Innovative biofilm control strategies
by Alex Martin Bargmeyer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
Montana State University

Abstract:
The drinking water industry is continually interested in finding new methods for preventing growth and/or eliminating biofilms in the water delivery system. Concern for reducing biofilms in public water distribution systems is due to problems such as undesirable taste and odor, corrosion of the infrastructure, and the possibility for harboring pathogens. These drinking water biofilms may be defined as a modular community of largely benign microorganisms attached to a surface within a microbe-derived hydrated matrix. The biofilm's basic structure and mode of growth enables resistance to antimicrobial-based removal strategies. The objectives of this research were to screen novel technologies or strategies in the laboratory using annular reactors and mixed population biofilms of drinking water origin and to test the best technology for application in a realistic setting.

This research investigated three strategies or technologies to enhance the removal or the prevention of growth of biofilm under drinking water conditions. The first was the use of specific chemical signalling compounds implicated in previous research, to have the ability to cause detachment of an established biofilm. The second, was the use of the bioelectric effect phenomenon in which the efficacy of antibiotics, has been shown to be enhanced through the application of weak electric fields. The final strategy in this investigation is a contact biocide which can be continually replenished by bulk fluid chlorine in the system.

The results of the cell signaling compounds did not show a significant effect on an established biofilm. The bioelectric effect proved to be corrosive to metal components in the reactor system and actually provided liberated metal ions that were more conducive for bacterial growth. The final strategy proved to be the best candidate for a drinking water system application. The contact biocide was able to facilitate faster and greater removal of biofilm given typical drinking water chlorine disinfectant concentrations. Experimental data suggests however, that low concentrations of residual chlorine in the system would not be adequate to render the surface biocidal.

INNOVATIVE BIOFILM CONTROL STRATEGIES

by

Alex Martin Bargmeyer

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Environmental Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2003

APPROVAL

of a thesis submitted by

Alex Martin Bargmeyer

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Dr. Anne K. Camper Anne K. Camper 8/22/03
(Signature) Date

Approved for the Department of Environmental Engineering

Dr. Brett Gunnink Brett Gunnink 8/22/03
(Signature) Date

Approved for the College of Graduate Studies

Dr. Bruce R. McLeod Bruce R. McLeod 8-22-03
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U. S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature 

Date 8/22/03

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The drinking water industry is continually interested in finding new methods for preventing growth and/or eliminating biofilms in the water delivery system. Concern for reducing biofilms in public water distribution systems is due to problems such as undesirable taste and odor, corrosion of the infrastructure, and the possibility for harboring pathogens. These drinking water biofilms may be defined as a modular community of largely benign microorganisms attached to a surface within a microbe-derived hydrated matrix. The biofilm's basic structure and mode of growth enables resistance to antimicrobial-based removal strategies. The objectives of this research were to screen novel technologies or strategies in the laboratory using annular reactors and mixed population biofilms of drinking water origin and to test the best technology for application in a realistic setting.

This research investigated three strategies or technologies to enhance the removal or the prevention of growth of biofilm under drinking water conditions. The first was the use of specific chemical signalling compounds implicated in previous research to have the ability to cause detachment of an established biofilm. The second was the use of the bioelectric effect phenomenon in which the efficacy of antibiotics has been shown to be enhanced through the application of weak electric fields. The final strategy in this investigation is a contact biocide which can be continually replenished by bulk fluid chlorine in the system.

The results of the cell signaling compounds did not show a significant effect on an established biofilm. The bioelectric effect proved to be corrosive to metal components in the reactor system and actually provided liberated metal ions that were more conducive for bacterial growth. The final strategy proved to be the best candidate for a drinking water system application. The contact biocide was able to facilitate faster and greater removal of biofilm given typical drinking water chlorine disinfectant concentrations. Experimental data suggests however, that low concentrations of residual chlorine in the system would not be adequate to render the surface biocidal.

CHAPTER 1

INTRODUCTION

Drinking water distribution system biofilms are an inevitable fact of our infrastructure. These biofilms are for the most part benign and pose no direct health threat. However, there are a number of problems associated with these biofilms that can arise if their growth is left unchecked. The problems that are associated with drinking water microorganisms and their biofilms are taste and odor, corrosion of metal structures and the potential for harboring pathogens.

Taste and odor primarily relates to the general aesthetics of the water and can be an indication of maintenance and operational problems of the treatment and/or distribution system. Corrosion of the infrastructure can reduce the systems efficiency and invoke costly repairs. Recently, it has been shown that distribution systems that have predominantly iron pipe have the greatest potential for biofilm regrowth.[1] The persistence of pathogens in these biofilms presents the most important concern. This is because biofilms are inherently resistant to disinfection by conventional means. Harbored pathogens within these biofilms may then be released into the system posing a possible public health threat.[2]

Current methods employed to combat nuisance biofilms include the use of disinfectants, reduction of organic material in the water, pipe materials that reduce the

amount of biofilm accumulation and modification of plant operations to discourage biofilm growth. In addition to these practices, other strategies or technology applied to the management of distribution systems may result in greater biofilm reduction and prevention or stalling regrowth events. Continuing research into the nature of biofilm growth/development and emerging technologies to enhance biofilm control may present additional strategies for this problem.

Goals

The goal of this research is to investigate and provide the drinking water industry with novel biofilm control strategies. Recommendations for application of promising technologies to distribution systems is not the intent of this study but rather to provide an exploratory look at possible complementary solutions to current biofilm control measures.

CHAPTER 2

LITERATURE REVIEW

Drinking Water Biofilms

Drinking water biofilms may be defined as a modular community of microorganisms embedded within a microbe-derived hydrated matrix of extracellular polymers that exists at an interface. This interface may be between the aqueous phase and a solid support such as polycarbonate, polyvinyl chloride (PVC), ductile iron and coated ductile iron pipe. It can also be between two different fluid phases such as air and water, or between two liquids of differing densities like oil and water.[3]

Biofilms in drinking water are typically in environments that do not provide optimum growth conditions, yet their persistence may lead to the problems mentioned previously. A distribution system's pipe network is the common interface for biofilm growth.[4] Conditions within these systems have variations in temperature, disinfectant residual, flow and chemical conditions such as pH, organic carbon, and nutrient levels. These variations may produce biofilm sloughing events which can increase the number of planktonic bacteria.[1] Therefore distribution system biofilm maintenance can be an arduous task.

Distribution systems can be colonized by a number of microorganisms that cause

a variety of problems. Organisms such as *Actinomycetes* or fungi can result in taste and odor problems.[5, 6, 7] Bacteria may grow on ferrous metal surfaces [8] and result in the presence of iron particulate in finished water.[9] Corrosion of distribution system pipe materials may be enhanced due to the presence of bacterial biofilms.[10] Coliform bacteria present in treated water may be an indicator of the prior growth and release of these organisms within distribution system biofilms.[11, 12, 13] Coliform regrowth events are generally proclaimed to be a regulatory nuisance rather than a hazard to public health. However, it has been noted that opportunistic pathogens, including *Aeromonas* spp., *Mycobacterium* spp., and *Legionella* spp. can and do grow in drinking water distribution system biofilms. These and other problematic microorganisms associated with distribution system biofilms are summarized in Table 1[14].

The biofilm mode of growth enables resistance to antimicrobial-based removal strategies. Previous research indicates that in order for disinfectants and antibiotics to be as effective against biofilm bacteria, concentrations of 500 to 5000 times greater than those required for killing planktonic strains of the same bacterial species are required.[15] One of the mechanisms by which inherent resistance to antimicrobial factors is mediated in biofilms is through very low metabolic levels and dramatically downregulated rates of cell division of the deeply embedded microbes.[16] Thus the strategies for disinfecting agents that depend upon robust and actively dividing microbes are often ineffective.[17] Another is that these structures demonstrate a

Table 1. Problematic Microorganisms in Distribution Systems.

Type of Microorganism	Infrastructure or Water Quality Problem
Coliforms	Positive samples may be a violation of the Total Coliform Rule.
Actinomycetes, Molds and Fungi	Produce earthy-musty-moldy taste and odor compounds and are commonly found in surface waters.
Iron Bacteria	Oxidize soluble iron to precipitate forms increasing the mass of corrosion products on pipe walls and pump casings. Excessive iron deposits cause increased pipe friction and lower pump efficiency.
Sulfate Reducing Bacteria	Reduces sulfate to hydrogen sulfide gas (rotten egg odor) and increases corrosion rates.
Nitrifying Bacteria	Oxidize ammonia to nitrate and consume alkalinity, which may result in pH reduction.
Protozoans	May reside in biofilms posing a health risk

(Abernathy, 1998)

physical barrier to penetration of the antimicrobial agents.[18] It has been previously shown that the polymeric matrix that encompasses the majority of biofilms retards the inward diffusion of a number of antimicrobial agents.[19, 18, 20] Also, biocides composed of common chlorine compounds used in water treatment such as hypochlorite, chlorine dioxide, monochloramine as well as other antimicrobial/antifouling agents may be deactivated in the outer layers of the biofilm faster than they can diffuse into the lower layers.[21, 22] Lastly, a number of studies have shown that the gene expression within biofilms is altered due to the physical action of attachment.[23] This

change in gene expression is a biologically programmed response to attachment and not due to nutrient deprivation. However, the link between antimicrobial resistance and altered gene expression is presently being studied.[24]

Cell Signaling Compounds

Research in bacterial biofilms has just begun to yield information about the complex nature of its survival strategies. One of these strategies increasingly studied since the 1960's, is the mechanism of cell-to-cell signaling or quorum sensing. This phenomenon was initially believed to be unique to bioluminescent marine bacteria such as *Vibrio fischeri* and *Vibrio harveyi*. In these organisms the exhibition of bioluminescence corresponds to high cell densities within high nutrient conditions in the light emitting organs of their aquatic hosts. The specific mechanism responsible for activation of bioluminescence was discovered to be the production and accumulation of a signaling molecule or "autoinducer" which is accumulated in response to high bacterial cell densities.[25] Since then there have been numerous other bacterial species identified to possess a quorum sensing system.[26] This mechanism for cellular communication has been implicated as instrumental in the cycle of bacterial attachment, biofilm formation/maturation and detachment.[27, 28]

Quorum sensing compounds associated with specific detachment signals for biofilm bacteria may be of value to the drinking water industry. If cells are chemically stimulated to detach from the biofilm mode of growth, it could potentially make them more

susceptible to disinfection by residual disinfectants. Furthermore, if cellular response to attachment and the formation of biofilm could be interrupted through the use of cell signaling compounds, biofilm could be stalled or prevented.

A comprehensive review by Shirliff et al.[24] covers the current understanding of molecular interactions within bacterial biofilms and the impact it has on biofilm development and phenotype. From the review there were three specific molecules that displayed properties that could applied to drinking water biofilms. These three potential cell-to-cell signaling molecules were evaluated (Figure 1. for structures) for their ability to promote biofilm detachment from mixed species drinking water biofilms.

The first of these molecules was 1,2 fluorodecal acyl homoserine lactone (FOdHSL), which had previously demonstrated efficacy in the prevention of *Pseudomonas aeruginosa* biofilm development.[29] This is a synthetic molecule and acts as a non-specific antagonist to bacterial signaling molecules that have previously been implicated in the formation of the fully mature biofilm phenotype. Therefore, by applying the bacterial signal antagonist FOdHSL to the biofilm, the hope is to disrupt this mature phenotype and promote biofilm detachment.[30, 31] Ideally, biofilm detachment would facilitate flow-mediated removal, and microbial death in the presence of residual disinfectants without the protective structure of the biofilm.

Another signaling molecule antagonist that was tested, wrs-I-51, was designed by

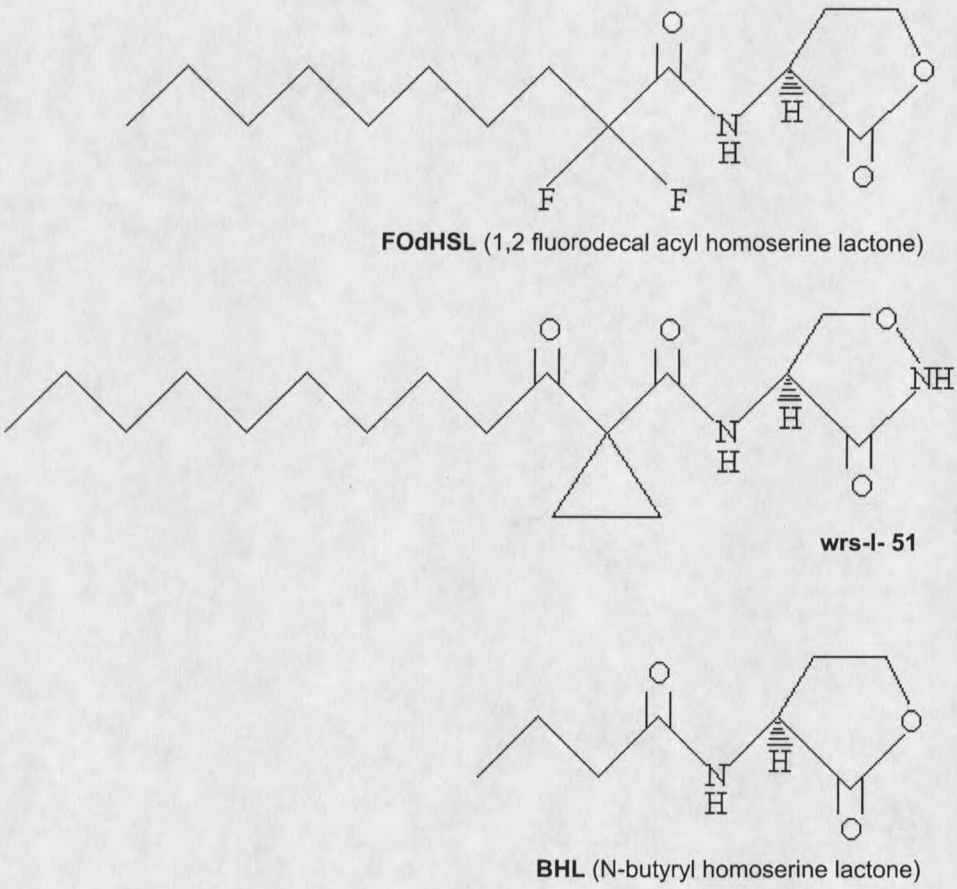

Figure 1. Structures of cell signaling molecules.

organic chemists at the Montana State University Chemistry Department as an irreversible inhibitor of the quorum sensing systems of microbes. These antagonists were used at greater than 100 times the concentration of the bacterial signaling molecules in biofilm environments. This molar excess of antagonist has been previously shown

to effectively inhibit quorum sensing in biofilms.[29]

The last signaling molecule that was evaluated was not an antagonist, but a native autinducing signaling molecule from the *P. aeruginosa* quorum sensing system, butyryl homoserine lactone (BHL)[32, 33, 34]. BHL has also been implicated as a more freely diffusible compound within a biofilm compared to the other HSL signaling compounds. The physiological BHL concentration at which quorum sensing-activated genes are upregulated is approximately 40 - 60 nM[35]. This signaling molecule has been implicated as a possible biofilm size limiting or detachment signal for *P. aeruginosa*[24]. Therefore, we tested the ability of BHL at 60 nM to promote detachment from mixed population biofilms in annular reactors. In addition, we evaluated the role of BHL to promote biofilm detachment at a concentration above (6000 nM) the physiological BHL concentration due to the effectiveness of the first BHL experiment.

Bioelectric Effect

The problem of biofilm-mediated resistance to disinfectants may be circumvented through the application of weak direct current fields. This "bioelectric effect" was initially discovered by Blenkinsopp et al.[36] and also by Costerton, et al.[37, 38] in which the efficacy of antibiotics was shown to be increased through the application of weak electric fields. In one particular study, biofilms were grown on dialysis tubing and immersed within a minimal media filled chamber. These membranes were either left untreated, treated with a weak electric field alone, treated with antibiotic

alone, or treated with a combination of antibiotic and electric field. In order to avoid the electrochemical generation of toxic products through electrolysis (discussed later), biofilms were formed in minimal salts medium that excluded chloride-containing compounds. The biofilms that were untreated, treated with a weak electric field alone, or treated with antibiotics alone were not affected. However, when an electric field was applied to the minimal salt medium containing antibiotics, the researchers found a dramatic effect on biofilm structure and bacterial concentration. It has also been found that the concentrations of antibiotics needed to be effective against biofilm bacteria in a weak electric field fell to only 1.5 - 4.0 times those necessary for planktonic bacteria.[39]

Another study conducted by Stewart et al.[40] found similar results of enhanced efficacy of applied antibiotics when coupled with a weak DC electric current. These experiments utilized *P. aeruginosa* biofilms grown on a polycarbonate substratum. When the biofilm was exposed to the antibiotic tobramycin the effective reduction in biofilm was 2.88 logs which was increased to 5.58 logs in the presence of the weak electric field. Because these experiments require an electric potential (not indicated) and current flow (2 mA) they realized that the potential electrolysis of water can create species such as oxygen and oxygen intermediates. These electrolysis reactions are shown in equations 2.1 through 2.3. Other electrolytically-generated oxygen compounds possible are superoxide anion, peroxide and hydroxyl radicals.

$$2H_2O \Rightarrow O_2 + 4H^+ + 4e^- \tag{2.1}$$

$$4H_2O + 4e^- \Rightarrow 2H_2 + 4OH^- \tag{2.2}$$

The overall net reaction becomes:

$$2H_2O \Rightarrow O_2 + H_2 \tag{2.3}$$

Oxygen did enhance the ability of tobramycin to reduce the biofilm by 4.68 logs. However, biofilm reduction was enhanced further by the addition of the electric current (5.58 logs), which implies the claimed bioelectric effect did have a synergistic effect on antibiotic effectiveness in this study.

Electrolysis or iontophoresis of other constituents when applying an electric potential to an electrolyte are also possible. It was found that the application of small electric currents in solutions that contain chloride ions can create chlorine gas shown in equation 2.4 .[41]

$$2NaCl + 2H_2O \Rightarrow 2NaOH + Cl_2 + H_2 \tag{2.4}$$

Chlorine gas quickly dissolves in water and forms hypochlorous acid and hypochlorite. These observations lead to the conclusion that in situations where there is a significant number of chloride ions present, antimicrobial efficacy is due to the iontophoretically produced chlorine based substances and not a synergistic bioelectric effect.[41]

Electric fields in direct contact with biofilm have been observed to dramatically change the structure of the biofilm. Biofilms grown on an electrode wire where the

electrical current could pass directly through the bacterial structure displayed synchronous expansion and contraction of the biofilm thickness with alternating current. This has been attributed possibly to the localized change in pH due to the change in current flow and charge reversal and also charged groups within the biofilm and the charge of the electrode. It was also thought that this induced structural "effect" may present a possible mechanism for the increasing the efficacy of biocides when combined with an electric field. From this research conjecture for the bioelectric effect's usefulness to reduce biofilms in industry has been made; but only if growing on a conductive surface in an aqueous environment and a counter electrode.[42]

Continued research by McLeod et al. in the bioelectric effect created a dose response curve for the effectiveness of applied electric current and antibiotic.[43] A current density within their system of $360\,\mu A/cm^2$ yielded consistent effectiveness for 5 times the minimum inhibitory concentration or MIC of tobramycin at 5 mg/L. These findings served as a starting point for drinking water biofilm experiments utilizing this strategy.

In review of the bioelectric effect, a number of possible explanations for the enhancement of antimicrobial efficacy have been proposed. These include electrophoretically-mediated augmentation of antimicrobial transport, cell membrane permeabilization, electrolytic generation of oxygen and potentiating oxidants, increased convective transport of antimicrobials due to contraction and expansion of the biofilm, and pH alterations in solutions to which current is applied. This portion of the study

evaluated the phenomenon of the bioelectric effect as a strategy to enhance the antimicrobial properties of chlorine against mixed population biofilms of drinking water origin.

Contact Biocides

The most promising novel strategy or technology for the reduction of biofilm within distribution systems is the contact biocide. Traditionally, reduction of bacterial growth in a water system is achieved through maintaining a residual concentration of disinfectant in the system. These disinfectants, mainly chlorine or monochloramine must then diffuse through the biofilm to effectively kill the cells. As mentioned previously, biofilms are resistant to disinfection, which is often due to the resulting mass transfer limitations of the disinfectant.[44] The effectiveness of typical chlorinated drinking water may be enhanced if the surface were to be inhospitable to the proliferation of microbial growth. A contact biocide which has been made available to this study comes from Vanson-HaloSource Inc. and is based on a group of organic compounds called N-halamines. These N-halamines and their derivatives have the ability to bind oxidative/reactive chlorine, thereby rendering a biocidal property.

A study of the comparative antimicrobial activities of these N-halamine derivatives was first published in 1976 by Kaminski et al.[45] The comparison evaluated a number of water soluble N-halamine compounds for their bacterial inactivation efficiencies. It was determined that these compounds could provide an alternative

stabilization method of the active Cl^+ ion and their subsequent use as a disinfectant.

More recently the application of this technology for the disinfection of water became apparent.[46] The compounds used in this study are similar to the N-halamine compounds described above but contain a heterocyclic carbon nitrogen or oxygen ring structure which has limited solubility in water. Again these compounds have a unique property that stabilizes the active chlorine or bromine species that makes it useful as a soluble disinfectant. In this study some of the chlorinated compounds tested were able to remain stable as an effective disinfectant over a period of 15 weeks.

Later development of insoluble polymeric N-halamine compounds suggested an application as a biocidal water filter.[47] This polymer has the same heterocyclic ring structure as previously described and is a derivative of a polystyrene hydantoin (poly1Cl, Figure 2). The chlorinated structure exists in solid form as pale yellow granules. In these filter experiments the solid granules were loaded into columns and challenged with water containing four bacterial species with concentrations greater than 10^6 colony forming units (CFUs) per mL. The filter's capacity for complete disinfection was about 160 mL contaminated water per gram of poly1Cl for *E. coli* at contact times of 1-2 s/mL.[48] The disinfectant properties of this filter could be renewed when flushed with a strong bleach solution to clean and reactivate the poly1Cl.

Other work in this area included coating various substrates with a poly-N-halamine compound.[49] Substrates used in these experiments were glass, plastic, cotton and a cotton polyester blend. The hard surfaces coated with the polymer achieved 6-log

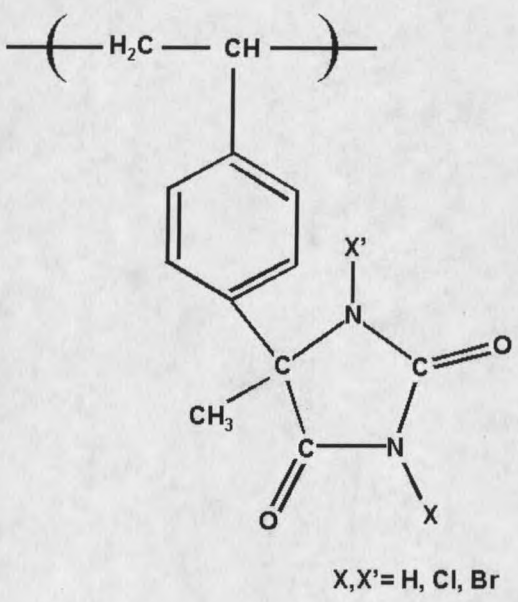

Figure 2. Structure of cyclic N-halamine polymer poly1Cl.

inactivation of *S. aureus* in 5-10 minutes. The textiles were tested for their zone of inhibition which was 0.5 mm for the cotton and 0.1mm for the cotton blend. Once again the unique properties of the poly N-halamine compounds is the ability to retain a stabile active N-Cl moiety until there is a collision or hit by a reactant i.e. bacterial cells, organic compounds, etc. The active chlorine is then used or donated from the structure. The antimicrobial properties can be regenerated by repeated exposure to Cl_2 or NaOCl.

A recently developed coating that incorporates an N-halamine hydantoin monomer into the backbone of a polyurethane polymer has promise for testing in drinking water surface applications. This particular coating is prepared by copolymerizing a

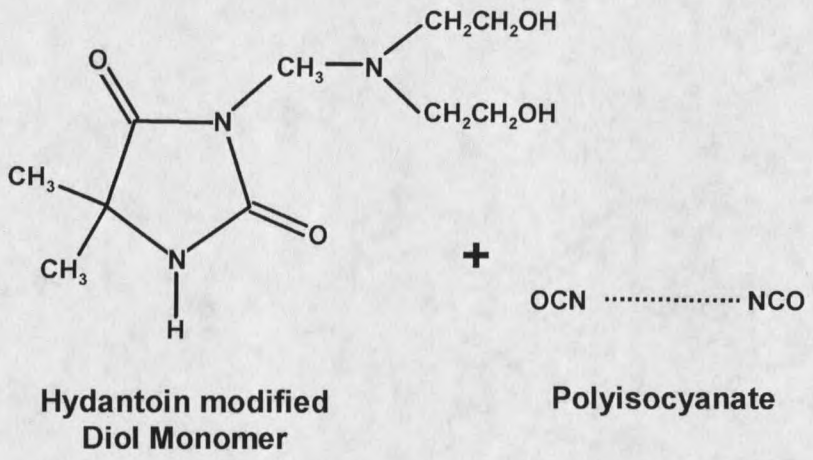

Figure 3. Preparation of biocidal polyurethane coating.

previously disinfection-proven, water-soluble, N-halamine derivative with commercially available polyurethane. The N-halamine used to create a diol monomer used in copolymerization is a 1,3-dihalo-5,5-dimethylhydantoin combined with diethanolamine and a methanol formaldehyde solution. The modified diol monomer is then combined with polyisocyanate (Figure 3) to create a surface active polyurethane . The coating is cured at room temperature and activated by exposure to a strong bleach solution (10% by volume) for 3-12 hours (Figure 4).[50]

The chemical structure of the N-halamine hydantoin is the key to the superior stability of the active nitrogen-chlorine bond. The electron donating alkyl groups adjacent to the nitrogen-chlorine moieties prevent significant release of chlorine into an aqueous solution. It has been shown that the coating is able to retain its biocidal