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Abstract

Microbes are generally thought of as unicellular organisms, but we know that many microbes live as parts of biofilms - complex, surface-attached microbial communities numbering millions of cells. Some authors have recently argued in favour of reconceiving biofilms as biological entities in their own right. In particular, some have claimed that multispecies biofilms are evolutionary individuals (Doolittle 2013; Ereshefsky & Pedroso 2015). Against this view, I defend the conservative consensus that selection acts primarily upon microbial cells.

Levels of selection in biofilms: Multispecies biofilms are *not* evolutionary individuals

We ordinarily think of as bacteria and other microbes as tiny unicellular organisms. Some of the great advances of microbiology were achieved thanks to the development of a laboratory methodology for purifying wild strains of microbes and growing them in perfectly clonal lineages where it can be assumed that a colony is just one cell multiplied many times. We have long known that naturally occurring microbes don't live like this. In the natural environment they are closely packed, with huge numbers of different species all living together. The default view is that the masses into which wild microbes aggregate are merely aggregates. But some microbiologists argue that the reductionist approach to microbial investigation has been a mistake and that biofilms should be thought of as multicellular individuals in their own right.

"In many ways an individual bacterium is more analogous to a component cell of a multicellular organism than it is to be a free-living, autonomous organism." (Shapiro 1988); "Biofilms resemble the tissues formed by eukaryotic cells" (Costerton et al 1995); "Microbial communities display emergent properties – the properties of the community are more than the sum of those of its component populations" (Marsh & Bowden 2000); "Bacteria have evolved the ability to form multicellular communities" (Fuente-Nunez et al 2013); "numerous types of multispecies biofilms have many features associated with evolutionary individuality." (Ereshefsky & Pedroso 2015).

Other researchers have been critical of this perspective, and have sought to undermine it: *"Biofilms are merely multiorganismal, similar in principle to a flock of birds, a school of fish or a swarm of insects."* (Nadell et al 2009).

My aim in this paper is to evaluate the claims that are made about biofilm individuality, especially the claim that multispecies biofilms are evolutionary individuals.

1.1 What is a biofilm?

Wild biofilms are as varied as the concatenations of different microbes that can constitute them and the niches which they can inhabit. Any generalisations made will fit actual cases only more or less well. Nonetheless, some generalities there must be, for it to be worth having a class term 'biofilm' at all.

A biofilm is a community of many sessile microorganisms. Biofilms, unlike mere aggregates of cells, always feature an extracellular matrix (ECM). This is a complex glue-like substance produced by the cells themselves. It holds the cells together, and forms a one way barrier with the environment, letting water and enzymes in but keeping threats out. Biofilms always develop on some sort of surface –such as the hull of a boat, the lining of an intestine or the air-water interface on the surface

of a liquid. I focus on multispecies biofilms. They will typically contain many different species of bacteria as well as other microorganisms such as archaea and protists, carrying out diverse metabolic functions. A mature biofilm contains many millions of cells.

Biofilms are ubiquitous, growing anywhere with nutrients and water, from oceanic thermal vents to gaps between soil particles, to drinking fountains (Schwering et al 2013). They love human bodies, growing on our teeth (Kolenbrander et al 2010), in our guts, on our skin, on medical implants such as catheters and even inside arteries.

Biofilms are not homogeneous lumps of slime, but delicate and spatially heterogeneous structures with complex and variable three dimensional architectures (Wolfaardt et al 1994) that are occasionally visible to the naked eye (Asally et al 2012). They are organised according to oxygen and nutrient gradients into different regions which specialise for diverse tasks (Stewart and Franklin 2008). Biofilm microbes use intercellular signals to coordinate colony behaviours and regulate gene expression (Parsek & Greenberg 2005). One signalling system, called 'quorum sensing', works by secretion of diffusible molecules which are monitored as a proxy for population density, allowing cells to tune their phenotype to the local abundance of cells of a particular genotype. Some quorum sensing systems are species-specific, but others support inter-species communication, for example in oral biofilms (Waters & Bassler 2005; Federle 2009), and even communication between bacteria and fungi (Bamford et al 2009; Elias & Banin 2012).

Biofilm microbes have a heightened capacity ('competence') for participating in lateral gene transfer (henceforth LGT), in which cells can absorb packets of Dna from the environment ('transformation'), swap DNA with another cell ('conjugation') or accept some DNA carried around by a virus or plasmid ('transduction') (Molin & Tolker-Nielsen 2003). Lateral transfer provides an extra route by which traits can be passed between cells, in addition to the normal vertical process of inheritance from a parent cell.

Biofilm life enables microbes to live in conditions where they cannot survive alone (Marsh & Bowden 2000). It provides defence against predators, against host immune defences and unpredictable environmental changes. It allows access to resources that individual cells cannot effectively obtain on their own, via metabolic divisions of labour (Kolter 2005). The study of microbial ecology has hugely important medical and industrial applications. Multispecies biofilms have been implicated in chronic infections, in the development of tooth decay and heart disease, in the clogging and poisoning of everything from waterways to dairy pumps to medical implants. Beneficial biofilms, on the other hand, can be used in food and pharmaceutical production, to degrade pollutants, to control pests, to maintain immune and digestive health, and more. A better understanding of the ways in which microbial cells interact is essential to harnessing these possibilities and controlling the risks (Boyle et al 2013; Crespi et al 2014).

1.2 Exclusions

"Some (bacteria) have adopted truly multicellular lifestyles and have abandoned unicellular growth" (Claessen et al 2014).

We must separate claims about multispecies biofilms from claims about other sorts of microbial aggregates. Some bacterial species form single-species colonies in the wild, while other species are

only known to form biofilms as parts of a multispecies community and never alone (Yamada et al 2005). Some form filaments or mats of connected cells. For example, filamentous cyanobacteria are always found in chains, but other species filament only facultatively (Rokas 2008). Obligately filamented species can be further divided into those that differentiate into sterile cell types and those that do not¹. Myxobacteria usually live as single cells, but under some conditions they form aggregate fruiting bodies in which some cells are sterile, in addition to other complex coordinated behaviours such as swarming and collective hunting (Dworkin 1996). Heterocystous cyanobacteria have cells that are specialised for nitrogen fixation.

The obligately filamented and differentiated bacteria are increasingly classified as ‘multicellular’ as a matter of consensus. In other words, even those that want to deny *general* claims about biofilm multicellularity think that obligately filamented and differentiated bacteria are a special case that belong with other paradigm multicellular organisms. It is interesting to consider what it is about the filamentous bacteria that has moved the consensus in favour of calling them multicellular². However, in the remaining discussion I set the filamentous and all single-species microbial aggregates aside and focus only on multispecies biofilms. Many of the vivid and colourful examples that Shapiro gave of complex bacterial behaviour – such as swarming, collective hunting and fruiting bodies – involved single-species bacterial aggregates, although he extended his claims about bacterial multicellularity much more widely (Shapiro 1988). Most studies of biofilms have focused on single –species biofilms, or on cocultures which differ at a single locus, but the results of such studies cannot be generalised to multispecies scenarios.

1.3 Some nearby views

Scientists can often be heard saying that definitions do not interest them. But none of us can decide whether it is right to ascribe some property to the world without first achieving some clarity about what the property is. In the absence of definitional work, rival interlocuters will often simply be ascribing different properties to the world and talking past one another. Table 1. collects some different claims which can underlie ascriptions of biofilm multicellularity.

| Claim | Authors | Status |
|---|--|--|
| 1. Certain bacterial species are obligately filamented and differentiated | Rokas 2008; Fisher et al 2013; Claessen et al 2014 | True, but not about multispecies biofilms. |

¹ A complication here lies in distinguishing programmed cell death from accidental death caused by competition for resources. Ratcliff et al argue that they saw the evolution of multicellular yeast floccules on the grounds that they exhibit sterile cells which apoptose in order to enhance the fitness of the group (Ratcliff et al 2012). But a different interpretation is that the cells in question merely starve to death as the surrounding cells obscure their access to scarce resources.

² Claessen et al are content to judge that some bacteria have lost the ability to survive and divide in a unicellular, planktonic state (Claessen et al 2014). Some authors write that a species will only be considered multicellular if its life cycle includes a multicellular stage necessarily (Fisher et al 2013). A problem with this view is that it makes a presupposition about what counts as a life cycle. From the point of view of view of a cell, the life cycle is complete once the cell has divided. If you argue that cells in a filament dont complete division, then you are assuming that physical attachment is enough to make two cells into one life. Although *Anabaena* always form chains, any cell separated from a chain will still divide. We can simplify matters if we rule that spore formation, but not cell fission, qualifies as completion of the life cycle. But this only makes it obvious that some circularity lies in defining multicellularity according to a life cycle.

| | | |
|---|--|--|
| 2. Certain bacterial species <i>sometimes</i> exhibit differentiated parts | Dworkin 1996; Shapiro 1998 | True, but not about multispecies biofilms |
| 3. Biofilms can be featured in attractive metaphors alongside multicellular organisms. | Watnick & Kolter 2000; Nikolaev & Plakunov 2007 | Not intended to have a literal truth value |
| 4. Biofilm cells are physically stuck together And 5. Biofilms cells communicate with one another | Lyons & Kolter 2015 Parsek & Greenberg 2005 | True |
| 6. Biofilms exhibit life cycles | Costerton et al 1995; O'Toole et al 2000; Stoodley et al 2002; Hall-Stoodley et al 2004; Singer et al 2010; Rendueles & Ghigo 2012 | Not an empirical claim |
| 7. Multispecies biofilms are organised/are physiologically unified systems | O'Malley & Dupré 2007; Dupré & O'Malley 2009; O'Malley 2014 | True |
| 8. Biofilms exhibit higher-level adaptations | Costerton et al 1995; Shapiro 1998; Stoodley et al 2002; Parsek & Greenberg 2005; Veening et al 2008; Ehrlich et al 2010; Wilking et al 2012; Kåhrström 2013 | Not a verifiable claim |
| 9. Single species bacterial colonies are social | Griffin, West & Buckling 2004; Kreft 2004; Diggle et al 2007; West et al 2007; Brockhurst et al 2008; Mitri, Xavier & Foster 2011; Celiker & Gore 2012; Boyle et al 2013 | True |
| 10. Multispecies biofilm cells interact synergistically | Marsh & Bowden 2000; Yamada et al 2005; Burmølle et al 2006; Mitri & Foster 2014; Ren et al 2015 | True |
| 11. Multispecies biofilms are evolutionary individuals | Ereshefsky & Pedroso 2013; 2015 Doolittle 2013 | False |

Claim three: Metaphor

"We liken the multispecies bacterial biofilm to a city" (Watnick & Kolter 2000)

We can separate out claims that are intended to be merely metaphorical or poetic in nature. In some cases the author means little more than metaphor, or literary flourish. Charlotte Werndl wrote that *"whether communities that show organism-like properties are called organisms or not is a matter of convention and is not very interesting."* (Werndl 2013). Similarly, Haber has said that most of the claims made about whether entity such and such is a superorganism are little more than

overblown analogies. Some colonies are similar to organisms in some ways, in other ways they are dissimilar. Since everything is similar to everything else in some sense or other, these are not questions we should seriously exercise ourselves with, he argues (Haber 2013).

Metaphors can be fruitful, especially when they draw attention to features of an object which might otherwise not be salient. Nevertheless, I focus my analysis on claims which yield more explicit empirical content. At least some of the time, the issue is discussed as if something substantive rests on it. See, for example, this claim - *"This perception of functional biofilm communitieswill usher in a new golden age of understanding in virtually all fields of microbiology"* (Costerton et al 1995).

Claims four and five: Adhesion and communication

Lyons & Kolter defend biofilm multicellularity in a 2015 paper, but they make it clear early on that what they mean by the claim is that biofilms meet both of two conditions. Firstly, there is cell-cell adhesion in virtue of the ECM. Secondly, there is 'intercellular communication leading to coordinated activity.' (Lyons & Kolter 2015, 21).

Each of these claims is empirically verifiable and true. They are, furthermore, interesting claims which connect to important questions about transitions and the evolution of multicellularity. What isn't so obvious is that we should *define* multicellularity by these two criteria. In fact, I think there are reasons why we should not, but these are not, and cannot be, empirical reasons. For present purposes it suffices to suggest that propositions featuring the term 'multicellular' can be replaced, throughout Lyons & Kolter's paper, with propositions featuring their two criteria, without any loss of content. Such a replacement will be a good thing in any instance where resistance to the claims is motivated by an alternative interpretation of the term 'multicellular'.

Claim six: Life cycle

Biofilms undergo cyclical changes, starting with initial colonisation of a surface by 'founder' cells, who are then joined by secondary colonisers (Bos et al 1999), moving through increased complexity and structured differentiation towards maturity, and ending with dispersal of 'propagule' cells (Stoodley et al 2002; Hall-Stoodley et al 2004). The changes are accompanied by dramatic changes in gene expression and phenotype on the part of individual cells, leading to many different forms of specialisation (Sauer et al 2002; Luppens et al 2008). For example, some cells specialise in anchoring the colony to a surface using extracellular pilli, like little grappling hooks. Others secrete glues or other useful products. Some become antibiotic resistant.

Stoodley and Hall-Stoodley have championed the view of changes in biofilm community structure as developmental stages of a life cycle, analogous to embryogenesis, beginning with initial colonisation and moving through maturation to dispersal (O'Toole et al 2000; Stoodley et al 2002; Hall-Stoodley et al 2004). The dispersal stage is said to enable biofilms to spread and colonize new surfaces – to achieve a sort of reproduction, in other words. Movement between the stages is regulated by chemical signalling and environmental cues. Hall-Stoodley et al provide detailed descriptions of

different developmental stages that biofilms move through and the different mechanisms that regulate them³.

Hall-Stoodley's claim that biofilms exhibit life cycles is not one that, like Lyons and Kolter's claims, can be settled empirically. Reference to various sorts of data and details about life cycle may make the claim appear empirical but all of the details can be made subject to a gestalt shift. A life cycle is one aspect of the picture that we see when we adopt a holistic perspective on biofilms. But there is no aspect of the data that we cannot alternatively see from a reductionistic perspective. The claims about life cycle stages can be conceptualised in terms of ecological succession, or changes in community structure (Alexander 1971; Barton & Northup 2011). Hansen et al liken the complexity found in biofilms to that found in a tropical rainforest (Hansen et al 2007). Ecological succession involves colonisation of a virgin territory by species that are well-adapted to be founders, and who then modify the environment in such a way that it becomes suitable for colonisation by later species. This does not move us to say that the first founders act for the benefit of the secondary colonisers in the ecological setting and we should be sceptical of parallel claims in the biofilm case.

None of the details that Hall-Stoodley et al describe is empirically decisive one way or the other. We can view a cell that leaves a biofilm as a propagule that develops into an offspring biofilm, or we can view it as a migrating individual that acts as a founder of a new ecosystem, without changing any empirical data. It is not that claims about life cycles can never be evaluated empirically. We could transform the distinction into an empirical one, for example by defining a life cycle as including terminally differentiated parts. But on that definition, biofilms will simply fail to qualify (Nikolaev & Plakunov 2007; Espinosa-Urgel 2009). Alternatively, we could build content into the claim about life cycles by including Godfrey-Smith's parameters (bottleneck, integration, germ separation) as necessary conditions, and then assessing whether or not biofilms meet these empirical conditions (Godfrey-Smith 2009). Ereshefsky & Pedroso show in their 2013 paper that biofilms, unlike paradigm metazoans, do not meet Godfrey-Smith's conditions. In the absence of such further conditions, the claim that biofilms can be viewed as if they exhibit life cycles says little more than the claim that biofilms can be viewed as if they are multicellular organisms.

Claim seven: Organisation

Another way that that we could interpret holistic claims about biofilms is as utilising the word 'organism' in its Kantian sense, to pick out a system that is organised in a particular way, or to a particular extent. 'Organism' is here contrasted with 'biological individual' where the latter picks out a unit defined by natural selection (Godfrey-Smith 2011). There are myriad ways in which we might make the property 'organisation' precise so that it can be empirically measured— in terms of homeostasis, or thermodynamics, or physiological complexity, or metabolic interdependence of parts (Godfrey-Smith 2011), for example. Sometimes claims about organisation are intended less as empirical claims, and more as a claim that we ought to foreground the organisation between a biofilm's parts ahead of, say selective competition between them (Dupré & O'Malley 2009).

2. Evolutionary individuals as levels of selection.

³ Although these papers were based on research carried out only on single-species biofilms, others make use of analogous 'life cycle stages' in multispecies settings (Singer et al 2010; Rendueles & Ghigo 2012).

“It is easy, then, to be drawn to the view that microbial communities, or at least those comprising persistent biofilms, are some kind of evolutionary entities in their own right, understandable as units of inheritance and targets of selection, however loose, with metabolic, structural and genetic “functions” (such as LGT) evolved by natural selection operating at this collective level. That is, they might indeed be reproducing individuals, showing heritable variation in fitness.” (Doolittle 2013)

Some authors explicitly claim that multispecies biofilms are evolutionary individuals⁴, defined in terms of natural selection and, in particular, Lewontin’s conditions for evolution by natural selection (Doolittle 2013; Ereshefsky & Pedroso 2013; 2015). These conditions – often summarised as heritable variance in fitness – appear in Lewontin’s 1970 statement as:

- i) Different members of the population express different values for a trait (phenotypic variation).
- ii) Variation in the trait value causes variation in fitness (differential fitness).
- iii) The trait value, along with its fitness effect, must be heritable (heritability) (Lewontin 1970).

To claim that biofilms are evolutionary individuals is to claim, therefore, that biofilms meet these conditions – that biofilms form populations, in which different biofilms express different phenotypic traits, that those traits cause differential biofilm fitness and are heritable.

I think this is a good way to think about evolutionary individuality, because it homes in on an object’s capacity to participate in selective competitions, and to respond to selection by evolving cumulative adaptations (Sober & Wilson 1998; Clarke 2013). This capacity is of great significance in evolutionary processes, because it determines which masses of living matter are separately selectable, so that they can found unique evolutionary lineages. Parts that vary from one another in the way Lewontin describes can be selected separately from one another. Parts that do not, can not. An object whose parts are unable to vary from one another in ways that are heritable and that affect their fitness can only be selected, if at all, together - as one single unit. So Ereshefsky and Pedroso are right to assert that Lewontin’s are the conditions that biofilms would have to meet if they are to function in a biofilm-level selection process, so that fitness differences between biofilms drive changes in the frequencies of biofilm traits over time.

However, I am not convinced that multispecies biofilms do meet Lewontin’s conditions – or at least, *not much*. Evolutionary individuality is continuous - living objects can manifest more or less of the properties that are essential to the evolutionary process. Paradigm examples of evolutionary individuals – such as mammals, and some eusocial colonies - have very high individuality, because their parts are completely co-selected: it is impossible for one part to do well, evolutionarily speaking, at the expense of another, because their parts are prevented from exhibiting differential heritable fitness. My arm cannot out-compete my leg⁵. But most living things, especially those from the plant and fungal domains, exhibit some intermediate degree of evolutionary individuality at multiple hierarchical levels. They have parts that are sometimes co-selected, but sometimes compete with one another.

⁴ A different use of the term ‘individual’ occurs when bacterial cells are claimed to be individuals - as in phenotypically different from one another (Davidson & Surette 2008; Ackermann 2013).

⁵ Cells, especially, cancerous cells, may occasionally attempt to proliferate at the expense of the rest. But they lack any means for passing their traits on to future generations – there is no heritability.

In my understanding, the hierarchical level at which selection acts in biofilms is not a matter of convention, any more than the question whether human beings are multicellular or unicellular entities is conventional. The objects at those levels either exhibit heritable variance in fitness or they do not. There is a difference between multicelled organisms and single celled organisms, even if it is not a sharp one. We should not accept a definition which trivialises or obscures the distinction.

My task is to find out where on this continuum a typical multispecies biofilm lies. Are biofilms selected mostly as wholes? Or are biofilm parts mostly selected independently of one another? According to the default, reductionist view, the answer is that selection acts on individual microbial cells, or not at all. Cells compete against one another by varying in those properties that affect their cellular fitness. And because cells pass on those traits to their mitotic daughters, populations of cells are able to respond to selection so that there is evolution – change in their frequencies of heritable traits that is driven by variance in cellular fitness. Microbial evolution, on this view, can be understood purely in terms of survival of the fittest cells.

The challenger, then, is a view according to which cells are mere parts in the real agents of microbial competition: biofilms. On this view, microbial evolution occurs as a consequence of competition between biofilms, with the more fit biofilms passing on their fitness-affecting traits to a greater number of descendants so that those traits increase in frequency over time. In other words, the view that multispecies biofilms are evolutionary individuals assumes that biofilm microbes evolve, at least in part, by *group selection* (Chuang et al 2009; Penn et al 2012; Roditi, Boyle & Xavier 2013).

Where is the truth, between these two extremes? Biofilms are different in one important respect from paradigm multicellulars: The cells that inhabit biofilms never undergo terminal differentiation. Their capacity to reproduce and to found their own lineages of cells is never epigenetically switched off. This means that there is always room for mutations, or other sources of heritable variation, to appear and to be passed on across cell divisions. If a mutant has an advantage it can proliferate and replace the wild type cells within the biofilm. Microbial cells can and do sometimes do well at the expense of their biofilm-coinhabitants and any account of the levels of selection at work in biofilms must accommodate this. If biofilms have any individuality at all, it is in addition to that possessed by their component cells.

However, there may be some reasons not to go entirely the other way, and conceive individuality as entirely cellular in biofilms.

2.2 Arguments in favour of viewing biofilms as evolutionary individuals

Heritability

A population that meets Lewontin's criteria is divided into discrete units, each of which reproduces with heritable variance in fitness. In most natural conditions it is difficult to see on what grounds we might delineate the boundaries of separate biofilms, which would be necessary in order to count what ever we decide qualifies as offspring. How many biofilms are there in the average mouth? How many on the ocean floor? It is hard to see how we would decide where one biofilm ends and another begins.

Supposing you do delineate groups, a response to selection on such groups will only occur if there is sufficient heritability of group traits: it needs to be the case that an offspring resembles its parent, in

respect of the focal trait, more than it resembles other biofilms in the population. There have been several demonstrations of evolution of biofilms in a laboratory setting, where heritability can be imposed on successive cultures of biofilms (Hansen et al 2007; Poltak & Cooper 2011; O'Rourke et al 2015) but it is unclear to what extent these can inform us about naturally occurring biofilms (Traverse et al 2013). There is some evidence that coevolution has occurred in a wild setting, in the lungs of cystic fibrosis patients (Elias & Banin 2012, 999). Consideration of the mechanisms by which wild biofilms are 'born' or founded give us further reason to doubt that variation in biofilm traits will tend to be passed on to biofilm 'offspring'.

Development of a mature biofilm from a propagule is aggregative. An initial founder will settle on the surface and shift from its planktonic to its biofilm mode. Then it will produce mitotic offspring, but also be joined by other cells from different places. Founders can sometimes exert some control over which following cells are accepted to join the community, using mechanisms of co-aggregation. But they cannot ensure that all of the species from the 'parent' biofilm make it into the 'offspring' (Kolenbrander et al 2010). So there is reason to doubt that biofilms are able to pass genes on to successive generations with sufficient reliability for competition between biofilms to produce any response. Furthermore, the species composition of the community can change over time in an open-ended fashion as its metabolic capacities change, and as interactions between species change, suggesting that gene frequency change occurring within the 'lifetime' of a biofilm will swamp any between-biofilm effects.

Ereshefsky & Pedroso say "*biofilms have adaptive traits that are transmitted with fidelity between ancestral and descendent biofilms.*" (Ereshefsky & Pedroso 2015, 10126) and "*microbiologists that study biofilms frequently talk about biofilm traits that occur over and over again....such as quorum sensing systems, metabolic interactions, aggregation patterns, cooperative behaviours, the mechanisms underlying lateral gene transfer and the production of EPS components.*" (Ereshefsky & Pedroso 2015, 10128). But we only consider transmission as giving rise to heritability if the traits transmitted vary in the population. It is not enough to resemble the parent with respect to traits that are universal/fixed in the population – these are invisible to selection anyway. So it is not enough to identify 'biofilm traits that occur over and over again'. To meet Lewontin's conditions, biofilms need to form lineages in which fitness-affecting novelties get transmitted from parents to offspring.

Ereshefsky and Pedroso note that the genetic basis of some of these traits has been identified. Genes are of course heritable, so this might seem like strong evidence that there is heritable variation for the traits at stake. However, recall that new biofilms are formed by the aggregation of cells from many different 'parent' biofilms. Possession of multiple parents is not in itself an obstacle to heritability, as long as the trait value of offspring is a simple sum of the parental values. But suppose that the founders do carry novelties, genetic variants that generated a fitness advantage in their parent context. When the founder settles in its new context, living with a different array of other microbial species, then there are two possibilities. Most likely, the new context will eliminate any adaptive value that the novelty conferred on the parent biofilm. In those cases in which the trait's function is relatively context-independent (antibiotic resistance, perhaps) then there is no reason to frame the trait as being a property of the whole biofilm, rather than as a property of one of the component cell lineages. That is, thanks to the way that combinations of microbes are

reshuffled between biofilm generations, fitness-enhancing novelties will either lose their fitness effect entirely when they are transmitted to offspring, or they are context-independent and so better conceived as cellular traits⁶. As an example, consider the production of the ECM, the glue that holds biofilm cells together and fixes them to their substrate. Some authors point to the advantages the ECM provides to biofilm cells, compared to planktonic cells – for example, increased resistance to antibiotics – as evidence that ECM production evolved for its benefits to biofilms. But if we find that ECM production is triggered by one lineage whenever it finds itself in the company of any other strain or species – if the trait is context-independent, in other words – then it makes more sense to view ECM production as a competitive adaptation *of that cell lineage* (Oliveira et al 2015). Heritable, yes, but not higher-level. Whole biofilms only exhibit heritable traits if their component lineages migrate collectively to new niches, and we do not see this happening (Kolenbrander 2010).

Doolittle argues instead that biofilms have a non-standard sort of heritability, in which community interactions patterns act as replicators (Doolittle 2013). A particular environmental niche will often be filled by a biofilm with a characteristic set of ‘guilds’ (Burke et al 2011) or ‘ecotypes’, where each guild carries out a particular metabolic role, but the occupiers of these roles can be very diverse, in terms of species and genotypes. So Doolittle’s suggestion is that biofilms have heritability in that the same variety of guilds will be re-established recurrently, so that biofilms inherit a ‘characteristic metabolic interaction systems’ but without inheriting any particular genes. *“It is (almost) as if this community were a superorganism, recruiting genes to maintain itself from a compositionally fluid collection of organismal lineages whose own evolutionary trajectories can be taken as largely irrelevant.”* (Doolittle & Zhaxybayeva 2010, 110). *“What would be being inherited is a characteristic metabolic interaction system, not (or not necessarily) a specific community of microbial lineages”* (Doolittle 2013).

If specific lineages are not co-inherited then there won’t be any genetic heritability. But genes are not the *only* source of heritable variance in fitness, even if they are a very good one. Epigenetics is capable of providing the heritability necessary for evolution by natural selection, psychological mechanisms for social learning are capable of supporting the heritability required for cultural evolution. What Doolittle suggests is that there is an analogous non-genetic source of heritability that could support evolution of biofilm phenotypes: recruitment. Some microbial species exhibit ‘co-aggregation mechanisms’, which allow the cells to recognise and then adhere to cells of particular species (Katharios-Lanwermer et al 2014). Several genes have been identified which play an active role in recruitment of other species during the colonisation of dental plaques (Xu et al 2003; Kuboniwa et al 2006). So Doolittle’s suggestion is that biofilm founders could use co-aggregation mechanisms to enable them to co-occur with others who will endow the biofilm, not with genetic heritability, but with similarity of metabolic function over time. In this case, changes to the recruitment mechanisms could act as mutations, bringing about recurrence of a slightly different metabolic phenotype.

⁶ It might be noted that analogous processes of shuffling during sexual reproduction are not thought to prevent the traits of sexual organisms from being heritable, even though we know that epistasis is significant. However, if those same epistatic effects were occurring between genes in organisms that had an unlimited number of parents, with no mechanisms for controlling which genes could pair with which and no mechanisms of developmental canalisation, then we might think again.

Note that mere similarity of guilds over generations of biofilms, generated by stability of ecological constraints, would not be enough to qualify as heritability. It needs to be the case that earlier biofilms *bring about* the traits that are similar in later generations: heritability is a causal notion⁷. I think Doolittle's suggestion is that ancestor biofilms do bring about the similarity by first sending founder cells to the new site, who then manipulate the traits of later joiners using their co-aggregation mechanisms.

The question is, are co-aggregation mechanisms successful in establishing sufficient heritability of this non-genetic sort? And would change brought about in this way be recognisable despite conflicting change occurring as a consequence of selection on microbial genes? These are interesting empirical questions, to which I don't believe we yet know the answer.

Adaptations

A promising source of argument in favour of the claim that multispecies biofilms are evolutionary individuals is the phenomenon of higher-level adaptation. To call a trait a higher-level adaptation is to say more than just that it is beneficial to the higher-level unit: it says something about the trait's selective history. Biofilms exhibit biofilm-level adaptations just in case they manifest traits that *have been selected* in virtue of the benefit they provide to the whole biofilm. Cellular adaptations, by contrast, have given a selective advantage to microbial cells. So if biofilms exhibit biofilm-level adaptations, we can use these adaptations as *evidence* that biofilms have in fact acted as evolutionary individuals. A biofilm adaptation is *proof* of whole-biofilm selection.

It is commonplace for microbiologists to advance adaptive hypotheses on behalf of single species biofilms. For example, mature biofilms often exhibit a structure in which cells grow in towers, separated by troughs or channels (Lawrence et al 1991; Wolfaardt et al 1994). Costerton et al suggest that the channels function as colony circulatory systems, allowing enhanced access to oxygen and nutrients as well as the efficient removal of waste (Costerton et al 1995). The implied claim is that circulatory channels are the outcome of a historical process of natural selection in which biofilms with channels outcompeted biofilms without channels. Veening et al characterise clonal microbial populations as adopting a collective bet-hedging strategy, in which a sub-population of cells go into a dormant, antibiotic-resistant 'persister' state, in order that the colony is insured against uncertain future environmental conditions (Veening et al 2008). Wrinkliness in *Pseudomonas aeruginosa* colonies has been described as an adaptation for maximising access to oxygen by increasing the colony surface area (Kåhrström 2013).

When it comes to multispecies biofilms, signalling systems have been described as biofilm-level adaptations, because the success of signalling depends upon the production of costly signalling molecules. The costliness of the molecules makes the communication system vulnerable to cheaters – free riders who reap the benefits of communication, or of the complexity it enables, without paying the costs (Parsek & Greenberg 2005). In the 'Distributed Genome hypothesis' Ehrlich et al argue that Lateral Gene Transfer is an adaptation in pathogenic biofilms that functions as a system of "mutagenesis to produce a cloud of similar strains to confuse and overwhelm the host's immune system" (Ehrlich et al 2010, 269).

⁷ With many thanks to Kim Sterelny for this point.

Unfortunately, it would be rash to suppose that we can use these examples as easy confirmation of biofilm individuality, because the status of biofilm traits as adaptations is not easy to settle. Nobody who denies that biofilms are multicellular individuals is likely to accept that biofilms exhibit adaptations. The question is, how would we recognise an adaptation if we saw one? We don't usually have access to the selective history of the trait. When we give an adaptive hypothesis we are inferring that the right sort of history took place, from the nature of the trait itself. We might say something like 'This trait is so complex/well-designed that it must have been selected for some purpose. I can imagine it playing purpose x, which benefits the whole biofilm. Therefore, it must have been selected by biofilm-level selection.'

The trouble is that these hypotheses are limited only by our imagination and often we will be wrong. Traits can look well-designed even when they appear purely as a consequence of physical or chemical necessity, without any need for a selective explanation. For example, Nadell et al used a computer simulation to demonstrate that you can get the formation of colonies with towers with empty channels between them just as a consequence of nutrient limitation, which imposes a bottleneck on the growing cell population (Nadell, Foster & Xavier 2009). The channels identified by Costerton et al as functioning for biofilm circulation, in other words, could emerge by mere physical necessity. The benefit to biofilm waste disposal is a mere side effect, rather than the explanation for the channels. Objections of this sort do not *prove* that any trait did not evolve in a process of biofilm-level selection. Yet they undermine the argument from adaptation in so far as they show that an adaptive hypothesis is not necessary for explaining the existence of a trait. Things can appear designed when they are not⁸.

Interactions

The cells in biofilms are known to engage in a variety of fitness-affecting interactions with one another, some competitive and some synergistic (Rendueles & Ghigo 2012; Elias & Banin 2012). A lot of work has been done, in the context of social evolution theory (West et al 2006), to understand how and when interactions between cells of a common genotype can be cooperative (Kreft 2004; Diggle et al 2007; West et al 2007; Brockhurst et al 2008; Nadell et al 2009; Boyle et al 2013; Van Gestel et al 2014). These results have relevance for single-species biofilms, but also for those multispecies biofilms, in so far as the latter as expected to contain clonal patches of cells as a consequence of clonal cell division (Mitri et al 2011; Mitri & Foster 2014).

Synergistic interactions are classified experimentally as those between two different strains that show a faster rate of increase, or higher biovolume, when co-cultured than when each is cultured alone⁹. A widespread class of synergistic interaction is syntrophy, a metabolic interaction in which species A depends on a waste product secreted by species B and vice versa (also known as 'by-product mutualism' or 'cross-feeding'). The pair together might then use resources more efficiently

⁸ This is not a problem that is specific to the biofilm case: adaptive hypotheses are generally flimsy constructions on which to rest arguments, although the problems are certainly exacerbated in contexts where there is potentially more than one hierarchical level of selection in play (Clarke Forthcoming). Methods are available for subjecting adaptive hypotheses to tests (eg Orzack & Sober 2001) but as far as I know these have not been applied to biofilm traits.

⁹ Note that this falls short of demonstrating that the relevant traits are social adaptations – i.e. were selected in virtue of their benefits to the recipient (Mitri & Foster 2013).

than either can alone. For example, biofilm communities often comprise a mixture of aerobic and anaerobic guilds situated at complementary depths within the three-dimensional structure.

Synergistic interactions are thought to be responsible for various biofilm advantages, such as greater biomass, increased resistance to antimicrobials and to host immune defences (Kolenbrander et al 2010; Elias & Banin 2012; Burmolle et al 2014). Some microbes have only been successfully cultured at all when in coculture and it is suspected that the underlying reason why many species identified by metagenomics methods have not yet been successfully cultured is that they depend for their survival on the secretions of other species.

There is ongoing controversy about whether competitive or cooperative interactions are dominant in multispecies biofilms, with some reviews suggesting the former (Foster & Bell 2012; Oliveira et al 2015) although critics point out that these tend to be based on investigations of culturable bacteria, which may be strongly biased towards an anti-social minority of all microbes.

How are interactions relevant to evolutionary individuality? To the extent that one strain is dependent on another for some ecological function then it is not free to evolve independently of that strain. If I need you, then your success affects me. What is bad for you is bad for me also. So even the slightest of interdependencies can set constraints on the extent to which two partners can enjoy differential fitness outcomes. However, the strength of the evolutionary alignment between two lineages depends upon how many options each maintains. If a cell can only get its needs met by one particular partner, then it is very dependent on that partner. But if there are a variety of different partners from which the resource can be taken, maybe even many different species, then the cell is not dependent on any one of them¹⁰. Metabolic integration is more compromising of evolutionary individuality when it is very specific – when there is only one provider of the relevant resource.

Cross-feeding interactions can be fairly stable, because the two species have very different ecological requirements and so avoid competition with one another, although the interaction can still be undermined by competition within either partner lineage if there is genetic variation (Mitri, Xavier & Foster 2011). However, the *evolution* of mutualism depends on the reliable presence of each partner. The most extreme examples of interdependence occur when there are mechanisms for co-inheritance between generations, so that the lineages run truly in tandem (Godfrey-Smith 2011). Endosymbiotic partnerships are a limit case, in which the partners often evolve reduced genomes, because they have undergone selection for loss of genes whose function is provided by the social partner (Morris et al 2012). There are some known cases in which cells of two separate species physically bind together (Mitri & Foster 2013). Because biofilms are aggregative the individual cell lineages have only limited control over the species with which they interact, so it is likely that they mostly maintain a plurality of different partners on which they can depend. However, mechanisms of co-aggregation, which control which pairs of species can bind together, may increase the probability of co-occurrence (Rickard et al 2003; Periasamy & Kolenbrander 2009).

So interdependency between cells in a biofilm really can undermine the evolutionary individuality of those cells, by limiting the extent to which they can evolve independently of one another. But interdependency is only expected to evolve when the dependence is specific, and when the cells are

¹⁰ Ecologists term this 'protocooperation' to indicate that the interaction is not essential.

in reliable interaction with one another over evolutionary time scales. In other words, the interaction must be heritable. Many cross-feeding mutualisms are not of this nature. Rather, mutualisms occur between metabolic types, where each actual partner is replaceable by any other species of that type.

Furthermore, synergistic interactions do not provide justification for the view that *whole biofilms* are evolutionary individuals, because most interactions take place across spatial scales that are much smaller than an entire biofilm (Mitri & Foster 2013). The scale across which an interaction occurs will be determined by variables such as how far a public good is able to diffuse over the relevant time scale, cell motility, growth rate, what sort of local population structure exists, environmental stability including level of disturbance, the nature of the substrate (eg its viscosity), nutrient density and resource gradients. Mature biofilms exhibit a fine grained structure as a consequence of resource gradients selecting for task specialisation in different areas¹¹. A typical biofilm will be divided into very many tiny microniches with different interactions occurring in different areas.

We might then use interspecific mutualisms and cooperation within clonal patches as cases of evolutionary individuality manifested by sub-biofilm but supra-cellular microniches? However, even this conclusion is not supported. Interactions between cells will often be neighbour-structured, rather than occurring within well-defined and non-overlapping groups of cells (Okasha 2006; Godfrey-Smith 2006; 2008). In other words, the cells in a biofilm may each have unique interaction networks so that there can be no non-arbitrary division of the population into groups. Without group structure there can be no group selection.

Back to reductionism?

If we reject the status of multispecies biofilms as evolutionary individuals it may look as if we are committed to a reductionistic view, in which microbes are conceptualised as unicellular atoms living independent lives. However, there are diverse approaches we can use to accommodate the rich interactions and evolutionary co-dependencies between different cells and different microbial species that occur in biofilms, *without* assuming that biofilm populations are structured into discrete groups.

Each of neighbour-modulated fitness (Frank 1998; Taylor et al 2007; Taylor et al 2013), contextual analysis (Goodnight et al 1992; Goodnight 2013) and kin selection theory (West et al 2006; West et al 2007; Driscoll & Pepper 2010) provides a way to model scenarios in which the fitness of a focal unit is dependent upon the character of its interaction partners – its social context (Okasha 2006). But because each understands fitness as a lower-level property - a cellular property, to be measured in the currency of cellular rate of increase, in the case of biofilms – they can all be applied to

¹¹ In fact Elias & Banin suggest that multispecies biofilms tend to exhibit one of three spatial architectures: a collection of neighbouring clonal microcolonies; co-aggregation in which cells of two species are mixed throughout the colony ('interdigitated cellular mosaics' (Katharios-Lanwermyer et al 2014)); and finally layered structures, in which different species occupy different layers (Elias & Banin 2012, 997). They note that these structures will have very different consequences for the ability of the different species to interact, especially in high flow conditions where diffusibles may not accumulate. There is some evidence, on the other hand, that the structure may be determined by the type of interactions between cells (Momeni et al 2013). In addition, more specific morphologies have been described for particular co-aggregations, such as 'corn-cob-like structures' and 'rosettes' (Katharios-Lanwermyer et al 2014).

neighbour-structured populations. Other useful tools can be borrowed from symbiosis research, such as biological market theory (Kiers et al 2011; Werner et al 2014). The ideal approach will combine elements of social evolution theory with ecology to capture the ways in which a microbial cell's fitness is affected by its particular local context, especially the properties of the cells with which it is engaged in fitness-affecting interactions (Hansen et al 2007; Mitri & Foster 2011; Coyte et al 2015).

Lateral gene transfer

Several authors have connected lateral gene transfer (LGT) to biofilm individuality. One argument is that LGT is a biofilm-level adaptation – that mechanisms for transfer have been selected in virtue of their benefits to biofilms. The postulated benefits include an enhanced ability to respond to changing threats (Ochman & Moran 2001), such as those from antibiotic treatment (Davies & Davies 2010) or host immune defences (Ehrlich et al 2010). The thought is that LGT gives microbes access to a sort of community resource, a genetic commons, from which they can draw in times of need (Dupré & O'Malley 2007; Doolittle 2013).

There are rivals to this explanation for LGT. Transfer by phage viruses ('transduction') could have been selected for its benefits to phage lineages, with the effects on cellular fitness being mere side effects. On this view cells are just bystanders in a process driven by competition between genetic elements which use cells as hosts. Transformation, in which cells take up free DNA from the extracellular environment and incorporate it into their own, may occur simply because that DNA constitutes a valuable source of scarce nutrients in densely packed biofilms (Redfield 2001). Each of these hypotheses is able to accommodate the enhanced rate of transfer observed in biofilm conditions, as well as the over-representation of cooperative phenotypes on transferred genes. However, conjugation, which some have found to be the dominant mode of transfer within biofilms (Wolska 2003), is not so easy to explain away.

In any case, it's not necessary for LGT to be a biofilm adaptation for it to have consequences for biofilm individuality. Ereshefsky & Pedroso argue that LGT interweaves the genomes of the parts of a biofilm together so that they are not merely parallel, unlike the members of a macroscopic ecosystem (Ereshefsky & Pedroso 2013). Even if LGT were simply an adaptation, it might still have the effect of raising relatedness between cells so that competition between them is eliminated.

The extent to which lateral transfer could act as a homogenising influence on biofilms is limited by the fact that transfer is trait-specific. It will not bring about the sort of across-trait relatedness that occurs as a consequence of common descent. Cooperation between cells that are related only at a single locus is expected to be unstable because it can act against the interests of all the other genes of those cells. They have a common evolutionary fate with respect to just one out of many traits, in other words. Perhaps, on the other hand, lateral gene transfer could play a role in facilitating genetic heritability between generations of biofilms. Supposing a key trait is missing from a biofilm because no cells bearing it happened to spread there from the parent – it is possible that a phage could transmit the trait to the biofilm later on. Much of this is still hypothetical however. More work needs to be done to investigate the extent to which LGT can alter the dynamics of social evolution by altering patterns of relatedness. Nonetheless, lateral transfer could have widespread and significant consequences for interaction patterns within biofilms.

Conclusions

Claims about biofilm individuality express a plurality of propositions, ranging from the innocuously metaphorical, at one extreme, to straightforwardly descriptive/empirical claims at the other. One claim that has relatively clear empirical content is the proposition that multispecies biofilms are evolutionary individuals, capable of responding to a process of selection between competing biofilms. Some ambiguity is implied by the fact that 'multispecies biofilm' is not a homogeneous kind, but a descriptor that collects together a variety of structures whose response to selection will vary according to various details. For example, biofilms which are frequently disrupted or experience fast flow likely to have less group structuring, in which case kin selection is likely to be more significant. Nonetheless, for any particular biofilm there will be an empirical fact of the matter about the extent to which the aggregate responds, as a whole, to natural selection, as opposed to responding only at the cellular level.

So, in general, are multispecies biofilms evolutionary individuals? Or are they instead mere colonial aggregates of cellular individuals? I found that the biggest obstacle standing in the way of a whole-biofilm response to selection is heritability. Thanks to the aggregative nature of biofilm formation, and to the retention of reproductive independence by cells, there will rarely be enough genetic heritability across biofilm generations to support a response to selection. Some possible exceptions to this verdict include cases where co-aggregation mechanisms are effective to secure co-occurrence of genotypes, or to secure co-occurrence of metabolic guilds if we assume that phenotypes can evolve in the absence of genetic heritability. I noted that mutualistic interactions between species can undermine the independence of cell lineages, but only in so far as the interaction is specific. For specific interaction partners to co-evolve, it is necessary once again for there to be some mechanism securing co-occurrence. These have evolved in respect of some symbiotic associations. However, none of these exceptions licence a general inference to whole-biofilm individuality, because they all concern units that are smaller than a whole biofilm – such as cell lineage pairs, clonal patches or cell interaction neighbourhoods. However, I do include a final question mark over the possible role of LGT in supporting cooperative interactions between parts of biofilms, and mediating heritability between them.

I conclude that on balance, there is little utility in treating wild biofilms as if they can function as evolutionary individuals. In other words, I doubt that wild biofilms generally evolve by group selection, where whole biofilms are taken as groups. The evolutionary fates of the cells that make up wild multispecies biofilms will not, generally, coincide.

This doesn't mean we are stuck with a reductionist understanding of biofilm cells as independent atoms or that population structure has not been critical to the evolution of their traits. We need not return to the Kochian paradigm in which microbial properties are discerned by investigation of individual cells. A biofilm is not one bacterium multiplied by a million, but a community whose members display as much variation and uniqueness as the trees of a forest, or the inhabitants of a city. Each cell develops its own phenotype, conditional on the precise conditions it finds itself in – who its neighbours are, where it sits on nutrient gradients and what messages it receives. Furthermore, biofilms exhibit synergistic phenomena which a reductive account of microbial evolution in terms of competition between selfish cells cannot explain – at least not without making reference to effects upon other cells.

Some of the traits of biofilm microbes will be comprehensible only if we take the context in which those cells are selected into account. I suggest that we utilise neighbour-structured, contextual, and social evolution models, which allow us to understand the fitness of microbial cells as constitutively social or context-dependent, without having to identify discrete groups or keep track of ancestry across generations of biofilms.

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Bibliography

Ackerman M (2013) Microbial individuality in the natural environment. *The ISME Journal* 7: 465-7.

Alexander M (1971) *Microbial Ecology*. New York, Wiley.

Asally M, Kittisopikul M, Rué P, Du Y, Hu Z, Çağatay T, Robinson AB, Lu H, Garcia-Ojalvo J and Süel GM (2012) Localized cell death focuses mechanical forces during 3D patterning in a biofilm. *Proceedings of the National Academy of Sciences*, 109(46): 18891-18896.

Bamford CV, d'Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF (2009) *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. *Infection and immunity* 77(9): 3696-704.

Barton LL, Northup DE (2011) *Microbial Ecology*. Wiley-Blackwell, New Jersey.

Bos R, Van der Mei HC, Busscher HJ (1999) Physico-chemistry of initial microbial adhesive interactions—its mechanisms and methods for study. *FEMS microbiology reviews* 23(2): 179-230.

Boyle KE, Heilmann S, van Ditmarsch D, Xavier JB (2013) Exploiting social evolution in biofilms. *Current opinion in microbiology* 16(2): 207-12.

Brockhurst MA, Buckling A, Racey D, Gardner A (2008) Resource supply and the evolution of public-goods cooperation in bacteria. *BMC Biology* 6(1):20.

Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ and Kjelleberg S (2006) Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. *Applied and environmental microbiology* 72(6): 3916-23.

Burmølle M, Ren D, Bjarnsholt T, Sørensen SJ (2014) Interactions in multispecies biofilms: do they actually matter?. *Trends in microbiology* 22(2): 84-91.

Celiker H, Gore J (2012) Cellular cooperation: insights from microbes. *Trends in Cell Biology* 23(1):9-15.

Chuang JS, Rivoire O and Leibler S (2009) Simpson's Paradox in a Synthetic Microbial System. *Science* 323, (5911): 272-275.

Claessen, Rozen, Kuipers, Sogaard-Andersen and van Wezel (2014) Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Mic* 12: 115-124.

Clarke E (2013) The multiple realizability of biological individuals. *The Journal of Philosophy* 110(8): 413-435.

Clarke E (Forthcoming) Adaptation, multilevel selection and organismality: A clash of perspectives. In Richard Joyce (Editor) *The Routledge Handbook of Evolution and Philosophy*.

Costerton, Lewandowski, Caldwell, Korber and Lappin-Scott (1995) Microbial Biofilms. *Annu Rev Microbiol* 49:711-45.

Coyte KZ, Schluter J and Foster KR (2015) *Science* 350(6261): 663-6.

Crespi B, Foster K, Ubeda F (2014) First principles of Hamiltonian medicine. *Phil Trans R Soc B* 369 (1642).

Damuth J, Heisler IL (1988) Alternative Formations of Multi-level Selection. *Biol Philos* 3: 407–430.

Davidson & Surette (2008) Individuality in Bacteria. *Annu Rev Genet* 42:253–68.

Davies & Davies (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev.* 74(3):417-33.

Diggie SP, Griffin AS, Campbell GS, West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. *Nature* 450(7168): 411-4.

Doolittle WF (2013) Microbial neopleomorphism *Biol Philos* 28:351–378.

Doolittle WF, Zhaxybayeva O (2010) Metagenomics and the units of biological organization. *Bioscience* 60(2): 102-12.

Driscoll WW and Pepper JW (2010) Theory for the evolution of diffusible external goods. *Evolution* 64(9): 2682-7.

Dupré JA O'Malley M (2009) Varieties of living things: life at the intersection of lineage and metabolism. *Philosophy & Theory in Biology* 1.

Dworkin M (1996) Recent advances in the social and developmental biology of the myxobacteria. *Microbiological Rev* 60: 70–102.

Ehrlich G, Ahmed A, Earl J, Hiller N., Costerton J, Stoodley P, Post C, De Meo P, Hu F (2010) The distributed genome hypothesis as a rubric for understanding evolution in situ during chronic bacterial biofilm infectious processes. *FEMS Immunol and Med Microbiol* 59:269–279.

Elias S, Banin E. Multi-species biofilms: living with friendly neighbors (2012) *FEMS Microbiology Reviews* 36(5): 990-1004.

Ereshefsky M & Pedroso M (2013) Biological Individuality: The Case of Biofilms *Biol Philos.* 28(2):331-49.

Ereshefsky M & Pedroso M. 2015. Rethinking evolutionary individuality. *PNAS USA* 112(33): 10126-32.

Espinosa-Urgel E (2009) Multicellularity, neoplasias and biofilms. *Research in Microbiology* 160(1):85-6.

Federle MJ (2009) Autoinducer-2-based chemical communication in bacteria: complexities of interspecies signaling. *Contrib Microbiol* 16: 18–32.

Fisher RM, Cornwallis CK, West SA (2013) Group formation, relatedness, and the evolution of multicellularity. *Current Biology* 23(12): 1120-5.

Foster KR, Bell T (2012) Competition, not cooperation, dominates interactions among culturable microbial species. *Current biology* 22(19): 1845-50.

Frank SA (1998) *Foundations of social evolution*. Princeton University Press.

de la Fuente-Núñez, Reffuveille, Fernández and Hancock (2013) Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr Op Microbiology* 16:580–589.

van Gestel J, Weissing FJ, Kuipers OP, Kovács ÁT (2014) Density of founder cells affects spatial pattern formation and cooperation in *Bacillus subtilis* biofilms. *The ISME journal* 8(10): 2069-79.

Godfrey-Smith P (2006) Local interaction, multilevel selection, and evolutionary transitions. *Biological Theory* 1(4): 372-80.

Godfrey-Smith P (2012) Varieties of population structure and the levels of selection. *The British Journal for the Philosophy of Science* 59(1): 25-50.

Godfrey-Smith P (2009) *Darwinian populations and natural selection*. Oxford University Press, Oxford.

Godfrey-Smith P (2011) Darwinian populations and transitions in individuality. In: Calcott B and Sterelny K (eds) *The major transitions in evolution revisited*. The MIT Press, Cambridge, pp 65–81.

Goodnight CJ, Schwartz JM and Stevens L (1992) Contextual analysis of models of group selection, soft selection, hard selection and the evolution of altruism. *Am. Nat.* 140: 743–761.

Goodnight C (2013) On multilevel selection and kin selection: contextual analysis meets direct fitness. *Evolution* 67(6): 1539-48.

Griffin AS, West SA, Buckling A (2004) Cooperation and competition in pathogenic bacteria. *Nature* 430(7003): 1024-7.

Haber M. (2013) Colonies are individuals: revisiting the superorganism revival. In: Bouchard F and Huneman P (eds) *From Groups to Individuals: Evolution and Emerging Individuality*. The MIT Press, Cambridge, pp 195-217.

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95–108.

Hansen SK, Rainey PB, Haagensen, JAJ, Molin S (2007). Evolution of species interactions in a biofilm community. *Nature*, 445, 533–536.

Kåhrström CT (2013) Biofilms: Survival of the wrinkliest. *Nature Reviews Microbiology* 11(3): 148-9.

Katharios-Lanwermeier S, Xi C, Jakobovics NS, Rickard AH (2014) Mini-review: Microbial coaggregation: ubiquity and implications for biofilm development. *Biofouling* 30(10): 1235-51.

Kiers ET, Duhamel M, Beesetty Y, Mensah JA, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart Mm, Bago A, Palmer TA West SA, Vandenkoornhuysse P, Jansa J and Bucking H (2011) Reciprocal rewards stabilise cooperation in the mycorrhizal symbiosis. *Science* 333(6044): 880-2.

Kolenbrander PE, Palmer RJ Jr, Periasamy S and Jakobovics NS (2010) Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 8, 471–480.

Kolter R (2005) Surfacing views of biofilm biology. *Trends in microbiology*, 13(1), 1-2.

Kreft JU (2004) Biofilms promote altruism. *Microbiology*, 150(8), 2751-2760.

Lewontin R (1970) The units of selection. *Ann Rev of Eco and Syst* 1:1–18.

Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE (1991) Optical sectioning of microbial biofilms. *Journal of bacteriology* 173(20): 6558-67.

Luppens SB, Kara D, Bandounas L, Jonker MJ, Wittink FR, Bruning O, Breit TM, Ten Cate JM, Crielaard W (2008) Effect of *Veillonella parvula* on the antimicrobial resistance and gene expression of *Streptococcus mutans* grown in a dual-species biofilm. *Oral microbiology and immunology* 23(3): 183-9.

Lyons NA, Kolter R (2015) On the evolution of bacterial multicellularity. *Current opinion in microbiology* 24: 21-8.

Marsh & Bowden (2000) Microbial community interactions in biofilms. In: Allison, D. G., Gilbert, P., Lappin-Scott, H. M. & Wilson, M. (eds). *Community Structure and Co-operation in Biofilms* (Vol. Society for Microbiology Symposium 59), Cambridge: Cambridge University Press. pp. 167–198.

Michod RE (1999) *Darwinian Dynamics: evolutionary transitions in fitness and individuality*. Princeton University Press.

Mitri S and Foster KR (2014) The Genotypic view of Social Interactions in Microbial Communities. *Annu Rev Genet* 47:247–73.

Mitri S, Xavier JB and Foster KR (2011) Social Evolution in Multispecies Biofilms. *PNAS* 108 (Supplement 2): 10839-46.

Molin S and Tolker-Nielsen T (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Op Biotech* 14 (3): 255–61.

Momeni B, Brileya KA, Fields MW, Shou W (2013) Strong inter-population cooperation leads to partner intermixing in microbial communities. *Elife* 2: e00230.

Morris JJ, Lenski RE and Zinser ER (2012) The Black Queen Hypothesis: Evolution of dependencies through adaptive gene loss. *MBio* 3(2): e00036-12.

Nadall CD, Xavier JB, Foster KR (2009) The sociobiology of biofilms, *FEMS Microbiol Rev* 1-19.

Nikolaev & Plakunov (2007) Biofilm—“City of Microbes” or an Analogue of Multicellular Organisms? *Microbiology* 76 (2): 125–138.

Ochman H and Moran NA (2001) Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science* 292 (5519): 1096-1099.

Okasha S (2006) *Evolution and the Levels of Selection*. Oxford: Clarendon Press.

Oliveira NM, Martinez-Garcia E, Xavier J, Durham WM, Kolter R, Kim W and Foster KR (2015) Biofilm formation as a response to ecological competition. *PLoS Biol* 13(7): e1002191.

Orzack SH and Sober E (2001) *Adaptationism and Optimality*. New York: Cambridge University Press.

O'Malley M (2014) *Philosophy of microbiology*. Cambridge University Press.

O'Malley MA and Dupré J (2007) Size doesn't matter: Towards a more inclusive philosophy of biology. *Biol Philos*, 22(2): 155–191.

O'Rourke D, FitzGerald CE, Traverse CC, Cooper VS (2015) There and back again: consequences of biofilm specialization under selection for dispersal. *Frontiers in genetics* 6.

O'Toole G, Kaplan HB, Kolter R (2000) Biofilm Formation as Microbial Development *Annu Rev Microbiol* 54:49–79.

Parsek MR, Greenberg EP (2005). *Sociomicrobiology: The connections between quorum sensing and biofilms*. *Trends in Microbiology* 13, 27–33.

Penn AS, Conibear TC, Watson RA, Kraaijeveld AR, Webb JS (2012) Can Simpson's paradox explain cooperation in *Pseudomonas aeruginosa* biofilms?. *FEMS Immunology & Medical Microbiology* 65(2): 226-35.

Periasamy S, Kolenbrander PE (2009) Mutualistic biofilm communities develop with *Porphyromonas gingivalis* and initial, early, and late colonizers of enamel. *Journal of bacteriology* 191(22): 6804-11.

Poltak SR, Cooper VS (2011) Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. *The ISME journal* 5(3): 369-78.

Ratcliff WC, Denison RF, Borrello M and Travisano M (2012) Experimental evolution of multicellularity. *Proceedings of the National Academy of Sciences* 109(5): 1595-600.

Ren D, Madsen JS, Sørensen SJ and Burmølle M (2015) High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. *The ISME Journal* 9: 81-9.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in microbiology* 11(2): 94-100.

Rendueles O, Ghigo JM (2012) Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS microbiology reviews* 36(5): 972-89.

Roditi LDV, Boyle KE and Xavier JB (2013) Multilevel selection analysis of a microbial social trait *Molecular Systems Biology* 9(1): 684.

Rokas A (2008) The origins of multicellularity and the early history of the genetic toolkit for animal development. *Annual review of genetics* 42: 235-51.

Sauer K, Camper AK, Ehrlich GD, Costerton JW and Davies DG (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184(4): 1140-54.

Schwering M et al. (2013) Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection. *Biofouling* 29: 917–928.

Shapiro JA (1988) Bacteria as multicellular organisms. *Sci Am* 258:82–89.

Singer SW, Erickson BK, VerBerkmoes NC, Hwang M, Shah MB, Hettich RL, Banfield JF and Thelen MP (2010) Posttranslational modification and sequence variation of redox-active proteins correlate with biofilm life cycle in natural microbial communities. *The ISME Journal* 4: 1398-409.

Smith J, Van Dyken JD and Zee PC (2010) A generalization of Hamilton's rule for the evolution of microbial cooperation. *Science* 328 (5986): 1700-3.

Sober E, Wilson DS (1998) *Unto others: The Evolution and Psychology of unselfish behaviour*. Harvard University Press.

Stewart PS, Franklin MJ (2008) Physiological heterogeneity in biofilms. *Nature Reviews Microbiology* 6(3): 199-210.

Stoodley P, Sauer K, Davies DG and Costerton JW (2002) Biofilms as complex differentiated communities *Annu Rev Microbiol* 56: 187-209.

Taylor PD, Day T, Wild G. (2007) Evolution of cooperation in a finite homogeneous graph. *Nature* 447(7143): 469-72.

Taylor TB, Rodrigues AM, Gardner A, Buckling A (2013) The social evolution of dispersal with public goods cooperation. *Journal of evolutionary biology* 26(12): 2644-53.

Traverse CC, Mayo-Smith LM, Poltak SR, Cooper VS (2013) Tangled bank of experimentally evolved *Burkholderia* biofilms reflects selection during chronic infections. *Proceedings of the National Academy of Sciences* 110(3): E250-9.

Veening, Smits & Kuipers (2008) Bistability, Epigenetics and Bet-Hedging in Bacteria. *Annu Rev Microbiol* 62:193–210.

Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* 21: 319-46.

Watnick & Kolter (2000) Biofilm, City of Microbes. *J Bacteriol* 182(10): 2675-9.

Werndl C (2013) Do microbes question standard thinking in the philosophy of biology?. *Analysis: ant017*.

Werner GD, Strassmann JE, Ivens AB, Engelmoer DJ, Verbruggen E, Queller DC, Noë R, Johnson NC, Hammerstein P, Kiers ET. (2014) Evolution of microbial markets. *Proceedings of the National Academy of Sciences* 111(4): 1237-44.

West SA, Griffin AS, Gardner A, Diggle SP (2006) Social evolution theory for microorganisms. *Nature Reviews Microbiology* 4(8): 597-607.

West SA, Diggle SP, Buckling A, Gardner A, Griffin AS (2007) The social lives of microbes. *Annual Review of Ecology, Evolution, and Systematics*: 53-77.

Wilking, Zaburdaev, De Volder, Losick, Brenner and Weitz (2012) Liquid transport facilitated by channels in *Bacillus subtilis* biofilms *PNAS* 110(3): 848-52.

Wolfaardt GM, Lawrence JR, Roberts RD, Caldwell SJ, Caldwell DE (1994) Multicellular organization in a degradative biofilm community. *Applied and Environmental Microbiology* 60(2): 434-46.

Wolska KI (2003) Horizontal DNA transfer between bacteria in the environment. *Acta Microbiologica Polonica* 52(3): 233-43.

Yamada M, Ikegami A, Kuramitsu HK (2005) Synergistic biofilm formation by *Treponema denticola* and *Porphyromonas gingivalis*. *FEMS microbiology letters* 250(2): 271-7.