Biomass distributions, activity, growth, and carbon utilization in heterotrophic bacterial communities
by Brian David Ellis

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

Abstract:
Bacteria can play a deleterious or beneficial role in the distribution or treatment of safe drinking water. The main mode of growth is sessile, attached to the distribution system pipelines or filter media, respectively. This report describes efforts to understand the activity of microorganisms growing in drinking water.

Methods including microscopy, activity measurements, carbon analysis, and rigorous statistical procedures were used to investigate bacterial growth parameters in model biofilm reactors (annular reactors). Amino acids, carbohydrates, and humic material were investigated as model growth compounds at three different concentrations. Control and chlorinated reactors were operated in parallel. Biologically filtered drinking water was used as inoculum and dilution water for the reactors. Long-term experiments were run, permitting for development of steady state conditions within the reactors.

Biomass/biovolume distributions of heterotrophic bacteria resulting from colonization of the reactors were investigated using mathematical probability distribution analyses. Sixty-eight of seventy-two datasets comprising approximately 36,000 individual cell volume measurements were found to follow a 3-parameter generalized Pareto distribution. The average biomass (mass/volume) conformed to the same distribution. These results show that biomass increases with decreasing cell size. An empirical non-linear model was developed that takes into account practical and theoretical limits for bacterial size to convert biovolume to biomass.

Activity measurements by microautoradiography and a suite of fluorescent stains were in good agreement. Substrate type had a larger effect on activity than did substrate concentration and chlorination had the largest impact.

Bacterial carbon production ranged from 0.003 - 1.74 grams carbon/L•day, yields from 0.034 - 0.25 grams cell carbon/gram carbon, and doubling times from 1 - 15.4 days. Specific growth rates and yields decreased with increasing carbon concentration. Doubling times were lower in the presence of chlorine and independent of the bulk fluid substrate concentration in both chlorinated and control biofilms. Therefore, biofilm growth was zero order. Although humic material is generally considered recalcitrant we found that control and chlorinated biofilm communities could remove 78% and 58% of the influent humic concentration at steady state and a hydraulic detention time of 2.1 hours. Biological filtration was as effective as chlorine in controlling bacterial growth in the annular reactors.

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APPROVAL

of a thesis submitted by

Brian David Ellis

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. Gordon A. McFeters _____ (Signature) 4/6/99 (Date)

Approved for the Center for Biofilm Engineering

Dr. Anne K. Camper _____ (Signature) 4/6/99 (Date)

Approved for the Department of Microbiology

Dr. Seth H. Pincus _____ (Signature) 4/6/99 (Date)

Approved for the College of Graduate Studies

Dr. Bruce R. McLeod _____ (Signature) 4-6-99 (Date)

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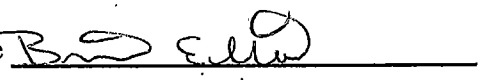
Signature 

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 3

LIST OF FIGURES

Chapter 3

Chapter 4

ABSTRACT

Bacteria can play a deleterious or beneficial role in the distribution or treatment of safe drinking water. The main mode of growth is sessile, attached to the distribution system pipelines or filter media, respectively. This report describes efforts to understand the activity of microorganisms growing in drinking water.

Methods including microscopy, activity measurements, carbon analysis, and rigorous statistical procedures were used to investigate bacterial growth parameters in model biofilm reactors (annular reactors). Amino acids, carbohydrates, and humic material were investigated as model growth compounds at three different concentrations. Control and chlorinated reactors were operated in parallel. Biologically filtered drinking water was used as inoculum and dilution water for the reactors. Long-term experiments were run, permitting for development of steady state conditions within the reactors.

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Bacterial carbon production ranged from 0.003 - 1.74 grams carbon/L•day, yields from 0.034 – 0.25 grams cell carbon/gram carbon, and doubling times from 1 - 15.4 days. Specific growth rates and yields decreased with increasing carbon concentration. Doubling times were lower in the presence of chlorine and independent of the bulk fluid substrate concentration in both chlorinated and control biofilms. Therefore, biofilm growth was zero order. Although humic material is generally considered recalcitrant we found that control and chlorinated biofilm communities could remove 78% and 58% of the influent humic concentration at steady state and a hydraulic detention time of 2.1 hours. Biological filtration was as effective as chlorine in controlling bacterial growth in the annular reactors.

CHAPTER 1

INTRODUCTION

The overall goal of the project was to determine the key biodegradable organic matter (BOM) constituents responsible for bacterial growth in drinking water distribution systems. Three scales of observation were included in this research plan: (1) full-scale monitoring to identify treatment processes which are responsible for the removal of BOM, (2) pilot scale studies to determine the fate of these BOM compounds in conventional and advanced treatment processes, and (3) laboratory studies designed around the key BOM compounds responsible for microbial growth and determination of the kinetics of growth on these constituents. Synthetic BOM amendments were used in the laboratory scale experiments in the presence of natural organic matter (NOM). Choice of carbon substrate amendments were based on their role in bacterial metabolism and common presence in a number of aquatic environments such as lakes, rivers, and oceans.

The primary objective of the bench scale experiments at MSU was to measure the response of heterotrophic bacterial biofilm communities to different types and concentrations of BOM in the presence or absence of chlorine. In conjunction with this primary aim, the measurement, prediction, and modeling of heterotrophic biomass was a principal objective of this study and is reported in Chapter 2. Chapter 2 is titled "Biomass Distributions in Heterotrophic Bacterial Communities". The authors are Brian D. Ellis, Phillip Butterfield, Warren L. Jones, Gordon A. McFeters, and Anne K. Camper. An

abbreviated and condensed version of this chapter has been submitted to the journal Microbial Ecology.

The relation of total biomass to active biomass comprises Chapter 3. This Chapter is titled "Relationships Between Total and Active Cells in Heterotrophic Bacterial Populations Subjected to Controlled Carbon Sources, Carbon Concentrations, and Chlorination." This chapter will be submitted to the journal Applied and Environmental Microbiology. The authors are the same as those for Chapter 2.

Chapter 4 reports on the growth rates, bacterial carbon production, and observed yields of the communities subjected to the various treatment factors. This chapter is titled "Effects of Carbon Source, Carbon Concentration, and Chlorination on Growth Related Parameters of Heterotrophic Biofilm Bacteria" and the authors are the same as for Chapter 2. An abbreviated and condensed version of Chapter 4 has been submitted to the journal Microbial Ecology.

We suspected that a significant amount of sampling would be required to detect differences in the response of bacterial communities to the different treatments. As such, we attempted to structure the experiments so that they were amenable to more powerful statistical tests than can be ordinarily performed in studies involving environmental microbiology. Three major treatment levels were applied: (1) substrate type (amino acids, carbohydrates, and humics), (2) substrate concentration (500 ppb C, 1000 ppb C, and 2000 ppb C), and (3) a base level treatment of chlorination. All experiments were performed in parallel annular reactors with one acting as a control and the other receiving chlorine. This experimental design is presented in Figure 1.1 and is amenable to a nested

analysis of variance (ANOVA). With this experimental design, treatment evaluation could be compared both within and between the various treatments and their levels. Nesting of the sampling strategy refers to the fact that all lower level treatments can be analyzed within the level or levels of treatment above that level. Thus, sampling level is nested within substrate concentration (at 3 levels) and both sampling and substrate concentration are nested within substrate (also at 3 levels). This allowed us to analyze the differential effects of 3 levels of treatment (sampling level, substrate concentration, and substrate type) simultaneously or separately.

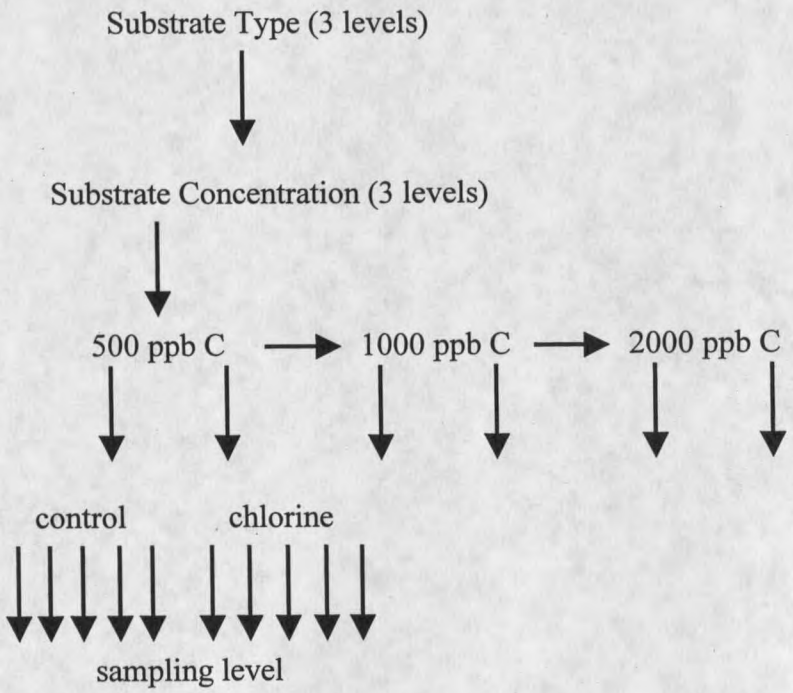

Figure 1.1 – Schematic of the experimental design used to evaluate substrate type, substrate concentration, and chlorination in this dissertation. Samples can be divided into control and chlorinated which can be nested within the next higher level of substrate concentration which contained three treatment levels. This middle treatment level of substrate concentration can also be nested within the upper treatment level of substrate type which also contains three treatment levels.

The experiments performed for this dissertation were funded by an American Water Works Association Research Foundation (AWWARF) grant to Dr. Anne Camper (Montana State University, Bozeman, MT), Dr. Peter Huck (University of Waterloo, Ontario, Canada), and Dr. Mark LeChevallier (AWWA Service Co., Voorhess, NJ). The project was titled "Investigation of Biological Stability of Drinking Water in Treatment Plants and Distribution Systems." The experiments performed at the Center for Biofilm Engineering, MSU, were the laboratory scale component of a larger study involving full scale treatment facilities in Canada and the United States.

CHAPTER 2

BIOMASS DISTRIBUTIONS IN HETEROTROPHIC BACTERIAL COMMUNITIES

Introduction

Bacteria play an important role in the turnover and cycling of elements, in addition to providing secondary carbon production in aquatic ecosystems. Microbial biomass is most commonly estimated from cell numbers and quite often from biovolume measurements (Norland, 1993; Bjornsen, 1986; Lee and Fuhrman, 1987; Nagata, 1986; Troussellier, et al., 1997; Fagerbakke, et al., 1996). Direct conversion of cell numbers to biomass through use of mass per cell or conversion of biovolume based on mass per volume are the standard methods of choice. In microbial communities comprising highly heterogeneous cell sizes, the number of volume measurements needed to adequately describe the distribution can become quite large. An ability to predict bacterial biomass that does not require an excessive number of observations of individual cells would be advantageous in elucidating the significance of heterotrophic bacterial activity in aquatic environments. Conversion factors used for adjusting biovolume or cell numbers to biomass influence the calculation of turnover rates and nutrient fluxes in almost all environmental microbiological studies.

Variability in the conversion factors for carbon per cell (C_c) or carbon per volume (C_v) range over more than an order of magnitude both between and within different studies (Fagerbakke, et al., 1996; Bratbak, 1985). Due to this large variability, most investigators have pointed out the advisability of determining the conversion factor for the particular environment under study. An excellent compilation of biovolume conversion factors through 1996 is given in Fagerbakke, et al. (1996). This paper provides values corresponding with environment (freshwater, marine, cultured, etc.) or organism (native communities, supplemented cells, cultured, etc.). Since 1996 a few more studies have added to the literature on conversion factors (Robertson, et al., 1998; Fukuda, et al., 1998; Loferer-Kroβbacher, et al., 1998; Troussellier, et al., 1997). Of particular interest is the study by Trousellier, et al. (1997), where previous speculation on the non-linear relationship between bacterial carbon content and cell volume was experimentally validated for 10 different Proteobacteria (marine and non-marine) under starvation conditions. This correlation, where smaller cells have a higher content of C_v, than larger cells had been tentatively confirmed in previous studies (Norland, 1993; Norland, et al., 1987; Simon and Azam, 1989; Lee and Fuhrman, 1987; Kroer, 1994; Psenner, 1990). Trousellier, et al. (1997) and Kroer (1994) expressed this relationship on a C_v basis, whereas Simon and Azam (1989), Norland, et al. (1987), and Lee and Fuhrman (1987) expressed the relationship on a C_c basis. Both methods produce different results and give differential weights (i.e. scaling factor) to organisms of different size.

While most of the investigations tend to infer that the relationship between carbon content and cell size is allometric, this occurs due to the common practice of

plotting the data on log-log scales. Allometry refers to the relationship between relative growth of a part in relation to the whole organism. Inherently, the distribution underlying the log-log transformation is non-linear and this should not be forgotten when attempting to use conversion factors. Given a large variability in conversion factors as well as the non-linear relationship it becomes important to determine whether a single conversion factor is appropriate or if a non-linear function that applies differential values to cells of different volumes is more appropriate. A simple allometric relationship would imply that the use of a single conversion factor for individual bacteria in communities and populations of variable biovolume is appropriate; however, doing so can result in either under or overestimation of the total biomass (Troussellier, et al., 1997; Lee and Fuhrman, 1987; Psenner, 1990; Verity, et al., 1992; Kroer, 1994; Vidondo, et al., 1997). Modeling of the biomass/biovolume interdependence is generally achieved with a power function due to the non-linear form of the relationship. The function normally used to describe this type of data is C_c or $C_v = b\text{Vol}^a$, where a is termed the location parameter and b is a constant that relates the amount of carbon present at unit volume. Underlying this power relationship is a probability distribution of the Pareto type (Johnson, et al., 1987; Vidondo, et al., 1997). Log-log plots of biovolume vs. C_v have generally been used to ascertain the correlation between these two variables. Trousellier, et al. (1997) found such a relationship between biovolume and C_v, implying that the use of a single value for the conversion of biovolume to biomass is appropriate for an accurate determination of biomass distributions in bacterial communities.

Similar to investigations of bacterial biomass, a variety of models based on biomass-size distributions in aquatic organisms such as fish, phytoplankton, and zooplankton have been derived (Boudreau, et al., 1991; Borgmann, 1987; Ahrens and Peters, 1991; Gaedke, 1993; Sheldon, et al., 1972; Dickie, et al., 1987; Harris, 1994). These models work quite well in describing predator/prey relationships and the transfer of energy between trophic levels. These models are also of the power form and generally presented as log-log plots. Vidondo, et al. (1997) recently showed that by using the normalized biomass-size spectra (NB-SS) models in analyzing size distributions, investigators have unknowingly been assuming that the underlying probability distribution follows a Pareto relationship. Originally, the Pareto distribution was developed in order to model income distributions in populations, but it has subsequently been used to model the distribution of the largest and smallest values for natural phenomena such as waves, pollutant concentrations, demographics, and floods (Castillo and Hadi, 1997; Vogel, et al., 1993; Johnson, et al., 1987; Zelterman, 1992; Lewis and Chatwin, 1997; Hosking and Wallis, 1987; Vidondo, et al., 1997). Since bacteria are considered the smallest of living organisms (neglecting viruses) and seem to demonstrate size extremes, then perhaps, biovolume/biomass can also be modeled by a Pareto distribution. As well as noting the equivalency of the NB–SS and the Pareto distribution, Vidondo, et al. (1997) stated that this type of data should be viewed and analyzed as a probability distribution. If the data conform to a probability distribution then we can take advantage of advances in the analysis, identification, and description of this type of data. For example, methodological advances have been made for identifying and describing the

parameters underlying different types of probability distributions using L-moment diagrams (Vogel and Fennessey, 1993; Hosking, 1990; Wang, 1996; Vogel, et al., 1993). L-moments are analogous to conventional moments (i.e. mean, variance, skewness and kurtosis), but they can characterize a larger variety of distributions and are more robust against outliers when estimated from samples. The ability to discriminate between different distributional hypotheses is made easier with L-moments. They are often superior alternatives for summarizing probability distribution in environmental samples. In this study we adopted methods used in hydrology for the analysis of the probability distributions of flood frequencies and applied these to bacterial biovolumes, C_c, and C_v.

While predictions based on the biomass-size spectra models used in macroecology are effective at higher trophic levels, bacteria have been difficult to model with this method (Gaedke, 1993). The first reason is that the models assume that carbon flow proceeds from small to large organisms. Trophic status in bacterial communities can be controlled either by top-down pressures due to grazing or bottom-up pressures due to resource limitation (Gaedke, 1993; Psenner and Sommaruga, 1992; Gasol, et al., 1997; Meyer, 1994; Pernthaler, et al., 1996; Felip, et al., 1996; Emerson, et al., 1994). Secondly, the models assume an allometric relationship, which has been investigated and shown to occur for cultured cells and bacteria from natural environments. However, data in the literature supporting this assumption are scarce. Thirdly, a coupling between metabolic activity and biomass, as is also implied by the allometric biomass–size spectra models used in oceanography and limnology, has generally not been detected in bacteria. Highly variable measurements on the active fraction of bacteria in aquatic environments

have been reported by a number of investigators (Tuomi, et al., 1995; Pernthaler, et al., 1996; Grossmann, 1994; Karner and Fuhrman, 1997).

In this study we investigate the biomass-size probability distribution of mixed heterotrophic bacteria in a semi-controlled, nutrient-supplemented and carbon-limited biofilm bioreactor originally intended to model drinking water distribution systems (Van Der Wende, et al., 1989). Oligotrophic drinking water with an assimilable organic carbon (AOC) content of approximately 25 μg acetate carbon equivalents/L was supplemented with 3 different carbon sources comprising those most frequently detected in aquatic systems. Investigated substrates included amino acids, carbohydrates, and humics supplied in separate experiments at three different carbon loading rates (250, 500 and 1000 μg carbon/L•hour) simulating different trophic states. Parallel reactors were run simultaneously with one acting as a control and the second receiving chlorine to investigate the effects of chlorine stress on the biomass distribution in the presence of different carbon sources. Probability distributions for biovolume and biomass were determined and found to follow a type II 3-parameter generalized Pareto distribution (GPD) in 68 of 72 datasets comprising a total of approximately 36,000 individual measurements. Carbon source, loading rate, and chlorination were found to significantly affect the mean and median biovolumes, as well as the parameters that describe the biovolume distribution. An empirical relationship between cell volume and C_v is derived that takes into account theoretical limitations on bacterial size and C_v. This relationship produces estimates that are more reasonable for C_v at the extreme small and large end of the biovolume spectrum when compared to models based on power functions.

Materials and Methods

Experimental System

The generation of mixed heterotrophic bacterial populations was accomplished with the use of a model biofilm reactor system comprised of rotating annular reactors (Van Der Wende, et al., 1989). Polycarbonate based, the reactors consist of a stationary outer cylinder with a rotating inner drum forming an annulus 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 total submerged surface area in the reactors was 0.18 m^2 and the liquid volume was 630 mL. The reactor top has twelve holes with stoppers that are not in contact with the liquid phase and can be removed to allow access to the slides without interrupting reactor operation. The rotational speed of the inner drum can be varied to create the desired shear stress on the wall of the outside cylinder. In this study 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 foot per second (0.3 meters per second). 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 2.1 hours for all experiments. The colonized areas of the annular reactors were immersed in a