Chapter 1

Viewing Biofilms within the Larger Context of Bacterial Aggregations

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Additional information is available at the end of the chapter

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Abstract

The 'Microbial Cities' vision of bacterial biofilms has dominated our understanding of the development and functioning of bacterial aggregations for the past 20 years, during which active sludge, clumps, colonies, flocs, mats, pellicles, rafts, slimes, zooglea, etc. have been largely forgotten or ignored. Although the medically inspired developmental model of human pathogen biofilms has merits including providing a rationale for the development of anti-biofilm therapeutics, it fails to provide links to other types of bacterial aggregation that are commonly found in a wide range of natural and man-made environments. Possibly as a result, applied and environmental microbiologists tend to avoid the term 'biofilm' and use others such as 'microbial mats' instead. Here we challenge the simplistic planktonic (independent and free-swimming bacteria)-biofilm (sessile and co-operative bacteria) dichotomy, and consider biofilms within the larger context of bacterial aggregations. By placing biofilms into context, which we see as a continuum of aggregations or communities with varying abiotic and biotic properties, fundamental physical, biological, and evolutionary ecological processes that effect community development and function can no longer be considered unique to biofilms, but may also be important in other aggregations that develop over time and change in nature depending on prevailing conditions. By doing this, we will be better able to distinguish those processes which govern bacterial colonisation and ecological success in a wider sense from those that are unique to particular environments and specialised strategies.

Keywords: Bacterial aggregations, Biofilms, Colonies, Communities, Planktonic and sessile bacteria

1. Introduction

Modern biofilm research often acknowledges the seminal reviews of Costerton et al. [1,2] in which a conceptual model of biofilm development and structure was first presented. In this model, biofilm development is described as a series of linked events, from the attachment of free-swimming planktonic bacteria to a submerged solid surface, the growth of microcolonies in simple conical structures, and subsequent maturation as larger mushroom-shaped structures which have been envisioned as 'Microbial Cities' (this appellation may derive from reviews entitled 'City of Microbes' and 'Microbial Metropolis' [3,4], but we are unsure). Equally important to this description was the dichotomous differentiation between independent free-swimming planktonic bacteria with the co-operative and co-ordinated communities of sessile bacteria forming biofilms, and the somewhat teleological suggestion that surface-attached communities allowed growth in harsh conditions which planktonic bacteria could not survive [2]. This view of complex bacterial behaviour and growth strategies was in contrast with the apparently contemporary idea that bacteria were unsophisticated organisms [5].

Since the publication of the Costerton et al. reviews, our understanding of biofilms has developed through the study of model bacteria as well as of natural communities forming multispecies biofilms (we direct the reader to the reviews cited in the following sections as a means of accessing recent biofilm research and current understanding). Model human pathogens forming biofilms important for virulence include *Escherichia coli* [6], *Pseudomonas aeruginosa* [7], *Salmonella enterica* [8], *Staphylococcus aureus* [9], *Vibrio cholera* [10], etc., though the archetype is probably *P. aeruginosa*, an opportunistic pathogen of the human respiratory tract and a key factor in cystic fibrosis patient mortality [11]. As a result of an understanding of pathogen biofilm formation, critical points in the developmental processes are now being scrutinised as possible targets for anti-biofilm therapeutics [7]. Biofilms are also recognised as having importance in a range of other natural and man-made environments, impacting on crop productivity, food technology, metal corrosion, veterinary medicine, etc. [12–15], and microbial mats, a term seemingly preferred by applied and environmental microbiologists, are found on rock surfaces, in caves, wetlands, sediments, salt marshes, lakes and seas, thermal springs, hypersaline ponds and lagoons, methane and petroleum seeps, oil wells, etc. [16–21]. Comparisons between pathogenic and environmental biofilm-forming bacteria highlighting commonalities suggest that biofilm developmental pathways or responses may not be unique to species or particular environments.

Investigations of biofilm-forming bacteria have revealed key sensory-regulatory pathways, including intercellular communication and intracellular regulation, required to control biofilm development by altering motility and attachment behaviour, physiology and metabolism, the production of extracellular polymeric substances (EPS) forming the matrix of biofilms, and dispersants required to release bacteria from mature structures [22–27]. The use of a variety of different experimental systems, including in vitro flow cells, microtitre plates, static microcosms, etc., as well as animal models [28–31], has also identified the impact of abiotic factors such as liquid flow and mass transport; O_2 and nutrient diffusion; surface physical-chemistry and topology; and biotic interactions between bacteria, surfaces, and matrix components; and

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so on, on biofilm formation and structure [23–26,32]. In addition, research focussed more on medical and environmental microbiology, rather than on biofilm formation per se [33–37], as well as on evolutionary ecology and social microbiology [11,38–41], are increasingly providing explanations for the role or function of biofilms in different environments and evidence of their ecological success.

Despite the obvious diversity in biofilm research as evidenced by publications in journals covering a wide range of disciplines including microbiology, microbial biotechnology, environmental science, and medical microbiology, our general understanding of biofilms is nonetheless dominated by a few human pathogens and the submerged solid-surface interface biofilms they produce in flow cells or microtitre plates [28,29,31] (here we refer to these as liquid-solid surface (L-S) interface biofilms to differentiate them from other types of biofilms or bacterial aggregations). Of these, the medically inspired developmental model of *P. aeruginosa*, often chosen to represent the 'Microbial Cities' vision, is perhaps the most persuasive, with bacteria growing in these structures almost exclusively compared to free-swimming planktonic bacteria. We are growing concerned that this vision is beginning to dominate biofilm research in a negative manner.

2. A continuum of aggregations

In this opinion piece, we challenge the simplistic planktonic-(sessile) biofilm dichotomy and advocate the inclusion of biofilms within the larger context of bacterial aggregations. We believe that by recognising biofilms within a continuum of aggregations or communities with varying properties, it will enable a more extensive investigation of bacterial colonisation, and in particular, allow us to distinguish those processes governing general colonisation and ecological success from those unique to particular environments and specialised strategies.

Costerton et al. [2] defined biofilms as 'matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces ... (and) includes microbial aggregates and floccules and also adherent populations within the pore spaces of porous media'. Although this definition is broad (i.e. sensu lato), there is a presumption by current researchers that biofilms are those structures formed on submerged solid surfaces (i.e. at the L-S interface) and that other structures associated with surfaces or interfaces are somehow different or inconsequential. We would suggest that L-S interface biofilms as observed in flow-cells and microtitre plates are a means to investigate biofilm formation independently of natural environments or context, as it is difficult or impossible to extrapolate from these simple in vitro systems to the more complex natural environments from which the bacteria of interest were first isolated [29–31]. We note that in some later reviews, the description of biofilms is extended with more examples. However, this has also lead to a more relaxed *(sensu amplo)* definition in which 'biofilm' is frequently used as a synonym of 'aggregation', even though the former is often defined by the latter (e.g. [42]). As a matter of etymology, 'aggregation' which originates in late Middle English (1150–1500 AD) should take precedence over 'biofilm' whose usage largely stems from the 1990s.

As an example of how the biofilm definition has been extended following the definition of Costerton et al. [1,2], we consider the inclusion of air-liquid (A-L) interface biofilms and agar plate-grown colonies as suggested by Branda et al. [42].

3. Biofilms at the air-liquid interface

Material, including bacteria, accumulates at the air-liquid interface of sea or fresh water to form surface films often subject to highly variable conditions [19]. A-L interface biofilms also form on the surface of static liquids in experimental microcosms, and are sometimes referred to as pellicles or floating biofilms. These are produced by a wide range of bacteria, including *Bacillus subtilis* [43], *Gluconacetobacter xylinus* (formerly known as *Acetobacter xylinum* and since reclassified as *Komagataeibacter xylinus*) [44], as well as numerous enteric bacteria and pseudomonads [44–46].

Although A-L interface biofilms may look superficially similar, the diversity of bacteria which form them would suggest that they vary in structure and other characteristics as well. We have been investigating this by comparing biofilms produced by environmental *Pseudomonas* spp. isolates, using relatively large static microcosms in 30-ml glass universal vials containing liquid growth medium [44,47,48]. These allow us to undertake combined biofilm assays which determine growth, attachment to the vial walls, and biofilm strength [49–51]. Using this approach, we have been able to quantitatively differentiate biofilms produced at the meniscus and A-L interface [50,52]. These include biofilms limited to the meniscus region, attached biofilms which extend across the A-L interface, and unattached 'floating' biofilms (as well as 'invisible' attached biofilms too thin or transparent to see by eye [52]). It is possible that the floaters and attached biofilms represent substantially different colonisation strategies, with the former recruiting planktonic cells directly from the liquid column to the A-L interface and growing from multiple loci, and the latter developing from sessile cells attaching in the meniscus region and subsequently growing out across the A-L interface [52].

Although floating biofilms have been reported in which buoyancy is the result of trapped CO_2 released by respiration (e.g. *G. xylinus* [44]), the two different A-L interface biofilms produced by our model environmental pseudomonad, *P. fluorescens* SBW25, known as the viscous mass and Wrinkly Spreader biofilms, are not buoyant per se and readily sink when disturbed [49,53,54]. It is likely that they are maintained at the A-L interface by hydrophobic cell surfaces, matrix components, and surfactant which pierce or weaken the A-L interface [53,54]. Interestingly, we have recently found that for the Wrinkly Spreader, a class of adaptive mutants of the wild-type strain which evolves in static microcosms, drip-fed glass bead columns, and soil [48,51,55], attached A-L interface biofilm growth can be seamlessly linked to swarming motility and colony growth using 'transitional microcosms' in which a layer of agar is set along the side of the vial, providing both liquid and dry agar surfaces for colonisation (C. Immoor, O. Moshynets , A. Spiers, Unpublished Observations).

In these transitional microcosms, we wonder whether there is more than just planktonic bacteria growing in the liquid column and a single, distinct structure colonising both the liquid and agar surfaces in these simple environments, as suggested by the simplistic planktonic-

(sessile) biofilm dichotomy. Instead, we think it is likely that there are different types of aggregation including L-S interface biofilms attached to the vial walls at the meniscus, attached biofilms extending out across the liquid surface, confluent colonies growing on the agar surface, and microcolonies developing in the swarm-front as bacteria move up the agar surface and away from the liquid media. We are interested in identifying which abiotic and biotic factors drive growth at different points across the transitional zone, and understanding how these might alter behaviour and gene expression patterns to produce the structures we can observe within a few hours and over several days.

4. Colonies are not biofilms

Whilst we approve of the inclusion of A-L interface biofilms in the *sensu amplo* Costerton et al. [1,2] definition, we resist the suggestion that colonies grown on agar plates should be included too, despite the fact that investigations of colony morphology have been presented as biofilm research (e.g. [43,56–61] etc.). In contrast, we have investigated the colony morphologies of Wrinkly Spreader mini-*Tn* mutants on agar plates as a means to identify the genes required for biofilm formation; importantly, we also tested the mutants in static microcosms to determine the impact on biofilm formation to confirm the identity of these genes as important in biofilm formation [48].

Our objection to the inclusion of colonies as biofilms is based on a consideration of the O_2 and nutrient gradients established in these aggregations, as well as liquid flow (**Figure 1**). Rather than argue that these are insignificant differences, we would suggest that colonies, A-L and L-S interface biofilms would be better presented within a larger continuum of aggregations in which O_2 and nutrient gradients (and other chemicals including communication signals and waste, etc.), and liquid flow can be used to differentiate between types of aggregation. In this way, it now becomes reasonable to ask whether the parallel O_2 and nutrient gradients observed

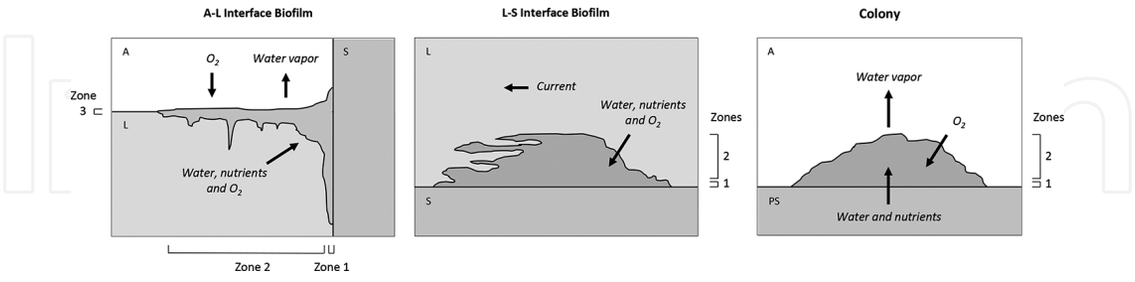

Figure 1. Bacterial aggregations include biofilms and colonies with significant similarities and interesting differences. Liquid-solid surface (L-S) interface biofilms (middle) are subject to physical stress and establish various chemical gradients. Similarly, air-liquid interface (A-L) biofilms (left) or colonies (right) also experience physical stress and establish gradients. In each type of aggregation, a layer of cells is attached to the solid surface (Zone 1) with distal regions are held in place by cell and matrix component interactions (Zone 2). In A-L interface biofilms, cells and matrix components may also break through the interface (Zone 3). However, nutrients are supplied by capillary flow (mass transport) from beneath colonies, whereas in A-L and L-S interface biofilms, they are transported or diffuse from the surrounding liquid. (A, air; L, liquid; PS, permeable or porous solid; S, solid.)

in L-S interface biofilms present a substantially different set of conditions for bacteria than the opposing gradients found in colonies. Similarly, it would be interesting to compare liquid flow within biofilms subject to external liquid currents, with the evaporation- and capillary-driven liquid flow within colonies subject to different drying regimes.

Notably, colonies show different growth patterns, have highly structured morphologies, are sometimes surrounded by EPS, and may show co-operative behaviour, so it is not unreasonable to consider that they are responding to abiotic and biotic conditions as do biofilms [6,42, 43]. In soil, water availability, often described by the matrix potential, is a significant factor restricting bacterial motility, the formation of aggregations, and colonisation through the pore network [62–64]. In such systems it is highly likely that contiguous bacterial populations colonise non-permeable solid surfaces covered or linked by thin films of water with slimes or swarms; permeable solids through which water is available with microcolonies and colonies; and partially and fully saturated pores with A-L and L-S interface biofilms, slimes, and planktonic bacteria.

5. Aggregations respond to different conditions

We would argue that bacteria colonising a range of environments should develop into different aggregations in response to local conditions and opportunities. These aggregations might appear to be superficially similar (e.g. A-L and L-S interface biofilms) or substantially different (cf. a colony), depending on bacterial responses and colonisation strategies aimed at maximising ecological success, as well as on our ability to recognise which abiotic and biotic factors have the greatest impact on the developing population.

Our observations of linked Wrinkly Spreader A-L interface biofilms and colonies in the transitional microcosms might suggest that these are very similar aggregations used to colonise two interfaces (the liquid and agar surfaces) which do not pose significantly different challenges to bacterial growth. However, competitive fitness assays in each environment suggests that Wrinkly Spreaders achieve substantially different levels of ecological success in colonising liquid and agar surfaces: they have a fitness advantage in static microcosms but are at a disadvantage in colonies compared to wild-type *P. fluorescens* SBW25 [65–68]. These fitness differences suggest that environmental conditions probably change across the transitional zone, with the initial Wrinkly Spreader population responding to these changes to colonise the A-L interface and agar surfaces in different manners.

We speculate that the fitness advantage of Wrinkly Spreaders is determined by the subtle trade-off in energy expenditure needed to produce A-L interface biofilms in static microcosms and the increased access to O_2 biofilm formation allows. The growth of *P. fluorescens* SBW25 is limited by O_2 and it drives the evolution of the Wrinkly Spreaders [69]. Relative small numbers of wild-type colonists rapidly generate an O_2 gradient through respiration, converting the homogeneous liquid column into a shallow upper zone having normal levels of O_2 and a deeper lower zone with rapidly diminishing O_2 levels (these colonists are in effect environmental engineers) [69]. As this population rapidly expands, random mutation results in

Wrinkly Spreader genotypes which are recruited to the A-L interface through the expression of attachment factor and cellulose which provides the main matrix component for the biofilm [49,53,68]. Those bacteria localised to the A-L interface have access to higher levels of O_2, compared with those lower down, and consequently grow faster [69]. Higher levels of O_2 might also induce a SOS response via reactive oxygen species (ROS) leading to the expression of an error-prone DNA polymerase, as in the case of *P. aeruginosa* PA01 [70], increasing mutation rates and the appearance of Wrinkly Spreader mutants.

As a result of access to higher levels of O_2, Wrinkly Spreaders have a fitness advantage over non-biofilm-forming competitors [69]. We speculate that in colonies where Wrinkly Spreaders still express attachment factor and cellulose, these components have no essential function and therefore pose a fitness cost to the growing population, explaining why the Wrinkly Spreaders are poorly adapted to growing on agar surfaces. Interestingly, improved O_2 access has also been suggested as an explanation for the wrinkled colonies produced by *P. aeruginosa* PA14. However, it appears that a more complex level of redox control regulating the use of different electron donors, including O_2 diffusing into the colony from above and redox-active phenazines produced by bacteria located at the base of the colony, may govern metabolism in *P. aeruginosa* PA14 and other bacteria producing wrinkled colonies including *B. subtilis* [57,71,72].

More generally, O_2 gradients determine the distribution of aerobic and anaerobic bacteria in a wide range of environments, including water columns, sediments and soils, where it effects growth, gene expression patterns and metabolism, and along with other competing electron acceptors, help define aerobic, micro-aerobic (transitional) and anaerobic niches [73–76]. It is therefore not surprising if O_2 gradients also played a central role in the development and function of a wider range of bacterial aggregations, and not just in biofilms and colonies.

6. Other terminology

As a slight digression, we present a non-exhaustive selection of vernacular and scientific terminology used to describe bacterial aggregations which includes active sludge, biofilms, clumps, colonies, flocs, mats, pellicles, rafts, slimes, zooglea, etc. (**Table 1**). We would expect that a more extensive review of the early microbiology literature, including French, German, and Russian publications, and of current microbiology, microbial biotechnology, environmental science, and medical microbiology publications, would result in more terms being identified. Although the first observations of biofilms (dental plaque) were made by van Leeuwenhoek (1683–1708) [34], microbial mats appeared much earlier, and are identified today as the fossilised remains of 3.5-billion-year-old stromatolites [77]. Arguably the first observation by a microbiologist was made by Pasteur (1864) [34], and by the end of the nineteenth-century, environmental microbiologists were investigating them as well (we list several early observations following Pasteur in **Table 2**). For example,Winogradsky (1895) [78] described jelly-like masses of bacteria as 'zooglea', whilst Egunov (1895) [79]) and Sorokina (1938) [80] more obviously referred to biofilms in the current sense, using terms that translate into bacterial 'plate' or 'plane', and 'film', respectively.

Abscesses, inclusion bodies and metastasis	A mass of bacteria growing within another structure, including sediments, soils, plant and animal tissues.
Active fluids	Formed by motile bacteria moving together at high density in a liquid.
Active sludge	A complex mixture or community of bacteria and other microorganisms produced and used in wastewater treatment.
Aggregates, blobs, clumps, colloids, lumps and masses	A mass of bacteria (and other microorganisms) having some sort of physical cohesion; these may have developed by growth, or they may be the result of physical mixing or disturbance.
Bacterial plates	A layer of bacteria formed in a water column at a certain depth depending on oxygen levels.
Biofilms	A mass of bacteria enclosed in a protective matrix of EPS and associated with a surface or interface; most often used to refer to liquid-solid surface (L-S) interface biofilms such as those developing in flow-cells and in microtitre plates.
Biolaminites, microbialites, stratifera and stromatolites	Living and fossilised microbial mats which may trap and bind sediments and/or cause mineral precipitation, sometimes intercalated by sediment laminae; sometimes referred to as microbial-induced sedimentary (MIS) structures.
Clusters	A zone of physiologically synchronised bacteria within a lager aggregation, or a small mass of bacteria such as a colony or floc.
Collapsed cakes	Formed by the collapse of clumps or flocs on membranes during filtration.
Colonies, macro- and micro-colonies	A mass of bacteria having some sort of physical cohesion and having developed by growth on a solid dry surface; micro-colonies are those associated with the biofilm development process on submerged solid surfaces, or small aggregations of bacteria not noticed as colonies unless observed with magnification on leaf surfaces, detritus, or agar plates.
Communities and consortia	A complex mixture of multiple bacterial species (or genotypes) and possibly other microorganisms in which biotic interactions define structure and function.
Crusts, dust particulates and aerosols	Microbial communities developing on the surface of soils and dessert sand; also the dried remnants of colonies etc.
Deposits and sediments	Mass or body of bacteria that accumulate on dry or submerged surfaces due to wind or water movement.
Desert varnish	A dark stain or coating covering rock surfaces and colonised by bacteria.
Filamentous structures and streamers	Long strands of material stretching out from the main mass of a biofilm subject to liquid flow.
Films, layers, planes, plates, volumes and zones	A thin layer or volume containing bacteria which may or may not be physically connected to one another, solid surfaces, or other interfaces, and occurring in liquids, porous or permeable solids.
Flocs and snow	Masses of bacteria formed by growth, self-association, hydrophobic interactions, or by attachment to suspended inert particles; flocs in sea water are referred to as snow.
Floaters	Biofilms at the air-liquid interface having no appreciable attachment to a solid surface; these may be localised at the liquid surface by buoyancy, penetration of the interface, or by hydrophobic surfaces.
Foams	Air-water emulsions containing high concentrations of bacteria and compounds such as polymers and surfactants, which may help stabilise the structure.
Granules	A mass of bacteria growing on small solid particles.

Jellies and zooglea (or zoogloea)	A slimy mass of bacteria encased in a gel-like material, often found floating in water or found on plant stems or leaf litter.
Laminae	Layers of microbial mat generations, or microbial mats overgrowing sediments.
Mats	Microbial communities, often with clear layering or stratification, found in streams, lake or sea beds; these may also contain, algae and plants, and trap small particles including sand and stones.
Meniscus growth	A mass of bacteria adhered to a solid surface in the meniscus region of static liquids (the air-liquid-solid surface (A-L-S) interface).
Microcenosis, microbial cenosis	Microbial communities formed in a particular niche or site.
Micro-zones and pelogens	Microbial communities growing in thin layers structurally segregated with different characteristics or activities in sediments or silt.
Pellicles	A thin film or gel-like coating surrounding of individual bacteria, as well as air-liquid (A-L) interface biofilms.
Phlegm balls	Flocs found in underground streams.
Plaque	Dental biofilms formed largely by anaerobic bacteria on the surfaces and cavities in teeth.
Rafts	Flat sections on the edges of colonies, often associated with twitching motility, or flat pieces of un-attached biofilm found at the air-liquid interface.
Remains and remnants	A mass of bacteria and cellular debris found at a site at which they probably did not develop or a portion of a larger mass which has been removed by physical disturbance, predation or decay.
Sediments	Bacteria from liquids no longer in suspension, or microbial communities developing in sediments or silt.
Slimes, glycocalyx and viscous liquids	Viscous liquids or regions of a larger volume of liquid containing high densities of bacteria and EPS.
Snottites and snoticles	Pendulous or dripping masses of bacteria developing on cave walls or at the bottom of stalactites, especially limestone cave speleothems.
Swarms	Bacteria showing a particular form of surface-associated motility, moving in high densities across moist or wet surfaces.

This is a non-exhaustive list where terms, meanings and usage varies between contexts, and grouped terms may not be synonymous.

Table 1. Vernacular and scientific terminology used to describe bacterial aggregations

1864 Pasteur describes slimy material called Mother of Vinegar [34].

1887 Winogradsky observed bacterial growth in ring-like structures in a liquid microcosm containing H_2S and covered with a glass slide to restrict O_2 diffusion [86].

1893 Beijerink observed bacteria growing in zones of enriched water microcosms, the positions of which could be altered depending on O_2 and H_2 levels. Described 'Bakterienniveau' or 'niveau' as an aggregation formed by motile bacteria [87].

1895 Winogradsky observes bacterial zoogleas on potato slices [78].

1895	Egunov observed bacterial plates forming in microcosms containing Black Sea sediments, the position of which depended on anaerobic conditions, O_2 and H_2S levels. Some of these bacteria were motile, and Egunov asked what forces drove them to form a stationary aggregation [79,88].
1900	Egunov describes bacterial attachment during plate formation, and his 'bioanisotropy' concept for environments (continuous matter exchange between an organism and its surroundings) [89].
1914	Isachenko describes 'pink water' caused by the aggregation of purple bacteria in sea water as well as bacteria forming cloud-like structures in liquid microcosms [36].
1933	Henrici observed that bacteria mostly grow on submerged surfaces, not in free flowing water [34].
1935	Zobell describes marine bacteria attachment to surfaces [34].
1938	Sorokina describes a bacterial film forming on a submerged slime surface in a liquid microcosm [80].

Table 2. Early observations of bacterial aggregations.

More recently, pendulous and dripping snottites have been described on cave walls and stalactites [81] and collapsed cakes are a problem in filtration [82]. More interesting, perhaps, are the reports that bacterial remains have been misidentified as dinosaur soft tissues [83] and desert varnishes are being used to train sensors for future planetary explorations [84]. Regrettably, we also note that a chance to create a more evocative science fiction term for biofilms on the International Space Station was missed [85].

We argue that such an extensive collection of terms used to describe bacterial aggregations should not be considered a plethora, but rather an indication that the diversity of aggregations we are aware of may reflect the multitude of ways bacteria to respond to differing ecological opportunities. However, we do not suggest that each of the terms are unique, as quite evidently different types of aggregations may be associated closely or more distantly from one another, depending on which abiotic or biotic factors are considered.

7. Key features linking aggregations

Bacteria interact with abiotic and biotic factors by responding to physical and chemical cues according to behavioural and adaptive strategies under constant selection to maximise fitness. When these cues and strategies are considered in the context of a larger continuum of aggregations, it is possible to identify key features that link aggregations within a larger continuum (**Table 3**). Here we briefly indicate how physical interactions, diffusion radii, and increasing genetic diversity might be used to compare different types of aggregations.

Chemical gradients	Controlling the behaviour of individuals and groups, defining zones of optimal and restricted growth, providing information about local conditions; of nutrients, O_2 and other electron acceptors and metabolites, chemosensory, regulatory and communication compounds, and waste.
Competition	Between genotypes or lineages for resources, drives adaptation, allows investment in public goods but also results in cheaters.
Complexity	Of abiotic and biotic interactions, of genotypes, metabolism, structures, etc.

Communication, co-operation and co-ordination	Linking individuals into a group through the exchange of communication signals and/or response to the same environmental signals, resulting in similar behaviours or activities, and the production of common goods such as EPS and other secreted products.
Developmental pathway	Guiding the behaviour individuals and groups through a series of defined stages and resulting in a specific type of aggregate; responding to abiotic and biotic factors including communication signals.
Environmental conditions and modification	Abiotic chemical and physical factors, biotic factors; niche, opportunities, substrates, resources, stress, variation and instability; local, large-scale and irreversible changes, depletion, contamination.
Gene expression	Controlling behaviour, communication, metabolism, the production of compounds required for the formation of aggregations, etc.
Genotypes and diversity	Aggregations may arise from a single individual or founder population of the same genotype, but diversity will develop over time with radiation and immigration; single or multi-species aggregations; cheaters, invaders and persisters.
Liquid flow	Around and within aggregations, effecting the development of the physical structure, deformation and breakage, boundary layers, mass transport and diffusion of molecules, as well as the movement of bacteria.
Mobility	Of individual bacteria, small groups and larger aggregations, over small and large-scale distances, across interfaces, surfaces and volumes, and through environments.
Physical interactions	With interfaces, surfaces, bacteria, EPS, etc., in terms of strength, elasticity or resilience, distances, duration and reversibility.
Resilience	To chemical and physical stress, external competition, predation, and in terms of physical structure.
Sensory zones	The ability of individuals to detect the presence of others using altered chemical gradients, metabolites and communication signals (diffusion radii), and the distance separating individuals or groups.
Stratification	From homogeneous mixtures to clustered or layered differences resulting from age, metabolism, diversity and function.
Structure and rheology	Aggregations having properties similar to Newtonian liquids, visco-elastic gels and solids.
Succession	The diversity and function of aggregations will develop over time and with changing environmental conditions.
Time	The time-scale for the development, presence or persistence of aggregations may vary considerably, effecting population size, radiation, succession, aggregation structure and stratification, and environmental impact.

Table 3. Features linking bacterial aggregations within a larger continuum.

Physical interactions involving bacterial surface coatings, appendages and matrix, solid surfaces and interfaces within A-L and L-S interface biofilms are known to be complex (**Figure 2**), but there is no reason to believe that all types of interactions are unique to these particular aggregations. We therefore propose that one feature of the continuum of aggregations could be expressed by a scale going from no interactions with solid surfaces or other interfaces, as in the case of a population of planktonic bacteria, through short-term, weak, or distant physical contacts with surfaces, interfaces, or material, to the complexity of interactions seen in A-L and L-S interface biofilms, and presumably in other aggregations such as flocs, granules, and snow.

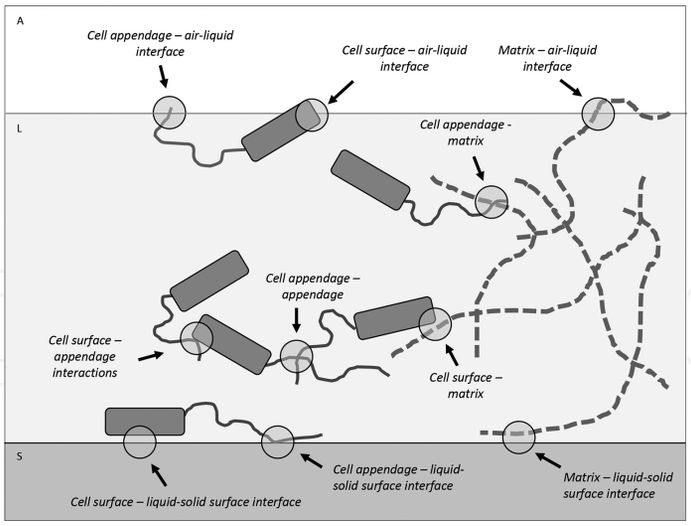

Figure 2. Bacterial aggregations are constructed by numerous and varied physical interactions. Bacterial cells within aggregations will interact with one another through close, intermediate, and long-range interactions involving surface coatings and extended appendages (e.g. flagella), matrix components, solid surfaces and interfaces (e.g. the air-liquid interface). (Key: A, air; L, liquid; S, solid; Matrix components or fibres are artistically depicted by dashed lines and bacteria as bacilli with a single flagella.)

Interactions with surfaces and interfaces also clearly limit the ability of an aggregation to develop, so the ability to expand across surfaces or to penetrate volumes, along with altered mass transport and diffusion characteristics, also present other scales with which to compare aggregations (**Figure 3**). In particular, diffusion radii will determine the ability of individual bacteria to detect the proximity of others and to respond competitively or with co-operation. Clearly, low-density planktonic and surface-attached bacteria may be beyond detection distances, but as bacterial numbers increase, their individual and collective impacts on local environmental conditions will lead to a situation where they are now within the same micro-environment, and similar conditions may result in coordinated changes in gene expression

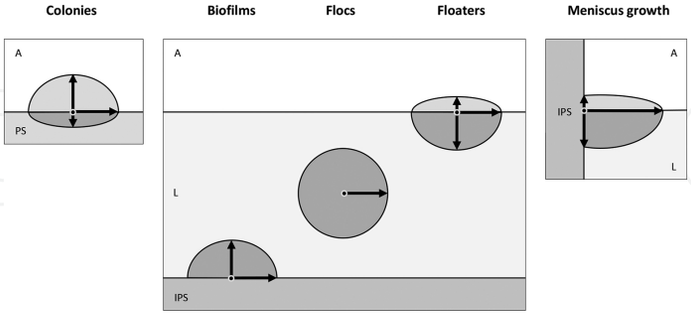

Figure 3. Bacterial aggregations develop at interfaces, within liquids, porous solids, and visco-elastic materials. Colonising bacteria may develop into communities restricted primarily by diffusion or liquid flow (mass transport) of chemicals and the ability to expand across interfaces or into spaces (radial arrows). Shown here (left to right) are colonies growing on and into a solid, biofilms growing on a solid submerged surface, flocs developing from a suspended particle, floaters forming at the air-liquid interface, and meniscus growth occurring at the air-liquid-solid surface interface. (Key : A, air; IPS, impermeable solid; L, liquid; PS, permeable or porous solid; S, solid; arrows indicate expansion radii and the opposite direction indicates diffusion gradients that limit growth.)

patterns, behaviour, and metabolism. We propose that separation distance, scaled in terms of chemical gradients, will also be a useful means of comparing and differentiating bacterial aggregations.

Aggregations developing over significant periods of time will also gain diversity by radiation and immigration, leading to multispecies communities subject to ecological succession, driven by internal competitive and co-operative interactions and changing environmental conditions (**Figure 4**). We propose that both diversity in terms of genotype or species composition and community function could also be used to consider bacterial aggregations, and this perspective is complementary to understanding the physical interactions and the molecular biology underlying the development of these structures.

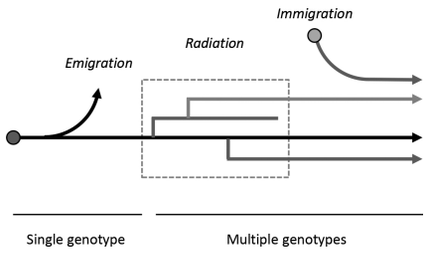

Figure 4. Bacterial aggregations gain diversity over time. An aggregation developing from a single genotype will gain diversity through radiation (mutation), immigration, and succession. Stochastic events and changing selective pressures will result in different genotypes within the community; some genotypes may become extinct and diversity may fall. (Key: Time progresses from left to right; colonising genotypes are shown as circles; mutation events as vertical lines; successful genotypes are indicated by arrows and an extinction event by a truncated line.)

By considering the ability of individual bacteria to respond to their local environments via different growth and colonisation strategies, the impact of abiotic conditions on individuals and the structures they create, and the longer-term development of the community, it is possible to speculate how altered environmental conditions and circumstance can lead to the cycling between different types of aggregation (**Figure 5**). The ability of bacteria to move between different types of aggregation with changing conditions or to exploit new opportunities will clearly differentiate those able to colonise a wide range of environments with those adapted to very specific niches.

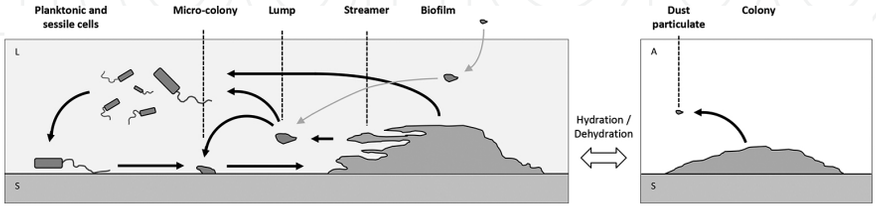

Figure 5. Bacterial aggregations change nature with altering environmental conditions. Although aggregations may develop and persist in one site, conditions may change resulting in an aggregation with a substantially different nature. The archetypal L-S interface biofilm developmental process is shown on the left. Biofilms may dry out to form slimes and colonies, and further drying might result in dust particulates which may rehydrate to form aggregations. (Key: A, air; L, liquid; S, solid; bacteria are artistically depicted as bacilli with a single flagella.)

8. Conclusion

In this opinion piece, we advocate the inclusion of biofilms within the larger context of a continuum of bacterial aggregations. We do this because we are growing concerned that the 'Microbial Cities' vision originating from the seminal reviews by Costerton et al. [1,2] is beginning to dominate biofilm research, and that such a narrow view limits our ability to better understand bacterial colonisation of a variety of different environments. In this continuum, we consider A-L and L-S interface biofilms to be biofilms, but argue that other aggregations such as colonies are significantly different and should not be referred to using this particular term. It is also possible that applied and environmental microbiologists prefer to refer to microbial mats rather than to biofilms, because the latter is too closely associated with experimental L-S interface biofilms produced in flow-cells or microtitre plates, and too far removed from the aggregations found in natural and other man-made environments.

We believe that the advantages of taking a wide view will allow us to distinguish those processes governing general colonisation through the formation of aggregations and ecological success, from those unique to particular environments and specialised strategies (as an apologia, we remind readers of the sensu lato definition of biofilms, secundum Costerton et al., which was inclusive of a number of different aggregations). By suggesting that biofilms are better considered as one of a variety of different aggregations, the simplistic planktonic-(sessile) biofilm dichotomy is also challenged, and perhaps the best reference or comparator for different aggregations will not always be logarithmic phase planktonic bacteria.

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