Edstrom INDUSTRIES, INC.
819 Bakke Ave., Waterford, WI 53185 USA
414-534-5181 • 800-558-5913
FAX 414-534-5184

Biofilm

Key to understanding and controlling bacterial growth in Automated Drinking Water Systems

What are biofilms?

Biofilms are a collection of microorganisms surrounded by the slime they secrete, attached to either an inert or living surface. You are already familiar with some biofilms: the plaque on your teeth, the slippery slime on river stones, and the gel-like film on the inside of a vase which held flowers for a week. Biofilm exists wherever surfaces contact water.

Why learn about biofilms?

As in any water system, 99 per cent of the bacteria in an automated watering system is likely to be in biofilms attached to internal surfaces. Biofilms are the source of much of the free-floating bacteria in drinking water, some of which can cause infection and disease in laboratory animals. One common biofilm bacteria, *Pseudomonas aeruginosa*, is an opportunistic pathogen which can infect animals that have suppressed immune systems. Besides being a reservoir of bacteria which can affect animal health, biofilms can also cause corrosion in stainless steel piping systems.

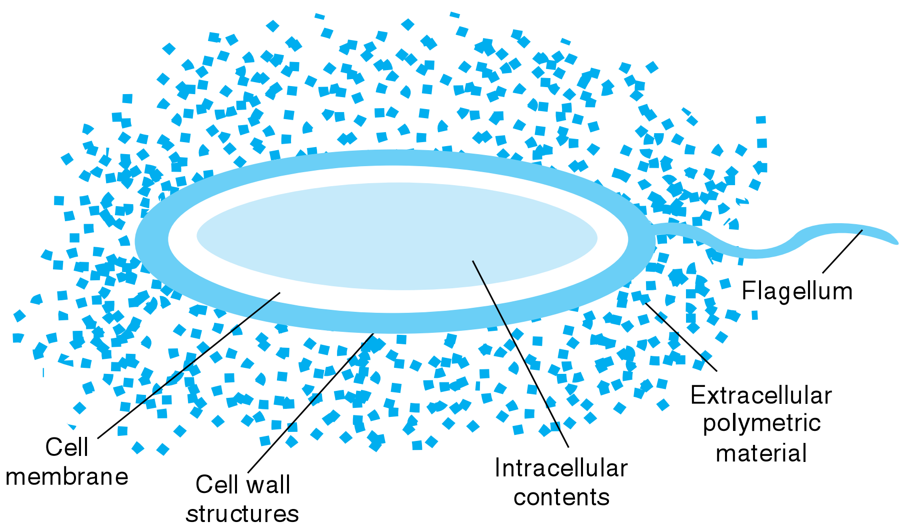

Figure 1.
Wild bacteria are "hairy" cells with extracellular polymers which stick to surfaces, concentrate nutrients, and protect bacteria from disinfectants.

Understanding bacteria in biofilms is one step toward preparing for the future. Animal facilities can meet the most demanding water quality requirements by supplying chlorinated reverse osmosis water and by maintaining water quality through flushing and sanitization. But what if chlorine use in animal drinking water is prohibited? Or what if water quality requirements become even more stringent with the use of new specialized animals? In order to design and operate automated watering systems that deliver the cleanest water possible we should understand how biofilms develop, some of the problems they can cause, and how biofilm growth can be controlled. This issue of UPDATE is devoted to understanding biofilms, since this information will direct enhancements to automated watering

Benefits to bacteria: food and protection

The association of bacteria with a surface and the development of a biofilm can be viewed as a survival mechanism. Bacteria benefit by capturing nutrients from the water and developing protection against disinfectants.

Nutrients

Potable water, especially high-purity water systems, are nutrient-limited environments, but even nutrient concentrations too low to measure are sufficient for microbial growth and reproduction. How does life in a biofilm help bacteria acquire nutrients?

- Trace organics will concentrate on surfaces.
- Extracellular polymers will further concentrate trace nutrients from the bulk water.
- Secondary colonizers use the waste products from their neighbors.
- By pooling their biochemical resources, several species of bacteria, each armed with different enzymes, can break down food supplies that no single species could digest alone.

Protection against disinfectants

Biofilm bacteria may be 150-3000 times more resistant to free chlorine than free-floating bacteria. In order to destroy the cell responsible for forming the biofilm, the disinfectant must first react with the surrounding polysaccharide network. The cells themselves are not actually more resistant, rather they have surrounded themselves with a protective shield. The disinfectant's oxidizing power can be used up before it reaches the cell. In fact, biofilm bacteria often produce more exopolymers after biocide treatment to further protect themselves.

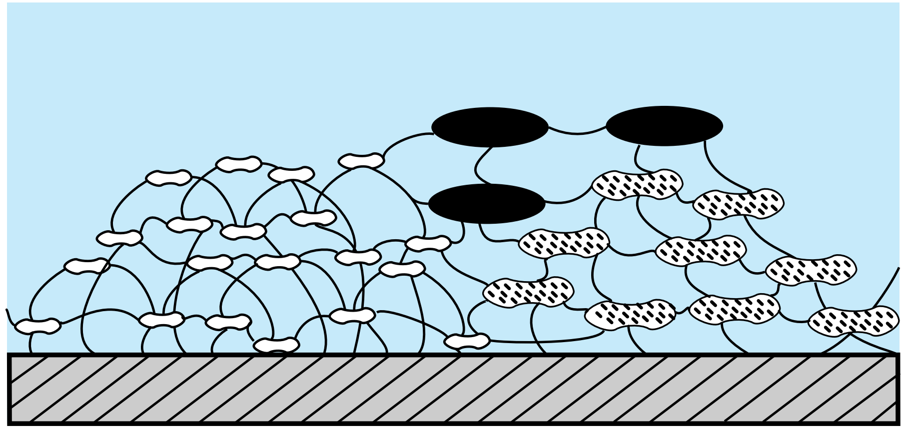

Figure 2.
Bacteria in biofilms bind together in a sticky web of tangled polysaccharide fibers which anchor them to surfaces and to each other.

Steps in biofilm development

1. Surface conditioning
Almost immediately after a clean pipe contacts water, organic molecules adhere to the surface. These organics neutralize the surface charge which may repel approaching bacteria.

2. **Adhesion of "pioneer" bacteria**
Planktonic (free-floating) bacteria first attach themselves by electrostatic attraction and physical forces. Some of these cells will permanently adhere to the surface with their *extracellular polymeric substances*, or sticky polymers.

3. "Slime" formation
The extracellular polymers consist of charged and neutral polysaccharide groups that not only cement the cell to the pipe wall, but also act as an ion-exchange system for trapping and concentrating trace nutrients from the water. As nutrients accumulate, the pioneer cells reproduce. The daughter cells then produce their own exopolymers, greatly increasing the volume of ion exchange surface. Pretty soon a thriving colony of bacteria is established. In a mature

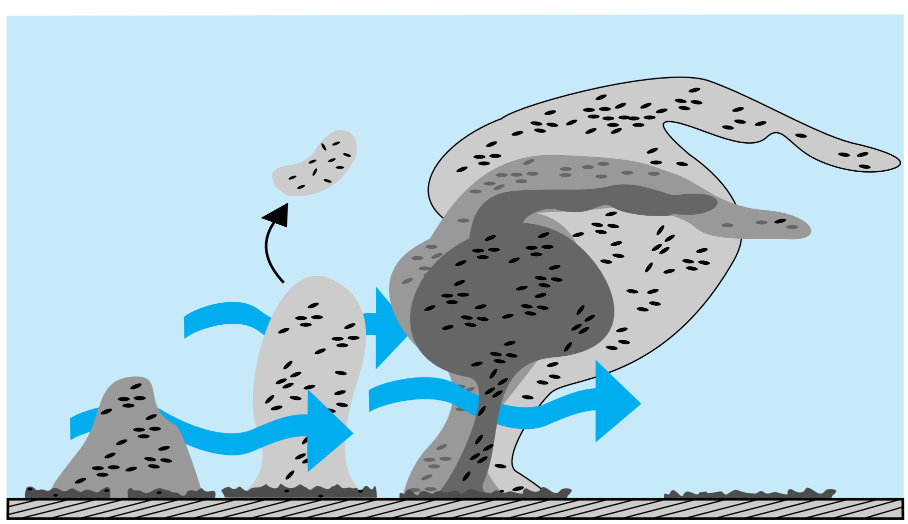

Figure 3.
Conceptual model of the architecture of a single-species biofilm based on direct observations using a confocal *microscope. (Costerton 1995)*

biofilm, most of the volume (75-95%) is occupied by the loosely organized polysaccharide matrix filled with water. This watery slime is what makes biofilm-covered surfaces gelatinous and slippery.

4. Secondary colonizers

Besides trapping nutrient molecules, the exopolymer web also snares other types of microbial cells through physical restraint and electrostatic interaction. These secondary colonizers metabolize wastes from the primary colonizers as well as produce their own waste which other cells then use.

5. Fully functioning biofilm

The mature, fully functioning biofilm is like a living tissue on the pipe surface. It is a complex, metabolically cooperative community made up of different species each living in a customized microniche. An anaerobic layer may develop underneath the aerobic biofilm. As the film grows to a thickness that allows it to extend through the quiescent zone at the pipe wall into zones of more turbulent flow, some cells will be sloughed off. These released cells can then colonize downstream piping.

A mature biofilm may take several hours to several weeks to develop, depending on the system. *Pseudomonas aeruginosa* is a common "pioneer" bacteria which can adhere to stainless steel, even to electropolished surfaces, within 30 seconds of exposure.

New discoveries

In the past, microbiologists assumed that biofilms contained disorderly clumps of bacteria located in no particular structure or pattern. New techniques to magnify biofilms without destroying the gel-like structures have enabled researchers to discover the complex structure of biofilms as if viewing a city from a satellite. See Figure 3.

Past researchers assumed that biofilm bacteria behaved much like solitary, free-floating microorganisms. Now they are discovering that while biofilm bacteria have the exact same genetic makeup as their free-floating cousins, their biochemistry is very different because they switch on a different set of genes. For example, up to 40% of cell wall proteins differ between biofilm and free-floating bacteria. In medicine, this makes biofilm bacteria difficult to kill because some of the targets for antibiotics are no longer there.

Bacterial evolution

Bacteria have evolved the means to find and attach to surfaces in order to increase the chances of encountering nutrients. Motile bacteria like *Pseudomonas aeruginosa* can swim along a chemical concentration gradient towards higher nutrient concentrations at the pipe wall. Many organisms will alter their cell wall to increase their affinity for surfaces. The cell wall becomes *hydrophobic*. Once at the surface, bacteria cells anchor themselves with their sticky polymers.

Biofilm development factors

Surface material

Surface material has little or no effect on biofilm development. Microbes will adhere to stainless steel or plastics with nearly equal enthusiasm.

Surface area

Surface area is one primary factor influencing biofilm development. Plumbing systems, unlike most natural environments (lakes and rivers), offer a tremendous amount of surface area. RO membranes, DI resins, storage tanks, cartridge filters, and piping systems all provide surfaces suitable for bacterial attachment and growth.

Smoothness

Although smoother surfaces delay the initial buildup of attached bacteria, smoothness does not significantly affect the total amount of biofilm on a surface after several days.

Flow velocity

High flow will not prevent bacteria attachment nor completely remove existing biofilm, but it will limit biofilm thickness. Regardless of the water velocity, it flows slowest in the zone adjacent to pipe surfaces. Even when water flow in the center of the pipe is turbulent, the flow velocity falls to zero at the pipe wall. The distance out from the pipe wall in which the flow rate is not turbulent is called the *laminar sublayer*. This distance can be considered equal to the maximum biofilm thickness.

Limited nutrients

Like other living creatures, bacteria require certain nutrients for growth and reproduction. Limiting these nutrients will limit bacteria growth, but even minute amounts of organic matter will support many bacteria. Theoretically, just 1 ppb of organic matter in water is enough to produce 9,500 bacteria/ml! Current technology cannot reduce nutrient levels completely, so total control of bacteria is not achievable by simply controlling nutrients. Similarly, very small quantities of oxygen will adequately support luxurious bacterial growth. Although it won't eliminate bacteria, nutrient-poor reverse osmosis water will support less biofilm than regular tap water supplies.

Biofilm in automated watering systems

To visualize biofilm in an automated watering system, it helps to compare the scale of cell size, biofilm thickness and microroughness on the pipe surface.

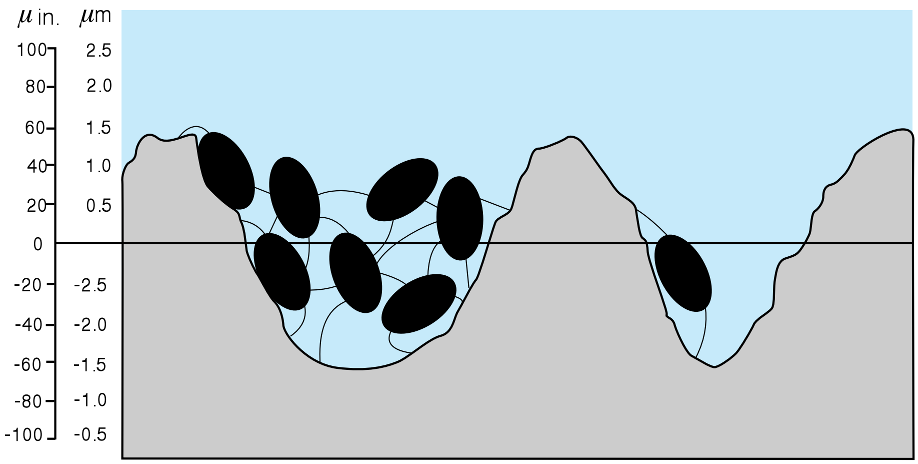

Figure 4.
Compare the size of Pseudomonas *cells to the profile of a 180 grit (32 microinch RA) stainless steel surface.*

The inside surface of stainless steel tubing used in room piping and manifolds is a rolled finish. Although not quantitatively defined, we can assume it is no smoother than a 32 microinch RA or 180 grit finish (which is considered sanitary for dairy, food, and pharmaceutical uses). Figure 4 shows that the surface irregularities in such a finish are large enough to harbor several layers of *Pseudomonas aeruginosa*.

Biofilm will reach a certain equilibrium thickness depending on flow velocity and nutrient levels. Assuming nutrients aren't limiting, biofilm thickness will be approximately the same as the depth of the laminar layer for a particular flushing flow rate. In current automated watering systems, piping is flushed at about 2 ft/sec. At 2 ft/sec, biofilm thickness is limited to approximately 125 microns. The goal for the future is to increase the flushing flow rate to 2.3 gpm which is an average velocity of 5 ft/sec. At 5 ft/sec, biofilm thickness should be limited to approximately 50 microns.

Figure 5 shows the maximum biofilm thickness for 2 and 5 ft/sec flushing velocities in Edstrom Industries' stainless steel RDS piping. Remember that biofilm is also limited by the available nutrients, so it could be thinner in pure water.

You can see that biofilm can grow to over 100 layers of individual bacterial cells. Also notice that irregularities in surface finish are small compared to the maximum biofilm thickness. This illustrates why surface smoothness has little impact on the total amount of biofilm on a pipe surface.

Figure 5 also shows that biofilm is a thin layer in a flushed pipe. In comparison, a much deeper biofilm could fill the crevice of an o-ring joint. These areas of deeper biofilm growth can have more corrosion problems and are harder to sanitize.

Aerobic bacteria near the outer surface of a biofilm consume oxygen. If biofilm is thick enough, oxygen will be depleted at the pipe surface creating an anaerobic zone. These zones inside a stainless steel pipe can cause corrosion.

Could the biofilm in automated watering systems be thick enough to have anaerobic zones? Possibly. The

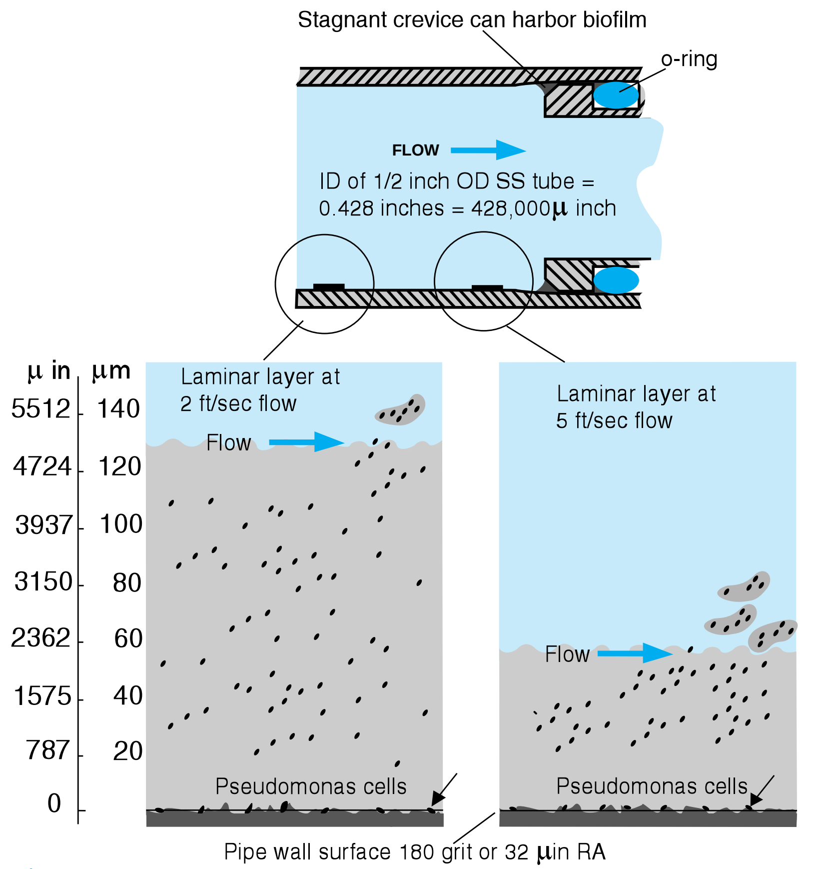

Figure 5.
Biofilm extending outside the depth of the laminar layer will be sheared off during flushing. Higher flow velocity results in less biofilm. Maximum biofilm thickness inside ½" stainless steel pipe compared to the crevice of an o-ring joint. Assumes biofilm thickness only limited by flushing, not by nutrients or sanitization.

depth of the oxygen gradient into the biofilm will vary, but one source indicates that oxygen can be depleted within 30-40 microns of the water/ biofilm interface. Anaerobic zones are most likely to occur in crevices at o-ring pipe joints and threaded fittings.

Microbiologically influenced corrosion

The physical presence of microbial cells on a metal surface, as well as their metabolic activities, can cause *Microbiologically Influenced Corrosion* or *biocorrosion*.

Nonuniform (patchy) colonies of biofilm result in the formation of *differential aeration cells* where areas under respiring colonies are depleted of oxygen relative to surrounding noncolonized areas. Having different oxygen concentrations at two locations on a metal causes a difference in electrical potential and consequently corrosion currents.

Stainless steel relies on a stable oxide film to provide corrosion resistance. When the oxide film is damaged or oxygen is kept from the metal surface, corrosion can occur.

Some biofilm bacteria produce corrosive chemicals like acids and hydrogen gas during their metabolism. Anaerobic zones in biofilm can have sulfate-reducing bacteria (SRBs). This group of bacteria reduce sulfate to hydrogen sulfide which corrodes metals. SRBs can grow in water trapped in stagnant areas, such as dead legs of piping. One way to limit SRB activity is to reduce the concentration of their essential nutrients: phosphorus, nitrogen, and sulfate. Thus, purified (RO or DI) water would support less SRBs.

Any practices which minimize biofilm thickness (flushing, sanitizing, eliminating dead-end crevices) will minimize the anaerobic areas which can cause biocorrosion.

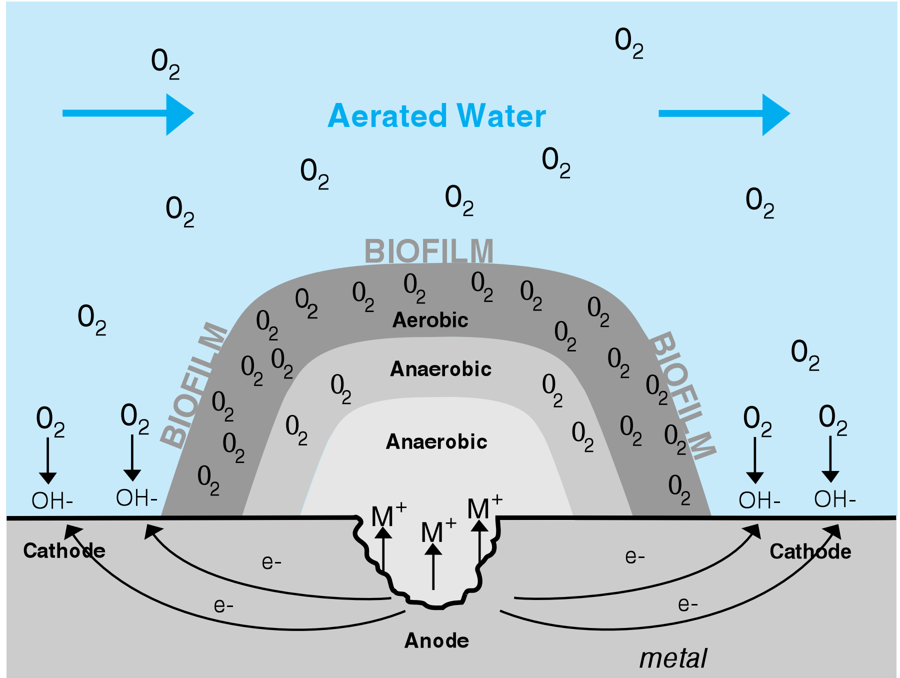

Figure 6.
Nonuniform colonization by bacteria results in differential aeration cells. This schematic shows pit initiation due to oxygen depletion under a biofilm. (Borenstein 1994)

Sanitizing biofilms

Biofilm can be removed and/or destroyed by chemical and physical treatments. Chemical biocides can be divided into two major groups: oxidizing and nonoxidizing. Physical treatments include mechanical scrubbing and hot water.

Oxidizing biocides

Chlorine

Probably the most effective and least expensive of all chemical biocides, chlorine not only kills free-floating and biofilm bacteria, but it also destroys the polysaccharide web and its attachments to the surface. By destroying the extracellular polymers, chlorine breaks up the physical integrity of the biofilm.

Higher concentrations of chlorine are needed to disinfect biofilm than to kill free-floating bacteria. As chlorine diffuses into a biofilm, it is used up reacting with bacteria cells and slime. At low chlorine levels, biofilm bacteria shield themselves by producing slime faster than chlorine can diffuse through. By increasing the concentration, chlorine will diffuse farther into the biofilm. When it comes to disinfection of biofilms, high chlorine concentration for short durations is more effective than low concentration for long durations. However, since it is corrosive to stainless steel, there is a limit on the recommended chlorine concentration for sanitization.

Chlorine dioxide

Chlorine dioxide has biocidal activities similar to those of chlorine. Because it is unstable, it must be mixed and prepared on-site. Like chlorine, chlorine dioxide is corrosive to metals and must be handled with care.

Ozone

As an oxidizer, ozone is approximately twice as powerful as chlorine at the same concentrations. However, because of its limited solubility, it is difficult to produce high concentrations of ozone in water. Chlorine can be used in higher sanitizing concentrations with equal disinfecting strength. Like chlorine dioxide, ozone must be generated on-site because of its high reactivity and relative instability. Systems must be designed with appropriate ozone resistant materials.

Peroxide

Hydrogen peroxide (10% by volume solution) is used as a biocide in microelectronic-grade water systems because it rapidly degrades to water and oxygen. It is less effective and more expensive than chlorine.

Non-oxidizing biocides

Quats

In addition to their biocidal activity, quaternary ammonium compounds are effective surfactants which help remove biofilm from surfaces. However, it requires exhaustive rinsing to flush them out of water systems.

Formaldehyde

Formaldehyde has been used to sanitize pharmaceutical-grade water systems. It is relatively noncorrosive to stainless steel, but its effectiveness against biofilm is questionable and it is a toxic carcinogen.

Physical Treatments

Heat

Pharmaceutical Water-for-Injection systems use continuously recirculating hot water loops (greater than 80°C) to kill bacteria. *Periodic* hot water sanitization can also be used to destroy biofilm, but since this requires a temperature of 95°C for 100 minutes, it's usually not practical in an animal drinking water system.

Detecting and counting

Routine monitoring of bacterial levels is an essential part of monitoring the quality of laboratory animal drinking water. The classic way to enumerate bacteria in water is to do a *plate count* which is to spread a known volume of sample on the surface of a laboratory medium and count the number of visible colonies that develop after a period of time. However, plate counts may underestimate the total number of bacteria present in a watering system.

Water samples only collect free-floating bacteria which are either sloughed off of the biofilm or pass through from the incoming water supply. A low plate count doesn't mean that bacteria are not present because more than 99% of bacteria in water systems are attached to pipe surfaces. If the integrity of a mature biofilm hasn't been disrupted by recent flushing or sanitization, it may not slough off many cells into the drinking water, but it is still there.

Plate counts are based on the ability of bacteria in a sample to grow on a defined nutrient medium and form distinct colonies. Theoretically, a colony is derived from a single bacteria cell, but some underestimation is caused by clumps of bacteria that form only one colony. In purified water samples, plate counts may be

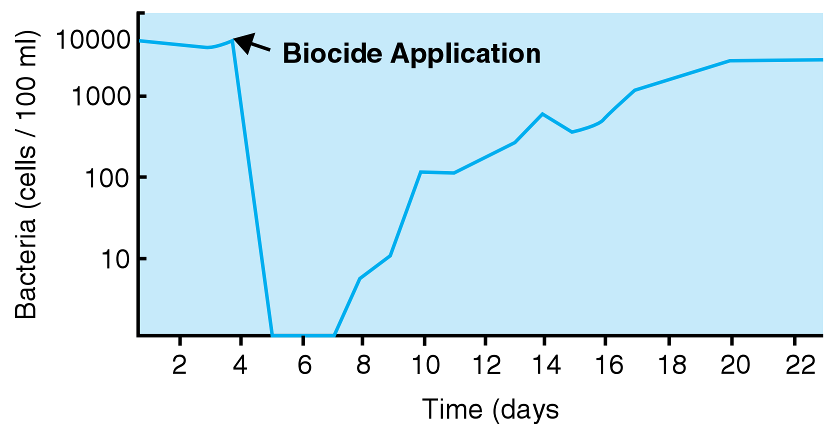

Figure 7.
Example of sanitization followed by biofilm recovery. Bacteria count samples were taken on a daily basis. (Mittelman 1986)

Biofilm recovery (regrowth)

Because they shield themselves in slime, bacteria associated with biofilms are much more difficult to kill than free-floating organisms. It is common to observe a rapid regrowth of biofilm immediately following chlorine sanitization as shown in Figure 7. Incomplete removal of the biofilm will allow it to quickly return to its equilibrium state, causing a rebound in total plate counts following sanitization.

Slime-producing organisms like *Pseudomonas* are less susceptible to biocides and may accumulate selectively in distribution systems that are sanitized.

Perhaps if sanitization could be automated, a watering system could be easily re-sanitized every few days before biofilm fully recovers.

low because the media is too rich to grow bacteria in a starved state.

Equilibrium biofilm thickness is dependent on water velocity and nutrients. As biofilms grow, single cells or "rafts" of cells are sloughed off during flushing. This results in random 'particle showers' of bacteria which can explain day-to-day fluctuations and occasional high bacteria count results.

What you can do

Bacteria constitute a very successful life form. In their evolution, they have developed successful strategies for survival which include attachment to surfaces and development of protective biofilms where they behave very differently than free-floating bacteria. Their successful strategies make it difficult to control biofilm growth in automated watering systems.

There is no "silver bullet" for controlling bacterial growth in automated watering systems. Unless a continuous chlorine level is allowed, it will take a combination strategy. But a multi-pronged strategy should result in a bacterial water quality which will satisfy the needs of animal research.

Questions or Comments

If you have any questions or comments on biofilms or if you'd like a copy of the complete Biofilm report, including references, please contact:

Edstrom Industries, Inc.
(800)-558-5913

e-mail: lab@edstrom.com

The biofilm assault...

We purify water to remove nutrients and ask "How could anything live in it?"

Biofilm bacteria use their polymer web to concentrate nutrients. They can live on nutrient levels we can't even measure.

We flush water lines trying to scour them off the pipes.

They cement themselves to surfaces with their sticky polymers under the laminar layer where shear forces are too weak to remove them.

We smooth the inside surfaces of fittings so they can't take shelter in crevices and crannies.

It doesn't matter. They will attach themselves speedily and inevitably anyway.

We sanitize piping with chlorine. They shield themselves in slime.

...and how to fight back.

Purify anyway!

It will limit nutrients somewhat, especially nutrients for microbes like sulfate-reducing bacteria which cause corrosion problems. Nutrient-poor RO water will support less bacteria than tap water. This means a thinner biofilm. Besides, the animals will be getting better quality drinking water.

Flush anyway!

Periodic flushing will minimize the thickness of the biofilm. Thinner biofilm have less anaerobic zones and sanitizing chemicals will have a shorter distance to diffuse through to reach the pipe surface.

Minimize crevices anyway!

Maybe surface finish doesn't matter much as far as total biofilm accumulation, but eliminating large crevices (like o-ring joints) will eliminate deep pockets of biofilm which are harder to sanitize and are more corrosive. Also, electropolishing will aid in resisting corrosion.

Sanitize anyway!

If biofilm recovers within 3 days after sanitization, knock it back down by sanitizing every 1-2 days. This could be done by automating chlorine or ozone sanitization.