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The role of metabolic interactions in spatial self-organization and diversity of expanding microbial communities

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A Viola

Senza te non sarebbe mai stato possibile.

Lebewesen leben einzeln,
leben als strukturdeterminierte Systeme.
So gesehen ist es ein konstellationsbedingter Zufall,
wenn das eine, obwohl es tut, was es tut,
dem anderen nützen kann.

— Niklas Luhmann, *Die Gesellschaft der Gesellschaft,* 1997

Contents

Summary

This thesis examines how metabolic interactions determine the spatial self-organization and diversity of expanding microbial populations. I have investigated the spatial dynamics of self-organization during a range expansion using experiments with a microbial model system, which allows for the selective manipulation of metabolic interactions between populations. In addition, I used spatial simulations of a mathematical model to make predictions about long-term developments and to draw conclusions about the underlying mechanisms.

In Chapter 1, I give a brief introduction to key concepts and present the experimental model system and the mathematical model. Chapter 2 is a review of the literature on synthetic microbial communities that have been used to study various interactions and their dynamics. In this review I was manly involved in the part about the role of spatial structure in such systems and the general discussion. Chapter 3 is a perspective about how metabolic specialization affects the composition of microbial communities. Metabolic specialization refers to the specialization of different microbial populations to certain metabolic degradation processes. My coauthors and I proposed that metabolic specialization is to be expected when biochemical conflicts exist between certain degradation processes. A more fundamental understanding of the metabolic specialization could be used to design synthetic microbial ecosystems with defined metabolic functions.

The following chapters 4-6 deal with my experimental research and constitute the main part of my thesis. In Chapter 4, I examine the consequences of successive range expansion on the spatial self-organization and diversity of a microbial model system. Previous investigations on range expansions have mainly studied primary expansions into uncolonized areas and have generally found a reduction of diversity during expansion. In contrast, I show that a successive expansion can increase spatial diversity in the secondary expansion zone because the secondary expansion front branches into dendritic structures. Furthermore, I examine the long-term impact of a secondary, dendritic expansion on genetic diversity by introducing mutations into the mathematical model. I then propose that a secondary, dendritic expansion can lead to a higher accumulation of mutations than a primary expansion, and thus accelerate evolutionary processes.

In Chapter 5, I examine the influence of a toxic metabolite on spatial diversity. I show that the spatial separation caused by successive expansion impedes direct interaction between the two populations, which prohibits the interaction to affect spatial arrangement. Furthermore, I show that toxicity leads to a slowdown of the primary expansion, which has the consequence that a larger proportion of the population can reproduce. This in turn reduces genetic drift on the expansion front and therefore maintains more of the initial diversity than in a rapidly expanding population without toxicity.

Chapter 6 deals with a phenomenon that I have observed in most of my experiments: while the larges part of the microbial colonies was expanding successively, there were often areas where the two populations expanded simultaneously. The study of this phenomenon revealed that the successive or simultaneous mode of expansion depends on the initial frequency of the secondary population. Such behavior is characteristic of chaotic systems in which differences in the initial conditions has a strong impact on the development over time. However, since the mechanism that causes these two different spatial organizations is not clear yet, further research is necessary to clarify the exact behavior of the system.

Finally, in Chapter 7 I discuss the results in a more general context, identify new questions raised by my research, and discuss direction of further research.

Zusammenfassung

Diese Dissertation untersucht wie Stoffwechselinteraktionen die räumliche Selbstorganisation und Diversität von expandierenden mikrobiellen Populationen beeinflussen. Ich habe die räumlichen Dynamiken der Selbstorganisation während einer Gebietsexpansion, welches das gezielte Manipulieren der Interaktionen erlaubt, untersucht. Darüber hinaus benutzte ich räumliche Simulationen eines mathematischen Modells, um Vorhersagen über längerfristige Entwicklungen zu machen und Rückschlüsse über die zugrundeliegenden Mechanismen zu ziehen.

In Kapitel 1 gebe ich eine kurze Einführung zu wichtigen Konzepten und stelle sowohl das experimentelle Modellsystem als auch das mathematische Modell vor. Kapitel 2 ist eine Rezension der Literatur über künstliche Mikrobielle Gemeinschaften, die dazu verwendet wurden um verschiedenste Interaktionen und deren Dynamiken zu studieren. Ich habe darin vor allem beim Teil über den Einfluss räumlicher Struktur auf solche Systeme und der generellen Diskussion mitgewirkt. Kapitel 3 behandelt das Thema wie metabolische Spezialisierung die Zusammensetzung von mikrobiellen Gemeinschaften beeinflusst. Meine Koautoren und ich stellen darin die These auf, dass metabolische Spezialisierung, d.h. die Spezialisierung von verschiedenen mikrobiellen Populationen auf bestimmte metabolischen Abbauschritte, vor allem dann zu erwarten ist, wenn gewisse Zwischenschritte des Abbaus aus biochemischer Sicht nicht gut miteinander vereinbar sind. Ein fundierteres Verständnis der metabolischen Spezialisierung könnte dazu verwendet werden künstliche mikrobielle Ökosysteme mit definierten metabolischen Eigenschaften zu entwickeln.

Die folgenden Kapitel 4-6 behandeln meine experimentelle Forschung und stellen den Hauptteil meiner Dissertation dar. In Kapitel 4 untersuche ich die Folgen einer Sukzessiven Gebietsexpansion auf die räumliche Anordung und Diversität des mikrobiellen Modellsystems. Vorhergehenden Untersuchungen zu Gebietserweiterungen, die vor allem Primäre Expansionen in unbewohntes Gebiet untersucht haben stellten dabei eine Reduktion der Diversität fest. Ich lege hier im Gegensatz dazu dar, dass eine Sukzessive Expansion zu einer Erhöhung der räumlichen Diversität in der sekundären Expansionszone führt, da sich die sekundäre Expansionsfront dendritisch verzweigt. Desweiteren untersuche ich den langfristigen Einfluss einer sekundären, dendritische Expansion auf die Genetische Diversität indem ich Mutationen in das mathematische Modell einführe. Damit mache ich die Vorhersage, dass eine sekundäre, dendritische Expansion langfristig zu einer höheren Ansammlung von Mutationen als in einer primaren Expansion führt.

In Kapitel 5 untersuche ich den Einfluss eines toxischen, metabolischen Zwischenprodukts auf die räumliche Diversität. Ich zeige dabei auf, dass die räumliche Trennung durch eine sukzessive Expansion eine direkte Interaktion zwischen den beiden Populationen erschwert, was verhindert, dass die Interaktion die räumliche Anordnung prägt. Desweiteren zeige ich, dass die Toxizität zu einer verlangsamung der primären Expansion führt, was zur Folge hat dass sich ein grösserer Teil der Population fortpflanzen kann. Dies wiederum verringert die genetische Drift an der Expansionsfront und erhält deshalb mehr der anfänglichen, räumlichen Diversität als in einer schneller expandierenden Population ohne Toxizität.

Kapitel 6 behandelt ein Phänomen, welches ich in den meisten meiner Experimente beobachtete habe: während der grösste Teil der mikrobiellen Kolonien sukzessiv expandierte, gab es meistens auch Stellen, wo die beiden Populationen gleichzeitig expandierten. Die Untersuchung dieses Phänomens brachte zum Vorschein, dass die sukzessive oder simultane Expansion von der Anfangsfrequenz der sekundären Population abhängt. Eine solches Verhalten

ist charakteristisch für chaotische Systeme, in denen unterschiede in den Anfangsbedinungen starke Auswirkungen auf die zeitliche Entwicklung hat. Da jedoch der Mechanismus der diese zwei unterschiedlichen räumliche Organisationsformen bewirkt noch nicht klar ist, ist weitere Forschungsarbeit nötig um das genau Verhalten des Systems zu klären.

Zum Schluss diskutiere ich in Kapitel 7 die Ergebnisse in einem allgemeineren Zusammenhang, identifiziere neue Fragen, die meine Forschung aufgeworfen hat, und bespreche in welche Richtung weitergehende Forschung auf diesem Gebiet gehen könnte.

1 Introduction

Microbial communities in nature are incredibly diverse. Studies using modern sequencing techniques have estimated that a single gram of soil can contain many thousands of microbial species[1,2]. However, it is not clear how this diversity emerges and how it is maintained in the environment[3]. Two factors that are thought to be important drivers of microbial diversity are spatial structure and metabolic interactions[4]. In this thesis I investigate how metabolic interactions shape spatial self-organization and, in turn, affect diversity in expanding microbial communities. I do so by using an experimental microbial model community and mathematical modelling. In the following I give a short introduction to the important concepts and then describe the experimental and mathematical model systems.

1.1 Metabolic interactions

Metabolic interactions, that is the exchange of metabolites that can enhance or decrease growth, are pervasive in microbial ecosystems[5]. Microbes degrade an extremely broad range of chemicals; they can metabolize nearly everything[6]. However, they often do not degrade metabolites completely but instead specialize in a certain step of degradation and release the remaining metabolites, which can then be further utilized by other microbes[7]. This process is called sequential cross-feeding and is thought to be important for microbial diversity because it enables co-existence of different species by niche specialization[4,8]. Cross-feeding thus allows several species to co-exist, even if only one initial metabolite is present in a system, because the microbes produce new metabolites and thus create new ecological niches. However, cross-feeding is just one example of microbial interactions. In Chapter 2 we give an extensive review of different mechansisms of interactions between different microorganisms.

1.2 Spatial structure

Spatial structure refers to the presence of surfaces and interfaces where microbes adhere to. This can be for example the liquid/solid or air/liquid interface, but spatial structure can also be created by the microbes themselves when they stick to each other[9]. Microbes that live in spatially structured habitats are called microbial biofilms. It is now widely recognized that the majority of microbes spend most of their lives in spatially structured systems, both in natural ecosystems and in pathogenic communities[10,11]. In biofilms, different microbial populations can be spatially organized based on their metabolic properties, which resembles the specialized tissues or even organs in higher organisms[11]. The spatial organization of the different populations in the biofilm is important because it can affect the functioning of the community as a whole, similar to the development of multicellular tissues where the exact positioning of specialized cell-types determines wheter the tissue is functional or not.

Spatial structure has several important consequences for microbial diversity. Spatial structure can potentially allow the co-existence of many species that use the same metabolite. The idea is that less competitive species, which would become exctinct in a well-mixed system because of competitive exclusion, can disperse into sites where the competitor does not yet exist[12]. This means that less competitive species might survive in a spatial niche as long as they live far enough from the competitors. Furthermore, spatial heterogeneity can itself create new niches to which species can specialize. It has for example been shown that *Pseudomonas fluorescence* rapidly evolved a polymorphism when propagated in a spatially structured system, where one of the newly arising morphotypes was specialized in colonizing the liquid air interface[13].

Another important consequence of spatial structure on microbial diversity is it's role in metabolic interactions. Mass transport in spatially structured systems is typically dominated by diffusion, while mass transport in well-mixed systems is typically dominated by advection. This has the consequence that concentrations of metabolites that are excreted in spatially structured systems are highest close to the origing of release, which leads to gradients. It is therefore beneficial for interacting microbial populations to remain close to each other. It has been shown that strong reciprocal interactions can lead to mixing of interacting populations and thus cause patterns of high spatial diversity[14,15]. However, there are also mechanisms which lead to demixing of spatially structured microbial populations.

1.3 Range expansion

One process that can spatially segregate populations is range expansion[16]. Range expansion in general is when a population colonizes new territory; it is therefore a general process that occurs in all domains of life[16,17]. Range expansion decreases diversity because of strong drift at the expansion edge. During range expansion, only a small part of the total population is positioned at the very front of the expansion. However, these individuals will constitute a large part of the founding population in the newly colonized area. During the expansion this process repeats itself many times and thus leads to a local reduction of diversity and segregation of previously mixed populations. This effect of segregation of populations has been described theoretically and has also been shown in experiments with microbial model systems[16,18].

Another important aspect of range expansion is how they affect the spatial distribution of mutations that occur in the populations. In well mixed systems, a newly occuring mutation competes with essentially the whole ancestral population because resources are equally available for all the individuals. If a mutation is advantageous, it will eventually spread and replace the whole population. A deleterious mutation, on the other hand, is unlikely to persist over longer times. During range expansion in spatially structured systems, however, the position at which a mutation appears can have a large effect on it's survival probability, because nutrients and other growth factors are only freely available at the front of the expansion. If a mutation occurs far behind the front, it is less likely to spread in the population simply because there is neither food nor space available. If a mutation occurs at the front, on the other hand, is in itself a growth advantage and the mutation is more likely to spread. It has been shown mainly theoretically that even deleterious mutations can establish at the front and remain there for longer times in a process called „surfing" of mutations[19–21]. It can thus be seen that range expansions can have important consequences for the spatial population structure and diversity, both on short time-scales, but also over longer evolutionary time-scales.

1.4 Experimental model system

The model organism that I've used in the experiments of this thesis is *Pseudomonas stutzeri* strain A1501, which is a Gram-negative bacterium that belongs to the class of *Gamma-proteobacteria*[22]. Cells of *P. stutzeri* are rod-shaped and 1-3 μm long and about 0.5 μm wide. They typically have a single flagellum that is located at a pole and they have type IV pili, which enables twitching motility[22]. Twitching motility is a mode of movement of bacteria on soft and wet surfaces such as semi-solid agar[23], which has an agar content that is less than half the agar content of standard agar plates (0.7% or lower instead of 1.5%)[24]. On standard hard agar plates, which were used in the experiments, bacteria are typically considered non-motile[25,18].

Strain A1501 of *P. stutzeri* is a facultative anaerobe and denitrifier, which means that it can use nitrate as electron acceptor in the absence of oxygen[22]. This is likely an adaptation to the

changing aerobic and anaerobic conditions in rice fields, where it was first been isolated[26]. The denitrification process is a pathway consisting of four successive steps, where different enzymes catalyze the reduction of nitrate (NO_3) via nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O) to dinitrogen gas (N_2)[27]. The complete denitrification apparatus is complex and is encoded in about 40 genes that are arranged in four gene clusters (*nar*, *nir*, *nor* and *nos*). Each of these clusters encode for the enzyme complex for one of the four above mentioned denitrification steps[27–29].

In strain A1501 the *nir* and *nor* gene clusters are located next to each other, while the *nar* and *nos* gene clusters are in separate positions[29]. This allowed us to delete the *nar* or the *nir* gene clusters to construct two new strains that are either not able to do the first step of denitrifcation (*nar* deletion, unable to degrade NO_3 to NO_2) or the second step (*nir* deletion, unable to degrade NO_2 to NO)[30]. If these two mutants are grown together under anaerobic conditions, they form a cross-feeding community where one strain degrades nitrate (NO_3) to nitrite (NO_2) and the other degrades nitrite (NO_2) to dinitrogen gas (N_2). To allow the observation of spatial patterns we introduced genes into the strains that encode for different fluorescent proteins (green-,red- or cyan-fluorescent protein). In addition we also tagged the ancestral strains, which can do the whole denitrification pathway, with the same fluorescent proteins. This allowed us to assemble different communities that were either cross-feeding nitrite or growing independently (rephrase).

An important feature of our cross-feeding system is that the intermediate metabolite nitrite is toxic at low pH conditions less than 6.8. Nitrite toxicity is unspecific and can act via several different mechanisms. At low pH, nitrite exists in it's protonated form nitrous oxide (HNO_2), which can act as a protonophore[31,32]. This means that it increases the permeability of protons across membranes, which consequently inhibits ATP synthesis. Another mechanism is that, at low pH, nitrite can form nitric oxide radicals. These radicals can oxidize the sulfhydryl groups of the amino acid cysteine and therefore cause enzyme damage[33]. By changing the pH of the growth medium, we can therefore change the toxicity of the cross-fed metabolite nitrite. This allows us to change the interdependence of the two cross-feeding strains from a one-sided interdependence at high pH where the nitrite-degrading strain was depends on nitrate production by the nitrite-producing strain (i.e., a commensalism), to a reciprocal interdependence where the nitrite-producing strain also benefits from a reduction of toxicity through the nitrite degradation activities by the second strain (i.e., a mutualsim).

1.5 Mathematical model

I have developed a mathematical model that simulates the spatial population dynamics of a microbial cross-feeding community. I used this model to make predictions about the long term effects of primary and secondary range expansions and to study the mechanism how toxicity influences spatial population diversity. The model is a spatially explicit reaction-diffusion type model. In this type of model, populations are assumed to be continous. This is obviously not true in real life, where organisms are discrete entitities. However, if the populations are sufficiently large, these models simulate real situations quite well. Furthermore, such models have been previously used to model spatial self-organization of microbes that were grown on the surfaces of agar plates[34,35].

The model is relatively simple, where the microbial populations grow with a Monod-type growth term that dependes on the local substrate concentration and they move with a diffusion term. However, the diffusion term is not spatially uniform, but instead varies locally and over time. It depends on the concentration of the nutrient, the concentrations of the two cross-feeding populations, and a local anisotropy term. The rationale for this rather complex diffusion term is connected to the observed movement characteristics of bacteria that grow on agar plates. On hard

agar surfaces, the bacteria mostly move by cell division, which occurs as long as they have nutrients available[34]. The dependency on the bacterial concentration reflects that movement is facilitated by the own local population densitiy[34] and hindered by the local population density of the other population. That is, the higher the own local population density, the harder a population can push forward. The anisotropic term represents the local alignment of packs of rod-shaped cells that is found in bacterial biofilms[36]. A more detailed description of the model including the equations can be found in Chapter 4. In Chapter 5 the model is extended to include toxicity of the cross-fed metabolite nitrite.

1.6 Thesis outline

In this thesis, I investigate how metabolic interactions influence spatial-self organization and diversity of expanding microbial communites. Chapter 2 is a literature review of studies that assembled synthetic microbial communities with different types of interactions. Chapter 3 is a perspective manuscript in which we propose that biochemical conflicts can promote metabolic specialization in microbes. In Chapters 4 to 6 I experimentally investigate different aspects of how metabolic interactions affect spatial self-organization and diversity. In Chapter 4 I examine the the consequences of succession on spatial population diversity. Furthermore I develop a model with wich I compare the differences between mutations that occur in a primary or secondary expansion. In Chapter 5 I investigate the effects of nitrite toxicity on spatial population diversity of the cross-feeding community. In Chapter 6 I show that local heterogeneity can lead to very different spatial self-organization and how this affects community productivity. Finally in Chapter 7 I discuss the findings in a broader context and identify potential future directions of research.

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2 Synthetic microbial assemblages and the dynamic interplay between microbial genotypes

A modified version of this chapter is under review as:
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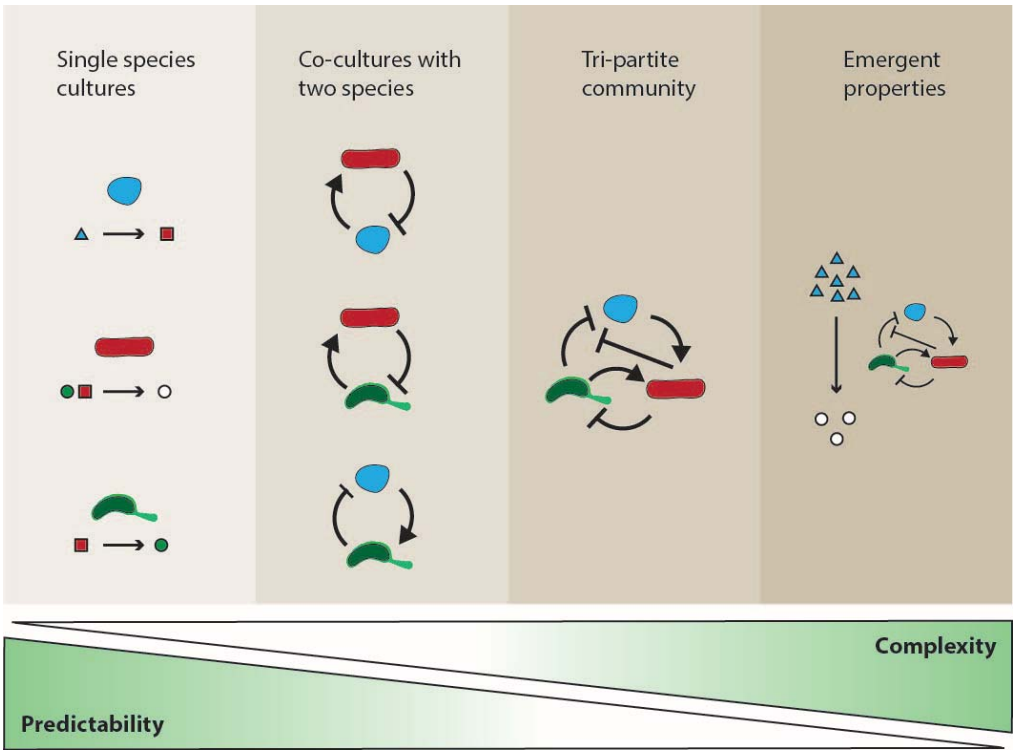
2.1 Abstract

Two microbial genotypes growing together can display surprisingly complex and unexpected dynamics and result in community-level functions and behaviors that are not readily expected from analyzing each genotype in isolation. This complexity has, at least in part, inspired a discipline of synthetic microbial ecology. Synthetic microbial ecology focuses on designing, building, and analyzing the dynamic behavior of "ecological circuits" (*i.e.*, a set of interacting microbial genotypes) and understanding how community-level properties emerge as a consequence of those ecological interactions. In this review, we discuss typical objectives of synthetic microbial ecology and the main advantages and rationales of using synthetic microbial assemblages. We next summarize the main methodologies of synthetic microbial ecology, including experimental, theoretical, and mathematical techniques. We then summarize recent findings of current synthetic microbial ecology investigations. In particular, we focus on the causes and consequences of the interplay between different microbial genotypes and illustrate how simple ecological interactions can create complex dynamics and promote unexpected community-level properties. We finally suggest that distinguishing between active and passive interactions and accounting for the pervasiveness of competition can provide an improved framework for designing and predicting the dynamics of microbial assemblages.



Graphical abstract

2.2 Introduction

Consider two human individuals that are married together. It is intuitively obvious that the interactions between these two individuals can be difficult to predict *a priori*. The interactions may also change readily and abruptly over time and give rise to complex dynamics. This complexity also exists among the smallest of organisms: microbes. Consider an ecosystem consisting of two different microbial genotypes that live within close spatial proximity to each other. Given the apparent simplicity of this ecosystem, one might presume that the dynamic behavior of these two microbial genotypes might be relatively easy to predict and to explain from basic principles and measurable properties. Recent theoretical and experimental investigations, however, suggest that even a simple assemblage of two microbial genotypes can exhibit surprisingly complex and unexpected dynamics[1–3]. Moreover, the dynamic interplay between the microbial genotypes can result in community-level functionalities and behaviors (*e.g.*, robustness, resilience, complementarity, facilitation, competition, antagonism, *etc.*) that might not be readily expected from analyzing each genotype in isolation[4–8]. Clearly, our understanding of the general rules and principles that govern the dynamics and emergent functionalities of microbial assemblages is at its infancy.

This knowledge gap has, at least in part, inspired the rapidly developing discipline of synthetic microbial ecology[6,9–17]. Synthetic microbial ecology can be viewed as an extension or sub-discipline of synthetic biology. While synthetic biology often focuses on designing, building, quantitatively analyzing, and predicting the dynamic behavior of metabolic and regulatory circuits (*i.e.*, a set of interacting molecules), synthetic microbial ecology focuses on designing, building, quantitatively analyzing, and predicting the dynamic behavior of "ecological circuits" (*i.e.*, a set of interacting microbial genotypes). In addition, whereas synthetic biology seeks to understand how cellular-level properties and behaviors emerge as a consequence of molecular interactions, synthetic microbial ecology seeks to understand how community-level properties and behaviors emerge as a consequence of ecological interactions.

From an experimental perspective, synthetic microbial ecology can be broadly delineated into bottom-up and top-down approaches (however, see alternative delineations by De Roy *et al.* (2013) and Großkopf and Soyer (2014)). Bottom-up approaches focus on the "design and build" principle, which is elegantly summarized by Richard Feynman in his statement that "What I cannot create, I do not understand." The main approach is to assemble different microbial genotypes with pre-determined properties together in order to achieve a desired set of interactions. The researcher then measures the dynamics and properties of both individual genotypes and the synthetic assemblage itself and attempts to understand or control specific features or behaviors that emerge as a consequence of those interactions. It therefore closely aligns with conventional synthetic biology, where the researcher often assembles genes, enzymes or other molecules together within a cell, measures the dynamics and properties of individual molecules and the cell itself, and attempts to understand or control specific features or behaviors that emerge as a consequence of the molecular interactions[18,19]. Top-down approaches, in contrast, are not based on designing and building a set of particular ecological interactions. Instead, genotypes from a predefined set are assembled together randomly to obtain a set of synthetic assemblages with certain compositional aspects (*e.g.,* assemblages with varying levels of functional, taxonomic, or phylogenetic diversity)[16,20–24]. The researcher then measures the properties of the synthetic assemblages (*e.g.*, resource consumption rates, biomass production, susceptibility to invasion, response to perturbations, *etc.*) and tests whether the controlled aspects relate to those properties. In some cases, the measured properties can provide insight into the interactions within those synthetic assemblages and how those interactions affect specific features

or behaviors of the assemblages (*e.g.*, Foster and Bell 2012; Zuppinger-Dingley *et al.* 2014). We note that synthetic microbial ecology is not an entirely new discipline, but instead builds upon many decades of research with microbial isolates and defined assemblages[27–29].

2.3 Goals and scope

The goals of this review are the following. First, we introduce typical objectives of synthetic microbial ecology and review the main advantages and rationales of using synthetic microbial assemblages. We primarily focus on bottom-up approaches, as in our view these approaches align most closely with the "design and build" principle that has been central to the broader field of synthetic biology[30,31]. Second, we summarize the main methodologies of synthetic microbial ecology, including experimental, theoretical, and mathematical techniques. We emphasize that synthetic microbial ecology often benefits when experimentation and mathematical modeling are combined. Third, we summarize the main findings of current synthetic microbial ecology investigations. Namely, we focus on the causes and consequences of the dynamic interplay between different microbial genotypes. We illustrate how simple ecological interactions can create complex dynamics and how those dynamic behaviors can lead to community-level functionalities that might not be readily predicted from analyzing each genotype in isolation. We do not attempt to define the scope of synthetic microbial ecology, but to instead provide a perspective for discussing the main concepts, approaches, and questions relevant to the field.

2.4 What are typical objectives of synthetic microbial ecology?

The specific objectives of a synthetic microbial ecology investigation can typically be assigned to one of the following two types.

a. Understand the general principles and rules that govern the dynamics, functioning, and higher-order community-level properties of microbial assemblages. For example, how do community-level behaviors emerge from the dynamic interplay between different microbial genotypes (*e.g.* ecological and evolutionary feedbacks)?
b. Rationally engineer synthetic microbial assemblages to enable, control, or optimize a desired biotransformation, such as the production of a valuable product from a low-cost source material or the transformation of a pollutant into an innocuous end product.

The objectives are therefore broad and encompass both fundamental questions and tangible applications. While these objectives are not mutually exclusive, the main focus of this review is on objective a. Objective b has been addressed in previous reviews and perspectives[15,16,32–35]

2.5 Why use synthetic microbial assemblages?

To achieve the objectives listed above, the following features of synthetic assemblages are of significant value:

a. Interactions can be controlled and monitored. Different microbial genotypes can be obtained or genetically engineered that interact with each other in a desired manner.
b. Experimental conditions can be controlled. Synthetic microbial assemblages can be propagated under carefully maintained experimental conditions where the physical and chemical properties of the environment are well defined. Experimental conditions can also be modified to perturb, promote or prohibit specific types of interactions between different genotypes, thus allowing for carefully controlled manipulation experiments.
c. Synthetic microbial assemblages are readily amenable to mathematical modeling. The growth and metabolic properties of each genotype can be measured in isolation and, in

some cases, within the assemblages themselves. It is therefore easier to obtain a comprehensive description of their dynamic properties, which is important for accurate and reliable model predictions. Moreover, because the frequencies of each genotype can typically be monitored over time, it is possible to compare model predictions with experimental observations at a high level of detail.

All three of these features are typically not available when investigating microbial assemblages in their natural environment. Natural microbial assemblages often contain many hundreds to thousands of different genotypes[36,37], most of which are not readily amenable to isolation or phenotypic characterization. The metabolic properties of individual genotypes and the interactions between them must therefore typically be deduced using predominantly indirect tools, such as (meta)genomic[38,39] and isotopic[40–42] methods. The interactions are consequently often hypothetical in nature, incompletely described, of high-dimensionality (*i.e.*, the number of interactions within a natural assemblage may be exceedingly large), and difficult to manipulate in a desired manner, thus creating confounding factors that make it difficult to understand how interactions affect community dynamics. In addition, environmental parameters available for manipulation are relatively limited when investigating natural microbial assemblages in their native environment. For example, one can add a resource to an existing nutrient pool to promote or prevent a particular interaction but it can be very difficult to completely remove a particular resource, which is less of an issue when working with synthetic microbial assemblages propagated in defined medium in the laboratory. Finally, it is typically more difficult to track the abundances or frequencies of different genotypes within natural microbial assemblages, and the recorded dynamics are therefore of coarser resolution.

2.6 Imposing interactions and analyzing synthetic microbial assemblages

There are two main approaches for imposing interactions between different microbial genotypes. One approach is to use different species or strains that mimic a simplified natural microbial assemblage of interest. For example, if one is interested in fermentation processes, one might assemble together a species that ferments an organic substrate to hydrogen with a species that consumes hydrogen[43]. While this approach may more accurately mimic a natural assemblage, it also suffers in that the two genotypes will likely have many genetic differences between them. This could create confounding factors and lead to unexpected or unwanted interactions, thus resulting in some loss of experimental control[44]. An alternative approach is to genetically engineer and assemble together a set of genotypes, all of which were derived from the same parental strain (*i.e.* isogenic mutants). The use of isogenic mutants minimizes unexpected or unwanted interactions that could emerge from numerous physiological differences between more distantly related genotypes. Interactions are therefore more controlled and effectively limited to a few well-defined types. For example, one can create interactions by deleting or inactivating genes encoding essential biosynthetic machinery, thus forcing two isogenic mutants to cross-feed specific biosynthetic building blocks (*e.g.* amino acids, nucleotides, *etc.*). A potential disadvantage of using isogenic mutants, however, is that they may not accurately mimic natural microbial assemblages, and thus provide less insight into natural processes.

After creating a synthetic microbial assemblage, the main behaviors of the assemblage are measured, such as resource consumption, growth, productivity, *etc*. Properties of the individual genotypes might also be measured, such as changes in their abundances or frequencies and their spatial positioning relative to each other. To quantify the abundances or frequencies of different genotypes, genetic or phenotypic traits (*e.g.*, fluorescent protein-encoding genes, antibiotic resistance genes, *etc.*) can be introduced into the genotypes, which are then assayed using

microscopic or phenotypic assays (*e.g.*, by selective plating). Alternatively, it may be possible to use native traits to distinguish and quantify different genotypes, such as the production of pigments, the ability to use certain resources, or the requirement for specific nutrients (*e.g.*, Lenski *et al.* 1991; Kassen *et al.* 2000). The decision about how to distinguish and quantify different genotypes depends on the main questions and objectives of interest. If spatial arrangement at the microscale is central for an investigation, then the use of fluorescent protein-encoding genes is especially powerful because one can readily quantify spatial metrics such as intermixing and co-occurrence patterns[44,47–49]. However, if one is interested in spatial arrangement at the macroscale or in behaviors in completely mixed systems, then the use of native traits may be sufficient and additionally avoids the possibility that the genetic markers themselves might impact the biology of the organisms (*e.g.*, Lenski *et al.* 1991; Kassen *et al.* 2000).

Mathematical models are often used to generate predictions and hypotheses that can be tested with synthetic microbial assemblages[17]. Mathematical models are powerful because interactions between different genotypes often result in non-linear behaviors and asynchronous growth of the different genotypes, which can lead to complex and non-intuitive predictions (*e.g.*, Yoshida *et al.* 2003). Dynamical models based on differential equations have been widely applied, where the goal is to predict how system features change over time. These system features may include the abundances of different genotypes, the concentrations of resources, or the abundances of specific biological molecules (*e.g.* enzymes, transcripts, ATP, toxins, *etc.*). While powerful, dynamical models typically require the estimation or measurement of a relatively large number of biological parameters, such as maximum reaction rates, half-saturation coefficients, inhibition coefficients, and carrying capacities. They also model population-level behaviors rather than individual-level behaviors. An advantage of dynamical models is that they have a fixed set of equations that are typically more tractable to mathematical analytics. The outcomes are therefore typically easier to generalize and the main drivers of system behavior are often easier to identify.

Other types of models include game theoretical and agent based models. In these models, individuals are treated as agents that obey by certain rules. These rules are the processes that govern how different individuals interact with each other and with their environment. They determine the types of strategies that individuals may take and the costs and benefits for implementing those strategies. For example, an individual may be a "cooperator" that secretes a molecule that is beneficial for the entire population or a "cheater" that consumes the secreted molecule but does not secrete it itself. If an individual engages in a strategy that has greater benefits than costs, then that individual is more likely to reproduce and, in turn, increase in frequency or abundance. Different strategies can therefore be competed against each other and the outcomes compared. Thus, they differ from dynamical models in that they focus on individual-level behaviors rather than on population-level behaviors. One disadvantage of agent based models, however, is that they are often complex. The outcomes can therefore be more difficult to generalize and the main drivers of system behaviors can be more difficult to identify.

More recently, metabolic models have been used to comprehensively predict a cell's metabolism and how that cell is likely to interact with others within synthetic assemblages[43,51–54]. These models differ from the previous two in that they are inherently stoichiometric and/or thermodynamic in nature, and they therefore do not require the measurement of kinetic parameters. They are also capable of generating accurate descriptions of metabolism using stoichiometric balancing and by considering thermodynamic properties of metabolic reactions (for reviews see *e.g.*, Ataman and Hatzimanikatis 2015; O'Brien, Monk and Palsson 2015). Continuing development of these models and ongoing translation of genomic data into mathematically described metabolic networks[55] hold great promise for identifying novel interactions and improving the rational design of synthetic microbial assemblages[17].

2.7 Types of synthethic microbial assemblages

An important objective of many synthetic microbial ecology experiments is to create a reduced ecosystem that nevertheless retains key functionalities of a more complex microbial assemblage. This reduces confounding factors and complexity that might otherwise prohibit detailed analyses and the direct testing of hypotheses and theoretical predictions. The first task of the synthetic microbial ecologist, therefore, is to define the biological properties of the genotypes that will be assembled together and how they are likely to interact in the context of the experimentally imposed environment. This may seem straightforward, but interactions are context dependent and can change in strength and nature over both time and space. The dynamics and emergent properties of a synthetic microbial assemblage are therefore sometimes difficult to predict. Nevertheless, defining initial interactions is a typical required starting point. We therefore summarize the types of interactions that have been imposed between two genotypes. We propose two major criteria to distinguish different types of interactions: whether the interaction is passive or active in nature (see below for definitions) and whether the interaction has positive or negative effects on each of the involved genotypes.

2.7.1 Passive unidirectional positive interactions

Passive unidirectional positive interactions refer to interactions where the growth of one genotype (designated as the beneficiary) is enhanced by the presence of a second genotype (designated as the passive promoter) but the growth of the passive promoter is not affected by the presence or abundance of the beneficiary (Fig. 1). We use the term "passive" to emphasize that the passive promoter does not actively invest metabolic resources into promoting the growth of the beneficiary, but instead promotes the growth of the beneficiary as an inadvertent consequence of its own metabolism. A typical example is unidirectional cross-feeding of a metabolic waste product, where one genotype consumes a resource and secretes a waste product that stimulates or supports the growth of another genotype. We emphasize that a strict passive unidirectional positive interaction is unlikely to occur, as the genotypes may simultaneously compete, at least to some extent, for other shared resources (*e.g.*, oxygen, nitrogen, phosphorous, see Fredrickson and Stephanopoulos 1981; Foster and Bell 2012). In order to achieve a strict passive unidirectional positive interaction, each genotype must occupy a separate and completely non-overlapping ecological niche that prevents competition for shared resources, which is experimentally difficult to implement (however see Weber, Daoud-El Baba and Fussenegger (2007) for a successful implementation).

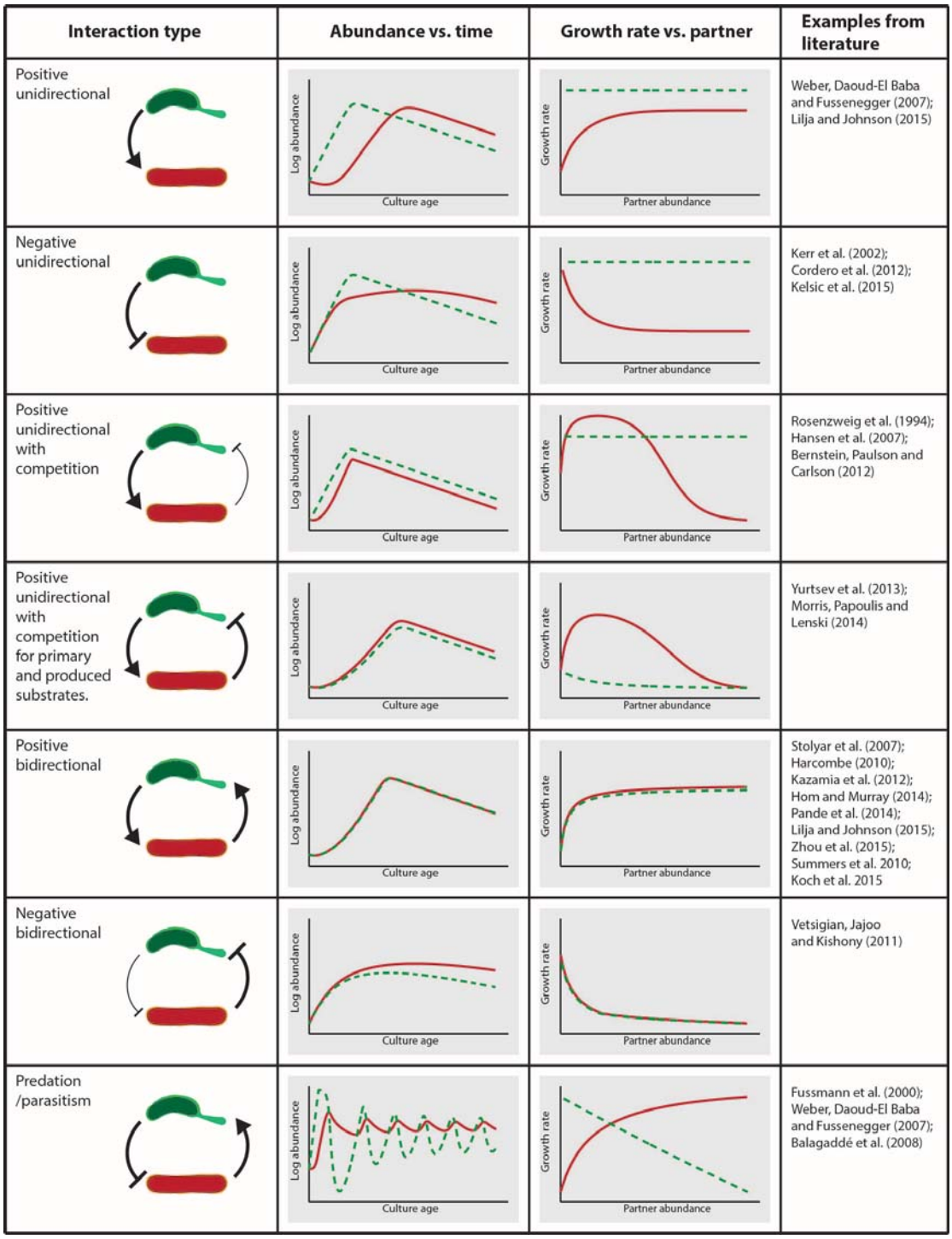


Figure 1 Dynamics of binary assemblages. We simulated population dynamics for binary assemblages with different interactions between the two distinct genotypes. The initial ratio of both genotypes was 1:1 (cell number:cell number). The first column describes the net interaction type, the second column shows how populations change over time, the third column shows how growth rate depends on the partner´s abundance, and the last column refers to studies where similar dynamics took place. Arrow thickness corresponds to the interaction strength. All models are based on the limited-substrate model proposed by Monod (Monod, 1950) for microorganisms growing in batch culture, with the exception of the predator-prey interaction where microorganisms are growing in chemostat culture. The model includes a constant mortality term, and thus results in the decline of all abundances at later time points. A full description of the models, an explanation of the underlying assumptions for each model, and further discussions of the dynamics are provided in the Supplementary Material.

2.7.2 Passive unidirectional positive interactions with competition

Passive unidirectional positive interactions are likely to contain, at least to some extent, competitive interactions at the same time, such as competition for shared resources (Fig. 1). Importantly, it may not be immediately clear whether the positive or the competitive interaction has the dominant effect on the dynamics and behaviors of the assemblage[44]. One of the most extensively investigated examples of a passive unidirectional positive interaction with competition is acetate cross-feeding within populations of *Escherichia coli*. When clonal populations of *E. coli* are propagated with glucose as the growth-limiting resource, metabolically specialized genotypes repeatedly evolve[58–62]. Experiments that assembled pairs of evolved genotypes together in synthetic microbial assemblages demonstrated that some pairs are maintained via negative-frequency dependent selection, thus enabling their coexistence[60,62]. In one case, two co-existing genotypes compete for glucose and acetate, but one genotype consumes glucose more effectively while the other consumes the secreted acetate more effectively [60,61]. Further experiments identified the genetic basis of these two genotypes, where genetic changes in a single gene are sufficient to increase glucose utilization while simultaneously decreasing acetate utilization[61]. Thus, the intrinsic tradeoff between glucose and acetate utilization creates a dynamic interplay between the two specialist genotypes that does not abolish competition for shared results but does result in accelerated glucose consumption via substrate cross-feeding.

Such dynamics were recently engineered *de novo* in synthetic assemblages of *E. coli* strains where the spatial structure of the environment was experimentally controlled. An acetate-consuming specialist genotype was genetically engineered in the laboratory, where glucose consumption was completely abolished by introducing four loss-of-function mutations into *E. coli*[63]. When the acetate-consuming specialist genotype was then assembled together with its glucose-consuming parental genotype, the two genotypes cross-feed acetate and other metabolites[63]. The cross-feeding assemblage achieved 15% greater biomass than the parental strain alone when grown in batch culture and 50% greater biomass than the parental strain alone when grown in biofilm. The two cross-feeding genotypes self-organized within the biofilm, where the upper well-oxygenated layer of the biofilm was populated predominately with the acetate-consuming genotype while the base of the biofilm was populated predominantly by the glucose-consuming genotype[63]. This structuring is therefore an emergent property of the interaction and likely improved the productivity of the biofilm, emphasizing the benefits of compartmentalizing different metabolic processes into different cell-types. Thus, if conflicts are known to exist between different metabolic processes (*e.g.*, in this case between glucose and acetate consumption; see Rosenzweig *et al.* (1994) and Treves, Manning and Adams (1998)), then one can engineer and assemble genotypes together that prevent the emergence of those conflicts and consequently accelerate community-level metabolic processes[63].

Passive unidirectional positive interactions need not always be based on cross-feeding of metabolic waste products. An example is the depletion of a growth-inhibiting molecule, such as an antibiotic[64]. Antibiotic resistant genotypes can create "antibiotic-free" local environments thorough their metabolic activities, where a resistant genotype secretes an enzyme such as β-lactamase that inactivates the antibiotic and enables the growth of a sensitive genotype (*e.g.*, Perlin *et al.* 2009). In one study with ampicillin-resistant and -sensitive strains of *E. coli*, the frequencies of the two populations reached equilibrium over time[64]. However, the equilibrium frequencies of the resistant strain were dependent on the initial ampicillin concentration (proportional dependence) and on the initial population size (inverse-proportional dependence). When resistant cells were initially rare, their relative abundance "overshot" the equilibrium point and then slowly descended back towards it. The authors proposed a model that recapitulated the system dynamics and revealed non-intuitive and unexpected behaviors. For example, the addition

of a β-lactamase inhibitor (which is used in clinical practice) increases rather than decreases the proportion of ampicillin resistant cells[64].

An important feature of the interaction described above is that it involves the production of a public good (*i.e.*, β-lactamase). There is therefore a "cooperative" and active intra-population interaction between genetically identical cells that produce β-lactamase. The inter-population interaction (*i.e.*, the interaction between the β-lactamase-producing and the non-producing genotypes), however, is nevertheless a passive unidirectional positive interaction with competition. The genotype that produces β-lactamase did not evolve this trait because it provides a benefit to the genotype that does not produce -lactamase. Instead, it likely evolved that trait to benefit itself. The interaction between the two genotypes is therefore passive, which in turn creates competition between the two genotypes for other metabolic resources. We therefore emphasize that the terminology discussed here refers to interactions between different genotypes and not to interactions between cells of a single genotype.

Passive unidirectional positive interactions based on the secretion or leakage of metabolites from one genotype and exploitation by other genotypes may be pervasive in the natural environment as predicted by the Black Queen hypothesis. The Black Queen hypothesis states that if one genotype provides a reliable source of a metabolite (*e.g.*, via passive cell leakage or secretion) or a service (*e.g.*, the consumption or inactivation of a growth-inhibiting molecule), then another genotype may exploit those metabolites and services and cease to biosynthesize or perform those services itself[66,67]. One outcome, then, is the origin of a passive unidirectional positive interaction between the two genotypes[66,67]. Synthetic microbial ecology has successfully tested some of the main predictions of the Black Queen hypothesis[67]. Namely, genes should be continuously lost from a community, and thus passive unidirectional positive interactions should originate (Fig. 2) as long as at least one genotype performs a leaky function that other genotypes can reliably exploit[66].

2.7.3 Passive bidirectional positive interactions with competition (non-cooperative)

Passive bidirectional positive interactions refer to interactions where the growth of one genotype (designated as passive promoter A) is promoted by the growth of a second genotype (designated as passive promoter B), while the growth of passive promoter B is also promoted by the growth of passive promoter A (Fig. 1). Thus, there are reciprocal beneficial effects. We again use the term "passive" to emphasize that the passive promoters do not actively invest metabolic resources into promoting the growth of others, but they instead promote the growth of others as inadvertent consequences of their own metabolism (*e.g.*, generating waste products or leakage of molecules that can be consumed by others). The interactions therefore do *not* constitute as cooperative interactions in the sociological sense, where active investments in partners are necessary (see below). A typical example is unidirectional cross-feeding of a growth-inhibiting waste product. One genotype produces a metabolic waste product that can inhibit its own growth if it accumulates to sufficient concentrations while another genotype consumes the waste product and relieves inhibition of the first genotype. The main dynamic behavior of such a system is typically the approximate convergence of growth rates between the two interdependent genotypes (Fig. 1).

A canonical example of this type of interaction is interspecies hydrogen transfer. Some microorganisms ferment organic substrates to hydrogen. However, if hydrogen accumulates to sufficient concentrations, then the metabolic reaction can become thermodynamically unfavorable and stop. Thus, a partner microorganism is required that consumes the hydrogen to sufficiently low concentrations to maintain thermodynamically favorable conditions[68,69]. In one synthetic microbial assemblage, the sulfate-reducing bacterium *Desulfovibrio vulgaris* was grown together with the methanogenic archaeon *Methanococcus maripaludis*[43]. In the absence of sulfate,

D. vulgaris can ferment lactate to hydrogen while the methanogen *M. maripaludis* maintains the metabolic activity of *D. vulgaris* by transforming the hydrogen into methane. Thus, the growth rates of the two genotypes spontaneously converge based on the production and consumption rates of hydrogen. Interestingly, when a large number of replicate synthetic assemblies were initiated and serially transferred, a subset of the assemblies went extinct[70,71]. Over evolutionary time, however, the remaining synthetic assemblies began to improve in productivity. Further experiments demonstrated that the improved performance was a consequence of genetic changes in both *D. vulgaris* and *M. maripaludis*, suggesting potential evolutionary responses to the passive bidirectional positive interaction.

Another example of this type of interaction is nitrite cross-feeding. Nitrifying communities are well known to cross-feed nitrite, where ammonia-oxidizing microorganisms convert ammonia to nitrite and nitrite-oxidizing bacteria consume the secreted nitrite[72,73]. Under low pH conditions, both microorganisms may benefit from this interaction because the cross-fed intermediate nitrite can become growth-inhibiting while also serving as a growth substrate for the nitrite-oxidizing bacteria. A similar scenario sometimes occurs within denitrifying communities. Many microorganisms are capable of using nitrogen oxides as terminal electron acceptors to support their growth[74]. While some can completely respire nitrate to nitrogen gas, others specialize at specific steps of the pathway and, in some cases, assemble together into nitrite or nitrous oxide cross-feeding consortia[75–77]. In one recent study, two isogenic mutant strains of *Pseudomonas stutzeri* were constructed, where one strain consumes nitrate to nitrite and another consumes nitrite to nitrogen gas [78]. The authors found that segregating the two parts of the pathway into different genotypes eliminated competition between the nitrate and nitrite reductases for intracellular resources and consequently reduced the accumulation of the intermediate nitrite. Moreover, under low pH conditions when nitrite has growth-inhibiting effects[79,80], nitrite cross-feeding accelerated substrate consumption, presumably because nitrite accumulated to lower concentrations and had reduced deleterious effects on growth[78]. This study again emphasizes that if conflicts are known between different metabolic processes (*e.g.*, in this case between the nitrate and nitrite reductases), then one can engineer and assemble genotypes together that avoid those conflicts and consequently accelerate community-level metabolic processes[15,81].

Passive bidirectional positive interactions have also been designed for biosynthetic applications, where metabolic interdependencies were engineered to control system dynamics. In one study, synthetic co-cultures were engineered to produce oxygenated taxanes, which are precursors for the anti-cancer drug taxol[82]. The co-cultures consisted of an *E. coli* strain that produced and secreted a precursor and an *S. cerevisiae* strain that oxygenated the precursor to produce the desired oxygenated taxane[82]. An important aspect of the system is that the two genotypes were not initially dependent on each other but instead had competitive and negative interactions. Both organisms competed for glucose while *S. cerevisiae* produced ethanol that inhibited the growth of *E. coli*. These competitive and negative interactions resulted in reduced taxane production. To overcome this, the authors re-engineered the environment to minimize these competitive and negative interactions. First, the authors provided xylose rather than glucose as a carbon substrate. Only *E. coli* could consume the xylose, thus preventing the main competitive interaction. Moreover, *E. coli* produced acetate as a waste product that *S. cerevisiae* then consumed as a growth-substrate, thus creating a passive positive interaction between the two genotypes. Finally, acetate has growth-inhibiting effects on *E. coli* if it is not consumed by *S. cerevisiae*, thus creating a second passive positive interaction in the opposite direction. Together, the minimization of competition and the promotion of the passive bidirectional positive

interaction resulted in improved taxane production, demonstrating that simple but well informed engineering of the environment can provide control over system behavior.

2.7.4 Active bidirectional positive interactions (cooperative)

Active bidirectional positive interactions refer to scenarios where each genotype actively invests resources into metabolic processes or behaviors that have positive effects on other genotypes (Fig. 1). For example, one genotype may divert cellular resources away from its own growth to produce a metabolite or provide a service that promotes or supports the growth of a second genotype, while the second genotype may divert cellular resources away from its own growth to produce a different metabolite or provide a service that promotes or supports the growth of the first genotype. There is therefore a reciprocal exchange of resources or services. Because each genotype diverts cellular resources that could otherwise be used to support its own growth, these interactions constitute as cooperative interactions. The main dynamic behavior is again typically the convergence of growth rates between the two genotypes (Fig. 1). Because of their cooperative nature, these interactions pose interesting evolutionary dilemmas. How do cooperative interactions between different genotypes emerge in the first place? What prevents genotypes that consume the secreted metabolites but do not pay any of the costs (*i.e.* "cheater" genotypes) from exploiting and disrupting the cooperative interaction? The susceptibility of cooperative interactions to cheating has therefore stimulated a large amount of research into how cooperative interactions originate and persist over time[83–85].

While posing interesting dilemmas, active bidirectional positive interactions are nevertheless found in nature. One such example is the vitamin B_{12} (cobalamin)-based interaction between many algal species and bacteria. Approximately one-half of cultivated algal species require vitamin B_{12} for their growth but cannot biosynthesize vitamin B_{12} *de novo*[86]. Instead, they depend on bacteria to provide vitamin B_{12}, which is metabolically costly to biosynthesize and secrete[87]. Kazamia *et al.* assembled the vitamin B_{12}-dependent green alga *Lobomonas rostrata* with the bacterium *Mezorhizobium loti* and found that the two species could be co-cultivated with each other in the absence of exogenous vitamin B_{12}[88]. The alga supported the growth of the bacterium by providing it with fixed carbon while the bacterium supported the growth of the alga by providing it with vitamin B_{12}. The authors further reported evidence that the bacterium actively diverts cellular resources from its own growth into supplying vitamin B_{12} to the algae. First, the bacterium produces more vitamin B_{12} when in the presence than in the absence of the algae[89]. Second, mathematical modeling indicated that bacterial lysis alone could not explain the extent of algal growth, as more bacterial cells would have needed to lyse than were present in the culture to support the observed growth of the algae[89]. Dissecting the vitamin B_{12}-based interaction between algae and bacteria further, Xie *et al.* demonstrated that the synthesis of the vitamin B_{12}-independent methionine synthase is repressed during heat stress in *Chlamydomonas reinhardtii*, and that survival of heat-stress depends on the functional vitamin B_{12}-dependent methionine synthase. By co-cultivating *C. reinhardtii* with diverse bacteria, they showed that algal stress tolerance is restored in the presence of vitamin B_{12}-producing bacteria, thus providing an example of an emergent community behavior (*i.e.*, stress resistance) that might not be readily predicted from analyzing each genotype in isolation[90].

While the example above attempts to mimic a natural system, likely the most widely engineered active bidirectional positive interaction (*i.e.*, cooperative interaction) is the reciprocal exchange of amino acids or nucleotides between genotypes with different auxotrophic requirements (*i.e.*, genotypes that require exogenous sources of different amino acids or nucleotides to support their growth). Genotypes that cannot biosynthesize certain amino acids or nucleotides are readily obtained from existing mutant libraries[91] or can be created by targeted

gene deletions. Interactions are then established by assembling two genotypes together that require different amino acids or nucleotides, thus forcing each genotype to provide the essential resource required by the other[48,51,92–98]. The questions addressed with these synthetic assemblages are diverse. They range from exploring conditions under which such interactions are likely to arise[51,92,97,98] to examining the factors that prevent the emergence and proliferation of mutants that exploit but do not contribute towards the cooperative interaction (*i.e.*, cheaters)[48,92].

The leitmotif that emerges from many of these studies is that costly metabolites are often not readily released, and genetically engineered auxotrophs do not typically release sufficient amounts of amino acids or nucleotides to support the growth of partner auxotrophs[92,98–100]. For example, pairs of *Saccharomyces cerevisiae* mutants that were deficient in either lysine or adenine biosynthesis repeatedly went extinct when they were grown together in the absence of lysine and adenine unless feedback inhibition mechanisms that prevent the overproduction of the shared metabolites were disrupted[92]. In a study discussed in more detail below[99], extensive mutagenesis was required to obtain a *Salmonella typhimurium* mutant that secretes sufficient methionine to support the growth of an *E. coli* genotype that cannot biosynthesize methionine. The necessity for engineered overproduction of exchanged amino acids was further elaborated in Pande *et al.* (2014), where four *E. coli* amino acid auxotrophs were assembled in pairwise combinations. They used metabolic modeling to identify genes that, upon deletion or inactivation, would contribute the most towards the overproduction of complementary amino acids. When the auxotrophic mutants were then assembled together in pairwise combinations, the majority of the synthetic assemblages had significantly higher growth rates when compared to the ancestral strain. Thus, while not easily achieved, segregating different amino acid biosynthetic pathways into different genotypes and promoting cooperative interactions via targeted genetic engineering can sometimes improve the overall productivity of synthetic assemblages[98].

An important point regarding the above-mentioned studies is that they only investigated the exchange of a limited number of metabolites, and the outcomes of bidirectional cross-feeding might therefore be quite different for alternative auxotroph pairs. This knowledge gap was partially addressed by Wintermute and Silver (2010a) and Mee *et al.* (2014). Wintermute and Silver constructed extensive libraries of *E. coli* auxotroph pairs and observed substantial growth for only 17% of the auxotrophic pairs[51]. They found that the metabolites most likely to be exchanged were those where the ratio of costs of secretion to benefits of uptake was a minimum, a finding that could be predicted via stoichometric modeling. Mee *et al.* (2014) performed a similar analysis and found a two-parameter predictor: the metabolites more likely to be exchanged in co-cultures of auxotrophic mutants were those that were costly to biosynthesize and needed in small amounts. Together, the relatively low prevalence of cross-feeding resulting in enhanced (or even possible) growth[51,97] seems to support the notion that, in general, costly metabolites are not readily shared.

A main question that arises, then, is the following: If segregating different amino acid biosynthetic pathways into different genotypes sometimes improves productivity[98], how prevalent are these types of reciprocal cooperative cross-feeding interactions in the natural environment? Mee *et al.* (2014) addressed this question by relating reciprocal amino acid cross-feeding in synthetic *E. coli* assemblages to the importance of such cooperative interactions in natural systems. Genome analyses of more than 6000 sequenced bacterial genomes identified numerous instances of apparent auxotrophies[14,97]. Additionally, genome analyses of 32 *E. coli* strains suggested high phenotype variability among closely related genotypes – more than half of the *E. coli* strains could not biosynthesize at least one amino acid and approximately one-third of the strains could not biosynthesize two or more amino acids[98]. Together, these studies suggest that, while somewhat difficult to impose in the laboratory, reciprocal cooperative cross-feeding

interactions might indeed be widespread in nature and contribute towards the assembly and functioning of microbial communities. It should be noted, however, that the isolation histories of laboratory strains were not considered in these studies, and amino acid auxotrophies are known to readily emerge during isolation and growth in rich medium[101].

2.7.5 Mixed interactions (positive and negative)

While positive interactions have received substantial attention, it is generally unclear whether positive or negative (*i.e.,* antagonistic but non-competitive) interactions dominate the dynamics and behaviors of natural microbial assemblages[25,102–104]. For example, inhibition by small molecules was common in pairwise co-cultures sampled from a set of 185 *Vibrio* isolates[102].

Predation provides a well-studied example of an interaction with a negative (*i.e.*, antagonistic but non-competitive) component where the predator consumes the prey. Predator-prey interactions are ubiquitous and occur among all kingdoms of life, including between different bacteria[105–107], between bacteria and protozoa[27,28,108,109], between rotifers and algae [50] and between bacteria and phage[110,111]. The typical dynamic that emerges is oscillations in the abundances of predator and prey. The prey population collapses as the predator population increases. However, when the prey reaches sufficiently low abundances, the predator population collapses and the prey population recovers (see Fig. 1).

While predator-prey dynamics have been studied for a long time and for all domains of life, studying these dynamics with microbial assemblages allows for more control and can be investigated using a range of novel approaches. One such novel approach allows to manipulate the strength of predator-prey-type interactions using quorum-sensing (QS) molecules to trigger specific predatory behaviors[112–114]. In one example, Balagaddé *et al.* (2008) engineered a predator genotype of *E. coli* based on the tunable expression of a toxic protein (CcdB) and its antidote (CcdA). They engineered two-way communication between the predator and prey by implementing well-characterized quorum-sensing systems. The engineered predator *E. coli* strain secretes an acyl-homoserine lactone (3OC12HSL) that is specifically recognized by the prey *E. coli* strain. This then triggers the prey genotype to secrete the *Vibrio fischerii*-native 3OC6HSL, which is specifically recognized by the predator genotype. In this system, only the presence of the prey will induce the synthesis of the antidote protein (CcdA) by the predator and rescue it from the toxin (which can be controlled by the IPTG induction). At the same time, the abundance of the predator will trigger the synthesis of the toxin CcdB in the prey, which provides the predator with a competitive edge. This interaction resulted in induction-dependent population oscillations that closely resemble the canonical predator-prey oscillations observed in nature. Interestingly, the study found that small population sizes are required in order to minimize the chance of evolutionary escape from the negative interaction. Accordingly, the experiments were performed in microchemostat reactors containing very small population sizes (approx. 10^2 - 10^4 cells) and were conducted over relatively short time scales[112].

Such oscillations are not unique to predator-prey-type interactions, but can instead occur for other types of mixed interaction systems. Weber *et al.* constructed a negative interaction between mammalian (CHO) cells and *E. coli*. In this system, the CHO cells produce β-lactamase that inactivates the antibiotic ampicillin. When the CHO and *E. coli* cells are cultivated within the same reactor amended with ampicillin, *E. coli* outcompetes CHO cells for resources (negative effect) but is at the same time dependent on CHO cells to degrade the antibiotic (positive effect). At continuous nutrient supply and intermediate antibiotic concentrations, this system results in predictable population oscillations that again resemble the canonical predator-prey oscillations observed in nature. The decline of CHO cell density as a result of the negative interaction causes the reduction in β-lactamase production and the eventual collapse of the *E. coli* population. After

the collapse of the *E. coli* population, the negative effect is relieved and the CHO cells recover. This then results in sufficient β-lactamase production such that *E. coli* can then grow and re-establish the negative interaction[57].

2.8 Interactions may not be readily defined

A central assumption of the terminology described above is that interactions between different genotypes can be precisely defined. This is not always the case. Distinguishing between passive and active positive interactions is especially challenging, yet this distinction has critical implications for predicting the dynamics of synthetic assemblages. Namely, active positive interactions are susceptible to cheating, while passive positive interactions based on unidirectional cross-feeding of an inhibitory waste product are not.

An example occurs between nitrite-reducing and carbon-oxidizing microorganisms[115]. In this interaction, one organism reduces nitrite to ammonia and provides the ammonia to the other organism that oxidizes carbon and provides CO_2 to the first partne[115]. There is therefore a division of labor where each microorganism provides an essential service to the other. Whether the nitrite-reducing organism actively diverts metabolic resources into the secretion of ammonia to stimulate the growth of the other, however, remains unknown. It is plausible that these secretions are not active processes but instead occur via inadvertent leakage from the cells or from cell turnover.

A similar situation occurs within some nitrifying communities consisting of an ammonia-oxidizing bacterium and a nitrite-oxidizing bacterium[116,117]. The ammonia-oxidizing bacterium converts ammonia to nitrite while the nitrite-oxidizing bacterium then consumes the secreted nitrite. When urea[117] or cyanate[116] were supplied to a community, they were decomposed to ammonia via the activity of the nitrite-oxidizing bacterium rather than the ammonia-oxidizing bacterium. The nitrite-oxidizing bacterium therefore depended on the ammonia-oxidizing bacterium for nitrite while the ammonia-oxidizing bacterium depended on the nitrite-oxidizing bacterium for ammonia. Whether the nitrite-oxidizing bacterium actively diverted cellular resources into the production of ammonia for the ammonia-oxidizing bacterium, however, again remains unclear. For example, cyanases may be required by the nitrite-oxidizing bacterium for the detoxification of its own metabolism[116]. It is therefore unknown whether this interaction is active or passive in nature and whether it is susceptible to the emergence and proliferation of "cheating" genotypes.

2.9 Interactions are themselves dynamic

While synthetic microbial assemblages are often designed to have a particular set of interactions, the interactions themselves are context dependent and can readily change over space and time[44,50,118]. Importantly, interactions may change in strength and nature as a consequence of the biological activities of the genotypes themselves (Fig. 2). One genotype may modify the environment by consuming or producing certain molecules, which in turn may affect how another genotype interacts with the first. This dynamical nature of interactions can lead to novel selection pressures, the emergence of new genotypes with different behaviors, and the further evolution of interactions (eco-evolutionary feedbacks).

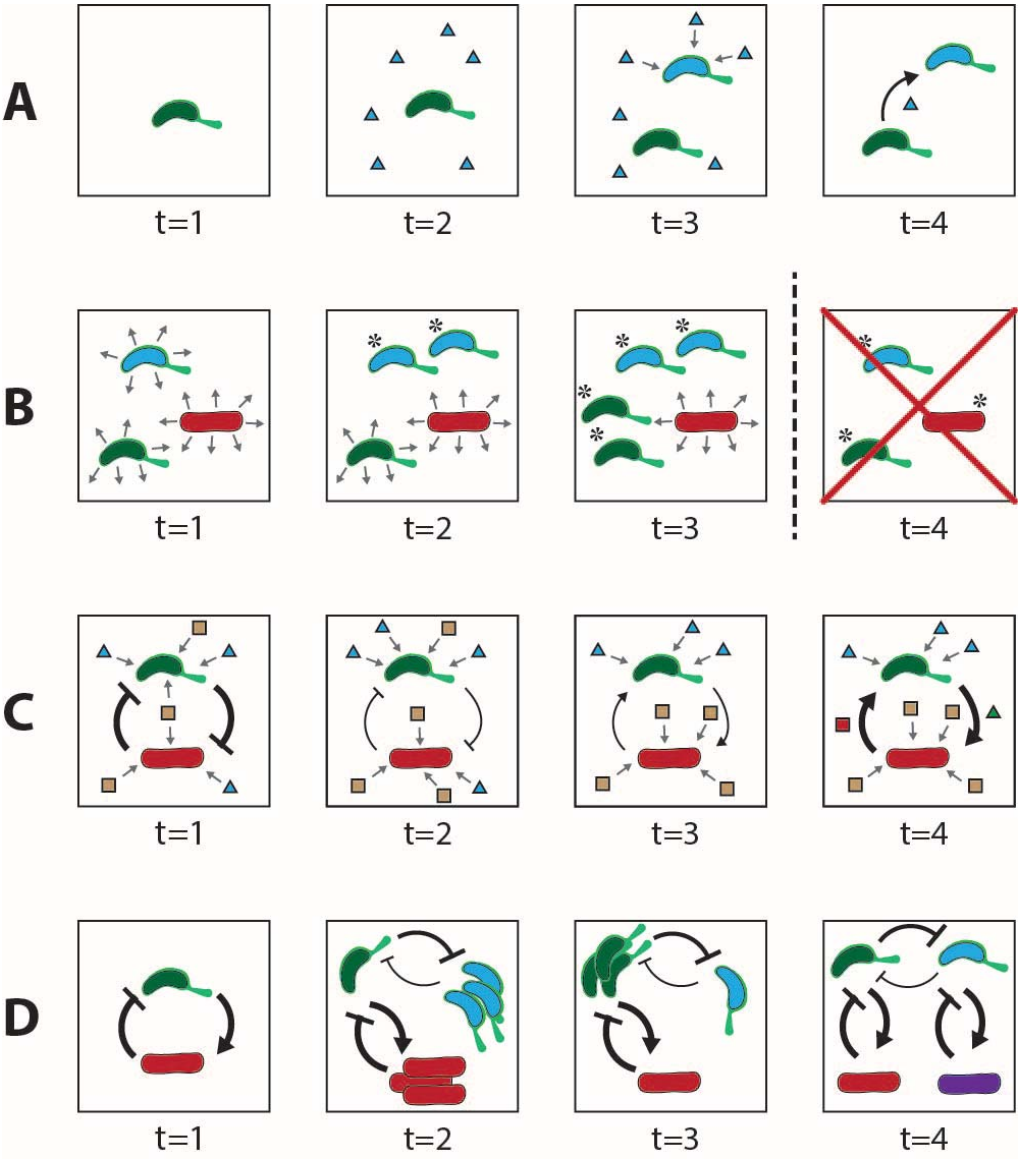


Figure 2 Effect of ecological and evolutionary feedbacks on dynamics. We consider some scenarios that illustrate how ecological and evolutionary feedbacks can affect ecosystem behaviors. **A)** Consider a genotype that consumes a substrate into a waste product. If the waste product accumulates to substantial concentrations (t=2, blue triangles), then this could promote the emergence of a mutant genotype that then consumes the metabolic waste product (t=3, blue genotype). Thus, the metabolic activities of the green genotype created a new niche allowing the emergence of the blue genotype. **B)** The Black Queen hypothesis predicts that genotypes will lose essential functions (grey arrows) whenever another genotype reliably provides those functions. However, as the number of service providers shrinks (t=2 and t=3, asterisks denotes those genotypes that have lost a previously shared function), the selective pressure on the remaining service providers increases, and ultimately any further loss of this function (*e.g.*, via environmental perturbations) will result in collapse of the entire ecosystem (t=4). **C)** Organisms residing in a similar environmental niche may show considerable overlap in resource use. Thus immediately after reassembly in a synthetic setting, resource competition is likely to be a predominant interaction between such organisms (t=1). However, after some rounds of experimental evolution, competitive interactions may weaken as resource specialization evolves (t=2). Subsequent evolution of positive interactions may then emerge (*e.g.*, via Black Queen dynamics) and shift the net negative interaction (t=1 and t=2) into a net-positive interaction (t=3 and t=4). **D)** Predator-prey interactions are particularly prone to evolutionary fluctuations. Predation can lead to the emergence of new prey genotypes that are resistant to the predator (t=2, blue genotype). However, this could create a competitive interaction between the two prey genotypes (t=3, blue and green genotypes), which in turn could affect predator abundance. Moreover, if the new prey genotype proliferates (t=4, blue genotype) then this could promote the evolution of a new predator genotype (t=4, purple genotype), thus accelerating diversification

A canonical example is Red Queen dynamics, which may occur when one genotype preys on another genotype. Predation rapidly selects for mutants of the prey that are no longer susceptible to predation. This, in turn, rapidly selects for mutants of the predator that can again predate on the prey (for recent review see Liow, Van Valen and Stenseth (2011)). In a series of studies with co-cultures of the predatory rotifer *Brachionus calyciflorus* and its algal prey *Chlorella vulgaris*, the predator reached its maximum abundance when the prey was at its minimum abundance and vice versa[50]. This is different from typical predator-prey oscillations, where the predator population reaches its maximum abundance shortly after the prey reaches its maximum abundance and the predator population reaches its minimum abundance shortly after the prey reaches its minimum abundance (Fig. 1, but see also Turchin (2003)). Mathematical modeling suggested that these unexpected oscillations could have resulted from diversification of the prey into two strains with different susceptibilities to predation[121]. Yoshida *et al.* (2003) then replayed these dynamics *in vitro*. In parallel synthetic assemblages, rotifers could either graze on clonal algal populations (resulting in a typical predator-prey oscillations) or on mixed algal populations (*i.e.*, an assemblage of more than one algal clone). The analysis of mixed algal populations showed that rotifers preferentially graze on the larger and faster growing algae strain and less on the smaller and slower growing strain, resulting in the same unexpected oscillations. Thus, predation pressure resulted in rapid algal diversification and the evolution of new interactions that resulted in atypical dynamics[50].

Another example of the dynamic nature of interactions is the benzoate cross-feeding interaction between strains of *Pseudomonas putida* and *Acinetobacter*[44,122]. The *Acinetobacter* strain can completely consume benzyl alcohol while the *P. putida* strain can only consume the intermediate benzoate. Above certain benzyl alcohol concentrations, *Acinetobacter* leaks benzoate out of the cell, thus allowing *P. putida* to consume the leaked benzoate and persist within the assemblage. When grown in co-cultures, the *Acinetobacter* strain typically had a positive effect on the *P. putida* strain by providing benzoate as a growth substrate. However, a mutant strain of *P. putida* emerged that had a deleterious effect on the *Acinetobacter* strain but a positive effect on the productivity of the assemblage as a whole. Thus, by accumulating particular mutations, an initially unidirectional positive interaction evolved into an exploitive interaction that was nevertheless more productive at the community level[44]. A similar outcome was observed between *S. cerevisiae* and *Rhizobium etli*, in that an initially unidirectional and positive interaction rapidly evolved to become competitive and antagonistic[118]. In both cases, a common feature is that the nature of the interactions changed rapidly, emphasizing that ecological and evolutionary processes may occur at similar and experimentally observable time-scales[123,124].

Interactions do not always evolve to become more competitive or antagonistic in nature. An opposite dynamic was recently observed in studies using randomly assembled synthetic assemblages containing species isolated from a common environment[125,126]. The authors found that the interactions were initially competitive. However, after approximately 60 generations of experimental evolution, the assemblages tended to improve in productivity. The authors demonstrated that species interactions within the assemblages evolved to become less competitive. While the underlying mechanisms that reduced competition were unclear, possible explanations include the emergence of mutants that consumed the waste products of others, thus providing an example of how the biological activities of some species can create novel ecological niches for different genotypes to occupy and thus reduce competition[125].

A single interaction may also change in strength rather than nature. For example, additional gene loss can lead to concomitant evolution of additional interdependencies. However, this needs not to be the only case. Such an alternative scenario was observed when *Geobacter metallireducens* and *Geobacter sulfurreducens* were combined in an environment that promoted the

evolution of more efficient electron transfer between the two genotypes[127]. Using an experimental evolution approach, resource consumption and productivity improved over time, but a previously planktonic co-culture began to form large aggregates that contained both genotypes. Genome re-sequencing and experiments with deletion mutants suggested that more efficient mechanisms for electron transfer had rapidly evolved. Electrons were initially transferred between the two genotypes via diffusible hydrogen. However, a new mechanism emerged, whereby electrons were transferred between the two genotypes via a conductive extracellular matrix. This change correlated with an increase in community-level productivity. Thus, the interaction rapidly changed at the mechanistic level in response to the necessity to transfer a metabolic intermediate between different genotypes (*e.g.*, in this case electron carriers).

2.10 Beyond binary interactions

An expanding number of studies have investigated the dynamics of assemblages that consist of more than two genotypes, and thus have more than a single interaction[53,97,128–130]. An important aspect of these studies is that they often result in unexpected dynamics that might not be readily predicted from interactions between any two-member community. One example is the synthetic construction of rock-paper-scissors dynamics[128,129]. In one case, three genotypes were investigated *in silico*, each of which produced a different antibiotic while being sensitive to the antibiotic produced by one of the other genotypes[129]. Thus, each genotype had a negative effect on only one other genotype. The authors discovered that all three genotypes could coexist in a spatially structured environment and undergo predictable frequency oscillations. However, they could not coexist in a homogeneous environment, as every genotype succumbed to the antibiotic produced by another genotype, but due to inherent fitness differences, one genotype eventually prevailed. This finding was in line with previous experimental and theoretical results [128]. Perhaps unexpectedly, however, when the system was extended such that each genotype had a negative effect on *one* genotype but a positive effect on *another* genotype (*i.e.*, when antibiotic degradation was "shared"), then the strains could coexist in a homogeneous environment (in this context see also Coyte, Schluter and Foster 2015).

Another example is the effect of cheating genotypes that exploit an active bidirectional positive interaction between two cooperating genotypes. Waite and Shou (2012) extended a synthetic reciprocal cross-feeding assemblage consisting of two yeast strains, each of which produced an essential metabolite (lysine or adenine) required by the other[92]. The authors then added a third strain that consumed lysine, but did not reciprocate by overproducing adenine, and could therefore be viewed as a "cheater"[96]. While theoretical considerations predicted that the ecosystem should collapse in the absence of spatial structure, cross-feeding unexpectedly persisted. This was a consequence of rapid evolution of the cooperating genotype that improved its metabolite uptake, which in turn reduced the relative fitness of the cheating genotype and thus prevented the cheating genotype from reaching sufficient abundance levels to disrupt the ecosystem.

Interactions between different microbial genotypes may also have important effects on their eukaryotic hosts. Samuel and Gordon constructed simple assemblages of *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* and inoculated them within the gut of gnotobiotic (*i.e.*, sterile) mice[132]. Both *B. thetaiotaomicron* and *M. smithii* can individually colonize the mouse gut. However, the addition of both species increased microbial biomass in the gut relative to the addition of either species individually. This is because *B. thetaiotaomicron* ferments saccharides to generate short-chain fatty acids as metabolic waste products. *M. smithii* then consumes the waste products and reduces their growth inhibiting effects. Indeed, metabolomic and transcriptomic analyses demonstrated that the addition of *M. smithii* shifts

sugar preferences of *B. thetaiotaomicron* toward fructans, shunts the fermentation pathway towards the production of formate (preferentially utilized by *M. smithii*) and acetate. This, in turn, had a profound effect on the mouse, which consumed the secreted acetate.

These experiments with more than two genotypes then raise a critical question: If all possible binary interactions are known for a set of genotypes, can the dynamic properties and behaviors of mixtures of more than two genotypes be predicted? Recent theoretical studies suggest that the answer might typically be no[133]. As the number of genotypes and strategies increases, then the set of binary interactions typically cannot be used to predict the dynamics of more complex assemblages. More specifically, the likelihood of successfully predicting the dynamics from binary interactions rapidly decreases as the number of genotypes within an assemblage increases. This could have profound implications for designing and applying synthetic microbial communities in concrete applications, as many applications require more than two genotypes to achieve a desired design objective (*e.g.*, see Kato *et al.* 2008).

2.11 Spatial structure can stabilize interactions

Spatial structure refers to the presence of surfaces, liquid-liquid interfaces, or gas-liquid interfaces that prevent complete mixing within a particular environment. The prevalence of spatial structure is intuitive in systems that typically lack turbulent flows and contain abundant surface area such as soils. However, spatial structure also occurs in environments that are often assumed to be spatially homogenous such as seawater, where incomplete mixing and particulate matter can create small-scale spatial structuring[135]. When one considers microbes residing in other spatially structured environments, such as plant and animal epithelia, sediments, stratified lakes, and the subsurface[136], it is clear that spatial structure is a general environmental factor that affects the majority of microbes in nature. There is therefore growing interest in understanding how spatial structure affects interactions and, in turn, community dynamics. In the section below, we review scenarios where spatial structure can impact interactions, community dynamics, and ultimately the evolutionary trajectories of microbial assemblages (Fig. 3). Synthetic ecology is especially useful for investigating the role of spatial structure on interactions and community dynamics because spatial structure can be experimentally manipulated in precise and quantifiable manners.

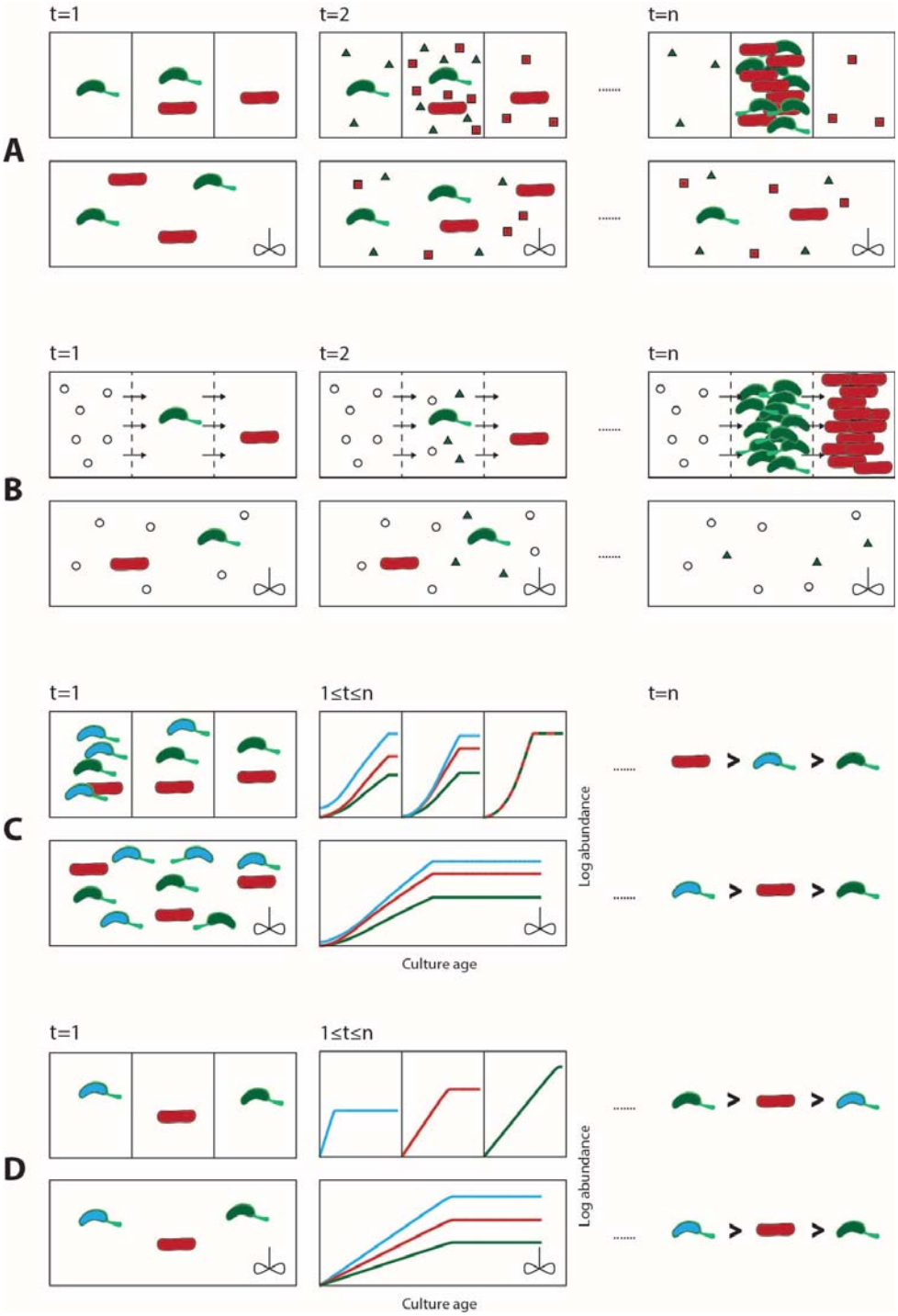


Figure 3 Effect of spatial structure on dynamics. We consider some scenarios where spatial structure can affect community-level dynamics. Upper scenarios in each panel show spatially organized environments (dashed lines separate different spatial patches, and solid lines separate different compartments) whereas lower scenarios depict well-mixed systems. **A)** For a bidirectional positive interaction between two genotypes, spatial structure can slow the loss of exchanged metabolites into the bulk medium (green triangles and red circles), and thus increase their local concentrations and promote growth (center scenario). However, spatial structure can also physically prevent different genotypes from interacting, thus reducing or preventing growth (left and right scenarios). **B)** Spatial structure can physically separate incompatible processes. In this case, the green genotype consumes oxygen (empty circles) while the red genotype is sensitive to oxygen. However, the red genotype depends on a molecule secreted by the green genotype. In a spatially structured environment, the green genotype consumes the oxygen before it encounters the red genotype, thus promoting growth. Analogous to sediments in nature, in this scenario oxygen penetrates only

from one direction (*i.e.*, from left to right). However, in a completely mixed environment, oxygen inhibits the red genotype and the green genotype suffers from product inhibition caused by the accumulation of the secreted metabolite. **C)** Physical barriers can effectively protect cooperating genotypes (red and green cells) from invasive "cheating" genotypes (blue cells). In a completely mixed system, the cheating genotype may achieve higher abundance than the cooperating genotypes. However, in a spatially fragmented system, cooperators may achieve higher abundances than the cheating genotype because of the localization of positive interactions. Note that the starting number of strains is equal for both scenarios. **D)** Spatial structure can modify the relative frequencies of different genotypes. Consider a mixture of three genotypes, competing for the same substrate, but whose rates and yields are negatively related to each other (*i.e.*, a rate-yield trade-off). At the completion of substrate consumption, the spatially structured environment could have greater biomass than the completely mixed environment. This is because the spatially structured environment may prevent competition between faster and slower growing genotypes, thus allowing the slower growing genotypes to proliferate and achieve higher cell densities.

2.11.1 Spatial structure can maintain positive interactions

In stirred or shaken liquid systems with extensive mixing, mass transfer of secreted or leaked molecules is dominated by convection rather than by diffusion. Each cell within the system therefore experiences approximately the same concentrations of those molecules. In contrast, in spatially structured systems, diffusion may become the main process governing mass transfer of secreted or leaked molecules. The concentrations of those molecules are therefore highest immediately adjacent to the producing cells and concentration gradients are created between producing cells and the bulk liquid or consuming cells.

The higher local concentrations of secreted or leaked metabolites in spatially structured environments can have important effects on interactions between different microbial genotypes. This is because the concentrations of the secreted or leaked molecules must often reach threshold concentrations before they can have significant effects on the dynamics of a microbial assemblage. This is particularly important when the secretion of the molecule occurs in limited amounts. In a completely mixed system, the rapid dilution of a growth-promoting molecule into the bulk medium may prevent the molecule from accumulating to sufficiently high local concentrations[137]. Therefore, at low cell densities and in completely mixed systems, the benefits of bidirectional cross-feeding may be lower than the costs, thus destabilizing and preventing the interaction from emerging. At high cell densities, on the other hand, the secreted molecule may accumulate to sufficiently high concentrations to enable the interaction, but competition for other shared resources can increase, which again can destabilize the interaction. Thus, theoretical and experimental studies suggest that, in the absence of spatial structure, intermediate cell densities are necessary to allow for the sufficient accumulation of cross-fed molecules while also preventing competition for other shared resources[137]. In a spatially structured system, however, diffusion limits substrate consumption, thus even dense localized populations will be able to compete only for the substrate in their immediate vicinity. However, distribution of such patches is important – when cell clusters are too close to each other, substrate competition might inhibit growth. But when they are too far apart, interactions based on secreted or leaked molecules might suffer from diffusion limitations [138].

Another critical point of interactions based on secreted or leaked molecules is that they are susceptible to "cheating". Genotypes may emerge that consume the secreted molecule but do not contribute towards the secretion of the molecule itself. They therefore exploit the interaction without paying any of the costs. In the absence of spatial structure, conceptual and experimental considerations suggest that such exploitation can cause the interaction to completely collapse and disappear[48,99,139]. Spatial structure and its localizing effects have been identified as one mechanism that could prevent cheating genotypes from disrupting positive secretion-based interactions (Fig. 3). The effects of spatial structure on preventing the emergence and proliferation of cheating genotypes were explored in detail using the yeast adenine-lysine cross-feeding system described

above[48,96]. When the environment was sufficiently structured (such as when the assemblages were grown on agar plates), the cooperative interaction persisted and was resilient towards the addition of cheating genotypes. Further experiments and theoretical simulations indicated that in environments where mixing is incomplete, patches of genotypes that secrete molecules were not displaced by cheating genotypes, and this was due to the preferential access of the secreting genotypes to the benefits of secretion (*i.e.*, spatial segregation prevented the cheating genotype from having equal access to the benefits of secretion, while local positive interactions between the cooperating genotypes ensured their faster growth). Therefore, spatial structure alone is sufficient to promote and maintain positive secretion-based interactions[48,140], and this result has been observed repeatedly in other systems [53,99,141].

2.11.2 Spatial structure can readily emerge itself

Spatial structure may also be created by interactions between the genotypes themselves via cell-cell contact, and thus maintain higher local concentrations of secreted or leaked metabolites[100]. In one example, synthetic assemblages of amino acid auxotroph pairs rapidly improved in productivity when grown within completely mixed environments. Upon microscopic analysis, the authors discovered that the different genotypes were connected via nanotube-like structures. Further experiments suggested that the nanotubes promoted transfer of cytosolic components from one genotype to the other (Pande *et al.* 2015, but see also Benomar *et al.* 2015) and that nanotube formation is regulated (*i.e.*, nanotubes did not form when amino acids were supplemented to the medium). Thus, if insufficient spatial structure is present in the abiotic environment, assemblages can sometimes rapidly create the necessary structure to maintain positive bidirectional interactions based on the exchange of essential metabolites (in this case by producing nanotube connections).

2.11.3 Spatial structure can mitigate competitive interactions

The diffusional gradients of molecules in spatially structured environments can create a diversity of quantitatively or even qualitatively different habitats for genotypes to proliferate within, and these niches may occur over very small distances. A canonical example occurs in sediments, where the chemical environment changes rapidly over depth as a consequence of the metabolic activities of the resident microorganisms. Physical conditions may also vary over small distances, such as the laminar boundary layer next to surfaces. Such spatial structure allows for the coexistence of species with different environmental adaptations. As an example, synthetic assemblages of different algal isolates were constructed where the isolates were allowed to compete in artificial flumes operated under homogenous or heterogeneous flow regimes[23]. System productivity was measured by the uptake rate of NO_3^- and primary production (algal biomass). When the assemblages were inoculated into a completely mixed environment, one of the genotypes rapidly displaced the others. In contrast, when the assemblages were inoculated into a spatially structured environment, more genotypes persisted within the system, presumably because they occupied different ecological niches created by diffusional gradients. Interestingly, the mixed assemblages always performed worse than monocultures in the completely mixed environment, pointing towards competitive and antagonistic interactions becoming dominant between species. On the other hand, the mixed assemblages generally performed better than monocultures in the heterogeneous environment, suggesting the maintenance of specialists via niche complementarity.

Environmental connectivity can also create different habitats that enable competing genotypes to coexist. A set of theoretical studies recently investigated how water availability affects environmental connectivity and, in turn, influences the coexistence of different genotypes[143,144]. In a wet and highly connected environment, a rapidly growing phenotype displaced a slower

growing phenotype within the assemblage. In dry conditions, however, where patches were less connected, the rapidly growing phenotype could co-exist with a slower growing phenotype over the time-course of the simulations. One reason for coexistence in dry environments is reduced cell motility (for experimental observations, see also Dechesne *et al.* 2010) which reduced the number of interconnecting channels for substrates to diffuse through. As a consequence, both cell types were spatially separated, effectively preventing competition. When a similar *in silico* community was designed to cross-feed a metabolic waste product on hydrated surfaces[146], the assemblages organized into persistent patterns. Importantly, the spatial organization depended on both the interaction network topology and on the connectedness of the environment.

2.12 Temporal heterogeneity can stabilize interactions

The activities of microorganisms themselves can rapidly change the environment over time, which can have consequences on microbial interactions. Such dynamic changes and their effects on ecosystem processes are readily observed within synthetic assemblages. These dynamic changes can create new habitats that allow otherwise competing genotypes to coexist. For example, while all cells experience the same environment at any given point in time, one genotype may have higher fitness at one time point while the other genotype may have fitness advantage at another time point. If conditions oscillate over time, then neither genotype may be able to completely displace the other. Examples of such dynamics include temporal oscillations in resource availability[60,62,82], chemical/physical parameters[134,147], and when growth alters density and/or frequency dependent interactions[5,57,64,148]. Below we highlight some examples of such dynamics.

A canonical example of the importance of temporal oscillations occurs when *E. coli* was evolved in batch cultures. During batch growth, resources were initially in excess but rapidly changed to become scarce. After serial transfers, at least two genotypes emerge. One genotype had higher fitness when resources were abundant while the other genotype had higher viability in stationary phase[58]. Synthetic assemblages of the two genotypes together combined with modeling demonstrated that both genotypes could be maintained by temporally segregated fitness benefits[58,62,149,150]. The temporal fitness segregation is context dependent. The genotype that had greater viability in stationary phase only benefits from this phenotype when the other genotype is present[58].

Another example occurs between organisms that exhibit different rate-yield properties. In one study, assemblages were constructed of a yeast strain that could both ferment and respire glucose with another strain that could only respire glucose[151]. The former strain grew faster but achieved lower yields while the latter strain grew slower but achieved higher yields[152]. When inoculated into a chemostat, the faster growing strain displaced the slower growing strain. However, in batch culture, both strains could co-exist. The explanation for co-existence was that the fermenting activity of the faster growing strain was more susceptible to product inhibition. They experimentally confirmed that when glucose was abundant, the intermediates accumulated and slowed the growth of the faster growing strain, thus providing a fitness benefit to the slower growing strain. Also, after the glucose was completely consumed, the slower growing but more efficient strain had already extracted energy from the metabolites, thus depleting the secondary resource pool available to the faster growing strain[151].

Temporal dynamics can also interact with spatial structure. In a recent modeling investigation[144], the simulation of temporal oscillations in a spatially structured environment yielded unexpected dynamics. Consider a spatially structured environment containing two competitor genotypes. If spatial patches are well connected with each other, the more competitive genotype increases in frequency. However, if spatial patches become increasingly disconnected,

the dense local populations of the faster growing genotype will have lower per capita substrate influx than those of less competitive genotype. The cellular maintenance requirements for the more competitive genotype are therefore less likely to be met, thus increasing the mortality of that genotype and increasing the frequency of the less competitive genotype. Under specific conditions, these temporal oscillations can prevent one genotype from completely displacing the other. Temporal oscillations combined with spatial structure were also critical for maintaining rock-scissor-paper-type dynamics with *E. coli*[128]. The colicin-resistant genotype could invade the spatial patch initially occupied by colicin-sensitive *E. coli*, but only after this spatial patch had been invaded by colicin-producing variant. The same was true for the other two genotypes; the invasion into a spatial patch occupied by an otherwise more competitive strain could proceed only in a periodic manner, thus requiring a particular succession history (*i.e.*, a particular ordering of immigration events).

2.13 Conclusions

The reader might have noticed that we took great care throughout this review to avoid using conventional terminology for interactions between different genotypes, such as commensalism and mutualism (Fig. 4). While this terminology is likely useful for macro-ecology, we believe that it is less useful for microbial ecology and can instead create confusion. The main problem is that conventional interaction terminology oversimplifies the true nature of interactions between different genotypes. We argue this on three points. First, the terms commensalism and mutualism do not indicate whether the positive interaction results from passive or active processes. Yet, whether the interaction is passive or active can profoundly affect the selection pressures that act on that interaction, and thus on its properties and stability over evolutionary time. The lack of such a distinction between passive and active processes can consequently create confusion, such as equating the terms mutualism and cooperation, which are not necessarily the same (*i.e.*, a mutualism need not be based on active processes while cooperation must be based on active processes). Second, the terms commensalism and mutualism suggest that the interaction is purely positive. However, a interaction is almost never purely positive. Instead, the interaction will almost always have competitive elements at the same time. Importantly, it is often unclear whether the positive or competitive elements have the dominant effect on ecosystem dynamics and community properties. Finally, the presentations similar to Fig. 4 give the appearance that interactions are static. However, interactions can change abruptly over both ecological and evolutionary time-scales. The challenge with this dynamical nature of interactions is enormous. Consider that the number of interactions within an assemblage scales exponentially with the number of genotypes, where the addition of an additional genotype adds n new potential interactions to already existing n*(n-1)/2 interactions within that system. If even one of these interactions changes in strength or sign (e.g., positive to negative) over time, then it could have cascading effects on all other interactions.

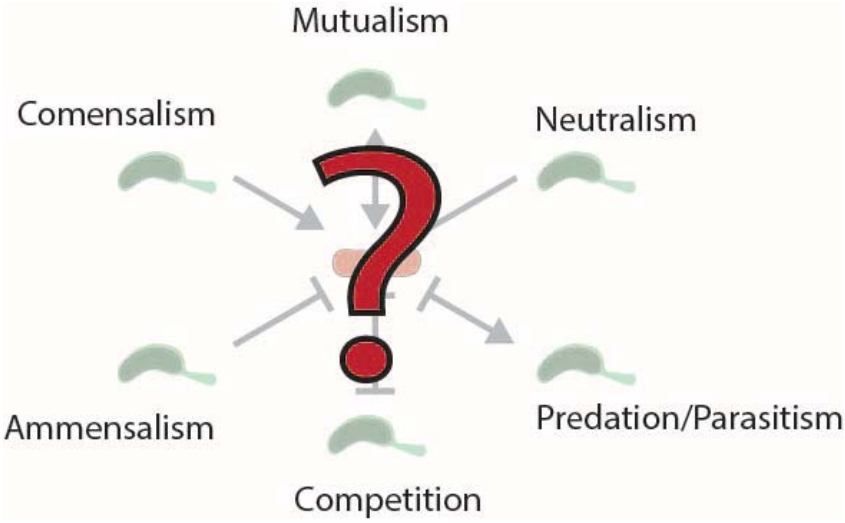


Figure 4 Interaction terminology common in microbial ecology can be misleading. We believe that microbial interactions can be rarely captured by simple terms such as mutualism, commensalism, or ammensalism. Some sort of competition is almost always present in microbial assemblages, thus microbial interactions are rather a sum of positive and negative components. These common terms also fail to capture the mechanisms behind an interaction (*i.e.*, whether the interaction is passive or active in nature). Furthermore, interactions are not static, but may instead change readily over time and space. We believe that it is important for the community to be aware of ecological mechanisms within a synthetic assemblage, as they can have a decisive impact on the interaction dynamics and stability. Thus, a more complete and precise terminology may be helpful.

Given these limitations, we argue that an alternative framework for describing interactions be adopted, and we propose a new set of terminology here. We refrained from simple single-word terminology and instead stated explicitly whether an interaction is active or passive and whether the interaction also contains competitive elements. Unfortunately, our terminology presented here is admittedly cumbersome and does not address the issue with the dynamical nature of interactions; further advances and insights are clearly needed. Perhaps bleakly, we note here that our arguments for developing a more precise terminology for interactions are not new, and similar suggestions regarding the nature of positive interactions and the pervasiveness and complexity of competition were made nearly 40 years ago[27,28], yet widespread adoption of more precise terminology generally remains absent.

Another important outcome of our review is that what we name today as "synthetic microbial ecology" is not a new discipline; it is rather an ongoing effort in microbiology. Perhaps the biggest advance in recent years is the predictive power of modern mathematical and metabolic modeling and the availability of elegant genetic systems to control and analyze the dynamic behavior of synthetic assemblages. Taken together, these tools allow us to premeditate and then impose interactions of our choosing, and here we are not necessarily limited by native constraints of microbes at hand. This then allows us to test long-standing hypotheses that have since been difficult to test. It is also stimulating a new generation of microbiologists that integrate strong analytical, mathematical, and experimental approaches to improve our basic understanding of microbial communities, which are expertise that have largely been distributed among different research fields rather than brought together within a single research effort.

2.14 Outlook

Synthetic microbial ecology will have continuing potential to understand how interactions between different microbial genotypes can lead to community level processes. This information is

not only important for basic scientific advances, but has potential to also be applied in concrete biotechnological applications. For example, we have emphasized the susceptibility of cooperative interactions to cheating as a consequence of their active (rather than passive) nature, which can lead to their eventual collapse. Thus, if one wishes to engineer a system to achieve a specific design objective, it is clear that one should avoid cooperative interactions or engineer environments that protect cooperative interactions from cheating. We have also emphasized how interactions can be engineered or imposed to control system dynamics for the production of valuable bioproducts. Continuing advances at the basic level should help to establish general engineering design principles that predict how best to distribute different metabolic processes across different genotypes to optimize a desired biotransformation. They will also help to identify how to impose new or additional interactions to control stability and resiliency, which should lead to important biotechnological advances.

Another great challenge for future research is to translate the advances in synthetic microbial ecology to natural microbial assemblages. This poses enormous challenges, as system complexity scales exponentially with the number of genotypes present in the system (*i.e.*, each additional genotype present within a community can, directly or indirectly, interact with every other genotype). Still, the general principles and rules derived from synthetic microbial ecology may be useful for understanding which interactions have a critical role within complex assemblages and which do not. This could help establish truly predictive and comprehensive models of system dynamics for real-world environmental communities, such as those residing in the human gut or in wastewater treatment plants. Thus, the bridging of synthetic ecology with natural systems will be a grand challenge that also could have profound effects on a variety of biological disciplines, including microbiology, ecology, evolution, and the environmental sciences.

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2.16 Supplementary Information

2.16.1 Comments on simulated dynamics summarized in Figure 1

In models describing unidirectional interactions, both genotypes occupy a separate niche, and do not compete for the same primary substrate. In subsequent models, both genotypes compete for the same primary substrate, but their growth is also affected by a secondary substrate. In all figures, positive interactions are modeled as an exchange of an essential growth-limiting substrate. Negative interactions (except predation) were modeled by simulating the secretion of a compound that inhibits the growth of a second genotype.

In situations where we included competition between cooperative genotypes, the sign of the interaction is density dependent. One genotype will initially grow more rapidly if its obligate partner genotype is abundant. However, at higher partner densities with more intense competition, the primary substrate rather than exchanged substrate becomes growth-limiting, and the interaction consequently changes from positive to negative. Please note that the abundance of dependent genotype has almost no impact on the growth of independent genotype when the later is rare, as the primary resource removal cannot proceed without the exchange substrate (*i.e.*, limiting substrate) being present in sufficient amounts. Comparing the outcomes to when interactions are positive unidirectional but without competition offers a useful guide for the construction of synthetic assemblages. Eliminating competition for the limiting substrate (e.g. by constructing a separate niche) will improve the growth of the dependent genotype; such a design allows each genotype to grow to effective numbers without simultaneously invading the ecological space of its partner.

Another interesting observation is the possible stabilization of growth dynamics, as observed in outcomes when modeled interactions were unidirectional and negative. It is unclear whether such outcomes are realistic in synthetic assemblages. They may be important in settings where metabolic waste produced by the uncontrolled growth of one genotype inhibits the functioning of an entire ecosystem, and constant population size would therefore lead to increased productivity.

We obtained a valuable insight when comparing both types of bidirectional interactions. In our simulations, one genotype had a minor advantage (slightly faster maximal growth rate) over another genotype. Whereas effective growth rates will synchronize to the slowest growing genotype in simulations describing positive bidirectional interactions, the negative bidirectional interactions have much more erratic outcomes. Even a minor increase in growth rate (or slightly faster inhibitor production) of one genotype will effectively tip the outcome of simulation to its favor.

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3 Metabolic specialization and the assembly of microbial communities

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3.1 Abstract

Metabolic specialization is a general biological principle that shapes the assembly of microbial communities. Individual cell types rarely metabolize a wide range of substrates within their environment. Instead, different cell types often specialize at metabolizing only subsets of the available substrates. What is the advantage of metabolizing subsets of the available substrates rather than all of them? In this perspective piece, we argue that biochemical conflicts between different metabolic processes can promote metabolic specialization and that a better understanding of these conflicts is therefore important for revealing the general principles and rules that govern the assembly of microbial communities. We first discuss three types of biochemical conflicts that could promote metabolic specialization. Next, we demonstrate how knowledge about the consequences of biochemical conflicts can be used to predict whether different metabolic processes are likely to be performed by the same cell type or by different cell types. We then discuss the major challenges in identifying and assessing biochemical conflicts between different metabolic processes and propose several approaches for their measurement. Finally, we argue that a deeper understanding of the biochemical causes of metabolic specialization could serve as a foundation for the field of synthetic ecology, where the objective would be to rationally engineer the assembly of a microbial community to perform a desired biotransformation.

3.2 Introduction

Metabolic specialization is a general biological principle that applies across every domain of cellular life. Consider a microbial cell residing within the human gut. This cell encounters a myriad of different substrates that could be metabolized to satisfy its energetic and elemental requirements[1]. Yet, even if this cell were near starvation, it would only metabolize a subset of the available substrates[1]. What is the advantage of metabolizing only subsets of the available substrates rather than all of them? What are the underlying causes of metabolic specialization? Can we predict which substrates are likely metabolized by the same cell type and which are likely metabolized by different cell types? Thus far, there are few general principles and rules that address these questions.

The questions posed above have relevance for one of the most perplexing enigmas in microbial ecology: why are some microbial communities so incredibly diverse? Advances in molecular ecology revealed that a single liter of seawater or gram of soil contain thousands of different microbial taxa[2–4]. Yet, the mechanisms that promote these levels of diversity are not fully clear[5]. Metabolic specialization provides one plausible explanation for how diversity could be promoted, and is therefore a likely general organizing principle that shapes the assembly of microbial communities.

A substantial body of research has greatly improved our understanding of the causes of metabolic specialization[5–7]. An important conclusion is that metabolic specialization readily evolves in nearly every conceivable environment and can often be explained by basic ecological and evolutionary principles. This research, however, has largely focused on the ecological and genetic causes of metabolic specialization rather than the biochemical causes[5]. It is clear that biochemical conflicts exist between different metabolic processes and likely promote the evolution of metabolic specialization. Our limited understanding about these biochemical conflicts therefore represents a significant gap in our knowledge.

In this perspective manuscript, we argue that a better understanding of the biochemical conflicts that exist between different metabolic processes could help reveal the general principles and rules that govern the evolution of metabolic specialization. We begin by discussing three types of biochemical conflicts that could promote specialization. We then demonstrate how knowledge about the consequences of biochemical conflicts can be used to predict whether different metabolic processes are performed by the same cell type or by different cell types. We conclude by discussing the major challenges in identifying and assessing biochemical conflicts and propose several approaches for their measurement.

3.3 Biochemical conflicts that could promote metabolic specialization

3.3.1 Conflicts resulting from competition for intracellular resources.

The synthesis and maintenance of metabolic enzymes requires the consumption of intracellular resources, including elemental building blocks (carbon and nitrogen), energy resources (ATP), mRNA synthesis machinery (RNA polymerase, sigma factors), protein synthesis machinery (amino acids, ribosomes, tRNAs, chaperones), and cellular space for housing enzymes. If one of these resources is limiting, then a cell that invests more resources in one metabolic process must invest fewer resources in other processes. Recent studies support the existence of such competitive resource conflicts[8,9], but the specific intracellular resources that were under competition were not identified. Below we discuss two that might be of relevance.

Cytoplasmic solvent capacity is one intracellular resource that could lead to competitive resource conflicts. The solvent capacity determines the maximum number of enzymes and other

macromolecules that can be contained within the cell[10]. If the solvent capacity is exceeded then the biochemical and biophysical properties of macromolecules can change, often with detrimental effects[10]. Recent combinations of experiments and modeling of *E. coli* showed that cells operate near their solvent capacity when grown with high substrate supply[11]. Under these conditions, cells that are provided with mixtures of different substrates produce enzymes for metabolizing only the most productive substrate[11], thus potentially leaving the less productive substrates available for uptake by different cell types. A likely explanation for this type of specialization is that, because the solvent capacity is near saturation, cells that produce more enzymes for less productive pathways must produce fewer enzymes for the most productive pathway. Competition for solvent capacity could therefore promote metabolic specialization under specific substrate supply conditions.

RNA polymerase is another intracellular resource that could lead to competitive resource conflicts. RNA polymerase interacts with different sigma factors to regulate the transcription of different genes[12]. Studies with *E. coli* showed that the *rpoS* and *rpoD* sigma factors compete for limited RNA polymerase[13]. *rpoS* regulates stress-response genes while *rpoD* regulates carbon metabolism genes that determine the metabolic versatility of *E. coli*. Increasing the expression of *rpoS* and stress-response genes must therefore coincide with decreasing the expression of *rpoD* and restricting the metabolic versatility of *E. coli*, thus resulting in metabolic specialization. Competition for limited RNA polymerase by different sigma factors could therefore promote metabolic specialization under specific stress conditions.

3.3.2 Conflicts resulting from inhibition.

The production of inhibitory intermediates and endproducts could also lead to biochemical conflicts between different metabolic processes[14–17]. One example is the antagonistic effect of oxygenic photosynthesis on nitrogen fixation. Oxygen is produced during photosynthesis but inhibits nitrogen fixation by irreversibly inactivating nitrogenase, which makes it challenging for a cell to perform both processes simultaneously[14]. A number of strategies have evolved to overcome this conflict, such as differentiation into different cell types where one type photosynthesizes and another type fixes nitrogen[14].

Inhibitory conflicts between metabolic processes could also occur if intracellular intermediates are growth inhibiting[15,16]. Theoretical models that consider growth-inhibiting intermediates predict that cross-feeding metabolic specialists are more likely to evolve as the inhibiting effects increase[15,16]. The explanation for this is that performing an additional metabolic conversion step of a substrate incurs two costs: the cost of synthesizing proteins for the additional step and the cost of producing additional intracellular intermediates. If these costs exceed the energetic gain from the additional step, then it is beneficial to not perform that step and only partially consume the substrate, thus allowing the intermediates to escape and be metabolized by other cell types. Although quantitative measures of these costs are lacking, these predictions are consistent with many empirical observations. For example, consortia of partially consuming cross-feeding cell types are often responsible for degrading pollutants that produce toxic intermediates[18,19].

3.3.3 Conflicts for enzyme specificity.

If the same enzyme interacts with different substrates, such as occurs with some membrane transporters[20], then conflicts could occur for enzyme specificity. In this scenario, improving the specificity for one substrate reduces the specificity for another substrate, thus leading to metabolic specialization. While specificity conflicts are intuitive and provide an explanation for why most enzymes have only a few substrates, there is little experimental support for these conflicts. For

example, artificial selection was used to select for improved utilization of alternative substrates for three different enzymes in *E. coli*[21]. After random mutagenesis and selection, improved utilization of the alternative substrates did not result in reduced utilization of the primary substrates, suggesting that specificity conflicts were not important for these enzymes[21].

3.4 The consequences of biochemical conflicts

Even though the biochemical conflicts that exist between different metabolic processes are often unknown, knowledge about their consequences can provide important insights into the evolution of metabolic specialization. The consequence of a biochemical conflict is often antagonistic pleiotropy, which describes an outcome where a single genetic change has beneficial effects on some processes but correlated and detrimental effects on others[22]. For example, if competitive resource conflicts exist between two processes, then a genetic change that increases the synthesis of one enzyme must simultaneously decrease the synthesis of other enzymes. These effects can be described graphically using constraint functions (Fig. 1). A constraint function is a type of trade-off function that describes how the activity levels of different metabolic processes are connected and influenced by biochemical conflicts. Examples of constraint functions for two processes are shown in Figure 1. In these examples, the activity levels are plotted on independent axes for every possible phenotype. A line is then drawn that connects the maximal activities of all possible phenotypes. This line does not define all phenotypes that could be expressed by a single genotype. Instead, it delimits the set of all possible phenotypes that could evolve after long-term selection in any particular direction, and thus encompasses a wide range of genotypes.

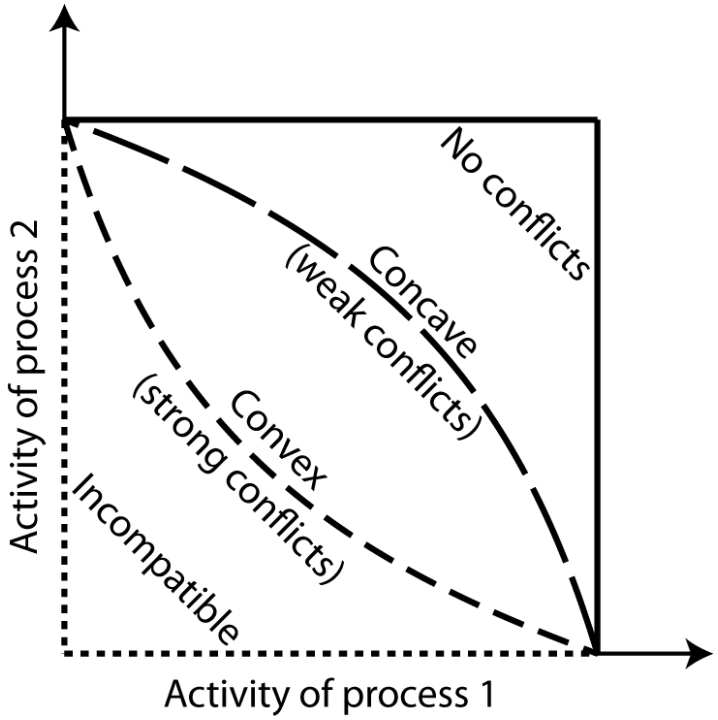

Figure 1 Constraint functions. The shape of a constraint function, which is a line that connects the maximal activities of all possible phenotypes, describes the consequences of biochemical conflicts on the activity levels of two different metabolic processes. A function consisting of vertical and horizontal lines with positive intercepts describes processes that are not in conflict with each other (solid lines). Note that the maximal activities of both processes are independent of each other. Alternatively, a function consisting of vertical and horizontal lines with zero intercepts describes processes that are in complete conflict with each other and cannot occur within the same cell type (short dashed lines). Lines between these two limiting cases describe processes that are in conflict with each other but can

still be performed by the same cell type. Concave functions indicate weak conflicts because the sum of activities when performing both processes is always greater than the maximum activity when performing only one (long dashed line). Convex functions indicate strong conflicts because the sum of activities when performing both processes is always less than when performing only one (medium dashed line).

What use are constraint functions for understanding the evolution of metabolic specialization? If the shape of the constraint function is known for a set of metabolic processes, then simple evolutionary models can be used to predict whether specialization is likely to evolve[23,24] (Fig. 2). For the models discussed in Figure 2, metabolic generalists evolve for concave constraint functions (weak conflicts between processes) while metabolic specialists evolve for convex constraint functions (strong conflicts between processes). For the latter case, the evolution of metabolic specialization occurs in two phases, under the assumption that mutations have small pleiotropic effects. A generalist cell type first evolves that performs both processes. Selection then turns disruptive and the population splits into two co-existing cell types, where each type specializes at only one of the processes[23].

The mathematical models discussed above can thus be used to predict whether different metabolic processes are likely to be performed by the same cell type or by different cell types. These predictions are general in that they are independent of the kinetic parameters of the model[23], and are therefore applicable to any pair of processes. In the simple scenario depicted in Fig. 2, the outcome of the model corresponds to what one might expect based on simple considerations: two processes will segregate into different cell types if they are in biochemical conflict. However, if more than two processes are involved[25] or if ecological or genetic aspects are considered[24], then verbal arguments are insufficient, and mathematical modeling offers a rigorous and objective way to predict evolutionary outcomes.

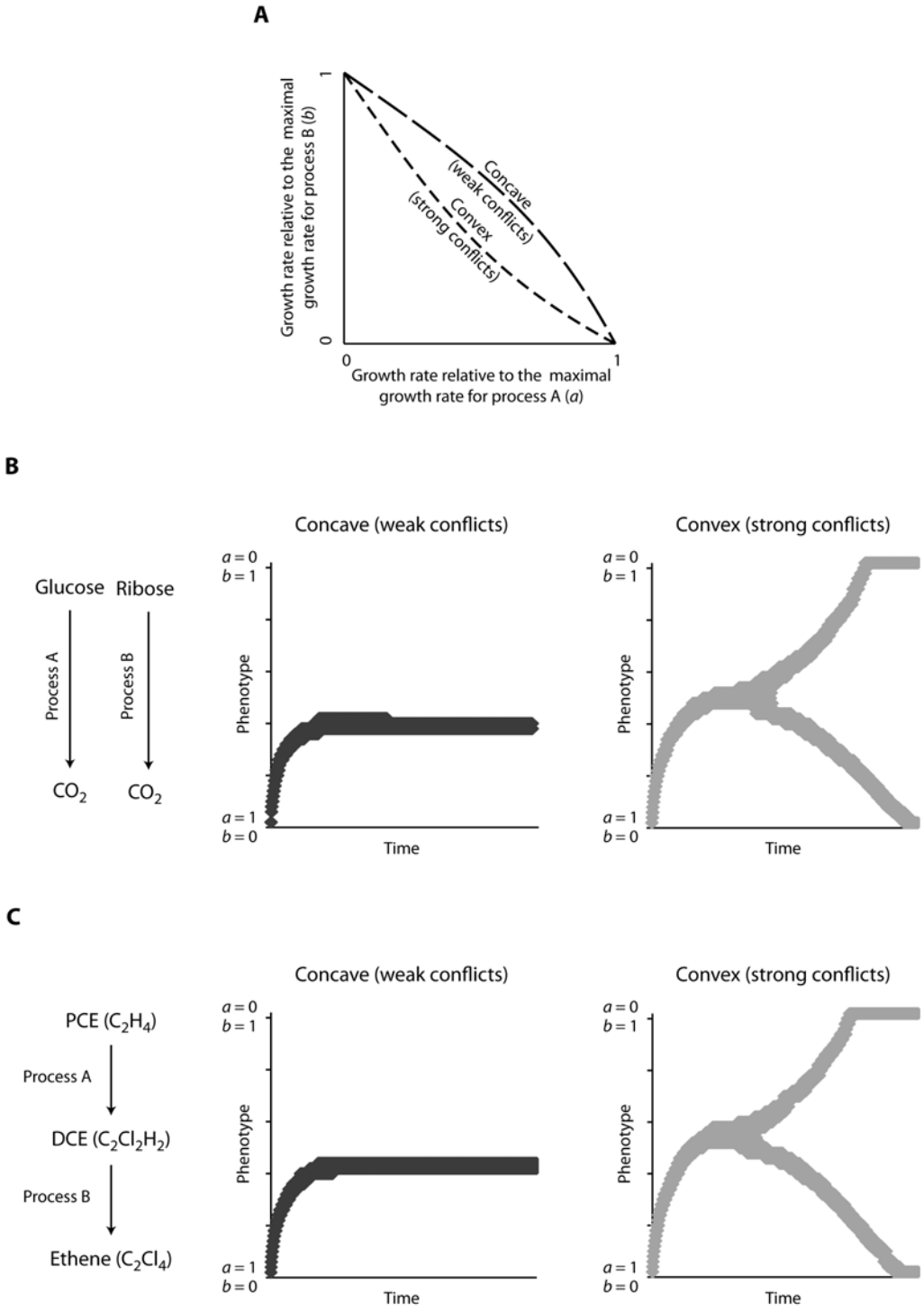

Figure 2 Predictions of metabolic specialization from constraint functions. The shape of a constraint function can be used to predict whether metabolic specialization is likely to occur. Consider the concave and convex functions shown in Panel A. We used the modeling approach described by Doebeli (2002) to predict the evolutionary fate of the glucose and ribose metabolic pathways (Panel B). The model predicts that the same cell type performs glucose and ribose metabolism if the function is concave, but that these pathways segregate into different cell types over evolutionary time if the function is convex (Panel B). This result is not unique to parallel pathways and also occurs within a single pathway (Panel C). Using the reduction of tetrachloroethene (PCE) to ethene as an example, our model predicts that the same cell type performs the complete pathway if the function is concave, but segregates into different cell types over evolutionary time if the function is convex (Panel C). For panels B and C, phenotypes are shown that are present in the population at frequencies greater than 5%.

3.5 Generating hypotheses about biochemical conflicts

We have demonstrated that qualitative information about the consequences of biochemical conflicts (*e.g.* the shapes of constraint functions) enable specific predictions about whether different metabolic processes are likely to be performed by the same cell type or different cell types (Fig. 2). This raises the question about how such constraints can be measured. In the following section, we discuss two main approaches for obtaining such information.

3.5.1 Genome comparisons across organisms

A first approach that generates hypotheses about possible biochemical conflicts is to analyze the distributions of metabolic processes across microbial genomes. The complete sequences of several thousand microbial genomes are currently available and automated gene annotation[26,27] enables the rapid assessment of co-occurrence patterns of different processes across different genomes. One could thus identify combinations of processes that are more often found in the same cell type or in different cell types. The latter are candidates for processes that are in biochemical conflict.

One main advantage of this approach is that it considers information from a large number of species and strains, of which an increasing number are not experimental model systems and cannot be grown in the laboratory. It thus allows rapid and comprehensive assessment of co-occurrence patterns of a large number of metabolic processes. Although promising, this approach has several major caveats. First, the approach assumes that automated genome annotation is sufficient to predict the complete set of metabolic pathways of microorganisms. Although this is increasingly possible for model microorganisms, this remains challenging for non-model microorganisms that contain unusual and poorly characterized enzymes and pathways. Second, this approach does not consider gene expression. Cells could maintain incompatible pathways by expressing them at different times or in different environments. Finally, this approach does not consider the environment in which microorganisms live. The absence of co-occurrence of two processes might result from the absence of their substrates in the same environment rather than a biochemical conflict. The influence of the latter two confounding factors could be lessened by analyzing groups of microorganisms that live in similar environments. For example, one could analyze metagenome information collected from a single environmental sample, provided that genes within the metagenome can be accurately assigned to different cell types[28].

3.5.2 Experimental evolution

A second and more direct approach to measure biochemical conflicts is through experimental evolution in the laboratory. If one is interested in measuring the constraint function between two metabolic processes, replicated populations can be selected to maximize the first process in isolation, the second process in isolation, or both processes simultaneously. This would provide a measure of multiple points on the constraint function (Fig. 1), which could then be used to estimate its shape.

Experimental evolution has, to the best of our knowledge, not been systematically used to investigate biochemical conflicts. Yet, we believe this method has potential. First, it offers experimental control. Confounding factors can be excluded by comparing different genotypes that have evolved under well-controlled conditions[6,29]. Experiments can also be conducted such that the only target of selection is the rate at which a strain grows on one particular substrate or combination of substrates[30]. Second, whole-genome resequencing and metabolic analyses can reveal detailed insights about the genetic changes that occurred over the course of experimental evolution[31,32], and can thus help generate hypotheses about the molecular basis of biochemical

conflicts. While the timescale of laboratory evolution experiments does not extend to the timescales of evolutionary processes in natural environments, such experiments can nevertheless give insights into the initial phase of metabolic specialization. This initial phase can determine further evolutionary processes[33–35], and studying this phase experimentally might thus provide information about how biochemical conflicts promote metabolic specialization.

3.6 Challenges with measuring biochemical conflicts

3.6.1 Conflicts likely affect many metabolic processes

Our discussion of biochemical conflicts thus far has focused on constraining relationships between two metabolic processes (Figures 1 and 2). In reality, constraining relationships likely involve more than two processes[36] (Fig. 3). To illustrate this, consider again one of the examples discussed above. We argued that RNA polymerase could be a limiting intracellular resource for which different sigma factors compete, and that this might lead to a conflict between stress response and metabolic versatility[13]. One obvious resolution to this conflict is to produce more RNA polymerase. However, the production of RNA polymerase, and gene expression in general, is metabolically costly[8], and this resolution might therefore have detrimental effects on other processes. A more realistic model would include a large number of metabolic processes and other cellular traits that are connected through complex interactions, both suppressive and facilitative.

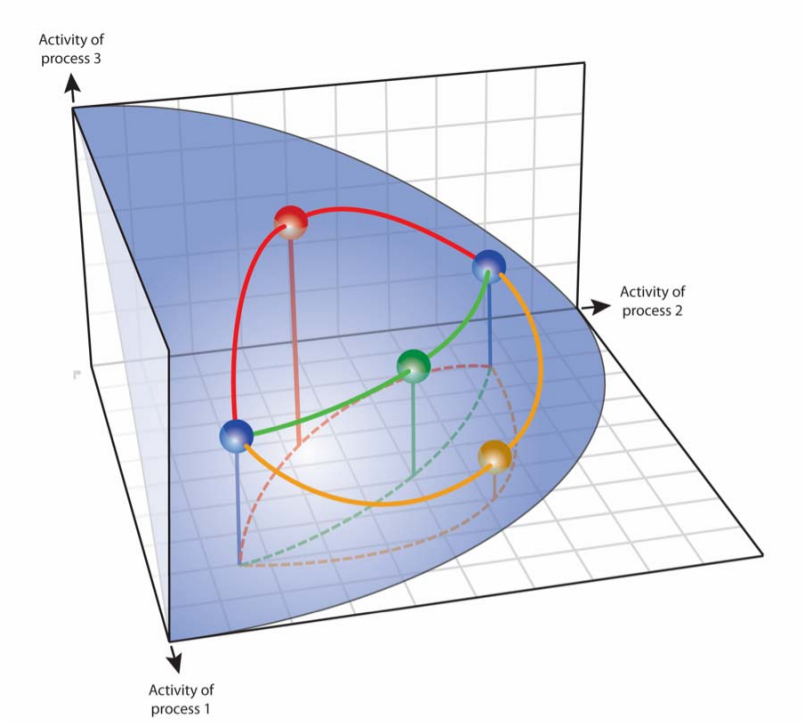

Figure 3 Constraints between several metabolic processes. Constraining relations are likely to involve more than two metabolic processes. For example, consider a situation where three processes are connected in a constraining relationship, but where an experimenter only measures two of them (process 1 and process 2); the experimenter thus only observes the projections of the constraint functions in the horizontal plane (dashed lines). If the activity of process 3 is kept constant during the experiment, the experimenter observes a concave constrain function between process 1 and process 2 (green dashed line). Changes in process 3 during the experiment can lead to the observation of qualitatively different constraint functions (red dashed line). Also, simultaneous improvements of process 1 and process 2 beyond their constraint function are possible at the cost of process 3 (orange).

One consequence of the involvement of multiple metabolic processes in constraining relationships is that constraints between two metabolic processes are not absolute; simultaneous improvements in both are possible at the cost of others (Fig. 3). The combinations of processes that are observed in a particular cell type represent evolutionary compromises across many different selection pressures. If the environment changes and the strength of selection on some processes increases, then one would expect that it is possible to simultaneously improve these at the cost of other processes that are subject to weaker selection.

Another consequence of the involvement of multiple metabolic processes is that complex interactions might generally promote metabolic specialization. A recent theoretical study showed that the probability of diversification increases as constraining relationships involve more traits[25]. While complex interactions between different metabolic processes are more difficult to experimentally measure, they might generally promote the diversification processes depicted in Fig. 2.

3.6.2 Dependence on the environmental conditions

A second challenge with measuring constraining relationships is their dependence on environmental conditions. Constraints are ultimately based on resource conflicts and interactions between different metabolic processes; the environment will affect both of these aspects, and is thus expected to influence constraining relationships both quantitatively and qualitatively. Given the wide range of natural environments, how can we make progress in determining constraining relationships and understanding how they promote metabolic specialization? In our opinion, the main goal is to establish basic principles about how different environmental factors affect biochemical conflicts and influence the main constraining relationships between different processes.

The emerging concept is thus a dynamic view on biochemical conflicts between metabolic processes, on how these conflicts are influenced by environmental factors, and on how they promote the evolution of metabolic specialization and shape the assembly of microbial communities. Each process in a cell is connected to a large number of other processes, and many of these connections will depend on the environmental conditions. Mutations that increase the activity of one process are likely to affect other processes. There might be combinations of mutations that would improve single processes without substantial impairments of other processes, but these genotypes might take a long time to evolve, or they might not be easily accessible by consecutive mutational steps that are all individually beneficial[37]. Such a situation can promote the emergence of mutants that specialize at metabolizing certain substrates. Genetic and ecological interactions might then lead to the consolidation of these differences and the evolution of specialized cell types that consume only subsets of the available substrates within their environment.

3.7 The foundation for synthetic ecology?

What is the broader importance of understanding the biochemical causes of metabolic specialization? A deeper understanding of these causes could serve as a foundation for establishing a field of synthetic ecology[38]In our view, the objective of synthetic ecology would be to elucidate basic design principles that enable the rational engineering of the assembly of microbial communities to perform desired biotransformations. For example, consider a biological process where a microbial population is used to transform a substrate into an intermediate and then into a value-added end product, such as a pharmaceutical or bioenergy source. One strategy would be

to engineer a single cell type that catalyzes the complete pathway[39]. An alternative strategy would be to engineer a community of specialized cell types, where one type transforms the substrate into the intermediate and another type then transforms the intermediate into the desired end product[40]. We currently lack basic design principles that predict which strategy maximizes the performance of such a process. A better understanding of the causes of metabolic specialization will likely help elucidate such engineering design principles and contribute towards establishing a discipline of synthetic ecology.

3.8 Acknowledgements

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4 Successive range expansions promote local population diversity in expanding populations of microorganisms

Felix Goldschmidt, Roland Regös, David R. Johnson

4.1 Abstract

During range expansions, genetic drift at the expansion front can segretate populations and lead to areas of low local population diversity. Range expansions have been studied mostly as primary expansions, where pioneering populations expand into previously uncolonized areas. However, the primary populations are typically succeded by secondary populations that have to establish in a landscape that is already inhabited. The effect of succession on local population diversity in the secondary populaitions are unknown. Here we use an experimental microbial model system to show that in contrast to primary range expansion, successive range expansion promotes local population diversity in the secondary population. We found that because of spatial constraints that are imposed by the presence of the primary population, the secondary population forms fractal, dendritic structures. This splits the advancing secondary population into many small sub-populations, and leads to high intermixing between the primary and the secondary population in the expansion zone. Furthermore, using mathematical simulations, we found that mutations are more likely to persist in the secondary, dendritic expansion. Our results thus show that successive range expansion can increase local population diversity between the primary and secondary populations on the short term, and can increase genetic diversity by accumulation of mutations in the secondary population during extended periods of expansion.

4.2 Introduction

One of the central objectives of spatial ecology is to explain patterns of local population diversity[1]. Local population diversity refers to the intermixing of two or more populations over a defined area. One factor that can profoundly affect local population diversity is the expansion of theacross a landscape (*i.e.*, range expansion[2]). Model microbial communities have been used to investigate the impact of range expansion on local population diversity. For example, in a seminal study by Hallatschek et al[3], the authors assembled two populations of *Escherichia coli* that expressed different fluorescent proteins but were otherwise genetically identical. They therefore interacted presumably only via competition for unoccupied space. The authors found that neutral drift caused the populations to segregate into sectors (*i.e.*, continuous spatial areas, each of which contains only one population) and to form inter-population boundaries parallel to the axis of expansion (Figure 4.1A). The consequence was the reduction of intermixing and thus local population diversity, as the population expanded.

An important feature of these range expansion studies is that the populations expanded simultaneously into uncolonized space; nutrients and space were thus freely available at the expansion edge. While there are some situations in nature where populations may expand into uncolonized space, e.g. following the retraction of glaciers after an ice-age[4], these situations are likely not the typical case. Instead, some populations likely expand first (referred to as primary colonizers) while other populations follow afterwards (referred to as secondary colonizers). This process is known as primary and secondary succession and is pervasive in nature[5]. For example, grasses expand before trees after forest fires. A key point is that because primary colonizers invade first, they change the environment. The secondary colonizers must therefore expand and establish within a landscape that is already modified by the primary colonizers, which might constrain and affect their potential expansion[5].

While successive range expansions have primarily been studied for plant expansions, such as the forest fire example described above, they likely occur for all organisms. For example, there is evidence for two major expansions of humans in the Paleolithic. The first expansion presumably consisted of hunters and gatherers that moved out of Africa. The second expansion consisted of farmers that expanded from the Middle East into areas that were already colonized by the hunters and gatherers[6,7]. Succession may also occur among the smallest of organisms: microbes. Bacterial biofilms on teeth are often periodically removed via brushing. Thereafter, primary colonizers attach to the surfaces of cleaned teeth via specialized surface proteins and begin colonization. Secondary colonizers then attach to those biofilms and invade the primary colonizers in a process called coaggregation[8,9].

While successive expansions are likely pervasive in natural microbial ecosystems, they have not been widely studied experimentally. Conceptually, succession can be seen as two temporally-segregated expansions of microorganisms (Figure 4.1B), which implies a complete segregation of the two populations in the direction of the expansion. The consequence is a reduction of intermixing of the two populations, and thus a reduction of local population diversity.

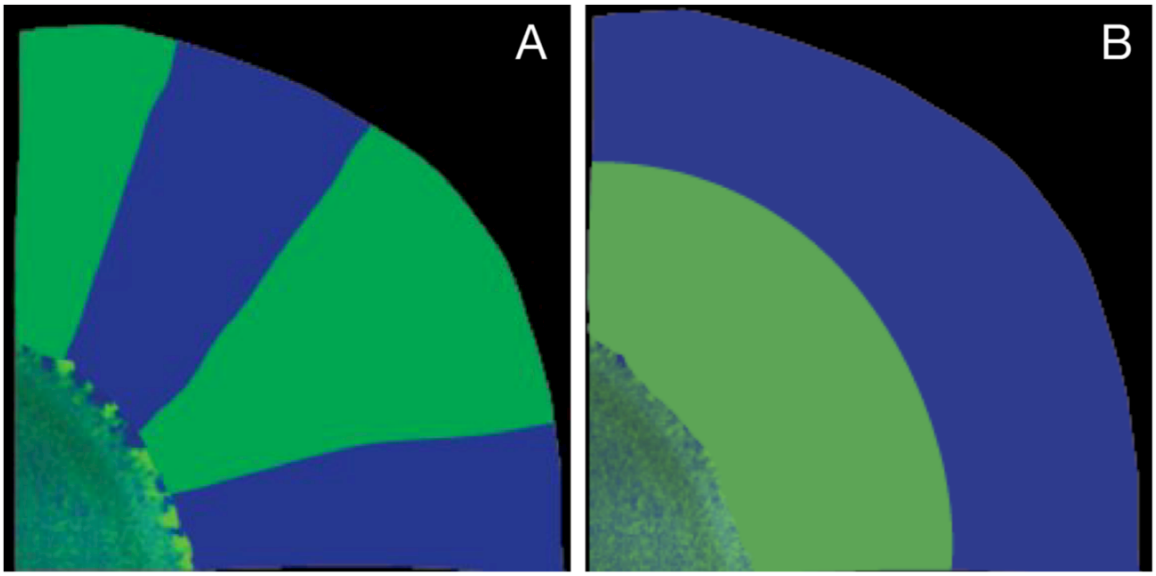

Figure 4.1. Schematic illustration of simultaneous (A) and successive (B) range expansion. In panel A the green and blue colored areas represent two populations that have the same growth characteristics. In the inoculation zone (lower left) the two populations are mixed. During the expansion genetic drift at the expansion front causes the two populations to demix into sectors, which reduces local populaion diversity. Panel B represents a successive range expansion of a primar colonizer (blue) and a secondary colonizer (green). The successive expansion could cause a demixing of the primary from the secondary colonizer in the direction of expansion, which would lead to two successive waves and an even further reduction in local population diversity.

The objective of this study was to quantitatively assess how succession affects local population diversity between primary and secondary colonizers, where we expect successive expansion to reduce intermixing between populations and, consequently, reduce local population diversity. To address this objective, we constructed an experimental system that allowed us to impose an interaction that should promote successive expansion of two populations of the bacterium *Pseudomonas stutzeri*. *P. stutzeri* is a facultative anaerobe that can use nitrate as an electron acceptor in the absence of oxygen in a process called denitrification[10]. *P. stutzeri* can perform the complete denitrification pathway (referred to as the completely degrading strain), in which nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide, and finally to dinitrogen gas[11].

We previously deleted specific genes within the *P. stutzeri* genome to construct two strains of *P. stutzeri* that cross-feed the metabolic intermediate nitrite[12]. One strain partially consumes nitrate to nitrite (referred to as the producer) and the other strain consumes nitrite to dinitrogen gas (referred to as the consumer). Because the consumer cannot grow before the producer when nitrate is provided as the growth-limiting substrate, we expected cross-feeding populations to undergo successive range expansion when grown together in the absence of oxygen, where the producer expands first and the consumer follows afterwards. Each population carries a gene encoding for a different fluorescent protein, thus allowing us to quantify the resulting spatial patterns and the magnitude of intermixing between populations using image analysis.

We then compared the results for co-cultures of the producer and consumer (i.e., two strains that cross-feed nitrite and presumably expand sequentially) with the results for co-cultures of two completely degrading populations (i.e., two ancestral strains that completely reduce nitrate to dinitrogen gas, have the same growth rates and therefore expand simultaneously). In addition, we developed a two-dimensional reaction-diffusion model that that allowed us to derive predictions about the long-term effects of successive range expansion on genetic diversity within each population.

4.3 Results

4.3.1 Primary expansion determines secondary expansion

We grew co-cultures of two complete degraders (simultaneous expansion) or co-cultures of the producer and the consumer (successive expansion) on anaerobic agar plates supplied with nitrate as the growth-limiting substrate. The complete degraders strains formed sectors with saw-tooth like boundaries between the two populations. These boundaries were oriented parallel to the axis of expansion, similar to the patterns described by Hallatschek et al.[3] for the expansion of metabolically identical populations of *E. coli* (see Figure 4.2A). In contrast, the producer and consumer expanded sucessively; the producer expanded first while the consumer followed (see figure 2B). The producer completely covered the expansion area and had a relatively continuous expansion front similar to that observed for the complete degraders. In contrast, the consumers unexpectedly formed branched dendrites that extended into the space occupied by the producers (Figure 4.2B).

The dendrites originated at a single point from the inoculation zone (referred to as stems) and branched into dendrites during expansion (referred to as tips). The tips were directly connected to a single stem and had clear boundaries with the producer. The dendrites typically did not touch each other, but were instead separated by a thin layer of the producer. While the dendrites generally did not touch each other, the number of dendrites nevertheless decreased with the direction of expansion. This was not a consequence of direct collisions between different dendrites, which generally did not occur. In contrast, the number of sectors between different completely degrading strains also decreased with the direction of expansion, but this only occurred after direct collisions between different sectors .

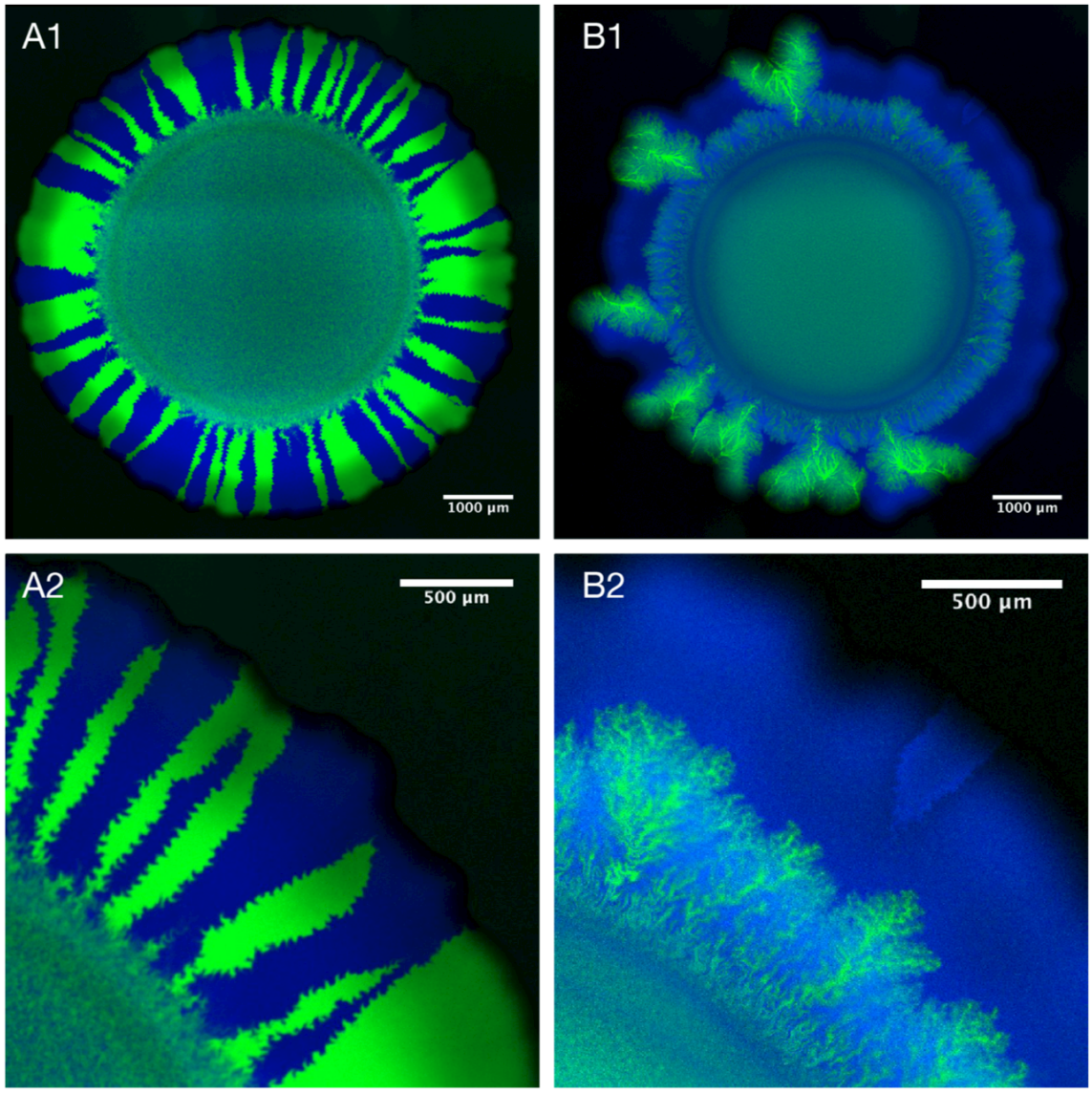

Figure 4.2 Expansion of co-cultures of complete degraders or co-cultures of producers and consumers. A1+2) In co-cultures of complete degraders sectors with boundaries lying parallel to the axis of expansion emerged. **B1+2)** In co-cultures of producers and consumers two successive expansions emerged, where the producers (blue) formed a continuos expansion front while the consumers (green) formed dendrites.

4.3.2 The consumer forms fractal patterns during expansion

The dendrites formed by the consumer qualitatively resemble the fractal branching-type patterns often generated by the diffusion-limited aggregation (DLA) process. DLA was first described by Witten and Sanders and is a computer model to generate dendritic patterns that typically have a fractal dimension of 1.71[13,14]. The fractal dimension of a pattern describes how the space filling properties change with the scale of observation and is used to quantify fractal patterns. DLA has since received increasing attention because similar patterns have been repeatedly observed in diverse natural systems, including a wide variety of geophysical processes ranging from small mineral deposits to large scale patterns of water injected into the ground for oil recovery (i.e., multiphase flow)[15]. To further investigate the structure of the secondary, dendritic expansion, we used image analysis to quantify the fractal dimension of the dendrites. We found that the consumer dendrites are statistically consistent with the fractal dimension of the DLA processes (mean D=1.73, standard deviation (SD)=0.03, N=8) (SI Figure 3). We acknowledge here,

however, that the fractal dimension alone is insufficient to determine the mechanism that generates the dendrites[16]. The mechanism of dendrite formation is therefore the focus of the next section of the manuscript.

4.3.3 Causes of dendrite formation

Dendritic DLA-type patterns have been described for the growth of bacteria like *Bacillus subtilis*[17]. The mechanisms causing the formation of dendrites have typically been attributed to diffusion limitation of the growth substrate or spatial constrains, such as agar hardness[18,19]. Our observed similarity of pattern formation during expansion of the consumer with DLA fractals thus suggests that one or both of these two mechanisms could have caused the formation of the observed dendrites. The first is that the expansion of the consumer is constrained by the availability of the growth-limiting cross-fed substrate nitrite while the second is that the expansion of the consumer is spatially constrained and must advance via cell shoving through the producer (i.e. mechanical shoving)[20]. Both mechanisms can produce patterns with the same fractal dimension as DLA and can therefore not be distinguished by simple observation of the pattern[21,22]. We therefore designed experiments to analyze the relative contributions of the two mechanisms to dendrite formation during the expansion of the consumer.

To test whether substrate limitation caused the formation of dendrites, we grew co-cultures of the producer and consumer on agar plates containing exogenous and excess supplies of both nitrate and nitrite, thus reducing the possibility that nitrite limitations caused the dendrite formation during expansion of the consumer. We found that, even when both nitrate and nitrite were provided exogenously, the two populations continued to form two successive wave fronts and the consumer still expanded after the producer. More importantly, even though the consumer had greater availability of its growth-limiting substrate than the producer (note that nitrite was provided exogenously and produced via the activity of the consumer), it nevertheless still produced dendritic patterns during expansion very similar to those observed when only nitrate was provided exogenously (Figure 4.3A).

To further exclude the possibility that the consumer is limited by the availability of nitrite, we grew two differently labeled consumers (i.e. they produced either blue or red fluorescent protein) together on agar plates containing nitrite as the only available electron acceptor. The consumer did not form dendrites when growing in the absence of the producer. Similar to the completely degrading strains, the consumers formed sectors with roughly straight boundaries parallel to the axis of expansion (Figure 4.3B). These results together indicate that substrate availability was not the main growth-limiting factor, and therefore not the main cause of dendrite formation.

This leaves the hypothesis that dendrite formation during expansion of the consumer was mainly caused by spatial constraints imposed by the consumer having to expand into space previously occupied by the producer. If this were a general mechanism, one would expect that different strains that grow into previously occupied space produce similar patterning. To test this hypothesis, we devised an experiment, where the producer had to expand into space occupied by the consumer (note that this is the reverse of the ordering imposed previously). To accomplish this, we inoculated the producer at low frequencies (below 100 cells per colony) on plates containing exogenous supplies of both nitrate and nitrite. The producer was thus completely surrounded by the consumer and had to grow through the consumer to expand. We found that the producer also formed dendrites when expanding into space occupied by the consumer (Figure 4.3C), indicating that spatial constraints were indeed the primary process causing dendrite formation in the secondary expansion.

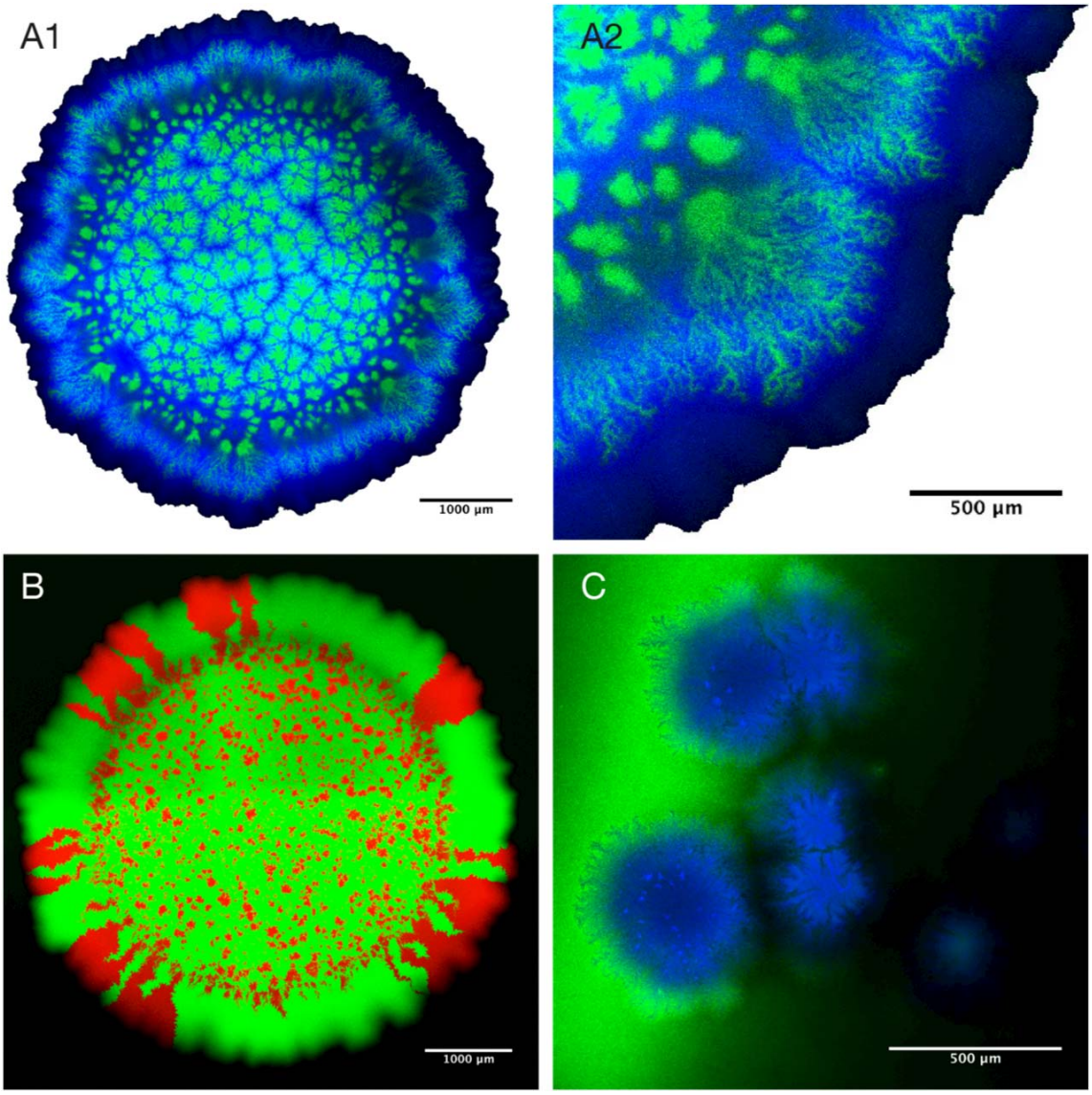

Figure 4.3 Microscopy images of range expansions with different growth limiting substrates that were devised to asses the causes of dendrite formation. A1+2) Co-cultures of the producing (blue) and consuming (red) strains grown together with exogenous supplies of both nitrate and nitrite. The area outside of the colony was colored white to visualize the expansion edge (**A2** magnification). The strains still succeeded each other and the consumer formed dendrites even though nitrite was added exogenously. **B)** Co-cultures of the consumer grown alone (in green and red) on plates containing nitrite, which do not form dendrites. **C)** The producer (blue) expanding into space occupied by the consumer (green) on plates containing exogenous supplies of both nitrate and nitrite, where the producer formed dendrites.

4.3.4 Intermixing increases in the secondary expansion

The dendritic nature of the secondary expansion causes increased local mixing of the two populations. We defined a intermixing index similar to Pielou et al.[23] and Momeni et al.[24] as the number of transitions between the consuming and producing populations divided by the expected value of transitions from a random distribution at a certain circumference (see Methods/SI). An intermixing index higher than one indicates more mixing than a random distribution while an intermixing index lower than one indicates more segregation than a random distribution. Note that in natural systems the latter is the predominant case[23]. Intermixing was

relatively constant in the inoculation zone at 0.161, but increased during expansion until it reached 0.174 and stayed stable until the edge of the expansion front of the consumer (Figure 4.4). Overall intermixing increased by 8.35% during expansion, which shows that the intermixing in the dendritic, secondary expansion was even higher than in the inoculation zone.

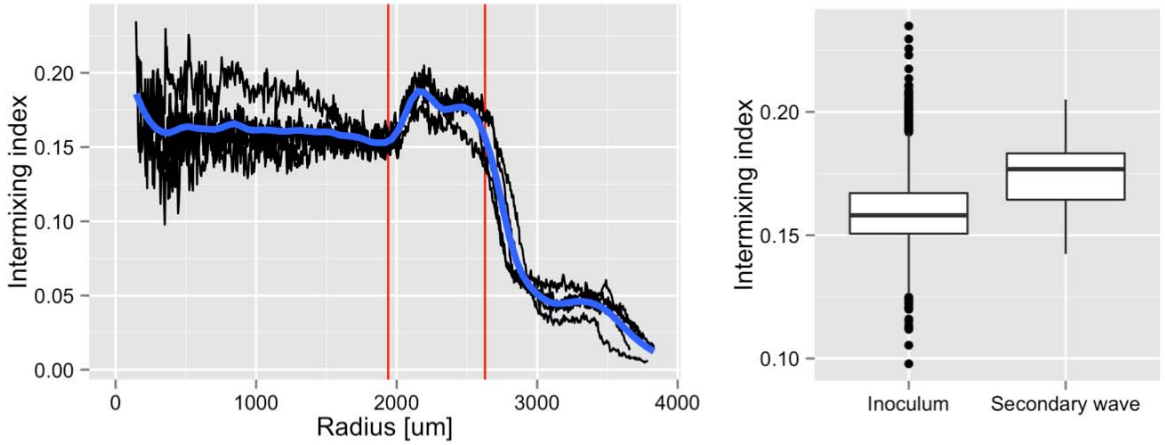

Figure 4.4 Measurement of intermixing from the center of colonies towards the edge of expansion. A value of 1 indicates the same diversity as a random distribution. **Left)** Intermixing index at radial position, the blue line is a loess smoother, the red lines indicate the beginning and end of the secondary expansion by the consumer (see supplementary methods). Intermixing is relatively constant in the inoculum, then increases and peaks in the expansion zone. **Right)** Intermixing in the secondary expansion is significantly higher than in the inoculum (Mann-Whitney test, p< 2.2e-16).

4.3.5 Evolutionary consequences of dendritic expansion

Besides the immediate effects on local-population diversity, the fundamentally different shape of the dendritic secondary expansion raises the question of whether the different shapes of the expansions cause differences in the long-term evolution of the primary and secondary strains. The idea here is that the probability of fixation of a mutant allele depends on the local effective (or active) population size, which in the case of range expansion is at least a sector[2]. During the primary expansion of the producing strain, a mutant allele competes with the entire active population of the sector. Furthermore, the increase in sector width during radial expansion causes the effective population size to increase over time. Therefore the probability of local fixation of a mutant allele decreases during the expansion. In contrast, the dendrite formation of the secondary expansion reduces local effective population size (down to a few cells) at the tips of the branches. The very small local populations at the dendritic tips are much more likely to be overtaken by a mutation. Also, we found that the number of branches increases with diameter during the expansion, so that the active population in a branch remains mainly constant, even during radial expansion. We therefore expect that mutant alleles are more likely to become fixed in the dendritic secondary expansion than in the non-dendritic primary expansion.

We developed a mathematical model that allowed us to study the dynamics of successive range expansions and the ramification of the secondary expansion on evolution. Following earlier approaches of modeling dendritic microbial expansion on agar plates[19,25], we chose a reaction-diffusion model with non-linear diffusion terms for the populations. The model consists of lattices for the two populations (producer and consumer) and the two corresponding growth-limiting substrates (nitrate and nitrite). As in the experiments, the strains were placed in a circular area at the beginning of the experiment and nitrate was provided as the growth-limiting substrate.

The model could qualitatively reproduce the dynamics of the two expansions and the formation of dendrites as was observed experimentally (Figure 4.6). The two populations underwent successive range expansion and the secondary expansion of the consumer formed dendrites with a fractal dimension of 1.798 (SD=0.002), which is higher than in the experimental data. If two completely consumers were inoculated together, they rapidly formed sectors as observed experimentally (Figure 4.6, SI movies). Note that our goal was not to exactly reproduce the patterns found in the experiments, but rather to develop a model that enabled studying how mutations establish in primary or dendritic secondary expansions.

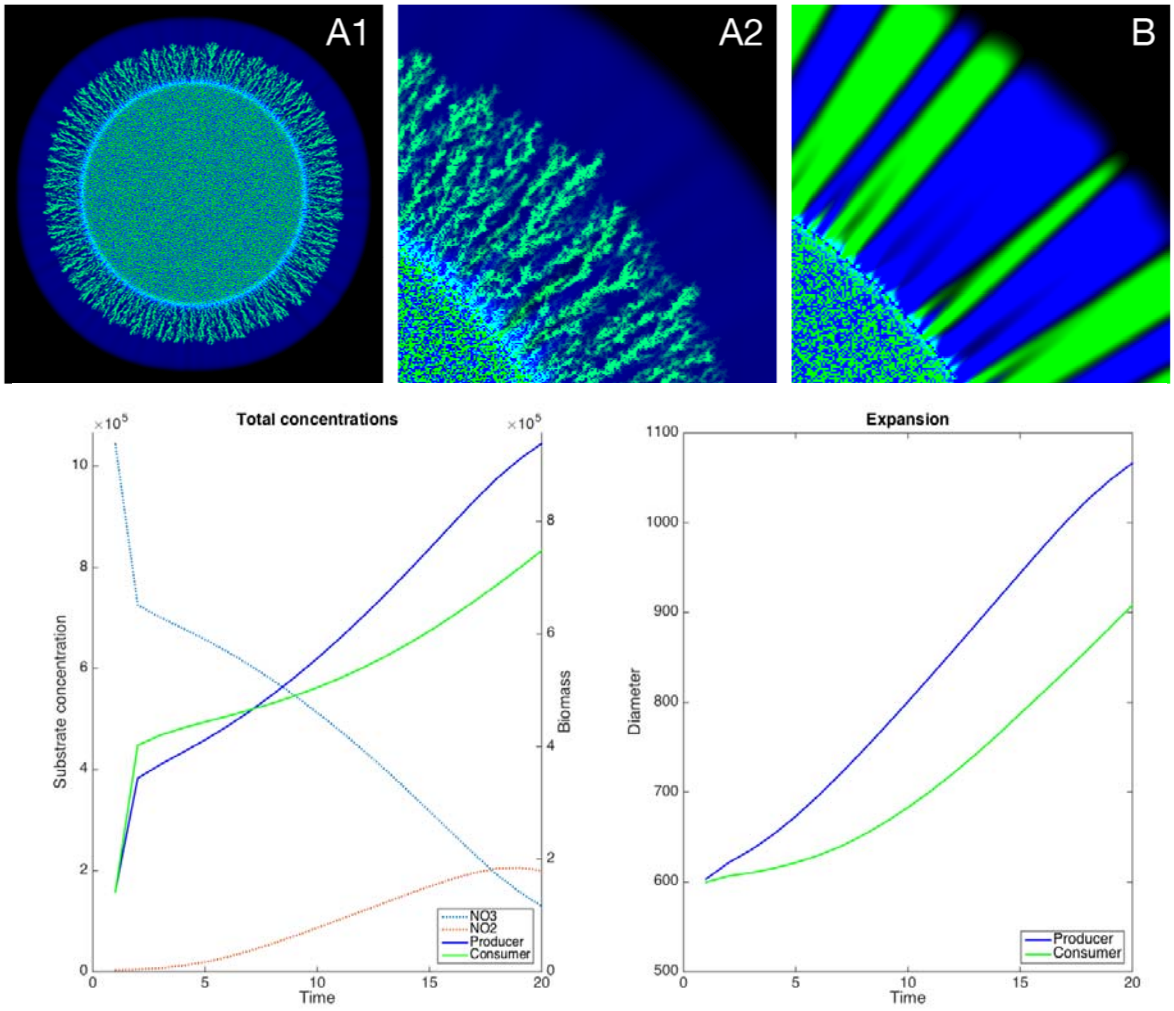

Figure 4.5 Modeling results. A1+2) The secondary expansion of the consumer produced fractal dendrites. **B)** If two producing or two completely degrading strains were inoculated together, they rapidly segregated into sectors. **Below)** Plot of the growth dynamics during the expansion. The two populations first grew in the inoculation zon, until the local substrate concentrations became sufficiently low. The populations then expanded and reached a steady state between expansion and local growth, which lead to a relatively constant population density (or height of the bacterial biofilm) in the expansion zone. The consumer initially expanded slower than the producer due to the initially low concentration of nitrite.

We introduced mutations into our mathematical model to compare the possible evolutionary differences between primary and secondary, dendritic expansions. At every time step the entire population of an active lattice site (i.e. that had substrate available for growth) could mutate with a certain probability (μ) from the ancestral population (A) to a new mutant population (B). The

new population then could grow and expand as the ancestral population. We introduced mutations in both the primary and the dendritic secondary expansions. We found that in our model mutant populations got extinct rapidly, if the growth rates were exactly the same as the ancestral population.

We thus introduced higher growth rates for the mutants. As the growth rate increased, we found that small patches of mutants first emerged and, if the growth rate was sufficiently high, the patches could increase in size. The mutants in the primary expansion typically formed patches that did not persist for longer times until they had a 50% higher growth rate than the ancestor, at which they rapidly displaced the ancestral population. The mutants in the dendritic secondary expansion, however, could establish dendrites that did not disappear throughout the simulations already at 20% higher growth rates. On the other hand it was more difficult for mutations within dendrites to displace the resident population, because narrow dendrites of the ancestral population could survive, even when they had a large growth disadvantage (SI Movies).

To quantify the difference between dendritic and non-dendritic expansion, we measured the fraction of mutants in the actively growing populations at the end of the simulations for different growth rates. The abovementioned dynamics is reflected in the shape of the Gompertz curves that were fitted to the model data (Figure 4.6): the lag-parameter (indicated as vertical lines) and the maximum slope of the secondary population were significantly lower than in the primary population (F-test, both $p<2.2e-16$), while the maximum proportion was not (F-test, $p= 0.379$). This had the consequence that at the point where the proportion of the mutant in the primary expansion started to increase substantially (i.e. at the end of the "lag-phase" and with a growth advantage of about 40% compared to the ancestor), the proportion of the mutants in the secondary dendritic expansion already accounted for about half of the active population. This thus supported our hypothesis that mutants are less likely to get extinct in the secondary, dendritic expansion than in the primary expansion.

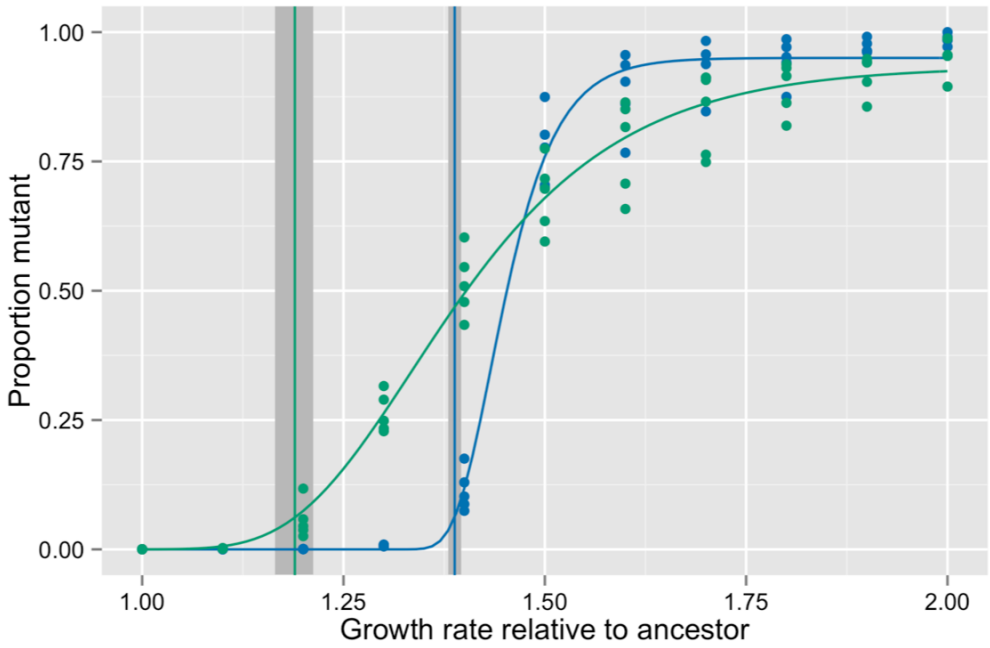

Figure 4.6 Model predictions. Y-axis is the proportion of the mutant in the actively growing part of the population at the end of a simulation. Blue is the primary non-dendritic expansion of the producer, green is the secondary dendritic expansion of the consumer. The curves show the Gompertz-model fits and the vertical lines represent the lag-parameter of these models with 95% confidence intervals in grey.

4.4 Discussion

We have found that, opposite to our initial expectations (Fig. 1), successive range expansion increases local population diversity rather than decreases local population diversity (Fig. 1). The cause for this increase in diversity is the production of dendritic patters by the secondary expansion. Dendrites increase intermixing of different populations, thus creating new local population diversity during expansion. In our case, this resulted in higher pattern diversity than was even observed in the intermixed inoculation zone. The main point here is that dendritic expansion leads to a zone where the two strains are intermixed in contrast to two segregated succeeding wave-fronts with a straight boundary, which would have very low intermixing. Increased intermixing signifies higher spatial heterogeneity, shorter average distances between the two populations and increased interface length. This could affect how the two populations interact with each other, e.g. by exchange of goods such as metabolites[9], and it could facilitate genetic exchange, e.g. via horizontal gene transfer[26,27].

Over longer time-scales, more genetic diversity might establish in the secondary dendritic expansion than in the primary non-dendritic expansion. Our modeling results show that mutations with smaller growth advantages can establish more easily during dendritic expansion. Because mutations that confer small fitness advantages are likely more common[28], we expect that more mutations can accumulate in the secondary dendritic expansion. The branching of the secondary dendritic expansion can therefore mitigate the loss of diversity due to two mechanisms: increased intermixing between the secondary and primary populations and higher maintenance of novel mutations in the secondary population during extended periods of expansion.

The main driver of the branching process is the reduced availability of resources to the secondary population, which must expand into areas already colonized by the primary population. Here we identified space as the main limiting resource when expanding into a previously colonized area. This suggests that the formation of the dendrites by the secondary population could be a general process that potentially affects many populations with limited dispersal capabilities. An obvious example could be tumors that penetrate into the surrounding tissue[29].

The exact shape of the expansion front does not necessarily have to be the same, the important point is that secondary expansion leads to splitting of the expansion front into small local sub-populations that would be affected by the same mechanisms that maintain or increase diversity as mentioned above[30]. For example plants grow usually in clustered groups[31]. A secondary expansion could thus resemble percolation clusters, which also consist of many small localized regions and exhibit fractal properties. But these processes could potentially also be important for our own species. A recent analysis of the native American population structure suggests that there were 3 successive immigration events into America and that there was extensive admixture between the first an the following populations[32].

Spatial constraints can of course also be present in primary expansions. For example, expansion into a river network will fragment the front into a dendritic shape. It has been shown that this process can increase biodiversity[33]. In the case of succession, however, the separation of the populations at the tips is likely not as absolute. Especially in the area behind the expansion front, gene flow between branches might occur over longer time scales[2], either through the primary population, e.g. plasmid exchange between bacteria, or if mobility of parts of the secondary population can be re-established. The effect of succession can therefore be seen as an intermediate case between complete separation of populations and complete overlap of ranges of populations.

4.5 Acknowledgments

We thank Fordyce A. Davidson for valuable insights and suggestions on how to design the non-linear diffusion term of the bacterial populations in the model; Lara Pfister, Selina Derksen-Müller and Anja Bernet for help with the strain construction; and Martin Ackermann and Will Macnair for helpful discussions and coments on early versions of the manuscript.

4.6 Materials and methods

4.6.1 Bacterial strains and growth conditions

For our experiments we used a previously developed experimental microbial system that is described in detail in Lilja et al.[12]. In short the *narG , nirS, and comA* gene were deleted from *P. stutzeri* A1501 using variants of the conditionally replicative plasmid pAW19[34]. In addition *comA*, which is a transporter required for competence[35], was deleted from all strains used in this study to prevent recombination during experimentation. The producer contains a deletion in the *NirS* gene, which encodes for the reduction of nitrite to nitrous oxide, and can only convert nitrate to nitrite[11]. The consumer contains a deletion in the *NarG* genecluter, which encodes the enzymes for the degradation of nitrate to nitrite, and can only convert nitrite to dinitrogen gas[11]. To enable fluorescence microscopy, the strains were tagged with different fluorescent proteins (green- or cyan-fluorescent protein) by site specific insertion of the mini-Tn7-transposon[36].

4.6.2 Expansion of colonies

We performed a modified anaerobic version of the expansion experiment described by Hallatscheck *et al.*[3]. First, overnight cultures of the different strains of *P. stutzeri* were grown separately in LB medium under aerobic conditions.. They were then centrifuged at 4200 rpm/ 25°C for 10 minutes and suspended in 500 µl saline (0.9% w/v NaCl solution). This dense mixture was diluted 1/20 in saline for OD measurements in order to be within the linear range of the spectrophotometer. The cultures were then adjusted to an optical density at 600 nm (OD_{600}) of 2 with saline. The adjusted cultures were mixed according to experimental needs as described in the results section (e.g. the producer and consumer at a ratio of 1:1 [cell number:cell number]) and transferred to the anaerobic glove box. A 2 µl drop of the mixtures was then inoculated onto the middle of anaerobic agar plates (see SI). The drops were allowed to dry for 1h and the plates were incubated at 21°C for up to 4 weeks.

4.6.3 Image acquisition

Images were taken under aerobic conditions with a confocal microscope (Leica TCS SP5 II). Most fluorophores (such as green fluorescent protein (gfp) and its variants) do not show fluorescence under anaerobic conditions, because they require molecular oxygen for their maturation[37]. Therefore, plates were taken out of the glove box and exposed to oxygen for 1h prior to imaging. To observe whole colonies, an array of images covering the whole colony was taken and stitched together in the Leica software (Leica LAS AF).

4.6.4 Quantitative image analyses.

To analyze and quantify the dendrites in the secondary expansions, the color channels of the microscopy images, which contained the consumer, were thresholded in imageJ using a local thresholding method (see SI). The locally thresholded image was then used as input for the Sholl plugin[38] of imageJ to measure the number of intersections between background and information containing parts of the image at each radial distance from 50 µm to the end of the colony using a stepsize of 5 µm.

4.6.5 Fractal dimension

We measured the fractal dimension with the fractal box count tool of imageJ, on the previously thresholded images of the consumer. To only measure the fractal dimension of the dendrites, we

removed the inoculation area and measured the fractal dimension of the expansion zone using box sizes ranging from 2-1024 pixels. The statistical analysis of the resulting data was done in R.

4.6.6 Intermixing index

We defined intermixing similar to the definition of Pielou et al[23] and the intermixing index of Momeni et al.[24]. Briefly, we divided the measured number of intersections at a given radius (N_r) between different populations by the expected number of intersections for a random spatial distribution of two populations ($E(N_r)$) (see SI). Intermixing at a given radius (I_r) was thus defined as:

$$I_r = \frac{N_r}{E(N_r)} = \frac{N_r}{\pi r/2} \tag{1}$$

4.6.7 Modeling

We developed a spatially explicit reaction-diffusion model to simulate successive expansion of two populations that exchange a metabolic intermediate. The model consists of a producer (P), a consumer (C), and two substrates nitrate (N_1) and nitrite (N_2), each of which is modeled on a separate square lattice of size 1024x1024. Initially, only substrate N_1 is present while substrate N_2 is produced by P. At the beginning of the simulations, P and C are randomly inoculated in a circular area in the middle of the grid and can then expand. The equations for the bacteria consist of a diffusion term and a growth term. The diffusion terms of the producer and consume are not constant, but instead depend on the local concentrations of the substrates, the densities of the two strains, and a local anisotropy term Ω. Diffusion of the cells is only possible as long as the respective substrate is present. Moreover, diffusion is facilitated by the own local population densitiy[19] and hindered by the local population density of the other population (note how P and C are swapped in equations 2b and 3b). Ω is a locally varying random number with values of either 0 or 100. The values and distribution of Ω can be used to change the properties of the dendritic pattern. σ is a scaling factor to balance the expansion speed between the two populations. Growth is modeled with a Monod-type term with maximum growth rates ($r_{p,c}$) and substrate specific half-velocity constants ($K_{1,2}$). A more detailed description of the model parameters and initial conditions can be found in the supplementary material.

Population P:

$$\frac{\partial P}{\partial t} = \sigma\nabla\left(D_p\nabla \mathrm{P}\right) + r_p P\frac{N_1}{K_1 + N_1} \tag{2a}$$

$$D_p = \frac{N_1(1+P)}{1+\Omega C} \tag{2b}$$

Population C:

$$\frac{\partial C}{\partial t} = \nabla(D_c\nabla \mathrm{C}) + r_c C\frac{N_2}{K_2 + N_2} \tag{3a}$$

$$D_c = \frac{N_2(1+C)}{1+\Omega P} \tag{3b}$$

The equations of the two substrates consist of a regular diffusion term (with diffusion constants D_{N1} and D_{N2}), a consumption term that is proportional ($\nu_{1,2}$) to the growth of the respective bacterial populations and for N_2 a production term that is equal to the consumption of N_1, reflecting the 1:1 stoichiometric relationship of the production of nitrite from nitrate.

Substrate N1:
$$\frac{\partial N_1}{\partial t} = D_{N1}\nabla^2 N_1 - \nu_1 r_p P \frac{N_1}{K_1 + N_1} \tag{4}$$

Substrate N2:
$$\frac{\partial N_2}{\partial t} = D_{N2}\nabla^2 N_2 + \nu_1 r_p P \frac{N_1}{K_1 + N_1} - \nu_2 r_c C \frac{N_2}{K_2 + N_2} \tag{5}$$

Generic parameters were used for the simulations (see SI Table 1).

4.7 Supplementary material

4.7.1 Model

The two populations move with a non-linear diffusion term that depends on the concentration of the corresponding limiting nutrient, the concentration of the own population and the concentration of the other population and a term for local anisotropy. The rationale for these terms are the following: The dependence on the substrate ensures that bacteria only move at the growing edge of the colony, where substrate is available[19]. The dependence on the bacterial concentrations has been shown stabilize dendritic growth[25]. Each population is slowed down by the presence of the other population and moves faster when the density of the own population is high (i.e. they can push harder together). This form was chosen to reflect the experimental findings suggesting that spatial constraints and mechanical shoving are the main drivers of ramification of the secondary wave. The anisotropic term represents the local alignment of packs of cells in bacterial biofilms[39], but anisotropy can be found in other systems as well[40]. It is linked to the presence of the other population, to ensure that it only matters when a population is growing into colonized areas.

To efficiently model the two populations and two substrates on large spatial grids, a part of the code was parallelized using GPU accelaration as described in van de Koppel[41]. The model is written in CUDA, which allows the execution of parts of the code on the graphics processor.

Table 1 Model parameters

Parameter	Ancestor	Mutants
$r_{p,c}$	1	1-2
σ	0.5	0.5
$K_{1,2}$	1	1
$D_{N1,N2}$	1	1
$\nu_{1,2}$	1	1

4.7.2 Anaerobic agar plates

The lysogeny broth (LB) agar plates were prepared with the same protocol as the anaerobic media in Lilja et al.[12]. 500 ml of liquid LB medium were prepared and supplemented with $NaNO_3$ to a final concentration of 1mM if needed. The pH of these solutions was adjusted to 7.5 with 1M HCl. The LB medium was then boiled for 10 minutes and cooled down under a constant flow of nitrogen gas to release the oxygen. The medium was then transferred to Schott bottles with polybutylene terephthalate (PBT) lids containing polytetrafluorethene (PTFE) coated silicon seals, mixed with agar to a final concentration of 1.5% and autoclaved. The liquid agar was then immediately transferred to an anaerobic glove box (Coy Laboratory Products, Grass Lake, USA) that was filled with a 3% H_2/97% N_2 atmosphere and supplemented with anaerobic isopropyl β - D-1-thiogalactopyranoside (IPGT) (0.1uM final) and gentamicin (10 ug/ml final) if necessary. Plates that contained nitrite were supplemented at this stage with anaerobic $NaNO_2$ solution to a final concentration of 1mM. The anaerobic $NaNO_2$ was prepared fresh for each experiment by mixing $NaNO_2$ crystals with anaerobic water to a final concentration of 1M and then filter sterilized in the glove box. The plates were poured by pipetting 10 ml of liquid medium into sterile, round 60x15 mm petri dishes (Greiner bio-one, Frickenhausen, Germany). Finally the plates were allowed to dry for 30 minutes with open lids.

4.7.3 Microscopy

Table 2 Basic microscopy settings

Channel	Excitation [nm]	Power	Emission [nm]	Gain	Mirror	Pinhole [um]
Blue	458	80%	467-489	1036	DD458/514	233.38
Green	488	38%	503-602	605	TD488/543/633	233.38
Reflected			483-493	366		
Transmitted				207		

4.7.4 Fractal box count

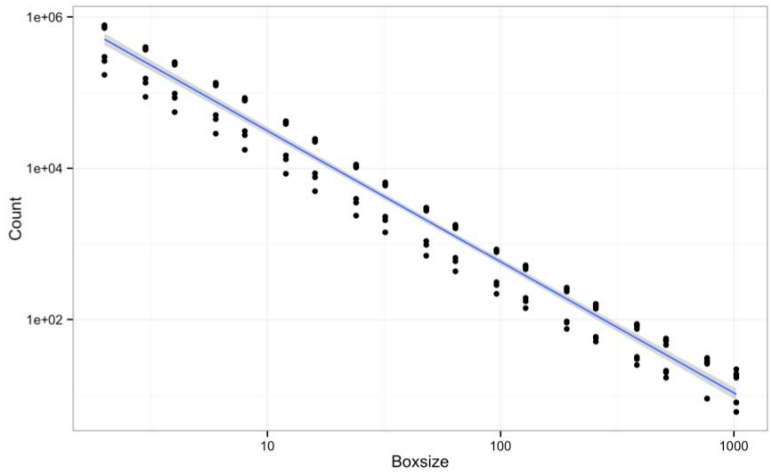

Figure 4.7 Log-log plot of box size vs. number of boxes necessary to cover the pattern, which provides a measure of the fractal dimension. Definitions: Blue line, linear regression; grey area, confidence interval. The slope of the curve represents the fractal dimension D (n=5, mean slope=1.73, SE=0.02).

4.7.5 Thresholding

Extracting the information of the branches poses an interesting problem, because the fractal properties of the branches make image segmentation inherently difficult. We chose an approach with a localized thresholding method in order to preserve the fine structure of the tips, but also to get the structure of thicker branches that have a stronger signal. This method, however, is very sensitive to background noise, because the local thresholding methods adapt to low signal areas and pick up these false positives. Therefore we did a thorough background removal that only gave us the signal of the cells, before thresholding locally. The image analyis was done in imageJ. The green channel was extracted from the images and thresholded using the Huang algorithm, which covered nearly all the area that contained cells. Remaining noise was removed using the 'remove outliers' method (radius=5, threshold=50, bright). The thresholded values were then normalized to values of 0 and 1 (containing information). This was then duplicated with the original, unthresholded green channel, which resulted in an image with all the grey-value information in areas with cells and 0 values for the background. This image was then thresholded locally using the Niblack algorithm (parameters: radius=15, k=0.2, offset=0).

4.7.6 Intermixing index

The expected value of intersections from a random distribution can be derived analytically. In a thresholded image of two populations expressing different fluorescent proteins, the sequence of pixels at a given circumference of n pixels is a vector of length n that consists of 0's and 1's. Such a sequence can be described as n Bernoulli trials. The number of sectors of a given color in a two-color system now corresponds to the number of runs of 1 with a length ≥1. The expected number of runs ≥1 of a sequence of n Bernoulli trials is approximately given as: $E(N_n) = n(1-p)p$ [42]. Thus, assuming an equal probability of being at a certain spot for both strains of ½, we expect a value of n/4 intersections.

4.7.7 Secondary wave

The beginning and end of the secondary wave were estimated from the intermixing index. A loess smoother was fitted to each intermixing index curve and the begining and end were determined as the maximum and minimum of the differential (with a lag of 50 to exclude the noisy beginning of the curves) of the smooth curve.

4.8 Literature

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5 Metabolite toxicity determines diversity during expansion of a microbial cross-feeding community

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5.1 Abstract

Metabolic interactions between species shape patterns of self-organization and spatial diversity within microbial communities. Strong interactions are thought to lead to intermixed patterns with high spatial diversity. A typical metabolic interaction between microbial populations is cross-feeding of intermediate products. Whereby one organism degrades a metabolite into an intermediate and another organism then further degrades the intermediate. These intermediate products can be toxic to the producing population, but can sometimes be further metabolized by a consuming population. Cross-feeding and the toxicity of intermediate products therefore cause different ecological interactions in microbial communities. The strength of the interaction between the cross-feeding populations should increase with the toxicity of the cross-fed metabolite. In this study, we varied the toxicity of an intermediate product in a synthetic microbial cross-feeding community and assessed the effect of toxicity on spatial diversity during range expansion. We find that toxicity did not increase spatial diversity between the two cross-feeding partners, but instead increased intra-population diversity. In our case, the spatial structure prohibited reciprocal interaction between the two populations, which uncoupled mixing of the two from the strength of the interaction. Intra-population diversity of the primary population on the other hand, increased because toxicity caused a larger part of those populations to grow at slow velocities, which in turn slowed the demixing effects of neutral drift. Our results show that spatial self-organization can be determined by non-intuitive mechanisms, emphasizing that care must be taken when analyzing cause and consequence of spatial patterns.

5.2 Introduction

The spatial self-organization of different microbial populations within communities can be an important factor affecting the survival and functionality of species, and therefore shape the diversity of microbial communities[1]. A key factor that determines spatial self-organization is the metabolic activities of different microbial groups[2,3]. Microbes excrete many energy-rich metabolites as side-products or end-products of their metabolism, which may then be further consumed by other species in a process called sequential cross-feeding (hereafter referred to as cross-feeding)[4–6]. Cross-feeding is thought to be an important driver of microbial diversity in nature because the sequential degradation of a single initial substrate can allow several species to co-exist by niche specialization[7]. This co-existence results in a broad range of ecological interactions between the different populations. For example, if the removal of the waste product by the second species also provides a benefit to the first one, cross-feeding interactions can be mutualistic (i.e., each population benefits from the presence of the other) and are sometimes referred to as reciprocal or cooperative cross-feeding[8–10]. An example for such a relationship is cross-feeding of toxic metabolites, where the second population can feed on the toxic metabolite, thus relieving the first population of the growth-inhibiting effects of accumulating the metabolite[6].

It is not clear how metabolite toxicity affects spatial self-organization and diversity in cross-feeding microbial colonies. In general during range expansions, initially mixed populations tend to demix into patches, where only one population prevails[11]. Experimental and theoretical considerations suggest that this demixing is a consequence of drift at the expansion front[12]. This effect can be mitigated to some extent by ecological interactions such as mutualism. It has been shown that strong mutualistic interactions can oppose drift and prevent demixing of the mutualistic partners[13,14]. These studies, however, have mainly investigated reciprocal exchange of nutrients between the obligately mutualistic partners. The partners must maintain close spatial proximity, because increasing distance between the populations will reduce the local availability of the exchanged nutrients and slow growth.

While this dynamics is intuitive, it is not clear how an interaction that is based on sequential cross-feeding of a toxic metabolite would affect spatial diversity during range expansion. Theoretical studies of such systems suggest that a stronger interaction that is imposed by high metabolite toxicity also prevents demixing[15]. However, theoretical studies[16] and our own empirical results have shown (Chapter 3) that sequential cross-feeding of a non-toxic cross-fed metabolite can lead to successive range expansion of the two populations. Successive range expansions result from non-identical expansion rates of the two populations, which cause a demixing of the two strains in the direction of the expansion. It is not immediately clear how toxicity would change the dynamics of such a system. We hypothesize that toxicity of the cross-fed metabolite slows down growth of the primary population, which could enable the succeeding population to catch up and promote mixing of the two populations.

Another aspect of expanding populations that beneficially interact with each other that has not yet been studied widely is the effect of such a growth dynamics on the diversity within each of the two interacting populations. As stated above, two alleles of a gene in one population are expected to demix during the expansion because of the abovementioned mechanism of drift. The strength of this demixing effect, however, depends on the growth dynamics of the population. It was proposed theoretically and recently shown empirically that nutrient levels and expansion velocity can impact the strength of drift during the expansion[17,18]. Faster expanding colonies tend to maintain diversity over longer distances. In a cross-feeding ecosystem, resource availability and growth rates are coupled to the partner populations. The growth dynamics are therefore an

emergent property of the whole system and are difficult to predict *a priori*. In general toxicity is dose dependent and inhibits growth[19], which should slow down the expansion velocity of the colonies. According to above-mentioned studies this thus suggests a stronger effect of drift causing a faster loss of diversity inside each population under toxic conditions.

In this study we investigated how a conditionally toxic metabolite affects demixing and diversity of two cross-feeding microbial populations. We investigated two main questions: First, does a stronger interdependence promoted by toxic conditions causes more mixing between the two populations? And second, do reduced growth rates caused by the toxicity lead to faster demixing of alleles within each population? We previously constructed a microbial cross-feeding model system that allowed the tuning of the toxicity of the cross-fed metabolite to test these hypotheses. The system consists of two strains of *Pseudomonas stutzeri* that differ in their ability to use nitrogen oxides. One strain consumes nitrate (NO_3) to nitrite (NO_2) while the other consumes the leaked nitrite (NO_2)[20]. These two cross-feeding strains will henceforth be called the producer and the consumer while the ancestral strain that completely degrades nitrate (NO_3) to nitrogen gas (N_2) will be called complete degrader.

Nitrite toxicity is pH dependent and can act via several unspecific mechanisms. At low pH nitrite also occurs in its protonated form nitrous acid (HNO_2). Nitrous acid can act as a protonophore, i.e. it increases proton permeability which inhibits ATP synthesis[21,22]. In addition, at low pH nitrite can spontaneously form nitric oxide radicals (NO) that can cause enzyme damage[23]. The pH dependence of toxicity allowed us to change the strength of the interdependence. We imposed an approximately commensal situation at pH 7.5 (low nitrite toxicity), where the consumer depends on the producer to provide its growth limiting resource nitrite while the producer does not depend on the consumer. We imposed a more mutualistic situation at pH 6.5 (high nitrite toxicity), where the consumer again depends on the producer to provide its growth limiting resource while the producer depends on the consumer to consume nitrite and prevent its toxic effects. The elegance of this system is that, in the absence of nitrite, pH itself has no growth-inhibiting effects at this pH range[20], thus allowing us to manipulate the toxicity of nitrite, and thus control the magnitude of interdependence, without creating any confounding factors by pH. We finally developed a mathematical, spatially explicit reaction-diffusion model of microbial growth under toxic conditions to assess the mechanism by which toxicity influences diversity in expanding systems.

5.3 Results

5.3.1 Nitrite is toxic at low pH, which reduces colony expansion velocity

To test the effect of nitrite toxicity in spatially structured systems, which may differ from completely mixed systems studied previously[20], we grew assemblages of our synthetic system of nitrite cross-feeding *P. stutzeri* strains on agar plates. At pH 7.5 nitrite is non-toxic to *P. stutzeri*, but at pH 6.5 it is toxic and reduces growth rates in liquid cultures[20]. To change the toxicity, we adjusted the pH of the agar medium prior to pouring the plates. To quantify the effect of toxicity, we used colony expansion velocity as a measure of growth dynamics[24]. We inoculated a large number of replicated plates at the same time and then sacrificed three replicates per pH condition at different time-points. This allowed us to measure the extent of colony expansion over time.

In general, the colony expansion velocity slowed over time regardless of the pH, and thus regardless of the growth-inhibiting effects of nitrite (Figure 5.1). The decrease in expansion velocity could be a combination of depletion of nutrients and hardening of the agar surface due to drying. We thus estimated the initial expansion velocities by a linear regression of the velocity profiles in the first week. The estimated expansion velocities and the corresponding calculated apparent diffusion coefficients of the cells are given in Table 3. Overall the expansion velocities were about 5-15 times slower than previously reported values for *Pseudomonas aeruginosa* grown aerobically at 21°C[25], which reflects the slower growth under anaerobic conditions.

The initial expansion velocity was pH dependent, and thus correlated with the growth-inhibiting effects of nitrite. Both the complete-degrading populations and the cross-feeding populations expanded slower at pH 6.5 (high nitrite toxicity) than at pH 7.5 (low nitrite toxicity) (Table 3, F-test, complete-degrading: p-value=8.628e-10, cross-feeding: p-value=8.525e-14), which would be expected given that nitrite can accumulate to substantial concentrations during denitrification[20,26,27]. The inhibition of the completely-degrading population is therefore consistent with previous observations that nitrite accumulates transiently during the growth of the complete-degraders[20].

Table 3 Estimated initial expansion velocities

Type	pH	Velocity [μm/d]	SE	D [cm²/s]
Complete-degraders	7.5	105.6	3.7	6.45E-10
	6.5	69.3	3.7	2.34E-10
Cross-feeders	7.5	59.9	1.8	2.08E-10
	6.5	42.7	2.2	1.06E-10

SE=standard error, D=apparent diffusion coefficient

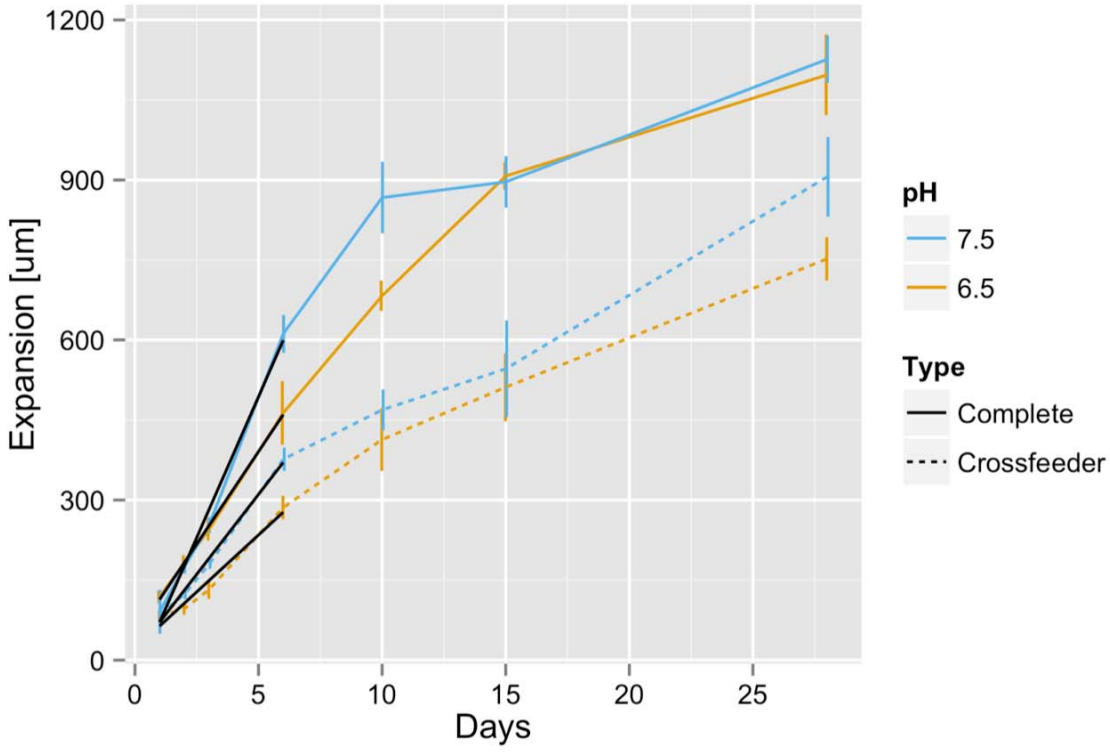

Figure 5.1 Expansion distances at different pH conditions. Shown are the expansion distances over time of complete-degraders and cross-feeders at different pH values. (N=3 per time point and condition, Vertical bars = 95% confidence intervals). The black lines represent the linear regressions that were used to calculate the maximal expansion velocities given in Table 3. Note that the primary front of the cross-feeders consisted solely of producers (see **Figure 5.2**).

5.3.2 Nitrite toxicity reduces intermixing of the two cross-feeders

To assess the effect of nitrite toxicity on spatial self-organization, we imaged the colonies after 3 weeks of growth using a confocal microscope. To distinguish the different sub-populations, we inoculated the producers and the complete degraders in two colors (blue and red) and the nitrite consumers in green. Figure 5.2 shows that the two colors of the producers and the complete-degraders demixed into sectors when expanding out of the inoculation zone, similar as previously reported for other organisms[12,18]. The consumers succeeded the primary wave of the producers and formed dendrites (as described in chapter 1) under both conditions. The final expansion width of the consumer was also independent of the pH (Figure S5.1). However, since the producers did not expand as far at pH 6.5, the distance between the producer and the consumer at the end of the experiment was shorter at pH 6.5 (strong nitrite toxicity) than at pH 7.5 (weak nitrite toxicity) (Wilcoxon test, p-value = 0.00806). This would be expected if nitrite accumulated within the primary wave, thus inhibiting growth until the secondary wave could advance further, consume nitrite, and relieve growth inhibition. Nevertheless the consumer did not completely catch up with the producer even after 4 weeks of growth and the dendrites formed by the consumer were qualitatively similar under both pH conditions. The fact that we did not observe simultaneous growth of the expansion fronts of the producer and the consumer suggests that they could not directly interact with each other, because they were physically separated.

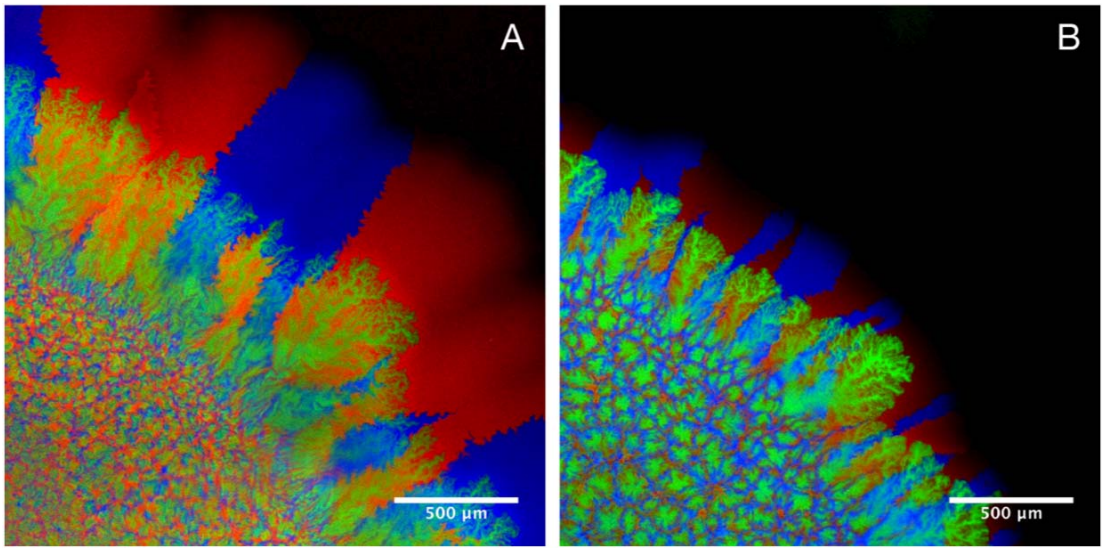

Figure 5.2 Confocal microscopy images of the cross-feeding populations. A) grown at pH 7.5 (low nitrite toxicity). **B)** grown at pH 6.5 (high nitrite toxicity). Both colonies are shown at the same magnification. The producers expressed cyan fluorescent protein (cfp, blue) or mcherry (red), the consumer green-fluorescent protein (gfp, green). The strains were inoculated at a ratio of 1:1:2 (cfp:mcherry:gfp), so that the total of the producers and the consumers had the same final concentration.

Intermixing of the cross-feeding populations is expected to be higher as the magnitude of interdependence increases . To assess a potential effect of interdependence, we therefore quantified the intermixing index, which is a measure of the number of transitions between the consumers and the producers. We measured intermixing on concentric circles from the center to the edge of the colonies at the different pH values. Overall intermixing was 19% lower at pH 6.5 than at 7.5 (Mann-Whitney test, p=0.00794), which was opposite to our initial expectations. This corresponds with the clumping of consumer patches that can be seen in the inoculation zone at toxic conditions upon visual inspection. It is not completely clear what caused the clumping, but taken together with the results above these results suggests that the interaction between the two populations was not substantially changed by nitrite toxicity, likely because the successive growth dynamics impeded reciprocal effects.

At both pH conditions, we observed that the intermixing was higher in the early expansion zone of the secondary wave than in the inoculum (Figure 5.3). The radial profile of the intermixing index was similar at both pH conditions, but the curves were shifted to lower values at toxic conditions. Intermixing was relatively constant in the inoculation zone and then increased in the secondary expansion zone, where the consumer was growing into the producer. This relative increase in intermixing in the early expansion zone compared to the inoculum was statistically the same for both pH values at about 10% (F-test, p-value=0.913), however, the absolute values the intermixing index were lower at toxic condtions. This result shows that the increase in intermixing during the expansion was independent of toxicity.

The intermixing in the secondary expansion zone is determined by the branching properties of the dendrites. We have previously shown that the dendrites have fractal properties, that is the dendritic branching pattern is similar when observing the pattern at different scales. Fractal dimension is a measure of how the properties of a fractal pattern change with the scale of observation and is used to classify fractal patterns[28,29]. The fractal dimensions of the observed

dendritic patterns (pH 6.5: D=1.70, SE= 0.02, pH 7.5: D=1.73, SE=0.03) was also statistically independent of the pH (F-test, p-value= 0.186) and it was similar to the fractal dimension of diffusion limited aggregation (DLA) patterns (D=1.71)[28,30]. This indicates that the process that caused the dendritic shape of the wave during the expansion was largely independent of the toxicity of nitrite, which corresponds with our previous finding that spatial constraints and cell shoving were the main drivers of dendrite formation (Chapter 1).

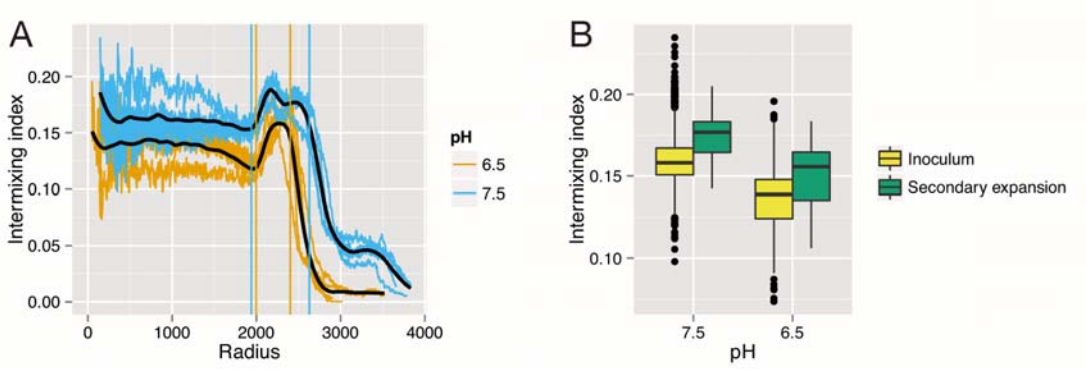

Figure 5.3 Intermixing index at different pH conditions. A) Intermixing with increasing radius from the inoculation zone. Each blue or orange line represents the data from one colony (N=5 per pH). The black lines are loess-smoothers of the combined data at a pH. The vertical lines show the estimated end of the inoculum and the end of the consumer wave at each pH (see Materials and Methods). The radii were normalized so that the expansion zone of all lines started at a radius of about 2000 micron. **B)** Boxplots that show the intermixing in the inoculation zones (yellow boxes) and the expansion zone of the consumer (green boxes).

5.3.3 Toxicity increases intra-population diversity

We used image analysis to measure the number of sectors for producers and complete degraders and the number of consumer dendrites to investigate the effect of toxicity on the intra-population diversity in each of the populations. The number of dendrites was not affected by the pH (Mann-Whitney test, p-value = 0.425), but we measured more sectors at pH 6.5 (toxic conditions) of both producers (Mann-Whitney test, p-value = 0.000215) and complete-degraders (Mann-Whitney test, p-value = 0.00158)(Figure 5.4). Toxicity thus increased local population diversity during the expansion and reduced the demixing effect of drift, but only in the producers or complete degraders. The strength of this effect (i.e. the difference between pH 7.5 and 6.5) was the same (F-test, p-value= 0.250) for both the producers in the cross-feeding communities and the complete-degraders that were growing without the consumers, suggesting that the demixing was independent of the presence of the consumers.

To further assess whether the presence of the consumers influenced sector numbers, we performed control experiments where we grew the producers and the complete-degraders with and without the consumers and measured the number of sectors. Overall we did not find a significant influence of the inoculation with the consumers (F-test, p-value= 0.865, Figure S5.2). These results show that the consumers did not significantly change the growth dynamics of the producers or complete-degraders that lead to reduced sector numbers. This was another indicator that, even under toxic conditions, the consumers did not substantially change the population structure of the producers.

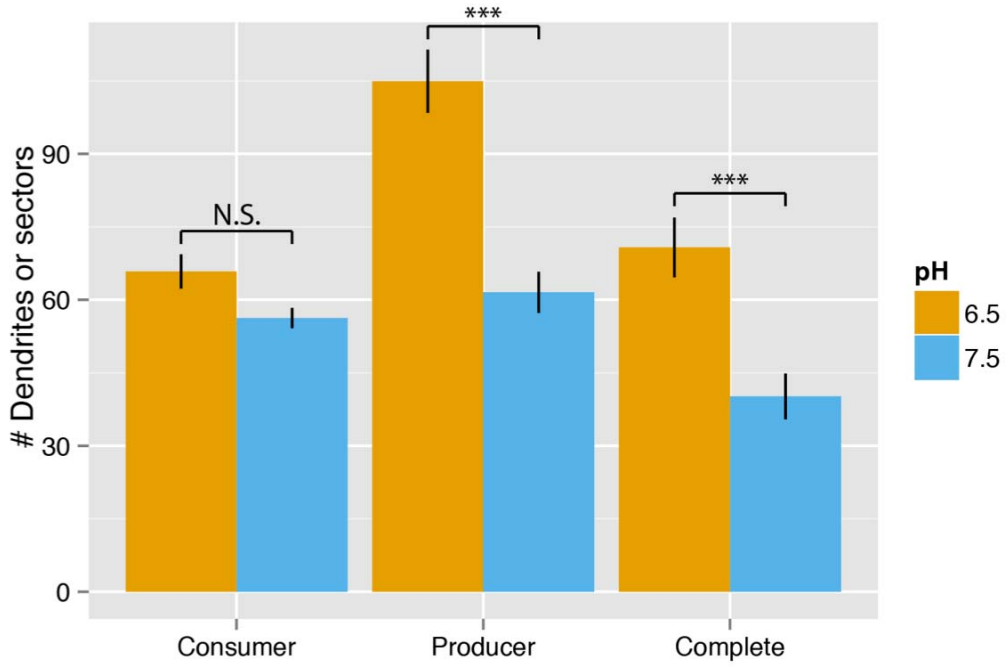

Figure 5.4 Mean numbers of sectors or dendrites. Producer and complete degrader sectors and consumer dendrites were measured radially at the position of the consumer front. The consumers and producers were measured on the same plates. N=13, vertical bars=standard error of the mean (SEM), ***=p-value of Mann-Whitney test below 0.01.

5.3.4 Negative correlation between expansion velocity and sector numbers

The above results showed two important effects of nitrite-toxicity on the producers and complete-degraders: first nitrite-toxicity slowed the expansion speed and second it increased sector numbers. Combining these two results, we found a negative correlation (Spearman rank correlation: rho= -0.681, p-value = 2.73e-08) between the number of sectors and the velocity of expansion. This seemed to contradict previous findings by Mitri et al[18], that showed a positive correlation between sector numbers and expansion velocity. The authors found that colonies growing on high nutrient agar were expanding faster and had more sectors than colonies on low nutrient agar. However, a more detailed analysis attributed this effect to the size of the actively growing population at the wave-front, which is equal to the effective population size in a growing colony[25]. The smaller effective population size, which is found at lower nutrient concentrations, is more affected by genetic drift that results in fewer sectors in the expansion zone.

In our system we did not manipulate the expansion speed by changing substrate concentrations, but we instead manipulated the expansion speed by changing nitrite toxicity. Because of spatial structure, the produced nitrite was locally inhibiting the expanding waves. This could have non-intuitive effects on the effective population size. We hypothesized that the inhibition could slow down the growth rate of the active population which would enable the primary substrate nitrate to diffuse further into the colony before being consumed, and thus enable more cells to grow simultaneously and increase the effective population size (see also Figure 5.7 in the discussion section for a schematic representation). This implies that the effective population size is in this case not just determined by nutrient availability, but also by the inhibition of nitrite, and that it should be larger in a slowly growing colony. The larger effective population should be less affected by drift, which could explain the higher sectors numbers in the slower growing colonies at pH 6.5.

5.3.5 Toxicity increases effective population size and heterozygosity

In the following section, we test the above hypothesis by using a mathematical model that incorporated toxicity of an intermediate product in a spatially growing cross-feeding community. This allowed us to study the mechanism by which toxicity influences local population diversity. The model is a spatially explicit reaction-diffusion model, which models cross-feeding microbial populations (producers and consumers) and their respective growth substrates (nitrate and nitrite). Each population and the substrates are modeled on a separate square lattice with 1024x1024 sites. The microbial populations grow with substrate dependent, Monod-type growth rate and move with a non-linear diffusion term. The substrates are consumed and produced proportionally to the microbial growth and diffuse with standard, linear diffusion rates (see methods section).

To this previously developed model (from chapter 1), we added a toxicity term, which slows the growth rates depending on the concentration of nitrite. The strength of the toxicity could be adjusted for each population by changing the specific toxicity constants $K_{inhib,P/C}$. As can be seen in the simulations (Figure 5.5), the patterns with and without toxicity were qualitatively similar. Under both conditions, the two populations of the producers demixed into sectors and were succeeded by the consumers that formed dendrites. However, under toxic conditions the colonies expanded slower than without toxicity. The model thus qualitatively reproduced the pattern and dynamics that we found in the experiments.

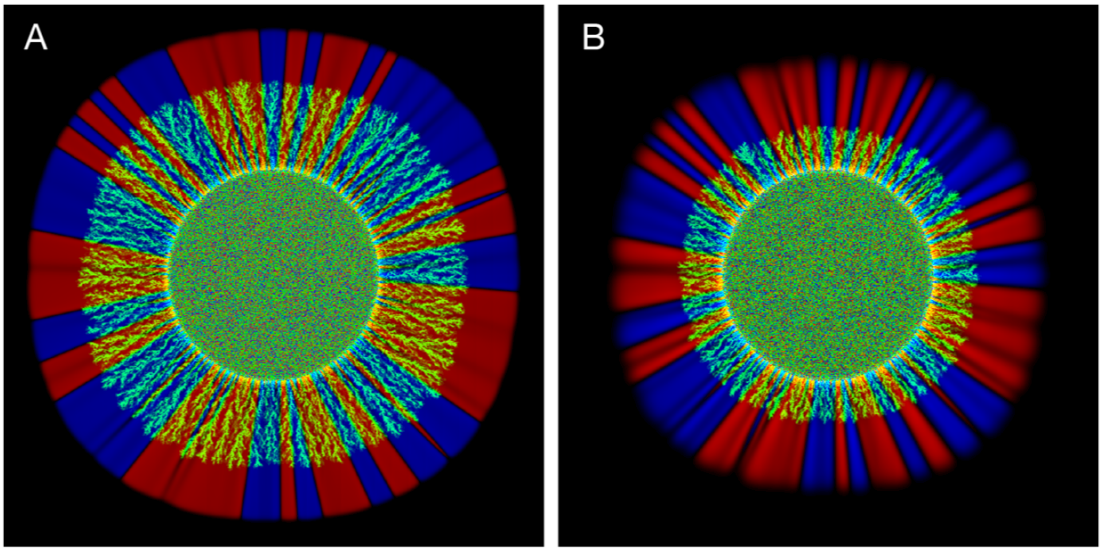

Figure 5.5 Simulated colonies. A) Without toxicity **B)** With toxicity.

To assess the effect of toxicity on drift, we quantified the intermixing of the two producer populations in the expansion area in the bacterial colonies and the simulations. For easier comparison with the previous findings of Mitri et al[18], we measured heterozygosity instead of the intermixing index. Heterozygosity is in principal a measure of co-occurrence of alleles and has a maximum of 0.5, which occurs when both alleles are locally present at equal ratios and maximum density. Heterozygosity is therefore higher at sector boundaries, where the two colors occur in close proximity and lower inside the sectors, where the two alleles are demixed. To quantify heterozygosity throughout the colonies, we measured it on concentric circles from the center of the colonies to the edges (see Mitri et al.[18] for a complete description of the definition and algorithm). Note that while heterozygosity does not measure exactly the same as sector numbers, it is also an indicator of demixing and shows qualitatively similar dynamics.

As can be seen in Figure 5.6, the model reproduced the experimentally observed dynamics of heterozygosity loss qualitatively. In both model and experiment, heterozygosity was higher under toxic conditions and heterozygosity decreased exponentially with distance from the inoculum. The initial heterozygosity of the model was about 4 times lower than the experimental data. This might be due to the fact that the initial mixing conditions were coarser in the model than in the experiments. In the model, each lattice site represented patches consisting of many cells. They were initiated with only one population per lattice site in the inoculation zone, which caused a stronger demixing of colors than in the experiments, where mixing in the inoculum was at a cellular level. The relative difference between heterozygosity under toxic and non-toxic conditions was the same for model and experiment at the beginning (about 58% (57.82%), F-test, p-value=0.650) and the end of the expansion zone (about 70% (69.97), F-test, p-value= 0.0565). We thus concluded that the model simulated the experimentally observed dynamics with sufficient accuracy to infer the mechanism by which toxicity affects intra-population diversity in the producer and complete-degrader.

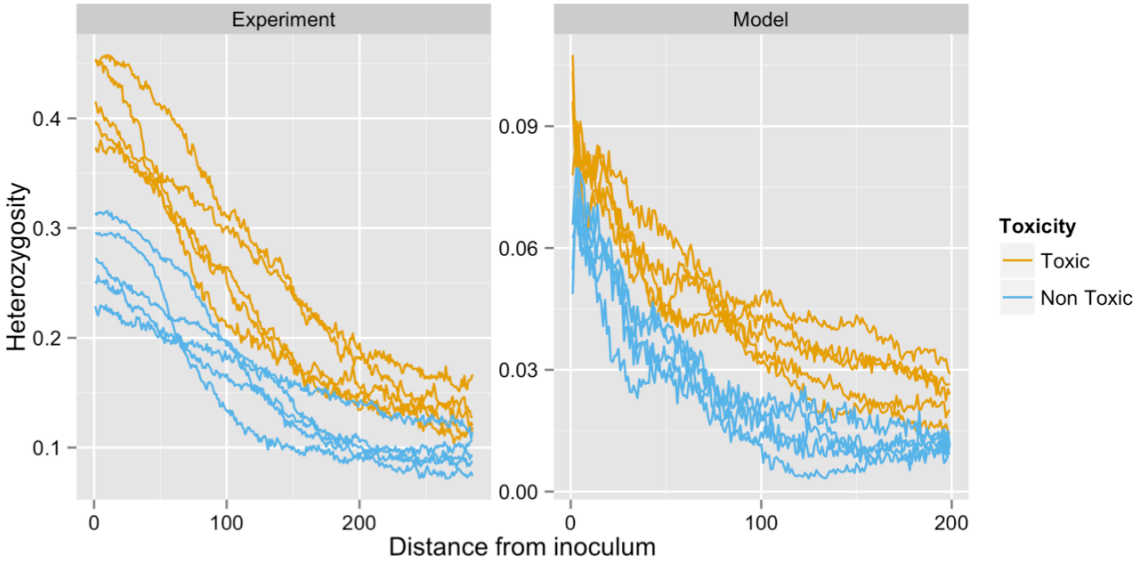

Figure 5.6 Decrease of heterozygosity with distance from inoculum (in pixels). Toxicity slows the loss of heterozygosity, resulting in higher inta-population diversity. Toxicity in the experimental data is equal to pH (6.5 for toxic and 7.5 for non toxic), while in the model it represents the strength of the inhibition term.

In the next step we quantified the size of the effective population in the model. As stated above the effective population size is equal to the actively growing layer at the edge of an expanding colony[25]. The effective population was thus defined as the part that had been growing since the last time-step. We found that the effective population was larger in simulations with toxicity (Mann-Whitney test: p-value = 0.00794, N=5) and also the number of active sites (i.e. the area that was covered by the effective population) was larger (Mann-Whitney test: p-value = 0.00794, N=5, see also Figure S5.3). This can also be seen in the simulations where only the actively growing part of the population was plotted (SI Movies). This showed that indeed toxicity reduced growth rates and also increased effective population size, which in turn reduced the effect of drift in the producer and increased heterozygosity. The results therefore supported our hypothesis about the mechanism that causes increased sector numbers at toxic conditions (see Figure 5.7 for a schematic representation of the mechanism).

5.4 Discussion

In contrast to our initial expectations, we found less intermixing between sequentially cross-feeding populations as toxicity increased. This can be explained by analyzing how intermixing of populations emerges during expansion. If both populations were obligately dependent on each other for growth, increasing patch size leads to increasing diffusion distances of the exchanged substrates and thus nutrient limitations. If the distances become too large, growth slows and may eventually stop. The average patch size is therefore limited by the diffusion properties of the substrates[13]. In our case, however, the producer is slowed by nitrite toxicity but is not obligately dependent on the consumer[31]. The producer initiates substrate consumption and, by expanding, may even evade the highest nitrite peak, which would be located behind the leading edge. The consumer, on the other hand, cannot grow under either non-toxic and toxic conditions until the producer produces sufficient levels of nitrite. The growth rate-reducing effect of toxicity was thus not sufficiently strong to promote simultaneous growth of the two populations. This prohibited a reciprocal feedback mechanism, which is a prerequisite for mutualistic interactions. The interaction was therefore non-equal; the consuming population benefitted from the production of nitrite but the producing did not equally benefit from the consuming population. These unequal benefits then lead to temporal segregation of the sub-populations, and intermixing mainly depended on the growth dynamics of the following consuming population rather than the producing population.

Our results show that, in this case, spatial structure hindered direct mutualistic interactions between the two populations rather than promoting them. This implies that intermixing here was not strongly correlated with how much these two populations interacted with each other. It was instead a measure of the growth dynamics and the environmental conditions of the consuming population. We therefore think that it is important to be careful when analyzing spatial patterns of microbial communities. It is fundamentally impossible to unequivocally infer a process based solely on an analysis of the pattern, because different processes can lead to the same pattern[32]. Without additional knowledge about the dynamics of the interactions themselves and biogeochemical processes of the system, the results of pattern analysis might be misleading.

We found that the toxicity of the intermediate product lead to a reduction in expansion velocity and higher diversity in the primary wave in the expansion zone. This resulted in a negative correlation between expansion velocity and diversity, which is opposite to previous findings[18]. However, mathematical modeling showed that both results could be reconciled by studying the mechanism that causes the loss of diversity during the expansion, which is genetic drift. Random sampling at the wave-front, where effective population size is low, causes a repeated founder effect which reduces diversity[33]. The strength of this effect depends on the effective population size. Mitri et al[18] varied substrate levels to change effective population size. More nutrients enable the growth of more individuals at the expanding front and therefore lead to a large effective population and less drift. The population dynamics in our system that contained toxicity caused less intuitive dynamics. By changing the pH, we made the cross-fed metabolite nitrite toxic, which caused slower colony expansion. This, however, did not reduce effective population size; on the contrary, it enabled more cells to grow and increased the size of the effective population size (Figure 5.7). The mathematical model showed that because the cells were growing slower, the cells at the front could not consume all the substrate fast enough, which enabled the primary substrate nitrate to diffuse further into the colony and increased the effective population size. The larger effective population size then reduced the demixing effect of drift, which maintained more of the initial diversity.

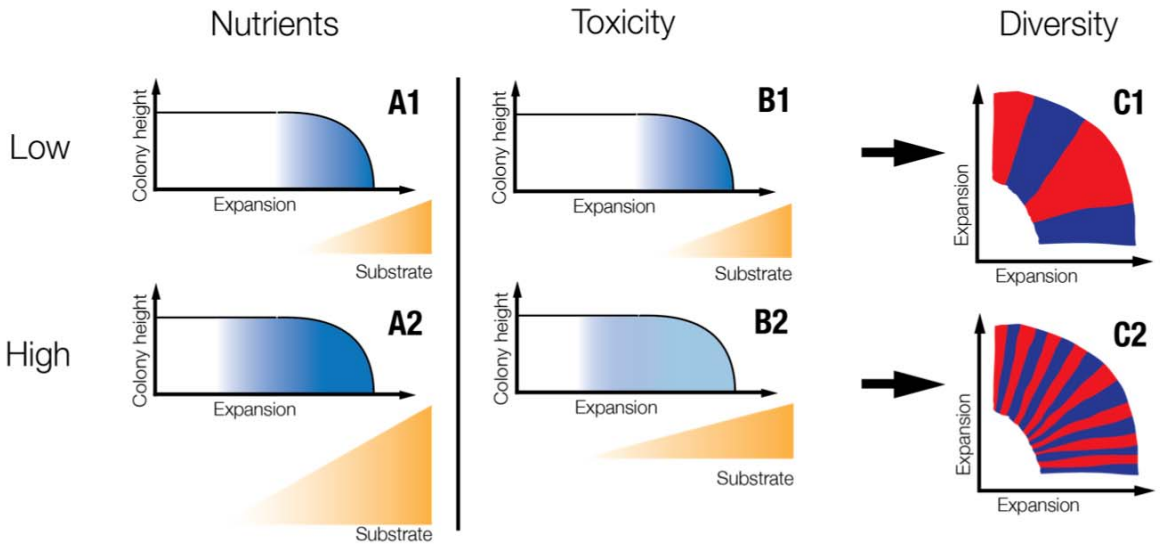

Figure 5.7 Schematic representation of the effect of substrate concentrations and toxicity on sector numbers and diversity. The graphs show cross-sections of expanding colonies. The blue shading represents the activity of the cells at the front. The size of the blue shaded area is thus equal to the effective population. Under low nutrient (**A1**) or non-toxic (**B1**) conditions the substrate is only available at a small edge of the colony. The effective population size is therefore small and drift is strong, which results in low diversity (i.e. sector numbers, **C1**). With high nutrient levels (**B2**), more substrate is available which increases effective population size. This reduces the effect of drift and increases diversity[18] (**C2**). Toxicity on the other hand slows down growth, which allows the substrate to diffuse deeper into the colony, because it is consumed at a slower rate. The result is a larger effective population size that has a low activity (B2) (indicated by a lighter shading). Nevertheless the larger effective population size reduces drift, which leads to higher diversity (**C2**). Note that in this study we only manipulated toxicity levels, while Mitri et al. manipulated nutrient levels[18].

In conclusion, we have investigated the effect of sequential cross-feeding of a conditionally inhibitory metabolite on spatial self-organization during a range expansion. We found two unexpected dynamics by which toxicity shapes spatial diversity. The first is that toxicity does not significantly affect the interdependence of the two cross-feeding populations. Therefore it also didn't increase mixing of the two populations. The second is that while toxicity did reduce expansion velocities, this did not decrease, but instead increased, spatial diversity. Both findings show that the community dynamics can lead to non-trivial emergent properties of seemingly simple microbial model systems. The sum and balance of the substrates that enhance or reduce growth in such systems are often difficult to determine without deeper knowledge of the biogeochemistry at work. It is thus difficult to predict the emerging properties of the complex microbial communities found in nature. A deeper knowledge of the fundamental ecological processes that govern simpler model systems could help us to understand and finally control microbial communities.

5.5 Acknowledgements

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5.6 Materials and methods

5.6.1 Bacterial strains and growth conditions

We used the same strains and growth conditions as in chapter 1. In addition to the green- and cyan-fluorescent tags we also used a strain that was marked with a red-fluorescent protein (mcherry). Also the anaerobic plates were prepared the same way as described in chapter 1. To adjust the pH of the medium, 1M HCl or 0.5M NaOH was titrated to the liquid lysogeny broth (LB) to a final pH of 6.5 or 7.5 prior to removing the oxygen by boiling under a nitrogen gas flow. The plates were then poured in an anaerobic glove box (Coy Laboratory Products, Grass Lake, USA).

For the colony expansion experiments a drop of a mixture of the liquid cultures was inoculated to the center of the plates. Liquid culture were inoculated from single colonies and grown aerobically at 30°C overnight. The cell densities where then adjusted by measuring the optical density at 600nm and diluting in sterile 0.9% (w/v) NaCl solution. The different strains were mixed at the ratios required in each experiment (1:1 or 2:1:1 for three color experiments) and a drop of 2ul was inoculated into the middle of each plate in an anaerobic glove box. The plates were then incubated for up to 4 weeks under anaerobic conditions at 21°C.

5.6.2 Confocal microscopy

The colonies were imaged with a Leica TCS SP5 II confocal microscope. The colonies were exposed to ambient air for 1 hour prior to imaging, to allow maturation of the fluorophores. The microscopy settings are shown in table 2.

Table 2 Basic microscopy settings

Channel	Excitation [nm]	Power	Emission [nm]	Gain	Mirror	Pinhole [um]
Blue	458	80%	467-489	1036	DD458/514	233.38
Green	488	38%	503-602	605	TD488/543/633	233.38
Reflected			483-493	366	TD488/543/633	233.38
Transmitted				207	TD488/543/633	233.38
Red	543	50%	574-727	677	DD488/543	233.38

5.6.3 Image analysis

5.6.3.1 Sectors and dendrites

The number of sectors and dendrites was measured at the position of the consumer wave-front. To measure the sectors the images were imported into imageJ and a line was drawn at the end of the secondary wave. Then the profile of the blue and red channels was measured along these lines with a line-with of 100 pixels to smooth the resulting data. This data was imported into R to calculate the number of sectors. The profiles typically showed areas of high or low signal intensities with sharp transitions between them. A sector was thus defined as an area where one

channel had a stronger signal than the other and the number of these areas was calculated. The number of dendrites was measured manually. A dendrite was only counted when it spanned the whole secondary front, from the inoculation zone to the line that was drawn to measure the sectors. To reduce manual counting bias all the images of both pH conditions were given random identifiers and two independent persons counted the dendrites in the first experiment. Because the results were very similar between the two persons, one person analyzed the remaining experiments manually.

5.6.3.2 Intermixing index

The intermixing index was calculated as in chapter 1. In short the green channel containing the consumers was thresholded using a local thresholding algorithm. Then the number of intersections was measured on concentric circles from 50 micron to the end of the expansion zone using the Sholl plugin in imageJ. The resulting data was analyzed in R. The beginning and the end of the consumer wave were determined by calculating the maximum and minimum of the differential of the loess-smoother curves. The differential was calculated with a lag of 50 to exclude the highly noisy data at small radii.

5.6.3.3 Fractal dimension

The fractal dimension was measured on the thresholded green channel. The inoculation zone was removed from the images and the fractal dimension of the remaining expansion zone was measured with the fractal box count routine of imageJ. The resulting data was analyzed in R.

5.6.3.4 Heterozygosity

Heterozygosity was measured with an adaptation of the algorithm of Mitri et al[18] in Matlab. In short the algorithm extracted pixel values of the blue and red channel along concentric circles from the edge of the inoculation zone to the end of the colonies. The pixel values were then divided by the mean pixel value of the respective channel and normalized between 0 and 1. From this data it calculated the fraction of red values and then the corresponding heterozygosity at each radial position. Heterozygosity was calculated on images of experiments where the producers and the complete-degraders were grown without the consumers. This was done because the dark areas, where the consumers had grown, that remained in the blue and red channels confounded the heterozygosity measurement. To analyze the data of the model the lattices of the nitrite-producing population at the end of the simulations were converted to red and blue channels of a tiff file. These tiff images were then analyzed like the experimental data.

5.6.4 Model

We used an extended version of the model developed in chapter 1. The model simulates two microbial populations (P: producer, C: consumer) and the two corresponding substrates (N1: nitrate, N2: nitrite) on a square lattice (1024x1024 sites) with the reaction-diffusion equations given below. The bacteria expand with a non-linear diffusion term, the substrates with a normal diffusion term (see chapter 1 for a detailed description and values of the parameters). Each population and substrate was modeled on separate lattices. To be able to measure heterozygosity in population P, two equal populations P_1 and P_2 were modeled on separate lattices. These populations were inoculated at ratios of 25% in a circle in the center of the lattice with radius 200 lattice sites, the remaining 50% of lattices sites was inoculated with population C.

To model toxicity we extended the Monod-type growth term of the bacteria with an inhibition term. This term depended on the concentration of the intermediate product nitrite and

could be tuned for each population separately via the toxicity constants $K_{inhib,\,P/C}$. $K_{inhib,P}$ was set to 0.1 and $K_{inhib,C}$ was set to 1 to reflect the experimental findings that the producer was inhibited stronger than the consumer. The dynamics of heterozygosity loss in population P were robust to changes in $K_{inhib,P}$, because population C always succeeded population P with sufficient distance.

Population $P_{1,2}$:

$$\frac{\partial P}{\partial t} = \sigma\nabla\left(D_p\nabla \mathrm{P}\right) + r_p P\frac{N_1}{K_1 + N_1}\times\frac{K_{inhib,P}}{K_{inhib,P} + N_2} \tag{1a}$$

$$D_p = \frac{N_1(1+P)}{1+\Omega C} \tag{1b}$$

Population C:

$$\frac{\partial C}{\partial t} = \nabla(D_c\nabla \mathrm{C}) + r_c C\frac{N_2}{K_2 + N_2}\times\frac{K_{inhib,C}}{K_{inhib,C} + N_2} \tag{2a}$$

$$D_c = \frac{N_2(1+C)}{1+\Omega P} \tag{2b}$$

Substrate N1:

$$\frac{\partial N_1}{\partial t} = D_{N1}\nabla^2 N_1 - \nu_1 r_p P\frac{N_1}{K_1 + N_1}\times\frac{K_{inhib,P}}{K_{inhib,P} + N_2} \tag{3}$$

Substrate N2:

$$\frac{\partial N_2}{\partial t} = D_{N2}\nabla^2 N_2 + \nu_1 r_p P\frac{N_1}{K_1 + N_1}\times\frac{K_{inhib,P}}{K_{inhib,P} + N_2} - \nu_2 r_c C\frac{N_2}{K_2 + N_2}\times\frac{K_{inhib,C}}{K_{inhib,C} + N_2} \tag{4}$$

5.6.4.1 Measuring the active layer

The active layer was defined as the part of the population that had changed since the last time step. The size of the active population and the number of active sites was measured at a colony diameter of 900 grid cells, which was at an intermediate size of the colony where the width of the active layer was constant over time.

5.7 Supplementary material

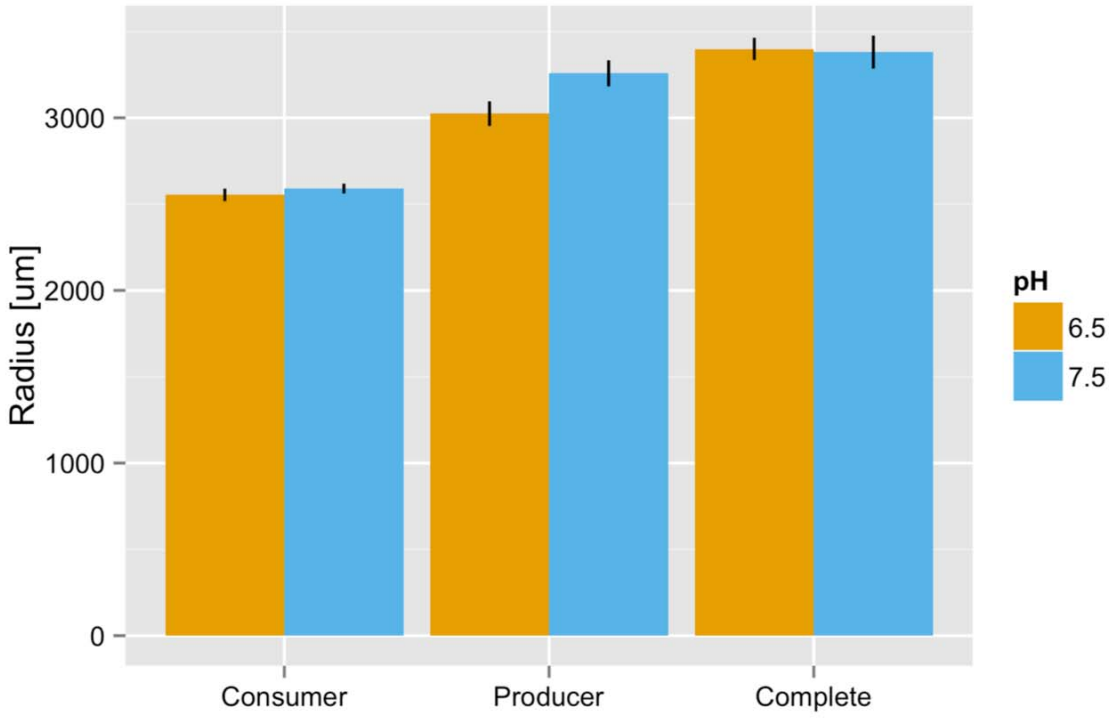

Figure S5.1 Final expansion widths. Expansion width was measured after 3 weeks of growth (Bars=SEM, N=13). Consumer and Producer were grown and measured on the same plates. The pH did not affect the consumers and the complete-degraders (p-value = 0.448, p-value=1). The producers were affected by pH (p-value = 0.0387, all Mann-Whitney tests). The distance between the producers and the consumers was significantly larger at pH 7.5 (Wilcoxon test (paired), p-value = 0.00806).

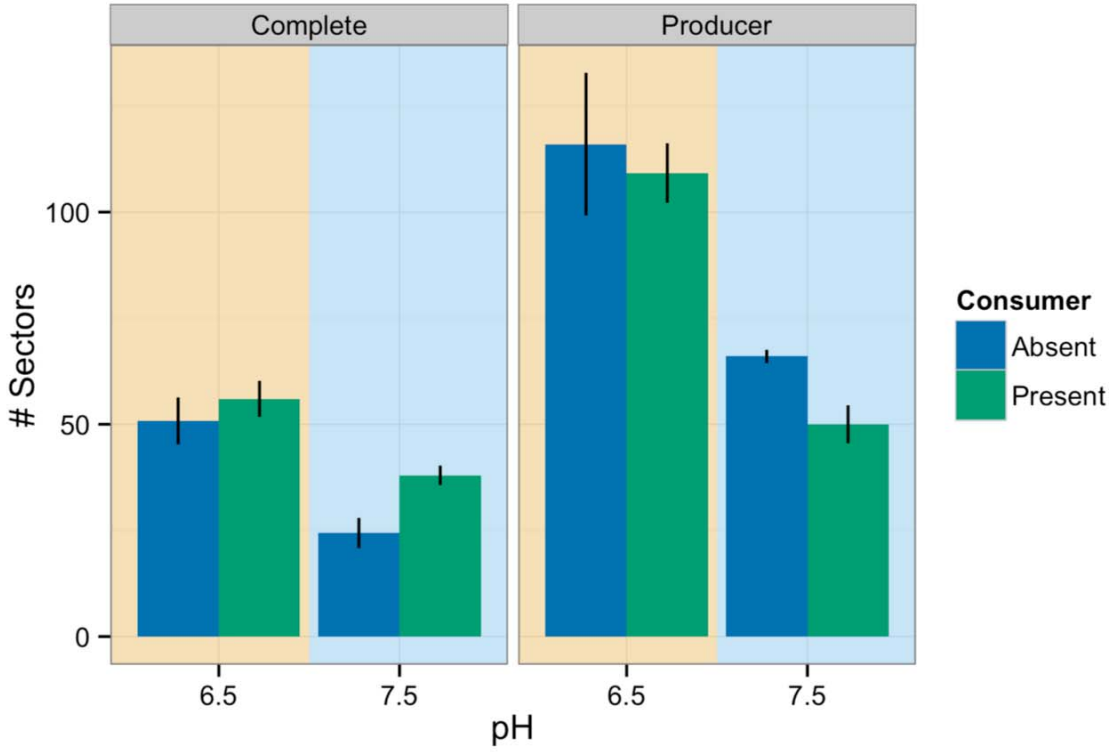

Figure S5.2 Sector numbers in the complete-degraders and the producers depending on presence and absence of the consumers and pH. N=5, Bars=SEM. The presence of the consumers and thus also a 50% lower initial inoculation density of the complete-degraders or the producers did not have a significant effect on sector numbers.

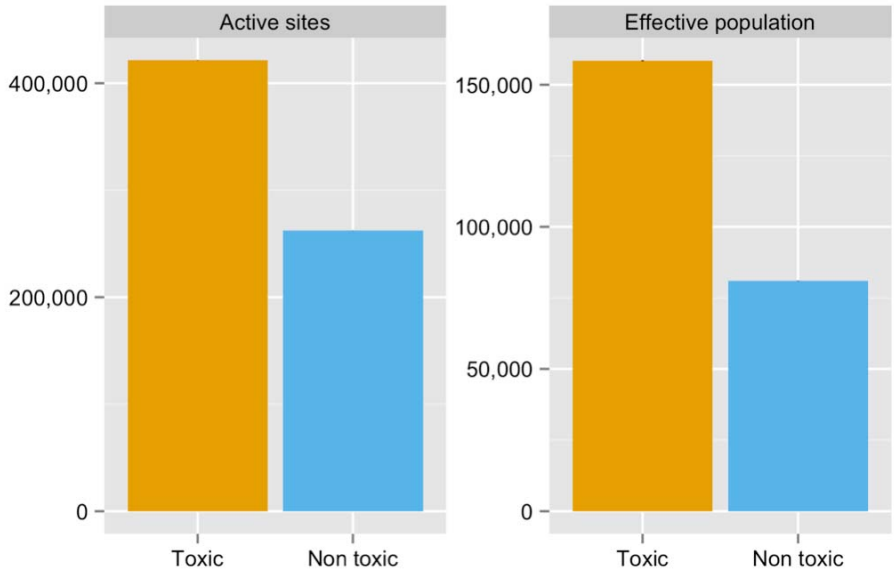

Figure S5.3 Modeled effective population size and number of active lattice sites depending on toxicity. The active sites are lattice sites where cells have been growing since the last time-step and the effective population is the sum of the population density at those sites. N=5, Bars=SEM, note that the standard errors are less than 200 and therefore too small to be seen in the plot.

5.8 Literature

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6 Spatial chaos? Local heterogeneity determines spatial self-organization of an interacting microbial community

Felix Goldschmidt, Lea Caduff, David R. Johnson

6.1 Abstract

Spatial chaos refers to two-dimensional deterministic systems where small differences in the initial conditions can lead to very different pattern formation. The outcome of pattern formation is thus seemingly chaotic. Spatial chaos has been studied largely with mathematical models. These models often consist of spatial versions of ecological models, such as predator-prey dynamics, or game theoretical models, such as the prisoner dilemma, and can produce an astonishing range of complex spatial patterns. However, experimental evidence of spatial chaos is scarce. Here we show that a sequentially cross-feeding community of microbes can self-organize in two distinct patterns. When the microbial colonies expand, the two populations can either grow successively, where the consuming strain follows the producing strain, or they grow simultaneously, where the consuming and producing strains advance at the same time. We further show that the difference in pattern formation is likely not caused by mutations, but that it is instead dependent on the initial frequencies of the two interacting populations. Our results thus demonstrate that differences in the initial conditions can lead to different pattern formations. However, we so far do not know why the different initial frequencies cause the different patterns. Further research is thus needed to determine the mechanism of pattern formation, to gain further understanding of the system dynamics that lead to this seemingly chaotic pattern formation.

6.2 Introduction

This chapter is devoted to a prominent feature of the expanding colonies of our system of cross-feeding *Pseudomonas stutzeri* strains. As can be seen in Figure 6.1, and as we have described before (Chapter 4), the two cross-feeding populations establish two succeeding expansions when expanding their range. However, there are also areas where the green consuming strain does not succeed the blue producing strain (see arrows in Figure 6.1A). In these areas, the two populations grew simultaneously and formed unexpected patterns. We thus observed two distinct local patterns that are distributed heterogeneously along the expanding edge. This suggests two fundamentally different hypotheses of the causes of local heterogeneity in pattern formation. One is that heterogeneity in the biological traits of individual cells (i.e., intrinsic heterogeneity) caused the different growth patterns. The other is that heterogeneity in the initial spatial positioning of individual cells and their abiotic environment (extrinsic heterogeneity) caused the different growth patterns. The difference between these two hypotheses lies in the assumptions they make; in one the source of heterogeneity is intrinsic (cellular) while in the other the source of heterogeneity is extrinsic (environmental).

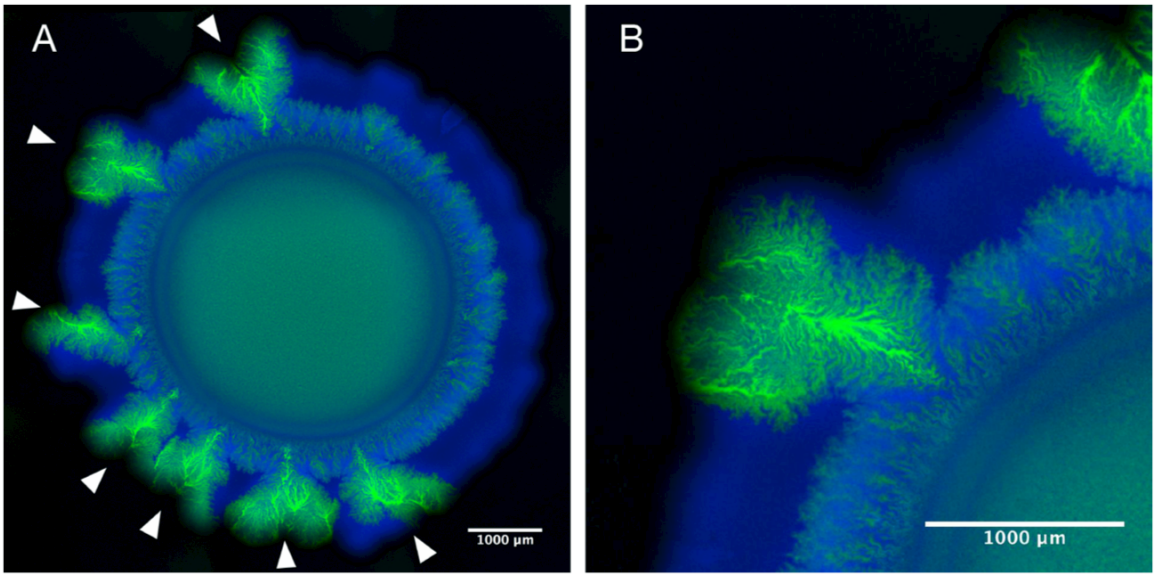


Figure 6.1 Areas of simultaneous growth. A) Colony growing mostly successively with isolated areas of simultaneous growth (arrows) **B)** Magnification.

Intrinsic heterogeneity could be caused either by a genetic change or by phenotypic variation through stochastic gene expression[1]. An obvious hypothesis is that the pattern of simultaneous growth is caused by mutation. In range expansions, mutants with higher productivity form a characteristic pattern; they protrude from the rest of the expansion-front and their interface with the neighboring population is not parallel to the expansion[2–4], as is the case in the previously described sectors (Chapter 4). The interface is typically curved outwards in the direction of expansion, such that the mutant area becomes larger during the expansion. Both features are intuitive consequences of the higher growth rates compared to the surrounding ancestral population. A visual inspection shows that both features are present in the simultaneous growth pattern in our colonies. However, this behavior is merely a sign of higher productivity of a local population compared to the surrounding population. It could also emerge in the absence of genetic change by phenotypes that are well adapted to the specific situation of expansion on an

agar plate. A more thorough test of the genetic content of the microbes that form the pattern is therefore necessary to test for mutations.

An alternative explanation could be that the pattern change is not caused by a change in the mode of growth, but instead by local heterogeneities in the initial conditions of spatial positioning. Such behavior has been theoretically described and is called spatial chaos[5,6]. In general, a chaotic system is defined as a deterministic system that is sensitive to variation in the initial conditions[7]. A classical example of deterministic chaos are predator-prey interactions that can cause seemingly chaotic oscillations in the abundance of predators and prey[8]. Furthermore, if predator-prey interactions are simulated in space, they can generate chaotic spiral patterns[9]. Other examples come from game theory such as the prisoners dilemma or the hawk-dove game that can generate „fractal kaleidoscopes“ when played in a two dimensional spatial system[5,6]. These studies have shown fascinating dynamics and patterns, however, direct experimental evidence for spatial chaos is still scarce.

6.3 Results

6.3.1 Occurrence of simultaneously growing phenotypes

In nearly all our cross-feeding range expansion experiments, we found several spatial areas where the consumer did not succeed the producer, but where the consumer instead expanded simultaneously with the producer (Figure 6.1). These areas are difficult to explain from simple growth dynamics, as the growth rates of the consumer and producer should not be synchronized and there is no reason why the producer should not expand before the consumer (i.e., the producer should depend on the consumer less than the converse). These areas typically began at a single point of the inoculation zone and extended until the edge of the colonies. They also grew progressively wider during the expansion, which is expected for sectors containing mutants with higher growth rates than the ancestors[3]. In these areas, the two strains were growing simultaneously and mixed. Mixing of expanding, mutualistic populations is associated with strong interactions[4] and can be beneficial to both partners, where reducing reduces diffusion distances and facilitates metabolite exchange. Furthermore, in expanding populations, the expansion velocity is a measure of productivity. As can be seen in Figure 6.1, the mixed growing populations typically extended further than successively growing areas. The simultaneously growing populations therefore presumably had a higher productivity during range expansion at the ecosystem level than the successively growing populations.

6.3.2 The pattern change is not caused by mutations

The findings above suggested that the areas of simultaneous growth consisted of mutants that were selected to the specific selection pressures imposed by colony expansion. To test whether these areas consisted of mutants, we isolated four pairs of the strains from these areas and sequenced their genomes. To do so, we picked the edges of simultaneously growing areas and streaked the strains on aerobic agar plates. The fluorescent markers allowed us to isolate single colonies of the producer or consumer populations within these areas. We then compared the sequenced genomes with the genomes of the ancestral strains. In some cases we did not find any new mutations that would lead to amino acid substitutions. In the other cases, the mutations were either already present in the ancestral strains or were sequencing errors (i.e. they could not be confirmed by re-sequencing the specific genomic region). However, we have not yet re-sequenced all potential mutations. Nevertheless, we have yet to find any evidence that the pattern change was caused by genetic mutations.

To further test the heritability of the pattern change, we repeated the range expansion experiments with the frozen stocks of the isolated strains. We found that in some cases all the expansion zone of the colonies grew simultaneously (Figure 6.2), but in others the amount of simultaneous growth was very similar to the ancestral pattern. This suggests that the emergence of simultaneous expansion was not heritable. To account for variability in the frozen stocks, we repeated this experiment and used 10 different colonies per strain as inocula. We compared these with 10 pairs of ancestral cross-feeding strains as controls. Interestingly, we also found colonies that grew simultaneously in the ancestral populations. But in comparison we did not find more completely, simultaneously growing colonies in the newly isolated populations than in the ancestral populations (Mann-Whitney test, p-value= 0.651). However, note that a more detailed image analysis might potentially reveal that a larger proportion of the expansion zone was covered with the simultaneously growing phenotype in the newly isolated strains (see images in Table S6.1). These results thus suggest that the trait is not genetically encoded because the patterns formed by the strains that were isolated from the simultaneously growing regions did not differ significantly from the ancestral patterns.

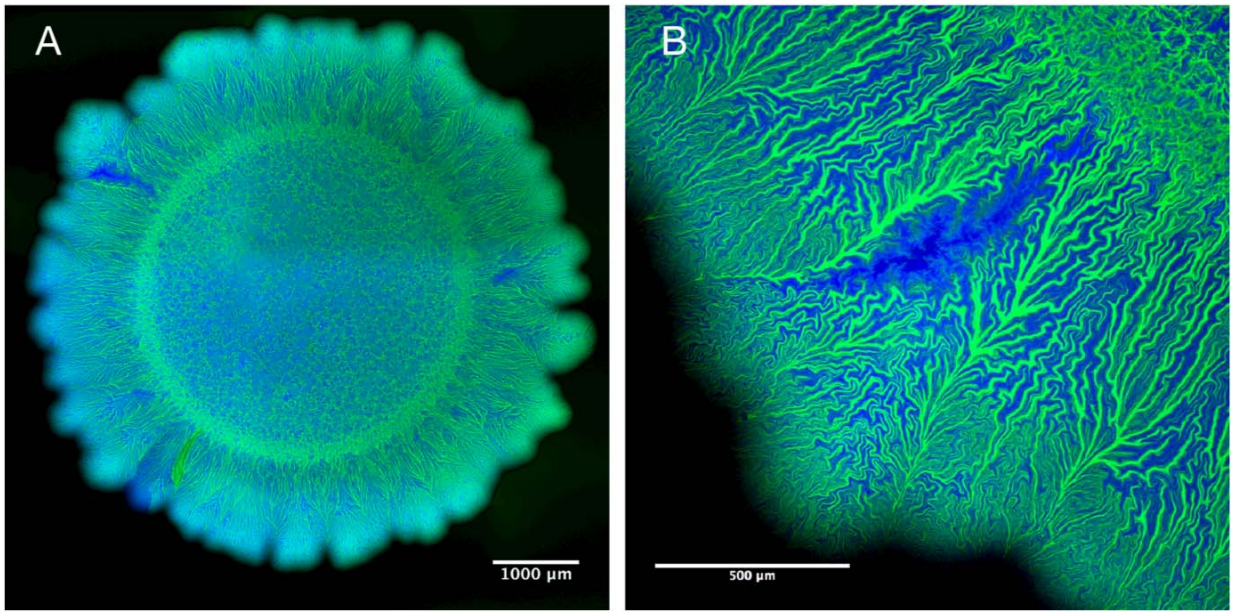


Figure 6.2 Simultaneously growing colonies. A) Colony that was growing simultaneously. **B)** Detail of the expansion zone (note: image from another colony from the same experiment).

6.3.3 Temporal dynamics of simultaneous growth

To observe the pattern development over time, we performed a time-course experiment where we inoculated a large number of plates and then measured three replicates at different time-points. Note that because the fluorescent proteins need oxygen for maturation and thus visualization with microscopy, we could not perform time-lapse imaging of the same colonies. We observed that after some days of growth, the consumer was growing ahead of the producer in some areas (Figure 6.3). Note that this is the opposite of what we usually observe or expect based on growth dynamics, where the producer should grow ahead of the consumer. The pattern that was formed in these areas was qualitatively similar as the pattern described initially in the simultaneously growing areas. Interestingly, the direction of branching of the green consumer population was reversed during simultaneous growth, where branching seemed to form towards the inoculation

zone. In contrast to the successive pattern, where the consumer branches outwards in the direction of expansion, the branching of the consumer during simultaneous growth was in the direction of the inoculum, i.e. against the direction of expansion (Figure 6.3).
It is not clear how the consumer was able to obtain a position at the expansion front and, more importantly, remain there during the expansion. A possible scenario is that the producer is pushed (or shoved) ahead by the producer and/or that it is feeding on nitrite that diffuses ahead of the producer wave front. The pattern could then be generated by the mixed dynamics of the producer expanding and branching into the consumer and backward growth of the consumer that is pushed ahead by the producer. However, the fact that we did observe the consumer growing in front of the producer can give indications about the underlying pattern forming process.

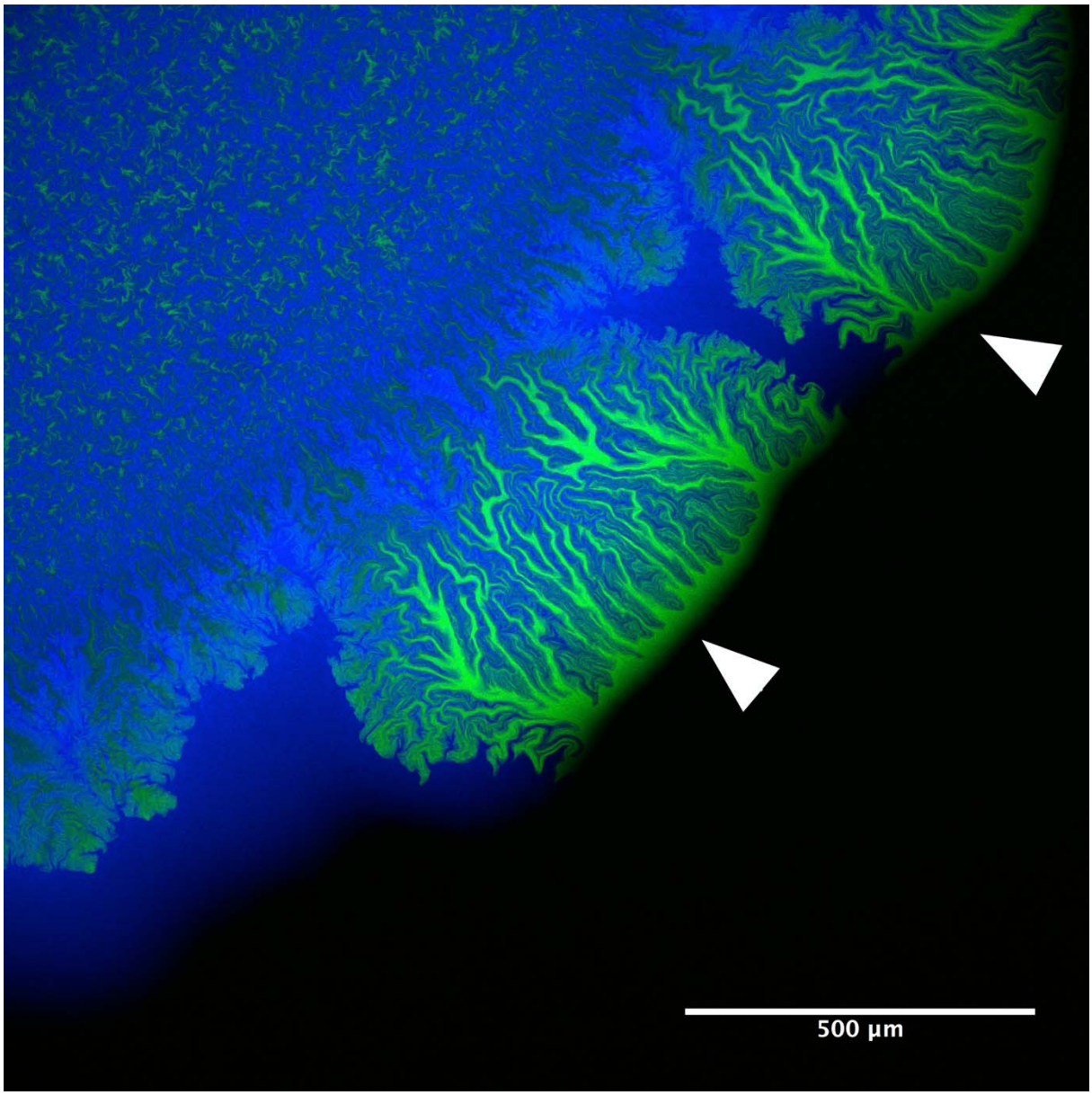


Figure 6.3 Consumer growing ahead of the producer. The image shows the edge of a colony after 8 days of growth. It can be seen that the green consumer grows slightly ahead of the blue producer in the areas indicated with arrows. In the wave behind these areas the characteristic river network patterns are emerging.

6.3.4 Simultaneous growth is frequency dependent

The results above showed that in the simultaneously growing areas, the consumer populations are able to stay at the front during the expansion, thus resulting in apparent simultaneous expansion. This suggests at least two possible explanations for the observed patterns. One is that the producer is from the start in a preferential position located in front of the producer. A possible scenario could, for example, be that by chance there are larger patches of consumers at some locations along the edge of the inoculum. The producers would then initially have to grow through a layer of the consumers. During this time the produced nitrite will diffuse in all directions, including ahead of the producer population. This could provide the consumer with sufficient time to begin growing. If these two phenomena, slowing of the producer by the consumer and diffusion of nitrite, are balanced, a new stable dynamics might emerge, which would allow both populations to grow nearly simultaneously. The result would be a chaotic system, where small changes in the initial conditions can lead to very different patterns[5,6].

The second explanation is that a part of the consumer population expresses a different phenotype that allows it to remain at the front of the expansion. It has, for example, been shown that smoother surface structures or production of lubricating polymers can lead to preferential positioning of cells at the front of expanding colonies. Although such behavior is mostly driven by mutants that are e.g. deficient in pili expression[10] or overproduce a surfactant[11], such behavior can also be seen at the phenotypic level[12]. It has been proposed that this could be an example of division of labor[13], where one population specializes in e.g. surfactant production and the other population pushes them ahead to gain new territory and nutrients[11].

Both these hypothetical mechanisms are intriguing, but they are also difficult to distinguish on a phenotypic level. For the first hypothesis, we expect that the effect should be frequency dependent. If we increase the proportion of the consumer, the likelihood for larger patches at the edge should increase and we expect more simultaneous growth. However, if the pattern is caused by a different phenotype that is expressed at a certain proportion of the population, the final outcome might be the same, because we also expect more of this phenotype to be at the edge when the consumer is inoculated at a higher proportion. To further assess these hypotheses we performed experiments where we increased the proportion of the consumer in the inoculum. We inoculated the producers and consumers at ratios of 1:1, 1:20, 1:100 and 1:1000. We found that indeed a higher frequency of consumers led to a larger fraction of the colony to expand simultaneously (Figure 6.4). These results show that simultaneous growth is frequency dependent and that a larger frequency of the consumer relative to the producer causes simultaneous growth.

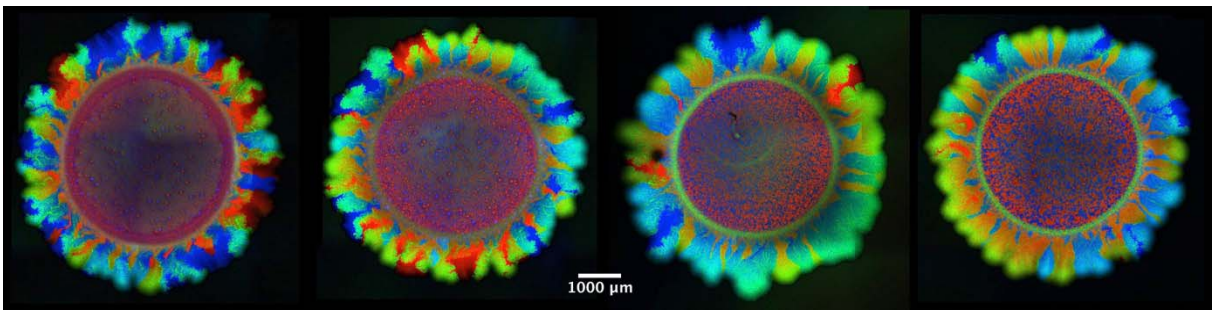


Figure 6.4 Increasing inoculation ratios. The images show colonies that were inoculated with an increasing ratio of the consumers. The consumer was inoculated in green and the producer in red and blue. The ratios from left to right are: 1:1, 1:20, 1:100, 1:1000 (density producer:density consumer). The proportion of the completely mixed areas increases with the increasing proportion of the consumers. However, after 1:100, the expansion zone is nearly saturated and the additional increase is marginal.

6.3.5 Toxicity reduces simultaneous growth

Intermixing and simultaneous growth should be beneficial for the two populations at a low pH. The nitrite-producing population profits from a reduction of the nitrite toxicity via nitrite consumption by the nitrite-consuming population. The nitrite consuming population, on the other hand, profits from high nitrite concentrations when being directly at the source of production and because the concentration is not reduced by diffusion of nitrite into the deeper agar layers. We thus expected to find more simultaneously growing areas at lower pH.

To assess this hypothesis, we measured the number of simultaneously growing areas per colony at both pH conditions. We found that at pH 6.5 there were significantly less areas where the strains were growing simultaneously (Mann-Whitney test, p-value = 0.000641). In some rare cases the highly mixed areas were completely absent at pH 6.5 and the nitrite-consuming population never reached the edge of the colony. This implies that they did not grow simultaneously with the nitrite-producing population in the primary front (Figure 6.5).

A possible explanation for this finding is linked to the frequency dependence of the intermixing pattern that we observed. At low pH, the producer was growing in large patches in the inoculation zone (see the center of the right colony shown in Figure 6.5). This indicates that only a part of the inoculated population managed to grow[14], while the rest was likely inhibited by nitrite toxicity. We can therefore assume that at the edge of expansion, only a part of the consumers was growing and overall the frequency of the consumers was therefore reduced under toxic conditions. This indicates that the simultaneous growth pattern was likely not an adaptation to nitrite toxicity, but presumably a consequence of locally varying initial conditions.

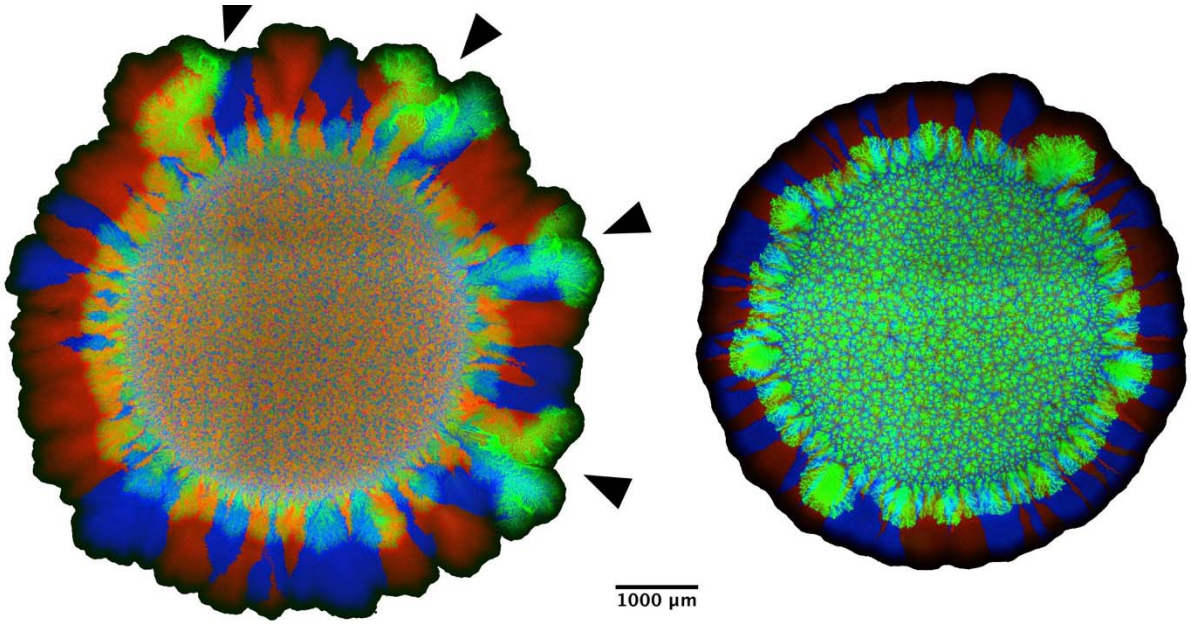


Figure 6.5 Influence of pH on the number of simultaneously growing areas. The left colony was grown at pH 7.5, the right one at pH 6.5. The area outside of the colonies was colored in white to precisely show the edge of the colony. The dark rim at the edge of the colony is due to a decline of the colony height and consequently a lower intensity of the fluorescent signal at the edge. The areas with the simultaneously growing pattern are indicated with black arrows and are only present in the left image. On the right image, it can be seen that although there were some green dendrites that protruded outwards further than the rest of the secondary front, they did not reach the edge of the colony and were not growing simultaneously with the nitrite-producing population at the primary front.

6.4 Discussion and outlook

We have shown that a sequentially cross-feeding community can spatially self-organize into two states: successive or simultaneous growth. Although the shape of the pattern suggests that is formed by mutants, to the best of our knowledge we did not find evidence of causal mutations in both the producer and consumer populations. We have, however, found that the pattern formation is frequency dependent. Different initial conditions (i.e. higher initial frequency of the consumer) had a strong impact on the amount of simultaneous growth in the expansion zone. This property is characteristic for chaotic systems; our observation of two different growth patterns could thus be an example for spatial chaos.

An alternative explanation would be that phenotypic heterogeneity, presumably in the consumer, leads to a division of labor during simultaneous growth. It is very difficult to disentangle these two proposed hypothesis experimentally, especially in our anaerobic system that makes time-lapse microscopy difficult. A possible way to gain further insights into these dynamics might therefore be mathematical modeling. We have so far not been able to reproduce the observed patterns with our previously developed model. An explanation for this might be that the complex dynamics of pushing and backward-growth require a more detailed modeling of the actual physical forces between the bacteria. We therefore currently collaborate with the group of Dani Or at ETH Zurich to develop a model that includes physical forces. The specific question we want to ask is whether cell shoving alone can explain the observed pattern, or if additional mechanisms, such as excretion of surfactants, are necessary to reproduce the patterns. This could in turn give more clear indications about how to design further experiments.

On the experimental side, it would be important to obtain a more detailed overview of the temporal dynamics of the pattern formation. To achieve this goal, it would be helpful to be able to find conditions where the producer and consumer either grow completely simultaneously or successively. This would allow to observe the early stages of pattern formation and open the route to finding the fundamental differences between the two situations. A way to achieve this goal is to determine the exact frequencies at which the whole expansion zone is growing either simultaneously or successively and then to perform a more detailed time-course experiment. We are currently working on experiments to establish these conditions. If we could grow them either simultaneously or successively, it would be important to determine at which stage the consumer manages to establish at the front ahead of the producer, because this could potentially help to infer the mechanism by which it reaches that position.

In general our results show that pattern formation can have opposite consequences depending on the boundary conditions. This is another example why one should be careful when drawing conclusions from pure pattern analysis without knowledge of the underlying mechanisms and boundary conditions of the system. The findings represented in this chapter are not yet fully conclusive, they represent a collection of the current status of our knowledge. Clearly more research is needed to work out the details of the mechanisms. Nevertheless the results are very intriguing and we hope to gain further insights soon.

6.5 Acknowledgments

We acknowledge Teresa Colangelo for helping with the re-sequencing lab work and Martin Ackermann and Dani Or for useful discussions.

6.6 Materials and methods

6.6.1 Strains and growth conditions

The same *Pseudomonas stutzeri* strains were used as in chapter 1 and 2. The strains were grown on the same anaerobic lysogeny broth (LB) agar plates as in chapter 1 and 2.

6.6.2 Restreaking experiments

To pick a pair of highly intermixed strains a spot at the edge of the colonies was chosen, where the strains were growing intermixed and were protruding from the successive expansion front. The edge was picked with a sterile toothpick under a stereomicroscope. The toothpick was then streaked on LB-agar plates that were supplemented with isopropyl β-D-1-thiogalactopyranoside (IPGT) (0.1 uM final) and gentamicin (10 ug/ml final). These plates were incubated over night at 30°C. Then colonies that were clearly separated and consisted only of one color were marked using a confocal microscope (Leica TCS SP5 II). Those colonies were then restreaked on LB-agar plates to provide inocula for experiments, sequencing, and to prepare frozen glycerol stocks. Glycerol stocks were prepared by mixing a liquid over night culture with a 50% glycerol solution to a final concentration of 15% glycerol and subsequently stored at -80°C.

6.6.3 Whole genome sequencing

The genomes of 4 pairs of strains that were isolated in the abovementioned way were sequenced using the Illumina MiSeq platform (Illumina Inc., San Diego, CA 92122 USA). The sequencing and data analysis was done at the Genomic Diversity Center at ETH Zurich. DNA was extracted from an over night culture that was inoculated with a single colony with the wizard genomic DNA extraction kit (Promega Corporation, Madison, WI 53711 USA) according to the protocol. RNA was digested using RNAseA from 5 Prime Inc. (Gaithersburg, MD 20878 USA). The sequencing libraries were prepared from the purified DNA with the Nextera XT Sample Preparation and Nextera XT Index kit from Illumina Inc. according to the manufacturers protocol. PhiX control library (Illumina Inc.) was added to 1% final concentration to the libraries prior to sequencing. The libraries were then sequenced using the Illumina MiSeq Reagent Kit v3. Because the MiSeq machine had a system failure during loading of the sequencing flow-cell, it had to be repaired and restarted, which lead to a ca. 3h interrupt between loading the flow cell and analyzing it on the MiSeq. Illumina Inc. therefore kindly replaced the flow cell and the same libraries, which were stored frozen at -20°C, were re-run 1 month later. Both data sets were combined for data analysis. The data was compared to previous whole genome sequencing of the original strains that were used for strain construction to remove mutations that were already present in the original strains, but not in the reference genome.

6.6.4 Re-sequencing regions of interest

All the potential non-synonymous mutations that were identified by the whole genome sequencing were verified using Sanger sequencing. Whole genome DNA was extracted from liquid LB over night cultures using the Wizard Genomic DNA kit. Then the target regions were amplified by PCR and the correct product length was checked using agarose gels. The PCR product was cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega Inc.) and the concentration of the clean DNA was quantified with a spectrophotometer (Nanodrop, ThermoFisher Scientific) or a fluorometer (Qbit, ThermoFisher Scientific). The DNA was mixed with the corresponding forward primer, diluted to the required concentration with sterile double distilled water, and sequenced at Microsynth (9436 Balgach, Switzerland).

6.6.5 Image acquisition and analysis

Images of the colonies and image analysis were done as in chapters 1 and 2. Images were acquired with a confocal microscope, exported as tif files and analyzed using ImageJ. To measure the intermixing index the green channel was thresholded using the previously described local thresholding algorithm and then measured using the Sholl plugin. The resulting data was analyzed in R.

6.6.6 Counting mixed areas

The number of highly mixed areas was counted by hand. Only areas where the green, nitrite-consuming population was growing from the inoculum to the edge of the colony. The data shown was collected from a single experiment. In this experiment every colony had at least one mixed area and there were not too many areas such that the overlap prohibited accurate counting.

6.6.7 Comparing different inocula

In this experiment 10 colonies of each an ancestral consumer, an ancestral producer, an isolated consumer, and an isolated producer were used as inocula for range expansion experiments. The frozen stocks of each of the abovementioned strains were streaked on LB-agar plates to single colonies. Then 10 of these colonies per strain were used to inoculate separate tubes containing liquid LB. These tubes were inoculated overnight at 30°C and the cell densities were then adjusted as mentioned above. Then 10 mixtures of cross-feeding strain pairs from the ancestral strains and 10 mixtures of the isolated strains were prepared and used to inoculate anaerobic agar plates. The plates were imaged after 4 weeks of anaerobic growth. The images were scored depending on whether the whole colony was growing mixed or not.

6.7 Supplementary information

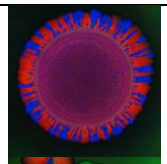
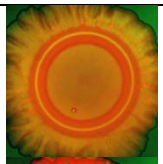
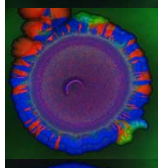
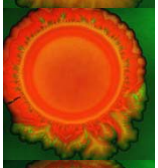
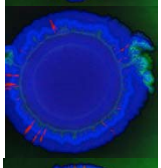
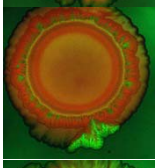
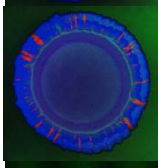
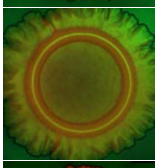
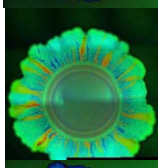
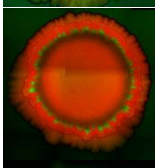
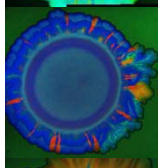
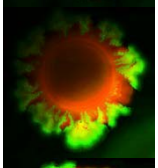
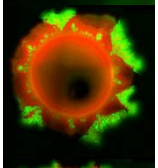
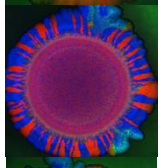
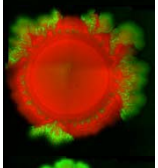
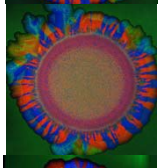
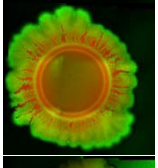
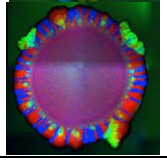
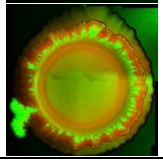
	Ancestral	Mixing		Isolated	Mixing
1		No			Yes
2		No			No
3		No			No
4		No			Yes
5		Yes			No
6		No			No
7		Yes			Yes
8		No			No
9		No			Yes
10		No			No

Table S6.1 Images showing colonies that were grown from different inocula. Each colony shown above was grown with a different inoculum of a blue or red producer and a green consumer (ancestral strains) or a red producer and a green consumer (isolated strains). Below each colony it is indicated whether it was growing completely mixed or not.

6.7.1 Resemblance with macroscopic water flow patterns

In spatial systems the pattern can be an indicator of the underlying process[15]. Because it was not clear how patches of simultaneous growth emerged, we analyzed the pattern dynamics in more detail. In the colonies where the two cross-feeding populations were growing simultaneously in the entire expansion zone, the consumers seemed to form structures that resembled rivers whose catchment area started at the inoculation zone and that coalesced in outwards direction into larger streams. Towards the end they resembled river deltas and the two populations mixed on a very fine scale (Figure 6.2). The flow of the pattern was therefore reversed to the regular, successive pattern, where the consumers formed outward branching dendrites (Figure 6.1).

To quantify the dynamics of intermixing during simultaneous expansion, we measured an intermixing index in concentric circles throughout the colony. As expected, intermixing was constant in the inoculation zone, and then it increased, decreased and increased again until it dropped at the edge of the colonies (Figure S6.1). By plotting the points of inflection of this curve onto the images of the colonies we found that this behavior corresponded to the three phases mentioned above: intermixing increased at the beginning of the expansion in the „catchment zone“ of the rivers, then in the intermediate zone of expansion it dropped (similar to the coalescence of small rivers into a stream) at the end in the delta-zone it went up again. This dynamic behavior of the intermixing pattern is surprisingly reminiscent of e.g. the erosion pattern of water flow (Figure S6.1).

The pattern dynamics therefore suggest that there might be an underlying force that changes during the expansion and leads to more or less intermixing. A possible driver of the intermixing dynamics is the expansion velocity. From previous experiments (Chapter 2), we know that the expansion over time follows a sigmoid shape. Expansion velocity is thus fastest in an intermediate stage and slower at the very beginning and after about a week of expansion. This potentially coincides with the observed pattern changes. However more detailed experiments where the expansion speed could be manipulated would be necessary to test this hypothesis. Nevertheless, the different intermixing dynamics at different stages of the expansion zone suggest that there is a temporal variation in the pattern forming process.

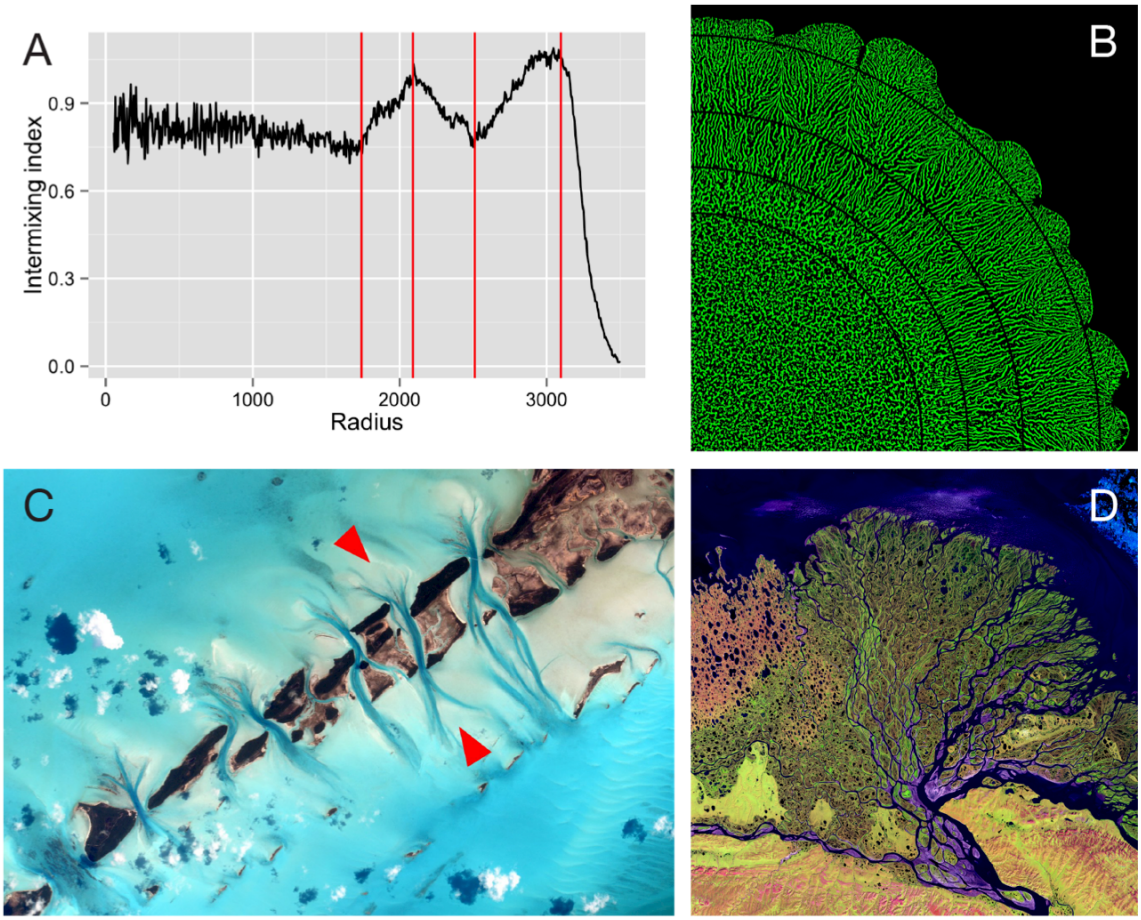


Figure S6.1 Branching dynamics. A) Intermixing at different radii and **B)** corresponding thresholded image of the colony. The vertical red lines indicate points of inflection of the curve and correspond with the black circles in the image of the colony. Intermixing increases at the beginning of the expansion. It then decreases in an intermediate phase, because the small „streams" coalesce into larger „rivers". In the end intermixing increases again, because the rivers branch into „deltas". Second row: Water flow can generate similar patterns. **C)** Photograph of part of the Bahamas seen from the International Space Station (NASA/Scott Kelly, public domain). The water gets accelerated when it flows between the small islands and forms dendritic patterns at the entrance and exits, which coalesce between the islands (see e.g. red arrows). **D)** Satellite image of the Lena river delta in false colors (NASA/Landsat, public domain). When the river reaches sea level, the flow velocity decreases and the transported sediments get deposited. With time the river has to push through the deposits and forms a branched dendritic structure.

6.7.2 Deleterious effect of simultaneous growth on the complete degraders

To determine the effect of the presence of the consumer on pattern formation in the complete degrader, we have previously inoculated the consumer together with the complete degrader (Chapter 5). We found that the patterning of the complete degrader is not significantly affected by the presence of the consumer, probably because it grows faster than the consumer and thus outcompetes it quickly. However, in some rare occasions we observed that the consumer could establish at the front together with the complete degrader. The pattern formed by the consumer was qualitatively the same as when growing simultaneously with the producer. The effect on the expansion velocity of the two populations, however, was the opposite. When the consumer was growing simultaneously at the primary front with the complete degrader, the expansion typically lagged behind the expansion front of the complete degrader growing alone (Figure S6.2).

This finding of an adverse effect of the presence of the consumer on the expansion of the complete degrader can potentially be explained by a more detailed analysis of the denitrification pathway. From previous studies we know that the complete degrader first utilizes nitrate before it starts using the nitrite, which results in an intermediate nitrite peak[16]. This is likely due to the regulation of the denitrification pathway and inter-enzyme competition for electron donors of the nitrate and nitrite reductases[17,18]. The presence of nitrate simultaneously induces the expression of both nitrate and nitrite reductases, so the complete degrader cells pay the full cost of the whole degradation pathway[18]. The exact time difference between the occurrence of nitrate and nitrite reductases is only about 30 minutes[19]. However, as long as the nitrate levels are sufficiently high the reduction of nitrite is ihnhibited[18]. This means that both enzymes are present in the cell, but initially mostly the nitrate reductase is active, because it has a higher affinity to electron donors[17]. The nitrate concentration should be highest at the expansion front, where new territory with abundant nitrate is constantly invaded. We thus expect that the complete degraders utilize mostly nitrate at the front. The consumer on the other hand can only grow if it reduces nitrite.

The continuous presence of the consumer at the wave front thus indicates an active nitrite degradation, which locally reduces nitrite concentrations. This in turn removes the nitrite from the complete degrader, who nevertheless pays the cost of producing the nitrite degradation enzymes. In total the growth rate of the two populations growing together might thus be reduced because the complete degrader pays the cost of producing nitrite reductases, without added benefit for it. This indicates that the highly mixed simultaneous growth pattern can have favorable or deleterious effects, depending on the composition of the two populations.

The presence of the consumer at the wave front has an opposite effect on the producer or the complete degrader. It accelerates expansion velocity when growing together with the producer and decelerates expansion when growing together with the complete degrader. However, the formed pattern between the consumer and the respective other population is qualitatively the same. In the case of growing with the complete degrader, the consumers can be seen as cheaters that profit from nitrite production but don't pay the cost of expressing the nitrate degradation enzymes. In general, the spatial constraints provided by the expansion purged the consumers efficiently from the front. However, in some rare instances it did not. It would be interesting to investigate the exact circumstances, which allow the cheating consumers to prevail at the front.

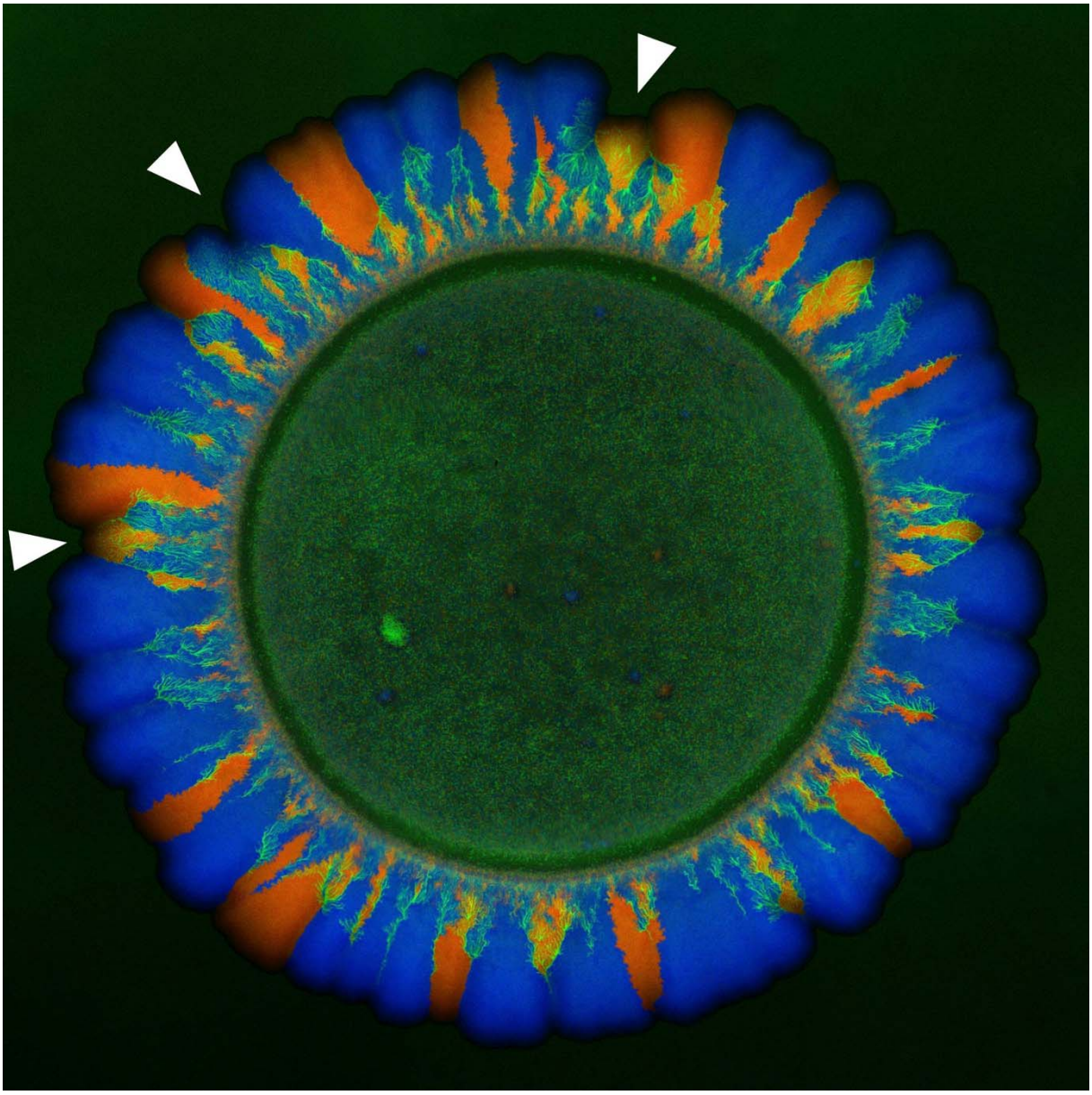


Figure S6.2 Simultaneous growth of complete degraders and consumers. The complete degraders generally outcompete the consumers quickly. However, at the positions indicated by the white arrows the consumers was growing simultaneously with the complete degraders and formed the same highly mixed pattern. As can be seen the two populations expanded less when growing together than the complete degraders alone.

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7 Discussion and outlook

In this thesis I have studied the consequences of metabolic interactions between microbial populations on spatial self-organization in expanding communities. In general I have shown that the spatio-temporal dynamics of growth can have important and sometimes non-intuitive consequences on the spatial self-organization of the different populations. In summary, the spatial self-organization of the interacting microbial populations is largely determined by who is growing when and where. This statement can be divided into two main parts: first, who is growing and how fast are they growing? And second, when and where are they growing? Of course these two aspects are intertwined and influence each other. However, I believe that this way of thinking is a good starting point when analyzing and predicting spatial self-organization.

7.1 The role of growth rate

When investigating only one population, an important quantity which determines spatial population diversity in an expanding population is the effective population size[1,2]. The size of the effective population at the edge of a colony influences how strong drift will act, which in turn determines the amount of demixing of allelels[3,4]. The effective population size is influenced by the amount of available nutrients[5,6]. If there are more nutrients available, the microbial cells at the front of the expansion will grow faster, and once they reach their maximum growth rate there will be more leftover nutrients for following cells.

However, as I have shown in Chapter 5, nutrient levels are not the only factors that determine effective population size. By increasing the toxicity of the cross-fed metabolite nitrite, I experimentally (and theoretically) reduced the maximum growth rate of the cells. This had the effect that the cells at the front of the expansion metabolized the growth limiting substrate nitrate slower, which again made more nitrate available for the following cells. This increased the effectively growing part of the population, which reduced the effect of drift and led to higher sector numbers. It can thus be seen that in order to understand the potential effect of range expansions on spatial population diversity, it is important to know the factors that enhance or reduce growth rates in the population.

To further assess the role of growth rates in spatial population diversity, one could manipulate other factors that determine microbial growth rates. A potentially important factor is e.g. the temperature at which the microbes are grown[7–9]. Microbes typically have an optimum growth temperature, at which the growth rate is maximal. By varying the temperature, it could therefore be possible to achieve similar effects than by manipulating toxicity. Considering my results from Chapter 5, I expect higher sector numbers at non-optimal growth temperatures, when the other growth determining parameters are kept the same.

The mechanism is likely the same as above: lower temperatures shift the growth rate away from the maximum, which has the consequence that more nutrients are available for cells that are not directly at the expansion front. Designing an experiment that allows temperature control should be fairly easy; however, it might be important to control for confounding factors, such as faster drying of the agar surface at higher temperatures. Testing this hypothesis thus seems feasible and the expectations are relatively clear. On the other hand, if the outcome is not as expected, new non-intuitive mechanisms might be revealed.

7.2 Spatio-temporal dynamics

Including different populations that interact via metabolites adds a layer of complexity to the spatial population dynamics, because the increase or inhibition of growth of a focal population depends also on the interacting partner. Especially with interactions, it is therefore also important to know when and where the different interacting populations grow. As I have shown in Chapter 4, the sequential nature of the cross-feeding system that we constructed led to successive growth of the two populations. This demixed the populations at the primary front of the expansion where only the producer was growing, but it increased intermixing of the two populations in the secondary expansion zone where the consumer was branching into the producer.

I have shown that succession on it's own had a strong effect on spatial population diversity. However, it also affected how the populations interacted with each other when the cross-fed metabolite was toxic. If the producer had grown simultaneously with the consumer, the toxic metabolite would presumably have caused a mutualistic relationship between the two. The producer would have provided nutrients and the consumer would have detoxified. However, as I have shown in Chapter 5, the toxicity did not strongly change the interaction between the two, presumably because the producer advanced too far ahead of the consumer to benefit noticeably from the reduction in toxicity. The spatio-temporal dynamics of the system thus had a strong influence on the self-arrangement of the interacting populations.

A future direction could be to study whether the spatial arrangement is an evolvable trait. As described in Chapter 5, the spatio-temporal segregation of the two cross-feeding strains led to slower colony expansion velocities compared to the complete-degraders under both non-toxic and toxic conditions. Interestingly, we have previously shown that in a well-mixed system in liquid batch cultures, the cross-feeding strains could grow relatively faster than the completely degrading strain at toxic conditions[10]. The reason for this discrepancy is likely that in the spatially structured system, detoxification was not occurring at the same place and time as nitrite production. This is because the producer and the consumer were not growing simultaneously at the same place, which impeded feedback between the two metabolic processes.

It would be interesting to investigate whether the two cross-feeders can evolve a more efficient spatial arrangement. One expectation is that simultaneous growth of the two cross-feeders could evolve, which should increase expansion velocity, especially under toxic conditions. As I have shown in Chapter 6, simultaneous growth is possible and often emerges spontaneously in the spatial system. However, the simultaneous pattern did not appear to be very stable. I would expect that during prolonged growth in a spatially structured system, simultaneously growing phenotypes should take over the expansion zone.

A possible way to approach this hypothesis experimentally could be to serially inoculate plates without going through the intermediate step of liquid pre-cultures that were used in this study. Because the simultaneously growing strains expand more rapidly than the successively growing strains, they should be able to outcompete the successively growing strains. If this trait would be genetically encoded, it would be possible to compare the adaptations to spatial structure with the adaptations to a well-mixed system that has been previously studied in our group. An interesting question that has not been studied largely, in my view, is whether there are trade-offs between the evolutionary trajectories of well-mixed and spatial growth, i.e. whether it is possible to increase fitness in both situations or whether a microbe has to specialize.

7.3 Mutations

In the second part of Chapter 4, I have analyzed how readily a mutation that occurs in a primary or dendritic secondary expanding wave becomes extinct during the expansion. The model that I've developed in Chapter 4 predicts that mutations in the primary wave require a higher relative fitness than in the dendritic wave in order not become extinct.

This prediction could be tested experimentally. It is not straight-forward to construct mutants that have a higher fitness compared to the ancestral populations. However, introducing genetic modification that have a cost, and therefore should reduce the relative fitness, seems more feasible. These mutants could then be used as a reference and the actual ancestors as the higher fitness variants. The expectation is that the relative proportion of the variants with higher fitness should be higher in the secondary dendritic expansion. Testing this hypothesis experimentally and quantifying the difference between a dendritic and a continuous expansion could help to understand the influence of succession on the genetic diversity of populations.

To gain further theoretical insights into the fate of mutations in dendritic expansions, it could be useful to develop a discrete and spatially explicit population model. The model that I used is a reaction-diffusion type simulation. A general problem with these models is that they assume a continuous population, while organisms in reality are discrete entities[11]. It has been shown with discrete models that mutations can reach high frequencies at the expansion-front in a process which is called surfing of mutations[12,13]. Interestingly, surfing can occur for neutral and even deleterious mutations[13,14]. In our model, we did not observe surfing of neutral mutations, which might be because of the continuous nature of the reaction-diffusion model. Studying the effects of dendritic expansion with discrete models could therefore provide broader insights into the fate of neutral and deleterious mutations at the wave front.

7.4 Dispersal

A general question is how the findings presented here about the effects of succession on range expansions apply to organisms other than microbes[15]. Microbes have been used as model system to study the spatial population dynamics during range expansions[4,16–18]. The experimental findings often matched the predictions of previously developed mathematical models well. As with every model system, the quality of the predictions depends on how well the model system reflects the dynamics of the natural system that one aims to study.

Besides effective population size, another important factor influencing the spatial population diversity of expanding populations is the migration or dispersal rate[1,2]. In the experiments presented in this thesis and also previous studies that used microbial growth on agar plates as model systems, the agar hardness was selected such that the bacteria were nearly immobile and expansion was thus mainly the result of growth and cell shoving[4,6,16,18,19]. I therefore think that an important factor that determines whether the results presented here apply to other organisms is if the organisms of interest have similar dispersal characteristics. As said before, the branching process depends on spatial constraints that are imposed by the primary colonizer. If dispersal is very high, such as for example in birds, it seems unlikely that the same rules apply, because the secondary organism can simply move to unoccupied space. If dispersal is limited, however, I expect that branching of the wave of the secondary colonizers can also occur in succession of other organisms.

7.5 Conclusion

During this thesis it became clear that even in a seemingly simple system consisting of two cross-feeding populations, relatively complex and sometimes non-intuitive population dynamics could emerge. Adding more interacting partners with new metabolic functions can lead to higher order interactions and indirect feed-back mechanisms, which adds additional layers of complexity. In natural microbial systems, there are often hundreds or more different metabolic functions present[20]. Understanding the dynamics of natural systems with sufficient rigor to make clear predictions will therefore likely remain challenging. However, studying the fundamental principles of metabolic interactions and their role in population dynamics, both in spatially structured and well-mixed systems, could be the foundation to understand and finally control microbial systems.

7.6 Literature

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