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Review

Bacterial adhesion and biofilms on surfaces

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Abstract

Bacterial adhesion has become a significant problem in industry and in the domicile, and much research has been done for deeper understanding of the processes involved. A generic biological model of bacterial adhesion and population growth called the bacterial biofilm growth cycle, has been described and modified many times. The biofilm growth cycle encompasses bacterial adhesion at all levels, starting with the initial physical attraction of bacteria to a substrate, and ending with the eventual liberation of cell clusters from the biofilm matrix. When describing bacterial adhesion one is simply describing one or more stages of biofilm development, neglecting the fact that the population may not reach maturity. This article provides an overview of bacterial adhesion, cites examples of how bacterial adhesion affects industry and summarises methods and instrumentation used to improve our understanding of the adhesive properties of bacteria.
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Keywords: Adhesion; Bacteria; Biofilm; Cleaning; Micromanipulation

1. Introduction

Bacteria generally exist in one of two types of population: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface or within the confines of a biofilm. Biofilms were observed as early as 1674, when Antonie van Leuwenhoek used his primitive but effective microscope to describe aggregates of "animalcules" that he scraped from human tooth surfaces [1]. Since then, many advances in technology and laboratory working practices have allowed more accurate descriptions of biofilms to be made, although even today there is still ambiguity: A biofilm consists of cells immobilised at a substratum and frequently embedded in an organic polymer matrix of microbial origin [2]. Biofilms are a biologically active matrix of cells and extra-cellular substances in association with a solid surface [3]. Biofilms are sessile microbial communities growing on surfaces, frequently embedded in a matrix of extracellular polymeric substances [4]. A biofilm may be described as a microbially derived sessile community characterised by cells that attach to an interface, embedded in a matrix of exo-polysaccharide which demonstrates an altered phenotype [5]. Microcolonies are discrete matrix enclosed communities of bacterial cells that may include cells of one or many species. Depending on the species involved, the micro-colony may be composed of 10–25% cells and 75–90% extracellular polymeric substances (EPS) matrix [6]. Bacterial cells within the matrix are characterised by their lack of Brownian motion, and careful structural analysis of many micro-colonies often reveals a mushroom-like shape [1].

Although descriptions of biofilms have varied over the years, the fundamental characteristics are frequently maintained. A biofilm is attached to a substrate and consists of many bacteria co-adhered by means of physical appendages and extra-cellular polymeric substances. The essential

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requirements for biofilm growth are the microbes themselves and a substrate. If one of these ingredients is omitted, a biofilm will not form [7]. However, it should be noted that without water bacterial motility and nutrient availability is reduced and osmotic pressures become less viable to most bacteria.

For bacteria, the advantages of biofilm formation are numerous. These advantages include: protection from antibiotics [8], disinfectants [9], and dynamic environments [10]. Intercellular communications within a biofilm rapidly stimulate the up and down regulation of gene expression enabling temporal adaptation such as phenotypic variation [11] and the ability to survive in nutrient deficient conditions [12]. About 99% of the world's population of bacteria are found in the form of a biofilm at various stages of growth [13] and the films are as diverse as the bacteria are numerous.

Over the past few decades biofilm growth has been observed in many industrial and domestic domains. Unfortunately, in most cases the growth of biofilms has been detrimental. Many industries suffer the ill-effects of biofilm growth of one type or another, which can result in heavy costs in cleaning and maintenance. Examples of such industries include the maritime [14], dairy [15], food [17], water systems [18], oil [19], paper [20], opticians [21], dentistry [22] and hospitals [23]. Perhaps the environment where people are exposed to biofilms most frequently is the domestic environment [24,25].

Product spoilage, reduced production efficiency, corrosion, unpleasant odours (malodours), unsightliness, infection, pipe blockages and equipment failure are examples of the detrimental effects of biofilms. For these reasons and the emergence of restrictive legislation regarding the effects of cleaning agents on the environment and to user health and safety (Commission Regulation EC No. 1048/2005), there is a lot of industrial interest in developing materials and methods which can remove and actively prevent the formation of biofilms.

2. Biofilms at home and in industry

The usefulness of biofilms is well known, especially in the field of bioremediation. The use of organisms to remove contaminants, e.g. metals and radio nuclides [26], oil spills [27], nitrogen compounds [28] and for the purification of industrial waste water [29], is now commonplace. Indeed the adhesive characteristics of natural human flora are now considered as a tool for preventing the adhesion of pathogenic bacteria to avert infection [30]. However, major problems due to the inappropriate formation of biofilms exist.

In the UK, it is estimated that 9 million cases of intestinal disease every year, much of which originates at home, where human excreta are the primary source of infection [31]. Estimates show that for every case of infectious disease reported to the Communicable Disease Surveillance Centre (CDSC), 136 unreported cases occur in the community causing considerable morbidity [24,31]. Global data on the incidence of infectious disease combined with concerns about emerging and re-emerging pathogens has led to a new governmental initiative to improve home hygiene [32], for example, the safe removal of bacteria from domestic surfaces. Approximately 16% of food poisoning outbreaks in England and Wales may be associated with meals prepared in private houses [33].

In the food industry biofilms cause serious engineering problems such as impeding the flow of heat across a surface, increases in fluid frictional resistance of surfaces and increases in the corrosion rate of surfaces leading to energy and production losses [16]. Pathogenic microflora grown on food surfaces and in processing environments can cross-contaminate and cause post-processing contamination [17]. If the microorganisms from food-contact surfaces are not completely removed, they can lead to mature biofilm formation and so increase the biotransfer potential. Examples of the food sectors that pay particular attention to the possibility of cross-contamination are the milk industry [34] and the slaughter industry [35].

Hospital-related infection (nosocomial infection) periodically provokes sensationalist headlines, for good reason. Surgical instruments and fluid lines, e.g. scalpels, drips and catheters, are common sources of biofilm growth and subsequent infection. Biofilm forming Methicillin-resistant *Staphylococcus aureus* (MRSA) is particularly important due to its ubiquity in the National Health Service (NHS) and repeated resistance to all but a few antibiotic programs [36]. Frequent sources of MRSA are the patients themselves [37].

Dentists have been under scrutiny in recent years due to some serious breaches of health and safety laws, in particular the sterility of instruments and Dental Unit Water Lines (DUWL) [38]. Water lines create optimal conditions for biofilm formation due to ideal surface chemistries, laminar flow and surface area. Potential sources of infection include mouth sprays with dysfunctional valves and contaminated hand pieces [39].

The oil industry has cited many problems resulting from biofilm formation by sulphate-reducing bacteria (SRB). Examples include pipe and rig corrosion, blockage of filtration equipment and oil spoilage. Contamination by SRB can result when oil reservoirs are subjected to water flooding for secondary oil recovery in fields found under the sea bed. Such contamination may arise from temperature-resistant organisms originating from hydrothermal vents [40]. Conversely, the effects of oil spills can result in shifts in the relative abundance of microbial flora which impacts fish and invertebrate mortality, growth and reproduction [41].

The implications of biofilm growth are enormous and they pose a potential threat to everybody and every surface. The sheer varieties of surfaces and environments that have been occupied by biofilms are almost infinite. It follows that combinations of the biofilm structural and temporal heterogeneity are just as numerous. Considering the threat to health and industry that biofilms pose, it is not

difficult to realise the magnitude of the problem. It is thought that further understanding of the mechanisms used by microorganisms to adhere to various surfaces, with the use of the techniques currently available to measure the adhesive strengths of various populations, will provide a basis for the development of better strategies for cleaning surfaces.

3. Current understanding of the mechanisms of bacterial adhesion and development

Biofilm growth is governed by a number of physical, chemical and biological processes. Attachment of a cell to a substrate is termed adhesion, and cell-to-cell attachment is termed cohesion. It is the mechanisms behind these forms of attachment, which ultimately determine the adhesive and cohesive properties a biofilm will exhibit.

Fletcher [42] described the accumulation of microorganisms on a collecting surface as a process of three stages: (1) adsorption, or the accumulation of an organism on a collector surface i.e. substrate (deposition); (2) attachment, or the consolidation of the interface between an organism and a collector, often involving the formation of polymer bridges between the organism and collector; (3) colonisation, or growth and division of organisms on the collector's surface.

Although useful as a snap shot of biofilm growth, this type of profile is limited when considering the intimate processes of cell–substrate/cell–cell interaction. Characklis and Marshal [2] later described an eight-step process which included the formation of an initial conditioning layer, reversible and irreversible adhesion of bacteria, and the eventual detachment of cells from a mature biofilm for subsequent colonisation.

3.1. The conditioning layer

The conditioning layer is the foundation on which a biofilm grows, and can be composed of many particles, organic or inorganic. Anything that may be present within the bulk fluid can through gravitational force or movement of flow settle onto a substrate and become part of a conditioning layer. This layer modifies substrata facilitating accessibility to bacteria. Surface charge, potential and tensions can be altered favourably by the interactions between the conditioning layer and substrate. The substrate provides anchorage and nutrients augmenting growth of the bacterial community.

3.2. Reversible adhesion

Initially, planktonic microbial cells are transported from bulk liquid to the conditioned surface either by physical forces or by bacterial appendages such as flagella. A fraction of the cells reaching the surface reversibly adsorbs. Factors such as available energy, surface functionality, bacterial orientation, temperature and pressure conditions, are local environmental variables which contribute to bacterial adhesion. If repulsive forces are greater than the attractive forces, the bacteria will detach from the surface. This is more likely to occur before conditioning of a substrate.

The activation energy for desorption of bacteria is low and so it is likely to occur, highlighting the weakness of the bonds. Physical forces associated to bacterial adhesion include the van der Waals forces, steric interactions and electrostatic (double layer) interaction, collectively known as the DVLO (Derjaguin, Verwey, Landau and Overbeek) forces [43]. DVLO theory has been used to describe the net interaction between a cell and a flat surface as a balance between two additive factors, van der Waals interactions (attractive) and repulsion interactions from the overlap between the electrical double layer of the cell and the substratum (repulsive due to negative charges of the cells) [44]. These are long range forces otherwise known as physical interactions or physisorption. An extended DVLO theory takes into consideration hydrophobic/hydrophilic and osmotic interactions [45] and has also been described in terms of thermodynamic interaction [46].

3.3. Irreversible adhesion

In real time, a number of the reversibly adsorbed cells remain immobilised and become irreversibly adsorbed. It has been argued that the physical appendages of bacteria (flagella, fimbriae and pili) overcome the physical repulsive forces of the electrical double layer [47]. Subsequently, the appendages make contact with the bulk lattice of the conditioning layer stimulating chemical reactions such as oxidation and hydration [17] and consolidating the bacteria–surface bond. Some evidence has shown that microbial adhesion strongly depends on the hydrophobic–hydrophilic properties of interacting surfaces [48].

3.4. Population growth

As the stationary cells divide (binary division), daughter cells spread outward and upward from the attachment point to form clusters [49]. Typically, such interactions and growth within the developing biofilm form into a mushroom-like structure. The mushroom structure is believed to allow the passage of nutrients to bacteria deep within a biofilm.

After an initial lag phase, a rapid increase in population is observed, otherwise described as the exponential growth phase. This depends on the nature of the environment, both physically and chemically. The rapid growth occurs at the expense of the surrounding nutrients from the bulk fluid and the substrate. At this stage the physical and chemical contribution to the initial attachment ends and the biological processes begin to dominate. Excretion of polysaccharide intercellular adhesion (PIA) polymers and the presence of divalent cations interact to form stronger bonding between cells [7].

Differential gene expression between the two bacterial states (planktonic/sessile) is in part associated to the adhesive needs of the population. For example, the production of surface appendages is inhibited in sessile species as motility is restricted and no longer necessary. Simultaneously, expression of a number of genes for the production of cell surface proteins and excretion products increases. Surface proteins (porins) such as Opr C and Opr E, allow the transport of extracellular products into the cell [50] and excretion materials out of the cell, e.g. polysaccharides. The structure of many Gram-negative bacterial polysaccharides is relatively simple, comprising either homopolysaccharides or heteropolysaccharides [51]. These molecules impart mechanical stability and are pivotal to biofilm adhesion and cohesion, and evasion from harsh dynamic environmental conditions. They consolidate the biofilm structure. Hall-Stoodley and Stoodley [49] identified the differences in gene expression of planktonic and sessile cells, and as many as 57 biofilm associated proteins were not found in the planktonic profile.

3.5. Final stages of biofilm development

The stationary phase of growth describes a phase where the rate of cell division equals the rate of cell death. At high cell concentration, a series of cell signalling mechanisms are employed by the biofilm, and this is collectively termed quorum sensing [52]. Quorum sensing describes a process where a number of auto inducers (chemical and peptide signals in high concentrations, e.g. homoserine lactones) are used to stimulate genetic expression of both mechanical and enzymatic processors of alginates, which form a fundamental part of the extracellular matrix.

The death phase sees the breakdown of the biofilm. Enzymes are produced by the community itself which breakdown polysaccharides holding the biofilm together, actively releasing surface bacteria for colonisation of fresh substrates. Alginate lyase produced by *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, *N*-acetyl-heparosan lyase by *Escherichia coli* and hyaluronidase by *Streptococcus equi* are examples of the enzymes used in the breakdown of the biofilm matrix [53]. Simultaneously, the operons coding for flagella proteins are up regulated so that the organisms have the apparatus for motility, and the genes coding for a number of porins are down-regulated, thus completing a genetic cycle for biofilm adhesion and cohesion.

4. Environmental factors influencing biofilm development

4.1. Effect of pH

Changes in pH can have a marked effect on bacterial growth and as such is frequently exploited in the production of detergents and disinfectants used to kill bacteria. Bacteria possess membrane-bound proton pumps which extrude protons from the cytoplasm to generate a trans-membrane electrochemical gradient, i.e. the proton motor force [38]. The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH [54]. Large variations in external pH can overwhelm such mechanisms and have a biocidal effect on the microorganisms.

Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes [55]. Studies have shown that a gradual increase in acidity increases the chances of cell survival in comparison to a sudden increase by rapid addition of HCl [56]. This suggests that bacteria contain mechanisms in place which allow the bacterial population to adapt to small environmental changes in pH. However, there are cellular processes which do not adapt to pH fluctuations so easily. One such process is the excretion of exopolymeric substances (polysaccharides). Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria [57].

4.2. Rheological and adhesive properties of biofilms

Both mixed species and pure culture biofilms behave like viscoelastic fluids. Biofilms exhibit both irreversible viscous deformation and reversible elastic response and recoil [58]. Extracellular polymeric substances like alginate, xanthan and gellan gum aggregate due to hydrogen bonding to form highly hydrated viscoelastic gels [59]. The presence of acetylated uronic acids in the bacterial alginate of *P. aeruginosa* biofilms increases its hydration capacity [17]. These properties provide the biofilm with mechanical stability [60].

The matrix formed by EPS responds to stress by exhibiting, (1) elastic tension due to a combination of polymeric entanglement, entropic, and weak hydrogen bonding forces; (2) viscous damping due to polymeric friction and hydrogen bond breakage; and (3) alignment of the polymers in the shear direction [61]. Such properties change with increased temperature. Increasing the temperature of polysaccharides produces a gel-like substance which gradually increases in strength until a critical point is reached. At the critical point the gel forms a solution [62]. Such behaviour affects the viscosity of the polysaccharides which can affect biofilm adherence.

4.3. Effect of temperature

The optimum temperature for a microorganism is associated with an increase in nutrient intake resulting in a rapid formation of biofilm [63]. Nutrient metabolism is directly associated and dependent on the presence of enzymes. So it may be fair to say that the formation of a biofilm is dependant on the presence and reaction rates of enzymes, which control the development of many physiological and biochemical systems of bacteria. Temperature is correlated with the reaction rate of enzymes and so has a

bearing on the development of the cells. Optimum temperatures result in the healthy growth of bacterial populations. Conversely, temperatures away from the optimum reduce bacterial growth efficiency. This is due to a reduction in bacterial enzyme reaction rates.

In addition to enzymes, environmental temperature affects the physical properties of the compounds within and surrounding the cells. Fletcher [64] reported the effect of temperature on attachment of stationary phase cells. Findings showed that a decrease in temperature reduced the adhesive properties of a marine Pseudomonad. It is believed that the effect was due to a decrease in the bacterial surface polymer at lower temperatures as well as effects such as reduced surface area. However, Herald and Zottola [65] observed that the presence of bacterial surface appendages was dependent on temperature. At 35 °C cells were shown to have a single flagellum whilst at 21 °C they had two to three flagella and at 10 °C, cells exhibited several flagella. This may suggest that the initial interaction between the bacteria and substrate may increase with a lowering of temperature, increasing the likelihood of adhesion. Perhaps the more uniform properties of polysaccharides at lower temperatures increase the possibility of biofilm adhesion, because many microbial polysaccharides undergo transition from an ordered state at lower temperatures and in the presence of ions, to a disordered state at elevated temperature under low ionic environments [66].

Although there is plenty of information describing the effect of temperature on bacterial growth in culture, the effect of temperature on removal of adhered microorganisms is not so well documented. The reports available describe fairly radical effects of temperature on adhered bacteria. Marion-Ferey et al. [67] observed the effect of high temperatures (80–90 °C) on the removal of biofilms. It was discovered that these temperatures were not effective for biofilm removal due to 'baking effects' at high temperature, apparently increasing the adherent nature of the biofilm to the surface.

5. Methods used to grow biofilms in the laboratory

Biofilm growth techniques tend to differ between each research group, producing biofilms often dissimilar both structurally and physiologically. Unfortunately, this can prevent direct comparison between experiments using alternative growth techniques. Wirtanen et al. [68] states that a series of methods should be employed to assess bacterial adhesion, for example, microscopy, plate techniques, and surface area fraction coverage analyses. The advantage of these techniques is that they are ubiquitous and standardised globally, enabling the direct comparison of the observations and measurements carried out by different groups using these techniques. Wirtanen et al. also described a slant technique for biofilm growth onto stainless steel. The benefit of this technique is that gravity plays a reduced role in biofilm formation, and thus a more traditional biofilm may be developed. Static growth techniques allow the culture to grow in media where there are few mechanical or thermal fluctuations [9]. However, nutrients become deplete over time, affecting biofilm growth. Such techniques are indicative of spillages left for periods of time, a common occurrence on various domestic surfaces.

Dynamic growth methods generally include those that grow biofilms with the use of liquid flow, where forces of attraction between bacteria and surfaces become greater than the shear forces of the flow for biofilm survival [69,70]. Flow techniques may be attached to chemostats by which nutrients and other chemical constituents of a culture may be constantly monitored and maintained over time [71]. However, this is not necessarily indicative of a natural or industrial process. Verran and Jones [16] describe the use of a Robbins device to grow a series of biofilms. The Robbins device enables easy extraction of studs from a flow rig onto which biofilms are attached providing an opportunity for direct analysis. However, the generation of biofilm gradients which develop from one side of the device to the other occurs, reducing consistency of biofilm growth between samples [16].

Biofilm growth techniques may or may not accommodate cytometric analysis or image analysis techniques. In some instances a flow cell can be incorporated into a rig facilitating in situ analysis of biofilm growth [60]. Although useful, direct measurement of biofilm adhesion is often made difficult, because access to the biofilm is often restricted.

6. Techniques used to observe and measure bacterial and biofilm adhesion

6.1. Background

Weiss [72] measured bacterial adhesion by allowing cells to settle onto a glass surface of a sealed chamber, counting was carried out with the aid of a microscope. After a period of incubation the chamber was turned upside down, the unattached cells fell from the surface and the remaining attached cells were recounted.

Weiss also described a disc shearing device which employed a static disc with cells attached and a second disc which spun above the attached cells. The shear stress is transmitted through a test fluid, which was dependent on the rotation rate of the disc, separation distance, fluid velocity and radial position. Other tests have employed gentle washing to remove adherent cells [73]. Christie et al. [74] used a water jet impinged vertically onto the test surface at a fixed velocity. Since then there have been a number of modifications to shearing techniques [75]. More recently, bacterial adhesion measurement has been aided by sophisticated technology such as Micromanipulation [10], Atomic Force Microscopy [76,77], and Optical Tweezers [78].

To measure the strength of bacterial adhesion it is necessary to remove them from a surface. Fowler and McKay

[79] described two types of adhesion measurement: (a) adhesion number (the counting of cells before and after an event) and (b) critical force (measurements during an event). Adhesion number techniques rely heavily on imaging equipment such as environmental scanning electron microscopy, optical microscopy and confocal laser microscopy. These techniques although powerful, are purely observational as they do not measure directly the adhesion of bacterial populations. Critical force techniques (atomic force microscopy and micromanipulation) directly interact with bacteria so that a force required to remove bacteria can be determined. Of these techniques only one enables the direct measurement of biofilm adhesion, micromanipulation.

6.2. Micromanipulation

In the late 1980s, there was a gap for an instrument to be developed capable of directly measuring cell mechanical properties, as previous attempts had fallen short of the requirements due to difficulties with the interpretation of data [80]. A system to measure such properties was developed by the Micromanipulation Group at Birmingham University, England. The system was based on micromanipulation of a probe onto a selected cell of interest. This technique has been used to acquire compression data using mammalian hybridoma cells [81] and yeast cells [82]. Currently, work is being carried out to characterise and model the mechanical properties of a wide range of biological and non-biological materials, including chondrocytes, microcapsules, and microspheres.

A second application of the micromanipulation technique is the direct measurement of the apparent adhesive strength of biofilms. A novel T-shaped probe was made and attached to the output tube of a force transducer. The transducer was then attached to a three-dimensional micromanipulator. The T-shaped probe is positioned just 1 μm above the surface of the substrate. As the T-shaped probe travels the surface of the substrate with biofilm, the biofilm is removed and the force exerted by the biofilm onto the probe is recorded and used to calculate biofilm adhesion. Chen et al. [10] used this technique to remove *P. fluorescens* biofilms grown within a biofouling rig.

The underlying principle is that the adhesion of biofilms to a surface may be used as an index of their strength. This is defined as the work required to remove the biofilm from a surface onto which it was originally attached [83]. Chen et al. [10] demonstrated the feasibility of the technique by showing that increased flow velocity (within a system a biofilm is growing) increases the adhesive strength of biofilms. Chen et al. [84] subsequently determined the effects of operating conditions on biofilm adhesion. In addition, this technique has been used to measure the forces of adhesion and cohesion of food fouling material [85]. Similar studies are currently being carried out using milk deposits.

The data produced using micromanipulation can be compared with data acquired using alternative techniques such as atomic force microscopy (AFM) and the flow techniques. These techniques complement each other by broadening the measurement range of force required to remove bacteria from a surface, thus allowing the observation of adhesive characteristics of a range of populations. For example, micromanipulation can measure forces within the N range, whereas AFM can measure forces within the nN range. Flow cell techniques can be used to observe the mechanical and cohesive properties of biofilms, but cannot be used to measure directly the forces required to remove biofilms from a surface. As the micromanipulation technique allow the measurement of adhesive and cohesive properties of biofilms directly, it is hoped that this technique can be used to fill the gap left by the limitations of techniques using AFM and flow cell devices.

7. Perspectives

Considering the number of industries negatively affected by biofilm development and also the increasing restrictions on the use of biocides, research into bacterial and biofilm adhesion is likely to maintain industrial interest at least to the foreseeable future. The reduction of biofilm adhesion and their suspension into bulk fluids renders the bacterial populations vulnerable to less toxic biocides. As bacterial and biofilm adhesion is still not fully understood in terms of genetic predispositions and environmental effects, techniques such as micromanipulation and atomic force microscopy are likely to become more frequently exploited. Currently, such techniques are only available off line, i.e. samples need to be taken from biofilm fields or cultures and then their adhesion analysed. Ideally, biofilm adhesion should be measured in situ. For example, biofilm rheology can be analysed online using confocal laser scanning microscopy. Stoodley et al. [86] designed a flow cell that could be mounted onto the stage of a confocal laser scanning microscope (CLSM) that allowed the biofilm to be observed in situ under flow conditions, and the elastic properties of biofilms could be clearly seen. Real-time observations of bacterial adhesion would perhaps provide a greater understanding of industrial situations.

Such work could be closely followed by bacterial adhesion fate analyses before, during and after exposure to chemicals designed to reduce bacterial and biofilm adhesion [87]. Constituent parts of such chemicals could be optimised for given industrial circumstances.

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