Chapter 3
Biofilms: Besieged Cities or Thriving Ports?

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Abstract From a humble beginning, with less than 50 articles published per year with the term "biofilm" in the title prior to 1990, research output on this topic has grown dramatically with concurrent improved understanding of this form of microbial existence. While remarkable advances in molecular techniques perhaps enabled the major share of this growing knowledge base, we argue in this chapter that due consideration of the biofilms' physical environment in our experimental design, measurements and interpretation of results, is needed. For instance, the effect of flow, and its effect on nutrient and metabolite flux, is markedly different for cells attached to the surface (impacted by both the hydrodynamic effect and physicochemical properties of the surface) compared to those in the quiescent zone of low flow close to the surface (impacted by only the hydrodynamic effect of the surface) and the moving bulk fluid further away (little or no effect of the surface). The quiescent zone bordering a biofilm presents an area where planktonic cells can remain, roam around the attached biomass, and increase in density due to reduced flow resulting in dilution rates that are lower than cell growth rate, thus potentially playing an important role in both immigration into and emigration from the biofilm. This may yield a notably different progression of biofilm development and maintenance and thus a higher degree of fluidity than the discrete stages as depicted by the classical view of biofilm development.

3.1 Introduction

The recognition that biofilms, defined as surface-associated communities of microorganisms, are the prevalent mode in which microbes exist has, in addition to the myriad of areas where biofilms intersect with human interests, resulted in an

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exponential increase in research efforts. A recent search of Elsevier's Scopus database (www.scopus.com) for articles published between 1976 and 2017 with the term "biofilm" in the title yielded 16,286 documents. Whereas 50 articles were published in 1990, this number grew consistently in subsequent years to 182 (2000), 504 (2005), 974 (2010), and 1790 (2016). As could be expected, the main subject areas represented by these publications were immunology and microbiology (36.1%), medicine (32.7%), biochemistry, genetics, and biology (29.7%), followed by environmental science (22.5%) and agricultural and biological sciences (17.9%). The remainder consisted of publications in fields as diverse as dentistry, chemical engineering, physics and astronomy, materials science, and mathematics, to name but a few. While this illustrates the multidisciplinary nature of biofilm-related research, it should also provide a sobering reality for newcomers to the field; the incorporation of fundamental principles from multiple, unrelated disciplines typically is required to adequately investigate biofilms. This naturally implies that investigators must not only be aware of but also become conversant in subjects that may fall outside of their primary training. Furthermore, by design and necessity, microbiological research remains firmly entrenched in homogenous, pure culture studies involving planktonic suspensions of microbes, despite the fact that spatially and temporally heterogeneous, surface-associated aggregates, consisting of multiple microbial species are more representative of prokaryote existence outside of the laboratory. In this chapter, we aim to illustrate how critical it is to foster an adequate awareness and understanding of the impact of the environment on biofilms. To achieve this, we focus on key physical aspects of aqueous environment and their influence on both individual bacterial cells and biofilms. Judicious use of analogies is advocated as tools to aid in the simplification of complex concepts to, firstly, convey information, secondly, facilitate understanding, and, thirdly, allow selection of the most crucial parameters to incorporate in experimental studies.

3.2 Rationale

3.2.1 Living Beings Interact with the Environment

Living beings are influenced by their environment and impact their environment in return. These interactions and resultant reciprocal changes may be minuscule or significant. In the field of biological sciences, we usually try to gain an increased understanding of a focus area by taking measurements during a carefully designed experiment. These collective measurements are then repackaged into descriptions, models, theories, and representations to (often incrementally) answer the questions of "what" and "how" that inspired our curiosity in the first place. For example, Quinn and Keough (2002) show in their first chapter that the scientific method involves stating a research hypothesis (as opposed to statistical hypothesis testing for inferential statistics) and how, with proper experimental design involving the measurement of copepod (predator) mortality, it is possible to postulate a model formulating a probable reason why dinoflagellates (prey) luminesce when the water is stirred.

The particular model mentioned by Quinn and Keough (2002) was referred to as a "burglar alarm" where the experimenters predicted that dinoflagellate bioluminescence would attract fish (copepod predators) leading to increased copepod mortality and indirectly to greater dinoflagellate survival. In this example, the experimenters seemingly considered sufficient role players (fish, copepods, luminescent and nonluminescent dinoflagellates) to address their research hypothesis after measuring copepod mortality. What was inherent, but perhaps invisible and therefore not considered in the experiment, was the particular environment where everything took place.

David Foster Wallace (2008) opened his Kenyon College commencement address of 2005 as follows:

> There are these two young fish swimming along and they happen to meet an older fish swimming the other way, who nods at them and says "Morning, boys. How's the water?" And the two young fish swim on for a bit, and then eventually one of them looks over at the other and goes "What the hell is water?"

As experimenters we remain vulnerable to let the obvious go unnoticed. In addition, we may also be lacking either in the ability to measure some (or all) environmental parameters, or being unable to accurately simulate these in an experiment. The latter could be because we do not fully understand which parameters, or interactions between parameters, are most relevant or because it is either physically impossible or too expensive to adequately re-create those relevant conditions within the laboratory. Naturally, the particular environment under which we conducted the experiment may not have influenced our design and eventual conclusion—but even in such cases it is best practice to describe the environment as thoroughly as possible rather than to appear ignorant about its influence. Since the influence from the environment is a given, the question is therefore simply whether the magnitude of influence is relevant or not. For small organisms such as microbes, a particular environment will affect the metabolism, growth, motility, gene expression, and behavior. This is true for single microbes as well as extracellular polymeric substance (EPS) matrix-encased biofilms which exist in close proximity as well as attached to surfaces. In this chapter we argue why the physical environment of aqueous biofilms should be considered in our experimental design, measurements, and interpretation of results.

3.2.2 Points of Consideration for the Next Generation of Biofilm Researchers

Biofilm research occurs and is perceived from many vantage points, including engineering, physics, and biological sciences, with each field making a unique and important contribution to the whole. Novice researchers entering the field of biofilm research will bring along with them an understanding and vocabulary native to their field. They will describe and interpret their findings through the lenses of their experience. While engineers and physicists are trained with an acute awareness of

physical and chemical environmental contributors, biologists often have minimal such awareness, and it is precisely this limitation that may hinder progress in the field.

When encountering any new field of study, students often are encouraged to use analogies as a method and tool for simplifying complex scenarios. This tool has deservedly stood the test of time as both a communication aid and a simplified means of fostering understanding. However, despite our best intentions, the potential exists that the similarities and correspondence with the original may break down at some point or that our intuition may fail us.

In this chapter we will indeed introduce analogies, while attempting to be mindful of our assumptions, to gain insight into the physical environment encountered by microbes in and around a biofilm. We hope to achieve this by first assembling a list of key aspects which will be considered parameters relating to the aqueous biofilm environment with special emphasis on those aspects most affecting of microbes. In the final section, these parameters will be applied to an example where awareness of the environment may aid in experimental design and interpretation of experimental results, thus leading to an increased understanding of the world around us.

3.3 Background

3.3.1 Microbiology Legacy

Human beings are inextricably intertwined with microbes—perhaps in no way more evidently than the fact that each living human body houses more microbial genes and cells than it does those of the human host (Qin et al. 2010). On any given day, the human path intersects with microbes or microbial functions that directly or indirectly influence the quality of life in areas as diverse as human health, enjoyment (food), and environmental sustainability (nutrient cycles). It is no wonder then that humans are motivated to understand and control microbial growth, death, and function. Although the collective effects of microbes have been observed for millennia, their existence went largely undetected until the invention of the microscope. Since microbes were originally interesting to humans by virtue of their effects (disease, fermentation, etc.), and measurable (noticeable) effects usually require large numbers of microbes, these organisms were and still are grown to sufficient quantities to satisfy the resolution of the experimental techniques of the day. For example, the well-mixed, pure culture batch flask allowed early researchers to learn more about a particular microbial species under specific growth conditions (e.g., at a specific time point) by assuming that the measured parameter of a homogenous collective was a representative average of each individual member. Even though the population's metabolism may change over time, for example, when switching from one carbon source to another (diauxic shift) or from one electron acceptor to another, the microbes will all display similar behavior on average. In contrast, systems that are not well-mixed such as static batch cultures and biofilms (more about the latter in

upcoming sections) and the responses of their constituent microbes to gradients of various forms (e.g., nutrients, redox potential) are expected to develop over time (Stewart 2003). It should be noted, however, that it may be hard to distinguish whether gradients develop first, followed by changes in microbial physiology, or whether microbial activity leads to gradients of environmental parameters (or a combination of both processes occurring simultaneously). Since each microbe is acutely affected by its immediate environment (e.g., temperature, solute concentration, viscosity, etc.), the assumption of spatial homogeneity is a very convenient one from an experimental perspective, especially given that an empirical result can be reproduced fairly predictably under similar conditions. It is therefore not surprising that the legacy of studying microbiology from a spatially homogenous viewpoint still remains in textbooks and undergraduate training. Outside the laboratory, however, microbiology occurs in spatially heterogeneous systems such as biofilms (Geesey et al. 1978).

3.3.2 Spatial and Temporal Heterogeneity Associated with Biofilms

Despite the observation that biofilms formed by various bacterial species proceed through similar steps and exhibit comparable properties, researchers have been unable to determine whether a biofilm-specific genetic program is in operation or if the resultant properties represent the culmination of the response and adaptation of individual cells to local environmental change (Kjelleberg and Givskov 2007). The inability to identify a biofilm-specific program may in fact be due to the acknowledged spatial heterogeneity of biofilms (Stewart and Franklin 2008). The physical, chemical, and biological properties of microenvironments within biofilms fluctuate continually due to a combination of various factors, which may include nutrient and oxygen diffusion gradients, sloughing, predation, etc. Individual cells respond and adapt to the prevailing conditions, leading to the establishment of physiologically differentiated subpopulations within a biofilm.

Due to its nature, a biofilm (even a pure culture biofilm) will be spatially heterogeneous in terms of:

- Chemical environment, e.g.:
  - Nutrients (both electron acceptor and donor)
  - Excreted products
  - Hydrogen ion (pH) concentrations
- Physiological manifestation
  - Gene expression
  - Mode of metabolism
  - Motility

The resulting spatial heterogeneity complicates a study since the emergent properties of the interactions encountered may be difficult or impossible to foresee or delineate. While this spatial heterogeneity is evident even in pure culture biofilms, it is not a big leap to conclude that the complexity may be much more pronounced in a multispecies environment. As a result, the strategy to research biofilm cells and their behavior has had to be adapted, when necessary, to address this lack of experimental resolution. Depending on whether viewed from an engineering (applied) or fundamental perspective, one could focus on global effects (overall mass balances or functional measurements) or conversely shift toward experimental techniques adapted to microscale to study local subpopulations within the biofilm with regard to their gene expression and immediate surroundings (De Beer et al. 1996; Rani et al. 2007). Fortunately, researchers are starting to recognize how heterogeneity stemming from single cell behavior affects collective function (Martins and Locke 2015).

Even more so than is the case for planktonic operation, explicit experimental conditions [e.g., experiments conducted under static flow conditions at solid-air interface (i.e., colony biofilms) or solid-liquid interface (i.e., microtiter plate biofilm assays), or flow conditions (i.e., flow cells or moving bed biofilm reactor)] should be taken into account when interpreting any biofilm-related experimental results given the added complexity resulting from granular spatial heterogeneity. Lewandowski and Beyenal (2013) argue for the need to consider the different levels of focus, whether it involves the very close scrutiny of microelectrodes or comparing the efficiency of different biofilm carriers in wastewater treatment.

While all of this might seem daunting to new researchers in the field, we hope to show that familiarization with a few key concepts will aid substantially in both understanding and communicating results and interpretations. In the next section, we will address certain areas related to how microbes respond to their physical environment, with a focus on aqueous systems, and how mastery of these building blocks can act as implements in a toolbox where the researcher can use some and neglect others to make sense of real-life observations.

3.4 Appropriate Analogies and Rubrics

A preferred means to convey new and complex concepts is by way of analogies. Analogies are based on prior knowledge, thereby making it easier for the reader (or listener) to categorize, classify, and position the new knowledge. In addition to facilitating understanding, analogies have the added advantage that complex concepts can be communicated parsimoniously, e.g., a bean being described as kidney-shaped and vice versa.

Once these analogies have been related, it can be assembled into rubrics or lenses for the researcher (or reader) to communicate. Given that the environment affects microbial behavior, it is important to avoid drawing conclusions from generalized inductive reasoning without regard for the experimental conditions that contribute toward the measured microbial behavior. Conversely, an undiscerning, exhaustive

listing of experimental conditions may be counterproductive in that it may limit understanding or be too cumbersome for the reader to wade through. The benefit of these rubrics is that areas of observation (whether on the scale of a research study or a measured parameter) can be evaluated rapidly and effectively allow for the capturing of the relevant important contributing factors without having to be exhaustive. For example, Flemming et al. (2007) describes the extracellular polymeric substances (EPS) as "The house of biofilm cells." With our understanding of a house, this mental picture makes it easier to conceptualize and explain different biofilm behaviors resulting from the presence of EPS such as structural stability, exchange of genetic material (communication and transfer of knowledge), and retention of nutrients and crucial enzymes. Similarly, Watnick and Kolter (2000) use the analogy of "biofilms being cities of microbes" to explain biofilm development and spatial arrangement by correlating it to geographical settling patterns and zoning laws found in a city.

Analogies can be of great benefit during the process of gaining understanding in an unknown or related field. However, caution should always be exercised in that one should remain aware of terminology use as well as the explicit and inherent assumptions—especially those that are valid in the progenitor field or system—that can perhaps indiscriminately be carried over to the new endeavor. For example, when we attempt to explain microbial behavior by using anthropomorphic terminologies such as altruism, it is often challenging to remain neutral about the human components associated with this term such as choice, compassion, goodwill, willing sacrifice, etc. Referring to microbial altruism and cooperation, West et al. (2007) stated:

> However, progress is often hindered by poor communication between scientists, with different people using the same term to mean different things, or different terms to mean the same thing. This can obscure what is biologically important, and what is not. The potential for such semantic confusion is greatest with interdisciplinary research.

With this in mind, an analogy to describe biofilm development under aqueous conditions is presented in the next section. Our aim is to provide a framework to evaluate biofilm experimental results by focusing on the differences in environmental conditions that result in many of the discrepancies observed between different biofilm studies conducted in aqueous environments. We hope to show that by simply being aware of whether or not the biofilm under consideration developed under flow versus stagnant conditions, the reader will become cognizant of how these differences influence viscosity, nutrient concentration, motility, and association with a surface and a biofilm, to name but a few parameters.

3.4.1 Thriving Port City or Besieged City?

According to this analogy, an aqueous biofilm surrounded by flowing conditions can be envisaged as a port city with its harbor. Pertinent to this analogy, one can consider

three distinct spatial zones related to the flow-surrounded biofilm and discuss how these zones interact via space and time to influence characteristic biofilm behavior. The three zones under consideration include the free-flowing bulk liquid, the fluid adjacent to the surface or biofilm, and the gel-like biofilm itself which consists of microbial cells and the EPS matrix. The characteristics of a port city naturally lends itself as a focal point for the initiation of colonization, but even long after the establishment of thriving communities, the same "gateway" character of these cities maintains their strategic importance. For example, when considering an established coastal country, we are usually aware of the importance of port cities especially as it relates to world commerce (as well as immigration and emigration during the time prior to air travel). But it is sometimes easy to forget about the strategic and crucial early roles that these port cities played in the establishment of the particular country or nation—e.g.:

- Regional trade between coast and hinterland
- Transit points for goods and people
- Possible entry points for invasions
- Immigration and emigration
- World commerce (Gilbert 2006)

During the days of naval exploration, *natural harbors* were prized as access points connecting the motherland (via the ocean) to the new land to be explored. The sheltered nature of the natural harbor provided safety and rest after being exposed and vulnerable during the ocean voyage, as well as access to the potential renewal of sustenance available on land. As previously mentioned, the comparison of a biofilm to a port city and its associated harbor will involve the recognition of three spatially differentiated zones. Each of these zones will be discussed in terms of physical characteristics (e.g., viscosity, diffusion, and advection), the impact of these characteristics on microbial motility, and how both the aspects of spatial zones and physical characteristics relate to emigrating from and immigration to microbial life on surfaces.

While port cities epitomize the concept of flow and facilitation of maintenance and growth, both in terms of resources and residents, a besieged city demonstrates the opposite. The absence of flow (or severely hindered movement) engenders conditions of stress, starvation, stagnation, refuse buildup, dormancy, and death. Compared to the flow system, its stagnant counterpart will conceptually be divided into two zones: the biofilm attached to a solid surface and the overlaying stagnant bulk fluid. Continuing with our analogy, the stagnant system could be considered a city in a swamp where all inhabitants and food will enter or exit at a much slower rate compared to a flow system, to the extent that stress and starvation conditions will be the eventual outcome if population growth is not controlled.

It should be noted that the two analogies of port cities and besieged cities were arbitrarily chosen and are by no means all-encompassing descriptions of biofilm environments but rather very commonly occurring scenarios. Prior to continuing with the above analogy and describing how the physical conditions in each of these zones impact on various aspects of microbial life at or near surfaces, it is essential to

first look at how a microbe experiences a watery environment. This may seem unnecessary if we inadvertently assume that a microbe will behave in a manner analogous to humans or another macroscopic organism's swimming behavior. However, in the course of the following sections, the relevance of doing so should become evident.

The selection of microbial swimming as a focus area is intended to demonstrate how this seemingly simple, often observed bacterial parameter can be influenced by its environment.

3.5 Key Physical Parameters

3.5.1 Microbes in Aqueous Solutions

General biology training usually does not include a comprehensive study of fluids either at rest or in motion (fluid mechanics), and even when included in the curriculum, it must be recognized that applying fluid mechanics to biological systems requires facing unique assumptions and challenges. For example, not all "rule of thumb" numbers such as transition values for Reynolds numbers and boundary layer thickness are applicable, as reviewed by Alexander (2016), and therefore this subject matter might be better taught with specific applications and constraints in mind (Loudon 1999) rather than as broad generalities only.

Here, we do not attempt to act as an authoritative text on fluid mechanics but will rather point the reader to worthwhile resources while at the same time hoping to raise an increased awareness of key concepts (Persat et al. 2015). As an example, due to the microscopic size of a microorganism, the impact of a watery environment is quite distinct from that experienced by a macroscopic organism (e.g., a fish or a human). The ability of a bacterium to propel itself relative to its surroundings when submersed in an aqueous solution is equivalent to a human being's ability to move around in honey. This is a prime example of an instance where attempting to interpret a scenario through the lens of a known experience, obtained either through observation or personal experience (e.g., swimming), could result in erroneous interpretation of experimental results and thus reaching an incorrect conclusion.

In the next few sections, the dimensionless Reynolds number will provide a lens to understand the challenges encountered by microbial swimmers.

3.5.2 The Reynolds Number

Physicists, mathematicians, and engineers have long attempted to understand their objects of study and translate this understanding into models, theories, or laws that could be encoded into a mathematical formula or theorem. Ideally, it would be possible to fully describe every object or process in the natural world with elegant

mathematical formulas for which all parameters and variables are known or could be measured. In practice, however, in many instances scientific efforts lead to the development of unwieldy equations with mathematical operators that require advanced training to understand and solve. To overcome these limitations, assumptions are made that allow simplification of the formulas to address relevant questions related to a particular aspect of the object of study. The choice of which parameters to include or exclude in experimental observations and measurements is usually dictated by some characteristic that will satisfy a practical need for the investigator, for example, excluding parameters that are not deemed to contribute significantly.

In the case presented here, the object of observation is either a fluid moving relative to a rigid object such as a fluid moving in a conduit such as a pipe or a swimming body moving relative to its surrounding fluid. The question that we wish to answer relates to whether the characteristics of the fluid and the characteristics of the rigid object influence the movement of one another (if at all). The Reynolds number, which describes the ratio of inertial to viscous forces of a fluid under defined conditions, can be applied to answer this question.

3.5.2.1 Inertial Force of a Fluid

When a fluid element (i.e., a small volume or unit of water) moves through space, an inertial force is ascribed to it which will resist a change in momentum; the latter could be either a change in direction or velocity. This inertial force is directly proportional to the density of the fluid and the square of the characteristic velocity meaning that when the fluid element has higher mass (as represented by the density term) or higher velocity, greater force will be required to stop it. The inertial force (F_{inertia}) is derived from Newton's second law of motion:

$$F_{\text{inertia}} = ma \tag{3.1}$$

$$m = \rho V = \rho l^3 \tag{3.2}$$

$$a = \frac{U}{t} = \frac{Ul}{tl} = \frac{U^2}{l} \tag{3.3}$$

$$F_{\text{inertia}} = \rho U^2 l^2 \tag{3.4}$$

where m is mass, a is acceleration, ρ is the liquid density, V is the volume, l is the characteristic length, U is the velocity of the fluid, and t is time.

3.5.2.2 Viscosity of a Fluid

Another force that may play a role in the movement of a fluid element is that of viscosity (F_{viscous}). Fluid sticks to itself, so if one pictures adjacent water elements as sheets of paper, the viscosity would be an indication of the stickiness between these

sheets or the "glueyness" of the fluid (Vogel 1994). Viscosity can also be viewed as fluid friction, with friction being an indication of the resistance generated upon the relative movement of two solid objects that are in contact with each other. Key to understanding viscosity as fluid friction is the realization that neighboring fluid elements are moving relative to each other at different velocities. For example, a sheet of water moving at a faster velocity will be slowed down by an adjacent, slower moving sheet. Conversely, the faster moving sheet will tend to drag the slower moving sheet at an increased velocity and if the adjacent sheets of water are moving at the same velocity, the viscous force between these will disappear.

With regard to viscosity, we have thus far only considered fluid moving relative to itself; however these observations can be extended to the case where a fluid moves relative to a solid object. In addition to fluid elements sticking to one another, the fluid will also stick to a solid object that it is in contact with. In fact, the extent of this affinity is so great that this phenomenon is referred to as the "no-slip" condition. This means that this layer of fluid is considered to have zero velocity relative to the surface of the object (i.e., both are moving at the same velocity). By implication, we can infer that a liquid velocity gradient will extend from the solid surface into the bulk liquid phase. A simple example to demonstrate this is by observing debris floating down a slow flowing river that travel slower when close to the bank compared to those floating in the middle of the stream. From this we can conclude that solid surfaces in contact with a fluid, whether a static pipe wall or a solid swimming body immersed in a fluid, will always result in viscous forces being present in the layers close to the surface where these velocity gradients exist.

The derivation for the equation for viscous force (F_{viscous}) can be found in most fluid mechanic textbooks and is given by

$$F_{\text{viscous}} = \frac{\mu U l^2}{l} \tag{3.5}$$

where μ is the dynamic viscosity of the fluid.

Terminology can sometimes be confusing, especially when a word might have one meaning in everyday speech but a different or nuanced meaning in a certain research field or when particular background knowledge is required to avoid misinterpretation. An example of the latter is found in fluid mechanics literature, where the fluid layer close to a surface is referred to as the viscous layer or the "region where viscous flow occurs" as opposed to inviscid flow further away from the surface. A person familiar with the field will realize that the dynamic viscosity for an incompressible Newtonian fluid such as water is indeed a constant (as is evident from the derivation of the shear stress equation—see any fluid mechanics textbook for an example), but a non-expert may assume that the viscosity decreases with increasing distance away from the surface.

When considering the movement of a fluid element, the analogies from Vogel (1994) are very useful: he describes inertial forces as reflecting the "individuality" of these fluid elements, while the viscous force reveals their "groupiness." In practice,

this can be viewed as follows: when inertial forces dominate, fluid movement can be described by the progress of a milling crowd, while the movement resembles that of a disciplined march when viscous forces prevail (Vogel 1994).

3.5.2.3 Reynolds Number Equation

For specific flow conditions and geometries, it is therefore possible to comment on whether the flow conditions are dominated by inertial or viscous forces by considering the ratio of these forces acting on a fluid element. This ratio, represented by the dimensionless Reynolds number (Re), is indeed frequently used in the field of fluid mechanics to make a distinction between inertia-dominated flow (turbulent flow) and viscosity-dominated flow (laminar flow).

$$\mathrm{Re} = \frac{F_{\mathrm{inertia}}}{F_{\mathrm{viscocity}}} = \frac{\rho l^2 U^2}{\mu l^2 U/l} = \frac{\rho l U}{\mu} \tag{3.6}$$

with the characteristic length, l, and characteristic velocity, U, being specific to different flow geometries and conditions.

3.5.2.4 Points for Consideration When Applying the Reynolds Number

In reference to the ideal scenario of having appropriate mathematical formulas to assist our understanding of the physical environment, Vogel (1994) praises the usefulness of the abovementioned ratio by calling the:

> Peculiarly powerful Reynolds number the centrepiece of biological fluid mechanics.

And Vogel goes on to say that this "almost magical variable" is "the nearest thing we have to a completely general guide to what is likely to happen when solid and fluid move with respect to each other."

After reading these commendations from an expert in the field of fluid mechanics, a biologist might conclude that he or she has encountered some sort of equational jackpot—which it indeed can be—but this is only true after careful consideration of how the Reynolds number should be understood and used.

In our case, we described the Reynolds number as the ratio of forces acting on a fluid element. However, it should be noted that there are various physical interpretations of the Reynolds number, four of which are mentioned by Lauga and Powers (2009). For the purpose described in this chapter, the main function of the Reynolds number is to provide an indication under which flow conditions inertial (turbulent) or viscous (laminar) forces will dominate, given the characteristics (e.g., density, viscosity, velocity, etc.) of the fluid under consideration. Rather than attaching importance to the specific numeric value of the Reynolds number, close attention should be paid to the critical Reynolds number, which indicates when flow

transitions from laminar to turbulent. For example, the transition Reynolds number for flow in a circular pipe starts at approximately 2000, and turbulent flow can be expected at a Reynolds number of 4000 (Brading et al. 1995). It is important to note that the transition Reynolds number is dependent on the geometry and flow conditions. For example, each geometry results in a different definition for the characteristic length, l, and the characteristic velocity, U; e.g., for a circular pipe, the characteristic length is equal to the inner diameter of the pipe, whereas the characteristic velocity is the average fluid velocity. In essence, the Reynolds number indicates the character of the flow regardless which of the contributing variables cause the change. A tenfold reduction in characteristic length will increase relative viscous effects by a factor of 10, similar to what would happen if the viscosity increased tenfold (Vogel 1994).

3.5.2.5 Reynolds Number for a Swimming Body Such as a Bacterium

Osborne Reynolds (after which the Reynolds number was named) investigated the transition from laminar to turbulent flow in pipes, which represent a fluid moving past a stationary surface. However, the same reasoning regarding the ratio of inertial forces to viscous forces can be applied to a rigid body moving through stationary fluid, given that the appropriate characteristic length and characteristic viscosity are defined. For a bacterial cell moving through a fluid, the characteristic length is taken as the greatest length of the swimming body in the direction of flow, and the characteristic velocity is the difference in velocity between the swimmer and the velocity of the bulk fluid. In stationary fluid, the bulk fluid velocity will be reduced to zero and the characteristic velocity used to calculate the Reynolds number will be equal to the velocity of the swimmer.

The Reynolds number for a swimming microorganism can therefore be determined by using the following approximate values (Lauga and Powers 2009); the density and dynamic viscosity of water can be given by $\rho \approx 10^3$ kg m^{-3} and $\mu \approx 10^{-3}$ Pa s, respectively, the characteristic length of microbes ranges between 1 and 10 μm, and the characteristic speed is ≈10–30 μm s^{-1}. These values will yield a Reynolds number of approximately 10^{-5} to 10^{-4}; these very low numbers indicate that a microorganism swimming in water will experience strong viscous resistance. In comparison, a 2-m-tall human, swimming at 1–2 m s^{-1} in water, would be equivalent to a Reynolds number in the order of 10^5 to 10^4, thus indicating a dominance of inertial forces. Only if a human would attempt to swim in honey, with a dynamic viscosity roughly 10,000 times that of water, would this person experience viscosity-dominated laminar swimming conditions similar to what bacteria experience when moving in water.

Related to whether viscous forces will dominate or not, we can use the same equations and reasoning to determine what distance a swimmer will coast (i.e., float) after all activity has stopped. Lauga and Powers (2009) derived equations to show that the coasting distance, d, under inertia-dominated flow conditions is given by

$$d \approx l \frac{\rho_{\text{swimmer}}}{\rho_{\text{fluid}}} \tag{3.7}$$

where l is the characteristic length of the swimmer and ρ the density of the swimmer and fluid, respectively. This equation indicates that a human swimmer will coast for a few meters in the water before coming to a standstill.

Under conditions where viscous forces dominate, the coasting distance is given by

$$d \approx l\text{Re} \frac{\rho_{\text{swimmer}}}{\rho_{\text{fluid}}} \tag{3.8}$$

where l is again the characteristic length and Re is the Reynolds number calculated as before, indicating that a bacterium will slow down after about 0.1 nm, within a time frame in the order of microseconds (Purcell 1977).

3.5.3 *Reduced Flow Velocity Near a Surface*

The liquid flowing near a wall, such as the inner surface of a pipe, will be dominated by viscous forces under both laminar and turbulent conditions. This region is associated with a gradient in flow velocity, with increasing fluid velocity observed further away from a surface. While the velocity profile in this region is strictly speaking parabolic, the assumption of linearity is acceptable if only a small distance is considered (Rao 2010). Engineers refer to this region as the hydrodynamic boundary layer, and conceptually it separates this fluid from the zones further away from the surface where viscous forces are negligible compared to inertial forces (Brading et al. 1995). Under turbulent flow conditions, the flow close to the surface is referred to as the "laminar sublayer" or "viscous sublayer" (Brading et al. 1995). From a biological perspective (e.g., biofilms growing on a pipe surface), the region close to the surface can be viewed as a "surface microenvironment" (Caldwell and Lawrence 1988; Watnick and Kolter 2000) as opposed to the macro-environment encountered in the bulk fluid flow further away from the surface.

For smooth surfaces a conservative thickness, δ, for this sublayer can experimentally be determined as

$$\delta = \frac{5v}{\sqrt{\tau_{\text{w}}/\rho}} \tag{3.9}$$

where $v(v = \mu/\rho)$ is the kinematic viscosity of the fluid, ρ is the density of the fluid, and τ_{w} is the shear stress at the wall (Rao 2010; Agrawal 2012). The thickness of δ is determined experimentally from velocity profile graphs (the regions where these profiles dominate and where they intersect) portraying the different layers that occur

under turbulent flow conditions. Depending on which portions of a particular equation or specific intersection of two profiles are used, some textbooks will provide a δ with a different constant (Allan 1995; Rubin and Atkinson 2001), e.g.:

$$\delta = \frac{11.6v}{\sqrt{\tau_w/\rho}} \tag{3.10}$$

More detailed explanations for this discrepancy can further be explored elsewhere (White 2010). Despite the use of these different constants, an approximation of the thickness of the viscous sublayer can be garnered from reported values, although scarce in the current literature. Rough approximations for industrially (Rubin and Atkinson 2001) and environmentally relevant conditions (Kumarasamy and Maharaj 2015) estimate this thickness to be in the order of 10^2 μm. This would suggest that the viscous sublayer may provide sufficiently retarded flow close to the surface for microbes to attach and form biofilms even under turbulent flow conditions. The latter has been reported for *Listeria innocua* (Perni et al. 2006), mixed species biofilms (Percival et al. 1999), and *E. coli* (Teodósio et al. 2011).

3.5.4 Flow in Non-Newtonian Fluids Such as a Biofilm

Up to now we have mostly considered Newtonian fluids (e.g., water) where the viscosity does not change when the rate of shear strain (change of strain or deformation with respect to time) is increased or decreased. However, the behavior of Newtonian fluids can change drastically when small particles are suspended in the liquid or when macromolecules are dissolved in the liquid (Brown and Jaeger 2011). The focus now turns to two aspects related to this nonlinear fluid behavior as it relates to biofilms, i.e., shear strain rate-induced nonlinearities (shear-thickening and shear-thinning) and viscoelastic behavior.

Some suspensions will be liquid-like when slightly perturbed, but once exposed to higher impact, an increase in viscosity will harden the liquid to such an extent that it could support a person running across it without sinking into it (Waitukaitis and Jaeger 2012). This phenomenon is termed shear-thickening, whereas a decrease in viscosity upon an increased rate of shear strain is called shear-thinning. Since a particular fluid may change its behavior depending on the shear rate applied, a fluid cannot be unequivocally classified as either shear-thinning or shear-thickening. These terms should therefore rather be considered as dependent on the flow conditions, as opposed to a characteristic of the fluid itself (Mewis 2012). Furthermore, the definitions of shear-thinning and shear-thickening (often portrayed as a graph of shear stress vs strain rate) represent another example where the terminology may be misleading since these terms refer to *shear*, while the definitions require strain rate to be the controlling factor (Mewis 2012). Biofilms or EPS matrix encapsulated microbial cells (Flemming and Wingender 2010) have been described as a complex

fluid (Wilking et al. 2011) and recorded to display shear-thinning behavior (Houari et al. 2008; Billings et al. 2015; Patsios et al. 2015).

Some sources in the literature may also refer to "strain hardening" or "shear stiffening" (functional definitions will be mentioned later in this paragraph) (Barai et al. 2016), but these terms should not be confused with shear-thickening or shear-thinning (Mewis 2012). Rather, the former terms are used to indicate the occurrence of viscoelasticity, which is normally determined from a plot of shear stress vs strain (Fabbri and Stoodley 2016), as opposed to using plots of shear stress vs strain rate to visualize shear-thickening or shear-thinning behavior (Stoodley et al. 1999a, 2002a; Klapper et al. 2002; Peterson et al. 2015). A viscoelastic material will display both viscous and elastic properties, meaning that it deforms when placed under stress. Once the stress is removed, the material returns to its previous state, which may seem similar, but not necessarily identical, to the original state (Peterson et al. 2015; Fabbri and Stoodley 2016).

3.6 Bacterial Locomotion

In the previous section, key interactions between the movement of fluids and solid surfaces or bodies relative to one another were discussed. In the following section, focus is shifted to bacterial motility with the aim of understanding how microbes reach and interact with surfaces, since this is a crucial step in biofilm development. It should be noted that while bacterial motility plays a role in surface association, not all microbes are capable of locomotion and furthermore that other factors, such as fluid hydrodynamics, do play a role in translocating microbes to surfaces.

Prokaryotes can propel themselves in different ways through liquids, including swimming, swarming, gliding, twitching, or floating, among others as reviewed by Jarrell and McBride (2008). Those authors detailed various mechanisms for movement, including the use of surface appendages such as flagella and pili, or internal structures such as the cytoskeleton and gas vesicles. The method of propulsion and mechanism does not always correspond; for example, swimming with or without flagella or gliding with or without pili is possible. Since the scope of this chapter is limited to aqueous environments, the main focus of this section will be flagellar swimming. Most bacterial flagella are thin (~20–50 nm diameter) helical structures (Berg 2003) that can extend from the cell body for several cell lengths. Information regarding flagellar structure, assembly (Macnab 2003), and regulatory genes has been reviewed by Jarrell and McBride (2008).

3.6.1 Single Cell Swimming

While flagellar-mediated swimming is a common means of microbial propulsion, there are a few subclassifications of this mode of locomotion that become relevant to

the discussion of near-surface swimming. In particular, we will consider differences in flagellar number, location or arrangement on the cell body, and swimming method.

The number and location of prokaryotic flagella vary among different species (Merino et al. 2006). Examples include single (monotrichous) or multiple (lophotrichous) polar flagella as found in *Pseudomonas aeruginosa* and *Pseudomonas putida*, respectively (Theves et al. 2013), and multiple flagella distributed uniformly over the cell body (peritrichous) such as seen for *Escherichia coli* and *Salmonella enterica* (Berg 2003; Merino et al. 2006). The specific swimming method strongly influences microbial behavior when an organism is interacting with its environment, such as when it encounters surfaces or nutrient gradients. Two important components of the method of swimming are speed and the ability to change direction, with the mechanism of the latter varying substantially among different flagellar arrangements.

Bacterial swimming speeds vary widely; e.g., *Bdellovibrio bacteriovorus* with a single polar flagellum (Lambert et al. 2006) was observed to swim at 160 μm s^{-1}, whereas *P. putida* with multiple polar flagella was able to swim at speed of 75 μm s^{-1} (Harwood et al. 1989), and *E. coli* was capable of reaching speed of 35 μm s^{-1} with peritrichous flagella (McCarter 2005).

Microbes with a single polar flagellum, such as *P. aeruginosa*, propel themselves forward in a pusher motion by counterclockwise (CCW) rotation of their flagella or reverse their direction in a straight trajectory by clockwise (CW) rotation (puller motion; Cai et al. 2016). Peritrichous bacteria, such as *E. coli*, bundle their flagella, and CCW rotation similarly drives the cells forward (called a "run"), while clockwise rotation disrupts the flagellar bundle and results in a "tumble" (Jarrell and McBride 2008; Cai et al. 2016) or "twiddle" as the original authors pointed out (Berg and Brown 1972). It should be noted that the direction of motion resulting from CW or CCW rotation depends on the handedness of the flagella of a particular species. CCW rotation of the left-handed filaments of *E. coli* drives the cell forward, whereas the single, right-handed helical filament of the bacterium *Caulobacter crescentus* move the cells forward by rotating CW and reverse the cell by CCW rotation (Lele et al. 2015). Thus, a switch between CCW and CW flagellar rotation brings about both propulsion and direction changes for peritrichous bacteria, while alternating between CCW and CW rotation for bacteria with a single flagellum will merely move the cells forward or backward in a straight trajectory (in the absence of a nearby surface).

While not a focus area in this chapter, eukaryotic flagella are considered to be distinct from prokaryotic flagella (Berg 2003) in terms of location, structure, function, and swimming patterns (Polin et al. 2009; Guasto et al. 2012; Moran et al. 2014). Among the eukaryotes, *Chlamydomonas* (algal unicellular flagellates) are capable of beating their flagella in two different ways: a breaststroke and an undulatory (waves travelling down the flagellum) stroke (Tam and Hosoi 2011).

3.6.2 Collective Swimming

While only single cell swimming has been considered thus far, collective swimming by numerous cells results in interesting deviations from the norm and may influence physical fluid parameters such as viscosity and diffusion. For example, Lushi et al. (2014) investigated self-organizing patterns exhibited by dense suspensions of confined bacteria. However, the interactions between microbes and fluid parameters are of greater relevance to the present discussion and will remain the focus area.

Sokolov and Aranson (2009) measured the shear viscosity in thin films resulting from swimming *Bacillus subtilis* using two complementary experimental approaches [shear viscosity can be determined experimentally as the ratio of the tangential shear stress and the shear rate (Sunthar 2010)]. The first approach involved measuring the deterioration of a large vortex, whereas the second determined the viscous torque on a rotating magnetic particle in the presence of various concentrations of *B. subtilis* cells. The authors report a decrease in liquid viscosity of up to a factor of 7; this decrease was relative to the viscosity of a fluid containing non-motile or no bacteria, and it was dependent on the bacterial concentration and swimming speed (Sokolov and Aranson 2009). It seems, however, that the above observation was particular to pusher-type swimmers such as *B. subtilis* since Rafaï et al. (2010) showed experimentally that puller-type swimming by the unicellular microalgae, *Chlamydomonas reinhardtii*, resulted in an increase in fluid viscosity compared to the same concentration of dead cells.

Ishikawa (2009) discussed how puller-type swimmers will repel each other when swimming side by side, while pusher-type swimmers will attract each other. The author goes on to describe how suspensions of swimming microbes can affect both fluid rheology and diffusive properties (enhancing the diffusion of dissolved chemicals in the suspension) by influencing these fluid properties on a mesoscale (between microscopic and macroscopic; Ishikawa 2009). Underhill et al. (2008) expand on the latter as it relates to the mode of swimming and showed that the pusher motion enhanced the diffusion of tracer molecules more so than did puller-type swimming.

Experimental simulations of a bath containing swimming *E. coli* indicated a significantly larger effective diffusion coefficient for a tracer molecule suspended in the fluid, compared to the thermal effective diffusion coefficient exhibited by a fluid containing only passive particles (Morozov and Marenduzzo 2014). The authors concluded that this increase in effective diffusion is dependent on the concentration of bacteria, their swimming speed, as well as a characteristic velocity field created by a single swimming bacterium (Morozov and Marenduzzo 2014). Wolgemuth (2008) investigated the formation of transient jet and vortex patterns in the presence of high densities of *B. subtilis* using a theoretic model. According to the simulations performed, active swimming by the bacteria resulted in turbulent mixing of the fluid, with fluid velocities exceeding the maximum swimming speed set for individual bacteria.

3.6.3 Surface Interaction

3.6.3.1 Swimming Close to a Surface

The tendency for swimming cells to remain in close proximity to surfaces in a time frame ranging from seconds to minutes has been ascribed to hydrodynamic effects (Vigeant et al. 2002; DiLuzio et al. 2005; Conrad 2012), as was shown for non-tumbling *E. coli* (Berke et al. 2008).

Confirmation of the role of bacterial locomotion in surface association was provided by Galajda et al. (2007). The authors established that swimming rather than non-swimming microorganisms were trapped along a wall with micro-fabricated funnel-shaped openings. Pusher cells strongly migrate toward nearby boundaries or surfaces when few in number. However, at higher cell concentrations, this coherence is disrupted by large-scale interaction between swimming cells (Hernandez-Ortiz et al. 2005).

The accumulation of cells at a surface has also been demonstrated to depend on swimming speed and cell length, with faster and longer cells accumulating to a greater degree than slower and shorter ones (Li et al. 2011). In addition, the tumbling frequency of peritrichous bacteria was reduced by 50% within 20 μm of a surface and has been proposed to contribute to near-surface cell trapping (Molaei et al. 2014).

The proximity of a surface is known to influence microbial swimming behavior such as a change in the microbe's trajectory or a reorientation of a cell. For example, *E. coli* swims in a CW, circular motion near a solid boundary; rotation of *E. coli*'s left-handed helix will cause the cells to turn to the right (CW) (Lauga and Powers 2009). The circular motion observed when monotrichous bacteria swim in reverse near a surface has been described as "run-and-arc" swimming (Karimi et al. 2015) and has been reported for a number of monotrichous bacteria including *Caulobacter crescents* and *P. aeruginosa*. Pusher cells have been found to reorientate themselves parallel to a surface, while pullers will orientate themselves at a right angle with respect to the wall, thereby appearing to swim into it (Lauga and Powers 2009). While the singly flagellated *Vibrio alginolyticus* usually swims backward and forward by alternating the rotation direction of its flagella, it behaves differently near a surface. Its forward and backward swimming speeds have been observed to differ significantly and the swimming motion to trace a more circular trajectory. The latter effect was explained by fluid dynamic interactions between the cell and the rigid boundary (Goto et al. 2005).

3.6.3.2 Surface Sensing

The question may arise as to how a bacterium senses that it is near a surface. Cells are able to sense direct contact with a surface or experience surface-associated changes in hydrodynamic conditions via different mechanisms, one of which

involves the restriction in flagellar rotation (Harapanahalli et al. 2015). Belas (2014) investigated the role of rotating flagella in surface sensing and found that rotating flagella are used as mechanosensors to detect subtle changes in the operation of their motors when they near a surface (Belas 2014), specifically via the motor stators (Lele et al. 2013). Obstruction of flagellar rotation may trigger adhesion and surface-associated motion (Ellison and Brun 2015). Cairns et al. (2013) discussed how the inhibition of flagellar rotation triggers a signal transduction cascade in *B. subtilis*. In addition to the differential expression of genes linked to motion, the association with surfaces and the related mechanical interactions may influence virulence factor-related gene expression. The latter was shown by Siryaporn et al. (2014) for *P. aeruginosa* after attachment to various, chemically distinct surfaces and is worth considering in the study of biofilms' role in infection.

However, it should be noted that not all surface-sensing pathways involve flagella, such as the case of non-motile bacteria (McClaine and Ford 2002; Belas 2014). Some bacteria sense a surface when experiencing adhesion force-induced deformation of the cell wall (Harapanahalli et al. 2015).

3.6.3.3 Shear Trapping

In the absence of flow, the density of a dilute suspension of non-tumbling, peritrichous *B. subtilis* was found to be uniform, whereas the introduction of flow led to the accumulation of cells at surfaces (Rusconi et al. 2014). An increase in flow rate or shear corresponded to an increase in the concentration of cells at the surface and was termed "shear trapping" (Rusconi et al. 2014; Rusconi and Stocker 2015). This phenomenon required motile cells, since dead cells did not show any variation in spatial density (similar to the instance of no-flow) (Rusconi et al. 2014). Shear trapping not only increases surface attachment but also suppresses chemotaxis (Rusconi et al. 2014) and the ability of a cell to respond to external stimuli (Bearon and Hazel 2015).

Similar results were observed for *P. aeruginosa* (Lecuyer et al. 2011) where the time that the bacteria remained adhered to the surface increased nearly linearly as a result of an increase in wall shear stress from zero to ~3.5 Pa. The authors furthermore observed that while the absence of type I pili, type IV pili, the flagellum, or EPS building block production affected the frequency with which cells could become either adhered or detached, none of these factors could account for the average increase in adhesion time correspondent with shear (Lecuyer et al. 2011). Since the same result was observed for tumbling *B. subtilis* and the monotrichous *P. aeruginosa*, it appears plausible that this behavior occurs irrespective of the swimming motion. Early reports by Molaei et al. (2014) indicated an increased incidence of shear trapping under conditions without flow (Molaei et al. 2014). In a subsequent study, the group (Molaei and Sheng 2016) used digital holographic microscopy to track swimming *E. coli* near surfaces under shear. Under these conditions, the authors found that shear mitigated the tumbling inhibition, which in turn reduced cell trapping close to the surface.

3.6.3.4 Swimming Upstream

Contrary to what may be assumed, microorganisms are able to swim and translocate over surfaces against the prevailing direction of flow (Hill et al. 2007; Rusconi and Stocker 2015). For example, *P. aeruginosa* cells are initially orientated along the direction of flow, after which the retraction of the polar type IV pili allow upstream twitching (Shen et al. 2012).

Kaya and Koser (2012) observed that *E. coli* can swim upstream under flow conditions at speeds exceeding 20 μm s^{-1}. In a previous section, it was mentioned that *E. coli* swims in circles when encountering a surface under quiescent conditions. In this mode the cell experiences an increased hydrodynamic drag which rotates and dips the front of the cell body, thereby keeping the bacterium pointed toward the surface with the flagella oriented away from the surface (Kaya and Koser 2012). Under flow conditions the drag on the flagella oriented further away from the surface than the cell body will rotate the cell to face upstream. The authors concluded that this upstream swimming behavior is not unique to the propulsion mechanisms of *E. coli* but merely requires a swimming microorganism moving freely over a surface under moderate flow conditions (Kaya and Koser 2012). Korber et al. (1989) compared surface colonization of motile and non-motile *P. fluorescens* under two flow velocities, 8 and 120 μm s^{-1}. They found that flagellated cells not only attached more rapidly than did nonflagellated cells under both velocities but also migrated upstream against the laminar flow.

3.7 Dissolved Nutrients

In this chapter the main focus is on how microbes interact with their physical aqueous environment. As a result, the question of how microbes interact with their chemical environment, especially as it pertains to breathables (electron acceptors) and edibles (electron donors) (Nealson 2003), the gradients of these chemicals and how it relates to chemotaxis will only be discussed briefly—its importance, however, should always be considered when interpreting biofilm behavior.

For biofilms growing on surfaces, a mass transport or diffusion boundary layer exists within the hydrodynamic boundary layer (Lewandowski and Beyenal 2013). This mass transport boundary layer can be demonstrated by the nutrient concentration gradient increasing with increasing distance from the surface (assuming that nutrients found in the bulk is being consumed as it approaches the biofilm on the surface); at distances close to the surface where fluid velocity is low, mass transport is diffusion dominated, whereas the higher flow velocities prevailing at greater distances from the surface result in convective mass transport.

3.7.1 Diffusion and Advection

If one considers a stationary, nutrient-filled fluid surrounding a spherical stationary microbial cell that assimilates all nutrients reaching its surface, a nutrient concentration of zero will result at the cell's surface (Dusenbery 2009). The difference in nutrient concentration between the cell surface and the bulk fluid will generate a concentration gradient which in turn will be the driving force for diffusion from the bulk to the cell surface. Given that the concentration of the particular nutrient is zero (at the cell surface) under these conditions, the question is whether the microbe would be able to increase its rate of nutrient uptake by swimming? To attempt an answer to the above question, some rudimentary calculations will provide insight into the relative contributions of the two main mechanisms of solute transport through a solution, namely, flow and diffusion. Under conditions of flow (advective transport) at velocity U, the time, t_a, required for a solute to move a distance l can be approximated (Purcell 1977; Dusenbery 2009) by

$$t_a \approx \frac{l}{U} \tag{3.11}$$

For diffusion the time, t_d (Purcell 1977; Dusenbery 2009), for transport can be approximated as

$$t_d \approx \frac{l^2}{D} \tag{3.12}$$

where D is the diffusion coefficient. One consequence of time being proportional to the squared distance is that diffusion times are very short over small distances; a small molecule will diffuse across a bacterial cell ($\sim$1 μm) in approximately a millisecond (approximating the diffusion coefficient in water as 10^{-9} m^2 s^{-1}). Both of the above approximations of transport time are derived from the one dimensional advective diffusion equation for an incompressible fluid where variable sizes have been estimated on the level of orders of magnitude rather than precise values. If the ratio of diffusion transport time to that of flow (advection) transport time is taken, we arrive at the dimensionless Péclet number (Vogel 2004; Dusenbery 2009):

$$Pe = \frac{t_d}{t_a} = \frac{Ul}{D} \tag{3.13}$$

Similar to the Reynolds number discussed earlier, the Péclet number is used to comment on the occurrence of certain regimes of flow (transport dominated by advection or diffusion) and exact values are of lesser importance, especially given that the Péclet number is not taking any geometrical details into account and has

been derived from order of magnitude assumptions rather than exact variables (Vogel 2004).

From our earlier discussions into how an individual swimming microbe experiences a watery environment, it is evident that "at low Reynolds numbers you cannot shake off your environment" (Purcell 1977). Purcell (1977) and Vogel (2004) approximated a Péclet number of 10^{-2} for a microbe swimming in water, thereby indicating that swimming will not increase nutrient transport to the cell. On a practical level this implies that a swimming microbe carries a layer of fluid around with it, and the only means whereby nutrients can cross the fluid is via diffusion. This phenomenon is succinctly summarized by Dusenbery (2009) as follows: "they carry a halo of fluid depleted of nutrient around with them."

3.7.2 Chemotaxis

Since tumbling by an *E. coli* cell will result in a random change of direction, the question arises on how this microbe manages to navigate in a desired direction, e.g., toward a higher concentration of a food source or another chemoattractant. Chemotaxis is facilitated by regulating the run length, which in effect means that the runs in between tumbling events will be longer when moving in the direction of a chemoattractant (Cai et al. 2016). In contrast, the presence of a chemorepellent will increase the tumbling frequency resulting in a net change in overall swimming direction. The chemotactic ability of monotrichous bacteria such as *P. aeruginosa*, especially as it relates to directional changes, has been ascribed to a number of different strategies. Firstly, a change in direction has been attributed to random derailing due to Brownian motion of the fluid (Li et al. 2008). Secondly, *P. aeruginosa* can increase the likelihood of moving toward a chemoattractant by prolonging movement in a particular direction (Cai et al. 2016). It was found that *P. aeruginosa* can use either the forward or reverse motion to move up a chemoattractant gradient (Cai et al. 2016). The switch between CCW and CW rotation is separated by a pause, with the length of the latter positively correlated with the size of the turning angle (Qian et al. 2013). Thirdly, some uniflagellate bacteria such as *Vibrio alginolyticus* can turn or "flick" (Son et al. 2013) by utilizing a strain-induced collapse in its flagellum, which the authors call "an example in nature of a biological function stemming from a controlled mechanical failure."

Taylor and Stocker (2012) argue that microbes should derive sufficient nutritional benefit given the energy expenditure required for chemotactic swimming motility (speed). Maintaining optimal swimming speeds to balance this trade-off will be particularly important in nutrient-poor environments such as ocean water (Stocker et al. 2008). The authors found that the monotrichous (Gauthier et al. 1995) marine bacterium *Pseudoalteromonas haloplanktis* was able to engage in a chemotactic response that was >10 times as rapid as *E. coli*. This swift response was mainly attributed to the faster swimming speed of *P. haloplanktis* ($\sim$68 μm s^{-1}). Stocker (2011) mentions that the chemotactic motility pattern of monotrichous bacteria

(a hybrid of forward and reverse movement with flicking) outperforms the chemotactic response of the run-and-tumble moving pattern exhibited by, for example, *E. coli*. Interestingly, of ~600 motile marine bacteria isolated, approximately 90% had a single polar flagellum (Leifson et al. 1964). The ability to respond rapidly may result in a competitive advantage given the transient microscale nutrient patches in the ocean.

3.8 Evaluating the Port City Analogy

To integrate the preceding discussions of the interactions between microbes and surfaces in a dynamic fluid environment, especially as it relates to bacterial motility, we will restrict the evaluation of our port city analogy to initial adhesion to a surface or biofilm (immigration), the association of motile cells with an existing biofilm, and detachment or dispersion from a biofilm (emigration).

When considering a port city with its harbor, we could recognize three zones, namely, the city, the harbor, and the ocean. These regions can in turn be related to the three zones of a biofilm in flowing conditions: the surface-attached biofilm, the quiescent zone of low flow close to the surface, and the moving bulk fluid further away from the surface (Fig. 3.1). Vigeant et al. (2002) similarly defined three zones but based these on the distance from the solid surface, namely, the near-surface constrained region, the near-surface bulk region, and the bulk fluid. The near-surface constrained zone is impacted by both the hydrodynamic effect and physicochemical

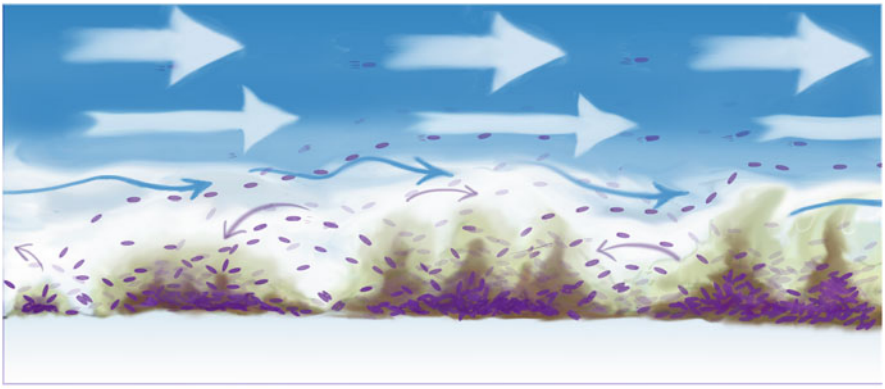

Fig. 3.1 A diagram of the proposed zones associated with a biofilm under flowing conditions (images taken from Bester et al. (2013) and not drawn to scale). According to the port city analogy, the biofilm corresponds to the city (purple cells and brown EPS matrix), whereas the region with reduced flow rates equates to the harbor (white background). In this zone, the reduced flow rates allow for freely motile cells to swim upstream and around the biomass. The zone furthest away from the surface (blue background) represents the ocean or bulk liquid flow where surface-associated hydrodynamics have no influence on the characteristics of the flow

properties of the surface, the near-surface bulk phase experiences only the hydrodynamic effect of the surface, while in the bulk fluid, no effect from the surface is evident.

According to the classical dogma, the formation of a biofilm is considered to be a developmental process which proceeds through four or five stages from attachment to maturation and detachment or dispersion (Sauer et al. 2002; Stoodley et al. 2002b). While this model provides a useful framework, it does not capture the complexity of surface-associated microbial growth. The particular focus of this model on the attached, sessile cells has the potential to restrict our perspective and understanding of surface-associated microbial communities.

For example, this model does not take into account the "harbor" region of the port city analogy where planktonic or freely motile cells can remain, meander around biofilm biomass, and multiply indefinitely due to reduced flow rates. This "low-flow" zone may be critically important in both immigration into and emigration from the biofilm. By extension, this may yield a notably different progression of biofilm development with a higher degree of fluidity than the discrete stages as depicted by the classical biofilm model.

An additional example of the inherent, but perhaps unrecognized bias of the biofilm developmental model is evident from an investigation into *Legionella pneumophila* biofilm formation. Mampel et al. (2006) found that continued planktonic cell replication (rather than sessile cell division) was necessary for biofilm formation under static conditions. *L. pneumophila* was furthermore unable to form robust biofilms under conditions of flow, but effectively associated with existing biofilms comprised of certain environmental bacterial species (*Microbacteria* and *Acinetobacter baumannii*), while other biofilms consisting of *Klebsiella pneumoniae*, *Pseudomonas* spp., or *Corynebacterium glutamicum* resisted *L. pneumophila* colonization (Mampel et al. 2006).

3.8.1 Biofilm Formation or Association: Immigration

Flow conditions can impact nutrient uptake and the rate of cell-to-cell encounters, while shear in particular can influence the attachment of bacteria to the surface as a first step in the process of microcolony and later biofilm formation (Brumley et al. 2015). Lemon et al. (2007) compared wild-type, flagellum-minus, and paralyzed-flagellum mutants of *Listeria monocytogenes* and found that flagellar motility was essential for initial surface attachment and biofilm formation in this species.

Vigeant et al. (2002) comments on the different forces at play when considering the classic biofilm development dogma. According to this theory, reversibly adhering cells originate from planktonic cells trapped by hydrodynamic forces after swimming close to the surface for extended periods. The subsequent, irreversible attachment of these cells can be explained by DLVO (Derjaguin, Landau, Verwey, and Overbeek) electrostatic interactions.

Much of the boundary effect theory is based on the no-slip wall condition which considers that at a solid boundary the fluid velocity relative to the boundary will be zero. Hu et al. (2015) predicted that *E. coli* can sense nanoscale surface slip as opposed to the usual no-slip assumption occurring at boundaries. Surface slip occurs at surfaces modified by, e.g., adsorbents and hydrophobic compounds (Lauga et al. 2007; Rothstein 2010). An increasing slip length, as could be achieved experimentally by coating a surface with the EPS polymer alginate, implies CCW circles of decreasing radius which may lead to increased surface adsorption due to a prolonged local residence time (Hu et al. 2015).

Reports such as the one by Barken et al. (2008) investigated whether the mere presence of certain organelles versus the presence of functional organelles influenced the outcome of a study deserve further consideration. The authors showed that both type IV pili and flagella are required for the development of mature multicellular structures in *P. aeruginosa* biofilms. Whereas motile, and thus functional flagella were necessary for this process, type IV pili were only required to be present but not necessarily functional, perhaps indicating a structural role in adhesion (Barken et al. 2008). Wood et al. (2006) reported similar findings in *E. coli* where the poorest biofilm formation, with respect to both architecture and thickness, was observed for strains with impaired motility.

Once a swimming cell adheres to a surface and biofilm formation is initiated, one could ask what happens to the appendages involved in motility. While a comparison of global gene expression between biofilm and planktonic *P. aeruginosa* only revealed a 1% difference, the expression of genes involved in pili and flagella synthesis were repressed in biofilms (Whiteley et al. 2001). In contrast Domka et al. (2007) showed that 20 motility-related genes are induced throughout all stages of *E. coli* biofilm development, ranging from early attachment stages to fully mature biofilms. Kalmokoff et al. (2006) demonstrated via proteomic analysis that the flagella motility complex plays an important role in initial attachment of *Campylobacter jejuni*. In addition, the continued expression of the flagella motility complex even into the stages of a mature biofilm was noted. While motility is important for the initial attachment of *Vibrio cholerae*, the expression of the flagella filament, *flaA* decreases in mature biofilms, as reviewed by Guttenplan and Kearns (2013). It is however unknown whether, after surface attachment, the flagellum remains functional or if it is lost and degraded or if it acts as a structural component of the biofilm (Teschler et al. 2015). Guttenplan and Kearns (2013) investigated the four model systems of *Bacillus*, *Vibrio*, *Pseudomonas*, and *Escherichia* and proposed that flagellar motility in the biofilm is regulated in two stages: firstly a fast inhibition stage at the level of flagellar function and, afterward, a slow inhibition at the level of flagellar gene transcription.

Nadell et al. (2015) investigated *V. cholerae* attached to surfaces and showed that the extracellular matrix prevented biofilm invasion, where non-motile cells were incapable of entering the existing biofilm and motile cells were only capable of colonizing and growing on the biofilm exterior.

3.8.2 Motility in or Around the Biofilm

Li et al. (2014) demonstrated that near-surface microbial swimming behavior differs in viscoelastic fluids, such as biofilms, compared to Newtonian fluids. Proteins and polysaccharides associated with the biofilm EPS matrix disperse into the surroundings, thereby imparting viscoelastic properties to the ambient fluid (Li et al. 2014). The viscoelastic environment allows puller swimmers to escape more readily from the biofilm environment, while the pusher swimmers appear to be perpetually trapped (Li et al. 2014). Disparate swimming strategies will interact differently with the viscoelastic environment, leading to increases or decreases in the swimming speed of both single and collective swimming bacteria. For example, helical bacteria demonstrate faster swimming speeds in a viscoelastic fluid compared to a Newtonian fluid with the same viscosity (Li et al. 2014).

Martinez et al. (2014) measured *E. coli* motility in polymer solutions and concluded that the flagella experienced a lower local viscosity than the cell itself. In this sense the flagella not only act as small rheometers able to detect local non-Newtonian behavior but also provide corridors of lower viscosity that will make it easier for another bacterium to follow in its wake (Martinez et al. 2014).

Planktonic bacteria in close proximity to a biofilm can tunnel deep into the biofilm by creating transient pores (Houry et al. 2012). These pores could benefit the biofilm by enhancing nutrient transport or harm it by allowing the penetration of antimicrobials (sometimes produced by the tunnelling bacteria themselves) leading to supplanting of the original biofilm occupants (Houry et al. 2012).

3.8.3 Biofilm Detachment: Emigration

The proposed advantages of detachment from biofilms are numerous and include (1) the ability to escape deteriorating environmental conditions once nutrients are depleted due to increased competition, or the presence of an antimicrobial agent, (2) the potential to reach and make use of new habitats, and (3) the opportunity to generate genetic variation (McDougald et al. 2012). To these advantages should be added the fact that detachment provides a mechanism to maintain metabolically active biofilms that are in equilibrium with the carrying capacity of the local environment, by balancing cell formation with accumulation. The continuous production and release of cells, both during biofilm development and after steady state is attained, has been proposed to be a mechanism for prokaryote proliferation and transmission (Bester et al. 2009). A prerequisite for the dissemination of microbes from biofilm-colonized habitats requires the capacity to detach and be actively propelled or passively transported to a different environment, whereafter reassociation with an existing biofilm or primary surface colonization can take place. Several biofilm detachment modes have been identified and described

empirically, but a clear distinction between the different mechanisms and consensus regarding the underlying mechanisms and ecological purpose remains to be reached.

3.8.3.1 Modes of Biofilm Detachment

The mechanisms of biofilm detachment have been defined both in terms of the particle size of the detached biomass and the frequency of detachment (Bryers 1988). Grazing by higher organisms such as protozoa or nematodes may result in the physical disruption of the biofilm, and additional loss of attached biomass can be expected to occur as a result of abrasion by solid particulates. Both of these mechanisms may remove biomass ranging in size from small clumps to large aggregates containing numerous microbial cells and EPS. Substantial reductions in the amount of biomass may also occur at random intervals, due to the sloughing off of large aggregates from the biofilm. While the underlying mechanisms resulting in sloughing have not been identified, it is thought to occur when shear forces exerted by flowing liquid exceeds the cohesive strength of the biofilm matrix.

Biofilms can move over surfaces (Stoodley et al. 1999b) and through porous media under both laminar and turbulent flow conditions (Stoodley et al. 2005). Under turbulent flow, ripple-like structures form and move downstream (Stoodley et al. 1999b). Furthermore, the formation of filamentous biofilm streamers has been reported under turbulent as well as laminar flow conditions (Stoodley et al. 1998; Rusconi et al. 2010, 2011), with the latter being dependent on complex flow patterns created by varied channel geometries. An increase in turbulent flow velocity led to the breakage or detachment of some filaments (Stoodley et al. 1998), whereas spontaneous sloughing off of filaments occurred at unpredictable intervals and completely constricted laminar flow (Drescher et al. 2013). While it was thought that the development of filamentous streamers under laminar flow required the presence of unusual channel geometries such as corners, Parvinzadeh Gashti et al. (2015) observed the formation of streamers in straight microchannels. The accumulation of dense ridges of biofilm biomass was correlated to the abrupt partial detachment of one end of a ridge, leading to the formation of streamers. The authors determine a corresponding detachment rate of 0.15 events mm^{-2} h^{-1} for a single species *Pseudomonas* biofilm.

3.8.3.2 Erosion, Planktonic Cell Yield, and Seeding Dispersion

The detachment of single cells from the biofilm has similarly been attributed to various processes, including liquid-mediated erosion of cells from the biofilm surface, active growth, cell division and release of progeny cells by the biofilm, and most recently seeding dispersion. Despite evidence to the contrary, the detachment of single cells from biofilms continues to be ascribed primarily to liquid-mediated erosive action. Under conditions where substrate availability is governed by flow velocity, the observed detachment rates are often better correlated to

substrate availability or biofilm growth rates, rather than shear removal forces (Peyton and Characklis 1992; Stewart 1993; Bester et al. 2009, 2013).

The rate at which single cells detached from various 120-h-old Pseudomonad biofilms, exposed to bulk liquid flow, was quantified at approximately 10^7 cells cm^{-2} h^{-1}, with an increase in the detachment rate evident as early as 6 h after the introduction of the cells into a sterile environment (Bester et al. 2009). The number of detached cells increased alongside biofilm development until both parameters reached a maximum after 96–120 h. The removal of the carbon source from 5-day-old Pseudomonad biofilms subjected to continuous flow resulted in a 40%, 94%, and 99% decrease in detachment rate after 1, 5, and 24 h, respectively. During the ensuing 8-day period of carbon starvation, the rate of cell detachment cells decreased by 1–2 orders of magnitude, compared to pre-starvation. A rapid increase in the number of detached cells was observed upon a reintroduction of carbon into the system, with a complete recovery of pre-starvation detachment rates within 24 h (Bester et al. 2011). The observed response in cell detachment was mirrored by the metabolic activity of the biofilm and clearly demonstrated the contribution of active biofilm carbon consumption and growth to the number of detached planktonic cells. To differentiate between shear-related erosion of attached biomass and active attached cell growth and release of progeny into the environment, the term "biofilm-derived planktonic cell yield" has been proposed (Bester et al. 2009).

Observation of the active dispersal of planktonic cells from within *P. aeruginosa* biofilm microcolonies (Sauer et al. 2002; Purevdorj-Gage et al. 2005) has been designated as the final stage of the biofilm development life-cycle model (Stoodley et al. 2002b). During dispersion, actively swimming planktonic cells are observed to exit from the interior of biofilm microcolonies, while a sessile, hollow structure remains behind (Purevdorj-Gage et al. 2005). Prior to cell dispersal taking place, an increase in cell lysis and death within the colonies have been reported. The latter is likely due to an accumulation of reactive oxygen and nitrogen species as well as bacteriophage-mediated cell lysis (Webb et al. 2003; Barraud et al. 2006). Interestingly, the dispersed population for both *P. aeruginosa* (Kirov et al. 2007) and *Pseudoalteromonas tunicata* (Mai-Prochnow et al. 2006) displayed significant variation in a number of phenotypic traits, such as colony morphology, motility, biofilm formation, metabolic activity, production of quorum sensing molecules, and virulence factors. The authors drew an analogy between this observed increase in phenotypic differentiation within a prokaryote and known eukaryotic dispersal strategies, which generate variation to enhance the probability of successful surface colonization under a variety of environmental conditions (Mai-Prochnow et al. 2006). While seeding dispersal has been documented for a number of bacterial species, not all strains within a species behave in this manner as evidenced by conflicting reports on the ability (or inability) of mucoid clinical isolates of *P. aeruginosa* to disperse (Purevdorj-Gage et al. 2005; Kirov et al. 2007). The relative contribution of each of these mechanisms to the extent of biofilm detachment will likely vary in space and time and depend on environmental conditions as well as biofilm-specific factors.

3.8.3.3 Rates and Extent of Biofilm Detachment

In addition to the abovementioned, others have attempted to quantify detachment rates from single species, defined consortia, and multispecies biofilms. In an elegant study by Stoodley et al. (2001), the rates at which aggregates detached from a defined four-species consortium biofilm and an undefined tap water biofilm, both cultivated under turbulent flow, were quantified. Detachment rates from the tap water biofilm were estimated at 3.7 detaching clusters mm^{-2} h^{-1} with an analysis interval of 1 h, up to 80.2 detaching clusters mm^{-2} h^{-1} when data was acquired at 20-min intervals. The majority of detached particles were single cells or small cell aggregates, whereas large clusters consisting of multiple cells detached less frequently but accounted for a significant fraction of the overall amount of biomass lost. The detachment rate from *P. aeruginosa* PAO1 biofilms has been estimated at 5.14×10^4 events min^{-1} mm^{-2}. Single cells accounted for a minimum of 70% of these events, whereas the remaining 30% corresponded to events consisting of 2 to >1000 cells, with aggregates consisting of >100 cells accounting for less than 0.28% of the total detachment events but containing up to 37% of the total number of detached cells for PAO1 (Wilson et al. 2004). A detachment rate of 1.8×10^7 CFU cm^{-2} h^{-1} quantified for 7-day-old *Staphylococcus aureus* biofilms. In contrast to *P. aeruginosa* biofilms, it was found that aggregates containing 11–100 cells detached at the greatest frequency (20% of all events) and contained the largest fraction of all detached biomass (54%) (Fux et al. 2004).

3.9 Conclusion

The recognition that microbes are predominantly associated with aggregates or biofilms in natural environments has naturally led to a shift in the perception of microbial processes, along with a concomitant increase in research efforts to address our lack of understanding. Extensive investigation into biofilm development by a selected number of "model" microorganisms has indeed greatly enhanced this understanding, and a notable outcome from these efforts was the establishment of the classical four- or five-stage model of biofilm development. This model, understandably, focuses on the attached, matrix-encased sessile cells and associated microenvironments, whereas planktonic cells are only seen to contribute to biofilm processes during initial attachment to an uncolonized surface and eventual detachment from a mature biofilm. This limitation has the potential to restrict our perspectives and understanding of surface-associated microbial communities.

Sessile cells acquire genetic and phenotypic characteristics during biofilm formation that distinguish them from planktonically derived cells. However, a number of authors have commented on the existence of a third phenotype (Bester et al. 2005). This phenotype is displayed by cells that detach from the biofilm and have been characterized with respect to differences in adhesion (Rollet et al. 2009),

virulence (Uppuluri and Lopez-Ribot 2016), and antimicrobial susceptibility (Boles and Horswill 2008).

In addition, the biofilm or surface-associated region with greatly reduced flow rates may facilitate the persistence of an independently-replicating planktonic population alongside the attached biomass. Delineating the interactions between these attached and motile subpopulations, along with determining their relative contributions to global processes, would require concerted future research efforts.

It can be expected that the particular environment experienced by bacterial populations, whether conditions of plenty or scarcity, will drastically influence their behavior and productivity. In order to learn more about an individual cell in its habitat, it is critical to be aware of the characteristics of the environment in which it is studied, a good example being consideration that different conditions can turn cooperator into cannibal (González-Pastor et al. 2003). It can be argued that learning about an individual or species in its environment is only of interest to fundamental researchers. However, in the case where humans are dependent on the well-being versus demise of a microbial community, the importance of understanding the relationship that the community has with its environment becomes much more than fundamental curiosity, especially if it must be manipulated to achieve a desired outcome.

List of symbols with definitions and units

Mathematical symbol	Definition	Units
a	Acceleration	m s^{-2}
l	Characteristic length	m
ρ	Density	kg m^{-3}
d	Coasting distance	m
δ	Thickness of viscous sublayer	m
D	Diffusion coefficient	m^2 s^{-1}
F	Force	N or kg m s^{-2}
F_{inertia}	Force inertial	N or kg m s^{-2}
F_{viscous}	Force viscous	N or kg m s^{-2}
m	Mass	kg
ν	Kinematic viscosity	m^2 s^{-1}
Pe	Péclet number	Dimensionless
Re	Reynolds number	Dimensionless
t	Time	s
t_a	Time required for a solute to move a distance l under advective transport	s
t_d	Time required for a solute to move a distance l under diffusive transport	s
τ	Shear stress	Pa
τ_w	Shear stress at the wall	Pa
μ	Dynamic viscosity	Pa s
U	Velocity	m s^{-1}
V	Volume	m^3

(continued)

Mathematical symbol	Definition	Units
Shear stress, τ	The parallel component of the applied force, $F_{\parallel}$, divided by the initial area of the sample, A_0	$\tau = \frac{F_{\parallel}}{A_o}$
Shear rate, $\dot{\gamma}$	Various definitions dependent on physical configuration but for a parallel plate system defined as the velocity of the top plate relative to the stationary lower plate divided by the distance between the plates	$\dot{\gamma} = \frac{U}{l}$
Newtonian fluid	For a Newtonian fluid the shear stress, τ, is directly proportional to the shear rate, $\dot{\gamma}$, with the dynamic viscosity, μ, as the slope of the straight line	$\tau = \mu\dot{\gamma}$

Compliance with Ethical Standards

Conflict of Interest Otini Kroukamp declares that he has no conflict of interest. Elanna Bester declares that she has no conflict of interest. Gideon M. Wolfaardt declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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