Biocide action of chlorine on Pseudomonas aeruginosa biofilm
by Ewout van der Wende

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
Montana State University

Abstract:
Biofouling by sessile microorganisms causes many problems for industry. Chlorine is often applied as a biocide to remove biofilm or prevent biofilm accumulation. The goal of this study was to determine the kinetics and stoichiometry of disinfection and detachment of Pseudomonas aeruginosa biofilm by chlorine. Much of the work was done on in situ biofilm in a RotoTorque reactor. Biofilm cell detachment and disinfection were observed during biocide treatment. Free chlorine reacted with biofilm and suspended cells in the RotoTorque reactor. The reaction rates were observed and described with separate kinetic expressions for the reaction with biofilm and suspended cells.

Results indicate that disinfection dominates detachment in eliminating viable cells from the biofilm, especially at higher chlorine doses. The reaction of free chlorine with biomass resulted in the formation of combined chlorine. Combined chlorine may play a significant role in biofilm detachment but is relatively ineffective in biofilm cell disinfection.

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APPROVAL

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Ewout van der Wende

This thesis has been read by each member of the thesis committee and 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.

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ABSTRACT

Biofouling by sessile microorganisms causes many problems for industry. Chlorine is often applied as a biocide to remove biofilm or prevent biofilm accumulation. The goal of this study was to determine the kinetics and stoichiometry of disinfection and detachment of Pseudomonas aeruginosa biofilm by chlorine. Much of the work was done on in situ biofilm in a RotoTorque reactor. Biofilm cell detachment and disinfection were observed during biocide treatment. Free chlorine reacted with biofilm and suspended cells in the RotoTorque reactor. The reaction rates were observed and described with separate kinetic expressions for the reaction with biofilm and suspended cells.

Results indicate that disinfection dominates detachment in eliminating viable cells from the biofilm, especially at higher chlorine doses. The reaction of free chlorine with biomass resulted in the formation of combined chlorine. Combined chlorine may play a significant role in biofilm detachment but is relatively ineffective in biofilm cell disinfection.

INTRODUCTION

The Problem

Accumulation of aquatic microbial cells on surfaces, usually referred to as biofilm, causes many problems for industry and often results in costly damage. Loss of heat transfer capacity in cooling towers and other heat exchangers is frequently a result of biofilm accumulation. Paper mills suffer biofilm-related damage when paper quality is affected by biofilm particles that slough from equipment surfaces and become entrained in the process stream. Hydraulic resistance in pipelines increases if biofilm is present, resulting in reduced flow rates or higher pumping costs. Corrosion is often attributed to the presence of sessile microorganisms in aerobic as well as in anaerobic environments although the mechanisms are not well understood.

Various methods are used to mitigate biofouling problems. Mechanical cleaning of pipelines includes the use of "pigs", i.e., brushes forced through a pipeline by water pressure. This practice is common for injection water pipelines used by the oil industry and is even used to clean drinking water mains. Anti-fouling coatings have been used with limited success. Chemical additives, biocides, are used in combination with mechanical procedures or as the sole treatment. Besides removing biofilm, many cells in the remaining biofilm are killed during this type of treatment resulting in increased biofilm recovery times. Oxidizing biocides such as chlorine can only be used effectively in predominantly aerobic systems. The application of chlorine is common because of its effectiveness and low price. However, its application in power plants in the United States has become limited after the U.S. Environmental Protection Agency lowered the allowable discharge concentrations of total chlorine residuals to 0.2 mg/l (as Cl_2) and restricted discharge to 2 hours a day per plant. The draft Clean Water Act of 1987 proposes even stricter regulations. Thus, application of chlorine and its influence on biofilm processes has become more important in recent years.

This thesis focusses on the relationship between chlorine dose and biofilm detachment and disinfection. Pseudomonas aeruginosa biofilms were grown in a continuous flow stirred tank biofilm reactor under strictly controlled conditions and treated with chlorine. Material balance analyses were employed in determining process rates. Preliminary studies were conducted to determine the advective fluid transport characteristics in the biofilm reactor and the free chlorine demand of system components.

Experiments with combined chlorine were conducted after the observation that combined chlorine and not free chlorine was the predominant chlorine species during treatment with free chlorine. The combined chlorine resulted from the reaction of free chlorine with biomass in the reactor.
Results indicate that the combined chlorine has little or no impact on biofilm disinfection but may play a role in biofilm detachment.

Research Goal

The goal of the research presented in this thesis is to establish a phenomenological basis for evaluating the effectiveness of a biocide on control of biofilm and biofilm activity.

Research Objectives

1) Develop and/or test methods necessary to adequately characterize the biofilm for the process analysis.

2) Compare the kinetics of disinfection of planktonic cells versus biofilm cells.

3) Determine the kinetics and stoichiometry of disinfection and detachment in the RotoTorque reactor due to chlorine addition.

LITERATURE REVIEW

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a bacterium commonly found in natural aquatic environments. A capsule of extracellular polymer substances (EPS) and attached growth on surfaces gives it some degree of protection against bacteriophages and predation by protozoa. It has been studied extensively in the form of suspended cells and in biofilms. Some important characteristics of this organism (Buchanan et al., 1974) are:

1) morphology:	rod shaped, 0.5 - 0.8 μm by 1.5 - 3.0 μm
2) gram stain:	negative
3) metabolism:	chemoorganotroph able to utilize many different substrates
4) respiration:	aerobe able to use nitrate as an electron acceptor under anaerobic conditions
5) motility:	through polar monotrichous flagellation

6) EPS composition:	polyuronic acids; mainly D-mannuronic acid and variable amounts of L-guluronic acid (Carlson and Matthews, 1966; Mian et al., 1978)
7) Optimal temperature:	35 - 37 C° (Buchanan and Gibbons; 1974)
8) Optimal pH:	6.8 (Buchanan and Gibbons; 1974)

Biofilm and Biofilm Processes

A bacterial biofilm consists of bacterial cells immobilized at a substratum and often embedded in a polymer matrix of bacterial origin. Inorganic solids may be entrapped in the biofilm.

Cell densities in biofilms can be remarkably low. Trulear (1983) calculated that the total volume of viable cells in a Pseudomonas aeruginosa biofilm was only 1 to 10 % of the biofilm volume. Characklis and Cooksey (1983) reported biofilm dry mass densities ranging from 10 - 50 kg/m3 in biofilms collected from various fluid flow systems.

Biofilm accumulation is the result of the following processes:

1) Adsorption of cells that are transported to the substratum.

2) Growth and reproduction of cells in the biofilm

3) Detachment of cells from the biofilm.

Microbial cells can be transported from the bulk fluid to a substratum surface by many different forces including: diffusion, gravity, taxis and fluid dynamic forces. Once cells have been transported to the substratum adsorption can take place. Some researchers have distinguished between reversible and irreversible adsorption (Marshall et al., 1971). In general adsorption becomes more irreversible when polymers produced by the cell anchor cells more firmly to the substratum. Cells obtain nutrients from the bulk fluid and/or from the substratum. Assimilation of these nutrients results in cell growth and cell reproduction. Biofilm activity is most prevalent in systems with fluid flow. Cell detachment from the biofilm occurs as a result of shear forces imposed on the biofilm by the flowing fluid. Steady state biofilm accumulation is reached when the total of biofilm accumulation processes results in no further change in biofilm areal density.

Biofilm detachment can result from erosion or sloughing. Erosion refers to the continuous removal of small biofilm particles and is highly dependent on the fluid dynamic conditions. Sloughing refers to massive removal of biofilm and has been attributed to oxygen or nutrient depletion in the biofilm (Howell and Atkinson, 1976). Bakke (1983) observed a drastic increase in detachment rates after the substrate loading rate was suddenly doubled. Turakhia et al. (1983) observed increased detachment rates after the addition of chelants. These researchers did not distinguish between biofilm erosion and sloughing. Most research on biofilm detachment has concentrated on indirect measurements including changes in frictional resistance and heat transfer resistance.

Chlorine Chemistry

Chlorine is a strong-smelling, greenish yellow gas and extremely irritating to mucous membranes. It received its name from the Greek word "chloros" meaning green. When chlorine is dissolved in water it rapidly forms the oxidizing agent hypochlorous acid (Morris, 1946).

$$Cl_2 + H_2O \text{------>} H^+ + Cl^- + HOCl \qquad [1]$$

The reaction is virtually complete at a pH > 3 and a total $[Cl_2]$ < 1000 mg/l. Hypochlorous acid is a weak acid, at pH 7.5 and 20 °C, only 50% dissociates into H^+ and the conjugated base, hypochlorite ion (Figure 1):

$$HOCl \text{<------>} H^+ + OCl^- \qquad [2]$$

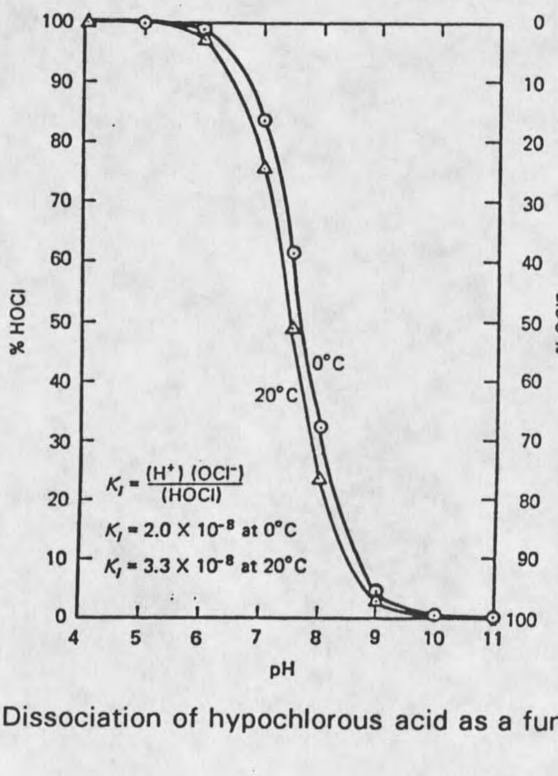

Figure 1. Dissociation of hypochlorous acid as a function of pH

The sum of hypochlorous acid and hypochlorite ion concentration form the free chlorine concentration. Free chlorine reacts quickly with reduced compounds commonly found in aqueous environments: ammonia, organic nitrogen (aminoacids, polypeptides, proteins and organic amines), iron (Fe^{++}), manganese (Mn^{++}), sulfide, and nitrite. Most of the reactions are simple and remove free chlorine quickly from the system. However, the most important aspects of aqueous chlorine chemistry, its reaction with various forms of nitrogen, is much more complex.

Free chlorine reacts with any compound containing a nitrogen atom with one or more hydrogen atoms attached.

The reaction product is a chloramine or N-chloro compound. There are two distinct classes of chloramines; organic and inorganic. Nitrogen appears in most natural waters and in varying amounts as either organic or inorganic nitrogen. In estuarine waters, Helz et al. (1978) measured (organic) amino nitrogen to be about ten times greater than inorganic nitrogen.

Inorganic chloramines are formed during the reaction with ammonia and organic chloramines during the reaction with organic nitrogen. The sum total of chloramine concentration is the combined chlorine concentration. The combination of free chlorine and combined chlorine is the total chlorine concentration. When free chlorine reacts with a relatively large amount of ammonia (molar ratio
[free chlorine]/[ammonia] = 2) almost 100 % monochloramine will be formed.

$$HOCl + NH_3 \text{------>} NH_2Cl + H_2O \qquad [3]$$

The resulting combined chlorine concentration will be about half the initial free chlorine concentration. When more free chlorine is added a disproportionation reaction begins forming dichloramine from free chlorine and monochloramine.

$$NH_2Cl + HOCl \text{ ------> } NHCl_2 + H_2O \qquad [4]$$

At the same time, the combined chlorine concentration starts to drop while the free chlorine concentration remains zero. This reaction proceeds in competition with reaction Eq.3 until the dose ratio of free chlorine to ammonia reaches 4. At this point, the so-called breakpoint has been reached (Figure 2). Monochloramine and dichloramine exist in approximately equal amounts, total chlorine is at a local minimum, free chlorine will start to appear while ammonia-nitrogen will start to disappear through oxidation by free chlorine. Ammonia-nitrogen is converted into elemental nitrogen. Further addition of free chlorine will add proportionally to the free chlorine residual.

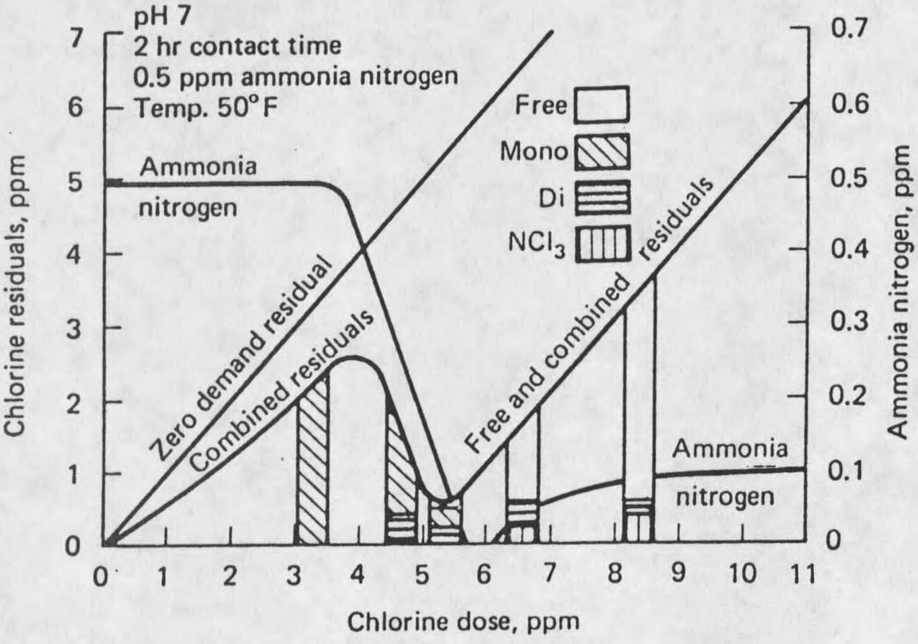

Figure 2. Chlorine residuals during the reaction between free chlorine and ammonia nitrogen. Source: G.C.White, Handbook of Water Chlorination (1972).

The reaction between free chlorine and organic nitrogen compounds results in the formation of organic chloramines. The rate and extent of the reaction depends largely on the structural complexity of the reactant (Taras, 1953). The more simple compounds are the unsubstituted amino acids. Formation of organic chloramines is relatively fast while much of the nitrogen content is lost during further oxidation. The carbon of some of these simple amino acids, such as cysteine and glycine, also exerts a free chlorine demand. For glycine, Palin (1950) noted that there are similarities to the chlorine-ammonia breakpoint phenomenon. However, after the breakpoint has been reached at a molar free chlorine/ammonia ratio = 1.5, significant amounts of free chlorine are not found until the molar chlorine/ammonia ratio reaches 4. Morris (1952) found it was carbon and not nitrogen that was being oxidized between the molar ratios 1.5 and 2. The original nitrogen was still present either as ammonia or organic nitrogen. Oxidized carbon was released as carbon dioxide. Stanbro and Smith (1979) found that the spontaneous decomposition products of N-chloroalanine (a rapidly formed chlorination product of alanine) depended on Ph and consisted of acetaldehyde, ammonia, carbon dioxide and chloride or, pyruvic acid, ammonia and chloride ion. The reaction was first order in N-chloroalanine with a half life of 46 min at pH 7 and 25 °C. The reaction rate coefficient increased by a factor of more than three for each 10 °C temperature increase. It was not affected by pH within the range of 5 to 9. Apparently, N-chloroalanine formed during the chlorination of natural waters will degrade in a few hours to substantially less toxic products.

Proteins have a complex structure and formation of organic chloramines from proteins is relatively slow. Loss of nitrogen, even after prolonged contact with free chlorine, is very small. Compounds with intermediate complexity (e.g., small polypeptides) show intermediate behavior. As a result, water containing considerable organic nitrogen does not demonstrate