

MERTON COLLEGE

Biodiversity and Ecosystem Functioning in Predator-Prey Communities

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Philosophy (DPhil)*

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Declaration of Authorship

I, Eric Topham, designed and carried out the experiments and analyses described in this thesis, the interpretation of which are my own. The authorship of this thesis is entirely my own, with help and guidance received listed below.

Dr Thomas Bell provided advice on the design of experiments, elements of analysis, interpretation, as well as editorial guidance on drafts of this thesis.

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Dr David Bass provided guidance on elements of experimental work.

This work has not been submitted, partially or in full, for any other qualification at this university or any other.

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Thesis abstract

Once regarded as little more than simple model systems, microbial communities are now understood to drive major earth processes and patterns of biodiversity. In order to more fully understand them, there is a need to investigate the mechanisms that determine the structure and function of microbial systems. Predator-prey interactions have been studied for a long time in macroecological systems and are known for their role in determining community structure and dynamics. Functional diversity has come to the fore of ecology to take a central role in understanding what links species to biodiversity and ecosystem function. In this thesis I investigated the biodiversity and ecosystem functioning of microbial predator-prey communities. I used naturally occurring bacterial communities in combination with co-occurring heterotrophic protists to perform manipulative experiments to investigate: a) the functional diversity of heterotrophic protists and their interactive contributions to ecosystem functioning; b) the effects of bacterial prey community diversity and composition on these attributes of protists; and c) the role of functional diversity in maintaining protist community diversity. I found that not only is it possible to classify functional diversity by species interactions, but that the effects of prey community diversity and composition highlight the flexible nature of this property, particularly in predators. I also found evidence of negative biodiversity-functioning relationships and strong competitive interactions among heterotrophic protists. However, their functional diversity allowed them to maintain higher diversity in disturbed environments. Taken together, my thesis has advanced the understanding of the role of predator-prey interactions and functional diversity in regulating interactions and ecosystem functioning in microbial systems.

Chapter 1 - Thesis introduction

Understanding the ecology of microorganisms is arguably one of the most compelling challenges facing modern ecology (Prosser et al. 2007). Microorganisms underpin many aspects of earth system processes and are recognized as being key to understanding patterns of biodiversity and ecosystem functioning. They perform important functional processes, including decomposition of ninety percent of all organic matter (Swift et al. 1979), cycling of carbon and nitrogen, and primary production (van der Heijden et al. 2008; Falkowski et al. 2008; Bell et al. 2009b; Jousset 2011). They are therefore relevant for understanding macroecological systems, as well as climate regulation and agriculture (Qaim & Zilberman 2003; Bardgett et al. 2008; Bell et al. 2009b).

Microorganisms are ubiquitous. Recent advances in DNA-based molecular techniques and next generation sequencing have revealed phylogenetic diversity many times greater than plants and animals (Whitman et al. 1998; Torsvik & Øvreås 2002; Zinger et al. 2012). Fingerprinting techniques such as terminal restriction length polymorphism (tRFLP), denaturant gradient gel electrophoresis (DGGE), and phospholipid fatty acid (PLFA) have also helped describe communities (Torsvik & Øvreås 2002). Though studies are to an extent still hindered by remaining difficulties of describing microbial biodiversity (large sample sizes, culturability of strains, sampling effects of DNA techniques), next generation methods are partly remedying the issue. It is therefore increasingly possible to describe ecological mechanisms that maintain diversity and functional structure that affect ecosystem functioning, rather than describe diversity alone.

Bacterial communities are at the base of most microbial food webs and are subject to strong top-down pressures through predation by a wide range of microbial eukaryotes in both aquatic and soil environments (Gasol et al. 2002; Rønn et al. 2002; Jousset 2011). Predation is a major cause of bacterial mortality (Pernthaler 2005). For instance, in aquatic food webs estimates are as high as eighty-eight percent of stock grazed per day (Sherr & Sherr 2002). Predation therefore plays

an important role in shaping the genetic and functional diversity, as well as the activity, of bacterial communities (Griffiths et al. 1999; Rønn et al. 2002; Bell et al. 2010). This microbial food web has been referred to as the ‘microbial loop’ (Pomeroy 1974; Azam et al. 1983) which involves the uptake of non-living organic matter by bacteria, primary production by algae, and the grazing of both by heterotrophic protozoans (Azam et al. 1983; Sherr & Sherr 1987; Sherr & Sherr 1988). The interaction between predators and prey has long been recognized as central to understanding the diversity, structuring, and functioning of communities and ecosystems (Hairston et al. 1960; Holling 1959; Levin 1970; Hassell 1976; Hassell 1978) and microorganisms have been used to test ecological theory since Gause (1934). However, with the increasing appreciation for the importance of microbial systems, there is an ever growing need for the testing of ecological theory in such systems (Prosser et al. 2007). While such efforts will coincidentally contribute to understanding of general ecological theory, it is vital to understand the interactions, functional diversity, and ecosystem functioning of microbial communities in their own right.

Protists are ubiquitous and abundant. They are found, like many microorganisms, in almost every known ecosystem (Sherr 2002). They are reported to be found from lakes in the dry valleys of Antarctica (Laybourn-Parry et al. 2000) to seawater under the arctic icecap (Sherr et al. 1997). Over the course of the last three decades, it has become increasingly clear that phagotrophic protists play vital ecological roles in both aquatic and soil communities and ecosystems (Sherr & Sherr 2002). Phagotrophy is an ancient mode of predation for protists and probably predates photosynthesis (Dyer & Obar 1994; Vickerman et al. 1998). As a result it is not surprising that protistan predators are so widespread (Sherr & Sherr 2002). Their distribution reflects that of protozoans at large, ranging from desert soils to seawater (Bouwman & Zwart 1994; Ekelund & Rønn 1994; Rodriguez-Zaragoza et al. 2005; Jousset 2011). Protists range from being of generally similar size to their microbial prey (bacteria, algae and other protists) up to two orders of magnitude bigger, and their population growth rates are comparable (Sherr & Sherr, 2002). A large fraction of protists are bacterivorous. Protistan bacterial

predators are a major cause of bacterial mortality, as well as having intra-guild predation effects on other heterotrophic protists, which therefore has wide ranging impacts on microbial community structure and function (Sherr & Sherr 2002).

In this thesis I will draw on these themes and use a group of recently described protistan predators (Howe et al. 2009; Bass et al. 2009) in microcosm experiments to answer questions arising from them. I will use an alternative approach to functional diversity examine the functional organisation and functional effects of microbial predators in microcosm communities. I will also examine the feedback effects of bacterial community structure on interactions, functional relationships, and contributions to community functional processes of heterotrophic protists. Finally I will examine the contribution of functional diversity to a mechanism of maintenance of diversity.

1.1 Predator-prey interactions between protists and prokaryotes

Hairston et al (1960) proposed that plant biomass is maintained indirectly by predators which consume herbivores, decreasing their abundance and thus controlling the mass of plant material eaten. Since this seminal work, understanding how the effects of predation cascade through communities to control plant (or more broadly, producer) density and biomass has been a central issue for ecologists (Kurle & Cardinale 2011). In the process, much attention has been given to the specific relationship between predators and prey (Holling 1959; Hairston et al. 1960; Levin 1970; Hassell 1976; Hassell 1978; Sih et al. 1985; Abrams 2000). The result is an understanding that such interactions can lead to trophic cascades with top-down and bottom-up effects, which can profoundly impact community structure (Estes & Palmisano 1974; Carpenter et al. 1985; Kurle & Cardinale 2011). The structure of bacterial communities is affected to a great degree by productivity (defined as resource availability), with peaks in diversity at intermediate levels of productivity, under simplified conditions at least (Kassen et al. 2000; Smith 2007). It is also regulated by mortality from heterotrophic protists through the ability of

these to do so is affected by productivity related life history trade-offs (Sherr & Sherr 2002; Stevens & Steiner 2006; Bell et al. 2010).

Productivity and bacterial defence

Bacteria have a diverse suite of defensive strategies to reduce their vulnerability to predation (Jürgens & Matz 2002; Jousset 2011). They may undergo morphological or physiological changes when subjected to protist predation though increases in cell size (Corno & Jürgens 2006), toxin release (Pernthaler 2005), or the formation of filaments (Pernthaler et al. 1997; Jürgens & Sala 2000; Hahn & Höfle 2001) among others (reviewed in Jousset 2011). However, there is a trade-off between predation resistance and competitive ability, since ultimately investment of resources in growth comes at the cost of defence.

One possible proximate mechanism is that small microorganisms are able to grow faster due to favourable surface area to volume ratio for nutrient acquisition, but at the cost of being more vulnerable to predation (Jürgens & Matz 2002; Jousset 2011). Conversely, investment in defence strategies such as cell wall thickness or increased size comes at the cost of rapid growth (Jousset 2011). In the absence of predators and in low productivity environments, palatable species out-compete resistant ones since they are better able to convert absorbed nutrients to population biomass (Jürgens & Matz 2002; Jousset 2011; Jousset et al. 2011). However, the presence of predators redresses this competitive balance in favour of inedible species (Jürgens & Matz 2002; Jousset 2011).

Predator feeding preferences

Defences and predator preferences form an integral part of microbial predator prey interactions. Studies have found contrasting but informative effects of the degree of specialism in predator-prey interactions on the bacterial diversity. Bell et al (2010) found that generalists did not alter the relative abundances of bacterial strains whilst Jiang and Morin (2005) found that generalists increased evenness. The presence or absence of different predator types can lead to different effects on community structure (Worsfold et al. 2009). For instance, Worsfold et al (2009)

found that the removal effect of a large generalist bacterivore depended on the presence or absence of a small and comparatively rarer specialist. This also illustrates the existence of facilitative interactions between protistan predators.

Protozoan predators can display a high degree of prey specificity which has been shown to contain phylogenetic signal (Pfandl et al. 2004; Glücksman et al. 2010). The identity of predators involved in predator-prey interactions has important impacts on bacterial community structure (Glücksman et al. 2010; Saleem et al. 2012). Protozoa that preferentially feed on competitively dominant edible prey types contribute to the maintenance of diversity in productive systems (Corno & Jürgens 2008; Bell et al. 2010) by promoting the spread of resistant, less competitive prey types (Bell 2002; Stevens & Steiner 2006; Jousset 2011). Such findings support Leibold's (1996) keystone predation model which predicts the coexistence of vulnerable and resistant prey types at intermediate productivity levels in the presence of predators.

Size and predation

Predators have size dependent structuring effects on communities. Body size affects the size of prey that predators can ingest (Vucic-Pestic et al. 2010), and this is true for unicellular microbial predators (Posh et al. 2001). Predators have optimal exploitation rates of prey of a certain body mass range (Schneider et al. 2012). Predators prefer prey one or two orders of magnitude smaller than themselves, decreasing along with feeding strength at smaller and larger sizes (Vucic-Pestic et al. 2010). The result is a unimodal relationship between body mass ratios of predators and prey, and per capita feeding rates (Otto et al. 2008). Pfandl et al. (2004) showed that in simple communities of *Ochromonas* sp. and *Spumella* sp. size selective feeding was dependent on cell dimensions with a tendency for selection against intermediate sizes. This may be a prominent size structuring effect of eukaryotes, driving shifts towards particularly small and large body sizes (Pernthaler 2005; Corno & Jürgens 2006). This relationship is most likely

driven by effects of handling time and attack rate (Vucic-Pestic et al. 2010), contact probability (Boenigk et al. 2002), as well as interference competition (Lang et al. 2012).

Size differences in predators also lead to differing effects on prey communities due to inherent size properties of predators. Small predators are more abundant than large predators (Peters 1983; White et al. 2007) due to the much greater metabolic demands associated with increased body size (Brown et al. 2004; Ehnes et al. 2011). As a result, large predators have large per capita effects while small predators have large population level effects (Schneider et al. 2012). This may be important in heterotrophic protists where ingestion rate is limited by vacuole content (Boenigk et al. 2002). Results from Glucksman et al (2010) showed that protist cell volume and plasticity were the two traits that most affected bacterial community composition, most likely because they have the greatest impact on feeding preferences.

Size selective feeding is therefore one of the major mechanisms by which protist predators impact bacterial populations, as evidenced by the range of prokaryote defence strategies devoted to countering it (Pernthaler 2005). In conjunction with competitive effects between bacteria and the productivity of the system, they promote shifts in bacterial community composition (Pernthaler et al. 1997; Posch et al. 2001; Rønn et al. 2002; Jousset 2011). Size selective feeding may also act as a driver of competition and intraguild predation amongst predators when variation in body size exists in prey population and numbers of suitable prey for predators are limited by their size (Woodward & Hildrew 2002).

Predation and diversity

Predation is thought to drive diversity by favouring coexistence of species that compete for the same resource (Gallet et al. 2007). Trade-offs between predation resistance and competitive ability amongst prey results in higher predation on susceptible but competitive prey types, with a mixture of direct and apparent competition allowing resistant but less competitive species to persist (Paine 1966; Holt et al. 1994; Bohannan & Lenski 2000). Predation may also reduce prey population density, reducing inter- and intraspecific competition, reducing the probability

of competitive exclusion (Paine 1969; Krivan 2003). However, predation may not always increase diversity, with its effects varying depending on predation strength and other conditions (Chase et al. 2002; Cadotte & Fukami 2005).

Evidence shows that through predator-prey interactions protistan predators have effects on bacterial diversity. For instance, Murase et al (2006) found that in soils t-RFLP and DGGE profiles showed increased diversity in the presence of protists. *B-proteobacteria* was preferentially preyed upon by cercozoans and gram-positive types became more abundant. Similar results were found by Rønn et al (2002). Similar effects from aquatic systems are also well reported (Šimek et al. 1997; Suzuki 1999; Hahn & Höfle 2001; Rønn et al. 2002; Bell et al. 2010). Hahn and Hofle (2001) found changes in bacterial composition, while Bell et al (2010) and Jiang and Morin (2005) reported contrasting but significant effects of protists on relative abundances of bacterial populations. Predation by protists can have positive effects on bacterial community diversity and yield, possibly through enhanced complementarity and evenness of bacterial species (Saleem et al. 2012). These effects have found to be greatest when protistan diversity is high, most likely reflecting differences in feeding modes and preferences (Saleem et al. 2012).

Less understood is how the diversity of prey influences predators. Meta-analyses of marine communities have shown that consumer effects are strongest at high prey diversities (Edwards et al. 2010). Prey diversity may increase the likelihood of communities containing resistant species (Duffy et al. 2007) and consumer resistant strains may provide a refuge for more palatable ones (Stachowicz 2001). Increased prey diversity may also positively affect predators though improved diet or negatively through reduced encounter rates (Root 1973; DeMott 1998). Prey diversity may also affect predator interactions, affecting niche overlap, competition, and intraguild predation (Woodward & Hildrew 2002; Snyder et al. 2006; Byrnes & Stachowicz 2009). However, investigations into this question remain relatively few and findings mixed, both within microbial ecology and more broadly (Fox 2004; Gamfeldt et al. 2005; Duffy et al. 2007; Schmitz 2007). Recent results using heterotrophic protists and bacteria suggested that

increasing prey richness enhances the production of single predators and enhanced predator evenness (Saleem et al. 2013). This effect was dependent on predator identity and predator complementarity was an important factor in predator production and prey reduction (Saleem et al. 2013). Simple communities of predatory bacteria and prey reveal divergent effects of prey community on predator community structure (Chen et al. 2011).

Thesis direction

The literature shows that there is a growing understanding of predator-prey interactions and their effects in microbial communities. However, what is clear is that this is generally from a top-down perspective of protozoan predators acting on bacterial prey. What is missing is an attempt to further understand the feedback effects of bacterial community structure on interactions and complementarity between predators.

1.2 Microbial ecosystem functioning and diversity

Understanding community structure and the role of predators is crucial to understanding the downstream effects on ecosystem functioning and services. Though studies have uncovered the consequences of losing plant species and primary consumers for ecosystem functioning, predators remain underrepresented in diversity studies addressing this question (Balvanera et al. 2006). The most likely reason for this is that it remains difficult to predict the effects and outcomes of single species losses, known as identity effects (Sih et al. 1998; O'Connor et al. 2008; Byrnes & Stachowicz 2009; Worsfold et al. 2009). Most research in this field has originated from terrestrial plant and animal communities, with the majority of focus on microbial services in relation to their effects on plants (Balvanera et al. 2006; Cardinale et al. 2012). Such work has shown that microbial diversity has an important effect (for instance Wagg et al. 2014). However, less attention has been paid to microbial diversity-functioning relationships in their own right, especially in relation to protozoan predators (Massana et al. 2009; Hillebrand & Matthiessen 2009; Peter et al. 2011)

Linking structure to functioning

A central tenet of microbial and general ecology is that composition and diversity of communities determines function (Frossard et al. 2012). Species richness can enhance the performance of ecosystems in terms of functioning and providing services (Loreau 2000). There are several reasons for this. Firstly, increasing species richness increases the probability that a community contains species with extreme trait values or that occupy complementary niches, known as the ‘selection effect’ (Loreau & Hector 2001). Secondly, if there are greater number of species that occupy distinct feeding niches, then productivity is increased since more of the resource pool can be utilized, and this is known as the ‘complementarity effect’ (Tilman et al. 1997; Loreau 2000; Loreau & Hector 2001). Studies have shown that different bacterial species consume complementary resources and that more diverse bacterial communities consume complex resources more efficiently (Jousset 2011).

Changes in microbial community structure, notably diversity, have been shown to affect ecosystem functioning (Bell et al. 2005; Bell et al. 2009b; Langenheder et al. 2010; Peter et al. 2011). Langenheder et al (2006) showed that specific enzyme activities varied in differently composed bacterial communities and Szabó et al (2007) showed that rare species were crucial for community functioning. Predation by protozoans may be important for microbial functioning due to their effects on bacterial community structure (Saleem & Moe 2014). This may be due to effects on bacterial evenness, or through recycling of nutrients and improving prey access to resources (Schmitz et al. 2010). Furthermore, measures of ecosystem functioning are more strongly influenced by predation than productivity (Corno & Jürgens 2008). Murase et al (2006) reported that the presence of protists in soil bacterial communities stimulated the level of nitrogen mineralisation and SO_4^{2-} reduction coinciding with changes in community structure. Shifts in genotype and phenotype distributions within bacterial communities exposed to predation lead to significant functional changes that affect overall carbon transformation and decomposition processes (Corno and Jurgens 2008; Jousset et al. 2011).

Some studies have failed to find such strong support for the relationship between community structure and functioning (Wertz et al. 2007; Langenheder et al. 2005; Frossard et al. 2012). This has been suggested to be due to differences between approaches that use carefully controlled assemblages and those that use indirect manipulations (Bell et al. 2009b; Frossard et al. 2012). The former may independently vary taxonomic composition and diversity, whereas the latter produces subsets of the original taxa (Frossard et al. 2012). These differences may lead to divergent results, but the former method may suffer from oversimplification whereas method such as dilution series may more accurately reflects realistic species losses.

The extreme diversity of microbial systems may mask effects of community structure on functioning (Frossard et al. 2012). This may be compounded by the fact that broad measures of ecosystem functioning commonly used in microbial communities such as respiration and nitrogen mineralisation are too coarse since they integrate across multiple processes (Frossard et al. 2012). However, other more specific measures such as the use of enzymatic pathways may be more tightly linked to aspects of particular functions such as leaf matter degradation and the populations that perform them (Langenheder et al. 2006; Frossard et al. 2012). Nevertheless, even here disconnects between community structure and function have been found (Jiang 2007; Frossard et al. 2012). Such disconnects have been proposed to be due to community structure being unrelated to function and negative selection effects of functionally unimportant but competitively dominant strains (Jiang 2007; Frossard et al. 2012) This therefore remains an area of microbial community functioning that needs to be addressed further.

Functional diversity

The relationship between community diversity and ecosystem functioning is suggested to be generally saturating and such a relationship suggests communities with high redundancy will lose functional capacity with decreasing species diversity (Petchey & Gaston 2006). Functional aspects of diversity are important for diversity-functioning relationships, though they remain conflated with species richness.

Functional diversity is a central concept in ecology since it has implications for a wide range of ecological questions centring - for the most part - on the prediction of rates and stability of ecosystem level processes (Tilman 2001; Díaz & Cabido 2001). It can be broadly defined as the range of ‘things that species do’ between species in a community (Tilman 2001), though a variety of similar alternative definitions can be found in the literature (Díaz & Cabido 2001; Tesfaye et al. 2003; Petchey et al. 2004). This has been more narrowly defined as ‘the value and range of those species and organismal traits that influence ecosystem functioning’ (Tilman 2001). A species trait is an attribute of a species that relates to morphological, physiological, or life-history features that can be measured at the individual level without reference to the external environment (Violle et al. 2007; Verberk et al. 2013). Functional traits are those that influence ecosystem functioning and the diversity of functional traits is referred to as functional trait diversity (Petchey & Gaston 2006; Verberk et al. 2013). A common but basic measure is the number of functional groups represented in a community, however functional diversity is often used vaguely and there is still some debate as to what functional diversity actually means and how it should be calculated (Tilman 2001; Loreau et al. 2001; Petchey & Gaston 2002b; Cadotte et al. 2011).

Methods of measuring functional diversity are also varied. Some use knowledge of the natural history of organisms to make subjective decisions regarding classification into categorical groups (Petchey & Gaston 2006). Others use continuous measures of functional trait variation to either produce continuous metrics of functional diversity, or partition them based on distance based approaches. Examples include summed length across dendrograms, average pairwise distances, functional attribute diversity, FD_{var} , FD , Rao’s entropy, $FAD2$ and more (Rao 1982; Walker et al. 1999; Heemsbergen et al. 2004; Ricotta 2005; Petchey & Gaston 2002b; Mason et al. 2003). All of these methods fundamentally operate by correlating functional trait diversity to observed ecosystem functioning.

Functional diversity of microorganisms

Functional diversity of microorganisms has been recognised to be key to the functioning of the world's ecosystems (Falkowski et al. 2008; van der Heijden et al. 2008). Microorganisms account for a large part of ecosystem metabolism and biomass (Whitman et al. 1998). Studies attempting to quantify and categorise microbial functional diversity have generally focussed on identifying different patterns of resource use. Methods tend to be comparative with studies correlating community changes along environmental gradients (Krause et al. 2014).

Quantification happens at a variety of scales, using catabolic response profiles, respiration and utilisation of carbon substrates, functional genes, and activity of specific enzymatic pathways among others (Zak et al. 1994; Torsvik & Øvreås 2002; Liang et al. 2011; Frossard et al. 2012). These reflect measures of functioning related to productivity, cellular activity, and the performance of specific functions such as carbon cycling, decomposition, fixing of inorganic compounds, and nitrogen and phosphorous utilisation (Sinsabaugh et al. 1991; Zak et al. 1994; Torsvik & Øvreås 2002; Langenheder et al. 2006; Sinsabaugh et al. 2008; Frossard et al. 2012; Krause et al. 2014). While studies have used manipulative approaches to show the relationship between microbial diversity and functioning, few have done so to expressly describe functional diversity.

While manipulative and comparative approaches have both demonstrated the existence of microbial diversity effects on functioning, these have tended to be at the scale of general patterns rather than the description and classification of functional groups or other measures of functional diversity. For instance, assemblage type experiments have demonstrated the ability to tie diversity to contributions of bacterial services (Bell et al. 2005) and broad scale community composition (PFLA) profiles have been correlated to enzymatic activities (Waldrop et al. 2000). However, there are examples where studies have identified specific functional groups. Ribes et al (2012) found that specific but different microbial assemblages were convergently responsible for nitrification, dissolved organic carbon and NH_4^+ uptake in different sponge species. Wertz et al (2007) identified the effect of disturbance on specific denitrifying and nitrite oxidising

functional groups, while there is also evidence of single phylotypes being responsible for metabolic functions such as chitin and cellulose degradation (Peter et al. 2011).

Of these studies elucidating microbial functional diversity, even fewer consider heterotrophic protists. Massana et al (2009) provide an example of one such study, using fluorescent *in situ* hybridisation (FISH) to determine functional groups based on feeding preferences of small heterotrophic marine nano-flagellates. Other work with heterotrophic protists has inferred diversity-functioning relationships and functional diversity related to feeding mode by partitioning complementarity and selection effects (Saleem et al. 2013). There has also been limited attention given to the functional trait diversity of these organisms in the context of coexistence (Jiang & Morin 2007). A combination of functioning and complementarity based approaches might yield new insights into the functional role of heterotrophic protists in microbial communities.

Problems of functional diversity

A number of studies do not find or find only weak relationships between functional diversity and ecosystem functioning (Dukes 2001; Stevens & Carson 2001; Díaz & Cabido 2001; van Kleef et al. 2006; Verberk et al. 2013). Determining the effects of functional diversity can be difficult since it is conflated with species richness. A number of explanations for weak or absent relationships have been proposed. These include the use of inaccurate or inappropriate trait information to define groups, inappropriate measures of functional diversity, and insufficient statistical power of experiments or greater importance of other ecological factors (Petchey & Gaston 2006).

The use of functional groups has been noted as being potentially particularly problematic (Petchey & Gaston 2006) and the use of more concrete functional diversity metrics or phylogenetic diversity is now being advocated (Petchey & Gaston 2006; Cadotte et al. 2011). At least two studies have found that groups defined *a priori* are often worse at explaining variation in experiments than when species were assigned to groups randomly (Petchey et al. 2004;

Wright et al. 2006). Phylogenetic diversity is thought to be a potentially better predictor of ecosystem functioning since it probably encompasses all of the trait variation in a community (Flynn et al. 2011). In a study of 29 plant experiments, Flynn et al (2011) found that phylogenetic diversity explained variation in response variables not explained by traits. However, in microbial ecology the extent to which traits are phylogenetically conserved is unclear, with contrasting results in correlations of functional traits and 16S rRNA phylogenies (Krause et al. 2014).

These issues associated to trait-based metrics are of particular note for studies using microorganisms where little information regarding the natural history or functional traits is known, or where functional traits may be hard to distinguish. Methods for measuring functional diversity generally use traits hypothesised *a priori* to be functionally important. However, in microbes traits can be difficult to observe directly. This is despite recent advances in technologies such as microscopy, flow cytometry, fluorescence techniques, spectroscopy, and isotope tracking (Krause et al. 2014). Much of microbial diversity is ‘hidden’ both in eukaryotes and prokaryotes and revealed only by DNA based molecular techniques (Pace 1997; Moreira & López-García 2002; Nannipieri et al. 2003; Venter et al. 2004). In addition, culturing of most microbial strains is challenging (Rappe & Giovannoni 2003). The number of individuals to survey, sampling effects, and definition of microbial species all add further difficulties identifying microbial diversity and are problematic for quantifying functional diversity using traits.

Finally, there is the developing notion that the function of a trait and therefore its contribution to ecosystem functioning, is context dependent (Verberk et al. 2013). There is a presumption that a single trait consistently conveys a functional adaptation across a particular gradient and analyses treat functional traits as having simultaneous independent actions (Poff et al. 2010; Verberk et al. 2013). However, traits may interact, with one changing the functional relevance of the other. Furthermore, the relevance of a trait to ecosystem functioning may vary according to the environmental conditions or habitat (Verberk et al. 2013). Measures of functional diversity that

account for species or trait interactions, as well as biological context are necessary and explaining context dependence may be the single greatest hurdle to developing theories of species diversity and ecosystem functioning (Schmitz 2007; Verberk et al. 2013)

Thesis direction

Despite these difficulties, there seems to be widespread consensus that functional diversity is an important factor in predicting ecosystem functioning (Loreau 1998a; Tilman 2000; Díaz & Cabido 2001; Petchey & Gaston 2006; Duffy et al. 2007; Griffin et al. 2009). There has been little explicit effort to quantify and categorise functional diversity in heterotrophic protists. Considering the important role that these are known to play in structuring bacterial communities, this is an area in the literature to address. In this thesis I will investigate new approaches for defining functional diversity that are tailored for microbes where natural history is unknown and traits are difficult to measure.

1.3 Coexistence in microbial communities

Mechanisms that allow coexistence and maintain diversity is a key question in microbial and general ecology (Houlahan et al. 2007; Loreau & de Mazancourt 2013). Ecosystem functioning is maintained by species diversity and therefore understanding the mechanisms that maintain diversity contribute to the understanding of ecosystem functioning. Since heterotrophic protists exert such strong pressures on microbial community structure and functioning, it is vital to understand the mechanisms that convey diversity and coexistence to them.

Coexistence mechanisms

Competition is an important interspecific interaction that is often a key determinant of community dynamics. It occurs whenever two or more individuals, populations or species must exploit a common resource, and has negative co-varying effects on competing parties (Chesson 2000b). When two (or more) species are limited by a common resource, the one which maintains a positive per capita growth rate at the lowest level of resource will outcompete the

other (Tilman 1982). This is known as the R^* rule and for coexistence to occur it must be broken (Tilman, 1982). Coexistence is the persistence of competing species in the same location and requires that competing species have ecological differences. These can be summed up as species' niches (Chesson, 2000b). Such differences are vital for coexistence because they allow species to depress their per capita growth rates more than their competitors (Chesson, 2000b) thus breaking the R^* rule. There are a number of mechanisms by which this can occur.

Mechanisms for coexistence through competition can either be dependent or independent of environmental variation. Independent mechanisms are concerned with the reduction of per capita growth rates, and therefore the R^* rule, through density dependent intra- and interspecific interference (Chesson 2000a; Chesson 2000b; Chesson et al. 2004). Mechanisms dependent on environmental variability operate through differences in species' responses to temporally or spatially autocorrelated changes in the abiotic environment, resulting in changes in relative competitive ability (Chesson 2000b).

Environmental variability dependent mechanisms

The vital rates of a population (metabolic rates, phenology, prey encounter rates, growth rates, etc.) that determine its response to the overall environment depend to varying degrees on a variety of environmental variables (Vitousek 1994; Ives & Hughes 1995; Ives 1995; Ives & Hughes 2002; Leary et al. 2012). However, population responses to environmental variability also depend on the surrounding community. The presence of one species may modify the effects of environmental variability on other species, and the effects of environmental variability may cascade through a community to influence further species not directly affected by the varying environmental factor (Ives et al. 1999; Loreau 2010).

Two mechanisms are proposed by which compensatory dynamics are conferred through environmental variability to populations, communities and ecosystem level processes. The first is known as the 'storage effect' of different species responses to variable environments (Chesson 2000a,b). Storage effects can be 'temporal' or 'spatial'. The former involves

mechanisms that allow organisms to maintain high species abundances through periods of non-optimal conditions and dampen the effects of increased competitive pressure (Chesson 2000b). Spatial storage effects refer to mechanisms that allow organisms to escape non-optimal conditions and increased competitive pressure due to variation in habitats across a landscape (Chesson 2000a).

The second mechanism is relative non-linearity of competition under variable environments. Competition is predicted to have stabilizing influences on communities and ecosystems due to negative covariance between populations (Tilman 1999). Under variable environments, periods of negative population growth and reduced abundance (temporally autocorrelated to environmental variability) in more competitive species, results in phases of relaxed competition for less competitively dominant species. Variable environments allow differences in resource use efficiency between competitors, leading to changes in dominance and coexistence of species over time (Long et al. 2007). Effectively, changes in competitive strengths of organisms due to environmental variability maintain species richness and evenness (Petchey and Gaston 2006).

The insurance hypothesis

Environmental variation can maintain diversity. A secondary consequence of this environment-dependent maintenance of diversity is the hypothesis that greater species richness confers a greater degree of stability on community and ecosystem processes (Yachi & Loreau 1999; Leary & Petchey 2009). This has been termed the ‘insurance hypothesis’ (Yachi & Loreau, 1999). The general mechanism proposed for the insurance hypothesis is that high species diversity provides an insurance against disturbance through the stabilizing effects of compensatory dynamics of different species’ responses to variability in the environment (Yachi & Loreau 1999; Leary & Petchey 2009). This has broad implications since variability in community properties has consequences for the reliability of ecosystem functions and the provision of ecosystem services which have conservation, environmental, and socio-economic values (Costanza et al. 1997; Gaston & Spicer 2003).

According to the insurance hypothesis, greater species richness confers stability through increasing diversity of species responses - and redundancy of responses - to environmental fluctuations (Loreau & Hector 2001; Chesson et al. 2004; Lennon & Jones 2011). Different species responses to environmental fluctuations may reduce temporal variability of community structure and composition, as well as ecosystem processes. Increases in one species may correspond to decreases in another, creating negative covariance between populations. Fluctuations in some species' population biomass will be compensated by opposite fluctuations in biomass of others which are better adapted to the current environmental conditions (Yachi & Loreau 1999). The idea of diversity in species' responses to environmental changes has been termed 'functional response diversity' by Leary and Petchey (2009) and they advocate using this approach when addressing questions of species diversity, environmental variability and the insurance hypothesis at the community level.

A growing number of studies have investigated the insurance hypothesis by measuring variability in community and ecosystem properties and comparing communities that differ in species richness (Leary & Petchey 2009). Studies on natural populations and communities have found that compensatory dynamics are rare and that environmental factors generally have synchronizing effects on populations (Houlahan et al. 2007; Mutshinda et al. 2009). On the other hand, other studies have suggested that compensatory dynamics are indeed present and that they may increase community stability (Descamps-Julien & Gonzalez 2005; Vasseur & Gaedke 2007; Downing et al. 2008; Leary & Petchey 2009). Whilst these studies point to the general importance of environmental fluctuations as a driver of community stability (Houlahan et al. 2007), they are limited in their insight into the mechanistic causes of these effects responsible for maintaining diversity, including niche differences, functional traits, and competitive interactions of component species (Ives et al. 1999; Loreau & de Mazancourt 2013; Leary & Petchey 2009). Indeed, Leary and Petchey (2009) note that a majority of studies have only addressed statistical and phenomenological approaches to uncovering mechanisms

Effects of environmental variability on microorganisms

Since environmental variability is proposed to be important for coexistence and diversity of microorganisms, it is important to measure the effects it has on them. Disturbances in the abiotic environment impact upon predators in a variety of ways. Environmental factors can fluctuate predictably and unpredictably, as well as over both spatial and temporal scales. Among others, these might include changes in pH, temperature, and medium viscosity which act upon predators through a number of effects on vital rates (Beveridge et al. 2010a,b). These include metabolic rates, phenology, prey encounter rates, growth rates, and swimming rates which apply to both multi- and unicellular organisms (Beveridge et al. 2010a, b, c).

However, there is currently a lack of information regarding how some of these vital rates scale with environmental factors (Beveridge et al. 2010a, b). For instance Beveridge et al (2010a, b) point out that especially for microbial predators, there is little understanding of temperature scaling of foraging traits such as handling times, attack rates and maximum ingestion rates. In particular, almost nothing is known about whether the energy demands of handling times and attack rates differ between taxa (Beveridge et al. 2010a, b, c). They suggest that such differences are plausible since attack rates and handling times have behavioural as well as biochemical components (Beveridge et al. 2010a, b, c).

Dormancy is a well-described response of microorganisms to environmental change (Lennon & Jones 2011). In particular, microbiologists have elucidated the mechanisms that underpin dormancy in a variety of clinically and environmentally important bacteria (Lennon & Jones 2011). Stevenson (1978) hypothesised that dormancy is widespread among microorganisms in order to contend with environmental conditions and that it contributes to stable abundances and functional processes of aquatic microbial communities. Technological advances such as fluorescence *in situ* hybridisation (FISH) and electron accepting fluorescence dyes have allowed the evaluation of microbial, and in particular bacterial, metabolic states (del Giorgio & Gasol 2008; Lennon & Jones 2011). It has also been possible to describe the active and total

composition of microbial communities using ribosomal RNA-based and ribosomal DNA-based profiles (Lennon & Jones 2011). Such studies have shown that microbial composition may be determined by dormancy strategies and broad environmental variability regimes such as seasonal temperature and photoperiod (Lennon & Jones 2011).

Dormancy in microbial communities is thought to be key to the maintenance of the particularly long tail of micro-eukaryote rank-abundance curves (Galand et al. 2009; Scheckenbach et al. 2010). A pressing question is whether such mechanisms maintain this ‘rare biosphere’ and its contribution to microbial ecosystem functioning (Lennon & Jones 2011). Two hypotheses exist: 1) either communities contain large reservoirs of genetic diversity allowing rapid adaptive response to environmental change, or 2) that they are characterised by transitions between active and dormant states that ultimately impact community structure (Lennon & Jones 2011).

The degree to which functional response traits such as dormancy affect the impact of environmental variability on microbial communities varies according to the degree of functional diversity and redundancy of traits and trait values (Leary & Petchey 2009). Functional redundancy requires a degree of overlap in functional response trait values but also requires that there are differences in species responses for stabilising effects to occur (Micheli et al. 2010; Allison & Martiny 2008; Leary & Petchey 2009). These concepts are integral to predictions of the insurance hypothesis and storage effects. There is already some evidence that these mechanisms are at work in microbial communities (Jiang & Morin 2007; Allison & Martiny 2008).

Thesis direction

This thesis will investigate how microbial predators are influenced by environmental variability and how the diversity of functional traits contributes to coexistence. Findings involving heterotrophic protists have wider implications since they regulate wider community structure and function. There is a need for studies that focus on experimental approaches with systems where precise knowledge of features of species’ biology and of environmental variables is

known (Ranta et al. 2008; Leary et al. 2012). There are three areas in the literature that need addressing. Firstly, there is a need for further empirical testing of diversity-disturbance relationships. Secondly, these must manipulate species richness and functional diversity. Thirdly, these manipulations must be based on measured differences in species responses to disturbances and environmental variables and used in the testing of predictions (Ranta et al. 2008; Leary & Petchey 2009; Leary et al. 2012). This thesis will aim to use such experimental approaches to elucidate mechanisms of maintenance of microbial community diversity.

1.4 Thesis aims and outline

Heterotrophic protists are important components of microbial ecosystems. However, their ecology and impacts on ecosystem functioning are poorly understood. This thesis aims to help remedy this by performing a series of experiments that explore the functional diversity and contributions to ecosystem processes of heterotrophic protists. More specifically, these experiments will try to understand how interactions and contributions to functioning are mediated by prey community characteristics. This thesis will also try to identify mechanisms that underpin the maintenance of protist diversity.

In Chapter 2 I discuss the current issues surrounding the use of trait-based approaches to functional diversity. I then propose an alternative method for defining functional groups using diversity-manipulation experiments. I demonstrate the utility of this approach to build a variety of single or multiple function hierarchical cluster dendrograms. I then perform a manipulative diversity experiment to evaluate the nature of the diversity-functioning relationships based on the interactions-based functional diversity in comparison to a trait-based approach and random assignment of functional diversity.

In Chapter 3 I use manipulations of protist community functional diversity to examine the way that functional diversity based on species interactions predicts diversity-functioning relationships. In particular, I test whether functional diversity based on species interactions

reveals different relationships between functional diversity and ecosystem functioning than expected from trait-based predictions. Furthermore, I investigate whether relationships are dependent on biological context, specifically the surrounding prey community. I discuss why non-positive diversity-functioning relationships might be found in this system, as well as the importance of interactions-based functional diversity in revealing them.

In Chapter 4, I use manipulative experiments to examine the degree to which bacterial community composition feeds back to affect protist predator contributions to microbial ecosystem functioning. I also ask whether differences in bacterial community composition feedback onto functional relationships and complementarity between protists. I discuss whether this ultimately affects the interactions-based method developed in Chapter 2.

I follow this up in Chapter 5 by attempting to elucidate how bottom-up bacterial diversity affects protist species interactions and contributions to ecosystem functioning. I also examine how they might affect functional complementarity and functional diversity of protists measured using the ecosystem process-based method developed in Chapter 2. Chapter 2 investigated the effect of bacterial composition using communities from different locations, whereas this experiment uses dilution to incur species losses on a single community

In Chapter 6 I investigate whether such bottom-up effects of microbial prey communities influence competitive mechanisms that affect protist community diversity. I test whether the same bottom-up effects of bacterial prey diversity as in Chapter 6 affect the competitive interactions and abundances of assembled protist communities.

In Chapter 7 I continue to address the issue of mechanisms that maintain protist predator diversity. I test predictions of the insurance hypothesis and the importance of dormancy for heterotrophic protists by testing for a mechanism of a temporal storage effect. I test the effect of a community level functional trait and functional trait diversity to maintain protist community diversity in the face of a variety of temperature perturbations during a long term experiment.

Chapter 2 – A novel approach for classifying protistan functional diversity

Abstract

A large body of work has developed regarding biodiversity-ecosystem functioning relationships. Most have used species richness as a predictor of ecosystem functioning, whilst others have tried to elucidate whether alternative metrics such as phylogenetic or functional diversity provide better prediction metrics. However, there are a number of problems with phylogenetic and trait-based approaches. In particular there is often little phylogenetic signal for functional traits, and trait-based approaches are difficult for groups whose natural history is poorly understood. Selection of traits is also problematic. These problems are often accentuated for those studying microorganisms. I used a new method to define functional groups of protists on the basis of the results of biodiversity-ecosystem functioning (BEF) experiments. This reverses the question; I looked to uncover functional groups within protist communities - and describe these - based on the results of BEF experiments. I empirically calculated the pairwise interactions of contributions to functioning of an initial pool of 22 species for seven measures of microbial ecosystem functioning. I used a quantitative method of to form functional groups based on similarity of species interactions. I then tested the predictive power of constructing communities based on my method, versus those constructed using functional traits and random assembly. I found diversity-functioning relationships similar to those previously reported, some interesting disconnects between functional diversity and certain functional measures, and some intriguing alternative patterns of functional diversity and functioning as a result of my new method.

Introduction

Ecologists are interested in ecosystem functioning because ecosystems regulate earth processes and provide services for human wellbeing (Loreau et al. 2001). Microorganisms are thought to play a central role in regulating ecosystem functions, particularly carbon cycling, decomposition, and nitrogen fixation (Sinsabaugh et al. 1991; Zak et al. 1994; Langenheder et al. 2006; Gessner et al. 2010). They also provide a range of services including bioremediation, biodegradation, and possible mitigation effects against global climate change (Singh et al. 2010; Saleem & Moe 2014).

Functional diversity is a key concept since it relates to a wide range of ecological questions centring on the prediction of rates and stability of ecosystem level processes (Tilman 2001; Diaz & Cabido 2001). It is thought to link organisms to ecosystems through mechanisms such as complementarity and facilitation, and is likely to be a driver of functional change in response to biotic, abiotic, or anthropogenic disturbance (Petchey & Gaston 2006). Functional diversity is confounded with species richness leading to difficulties in partitioning the effects of each on ecosystem functioning (Petchey et al. 2004). Some studies have attempted to address this (for instance Reich et al. 2004). However, part of the problem lies in the difficulty of both defining functional diversity and choosing traits in a standard, objective, and quantitative method (Petchey & Gaston 2006). This chapter will address this issue.

The use of the term ‘functional diversity’ has attained a position of importance within ecology in the last three decades. However, the concept is in many ways still difficult to define and has failed to crystallise in any one single form (Mason et al. 2005; Petchey & Gaston 2006; Villéger 2008; Mouchet et al. 2010; Cadotte et al. 2011). In its broadest sense, functional diversity relates biodiversity to what organisms do – their range of ecological functions rather than their evolved taxonomic identities. However, such a definition is vast. Through improvements in understanding of the effects of biodiversity on ecosystem processes, a more narrow definition has come in to common usage, most succinctly presented by Tilman (2001) as “the value and

range of those species and organismal traits that influence ecosystem functioning". For the most part, functional diversity has been treated, tested, quantified, and observed as the measuring of functional trait diversity where traits are components of an organism's phenotype that affects ecosystem level processes (Petchey & Gaston 2006).

Current approaches to measuring and quantifying functional diversity are varied and there has been a lack of a single standardised method for choosing functional traits and defining functional groups. This seems to have been due to the problems associated with choosing traits; what type, at what scale, which traits, how many, and how exactly to obtain trait values. Restrictions within systems or differences of opinion seem to have led to varied outcomes in terms of approaches taken. For instance, some approaches have focussed on categorical classification methods such as plants into C3 grasses, C4 grasses, N-fixing legumes and non-N-fixing herbaceous plants (Gitav & Noble 1997). However, categorical methods reduce pairwise distance between species to being entirely binary and may overly rely on subjective classification (Cadotte et al. 2011).

Use of continuous measurements involves the separation of species in n -dimensional trait space (Mason et al. 2005; Petchey & Gaston 2006). One advantage of continuous measures is that, unlike categorical ones, continuous measurement of traits allows for continuous variation in trait distances (Petchey & Gaston 2006). Subsequent classification into categorical groups can be achieved if desired with reduced risk of subjectivity (Petchey & Gaston 2006). However, the formation of groups often requires relatively arbitrary methods of defining groups and excluding differences, and there seems to be a very large number of ways that pairwise distances can be summarised (Petchey & Gaston 2006). Amongst others, continuous measurements of functional trait diversity include summed length across dendrograms, average pairwise distances, functional attribute diversity, FD_{var} , Rao's entropy, FAD2 and more (Rao, 1982; Walker et al. 1999; Heemsbergen et al. 2004; Ricotta 2005; Petchey & Gaston, 2002; Mason et al. 2003). These approaches analyse functional diversity by correlating functional diversity with the amount of ecosystem functioning observed. Others have correlated functional

diversity to overyielding (Marquard et al. 2009; Griffin et al. 2009; Flynn et al. 2011; Cadotte et al. 2011). Like taxonomic diversity, functional diversity can be turned into measures analogous to species evenness and divergence through the inclusion of relative abundances of constituent species (Diaz & Cabido, 2001; Mason et al. 2005).

Functional diversity of microorganisms has been the subject of research for some time and has been recognised to be pivotal to the functioning of the world's ecosystems (Falkowski et al. 2008; van der Heijden et al. 2008). They account for a large part of ecosystem metabolism and biomass, reportedly accounting for fifty percent of total global protoplasm (Whitman et al. 1998). Approaches to microbial functional diversity have generally focussed on identifying different patterns of resource use using approaches such as catabolic response profiles, respiration and utilisation of carbon substrates, and enzymatic activities of specific enzymes (Zak et al. 1994; Torsvik & Øvreås, 2002; Frossard et al. 2012). These are generally taken to represent elements of functioning relating to productivity, cellular activity, and the performance of specific functions such as carbon cycling, decomposition, nitrogen fixation and nitrogen and phosphorous utilisation (Sinsabaugh et al. 1991; Torsvik & Øvreås 2002; Langenheder et al. 2006; Sinsabaugh et al. 2008; Frossard et al. 2012). Mineral and organic compounds are important for microbial communities (Alexander 1999). Direct functions such as leaf matter degradation can be constrained by availability of such resources (Smith 2002) and there is evidence of differential resource use that can alter ecosystem processes. Quantifying fluxes of these substances can therefore provide a way of measuring functioning and subsequently functional diversity in experimental systems and *in situ*. However, some studies have reported a poor correlation between such measures of functional processes and attributes of community structure, complicating their use for understanding microbial BEF relationships (Frossard et al. 2012). This is an area that requires addressing.

There are a number of further challenges specific to microorganisms regarding quantifying functional diversity. Methods of quantifying functional diversity are generally used with functional traits hypothesised to be relevant *a priori*. However, in microbes traits can be

difficult to observe directly. Phenotypic traits in microbes are notoriously hard to identify, a fact highlighted by the realisation that most microbial diversity is 'hidden' both in eukaryotes and prokaryotes and revealed only by DNA based molecular techniques (Pace 1997; Moreira & Lopez-Garcia 2002; Nannipieri et al. 2003; Venter et al. 2004). In addition, culturing of most microbial strains is challenging (Rappe & Giovannoni 2003). The number of individuals to survey, sampling effects, and definition of microbial species all add further difficulties identifying microbial diversity and are problematic for quantifying functional diversity using *a priori* selected traits. Furthermore, there is evidence to suggest that *a priori* methods may not predict functioning any better than when species are assigned to groups randomly (Petchey et al. 2004; Wright et al. 2006).

There is a requirement in microbial ecology to consider novel approaches to quantifying functional diversity. I therefore propose an alternative approach to quantifying functional groups in these organisms. Instead of relying on trait data, I defined functional groups based on how species impacted several functional processes in diversity manipulation experiments. This allowed me to generate functional groups based solely on experimental data and without the need to define functional traits *a priori*. I assessed this approach for quantifying functional diversity by comparing the diversity-functioning relationships found using my method and conventional measures of functional diversity. I used a group of heterotrophic protists as my study system. The Glissomonada and Cercomonada are particularly useful for three reasons. Firstly, they are voracious bacterivores with strong impacts on prey communities (Glucksman et al. 2010). Secondly they have cryptic morphology making them a good candidate system for defining functional diversity without traits (Bass et al. 2009; Howe et al. 2009; Cavalier-Smith & Oates 2012). Thirdly, they are phylogenetically diverse (Bass et al. 2009).

Methods

Section 1 – Response ratio (RR) based dendrograms

Bacterial tree-hole community and protist species pool

The naturally occurring bacterial tree-hole community was collected from a single beech tree hole filled with rain water on the Ashridge Estate (51 47'.683 N, 00 35'.079W) as part of a survey in 2010. Samples were stored frozen in 30% v/v glycerol at -80°C and sub-samples re-grown as required for the experiment. While I expected the freezing process to alter bacterial communities to some degree, the procedure allowed the major benefit of enabling me to conduct repeatable experiments using the same complex mixture of bacteria through my thesis.

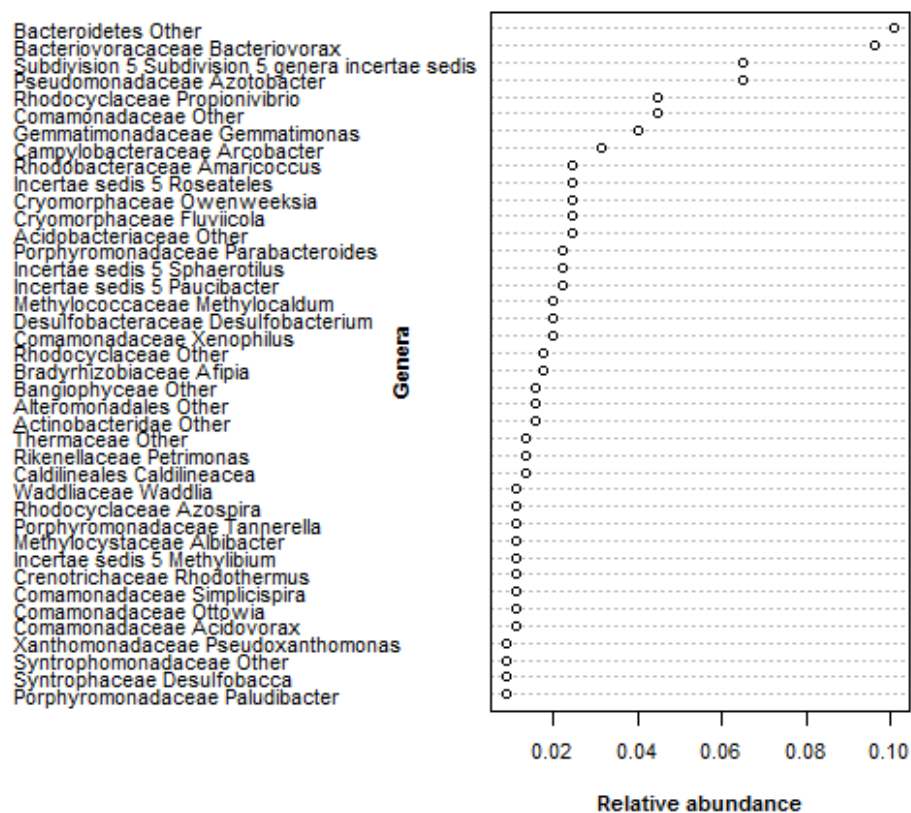


Figure 1. Dot chart showing the forty most abundant prokaryote genera in the treehole sample T143. Relative abundances are shown on the x axis. Sequence data was collected using Roche 454 sequencing (Bell pers. comm.).

Protist strains were collected as environmental isolates from a variety of geographical locations and habitats prior to this study (Bass, pers. comm). Two major taxonomic groups were represented. These were the Cercomonadida (=Cercomonads informally) and Glissomonadida (=Glissomonads informally). The first group are gliding biflagellate protozoa (Bass et al. 2009). They are heterotrophic and extremely abundant and diverse in both freshwater and soil (Bass et al. 2009; Bass & Cavalier-Smith 2004). Indeed they are one of the most common zooflagellate groups found in soils (Bass & Cavalier-Smith 2004). They are generally larger than the glissomonads, and have greater intra-clonal variability in both behaviour and morphology (Cavalier-Smith & Oates 2012). Much of the diversity of these organisms is morphologically cryptic and large parts of their diversity has been described using 18s rDNA sequences (Howe et al. 2009; Bass et al. 2009).

The second group – the glissomonads - are a relatively new order that was established due to the marked phylogenetic and morphological differences between this group of organisms and the order Cercomonadida to which they previously belonged (Howe et al. 2009). They are abundant in both freshwater and soils (Howe et al. 2009). Similarly to the cercomonads, the glissomonads are largely able to form cysts during periods of sub-optimal conditions.

Strains were selected because they represented two important, understudied but related taxonomic groups with a number of identifiable and quantifiable traits and that are widespread in soil and aquatic habitats (Howe et al. 2009; Bass et al. 2009). Both taxonomic groups display a wide diversity of behaviours in terms of swimming or gliding speed, position in the water column (or substrate surface), predation mode, prey choice, and cyst formation (encystment). All were known to be heterotrophic and bacterivorous (Howe et al. 2009; Bass et al. 2009). While the range of habitats that protists were isolated from may suggest a lack of direct relevance to beech tree holes specifically, they are sufficiently common in most environments to be appropriate organisms for inclusion.

Protist strains were kept long term in nutrient poor conditions. Long term cultures were kept in 250ml culture flasks filled with Volvic™ mineral water and a boiled wheat grain. These cultures consisted mainly of metabolically inactive cysts, with an extremely low density of active cells. They were not grown on bacterial communities used for experiments prior to the construction of experiments and growth of cultures to experimental densities for inoculation into microcosms. Long term stocks were regularly checked for contamination. A total of 30 strains were available from long term stocks, though only 22 were eventually used for experiments due to culturing problems and one case of contamination. The strains used displayed significant interspecific trait variation.

Microcosm construction and strain growth

A total of 22 protist strains were grown in both monoculture and all pairwise combinations in beech leaf tea (BLT) and on a single background bacterial community. I refer to this community as T143 throughout this thesis. This was used to inoculate fresh media for use in experimental microcosms. Beech leaf tea is media produced in order to replicate the nutrient and energy inputs to microbial communities in beech tree holes from beech tree litter. Beech leaves were collected from Wytham woods in October 2011 and stored at -20°C. Media was produced by combining 1g of beech leaf with 10ml of deionised water which was then autoclaved before being filtered using Whatman™ grade 1 0.22µm filter paper to produce concentrate. This was then diluted 1:32 and filtered again using Whatman™ grade 1 0.22µm filter paper and re-autoclaved.

15µl of frozen bacterial community stock were introduced into 50µl falcon tubes of BLT. Bacteria were grown for two days at 21°C to reach equilibrated abundances of $\sim 10^6$ cells ml⁻¹ (flow cytometry mean count = $2.1 \times 10^6 \pm 1.1 \times 10^4$ cells ml⁻¹) before inoculation of 150µl into the experimental microcosms. Protist strains were grown in monoculture on the bacterial community for 7 days at 17°C in order to reach carrying capacity before combination and inoculation in to all pairwise combinations. The experimental design then consisted of four replicates of each experimental monoculture microcosm and three replicates of each

experimental pairwise mixture microcosm. A total of 180µl of protist inoculate was used. This produced a total experiment size of 748 microcosms. These were then allowed to grow for 15 days at 17°C. All culturing of protists was carried out in 24 well plates containing 2ml of protist and bacteria inoculated BLT.

Bioassays and measures of ecosystem functioning (EF)

After fifteen days I performed seven bioassays to assess different measures of ecosystem functioning in each microcosm. These were as follows:

- a) A BacTiter-Glo ATP Cell Viability Assay (Promega Corporation, USA) on a FluoStar Optima (BMG LabTech) luminometer to assess ATP concentration as a measure of metabolic activity and productivity. 25µl of BacTiter-Glo reagent was added to 25µl of experimental sample in white opaque 384 well plates. These were then read in five minute kinetic reads at room temperature and the maximum luminescence was recorded. This is an accepted method for detecting microbial ATP (Sule et al. 2008). Luminescence was converted to nM ATP using the calibration equation $nM\ ATP = 0.0002x$ where x was luminescence. For additional information see Appendix 1 (page 198) and Appendix 2 (page 205).
- b) A MicroResp CO₂ concentration colormetric absorbance assay on a Synergy HT (BioTek) absorbance plate reader to assess CO₂ production levels between days 14 and 15 of the experiment, as a measure of total community respiration rates. Assays were carried out in 96 well deep well plates containing 1425µl of sample. Indicator was calibrated to give an $R^2 = 0.936$ between the mass of CO₂ present and change in absorbance at 572 nm. Absorbance was measured at 572 nm. Indicator solution was composed of 12.5µg ml⁻¹ cresol red, 150mM potassium chloride, and 2.5mM sodium bicarbonate. This was combined with purified agar in 1:2 agar:indicator ratio to achieve a final 1% concentration of agar and poured into 96 well plates, as per the manufacturer's instructions. Absorbance was converted to mg ml⁻¹ using the equation

$mgCO_2 = e^{(\ln(\Delta\lambda_{572})+0.305414)/0.282164}$ where $\Delta\lambda_{572}$ is the difference in absorbance between the beginning and the end of the experiment. For further details see Appendix 1 (pages 199 – 201).

- c) A phosphate concentration colorimetric absorbance assay on a Synergy HT (BioTek) absorbance plate reader to assess phosphate levels in experimental communities as a measure of phosphate available within a system. The assay consists of combining 2.91M sulphuric acid, 0.026M ammonium molybdate, 0.31M ascorbic acid, and 0.001M potassium antimony tartrate in a 6:2:2:1 ratio to produce the reagent, of which 30µl is added to each 170µl of sample in clear 96 well plates. These were then allowed to stand for ten minutes to allow colour to develop before absorbance was read at 880nm. The assay has a detection threshold and sensitivity of 0.2mg ml⁻¹. Chemistry for the assay is based on Murphey and Riley, 1962. Absorbance was calibrated to mg ml⁻¹ using the equation $mg\ ml^{-1}\ PO_4^{3-} = 0.2252x + 0.0818$. Further details are provided in Appendix 1 (page 198) and Appendix 2 (page 205).
- a) Four enzymatic activity assays. These quantified the degree to which each community or monoculture utilised four enzyme pathways to utilise substrates likely to be available during leaf degradation (Sensibaugh et al. 1991; De Angelis et al. 2012). 25µl sub-samples of each microcosm were taken and diluted to 100µl with sterile water to reduce quenching of the fluorescence in white opaque 96 well plates. These were then inoculated with 25µl fluorescently tagged substrate analogues (4-methylumbelliferone) for four enzymes involved in the metabolic pathways for the breakdown of phosphomonoesters, glucosamines, cellulose, and hemicellulose. They were then incubated in the dark at room temperature for 90 minutes. The enzymes, their function and their analogues are given in table 1. 10µl 1M NaOH was added 2 minutes prior to fluorescence readings. These were taken in 3 minute reads on a Synergy HT (Biotek) fluorescence plate reader at excitation wavelength of 360nm and emission at 460nm. Fluorescence was calibrated to nM 4-MUB using the equation: $nM\ 4 - MUB =$

0.0004x. Further details are provided in Appendix 1 (page 200) and Appendix 2 (page 205).

Table 1. List of fluorescently tagged enzyme substrate analogues, corresponding enzymes and their function. Acronyms used throughout the thesis are provided

<i>Enzyme</i>	<i>Acronym</i>	<i>Substrate analogue</i>	<i>Function</i>
β -Glucosidase	MUG	4-MUB- β -D-glucopyranoside	Cellulose degradation
β -Xylosidase	Xylo	4-MUB- β -D-xylopyranoide	Hemicellulose degradation
B – N - acetylglucosaminida se	MUN	4-MUB-N-acetyl- β -D-glucosamine	Degradation of β -1,4 glucosamines
Phosphatase	MUP	4-MUB-phosphate	Phosphomonoester degradation

The Response Ratio (RR)

I defined species as belonging to the same functional group if they contributed to a functional process in a similar way, while species that belonged to different functional groups contributed differently to the functional process. I estimated this indirectly by comparing the polyculture level of functioning to what would be obtained if all of the species in the community contributed independently to ecosystem functioning. For each community I calculated a response ratio (RR):

$$RR = \frac{p}{\sum_{i=1}^n m_i}$$

Where p is the value of the ecosystem functioning measure of the polyculture community, and m is the value of the ecosystem functioning measure for the i^{th} monoculture of n monocultures that make up the polyculture. This has similarities with D_{max} , D_T , relative yield total (RYT), and $\ln(rr)$ (Loreau 1998b; Goldberg et al. 1999). It is also directly analogous to Loreau's formulation of Jolliffe's Relative Land Output (RLO) (Jolliffe 1997; Loreau 1998b) and Fiegna et al's (2015) method of inferring species interactions. The sum of the monocultures is an important value since it provides a null situation in which all species contribute perfectly additively to ecosystem functioning with complete complementarity. Response ratio values smaller than 1 indicate that polyculture component species have negative effects on the functional process relative to the additive expectation. Larger response ratio values indicate increasing complementarity. A value of 1 indicates complete complementarity with no interaction and completely independent effects on functioning. Values greater than 1 indicate that polycultures have positive complementarity or facilitative effects on functional processes. In this experiment polycultures were pairwise combinations of protists. However, response ratios can be calculated for more diverse polycultures (see Chapters 4 and 5). A conceptual representation is provided in figure 2 (page 45).

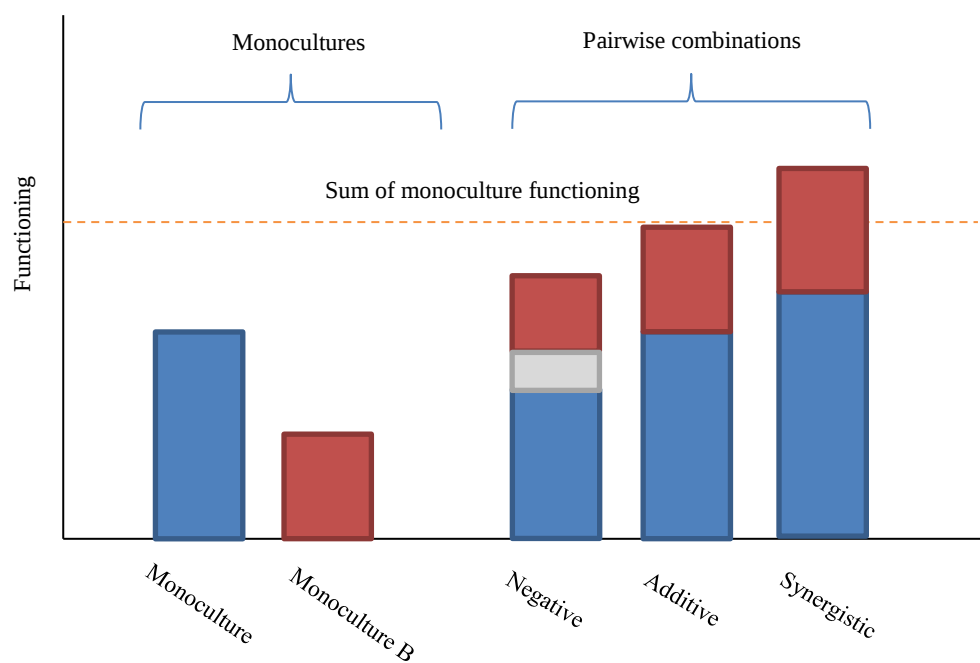


Figure 2. This is a conceptual representation of the response ratio. The three situations are represented where pairwise polyculture functioning is less than the sum of the monocultures, equal to the sum of the monocultures, and exceeds the sum of the monocultures. These indicate negative interactions (grey represents the interaction), complete complementarity, and positive complementarity (synergistic) respectively. The diagram is reproduced and adapted from (Fiegna et al. 2015).

Construction of response ratio-based functional dendrograms

The response ratios of the pairwise interactions were then used to produce cluster dendrograms of the 22 protist strains. First, a matrix of response ratios (response matrix) was constructed. It is symmetrical around the 0.5 diagonal. It was then scaled and converted into a distance matrix using the scale and dist functions in R v3.0.3 (R Development Core Team 2014). This matrix contained distances between response ratios of pairs of species. The distance matrix was then hierarchically clustered using the hclust function in R v3.0.3 (R Core Development Team, 2014) to produce a cluster dendrogram. The hierarchical clustering method used complete linkage to find clusters.

Defining groups

In order to define functional groups, it is necessary to apply method of cutting the functional dendrogram so as to leave a number of functional clusters. Previous methods take an arbitrary cut off in order to produce groups while noting that differences in cut-off points will produce different groups. It would be better to apply an objective method of cutting the dendrogram to form groups of protists according to their functional relationships.

The height of the tree allowed me to see the height of each bifurcation in the cluster dendrogram and to therefore see the cumulative number of branches at each height along the tree. Most of the height of the tree is accumulated by some saturation point, after which little additional bifurcation adds any extra height to the tree. Biologically this means that differences in species effects on functional processes past this point become fewer and the rate of accumulation of these differences slows. They can be considered as sufficiently similar to each other at this point as to be grouped.

I created a plot of tree height against clusters. If this had produced a straight $y = -mx + c$ line, then this would have shown that species branched continuously with no clusters. The more the curve tends away from this situation, the greater the degree of functional dissimilarity can be

thought to exist within the community, with greater numbers of nodes and bifurcations implying greater clustering.

To apply a cut-off point to the dendrogram in order to define clusters, it seemed appropriate to apply this at the point at which the maximum rate of accumulation of nodes had been achieved along the height of the tree and the rate of accumulation slows thereafter i.e. the point at which the curve of tree height against number of clusters is the furthest from the $y = -mx + c$ line of functional unity.

I calculated this point as the point of furthest perpendicular distance between the $y = -mx + c$ line and the curve of tree height against number of clusters. I then took the height of the tree at this point to apply the cut-off on the tree. Clusters that were then formed from nodes below this cut-off were taken to be functionally related groups of protists.

Combining dendrograms of individual EF measures into a single dendrogram

I then sought to combine trees across the multiple EF measures I had calculated RRs for. This was so that I could construct a multifunctional dendrogram to represent functional relationships between protists based on their effects on several functional processes. This would allow comparison of functional organisation based on different functions, as well as a basis for determining functional diversity of communities in future experiments where the same suite of functions would be measured. I achieved this by averaging across the matrices of RRs for the 7 different measures used. The matrix of average response ratios was then treated as previously described to produce a distance matrix and finally a cluster dendrogram. The dendrogram produced is shown in figure 7.

**Box 1 – Response ratio (RR)
dendrogram construction**

Direct measurement of functional processes from microcosms of monocultures or pairwise communities (figure A)

Measurements were used to calculate response ratios which were then combined into a matrix (figure B)

This was then converted into a distance matrix of distances between species' response ratios with other species (figure C)

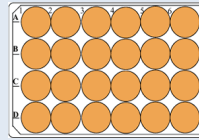
Distances were then used to hierarchically cluster species based on RRs with other species (figure D). The dendrogram presented here was based on random values for ecosystem functioning and eight hypothetical species

The number of clusters or bifurcations along the height of the tree was then plotted and a predictive smoothed curve fitted. The $y = -mx + c$ line connecting the minimum and maximum heights of the tree represented the expected line if there were no clusters – the line of functional unity (figure D).

The point of furthest distance between the curve representing the numbers of clusters along the height of the tree and the line of functional unity was found by finding the difference between the two at each height and represented with a red dot (figure D).

The dendrogram was cut at the height at which this maximum difference occurred (figure E)

A

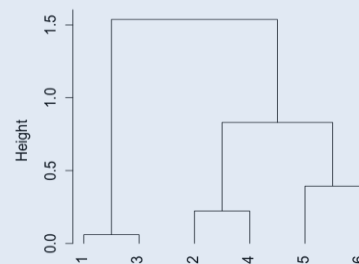


$$RR = \frac{p}{\sum_{i=1}^n m_i}$$

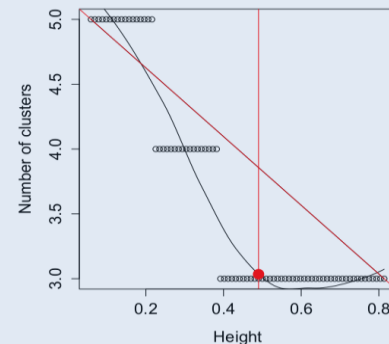
B

	1	2	3	4	5	6
1		RR	RR	RR	RR	RR
2	RR		RR	RR	RR	RR
3	RR	RR		RR	RR	RR
4	RR	RR	RR		RR	RR
5	RR	RR	RR	RR		RR
6	RR	RR	RR	RR	RR	

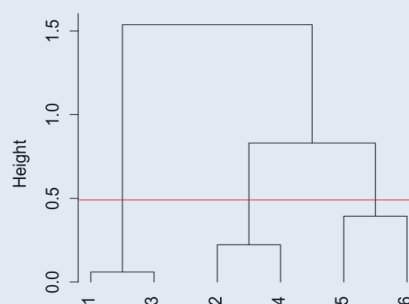
C



D



E



Section 2 –Trait-based dendrograms

Selection and quantification of traits

Having constructed hierarchical cluster dendrograms based on contributions to functional processes and response ratios, it was necessary to compare these results to what could be achieved using a more conventional trait-based approach. I therefore produced a combined cluster dendrogram based on measures of 4 morphological functional trait measures; size, linear motility, estimated growth rate, and propensity to encyst. These traits were chosen primarily because they were the easiest to quantify in these morphologically similar species. They were also anecdotally known to show interspecific variability and I could hypothesise that all would have functional relevance. I obtained data for 24 strains.

i. Size

All strains were measured at the end of the initial growth of the monocultures using a bifocal inverted microscope with an HD camera connected to a PC running Olympus software. Sizes of strains in monoculture were then measured by photographing live samples and later measuring cell sizes using a calibrated electronic tool included in the software. Twenty cells were measured from each monoculture. Sizes found were then, where possible, cross checked with those reported in previous literature (Bass et al. 2009; Howe et al. 2009) to verify the consistency in trait variability with previous observations and ensure that the range of trait values matched those expected.

ii. Estimated growth rate

I estimated growth rate as the per capita increase or decrease in cells over five days after an initial growth period of 3 days. Strains were grown at 17°C, which is considered suitable for all the species to grow in. Cells were counted in three fields of view at 400x magnification on a bifocal inverted microscope in three replicates for each strain, on days 1 and day 5 following the initial growth period.

The growth rate was then estimated using the equation:

$$r = \frac{\ln(N_2 - N_1)}{t_2 - t_1}$$

Where r is growth rate, N is the number of cells and t is the time point.

iii. Linear motility

I estimated the degree of motile behaviour shown by each strain. I measured this as the distance travelled across the field of view in a single linear distance in a given time period. The Olympus bifocal inverted microscope used in conjunction with Olympus software and HD camera was able to photograph microcosms at the same point at set time intervals. I used this feature to photograph one field of view in each of two microcosms of each monoculture. The photographs were taken 5 seconds apart at 200x magnification with an exposure of 51 milliseconds using the autofocus feature in a range of 200µm. I then measured the distance between two fixed points of the same protist between the two photographs. This was repeated ten times. Finally, the distances travelled were standardised relative to body size by dividing the mean linear distance travelled by the mean body size of the strain.

iv. Cysting propensity

The cercomonads and glissomonads used in this study characteristically form cysts when subjected to non-optimal changes in environmental conditions such as change in pH, temperature, and prey availability. I decided to characterise this trait for each of the strains in response to temperature changes. I grew 9 replicates at 17°C for 8 days (growth data was collected for three of these from days 5 – 8 as previously described). At the end of day 8, three replicates were moved to an incubator at 25°C and three to another at 10°C. Three were maintained at 17°C. These temperatures are considered to be at the extremes of the temperature tolerances for the strains used based on observations (Bass, pers. comm.) and preliminary data. On days 9, 10 and 12, the number of cysts in three fields of view of each

of the three replicates at each temperature was counted and averaged. The proportion of cells converted to cysts at each day was estimated by dividing the mean number of cysts on each of days 9, 10 and 12 by the maximum number of cells counted in the microcosm. I then took the mean proportion of cells converted to cysts over days 9, 10 and 12 for each temperature transition. I then summed these across the three transitions to give a ‘cyst score’ to each strain. This is summarised in the equation:

$$C = \sum_{i=1}^3 \frac{\bar{c}}{C_{max.i}}$$

Where C is the cyst score for the strain, $\bar{c}$ which is the mean number of cysts counted on an observation day divided by $C_{max.i}$ the maximum number of cells observed, summed across i number of observation days.

Construction of trait-based dendrograms

I then produced cluster dendrograms for individual traits, as well as a combined multivariate cluster dendrogram of all four traits. Trait matrices were first scaled to have $\bar{x} = 0$ and $\sigma = 1$, then derived into distance matrices using the `dist` function in R v3.0.3 (R Development Core Team 2014). Distance matrices contained distances between species’ trait values. The distance matrices are then used to produce dendrograms through hierarchical clustering using the `hclust` function from the R package `Vegan` (Oksanen et al. 2015). Clustering used complete linkage. The method used is identical to that of (Petchey & Gaston 2002b). I also produced a cluster dendrogram based on both direct measurements of ecosystem functioning and on measurements of morphological functional traits. This was done following the method described above in the previous sub-section. Functional processes were treated as traits, and mean monoculture values of these processes were treated as trait values.

Section 3 – Validation and comparison

Validation and exploration

In order to test the validity of my method, I conducted an experiment to test the diversity-functioning relationships based on a number of functional dendrograms produced by the methods described above. I tested the relationships between species richness, functional diversity and different ecosystem functioning measures by constructing protist communities from a pool of 18 species and independently varying functional group richness (FGR) and species richness. FGR was determined and communities assembled using either the combined trait-function dendrogram, trait-based dendrogram, or response ratio-based dendrogram. Treatment levels were combinations of FGR 1 – 5 and species richness 1, 2, 3, 6, 9, and 18, according to the ‘random partitions design’ (Bell et al. 2009a). There were a total of 19 treatment combinations for communities assembled by the response ratio-based dendrogram and 16 treatment combinations for those assembled by the combined and trait-based dendrograms. There were 20 replicates of each treatment combination with species randomly assigned to each replicate, though monocultures were only replicated four times per dendrogram FGR treatment. A total of 1056 communities were assembled. I also assembled a further 261 communities of 1, 2, 3, 6, 9, or 18 species using the random partitions design (Bell et al. 2009a; see Chapter 3, page 79) with three partitions and three replicates. Monocultures were only replicated four times. Functional group identities (1 – 5) were randomly assigned to species in order to assign random FGR. Communities were grown and assayed as previously described. The same bioassays as previously described were used except for Xylo enzymatic activity.

Statistical analyses

In addition to the six measures of ecosystem functioning already described, I calculated a metric of multifunctioning. To do this I standardised measures of functions to have $\bar{x} = 0$ and $\sigma = 1$. Measures were then averaged to produce a single metric of average multiple functioning. Data were analysed by generalised linear model (GLM) in R v3.0.3 (R Core Team 2014) with gaussian errors and identity link function. Data were log transformed for reasons of variance stability. The maximal model included main effects of species richness, functional group richness (FGR), and method of functional dendrogram assembly and all interactions (see Appendix 3, supplementary table 1). Model criticism was carried out by inspection of plots of residuals against predicted values, standard deviance of residuals against theoretical quantiles, and leverage. Model simplification was carried out by manual stepwise removal of least significant terms and model comparison using ANOVA until an adequate minimal model was achieved (Crawley 2007).

Results

Ecosystem function response ratio-based dendrograms

I produced cluster dendrograms with all 22 strains for all 7 ecosystem functioning measurements based on the response ratios calculated for all of the pairwise communities (figure 4). These showed a pattern of branching that indicated that the strains used resolve into discernible clusters, rather than continuous subsequent branches. Applying my method of cutting the dendrogram resolved groups in all cases (figures 3, 5, and 7). CO₂ production produced the tree with the fewest groups – just two. The enzymatic activity measures produced between four and six groups, while ATP production and phosphate concentration produce six and five respectively. I was then able to produce a cluster dendrogram based on response ratios combining all seven ecosystem function measures (figure 7). In this case my approach for defining clusters within the dendrogram produced six groups of between one and five protist strains. The dendrograms produced for response ratios of functional processes seemed to diverge far more than trait-based dendrograms in composition of functional groups and position of species on the dendrogram relative to the combined cluster dendrogram (figures 3, 5, and 7).

Trait-based dendrograms

I also produced cluster dendrograms for each of the four pre-determined functional traits. Though I attempted to collect data for all twenty-four strains, some dendrograms had to have strains omitted due to missing or incomplete data, or an inability to collect the data sufficiently accurately. I successfully collected cysting data for all twenty-four strains, growth for twenty-two strains, size data for twenty-one strains, and motility data for twenty strains. Estimated growth rates for C18, W70 and Wa57 were found to be negative. I observed that these strains grew consistently very quickly during the initial growth phase when no counting took place and were already declining by the time counting was carried out. These three strains were retained since they formed their own group with only one other strain and there was little effect on the dendrogram when they were removed. Normally very low densities and slow growth are

observed in the vast majority of strains which is why it was decided that counting would not occur until day 3 after inoculation. In general my method for cutting the tree did not resolve groups as distinctly or into as many groups on these trait-based dendrograms (figures 3 - 7). The combined multivariate dendrogram based on the functional traits was cut into five groups of between one and six strains. In these trait-based trees, species that clustered in the combined dendrogram also tended to cluster more readily together in the dendrograms for individual traits, in particular those in the orange and red groups (see figure 4).

Table 2. Table showing the trait values obtained for the four pre-determined functional traits; linear motility relative to body size, cysting score, growth rate, and size. N/A denotes species for which data was not successfully collected

	<i>Linear motility ($\mu\text{m s}^{-1} \mu\text{m}^{-1}$)</i>	<i>Cysting score (C)</i>	<i>Growth rate (r)</i>	<i>Size (μm)</i>
Spain 1	3.7	2.0	0.20	4.5
C18	1.7	1.2	-0.18	8.0
Buff H5	1.1	0.7	0.26	9.5
W36	2.7	0.2	0.28	4.0
IB3	1.1	0.3	0.16	12.5
W70	2.0	1.9	-0.26	10.0
B13	0.6	0.03	0.25	18.0
IVY20	0.5	1.6	0.15	14.0
SW2	1.9	0.03	0.10	7.0
G9	3.6	0.3	0.26	6.0
G11	4.3	0.04	0.11	4.5
Giraffe G9	3.5	2.0	0.17	4.8
Wa42	6.5	2.0	0.08	4.5
W80	3.9	2.0	0.07	5.0
IVY 7a	1.0	0.4	0.06	21.0
KuHF	3.4	0.06	0.28	4.0
II Grass A	1.6	1.3	0.01	8.5
Wa8	2.3	0.4	0.16	12.0
Wa46	5.6	1.9	0.08	4.5
C7	1.8	5.6	0.18	15.0
Wa57	N/A	2.5	-0.18	N/A
C43	N/A	2.2	0.10	N/A
Casph II	N/A	1.3	N/A	36.4
Ivy 11	N/A	1.3	N/A	N/A

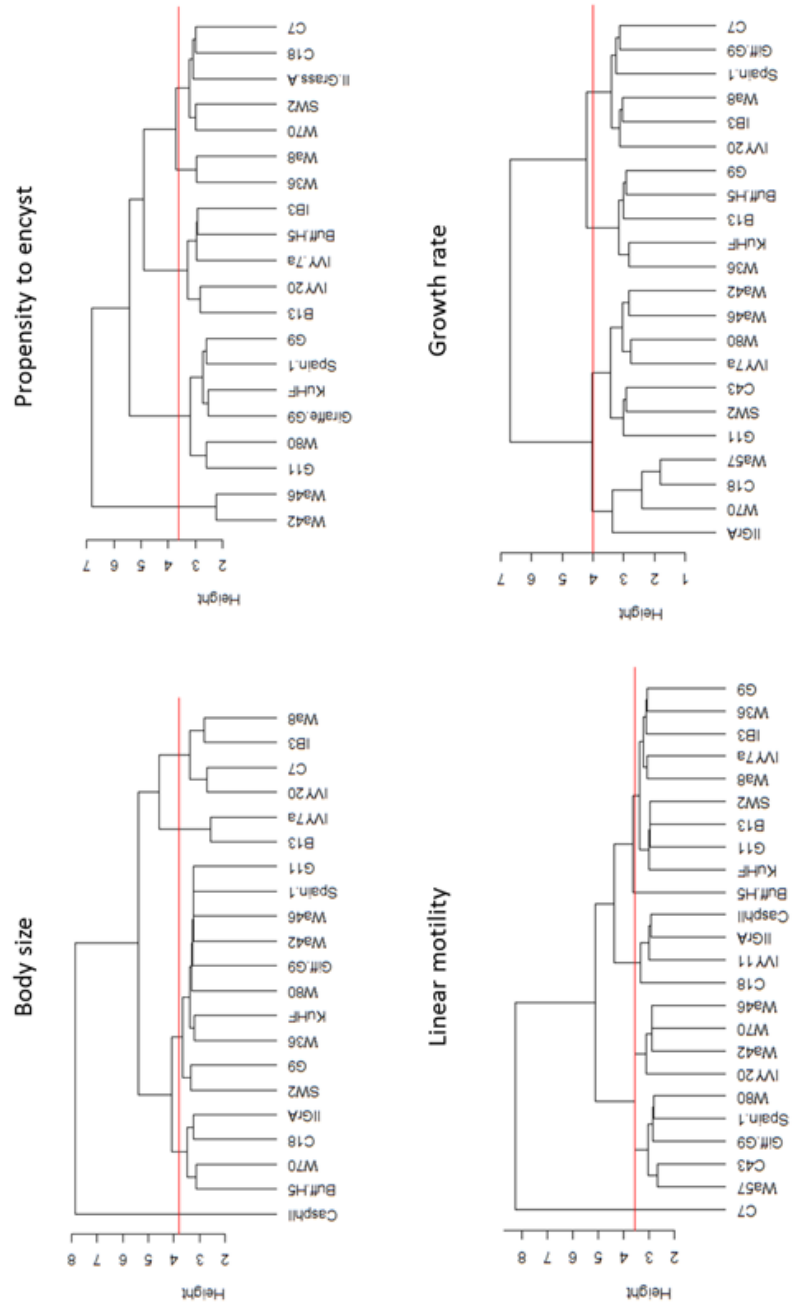


Figure 3. Dendrogram of relationships between protist strains based on hierarchical clustering of top left: body size trait values; bottom left: propensity to cyst (cyst score) trait values; top right: linear motility trait values; bottom right: estimated growth rate trait values

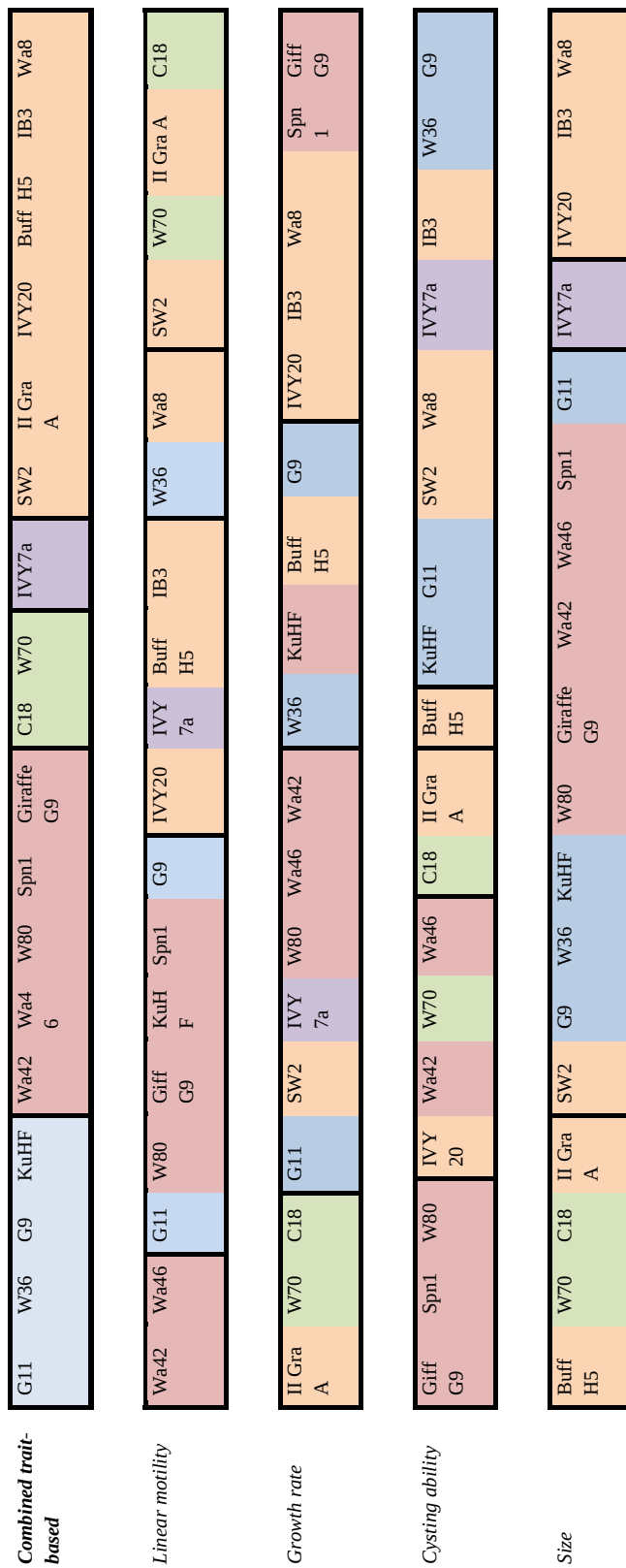


Figure 4. Diagrammatic representation of changes in functional group compositions and species positions on dendrograms for trait-based hierarchical cluster dendrograms of the four functional traits, relative to the combined trait-based cluster dendrogram. Colours represent functional groups defined in the combined dendrogram. Black borders denote functional groups for the dendrogram of the functional trait in question

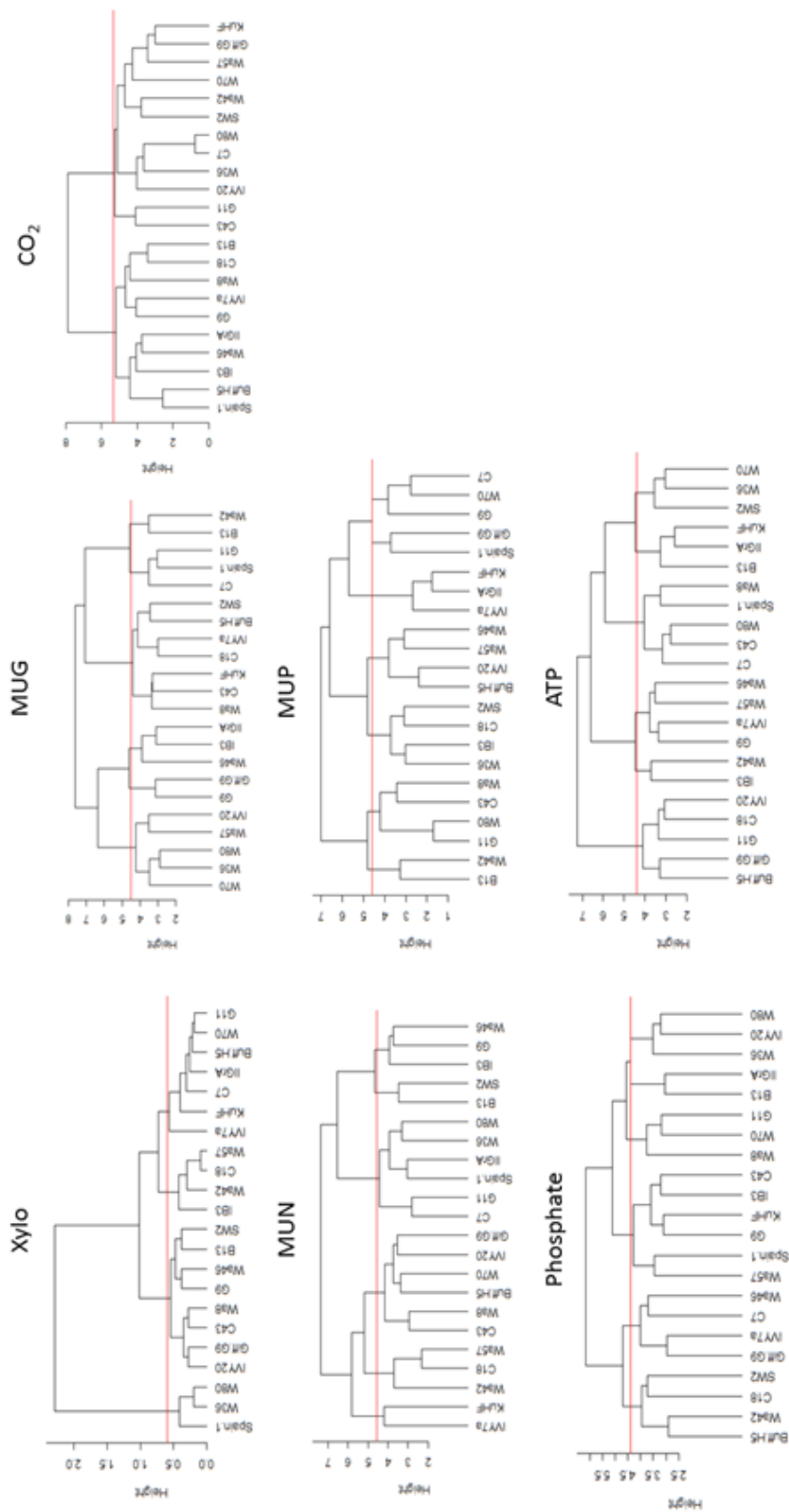


Figure 5. Dendrogram of functional relationships between protist strains, based on distances in response ratios, for; top left: microcosm Xylo enzymatic activity; middle left: MUN enzymatic activity; bottom left: phosphate concentration top middle: MUG enzymatic activity; middle middle: ATP concentration; top right: CO₂ concentration

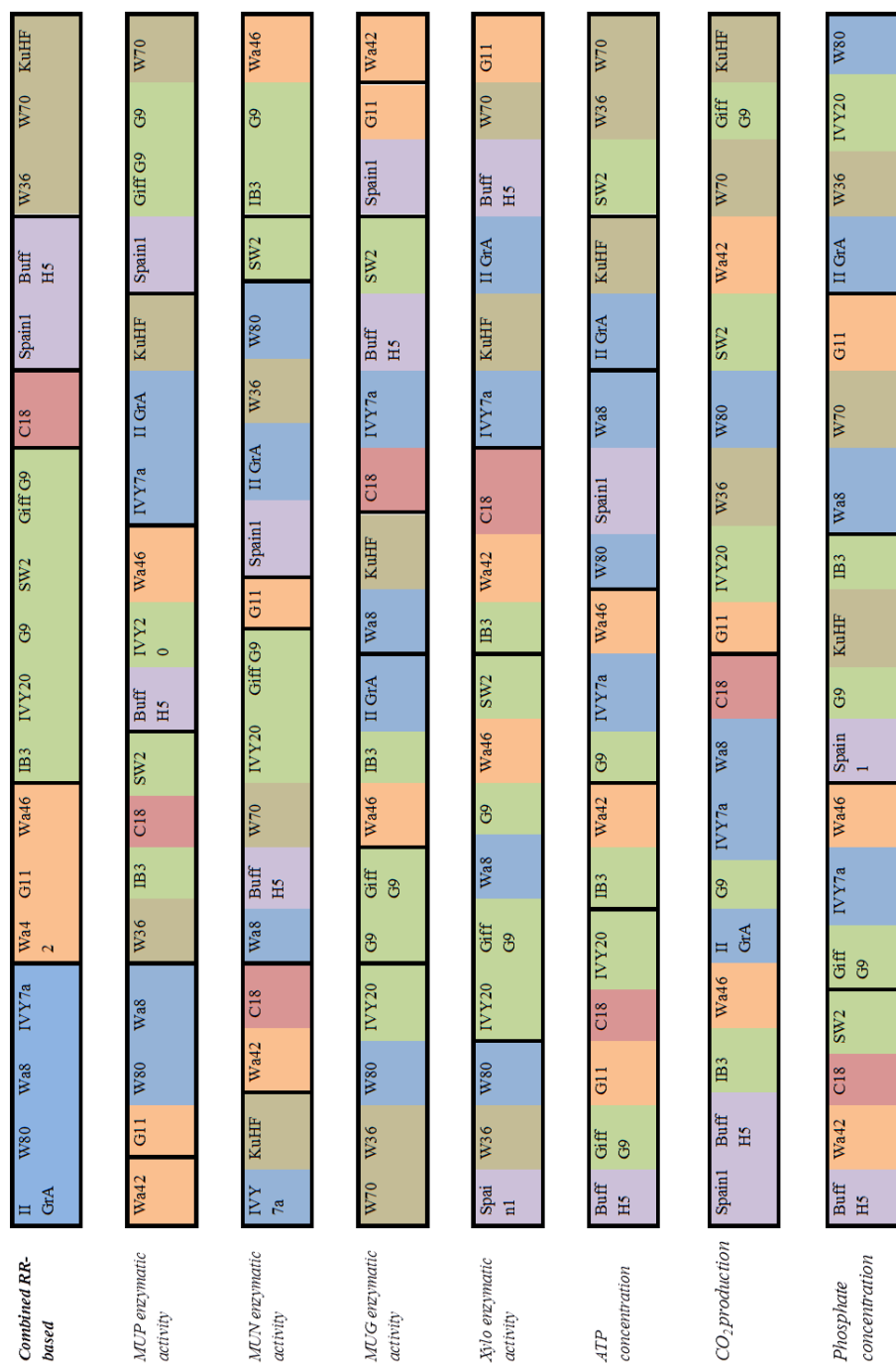


Figure 6. Diagrammatic representation of changes in functional group compositions and species positions on dendrograms for RR-based hierarchical cluster dendrogram for each ecosystem function measure relative to the combined RR-based cluster dendrogram. Colours represent functional groups defined in the combined dendrogram. Black borders denote functional groups for the dendrogram of the ecosystem function measure in question

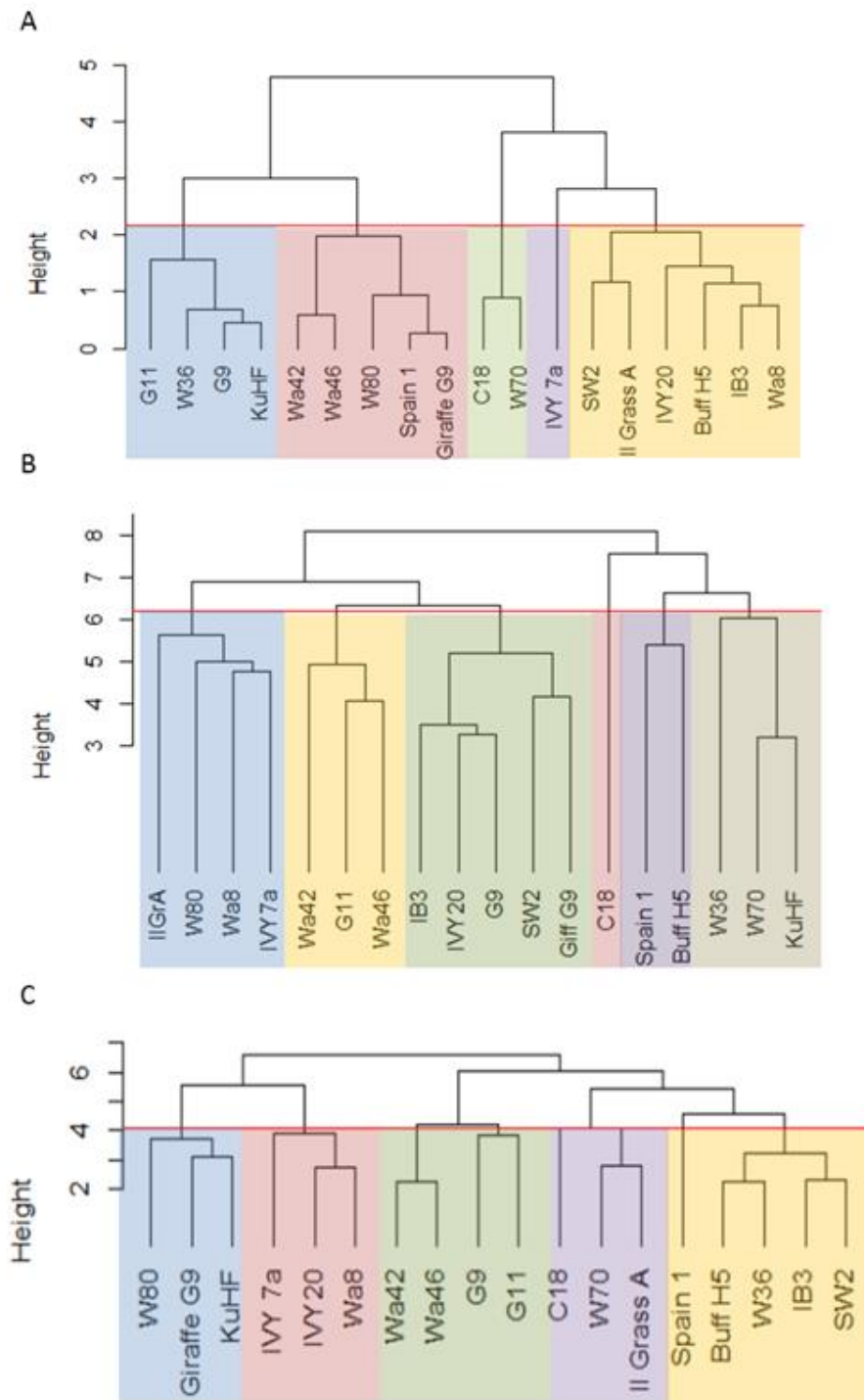


Figure 7. Dendrogram of relationships between protist strains based on; **A:** hierarchical clustering of combined trait values of the four phenotypic functional traits (estimated growth rate, propensity to cyst, body size, and linear motility); **B:** clustering of combined distances in response ratios across the 7 ecosystem function measures (MUG, MUP, MUN and Xylo enzymatic activity, as well as CO₂ and ATP production, and microcosm phosphate concentration); **C:** combined across monoculture functioning and traits. Colours of functional groups for **A** and **B** correspond to those in figures 4 and 6.

Validation and exploration

I found that there was a significant positive linear relationship between species richness and ecosystem functioning for all functions and multifunctioning. I found that the trait-based assembly of functional groups performed significantly worse than random assignment of groups in terms of CO₂ production. This was indicated by a significant negative interaction between species richness, FGR, and assembly method, but with no significant difference in overall functioning (GLM: estimate = -0.17, $t = -2.61$, $p = 0.0093$). This was also the case for MUP enzymatic activity at FGR2, 3 and 5 (GLM: estimate = -0.24, $t = -0.25$, $p = 0.048$; estimate = -0.25, $t = -2.41$, $p = 0.015$; and estimate = -0.21, $t = -2.06$, $p = 0.039$). For all other measures, functional diversity assembled by the trait-based method did not predict functioning differently from random assembly.

Response ratio-based assembly predicted decreasing ecosystem functioning with increasing FGR relative to random assembly, despite having no significant difference in overall levels of functioning. This was the case for MUG enzymatic activity where there were significant negative interactions between RR-based assembly and FGR despite significantly greater overall functioning (GLM: estimate = 0.44, $t = 5.01$, $p < 0.001$). Similarly, there was a significant negative interaction between RR-based assembly method and species richness for MUN enzymatic activity at FGR2. Increasing FGR under the response ratio-based assembly method predicted decreasing MUG enzymatic activity compared to the trait-based method. For statistical outputs see Appendix 4, supplementary table 2.

Response ratio-based and combined trait-ecosystem functioning based approaches predicted greater microcosm cellular activity than random assignment at higher FGRs, indicated by significant positive three-way interactions between group assignment method, FGR and species richness. There was no significant difference between trait-based and response ratio-based assignment methods. All methods of functional group assignment interacted positively with FGR for CO₂ production. For statistical outputs see Appendix 4, supplementary table 2.

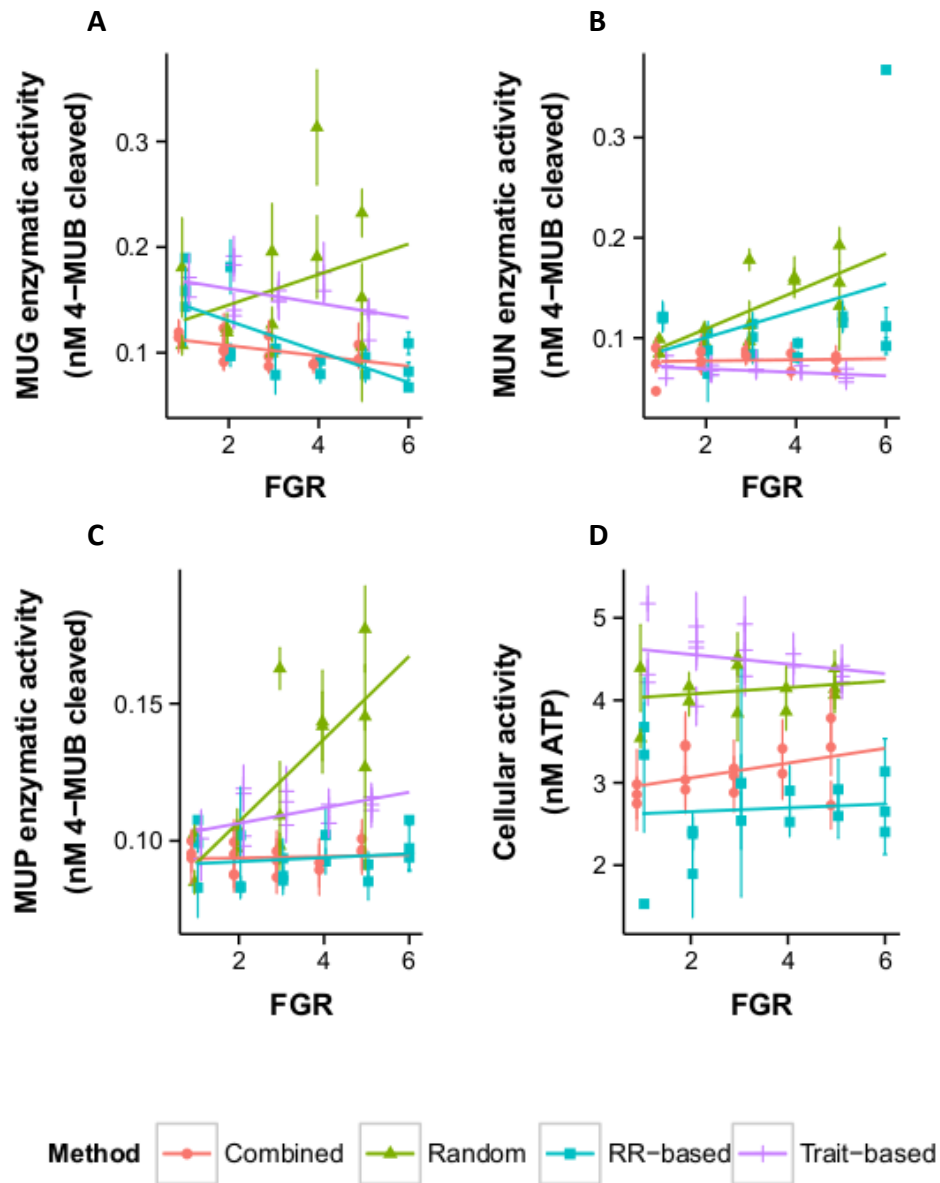


Figure 8. Plots of ecosystem functioning measures against functional group richness (FGR). Plots are **A)** MUG enzymatic activity, **B)** MUN enzymatic activity, **C)** MUP enzymatic activity, and **D)** cellular activity (ATP). Enzymatic activities are rates of cleavage of substrate in 90minutes. Points are means and error bars are standard errors. Regression lines are fitted by GLM. Colours and shapes correspond to the four methods of assembling communities according to the four methods of classifying functional diversity. These are; combined morphological functional trait and ecosystem functioning measure (Combined), random assignment of species to groups (Random), response ratio (RR-based), and morphological functional trait-based (Trait-based).

Discussion

In this chapter I attempted to classify the functional diversity of a group of heterotrophic protists using a novel approach tailored to circumnavigate some of the problems associated with quantifying functional diversity, in particular in microorganisms. My method combined four main components; a) using direct measures of ecosystem functioning to determine groups rather than traits predicted *a priori*, b) measures relevant to microbial functioning, c) response ratios that contain information about the interactions between species, and d) an objective method of cutting the dendrogram based on the accumulation of nodes along the tree. I was able to produce cluster dendrograms based on direct measures of functional processes and compare these to the results of trait-based dendrograms similar to those of Petchey and Gaston (2002).

Response ratio dendrograms

In general dendrograms based on direct measurements of functional processes delineated groups more clearly. Branching was balanced with well resolved nodes and few species branching by themselves or successively branching from one another. MUB, MUP and MUN enzymatic activity assays seemed to form groups best. These fine scale measures are likely to do so as they are more tightly linked to bacterial populations constituting an assemblage (Langenheder et al. 2006; Peter et al. 2011). These sorts of biochemical functional measures therefore reflect more accurately predator effects on the community. In contrast, ATP cell viability, microcosm CO₂ production, and phosphate concentration absorbance assays resolved groups least well, most likely because these coarse measures of functioning integrate across many processes and do not reflect more subtle changes due to predation of bacterial populations by protists (Langenheder et al. 2005; Frossard et al. 2012). This supports the argument that some studies may have failed to detect effects of community composition on functioning due to insufficiently high resolution measures of functional processes. My results support studies that have found that community structure has important effects on ecosystem processes (Bell et al. 2005; Langenheder et al. 2010; Peter et al. 2011).

Trait-based dendrograms

I found that dendrograms based on phenotypic functional traits did not differentiate between species as well as response ratio-based dendrograms that use species interactions. There were greater numbers of species branching alone and dendrograms also tended to be cut to form fewer groups. The multiple trait dendrogram had more even branching with a greater number of balanced groups than dendrograms based on single traits. The multiple functional measure dendrogram contained the median number of groups found in the single function dendrograms with a more even distribution of species also, though the effect was less pronounced. This was because combining traits or measures that showed little variation between species, allowed greater variation to be introduced. This provided a better mean estimate of pairwise distances and therefore resolved relationships better. If the measures contain large variation, increasing the diversity of traits or measures may be redundant. In either case, it is clear that the number of traits or measures included to determine functional diversity is still important and careful consideration to the number of traits used is necessary (Lefcheck et al. 2015b; Sirot et al. 2015).

In large macro-organisms, it is relatively easy to make predictions about which traits are likely to be functionally important, quantify them and classify or measure them. However, in microorganisms this is far more challenging. While it is possible identify variation in phenotype, most of the diversity currently understood in the Glissomonada and Cercomonada is cryptic and phylogenetic diversity only revealed through molecular DNA techniques and next generation sequencing (Howe et al. 2009; Bass et al. 2009). There have been improvements in attempts to use traits in approaches to microbial biodiversity and functioning (reviewed in Krause et al. 2014). These include recording of physiological traits such as nutrient use efficiency and affinity (Edwards et al. 2012), cell specific metabolic rates, and substrate use and isotope tracking (Nielsen et al. 2003; Hoehler & Jørgensen 2013; Krause et al. 2014), as well as advances in microscopy and biomolecular imaging (Haagensen et al. 2011).

Performance of tree cutting method

Previous methods have often delimited functional groups arbitrarily (Petchey & Gaston 2002b). Our method cuts the tree at the point where increased height ceases to accumulate more nodes. Biologically this means that it is the point on the tree beyond which pairwise distances in response ratios cease to increase and accounts for most of the variation in species distribution in niche/functional space. The results show that the method of cutting the dendrograms works well, with cutting points often close to nodes but with little ambiguity. The dendrogram of CO₂ production is cut very high along the branch lengths, producing two large groups. This supports the notion that measures such as this are too coarse (Frossard et al. 2012) to resolve differences between predators and their structuring effects that underlie their functional impacts. Alternatively, this could be interpreted as a result of functional redundancy. Predators may have different structuring effects, but using broad scale functional measures, these are redundant to each other and are not detected.

Measures such as enzymatic activity which better reflected functional differences in the bacterial prey population will resolve differences in the predators that structure them. This was evident from dendrograms based on measures of enzymatic activity that are cut much lower. Clusters accumulated maximally much lower down the tree, indicating less functional unity and greater functional difference. As a result I found between 5 and 7 groups for these. This showed that just as for traits the types of measures selected are still important (Petchey & Gaston 2006).

Validation and comparison

The results from this part of the experiment are interesting. They mostly show the expected relationship between species richness and functioning. Random assignment of functional group richness predicted ecosystem functioning at least as well as assignment from trait-based dendrograms. This was consistent with the findings of other studies that have found that trait-based functional diversity performs often no better than random or species richness (Cadotte et al. 2011). This is unsurprising for coarse measures of functional processes such as cellular

activity (ATP) or respiration (CO₂) production since they are unlikely to reflect changes in bacterial populations brought about by differences in trait functional diversity of protist predator (Frossard et al. 2012). However, it would have been reasonable to expect trait-based functional group richness to have a discernible effect in terms of measures of biogeochemical processes such as activities of enzymatic pathways since they are more likely to be linked to bacterial populations (Langenheder et al. 2006; Frossard et al. 2012).

Traits such as size are known to affect the predation modes and community level effects of protists on bacterial populations (Posch et al. 2001; Corno & Jurgens 2006; Petchey et al. 2008; Glucksman et al. 2010). However, a lack of correlation between protist functional diversity and functioning would seem to suggest three possibilities; firstly that bacterial diversity is so great that while predation may have structuring effects it is not strong enough to impact functional processes and this is reflected in effects of related functional trait diversity; secondly the traits chosen may be functionally irrelevant (particularly if they are not directly related to predation); thirdly, community structure is not related to function (Burke et al. 2011; Frossard et al. 2012). Alternatively it may be that because FGR and species richness are correlated and explanatory power overlaps, then FGR based on traits is no more predictive than groups based on random groups (Hector 2002; Naeem 2002; Petchey et al. 2004).

In contrast to species richness and trait-based assignment of functional diversity, FGR assigned by the response ratio interactions-based method predicted decelerating relationships between species richness and functioning for all measures. In fine scale measures of enzymatic activity, I found a trend of decreasing functioning with increasing FGR compared to random and trait-based assignment methods. This seems contradictory to established expectations of diversity-functioning relationships but can be reconciled when considering the biological relevance of interactions-based functional group richness.

Functional groups formed from response ratios represent groups of protists that have similar pairwise response ratios with other species in the pool. In the absence of functional traits, the

functional impacts of predators can be understood to be their structuring effects through predation on lower trophic levels of a community (Duffy 2002). Changes in community attributes, including those of microbial communities, have been shown to affect ecosystem processes (Bell et al. 2005; Bell et al. 2009b; Langenheder et al. 2010; Gessner et al. 2010; Kurle & Cardinale 2011). Protistan predators are known to have strong effects on community structure (Posch et al. 2001; Jurgens & Matz 2002; Glucksman et al. 2010; Saleem et al. 2012) and it is therefore reasonable to view functional differences in predatory protists in terms of their community structuring effects that impact functional processes.

Response ratios reflected the species' interactive effects on ecosystem processes by quantifying the interaction strength or degree of over-or under-yielding (Goldberg et al. 1999; Weigelt & Jolliffe 2003; Fiegna et al. 2015). Functional groups based on response ratios grouped species with similar interactive effects on contributions to functional processes. It would be expected for these species to be impacting similar prey and therefore be less complementary. However, if negative complementarity exists, then species in a group could also be more likely to have more positive (or less negative) interactions under certain conditions (Eisenhauer 2012; Becker et al. 2012). Such relationships are expected when there is redundancy between species or maximum functioning is achieved by the presence of only a few (Bell et al. 2009b). They might also have opposing impacts on the bacterial community, either directly through predation or indirectly through the release and cycling of nutrients that favour competing bacterial species (Saleem and Moe 2014). This relationship might only be predicted by functional diversity based on interactions because this measure of diversity would account for interactions between traits and species (Verberk et al. 2013). Such interactions may affect their relevance to ecosystem functioning depending on the other functional traits present, meaning that the functional effects of species may depend on those species that they interact with (Duffy 2002; Hooper et al. 2005; Verberk et al. 2013). Therefore species richness and functional trait diversity might continue to predict positive relationships based on positive complementarity and simultaneous actions of

single traits, while functional diversity based on interactions of species and their traits could reveal different relationships (Verberk et al. 2013).

Limitations and improvements

There are a number of minor limitations to our method. The complete linkage clustering method is as arbitrary as any other, for example average linkage or minimum linkage and prone to the same problems of distortion of trait space (Mouchet et al. 2008). Also, the Euclidean distance metric is equally arbitrary. The method of combining trees may also require some improvement. The averaging of distances across functional measures is a simple and effective approach, especially as the use of response ratios doesn't require weighting of traits. However, depending on how the method is adapted or developed it may require more sophisticated approaches.

In some ways the dendrograms do not offer much more than existing approaches. While I believe that the method provides improvements with regard to microorganisms, the dendrogram that is the end product is not necessarily in itself more informative, though being familiar it allows measures such as FD, FGR, FD_{var} etc. to still be applied. Response ratios and measures of functional diversity based on their pairwise distances and hierarchical cluster dendrograms could be combined with abundance data in order to capture functional richness, evenness and divergence (Mouchet et al. 2010). This would address the fact that functional diversity should ideally take into account the distribution of species across niche space which is dictated by the resources available, intraspecific genotypic variation, phenotypic plasticity, and character shifts (Kark et al. 2002; Day & Young 2004; Mason et al. 2005; Mouillot et al. 2013a). There are a number of small additional advantages to using response ratios, including the fact that no weighting is required for multivariate methods.

Beyond the dendrogram

The plot in figure D of Box 1 possibly provides a useful way of visualising the functional organisation of communities. It could provide a ‘functional fingerprint’ of the community as a whole and instead of calculating various measures of functional diversity *post hoc*, this ‘functional fingerprint’ could be used to quantify the amount of functional diversity in the community and the way that it is distributed. The distance between the $y = -x$ line of functional unity and the point on the curve that is furthest from this line might be used as a measure of community functional diversity. The shape of the curve provides information about how that diversity is distributed and the exponent of the curve might be used as a metric. The line of functional unity itself provides a potentially useful null model to test against. However, both of these suggestions remain just that and require further investigation.

Chapter 3 – Manipulations of interactions-based functional diversity reveals context dependence

Abstract

The saturating relationship between species richness and ecosystem functioning is well studied and investigations have progressed to trying to resolve this further in terms of other metrics. Most prominent has been the use of trait-based functional diversity. A central tenet of functional ecology has been that as trait-based functional diversity increases, so does ecosystem functioning. My method based on interactions of species' contributions to functioning is fundamentally different to trait-based functional diversity. I tested the behaviour of the relationship between commonly used measures of functional diversity applied to my interactions-based method and ecosystem functioning. I used expected patterns from trait-based relationships as a null hypothesis. I also tested whether this measure of functional diversity reflects greater context dependence, in particular with regards to 'composition effects'. The results allowed me to make inferences regarding the nature of interactions-based protistan functional diversity, what it represents, and the relative predictive importance of continuous and discontinuous measures. More importantly, the results highlighted the lack of emphasis placed on interactions and context dependence in trait-based functional diversity while at the same time supporting the importance placed on complementarity as a fundamental mechanism for biodiversity-ecosystem functioning relationships.

Introduction

Since biodiversity has been recognised to comprise multiple components, functional diversity has come to the fore in biodiversity-ecosystem functioning (BEF) research (Mouillot et al. 2011). In addition, a trait-based approach to determining causal mechanisms linking biodiversity and ecosystem functioning has driven some ecologists to treat functional diversity as more informative than species richness (McGill et al. 2006; Cadotte et al. 2011). Functional diversity has been broadly defined as “the value and range of those species and organismal traits that influence ecosystem functioning” (Tilman 2001) though is often regarded as the diversity of species niches and functions (Petchey et al. 2004; McGill et al. 2006; Villéger 2008). However, the concept has always been rather diffuse – even described as “slippery” (Diaz & Cabido 2001) – due to the variety of metrics and approaches to its quantification (Petchey & Gaston 2006). Definitions of functional traits therefore also tend to be quite broad, relating to well-defined and measurable properties that link performance and contribution to one or several ecosystem functions (Violle et al. 2007; Krause et al. 2014). As a result, a wide range of physiological, morphological, or genetic properties that affect fitness of organisms and function can be regarded as functional traits (Violle et al. 2007; Krause et al. 2014).

A wide range of measures have been proposed including but not limited to weighted trait variance (FD_{var}), distances in trait space (FAD), sum of branch lengths on cluster dendrograms (FD) and the number of functional groups in a community (FGR) (Mason et al. 2003; Walker et al. 1999; Petchey & Gaston 2002b). In addition, functional diversity can be decomposed into functional richness, evenness and divergence (Mouillot et al. 2005; Mason et al. 2005). These are defined by Mason et al (2005) as; a) the amount of niche space filled by species in the community (functional richness), b) the evenness of abundance distribution in filled niches space (functional evenness), and c) the degree to which abundance distribution in niche space maximises divergence in functional characteristics within the community (functional divergence). The justification for all of these measures is based on the assumption that they

provide a representation of niche differences because the range of trait values is likely to be more informative and provide greater explanatory power in BEF experiments than species diversity measures such as richness and evenness (Cadotte et al. 2011). However, there remains a lack of consensus as to which method is the best.

The trait-based approach to functional diversity seems intuitive in linking biodiversity to ecosystem functioning. However, regardless of exactly which functional diversity metric provides the most insightful approach to BEF relationships, all of these measures of functional diversity suffer from a number of intrinsic problems derived from the use of traits. The *a priori* selection of traits requires traits to be identifiable and measurable. This is not always possible - for instance in microorganisms -though technological advances are making this increasingly possible (Krause et al. 2014). These include advances in microscopy, flow cytometry, nutrient use profiles, spectroscopy and fluorescence visualisation techniques (Edwards et al. 2012; Behrens et al. 2012; Garcia et al. 2012; Haagensen et al. 2011; Watrous et al. 2013; Krause et al. 2014). Traits have to be hypothesised to be functionally relevant and when considering multiple traits some degree of weighting has to be applied (Petchey & Gaston 2006). However, while there has been a push for greater integration of functional traits into microbial BEF studies, such problems have resulted in many more microbial studies examining functional diversity through experiments involving environmental gradients or treatments, rather than by direct manipulation (Krause et al. 2014). In addition, approaches that produce dendrograms apply different clustering methods (such as single or complete linkage) that produce different clustering patterns (Petchey & Gaston 2006; Mouchet et al. 2008; Poos et al. 2009). Once clustered, different arbitrary methods (Euclidean, Gower, etc.) to define groups are applied (Petchey & Gaston 2006; Mouchet et al. 2008; Poos et al. 2009). Discontinuous methods of classification face the issue of at what scale of traits should classification be applied (Petchey & Gaston 2006).

In cases where functionally relevant traits are not easily identifiable, it has been suggested that phylogenetic diversity may encompass continuous trait variation along a phylogeny and that

therefore phylogenetic diversity can operate as well, if not better, than functional diversity in predicting ecosystem functioning (Flynn et al. 2011; Srivastava et al. 2012; Venail & Vives 2013). However, while ‘trait-free’ approaches have concentrated primarily on phylogenetic diversity (Flynn et al. 2011), there are reasons why it is unlikely to be more useful than functional diversity. Large functional differences may not always reflect phylogeny or vary continuously along it. The fact that trait and genetic diversity varies greatly within species populations reduces the usefulness of phylogenetic diversity and this is of particular consideration in microbial systems with high rates of horizontal gene transfer (Polz et al. 2013). For instance, variations in functional traits were found not to correlate with 16S rRNA-based phylogenies of denitrifying bacteria (Jones et al. 2011; Salles et al. 2012). In addition, directional selection may quickly eliminate phylogenetic effects. Without some way of incorporating phenotypic information phylogenetic diversity is likely to remain of limited usefulness. However, while there have been calls for these findings to lead to a greater shift towards trait based ecology (Messier et al. 2010; Cadotte et al. 2011; Krause et al. 2014), this might be viewed as unnecessarily complicating.

In light of these problems with trait-based functional diversity and phylogenetic diversity, I sought in Chapter 2 to develop an alternative approach to classifying functional relationships between organisms, in particular a group of heterotrophic biflagellate protists (see Chapter 2). My method circumvented the need for *a priori* selected traits and classified functional relationships between protists according to their pairwise interactions, quantified as “response ratios”. These were obtained using direct measures of ecosystem functioning and the subsequent classification of the organisms reflected similarities in interactions with other species. Dendrograms no longer represented diversity of traits, but diversity of interactions and contributions to rates of functional processes. Functional groups of predators represent those that have similar interactions with others.

A central tenet of functional ecology is that increasing functional diversity increases functioning due greater resource use partitioning and efficiency in heterogeneous environments (Diaz and

Cabido 2001). Species that have complementary functional traits will occupy non-overlapping niches and that total occupied niche space increases with increasing species richness (Loreau 1998a; Loreau & Hector 2001). Studies over the last two decades have - using trait-based functional diversity - tended to support this hypothesis, though consensus has been lacking and the relationship viewed as somewhat unresolved (Naeem 2002; Cadotte et al. 2011). However, what functional diversity represents when estimated using species interactions and complementarity of contributions to ecosystem functioning is fundamentally different.

Heterotrophic protists have important impacts on bacterial communities which in turn underpin functional processes (Posch et al. 2001; Langerhans et al. 2005; Bell et al. 2005; Corno & Jurgens 2008; Jürgens & Massana 2008; Saleem et al. 2013). These functional differences are likely to be due to differences in feeding mode, prey selectivity, susceptibility to bacterial defences (Sherr & Sherr 2002; Corno & Jurgens 2008; Massana et al. 2009; Jousset 2011). Their niches are broadly defined by their available prey resources and impacts upon them (Tilman et al. 1997). Furthermore, the degree of niche overlap and strength of interactions between predators depends on prey community structure (Finke & Denno 2005; Snyder et al. 2006; Byrnes & Stachowicz 2009). Therefore the nature of the interactions between protists and their contributions to functioning is in part determined by the complementarity of their effects on the prey community. Functional diversity estimated using this method, group's together species that have similar interactions. We would expect similar interactions to result from similar trophic interactions and be less complementary (Duffy 2002). Alternatively, species from different groups might have contrasting effects on community structure and function that promote negative interactions between bacterial populations and reduce functioning.

This rationale led me to make two testable hypotheses. Firstly, that unlike trait-based functional diversity increasing functional diversity based on species interactions may not predict increasing ecosystem functioning. Secondly, functional diversity based on species interactions in the presence of one bacterial prey community will not predict the same relationship with

functioning in another. Using a microcosm approach, I tested the first hypothesis by manipulating two measures of functional diversity – functional group richness and mean pairwise dissimilarity – based on the combined functional dendrogram in Chapter 2 (figure 7, panel B) and examining the relationship with seven measures of ecosystem functioning and an index of multifunctioning. To test the second hypothesis I repeated the experiment across four bacterial community treatments and again compared the diversity-functioning relationships.

Methods

Bacterial community treatments

Four bacterial background communities were used as experimental treatments to test the effect of variation in surrounding community composition on response ratio based relationships between protist predators i.e. complementarity of contributions to community functional output. These bacterial communities were sampled from tree holes in Wytham Woods (Oxfordshire, UK) and Silwood Park (Berkshire, UK), Burnham Beeches (Berkshire, UK), and the Ashridge Estate (Bedfordshire, UK). These are referred to as Wyt116, T143, BB88 and AE155 respectively, in this chapter. Composition and abundance data was previously obtained by next generation sequencing. Prior to use they were stored as frozen stocks at -80°C. Culture for inoculation into experimental microcosms was grown in falcon tubes of beech leaf tea (BLT). BLT was inoculated with 15µl frozen stock and left to grow for two days before inoculation of 150µl into each experimental microcosm. BLT was brewed as described in Chapter 2.

Protist strain growth

I grew protist strains in order to construct communities with manipulated levels of functional diversity and species richness. Eighteen protist strains (see figure 10) were grown as monocultures from pure stock cultures prior to the assembly of the experiment. These were grown for 7 days in 2ml of beech leaf tea (BLT) in 24 well culture plates pre-inoculated with T143 bacterial tree-hole community to check that they would produce active cells. To ensure that strains were at the same growth phase at inoculation into experimental microcosms, they were categorised into four broad categories based on estimated growth rates in Chapter 2; slow, medium, fast, and very fast. Slow strains were grown for six days before inoculation to experimental microcosms, medium five days, fast four days, and very fast three days beforehand.

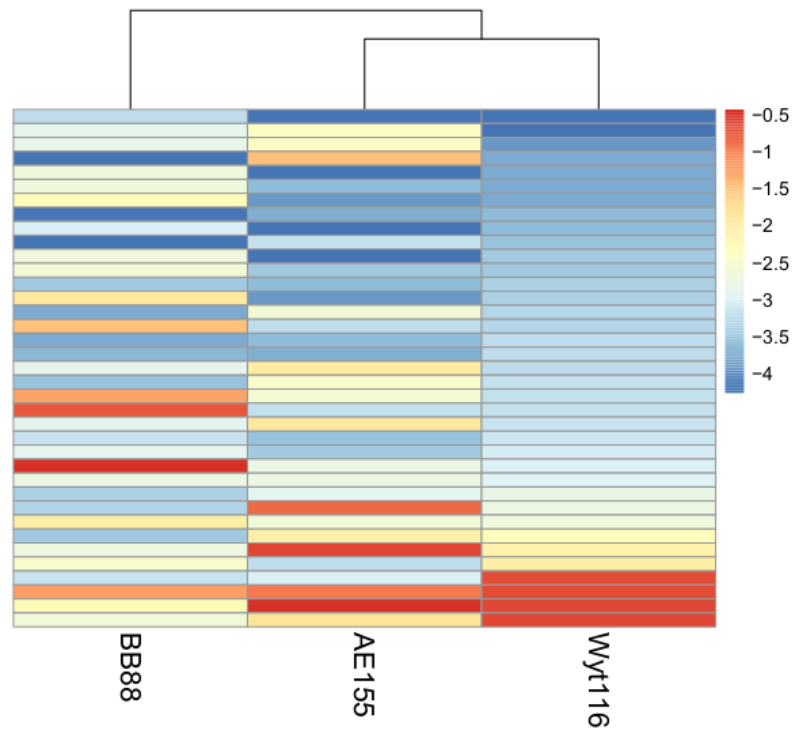


Figure 9. Heat map showing the relative abundances of bacterial strains in communities BB88, AE155, and Wyt116. Relative abundances have been $\log_{10}$ transformed. Only genera occurring in all three communities are shown. Rows are genera but these are unlabelled for display. The heat map is clustered by bacterial community. Relative abundances are given in the key on the right. This figure is to illustrate the differences in community composition. These communities were sequenced using Illumina MiSeq. T143 is not presented with them as Roche 454 was used and compositions shown are not comparable.

Random partitions design (RPD) and protist community assembly

Protist communities were assembled according to the random partitions design for community and biodiversity-ecosystem functioning experiments designed by Bell et al (2009a). The random partitions design of the experiment was generated using R v3.0.3 (R Core Team 2014). This design allows for large scale diversity experiments without the need for all possible combinations of constituent species at all levels of diversity. Random selection of species is suitable, but the RPD design has additional desirable properties. Notably, the RPD samples the species pool without replacement at each level of diversity, which results in species being selected the same number of times at each level of diversity and across the experiment as a whole. Furthermore, experiments can then be analysed through linear modelling approaches without the need for unmanageably large experiments. The random partitions design of the experiment was generated using R v3.0.3 (R Core Team 2014). The experiment consisted of two random partitions of 1, 2, 3, 6, 9 and 18 strains, each replicated twice. These were then replicated again four times, once for each of the four bacterial background communities used in the experiment. For the purpose of this experiment, communities of greater than two initial strains were then discarded from the design of the experiment.

Experimental communities were assembled by pipetting transfer from 96 well deep well plates to 96 well shallow well plates by Hamilton (Starlet) pipetting robot. I used a substitutive design for the inoculation of protists, with monocultures containing 180µl of isolate, two species communities containing 90µl of each isolate, three species communities 60µl, and so forth. 20µl of BLT was then also added. Once assembled in shallow 96 well plates, the mixtures were transferred to experimental microcosms containing 1800µl of BLT and bacterial culture. The microcosm communities were then left to grow for 15 days at 17°C.

Protist functional diversity manipulations

For each of the monocultures and two-species communities determined following the RPD design of the experiment, another two protist communities were included into the design. One

of these would be the original monoculture or two species community plus an extra species from within the same functional group as the monoculture or one of the two other species, or from a different functional group to the monoculture or either of the two other species. These treatments were termed ‘within’ and ‘between’ additions. Due to the two extra communities for addition manipulations per RPD community, the experiment had a total size of 1,248 microcosms. These manipulations increased community species richness while either maintaining functional group richness (FGR) or increasing it by one unit. There were three levels of FGR: FGR1, FGR2, and FGR3. Functional groups were determined in Chapter 2 using the response ratio-based cluster dendrogram combining response ratios of protists contributing to seven ecosystem functions (see figure 7). Protist communities used in the experiment to do this were growing on bacterial community T143 (see Chapter 2, pages 37 and 39).

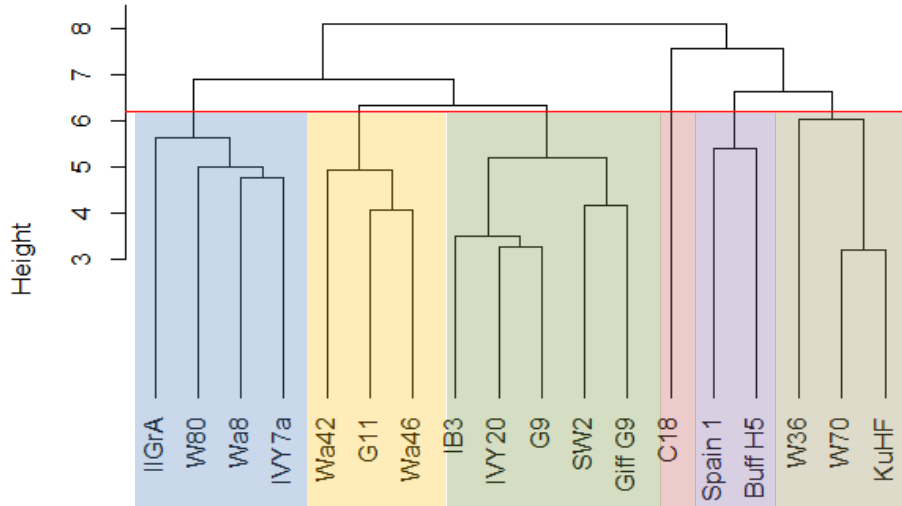


Figure 10. Dendrogram of relationships between protist strains based on hierarchical clustering of distances between response ratios of ecosystem functioning associated to each strain, combined across the 7 ecosystem function measures (MUG, MUP, MUN and Xylo enzymatic activity, as well as CO₂ and ATP production, and microcosm phosphate concentration). Coloured boxes show functional groups

I calculated community mean pairwise dissimilarity (MPD) of response ratios as a continuous measure of community functional diversity, representing the average distance in species interactions. I did this by calculating the average distance between pairs of species that composed communities:

$$MPD = \frac{\sum_{i=1}^n d_i}{n}$$

Where d is the pairwise distance between two species and n is the number of pairwise distances in the community. This is the same metric as Heemsbergen et al's (2004) 'mean dissimilarity' for trait-based functional diversity. Distances were obtained from the distance matrix of distances between protist response ratios. Manipulated increases in mean pairwise dissimilarity relative to the initial monoculture or two species communities were also calculated.

Ecosystem functioning bioassays

Again, I used the same 7 bioassays as previously described in Chapter 2 (pages 40 and 41). These included four measures of enzymatic activity, respiration (CO₂ production), cellular activity (ATP concentration) and phosphate concentration. They were performed after 15 days from inoculation of the experimental microcosms. Assays were again carried out on days 15, 16, and 17 of the experiment, and carried out in 96 well shallow well plates. Subsampling of the experimental microcosms was carried out by Hamilton (Starlet) pipetting robot after manual transfer from 24 well plates to deep well 96 well plates. The measures were then standardised using calibration curves (see Appendix 2, supplementary figure 3)

These assays are useful since they represent two groups of assays that detect either coarse or fine grained measures of functioning. Broad ecosystem processes such as respiration, productivity, and cellular activity integrate across many processes and may be too coarse to reveal patterns of functional diversity and ecosystem functioning (Frossard et al. 2012). Such measures have been argued to falsely report functional redundancy in microbial communities (Peter et al. 2011). Measures of finer scale biogeochemical processes such as enzymatic activity

assays are thought to be more tightly linked to bacterial populations (Langenheder et al. 2006; Peter et al. 2011). These may provide an interesting contrast in revealing diversity-functioning patterns of heterotrophic protists that feed upon them.

I also used the bioassay results to calculate an index of combined multiple functioning. I did this by standardising assay results to have $\bar{x} = 0$ and $\sigma = 1$ (z-score). I then averaged across measures to obtain an index of average multiple functioning (mean z-score).

Statistical analyses

Statistical analyses were carried out using the statistical package R v.3.0.3 (R Development Core Team 2014). Analyses involved generalised linear modelling (GLM) to model ecosystem functioning in response to protist community mean pairwise dissimilarity (MPD), protist functional group richness and bacterial community. The effect of increasing or maintaining functional diversity while increasing species richness on ecosystem functioning was also modelled by GLM. Models included difference in functioning relative to unmanipulated communities (where increase in both species richness and functional diversity was zero) as the response variable. The explanatory variables included increase in protist community mean pairwise dissimilarity relative to unmanipulated communities, bacterial community, initial mean pairwise dissimilarity (of unmanipulated communities), and functional group richness manipulation. A full list of maximal models can be found in Appendix 3, supplementary table 1.

Models treated data with Gaussian errors and identity link function. Ecosystem functioning data was log transformed to increase variance stability and normalise residuals where appropriate (all enzymatic activity data except for MUN). Cellular activity (ATP) data was +1 translated before log transformation. Species richness was included as a covariate. Model simplification was carried out manually through removal of least significant complex terms (Crawley 2007). Model comparison was carried out by ANOVA. Post hoc contrasts were carried out by Tukey HSD using the 'glht' function from the R package 'multcomp' (Bretz et al. 2015). All GLM models are given in Appendix 3, supplementary table 1.

Results

Functioning of bacterial communities

There were significant differences in ecosystem functioning between background bacterial communities across all measures (see figure 11). Post hoc tests by Tukey HSD found that there were significant contrasts between all comparisons of bacterial community phosphate concentrations (Tukey HSD: $p < 0.001$). There were significant differences in cellular activity between BB88 (mean = 2.24 ± 0.1 nM ATP) and T143 (mean = 1.47 ± 0.07 nM ATP; Tukey HSD: $p = 0.001$), and between BB88 and Wyt116 (mean = 1.77 ± 0.1 nM). There were also significant differences in respiration rates between bacterial communities between BB88 (mean = 0.19 ± 0.003 $\mu\text{g CO}_2 \text{ ml}^{-1} \text{ d}^{-1}$) and AE155 (mean = 0.21 ± 0.002 $\mu\text{g CO}_2 \text{ ml}^{-1} \text{ d}^{-1}$; Tukey HSD: $p < 0.001$), AE155 and T143 (mean = 0.19 ± 0.003 $\mu\text{g CO}_2 \text{ ml}^{-1} \text{ d}^{-1}$; Tukey HSD: $p < 0.001$), as well as AE155 and Wyt116 (mean = 0.19 ± 0.002 $\mu\text{g CO}_2 \text{ ml}^{-1} \text{ d}^{-1}$; Tukey HSD: $p < 0.001$). There were significant differences in all contrasts of bacterial communities for MUP, MUN, and Xylo enzymatic activities (all Tukey HSD: $p < 0.001$). For MUG enzymatic activity there were significant differences in contrasts between Wyt116 (mean = 0.10 ± 0.003 nM) and AE155 (mean = 0.12 ± 0.002 nM; Tukey HSD: $p < 0.001$), Wyt116 and BB88 (mean = 0.13 ± 0.003 nM; Tukey HSD: $p < 0.001$), and Wyt116 and T143 (mean = 0.11 ± 0.002 nM; Tukey HSD: $p < 0.001$).

Continuous measure of functional diversity (MPD)

I modelled whether protist community mean pairwise dissimilarity (MPD) predicted ecosystem functioning measures and tested whether this relationship was affected by the bacterial community protists were feeding on. I found that increasing MPD predicts significantly increasing overall levels of cellular activity (ATP production) (GLM: estimate = 0.047, $t = 3.41$, $p < 0.001$), phosphate concentration (GLM: estimate = 0.00014, $t = 2.36$, $p = 0.019$), and the enzyme activities of MUP (GLM: $t = 2.63$, $p = 0.0086$), MUN (GLM: estimate 0.001, $t = 2.57$, $p = 0.01$) and Xylo (GLM: estimate = 0.020, $t = 4.26$, $p < 0.001$). On the other hand, increasing

MPD had a significant negative effect on respiration (CO₂ production) (GLM: estimate = -0.0020, $t = -2.72$, $p = 0.0067$).

There were significant interactions between MPD and bacterial community treatment for cellular activity, respiration, and MUP and MUG enzymatic activities. MUG (GLM: estimate = -0.027, $t = -1.96$, $p = 0.05$) and MUP (GLM: estimate = -0.028, $t = -3.64$, $p < 0.001$), as well as ATP production (GLM: estimate = -0.077, $t = -3.90$, $p < 0.01$), decreased with increasing MPD in the presence of bacterial background T143. This was also the case for MUP in the presence of bacterial background Wyt116 (GLM: estimate = -0.23, $t = -2.96$, $p < 0.001$). It should also be noted that though there were no significant interactive effects of MPD and bacterial community for Xylo and MUN enzymatic activities, in both cases enzymatic activity also decreased with increasing MPD under the T143 community treatment and effects were close to significant (see figure 9).

I found that there was a positive significant effect of MPD on the multifunctioning index (GLM: estimate = 0.019, $t = 2.21$, $p = 0.027$), as well as significant differences in overall ecosystem functioning between background bacterial communities (Tukey HSD: $p < 0.001$ for all contrasts; see figure 9). The effect of MPD was reversed in the significant interaction with bacterial community T143 (GLM: estimate = -0.031, $t = -2.53$, $p = 0.011$)

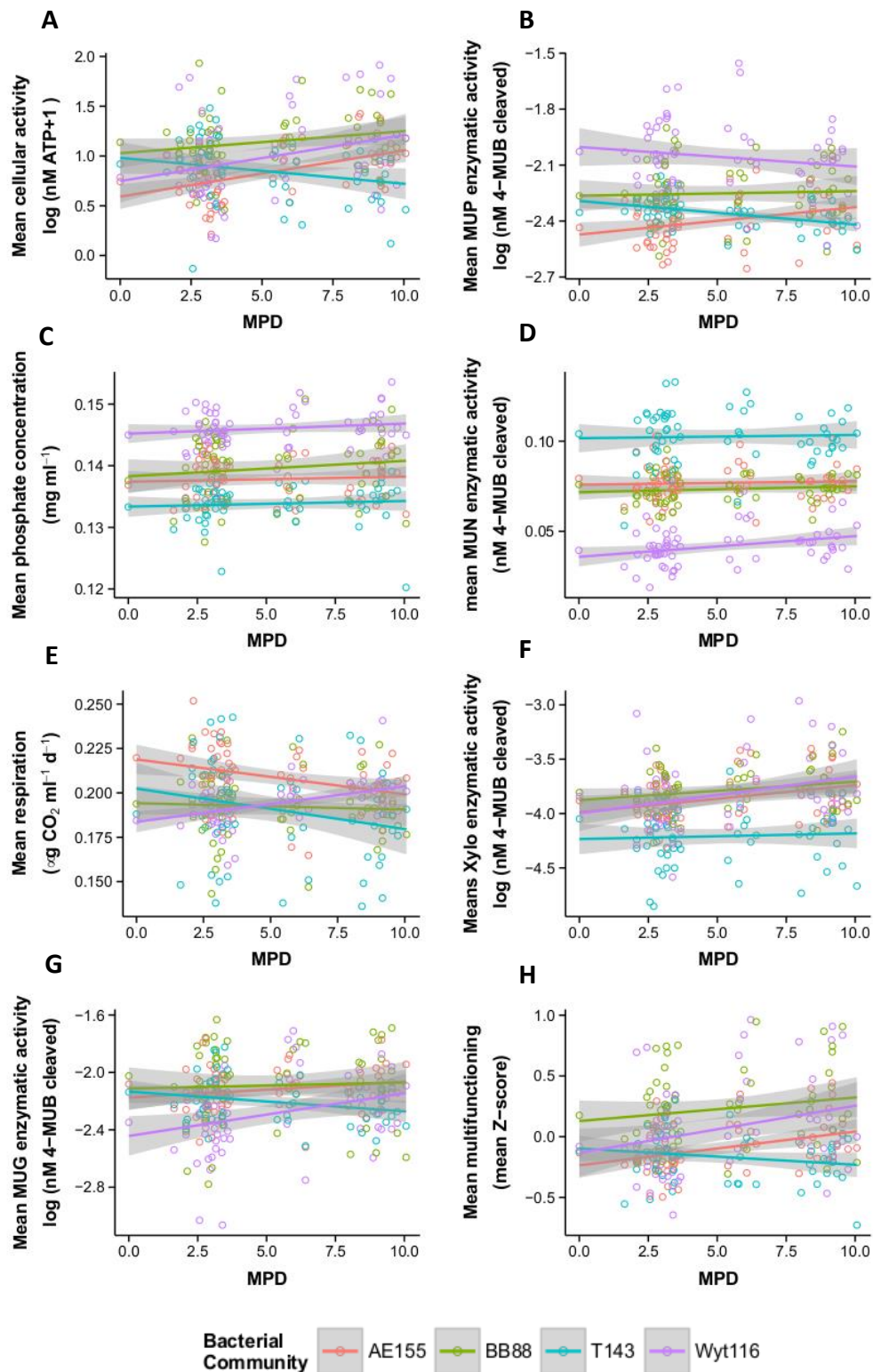


Figure 11. Scatter plot of mean ecosystem functioning measures and multifunctioning index against protist community mean pairwise dissimilarity of response ratios (MPD). Colours represent the four bacterial community treatments. Regression lines are fitted by GLM. Panels are; **A)** Cellular activity, **B)** MUP enzymatic activity, **C)** phosphate concentration, **D)** MUN enzymatic activity, **E)** respiration, **F)** Xylo enzymatic activity, **G)** MUG enzymatic activity, **H)** multifunctioning metric.

Discrete measure of functional diversity (FGR)

In order to test differences in predictions by continuous and discrete measures of functioning, I modelled whether protist community functional group richness predicted ecosystem functioning. I also whether this relationship was affected by the bacterial community protists were feeding on.

Protist community functional group richness of two (FGR2) had significant positive effects on MUG enzymatic activity compared to a functional group richness of one (FGR1) (GLM: estimate = 0.30, $t = 3.82$, $p < 0.001$). There were also significantly lower rates of MUN enzymatic activity under the FGR2 treatment (GLM: estimate = -0.04, $t = -2.74$, $p = 0.006$) and Xylo (GLM: estimate = 0.29, $t = 1.84$, $p = 0.05$), with activity increased again under the FGR3 treatment (see figure 12)

There was a negative interaction between FGR3 and bacterial community for MUP enzymatic activity (FGR*BB88 GLM: estimate = -0.30, $t = -3.11$, $p = 0.020$). The interaction was also found to be significant for MUG enzymatic activity (FGR*BB88 GLM: estimate = -0.25, $t = -2.13$, $p = 0.03$; FGR*Wyt116 GLM: estimate = -0.25, $t = -2.08$, $p = 0.04$).

Interaction of discrete and continuous functional measures

Functional group richness (FGR) had significant negative interactive effects with mean pairwise dissimilarity on MUG enzymatic activity, (FGR2*MPD GLM: estimate = -0.08, $t = -5.70$, $p < 0.001$). This interaction was also significant for MUN enzymatic activity (FGR2*MPD GLM: estimate = -0.0033, $t = -4.11$, $p < 0.001$) and Xylo enzymatic activity (FGR2*MPD GLM: estimate = -0.10, $t = -2.91$, $p = 0.0036$). In all cases the positive effect of increasing MPD is most pronounced in FGR1 treatment microcosm communities (see figure 13). In addition, phosphate concentration was less under bacterial community T143 treatments with protist FGR3 and increasing MPD (GLM: estimate = -0.0049, $t = -2.28$, $p = 0.023$).

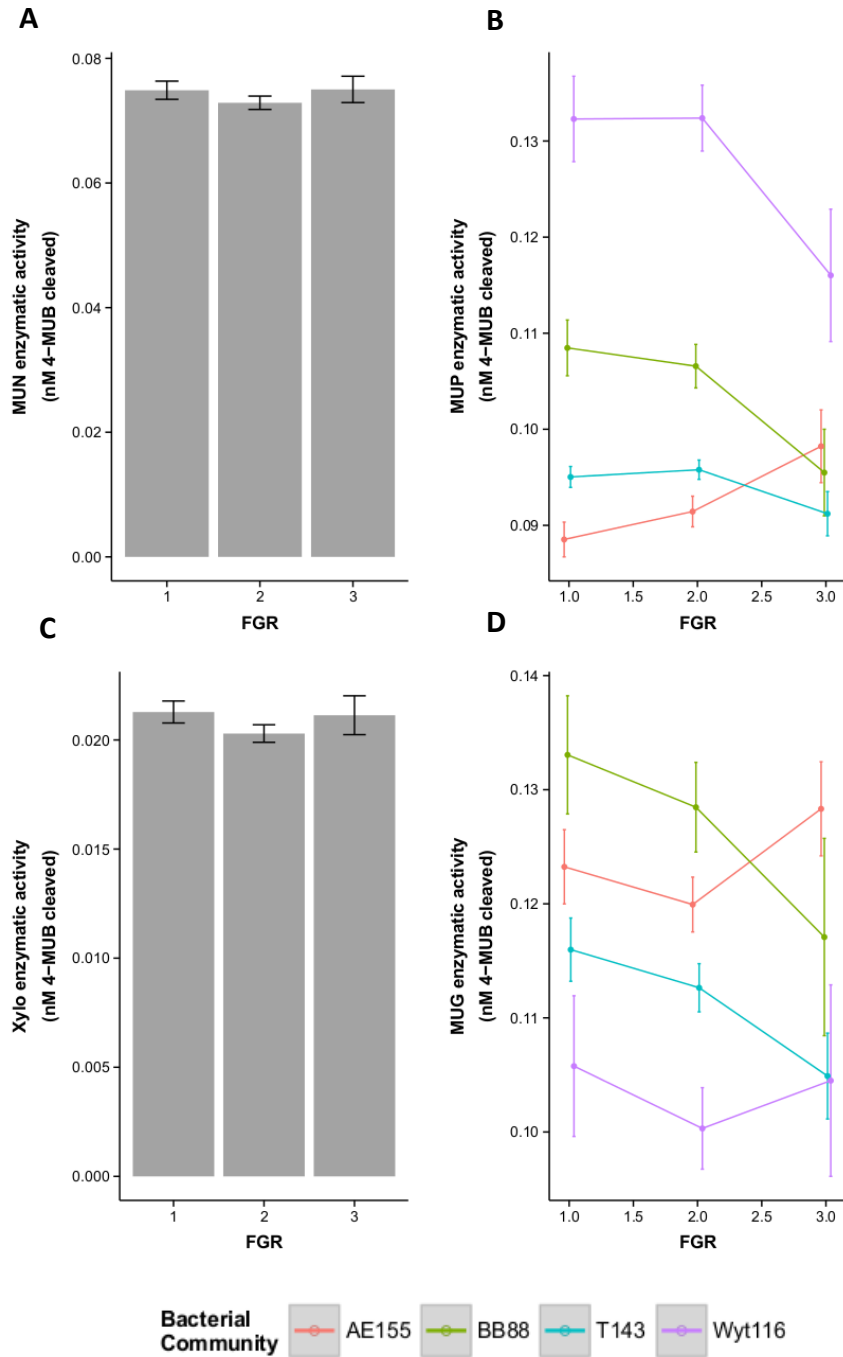


Figure 12. Bar plots of **A)** mean MUN and **C)** Xylo enzymatic activities at three levels of functional group richness (FGR). Error bars are standard errors. Line graphs show means and standard errors of **B)** MUP and **D)** MUG enzymatic activities, coloured by bacterial community.

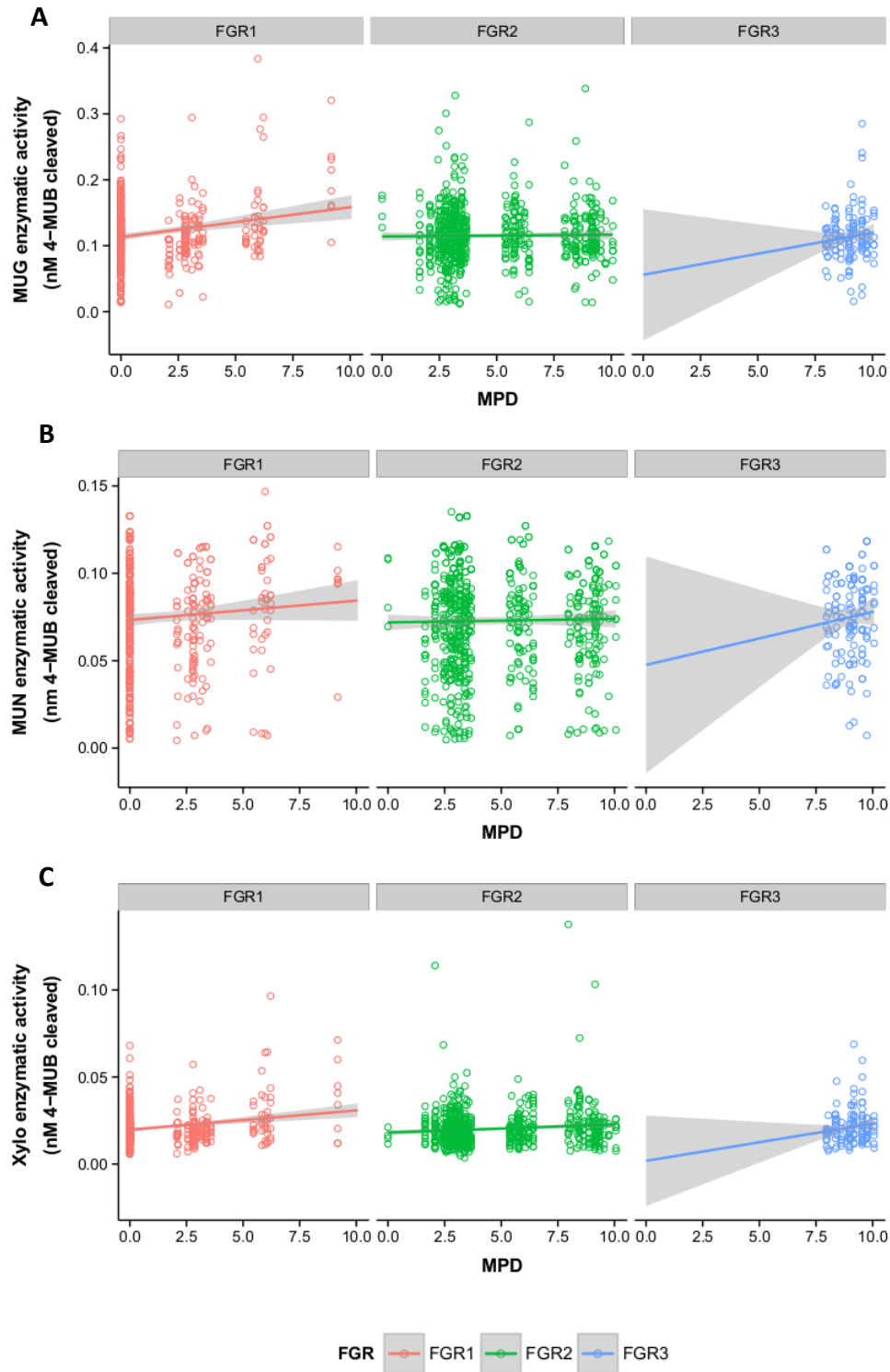


Figure 13. Scatterplots of **A)** MUG, **B)** MUN and **C)** Xylo enzymatic activities against protist community mean pairwise dissimilarity (MPD). Panels are faceted by protist community functional group richness (FGR). Regression lines are fitted by GLM and shaded areas represent the standard error.

Manipulated increases of continuous functional diversity

I modelled the effect of manipulated increases in protist community MPD and initial (prior to protist addition) community mean RR pairwise distance on differences in ecosystem functioning relative to unmanipulated protist communities. Increases in MPD led to significantly smaller or more negative differences in MUG (GLM: estimate = -0.022, $t = -4.22$, $p < 0.001$), MUN (GLM: estimate = -0.015, $t = -5.88$, $p < 0.001$), and Xylo (GLM: estimate = -0.004, $t = -2.19$, $p = 0.029$) enzyme activities, respiration (GLM: estimate = -0.027, $t = -2.15$, $p = 0.032$), and multifunctioning (GLM: estimate = -0.17, $t = -2.88$, $p = 0.004$).

Higher initial community MPD predicted smaller differences in MUN enzymatic activity (GLM: estimate = -0.005, $t = -2.58$, $p = 0.01$). Increases in MPD predicted more positive differences in enzymatic activity when the initial MPD of the community was high (GLM: estimate = 0.0018, $t = 3.59$, $p < 0.001$) and when protists were feeding on bacterial community T143 (GLM: estimate = 0.02, $t = 2.61$, $p = 0.009$; see figure 14) or BB88 (GLM: estimate = 0.015, $t = 2.23$, $p = 0.03$; see figure 14).

For respiration, greater increases in community MPD led to greater differences in CO₂ production relative to unmanipulated protist communities in those with protists feeding on T143 (GLM: estimate = 0.0057, $t = 2.17$, $p = 0.031$) and BB88 (GLM: estimate = 0.047, $t = 2.78$, $p = 0.006$) bacterial assemblages. However, this effect was reversed in a three way interaction with initial community MPD, such that as initial MPD increased, then predicted differences in CO₂ production were smaller or more negative (GLM: estimate = -0.002, $t = -2.13$, $p = 0.033$). The interaction existed for MUG enzymatic activity also (GLM: estimate = -0.0028, $t = -2.47$, $p = 0.014$).

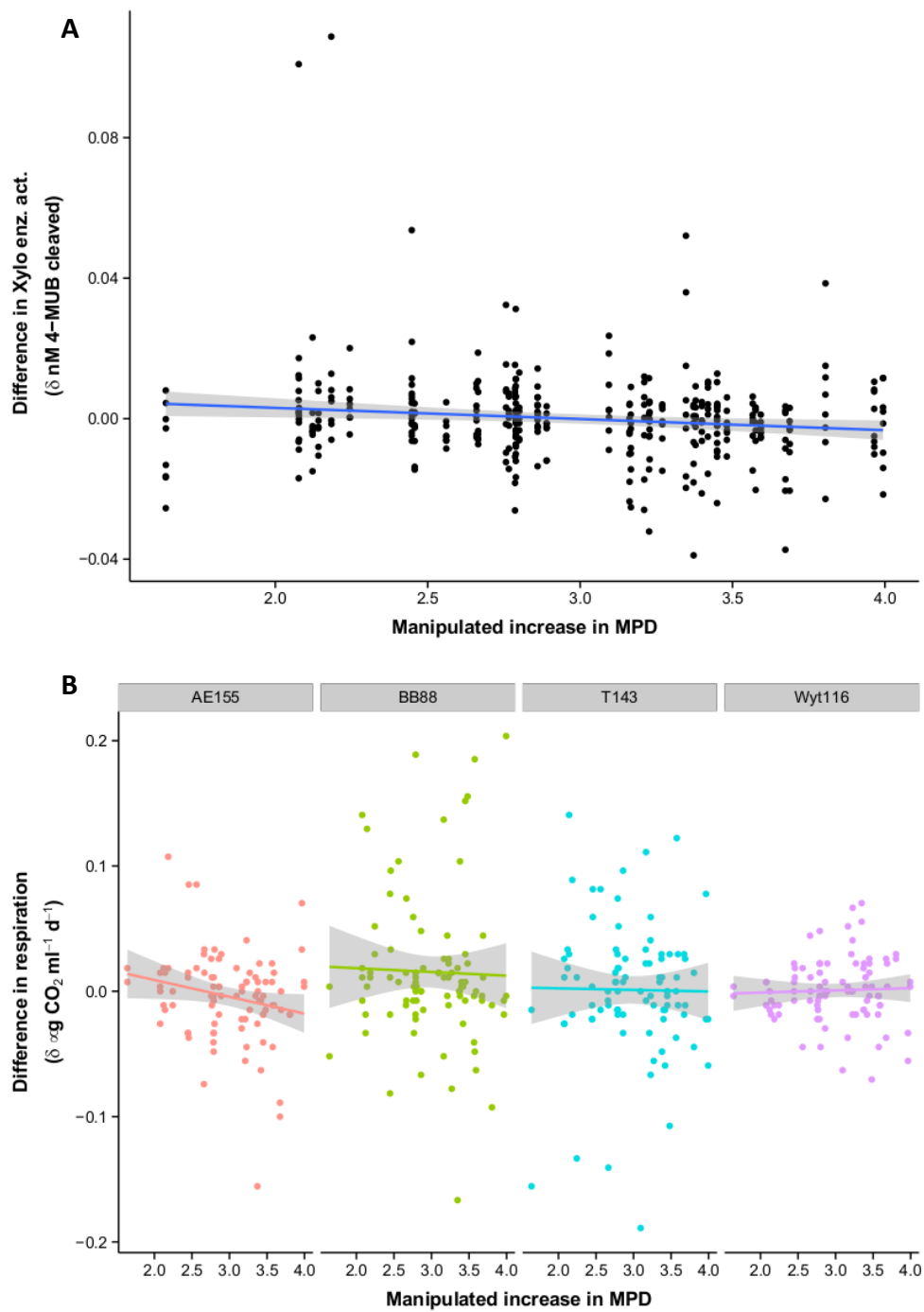


Figure 14. Scatterplots of difference in **A)** respiration (CO₂ production) and **B)** Xylo enzymatic activity relative to un manipulated communities, against manipulated increase in protist community mean pairwise dissimilarity (MPD). Panels of respiration plots are faceted by bacterial community. Regression lines are fitted by GLM and shaded areas represent the standard error. Panel B is faceted by bacterial community.

Manipulated increases of discrete functional diversity

I found no significant main or interactive effects of addition treatment on changes in any of the measures of functioning except for MUN and MUG enzymatic activities. For MUN enzymatic activity, addition of strains from previously represented functional groups ('within' treatment) led to greater (or less negative) differences in enzymatic activity (GLM: estimate = -0.074, $t = -4.45$, $p < 0.001$; see figure 15). Differences in functioning as a result of additions of protists from within already represented groups were also less negative when feeding on T143 (GLM: estimate = 0.095, $t = 2.91$, $p = 0.004$) or BB88 (GLM: estimate = 0.097, $t = 2.99$, $p = 0.003$). Differences in functioning as a result of additions of protists from within already represented groups were also less negative when increases in MPD were greater (GLM: estimate = 0.03, $t = 3.27$, $p = 0.001$).

Differences in MUG enzymatic activity were less negative with the addition of a species from an already represented functional group (GLM: estimate = -0.13, $t = -2.00$, $p = 0.046$; see figure 16). The change in MUG enzymatic activity resulting from the addition of a species from an already represented group was more positive the greater the increase in MPD (GLM: estimate = 0.045, $t = 2.00$, $p = 0.048$). Furthermore, if the addition of a species from an already represented group was in combination with bacterial community T143, then changes in functioning were also more positive (GLM: estimate = 0.19, $t = 2.25$, $p = 0.025$).

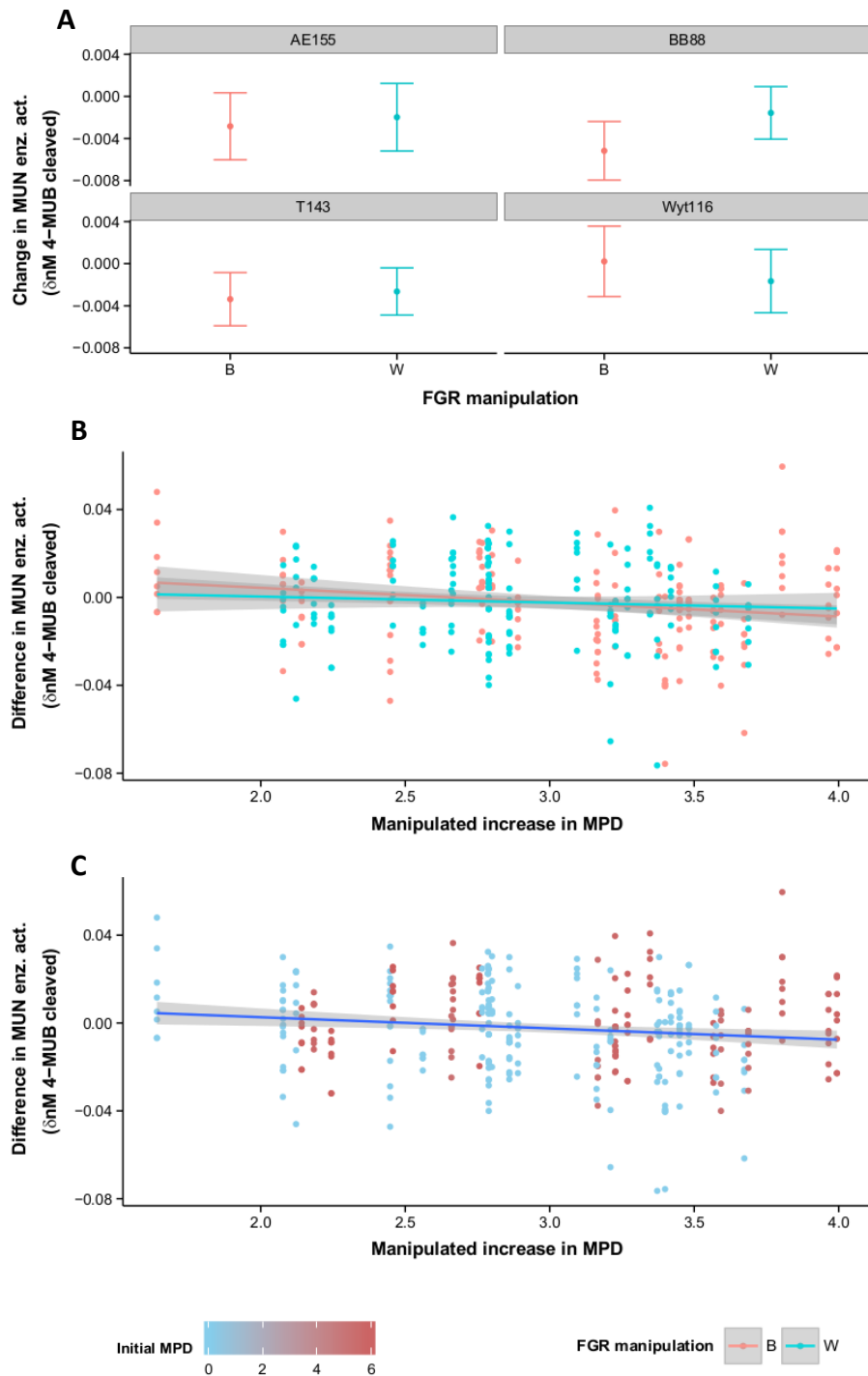


Figure 15. Plot **A**) shows mean and standard errors for the difference in MUN enzymatic activity relative to unmanipulated protist communities for manipulated communities. These had had functional group richness increased by 1 (B = ‘between’ group addition treatment) and those whose species richness had increased by 1 but functional group richness had not (W = ‘within’ group addition treatment). Plot **B**) shows a scatterplot and regressions of difference in MUN enzymatic activity relative to unmanipulated protist communities, against manipulated increases of protist community mean pairwise dissimilarity (MPD). Regression lines are fitted by GLM for the ‘within’ and ‘between’ treatments. Shaded areas represent the standard error. Plot **C**) shows the same data as B) but with points coloured by initial mean pairwise dissimilarity (i.e. MPD of unmanipulated communities mapped on to corresponding manipulated communities and themselves). The regression line is fitted by GLM for the main effect of MPD.

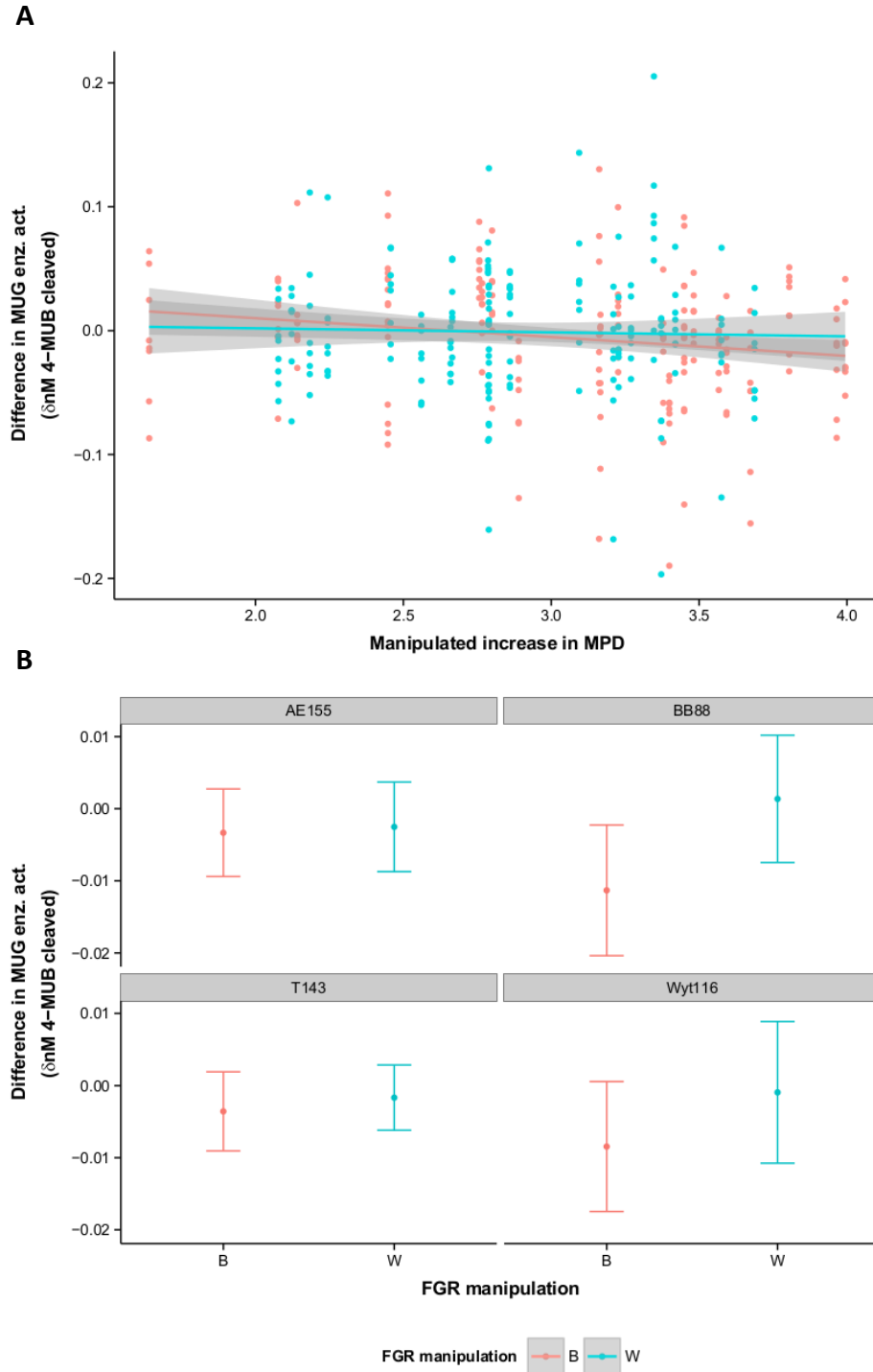


Figure 16. Plot **A**) shows the interaction between FGR manipulation type mean pairwise dissimilarity of protists. The difference in MUG enzymatic activity relative to unmanipulated protist communities, against manipulated increase in protist community mean pairwise dissimilarity is plotted. The regression lines are fitted by GLM and coloured by functional group richness (FGR) addition manipulation (‘within group’ or ‘between group’). Shaded areas represent standard errors. Plot **B**) shows mean and standard errors of difference in MUG enzymatic activity relative to unmanipulated communities against the type of FGR manipulation (‘between’ or ‘within’). Panels are organised by bacterial community.

Discussion

Main findings

My results supported the hypothesis that a non-positive relationship between interactions-based functional diversity and functioning would exist. This relationship was pronounced when protists fed on the bacterial community used to establish functional diversity measures. This supported my second hypothesis that the nature of the relationship is dependent on the bacterial community present. Furthermore, greater increases in measures of functional diversity led to more negative changes in functioning. However, this effect was less pronounced if increases in functional diversity occurred from within the same functional group. There was a tendency for finer measures of functioning such as enzymatic activities to be more closely related to community structure.

Contrast with previous findings

The results of this experiment provided interesting contrasts with the results of previous theoretical and empirical studies that have generally established a positive linear relationship between functional diversity and ecosystem functioning (Diaz & Cabido 2001; Petchey et al. 2006; Mouchet et al. 2010; Cadotte et al. 2011). This relationship is hypothesised to exist because increasing functional diversity increases complementarity of traits and resource use efficiency in heterogeneous environments (Loreau, 1998; Diaz & Cabido 2001; McGill et al. 2006; Griffin et al. 2009). Early biodiversity-ecosystem functioning research focused on manipulations of species richness (Naeem et al. 1994; Tilman & Downing 1994). However, the recognition of the role of functional diversity in explaining biodiversity-ecosystem functioning relationships has grown (Tilman et al. 1997; Petchey et al. 2004; Griffin et al. 2009; Mouchet et al. 2010). For instance, functional diversity metrics have been shown to be more powerful than species richness at explaining BEF relationships and can better explain over yielding of productivity (Petchey et al. 2004; Griffin et al. 2009). Analysis has also shown that certain measures of functional diversity have greater explanatory power than others (Petchey et al.

2004; Petchey et al. 2009). My results support the finding that functional group richness has less explanatory power than continuous measures or dendrogram based measures such as mean dissimilarity, FAD or FD (Walker et al. 1999; Petchey & Gaston 2002b; Petchey et al. 2004; Heemsbergen et al. 2004; Petchey et al. 2009).

My results are interesting for a number of reasons. There is an overall positive linear effect of functional diversity on ecosystem functioning as predicted by theory and in line with established results (Diaz & Cabido. 2001; Petchey et al. 2006; Mouchet et al. 2010; Cadotte et al. 2011; see figure 11). However, for most functions there was a negative linear relationship when protists were feeding on the T143 bacterial community which was also used in the experiments to determine the interactions between protists that underlie the functional diversity metric. This suggests that while functional diversity has tended to be viewed as a static property, there is a flexibility and context dependence which has been under emphasised in the literature (Duffy 2002). Furthermore, while theory predicts that greater increases in trait-based functional diversity result in increased complementarity, resource use efficiency and functioning, the results of this study show negative effects of measures of functional diversity. This suggests that novel aspects of the relationship between complementarity and ecosystem functioning may be revealed when using interactions-based functional diversity because it accounts not only for diversity of traits but also the interactions between species.

Context dependence

My results suggest that functional diversity measured using species interactions and contribution to functioning is not a static property. Relationships between MPD and functioning in microcosms that contained the bacterial prey community used to determine protist functional diversity (see Chapter 2 methods) were generally negative, but were positive in ones that did not. The implication is that findings from previous studies of the relationship between trait-based functional diversity and ecosystem functioning may not be completely generalizable. Results may depend not only on the traits selected, but also on species interactions which are affected by the surrounding community (Petchey & Gaston 2006; Petchey et al. 2009). Indeed

there is an assumption in trait-based approaches that trait-environment relationships are general and that single traits are consistent in the functional adaptation that they convey (Horrigan & Baird 2008; Verberk et al. 2013). Furthermore, traits and species may interact to change their relevance to ecosystem functioning, as well as have simultaneous independent impacts (Poff et al. 2010; Verberk et al. 2013).

Context dependence of functional diversity is predicted by theory and has been demonstrated in studies that vary abiotic conditions at a single site (Cardinale et al. 2000; Jonsson et al. 2001; Fridley 2002; Petchey et al. 2004). Petchey et al (2004) found that the diversity of nitrogen fixing ability in plants varied by geographical location and that functional group richness and species richness varied in their explanatory power between sites (Naeem 2002; Petchey et al. 2004). More recent evidence has also shown that habitat structure influences species contributions to ecosystem properties (Godbold et al. 2011).

Context dependency adds to the list of possible reasons for non-significant relationships between functional diversity and ecosystem functioning (Petchey & Gaston 2006).

Inappropriate choice of traits is one common explanation (Petchey et al. 2004), but the reverse of this is that traits thought to be generally relevant in one context may not be in another.

Changes in biodiversity-functioning relationships might occur without great changes in trait-based functional diversity or species richness. Furthermore, not accounting for context may cause problems for predictions and obscure causal mechanisms because the functional relevance of a trait is assumed to be equal across species (Verberk et al. 2013).

Mechanisms for context dependence

Functional diversity is hypothesised to connect species to ecosystem functioning through mechanisms such as complementarity (Tilman et al. 1997; Loreau 1998a,b). Complementarity depends on niche differentiation between species (Tilman et al 1997; Tilman 1999; Loreau & Hector 2001). Functional diversity of traits is assumed to reflect complementarity and is fundamental to determining species interactions and contributions to ecosystem processes

(Davies et al. 2007; Hillebrand et al. 2008; Mokany et al. 2008; Lavorel et al. 2011; Cadotte et al. 2011)

Traits do not exist in isolation. In food webs, functional traits manifest themselves as trophic interactions (Duffy 2002). In the context of predator-prey interactions, functional diversity can be defined in terms of the number of combinations of prey species that are significantly impacted by consumers (Duffy 2002). The net effect of a predator on prey is the sum of its direct feeding and indirect effects of intraguild predation and therefore depends on food web context (Schneider & Brose 2013). Interactions between species and their contributions to ecosystem functioning are a product of complementarity in terms of resource availability and use (Tilman et al. 1997; Loreau 2000). For predators, these resources are their prey and changes in communities of these affect niche overlap and interspecific interactions (Woodward & Hildrew 2002; Snyder et al. 2006; Byrnes & Stachowicz 2009). Therefore the functional diversity of predators is inherently context dependent and subject to ‘composition effects’ of the surrounding community (Schneider & Brose 2013). As a result, functional diversity measures in my experiment were only meaningful when protists fed on the T143 community.

Both theory and emerging empirical evidence support the hypothesis that protist predator interactions, structuring effects, and contributions to ecosystem processes are affected by feedbacks of the prey community (Snyder et al. 2006; Byrnes & Stachowicz 2009; Bell et al. 2010; Apple et al. 2011; Chen et al. 2011; Saleem et al. 2012, 2013; Sintes & del Giorgio 2014). Protists have a great diversity of functionally important phenotypic traits relating to size, swimming speed, grazing rates, and size selective feeding among others (Boenigk 2000; Sherr & Sherr 2002; Boenigk et al. 2002; Weisse & Weisse 2002; Massana et al. 2009; Caron et al. 2009; Beveridge et al. 2010c; Apple et al. 2011). As a result, they have a wide range of effects on community structure and function (Suzuki 1999; Hahn & Höfle 2001; Jürgens & Matz 2002; Thebault & Loreau 2003; Murase et al. 2006; Massana et al. 2009; Saleem et al. 2013; Saleem & Moe, 2014). However, these effects are constrained by feedbacks from the bacterial prey community (Massana et al. 2009; Saleem et al. 2012; Saleem et al. 2013; Sintes & del Giorgio

2014) and such feedbacks by prey may be mediated through diversity, abundance and defence (Pernthaler 2005; Chen et al. 2011; Saleem et al. 2013). Feedbacks of bacterial community composition have been shown to directly affect important functional traits such as species specific grazing rates of protists (Massana et al. 2009).

The results of this study and the hypothesised explanation are not contradictory to current understanding of BEF relationships found using trait-based functional diversity. On the contrary, since the importance of context dependence is the link with complementarity, using a different approach has served to highlight and support the hypothesis that complementarity is a fundamental mechanism underlying BEF relationships. However, using interactions-based measures of functional diversity may be one of the most meaningful ways of investigating consumer functional diversity since it inherently accounts for context dependence and interactions between species and traits. Indeed, since trophic interactions of predators constitute their functional traits, interactions-based measures of functional diversity may account for the effects of trait interactions that have been under-represented in trait-based approaches (Verberk et al. 2013).

Negative diversity functioning relationship

Interactions-based functional groups represent species that have similar interactions with other species and similar contributions to functioning. These result from trophic interactions and indirect impacts on bacterial prey populations which can be defined as their functional traits (Duffy 2002; Julia et al. 2014; Saleem & Moe 2014). Functional diversity based on interactions and contributions to functioning reflect these traits. Thus, protists that are in the same functional group, or have lower dissimilarity, are less functionally diverse. The expectation is that greater functional diversity and increases in functional diversity to lead to higher levels of functioning and change in functioning due to increased complementarity of impacts on bacterial prey species (Duffy 2002). This should lead to better prey resource partitioning, increased evenness and ultimately improved functioning (Tilman et al. 1997; Loreau & Hector 2001)

However, the results showed negative changes in functioning when mean pairwise dissimilarity increased, and negative effects of MPD were compounded when functional group richness also increased (see figures 13 and 14). Ecosystem functioning also decreased with increasing MPD when protists were feeding on bacterial community T143 (figures 11 and 12). MPD also predicted increasing ecosystem functioning when $FGR = 1$, but does not when $FGR = 2$ (figure 13). These results are in contrast to those using trait-based functional diversity that find a positive relationship with ecosystem functioning (Balvanera et al. 2006; Petchey et al. 2009; Cadotte et al. 2011). These studies show patterns of positive complementarity which are predominant in the BEF literature. However, my results support a smaller body of works that have suggested that ‘negative complementarity’ effects are also important (Loreau & Hector 2001; Eisenhauer 2012; Becker et al. 2012). Complementarity and facilitation drive positive BEF relationships, while antagonistic interactions and competition may underlie negative relationships (Loreau & Hector 2001; Foster & Bell 2012; Becker et al. 2012; Fiegna et al. 2015). Trait-based functional diversity will reflect positive complementarity due to resource partitioning but grouping species by similarity of interactions may highlight opposite, negative complementarity effects driven by antagonistic interactions.

A number of studies have reported negative effects of biodiversity on community productivity (Naeem & Li 1997; Polley et al. 2003). Diversity affects competition between organisms, either through indirect competition for resources or direct competition such as interference (Jousset et al. 2011; Jousset 2011; Becker et al. 2012). Interference might have a negative effect on functioning by inhibiting productive competitors (Massey et al. 2004). These mechanisms are likely to be particularly strong when organisms are competing for limited resources (Jousset et al. 2011). For instance, in bacterial biofilms, strong competition for space and nutrients may favour antagonisms and reduce functioning at higher diversities (Tait & Sutherland 2002; Becker et al. 2012).

Protist communities may be dominated by these sorts of negative interactions. There is evidence that competition and competitive exclusion drive negative complementarity among functionally

similar protists (Carrara et al. 2015). A similar relationship was found between phylogenetic distance and community productivity (Venail & Vives 2013). Protists exert strong grazing pressures on bacterial communities, reducing them to very low abundances and protist populations can become very abundant, driving stronger resource competition and reduced niche partitioning (Sherr & Sherr 2002; Jousset et al. 2011; Jousset 2011; Becker et al. 2012). Under these conditions, interference is likely to be strong particularly due to the possible existence of intraguild predation (Polis & Holt 1992; Arim & Marquet 2004; Ives et al. 2005). Intraguild predation between protists may negatively affect functioning since it would reduce their impact on bacterial communities allowing competitive species to dominate causing negative selection effects, reducing evenness, and excluding functionally important rare species (Jiang et al. 2008; Peter et al. 2011; Saleem et al. 2013).

However, in my opinion the results do not support negative complementarity resulting directly from negative interactions between protists. Species richness had positive effects, as did functional diversity overall. When not in the context of bacterial community T143, increases in interactions-based functional diversity was no different to increases in species richness. The fact that the effect was most pronounced in results of enzymatic activity measures (figures 11 - 14) supports this. These assays are finer grained and thought to be more tightly linked to bacterial populations and reflect impacts upon them (Langenheder et al. 2006).

The alternative is that protists may have negative complementarity effects on ecosystem functioning through impacts that drive negative complementarity in the bacterial community. I hypothesise that protists may promote contrasting combinations of bacteria which in turn may have strong antagonistic effects on each other. Protists show strong prey selectivity and preference, as well as a wide range of feeding traits and modes that may directly relate to their structuring of the bacterial community (Posch et al. 1999; Corno & Jurgens, 2008; Massana et al. 2009; Bell et al. 2010; Saleem et al. 2013). If predation impacts drive divergent combinations of assemblages then their biochemical defence mechanisms might negatively affect the each other. There is plenty of evidence for antagonistic mechanisms leading to negative

complementarity in bacteria. For instance, bacteria can use toxins to increase competitive ability (Czárán et al. 2002) or prevent invasion (Vos & Velicer 2009). Bacteria may also sense and respond to competitors through quorum sensing and upregulation of secondary metabolism, mechanisms that are proposed to be largely responsible for bacterial negative complementarity (Haas & Keel 2003; Garbeva et al. 2011; Becker et al. 2012). Beach leaf tea is also a relatively nutrient poor medium which might limit niche partitioning and reduce complementarity (Jousset 2011; Lawrence et al. 2012). In such an environment increased evenness due to predation might also increase competition for resources. Increased evenness might also result in reduced functioning if few bacterial species contribute most of the observed ecosystem functioning.

Conclusions and future direction

This chapter has highlighted the importance of context dependence in functional diversity, especially that of consumers whose functional traits are manifested in – and functional diversity defined by – trophic interactions. Greater consideration needs to be given to interactions between traits, species, and their context dependent consequences for biodiversity-ecosystem functioning relationships.

My work has shown that dissimilarity of interactions and presumed increased complementarity of impacts on bacterial populations do not always lead to positive biodiversity-ecosystem functioning relationships. This warrants further investigation into the drivers of protist contributions to diversity. In particular, the ‘composition effects’ and feedbacks of the bacterial community on protist interactions and contributions to functioning will be investigated in the following chapters.

Chapter 4 – Bacterial community composition effects on protist species' functional relationships and ecosystem functioning

Abstract

Biodiversity-ecosystem functioning relationships have been well studied in a number of micro- and macroscopic systems. Biodiversity-ecosystem functioning research has largely focussed on top-down effects of predation on lower trophic levels or bottom up effects of resources on primary producers. There has been little or no effort to elucidate how the bottom-up effects of prey community composition feedback on the complementarity of predators in their contributions to ecosystem functioning. Previous studies provide mixed evidence as to the dominance of composition or diversity in determining ecosystem functioning. I used a large scale microcosm study of 1200 microcosms containing bacterial treehole communities and protistan predators along with a number of functional bioassays to test the relationship. I found that ecosystem functioning varied significantly between bacterial communities. Predator interactions predicted different levels of functioning between bacterial communities. I also found that contributions to complementarity by individual protist species were correlated between some bacterial communities and uncorrelated between others. A small number of species had significantly above average contributions to complementarity and I propose that these might be viewed as keystone or cornerstone species. I conclude that predator contributions to functioning are context dependent and that the nature of interactions-based functional diversity is too.

Introduction

Studies of microbial ecology have increasingly addressed the role of aspects of community structure in contributing to ecosystem functioning (Torsvik & Øvreås 2002). In particular, there are notable examples of contributions by bacterial diversity to functional processes (Bell et al. 2005; Szabó et al. 2007; Bell et al. 2009b; Langenheder et al. 2010). However, there have been noticeably contrasting results and it remains unclear which characteristic of community structure most underpins functioning. Some studies have found clear effects of diversity (Bell et al. 2005; Szabó et al. 2007; Wagg et al. 2014) while others have found none (Wertz et al. 2007). To complicate matters, this contrast appears to be due to differences in experimental methods (Frossard et al. 2012). Experiments using assembled communities tend to find effects while those using indirect manipulations do not (Bell et al. 2005; Wertz et al. 2007; Langenheder et al. 2010; Östman et al. 2010). This may be the result of assembled communities separately varying composition and diversity, diverging levels of diversity produced by the two methods, and the patterns of diversity along a dilution gradient (Franklin et al. 2001; Bell et al. 2009b; Frossard et al. 2012). The importance of composition is equally debated with some studies finding connections between community composition and function (Langenheder et al. 2006; Peter et al. 2011) where as others find a disconnect (Langenheder et al. 2005; Burke et al. 2011). In addition, there is a suggestion that the patterns observed may depend on the coarseness of the measures of functioning used (Langenheder et al. 2006; Frossard et al. 2012).

Heterotrophic protists have an important role in shaping bacterial community diversity, composition, productivity and functioning (Murase et al. 2006; Corno & Jurgens 2008; Bell et al. 2010). Most work has focused on top-down regulation by protists but recently attention has been paid to bottom-up impacts of bacterial prey of both bacterial and protistan predators (Suzuki 1999; Jurgens & Massana 2008; Bell et al. 2010; Apple et al. 2011; Chen et al. 2011; Saleem et al. 2012; Saleem et al. 2013). While this most recent work has focussed on a number of hypotheses concerning diversity effects (reviewed in Duffy et al. 2007 and Bell et al. 2009b),

there has been no attention paid to feedbacks by bacterial community composition rather than richness, or how such feedbacks affect protistan complementarity of contributions to functional processes.

Species niches can be defined in terms of the resources they require and their impacts upon them (Tilman et al. 1997; Tilman 1999). For consumers such as heterotrophic protists, niches can be viewed in terms of their preferred bacterial prey types, their availability within the community, and their trophic interactions with them (Duffy 2002). Prey species richness affects predator niche complementarity and interspecific interactions (Snyder et al. 2006; Byrnes & Stachowicz 2009). A species' contribution to functioning is thought to be related to its niche. In a food web based view of niches and functioning, the functional traits of a predator manifest themselves as its trophic interactions, both directly and indirectly through intraguild predation (Duffy 2002; Schneider & Brose 2013). Therefore the impact of protists on bacterial resources both defines their niches and links them to functioning. This is because heterotrophic protists structure bacterial communities and this structure underpins functional processes (Posch et al. 2001; Murase et al. 2006; Corno & Jurgens 2008; Jürgens & Massana 2008). However, though protists have impacts on functional processes, these are in turn affected by their interactions with bacteria (Saleem and Moe, 2014). The degree of shared prey resource, diet breadth and contrasting effects on prey community structure is likely to determine the degree of complementary niche occupancy and contribution to functional processes (Woodward & Hildrew 2002; Snyder et al. 2006; Julia et al. 2014). I hypothesised that changes in prey community composition affect the complementarity of protist functional interactions and contributions to functioning.

Complementarity between species in a community has been quantified in various ways (Loreau & Hector 2001; Fox & Harpole 2008; Fox & Kerr 2012), as have species contributions to functioning (Kirwan et al. 2009; Connolly et al. 2013) and competitive interaction strengths (Goldberg et al. 1999). In Chapter 2 I used the 'response ratio' (RR) to quantify the difference between expected species contributions to functioning and observed functioning in pairwise

combinations. I used this to infer the degree of complementarity of species' contributions to functioning and build dendrograms to represent the functional diversity of these relationships. In Chapter 3 I tested the hypothesis that interactions-based functional diversity and the relationships it predicted was context dependent because it captured both trophic and functional trait interactions that define consumer functional traits and affect their relevance to ecosystem functioning (Duffy 2002; Schneider & Brose 2013; Ritchie et al. 2012; Verberk et al. 2013) . Using interactions-based functional diversity also revealed negative complementarity effects. I suggested that this might be due to negative interactions between protists or, perhaps more likely, that predation caused negative complementarity effects between bacterial species. However, I discussed little the possibility of bacterial community composition effects that feedback on protist interactions and contributions of individual species to functioning.

In this Chapter I followed on from my investigation of composition effects on diversity-functioning relationships and interactions-based functional diversity and tested the hypothesis that community composition is an important feedback on individual protist species complementarity and contributions to functional processes. Species contributions, whether positive or negative, might be context dependent. This is because changes in composition affect resource availability, niche overlap, and trophic interactions of predators (Duffy 2002; Woodward & Hildrew 2002; Ritchie et al. 2012; Julia et al. 2014). Predator functional traits can be defined by their trophic interactions (Duffy 2002) and functional traits are hypothesised not to retain functional relevance across species and environmental gradients (Verberk et al. 2013).

I tested these hypotheses using 1,200 bacteria-protist treehole community microcosms. I estimated ecosystem functioning using seven different measures of microbial respiration, cellular and enzymatic activity, and used these to calculate protist community response ratios across four different bacterial treehole communities. Using this array of measures allowed me to test the hypothesis that coarse and fine scale measures of ecosystem functioning reflect differently the link between community structure and functioning (Langenheder et al. 2006; Frossard et al. 2012). Furthermore, multifunctionality - whereby species perform multiple

functions resulting in high redundancy of single ones - is thought to be important in microbial communities (Gamfeldt et al. 2008). However, this is disputed on the basis that this has been due to the use of very broad measures (Peter et al. 2011).

Methods

Protist strain growth

This experiment was carried out as part of the same set of experiments as Chapter 3. The eighteen protist strains from the combined response ratio dendrogram in Chapter 2 were grown as monocultures from pure stock cultures prior to the assembly of the experiment. They were grown for 7 days in 2ml of beech leaf tea (BLT) in 24 well culture plates pre-inoculated with naturally occurring bacterial tree-hole community to check that they would produce active cells. Strains were categorised into four broad categories based on estimated growth rates in Chapter 2 (pages 49 and 55); slow, medium, fast, and very fast. Slow strains were grown for six days before inoculation to experimental microcosms, medium five days, fast four days, and very fast three days beforehand. This was to ensure that all strains were inoculated into experimental microcosms at a similar stage of growth.

Bacterial background community treatments

Four bacterial background communities were used as experimental treatments to test the effect of variation in surrounding community composition on response ratio based relationships between protist predators i.e. complementarity of contributions to community functional output. These bacterial communities were the same as in Chapter 3 and sampled from tree holes in Wytham Woods (Oxfordshire, UK) and Silwood Park (Berkshire, UK), Burnham Beeches (Berkshire, UK), and the Ashridge Estate (Bedfordshire, UK). These are referred to as Wyt116, T143, BB88 and AE155 respectively, in this Chapter. Storage of frozen stocks, sampling and growth of live culture from frozen stock was as described in Chapter 3.

Random partitions design (RPD) and protist community assembly

I used the same random partitions design for community and biodiversity-ecosystem functioning experiments devised by Bell et al. (2009a) as in Chapter 3 (page 79). The justification for this was as outlined in Chapter 3. The experiment consisted of two random

partitions of 1, 2, 3, 6, 9 and 18 strains, replicated twice. These were then replicated again four times, once for each of the four bacterial background communities used in the experiment. Experimental communities were assembled according to the RPD generated compositions by pipetting transfer from 96 well deep well plates to 96 well shallow well plates by Hamilton (Starlet) pipetting robot. I used a substitutive design for the inoculation of protists, with monocultures containing 180µl of isolate, two species communities containing 90µl of each isolate, three species communities 60µl, and so forth. 20µl of BLT was then also added. Once assembled in shallow 96 well plates, the mixtures were transferred to the experimental microcosms containing 1800µl of BLT and bacterial culture. The microcosms were then left to grow for 15 days at 17°C.

Ecosystem functioning bioassays

I used the same 7 bioassays as previously described in Chapter 2 (pages 41 and 42). These were performed after 15 days from inoculation of the experimental microcosms. Assays were again carried out on days 15, 16, and 17 of the experiment, and carried out in 96 well shallow well plates. Subsampling of the experimental microcosms was carried out by Hamilton (Starlet) pipetting robot after manual transfer from 24 well plates to deep well 96 well plates. Ecosystem function measures were then standardised using calibration curves (see Appendix 2, supplementary figure 4B)

These seven assays are useful since they represent two groups of assays that can be grouped according to the coarseness of the ecosystem processes they detect. Broad ecosystem processes such as microbial respiration, productivity, and cellular activity integrate across many processes and may be too coarse to reveal changes in functioning due to impacts of community structure (Frossard et al. 2012). While enzymatic activities of specific metabolic enzymes may be more tightly linked to bacterial populations, even here there have been contrasting results with some studies showing a tight association of structure and function, and others finding a disconnect (Langenheder et al. 2005; Langenheder et al. 2006; Wertz et al. 2007; Frossard et al. 2012). These assays therefore provide an interesting contrast and range for investigation.

Calculating response ratios (RR)

Response ratios were calculated for all communities in all backgrounds. These were calculated as described in Chapter 2 (page 44). The response ratios form the basis my method for drawing functional hierarchical cluster dendrograms and defining functional groups in Chapter 2. They allowed me to infer the degree of complementarity between species within a community in terms of contribution to ecosystem processes. Using them, I could examine whether bacterial community composition affects complementarity and the functional organisation of the protist species pool.

Statistical analyses

Statistical analyses were carried out using the statistical package R CRAN v.3.0.3 (R Development Core Team 2014). Analyses initially involved generalised liner modelling (GLM) of protist community measures of ecosystem functioning and multifunctioning across experimental bacterial background community treatments. Models treated data with Gaussian errors and identity-link function. They included an interaction term between protist community identity, bacterial community, and response ratio, as well as protist species richness as a covariate. Xylo enzymatic activity data, as well as ATP and multifunctioning data were log transformed for reasons of variance stability. Multifunctioning scores were calculated as average Z-scores. Z-scores with a mean of zero and standard deviation of 1 were calculated for each function and then averaged across functions. Monoculture data was also removed from the dataset for analysis of response ratios as these are not informative since the RR of a monoculture is always 1 or 0.5 (depending on how it is calculated).

I also analysed the effect of presence and absence of strains on response ratios using the analysis developed by Bell et al (2009a) for the analysis of RPD designed experiments. This method sequentially apportions variance to the linear effects of species richness, the effect of the presence or absence of each component species, the effect of the number of partitions and replicates, and finally the non-linear effects of protist community species richness. This allowed

me to determine the effect of each species on changes in response ratios in each of the measures of ecosystem functioning under each of the bacterial background community treatment levels. The non-linear richness term can be used to interpret the importance of species interactions and complementarity effects (Bell et al. 2009a).

Using the coefficients of effect on response ratio from the RPD analysis, I then performed Mantel tests between pairs of bacterial background communities using R package ‘vegan’ (Oksanen et al. 2015). The Mantel statistic was based on Pearson’s product moment coefficient and the test performed nine hundred and ninety nine permutations. I also used Pearson’s correlation coefficient to examine the coefficients of contributions to response ratios of individual species for each of the seven ecosystem function measures across different bacterial community treatments. The heat maps produced in the analysis used Euclidean distances and complete linkage to produce dendrograms with the R package ‘gplots’ (Warnes et al. 2015).

Results

Bacterial composition and ecosystem functioning

I found significant differences in levels of ecosystem functioning between bacterial communities for MUN, MUP, and Xylo enzymatic activities (all Tukey HSD contrasts $p < 0.001$). This was also the case for microcosm ATP concentrations, phosphate concentrations, CO_2 production, and multifunctioning (Tukey HSD contrasts, $p < 0.001$). There were significant interactions between bacterial community and protist community compositions for MUN (driven by BB88 GLM: estimate = -0.0001, $t = -4.53$, $p < 0.001$; and T143 GLM: estimate = 0.00007, $t = -2.88$, $p < 0.001$), MUP (BB88 GLM: estimate = 0.00016, $t = 3.99$, $p < 0.001$; T143 GLM: estimate = 0.00013, $t = 3.38$, $p < 0.001$), and Xylo enzymatic activities (T143 GLM: estimate = 0.0033, $t = 6.84$, $p < 0.001$; Wyt116 GLM: estimate = 0.0014, $t = 2.91$, $p = 0.0037$). The effect was also significant between all bacterial and protist community compositions for microcosm cellular activity (ATP concentration). For phosphate availability this was driven by BB88 and Wyt116 (GLM: estimate = -0.00002, $t = -2.69$, $p = 0.0072$; and GLM: estimate = -0.00003, $t = -3.17$, $p = 0.0016$), and both T143 and Wyt116 for respiration (CO_2 production) (GLM: estimate = 0.045, $t = -3.28$, $p = 0.0012$; and GLM: estimate = 0.049, $t = -2.72$, $p = 0.0066$ respectively). This indicates that differences in functioning were exacerbated by differences in contributions to functioning by different protist community compositions.

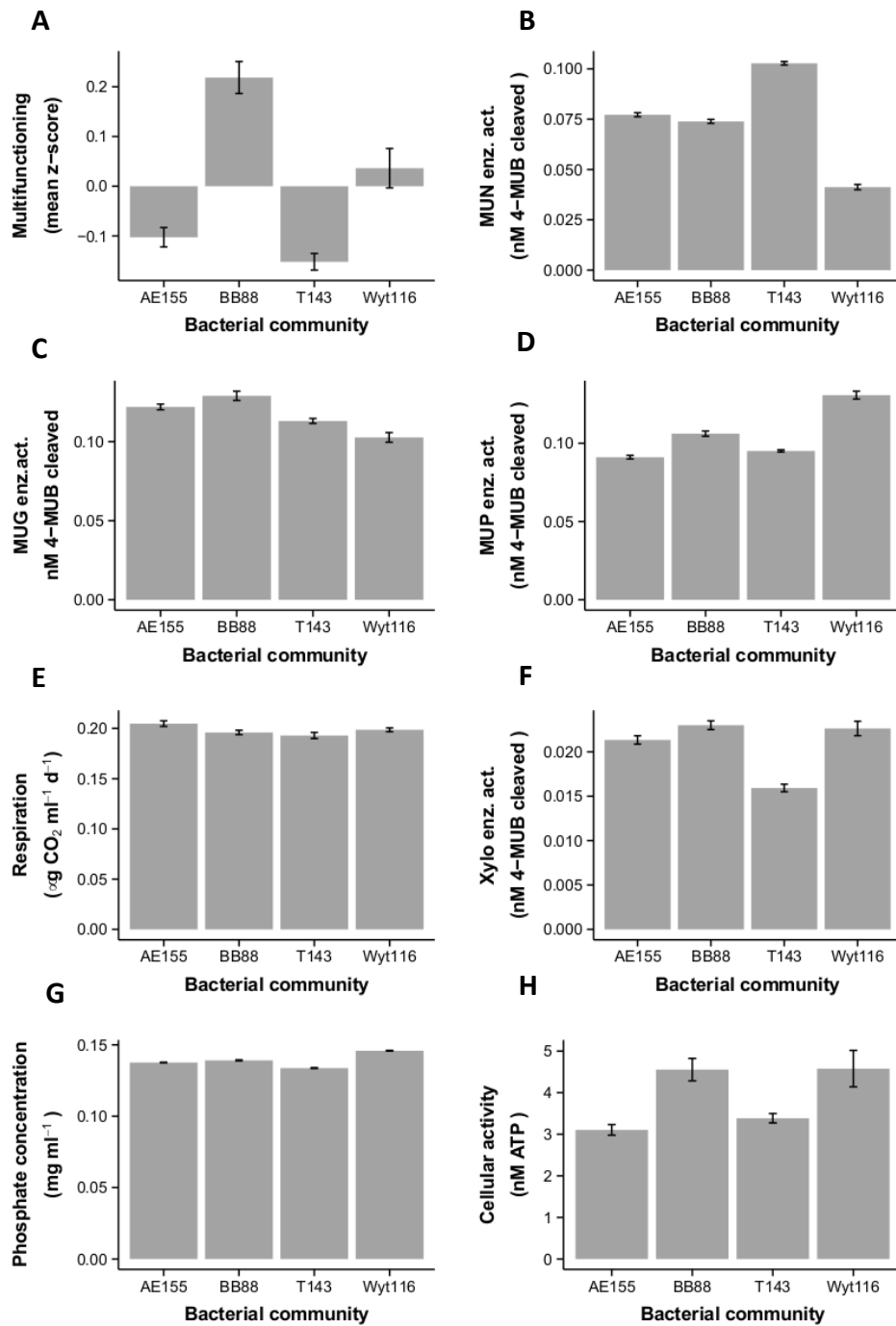


Figure 17. Bar plots of mean functioning on each of the four backgrounds for **A)** multifunctioning, **B)** MUN enzymatic activity, **C)** MUG enzymatic activity, **D)** MUP enzymatic activity, **E)** respiration (CO_2 production), **F)** Xylo enzymatic activity, **G)** available phosphate concentration, and **H)** cellular activity (ATP). Error bars are standard errors. Measures of average multifunctioning are positive and negative because they are calculated using average Z-scores across the seven functions. These have mean of 0 and standard deviation of 1.

Protist complementarity and ecosystem functioning

Response ratios significantly predicted levels of ecosystem functioning for MUN (GLM: estimate = 0.062, $t = 8.69$, $p < 0.001$), MUG (GLM: estimate = 0.13, $t = 5.17$, $p < 0.001$), MUP (GLM: estimate = 0.004, $t = 3.13$, $p < 0.001$), and Xylo enzymatic activities (GLM: estimate = 0.98, $t = 23.5$, $p < 0.001$). The relationship was also significant for ATP (GLM: estimate = 0.12, $t = 4.26$, $p < 0.001$) and phosphate availability (GLM: estimate = 0.0027, $t = 2.25$, $p = 0.025$). Multifunctioning response ratios predicted multifunctioning (GLM: estimate = 0.053, $t = 3.24$, $p = 0.001$)

However response ratios predicted functioning significantly differently between bacterial communities as indicated by significant interactions for MUN (RR*Wyt116 GLM: estimate = -0.027, $t = -3.53$, $p < 0.001$) and MUP (RR*BB88 GLM: estimate = 0.071, $t = 5.31$, $p < 0.001$; RR*Wyt116 GLM: estimate = 0.055, $t = 4.73$, $p < 0.001$) enzymatic activities. The effect was also significant between all bacterial community identities and response ratios for microcosm ATP concentration and CO₂ production. There was also a significant interaction between bacterial community and response ratios for multifunctioning (T143*RR GLM: estimate = -0.038, $t = -2.16$, $p = 0.03$; Wyt116*RR GLM: estimate = -0.053, $t = -3.2$, $p = 0.001$)

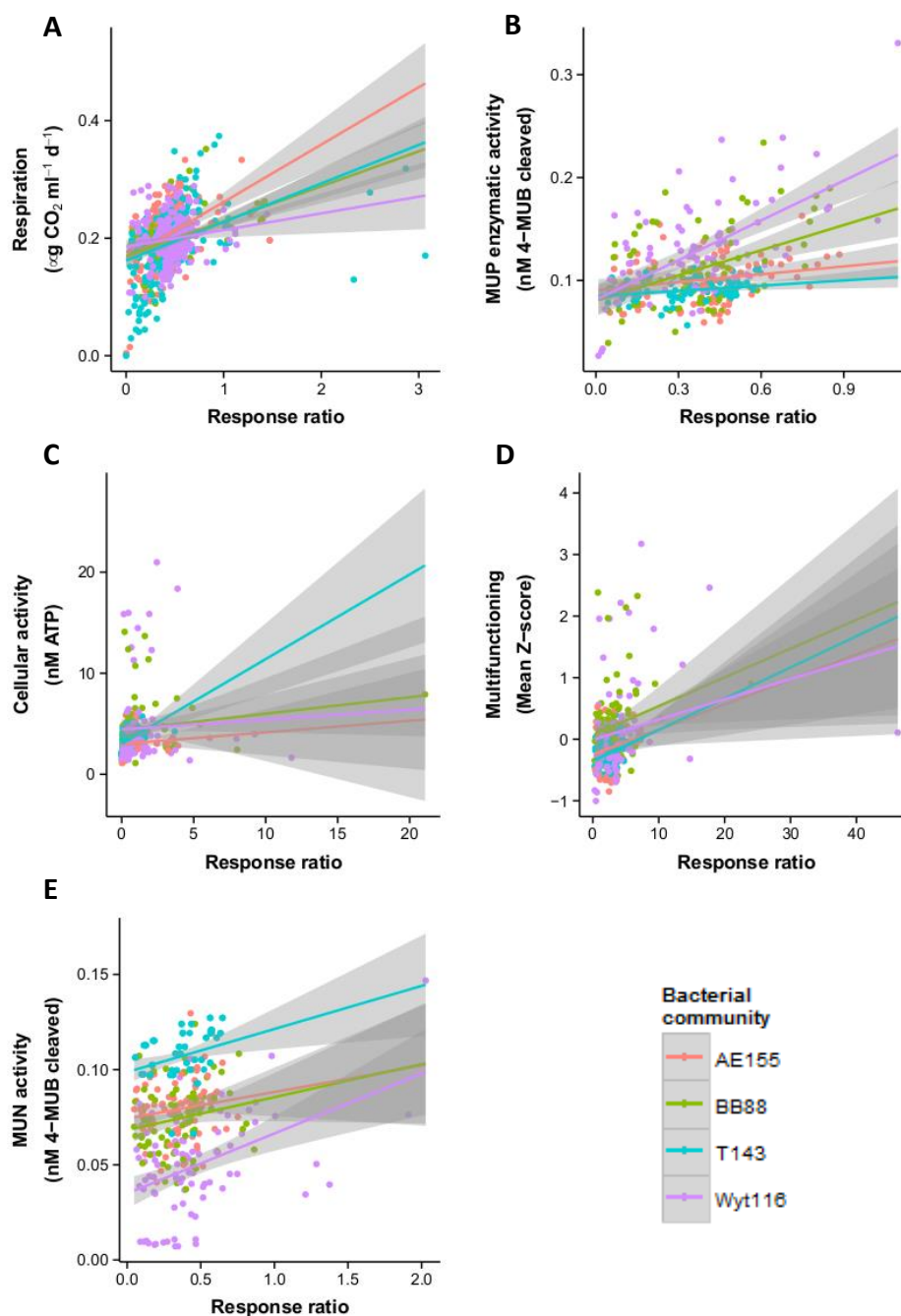


Figure 18. Scatterplots plots of functioning against response ratios coloured by each of the four bacterial communities. Shown are **A)** respiration, **B)** MUP enzymatic activity, **C)** cellular activity, **D)** multifunctioning, and **E)** MUN enzymatic activity, all for which the interaction between response ratio and bacterial community was found to be significant. Shaded areas are standard errors. Measures of average multifunctioning are positive and negative because they are calculated using average Z-scores across the seven functions. These have mean of 0 and standard deviation of 1.

Bacterial composition and individual protist contributions to complementarity

The linear model analysis for RPD designs (Bell et al. 2009a) found that non-linear richness terms in the analysis were also significant in all measures of functioning and across all bacterial communities. In addition, very few protist strains contributed significantly above or below average to differences in response ratios of different functions. A total of eight species made significant contributions across all functions and bacterial communities. In the presence of the AE155 bacterial community, one species made a significant contribution to differences in response ratios for cellular activity (ATP). Two species also made significant contributions to response ratios for MUG enzymatic activity. One of these also had a significant effect on contributions to MUP enzymatic activity response ratios. In mixture with the BB88 bacterial community, two species significantly contributed to response ratios of cellular activity (ATP), while one significantly contributes to differences in response ratios of respiration (CO₂ production).

Combined with the T143 bacterial community, two species significantly contributed to response ratios of respiration while one also did for MUN enzymatic activity. Two species contributed to response ratios of respiration when feeding on the Wyt116 bacterial community, one of which again contributed significantly but this time to Xylo enzymatic activity response ratios. Species that made significant contributions are indicated in figures 17 and 18 which also show whether the contribution was positive or negative. No species made significant contributions to multifunction score response ratios.

For each of the eighteen species I also examined the correlation between coefficients of contributions to response ratios by protist species between pairs of bacterial communities. A strong positive correlation indicated that coefficients of contributions to response ratios differ very little between the two bacterial communities across all ecosystem functioning measures and *vice versa*. A lack of correlation indicated that some ecosystem functioning measures did differ greatly while others did not. Results are given in table 3.

Table 3. For each protist strain the results of a Pearson's correlation coefficient test between coefficients of effect on RRs for each function, for each of the pairwise combinations of each background bacterial community. Green cells indicate a significant positive correlation, red cells a significant negative correlation, and white indicates no significant correlation.

Species	Ae155 – BB88		Ae155 – T143		Ae155 – Wyt116		BB88 – T143		BB88 – Wyt116		T143 – Wyt116	
	r	p	r	p	r	p	r	p	r	p	r	p
<i>Bufl/H5</i>	0.97	<0.001	0.81	0.016	0.96	<0.001	0.85	0.0075	0.91	0.0015	0.66	0.076
<i>Cl8</i>	-0.81	0.014	0.97	<0.001	0.96	<0.001	-0.67	0.054	-0.93	<0.001	0.89	0.003
<i>Gl1</i>	-0.87	0.0054	0.95	<0.001	0.94	<0.001	-0.88	0.0036	-0.98	<0.001	0.94	<0.001
<i>G9</i>	0.55	0.16	-0.26	0.54	-0.45	0.42	-0.12	0.78	0.19	0.65	0.35	0.38
<i>Giff/G9</i>	0.34	0.41	-0.30	0.48	0.28	0.50	-0.68	0.064	0.94	<0.001	-0.81	0.015
<i>IB3</i>	0.99	<0.001	0.87	0.0052	-0.99	<0.001	0.85	0.0072	-0.98	<0.001	-0.834	0.0095
<i>IGRA</i>	-1.0	<0.001	-0.89	0.0023	-0.83	0.011	0.89	0.0032	0.83	0.01	0.67	0.070
<i>IPY20</i>	1.0	<0.001	0.84	0.0087	0.84	0.0087	0.86	0.0058	0.86	0.0058	1.0	<0.001
<i>IPY7a</i>	-0.83	0.012	0.89	0.0031	0.28	0.50	-0.94	<0.001	-0.72	0.045	0.53	0.17
<i>Ku/HF</i>	-0.93	0.001	0.93	<0.001	-0.92	0.0010	-0.91	0.0015	1.0	<0.001	-0.92	0.0013
<i>Spain1</i>	0.91	0.0018	0.39	0.34	-0.89	0.0033	0.39	0.34	-1.0	<0.001	-0.41	0.32
<i>SW2</i>	-0.95	<0.001	0.83	0.010	-0.99	<0.001	-0.68	0.062	0.97	<0.001	-0.77	0.025
<i>W36</i>	-0.99	<0.001	0.39	0.86	-0.98	<0.001	-0.23	0.59	0.98	<0.001	-0.26	0.54
<i>W70</i>	0.88	0.0033	0.72	0.045	0.88	0.0042	0.91	0.0019	0.77	0.025	0.60	0.12
<i>W80</i>	-0.82	0.013	0.46	0.25	0.97	<0.001	-0.65	0.079	-0.93	<0.001	0.55	0.16
<i>Wa42</i>	0.63	0.093	0.25	0.45	0.98	<0.001	0.62	0.10	0.73	0.041	0.34	0.33
<i>Wa46</i>	-0.12	0.77	0.12	0.78	-0.26	0.54	0.84	0.010	0.18	0.67	0.27	0.53
<i>Wa8</i>	0.98	<0.001	0.12	0.77	-0.83	0.010	-0.004	0.99	-0.77	0.021	-0.32	0.44

Broad patterns of species' contributions to complementarity

To analyse overall patterns of contribution to response ratios by protists between bacterial communities, I examined the coefficients of each species from the RPD analysis. I examined correlated similarities in contributions to bacterial community functioning, across the seven measures of ecosystem functioning and the multifunctioning metric. I found that coefficients of contributions to response ratios varied greatly between bacterial communities for all protist species, as seen in figures 19 and 20.

Mantel tests revealed significant overall differences in coefficients of contributions to response ratios between bacterial communities for only four measures of ecosystem functioning. There were significant correlation between coefficients of contribution to cellular activity (ATP) response ratios (Mantel test; mantel $r = 0.283$, $p = 0.046$), MUP enzymatic activity (Mantel test; $r = 0.1535$, $p = 0.05$), and MUN enzymatic activity (Mantel test; $r = 0.5467$, $p = 0.002$) by species feeding on BB88 and Wyt116 bacterial communities. There was also a significant correlation between coefficients of species contributions to response ratios for MUN enzymatic activity between T143 and Wyt116 bacterial communities (Mantel test: $r = 0.2$, $p = 0.031$). Coefficients of effect on response ratios were also significantly correlated between AE155 and BB88 bacterial backgrounds for Xylo enzymatic activity (Mantel test: $r = 0.24$, $p = 0.043$). There were a greater number of significant correlations between communities for the multifunctioning metric. These were between AE155 and BB88 (Mantel test: $r = 0.36$, $p = 0.01$), AE155 and Wyt116 (Mantel test: $r = 0.2$, $p = 0.03$), and BB88 and Wyt116 (Mantel test: $r = 0.5$, $p = 0.001$)

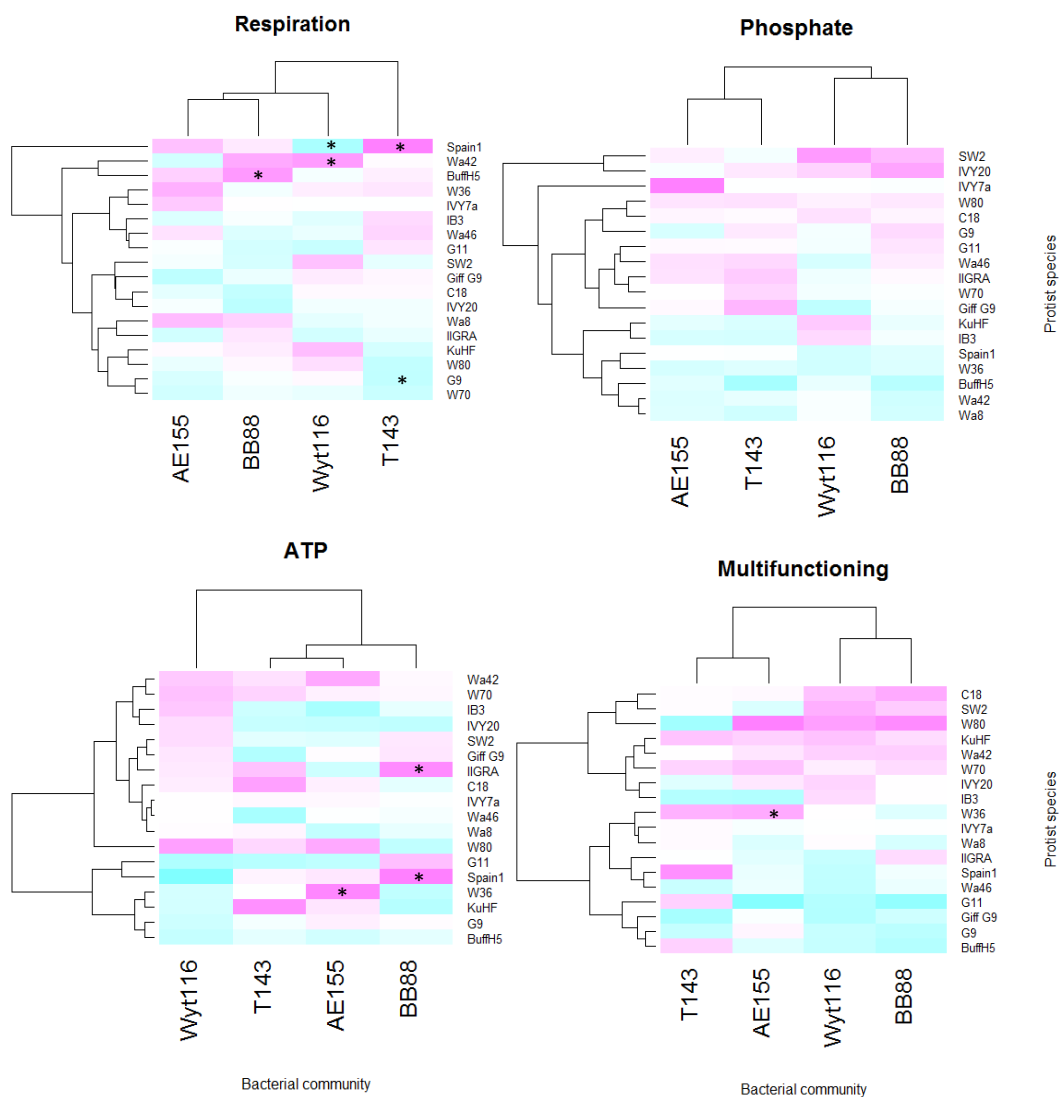


Figure 19. Heat maps of species' contributions to response ratios for respiration (CO₂ production), cellular activity (ATP), phosphate concentration, and multifunctioning Z-score measures. Purple indicates positive contributions to response ratios and an increase in complementarity through species presence. Blue indicates the opposite. Species are clustered by similarity of contributions. Bacterial communities are grouped by similarity of effects by protists. Groupings do not indicate significance of correlations between communities using mantel tests

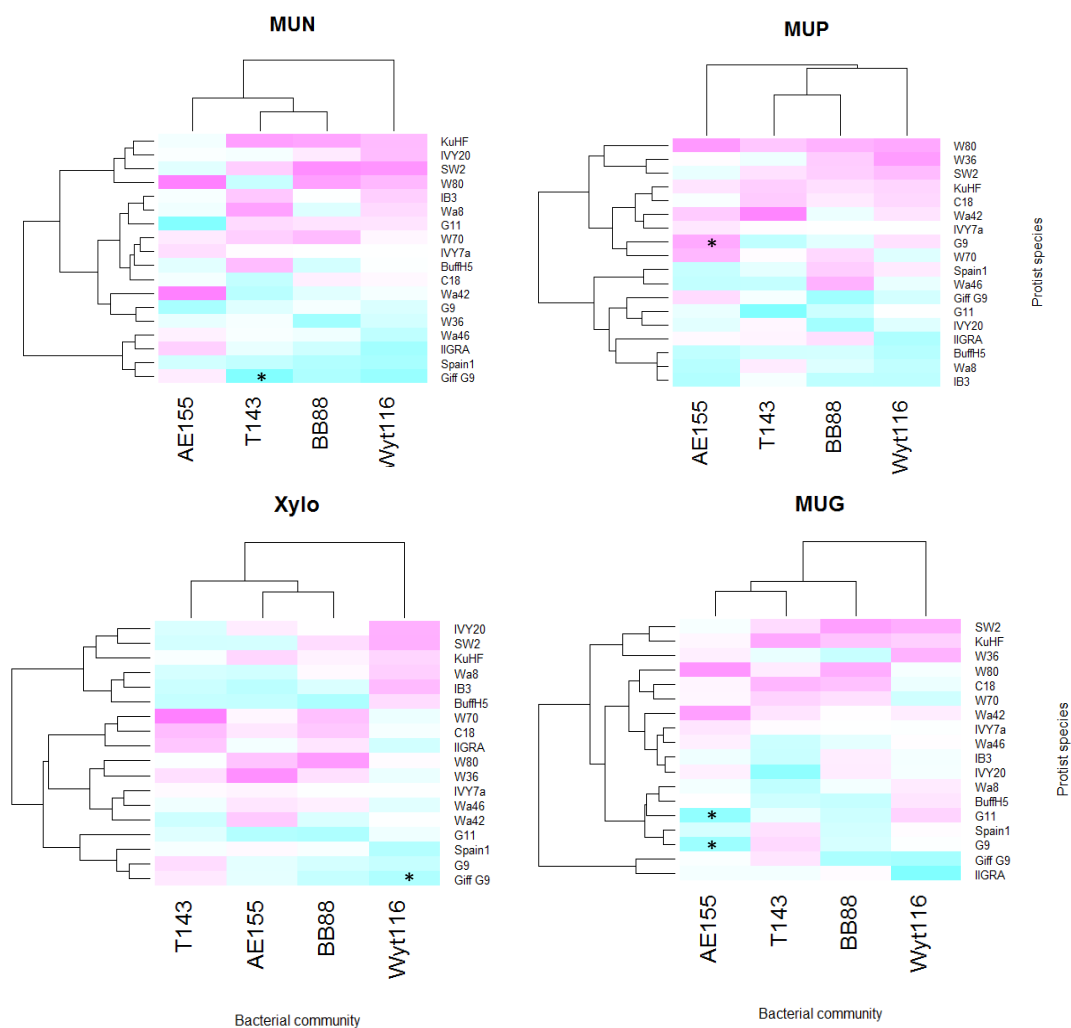


Figure 20. Heat maps of species' contributions to response ratios for the four enzymatic activity measures. Purple indicates positive contributions to response ratios and an increase in complementarity through species presence. Blue indicates the opposite. Species are clustered by similarity of contributions. Bacterial communities are grouped by similarity of effects by protists. Groupings do not indicate significance of correlations between communities using mantel tests

Discussion

Main findings

In this Chapter I tested whether ecosystem functioning and the contributions of protist species to it varied with bacterial community composition. I found that composition had important impacts for all types of functioning and multifunctioning. I also found that response ratios predict functioning differently in different bacterial communities and that protist species' contributions to interactions (response ratios) is affected by bacterial community composition.

Ecosystem functioning

My analysis of ecosystem functioning showed that there were significant differences in ecosystem functioning between bacterial communities (figure 15) and supports the hypothesis that community composition is important for functioning. This is supported by the fact that protistan response ratios predicted ecosystem functioning differently according to the bacterial community (figure 18). These findings are in line with results that have found that composition affects patterns of functioning (Langenheder et al. 2006; Szabó et al. 2007; Strickland et al. 2009; Peter et al. 2011) and contrast with those that suggest disconnect (Langenheder et al. 2005; Wertz et al. 2007; Burke et al. 2011; Frossard et al. 2012). It has been suggested that this lack of consensus is a consequence of methodological differences between studies. These include three broad types; controlled assembly studies (Bell et al. 2005; Peter et al. 2011), field-based studies that examine simultaneous differences in composition and function along an environmental gradient (Findlay & Sinsabaugh 2006; Comte & Del Giorgio 2009), those that indirectly manipulate diversity (Griffiths et al. 2000; Östman et al. 2010). This study adds an interesting perspective since it uses different natural assemblages, does not manipulate bacterial community structure (before the addition of protists), and retains the control inherent to laboratory microcosm studies.

My results are consistent with those of Strickland et al (2009) who found that rates of CO₂ production varied significantly between bacterial communities in the same environment. Szabó et al (2007) showed that removal of rare species has large impacts on functioning. Rare species by definition are more likely to be absent from - and account for differences in - community compositions and therefore may account for differences in patterns of ecosystem functioning (Mouillot et al. 2013b). Other results have suggested that large proportions of observed functioning such as decomposition can be due to single strains (Jiang & Krumins 2006).

Multifunctionality means that species perform multiple functions at the same time (Hector & Bagchi 2007; Lefcheck et al. 2015b). If a group of species within a community account for most of the contribution to ecosystem functioning, this introduces high redundancy (Lefcheck et al. 2015a). On the other hand, species may perform specific functions requiring greater biodiversity to maintain them all (Hector and Bagchi 2007; Lefcheck et al. 2015a). Langenheder et al (2006) suggested that the degree of functional redundancy detected in bacterial communities is dependent on the type of measure used. My results showed that there were significant pairwise comparisons between communities for all measures, but were far fewer pairwise differences for cellular activity (ATP) or respiration (CO₂) than for measures of enzymatic activities. Such differences are suggested to be due to the integration of multiple processes by coarse measures of cellular activity or respiration, as opposed to enzymatic activities of specific biogeochemical pathways that may be more tightly linked to populations (Peter et al. 2011; Frossard et al. 2012). This result supports the suggestion by Peter et al (2011) that multifunctionality may need to be reassessed for microbial communities which have been considered to have redundancy (Gamfeldt et al. 2008). The context dependence of contributions by consumers such as heterotrophic protists adds an extra dimension to this, since they not only have different contributions to functioning, but these are subject to composition effects.

Composition interactions between trophic levels

The structuring effects of protist predators on bacterial community structure and function are relatively well documented (Pernthaler 2005; Jiang & Krummins 2006; Murase et al. 2006; Corno & Jurgens 2008; Worsfold et al. 2009; Bell et al. 2010). The results of my study show that there are significant interactions between bacterial prey and protist predator community compositions in determining ecosystem functioning. Such results are still sparse in microbial ecology literature. While some studies have investigated compositional effects in bottom up and top down control of microbial communities (for example: Balcionas & Lawler 1995; Stevens & Steiner 2006; others reviewed in Duffy et al. 2007), only a small number of studies have explicitly addressed the feedbacks of prey community composition on higher trophic levels (Fox 2004; Gamfeldt et al. 2005; Chen et al. 2011; Saleem et al. 2013). Results of predator – prey experiments are mixed, with some finding no effect of adjacent trophic levels (Fox 2004) and others that do (Gamfeldt et al. 2005; Chen et al. 2011; Saleem et al. 2012; Saleem et al. 2013). However, even these have not explicitly addressed impacts on ecosystem functioning.

Bacterial composition and protist contributions to functioning

Two results support the hypothesis that ecosystem functioning responses to protists depend on bacterial community composition. Firstly, response ratios predict levels of functioning differently between different bacterial community compositions. Secondly, the effect of protist community identity on ecosystem functioning varies significantly between bacterial communities. In addition, three pieces of evidence support the hypothesis that protist coefficients of contribution to complementarity (making them more positive or negative) depend on the composition of bacterial community they are feeding on: 1) protist contributions to response ratios only correlated in three combinations of bacterial communities across all seven individual functional measures; 2) species that made significant contributions to complementarity differed between bacterial communities (figures 19 and 20); and 3) there was variation in individual species' correlations of contributions, across functions, between pairs of bacterial communities (table 3).

Changes of protist contribution to response ratios between bacterial communities might reflect availability of prey types, ability of bacteria to constrain predation, and feeding modes or preferences of protists. Protists are known to display different degrees of generalism and specialism (Bell et al. 2010; Saleem et al. 2012) due to selectivity mechanisms (Boenigk 2000; Boenigk et al. 2001; Boenigk et al. 2002). Active mechanisms are related to feeding traits - for instance size and motility – or can be related to prey abundance and quality mediated by controls at stages of capture, ingestion, and egestion (Boenigk 2000; Pfandl et al. 2004; Beveridge et al. 2010a, b, c). Passive mechanisms relate to contact probabilities (Boenigk et al. 2002). Bacteria display a wide range of defences in response to protistan predators, including killing them, forming biofilms or filaments, and causing release post phagocytosis and the efficacy of these against predators depends on the composition and diversity of the community (Boenigk et al. 2001; Hector et al. 2002; Pernthaler 2005; Lainhart et al. 2009; Jousset 2011; Jousset et al. 2011). Changes in the prey resource community, predator diversity, and the ability of protists to impact on them will alter their niche complementarity and interspecific interactions which in turn will alter their contributions to functioning (Tilman et al. 1997; Sinclair et al. 2003; Balcionas & Lawler 1995; Finke & Denno 2005; Ives et al. 2005; Snyder et al. 2006; Byrnes & Stachowicz 2009).

Consequences for functional diversity

The results support the hypothesis that the interactions-based functional diversity of protists is dependent on prey community composition. The functional traits of predators manifest themselves as trophic interactions (Duffy 2002). Interactions-based functional diversity using protist ecosystem-functioning response ratios captures differences in protist trophic interactions with the prey community underpinned by phenotypic functional traits (Murase et al. 2006; Corno & Jurgens 2008; Massana et al. 2009). The results show that contributions to response ratios can vary from positive to negative according to the surrounding prey community. This is because changes in prey composition affect the resource availability, niche overlap, trophic

interactions, and complementarity of predators (Duffy 2002; Woodward & Hildrew 2002; Ritchie et al. 2012; Julia et al. 2014).

Functional traits have also recently been suggested to not retain functional relevance across species and environmental gradients (Verberk et al. 2013). Changes in species contributions to response ratios demonstrates that the relevance of the phenotypic functional traits of an organism is not static (Verberk et al. 2013). Key aspects of biological context are crucially not taken into account by functional diversity based on morphological feeding traits, a key element to understanding the functional diversity of predators the sign of whose effect should depend on the food-web context (Duffy 2002; Ritchie et al. 2012; Schneider & Brose 2013). Indeed, I would expect to find different protist functional dendrogram structures and functional group compositions when applying interactions-based functional diversity, but no such variation when based on functional feeding traits for instance. These differences in protist contributions to functioning both within and between different prey communities indicate that heterotrophic protists should not be treated as a functionally substitutable trophic level (Saleem et al. 2012).

Protists may also have an equally context dependent indirect ‘predator partitioning’ effect on the bacterial community (Thebault & Loreau 2003; Saleem et al. 2013). In the presence of predators that exert selective feeding preferences, prey may avoid predators differently and complementarily (Thebault & Loreau 2003). The extent to which predators may contribute to functioning may therefore not only rely directly on complementarity of feeding preferences but also indirectly on the presence of bacterial strains that can avoid them in a complementary manner, enhancing evenness and functioning (Duffy et al. 2007; Saleem et al. 2012.)

Functional characteristics of protists

The response ratio does not allow me to partition complementarity or selection effects as in the methods of Fox and Harpole (2008) or Loreau and Hector (2001). Response ratio values smaller than 1 indicate that polyculture component species have negative effects on the functional process relative to the additive expectation. Larger response ratio values indicate increasing

complementarity. In addition, the method of Bell et al, (2009a) used to examine species contributions to response ratios does not explicitly examine the relative importance of selection effects. With these caveats in mind, the results allow for speculation about functional characteristics of protist species.

Firstly, the small number of species that contributed significantly more positively or negatively than the average to response ratios might be viewed as functionally significant species that have dominant, selection-like effects. They might also contribute through ‘keystone’ like effects on the surrounding community (Paine 1969; Power et al. 1996; Brose et al. 2005). The results would also fit the theory that keystone effects are context dependent (Brose et al. 2005). Significant non-linear richness terms in the RPD analyses indicated that species interactions were important, but the large coefficients indicated that complementarity was low (Bell et al. 2009a). According to Bell et al (2009a) this indicates that there are large selection effects in which they include facilitation.

Secondly, significant positive correlations between individual species’ coefficients of contribution to response ratios for contrasts of bacterial communities (see table 3) indicated that the species in question had the same effects when feeding on either prey community. A significant negative correlation showed that the species had highly opposed effects in the different community backgrounds across all functions. A lack of correlation indicated that for some functions the species had the same effect on response ratios and for others it differed according to prey community. Those such as species G9 that had mostly significant positive correlations (see table 3) might be considered functional generalists, since they seem to have broadly the same effects on functional measures across most background communities. Those with a greater number significant negative correlations can be viewed as more functionally specialist, with strong contrasting effects on response ratios between background bacterial communities This might form the basis of future approaches to functionally characterising species across multiple functions

Correlating communities and functioning

The results support the hypothesis that single functions may be correlated to bacterial community composition and that by extension, protist contributions are too (Langenheder et al. 2006; Jiang & Krummins 2006; Szabó et al. 2007; Peter et al. 2011; Frossard et al. 2012). There were a greater number of significant correlations of protist contributions to response ratios between bacterial communities for multifunctioning. In contrast, single functions had few significant positive correlations, particularly for measures of enzymatic activity. This supports the argument that functional redundancy may not be as pervasive in microbial communities as previously thought (Langenheder et al. 2006; Hector and Bagchi, 2007; Szabó et al, 2007; Gamfeldt et al. 2008; Mouillot et al. 2013b). Furthermore, it supports the notion that coarse measures integrate across multiple processes (Langenheder et al. 2006; Frossard et al. 2012).

Chapter 5 – Bacterial diversity effects on protist predator functional relationships and community functioning

Abstract

The saturating relationship between levels of ecosystem functioning and species richness has been well documented in a number of systems, both macroscopic and microscopic. In microbial predator-prey systems most attention has been paid to top-down diversity effects of protist predators on bacterial communities. Only recently has work begun to address bottom-up diversity effects of prey on predator abundance and performance. There has been little attempt to test how these bottom-up effects impact functional relationships and complementarity between heterotrophic protists, or how they feedback on ecosystem functioning. Furthermore, I was interested in how such differences in prey community structure might affect estimations of functional diversity based on species' contributions to functioning since it is based on interactions between pairs of protists. I used a large scale microcosm study of 2232 microcosms containing bacterial treehole communities and protistan predators along with a number of functional bioassays to test the relationship. I found that ecosystem functioning varies predictably along prey diversity gradients. However, I found that prey diversity has no overall effect on trends in complementarity between protists though the contributions of individual species vary. A small number of species have significantly more positive or negative contributions on complementarity than the average. I propose that these might be viewed as either species performing keystone roles or that are disproportionately affected by changes in bacterial diversity.

Introduction

Changes in species diversity and abundance are non-random, with rarer species and predators at particular risk (Hutchinson 1959; Menge & Sutherland 1987; Cardillo et al. 2005; Bracken & Low 2012). Species losses from communities have complex, cascading effects on community structure and functioning (Terborgh et al. 2001; Dulvy et al. 2004; Finke & Denno 2004; Bruno & Cardinale 2008; Thébault et al. 2007). Consequences of such losses have been intensively investigated in macroscopic systems (Duffy et al. 2007; Estes et al. 2011). Microbial diversity and ecosystem functioning have almost exclusively been studied in the context of productivity effects on bacterial communities, bacterial community diversity effects on functioning, and the role of protists in regulating bacterial community structure (Zak et al. 1994; Sherr & Sherr, 2002; Jiang & Morin 2005; Bell et al. 2005; Corno & Jurgens, 2008; Bell et al. 2010; Frossard et al. 2012).

A number of hypotheses exist regarding the effect of prey diversity on predator interactions. These include the variable edibility hypothesis (Leibold 1989), the balanced diet hypothesis (DeMott 1998; Gamfeldt et al. 2005), and resource concentration hypothesis (Root 1973). Despite recognition of prey community effects on predators, investigation of these predictions in terms of bacterial diversity feedbacks on protistan communities is still lacking (Woodward & Hildrew 2002; Gamfeldt et al. 2005; Ritchie et al. 2012; Saleem et al. 2013). A limited number of studies have addressed interactions of prey and predator diversity on microbial community performance and interactions, but though they discuss possible impacts on ecosystem processes they do not measure them experimentally (Gamfeldt et al. 2005; Saleem et al. 2012; Saleem et al. 2013).

The nature of functional interactions between pairs of protists depends on the degree to which they promote community structures that are complementary in terms of functional output. Indeed, the functional traits of consumers are manifested as their trophic interactions and their functional diversity as the range of unique combinations of prey that they impact (Duffy 2002;

Schmitz 2007; Schneider & Brose 2013). These are not static properties of predators and are likely to depend on the overlap of prey types they predate on, how generalist or specialist predators are, the abundance of suitable prey, and the presence of intraguild predation (Arim & Marquet 2004; Schmitz et al. 2010; Ritchie et al. 2012). This is likely to be partly constrained by prey diversity.

Protists are known to have differences in prey selectivity due to - among others – specialism or generalism, size, and swimming speeds (Boenigk & Arndt 2000; Boenigk et al. 2002; Sherr & Sherr 2002; Bell et al. 2010; Glücksman et al. 2010; Beveridge et al. 2010c; Julia et al. 2014). Their complementarity and effects on functioning are therefore directly related to their feeding niches and trophic interaction functional traits which are determined by the availability of prey resources (Duffy 2002; Snyder et al. 2006; Byrnes & Stachowicz 2009; Saleem et al. 2013).

Protists are known to affect measures of ecosystem processes such as nitrogen mineralisation, SO_4^{2-} reduction, decomposition, and carbon transformation (Murase et al. 2006; Corno & Jurgens 2008; Saleem & Moe 2014). This is thought to be due to both non-consumptive effects of nutrient release and consumptive effects on bacterial community structure (Mattison & Harayama 2001; Saleem & Moe 2014). However, investigating bacterial diversity effects on functioning in isolation or considering only top down effects of protists is only part of the story. It is important to understand how changes in prey diversity affect relationships within communities of predators in terms of their effects on functioning.

There is a contrast between studies that find a connection between bacterial community structure and functioning (Bell et al. 2005; Langenheder et al. 2006; Langenheder et al. 2010; Peter et al. 2011) and those that do not (Langenheder et al. 2005; Wertz et al. 2006; Östman et al. 2010; Frossard et al. 2012). These can be categorised in to studies that used tightly controlled assemblages (Bell et al. 2005; Langenheder et al. 2010; Peter et al. 2011) and those that have used indirect manipulations such as dilution series (Langenheder et al. 2005; Langenheder et al. 2006; Wertz et al. 2007; Östman et al. 2010). Such differences and disconnects are suggested to

be due to a number of reasons, ranging from coarseness of ecosystem processes; divergence of diversity considered, and simplification of experimental assemblages (Frossard et al. 2012).

In Chapters 2 and 3, I measured and tested the context dependence of the functional diversity of my study group of protists based on their interactions of contributions to ecosystem processes.

In Chapter 4 I investigated the effects of bacterial community composition on contributions to functioning of individual species. This interactions-based functional diversity of protists is founded on the premise that protist predators have different effects on bacterial community structuring and are important in regulating bacterial community abundance, composition, and processes (Šimek et al. 1997; Pernthaler 2005; Bell et al. 2010; Glücksman et al. 2010). In this chapter I moved from testing the effects of composition of natural bacterial communities to the effects of manipulation of a single community's diversity on protist functional interactions.

Using 2232 microcosms, I tested a number of hypotheses: 1) ecosystem functioning and complementarity of protists was affected by bacterial diversity, indicating that community structure not only affected functioning directly, but also protist functional effects and diversity by altering their functional feeding niche space; 2) neither are affected, indicating that ecosystem functioning and complementarity of protist effects are unrelated to bacterial community diversity; 3) ecosystem functioning is affected while complementarity and functional organisation of protists is not, indicating that ecosystem functioning is linked to bacterial diversity, but that protist functional effects are insulated from it by compensatory mechanisms; 4) ecosystem functioning is unaffected but bacterial diversity affects protist complementarity indicating that protistan functional diversity is dependent on bacterial community structure but ecosystem functioning itself is not. These hypotheses were similar to those of Frossard et al. (2012) regarding the investigation of communities and processes along gradients of environmental conditions. I also tested the hypothesis that individual protist species' contributions to complementarity will increase in variability at ends of the bacterial diversity gradient as species face increased interspecific interactions and constraints on structuring from extremes of high and low prey diversity.

Methods

Protist strain growth and bacterial diversity community manipulation

The same eighteen protist strains as in Chapter 3 were grown as monocultures from pure stock cultures prior to the assembly of the experiment. They were grown for 7 days in 2ml of beech leaf tea (BLT) in 24 well culture plates inoculated with T143 bacterial tree-hole community to for growth. BLT production and inoculation of bacterial culture and protist strains was carried out as in Chapter 2. Strains were categorised into four broad categories based on estimated growth rates in Chapter 2; slow, medium, fast, and very fast. Slow strains were grown for six days before inoculation to experimental microcosms, medium five days, fast four days, and very fast three days beforehand. This was to ensure that all strains were inoculated into experimental microcosms at a similar stage of growth.

Naturally occurring bacterial communities (T143) were inoculated into and grown in 50ml falcon tubes of BLT for two days at 21°C and then diluted in BLT over six orders of magnitude by serial dilution to give a dilution gradient of 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . This manipulated the bacterial community diversity by progressively excluding rarer species from the community (Garland & Lehman 1999; Franklin et al. 2001). These were then left to grow back to carrying capacity ($\sim 10^6 \text{ ml}^{-1}$) for three days. Dilutions were carried out in 50ml falcon tubes to ensure enough inoculum was produced for the entire experiment. After three days the experimental microcosms were inoculated with 150µl of diluted T143 bacterial culture.

Random partitions design (RPD) and protist community assembly

Protist communities were assembled according to the random partitions design for community and biodiversity-ecosystem functioning experiments as previously described in Chapter 3. The random partitions design of the experiment was generated using R CRAN (v3.0.3). The experiment consisted of two random partitions of 1, 2, 3, 6, 9 and 18 strains, replicated twice. The whole experiment was replicated at each bacterial dilution treatment level. This produced a total experiment size of 2232 microcosms. The same strains were used as in Chapter 3.

Experimental communities were assembled following the same assembly process as described in Chapter 4 using a Hamilton (Starlet) pipetting robot into shallow well 96 well plates from pure cultures in deep well 96 well source plates. I used a substitutive design for the inoculation of protists, with monocultures containing 180µl of isolate, two species communities containing 90µl of each isolate, three species communities 60µl, and so forth. Finally, 20µl of BLT was then also added. Once assembled in shallow 96 well plates, the mixtures were added to the experimental microcosms containing 1800µl of BLT and bacterial culture. The microcosms were then left to grow for 15 days at 17°C.

Ecosystem functioning bioassays

I used the same 7 bioassays as previously described in Chapter 2 (pages 40 and 41). These were performed after 15 days from inoculation of the experimental microcosms. Assays were carried out on days 15, 16, and 17 of the experiment, and carried out in 96 well shallow well plates. Subsampling of the experimental microcosms was conducted using Hamilton (Starlet) pipetting robot after manual transfer from 24 well plates to deep well 96 well plates. The measures were then standardised using calibration curves (see Appendix 2, supplementary figure 4) before calculation of response ratios. These seven assays were chosen for the reasons described in Chapter 4. They represent two groups of assays that can be grouped according to the coarseness of the ecosystem processes they detect and are thought to reflect bacterial populations and community structure differently (Langenheder et al. 2006; Peter et al. 2011).

Calculating response ratios (RR)

Response ratios were calculated for all communities across the dilution diversity gradient. These were calculated as described in Chapter 2. The response ratios formed the basis of my method for drawing functional hierarchical cluster dendrograms and defining functional groups in Chapter 2. They allowed me to infer the degree of complementarity between species within a community in terms of their effect on ecosystem processes. Using them, I can examine whether

bacterial diversity affects contributions to ecosystem processes and functional diversity of species.

Statistical analyses

Statistical analyses were carried out using the statistical package R v.3.0.3 (R Development Core Team 2014). Analyses initially used general liner modelling (GLM) of community response ratios and ecosystem functioning values across the bacterial diversity dilution gradient. The response variables and dilution treatment variables were log transformed and models analysed using a Gaussian error distribution and identity link function. Residuals were found to be normally distributed after transformation and variance of errors constant.

Maximal models with quadratic terms were fitted and subsequently simplified where appropriate (see Appendix 3, supplementary table 1). Model simplification was carried out manually by sequential removal of least significant terms and comparison of models by ANOVA until removal resulted in a significant reduction in sums of squares (Crawley 2007). The minimum adequate model was retained.

CO₂ production in two plates was found to be very low (almost 0 µg CO₂ ml⁻¹ d⁻¹). Inspection of the data suggested that this is due to experimental failure of the MicroRespTM setup, with leakage around the seals accounting for such low CO₂ detection. These data were removed. A plot showing this is given in Appendix 1. Monoculture data was also removed from the dataset for analysis of response ratios as these are not informative since the RR of a monoculture is always 1 or 0.5 (depending on how it is calculated).

I used the analysis developed by Bell et al. (2009a) for analysis of random partitions designed experiments to sequentially apportion variance to the linear effects of species richness, the effect of the presence or absence of each component species, the effect of the number of partitions and replicates, and finally the non-linear effects of protist community species richness. This allowed me to determine the effect of each species on changes in response ratios in each of the measures of ecosystem functioning at each of the diversity dilution treatment levels.

Results

Bacterial diversity and ecosystem functioning

There were no significant interacting effects between protist and bacterial diversity for any of the ecosystem functions measured. Bacterial diversity predicted a significant negative quadratic relationship with MUG enzymatic activity (GLM: estimate = -0.02; $t = -19.2$, $p < 0.001$), Xylo enzymatic activity (GLM: $y = -0.1$; $t = -6.03$, $p < 0.001$), phosphate availability (GLM: estimate = -0.0003, $t = -4.0$, $p < 0.001$), and bacterial respiration (CO_2) (GLM: $y = -0.02x^2 - 0.22x - 1.4$; $t = -32.6$, $p < 0.001$).

Bacterial diversity had a significant positive quadratic relationship with MUN enzymatic activity (GLM: estimate = 0.01; $t = 15.7$, $p < 0.001$), MUP enzymatic activity (GLM: $y = 0.003$; $t = 6.55$, $p < 0.001$), and cellular activity (GLM: estimate = 0.006; $t = -8.9$, $p < 0.001$).

Conversely to all the other patterns of enzymatic activity observed, there was a decrease in MUN cleaved at the 10^{-1} and 10^{-2} bacterial community dilutions (means 0.07 ± 0.001 and 0.08 ± 0.001 nM respectively). There was a similar trend for MUP enzymatic activity, though the minimum of the curve was found at lower bacterial diversity than MUN.

Protists diversity and ecosystem functioning

Protist community diversity and response ratios were highly correlated. There were significant positive interactions between response ratio and protist species richness for all measures of functioning. Response ratios were low when richness was high. Higher response ratios and greater richness both predicted greater functioning for all measures. In order to partition protist species richness effects I used the RPD analysis developed by Bell et al (2009) (see methods). I found both linear and non-linear significant effects of protist richness on all measures of ecosystem functioning.

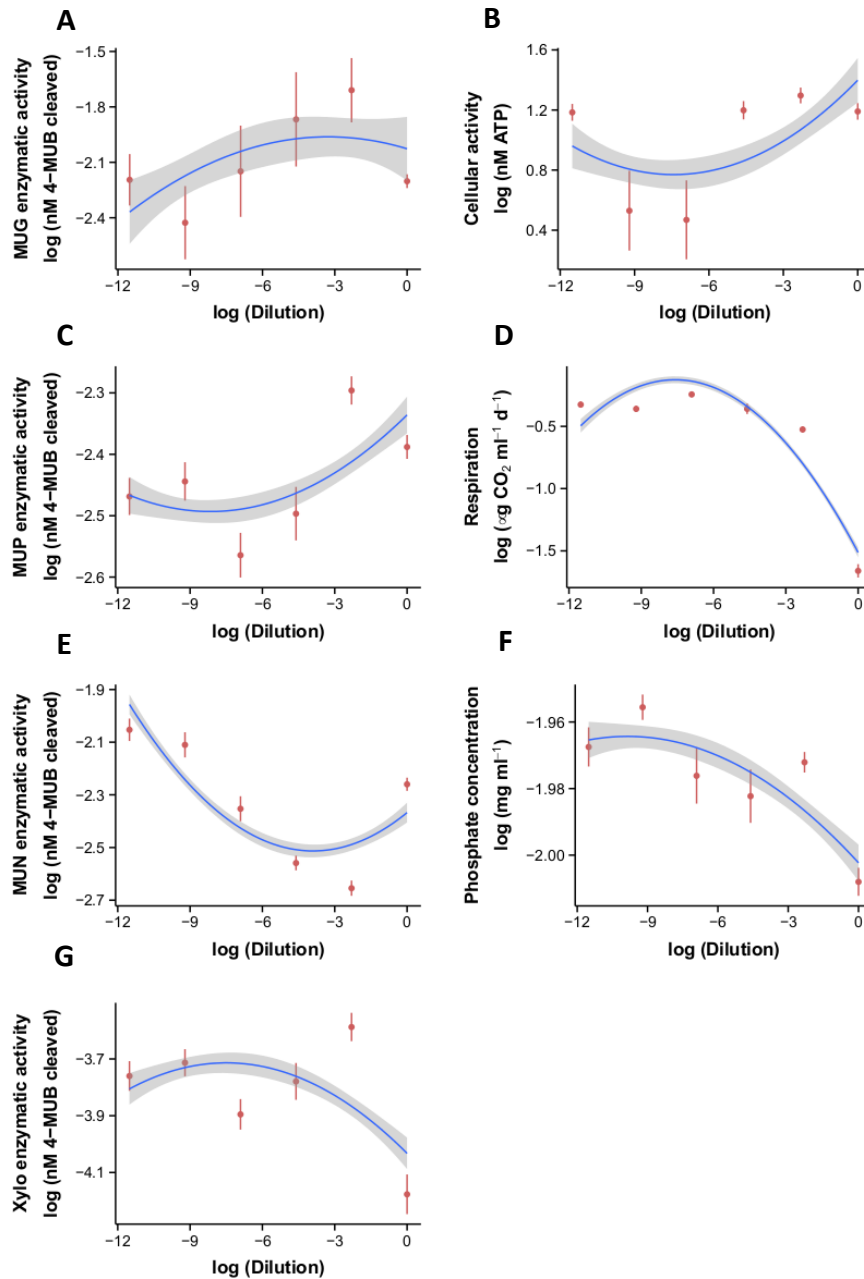


Figure 21. Scatter plots of ecosystem function against dilution. The functions are **A)** MUG enzymatic activity, **B)** cellular activity, **C)** MUP enzymatic activity, **D)** respiration, **E)** MUN enzymatic activity, **F)** available phosphate concentration, and **G)** Xylo enzymatic activity. Blue fitted lines represent the quadratic relationships fitted by GLM. Red points represent mean and 95% CI. Points are jittered for display and coloured by response ratio of the community

Bacterial diversity and overall protist complementarity

I found that there were no significant effects of bacterial diversity on protist community response ratios across the bacterial diversity gradient for any of the enzymatic activity assays. However, there was a significant quadratic relationship for ATP ($y = -0.06x^2 - 0.06x - 1.3$; GLM: estimate = -0.06, $t = -2.2$, $p = 0.026$), with greatest response ratios indicating greatest complementarity at intermediate levels of bacterial diversity.

Bacterial diversity and protist species' contributions to complementarity

Using the RPD analysis, I analysed the contributions of each species to response ratios at different bacterial diversity treatments. I found that species have differing coefficients of effects on community response ratios depending on the diversity dilution level and the functional measure in question (see figure 20). However there is great heterogeneity in the responses among species.

Across all functions, only eight protist species had significant contributions to response ratios (see figure 20). For each ecosystem function, response ratios were significantly affected by between two and four species (see figure 22). There was little pattern to the distribution of species with significant above or below average effects on response ratios across the diversity gradient.

Maximum mean magnitudes of species' contributions to response ratios were reached at either the most diverse (10^{-5} and 10^{-4}) or least diverse (10^0 and 10^{-1}) bacterial diversity treatments (see figures 22 and 23). However, inspecting the median contributions, I found that while the greatest median contributions were found at either the most diverse (10^{-5} and 10^{-4}) or least diverse (10^0 and 10^{-1}) bacterial diversity treatments, all but one of the most positive median contributions were found in the least diverse bacterial diversity treatments (10^{-5} and 10^{-4}). The most negative median contributions were found between the 10^0 and 10^{-3} bacterial diversity treatments. For means, standard errors, and medians see Appendix 4, supplementary table 2.

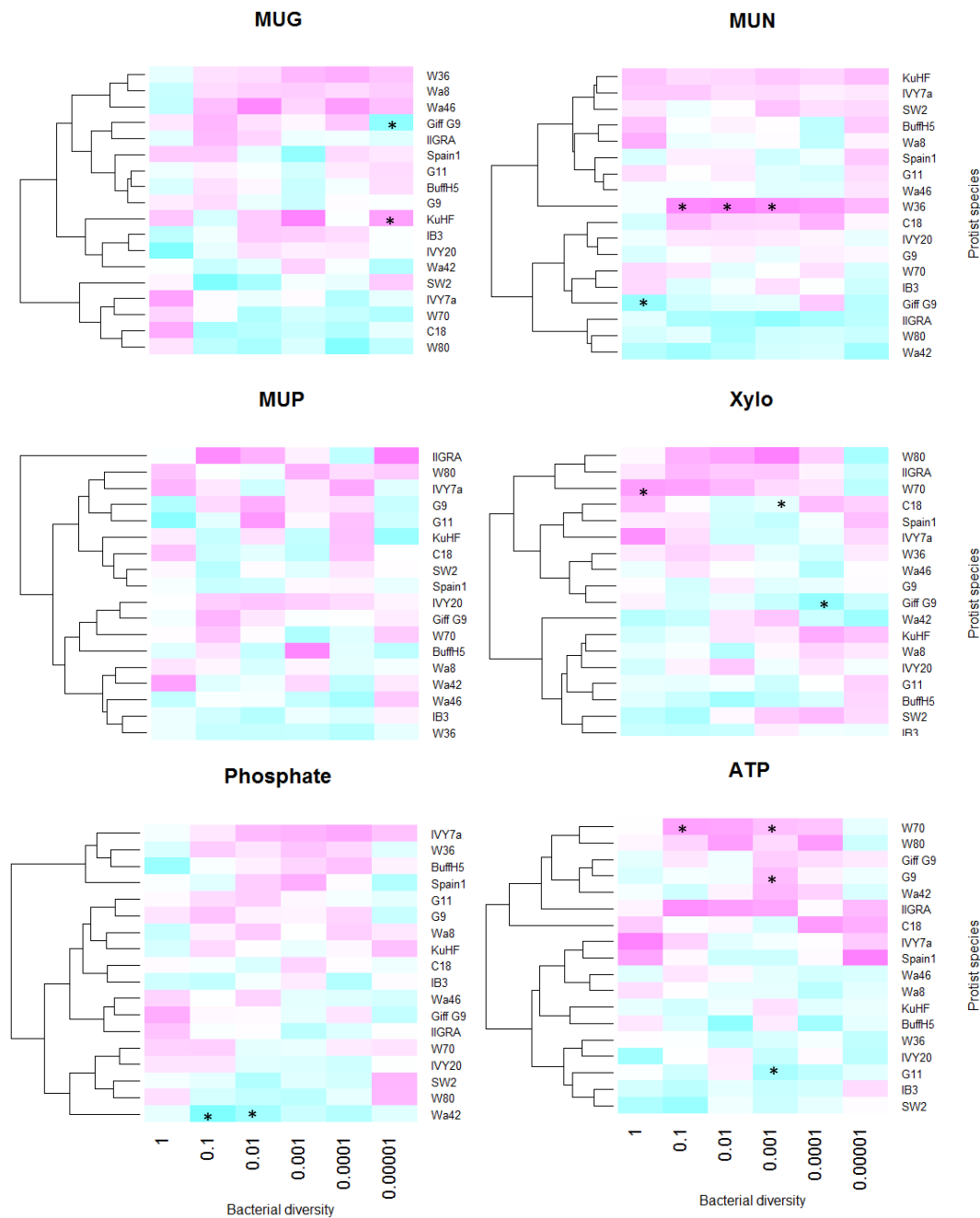


Figure 22. Heat maps representing the coefficients of species' contributions to response ratios across the bacterial diversity gradient for the four enzymatic activity measures of ecosystem functioning, as well as microcosm phosphate concentration and metabolic activity (ATP). Pink represents positive coefficient and blue represents negative ones - the stronger the colour the greater the magnitude of the coefficient. Black asterisks denote dilutions at which individual species had significant effects on response ratios.

Contributions to response ratios were most often significantly correlated between intermediate levels of bacterial diversity or between the extremes (see Appendix 3, supplementary table 4). Between diversity levels that were significantly correlated, coefficients appeared mostly to be more positive in the higher bacterial diversity treatments. This was inferred because most regressions had slopes less than 1 when the more diverse treatment level was plotted on the x axis (see figure 23 for an example). The 0.1 to 0.001 dilution levels showed the greatest number of significant correlations across the different functional measures. This indicated that contributions to response ratios were most similar either at intermediate levels of the diversity dilution gradient, or between the extremes.

Analysis by GLM of coefficients of effect on response ratios found that there were no significant overall trends across the diversity gradient (see figure 24). However, a total of five positive interactions were found between species identity and increasing bacterial diversity on contributions to Xylo enzymatic activity response ratios (see figure 24)

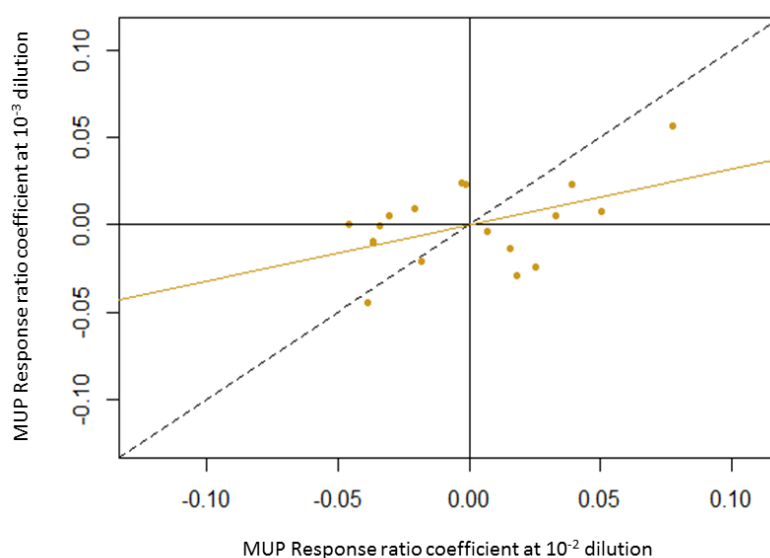


Figure 23. Correlation of species coefficients of effect on response ratios of MUP enzymatic activity. The x-axis shows coefficients in the 10⁻² diversity treatment and the y-axis in the 0.001 diversity treatment. Points represent species. The dashed line is $y = x$. Other correlations are not shown due to the large number of contrasts.

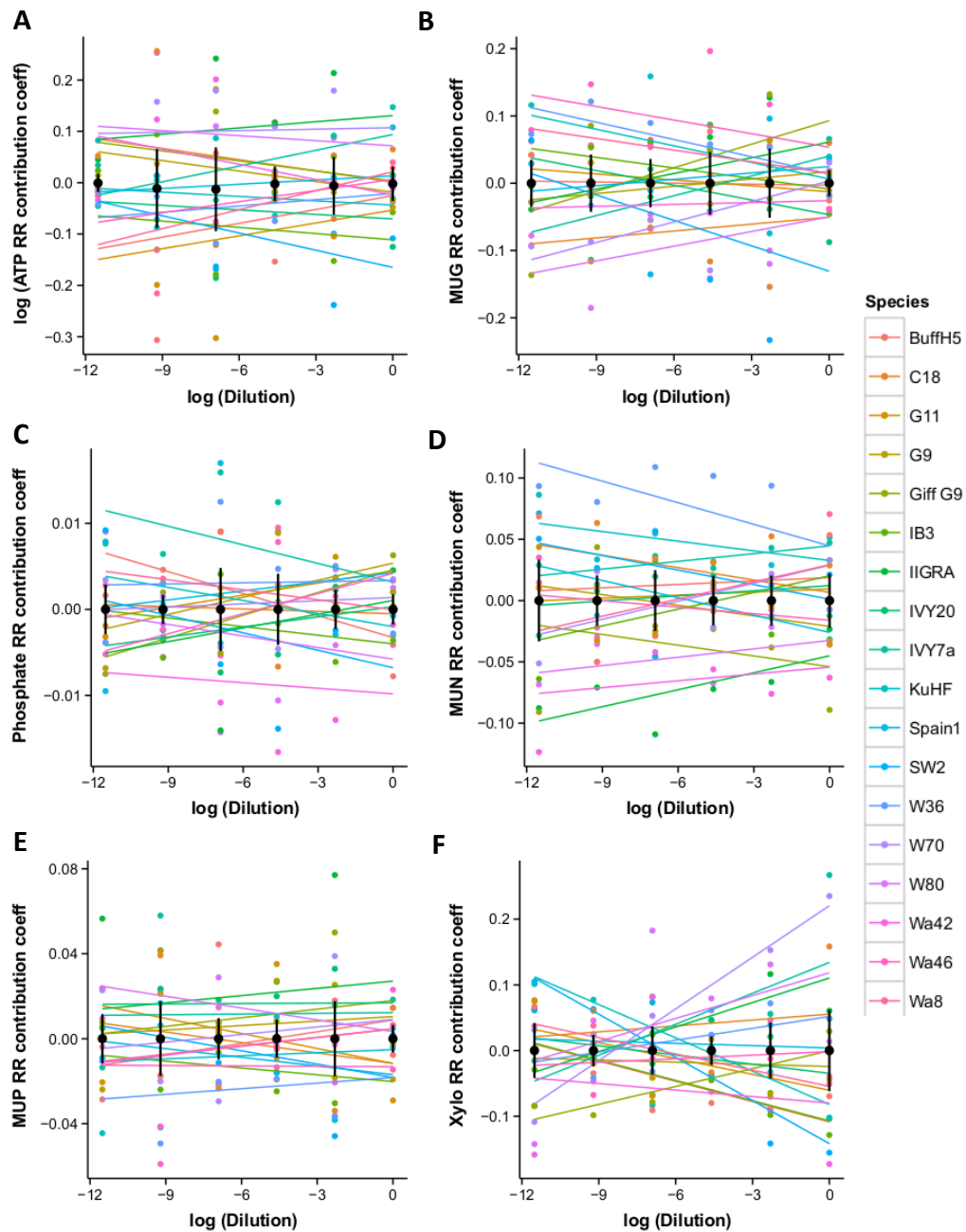


Figure 24. Species' coefficients of contribution to response ratios plotted against bacterial diversity treatment (dilution). Panels correspond to coefficients of effect on response ratios for A) ATP, B) MUG enzymatic activity, C) phosphate concentration, D) MUN enzymatic activity, E), MUP enzymatic activity, and F) Xylo enzymatic activity. Dilution has been log transformed. Black points represent means and bars are 95% CI. Points and lines are coloured by species (see legend)

Discussion

Main findings

The results support the hypothesis that bacterial diversity affects ecosystem functioning and protist complementarity at the level of single species contributions. However, they also show that though ecosystem functioning is affected there are no significant trends in protist community response ratios across the bacterial diversity gradient. The hypothesis that the strongest effects of species contributions are found at extremes of bacterial diversity is supported in some measures of functioning but not others.

Bacterial diversity and community level response ratios

There was a lack of correlation between bacterial diversity and protist community complementarity. Cellular activity (ATP) was an exception, with small but significant decreases in response ratios at the extremes of bacterial diversity. Nevertheless, the results broadly do not support the hypothesis that changes in bacterial diversity lead to predictable changes in complementarity of protist effects on functional processes. This hypothesis was based on the premise that a reduction in prey resources at low prey diversities (Garland & Lehman 1999; Franklin et al. 2001) would lead to reduced niche partitioning, complementarity, and increased competition due a reduction in available resources (Chesson 2000b; Loreau 2000; Hector & Loreau et al. 2001; Chase & Leibold 2003).

At low prey diversity, contributions to functioning by predators were hypothesised to be more negative due to decreased niche complementarity, increased competition and interspecific interference, and increased predation pressure on decreasing numbers of prey types (Woodward & Hildrew 2002; Snyder et al. 2006; Byrnes & Stachowicz 2009). At high prey diversities, reduced foraging success by (Root 1973; Leibold 1989) might result in predator effects on community function to be increasingly less additive than at intermediate levels of diversity. Predator communities with higher numbers of specialists are expected to be particularly affected

by interference by unsuitable prey types (Root 1973; Ostfeld & LoGiudice 2003; Joshi et al. 2004; Keesing et al. 2006) and increased selectivity by predators when bacterial abundances positively co-vary with diversity (Boenigk et al. 2002). In addition, effective predation is reduced due to increased bacterial abundance and defences, which in turn reduces the ability of protists to enhance processes such as biodegradation (Matz & Kjelleberg 2005; Saleem et al. 2012; Saleem et al. 2013). A combination of body size constraints and resource availability may also lead to negative interactions such as intraguild predation (Williams & Martinez 2000; Woodward & Hildrew 2002; Poisot et al. 2011)

In the absence of bacterial diversity effects on community level response ratios in six of the seven measures, it is possible to come to one of two conclusions. On one hand, the mechanisms suggested to link bacterial diversity to protist community complementarity of effects on functional processes are not operating. The alternative is that these feedbacks from bacterial diversity do impact individual protist species, but that compensatory mechanisms maintain protist community level effects on functional processes.

Protist species effects and compensatory mechanisms

Bacterial diversity affected individual protist species' contributions to complementarity of functional interactions but not the overall complementarity of protist communities. This suggests that there were compensatory mechanisms that maintained protist community complementarity in spite of effects on individual species. Figure 22 shows that coefficients of effect on response ratios varied from positive to negative across the bacterial diversity gradients and that this variation was different for different species. Notably there was clustering in the MUG, MUN and Xylo enzymatic activities of species into groups that have opposing trends of positive or negative effects along the diversity gradient (see figure 22). The small number of species that contributed significantly above or below average to response ratios also changed with bacterial diversity between functions (see figure 22). These differences between species' responses to bacterial diversity might have had compensating effects on response ratios at the

community level. However, what is less clear is why these species had such a strong effect and whether they acted to compensate for changes in other species contributions to response ratios. They might have played an inherent ‘keystone’ role through their impacts on bacterial populations (Mills et al. 1993; Power et al. 1996), or they might have been particularly strongly affected by bacterial diversity.

Bacterial diversity is known to have a number of impacts on protists and differences between protist species responses to these are likely to drive compensatory mechanisms (Saleem et al. 2013). Bacterial diversity can impact protistan predation through changes in bacterial abundance (Saleem et al. 2013), defences (Pernthaler 2005; Matz & Kjelleberg 2005), availability of suitable prey (Leibold 1989; Saleem et al. 2013), and detection or availability of suitable prey (Root 1973; Gamfeldt et al. 2005; Jousset 2011; Saleem et al. 2013). Different protists can be expected to respond differently since they show differences in feeding mode related to prey selectivity, size, swimming speed, and predation mode (Posch et al. 2001; Boenigk et al. 2002; Sherr & Sherr 2002; Pfandl et al. 2004; Bell et al. 2010) which may lead to reversals of competitive interactions (Saleem et al. 2013). In addition, if species’ niches are determined by resources and species impacts upon them (Tilman 1982; Chase & Leibold 2003), then changes in prey resource availability affect niche occupancy of protist predators. This might affect niche complementarity with regard to feeding modes and affect interspecific interference levels (Snyder et al. 2006; Byrnes & Stachowicz 2009). As some species are affected and become more constrained, other species may be able to re-occupy newly available niche space or niches that have become vacant (adaptive zones) through ecological sorting if they have the genotypic plasticity to do so (Wellborn & Langerhans 2015). Total occupancy of niche and functional space is unaltered, but how species contribute has.

Consequences for protist functional diversity

Measures of protist interactions-based functional diversity applied with metric such as FGR, FD, and mean dissimilarity are unlikely to change substantially in response to changes in community structure because overall, response ratios were unaffected by prey diversity. This would leave distances between response ratios (see Chapter 2, page 48, box 1) the same on average. However, the contributions of species to response ratios will undoubtedly affect the precise form of dendrograms and composition of functional groups.

The results imply a degree of protistan functional redundancy in which predators can compensate each other's contributions to functional processes different responses to bacterial diversity. Functional diversity is expected to insure ecosystem functioning against disturbances through redundancy of functional traits and differences in species responses to the environment (Tilman 1999; Yachi & Loreau 1999; Petchey & Gaston 2006; Loreau & De Mazancourt 2013). This experiment did not explicitly test effects of disturbances on bacterial diversity and subsequent protist functional effects. However, the results suggest that differences in protist contributions to ecosystem processes may insure community level contributions against changes in prey diversity which could result from disturbance.

Functional traits of consumers manifest themselves as their trophic interactions (Duffy 2002). Functional diversity in this context is the number of unique combinations of prey species impacted by predators (Duffy 2002). At least in the special case of predators (and perhaps consumers more widely), this is a flexible and context dependent quality (Duffy 2002; Duffy et al. 2007; Schmitz 2007; Schneider & Brose 2013; Verberk et al. 2013). This flexible nature of protist predator functional diversity may contribute to the compensatory mechanisms discussed. I speculate that this is due to the interplay between differences in predation traits of protists and bacterial diversity impacts on predation (Sherr & Sherr 2002; Posch et al. 2001; Pfandl et al. 2004; Pernthaler 2005; Saleem et al. 2013). There is evidence that differences in protist traits and responses to abiotic disturbances have stabilising effects on communities (Leary et al.

2012). There is also direct evidence from macroscopic systems that show stabilising effects of predator trait diversity on prey populations during abiotic disturbances (Griffin & Silliman 2011). It is therefore reasonable to propose that analogous biotic mechanisms exist.

Bacterial diversity and magnitude of protist species' contributions

Individual species' contributions to response ratios of MUG, MUN, and Xylo enzymatic activities were greatest in magnitude and variability at the extremes of the diversity gradient. Protist contributions were also more correlated between extremes of the bacterial diversity gradient or between moderate levels. Changes in diversity at moderate dilutions are likely to be small due to counteracting effects of evenness and richness until the dilution factor surpasses the number of bacterial strains (Franklin et al. 2001). If protist impacts on functioning depend on prey resource availability and diversity, then contributions to functioning will be most pronounced at extremes of prey diversity when dilution most severely impacts diversity. This is because bacterial diversity affects strength of consumer impacts by protists most strongly at these diversities through pronounced effects on bacterial interactions, defence, and predation success (Pernthaler 2005; Jousset 2011; Saleem et al. 2012).

Unlike enzymatic activity, magnitudes of contributions to ATP and phosphate response ratios were greatest at moderate levels of diversity. The difference is that broad measures such as these are coarser measures of functioning since they integrate many processes of cellular metabolic activity and productivity (Frossard et al. 2012). Such measures are known to be enhanced by protists due to consumptive effects of the microbial loop and non-consumptive effects of nutrient release (Mattison & Harayama 2001; Corno & Jürgens 2006; Saleem & Moe 2014). Since protist predation is expected to perform best at intermediate to low levels of prey diversity, effects on complementarity of effects on functioning were most pronounced here too.

Bacterial diversity effects on ecosystem function

The results showed strong effects of bacterial community diversity on ecosystem functioning. This supports previous work that shows that bacterial community structure is a key determinant

of ecosystem functioning (Langenheder et al. 2010; Peter et al. 2011). However, it contrasts with many studies that do not find such relationships when experimental manipulations such as dilution gradients are applied (Langenheder et al. 2005; Wertz et al. 2007; Östman et al. 2010). The results show significant unimodal relationships, four of which are negative with lowest functioning at the extremes of the bacterial diversity gradient. This was least pronounced in phosphate concentration, and MUP enzymatic activity and most in CO₂ production. Of the three with positive quadratic functions fitted, two (MUP and ATP) show decreased functioning in the most diverse communities and highest in the moderate 10⁻¹ or 10⁻² dilutions with the relationship driven by the subsequent diversity treatments.

The results are in line with previous works that commonly find decreased functioning at high bacterial diversity. For instance, Bell et al (2005) found that at high bacterial diversity, community respiration was lower. High functional redundancy and low complementarity leads to resource and interference competition which reduces ecosystem functioning (Bell et al. 2005). Additionally, high bacterial abundances at high diversity levels are known to lead to greater competition for resources and stronger antagonistic interactions, which in simple environments are the main architects of community functioning (Inglis et al. 2009; Jousset 2011; Saleem et al. 2012; Saleem et al. 2013).

Becker et al (2012) found that strong antagonistic effects caused bacterial population collapses, losses in plant host protection, and decreases in root colonisation. My results support this idea of “negative complementarity effects” in bacterial communities (Becker et al. 2012). These may be mediated by the production of toxins to enhance competitive ability by multiple strains resulting in high levels of mutual inhibition (Kerr et al. 2002; Czárán et al. 2002). Strains such as *Jathinobacterium* sp. are thought to be particularly competitive due to their ability to produce antimicrobial metabolites (Brucker et al. 2008). Bacteria also sense and respond to competitors through quorum sensing, resource limitation, cross-talk and chemical cues (Haas & Keel 2003; Dubuis & Haas 2007; Garbeva et al. 2011). Becker et al (2012) propose that this plasticity accounts for the prevalence of negative complementarity effects. These effects support the

explanation in Chapter 3 that negative complementarity of protist functional diversity on ecosystem functioning might be caused by predation induced negative complementarity in the bacterial community.

At high bacterial diversity it is more likely that the community will contain more resistant strains, as described by the 'variable edibility hypothesis' (Leibold 1989). Predation by protists may also be less effective due to resource dilution effects (Root 1973). These effects on functioning are likely to be important if rarer, less competitive species perform key functions but face strong competition from predation resistant strains at high diversities when predation is less effective (Szabo et al. 2007).

At moderate levels of bacterial diversity ($10^{-1} - 10^{-2}$) the results show the highest levels of functioning for all measures except MUN enzymatic activity. Reduced diversity increases positive complementarity and increases evenness and bacterial performance (Becker et al. 2012; Saleem et al. 2012). Increased evenness, weaker bacterial interactions and predator defence strategies all allow for more efficient predation by protists resulting in stronger and enhancing effects on ecosystem processes (Mattison & Harayama 2001; Mattison et al. 2005; Pernthaler 2005; Saleem et al. 2012; Saleem et al. 2013; Saleem & Moe 2014). This has shown to feed back through protist grazing of dominant bacteria, further enhancing bacterial evenness (Jiang & Krummins 2006).

Reduced functioning at low diversity is due to a loss in functional diversity conflated with losses in species richness, reducing efficient resource use and partitioning (Franklin et al. 2001; Petchey & Gaston 2006; Cadotte et al. 2011). In addition, rare species that are lost by dilution are shown to be important for functioning (Szabó et al. 2007; Mouillot et al. 2013b). Higher predation pressure and very low bacterial abundance may compound the effect of bacterial species loss on functioning, outweighing the positive effects of protist predation such as protist mediated nutrient release (Mattison & Harayama 2001; Mattison et al. 2005).

Chapter 6 – Bacterial diversity effects on protist competitive interactions

Abstract

Phagotrophic protozoa are a major cause of microbial mortality but the inter-relationships between protozoa and their prey communities remains poorly understood. I investigated the effects of changing bacterial prey community diversity on the interactions between heterotrophic protist predators. I used laboratory microcosms of naturally occurring communities of bacteria and co-occurring protists to understand how manipulations of bacterial diversity impacted predator communities. Bacterial diversity was manipulated using serial dilution, which allowed me to retain the complex prey communities. I found that the manipulation of bacterial diversity had a significant impact on competitive relationships among protist strains and on the total biovolume of the protist communities. I also found that the effect of the type of species mixture a particular protist found itself in was also significant. The bacterial diversity treatment also impacted initial bacterial abundance, thus I could not distinguish whether the bacterial diversity or bacterial abundance were responsible for altering the competitive relationship among the predators.

Introduction

Changes in species diversity and abundance in response to disturbance is non-random, with rarer species and predators at particular risk of extinction (Hutchinson 1959; Menge & Sutherland 1987; Cardillo et al. 2005; Bracken & Low 2012). Species losses from communities can have cascading effects on community structure and functioning leading to further species loss (Terborgh et al. 2001; Dulvy et al. 2004; Finke & Denno 2004; Bruno & Cardinale 2008; Thibaut & Connolly 2013). Consequences of diversity loss have been intensively investigated in macroscopic systems (Duffy et al. 2007; Estes et al. 2011). However, far less is understood about their consequences for microbial interactions (Saleem et al. 2013).

Studies of macroscopic systems have investigated the effects of both consumer and plant diversity loss (Lubchenco 1978; Novotný & Basset 2000; Duffy 2002; Haddad et al. 2009; Bracken & Low 2012; Ebeling et al. 2014). For instance, Ebeling et al (2014) showed that plant diversity had positive effects on invertebrate herbivore diversity and abundance. In a different system, Bracken and Low (2012) found that in a diverse assemblage of seaweeds and sessile invertebrates, realistic losses of rare species resulted in large percentage declines in consumer biomass (Bracken & Low 2012). Meta-analyses by Edwards et al. (2010) also revealed that prey diversity could weaken the top-down control of sessile marine organisms by predators.

In microbial systems, the effects of top-down control of bacterial prey community structure and functioning have been well studied (Pernthaler 2005, Corno & Jurgens 2008; Bell et al. 2010; Glucksman et al. 2010). However, there is evidence that in microbial systems bacterial prey communities can influence predator communities, not least through mechanisms of bacterial defence (Jousset 2011). Sintes and del Giorgio (2014) also showed that changes in community structure of the bacterial prey community had negative feedback effects on heterotrophic protist single cell activity. There has also been recent evidence specifically pointing to influences of bacterial diversity on protistan predator performance and community stability (Saleem et al. 2013). This study found positive influences of prey diversity on the evenness and abundance of

predators, with the degree to which these effects occurred varying between predators (Saleem et al. 2013). Understanding these effects of bacterial prey diversity on protistan predator competitive interactions is important since competition is a mechanism for coexistence and determines community structuring (Chesson 2000b). Elucidating the factors influencing competition may clarify the role it plays in maintaining the high diversity of heterotrophic protists found in microbial communities (Lennon and Jones 2011).

Changes in prey diversity are predicted to affect predators through a number of hypothesised mechanisms. First, the dilution or resource concentration hypothesis predicts that generalist predators should be favoured in high diversity communities. In communities with highly diverse prey assemblages, the effects of specialists should decrease due to poorer foraging efficiency, due to the decreasing likelihood of encountering suitable prey (Root 1973; Ostfeld and Lo Giudice 2003; Joshi et al. 2004; Keesing et al. 2006). This effect on predator biomass could be compounded by an increase in inter- and intraspecific competition between predators. Secondly, the variance in edibility hypothesis postulates that a resource base with greater diversity is more likely to contain species that are resistant to predation and can dominate in the presence of consumers (Leibold 1989; Duffy 2002; Hillebrand & Shurin 2005). Thirdly, the balanced diet hypothesis proposes that a more diverse resource base will provide a greater proportion of the range of nutritional resources required by species, thus increasing predator biomass and potentially strengthen top-down pressures on lower trophic levels (DeMott 1998). Despite these hypotheses, the upward effects of prey on predator interactions remains little investigated and results mixed (Fox 2004; Gamfeldt et al. 2005; Saleem et al. 2013; Edwards et al. 2010)

In previous chapters I investigated the effects of bacterial community composition and diversity on functional interactions of protists and protist functional diversity. However, I did not directly investigate the effect of bacterial communities on protist interspecific interactions such as competition that might underlie some of the results. Furthermore, investigating interspecific interactions - and in particular competition – is important for understanding what drives the coexistence of such diverse organisms.

In this chapter, I investigated the effects of bacterial community diversity on the interactions between heterotrophic protists. I used laboratory microcosms of naturally occurring communities of bacteria and co-occurring protists to bridge the gap between simple microcosm experiments and field studies. Using serial dilutions of bacterial communities to manipulate diversity also provided me with a simple method of replicating losses of rarer species (Franklin et al. 2001). This approach allowed me to retain the natural complexity of ecosystems, as well as the control and replication necessary to partition effects of prey and predator diversity changes on predator interactions and community structuring.

Methods

Experimental setup and method

Four strains of protists were selected to be used in the experimental set up. These were; SW2 (*Paracircomonas* (= *Cercobodo*) *minima*), F2 (*Allapsa brevifila*), CasphII (*Cercomonas pigra*), and Ivy20 (*Cercomonas hederæ*) (Bass et al. 2009). For simplicity I refer to them as species A, B, C and D respectively from here on.

The experiment included the following steps: On day 1, six wells of a 24-well plate were filled with 1485µm of beech leaf tea (BLT). Each was inoculated with 15µm frozen stock of a naturally occurring tree hole bacterial community. This introduced around 1500 cells, assuming a bacterial density of the order of 10^5 cells ml⁻¹. The bacteria were allowed to grow at 21°C for two days. On day 3 each of these wells the growing bacterial cultures were diluted up to six orders of magnitude (i.e. 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5}) in BLT. This dilution series served to manipulate the bacterial diversity at each dilution by removing rarer species (the tail of the species abundance curve). These were then left to grow for two days, after which total metabolic activity was measured using a BacTiter-Glo™ ATP Cell Viability Assay (Promega Corporation, USA) on a FluoStar Optima (BMG LabTech) luminometer.

On day 5, nine 24-well plates were filled with 1350µl of sterile BLT per well. These were inoculated with 150µl of the re-grown diluted bacterial culture. These were again allowed to grow for two days. After two days the protist strains were added to the bacterial communities. These were added as four monocultures, all pair-wise combinations, and one full four-way combination. The inoculated biovolume of each protist strain was equilibrated at the initiation of the experiment. I used an additive design where the starting biovolume was the same for each protist independent of the number of protist strains that were inoculated. One negative control with no protists was also retained. This provided 12 combinations of protists, across 6 levels of bacterial prey diversity. Each experiment unit was replicated 3 times, giving a total of 216 microcosms. Prior to the addition of the protists, a Bactiter-glo™ ATP Cell Viability (Promega

Corporation, USA) was used to determine the initial bacterial metabolic activity. The protists were allowed to grow for two days prior to counting using a bifocal inverted microscope. Further details of the counting protocol are given in Appendix 1. From day 9 onwards, the protist abundance of each species in mixture or monoculture was counted every 24 hours for five days.

Statistical analysis

All statistical analyses were carried out using the statistical package R CRAN v.2.12.0 (R Development Core Team, 2010). ATP luminescence relative light units (RLU) from the BacTiter-Glo Cell Viability assay data were analysed using a generalised liner model (GLM) with gamma errors and log link function. Protist biovolume was analysed as a function of the interaction between bacterial and protist diversity using a generalised liner model (GLM) with a log-link function. Biovolume data was +1 transformed to allow analysis despite 0 scores in the raw data. Count data were analysed using a GLM with quasipoisson errors and log-link function. Where quadratic terms were included in models, models with and without these terms were compared by ANOVA. Presence-absence data were also used to model species identity effects and interactions between protist species. Due to the significant effect of bacterial diversity manipulations on ATP concentrations I tried to account for this during statistical modelling in order to avoid confounding effects of bacterial diversity and abundance on protist biovolumes. I achieved this by adding ATP as a covariate of bacterial diversity to the GLM used to analyse the interactive effects of bacterial diversity and protist diversity.

Results

Bacterial diversity effects on microcosm ATP

Analysis found that there were significant effects of bacterial diversity manipulations measures of microcosm ATP concentration (figure 22). The minimal model contained a significant quadratic term (GLM: estimate = 3.75, $t = 4.00$, $p < 0.001$) and linear term (GLM: estimate = -3.50, $t = -3.66$, $p < 0.001$) for prey diversity (dilution treatment).

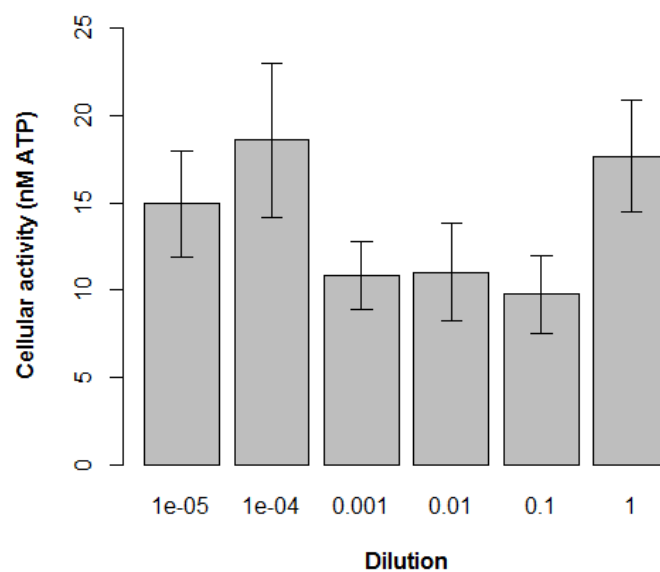


Figure 25. Bar plot of ATP concentration against bacterial diversity treatment. Error bars are standard error. Means and standard errors are as follows; $10^0 = 17.6 \pm 3.21$, $10^{-1} = 9.78 \pm 2.23$, $10^{-2} = 11.0 \pm 2.82$, $10^{-3} = 10.8 \pm 1.97$, $10^{-4} = 18.6 \pm 4.42$, $10^{-5} = 14.9 \pm 3.06$

Bacterial and protist diversity effects on total protist biovolume

Modelling by GLM of effects of protist diversity and bacterial diversity treatments on protist predator biovolumes (see figure 26A), found that the interaction between protist diversity and bacterial diversity treatment was significant. The interaction was driven by the disproportionate positive effect of increasing bacterial species richness on biovolumes in four species protist communities (GLM: estimate = 1.02, $t = 2.54$, $p = 0.011$). There was a significant effect of protist diversity on total protist community biovolume. Both four and two species communities had significantly smaller total biovolumes than monocultures (four species GLM: estimate = -0.92, $t = -5.42$, $p < 0.001$; two species GLM: estimate = -0.48, $t = -3.47$, $p < 0.001$). Bacterial diversity dilution also had a significant effect and the model retained a significant quadratic term (GLM: estimate = -3.48, $t = -2.43$, $p < 0.001$). The model fitted the quadratic curve $y = -3.48x^2 + 2.33 + 10.1$. ATP concentrations were found to be a non-significant covariate in all models.

Bacterial and protist diversity effects on total protist abundance

Similar results were found for effects bacterial diversity treatment and predator diversity on total protist abundances. There was a significant negative effect of prey bacterial diversity on total protist abundances (GLM: estimate = -2.15, $t = -5.83$, $p < 0.001$). When dilution treatment was treated as a categorical variable to examine pairwise differences in prey diversity treatment level effects on total protist abundances, I found that this relationship was driven by a strong significant difference between the 10^0 dilution treatment and all others (Tukey HSD: $p < 0.001$). No quadratic term was fitted. There was also a significant effect of protist diversity on total biovolumes. Four species and two species communities had significantly lower total abundances than monocultures (four species GLM: estimate = -1.54, $t = -10.8$, $p < 0.001$; two species GLM: estimate = -0.69, $t = -8.89$, $p < 0.001$). Increasing protist community diversity flattened the slope of the protist diversity*bacterial diversity relationship significantly. Two and four species protist communities had smaller decreases in protist abundance with increasing bacterial

diversity (four species*dilution GLM: estimate = 1.67, $t = 3.18$, $p < 0.001$; two species*dilution GLM: estimate = 0.91, $t = 2.17$, $p = 0.03$; see figure 26B).

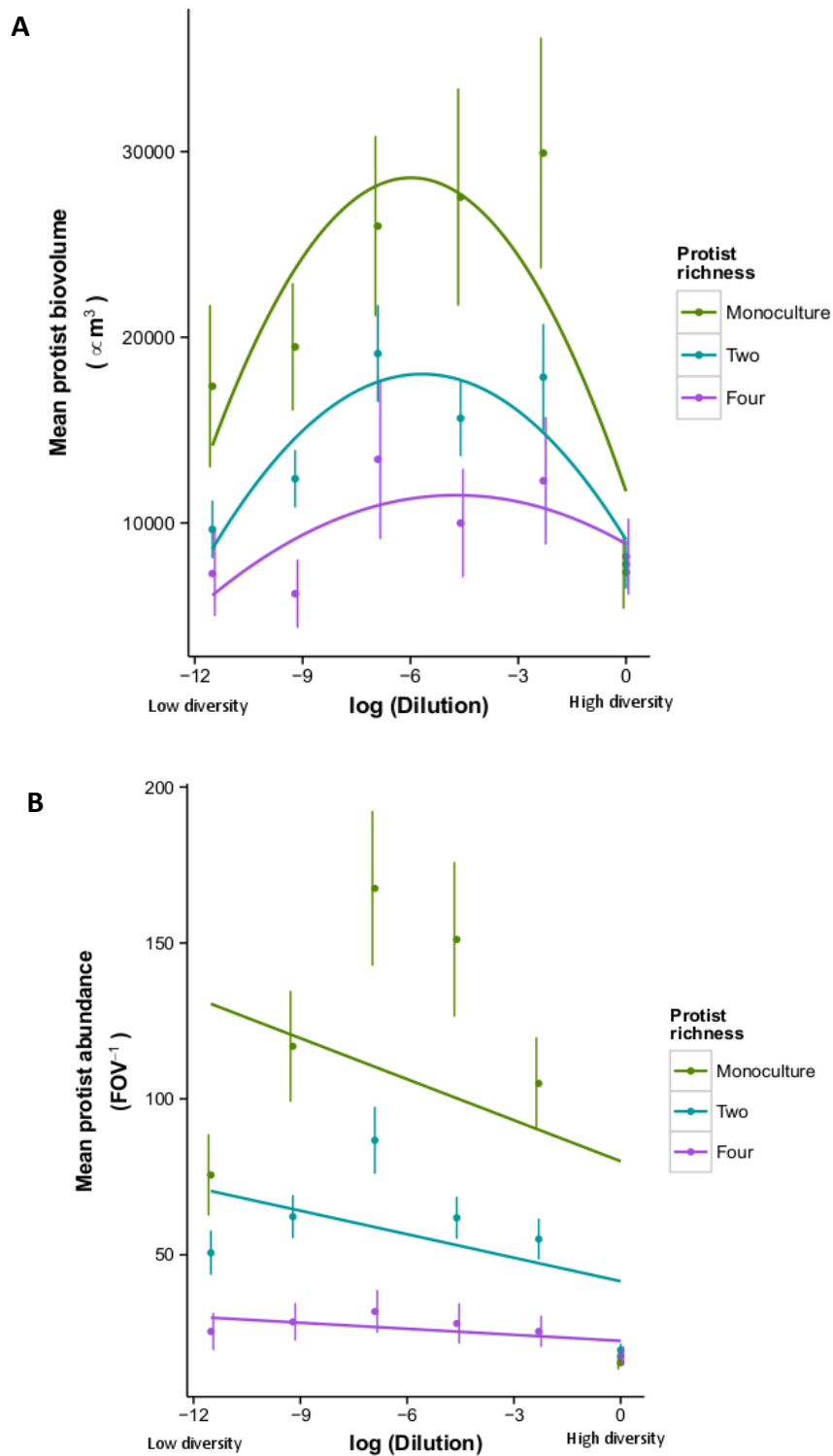


Figure 26. Interactions between protistan community richness and bacterial community diversity (dilution) for **A)** protistan biovolume and **B)** protist abundance. Points are means and error bars are standard errors. Regression lines are fitted by GLM.

Interacting effects of species identity, bacterial and protistan diversity

There were significant differences in biovolumes of species. Species B had the lowest total biovolume (GLM: estimate = -1.34, $t = -7.38$, $p < 0.001$) and C the greatest (GLM: estimate = 2.47, $t = 13.6$, $p < 0.001$). There was no significant difference between A and D. The larger species D (GLM: estimate = -3.10, $t = -2.05$, $p = 0.040$) and C (GLM: estimate = -3.10, $t = -2.05$, $p = 0.040$) had significant unimodal relationships between biovolume and bacterial diversity. Smaller species only retained a significant linear term in their models.

All species individually performed better in increasingly less diverse protist communities (see figure 27). However, this effect was particularly pronounced for species B relative to A in monoculture (GLM: estimate = 0.84, $t = 3.36$, $p < 0.001$) and two species combinations (GLM: estimate = 0.46, $t = 2.25$, $p = 0.024$). Species C benefited significantly less from two species mixtures (GLM: estimate = -0.47, $t = -2.27$, $p = 0.023$) while species D benefited significantly more (GLM: estimate = 0.41, $t = 2.00$, $p = 0.045$).

The negative effects of increasing bacterial diversity on species A were increasingly pronounced in two species mixtures (GLM: estimate = -0.68, $t = -2.17$, $p < 0.001$) and monoculture (GLM: estimate = -1.93, $t = -5.02$, $p < 0.001$) compared to four species mixtures. The same result was found for species B (GLM two species*dilution: estimate = -1.05, $t = -2.30$, $p = 0.022$; monoculture*dilution: estimate = -1.56, $t = -2.80$, $p = 0.0055$). Species C (GLM monoculture*dilution: estimate = -0.85, $t = -1.97$, $p = 0.049$) and species D (GLM monoculture*dilution: estimate = -1.03, $t = -2.43$, $p = 0.015$) only suffered increased negative effects of bacterial richness when in monoculture.

The effects of increasing bacterial richness had less negative effects on species C and D compared to A and B, as indicated by the positive interaction between species identity and bacterial diversity (GLM dilution*species C: estimate = 1.00, $t = 4.59$, $p < 0.001$; GLM dilution*species D : estimate = 0.75, $t = 3.44$, $p < 0.001$). Results also showed that species C biovolume benefited proportionately less at intermediate bacterial diversities than other species.

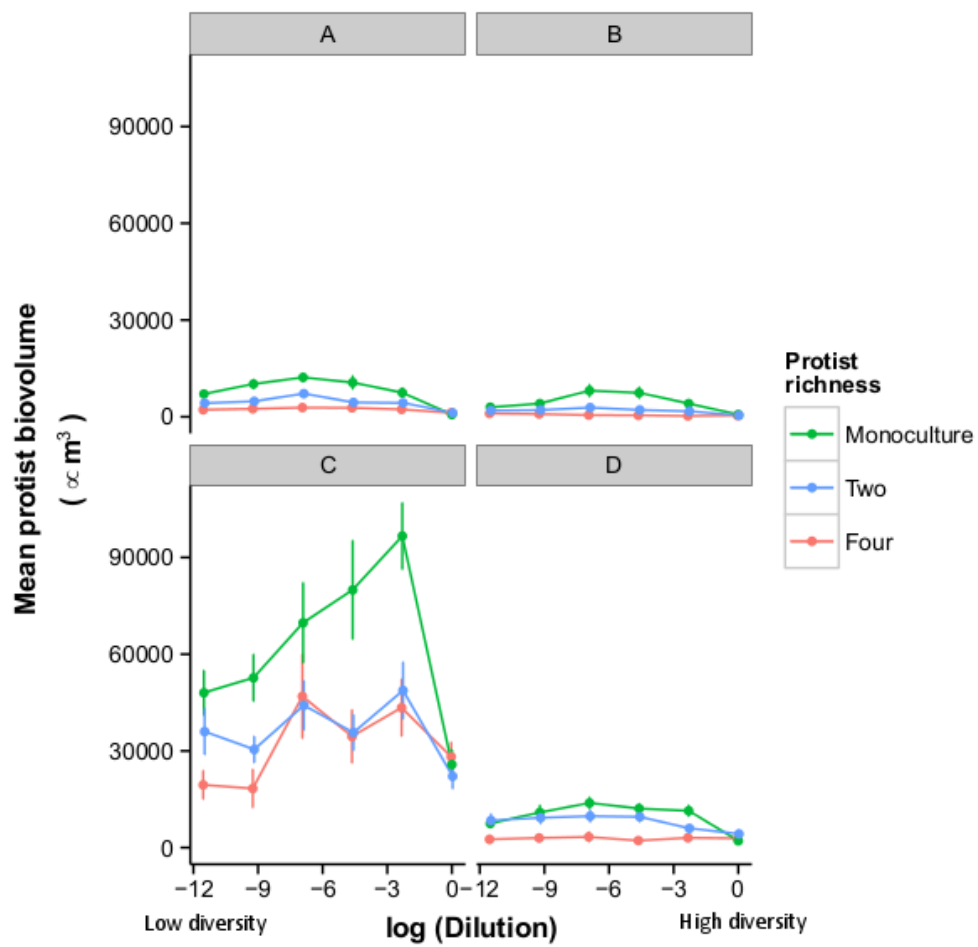


Figure 27. Interaction plots of the number of protist species in mixture and bacterial diversity treatment (dilution) for **A** (*Paracircomonas (=Cercobodo) minima*), **B** (*Allapsa brevifila*), **C** (*Cercomonas pigra*), and **D** (*Cercomonas hederæ*). Points are means and error bars are standard errors.

Interactions between protists

I found a number of significant interactions between species that affected biovolumes. There were significant negative effect of species C with species A (GLM: estimate = -0.32, $t = -2.14$, $p = 0.033$) and species D (GLM: estimate = -1.53, $t = -11.0$, $p < 0.001$). However, there were significant positive interactions between species D and species A (GLM: estimate = 0.76, $t = 4.09$, $p < 0.001$) and species D and species B (GLM: estimate = 0.85, $t = 4.74$, $p < 0.001$).

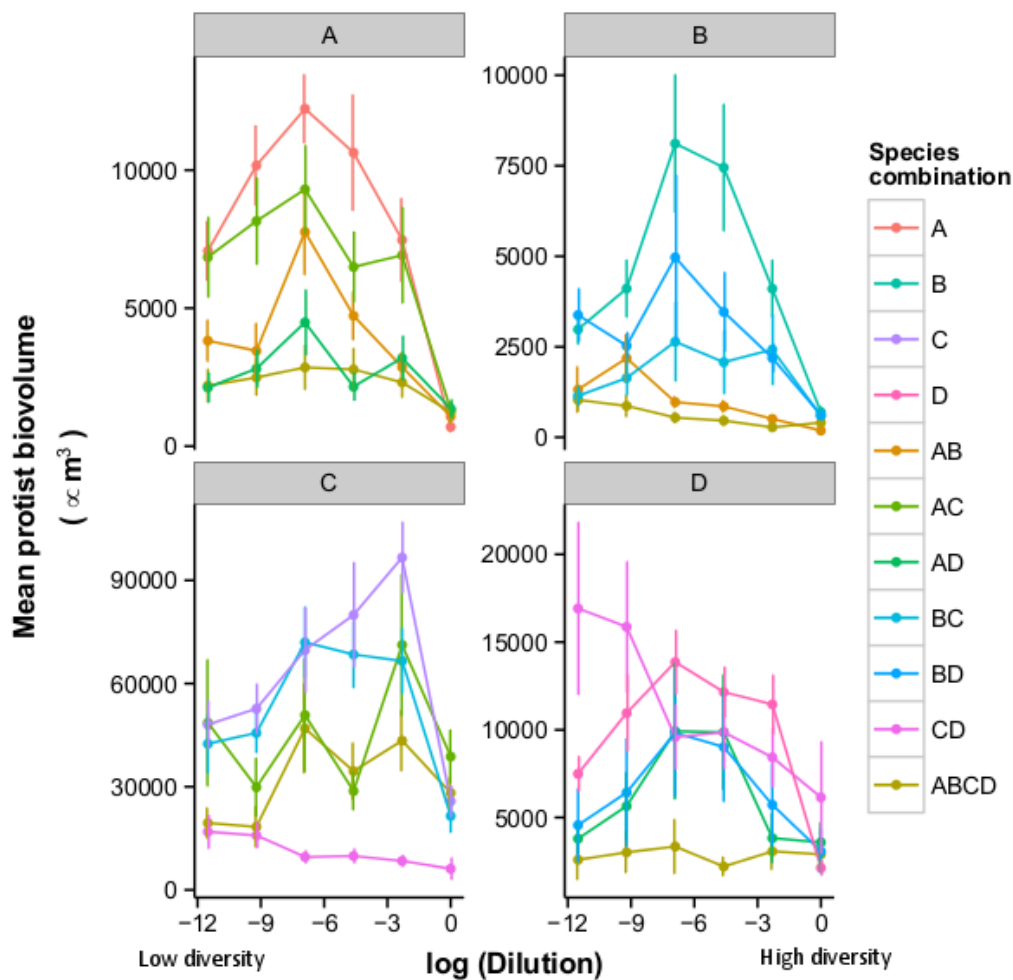


Figure 28. Plots showing species interactions effects on biovolumes of single species across bacterial community diversity. Panels show **A** (*Paracircomonas* (= *Cercobodo*) *minima*), **B** (*Allapsa* *brevifila*), **C** (*Cercomonas* *pigra*), and **D** (*Cercomonas* *hederae*). Points are means and error bars are standard errors. Colours represent species combinations which are given in the key.

Discussion

In this chapter I tested the feedback effects of bacterial community diversity on protist biovolume, abundance, and interactions. I also investigated the effects of protist community diversity and protist interactions on protist biovolumes. I found strong effects of bacterial community diversity and protist diversity, as well as a number of interspecific interactions.

Bacterial diversity feedbacks

Bacterial community diversity had a significant impact on total biovolumes of protists. Total protist biovolumes (figure 26) increased from high to intermediate levels of bacterial diversity and subsequently decreased at low bacterial diversity. The relationship was significantly better explained fitting a quadratic rather than a linear relationship to the data. Notably, this pattern appeared to inversely match bacterial metabolic activity across bacterial diversity treatments at the start of the experiment. These results showing negative effects of high as well as low bacterial prey diversity were in contrast to those of Jiang (2007) who found positive effects of bacterial diversity on protist biovolumes in a four species system. However, the results did show a significant positive interaction of increased bacterial diversity and protist diversity, with increased biovolumes of four species communities relative to monocultures at higher diversities. This indicated a decrease in under-yielding and an increase in complementarity (figure 26) (Leibold 1995; Loreau & Hector 2001; Gamfeldt et al. 2005; Saleem et al. 2013). However, considering the shape of the relationship (figures 26A and 28) this is more likely to be due to the ability of diverse protist communities to compensate the negative effects of bacterial diversity, rather than bacterial diversity having positive effects on protist diversity.

There are a range of non-mutually exclusive explanations for these results. While many protozoans are generalist and consume a wide range of bacteria (Lekfeldt & Rønn 2008), many are also capable of a high degree of selectivity (Weekers et al. 1993; Boenigk et al. 2002; Pfandl et al. 2004; Massana et al. 2009). Prey selection by protists can increase at higher prey densities (Boenigk et al. 2002) and therefore prey adaptations for unpalatability at high prey densities are

more advantageous and more likely to be selected for (Jousset 2011). In the very high and low bacterial diversity treatments where initial bacterial abundance was higher, bacteria formed much thicker biofilms. This might be a direct consequence of bacterial community diversity, with greater expression of positive interactions at high diversity (Saleem et al. 2012) or enhanced predator defence strategies (Matz & Kjelleberg 2005; Pernthaler 2005; Edwards et al. 2010; Saleem et al. 2013). This would be compounded by reduced encounter rates of suitable prey by predators in high diversity communities according to the resource concentration hypothesis (Root 1973), lowering predation pressure and allowing greater prey population growth. As prey diversity decreases past intermediate diversities the number of available suitable prey declines, niche overlap increases and predator biovolume and abundance is reduced, according to the variance in edible prey hypothesis (Leibold 1989; Leibold 1995).

Protist predation performance is affected by density-dependent defence mechanisms such as structural defences and increased expression of quorum sensing regulated traits (e.g. secondary metabolite production) (Pernthaler 2005; Jousset 2011). My results suggest that while non-significant, bacterial abundance may have a role in mediating diversity effects on protist biovolume. I suggest that species require areas of low bacterial density to establish them into the biofilm in order to begin engulfing prey. Smaller bodied species would have been more severely impacted since they were less able to engulf either resistant prey with structural defences, or large chunks of dense bacterial biofilm mat. The importance of size limited predation has been demonstrated in a variety of systems (Woodward & Hildrew 2002; Pfandl et al. 2004).

This conclusion was supported by the data showing that the larger species C and D were less impacted by bacterial diversity than the smaller species A or B. Furthermore, C benefited less from the intermediate bacterial diversity levels in comparison to the smaller species. This suggests that its size related competitive advantage was reduced at these diversities, possibly because other species were then able to break down the biofilm and defensive structures. Resulting enhanced performance by the smaller species increased competitive pressure on C. This supported the observation that structural defences such as biofilms provide only limited

protection, which is in turn supported by the diversity of amoebae living on bacterial biofilms (Thomas et al. 2008; Jousset 2011). This might explain why four species protist communities performed better at high diversity. Greater diversity of protist feeding traits allowed them to overcome bacterial defences, which in turn allowed greater resource partitioning of the more diverse prey community.

The nature of the diversity manipulation may have influenced the relationships. Dilutions to manipulate microbial diversity operate on the premise that dilution and regrowth of bacterial communities systematically alters diversity through the removal of rarer species (Franklin et al. 2001). However, if species are unevenly distributed then communities resulting from the first dilution would not only undergo a decrease in richness but also a simultaneous increase in evenness resulting in little change in overall diversity (Franklin et al. 2001). This has important implications for understanding the resulting trophic interactions. An increase in evenness could result in an increase in availability of less competitive palatable prey types, thus leading to the change in observed protist relative abundances and competition. Very diluted communities undergo real reductions in overall diversity with losses of rarer types causing a loss in evenness as competitive strains dominate (Franklin et al. 2001). The loss of edible prey types frees competitive unpalatable types to increase their abundances and occupy newly available niche space resulting in the negative impacts on protist biovolume described (Leibold 1989; Franklin et al. 2001; Wellborn & Langerhans 2015). However, the effect of bacterial evenness on trophic interactions remains completely untested and this result highlights the future need to partition the effects of components of diversity.

Protist diversity and performance

Significant differences in total biovolumes between protist species showed clear differences in competitive ability and performance. There were similarities, notably between species A and D. Reduced abundances of all species in the presence of others was an indication of strong competitive effects on all four species. This suggested a general lack of complementarity

(Hector & Loreau, 2001). Importantly however, the significant positive interaction between protist and bacterial diversity showed that biovolumes of four species communities were increased in more diverse prey communities. This suggested some increased complementarity and reduced niche overlap with a greater diversity of prey types (Leibold 1995; Loreau & Hector 2001; Saleem et al. 2013)

Species A and B performed better in low diversity protist communities and were less able to cope with increasing competitive pressure from increasing numbers of competitors. Species C benefited least from lower protist diversity, indicating that it suffered least competition and that its effects on other species were asymmetric. I suggest that the effect of protistan diversity on species is related to body size, particularly if prey resource size distributions constrain protist diet and result in intraguild predation (Woodward & Hildrew 2002; Jousset 2011; Schneider et al. 2012). Larger species were better able to cope with effects of bacterial diversity, and exert intraguild predation on smaller strains (Woodward & Hildrew 2002). The findings support the established idea that intraguild predation, inter-predator competition, and negative interactions are predominant features of predator communities (Polis & Holt 1992; Arim & Marquet 2004). If such negative interactions exist, then increasing predator diversity may reduce their impact on prey abundance (Finke & Denno 2004; Schmitz 2007) which in turn feeds back to reduce predator abundance further. This reiterates not only bacterial community effects on protist competition and coexistence, but also the ensuing interactions and feedbacks that determine wider microbial community structure.

Protist competitive interactions

The results showed a range of competitive interactions affecting protist biovolumes. There is a clear selection effect of CasphII and a likely facilitation effect between species D and the smaller strains, A and B (Hector & Loreau 2001). The significant negative effects of C when in mixture with any of the other three strains suggests that there are either strong asymmetric competitive effects between it and the smaller bodied protists, or that intraguild predation is

occurring. In particular, this result seems consistent with a dominance effect (Fox & Harpole, 2008). C may have a particularly strong effect on A and B because it can compete for all prey accessible to these smaller species and has a greater spectrum of potential prey due to its size, whilst they cannot (Woodward & Hildrew 2002). This is important if bacterial diversity and size related defence strategies are important features of the community. The interactions between the similar sized small species were broadly neutral. In addition, the negative effects of bacterial diversity were strongest in the least diverse protist communities. This is in contrast to the finding by Gamfeldt et al (2005) that prey richness had strong positive effects on consumer diversity and biomass.

The negative effects of A and B in mixture indicates symmetric direct competition, while the positive interactive effects between D and A, and D and B points to facilitation by D. This may have been due to grazing effects by D, either reducing bacterial density and preventing the dominance of bacterial strains which were unpalatable to A and B or by grazing the bacterial biofilm creating areas of reduced density and allowing A and B to more effectively establish themselves. However, unlike C, D was unlikely to be exerting intraguild predation on smaller species due to insufficient differences in body size (Woodward & Hildrew 2002; Arim & Marquet 2004; Vance-chalcraft et al. 2007). This would be indicative of the sort of synergistic interaction most prominent at intermediate diversity levels, as suggested by Saleem et al (2013). D had negative interactions with C, probably due to greater competition since they were of more similar size and may have shared a greater range of potential prey (Woodward & Hildrew 2002).

Concluding remarks

The lack of bacterial abundance data once predation had started made it hard to distinguish abundance effects that were independent from diversity. This was complicated somewhat by later work that showed weak correlation between ATP concentration and abundances of these natural bacterial communities measured by flow cytometry (see Appendix 4, supplementary

figure 6). Protistan diversity was also artificially low compared to natural assemblages. Nevertheless, the results supported the hypothesis that bacterial diversity impacted on protist community performance. In particular, evenness resulting from dilutions may be key to understanding prey diversity effects on predators and requires explicit investigation. There was clear evidence for negative interactions and under-yielding by protists. Competitive interactions between protists were likely to be determined by size differences, diet breadth and ability to overcome bacterial defences (Julia et al. 2014). However, not all interactions were negative, with evidence of facilitative effects also.

Data Chapter 7 –Functional diversity and coexistence in fluctuating environments

Abstract

The impact of biodiversity on ecosystem functioning is dependent on local environmental conditions, but conditions are known to vary spatially and temporally. Changes in functional trait diversity affect the probability that organisms can persist in a range of conditions and therefore the ability of communities to buffer against fluctuations. There are a number of hypothesised mechanisms by which community diversity can be maintained. Storage effects are hypothesised to be one possible mechanism for maintaining community diversity in the face of environmental variability. These are mechanisms by which an organism may withstand non-optimal periods, depressing the effect of competitors and buffering against depressing effects on population numbers. Many protists of the Cercozoa can form metabolically inactive cysts during periods of environmental stress, only to return to an active form later. I hypothesised that this is a potential temporal storage effect mechanism which maintains community diversity among the Cercozoans. I identified a functional trait that showed interspecific variability and is a biologically relevant candidate for a temporal storage effect mechanism. I then experimentally manipulated both experimental communities and their environment in order to elucidate the role of the proposed mechanism in conveying compensatory dynamics to these communities in the face of environmental disturbance regimes. Using microcosm communities, I tested over 72 days whether the propensity of a community to form cysts led to increased community diversity across a range of temperature fluctuation regimes. I also tested whether the functional diversity (functional group richness and functional dissimilarity) of this trait predicted the ability of the community to buffer against fluctuations. I found that the overall ability of the community to cyst did increase community diversity. I also found that increasing levels of functional dissimilarity also led to higher levels of community diversity by the end of the experiment.

Introduction

In Chapter 6 I investigated how prey diversity affected competitive interactions between heterotrophic protists. Competition is an important interspecific interaction is considered a key determinant of community dynamics. It occurs whenever two or more individuals, populations or species must exploit a common resource, and has negative co-varying effects on competing parties (Chesson 2000b). When two (or more) species are limited by a common resource, the one which maintains a positive per capita growth rate at the lowest level of resource will outcompete the other (Tilman 1982). In order for coexistence to occur this effect must be counteracted (Tilman 1982). Coexistence is the persistence of competing species in the same location and requires that competing species have ecological differences (Chesson 2000b). Such differences are vital for coexistence because they allow species to depress their per capita growth rates more than their competitors (Chesson 2000b). There are a number of mechanisms by which this can occur and these fall into two categories – environmental variability dependent and independent (Chesson 2000a,b).

Functional diversity commonly invoked to answer ecological questions regarding the prediction of rates and reliability of ecosystem processes (Mason et al. 2005). In the case of heterotrophic protists, their wide variety of functionally important traits are generally considered to be those that relate to their top-down effects on bacterial communities (Sherr and Sherr 2002; Massana et al. 2009). For instance, traits such as motility, size, generalism, preference for prey according to surface properties, phylogenetic affiliation and quality may all contribute to coexistence (Sherr & Sherr 2002; Matz & Kjelleberg 2005; Jezbera et al. 2005; Shannon et al. 2007; Massana et al. 2009). Functional diversity of this sort is important for the coexistence of heterotrophic protists and ecosystem functioning because it affects complementarity and competition for resources which falls under the category of coexistence mechanisms that are independent of environmental variability (Tilman et al. 1997; Loreau 1998b; Chesson 2000a; Chesson 2000b).

A second suite of competition related mechanisms that maintain species coexistence depend on environmental variability (Chesson 2000b). These operate through differences in species' responses to temporally or spatially auto-correlated changes in the abiotic environment, which results in changes in relative competitive ability (Chesson 2000b). Functional diversity may reflect a greater array of responses to environmental variability so that niche complementarity helps buffer diversity and ecosystem functioning against environmental fluctuations (Tilman et al. 1997; Mason et al. 2005; Adler et al. 2013). This falls under the predictions of the insurance hypothesis which predicts that greater species richness confers a greater degree of stability on community and ecosystem processes (Yachi & Loreau 1999). Diversity of species' responses to environmental change has been termed 'functional response diversity' and traits 'functional response traits' (Diaz & Cabido 2001; Leary & Petchey 2009).

Diversity of traits may convey a 'storage effect' which allows organisms to maintain high species abundances through non-optimal conditions dampening the effects of increased competition (Tilman 1999; Chesson 2000a, b). This can be broken down into 'spatial' and 'temporal' storage effects; the former concerns within-habitat covariance of the quality of the environment and competition intensity while the latter concerns this relationship through time (Chesson 2000a, b; Sears & Chesson 2007; Chesson & Kuang 2008). Other traits may cause asymmetric or negative covariance in competitive ability through reduced abundance and growth correlated with environmental change, an effect termed 'relative non-linearity of competition' (Chesson 2000b; Chesson & Kuang 2008). Such traits in protists that affect responses to environmental change might include those related to motility, activation energy of metabolic processes, and dormancy (Beveridge et al. 2010c; Lennon & Jones 2011; Leary et al. 2012).

Functional diversity, the insurance hypothesis, and storage effects have particularly received attention in plant community ecology (Kelly & Bowler 2002; Adler et al. 2006; Angert et al. 2009; Chesson & Kuang 2008; Usinowicz et al. 2012; Adler et al. 2013; Holt & Chesson 2014; Garcia De Leon et al. 2014). However, results have been mixed and studies have not always

directly established mechanisms (Naeem & Li 1997; Houlihan et al. 2007; Mutshinda et al. 2009; Valone & Barber 2008; Adler et al. 2013; Loreau & de Mazancourt 2013). Mixed evidence for the insurance hypothesis and the mechanisms that underpin it may be due to a measuring of unsuitable aspects of species responses to environmental disturbances (Leary & Petchey 2009).

There is great potential to uncover the role of the insurance hypothesis and contribution of storage effects in microbial ecology. The insurance hypothesis may play an important role in understanding the functioning of microbial biofilms and human disease (Boles et al. 2004; Costerton 2004). Recent studies have demonstrated stabilising insurance mechanisms in microbial communities (Becker et al. 2012; Awasthi et al. 2014). Furthermore, dormancy is considered to be an important mechanism for coexistence in microbial communities (Stevenson 1978; Cáceres 1997; Lennon & Jones 2011; Shade et al. 2012). It is an ideal candidate mechanism for conveying a temporal storage effect because it is initiated by environmental change, varies between species, and the differentiation into resting structures allows populations of bacteria to maintain abundances during non-optimal periods (Lennon & Jones 2011).

Dormancy in bacteria is known to occur as a result of a number of stimuli such as light, pH, osmotic pressure, and temperature, and resource limitation (Lennon & Jones 2011). The variety of resting structures is equally great, including but not limited to; spores, conidia, cysts, reduced cell size, reduced nucleic content, altered quantity and composition of proteins, lipids and fatty acids, and changes in structural features (Novitsky & Morita 1976; Choi et al. 1996; Choi et al. 1999; Kieft et al. 1997; Suzina et al. 2004; Archuleta et al. 2005).

Dormancy exists in protists and may provide a mechanism for temporal storage effects. These dormant forms take the shape of cysts, phenotypically distinct resting structures reported in a wide range of taxa (Faber & Preisig 1994; Aksozek et al. 2002; Bass et al. 2009; Khan et al. 2015). Cysts form calcified structures which convey resistance to environmental conditions and scale to the body size of active forms (Aksozek et al. 2002; Donlan et al. 2005; Bass et al. 2009; Khan et al. 2015). Cyst banks of dormant predators can reach densities orders of magnitude

above the active population, and encysted protozoa can rapidly resume their activity when conditions return to their optimal range (Lloyd et al. 2001; Eichinger 2001). This has been proposed as a possible mechanism to explain the extremely long tail of the rank abundance curve of micro-eukaryotes, allowing large numbers of rare species to persist (Lennon & Jones, 2011). In addition, there are implications for human health since protists can not only act as pathogens themselves, but vectors and refuges for pathogenic bacteria (Khan et al. 2015).

A number of studies have used protists to investigate the role of functional diversity in the insurance hypothesis and storage effects (Eddison & Ollason 1978; Naeem & Li 1997; Descamps-Julien & Gonzalez 2005; Jiang & Morin 2007; Leary & Petchey 2009; Leary et al. 2012). Naeem and Li (1997) used a microcosm approach to show that increasing functional diversity and redundancy improved consistency in biomass and density. Leary and Petchey (2009) showed that greater differences in protist species responses to environmental fluctuations had lower temporal variability in biomass and greater negative covariance between populations. Furthermore, Jiang and Morin (2007) demonstrated that a storage effect most likely drove coexistence in autocorrelated temperature fluctuations.

In this chapter I attempted to show experimentally that the cysting behaviour of protists promotes community diversity in the face of temperature fluctuations of a variety of frequencies and autocorrelative structures. I not only tested this mechanism at different community diversity levels, but also whether diversity of cysting traits predicts community diversity in the face of perturbations. I built on the approach suggested by Leary et al. (2012), using functional response traits and apply fluctuations over the range that these traits operate. I test the hypotheses that; i) communities with a greater overall propensity to encyst maintain greater diversity, ii) communities with greater cyst forming response trait diversity maintain greater diversity in more variable environments, iii) greater diversity is maintained in autocorrelated environments, iv) there is reduced diversity in high frequency variable environments, and v) low frequency variability and autocorrelated environments have a compounding negative effect on community diversity.

Methods

Bacterial community and protist strain growth

Bacteria from tree-hole community T143 were grown for two days at 21°C in beech leaf tea (BLT) before inoculation into experimental microcosms. Experimental microcosms were inoculated with 50µl of pre-grown stock bacterial community in BLT into 1750µl of BLT in the experimental microcosm. Fifteen protists species were grown for between three and seven days before inoculation, depending on their estimated growth rates in order to ensure the same stage of growth (see Chapter 2). The protists used are shown in figure 27. Protist communities contained either two or four species. Four species communities had 50µl of each monoculture added to the experimental microcosms, while two species communities had 100µl added. Microcosms consisted of a total of 2ml BLT containing both bacteria and protists

Quantifying cysting traits and cyst scores

I estimated a ‘cyst score’ for each species (see Chapter 2), which was the per capita cysting rate in the absence of competition (Adler et al. 2013). The species for which cyst scores were calculated formed the pool of strains for use in assembling communities (see figure 29). I then calculated the overall mean cysting ability of the species in a community. I termed this the ‘community cyst score’. In addition, the mean dissimilarity (Heemsbergen et al. 2004) in cyst scores between the constituent species in each community was also calculated. This is given by the equation:

$$C_{pd} = \frac{\sum(S_1 - S_2) + \dots + (S_{n-1} + S_n)}{n}$$

Where C_{pd} is community mean pairwise dissimilarity in cyst scores, S is cyst score of each of the n strains in the community.

Flow cytometry

Flow cytometry was used to count protist abundances in mixed polycultures. I used a BD Accuri™ flow cytometer with a robotic arm to sample microcosms throughout the experiment. Flow rate was set to $66\mu\text{l s}^{-1}$ and a core size of $33\mu\text{m}$. Experimental microcosms - in 24 well plates - were sub-sampled into 96 well shallow well plates. $200\mu\text{l}$ of sample into the 96 well plates and $150\mu\text{l}$ of this was sampled by the flow cytometer. Counts were then recorded in R v3.0.3 (R Core Team 2014) at the known peaks of forward scatter (FS-H) corresponding to the constituent strains of the community.

Protist community composition and selection

Cell sizes were originally obtained using microscopy as described in Chapter 2 (page 49). Cell sizes are given in table 2 (Chapter 2, page 56). I then obtained the distribution of cell sizes of each protist species in monoculture after seven days of growth using the forward scatter channel of the flow cytometer. All the possible communities of two and four species combinations were then tested *in silico* for their ease of distinguishing between the constituent strains by overlaying the smoothed curves of their forward scatter size histograms in R v3.0.3 (R Core Team 2014; see figure 30 A). Following this a list of distinguishable two and four species communities was obtained. The final communities to be used in the experiment were selected to ensure that protist species were represented as evenly as possible throughout the experiment and that species were included in communities with relatively larger and smaller mean pairwise differences in cysting scores. Twenty two-species and twenty four-species communities were chosen, with fifteen out of the pool twenty two species represented.

Temperature manipulations

Temperature manipulations were predetermined as low variability, medium variability or high variability. Communities subjected to low variability conditions would be subjected to a temperature change every eight days, medium variability every four days, and high variability every two days. Temperatures were varied by moving experimental microcosms between four

incubators set at 10°C, 15°C, 20°C, and 25°C. In addition to low, medium and high variability environments, microcosms were subjected to either redshifted or white shifted temperature variations. Red shifted series are those that have some autocorrelation structure to the transitions, while white shifted do not. In red shifted conditions, the probability of shifting to another temperature is not even, but dependent on the temperature currently experienced. For this experiment I used a Markov chain to produce red-shifted temperature regimes. A Markov chain is a sequence of random transitions between states (temperatures). The process is ‘memoryless’ in the sense that the probability of transition depends only on the current state and not on the sequence of previous states. For this experiment the transition probabilities were; $p(\text{no transition}) = 0.5$, $p(\text{one temperature step}) = 0.35$, $p(\text{two temperature steps}) = 0.1$, and $p(\text{three temperature steps}) = 0.05$. I produced two temperature series for each combination of temperature treatments (see figure 30 B). Mean temperature was 17°C for all temperature time series.

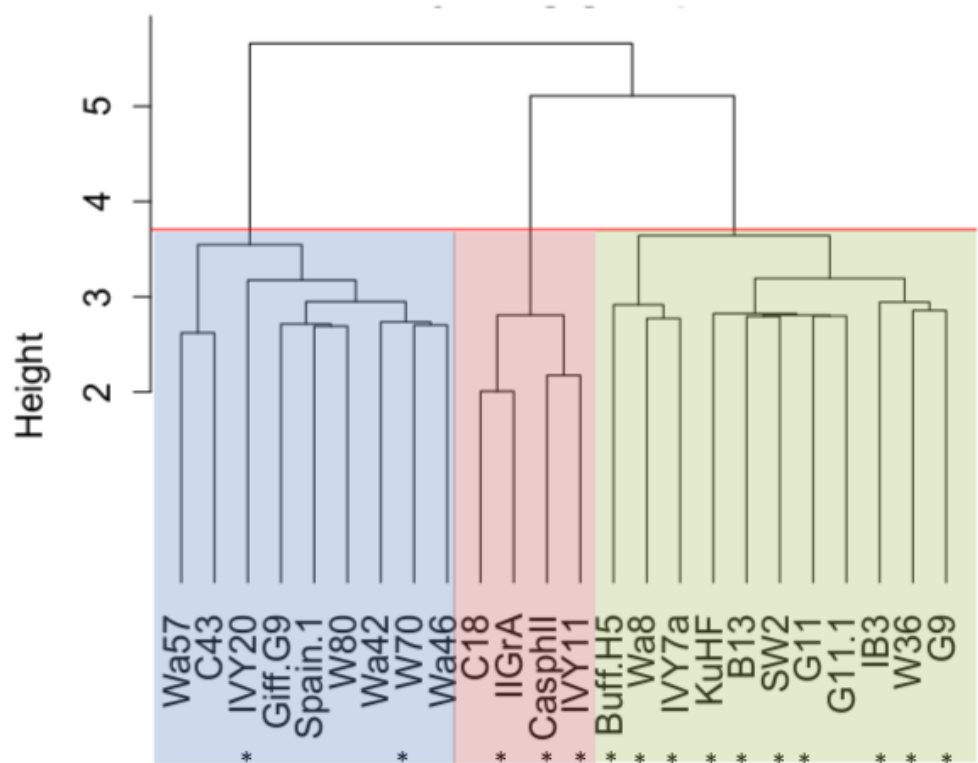


Figure 29. Hierarchical cluster dendrogram of protist predators according to pairwise distances in cysting score. The red line represents the cut-off point below which functional groups are defined. Black asterisks denote species used in the experiment

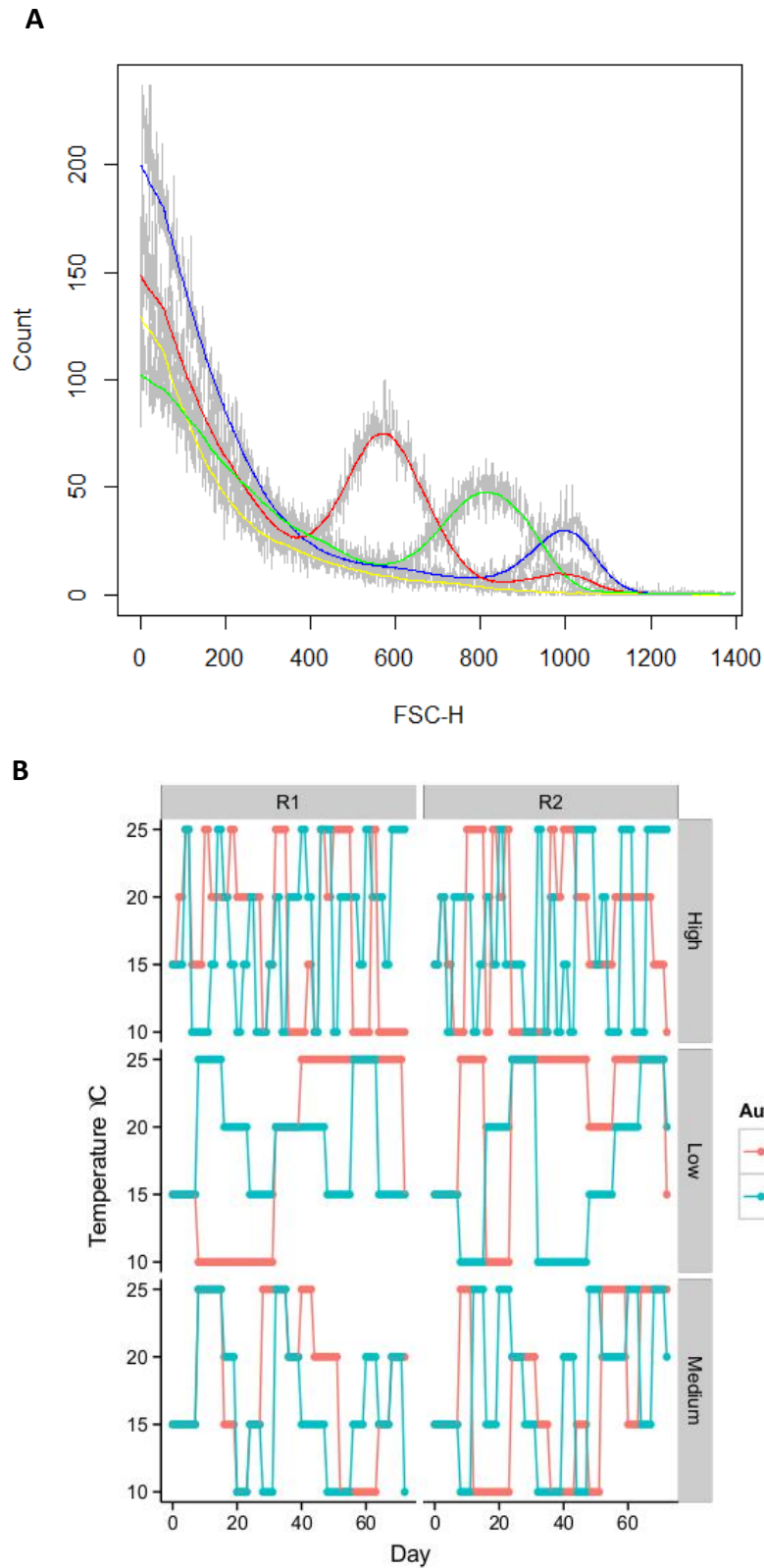


Figure 30. Panel **A**) shows overlaid forward scatter against counts for three different protist strains. **B**) Shows the temperature time series applied during the experiment. R1 and R2 are the two series. Variability frequency treatments are labelled 'High', 'Medium', and 'Low'. Autocorrelation treatments are labelled 'Red' and 'White' and are coloured red and blue respectively.

Experimental procedure

Communities were replicated three times under each combination of autocorrelation and variability frequency treatments. This produced an experiment size of 1440 microcosms. Once the communities were assembled, they were left to grow whilst being subjected to the experimental treatments described above. Flow cytometry counts were then taken at day eight of the experiment and at day 72, at which point the experiment ended. Microcosms were well mixed before sampling. After sampling the experimental microcosms, 200µl of fresh beech leaf tea was added to each to keep the total volume at 2ml. Counts of each strain were then converted to counts per microliter.

Statistical analyses

The species abundances were analysed as Shannon Diversity indices for each community. These were calculated as:

$$H = - \sum_{i=1}^s p_i \ln p_i$$

H is Shannon's Diversity Index, s is the total number of species (species richness) and p is the proportion of s made up by the *i*th species. The indices were then +1 transformed and then normalised by log₂ transformation. I also calculated Shannon's Equitability (*E_H*) as a measure of community evenness. This is given by the equation:

$$E_H = \frac{H}{\ln(S)}$$

E_H is Shannon's Equitability, s is species richness and H' is Shannon's Diversity Index. Analysis was carried out in R v3.0.3 (R Core Team 2014) and using package Vegan (Oksanen et al. 2015). Shannon's Diversity Index was analysed by GLM with quasipoisson errors and log-link function. I modelled the effect overall ability of the community to form cysts (community cyst score), mean dissimilarity of cyst scores, the variability regime of the environment and the autocorrelative structure of the variability on protistan community diversity. Maximal models

are given in Appendix 3, supplementary table 1. Model simplification was carried out manually by removal of least significant complex terms. Model comparison was carried out by ANOVA.

Results

Temperature regimes

I found that there were significant main effects of the environmental variability treatment, the autocorrelative structure of this variability, and the community cyst score. Low variability environments led to lower diversity (mean = 0.486 ± 0.007) than the intermediate (mean = 0.542 ± 0.0069 , Tukey HSD: $p < 0.001$) or high variability environments (mean = 0.55 ± 0.0072 , GLM: estimate = -0.324 , $t = -6.54$, $p < 0.001$). There was a significant effect of autocorrelation structure of the temperature series, with white-shifted (mean = 0.53 ± 0.012) environments having significantly lower diversity than red-shifted ones (mean = 0.52 ± 0.01 ; GLM: estimate = -0.08 , $t = -2.56$, $p = 0.01$; see figure 29). I also found a significant interaction between autocorrelative structure and frequency of temperature variation, with low frequency environments with a white-shifted autocorrelative structure leading to significantly increased levels of diversity (GLM: estimate = 0.432 , $t = 6.27$, $p < 0.001$).

Low variability (mean = 0.45 ± 0.012) led to significantly less even communities than high variability disturbance (mean = 0.52 ± 0.012 ; GLM: estimate = -0.65 , $t = -4.51$, $p < 0.001$) and intermediate variability (mean = 0.52 ± 0.012 ; Tukey HSD: $p < 0.001$). There was no significant difference between high and intermediate disturbance. There was no significant difference in evenness between red and white-shifted environments. However, white-shifted autocorrelation relieved the negative effects of low variability treatments (GLM: estimate = 0.80 , $t = 3.40$, $p < 0.001$).

Community cysting ability and functional diversity

Community cyst score had a non-significant effect on overall diversity (H') but had a significant effect on evenness (E_H GLM: estimate = 0.4 , $t = 2.92$, $p = 0.004$). Mean dissimilarity of cysting ability had a significant non-linear effect on H' ($y = 0.01x^2 - 0.1x + 0.01$; GLM: estimate = 0.014 , $t = 2.91$, $p = 0.004$) and a significant positive effect on E_H (GLM: estimate = 0.3 , $t =$

4.39, $p < 0.001$). There was a significant positive interaction between the overall community cyst score and the mean dissimilarity of cyst scores for both diversity measures (H' GLM: estimate = 0.04, $t = 2.72$, $p = 0.007$; E_H GLM: estimate = 0.20, $t = 5.18$, $p < 0.001$). Mean dissimilarity also had positive interactions with richness on evenness (GLM: estimate = 0.17, $t = 2.28$, $p = 0.02$).

Interactions between diversity and disturbance

There was a significant four way interaction between the community cyst score, mean dissimilarity of cyst score, autocorrelation of the temperature series, and the frequency of temperature variability on both diversity measures (H' GLM: estimate = 0.07, $t = 2.09$, $p = 0.04$; E_H GLM: estimate = 0.26, $t = 3.56$, $p < 0.001$). This showed that communities subjected to the low variability treatment had improved levels of diversity when they had high diversity of cysting traits (mean dissimilarity), a high ability to cyst (community cyst score), and that the autocorrelation structure of the temperature regime was white-shifted. The negative effects on evenness of low diversity were also reduced in more species rich, functionally diverse communities, with white shifted autocorrelation structure to disturbances (GLM: estimate = 0.6, $t = 2.72$, $p < 0.001$).

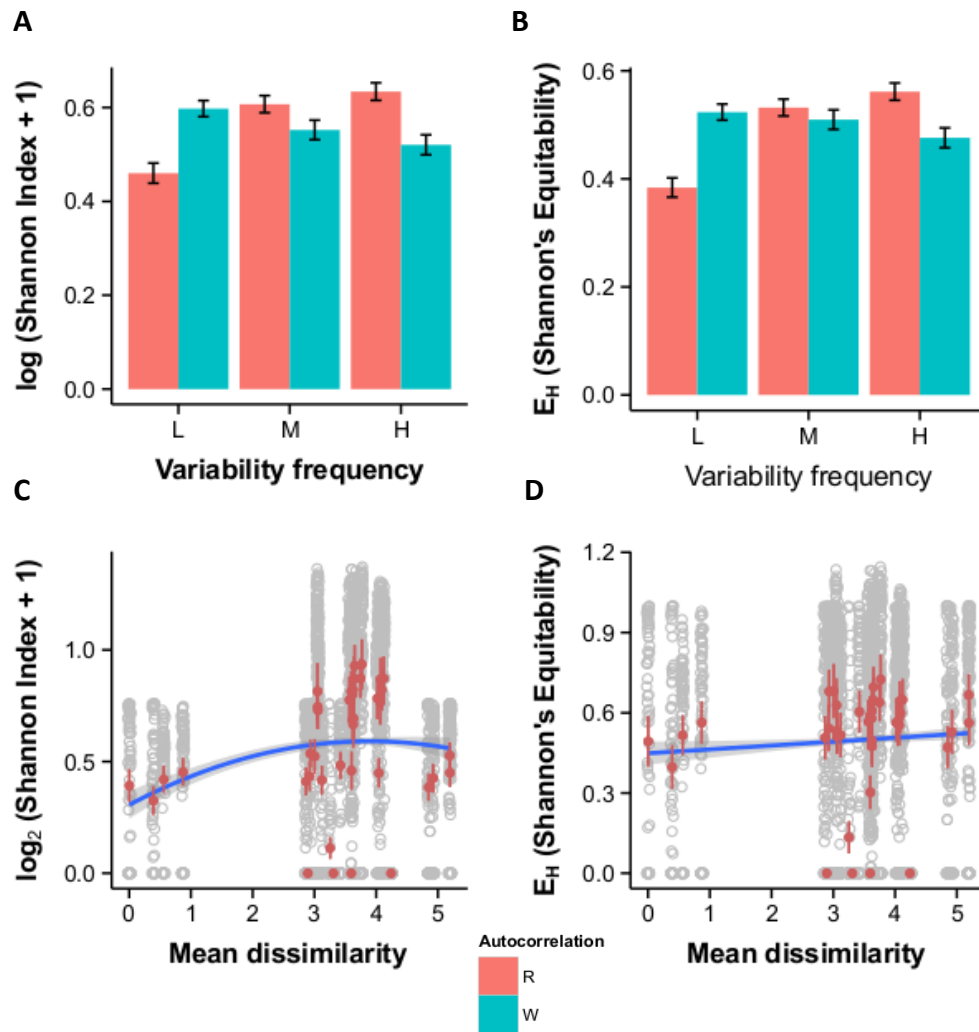


Figure 31. Panel **A**) Bar plot with standard error bars of environmental variability frequency against $\log(\text{Shannon Index} + 1)$. “R” and “W” stand for red and white shifted autocorrelation treatments. “H”, “M”, and “L” stand for high, medium, and low frequency disturbance treatments. **B**) Barplot with standard error bars for Shannon’s equitability. Panels **C**) and **D**) are scatter plots of diversity against mean dissimilarity of cyst scores and evenness against mean dissimilarity of cyst scores. The quadratic is fitted by GLM. The grey shaded area represents the 95% confidence interval. Red points are means and 95% confidence interval.

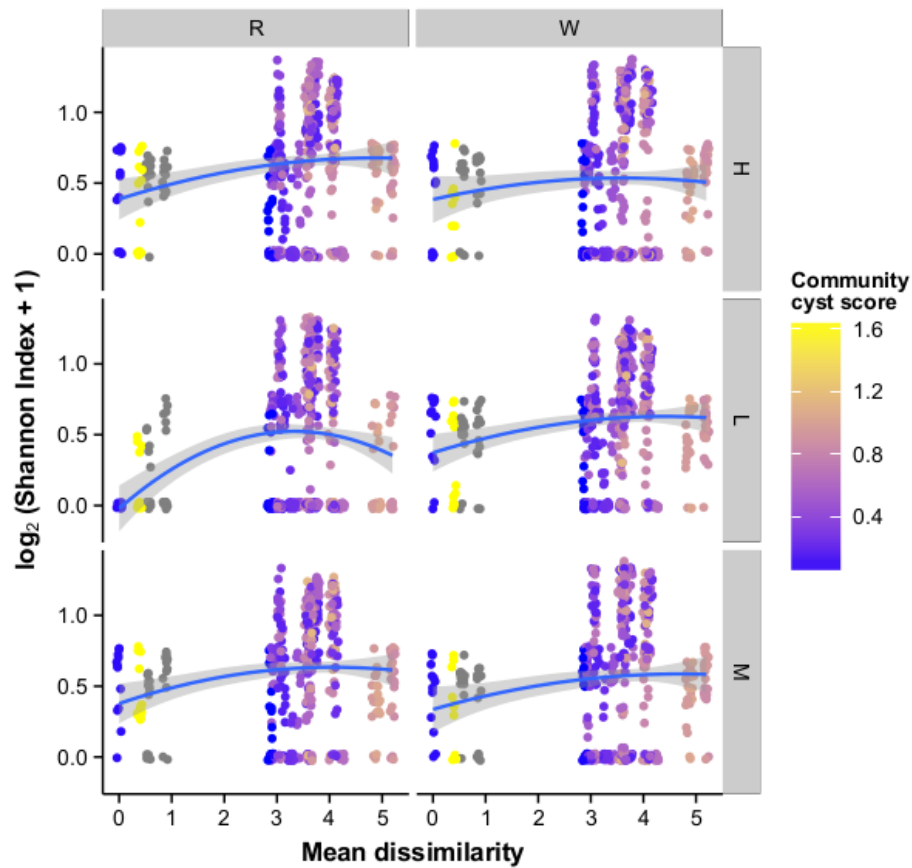


Figure 32. Scatter plot of interactions of diversity against mean dissimilarity of cyst scores, variability frequency, and temperature time series autocorrelation structure. The quadratic regressions are fitted by GLM. The grey shaded area represents the 95% confidence interval. Points are coloured by community cyst score. “R” and “W” stand for red and white shifted autocorrelation treatments. “H”, “M”, and “L” stand for high, medium, and low frequency disturbance treatments.

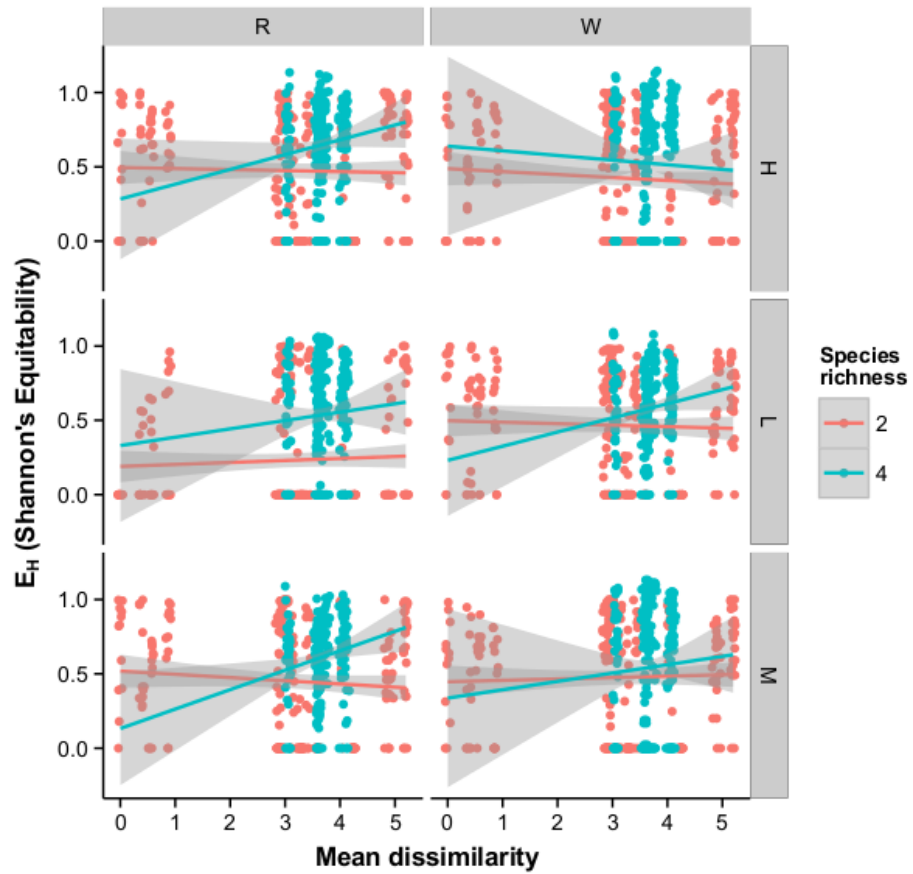


Figure 33. Scatter plots of interactions of evenness (Shannon's equitability) against mean dissimilarity of cyst scores, faceted by variability frequency and temperature time series autocorrelation structure. The regressions are fitted by GLM. The grey shaded area represents the 95% confidence interval. Points are coloured by species richness. "R" and "W" stand for red and white shifted autocorrelation treatments. "H", "M", and "L" stand for high, medium, and low frequency disturbance treatments.

Discussion

Main findings

The results showed that functional trait diversity had a significant positive effect on overall diversity and evenness. The overall propensity of the community to cyst (cyst score) had a non-significant effect on protist diversity. However, it had a significant positive interactive effect with functional diversity on protist community diversity. They also showed that frequency of disturbances had significant effect on diversity with greatest diversity at intermediate disturbance levels. In addition, diversity was generally significantly higher in white-shifted temperature disturbance regimes. However, these two disturbance treatments interacted significantly to either compound or compensate each other's effects on diversity. In particular, diversity in low frequency disturbance regimes were more diverse if temperature shifts were random (white-shifted). However, white-shifted regimes compounded the negative effects of high frequency treatments. The findings generally support those that have previously reported stabilising effects of environmental fluctuations and storage effect mechanisms on community diversity (Adler et al. 2006; Jiang & Morin 2007; Jiang & Patel 2008; Adler et al. 2009; Angert et al. 2009; Usinowicz et al. 2012).

The intermediate disturbance hypothesis

The results support the intermediate disturbance hypothesis (Connell 1978). According to the hypothesis, low variability environments lead to less diverse communities through competitive exclusion. High variability environments lead to reduced diversity due to the inability of species to withstand disturbance, leaving only the most resistant to dominate. This produces a unimodal relationship between diversity and disturbance, with highest diversity at intermediate levels of disturbance. However, these results are presented in the context of mixed support for the generality of this relationship.

Studies have been unable to consistently predict diversity-disturbance relationships (Hubbell et al. 1999; Kondoh 2001; Mackey & Currie 2001; Schnitzer & Carson 2001; Carreño-rocabado et al. 2012). There may be a number of reasons why the diversity-disturbance relationship varies from predictions. These include biotic factors such as productivity, trophic complexity and predation, as well as abiotic effects of disturbance frequency and intensity (Kondoh 2001; Gallet et al. 2007; Miller et al. 2011; Hall et al. 2012). However, my results are consistent with the smaller number of microbial studies that have found support (Kassen et al. 2000; Morgan & Buckling 2004; Gallet et al. 2007). The poor performance of the intermediate disturbance hypothesis and plurality of possible alternative diversity-dependence relationships suggest that the existence of the unimodal relationship found here may be dependent on specific characteristics of the system used. For instance, in high productivity environments strong disturbances are required to counteract high rates of growth and competitive exclusion (Huston 1979; Kondoh 2001; Svensson et al. 2012). Beach leaf tea is a relatively low productivity environment (supporting around 10^5 cells ml⁻¹) and therefore the disturbances applied were perhaps more able to generate the unimodal relationship.

The unimodal disturbance-diversity relationship found between disturbance and Cercomonads and Glissomonad diversity is perhaps surprising. The results contradict the prediction that unimodal diversity-disturbance relationships may be less common in predatory or mobile species (Haddad et al. 2008). Furthermore, the hypothesis assumes a trade-off between competitive ability and disturbance resistance, with rarer less competitive resistant species and more competitive less resistant species (Jiang & Patel 2008). The Cercomonads and Glissomonads are common in soils and aquatic systems, implying that they have relatively high competitive ability in natural communities (Howe et al. 2009; Bass et al. 2009; Cavalier-Smith & Oates 2012). Though there is variation in their cyst forming ability and growth rates, the existence of sufficiently large differences in trade-offs seems surprising (Jiang & Patel 2008; Howe et al. 2009; Bass et al. 2009; Cavalier-Smith & Oates 2012).

Functional diversity, dormancy and storage effects

Dormancy has been hypothesised to be an important mechanism for storage effects in microbial communities since Stevenson's (1978) original formulation of the theory. The ability of such mechanisms to maintain diversity under the insurance hypothesis is well documented (Shade et al. 2012; Holt & Chesson 2014). My results support the hypothesis that cysting allows species to buffer the effects of competition during longer non-optimal periods by maintaining highly populous 'cyst banks', rather like seeds in plant communities (Adler et al. 2013). These cyst banks can supplement the active population during periods of optimality, increasing their geometric mean fitness, and having a stabilising effect on the community (De Jong et al. 2011; Adler et al. 2013). This is supported by evidence from Berga et al. (2012) that showed that communities of bacteria recovered best in low disturbance environments, most likely due to dormancy mechanisms. My results are consistent with this, showing that diversity - and in particular evenness - benefited most from cysting ability in low variability treatments. Similar results were found in zooplankton with long lived egg stages with overlapping generations (Cáceres 1997). These results support the hypothesis that dormancy mechanisms in variable environments may account for the long tails of species' rank abundance curves, not only in bacterial communities, but microbial eukaryotes too (Lennon & Jones 2011).

Predator-prey interactions are predicted to have stabilising though complex effects on community dynamics in response to environmental fluctuations (Ives & Gilchrist 1993; Ives 1995; Jiang & Kulczycki 2004). Despite the prevalence of dormancy strategies, microbial communities are known to have low resistance and resilience to disturbance (Allison & Martiny 2008; Lennon & Jones 2011; Berga et al. 2012; Shade et al. 2012). While most of this work has been carried out on bacterial communities, disturbance may have effects on protists both directly and indirectly through changes in bacterial prey community structure and quality (Shade et al. 2012; Saleem et al. 2013). This is important since the insurance hypothesis has been noted to be context dependent and effects of disturbance on community stability may depend on community composition (Gallet et al. 2007; Leary et al. 2012). In particular,

disturbance may modulate the effect of predation due to trade-offs in prey populations of disturbance resistance and competitive ability that affect their susceptibility to predation after a disturbance (Gallet et al. 2007). Protist community dynamics might therefore lead to stabilising influences on the bacterial community post disturbance, allowing less competitive but resistant prey species to recover (Allison & Martiny 2008; Gallet et al. 2007). Differences in species responses may also strengthen top-down controls by predators, with increased species richness conveyed stabilising community dynamics through differences in species responses to seasonal variation (Griffin & Silliman 2011).

On initial inspection, the results do not support the prediction that storage effects should be more pronounced in autocorrelated environments (Jiang & Morin 2007). While there is no significant main effect of autocorrelation, red-shifted temperature series compound the negative effect of low frequency on evenness and total diversity. Low frequency variability and autocorrelation could both be considered different forms of ‘press’ disturbances (those that involve a change to a long term state rather than a reversion to a previous one) (Bender et al. 1984; Shade et al. 2012). If so, then the positive effect of cyst score and cysting mean dissimilarity in the low frequency treatment microcosms suggests some consistency with Jiang and Morin’s (2007) predictions.

This study adds to the growing list of works indicating an important role for temperature disturbances on coexistence and community dynamics of microbial communities as well as other higher organisms (Eddison & Ollason 1978; Cáceres 1997; Whitesides & Oliver 1997; Descamps-Julien & Gonzalez 2005; Jiang & Morin 2007; Leary & Petchey 2009). Such studies are particularly interesting because of the ubiquity of temperature effects, particularly in aquatic microorganisms. Temperature has important seasonal and diurnal cycles as well as deviations from these trends (Jiang & Morin 2007). However, my results suggest that cysting may be an adaption against long term cyclical press disturbances, providing a buffering effect and maintaining diversity in these conditions only (Müller et al. 2002; Jiang & Morin 2007; Shade et al. 2012).

The ability of mechanisms such as encystment to convey resistance and resilience to disturbance is dependent on both the strength of the disturbance and the strength of the species response (Shade et al. 2012). Organisms whose traits are insufficiently plastic or that are adapted to more stable environments are also more likely to suffer (Shade et al. 2012). The results support this as neither propensity to cyst nor mean dissimilarity in cysting ability showed interactive effects in the higher disturbance regimes. The mechanism may not be quick enough to counter these, possibly due to lag induced by physiological constraints.

Modelling and observations of global climate change indicate a rise in frequency of extreme weather events, such as heatwaves, storms and droughts in the future (Karl & Trenberth 2003; Jain et al. 2005; Salinger 2005; Allan & Soden 2008). With predicted microbial community responses to climate change, the ability of microorganisms to buffer the effects of these through dormancy is an interesting avenue of research. Their resistance and resilience in response to climate change related variables such as temperature and CO₂ levels have been found to be highly variable and debate surrounds the possible feedbacks they may have on global carbon fluxes as well as on above ground plant communities (Allison & Martiny 2008; Singh et al. 2010; Schnitzer et al. 2010). Furthermore, the ability of protists to cyst in response to disturbances and maintain diversity has public health implications beyond ecology (Khan et al. 2015). Free living pathogenic protists can remain encysted for up to twenty years without losing their pathogenicity (Mazur et al. 1995; Sriram et al. 2008). Furthermore, protists can act as refuges for pathogenic bacteria during periods of stress (Greub & Raoult 2003; Khan et al. 2015). Understanding mechanisms that convey storage effects may contribute to understanding how they may contribute to the persistence of free living pathogenic protozoans. This may be an important avenue for understanding the effects of pathogenic protists on human health (Eichinger 2001; Aguilar-Díaz et al. 2015; Khan et al. 2015).

Functional diversity and the insurance hypothesis

The results of this experiment support the hypothesis that functional diversity contributes to coexistence in variable environments, accounting for the insurance effect of species richness (Yachi & Loreau 1999; Loreau & Hector 2001; Petchey & Gaston 2002a). Notably, functional diversity interacted significantly with species richness, with more positive effects of functional diversity in the more species rich communities, reflecting the greater complementarity of functional traits within the community.

The results are consistent with previous studies showing that greater diversity of functional traits and responses to disturbance are important in explaining community diversity-disturbance relationships (Jiang & Morin 2007; Haddad et al. 2008; Leary et al. 2009; Carrera et al. 2014). This has also been shown in natural marine phytoplankton using laboratory tested traits (Edwards et al. 2012). Communities with low mean dissimilarities of traits are less complementary and have increased functional redundancy and niche overlap leading to synchronising dynamics and reduced coexistence (Loreau & Hector 2001; Petchey & Gaston 2006). Greater functional response trait diversity predicted a non-linear relationship with community diversity and an overall positive linear effect on community evenness (figures 31 – 33)

Diversity-disturbance relationships pose a “chicken and egg” problem for ecology (Hughes 2012). Diversity may modulate the impacts of disturbance because greater species richness - especially of rarer species - ensures greater functional trait diversity and redundancy, whilst at the same time disturbance is a driver of diversity by preventing competitive exclusion (Sousa 1979; Lyons et al. 2005; Hughes et al. 2007; Hughes 2012). Not only have these individual elements of this reciprocal relationship been recorded, there is evidence that they can occur within the same system and on similar scales (Hughes et al. 2007). The results in this experiment illustrate this problem. Both overall diversity and evenness are affected by disturbance. However, evenness is positively affected by species richness in low variability

treatments, indicating that it in turn modulates the impact of disturbance. In addition, the independent effect of functional diversity (mean dissimilarity) and the interaction between it and species richness shows that the benefit of species richness is at least in part due to the co-varying increase in diversity of cysting response traits. While a functional approach may help to detangle the mechanisms underlying community responses to disturbance, there is evidence that disturbances can also affect levels of functional diversity in communities (Carreño-rocabado et al. 2012; Mouillot et al. 2013a).

Limitations

My study has a number of limitations. Firstly, though it satisfies two criteria set out in order to demonstrate a storage effect (species must have a system for persisting in non-optimal conditions and species must respond differently to the environment), it did not explicitly address the third (the effects of competition must co-vary with the environment such that competition limits growth more in more optimal conditions) (*sensu* Chesson 2000b). Though I did not empirically address this third criterion, Adeler et al (2013) state that studies that focus on differences in species responses are just as tractable, if perhaps a little less conclusive. I also acknowledge that the effect of autocorrelation is difficult to delineate from the effect of frequency, an effect compounded if the temporal scales of disturbances and protist generation times do not match.

There also limitations to the way that I have quantified the propensity of strains to cyst. For instance, the score doesn't directly reflect the temperature niches of species only that within the temperature shifts used there are differences between them in cysting. Some of the variation in scores is due to differences in intrinsic cysting abilities and temperature niches. Despite this, because the experiment uses the full range of temperatures over which strains were found to form cysts, this issue is minimised.

Finally, my trait measure suffers from one of the main weaknesses highlighted by Petchey and Gaston (2006). The trait value is a single value, with no information contained regarding

intraspecific variation in the trait which has been averaged away. Furthermore, due to the nature of our study organisms, raw trait data was obtained at the population level rather than the individual level. It would be interesting to understand the effect of intraspecific response trait variation on the storage mechanism, particularly in light of the re-emergence of the importance of intraspecific variability in functional ecology (Violle et al. 2012; Albert et al. 2012).

Chapter 8 – General discussion

Synthesis of results and conclusions

My novel method for quantifying functional diversity produced dendrograms of species clustered by species interactions and contributions to functioning (Chapter 2). Testing diversity-functioning relationships highlighted negative relationships between interactions-based functional diversity and functioning (Chapter 3). The results showed that species' interactions and contributions to functioning were dependent on feedback effects of the surrounding bacterial community (Chapters 3, 4, and 5). Community structure affected the contribution to functional complementarity of protist species and overall community functional output (Chapters 4 and 5). However, average complementarity of protist communities remained unaffected by diversity or compositional differences, but predicted functioning differently according to compositional differences in the bacterial community (Chapters 4 and 5).

The results of Chapter 6 also showed that competition between protists was strong, with reduced biovolume and abundance in polycultures. Bacterial community diversity also affected biovolume and abundance of protists. This suggested that negative complementarity found in previous chapters might be in part due to strong interspecific competition and intraguild predation between protists (Chapters 2, 3, and 6). However, competition between protists did not change significantly across bacterial diversity gradients and species richness had a positive effect on ecosystem functioning (Chapter 3 and 6).

Finally, the results showed that competitive interactions underpinning coexistence were stable across prey diversity gradients (Chapter 6). Facilitation effects in more diverse protist communities buffered them against the negative impacts of high bacterial diversity, maintaining higher biovolumes than less diverse protist communities (Chapter 6). This suggested the importance of high size or feeding related functional trait diversity. Functional diversity of a dormancy trait conveyed a likely storage effect to protist communities in accordance with the

insurance hypothesis (Chapter 7). Protist diversity also responded to disturbances according to predictions of the intermediate disturbance hypothesis (Chapter 7).

Together, these results led to the following conclusions: 1) functional processes were primarily driven by bacterial community attributes; 2) individual protist species' functional contributions and complementarity were context dependent. Therefore the functional diversity of this group of predators was not independent of the surrounding community; 3) overall protist community complementarity was on average unaffected by bacterial community structure, therefore changes in functional complementarity and contribution to functioning by some species was compensated for by others. This provided functional redundancy; 4) competition between protists can lead to negative complementarity effects; 5) since not all protist competitive interactions were negative, some negative complementarity was the result of contrasting community structuring effects that led to negative interactions and reduced functioning in bacterial communities; 6) strong mechanisms of coexistence among protist communities suggested that they might have strong stabilising effects on microbial communities and ecosystem processes.

Broad themes and conclusions

My thesis has contributed to the understanding of predator-prey interactions and their consequences for ecosystem functioning in microbial systems. In particular, the results have added to the understanding of effects of bacterial community structure on protistan predators. Empirical investigation of this question had been lacking in microbial ecology (Chen et al. 2011; Saleem et al. 2012; Saleem et al. 2013). Studies have tended to focus on the top-down effects of protistan predators or bottom-up effects of productivity on bacterial communities (for instance Bell et al. 2010) and neglected impacts of prey upon predators. This gap in knowledge is important for understanding microbial systems since bacteria have evolved complex antipredator strategies. The results found support for some long standing hypotheses regarding prey diversity effects on predator dynamics (Root 1973; Leibold 1989), as well as predictions of

how bacterial diversity may affect protist performance and contributions to functioning through defence and abundance.

This thesis has advanced the understanding of the functional ecology of microbial communities and more specifically of a group of prominent bacterivorous biflagellates, some of which were only recently described and the ecology little known (Bass & Cavalier-Smith 2004; Bass et al. 2009; Howe et al. 2009; Cavalier-Smith & Oates 2012). While previous studies have investigated the functional role of protists in natural communities, noted functional characteristics, and grouped them into guilds, none have used BEF experiments to classify them based on interactions or contribution to functioning (Treseder et al. 2012). Furthermore, I used a novel objective method for delineating functional groups which may be useful for future investigations.

Testing of the BEF relationship found negative complementarity effects, an aspect of BEF relationships that appears under-investigated and may require greater incorporation into the BEF framework (Becker et al. 2012). Furthermore, the results agree with parts of the literature that show that the BEF relationships observed may depend on the resolution of measures used, and that levels of functioning are linked to - and dominated by - bacterial community structure (Langenheder et al. 2006; Peter et al. 2011).

Functional diversity based on traits has been considered an important static property of communities (Petchey & Gaston 2006; Cadotte et al. 2011; Verberk et al. 2013). However, by using an interactions-based method I have highlighted that functional diversity may have a more flexible nature than emphasised in the literature. Current understanding equates the likely contribution of a species to functioning to the degree of complementarity it has with other species based on trait differences (Tilman et al. 1997; Tilman 1999; Hector & Loreau 2001). However, the interactions-based approach to functional diversity has shown that species' contributions to functioning are not independent of biotic factors that affect complementarity. Explicitly, protists may have a range of feeding related functional traits, but the

complementarity conferred by these is also dependent on the attributes of the surrounding prey bacterial community as well as the trait values themselves (Duffy 2002).

This is not contradictory to the current understanding of functional diversity and BEF relationships. Complementarity is still at the heart of the relationship between species and ecosystem functioning - only the perspective taken is different. Trait-based functional diversity predicts rates of functional processes based on a single determinant (traits) of species complementarity. The interactions-based method focusses directly on contributions to functioning which result from species complementarity of phenotypic traits, trophic interactions, and resource availability. It captures the inherently context dependent nature of consumer functional traits and functional diversity when defined by their range of trophic interactions (Duffy 2002; Woodward & Hildrew 2002; Snyder et al. 2006; Schneider et al. 2012; Ritchie et al. 2012; Schneider & Brose 2013). This is important for interpreting the results of experiments that show BEF relationships that do not fit predictions, vary between sites, vary at different scales, or are found to be non-significant (Petchey et al. 2004). This is important for the generality of results and may have consequences as important as the choice of appropriate functional traits (Petchey & Gaston 2006).

My thesis also demonstrated the use of trait-based measures of functional diversity in explaining microbial community dynamics. I showed that dormancy strategies that convey storage effects and buffer against disturbance are not restricted to bacterial communities where they are known to be common (Lennon & Jones 2011). This contributed to the growing evidence that functional diversity is a fundamental component underlying the insurance effects of species richness (Yachi & Loreau 1999). The results also contributed to the ongoing debate regarding the validity of predictions of the intermediate disturbance hypothesis (Connell 1978).

Finally, the thesis generally suggests that protist predators confer stabilising dynamics in microbial communities. Strong disturbance resistance mechanisms are likely to allow protists to have stabilising effects on less resistant or resilient bacterial communities (Gallet et al. 2007).

Variation in traits may strengthen top-down controls and improve bacterial community diversity and functioning (Griffin & Silliman, 2011). Such mechanisms also prevent the loss of protist functional diversity, further reinforcing resistance and resilience (Mouillot et al. 2013a,b). Furthermore, changes in prey community structure do not affect overall levels of protist contributions to functioning despite impacts on individual species, suggesting that protist communities exert stable pressures on bacterial community structure and functioning.

Future directions

Future work into the ecology of microbial systems should focus on a number of themes. Firstly, studies should try to quantify the reciprocal impacts of predators and prey on community structure and dynamics, using next generation sequencing techniques to link these to specific functions. Furthermore, interactions-based functional diversity could be used in conjunction with phylogenetic methods and trait-based functional diversity to try and understand the links between traits, species contributions to functioning, functional organisation, phylogeny, and community structure (Treseder et al. 2012). Investigations should focus on linking protist feeding traits, trophic interactions, bacterial diversity and defence strategies. Advances in methods such as transcriptomics, fluorescence techniques, microscopy, and flow cytometry will help to open the ‘black box’ of microbial biodiversity and ecosystem functioning (Krause et al. 2014). Additionally, there should be greater attention paid to negative complementarity effects in microbial systems where high diversity and/or low resource complexity may make them prevalent (for instance in biofilms; Becker et al. 2012).

Dormancy should also be heavily incorporated into these studies since it is likely to explain the high diversity of rare but functionally important species of bacteria and protists, as well as conveying top-down stabilising effects of protist predation (Lennon & Jones 2011; Griffin & Silliman, 2011). This would provide the ideal context for investigating the link between predator-prey interactions, BEF relationships, and diversity-disturbance relationships using microbial trait-based and interactions-based functional diversity

Research into these aspects of microbial ecology also has implications for a number of more applied fields. Firstly, microorganisms play a central role in global fluxes of biogenic greenhouse gasses and are likely to respond rapidly to climate change (Singh et al. 2010). It is currently unclear whether microbial communities will have negative or positive feedbacks, but it will be important to understand the reciprocal effects of predators and prey on community dynamics that link microbial communities to biogeochemical cycles and ecosystem functioning (Treseder et al. 2012; Singh et al. 2010; Nie et al. 2013). In particular, storage mechanisms and disturbance resistance of protists, as well as their ability to convey stability, maintain functional diversity and species contributions to functional processes across multiple trophic levels are central to this issue. The consequences of disturbances for functional diversity and complementarity of interactions both within belowground and between below- and aboveground systems as a result of disturbances are also of pressing interest, in particular the drivers of negative complementarity effects (Eisenhauer 2012).

Secondly, this line of research may be able to make contributions to research into microbial communities and human health. In particular, both protists and bacterial biofilms can be important pathogens (Costerton, 2004; Boles et al. 2004; Thomas et al. 2008; Kahn et al. 2015). Knowledge of the mechanisms that maintain the diversity and functioning of microbial systems existing within the human body or that invade it (for instance within the gut microflora or the biofilms of cystic fibrosis patients) is important for working towards successful treatments (Costerton 2004). Understanding protist predation on biofilms may help advance the understanding of the consequences of phagotrophic host defences. Conversely, understanding the effect of bacterial diversity and defence on predation may lead to increased understanding of pathogen resistance to immune systems. Furthermore, identifying mechanisms of disturbance resistance in pathogenic protists and bacteria might improve knowledge of resistance to treatment on ecological and evolutionary scales (Kahn et al. 2015).

Finally, microbial systems provide services for human health and wellbeing, such as biodegradation and improvements in water quality and plant growth (Saleem & Moe 2014).

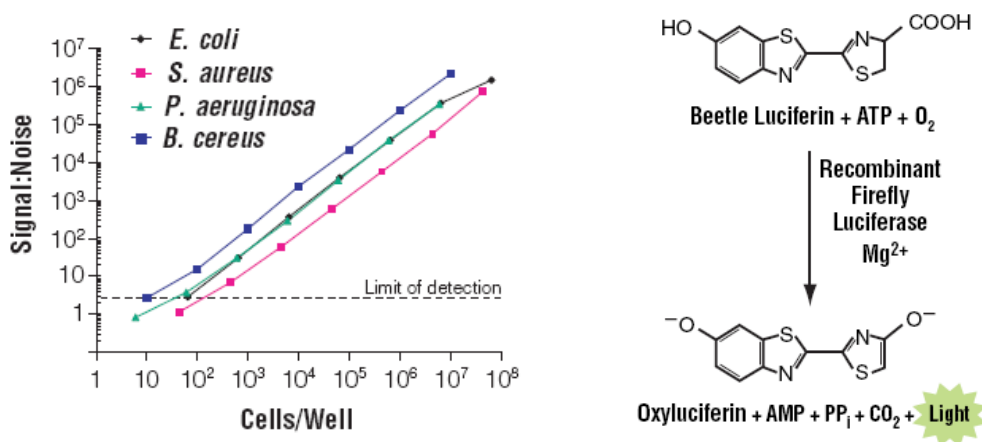
Knowledge of multilevel microbial loop interactions between bacteria, protists and viruses is gaining increased recognition. Protists have both positive and negative consumptive and non-consumptive effects on bacterial communities, functional processes, and therefore the services they provide (Wang et al. 2009; Schmitz et al. 2010; Saleem & Moe 2014). There is therefore a need to further understand how protist species interactions and functional diversity are linked to ecosystem processes such as bioremediation, wastewater treatment, nutrient cycling and plant growth. Due to the developing knowledge of the key role played by protists in these processes, there is a need to link this biotechnological perspective to general ecological theory, predictions of microbial diversity losses to climate change, functional diversity, and the mechanisms underlying community dynamics (Moe & Saleem 2014). With growing knowledge of microbial systems and services they provide in the face of a changing natural world, the consequences of the loss of these organisms and even whether steps need to be taken to conserve them will become of increasing concern (Cotterill et al. 2008).

Appendices

Appendix 1 – Supplementary methods

BacTiter-Glo cell viability assay

The BacTiter-Glo Microbial Cell Viability Assay (Promega Corporation, USA) involves the addition of a single reagent to a cell culture and measuring luminescence. The number of viable cells is determined through the quantitation of present ATP within the sample. It is a high throughput method which allows for the rapid screening of multiple samples (Promega Corporation Technical Bulletin 2010). The luminescent signal produced by the addition of the reagent, is proportional to the quantity of ATP present in the sample, which is in turn directly proportional to the number of viable microbial cells in the mixture (see supplementary figure 1). (Promega 2012). The reagent in question relies on a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase; Promega Corporation, USA) which produces a signal following the luciferase reaction shown in supplementary figure 1.



Supplementary figure 1. Reproduced from the Promega Corporation technical bulletin, 2010

Microcosm phosphate concentration colormetric assay

This method allows the rapid determination of available phosphate within a system using a colorimetric 96-well plate based assay. The chemistry is based on Murphy & Riley (1962).

The following reagents are made:

- Reagent 1 - 2.91 M Sulphuric acid = 2 ml of concentrated sulphuric acid (18 M) in 13ml de-ionised water (dH₂O)
- Reagent 2 - 0.0258 M Ammonium molybdate = 0.1562g in 4.9ml dH₂O
- Reagent 3 - 0.307 M Ascorbic acid = 0.2650g in 4.9ml dH₂O
- Reagent 4 - 0.001 M Potassium antimony tartrate = 0.001506g in 2.4545ml dH₂O

Ascorbic acid was made on the same day as it was required. Potassium phosphate stock was made by dissolving 0.0439g in 100 ml of dH₂O. This gave a stock standard solution of 100 mg ml⁻¹ (5.26 mM) phosphate. Standards were diluted between 0 - 20 mg ml⁻¹. The final calibration equation was:

$$mg\ ml^{-1} PO_4^{3-} = 0.2252x + 0.0818$$

The four reagents R1, R2, R3, and R4 were then mixed in the ratio 6 : 2 : 2 : 1 and 170 µl of standards and samples were aliquoted into the wells of a 96-well shallow transparent plate. 30 µl of the reagent mixture was dispensed into each of the wells. Plates were then incubated at room temperature for 10 minutes for the colour to develop. Colour develops due to the addition of antimony (as potassium antimonyl tartrate solution) to phosphate solution causes a reaction resulting in a colorimetric change of the solution to a blue colour (Murphey and Riley, 1962). The absorbance of the wells of the wells was read at a wavelength of 880 nm in a Biotek Synergy HT plate reader.

Enzymatic activity assays

These assays allowed the assessment of differences in enzyme expression. This was accomplished by using substrates that are covalently linked to the fluorescent moiety 4-methylumbelliferone (4-MUB). Once cleaved the 4-MUB could fluoresce and fluorescence measured by Synergy HT (BioTek). These methods were adapted from Frossard et al. (2012). Powdered substrate (Sigma) was dissolved in DMSO to achieve a 200mM stock solution of substrate as per the manufacturer's instructions. This was then diluted again 1:999 to achieve a working stock dilution of 200µM. 100µl of experimental sample was transferred to wells of a white, shallow flat-bottomed 96-well plate. For beach leaf tea this was reduced to 25µl of experimental sample which was then diluted to 100µl. This reduced quenching of fluorescence. 25µl of the working stock was then added to the wells. Plates were then incubated at room temperature for ninety minutes in the dark. After incubation, 10µl of 1M NaOH was added to wells. Plates were incubated again for no more than two minutes. Fluorescence was then read on a Biotek Synergy HT at 460/40nm (emission) and 360/40nm (excitation) which equates to an excitation wavelength of 365nm and emission of 465nm. Enzymatic activities were a rate of cleavage, but since cleavage time is held constant at ninety minutes only the concentration of the 4-MUB is given. Fluorescence was converted to nM using the calibration equation:

$$nM\ 4 - MUB = 0.0004x$$

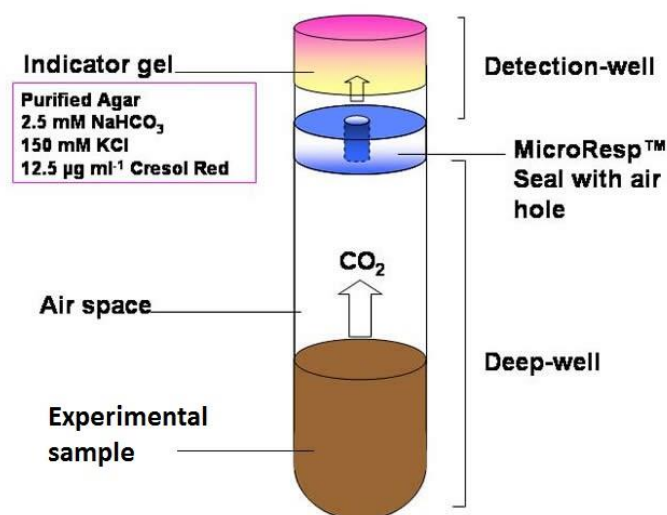
MicroResp™ CO₂ detection colorimetric assay

The MicroResp CO₂ detection colorimetric assay (Macaulay Scientific Consulting Ltd) provided a microplate-based respiration system that allowed the analysis of up to 96 samples simultaneously. The details of the assay are provided in the methods to chapter 2. However some additional details are given here.

The detection plates were produced using the following method as per the manufacturer's instructions. Indicator solution was made by dissolving 18.75mg cresol red, 16.77g potassium

chloride (KCl), and 0.315g sodium bicarbonate (NaHCO₃) in 900ml of de-ionised water (dH₂O) in a volumetric flask at less than 65°C.

This was then made up to 1000ml. This gave final concentrations of 12.5µg ml⁻¹ cresol red, 150mM KCl, and 2.5mM NaHCO₃. An appropriate amount of 3% agar was then prepared in dH₂O (3g per 100ml) and dissolved by microwaving at a low power setting. Agar was then allowed to cool to 65°C in a water bath. Indicator and agar were then mixed in a 2:1 ratio in a beaker and mixed thoroughly at 60°C with constant stirring. Finally, 150µl aliquots were dispense into flat bottom shallow 96 well plates and allow to cool and solidify. Plates were stored in the dark at room temperature in a sealed plastic box containing a falcon tube of water and a falcon tube of soda lime. Plates were not re-used within 48 hours of previous use to allow equilibration.



Supplementary figure 2. Setup of the MicroResp™ CO₂ detection colormetric assay. The diagram is reproduced and adapted from the MicroResp™ website (<http://www.microresp.com/Science.html>)

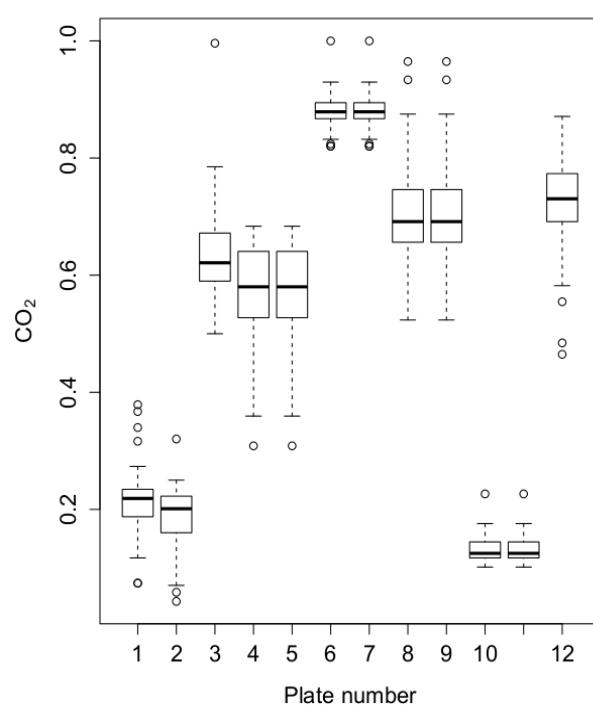
Detection plates were read at 572nm before assembly with MicroResp rubber seal and deep well plates containing experimental samples. Once assembled the whole unit was clamped tightly and stored in incubators at the appropriate experimental temperature. Incubation for the detection period was for twenty-four hours which is longer than the six hours described in the manufacturer's protocol. However, this states that it is acceptable to run the assay for longer periods given re-calibration. Detection plates were read again after twenty-four hours at 572nm. A diagram of the MicroResp set up is given (supplementary figure 2).

Calibration was carried out in the following way. Stock solutions of 1M sodium bicarbonate (NaHCO_3) and 1M HCl were pre-prepared. A one in ten dilution series of NaHCO_3 was produced and replicated in a 96 deep well plate. HCl was then added in excess such that the total volume was 600 μl . Detection plates, rubber seal and clamp were assembled with the 96 deep well plate containing the calibration samples. The detection plate was read after twenty-four hours and a standard curve produced. Percentage CO_2 calculations and conversion to CO_2 production rates was carried out as per the MicroResp™ technical manual. The final calibration equation was:

$$mg\text{CO}_2 = e^{(\ln(\Delta\lambda_{572})+0.305414)/0.282164}$$

$\Delta\lambda_{572}$ is the change in absorbance (572nm) between the beginning and the end of the experiment.

I reported in chapter 5 that data was discarded due to the failure of two MicroResp™ plates. This would have allowed seepage from the deep well and detection plates, resulting in no colorimetric reaction. A graph illustrating this is given on the next page.



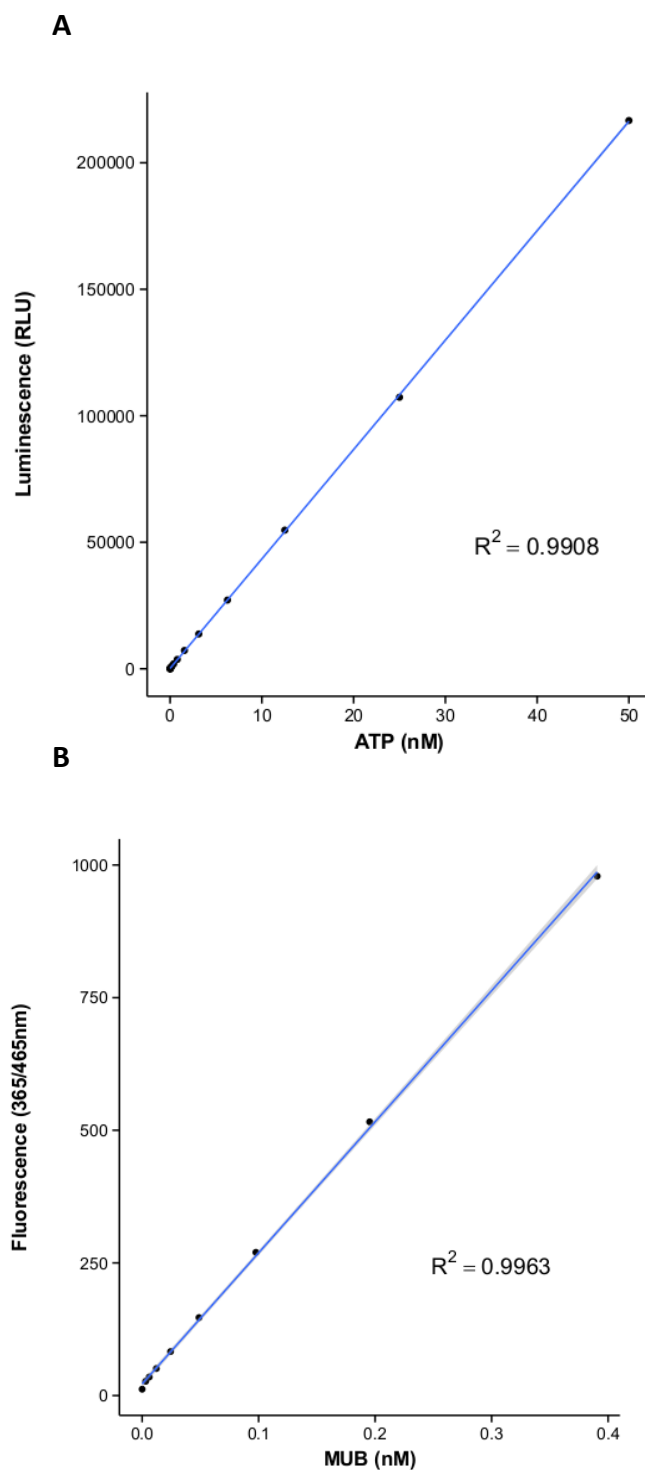
Supplementary figure 3. Box and whisker plots showing CO₂ production in each experimental plate. In A, plates 10 and 11 were considered to have failed due to the very small amount of CO₂ production detected in all wells.

Protist cell counting and biovolume calculation

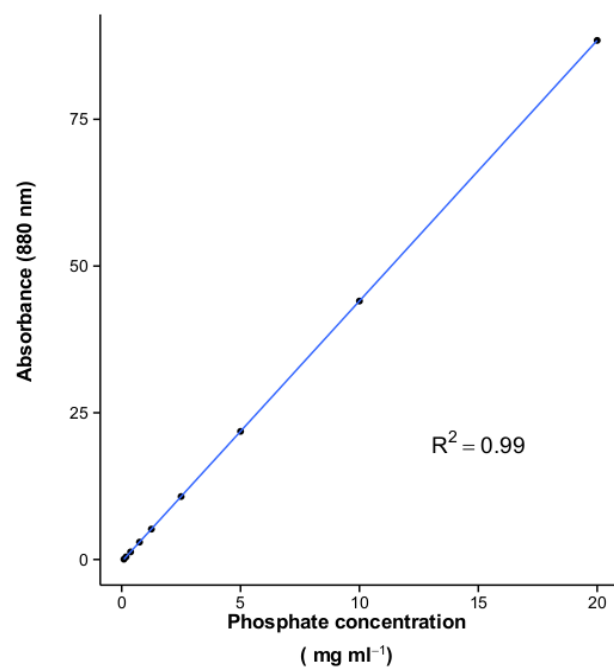
In order to ensure that initial protist bio-volumes were equivalent between strains at the start of the experiment, these were calculated for single cells of each strain and inoculating volumes adjusted accordingly. Calibration for measurements of cell diameters under a stereoscopic microscope was achieved using 6µm microsphere standards. This allowed the measurement of cells, and subsequently the calculation of mean diameters for each strain. These were then cross referenced with mean diameters obtained from Bass et al. 2009 and Howe et al. 2009. From this, mean volumes of cells were calculated for each strain.

Cell densities (cells µm⁻²) per field of view (FOV) for each strain were then approximated by assuming an even distribution, counting cells in three fields of view, calculating the mean, and then dividing by the surface area of one FOV. The surface area one well was calculated by π^2 . Using the surface area of one well and the estimated cell densities per field of view, a total number of cells per well was estimated. Using the known mean volume of medium per well (1503µl), mean cell density (cells µl⁻³) was calculated. Knowing the estimated mean number of cells per µl, and the estimated mean biovolume of each strain, adjusted volumes of cultures could be combined to produce mixed protist predator communities.

Appendix 2 – Calibration curves



Supplementary figure 4. Calibrations of A) ATP concentration and B) enzymatic activity (cleavage of 4-MUB). The equation for the ATP calibration is $y = 0.0002x$. The equation for 4-MUB substrate cleavage is $y = 0.0004x$.



Supplementary figure 5. Calibration curve of phosphate concentration colormetric assay. The calibration equation is given by $y = 0.2252x + 0.0818$

Appendix 3 – Statistical models

Supplementary table 1. This table shows the maximal generalised linear models (GLM) used throughout this thesis, organised by chapter

Chapter	Data analysed	Model	Response	Main effects	Covariates	Error distribution	Link function
2	FD method effect	GLM	Functioning	Protist richness*FD*FD method	N/A	Gaussian	Identity
3	Change in functional diversity effects	GLM	Difference in functioning	$\Delta \text{Mean dissimilarity} * \text{Initial mean community ID}$	Protist richness	Gaussian	Identity
	Functional diversity effects	GLM	Functioning	Mean dissimilarity*FGR*Bacterial community ID	Protist richness	Gaussian	Identity
4	Composition effects	GLM	Functioning	Bacterial community ID*Protist community ID*Response ratio	Protist richness	Gaussian	Identity
5	Diversity effects	GLM	Functioning	Protist richness*Response ratio*Bacterial diversity*(Bacterial diversity) ²	N/A	Gaussian	Identity
	Diversity effects	GLM	RR	Bacterial community diversity*(Bacterial community diversity) ²	N/A	Gaussian	Identity
6	Diversity effects	GLM	Total biovolume	Protist richness*Bacterial diversity*(Bacterial diversity) ²	ATP concentration	Gamma	Log
	Diversity effects	GLM	Total abundance	Protist richness*Bacterial diversity*(Bacterial diversity) ²	ATP concentration	Quasipoisson	Log
	Presence/absence effects	GLM	Species biovolume	Presence SpA*Presence SpB*Bacterial diversity (monoculture and four species combinations also)	N/A	Gamma	Log
	Diversity effects	GLM	Species A biovolume	Protist richness*Bacterial diversity	N/A	Gamma	Log
7	Functional diversity and disturbance	GLM	Shannon's Index (H')	Disturbance frequency*Disturbance autocorrelation*Cyst score*Mean dissimilarity + (Cyst score) ² + (Mean dissimilarity) ²	N/A	Quasipoisson	Log
	Functional diversity and disturbance	GLM	Shannon's Equitability (EH)	Disturbance frequency*Disturbance autocorrelation*Cyst score*Mean dissimilarity + (Cyst score) ² + (Mean dissimilarity) ²	N/A	Quasipoisson	Log

Appendix 4 – Supplementary results

Supplementary table 2. Table of GLM outputs for analysis of the experiment in Chapter 2 that compared the diversity-functioning relationships of communities assembled according to four methods of building dendrograms and classifying functional group richness (FGR). The four methods are response ratio (RR) based, trait-based, and combined trait and ecosystem functioning measure-based, and random assignment based.

<i>EF measure</i>	<i>Intercept</i>	<i>Interaction</i>	<i>Estimate</i>	<i>t-value</i>	<i>p-value</i>
<i>MUG</i>	Random method*FGR1	RR method*FGR2	-0.37	-2.98	0.0031
		RR method*FGR3	-0.38	-2.28	0.023
		RR method*FGR4	-0.86	-3.6	< 0.001
<i>MUN</i>	Random method*FGR1	RR method*FGR2	-0.51	-1.99	0.047
<i>CO₂</i>	Random method*FGR1	RR method*FGR2	0.34	2.50	0.013
	Random method*FGR1	Trait method*FGR2	0.48	4.19	< 0.001
	Random method*FGR1	Combined trait method*FGR2	0.52	4.53	< 0.001
<i>Cellular activity (ATP)</i>	Random method*FGR1*Species richness	RR method*FGR4*Species richness	0.43	1.94	0.049
		Trait method*FGR3*Species richness	0.30	1.97	0.05
<i>MUG</i>	Trait method*FGR1	RR method*FGR2	-0.34	-2.69	0.0074
		RR method*FGR3	-0.42	-2.63	0.0087
		RR method*FGR4	-0.72	-3.48	< 0.001
<i>MUP</i>	Trait method*FGR1	RR method*FGR2	-0.23	-2.74	0.0063
		RR method*FGR3	-0.27	-2.61	0.0094
		RR method*FGR5	-0.34	-2.26	0.024

Supplementary table 3. Table A shows mean and standard errors for species' coefficients of effect on response ratios experimental ecosystem functioning measures. Cells coloured green show the diversity level with the highest mean coefficient for a given function. Table B shows median and inter-quartile ranges of coefficients across the diversity gradient for each of the functions. Red cells show the most negative median coefficients and green cells the most positive. IQR is useful for looking at the magnitude of coefficients at different levels of diversity

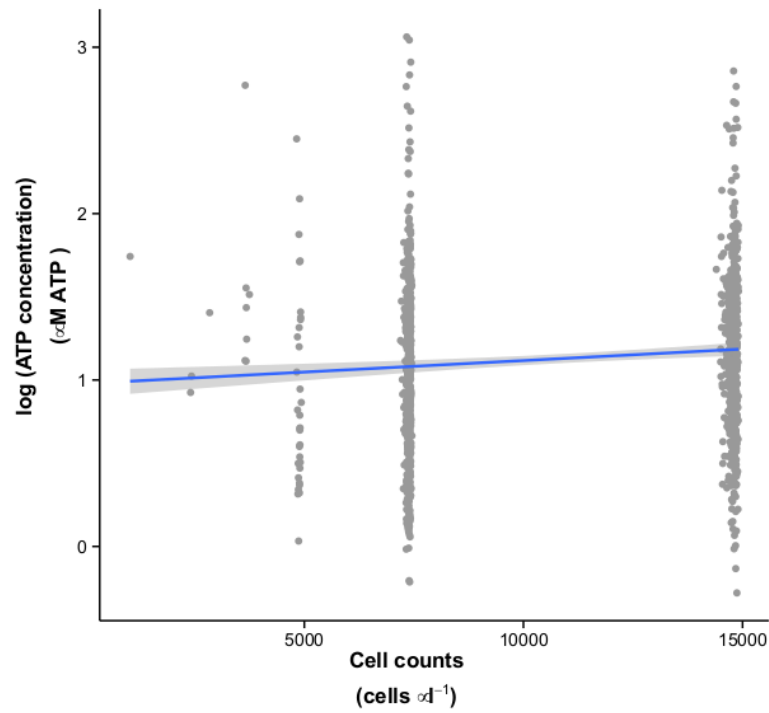
Table A	Coefficient of effect on response ratio ; means and standard errors by community diversity: dilution level											
	10^0		10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Functional measure												
MUG	0.032	0.0053	0.083	0.014	0.076	0.012	0.058	0.0095	0.065	0.013	0.056	0.0091
MUN	0.038	0.011	0.030	0.0063	0.029	0.0065	0.039	0.0072	0.034	0.0053	0.057	0.0076
MUP	0.0093	0.0021	0.029	0.0044	0.014	0.0024	0.015	0.0028	0.030	0.0040	0.017	0.036
Xylo	0.096	0.018	0.071	0.010	0.036	0.0056	0.059	0.0097	0.038	0.0063	0.069	0.01
ATP	0.049	0.01	0.083	0.02	0.049	0.01	0.15	0.02	0.12	0.02	0.029	0.005
Phosphate	0.0027	0.00049	0.0034	0.00072	0.0066	0.0011	0.0080	0.0012	0.58	0.045	0.0047	0.00077
Table B	Coefficient of effect on response ratio; medians and IQRs by community diversity											
	10^0		10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Functional measure												
MUG	0.0030	0.052	0.015	0.13	0.0027	0.11	-0.0035	0.11	-0.00063	0.084	-0.0036	0.094
MUN	-0.0074	0.065	-0.038	0.041	0.0056	0.038	-0.00017	0.063	0.0032	0.058	0.013	0.11
MUP	-0.0004	0.010	-0.0020	0.057	-0.0037	0.025	0.0026	0.025	0.0030	0.046	0.00017	0.022
Xylo	-0.018	0.14	-0.0044	0.12	0.0022	0.060	-0.016	0.11	-0.0043	0.066	0.018	0.14
ATP	-0.0022	0.061	-0.0030	0.14	-0.017	0.050	-0.053	0.28	0.0088	0.20	-0.015	0.045
Phosphate	-0.0001	0.049	0.00032	0.0054	0.00058	0.012	-0.0020	0.014	0.58	0.23	-0.0001	0.0049

Supplementarytable 4. r and p values of Mantel tests that found significant correlations of coefficients of effect on response ratios from the RPD linear model analysis. Correlations are between diversity dilution gradient levels for the six EF measures for which response ratios were calculated

Diversities	Ecosystem functioning measures																	
	ATP			Phosphate			MUG			MUN			MUP			Xylo		
	r	p		r	p		r	p		r	p		r	p		r	p	
$y \sim x$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.32	0.006	
$10^{-1} - 1$																		
$10^{-5} - 1$	0.38	0.01	-	-	-	-	-	-	-	0.30	0.04	-	-	-	-	-	-	-
$10^{-2} - 10^{-1}$	0.33	0.02	0.33	0.02	0.35	0.009	0.76	0.001	-	-	-	-	0.23	0.02	-	0.23	0.02	
$10^{-2} - 10^{-1}$	0.23	0.01	-	-	-	-	0.55	0.002	-	-	-	-	0.23	0.02	-	0.23	0.02	
$10^{-4} - 10^{-1}$	-	-	-	-	-	-	0.47	0.001	-	-	-	-	0.40	0.002	-	0.40	0.002	
$10^{-5} - 10^{-1}$	-	-	-	-	-	-	0.46	0.001	-	-	-	-	-	-	-	-	-	
$10^{-3} - 10^{-2}$	-	-	0.23	0.04	-	-	0.64	0.001	-	-	-	-	-	-	-	-	-	
$10^{-4} - 10^{-2}$	0.29	0.03	-	-	0.51	0.001	0.45	0.002	-	-	-	-	-	-	-	-	-	
$10^{-5} - 10^{-2}$	-	-	-	-	-	-	0.53	0.001	-	-	-	-	0.23	0.03	-	0.23	0.03	
$10^{-4} - 10^{-3}$	-	-	-	-	-	-	0.52	0.001	-	-	-	-	-	-	-	-	-	
$10^{-5} - 10^{-3}$	-	-	-	-	-	-	0.23	0.04	-	-	-	-	-	-	-	0.42	0.001	

Supplementary table 5. Intercepts and gradients of regression lines of coefficients of effect on response ratios. Regressions were plotted between significantly correlated diversity levels for the six EF measures for which RRs were calculated. The gradients correspond to the most diverse diversity level plotted on the x axis and the least on the y. A positive relationship less than 1 therefore indicates a positive correlation but where coefficients are more positive in the more diverse community on the x axis

Diversities ($y \sim x$)	Ecosystem functioning measures											
	ATP		Phosphate		MUG		MUN		MUP		Xylo	
	Est.	Int.	Est.	Int.	Est.	Int.	Est.	Int.	Est.	Int.	Est.	Int.
$10^{-1} - 1$	-	-	-	-	-	-	-	-	-	-	0.61	0
$10^{-2} - 1$	-	-	-	-	-	-	0.27	0	-	-	-	-
$10^{-5} - 1$	0.3	0	-	-	-	-	-	-	-	-	-	-
$10^{-2} - 10^{-1}$	0.35	0	0.99	0	0.56	0	-	-	-	-	0.3	0
$10^{-3} - 10^{-1}$	0.73	0	0.69	0	0.71	0	0.91	0	-	-	0.23	0
$10^{-4} - 10^{-1}$	-	-	-	-	-	-	0.68	0	-	-	-	-
$10^{-5} - 10^{-1}$	-	-	-	-	-	-	1.2	0	0.32	0	-	-
$10^{-3} - 10^{-2}$	-	-	-	-	-	-	0.99	0	-	-	1.1	0
$10^{-4} - 10^{-2}$	1.4	0	-	-	-	-	0.67	0	-	-	-1.1	0
$10^{-5} - 10^{-2}$	-	-	-	-	-	-	1.3	0	-	-	-0.72	-
$10^{-4} - 10^{-3}$	-	-	-	-	-	-	0.61	0	-	-	-	-
$10^{-5} - 10^{-3}$	-	-	-	-	-	-	0.73	0	-	-	-	-



Supplementary figure 6. Scatter plot of microcosm ATP concentration against bacterial cell counts per microliter. Counts were obtained by flow cytometry and ATP concentrations by BacTiter-Glo cell viability assay. The regression is plotted by GLM and has the equation $y = 0.00014x + 1$

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