



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

Montana State University

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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.

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APPROVAL

of a thesis submitted by

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.

Dr. Warren L. Jones

Warren L. Jones
(Signature)

11/25/98
Date

Approved for the Department of Civil Engineering

Dr. Donald A. Rabern

Don Rabern
(Signature)

11/25/98
Date

Approved for the College of Graduate Studies

Dr. Joseph J. Fedock

Joseph J. Fedock
(Signature)

11/30/98
Date

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Date November 25, 1998

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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.

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):
 - Amino acids
 - Carbohydrates
 - 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 Chapter 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 inoculum 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 ($\mu\text{g C/L}$)

kept the plate count on agar medium less than 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 Jørgensen 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 $\sim 1\mu\text{M}$, increasing to $\sim 5\mu\text{M}$ in the marsh, and $\sim 8\mu\text{M}$ 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 $\approx$ 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, S ndergaard et al. (S ndergaard, 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 $\mu\text{g/L}$) 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 $\mu\text{g/liter}$ 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 $\mu\text{g/liter}$. 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 $\mu\text{g/liter}$ 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.

Lovley et al. (1996) provided evidence of humic substances acting as electron acceptors for anaerobic microorganisms degrading organic compounds and hydrogen. The process yielded energy for growth. A possibility exists that humics act as an electron shuttle between microorganisms and metals such as Fe(III) and Mn(IV). The shuttle of electrons could enhance the ability of Fe(III)-reducing bacteria to pass electrons to Fe(III) oxides.

Limited information is available on the growth of biofilm using humic material as substrates. Namkung and Rittmann (1987) utilized a packed bed biofilm reactor to evaluate the ability of a biofilm grown on humic materials to remove taste- and odor-causing substances. The packed bed reactor (3 millimeter glass beads) was initially inoculated with water from an oligotrophic lake, then fed a mixture of peat fulvic acid and an inorganic nutrient medium. The packed bed reactors had a hydraulic retention time of 19.8 minutes based on void volume (empty bed contact time = 49.5 minutes). Overall removal of the peat fulvic acid, measured as dissolved organic carbon, was approximately 10 percent over a one-year period (at 22 °C) when the influent feed concentration was 1 mg C/L. Short-term nonsteady-state experiments were performed to determine the ability of the biofilm to utilize phenol, geosmin, MIB, and naphthalene. At concentrations of 100 µg/L the removal was 40 to 50 percent for geosmin and MIB, and 90 percent for phenol and naphthalene. The ability of the biofilm reactor to remove a high percentage of phenol may have indicated adaptation of the biofilm to degradation of humic-type substances that typically contain aromatic structures.

Influence of Cell Attachment on Growth

In a review by van Loosdrecht et al. (1990), numerous references were made to research indicating there was a change in microbial activity in cells when attached to a surface. Although a large number of research articles were reviewed by van Loosdrecht et al., there was no consensus on the effect of bacterial adhesion on growth rate or activity of bacteria. For example, the work by Bright and Fletcher (Bright, J. J. and Fletcher, M., 1983a; Bright, J. J. and Fletcher, M., 1983b; Fletcher, M., 1979) found the substratum and environmental conditions influenced how bacteria respond after attachment. Work by others (Jeffrey, W. H. and Paul, J. H., 1986) have shown changes in both the substrate affinity and maximum specific growth rate for attached cells. Macromolecules, such as humic substances, are adsorbed to the biofilm surface and biodegradation takes place by exoenzyme activity (van Loosdrecht, M. C. M. et al., 1990). Early work by ZoBell (1943) concluded the adsorbed molecules might retard movement of the exoenzymes into the bulk liquid, therefore aiding the metabolism of the attached bacteria. The review by van Loosdrecht et al. concluded that attachment to a surface has been shown to both increase and decrease microbial activity; there are many factors which influence activity of biofilm cells and these must all be considered when assessing growth of biofilm compared to planktonic cells.

Kinetics of Drinking Water Biofilm

Kinetic Parameter Estimation for Biofilm

Rittmann et al. (1986) determined the Monod kinetic parameters for an oligotrophic biofilm utilizing an in-situ technique. The biofilm was developed on glass bead packed column reactors utilizing environmental cultures from a lake and soil. Substrates included sodium salicylate, sodium acetate, and phenol; each reactor received a different substrate. In-situ kinetics were determined by performing a series of short-term, non-steady-state experiments once the reactors has reached steady-state conditions as determined by monitoring effluent substrate concentrations. Each short-term experiment utilized a different substrate concentration and was performed for a duration of 4 hours. Substrate concentration in the reactor effluent was monitored using a ^{14}C -labeled radiotracer. Kinetic parameters were determined by using a curve-fitting technique based upon the steady-state model developed by Rittmann and McCarty (1978; Rittmann, B. E. and McCarty, P. L., 1980a; Rittmann, B. E. and McCarty, P. L., 1980b). Basic parameters required for the model and technique include average biofilm thickness (L_f), biofilm density (X_f), effective diffusion layer (L), substrate molecular diffusivity in the liquid phase (D) and in the biofilm (D_f), influent substrate concentration (S_m), effluent substrate concentration (S_e), and the liquid phase volumetric flow rate (Q). The biofilm model equations for simultaneous reaction and diffusion were solved for a variety of conditions. A family of curves was developed based on the solutions and the

experimental data was visually fit to one of the curves to determine the appropriate kinetic parameters K_S , the Monod half-velocity constant, and k , the maximum specific substrate utilization. In terms of grams of chemical oxygen demand (COD) per cubic meter, K_S values ranged from 0.0005 for salicylate to 0.018 for acetate. Values of k ranged from 0.15 grams COD per gram biomass per day (g/g-day) for salicylate to 1.7 g/g-day for acetate.

Another in-situ technique, based on the method developed by Rittmann et al. (1986) was used by Frías et al. (1994) to estimate kinetic parameters for biofilm developed in glass bead packed columns. The reactor used by Frías et al. consisted of two columns operated in series, originally developed by Lucena et al. (1990) to measure biodegradable dissolved organic carbon (BDOC) content in drinking water. Dechlorinated drinking water was the substrate and nutrient source for the biofilm in the columns. Kinetic experiments were performed using both drinking water and the effluent from the second column as sources of dissolved organic carbon (DOC). Kinetics experiments were performed for both suspended bacteria and the in-situ biofilm in the columns. Two strains of bacteria were isolated from the columns and grown in batch culture for the use in the suspended growth experiments. Substrate concentration was measured as DOC for all experiments.

Frías et al. (1994) found biofilm bacteria had a greater affinity (lower values of K_S) for drinking water compared to the water from the outlet of the first column, and the K_S values for the biofilm were greater than those measured for the suspended bacteria.

K_s for the biofilm in the first column (hydraulic detention time of 1.10 hours based on void volume [EBCT=1.8 hours]) was 0.16 mg DOC per liter and 0.92 mg DOC per liter for the second column in series. Because the experiments were one day in duration, it was believed soluble microbial products were present as part of the DOC measured in the effluent. No measurement of CO_2 was made during the work. The work did show that biofilm that had been acclimated to drinking water had a greater affinity for drinking water substrates than for the more refractory substrates in water that had been treated biologically.

Rotating annular reactors (Rotatorques) were utilized by van der Wende et al. (1989) to estimate kinetic parameters for biofilm in drinking water conditions. Four reactors in series were used to model the plug-flow conditions in a pipeline. Shear stress on the wall of the fixed cylinder was equal to that in a 6-inch-diameter pipeline with an average flow velocity of 3 feet per second. Heterotrophic plate counts using R2A medium were used to determine growth rates for the biofilm. As the dilution rate (inverse of the hydraulic detention time and proportional to the system flow rate) increased from 0.14 per hour (h^{-1}) to 0.43 h^{-1} , specific growth rates for the biofilm cells also increased from 0.00025 h^{-1} to 0.006 h^{-1} , respectively. Increasing the dilution rate (flow rate) apparently provided a higher substrate loading to the biofilm and resulted in higher specific growth rates.

Kinetics of Bacterial Growth

Lewis et al. (1988) investigated the kinetics of mixed microbial assemblages under a wide range of organic substrate concentrations. Experiments were performed using detached biofilm cells grown using natural water samples. Substrate removal rates were enhanced at low substrate concentrations. Enhanced removal was believed to be the result of very efficient uptake systems at low substrate concentrations. Natural mixed populations are kinetically heterogeneous, having a large variety of substrate uptake systems that also have a wide range of characteristic kinetic parameters. Based on experimental work, Lewis et al. state that $S(V_{\max}/K)$ is more appropriate for predicting substrate uptake (v) than the Michaelis-Menten equation when $S > K$ (S and $V_{\max}$ determined at low S). At low concentrations of S where $V_{\max}/K$ is changing, Lewis et al. states neither Michaelis-Menten nor $S(V_{\max}/K)$ are good predictors of v . Therefore, in a heterogeneous kinetic system, kinetic parameters determined at high substrate concentrations are not appropriate for predicting v at low substrate concentrations and vice versa.

Wright and Hobbie (1965) developed a method for determining the uptake kinetics for planktonic bacteria and algae using radiolabeled compounds. They utilized radiolabeled glucose and acetate to assess if different uptake mechanisms existed in natural populations of lake bacteria. Michaelis-Menten enzyme kinetics was present at low substrate concentrations indicating transport systems dependent on substrate concentration. The maximum uptake velocity and the half-saturation constant K were

determined. Another method of uptake applicable to algae, diffusion, was found at higher substrate concentrations. Uptake in the range of 1-100 $\mu\text{g/L}$ of substrate followed Michaelis-Menten kinetics while at higher substrate concentrations, 0.5-5 mg/L , the uptake depended on the substrate concentration, increasing linearly with increasing substrate concentration. At low substrate concentrations bacteria dominated uptake while algae dominated uptake at higher substrate concentrations. Wright and Hobbie stressed the necessity to measure kinetic parameters over a range of substrate concentrations. Later work by Wright and Hobbie (1966) utilized the same methodology of their previous work and the Lineweaver-Burk transformation to determine the uptake kinetic parameters using radiolabeled substrates. At low concentrations of acetate and glucose bacteria were shown to have extremely effective transport systems.

Measurement of Bacterial Growth and Production

A review article by Riemann and Bell (1990) presents four of the most widely used methods of determining bacterial growth: 1) [^3H]thymidine incorporation into DNA, 2) [^3H]leucine incorporation into protein, 3) [^3H]adenine incorporation into DNA and/or RNA, and 4) the frequency of dividing cells. The two methods gaining widespread use in microbial ecology are the thymidine and leucine techniques.

Radioisotope techniques have been utilized by microbial ecologists to measure bacterial growth and production for many years (Pollard, P. C. and Moriarty, D. J. W., 1984; Servais, P., 1995; Thomaz, S. M. and Wetzel, R. G., 1995; Kirchman, D. L. and Hoch, M. P., 1988; Simon, M. and Rosenstock, B., 1992; Wright, R. T. and Hobbie, J. E.,

1966; Fuhrman, J. A. and Azam, F., 1982; Riemann, B. and Bell, R. T., 1990). The two primary isotopes used are tritiated thymidine ($[^3\text{H}]$ thymidine) and $[^3\text{H}]$ leucine. $[^{14}\text{C}]$ leucine has also been used for the same purpose (van Looij, A. and Riemann, B., 1993). Thymidine is incorporated into DNA whereas leucine incorporated into protein. The use of either of these isotopes requires conversion factors to convert isotope incorporation into production or growth (Bjornsen, P. K. and Kuparinen, J., 1991; Servais, P., 1995; Chin-Leo, G. and Kirchman, D. L., 1988; Riemann, B. and Bell, R. T., 1990). Both methods require analysis of both extracellular and intracellular isotope dilutions. Researchers have found that by overwhelming the extracellular pool with added thymidine, *de novo* synthesis can be minimized (Riemann, B. and Bell, R. T., 1990). Similar techniques have been used when $[^3\text{H}]$ leucine is used as the isotope (Servais, P., 1995; Riemann, B. and Azam, F., 1992; van Looij, A. and Riemann, B., 1993). Excess isotope within the cell that is not incorporated into macromolecules is removed during rinsing with ice-cold trichloroacetic acid (TCA) (Riemann, B. and Bell, R. T., 1990; van Looij, A. and Riemann, B., 1993). Conversion factors are determined using either theoretical or empirical methods (Riemann, B. and Bell, R. T., 1990; Bell, R. T., 1990; van Looij, A. and Riemann, B., 1993). Most researchers prefer the development of empirical conversion factors based upon the environment being investigated (Servais, P., 1989; Servais, P., 1995; van Looij, A. and Riemann, B., 1993). Empirical factors are typically determined by measuring cell growth and isotope uptake over time in a dilute batch culture of the microbial population in question. Dividing the

increase in cells, or biomass, by the integrated isotope uptake over time provides the conversion factor (Riemann, B. and Bell, R. T., 1990; Bjornsen, P. K. and Kuparinen, J., 1991). When using leucine in freshwater conditions, isotope dilution must be taken into account to accurately predict uptake of the isotope (Servais, P., 1995; Simon, M. and Rosenstock, B., 1992). Two advantages of the leucine procedure (Riemann, B. and Bell, R. T., 1990) are: 1) the method is an order of magnitude more sensitive than the thymidine method, making the leucine method appropriate for oligotrophic environments, and 2) in some cases non-specific incorporation of thymidine has been discovered (Brittain, A. M. and Karl, D. M., 1990). Chin-Leo and Kirchman (1988) have employed a dual-labeling approach using [^{14}C]leucine and [^3H]thymidine; Simon (1990) gives a review of the method. When leucine incorporation is measured, bacterial carbon production can be determined without knowing actual cell volumes (Simon, M. and Azam, F., 1989; Riemann, B. and Azam, F., 1992).

The work of Simon and Azam (1989) presented valuable information on the use of [^3H]leucine to determine bacterial production in a coastal marine environment using protein content and protein synthesis rates. Protein was correlated to cell volume using a power function ($y = 88.6x^{0.59}$; y = protein per cell [fg], x = cell volume [μm^3]). Leucine was determined to be 7.3 ± 1.9 mole percent. Cell carbon per cell volume, computed from protein incorporation, was determined to vary with cell size. Smaller cells had the highest carbon to volume ratio. Cell carbon varied from $400 \text{ fg } \mu\text{m}^{-3}$ at a cell volume of $0.026 \mu\text{m}^3$ to $133 \text{ fg } \mu\text{m}^{-3}$ at a cell volume of $0.400 \mu\text{m}^3$. The amino acids content of

proteins as mole percents were relatively constant, making the use of labeled leucine an accurate tool for measurement of bacterial production, given the intracellular isotope dilution can be corrected (Simon, M. and Azam, F., 1989). Bacterial carbon production (BCP) was related to bacterial protein production as follows: $BCP = 0.86 \times BPP$.

Kirchman et al. (1985) showed that greater than 90 percent of the leucine added to marine cultures was incorporated into protein. The addition of extracellular leucine inhibited leucine biosynthesis, showing leucine uptake to be a valid technique for measuring protein production.

Servais (1995) has recently shown the utility of measuring amino acid uptake to determine bacterial production. His work evaluated four labeled amino acids, leucine, tyrosine, lysine and alanine, in natural freshwater systems. Utilizing both a cold- and hot-TCA method for extracting the proteins, Servais found a small overestimation of protein using the cold method. A factor of 0.93 was used by Servais to correct for the cold method. Similar results using the cold-TCA technique were found by others (Kirchman, D. et al., 1985; Riemann, B. and Azam, F., 1992). A kinetic approach proposed by Kirchman et al. (1986) was used by Servais to determine isotope dilution. By plotting the amino acid incorporation rates against the added concentration, a hyperbolic response is typically seen. Nonlinear regression of this data can determine a value of the maximum uptake velocity (V_{max}) and isotope dilution can be determined. This method has been used successfully by other researchers (Riemann, B. and Azam, F., 1992; Servais, P., 1989).

Epiphytic bacterial biomass production was measured utilizing the [^3H]leucine method by exposing intact biofilm on leaf detritus to [^3H]leucine (Thomaz, S. M. and Wetzel, R. G., 1995). Leucine uptake saturation did not occur at values less than 400 nM leucine, 10 to 40 times greater than values found using planktonic bacteria. Bacterial biomass production of cells removed from biofilm was compared to production of the intact biofilm. Bacterial biomass production for the two "thin" biofilm (1.17 to 2.29 mg dry weight cm^{-2}) compared well to production of their planktonic cells; planktonic cells from the thick biofilm (5.98 mg dry weight cm^{-2}) had greater production rates than the biofilm. Diffusion resistance was believed to be the cause of poor uptake in the thick biofilm. Because the biofilm cultures were incubated up to 180 minutes, it was hypothesized that internal recycling of the leucine may be taking place within the biofilm.

Kirchman et al. (1982) estimated bacterial growth based upon substrate uptake kinetics and biomass. Changes in the incorporation of tritiated thymidine ([^3H]thymidine) into cell DNA were utilized to calculate growth of a natural freshwater microbial population. Kirchman's equations for determining the conversion factor and growth rate take into account increases in cell volume and specific activity with time.

Pollard and Moriarty (1984) demonstrated that the use of [^3H]thymidine to calculate growth rates was valid if isotope dilution was taken into account in the experimental design. By plotting the total concentration of added thymidine against the reciprocal of the radioactivity in the cellular DNA, isotope dilution was represented by the plot's intercept of the ordinate. If the plot passed through zero then there was no

isotope dilution, i.e., no thymidine was incorporated from sources other than the pool added as extracellular thymidine. A negative intercept of the ordinate indicated some pool of thymidine other than the added amount, most likely from biosynthesis within the cell. Their one-step transfer model assumed two things: 1) the concentration of precursors does not affect the rate of labeling of the macromolecule, and 2) isotope transfer rate from the precursor pool into the macromolecule is constant. The rate-limiting step in isotope dilution analysis is the final step of incorporation into the macromolecule.

Van Looij and Riemann (1993) found that isotope dilution should be determined for each sampling time when using [^{14}C]leucine to measure bacterial production in a marine environment. Leucine concentrations of 200 nM were required to correct for external isotope dilution, a value many times greater than determined by other investigators (Simon, M. and Azam, F., 1989; Servais, P., 1995). Isotope dilution was determined by measuring the uptake rate of leucine for a wide range of concentrations, using nonlinear regression to determine a value for V_{max} and then dividing V_{max} by the incorporation rate at the actual leucine concentration in the extracellular pool. Others (Riemann, B. and Azam, F., 1992; Servais, P., 1995) have used a similar technique. Van Looij and Riemann also found that ethanol rinsing of the TCA precipitate in the labeled leucine method did not improve recovery of the labeled proteins.

Utilizing the techniques developed by Simon and Azam (1989) for determining intracellular isotope dilution, Simon (1991) investigated the importance of various DFAA

to the biosynthesis of protein by subarctic Pacific bacteria. The intracellular pool was dominated by glutamate (41 mole percent) and glutamate had a high intracellular isotope dilution, meaning *de novo* synthesis of glutamate using ammonium and organic carbon was important to the bacterial production process. The subarctic Pacific has relatively high concentrations of nitrogen as reported by Simon.

Temperature was demonstrated to impact the incorporation rates of thymidine and leucine by bacteria in marine, estuarine and freshwater habitats (Tibbles, B. J., 1997). Temperature had a greater impact on leucine incorporation, resulting in higher rates of leucine incorporation as temperature increased.

Bacterial Biovolume and Biomass

The determination of bacterial growth and yield parameters often requires the conversion of cell number to biovolume and/or biomass. Numerous researchers have performed experiments to determine biovolume and biomass in a number of different environments. The ratio of cell carbon to cell volume has been shown to increase as cell size decreases (Simon, M. and Azam, F., 1989; Bratbak, G., 1985; Nagata, T., 1986; Lee, S. and Fuhrman, J. A., 1987; Watson, S. W. et al., 1977). Simon and Azam (1989), utilizing [^3H]leucine incorporation into protein and epifluorescence microscopy to enumerate and size cells, determined the carbon-to-volume ratio, dry weight, biomass density, cell density and percent water volume for marine bacteria in the size range from 0.026 to 0.400 μm^3 . Carbon content ranged from 400 fg C μm^{-3} for a cell volume of 0.026 μm^3 to 133 fg C μm^{-3} for a cell volume of 0.400 μm^3 . Lee and Fuhrman (1987)

measured the volume and carbon content of marine cells using glass fiber filtration and a CHN analyzer. Cells were sized using epifluorescence microscopy for calculating biovolume. The average value for the marine bacteria evaluated was $0.38 \pm 0.05 \text{ g C cm}^{-3}$ ($380 \text{ fg C } \mu\text{m}^{-3}$) based on average cell volumes ranging from 0.036 to $0.073 \text{ } \mu\text{m}^3$. Using a technique almost identical to that used by Lee and Fuhrman, Nagata (1986) determined conversion factors of $106 \text{ fg C } \mu\text{m}^{-3}$ for natural bacterial populations from a mesotrophic lake with mean cell volumes of 0.13 to $0.14 \text{ } \mu\text{m}^3$. Bratbak (1985) determined the biovolume and biomass conversion factors for a pure culture of *Pseudomonas putida* and a mixed culture from a marine estuary. The culture media consisted of $0.2\text{-}\mu\text{m}$ pore size filtered seawater amended with glucose, nitrate and phosphate such that one nutrient was made limiting by increasing the concentration of the other two nutrients by a factor of 10 over ambient concentrations. Cells for sizing and carbon content were taken from the cultures during the stationary phase of growth. Cell size and carbon content for the carbon limited natural population were 0.11 to $0.19 \text{ } \mu\text{m}^3$ and 580 to $800 \text{ fg C } \mu\text{m}^{-3}$, based on the method used to determine cell volume. Bratbak's values for cell carbon to volume ratios are greater than found by other investigators (Simon, M. and Azam, F., 1989; Nagata, T., 1986; Lee, S. and Fuhrman, J. A., 1987).

Analysis of biomass distributions by size class for marine seston indicated the underlying distribution was Pareto in nature (Vidondo, B. et al., 1997). The Pareto I and II modifications were shown to provide a better description of the underlying size distribution.

Models to Predict Biofilm Accumulation

The drinking water industry desires mathematical models capable of predicting biofilm growth and substrate uptake in drinking water biological treatment and distribution systems. Models could then be used to estimate treatment system size or evaluate the impacts of different treatment options on biofilm growth in the distribution systems. Understanding the basic relationships between carbon and energy sources in drinking water and growth kinetics is one step in development of desired models. Models require an understanding of the relationships between substrate type/concentration with growth and uptake in the biofilm (kinetic parameters). The following paragraphs review current models that may be applicable to biofilm in drinking water systems.

Atkinson and Davies (1974) combined the basics of diffusion with the Monod equation for growth (reaction) to develop a combined rate equation. The model assumes a one-dimensional biofilm of a constant "thickness" and homogeneous throughout. By equating diffusion with reaction and introducing dimensionless terms, an equation that can be solved numerically was derived. Further simplifications of the kinetics of reaction were used to develop generalized solutions using equations for the effectiveness factor, λ , the ratio of actual substrate flux in a biofilm to the flux that would occur if the entire biofilm were fully penetrated by the substrate at the concentration found at the biofilm-liquid interface.

Building upon the work of Williamson and McCarty (1976a; Williamson, K. and McCarty, P. L., 1976b), Rittmann and McCarty (1978) presented a variable-order model for biofilm growth kinetics. The model accounts for one-dimensional diffusion through both a liquid boundary layer and a homogeneous biofilm layer, equating diffusion of the substrate with Monod growth kinetics. Utilizing dimensionless terms, the equations were simplified to allow evaluation of deep biofilm. In a further development of the variable-order model, Rittmann and McCarty (1980b) developed a model for a steady-state biofilm, one where growth was equal to decay. No specific term for net bacterial detachment was included in the model. The minimum substrate concentration that must be available for steady-state biofilm growth was defined in terms of dimensionless variables. Rittmann and McCarty (1980a) presented laboratory data to validate their steady-state biofilm model. Their model was further refined to allow evaluation of biofilm of any thickness and to calculate the substrate flux in a nonsteady-state biofilm. The model incorporated the effectiveness factor concepts developed by Atkinson and Davies (1974).

Utilizing the model developed by Rittmann and McCarty (1981), Suidan and Wang (1985) developed an algebraic expression for the substrate flux, providing a simplified solution to the more complex model previously developed. Suidan (1986) developed expressions for the flux in completely mixed and plug flow, thick biofilm reactors. Monograms were presented to aid in design of such reactors. Sáez and Rittmann (1988) developed improved algebraic expressions for substrate flux in biofilm,

providing solutions that corrected potential errors in earlier work (Rittmann, B. E. et al., 1986). Suidan et al. (1987) developed a graphical procedure for evaluating the substrate penetration inside a one-dimensional biofilm. A rate modulus, Q , was defined as the ratio of the dimensionless substrate utilization rate at the substratum to the dimensionless substrate utilization rate for the bulk substrate concentration. Based upon the given definition, $Q = 0$ for a thick biofilm which is reaction limited and $Q = 1$ for a fully penetrated biofilm. Analysis of limiting cases of the steady-state biofilm model (Rittmann, B. E. and McCarty, P. L., 1980b; Rittmann, B. E. and McCarty, P. L., 1980a; Rittmann, B. E. and McCarty, P. L., 1981) were performed by Sáez and Rittmann (1990) for thick, fully penetrated, first-order and zero-order biofilm. They concluded fully penetrated solutions were rarely accurate and that limiting case solutions to the steady-state model introduces errors in substrate flux computations.

Although the steady-state biofilm model developed by Rittmann and McCarty (1981) predicts the minimum substrate concentration, $S_{\min}$, for a steady-state biofilm, a nonsteady-state biofilm is capable of utilizing substrates at concentrations below those predicted for $S_{\min}$. Rittmann and Brunner (1984) developed a model to approximate the nonsteady-state biofilm process and performed experiments to evaluate the response of a packed-bed biofilm reactor to substrate concentrations less than predicted $S_{\min}$. The reactor removed greater than 85 percent of the influent substrates for a 1-year period. The proposed model was not able to predict the biofilm growth based on the measurement techniques used in the study. Although not discussed in the article, a shift in population

kinetics appears to be an explanation for the inability of the model to predict biofilm growth at the low substrate concentrations used in the study. In a similar study by Stratton et al. (1983), a packed-bed biofilm reactor was initially fed primary substrates (acetate, galactose, alanine, phenol, and thymine) as single substrates at concentrations of 3 mg/L to develop a biofilm unique to that particular substrate. Secondary substrates were introduced to evaluate the response of the biofilm to their uptake. A thymine-fed biofilm showed little initial uptake of galactose and phenol; an alanine-fed biofilm little uptake of acetate; a galactose-fed biofilm little uptake of phenol and acetate; an acetate-fed biofilm little uptake of alanine; and a phenol-fed biofilm little uptake of thymine. Conversely, for those secondary substrates that were utilized, the effluent concentrations were less than the $S_{\min}$ values presented in the paper. Different kinetics applied to various substrate groups. Thymine and galactose had the slowest uptake rate while acetate, alanine and phenol had the fastest uptake rates. Experimental values of the biofilm flux were plotted against influent substrate concentration on a log-log scale to evaluate the relationship between substrate loading and flux to the biofilm.. Rapidly utilized secondary substrates (alanine and galactose on the acetate biofilm and alanine and acetate on the thymine biofilm) showed a linear, first-order response. Less rapidly utilized secondary substrates (galactose and thymine) showed first-order kinetics at low substrate concentrations, moving to a reaction order less than one (non-linear) at higher substrate concentrations.

Wanner and Gujer (1986) presented a mathematical model which was capable of taking into account multiple species and associated substrates in a biofilm. Based upon conservation principles, the model assumes the biofilm properties only change in the direction perpendicular to the substratum and the biofilm is a continuum. Biomass is characterized by average concentration using density and volume fraction; the density of a species is assumed constant with time and the volume fraction occupied by that species is allowed to change. A pseudo steady-state solution is created from the model results utilizing a net detachment term to counter growth. The detachment term can be formulated in a number of ways, such as a function of the liquid-biofilm interface velocity. Wanner and Gujer's basic model was modified for use on a personal computer (BIOSIM) (Reichert, P. et al., 1989). A similar model was developed by the Center for Biofilm Engineering at Montana State University (Biofilm Accumulation Model-BAM) (1992). BAM incorporated improved output graphics along with the option of modeling the effects of biocide addition to the CSTR biofilm reactor.

Reichert (1994) describes the model AQUASIM developed at the Swiss Federal Institute for Environmental science and Technology (EAWAG). AQUASIM is capable of modeling a number of aquatic environments utilizing model compartments that include mixed reactors, biofilm reactor and a river section. By introducing advective and diffusive links between the compartments a number of potential aquatic systems can be modeled, e.g., activated sludge with nitrification. The basis for the biofilm compartment followed closely the capabilities of the earlier BIOSIM (Reichert, P. et al., 1989) model.

The model provided the capability of modeling a diffusive substratum for the biofilm compartment. The addition of user defined variables, parameter estimation capabilities, and sensitivity analysis capabilities made AQUASIM a useful tool for laboratory studies. A graphical user interface and improved graphics capabilities made the program more user friendly and improved interpretation of the output.

The above models were developed for use with any biofilm over a wide variety of conditions. Several models have been developed specifically for use in drinking water applications. These models will be discussed in the following paragraphs.

The SANCHO model was developed based on kinetics for enzyme-controlled degradation of BDOC by microorganisms in natural environments. Developed by Servais et al. (Servais, P. et al., 1995; Servais, P. et al., 1994), the SANCHO model relies on Michaelis-Menten kinetics for growth and degradation of BDOC. Utilizable substrate comes from three fractions according to this model. Substrate can be either easily utilizable (such as free amino acids), a rapidly hydrolyzed fraction of BDOC, or a slowly hydrolyzed fraction of BDOC. Cell attachment/detachment is modeled based on Langmuir adsorption and desorption models. The cell population is divided into fixed biomass (irreversibly attached), sorbed biomass (reversibly attached) and free biomass. The model can account for recycle of dead biomass back to the BDOC pool available for growth. Attachment assumes a fixed number of sites available for sorption of cells. The effects of chlorine are modeled using first-order kinetics with threshold values for both fixed and free bacteria. The impact of chlorine in the model is to reduce the maximum

growth and enzyme production rates. Residence time calculations from hydraulic models must be input to the SANCHO model to perform analyses of actual distribution systems or pipelines. Evaluation of data from seven full-scale systems indicated the model performed well at predicting biofilm (Laurent, P. et al., 1997). The model requires estimates for sixteen parameters, making application of the model problematic.

A model for biofilm in drinking water systems was developed by Dukan et al. (Dukan, S. et al., 1996) that attempts to take into account the many interactions of free chlorine with biofilm cells and planktonic cells. The model accounts for resistance to chlorine by biofilm cells by assuming the biofilm consists of an outer layer of biomass impacted by chlorine and an inner layer of biomass not impacted by chlorine. Michaelis-Menten substrate uptake kinetics is assumed in the model. The model was designed to determine the level of BDOC at which biofilm biomass can be controlled without using chlorine.

Effects of Chlorine on Bacterial Cells

Chlorine has for many years been considered the most efficient oxidizing disinfectant for drinking waters. The interaction of chlorine with bacterial cells may be important to understanding how chlorine impacts biofilm in drinking water systems. The following paragraphs present some findings of research in that subject area.

Chlorine has been shown to damage the membrane structure of cells (Haas, C. N. and Engelbrecht, R. S., 1980; Venkobachar, C. et al., 1977) making the cell membrane

more permeable and allowing leakage of cell constituents into the environment (Samrakandi, M. M. et al., 1997; Venkobachar, C. et al., 1977). Hypochlorous acid has been shown to inhibit cell transport systems (Thomas, E. L., 1979a; Thomas, E. L., 1979b) and inhibit electron transport (Barrette, W. C. Jr. et al., 1987) in *Escherichia coli* cells. Hypochlorous acid can impair uptake systems capable of transporting amino acids to the cell. Hypochlorous acid reacted with peptides to produce nitrogen chlorine derivatives (Thomas, E. L., 1979a; Thomas, E. L., 1979b) when *E. coli* cells were exposed to hypochlorous acid. The presence of any nitrogen-chlorine derivatives was extremely detrimental to the cell since these derivatives contained chloramine- and chloramide-type substances. These N-Cl derivatives had a long-lasting effect, oxidizing peptide bonds in the membrane, oxidizing bacterial sulfhydryls (iron-sulfur proteins associated with the cell's electron transport system) or oxidizing other cell components. Oxidation of the N-chloro compounds to an aldehyde could make these initial reaction products with chlorine less toxic.

The extracellular polymeric substances (EPS) produced by biofilm cells have been implicated in the protection of biofilm cells from oxidizing disinfectants such as chlorine (Stewart, P. S. et al., 1996). Samrakandi (1997), working with *Escherichia coli* biofilm, found that the substrate used by the biofilm may be important to its ability to produce EPS and develop protection against chlorine effects. When sucrose was substituted for lactose in a growth medium containing ammonium nitrate, *Escherichia coli* cells increased their production of EPS.

E. coli cells with intracellular glutathione were found to be more resistant to the oxidative effects of chlorine and chloramine than a similar *E. coli* strain replete in glutathione (Chesney, J. A. et al., 1996). The sulfur containing glutathione was proposed as a sacrificial compound against the oxidizing effects of hypochlorous acid. Glutathione was effective against hypochlorous acid in both its oxidized and reduced states. Recycling of oxidized glutathione to glutathione was proposed to play a role in protection of the cell from less reactive compounds such as organic chloramines.

CHAPTER 3

MATERIALS AND METHODS

General

The approach was designed to evaluate how biofilms in model drinking water systems respond to three basic groups of biodegradable compounds commonly found in water supplies and utilized by biofilm microorganisms as carbon and energy sources. Based upon a review of the literature (Camper, A. K. et al., 1994) three primary groups of substrates were selected for investigation: amino acids, carbohydrates and humic substances. These three groups also represent compounds found in source water that has received pollutants from both point and nonpoint discharges. Amino acids, primarily in the form of proteins, are commonly associated with discharges from municipal wastewater treatment plants or agricultural runoff. Carbohydrates can be released following algal blooms in rivers and lakes. Since most carbohydrates and amino acids in natural waters are bound to humic substances, this undefined substrate group was also investigated.

Rotating annular reactors were used to develop model drinking water system biofilms. Rotating annular reactors, described in detail later in this section, are laboratory-scale devices that can be used to grow biofilms under hydraulic conditions similar to those found in a pipeline, and provide an easy means for routine sampling of the biofilm without interrupting the operation of the reactor. The term 'model drinking

water system biofilms' has been used to describe the biofilms developed in annular reactors under the conditions utilized in this study.

The overall approach for the first phase of the experiment is summarized in Figure

1. The reactors were first seeded with coliforms followed by introduction of heterotrophic bacteria indigenous to a drinking water system. The result was development of a heterotrophic biofilm containing coliforms. One reactor served as a control (control reactor) while the other eventually received chlorine (chlorinated reactor) to model a biofilm under conditions of free-chlorine disinfection. After a series of substrate and chlorine feed concentration increases, the biofilm on the reactor surfaces was removed and placed into batch cultures for measurement of substrate uptake and cell numbers over time (see Phase I – Batch Culture Uptake Experiments below).

The second phase of the experimental plan included development of biofilms using the same protocol as for the first phase with the exception that parameters for growth kinetics were determined using radiolabeled substrates (amino acids experiment only) and radiolabeled leucine. The second phase was also different because attached biofilms were used for measurement of kinetic parameters rather than scraped biofilm cells in planktonic batch cultures.

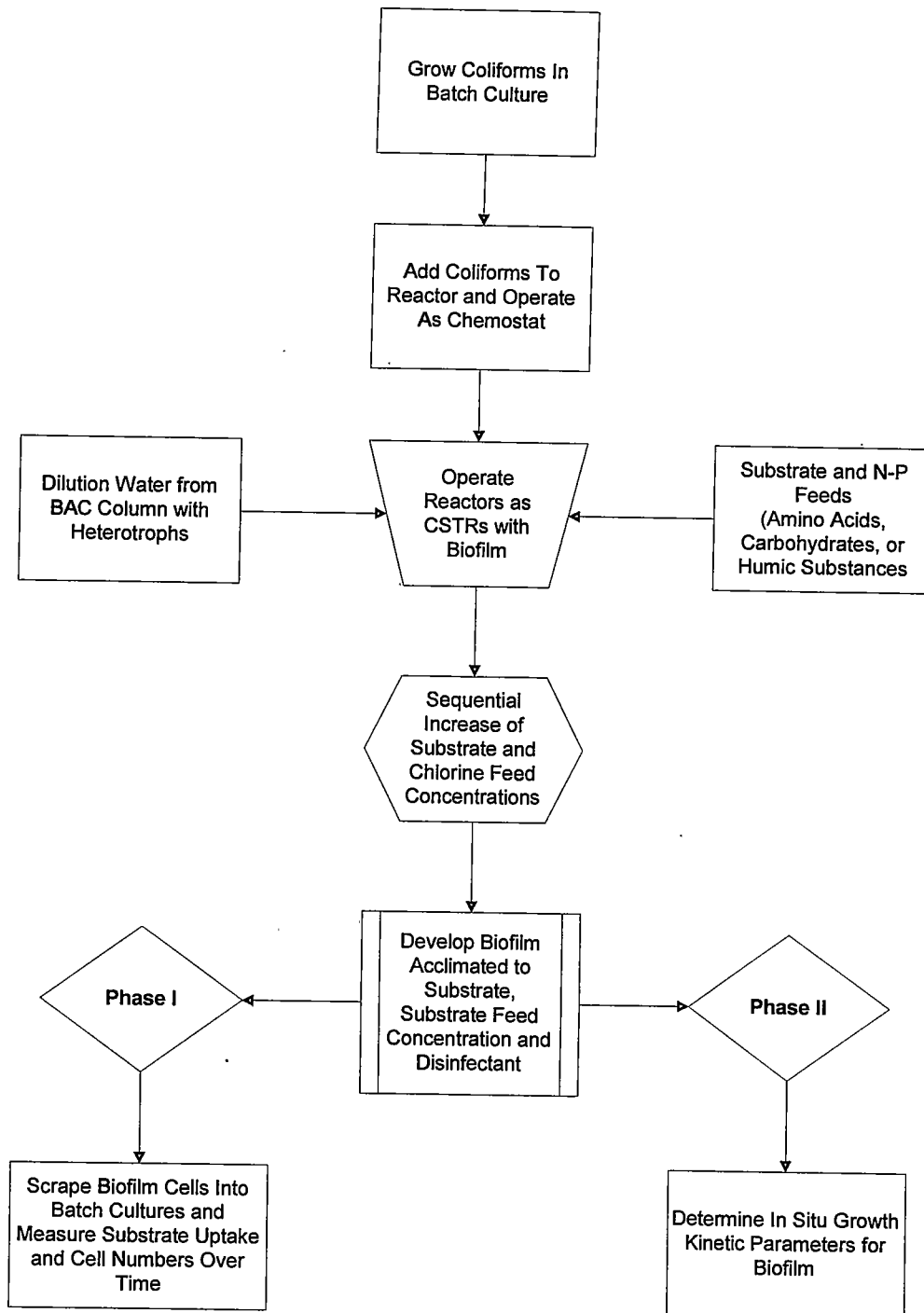


Figure 1. Flow diagram for phases I and II of the experimental plan.

Reactors

Model drinking water system biofilms were grown in rotating annular reactors developed at Rice University by W. G. Characklis and others. Constructed of polycarbonate, the reactors consist of a stationary outer cylinder with a rotating inner drum forming an annular space between the inner drum and the outer cylinder. Twelve removable polycarbonate slides are located in machined grooves in the inside wall of the outer cylinder, forming a relatively smooth, even surface between the cylinder wall and the slides. The reactor top has twelve holes with stoppers that can be removed to allow access to the slides without interrupting reactor operation; the biofilm can be sampled at any time during an experiment by pulling one or more of the slides from the reactor.

The rotational speed of the inner drum can be varied to create the desired shear stress on the wall of the outside cylinder. A rotational speed of 30 revolutions per minute was used in all work, simulating the shear stress at the wall of a 4-inch-diameter (102-mm-diameter) pipe with a flow velocity of 1.0 feet per second (0.3 meters per second). Vertical draft tubes in the inner rotating drum assist in providing complete mixing within the reactor. These reactors can be modeled as continuously stirred tank reactors (CSTRs). The total flow rate to the reactor was set to provide a hydraulic detention time of approximately 2.1 hours for all experiments. This residence time allows coliforms to persist in a heterotrophic biofilm (Camper, A. K. et al., 1996) and minimizes planktonic growth of both the coliform microorganisms (Camper, A. K. et al., 1991) and the suspended heterotrophic organisms (Camper, A. K., 1995; Servais, P. et al., 1994). The

lower portion of the rotating annular reactors was immersed in a water bath maintained at $20 \pm 1^\circ \text{C}$ utilizing an external cooling device.

The influent flow to each reactor consisted of dilution water (referred to as "AOC 'free' " or reactor dilution water) from a biologically activated carbon (BAC) column, the substrate of interest, and a nitrogen/phosphorous solution. City of Bozeman, Montana, drinking water (treated using iron and polymer coagulation, direct filtration and free chlorine disinfection) was treated in two columns containing granular activated carbon media to provide the AOC "free" dilution water. The first column provided chlorine removal from the water and contained granular activated carbon (12x40 mesh Nuchar, Westvaco). The second column contained biologically activated carbon originally obtained from a full-scale water treatment plant (City of Laval, Quebec, Canada), and had been receiving Bozeman drinking water for over three years prior to the experiments. The assimilable organic carbon (AOC) level of the effluent from the BAC column was typically less than 25 micrograms per liter (μg acetate equivalents/L) as determined by the method of van der Kooij et al. (1982) as modified by the method of LeChevallier et al. (1993). The BAC column effluent contained a mixed population of heterotrophic bacteria (ca. 10^4 to 10^5 colony forming units per milliliter [cfu/ml] on R2A medium) that colonized the reactor surface. No coliform organisms were detected in the BAC column effluent. Substrate and nitrogen/phosphorous feed solutions were made in glass carboys (combusted at 500°C for 4 hours to remove residual carbon) using Nanopure reagent grade water. The glass carboy and water were autoclaved prior to use. Sterile substrate

or nutrient solutions were added by syringe to the carboys after the water had been autoclaved. The concentration of nitrate (NO_3) and phosphate (PO_4) in the reactors was approximately 600 $\mu\text{g/L}$.

Prior to each experiment the reactors were cleaned and disinfected using a 50 milligrams per liter (mg/L) free chlorine solution placed in the reactor for at least 24 hours. The reactor top assembly/inner-drum and all tubing (Masterflex L/S silicone) were autoclaved prior to reactor assembly and chlorine disinfection. After 24 hours with the inner drum rotating, the chlorine solution was drained from the reactor and a sterile feed of Nanopure water was commenced for 24 hours. Following the 24-hour flushing period the reactors' effluent was sampled to insure there was no remaining chlorine residual. A sterile sodium thiosulfate solution was added to remove any remaining free chlorine prior to inoculation.

Coliform Inoculation

Prior to introduction of the BAC column dilution water and the substrate and nutrient feeds, the reactors were inoculated with a combination of five coliform strains originally isolated from a drinking water distribution system. The five coliforms, *Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter aerogenes*, *E. cloacae*, and *Escherichia coli* were first grown in batch cultures using a 5000 μg carbon/L substrate consisting of equimolar as carbon concentrations of all substrates used in the experiments (see Substrates below and Table 1) and 1000 $\mu\text{g/L}$ each of nitrate (NO_3) and phosphate (PO_4).

Incubation of the batch cultures was for 48 hours at 20° C in a temperature controlled shaking table incubator.

Substrate Group	Individual Compounds
Amino Acids	L-glutamic acid (Sigma) L-aspartic acid (Sigma) L-serine (Sigma) L-alanine (Sigma)
Carbohydrates	D(+)glucose (Sigma) D-galacturonic acid (Sigma) D(+)galactose (Sigma) D(-)arabinose (Sigma)
Humic Substances	Elliot Silt Loam Soil, BS102M, Joliet, IL: Mollic Horizon (IHSS)

Table 1. Compounds utilized as substrates. IHSS = International Humic Substances Society.

Five milliliters (ml) of the coliform batch culture were added to each of two 500-ml sterile chemostats that were fed the same substrate and nutrient concentrations as the batch cultures. The chemostats were aerated and mixed using compressed air passed through a 0.22- μ m pore-size air filter. The chemostats were operated at a residence time of 20 hours (growth rate of 0.05 hr⁻¹) which has been found to be optimal in allowing coliforms to successfully persist in mixed-population biofilms (Camper, A. K. et al., 1996). After a period of approximately 3 residence times the contents of the two chemostats were mixed in a sterile flask and equal volumes were then added to each of the empty, sterile rotating annular reactors. Approximately 100 ml of sterile Nanopure water was added to each reactor to bring the total volume to approximately 600 ml, the inner drum rotation was started, and the reactors were then operated for 20 hours as

chemostats with the same substrate/nutrient feed solution that was used for the chemostats. Following the 20-hour operation of the reactors as chemostats, the chemostat feed solution was terminated and the reactor dilution water, substrate, and nutrient feed solutions were commenced.

Substrates

Three substrates groups, amino acids, carbohydrates and humic substances, were utilized as substrates for the experiments. Table 1 lists the specific compounds used. These compounds were selected based on their presence in a variety of natural and drinking waters, (Jørgensen, N. O. G. and Jensen, R. E., 1994; Tranvik, L. J., 1988; Pantoja, S. and Lee, C., 1994; Tranvik, L. J., 1993; Tranvik, L. J. and Höfle, M. G., 1987; Amon, R. M. W. and Benner, R., 1996; Amon, R. M. W. and Benner, R., 1994; Volk, C. J. et al., 1997; Jørgensen, N. O. G. et al., 1998; Gremm, T. J. and Kaplan, L. A., 1997; Kaplan, L. A. and Newbold, J. D., 1993; Cherrier, J. et al., 1996; Boon, P. I., 1990), the variation each compound has in the metabolic pathways of heterotrophic microorganisms, and the ability to detect the compounds at low concentrations. The three primary groups of compounds are also found in waters that have received both point and nonpoint discharges of pollutants. Amino acids have been associated with agricultural runoff and discharges from wastewater treatment plants. Carbohydrates and amino acids are released following algal blooms in natural waters.

The amino acids and carbohydrates experiments utilized equimolar concentrations as carbon of the compounds in each group. The humic substance concentration was based on total organic carbon analysis of a concentrated stock solution. The concentrated stock solution was prepared by adding International Humic Substances Society (IHSS) humic substance to a 0.1 normal (N) NaOH solution, mixed for 24 hours, and then centrifuged at 10,000 g at 4° C to remove particulates. The concentrated humic stock solution was not autoclaved and was stored in the dark at 4° C. After addition of the humic stock solution to the sterile Nanopure water, the pH was adjusted to ca. 7 using 2N HCl. Nitrate and phosphate were fed at concentrations of 612 and 591 µg /L (9.9 and 6.2 µM), respectively. The reactors were carbon limited under all substrate feed concentrations.

Reactor Operation

Amino Acids, Carbohydrates and Humic Substances

Figure 2 shows the substrate and chlorine feed protocols used during each experiment. Initially each reactor was fed a substrate concentration of approximately 500 µg C/L until the reactors reached a pseudo steady state based on total and culturable cell counts. The biofilm was sampled and then the substrate concentration was increased to 1000 µg C/L, the reactors operated until steady-state was obtained, and then chlorine addition to one of the two reactors was started. The chlorine dose was slowly increased until a free chlorine residual of 0.15 to 0.2 mg/L was obtained in the effluent.

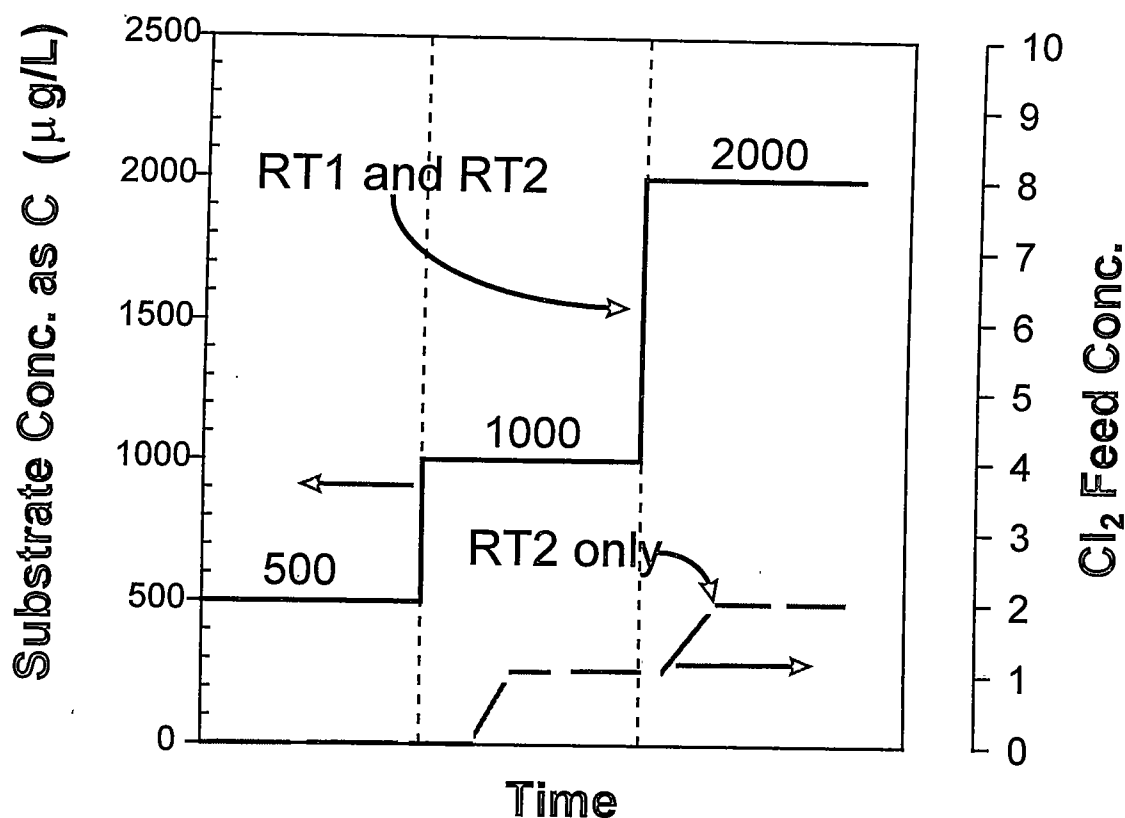


Figure 2. Protocol for substrate and chlorine concentration step increases for rotating annular reactor experiments.

The reactors were operated until another pseudo steady-state was reached, the biofilm sampled and then the substrate concentration was increased to 2000 $\mu\text{g C/L}$, the reactors operated until pseudo steady-state was again reached, and then the chlorine feed dose was slowly adjusted until the effluent residual of the chlorinated reactor was 0.15 to 0.2 mg/L. Duplicate experiments were performed for the humics substrate group and triplicate experiments for the amino acids and carbohydrates substrate groups.

Mixed Substrate

A single experiment was performed for the mixed substrate experiment.

Operation of rotating annular reactors in series was utilized to predict laboratory growth parameters to a pipeline situation (plug flow reactor). Two rotating annular reactors were operated in series, one pair served as the controls and one pair served as the chlorinated reactors. Startup and operation was as described above reactors using the other substrate groups. Substrate, buffer/nutrient, dilution water and chlorine were added only to the first of the two reactors in series and the effluent of the first reactor flowed directly to the second reactor. Hydraulic detention time in each reactor was approximately 2 hours for a total of 4 hours for each pair of reactors. Polycarbonate biofilm sample slides were utilized for this work to allow comparison to the laboratory reactor data and allow measurement of growth parameters for the attached biofilm. The substrate and chlorine protocol were similar to that shown in Figure 1 with the exception that the 500 $\mu\text{g C/L}$ phase was extended to have a chlorination phase at that substrate concentration. This provided three substrate concentration sampling points for the chlorinated reactors.

Series reactor sampling was performed at the end of the 500, 1000 and 2000 $\mu\text{g C/L}$ substrate concentration phases. One slide was pulled from each reactor and sampled at the top, middle and bottom for TDC, HPCs and coliforms. The remainder of the slide was scraped into carbon free water and NPOC used to determine the carbon bound to the biofilm. A second slide was pulled from the first reactor in series and a determination of V_{max} for leucine uptake was performed as described below.

One slide was pulled from each reactor and the attached growth rate was determined using the technique described below for the amino acids experiment. The influent and effluent to each reactor was sampled for NPOC to determine overall reactor carbon removal rates.

Reactor Sampling

The sampling protocol for the reactors during their operation included grab samples of the dilution water and effluent from each reactor for total cell counts, culturable cell counts, reactor effluent total coliforms, and chlorinated reactor effluent free and total chlorine concentration. The reactor dilution water, effluent, substrate feed and nitrate/phosphate feed were sampled for total organic carbon (TOC) analysis. The first two amino acids experiments were not sampled for TOC as the appropriate instrument was not available during the time of those two experiments.

Total Direct Cell Counts (TDC)

Samples for total direct cell counts were fixed using formaldehyde (0.7% final concentration) for 24 hours at approximately 4 °C. Fixed samples were filtered onto 0.2- μ m pore-size black polycarbonate filters (Poretics) then stained on the filter using 0.5 ml of 100 mg/L - 4',6-diamidino-2-phenylindole (DAPI) (Sigma) for a minimum of 10 minutes. Total direct counts were made using an Olympus BH-2 microscope with an ultra-violet light source for epifluorescence of the sample.

Culturable Cell Counts

Culturable cell counts (heterotrophic plate counts [HPC]) were determined by spread plating in triplicate on R2A agar medium (Difco) and incubating at room temperature for 7 days.

Total Coliforms

Total coliforms were determined either by spread plating in triplicate on mT7 medium (prepared in-house) or filtering samples, in triplicate, onto 0.22- μ m pore-size acetate filters (MSI) and placing the filter onto the surface of the mT7 medium.

Incubation of coliform samples was at 35° C for 24 hours.

Chlorine

Free and total chlorine concentration were determined using the DPD colorimetric method and a digital colorimeter (LaMotte).

Total Organic Carbon Analyses

Samples for TOC were processed immediately upon sampling. Filters used to determine dissolved organic carbon were sterile 0.2- μ m pore-size nylon syringe filters (Fisher Scientific), prewashed with 30 ml of 0.1N HCl then 120 ml of Nanopure reagent grade water using a 30 ml sterile, disposable syringe that was also used for the sample. The first 10 to 20 ml of the filtered sample was wasted. All total organic carbon samples were acidified prior to analysis using 0.2 ml of 2N HCl in ca. 40 ml of sample. All

samples were analyzed for non-purgeable organic carbon (NPOC) using a Shimadzu TOC 5000A with high sensitivity catalyst and an auto-sampler. Standard curves for the TOC 5000A were developed using 4 concentrations of potassium hydrogen thalate, and the resulting linear calibration curve typically had a coefficient of determination (R^2) of 1.00 and never less than 0.98. An internal standard (from the standards used to develop the standard curve) was always run with each group of samples to correct for any variations in carrier gas flow rate. All glassware was precombusted at 500 °C for 4 hours, minimum. Auto-sample vials were acid washed, rinsed 6 times with Nanopure reagent grade water, and precombusted at 300 °C for 6 hours, minimum. The auto-sample vial glass-type prevented combustion at temperatures greater than 300 °C. Glass volumetric flasks and pipettes were acid washed and rinsed 6 times with Nanopure water.

Biofilm Sampling

The biofilm in the reactors was sampled by removal of a slide (area of ca. 30 square centimeters [cm^2]) and aseptically scraping the biofilm from the slide into a sterile phosphate buffer solution using a sterile, straight razor blade. The biofilm/buffer solution was then homogenized at 20,000 rpm for 1 minute using a tissue homogenizer (Tekmar). Samples of the biofilm suspension were then taken and analyzed for total and culturable cell counts and coliforms as described above.

Amino Acids Analysis

Amino acids were analyzed as o-phthaldialdehyde (OPA) derivatives by High Performance Liquid Chromatography (HPLC) according to the technique of Lindroth and Mopper (1979) with the modifications described below. A DX-300 (Dionex, Sunnyvale, CA) HPLC was used equipped with a Supelcosil LC-18, 15cm x 4.6mm ID, 5 μ m particles, reverse phase chromatography column (Supelco, Bellefonte, PA). The mobile phase consisted of two components: (A) 0.5% tetrahydrofuran (THF) in 50 millimolar (mM) NaOAc, pH 5.7 and (B) absolute methanol. All buffers and eluants were HPLC grade. The flow rate was maintained at 1.5 milliliter per minute while the gradient was optimized (salt concentration, start and stop points for isocratic gradients) for each amino acid as well as the mixture of the 4 amino acids. Detection was by fluorescence (excitation 335 nanometers (nm), emission 435 nm) on a Jasco FP-920 Intelligent Fluorescence Detector (Jasco, Tokyo, Japan). An internal standard using β -amino-butyrate was used for each sample group to verify the standard curve values.

Derivatization was performed in 2 ml Target vials (National Scientific Company, Lawrenceville, GA) using 0.1 ml sample (0.2- μ m pore-size filtered using nylon syringe filters) plus 0.1 ml internal standard (1:3, Na borate (pH 9.6):methanol) plus 0.2 ml OPA (20 mg/ml in absolute ethanol) plus 0.1 ml 2-mercaptoethanol (40 μ L/ml in absolute ethanol) and injected within 2 minutes via an auto-injector equipped with a 15 μ L loop. Standards were all $\geq 99\%$ pure by TLC, except alanine which was $>98\%$ pure by TLC,

and were purchased from Sigma (St. Louis, MO). 2-mercaptoethanol was purchased from Pierce (Rockford, Illinois) while absolute ethanol was purchased from McCormick Distilling Co. Inc. (Weston, Missouri).

Cell Volume and Carbon Estimates

Samples used for total direct cell counts were also used for determining cell size. Digital images of the stained cells were gathered from random fields of view using an Olympus BH-2 microscope and a charge coupled device (CCD) camera and Image-Pro Plus™ software. Cell sizes were determined utilizing ImageTool software developed at the University of Texas Health Science Center at San Antonio, Texas. Images for the amino acids replicate 1 and 2 experiments were obtained by first photographing the desired stained cell sample using a 35 millimeter (mm) camera, then having the slides converted to Kodak Photo CD images. Upon acquisition of Image-Pro software the digital images were saved and utilized directly to determine cell sizes. Digital images saved as TIFF files using Image-Pro software were converted to greyscale images using ImageTool software. After adjustment of contrast and brightness, image thresholding was accomplished manually to provide precise cell size measurements. Cell volume was calculated using the formula $(\pi/4)W^2((L-W)/3)$, where W is the cell width (minor axis length) and L is the cell length (major axis length) (Bratbak, G., 1985). Carbon per cell measurements were made for the influent and effluent cells from the amino acids replicate 3 experiment, both humics replicate experiments, the mixed substrate

experiment and the carbohydrates replicate 3 experiment. The conversion of cell volume to cell biomass (as carbon) requires actual measurements or can be obtained from the literature (Bratbak, G., 1985; Troussellier, M. et al., 1997; Bjornsen, P. K., 1986; Bjornsen, P. K. and Kuparinen, J., 1991; Nagata, T. and Watanabe, Y., 1990; Lee, S. and Fuhrman, J. A., 1987; Nagata, T., 1986).

Where possible, actual measurements were performed to estimate carbon per cell and carbon per volume for the experiments in this study. Researchers have found that the carbon per volume value increases as cell volume decreases for cells in low carbon environments and can be estimated using a power function for the cell carbon to cell volume relationship (Simon, M. and Azam, F., 1989; Troussellier, M. et al., 1997). Based on mean cell volume, total direct count cell numbers, and the NPOC difference between 0.2- μm pore-size filtered and unfiltered samples, mean carbon per volume data were determined and used to estimate a power function describing cell carbon per volume. The power function was applied to each measured cell volume to estimate a carbon per cell value. The mean of all calculated carbon per cell values was then used to estimate the carbon per cell value for the sample. This technique provided a more representative value of the population mean compared to using a carbon per volume value and applying it to the mean cell volume measurement. Additional mean carbon per volume values were determined for the mixed substrate and carbohydrates replicate 3 experiment. Analysis of the data ($n=61$) using a power function indicated the function derived from the data and by others resulted in unrealistically high carbon per cell values

for small cells. The carbon per cell volume data was re-analyzed using the three parameter Generalized Pareto distribution function (Aroner, E. R., 1996) using TableCurve 2D software to perform a least-squares regression to estimate the parameters for the fit. The Generalized Pareto distribution function provides an upper limit to the carbon per volume estimate for extremely small cells while at the same time being sensitive to large cell volumes. The Generalized Pareto distribution function is:

$$\alpha^{-1} [1 - k(X - \beta) / \alpha]^{1/k-1} \quad (1)$$

where the parameters of the function are α , β and k ($k < > 0$), and X is cell volume. The following parameter values were determined from the nonlinear regression:

$$\alpha = 0.00780$$

$$\beta = 0.499$$

$$k = -0.0122$$

Leucine Uptake to Measure Growth Rates

One method utilized for determining the kinetic parameters for growth involved measuring the uptake rate of radiolabeled leucine combined with parallel measurements of substrate uptake using radiolabeled substrates. This technique was utilized for the second humics replicate experiment batch cultures, the third amino acids and carbohydrates replicate experiments with attached biofilms and the mixed substrates experiment with attached biofilm.

The amino acid leucine can be taken up by bacterial cells and utilized in the synthesis of protein. Since the mole fraction of leucine in cell protein is nearly constant (7.3%) (Simon, M. and Azam, F., 1989), and the ratio of cell carbon to protein mass is nearly constant (0.86) (Simon, M. and Azam, F., 1989), the cell production rate in terms of carbon can be determined by measuring the uptake of leucine. This technique has been used extensively in aquatic microbiology (Simon, M. and Azam, F., 1989; Servais, P., 1995; Hollibaugh, J. T., 1994; Kirchman, D. et al., 1985; Hollibaugh, J. T. et al., 1992; Riemann, B. and Azam, F., 1992; Thomaz, S. M. and Wetzel, R. G., 1995; Kirchman, D. L., 1992; Kirchman, D. L. and Hoch, M. P., 1988; Kirchman, D. et al., 1986; Bjornsen, P. K. and Kuparinen, J., 1991; Simon, M. and Rosenstock, B., 1992; Chin-Leo, G. and Kirchman, D. L., 1988; Riemann, B. and Bell, R. T., 1990; Simon, M., 1990; van Looij, A. and Riemann, B., 1993; Riemann, B. et al., 1990), but has been used less extensively for biofilms (Servais, P. et al., 1994; Thomaz, S. M. and Wetzel, R. G., 1995).

The uptake of radiolabeled leucine can be converted to bacterial carbon production utilizing the following equation (Servais, P., 1995):

$$\text{BCP} = \text{Leu}_{\text{inc}} \times 1,080 \times \text{F1} \times \text{ID} \quad (2)$$

BCP = bacterial carbon production

Leu_{inc} = (mole Leu incorporated)(volume⁻¹)(time⁻¹)

F1 = ratio Leu in "hot TCA" fraction to Leu in "cold TCA" fraction

ID = isotope dilution

The conversion factor of 1,080 was developed as follows (Servais, P., 1995):

$$1,080 = (131-18) \times (100/9) \times 0.86 \quad (3)$$

131 = molecular weight of leucine

18 = molecular weight of water removed from the leucine molecule when the peptidic bond is formed

9 = weight percent of leucine in protein

0.86 = ratio cell carbon to protein mass

Bacterial cells removed from a liquid medium by filtration can be treated with trichloroacetic acid (TCA) to separate macromolecules from other cell components. The cold TCA method (Servais, P., 1995; Riemann, B. and Azam, F., 1992; Chin-Leo, G. and Kirchman, D. L., 1988) was used in this work with no correction factor ($F1=1$).

Isotope dilution via *de novo* synthesis must be considered when using leucine to measure growth rates. The ideal situation when utilizing the leucine uptake technique is that all extracellular leucine taken up by the cell be converted to protein. By saturating the uptake mechanisms of the cells, *de novo* synthesis of leucine by the cell is reduced and isotope dilution can be minimized. Leucine saturation studies are required to determine the leucine concentration at which maximum uptake rate is obtained ($V_{\max}$). Leucine concentrations at $V_{\max}$ vary from 10 to 200 nanomolar (nM) for planktonic marine and freshwater cells (Servais, P., 1995; Riemann, B. and Azam, F., 1992; Simon, M. and Azam, F., 1989) and as high as 400 to 800 nM for biofilms (Thomaz, S. M. and Wetzel, R. G., 1995). All leucine uptake rates were determined using the measured leucine concentration at or near $V_{\max}$ to minimize isotope dilution. No correction factor

was used for isotope dilution in the calculation of BCP. The limited number of samples available at the end of an experiment coupled with the time requirement to develop the biofilm for sampling (60 to 120 days) did not allow sufficient replicates for determining isotope dilution using standard techniques.

Growth Rates in Suspended Cell Cultures

The leucine concentration required to saturate uptake was determined as shown in Figure 3. Liquid samples with 4 to 5 different leucine concentrations were analyzed as

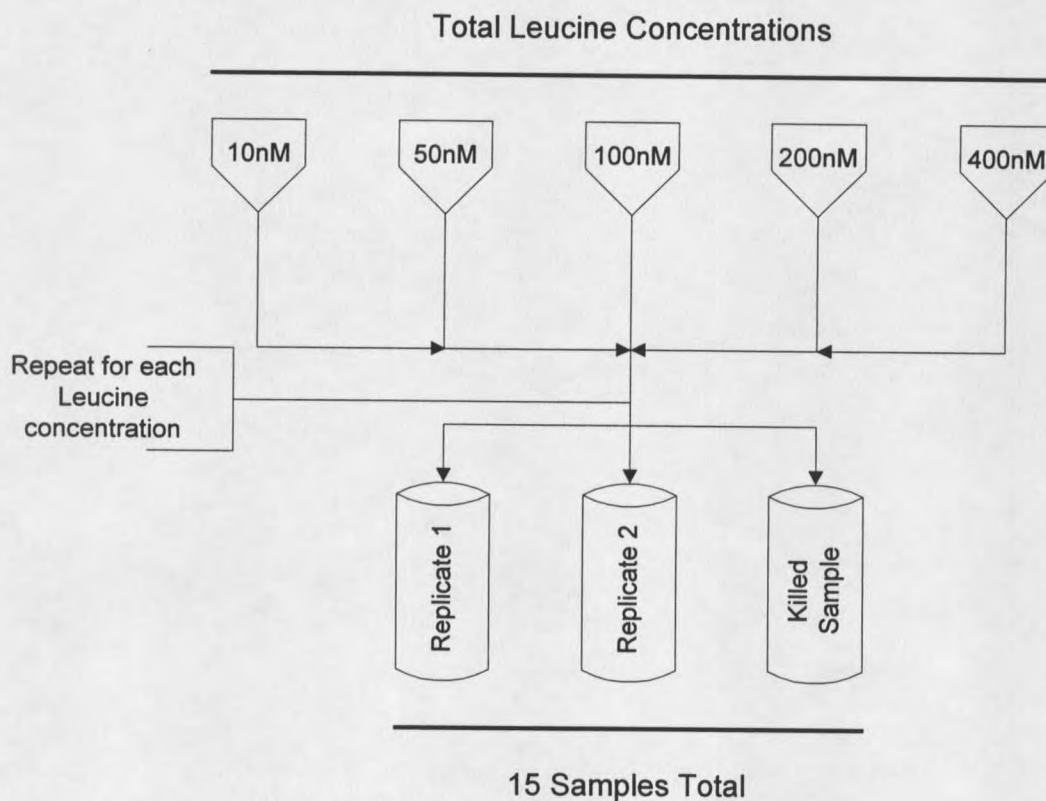


Figure 3. Sampling protocol for determining V_{max} for leucine uptake in suspended cell cultures.

described below. The resulting uptake rate of leucine (picomoles per milliliter per hour [$\text{pmol ml}^{-1} \text{ hr}^{-1}$]) by the suspended cells was plotted against leucine concentration and a saturation function (hyperbolic curve) was fit to the data using a nonlinear regression technique (TableCurve 2D software) to estimate V_{max} for leucine. A leucine concentration near V_{max} was used in subsequent uptake experiments to minimize the effects of isotope dilution by *de novo* synthesis.

A 5 ml sample of bacterial cells suspended in the liquid medium was incubated for 60 minutes after addition of commercially prepared L-[4,5- ^3H]leucine (Amersham, 126 – 184 Curies per millimole [Ci/mmol]) (approximately 10 nM labeled leucine) plus enough cold-leucine to saturate the sample as determined from the saturation experiment. The samples were incubated on a rotary shaking table and the incubation stopped by adding ice-cold TCA (5% final concentration) to the vial. The vials were immediately placed on ice, allowed to sit for 30 minutes for cold-TCA treatment of the cells. The liquid was filtered using a 0.2- μm pore-size polycarbonate filter (Nuclepore) mounted in a 12-place filtration apparatus (Millipore). The filter was rinsed repeatedly with ice-cold 5% TCA (2ml times 5), followed by a final rinse with cold, sterile phosphate-buffered saline (PBS, pH 7.2) with 0.1M non-radiolabeled cold-leucine. The filter was placed into a clean scintillation vial, 10 ml scintillation cocktail (Packard Ultima Gold) added, the vial vigorously mixed and allowed to sit overnight. Prior to measurement using a liquid scintillation counter (Packard 1900A), the vials were again vigorously mixed by shaking. Killed controls were prepared for each sample by adding the liquid sample to ice-cold

TCA (5% final concentration) with only the cold-leucine added. Killed samples were allowed to react for approximately 30-minutes before addition of the tritiated leucine. In addition to the killed controls, two filters were placed in 10 ml of the scintillation cocktail for determining background counts. A sample of the labeled leucine used in the uptake experiments was added to 10 ml of cocktail to check the original count of the label added to the sample and allow adjustment of the calculated activity based on the actual count. Samples were measured in triplicate and killed controls in duplicate.

Growth Rates for Attached Biofilm

Using aseptic techniques, a reactor sample slide was removed from a reactor, the back side of the slide scraped to remove any biofilm that may have developed, rinsed with sterile buffer, cut into equal length pieces (~ 1 cm in length = 1.74 cm²) and each piece placed biofilm-side-up into a 20 ml scintillation vial (clean and fired at 300 °C for 6 hours). The liquid medium was filter sterilized (0.2 - μ m pore-size nylon) AOC "free" reactor dilution water (4.75 ml) plus sterile nitrate+phosphate buffer (0.25 ml) plus the substrate and leucine. Substrates and leucine were added in microliter quantities and all concentrations were corrected for any dilution resulting from the sample size not being exactly 5 ml in volume. A liquid volume of 5 ml was selected for the experiments. ³H-labeled leucine (L-[4,5-³H]leucine, Amersham) (10 nM labeled + cold, non-labeled) leucine was added to the vials at a total concentration as determined from the saturation experiment (see below). Two biofilm samples and a killed control (5% final concentration TCA) were utilized for each analysis.

The biofilm samples were incubated for 60 minutes on a temperature controlled rotary shaking table ($24 \pm 1^\circ\text{C}$, 210 rpm) and the incubation stopped by adding ice-cold TCA (5% final concentration) to the vial. The vial was immediately placed on ice, allowed to sit for 30 minutes for cold-TCA treatment of the biofilm cells. Killed controls were allowed to react with the cold-leucine for 30 minutes prior to the addition of labeled leucine.

Following the period of 30 minutes on ice, the biofilm on the slide was removed and dispersed into the liquid by adding 7 to 8 μl of 1%-Triton X-100 (Jeffrey, W. H. and Paul, J. H., 1986) followed by probe sonication (Kontes) at approximately 40W for 1 minute (Jeffrey, W. H. and Paul, J. H., 1986; Thomaz, S. M. and Wetzel, R. G., 1995). Following sonication the liquid was filtered using a 0.2- μm pore-size polycarbonate filter (Nuclepore) mounted in a 12-place filtration apparatus (Millipore). The biofilm slide was placed on top of the filter, the filter and slide rinsed once with 5 ml of ice-cold 5% TCA, the slide removed and manually scraped with a straight razor blade, the blade rinsed with 1 ml of ice-cold 5% TCA, the slide rinsed with 1 ml of ice-cold 5% TCA, the slide and filter rinsed again with 3 ml of ice-cold 5% TCA, followed by a final rinse with 5 ml cold, sterile phosphate-buffered saline (PBS, pH 7.2) with 0.1M cold leucine. The slide and filter were placed into a clean scintillation vial, 10 ml scintillation cocktail (Packard Ultima Gold) added, the vial vigorously mixed and allowed to sit overnight. Prior to measurement using a liquid scintillation counter (Packard 1900A), the vials were again vigorously mixed by shaking and the vial checked to assure the slide piece was on the

bottom of the vial. Preliminary tests showed the polycarbonate slide did not interfere with liquid scintillation counting (LSC) when it was on the bottom of the vial. In addition to the killed controls, two filters were placed in 10 ml of the scintillation cocktail for determining background counts. A sample of the labeled leucine used in the uptake experiments was added to 10 ml of cocktail to check the original count of the label added to the sample and allow adjustment of the calculated activity based on the actual count. Following LSC, the slide was removed from the vial and the cut length measured to determine the biofilm area of the individual sample.

To determine $V_{\max}$ for the intact biofilm, one reactor sample slide was cut into 18 equal pieces (~ 1 cm in length = 1.74 cm^2) and each piece placed biofilm side up into a 20 ml scintillation vial for incubation with labeled leucine. The liquid medium for the $V_{\max}$ determination was filter sterilized ($0.2\text{-}\mu\text{m}$ pore-size nylon) reactor effluent in the amino acids experiment and filter sterilized reactor dilution water with a known substrate concentration added for the mixed substrate and carbohydrates experiments.

Six different total leucine concentrations (duplicate samples) and a killed control (5% TCA final concentration) for each concentration were evaluated. The leucine concentrations were typically 50, 100, 200, 400, 800, and 1200 nM. The resulting uptake rate of leucine ($\text{pmol cm}^{-2} \text{ hr}^{-1}$) by the biofilm was plotted against leucine concentration and a saturation function (hyperbolic curve) was fit to the data using a nonlinear regression technique (TableCurve 2D software) to allow estimation of $V_{\max}$ for leucine.

A leucine concentration near $V_{\max}$ was used in subsequent activity, growth and substrate uptake experiments to minimize the effects of isotope dilution by *de novo* synthesis.

Phase I—Batch Culture Uptake Experiments

Amino Acids and Carbohydrates, Replicate Experiments 1 and 2

Upon reaching steady-state at the 2,000 $\mu\text{g C/L}$ substrate feed concentration, all slides were pulled from a reactor, the biofilm scraped into a sterile buffer solution, the cell suspension homogenized, and the suspended cells used for performing batch culture substrate uptake experiments. Batch cultures for amino acids and carbohydrates were prepared for each individual compound in the substrate mixture and for the combination mixture using a total concentration of 2,000 $\mu\text{g C/L}$ for all cultures. Sterile buffer/nutrient and substrate solutions were added to 100 ml total volume in covered, sterile precombusted (500 °C) 250 ml Erlenmeyer flasks, the contents sampled for substrate concentration at time zero, then 1 to 2 ml of the biofilm cell suspension added as inoculum. The objective was to provide an initial total cell concentration of ca 10^6 per ml in the batch culture, dependent on the available cells from the reactor biofilm slides. Batch cultures were sampled at time zero and throughout the experiment at various time points for total and culturable cells counts and substrate concentration in the liquid phase. The flasks were incubated at 24 ± 1 °C in a temperature controlled shaking table incubator. Experiment duration was 3 days for amino acids batch cultures and up to 7 days for carbohydrates. Samples for substrate concentration measurements, amino acids and

carbohydrates, were withdrawn from the batch cultures using a disposable sterile syringe, then filtered using 0.2- μ m pore-size nylon syringe filters (MSI Cameo) into precleaned glass vials, capped and frozen for subsequent analysis.

Humic Substances

At the end of the first humic substances experiment, duplicate batch cultures were prepared containing 2000 μ g C/L of the humic material plus the buffer/nutrient solution. Duplicate samples for total organic carbon analysis, UV light absorbence, total direct cell counts and culturable cells were taken daily over a 10-day period. Blanks without cells added were also sampled at various points throughout the experiment.

Samples for TOC (NPOC) were processed immediately upon sampling. Ultraviolet (UV) light absorbence was used as a surrogate parameter to evaluate humic substance uptake in batch cultures and the reactors. UV-absorbence was measured at 254- and 285 nm wavelength using Standard Method 5910B and a Shimadzu UV-2101PC UV-Vis Scanning Spectrophotometer. Samples for total direct cell counts and culturable cell counts were handled as described above.

Batch cultures for the second humic substances replicate experiment were prepared and analyzed differently than the first replicate, based on the limited information obtained during the first experiment. Figure 4 shows the flow diagram for analysis of batch cultures from the second humics experiment. Batch cultures were prepared by adding scraped biofilm cells to batch cultures containing different concentrations of

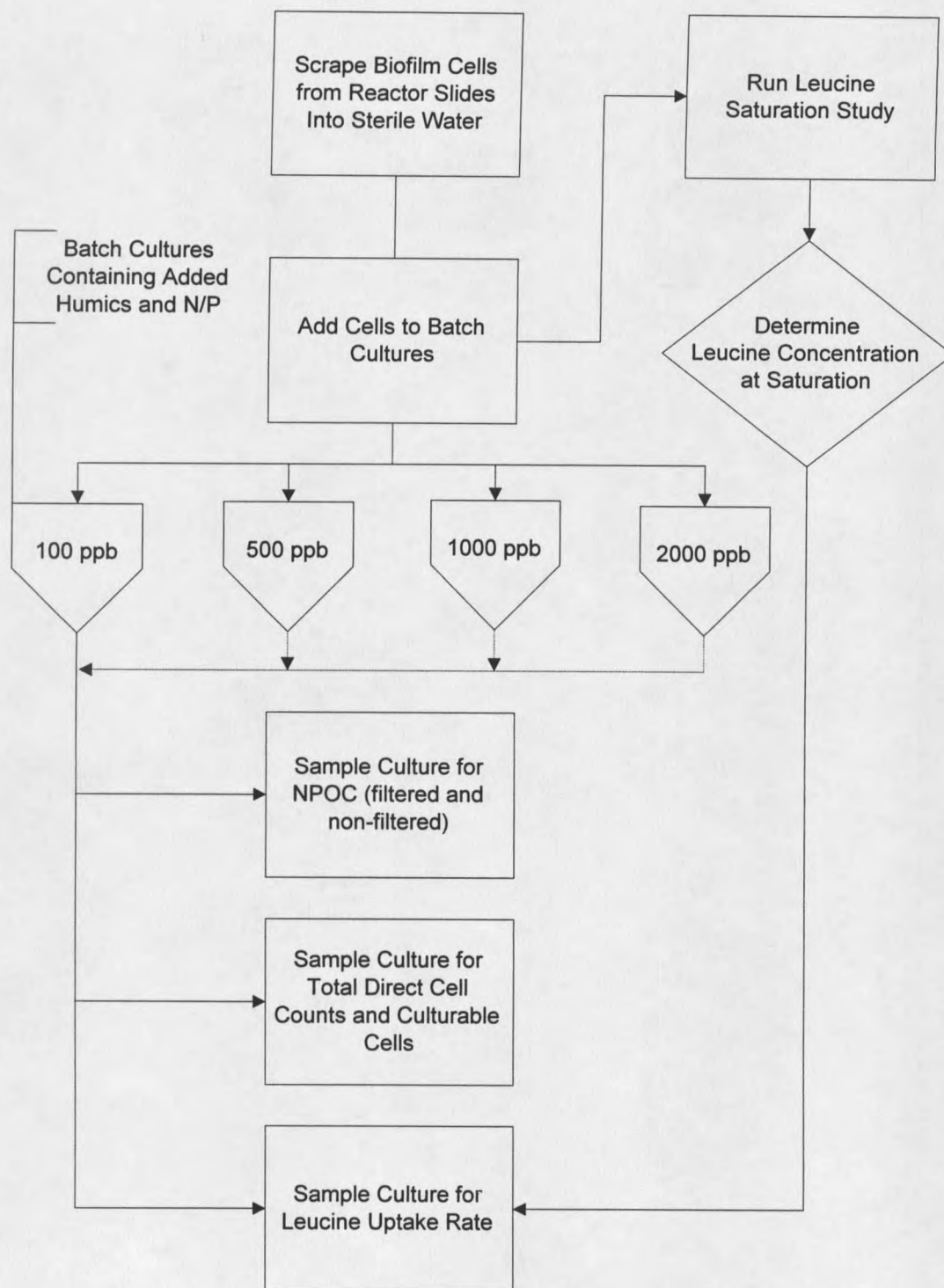


Figure 4. Flow diagram for determining growth kinetic parameters for biofilm cells grown on humic substances.

added humic substances: 100, 500, 1,000 and 2,000 $\mu\text{g C/L}$. A sufficient number of batch cultures were prepared to allow a batch culture from each prepared concentration to be sampled 10 to 11 times during the 10 day incubation period, along with blank (no cells) controls which were sampled three times during the course of the experiment. At each time point, the batch cultures were sampled for NPOC (filtered and unfiltered), total and culturable cells, and leucine uptake rate.

Phase II—Measurement of Biofilm Growth Kinetic Parameters

Growth kinetics for the attached biofilm were determined for the third amino acids replicate experiment. Biofilm development in rotating annular reactors took 60 to 120 days depending upon a number of factors such as response of the chlorinated reactor to chlorine or coliform growth in the reactors. Since the goal of the proposed work was to investigate growth kinetics for each substrate group, the time required for developing the biofilm for analysis limited the number of tests that could be performed and the number of substrates that could be evaluated.

A novel approach was developed to determine growth rates for biofilm attached to the sample slides. The polycarbonate sample slides with attached biofilm were cut into pieces approximately 1 centimeter (cm) long (approximately 1.74 square centimeters [cm^2]), placed into a 20 ml scintillation vial with 5 ml of media, and incubated to determine either the radiolabeled leucine or substrate uptake. The details of the media

and incubation techniques were presented above. Details of the experimental design are presented with the discussion of the results.

The kinetics study utilized all twelve biofilm slides in a reactor as described below.

- Two slides had been pulled previously to sample the biofilm for TDC and HPC.
- One slide was used to characterize the biofilm. Biofilm characterization included the following analyses:
 - Total direct cell counts (DAPI)
 - Cell size measurements (digital images saved using Image-Pro software and cell size measurements using ImageTool software)
 - Culturable cell counts (R2A media)
 - Staining of biofilm on polycarbonate slide using FITC and examination with confocal laser scanning microscope to determine approximate thickness and areal density
- One slide was cut into equal length sections for determination of $V_{\max}$ for leucine uptake.
- One slide was sampled to determine the vertical variation in biofilm activity based on the uptake of ^3H -leucine.
- Six slides were cut into 1 cm length sections and utilized for measurement of growth (utilizing ^3H -leucine) and substrate uptake (utilizing ^3H -labeled compounds) at different substrate concentrations.

- One slide was held in reserve for analysis in case an error was made using other slides.

External mass transfer may be a limiting factor when incubating the biofilm slides in scintillation vials; calculations of mass transfer by diffusion only show external mass transfer to be an issue. Tests performed to determine the impact of external mass transfer in the scintillation vials showed mixing at 210 rpm was sufficient to overcome mass transfer limitations. Mixing at greater rpm's tended to cause the slide to float during the incubation.

Determining Variation in Biofilm Activity

Vertical variations in biofilm activity were taken into account by mapping the leucine uptake rate of a slide utilizing small pieces of the biofilm sample slide as described above. Gjaltema et al. (1994) determined vertical variations existed in the biofilm on polycarbonate sample slides from rotating annular reactors. Gjaltema et al. also noted the biomass was greater on slides near the substrate influent port. Although ideal mixing occurs in the liquid within the annulus (Gjaltema, A. et al., 1994), there appears to be a vertical concentration gradient in the reactor. This can be a particular issue with nutrient limited and chlorinated systems such as those used in this work. The technique involved mapping a representative slide pulled from each reactor using ^3H -leucine uptake as the indicator of biofilm activity and including similar measurements of the top and bottom piece of each slide used for growth and substrate experiments to account for potential variation between slides. The technique was identical to the above-

described method for determining $V_{\max}$ with the exception that the saturating concentration of leucine was used for each sample vial. Figure 5 outlines the sampling technique.

Measurement of Growth and Substrate Uptake (Amino Acids Replicate 3 Experiment)

Slides with biofilm were removed from the annular reactor and cut into pieces approximately 1 cm long and placed into 20 ml glass scintillation vials with 5 ml of liquid medium. The liquid medium for all growth experiments consisted of 4.75 ml filter sterilized AOC "free" (0.2- μ m pore-size nylon) water and 0.25 ml sterile buffer/nutrient solution; final concentration of buffer solution in the vial was the same as in the reactor influent. Vials for growth contained ^3H -leucine in addition to cold-leucine and cold-amino acids. Vials for substrate uptake contained ^3H -amino acid in addition to cold-amino acids and cold-leucine. The sum of the labeled and unlabeled leucine concentrations was the same for each vial and equal to the concentration near saturation as determined by the $V_{\max}$ experiments. The amino acids concentrations were selected based on the experimental design, discussed in detail with the results. Following an incubation time of 60 minutes, the samples were handled as described above. The filters for the samples used to measure substrate uptake were rinsed with PBS containing 0.1 M non-labeled substrate.

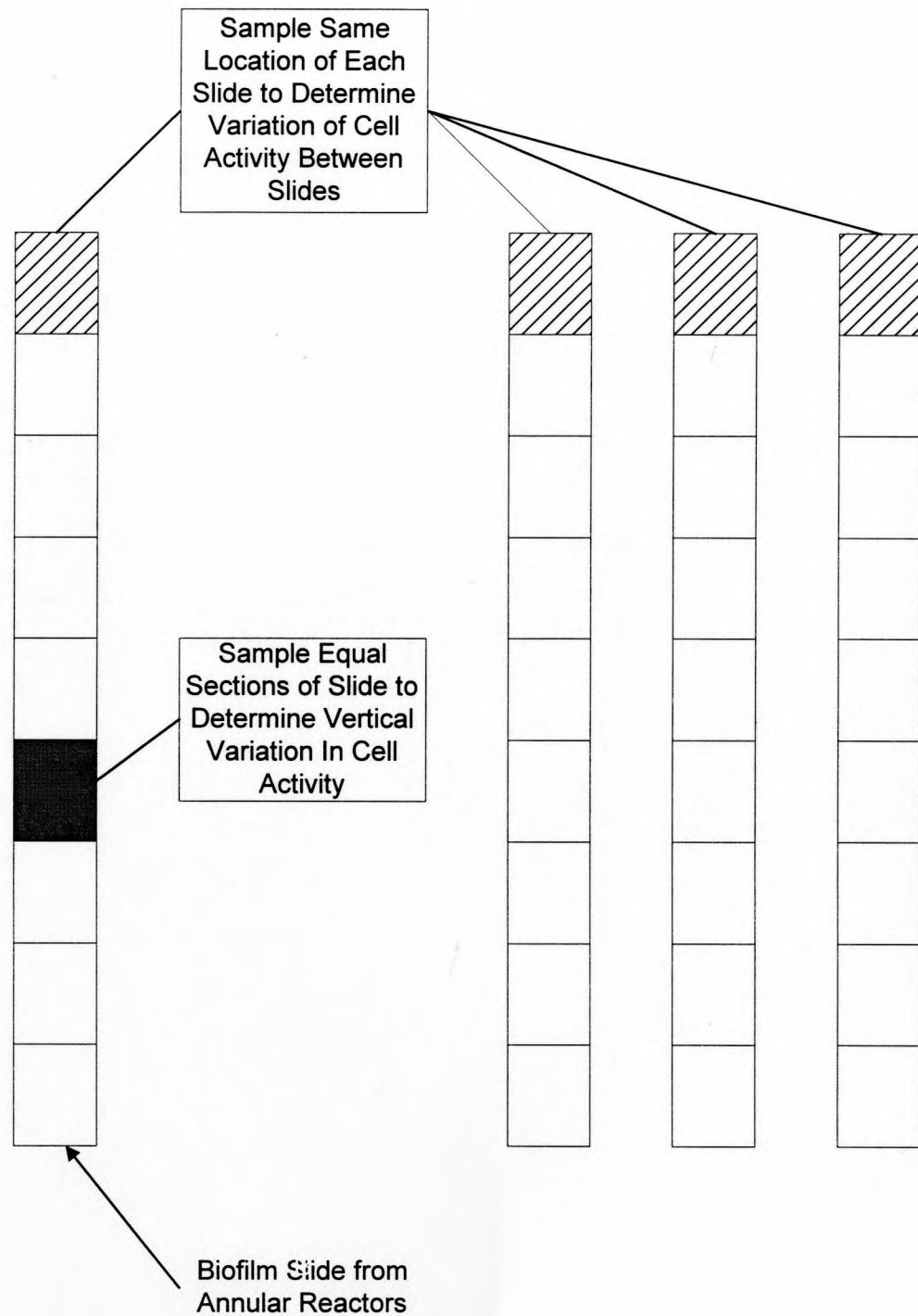


Figure 5. Biofilm slide sampling protocol to determine variations in biofilm cell leucine uptake activity.

Experimental Design for the Amino Acids Replicate 3 Experiment

The experimental design for evaluation of biofilm growth kinetics of the amino acids third replicate experiment involved utilizing ^3H -leucine to determine biofilm growth and ^3H -labeled amino acids to estimate substrate uptake. The experiment was performed utilizing biofilm sample slides taken from a reactor with an influent feed concentration of approximately 2000 $\mu\text{g C/L}$.

As discussed above, the number of biofilm sample slides limited the size of the experiment. The experimental design selected was a response surface analysis utilizing the Box-Behnken design with 3 factors. This design was used to evaluate the biofilm growth rate at three amino acids concentrations using three of the four amino acids. Although it would have been desirable to use all four amino acid substrates, the addition of another substrate to the experimental design would have increased the number of samples from 15 for a 3-factor design to 27 for a 4-factor design, for which there were insufficient biofilm sample slides.

A 2×2 factorial design was nested within the Box-Behnken design to assess the effects of individual and total amino acids concentration on uptake rates of specific amino acids. The amino acids selected for the experiment were glutamate, a 5-carbon amino acid, aspartate, a 4-carbon amino acid, and alanine, a 3-carbon amino acid. Serine was not selected as the 3-carbon amino acid because when compared to alanine, serine was a more utilizable amino acid and did not represent what was believed to be the low range of uptake for the group of four amino acids.

The amino acids concentrations used in the growth experiments were selected to represent a range of high to low concentrations the biofilm would be exposed to in the annular reactors. The computer software program AQUASIM (Wanner, O. and Reichert, P., 1996; Reichert, P., 1994) was used to estimate the substrate concentration at the biofilm-liquid interface. The three concentrations selected were 5, 35.36 and 250 $\mu\text{g C/L}$ for each amino acid, which allowed the use of the $\log_{10}$ values in the response surface model (the $\log_{10}$ values of 0.69897, 1.548455 and 2.39794 meet the model requirements since equal differences are required between factor values). Total amino acids concentrations in the experimental design ranged from 45.36 to 535.36 $\mu\text{g C/L}$.

Correction for Biomass and Activity Variation (Amino Acids and Carbohydrates Replicate 3 Experiments)

Leucine uptake by the biofilm was used to estimate the variation in both biomass and activity for each reactor. One biofilm sample slide was pulled from each reactor, cut into approximately 18 equal pieces, and the uptake of leucine determined for each piece. This provided a map of leucine uptake activity from the bottom to the top of a slide. Also, for the amino acids replicate 3 experiment, the top and bottom pieces of each slide pulled for the experiments were used to measure leucine uptake under conditions identical to those used when the slide was mapped.

Mixed Substrate Series Reactor Experiments

Series reactor sampling was performed at the end of the 500, 1000 and 2000 $\mu\text{g C/L}$ substrate concentration phases. One slide was pulled from each reactor and sampled

at the top, middle and bottom for TDC, HPCs and coliforms. A second slide was pulled from the first reactor in series and a determination of $V_{\max}$ for leucine uptake was performed as described above.

One slide was pulled from each reactor and the attached growth rate was determined using the technique described above for the amino acids replicate 3 experiment using ^3H -leucine. A minimum of three different concentrations as carbon of mixed substrate was used for the growth rate measurements, each measurement consisting of one replicate and a killed sample. Since a suitable method was not available to radiolabel humic substances, no direct measurement of substrate uptake was made for the mixed substrate reactor experiments.

Measurement of Biofilm Growth (Carbohydrates Replicate 3 Experiment)

Specific growth rates for the attached biofilm were determined using ^3H -leucine uptake as described above for the amino acids replicate 3 experiment.

Kinetics of Growth and Uptake

One goal of the work reported herein was to investigate parameters defining the growth kinetics of biofilms in drinking water systems for different substrate groups. It would be desirable to have the capability to model drinking water biological treatment systems or biofilm growth in distribution systems. These models could then be used to estimate treatment system size or evaluate the impacts of different treatment options on biofilm growth in the distribution systems. Understanding the basic relationships

between carbon and energy sources in drinking water and growth kinetics is one step in development of desired models.

Reactor Operation

Data collected during operation of the rotating annular reactors (total direct cell counts, cell size and volume measurements, NPOC) were used to indirectly estimate kinetic parameters for the biofilms in the reactors. These estimated parameters rely on measurements of cells entering and leaving the reactors and the number of cells in the biofilm. Difficulty in accurately determining these measurements can lead to variability in estimating parameters when using the natural, mixed-culture biofilm populations in these experiments.

Analysis of reactor data requires development of mathematical expressions for mass balances around the reactors assuming continuously stirred tank reactors (CSTRs) with biofilm on the reactor wetted surfaces. The reactor mass balance for planktonic cells gives the following equation:

$$V \frac{dX_1}{dt} = F(X_0 - X_1) + \mu_p X_1 V + r_d X_b A \quad (4)$$

where,

V = liquid volume (L^3)

X_1 = planktonic cell concentration in reactor liquid ($M_x L^{-3}$)

X_0 = planktonic cell concentration entering the reactor ($M_x L^{-3}$)

X_b = biofilm cell density ($M_x L^{-2}$)

- F = volumetric flow rate through reactor ($L^3 T^{-1}$)
 μ_p = specific growth rate of planktonic cells (T^{-1})
 r_d = specific biofilm detachment rate (T^{-1})
 A = surface area for biofilm accumulation in reactor (L^2)
 M = mass
 L = length
 T = time

A mass balance for the biofilm cells gives the following equation:

$$A \frac{dX_b}{dt} = \mu X_b A - r_d X_b A \quad (5)$$

where,

$$\mu = \text{specific growth rate of biofilm cells } (T^{-1})$$

At steady state the derivatives are assumed to equal zero and Equations 4 and 5 simplify to:

$$\frac{F}{V}(X_1 - X_0) = \mu_p X_1 + r_d X_b \frac{A}{V} \quad (6)$$

$$\mu = r_d \quad (7)$$

If $\mu_p X_1 \ll r_d X_b A/V$, then the term $\mu_p X_1$ can be ignored and Equations 6 and 7 can be solved. Assuming μ_p and r_d are nearly equal, X_1 and $X_b A/V$ can be compared for the annular reactors. Assuming the concentration of cells in the effluent is 10^6 cells per ml, the concentration of cells in the biofilm is 10^7 cells per cm^2 and a value of 35 femtograms

of carbon per cell, $X_1 = 3.5 \times 10^{-8}$ g C/ml and $X_b A/V = 9.5 \times 10^{-7}$ g C/ml and therefore $\mu_p X_1 \ll r_d X_b A/V$. This assumption is strengthened by the fact that since growth rates are generally low ($< 0.1 \text{ hr}^{-1}$) the hydraulic residence time is short enough to preclude significant planktonic growth. The term r_d can be solved using the following equation:

$$\frac{F}{V}(X_1 - X_0) = r_d X_b \frac{A}{V} \quad (8)$$

Equation 8 can be used to solve for the specific growth rate of the biofilm cells (μ_b).

Assuming steady state and minimal planktonic growth an observed yield can be calculated for the reactor using the following equation:

$$Y_{\text{obs}} = \frac{X_1 - X_0}{S_0 - S_1} \quad (9)$$

where,

S_0 = Substrate concentration in the influent to the reactor, $M_s \text{ L}^{-3}$

S_1 = Substrate concentration in the effluent from the reactor, $M_s \text{ L}^{-3}$.

Batch Culture Uptake Experiments

Data from the batch uptake experiments (biofilm cells in planktonic phase) provided substrate preference information by a comparison of the rates of substrate uptake and cell growth in batch culture. By using the measurements of cell number and cell volume along with changes in the substrate concentration, an estimate of cell yield ($Y_{X/S}$) was made for each substrate group and the combined substrate cocktail at one initial substrate concentration (2000 $\mu\text{g C/L}$). The uptake experiments provided

information used to compare uptake and cell growth differences between the control and chlorinated reactors.

The batch culture experiments were also analyzed to determine parameters for growth kinetics. The following additional definitions apply to these analyses:

Maximum specific growth rate ($\mu_{\max}$): $\frac{\text{maximum mass cells produced}}{\text{original cell mass} \cdot \text{time}} = \frac{M_{Xp(\max)}}{M_{X0} \cdot T}$

Monod half-saturation coefficient (K_s): value of S at $\frac{1}{2} \mu_{\max}$ when μ is plotted as the dependent variable against S as the independent variable, assuming Monod saturation kinetics apply.

Yield

Yield was determined using the change in cell mass (dX) for a measured change in substrate (dS), or $Y_{X/S} = dX/dS$. The approximation of dX/dS was made by using the following relationship:

$$Y_{X/S} = \frac{dX}{dS} \approx \frac{X_{t2} - X_{t1}}{S_{t1} - S_{t2}}, \quad (10)$$

where the subscripts $t1$ and $t2$ refer to the times used to evaluate X and S . Based on curves of S and X versus time, points were selected when the rates of substrate utilization and cell mass increase are near zero. The change in substrate concentration was determined using either the measured substrate concentrations (amino acids and carbohydrates) or the filtered non-purgeable organic carbon (NPOC) values for humics substances.

As discussed below for the humics batch cultures, determination of cell mass (X) can be made using several different approaches given the data collected.

1. The first approach possible is to use the culturable cell counts as determined using R2A media.
2. The second approach is to use total direct cell counts.

All approaches require a method to estimate the cell mass (biomass) per cell. Biomass should ideally be determined in terms of carbon so the mass terms in Equation 10 have the same units. Biomass was calculated based on cell volume measurements where data was available. The digital images of DAPI stained cells were used to first determine the cell size measurements of 200 or more cells per sample. Carbon per cell values were calculated as described above.

Analysis of Individual Batch Culture Data

Batch reactor data can be analyzed using either the differential or the integral methods (Grady Jr., C. P. Leslie and Lim, H. C., 1980)(p.128). The individual batch culture data was analyzed assuming first order growth kinetics. The integral method, commonly used to evaluate batch culture kinetics, was utilized to determine the specific growth rate. If a first-order reaction for the increase in cell mass is assumed,

$$\frac{dX}{dt} = \mu X. \quad (11)$$

When Equation 11 is integrated, the following relationship is obtained:

$$\ln \frac{X}{X_0} = \mu t. \quad (12)$$

Equation 12 can be placed in a linear form,

$$\ln X - \ln X_0 = \mu t. \quad (13)$$

A plot of $\ln X$ versus time (t) will be linear if the reaction is first-order and the slope of the line will be the specific growth rate μ . Typical batch culture growth curves demonstrate a lag phase where no growth occurs, followed by an exponential or log growth phase, followed by a decrease in growth rate to a stationary phase where none or negative growth occurs. Growth in the exponential phase was utilized to determine the specific growth rate.

The rate of substrate utilization in a batch culture is,

$$r_s = \frac{dS}{dt}. \quad (14)$$

If first order kinetics apply to the reaction, the substrate utilization rate can be related to cell growth using yield,

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}}. \quad (15)$$

which can be rearranged as,

$$\mu = -\frac{dS}{dt} \cdot \frac{Y_{X/S}}{X}. \quad (16)$$

Because yield may be dependent upon the growth rate (Button, D. K., 1993; Søndergaard, M. and Theil-Nielsen, J., 1997), direct measurement of substrate depletion was utilized to determine substrate uptake rate. The uptake of substrate by microbial

cells has been described using Michaelis-Menten saturation kinetics. Button (1991) showed that kinetics of substrate uptake for planktonic cells remain hyperbolic (saturation kinetics) even when multiple enzymes were controlling the rate of uptake. Substrate uptake can be described by the relationship:

$$v = \frac{V_{\max} S X}{K_T + S}, \quad (17)$$

where v is the concentration-dependent uptake rate ($\mu\text{g C L}^{-1} \text{ hr}^{-1}$), S is the substrate concentration outside the cell ($\mu\text{g C/L}$), $V_{\max}$ is the maximum rate of uptake (hr^{-1}) and K_T is the whole cell Michaelis constant for transport (Button, D. K., 1991). The concentration-dependent rate of uptake v can be determined directly from the measurement of substrate over time in the batch cultures. The rate was determined by first fitting a transition-type curve (a curve with a characteristic S-shape) to the substrate uptake data using nonlinear regression (TableCurve™ 2D). The slope of the straight line portion of the depletion curve was determined using linear regression to obtain a value for the rate of substrate depletion, dS/dt , or v . Dividing v by the mean value of X over the same time period gave the specific substrate utilization rate, v/X (hr^{-1}).

Parameter Estimation Using Nonlinear Regression. Kinetic parameters for growth and substrate utilization can be estimated using substrate depletion curves resulting from batch cultures (Robinson, J. A. and Tiedje, J. M., 1983; Simkins, S. and Alexander, M., 1984; Smith, L. H. et al., 1998; Templeton, L. L. and Grady Jr., C. P.

Leslie, 1988). Monod growth kinetics are described by the hyperbolic saturation equation:

$$\mu = \frac{\mu_{\max} S}{(K_s + S)} \quad (18)$$

The Monod relationship between growth and substrate concentration can be substituted into Equations 11 and 15 to give the following two relationships:

$$\frac{dX}{dt} = \mu_{\max} \left[\frac{S}{K_s + S} \right] X \quad (19)$$

$$-\frac{dS}{dt} = \frac{\mu_{\max}}{Y_{X/S}} \left[\frac{S}{K_s + S} \right] X \quad (20)$$

Equations 19 and 20 can be combined to provide a relationship between substrate uptake and growth, giving the following equation:

$$\frac{dX}{dt} = -Y_{X/S} \frac{dS}{dt} \quad (21)$$

Equation 21 can be simplified to provide a function relating cell mass to initial cell mass, change in substrate concentration, and yield as follows:

$$X = X_0 + Y_{X/S}(S_0 - S) \quad (22)$$

Substitution of Equation 22 into Equation 20 provides a relationship for substrate concentration over time without the variable X:

$$\frac{dS}{dt} = - \left[\frac{\mu_{\max} S}{K_s + S} \right] [X_0 + Y_{X/S}(S_0 - S)] \frac{1}{Y_{X/S}} \quad (23)$$

Integration of Equation 23 provides the integrated Monod equation that can be used to determine kinetic parameters based on substrate depletion curves. The integrated Monod equation is (Robinson, J. A. and Tiedje, J. M., 1983):

$$C_1 \ln \{ [Y_{X/S}(S_0 - S) + X_0] / X_0 \} - C_2 \ln(S/S_0) = \mu_{\max} t \quad (24)$$

where,

$$C_1 = (Y_{X/S}K_S + Y_{X/S}S_0 + X_0) / (Y_{X/S}S_0 + X_0) \quad (25)$$

$$C_2 = (Y_{X/S}K_S) / (Y_{X/S}S_0 + X_0) \quad (26)$$

Equation 24 cannot be solved explicitly for S and must therefore be solved using numerical techniques. The nonlinear regression capabilities of the software TableCurve™ 2D were utilized to approximate solutions to the integrated Monod equation. Equation 24 was rearranged to equal zero and the implicit solution capabilities of the software were used to perform nonlinear regression of the substrate-time data. TableCurve employs the Levenberg-Marquardt method using least-squares to solve the nonlinear equation and estimate best-fit parameters. The user-defined equation function of TableCurve allows the user to define the initial guess for parameters and minimum-maximum constraints for the parameters. A major advantage of TableCurve was the ability to graphically view the regression curve and data based on the initial estimates for the parameter and to interactively adjust the parameter values to provide a reasonable first-guess.

One disadvantage of using TableCurve to perform the nonlinear regressions was that sensitivity functions (Simkins, S. and Alexander, M., 1985; Robinson, J. A. and

Tiedje, J. M., 1983) are not used to adjust parameter estimates during the regression. This disadvantage was overcome by performing numerous regressions, setting the parameter estimates to different values, and plotting the residual sum of squares [RSS = $\Sigma(Y_{\text{obs}} - Y_{\text{pred}})^2$] as a function of that parameter. The parameter value that resulted in the global minimum RSS was then selected (Robinson, J. A., 1985; Panikov, N. S., 1995).

Growth Based On ^3H -Leucine Uptake. The biomass carbon production (BCP) rate, determined from the radiolabeled leucine uptake measurements, was used to estimate the growth rate in the batch cultures of the second humics experiment and growth rate for attached biofilm in the amino acids and carbohydrates replicate 3 experiments and the mixed substrate experiment. BCP was calculated using the equation presented previously:

$$\text{BCP} = \text{Leu}_{\text{inc}} \times 1,080 \times \text{F1} \times \text{ID} \quad (2)$$

where ID was taken as 1 since the concentration of leucine was near saturation; F1 was also assumed to be 1.

The BCP rate was determined at several time points throughout the batch culture incubation. Growth rates for the biofilm samples were determined for each substrate concentration. The specific growth rate μ was determined from the BCP rate. The rate of change in the BCP rate for a batch culture is the specific growth rate (Kirchman, D. et al., 1982). The most direct method of analysis of the data, assuming first-order dependence on biomass, is to plot the natural logarithm of the BCP rate against time and perform a linear regression. The slope of the line is the specific growth rate μ . The differential

method can also be used to analyze the data as was described above using curve fitting techniques.

Growth rates for the biofilm samples require both the BCP rate and a measurement of the biomass in the biofilm (Pollard, P. C. and Greenfield, P. F., 1997). If the BCP rate is r_{BCP} ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) and the biomass on the slide is X ($\mu\text{g C cm}^{-2}$), then the specific growth rate can be determined as follows:

$$\mu = r_{\text{BCP}} \frac{1}{X} = \frac{dX}{dt} \cdot \frac{1}{X}. \quad (27)$$

The biofilm biomass was determined using total direct cell count, measurement of the cell size, calculation of cell volume and conversion of cell volume to cell carbon.

Analysis of Combined Batch Culture Data. Analysis of the batch culture data for the amino acids and carbohydrate experiments was performed for each of the four individual substrate cultures and for the combined substrate culture, but only at one initial substrate concentration - 2000 $\mu\text{g/L}$ as carbon. Yield, cell growth rate and substrate utilization rate were determined for the four substrates in the cocktail and for the combined substrate cocktail using the techniques outlined above. Because analysis of the carbohydrates could not be performed, no data exists for the substrate uptake in those cultures.

Analysis of the batch culture data for the first humics experiment was performed using the same technique as described above for the amino acids and carbohydrates

experiment with the exception that only one substrate was evaluated, the filtered NPOC data.

The batch culture data for the second humics experiment was analyzed to determine the impacts of different initial substrate concentrations on kinetic parameters. The relationship between cell growth and substrate concentration was developed for the second humics experiment. Specific growth and substrate uptake rates were determined as described above for the individual batch cultures.

CHAPTER 4

RESULTS

Introduction

The results of the research presented in this chapter have been organized into two major sections. The first section presents the results from operation of annular reactors using each of the three substrate groups and for the mixture substrate experiment. Included in the first section are results from experiments using ^3H -leucine and ^3H -labeled amino acids to estimate growth and substrate uptake for attached biofilm samples. The second section covers the results from batch culture experiments performed for the amino acids, carbohydrates and humic substance substrate groups.

Kinetic parameters determined for the annular reactor biofilm were specific growth rate, yield and substrate uptake. Reactor-based biofilm kinetic parameters were developed for each substrate group for both the control and chlorinated reactor. The same kinetic parameters were determined using the data from the batch culture experiments. Batch culture kinetic parameters for the amino acids and carbohydrates were developed for each individual substrate within the amino acids and carbohydrates groups and the combination substrate for each group.

Kinetic parameters presented below are for the biofilm. Some would argue that kinetics of growth and substrate uptake applies only to the individual cells within the biofilm. Since the biofilm investigated were mixed natural populations of microorganisms indigenous to drinking water (at least within the City of Bozeman,

Montana), the question becomes are the kinetics relevant to the cells or the group of cells? In this dissertation the kinetic parameters were determined for the biofilm or group of microorganisms. Therefore the term biofilm kinetics has been used throughout this dissertation.

Reactor Biofilm Kinetics

The following section presents the kinetic parameters derived from data collected during operation of the annular reactors.

General

The objectives of the reactor experiments were to determine kinetic parameters for the biofilm and utilize those parameters to compare the response of the biofilm to different substrate groups, carbon loading rates and free chlorine disinfection.

Data were collected during operation of the annular reactors to provide the information necessary to determine growth rates, observed yields and substrate uptake rates for the biofilm. The data collected during operation of the reactors included influent, effluent and biofilm cell numbers and cell sizes; influent and effluent carbon concentrations (as NPOC), and free chlorine concentrations to determine both chlorine dose and residual in the reactor effluent. Specific growth rate, substrate uptake rate and observed yield were calculated as described in Chapter 3, MATERIALS AND METHODS.

The uptake of radioisotope-labeled leucine was used to estimate biofilm growth rates for the amino acids and carbohydrates third replicate experiments (replicate 3) and

the mixed substrate experiment (no replicates). The experimental design for evaluation of biofilm growth kinetics of the amino acids replicate 3 experiment involved utilizing ^3H -leucine to determine biofilm growth and ^3H -labeled amino acids to estimate substrate uptake. The experiment was performed utilizing the reactor biofilm sample slides taken from a reactor with an influent feed concentration of approximately 2000 micrograms as carbon per liter ($\mu\text{g C/L}$). The goals of the amino acids replicate 3 experiment were as follows:

- Assess the attached biofilm growth rate over a range of substrate concentrations.
- Evaluate substrate uptake rates associated with the biofilm growth rates.
- Estimate the interactions between the growth and substrate uptake rates with the various substrates.

Although the experimental design used for the amino acids replicate 3 experiment was intended also for the carbohydrates replicate 3 experiment, time and budget constraints required an experimental design for the carbohydrates replicate 3 experiment that measured only the biofilm growth rate using ^3H -leucine uptake.

In the following paragraphs, control data or control reactor refers to the reactor that received no chlorine. Chlorinated data or chlorinated reactor refers to the reactor that received chlorine in addition to the same nutrients received by the control reactor. It is important to note that at the 500 carbon levels neither the control or chlorinated reactors received chlorine for the amino acids, carbohydrates and humic substance experiments. It was believed that chlorination at the 500 level might reduce or inhibit the biofilm

development so much that data at the 1000 and 2000 carbon levels would be compromised. However, both reactors were chlorinated at the 500 level for the mixed substrate reactor experiment.

Comparisons between reactors refer to a comparison of data from the control and chlorinated reactors. Comparisons between reactors within a carbon level refer to a comparison of the control and chlorinated reactors at the same substrate concentration level. Carbon levels within a substrate group refer to a comparison between the three carbon levels for a single substrate group. Reactors within carbon levels and substrate groups refer to a comparison between reactor types for a carbon level within a substrate group (e.g., control compared to chlorinated at the 500 level for the amino acids substrate group). Reactor group or substrate group refers to data for a single group, such as the control reactors or humics reactors. See Chapter 3, MATERIALS AND METHODS for a description of the control and chlorinated reactors and their operation prior to sampling.

Specific growth rates, observed yield and substrate uptake across the reactor for the biofilm were calculated for at least four days of influent and effluent total direct count (TDC) data prior to a change in substrate level or at the end of the experiment (500, 1000 and 2000 $\mu\text{g C/L}$ nominal carbon levels). TDC data used to determine kinetic parameters were taken on the same days when NPOC and biofilm characteristics were determined. Kinetic parameters were compared between reactors at the various carbon levels and different substrate types. Kinetic parameters at each carbon level (500, 1000 and 2000) were pooled *a priori* for all experiments for all analyses except the one-way analysis of

variance (ANOVA) where reactor type (control or chlorinated) were pooled *a priori* for certain analyses.

The data were compared using standard ANOVA techniques utilizing MiniTab® statistical software. The experiments analyzed were for the four substrate groups: amino acids, carbohydrates, humic substances and mixed substrate. Treatment types were the control and chlorinated reactors. Carbon levels for each kinetic parameter were the nominal substrate feed concentrations as carbon (500, 1000 and 2000 µg C/L nominal carbon levels).

Kinetic parameters for a single reactor type within an experiment were analyzed utilizing a one-way ANOVA at an α -level of 0.05 and Tukey's pairwise comparison at the three carbon levels. The purpose of the one-way ANOVA was to determine if there were differences in the mean kinetic parameter between substrate carbon levels for each reactor type. A balanced ANOVA or the General Linear Model (GLM) ANOVA (α -level of 0.05) was used to compare the biofilm kinetic parameters between reactors for a single experiment (substrate group) and for all experiments (all substrate groups).

Carbon levels and reactor type (control or chlorinated), nested within carbon level (e.g., control reactor within the 500 carbon level), were the factors for the ANOVA used to assess the experiment for a single substrate group. Substrate group, carbon level and reactor type were the factors in the ANOVA used to assess all substrate groups (amino acids, carbohydrates, humics and mixed substrate); carbon level was nested within substrate group and reactor was nested within carbon level and substrate group. A natural log transformation of the specific growth rate data was necessary to provide

homogeneity of variance. ANOVAs for specific growth rates were based on analysis of natural log transformed data. Means and statistics for the means were reported for the non-transformed data. All other ANOVAs were performed on non-transformed data unless noted otherwise.

Specific Growth Rates

Specific growth rates for the biofilm were determined using two basic approaches or techniques. The first approach presented below was based on mass balances around the reactor. The second approach was based upon the rate of uptake of ^3H -leucine to estimate growth rates of the biofilm.

The following paragraphs present the results of the specific growth rate determinations using the two approaches. Results are presented for each substrate group by approach. Specific growth rates were compared between reactor types and nominal carbon levels using statistical methods described above. The relationships between specific growth rate and effluent substrate concentration were analyzed and the results are presented following the results of the statistical comparisons.

Amino Acids-Reactor Mass Balances. Statistical analysis of the amino acids reactor biofilm growth rates indicated the means were not equal between carbon levels ($p < 0.0005$) for the combined control and chlorinated reactor data, the means were equal between reactors within the carbon levels ($p = 0.102$), and the means were not equal between carbon levels within a reactor group ($p < 0.0005$). Analysis of the data for each

reactor using a one-way ANOVA showed the means were not equal between carbon levels for the control ($p=0.041$) and chlorinated ($p<0.0005$) reactors.

Specific growth rates at the 500 carbon level were significantly greater than at the 1000 and 2000 carbon levels for both the control and chlorinated reactors. Tukey's pairwise comparison for the chlorinated reactor biofilm growth rates indicated a significant difference between the growth rate at the 500 level and the growth rates at the 1000 and 2000 carbon levels, likely a result of chlorination at the 1000 and 2000 carbon levels. The significant difference for the control reactor was between growth rates at the 500 and the 1000 carbon levels. When only the 1000 and 2000 carbon levels were considered using a GLM ANOVA, the means were statistically equal when compared between the control and chlorinated reactors ($p=0.425$) and between the two carbon levels within a reactor group ($p=0.391$). Figure 88 in the Appendix shows the data used for the analysis. Mean values for the biofilm specific growth rates by reactor and carbon level are shown in Table 2 and Figure 6.

Amino Acids–Leucine Uptake for Replicate 3. The experimental design for evaluation of biofilm growth kinetics of the amino acids third replicate experiment involved utilizing ^3H -leucine to determine biofilm growth.

Correction for Biomass and Activity Variations. Leucine uptake by the biofilm was used to estimate the variation in both biomass and activity for each reactor. One biofilm slide was pulled from each reactor, cut into approximately 18 equal pieces, and the uptake of leucine determined for each piece. This provided a map of leucine uptake

Substrate	Reactor	Carbon Level ($\mu\text{g/L}$)	Mean (hr ⁻¹)	95% C.I.		n
				Lower	Upper	
Amino Acids	Control	500	0.0440	0.0165	0.0715	12
		1000	0.0456	0.0181	0.0730	12
		2000	0.0410	0.0135	0.0684	12
	Chlorinated	500	0.0978	0.0728	0.123	12
		1000	0.0259	0.00092	0.0509	12
		2000	0.0450	0.0201	0.0700	12
Carbohydrates	Control	500	0.0616	0.0454	0.0778	16
		1000	0.0165	0.00036	0.0327	16
		2000	0.0319	0.0157	0.0481	16
	Chlorinated	500	0.0556	-0.00158	0.113	16
		1000	0.113	0.0560	0.170	16
		2000	0.119	0.0620	0.176	16
Humics	Control	500	0.0784	0.0496	0.107	8
		1000	0.00238	-0.0265	0.0312	8
		2000	0.00266	-0.0262	0.0315	8
	Chlorinated	500	0.0760	0.0492	0.103	8
		1000	0.0449	0.0181	0.0717	8
		1000(a)	0.00537	-0.0283	0.0390	4
		2000	0.00589	-0.0209	0.0327	8

(a) Mean without replicate 1 data.

Table 2. Mean biofilm specific growth rates for the control and chlorinated reactors at each carbon level using the mass balance approach.

activity from the bottom to the top of a slide. Also, the top and bottom pieces of each slide pulled for the experiments were used to measure leucine uptake under conditions identical to those used for the mapping slide.

The control reactor demonstrated some variation in leucine uptake between individual samples from the single mapping slide (Figure 7). A linear regression of the data indicated no trend from top to bottom ($R^2=0.008$). There was little variation in top and bottom values around the circumference of the reactor (data not shown). Because no clear trend in variability was found with position of the biofilm within the reactor, no correction for biomass and activity was applied to the individual data values for the control reactor.

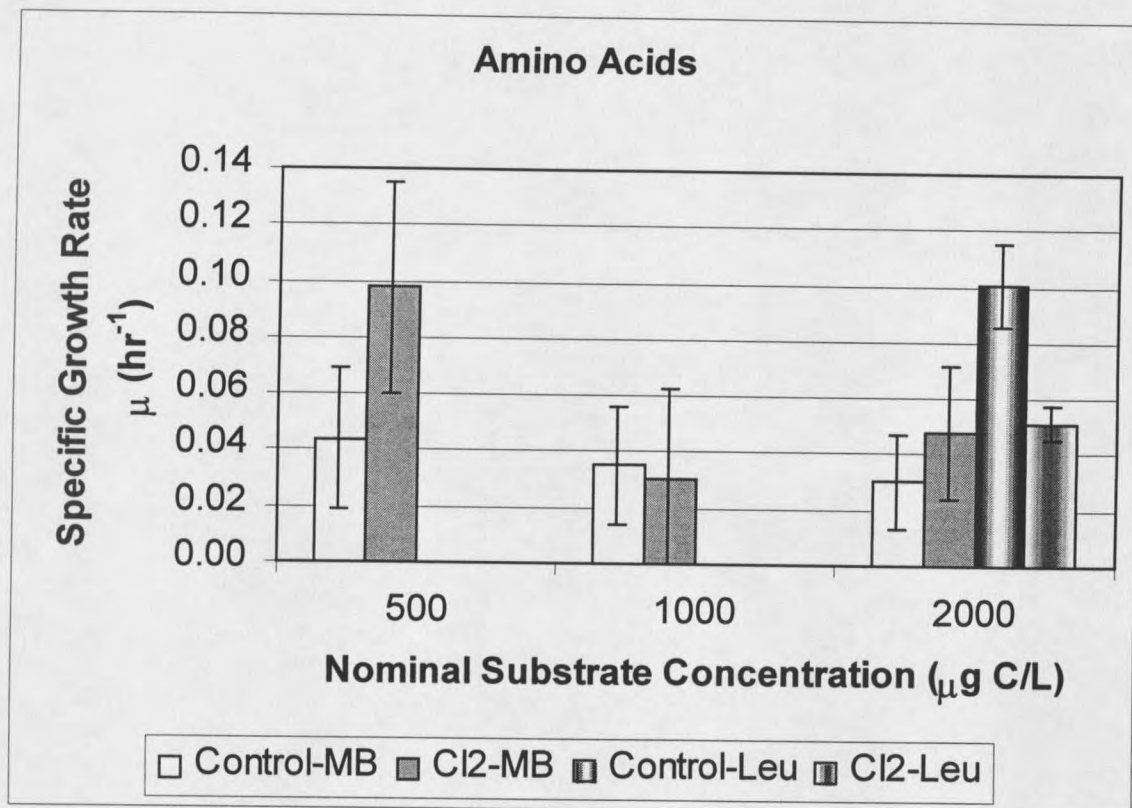


Figure 6. Comparison of mean reactor specific growth rates at the three carbon feed levels for the amino acids experiments using the mass balance (MB) and leucine uptake (Leu) methods. Leucine uptake data were from the replicate 3 experiment only. Error bars are 95% confidence intervals based on one-way ANOVA pooled standard deviations.

The chlorinated reactor had a measurable variation in activity from top to bottom as shown in Figure 8. The two slides mapped for leucine uptake activity showed an increasing activity from top to bottom. Although annular reactors have been shown to provide complete mixing, it is apparent that the interaction of chlorine with the amino acids and biofilm leads to biomass and activity variations within the reactor. Variation was also noted in the top and bottom pieces around the circumference of the reactor. Using the data from the two mapping slides and the top and bottom values from each

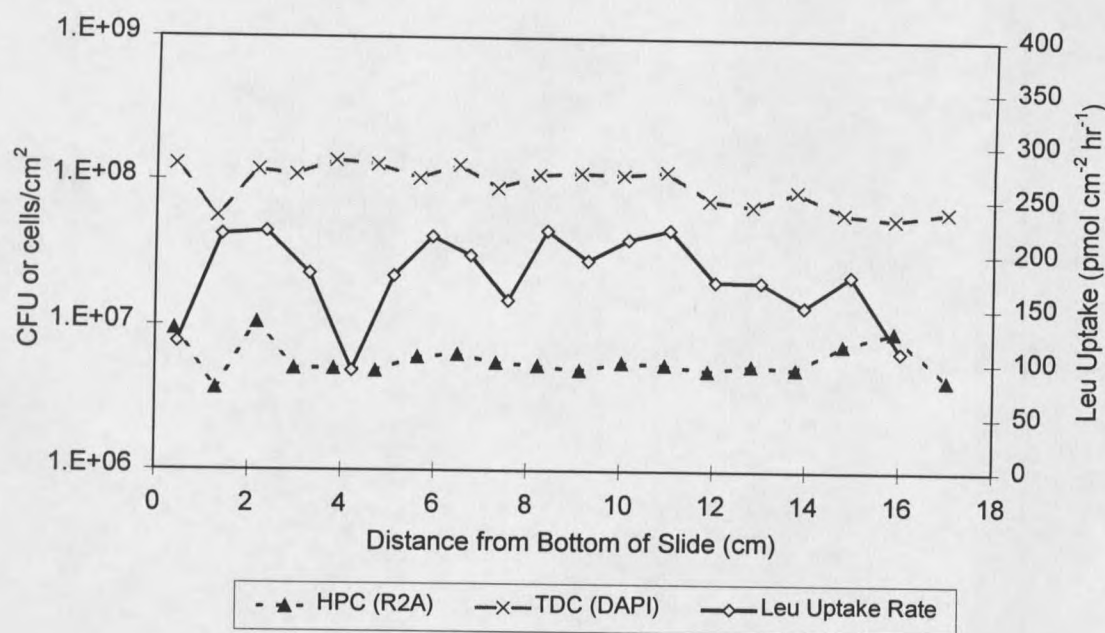


Figure 7. Variation in culturable cells (HPC), total direct count cells (TDC), and leucine uptake for a biofilm sample slide from the control reactor, amino acids replicate 3 experiment.

sample slide, Figure 9 was constructed utilizing 3-D interpolation software (SigmaPlot) to help visualize the variation in the outer wall of the reactor if it were a flat surface.

Utilizing the activity values created for Figure 9, the leucine uptake values for each chlorinated reactor sample point was corrected based on its position in the reactor. This correction was necessary to allow comparison of data taken from biofilm samples at different points within the reactor.

Control Reactor Biofilm Growth Rates. The data collected from the control reactor biofilm samples was analyzed using the response surface analysis capabilities of

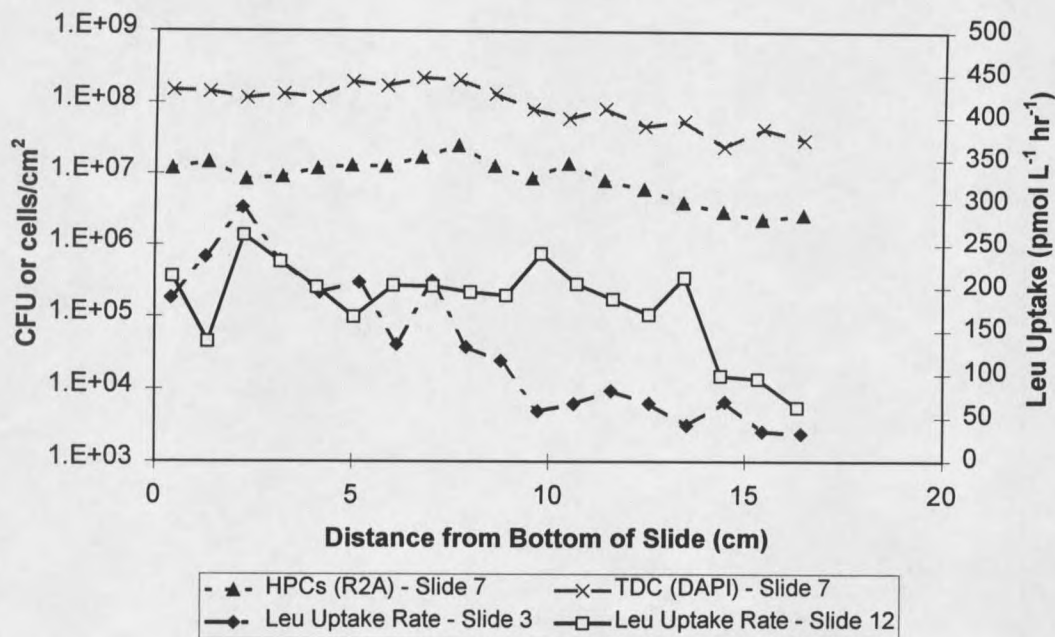


Figure 8. Variation in culturable cells (HPC), total direct count cells (TDC), and leucine uptake for biofilm sample slides from the chlorinated reactor, amino acids replicate 3 experiment.

the computer software MiniTab®. A linear model was found to best describe the response of the specific growth rate μ to the concentration of the specific amino acids.

The model equation determined from the regression was:

$$\mu \text{ (hr}^{-1}\text{)} = a + b(S_{\text{Glu}}) + c(S_{\text{Asp}}) + d(S_{\text{Ala}}) \quad (28)$$

where S is the $\log_{10}$ concentration of the amino acid as $\mu\text{g C/L}$. Table 3 presents the data for the model constants and statistics of the fit.

Examination of the t -values in Table 3 for the various amino acids indicates glutamate had the greatest effect on μ (highest t -value) compared to the other two amino

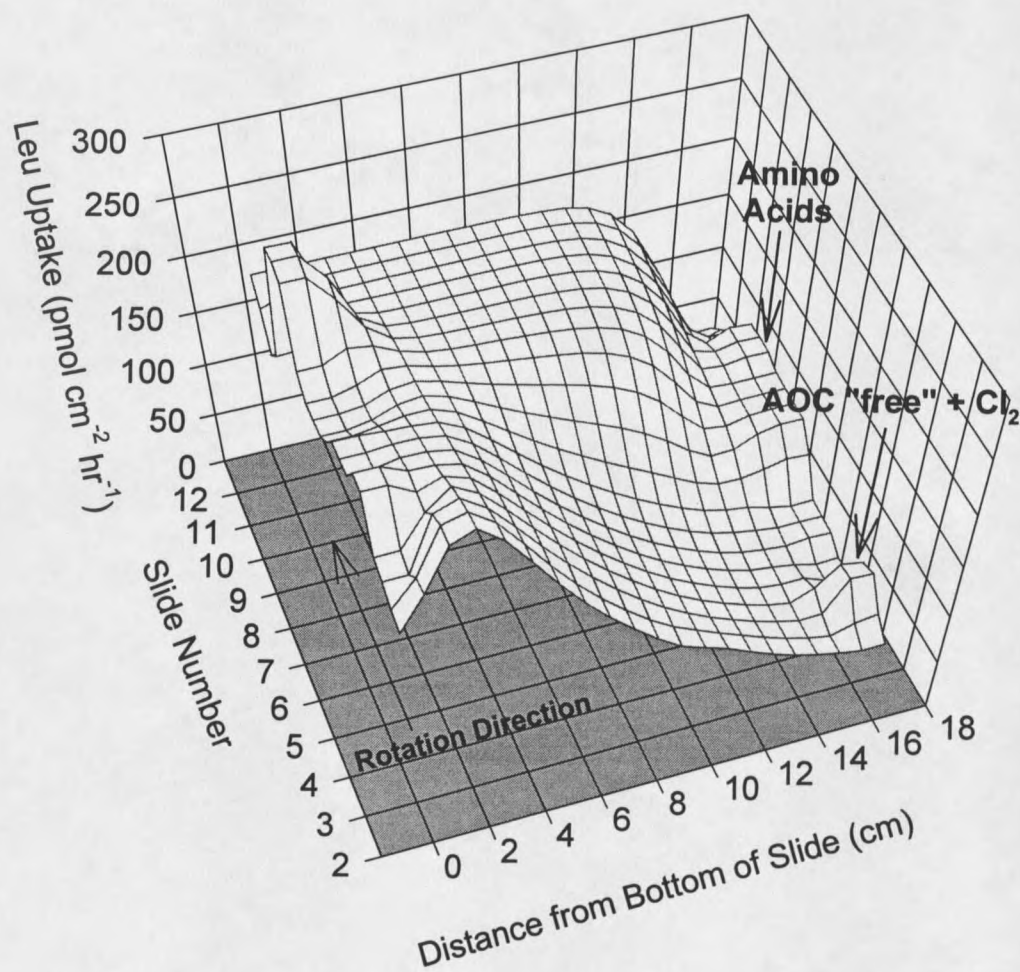


Figure 9. Interpolated leucine uptake rate for the biofilm slide surface of the chlorinated reactor, amino acids replicate 3 experiment.

Term	Coefficient	St Dev	t-value	p
Constant (a)	0.05384	0.018044	2.984	0.012
Glutamate (b)	0.03258	0.006555	4.971	0.000
Aspartate (c)	0.00971	0.006555	1.481	0.167
Alanine (d)	-0.01231	0.006555	-1.878	0.087
$R^2 = 0.734$				
Adj. $R^2 = 0.662$				
p (regresn.) = 0.002				
Note: Amino Acids Concentrations are $\log_{10}$ as $\mu\text{g C/L}$				

Table 3. Linear model for biofilm specific growth rate μ (hr^{-1}) – amino acids replicate 3 control reactor based on a response surface analysis of data from a Box-Behnken design.

acids, and alanine had a negative effect. In order to visualize the effects of each amino acid on the specific growth rate a main effects plot for the mean predicted specific growth rate values is presented in Figure 10.

The use of the above linear model has limited application since it requires specific amino acid concentrations and only included three amino acids. Therefore, an analysis of the specific growth rate as a function of the total amino acids concentration was performed for the control reactor. The data are shown in Figure 11. A linear regression (Figure 11) indicated no significant linear relationships between substrate concentration and growth rate ($R^2 = 0.054$, $p=0.405$). The mean value of the measured specific growth rate was 0.100 hr^{-1} ($0.0853 - 0.115$, 95% confidence intervals). The mean specific growth rate using leucine uptake is shown in Figure 6 for comparison with specific growth rates determined using the mass balance approach. The control reactor's biofilm growth rates using leucine uptake were greater than those calculated using the mass balance approach.

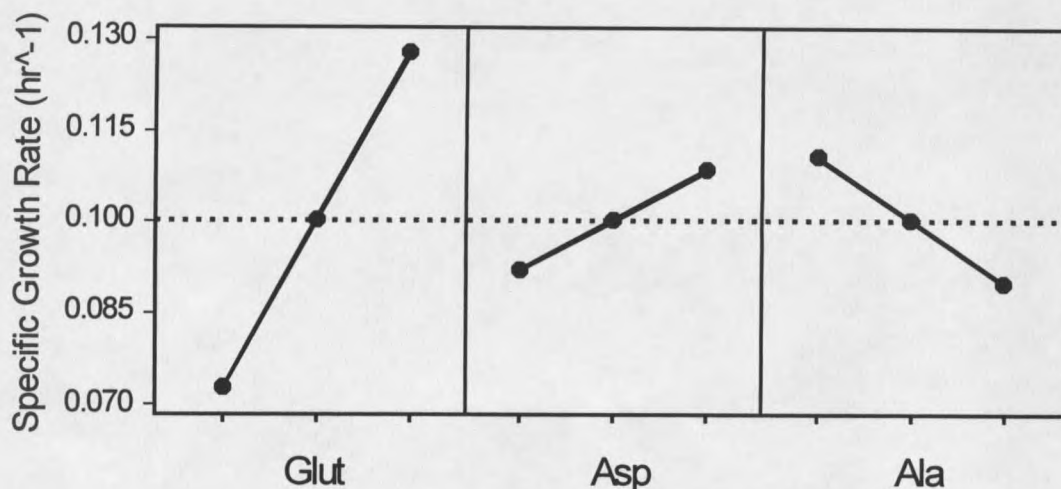


Figure 10. Main effects plot for amino acids replicate 3 control reactor. The values for growth rate are the mean at each substrate level based on fit values using the response surface model. The three substrate levels for each amino acid (x-axis) are 5, 35.36 and 250 $\mu\text{g C/L}$. The dashed line indicates the mean value for the reactor.

Chlorinated Reactor Growth Rates. A linear model that included the interactions between amino acids was found to best describe the response of the specific growth rate μ to the concentration of the specific amino acids for the chlorinated reactor. The model equation based upon the regression was:

$$\mu \text{ (hr}^{-1}\text{)} = a + b(S_{\text{Glut}}) + c(S_{\text{Asp}}) + d(S_{\text{Ala}}) + e(S_{\text{Glut}} S_{\text{Asp}}) + f(S_{\text{Glut}} S_{\text{Ala}}) + g(S_{\text{Asp}} S_{\text{Ala}}) \quad (29)$$

where S is the $\log_{10}$ concentration of the amino acid as $\mu\text{g C/L}$. Table 4 presents the data for the model constants and statistics of the fit.

Amino Acids Replicate 3 - Control

$$Y = 8.82E-02 + 3.54E-05X$$

$$R-Sq = 5.4\%; p=0.405$$

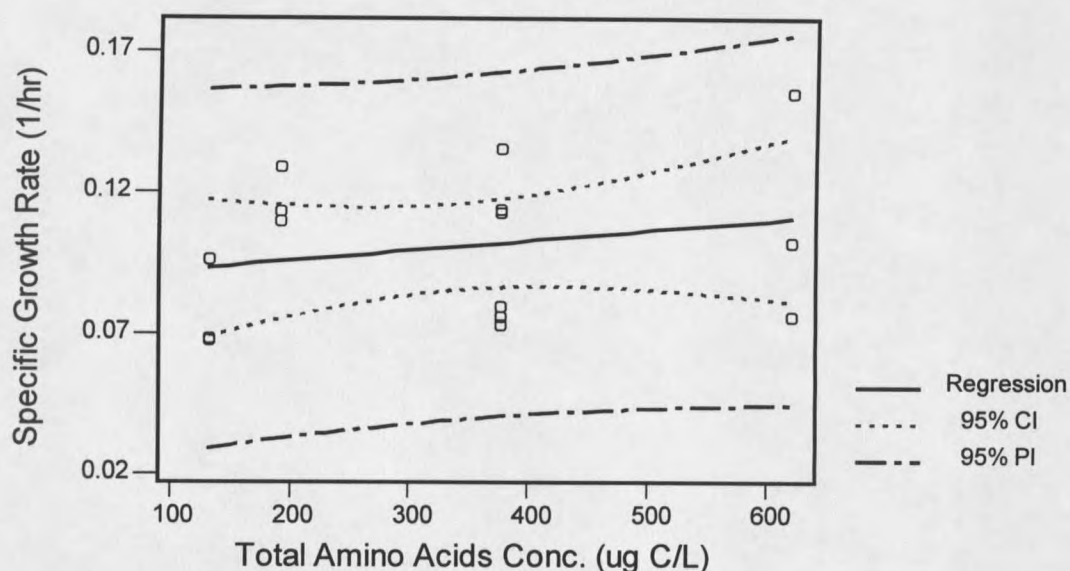


Figure 11. Specific growth rate based on leucine uptake as a function of total amino acids concentration for the amino acids replicate 3 control reactor. Added leucine concentration was 87 $\mu\text{g C/L}$.

Term	Coefficient	St Dev	t-value	p
Constant (a)	0.00174	0.023608	0.074	0.943
Glutamate (b)	0.03347	0.011992	2.791	0.024
Aspartate (c)	-0.01018	0.011992	-0.849	0.421
Alanine (d)	0.04554	0.011992	3.798	0.005
Glut $\times$ Asp (e)	0.00725	0.005281	1.373	0.207
Glut $\times$ Ala (f)	-0.02816	0.005281	-5.331	0.000
Asp $\times$ Ala (g)	-0.00287	0.005281	-0.543	0.602
$R^2 = 0.802$				
Adj. $R^2 = 0.654$				
p (regrsn.) = 0.016				
Note: Amino Acids Concentrations are $\log_{10}$ as $\mu\text{g C/L}$				

Table 4. Linear + Interactions model for biofilm specific growth rate μ (hr^{-1}) – amino acids replicate 3 chlorinated reactor based on a response surface analysis of data from a Box-Behnken design.

Glutamate and alanine had the greatest effect on the linear portion of the growth model while the interaction between glutamate and alanine had a large negative effect on the model. Overall the effect of glutamate was slightly positive while both aspartate and alanine were negative as shown in the main effects plot for the predicted mean specific growth rate for the chlorinated reactor, Figure 12.

An analysis of the specific growth rate as a function of the total amino acids concentration was also performed for the chlorinated reactor. The data are shown in Figure 13. A linear regression (Figure 13) indicated no significant linear relationships between substrate concentration and growth rate ($R^2 = 0.019$, $p=0.625$). The mean value of the measured specific growth rate was 0.0513 hr^{-1} ($0.0449 - 0.0578$, 95% confidence intervals). A comparison of the control reactor biofilm specific growth rates to those of the chlorinated reactor indicated the means were not equal ($p=0.0000$, 2-Sample t-test). The mean specific growth rate using leucine uptake is shown in Figure 6 for comparison with specific growth rates determined using the mass balance approach. The chlorinated reactor's biofilm growth rates using leucine uptake were nearly the same as those calculated using the mass balance approach (one-way ANOVA, $p=0.853$).

Carbohydrates-Reactor Mass Balances. Because the specific growth rate data for the carbohydrates experiment were not balanced (the number of data points varied by carbon level), the General Linear Model (GLM) was utilized to perform the ANOVA. When the control and chlorinated reactor data were combined, the means were equal between carbon levels for the carbohydrate reactor biofilm growth rates ($p=0.194$), but the means were not equal between reactors within the carbon levels ($p<0.0005$). Further

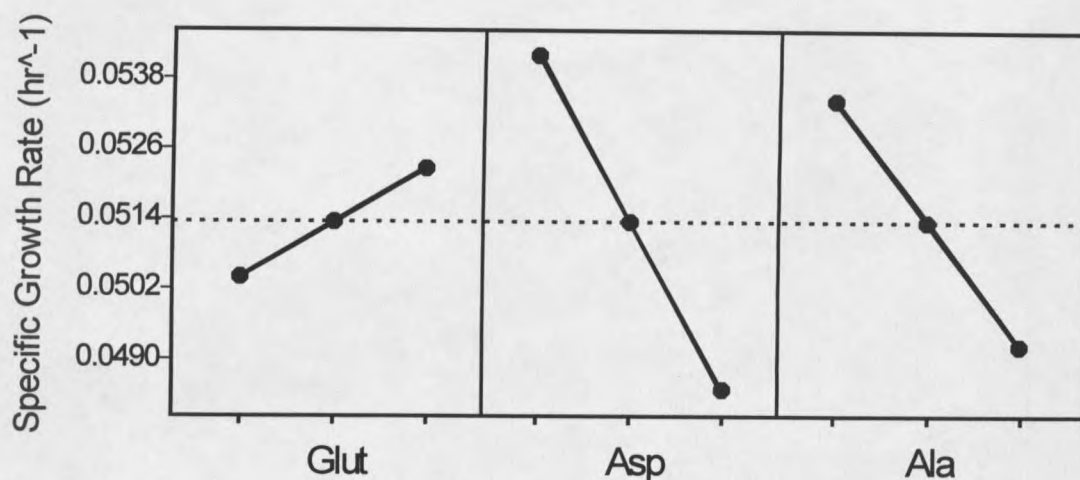


Figure 12. Main effects plot for amino acids replicate 3 chlorinated reactor growth rates. The values for the growth rate are the mean at each substrate level based on fit values using the response surface model. The three substrate levels for each amino acid (x-axis) are 5, 35.36 and 250 $\mu\text{g C/L}$. The dashed line indicates the mean value for the reactor.

analysis of the data for each reactor using a one-way ANOVA indicated the mean growth rates were not equal between carbon levels for the control reactor ($p=0.002$) and were equal between carbon levels for the chlorinated reactor ($p=0.324$). The control reactor biofilm growth rate at the 500 level was significantly greater than at the 1000 and 2000 carbon levels (Tukey's pairwise comparison), and there was no significant difference between the 1000 and 2000 carbon levels for the control reactor.

Growth rates for the chlorinated reactor biofilm were greater than for the control reactor biofilm at the 1000 and 2000 carbon levels. When only the 1000 and 2000 carbon levels were considered, the mean growth rates were not statistically equal between the two reactors ($p<0.0005$). The mean growth rates were equal at the two carbon levels

Amino Acids Replicate 3 - Chlorinated

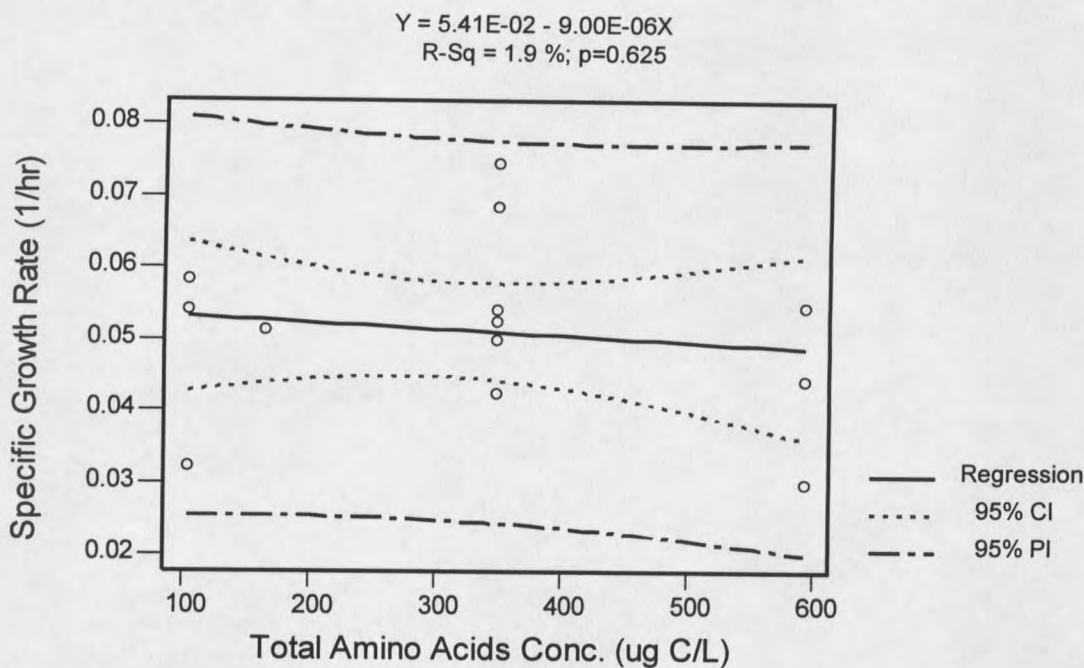


Figure 13. Specific growth rate based on leucine uptake as a function of total amino acids concentration for the amino acids replicate 3 chlorinated reactor. Added leucine concentration was 58 $\mu\text{g C/L}$.

(1000 and 2000; $p=0.488$), but were not equal between reactors within each carbon level (1000 and 2000; $p<0.0005$). Figure 89 in the Appendix shows the data values used for the ANOVA. Mean values for the biofilm growth rates at each nominal carbon level are shown in Table 2 and Figure 14.

Carbohydrates–Leucine Uptake for Replicate 3. Specific growth rates for the reactor biofilm were determined using leucine uptake at the 1000 and 2000 carbon levels. The biofilm was not sampled at the 500 carbon level for leucine uptake (neither reactor was chlorinated at the 500 carbon level). At each sampling (1000 and 2000 carbon

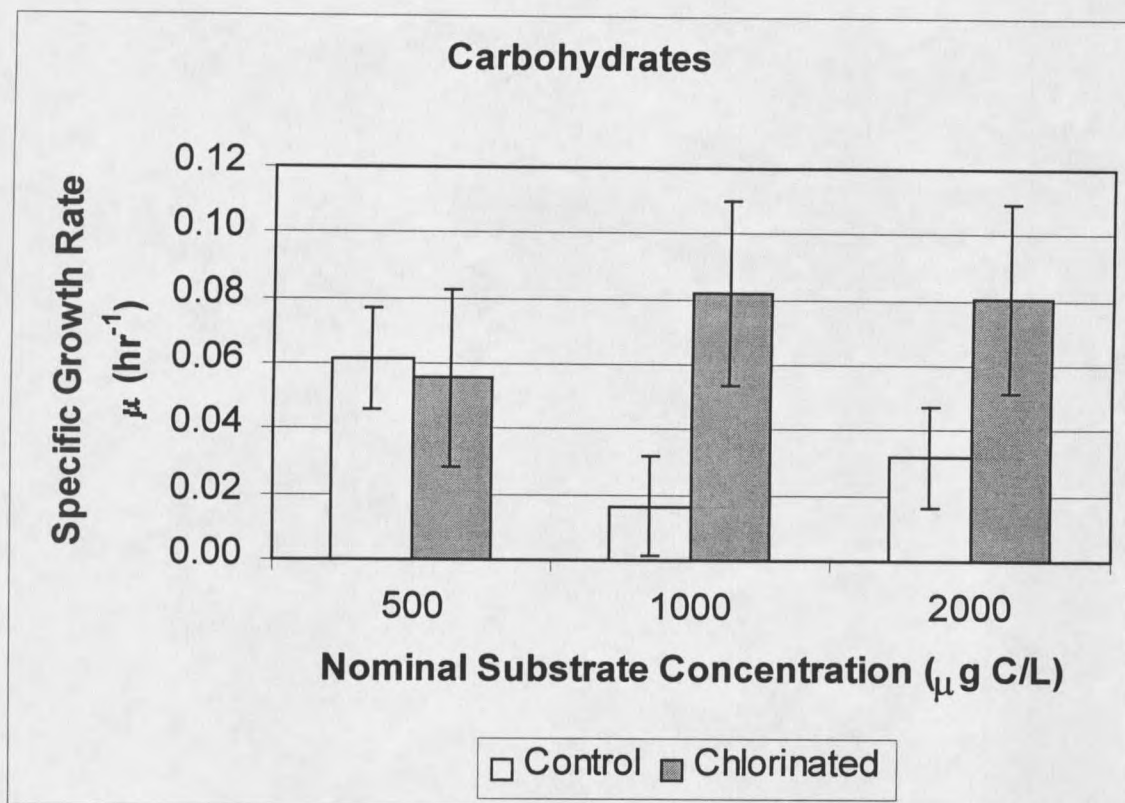


Figure 14. Comparison of mean biofilm specific growth rates using the mass balance approach at the three carbon feed levels for the carbohydrates experiments. Error bars are 95% confidence intervals based on one-way ANOVA pooled standard deviations.

levels) leucine uptake was determined at four to five different substrate concentrations to determine if substrate concentration impacted the uptake of leucine and the growth rate at a particular reactor carbon level.

A one-way analysis of variance was used to determine if there were differences between the biofilm specific growth rates at the 1000 and 2000 carbon levels for each reactor (the nominal carbon levels when the biofilm samples were taken). The mean growth rates were statistically equal for the chlorinated reactor biofilm at the 1000 and 2000 carbon levels ($p=0.587$), but there was a significant difference at the two carbon

levels for the control reactor ($p=0.005$ and Tukey's pairwise comparison); the growth rates were greater at the 1000 carbon level for the control reactor (see Figure 90 in the Appendix). An ANOVA of the combined data using the GLM indicated the mean growth rates were not equal between growth rates by reactor ($p<0.0005$); the chlorinated reactor growth rates based on leucine were less than those for the control reactor which was opposite to that using the mass balance approach. The mean growth rates at each carbon level are summarized in Table 5 and shown in Figure 15 along with the mean growth rates using the mass balance approach.

Humics-Reactor Mass Balances. Analysis of the combined control and chlorinated humics reactor data indicated the mean growth rates were not equal between carbon levels and between reactors within the carbon levels ($p<0.0005$ and $p=0.001$, respectively). A one-way ANOVA for the control reactor data indicated the mean growth rates were not equal between carbon levels ($p<0.0005$), and the difference between the 500 level and the 1000 and 2000 levels was significant (Tukey's pairwise comparison). There was no significant difference between growth rates at the 1000 and 2000 levels for the control reactor.

The mean growth rates for the chlorinated reactor biofilm were not equal when compared between carbon levels based on a one-way ANOVA ($p=0.001$), and growth rates at the 1000 and 2000 carbon levels were significantly less than at the 500 carbon level (Tukey's pairwise comparison). A one-way ANOVA of the chlorinated reactor growth rates without the 500 carbon level data indicated no significant difference

Substrate	Reactor	Carbon Level ($\mu\text{g/L}$)	Mean (hr^{-1})	95% C.I.		n
				Lower	Upper	
Carbohydrates Replicate 3	Control	1000	0.0262	0.0236	0.0288	14
		2000	0.0228	0.0207	0.0249	20
	Chlorinated	1000	0.00512	0.00363	0.00662	18
		2000	0.00542	0.00392	0.00691	18

Table 5. Carbohydrates replicate 3 mean biofilm growth rates based on leucine uptake for the control and chlorinated reactors.

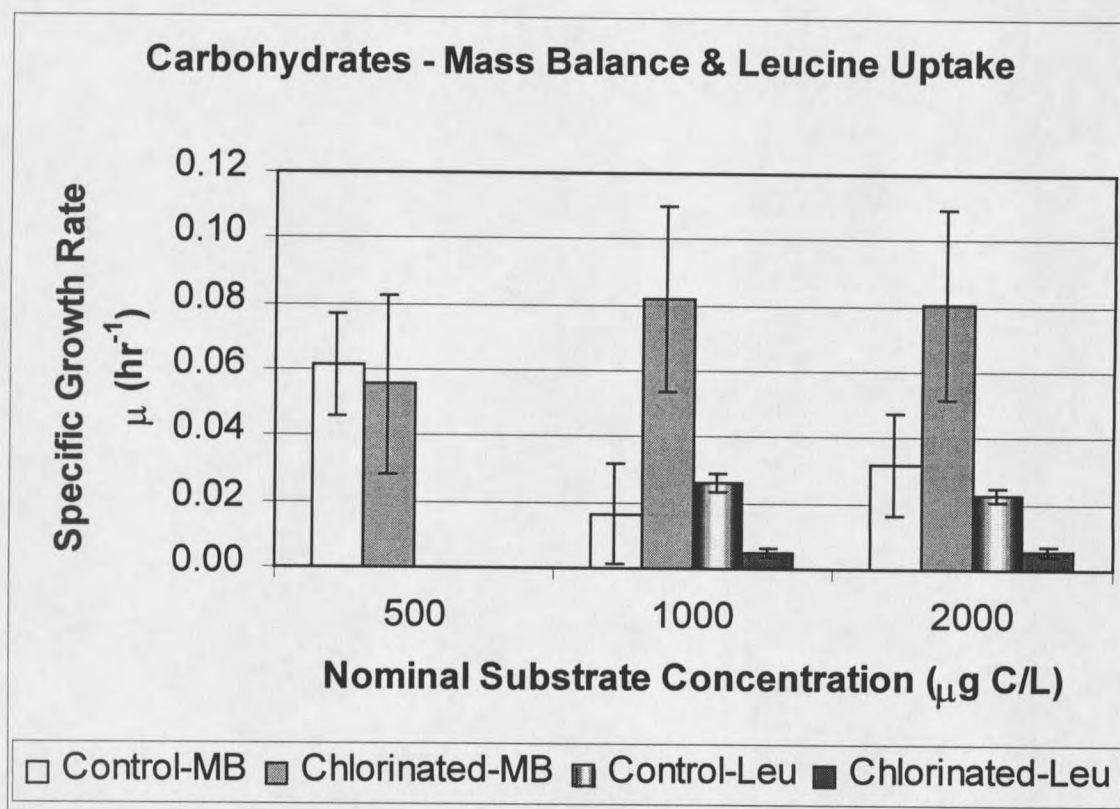


Figure 15. Comparison of mean reactor biofilm specific growth rates measured using leucine uptake (Leu) and mass balance (MB) at each carbon feed level for the carbohydrates experiment. Leucine uptake data are from the Carbohydrates replicate 3 experiment. Mass balance data is from all carbohydrates experiments. Error bars are 95% confidence intervals based on one-way ANOVA pooled standard deviations.

between the 1000 and 2000 carbon levels ($p=0.080$). A one-way ANOVA between reactors without the 500 carbon level data showed the mean growth rates were not equal ($p=0.001$), and the chlorinated reactor biofilm growth rates were greater than the control reactor rates.

It was noted that the chlorinated reactor growth rates for replicate 1 at the 1000 carbon level were much greater than measured for the replicate 2 experiment. The impact of removing from the analysis the replicate 1 data at 1000 is shown in Figure 16. When the replicate 1 data at 1000 was excluded from the ANOVA the mean growth rates for the chlorinated reactor biofilm were equal when compared between the 1000 and 2000 carbon levels ($p=0.503$). The chlorinated reactor growth rates were significantly greater than the control reactor growth rates for the combined 1000 and 2000 carbon level data ($p=0.007$). Figure 91 in the Appendix shows the data used in the ANOVA analysis. Mean values for the biofilm growth rates are shown in Table 2 and Figure 16.

Mixed Substrates-Reactor Mass Balances. The mixed substrate experiment consisted of two annular reactors operating in series for the control group and two for the chlorinated group. The first reactor in each series (the "a" reactor) received the substrate, nutrient and dilution water feed, and the second reactor (the "b" reactor) received only the effluent from the first reactor. Only the first reactor received chlorine for the chlorinated reactors. Unlike the other experiments, chlorination of the chlorinated reactor started at the 500 carbon level.

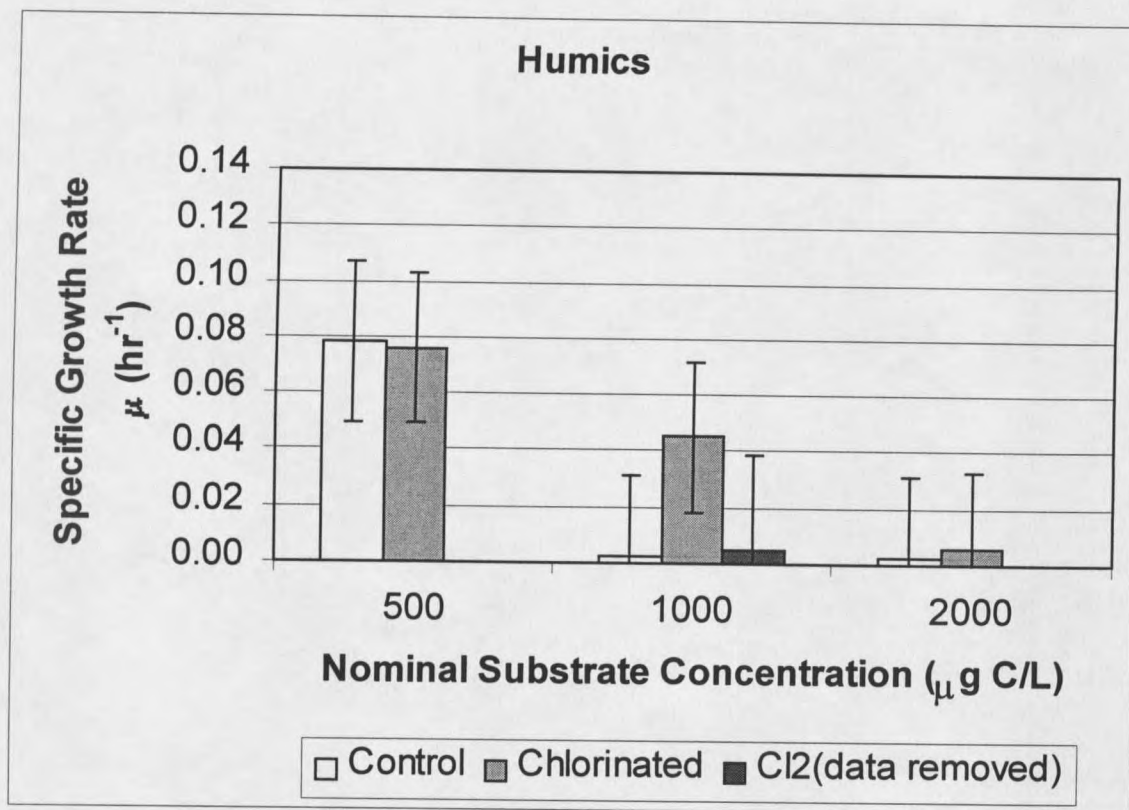


Figure 16. Comparison of mean reactor specific growth rates at the three carbon feed levels for the humics experiments. Data shown for chlorinated reactors at 1000 $\mu\text{g/L}$ carbon level without replicate 1 data (Cl2-data removed). Error bars are 95% confidence intervals based on one-way ANOVA pooled standard deviations by carbon level.

"a" Reactors. Analysis of the combined control and chlorinated reactor data indicated the mean growth rates were not statistically equal between carbon levels and between reactors within the carbon levels ($p=0.002$ and $p<0.0005$, respectively) for the mixed substrate "a" (first) reactors. A one-way ANOVA for the control reactor data indicated the mean growth rates were not equal between carbon levels ($p=0.001$), and there was a significant difference between the 500 and 2000 carbon levels and between the 1000 and 2000 carbon levels (Tukey's pairwise comparison). Growth rates for the

control reactor 500 carbon level were greater than at the 1000 carbon level, and the growth rate at the 1000 carbon level was greater than at the 2000 carbon level.

Based upon results of a one-way ANOVA ($p=0.021$) the mean growth rates for the chlorinated "a" reactor biofilm were not equal between carbon levels. The difference in growth rates between the 500 and 1000 carbon levels was not significant, but the difference between the growth rates at the 500 and 2000 carbon levels and the 1000 and 2000 carbon levels was significant (Tukey's pairwise comparison). In general, the growth rate in the chlorinated "a" reactor was the lowest at the 500 carbon level and increased as the carbon concentration increased, a trend opposite to that seen in the control "a" reactor. Figures 92 and 93 in the Appendix show the data used in the mixed substrate ANOVA analysis. Mean values for the biofilm growth rates are shown in Table 6 and Figure 17.

"b" Reactors. Growth rates in the control "b" reactor were very low and difficult to determine using the mass balance technique. Where growth rates were computed to be less than zero, a value near zero (0.000001 hr^{-1}) was assumed to allow use of the natural log transformation required to obtain homogeneity of variance for the ANOVA. Using the GLM to perform the ANOVA for the combined control and chlorinated "b" reactor data, the mean growth rates were not equal when compared between reactors and carbon levels within reactor groups. A one-way ANOVA for the control "b" reactor indicated the mean growth rates were statistically equal between carbon levels ($p=0.397$). A one-way ANOVA for the chlorinated "b" reactor indicated the mean growth rates were not equal between the three carbon levels ($p=0.004$), and the difference between growth rates

Substrate	Reactor	Carbon Level ($\mu\text{g/L}$)	Mean (hr^{-1})	95% C.I.		n
				Lower	Upper	
Mixed Substrate	Control-a	500	0.0869	0.0510	0.123	5
		1000	0.0363	0.00042	0.0722	5
		2000	0.00530	-0.0306	0.0412	5
	Chlorinated-a	500	0.0283	-0.0332	0.0898	5
		1000	0.0894	0.0279	0.151	5
		2000	0.107	0.0450	0.168	5
	Control-b	500	0.0153	-0.00740	0.0380	5
		1000	0.00000	-0.0227	0.0227	5
		2000	0.00000	-0.0227	0.0227	5
	Chlorinated-b	500	0.211	0.124	0.298	5
		1000	0.0112	-0.0757	0.0982	5
		2000	0.00886	-0.0781	0.0958	5
	Control – Combined	500	0.0432	0.0335	0.0530	5
		1000	0.00511	-0.00465	0.0149	5
		2000	0.00231	-0.00745	0.0121	5
	Chlorinated – Combined	500	0.117	0.0735	0.160	5
		1000	0.0223	-0.0211	0.0656	5
		2000	0.0366	-0.00672	0.0800	5

Table 6. Mixed substrate mean biofilm growth rates for the control and chlorinated reactors at each carbon level based on the mass balance approach. First reactor in series denoted as “a”, second reactor in series denoted as “b”, and a+b denoted as “combined.”

at the 500 and 2000 carbon levels was significant. In general, for both the control and chlorinated mixed substrate “b” reactors the growth rate at the 500 level was greater than at the 1000 and 2000 carbon levels and growth rates at the 1000 and 2000 carbon levels were nearly the same. Figures 92 and 93 in the Appendix show the data used in the mixed substrate “b” reactors ANOVA. Mean values for the biofilm growth rates are shown in Table 6 and Figure 17.

Combined Reactors. The data from the mixed substrate reactors was analyzed by using data from the influent of the “a” reactors and effluent from the “b” reactors, referred to as the combined reactor data, thus providing data for each reactor type over a

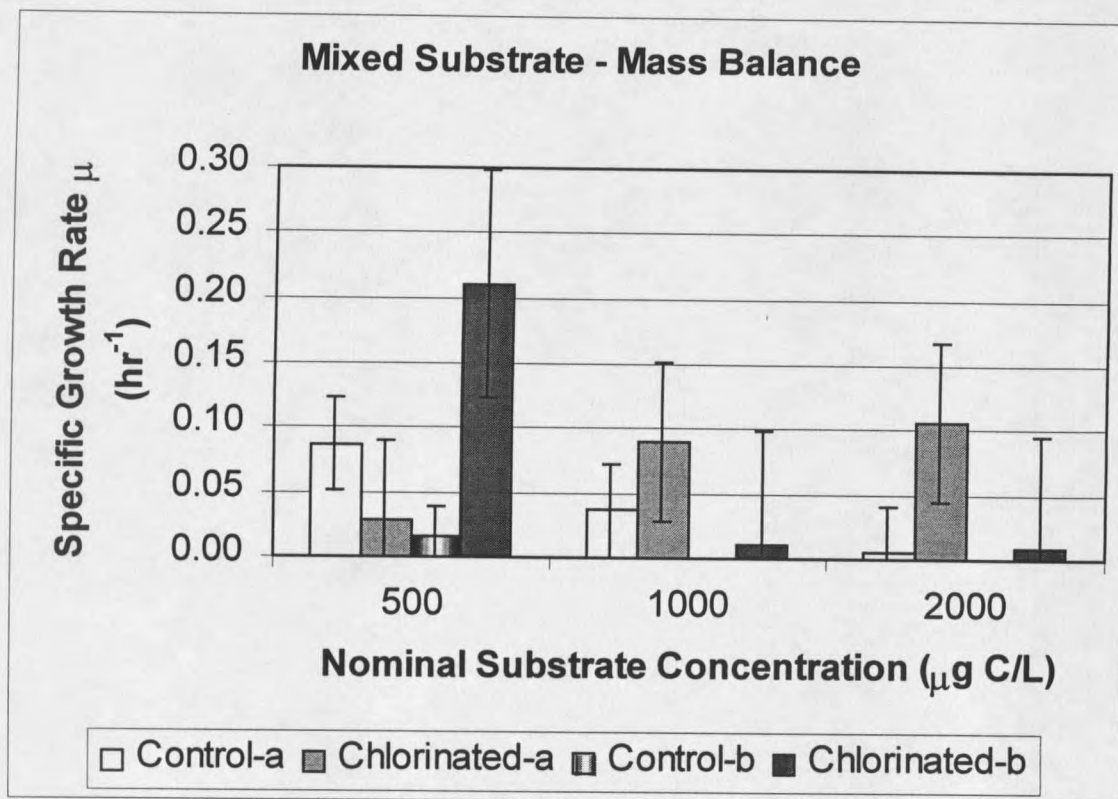


Figure 17. Comparison of reactor mean specific growth rates at the three carbon feed levels for the mixed substrate experiments. Growth rates were not detectable for the Control b reactors at the 1000 and 2000 $\mu\text{g/L}$ carbon levels. Nominal substrate concentration is that fed to the first (a) reactor in series. Second reactor in series denoted as "b". Error bars are 95% confidence intervals based on the one-way ANOVA pooled standard deviations.

detention time twice that of a single reactor (approximately 4 hours). Using the combined control and chlorinated reactor data, an ANOVA utilizing the GLM indicated the mean growth rates were not equal when compared by reactor ($p < 0.0005$), by carbon level ($p < 0.0005$), and by carbon level within the reactor group ($p < 0.0005$). A one-way ANOVA of the combined control reactor data indicated the mean growth rates were not equal when compared by carbon level ($p < 0.005$), and the differences between growth rates at the 500 and 1000 carbon levels and the 500 and 2000 carbon levels were

significant (Tukey's pairwise comparison). The difference between growth rates at the 1000 and 2000 carbon levels was not significant for the combined control reactor. A one-way ANOVA of the chlorinated combined reactor data indicated the mean growth rates were not equal when compared by carbon level ($p < 0.005$), and the differences between growth rates at the 500 and 1000 carbon levels and the 500 and 2000 carbon levels were significant (Tukey's pairwise comparison). The difference between growth rates at the 1000 and 2000 carbon levels was not significant for the combined chlorinated reactor. In general, the specific growth rates in the control and chlorinated combined reactors followed the same trend. Growth rates were greatest at the 500 carbon level. Growth rates at the 1000 and 2000 carbon levels were nearly the same when compared within a reactor group. Overall, growth rates in the chlorinated combined reactors were greater than in the combined control reactors. Mean values for the biofilm growth rates are shown in Table 6 and Figure 18 for the combined mixed substrate reactor data.

Mixed Substrates–Leucine Uptake. Biofilm growth rates were determined using the leucine uptake method at each carbon level for each of the four reactors in the mixed substrate experiment. As was mentioned previously, the chlorinated reactor was receiving a free chlorine dose at the 500 carbon level. Biofilm leucine uptake rates were determined at a minimum of three substrate concentrations for each carbon level.

There were no significant differences between biofilm growth rates at the various substrate concentrations used to measure biofilm growth rates for a single reactor carbon level (ANOVA using GLM). This fact allowed the data to be combined for analysis by carbon level for each reactor.

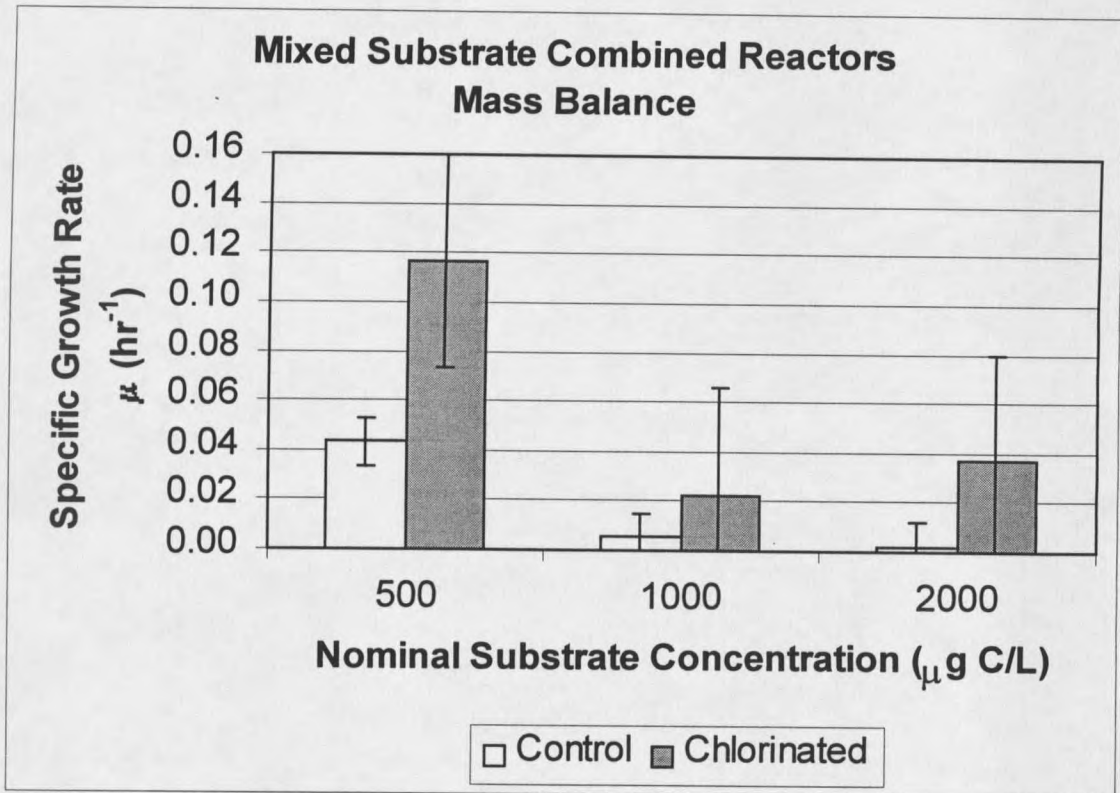


Figure 18. Comparison of mean reactor specific growth rates at the three carbon feed levels for the mixed substrate combined reactors. Error bars are 95% confidence intervals based on the one-way ANOVA pooled standard deviations.

“a” Reactors. The mean growth rates were not statistically equal when compared between carbon levels and between reactors within the carbon levels ($p < 0.0005$ and $p < 0.0005$, respectively) for the mixed substrate “a” reactors (“a” refers to the first and “b” to the second reactor in series). The mean biofilm growth rates by carbon level were not equal for the control “a” reactor based upon a one-way ANOVA ($p < 0.0005$), and the difference in growth rates was significant between each of the three carbon levels (Tukey’s pairwise comparison). In general, the growth rate in the control “a” reactor was the greatest at the 500 carbon level and decreased as the carbon concentration increased.

Figures 94 and 95 in the Appendix show the data used in the mixed substrate ANOVA analysis. Mean values for the biofilm growth rates are shown in Table 7 and Figure 19.

Substrate	Reactor	Carbon Level ($\mu\text{g/L}$)	Mean (hr^{-1})	95% C.I.		n
				Lower	Upper	
Mixed Substrate	Control-a	500	0.146	0.129	0.162	6
		1000	0.0433	0.0307	0.0559	9
		2000	0.0127	0.00008	0.0253	9
	Chlorinated-a	500	0.00885	0.00557	0.0121	9
		1000	0.00540	0.00251	0.00830	11
		2000	0.00143	-0.00186	0.00472	9
	Control-b	500	0.0340	0.0202	0.0478	6
		1000	0.0533	0.0429	0.0637	9
		2000	0.0481	0.0377	0.0585	9
	Chlorinated-b	500	0.0214	0.00897	0.0339	10
		1000	0.0622	0.0489	0.0755	9
		2000	0.0263	0.0130	0.0396	9

Table 7. Mixed substrate mean biofilm growth rates for the control and chlorinated reactors at each carbon level based on the leucine uptake approach.

A one-way ANOVA for the chlorinated reactor "a" data indicated the mean growth rates were not equal when compared between nominal carbon levels ($p < 0.0005$). Based on Tukey's pairwise comparisons, the growth rates at the 500 and 1000 carbon levels were significantly greater than at the 2000 carbon level. The trend in growth rates between carbon levels was the opposite of that determined for the chlorinated "a" reactor using the mass balance technique.

"b" Reactors. The mean growth rates were not statistically equal when compared between reactors and between carbon levels within a reactor group ($p = 0.051$ and $p < 0.0005$, respectively) for the mixed substrate "b" reactors. A one-way ANOVA for the control "b" reactor data indicated the mean growth rates were equal between carbon

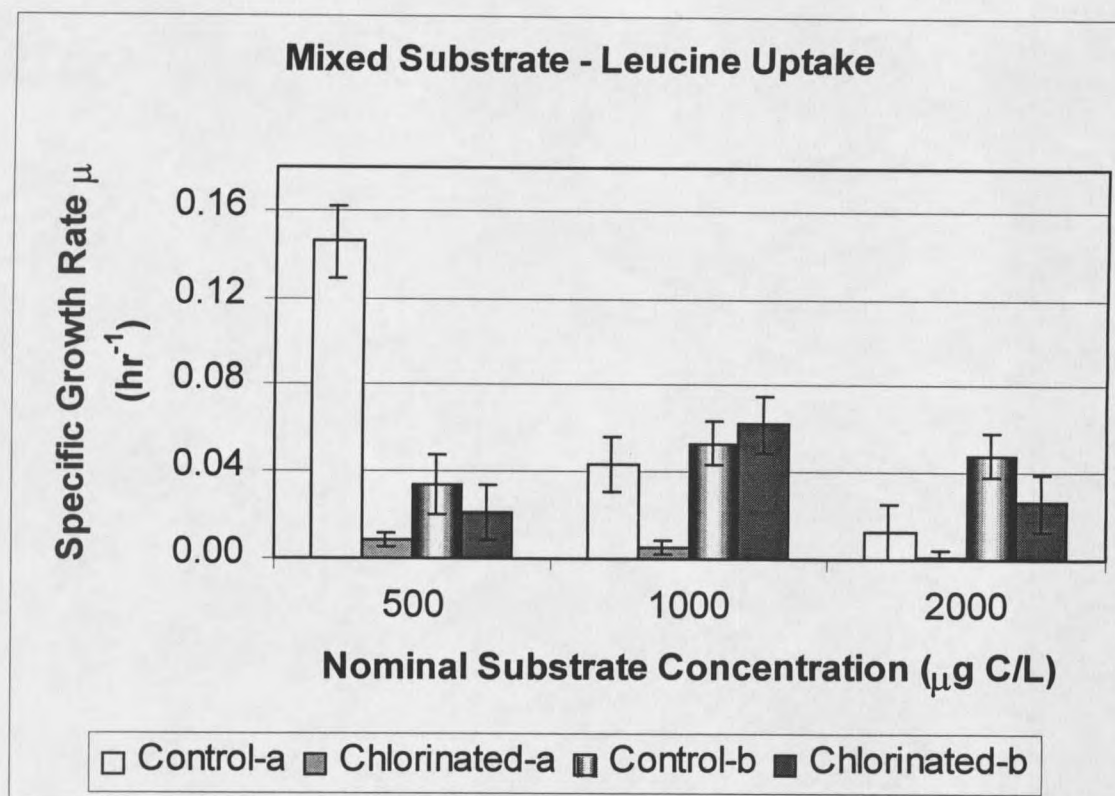


Figure 19. Comparison of mean biofilm specific growth rates based on leucine uptake at the three carbon feed levels for the mixed substrate experiment. Nominal substrate concentration is that fed to the first (a) reactor in series. Second reactor in series denoted as "b". Error bars are 95% confidence intervals based on the one-way ANOVA pooled standard deviations.

levels ($p=0.060$). The trend in control "b" reactor biofilm growth rates between carbon levels was slightly positive. Since the mass balance approach did not yield data for the control "b" reactor it was not possible to compare the leucine uptake data to the mass balance data.

The mean growth rates were not statistically equal when compared between carbon levels for the chlorinated "b" reactor biofilm growth rates based upon a one-way ANOVA ($p<0.0005$). The difference in growth rates was significant between the 1000

carbon level and the other two levels (Tukey's pairwise comparison) with the growth rates at the 1000 carbon level greater than for the 500 and 2000 carbon levels, and no significant difference between the 500 and 2000 carbon levels. The inconsistency of the data, particularly when compared to the mass balance data for the same reactor makes it difficult to assess the chlorinated "b" reactor biofilm growth rates based on leucine uptake.

A comparison of mean specific growth rate by reactor using the mass balance and leucine uptake techniques is presented in Figure 20. The most notable differences between the two methods are for the chlorinated "a" and control "b" reactors. The leucine uptake method for the chlorinated reactor resulted in a lower specific growth rate compared to the mass balance approach, a result also seen for the carbohydrates data (see Figure 15).

Comparison of Specific Growth Rate Data by Substrate-Mass Balance. The combined data for all substrate groups was modified to include in the control reactor group the 500 carbon level data of both reactors, except for the mixed substrate experiment where the chlorinated reactor received chlorine at the 500 carbon level. The specific growth rate data was natural log transformed to provide homogeneity of variance for statistical analysis.

A one-way ANOVA of the control reactor data analyzed by substrate group indicated the mean biofilm growth rates were not equal ($p < 0.0005$) and that there was no significant difference between amino acids, carbohydrates and the mixed substrate, but

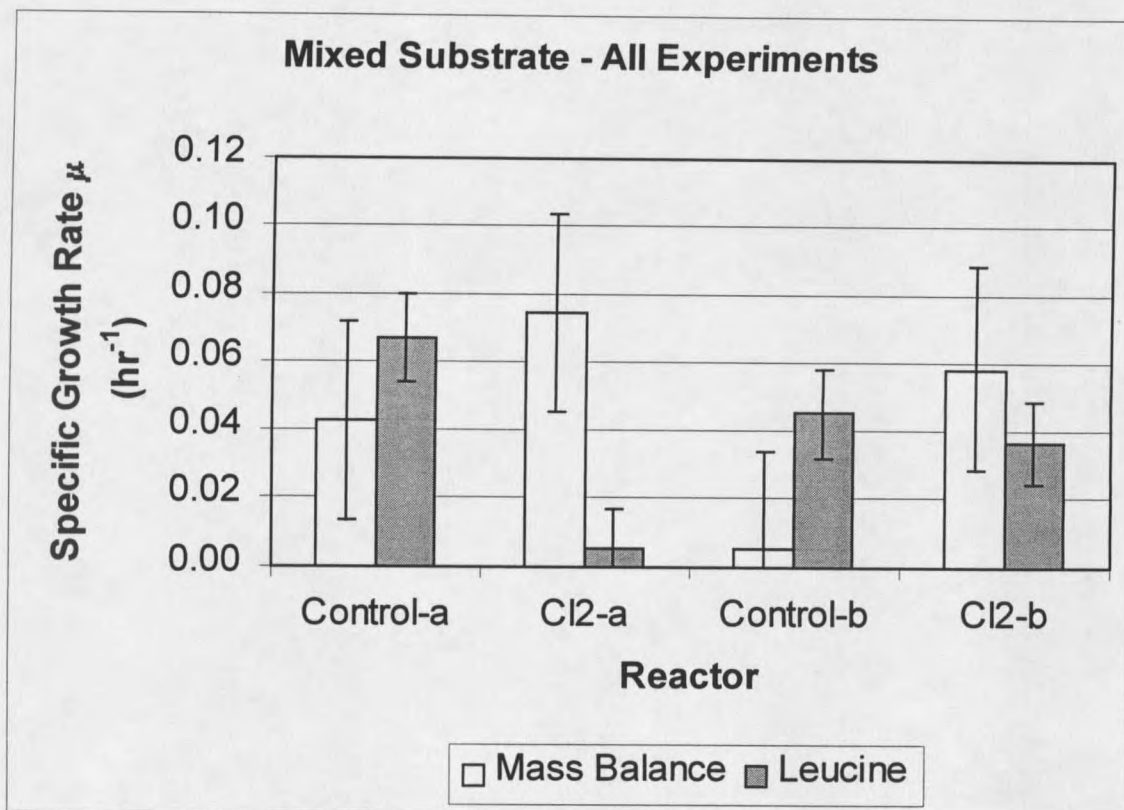
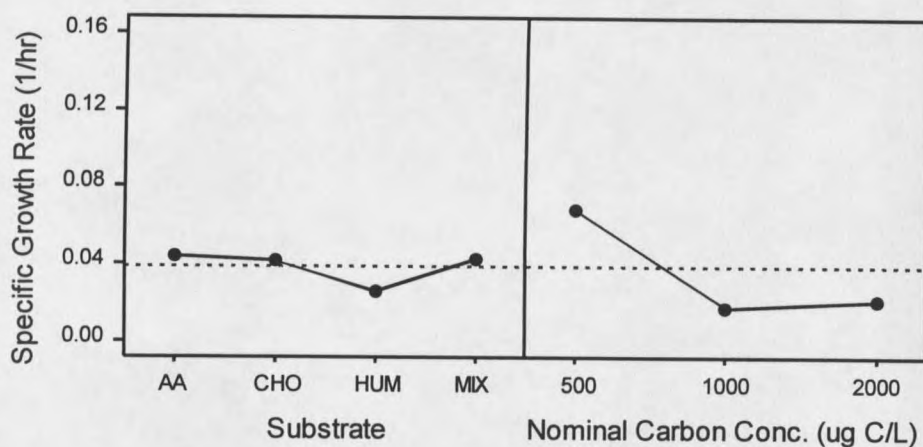


Figure 20. Comparison of mean biofilm specific growth rates by mixed substrate reactor for growth rates based on mass balance and leucine uptake. Mean values for a reactor are for all carbon levels. First reactor in series denoted as "a" and second reactor in series denoted as "b". Error bars are 95% confidence intervals based on one-way ANOVA pooled standard deviations.

the mean growth rate for humics was less than for the other three substrate groups (Tukey's pairwise comparison). Analysis of the chlorinated reactor data by substrate group indicated the mean growth rate for humics was statistically less than the mean carbohydrates and mixed substrate growth rates (Tukey's pairwise comparisons).

Although the mean growth rate for humics chlorinated biofilm was less than the mean for amino acids, there was no statistical difference between the two. Figure 21 presents

Main Effects Plot - Mean Control Reactor Data



Main Effects Plot - Mean Chlorinated Reactor Data

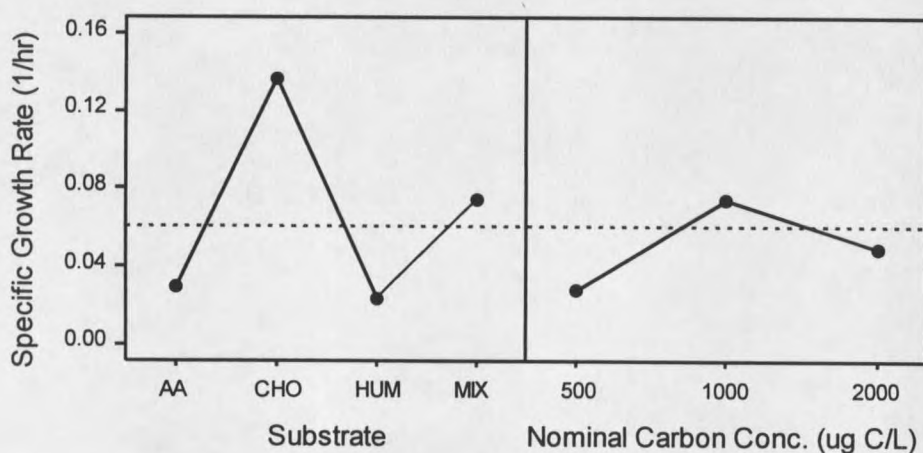


Figure 21. Main effects plot for all control and chlorinated reactor mean biofilm specific growth rates μ (hr^{-1}). Main effects are substrate group and carbon level. Chlorinated reactor group does not include data at 500 carbon level except for mixed substrate experiment data. Dashed horizontal line represents mean for all data. AA = amino acids; CHO = carbohydrates; HUM = humics; and MIX = mixed substrates.

main effects plots for the control and chlorinated reactor mean specific growth rate by substrate group and carbon level.

When the data for both reactor types was combined and analyzed by carbon level the mean growth rates were not equal ($p < 0.0005$). The mean growth rate at the 500 level was significantly greater than at the 1000 and 2000 levels (Tukey's pairwise comparison) and there was no significant difference between the 1000 and 2000 levels. Figure 21 presents the means by carbon level for each reactor. Control reactor biofilm growth rates at the 500 level were significantly greater than at the 1000 and 2000 levels. Based only on the data from a single mixed substrate experiment, the growth rates at the 500 carbon level for the chlorinated reactor were less than at the 1000 and 2000 carbon levels.

When only the 1000 and 2000 carbon levels were considered for each reactor type in a one-way ANOVA the mean chlorinated reactor growth rate was greater than the mean control reactor growth rate (Tukey's pairwise comparison). There was no significant difference between specific growth rates at the 1000 and 2000 carbon levels for the control reactors ($p = 0.219$) but the difference was significant for the chlorinated reactors ($p < 0.0005$), the mean growth rate at the 1000 carbon level was greater.

Biofilm specific growth rates were greatest in the mixed and carbohydrates substrate groups. Growth rates were the lowest in the humics substrate group. Biofilm specific growth rates in the chlorinated reactors were generally greater than for the control reactor. Increasing carbon levels resulted in lower growth rates for the control reactors and (comparing only the 1000 to the 2000 carbon level) in the chlorinated reactors.

Comparison of Specific Growth Rate Data by Substrate–Leucine Uptake.

Growth rates using leucine uptake were determined for three substrates: amino acids, carbohydrates and mixed substrates. Comparison between these three substrates was possible only at the 2000 carbon level since amino acids data were collected only at that carbon level (replicate 3). Comparisons by substrate for each reactor are presented as main effect plots in Figure 22. The order of substrates from high to low mean growth rates for the control and chlorinated reactors biofilm was:

- amino acids>carbohydrates>mixed substrates.

The difference in growth rates between each substrate was significant for the control and chlorinated data (Tukey's pairwise comparison).

Main effects for mean biofilm growth rate data are presented in Figure 23 by substrate group for reactor type and nominal carbon level. Mean growth rates based on leucine uptake for control reactor biofilm were greater than for chlorinated reactor biofilm for each substrate. This relationship was opposite that observed using the mass balance technique in the carbohydrates and mixed substrates reactors where chlorinated growth rates were greater than for control.

Overall, influent carbon level had little impact on mean growth rates for the carbohydrates reactor between the 1000 and 2000 carbon levels, but did have an influence on growth rates for the mixed substrates reactors. Mean growth rates for the mixed substrate reactors decreased with increasing carbon levels for both reactors. The negative trend in growth rates measured using leucine uptake was similar to that seen

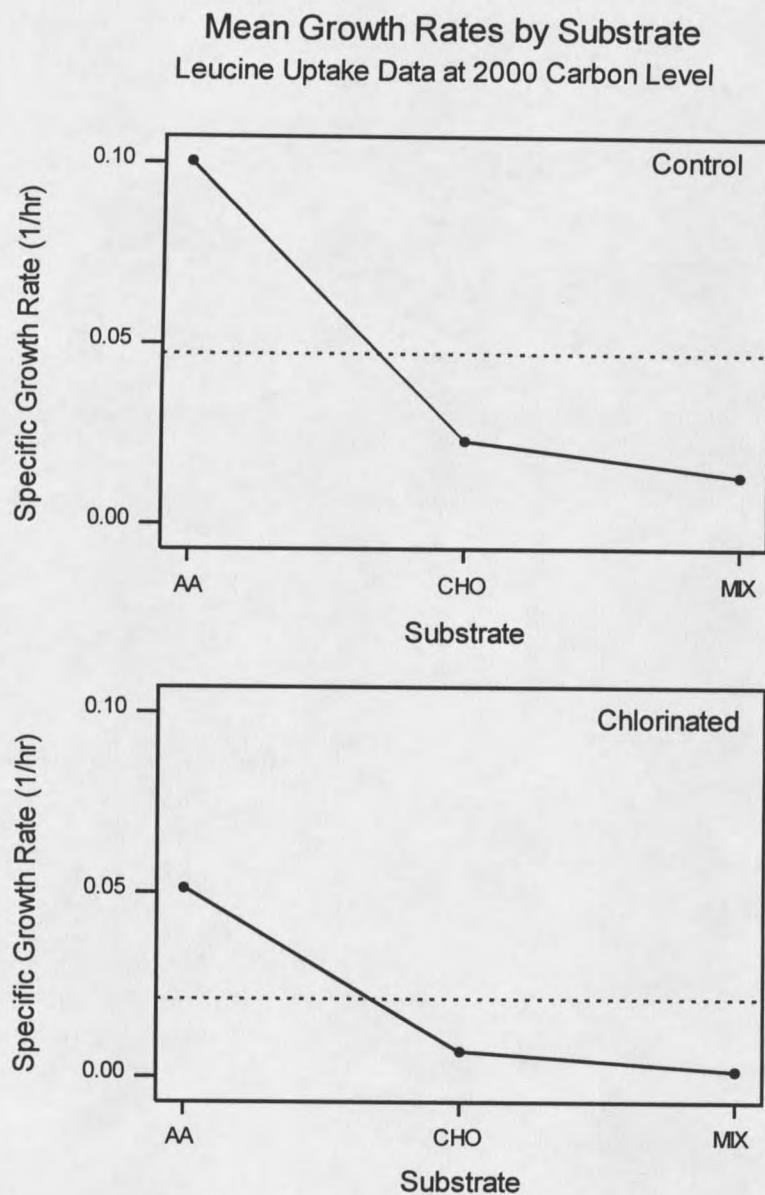


Figure 22. Main effects plots for biofilm specific growth rate by substrate at the 2000 carbon level based on leucine uptake. Horizontal dashed line represents mean for data in figure. AA = amino acids; CHO = carbohydrates; and MIX = mixed substrates.

Main Effects by Reactor and Carbon Level - Leucine Uptake Data

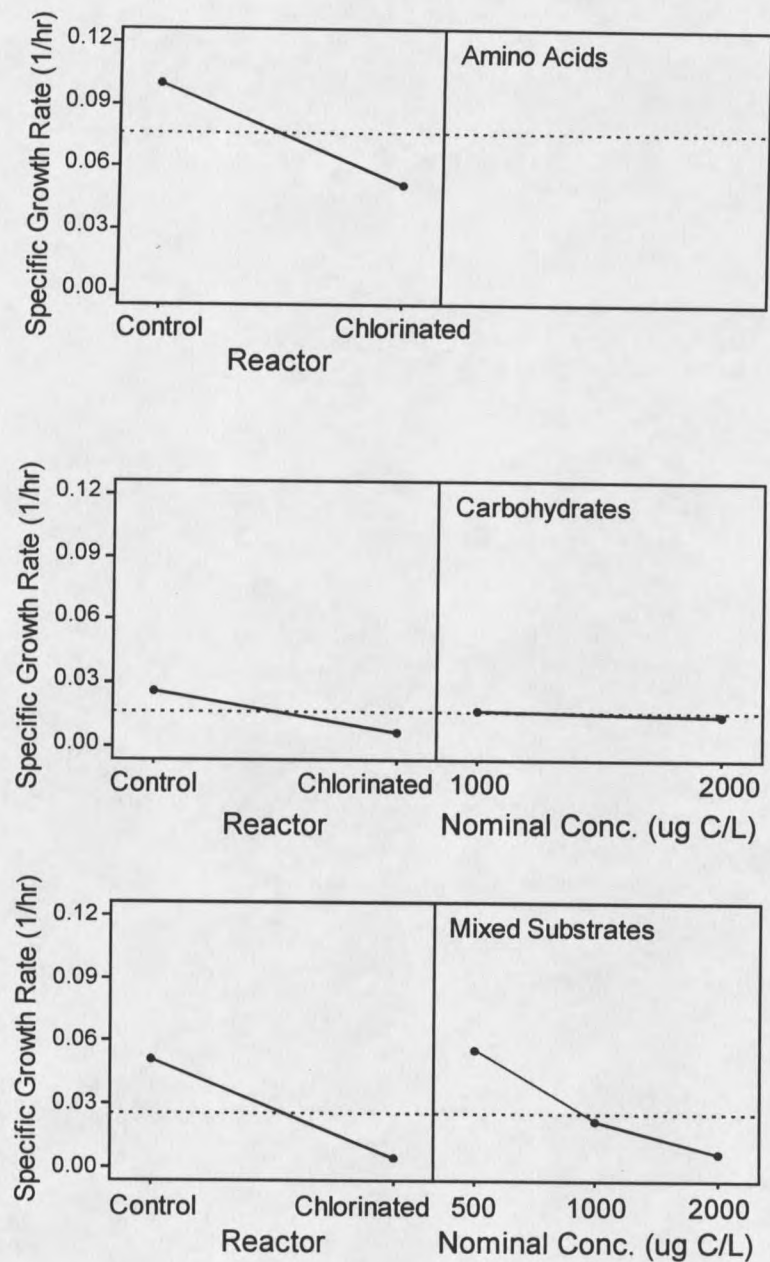


Figure 23. Main effects plots for biofilm specific growth rate by substrate and reactor based on leucine uptake. Main effects are reactor type and nominal carbon concentration. Horizontal dashed line represents mean for data in figure.

using the mass balance approach for the control "a" reactors, combined control and combined chlorinated reactors. The negative trend in growth rates with increasing carbon feed concentrations was similar to that seen using the mass balance approach for control reactors in each substrate group and chlorinated reactor in the amino acids and humics groups. As will be discussed in Chapter 5, adsorption of leucine by humic substances in the sample liquid may have been the cause for decreasing growth rates with increasing carbon levels.

Specific Growth Rate-Substrate Relationships. Because carbon was assumed to be growth limiting for drinking water biofilm, it was hypothesized that biofilm specific growth rate (μ) was dependent upon the type and concentration (S) of the limiting carbon substrate. Therefore, relationships were evaluated with specific growth rate as the dependent variable and substrate concentration the independent variable. The general trend for the specific growth rate as a function of the nominal influent substrate concentration was described above for each reactor type within a substrate group. The above analyses help define general trends between carbon loading levels and reactor types. However, the effluent substrate concentration best describes the substrate concentration seen by the biofilm. The true substrate concentration seen by the biofilm would require taking into account mass transfer resistance across the laminar boundary layer just above the biofilm and between it and the bulk fluid. Since the mass transfer resistance was not determined as part of this research, the μ -S relationships were evaluated using the bulk fluid substrate concentration. Those trends were evaluated to determine if a μ -S relationship could be mathematically described. Three possible

models were formulated based upon examination of the results presented above. The models evaluated were:

1. Zero-order
2. Substrate inhibition
3. Hyperbolic (Monod)

A zero-order model implies there was no relationship between substrate concentration and specific growth rate as expressed in the following equation:

$$\mu = k_1, \quad (30)$$

where μ is the specific growth rate and k_1 is a constant. In those cases where the mean μ by carbon level was statistically equal a zero-order relationship could be possible.

The specific growth rate for many of the control reactors showed a decrease between the 500 carbon level and the 1000 carbon level and, for some substrates, there was little difference between the 1000 and 2000 carbon levels. This type of trend could indicate some form of inhibition related to the substrate concentration. The model used for substrate inhibition was analogous to the Andrews (Andrews, J. F., 1968) model. The model equation was:

$$\mu = \frac{a \cdot S}{(b + S + S^2/c)}, \quad (31)$$

where the variable a is a constant k_2 , the variable b is analogous to the term K_S , and c is K_I , a constant related to inhibition by the substrate. The maximum rate of growth can be

determined by taking the derivative of Equation 31 and setting it equal to zero, resulting in the following expression:

$$\mu_{\max} = k_2 / \left[1 + 2(K_S/K_I)^{0.5} \right]. \quad (32)$$

Finally, traditional Monod (Monod, J., 1949) kinetics describe the relationship between μ and S using a simple hyperbolic function based on saturation kinetics expressed as follows:

$$\mu = \frac{a \cdot S}{(b + S)} \quad (33)$$

where the variable a is the maximum specific growth rate, $\mu_{\max}$, and the variable b is the substrate concentration at one-half $\mu_{\max}$, or the half-saturation constant for growth, K_S .

The models were evaluated using the actual measured substrate effluent concentration in terms of carbon (measured as NPOC). The inhibition and hyperbolic models were evaluated by using nonlinear least squares regression to fit the desired function to the data. The software TableCurve™ 2D was used to perform the regressions and compute the model parameters and statistics.

The results of the model evaluation are presented in Table 8 by substrate group and reactor type. The mean value for μ and the upper and lower 95% confidence interval values are shown for the zero-order model where μ is the zero-order constant k_1 . Parameters a , b and c for the various models are as defined above. Three statistics are presented to compare how well the models fit the raw data. The statistic "p(regression)" is based on the ANOVA for the regression and represents the probability of the F-statistic

Substrate	Reactor	Model	Mean μ (hr ⁻¹)	95% CI Lower (hr ⁻¹)	95% CI Upper (hr ⁻¹)	Model Parameters			$\mu_{\max}$ Inhibtn (hr ⁻¹)	p (regrsn)	Adj. R ²	RSS
						a (hr ⁻¹)	b ⁽¹⁾	c (ug C/L)				
AA	Control	Zero-order	0.0199	0.0147	0.0251							0.0221
		Monod				0.0222	5.65			0.664	0.000	0.0220
		Inhibition				0.192	201	15.3	0.0229	0.652	0.000	0.0217
	Cl ₂	Zero-order	0.0156	0.0118	0.0192							0.0040
		Monod				0.0175	13.5			0.376	0.000	0.0039
AA 3 – Leucine	Control	Zero-order	0.1003	0.0853	0.1153							0.0103
	Cl ₂	Zero-order	0.0513	0.0449	0.0578							0.0019
CHO	Control	Zero-order	0.0334	0.0249	0.0419							0.0371
		Monod				0.0430	105			0.465	0.000	0.0367
		Inhibition				0.0535	203	8408	0.0408	0.844	0.000	0.0368
	Cl ₂	Zero-order	0.0815	0.0564	0.107							0.0893
		Monod				0.0833	33.0			0.004	0.000	0.0893
CHO 3 Leucine	Control	Zero-order	0.0264	0.0234	0.0294							0.0029
	Cl ₂	Zero-order	0.0067	0.00503	0.00846							0.0012
HUM	Control	Zero-order w/ 500 data	0.0263	0.0136	0.0389							0.0973
		Zero-order w/o 500 data	0.0023	0.0014	0.0032							0.00022
		Inhibition				0.338	7.93	19.2	0.148	0.999	0.000	0.0973
	Cl ₂	Zero-order	0.0237	0.0115	0.0359							0.0454
		Zero- order ⁽²⁾	0.0051	0.00370	0.00648							0.00032
		Monod				0.0233	1			0.999	0.000	0.0454
		Monod ⁽²⁾				0.0051	1			0.999	0.000	0.00032

⁽¹⁾ b (Monod or Inhibition) = $\mu\text{g C L}^{-1}$.

⁽²⁾ Humics replicate 1 chlorinated data at 1000 carbon level not included.

Table 8. Kinetic models for biofilm specific growth rate as a function of effluent substrate concentration as carbon for annular reactors. (AA=amino acids; CHO=carbohydrates; HUM=humic substances; MIX=mixed substrates; Cl₂=chlorinated)

Substrate	Reactor	Model	Mean μ (hr ⁻¹)	95% CI Lower (hr ⁻¹)	95% CI Upper (hr ⁻¹)	Model Parameters			$\mu_{\max}$ Inhibtn (hr ⁻¹)	p (regrsn.)	Adj. R ²	RSS
						a (hr ⁻¹)	b ⁽¹⁾	c (ug C/L)				
MIX	Control	Zero-order	0.0428	0.0178	0.0679							0.0286
		Inhibition				12.0	2774	0.720	0.0962	0.006	0.461	0.0121
	Cl ₂	Zero-order	0.0747	0.0412	0.1082							0.0512
		Monod				0.307	1892			0.031	0.194	0.0377
MIX- Comb.	Control	Zero-order	0.0169	0.0053	0.0284							0.0061
		Inhibition				0.0337	0	165	0.0337	0.002	0.457	0.0028
	Cl ₂	Zero-order	0.0586	0.0279	0.0893							0.0431
		Inhibition				0.0895	0	252	0.0895	0.005	0.377	0.0230
MIX – Leucine	Control	Zero-order	0.0512	0.0304	0.0720							0.0509
		Inhibition				0.1813	1.00	128.6	0.154	0.000	0.625	0.0165
	Cl ₂	Zero-order	0.0046	0.00310	0.00609							0.00040
		Inhibition				0.0088	1.00	631.3	0.00816	0.022	0.170	0.00030

⁽¹⁾ b (Monod or Inhibition) = $\mu\text{g C L}^{-1}$.

Table 8 (continued). Kinetic models for biofilm specific growth rate as a function of influent substrate concentration as carbon for annular reactors.

for the regression being greater than the critical F-statistic at an α -level of 0.05. p values greater than 0.05 indicate the regression was not significant at the specified α -level. The adjusted R^2 (adjusted for the degrees of freedom associated with the regression) allows comparison of the regressions where the greater value of adjusted R^2 indicates a more significant regression. The residual sum of squares (RSS) is the sum of the squares of the difference between the predicted and actual values of the dependent variable. While not a powerful parameter for comparing regressions, the RSS allows the zero-order model to be compared to the results of the other models. Lower values of the RSS indicate less variation between the predicted and actual values.

The models presented in Table 8 should be considered applicable only over the range of substrate concentrations used during this work. Growth rates using leucine uptake were measured at substrate concentrations that ranged from below to above the reactor effluent concentration. Models for chlorinated reactors include only data from the 1000 and 2000 carbon levels because with the exception of the single mixed substrate experiment, chlorine was not added to reactors at the 500 carbon level. Figures 24, 25, 26, 27 and 28 present the mean growth rate and associated 95-percent confidence intervals as a function of the mean effluent substrate concentration for amino acids, carbohydrates, humics, mixed substrate "a" reactors and mixed substrate combined reactors, respectively.

The zero-order model appeared most suitable for all substrate groups except the mixed substrates. The inhibition model provided a good fit to the mixed substrate control "a" data and the Monod model fit the chlorinated "a" data. Inhibition models were good

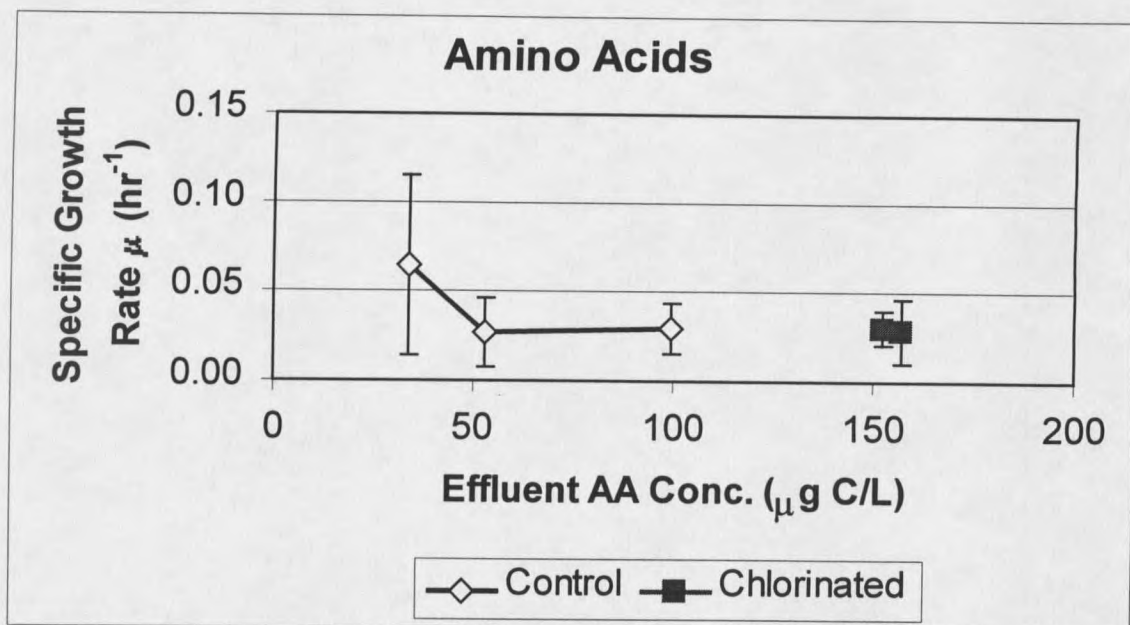


Figure 24. Biofilm specific growth rate based on mass balance technique as a function of effluent amino acids (AA) concentration as carbon. Error bars are 95 percent confidence interval data means.

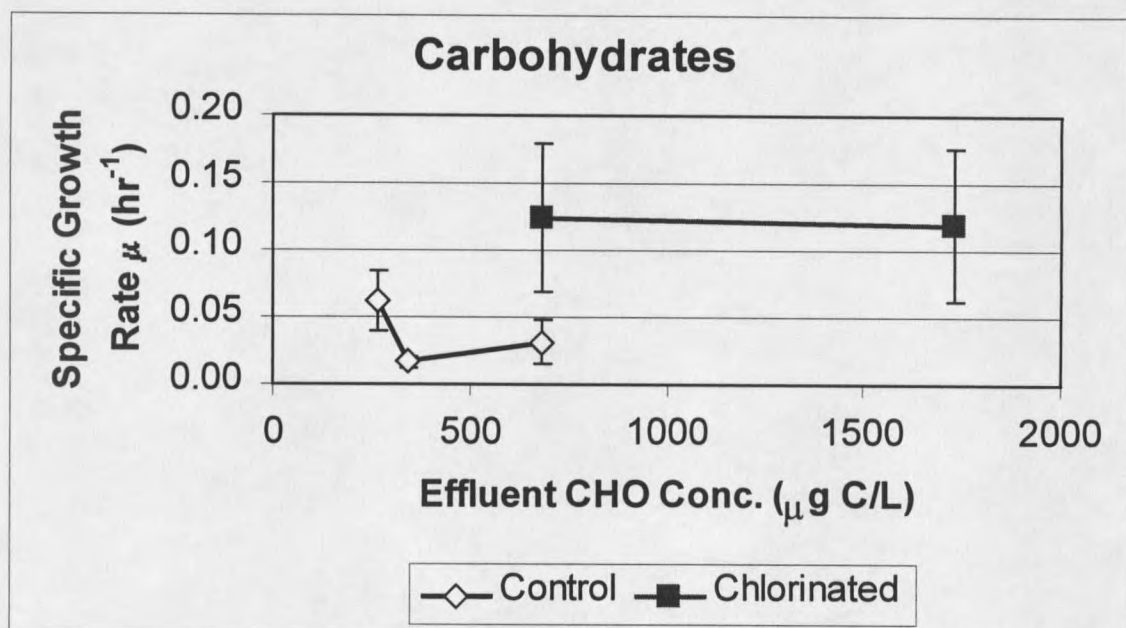


Figure 25. Biofilm specific growth rate based on mass balance technique as a function of effluent carbohydrates (CHO) concentration as carbon. Error bars are 95 percent confidence intervals for data means.

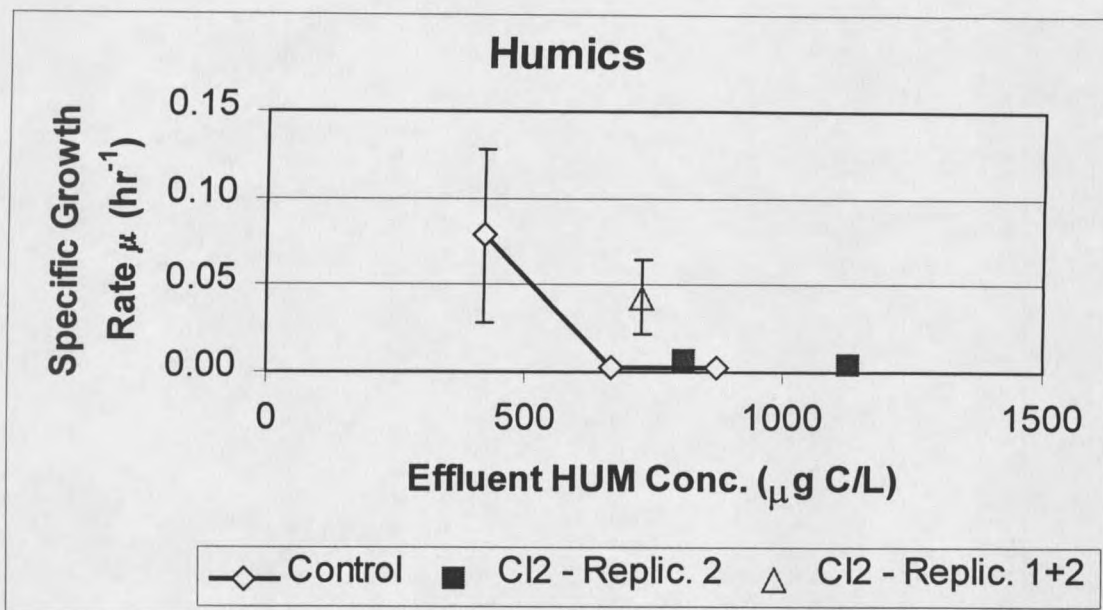


Figure 26. Biofilm specific growth rate based on mass balance technique as a function of effluent humics (HUM) concentration as carbon. Chlorinated (Cl2) data shown for both replicate 2 and replicates 1 plus 2. Error bars are 95 percent confidence intervals for data means.

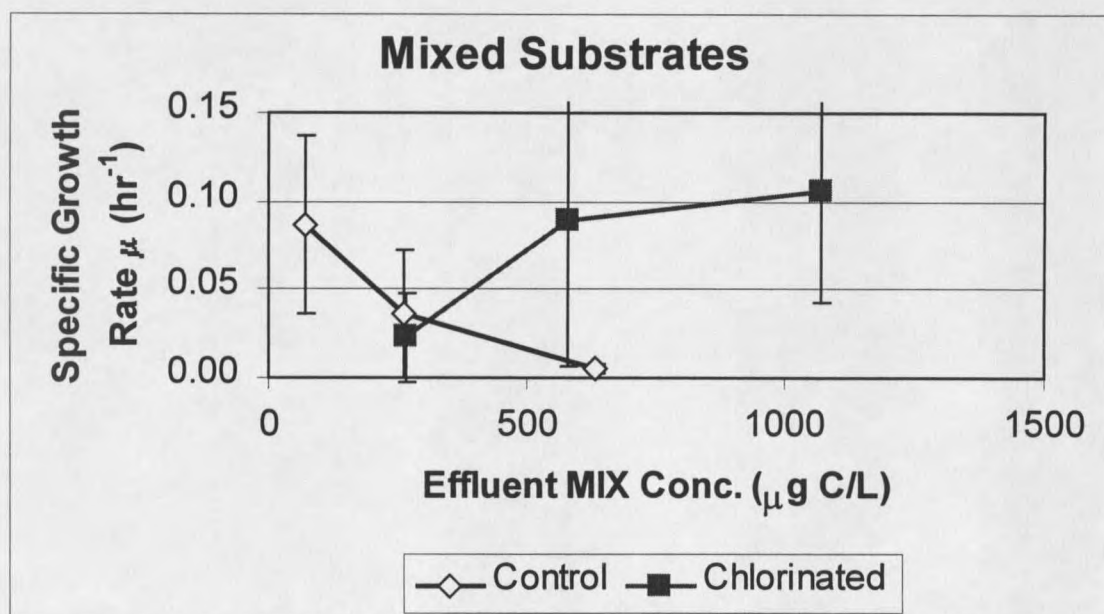


Figure 27. Biofilm specific growth rate based on mass balance technique as a function of effluent mixed substrate (MIX) concentration as carbon. Error bars are 95 percent confidence intervals for data means.

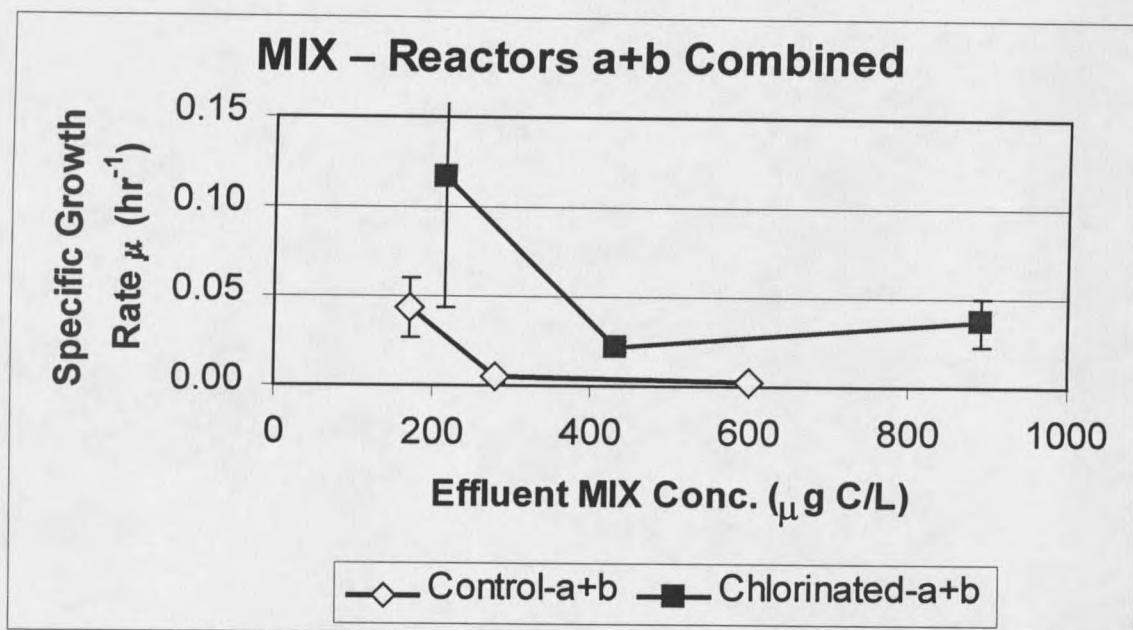


Figure 28. Biofilm specific growth rate based on mass balance technique as a function of effluent mixed substrate (MIX) concentration as carbon for reactors a+b combined. Error bars are 95 percent confidence intervals for data means.

fits for the combined ("a+b") control and chlorinated data. The effect of the organic carbon content of the dilution water (biologically activated carbon filter effluent) will be discussed, along with a comparison of models, in Chapter 5, DISCUSSION.

Reactor Carbon Removal

Carbon removal across reactors using filtered non-purgeable organic carbon (NPOC) data was determined for the amino acids replicate 3 experiment, all replicates of the carbohydrates and humics experiments, and the single mixed substrate experiment. Data analysis for the chlorinated reactors excluded the 500 carbon feed level ($\sim 500 \mu\text{g C/L}$) data since the reactor was not chlorinated at that point in the experiment. Data for the mixed substrate experiment chlorinated reactor includes the 500 carbon level data

because the reactor was receiving chlorine at that carbon level. Carbon removal was determined as a flux ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) and as a normalized (or specific) carbon removal rate (hr^{-1}), normalized to the carbon content of the biofilm's biomass. Specific calculation of carbon removal was presented in Chapter 3, MATERIALS AND METHODS. Carbon flux represents the carbon removed per unit area of biofilm per time. The normalized or specific carbon removal rate represents the carbon removed per mass of biofilm, in terms of carbon, per unit time.

When carbon removal flux ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) was plotted against the influent loading rate ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) a linear relationship was seen in most data. Linear regressions were performed on the carbon flux versus loading data using both an intercept version and one that assumed the regression line went through the origin. The dimensionless slope of the regression line for carbon flux versus loading rate indicates the fraction of the influent carbon removed within the reactors (detention time of 2.1 hours).

A linear relationship did not always occur for the normalized carbon removal rate when plotted as a function of the effluent substrate concentration. Some data sets exhibited notable variability, particularly for the chlorinated reactors. Data outliers (data points representing unusually high or low observations) were noted for the carbon removal data. Data outliers are not unusual for this type of data where natural systems are investigated. Including the unusual observations can lead to misrepresentation of the majority of the data. For example, a single high data point included in a linear regression could significantly alter the slope of the regression line when it is clear from looking at

the data a linear relationship applied to all data except the one point. Data outliers were initially identified as those data outside the $1.5 \times$ interquartile range (Aroner, E. R., 1996). Correlation analyses were performed for the normalized data with outliers removed. Data for the chlorinated reactor analysis did not include data at the 500 carbon level since chlorination had not commenced. Data for the mixed substrate chlorinated reactor, however, includes the 500 carbon level data because the reactor was receiving chlorine at that carbon level. Correlation analyses were performed using normal scores transformed data and WQHYDRO software (Aroner, E. R., 1996) or MiniTab® software. Linear least squares regressions using TableCurve™ 2D software were performed where a strong correlation was noted (high absolute value of the correlation coefficient and low probability values).

Amino Acids–Replicate 3 Reactors. Carbon removal was determined across reactors for the amino acids replicate 3 experiment using filtered NPOC data (data was not available for the first two replicate experiments). Figures 29 and 30 present the data for the control and chlorinated reactors, respectively. A linear regression of carbon removal flux as a function of influent substrate loading rate was significant at the $\alpha=0.05$ level for the control and chlorinated reactors (Table 9). Little difference in carbon removal existed between the two amino acids reactors; nearly all of the influent amino acids (as carbon) were removed within both reactors. Correlation between the normalized carbon removal rate and effluent substrate concentration (Table 10) was not

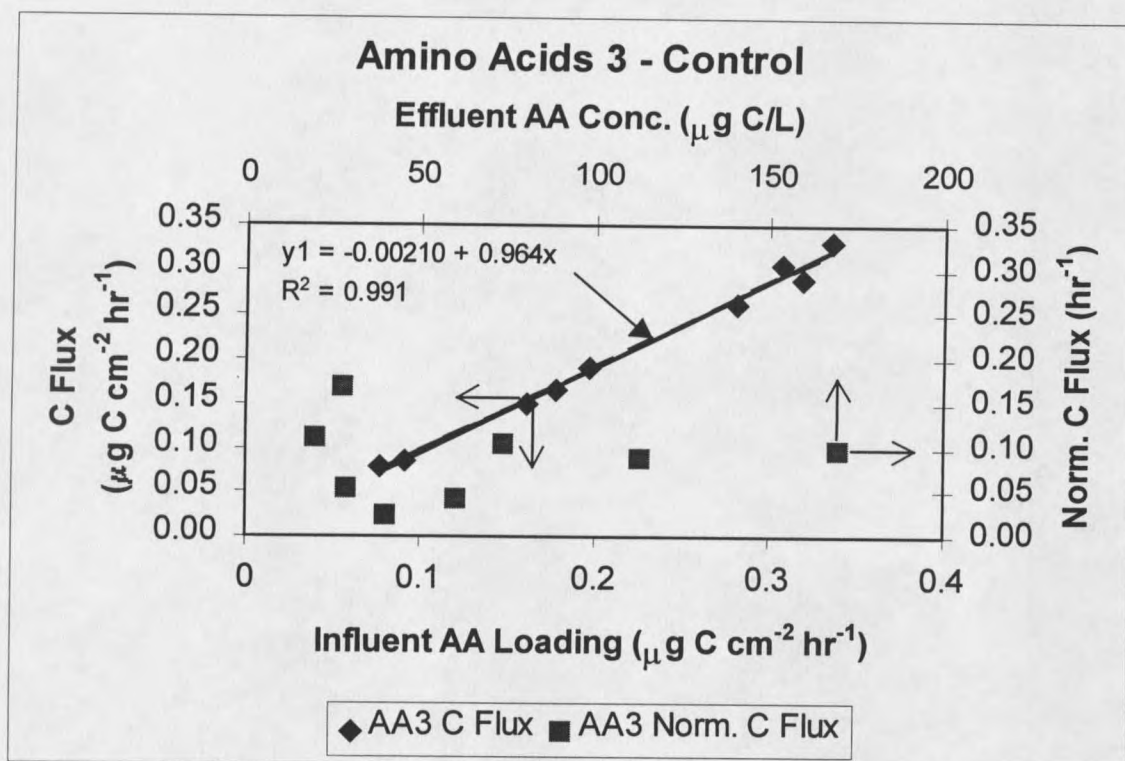


Figure 29. Carbon removal flux (C Flux) and normalized carbon removal rate (Norm. C Flux) for amino acids replicate 3 (AA3) control reactor. Carbon removal rate normalized to biofilm biomass carbon. $y1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{ hr}^{-1})$.

significant for the control and chlorinated reactors. Correlation of the chlorinated reactor normalized data indicated a negative trend with increasing effluent substrate concentration. Mean values for the normalized carbon removal rate were 0.0741 and 0.487 hr^{-1} for the control and chlorinated reactors, respectively (Table 11).

Amino Acids- ^3H -Substrate Uptake for Replicate 3. The experimental design to evaluate substrate uptake in the amino acids third replicate experiment involved utilizing ^3H -amino acids to determine their uptake by the biofilm. Biomass and activity variations in the biofilm were corrected as described above in section "Specific Growth

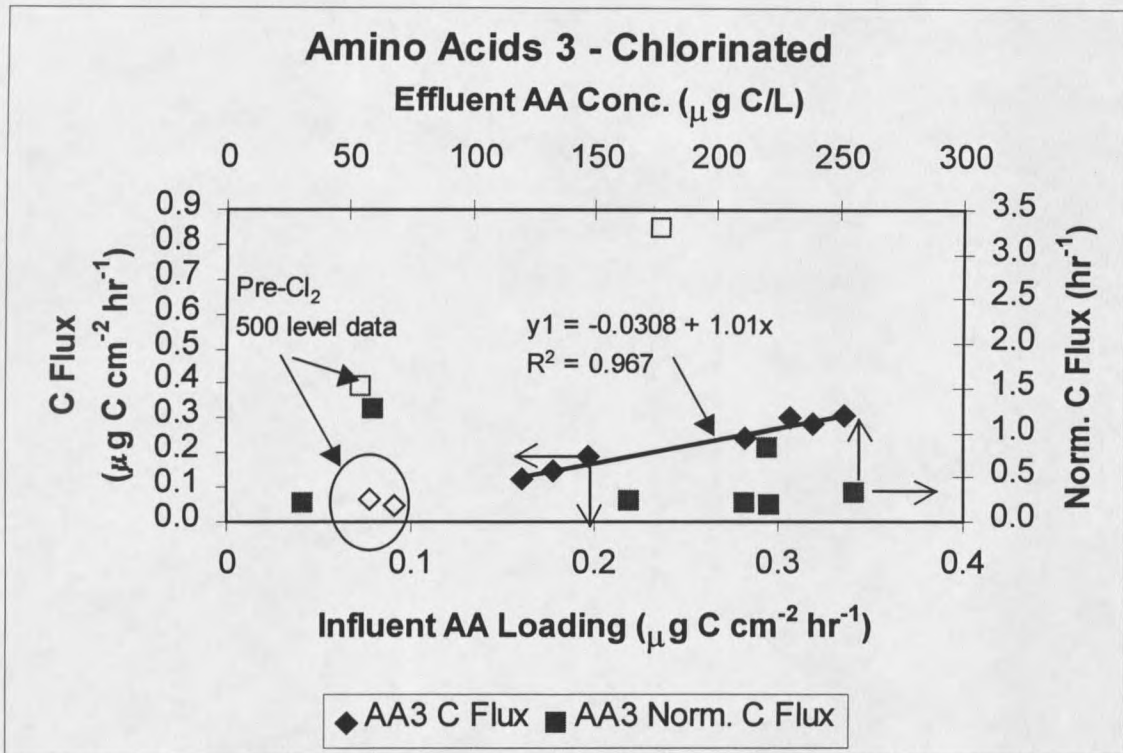


Figure 30. Carbon removal flux and normalized carbon removal rate for amino acids replicate 3 (AA3) chlorinated reactor. Carbon removal rate normalized to biofilm biomass carbon. Open data symbols indicate data not included in the analysis. $y1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{ hr}^{-1})$.

Rates, Amino Acids–Leucine Uptake for Replicate 3". The factorial experimental design provided data for analysis of the uptake of each amino acid with respect to the other two amino acids. Also analyzed was the uptake of each amino acid and the total amino acids with respect to the concentration as carbon of amino acids in the sample vials. Results for the substrate uptake experiment are presented below for the control and chlorinated reactor biofilm.

Substrate	Reactor	a (intercept) ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$)	b (slope)	R ²	P (regsrn)
Amino Acids	Control	0	0.956	0.991	<0.0005
		-0.00210	0.964	0.991	<0.0005
	Chlorinated	0	0.901	0.954	<0.0005
		-0.0308	1.01	0.967	<0.0005
Carbohydrates	Control	0	0.657	0.817	<0.0005
		-0.0107	0.694	0.821	0.001
	Chlorinated ⁽¹⁾	0	0.170	0.000	0.999
		----	----	----	----
Humics	Control	0	0.507	0.814	<0.0005
		-0.0689	0.759	0.940	<0.0005
	Chlorinated	0	0.409	0.687	0.006
		-0.0692	0.649	0.810	0.002
Mixed Substrate	Control	0	0.472	0.764	0.001
		-0.00165	0.671	0.997	<0.0005
	Chlorinated	0	0.303	0.819	<0.0005
		-0.00606	0.397	0.821	<0.0005
⁽¹⁾ Regressions not significant.					

Table 9. Linear regression data for carbon flux ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) as a function of influent substrate loading rate ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$). Linear regressions shown with and without an intercept.

Substrate	Reactor	r	2×p	Significance
Amino Acids	Control-Norm.	-0.073	0.877	<80%
	Chlorinated-Norm.	-0.469	0.348	<80%
Carbohydrates	Control-Norm.	0.099	0.800	<80%
	Chlorinated-Norm.	0.237	0.763	<80%
Humics	Control-Norm.	-0.479	0.161	84%
	Chlorinated-Norm.	-0.795	0.032	96%
Mixed Substrate	Control-Norm.	-0.742	0.056	94%
	Chlorinated-Norm.	0.337	0.341	<80%

Table 10. Summary of correlation results for normalized (specific) carbon removal rates for the reactors. Correlation values (r) for chlorinated reactor are shown for data censored by removal of prechlorination data at the 500 carbon level.

Substrate	Reactor	Mean Norm. Carbon Removal Rate (hr^{-1})	95% C.I. for Mean
Amino Acids	Control	0.0741	0.0436-0.104
	Chlorinated	0.487	0.0286-0.946
Carbohydrates	Control	0.304	0.196-0.412
	Chlorinated	2.69	1.23-4.15
Humics	Control	0.141	0.102-0.181
	Chlorinated	0.382	-0.070-0.835
Mixed Substrate	Control	0.0972	0.0219-0.173
	Chlorinated	0.316	0.184-0.449

Table 11. Mean normalized reactor carbon removal rates (hr^{-1}) and 95-percent confidence intervals for the mean.

Control Reactor Substrate Uptake. The effect of varying the concentration of two amino acids on the uptake of the third amino acid is presented in Figure 31 for biofilm from the control reactor. Amino acid concentrations are in terms of equivalent carbon concentrations and in Figure 31 the abscissa values are the $\log_{10}$ of the concentration.

- Glutamate: Aspartate concentration had a significant main effect on the uptake of glutamate. Alanine had little effect and the interactions between aspartate and alanine were not significant.
- Aspartate: Glutamate and alanine concentrations both had positive and significant main effects on the uptake of aspartate. The interaction between glutamate and alanine was also significant for the uptake of aspartate.
- Alanine: Aspartate concentration had a positive and significant effect on the uptake of alanine; glutamate had no effect on alanine uptake and the interactions between glutamate and aspartate were not significant to the uptake of alanine.

Control Reactor Biofilm Specific Uptake

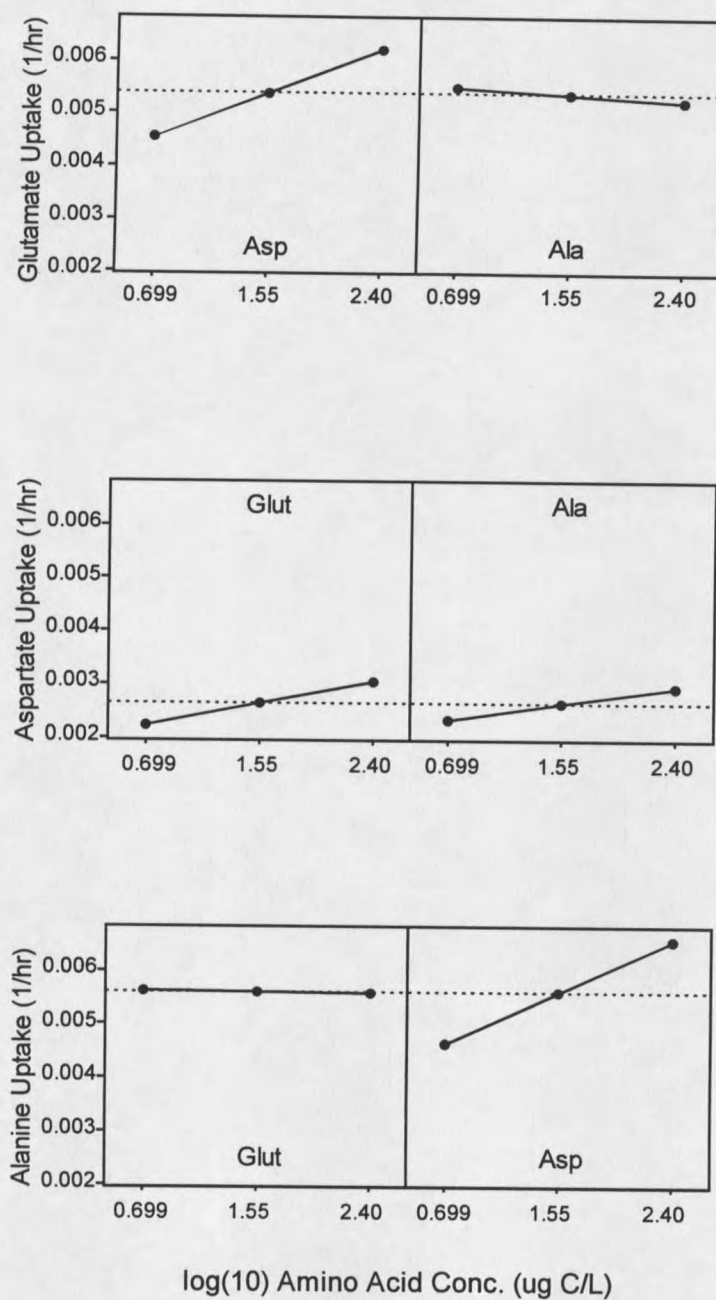


Figure 31. Main effects plots for specific uptake of isotope-labeled amino acids, control reactor biofilm. Main effects are based on fitted means. Glut = glutamate; Asp = aspartate; and Ala = alanine.

A one-way ANOVA of all uptake rates by amino acid indicated the means were not equal ($p=0.001$) and mean uptake rates for glutamate and alanine were greater than for aspartate for the control reactor biofilm (Tukey's pairwise comparison). Figure 32 presents boxplots comparing the uptake rates by amino acid for the control reactor biofilm.

Chlorinated Reactor Substrate Uptake. Main effect plots for specific uptake of the three amino acids are presented in Figure 33 for biofilm from the chlorinated reactor.

- Glutamate: Neither aspartate or alanine concentration had a significant main effect on the uptake of glutamate ($p[\text{main effects}]=0.312$). Alanine had a slight positive effect and aspartate a slight negative effect. The lack of a significant main effect indicates glutamate uptake was not impacted by the concentration of the other two amino acids for the chlorinated reactor biofilm.
- Aspartate: Increasing glutamate concentration had a positive main effect on the specific uptake of aspartate while increasing alanine concentration had a negative main effect ($p[\text{main effects}]=0.169$). The interaction between glutamate and alanine was significant at the $\alpha=0.10$ level for the uptake of aspartate.
- Alanine: Increasing glutamate and aspartate concentration had a significant negative effect on the uptake of alanine ($p[\text{main effects}]=0.022$). The interactions between glutamate and aspartate concentration on the uptake of alanine were significant at the $\alpha=0.10$ level.

Control Reactor Isotope-Labeled Amino Acid Uptake

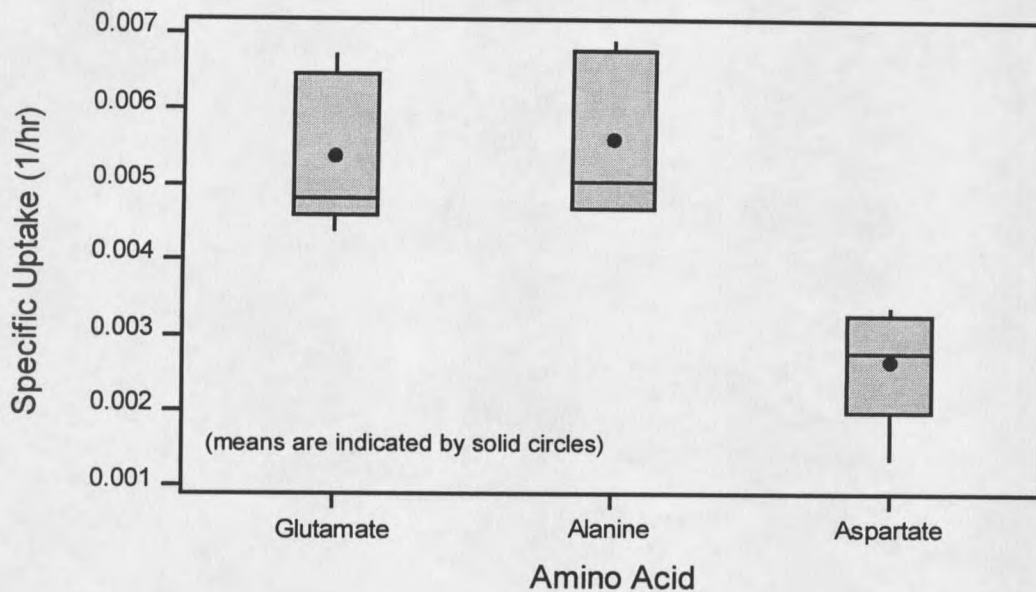


Figure 32. Boxplots for specific uptake of isotope labeled amino acids, control reactor biofilm. Boxes represent interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend to $1.5 \times$ interquartile range.

A one-way ANOVA of all uptake rates by amino acid indicated the means were not equal ($p=0.010$) and there was a significant difference between glutamate and alanine (Tukey's pairwise comparison). The specific uptake rates for alanine were highest, followed by aspartate then glutamate. Figure 34 presents boxplots comparing the uptake rates by amino acid for the chlorinated reactor biofilm.

Comparison of Control and Chlorinated Uptake Rates. As shown by the boxplots in Figure 35, the specific uptake rates for amino acids in the control reactor biofilm

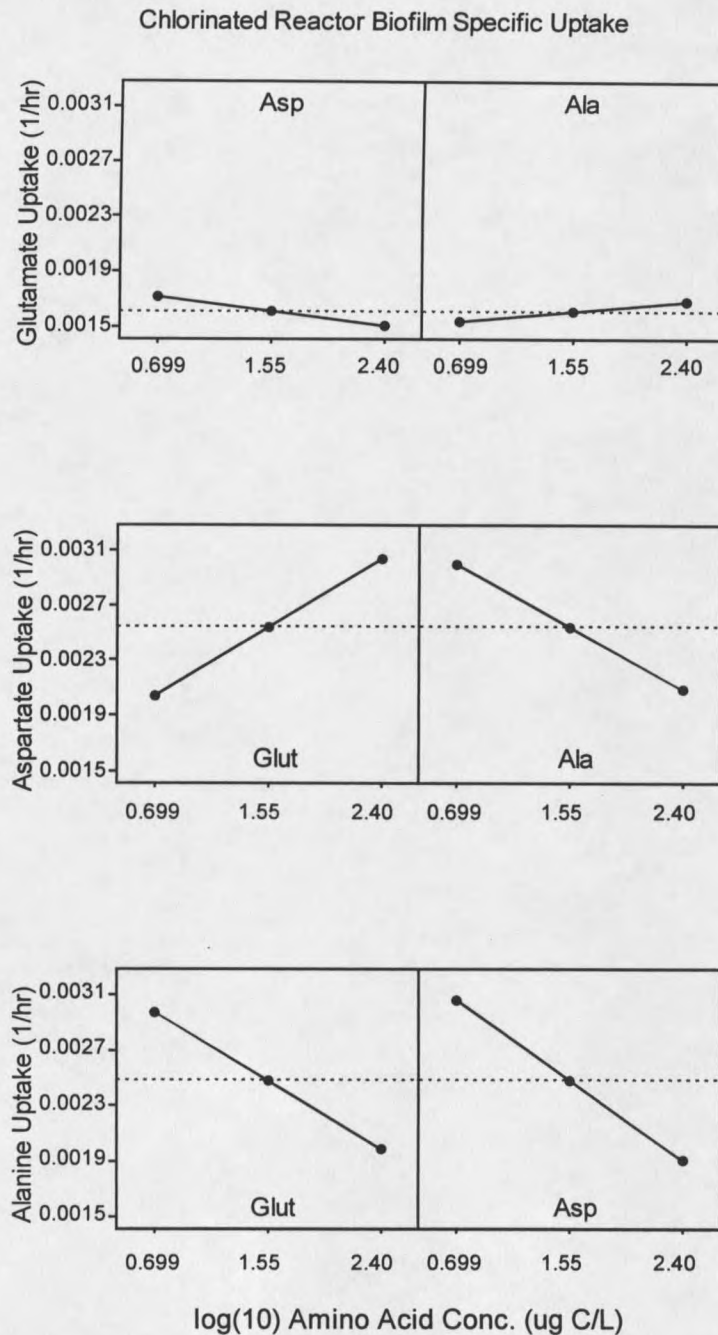


Figure 33. Main effects plots for specific uptake of isotope-labeled amino acids, chlorinated reactor biofilm. Main effects are based on fitted means. Glut = glutamate; Asp = aspartate; and Ala = alanine.

Chlorinated Reactor Isotope-Labeled Amino Acid Uptake

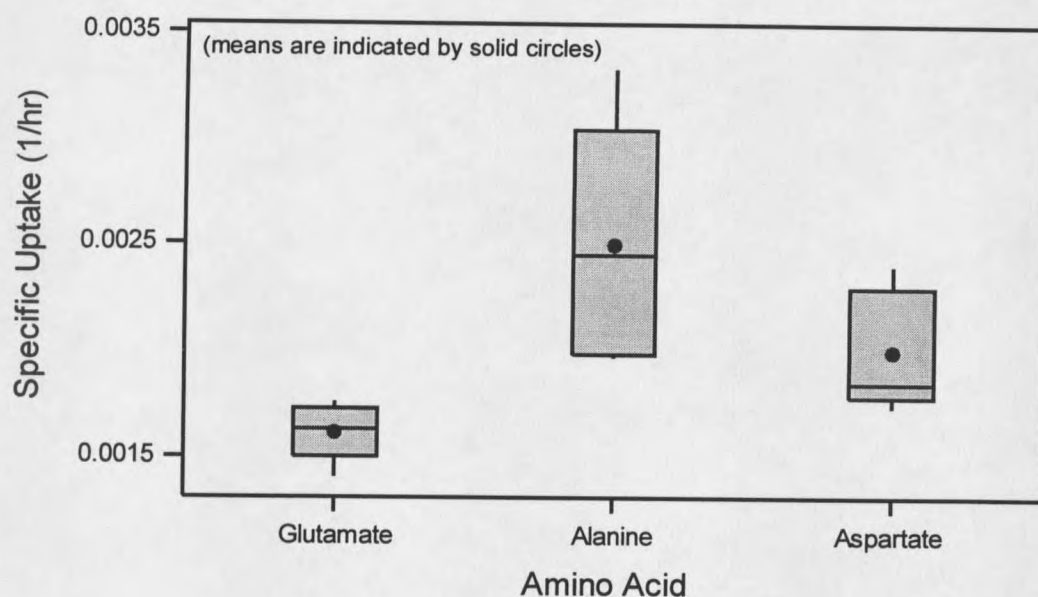


Figure 34. Boxplots for specific uptake of isotope labeled amino acids, chlorinated reactor biofilm. Boxes represent interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend to $1.5 \times$ interquartile range.

were more than double the rates in the chlorinated reactor biofilm, and the differences were significant (one-way ANOVA, $p < 0.0005$). The uptake rates for glutamate and alanine were significantly greater in the control reactor compared to the chlorinated reactor. While the uptake rate of aspartate was greater in the control reactor than in the chlorinated reactor, the difference was not significant (one-way ANOVA $p = 0.120$).

Relationship between Uptake and Carbon Loading Rate. Total amino acid concentration was converted to an equivalent reactor loading rate and the relationship

Boxplots of Total Isotope-Labeled Amino Acid Uptake by Reactor

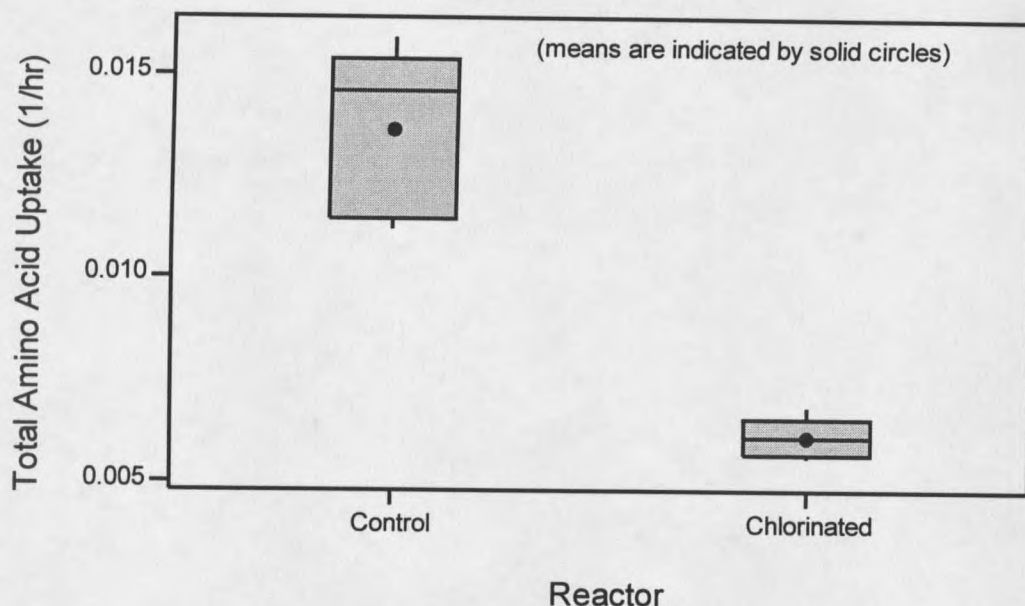


Figure 35. Boxplots showing comparison of total amino acid specific uptake by reactor for isotope-labeled amino acids experiment. Boxes represent interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend to $1.5 \times$ interquartile range.

between specific uptake rates and loading rate was examined. Models relating specific uptake to carbon loading were examined for each amino acid and for the total amino acid uptake (sum of three amino acids). Models examined included zero-order, linear and hyperbolic. Table 12 presents the results for the model fits. The linear model fit to the data was:

$$v = a + b \cdot S_L \quad (34)$$

Reactor	Amino Acid	Model	Mean (hr ⁻¹)	Upper 95% CI (hr ⁻¹)	Lower 95% CI (hr ⁻¹)	Model Parameters			p (regsrn)	adj. R ²	RSS
						a (hr ⁻¹)	b ⁽¹⁾	b (μg C L ⁻¹)			
Control	Total(3)	Zero-order	0.0136	0.0110	0.0162						1.75E-05
		Linear				0.0102	0.0384		0.012	0.877	1.60E-06
		Hyperbolic				0.0178	0.0214	122.23	0.005	0.931	8.99E-07
Chlorinated	Total(3)	Zero-order	0.00610	0.00550	0.00670						9.36E-07
		Linear				0.00647	-0.00490		0.215	0.266	5.15E-07
⁽¹⁾ b (Linear) = cm ² (μg C) ⁻¹ ; b (hyperbolic) = μg C cm ⁻² hr ⁻¹											

Table 12. Specific substrate uptake models for amino acids replicate 3 experiment, isotope-labeled amino acids. Models are for total amino acids uptake (glutamate + aspartate + alanine).

where v is the specific uptake rate (hr^{-1}), a is the intercept (hr^{-1}) and b is the slope ($\text{cm}^2/\mu\text{g C}$) and S_L is the equivalent carbon loading rate per unit area per time ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$). A hyperbolic model represents the traditional Michaelis-Menten substrate uptake equation:

$$v = \frac{a \cdot S_L}{b + S_L} \quad (35)$$

where a is the maximum specific uptake ($V_{\max}$, hr^{-1}) and b is the half-saturation constant for uptake (K_m , $\mu\text{g C cm}^{-2} \text{ hr}^{-1}$). In Table 12 the variable b (K_m) is also given in terms of amino acids concentration, $\mu\text{g C/L}$.

The zero-order model assumes no relationship between carbon loading rate and the specific uptake rate. A zero-order model adequately described specific substrate uptake for the chlorinated reactor biofilm. The low adjusted R^2 (0.266) and high regression statistic ($p=0.215$) indicate the linear model was not appropriate because there was no trend in the data. A hyperbolic model was also not appropriate for the chlorinated reactor and the data was not presented. Significant regressions resulted for the linear and hyperbolic models when fit to the control reactor biofilm specific uptake data.

Carbohydrates. Carbon removal data for all carbohydrate replicate experiments are presented in Figures 36 and 37 for the control and chlorinated reactors, respectively. Linear regression of carbon removal flux as a function of influent substrate loading rate was significant at the $\alpha=0.05$ level for the control reactor and was not

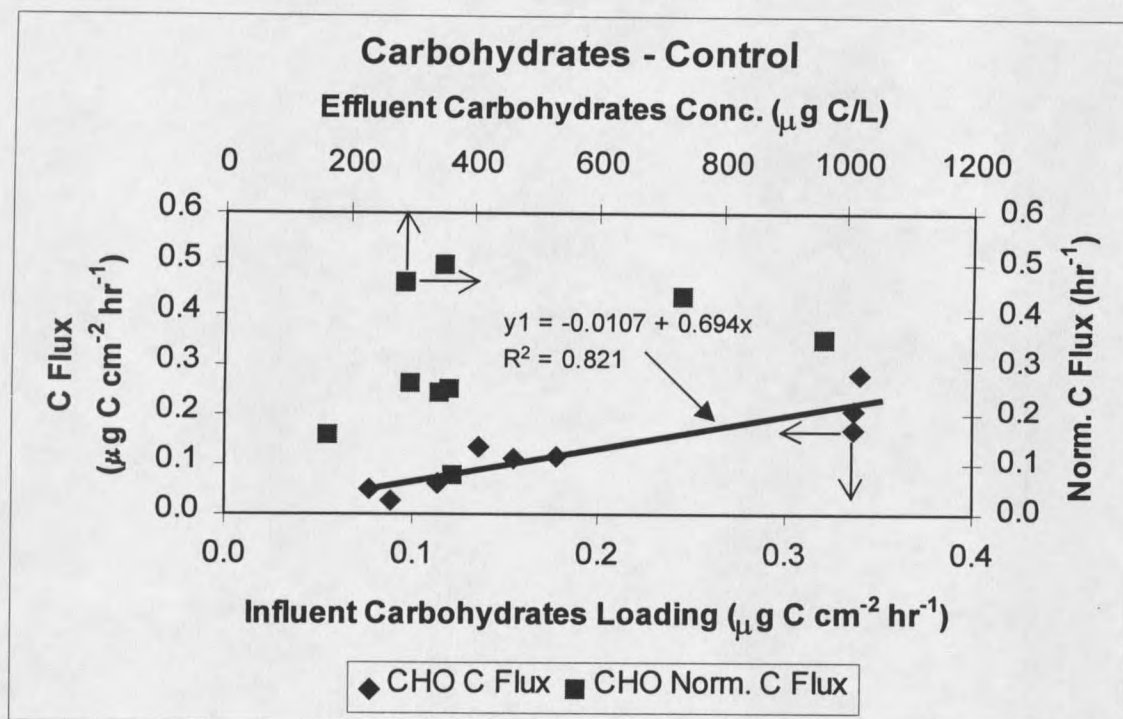


Figure 36. Carbon removal flux and normalized carbon removal rate for carbohydrates (CHO) control reactors. Carbon removal rate normalized to biofilm biomass carbon. $y1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{hr}^{-1})$.

significant for the chlorinated reactor data (Table 9). The removal fraction for the control reactor (0.657) was greater than for the chlorinated reactor (0.170), indicating lower overall removal of carbohydrates in the chlorinated reactor. Correlations were positive but not significant for the control and chlorinated reactor normalized carbon removal rates. Mean values for the normalized carbon removal rate were 0.304 and 2.69 hr^{-1} for the control and chlorinated reactors, respectively (Table 11). Results of the linear regressions with and without an intercept are presented in Table 10.

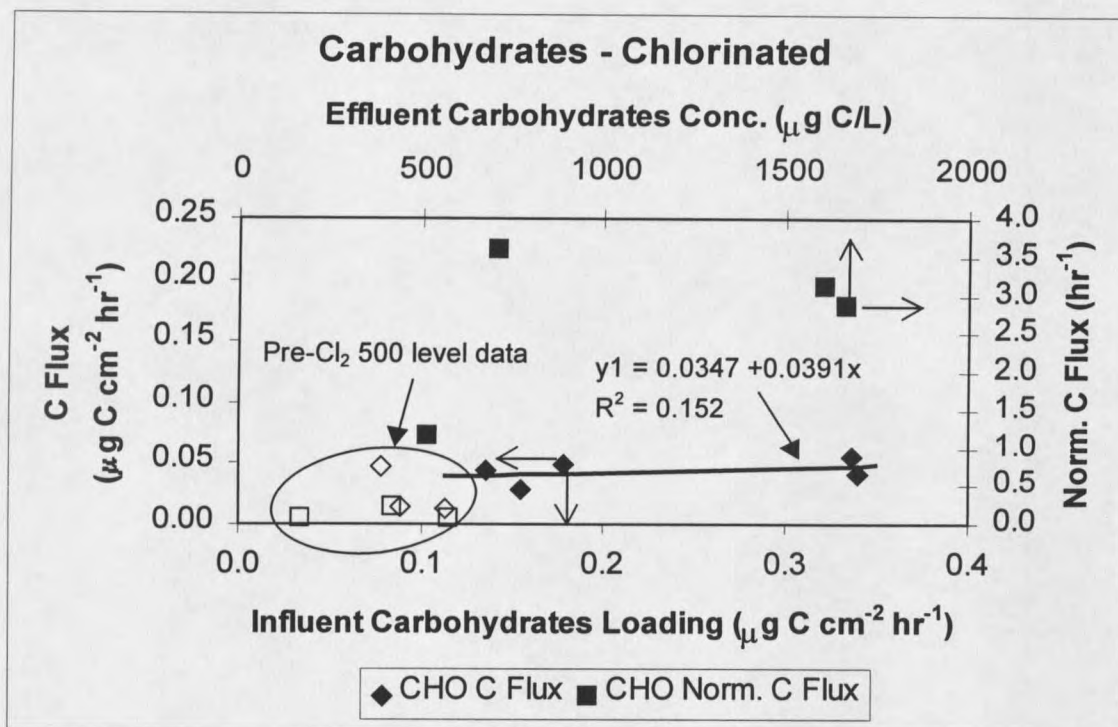


Figure 37. Carbon removal flux and normalized carbon removal rate for carbohydrates (CHO) chlorinated reactors. Carbon removal rate normalized to biofilm biomass carbon. Open data symbols indicate data not included in the analysis. $y_1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{ hr}^{-1})$.

Humics. Carbon removal data for the humics experiments are presented in Figures 38 and 39 for the control and chlorinated reactors, respectively. As shown in Table 9, linear regressions for both reactors were significant at the $\alpha=0.05$ level. The control reactor fractional removal (slope of regression line) (0.507) was greater than in the chlorinated reactor (0.409). Correlation of the normalized carbon removal rate with effluent substrate concentration was negative for the control and chlorinated reactors and the correlations were significant ($>80\%$). Mean values for the normalized carbon removal rate were 0.141 and 0.382 hr^{-1} for the control and chlorinated reactors, respectively (Table 11).

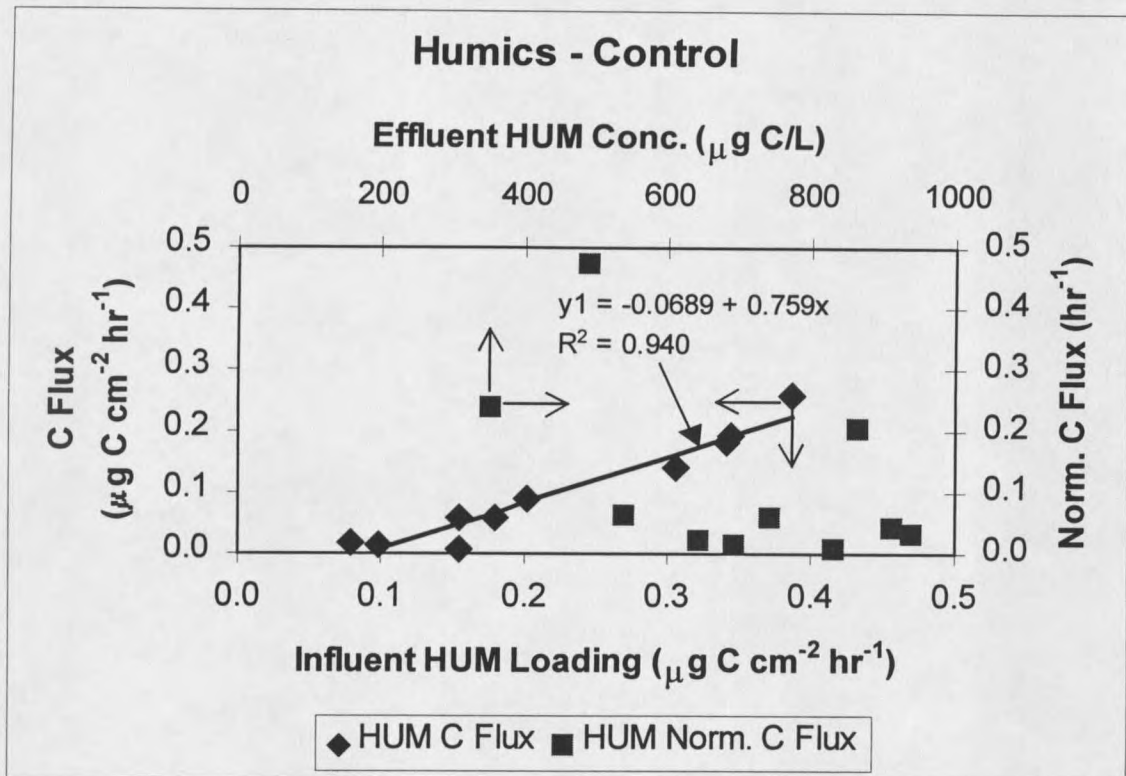


Figure 38. Carbon removal flux and normalized carbon removal rate for humics (HUM) control reactors. Carbon removal rate normalized to biofilm biomass carbon. $y1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{hr}^{-1})$.

Mixed Substrates. Carbon removal data for the single mixed substrates replicate experiment are presented in Figures 40 and 41 for the control and chlorinated reactors, respectively. Linear regressions for carbon removal flux versus carbon loading data were significant for both reactors at the $\alpha=0.05$ level (Table 9). Correlation of the normalized carbon removal rate with effluent substrate loading was negative ($r = -0.749$) and significant for the control reactor, but was positive ($r = 0.337$) and not significant for the chlorinated reactor (Table 10). Mean values for the normalized carbon removal rate

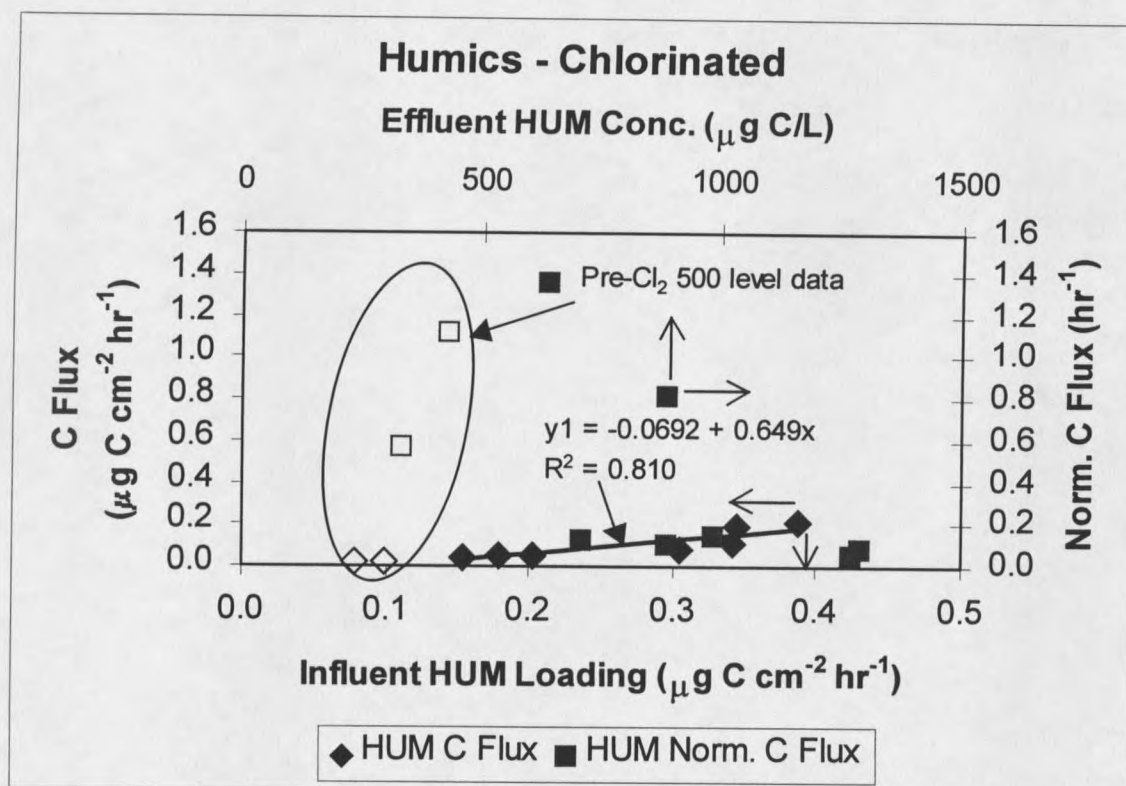


Figure 39. Carbon removal flux and normalized carbon removal rate for humics (HUM) chlorinated reactors. Carbon removal rate normalized to biofilm biomass carbon. Open data symbols indicate data not included in the analysis. $y_1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{ hr}^{-1})$.

were 0.0972 and 0.316 hr^{-1} for the control and chlorinated reactors, respectively (Table 11).

Overall Comparison of Combined Data. The carbon flux data was combined (control + chlorinated) and analyzed by substrate group to determine the overall impact of substrate-type on carbon flux. Figure 42 presents boxplots for the combined carbon flux data analyzed by substrate group. A Box-Cox transformation of the carbon flux data was required to obtain homogeneity of variance. A one-way ANOVA of the transformed

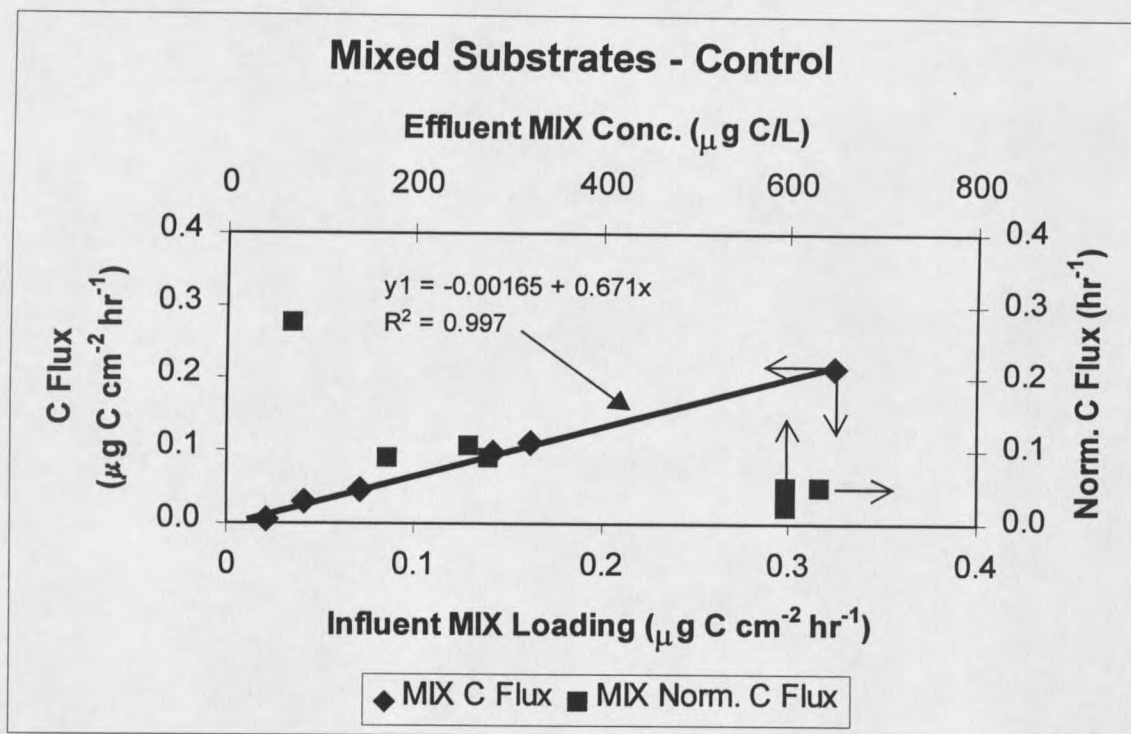


Figure 40. Carbon removal flux and normalized carbon removal rate for mixed substrates (MIX) control reactors. Carbon removal rate normalized to biofilm biomass carbon. $y1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{hr}^{-1})$.

data indicated the mean carbon flux was not equal when compared by substrate group ($p < 0.0005$) and carbon flux data for amino acids was significantly different (greater) compared to data for the other three substrate groups (Tukey's pairwise comparison).

There was no significant difference in carbon flux data for carbohydrates, humics and the mixed substrate groups (Tukey's pairwise comparison).

The normalized carbon flux data was combined (control + chlorinated) and analyzed by substrate group to determine the overall impact of substrate-type on

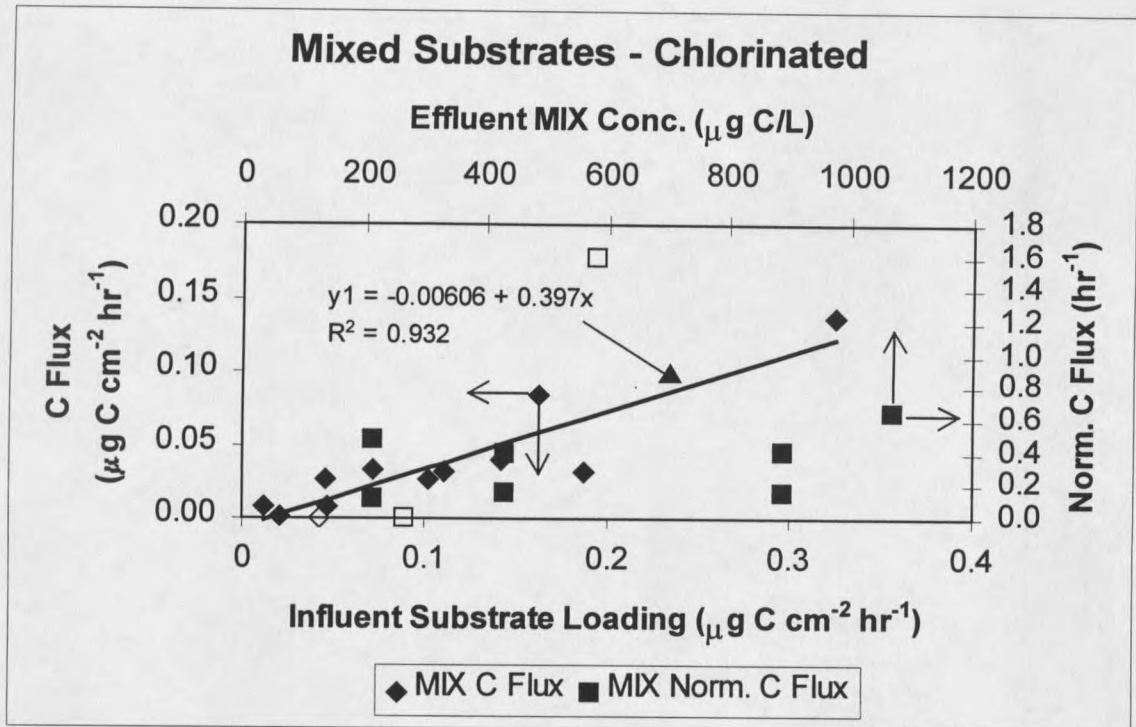


Figure 41. Carbon removal flux and normalized carbon removal rate for mixed substrates (MIX) chlorinated reactors. Carbon removal rate normalized to biofilm biomass carbon. Open data symbols indicate data not included in the analysis. $y_1 = \text{C flux } (\mu\text{g C cm}^{-2} \text{ hr}^{-1})$.

normalized carbon flux. Figure 43 presents boxplots for the combined normalized carbon flux data analyzed by substrate group. A Box-Cox transformation of the carbon flux data was required to obtain homogeneity of variance. A one-way ANOVA of the transformed data indicated the mean normalized carbon flux values were not equal when compared by substrate group ($p=0.001$) and that difference existed only between carbohydrates and humics (Tukey's pairwise comparison). Carbohydrates had the greatest normalized carbon flux value compared to the other three substrate groups.

Boxplots of Combined Carbon Flux by Substrate

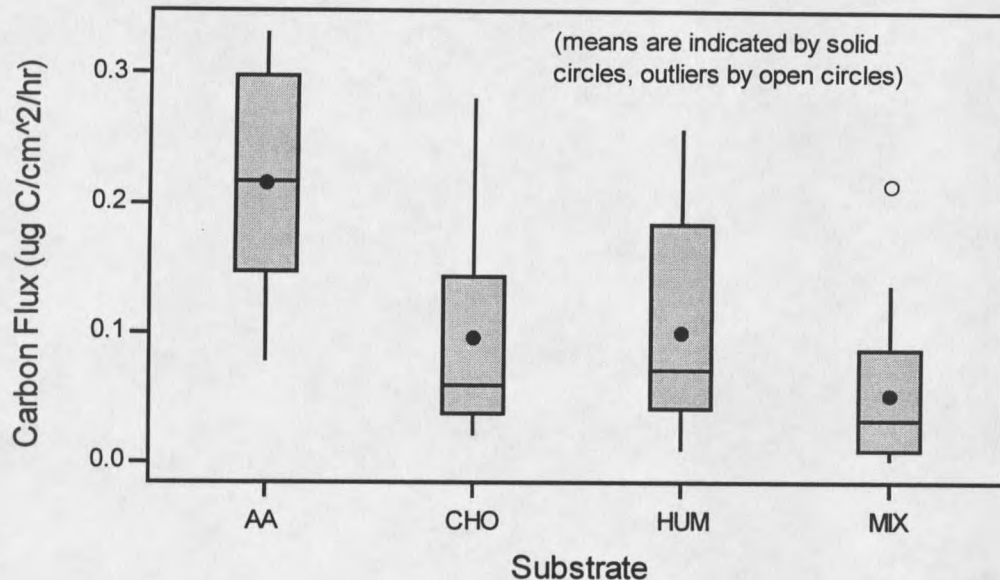


Figure 42. Boxplots showing combined carbon flux data by substrate. Boxes represent interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend to lowest or highest data point within the range defined by $1.5 \times$ interquartile range. AA = amino acids; CHO = carbohydrates; HUM = humic substances; and MIX = mixed substrate.

It is apparent from the number and magnitude of the outliers in Figure 43 that there was a great deal of variability in the normalized carbon flux data.

There was a strong linear relationship between carbon flux and influent carbon loading rate for all substrates and reactor type except the carbohydrates chlorinated reactor. In this case the carbon flux was nearly constant over the range of influent loading values examined, resulting in a non-significant regression ($p=0.999$).

Boxplots of Combined Normalized Carbon Flux by Substrate

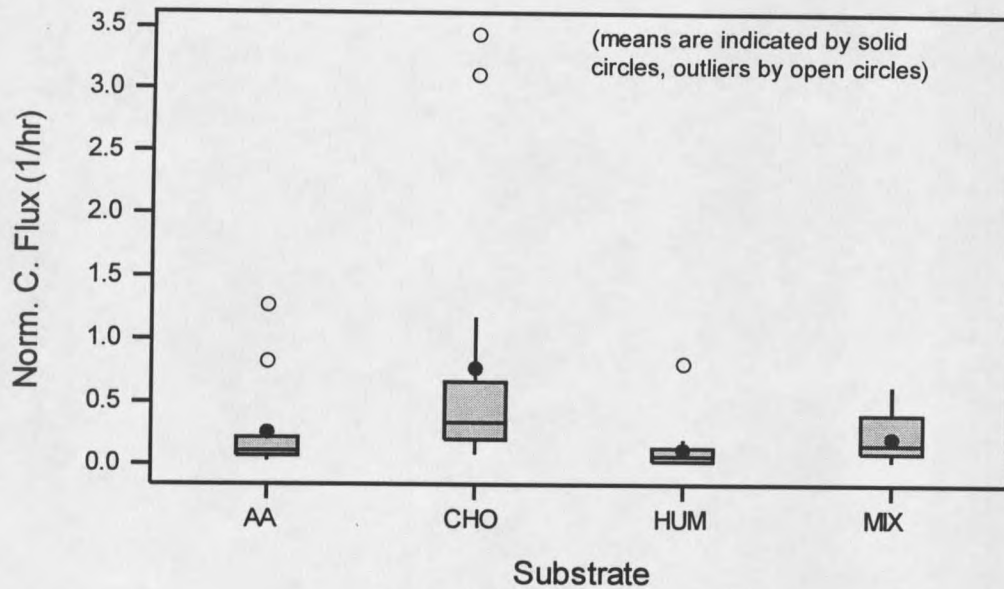


Figure 43. Boxplots showing combined normalized carbon flux data (hr^{-1}) by substrate. Boxes represent interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend lowest or highest data point within the range defined by $1.5 \times$ interquartile range. AA = amino acids; CHO = carbohydrates; HUM = humic substances; and MIX = mixed substrate.

Figure 44 presents a bar chart showing the fractional carbon removal values by substrate for the control and chlorinated reactors (fractional removal was the slope of the regression lines for carbon flux versus influent carbon loading rate, regression forced through the origin). Amino acids control reactors had the highest fractional carbon removal (0.956) and the chlorinated carbohydrate reactors had the lowest fractional removal (0.170). Significant differences existed between the control and chlorinated

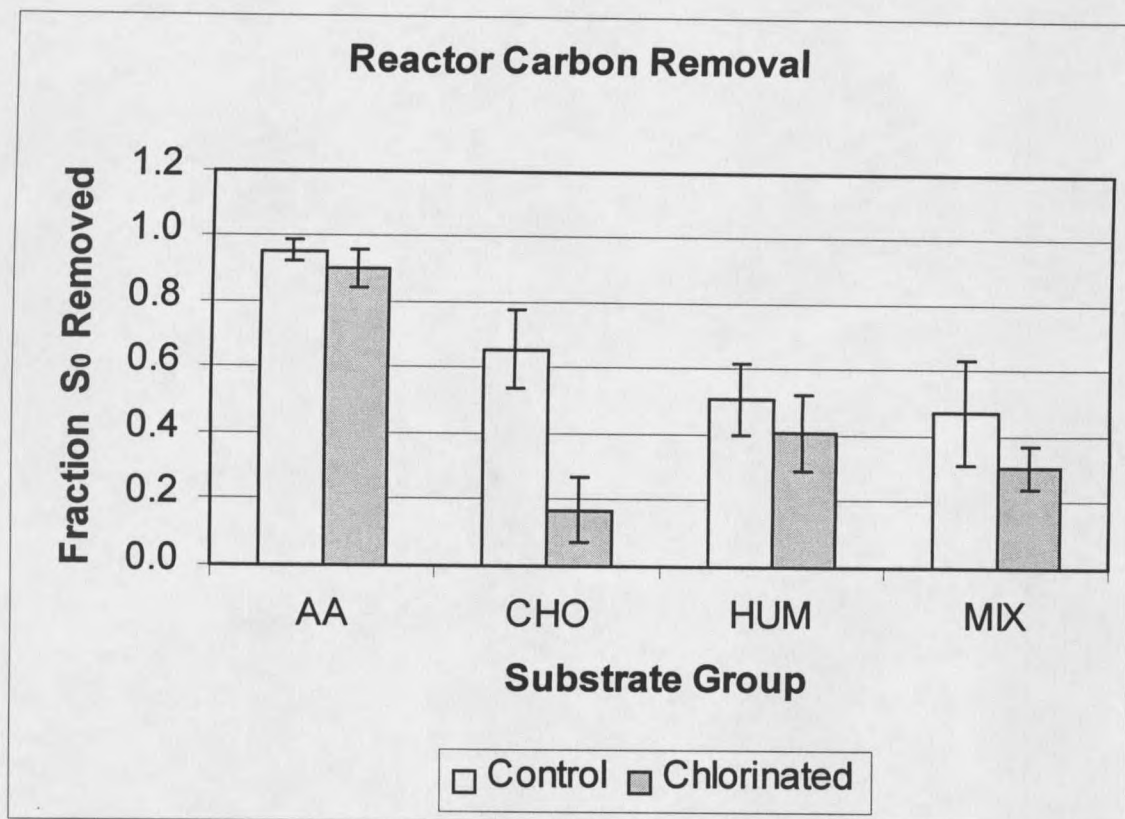


Figure 44. Summary of reactor carbon removal as a fraction of the influent carbon loading rate ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$). Error bars represent 95% confidence intervals. AA = amino acids; CHO = carbohydrates; HUM = humic substances; and MIX = mixed substrate.

reactors for carbohydrates. There was no significant statistical difference between fractional carbon removal values for the carbohydrates, humics and mixed substrate control reactors.

A one-way ANOVA for all normalized carbon flux data combined by reactor type indicated the mean values were not equal ($p=0.001$). The mean chlorinated reactor data (0.360 hr^{-1} ; $0.220\text{--}0.500$, 95% CI) was greater than for the control reactor (0.136 hr^{-1} ; $0.0837\text{--}0.188$, 95% CI). The combined normalized data for each reactor was analyzed to

determine if differences existed between the means by carbon level. The mean normalized carbon flux values were statistically equal between carbon levels for the control reactors ($p=0.168$) and the chlorinated reactors ($p=0.252$). Based on this information it can be assumed that for the combined normalized carbon flux data there was no difference in values by carbon level within a reactor group.

Reactor Biofilm Observed Yield

The kinetic parameter yield (Y or $Y_{X/S}$) is commonly used in functions relating growth to substrate uptake. Yield is the amount of biomass produced for a given mass of substrate utilized. An observed yield was calculated for the reactor biofilm using the methods described in Chapter 3, MATERIALS AND METHODS. The term "observed" yield is used because the calculated yield includes substrate used for purposes other than biomass production, such as endogenous respiration. The data utilized to determine reactor biofilm growth rates and carbon removals were also utilized to compute the observed yields, allowing comparison of the three measurements. Data for the chlorinated reactors at the nominal carbon concentration of $500 \mu\text{g C/L}$ (prior to commencement of chlorine addition) were not included in the analysis for the chlorinated reactors. Correlation analyses were performed using normal scores transformed data and WQHYDRO software (Aroner, E. R., 1996) or MiniTab® software.

Amino Acids. Data for the amino acids replicate 3 experiment are shown in Figure 45 where observed yield was plotted as a function of the effluent amino acids

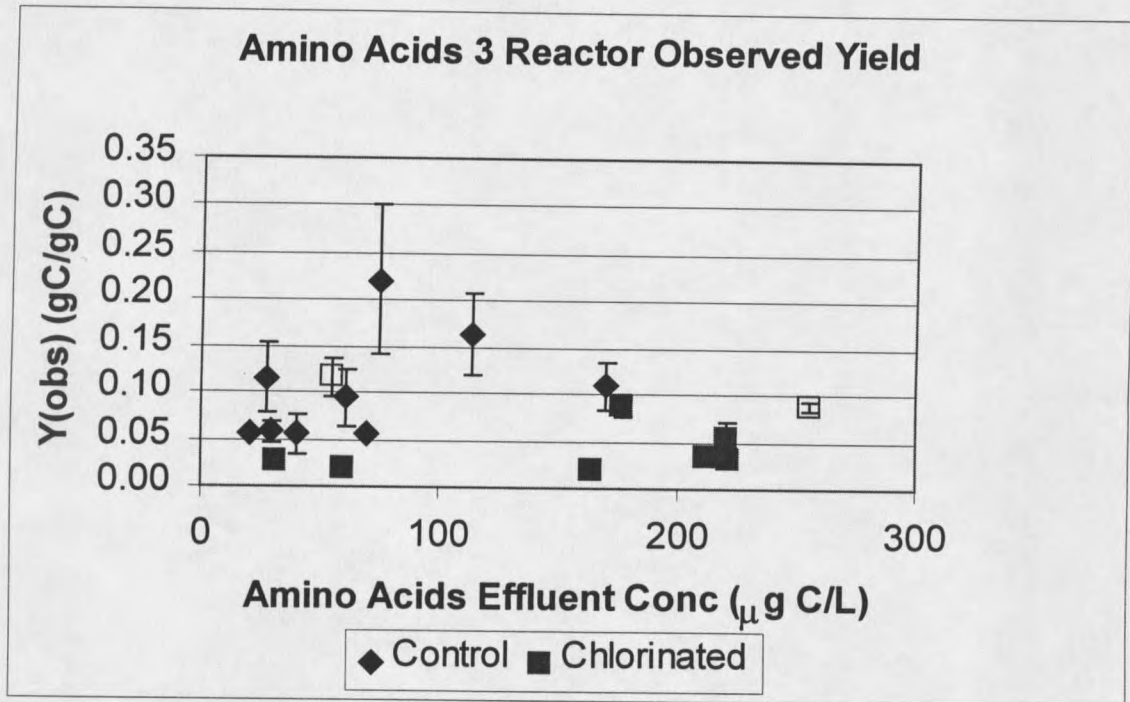


Figure 45. Observed yield for biofilm in amino acids replicate 3 reactors as a function of effluent amino acids concentration. Error bars represent $\pm$ standard error of the mean. Open symbols indicate data not used in the analysis for the chlorinated reactor (500 carbon level reactors were not chlorinated).

feed concentration as carbon. The data for the control reactor showed a positive trend with increasing effluent substrate concentration (correlation coefficient $[r] = 0.394$) but the correlation was not significant ($2 \times p = 0.294$, or $<80\%$ significant) (see Table 13).

The correlation remained positive ($r=0.305$) between observed yield and effluent substrate concentration for the control reactor data with the chlorinated reactor data at the 500 carbon level included. The chlorinated reactor data also showed a positive trend with increasing effluent substrate concentration ($r = 0.435$) but the correlation was not significant. Figure 46 presents both the raw data and boxplots for the raw data. In the boxplots, open dots represent data points, boxes represent 95% confidence intervals for

Substrate	Reactor	r	2 × p	Significance
Amino Acids	Control	0.394	0.294	<80%
	Chlorinated	0.435	0.329	<80%
	Control – all 500	0.305	0.361	<80%
Carbohydrates	Control	0.047	0.904	<80%
	Chlorinated	0.306	0.556	<80%
	Control – all 500	0.245	0.442	<80%
Humics	Control	-0.176	0.626	<80%
	Chlorinated	-0.189	0.654	<80%
	Control – all 500	-0.167	0.604	<80%
Mixed Substrate	Control “a”	-0.500	0.667	<80%
	Chlorinated “a”	1.000	***	>99%

Table 13. Summary of correlation data (r = correlation coefficient) for observed yield and effluent substrate concentration for the reactor biofilms. Correlations are shown for the control reactor including the 500 carbon level data from the chlorinated reactor (prior to start of chlorination) for amino acids, carbohydrates and humics. “a” = first reactor in series for mixed substrates.

the median, solid horizontal line across the box represents the median, whiskers extend to the lowest or highest point within the range defined by the quartile value plus or minus $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and solid dots beyond whiskers represent possible outliers. The mean observed yield for the chlorinated reactors (0.0393) was less than in the control reactor (0.104) and the means were not equal based on a 2-sample t-test with $\alpha=0.05$ ($p=0.012$). The statistics for the means are presented in Table 14.

Carbohydrates. Observed yield data for the carbohydrate experiment are shown in Figures 47 and 48. The data for the control reactor as a function of effluent substrate concentration indicates a slightly positive correlation ($r = 0.047$) but the

Amino Acids

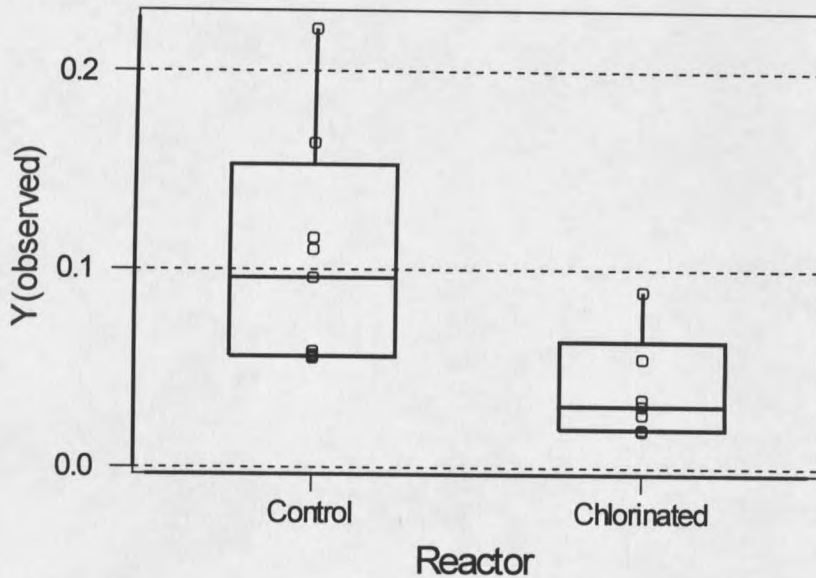


Figure 46. Observed yield (g C/g C) for biofilm in the amino acids replicate 3 reactors. Open dots represent data points. See text for description of boxplot characteristics.

Substrate	Reactor	Mean Y_{obs} (g C/g C)	95% C.I. for Mean	p (2-sample t-test)
Amino Acids	Control	0.104	0.0592 – 0.148	0.012
	Chlorinated	0.0393	0.0164 – 0.0621	
Carbohydrates	Control	0.187	0.0700 – 0.304	0.320
	Chlorinated	0.113	-0.0086 – 0.235	
Humics	Control	0.111	0.0206 – 0.201	0.160
	Chlorinated	0.0472	0.0255 – 0.0689	
Mixed Substrate	Control "a"	0.255	-0.0642 – 0.5750	0.130
	Chlorinated "a"	0.0738	-0.1355 – 0.2832	

Table 14. Observed yield data for reactor biofilm. Comparison of means (2-sample t-test, equal means) based on $\alpha=0.05$. "a" = first reactor in series for mixed substrates.

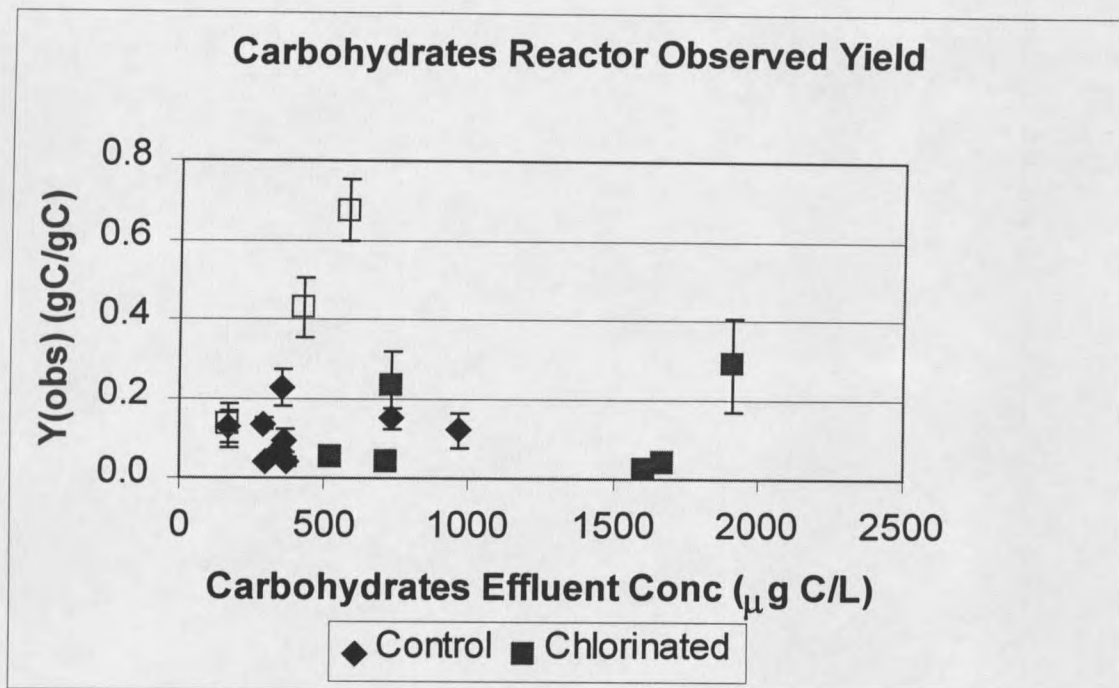


Figure 47. Observed yield for biofilm in carbohydrates reactors as a function of effluent carbohydrates concentration. Error bars represent $\pm$ standard error of the mean. Open symbols indicate data not used in the analysis for the chlorinated reactor (500 carbon level reactors were not chlorinated).

correlation was not significant ($2 \times p = 0.904$) (see Table 13). Data for the chlorinated reactors indicated stronger positive trend than did the control reactor data ($r = 0.306$, chlorinated) but the correlation was not significant. There was greater correlation between observed yield and effluent substrate concentration ($r = 0.245$, $2 \times p = 0.442$) for the control reactor data with the chlorinated reactor data at the 500 carbon level included. Figure 48 presents both the raw data and boxplots for the raw data. Mean observed yield for the chlorinated reactors' biofilm (0.113) was statistically equal to the control reactors' biofilm yield (0.187) at the $\alpha=0.05$ level (2-sample t-test, $p=0.32$). The statistics for the means were presented in Table 14.

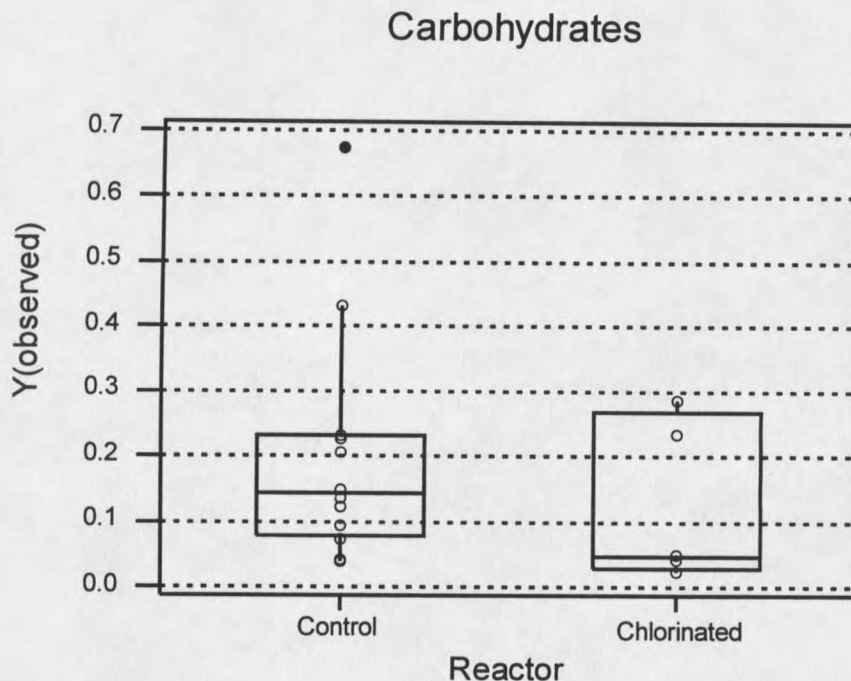


Figure 48. Observed yield (g C/g C) for biofilm in the carbohydrates reactors. Open dots represent data points and solid dots represent possible outliers. See text for description of boxplot characteristics.

Humics. Observed yield data for the humics experiments are shown in Figures 49 and 50. The data for the control reactor as a function of effluent substrate concentration indicates a negative trend with increasing effluent substrate concentration ($r=-0.176$) but the correlation of the data was not significant ($2 \times p = 0.626$) (see Table 13). A similar trend in the results were found for the chlorinated reactor: negative correlation ($r=-0.189$), but again the correlation was not significant ($2 \times p = 0.654$). The correlation was negative and not significant ($r=-0.167$, $2 \times p = 0.604$) for the control reactor data with the chlorinated reactor data at the 500 carbon level included.

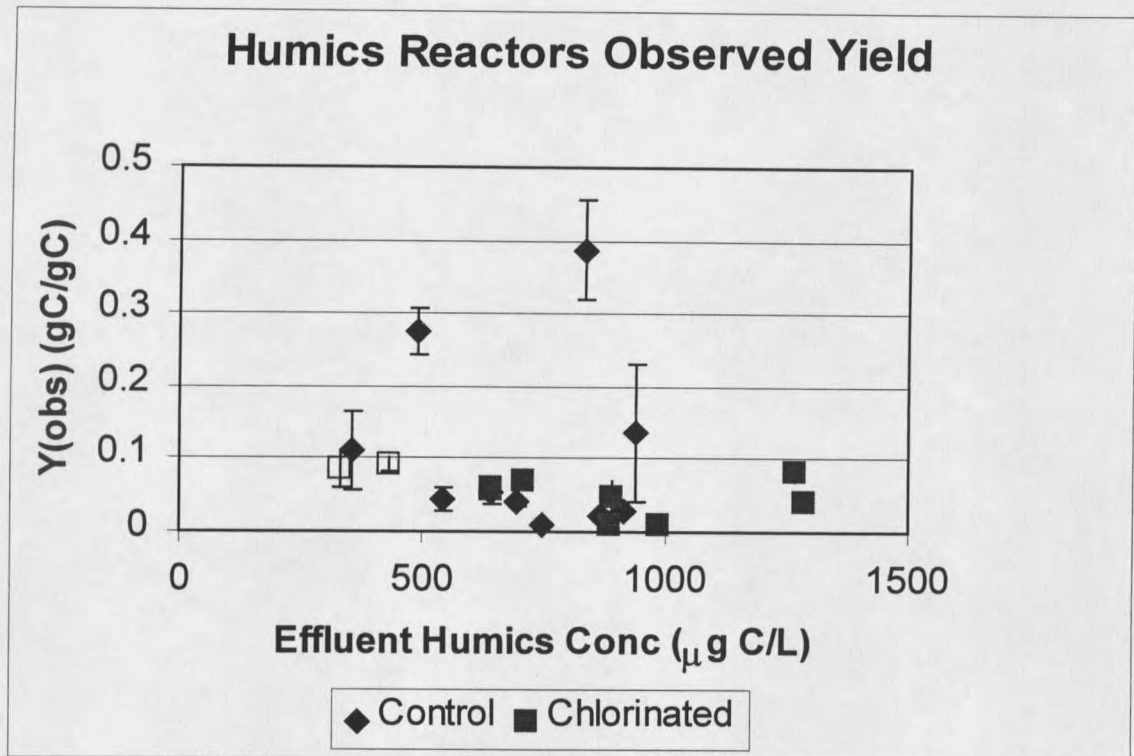


Figure 49. Observed yield for biofilm in humics reactors as a function of effluent humics concentration. Error bars represent $\pm$ standard error of the mean. Open symbols indicate data not used in the analysis for the chlorinated reactor (500 carbon level reactors were not chlorinated).

Figure 50 presents both the raw data and boxplots for the raw data. Observed yield for the chlorinated reactors (0.0472) was less than in the control reactor (0.111), but the difference in the means was not significant at the $\alpha=0.05$ level (2-sample t-test, $p=0.16$).

The statistics for the means are presented in Table 14.

Humics

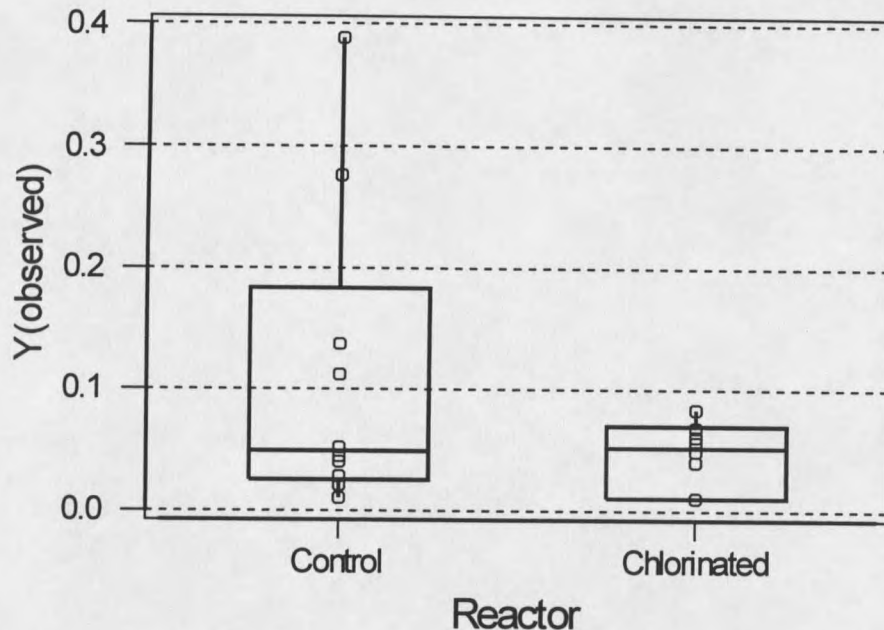


Figure 50. Observed yield (g C/g C) for biofilm in the humics reactors. Open dots represent data points. See text for description of boxplot characteristics.

Mixed Substrate. Observed yield data for the mixed substrate experiments are shown in Figure 51. Data for the “a” reactors (first reactor in series) were analyzed to allow comparison with yield data from other substrate experiments. The data for the control reactor as a function of effluent substrate concentration indicates a negative trend with increasing effluent substrate concentration ($r=-0.500$) but the correlation of the data was not significant ($2 \times p = 0.667$) (see Table 13). Analysis of the chlorinated reactor yield data indicate a significant positive correlation ($r=1.000$) with effluent substrate

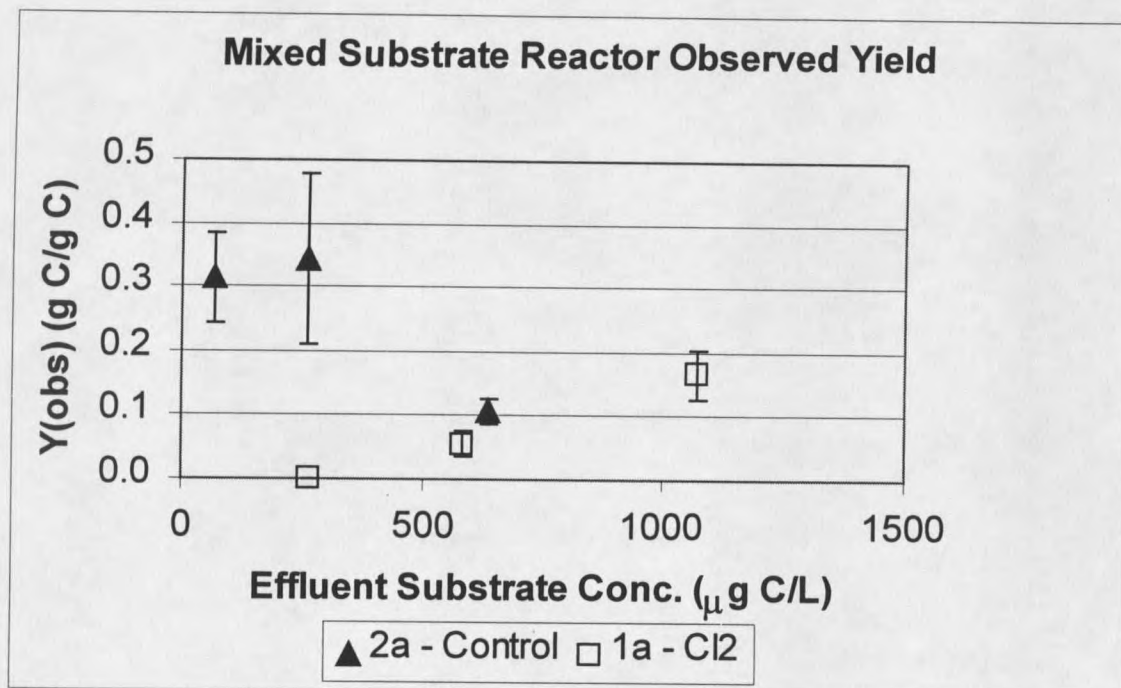


Figure 51. Observed yield for biofilm in mixed substrate “a” reactors (first of two in series) as a function of effluent mixed substrate concentration. Error bars represent $\pm$ standard error of the mean. Both reactors received chlorine at the 500 carbon level.

concentration. Observed yield for the chlorinated reactors (0.0738) was less than in the control reactor (0.255), but the difference in the means was not statistically significant at the $\alpha=0.05$ level (2-sample t-test, equal means, $p=0.13$). The small sample size ($n=3$ for each reactor) reduces the power of the statistical comparison. The statistics for the means are presented in Table 14.

Overall Comparison of Combined Observed Yield Data. The combined data (control + chlorinated) for each substrate groups were modified to include in the control reactor group the 500 carbon level data of both reactors, except for the mixed substrate experiment where the chlorinated reactor received chlorine at the 500 carbon level. The

observed yield data was Box-Cox transformed to achieve homogeneity of variance for statistical analysis.

A one-way ANOVA of the combined yield data analyzed by reactor indicated the means were not equal ($p < 0.0005$); the control reactor mean was greater than for the chlorinated reactor. A one-way ANOVA of the combined yield data analyzed by substrate group indicated the mean observed yield was not equal when compared between substrate groups ($p = 0.039$) and that the mean yield for mixed substrates was significantly greater than for carbohydrates and humics (Tukey's pairwise comparison) (Figure 52).

The statistical comparison of observed yield by substrate group for all control reactor data indicated the mean values were statistically equal ($p = 0.096$, $\alpha = 0.05$), but the control mixed substrate reactor observed yield was significantly greater than all other substrates when analyzed using the less conservative Fisher's pairwise comparison at $\alpha = 0.10$ (Figure 53). Mean observed yield values were statistically equal when compared by substrate group for the chlorinated reactors ($p = 0.609$), indicating less variation for the chlorinated reactor (Figure 53).

The difference between mean observed yield by carbon levels (Figure 53) was not significant for the combined data ($p = 0.126$), but when the data for the mixed substrate chlorinated reactor at the 500 carbon level was removed from the analysis the difference was significant ($p = 0.013$) with the 500 level significantly different from the 1000 and 2000 levels (Tukey's pairwise comparison) and no significant difference between the 1000 and 2000 levels. Biofilm observed yield at the 500 level was significantly different

Observed Yield by Substrate

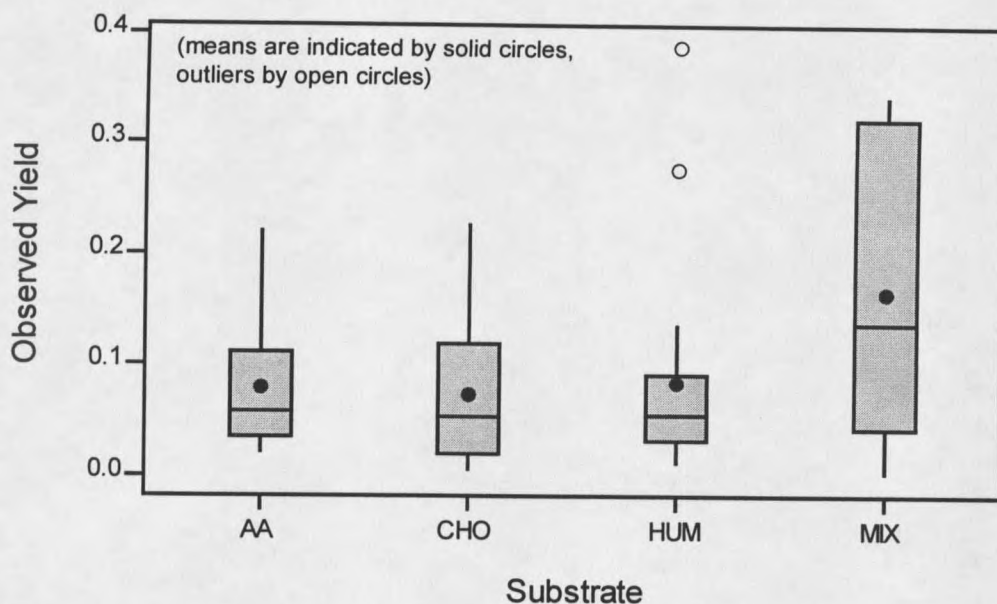
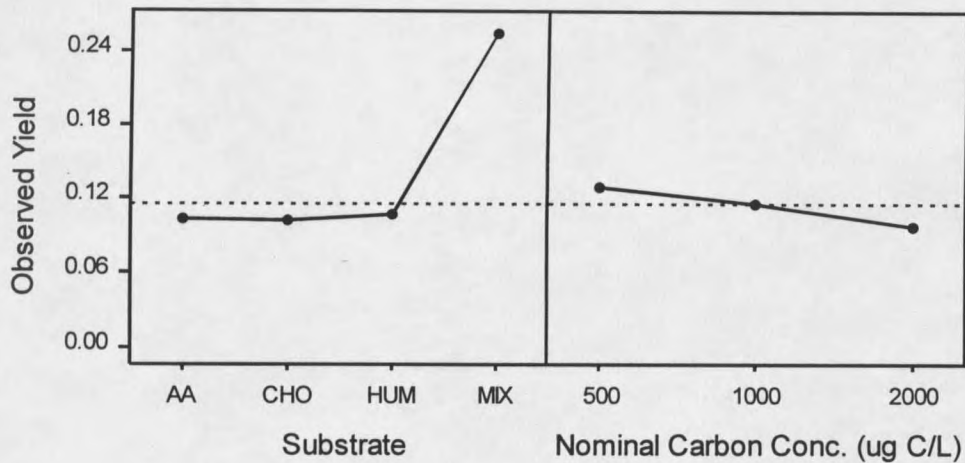


Figure 52. Boxplots showing observed yield (g C/g C) for biofilm by substrate group. Boxes represent the interquartile range ($Q_{75} - Q_{25}$), solid horizontal line in box represents the median, and whiskers extend to the lowest or highest data point within the range defined by the quartile level $\pm 1.5 \times$ interquartile range. AA = amino acids; CHO = carbohydrates; HUM = humics; and MIX = mixed substrates.

(less) than both the 1000 and 2000 levels for the chlorinated reactors. However there was only one data point for observed yield at the 500 carbon level from the chlorinated mixed substrate reactor. There was no significant difference between the mean observed yield by carbon level for the control reactor data ($p=0.483$). When the mixed substrate chlorinated reactor data at the 500 carbon level was included, there was a difference between mean observed yield by carbon levels ($p=0.007$), but there was no significant

Main Effects Plot - Mean Control Reactor Yield



Main Effects Plot - Mean Chlorinated Reactor Yield

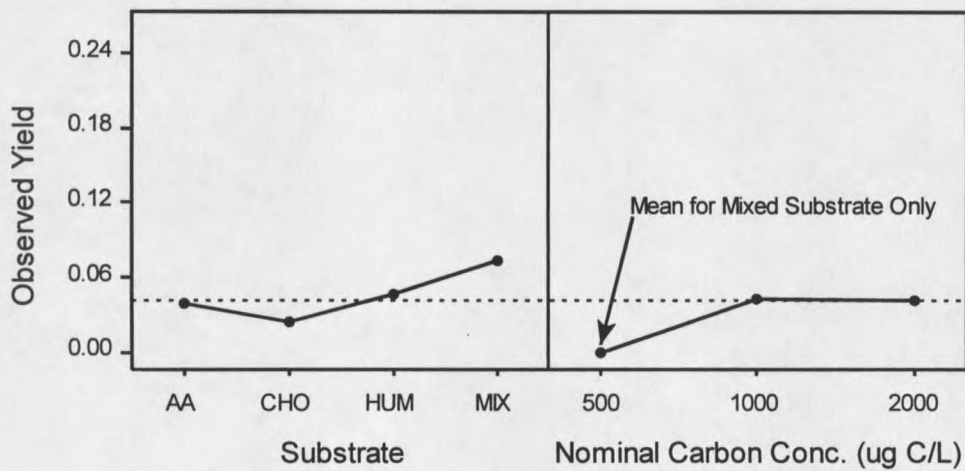


Figure 53. Main effects plot for all control and chlorinated reactor mean biofilm observed yield(g C/g C) by substrate group and carbon level. Dashed horizontal line represents mean for all data. AA = amino acids; CHO = carbohydrates; HUM = humics; and MIX = mixed substrates.

difference between mean observed yield at the 1000 and 2000 carbon levels (Tukey's pairwise comparison or one-way ANOVA, $p=0.599$).

The major factor influencing biofilm observed yield appears to be chlorination. Substrate group did not have a key role in observed yield with the exception of the mixed substrate group where observed yield was higher than for the other three substrate groups. Carbon level has some influence on observed yield, but does not appear to be a significant factor.

Batch Culture Kinetics

General

Determining kinetic parameters for biofilm can be difficult, requiring special reactors and/or techniques such as those used for the annular reactors. The research approach herein included using suspended biofilm cells in batch culture to determine if the batch culture technique would be a suitable approach for estimating kinetic parameters for biofilm in drinking water systems. Kinetic parameters determined from batch culture were compared to those obtained using other techniques to see if the batch culture technique was appropriate. Batch cultures of suspended biofilm cells were also utilized to detect biofilm response to specific substrates in the reactors, e.g., growth rates for each of the four amino acids.

At the end of the first two replicate experiments for amino acids, carbohydrates and humic substances, biofilm cells were removed from reactor sample slides, placed in batch cultures with different substrates, and sampled over time. Samples were analyzed

for total direct count (TDC) cells, culturable heterotrophic plate count (HPC) cells on R2A medium, and substrate concentration. Leucine uptake rates were measured over time in batch cultures for the second humics experiment. Growth rates were determined for all batch cultures using cell numbers (TDC and HPC). Growth rates determined using the integrated Monod equation and nonlinear regression techniques (see Chapter 3 for details) were estimated for only the amino acids combination and humics replicate 2 batch cultures. Results of parameter estimation using the integrated Monod equation were compared to results based on reactor mass balances. The following paragraphs describe the results of the work for the amino acids, carbohydrates and humics batch culture experiments. See Chapter 3, MATERIALS AND METHODS, for a description of the techniques used for taking samples and their analysis.

In the following paragraphs, control data or control reactor refers to the batch cultures using biofilm cells scraped from the control reactor. Chlorinated data or chlorinated reactor refers to the batch cultures using biofilm cells scraped from the chlorinated reactor. Comparisons between reactors refer to a comparison of data from the control and chlorinated reactors. Comparisons between reactors within a substrate group refer to a comparison of the control and chlorinated reactor using the same substrate. Comparison of the data between substrate groups without consideration of the reactor refers to a comparison of all data from both reactors for a single substrate. Reactor group or substrate group refers to data for a single group, such as the control reactors or glutamate batch cultures. The statistical software MiniTab® was used to analyze the data by one-way analysis of variance (ANOVA) to determine possible differences between

substrates within a reactor group. A balanced ANOVA or an ANOVA using the General Linear Model (GLM) was performed to assess differences between reactors, substrates and reactors within substrate groups (comparison of the control to the chlorinated reactor for an individual substrate). The α -level for the ANOVAs was 0.05, meaning p-values less than 0.05 indicate the means being compared cannot be assumed equal. See Chapter 3, MATERIALS AND METHODS, for a description of the two reactors and their operation prior to sampling.

Batch Culture Growth Rates–Cell Number and Leucine Uptake (Humics 2)

The purpose of determining growth rates in the batch cultures was to ascertain if there were differences in the way that the biofilm population in the reactor responded to specific substrates. First-order specific growth rates were determined for each batch culture as described in Chapter 3, MATERIALS AND METHODS. Growth rates for the TDC and HPC method were based upon the change over time of cell numbers.

Specific growth rates were determined by first fitting a curve to the cell number data as a function of time. The software TableCurve™ 2D was utilized to perform the nonlinear regressions. Transition-type curves, such as a logistic-type curve, typically provided the best fit, based upon the adjusted degrees-of-freedom R^2 value. Other curves were selected where appropriate. Visual verification of the curve-fit was always made to prevent use of erroneous curve equations. Because data from this type of work typically had outliers (data points that did not appear to fit a defined growth curve), several curve fits were performed to define possible growth rates. Once the curve-fit was performed, the natural log of the predicted cell numbers was plotted as a function of time and the

slope of the straight line portion of the curve (exponential phase) was determined to provide an estimate of the specific growth rate.

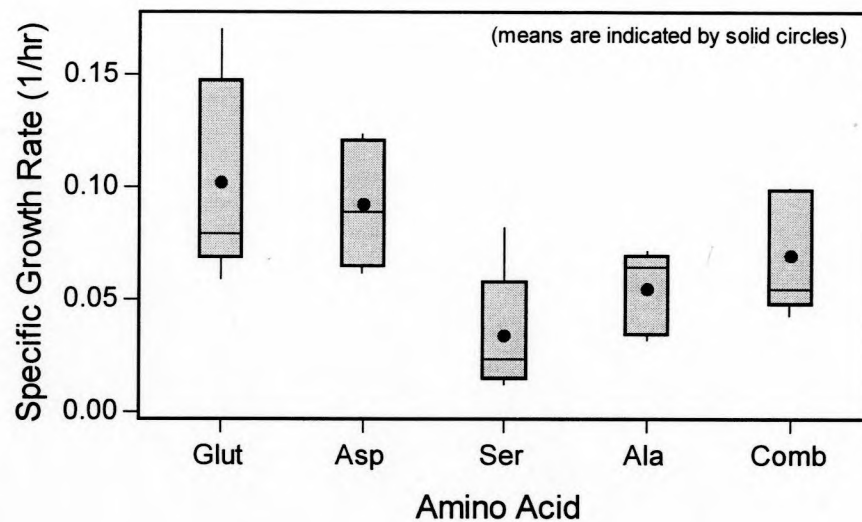
The following paragraphs present the results by substrate group using both TDC and HPC data from batch cultures. Specific growth rates based on ^3H -leucine uptake for the humics replicate 2 experiment batch cultures are also presented. Boxplots presented in the figures represent the interquartile range of the data ($Q_{75} - Q_{25}$), the solid horizontal line within the box is the median, the solid circle is the mean, and whiskers extend to the lowest or highest data point within the range defined by the quartile level $\pm 1.5 \times$ interquartile range.

Amino Acids. The substrates utilized for the amino acids experiment were the same four individual amino acids used in the reactor feed (glutamate, aspartate, serine and alanine) plus a combination of the four (combination). All initial concentrations were approximately 2000 $\mu\text{g/L}$ as carbon.

Growth Rates for Amino Acids Based on TDC. Sufficient determinations of the specific growth rate were made to allow analysis of five values for each substrate within a reactor group (control or chlorinated). The results shown in Figure 54 for the control reactor batch cultures indicate the following ranking of specific growth rates by amino acids using TDC data:

- Control: glutamate $\cong$ aspartate > combination > alanine > serine

Amino Acids Control Reactor Batch Cultures - TDC



Amino Acids Chlorinated Reactor Batch Cultures - TDC

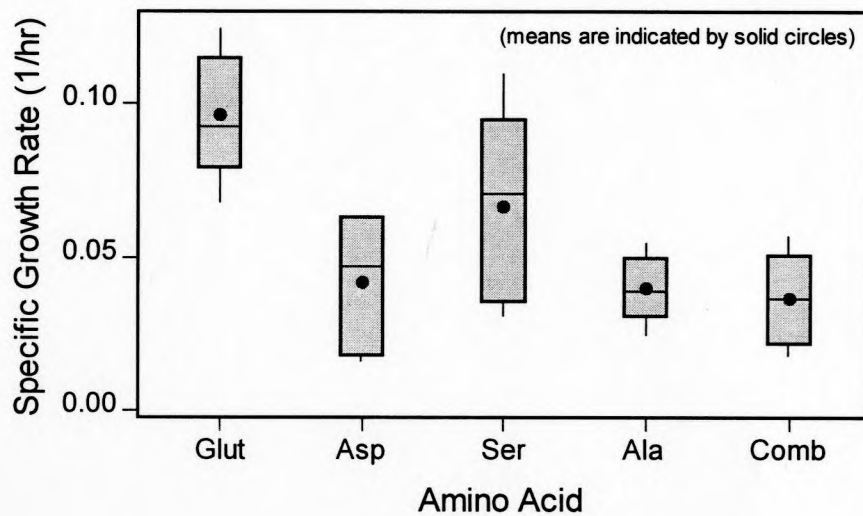


Figure 54. Boxplots of specific growth rate data by amino acid and the combination based on total direct cell counts (TDC) for batch cultures of biofilm cells from the amino acids control (top plot) and chlorinated (bottom plot) reactors. $n=5$ for each substrate. Glut = glutamate; Asp = aspartate; Ser = serine; Ala = alanine; and Comb = combination.

A one-way ANOVA for the control data indicated the mean growth rates were not statistically equal when compared by substrate ($p=0.013$). Tukey's pairwise comparisons showed that growth rates for glutamate and aspartate were significantly greater than serine and there was no difference between the growth rates for serine, alanine and the combination.

The results shown in Figure 54 for the chlorinated reactor batch cultures indicate the following ranking of specific growth rates by amino acids using TDC data:

- Chlorinated: glutamate > serine > alanine $\cong$ aspartate $\cong$ combination

The mean growth rates were not statistically equal when compared by individual substrate ($p=0.001$) based on a one-way ANOVA. Tukey's pairwise comparisons showed that glutamate was significantly greater than aspartate, alanine and the combination. There was no significant difference between aspartate, alanine and the combination. Fisher's test showed the mean growth rate for glutamate was greater than all other substrates.

A balanced ANOVA was performed to evaluate the differences in the combined data between the control and chlorinated reactors. A comparison of growth rates between reactors without consideration of the substrate groups indicates the mean growth rates were statistically equal ($p=0.093$) even though the mean growth rate for the control reactor was greater than for the chlorinated reactor.

A comparison of growth rates by reactor for the combination substrate batch culture showed the growth rate for the control reactor was significantly greater than in the chlorinated reactor ($p=0.053$). Boxplots for the amino acids combination growth rate

data are shown in Figure 55. Later in this chapter, the combination batch culture kinetic parameters will be compared to growth rates using the reactor mass balance technique.

Growth Rates for Amino Acids Based on HPCs. Reliable heterotrophic plate count data was available for only the second amino acids batch culture experiment. Only 2 measurements ($n=2$) of the specific growth rate were available for each substrate group, greatly reducing the power of the statistical analyses. The results shown in Figure 56 for

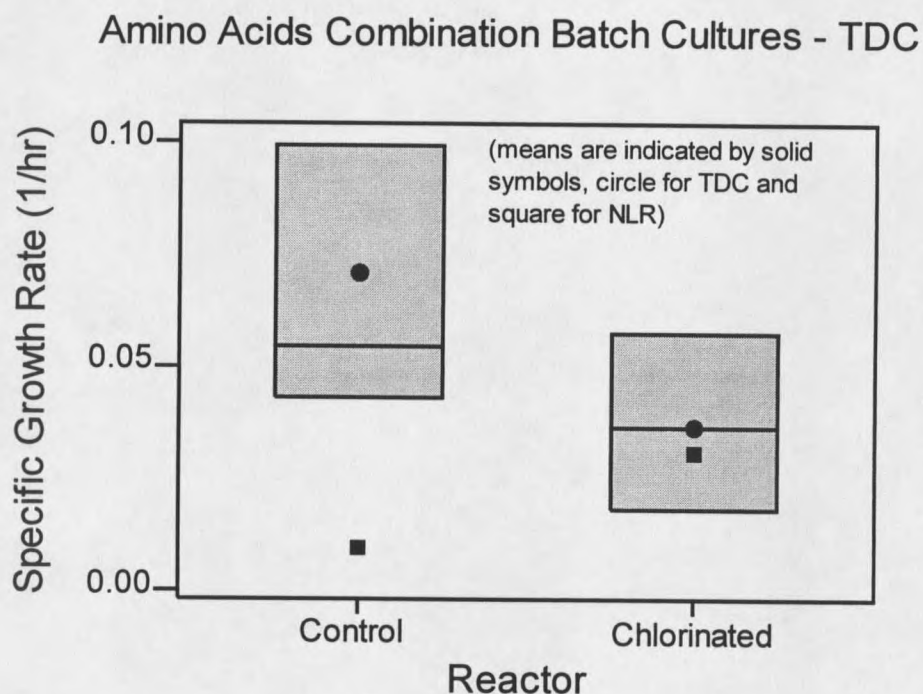
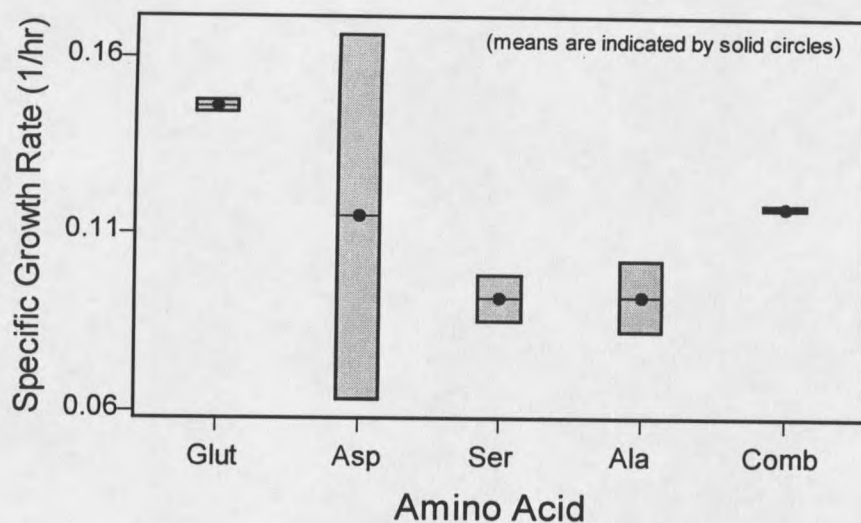


Figure 55. Boxplots of specific growth rate data for the combination substrates based on total direct cell counts (TDC) for batch cultures of biofilm cells from the amino acids control and chlorinated reactors. NLR = mean based on nonlinear regression of integrated Monod equation.

Amino Acids Control Reactor Batch Cultures - HPCs



Amino Acids Chlorinated Reactor Batch Cultures - HPCs

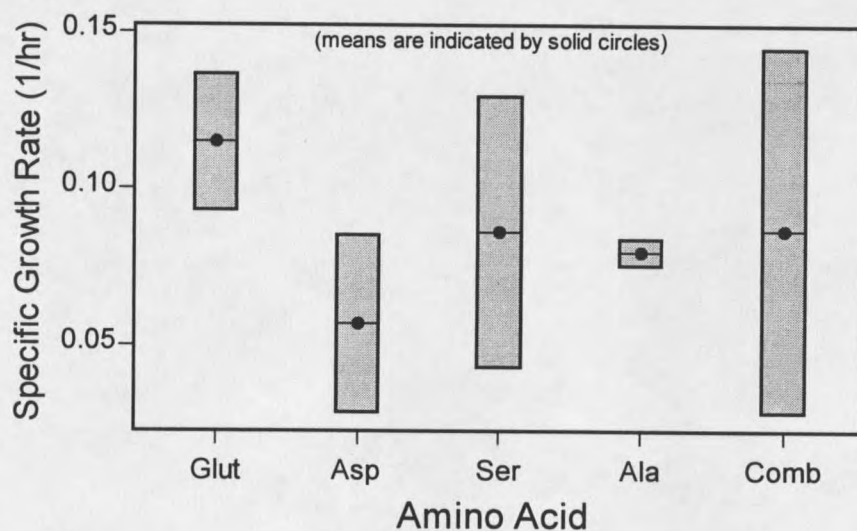


Figure 56. Boxplots of specific growth rate data by amino acid based on heterotrophic plate counts (HPC) for batch cultures of biofilm cells from the amino acids control (top plot) and chlorinated (bottom plot) reactors. Boxes represent the upper and lower range of the data, solid horizontal line within the box is the median, and the solid circle is the mean. $n=2$ for each substrate.

the control reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using HPC data:

- Control: glutamate > aspartate $\cong$ combination > alanine $\cong$ serine

The mean growth rates were statistically equal when compared by amino acid for the control reactor batch cultures ($p=0.534$) based on a one-way ANOVA ($n=2$ for each amino acid).

The results shown in Figure 56 for the chlorinated reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using HPC data:

- Chlorinated: glutamate > serine > alanine $\cong$ combination $\cong$ aspartate

A one-way ANOVA of the data for the chlorinated reactor indicated the mean growth rates were statistically equal when compared by amino acid ($p=0.849$, $n=2$).

A one-way ANOVA was used to compare growth rates between the two reactors using combined HPC data. The mean growth rates were statistically equal when compared by reactor type only ($p=0.122$). However, the mean growth rate for the chlorinated reactor was greater than for the control reactor.

A comparison of growth rates between reactors for the combination substrate batch cultures showed the mean growth rates were statistically equal ($p=0.69$). Boxplots for the amino acids HPC combination batch culture data are shown in Figure 57. Means and statistics for the amino acids first-order specific growth rates in batch cultures are listed in Table 15.

Carbohydrates. The substrates utilized for the carbohydrate experiments were the same four individual carbohydrates used in the reactor feed (galacturonic acid,

Amino Acids Combination Batch Cultures - HPCs

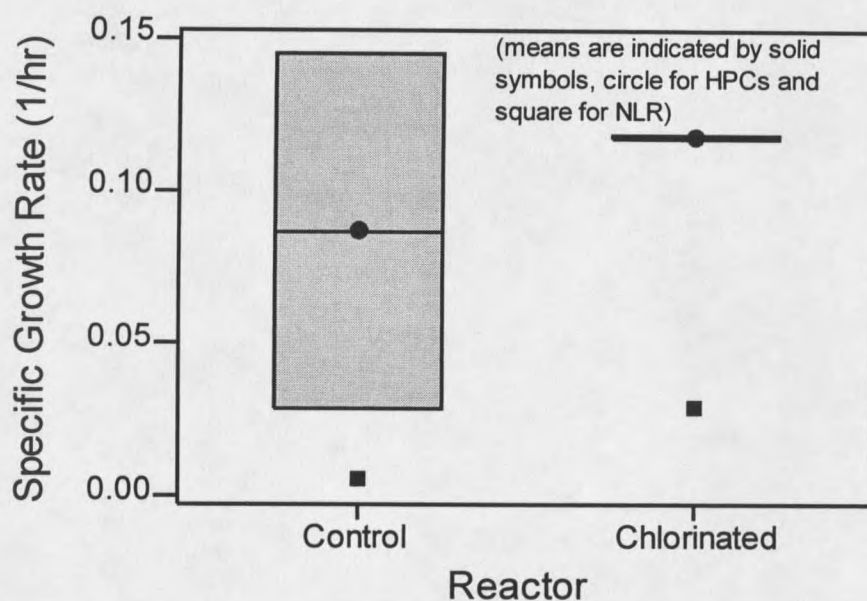


Figure 57. Boxplots of specific growth rate data for the combination substrates based on heterotrophic plate counts (HPC) for batch cultures of biofilm cells from the amino acids control and chlorinated reactors. Boxes represent the upper and lower range of the data, solid horizontal line within the box is the median, solid circle is the mean for HPC and solid square is mean for NLR. $n=2$ for each substrate. NLR = mean based on nonlinear regression of integrated Monod equation.

galactose, arabinose and glucose) plus a combination of the four (combination). All initial concentrations were approximately 2000 $\mu\text{g/L}$ as carbon.

Growth Rates for Carbohydrates Based on TDC. Sufficient determinations of the specific growth rate were made to allow analysis of seven values for each substrate within a reactor group (control or chlorinated) except for galacturonic acid which had six

Substrate Group	Reactor	Method	Substrate	n	Mean	StDev	SEM	95%CI	
								Lower	Upper
Amino Acids	Control	TDC	Glutamate	5	0.103	0.0449	0.0201	0.0320	0.172
			Aspartate	5	0.0921	0.0282	0.0126	0.0480	0.136
			Serine	5	0.0336	0.0280	0.0125	-0.0102	0.0774
			Alanine	5	0.0544	0.0187	0.0084	0.0252	0.0836
			Combination	5	0.0697	0.0268	0.0120	0.0278	0.112
Amino Acids	Chlorinated	TDC	Glutamate	5	0.0963	0.0207	0.0092	0.0639	0.129
			Aspartate	5	0.0418	0.0228	0.0102	0.0062	0.0774
			Serine	5	0.0664	0.0318	0.0142	0.0168	0.116
			Alanine	5	0.0400	0.0110	0.0049	0.0228	0.0572
			Combination	5	0.0364	0.0153	0.0069	0.0125	0.0604
Amino Acids	Control	HPC	Glutamate	2	0.146	0.0023	0.0016	—	—
			Aspartate	2	0.115	0.0733	0.0518	—	—
			Serine	2	0.0918	0.0094	0.0067	—	—
			Alanine	2	0.0923	0.0140	0.0099	—	—
			Combination	2	0.118	0.0008	0.0005	—	—
Amino Acids	Chlorinated	HPC	Glutamate	2	0.115	0.0307	0.0217	—	—
			Aspartate	2	0.0569	0.0400	0.0283	—	—
			Serine	2	0.0862	0.0612	0.0433	—	—
			Alanine	2	0.0797	0.0061	0.0043	—	—
			Combination	2	0.0865	0.0824	0.0583	—	—

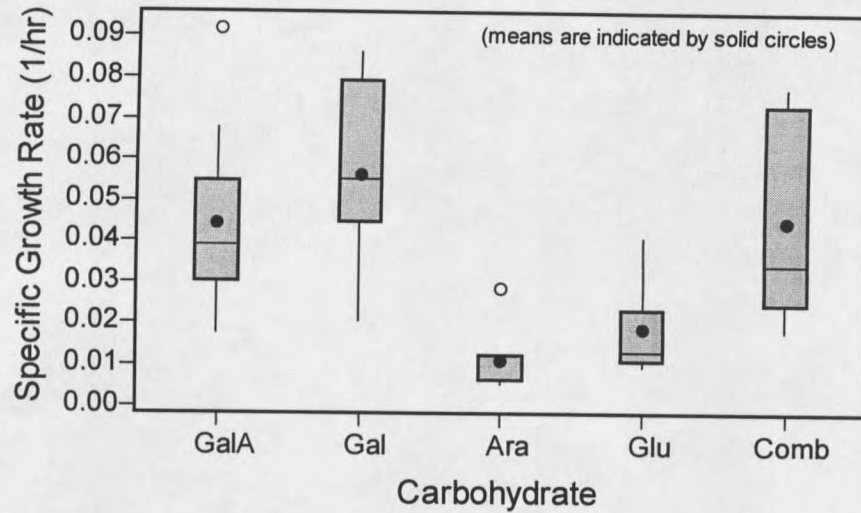
Table 15. First-order specific growth rates for amino acids batch cultures. TDC is the total direct count method and HPC is the heterotrophic plate count method.

values. The results shown in Figure 58 for the control reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using TDC data:

- Control: galactose > galacturonic acid $\cong$ combination > glucose > arabinose

A one-way ANOVA for the control data indicated the mean growth rates were statistically not equal when compared between individual substrates ($p < 0.000$). Tukey's pairwise comparisons ($\alpha = 0.05$) showed that the growth rate for arabinose was statistically less than galacturonic acid, galactose and the combination, but there was no

Carbohydrates Control Reactor Batch Cultures - TDC



Carbohydrates Chlorinated Reactor Batch Cultures - TDC

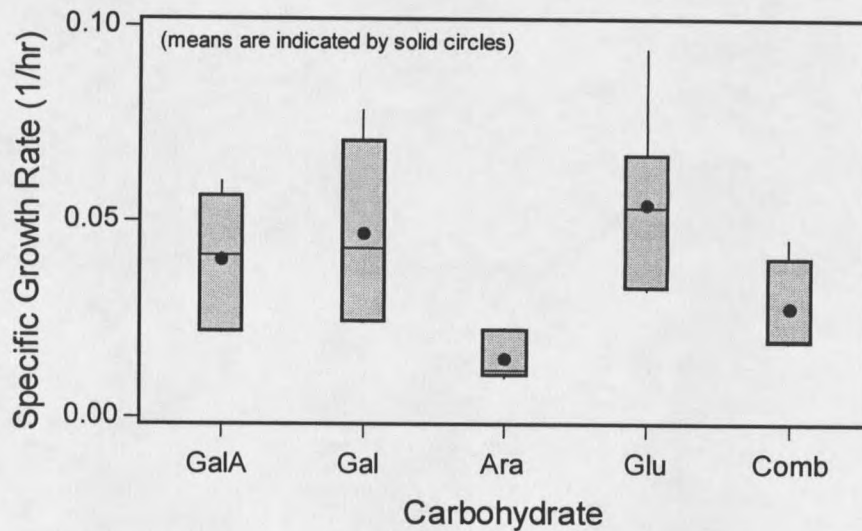


Figure 58. Boxplots of specific growth rate data by carbohydrate and the combination based on total direct cell counts (TDC) for batch cultures of biofilm cells from the carbohydrates control (top plot) and chlorinated (bottom plot) reactors. Open circles represent possible outliers. $n=6$ for galacturonic acid, $n=7$ for other substrates. GalA = galacturonic acid; Gal = galactose; Ara = arabinose; Glu = glucose; and Comb = combination.

statistically significant difference between the data for arabinose and glucose. Galactose as a substrate resulted in the highest mean growth rate for the control group (0.0562 hr^{-1})

The results shown in Figure 58 for the chlorinated reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using TDC data:

- Chlorinated: glucose > galactose > galacturonic acid > combination > arabinose

The mean growth rates were statistically not equal when compared by individual substrate for the chlorinated reactor batch cultures ($p=0.009$) based on a one-way ANOVA. Tukey's pairwise comparisons showed galactose was significantly greater than arabinose, and glucose was significantly greater than arabinose. There was no significant difference between galacturonic acid, galactose, glucose and the combination. Glucose as a substrate resulted in the highest mean growth rate for the chlorinated group (0.0542 hr^{-1}).

An ANOVA using the GLM was performed to evaluate the differences in the data between reactors. A comparison of growth rates between the control and chlorinated reactors without consideration of the substrate groups indicates the means were statistically equal ($p=0.640$). When growth rates were compared between reactors within substrate groups the results indicated the means were not equal ($p=0.018$). The most notable difference between reactors was the growth rate for glucose, which was greater in the chlorinated reactor batch culture compared to the control.

A comparison of growth rates between reactors for the combination substrate batch culture showed the rate for the control reactor was greater than in the chlorinated reactor, but a 2-sample t-test of the data indicates the difference was not significant

($p=0.14$). Boxplots for the combination data are shown in Figure 59. It is interesting to note that when specific growth rates were determined for the biofilm using leucine uptake the trend was the same shown in Figure 59; but the trend was opposite for growth rates determined using reactor mass balances, i.e., the chlorinated data mean was greater than the control data mean.

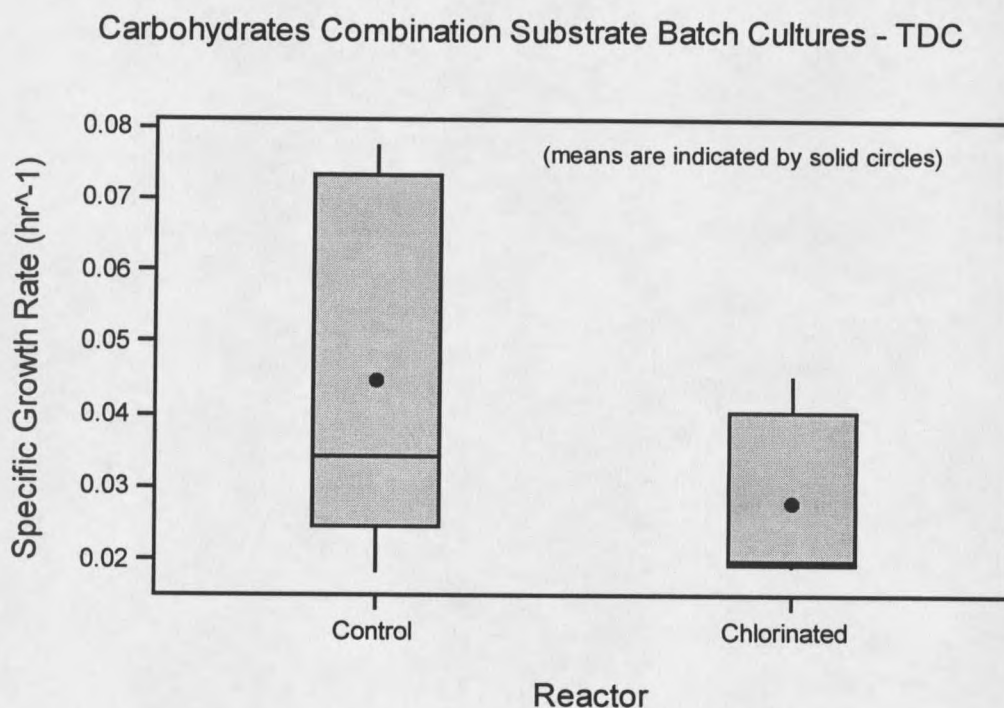


Figure 59. Boxplots of specific growth rate data for the combination substrates based on total direct cell counts (TDC) for batch cultures of biofilm cells from the carbohydrates control and chlorinated reactors. $n=7$ for each reactor.

Growth Rates for Carbohydrates Based on HPCs. Reliable heterotrophic plate count data were available for only the first carbohydrate batch culture experiment. Therefore, statistical comparisons have little power and conclusions are difficult to make.

With this caveat in mind, the mean growth rates were not statistically equal when compared by carbohydrate for the control reactor batch cultures ($p=0.006$) based on a one-way ANOVA ($n=2$ for each carbohydrate). The results shown in Figure 60 for the control reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using HPC data:

- Control: galactose > arabinose > glucose > combination > galacturonic acid

Based on Tukey's pairwise comparisons, the growth rate for galactose was significantly greater than for the other four substrates and there was no significant difference between galacturonic acid, arabinose, glucose and the combination. The growth rate for galacturonic acid was the lowest of the five substrates (0.0129 hr^{-1}) and galactose resulted in the highest growth rate (0.0260 hr^{-1}).

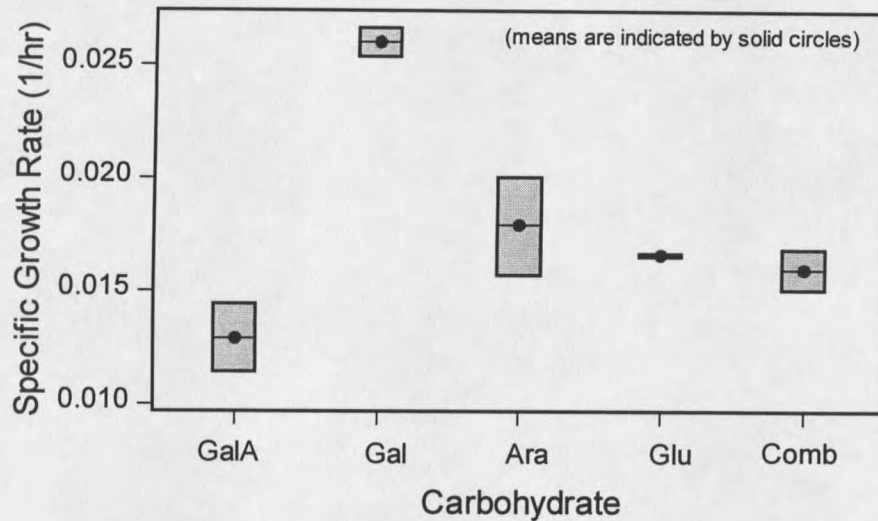
The results shown in Figure 60 for the chlorinated reactor batch cultures indicate the following ranking of specific growth rates by individual substrate using HPC data:

- Chlorinated: combination > galacturonic acid > arabinose $\cong$ galactose > glucose

A one-way ANOVA of the chlorinated reactor growth rate data indicated the means were statistically equal between carbohydrates ($p=0.345$, $n=2$ for each carbohydrate). A one-way ANOVA was used to compare growth rates between the two reactors using HPC data. Mean growth rates were not statistically equal when compared between reactors with no consideration to carbohydrate ($p<0.0005$). The mean growth rate for the chlorinated reactor (0.137 hr^{-1}) was greater than for the control reactor (0.0159 hr^{-1}).

A comparison of growth rates between reactors for the combination substrate batch culture, Figure 61, showed the data for the chlorinated reactor was greater than

Carbohydrates Control Reactor Batch Cultures - HPCs



Carbohydrates Chlorinated Batch Cultures - HPCs

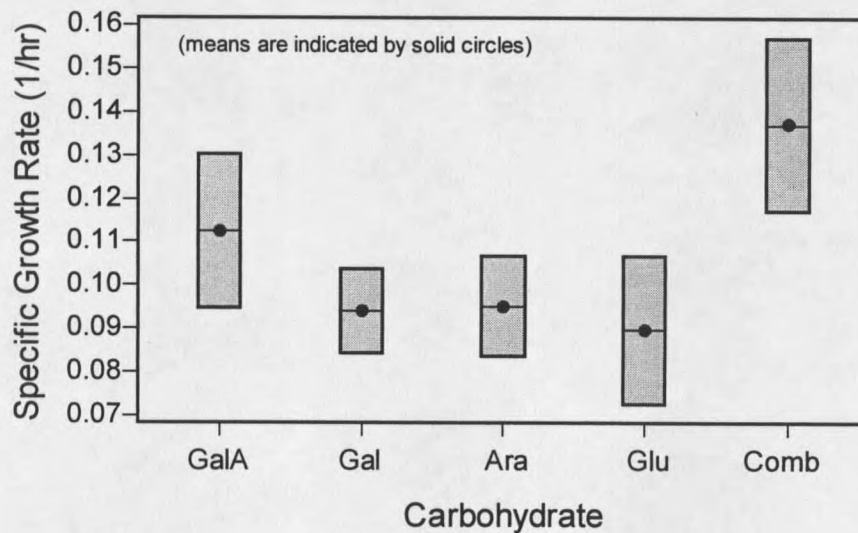


Figure 60. Boxplots of specific growth rate data for the five carbohydrates based on heterotrophic plate counts (HPC) for batch cultures of biofilm cells from the carbohydrates control (top plot) and chlorinated (bottom plot) reactors. Boxes represent the upper and lower range of the data, solid horizontal line within the box is the median, and the solid circle is the mean. $n=2$ for each substrate.

Carbohydrates Combination Batch Cultures - HPCs

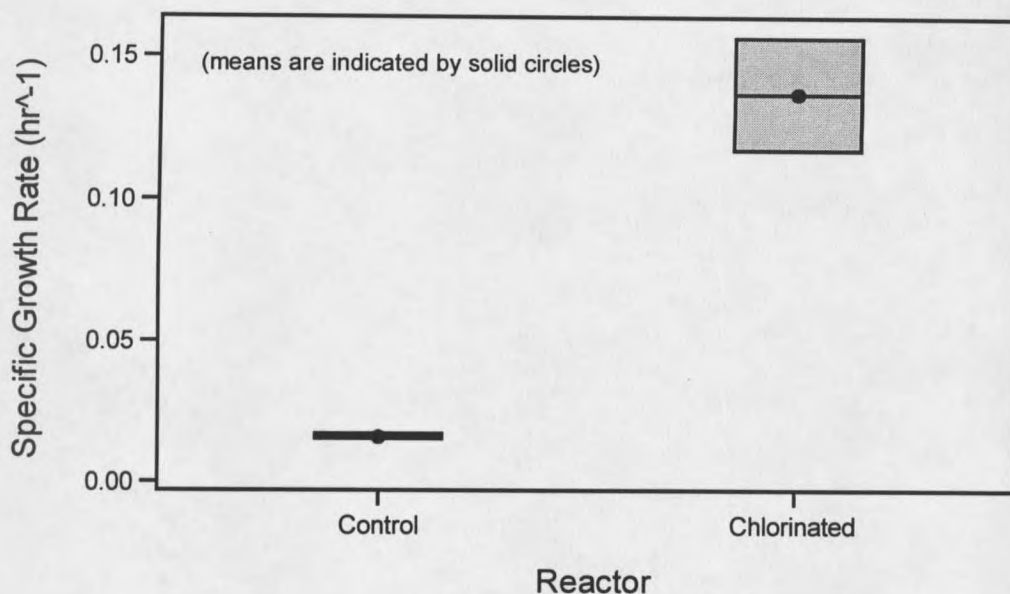


Figure 61. Boxplots of specific growth rate data for the combination substrates based on heterotrophic plate counts (HPC) for batch cultures of biofilm cells from the carbohydrates control and chlorinated reactors. Boxes represent the upper and lower range of the data, solid horizontal line within the box is the median, and the solid circle is the mean. $n=2$ for each substrate.

for the control. Means and statistics for first-order specific growth rates from the carbohydrates batch cultures are listed in Table 16.

Humic Substances. The humic substances (humics) batch cultures utilized only one substrate, the Elliot Silt Loam Soil humic material. All batch cultures for the first humics experiment had an initial humics concentration of 2000 $\mu\text{g/L}$, as carbon, based on NPOC measurements. Batch cultures for the second humics experiment had initial target substrate concentrations of 100, 500, 1000 and 2000 $\mu\text{g C/L}$. Actual initial humics

Substrate Group	Reactor	Method	Substrate	n	Mean	StDev	SEM	95%CI	
								Lower	Upper
Carbohydrates	Control	TDC	Galacturonic Acid	6	0.0436	0.0253	0.0103	0.0109	0.0762
			Galactose	7	0.0562	0.0220	0.00831	0.0316	0.0809
			Arabinose	7	0.0110	0.00825	0.00312	0.00173	0.0203
			Glucose	7	0.0187	0.0110	0.00417	0.00632	0.0311
			Combination	7	0.0447	0.0236	0.00892	0.0182	0.0712
Carbohydrates	Chlorinated	TDC	Galacturonic Acid	6	0.0403	0.0176	0.00716	0.0176	0.0629
			Galactose	6	0.0469	0.0251	0.0102	0.0146	0.0793
			Arabinose	6	0.0149	0.00588	0.00240	0.00728	0.0225
			Glucose	6	0.0542	0.0226	0.00921	0.02505	0.0833
			Combination	5	0.0278	0.0120	0.00537	0.00905	0.0466
Carbohydrates	Control	HPC	Galacturonic Acid	2	0.0129	0.00211	0.00149	—	—
			Galactose	2	0.0260	0.00090	0.00064	—	—
			Arabinose	2	0.0179	0.00312	0.00221	—	—
			Glucose	2	0.0165	0.00009	0.00006	—	—
			Combination	2	0.0159	0.00129	0.00091	—	—
Carbohydrates	Chlorinated	HPC	Galacturonic Acid	2	0.113	0.0250	0.0177	—	—
			Galactose	2	0.0941	0.0139	0.00980	—	—
			Arabinose	2	0.0952	0.0163	0.0115	—	—
			Glucose	2	0.0897	0.0241	0.0171	—	—
			Combination	2	0.137	0.0281	0.0200	—	—

Table 16. First-order specific growth rates for carbohydrates batch cultures. TDC is the total direct count method and HPC is the heterotrophic plate count method.

concentrations in batch cultures were greater than the target values because biofilm scraped from the sample slides contained attached humics and other carbon-containing substances, such as extracellular polymeric substances (EPS), that increased the initial concentrations. Over an incubation period of 10 days, the batch cultures were monitored for total direct counts (TDC), heterotrophic plate counts (HPC), and both filtered and

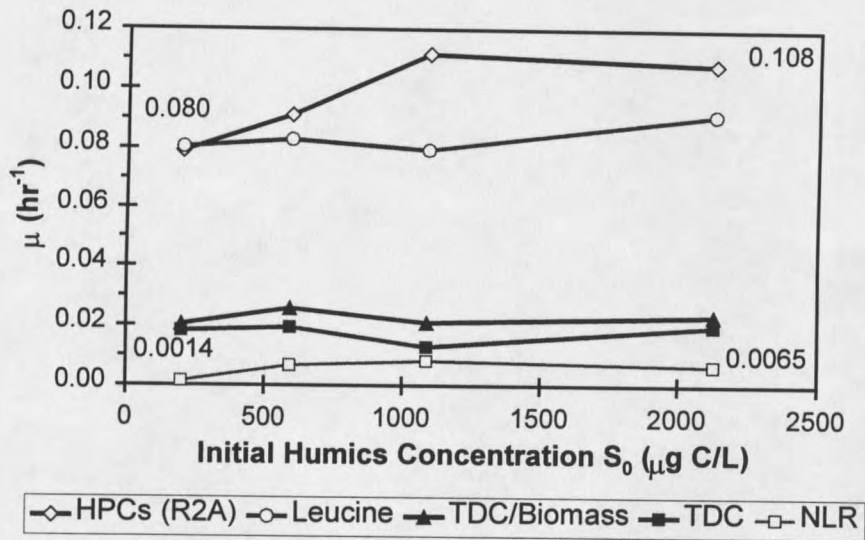
unfiltered NPOC. For the second humics experiment, cell size was determined for each batch culture at each sample time. Cell size was converted to cell volume and biomass. By multiplying the mean biomass per cell value by the TDC, an estimate of total biomass was made for each sample time and a growth rate was estimated based upon the change in biomass, noted as TDC/Biomass method. At each batch culture sampling time for the second humics experiment, samples were analyzed for tritiated leucine uptake to provide an additional measurement of the growth rate. Details of the experiment and analysis techniques are described in Chapter 3, MATERIALS AND METHODS.

Specific Growth Rates. Specific first-order growth rates were determined using data from four measurement techniques:

- Total direct counts (TDC)
- Heterotrophic plate counts (HPC)
- Leucine uptake
- Biomass based on total direct counts, cell volume measurements and estimates of the cell carbon per unit volume (TDC/Biomass)

The specific growth rates for the control and chlorinated reactor biofilm cell batch cultures are presented in Figure 62, where growth rate was plotted as a function of the initial humics concentration. Growth rates were different based on the measurement technique utilized. Growth rates calculated from HPC and leucine data were higher than when TDC and TDC/Biomass were used. Within a measurement method there was little change in specific growth rate for both reactors over the range of initial humics

Humics(2) Control Reactor Batch Cultures



Humics(2) Chlorinated Reactor Batch Cultures

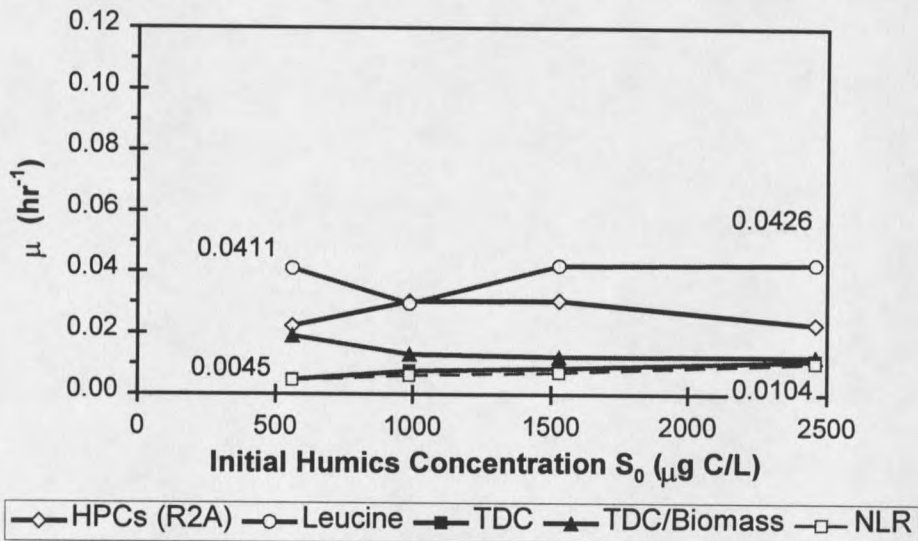


Figure 62. First-order specific growth rates (μ) versus initial humic substances concentration (S_0) in the humics control (top) and chlorinated (bottom) reactor batch cultures. NLR = nonlinear regression; TDC = total direct cell counts; and HPC = heterotrophic plate counts.

concentrations used for the experiment. This relationship yields a straight line with no significant slope, and can be described as zero-order kinetics with respect to initial batch culture substrate concentration, i.e., the growth rate was independent of the initial substrate concentration.

Growth rates determined utilizing leucine uptake or HPC consistently gave higher values than techniques utilizing TDC. Assuming growth rates by measurement technique to be a factor, or group, a one-way ANOVA was performed to verify differences. The ANOVA indicated the mean growth rates were statistically not equal when compared by measurement technique ($p < 0.000$) and growth rates determined using leucine uptake and HPC were significantly greater than rates determined using TDC or TDC/Biomass. There was no significant difference between growth rates determined using leucine uptake and HPC, and no significant difference between growth rates using TDC and TDC/biomass (Tukey's pairwise comparisons).

Using a similar grouping as was assumed for the one-way ANOVA, a balanced ANOVA was performed with the factors being reactor, type of growth rate measurement technique and initial humics concentration grouped within reactors. Mean growth rates were statistically not equal when compared between reactors and measurement type ($p < 0.0005$). Mean growth rates were statistically equal when compared by initial humics concentration for a reactor group ($p = 0.986$), indicating there was no significant variation in growth rate over the range of initial substrate concentrations evaluated. The ANOVAs verified there were significant differences in growth rates between the two reactors and measurement techniques. Mean growth rates by all methods for the control reactor were

greater than for the chlorinated reactor. Means and statistics for the specific growth rates in humics batch cultures are listed in Table 17.

Batch Culture Growth Rates–Nonlinear Regression

The Monod parameters $\mu_{\max}$ and K_S were estimated for the amino acids combination and humics replicate 2 batch cultures. The estimated kinetic parameters were then used to estimate specific growth rate at a substrate concentration of 2000 $\mu\text{g C/L}$ and the results compared to growth rates based on reactor mass balances. The results are presented below by substrate group.

Reactor	Method	Culture	Mean	95%CI		StDev	SEM	n
				Lower	Upper			
1	HPC	All	0.0975	0.0732	0.1220	0.0153	0.00763	4
1	Leucine	All	0.0835	0.0749	0.0921	0.00540	0.00270	4
1	TDC/Biomass	All	0.0225	0.0187	0.0264	0.00244	0.00122	4
1	TDC	All	0.0175	0.0124	0.0225	0.00318	0.00159	4
2	HPC	All	0.0266	0.0194	0.0338	0.00452	0.00226	4
2	Leucine	All	0.0388	0.0290	0.0487	0.00618	0.00309	4
2	TDC/Biomass	All	0.0142	0.00909	0.0193	0.00322	0.00161	4
2	TDC	All	0.0081	0.00384	0.0123	0.00265	0.00132	4
1	TDC	2000	0.0137			0.00853	0.00604	2
1	HPC	2000	0.0778			0.0431	0.0305	2
2	TDC	2000	0.0152			0.00565	0.00399	2
2	HPC	2000	0.0969			0.105	0.0739	2

Table 17. First-order specific growth rates for humics batch cultures. All cultures refers to the mean of the 100, 500, 1000 and 2000 $\mu\text{g C/L}$ batch cultures. TDC is the total direct count method, HPC is the heterotrophic plate count method, Leucine is the tritiated leucine uptake method, and TDC/Biomass is the biomass method using TDC and cell carbon/volume.

Amino Acids. Total amino acid uptake over time was analyzed for the combination batch cultures from amino acids replicate 1 and 2 experiments. Estimated

parameters are presented in Table 18 based on nonlinear regression (NLR) of the integrated Monod equation. Using the estimated parameters, specific growth rates were

Replicate	Reactor	X_0 ($\mu\text{g C/L}$)	$Y_{X/S}$	$\mu_{\max}$ (hr^{-1})	K_S ($\mu\text{g C/L}$)	$S_0^{(1)}$ ($\mu\text{g C/L}$)	Adj. R^2
1	Control	416	0.80	0.0540	757.0	1999	0.956
2	Control	9.60	0.25	0.303	2895	2031	0.916
1	Cl_2	327	0.37	0.0267	202.8	2035	0.956
2(1)	Cl_2	7.58	0.37	0.109	218.3	1653	0.959
2(2)	Cl_2	2.15	0.21	0.163	589.1	2341	0.997

⁽¹⁾ S_0 included as regression parameter.

Table 18. Estimated kinetic parameters for amino acids combination batch cultures using nonlinear regression of the integrated Monod equation.

computed using the estimated effluent substrate concentrations of 83 and 147 $\mu\text{g C/L}$ for the control and chlorinated amino acids reactors, respectively. Calculated growth rates based on regression parameters and growth rates based on TDC and mass balance around the reactors are compared in Figure 63. Error bars for the batch culture values shown in Figure 63 were estimated by the following method. Based on a bootstrap analysis of error in the humics batch culture growth rates, the standard deviation averaged 54 % of the mean value for 100 analyses of simulated data sets assuming Gaussian errors. Recognizing error exists in the estimated growth rates using parameters from the nonlinear regression, the standard deviation for the batch culture estimates shown in Table 18 are based on 50% of the estimated value. While this error is only a guess, it is probably not unreasonable given that the analysis of humics batch culture data also relied on nonlinear regression techniques.

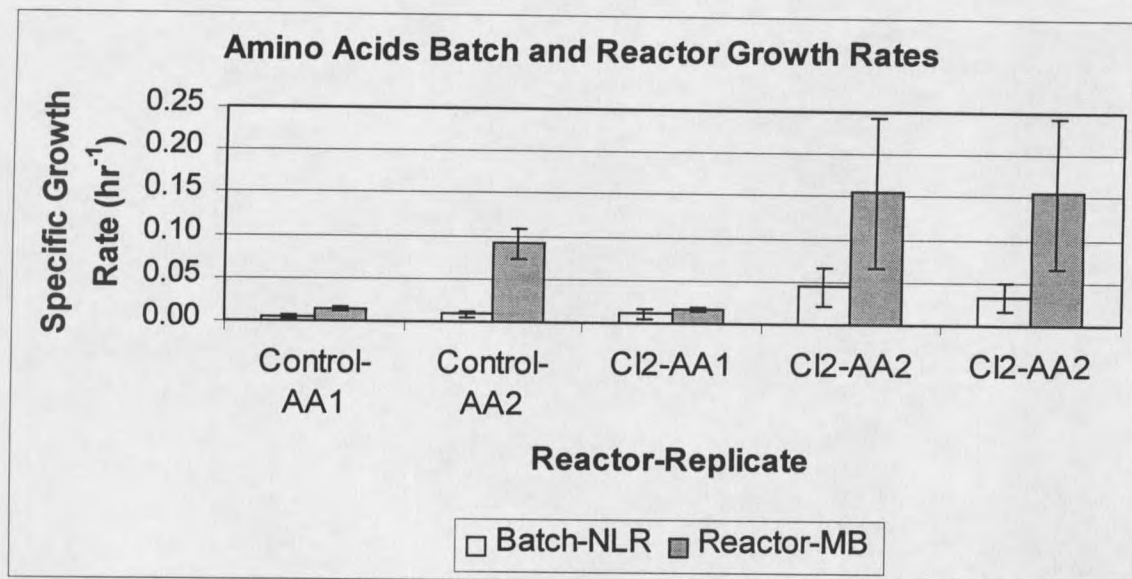


Figure 63. Comparison of calculated growth rates for amino acids combined batch cultures based on NLR to growth rates for reactors using mass balance (MB). Error bars indicate $\pm$ one standard deviation (see text). Two batch cultures were available for the chlorinated amino acids 2 replicate experiment. AA1=amino acids replicate 1; AA2=amino acids replicate 2; and Cl2=chlorinated.

The results presented in Figure 63 indicate that specific growth rates determined using nonlinear regression of the batch culture substrate depletion data gave growth rates that were less than those from the reactor mass balance approach. For example, the NLR-based growth rate for the control amino acids replicate 2 was 0.0085 hr^{-1} and the reactor mass balance growth rate was 0.091 hr^{-1} . Mean values for growth rates based on NLR were shown in Figures 55 and 57 where the means could be compared to results based upon TDC and HPC for the amino acids combination batch cultures. The NLR-based growth rates were much less than TDC and HPC methods for all but the chlorinated TDC method (Figure 55). Growth rates calculated using parameters based on the NLR method

were lower than growth rates determined using the reactor mass balance approach and suspended biofilm cell batch culture approach.

Humic Substances. Humic substance uptake over time (measured as filtered NPOC) was analyzed for each of four batch cultures (for each reactor) for biofilm cells scraped from surfaces in the humics replicate 2 experiment. Estimated parameters are presented in Table 19 based on nonlinear regression of the integrated Monod equation. Since the effluent substrate concentration from the control and chlorinated humics reactors was approximately 870 and 1130 $\mu\text{g C/L}$, respectively, these concentrations were used to calculate NLR-based growth rates for each batch culture.

Reactor	Culture	X_0 ($\mu\text{g C/L}$)	$Y_{X/S}$	$\mu_{\max}$ (hr^{-1})	K_S ($\mu\text{g C/L}$)	$S_0^{(1)}$ ($\mu\text{g C/L}$)	Adj. R^2
Control	100	135	0.370	0.0708	9981	209.7	0.566
Control	500	92.4	0.454	0.0732	6000	595.1	0.760
Control	1000	102	0.425	0.0529	5986	1106	0.719
Control	2000	95.6	0.199	0.0372	10000	1993	0.833
Cl_2	100	43.0	0.199	0.00534	100.0	605.4	0.713
Cl_2	500	41.8	0.207	0.0375	4998	1017	0.723
Cl_2	1000	32.1	0.205	0.00970	538.1	1610	0.768
Cl_2	2000	20.4	0.232	0.0316	5000	2506	0.842

⁽¹⁾ S_0 included as regression parameter.

Table 19. Estimated kinetic parameters for humics replicate 2 batch cultures using nonlinear regression of the integrated Monod equation.

Calculated growth rates based on regression parameters and growth rates based on TDC and mass balance around the reactors are compared in Figure 64. Mean growth rates based on reactor mass balance are presented for the humics replicate 2 experiment and for the humics replicate 1 and 2 experiments combined. A comparison of specific growth rates determined using NLR to the humics replicate 2 data indicates the

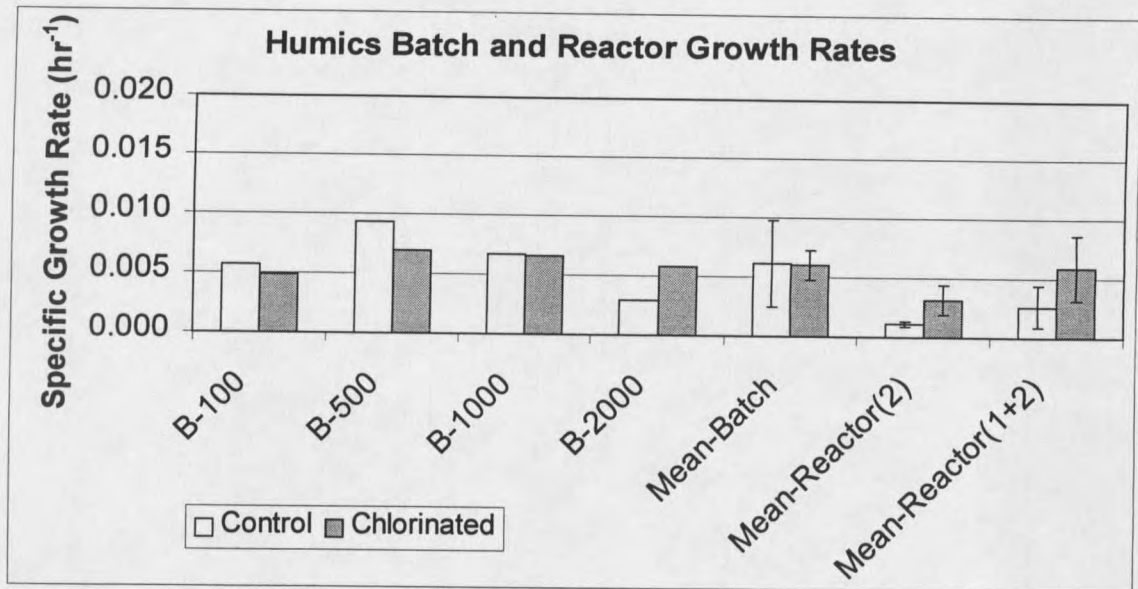


Figure 64. Comparison of calculated growth rates for humics replicate 2 batch cultures to growth rates for reactors using mass balance. Error bars indicate 95% confidence intervals for the mean. Reactor data shown for replicate 2 only [Mean-Reactor(2)] and for replicates 1 and 2 combined (1+2). B-100 = batch culture 100, typical.

NLR-based growth rates were greater than the reactor mass-balance-based growth rates (470% greater for the control and 90% greater for the chlorinated). When the mean NLR-based growth rates were compared to the reactor mass balance data for replicates 1 and 2 combined the NLR-based growth rates were 130% greater for the control and 3% greater for the chlorinated. Based on the 95% confidence intervals for the mean shown in Figure 64, the NLR-based growth rates were statistically equal to the reactor mass balance growth rates, replicates 1 and 2 combined.

Growth rates calculated using the nonlinear regression (NLR) parameters (Table 19) were shown in Figure 62 where the results can be compared to the other techniques used to calculate growth rates in the humics reactors. Results using the NLR technique compared

well to the TDC method for the chlorinated reactor. NLR based growth rates were lower than any other technique for the control reactor.

Batch Culture Substrate Uptake Rates

The purpose of determining substrate uptake rates in the batch cultures was to ascertain if there were differences in the way that the biofilm population in the reactor responded to specific substrates. Substrate uptake rates determined from batch culture were compared to those determined using other techniques to see if the batch culture technique, using biofilm cells, was an appropriate technique to determine kinetic parameters for drinking water biofilm. The following paragraphs describe the results of the work for the amino acids and humics experiments. Substrate concentration data was not available for the carbohydrates batch culture experiment.

Amino Acids. The substrates utilized for the amino acids experiment were the same four individual amino acids used in the reactor feed (glutamate, aspartate, serine and alanine) plus a combination of the four (combination). All initial concentrations were approximately 2000 $\mu\text{g/L}$ as carbon. Two values for the specific uptake rate were available for each amino acid and the combination, one value from each of the first two amino acids replicate experiments. Additionally, two uptake values were determined for each specific amino acid in the combination culture. Figure 65 presents the specific uptake data for the amino acids control and chlorinated reactor batch cultures.

Although statistical comparison of the data has little power due to the small sample sizes, comparison were made using ANOVA. Mean substrate uptake rates were

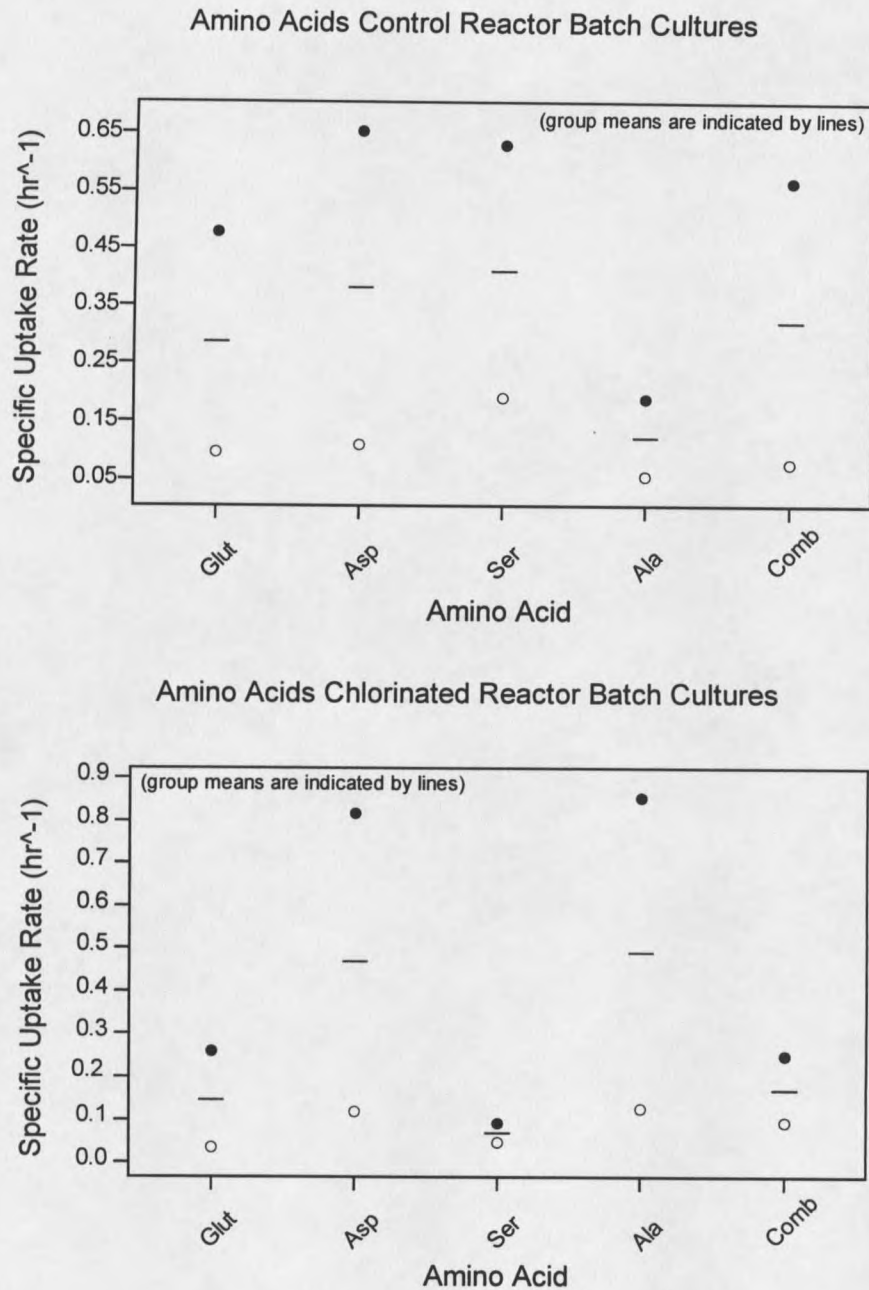


Figure 65. Specific amino acid uptake rates for the control (top) and chlorinated (bottom) reactor biofilm batch cultures. Solid circles are data from replicate 2 and open circles are data from replicate 1. Solid lines represent the mean. Glut = glutamate; Asp = aspartate; Ser = serine; Ala = alanine; and Comb = combination.

not statistically equal when compared by amino acid between replicates 1 and 2 (GLM-ANOVA, $p < 0.0005$). However, the trends in the data were consistent between replicates, allowing conclusions to be drawn regarding differences in uptake rates between amino acids. Aspartate and serine were taken up at the highest rates for the control reactor batch cultures (0.380 and 0.409 hr^{-1} , respectively), and aspartate and alanine had the highest uptake rates in the chlorinated reactor batch cultures (0.468 and 0.490 hr^{-1} , respectively). Alanine was taken up at the lowest rate in the control batch cultures (0.121 hr^{-1}), while serine was lowest in the chlorinated batch cultures (0.070 hr^{-1}). Glutamate and the combination were taken up at nearly the same rate within each set of reactor batch cultures. In summary, for the amino acids batch culture specific substrate uptake rates:

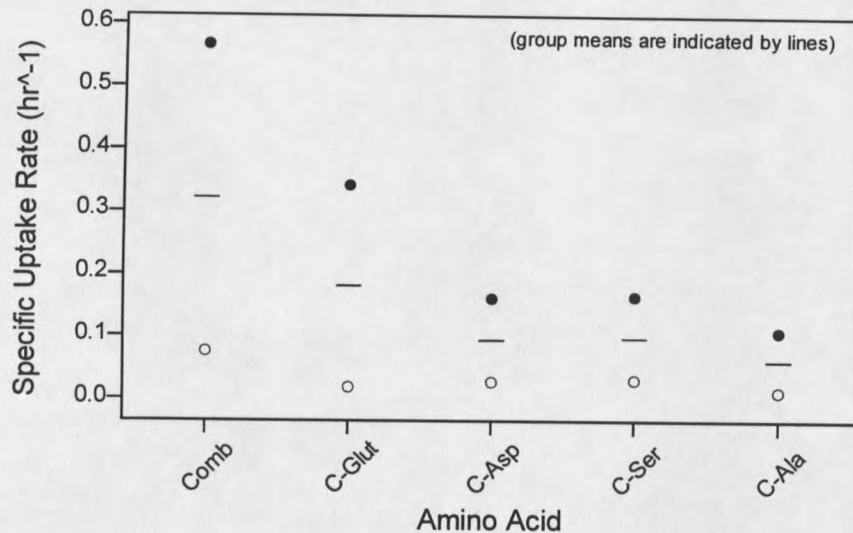
- Control: serine $\cong$ aspartate $>$ combination $\cong$ glutamate $>$ alanine
- Chlorinated: alanine $\cong$ aspartate $>$ combination $\cong$ glutamate $>$ serine

An ANOVA using the GLM model for the combined uptake data analyzed by reactor indicated the mean uptake rates were equal when compared by reactor ($p = 0.803$), which was confirmed by the nonparametric Kruskal-Wallis and Friedman's tests.

Specific uptake data for only the combination batch cultures (for later comparison to reactor data) are presented in Figure 66 for the control and chlorinated reactor. Uptake rates were calculated for each individual amino acid in the combination and for the total of the four amino acids (combination-all). The order of substrate uptake rates from highest to lowest for the amino acids combination batch culture was:

- Control: glutamate $>$ aspartate $\cong$ serine $>$ alanine
- Chlorinated: aspartate $>$ glutamate $>$ serine $>$ alanine

Amino Acids Control Reactor Combination Batch Cultures



Amino Acids Chlorinated Reactor Combination Batch Cultures

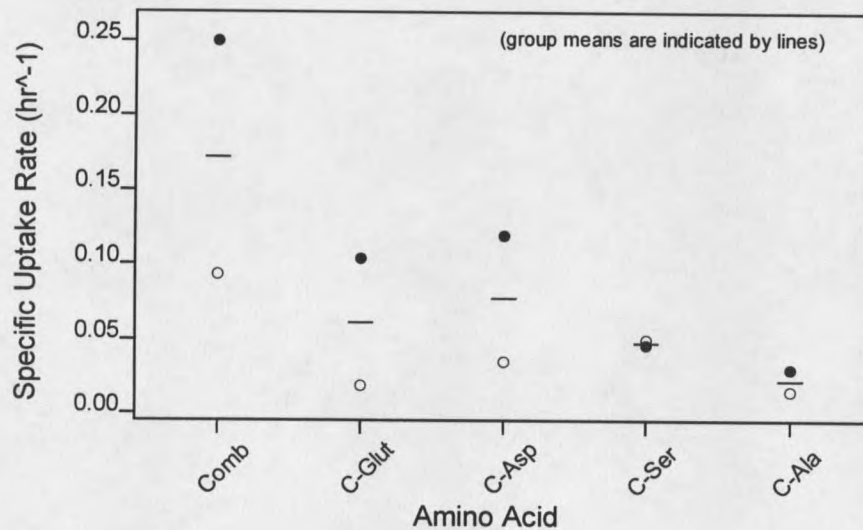


Figure 66. Specific amino acid uptake rate for the control (top) and chlorinated (bottom) reactor biofilm combination batch cultures. Solid circles are data from replicate 2 and open circles are data from replicate 1. Solid bars represent the mean. Data for the combination-all (Comb) represents the uptake rate based on the total of the individual substrates over time. C-glut = glutamate in the combination culture, typical.

The uptake rate for alanine was the lowest for the four amino acids in both the control and chlorinated combination batch cultures (0.060 and 0.022 hr⁻¹, respectively). Uptake rates for glutamate, aspartate and serine were greater than for alanine in both reactors, but variation in the uptake rates between the two experiments made it difficult to spot trends. Based on mean values for the two experiments, uptake rates for aspartate and serine were nearly the same in the control batch cultures, aspartate was the highest in the chlorinated combination batch culture (0.077 hr⁻¹) and glutamate was the highest in the control combination batch culture (0.180 hr⁻¹). Table 20 presents means and statistics for specific uptake rates from the amino acids batch cultures.

Reactor	Amino Acid	Mean Uptake Rate (hr ⁻¹)	StDev	SEM	n
Control	Glutamate	0.287	0.270	0.191	2
	Aspartate	0.380	0.384	0.272	2
	Serine	0.409	0.310	0.219	2
	Alanine	0.121	0.0941	0.0665	2
	Combination-All	0.321	0.346	0.245	2
	Comb.-Glut	0.180	0.227	0.161	2
	Comb.-Asp	0.094	0.0943	0.0667	2
	Comb.-Ser	0.097	0.0939	0.0664	2
	Comb.-Ala	0.060	0.0672	0.0475	2
Chlorinated	Glutamate	0.147	0.158	0.112	2
	Aspartate	0.468	0.493	0.349	2
	Serine	0.070	0.0304	0.0215	2
	Alanine	0.490	0.514	0.363	2
	Combination-All	0.172	0.111	0.0786	2
	Comb.-Glut	0.061	0.0601	0.0425	2
	Comb.-Asp	0.077	0.0593	0.0420	2
	Comb.-Ser	0.048	0.00262	0.0019	2
	Comb.-Ala	0.022	0.0102	0.0072	2

Table 20. Summary of mean specific uptake rates for batch cultures using scraped biofilm cells from the amino acid reactors.

Humics. Based on measurements of filtered NPOC, humic substance uptake rates were determined for the batch cultures in the second humics experiment. Data was not available for the first humics experiment due to equipment problems. Attempts to correlate humic substance concentration in the effluent using UV-absorbance were not successful; suspended cells in the sample may interfere with the absorbance measurement. Specific uptake rates were calculated as described above for the amino acids batch cultures.

The relationship between specific substrate uptake rate and initial humics concentration in the batch cultures followed hyperbolic kinetics according to the Michaelis-Menten equation:

$$v/X = \frac{V_{\max} \cdot S}{K_T + S}, \quad (36)$$

where v is the concentration-dependent uptake rate ($\mu\text{g C L}^{-1} \text{ hr}^{-1}$), X is the cell biomass ($\mu\text{g C/L}$), S is the initial substrate concentration outside the cell ($\mu\text{g C/L}$), $V_{\max}$ is the maximum rate of uptake (hr^{-1}) and K_T is the whole cell Michaelis constant for transport (Button, D. K., 1991). Dividing v by the mean value of X over the same time period as used to determine dS/dt gave the specific substrate utilization rate, v/X (v , hr^{-1}). Specific substrate uptake rates, curve fits assuming Michaelis-Menten uptake kinetics and specific affinity ($a^0 = V_{\max}/K_T$), a measure of how well cells can acquire substrates in low nutrient environments, are shown in Figure 67.

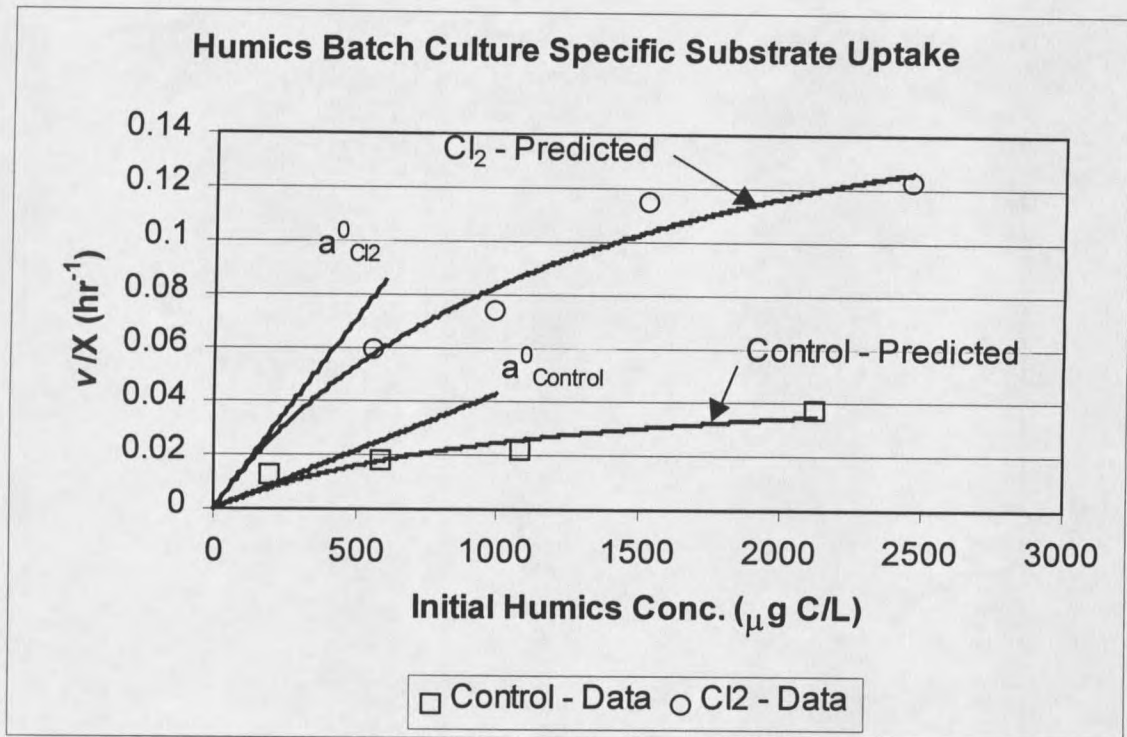


Figure 67. Specific substrate uptake versus initial humics concentration in the humics batch cultures. Predicted lines represent curve fit of a hyperbolic equation to the data. The slope of the lines tangent to the predicted curves at their origin ($a^0 = V_{max} / K_T$) are an indication of the oligotrophic characteristics of the cells in the culture. Cl₂ = chlorinated.

The chlorinated reactor biofilm cells exhibited a high specific affinity and high maximum specific uptake rate (V_{max}) compared to the control reactor, indicating biofilm cells from the chlorinated reactor had a greater ability to rapidly uptake humics with a greater maximum capacity than the control reactor biofilm cells. V_{max} for the control reactor (0.055 hr⁻¹) was less than for the chlorinated reactor (0.194 hr⁻¹). The greater specific affinity value for the chlorinated reactor biofilm cells indicates the cells were better adapted to the low nutrient environment, presumably due a substrate limitation

within the chlorinated reactor environment. Table 21 presents the specific uptake rate data shown graphically in Figure 67.

Reactor	Batch Culture	Initial Humic Conc. S_0 ($\mu\text{g C/L}$)	v/X (hr^{-1})	$V_{\max}$ (hr^{-1})	K_T ($\mu\text{g C/L}$)	Adj. R^2	p (regrsn)	a^0 ($\text{L } \mu\text{g-C}^{-1} \text{hr}^{-1}$)
Control	100	199.6	0.0120	0.0549	1240	0.632	0.0633	4.29E-05
	500	592.0	0.0179					
	1000	1089	0.0215					
	2000	2128	0.0367					
Chlorinated	100	559.9	0.0595	0.198	1324	0.773	0.0385	1.42E-04
	500	986.5	0.0738					
	1000	1529	0.114					
	2000	2458	0.122					

Table 21. Humics batch culture specific substrate uptake rates, maximum rate of uptake ($V_{\max}$), half-saturation constant (K_T), degrees-of-freedom adjusted R^2 for the curve fit of the hyperbolic Michaelis-Menten equation, p-value for the regression, and specific affinity (a^0).

Batch Culture Yield

Yield (mass of cell carbon produced per mass of substrate carbon utilized) was determined for each batch culture as described in Chapter 3, MATERIALS AND METHODS. The following paragraphs describe the results of the work for the amino acids and humics experiments. Yield based on HPC/Biomass was only available for the second amino acids batch culture experiment. Substrate concentration data was not available for the carbohydrate experiment; therefore yield could not be determined for carbohydrates batch cultures.

Amino Acids Yield. The substrates utilized for the amino acids experiment were the same four individual amino acids used in the reactor feed (glutamate, aspartate, serine

and alanine) plus a combination of the four (combination). All initial concentrations were approximately 2000 µg/L as carbon.

Yield data based on TDC/Biomass for biofilm cells in batch culture from the amino acids control reactor are presented in Figure 68. Yield values for the first experiment were greater than for the second experiment. However, trends between amino acids were the same for the two experiments, allowing a comparison using the mean values. Yield for the alanine and combination batch cultures (mean of 0.672) were greater than for the other three (glutamate, aspartate and serine – mean of 0.302). The following ranks yield from high to low for the amino acids batch culture data using the TDC/Biomass method of determining cell mass:

- Control: alanine > combination > glutamate > aspartate > serine

Yield data based on TDC/Biomass for the amino acids chlorinated reactor biofilm cell batch cultures are presented in Figure 68 (bottom). The following ranks yield from high to low for the amino acids chlorinated batch culture data using the TDC/Biomass method of determining cell mass:

- Chlorinated: alanine > combination > glutamate $\cong$ aspartate > (?) serine

There was little difference in mean values between amino acids, but the data by experiment was highly variable, particularly for serine. Review of the raw data indicated the high value for serine may have been due to insufficient incubation time (>72 hours may have been required) for complete uptake of serine. Assuming the value for serine was near the lower data point shown in Figure 68, the overall trend for the chlorinated reactor culture would be similar to the trend in the control reactor culture; alanine had

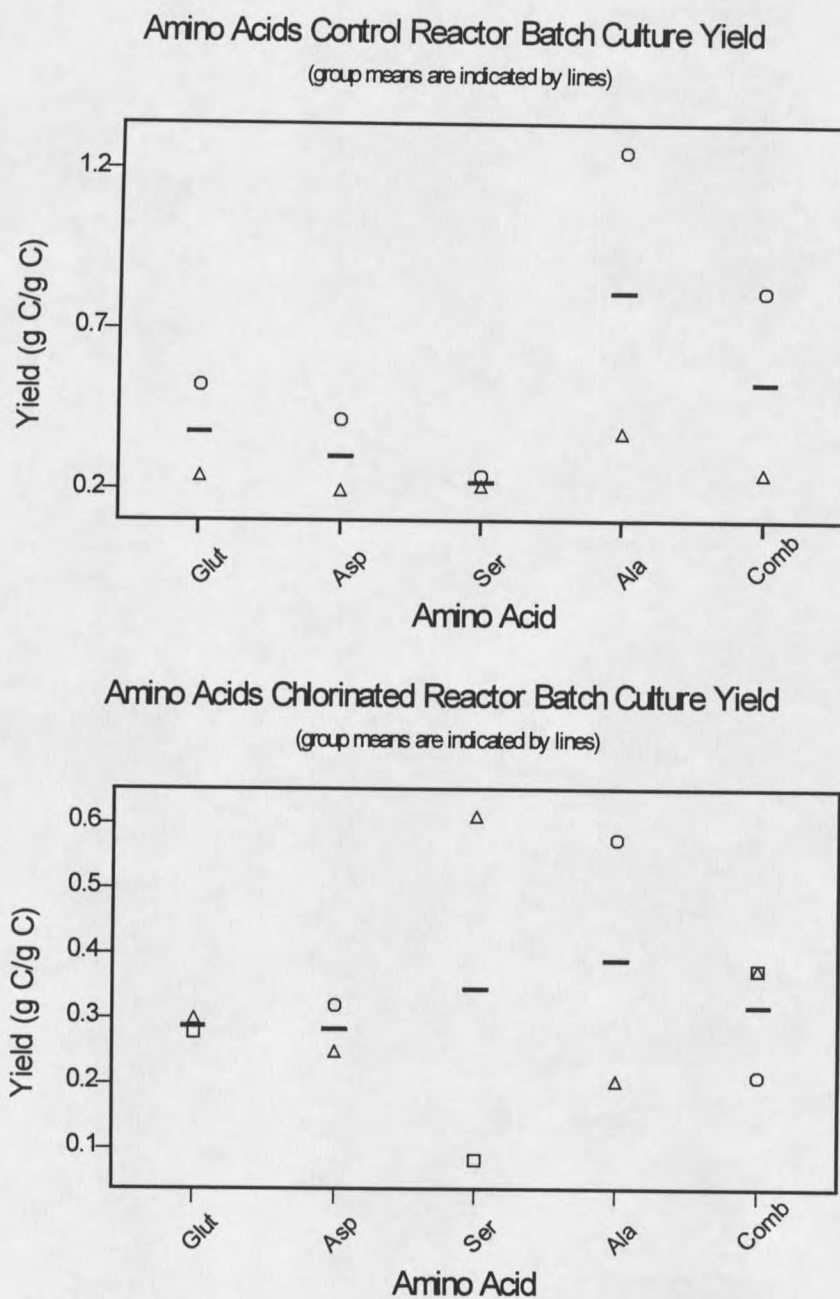


Figure 68. Yield data for biofilm cell batch cultures from the amino acids control (top) and chlorinated (bottom) reactors based on TDC/Biomass. Open circles are data from replicate 1, open triangles and squares are data from replicate 2, and solid bars are the mean of the data points.

the highest yield (0.389), followed by the combination (0.319), and serine the lowest yield (0.0816).

A comparison of all yield data combined by reactor (yield based on the TDC/Biomass measurement technique) indicated the mean yield was statistically equal between reactors ($p=0.415$) and between amino acid groups ($p=0.332$). The primary reason for any lack of significant differences was most likely the large spread in the data between replicate experiments. The yield data for the chlorinated reactor was much more variable than for the control reactor, indicating individual substrates or replicate experiment had more effect on yield compared to the control batch cultures. Yield values for alanine and the combination showed the greatest variation between control and chlorinated reactor batch cultures, and the control yield values for these two amino acids (0.813 and 0.513, respectively) were greater than values for the chlorinated batch cultures (0.389 and 0.319, respectively).

Figure 69 presents boxplots for the combined control and chlorinated reactor TDC/biomass yield data by amino acid group. The data in Figure 69 show that alanine and the combination generally resulted in higher yield values than for glutamate, aspartate and serine. The mean yield values shown in Figures 68 and 69 are summarized in Table 22.

The raw yield data and means based on HPC/Biomass and TDC/Biomass are presented in Figure 70. Yield based on HPC/Biomass correlated well with the data based on TDC/Biomass for the control reactor. A similar conclusion can be drawn for the chlorinated reactor with the exception of the data for serine where the difference between

Amino Acids Control and Chlorinated Reactors' Batch Cultures Combined

(means are indicated by solid circles)

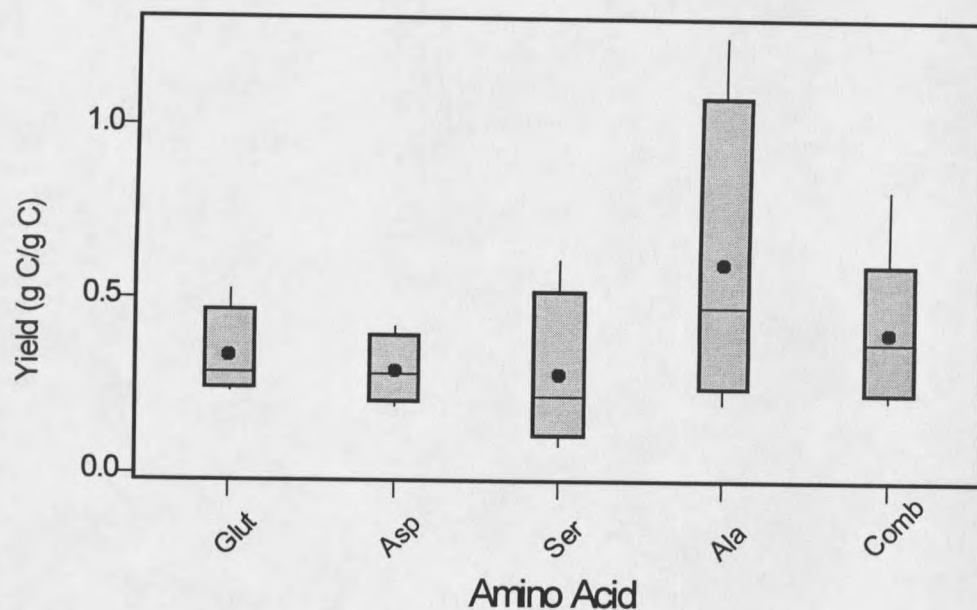


Figure 69. Boxplots for yield data from amino acids control and chlorinated reactor biofilm cell batch cultures based on TDC/biomass. Data was combined by amino acid.

yield from the two methods was significant. Using the GLM-ANOVA to compare the data between reactors without consideration of the amino acid group, it was determined the mean yield values were statistically equal between reactors ($p=0.365$), and between measurement techniques ($p=0.277$). However, with the exception of alanine, the TDC/Biomass technique generally resulted in lower yield values than the HPC/Biomass technique.

Reactor	Amino Acid	Mean Yield (g C/ g C)	StDev	SEM	n
Contol	Glutamate	0.380	0.203	0.144	2
	Aspartate	0.304	0.158	0.112	2
	Serine	0.223	0.026	0.018	2
	Alanine	0.813	0.624	0.441	2
	Combination	0.531	0.400	0.283	2
Chlorinated	Glutamate	0.286	0.014	0.010	2
	Aspartate	0.284	0.052	0.037	2
	Serine	0.345	0.372	0.263	2
	Alanine	0.389	0.264	0.187	2
	Combination	0.319	0.094	0.054	3
Combined Data (Control & Chlorinated)	Glutamate	0.333	0.130	0.065	4
	Aspartate	0.294	0.097	0.048	4
	Serine	0.284	0.227	0.113	4
	Alanine	0.601	0.462	0.231	4
	Combination	0.404	0.241	0.108	5

Table 22. Mean cell yield for amino acids scraped biofilm cell batch cultures based on TDC/Biomass measurements. TDC = total direct cell count.

Humics Yield. Based on measurements of filtered NPOC, TDC, HPC, and cell volume/biomass, yield was calculated for the batch cultures in the humics replicate 2 experiment. Data was not available for the first humics experiment due to TOC analyzer equipment problems.

In scraped biofilm cell cultures from both reactors, yield values determined using TDC/Biomass were greater than values determined using HPC/Biomass. Figures 71 and 72 present the yield data by batch culture and measurement technique for the control and chlorinated reactor batch cultures, respectively. The difference in mean yield

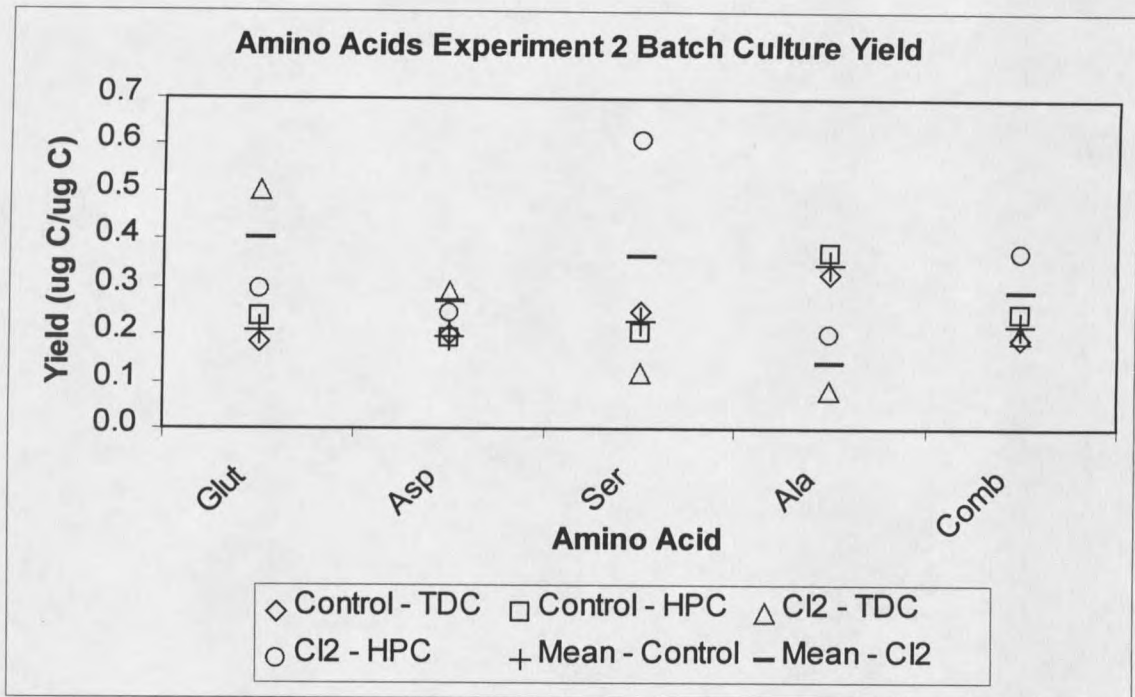


Figure 70. Amino acids replicate 2 batch culture yield data comparing measurement technique. TDC = total direct counts/biomass; HPC heterotrophic plate count/biomass; CI2 = chlorinated; Glut = glutamate; Asp = aspartate; Ser = serine; Ala = alanine; and Comb = combination.

between the two measurement techniques was significant at the $\alpha=0.05$ level (2-sample t-test) for the chlorinated reactor batch cultures, but not for the control reactor batch cultures (2-sample t-test). There appears to be a reduction in yield for the control reactor batch culture at an initial humics concentration of 2000 $\mu\text{g C/L}$. Little variation between cultures within a measurement technique was evident for the chlorinated reactor cultures. The mean yield based on TDC/Biomass was 0.362 and 0.211 g cell C/g humics C for the control and chlorinated batch cultures, respectively. The mean yield based on HPC/Biomass was 0.215 and 0.0452 g cell C/g humic C for the control and chlorinated batch cultures, respectively.

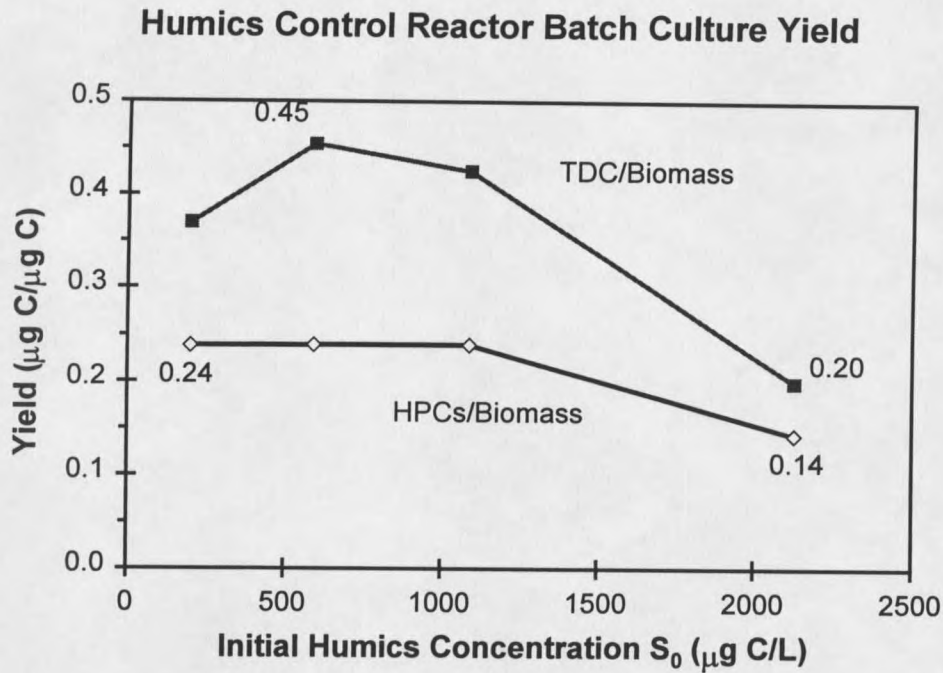


Figure 71. Yield from the humics control reactor replicate 2 biofilm cell batch cultures. TDC = total direct cell count; HPC = heterotrophic plate count.

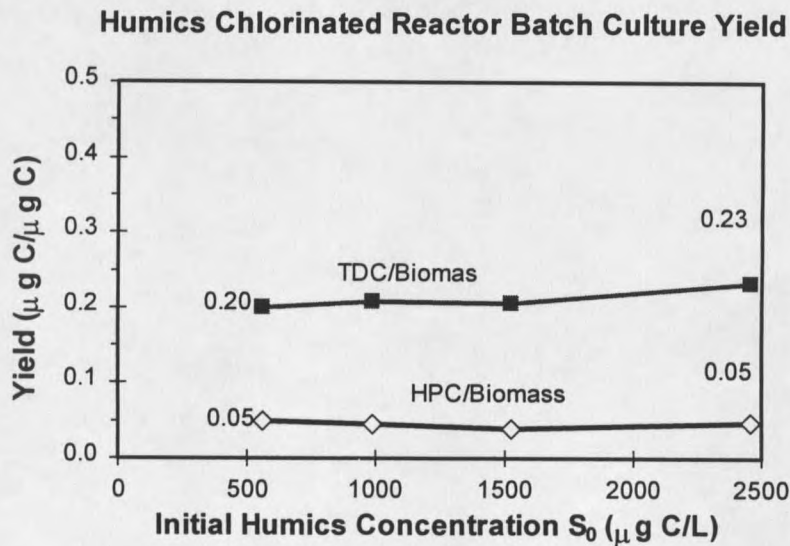


Figure 72. Yield from the humics chlorinated reactor replicate 2 biofilm cell batch cultures. TDC = total direct cell count; HPC = heterotrophic plate count.

The difference between reactors for yield determined using the TDC/Biomass method was not significant at the $\alpha=0.05$ level (2-sample t-test), but the difference between reactors using the HPC method was significant ($p=0.006$). Yield data for the humics batch cultures are summarized in Table 23.

Reactor	Culture	Initial Humics Conc. S_0 ($\mu\text{g C/L}$)	Yield TDC/Biomass ($\mu\text{g C/ } \mu\text{g C}$)	Yield HPC/Biomass ($\mu\text{g C/ } \mu\text{g C}$)
Control	100	200	0.370	0.238
	500	592	0.454	0.239
	1000	1089	0.425	0.239
	2000	2128	0.199	0.143
	Mean		0.362	0.215
	Standard Deviation		0.114	0.048
	SEM		0.057	0.024
	Lower 95 % CI		0.180	0.139
	Upper 95 % CI		0.544	0.291
Chlorinated	100	560	0.199	0.0487
	500	987	0.207	0.0453
	1000	1529	0.205	0.0393
	2000	2458	0.232	0.0475
	Mean		0.211	0.0452
	StDev		0.0146	0.0042
	SEM		0.0073	0.0021
	Lower 95 % CI		0.1876	0.0386
	Upper 95 % CI		0.2339	0.0519

Table 23. Yield data for the humics replicate 2 biofilm cell batch cultures based on TDC/Biomass and HPC/Biomass measurement techniques. TDC = total direct cell count; HPC = heterotrophic plate count.

CHAPTER 5

DISCUSSION

Introduction

Seasonal changes in the organic carbon content and make-up of natural water supplies, or changes caused by man-made actions such as agricultural runoff or wastewater treatment plant discharges, will create changes in biofilm growth rates and the make-up of the microorganisms in the biofilm. The challenge for drinking water suppliers will be to understand the make-up of the carbon loading in the water, a parameter typically controlled by natural processes within the watershed. Humic substances make up the majority (50 – 75%) of the dissolved organic carbon (DOC) in all natural water supplies. Amino acids and carbohydrates, found both as free compounds and combined with humic substances, are readily utilized by microorganisms but make up a small percentage of the total DOC. Monitoring a greater number of chemical constituents in natural waters will provide more information on the types of carbon compounds in the water supply and help predict performance of treatment systems.

Biological treatment systems for drinking water must be designed to meet potential organic loading much in the same manner as wastewater systems have been designed for many years. Since not all water utilities will employ biological treatment, it would be desirable to understand both the variations in the type of carbon compounds entering the distribution system and their impacts on the biofilm and microbial quality of

the water. This process is again analogous to predicting the impacts of a change in quality or quantity of a wastewater treatment plant's discharge on a receiving water body, only here the treatment plant is the water treatment plant and the receiving water body is the distribution system. Water systems that do not employ conventional water treatment and only use disinfection may be more vulnerable to seasonal water quality variations and their impact on water quality in the distribution system.

Rotating annular reactors were utilized to develop biofilm under hydraulic shear stress conditions similar to a pipeline. The reactors provided a tool to both develop biofilm relevant to drinking water conditions and measure parameters that describe the biofilm growth kinetics. Kinetic parameters such as specific growth rate, specific substrate uptake rate and observed yield provided a means to compare the biofilm response to the various substrates, as well as parameters required for water quality models that incorporate biofilm growth.

Biofilm cells exist in clusters attached to a surface, such as filter media or a distribution pipe wall. Techniques to study the growth of biofilm cells without removing them from their environment are only now being developed and are typically very detailed in nature. Batch cultures have been used extensively to study growth, substrate uptake and yield in microorganisms; but the microorganisms must be homogeneously suspended in a growth medium. At the outset of this project it was hypothesized that if a sufficient number of biofilm cells could be removed from their surface of attachment and placed in batch cultures containing the same substrate(s) as in the biofilm environment,

substrate uptake could be measured without appreciable growth. If substrate uptake could be determined before significant growth occurred, then substrate uptake may be representative of the biofilm and not the population of microorganisms that would eventually develop in the batch cultures.

The initial batch culture experiments showed that when biofilm cells were placed in batch cultures there was a lag period for both growth and substrate uptake, particularly for the chlorinated reactor biofilm cells. Since the biofilm in the reactors had a limited number of cells (typically 10^6 per square centimeter) it was not possible to put a high enough concentration of cells in the batch cultures and have limited growth as was hypothesized. Therefore all batch cultures showed both an increase in cell numbers and associated substrate depletion.

Knowing there would be an increase in cell number, the batch culture experiments were performed to determine if the growth and substrate uptake rates in batch culture would be different from the growth and substrate uptake rates measured for biofilm in the reactors using different techniques. If growth rates determined using batch culture were nearly the same as those determined for the biofilm in the reactors, then the simpler batch culture method could be used for determining the kinetic parameters. Growth rates for batch cultures using the individual compounds in the substrate group were monitored to determine the impact of the particular on growth and provide a parameter for use in comparing between batch cultures using different substrate groups.

In the following paragraphs a detailed discussion of the results is presented. An outline of this chapter is as follows:

Specific Growth Rates

- Biofilm Growth Rates Based On Mass Balances

- Biofilm Growth Rates Based On Leucine Uptake

- Batch Culture Growth Rates

- Comparison by Substrate

- Comparison of Reactor to Batch Culture Growth Rates

Substrate Uptake

- Reactor Carbon Removal Rates

- Batch Culture Substrate Uptake

- Comparison of Reactor to Batch Culture Substrate Uptake

Yield

- Reactor Biofilm Observed Yield

- Batch Culture Yield

- Comparison of Reactor to Batch Culture Yield

- Relevance to Drinking Water

- Impacts on Biofilm Models

Specific Growth Rates

Biofilm Growth Rates Based On Mass Balances

Specific growth rates determined using the mass balance approach are discussed below for each substrate group.

Amino Acids—Reactor Mass Balance. In general, chlorination and substrate concentration did not have a significant impact on biofilm specific growth rates (μ) when amino acids were the substrate. When data for the 1000 and 2000 carbon levels were analyzed without consideration of the 500 carbon level data, there were no significant differences between the two carbon levels and between reactors within each carbon level. Both reactors had a decreasing growth rate between the 500 carbon level and the 1000 carbon level. Since the chlorinated reactor was not receiving chlorine at the 500 carbon level the reduction in growth rates at the higher carbon levels could be due to the interaction of chlorine with the amino acid or the interaction of chlorine with the biofilm and extracellular polymeric substance (EPS).

Amino acids react with chlorine quite rapidly, forming N-chloro compounds such as chloramines (Trehy, M. L. et al., 1986; de Leer, E. W. B. et al., 1990; Choshen, E. et al., 1990) or creating the corresponding aldehyde or nitrile (Trehy, M. L. et al., 1986). Despite these potential interactions, either chlorinated biofilm were able to grow utilizing the oxidation products, amino acids were taken up by the biofilm before complete reaction with the chlorine, or a combination of both was occurring within the reactor.

The uneven distribution of leucine uptake by attached biofilm on slides taken from the reactors, as shown in Figure 9, indicated possible uneven mixing or variations in biofilm within the reactor in response to chlorine. Biofilm further from the chlorine addition point (top of reactor) exhibited higher leucine uptake rates. The scope of this research did not include investigation of the true interactions that might explain why there were no differences in growth rates between the control and chlorinated reactors.

Models that relate specific growth rate (μ) to the bulk fluid (effluent) substrate concentration (S) were presented in Table 8. Comparison of the zero-order model to the other models investigated indicates that the zero-order model was appropriate for both the control and chlorinated reactor biofilm. Although there was a reduction in growth rate for the control reactor between the 500 and 1000 carbon levels, the inhibition model did not provide a good fit to the data as evidenced by the low adjusted R^2 value and high p(regression) value.

Carbohydrates-Reactor Mass Balance. Chlorination had a significant impact on biofilm growth rates for reactors with carbohydrates as the substrate group. Growth rates in the chlorinated reactors at the 1000 and 2000 carbon levels were significantly greater than growth rates for the control reactors.

Unlike amino acids, carbohydrates do not typically react with chlorine as indicated by their low THM formation potential (Bruchet, A. et al., 1990). It would be logical to assume that in the chlorinated reactor there was free available chlorine that could interact with the biofilm, thereby decreasing biofilm growth rates. It appears from

the data summarized in Figures 14 and 25 that the effect of chlorine was to increase biofilm growth rates. As can be seen in Figure 25, when substrate concentrations were equal the chlorinated reactor biofilm growth rate was greater than for the control.

Possible explanations for the increased growth rates in the chlorinated reactor include interactions of chlorine with the biofilm EPS or natural selection of biofilm cells more resistant to chlorine. The EPS matrix, consisting of proteins and polysaccharides, has been implicated in the protection of biofilm cells from the oxidizing effects of free chlorine (Characklis, W. G., 1990; Koudjonou, B. K. et al., 1997; Samrakandi, M. M. et al., 1997). Chlorine could oxidize the EPS matrix, decreasing biofilm thickness and at the same time provide protection against free chlorine. A reduction in EPS could decrease mass transfer resistance imposed by the EPS matrix, allowing carbohydrates in the bulk fluid to more easily diffuse to the biofilm cells, increasing growth and additional EPS production. Characklis (1990) indicated biofilm exposure to chlorination leads to selection of microorganisms that are more resistant to chlorine and added resistance has been associated with increased EPS production.

LeChevallier et al. (1988) found biofilm age to be an important factor in biofilm resistance to chlorine. This finding may reflect acclimation and selection of microorganisms more resistant to chlorine. In the research being reported herein, biofilm age increased along with carbon concentration, making the two factors inseparable in the experiments. Biofilm age (physiological adaptation) may be responsible for the higher growth rates seen in the chlorinated reactors.

Research to determine the effects of chlorine on cells has shown chlorine can damage or kill cells but some cells have mechanisms to protect the cell from chlorine. Free available chlorine (hypochlorous acid) has been shown to damage the membrane structure of cells (Haas, C. N. and Engelbrecht, R. S., 1980; Venkobachar, C. et al., 1977). It would not seem logical that damaging the membrane would improve growth rates. Certain strains of *E. coli* cells have been found to provide protection against hypochlorous acid using intracellular glutathione as a sacrificial compound against the oxidizing effects of hypochlorous acid (Chesney, J. A. et al., 1996).

Other possible explanations for the observed high growth rates in the chlorinated reactor data could be errors in how the growth rates were calculated. Potentials for errors of this type are discussed in detail later in this chapter.

As with the amino acids, zero-order models best describe the μ -S relationship for biofilm from the carbohydrate reactors (see Table 8). There was little difference in growth rates at the 1000 and 2000 carbon levels for the chlorinated reactors even though there was a significant difference in the effluent concentrations (Figure 25). Control reactor growth rates did decrease between the 500 and 1000 carbon levels in the same manner as seen for the amino acids control reactor, but an inhibition model did not provide a good fit to the data.

Humic Substances-Reactor Mass Balances. Influent carbon concentration and chlorination both impacted specific growth rates for biofilm in the humics reactors. Reactor biofilm growth rates for the control and chlorinated humics reactors showed a

significant decrease between the 500 carbon level and the 1000 carbon level; there was little difference in growth rates between the 1000 and 2000 carbon levels. As in the chlorinated carbohydrate reactor, chlorination of the humics reactor appeared to result in greater biofilm growth rates than found in the control reactor. The one-way ANOVA using just data for the 1000 and 2000 carbon levels confirmed that the chlorinated reactors had growth rates significantly greater than the control reactors.

The interaction of chlorine with humic substances may make humic substances more available to the biofilm, thus increasing biofilm growth rates. A study of the effects of chlorine on humic substances indicated a decrease in phenols, methoxyl carbon, aryl-C carbon and ketones, and an increase in carbohydrate and alkyl carbon (Hanna, J. V. et al., 1991). Chlorination of humic substances can also rupture the aromatic ring structure of carbon compounds, creating haloacetic acids, dicarboxylic acid and α -chloropropionic acid (Christman, R. F. et al., 1980). These and other carbon compounds may be more available to microorganisms than the original humic substances without the effects of chlorine.

The general relationship between control and chlorinated reactor biofilm growth rates and substrate concentrations at the 1000 and 2000 nominal carbon levels was zero-order, meaning the growth rate did not appear to be related to effluent substrate concentration (Figure 26). Control reactor biofilm growth rates exhibited a significant decrease between the 500 and 1000 carbon levels, but an inhibition model did not provide a good fit to the data (Table 8).

The zero-order model parameter (biofilm specific growth rate) for the control and chlorinated reactors was shown in Table 8 for two possible scenarios. The growth rate for the control reactor was 0.0263 hr^{-1} if the 500 carbon level data was included and 0.0023 hr^{-1} when the 500 data was excluded. For reasons that will be discussed below, the zero-model without the 500 carbon level data may represent a more realistic biofilm growth rate for the control humics reactor.

It was noted in Chapter 4 that growth rates for the chlorinated humics reactor at the 1000 carbon level were greater in the first replicate experiment than in the second experiment. The impact of the difference in growth rates between experiments was presented in Figures 16 and 26. When the replicate 1 data are included the zero-order model growth rate was 0.0237 hr^{-1} and without the replicate 1 data the growth rate would be 0.0051 hr^{-1} (Table 8). One difference between the two experiments was the time between sampling at the 500 and 1000 carbon levels (26 days for replicate 1 and 56 days for replicate 2). The increased age of the biofilm for replicate 2 may be why the growth rate was less than for replicate 1. Humic substances may adsorb or attach to the surface of the biofilm and thus influence biofilm growth rates.

Marshall (1988) cites work by himself and others showing that when humic substances and other hydrophobic compounds adsorb to surfaces, biofilm develops at those surfaces, using the adsorbed molecules as nutrients. Attachment of humic substance molecules to the biofilm matrix could make the biofilm growth rate a function of how fast the biofilm cells could break down the attached humic substances rather than

a function of how much humic substance was in the bulk fluid. Humic substances attached to the biofilm were clearly visible when sample slides were pulled from the humics reactors. NPOC measurements of the biofilm attached to slides from the humics replicate 2 reactors at the 2000 carbon level found 8.33 μg of non-cell carbon per square centimeter in the chlorinated reactor and 30.1 μg non-cell C/cm² in the control reactor. This could explain why there was no net increase in growth rate with increased humics concentration.

The ratio of non-cell carbon to cell carbon was 37 and 3.3 for the chlorinated and control humics reactors, respectively. The high ratio of non-cell carbon to cell carbon for the chlorinated reactor biofilm could account for the difference between the control and chlorinated humics reactor growth rates at the higher carbon loading rates by (1) providing more attached carbon available per cell compared to the control reactor, thus leading to higher growth rates or, (2) it could provide protection against the effects of chlorine in the bulk fluid (LeChevallier, M. W. et al., 1988), or (3) providing a localized source of nutrients released by the interaction of chlorine with the humic substances.

If humic substances were concentrated at the biofilm – bulk fluid interface, then it would be logical to expect higher growth rates in the biofilm cells due to the apparent large supply of carbon near the cells. A possible explanation could be that the humic substances nearest the biofilm cells become less amenable to biodegradation over time. Enzymatic degradation of the more labile components of the humic substances could occur first, leaving the more refractory polymers. Growth rates could become limited by

the rates at which the refractory components of humic substances are broken down by enzymes combined with the rates at which enzymes and substrate can diffuse through the attached humic substance matrix.

Investigation of microbial growth on naturally occurring dissolved organic carbon has shown that the high molecular weight (HMW) fraction (molecular weight > 1000 Daltons) resulted in higher carbon utilization rates and growth efficiencies compared to the low molecular weight (LMW) fraction (Amon, R. M. W. and Benner, R., 1996; Tranvik, L. J., 1990). It was believed the HMW compounds were more bioreactive because the material was less diagenetically altered than the LMW fraction; the HMW fraction contained more easily degradable compounds. As biodegradation altered the humic substances attached to the biofilm in the humics reactors, the humic substances may have become less bioavailable to the biofilm cells resulting in lower growth rates.

Mixed Substrate-Reactor Mass Balances. Chlorination and substrate concentration had significant impacts on growth rates in the mixed substrate reactors. The mixed substrate "a" reactors were operated identically to reactors in the other substrate experiments except chlorine addition was started at the 500 carbon level. The μ -S relationship for the control "a" reactors showed a significant decreasing trend in μ with increasing effluent substrate concentration and the chlorinated "a" reactor data showed the opposite trend (Figure 27). An inhibition-type model provided a good fit to the control reactor data (Table 8, $p(\text{regression}) = 0.006$). A Monod-type model provided a good fit to the chlorinated reactor data (Table 8, $p(\text{regression}) = 0.031$).

Growth rates were greater in the chlorinated biofilm than in the control. The maximum specific growth rate ($\mu_{\max}$) for the control reactor biofilm based on the inhibition-type model was 0.0962 hr^{-1} and $\mu_{\max}$ for the chlorinated reactor biofilm was 0.307 hr^{-1} based on the Monod-type model. Mean growth rates based on the zero-order model were also greater for the chlorinated reactor biofilm than for the control (0.0747 and 0.0428 hr^{-1} , respectively). However, zero-order models did not provide good fits to the mixed substrate "a" reactors' data as noted by the higher residual sum of squares (RSS) in Table 8 for the zero-order models compared to the other models investigated.

Growth rates were computed for the combined "a" plus "b" reactors and shown as a function of effluent substrate concentration in Figure 28. Combined a+b growth rates were based on the influent to the "a" reactor and effluent from the "b" reactor. Trends in growth rate with increasing effluent substrate concentration for both combined reactors were the same as seen for the control "a" reactor, decreasing growth rate with increasing substrate concentration. The inhibition-type model provided a good fit to the control and chlorinated combined reactor data (refer to Table 8) based on low p(regression) values (0.002 and 0.005 , respectively) and a lower RSS value than for the zero-order model.

Trends in specific growth rate with effluent substrate concentration for the mixed substrate "b" reactors showed a decreasing growth rate with increasing substrate concentration. Recall that only the "a" reactor received substrate; the "b" reactor was fed only the effluent from the "a" reactor. Growth rates in the "b" reactors were non-detectable at the 1000 and 2000 carbon levels for the control reactor and growth rates

were low (0.0100 hr^{-1}) in the chlorinated reactor "b" reactor compared to the chlorinated "a" reactor (0.0982 hr^{-1}).

At the 500 carbon level, the chlorinated "b" reactor had the highest growth rate measured over the course of the mixed substrate experiment. It cannot be determined for certain why this result occurred, but several reasons are possible. Potential measurement errors, as will be discussed below, could have been the cause. However, growth rates at each carbon level for the mixed substrate experiment were based on five individual measurements over a period of one to two weeks, making it unlikely that measurement error could be suspect. One reason for the higher growth rate could be that very little carbon was used in the "a" reactor, where chlorine limited biofilm development and thus limited overall uptake of substrate. This resulted in the "b" reactor receiving a high carbon load with less influence by free chlorine (there was no residual in the effluent of the "b" reactor).

The chlorinated "b" reactor represents a condition where a free chlorine residual was maintained in the upstream "a" reactor, but no residual was maintained in the effluent of the "b" reactor. The interaction of chlorine with the humic substances while in the "a" reactor may have also helped stimulate growth in the "b" reactor by breaking-down the humic molecules into more biodegradable components. Because the control "a" reactor did not have growth rates as high as the chlorinated "b" reactor, it is believed the action of chlorine on the substrates played a role in the stimulated growth in the chlorinated "b" reactor. The higher growth rates in the combined chlorinated reactor

compared to the combined control reactor (Figure 28) would support the hypothesis that the interaction of chlorine with the substrates may have resulted in higher overall growth rates compared to the combined control reactors.

The interaction between the chlorinated "a" and "b" reactors illustrate a condition that can develop in drinking water distribution systems. As water moves through the distribution system free chlorine can be consumed by its interaction with the bulk fluid, pipe materials and biofilm. In the upstream parts of the distribution system the development of biofilm may be limited by chlorine while in downstream stretches there may be a redevelopment or regrowth of biofilm, possibly stimulated by factors occurring in the upstream portions of the system. The terms "recovery" and "regrowth" have been applied to conditions seen in the chlorinated "b" reactor (Characklis, W. G., 1990) where cells exposed to chlorine become more resistant to its effects and are capable of establishing new biofilm.

Comparison of Growth Rates by Type of Substrate. Growth rates for biofilm in control reactors using humics as the substrates were significantly less than those for the other three substrate groups (refer to Figure 21). When all control reactor data was combined and compared by carbon level, growth rates at the 500 carbon level were significantly greater than at the 1000 and 2000 carbon levels. This result was noted above for control reactors in each substrate group.

Growth rates for chlorinated reactors using carbohydrates and mixed substrates were greater than for other substrates; biofilm from the chlorinated carbohydrate reactors

had the highest growth rates. Based on growth rates using the reactor mass balance approach it would appear that carbohydrates might result in higher growth rates when free chlorine is used for disinfection. Growth rates for chlorinated reactors using amino acids or humics were not statistically different, but mean growth rates for humics were lower than for amino acids.

In most natural water supplies, humic substances will dominate the dissolved organic carbon fraction, with most amino acids and carbohydrates bound to the humics (Malcolm, R. L., 1991; Kaplan, L. A., 1993). The organic carbon makeup of most water supplies can be impacted seasonally by factors such as algal blooms (de Haan, H. and de Boer, T., 1979) or heavy runoff. The content of free or combined amino acids and carbohydrates can then be variable, perhaps impacting to a significant extent the growth rate of biofilm. Treatment processes such as chemical coagulation and sedimentation or ozonation impact the organic carbon makeup of finished drinking water. Chemical coagulation and sedimentation can typically remove a large fraction of the humic substances. Although coagulation and sedimentation can reduce the overall carbon load to the distribution system, humic substances will continue to dominate the makeup of the dissolved organic carbon. Ozonation will oxidize organics into smaller, potentially more biodegradable compounds. Biofilm growth rates for ozonated water may more closely represent those in this research where amino acids or carbohydrates were the substrate.

Overall Discussion of Growth Rates Determined Using Mass Balances.

Examination of the control reactor data in Figures 6, 14, 16 and 17 indicated that reactor

specific growth rates at the 500 carbon level were greater than for the two higher carbon levels. This same trend was noted when growth rates were presented as a function of effluent carbon concentration (Figures 24, 25, 26 and 27). An inhibition-type model was tested for those conditions as a means for describing the noted relationship. While the work in this project does not provide definitive answers as to why the perceived inhibition occurred, some possible explanations have been postulated.

The BAC column using de-chlorinated Bozeman tap water provided the heterotrophic bacteria that initially attach and form a biofilm community in the reactors. These heterotrophs have been acclimated to a low carbon environment, and can be generally described as oligotrophs. Concentrations of dissolved free amino acids in natural waters have been found to range from 78 nM in a lake to 8 μ M in a river (Jørgensen, N. O. G. and Jensen, R. E., 1994; Lytle, C. R. and Perdue, E. M., 1981; Simon, M. and Rosenstock, B., 1992; Jørgensen, N. O. G., 1987; Volk, C. J. et al., 1997). In the amino acids reactors at the 500 carbon level the total amino acid concentration was 11.6 μ M, a value greater than typically seen for dissolved free amino acids, but possibly low enough for bacteria adapted to a low nutrient environment to maintain high growth and uptake rates. When the concentration of amino acids was doubled to 23.2 μ M (1000 carbon level), the population may have adapted to the higher concentrations of substrates by slowing growth and uptake rates, even though the biofilm bacterial density had increased at the higher carbon level. Oligotrophs are believed to have high affinity substrate uptake systems such that if exposed to high substrate concentrations growth is

prevented by a process termed 'substrate accelerated death' (Höfle, M. G., 1984). It may be possible for the bacteria to switch to a lower uptake affinity to prevent 'substrate accelerated death' (Schut, F. et al., 1997).

Biofilm age and concurrent attachment of humic substances to the biofilm, as discussed above for the humics reactor growth rate data, may be a possible reason for the decrease in control reactor biofilm growth rates as the carbon level increased for the humics and mixed substrates. Non-cellular attached carbon (as NPOC) was measured for the biofilm during the mixed substrate experiment at each nominal carbon feed level as shown in Figure 73. The amount of non-cell carbon continually increased in the

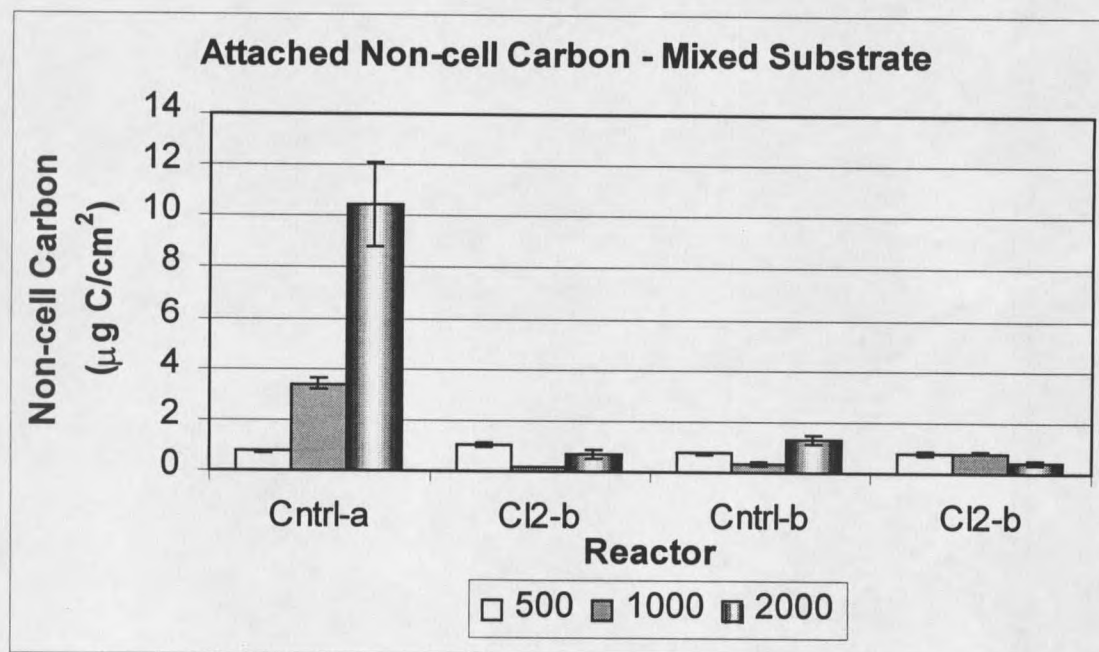


Figure 73. Attached non-cell carbon in the mixed substrate reactor biofilm at each nominal carbon feed level (500, 1000 and 2000 $\mu\text{g C/L}$). Error bars represent 95% confidence limits. Cntrl = control, Cl2 = chlorinated, "a" is first of two reactors in series and "b" the second.

control "a" reactor biofilm as carbon concentrations increased. As was noted above for the humic substance reactors' biofilm, the mixed substrate control reactor biofilm sample slides also appeared to have humic substances attached to the biofilm matrix. The declining growth rate in the control "a" reactor may be due to the attached humic substances. The attached humic substances could impede diffusion of amino acids and carbohydrates as more humics attach to the biofilm, indicated by the increase in attached non-cellular carbon. The biofilm would become increasingly reliant on the attached carbon material, which in turn could become less biodegradable with time, resulting in lower growth rates.

The attachment of humic material to the biofilm matrix could also have occurred in the amino acids and carbohydrates reactors where the source of humic material was the dilution water from the BAC column. While the dilution water was very low in assimilable organic carbon (AOC) it was not free of organic carbon (0.5 to 3 mg/L as NPOC). Biological filtration removes much of the readily assimilable carbon compounds, such as those measured using the AOC test. Less easily degradable carbon compounds such as humic substances are not easily removed by biofilm in the BAC column. As determined by data collected during this study (data not presented) and by many other (de Leer, E. W. B. et al., 1990; Reckhow, D. A. and Singer, P. C., 1990) humic substances have a high potential for trihalomethane formation. The BAC column effluent was found to have an average trihalomethane formation potential of 39 $\mu\text{g/L}$ and this formation potential is believed to be associated with humic substances.

Biofilm growth rates determined using the mass balance approach require numerous measurements, each with its associated error. Two areas of concern that were investigated are errors in the measurement of biofilm biomass and growth of cells in the reactor bulk fluid.

Error in Biofilm Biomass. The mass balance around a rotating annular reactor uses Equation 8 (presented in Chapter 3) to determine biofilm specific growth rate:

$$\frac{F}{V}(X_1 - X_0) = r_d X_b \frac{A}{V} \quad (8)$$

where the term r_d equates to the biofilm specific growth rate μ . Rearranging Equation 8 yields the equation used to determine μ .

$$\mu = \frac{F}{A} \cdot \frac{(X_1 - X_0)}{X_b} \quad (37)$$

An underestimation of X_b could lead to an overestimation of μ . Measurement of biofilm total direct count cells requires that cells first be scraped from the biofilm slide followed by staining and microscopy. Based upon biofilm cell measurements made at each carbon level, there typically were fewer cells at the lowest carbon concentrations and numbers increased with increasing carbon concentration. If there were errors in determining total cells caused by incomplete removal from the slide, the error could be exaggerated at the lower carbon levels, leading to greater calculated growth rates. Therefore, sensitivity of the model in Equation 37 to errors in attached cell numbers ($\sim X_b$) was assessed using data from the carbohydrates reactors.

The impacts of errors in the number of biofilm cells are presented graphically in Figures 74 and 75 for the carbohydrates replicate 3 reactors at the 500 and 2000 carbon levels, respectively. To result in a growth rate near that measured at the 1000 carbon level, an error factor of greater than 10 would be required for the 500 carbon level biofilm cell determination. The same error factor would be required at the 2000 carbon level to bring the chlorinated growth rate equal to the control growth rate. While scraping cells from the polycarbonate slide surface does not result in removal of all cells, an order of magnitude error seems implausible – certainly more than 10% of the cells were removed from the slides. Occasional microscopic examination of scraped slides supports this conclusion.

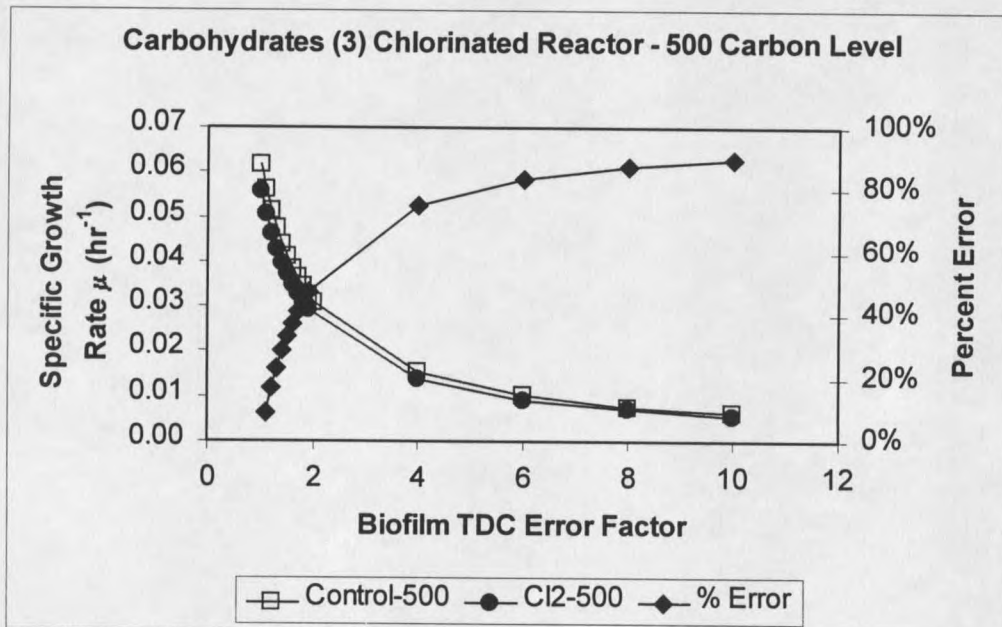


Figure 74. Effect of error in biofilm total direct cell count (TDC) on calculated biofilm specific growth rate at the 500 carbon level for the carbohydrates replicate 3 experiment.

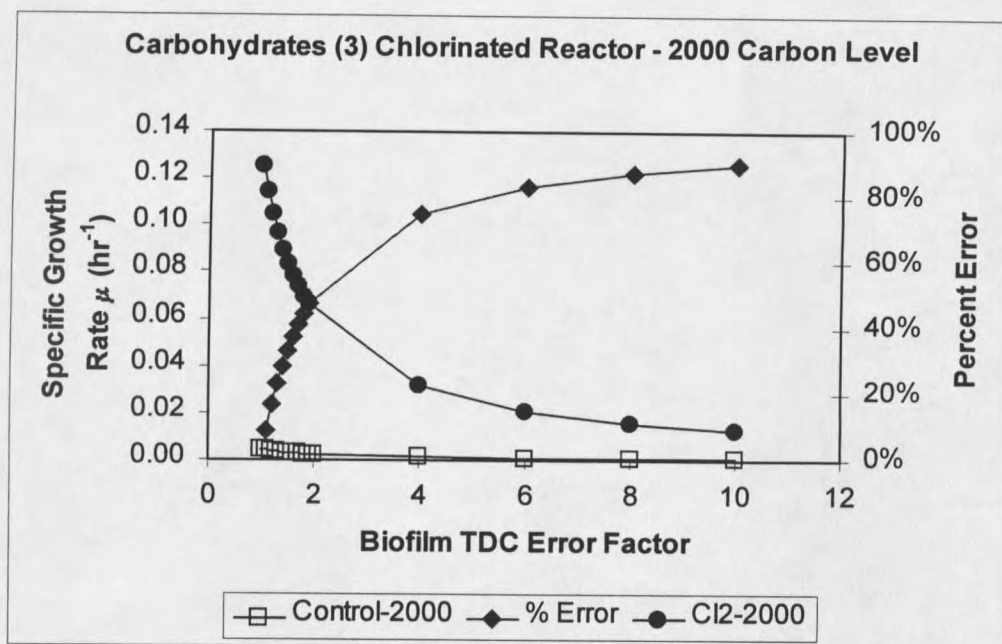


Figure 75. Effect of error in biofilm total direct cell count (TDC) on calculated biofilm specific growth rate at the 2000 carbon level for the carbohydrates replicate 3 experiment.

Bulk Fluid Growth. Another possible error in the mass balance approach for determining specific growth rate was the assumption that growth of cells in the bulk fluid was negligible. Referring back to the original mass balance equation (6) presented in Chapter 3

$$\frac{F}{V}(X_1 - X_0) = \mu_p X_1 + r_d X_b \frac{A}{V}, \quad (6)$$

it can be seen that including the term $\mu_p X_1$ will result in a reduced value for $r_d (= \mu)$. The bulk fluid growth rate (μ_p) can be assumed equal to the biofilm growth rate if certain assumptions can be made (Camper, A. K., 1995): 1) no disinfectant, 2) bulk fluid and biofilm consist of the same population and 3) little mass transfer resistance between the

biofilm and bulk fluid. Assuming the bulk fluid growth rate was equal to that in the biofilm, Equation 6 can be solved for the biofilm growth rate as shown below.

$$\mu = \frac{(F/V)(X_1 - X_0)}{X_1 - X_b(A/V)} \quad (38)$$

Growth rates were calculated for the carbohydrates experiments (the largest data set available) using Equation 38 and compared to growth rates calculated using Equation 37 (minimal growth in the bulk fluid). Figure 76 presents the mean specific growth rates by reactor and nominal carbon level for each method of calculating biofilm growth rate.

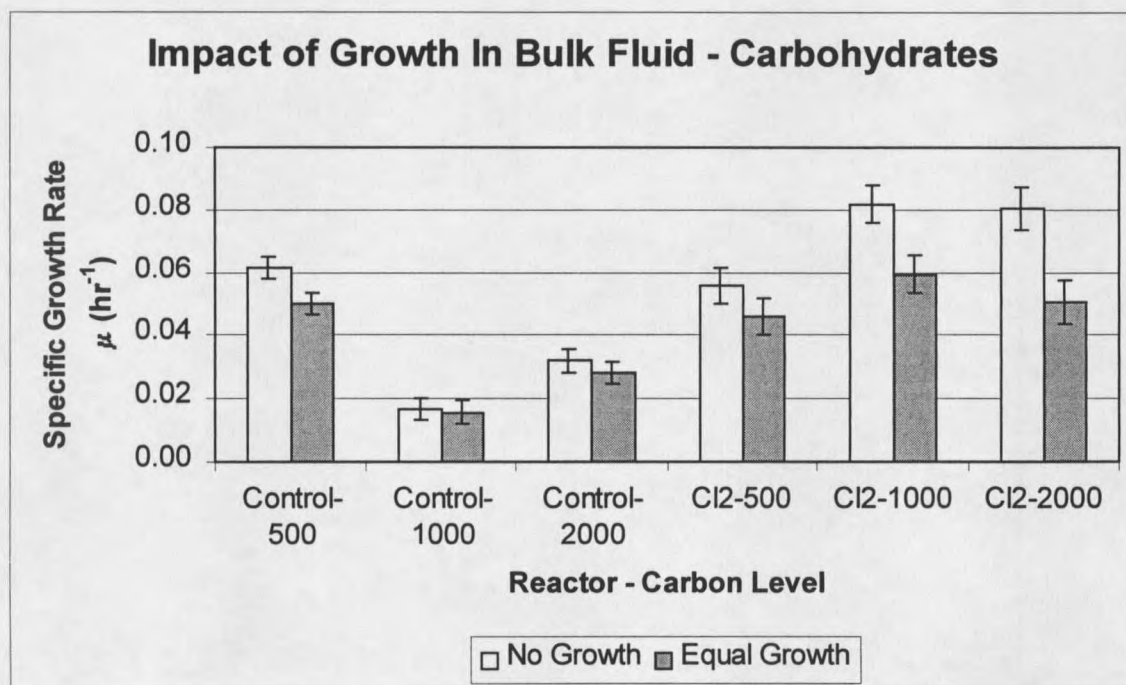


Figure 76. Impact of assuming equal growth rates in the bulk fluid and biofilm on the specific growth rate for the carbohydrates data set. Error bars represent 95% confidence intervals based on the General Linear Model used to compare the data. Carbon levels are nominal carbon concentration ($\mu\text{g C/L}$). Cl2=chlorinated.

Results from the two methods were compared statistically using General Linear Model ANOVAs for data at each carbon level for a particular reactor type, and one-way ANOVAs to compare the results by carbon level for a particular reactor and method. The ANOVA results indicated there was no change in conclusions drawn from analysis of the data set excluding bulk fluid growth as was presented in Chapter 4. The greatest impact on specific growth rates appears to be in the chlorinated reactors where the mean growth rates were less when growth in the bulk fluid was included in the analysis (refer to Figure 76). Neglecting growth in the bulk fluid is therefore appropriate in the overall analysis. The growth rates presented herein were not adjusted for potential growth in the bulk fluid.

Biofilm Growth Rates Based On Leucine Uptake

Specific growth rates determined using the ^3H -Leucine uptake method are discussed below by substrate group followed by an overall discussion.

Amino Acids–Leucine Uptake. For biofilm samples at the nominal 2000 $\mu\text{g/L}$ carbon feed level, the initial concentration of amino acids as carbon did not have a significant impact on specific growth rate. When biofilm growth rates were determined using leucine uptake the growth rates for the control reactor were significantly greater than the chlorinated reactor were.

Biofilm growth rates measured using leucine uptake showed little variation over the range of substrate concentrations used in the experiments (45 to 535 $\mu\text{g C/L}$ for the

added substrates, 103 to 622 $\mu\text{g C/L}$ when the added leucine was included) and the μ -S relationship was zero-order for biofilm in the control and chlorinated reactors. The biofilm samples used for these assays were from reactors receiving an approximate influent amino acids concentration of 2000 $\mu\text{g C}$ per liter. Repetition of the experiment at each of the carbon levels may have shown different growth rates at each carbon level in the reactor. However, examination of the reactor biofilm growth rates, discussed above, indicated zero-order kinetics may be applicable within the constraints of these experiments.

Biofilm growth rates measured utilizing the leucine uptake method were greater than growth rates determined utilizing mass balances for the control reactor when all reactor mass balance data were considered. There were no differences between the leucine uptake and mass balance methods for the chlorinated reactor. The mean biofilm growth rates at the 2000 carbon level based on mass balances were 0.0301 and 0.0475 hr^{-1} for the control and chlorinated amino acids reactors, respectively, and the biofilm growth rates utilizing the leucine uptake method were 0.1003 and 0.0513 hr^{-1} for the control and chlorinated reactors, respectively.

When only data from the amino acids replicate 3 experiment were considered (leucine uptake data were from the replicate 3 reactors), growth rates using the leucine uptake method were 7 to 9 times greater for both the control and chlorinated reactor biofilm than growth rates determined using the mass balance approach (see Figure 77).

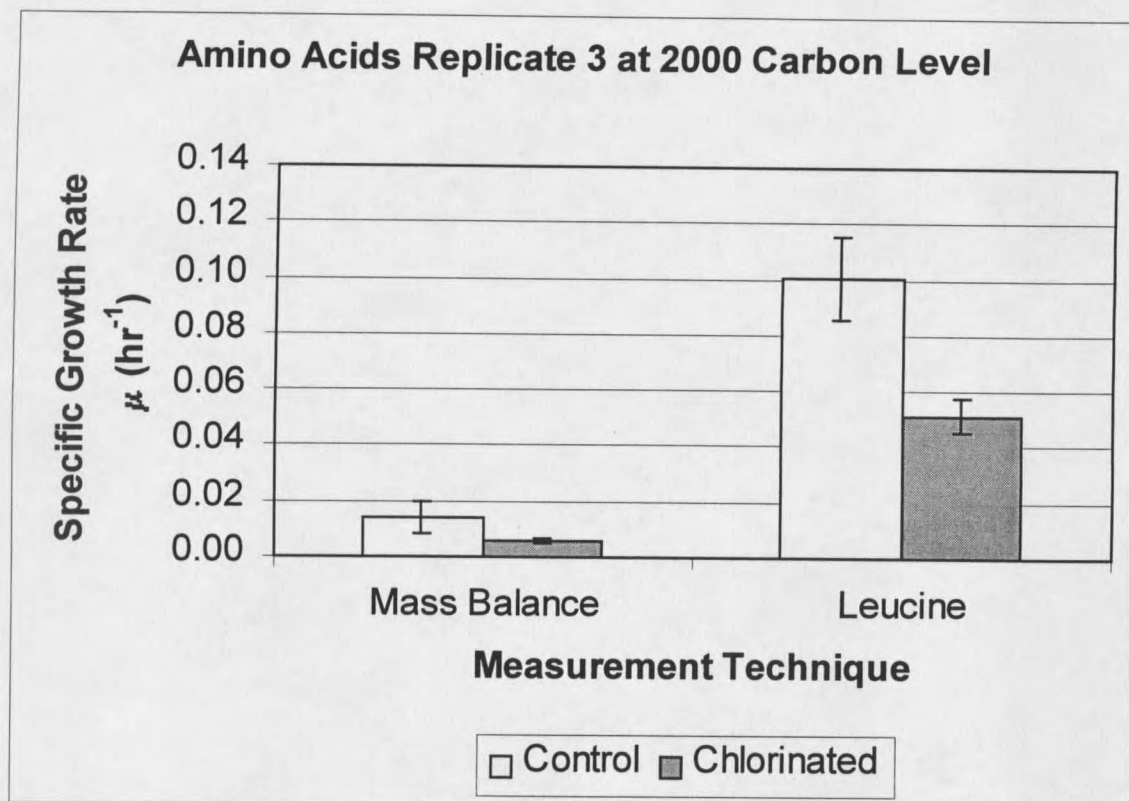


Figure 77. Comparison of first-order specific growth rates for the amino acids replicate 3 control and chlorinated biofilms using the mass balance and leucine uptake methods. Reactor influent amino acids concentration was $\sim 2000 \mu\text{g C/L}$ carbon level. Error bars indicate the 95% confidence limits.

In addition, the control reactor growth rate was greater than for the chlorinated either technique.

One possible explanation for the difference in data from the two techniques may be that biofilm cells that used amino acids as the primary substrate had developed uptake mechanisms capable of efficient amino acid uptake, including leucine, leading to an over-estimation of the growth rate. On the other-hand, the mass balance approach could lead to an under-estimation of the biofilm growth rate.

Carbohydrates–Leucine Uptake. Chlorination had a significant impact on specific growth rates based on leucine uptake. Specific growth rates were greater for the control reactor biofilm than for the chlorinated reactor biofilm when leucine uptake was the basis; the opposite was true for growth rates determined using the mass balance approach (Figure 15). While there was little difference between approaches for the control reactors, there was a significant difference between the two methods for the chlorinated reactor biofilm growth rates (Figure 15); growth rates based on the mass balance technique for the chlorinated reactor biofilm were greater than those based on leucine uptake.

A comparison of growth rates based on the mass balance and leucine uptake methods for the replicate 3 experiment (the only replicate with leucine uptake data) is presented in Figure 78. As shown in the figure, the leucine uptake technique resulted in significantly lower values of μ for the chlorinated reactors than did the mass balance technique; a result consistent with the comparison of growth rates using leucine uptake with growth rates based on data from all replicate experiments for the mass balance technique.

Zero-order models provided good fits to the specific growth rates for the control and chlorinated biofilm using leucine uptake because there was little variation in values between the two carbon levels investigated (refer to Table 8).

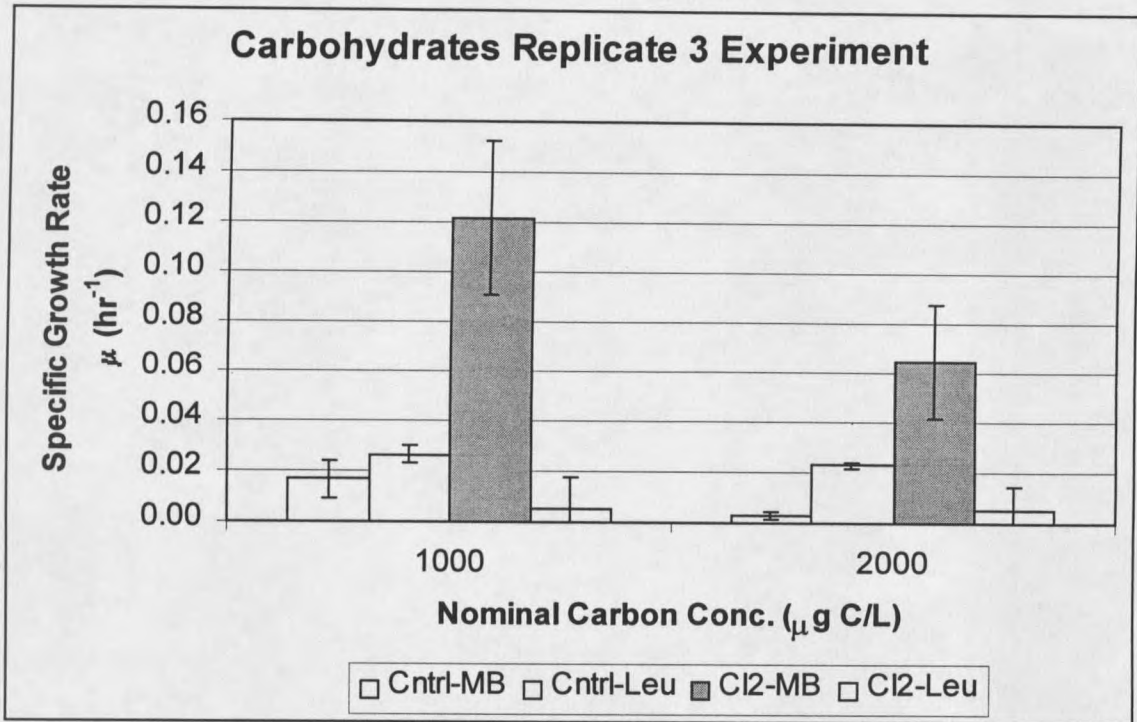


Figure 78. Comparison of first-order specific growth rates for the carbohydrates replicate 3 control and chlorinated biofilms using the mass balance (MB) and leucine uptake (Leu) methods. Error bars are based on one-way ANOVAs between measurement techniques at each carbon level and indicate the 95% confidence limits.

Mixed Substrate–Leucine Uptake. The trend in growth rates based on leucine uptake for the control “a” reactor were the same as for the chlorinated “a” reactor; growth rates decreased with increasing substrate concentration. The trend in leucine-uptake-based growth rates for the control “a” reactor was the same as determined using the mass balance approach, but the trend in leucine-uptake-based growth rates for the chlorinated “a” reactor was opposite that determined using the mass balance approach (Figure 79). Similar trends were noted above for leucine-uptake-based growth rates for the carbohydrates reactors; growth rates for the chlorinated reactor biofilm using the mass

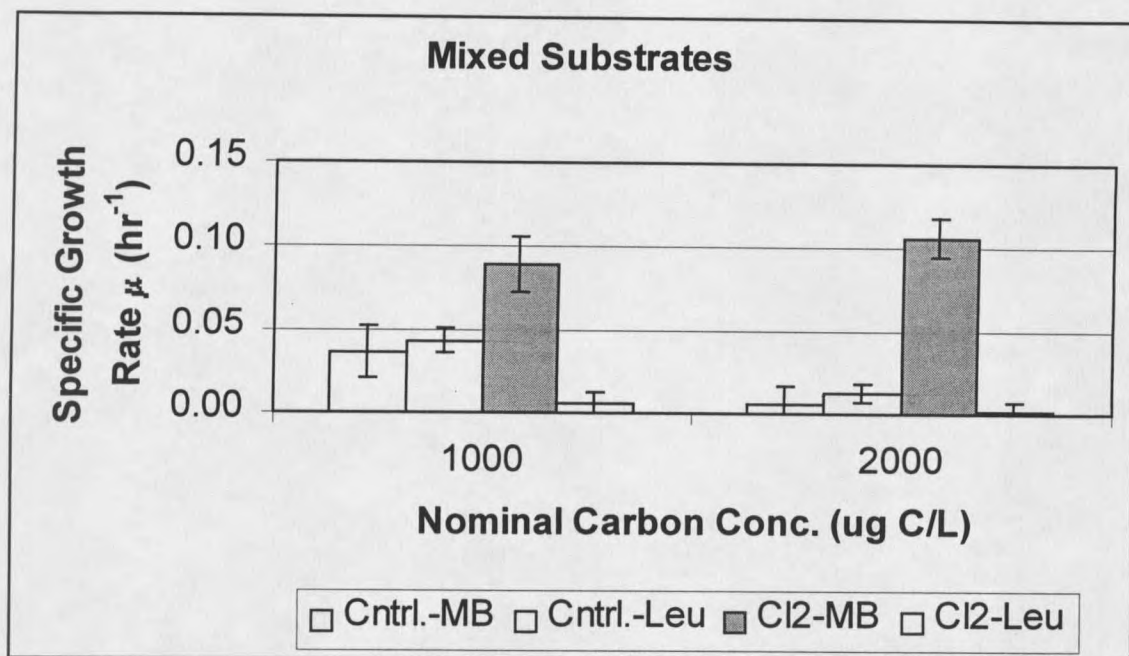


Figure 79. Comparison of first-order specific growth rates for the mixed substrates control and chlorinated biofilms using the mass balance (MB) and leucine uptake (Leu) methods at the 1000 and 2000 carbon levels. Error bars are based on one-way ANOVAs between measurement techniques at each carbon level and indicate the 95% confidence limits.

balance technique resulted in a positive μ -S correlation and the leucine uptake method resulted in a negative μ -S correlation. In the "a" reactors leucine-uptake-based growth rates were less in the chlorinated reactor than in the control, a finding consistent with leucine-uptake-based results for amino acids and carbohydrates reactors. A comparison of specific growth rates by reactor and measurement technique is presented in Figure 79.

At a given carbon feed level, leucine-based growth rates were not dependent upon the substrate concentration used during sample incubation. This was also seen in samples for amino acids and carbohydrates; zero-order kinetics applied at a specific carbon feed

level. However, since leucine-uptake-based growth rates generally decreased with increasing substrate concentration, an inhibition model as shown in Table 8 could describe the negative trend in μ versus S . As will be discussed below, this trend may be explained by failure to account for adsorption of leucine by humic substances.

Overall Discussion of Growth Rates Determined Using Leucine Uptake. Two trends were seen in growth rates determined using leucine uptake that were not consistent with growth rates based on the mass balance approach. First, chlorinated reactor biofilm growth rates were less than for the control reactor biofilm. Second, growth rates decreased with increasing substrate concentration (as carbon) for the carbohydrates and mixed substrates. This was most noticeable in the mixed substrate chlorinated reactor. These two trends in the findings will be discussed in further detail below.

Leucine uptake tests were designed to match conditions within the annular reactor as closely as possible. The two conditions that were different in the sample vials were hydraulics (shear stress and mass transfer limitations) and, for biofilm from the chlorinated reactor, no chlorine in the liquid. The following paragraphs summarize the conditions used for measuring leucine uptake by the attached biofilm:

The liquid mixture in the sample vials matched that in the reactors with varying concentrations of substrate. However, the sample vials were free of chlorine. Shear stress and mass transfer conditions in the sample vial were different from those in the reactor. Tests performed to evaluate if mass transfer limitations

would occur in the sample vials indicated that incubation on the shaking table would overcome such limitations.

Substrate concentrations were selected to provide a range around that in the reactor effluent; typically one concentration was about the same as the effluent and the others ranged lower and higher, up to the amount being fed to the reactor.

Minimal disturbance of the attached biofilm while making a direct measurement of leucine uptake. Biofilm were kept wet at all times while cutting slides into smaller sample pieces. Biofilm samples were placed face down and floating in sterile buffer until the sample was placed into the sample vial.

The 60-minute incubation time utilized for measuring leucine uptake, typical of research using this technique, was short enough to minimize changes in the physiological characteristics of the biofilm cells, which has doubling times of 7 hours or longer.

Knowing the conditions used for determining leucine uptake by the attached biofilm, possible explanations are presented below for the differences and similarities between leucine-uptake-based growth rates and those based on the mass balance technique.

Impacts of Chlorination. Leucine-uptake-based growth rates for chlorinated reactor biofilm was always less than for the control reactor biofilm. The opposite relationship was found for growth rates based on the mass balance technique. The results

of this research indicate that factors related to the biofilm history may influence the ability of biofilm cells to assimilate leucine. Two factors in the history of the biofilm that may be important are (1) the dominant substrate type and (2) the presence of chlorine.

Growth rates determined using leucine uptake for the amino acids were significantly greater than growth rates based on mass balance (see Figures 6 and 77). Leucine-uptake-based growth rates for biofilm using amino acids were greater than growth rates for carbohydrates and mixed substrates (refer to Figure 22). Using the mass balance approach growth rates for carbohydrates and mixed substrates were much greater than those from the amino acids reactors under chlorinated conditions (see Figure 21). Biofilm cells from the amino acids reactors may have developed uptake systems that had adapted for efficient amino acid uptake. This may explain why the leucine-uptake-based growth rates for amino acids were higher when compared to leucine-uptake-based growth rates for carbohydrates and mixed substrates. Biofilm cells from the carbohydrates and mixed substrates reactors may not have been as well adapted for uptake of free amino acids. Although free amino acids were mixed with carbohydrates and humic substances to form the mixed substrate, the amino acids may have attached to the humic material, making them less available to the cells, or the attached humic material may have retarded their diffusion.

The chlorinated biofilm growth rates based on leucine uptake were always less than the corresponding control biofilm growth rate, suggesting that chlorination may create an environment where the ability of biofilm cells to take up leucine and/or the

cells' need to take up leucine, have been altered. Hypochlorous acid has been shown to inhibit cell transport systems (Thomas, E. L., 1979a; Thomas, E. L., 1979b) and inhibit electron transport (Barrette, W. C. Jr. et al., 1987) in *Escherichia coli* cells.

Hypochlorous acid can impair uptake systems capable of transporting amino acids to the cell. If biofilm cells had been directly exposed to hypochlorous acid, then those cells could have reduced ability to transport leucine.

If cells attach to surfaces and create biofilm as a protective measure against chlorine then the action of free chlorine on the cell may not be a factor in the majority of the cells (i.e., the cells do not "see" chlorine). Free chlorine will react rapidly with free amino acids to form N-chloro compounds. Amino acids and free chlorine outside a biofilm cell could lead to killing conditions. The work by Thomas (1979b; 1979a) with *E. coli* cells indicated that the presence of any nitrogen-chlorine derivatives were extremely detrimental to the cell since these derivatives contained chloramine- and chloramide-type substances. These N-Cl derivatives had a long-lasting effect, oxidizing peptide bonds in the membrane, oxidizing bacterial sulfhydryls (eventually killing the cell) or oxidizing other cell components. Biofilm cells in such a chlorinated oligotrophic environment may not need or possess efficient amino acid uptake systems since the concentration of free amino acids should be minimal. This may explain why leucine uptake rates were low for all chlorinated biofilm samples.

Impacts of Carbon Concentration. Leucine-uptake-based growth rates decreased with increasing carbon concentrations, particularly in the mixed substrate data, but also in

the carbohydrate data. The same reasons discussed above for the mass balance approach could apply to the data based on leucine uptake: (1) attachment of humic substances to the biofilm matrix, increasing with biofilm age, and/or (2) inhibition by increasing concentrations of substrates. The coupling of either of these two effects with a reduction in leucine uptake capability by chlorinated cells could account for the wide disparity in growth rates for the two methods when applied to the chlorinated reactor data.

Leucine adsorption to humic substances could also account for decreasing growth rates with higher substrate concentrations for the mixed substrate reactor biofilm. Researchers working with humics have noted this problem previously with tritiated thymidine (Tranvik, L. J. and Höfle, M. G., 1987). The mixed substrates contained 50% humic substances as carbon, which may have adsorbed increasing amounts of leucine with increasing concentration of mixed substrates. Leucine available for uptake and calculated growth rates would then be reduced.

Evidence for leucine adsorption by humic substances was seen by examining the effect of substrate concentration on counts for the killed samples. Killed samples containing higher concentrations of mixed substrates typically had lower counts (as disintegrations per minute, dpm). The protocol for killed samples delayed the addition of ^3H -leucine to allow cold-leucine to saturate adsorption sites. When this was not done killed sample counts, particularly for chlorinated biofilm samples, were extremely high. If sufficient concentrations of cold-leucine were not added, leucine could have adsorbed to humic substances making less leucine available to adsorb to the killed biofilm,

resulting in lower counts for the killed sample. In non-killed samples used to determine growth there would be less leucine available for uptake and calculated growth rates would be low. Adsorption of leucine by humic substances could have been taken into account by using in the saturation experiment the highest substrate concentration to be tested for growth rates, thus assuring enough leucine was added to overcome any adsorption by the substrate. Unfortunately this was not recognized during the experiments.

While leucine adsorption to humics may partially explain the discrepancy in leucine-uptake-based growth rates for the mixed substrates, humic substances were not added to the sample liquid for carbohydrates. The variations in killed-sample counts were much less for carbohydrate samples, indicating that interference between the substrate and leucine was most likely not a factor for the carbohydrate experiment.

Batch Culture Growth Rates

In Chapter 4, batch culture growth rates were presented based on total direct cell count (TDC), heterotrophic plate count (HPC) and nonlinear regression (NLR) of the substrate depletion data. The following paragraphs will discuss the information provided by batch culture growth rates using these methods, as well as differences and similarities between growth rates using these methods. Comparison of growth rates for individual compounds within a substrate group provided insightful information on differences between biofilm cells grown in chlorinated conditions compared to those from a non-chlorinated environment. The combination substrate batch culture was used to compare growth rates from the amino acid and carbohydrate batch cultures to growth rates

determined for reactors using the mass balance method. Comparisons were made between growth rate measurement methods by examining the 95% confidence limits on the means.

Amino Acids – Batch Culture. Glutamate as a single substrate resulted in the highest growth rate based on TDC for both the control and chlorinated batch cultures, and growth rates determined using HPC data indicated the same trends within each set of reactor cultures. A comparison of growth rates for the combination substrate cultures (Figures 55 and 57) showed the growth rate for the control reactor culture was greater than the chlorinated reactor culture based on TDC and the difference was significant, but there was little difference between the chlorinated and control reactor cultures using HPC.

Growth models based on ^3H -leucine uptake also indicated that glutamate, when compared to aspartate and alanine, was the only amino acid with a positive effect for both reactor types. The growth rate response of attached biofilm cells to the individual substrates was similar to the response determined by using biofilm cells in batch culture. However, the attached biofilm tests using ^3H -leucine gave considerably more information than the batch culture method regarding possible interactions between substrates. For instance, interactions between amino acids were important for the chlorinated biofilm growth model, providing an indication that substrate uptake mechanisms for chlorinated biofilm cells were different than for control biofilm cells.

Growth rates based on ^3H -leucine uptake by attached biofilm compared well with the batch culture growth rates calculated using TDC. The limited data using the batch-

HPC method gave values similar to the leucine and batch-TDC methods for the control reactor, but resulted in much higher values for the chlorinated culture. It was noted that cells from chlorinated biofilm were typically hard to culture initially using R2A medium, and the lag time was longer for chlorinated biofilm batch cultures than for the control cultures. The lag period was most likely required for cell repair and/or physiological acclimation to the batch culture medium. Based on the HPC data, once growth started it was quite rapid, resulting in high growth rates. A similar response to the effects of chlorine on biofilm was seen in the actual reactors. In the mixed substrate sequential reactor experiment, cells that had been exposed to high doses of chlorine ("a" reactor) and placed in a low to zero chlorine environment ("b" reactor) were able to develop biofilm and grow at rates equal to biofilm in a nonchlorinated environment. Therefore, growth of cells in the chlorinated biofilm batch culture based on HPC may be more representative of recovery of biofilm cells after repair and acclimation rather than growth following adaptation to a chlorinated biofilm environment.

Growth rates based on nonlinear regression using the integrated Monod equation were presented in Figures 55 and 57. With the exception of the chlorinated batch culture growth rates calculated using TDC, growth rates based on NLR results were generally much less than mean growth rates for the batch cultures using TDC or HPC. Based on comparison of growth rates using NLR results to growth rates for the reactors using mass balance (see Figure 63), NLR did not result in comparable growth rates for amino acids for either reactor.

Carbohydrates – Batch Culture. With the exception of growth rates for the glucose culture, general trends in growth rates between control and chlorinated batch culture growth rates were similar when TDC was used to estimate growth. Batch cultures with galactose and galacturonic acid resulted in growth rates higher than for the other compounds in both the control and chlorinated reactor. Glucose gave one of the highest growth rates among the chlorinated batch culture and a low growth rate in the control batch culture. Arabinose resulted in the lowest growth rate for both the control and chlorinated batch cultures.

Growth rates based on HPC data resulted in different trends between compounds and between reactors compared to growth rates using TDC. The major difference between the two methods was most likely the lack of sufficient data plus the phenomenon noted above for the chlorinated batch cultures, a long lag phase followed by very rapid growth. Growth rates for the carbohydrate combination control and chlorinated batch cultures were nearly the same based on TDC, but significantly different (control < chlorinated) using HPC. As noted above for the amino acids batch cultures using cells from the chlorinated biofilm, the HPC method did not appear feasible for determining growth rates batch cultures of previously chlorinated cells.

In low nutrient environments, such as drinking water, microorganisms are capable of simultaneous utilization of a number of different substrates. Lendenmann et al. (1996) has shown this to be the case for mixtures of carbohydrates. The presence of mixtures of sugars can enhance the ability of microorganisms to take up low concentrations of sugars

(Egli, T. et al., 1993; Lendenmann, U. et al., 1996). Lendenmann et al. (1996) found that *Salmonella typhimurium* was able to take up glucose at low concentrations if arabinose was present. When *Escherichia coli* cells grown in chemostats using glucose as a medium were placed into batch culture containing only arabinose, there was a lag period before growth was observed in the culture, but no lag period occurred for cultures that contained both glucose and galactose (Lendenmann, U. and Egli, T., 1995). In the carbohydrates batch cultures, low growth rates were noted for the biofilm cell batch cultures using arabinose as the substrate. Only in the chlorinated batch culture using HPC data were growth rates for the combination culture greater than in all the individual compound batch cultures.

The means for the combination control and chlorinated batch cultures using TDC were not statistically different (Figure 59). Chlorination may not have had as great an impact on scraped biofilm cell growth rates as for the amino acids. Biofilm growth rates in the reactors based on mass balance using TDC also indicated chlorination did not reduce growth rates.

Humics – Batch Culture. When placed in batch cultures, the growth rate for biofilm cells from the humics reactors was nearly independent of the initial humic substance concentration in the batch cultures. A zero-order relationship between specific growth rate and initial substrate concentration applies to the growth of biofilm cells placed in batch cultures as well as to growth rates based on mass balance around the reactors if data from the 500 carbon level are not included. Actual growth rates for the

control reactor biofilm cells were higher than for chlorinated reactor biofilm cells by the batch culture method, but the opposite was true for the mass balance method. This finding makes use of batch cultures questionable when determining growth rates for biofilm where humic substances are the substrate. As discussed above, the attachment of humic substances to the biofilm at the biofilm/water interface is an important factor in growth in natural environments, and it cannot be duplicated in a batch culture.

Growth rates based on results from NLR of the integrated Monod equation were presented in Figure 62. Of the techniques used to estimate growth rates in the humics batch cultures, NLR resulted in the lowest growth rates, but NLR results in the chlorinated cell batch cultures were nearly the same as for the TDC method (see Figure 62, bottom).

Kaplan and Bott (1983) reported mean generation times of 12.5 to 46.2 hours ($\mu = 0.056$ to 0.015 hr^{-1}) at 15°C for bacteria in stream sediments exposed to a variety of high molecular weight compounds. Tranvik (1988), investigating growth of natural microbial communities in lakes of various humic content, determined growth rates from 0.05 to 0.11 hr^{-1} at 17°C . Bano et al. (1997) determined growth rates from 0.026 to 0.044 hr^{-1} for natural microbial communities utilizing humic substances as the substrate. Growth rates ranged from 0.027 to 0.054 hr^{-1} when a natural microbial community from a humic lake was incubated in batch culture with humics from the same lake (Tranvik, L. J. and Höfle, M. G., 1987). Growth rates determined in humics batch cultures for this project compare well with the range of values seen in the literature for planktonic cells. However, growth

rates based on mass balance around reactors were much lower than the reported values for planktonic cells. This indicates the natural environment created by the biofilm and attached humic substances are important when determining growth rates for biofilm exposed to humic substances. Biofilm cells placed in suspended cell batch cultures are removed from the natural biofilm community and environment and most likely do not exhibit the same growth characteristics.

Comparison by Substrate – Batch Culture

In batch culture, amino acids as a substrate resulted in the highest growth rates and humics the lowest (Figure 80). Growth rates in batch cultures using biofilm cells from the chlorinated reactor were always less than for the control batch cultures. Although only the humics batch cultures showed a significant difference between the means for the control and chlorinated cultures (95% CI), there were no statistical differences between mean control versus chlorinated growth rates for the amino acids and carbohydrates cultures.

Growth rates based on reactor mass balances indicated carbohydrates had the highest growth rates (see Figure 21), and the chlorinated humics growth rates were greater than for the control (see Figure 16). Batch cultures using chlorinated biofilm cells did not provide results relevant to the environment within the reactor and this was particularly evident for the chlorinated cultures.

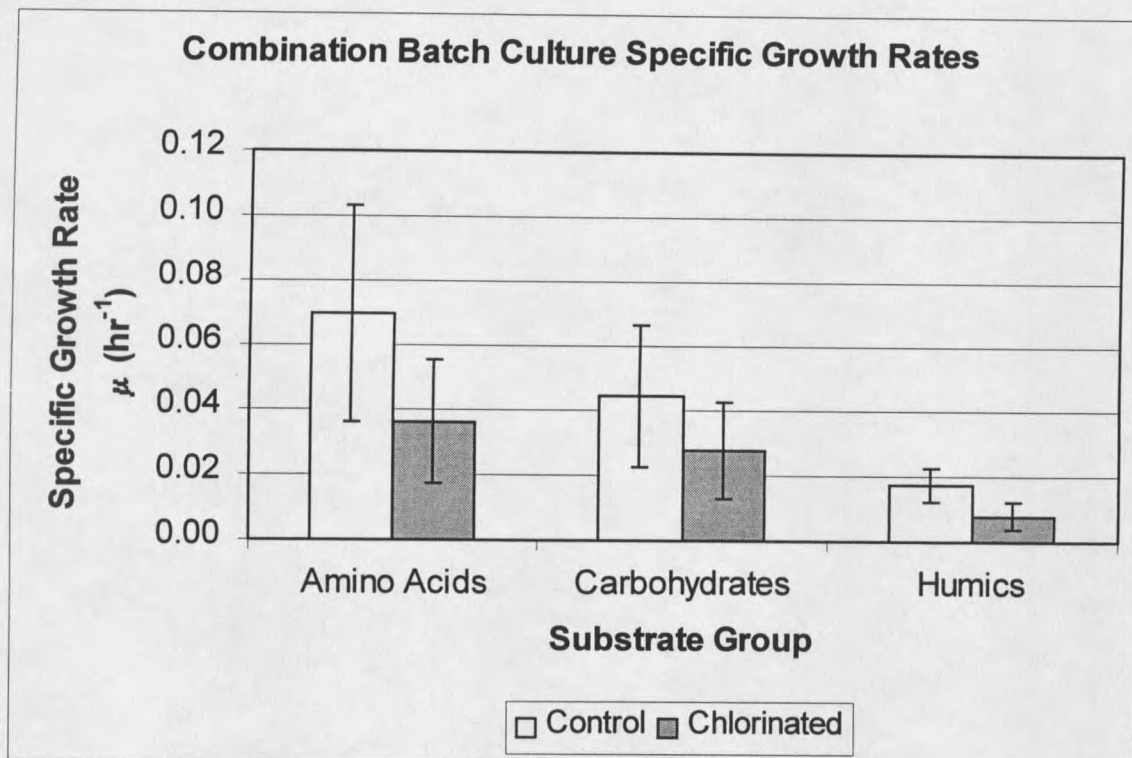


Figure 80. Comparison of mean specific growth rates for the amino acids and carbohydrates combination batch cultures and the humics batch cultures. Growth rates are based on total direct cell count data. Error bars represent 95% confidence intervals for the mean.

Comparison of Reactor to Batch Culture Growth Rates

It was desired to determine if the batch culture technique, utilizing biofilm cells in suspended culture, was an appropriate method for estimating the specific growth rate of the biofilm. Therefore, the specific growth rates for the combined substrate batch cultures were compared to growth rates determined for the reactors utilizing the mass balance approach. Only growth rates from the combination substrate batch culture could be compared to reactor mass balance growth rates since the combination substrate was the

same as was fed to the reactors. The comparisons are discussed below for each substrate group.

Amino Acids. Growth rates determined for the amino acids combination batch cultures were compared to growth rates for the at the 2000 $\mu\text{g/L}$ carbon feed level, replicates 1 and 2 only. The data, shown in Figure 81, indicate that there were no significant differences in growth rates for the various methods when measurement error was taken into account with the exception of the growth rate for the control based on NLR. The NLR-based growth rate was less than for all other methods (Figure 81). Although 95% confidence limits were not available for the batch-HPC method, even a conservative estimate of 50% of the mean would result in no significant difference for the four methods other than noted. The mean growth rates using NLR do not appear to compare well with the reactor mass balance data. The leucine uptake based growth rates are not shown in Figure 81 because the data was from replicate 3, but the data, if shown, would be within the error ranges of all other data shown in Figure 81.

While growth rates noted above may have compared well based on a statistical basis, the relationship between control and chlorinated biofilm growth rates, as determined using mass balance around reactors, was not always valid for the batch culture techniques. Growth rates for chlorinated biofilm cells in batch culture were less than in the control, a result opposite that seen using the reactor mass balance data. The interactions between chlorine, amino acids, dilution water, and biofilm cannot be

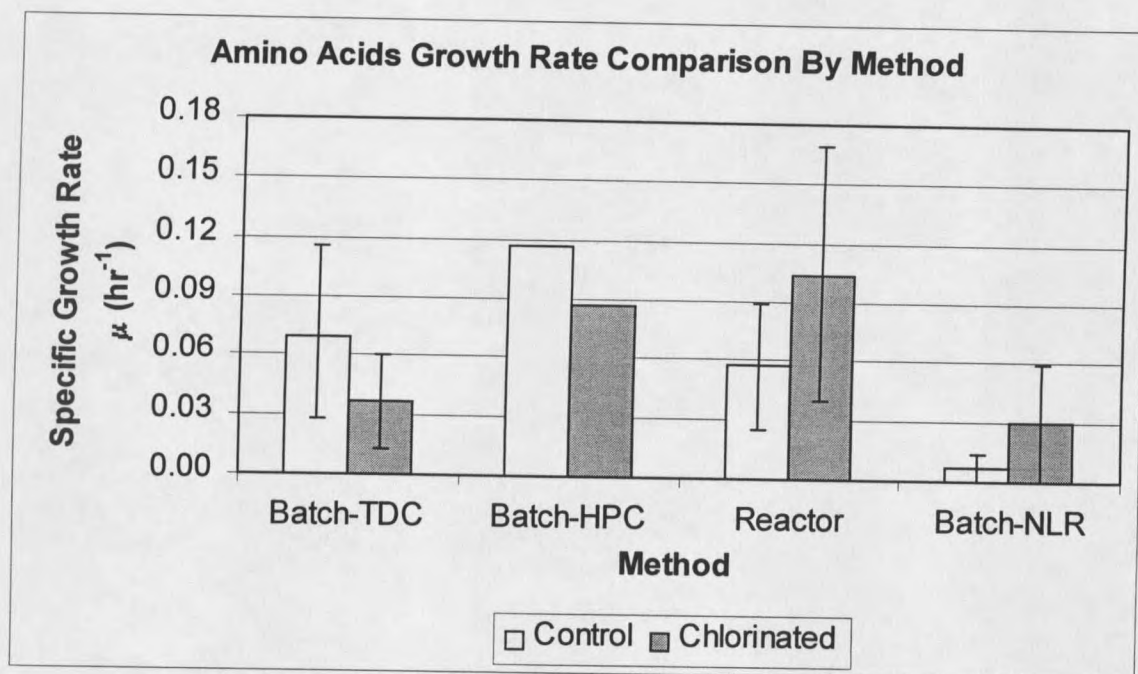


Figure 81. Comparison of specific growth rates for the amino acids control and chlorinated biofilms using various methods. The Batch-TDC, Batch-HPC and Batch-NLR methods are for the biofilm cell combination substrate batch cultures, reactors at 2000 carbon level, means for replicates 1 and 2. Reactor values are means based on reactor mass balances at the 2000 carbon level for replicates 1 and 2. Vertical error bars indicate 95% confidence limits. TDC = total direct cell count; HPC = heterotrophic plate count, and NLR = nonlinear regression.

duplicated in batch culture. These interactions appear important when determining growth rates for chlorinated biofilm.

Carbohydrates. A comparison of biofilm growth rates by method is shown in Figure 82 for the combination carbohydrates batch culture using TDC and HPC, and for the reactors using the mass balance approach. There were no significant differences in

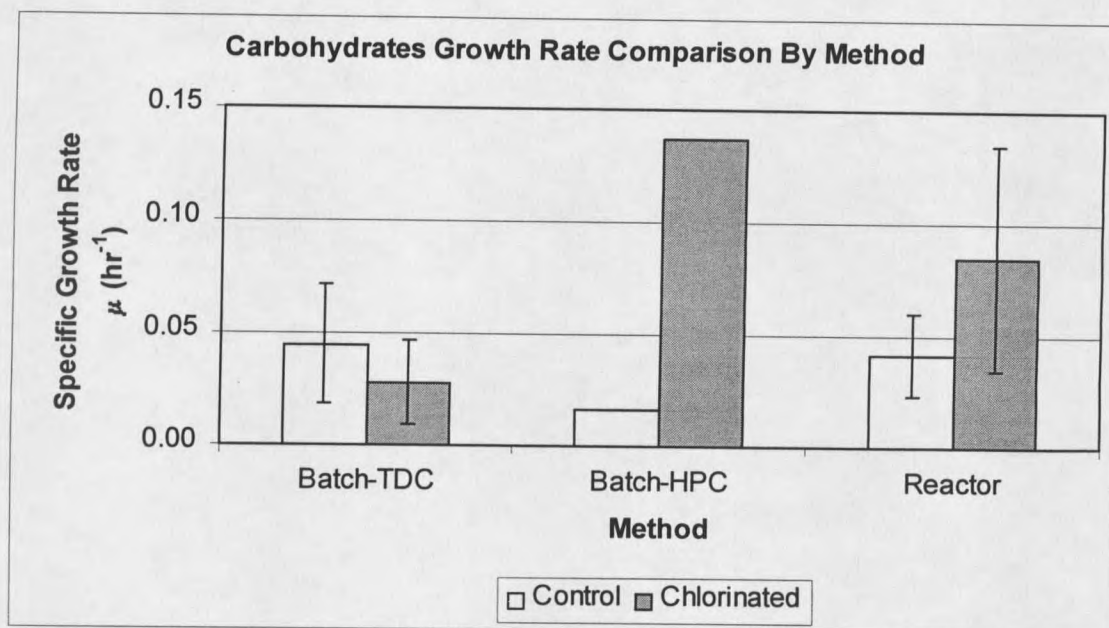


Figure 82. Comparison of specific growth rates for the carbohydrates control and chlorinated biofilms using various methods. The Batch-TDC and Batch-HPC methods were used for the biofilm cell combination substrate batch cultures, reactors at 2000 carbon level, replicates 1 and 2. Reactor values are based on reactor mass balances at the 2000 carbon level, replicates 1 and 2. Vertical errorbars indicate 95% confidence limits. TDC = total direct cell count and HPC = heterotrophic plate count.

specific growth rates using the batch-TDC method and growth rates determined for the reactors. The batch HPC method resulted in an extremely high estimate of growth rate for the chlorinated biofilm and the lowest growth rate of the three methods for the control biofilm. The limited data available for the carbohydrates batch HPC method is most likely the reason for the variable results. Problems with culturability of chlorinated biofilm cells, as noted above, may have been the reason for the high growth rate in the chlorinated batch culture using the HPC method.

The batch-TDC method results indicated chlorinated culture growth rates were less than in the control, opposite the results from the reactor mass balance results. The interactions between chlorine, carbohydrates, dilution water and biofilm appear to be important for carbohydrates also.

Humics. The reactor mass balance approach resulted in the lowest specific growth rates for biofilm utilizing humic substances (see Figure 83). The TDC and NLR methods for batch cultures resulted in growth rates closest to those based on reactor mass balance. However, within the limits of the 95% confidence intervals, the batch culture mean values were not equal to those from the mass balance approach. All batch culture techniques for growth rate resulted in lower values for the chlorinated biofilm batch cultures than for the control, opposite to the result seen for the reactor mass balance approach. The leucine and HPC methods were similar to each other and the growth rates were significantly greater than for the other methods (TDC, TDC/Biomass, NLR and reactor).

Assuming the reactor mass balance approach to be more representative of actual biofilm growth rates, the significant difference in batch culture growth rates compared to reactor growth rates suggests that batch cultures are not suited for determining growth rates for biofilm cells utilizing humic substances as the substrate. Attachment of humic substances to the biofilm was noted in both reactor biofilm samples. The interaction

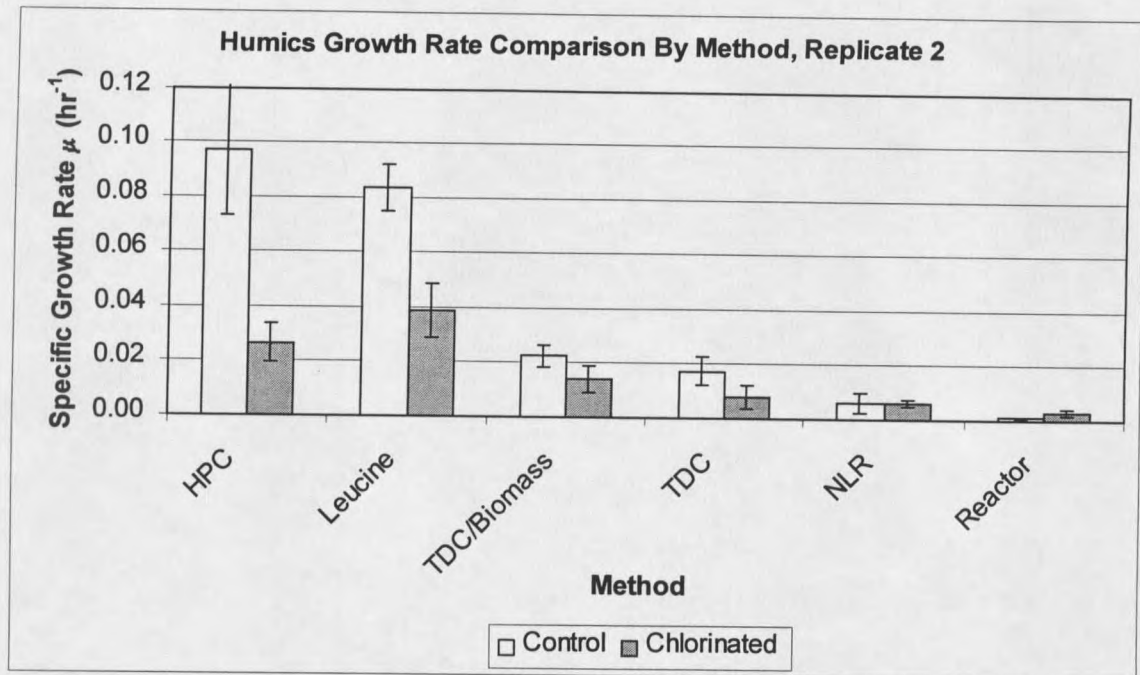


Figure 83. Comparison of specific growth rates for the humics control and chlorinated biofilms using various methods. HPC, leucine uptake, TDC/Biomass, TDC and NLR are mean values for the humics replicate 2 batch cultures. Growth rates for the reactor are based on mass balances at the 2000 $\mu\text{g C/L}$ carbon level, replicate 2. Vertical error bars indicate 95% confidence limits. TDC = total direct cell count; HPC = heterotrophic plate count, and NLR = nonlinear regression.

between the biofilm cells and the attached or sorbed humic materials may have controlled growth of the biofilm, with little effect by bulk fluid humics on the biofilm growth rate once the humic substance functional groups bound to all available sites. It is speculated the biofilm produced enzymes capable of breaking away from the humic structure the carbon, nitrogen and phosphorous moieties required for energy and growth. Chlorination may have aided the process by making the humic material more amenable to enzymatic attack or by providing substrates the biofilm can use directly (Hanna, J. V. et al., 1991; Bruchet, A. et al., 1990). When biofilm cells were placed in batch culture the constraints

found in a biofilm (mass transfer limitation from the bulk fluid to the biofilm, limitation of substrate to the sorbed or attached humic material) were removed. Based on the data summarized in Figure 83, the cells in batch culture were able to more readily utilize the humic material.

Attachment of humic substances to the biofilm matrix does not necessarily mean biofilm cells will have an infinite supply of nutrients, resulting in higher growth rates than when cells are placed in a suspended culture. Sites for humics attachment to the biofilm are limited, thus restricting the amount of humics attached and the supply of substrate. In natural water environments humic substances are attached to the surfaces of all particles (Beckett, R., 1990). Models for adsorption of humics to both positive and negatively charged surfaces have been developed (Beckett, R., 1990). In addition to surface charge characteristics, factors that influence humics adsorption are pH and the concentration of divalent cations, particularly Ca^{2+} and Mg^{2+} (Tipping, E., 1981). A biofilm cell adept at producing extracellular enzymes, when placed in an environment where mixing promotes surface collisions with and adsorption of humic substances, may be capable of greater rates of growth than when in the biofilm environment. The zero-order nature of growth rates in the batch cultures indicate that while conditions for growth may be improved in suspended culture, there were still limiting factors to growth independent of substrate concentration. This can be expected if adsorption of humic molecules to the cell surface and enzymatic breakdown becomes controlling factors.

Substrate Uptake

Reactor Carbon Removal Rates

The general relationship between carbon removal flux (carbon removed per unit area of biofilm per time) and carbon loading rate was linear in all reactors. The slope of the linear relationship indicates the fraction of the influent carbon removed within reactors that had detention times of approximately 2.1 hours and area-to-volume (A/V) ratios of $2.72 \text{ cm}^2/\text{cm}^3$. Figure 44 in Chapter 4 showed a comparison of the removal fraction for the cases investigated (see Table 10 for details of the regressions). The reactors with amino acids as the substrate showed complete or nearly complete removal of the influent amino acids for both the control and the chlorinated reactors. A large difference in carbon removal was seen in the carbohydrate reactors; removal in the chlorinated reactor was lower than for the control and the lowest for any substrate group. Conversely, on a normalized or specific substrate uptake basis (μg substrate C/ μg cell C/hr) the chlorinated biofilm using carbohydrates had the greatest normalized uptake rate.

Amino Acids. The carbon removal fractions in the amino acids reactors were very high, 0.956 and 0.901 for the control and chlorinated reactors, respectively, indicating that the biofilm cells were able to utilize nearly all added amino acids. The mean normalized carbon removal rate for the chlorinated reactor was nearly 7 times greater than in the control reactor (0.487 and 0.0741 hr^{-1} , respectively). Normalized carbon removal rates for the amino acids control and chlorinated reactors exhibited

negative correlation with the effluent substrate concentration (-0.073 and -0.469, respectively), but the correlations were not significant.

High removal fractions in the amino acid reactors were expected. Free amino acids are labile substrates, very amenable to biodegradation by natural microbial populations. Free amino acids can be used directly by the cell without extracellular enzymatic action by the cell. Researchers have noted that attached bacteria have higher uptake rates for amino acids than free-living bacteria in marine environments (Simon, M., 1985; Bright, J. J. and Fletcher, M., 1983a). In natural water systems free amino acids have been proven to actually be present and free (not all amino acids are complexed with humic substances), readily available to bacteria for uptake (Jørgensen, N. O. G. and Søndergaard, M., 1984). Pedogenic and aquagenic proteinaceous compounds make up 5 to 10 percent of natural organic matter in water (Krasner, S. W. et al., 1996). The concentration of proteinaceous compounds can be greater in natural waters receiving discharges from municipal wastewater treatment plants or agricultural runoff. Combined amino acids in the form of proteins are common in natural waters and are easily degraded by enzymes excreted by natural microbial populations (Billén, G., 1991). Dissolved free amino acids (DFAA) may be a small percentage of the total dissolved organic carbon pool in natural waters. Volk et al. (1997) found amino acids were 2-6% of the total biodegradable dissolved organic carbon (BDOC) in streamwater and 94-100% of the amino acids associated with BDOC were bound to humic substances. Amino acids in drinking water can be expected to be easily and readily utilized by biofilm.

There was nearly complete removal of amino acids across the chlorinated reactor. This was not expected because amino acids react quickly with chlorine to form N-chloro compounds such as mono- and dichloramines with further reaction to form the aldehyde and nitrile of the amino acids (Trehy, M. L. et al., 1986; Choshen, E. et al., 1990; Trehy, M. L. and Yost, R. A., 1989; Le Cloirec, C. and Martin, G., 1985; Issac, R. A. and Morris, J. C., 1980). Two possible scenarios could help explain why all amino acids were removed in the chlorinated reactor: (1) although chlorine reacts with the amino acids the oxidation products may still be suitable substrates for the biofilm, and (2) amino acids were able to react with the biofilm before complete reaction with the chlorine. The uneven uptake of leucine as shown in Figure 9 may indicate the second case was possible in the chlorinated reactor.

Carbohydrates. A distinct difference was seen in the carbon removal fractions of the control and chlorinated carbohydrate reactors (0.657 and 0.170, respectively). Chlorination appeared to significantly reduce the overall uptake of carbohydrate carbon compared to the control reactors based on a fractional removal basis. However, the mean normalized carbon uptake rate for the chlorinated reactor biofilm was approximately 9 times greater than for the control biofilm (2.69 and 0.304 hr⁻¹, respectively). There was a positive, but not significant, correlation between normalized uptake rates and the effluent substrate concentration for both the control and chlorinated reactor data (0.099 and 0.237, respectively). The more positive correlation for the chlorinated reactor normalized uptake rates may have been the result of a 50% decrease in biofilm biomass for the

chlorinated reactors between the 1000 and 2000 carbon levels (0.0274 to 0.0128 $\mu\text{g cell C/cm}^2$). The increase in chlorinated reactor biofilm growth rates between the 1000 and 2000 carbon levels would follow the trend of increasing specific carbon removal rate for the chlorinated reactor biofilm. The carbohydrate reactors' biofilm appeared to compensate for the actions of chlorine by increasing its specific carbon uptake and growth rates without increasing biomass.

The high normalized substrate uptake rates for the chlorinated reactor biofilm were examined to determine if potential errors could have influenced the results. As was stated for the growth rate determination, errors in biofilm biomass estimation could potentially account for the apparent high values for uptake (low estimates of the biofilm biomass would lead to overestimation of normalized uptake rates). The high normalized uptake values were noted after analysis of data from the first two replicate experiments. A third replicate experiment was performed to help verify results of the first two experiments. Additional NPOC and total direct count data were collected and analyzed to insure errors were minimized. As was done for the amino acids replicate 3 and mixed substrate experiments, biofilm sample slides were cut into three sections, each section scraped separately and total direct count and cell size information determined for each section of the sample slide. This technique was implemented to help minimize errors in determining biofilm biomass. The results of the carbohydrates replicate 3 experiment verified the previous results, i.e., the normalized substrate uptake in the chlorinated reactor was high compared to the carbohydrates control reactor.

One mechanism discussed above for protection of biofilm cells against the actions of free chlorine was the production of EPS to either protect the cell from chlorine by creating a diffusion barrier or providing a sacrificial compound for the oxidation effects of chlorine. EPS consists primarily of polysaccharides that have been shown to react with hypochlorite (Characklis, W. G., 1990). Given an ample supply of free carbohydrates, it may be possible that the biofilm cells had adapted to produce large amounts of EPS as sacrificial compounds, providing protection against the effects of chlorine. A survival scheme such as this could require uptake and conversion of large amounts of carbohydrates into EPS.

In general, carbohydrates compose a larger fraction of dissolved organic carbon than amino acids in natural waters. Volk (1997) found that carbohydrates were 22-43% of the total BDOC in a stream, and 62-65% of the carbohydrates associated with BDOC were polysaccharides bound to humic substances. The results presented previously show that dissolved free carbohydrates (DFCHO) are readily taken up by drinking water biofilm when chlorine is not present, but that chlorine plays a role in carbohydrate uptake, apparently causing an increase in specific carbon uptake rates.

Humic Substances. Fractional carbon removal was greater in the humics control reactors than in the humics chlorinated reactors but the difference was not significant (see Figure 46). Normalized carbon removal rates, however, were greater in the chlorinated humics reactors than in the control reactors (0.382 and 0.141 hr^{-1} , respectively). This follows the trend seen in biofilm growth rates for the humics reactors; higher in the

chlorinated reactors than in the control. Total biofilm biomass in the humics chlorinated reactor biofilm was less than in the humics control biofilm (2.56 and 0.549 $\mu\text{g cell C/cm}^2$, control and chlorinated biofilm, respectively). Chlorination of biofilm using humic substances appears to stimulate both the specific carbon uptake rate and growth rates in drinking water biofilm. However, in the humics reactors chlorination reduced the number of total biofilm cells and biomass compared to the control situation where no chlorine was present.

Possible reasons for higher normalized uptake of substrate in chlorinated humics reactors are similar to the reasons discussed above for growth rates. The interaction of chlorine with humic substances to create more easily degradable compounds may be one reason greater uptake rates are possible. The measurement of attached carbon indicated a higher ratio of attached non-cellular carbon to cell carbon in the chlorinated biofilm than in the control. The greater amount of attached carbon, the exact composition of which is not known, could provide protection of the biofilm cells from chlorine. It may also be a source of nutrients, with some being released by the action of chlorine on the humic molecules and its functional groups. As was discussed for the carbohydrates chlorinated reactor, biofilm cells in a chlorine environment may require more substrate for production of additional EPS as a protective mechanism against the action of chlorine.

Humic substances comprise the majority of the dissolved organic carbon in natural surface waters, making up 50-75% of the natural organic matter (NOM) (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. Volk et al. (1997) found humic substances to comprise approximately 75 percent of the total dissolved organic carbon (DOC) in a freshwater stream. Humic substances are a major contributor to the organic carbon found in drinking water and are therefore an important substrate to consider in assessing biostability.

Mixed Substrates. Fractional carbon removal was greater in the mixed substrates control reactors than in the chlorinated reactors (0.472 and 0.303, respectively), but the difference was not significant (see Figure 44). Normalized carbon removal rates, however, were greater in the chlorinated mixed substrate reactors than in the control reactors (0.316 and 0.0972 hr⁻¹, respectively). This follows the trend seen in biofilm growth rates for chlorinated mixed substrates, higher in the chlorinated reactors than in the control. Chlorination of biofilm using mixed substrates appears to stimulate both the specific carbon uptake rate and growth rates in drinking water biofilm.

Comparison by Substrate. Normalized substrate uptake rates for chlorinated reactors were greater than in the control reactors, and this difference was significant. This does not imply there was more biofilm biomass in the chlorinated reactors. Measurements indicated the opposite was true for chlorinated biofilm, less biomass than found in control biofilm.

Amino acids resulted in the highest carbon flux values and subsequent fractional removals of any substrate tested. The overall fractional removal in the amino acids reactors was statistically greater than measured for the other three substrates. A comparison of overall fractional removal in the carbohydrates, humics and mixed substrate reactors found there were no significant differences. Although amino acids are not typically a large percentage of dissolved organic matter, they can be easily degraded and are an important substrate in environments such as drinking water where nitrogenous forms of pollution may be occurring in the source water.

The overall comparison of normalized substrate uptake rates indicated the rates for biofilm in the carbohydrates reactors were significantly greater than for biofilm using other substrates. There were no significant differences between normalized uptake rates for the amino acids, humics and mixed substrate reactors. Very little difference existed between the humics and mixed substrates reactors. While free amino acids and carbohydrates were added to humic substances for the mixed substrate, these compounds did not appear to impact substrate uptake in the same manner as for the reactors with only the free amino acids or carbohydrates. No tests were performed to determine if the compounds remained "free" once mixed with the humics. Based on the uptake data it would appear either the compounds did not remain "free," complexing with the humics, or the effects of the humics were more significant to the biofilm environment and subsequent uptake of substrate.

Comparison by Reactor. For all substrate groups, normalized substrate uptake rates were greater by biofilm in chlorinated reactors than in control reactors, and the differences were significant. This does not imply there was more biofilm biomass in the chlorinated reactors. Measurements indicated the opposite was generally true for chlorinated biofilm, less biomass than found in the parallel control biofilm. Higher rates of carbon uptake by the chlorinated biofilm in all substrate groups indicated that when chlorine was present the biofilm cells required greater amounts of carbon per unit of biomass. The reasons for increased carbon requirements, as have been previously discussed, may be for increased EPS production or other cell functions associated with protection against the potential actions of chlorine on the cell.

Batch Culture Substrate Uptake

The original intent of the project was to assess substrate preference by measuring uptake in batch cultures. Traditional kinetics of microbial growth relates substrate uptake to growth using the term called yield, mass of cells per mass of substrate utilized (see Equation 10). Determining the rate of substrate uptake should therefore provide an indication of substrate preference by biofilm cells in batch culture as well as data for the proposed relationship between substrate uptake and growth. Substrate uptake rates were determined for the amino acids and humics batch cultures. Individual substrate measurements were done for the amino acids but not completed for the carbohydrates batch cultures. The humics batch culture provided data only for one substrate, humic substances, as filtered NPOC.

Amino Acids. Aspartate and serine were taken up at the highest rates in the control batch cultures while aspartate and alanine were taken up at the highest rates in the chlorinated reactor batch cultures. Alanine resulted in the lowest uptake rate in the control and serine had the lowest uptake rate in the chlorinated batch cultures.

A comparison of the batch culture uptake data with that from the work using tritiated amino acids and attached biofilm indicated differences in the results. The only similar conclusion was that both techniques measured high uptake rates for alanine using chlorinated biofilm. The results from the tests using tritiated amino acids have an advantage of being able to measure individual amino acid uptake while exposing the biofilm to a mixture of amino acids. The tritiated amino acid tests indicated uptake rates for alanine and glutamate were greater than for aspartate for the control biofilm, and alanine uptake was the highest for the chlorinated culture with glutamate uptake being the lowest.

Simon and Rosenstock (1992) determined dissolved free amino acid composition in a mesotrophic lake and within the cytoplasm of planktonic bacteria. Serine, glycine + threonine, and alanine were dominant dissolved free amino acids. Serine and glutamate, which varied in concentration by season, were dominant amino acids in the intracellular pool; indicating serine and glutamate were taken up to a greater extent by the bacteria in their natural environment. In the amino acids batch cultures the uptake of serine was the highest in the control cultures but alanine uptake was the lowest. Alanine and glutamate uptakes were the highest for the tritiated-amino acid test.

Humics Substances. The approach for measuring substrate uptake in the humics batch cultures was different than that utilized for the amino acids since there was only one substrate. A traditional batch culture technique was employed using different initial concentrations of the humics in each of four batch culture sets. The results provided data that were analyzed to determine if the biofilm cells in batch culture demonstrated different uptake characteristics compared to biofilm in the reactors. The results previously shown in Figure 67 indicate basic differences in the two biofilm cell populations evaluated in batch culture. Control reactor biofilm cells had a lower $V_{\max}$ than did the chlorinated reactor cells, indicating a lower maximum uptake rate at high substrate concentration. Both reactor cultures had similar half-saturation constants (K_T) when the data was fit using a hyperbolic function.

The slope of a line tangent to the hyperbolic curve near its origin (Figure 67) provides an indication of how well the cells are able to take up substrates in a low concentration environment. The greater the slope of the line (termed specific affinity (Button, D. K., 1983; Button, D. K., 1993; Button, D. K., 1985)) the better suited are the cells to a low nutrient environment. Specific affinity (a^0) is defined for a hyperbolic function as $V_{\max}/K_T$. The specific affinity of the chlorinated reactor cells was greater than for the control reactor, indicating the chlorinated reactor biofilm cells may be better suited to low nutrient conditions. The nutrient limited transport theory of Button (1983) would indicate the chlorinated cells have a greater number of membrane transporters, resulting in different $V_{\max}$ and specific affinity values without changing the Michaelis

constant K_T . This finding was consistent with the reactor data where normalized substrate uptake for the chlorinated biofilm was greater than for the control biofilm.

Contrary to what would be expected if traditional microbial growth kinetics applied, the humics batch culture substrate uptake data did not coincide with the batch culture growth data. Substrate uptake was greater in batch culture for biofilm cells from the chlorinated reactor, but growth rates in batch culture were greater for biofilm cells from the control reactor. However, growth rates based on mass balance were greater for the chlorinated reactors than for the control reactors. TDC data for the humics batch cultures showed a lag period of 48 to 72 hours for the chlorinated reactor biofilm cells and 20 to 29 hours for the control reactor biofilm cells. The substrate uptake data showed a lag period of 80 to 150 hours for the chlorinated reactor biofilm cells and 0 to 48 hours for the control reactor. Substrate uptake and growth occurred over the same general time period for the control batch cultures. However, in the chlorinated biofilm cell batch cultures, growth appeared to start much earlier than did substrate uptake. Growth and substrate uptake did not coincide in the humics chlorinated biofilm cell batch cultures. One possible explanation for the discrepancy in the batch culture findings would be that biofilm cells from the chlorinated reactor, when placed in the chlorine-free batch culture required time to repair the cells or acclimate to their new environment. It has been shown that one effect of chlorine on cells is to damage the membrane structure (Haas, C. N. and Engelbrecht, R. S., 1980) or to impair transport functions (Thomas, E. L., 1979a; Thomas, E. L., 1979b; Venkobachar, C. et al., 1977). Another explanation may be that in

the biofilm environment chlorine might provide lower molecular weight compounds for the biofilm cells. When the biofilm cells were placed in the batch culture environment they may have been required to adapt by producing extracellular enzymes capable of degrading the humic substances. The control reactor biofilm cells did not show this response and were able to immediately grow and take up nutrients.

Substrate uptake results for the chlorinated biofilm batch culture provide insight into what can happen in a drinking water distribution system when cells detach from biofilm in a part of the distribution system with a chlorine residual and are transported to areas with little or no chlorine. Batch culture substrate uptake data for the humics chlorinated reactor biofilm cells showed a lag period of 80 to 150 hours compared to 0 to 48 hours for the control biofilm. Substrate uptake rates were greater in batch culture for biofilm cells from the chlorinated reactor compared to the control. One possible explanation for the long lag phase in batch culture would be that biofilm cells from the chlorinated reactor, when placed in the chlorine-free batch culture medium, required time to repair the cells or acclimate to their new environment. Another explanation may be that in the biofilm environment the action of chlorine on humics might provide lower molecular weight compounds for the biofilm cells. When the biofilm cells were placed in the batch culture environment they may have been required to adapt by producing extracellular enzymes capable of degrading the humic substances. The control reactor biofilm cells did not show this response and were able to immediately grow and take up nutrients. Chlorine may retard overall biofilm development but that does not mean the

cells are not viable and capable of re-establishing a biofilm with subsequent growth and substrate uptake.

Comparison of Reactor to Batch Culture Substrate Uptake

Amino Acids. Figure 84 presents a comparison of specific uptake rates for the amino acids combination batch cultures and reactors, replicates 1 and 2. The difference between uptake rates was statistically significant in the control and chlorinated reactors, but this was not true for the combination batch cultures. The batch culture data contains only two data points for each reactor type with a high degree of variability, making comparison to the reactor data difficult. Based on the limited data, however, it appears the batch culture technique resulted in more variation and higher uptake rates than found in the reactor data. Both the batch culture and reactor uptake data are sensitive to measurements of biomass, and this measurement can have error due both to analyst and basic technique. Biomass measurement may be responsible for the variation seen in the data.

Several researchers have found the uptake of amino acids by attached bacteria can exceed that for free-living bacteria. Simon (1985) found the attached bacteria in a freshwater lake took up amino acids at a rate greater than for free-living bacteria. When Simon's data was corrected for active cells, however, there was no significant difference between attached and free-living bacterial uptake of amino acids. Bright and Fletcher (1983b) determined that uptake of amino acids by attached bacteria was greater than

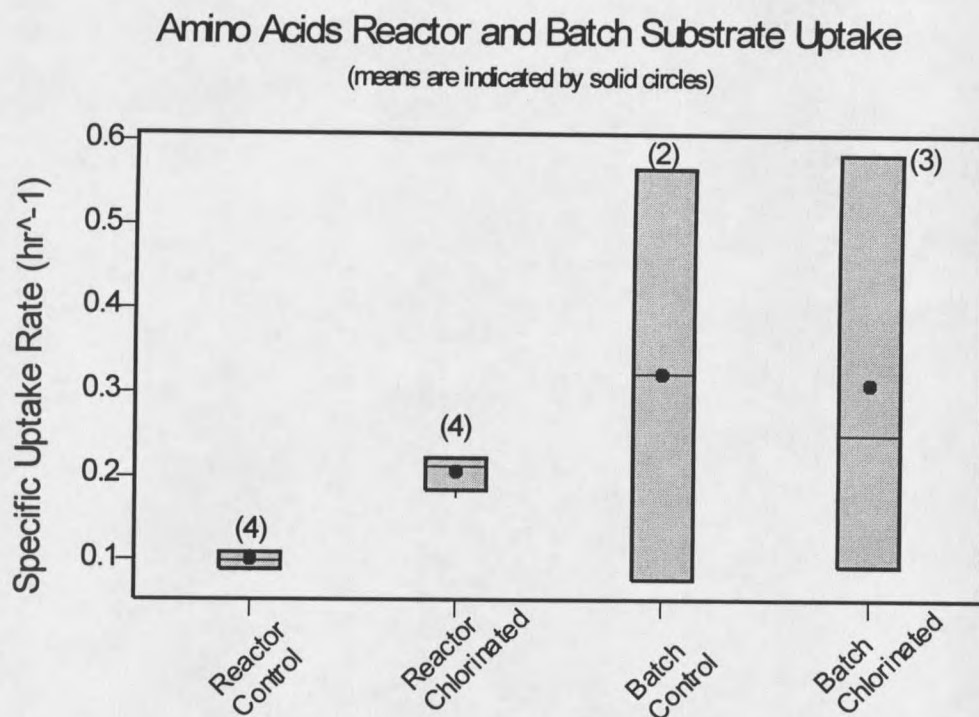


Figure 84. Boxplots for the amino acids control and chlorinated reactor and combination batch culture specific substrate uptake rates. Reactor data is from the amino acids reactors at the 2000 $\mu\text{g C/L}$ level. Numbers in parenthesis indicate number of data points.

for free-living cells for a marine *Pseudomonas* sp. The data shown in Figure 84 indicates batch culture (planktonic) cells had greater uptake rates than did the reactor biofilm cells, a finding opposite that of other researchers. The low concentration of amino acids in the reactor bulk fluid compared to the batch cultures could account for the measured differences.

Humic Substances. Figure 85 presents a comparison of specific uptake rates for the humics replicate experiment 2 batch culture and reactor data. The data for the batch

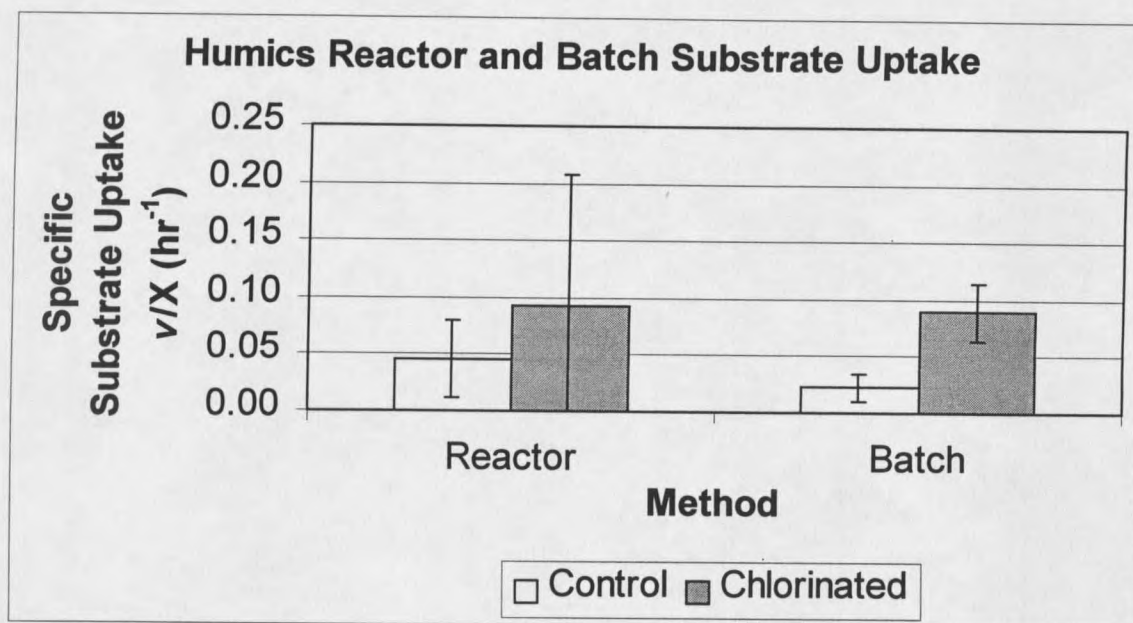


Figure 85. Comparison of the humics replicate experiment 2 control and chlorinated reactor and batch culture specific substrate uptake rates. Data are from the humics reactors at the 2000 $\mu\text{g/L}$ carbon feed level. Error bars represent 95% confidence intervals for the mean.

culture was based on the parameters from the curve fits shown in Figure 67 and the reactor effluent substrate concentrations. There was no statistical difference between the mean uptake rates determined in batch culture to mean uptake rates determined for biofilm in the reactors. Uptake rates for the chlorinated reactor and batch cultures were greater than the control reactor and batch culture uptake rates, respectively. The error associated with the chlorinated data was such that there was no statistical difference between the control and chlorinated uptake rates for reactor biofilm at the $\alpha=0.05$ level. The statistical difference between the batch culture control and chlorinated uptake rates was significant. Chlorination appears to have a notable influence on the rate of substrate

uptake when humics are the substrate. Physiological adaptation of biofilm cells to the chlorine environment appears to result in cells with greater capacity uptake systems. Carbon requirements for production of EPS to protect the biofilm from chlorine may be a factor in explaining the greater substrate uptake rates for chlorinated biofilm. Bacteria from limited nutrient environments have been shown to have low affinity, high capacity uptake systems capable of transporting a wide variety of substrates simultaneously (Schut, F. et al., 1997) (Schut, F. et al., 1995), a requirement for microbial survival using humic substances. Other researchers indicate 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).

Yield

The kinetic parameter yield was determined for the biofilm in the reactors as well as for the amino acids and humics batch cultures.

Reactor Biofilm Observed Yield

The correlation between observed yield and effluent substrate concentration for a specific substrate and reactor group indicated there were no significant correlations (Table 13) with the exception of the chlorinated "a" mixed substrate reactor which had a significant positive correlation. Both humics reactors and the control "a" mixed substrate reactor had negative correlation while correlation coefficients were positive for all other reactors and substrate groups. The data support an overall negative (decreasing trend) in

observed yield with increasing carbon concentration when humic substances are the substrate and an opposite trend when amino acids or carbohydrates are the substrate.

When data for each reactor was combined and analyzed by carbon level, the means were statistically equal between carbon levels for the control and chlorinated reactors, as was also indicated by the insignificant correlations.

Tranvik (1990) reported a decreasing bacterial yield with increasing DOC in lakes of varying humic content. One explanation given by Tranvik was a possible inhibitory effect of humic substances on bacterial utilization of DOC as found by Shimp and Pfaender (1985) when investigating the degradation of phenols by aquatic bacteria. Tranvik's findings may help explain the negative trend in yield with increasing humics concentration. Biofilm age and associated age of the humics attached to the biofilm, resulting in less easily degradable substrate for the biofilm cells, may also explain the negative trend. An explanation for the positive correlation between yield and carbon concentration in the chlorinated mixed substrate reactor cannot be explained; data was available for only one experiment and additional experiments were needed to verify the results.

The mixed substrate resulted in yield that was greater than for the other substrates in both the control and chlorinated reactors. Geller (1986) found that the addition of glutamic acid to cultures improved biodegradation of natural 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. The effects of mixing amino acids and carbohydrates with humics (mixed substrate) appeared to be improved degradation of the substrate and increased yield. Simultaneous removal of a large variety of substrates has been found for most oligotrophic microorganisms (Schut, F. et al., 1997).

The observed yield for control reactor biofilm was greater than the chlorinated reactor biofilm yield for all substrate groups, but the difference was significant for only the amino acids group. When yield data was combined and analyzed by substrate group for each reactor type there were no significant differences between observed yield by substrate group. Mean values for the biofilm observed yield for the control and chlorinated reactors was 0.112 (0.0753 – 0.149, 95% C.I.) and 0.0656 (0.0305 – 0.101, 95% C.I.), respectively.

Compared to the control, chlorinated biofilm was determined to have greater substrate uptake rates and growth rates, but the observed yield was less. These results indicate that the chlorinated biofilm, while replicating at rates faster than the control biofilm, required more substrate for metabolism, repair and other purposes. As discussed above, one mechanism biofilm cells use to protect against the action of chlorine is to produce additional EPS. EPS can serve as both diffusion and a reaction barrier, inactivating chlorine that might otherwise damage biofilm cells. Production of additional EPS would require the cell to take up more substrate and manufacture the polysaccharide and proteins typical of EPS structure.

Batch Culture Yield

Cell yield values were determined for the amino acids and humics batch cultures. While the amino acids batch cultures data gave results making comparison of yield by individual amino acid possible, the humics batch culture provided only data for the one substrate, humic substances, as filtered NPOC. Batch culture yield results are discussed below for each substrate group.

Amino Acids. Yield values for the alanine and combination batch cultures were greater than for the glutamate, aspartate and serine batch cultures both for the control and chlorinated biofilm cells. Serine resulted in the lowest yield for the control biofilm cells in batch culture and aspartate resulted in the lowest yield for the chlorinated biofilm cells. Aspartate has been shown to be very reactive with chlorine (Trehy, M. L. et al., 1986; Trehy, M. L. and Yost, R. A., 1989) thus the cells may not be acclimated to aspartate uptake as little would have existed in the chlorinated reactor environment. There were no significant differences between yield values for the various amino acid batch cultures when data between the control and chlorinated batch cultures were compared. The same general observation applies to growth rates and substrate uptake rates for the amino acids reactors. The data indicates chlorination may have had minimal impact on biofilm cells in reactors where dissolved free amino acids were the primary substrate.

Humic Substances. There was variation in the yield by initial substrate concentration for the control reactor batch cultures, and there was little variation in yield

values over the range of initial humics concentrations used for the chlorinated batch cultures (refer to Figures 71 and 72). The decrease in yield for the control 2000 culture occurred for both measurement techniques (TDC/Biomass and HPC/Biomass). Since growth rate did not increase for the control 2000 batch culture compared to the other three cultures, the lower yield values result from of the higher substrate uptake in that culture (refer to Figure 67). Also, the lower yield could be an experimental artifact. Since time restrictions did not allow repetition of the batch culture experiment, it was difficult to determine the reason for the decreased yield. Tranvik (1990) reported a decreasing bacterial yield with increasing DOC in lakes of varying humic content. One explanation given by Tranvik was a possible inhibitory effect of humic substances on bacterial utilization of DOC.

Yield measured in the batch cultures for humics was similar to those measured by microbial ecologists using natural bacterial populations found in streams or lakes. Tranvik (1988) reported an average growth efficiency of 26% (yield of 0.26 g cell C/g DOC) in 10 oligotrophic lakes with varying concentrations of humics. In a study of humic and clearwater lakes, Tranvik and Höfle (1987) reported an average growth efficiency of 20% based on DOC. Amon and Benner (1996) determined growth efficiencies of 8 to 39% for dissolved organic matter with a molecular weight of greater than 1 kilo-Daltons in Amazon River water. The molecular weight fraction greater than 1000 Daltons would encompass most humic substances. An investigation of the growth of natural bacterial communities from a variety of aquatic environments found an average

growth efficiency of 22% on lake humics (Moran, M. A. and Hodson, R. E., 1990). A similar study using bacteria and humics from a swamp environment found the growth efficiency to average 22% (Bano, N. et al., 1997). Observed yields based on mass balances around the reactors (0.047 to 0.111) were less than those reported in the literature for planktonic cells. This finding reflects the unique environment of the humics biofilm compared to a planktonic environment or differences in the chemical nature and structure of the humic substances in natural waters versus the humics used in this work. These results point out the difficulty of determining kinetic parameters for biofilm in batch culture conditions, particularly for humics.

Comparison of Reactor to Batch Culture Yield

Amino Acids. Figure 86 presents a comparison of the mean observed yield for the amino acids reactors at 2000 $\mu\text{g/L}$ carbon feed level and the combination batch cultures ($S_0 \approx 2000 \mu\text{g C/L}$). Yield values determined by batch culture were greater than those determined based on reactor mass balances. Yields for the control batch culture and reactor were greater than for the chlorinated batch culture and reactor yield, respectively.

The initial substrate concentration in the batch cultures was $\sim 2000 \mu\text{g/L}$ as carbon while to bulk fluid concentration in the reactors was $100 \mu\text{g C/L}$ for the control reactor and $157 \mu\text{g C/L}$ for the chlorinated reactor. The large difference in substrate concentration could be why yield was higher in the batch cultures.

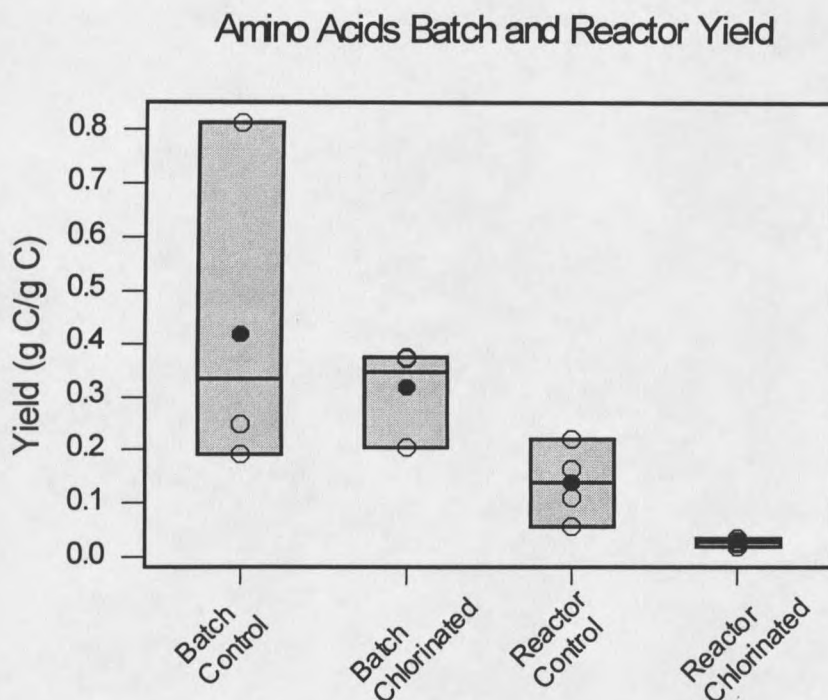


Figure 86. Boxplots of the amino acids batch culture and reactor yield data. Data for the reactors is the observed yield when amino acids feed was 2000 $\mu\text{g C/L}$. Boxes represent the 95% confidence interval, line across the box is the median, open circles are data points, and solid circles are the mean.

The reactors were operated for over 1 year for the amino acids replicate experiment 3, the experiment from which the yield data was obtained. As determined by confocal laser scanning microscopy, the biofilm matrix in the reactors was from 35 to 60 microns (μm) thick in the chlorinated reactor and 50 to 80 μm thick in the control reactor. Humic substances in the AOC “free” water may have attached to the biofilm matrix in the mature reactors creating a diffusion barrier, thus slowing the movement of amino acids to the biofilm cells and accounting for the lower values of yield in the reactors compared to

the batch cultures. The EPS matrix also imposes a resistance to mass transfer and could account for reduced yield in the biofilm compared to the batch culture where mass transfer resistance was less.

Humic Substances. Batch culture techniques are not well suited for measurement of yield for biofilm utilizing humic substances as the substrate. Mean observed yield for the humics reactors at the 2000 $\mu\text{g C/L}$ level and the mean for all humics batch cultures based on the TDC/Biomass measurement technique are presented in Figure 87. Yield measured in batch culture was statistically different and greater than

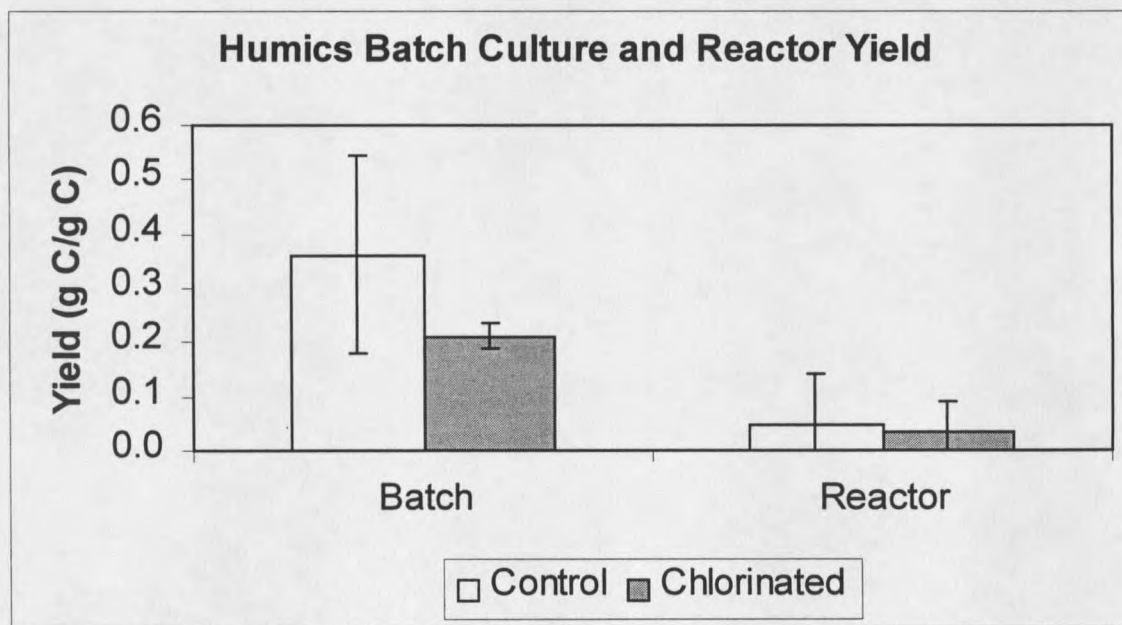


Figure 87. Comparison of humics biofilm cell batch culture yield with reactor observed yield. Batch culture data represents mean of all humics batch cultures using the TDC/Biomass measurement technique. Reactor data is the observed yield using mass balances at the 2000 $\mu\text{g C/L}$ carbon feed level. Error bars represent 95% confidence levels for the mean. TDC = total direct cell count.

observed yield for the reactors. Within the batch culture or reactor groups, there was no statistically significant difference between the control and chlorinated reactor biofilm cells although the mean for the chlorinated biofilm was less than for the control biofilm. Attachment of humic substances to the biofilm matrix, creating a diffusion barrier, may be the reason for lower yield in the reactor biofilm compared to batch cultures where cells are suspended freely in the humics solution. As discussed above for growth rates in the humics batch cultures, attachment of humics to the biofilm does not mean there was an infinite supply of carbon. Attachment sites for humics and the production and action of extracellular enzymes were likely the major factors controlling uptake and growth. When biofilm cells were placed in suspended batch cultures mixing provided numerous collisions between the humic substance molecules and the cell and only the degradation and uptake process may have been controlling. If this reasoning stands correct, then interaction and attachment of humic substances to the biofilm matrix may be a limiting factor in biofilm growth and uptake.

Yield for biofilm cells should logically be less than for cells in suspended culture. Biofilm cells use substrates to produce the EPS matrix whereas cells in suspended culture do not have that energy requirement. This factor alone could account for the difference in yield between reactors and batch cultures.

Relevance to Drinking Water Biofilm

The results of this research have pointed out several important factors influencing drinking water biofilm. First, humic substances can support a viable biofilm in drinking water systems. Second, chlorination produced greater growth rates and substrate uptake and lower yield than in the control. Third, attachment of humics to the biofilm matrix was most likely a controlling factor of growth and uptake. Fourth, the interaction of chlorine with the biofilm and substrate will impact kinetics of biofilm growth and substrate uptake.

Humic substances were capable of supporting biofilm in chlorinated and non-chlorinated conditions. This finding is relevant because humic substances comprise the majority of dissolved organic carbon in most drinking water. The ability of heterotrophic biofilm to degrade and utilize humic substances is important to the understanding of what constitutes biostability in drinking water. Removal of humic material by water treatment processes has been emphasized in the past few years, but the reason has been to reduce the formation of disinfection by-products created by the interaction of chlorine with humic material. This research has demonstrated that removal of humic material will also benefit biostability by reducing a major source of carbon and other nutrients required for biofilm growth and maintenance.

Chlorination will reduce the number of cells in the biofilm but those cells that persist have growth and substrate uptake rates greater than biofilm cells in non-

chlorinated environments. The greater growth and substrate uptake rates are most likely indicators of a biofilm population that has adapted various mechanisms to survive in an environment containing chlorine. One well-known mechanism is production of EPS to serve as a barrier to chlorine and/or serve as a sacrificial reactant with chlorine to reduce the amount of chlorine reaching the cell. In the case of humic substances chlorine can react with humics to make smaller groups that are more easily utilized by the biofilm cells.

Certain results from this research can be better explained if the biofilm matrix (cells and EPS) served as attachment sites for humic materials. Attachment of humics would help explain the decrease in growth rates with increasing substrate concentration, particularly between the 500 and the 1000 nominal carbon feed levels ($\mu\text{g C/L}$). Data indicated increasing amounts of attached non-cellular carbon with increasing carbon concentration, and in these experiments, biofilm age. While attached carbon also includes EPS and other material in the biofilm matrix, it is known that humic substances attach to and cover most surfaces in natural environments. Visual examination indicated the brown-colored humic material was attached to the biofilm sample slides. Attached humic material would create a diffusion barrier for nutrients and chlorine, and serve as a substrate source for the biofilm. Chlorine could enhance growth by making the humics easier to degrade while at the same time the action of chlorine on humics would help protect the biofilm cells, the humics acting as a sacrificial compound. Limited adsorption

sites on the biofilm matrix could limit the amount of substrate available, thus serving to control growth and uptake processes.

The presence of chlorine in the biofilm environment will likely lead to selection of a population that has adapted to survive its negative effects. Survival may include increased production of EPS to serve as a sacrificial barrier to chlorine; polysaccharides and proteins in the EPS would react with chlorine before the chlorine could reach the biofilm cells. The high growth and substrate uptake rates for the chlorinated carbohydrate reactor indicated such a process may be occurring.

Impacts On Biofilm Models

Numerous models have been developed to help describe the biofilm growth process. The impact of the results of this work on these models will be discussed following a general review of the kinetic parameters found in this research.

Specific Growth Rate

Two general trends were seen in the specific growth rates depending upon whether chlorination was employed. First, when there was no chlorine added (control reactor) there was a significant decrease in growth rates between the 500 and 1000 carbon levels, and there was less difference between the 1000 and 2000 carbon levels. There was little difference in growth rates between the 1000 and 2000 carbon levels for the chlorinated reactors. If the response seen in growth rates were related to aging of the biofilm (attachment of humic materials to the biofilm and/or development of EPS), then a

zero-order model would provide a good description of growth for the carbon concentrations used in this work. A similar conclusion can be drawn for the chlorinated reactor: zero-order kinetics applies. The advantage of this finding is that kinetic models can be simplified. The disadvantage is that growth rates may be system specific.

Substrate Uptake and Yield

Growth and substrate uptake can be related using the parameter yield. Yield was not always constant over the range of substrate concentrations evaluated but the statistical differences were not significant. Within the context of drinking water biofilm it appears a constant yield value may apply over the substrate concentrations evaluated. Yield for non-chlorinated environments were different than for chlorinated environments and different values of yield should be utilized.

Current Models and Their Application

Water Quality Models Linked to Distribution Models. Several current models to determine hydraulic characteristics of water distribution systems have the added capability to model water quality. These models generally have at least a first-order kinetics capability and mass balance at each pipe node (pipe connection point). One example of this type of model is EPANET, a public domain software package developed through funding by the U.S. Environmental Protection Agency. EPANET's water quality capabilities include reactions within the bulk flow, reactions at the pipe wall, and mass transport between the bulk flow and the pipe wall.

The water quality components of EPANET and similar models were designed to model processes such as chlorine consumption within the distribution system. Several researchers have developed chlorine models for water distribution systems (Dukan, S. et al., 1996; Vasconcelos, J. J. et al., 1997). Attempts to model biofilm using EPANET have not been successful due to type of reactions and kinetics available. The water quality models can be adapted to model substrate uptake or growth, but are not well suited to model functions such as detachment. Mass transfer limitations have to be combined with other kinetic parameters as was done for the chlorine model proposed by Vasconcelos et al. (1997). Overall, simplified kinetic expressions such as zero-order growth kinetics enhance the possibility of using these water quality models for biofilm, but additional work and modifications will be required.

SANCHO Model. The SANCHO model was developed based on kinetics for enzyme-controlled degradation of BDOC by microorganisms in natural environments. Developed by Servais et al. (1995; 1994), the SANCHO model relies on Michaelis-Menten kinetics for growth and degradation of BDOC. Utilizable substrate comes from three fractions: (1) substrate that can be easily utilizable, such as free amino acids, (2) a rapidly hydrolyzed fraction of BDOC and (3) a slowly hydrolyzed fraction of BDOC. Cell attachment/detachment are modeled based on Langmuir adsorption and desorption models. The cell population is divided into fixed biomass, sorbed biomass and free biomass. The model can account for recycle of dead biomass back to the BDOC pool available for growth. Attachment assumes a fixed number of sites available for sorption

of cells. The effects of chlorine are modeled using first-order kinetics with threshold values for both fixed and free bacteria. The impact of chlorine in the model is to reduce the maximum growth and enzyme production rates. Residence time calculations from hydraulic models must be input to the SANCHO model to perform analyses of actual distribution systems.

The degradation component of the SANCHO model may be one of its strong points in modeling drinking water biofilm. The model recognizes that little of the dissolved organic matter (DOM) in natural environments is readily utilizable. Roughly 80 to 90 percent of DOM is polymeric in nature and only a small percentage (5 to 10%) of DOM can be utilized directly by microorganisms (Münster, U. et al., 1992). Enzymatic hydrolysis of the polymeric substances is a rate-limiting factor in DOM utilization in natural aquatic environments (Chróst, R. J., 1990). Modification of the enzyme degradation component of SANCHO could provide a control factor in the model for substrate availability, thus controlling the overall growth of biofilm regardless of the concentration of DOM in the bulk fluid. If substrate is adsorbed to the biofilm matrix and the amount of carbon adsorbed exceeds the biofilm requirements, then a control step like enzymatic degradation could be appropriate. Using the concept of rapidly and slowly hydrolyzable fractions of BDOC could also help model the interaction of chlorine with humics and the possible creation of more utilizable compounds through that interaction. The rapidly hydrolyzable fraction could be increased when chlorine was present. Recycle

of dead cell mass could be important when chlorine is present to account for a high growth rate to produce biomass as part of the overall protection mechanism.

The effect of chlorine in the SANCHO model is to retard the maximum specific growth and enzyme reaction rates. This research indicates an opposite effect: greater growth and substrate uptake rates when chlorine is present. This brings up the question as to how the biofilm population regulates biomass. This question is beyond the work reported here but must be taken into account when chlorine is present in the biofilm environment. Higher growth and substrate uptake rates, combined with lower yield, indicate the biofilm cells are creating new cells faster, using more substrate to produce new biomass and possibly EPS, but the yield of new cells per unit substrate consumed is lower than in the control (chlorinated biofilm cells are performing additional non-growth functions). The production of EPS and loss of cells to chlorine effects do not appear in models except for the single yield factor.

Mass transfer resistance from the bulk fluid to the biofilm and diffusion within the biofilm are not included as processes in the SANCHO model. Data from reactor mass balances at the 2000 carbon level were analyzed to determine if mass transfer resistance and diffusion were important for the drinking water biofilm evaluated in this research. The importance of diffusion was evaluated by calculating the Thiele modulus (Stewart, P. S., 1993) for zero-order kinetics. The Thiele modulus (ϕ) is a dimensionless parameter that describes the rate of diffusion to the rate of reaction for simplified homogeneous biofilm with a constant thickness or depth. When the Thiele modulus is less than one (ϕ

< 1) diffusion is fast compared to the reaction rate, implying the growth rate is uniform throughout the biofilm and diffusion is not a rate-limiting process. The zero-order kinetics Thiele modulus is given by the following equation:

$$\phi_0^2 = \frac{\mu_{\max} \rho_X L_f^2}{2Y_{X/S} D_e S_0} \quad (39)$$

where,

ρ_X = cell density in the biofilm

D_e = effective diffusivity of substrate in the biofilm

S_0 = bulk substrate concentration (reactor effluent concentration)

Mass transfer resistance was estimated by equating the carbon flux ($\mu\text{g C cm}^{-2} \text{ hr}^{-1}$) to the relationship for the difference in substrate concentration across a laminar sublayer:

$$J = k_L (S_0 - S_b) \quad (40)$$

where,

J = carbon flux to the biofilm

k_L = mass transfer coefficient

S_b = Substrate concentration at the biofilm/water interface

The mass transfer coefficient was estimated as the ratio of the laminar sublayer thickness (L_L) to the diffusivity of the substrate in aqueous solution (D_{aq}). The calculated value for S_b was used in the equation for the Thiele modulus. Thickness of the laminar sublayer for turbulent flow was calculated as presented in Daugherty and Franzini (1965) and was equal to 0.03 centimeters. Biofilm thickness was determined for the amino acids

replicate 3 experiment using confocal laser scanning microscopy. Biofilm thickness for other substrates was estimated. Ranges of biofilm thickness were evaluated to determine if gross changes in the estimated thickness values would change the Thiele modulus.

Diffusion coefficients for humics were estimated using values determined by Dixon and Larive (1997) for Suwannee River fulvic acids at concentrations relevant to this research.

The analysis of the data for all reactors at the 2000 carbon feed level indicated a range of Thiele modulus from 0.01 to 0.57. These low Thiele modulus values (< 1) indicate biofilm growth was not limited by diffusion. The impact of mass transfer resistance was a 2 to 60% reduction in the substrate concentration at the biofilm interface compared to the bulk fluid. It would appear that mass transfer resistance should not be neglected in biofilm models for drinking water systems.

Reactor or Compartment Models: Aquasim and BAM. Aquasim and BAM (Biofilm Accumulation Model) are models based on reaction-diffusion phenomena with the capability of modeling multiple species (Camper, A. K. et al., 1994; Reichert, P., 1994; Wanner, O. and Reichert, P., 1996). The two models are very similar; Aquasim is a newer, more flexible version than BAM. These models are based on a reactor or compartment with a specified biofilm area, liquid volume and rate of flow. Reactors or compartments must be placed in series to accurately model plug-flow in a pipe. Implementation of Aquasim or BAM to actual pipe systems requires input of residence time and other system characteristics (area to volume ratio) into a model containing reactors in series. Aquasim allows the user to select kinetic models for various processes.

Zero-order growth kinetics and chlorine interactions could be implemented using Aquasim.

Aquasim and BAM include mass transfer kinetics and diffusion theory. The bulk fluid is assumed completely mixed and a boundary layer exists between the bulk fluid and the biofilm, creating mass transfer resistance. The model accounts for diffusion resistance and reaction of substrate and biomass within the biofilm. Processes found important to biofilm growth under conditions of chlorination in this research are not currently found in these models. Adsorption or attachment of humics may be a rate-limiting step and would be difficult to model directly but it may be possible to model indirectly by manipulation of other model parameters.

pBAM. This model is functionally the same as BAM with the exception that the reactor is a pipe with characteristics such as length, diameter and roughness. Radial dispersion and resistance across a turbulent boundary layer control mass transfer from the bulk fluid to the biofilm. Lateral dispersion controls the movement of substrates and particulate along the length of the pipe. Diffusion and reaction equations for substrate and biomass are solved to determine growth and substrate uptake, a similar approach as for Aquasim and BAM. Detachment can be modeled as a function of biofilm biomass (biofilm thickness in this case). The advantage pBAM has over Aquasim or BAM is that pipes can be modeled rather than a series of completely mixed reactors.

pBAM has the same modeling limitations as Aquasim and BAM with respect to the effects humic substances and chlorine have on biofilm. The model can utilize zero-

order kinetics but does not currently have the ability to model chlorine effects. Use of parameters derived in this research for chlorinated conditions would not give accurate results. A function relating humic substance attachment to the biofilm, degradation of the humic substance, growth, EPS production, and chlorine consumption would be required.

CHAPTER 6

SUMMARY

Biofilm Growth Rates

Reactor Mass Balance

While chlorination may reduce the number of cells in a drinking water biofilm, it may also lead to higher growth rates for those biofilm cells. In the carbohydrates, humics and mixed substrate reactors growth rates for the chlorinated biofilm were greater than for the control. Chlorination had the greatest impact on growth rates for the carbohydrate and mixed substrate reactors; these had the highest growth rates of all substrates for chlorinated biofilm. However, biofilm growth rates in the chlorinated amino acid reactors were statistically the same as for the control reactor based on data from three replicate experiments.

Possible explanations for greater growth rates in the chlorinated carbohydrates biofilm include interactions of the chlorine with the biofilm extracellular polymeric substance (EPS), selection of biofilm cells more resistant to chlorine, or production of new cells to overcome the effects of chlorine on the population as a whole.

Chlorine does not readily react with carbohydrates in the bulk fluid, possibly leading to more free chlorine for interaction with the biofilm. Reaction of chlorine with the EPS matrix could oxidize EPS compounds, decreasing EPS and at the same time provide protection against free chlorine. A decrease in EPS could reduce mass transfer

resistance imposed by the EPS matrix, allowing carbohydrates in the bulk fluid to more easily diffuse to the biofilm cells, increasing growth and more production of EPS.

Biofilm exposure to chlorination may lead to the acclimation or selection of microorganisms that are more resistant to chlorine, which would increase as the biofilm aged. In all reactors, biofilm age increased along with carbon concentration, making the two factors inseparable in these experiments. It is likely the two factors, age (physiological adaptation/selection) and carbon, both influenced the higher growth rates seen in the chlorinated reactors.

Humic substances have been shown to react with chlorine, altering the structure of the macromolecules that in turn might create more easily biodegradable compounds. The action of chlorine on humics, along with the protection afforded by attached humic substances and selection for biofilm cells resistant to chlorine could be factors in explaining the higher growth rates in the chlorinated humics and mixed substrate reactors compared to the control reactors.

Relationships between specific growth rate and effluent substrate concentration were zero-order for the amino acids, carbohydrates and humics control and chlorinated experiments, inhibition-type kinetics for the mixed substrate control experiment and Monod-type kinetics for the mixed substrate chlorinated experiment. If the effects of growth rates at the 500 $\mu\text{g C/L}$ nominal carbon feed level were ignored, zero-order kinetics could provide reasonable estimates of specific growth rates for all substrates with the possible exception of the mixed substrate control experiment.

Specific growth rates at the 500 $\mu\text{g C/L}$ nominal carbon level were typically greater than at the 1000 and 2000 $\mu\text{g C/L}$ nominal carbon levels. Potential errors in the calculation of growth rates did not explain the difference in growth rates. Most pronounced in the humics and mixed substrate experiments, this result may be due to either a true response of biofilm cells to low nutrient conditions and/or the "younger" age of the biofilm. The biofilm age factor refers to the attachment of humic substance molecules to the biofilm matrix as the biofilm developed, creating a diffusion barrier and making biofilm growth a function of degradation rates of the attached material rather than the concentration of the substrate in the bulk fluid. Humics attachment was less significant at the 500 $\mu\text{g C/L}$ nominal carbon level because the biofilm was typically only two to four weeks old. The longer reactors were operated the more humic substances that may have attached to the biofilm matrix, up to the point of saturating all possible attachment sites. Measurement of attached non-cellular carbon for the mixed substrate reactors indicated this scenario was possible. In the amino acids and carbohydrates experiments, humic substances were only available from the dilution water and were therefore much less a factor than for the humics and mixed substrate experiments.

A zero-order model would not apply at extremely low substrate concentrations since it is logical to assume some transition to zero growth at zero substrate concentration. A problem with these experiments was that it was difficult to know where zero substrate would be due to the possible contributions of the dilution water.

Growth rates based on control reactor mass balances were lowest for humic substances. Amino acids and humic substances resulted in the lowest growth rates for chlorinated reactors. Mixed substrates and carbohydrates reactors had the greatest growth rates for the chlorinated reactor type.

Leucine Uptake

Growth rates measured using leucine uptake by the attached biofilm were not consistent with growth rates based on mass balances. The major inconsistency between the mass balance and leucine methods was for chlorinated biofilm growth rates. Chlorinated biofilm growth rates using leucine uptake were always less than for the control biofilm and much lower than those measured using reactor mass balance. Based on leucine uptake, growth rates for amino acids were greater than those based on reactor mass balance. Leucine-based growth rates for carbohydrates and mixed substrates were comparable for control biofilm and much lower for chlorinated biofilm. The variation between the two methods could be because the leucine uptake method relies upon the leucine uptake capabilities of the biofilm cells, which may be impacted by chlorine or be inherently different for cells adapted to the chlorine environment. If the latter were the case, short-term leucine uptake would be much less for chlorinated biofilm cells, resulting in lower apparent growth rates. Acclimation of biofilm cells to amino acid uptake in reactors receiving only amino acids could have resulted in enhanced amino acid uptake capabilities and greater apparent growth rates for both reactor types. Models relating growth rate to substrate concentration for the leucine uptake method were developed for

the carbohydrates and mixed substrate biofilm. Models were zero-order for carbohydrates and inhibition-type for the mixed substrates.

A factor that may have impacted leucine-uptake-based growth rates for the mixed substrate biofilm was adsorption of leucine by humic substances in the sample vials, making less leucine available for uptake and resulting in lower apparent growth rates as the concentration of substrate was increased. This problem was not recognized when performing the tests and correction was therefore not done.

Batch Cultures

In the humics batch cultures specific growth rate was independent of the initial substrate concentration resulting in zero-order kinetics over the range of concentrations used in the experiments, regardless of the technique used to determine growth rates. While growth rates in batch culture were greater than for reactor mass balance methods, zero-order kinetics for the growth rate–substrate concentration relationship applied to both when data from reactors at the 1000 and 2000 carbon levels were analyzed.

In general, using HPC data for determining specific growth rates in batch cultures gave higher growth rates than other methods and was not suited for determining growth rates for biofilm cells from a chlorinated environment. Following a lag phase chlorinated biofilm cells placed in a suspended-cell, chlorine-free environment show a greater growth rate based on HPC than do cells from a nonchlorinated environment. This indicated the ability of chlorinated cells to acclimate and recover, a condition noted as recovery or regrowth.

Batch cultures can provide estimates of the biofilm growth rate when the substrate consists of dissolved free amino acids or carbohydrates. Only the total direct count (TDC) method for determining growth rates in batch culture gave similar results to the reactor mass balance approach for carbohydrates, whereas several methods were comparable to the reactor mass balance data for amino acids. When complex substrates exist, such as humic substances, batch culture based growth rates will most likely not be representative of actual conditions. Growth rates for complex polymeric substrates require that the conditions be nearly identical to those found in the actual environment being studied, such as in a distribution pipeline or granular media biofilter. The attachment of humic substance molecules to the biofilm matrix and interaction of chlorine with the humic substances may be major factors controlling the growth rate of the biofilm cells.

Biofilm Substrate Uptake

The relationships between carbon removal flux (carbon utilized by the biofilm per unit area per time) and the carbon loading rate (same units) were linear in all reactors for all substrates. Regressions for carbon removal flux and influent loading rate were significant for all reactors and substrates with the exception of the chlorinated carbohydrate reactor. On the basis of fractional removal of the influent carbon, biofilm receiving amino acids as the substrate had the highest fractional removal (all amino acids were utilized), and there was no statistical difference between the mean for the other

substrates tested (carbohydrates, humic substances, and mixed substrates). These results indicate the biofilm adapted to increasing carbon concentrations by either increasing the amount of biofilm biomass (typical of the control reactors) or increasing the normalized uptake rate (typical of the chlorinated reactors).

Normalized carbon removal rates (mass of carbon removed per mass of cell carbon per time) were greater for chlorinated reactor biofilm than for the control. The chlorinated carbohydrate reactors showed the highest normalized removal rates. There was no statistical difference between removal rates for the other substrates. Although there was generally a decrease in biofilm biomass with increasing carbon concentrations for chlorinated biofilm, the normalized substrate uptake of the biofilm increased to maintain the same fractional carbon removal. Potential errors in measurement of biofilm biomass could impact the normalized carbon removal rates by lowering the calculated values.

Substrate uptake rates determined using suspended biofilm cells in batch cultures were greater than uptake rates determined using reactor mass balances for amino acids and nearly the same for humic substances. When placed in batch culture, chlorinated biofilm cells from the humics reactors exhibited greater specific affinity than biofilm cells from the control reactor. Specific affinity has been proposed as an indicator of cells that have better adapted to low nutrient environments by increasing the number of membrane transporters. Cells with greater specific affinity are better adapted to environments low in nutrients.

Biofilm Yield

Carbon concentration did not have a significant influence on observed yield for the biofilm in the reactors. With the exception of the chlorinated mixed substrate reactor, correlation between observed yield and effluent substrate concentration for a specific substrate and reactor group was not significant (line without a slope). When data for each reactor was combined and analyzed by carbon level, the means were statistically equal between carbon levels for the control and chlorinated reactors.

The observed yield for biofilm in control reactors was greater than for the chlorinated reactors for all substrates evaluated. Although substrate uptake and specific growth rates were greater for chlorinated reactors compared to control reactors, yield was lower in chlorinated reactors indicating the biofilm cells had greater needs for metabolism and other processes. Additional substrate may have been required for production of greater amounts of EPS per cell compared to control biofilm cells.

The negative correlation between yield and effluent carbon concentration for the humics reactors may be indicative of the effects of humics attachment to the biofilm. The possible interaction would be similar to an inhibition effect by increasing humics concentration that has been suggested by research in aquatic environments. Assuming a limited number of humics molecules can attach to the biofilm matrix, degradation of humics over time results in an increasing fraction of less biodegradable humic material.

Yield determined using batch cultures for amino acids and humics was greater than values determined based on observed yield for reactors. Batch cultures did not provide representative values of yield for biofilm based on their comparison with reactor data.

Relevance to Drinking Water Biofilm

The results of this research have pointed out several important factors influencing drinking water biofilm. First, humic substances can support a viable biofilm in drinking water systems. Second, the effects of chlorination were greater growth rates and substrate uptake than in the control, but lower yield than in the control. Third, attachment of humics to the biofilm matrix was most likely a controlling factor of growth and uptake. Fourth, the interaction of chlorine with the biofilm and substrate will impact kinetics of biofilm growth and substrate uptake.

Impacts on Biofilm Models

Most current models for biofilm development and substrate uptake do not provide a suitable model structure for the interaction between chlorine, the substrate and the biofilm cells. These interactions are important to drinking water biofilm since chlorine is the primary disinfectant currently used to protect consumers from microbial contaminants. Many models include chlorine reactions but the models assume chlorine retards biofilm growth rates and substrate uptake, a result contrary to the findings of this

research based on reactor mass balance data. While the results of current models correctly predict less biomass and overall substrate use by chlorinated biofilm, a result also seen in this research, the fundamental biofilm processes may not be accurately modeled. Additional research will be required to investigate these interactions in more detail.

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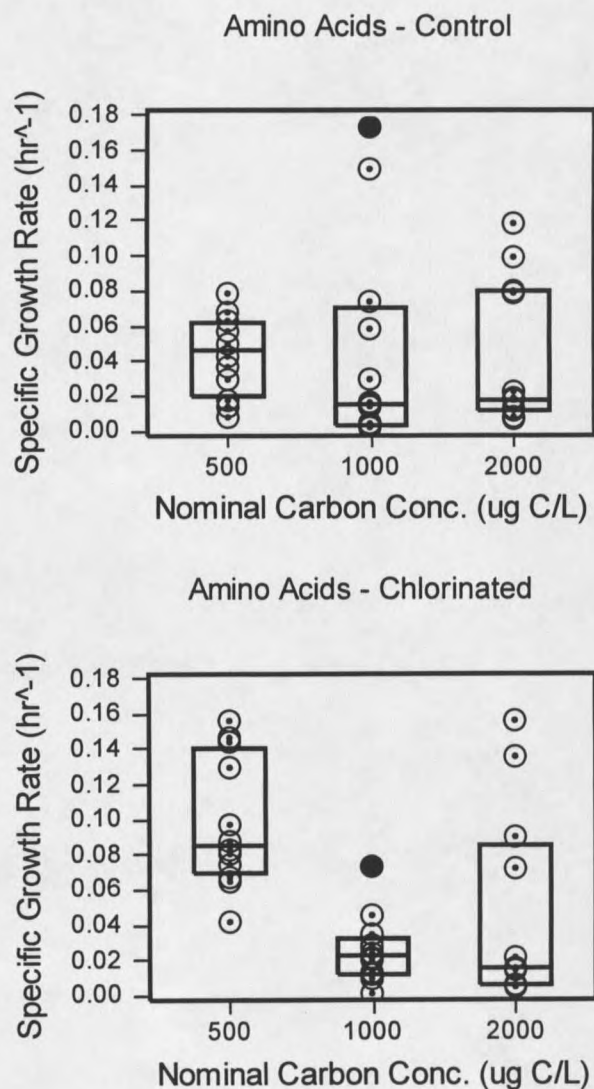
Appendix

Figure 88. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the amino acids reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

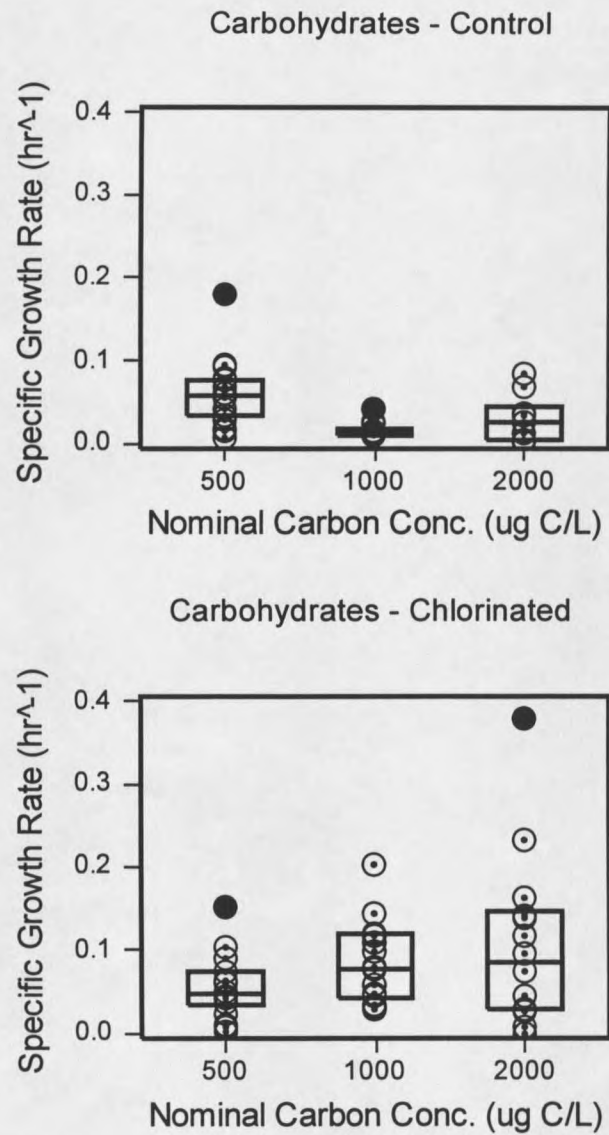


Figure 89. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the carbohydrates reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

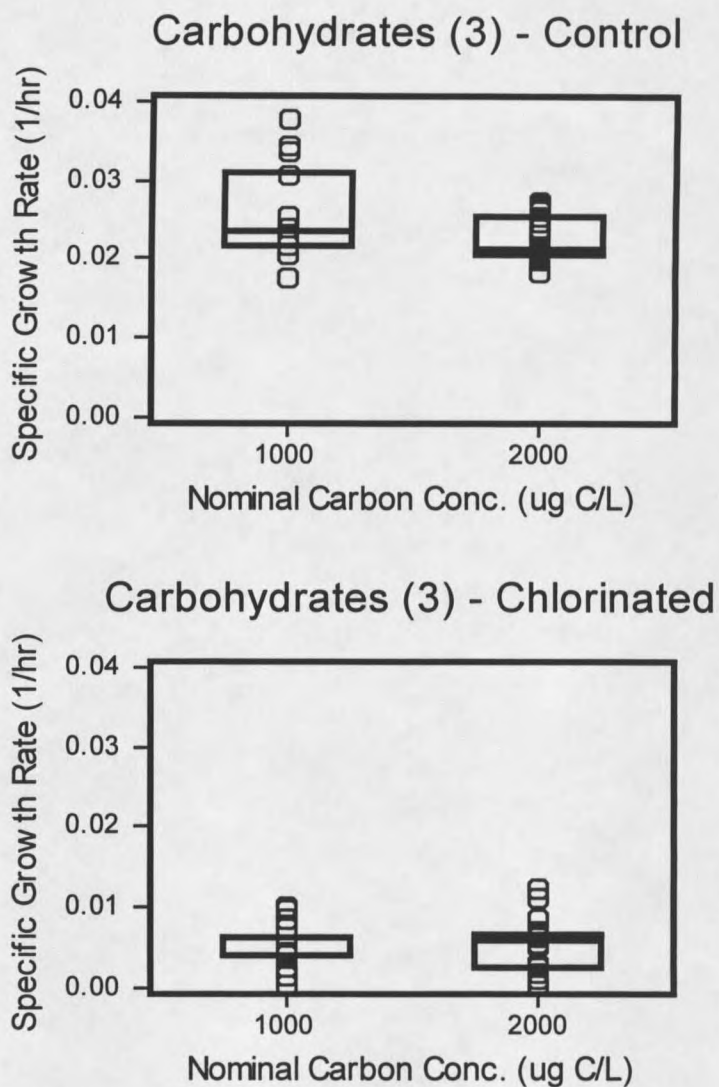


Figure 90. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the carbohydrates replicate 3 reactors using leucine uptake. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

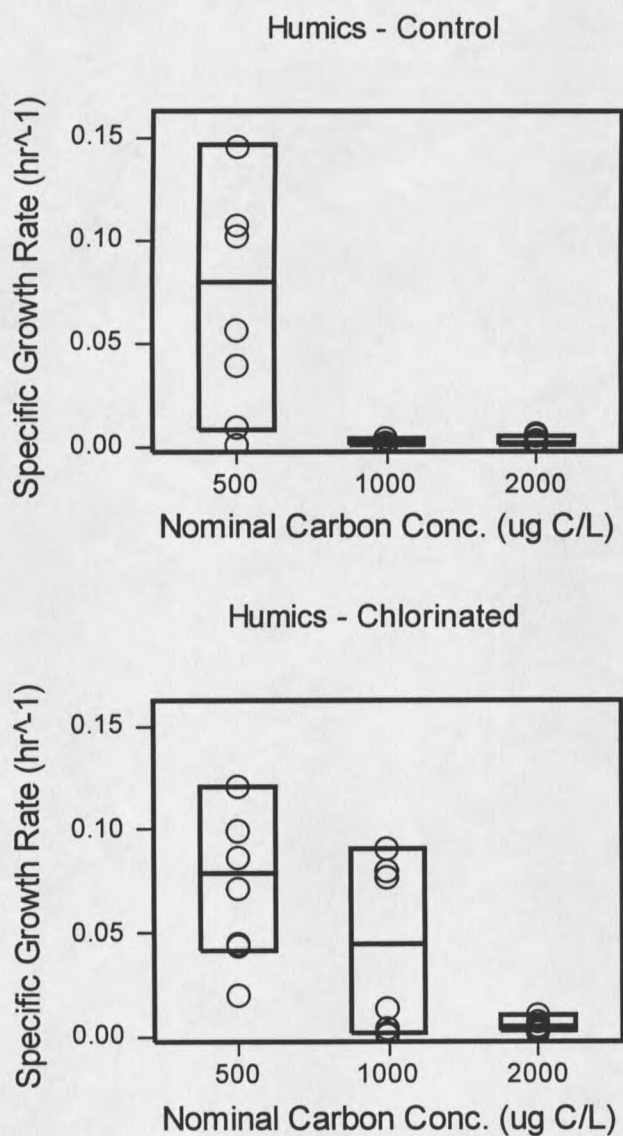


Figure 91. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the humics reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

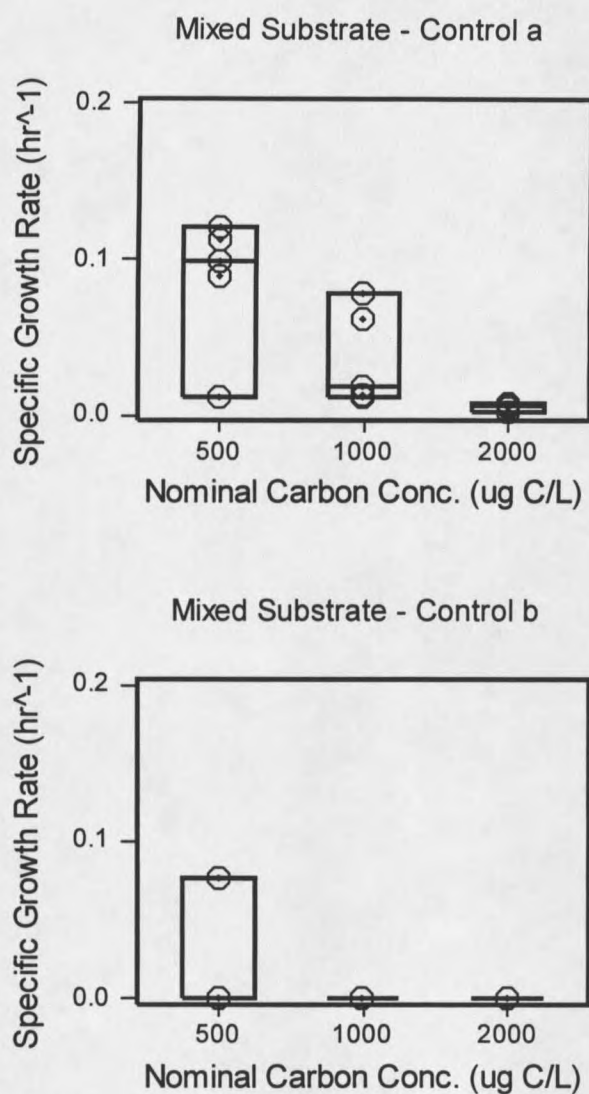


Figure 92. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the control mixed substrate reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

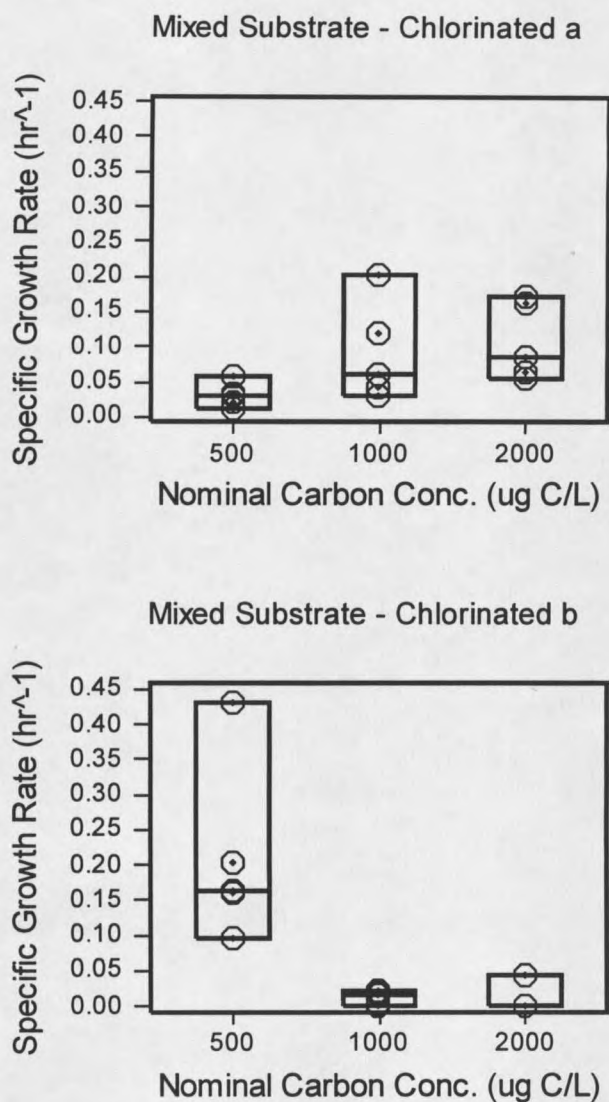


Figure 93. Raw specific growth rate data (circles) and boxplots for each nominal carbon feed concentration for the chlorinated mixed substrate reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

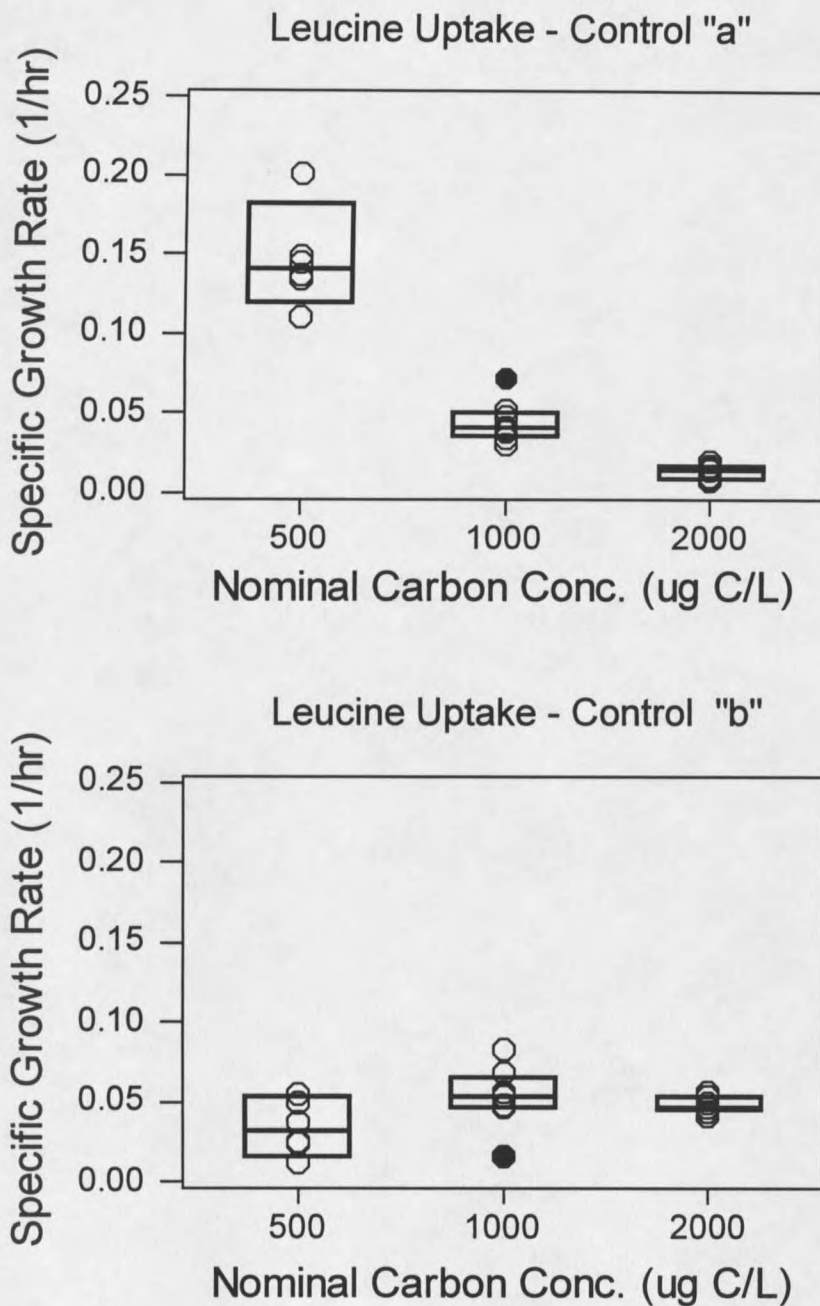


Figure 94. Specific biofilm growth rate data based on leucine uptake (circles) and boxplots for each nominal carbon feed concentration for the control mixed substrate reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

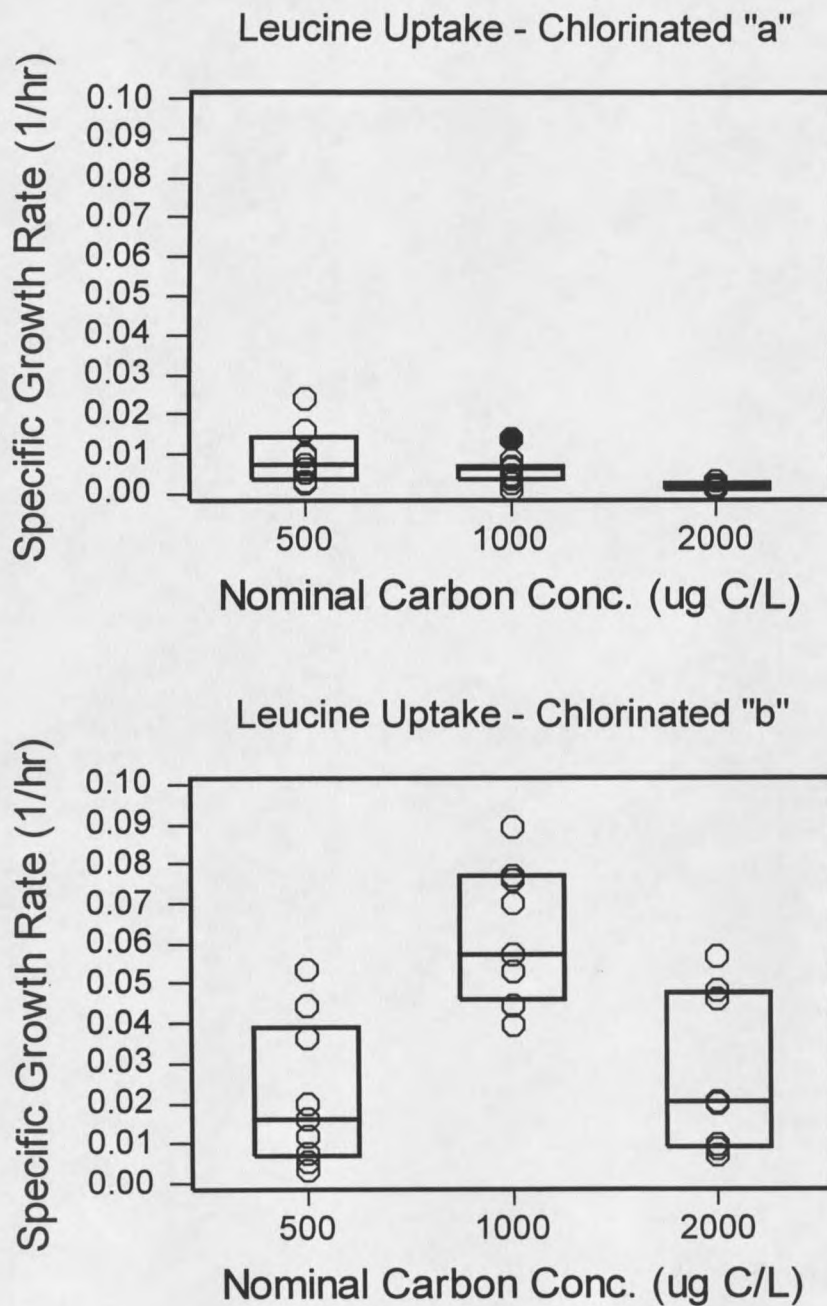


Figure 95. Specific biofilm growth rate data based on leucine uptake (circles) and boxplots for each nominal carbon feed concentration for the chlorinated mixed substrate reactors. Boxes represent 95% confidence interval for the median, solid horizontal line in box represents the median and solid dots are data outside $1.5 \times$ interquartile range ($Q_{75} - Q_{25}$) and represent possible outliers.

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