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The Formation and Stability of a Microbial Community

Submitted by Joseph Pennycook, to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences, March 2019.

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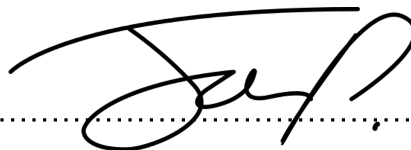

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Abstract

New communities form regularly in nature, as many species rush to colonise a freshly formed island, pool, or microbiome, but it is unclear what rules govern the arrangement of these founders into a smaller, stable community, or whether the process is predictable.

I simultaneously inoculated a master mix of bacterial colonisers into 45 identical environments, and allowed them to compete and evolve for around three months. By the end of the experiment, the species compositions of these communities had split into two broad groups, defined mostly by the mutual exclusivity of two *Pseudomonas* species, which may represent the ecological equivalence of the two species. Due to this functional similarity, I propose that community formation may be predictable at an ecological level, if not a taxonomic level.

I also explored one of the communities formed in this experiment in further detail, investigating the maintenance of its diversity and stability. The community was fairly stable, as every species was able to persist even when it began at a much lower population size than its competitors, and no diversity was lost after 4 weeks of culture. I grew the species from this community in monoculture, as well as in every possible pair, triplet, and quartet, to fully assess the network of interactions, and found evidence for many significant higher-order interactions, which have been shown to have a stabilising effect in theoretical models.

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Introduction

Community ecology, the study of how multiple species interact within a single environment, is at the core of many problems currently facing humanity. For example, the microbial communities that colonize the human body are regarded as increasingly important for host health (Lynch and Pedersen, 2016), conservation efforts are often targeted on keystone species such as predators which safeguard biodiversity within their communities (Sergio *et al.*, 2006), and even agricultural yields are dependent on a healthy microbial community within the soil (Wood and Bradford, 2018). However, natural communities can be incredibly complex. For example, despite decades of intensive research, many of the hundreds of bacteria species that colonise the human gut remain uncharacterised (Rajilić-Stojanović and de Vos, 2014), and the diversity of the bacteriophages, viruses which play a predatory role in the gut ecosystem, is even less well understood (Manrique et al. 2017). The Amazon rainforest contains at least 6727 species of tree (Cardoso et al. 2017), all competing for the same basic resources of light, water, and pollination. Even much simpler communities can function in surprisingly complex ways, such as a collection of riverbed grazers in a North American stream where the removal of one grazer has cascading effects through two competing algae species to inhibit a second grazer (Creed, 1994). For this reason, community ecology has historically been a difficult field to study (McGill *et al.*, 2006).

Perhaps due to the difficulty of direct experimental work, there exists a rich history of theoretical investigation of community ecology. The classic view of community assembly is based on the concept of niches, where resources are divided up by different species that are all highly adapted to compete most effectively within their own spatial, temporal, and ecological window (MacArthur, 1970). Diversity under this system is therefore maintained by the fact that no species can be the dominant competitor for all available resources, and must specialise on a specific subset. A core principle of this theory is the idea that two species cannot be completely equivalent competitors in the same environment; one must always drive the other to find a different niche, or else to extinction (Levin, 1970). However, there is increasing support for an opposing view, known as the neutral theory, where many species are ecologically equivalent, and able to maintain competing populations for at least long enough

for processes of speciation and migration to maintain diversity in an environment (Hubbell, 2001), and theoretical work has shown that neutral processes can result in patterns of abundance similar to those observed in nature (Bell, 2001).

Compelling evidence exists for both perspectives. There are clear examples of competitive exclusion, such as the diverging perch heights of Carribean anoles (Losos, 1998) or the differentiation in host species for North American fruit flies (Feder, et al., 1988). Other environments, however, contain far more diversity than seems to reasonably reflect distinct niches, such as the near identical freshwater shrimp species inhabiting a North American glacier (Witt and Hebert, 2000). Coral reef fish species have been found to overlap far more than expected in food preferences and habitat use, and this observation prompted the neutral-based ‘lottery hypothesis’, where diversity is maintained in highly unpredictable environments because no species is competitively superior enough to win the ‘lottery’ 100% of the time (Sale, 1977). Still, it remains possible that the diversity of available niches is being underestimated in these cases; Falster et al. (2017) showed that by including several important, but perhaps less obvious, axis of niche differentiation in their models, predictions that resemble real, natural communities could be obtained. Both theories likely have a role to play in building a comprehensive understanding of community ecology, and recent theoretical approaches have synthesised aspects of each (e.g. Vellend, 2017), but the exact balance is still a matter of debate.

Taking the first steps into experimental exploration, many fields of study first establish broad principles with very simple model systems, such as Mendel’s peas (Mendel, 1965) or Darwin’s finches (Grant, 1986), which can then be tested and refined with more complex experiments. A similar approach has been taken with community ecology, where microbiological study allows far higher replication and ease of manipulation than other systems. In the very simplest cases, multiple strains or morphs of the same species have been used to approximate a community (Kassen *et al.*, 2000), and these approaches have their uses, but it is unclear whether results from these systems can be extrapolated and applied to true communities. Great success has been found by assembling simple collections of bacteria or protists, and these models have been used to explore areas like the sequential assembly of communities

(Warren, Law, and Weatherby, 2003), or the effect of global warming on food webs (Petchey et al. 1999). In recent years, with molecular techniques such as 16S sequencing and shotgun sequencing becoming easier and cheaper, the focus of microbial community ecology has shifted towards whole-community surveys of gut or soil biomes (e.g. Reichardt *et al.*, 2017; Goldford *et al.*, 2018), but even when these studies produce valuable experimental results, it can be very difficult to investigate the mechanisms that underlie them, as in many cases, integral species have not been isolated or well-described, and the communities are once again incredibly complex. There is room in the field to supplement modern, molecular techniques with a return to simple, well-understood community models, especially where the interactions between all species are well characterised, and the community is shown to have inherent stability. Such a model could be used to test the response of a communities to factors such as predation, invasion, or environmental disturbance, with a large number of replicates and very quickly, and allow a far deeper insight into underlying mechanisms than other methods.

One of the core advantages of a simple model community would be the possibility of exhaustively characterising the relationships between species. The number of potential relationships in a community rises exponentially with the total number of species, and most natural systems are far too complex to study more than a tiny subset of relationships, even though they are essential to the ecology of the community. Some species may not interact at all, or their interactions may be as distant and indirect as sharing a common resource or predator, but other relationships based on cross-feeding, parasitism, or predation may be essential to the fitness of one or both species. Increasing the complexity even further, on top of these direct interactions between pairs of species, higher-order interactions between triplets or larger groups can exist (Levine *et al.*, 2017). These may take the form of interaction chains, where the mechanisms remain the same as the direct interactions, but where changing a population size changes the size of the effect, leading to a complicated cascade of influence. An example is the effect of the black rush *Juncus geradi* on the aphid species *Uroleucon ambrosiae*, where the rush has a positive effect on the aphid not because of any direct interaction, but because the aphid feeds on an elder species which benefits from the way the rush structures the soil (Levine *et*

al., 1998). Other higher-order interactions, sometimes described as *true* higher-order interactions, even operate distinctly from the corresponding direct interactions. For example, the red-breasted nuthatch *Sitta canadensis* eavesdrops on the alarm calls of the black-capped chickadee *Poecile atricapillus*, which warn the birds of predation from raptors, and so the interaction between raptors and the nuthatch is distinctly different in the presence or absence of the chickadee (Templeton and Greene, 2007). Despite these charismatic examples, there still exists debate as to how common and important interspecific interactions are in natural communities. A recent study by Messier, McGill and Lechowicz (2010) found that in multiple surveyed natural communities of plants, within-species variation in key functional traits was just as high as between-species diversity. If this is true for most natural communities, can interspecific interactions be considered any more important than simple competition between any two individuals, regardless of species? By using a community simple enough to map every interaction, it would be possible to understand not only whether the species were interacting (directly and via higher-order effects), but also how important these interspecific interactions were for the species' fitness.

In this study, I have attempted to establish a simple model bacterial community, and used the opportunity to investigate the mechanisms underpinning its structure. In the first chapter I will detail the long-term co-culture of many bacteria species, and the variability of the resulting communities. Then, in the second chapter, I will focus on a single one of these communities and examine the mechanisms behind its stability by characterising the species interactions. Finally, I will provide a short summation of the study.

Chapter 1: *The variability of community formation.*

Almost all biological species exist in the context of a community, and while the complexity of community ecology has historically slowed the establishment of broad principles (McGill *et al.*, 2006), technical advances such as metagenomic analysis, and recent key publications such as Vellend's (2016) synthesised theory have allowed some real progress in the field (e.g. Mouillot *et al.*, 2013; Goldford *et al.*, 2018). Historically, theoretical models (e.g. MacArthur, 1970; Hubbell, 2001) and simple, microbial systems (e.g. Kassen *et al.*, 2000; Petchey et al. 1999) have been used reduce the complexity to such a degree that basic questions can be answered, and conclusions can then be explored in more complex, natural communities (Mayfield and Stouffer, 2017; González-Barrios and Álvarez-Filip, 2018). In many cases, these simple models still present the best opportunity to directly study the complex mechanics of interacting species. One of the most fundamental questions of community ecology is this: what rules govern the formation of biological communities?

It may seem irrelevant in modern biology to investigate the formation of communities; at first glance, every environment on the planet has long since been colonised, and therefore it would seem far more relevant to study the development and succession of existing communities. However, on closer examination, the formation of novel communities is a perpetually relevant question, with immense importance to matters of health and conservation. Humans, and many other animals, are born with no microbiome, and are then colonised by microbial life from their parents and the environment (Bäckhed *et al.*, 2015), the composition of which has a large impact on future health outcomes (Tamburini *et al.*, 2016). Could we identify poorly forming communities early and intervene? Furthermore, not only do new volcanic islands rise from the sea, but other isolated environments such as lakes and coral reefs, which can follow the same ecological principles as islands (MacArthur and Wilson, 1967), form regularly and await colonisation. González-Barrios and Álvarez-Filip (2018) found that certain species of coral are more effective at supporting a healthy reef than others. Would it be possible to manipulate the formation of a reef to favour these beneficial species? To answer questions such as these, we must first understand whether community formation is a fundamentally variable or invariant process.

Goldford *et al.* (2018) recently investigated the formation of microbial communities in very simple environments, by inoculating mixtures of bacteria species into single-nutrient media. These microcosms developed into surprisingly diverse communities, the composition of which they found to be variable at the species level, but invariant when species were grouped by family. 3 types of nutrient media were tested, and each promoted a different yet invariant composition at the family level. However, although the study confirmed that multiple taxa were indeed present in each community by plating on agar, they only used 16S sequencing to measure the actual composition of these communities, which can incorrectly estimate the proportions of species based on copy number (Louca *et al.*, 2018). If this conclusion, that community formation is variable at the species level, but invariant at higher taxonomic ranks, could be replicated in multiple systems with multiple sampling methods, it could become an important facet in our understanding of community formation.

Taken to its furthest extremes, however, this conclusion would not be particularly helpful or surprising, as a community will almost always be variable at the strain level, but invariant at the level of the Domain. The taxonomic similarity of species is not inherently meaningful in this context, but only as an approximation of phenotypic similarity; it is a reasonable assumption that species of the same genus will share phenotypic traits that are not shared with species of different genera (Martiny *et al.*, 2012). Traditional niche theory suggests that environments contain certain niches that can be filled by organisms of a specific phenotype (Vandermeer, 1972). It may be that the specific species colonising a new environment will always be variable, but that it would be possible to predict and manipulate the *ecological roles* that colonising species will fill based on the environmental conditions. Goldford *et al.* (2018) did not directly investigate the mechanisms behind the variability in their communities, but it would be useful to determine whether species that appeared mutually exclusive between communities were in fact functionally equivalent.

In this study, I carried out a similar experiment to Goldford *et al.* (2018), measuring the variability of community formation at multiple taxonomic ranks, with multiple sampling methods, then investigating the underpinnings of the variability in these communities.

Methods

Experiment 1.1: Is community formation variable?

To investigate the variability of community formation, I set up multiple communities under identical conditions, with identical founders, and compared their species composition over time.

I inherited 27 bacteria species from another researcher in the same lab, originating from soil samples. Each species was from a unique genus, and was not identified below genus level. The genera covered were: *Achromobacter*, *Acidovorax*, *Agromyces*, *Arthrobacter*, *Bacillus*, *Bordatella*, *Brevundimonas*, *Candidamonas*, *Cupriavidus*, *Devosia*, *Flavobacterium*, *Lysinibacillus*, *Microbacterium*, *Ochrobactrum*, *Oersboria*, *Paenibacillus*, *Paracoccus*, *Pedobacter*, *Pigmentiphaga*, *Pseudomonas*, *Pussillimonas*, *Rhizobium*, *Rhodococcus*, *Shinella*, *Staphylococcus*, *Stenotrophomonas*, *Variovorax*. I only used these samples in the experiments covered here. However, later molecular work showed that multiple operational taxonomic units (OTUs) belonging to a single genus were present in the resulting communities, e.g. at least 3 separate OTUs of *Pseudomonas* were detected. It's possible that the original isolates were not pure strains, or else the stocks were contaminated by additional species at some point. Nonetheless, due to the presence of these extra strains in almost every replicate of the experiment, it is clear that any potential contamination occurred before the experiment began, and should have affected all communities equally.

These 27 species were used to create a master mix, then this mixture was inoculated into 48 separate 6 mL microcosms of TSB (Tryptone Soya Broth) media at 1/64th dilution, which were incubated statically at 28 ^{o}C. It was vital to ensure that multiple species would persist, or else the resulting cultures would not be communities, so I took several steps at this stage to encourage the coexistence of species. I expected that a complex medium such as TSB would provide many resource-based niches, while a static environment would provide multiple spatial niches, increasing the likelihood of multiple species coexisting. I also left these microcosms to grow for 5 weeks without the addition of any nutrients, before any data collection took place, which, along with the highly

diluted media, I hoped would encourage cross-feeding relationships to develop between species.

After this 5-week 'settling' period, I began to passage 1% (60 µL) of each community into a fresh 6mL microcosm of 1/64th TSB every week, for 7 weeks. To measure species composition, I grew diluted samples of each community on KB (King's medium B) agar plates, and counted the number of colonies of every morph (see *Morph Definitions* below) in every community, before the passage each week.

To produce a second measure of community composition, I extracted DNA from all communities at weeks 3 and 6 of the experiment and performed a metagenomic analysis. All samples were subjected to 3 freeze-thaw cycles prior to help lyse the cells, then DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit with pre-treatment for gram-negative bacteria. The extracted DNA was then tested for quality and quantity using 1% agarose gel electrophoresis, and the dsDNA HS kit with a Qubit Fluorometer. The 16S gene was amplified using PCR with 515F and 806R primers, then the sequencing was carried out using the standard protocols of an Illumina MiSeq machine. Paired-end reads were used with the Miseq Reagent Kit v2 (500 cycles) and run metrics of 250 cycles using Miseq Control Software 2.2.0 and RTA 1.17.28. This sequence data was then analysed using the full stack workflow for microbiome analysis (Callahan *et al.*, 2016) using the dada2 and phyloseq packages in R. Samples were pooled for species inference to improve the detection or rare variants, and taxonomy was assigned to Amplicon Sequence Variants (ASVs) using the Ribosomal Database Project (Cole *et al.*, 2013). Sequencing and analysis was successful for all 96 samples, and 41 ASVs were assigned, with a mean of 75565 reads per sample (minimum of 57801 reads, maximum of 92482 reads).

Morph Definitions

To measure the species composition of my communities as accurately as possible from plate counts, I first defined specific, visually distinct morphs to categorize colonies. Then, I counted the prevalence of each morph in each community, and took steps to see how closely my morph definitions aligned with actual species. I plated a single community at 3 weeks into the 5-week settling period, and defined as many morphs as I could, taking 2 isolates of each morph and growing them in monoculture. I then plated the monocultures and merged

morph definitions when two morphs appeared identical to each other in monoculture, or appeared on each other's plates in monoculture, indicating that the difference between the morphs was either non-heritable or extremely plastic. At this point I had 13 morphs, of which 2 never appeared in plate counts after the settling period, leaving 11 morphs to comprise the plate count data.

After the main body of the experiment, I needed to test how well the true species of a colony was estimated by morph identification, which I approached by sequencing isolated colonies. I picked 6 communities from the 48 available, and took isolates of every morph present in those communities. I then took isolates from additional communities to ensure that I had at least 3 samples from each time point (weeks 3 and 6) for each morph. One community was never observed from the second week of data collection onwards, so could not be confirmed by 16S sequencing, but was included in the analysis for the weeks where it was present. I used PCR on these samples to amplify the region of the 16S gene between the 515F and 806R primers, and sequenced the results. Sequences were successfully extracted from 82 out of 95 samples, leaving at least 2 samples from each time point for each morph.

Morph identification was mostly able to distinguish unique species, with 3 major exceptions (Figure 1). First, Morph B and Morph J represented the same *Pseudomonas* species, so the counts of those morphs were merged for the remainder of the analysis. A *Cupriavidus* species was only ever identified as Morph G, but the *Pseudomonas* species normally identified as Morph I was often also identified as Morph G. Similarly, an *Achromobacter* species was only ever identified as Morph D, but the *Stenotrophomonas* species normally identified as Morph C was also often identified as Morph D. Morph D and Morph G, therefore, were poor tools to measure true species abundance, as I was unable to accurately distinguish them by eye from other morphs during this experiment. After consideration, I decided to continue with the analysis, bearing this bias in mind, and interpreting counts of Morph D and Morph G as the species uniquely associated with them. The plate count data and 16S rDNA sequence data were both imperfect lenses through which to view these communities, but their biases (morph misidentification and copy number respectively) were independent of each other, so interpreting these datasets in tandem should lend clarity to the analysis.

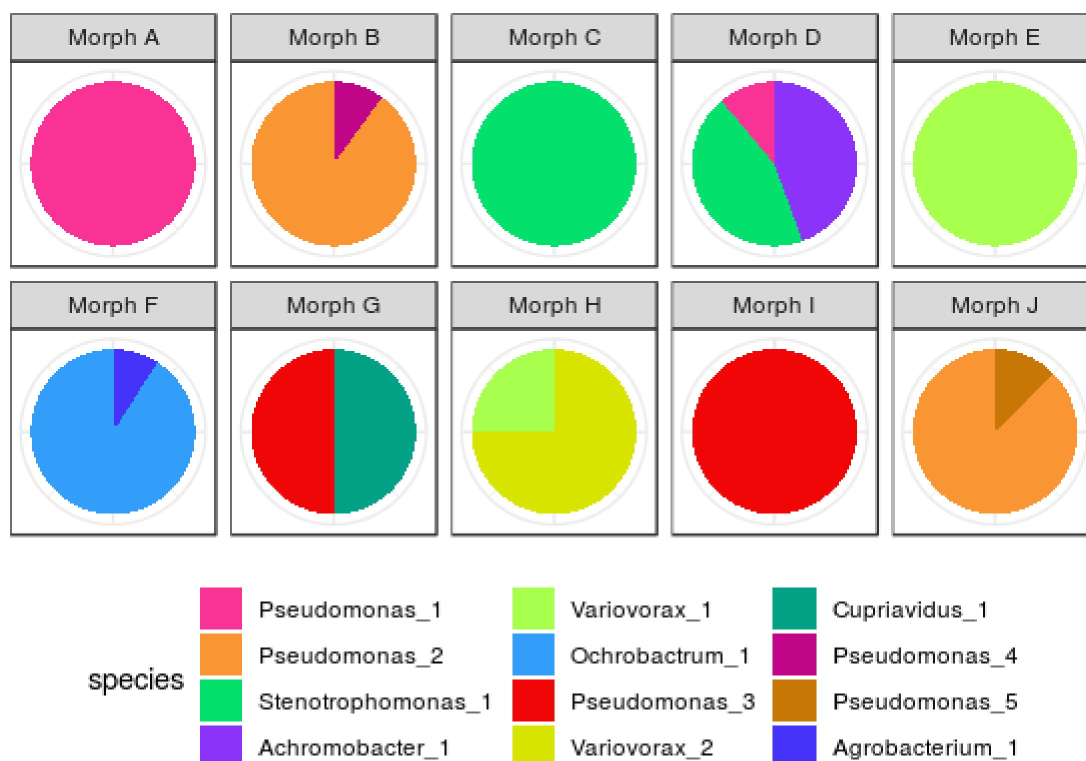

Figure 1: True species identity for each morph in Experiment 1.1, derived from 16S rDNA sequencing of clonal colony samples. Number of samples of each morph = 9(A); 10(B); 6(C); 9(D); 9(E); 11(F); 6(G); 8(H); 6(I); 8(J).

Data Analysis

If all 48 communities were very similar at the end of this experiment, the formation of these communities could be described as invariant, and if they were very different, or could be sorted into multiple distinct groups, their formation could be described as variable. In other words, if they formed a single cluster, they could be described as invariant, and if they formed multiple clusters, they could be described as variable, following Liu *et al.* (2008)'s definition of a cluster as data falling within a single, multivariate, normal distribution.

I used Ward's (1963) algorithm, along with the Bray-Curtis distance metric, to build a hierarchical tree of clusters and the sigclust2 algorithm (Kimes *et al.*, 2017) to move down the tree and determine significance at each node, in order to sort communities from any given time point or sampling method into significant clusters. I also wanted to test whether communities remained in the

same clusters across multiple weeks, or if they were randomly sorted into clusters each week. If clusters were present but not consistent across time points, these clusters could represent different states in a fluctuating system that was nonetheless very similar between replicates, just out of step. To test this, I developed a ‘consistent clustering’ algorithm, which builds hierarchical cluster trees for data over multiple time points, then builds a single, combined tree, based on how often pairs of communities are in the same cluster across all time points (Box 1).

Box 1: ***The Consistent Clustering Algorithm***

Many methods exist to build hierarchical trees of nested clusters based on the similarities within a single dataset (Ward, 1963; Kaufman and Rousseeuw, 1990). The consistent clustering algorithm is an attempt to build a hierarchical tree of nested clusters, incorporating how *consistently similar* labelled communities are across multiple observations (such as multiple time points). The algorithm builds hierarchical trees using any desired method for each observation individually (this study uses Ward's (1963) method, then calculates the number of times each pair of communities shares a cluster across all time points). The inverse of this cluster-sharing count can then be used as the new distance matrix to build a new hierarchical cluster tree (again using any desired algorithm). The combined tree, in theory, should identify clusters more accurately than standard methods, by incorporating the consistency of clusters across time, and can then be used with significance tests such as sigclust2 (Kimes *et al.*, 2017).

I carried out many tests of both standard Ward clustering and consistent clustering, both along with sigclust2, on simulated datasets where the number of true clusters was already known (Figure B1). Consistent clustering was slightly less successful than standard clustering when only 2 time points were considered, although this difference was not significant (t-test; $t_{3893.7} = 0.836$, $p = 0.403$). However, consistent clustering was significantly more successful than standard clustering when there were 4 or 8 time points (t-test; $t_{3693.8} = -8.837$, $p < 0.001$; $t_{3694.1} = -17.536$, $p < 0.001$). For these latter treatments, consistent clustering was particularly more effective when the clusters were roughly 1 standard deviation apart. Furthermore, consistent clustering was much less prone to overestimating the number of clusters (0.5% type 1 error) than standard methods (6.3% type 1 error), regardless of how many time points were used (t-test; $t_{8483.8} = 28.114$, $p < 0.001$).

Consistent clustering was less successful than the standard Ward method when the clusters were distinct by less than a standard deviation (t-test; $t_{4331.9} = 3.807$, $p < 0.001$), although neither method was at all successful in these cases (2.0% success and 3.4% success respectively). However, consistent

clustering was more successful than the standard ward method when the clusters were distinct by at least a standard deviation (t-test; $t_{6879.8} = -21.587$, $p < 0.001$), with much more respectable success (73.9% and 53.6% respectively).

In conclusion, the simulation data suggests that the consistent clustering method is more effective than standard clustering at accurately identifying the number of clusters in a dataset, so long as data was sampled on at least 4 time points, and the clusters are distinct by at least a standard deviation.

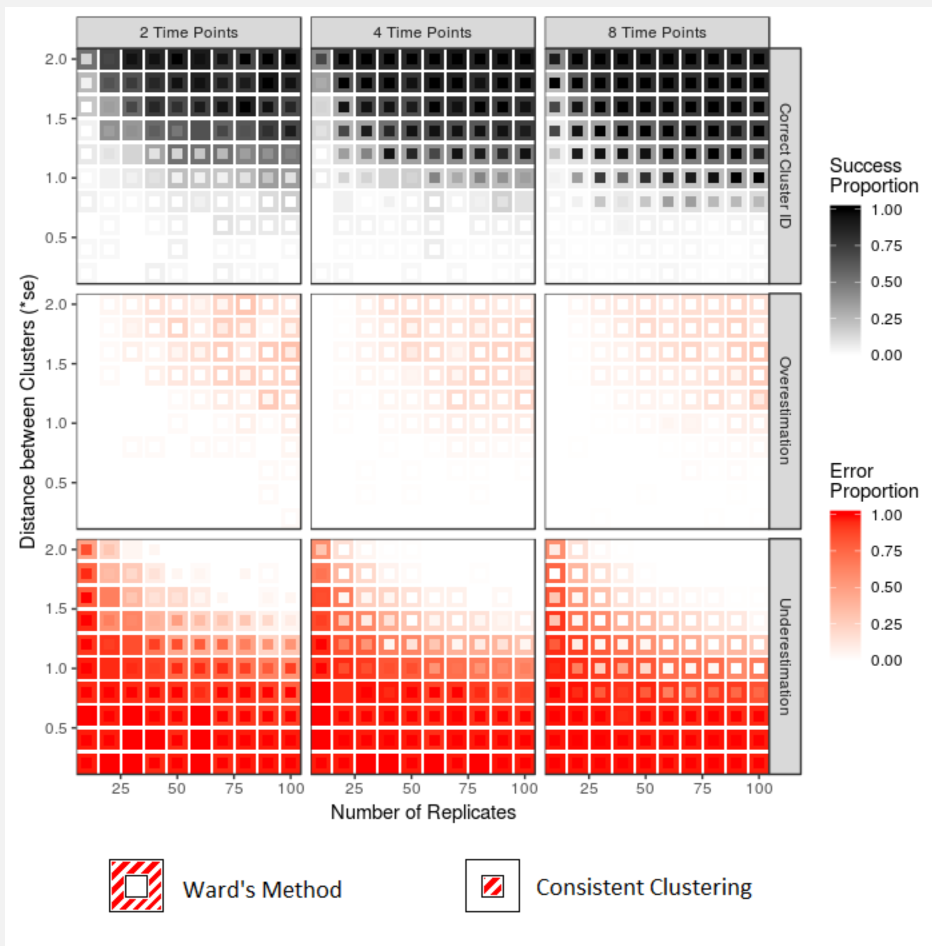

Figure B1: The cluster detection success of both Ward's method (outer square) and the newly developed consistent clustering method (inner square), both using the sigclust2 algorithm to determine significance, in correctly identifying clusters in a simulated dataset. Simulated datasets comprised 8 variables, all sampled from normal distributions, with between 10 and 100 values (replicates). All datasets comprised of 2 true clusters, meaning that each replicate was randomly drawn from 1 of 2 different normal distributions, which were between 0.2 and 2 standard deviations apart. Simulated datasets were recalculated either 2, 4, or 8 times to simulate sampling communities at multiple time points. Each combination of replicate number, cluster distance, and time points was calculated 20 times for each algorithm, to determine the proportion of times each method would overestimate, underestimate, or correctly identify the number of clusters.

Goldford *et al.* (2018) found that community formation in their experiments was variable at higher taxonomic levels, but invariant at lower taxonomic levels. As a further test of this conclusion, I performed all analyses at both the genus and species level, and compared the results.

3 out of 48 communities produced plates with no colonies at least once, probably due to a dilution error during plating, and since these communities were missing data for at least one time point, they were excluded from analysis.

Results

Experiment 1.1:

Community composition appeared to be broadly similar between communities, although quite different between sampling methods (Figure 2). The count data showed a much larger proportion of *Achromobacter*, while the 16S rDNA sequence data showed a much larger proportion of the species *Pseudomonas* 1 and *Pseudomonas* 2. It was also apparent that these two *Pseudomonas* species were mutually exclusive.

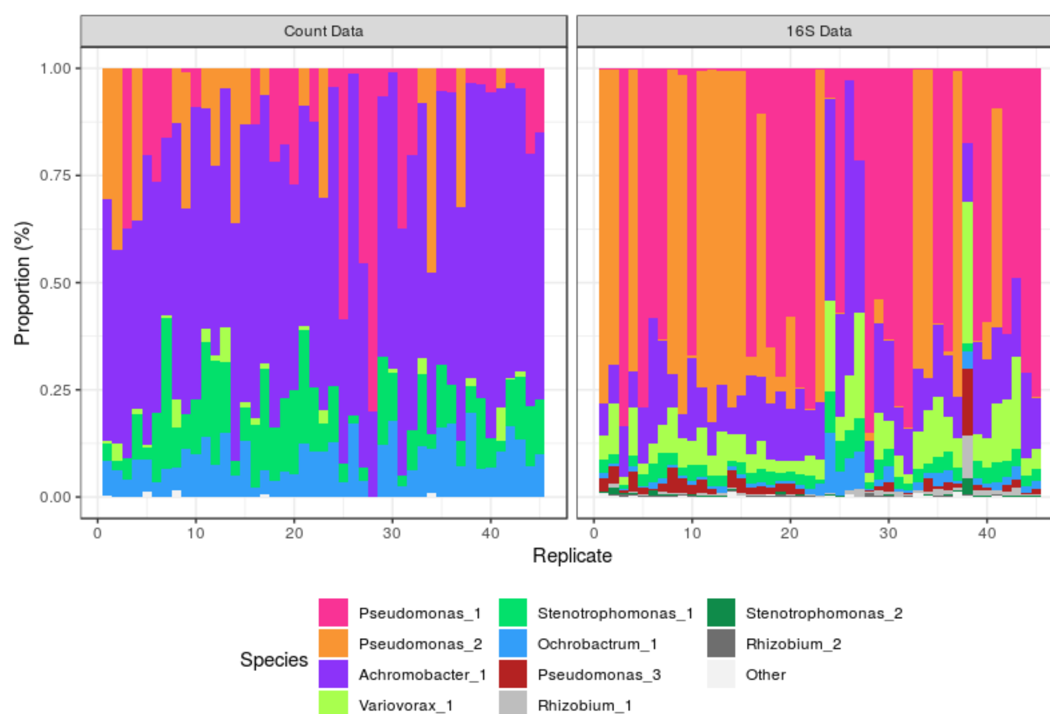

Figure 2: Species composition of each community at week 6 of Experiment 1.1, measured by both plate counts and 16S rDNA sequencing. Species shown are the 10 most abundant species from the sequence data, with all rarer species grouped into 'Other'.

Standard Ward (1963) clustering and sigclust2 (Kimes *et al.*, 2017) analysis showed distinctly different results depending on the sampling method (Figure 3; Table 1; Table 2). Analysis of the count data found only a single cluster most weeks, but 2 or 3 significant clusters in some instances. In week 2 of the count data, there were more clusters at the genus level, and in week 3, there were more clusters at the species level, showing no clear support or denial of Goldford *et al.* (2018)'s conclusion that community formation was less variable

at higher taxonomic ranks. Conversely, analysis of the 16S rDNA sequence data showed 2 significant clusters at the species level, but a single cluster at the genus level, across both sampled weeks, showing strong support for Goldford *et al.* (2018)'s conclusion.

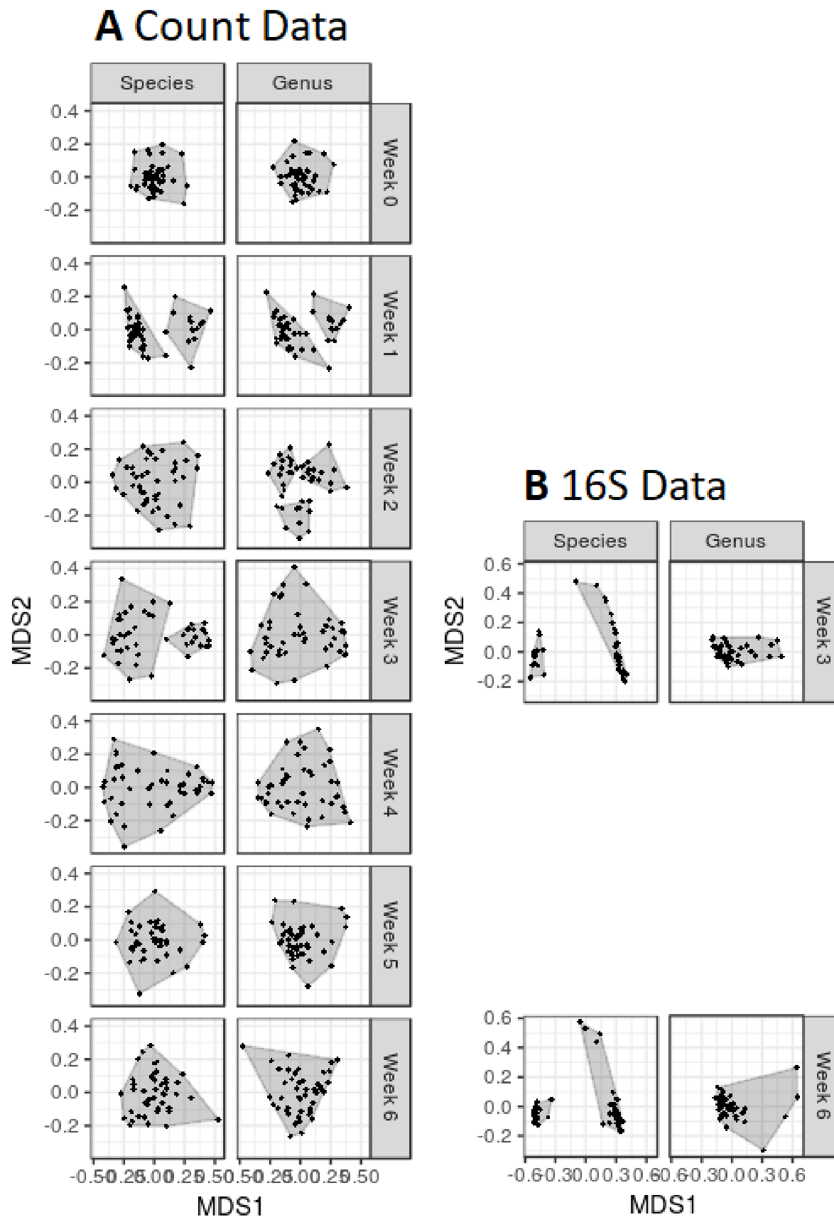

Figure 3: NMDS projections of the dissimilarity between communities at each week of **A:** plate count data and **B:** 16S rDNA sequence data, analysed at both the genus and species level. Each significant cluster found by the sigclust2 algorithm is indicated by a distinct grey polygon, so points falling within the same polygon represent communities that belong to the same cluster, and points within different polygons belong to different clusters.

Table 1: Statistical results from sigclust2 analysis of plate count data at each week of Experiment 1.1, showing the number of significant clusters, the p values at each significant split of the hierarchical clustering, and the cluster indices at each significant split of the hierarchical clustering. Results are shown grouping data both by species and genus. Cluster Index (CI) values are the test statistic of sigclust2, which show the degree of difference between two compared clusters, and are used to calculate the p-value. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Species Level			Genus Level		
Week	**n clusters**	**p values**	**CI values**	**n clusters**	**p values**	**CI values**
0	1	0.380	0.720	1	0.078	0.695
1	2	**0.003** *	0.426	2	**0.016** *	0.521
2	1	0.284	0.658	3	**0.002;** **0.005** *	0.657; 0.441
3	2	**0.028** *	0.479	1	0.124	0.563
4	1	0.114	0.524	1	0.194	0.555
5	1	0.921	0.727	1	0.915	0.727
6	1	0.316	0.658	1	0.716	0.679

Table 2: Statistical results from sigclust2 analysis of 16S rDNA sequence data at each week of Experiment 1.1, showing the number of significant clusters, the p values at each significant split of the hierarchical clustering, and the cluster indices at each significant split of the hierarchical clustering. Results are shown grouping data both by species and genus. Cluster Index (CI) values are the test statistic of sigclust2, which show the degree of difference between two compared clusters, and are used to calculate the p-value. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Species Level			Genus Level		
Week	**n clusters**	**p values**	**CI values**	**n clusters**	**p values**	**CI values**
3	2	**< 0.0005**	0.161	1	0.168	0.384
6	2	**< 0.0005**	0.220	1	0.239	0.419

Use of the consistent clustering algorithm along with sigclust2 analysis showed much more consistent results between sampling methods (Figure 4; Table 3). Both sampling methods showed 2 consistent, significant clusters at the species level, and a single consistent cluster at the genus level, showing support for Goldford *et al.* (2018)'s conclusion.

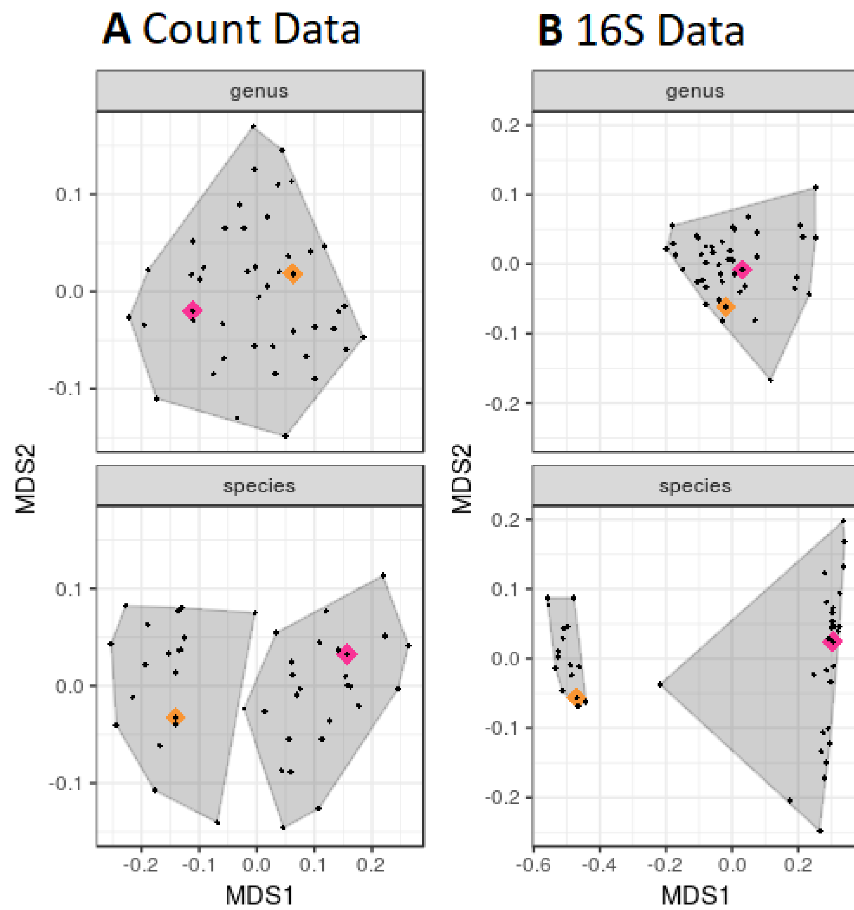

Figure 4: NMDS projections of the mean dissimilarity across all time points between communities from **A:** plate count data and **B:** 16S rDNA sequence data, analysed at both the genus and species level. Points within the same grey polygon represent communities that belong to the same significant cluster according to the sigclust2 algorithm and the consistent clustering algorithm, and points within different polygons belong to different clusters. In each panel, the point highlighted in pink represents the community sampled and used as P1 in Experiment 2.1, and the point highlighted in orange represents the community sampled and used as P2 in Experiment 2.1.

Table 3: Statistical results from sigclust2 and consistent clustering analysis of plate count data and 16S rDNA sequence data from Experiment 1.1, showing the number of significant clusters, the p values at each significant split of the hierarchical clustering, and the cluster indices at each significant split of the hierarchical clustering. Cluster Index (CI) values are the test statistic of sigclust2, which show the degree of difference between two compared clusters, and are used to calculate the p-value. Results are shown grouping data both by species and genus. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Species Level			Genus Level		
Method	**n clusters**	**p values**	**CI values**	**n clusters**	**p values**	**CI values**
Count	2	**0.013 ***	0.444	1	0.989	0.818
16S	2	**< 0.0005**	0.127	1	1.000	0.852

The independent clustering of communities was very similar between plate count and 16S rDNA sequencing data, with only 3 out of 45 communities assigned to different clusters depending on the sampling method (Figure 5). Species composition was similar between the two clusters, according to both sampling methods, except for a pair of *Pseudomonas* species, each of which hugely outnumbered the other in one of the clusters (Figure 6).

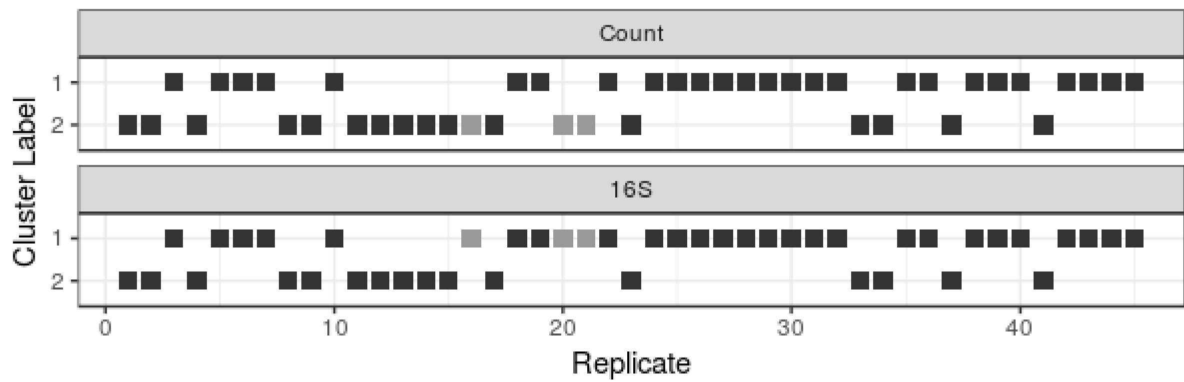

Figure 5: Cluster identity of all replicates of Experiment 1.1 for both plate count data and 16S rDNA sequencing data, showing whether each individual replicate community was assigned to the first or the second of two significant clusters. Dark grey squares indicate that the cluster identity of that replicate was the same according to both plate count data and 16S rDNA sequencing data, while lighter grey squares show that the cluster identity differed between sampling methods.

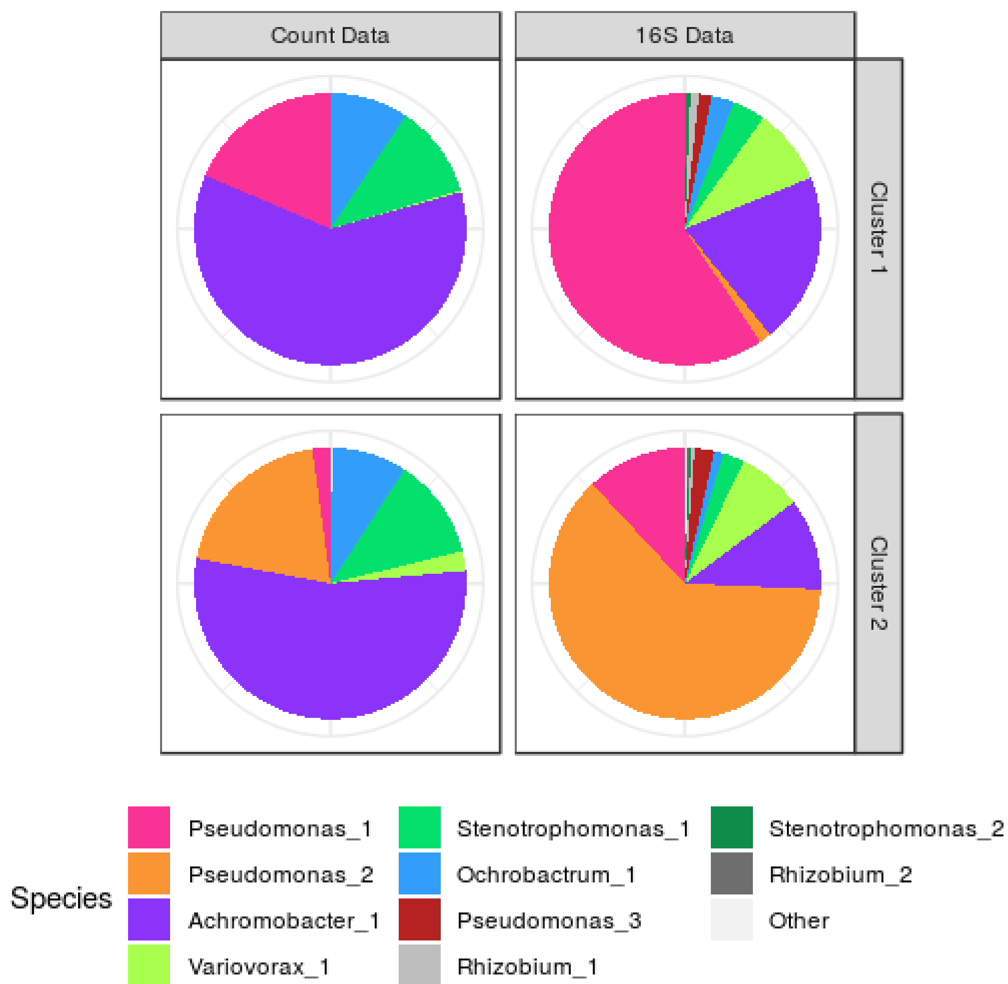

Figure 6: Mean species composition of each significant cluster found with the consistent clustering algorithm and the sigclust2 algorithm, for each sampling method used in Experiment 1.1.

Discussion

Different sampling methods and analyses led to significantly different results in this experiment (Figures 2, 3, 4, & 6). For example, standard Ward clustering of the count data unusually showed that genus level had more clusters than the species level at some time points (Figure 3). These genus level clusters may have represented legitimate differences between communities that were obscured by noise at the species level, or else may have been caused by the clustering algorithm. Comparison of the count and 16S rDNA sequencing data seems to confirm that copy number bias (Louca *et al.*, 2018) exaggerated the proportions of some species, most notably the *Pseudomonas* species (Figures 2 and 6). This led to unwarranted statistical confidence in clusters based just on the 16S data (Figure 3), suggesting that caution should be taken when interpreting studies that rely entirely on 16S rDNA sequencing, such as Goldford *et al.*'s (2018). Nonetheless, when looking at patterns across all sampling methods and analyses, our results seem to support the same conclusion as Goldford *et al.*'s. It would have been impossible to predict, at the start of the experiment, which community would fall into which cluster, as all 45 communities grew under identical conditions, but developed into distinctly different species compositions (Figure 4). However, the composition of these communities was invariant at the genus level, which suggests that it may be possible to predict and manipulate the formation of communities at broad taxonomic levels.

As previously discussed, functional predictability may be the underlying cause of this taxonomic predictability, assuming that closely related species share similar phenotypes. This assumption may not always be true, particularly in bacterial communities due to their capacity for horizontal gene transfer, but at least in general, closely related bacteria species share traits more often than would be expected through chance alone (Martiny *et al.*, 2015). However, the link between taxonomy and phenotype varies in strength depending on the trait in question. For example, the ability to metabolise particular carbon sources has been found to vary even between closely related species (Martiny *et al.*, 2012), so if the formation of a community was primarily governed by the available metabolites, the metabolite-imposed structure may not be apparent from the taxonomy of the community.

The two clusters in Experiment 1.1 differed primarily in their proportions of two *Pseudomonas* species (Figure 6), which appeared to grow almost exclusively in communities where the other did not (Figure 2). This prediction could be confirmed by exploring the ability of the two species to invade communities both where the other is present, and where the other is not. If each species was able to prevent the other from invading into its community, that would be strong evidence for true mutual exclusivity. In fact, this experiment was attempted, but suspected contamination issues undermined confidence in the results, so the experiment is being repeated and will be reported elsewhere.

If this apparent mutual exclusivity were to be confirmed, it would be evocative of the competitive exclusion principle (Levin, 1970), which suggests that close competitors are unable to coexist, and one will always drive the other to extinction, or promote divergent evolution (eg. Stuart *et al.*, 2014), making it highly likely that these two *Pseudomonas* species would share a similar niche and a similar phenotype. This would seem to confirm that the invariant, genus level structure of communities formed in Experiment 1.1 (Figure 4) was based on an invariant set of niches, or ecological roles, provided by the environment, which species were able to slot into as colonisation occured. This would line up with several existing natural observations. Sale's (1977) observation of coral reef fishes gave rise to the lottery hypothesis, where individuals of competitively equivalent species live or die based on luck-of-the-draw resource assignment. The two *Pseudomonas* species in this model may be such species, only dominating in any given community by random chance, and as in the lottery hypothesis a stable equilibrium between the two may be found in a more complex model that involved dispersal and multiple environments to colonise. Messier, McGill and Lechowicz (2010) also found that plant communities had very variable species composition between sites in the same environment, but very low trait variation, suggesting again the kind of functional community assembly that may be at work in this model.

If this principle holds true, predicting community formation would still be a daunting task with great complexity, particularly as many species open up new niches by creating spatial structure (eg. Foster *et al.*, 2013) or producing waste that other species can utilise (eg. Belenguer *et al.*, 2006), so the task would not be as simple as merely understanding the abiotic environment. Nonetheless,

the results of this study suggest that it would be at least possible to predict the ecological composition of a forming community, so long as the raw environment and the pool of possible founders were well understood.

In this experiment, every species was introduced to the sterile environment simultaneously, but in nature this is unlikely to be the case. Species which arrive first are able to adapt to the local environment before they are forced to compete with other community members, which can significantly impact the composition of a resulting community (Gómez *et al.*, 2016), and could in theory increase or decrease the variability of community formation. Some species depend on being the first to reach a new environment, and invest energy into strategies to achieve this, such as pioneer tree species which grow seeds with wind-catching sails or bribe animals into carrying their seeds (Dalling *et al.*, 2002). Similarly, the first species to colonise the human microbiome, at least for traditionally delivered babies, are those found in the mother's vaginal tract (Dominguez-Bello *et al.*, 2010). Given the importance of a community's founders to its eventual composition, a consistent set of pioneer species might greatly decrease the variability of community formation. Conversely, if there is no structure to which species arrive first, founder effects could simply serve to magnify the randomness of the process. Investigating the effects of colonisation order on the variability of community formation might be an interesting area for future study.

In conclusion, by replicating a community formation event in bacteria, I was able to determine that the structure of the resulting community was variable at the species level, but invariant at the genus level. This variability was mostly underpinned by the near mutual exclusivity of two *Pseudomonas* species, which may be functionally equivalent competitors. These results suggest that the ecological structure of a community could be predicted from its founders and the environment.

Chapter 2: *Higher-order interactions within a stable community.*

The diversity of communities in the natural world is significantly higher than we would expect based on classical ecological theory. The principle of competitive exclusion predicts that closely competing species cannot coexist (Hardin, 1960), yet we can observe far larger numbers of species in stable communities than we can reasonably assume to not compete with each other (Hutchinson, 1961). How is this diversity maintained?

Several theories have been put forward to explain the high degree of diversity in nature. The first, unstable coexistence, proposes that species of very similar fitness are not truly coexisting, but simply driving each other to extinction so slowly that speciation and migration are able to maintain diversity (Hubbell, 1997). Conversely, theories of stable coexistence suggest that multiple species, even close competitors, can coexist indefinitely without outside influence (Chesson, 2000). One such theory is the idea of intransitive networks (Allesina and Levine, 2011), where organisms are competing for multiple resources, but different organisms are the dominant competitors for different resources. A theoretical model has shown that intransitive networks can support a large number of competitors, although the model also led to some unusual results, such as only supporting an odd number of species (Allesina and Levine, 2011). However, intransitive networks have indeed been shown to maintain diversity in competing plant species (Lankau *et al.*, 2010).

The interactions between multiple trophic levels can also have a stabilising influence on communities. One example is cross-feeding (Ribeck and Lenski, 2015), where organisms feed off the waste metabolites of other community members, which can increase the diversity of available resources, creating more niches and higher species diversity. Cross feeding is well established in laboratory studies (Pfeiffer and Bonhoeffer, 2004; Ribeck and Lenski, 2015), but it has unclear relevance outside of the microbial world. Both predator-prey interactions and parasitism may also help to reduce the pressure of direct competition, as dominant competitors will find themselves targeted more by their enemies as their population sizes grow, and rarer species targeted less (Hastings and Godfray, 1999; Murdoch, 1969). This kind of dynamic is known as negative frequency-dependent selection, where the fitness of any individual

species increases as it gets rarer (Levin, 1988), and is a hallmark of any stable community.

Another promising, but relatively untested, explanation for diversity maintenance is the concept of higher-order interactions (Levine *et al.*, 2017). Even if the direct interactions between species are competitive and reduce the fitness of both competitors, it is plausible that the nature of these interactions change in the presence of other species, reducing the pressure of competition or even having a positive effect on fitness. This could be through multiple competitive interactions reducing the population size of a species to the point where its competitive effects on any other species would be weakened (known as interaction chains), or a more complex mechanism where one or more species alters the relationship between two others (true higher-order interactions). Theoretical models have shown that even random higher-order interactions can have a stabilising influence on high-diversity communities (Bairey, Kelsic and Kishony, 2016), but the prevalence of these effects in nature is difficult to test, due to the complexity of measuring higher-order interactions within large communities.

The simple microbial communities formed in Chapter 1 present a valuable opportunity to investigate the role of higher-order interactions in maintaining diversity. Previous studies have only been able to study either a fraction of the possible interactions within a natural community, or all interactions within a subset of an existing community. Mayfield and Stouffer (2017) found compelling evidence for the presence of higher-order interactions within a natural community of annual plants, but were only able to study interactions between species they observed in close proximity, and interactions between groups of species of four or more were not considered. Sanchez-Gorostiaga *et al.* (2018) found multiple higher-order interactions within a consortia of soil bacteria, but these species (6 out of 7 of which shared a genus) were removed from their natural context, and it seemed that all significant higher-order interactions were caused by a single redundancy effect, based on one species lacking a vitamin required for growth. Within a 5-species community, there are enough combinations of more than one species (26) to thoroughly explore the concept, but few enough that an exhaustive investigation would be possible.

I performed a series of experiments to test for negative frequency-dependent selection, to determine if species were stably coexisting, in the resulting communities from Chapter 1, and then investigated whether higher-order interactions could explain the maintenance of diversity in these communities.

Methods

Experiment 2.1: Are the focal communities stable?

To test if the focal communities were truly stable, I tested to see if individual species would be able to recover from a much lower starting frequency than the rest of the community, which would indicate the stabilising presence of negative frequency-dependent selection.

I picked a community from Experiment 1.1 which showed the presence of P1 (*Pseudomonas_*1 in Figure 2) but not P2 (*Pseudomonas_*2 in Figure 2), and isolated a clone of each visually identifiable species. In addition to P1, 4 other species were isolated: a *Stenotrophomonas* species; an *Achromobacter* species; a *Variovorax* species; and an *Ochrobactrum* species. I grew these species for 2 days in 6 mL static microcosms of 1/64th TSB at 28°C. I had previously calculated the relationship between optical density and cell density in these species, so I used these formulas to estimate the density of cells in each monoculture. I then set up communities with 3 replicates in each of 5 treatments: -P, where an equal density of *Stenotrophomonas*, *Achromobacter*, *Variovorax*, and *Ochrobactrum* were inoculated into a fresh microcosm, along with *Pseudomonas* at 0.01 times the density; -S, where *Stenotrophomonas* was 0.01 times the density of the other 4 species; and so on for -A, -V, and -O treatments. I then repeated the entire process, but for a community showing the presence of P2 but not P1. The same 4 additional species were present in the P2 communities, but they were likely to be slightly different than the P1 community species after many weeks of separate coevolution.

All 5 treatments from each community were grown at 28°C and plated out every day for 3 days, with the first plating taking place 24 hours after the communities were set up.

Data Analysis

To determine if invasions were successful in any given treatment, I first calculated the proportion of the invader at the end of the experiment relative to its proportion at the beginning, using the formula:

$$v = \frac{p1(1 - p0)}{p0(1 - p1)}$$

where $p0$ was the starting proportion of the invader, and $p1$ was the final proportion of the invader. I then used a one sample *t*-test to compare the values of v for each treatment to a mean of 1. If, for example, the *Pseudomonas* species grew to be significantly more abundant than its initial 100-fold lower density in the -P treatment, that would be strong evidence for the presence of negative frequency-dependent selection, where the fitness of an individual *Pseudomonas* cell is higher as the species becomes rarer (Levin, 1988). If negative frequency-dependent selection acted upon every species within a community, that would be strong evidence for the stability of that community.

Experiment 2.2: Are higher-order interactions present?

To test whether the stability of the focal community was mediated entirely by pairwise competition, or whether there was a significant influence of higher-order interactions, I grew and observed the species in every possible combination, then tested to see which model best fit the abundances of each species in each treatment.

I selected the P2 community from Experiment 2.1 as a focus, due to its greater species persistence in Experiment 2.1 (Figure 7). I grew in monoculture all 5 species from this community, for 2 days in 6 mL static microcosms of 1/64th TSB at 28°C. I then used these monocultures to set up 3 replicates for each of 30 treatments: P, where 20 μL of *Pseudomonas* was inoculated into a fresh microcosm; PS, where 20 μL of both *Pseudomonas* and *Stenotrophomonas* were inoculated; PSA, where 20 μL of *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* were inoculated; and so on for the treatments S, A, V, O, PA, PV, PO, SA, SV, SO, AV, AO, VO, PSV, PSO, PAV, PAO, PVO, SAV, SAO, SVO, AVO, PSAV, PSAO, PSVO, PAVO, and SAVO. 6 replicates of the full community (PSAVO) treatment were also set up, to collect more data on the stability of the full community. All of these microcosms were left to grow for a week at 28°C before they were plated and 1% (60 μL) of each was passaged into a fresh microcosm. This process was repeated for 4 weeks.

Data Analysis

I constructed a data table where the counts of all species in every treatment were recorded, even when the treatment did not include every species. For example, for the P treatment, where only *Pseudomonas* was inoculated, I listed the counts of *Stenotrophomonas*, *Achromobacter*, *Variovorax*, and *Ochrobactrum* as 0. In this manner, I synthesised the results of all treatments into a single set of data, which could be used to construct predictive models.

I approached this analysis by treating each of the 5 species in turn as the focal species, and testing whether higher-order interactions were necessary to explain the abundance of the focal species across different treatments. In other words, I produced 5 sets of models, where for each set, the counts of the focal species were considered the dependent variable, and the counts of the other 4 species were considered the dependent variables.

I followed the framework of Mayfield and Stouffer (2017) by constructing negative binomial models, with a additional adjustments. Firstly, Mayfield and Stouffer used a single focal individual, while I am using a focal population, and they observed individual plants as competitors, while I observed competitor populations. As a result, I used estimated population size as a measure of fitness, rather than fecundity, and I removed terms in the model relating to intraspecific competition, which I was unable to measure. Secondly, Mayfield and Stouffer only included first and second-order interactions in their model (i.e. direct effects, and interactions between pairs of competitors), but I have sufficient data to also estimate third and fourth-order effects (interactions between triplets and quartets of competitors), so I added appropriate terms to the model. The new model took the general form:

$$P_i|\{N\} = \lambda_i e^{A_i|\{N\}} e^{B_i|\{N\}} e^{C_i|\{N\}} e^{D_i|\{N\}} e^{E_i|\{N\}}$$

where P_i was the population size of the focal species i when grown alongside the multiple listed species included in the term $\{N\}$, λ_i was the population size of the focal species i when grown in monoculture, and the exponentials captured the direct ($A_i|\{N\}$) and higher-order ($B_i|\{N\}$, $C_i|\{N\}$, …) effects of the other species $\{N\}$ on the population size of focal species i.

The direct effects were described by:

$$A_i|\{N\} = -\sum_{j=1}^{S} \alpha_{ij} P_j$$

where P_j was the population size of the individual competitor species j, α_{ij} was the effect of the individual competitor species j on the population size of the focal species i, and the sum was across all competing species S. The second-order effects were described by:

$$B_i|\{N\} = -(\sum_{j=1}^{S} \sum_{k=j+1}^{S} \beta_{ijk} P_j P_k)$$

where any repeated terms were the same as the direct effect equation, P_k was the population size of the second individual competitor species k, and β_{ijk} was the effect of the interaction between species j and k on the population size of species i. The third and fourth-order effects followed the same pattern, adding terms l and m for the third and fourth interacting species.

Again taking the lead from Mayfield and Stouffer (2017), I tested the fit of a selection of models against my data, for each focal species. The models tested were: a null model, where competing species had no effect on the population size of the focal species; a direct interaction model, where only the direct effects were included; and second, third, and fourth order interaction models. I then used the Akaike Information Criterion (AIC), which tests the fit of models and penalises them more as they increase in complexity (Bozdogan, 1987), to evaluate each model, and selected the model for each focal species with the lowest AIC value as the most parsimonious. For example, I produced a set of models with *Pseudomonas* as the focal species: a null model, a direct interaction model, a second-order interaction model, and so forth. Whichever of these models had the lowest AIC value was considered the most effective model in explaining the population size of *Pseudomonas*, when given the population sizes of the other species.

Results

Experiment 2.1:

After correction for multiple t-tests using the Sequential Bonferroni correction (Rice, 1989), in the P1 community, *Stenotrophomonas*, *Achromobacter*, and *Ochrobactrum* were significantly successful in invading a community of the other species (Figure 6; Table 4). The proportion of *Variovorax* also fell drastically in every other treatment for this community, and was not observed in 9 out of 15 total communities.

In the P2 community, only *Pseudomonas* was significantly successful in invading a community of the other species (Figure 6; Table 4). However, every species, including invading species, was observed in every replicate of every treatment by day 3.

When combining the results of the two communities, *Stenotrophomonas*, *Achromobacter*, and *Ochrobactrum* were significantly successful invaders, while the other two species were not (Table 5).

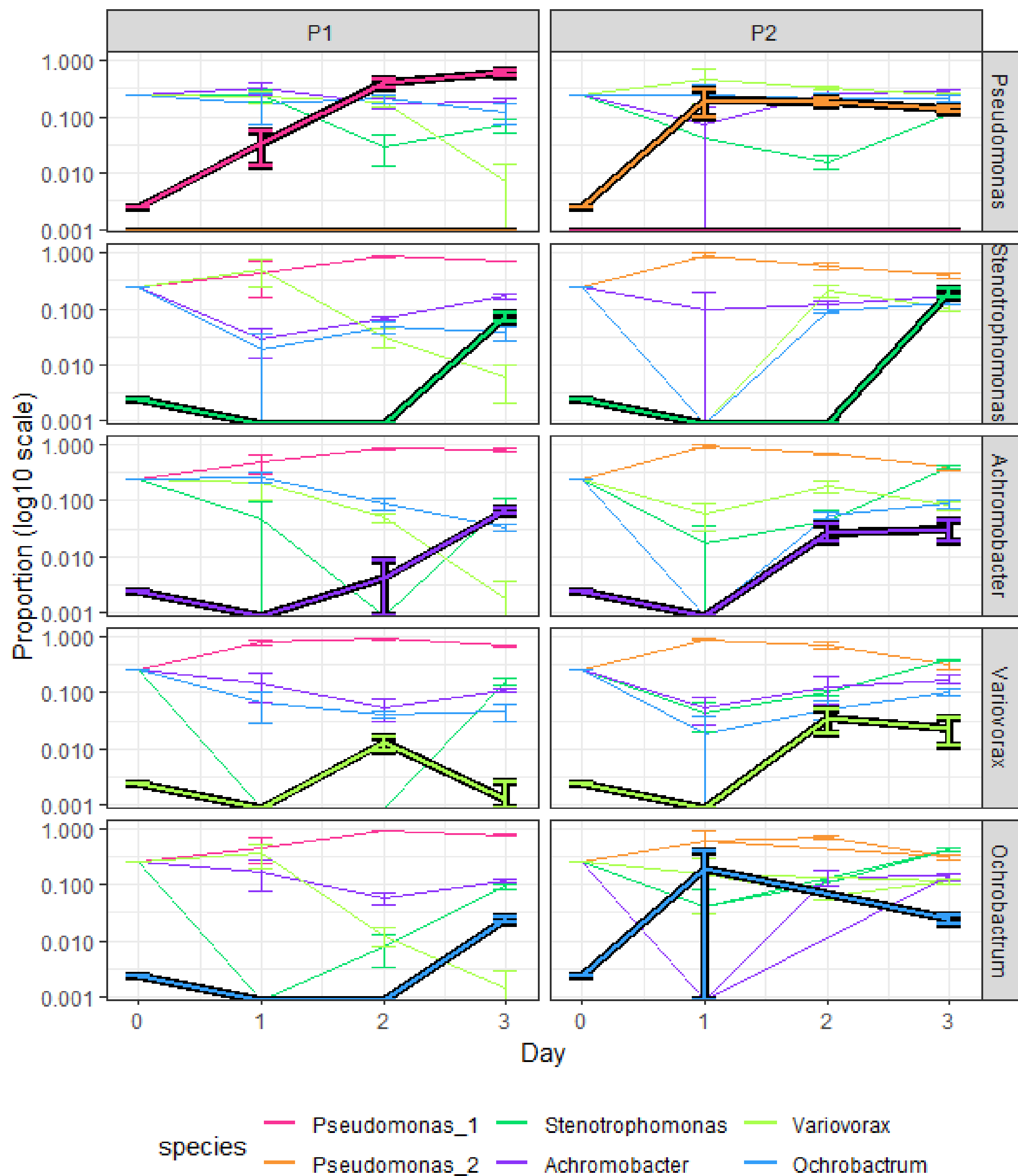

Figure 7: The mean proportion of each species in each treatment in each community of Experiment 2.1, across 4 days, with ±1 standard error bars. In each panel, the thicker line with the dark grey border shows the invading species. n = 3 for each combination of invading species and community.

Table 4: Statistical results from one-tailed t-tests comparing the values of the invasion success parameter *v* to a mean of 1, for each species invading into each community in Experiment 2.1, where *v* greater than 1 indicates a successful invasion. Significant p-values after Sequential Bonferroni correction (Rice, 1989) are shown in bold and with an asterisk.

	P1			P2		
Genus	**t-value**	**df**	**p-value**	**t-value**	**df**	**p-value**
Pseudomonas	3.579	2	0.035	7.879	2	**0.008** *
Stenotrophomonas	5.963	2	**0.014** *	4.744	2	0.021
Achromobacter	7.340	2	**0.009** *	2.360	2	0.071
Variovorax	-0.938	2	0.776	1.712	2	0.115
Ochrobactrum	6.779	2	**0.011** *	5.747	2	0.014

Table 5: Statistical results from one-tailed t-tests comparing the values of the invasion success parameter *v* to a mean of 1, for each species invading combined treatments in Experiment 2.1, where *v* greater than 1 indicates a successful invasion. Significant p-values after Sequential Bonferroni correction (Rice, 1989) are shown in bold and with an asterisk.

	P1 + P2		
Genus	**t-value**	**df**	**p-value**
Pseudomonas	2.296	5	0.035
Stenotrophomonas	3.572	5	**0.008** *
Achromobacter	4.400	5	**0.003** *
Variovorax	1.340	5	0.119
Ochrobactrum	9.796	5	**< 0.0005**

Experiment 2.2:

Simpson's diversity of the full community treatment remained stable over the course of this experiment, and in fact increased slightly (Figure 8; linear regression; $R^2 = 0.230$, $p = 0.018$). This slight increase was probably just an artefact of the community settling in week 1, as, if the data from week 1 is excluded, the effect disappears (linear regression; $R^2 = 0.052$, $p = 0.362$).

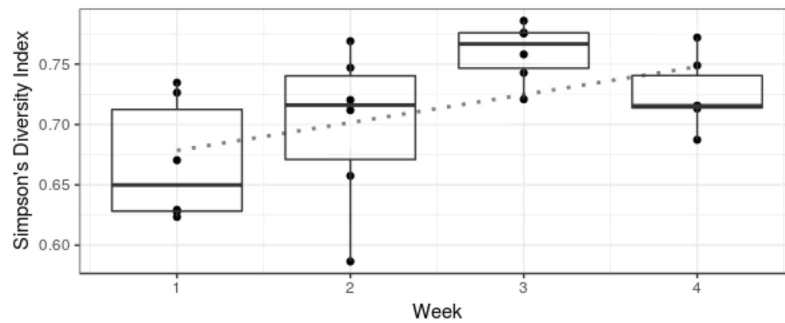

Figure 8: The Simpson's diversity index of the full community treatment (PSAVO) in Experiment 2.2 across 4 weeks, with a dotted grey regression line. Dots for each week show the diversity of individual replicates.

According to the Akaike Information Criterion, the third-order interaction models were selected as the most parsimonious in explaining the populations of every focal species (Table 6). Multiple higher-order terms were significant in the most parsimonious models for every focal species (Figure 10; Supplementary Tables 1-5). No significant direct interactions were positive, while 16.7% of significant second-order interactions were positive and 60.0% of third-order interactions were positive.

Table 6: Akaike Information Criterion values for each model for each focal species in Experiment 2.2, which indicates model parsimony and penalises models as they become more complex. The lowest AIC value for each focal species, indicating the most parsimonious model, is in bold and with an asterisk.

	Akaike Information Criterion (AIC)				
Model	**Null**	**Direct**	**2nd Order**	**3rd Order**	**4th Order**
Pseudomonas	420.8	383.3	374.3	**372.3 ***	372.5
Stenotrophomonas	599.5	572.0	519.5	**500.7 ***	501.3
Achromobacter	539.3	519.3	493.5	**480.2 ***	481.7
Variovorax	421.8	413.3	402.4	**401.2 ***	403.2
Ochrobactrum	502.1	467.7	454.7	**443.5 ***	444.5

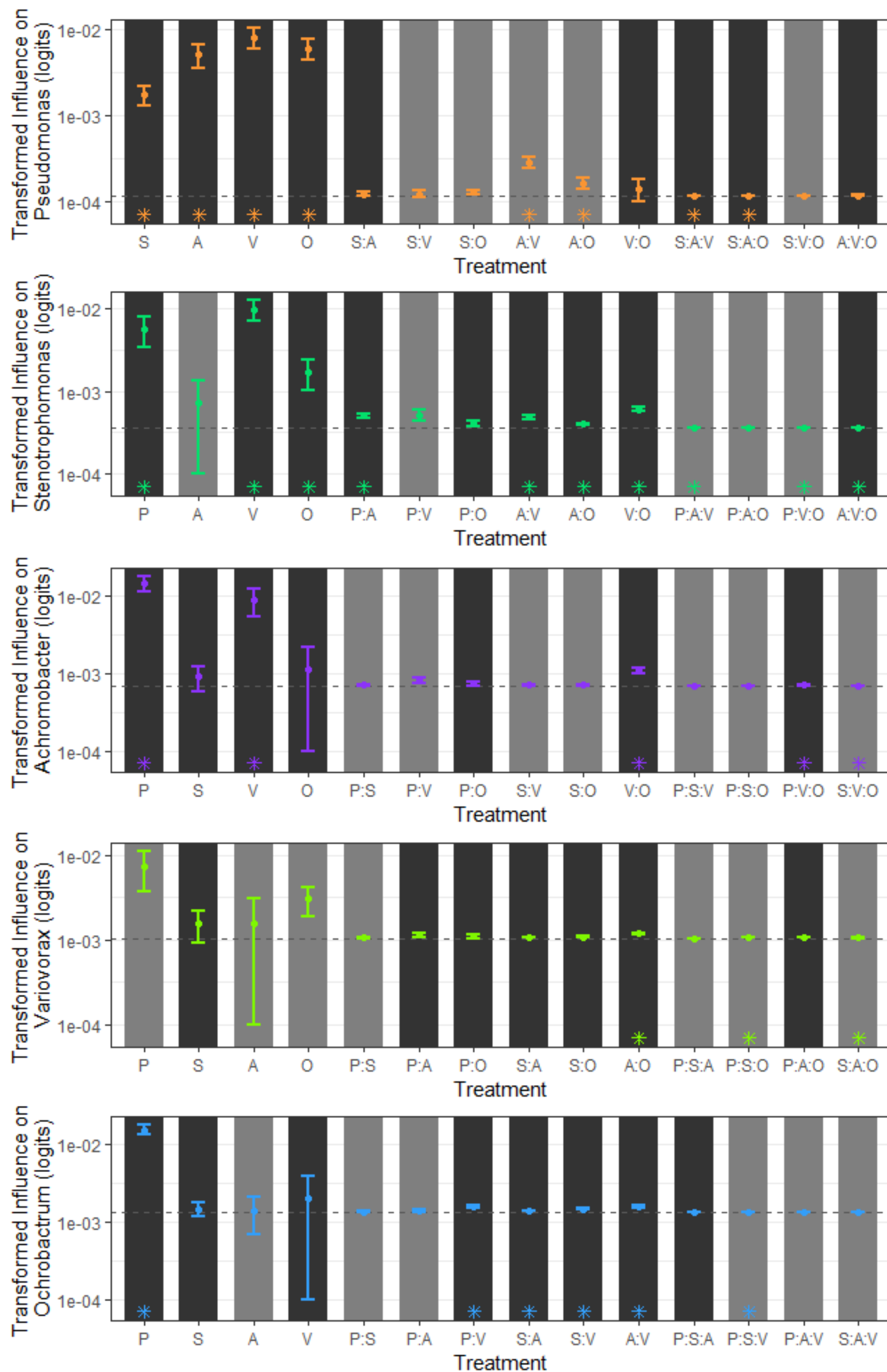

Figure 9: The model parameters of the most parsimonious model for each species, showing the influence of each direct and higher order interaction on the population size of the focal species, in logits. All data is displayed as *absolute* values, where dark grey bars indicate a negative effect on the focal species and light grey bars indicate a positive effect. Data was also transformed by addition, so that the minimum value on each plot was raised above zero to 10^{-4}, allowing data to be displayed on a log10 scale for easier comparison. Significant parameters are indicated by asterisks. The dotted horizontal line on each plot shows the level of zero influence.

The direct relationships between species comprised of 3 -/- relationships (P:S, P:A, & P:O), 4 -/0 relationships (V:P, V:S, V:A & O:S), and 3 0/0 relationships (S:A, A:O, V:O)). *Pseudomonas* is the only species to interact directly with every other species, and is further affected by two positive second-order interactions, and 2 negative third-order interactions. *Stenotrophomonas* does not interact directly with *Achromobacter*, but is affected by four negative second-order interactions, and three third-order interactions. *Achromobacter* is mostly negatively affected by both direct effects and higher-order interactions, with the exception of a positive influence from the triplet SVO. *Variovorax* is significantly affected by the least interactions, only by a single negative second-order interaction, and 2 positive third-order interactions. *Ochrobactrum* is only directly affected by *Pseudomonas*, but is affected by 4 negative second-order interactions, and a single positive effect from the triplet PSV (Figure 10).

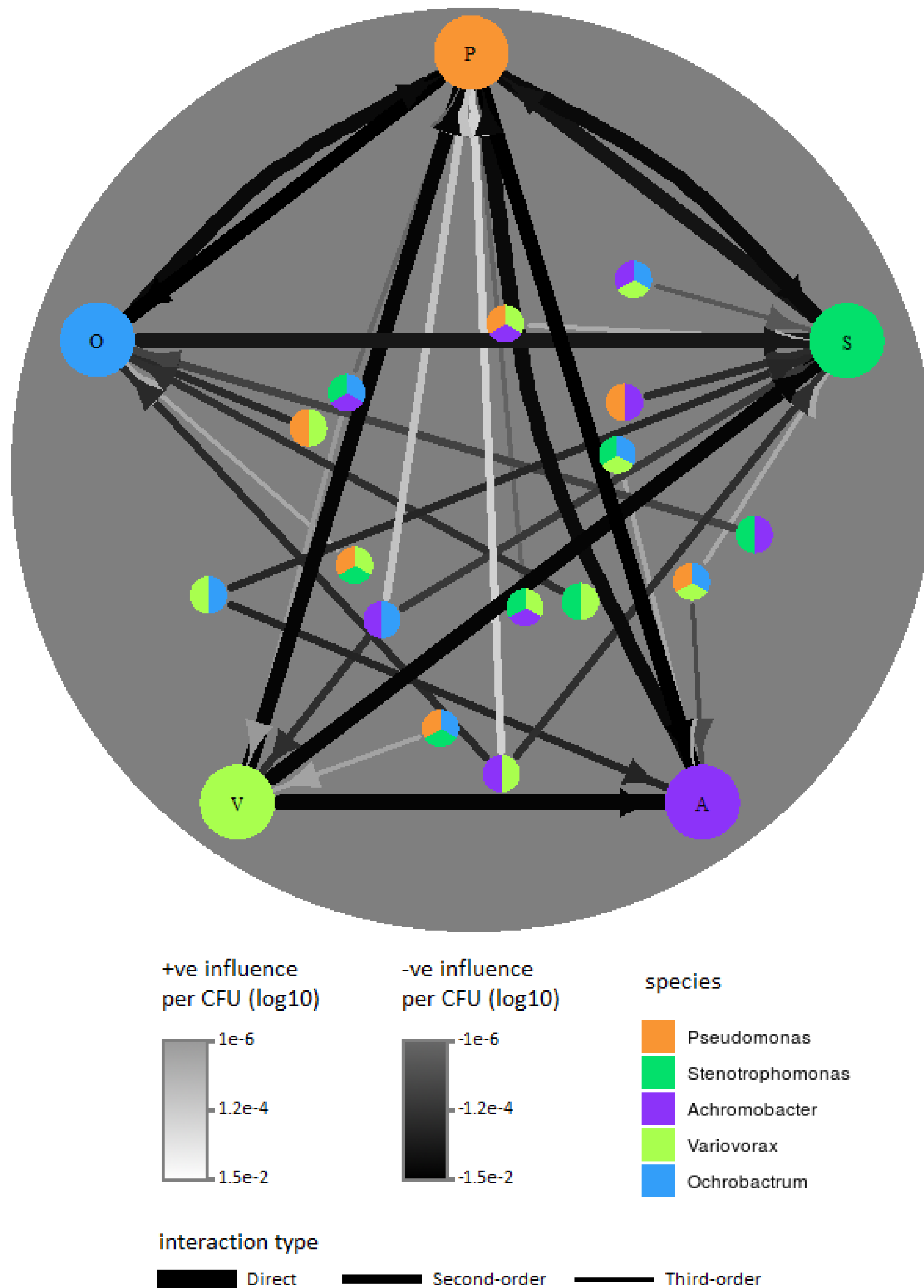

Figure 10: Full interaction chart of significant interactions in the 5-species community, including significant higher-order effects. Arrows from a labelled, solid-colour circle to another labelled, solid-colour circle indicate the direct influence of the first species on the second. Arrows from smaller, multicoloured circles to labelled, solid-colour circles indicate the higher-order influence of the pair or triplet of species on the individual species. Arrows lighter than the background indicate a positive influence, and arrows darker than the background indicate a negative influence.

Discussion

The 5-species community shows a certain degree of stability, but the results of this experiment are not entirely conclusive. Experiment 2.1 shows that all species were able to persist when invading-from-rare (Figure 7), and in Experiment 2.2 the diversity of the full community remained constant for the full 4 weeks (Figure 8). However, only *Pseudomonas* was able to significantly increase in proportion when invading-from-rare in community P2, and this only increased to three out of five species when the results were combined with community P1. Therefore, the possibility that this community is unstable and would lose diversity incredibly slowly cannot be dismissed, although Experiment 2.2 shows that it is stable enough to maintain diversity long enough for use as a model system. Even if this community would very slowly drive itself towards a monoculture, some theories suggest that the same would be true of many natural communities without the diversifying influences of speciation and migration (Hubbell, 2001).

The significant direct interactions between species were entirely competitive (Figure 10), although the positive effect of *Pseudomonas* on *Variovorax* came close to significance ($p < 0.1$). It is possible that *Variovorax* cross-feeds from *Pseudomonas*, or that it can increase its fitness by exploiting a biofilm or other common-good that another *Pseudomonas* creates. Despite the competitive interactions in this system, even when the species were grown in pairs, no species was completely driven to extinction by any other (Figure 9), which may suggest that none of the species' resource-requirements completely overlap with any other (unlike the two competing *Pseudomonas* species in Experiment 1.2). This could be explained by evolutionary trade-offs in resource use (Kneitel and Chase, 2003), where species have specialised to most effectively use a specific subset of resources, creating a stabilising intransitive network as described by Allesina and Levine (2011). Additional invasion-from-rare experiments using pairs of species could confirm the separate niches of these species. However, neutral theory (Hubbell, 2001) casts doubt on the prevalence of competitive exclusion in natural systems, and could also explain the observed coexistence between these species if they were simply ecologically equivalent competitors.

The higher-order interactions within the 5-species community had more complex mix of positive and negative effects. The significant second-order effects remained mostly negative, while the significant third-order effects had a mostly positive influence on other species, which supports Bairey, Kelsic and Kishony (2016)'s finding from theoretical models that third-order effects specifically have a stabilising influence and allow for greater diversity. This shows that higher-order interactions are likely responsible for some degree of the stability of this 5-species community, although other mechanisms are almost certainly at work. *Achromobacter* and *Ochrobactrum*, for example, are each only significantly influenced by a single, relatively weak, positive effect, compared to a large number of strong negative effects (Figure 9), which does not seem to explain their stability in the community (Figure 7). An intransitive network, as described above, or some other mechanism, likely supports these species.

Experiment 2.2 showed compelling evidence for the presence of many significant high-order interactions within the 5-species community. However, recent results from Messier, McGill and Lechowicz (2010) found that in some communities, the variation within-species can be just as large as the variation between-species, casting doubt on the importance of interspecies interactions in general. It is possible that the strong interspecific interactions observed here are merely the result of a very young community, where all the species had recently gone through a severe bottleneck (inoculation) and were not as diverse as species within a natural community would be. In that case, theoretically, as the intraspecific diversity in this community increased, the interspecific interactions would gradually fade away. Nonetheless, it is worth remembering that even relatively simple organisms like bacteria have a large variety of traits, such as cell-wall type, motility, replication time, resource use, oxygen tolerance, and so on. Even if intraspecific variation is so high that species are barely distinct in any one of these categories, the combination of all of them leaves a lot of space for species to hold a unique identity. Along with the findings of Mayfield and Stouffer (2017) and Sanchez-Gorostiaga *et al.* (2018), these results begin to suggest that higher-order interactions could be important ecological features in many communities.

In conclusion, I found evidence for the stability of a 5-species community from Chapter 1, in the form of negative frequency-dependent selection affecting some species, and persistent diversity over multiple weeks. Furthermore, I have begun to uncover the mechanisms maintaining this stability, with evidence shown for higher-order interactions and a possible intransitive network.

Conclusion

Over the course of this study, I used a simple microbial model to investigate several facets of community ecology. In this process, I have begun to provide answers to some open questions in community ecology, such as the balance between niche and neutral assembly, and the prevalence of higher-order interactions, as well as establishing a valuable model system for future work.

I found that the focal microbial communities assembled into a broadly predictable structure at the genus level, but that the structure was variable at the species level. This variation was underpinned by two almost mutually exclusive *Pseudomonas* species, which may have been ecologically equivalent. This lends support to the niche-determined theory of community assembly, as community formation was more predictable than the random processes predicted by the neutral model, and at least partially based on ecological roles. I studied one of the communities in further detail, and found it to be fairly stable, as it was able to maintain its diversity for at least 4 weeks, and every species was able to persist in an invasion-from-rare experiment. The interactions between species in this community were exhaustively characterised, and interestingly multiple higher-order interactions were found to have a significant influence on the abundances of species. These higher-order interactions were partially able to explain the maintenance of the community's diversity, although it is likely that an intransitive network or other mechanisms were also at work.

The model system established in these experiments, where a 5-species community is cultured in 1/64th TSB medium and subject to a 1% bottleneck every 24 hours, has great potential as a model system for community ecology studies. The model is simple, with a small number of species, yet still a true community, with a demonstrated degree of stability, and it can be easily cultured and measured with simple laboratory techniques. In the first chapter, I have shown that the structure of this community is likely based on ecological niches, and in the second chapter I have characterised the complex relationships between species. All this information will be invaluable in interpreting the results of future studies. This model could be used to quickly and effectively investigate phenomena such as the effect of phage predation on

stability, the factors that mediate a community’s resilience to invasion, or its response to regular disturbance.

In conclusion, this study has shed some light on the structure of biological communities, and shown one of the strongest examples to date of higher-order interactions at work within a community. In addition, it has provided a valuable model system for use in future research, which should lead to many more discoveries.

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Supplementary Tables

Table S1: The estimates and p-values of all model terms in the most parsimonious model to explain the abundance of *Pseudomonas* in the presence of other species. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Influence on *Pseudomonas*	
Treatment	**Estimate (per CFU)**	**p-value**
Monoculture	4.512	**< 0.0005**
S	-1.612e-03	**< 0.0005**
A	-4.915e-03	**0.001** *
V	-7.928e-03	**< 0.0005**
O	-5.854e-03	**< 0.0005**
S:A	-5.833e-06	0.379
S:V	6.233e-06	0.541
S:O	1.254e-05	0.114
A:V	1.684e-04	**< 0.0005**
A:O	4.635e-05	**0.046** *
V:O	-2.451e-05	0.533
S:A:V	-4.733e-07	**0.045** *
S:A:O	-3.459e-07	**0.019** *
S:V:O	3.216e-08	0.920
A:V:O	-1.978e-06	**0.071** *

Table S2: The estimates and p-values of all model terms in the most parsimonious model to explain the abundance of *Stenotrophomonas* in the presence of other species. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Influence on *Stenotrophomonas*	
Treatment	**Estimate (per CFU)**	**p-value**
Monoculture	6.224	**< 0.0005 ***
P	-5.365e-03	**0.022 ***
A	3.762e-04	0.553
V	-9.443e-03	**< 0.0005**
O	-1.346e-03	**0.042 ***
P:A	-1.450e-04	**< 0.0005 ***
P:V	1.573e-04	0.059
P:O	-4.829e-05	**< 0.0005 ***
A:V	-1.272e-04	**< 0.0005 ***
A:O	-4.272e-05	**< 0.0005 ***
V:O	-2.482e-04	**< 0.0005 ***
P:A:V	5.026e-06	**< 0.0005 ***
P:A:O	4.404e-08	0.939
P:V:O	3.465e-06	**0.008 ***
A:V:O	-1.840e-06	**0.001 ***

Table S3: The estimates and p-values of all model terms in the most parsimonious model to explain the abundance of *Achromobacter* in the presence of other species. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Influence on *Achromobacter*	
Treatment	**Estimate (per CFU)**	**p-value**
Monoculture	5.249	**< 0.0005**
P	-1.351e-02	**< 0.0005**
S	-2.135e-04	0.508
V	-8.143e-03	**0.020** *
O	-4.182e-04	0.677
P:S	9.962e-06	0.657
P:V	1.241e-04	0.108
P:O	-5.104e-05	0.248
S:V	8.193e-06	0.716
S:O	8.670e-06	0.235
V:O	-3.877e-04	**< 0.0005**
P:S:V	6.748e-09	0.994
P:S:O	4.502e-07	0.507
P:V:O	-7.380e-06	**0.002** *
S:V:O	3.165e-06	**< 0.0005**

Table S4: The estimates and p-values of all model terms in the most parsimonious model to explain the abundance of *Variovorax* in the presence of other species. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Influence on *Variovorax*	
Treatment	**Estimate (per CFU)**	**p-value**
Monoculture	3.864	**< 0.0005**
P	6.232e-03	0.084
S	-5.195e-04	0.416
A	5.229e-04	0.722
O	1.975e-03	0.089
P:S	1.072e-05	0.604
P:A	-9.216e-05	0.073
P:O	-5.790e-05	0.372
S:A	-1.249e-05	0.131
S:O	-2.571e-05	0.161
A:O	-1.384e-04	**< 0.0005** *
P:S:A	2.436e-07	0.474
P:S:O	2.187e-06	**0.008** *
P:A:O	-3.797e-06	0.066
S:A:O	9.071e-07	**0.024** *

Table S5: The estimates and p-values of all model terms in the most parsimonious model to explain the abundance of *Ochrobactrum* in the presence of other species. Significant p values (< 0.05) are shown in bold and with an asterisk.

	Influence on *Ochrobactrum*	
Treatment	**Estimate (per CFU)**	**p-value**
Monoculture	5.154	**< 0.0005**
P	-1.432e-02	**< 0.0005**
S	-1.205e-04	0.698
A	5.468e-05	0.941
V	-6.422e-04	0.756
P:S	1.282e-06	**0.906**
P:A	2.889e-05	0.452
P:V	-2.290e-04	**0.002** *
S:A	-1.457e-05	**< 0.0005**
S:V	-1.220e-04	**< 0.0005**
A:V	-2.246e-04	**0.002** *
P:S:A	-5.889e-07	0.080
P:S:V	3.805e-06	**< 0.0005**
P:A:V	4.269e-06	0.088
S:A:V	4.018e-07	0.664