MICROBIAL BIOFILMS

Biofilms: an emergent form of bacterial life

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Abstract | Bacterial biofilms are formed by communities that are embedded in a self-produced matrix of extracellular polymeric substances (EPS). Importantly, bacteria in biofilms exhibit a set of 'emergent properties' that differ substantially from free-living bacterial cells. In this Review, we consider the fundamental role of the biofilm matrix in establishing the emergent properties of biofilms, describing how the characteristic features of biofilms — such as social cooperation, resource capture and enhanced survival of exposure to antimicrobials — all rely on the structural and functional properties of the matrix. Finally, we highlight the value of an ecological perspective in the study of the emergent properties of biofilms, which enables an appreciation of the ecological success of biofilms as habitat formers and, more generally, as a bacterial lifestyle.

Grazing
A form of predation, such as when protozoa feed on bacteria.

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Biofilms have been defined as 'aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface' (REF. 1). The term 'aggregate' accounts for the fact that most cells in multilayered biofilms experience cell-to-cell contact, either in surface-attached biofilms, in which only one layer is in direct contact with the substratum, or in flocs, which are mobile biofilms that form in the absence of any substratum. Through intercellular interactions, both social and physical, together with the properties of the matrix, the biofilm lifestyle is clearly distinct from that of free-living bacterial cells. Thus, biofilm communities have emergent properties; that is, new properties that emerge in the biofilm that are not predictable from the study of free-living bacterial cells[2].

Biofilms are one of the most widely distributed and successful modes of life on Earth[3], and they drive biogeochemical cycling processes of most elements in water, soil, sediment[4] and subsurface[5] environments. Biotechnological applications of biofilms include the filtration of drinking water, the degradation of wastewater and solid waste, and biocatalysis in biotechnological processes, such as the production of bulk and fine chemicals, as well as biofuels[6]. All higher organisms, including humans, are colonized by microorganisms that form biofilms[7], which can be associated with persistent infections in plants and animals, including humans[8], and with the contamination of medical devices and implants[9]. Furthermore, biofilms are responsible for biofouling and contamination of process water[10], deterioration of the hygienic quality of drinking water[11] and microbially influenced corrosion[12].

Biofilms are complex systems that have high cell densities, ranging from 10^8 to 10^{11} cells g^{-1} wet weight[13,14], and typically comprise many species. A further source of heterogeneity is the ability of cells in biofilms to undergo differentiation, which can be triggered by local conditions, and coordinated life cycles that include stage-specific expression of genes and proteins, as is typical for the growth and development of microorganisms in spatially heterogeneous ecosystems[15]. The emergent properties of biofilm communities comprise 'novel structures, activities, patterns and properties that arise during the process, and as a consequence, of self-organization in complex systems' (REF. 16), occur concomitantly and lead to biogenic habitat formation (BOX 1; FIG. 1). Fundamental to these emergent properties — which include the formation of physical and social interactions (such as in synergistic microconsortia), an enhanced rate of gene exchange and an increased tolerance to antimicrobials — is the role of the self-produced EPS matrix that encases the cells of the biofilm and is mainly composed of polysaccharides, proteins, lipids and extracellular DNA (eDNA)[17].

The formation of the matrix is a dynamic process and depends on nutrient availability, the synthesis and secretion of extracellular material, shear stress, social competition and grazing by other organisms. Not surprisingly, the production of the matrix incurs a considerable energetic cost[18]; however, this cost may be evolutionarily justified, owing to the structural and physicochemical centrality of the matrix to the formation and function of the biofilm, without which the beneficial emergent properties of biofilms would not arise (BOX 1). The matrix is an interface, or rather an 'interspace', between

Box 1 | **Biofilms as biogenic habitat formers**

Much of the physical structure that we see in the natural world is biogenic — that is, created by organisms[128,129]. Habitat formation by organisms occurs throughout natural systems, with prominent examples at large scales (hundreds of kilometres or more) including trees and other plants, corals and other marine invertebrates, and marine and aquatic macrophytes[130]. Many other organisms create habitats at somewhat smaller scales[129], including beavers and their ponds, social insects (such as ants, bees and termites) and their hives or nests, and bromeliads and their small pools. Biogenic habitat formers have profound effects on the communities in these systems through the modulation of both the physicochemical environment and biotic interactions. Indeed, the removal of key habitat-forming organisms from a community completely changes the structure and functioning of that community[131], sometimes at a global scale[132].

Many of the emergent properties of natural communities rely on the creation of biogenic structures by habitat-forming organisms, and this concept may similarly provide a general framework for understanding the emergent properties of biofilms. Arguably, bacteria in biofilms can be viewed as biogenic habitat formers at a microscale. By generating a matrix, bacteria in biofilms create a physically distinct habitat that provides shelter, promotes the accumulation of nutrients and fundamentally alters both the physicochemical environment of the biofilm and interactions among the organisms therein. In some instances, emergent properties deriving from biogenic habitat formers in biofilms extend to the macroscale, such as in structured biofilms that take the form of microbial mats or that can be found in the rhizosphere, in which microbial communities have an essential role in the cohesion of soil particles.

The increasing number and breadth of macroecological studies of the role of habitat-forming organisms[128] in natural communities provide a conceptual underpinning for understanding biofilms. For example, an increased appreciation of the role of habitat-forming organisms has led to a much greater emphasis on the role of facilitation (that is, positive interactions between species) in the structuring of natural communities[128,133], which parallels the recent emphasis from experimental evidence on positive interactions between species in biofilm communities. The fundamental role of the physical structure of the biofilm in regulating nutrient dynamics and the physicochemical environment inside the matrix can also be viewed as analogous to similar effects that have been observed in canopies of forest or marine macrophytes, including the intriguing recent suggestion that these habitat formers ameliorate ocean acidification at the surfaces of the canopies[134]. As we have argued previously[135], the application to biofilms of these and other general ecological concepts that are derived from eukaryotic systems has considerable potential for enhancing our understanding of the ecology of biofilms.

the biofilm and its environment that defines processes inside the biofilm and interactions with the external world (BOX 2). The matrix also confers a spatial organization on biofilms, from which they derive steep gradients, high biodiversity, and complex, dynamic and synergistic interactions, including cell-to-cell communication and enhanced horizontal gene transfer. In this Review, we contend that the concept of the biofilm as an emergent form of microbial life relies on the supracellular organization that arises from the formation of the EPS matrix. To support this view, we describe how the matrix provides structural and functional benefits to the biofilm, such as hydration, resource capture, digestive capacity and protection from antimicrobials, in addition to facilitating intercellular interactions that can enhance the metabolic capacity of cells in the biofilm and resistance to antimicrobials. Finally, we consider the role of ecological theory in understanding the social interactions that exist in biofilms, and, conversely, the potential of the study of biofilms to inform ecological theory (see BOX 1). This ecological perspective highlights the importance of distinguishing between single-species and multispecies biofilms, and the need to study biofilms that more closely reflect the complex communities that are frequently found in nature rather than the single-species biofilms that are most often studied in the laboratory.

The biofilm matrix

Most of the biomass of the biofilm comprises hydrated EPS rather than microbial cells[17]. The self-organization of EPS molecules in the matrix is based on intermolecular interactions between EPS components, which also determine the mechanical properties of the matrix, and the physiological activity of the organisms in the biofilm[17]. EPS molecules mediate the formation of the biofilm architecture, which is a continuous, dynamic process that produces a spatial organization in which cells in the biofilm cluster in microcolonies[19]. A very elegant recent study described microbial clusters in *Escherichia coli* biofilms with a complex supracellular architecture that is responsible for spatial physiological differentiation[20]. Single-particle tracking of functionalized microbeads in combination with microrheology revealed that *E. coli* biofilms have a height-dependent charge density that changes over time[21]. Furthermore, EPS molecules fill and shape the space between the cells of the biofilm, directly determining the environment and living conditions of the cells and providing mechanical stability to the biofilm[22]. Particularly interesting is the role of eDNA; for example, the cationic exopolysaccharide Pel crosslinks eDNA in *Pseudomonas aeruginosa* biofilms[23], which provides structural integrity to the matrix, and DNABII binding proteins are thought to enable the formation of uropathogenic *E. coli* biofilms by stabilizing the secondary structure of eDNA[24]. eDNA was also found to support the formation of a stable filamentous network structure in biofilms of an aquatic bacterium[25]. The main component of the matrix is water (up to 97%), which contains the structural and functional components of the matrix: soluble, gel-forming polysaccharides, proteins and eDNA[17], as well as insoluble components such as amyloids[26], cellulose[20], fimbriae, pili and flagellae[26]. Pores and channels between microcolonies that form voids in the matrix[27] were recently shown to facilitate liquid transport[28], inspiring the concept of a 'rudimentary circulation system' for the biofilm[29].

In some cases, structural components of the matrix may also have other functions that benefit the biofilm. For example, in biofilms that are formed by *E. coli*, the main structural component of the matrix is the curli protein, which together with cellulose contributes to the desiccation tolerance of the biofilm (see below), and *Bacillus subtilis* uses proteins called hydrophobins to form highly hydrophobic biofilms that float at the air–liquid interface[26]. Other functional components of the biofilm matrix include proteinaceous filaments and nanowires that are capable of electron transport[30], and methods from materials science and biophysics are increasingly being used to interrogate the physical properties of biofilms, including the use of rheological[22,27], ecomechanics and electrochemical[31] methods to investigate electrogenic properties of biofilms. In biofilms that are formed by *P. aeruginosa*, the EPS matrix self-assembles into a

Macrophytes
Aquatic photosynthetic organisms that are visible to the naked eye. In marine systems, 'macrophytes' is often used as a general term that includes macroalgae, such as kelps, and coastal plants, such as seagrasses or mangroves.

Bromeliads
A family of flowering plants that has leaves that often form in such a way so as to enable the persistence of pools of water that form distinct habitats and ecological communities.

Microrheology
The study of the rheological properties of a material at the micrometre scale.

Desiccation tolerance
The ability to survive water limitation.

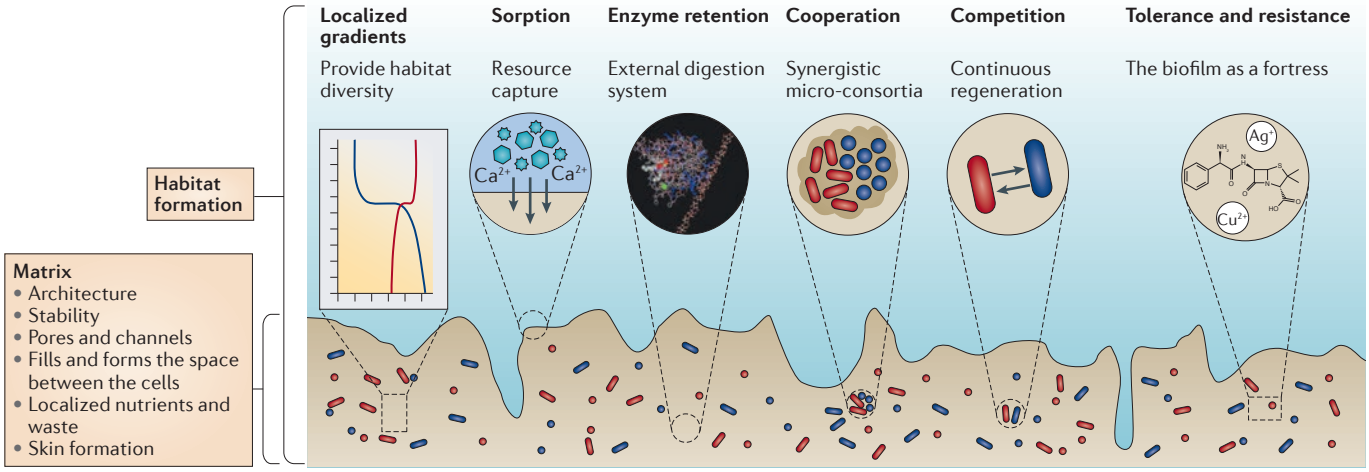


Figure 1 | **Emergent properties of biofilms and habitat formation.** Bacterial cells in biofilms can be considered to be habitat formers, owing to their generation of a matrix that forms the physical foundation of the biofilm. The matrix is composed of extracellular polymeric substances (EPS) that provide architecture and stability to the biofilm. Nutrients and other molecules can be trapped both by sorption to EPS molecules and to the pores and channels of the matrix, whereas skin formation by hydrophobic EPS molecules enhances the ability of the biofilm to survive desiccation. Biofilms derive several emergent properties — that is, properties that are not predictable from the study of free-living bacterial cells — from the EPS matrix. These properties include localized gradients that provide habitat diversity, resource capture by sorption, enzyme retention that provides digestive capabilities, social interactions and the ability, through tolerance and/or resistance, to survive exposure to antibiotics.

Nanowires
Electrically conductive structures that are produced by microorganisms.

Rheologocial
Pertaining to the study of the flow of matter, primarily in a liquid state but also as 'soft solids' or solids under conditions in which they respond to an applied force with plastic flow rather than elastic deformation.

Ecomechanics
The biomechanical mechanisms by which organisms interact with their environment.

Electrogenic
Capable of generating an electric current.

Microbial mats
A coherent, layered organization of microorganisms with complementary metabolic capacities. Microbial mats are typically found in aquatic environments, anchored to a surface.

Mass transfer
The net movement of compounds or chemical species from one position to another.

Streamers
Portions of the biofilm extracellular polymeric substances (EPS) that extend out from the biofilm surface into the liquid flow.

Sorption
Adsorption or absorption, or a combination of both processes.

liquid crystal structure through entropic interactions between polymers, and the viscosity of the liquid crystal is strongly enhanced by filamentous Pf phages[32]. Finally, in Gram-negative bacteria, enzymes that are packaged in extracellular membrane vesicles can contribute to the degradation potential of the matrix[33]. Thus, the matrix is not simply an amorphous gel that is composed of polysaccharides, but instead has a very heterogeneous — yet highly ordered — composition that includes a wide range of biopolymers that contribute to its function and emergent properties[26].

The non-rigid structure of the biofilm, in which distinct zones (which can be microscopic in scale) have substantially different viscosities, allows for the movement of cells in the matrix, with consequences for porosity, mechanical properties and microrheology[22,34]. Common observations include the vertical migration of bacterial populations, such as in hypersaline microbial mats[35], and migration as a collaborative effort of populations that involves the division of labour[36]. This is particularly well exemplified by recent observations that showed that a subpopulation of motile, planktonic *Bacillus thuringiensis* cells is able to tunnel deep into the biofilm structure at high speed (up to 20 µm sec^{-1})[37]. Swimmers that tunnel through the biofilm matrix create transient pores that increase mass transfer in the biofilm[37]. Other recent observations showed that active dispersal of the biofilm can occur by partial matrix degradation[38], and together these observations lead to a conclusion that the highly organized biofilm matrix is not an end-point, but that instead the biofilm matrix is continuously remodelled[39]. This remodelling is essential for the biofilm to respond to changes in the environment, such as hydrodynamic shear, or to form streamers that facilitate the colonization of a surface. In the remodelling process, specific enzymes degrade and reconfigure the biofilm, which not only results in passive sloughing but also in active dispersal of the biofilm and subsequent surface recolonization[39].

An important emergent property of the biofilm that is conferred by the matrix is tolerance to desiccation (FIG. 2a), as microorganisms in the environment regularly experience water stress. Indeed, bacteria in the biofilm actively respond to desiccation by the production of EPS molecules[40], which, owing to the high proportion of hydrated polymers in the EPS matrix, protects the biofilm from desiccation by acting as a hydrogel that retains water[17]. Furthermore, skin formation by the uppermost EPS layers leads to an effective evaporation barrier[41]. In a study that investigated the effect of desiccation on groundwater biofilms, the enzymatic activity of desiccated samples was fully restored following a return to wet conditions[42]. Thus, the biofilm mode of life is expected to provide much better protection against desiccation than that of free-living bacterial cells, which lack the benefits of the EPS matrix.

Resource capture by biofilms. The matrix enables the biofilm to capture resources such as nutrients that are present in the water phase of the biofilm or that are associated with the substratum on which the biofilm is growing (FIG. 2b). Nutrient acquisition is an essential process for all organisms, and biofilms have developed a very efficient capture strategy for nutrients that exceeds that of free-living bacterial cells. The strategy relies on the passive sorption properties of the sponge-like EPS matrix, which influence the exchange of nutrients, gases and other molecules between the environment and biofilms on a global scale[34]. Substances that are sequestered from the water phase are retained in the biofilm and regarded

Box 2 | **Biofilms as physically bounded systems — a framework for understanding emergent properties?**

Biofilms encompass several levels of the traditional biological hierarchy (that is, from individual cells to communities), which adds to the challenge of arriving at a holistic understanding of biofilm biology. In particular, biofilms can be composed of either a population or a community, which are fundamentally different levels of ecological organization. As populations are groups of organisms of the same species, whereas a community is a collection of several species, the ecological and evolutionary bases for interactions among organisms can differ substantially between populations and communities (although see recent attempts at unification, such as REF. 136), and this distinction is also likely to be fundamental for the study of biofilms.

BUBBLES, CRYSTALS and WAVES have been proposed as models for understanding emergent properties of biological systems based on physical properties[137]. Importantly, for the study of biofilms, these models traverse the various levels of the biological hierarchy, and thus potentially unify emergent properties of biofilms across populations and communities of bacteria. In particular, BUBBLES, which are defined as 'systems whose most important properties are conditioned by their external envelope', seem particularly suitable as the basis of a model for biofilms, with the outer layer of the biofilm matrix as the external envelope or boundary of the system.

Systems that are organized as BUBBLES contain and enhance processes inside the envelope of the system, and filter interactions between the system, the environment and other systems. The envelope facilitates chemical, visual and/or electrical signalling in the system, enhances resistance to external stressors and provides an enclosed space for biotic interactions, such that BUBBLES have been proposed to be a site of co-evolution for organisms in a community[137]. BUBBLES found in nature include beehives, termite nests and other examples of social or colonial organisms, which, similarly to biofilms, are examples of cooperative behaviour that occur in an enclosed physical environment. Forests and their canopies are BUBBLES that have a particular resonance with biofilm communities, owing to the development of complex physical structures and a community composition that encompasses a wide variety of species that occupy different niches to maximize the use of resources. Similarly to the biofilm matrix, the forest canopy regulates emergent processes, such as light penetration, nutrient cycling and water dynamics, and imposes physicochemical gradients that are crucial to the functioning of the system, in this case vertically from the top of the canopy to the forest floor[138]. Also similarly to biofilms[139], the canopy regulates the effect of biological processes, such as competition or predation, on the forest[140]. Finally, light gaps in the canopy are fundamental to forest ecology, as they enable the recruitment of new individuals, increase the availability of resources such as water, nutrients and light on the forest floor, and increase exposure to predators. The dynamics of light gap formation are one of the keystones of forest ecology[141], but the dynamics of the analogous process in biofilms—that is, the formation and removal of the matrix —are currently not well understood. A dynamic matrix would probably generate a very heterogeneous physical context for the cells of the biofilm, which would correspond to a dynamic physicochemical and biological environment such as that seen in forests.

as 'sorbed', which includes both absorption in the water phase of the matrix and adsorption to matrix biopolymers and biofilm cells[17], and surface materials that are biodegradable can be used by colonizing biofilms as nutrients. For example, decomposers that degrade organic matter can drive decontamination processes at a global scale, whereas biodegradation of solid materials that occurs at the wrong place and the wrong time causes economically damaging biodeterioration[43]. As nutrients from surface materials are most highly concentrated at the base layer of the biofilm, the nutrient gradient is reversed compared with the gradient of nutrients acquired from the water phase.

Biofilms are complex sorbent systems with different sorption mechanisms and binding sites in the cytoplasms of biofilm cells, the cell walls of biofilm cells and the EPS of the matrix. These binding sites include both anionic and cationic exchangers, which means that a very wide range of substances can be trapped and accumulated for possible consumption by cells in the biofilm[44], even when such compounds are present at very low concentrations. This potent sorptive capacity enables biofilms to grow even in highly oligotrophic environments[45]. Sorption by the biofilm is not compound specific, which means that not only nutrients, but also toxic substances, can accumulate in biofilms, and compounds such as erythromycin, ethylsuccinate, acetaminophen[46], acidic pharmaceuticals[47], steroidal hormones and 4-nonylphenol compounds have been found in biofilms[48]. Surprisingly, even non-polar substances, such as benzene, toluene and xylene, can accumulate in the EPS matrix, even though it is highly hydrophilic and has no obvious lipophilic binding sites[49]. If they are not degraded, sorbed substances will be released into the water phase from the matrix if there is a concentration gradient towards water, or they will otherwise remain in the biofilm until it decomposes. Therefore, biofilms act both as a sink and a source of contaminants[44]. Interestingly, biofilms respond dynamically to sorbed substances. For example, in response to exposure to toluene, biofilms of *Pseudomonas putida* produce EPS with a greater number of carboxyl groups[50], which, as anions, can lead to an increased ion exchange capacity for cations. Other anions can also be deposited in biofilms, such as the phosphate ions that enhance the mechanical stability of the highly structured, multigenus biofilms in dental plaques[51].

When cells decay and lyse, their debris remains in the matrix to be 'cannibalized' as nutrients by surviving cells. This process has been investigated in detail in *B. subtilis* biofilms[52], which showed that DNA from lysed cells is a source of phosphorus, carbon and energy[53], and *P. aeruginosa* has been shown to specifically produce extracellular DNases in biofilms to exploit DNA from lysed cells as a nutrient resource[54]. Although other EPS compounds are relatively recalcitrant to degradation and persist longer than the cells that are responsible for

Oligotrophic
An environment that is characterized by low nutrient concentrations.

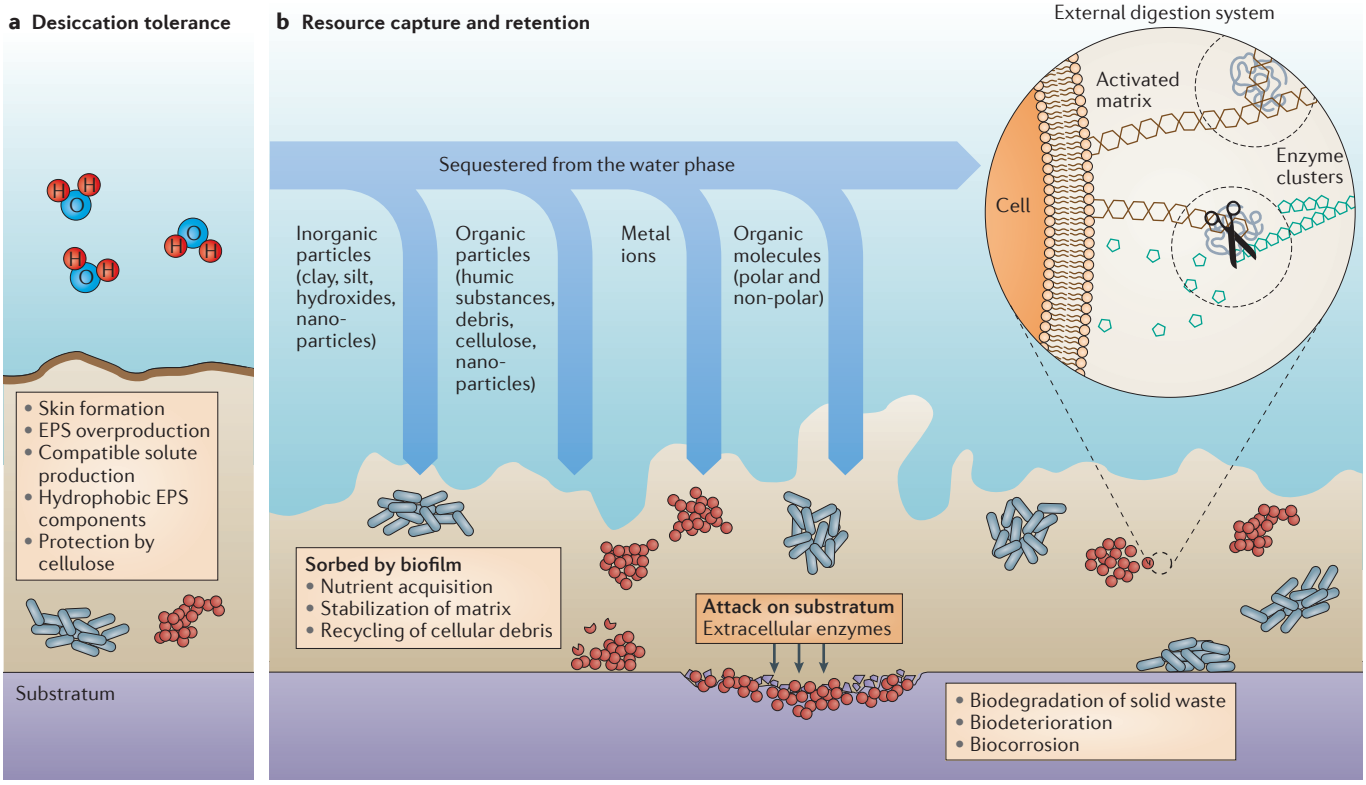


Figure 2 | **Physical and chemical properties of the biofilm matrix. a** | The biofilm can be viewed as a fortress that, through several properties of the matrix, enables constituent cells to survive desiccation. **b** | The biofilm is a sponge-like system that provides surfaces for the sorption of a diverse range of molecules that can be sequestered from the environment. This confers several benefits to the biofilm, such as nutrient acquisition and matrix stabilization. Similarly, the physicochemical properties of the matrix enable biofilms to retain and stabilize extracellular digestive enzymes that are produced by biofilm cells, which turns the matrix into an external digestive system. Surface-attached biofilms are not only able to take up nutrients from the water phase but can also digest biodegradable components from the substratum, which is exposed to enzymes in the matrix.

their production[55], such molecules can also eventually be used as a nutrient source[56]. Indeed, as all, or nearly all, biofilm components are retained in the matrix, the biofilm can be viewed as a highly effective recycling yard of cellular debris.

Essential metal ions, such as calcium, iron and manganese, accumulate in biofilms[57] and contribute to the stabilization of the biofilm matrix through the bridging of carboxyl groups across EPS molecules[58]. Indeed, EPS are standardly isolated using cationic ion exchange resin treatment, the efficacy of which is based on the solubility of EPS once calcium ions are removed[59]. Cell surfaces can also provide binding sites for metals[49]. When calcium is deposited as carbonate[60], capture by biofilms contributes to crust formation, lithification and the formation of stromatolites[61], and iron deposits have been generated by biofilms of iron-oxidizing bacteria, such as *Pedomicrobium* spp., on a global scale[62]. The matrices of biofilms that are formed by *B. subtilis* accumulate metals such as Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} (REF. 63), which protects the biofilm matrix from erosion and the biofilm cells from the toxicity of the metal ions, which can be present at concentrations that are toxic to free-living cells. The metal sorption capacity of biofilms has been used in biotechnology for applications such as the decontamination of uranium from groundwater[64]. In activated sludge, the accumulation of metal ions such as Pb^{2+}, Cd^{2+} or Cu^{2+} has been reported to cause contamination problems when the sludge is used as a fertilizer[65].

In addition to dissolved compounds, suspended solids can be trapped by biofilms and incorporated into the matrix[61], including biodegradable material that can be used as a source of nutrients. A large proportion of the organic material in raw wastewater consists of solid particles and is eliminated by biofilms to which the particles attach[66], forming flocs and sludge. Large particles with a diameter of a few micrometres that are captured by the matrix can traverse thick biofilms through channels in the matrix[67]. Both organic and inorganic particles are often trapped in biofilms, including clay and silicate, and the capture of inorganic particles by biofilms contributes to lithification on a global scale[4]. Inorganic particles in biofilms also include electrically conductive particles that can support interspecies electron transfer (IET), as has been shown for graphite particles, granular activated carbon, charcoal and carbon cloth[68]. Interestingly, electric signals are also used for intercellular communication, which further supports

Lithification
A process in which sediments compact under pressure and gradually become solid rock. The biogenesis of carbonate can support this process.

Activated sludge
The microbial biomass in the aerobic portion of a wastewater treatment system.

Carbon cloth
A soft, flexible cloth-like material made from carbon fibre.

Box 3 | **Intercellular communication in biofilms**

Since the first suggestion that bacteria were capable of intercellular communication[142], numerous studies have investigated the signalling mechanisms that are used by bacteria to control phenotypes at the population or community level. These mechanisms include the exchange of small organic molecules or proteins, and even the transmission of electrical signals. The majority of studies have focused on systems that use chemical signalling (also known as quorum sensing), which relies on the release of signalling molecules by bacteria in response to population size, and the sensing of these molecules by neighbouring cells, to induce the coordinated expression of specific genes[143]. Most laboratory studies of quorum sensing use batch cultures, which are closed systems in which signals are contained and can accumulate to high concentrations. However, in natural environments, such as the open ocean, individual planktonic cells are not thought to experience such high concentrations of signalling molecules. By providing a closed system in which signalling molecules can be concentrated, the biofilm is an environment that facilitates intercellular signalling, which may, in part, explain why so many signalling phenotypes are specific to cells in biofilms.

Matrix components, such as amyloid fibres, exhibit weak, but functional, binding affinity for quorum sensing signalling molecules, thus providing a continuous on–off mechanism for modifying the concentration of these molecules in the biofilm matrix[144]. This mechanism enables quorum sensing signalling molecules to be spatially restricted such that they reach sufficiently high concentrations to be sensed[145]. Indeed, the concentrations of acyl homoserine lactones (AHLs) can be up to 1,000-fold higher in biofilms than in environments that are inhabited by planktonic cells, which highlights the potency with which biofilms can concentrate signalling molecules to facilitate quorum sensing[146,147]. Remarkably, external flow can influence the activation of quorum sensing systems inside the biofilm, which leads to spatially structured quorum sensing that produces spatial and temporal heterogeneity in the phenotypic response of individual cells in the biofilm to the quorum sensing signal[148]. The ability of external flow to influence quorum sensing is an example of how external conditions can modify the emergent properties of biofilms.

Recently, it has been suggested that, as an alternative to chemical signalling, some bacteria can respond to electrical signals to elicit coordinated, population-based behaviours. For example, *Bacillus subtilis* cells in the periphery of the biofilm can use long-range electrical signals to cooperate with cells in the centre of the biofilm, such that collective oscillations are generated that coordinate competing metabolic demands[149,150]. The electrical signals are passed through potassium ion channels in the matrix[151], which may be facilitated by conductive particles of ferric iron that act as bridges between cells that are in close proximity, such as cells in biofilms. The use of conductive particles of ferric iron for interspecies electron transfer has been described in microconsortia of *Geobacter sulfurreducens* and *Thiobacillus denitrificans*[152].

In summary, biofilm formation enables effective intercellular communication, whether using chemical or electrical signals, even in habitats in which signalling molecules that are not contained by the biofilm would be readily diffused, such as granules in wastewater treatment systems[148]. By contrast, the use of signal-driven processes is not possible for planktonic cells, which lack the inherent organization and matrix components of a biofilm.

Planktonic cells
Free-living cells (that is, not in a biofilm) in the liquid phase.

Humic substances
The combination of compounds, generally humic acids, that make up the organic components of soils, peat and coal.

Molecular modelling
Methods that encompass theoretical methods and computational techniques that are used to model or mimic the behaviour of molecules.

the role of the matrix in cell-to-cell interactions (BOX 3). Finally, nanoparticles accumulate in biofilms, and complex chemical transformations of nanoparticles have been observed following absorption[69].

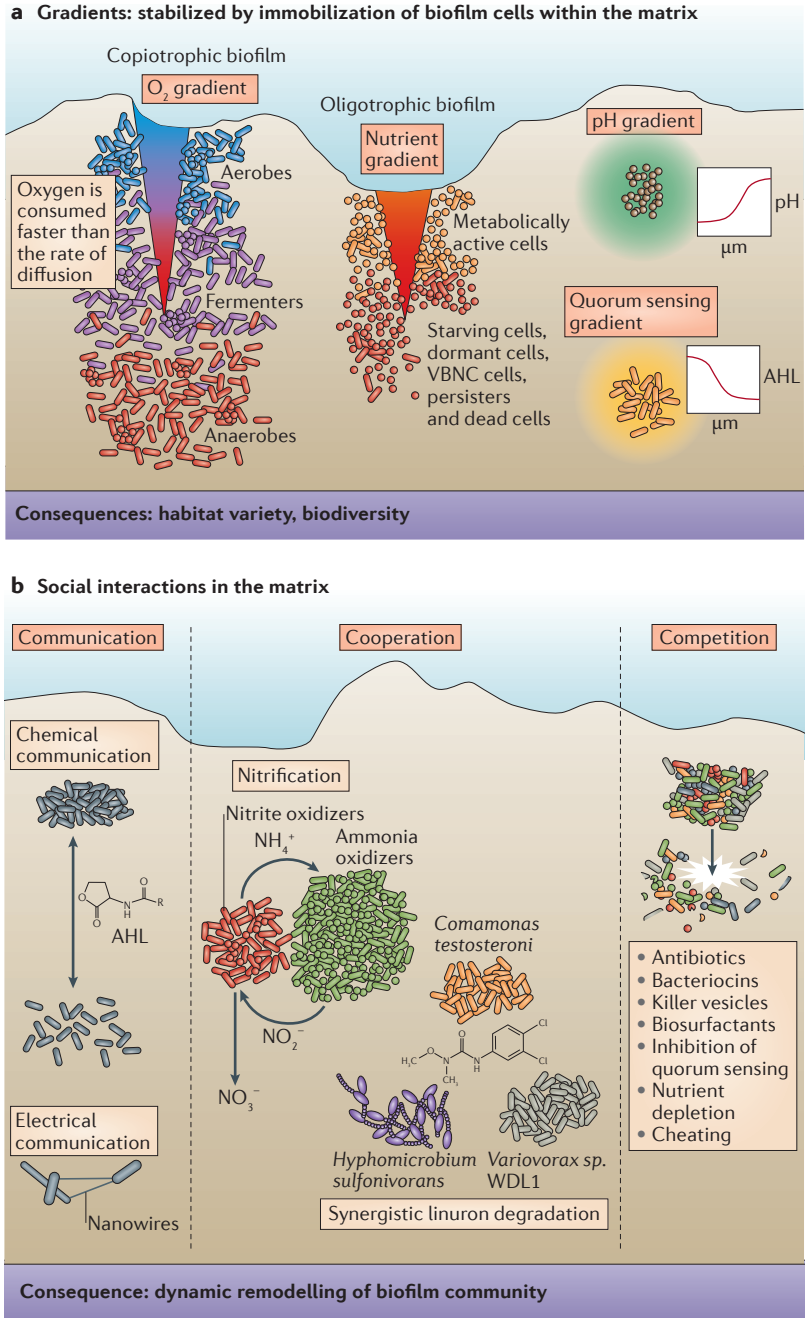
The matrix as a communal external digestion system. Resource capture by biofilms not only includes external resources, but also enzymes that are secreted by cells in the biofilm. Indeed, cells in the biofilm make much more effective use of their extracellular degradative enzymes than free-living bacterial cells. Whereas extracellular enzymes that are secreted by planktonic cells diffuse away from the producing cell and become diluted in the aqueous environment, extracellular enzymes that are secreted by cells in the biofilm are retained in the biofilm, where the enzymes interact with EPS components, such as polysaccharides, and accumulate in the matrix[17]. Thus, an activated matrix is generated that can be considered to be an external digestion system[70], which is a notion that was suggested as early as 1943 (REF. 71). A wide variety of extracellular enzymes have been found in biofilms, in both natural terrestrial and aquatic ecosystems[72]. In such biofilms, abundant humic substances[73] and extracellular enzymes form stable complexes that are extremely resistant to thermal denaturation, dehydration and proteolysis[74]. The interaction of extracellular enzymes with matrix components leads to the long-term stabilization of the enzymes and the persistence of enzymatic activity, which buffers the biofilm community from sudden changes in the composition and concentration of dissolved organic matter in the bulk water phase.

Matrix-associated proteins are constantly produced and degraded in response to changing conditions in the biofilm. The matrix proteome of *P. aeruginosa* biofilms was found to consist of secreted proteins, proteins derived from cell debris, and a substantial number of proteins associated with extracellular membrane vesicles[75]. Some evidence suggests that the matrix proteome of *P. aeruginosa* biofilms forms a well-regulated system that contributes not only to nutrient acquisition but also to stress resistance, pathogenesis and stability of the biofilm[76], which are similar to the functions of flagellae in *E. coli*[20]. For example, during infection with *P. aeruginosa*, the extracellular enzymes elastase and lipase (LipA) have important roles in providing nutrients to the biofilm by degrading host tissue, and, in this capacity, the enzymes act as virulence factors. *In vitro* studies that used molecular modelling showed that LipA secreted by *P. aeruginosa* binds to the EPS matrix by forming electrostatic interactions with alginate[70], with a concomitant increase in heat tolerance and protection against enzymatic degradation. Thus, the matrix is able to alter the properties of secreted enzymes in an unexpected way.

Matrix-bound enzymes are not only a resource for the cells that produce them, but also become a resource that is available to all members of the biofilm community, even when the community is a mixed-species consortium. In a population-based biofilm model (see BOX 2) that consisted of both proteolytic and non-proteolytic strains of *Pseudomonas fluorescens*, the protein hydrolysates that were produced by degradative enzymes that were secreted by the proteolytic strain were found to be available to both strains[77]. Thus, secreted enzymes that are retained in the matrix provide nutrients for members of the microbial community other than the secreting cells and represent a potential shared resource that arises from what has been termed the 'social function of extracellular hydrolysis' (REF. 78).

Heterogeneity and social interactions

The organization of bacterial biofilms, based on the matrix, allows for a myriad of organisms to interact and to do so in close proximity. This enables the exchange of metabolites, signalling molecules, genetic material and



◂ Figure 3 | **Biofilms are characterized by heterogeneity and social interactions. a** | The formation of the extracellular polymeric substance (EPS) matrix leads to the establishment of stable gradients that provide different localized habitats at a small scale. In an aerobic copiotrophic biofilm, organisms are stratified according to oxygen availability, which becomes depleted in the lower layers of the biofilm, as the consumption of oxygen by aerobic organisms in the higher layers of the biofilm is faster than the rate of diffusion. Similarly, in aerobic oligotrophic biofilms, nutrient consumption by organisms in the upper layers results in the starvation of organisms in the lower layers, which may lead to the adoption of slow growth states, such as those found in dormant cells, or even in cell death. Other gradients that are present in biofilms include pH gradients, which are produced by heterotrophic metabolism, and gradients of signalling molecules, in which the concentration of quorum sensing molecules varies according to the distance from producing cells. **b** | Social interactions in biofilms involve cooperation or competition between cells and can result in the dynamic remodelling of the biofilm community. Cooperation can be mediated by chemical communication (for example, using AHL) or electrical communication (for example, using nanowires) and/or it can involve cooperative metabolism, such as that seen for the process of nitrification, in which ammonia-oxidizing bacteria produce nitrite that is further oxidized by nitrite-oxidizing bacteria. A metabolic interaction that precedes nitrification can occur when nitrite-oxidizing bacteria supply ammonia to ammonia-oxidizers, as has been shown for the nitrite-oxidizing bacterium *Nitrospira moscoviensis*. These interactions rely on the close proximity of cells that exchange metabolites, to enable efficient exchange by diffusion. Another example of cooperation in biofilms is the synergistic degradation of the toxic herbicide linuron by mixed-species biofilms that are formed by *Comamonas testosteroni*, *Hyphomicrobium sulfonivorans* and *Variovorax* sp. WDL1, which enables higher concentrations of linuron to be tolerated than can be tolerated by the respective single-species biofilms. Negative interactions, in the form of competition or cheating, have also been observed in biofilms. Competition between cells in biofilms can involve killing mechanisms, such as those using antibiotics, bacteriocins or extracellular membrane vesicles (which can contain enzymes that kill or impede the growth of competing organisms)[153], or strategies that compromise growth, such as nutrient depletion or the inhibition of quorum sensing. AHL, acyl-homoserine lactone; VBNC cells, viable-but-nonculturable cells.

defensive compounds, all of which dictate interactions between organisms. Furthermore, heterogeneity, such as in the form of cells with different metabolic capacities or physiological gradients, provides opportunities for cooperation.

Heterogeneity in biofilms. The heterogeneous physiological activity of biofilms produces steep gradients of electron acceptors and donors, as well as of pH value and redox conditions[79] (FIG. 3a). These unique features of biofilms are not only observed in thick, multilayer biofilms, but have already emerged after only a relatively small number of cells have attached to a surface[80]. Heterogeneity is observed even in monospecies biofilms[81], which is most likely the result of phenotypic variation that arises from fluctuating gene expression over time in individual cells and differential gene expression between different cells. The localized physiological activity of these spatially separated, immobilized cells contributes to the formation of gradients and other spatial heterogeneities, which further increases in multilayered biofilms[17], such as microbial mats or flocs.

One of the most important external triggers of the establishment of gradients is the availability of electron acceptors such as oxygen. In aquatic habitats, in which oxygen is present in the water phase, the upper layer of the biofilm is aerobic. Actively respiring aerobic microcolonies can consume oxygen faster than it diffuses

Copiotrophic
An environment that is characterized by high nutrient concentrations.

Heterotrophic
Refers to the use of organic compounds for nutrition.

through the biofilm, which results in the formation of anaerobic zones in deep layers of the biofilm, whereas upper layers remain aerobic[82,83]. Gradients of oxygen availability can occur over a small distance such that aerobic and anaerobic areas of the biofilm are separated by only a few micrometres[82].

Physiological stratification and heterogeneity in biofilms enable the spatial organization of mixed-species (as well as monospecies) biofilms. For example, phototrophic microorganisms, such as algae, cyanobacteria and anoxygenic phototrophic bacteria, generate and release organic substrates as exudates, and neighbouring species in close proximity to the producing cells benefit from these substrates and show enhanced metabolic activity[84]. These metabolic interactions enable the development of spatially organized biofilms that are complex interactive systems, such as microbial mats[84] or river biofilms[85]. The microscopic details of such biofilms only became experimentally accessible with the availability of specific microelectrodes that enabled parameters such as oxygen and pH to be measured at a microscale resolution (comprehensively reviewed in REF. 34).

Cooperation and competition — all together now or everyone for themselves? The complex network and coordinated division of labour[36] that emerges from the close proximity between cells in the biofilm matrix has inspired the introduction of the anthropomorphic term 'sociomicrobiology' (REF. 86). One of the enabling mechanisms of sociomicrobiology is the process of intercellular signalling, which is itself strongly influenced by the properties of the biofilm matrix (BOX 3), but metabolic activity is also an important feature of social interactions in biofilms. Indeed, given the high cell densities and species diversity of many biofilms, it is not surprising that biofilms are the primary sites for the exchange of metabolic by-products between species[87]. Such processes are not possible for suspensions of planktonic cells. Amino acid auxotrophy is a common strategy by which microbial communities lessen the collective metabolic burden of biosynthesis and stabilize cooperation[88], and it is likely that this is also true for sugars and nucleotides. Thus, the exchange of amino acids and sugars can be considered to be common mutualistic interactions in subcommunities that exist in parallel to one another[89]. The formation of synergistic multispecies consortia is most prominent when metabolic substrates and intermediates have short diffusion distances to minimize loss[90], which reinforces the importance of high cell densities for social interactions in the biofilm. An interesting example of metabolic interactions between different species in biofilms is the process of nitrification, in which ammonia-oxidizing bacteria convert ammonium into nitrite, which is then oxidized by nitrite-oxidizing bacteria (FIG. 3b). As a preceding step, the nitrite-oxidizing bacterium *Nitrospira moscoviensis* uses urease to produce ammonia for oxidation by ammonia-oxidizing bacteria that lack this enzyme[91]. Thus, the metabolic interaction is reciprocal, as *N. moscoviensis* exchanges ammonia for its oxidation product, nitrite. Owing to the close proximity between nitrite-oxidizing bacteria and ammonia-oxidizing bacteria in the biofilm, metabolites are exchanged using short diffusion pathways that minimize loss and maximize effective substrate use. Genes that are predicted to encode ureases have been found in other nitrite-oxidizing bacteria and in several metagenomic datasets, which suggests that many of these bacteria are not merely recipients of nitrite produced by ammonia-oxidizing bacteria but instead form reciprocal metabolic interactions in which ammonia is exchanged for nitrite. Another highly interesting synergistic biofilm consortium is that composed of cyanobacteria and fungi in biofilms on desert rocks or on the surface of buildings. In this consortium, the cyanobacteria provide nutrients for the fungi, which, in turn, release essential metals from the rock that benefit the cyanobacteria[92].

The distinction between populations (groups of individuals of one species) and communities (groups of individuals of several species) is fundamental to the study of ecology (BOX 2). As such, biological outcomes — such as resource partitioning, cheating, cooperation and competition — that occur in mixed-species biofilms should be framed in the context of a community, rather than in the context of a population of individuals from a single species, as distinct ecological theories apply in each case. However, although most natural biofilms exist as very diverse communities, we note that most laboratory experiments study biofilms that are single-species populations rather than mixed-species communities. Recent studies of models of mixed-species biofilms clearly demonstrate the occurrence of cooperative behaviour[93], and enable such behaviour to be experimentally investigated. For example, a study of a biofilm formed by *P. aeruginosa, Pseudomonas protegens* and *Klebsiella pneumoniae* found that stress tolerance was present to an equal extent in all three community members. Furthermore, a biofilm community with three species was shown to tolerate exposure to the phenylurea herbicide linuron by synergistic degradation of the toxin, which none of the cognate monospecies biofilms was able to degrade[94]. Indeed, stress tolerance at a community level is a key feature of mixed-species biofilms and partially explains why such communities can tolerate the accumulation of toxic compounds, whereas equivalent planktonic cultures cannot. However, the underlying mechanisms that mediate stress tolerance in mixed-species biofilms seem to be complex and are not yet fully understood.

A study that intensively investigated co-evolution and the development of cooperation in biofilms showed that an intimate and specialized association was formed by genetic adaptation in a biofilm that was formed by two species[95]. Specifically, the genome of one species had a small number of mutations that seemed to be adaptive to the presence of the other species. The derived community with the adaptive mutations was reported to be more stable and more productive than the ancestral community, which lacked the adaptive mutations. The concept of co-evolution is further supported by a recent study of isolates from tree-hole rainwater pool communities. Species in these communities tend to use similar resources to one another, which might be expected to lead to competition. Using growth media that reflected

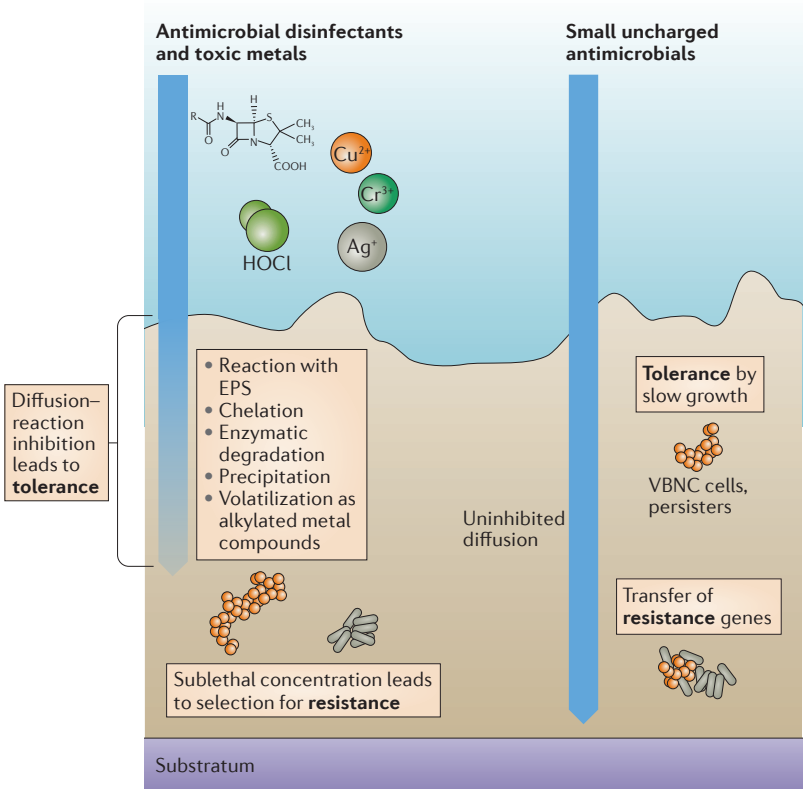


Figure 4 | **Tolerance of, and resistance to, antimicrobials.** In the context of human health, an important emergent property of biofilms is an increased ability to survive exposure to antimicrobial compounds, including disinfectants, toxic metals and small-molecule antibiotics, which can occur by several mechanisms. Tolerance, which is a non-heritable phenotype, can arise when extracellular polymeric substance (EPS) molecules in the matrix quench the activity of antimicrobials using diffusion–reaction inhibition, or as a consequence of the slow growth states that are adopted by many biofilm cells, which enables tolerance of the numerous antimicrobial drugs that target metabolic (or other) processes that occur during growth. Furthermore, diffusion–reaction inhibition that decreases the concentration of antimicrobials to sublethal concentrations can lead to the survival of exposed cells and to the development of antimicrobial resistance. Resistance to antimicrobials can also increase in biofilms as a result of the dissemination of resistance genes between cells by horizontal gene transfer, which is facilitated by the close proximity of biofilm cells to one another and, it has also been suggested, by the presence of extracellular DNA in the matrix (not shown). VBNC cells, viable-but-nonculturable cells.

the natural habitat of the tree-hole, the study showed that mixed-species biofilms ultimately evolve such that negative interactions between community members are decreased and co-metabolism or metabolic sharing is increased, which leads to more efficient resource partitioning between community members[96]. As might be expected, cooperation in biofilms is strongest when community members originate from the same habitat and cooperation is weakest when they originate from different habitats[97].

Cooperation does not necessarily occur in all biofilms, and it has even been suggested that the majority of species–species interactions in biofilms are negative (that is, either competitive interactions or interactions that are undesirable for one partner and neutral for the other)[98]. According to this argument, observations that attribute a larger number of social interactions to cooperative effects may do so owing to prior selection for cooperative interactions and/or the use of a definition for cooperation that lacks stringency. The mechanisms that mediate competition in biofilms have been comprehensively reviewed elsewhere (see REF. 99) and include the use of antibiotics, bacteriocins, extracellular membrane vesicles[99] and type VI secretion systems (as is the case for *Vibrio cholerae*[100]). These weapons of competition drive competitive strategies that include the inhibition of initial adhesion to the biofilm, surface blanketing (for example, the spread of *P. aeruginosa* cells on the surface by twitching motility, thereby preventing the adhesion of competing *Agrobacterium tumefaciens* cells) or the production of biosurfactants with antimicrobial properties[99]. Furthermore, invaders can inhibit the maturation of a biofilm and promote its dispersal through downregulation of the production of adhesin, inhibition of cell-to-cell communication, or the degradation of matrix polysaccharides, nucleic acids and proteins[38,100]. However, such defensive mechanisms seem to be specific cases that are not generalizable to most environmental biofilms, which usually contain many species and are dynamic systems in which invasion by new members can be of mutual benefit and may increase biodiversity to provide flexibility to environmental changes. Thus, for most biofilms, the majority of social interactions may indeed be cooperative.

The biofilm as a fortress

Enhanced resistance or tolerance to antibiotics and other antimicrobial agents compared with free-living bacterial cells are typical examples of the emergent properties of biofilms. Both 'resistance' and 'tolerance' are terms that are used to refer to an enhanced ability of an organism to survive exposure to compounds that are lethal to susceptible organisms. In this Review, we use the term 'resistance' to denote a genetic, heritable characteristic that is acquired either by mutation or by gene exchange and that remains even when cells in the biofilm are dispersed. By contrast, we use the term 'tolerance' to denote a characteristic that is specific to biofilms[101,102] and that is lost following dispersal to free-living bacterial cells[103,104] (FIG. 4). Owing to the ability of cells in the biofilm to survive exposure to antibiotics, together with their enhanced ability to survive desiccation, we suggest that the biofilm can be viewed as a fortress, in which antimicrobial resistance, antimicrobial tolerance and survival of desiccation form the buttresses.

Tolerance. Tolerance in biofilms can be a product both of the properties of the biofilm matrix, through the entrapment or inactivation of antimicrobials, and of the slow growth that can occur in biofilms. Intuitively, the EPS matrix might seem to plausibly represent a diffusion barrier. However, antimicrobials that do not interact with EPS molecules have been shown to diffuse through biofilms as easily as through water[105], and the diffusion barrier alone is not nearly large enough to account for the reduced susceptibility of biofilms to antibiotics. If not by inhibition of diffusion, how does the quenching of antimicrobial activity in biofilms occur? Although it seems not to be a physicochemical barrier to the diffusion of antimicrobials, EPS components of the matrix

Type VI secretion systems
Multiprotein complexes that use a one-step mechanism to inject effector proteins, such as virulence factors, from the interior of a bacterial cell into a target cell. These systems have been found in a quarter of all proteobacterial genomes, including those that encode animal, plant and human pathogens, as well as soil, environmental and marine bacteria.

can substantially quench the activity of antimicrobial substances that diffuse through the biofilm[34] in a form of inhibition known as diffusion–reaction inhibition[105], which can involve chelation by complex formation, enzymatic degradation of antimicrobials or even sacrificial reaction of EPS (for example, with oxidizing disinfectants)[105]. By decreasing the effective concentration of antimicrobials to sublethal concentrations, diffusion–reaction inhibition may promote selection for antimicrobial resistance in biofilm cells that are exposed to, but can survive, antimicrobial stress. Antimicrobials that are subject to diffusion–reaction inhibition by the matrix include toxic metals, such as copper, which is complexed by polysaccharides in the matrix of *Erwinia amylovora* biofilms to protect the biofilm from copper stress[106]. One study[107] integrated the mechanisms of metal detoxification in biofilms into a multifunctional model that suggested that numerous mechanisms can contribute to tolerance, including metabolic heterogeneity, extracellular signalling, metal immobilization and complexing, reaction with siderophores, genetic mutations and phenotypic variations. EPS molecules have also been suggested to have a role in conferring tolerance to aminoglycosides[108,109].

Slow growth rates and dormancy have long been recognized as means of survival for bacteria in biofilms that are exposed to antimicrobials[110]. Biofilms contain substantial numbers of cells in stationary phase and these cells have a reduced susceptibility to the many antimicrobials that rely on the metabolism of bacterial cells for their activities[111]. Indeed, for bacterial cells in biofilms that are in stationary phase, at least 1% become tolerant to antibiotics[112]. Over time, a larger number of cells in the biofilm enter the stationary phase. Accordingly, some antibiotics (vancomycin, but not rifampicin or tetracycline) show substantially reduced killing efficiency as the biofilm ages (from 6 h to 24 h or 48 h)[113], which indicates that older biofilms show higher tolerance for these antibiotics. Little or no reduction in ATP content was observed following exposure to silver nanoparticles in late stages, as compared with early stages, of biofilm development[105], which means that the cells were not killed but were prevented from multiplying, suggesting that the increased tolerance of older biofilms applies to silver nanoparticles as well as antibiotics. Slow growth rates can lead to the viable-but-nonculturable state (VBNC state) of microorganisms[114], or to other forms of dormancy[115], but metabolic activity and membrane integrity are still maintained during dormancy[115]. For example, tolerance to silver ions and silver nanoparticles was associated with cells that were entering a VBNC state[103,104], which is considered to be one of two dormancy states of non-sporulating bacteria. In the other dormancy state, cells are known as 'persisters', which are multidrug tolerant subpopulations that are phenotypic, rather than genetic, variants[116,117]. Given the high proportion of stationary cells in biofilms[118], persisters might be expected to be prevalent in biofilm communities.

Dissemination of resistance by horizontal gene transfer. One mechanism by which the resistance of cells in the biofilm to antimicrobials can be enhanced is the uptake of resistance genes by horizontal gene transfer[119]. The high cell density, increased genetic competence and accumulation of mobile genetic elements that occur in biofilms have been suggested to provide an ideal set of factors for efficient horizontal gene transfer, including the uptake of resistance genes[109]. Furthermore, the matrix provides a stable physical environment for cell-to-cell contact, which is required for some mechanisms of gene transfer, and is a source of DNA in the form of eDNA (see below)[120]. A common mechanism of horizontal gene transfer in biofilms is plasmid conjugation. For example, plasmids with genes that confer resistance to several antibiotics were readily transferred in dual-species biofilms of *P. putida* and *E. coli*[121]. More generally, conjugation has been shown to be up to 700-fold more efficient in biofilms compared with free-living bacterial cells[122]. Indeed, a study of *Staphylococcus aureus* showed that conjugal plasmid transfer occurred in biofilms but not in cultures of free-living bacterial cells, providing another example of a behaviour that occurs in a biofilm but that is not possible for free-living bacterial cells[123].

In *V. cholerae* biofilms, type VI secretion systems provide an alternative mechanism of horizontal gene transfer[124]. These secretion systems require cell-to-cell contact, which provides another example of why the close proximity and high cell density that are inherent to biofilms are important for horizontal gene transfer. Interestingly, *V. cholerae* uses its type VI secretion system to acquire the DNA of other cells by lysing them and uptaking the released DNA by competence and/or natural transformation mechanisms[124]. eDNA in the matrix can also be a source of DNA for horizontal gene transfer, and *Streptococcus gordonii* has been shown to take up plasmid DNA when present in biofilms formed by *Treponema denticola*[125]. A study of biofilms formed by *Acinetobacter baylyi* found that genetic transfer by transformation was strongly influenced by the EPS architecture of the biofilm[126], which led the authors of the study to propose that a major role of the matrix is to facilitate binding and stability of plasmid DNA for uptake by cells in the biofilm.

Conclusions and future directions

Our understanding of biofilms has progressed tremendously since they were first formally defined in the mid-1980s. Much of this new knowledge has been based on the elucidation of genetic pathways, physiological responses and intracellular signal transduction pathways, such as those that are regulated by cyclic dimeric guanosine monophosphate (c-di-GMP), that underpin biofilm development and that have been reviewed extensively elsewhere (see, for example, REF. 127). By contrast, we are only now beginning to scratch the surface of the properties of the biofilm matrix, even though the matrix represents the largest constituent, as well as the defining feature, of biofilms: the 'house' of biofilm cells. Studies have shown that EPS molecules comprise many different biopolymers that impart their individual physical properties to the matrix, which provides the biofilm with strength, cohesion and the capacity to retain large and small molecules alike. As biofilms retain or capture many

Aminoglycosides
A class of antibiotics that typically have a cyclohexane ring and amino sugars.

Stationary phase
A slow or non-growth phase of the bacterial growth cycle that typically results from a lack of electron donors or acceptors.

Viable-but-nonculturable state
(VBNC state). A state of dormancy that is defined by failure of an organism to be cultured on media that normally supports its growth, while retaining measurable indications of viability, such as respiratory activity, the presence of rRNA and integrity of the cell membrane.

Plasmid conjugation
The transfer of a specialized type of plasmid from one cell to another by a pilus that is encoded within the plasmid genome.

different compounds, future studies will need to address how the production and variability of EPS molecules are regulated, as well as how the biofilm makes use of the captured resources, including signalling molecules, at scales that are relevant to cells in the biofilm, whether by resource sharing and partitioning or diffusion of resources. For applied purposes, the manipulation of EPS production in biofilms may be of interest, such as in the mitigation of biofouling[10], although the potential utility of such a manipulation has not yet been well explored.

Particularly intriguing is the dependency of antimicrobial tolerance on the biofilm lifestyle, as this phenotype is lost following dispersal[103,104]. This dependency seems to be explained, in part, by the concealment of cells in the matrix, which provides protection from antimicrobials, although how this protection occurs is not yet fully understood, as antimicrobial tolerance remains at concentrations of antimicrobials that are above the saturation point of diffusion–reaction inhibition. Slow growth states, such as dormancy, are also expected to contribute to the tolerance that is observed in biofilms; however, future studies will need to establish whether dormancy is a common feature of biofilms for many organisms, rather than the small number of organisms studied in the laboratory to date, and, thus, whether biofilms are commonly a reservoir of cells in a VBNC state, possibly in starvation zones of the biofilm. Such a finding would have immense implications for the treatment of microbial infections, for the disinfection of medical devices and for an improved understanding of microbial ecology, although these applications would require further work to identify the conditions that initiate dormancy or resuscitation of cells in a biofilm. An increased emphasis on biofilm community biology will also need to address the role of interkingdom interactions between the diverse array of microbial organisms that can be present in mixed-species biofilms. Indeed, by taking into account the structure–function contributions that can be made to interkingdom biofilms by organisms such as viruses, archaea, protozoa and fungi, studies of biofilm communities will more closely reflect the true compositions of biofilms in many natural habitats.

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