Kinetics of biofilm growth and substrate uptake in model drinking water systems
by Phillip Wesley Butterfield

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
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Abstract:
The effects of different substrates on kinetics of biofilm growth and substrate uptake in model drinking water systems were investigated. Biofilm were grown using rotating annular reactors at 20°C and influent substrate concentrations from 500 to 2000 μg C/L. Substrate groups were amino acids, carbohydrates, humic substances and a mix of the three (mixed substrates). Two reactors were operated in parallel; one was the control and the other was chlorinated.

Growth rates were determined using three methods: 1) mass balance for biomass and substrate across reactors, 2) batch cultures using suspended biofilm cells and, 3) uptake of 3H-leucine by attached biofilm on reactor sample slides. Substrate uptake and yield were evaluated for biofilm in reactors and batch culture.

Specific growth rates based on mass balances for chlorinated reactors were greater than for the control. Chlorinated reactors using carbohydrates or mixed substrates had growth rates greater than for amino acids and humic substances. Growth rates based on mass balance for the control reactor biofilm were statistically the same for all substrates except humic substances, which had the lowest growth rate.

Kinetic parameters determined using biofilm cells in batch culture did not generally apply to biofilm in reactors. Removing cells from the biofilm structure alters important parameters such as mass transfer, impact of nutrients that attach to the biofilm matrix, cell physiology, and the influence of chlorine.

Growth rates determined using leucine uptake were comparable to those based on mass balance for control reactor biofilm, but much less than mass balance based growth rates for the chlorinated biofilm. Chlorination may influence the ability of biofilm cells to utilize amino acids such as leucine.

Fractional carbon removal across reactors was constant by substrate and reactor type over the range of substrate concentrations used. Substrate uptake normalized to biomass was greater for chlorinated biofilm than for control. Yield was less for chlorinated biofilm than for control. Although chlorination reduced biomass in the biofilm, the biomass had greater rates of growth and substrate uptake than in the control. Lower yield in chlorinated biofilm may indicate biofilm cells' need to produce additional exopolymeric substances.

KINETICS OF BIOFILM GROWTH AND SUBSTRATE UPTAKE IN MODEL

DRINKING WATER SYSTEMS

by

Phillip Wesley Butterfield

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Phillip Wesley Butterfield

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.

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Date *November 25, 1998*

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ABSTRACT

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CHAPTER 1

INTRODUCTION

General

Drinking water utilities have become increasingly concerned about the microbiological stability of drinking water in distribution systems. Drinking water quality deterioration between the treatment facility and the customer can be the result of actions by microbial biofilm attached to pipe surfaces within the distribution system. Water quality characteristics potentially impacted by biofilm in the distribution system are disinfectant and disinfection by-products concentrations, corrosion products, taste and odor characteristics and microbiological quality. The term "regrowth" is used by the drinking water industry to describe the occurrence of coliform bacteria in water samples arising from growth or "regrowth" in biofilm when no other source of system contamination can be implicated. New drinking water quality regulations, particularly with respect to disinfection by-products and microbial regrowth, have prompted the drinking water industry to investigate factors causing regrowth and the growth of biofilm in distribution systems.

Microorganisms are found in extremely well treated drinking water and are capable of attaching to pipe wall surfaces and forming a microbial community called biofilm. The biofilm utilizes dissolved nutrients found in drinking water for the purposes of energy and growth. Carbon is the nutrient limiting microbial growth in most drinking

water systems; nitrogen, phosphorous and trace elements are typically in sufficient quantity to not limit growth.

The Total Coliform Rule of the federal Safe Drinking Water Act establishes stringent criteria for the number of coliform positive samples a drinking water utility may have before measures such as public notification or a "boil order" take effect. The presence of coliform bacteria in a drinking water sample should indicate that some form of contamination has taken place. Many times no evidence of contamination such as backflow from a sanitary sewer, broken water main, or loss of disinfectant can be found by utility operators. In the case of no known contamination, the presence of coliform bacteria in drinking water has been associated with the release of coliform bacteria growing in the biofilm within the distribution system (Camper, A. K., 1995). Whether the coliform bacteria come from a true contamination event or from the biofilm, the microbial quality of the drinking water must be assumed compromised and appropriate steps taken to protect public health.

The drinking water industry has begun to investigate regrowth and biofilm growth in general to better understand how to minimize regrowth events and biofilm in distribution systems. Addressing issues such as microbial regrowth requires a basic understanding of how biofilm found in drinking water treatment and distribution systems respond to various types of organic compounds naturally found in water. Since the same compounds in drinking water thought responsible for biofilm growth are also the precursors of disinfection by-products, water treatment processes that remove those

carbon compounds can minimize regrowth, thus improving microbiological stability, and reduce disinfectant demand and formation of disinfection by-products.

Research Goal and Objectives

The goal of the research reported in this dissertation was to broaden the understanding of which organic carbon compounds in drinking water most impact biofilm growth in drinking water systems. Specific objectives of the research were as follows:

1. Investigate the growth response of biofilm in a model drinking water system to three major groups of naturally occurring organic compounds (substrates):

   o Amino acids

   o Carbohydrates

   o Humic substances

2. Determine parameters describing growth and substrate uptake kinetics of biofilm cells grown in a model drinking water system utilizing each of the three major substrate groups.

3. Compare kinetic parameters for biofilm cells from a chlorinated and non-chlorinated (control) system for each of the three major groups of substrates.

Research Approach

The approach to the research involved development of biofilm in reactors capable of simulating conditions in a drinking water distribution pipeline. One or a combination

of the substrate groups was fed to two reactors operating in parallel, one reactor receiving free chlorine as a disinfectant and the other reactor serving as the control. The reactors were monitored to assess the response of the biofilm to varying concentrations of the substrate group and disinfectant. Batch cultures utilizing biofilm cells from reactor surfaces were used to assess the response to different individual compounds within the substrate group. For example, the amino acids substrate group consisted of four individual amino acids fed in combination to the reactors and assessed individually and in combination using the batch cultures.

A technique was developed and utilized to assess the growth rate of attached biofilm cells by determining the microbial uptake of leucine labeled with the radioisotope tritium. This technique was applied to biofilm from reactors and was used to develop growth rates for the biofilm under different substrate concentration conditions. The approach is described in detail in Chapter 3, MATERIALS AND METHODS.

Data collected from the reactors were utilized to determine biofilm growth and substrate uptake parameters. Cell concentration, cell size, and dissolved organic carbon were monitored and utilized to assess reactor biofilm growth kinetics. Batch culture cell number, cell size and dissolved organic carbon concentrations were monitored over time to allow determination of comparative kinetic parameters. The techniques used to determine kinetic parameters are described in detail in Chapter 3, MATERIALS AND METHODS.

The results of the research are reported in Chapter 4, RESULTS. The results for each major substrate group are presented in two major categories: 1) reactor biofilm kinetics data and 2) batch culture kinetics data. Each category of data is evaluated to determine kinetic parameters for growth and substrate uptake. Comparisons are made between data for different substrate groups for both the chlorinated and control reactors to investigate which substrate group has the greatest impact on particular kinetic parameters.

Chapter 5, DISCUSSION, contains a discussion of the results, organized in a manner similar to Chaper 4. Chapter 6, SUMMARY, presents a summary of the results and the primary conclusions drawn from the reported research.

CHAPTER 2

LITERATURE REVIEW

Impacts of NOM on Drinking Water Quality

Heterotrophic bacteria, including coliforms and other potentially pathogenic organisms, utilize the biodegradable fraction of naturally occurring organic matter (NOM) as a carbon and energy source and form biofilm on surfaces in drinking water systems (Characklis, W. G., 1988). Reaction of NOM and biofilm with disinfectants can lead to a reduction in the disinfectant residual concentration, posing a possible risk to public health. Biofilm regrowth has been correlated with the occurrence of suspended coliforms which are detected during routine monitoring of the water (LeChevallier, M. W., 1990) and have been shown to be part of the biofilm (Camper, A. K., 1995). Conditions that promote biofilm growth in a distribution system can also lead to increased biocorrosion of the pipe and the production of metabolites that cause offensive tastes and odors (Dukan, S. et al., 1996). A trophic food web can develop within the distribution system under extreme circumstances, leading to higher-order problem organisms such as *Asellus* (AWWA, 1995).

Biological stability of drinking water has become an issue of increasing importance to the drinking water industry. No single definition or criteria exists to describe what makes water biologically stable. Rittmann and Snoeyink (1984) describe a biologically stable water as one that does not support a significant amount of microbial

growth. A definition does not exist for what is an acceptable amount of microbial growth. Most efforts by researchers have been to define a single parameter that can be used to determine biological stability. As a single parameter cannot describe the inorganic characteristics of water, neither can a single parameter be used to describe biological stability.

Several techniques have been developed to measure the biological stability of drinking water. Most known of those methods is the test for assimilable organic carbon (AOC) developed by van der Kooij (1992; van der Kooij, D. et al., 1982). AOC was defined as the portion of the biodegradable carbon that can be converted to biomass and expressed as a carbon equivalent. The method of van der Kooij used a batch culture technique with an innoculum of *Pseudomonas fluorescens* (strain P17) and a *Spirillum* species (strain NOX). Another measurement of biological stability is biodegradable dissolved organic carbon (BDOC). The measurement of BDOC involves determining the change in dissolved organic carbon (DOC) when a water sample is incubated with an indigenous mixed microbial biofilm for a set period of time. The biofilm can be on sand from a biologically active sand filter as in the methods of Joret-Lévi (1988) or Billén-Servais (Servais, P. et al., 1987), or developed on glass beads in a column reactor (Lucena, F. et al., 1990; Ribas, F. et al., 1995; Kaplan, L. A. and Newbold, J. D., 1995).

Significant work has been performed to determine the acceptable level of AOC or BDOC in drinking water and still ensure biological stability. van der Kooij (1992) reported that AOC concentrations less than 10 micrograms as carbon per liter (µg C/L)

kept the plate count on agar medium less the 100 colony forming units (cfu) per milliliter (ml). LeChevallier et al. (1991) investigated the occurrence of coliforms in a full-scale distribution system. It was proposed that AOC levels should be less than 50 μg acetate carbon equivalents per liter to minimize the occurrence of coliform regrowth events. Servais et al. (1991) has estimated biological stability can be achieved when BDOC levels are less than 0.2 mg/L. Camper (1995) found no association between AOC levels and coliform or heterotroph regrowth in a pilot distribution system consisting of unlined mild steel pipe.

A universal method to determine biological stability in drinking water systems does not exist. Apparently the interactions between biofilm cells and the numerous possible substrates in drinking water are too complex to measure with a single parameter. A more comprehensive approach has been undertaken in the research reported herein. Basic groups of utilizable substrates have been identified for further investigation to determine the response of biofilm in drinking water systems in terms of kinetic parameters such as growth and substrate uptake. The approach is a first step in determining if certain groups of substrates are more important than others in the assessment of biological stability. This literature review focuses on naturally occurring organic matter, its occurrence and impact on microbial growth and substrate uptake. The kinetics of drinking water biofilm will be reviewed as it relates to the reported research. Models used to predict biofilm accumulation and substrate uptake are reviewed, including models used for drinking water systems. Because chlorine is the most widely used

disinfectant in drinking water and was used in this research, a review of its effects on bacterial cells has been included.

Naturally Occurring Organic Matter in Water

Naturally occurring organic matter (NOM) in surface waters includes many compounds; most are complex in nature and difficult to characterize. NOM results from the interactions between soil, plants, microorganisms and water in the environment. NOM has traditionally been characterized by many into two general categories, labile and refractory (Krasner, S. W. et al., 1996). To provide a source of carbon and energy microorganisms can easily degrade the labile fraction. The refractory fraction is believed to not be as amenable to biodegradation and consists of compounds that have been recycled in the aquatic environment.

The sources of NOM can be characterized as pedogenic (derived from the soil) and aquagenic (derived from the aquatic environment) (Krasner, S. W. et al., 1996). The proportions of pedogenic and aquagenic NOM are variable depending upon the source of the surface water. Surface water contains 5-10 percent proteinaceous compounds, 10-20 percent polysaccharides, 5-20 percent aquagenic refractory organic matter, and 50-80 percent pedogenic refractory matter (Krasner, S. W. et al., 1996). Most refractory matter can be classified as humic substances.

The watershed and uses of water and land therein impact the nature of the NOM in a water supply. Aiken and Cotsaris (1995) described the importance of the soil

chemistry, hydrology and source material in the make up of NOM. Water sources from areas with wetlands and marshes can be expected to have a high amount of humic substances as part of the total NOM. Important sources of dissolved free carbohydrates in freshwaters are enzymatic degradation of detritus and dissolved organic material, and extracellular organic matter released by phytoplankton (Münster, U. and Chróst, R. J., 1990). Water sources with a high algal activity may have higher than normal concentrations of carbohydrates and amino acids. Source waters with municipal and/or agricultural wastes may be considered to have higher than normal contents of nitrogen compounds, modeled in this project using amino acids as the substrate.

Organic carbon in water consists of both dissolved organic carbon (DOC) and particulate organic carbon (Aiken, G. and Cotsaris, E., 1995). An operational definition for DOC is the fraction passing a 0.45-micron-pore-size (μm) filter, the amount retained on the filter being the particulate fraction. The particulate fraction found in most surface waters consists primarily of microorganisms and detritus material (Aiken, G. and Cotsaris, E., 1995). Typically DOC makes up over 90 percent of the organic matter in most waters (Thurman, E. M., 1985; Owen, D. M. et al., 1995).

Wetzel (1991; 1992; 1990) indicates the source of most dissolved organic matter (DOM) in lake and river systems is from photosynthesis by plants followed by microbial degradation of the plant products. Photosynthesis by phytoplankton provides a small portion of the DOM. The action of attached microorganisms at interface zones (wetland and littoral zones near lakes and rivers) produces most DOM. The input of DOM from

soil and detritus due to runoff is very sporadic and does not constitute the major source of DOM. Pelagic sources of DOM from the action of phytoplankton and bacteria are small compared to the inputs from the wetland and littoral zones.

The source of DOC in an aquatic environment can be allochthonous, entering from a terrestrial watershed, or autochthonous, derived from microorganisms and plants within the water body (Aiken, G. and Cotsaris, E., 1995). Biodegradation and leaching of organic detritus in soils of the watershed are the major source of DOC (Aiken, G. and Cotsaris, E., 1995).

Leenheer (1981) indicated DOC could be divided into six fractions based on chromatographic techniques. The six fractions are hydrophobic acids, bases and neutrals; and hydrophilic acids, bases and neutrals. Humic, fulvic and low molecular weight acids make up approximately 75 percent of the total DOC, neutrals approximately 21 percent, and bases the remaining approximately 5 percent (Malcolm, R. L., 1991). Refractory organic compounds, defined as aquatic fulvic acids and hydrophilic acids, make up the majority of the DOC compounds (Aiken, G. and Cotsaris, E., 1995).

Amon and Benner (1996) investigated the bacterial utilization of high-molecular weight (>1 kDa) dissolved organic carbon in freshwater and marine environments. High molecular weight (HMW) compounds made up over 80 percent of the DOC in a freshwater environment (Amazon River) whereas 70 percent of the marine DOC was low-molecular weight (LMW) compounds less than 1 kDa in size. Studies of the size and diagenetic state of organic matter in the environment have found that compounds have

been increasingly degraded as their size decreases (Hedges, J. I. et al., 1994). Dissolved

organic matter (DOM) in river environments has been found to be composed of HMW

compounds (Hedges, J. I. et al., 1994) and can therefore be considered diagenetically

young. Based on bacterial growth efficiency and direct measurements, Amon and Benner

(1996) indicate that LMW DOM has a lower carbon to nitrogen ratio (C:N) than the more

reactive HMW DOM, possibly due to the higher organic nitrogen or amino acids

composition of LMW DOM. In a study of 10 oligotrophic lakes with varying degrees of

humic content, Tranvik (1990) found a higher percentage of HMW compounds (size

>10,000) as a fraction of total DOC with increasing humic content and DOC.

Humic Substances

Chemically complex polymers called humic substances are found in both surface

and ground waters. Humic substances, a general and operational term, includes two

categories of organic acids, humic and fulvic acids (Malcolm, R. L., 1985; Aiken, G. and

Cotsaris, E., 1995). The definitions given humic and fulvic acids are operationally based

on the techniques used to separate each fraction. The definition for each acid is as

follows (Malcolm, R. L., 1985):

- ○ Humic acids —insoluble and form precipitates at pH 1
- ○ Fulvic acids—soluble at pH 1.

Fulvic acids comprise the major component of humic substances in surface waters

(Malcolm, R. L., 1985; Aiken, G. and Cotsaris, E., 1995) and are approximately 20-80

percent of the DOC (Aiken, G. and Cotsaris, E., 1995).

Schnitzer (1980) defines humic substances as "heterocondensation polyphenolic polymers of reactive simple monomers." The reactive monomers consist of polyphenols, amino acids, simple sugars and quinones (Malcolm, R. L., 1985; Thurman, E. M., 1985) resulting from microbial degradation of natural organics such as plants, animals and detritus or from cell lysis or exudates. Aquatic humic and fulvic acids have typical molecular weights of 500-1,000 for fulvic acid and 3,000 for humic acid (Malcolm, R. L., 1985). The degree of aromaticity of humic substances is dependent on its source. Lignin derived organic matter has a high content of aromatic carbon, high in phenolic content, and low in nitrogen content whereas microbially derived substances are low in aromatic carbon and phenols but high in nitrogen content (Aiken, G. and Cotsaris, E., 1995). Monomeric and polymeric substances are released by phytoplankton (Münster, U., 1993). It therefore follows that pedogenic humic substances are more aromatic (Schnitzer, M., 1980; Zumstein, J. and Buffle, J., 1989) and have higher C:N ratios compared to aquagenic humic substances which are more aliphatic (Gagosian, R. B. and Stuermer, D. H., 1977; Zumstein, J. and Buffle, J., 1989) and have lower C:N ratios.

The humification process model presented by Hatcher and Spider (1988) involves the rapid utilization of labile, LMW macromolecules during early diagenesis. Refractory compounds are not utilized early and become what is termed humin. Humic and fulvic acids are formed during microbial degradation of humin, leading to more oxidized compounds of lower molecular weight and increasing solubility.

Humic substances make up the major fraction of the so-called refractory DOC pool in most freshwaters. Humic substances make up 50-75 percent of the NOM found in surface waters (Malcolm, R. L., 1991). Kaplan (1993) presented information for the NOM composition of a typical surface water indicating humic substances make up approximately 69 percent of its composition, free and humic bound amino acids 2 percent and free and humic bound carbohydrates 16 percent, the remaining being other compounds.

Humic substances, a major component of DOM, have been shown to form complexes with extracellular enzymes, inhibiting the action of those enzymes (Wetzel, R. G., 1992). The humic substance-enzyme complexes can be transported to other parts of the ecosystem, such as the pelagic zone, where UV radiation from sunlight can cleave the enzyme-humic complex and make the enzymes available. Humic substances in soft waters with lower concentrations of divalent cations (Ca^{2+}, Mg^{2+}) are better able to form the humic-enzyme complex. The presence of divalent cations appears to suppress formation of humic-enzyme complexes. Productivity using humic substances has been found to be greater in hard waters than in soft since extracellular enzymes produced by microorganisms do not complex with the humic material. Polymeric substances are partially recalcitrant to degradation and their assimilation requires extracellular hydrolysis (Billén, G., 1991)

Humic substances actively attach to most surfaces and particulate matter in natural water, creating the negative charge found on most colloids, particulates and

surfaces (Beckett, R., 1990). The charge density associated with functional groups (primarily carboxyl groups) controls the attachment or adsorption of humics to surfaces. In aqueous solutions the functional groups are negatively charged. Humics can bind to surfaces through electrostatic attraction or covalent bonds (Tipping, E., 1981). The presence of divalent cations (Ca^{2+}, Mg^{2+}) can increase the adsorption of humic substances to iron oxides (Tipping, E., 1981) but phosphates and silicates decrease humic adsorption (Tipping, E. and Cooke, D., 1982).

Carbohydrates and Related Substances

Algogenic material found in surface waters is derived from algae, either directly from algal cells or as extracellular organic matter (EOM) given off by algal cells. Algogenic EOM is polar, primarily aliphatic, and is similar to polysaccharides. Polar compounds comprising EOM include glycols, glycoses, deoxyglycoses, glyconic acids, glycuronic acids and glycaric acids (Hoyer, O. et al., 1986; Hoyer, O. et al., 1987). EOM consists of neutral and acidic polysaccharides (20-40 percent) and uronic acids (2-10 percent). The algal species and their growth phase are factors influencing the composition and properties of EOM (Hoyer, O. et al., 1986; Hoyer, O. et al., 1987).

Carbohydrates in freshwater systems consist of dissolved combined and free carbohydrates (DCCHO and DFCHO, respectively) (Jørgensen, N. O. G. and Jensen, R. E., 1994), making up from 1 to 30 percent of the total DOC (Jørgensen, N. O. G. and Jensen, R. E., 1994). In studies of carbohydrates in a mesotrophic lake in Denmark by Jorgensen and Jensen (1994) the concentrations of individual free carbohydrates were

typically in the range of 5-50 nM, and total dissolved free carbohydrates were from 67 to 224 nM. Dominant dissolved free carbohydrates were galactose, glucose, fructose and mannose/xylose. During incubation of 1.0 micron (μm) pore size filtered lake water it was found that carbohydrates were most likely excreted by bacteria. Important sources of DFCHO in freshwaters are enzymatic degradation of detritus and dissolved organic material, and extracellular organic matter released by phytoplankton (Münster, U. and Chróst, R. J., 1990). Jorgensen and Jensen (1994) found glucose and galactose to be the primary species of DCCHO in lake water, both of which are found as exopolysaccharides (Whitfield, C., 1988). Glucose is a major component of many polysaccharides and galactose is found in structural carbohydrates (Wicks, R. J. et al., 1991). In batch culture studies Jorgensen and Jensen (1994) found DCCHO were degraded to DFCHO by bacterial enzymatic activity.

A review article by Münster (1993) found the labile organic carbon pool, consisting of dissolved free carbohydrates and amino acids, to form 10 to 30 percent of the total dissolved organic carbon (DOC). However, 70 to 90 percent of the DOC consisted of material apparently recalcitrant to microbial degradation, most likely in the form of humic material.

Amino Acids

Pedogenic and aquagenic proteinaceous compounds make up 5 to 10 percent of natural organic matter in water (Krasner, S. W. et al., 1996). Lytle and Perdue (Lytle, C. R. and Perdue, E. M., 1981) investigated the amino acid content of the Williamson River

before and after it flowed through the Klamath Marsh in southwestern Oregon. Amino acids concentration before the marsh were ~1μM, increasing to ~5μM in the marsh, and ~8 μM in the river after the marsh. The relative abundance of amino acids did not change throughout the river system or seasonally, and the amino acids in order of abundance were glycine>aspartic acid>alanine>glutamic acid≅serine. Greater than 96 percent of the amino acids were associated with the aquatic humic substances. Seasonal variations in total amino acids and humic carbon indicated higher concentrations of both during the winter and spring months when surface runoff into the river system was high. Lytle and Perdue concluded that most humic carbon and amino acids were derived from soil humic substances flushed into the river system during periods of high runoff.

Much of the dissolved organic matter produced by phytoplankton primary production enters the dissolved phase by processes such as autolysis and exudation of algae and sloppy feeding by zooplankton (Simon, M. and Rosenstock, B., 1992). This process produces much of the dissolved combined- and free-amino acids content of lakes (Søndergaard, M. et al., 1988). High concentrations of dissolved amino acids (up to 13 percent of the DOM) have been seen in eutrophic lakes with senescent phytoplankton blooms (de Haan, H. and de Boer, T., 1979). Most of the dissolved amino acids in the eutrophic lake, Tjeukemeer, were found in the apparent molecular weight fraction > 5,000; ca. 15 percent were free amino acids and/or simple peptides and most dissolved amino acids were associated with the fulvic acids (de Haan, H. and de Boer, T., 1979). In the study of one oligotrophic and two eutrophic lakes in Denmark, Jørgensen (1987)

determined phytoplankton degradation was a major source of dissolved free amino acids (DFAA). DFAA concentrations varied from 216 to 3,672 nM in the oligotrophic lake and from 78 to 1,509 nM in the two eutrophic lakes studied by Jørgensen (1987). Serine, glycine, alanine and ornithine were the dominant DFAA in Jørgensen's study (1987). Jørgensen and Jensen (1994) found the total DFAA concentration in a mesotrophic lake to be from <100 to 455 nM, with serine, glycine and alanine the dominant compounds.

Bacterial Response to Substrates

Microbial ecologists have studied the response of indigenous aquatic microbial communities to natural organic matter found in freshwater and marine environments. The goal of the microbial ecologists' work has been to understand the role of microorganisms in cycling of carbon and other nutrients in the environment. The relevance of the ecologists' work is that it involves oligotrophic environments, and the primary groups of carbon substrates studied have been amino acids, carbohydrates and humic substances.

General Bacterial Response to Oligotrophic Environments

Drinking water distribution systems are man-made environments where there is purposely a deficiency of nutrients for microbial growth and oxidizing disinfectants have been added to inhibit microbes. However, microbial growth does occur even when treatment processes efficiently remove organic compounds. Microbiologists define growth as the increase in the number of viable cells. Energy is a requirement of cell

growth. In nutrient-limited oligotrophic natural or man-made environments, energy is in short supply or may only be available for short periods of time. In a natural environment only a portion of the microbial population may be active while others are in a state of starvation (Morita, R. Y., 1988). When microorganisms are not growing due to a lack of energy, then the cells must be in a physiological state called "starvation survival" (Morita, R. Y., 1988). Maintenance energy requirements found in laboratory cultures are much greater than the available carbon supply found in nature, yet microorganisms are able to survive in oligotrophic soil and aquatic environments (Morita, R. Y., 1988). Morita (1988) reported four possible starvation survival responses for populations where insufficient energy exists: 1) cells increase in number then their numbers decline to a constant value, 2) cell numbers are constant, 3) cell numbers increase and remain constant, and 4) cell numbers decrease to a constant value. Starvation conditions lead to very small cells being formed, called ultramicrobacteria (Morita, R. Y., 1988). The small size gives the cell an advantage in an oligotrophic environment by increasing the cell surface area to volume ratio, allowing the cell to contact more substrate. The starvation process can cause cells to shift to a high affinity transport system to acquire substrates from a dilute concentration environment (Geesey, G. G. and Morita, R. Y., 1979). Other starvation survival changes include decrease in the cell lipid content and changes in cell protein (Morita, R. Y., 1988).

Harder and Dijkhuizen (1983) reviewed the structural and functional changes in planktonic bacteria under nutrient limited growth conditions. General oligotrophic

strategies include increasing the transport rate of existing uptake systems or synthesizing

new high-affinity systems for the nutrient that is growth limiting. Another strategy is to

increase the internal metabolism of a nutrient the cell has stored when the concentration

of the nutrient in the cell becomes low. The cell can rearrange the chemical composition

of cellular structures to more efficiently respond to available nutrients. How a cell

responds to a growth-limiting nutrient depends on the growth rate and the concentration

of the nutrient in the environment. *Pseudomonas aeruginosa* responds to glucose

limitation by expressing a high-affinity uptake system (Harder, W. and Dijkhuizen, L.,

1983). Organisms faced with a decreasing nutrient concentration respond by increasing

synthesis of catabolic enzymes or synthesizing a new enzyme to more efficiently capture

the growth limiting substrate (Harder, W. and Dijkhuizen, L., 1983). As discussed

below, simultaneous utilization of substrates is another bacterial survival response to

nutrient limited environments.

In a review article, Koch (1996) proposes that obligate oligotrophs are not capable

of extruding or passively leaking small compounds from the cytoplasm, a process called

overflow metabolism commonly found in most microorganisms growing in a nutrient

sufficient environment. Because oligotrophic bacteria have a high density of transport

mechanisms per unit area of membrane and their internal volume is small, exposure of

the cell to an abundant nutrient concentration can lead to high internal concentrations

which may damage the cell, a process called substrate activated or accelerated death.

Amino Acids

Simon (1985) investigated the specific uptake rates of amino acids for free-living and attached bacteria in a mesotrophic lake. Generally the specific uptake rates for attached bacteria were greater than for free-living bacteria. Measured uptake rates for amino acids were 9.41×10^{-11} to 6.11×10^{-8} nanograms of carbon per cell per hour (ng C cell^{-1} h^{-1}).

In a study of oligotrophic Lake Almind in Denmark, Søndergaard et al. (Søndergaard, M. et al., 1988) determined that the uptake of DFAA was responsible for 52 to 62 percent of the gross bacterial production and that the amino acids were derived primarily from extracellular organic carbon released by phytoplankton.

Results of an investigation of the incorporation of leucine and methionine into cell protein by Kirchman et al. (1986) indicated the regulation of uptake and biosynthesis of amino acids, influenced by the concentration of dissolved amino acids, could impact the rates of uptake and mineralization of other dissolved compounds and bacterial growth.

In a study of a marine *Pseudomonas* sp. strain, Bright and Fletcher (1983a) found that cells attached to some surfaces took up amino acids at higher rates than planktonic bacteria. Studies of activity using microautoradiography and a marine *Pseudomonas* sp. indicated those cells attached to a glass surface were more active in taking up amino acids compared to unattached cells (Bright, J. J. and Fletcher, M., 1983b; Fletcher, M., 1979), but the amino acid, its concentration and the attachment surface influenced activity of the biofilm cells. The adsorption of dissolved substrates to surfaces (Marshall, K. C., 1988)

may be one explanation for the observed higher activity of attached bacteria. However, Marshall (1988) indicates adsorption of hydrophobic molecules, macromolecules and humic substances to surfaces may be more important in a natural environment since the more labile monomers are quickly metabolized by the planktonic bacteria. Simon (1985) found high fluctuations in the specific uptake rates for amino acids in attached bacteria and in general the activity of attached bacteria was greater than for suspended bacteria. In a study of growth using biomass turnover times, Simon (1985) did not find major differences in growth between suspended and attached bacteria even though the attached bacteria had greater uptake rates for organic compounds (Simon, M., 1988).

Jørgensen (1987) studied uptake of amino acids by natural bacterial populations from a mesotrophic lake using batch cultures. The bacteria readily assimilated the DFAA with a 69 percent reduction during the period from 6 hours to 24 hours. Serine, glycine and alanine were the dominant DFAA.

The four known pathways for amino acids to become part of the intracellular pool are (Simon, M., 1991): 1) direct uptake of dissolved free amino acids; 2) intracellular hydrolysis of oligopeptides (2 to 5 amino acids) that have been directly taken up; 3) uptake of dissolved combined amino acids using cell-surface-mediated hydrolysis combined with direct uptake of dissolved free amino acids and oligopeptides; and 4) intracellular *de novo* synthesis of amino acids using ammonium and organic compounds such as carbohydrates. In the study of a thin wastewater biofilm, Eighmy and Bishop (1984) showed that aspartate was transported to the biofilm via a high-affinity, low-

capacity transport system with an apparent Michaelis-Menten transport constant (K_t) of 4.3 to 4.6 μM, and by a low-affinity, high-capacity system with a K_t of 116.7 μM. The high-affinity transport system was a membrane bound proton symport and the low-affinity system was a binding proton-mediated system using phosphate bond energy.

Carbohydrates and Mixed Substrates with Carbohydrates

In a study of the kinetics of uptake of glucose and acetate by planktonic bacteria, Wright and Hobbie (1966) found low concentrations of the substrates (1-10 μg/L) in a eutrophic lake. They speculated that planktonic bacteria utilized these substrates very fast (high rates of uptake), therefore the concentrations remained low in the natural aquatic environment.

Extracellular organic matter derived from algal blooms can be a source of carbohydrates in natural waters. Bell (1980) studied the uptake of radiolabeled extracellular products produced during an algal bloom in the Trondheimsfjord, Norway. The native bacterial population was shown to utilize the extracellular products. Maximum bacterial activity occurred during the senescent phase of the bloom. Kato (1994), utilizing [^{14}C]bicarbonate and enclosures within a eutrophic lake, determined extracellular dissolved organic carbon released from the photosynthetic phytoplankton was an important source of carbon for the planktonic bacteria; 50 to 60 percent of the labeled extracellular DOC was transformed into bacterial macromolecules. De Haan and de Boer (1979) found increased concentrations of dissolved carbohydrates just after blooms of diatoms, green and blue-green algae.

Jørgensen and Jensen (1994), in a study of natural populations from a mesotrophic lake, found that glucose and fructose incorporation, in terms of carbon, equaled 44 to 92 percent of the amino acid incorporation. Fructose and glucose assimilation was similar in experiments performed in the dark to minimize impacts of photosynthetic bacteria.

Several investigations have been performed to assess the impacts of multiple-substrates, including carbohydrates, on growth kinetics of bacteria in oligotrophic environments. Law and Button (1996) investigated growth kinetics of a marine coryneform bacterium under various substrate-limited regimes. Continuous cultures using glucose only were compared to cultures with multiple substrates consisting of glucose and an amino acid(s). At glucose concentrations as low as 0.3 µg/liter the bacterium was able to utilize the substrate as a nutrient source even though the threshold value (S_{min} or minimum substrate concentration that can support cell growth), determined using kinetic considerations from the continuous cultures was 210 µg/liter. The addition of arginine, arginine-glutamate and a mixture of 20 amino acids stimulated the growth rate and reduced the threshold values for glucose and the amino acids. Cell yield was found to increase with decreasing growth rates. The bacterium was able to survive in a starvation mode, utilizing substrates at concentrations in the µg/liter level. By providing a multiple carbon source, the concentrations required for growth were reduced substantially over those found from single substrate cultures.

Escherichia coli were used in continuous culture to assess kinetics of a multiple carbohydrate substrate (Lendenmann, U. et al., 1996). The carbohydrates were glucose,

galactose, maltose, ribose, arabinose and fructose. Steady-state concentrations of the carbohydrates were lower when multiple substrates were present compared to single substrate cultures. All six carbohydrates were utilized simultaneously under carbon-limited conditions. It was proposed that under environmental conditions microorganisms utilize carbon substrates at concentrations less than those found during single-substrate studies. When *E. coli* cells were grown in glucose-limited continuous cultures at dilution rates of 0.2, 0.3 and 0.6 h^{-1}, the cells were able to immediately uptake other carbohydrates without lag (Lendenmann, U. and Egli, T., 1995). The sugars fructose, mannose, maltose and ribose were taken up immediately, galactose was taken up immediately but growth was delayed after a lag period. When *E. coli* was grown in continuous cultures at high glucose concentrations the uptake of other sugars was repressed. At moderately low glucose concentrations *E. coli* was able to immediately uptake other substrates. Such uptake capabilities would be relevant in oligotrophic environments.

Schmidt and Alexander (1985) investigated the impacts of multiple substrates on the biodegradation of organic compounds at low concentrations. When substrates such as phenol and aniline were present at high concentrations, the uptake was diauxic in nature for the bacterium *Pseudomonas acidovorans*; the most easily utilized substrate was preferentially utilized before the other. However, when both substrates were at initially low concentrations the uptake of the substrates occurred simultaneously. Growth of a *Pseudomonas* sp. in a glucose and aniline substrate solution of 3.0 µg/liter of each compound resulted in the bacteria first using unidentified carbon compounds in the salt

medium, followed by simultaneous uptake of the glucose and aniline. When the same bacterium was grown in medium with 300 µg/liter of each compound the growth was diauxic.

In batch culture studies of a facultatively oligotrophic ultramicrobacterium, Schut et al. (1995) reported that the presence of alanine in an alanine-glucose culture did not have the same impacts as seen by Law and Button (Law, A. T. and Button, D. K., 1996). Half-saturation constants were not lower and the specific affinities for glucose did not increase. However, dual-substrate-limited growth using alanine demonstrated alanine utilization rates 50 percent greater than when alanine alone was present, and the maximum specific growth rate of cells for dual-substrate-limited cultures were greater than the maximum attained using a single substrate culture.

Humic Substances

Humic substances have often been regarded as refractory or inert to bacterial degradation (Moran, M. A. and Hodson, R. E., 1990). Amon and Benner (1996) proposed a size-reactivity continuum model based on the investigation of bacterial utilization of both high molecular weight (HMW) and low molecular weight (LMW) compounds found in both freshwater and marine environments. Their work indicated diagenetically young organic matter is the most reactive (biodegradable) and that organic matter becomes less reactive and diagenetically older as its size decreases. In their tests HMW compounds were utilized to a greater extent by planktonic cells than LMW compounds, but the bacterial growth efficiency was greatest for LMW compounds. In a

study of a marine environment, Amon and Benner (1994) found higher and faster utilization of HMW dissolved organic carbon derived during a diatom bloom compared to LMW compounds. In a similar study of Amazon River water, Amon and Benner (1996) documented higher growth efficiencies in the LMW fractions, but planktonic bacterial growth rates were higher in the HMW fraction in all cases investigated. The size-reactivity continuum model appeared to describe the decrease in bioreactivity with size of dissolved organic compounds, also referenced by Hedges et al. (1994). Tranvik (1990) also found greater bacterial production per unit of carbon in HMW compounds compared to LMW compounds in a study of 10 oligotrophic lakes. As the humic content of the HMW component increased the availability to bacteria for growth decreased (Tranvik, L. J., 1990).

Geller (1986) investigated bacterial degradation of macromolecular DOM with apparent molecular weights > 1,500. Using 0.1-μm-pore-size filtered lake water as a medium and two different bacterial species isolated from the lake water, Geller investigated the impacts of aging and nutrient additions on bacterial degradation of the DOM. Batch culture experiments were carried out to six weeks with the following parameters being monitored: UV absorbance, DOC, total cell counts using acridine orange and epifluorescence microscopy. Biodegradation of the DOC took place primarily within the first week of incubation, after which minimal changes in the DOC concentration were observed in cultures without addition of a co-substrate. Aging of the DOM (exposure to natural light) made it more available to one species (*Pseudomonas*).

Addition of glutamic acid to the cultures improved biodegradation of the DOM over the last five weeks of incubation, enhancing biodegradation by 0 to 20 percent. The addition of a pulsed dose of nutrient may have provided cells the required energy to produce extracellular enzymes required for DOM degradation. UV absorbance was not helpful in determining degradation of the DOM since UV-absorbing microbial metabolites produced from glutamic acid or airborne organic substances interfered with measurements.

Tranvik and Höfle (1987) investigated bacterial growth in water from both a humic and a clear lake. The humic water cultures had twice the biomass production of the clear water cultures. Both cultures consumed the same amount of the total DOC. When glucose was added to the cultures it was taken up rapidly during the exponential growth phase. Degradation of phenol, added to certain cultures, was minimal by bacteria in the clear water cultures, but phenol degradation occurred in the humic cultures during the stationary phase. Degradation of phenol was used as a surrogate for humics degradation.

Moran and Hodson (1990) were able to show that natural bacterial populations were capable of utilizing the humic fraction of DOC as a carbon and energy source. Humic substances supported four times less bacterial production than the corresponding nonhumic fraction from the same environment. However, humic substances supported a significant amount of the total bacterial growth on the available DOC.