PROPOSED RESEARCH

EARLY CAREER APPLICATION	*Application Reference* RGY0064/2022

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Background

Predicting how organisms respond to novel environments is a fundamental challenge[1]. Will coral reefs withstand global warming and increasing ocean acidity? How do pollinator populations decline with habitat degradation? What are the effects of antibiotics on the human microbiome and consequent health? Immigration, phenotypic plasticity, and adaptive evolution are the primary mechanisms that allow populations to be 'rescued' from extinction or to establish in new habitats following changes in environmental conditions[2]. Immigration increases abundance, providing more time for adaptive evolution, and increases genetic variation on which selection can operate[2]. Phenotypic plasticity is the ability of genotypes to adjust their phenotype according to the conditions they experience[3]. It operates on standing genetic variation and thus offers a rapid ability to withstand environmental changes. However, it may be insufficient if the mismatch between plastic traits and new environmental conditions is too large[4]. Evolution, on the other hand, involves genetic changes via mutation, horizontal gene transfer (HGT), or other mechanisms and provides long-term adaptation[5]. For example, in populations of *Pseudomonas fluorescens* exposed to antibiotics, phenotypic plasticity was evident in genetically diverse populations, providing more time for antibiotic-resistant mutations to evolve[6].

This theory of rescue effects[2] is fundamental for predicting the response of individual organisms to environmental change[7–9]. Nevertheless, organisms do not occur in isolation but instead form part of diverse communities in which they interact in multiple ways. Interactions both regulate populations (e.g., via competition) and create co-dependence. For example, Goldford et al.[10] have shown that bacteria competing for a single carbon source coexisted because each strain grew on the secretions of the others, creating a cross-feeding network. On the other hand, individual organisms will be influenced by their surrounding community[11,12]. Continuing the example[10], cross-feeding networks have collective properties that allow individual strains to persist, possibly via higher-order interactions (HOI)[13].

In the language of statistical physics, organism-level processes such as rescue effects and pairwise interactions such as competition are broadly referred to as *microscopic-level rules*. These rules influence higher levels of system description: the *meso-* and *macroscopic scales*, where units stop functioning as individual parts and start exhibiting collective behavior[14] (Fig. 1). Emerging mesoscale structures have collective properties that cannot be attributed to any individual constituent[15]. Specifically, their stability—a multifaceted concept that broadly refers to their ability to withstand perturbations[16]. Networks are an ideal mathematical tool for investigating the mesoscale structures

emerging from microscopic-level processes and have been widely applied to describe the structure and dynamics of ecological communities[12,17–20]. An early insight was that diversity alone does not provide stability[21], leading to a rich history of studying the link between the structure of species interaction networks and their stability[16,18]. For example, modular networks partitioned into groups of strongly-interacting species are more resilient to perturbations than networks of randomly interacting species[22,23].

Advances in ecology go hand in hand with recent developments in statistical physics that allow coping with increasing levels of complexity such as multiple kinds of relationships[24,25]. Such complexity can be modeled using multilayer networks, whose structure and stability result from processes that operate across multiple networks (layers) that form the same system[26–28]. For instance, in bacteria, community stability is affected by both competition and mutualism[29]. Nevertheless, how the interplay between HGT networks (who donates genes to whom[30]) and other interaction networks (e.g., competition) affects stability remains unstudied. This is a major gap because HGT is a major driver of genetic change and adaptation in microbes.

Another knowledge gap is the mechanistic link between environment, structure and stability. The type, rate, and strength of the environmental change will mediate rescue mechanisms[5], affecting the structure and hence community stability[31,32]. Therefore, particular communities will be more stable than others in specific environments due to their collective, irreducible system properties[31–33]. While the structure-stability debate has attracted extensive research[16], how the environment mediates it is understudied. The type of environmental change is crucial because communities can be stable in one environment and not the other. For example, the composition of bacterial soil communities gradually shifts with increasing salinity[34]. Recent work has shown a correlation between environmental variability and the stability of ecological networks but did not include individual species[32].

Overall, this line of evidence indicates that in the face of environmental change, the interplay between species-level processes (in particular HGT), community structure and the environment will determine the stability of communities and the persistence of their constituents (Fig. 1). Nevertheless, the study of these three components has been largely disconnected. **This proposal aims to develop a systematic research program to address this knowledge gap by combining theory and experiments.** To this end, we have defined three aims with individual work packages (WP). In Aim 1, we will develop a model to link the three components that will allow us to test how specific processes affect response to different environmental changes. We will particularly test the role of HGT and its interplay with competition. In Aim 2, we will experimentally test the theory. First, we will establish a benchmark for community structure using soil bacteria to quantify how pairwise interactions respond to environmental change. Then, we will scale up to test the response of entire communities to multiple kinds of environmental change. We will also identify genetic changes underlying adaptations. Aim 3 will experimentally address the HGT part of Aim 1. We will use the communities from Aim 2 together with plasmids to build experimental HGT networks. This will allow us to test how the structure of a multilayer network coupling an HGT and competition layers affects the response of bacterial communities to environmental change.

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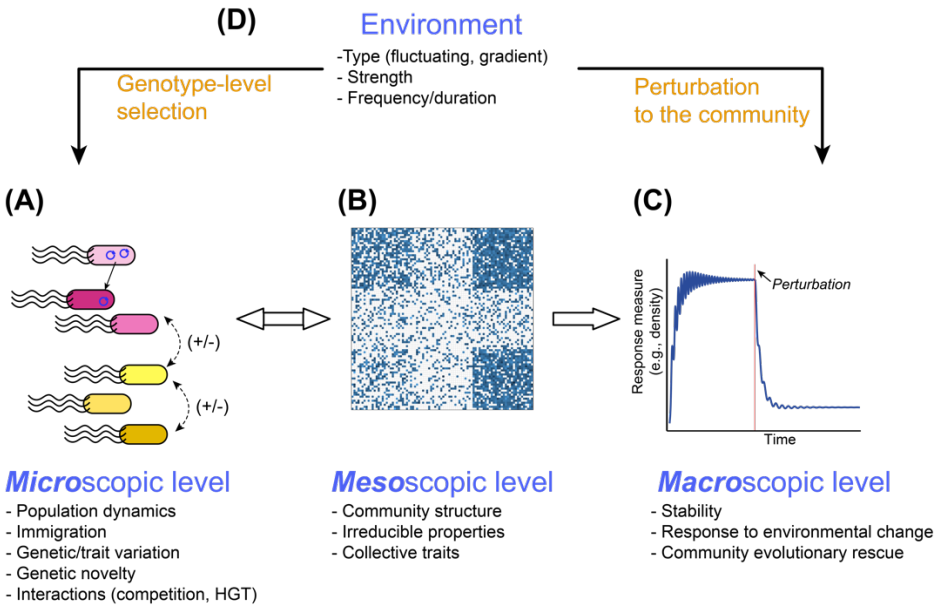

Fig. 1. Proposal's hypothesis. **(A)** Microscopic processes operate at the genotype/population level. **(B)** These processes create, and are at an interplay with, mesoscopic community structures. **(C)** Mesoscopic structures determine the response of the community to environmental change. **(D)** the environment affects the microscopic level via selection but changes in the environment can be considered perturbations.

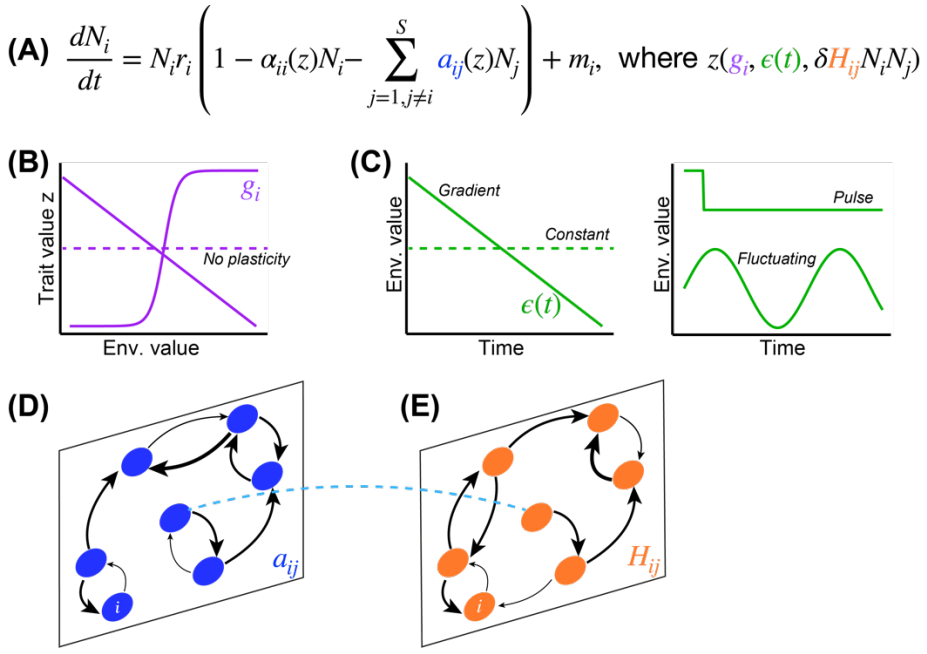

Fig. 2. Theoretical model framework. **(A)** A dynamical model in which competition coefficients depend on a trait z. **(B)**, The trait exhibits phenotypic plasticity, modeled as reaction norms, determining its expression value in a given environment. **(C)** Different classes of environmental changes can be introduced (two plots for clarity). **(D)** Competition network is an emergent collective feature. **(E)** HGT network will affect z. The blue dashed edge (only one shown for clarity) encodes the interplay between competition and HGT, creating a functional multilayer network.

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Aim 1 (SP/MDD): Develop an overarching theoretical framework

We propose a modeling framework that considers four main components: (1) plastic variation in the value of a trait; (2) evolution; (3) mesoscopic structure; and (4) environmental heterogeneity and perturbations (Fig. 2). This approach leads to a nonlinear system with analogs in physics of collective behavior[35,36]. The design of our computational experiments (Table 1) will allow us to compare the relative contribution of different model components to emerging mesoscopic structure and stability. We will implement the modeling framework using deterministic models (but see Risk Analysis). In WP1a, we will investigate the evolution of mesoscopic structures emerging under different conditions. In WP1b, we will explore the stability of evolving communities and how those that have evolved in one environment respond when exposed to another.

WP1a: How is community evolution mediated by the environment?

The model (Fig. 2A) tracks the abundance N_i of each genotype (=species) i with growth rate r_i in a system with S genotypes (S changes in time). Genotype populations are regulated by intraspecific (per-capita effect of i on itself, a_{ii}) and interspecific (per-capita effect of j on i, a_{ij}) competition. The terms a_{ii} and a_{ij} depend on a trait z such that reducing intraspecific competition comes at the expense of another (not modeled) trait that increases interspecific competition[37]. For example, increased intake rates of a limiting nutrient (advantageous when the abundance of conspecifics is high) can come at the expense of antibiotic release (advantageous when the abundance of heterospecifics is high) because limited cell resources cannot be diverted to both. This tradeoff directly addresses our hypothesis because investment at the genotype level comes at the expense of the community level.

Phenotypic plasticity is typically modeled using 'reaction norms' to describe changes in trait values as a function of environmental conditions for a specific genotype[38]. The value of z depends on a reaction norm g_i (Fig. 2B). *Candidate* forms of reaction norms are sigmoidal[39], linear[40], and gaussian[41]. Conditioning the environmental value (e.g., resource concentration) ϵ on t allows us to manipulate the environmental factor's duration, frequency, strength, and type (Fig. 2C). We will calibrate the environmental change to the growth rate. We will also consider the effects of demographic rescue via migration rate m_i from a regional pool[42] (metapopulations are beyond the scope).

Heritability plays an essential role in determining the robustness of communities to environmental perturbations[41]. Hence, we will allow the evolution of trait means using a quantitative genetics framework that includes parameters for defining heritability in g_i, leading to evolution of the main trait value (with variance) in genotype i[41]. The trait z will further depend on the HGT network H_{ij}, encoding the additive genetic value that i receives from j (Aim 3). The parameter δ controls the contribution of HGT (0=none, 1=full). We will investigate the multilayer structure created by H_{ij} and a_{ij} to assess the relative contribution of structural (i.e., multilayer) and dynamical (i.e., feedback loops) effects in response to environmental shifts. Specifically, by conditioning z on H_{ij} we can create a tradeoff between competition and the ability to obtain accessory genes that provide an advantage in a given environment[43], in line with Aim 3.

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WP1b: How does variation in evolved structures affect response to perturbations?

Complementing WP1a, we will test the stability of evolved communities to changing environments in two ways. **First**, we will measure multiple system-level (e.g., global stability) and genotype-level (e.g., richness, persistence) indices of stability of our evolved communities, following guidelines by [16,41,44]. The structure of a_{ij} is an emerging property that we will measure using multiple network properties[45]. We will compare the stability of emerging structures to those theoretically known to provide stability, such as modularity or nestedness[23,46] using a version of the model with no evolution and imposed structures (Table 1). Testing against null models (Table 1) will allow us to rule out stability resulting from by-products of assembly processes[44,47]. **Second**, in line with Aim 2, we will expose communities that have evolved in one environment to different kinds of environmental changes after a simulation has converged[42] (Fig 1D). For example, we can expose a community that has assembled under fluctuating dynamics to a press perturbation that reduces the amplitude of the original environment or to another fluctuating environment with a different frequency.

Synergism with experiments

We have specified model contributions to the experiments above. In addition, as detailed in Aims 2+3, we will use the model to generate predictions and to ameliorate potential risks.

Expected outcomes

We will obtain insights into the relative importance of genotype-level, community-level, and environmental factors in determining community structure and multiple aspects of stability. We will also quantify the contribution of horizontal and vertical genetic change to structure and stability.

Risk Analysis

We foresee three main challenges. **First**, a limitation of the deterministic modeling framework is that trait values may not be continuous. A solution to that would be an agent-based model in which each species i is composed of a set of explicitly modeled genes. This will also enable modeling allele exchange via HGT. We opted to start with a deterministic version because we can draw inspiration and methodology from previous studies[37,40,41,44] that provide a foundation and alternative approaches. For example, if the quantitative genetics framework of [41] will not fit the modeling scheme, we can use the approach by [44], directly manipulating values of a_{ij}. However, if resources allow we will develop an ABM. **Second**, exploring the outputs of the model to establish which inputs determine which output—a problem tied to parameterization and model initialization rules. Previous studies and our experiments can guide such caveats. Exploration analyses that focus on one or a few parameters while holding others constant will be of further assistance, specifically when selecting reaction norms. **Third**, deciding on biologically relevant features for environmental changes and categories (e.g., exposure to antibiotics is not the same as nutrient limitation). A first step would be to conduct a literature review of environmental change scenarios (Kéfi et al.[16] can provide a starting point). To focus our efforts, we will prioritize scenarios relevant for testing our Aims 2+3.

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Table 1. Design of computational experiments.

Level	Component	Null model	Biological mechanism
Genotype	Reaction norm	constant	Phenotypic plasticity
	Evolution	No heritability	Adaptive evolution
		$H_{ij} = 0$/uniform	HGT
	Migration	$m = 0$/uniform	Demographic rescue
Community	a_{ij}	$a_{ij} = 0$/random	Community-level rescue
System	Environment	$\epsilon(t) =$ constant/noise	

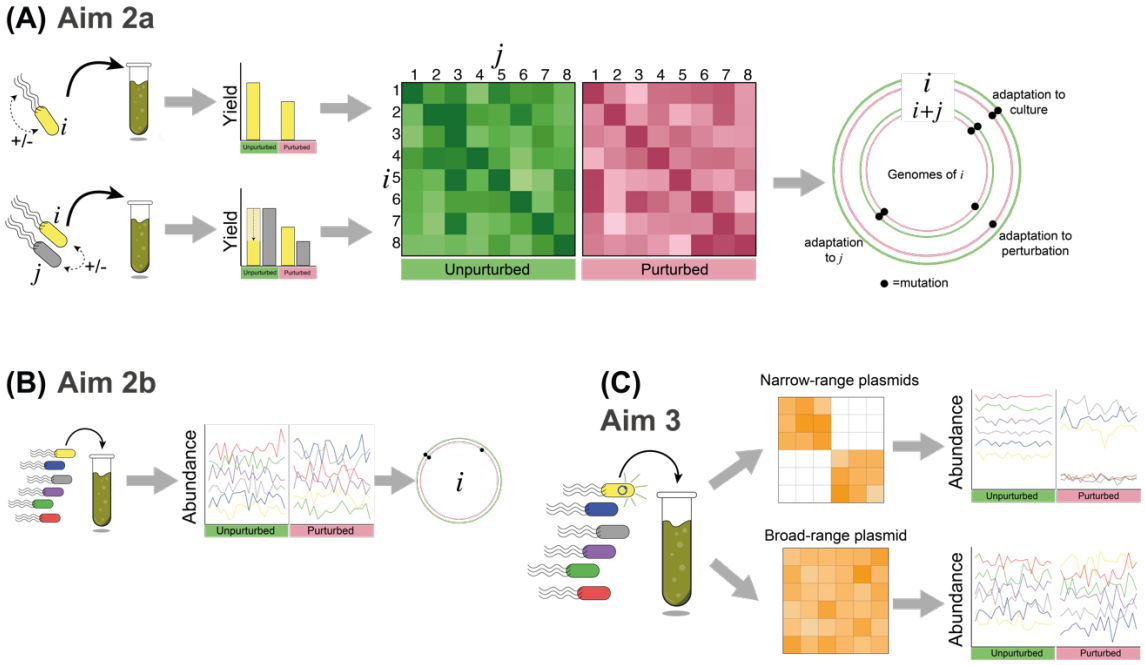

Fig 3. (A) Individual species i will be cultured, or co-cultured (with j) in unperturbed and perturbed environments. We will quantify interactions by comparing the total yield of i when grown alone or with j, each growth cycle. We will quantify environmental effects by comparing yields between treatments. Genomes from co-cultured isolates will be re-sequenced to identify adaptations to ecological treatments. **(B)** To test how species interactions affect the stability of multi-species communities under environmental change, we will subject six- or eight-species assembled communities to perturbations and track relative abundances over time. Metagenomes will reveal if species' adaptations are similar to those from pairwise and single-species culture. **(C)** Communities will be cultured with conjugative plasmids that vary in host range across community members. Community composition and plasmid carriage will be tracked to assess how HGT and competition affect response.

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Aim 2: (JPH/SP): Investigate the response of bacterial communities to environmental change

Experimental work in bacterial systems has shown that species interactions affect community structure and dynamics[10,48] and adaptation to environmental change[49–52]. However, a significant limitation is that, to increase tractability, communities are typically grown in well-mixed, homogeneous media. Furthermore, growth media tends to be either nutrient-rich and labile or with limited and defined carbon sources[10]. This limits the ecological and evolutionary responses available to the resident communities and the relevance of laboratory experiments to the natural world. By contrast, most naturally occurring bacteria of interest exist in spatially-structured, nutrient-complex habitats, such as the soil[53]. Here, we will port an established soil microcosm system[54,55] to a tractable deep-well microplate format, enabling hundreds of independent communities to be cultured as biofilms in sterilized soil media. **This approach will allow us to explore community adaptation across a wide range of community compositions under conditions close to nature, with and without various environmental perturbations.** Leveraging experimental 'evolve-and-resequence' approaches, we will unpick how ecological factors (abiotic perturbation, community interactions) affect genetic adaptation in several coexisting species. The experiment will therefore focus on community adaptations rather than on phenotypic plasticity. In WP2a we will aim to understand the interplay between bacterial interactions and the environment using pairwise experiments. In WP2b we will use this benchmark to understand how whole communities respond to perturbations.

WP2a. How do pairwise interactions in soil communities vary with environmental change?

Our model proposes that the environment mediates interactions and their structure. For example, biotic interactions can inhibit adaptation to an abiotic environment due to selection against the loss of costly nutrient transporters[54]. However, the interplay between adaptation to coexisting species and environmental perturbations is likely to vary between species. We will therefore investigate how pairwise interactions are affected by perturbation. This will provide valuable insights into the genetic bases of adaptation and will generate a benchmark against which full-community experiments (WP2b) and experiments with horizontal gene transfer (Aim 3) will be compared.

Design (Fig. 3A): We will perform pairwise co-culture experiments for eight soil bacterial isolates (28 pairwise combinations + 8 single-species controls), with four replicates per condition (n = 288 populations). The bacteria will be cultured under unperturbed conditions, and under low, selective levels of tetracycline antibiotic, a common soil pollutant[56]. We will focus on Pseudomonadaceae and Enterobacteriaceae, which commonly co-occur in soil. Pairs will be cultured for approximately 300 generations, with continued growth enabled by media replacement at each sampling point. Abundances of each competitor will be measured weekly by qPCR and by selective plating. We will assess the interactions of each competitor as differences in population size compared with single-species controls.

At the end of the experiment, we will perform population resequencing for each evolved population to identify genetic adaptations to the experimental conditions. We will look for treatment-specific parallel mutations, i.e. locus-level mutations that occur in multiple replicates cultured under the same conditions, indicative of adaptation[57]. We will infer the evolutionary response to different factors by comparing competition and environmental treatments.

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We anticipate five classes of mutation: (1) general adaptation to the culture regime, observable across all treatments; (2) general adaptation to the perturbation, or to non-perturbed conditions; (3) general adaptation to the presence of a competitor (observed across all competitors *j* for species *i*); (4) species-specific adaptations, observed only for particular species; and (5) species-by-perturbation adaptations. This experimental design enables us to disentangle general adaptations from those that occur due to interactions and the environment and to determine the reproducibility of such general adaptations across different genetic backgrounds.

WP2b. How do the evolutionary responses of communities vary with composition and environmental change?

Though pairwise interactions may predict the outcomes of more diverse communities[10], it is not clear whether such predictions can accommodate the potential disruptions associated with environmental change. While studies tend to explore single disturbances and extrapolate general ecological and evolutionary consequences, environmental perturbations can take several forms, which can vary over time (fluctuating vs. press), and in nature (antibiotics vs resources). In particular, while most studies focus on detrimental environmental change, community disturbance can potentially occur from a surfeit of resources, such as over-fertilization. We will directly test how adaptation to environmental change depends on species composition, using assembled communities based on the results of WP2a.

Design (Fig. 3B): We will construct three communities: two six-member communities, whose composition will vary depending on the strength of interspecific interactions measured in WP2a (low-interaction and high-interaction communities), and an eight-member community comprising all strains tested in WP2a. We will also test single-species controls. Four replicate communities for each type will be assembled and subjected to one week's growth in unperturbed conditions before being split into perturbed or unperturbed conditions for 150 generations.

Perturbations will include a tetracycline antibiotic and supplementing the soil with high nutrient broth (resource), which we will apply in different forms: constant, pulse and fluctuating. This design will allow us to monitor responses of the same communities to different environmental changes. We will determine relative abundances of species by 16S rDNA amplicon sequencing each week to track composition and stability and will test overall community size weekly by selective plating.

We will perform full community shotgun metagenomics at the end of the experiment to identify genetic changes within each species associated with culture under different community compositions, and in response to perturbations using the same approach as in WP2a. We will also compare mutations occurring in the multispecies communities with those identified from the pairwise experiments to test whether higher-order species interactions affect adaptation.

Synergism with theory

The experiments will allow measuring resistance of community members to perturbations (a proxy for the function of z), and the theory will be used to gain insights into the underlying (nonlinear) response of stability. The experiments will further provide insights into the genetic nature of adaptations, informing the genetic framework of the model. We may also be able to identify tradeoffs such as environmentally-adaptive mutations that do not arise in

the presence of other species[54]. Quantifying such genetic changes will allow us to test the heritability framework we will apply in Aim 1. The experimentally determined interaction matrices (WP2a) will allow us to enhance parameterization of Aim 1 by providing biologically-relevant distribution of competition effects.

We will generate predictions for experiments by modifying the framework of Aim 1. Specifically, we will: (1) match the number of species; (2) exclude the terms for HGT and migration; (3) use values for a_{ij} that correspond with what is expected of the species (e.g., species from the same bacterial family should experience stronger competition); (4) modify the environmental values to match those of the experiments.

Expected outcomes

An integrated, comprehensive picture of how species interactions affect genome evolution and adaptation to environmental change under conditions that closely represent the natural environment. The results of WP2b will show how different modes of perturbation affect stability and evolutionary dynamics. We will also understand the genetic changes behind observed phenotypic adaptations.

Risk Analysis

We foresee three main challenges. **First**, our experimental work program is ambitious. We have therefore designed it such that even if not all is fulfilled, we still gain the insights we aim for. For instance, we can reduce the number of pairwise interactions to 6 instead of 8 in WP2a and the types of environmental perturbations in WP2b. In addition, as the aim of the proposal focuses on phenotypic responses, we can consider reducing the extent of metagenomic analyses. **Second**, one or more of our novel approaches (e.g., high-throughput soil culture) could prove unfeasible. This risk is alleviated because we build on proven techniques, as demonstrated by JPH's long experience working with experimental soil microbial communities (also alleviating the risk of not being able to co-culture multiple species). Also, we can still complete the experiments by falling back on lower-resolution approaches. **Third**, assembling communities with more than two species can involve indirect effects on competition via higher-order interactions—a generic problem in experiments of this kind that is difficult to address without specific experiments[58]. One way to get a handle on HOI is to use recent approaches for assessing HOI effects[59] in combination with our theoretical framework. Nevertheless, because our goal is to test communities' response to perturbations, even if we do not fully understand the role of HOI in the system, we still gain valuable insights.

Aim 3: (JPH/MDD): Investigate the multilayer effect of HGT and competition on community response

Plasmids are one of the most important entities for HGT. Plasmids often carry genes conferring adaptation to environmental change (e.g., antibiotic resistance), but carrying plasmids in the absence of selection confers a fitness cost[43]. Hence, HGT can interact with competition, particularly in a changing environment. For example, the acquisition of resistance via HGT enables otherwise susceptible species to remain competitive in the presence of antibiotics [55]. In contrast, costs imposed by carrying a plasmid in an environment with no antibiotics will make bacteria less competitive.

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HGT is a community-level process connecting bacteria, but it is typically not considered an interaction. Hence, very few studies have investigated networks of HGT[30] and no study that we know of has explored the interplay between the structure of competition and HGT networks theoretically or experimentally, particularly in the context of response to perturbations. One way to model this interplay is with multilayer networks. A hallmark of multilayer networks is that mesoscale structures exist in multiple layers and can dynamically affect each other. In this aim, we will use our soil microbial community approach to experimentally design multilayer networks, with layers encoding the functional coupling between competition and HGT relationships. We will complement experimental results with theory.

Design (Fig. 3C): Plasmids vary in their host ranges. Broad host-range plasmids such as RP4 are capable of replicating in diverse gammaproteobacteria including Enterobacteriaceae and Pseudomonadaceae, whereas narrow host-range plasmids such as R6K and pOZ176 appear largely restricted to Enterobacteriaceae and Pseudomonadaceae respectively . We will leverage plasmid specificity to engineer the links in a HGT network layer (Fig. 2E), which we will confirm using epicPCR[55]. We will use our insights from Aim 2 as a basis for assembling the competition layer (Fig. 2D). Ideally, we will use the same communities as in WP2b. The experiment will include four populations for each plasmid type (n=12 populations).

We will allow communities to establish over two weeks before either exposing them to a tetracycline perturbation or leaving them unperturbed. The plasmids will carry a gene that provides resistance to tetracycline and we will match the perturbation scheme to that of tetracycline in Aim 2. Communities will be cultured for approximately 150 generations, and we will track community composition each week as in Aim 2. We will follow plasmid carriage by epicPCR[55].

Synergism with theory

This aim will inform the model on (1) the relative contribution of HGT (controlled with parameter δ) compared with mutations; and (2) the extent to which H_{ij} and a_{ij} are intertwined. We will generate predictions for experiments by modifying the framework developed for Aim 1 similarly as we did in Aim 2, but here we will match H_{ij} instead of excluding it. In addition, the model will allow us to explore a large space of network configurations varying in the structure of interlayer interactions (effect of competition on HGT), to which we can compare the data.

Expected outcomes:

Insights into the effect of the interplay between HGT and competition on the ability of communities to withstand perturbations. Comparison with Aim 2 will allow us to distinguish between adaptations that are horizontally transmitted to those that have evolved via mutations.

Risk Analysis

The risks are similar to those of Aim 2, with the addition of the use of epicPCR to follow plasmids in multispecies communities. epicPCR was previously used by Hall[55] but if it fails to establish in the current system, we will use

fluorescent protein-based plasmid tagging[60] and/or culture-based techniques (e.g., species-specific PCRs[61]). As with Aim 2, it is possible that HOI will affect community dynamics. For instance, that plasmid carriage affects competitive interactions in a species-specific manner or the way that competitive interactions respond to perturbation. This may complicate the issue of disentangling the causal factors because our measurements will be made in the context of a community. However, we can assess, to some degree, the effects of HOI in multilayer networks theoretically and computationally[62,63] using our model and by comparing between treatments and to results of Aim 2.

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