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14. ABSTRACT
Pthogenic bacteria can be trapped and retained in biofilms in drinking wter distribution systems for times that exceed their persistence in the water. Consequently, biofilms can be used as a tool for interrogating a drinking water system to determine if an agent has been added. Basic and applied research has provided insight on how to detect pathogens, how they are retained in biofilms, the behavior of these organisms in biofilms, a prototype device for capturing pathogens, and models for predicting pathogen presence and potential growth.

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Project Statement

This multi-year project was focused on understanding the integration of pathogenic bacteria into biofilms in simulated drinking water systems. Because one of the major problems with monitoring pathogens in the environment revolves around adequate detection, there was considerable effort expended on developing methods to determine the presence and potential viability of the organisms. Parallel work was done in model reactor systems designed specifically to grow biofilms to trap the pathogens and the operational parameters that optimized pathogen capture. There was also basic research done to advance our understanding of how frank and opportunistic waterborne bacterial pathogens behave in biofilms. Finally, was a modeling component where the experimental data were integrated into a cellular automata model that has in turn been integrated into a flow model with the intent being to predict the behavior of pathogens in biofilm systems. To complete this work, a diverse and multidisciplinary group of faculty, staff, and students (graduate and undergraduate) from groups across campus and at different institutions participated in the research.

Methods Development

A significant issue related to the ability to track pathogens in environmental systems is reliable detection methods. Conventional plate counts are too slow and rely on selective media, and since pathogens are often present in low numbers, complex concentration methods may be required. It has also been observed that bacteria persist in biofilms even though they are no longer culturable, so other detection methods are required to assess the number of organisms.

Detecting Viable Cells

An issue of critical concern in microbiology is the ability to detect viable cells by methods other than culturing. Culturing can be selective, decrease diversity, and the results may not represent the natural community. Alternative approaches that reduce selectivity, decrease bias from sample storage and incubation, and reduce assay time are needed. These issues are particularly critical when considering the presence of pathogens in water systems.

The project resulted in the development of a new method for detecting cells with intact membranes (and hence most likely viable) using the novel chemical propidium monoazide (PMA). Treatment of bacterial populations comprising both live and dead cells resulted in the selective detection of only live cells using PCR-based diagnostic tools. PMA was shown to be superior to ethidium monoazide (EMA), a substance which was used before for the same purpose (Nocker and Camper, 2006; Hein et al., 2006; Nocker et al., 2006). The main advantage of PMA is its higher selectivity for dead cells. Detection of live cells was not affected by the treatment. PMA proved useful for molecular monitoring of disinfection efficacy using disinfectants which compromise the cell membrane (e.g. chlorine, benzalkonium chloride, heat, alcohol) (Nocker et al., 2007a). Molecular diagnostics was successfully limited to live cells, whereas killed cells were excluded from analysis. PMA was also evaluated for its use in microbial ecology and was shown to have the potential to change the genotypic profiles of bacterial communities due to removal of dead cells from analysis (Nocker et al., 2007b). The live-dead distinction method was filed as a patent. The patent was licensed to QIAGEN Inc. In addition, a review about PCR-

based microbial community profiling was written together with Dr. Mark Burr and Dr. Anne Camper.

This method was extended to microarray assays as part of a collaboration with Dr. Roland Brousseau and Dr. Luke Masson at the National Research Council of Canada, Biotechnology Research Institute, 6100 Ave. Royalmount, Montreal, Quebec, Canada. Microarrays were designed for an experiment using defined mixtures of 5 bacterial pathogens, *Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella typhimurium, Serratia marcescens,* and *Escherichia coli* 0157:H7. Experiments were conducted where one of the five organisms was subjected to treatment by isopropanol and a portion of the organisms were killed. The killed cells were then mixed with the other species and detected using the microarray. The results from the microarray corresponded well with information obtained with quantitative PCR and showed that there was a significant signal reduction when the cells were killed. This illustrates the usefulness of PMA treatment for molecular methods beyond PCR (Nocker et al., 2009).

Another approach has been the detection of live vs dead cells using flow cytometry instead of molecular methods. Flow cytometry (FCM) is a sensitive analytical technique that can rapidly monitor physiological changes of microorganisms, especially when related to membrane integrity. In this project, optimized flow cytometry was used for detecting the viability of several bacterial species through the selection of appropriate dyes and operating conditions. A protocol was developed by which the proper stains, staining protocols, and instrument settings for optimal results could be obtained. Organisms tested included gram negative and gram positive bacteria and yeast cells. The optimized methods were then used to determine the viability of several types of cell suspensions. When cells grown to mid-log phase were analyzed using plate counts and flow cytometry, it was shown that 1 to 64% of cells were non-culturable (were recovered by flow cytometry but not plate counts), 40 to 98% were culturable (by plate counts), and 0.7 to 4.5% had damaged cell-membranes and were therefore theoretically dead (using flow cytometry). The results illustrate the usefulness of flow cytometry for the rapid detection of bacteria in various states of membrane damage (Khan et al., submitted).

Microbial Community Analysis

In addition to the research done to detect viable bacteria, work also focused on pathogens as a member of the microbial community in the environment. This has important implications because competition within the community is one factor that affects a pathogen's ability to persist. A protocol was developed for profiling the total bacterial community in an environmental sample. PCR is used to amplify 16S rDNA sequences from all members of the community, including pathogens. A cloning reaction is then performed to separate the individual sequences, that is, a single sequence (individual species) is packaged into each clone. Pools of clones are randomly selected to reconstruct the original community, and a profile of this pseudo community is created using denaturing gradient gel electrophoresis (DGGE). By knowing the location in the profile of a band that corresponds to a pathogen, we can track the prevalence of the pathogen relative to the rest of the community. Furthermore, by enumerating clones containing the 16S rDNA sequence of the pathogen vs. clones corresponding to non-pathogens, we can quantify the relative prevalence of the pathogen within the bacterial community (Burr et al., 2006).

This work was extended to other natural communities, including constructed wetland plant rhizospheres and agricultural soils. These environments are genetically much more complex and therefore more challenging to analyze than laboratory biofilm reactors or water systems; therefore, they serve as a good model for testing methods that must work well in complex environments. For agricultural soils in particular, we have evidence that microbial communities are fairly stable despite differences in treatment (crop rotation, fertilizer regime, tillage, etc.) and that rather drastic disturbances (e.g., compaction and total removal of plant cover) are required in order for community changes to be reflected in genetic fingerprints of ribosomal DNA. We had success in profiling specific functional genes of the nitrogen cycle and believe these genes are more likely to respond to differences in the environment or changes in treatment (crop rotation, fertilizer regime, tillage, etc.) than the phylogenetic 16S rRNA gene. All of this work depends upon the use of appropriate methods and approaches for DNA isolation, amplification and detection, and these topics were covered in a review article (Nocker et al., 2007c). This review led to initial efforts to purify total RNA from environmental samples in order to have the option of both DNA- and RNA-based microbial community analyses.

Observations and Descriptions of Cell Behavior in Biofilms

There have been no previous studies where quantitative descriptions of biofilm growth and dynamics have been obtained. These data are needed to advance the next set of biofilm models where the behavior (growth, retention, loss) of bacteria can be predicted. This researchwas done to describe growth, displacement of neighboring cells, and accumulation of organisms in a *Pseudomonas aeruginosa* biofilm containing two subpopulations labeled with green or cyan fluorescent proteins. Cells in microcolonies accumulated exponentially and then entered into a stationary phase. Larger clusters accumulated volume more slowly, presumably due to nutrient limitation. The background cells that were not in discrete microcolonies did not seem to grow at all, even though it is likely that they were not nutrient limited. Growing clusters displaced neighboring cells in the background biofilm on the surface, often by accumulating them on the sides of the cluster (Klayman et al., 2008 and Klayman, Ph.D. dissertation). The data should help biofilm modelers validate recent generations of computer models. These models include those that seek to predict the fate of pathogens in drinking water biofilms.

Pathogens in Biofilms

Biofilm Trap

Drinking water distribution systems pose the potential to transport biological and chemical contaminants to the consumers' tap that can be responsible for widespread waterborne disease outbreaks (WBDO). A need exists to improve the ability to monitor contaminants that can attach to the distribution system's interior surfaces and to obtain samples for diagnosing both the cause of a WBDO and the extent of contamination within the system.

A senior design group (senior engineering students) led by Warren Jones developed a prototype biofilm trap. In their design, there were a series of assumptions made, and some of these assumptions were tested experimentally. The reactors were designed to operate using a venturi and be installed as a side-stream on a distribution system main. The reactors were packed with glass beads of the same size as those used in the laboratory experimental reactors. Because it was not feasible or to test the systems in full scale distribution systems (particularly because it is not realistic to add pathogens), several laboratory simulations were run. This included the design

of reactors, the development of biofilm in the reactor systems under conditions relevant to drinking water, and the challenging of these reactors with either particles or bacteria. Initial work was done using fluorescent beads as surrogates for bacteria to determine if the biofilm had a defined capacity for particle capture. This work was being completed while the detection methods needed for experiments with actual pathogens in the same system were being perfected. *E. coli* 0157:H7 was introduced into the same systems operated under a set flow rate and its retention followed under conditions that produced natural biofilms of different thicknesses. Parallel to this work, biofilms are being developed in constant head conditions so that the influence of varying flow on pathogen capture using *Salmonella typhimurium* can be determined.

Particle Capture in Biofilms

One of the difficulties in investigating particle capture using organisms is that they are influenced by the environment, which affects their behavior in the system as well as recovery methods. Previous work has found that mass balances using organisms are typically poor, with recoveries often below 10%. Consequently, we have used fluorescent latex beads of a size (1 μm diameter) and charge (negative) similar to that of many bacteria in water systems. The experiments used porous media reactors designed specifically for these experiments. The reactors have an entrance area that contains a small stir bar and tangential flow to ensure instantaneous mixing. The reactors are operated in an upflow mode. Tracer studies demonstrated that there was no preferential flow. In initial experiments, a 10^3 inoculum of beads was added to reactors with no biofilm, a biofilm developed on 0.5 mg/L carbon, and a biofilm grown on 1.5 mg/L carbon. These conditions were used to simulate situations with varying levels of pre-existing biofilm growth. Five pore volumes of water were collected at half-volume increments and the beads concentrated by filtration. The columns were then drained and destructively sampled to the beads to determine the number of beads retained on the porous media and reactor walls. The fluorescent beads were enumerated using a ChemScanRDI instrument that uses laser detection. Mass balances on the beads could be closed, demonstrating that the method was proving us good recoveries. Most of the beads passed through the porous media, but there was an increasing trend of retention with the thicker biofilms. Maximal retention in the biofilms was approximately 7% in the control and low nutrient biofilm, and 11% in the thicker biofilm. In subsequent experiments, either 10^5 or 10^6 beads were added to the same porous media reactors precolonized with a biofilm grown on 0.5 mg/L carbon. Sampling protocols were identical to those used earlier. The method was more prone to error with higher particle numbers, and total recoveries tended to be up to 130%. Interestingly, the percent captured seemed to be approximately the same for each situation. Seven percent were retained in the biofilm when 10^5 beads were added, and 10% were retained when ten-fold more were used. These results suggest that the biofilm surface is not saturated with the particles under any of the test conditions. However, the number captured appears to be proportional to the quantity that is added. These results were compared with experiments described below using actual bacteria to determine if the beads can be used as a surrogate for their capture in biofilms.

In two subsequent studies, porous media reactors colonized with a mixed-species drinking water biofilm were used to study the capture of *Salmonella typhimurium* or *Escherichia coli* O157:H7 as model pathogens. These porous media reactors could be easily adapted as side stream devices in a water system and used forensically to determine if a contamination event had occurred.

In one study (Bauman et al., 2009 and Bauman, M.S. thesis), reactors were colonized with indigenous drinking water biofilms for two or three weeks under constant flow conditions with 0.5 mg/L organic carbon to form thin and thick biofilms, respectively. The colonized reactors were then injected with slug doses of ca. 1 x 10^9 CFU/ml of *Escherichia coli* O157:H7. Interestingly, plate counts proved to be the most efficient detection method; there were interferences from molecular methods that underestimated pathogen detection. Because plate counts could close the mass balance around the reactor (could account for 100% of the cells added) they were used to measure the fraction of the inoculum immobilized within the reactors. The issue of the inadequacy of molecular methods is being addressed in other on-going research. Compared with the control reactors (no biofilm) that immobilized only 0.22% of the inoculum, the thin reactors immobilized more cells (0.75%) and the thick biofilm reactors were even more efficient (9.37%). Effluent cells from the reactors were also collected so that breakthrough curves could be calculated. This showed that cells were both reversibly and irreversibly attached during the time period of operation. The organisms showed both accelerated breakthrough and tailing when compared to an inert tracer.

In another study (Grabinski, M.S. thesis, manuscript in preparation), the same types of reactors were operated under both constant flow (CF) and constant head (CH) to compare flow-regime induced spatial variations in biofilm accumulation and the resulting pathogen capture. Parallel test reactors were operated with 0.5 mg/L supplemental carbon as above until the accumulation of biofilm in the CH reactor reduced the flowrate to the target sampling point (CF flowrate). Both test reactors were then inoculated with slug doses of approximately 3x10^9 CFU *S. typhimurium*. Effluent water samples were collected for five pore-volumes, followed by the destructive sampling of the reactor. Plate counts were used to enumerate *S. typhimurium* present in effluent samples and captured within the reactor. Cell counts in effluent samples displayed an accelerated breakthrough compared with a non-reactive tracer. Compared with uncolonized control reactors (0.13%), colonized reactors (0.96%) captured significantly more cells. Despite spatial variations in biofilm accumulation, colonized CH and CF reactors captured comparable amounts of *S. typhimurium*. Increasing sampling duration to twenty pore volumes demonstrated greater retention of captured cells in the colonized reactors over the control reactors. *S. typhimurium* transport and capture was also observed in a 0.9 mm square flowcell packed with 100 micrometer beads using a confocal microscope. Interception and straining were responsible for capture on clean beads while biofilm accumulation narrowed pore throats sufficiently to allow for mechanical filtration to occur.

Both of these studies demonstrated that using biofilm colonized porous media may be an effective tool to capture pathogens for monitoring drinking water distribution systems.

Pathogen Capture and Retention in a Biofilm

In addition to the above work centered on the efficacy of a device for capturing pathogens, fundamental work was done to determine if a biofilm was necessary for the entrainment of certain pathogens. In other words, is a clean system better for capture or is it preferable to have a biofilm of indigenous organisms present? The above research suggested that a biofilm was more likely to capture pathogens in a porous media system, but there was also a need to examine a plug-flow condition. In this work (Klayman et al., 2009 and Klayman, Ph.D. dissertation), a background biofilm of *Pseudomonas aeruginosa* labeled with a fluorescent protein was grown in

a flow cell that could be observed under the confocal microscope. A fluorescent protein labeled *E. coli* O157:H7 (same parent strain as work above in biofilm trap section) was also added to these systems. Three conditions were used: (1) *E. coli* alone, (2) the two organisms co-inoculated, and (3) the system was precolonized with *P. aeruginosa* (established biofilm) and then inoculated with *E. coli*. *E. coli* was unable to attach to the reactor after 48 hr of continuous inoculation. When added to the system containing an established *P. aeruginosa* biofilm, they could attach, and at a level approximately 10 fold higher than when added to the clean reactors along with *P. aeruginosa*. However, the pathogen only developed into microcolonies when co-inoculated. In this case, there was a repeatable pattern of colonization in the two organisms. *E. coli* could only persist in the low shear areas of the reactor. This relationship was confirmed by modeling the hydrodynamics of the system. In the low-flow areas, the pathogen comprised 50% of the total biovolume, even though it was less than 1% of the biovolume of the entire reactor.

Gene Expression of Pathogens in a Biofilm

It is known that biofilm organisms are much less susceptible to disinfectants and environmental stress. It has also been observed that pathogens tend to lose their culturability in a biofilm over time, even though they persist. It is possible that these responses are due to differences in gene expression over time. An investigationwas done (Clark and Camper, submitted; Clark, Ph.D. dissertation) to determine the behavior of *Salmonella typhimurium* in a biofilm at a genomic level using microarrays supplied by the Pathogen Functional Genomics Research Center (PFGRC) at the J Craig Venter Institute (JCVI) formerly The Institute for Genomics Research (TIGR). The same environmental isolate of *S. typhimurium* used in the previously described experiments is was grown in a biofilm reactor reactor. Biofilm has been harvested from the reactor surfaces and the RNA extracted and purified. Using the same strain of *S. typhimurium*, planktonic cultures were grown and RNA purified from harvested cells. The RNA from these two cultures was then converted to cDNA and labeled with a fluorescent dye and hybridized to the whole-genome microarrays. These comparisons showed that there were differences in gene expression associated with virulence factors and other known attachment factors, illustrating that there is differential gene regulation in the two modes of growth. The gene expression pattern of these organisms was also be compared with planktonic and biofilm-grown cultures of the type strain of *Salmonella typhimurium* from ATCC, designated LT2. This strain of the pathogen exhibits an extremely weak ability to form biofilm when compared to the environmental isolate. Growth curve comparisons showed that the environmental strain grows faster than the type strain. The gene expression pattern of these two organisms was compared, and there were differences in virulence factor expression in biofilm formation between the two strains.

Disinfection of Pathogens

The disinfection susceptibilities of suspended planktonic cells have been well-studied for a large variety of disinfectants. These disinfectants have been found to be much less effective against cells in biofilms, requiring concentrations orders of magnitude higher than those necessary to kill suspended planktonic cells. Although the detachment of aggregated cells from biofilms, and in particular pathogens, is of fundamental importance to the dissemination of contamination and infection in both public health and clinical settings, the efficacies of commonly used disinfectants on detached particles have not been investigated yet. We hypothesize that detached particles are less susceptible to biocides than planktonic cells. For these experiments, the focus is on the efficacy of disinfectants against pathogens grown in biofilms, aggregates of cells naturally

detaching from these biofilms, and the same organism grown planktonically. Our environmental *S. typhimurium*, as a model pathogen, is grown in standardized laboratory reactors for enhanced repeatability. Planktonic cultures are grown in a continuously stirred chemostat, while biofilm is obtained from a tubing reactor. Detached aggregates can be sampled from the outflow of the biofilm tubing reactor. Disinfection experiments are being performed with sodium hypochlorite concentrations from 1 – 40 ppm to calculate log reduction rates for each scenario. There were significant differences in susceptibility of the different cell preparations. As expected, the planktonic cells were the most susceptible, the biofilm the least susceptible, and the aggregates were intermediate. These results are particularly interesting because the chemostat grown planktonic cells also form small aggregates of cells (1-5 cells per cluster) which is in the same size range as the clusters released from the biofilm reactor. This work is now being funded by another source and will be published when the project is completed.

Opportunistic Pathogen Mycobacterium avium

The above pathogens are not generally believed to grow in biofilms in the environment. In contrast, opportunistic pathogens including several *Mycobacteria* spp. are known to grow and persist in drinking water biofilms. There have been two projects where this organism has been investigated. In one set of experiments (Karmacharya, M.S. thesis), the ability of this organism to persist long-term in biofilms in a simulated drinking water system was investigated. The organism was shown to become incorporated into existing heterotrophic biofilms or to form biofilms on its own and to grow under very low nutrient conditions. Other investigations have focused on possible signals that are produced by other biofilm organisms that may support the growth of *Mycobacterium avium* in biofilms (Geier et al., 2008; and Geier, Ph.D. dissertation). Microarray studies comparing the response of planktonically grown *Mycobacterium avium* in the presence or absence of the autoinducer molecule AI-2, which is produced by gram negative bacteria, showed the up-regulation of five genes in the presence of AI-2. Primers were designed for these genes and the housekeeping gene sigA and RT-PCR was performed to confirm that these genes were indeed up-regulated in the presence of AI-2. Since the up-regulated genes belong to the oxidative stress response, microtiter assays were done to show that hydrogen peroxide can induce biofilm formation. It was also confirmed that the same five genes were up-regulated in the presence of hydrogen peroxide by RT-PCR (although this is well known from the literature). To confirm that there is signaling cross-talk, the influence of cell-free culture supernatants from 4 bacterial strains on *M. avium* biofilm formation was tested. The results showed that *E. coli* is unable to induce biofilm formation in *M. avium* even though it produces AI-2. *V. harveyi* (even the luxS mutant) and *P. aeruginosa* PAO1 (does not have luxS) are able to induce biofilm formation in *M. avium*. This shows that bacteria are able to stress one another and that *M. avium* is able to distinguish between different bacterial species.

Modeling

A key component of our research is integrating the experimental data with models that describe the behavior of pathogens in biofilms. The data acquired in the experiments described above, particularly those where direct microscopic observation has been used, are used as input to the models, and those models are then used to predict the behavior seen in subsequent experiments.

The CBE cellular automata modeling group developed a generalized, interactive cellular automata model for application to biofilm system analysis. This three-dimensional model was used to explore theoretical conjectures on biofilm systems. Processes which can be incorporated in the model include substrate and antimicrobial diffusion, substrate consumption, cell growth, death, conversion, antimicrobial killing, phenotypes, movement, and detachment. Simulations can be configured by a graphical user interface removing programming knowledge as a requirement for use. Results were analyzed quantitatively as well as qualitatively through visualization. The cellular automata model was applied to a data set developed by Klayman. A *Pseudomonas aeruginosa* PAO1 biofilm, tagged with a constitutively expressing GFP was grown in a capillary flow cell under continuous laminar flow conditions. The *P. aeruginosa* biofilm reached a quasi-steady state thickness of 75-100 micron (with considerable spatial heterogeneity) after 72 hours, at which time an *E. coli*, tagged with a constitutively expressing DsRed plasmid, was introduced to the reactor. These experimental data were modeled using the cellular automata model. The primary goal was to determine model parameters and operating rules so as to reproduce the major features of the spatial and temporal distribution of the two species in the flow cell along with the detachment-related suspended cell concentrations leaving the reactor. After varying the detachment parameters by trial and error, it was found that an n value of 6 allowed the cellular automata model to reproduce the structural features of the two-species biofilm as well as the average, steady state, biofilm thickness (approximately 100 microns). Effluent suspended cell measurements were also reproduced by varying the detachment parameters in the model. These results will be compared with two other detachment models based on 1) substrate concentration and 2) biofilm thickness. The introduction of *E. coli* to an established *P. aeruginosa* biofilm represents a model system for studying the introduction of pathogenic bacteria to water supply systems containing biofilms. The results of our study support the observations that: 1) *E. coli* persistence in an established *P. aeruginosa* biofilm and subsequent detachment into the bulk fluid can be modeled using cellular automata. 2) Structure and density of *P. aeruginosa* biofilm significantly influenced the initial colonization, morphology, and colony structure of the invading *E. coli.* A significant modeling breakthrough occurred when the modeling team developed an algorithm which allows the digital confocal microscope image of the biofilm to be imported directly into the cellular automata model as the starting condition. This allows for meaningful comparison of experimentally observed biofilm behavior with computationally simulated behavior. This approach is still being finalized and will be submitted for publication when completed.

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