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Development and structure of drinking water biofilms and techniques for their study

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1. SUMMARY

Drinking water systems are known to harbour biofilms, even though these environments are oligotrophic and often contain a disinfectant. Control of these biofilms is important for aesthetic and regulatory reasons. Study of full-scale systems has pointed to several factors controlling biofilm growth, but cause-and-effect relationships can only be established in controlled reactors. Using laboratory and pilot distribution systems, along with a variety of bacterial detection techniques, insights have been gained on the structure and behaviour of biofilms in these environments. Chlorinated biofilms differ in structure from non-chlorinated biofilms, but often the number of cells is similar. The number and level of cellular activity is dependent on the predominant carbon source. There is an interaction between carbon sources, the biofilm and the type of pipe material, which complicates the ability to predict biofilm growth. Humic substances, which are known to sorb to surfaces, appear to be a usable carbon source for biofilms. The finding offers an explanation for many of the puzzling observations in full scale and laboratory studies on oligotrophic biofilm growth. Pathogens can persist in these environments as well. Detection requires methods that do not require culturing.

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2. INTRODUCTION

A drinking water distribution system would appear to be a hostile environment for microbial growth. The low organic carbon concentrations, presence of a disinfectant, generally low temperatures, and flow regimes do not seem conducive to bacterial proliferation. In spite of these conditions, the growth and persistence of bacteria in distribution systems is well documented. The bacteria typically grow on surfaces, including the pipe walls and sediments. Sediment accumulations are more probable in reservoirs and storage tanks, while pipe wall growth appears to predominate in distribution systems. In both cases, the bacteria attach themselves to the surfaces and persist as biofilms. Biofilm accumulation in drinking water distribution systems has been of interest to the water industry for a number of reasons. Colonization by organisms such as *Actinomycetes* or fungi may result in taste and odor problems (Burman 1965, 1973; Olson 1982). Iron bacteria may grow on ferrous metal surfaces (Ridgway *et al.* 1981) and result in the presence of iron particulates in finished water (Victoreen 1974). Corrosion of distribution system pipe materials may be enhanced due to the presence of bacterial biofilms (Lee *et al.* 1980). There is the potential for coliform and heterotrophic regrowth, where biofilm organisms are shed into the water and place a utility in violation of regulations. Another problem is the potential for nitrification in systems that use chloramines as disinfectants (Wolfe *et al.* 1990). An area of increasing concern is the ability for frank and opportunistic pathogens to colonize pipe surfaces, result-

ing in cases of gastroenteritis or other nosocomial infections (United States Environmental Protection Agency 1992). Nutrients tend to adsorb to surfaces, making them more available to surface-attached cells (Fletcher and Marshall 1982). Attachment has been shown to be a major factor in resistance to disinfection (LeChevallier *et al.* 1988), potentially protecting attached cells from a chlorine residual. Attached cells could serve as a reservoir for subsequent spread through the system, following detachment or biofilm sloughing caused by changes in nutrient, disinfectant, or hydrodynamic status. Pipe wall biofilms and sediments are rarely examined in routine microbial assessment of a distribution system. The tendency is to attempt to infer biofilm presence and activity by using suspended cell information; this approach is tenuous at best unless the data are obtained from carefully controlled experiments.

To adequately determine potential cause-and-effect relationships between the environment and biofilms grown under low nutrient conditions, it is necessary to use experimental systems so that variables can be controlled. Full scale systems can be studied and correlations made, but there are often multiple effectors that result in a response. Therefore, it is necessary to use appropriate physical models to simulate distribution system processes. An overview of some models is given below, with more details available in Camper (1996).

3. BIOFILM REACTORS

Laboratory and pilot-scale physical models are being used to simulate the impact of various water quality parameters on biofilm growth in distribution systems. Although these models typically cannot simulate actual flow directions and pressure gradients, they can be operated to represent temperatures, hydraulic residence times, shear stresses, substrate concentrations, disinfectant types and concentrations, corrosion control strategies, and materials found in full-scale distribution systems. Physical models provide researchers and utilities with the opportunity to view processes occurring on pipe surfaces; this is difficult if not impossible in full-scale systems.

The laboratory annular reactor has been used extensively in the Center for Biofilm Engineering to assess biofilm formation and response to substrates, shear stress, disinfectants, addition of inert particles, and other parameters. The reactor consists of a stationary outer cylinder and a rotating inner cylinder with four draft tubes to provide radial and axial mixing and a calculated shear stress (Fig. 1). Twenty slides of any material (ductile iron, mild steel, cement coated steel, plastics, etc.) are flush mounted on the inner cylinder. Access ports at the top of the reactor allow for the input of substrates, dilution water, and other additives. Residence time is controlled by the composite volumetric flow rate into the reactor. Because the reactors are well mixed, they can be modelled as continuously stirred tank reactors (CSTRs). If residence time is sufficiently short (less than the replication time of the organisms in the bulk), the mass balance on cells across the reactor will reflect only biofilm processes. The outer cylinder of the reactor is encased in a water tight compartment that can be used for temperature control.

The last two reactors discussed here are classified as pilot systems. Pilot-scale distribution facilities are used to estimate the influence of water quality parameters on full-scale distribution system performance. Pilot facilities are used when access to the full scale system is limited, when the parameters to be tested may significantly alter the quality of water delivered to the consumer, or if more tightly controlled conditions are required (flow, water quality, pipe composition, etc.). Ideally, the reactors should be relatively compact, easily controlled, have minimal water demand, and be inexpensive to construct, maintain, and operate.

The pipe annular reactor used as pilot reactors are a modification of the laboratory system. The major difference is that the outer cylinder is a section of 152 mm (6″) pipe of industrial relevance. The reactors can be operated independently or in series to simulate increasing hydraulic residence time. As with the laboratory systems, shear stress is independent of residence time and the reactor can be modeled as a CSTR. Temperature is controlled by housing the reactors in a heated polyethylene 'tent'. Mild steel reactors have been used in parallel with a pipe loop system to assess the importance of residence time, temperature, carbon substrate concentration, chlorine, and monochloramine on coliform-containing biofilms grown under conditions relevant to drinking water distribution systems. A large number of ductile iron reactors have also been used in house and at several utilities.

We have also constructed a five loop pipe loop system at the City of Bozeman Drinking Water Treatment Plant. Each loop is 40 ft long and contains 20 sections of 102-mm (4″) mild steel pipe in 2-ft lengths. Sections have four flush-mounted coupons of the same material. A return line connects the test length to an insulated drum with a heater or cooling coil. The residence time in the loop is controlled by an overflow on the drum. Water is pumped from the drum through a section of return line, past a flow meter, and into the test section. There is a high recycle rate on the loops, allowing for modeling as a CSTR. Each loop also contains a short dead end section with flush-mounted coupons. As with the annular reactors, the loops can be operated in series or parallel, depending on experimental design.

4. BIOFILM MEASUREMENT TECHNIQUES

The methods used to study and describe biofilms are dependent on the information sought. In many cases, regulatory concerns dictate that simple, reliable methods like plate counts be used. For the research microbiologist, the interest

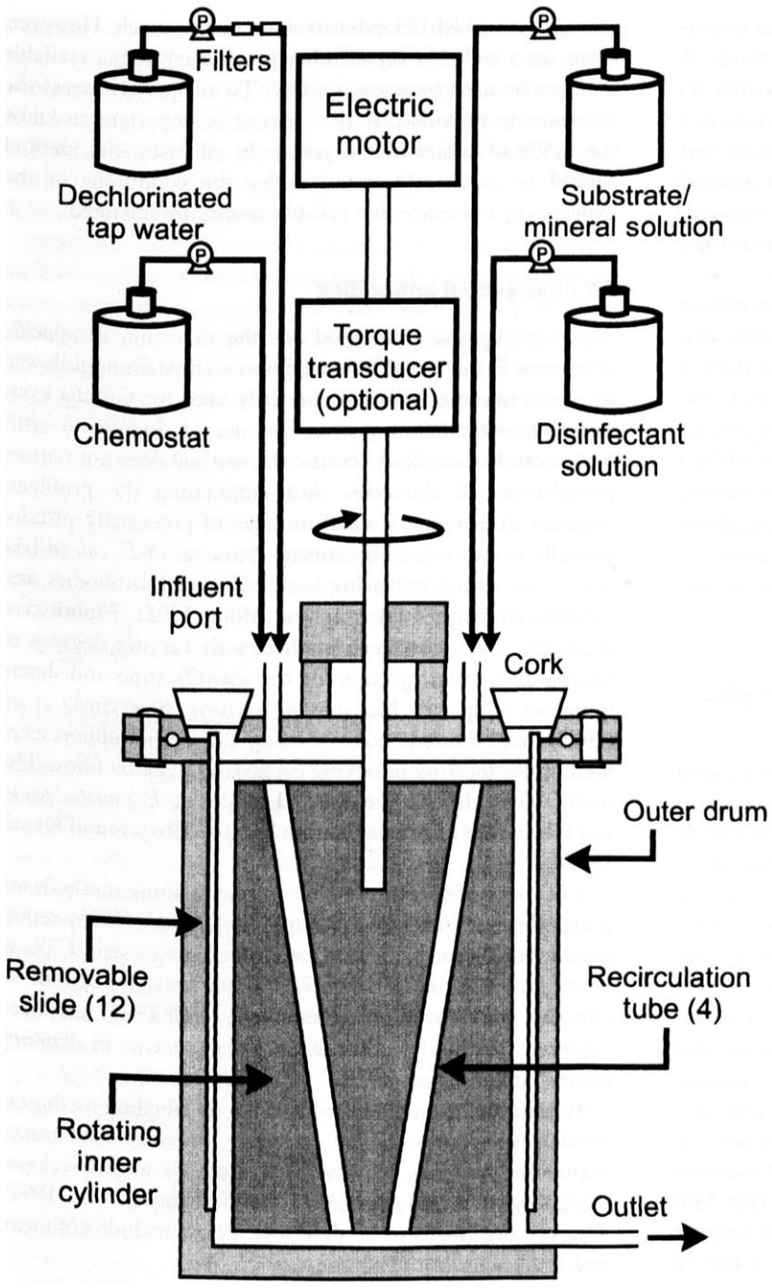

Fig. 1 Schematic of the annular reactor.

may be a sophisticated description of the microbial ecology and spatial distribution of cells in biofilms. The second approach will rely more heavily on technologies emerging from the fields of molecular biology and microbial ecology.

Traditionally, environmental bacteria and biofilms have been studied by culture-dependent methods or direct microscopy. Both approaches have limitations. It is now well established that cultural methods underestimate the numbers and diversity of environmental bacteria. Amann *et al.* (1995) suggested that cultural techniques fail to enumerate two classes of organisms: (1) known and previously cultured species that have entered a state in which they can no longer be cultured, or not by the method being used; and (2) novel species for which no suitable culturing method has been developed. They noted, however, that nature is capable of culturing all species. While direct microscopy detects greater numbers of cells than culturing, species generally can not be identified under the microscope based on morphology alone

(Trebesius *et al.* 1994). Increasingly, environmental microorganisms are detected by so-called 'molecular' methods. A broad interpretation of the term includes methods that do not require bacterial growth on selective or non-selective media. A narrow interpretation is limited to methods that target nucleic acids or proteins. Either whole cells or purified cell extracts of DNA, RNA, or proteins can be analysed. Whole cells can be extracted and/or concentrated from samples, or analysed *in situ*.

In-situ detection of undisturbed whole cells, especially in biofilms, is essential in order to determine the spatial distribution of species. A list of molecular tools includes nucleic acid and protein stains, physiological indicators, labelled antibodies, nucleic acid amplification, nucleic acid probes, and gel electrophoresis of nucleic acids and proteins. An integrated molecular approach to studying planktonic or biofilm bacteria would include methods for determining their identity, abundance, and physiological status in single or parallel assays.

An overview of some of the methods used at the Center for Biofilm Engineering (CBE) are described below.

4.1 Nucleic acid stains and fluorescent physiological indicators

Many investigations at the CBE require information on total cells present in a sample. The two most commonly used stains are acridine orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI). Both are DNA stains, although in some instances AO has been used to indicate activity based on the observation that single-stranded RNA bound by AO fluoresces red/orange, while double-stranded DNA fluoresces green. Therefore, red/orange fluorescence has been considered an indication of high RNA content and high metabolic activity. McFeters *et al.* (1991) found that AO fluorescence depended on several factors including staining and fixation procedures.

Physiological stains are also often used in conjunction with total cell stains to determine the active portion of a population. Yu and McFeters (1994) reported that 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and rhodamine 123 (Rh 123) were effective indicators of metabolically active cells in biofilms, but Morin and Camper (1997) reported a lack of sensitivity with CTC in chlorinated biofilms.

Nucleic acid stains and physiological fluorochromes do not identify species but may be used in conjunction with specific antibodies or nucleic acid probes, provided the emission spectra of the different fluorochromes are dissimilar enough to allow separate detection of each component. For example, Hicks *et al.* (1992) combined DAPI for total cell counts with a specific oligonucleotide probe labeled with tetramethyl rhodamine isothiocyanate (TRITC). Pyle *et al.* (1995) used CTC and a fluorescent antibody for *Escherichia coli* O157:H7 in the same assay to detect actively respiring cells.

We have used AO, DAPI, cyanoditolyl tetrazolium chloride (CTC) and Rh123 extensively in our research. However, there are a wide variety of other proprietary stains available that can be used to assess activity. To adequately assess the information provided by the stain, it is important to know the mode of action of the probe. In all cases, the method should be rigorously tested under the conditions of the experiment to ensure that reliable results are obtained.

4.2 Fluorescent antibodies

A potentially useful method for the detection of specific organisms is the use of immunofluorescent staining followed by direct microscopy, as is currently used for *Giardia* cysts and *Cryptosporidium* oocysts. The use of fluorescent antibodies can be beneficial because the method does not require preculturing of the cells, thus eliminating the problems inherent in growing a small number of potentially physiologically compromised organisms. Survival of *E. coli* in lake water was monitored using both polyclonal antibodies and cultural techniques (Brettar and Höfle 1992). Fluorescent antibodies have also been applied with varying degrees of success to biofilm systems for the identification and determination of specific locations of bacteria. Szwerinski *et al.* (1985) had limited success using an immunofluorescent method for locating nitrifying bacteria in thick biofilms. The method has also been employed to detect *Legionella pneumophila* in thin mixed population biofilms (Rogers and Keevil 1992).

Variations of the immunofluorescent staining methods are under development. For example, Pyle *et al.* (1995) report combining a specific antibody with the activity stain CTC to detect active *E. coli* O157:H7. Alternatively, the use of an enzyme-linked immunosorbent assay (ELISA) has been explored for the detection of *Enterobacteriaceae* in drinking water (Hubner *et al.* 1992).

We have used the fluorescent antibody labelling method to identify specific organisms in intact and dispersed mixed population biofilms. However, the methods were developed using defined mixed population biofilms (Stewart *et al.* 1997). The key organisms to be described below include coliforms and frank waterborne pathogens.

4.3 Molecular probes in biofilm research

There have been few published reports to date in which molecular probes have been used to study biofilms, and fewer still involving drinking water biofilms specifically. Manz *et al.* (1993) inserted glass slides into a Robbins device installed in a drinking water distribution system about 30 km (and 60 h) from the waterworks. After 3–8 weeks some single cells and microcolonies were visible on the slides with phase contrast microscopy. Attached cells were hybridized directly on the glass slides. Planktonic cells were concentrated by cen-

trifugation and spotted onto slides. Both kinds of samples were hybridized with oligonucleotide probes and subsequently stained with DAPI. The universal probe (EUB 338) was detected in about 70% of the attached cells but only about 40% of the planktonic cells, based on DAPI total counts. This disparity was considered evidence of higher rRNA content of attached cells.

Szewzyk *et al.* (1994) used a gram positive bacterium (isolate B-4) capable of using benzoate as a sole carbon and energy source to produce a biofilm on glass slides in a laboratory reactor fed only with benzoate. Once the biofilm was established, it was inoculated with an *E. coli* strain incapable of using benzoate. *In-situ* hybridization of the biofilm with differently labelled probes for both species at different times following incubation revealed that the *E. coli* progressively colonized the B-4 biofilm and was contaminating the effluent by shedding cells. Their study also suggested that oligonucleotide probes can penetrate biofilms of a thickness typically encountered under oligotrophic conditions.

We have been using universal probes to provide insight on the distribution of cells in intact biofilms.

4.4 Other activity measurements

Textbooks on aquatic ecology will often reference the use of radiolabelled thymidine to determine cell activity. The thymidine is ideally incorporated into the DNA of actively growing cells which can then be detected using a liquid scintillation counter or other detection methods. A disadvantage of this method is that the cell must be producing DNA. In oligotrophic environments, it is possible that cells are actively respiring, but not necessarily replicating. In these instances, the radiolabelled thymidine method will underestimate the activity of the population.

An alternative to thymidine is the use of radiolabeled leucine. When cells are exposed to this amino acid in appropriate concentrations, it is taken up and used analogically for protein production with no net use as an energy source. Therefore, the radiolabel can be used to 'tag' metabolically active cells.

One method for assessing activity of leucine labeled cells is with microautoradiography. The method has been used successfully to assess activity of natural bacteria in aquatic systems (Meyer-Reil 1978; Tabor and Neihof 1982) and was adapted for similar research done at the Center for Biofilm Engineering. If dispersed biofilm is used, the cells are exposed to a known concentration of labelled leucine for a predetermined amount of time. The bacteria are then filtered onto a 0.2 μ pore size polycarbonate membrane. The retained cells are then permeabilized and washed to remove any unincorporated isotope. The filters are placed on a microscope slide coated with photographic emulsion and incubated in the dark to expose the silver grains. Emitted beta radiation causes exposure of the emulsion with formation of silver grains. The emulsion on the slides is developed, treated with DNA stain DAPI and the filters removed. The DAPI stained bacteria that were on the filter are retained in the emulsion. Both light microscopy for detection of silver grains and epifluorescence microscopy for detection of the DAPI stained cells on the emulsion surface are used to quantify the number of total cells as well as bacteria in the proximity of exposed silver grains. These are compared to give the percentage of active cells. The method can also be used for intact biofilms. In this case, the biofilm is incubated in the leucine, the excess washed away, and the biofilm covered with the photographic emulsion.

4.5 Epifluorescence and confocal microscopy

All of the above described methods rely on direct microscopy for detection of individual cells. The microscopic instrumentation used to detect whole-cell stains and probes is as important as the staining or hybridization protocols (Amann *et al.* 1995). Most detection of whole-cell probes has been done using epifluorescence microscopy, in which a fluorochrome is excited at a wavelength near its absorbance maximum and emits visible light of a longer wavelength. Filters are used to narrow the excitation and emission spectra. Using a combination of filters, different fluorochromes can be detected on the same slide. For example, Ramsing *et al.* (1993) used three different filters sets to detect cells stained with DAPI and bound by either a fluorescein isothiocyanate (FITC)- or a (TRITC)-labelled probe. In any case, information can be collected using image analysis software and the data analysed to determine size, number, and distribution of cells.

Confocal microscopy has some advantages over epifluorescence microscopy, but it is also limited to excitation at specific wavelengths. For example, a Kr-Ar laser excites at 488, 568, and 647 nm (our laboratory). Confocal microscopes are linked to computer image analysis capabilities. Images obtained from the separate excitation wavelengths can be compiled and enhanced. In addition, objects on the slide can be optically sectioned in the z-plane, which means that thick objects (like biofilms), that can not be brought into focus in their entirety by epifluorescence, can be visualized after reconstruction of the individual thin-section confocal images. Amann *et al.* (1996) used a confocal microscope to detect three probes labelled with different fluorochromes on the same slide.

Both conventional fluorescent and confocal microscopes have been used extensively by our group for observation of planktonic bacteria, disrupted biofilms, and intact biofilms.

4.6 Polymerase chain reaction methods

The polymerase chain reaction (PCR) has become a staple of research laboratories in all fields of biology. In water research

PCR has been useful for detecting pathogenic bacteria (Bej *et al.* 1990). Specific PCR primer sequences for a wide range of pathogens have been published or can be designed from gene sequences obtained from DNA sequence databases (Olsvik and Strockbine 1993; Bej *et al.* 1994; Dorsch *et al.* 1994). PCR has generally been used as an indicator of bacterial DNA presence-absence. Semi-quantitative enumeration of the bacterial cells in a sample has been based on the observation that the intensity of a PCR product in an agarose gel is related to the amount of initial template. Known concentrations of template DNA can be serially diluted before PCR is performed in order to produce a series of bands of decreasing intensity in a gel, which is essentially a standard curve for estimating the initial template or number of bacterial cells in actual samples. Alternatively, initial template has been estimated by co-amplifying internal standards along with the target sequence (competitive PCR) (Reilly and Attwood 1998), or by constructing standard curves for PCR products detected by high performance liquid chromatography (Marlowe *et al.* 1997).

PCR does not indicate the physiological state of bacterial cells in a sample. DNA from active, viable, non-viable, or dead cells, as well as extracellular DNA can be amplified. This has been both an advantage and a disadvantage of the method. PCR has been used to indicate a health risk that was not detected by culturing, for example, when pathogens have become viable but unable to be cultured (Islam *et al.* 1993). On the other hand, the ability of PCR to amplify extracellular DNA, or DNA from dead or non-viable cells may create the potential for false positives; e.g., when cells incapable of causing disease are detected by PCR.

5. FACTORS CONTROLLING DRINKING WATER BIOFILM FORMATION

An indication of the substantial evidence for interactions is given in the literature about regrowth in distribution systems. Key conditions believed to be conducive to the proliferation of both coliforms and heterotrophs in distribution biofilms have been: (1) temperature effects, especially warm water conditions; (2) the amount of usable carbon for substrate; (3) inefficiencies in the removal/disinfection of organisms in treatment; (4) the presence of corrosion products in distribution systems; (5) disinfectant dose/type; and (6) distribution system hydrodynamics.

The variables that have received the most emphasis in prior publications are usable carbon concentrations as AOC or BDOC, hydraulic residence time, and disinfectant. Potential threshold concentrations of assimilable organic carbon have been set at 10 mg C/L for heterotrophs (van der Kooij 1992) and 50 mg C/L for coliforms (LeChevallier *et al.* 1991). Servais *et al.* (1991) have associated biological stability of water with a BDOC level of 0.2 mg/L. Hydraulic residence time is related to carbon, as concentrations of organic carbon capable of assimilation have been shown to be higher at the plant than at increased travel times in the distribution system (LeChevallier *et al.* 1987, 1991; van der Kooij 1992). Decreases in AOC with water age has been noted (van der Wende *et al.* 1988); high AOC levels were associated with heterotrophic regrowth. However, there are instances where there has not been a clear cut correlation between AOC/BDOC and biofilm development. Pilot experiments at Montana State showed a weak correlation between biofilm and influent AOC concentrations, but no correlation with the concentration of AOC in the reactors (Camper 1996). Conflicting information was also obtained in field studies; some systems with AOC levels less than 100 mg/L experienced regrowth while others with average values less than 100 mg/L also had regrowth events (LeChevallier *et al.* 1996a).

One reason why AOC levels may not be directly correlated with regrowth in distribution systems is that bacterial growth is believed to be balanced with the decay of disinfectant. Since disinfectant concentrations and AOC are often highest at the plant, the growth of organisms may be limited until the disinfectant has decayed sufficiently. Intuitively, elevated levels of chlorine should control regrowth, but this is often not the case (Centers for Disease Control 1985; LeChevallier *et al.* 1987). Historically, utilities have relied on increased disinfectant doses to control regrowth events, with mixed success. It is known that biofilm organisms are less susceptible to disinfectants than suspended cells, especially if they are present on reactive iron surfaces (LeChevallier *et al.* 1990; Chen *et al.* 1993). LeChevallier *et al.* (1993) described results where increased corrosion rates decreased the efficacy of free chlorine on biofilm bacteria.

Another issue related to regrowth and loss of disinfectant is the presence of unlined iron-containing pipe materials in the distribution system. Many older distribution systems contain unlined cast or ductile iron pipes frequently characterized by accumulations of corrosion products or tubercles that can nearly occlude the pipe diameter. Iron surfaces are particularly reactive and contribute the deterioration of water quality through a variety of processes. It has been noted that iron surfaces are prone to substantial microbial colonization and have been implicated as a key component in microbial regrowth in distribution systems (Camper 1996; Camper *et al.* 1996; LeChevallier *et al.* 1993, 1996b). Utilities with a large proportion of unlined ferrous metal pipes that have had coliform regrowth problems are Vancouver British Columbia, Boston, and Washington, DC; and a utility survey has shown a positive relationship between the number of miles of unlined metal pipes and coliform occurrences (LeChevallier *et al.* 1996b). In pilot distribution systems with varied materials, organisms growing on ferrous metal surfaces were less susceptible to free chlorine than when present on other materials (LeChevallier *et al.* 1987, 1990), presumably because the

metal exerts a chlorine demand. The ability for reduced iron to react with disinfectants has been documented (Knocke 1988; Knocke *et al.* 1994; Vasconcelos *et al.* 1996). The importance of surface material on organism numbers, including coliforms, was substantiated in our research. Even in the absence of a disinfectant, mild steel surfaces were consistently colonized by nearly 10-fold more heterotrophs and two to 10-fold more coliforms than polycarbonate when the reactors were operated under the same conditions. The impact extended to the effluent bacterial concentrations as well; elevated counts were found in reactors with mild steel even though only 10% of the reactor surface area is encompassed by the slides. Further, the presence of mild steel affected population densities on polycarbonate surfaces in the same reactor. These surfaces supported the same numbers of bacteria as seen on the steel itself. It therefore appears that the mild steel surface is capable of enhancing biofilm growth, rather than only protecting it from the action of a disinfectant (Camper *et al.* 1996).

It is also known that reactions on ferrous metal surfaces are affected by corrosion control methods (pH adjustment and phosphate addition). Laboratory and pilot work at Montana State University has shown that the number of biofilm bacteria is directly related to the mass of corrosion products present; reduction in biofilm and corrosion product accumulation can be achieved by corrosion control schemes or altering the disinfectant to produce less corrosion. There are reports where implementation of corrosion control in full-scale distribution systems apparently has lead to mitigation of coliform regrowth (Martin *et al.* 1982; Hudson *et al.* 1983; Schreppel *et al.* 1997). The mechanism for the ability for corrosion control measures to influence biofilm numbers is not clear cut. LeChevallier *et al.* (1993) showed that corrosion control reduced biofilm numbers but attributed the response to increased chlorine efficacy due to decreased corrosion rates. However, we have noted that at near neutral pH, the presence of low levels of disinfectant actually increases biofilm density on ductile or steel surfaces, presumably because corrosion was enhanced and the disinfectant consumed at the surface (Camper 1996, unpublished data). Martin *et al.* (1982) noted an interaction between pH and chlorine by showing that an elevated pH of 9 reduced bacterial counts substantially. Since chlorine is less effective at high pH, it may be inferred that in this case the reduction in organism numbers was the result of corrosion control and not increased disinfection efficacy.

Another reactive characteristic of iron corrosion products (iron oxides) is that they have a large potential for the adsorption of natural organic matter (NOM; Parfitt *et al.* 1977; McCarthy *et al.* 1993; Zhou *et al.* 1994). Distribution system deposits have been shown to include a variety of iron oxides as well as manganese, carbonate species, and silica (Robinson 1981; Carlson and Schwertman 1987). Under abiotic conditions, humic material is irreversibly held on the surface of iron oxides (Gu *et al.* 1994, 1996). In fact, this property has been used to develop a technique for the removal of NOM from water by coating sand particles used in slow sand filter beds with iron oxides (McMeen and Benjamin 1997). Circumstantial evidence indicates that the bound organic matter is potentially available for biofilm bacteria when these same investigators mentioned that the iron oxide-coated olivine used in their filtration studies continued to remove NOM for a 16-month time period; they suggested that the adsorption sites were being 'bioregenerated'. There is evidence that corrosion products removed from distribution systems are capable of supporting bacterial growth with no other added carbon (Martin *et al.* 1982). Experiments to examine the potential for corrosion products to support microbial growth were done where corrosion products were removed from ductile iron reactors, packed into columns, and fed a sterile humic acid solution. The corrosion products were then placed in sterile flasks and a population of suspended bacteria in biologically active carbon column effluent added. There was a two log increase in bacterial numbers over a three day time period, again providing circumstantial evidence that the adsorbed humic material was available for microbial metabolism.

Humic substances is the general term for both humic and fulvic acids. The NOM from surface waters has been classed into the general constituents (Malcolm 1991) which indicate that humic substances can make up approximately 50–75% of the NOM in surface waters. These concentrations are less in groundwater. NOM is responsible for chlorine demand in the bulk fluid and is a precursor for disinfection by-products. Humic substances are generally considered to be poorly biodegradable, because of their large molecular size. However, Namkung and Rittmann (1987) have shown that humic substances are in fact biodegradable. Volk *et al.* (1997) have shown that biofilm bacteria in their BDOC columns are capable of using humic substances; these substances are then considered a component of the BDOC. To address this concept, a series of experiments was completed where humic materials were the sole carbon and energy source for biofilm bacteria. The dilution water was biologically treated tap water. Compared to control reactors fed only biologically treated water, there was a two log increase in cell numbers. This is again evidence for biological use of humic substances.

The confusing aspect of NOM utilization by organisms is the relative recalcitrance of the material in the bulk phase. Therefore, it is probable that there must be another mechanism that increases the bioavailabilty of the humic substances. We believe that the humic substances become utilizable when they are adsorbed to surfaces. These molecules can then undergo a conformational change and expose the utilizable attached functional groups. The immobilization on the surface is also likely to permit the cells to use exoenzymes to attack the bonds between the bound amino acids, sugars, etc.

and the backbone of the humic molecule. There is strong evidence to suggest that the adsorption of humic substances allows them to become available for biofilm use. When an assessment of the growth rates of biofilm bacteria grown on humic materials was made, it was found that the growth rate was independent of the added humic carbon concentration (zero order kinetics). This is because of the large amount of humic materials bound in the biofilm; supplementation of additional humic material did not influence the growth rate. There was also visual evidence that the humic material was adsorbed, as these biofilms were a characteristic brown color. This mechanism has profound implications for the water industry. If adsorbed humic materials are utilizable, the prediction of bacterial proliferation using the assimilable organic carbon and biodegradable organic carbon analyses may need to be readdressed, since these methods are believed to measure the quantities of readily available organic carbon in solution. In many conversations with researchers and utility personnel, there has been the feeling that levels of AOC and BDOC in the distribution system may not be associated with bacterial growth, particularly in the presence of ferrous materials. Since iron oxides have such a propensity for adsorption of NOM, the interaction of organics, iron oxides, and bacteria may help explain many of the observations on regrowth in distribution systems. In subsequent experiments using humic material as the sole carbon and energy source, there was no correlation between increased concentrations of added humic material and bacterial production. In these same experiments, it was noted that the humic materials were adsorbed to the biofilm as well as to the surface.

6. STRUCTURE OF DRINKING WATER BIOFILMS

6.1 General morphology

The structure of biofilms from drinking water distribution systems is often very difficult to study because of the large amounts of detritus, corrosion products, and other inorganic matter. These compounds severely limit the number of techniques that can be used. For example, direct microscopy is often impossible because of autofluorescence, inability of stains to penetrate the deposit, and blocking of light by the inorganic matter. In most instances, the information on the structure of biofilms grown under low nutrient environments has come from laboratory experiments where some of the interfering substances can be eliminated from the system.

We have noticed that disinfection has a profound influence on the general structure of the natural mixed-population biofilms. In control reactors, the biofilm cells are distributed as clumps and single cells across the entire surface. Initially, the majority of the population is present as single cells in direct contact with the substratum, but a few clumps up to 20 mm in height and strings of cells are present. At this stage of development, the surface has approximately 60% surface coverage (Camper *et al.* 1996). As the biofilm ages, the number of cells per unit surface area does not change appreciably, but biofilm thickness increases. An interesting observation is that the lower layer of the biofilm is separated from the substratum by a space several microns thick. Bacteria are also found in association with small pieces inorganic matter in the biofilm. When activity staining or microautoradiography after exposure to tritiated leucine is done, we find that between 60% and 80% of the cells are metabolically active. When a EUB probe is used, approximately 80% produce a signal.

Intact chlorinated biofilms are physically different in appearance. The cells tend to remain only in clumps, while the individual organisms disappear. The biofilm is more patchy and the cells are approximately 50% smaller than in control reactors. There is also a shift in morphology from longer rod shaped cells to more rounded organisms. When plate counts are done, there is also change in dominant colony morphology. The chlorinated biofilm is much more difficult to image with the EUB probe. When microautoradiography is done after exposure to tritiated leucine, nearly all of the cells in clumps are active, while there are variable responses in the single cells. The same percentage of active cells is obtained if the biofilm is dispersed before exposure to the leucine. Since this procedure is much easier to perform, we feel confident that the use of dispersed biofilm samples to determine activity using labelled leucine is appropriate to obtain quantitative information.

This method has been used to assess the differences in activity between parallel control and chlorinated reactors. When compared to the control biofilms, the activity in the chlorinated systems is lower, but the difference depends upon the substrates the cells are using. For example, if low concentrations of a mixture of amino acids is fed to both reactors, 80% of the control cells are active vs. 60% for the chlorinated system. When humic substances are the sole carbon source, 60% of the cells in the control biofilm are respiring while this is reduced to 20% in the chlorinated system. When radiolabelled leucine is used to measure population growth rates, it appears that the rate is higher for chlorinated biofilm. We hypothesize that the chlorinated cells are incorporating leucine to repair damage due to disinfection.

6.2 Importance of particles

As stated above, biofilms found in distribution systems and other natural aquatic environments contain inorganic particulate material. There has been very little work published on the importance of the particles, with usual references being to generalized responses to known compounds added to defined population biofilms. For example, Srinivasan *et al.* (1995) describe experiments where the addition of kaolin or

calcium carbonate decrease the efficacy of a disinfectant against a two-species biofilm. In contrast, we have chosen to investigate the manner in which particles influence mixed population biofilms grown in low nutrient environments.

In the case of corrosion products, it was shown that the incorporation of iron oxides into biofilms grown on plastics produced the same elevated counts as seen when the biofilms were grown directly on mild steel surfaces. This observation suggests that inert pipe surfaces exposed to water carrying corrosion products from upstream pipes or to water high in iron can be at risk for elevated microbial counts (Camper 1996).

Other work has shown that colonized granular activated carbon particles become incorporated into biofilms and may act as a carrier for the particle associated bacteria. In general particles with an average size of 8 μ were more likely to be retained than smaller particles. Disinfection assays on biofilms containing particles were performed with chlorine (0.5 mg/l) and monochloramine (1 mg/l). The disinfectants had limited influence on the bacteria, even though there were large numbers of carbon particles (approximately equivalent to the number of bacteria). Direct microscopic observation after staining with DAPI and the activity stain Rh 123 showed: (1) there was no evidence of increased numbers or enhanced activity of bacteria next to carbon particles in reactors without disinfectant; and (2) there was no preferential retention of total or active bacteria associated with carbon particles when disinfectant was added (Morin and Camper 1997).

Similar experiments were done with carbon particles colonized with the indicator organism *Klebsiella pneumoniae* introduced to mixed population biofilms. Biofilms were monitored using total cell counts and specific monoclonal antibody staining for the indicator organism. The particles acted as a mode of transmission for the coliform from the bulk fluid to the biofilm. When chlorine (0.5 mg/l) was then applied, there was a loss of 90% of the indicator bacteria and 80% of the carbon particles (Morin *et al.* 1996).

6.3 Monitoring target organisms in biofilms

Often it is desirable to monitor discrete members of the biofilm to determine specific responses to environmental conditions. The initial work was done with mixed populations colonized with coliforms, particularly *Kleb. pneumoniae*. Direct microscopic evidence after staining biofilms with a monoclonal antibody showed that the coliform was generally present as discrete microcolonies rather than single cells (Camper *et al.* 1996). When transported by carbon particles to the surface, many remained as single cells rather than replicating into microcolonies (Morin *et al.* 1996).

Fluorescent antibodies were also used to monitor frank pathogens (*Salmonella typhimurium* and *E. coli* O157:H7) introduced to mixed population biofilms grown under conditions relevant to drinking water distribution systems. Low levels of disinfectant were also applied. *Salmonella* cells were found to attach and persist in biofilms for over 50 days, even in the presence of chlorine. The cells in the biofilm became impossible to culture on selective media over time, but could be resuscitated if the biofilm was incubated in a non-selective medium. Direct observation of the biofilms revealed no microcolonies. In contrast, *E. coli* O157:H7 was eliminated within 2 weeks of introduction to the reactors regardless of the presence of chlorine. Both of the tested organisms showed a change in physiology over time as evidenced by a reduction in colony counts on selective media (Camper *et al.* 1998).

We have also seeded pathogenic bacteria into laboratory columns containing drinking water filter media (biologically activated carbon, BAC) and determined their persistence by various methods including culturing and PCR. Seed bacteria (*S. typhimurium*, *Aeromonas hydrophila*, *Kleb. pneumoniae*, and *E. coli* O157:H7) were grown in chemostats and enumerated on selective media. Following seeding, pathogens were virtually undetected when column effluents were plated on selective media. Explanations for this observation include injury to the cells causing them to enter an unculturable state, predation by other organisms, or attachment to the filter media. PCR was used to confirm the results obtained by culturing. A seed stock was prepared by adding 10^7 c.f.u. of each seed organism (based on counts on R2A medium) to 500 mL dechlorinated tapwater. This seed stock was recycled through the column for 24 h, after which dechlorinated tapwater flowed through the column and effluents were collected at various times. Template DNA was prepared by concentrating bacterial cells on polycarbonate membranes by filtration, followed by a direct DNA extraction from the membrane. PCR was performed on the seed solution before and after the 24 h of recycling, and on subsequent column effluents. Preliminary results obtained by comparing band intensities in the seed solution before and after recycling and in the column effluents indicates that bacterial cells have not remained in solution, and may have attached to the filter media or been consumed. Direct DNA extraction from the filter media has been used to detect attached cells.

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