



The Problem: Not Just Bacteria–Bacterial Biofilms

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Bacteria will stick to almost any surface in an industrial water system and will almost always produce a slimy polysaccharide matrix. This state of microorganism activity is now universally known as biofilm. Biofilm poses numerous problems in water processes, including corrosion, loss of process efficiency such as heat transfer, and health issues such as Legionella infection. Biofilm is not only ubiquitous and costly; it has also exhibited a recalcitrance to treatment and control that has been frustrating industrial engineers for decades.

The immediate practical difficulty posed by biofilm resistance to chemical challenge is that most real-world microbiological problems are rooted in biofilms, whereas most existing methods for studying microbial susceptibility are based on the culture of free-floating or "planktonic" cells. Recent research advances at the Center for Biofilm Engineering (CBE) at Montana State University-Bozeman have laid the groundwork for envisioning novel strategies to control biofilms in industrial water processes. The following are key CBE research developments and their implications for effective mechanisms of biofilm control:

Biofilm Resistance to Antimicrobials

When microorganisms attach to a surface and grow as biofilm, they become less susceptible to biocides and disinfectants than are the same microbes when studied in a conventional suspension culture. This resistance in the biofilm state has been demonstrated for many different bacteria and fungi. Biofilm resistance has also been documented for chemically diverse biocides, including many of those commonly used in water treatment. The mechanisms by which biofilms evade killing by antimicrobial agents are now beginning to emerge, thanks in part to recent discoveries at the CBE.

Some antimicrobials fail to penetrate the biofilm rapidly or completely. Because the delivery of an antimicrobial agent into the nether regions of a biofilm depends on the transport process of diffusion, an understanding of the nature of diffusion in biofilms is essential to addressing the question of biocide penetration. Biofilms are mostly water; but the presence of microbial cells, extracellular polymers, and abiotic particles such as silt, scale, corrosion products or paper fibers, will obviously retard diffusion inside a biofilm. Measurements of effective diffusion coefficients in biofilms confirm this expectation.

Diffusion in a biofilm proceeds at rates approximately 20 to 80 percent of those in pure water for solutes comparable in size to common biocides (Stewart, 1998). One important interpretation of these values is that there is no inherent physical barrier to the entrance of an antimicrobial agent into the biofilm. Solutes with molecular weights up to about 1000 daltons can move freely into and out of biofilms, albeit at somewhat slower rates than in pure water.

The real barrier to biocide penetration of a biofilm occurs when the biocide is reactively neutralized in the surface layers of the biofilm faster than it diffuses. If an antimicrobial agent reacts with some constituent of the biofilm, then its penetration will be slowed or even completely halted. Reactive oxidants such as chlorine and hydrogen peroxide are particularly prone to transport breakdown by this mechanism (*de Beer et. al., 1994a; Chen and Stewart, 1996; Xu et. al., 1996; Liu et. al., 1998*), but recent experiments suggest that non-oxidizing biocides can also be limited in this way (*Stewart et. al., 1998*). Key products of the CBE's research effort in biofilm control have been theories of antimicrobial penetration incorporating the reaction-diffusion interaction (*Stewart and Raquepas, 1995; Stewart et. al., 1996; Stewart, 1997*) and methods for diagnosing such transport limitation (*Stewart et. al., 1998*).

Another powerful explanation for biofilm-reduced susceptibility hinges on the likely presence in the biofilm of slow growing or non-growing microbial cells. Slow growing or starved bacteria are known to be less sensitive to a wide variety of environmental insults than are their rapidly growing counterparts. In addition to better tolerating desiccation, heat shock, and oxidative stress, nutrient-limited cells are found to be less susceptible to disinfectants and antibiotics. Within a biofilm, gradients in the concentration of nutrients can lead to zones of substrate limitation. For example, CBE researchers have used oxygen microelectrodes to confirm the presence of anaerobic niches in the depths of biofilms growing in aerated waters (*de Beer et. al., 1994b*). New techniques are now allowing us to directly visualize the resulting "physiological heterogeneity" for the first time. The fact that a biofilm contains cells in a broad spectrum of metabolic states, from rapidly proliferating to absolutely dormant, certainly frustrates efforts to control biofilms with antimicrobial agents. The agents now in widespread use have been selected and given regulatory approval on the basis of their ability to kill cells grown in planktonic culture in a narrow, and relatively susceptible, phenotypic state.

These insights into the fundamental basis of biofilm resistance to antimicrobial agents suggest new approaches to biofilm control. For example, it is critical to weigh both biocide disinfecting power and penetrating ability when selecting a biocide for a biofilm application. CBE mathematical models predict that a weak disinfectant, if it penetrates the biofilm, will easily outperform a strong disinfectant that fails to completely penetrate. Where nutrient-limited physiologies foil current antimicrobial agents, it might pay to search for alternative biocides. There may be some biocides previously dismissed as inferior based on tests against planktonic microorganisms whose relative efficacy against biofilms would make them superior for this application.

Progress in biofilm control technology requires good biofilm-based testing and modeling methods. Even with the recent advances in biofilm science, it is still impossible to predict the efficacy of an antimicrobial agent against biofilm from data collected using conventional planktonic cultures. Essential biofilm phenomena, such as penetration and physiological heterogeneity, are simply not captured by tests that use microorganisms in suspension (*Zelver, et. al., 1999*). A real barrier to the discovery, understanding, and development of practical biofilm control technologies is the current lack of recognized standard biofilm testing methodologies. A major initiative now underway at the CBE is to develop reliable biofilm testing methods, to validate them by comparison with field studies, and to lobby for their recognition and acceptance by regulatory agencies.

Using Biofilm Fundamentals to Treat Water Biological Pretreatment

The water treatment industry has traditionally relied on the use of chemicals to reduce microbial counts. Very often, disinfectants are applied continuously throughout a water distribution system and quenched or removed immediately upstream of a process where these chemicals are not tolerated. A case in point involves the use of reverse osmosis membranes. Many membranes cannot tolerate oxidizing agents such as chlorine, so the chlorine must be neutralized before the water comes in contact with the element. Because this water is rarely sterile, and the disinfectant has now been removed, the Membrane is the first place where microbial growth is not repressed. As a consequence, biofouling of membranes occurs, which results in increased pressure drop and decreased productivity. Membrane maintenance is then required, which may again rely on the use of chemicals.

Because of increasing constraints on environmental discharge of disinfectants, as well as a desire to reduce costs, the water treatment industry has been looking for alternative methods to reduce biofilms and microbial counts in industrial waters. One promising technology, used in the drinking water industry for over a decade, is biological treatment. Biological treatment uses biofilm to reduce or remove the constituents in water that foster the growth of bacteria in downstream distribution systems or process Equipment. The premise is to encourage bacterial growth in a location where it can be controlled and optimized, rather than having it happen ad hoc and in an uncontrolled manner in critical downstream elements. Biological treatment uses naturally occurring bacteria adapted to the types of organic carbon found in the water. The chance for further microbial growth is limited by the depleted food source, so less or no disinfectant is required. The CBE has been applying these principles to the pretreatment of water for reverse osmosis membranes.

The premise of biological treatment prior to RO membranes is to immobilize organic matter that would otherwise accumulate on reverse osmosis membranes and (2) to use indigenous organisms to metabolize the sorbed organics as well as other bulk phase carbon compounds that could support biofouling on the membranes. For industrial purposes, water is allowed to flow through a column containing filtration media (sand, anthracite, granular activated carbon). The surfaces of the filter media act as a support for microbial attachment and growth, resulting in a biofilm adapted to using the organic matter found in that particular water. Total organic carbon removals in filters used for drinking water range from 5 to 75% (*Bouwer and Crowe, 1988*). Since natural organic matter is also a constituent in membrane fouling, both biofilm development and natural organic carbon deposition on the membrane can be decreased via biological treatment.

Interestingly, the use of granular activated carbon columns upstream of RO membranes is generally discouraged (*Kucera, 1997*) because they are perceived as "bug farms" with only deleterious effects. By contrast, CBE researchers have been able to demonstrate that 40-50% of the total organic carbon present in humic substance amended water can be removed by biofilm-colonized granular activated carbon columns followed by nanofiltration. Moreover, this treatment has resulted in downstream surface biofilm cell count reductions of over a hundred-fold, as compared to water with nobiological pretreatment. These results demonstrate that properly operated biological treatment has the potential for improving the longevity of downstream membrane processes as well as for reducing biofilm development in distribution systems. A favorable outcome of our research would be a cost effective treatment method to reduce fouling and chemical use for RO membranes and other biofouling prone processes.

Thoughts for the Future - Biofilm Signaling

One of the early advances in the understanding of biofilm structure and function was the demonstration that, while they appear to the naked eye to be homogeneous accumulations of slime, biofilms are remarkably complex structurally. Subsequent research has pointed to the role of signaling mechanisms in the development of the bacterial micro colonies and water channels that characterize biofilm structure. Prior to this biofilm work, signals had been identified elsewhere in the bacterial world, in bacterial populations which formed demonstrable structures (e.g. fruiting bodies) or temporary associations with higher organisms (e.g. luminescent bacteria with deep-sea fish). Greenberg (*Fuqua and Greenberg, 1998 and Iglewski Passador et al, 1993*) then showed that other bacterial activities, such as toxin and enzyme production, were controlled by signals with a similar chemical composition, in that they were all acyl homoserine lactones (AHLs). Davies et. al. (*Science, 1998*) reasoned that mutants lacking the ability to either produce or perceive these signal molecules might be altered in their ability to form biofilms, and this prediction was found to be correct. Mutants of *Pseudomonas aeruginosa* that lack the ability to synthesize N-(3-oxododecanoyl)-L-homoserine lactone (ODdHL) cannot produce the biofilm matrix and cannot form structured biofilms. The addition of ODdHL

to cultures of this mutant restores their ability to make fully structured biofilms. The pivotal role of this signal molecule in biofilm formation suggests that its chemical analogues may be able to block the process in a way that would leave the cells able to live and metabolize, but not to form biofilms.

Kolter and Losick (1998) suggest that this discovery of the control of biofilm formation by AHL signals heralds a new era, in which indiscriminate toxic agents that simply kill bacteria will be replaced by signals and signal analogues that manipulate specific bacterial "behaviors." For example, reverse osmosis membranes might be treated with universal signal inhibitors that would protect them from fouling by marine organisms, and the use of oxidizing cleaners such as chlorine could be entirely obviated. In instances in which we would like to retain beneficial biofilms on a surface, as in sewage systems and chemical reactors, the beneficial organisms could be made non-reactive to a biofilm inhibitor that would preclude colonization by all extraneous organisms.

Recent work at the CBE has shown that most natural biofilms exhibit a phenomenon that we call programmed detachment. This common biofilm behavior involves the mobilization of planktonic cells, which may be mobile by flagellar activity in some species within specific microcolonies that comprise the biofilm. The environmental control of this phenomenon is not fully understood but, in *Pseudomonas aeruginosa*, it may be induced by the cessation of flow. After an interval, all of the sessile cells in a microcolony may revert to the planktonic phenotype, and produce the enzyme (lyase, in *Pseudomonas aeruginosa*) necessary to degrade the biofilm matrix, so that cells are released into the bulk fluid. If, as we suspect, this programmed detachment is under the control of a signal molecule, the natural signal or an analogue may someday be used to induce the wholesale detachment of established biofilms from colonized surfaces.

Summary

The more we learn about biofilms, the more intrigued we become with the profound differences between the behavior and structure of the community of biofilm microorganisms compared to their planktonic counterparts. Of paramount concern to the water treatment industry, overwhelming evidence has shown that microorganisms in the biofilm state are significantly more difficult to disinfect than planktonic microorganisms. To address this problem we need a better understanding of the mechanisms for such resistance, methods for analyzing susceptibility of biofilms to disinfectants and innovative ways to control biofilm growth and persistence in water systems. Research results from the Center for Biofilm Engineering (CBE) have shown promising new directions and strategies that may someday be applied in the control and removal of biofilm in industrial water systems. These strategies could include:

- (1) Antimicrobials that penetrate deep within a biofilm. To develop better antimicrobials, we need to be able to model and measure the ability of an antimicrobial to penetrate the biofilm. The CBE has developed several techniques that can be used to screen antimicrobials for their ability to penetrate biofilm. For example, CBE mathematical models predict that a weak disinfectant, if it penetrates the biofilm, will easily outperform a strong disinfectant that fails to completely penetrate.
- (2) Biological pretreatment. To reduce reliance on disinfection, an alternative control strategy may be to starve the biofilm microorganisms by biologically pretreating water upstream of the industrial process. A pretreatment filter consisting of a biofilm grown on a carbon filter has been shown to remove 40-50% of the total organic carbon.
- (3) Biofilm Signaling. Cell-to-cell signaling between biofilm microorganisms has been shown to exist and to be related to development of the biofilm structure and detachment. Mimicking the intercellular communication signals to induce biofilm detachment may someday be used to strip a water process of problematic biofilm without the use of disinfectants. As biofilm researchers at the CBE and elsewhere probe further into the unique nature of biofilm, we anticipate further innovative possibilities for controlling biofilm in industrial water systems. We can look forward to a day when plants run more efficiently with better and "greener" biofilm control technologies. Perhaps by then, today's approaches to controlling biofilm microorganisms will seem as crude as using a sledge hammer to kill an insect.

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