289, P = 0.084	t = 2.224, P = 0.090	t = 0.115, P = 0.914
Community 6	t = 1.491, P = 0.210	t = -2.955, P = 0.042	t = 2.263, P = 0.086	t = 0.279, P = 0.794

Appendix 3

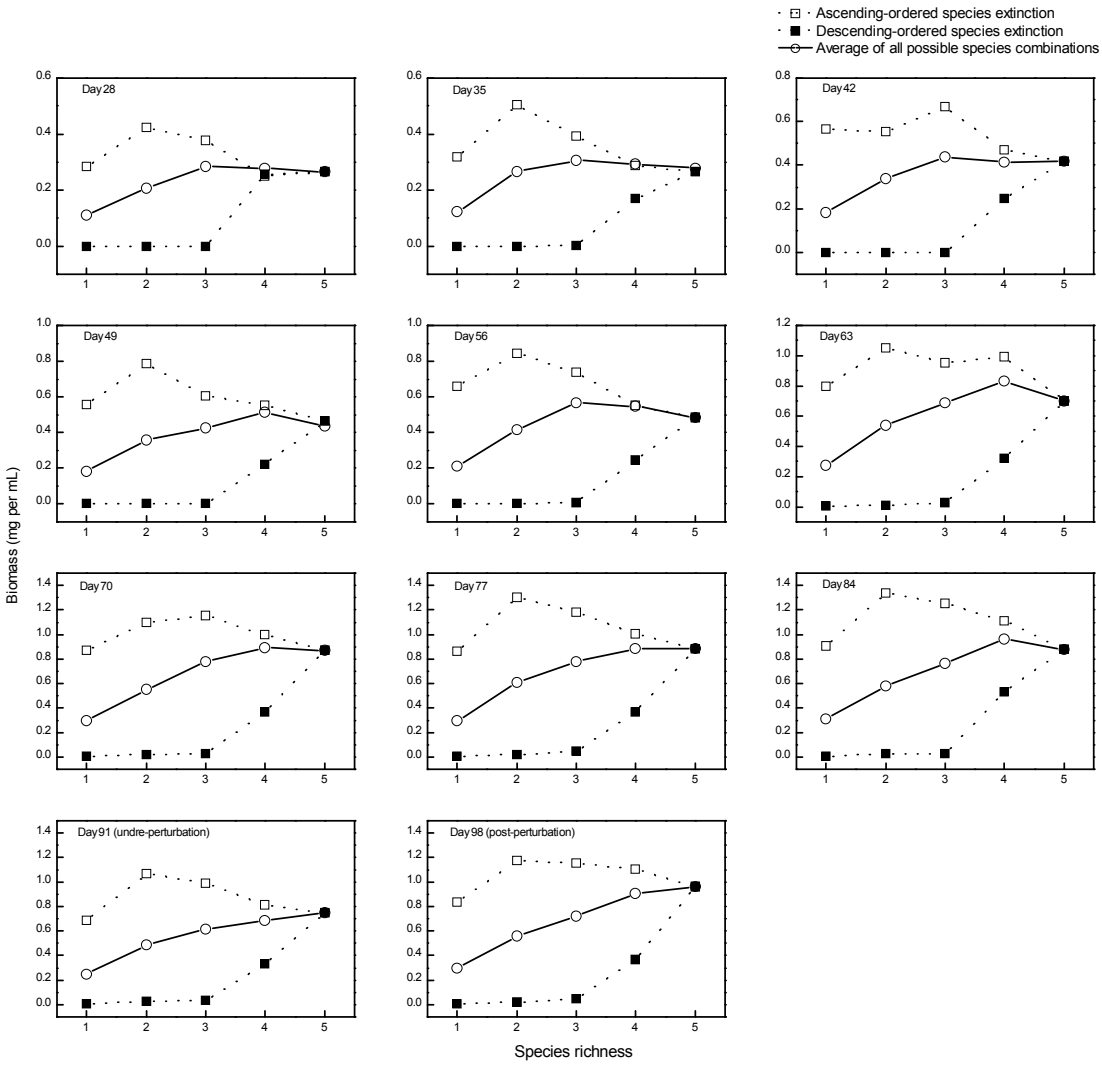

Figure 5.A1 The observed diversity-biomass relationships under the ascending and descending species extinction scenarios and that produced by the average of all possible species combinations (i.e. all possible species extinction scenarios) in the algal experiment.

Appendix 4

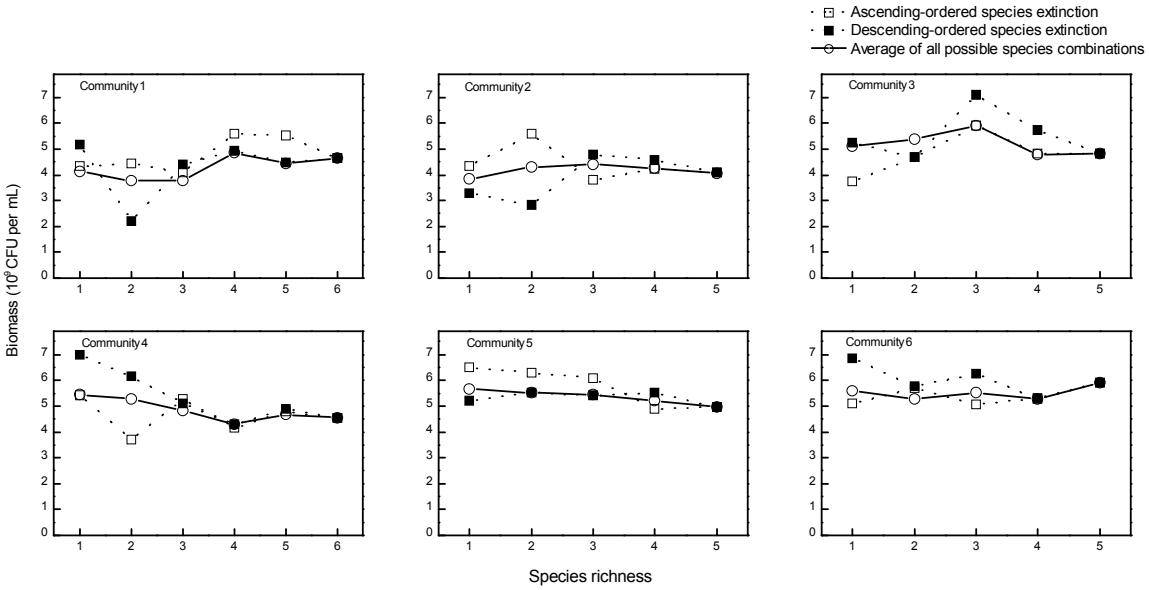

Fig. 5.A2 The observed diversity-biomass relationships under the ascending and descending species extinction scenarios and that produced by the average of all possible species combinations (i.e. all possible species extinction scenarios) in the bacterial experiment.

Chapter 6: The effect of migration on diversity and productivity of populations along a continuous environmental gradient

Summary

Migration may play an important role in the evolutionary adaptation of local populations, and hence influence the emergence and maintenance of biodiversity and ecosystem properties at the regional scale. I address the effect of local (neighbouring) and global migration on diversity and productivity of experimentally evolved bacterial populations along a continuous environmental gradient. Regional diversity (diversity within metapopulations) was not affected by migration, while regional productivity was slightly enhanced by global migration but unaffected by local migration; there is no correlation between diversity and productivity across metapopulations. At the local scale, the overall effect of migration on diversity was negligible, but migration did increase local diversity in some environments; the relationship between diversity and productivity across local populations was positive. The results imply that there lacks a strong trade-off in using different carbon substrates among bacterial genotypes, that evolution under every migration scenario leads to imperfect generalists with considerable overlapping niche space, and that global migration may have promoted the fitness evolution of some generalists and thus enhanced the regional productivity.

6.1 Introduction

Migration has long been studied by ecologists and evolutionary biologists because of its role in the emergence and maintenance of species diversity in metacommunities or genetic diversity metapopulations (MacArthur & Wilson 1967; Hanski & Gilpin 1997; Holyoak *et al.* 2005). Through source-sink dynamics, migration prevents the low-performance populations from going extinct (i.e. demographical rescue); and immigration may also introduce more genetic variation into local populations and thus promote the adaptation of populations to local environments (evolutionary rescue); but very strong immigration may impede such local adaptation processes by excluding the locally adaptive genotypes. Local adaptation and diversity at the regional scale are often predicted to be enhanced by low or moderate levels of migration (McLaughlin & Roughgarden 1993; Holt 1996; Lively 1999; Gandon 2002; Morgan *et al.* 2005; Habets *et al.* 2006; Venail *et al.* 2008).

At the same time, biodiversity is believed to affect ecosystem properties such as productivity (e.g. Tilman 1999; Loreau 2000; Hooper *et al.* 2005; Spehn *et al.* 2005; Balvanera *et al.* 2006; Cardinale *et al.* 2006; Cardinale *et al.* 2007). Most experimental studies on this question have been done on local scales and generally found a positive relationship between biodiversity and productivity which could be explained by either a biological mechanism 'niche complementarity' (i.e. resource/habitat partitioning or facilitation among species) or a statistical process 'selection effect' (in which more diverse communities have higher chances to contain some particular productive species that govern the property of communities). Ecologists have achieved a general understanding of biodiversity-ecosystem functioning relationship, and begun to predict

the consequences of species loss in natural systems (Jonsson *et al.* 2002; Zavaleta & Hulvey 2004; Cross & Harte 2007; Bracken *et al.* 2008). However, it is yet unclear how the mechanisms of species maintenance are linked to biodiversity-ecosystem functioning relationship (Kinzig & Pacala 2001; Mouquet *et al.* 2002; Fox 2003; Loreau 2004). A very recent study (Venail *et al.* 2008) confirmed such a link at an evolutionary time scale: in experimentally evolved metapopulations of a bacterium (*Pseudomonas fluorescens*), intermediate level of migration promoted local adaptation in poor environments and thus niche differentiation among genotypes within metapopulations; and the niche differentiation led to higher regional productivity.

Here I investigate diversity and productivity in both local and meta- populations of a bacterium (*P. fluorescens*) that evolved under three migration scenarios, no migration, local (neighbouring) migration, and global migration. Each metapopulation consisted of 15 local populations along a continuous niche axis. Several predictions are tested. (1) Regional (metapopulation) diversity and productivity may peak at local migration. Local migration is of low magnitude and is unlikely to homogenize all the local populations, but may promote local adaptation by introducing more genetic variation into local populations. Such local adaptation can lead to greater niche differentiation at the regional scale which increases regional productivity. (2) The mean diversity of local populations, however, should peak at global migration regime which readily introduces all regional genotypes into every local population. (3) The relationship between local diversity and local productivity may be distinct among migration scenarios. In the absence of migration, both diversity and productivity should be determined mainly by the physical environment and the correlation between them could be null, positive or

negative. In the presence of migration, high-yielding local populations import more individuals than did the low-yielding populations. The diversity in high-yielding populations is enhanced by migration to a less extent than that in low-yielding populations; so the correlation between diversity and productivity may be more negative than that in the absence of migration (e.g. if the correlation was positive in the absence of migration, migration may make it less positive).

Table 6.1 The composition of carbon substrates in the selection environments. The total concentration of carbon source in every environment was 1.2mM of carbon moles. The percentage numbers in this table show the relative amount of each carbon substrate in each environment, e.g. the culture media in environment 2 consisted of 0.8 × 1.2mM carbon moles of glucose and 0.2 × 1.2mM carbon moles of galactose.

Selection environments	Carbon substrates		
	Glucose	Galactose	Trehalose
1	100%		
2	80%	20%	
3	60%	40%	
4	40%	60%	
5	20%	80%	
6		100%	
7		80%	20%
8		60%	40%
9		40%	60%
10		20%	80%
11			100%
12	20%		80%
13	40%		60%
14	60%		40%
15	80%		20%

6.2 Methods

Selection experiment

Fifteen selection environments were set up using three carbon substrates, glucose, galactose, and trehalose, which differ from each other in the mechanisms of transport

through the outer (OmpF vs. LamB) and inner (PTS vs. non-PTS) membranes in the Gram-negative bacteria (Lin 1987). The selection environments differed from each other in carbon substrate ratio (Table 6.1), and can be considered as habitats along a continuous and circular niche axis (the right end point of the niche axis is the neighbour of the left end). The bacterium *P. fluorescens* SBW25 (Rainey & Bailey 1996) evolved over 160 generations in the 15 environments, without migration among environments, or with local or global migration. The selection experiment was replicated in five blocks. Such a design yielded 225 selection lines (15 environments × 3 migration regimes × 5 blocks).

The bacteria were grown in 96-well micro-plates in a shaking incubator (200 rpm) at 28°C, with each well containing 0.2 mL of M9 salt solution (Na_2HPO_4, 6 g L^{-1}; KH_2PO_4, 3 g L^{-1} NH_4Cl, 1 g L^{-1}; NaCl, 0.5 g L^{-1}) supplemented with appropriate carbon sources. Every 48 h, I transferred the selection lines into fresh media. Under local migration regime, each new microcosm (well) was initialized by a mixture of cultures from three neighbouring environments (with equal amount of culture from each environment), e.g. the new microcosm in selection environment 1 received a mixture of cultures from environment 15, 1 and 2. Under global migration regime, every new microcosm received a mixture of cultures from all the 15 environments. The total population size in each selection line was diluted 100 times at each transfer. Twenty-four transfers (~160 generations) later, all selection lines were frozen at -80°C in 50% glycerol.

Assays

I assayed the growth profile of bacterial genotypes from every selection lines in each of the 15 environments used as selection environments (i.e. the 15 assay environments were the same as the 15 selection environments). A sample of culture from

each selection line (local population) was streaked on a KB agar plate; six randomly chosen colonies, considered as six genotypes, were grown in their selection environments for 48 h; the cultures were diluted and starved in M9 salt solution for at least 2 h; then ~10^6 starved cells were added to each of the 15 assay environments, grown for 48 h (culturing conditions as in the selection experiment). Optical density (OD) at 600 nm was measured as an estimate of population density. Therefore, I measured the growth performance of six genotypes across 15 environments for every local population, and 90 genotypes for every metapopulation (note that the 90 genotypes were not randomly chosen from a metapopulation, but equally chosen from all constituent local populations).

Measurement of diversity and productivity

The variance in growth performance of genotypes from each local or metapopulation across different environments was decomposed into genotypic (G), environmental (E) and genotype-by-environment ($G \times E$) interaction components. The genotypic variance is the variance of average performances of a given genotype across all environments, and the environmental variance indicates the deviation of each phenotypic performance value from the mean performance of the genotype across all environments. The interaction component reflects environment-dependent difference among phenotypes. $G \times E$ variance was further partitioned into 'responsiveness' and 'inconsistency' components (Bell 1990; Barrett *et al.* 2005), $\sigma_{GE}^2 = \frac{\sum(\sigma_{Ei} - \sigma_{Ej})^2}{2G(G-1)} + \frac{\sum \sigma_{Ei}\sigma_{Ej}(1-\rho_{EiEj})}{G(G-1)}$

where σ_{Ei} and σ_{Ej} are the environmental standard deviation of environmental response of any pair of genotypes and ρ_{EiEj} is the environmental correlation among this pair of genotypes. The first component is responsiveness (R) due to differences in environmental

variance among genotypes, with a high value suggesting the co-occurrence of generalists and specialists. The second component is inconsistency (*I*) due to lack of correlations between genotypes over environments, indicating niche differentiation (Hall & Colegrave 2007; Venail *et al.* 2008). The proportion of total variance attributable to *I* was used as a measure of functional diversity (Venail *et al.* 2008). As the absolute quantities of the variance terms (*G*, *E*, *G* × *E*, *R*, and *I*) were dependent of the absolute values of population density, I also normalized each variance term by dividing it by the squared mean value (i.e. $[\text{mean}]^2$).

The productivity of a local population was measured as the average population density of genotypes from that population when grown in their selection environment; and the productivity of a metapopulation was the average productivity of its constituent local populations.

The variance terms and productivity were analyzed using ANOVA, with migration regime as a categorical explanatory variable and block as a random factor; for the analyses of local populations, selection environment was also included as a categorical variable. The relationship between local diversity and local productivity was analyzed using ANCOVA, with functional diversity (arcsine-transformed) as a continuous explanatory variable and migration scenario as a categorical variable.

6.3 Results

Metapopulations

Migration regime was a significant predictor for genotypic variance, *G* ($F_{2, 8} = 5.01$, $P = 0.039$) or genotype-by-environment variance, *G* × *E* ($F_{2, 8} = 12.32$, $P = 0.004$),

but not for environmental variance, E ($F_{2, 8} = 51.93$, $P = 0.207$; Fig. 6.1a). When these variance terms were normalized by the squared mean values, none of them significantly differ among migration scenarios.

The functional diversity did not differ among migration treatments ($F_{2, 8} = 3.77$, $P = 0.070$; Fig. 6.1b). There was a significant effect of migration regime on regional productivity ($F_{2, 8} = 20.42$, $P = 0.001$), however, only < 2% of the variation in productivity was accountable for by migration regime. Metapopulations with global migration had higher productivity (0.132 ± 0.007, mean ± SE.) than those without migration (0.101 ± 0.004) or with local migration (0.113 ± 0.002), but there was no significant difference between local and no migration scenarios (Fig. 6.1c). There was no significant correlation between diversity and productivity across all the metapopulations (Pearson's, $r = 0.172$, $P = 0.541$).

Local populations

Genotypic variance (G) within local populations was unaffected by migration treatment ($F_{2, 8} = 0.786$, $P = 0.488$), but differed among selection environments ($F_{14, 56} = 4.61$, $P < 0.001$; Fig. 6.2a). E was affected by migration regime ($F_{2, 8} = 7.97$, $P = 0.012$), selection environment ($F_{14, 56} = 9.27$, $P < 0.001$) and migration × environment interaction ($F_{28, 112} = 4.84$, $P < 0.001$; Fig. 6.2b). For $G \times E$, both migration treatment ($F_{2, 8} = 12.57$, $P = 0.003$) and selection environment ($F_{14, 56} = 13.67$, $P < 0.001$; Fig. 6.2c) were significant predictors. After being normalized by the squared mean values, G was affected by selection environment ($F_{14, 56} = 5.62$, $P < 0.001$) but not migration regime ($F_{2, 8} = 0.53$, P = 0.606); for E, the main effect of migration treatment was non-significant ($F_{2, 8} = 1.98$, $P = 0.20$), but the effects of selection environment ($F_{14, 56} = 6.99$, $P < 0.001$) and

migration × environment interaction ($F_{28, 112} = 3.88$, $P < 0.001$) were significant; $G \times E$ was affected by both migration regime ($F_{2, 8} = 7.08$, $P = 0.017$) and selection environment ($F_{14, 56} = 16.16$, $P < 0.001$).

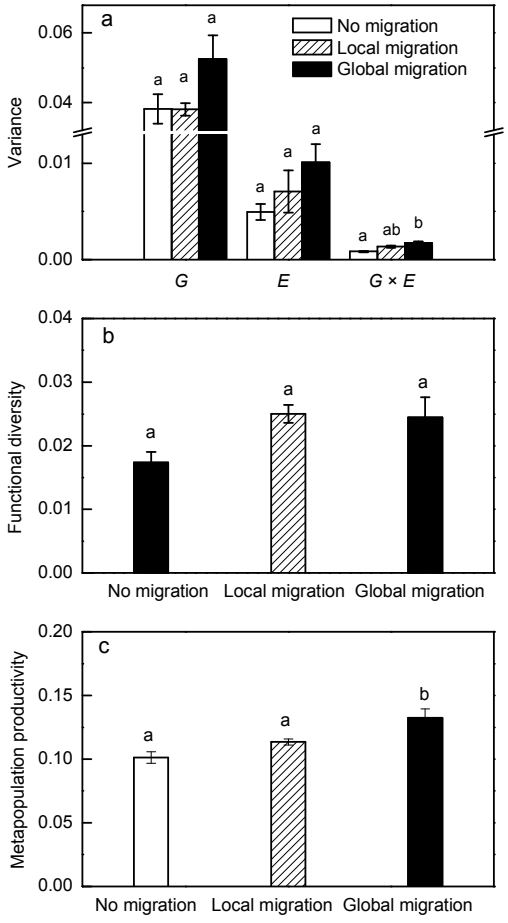

Figure 6.1 Phenotypic variance partitioning into *G*, *E* and *G* × *E* components (a), functional diversity (b) and productivity (c) of metapopulations under different migration treatments. In (b), (c) or each variance term in (a), the bars sharing a same letter have non-significant difference. Data show mean ± SE.

The functional diversity was significantly affected by selection environment ($F_{14, 56} = 4.09$, $P < 0.001$) and migration × environment interaction ($F_{28, 112} = 1.85$, $P = 0.013$), with the main effect of migration treatment being non-significant ($F_{2, 8} = 0.45$, $P = 0.656$; Fig. 6.2d). Migration regime and selection environment both were significant predictors for the productivity of local populations (migration, $F_{2, 8} = 20.42$, $P = 0.001$; selection

environment, $F_{14, 56} = 42.7$, $P < 0.001$; migration × environment, $F_{28, 112} = 5.87$, $P < 0.001$; Fig. 6.2e). Local productivity showed an overall positive relationship with local diversity ($F_{1,219} = 15.64$, $P < 0.001$), and differed among migration scenarios ($F_{2,219} = 3.56$, $P = 0.03$); the slope of the local productivity-diversity relationship in the absence of migration was not different from that under local migration ($P = 0.319$), but marginally greater than that under global migration ($P = 0.095$).

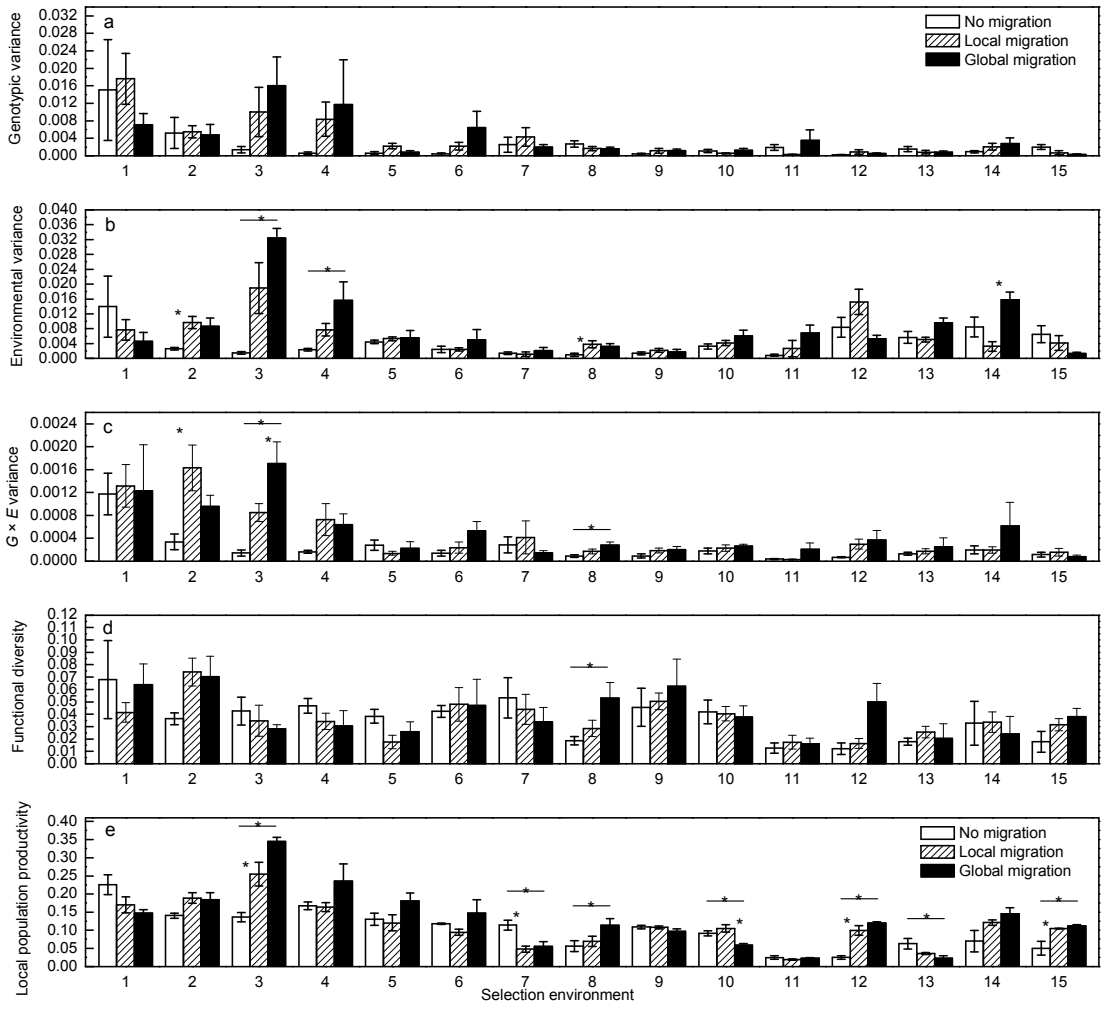

Figure 6.2 Phenotypic variance partitioning into *G* (a), *E* (b) and *G* × *E* (c) components, functional diversity (d) and productivity (e) of local populations. Within each selection environment, an asterisk indicates a significant difference between two neighbouring bars (no migration versus local migration, or local versus global migration), and an asterisk together with a section of horizintal line indicates a significant difference betwen no migration and global migration treatments. Data show mean ± SE.

6.4 Discussion

My predictions are not well supported by the results. First, at the regional scale, diversity of metapopulations was not affected by migration treatment at all; productivity was slightly higher in metapopulations with global migration than in those without migration or with local migration; I also failed to observe a correlation between functional regional diversity and regional productivity. Second, at the local scale, genotypic diversity was not affected by migration regime; the functional diversity in some selection environments was increased by migration but the overall effect of migration treatment was not statistically detectable. Third, my prediction that migration should make the relationship between local diversity and local productivity more negative is poorly supported: compared with no migration treatment, global migration made this relationship less positive (only marginally significantly) and local migration had no impact.

Niche differentiation among phenotypes within my metapopulations was very weak, with a functional diversity index being near 0.02 under any migration treatment; whereas a recent study (Venail *et al.* 2008) with the same bacterium found functional diversity of 0.1-0.2 in metapopulations each of which colonized discrete patchy environments (with each patch containing a distinct carbon substrate). The difference in niche differentiation between the two experiments is most likely explained by (1) the different selection pressures for specialization. In my experiment the local habitats are along a continuous and shallow environmental gradient that may select for generalists, and the local habitats in the pervious study (Venail *et al.* 2008) are completely different from each other, and such steep environmental change among habitats should have

selected for specialists. Also plausible are the following explanations: (2) a lack of evolutionary trade-off in using the three different carbon substrates explaining the evolution of generalists in the two-resource environments, and (3) positive correlated response to selection for using different carbon sources (which means that evolutionary adaptation to one carbon source also increase the performance on another substrate) explaining the evolution of generalists in the single-resource environments. An analysis of relative fitness evolution of populations in environments containing glucose and/or trehalose (environment 1 and 11-15) without migration confirmed a lack of evolutionary trade-off in using these two carbon substrates, and found that the bacteria in some two-resource environments could even adapt simultaneously to the two substrates to a greater extent than those in single-resource environments adapted to their only carbon sources (Appendix 1 of this chapter). The third explanation is supported by an absence of difference in local functional diversity between the single- and two-resource environments ($P > 0.2$ under any migration scenarios; Fig. 6.2). However, the generalists are unlikely to be perfect; if a perfect (super) generalist emerged, it may dominate all local populations in the presence of migration, and the local populations under global migration regime should have G, E, $G \times E$ variance and functional diversity index independent of selection environments; this is not the case (Fig. 6.2). Elsewhere, a study with the same bacterium also found the evolution of imperfect generalists in multi-carbon substrate environments and positive correlated response to selection for using different carbon sources (Barrett *et al.* 2005); another study with this bacterium found the evolution of generalists in multi-carbon substrate environments only when resource

availability was low, 0.3-1.2mM of carbon moles (Hall & Colegrave 2007); note that the carbon source availability in my experiment was also low (1.2mM of carbon moles).

Although having no effect on niche differentiation, global migration slightly enhanced regional productivity. This suggests that migration may have speeded up the fitness evolution of some or all generalists in the metapopulations. Examination of the individual local populations shows that the productivity in some of the glucose-galactose environments (selection environment 2-5) was increased by migration (Fig. 6.2e); migration may have promoted the fitness evolution in these environments, and thus increased the regional productivity.

No relationship was found between diversity and productivity across metapopulations. At the local scale, the diversity-productivity relationship was positive, although this relationship was less steep under global migration. However, the positive diversity-productivity relationship at the local scale cannot be considered as evidence for a biodiversity effect on productivity, as the local populations differ from each other in environmental resources (Abrams 1995; Loreau *et al.* 2001; Schmid 2002); rather, it is likely that both population productivity and diversity were determined by the environmental properties, with rich environments (that contain glucose; Fig. 6.2e) supporting both larger populations and higher genotypic diversity. The less steep relationship of local productivity and diversity under global migration can be explained by the fact that the high-yielding local populations exported more individuals than did the low-yielding populations and the global migration treatment may have increased the local diversity only in some low-yielding populations (Fig. 6.2d).

Population and community ecologists often consider environmental heterogeneity and migration as two major forces in determining the diversity within populations or communities (McLaughlin & Roughgarden 1993; Hanski & Gilpin 1997; Amarasekare & Nisbet 2001; Mouquet & Loreau 2002; Cadotte & Fukami 2005; Holyoak *et al.* 2005; Habets *et al.* 2006). In doing so, ecologists assume that species (or genotypes within species) exhibit trade-offs in using different habitats (Tilman & Pacala 1993; Rainey *et al.* 2000; Chase & Leibold 2003) so that environmental heterogeneity directly determines the magnitude of diversity. However, many studies to date, including the present one, have suggested that such trade-offs may be quite rare. For instance, experimental work with the bacterium *P. fluorescens* often failed to detect trade-offs in consuming different carbon substrates. In simple environments with a single carbon source, adaptation often leads to more rapid growth on many other carbon sources as a correlated response to selection (MacLean 2002; Barrett *et al.* 2005), while in complex environments with multiple carbon sources, the bacteria often evolve generalist strategy to better consume many, if not all, of the substrates available (Kassen 2002; Barrett *et al.* 2005; Hall & Colegrave 2007). Experimental work with the bacterium *E. coli* found mixed evidence for trade-offs in using different carbon substrates (Travisano *et al.* 1995; Travisano & Lenski 1996) or surviving different temperatures (Bennett *et al.* 1992; Bennett & Lenski 1993; Mongold *et al.* 1996; Bennett & Lenski 2007). Taken together, these studies suggests that a lack of strong trade-off among species (or genotypes within species) in colonizing heterogeneous environments may allow the evolution of generalists; in such situations, both environmental heterogeneity and migration may have little effect on biodiversity.

Appendix of Chapter 6

Appendix 1: Evolution of relative fitness in environments with substitutive resources

To test for the existence of an evolutionary trade-off in using different carbon substrates, I also investigated the evolution of relative fitness of bacteria in environments containing glucose and/or trehalose (i.e. environment 1, 11-15) that evolved in the absence of migration. Glucose is a high-quality carbon source and trehalose a poor one (Fig. 6.2e).

Fitness assay

A sample of culture from each population was streaked on a KB agar plate; five colonies (genotypes) were randomly chosen for relative fitness assay. Every evolved genotype and the ancestor (*P. fluorescens* SBW25) were competed with a standard competitor, *P. fluorescens* SBW25EeZY6KX (Bailey *et al.* 1995), in two assay environments that contained either a single carbon substrate glucose (the same as selection environment 1) or a single substrate trehalose (the same as selection environment 11). These competitors were separately grown in the assay environments for two days in 96-well micro-plates in a shaking incubator at 28°C (culturing conditions as the selection experiment); the cultures were diluted and starved in M9 salt solution for at least 2 h; then ~10^6 cells of each pair of competing isolates were added together to the assay environments, with the initial and final density of each competitor measured by KB agar plates containing 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal; 0.004%, w:v) where the colonies of SBW25EeZY6KX showed blue colour and were easily distinguishable from those of SBW25. The fitness of every SBW25 genotype relative to

the common competitor (SBWEeZY6KX) was calculated as the ratio of their estimated Malthusian parameters (m); $m = \ln (N_f/N_0)$, where N_0 is the starting density and N_f the final density (Lenski *et al.* 1991). The fitness increment of the evolved clones was measured as the difference in relative fitness between them and the ancestral clone. The phenotypic variance in fitness increment was partitioned into G, E and $G \times E$ components; responsiveness (R) and inconsistency (I) indices were also calculated.

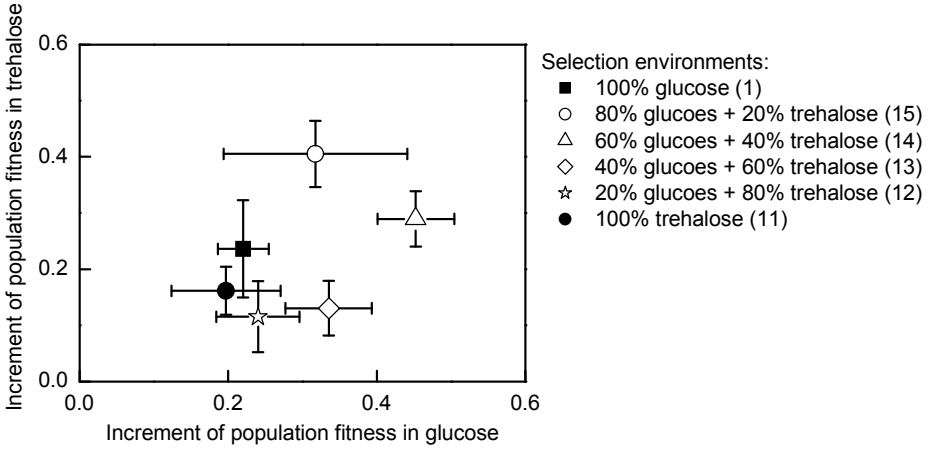

Figure 6.A1 The increase of population fitness in glucose and trehalose environments. Data show mean ± SE. In glucose assay environment, populations from environment 14 had higher fitness increment than those from environment 1, 11, and 12 (two-sample *t* test, $P < 0.05$). In trehalose, populations from environment 15 had higher fitness increment than those from 11, 12, and 13; populations from environment 14 had higher fitness increment than those from environment 12.

Results and discussion

The population fitness increment significantly differed among selection lines ($F_{5, 46} = 3.20$, $P = 0.015$) but not among assay environments ($F_{1, 46} = 3.27$, $P = 0.077$; Fig. 6.A1), suggesting that populations from some selection environments evolved higher performance in using both resources than those from the other selection environments. Specifically, populations from selection environment 15 (80% glucose + 20% trehalose)

and 14 (60% glucose + 40% trehalose) had higher fitness increment in either assay environment.

Neither of the genotypic (G), environmental (E), genotype-by-environment ($G \times E$), responsiveness (R), or inconsistency (I) variance term differed among selection environments ($P > 0.10$), suggesting that the populations from the two-resource environments did not have higher diversity than those from the single-resource environments.

Therefore, the bacteria may not have an evolutionary trade-off in utilizing glucose and trehalose as carbon sources, although previous study suggested that these two sugars involve different membrane transport mechanisms in the Gram-negative bacteria (Lin 1987; Travisano & Lenski 1996). Across the 30 populations (six selection environments × five blocks), the fitness increment in glucose environment was positively correlated to that in trehalose environment (Pearson's, $r = 0.52$, $P = 0.005$). Populations from two selection environments with mixed carbon substrates (selection environment 14 and 15) showed greater fitness increases in using both carbon sources (Fig. 6.A1). It is surprising that populations selected in the two-carbon substrates environments could simultaneously adapt to both substrates to a greater extent than those selected in the single-substrate environments adapted to their single carbon source. One possible explanation is the following. The populations in my study have not evolved to be optimal to their selection environments (the duration of the selection experiment was not long enough), and the populations in selection environment 14 and 15 may have evolved more rapidly than the others, probably because the fitness landscapes (which are used visualize the relationship between genotypes or phenotypes and fitness (Wright 1931; Simpson 1953)) of the

bacteria in these two environments were smoother than in other environments. If this is the case, populations from environment 14 and 15 may only transiently have higher fitness in using both substrates than those from the other environments. Given long enough time, the populations selected in a single-carbon substrate should be the best at using that substrate. If so, the absence of an evolutionary trade-off in utilizing glucose and trehalose may also be a transient phenomenon.

The results demonstrate that the evolution of bacteria in two-carbon substrate environments was not totally dominated by the evolutionary response to the high-quality resource (glucose), contradicting a previous study with the same bacterium (Jasmin & Kassen 2007b). The low-quality carbon substrate trehalose did influence the evolution of the bacteria, as populations selected in two-carbon substrate environments could have different fitness changes from those selected in glucose environment. I did not detect the evolution of specialists in any environment; no evolutionary trade-off was found in utilizing the two substrates. However, the result show that the relative amount of substitutive resources could influence the evolutionary adaptation of bacteria, possibly by changing the shape of fitness landscapes and hence the speed of adaptation. My results here, together with previous studies (Travisano & Lenski 1996; Barrett *et al.* 2005; Hall & Colegrave 2007; Jasmin & Kassen 2007a, b), suggest that adaptation of bacteria to environments with alternative resources may be more dynamical than previously thought, and the relative amount of substitutive resources deserves more attention in future studies.

Appendix 2: A test of the evolution of phenotypic clusters along an environmental gradient

The initial aim of the experiment presented in this chapter is to test a hypothesis that species show clustered distribution along continuous niche axes. There are two alternative ways for different species to coexist, being sufficiently different (niche differentiation) or being sufficiently similar (neutrality); for this reason, species may show clustered distribution along a niche axis; each cluster contains several similar species that coexist neutrally, and the different clusters are regularly spaced, being sufficiently different from each other (Doebeli & Dieckmann 2003; Scheffer & van Nes 2006; Leimar *et al.* 2008). I performed the experiment reported in this chapter, to test this hypothesis by investigating the evolution of phenotypic profile of *P. fluorescens* along an environmental gradient. The predictions were: (1) in the absence of among-population competition (migration), gradual variation in environmental conditions along a niche axis leads to gradual variation in phenotypic profile of the evolved bacteria, with each phenotype being the best-adapted in its home environment; and (2) in the presence of competition (migration), the phenotypic profiles of the evolve bacteria break up into (possibly regularly spaced) clusters along the niche axis (Leimar *et al.* 2008).

In the present experiment, the bacterial populations that evolved without among-population competition did not show local adaptation to their selection environments (Fig. 6.A2), so the first prediction (i.e. in the absence of competition, gradual variation in environmental conditions along a niche axis leads to gradual variation in phenotypic profile of the evolved bacteria) was rejected. Therefore, a key assumption of the modelling work (Doebeli & Dieckmann 2003; Scheffer & van Nes 2006; Leimar *et al.*

2008) that each species/phenotype should be the best-adapted to its home environment could not be met in this system; my data could not test the prediction on the clustered distribution of species along niche axes.

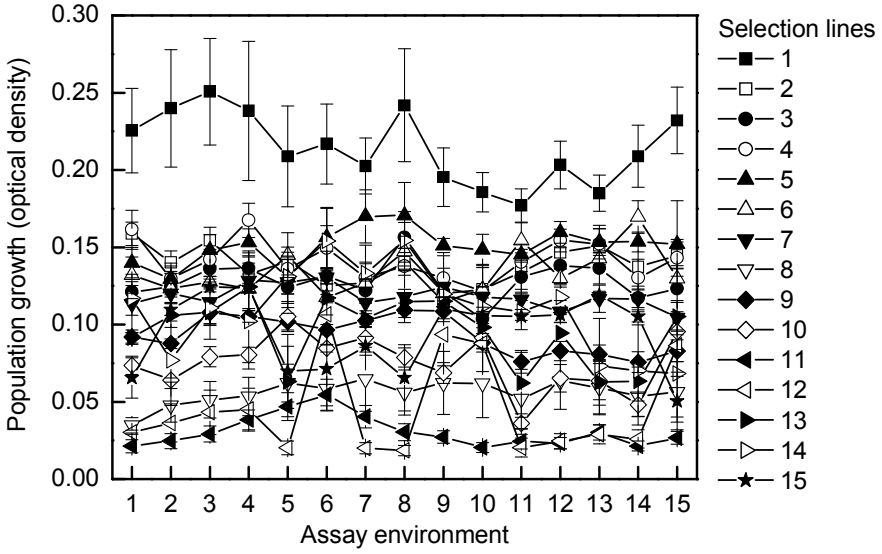

Figure 6.A2 Mean growth score in 15 assay environments of the populations that evolved without among-population migration.

Chapter 7: Coevolution between cooperators and cheats in a microbial system

Summary

A conflict between cooperators and cheats may select for improved resistance of the cooperators to the cheats and increased cheating performance of the cheats, resulting in arms race-like coevolution. I here report such escalatory antagonistic coevolution in a microbial system, *Pseudomonas fluorescens*. When grown in static tubes of nutrient media, a category of phenotypes of this bacterium, wrinkly spreaders (WS), perform a cooperative behaviour, to form a biofilm at the air-broth interface by overproducing cellulosaic polymer. This behaviour is costly to individuals but confers group benefit by improving access to oxygen. Another group of phenotypes, smooth morphs (SM), could invade the biofilms to enjoy the benefit of oxygen acquisition but make only small contributions to the biofilm construction. I examined the evolution of the ability of SM to invade WS biofilms and the resistance of WS to SM invasion, and found escalatory antagonistic coevolution between these two traits. The results suggest that the cooperator-cheat systems, like the classical victim-exploiter systems (plant-herbivore, prey-predator, and host-parasite), may show long-term arms race-like coevolutionary dynamics.

7.1 Introduction

Cooperation occurs when an individual performs costly behaviours that benefit other individuals. Such behaviours are subject to exploitation by cheats who do not perform the cooperative behaviours but enjoy the benefit. Explaining the maintenance of cooperation (in either humans or other organisms) has been a major challenge to evolutionary biologists (Hamilton 1964; Maynard Smith 1964; Maynard Smith & Szathmary 1995). Theoretical research has offer a general understanding of this problem (reviewed by Sachs *et al.* 2004; Lehmann & Keller 2006; West *et al.* 2006; Rankin *et al.* 2007; West *et al.* 2007). In empirical studies, biologists have often been concerned with the short-term success of cooperators versus cheats, based on which the underlying mechanisms such as kin selection or dispersal limitation were inferred (e.g. Rainey & Rainey 2003; Griffin *et al.* 2004; Kerr *et al.* 2006; MacLean & Gudelj 2006; Diggle *et al.* 2007; Ross-Gillespie *et al.* 2007; Sandoz *et al.* 2007). Meanwhile, the long-term evolutionary dynamics of cooperator-cheat interactions, although having motivated some theoretical thinking (e.g. Riolo *et al.* 2001; van Baalen & Jansen 2003; Jansen & van Baalen 2006), have received little empirical investigation.

The interaction between cooperators and cheats (either within or between species) is to some extent analogous to that between victim and exploiter species (such as prey and predator or host and parasite). The conflict between such victims and exploiters may lead to antagonistic coevolution, whereby the exploiters impose selection for defence in the victims that in turn imposes selection for counter defence by the exploiters. Such coevolutionary interactions have important consequences for population dynamics and maintenance of biodiversity (van Valen 1973; Chao *et al.* 1977; Anderson & May 1982;

Hamilton *et al.* 1990; Thompson 1998; Abrams 2000; Buckling & Rainey 2002a; Brockhurst *et al.* 2003; Morgan *et al.* 2005). Here I report antagonistic coevolution between cooperators and social cheats observed in a laboratory microbial system.

I focus on biofilm formation in a plant-colonizing bacterium, *Pseudomonas fluorescens,* as a cooperative behaviour. Initially isogenic *P. fluorescens* populations can rapidly diversify in spatially heterogeneous environments (static tubes of growth media), generating by mutation various phenotypes that are distinguishable by their heritable colony morphologies and niche occupancy. The phenotypes fall into three categories: smooth morphs (SM) that resemble the ancestral type and inhabit the liquid phase, wrinkly spreaders (WS) that form a biofilm at the air-liquid interface, and fuzzy spreaders (FS) that colonize the bottom of the tubes (Rainey & Travisano 1998; Buckling *et al.* 2000; Kassen *et al.* 2000). FS phenotypes are usually very rare. WS phenotypes form a biofilm at the air-broth interface through overproduction of cellulosaic polymer. Polymer overproduction is costly to individuals, as evidenced by the reduction in growth rate of WS relative to SM in exponential phase (Rainey & Rainey 2003; MacLean *et al.* 2004), but a biofilm provides group benefit to WS presumably by enhancing access to oxygen (Rainey & Rainey 2003). This cooperative behaviour of WS can be exploited by SM phenotypes, which invade the biofilms, gaining the benefit of oxygen access without paying the cost of overproducing the polymer. A biofilm carrying SM cells is less robust than a pure-WS one, and thus has a higher probability of collapsing and sinking into the broth phase where the group suffers the consequences of anoxia (Rainey & Rainey 2003; Brockhurst *et al.* 2006; Brockhurst *et al.* 2007a). In this context, WS are cooperators and

SM are cheats. By measuring interactions between SM and WS over time, I provide evidence for a cooperator-cheat arms race.

7.2 Methods

I used both the wild type of *Pseudomonas fluorescens* SBW25 (Rainey & Bailey 1996) and a modified isolate SBW25EeZY6KX (Bailey *et al.* 1995), into which has been inserted two constitutively expressed marker gene cassettes: a KX cassette (kanamycin resistance and catechol 2,3-dioxygenase) and a ZY cassette (lactose utilization). Six lines of SBW25EeZY6KX populations were grown in 30 mL universal containers with loose lids (microcosms) containing 6 mL of King's Medium B (KB). Initially, 10^8 cells from an isogenic culture were added to each microcosm which were then incubated statically at 28°C. 60 µL (1%) of each culture was transferred to fresh media every seven days for five transfers. At each transfer every microcosm was vortexed, and a sample was stored at -80°C in 50% glycerol. Each culture was plated onto KB agar; the numerically most dominant SM and WS phenotypes were isolated, and stored at -80°C.

The effect of SM invasion on the quality of WS biofilms

I tested whether the presence of SM could really decrease the strength of WS biofilms. The SM and WS phenotypes from each selection line at transfer 1 or 5 were grown in isolation and in combination in static microcosms for seven days (the initial abundance of each isolate in each microcosm was 10^8 cells), with twelve replicates for each phenotype composition. I examined the number of microcosms in which the biofilms collapsed within the seven days. If the biofilms in microcosms comprising of SM and WS had higher probability of collapse than those in pure WS microcosms, I

concluded that the invasion of SM compromised the performance of the biofilms. Paired-sample *t* tests were used to analyze the difference in probability of biofilm collapse among different types of microcosms (SM, WS, or SM-WS), with the probability data (proportional data) arcsine-transformed.

Measurement of SM cheating ability and WS resistance

I examined whether the evolved SM and WS had increased performance within a biofilm, with respect to a reference WS and a reference SM phenotype, respectively. I grew *P. fluorescens* SBW25 (Rainey & Bailey 1996) in a static microcosm for seven days and isolated the most dominant SM and WS phenotypes, which were then used as the standard reference isolates. Every evolved SM was inoculated into a microcosm together with the reference WS (10^8 cells of each isolate), which was incubated for two days. A sample of the biofilm was collected with a wire loop, and vortexed in M9 salt solution (Na_2HPO_4, 6 g L^{-1}; KH_2PO_4, 3 g L^{-1} NH_4Cl, 1 g L^{-1}; NaCl, 0.5 g L^{-1}), which was then diluted and plated onto KB agar. I measured the proportion of SM cells in the biofilm as an estimate of the cheating ability of SM (Rainey & Rainey 2003; Brockhurst *et al.* 2006). The ability of the reference SM to invade the biofilm of every evolved WS was also determined, to study the resistance of the evolved WS. Each assay was replicated three times. The change in cheating ability over time was statistically estimated by mixed-model ANCOVA, with transfer number as a continuous explanatory variable and selection line as a random factor. Cheating ability data (proportional data) were arcsine-transformed before analyses.

Measurement of coevolution between SM and WS

I next determined whether there was reciprocal evolution of WS defence and SM invasion ability using a method developed for bacteria-phage coevolution (Buckling & Rainey 2002a; Brockhurst *et al.* 2003). For an evolved WS isolate, I measured the cheating ability (proportion of SM in biofilms) of past (one transfer previous), contemporary, and future (one transfer subsequent) SM. The slope of SM cheating ability against the time when the SM was isolated approximates the temporal change in this trait, with a positive value suggesting increased cheating ability. This measurement was repeated for every dominant WS at each transfer in each replicate selection line. The equivalent measurements were carried out for the evolution of short-term WS resistance to SM; I analyzed the cheating ability of every evolved SM isolate when tested with its past (one transfer previous), contemporary, and future (one transfer subsequent) WS. A negative slope of SM cheating ability against the time when the WS was isolated indicates increasing resistance of WS over time. The sign (positive, zero, or negative) of slopes of SM cheating ability against time (past versus contemporary, or contemporary versus future transfers) were determined by mixed-model ANCOVA, with time as a continuous explanatory variable and selection line as a random factor.

7.3 Results

I observed no obvious change over time in the colony morphology of the most dominant SM or WS phenotypes. From transfer 2 onwards, every SM isolate, when grown alone, could form an observable biofilm at the air-broth interface, as well as colonize the broth phase. The biofilms formed by SM from transfer 2 were very thin; at transfer 3-5 the SM biofilms were not thinner than WS biofilms. The SM biofilms were

less robust than WS biofilms: they were easily broken when collected by a wire loop, whereas the WS biofilms could often be collected by a wire loop as integrative units. I examined whether the presence of SM increased the probability of biofilm collapse, by using the SM and WS isolates from transfer 1 and 5. The biofilms in microcosms comprising SM and WS did have higher probability to collapse within seven days than those in pure WS microcosms (Fig. 7.1). SM from transfer 5 could form biofilms when grown alone, which had non-significantly higher probability of collapse than SM-WS biofilms (Fig. 7.1).

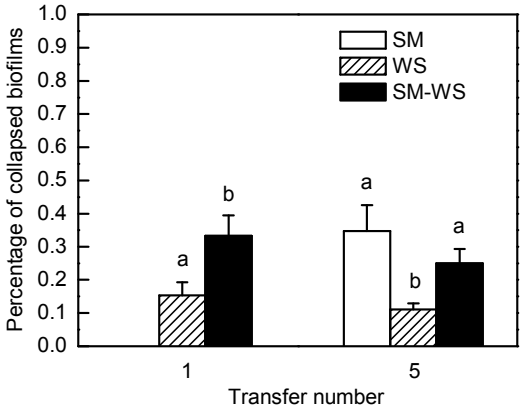

Figure 7.1 The probability of biofilms to collapse within seven days in microcosms composed of SM, WS, or SM-WS from transfer 1 or 5. Note that SM from transfer 1 did not form observable biofilms. Within each transfer, the different letters on the bars indicate significant difference (paired-samples *t* test, $P < 0.05$). Data show mean ± SE. ($N = 6$).

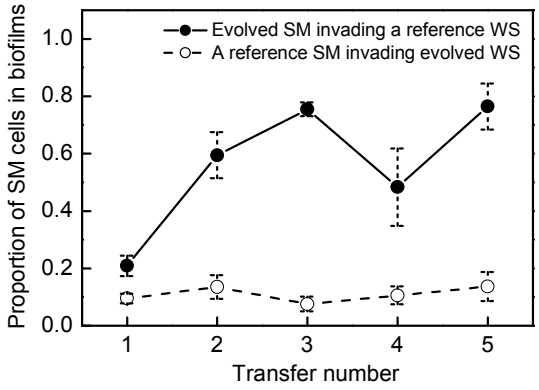

Figure 7.2 The ability of evolved SM to invade biofilms of a reference WS and that of a reference SM to invade biofilms of evolved WS (measured as the proportion of SM cells in biofilms) over time. Data show mean ± SE. ($N = 6$).

The ability of the evolved SM to invade a reference WS, measured as the proportion of SM cells in the biofilms, increased over time (ANCOVA, $F_{1, 23} = 9.56$, $P = 0.005$). The proportion of SM cell in the biofilms could be surprisingly high (even near 80% for SM from transfer 3 and 5; Fig. 7.2). The ability of a reference SM to invade biofilms of evolved WS was fairly low (around 10%), showing no significant change against the time when the WS was isolated ($F_{1, 23} = 0.237$, $P = 0.631$; Fig 7.2).

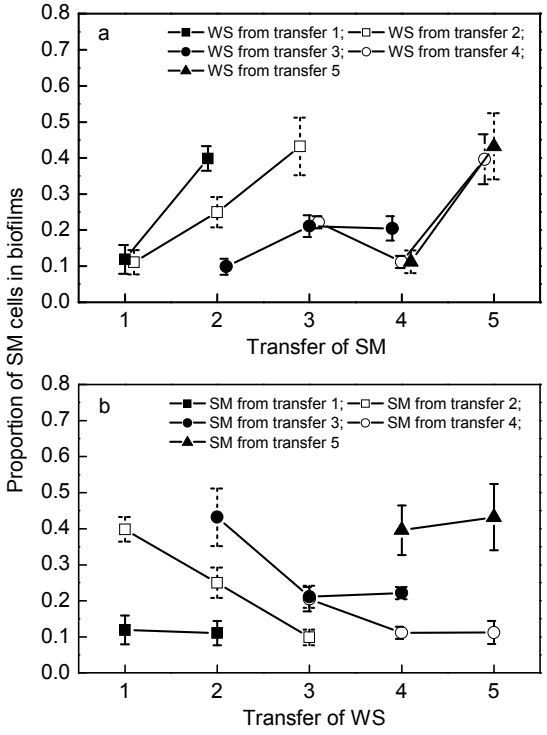

Figure 7.3 Evolution of SM cheating ability and WS resistance. In a, each set of lines shows (from left to right) the ability of SM from one transfer in the past, contemporary transfer, and one transfer in the future to invade the biofilm of the contemporary WS. In b, each set of lines shows (from left to right) the ability of contemporary SM to invade the biofilms formed by WS from one transfer in the past, contemporary transfer, and one transfer in the future. Data show mean ± SE. ($N = 6$).

Antagonistic coevolution between SM and WS was observed. In general, future SM were better than contemporary SM, and contemporary SM were better than past SM, at invading the contemporary WS biofilms, as indicated by the mainly positive slopes of SM cheating ability against the time when SM was isolated (six positive, one zero and one negative; $\chi^2 = 6.25$, $P = 0.044$; Fig. 7.3a). The resistance of WS biofilms to SM invasion increased, or did not change, over time, as demonstrated by the negative (four

out of eight cases) or zero (four cases) slopes of SM cheating ability against the time when WS was isolated (Fig. 7.3b).

7.4 Discussion

Resource competition versus cooperator-cheat conflict

The SM and WS phenotypes in this microbial system show resource competitive interactions as well as a cooperator-cheat conflict. Several findings demonstrate the presence of a cooperator-cheat conflict between the WS and SM: (1) a biofilm at the air-broth interface does confer fitness benefits to its occupants, as the biofilm-forming WS can invade a population from rare (Rainey & Travisano 1998); (2) biofilm formation is costly to individual cells of WS (Rainey & Rainey 2003; MacLean *et al.* 2004); (3) both SM and WS can be observed in biofilms (Rainey & Rainey 2003; Brockhurst *et al.* 2006; Brockhurst *et al.* 2007a); (4) within a biofilm, the per cell contribution of WS to biofilm integrity is higher than that of SM (as the SM biofilms are more likely to collapse than WS biofilms; Fig. 7.1); and (5) the presence of SM compromises the performance of WS biofilms (Rainey & Rainey 2003 and the present study).

Resource competition and social conflict could both potentially shape the evolution of SM and WS phenotypes. However, the evolution of SM-WS interactions in the present study is inconsistent with the prediction of a traditional character displacement hypothesis (Brown & Willson 1956; Connell 1980; Schluter 1988, 1993, 2000b; Grant & Grant 2006; Steiner *et al.* 2007), which here predicts that within-microcosm (sympatric) SM and WS should evolve to be more divergent in resource use

profile over time and thus show decreased competitive strength. I observed increased negative effects of SM and WS on each other (Appendix of this chapter).

In the present study I measured SM cheating ability as the proportion of SM cells in biofilms. The proportion of SM cells in biofilms may be determined by the population size of SM (simply because larger populations of SM have more cells in contact with the biofilms) and/or the ability of SM to integrate themselves to the biofilms. I did not measure the actual population size of SM and WS when assaying SM proportion in biofilms; however, I found no change over time in the monoculture yields of either SM (ANCOVA, $F_{1, 23} = 0.71$, $P = 0.409$) or WS ($F_{1, 23} = 1.01$, $P = 0.326$), suggesting that the proportion of SM in biofilms was likely to only reflect SM's ability to invade the biofilms.

SM phenotypes evolved higher abilities to invade WS biofilms, and the ability to form their own biofilms when grown alone. The results may be explained by either the coevolution between SM cheating and WS resistance, or the evolution of generalist SM phenotypes that colonize both the broth phase and the air-broth interface. I favour the former explanation. In the presence of a biofilm at the air-broth interface, any SM mutants that are better at gaining access to oxygen should be selected for; and to invade the WS biofilms is an efficient means to obtain oxygen. The SM mutants that pay costs to produce small amount of cellulosaic polymer can be better than the ancestral SM at adhering to the WS biofilms. Such SM mutants colonize the liquid phase as well as the WS biofilms, and may have an advantage over the ancestral type. When grown alone, such SM can form their own biofilms at the air-broth interface which are less robust than WS biofilms. In the sense of cooperator-cheat interaction, the evolved SM phenotypes in my experiment were selected to invade WS biofilms, while their ability to form biofilms

when grown alone is a by-product. It is also possible that weak biofilm evolution in SM represents the emergence of a generalist phenotype that can colonize both the liquid medium and the air-broth interface; in this case, the observed patterns in Figure 7.3 can be explained by that both SM and WS evolved higher ability to compete for the niche of air-broth interface. However, the extent to which the presence of SM increased the probability of collapse of WS biofilms (i.e. the difference in probability of collapse between pure WS biofilms and mixed biofilms) did not differ between transfer 1 and 5 (t = 0.46, df = 5, P = 0.666; Fig. 7.1), suggesting that SM did not evolve improved performance of biofilm construction over time. Therefore, the ability of evolved SM to form biofilms when grown alone is most likely a by-product of improved cheating performance.

It is difficult to more rigorously test the relative importance of resource competition and social conflict in the evolution of SM and WS due to some methodological issues. For instance, I could not quantitatively estimate how much benefit SM can get from invading WS biofilms; if SM and WS could be grown in the same microcosm, with or without some kind of physical barrier that confines WS to the top layer of the medium and SM to the rest but which does not block the diffusion of nutrients, the benefit of invading WS biofilms could be assessed. But such experiments are very hard to perform. Furthermore, I was not able to measure the total number of bacterial cells in the biofilms (it is difficult to collect an entire biofilm from a microcosm as the biofilm is often broken when collected) and thus I do not know how many SM cells occupy the liquid niche and how many inhabit the WS biofilms.

The behavioural mechanisms underlying cheating and resistance

I observed reciprocal coevolution between SM and WS in cheating and resistance performance, although this was not perfectly symmetrical. SM often evolved a higher ability to invade WS biofilms over time (Fig. 7.3a), while WS evolved higher or unchanged resistance over time (Fig. 7.3b). The mechanisms behind the cheating and resistance behaviours are, however, unclear. I think it most likely that the evolved SM produced small amounts of cellulosaic polymer to adhere to the WS biofilms (as the evolved SM can form their own biofilms when grown alone). It is possible that WS phenotypes resist the invasion of SM by producing allelochemicals (which keep the SM cells away from the biofilms), or by cell suicide (in which the dead cells release extracellular DNA to thicken the biofilms so that the SM cells adhering to the bottom of biofilm mats receive smaller benefit). Indeed, the resistance of WS itself could be a second-order cooperative behaviour; this behaviour may be costly to the individuals but benefit all the WS individuals (first-order cooperators); such behaviour is subject to the exploitation of the second-order cheats (Rankin *et al.* 2007) that do not pay the cost of resisting the (first-order) cheats but enjoy the benefit of the resistance behaviour. In the present study the resistance behaviour of WS was maintained, which may probably be explained by kin selection and dispersal limitation (Griffin *et al.* 2004; Sachs *et al.* 2004; Kerr *et al.* 2006; Lehmann & Keller 2006; West *et al.* 2006; West *et al.* 2007) as WS cells in biofilms are very likely to be neighboured by close relatives.

In summary, I document escalatory antagonistic coevolution between cooperators and cheats in a bacterial system, in which the former evolved higher resistance and the latter evolved higher cheating performance over time. The results argue for more attention to the long-term evolutionary dynamics of cooperator-cheat systems. The

maintenance of resistance of cooperators to cheats, a second-order cooperative behaviour, may be a very interesting question in future studies. Furthermore, the results support the notion that the cooperator-cheat conflict is to some extent analogous to the classical victim-exploiter (plant-herbivore, prey-predator, and host-parasite/parasitoid) interactions, which highlights the significance to study the common features shared by different biotic interactions in the light of evolution.

Appendix of Chapter 7

Appendix 1: Competition between the evolved SM and WS

I here define 'competition' as the reduction in the abundance of one species (phenotype) by the presence of the other species (phenotype). Such a negative effect among SM and WS in the present experiment could be caused by resource competition or the cooperator-cheat conflict. Under an assumption that resource competition is the single interaction between SM and WS, the negative effect among within-microcosm (sympatric) SM and WS should decrease over time, as predicted by the character displacement hypothesis (Brown & Willson 1956; Schluter 1988, 1993, 2000b; Grant & Grant 2006; Steiner *et al.* 2007).

The SM and WS isolates from each selection line at each transfer were grown in monocultures and a mixture, each of which was initialized by 10^8 cells of SM and/or WS, and grown in a static incubator for two days at 28 °C. Each monoculture or mixture was replicated three times, and the average across the three replicates was used in analyses. The intensity of competition on a phenotype was measured by a log response ratio index lnRR (Goldberg *et al.* 1999; Hedges *et al.* 1999), which was calculated as the log-transformed ratio of yield in the absence of competitors to that in the presence of competitors (i.e. ln [yield in monoculture/yield in mixture]). Zero lnRR indicates no competition on the focal phenotype, and greater lnRR suggests stronger competition. Furthermore, the intensity of competition between SM and WS was measured by the relative yield total (RYT) index (Trenbath 1974; Harper 1977). Relative yield (RY) of a phenotype in a mixture is the proportion of its yield in the mixture to that in monoculture,

and the sum of RYs of SM and WS is RYT. RYT equal to one indicates perfect niche overlap (strong competition); RYT greater than one suggests niche differentiation among phenotypes (weak competition).

SM showed negligible competitive effect on WS at transfer 1-4 (competition intensity did not differ from zero; one-sample *t* test, $P > 0.10$), but decreased the density of WS marginally significantly at transfer 5 ($P = 0.067$). Across the five transfers the intensity of competition of SM on WS increased over time ($F_{1, 23} = 14.1$, $P = 0.001$). The presence of WS always decreased the density of SM (competition intensity was higher than zero at every transfer, $P < 0.01$); but the competition intensity did not change over time ($F_{1, 23} = 0.110$, $P = 0.743$; Fig. 7.A1a).

Relative yield total (RYT) of mixtures of SM and WS was higher than one at transfer 1-3 ($P < 0.050$), and not different from one at transfer 4 and 5 ($P > 0.100$). RYT decreased over time ($F_{1, 23} = 22.0$, $P < 0.001$; Fig. 7.A1b), suggesting that the negative effect between the two types increased over time.

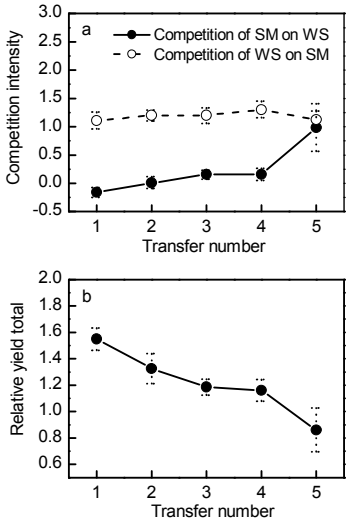

Figure 7.A1 The intensity of competition between within-microcosm (sympatric) SM and WS. Competition on WS or SM (a) was calculated by a log response ratio, ln (yield when competitor absent / yield when competitor present), where a value of zero suggests zero competition, and higher values indicate stronger competition. Competition between SM and WS (b) was inferred by a relative yield total (RYT) index, with higher values indicating weaker competition. Data show mean ± SE. ($N = 6$).

Chapter 8: Discussion

The primary goal of this thesis is to experimentally test some well-established or currently debated hypotheses on the origin, maintenance, and functional consequences of biodiversity. I used a model microbial system, *Pseudomonas fluorescens*, in my studies.

Chapter 2 tested a long-standing idea in evolutionary biology that adaptive radiation in a lineage should be impeded by competition from the other lineages. I tracked the process of diversification of *P. fluorescens* populations in the presence and absence of a bacterium *P. putida* in both spatially heterogeneous and homogeneous environments. It was found that the extent of radiation in *P. fluorescens* was not affected by the competitor in either environment, but the initial stage of diversification in homogeneous environment was speeded up by the competitor. The results suggest that there may not be a general pattern in the effect of competitors on a radiating lineage's diversification. The effect may depend on the competition intensity and the nature of the competitors.

Chapter 3 investigated the relative importance of stabilizing (niches) and equalizing mechanisms (neutrality) for the maintenance of phenotypic diversity in *P. fluorescens* populations. I measured the relative growth rate when rare of 32 phenotypes from six microcosms, to detect negative frequency-dependent selection which reflects the operation of niche processes. I found that ~2/3 of the phenotypes persisted neutrally in their communities while the remaining 1/3 persisted stably. Furthermore, I used the same system to investigate the relationship between diversity and productivity (biomass production), finding no effect of diversity on productivity. The results suggest that both niches and neutrality mechanisms contribute to phenotypic coexistence in this system,

and the operation of niche processes does not necessarily lead to positive effect of biodiversity on ecosystem properties.

Chapter 4 used the data from Chapter 3 and a published study with laboratory algal communities, to address the potential of a 'competitive hierarchies' framework to predict the structure of multi-species communities. This framework assumes that the competitive interactions among species are transitive, and proposes that the structure of multi-species communities can be predicted by pair-wise competition experiments. In either of the experiments used in Chapter 4, species (or phenotypes) from a species pool (or phenotype pool) were grown in all possible monocultures and all possible species/phenotype combinations. The data allowed me to construct competitive hierarchies based on pair-wise and multi-species competitions. I found that the competitive interactions among species/phenotypes were generally transitive, and the competitive hierarchies inferred from pair-wise competitions were consistent with those from multi-species competitions, suggesting that the 'competitive hierarchies' framework could be a working approach to understanding the structure of natural communities.

Chapter 5 employed the same data as Chapter 4, to test whether the random assembly biodiversity experiments can be used to predict the effect of non-random species loss. As experiments used in the analyses contained all possible species/phenotypes combinations from a species/phenotype pool, I can assess the effect of all possible extinction events based on the communities containing all species/phenotypes. My analyses suggested that the complementarity and selection effects estimated by the random assembly biodiversity experiments could be helpful to predicting the consequences of particular extinction scenarios. Greater niche

complementarity effects indicate smaller niche overlap among species, and thus weaker compensatory growth following species extinction; and smaller complementarity effects suggest higher levels of compensatory growth among species. The selection effect values show the relation between species' abundance in multi-species communities and their functional performance, and are helpful in predicting the magnitude of functional recovery due to species compensatory growth under specific extinction scenarios. I obtained the observed diversity-biomass relationships under two extinction scenarios, by comparing the biomass of communities with certain species compositions. The observed diversity-biomass relationships were generally consistent with predicted by an analysis of niche complementarity and selection effects.

Chapter 6 addressed the effect of migration on diversity and productivity of experimentally evolved bacterial populations along a continuous environmental gradient. The results suggested a lack of strong trade-off in using different carbon substrates among bacterial genotypes, and evolution of imperfect generalists with considerable overlapping niche space either in the absence of migration or in the presence. Migration showed an overall non-significant effect on diversity at the local (local population) or the regional (metapopulation) scale; but one migration scenario (global migration) may have accelerated the fitness evolution of some generalist bacterial genotypes, and thus slightly increased regional productivity.

Chapter 7 revealed arms race-like coevolution between cooperators and cheats in a microbial system. Here the cooperators are wrinkly spreader phenotypes in *P. fluorescens* populations, which form a biofilm at the air-broth interface. Biofilm formation is costly to individuals but benefits all the occupants by enhancing access to

oxygen. Smooth morph phenotypes in the populations may act as cheats, which can invade the biofilm to get the benefit but pay no cost. I measured the cheating performance of smooth morphs to past, contemporary, and future wrinkly spreaders, and found that the cheats generally evolved higher cheating performance, and the cooperator evolved higher or unchanged resistance to cheats, over time.

The work presented here suggests several avenues for further research. First, Chapter 3 found that niche and neutral processes could operate simultaneously to promote the maintenance of diversity. However, my experimental system differs from natural communities in many aspects. Most importantly, my microcosms accommodate very large communities, without immigration. It would be interesting to investigate the role of niche and neutral processes in small communities with migration (meta-communities). Smaller communities may contain smaller number of neutral species where drift may reduce diversity more efficiently; but the spatial turnover in species composition (β-diversity) may be greater across small communities as small communities are less likely to show predicable competitive outcomes among neutral species. Migration may counterbalance the effect of small community sizes; extremely high migration rate, in effect, integrates different local communities into a single large community. Second, Chapter 5 suggests that more work should be done to use existing data for understanding the consequences of non-random biodiversity changes for ecosystem functioning. Third, the evolution of cooperator-cheat interaction should be given more attention. The results of Chapter 7 suggest that the cooperator-cheat system where social interactions are involved in, as the conventional prey-exploiter systems (plant-herbivore, prey-predator, and host-parasite/parasitoid), can show arms race-like coevolution. It may be interesting

to study the resistance behaviour in cooperators. The resistance can be considered as a second-order cooperative behaviour which may be costly to individuals but benefit the group by reducing the invasion of the first-order cheats. It is unclear whether a second-order cheats (which do not pay cost to resist the first-order cheats) can evolve and invade the populations.

In conclusion, this thesis has provided experimental tests of several hypotheses concerning the origin, maintenance and functional role of biodiversity, and also revealed arms race-like coevolution in a cooperator-cheat system. In doing this the thesis has also confirmed the value of using laboratory bacterial populations for ecological and evolutionary studies.

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Appendix

Appendix 1: A miscellany of pilot experiments with *Pseudomonas fluorescens*

I did some trial experiments using *P. fluorescens* SBW25, which were aimed at better understanding this system. These preliminary results are not included in the main text, but presented here.

Diversification of P. fluorescens strains

I found that *P. fluorescens* SBW25R::RFP did not diversify into different phenotypes in static KB microcosms, but SBW25, SBW25EeZY6KX, SBW25R (pQBR103::GFP), and SBW25B728a::GFP diversified.

Coexistence of several strains

To prepare for the experiment in Chapter 2 (the effect of an interspecific competitor on diversification), I looked for some bacterial strains that coexist with *P. fluorescens* SBW25EeZY6KX. It was found that *Pseudomonas putida* UWC1::GFP could coexist with SBW25EeZY6KX, whereas *P. putida* MES300, *P. fluorescens* A506, SBW25R (pQBR103::GFP), and B728a::GFP could not.

A failure to detect allelopathic effect in P. fluorescens populations

A previous study (Rainey & Travisano 1998) showed that fuzzy spread (FS) phenotypes in diverged *P. fluorescens* populations could not invade abundant wrinkly spreader (WS) phenotypes but WS could invade FS, although they occupy apparently distinct spatial niches in static microcosms. It is possible that WS produces some allelochemicals which suppress the growth of FS. I did a trial experiment, but did not detect an allelopathic effect.

I isolated four WS and four FS colonies, and did two experiments. In the first experiment, four FS isolates were grown in two environments: (1) control environment, 5 mL of KB medium plus 1 mL of PBS buffer, and (2) treatment environment, 5 mL of KB plus 1 mL of media consumed by WS for two days (WS cells were removed). In the treatment environments the media consumed by WS was diluted by 6 times. Every FS isolate was grown in one microcosm with control environment and two microcosms with treatment environment (the two microcosms contained media consumed by two different WS isolates). The nutrient in the treatment environment was 1~1.2 times of that in control environment. If the yields of FS in treatment environment were lower than those in control environment, the allelopathic effect manifested itself. In the second experiment the control environment consisted of 5.4 mL of KB plus 0.6 mL of PBS buffer, and the treatment environment was 5.4 mL of KB plus 0.6 mL of media used by WS, where media used by WS were diluted by 10 times. In the first experiment, the yield of FS in treatment environment relative to that in control environment was 0.97 ± 0.075, not different from one (one-sample t test, $P = 0.74$). In the second experiment this relative yield was 1.04 ± 0.10, not different from one ($P = 0.72$). The results suggest that WS does not have allelopathic effect on FS, or the effect is too weak to detect.

The effect of biofilm formation on resistance of bacteria to phages

Aggregation of host individuals may promote the outbreak of parasites, by shortening the distance for a new parasite to find a new host. Therefore, the biofilm formation of WS in *P. fleorescens* could be a behaviour selected against by phages. I did a pilot experiment on the effect of biofilm formation of WS on bacterial/phage fitness. Six SM isolates were used to found six SM populations, and six WS clones were used to

found 12 populations (6 clones × 2 replicates). After one-day growth, the SM populations were vortexed and inoculated with 10^7 phage particles (SBW25Ø2); six WS populations were vortexed and inoculated with phages, and the other six WS populations were introduced with phages without being vortexed. Another 24 h later, the density of bacteria and phages in each microcosm was measured. There was no significant difference in phage or bacterial density between the SM microcosms and any other type of microcosms (independent samples *t* test, $P > 0.30$), or between vortexed WS and unvortexed WS microcosms (paired samples *t* test, $P > 0.50$). The ratio of phage to bacterial density showed no difference among any two types of microcosms. Therefore, biofilm formation of WS showed no effect on the bacteria/phage population dynamics. Possible reasons are as the followings. (1) The density of bacterial populations was very high (~10^9 per mL), and the phages can transmit efficiently in either broth cultures or biofilms. (2) The biofilms of WS can either promote phage transmission by reducing the distance for a phage to find new host, or impede phage transmission because the polymer in the biofilms may decreases mobility of the phages.