Drivers of spatial structure in social microbial communities

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Microbes are social organisms, interacting primarily through secreted biomolecules. Many traits have evolved for the express purpose of affecting others, and even those traits that haven't often create social side-effects that are mediated by spatial population structure. Predicting the evolution of many microbial traits thus requires a comprehensive understanding of their social consequences. In this review, we examine the critical role of population spatial structure in microbial social evolution. We briefly review key mechanisms structuring microbial communities, focusing primarily on the universal roles of cellular death and reproduction. Finally, we explain how spatial assortment can be efficiently calculated in 2D surface-attached populations.

cooperation | conflict | microbial interaction | social evolution | public goods | assortment | biophysics

Introduction

Until relatively recently, microbes were thought to be solitary organisms [1] - molecular machines too simple to express the complex social interactions characteristic of larger, more charismatic taxa. Research over the last several decades has flipped this view, revealing that microbes are in fact extremely social: communicating, cooperating, and competing through a diverse and rapidly-expanding set of behaviors [2]. Much like classic examples of cooperation and conflict in animals, many of these social traits have doubtless evolved because of their social consequences (e.g., antibiotics increase the fitness of the actor by harming competitors).

In contrast to explicitly social traits, otherwise individually-beneficial behaviors can also exhibit social side-effects, commonly occurring whenever a trait's evolution is influenced by its effects on other members of the population [3]. For example, bacteria growing on a solid chitin substrate consume it by producing chitinase, freeing short chains of N-acetylglucosamine that can readily be consumed [4]. Chitinase production would appear to be a classic individually-beneficial behavior, necessary for consumption of the food source. But if nearby bacteria consume freed oligomers as well, then chitinase production can incur a social side-effect, as the subsequent reproduction of neighbors increases or decreases the frequency of genes coding for chitinase-production in the population (Figure 1). As microbes have a penchant for dense packing and reliance on extracellular metabolism, many microbial traits will thus evolve socially. In this review, we will examine why spatial structure matters for microbial cooperation, review key biophysical mechanisms structuring microbial populations, and describe how to quantify the degree of structuring in models and experimental images via spatial assortment.

Cooperation, conflict, and population spatial structure

Few topics in evolutionary biology have received as much attention as the evolution of cooperation. Cooperation, in contrast to social traits that directly benefit the actor like killing competitors, is susceptible to exploitation by non-cooperating 'cheats.' This is especially true of altruism, where the actor does not receive a direct fitness benefit. Three general conceptual frameworks have been developed that allow us to explain the evolution of cooperation: inclusive fitness [5, 6], multilevel selection [7] , and ecological mechanisms of cheater avoidance (e.g., partner choice [8], sanctions [9], vertical transmission [10], etc.). In all three frameworks, cooperation can only persist if individuals carrying genes for cooperation get more of the benefits from cooperation than do cheats. Put another way, there must be positive assortment between the benefits of cooperation and its underlying genetics [11]. How does this occur in microbial populations?

Microbial populations are rarely well-mixed. Instead, they tend to be structured in space, with individual microbes living more closely to relatives than would be expected by chance [12] (Figure 2). While some microbes have evolved sophisticated mechanisms for directing resources towards specific cells [13], the majority of secreted biomolecules move via diffusion and bulk transport. In these cases, the spatial structure of a population will determine the extent to which cooperative goods will be consumed by relatives with the genes for cooperation.

Work exploring the evolutionary consequences of spatial structure has deep roots in mathematical biology [14, 15] (beautifully-reviewed in [16]). Indeed, microbial experimental systems offer a maximally-tractable meeting ground for theory and experiments [17, 2], which has strengthened our understanding of social evolution more broadly. While mathematical models typically examine the consequences of population spatial structure on evolutionary processes, in this mini review we focus on the mechanisms generating spatial structure in the first place.

We can mathematically describe microbial spatial structure by calculating the clonal assortment of a focal genotype over a range of spatial scales. For instance, we can define assortment in a scale that goes from 1 (all goods consumed by cooperators) to -1 (all goods consumed by non-producing competitors), with an assortment value of 0 representing a well-mixed population (Figure 2). This measure of assortment is analogous to the 'relatedness' parameter in Hamilton's rule [5]. Spatially-structured populations with high clonal assortment thus strongly favor cooperation among related cells [11]. Note that high clonal assortment is not a universal driver of cooperation: while it favors cooperation among relatives, it inhibits interclonal/interspecies mutualism, which requires close physical proximity between trading partners [18]. In this review we focus on positive spatial structure, which favors cooperation among relatives.

A common theme in social evolution is the production and consumption of 'public goods', which are defined as "A resource that is costly to produce, and provides a benefit to all the individuals in the local group or population." [19]. Incidentally, while evolutionary biologists co-opted this language from economics, we did so incorrectly. In addition to being equally available to others in the population, public goods are defined in economics as being non-rivalrous, such that their use by one individual does not diminish their ability to be used by others. Common goods, on the other hand, are rivalrous [20]. Most goods in microbiology will be common rather than public, though there may be interesting exceptions (i.e., when information is shared, not resources). Terminology aside, high clonal assortment strongly favors investment in common or public goods by increasing the proportion of goods that are utilized by relatives bearing goods-producing genes.

Mechanisms structuring microbial populations

Many organisms live in structured environments [12]. Relative to macro-organisms, however, microbes may be especially predisposed to generating population structure. Clonal reproduction increases the genetic identity of reproducing individuals, relative to sexual recombination, and fast reproductive rates increase the degree to which clonemates are spatially autocorrelated. In this review we only consider surface-attached microbial populations, which are common and easy to analyze quantitatively, but the principles we examine can be applied to any spatially-resolved system (e.g., free-floating aggregates [21]).

Dispersal is a fundamental driver of spatial population structure. Colonization of novel territory rapidly partitions genetic variation among cellular patches in space, structuring populations and ensuring that the majority of cell-cell interactions are with clonemates. This process can be driven by both passive (e.g., being carried on a water or air current) and active processes (e.g., active motility or attraction to chemically or mechanically favorable attachment sites [22]). Indeed, some microbes have even evolved a simple multicellular life cycle, characterized by the growth of a densely-packed multicellular biofilm that reproduces by shedding motile propagules, which can consist of either single cells or small groups. These ultimately disperse away from their parent biofilm, colonize a surface, and form a new biofilm [2, 23].

Many microbial behaviors directly generate spatial structure. Active migration towards a common cue, for example, can spatially co-localize microbes, concentrating clonemates with the same behavior into discrete patches [24]. Secretion of extracellular matrix products can suffocate or displace competitors, cutting them off from nutrient access while simultaneously structuring populations [2, 25]. Cellular shape and chirality can change how cells pack within biofilms, causing a heterogenous population to phase separate into clonal domains [26]. For example, rod shaped cells form wedge shaped growths that tend to get buried beneath round shaped cells, leading to layered structures in biofilms that segregate into distinct patches based on cell shape [27]. Finally, spatial structure is a common outcome of differential growth of different genotypes within a patchy environment. For example, deep sea methanotrophic bacterial mats can have a layered biofilm structure due to gradients in methane seeping to the sediment surface [28, 29], and spatially-varying responses of the host immune system can structure the biofilms of human pathogens [30]. Growth can also induce mechanical stresses that favor cooperation [31], and slow down the rate that structure is dissipated by natural selection [32]. Of course, processes generating structure are often dependent on regulatory mechanisms mediating large suites of cell-cell interactions, such as quorum sensing [33].

Rather than provide a cursory review of the constellation of specific microbial behaviors that can create population structure, with the remainder of our review we focus on two universal biophysical mechanisms that structure microbial communities: birth and death. Birth and death generate spatial structure by preferentially locating new individuals near their parents. If a population is below its carrying capacity and free space is available for growth, then local reproduction (i.e., progeny that do not disperse a long distance from their parents) will create spatial structure. Alternatively, if a population is at its carrying capacity and there is little demographic turnover, then free space must first be created before individuals can reproduce. Microbial warfare, while directly benefiting the victor, can preferentially kill non-relatives and also creates the free space necessary for reproduction. As a result, it can be a powerful driver of spatial structure.

Physical structuring via birth: reproduction into free space. As cells grow along a surface into unoccupied space, simple physical interactions rapidly drive even well-mixed populations into a highly-structured state [37]. As growth along a surface is primarily led by the small fraction of the population at the colony edge, expansion proceeds through a continuous physical bottleneck. This rapidly purges genetic diversity along the growing front, resulting in two-dimensional clonal sectoring (Figure 3 A). As the circumference of the colony increases, all of the clonal patches at the edge either expand along with it or go extinct–leading to larger and more separated clonal microcolonies. This microcolony expansion also introduces a selective effect, as patches of cells with higher growth rates expand more

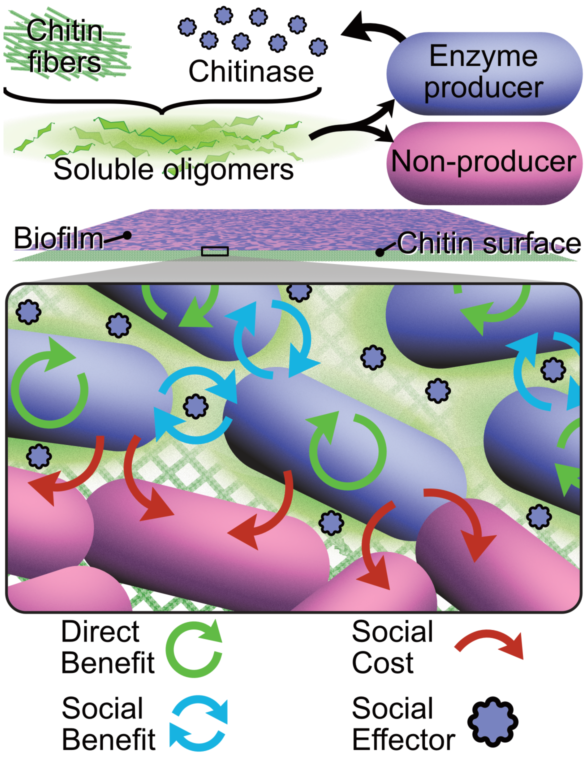


Fig. 1. Social interactions in a spatially-structured population. Microbial behaviors that rely on extracellular secretion will often evolve socially. For example, before a microbe can consume chitin, it must first break it down into short chains of N-acetylglucosamine via chitinase. While chitinase production can thus directly benefit a cell, consumption of chitin oligomers by neighboring cells creates a social fitness side-effect. Preferential consumption by clonemates (with genes for chitinase production) can further increase the frequency of chitinase genes in the population, while preferential consumption by unrelated competitors can exert a social cost. Social benefits are maximized in highly structured populations.

quickly and increase their frequency around the perimeter of a colony (Figure 3 B& C). Importantly, this can provide a strong advantage to genotypes that produce beneficial common goods. As a result of these phenomena, microbial colonies typically become highly structured when they grow along a surface into free space.

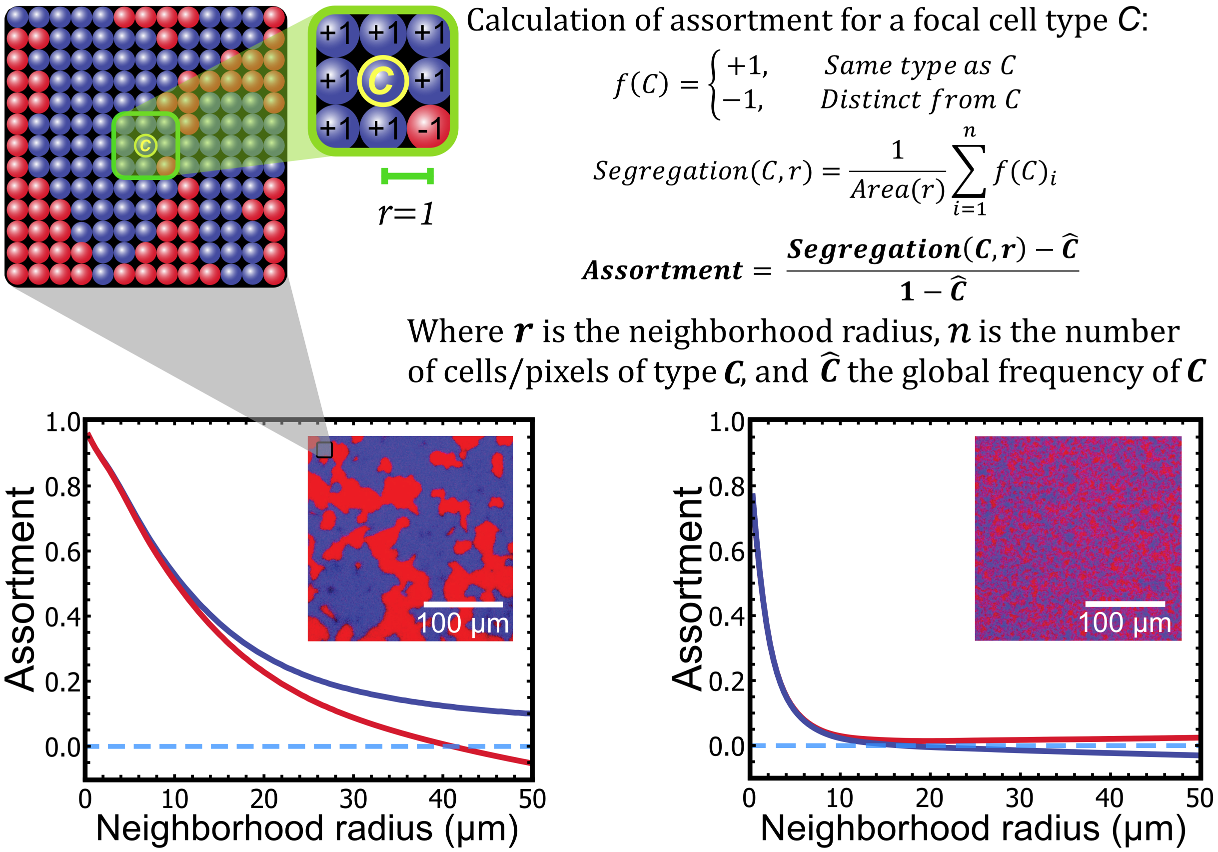
Physical structuring via death: opening free space and preferential killing. Intercellular killing can restructure populations through two related effects. First, killing competitors but not clonemates directly increases assortment, as the killers subsequently occupy a larger fraction of the space in their local neighborhood. Second, killing opens up space for growth. Microbes often live and compete at or near their carrying capacity, such that their population is neither expanding nor shrinking and the amount of cell division and death must be roughly equivalent. In this zero-sum game, for one genotype to expand, another must first lose its territory. While free space from cell death can arise spontaneously due to background cell death or environmental perturbations, it is often created by warfare.

While intercellular killing facilitates the transition from a well-mixed population to one that is highly structured, the emergent population structure and the kinetics of this transition depend on the character of intercellular killing. For example, secreted bacteriocins can diffuse into aqueous media and kill competitors over long distances. In contrast, the type VI secretion system (T6SS) uses a modified phage tail spike to inject antibiotics directly into adjacent cells, thus requiring killer cells to be in direct contact with their targets. While microbes have evolved a wide variety of antibiotic delivery mechanisms, they can largely be described as acting locally–via contact dependent growth inhibition–or non-locally–via diffusible toxins.

Structuring via local killing. Antagonism in nature frequently acts locally. For example, predation in animals involves direct contact between individuals, and allelopathy in plants usually acts close to the toxin producer [39]. In microbial populations, several local killing mechanisms have been identified. Contact-dependent growth inhibition was first observed in bacteria in 2005 in an *E. coli* [40] that uses the type V secretion system to apply a growth inhibiting compound onto adjacent competitors. Since then, a number of locally-acting antibiotics have been discovered, including the type IV, type VI, and type VII secretion systems, outer membrane exchange, and others [41]. These local killing mechanisms are typically targeted, with strains expressing weaponry being themselves immune to that weaponry. Contact-dependent antibiotic delivery mechanisms are a rapidly-developing field within microbiology, but it is clear that they are diverse, modular, relatively conserved, and phylognetically widespread [42, 43].

As this class of killing mechanisms requires close contact with competitors, cell death only occurs at the interface between strains. There, microbes open up space by killing genetically disparate neighbors and replacing those neighbors with genetically identical daughter cells (Fig. 3 D). Through this process, a cell can eventually surround itself with clonemates. However, at this point it ceases killing, thus limiting the rate at which spatial coarsening can occur. In fact, the long-time rate of coarsening is predictable; the growth of clonal domains via contact killing appears to be a member of the broad “Model A” Ising universality class, typical of ferromagnetic materials [44]. In Model A transitions, the size of clonal domains increases with the square root of time. For example, if it takes time t_0 for a clonal patch of size L_0 to grow from well-mixed conditions, it will take $4t_0$ for a clonal patch with twice the size $(2L_0)$ to form. In summary, the fact that killing only occurs at interfaces slows coarsening and mitigates the advantage ‘superior’ killers hold over ‘inferior’ killers. Thus, local killing at interfaces facilitates the creation of durable clonal patches that are large relative to the diffusion length of excreted common goods–favoring cooperation–but it also takes a long time to completely displace a competitor.

Structuring via diffusible toxins. Microbial production of diffusible antibiotic compounds is also widespread [24, 45]. For example, small antimicrobial compounds are produced by as



$$f(C) = \begin{cases} +1, & \textit{Same type as } C \\ -1, & \textit{Distinct from } C \end{cases}$$

$$Segregation(C,r) = \frac{1}{Area(r)} \sum_{i=1}^{n} f(C)_i$$

$$\boldsymbol{Assortment} = \frac{\boldsymbol{Segregation(C,r) - \widehat{C}}}{\boldsymbol{1 - \widehat{C}}}$$

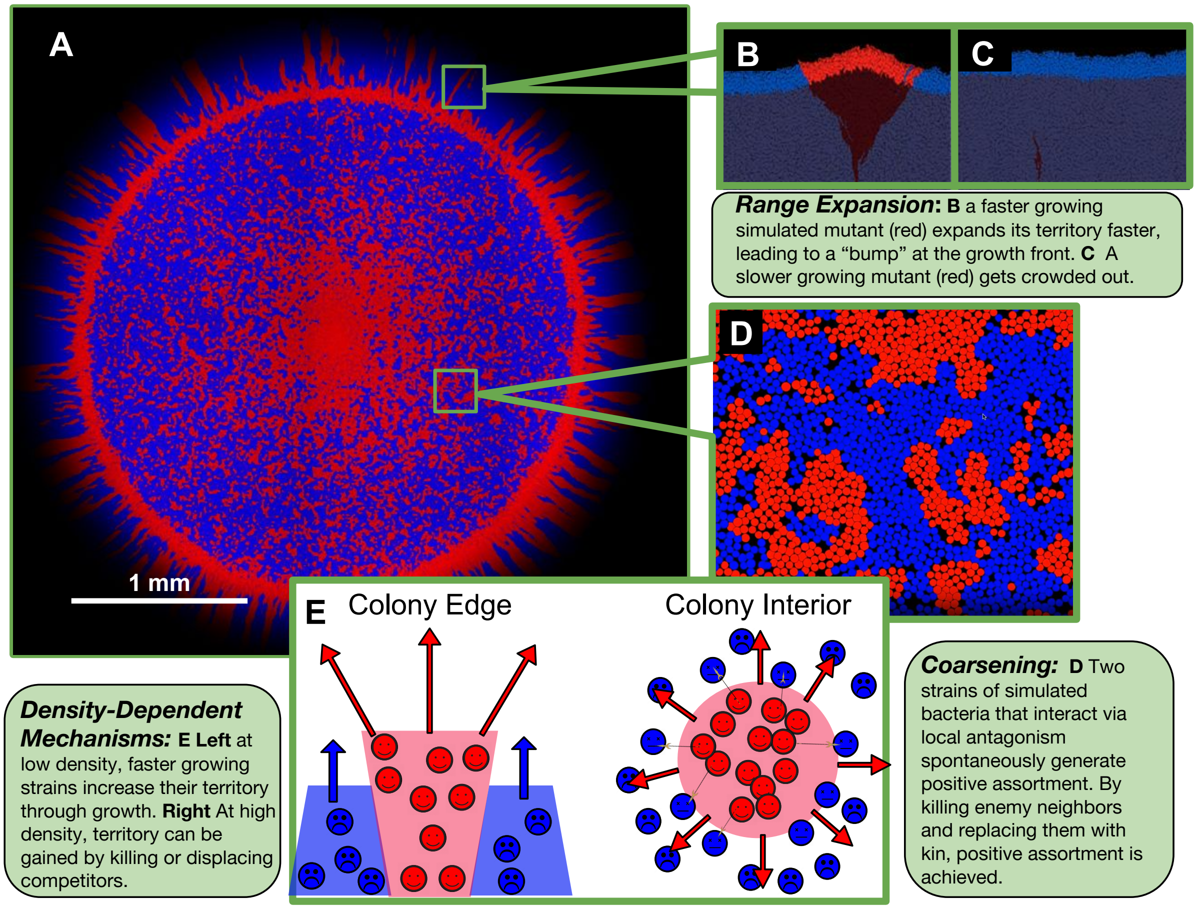
Where $\boldsymbol{r}$ is the neighborhood radius, $\boldsymbol{n}$ is the number of cells/pixels of type $\boldsymbol{C}$, and $\boldsymbol{\widehat{C}}$ the global frequency of $\boldsymbol{C}$

Fig. 2. Calculating spatial assortment. Spatial assortment (analogous to the relatedness parameter often used in social evolution [34, 23, 35, 36]) is easily calculated for populations imaged in 2D. Here we plot each genotype's mean assortment for a range of length scales, from 1-50 µm. The assortment index presented here was adapted from [34] and is bounded between -1 and 1. Assortment values of zero reflect a well mixed population with no preferential interactions between clonemates (dashed blue lines in the plots). Our examples here are two strains of *Vibrio cholerae*, grown for 24 hours on solid media. On the left, we show two strains that can kill each other using the type VI secretion system (T6SS), which creates a highly structured population (bottom left panel). On the right, we show T6SS-deficient knockouts of the same two strains, which produce populations that are structured only over very short distances (bottom right panel). The Python script used to generate these assortment calculations is available here.

much as 50% of *Escherichia coli* [46] and more than 90% of *Pseudomonas* strains [47]. Relatively few studies have directly examined the effect of diffusible antibiotics on microbial spatial structure, but in principle it should work similarly to local killing: displacing killed competitors with clonemates should drive the emergence of spatial structure. Theoretical work suggests that diffusible toxins should generate a high degree of spatial structure when they act locally [48]. However, toxins may instead diffuse far from the secreting cell and its clonemates before killing; the distance a toxin diffuses is critical as local and non-local killing do not generate structure equally well. When microbes in a densely-packed biofilm are killed, their space is often taken by a neighbor through either cellular migration or reproduction. Toxins that act locally will often allow the toxin-producing strain to claim this space, increasing spatial structure. When competitors are killed far from the toxin-producing cell, however, it is far less likely that it (or its clonemates) will claim this free space.

Antibiotic production can also structure populations through non-transitive ecological processes. Like a game of 'Rock-Paper-Scissors' in which there is no universal winner, microbes can coexist in structured populations characterized by stable temporal oscillations. The classic example of microbial Rock-Paper-Scissors occurs with three strains: a toxin-producer that is also resistant to the toxin, a resistant non-producer, and a susceptible non-producer. Here the toxin producer kills the susceptible strain, but is beat by the non-producing resistant strain (which grows faster due to not making the toxin), but this is beat by the sensitive strain (which grows faster by not making resistance factors). In experiments [49] and simulations [50], these non-transitive interactions create highly-structured populations, albeit with constantly-moving clonal domains.

Conflict drives cooperation. Perhaps surprisingly, the evolutionary outcome of ruthless conflict appears to be, paradoxically, increased cooperation. Microbial antagonism strongly drives the creation of spatially-structured populations in which individuals interact primarily with close relatives. This, in turn, stabilizes the evolution of cooperative behaviors by allowing the social benefits of individual traits to be directed preferentially back to relatives with genes that code for these cooperative traits. McNally, *et al.*, (2017) examined this bioinformatically with the Type VI Secretion System (T6SS). They constructed a Bayesian phylogeny of



***Range Expansion*:** **B** a faster growing simulated mutant (red) expands its territory faster, leading to a “bump” at the growth front. **C** A slower growing mutant (red) gets crowded out.

Coarsening: **D** Two strains of simulated bacteria that interact via local antagonism spontaneously generate positive assortment. By killing enemy neighbors and replacing them with kin, positive assortment is achieved.

Density-Dependent Mechanisms: **E Left** at low density, faster growing strains increase their territory through growth. **Right** At high density, territory can be gained by killing or displacing competitors.

Fig. 3. Generating positive assortment through birth and death in a growing colony. A Confocal microscope image of a colony containing two mutually-killing strains of *Vibrio cholerae*, as described in [34]. Large clonal domains are visible on the periphery, due to stochastic bottlenecking during a range expansion. Tracing a ring around the colony periphery reveals a similar degree of genetic diversity regardless of the radius of the ring, while the number of organisms increases with the radius of the ring. Thus, many individuals are descended from a few— indicating a spatial population bottleneck. **B, C** Simulation of microbes at a growing interface, reprinted from [38], shows how selection can act to increase or decrease domain size. **D** Within the cluster interior, assortment is generated through a "Model A" phase transition, driven by contact-mediated killing using the type VI secretory system. The inset shows the result of an individual based model that was initialized with a well-mixed population (code available here). **E** Schematic showing density dependence of mechanisms for generating positive assortment through birth and death.

439 bacterial genomes from 26 genera, sampled from lineages in which at least some members produce a T6SS. They used the number of T6SS apparatuses and distinct toxins these systems deliver (called effectors) as a proxy for how effective T6SS is at generating spatial structure- a single toxin may not result in a very highly structured population, simply because competitors may acquire the antitoxin through horizontal gene transfer or be otherwise invulnerable. Genotypes that possess a larger number of ways of killing competitors, should, all else equal, generate more structured populations. McNally, *et al.*, used the proportion of genes encoding secreted products (i.e., the relative size of the secretome) as a proxy for the degree to which a given genome invests in social products, the logic being that once a molecule is outside of the cytoplasm, it has the potential to be used by neighbors. While these measures are somewhat crude, they found a strong, positive relationship between T6SS repertoire and overall secretome size: after accounting variation explained by phylogeny, variation in the number of T6SS systems and effectors explained $\sim 90\%$ of the variation in secretome size. While this analysis cannot disentangle the order of evolutionary events (i.e., whether increased T6SS specificity favored the evolution of increased cooperation, or whether more cooperative lineages benefited from expanding their T6SS repertoire), it nonetheless demonstrates the degree to which cooperation and conflict embody two sides of the same coin.

Future outlook. Due to their dense packing and reliance on extracellular metabolism, few microbial behaviors occur in a social vacuum. Understanding and quantitatively predicting the evolution of many microbial traits will thus require an accurate accounting of their social consequences. To accomplish this, we will need to develop and employ new experimental approaches that embrace the complexity of population structure and ecological interactions, as well as new analytical tools to quantify these interactions. Biophysics has much to offer microbial ecology and evolution- both because the mechanisms structuring microbial populations are physical in nature, and because biophysical techniques have already been developed for measuring and manipulating matter at microbial length scales.

Quantifying the assortment of your own populations. While measuring the spatial structure of some microbial populations may be difficult (particularly in complex 3D media such as soil or a water column), it is relatively straightforward to calculate for microbes growing in roughly 2D surface-attached populations. To run the assortment calculations presented in (Figure 2) on your own data, please download our script (written in Python) from https://github.com/PedroSapichu/Yanni_etal_CB2019. Instructions for use can also be found at the above URL.

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AUTHOR CONTRIBUTIONS

All authors contributed to the conception and creation of the manuscript.

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