University of Montana

ScholarWorks at University of Montana

2001

Subsurface microbiology: Viral transport studies and the microbial ecology of landfill environments

Patrick N. Ball
The University of Montana

Follow this and additional works at: https://scholarworks.umt.edu/etd

Let us know how access to this document benefits you.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Maureen and Mike
MANSFIELD LIBRARY

The University of

Montana

Permission is granted by the author to reproduce this material in its entirety, provided that this material is used for scholarly purposes and is properly cited in published works and reports.

Please check "Yes" or "No" and provide signature

Yes, I grant permission   ✓
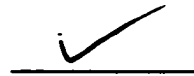
No, I do not grant permission   _____

Author's Signature: _____

Date: _12/2/01_

Any copying for commercial purposes or financial gain may be undertaken only with the author's explicit consent.

8/98

Subsurface microbiology: Viral transport studies and the microbial ecology of landfill environments.

By

. **Patrick N. Ball**

B.S. The University of Montana, 1991

Presented in partial fulfillment of the requirements for the degree of
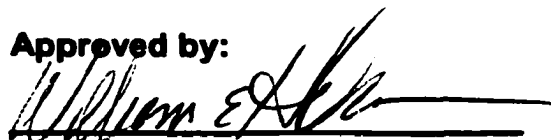
Doctor of Philosophy

The University of Montana

2001

Approved by:

_____
Chairman, Board of Examiners

_____
Dean, Graduate School

12-28-01
_____
Date

UMI Number: 3034897

UMI®

Subsurface microbiology: Viral transport studies and the microbial ecology of landfill environments.

Director: Dr. William E. Holben WEH

To investigate the transport behavior and fate of viruses in subsurface environments field experiments were conducted in an unconfined, cold-water aquifer by seeding viruses into established monitoring well networks. Both natural and forced gradient experiments were conducted using bromide, bacteriophages MS2, ΦX174, PRD1 as well as, poliovirus (Chat 1 strain). Breakthrough curves were established and percent relative breakthrough (RB), attenuation (RA), collision efficiencies (α) and aqueous mass balance data was calculated. A third experiment was conducted in a sewage impacted aquifer. Here an additional forced gradient experiment was conducted in the established well network. During this study bromide and MS2 were seeded into the groundwater and both aqueous and solid phase data was collected and a total system mass balance was determined.

Information from these studies lead to the realization that high concentrations of virus are capable of moving with the average velocity of the groundwater and capable of surpassing setback distances commonly used between source wells and septic systems. Additionally, viral isoelectric points (pI) have a major influence on adsorption (effect α values), which in turn, appears to be a major controlling factor for attenuation. However, adsorbed viruses may have lower inactivation rates and are capable release back into the aqueous portion of the aquifer. Further, under forced gradients the bacteriophage % RB is increased relative to natural gradients, while that of poliovirus is decreased. Also, pore exclusion transport may be enhanced under a forced gradient. By performing a total mass balance (solid and aqueous phase) the validity of the parameters RB, RA, and α were tested. Although calculated values were consistent with measured values, the attenuated portion of the virus proved difficult to quantify.

A Multi-tiered approach using a combination of molecular techniques was used to access gross microbial community structure and diversity and identify possible key members of landfill environments. Phylogenetic analysis identified microorganisms whose "best match" in established databases distinguished them as candidates in a proposed model of landfill microbial community members. Physical and chemical environments of landfills and their relative unexplored nature suggest some of the "best match" identities could be undescribed bacterial species or sub-species.

ACKNOWLEDGMENTS

My sincere thanks to Dr. William Holben for his encouragement and support during the final years of my studies. I am also indebted to Dr. William Woessner from the U of M Geology department for his tremendous support. I am also very grateful to my committee members, Dr. Mike Minnick, Dr. Don Christian, and Dr. Scott Manning. I would also like to thank Dr. Dan DeBorde. I am also grateful to the many "hands" that worked so hard in the field and laboratory lending their assistance. Thanks to the many great people in the DBS. A special thanks you to my father who taught me to hang in there. Last, but most important, to my wife Esther who has supported me with her understanding, encouragement, inspiration and love throughout this process.

TABLE OF CONTENTS

iv

LIST OF TABLES

LIST OF FIGURES

Chapter 1

General Introduction

It is estimated that between 70 and 110 million people, approximately 50% of the population of the United States, use groundwater as their main source for drinking water supplies (Rose, 1998). Aquifers that are shallow, cold, unconfined and prolific are especially susceptible to contamination with various pathogenic microorganisms. Groundwater contamination has been implicated as the source in about half of all microbial disease outbreaks, accounting for several million illnesses per year (Keswick and Gerba, 1980). Sources of pathogens include leaking sewer lines, land application of sewage, and septic system effluent

Septic effluents can contain pathogenic human viruses (>100 enteric viruses) which may be shed in fecal matter during episodes of infection (Rao and Melnick, 1987). These types of pathogens are especially noteworthy because their infectious dose can be exceedingly low; in some cases a few viruses may cause disease onset (Abbaszadegan et al., 1999). These viruses are generally quite robust; capable of withstanding low pH and organic solvents, conditions that may prevail within septic systems. Such characteristics allow for survival in saturated zones of cold water aquifers for long periods of time. In turn, this provides an opportunity for viral transport over tens to hundreds of meters from a source.

From research conducted in our own laboratory we have isolated seeded coliphage (acknowledged as an acceptable surrogate for modeling the behavior of enteric viruses in field studies) in monitoring wells over 40 m from the site of injection, and

1

recovered bacteriophage at measurable titers nine months after the time of input. These inherent capabilities highlight the threat of viral disease(s) being contracted via water transmission. In fact, many cases of gastroenteritis have reportedly been contracted through this route, with the likelihood that a great number of illnesses go unreported or that the source of origin is unknown (Bitton and Gerba, 1984; Moore, 1982). To assume that this occurs only in third world countries is naive. In 1993, this laboratory found titers of roughly 150 PFU/ ml of virus, from raw sewage coming into the Missoula wastewater treatment plant. This may account for the three to five unexplained cases of Hepatitis A that occur annually in Missoula County (Greg Oliver).

Specific concentrations of human viruses allowable in drinking water are as yet undefined. The World Health Organization (WHO) has suggested that drinking water should contain less than one infectious particle per 1,000 liters, while the Environmental Protection Agency (EPA) has suggested a limit of no more than two viruses in 10,000,000 liters (Regli, 1991). Despite these recommendations, laboratory methods for detection of viruses to such high levels of sensitivity do not currently exist. At best, even under ideal conditions, approximately one virus in 1,000 liters is the lowest level of detection currently capable of being measured. In view of these facts the EPA has proposed a draft Ground Water Disinfection Rule (GWDR); (National Primary Drinking Water Regulations: Ground Water Rule – EPA), with work currently underway to complete a final version. In the current GWDR, water providers must demonstrate that the number of infectious particles in drinking water reaching a wellhead is such that less than one infection per 10,000 people occurs per year. This roughly translates into the aforementioned < 2 viruses/10^7 liter, based on a risk assessment performed by Regli et al.

2

in 1991. Also stipulated under the draft GWDR, potable water providers would not be required to chemically treat groundwater if such water, through the process of "natural disinfection", meets the above requirement.

Adequate delivery of groundwater to wells under "natural disinfecting" conditions is an attractive alternative to conventional chemical or physical means of treatment and supply (Maclar, 1995). Although the risks are not completely defined, products or byproducts associated with chemical treatment can affect human health adversly. The cost of providing safe drinking water by these methods can be astronomical, and providers required to continue service to the public must recover that cost by increasing fees to customers. A proposed method for granting disinfection variances is based on models predicting concentrations of virus that would enter the capture zone of a supply well. However, to date, the models proposed have generally under-predicted concentrations and over-predicted travel times of viruses. (Yates and Jury, 1995). It is obvious that to use this sort of predictive tool more information on virus fate and transport must be gathered and analyzed. Of particular interest are data from laboratory studies that can be correlated with field scale studies. Integrating these approaches would seem to provide the most reliable information concerning these questions.

There is a general lack of information that describes transport parameters of virus in a presumptive high-risk aquifer, such as the groundwater system found in the Missoula valley. Due to its nature, this highly conductive, cold, coarse-grained groundwater system, the sole source supply to city and rural residents, represents a "worst case scenario", when considering the potential for subsurface virus transport and survival. Knowledge of the partitioning behavior of viruses between the bound and unbound

3

phases in this type of setting is vital. Such information may be used to re-evaluate safe setback distances between septic system drain-fields and tanks and potable water sources, and possibly aid in implementing appropriate remediation strategies. This information will also provide insight for evaluation of the controls and constraints on the natural disinfection process. Major variables to be considered will be maximum concentrations and travel times.

Field and column/batch experiments cited in the literature suggest that adsorption and inactivation are the principal mechanisms that control the fate and transport of viruses in subsurface environments (Yates et al., 1987; Goyal and Gerba, 1979; Bales et al., 1993; Gerba, 1984). Additionally, dispersion may account for the reduction of virus concentrations by a dilution effect. Dispersion is generally controlled by the hydrogeological properties of an aquifer. Factors such as groundwater velocity and sediment dispersivity result in this dilution process.

Adsorption seems to be the most critical factor controlling the transport and fate of viruses in groundwater systems, especially in systems with similar hydrogeologic characteristics and cold water temperatures as in the Missoula valley. A number of factors influence virus adsorption to soil particles, including soil type and chemistry, pH, ionic strength, hydrophobic interactions and type and strain of virus (Goyal and Gerba, 1979; Keswick and Gerba, 1980; Gerba, 1984; Jansons et al., 1989a; Bales et al., 1993; Yeager and O'Brien, 1979). Researchers have gone to great effort in trying to describe the interactions that occur between viruses and adsorbent matrices (Gerba, 1984). Two of the most important results of this research are the realization that the binding event is reversible and possibly protective in nature (Bales et al., 1993; Bales et al., 1995; Bitton,

4

1975; Grant et al., 1993; Herbold-Paschke et al., 1991; Hurst et al., 1980). The former

effect is evident, based on our previous research at two separate experimental sites

(Erskine Fishing Access site and Frenchtown High School) during several transport

experiments in which the bacteriophages MS2, PRD1 and ΦX174 and attenuated polio

virus type I (chat strain) were used (Fig. 1).

P2-9, 25 ft
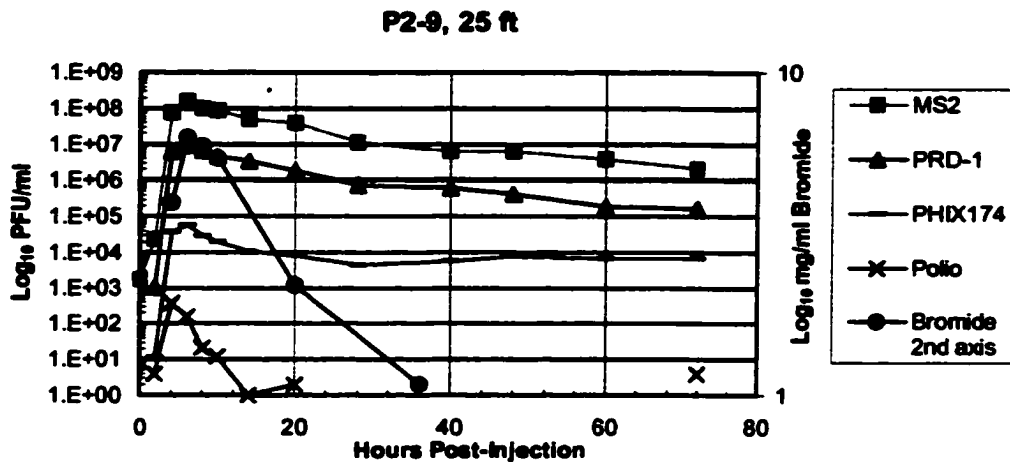
Figure 1. Breakthrough curve constructed from data obtained from monitoring well (from Erskine field site, natural gradient experiment).

The long tailing that follows the peak concentration of the virus that passes the

monitoring wells indicates that the main mass of the virus is most likely adsorbed and

then slowly released over time back into the flow system (Fig 1). This phenomenon poses

a potential threat to downgradient wells. It has also been proposed that a portion of the

tailing may be the result of unabsorbed aqueous phase virus entering lower velocity

portions of the aquifer.

Though not well understood, the main mode of inactivation of virus particles

appears to be temperature controlled, with inactivation having a positive correlation with

increases in temperature (Yahya et al, 1993, Yates and Yates, 1987, Snowdon et al.,

5

1989). However, there may be other contributing factors such as chemical (e.g., enzymatic degradation and low levels of dissolved oxygen) or physical events (e.g., conformational changes on virus surface) that cause either permanent or temporary loss of infectivity, thus contributing to inactivation (Bales et al., 1993). Most previous concern about subsurface transport has focused on the portion of the virus that is transported in the aqueous phase. This fraction should be found first and farthest down gradient, entering well capture zones. The fact that this portion may represent a relatively small concentration is not trivial. With sources reaching values greater than 10^{10} PFU/ml (Abbaszadegan et al., 1999), one can easily see that even small percentages still represent large quantities of potentially infectious particles. To meet the proposed limitations of < 2 viruses/liter reaching the wellhead, a 13 log reduction in the virus concentration from source would be required. Under the influence of a pumping well there may be an increase in viral concentrations arriving at capture zones. In addition, this elevated level may persist for longer periods of time than would be normally encountered under natural conditions.

The research that conducted provides data useful in a number of relevant areas. For issues concerning water quality and disinfection, these studies provide data relevant to developing natural disinfection criteria necessary for well and aquifer protection. Protective regulations provide health, and cost benefits, by reducing chemical or physical groundwater disinfection treatments. In addition, this research also provides information related to the potential health risks posed to the public in areas that could be considered under threat of contamination by defining transport parameters in "high risk" environments (cold and highly conductive aquifers).

6

To summarize, increasing human population densities put a burden on local water supplies, both in a consumptive and additive manner. In areas where groundwater supplies the majority of domestic water, the risk of groundwater contamination by pathogenic microorganisms appears to be increasing. It is ironic that the same features that make an aquifer desirable for obtaining quality drinking water (e.g., high transmissivity, cold) also make it more susceptible to becoming contaminated with microbial pathogens such as human enteric viruses. Unfortunately, there is a paucity of information available to the public and policy makers concerning these issues. Insufficient data on primary factors that control the transport and fate of microorganisms are the limiting element impeding this process of information acquisition and dissemination. Understanding controlling parameters is paramount to gaining insight into natural disinfection processes for viruses.

Dissertation organization, research aims and the scope and nature of research project.

Dissertation organization:

Chapter 1, presented above, is a general introduction to the topic of subsurface viral transport and presents the conceptual framework for the subject-matter of this dissertation. Chapter 2 provides an introduction to concepts and factors controlling viral transport. Chapter 3 is a review of supporting research and background information from a previous experiment, which sets the stage for the original research that was conducted. It highlights the main points of a manuscript published in the journal *WATER*

7

RESEARCH. Chapter 4 is a draft of a manuscript entitled "Viral Attachment During Groundwater Transport Under Field Pumping Conditions: Mass Balance Approach" submitted for publication. Chapter 5 is a draft of a manuscript entitled "Partitioning of MS2 During Transport in a Septic Effluent Impacted Unconfined Aquifer" and in the process of being submitted for publication.

Research Aims:

The specific aims of this research are to address and quantify the processes affecting virus transport through an aquifer, the fate of the viruses and how working wells further influence these processes. The information gathered from this study further define critical processes controlling virus concentrations in groundwater systems. Our approach uses field experiment data to examine the validity of laboratory observations. There have been few experiments using viruses (bacteriophage and particularly human enteric viruses) at field scale.

Scope and nature of research project:

The nature of this project bridges the gap between hydrogeology and microbiology and in that sense required an interdisciplinary approach. The findings gathered during the natural gradient experiment helped identify critical areas yet to be addressed. The following approach was used:

1) A forced gradient, multiple-virus field experiment was conducted, and a mass balance based on the aqueous phase mass was computed. This measured quantity was compared to theoretic viral mass values computed using relative breakthrough analysis.

8

2) I describe and quantitatively examine the partitioning processes of a virus between the aqueous phase and solid phase during transport at the field scale. The petitioning process was examined by seeding the bacteriophage MS2 through an injection into the capture zone of a pumping well and computing both an aqueous and bound phase mass balance. Then results of these computations were compared with theoretical values computed using relative breakthrough and attenuation analysis.

9

Chapter 2

General Concepts in Viral Transport:

This chapter addresses critical factors influencing virus fate and transport in subsurface environments. These include physical, chemical and biological components.

General hydrodynamic factors that may affect virus fate and transport:

The transport of a solute through saturated subsurface porous media is principally controlled by advection and dispersion. The main mass of a conservative solute is transported at approximately the average flow velocity of the groundwater. This process is referred to as advection. The distribution of the solute mass can also be altered during transport by mechanical dispersion and molecular diffusion. Together these spreading mechanisms, hydrodynamic dispersion, have a tendency to cause solute mass to spread three-dimensionally along their flow paths.

Mechanical dispersion results from the composition of the subsurface substrate and its innate properties. Three-dimensional spreading occurs when the solute enters pores of different sizes and pore channels of different lengths, as the velocities field is effected so is the rate of solute transport. Spreading in the direction of flow is termed longitudinal dispersion (Fetter, 1994). The three-dimensional mechanical dispersion is equal to the product of the average linear velocity of the solution and the directional dynamic dispersivity. Dynamic dispersivity is defined as a factor that accounts for the scale effect observed at field scales (Fetter, 1994). This mechanical process results in the mass of the solute becoming increasingly diffuse (concentration becomes lower) as the length of the flow path increases.

10

The second component of hydrodynamic dispersion is molecular diffusion. However, molecular diffusion is usually considered independent of solution movement and is a function of a chemical gradient. Molecular components move due to kinetic energy or Brownian motion (random motion) along a gradient of concentration. It is considered the primary factor in solute movement in the vicinity of sediment particles (Penrod et al. 1996) and when flow velocities are extremely small.

Solutes that are not conservative react or interact in several different manners. Under conditions where the solute is reactive (usually charged particles) and/or the matrix of the porous media is charged (such as clay particles) solute transport can be affected by retardation. Under these conditions, the solute may become adsorbed causing the rate of the solute mass travel to fall below the average flow velocity. The amount of solute adsorbed per unit of adsorbing material may be plotted as an adsorption isotherm. This type of information is used to describe the relationship that exists between solutes and matrices. It is the basis from which a distribution coefficient (K) can be determined, which in turn can be used to compute a retardation factor (R) that describes the impact adsorption has on solute transport.

Real geologic porous media are normally not isotropic and homogeneous in their composition and distribution of these properties affects solute transport. This means that variations in grain shape, composition, and distribution can have significant effects on solute transport. Harvey et al. (1993) cited physical heterogeneities as being responsible for differential transport of bacteria and bromide. LeBlanc et al. (1991) reported similar findings for transport of a conservative tracer at the same site. Woessner et al. (1998) found that local heterogeneities created a zone of preferential flow within the capture

11

zone of a pumping well producing increased transport rates and higher than predicted concentrations of virus at a withdrawal well head during a bacteriophage tracer experiment.

Non-hydrodynamic factors that affect virus fate and transport:

Movement of viruses in ground water environments is generally controlled by colloidal deposition that can be viewed in two parts; transport and attachment (Loveland et al. 1996). Attachment of colloids is thought to be controlled by the hydrodynamic approach and surface chemical interactions (Loveland et al. 1996). The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory attempts to quantify particle/collector interactions by examining the balance between the van der Waals forces (responsible for attractive forces) and double-layer interactions (responsible for repulsive forces; Gerba, 1984). Factors that influence virus attachment may include, but are not limited to, ionic strength and valency, organic matter, pH, dissolved oxygen and soil composition. **Ionic strength and valency** – High ionic strength solutions generally lead to increased virus attachment to surfaces because they constrict the thickness of the double layer that exists between particles of like charge (Gerba, 1984). Additionally, the valency of cations is important, and increased adsorption is positive correlated with valency (trivalent > divalent > monovalent ; Bitton, 1975). This is only true when the pH of the solution is above the pI of the virus and sediment, which is typically the case in aqueous environments with near neutral pH (Lance and Gerba, 1984). They also indicated that high salt content in sewage and tap water increased virus attachment. A similar result was reported by Kinoshita et al. (1993) for the bacteriophage PRD1 in column studies.

12

Viruses in wastewater effluents with conductivities of $500 - 600$ μmhos/cm^3 demonstrated higher retention to soils when compared to experiments with similar soils in which distilled water with conductivity of approximately 10 μmhos/cm was used (Bitton and Gerba, 1984).

Organic matter - Evidence suggesting that the presence of organic matter in groundwater hinders virus adsorption to sediment can be found in the literature (Bitton and Gerba, 1984; Powelson et al., 1991; Bales et al., 1989,1993). Although most of these studies were conducted in laboratories, two recent field experiments in sewage-impacted areas report similar findings (Pieper et al., 1997; Schijven et al., 1999). It is believed that the organic materials compete for similar binding sites on sediments (Bitton and Gerba, 1984) and also coat the surface of viruses altering their adsorption behavior to solids (Gerba, 1984). Sobsey et al. (1980, 1995) and Moore et al. (1982) concluded that muck soils (high organic content) had a lower adsorption capacity then typical mineral soils, such as sand.

pH – Because most viruses have a capsid (outer coat) constructed of ionizable amino acids (in the form of proteins), pH of the environment plays a significant role on the charge that can be found on the capsid. However, each virus is characterized by its own isoelelectric point (pI) value (i.e., the pH where total negative and positive charges on the capsid are equal), which is ultimately affected by pH of the surrounding environment. A number of experiments suggest that pI appears to be one of the most important factors affecting virus transport in subsurface media (Bales et al., 1993; Loveland et al., 1995; Jin et al., 1997; Pieper et al., 1997; Dowd et al., 1998; DeBorde et al., 1999; Ball et al., 1999 submitted). This argument also holds true for the surface of the sediment, whose

13

exposed charged groups can be modified by the pH of the aqueous environment.

Typically, the net charge of the virus will be negative at pH values above neutrality. This

appears to also be true for most major soil components, including sands, clays and

organics.

Both attachment and detachment of bacteriophage are pH sensitive. Bales et al.

(1991) and Kinoshita et al. (1993) report that bacteriophage attachment to sand was

reduced at pH ranges between 5.5 and 8.0. Loveland et al. (1995) and Schijven et al.

(1999) demonstrated in laboratory settings that viral detachment was pH susceptible,

increasing with rising pH values, probably due to changes related to hydrophobic

characteristics existing on the sediment. In field studies conducted by Bales et al. (1995)

and Ryan et al. (1999), the bacteriophage PRD1 recoveries were enhanced after pulse

injections of high pH solutions (pH >8.3). Conversely, lower pH conditions have been

shown to enhance virus adsorption (Goyal and Gerba, 1979, Sobsey et al., 1980,

Loveland et al., 1995).

Dissolved oxygen (DO) – Under increased dissolved oxygen (DO) conditions an oxic

environment predominates. This condition is considered conducive to the formation of

metal-oxide rich environments. Oxide species, such as Fe_2O_3, are positively charged and

thus can promote virus adsorption (Gerba, 1984, Loveland et al., 1995, Pieper et al., 1997

and Ryan et al., 1999) through electrostatic interactions. In 34 soils tested by Moore et

al. (1982), those containing metal oxide species were determined to be the most effective

at virus adsorption.

Soil types – Soils are extremely complex environments and may contain a number of

components such as sand, silt, gravel, clay and organic matter. Each of these components

14

can influence on how viruses sorb. When Moore et al. (1981) examined 34 different soils they found that poliovirus adsorption varied was 16 - 79% in muck type soils and as high as 99.99% for sand-rich sediments. Their general conclusions indicated that there was a significant correlation between the total amount of negatively- charged soil material and adsorption. This was a confirmation of the findings of Sobsey et al. (1980) using poliovirus in various soils and adsorption conditions. Bitton and Gerba (1984) summed up data from a number of studies by saying that, in general, fine textured soils were the most effective at adsorbing viruses and that muck type soils were typically the least effective.

Burge and Enkiri (1978) used the bacteriophage ΦX174 to examine the adsorption characteristics of five soils with various cation exchange capacities (CEC), clay contents, specific surface areas (SSA) and organic carbon (OC) content. In general, their results indicate that virus adsorption capacities increased with corollary increases in CEC, OC, SSA and clay content. The soil that demonstrated the least virus adsorption capability was also the coarsest and had the highest pH, which appears to be consistent with later research results. Meschke and Sobsey (1998) conducted batch studies using six different soil types ranging from very high clay content to pure sand and including one with organic muck soil. In this study the soils containing clays exhibited the greatest adsorption of viruses, while the lowest adsorption observed was for soils containing organic muck and coarse sand.

Virus type - Different types and strains of viruses behave differently during their transport in subsurface environments. This heterogeneity in transport behavior came to

15

light when researchers were attempting to identify suitable surrogates for enteric viruses that were safe, easy to handle and allowed for efficient assays. Goyal and Gerba (1979) examined the adsorption of 33 different viruses including many enteric viruses and bacteriophages. They concluded that, although a number of the viruses behaved similarly, some closely related species did not have similar adsorption properties. It also appeared that most human enteric viruses were more readily adsorbed then most bacteriophages. Some researchers have classified viruses into three distinct groups depending on the adsorptive nature (Goyal and Gerba, 1979; Gerba et al., 1980, 1981; Bales et al., 1991). Group I, includes many of the commonly used surrogates like MS2 and ΦX174; they exhibited relatively low adsorption to test soils. Group II, which includes poliovirus I, exhibited a high adsorption behavior. The f2 bacteriophage was categorized separately in Group III based on its variability of adsorption behaviors, unique to most test soils.

Blanc and Nasser (1996) showed in laboratory tests that poliovirus and bacteriophages MS2 and PRD1 all had different binding characteristics. In batch studies, Meschke and Sobsey (1998) using poliovirus, Norwalk virus and bacteriophage MS2, also demonstrated that adsorption was dependent on virus type. Using six different soils ranging from very high clay content to pure sand, they showed that the general trend in adsorption for all cases was of the following order: poliovirus > Norwalk virus> MS2. Field studies conducted by DeBorde et al. (1999) confirmed that the adsorption behaviors of poliovirus and the bacteriophages PRD1, MS2 and ΦX174 differed, with poliovirus exhibiting the greatest degree of adsorption. Powelson and Gerba, (1994) concluded that poliovirus was significantly retarded as compared to MS2 and PRD1 during saturated flow in column experiments, again indicating the highest rate of adsorption. Most likely,

16

the individual biological characteristics (e.g., amino acid composition of the capsid) and perhaps to some extent the capsid geometry, play a major role in the adsorption behavior of viruses (Powelson et al., 1993; Dowd et al., 1999; Ball et al., 1999 submitted).

Virus Survival:

As for virus activity and behavior in the subsurface, one must consider two distinctly different environments: groundwater and sediment/soil matrix. Clearly temperature and soil type are two of the most dominant factors governing fate and transport of viruses. However, in soils and sediments other factors such as the degree of soil saturation, soil chemistry and particulate association exert additional influences. Typically daily inactivation rates for viruses are expressed in terms of the following rate equation (measured in day^{-1}):

$$C_t = C_o (e^{-Kt})$$

Where C_t is virus concentration at time t, C_o is initial concentration and K is the inactivation rate.

Virus survival in groundwater: Inactivation is a major factor contributing to the loss of viable virus mass during subsurface transport. Inactivation, or loss of replication ability, could occur due to a number of reasons that may include but are not limited to; physical or chemical alteration/destruction of genetic material or capsid proteins and interference with receptors due to geometric distortion or hindrance. While a number of researchers (Yahya et al., 1993; Yates et al., 1985; Powelson et al., 1990; Enriquez et al., 1995) have found temperature to be the single most important factor responsible for inactivation in groundwater, Jansons et al., (1989) reported dissolved oxygen to be the most critical factor leading to loss of infectivity for a suite of enteric viruses. They

17

postulated that dissolved oxygen might be directly responsible for oxidative damage to viruses. Snowdon et al., (1989) established that temperature was indeed important to virus inactivation, but the chemical and particulate composition of the liquid had a greater overall negative effect on survival. Experimentally determined inactivation rates for a number of different viruses in groundwater differ over 2 orders of magnitude (Gerba and Bitton, 1984) depending on the virus type. However, Sobsey et al., (1995) compared survival of two bacteriophages with two enteric viruses found in sewage contaminated seawater. They found that a 4 log unit reduction was reached in time intervals that ranged from one to 10 weeks under similar experimental conditions. This would suggest that there is a wide range of inactivation rates for viruses depending on their type.

   Virus survival in soils: The literature contains conflicting information regarding the relationship between the attachment process and viral inactivation. A number of studies indicate that inactivation is inhibited by adsorption (Moore et al., 1975; Bitton et al., 1975; Babich and Stotzky, 1980; Liew and Gerba, 1980; Sobsey et al., 1980). However, other investigation indicate that inactivation is augmented by attachment (Bitton, 1974; Moore et al., 1982; Gerba, 1991). Clearly, a number of factors must influence virus survival in soils. As with groundwater, temperature is critical to inactivation rates on soils and sediments and shows a positive correlation (Duboise et al., 1976; Yeager and O'Brien, 1979; Hurst et al., 1980; Hurst, 1988). Typically the average inactivation rate in the 1 to 8° C range was four times longer than at 20 to 25° C.

   Soil moisture content is also a very important parameter of virus inactivation in soil zones above the saturated soil (i.e., in the vadose zone). Yeager and O'Brien (1979) found that the desiccation of eight various soils containing viruses accounted for an

average reduction in the titer of active viruses of 5 log units as compared to their saturated counterparts. Sagik et al. (1980) reported 99% inactivation of poliovirus in soil when the soil moisture dropped approximately 10% below saturating conditions. The same degree of inactivation took 8 to 10 weeks to occur under saturated conditions. Bitton and Gerba (1984) reported that virus survival in sludge-amended soils was greatly affected by desiccation (8 days in dried- vs. 35 days in wetted-soils).

Although temperature and moisture content have been identified as critical factors affecting inactivation in partially saturated soils, soil characteristics and biological factors also play a role in saturated media. Hurst et al. (1980) determined that pH extremes and exchangeable cations affected activity and survival. It is assumed that pH variability affects adsorption and therefore survival (Hurst et al. 1980; Gerba, 1984). The cation content of the soil may also affect adsorption, but more directly may increase the thermal stability of virions via electrostatic bridging interactions (Yeager and O'Brien, 1979b).

Soil type has also been linked to virus inactivation in a more indirect matter (Yeager and O'Brien 1979a; Moore et al. 1981; Hurst 1980; Sobsey et al. 1980). Because the specific characteristics of the soil that seem to have a positive effect on survival also correlate with increased adsorption, investigators speculate that adsorption plays a key role in diminishing inactivation rates. Murray and Laband (1979) reported that the presence of oxidized mineral species in certain soil types enhanced viral infectivity after desorption. Hurst et al. (1980) and Sobsey et al. (1980, 1986) reported that sorption variability of viruses led to differences in inactivation rates in similar soils and in sewage. This notion was later confirmed by Blanc and Nasser (1996), who demonstrated differences in inactivation between poliovirus, MS2 and PRD1 when incubated in soils

19

with similar saturating conditions and temperatures. Although these same experiments reported microbial activity as a possible explanation for virus inactivation, it was difficult to conclude this due to other confounding factors.

The mechanisms responsible for attachment and inactivation of viruses have yet to be fully characterized or identified. Nonetheless, a number of studies have at least recognized some of the factors that affect both activities.

The following portions of the dissertation examines transport processes in field settings with a suite of viruses that have been identified as having differing attachment and survival characteristics. By examining the transport processes and fate of this suite of viruses, I hope to provide valuable data that allows me to more fully understand how viral pathogens behave during subsurface transport in saturated porous media.

REFERENCES

Abbaszadegan, M., Stewart, P., and LeChevallier, M. 1999. A strategy for detection of viruses in groundwater by PCR. *Appl. Environ. Microbiol.* 65: 444-449.

Babich H. and Stotzky, G. 1980. Reductions in inactivation rates of bacteriophages by clay minerals in lake water. *Water Res.* 14: 185-187.

Bales, R. C., C. P. Gerba, G. H. Grondin and Jensen., S. L. 1989. Bacteriophage transport in sandy soil and fractured tuff. *Appl. Environ. Microbiol* 55: 2061-2067.

Bales, R. C., Li, S., Maguire, K.M., Yahya, M.T. and Gerba., C. P. 1993. MS-2 and poliovirus transport in porous media: hydrophobicity and chemical perturbations. *Water Resources Res.* 29: 957-963.

Bales, R.C., Li, S., Maguire, K.M., Yahya, M.T., Gerba, C.P., and Harvey, R.W. 1995. Virus and bacteria transport in a sandy aquifer, Cape Cod, MA. *Ground Water.* 33, No. 4: 653-661.

Ball, P. N., Woessner, W. W., DeBorde, D. C. and Troy, T. 1999. Viral attachment during groundwater transport under field pumping conditions: mass balance approach. Submitted to *Environmental Science and Technology*.

Bitton, G. and R. Mitchell. 1974. Effect of colloids on the survival of bacteriophages seawater. *Water Res.* 8: 227-229.

Bitton, G. 1975. Adsorption of viruses onto surfaces in soil and water. *Water Res.* 9: 473-484.

Bitton, G. and Gerba, C.P. 1984. Groundwater Pollution Microbiology. p.1-7; 65-88. In G. Bitton and C. P. Gerba, (ed.), *Groundwater Pollution Microbiology*, John Wiley and Sons, New York.

Burge, W.D. and Enkiri, N.K. 1978. Adsorption kinetics of bacteriophage ΦX-174 on soil. *Journ. Environ. Qual.* 7: Num. 4, 536-542.

DeBorde, D. C., Woessner, W. W., Kiley, Q.T. and Ball, P. 1999 Rapid transport of virus in a floodplain aquifer. *Water Research.* 33: 2229-2238.

Dowd, S.E., Pilla, S.D, Wang, S. and Corapcioglu, M.Y. 1998. Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. *Appl. Envrion. Microbiol.* 64: 405-410.

Duboise, S.M., Moore, B.E. and Sagik, B.P. 1976. Poliovirus survival and movement in a sandy forest soil. *Appl. Envrion. Microbiol.* 31: 536-543.

21

Enriquez, C.E., Hurst, C.J., and Gerba, C.P. 1995. Survival of the enteric Adenovirus 40 and 41 in tap, sea and waste water. *Water Research*. 29: 2548-2553.

Fetter, C.W., 1995. Applied Hydrogeology: Charles E. Merritt Publishing Company. Columbus, Ohio.

Gerba, C.P., Goyal, S. M., Cech, I. and Bogdan, G.G. 1980. Quantitative assessment of the adsorptive behavior of viruses to soils. *Environ. Sci. Technol*. 5: 940-944.

Gerba, C.P.; Wang, D.S.; Lance, J.C. 1981. Effect of soil permeability on virus removal through soil columns. *Appl. Environ. Microbiol*. 42: 83-88.

Gerba, C.P., 1984. Applied and theoretical aspects of virus adsorption to surfaces. *Advances in Applied Microbiology*. 30: 113-168.

Gerba, C.P., Powelson, D.K, Yahya, M.T., Wilson, L.G. and Amy G.L. 1991. Fate of viruses in treated sewage effluent during soil aquifer treatment designed for wastewater reclamation and reuse. *Water Sci. Tech*. 24: 95-102.

Goyal, S. M. and C. P. Gerba. 1979. Comparative adsorption of human enteroviruses, simian rotavirus, and selected bacteriophages to soils. *Appl. Environ. Microbiol*. 38: 241-247.

Grant, S.B., List, E.J. and Lidstrom, M.E. 1993. Kinetic analysis of virus adsorption and inactivation in batch experiments. *Water Resources Research*. 29: 2067-2085.

Harvey, R.W., Kinner, N.E., MacDonald, D., Metge, D.W. and Bunn, A. 1993. Role of physical heterogeneity in the interpretation of small-scale laboratory and field observations of bacteria, microbial-sized microspheres, and bromide transport through aquifer sediments. *Water Resources Research*. 29: 2713-2721.

Herbold-Paschke, K., Straub, U., Hahn, T., Teutsch, G. and Botzenhart K. 1991. Behavior of pathogenic bacteria, phages, and viruses in groundwater during transport and adsorption. *Water Sci. Tech*. 24: 301-304.

Hurst, C.J., Gerba, C.P. and Cech, I. 1980. Effects of environmental variables and soil characteristics on virus survival in soil. *Appl. Environ. Microbiol*. 40: 1067-1079.

Hurst, C.J. 1988. Influence of aerobic microorganisms upon virus survival in soil. *Can. Jour. Microbiol*. 34: 696-699.

Jansons, J., Edmonds, L.W., Speight, B. and Bucens, M.R. 1989a. Survival of viruses in groundwater. *Water Resources*. 23: No. 3. 301-306.

Jin, Y., Yates, M.V., Thompson, S.S. and Jury, W. 1997. Sorption of viruses during flow through saturated sand columns. *Environ. Sci. Technol.* 31: 548-555.

Keswick, B.H. and Gerba, C.P. 1980. Viruses in groundwater. *Environ. Sci. Technol.* 14: 1290-1297.

Lance, J. C. and Gerba C. P. 1984. Virus movement through soil during saturated and unsaturated flow. *Appl. Environ. Microbiol.* 47: 335-337.

LeBlanc, D.R., Garabedian, S.P., Hess, K.M., Gelhar, L.W., Quadri, R.D., Stollenwerk, K.G. and Wood, W.W. 1991. Large-scale natural-gradient tracer test in a sand and gravel aquifer, Cape Cod, Massachuestts: experiment design and tracer movement. *Water Res.Research*, 27: 895-910.

Loveland, J., Ryan, J., Amy, G. and Harvey, R. 1996. The reversibility of virus attachment to mineral surfaces. *Colliods Surf. A: Physiochem. Eng Asp.* 107: 205-221.

Liew, P. and Gerba, C.P. 1980. Thermostabilization of enteroviruses by estuarine sediment. *Appl. Environ. Microbiol.* 40: 305-308.

Macler, B.A., 1995. Developing a national drinking water regulation for disinfection of groundwater. *Ground Water Monitoring and Remediation.* Autumn: 77-84.

Meschke, J.S. and Sobsey, M.D. 1998. Comparative adsorption of Norwalk virus, poliovirus 1 and F+ RNA coliphage MS2 to soil suspended in treated wastewater. *Water Sci Tech.* 38: 187-189.

Moore, B.E., Sagik, B.P and Malina, J.F. 1975. Viral association with suspended solids. *Water Res.* 9: 197-203.

Moore, M. 1982. Enteroviral disease in the United States, 1970-1979. *Journ. Infect. Dis.* 146: 103-108.

Moore, R.S., Taylor, D.H., Struman, L.S., Reddy, M.M. and Fuhs, G.W. 1981. Poliovirus adsorption by 34 minerals and soils. *Appl. Environ. Microbiol.* 42: 963-975.

Moore, R.S., Taylor, D.H., Reddy, M.M., Struman, L.S. 1982. Adsorption of reovirus by minerals and soils. *Appl. Environ. Microbiol.* 44: 852-859.

Murray, J.P. and Laband, S.J. 1979. Degradation of poliovirus by adsorption on inorganic surfaces. *Appl. Environ. Microbiol.* 37: 480-486.

Penrod, S.P., T. M. Olson and S.B. Grant (1996) The deposition kinetics of two viruses in packed beds of quartz granular media. *Langmuir*, 12 (23): 5576-5587.

23

Pieper, A.P., Ryan, J.N., Harvey, R. W., Amy, G.L., Illangasekare T.H. and Metge, D.W. 1999. Tranport and recovery of bacteriophage PRD1 in a sand and gravel aquifer: effect of sewage-derived organic matter. *Environ. Sci. Technol.* 31: 1163-1170.

Powelson, D.K., Simpson, J.R., and Gerba, C.P. 1990. Virus transport and survival in saturated and unsaturated flow through soil columns. *Journ. Environ. Qual.* 19: 396-401.

Powelson, D.K., Simpson, J.R. and Gerba, C.P. 1991. Effects of organic matter on virus transport in unsaturated flow. *Appl. Environ. Microbiol.* 57: 2192-2196.

Powelson, D.K., Gerba, C.P., and Yahya, M.T. 1993. Virus transport and removal in wastewater during aquifer recharge. *Water Res.* 27: 583-590.

Powelson, D.K. and Gerba, C.P. 1994. Virus removal from sewage effluent during saturated and unsaturated flow through soil columns. *Water Res.* 28: 2175 - 2181.

Rao, C.V. and Melnick, J.L. 1987. Human Viruses in Sediments, Sledges, and Soils. CRC Press Inc., Boca Raton, Fl.

Rose, J.B.; Altas, R.M.; Gerba, C.p,; Gilchrist, M. J.; LeChevallier, M. W.; Sobsey, M. D.; Yates, M.V. In Microbial Pollutants in our Nation's Water; Environmental and Public Health Issues. American Society of Microbiology. Washington, D.C.

Regli, S., Rose, J.B., Hass, C.N. and Gerba, C.P. 1991. Modeling the risk of Giardia and viruses in drinking water. *Journ. Amer. Water. Works. Assoc.* 83: 76-84.

Ryan, J.N., Elimelech, M., Ard, R.A., Harvey, R.W. and Johnson, P.R. 1999. Bacteriophage PRD1 and silica colloid transport and recovery in an iron oxide-coated sand aquifer. *Environ. Sci. Technol.* 33: 63-73.

Sagik, B.P., Moore, B.E. and Sorber, C.A. 1980. Human Enteric Virus Survival in Soil Following Irrigation with Sewage Plant Effluents. Environment Protection Agency EPA-600/1-80-004. Cincinnati, OH.

Schijven, J.F., Hoogenboezem, W., Hassanizadeh, S.M. and Peters, J.H. 1999. Modeling removal of bacteriophages MS2 and PRD1 by dune recharge at Castricum, Netherlands. *Water Resources Res.* 35: 1101-1111.

Snowdon, J.A., Cliver, D.O., and Converse, J.C. 1989. Inactivation of poliovirus 1, as a function of temperature in mixed human and dairy animal wastes. *Water Manage. & Res.* 7: 135-142.

Sobsey, M.D., Dean, C.H., Knuckles, M.E. and Wagner, R.A. 1980. Interactions and survival of enteric viruses in soil materials. *Appl. Environ. Microbiol.* 40: 92-101.

24

Sobsey, M.D., Shields, P.A., Hauchman, F.H., Hazard, R.L. and Caton, L.W. 1986. Survival and transport of hepatitis a virus in soils, groundwater and wastewater. *Water Sci. Tech.* 18: 97-106.

Sobsey, M.D., Hall, M.H. and Hazard, R.L. 1995. Comparative reductions of hepatitis a virus, enteroviruses and coliphage ms2 in miniature soil columns. *Water Sci. Tech* 31: 203-209.

Woessner, W. W., Troy, T., Ball P.N. and DeBorde D. C. 1998. Virus transport in the capture zone of a well penetrating a high hydraulic conductivity aquifer containing a preferential flow zone: challenges to natural disinfection. *Proceeding of Source Water Protection, International 98.*

Yahya, M.T., Galsiomes, L., Gerba, C. P. and Bales, R. C. 1993. Survival of bacteriophages MS2 and PRD1 in ground water. *Water Sci. Tech.* 27: 409-412.

Yates, M.V., Gerba, C.P., and Kelley, L.M., 1985. Virus persistence in groundwater. *Appl. Environ. Microbiol.* 49: 778-781.

Yates, M.V. and Jury, W.A. 1995. On the use of virus transport modeling for determining regulatory compliance. *Journ. Eviron. Qual.* 24: 1051-1055.

Yates, M.V. and Yates, S.R. 1987. A comparison of geostatistical methods for estimating virus inactivation rates in ground water. *Water Res.* 21: No. 9. 1119-1125.

Yeager, J.G. and O'Brien, R.T. 1979a. Enterovirus inactivation in soil. *Appl. Environ. Microbiol.* 38: 694-701.

Yeager, J.G. and O'Brien, R.T. 1979b. Structural changes associated with poliovirus inactivation in soil. *Appl. Environ. Microbiol.* 38: 702-709.

Chapter 3

Supporting Research and Background

Natural gradient seeding experiment at the Erskine Fishing Access site:

Literature observations:

The effects of differing hydrogeologic settings on viral transport are poorly documented. Difficulties characterizing the physical properties governing transport in sand, gravel and cobble dominated aquifers have also confounded our ability to understand and predict virus transport processes in these types of high velocity groundwater systems. Virus transport studies in coarse-grained aquifers have documented viruses traveling over 900 m (Noonan and McNabb, 1979). Rates of virus transport in sand and gravel aquifers, measured by monitoring peak concentrations from viral injections have been reported at 0.2 to 1 m/d in the glacial outwash of Cape Cod (Bales et al., 1995; Pieper et al., 1997), 1 to 2.9 m/d in fluvial sand and gravel near Frenchtown, MT. (DeBorde et al., 1998a,b), 11 to 132 m/d in a floodplain aquifer of the Emme Valley, Switzerland (Rossi et al., 1994) and over 300 m/d in highly permeable alluvial aquifers of the Canterbury Plains of New Zealand (Noonan and McNabb, 1979). However, few field experiments have been described in aquifers dominated by gravel and cobbles, such as the Erskine site. Thus, predicting virus fate and transport under such conditions is uncertain.

26

Objectives:

To address the uncertainties associated with predications in these types of hydrogeologic settings, a natural gradient experiment was conducted in an attempt to characterize the movement and fate of four viruses (the bacteriophages MS2, PRD1, and $\varnothing$X174, and attenuated polio virus type-1 (CHAT strain)) during transport through 40.5 m of the gravel-dominated floodplain aquifer at Erskine. The behavior of viruses during transport was compared and the study results in the context of proposed natural disinfection criteria were also examined.

To achieve these goals, the data obtained were analyzed by a number of different methods. Breakthrough curves (time vs. concentrations) at sampling wells in the center of the viral plumes were constructed to examine and compare arrival times, concentrations and compute velocities for each virus.

Relative virus breakthrough (RB) at monitoring wells, and the degree of virus attenuation by attachment to the aquifer material were calculated using the procedure described by Harvey and Garabedian (1991). RB is calculated using concentration versus time data from a sampling point centered in the tracer plume. It is a comparison between the ratio of the measured and source virus concentration and the same ratio of the conservative tracer (bromide). RB is calculated by the following expression:

$$RB = \int_{t_0}^{t_f} \frac{C_t}{C_0} \, dt \Big/ \int_{t_0}^{t_f} \frac{Br_t}{Br_0} \, dt$$

Where C_0 and Br_0 are the initial virus and bromide concentrations at the injection well (PFU/ml (Plaque Forming Units) and mg/L), and C_t and Br_t are the concentrations at a monitoring well at some time t after the tracer injection. The terms t_0 and t_f are the times representing the beginning and end of the breakthrough curve. The percent of

27

relative attenuation (RA) is derived by converting RB to a percent and subtracting the result from one hundred (RA = 100-RB).

In addition, the collision efficiency factor α (a parameter in filtration theory representing the collision between particles (virus) and collector grains) was determined. α represents the ratio of the rate of collisions resulting in attachment to the total rate of collisions (Harvey and Garabedian, 1991) and is defined as follows:

$$\alpha = d\{[1-2(\alpha_L/x)\ln(RB)]^2 -1\}/ 6(1-\theta)\eta\alpha_L$$

where d is the average grain diameter (L), α_L is the longitudinal dispersivity (L) , x is the transport distance (L), θ is the porosity and η is the single collector efficiency caused by Brownian motion (dimensionless). In the work described here, the value η was determined as presented by Harvey and Garabedian (1991) and defined by Pieper et al. (1997):

$$\eta = 0.9 \ A_s^{1/3} \ [(k_B T/\mu d_p dv)]^{2/3}$$

where A_s is the Happel sphere-in-cell model correction factor, k_B is the Boltzmann constant $(1.38 \ X \ 10^{-23} \ J \ mol^{-1}K^{-1})$, T is absolute temperature (K), μ is the dynamic viscosity (mass/(Lt)), d_p is the virus diameter (L), d is the average grain diameter (L) and v is the fluid velocity (L/t). A_s is calculated where $\varepsilon = (1-\theta)^{1/3}$:

$$A_s = 1 - \varepsilon^5 /(1-1.5\varepsilon + 1.5\varepsilon^5 - \varepsilon^6).$$

Finally, a mass balance of the viruses and bromide was estimated from the 8 h data. These values were used to approximate the mass of each virus present in the aquifer.

28

Experiment preparation and characterization:

We completed a rigorous site characterization that included monitoring of rhodamine dye (conservative tracer), bromide (conservative tracer), and the bacteriophage MS2 (reactive tracer), throughout the instrumented field site at Erskine fishing access (Fig. 1). Although the conservative chemical tracers were useful for determining many aquifer parameters, their limits of detection were not sufficient to monitor and characterize long flow paths within the instrumented site. For increased sensitivity (lower detection limits), the bacteriophage MS2 was injected into the aquifer at concentrations exceeding 10^{10} PFU/ml. Despite the adsorptive nature of virus particles, their use as tracers for long distances exceeded the capabilities of either bromide or rhodamine. These experiments were necessary to fully characterize the hydrogeologic properties and stratigraphy of the flow field, as well as to test the validity of preliminary data gathered at the Erskine field site. Due to the dynamic and complex nature of groundwater flow, it was essential to establish as completely as possible a model of the physical system so that data collected would represent the true fate and transport parameters of viruses in these settings.

The shallow unconfined aquifer that underlies the Erskine field site is composed of Quaternary fluvially derived fine-to coarse-grained sands, gravels and cobbles. This complex stratigraphy forms an anisotropic and heterogeneous aquifer that is extremely prolific. Parameters and water chemistry data for Erskine site are represented in Table 1.
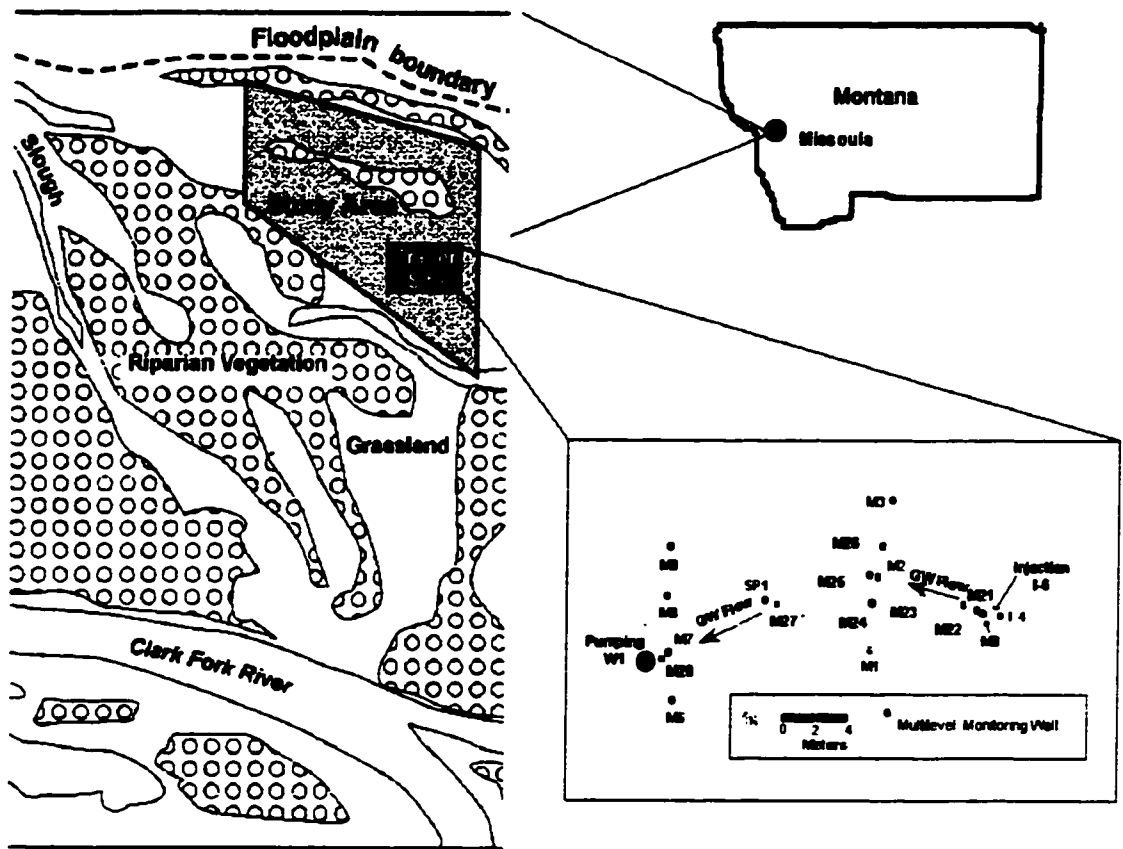
Figure 1. Erskine experimental site map. W1 – withdrawal well; I4 – injection well; numbered wells designated M are multi-level sampling wells

Table 1– Aquifer properties and water data from Erskine Fishing Access

AVERAGE K (HYD COND)	900-1.38x10⁴ m/d
AVERAGE VELOCITY (BR)	22-29.3 m/d
VIRUS VELOCITY	33.8 m/d
HYDRAULIC GRADIENT	5.0x10⁻⁴
AVERAGE TEMPERATURE	10.3° C
pH	7.4
TOTAL DISSOLVED SOLIDS	3.5 mg/L
WATER TYPE	Ca, HCO₃
POROSITY	0.15-0.2
SPC. CONDUCTANCE.	288 mS/cm²

30

Figure 1. Erskine experimental site map. W1 – withdrawal well; I4 – injection well; numbered wells designated M are multi-level sampling wells

Table 1– Aquifer properties and water data from Erskine Fishing Access

AVERAGE K (HYD COND)	$900\text{-}1.38 \times 10^4$ m/d
AVERAGE VELOCITY (BR)	22-29.3 m/d
VIRUS VELOCITY	33.8 m/d
HYDRAULIC GRADIENT	5.0×10^{-4}
AVERAGE TEMPERATURE	$10.3°$ C
pH	7.4
TOTAL DISSOLVED SOLIDS	3.5 mg/L
WATER TYPE	Ca, HCO_3
POROSITY	0.15-0.2
SPC. CONDUCTANCE.	288 mS/cm^2

30

Instrumentation of the Erskine site resulted in the establishment of 89 monitoring wells and 20 four-port multi-level samplers. Each multi-level sampling well was constructed with three 0.5 cm diameter high-density polyethylene (HDPE) tubes affixed to a 1.3 cm diameter PVC pipe and extending to different depths. The HDPE sampling ports extended to 0.8, 2.7, 3.6 m below ground surface and the PVC pipe extended to 4.5 m below the ground surface. The ends of the tubing and PVC were perforated over 5 cm and covered with nylon mesh. A dedicated 0.3 m long flexible tube was attached to the above ground end of each HDPE tube port, and a separate dedicated HDPE tube placed in the PVC pipe. This arrangement allowed sampling with portable peristaltic pumps equipped with MASTERFLEX quick release heads (Cole-Parmer, Vernon Hills, IL). This well construction and network arrangement insured that virus seeded at well I4 (screened from 1.8 to 2.3 m below land surface) would pass through the arcs of multi-level monitoring wells located 7.5, 19.4, 30, and 40.5 m downfield from that point (Fig. 2).

Following site characterization and instrumentation, bromide, the bacteriophages MS2, ΦX174 and PRD1, and attenuated poliovirus type I (CHAT strain) were injected as a slug source under natural gradient conditions into the injection well I4, in the autumn of 1996. Table 2 shows the concentrations of tracer and diameter of each virus used in this multiple virus seeding experiment. A 72 h sampling regime was implemented in which approximately 245 samples were collected and analyzed to determine bromide, bacteriophage and poliovirus concentrations. Only those samples perceived as being directly in the flowpath were analyzed for poliovirus. Based on the results of the prior bacteriophage tracer study, we were able to limit the number of samples analyzed to those deemed most relevant for the purposes of this multi-virus study.

31
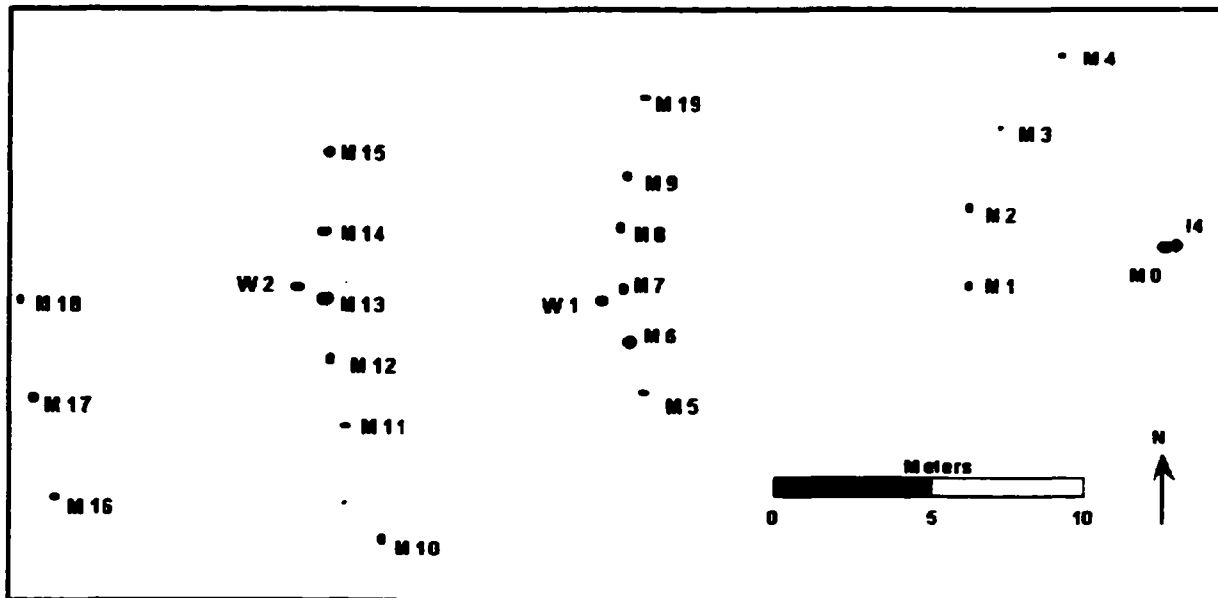
Figure 2- Well Network - I4-Injection well; M0-M19 Multilevel wells; W1 &W2 Pumping wells

Table 2 – Virus injection concentrations and diameter.

VIRUS TYPE	PFU/ML	DIAMETER
MS2	5.60×10^{10}	24 nm
φX174	2.90×10^7	32 nm
PRD1	5.40×10^9	65 nm
Polio Clon 1	3.40×10^6	28 nm
Ionic Tracer	mg/L	
Bromide	1143	

Summary of results:

Breakthrough curves were constructed for wells along the line of preferential flow within the site. Breakthrough curves for monitoring wells M2, M7 and M14 are presented in Fig 3. Analyses of breakthrough curves revealed that, while a portion of the seeded viruses

32

traveled at rates similar to that of bromide, the majority attached to the aquifer material in the vicinity of the injection well and along the flow path. Thus, virus attachment to the aquifer material represents an important process affecting virus transport. These data also indicate a heterogeneous nature in the virus population with regards to transport behavior.

Examination of relative breakthrough (RB), relative attenuation (RA) and C/C_o curves indicated that a significant portion of the virus mass remained attached to the aquifer material, and that the attenuated poliovirus attached at proportionally higher rates than the other injected viruses. Poliovirus also had a significantly higher collision efficiency (α) then the other viruses (Table 3), which is consistent with the higher attachment rates observed in the field.

33
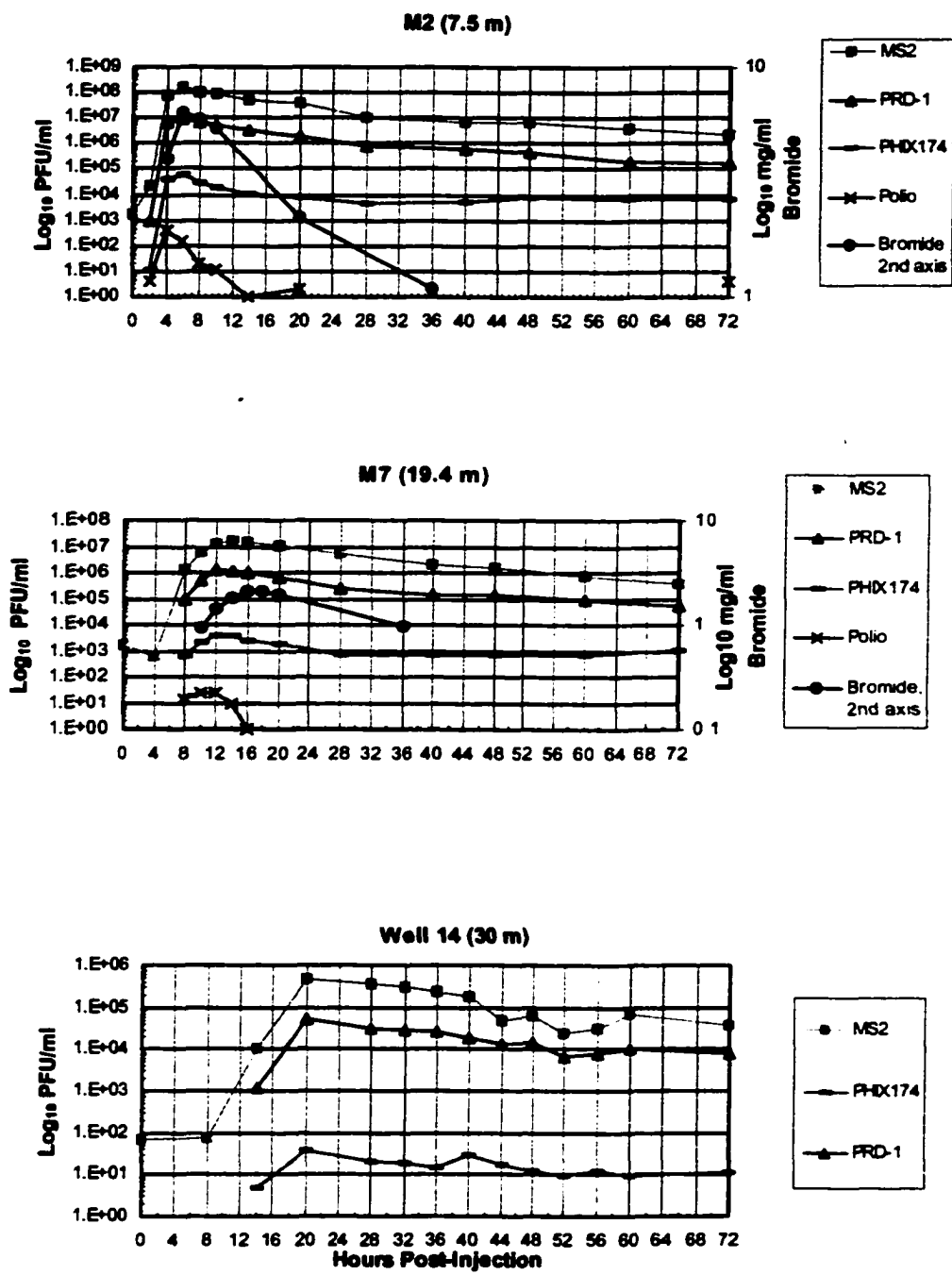
Figure 3 – Breakthrough curves of wells M2, M9 and M14

34

Table 3. Relative Breakthrough (RB), Relative Attenuation (RA) and Collision Efficiency (α) of Viruses After 7.5 and 19.4 m of Transport.

Virus	Well M2-9 (7.5 m from I4)			Well M7-9 (19.4 m from I4)		
	RB%	RA%	α	RB%	RA%	α
MS2	51 (29-92)*	49 (8-71)	0.004 – 0.182	15 (8-26)	85 (74-92)	0.004 – 0.202
PRD1	29 (16-51)	71 (49-84)	0.014 - 0.632	12 (7-21)	88 (79-93)	0.005 – 0.385
ØX174	35 (19-62)	65 (38-81)	0.006 – 0.311	6 (4-12)	94 (88-96)	0.007 – 0.317
Polio Type 1 (Chat)	1 (0.6-1.9)*	99 (98.1-99.4)	0.047 – 2.108	0.2 (0.1-0.5)	99.8 (99.5-99.9)	0.019 – 0.866

*Range values (given in parentheses) were calculated using 95% confidence limits of ±14% for bromide, ±15% for the bacteriophage, and ±15% for virus measurements. Collision efficiency range calculated using a mean grain size of 0.00125 m and 0.012m.

From these data, a range of average velocities was calculated using peak arrival

time and distance from the injection point, (Table 4).

Table 4 - Apparent transport velocities (m/d) calculated from breakthrough curve peaks.

Well Distance	M2 7.5m	M7 19.4m	M14 30m
Bromide	22.5-30	26-29.25	NC*
MS2	30	23.4-39	25.7-36
PRD1	30	26-39	36
ØX174	30	33.4-39	18-36
Attenuated Polio	45	33.4-58.5	NC

*NC – Not completed

The viruses and bromide appear to have at least overlapping (within a range) transport rates, with one significant exception. At M2 (7.5 m) poliovirus arrived before bromide and the other viruses. Similar particulate reactive tracer behavior has been reported in the literature (Rossi et al., 1994) and was observed in the field in prior

experiments completed at this site (data not shown). This phenomenon has also been reported for laboratory experiments using coliphage MS2 and f2, in which transport rates in columns were interpreted to be 1.6 to 1.9 times that of bromide (Bales et al., 1989). To reconcile this observation, the following hypothesis was proposed by DeBorde et al. (1999). "Although poliovirus is being transported at similar rates as the bacteriophage and bromide, the high attachment rate during the early stage of transport results in a truncation of the virus breakthrough curve. This causes an apparent peak to arrive before the bromide or bacteriophage peak". The truncation process can be defined by applying a simple algorithm to the conservative Br (C/C_0) data:

$$Br_tADJ = [Br_t - (K^* t^* Br_t)]$$ $\boxed{{}^* t \text{ is time in (h) hours}}$

where, Br_t = bromide concentration (C/C_0) at time t (h); t = time of arrival at well M2 (h); K = net virus attachment value/h; and Br_tADJ = the attachment transformed Br data. Calculation of K from a set of conservative tracer and virus tracer field data is accomplished by substituting virus concentrations for BrADJ. The application of this equation results in a peak shift to the left, as was observed for the poliovirus in the field experiment.

Viral transport plumes constructed from "snapshot" data analysis for all the wells at given depth intervals allowed for the examination of virus distribution throughout the flowfield (data not shown). From this it was determined that the highest observed concentrations of tracer remained in the injection well throughout the experiment,

36

suggesting that the viral tracer is being held up at the injection site either by adsorption and attachment or hydrogeological conditions.

Data also suggested that using bromide as a tracer in these types of hydrolgeological settings is limited because the high dispersal rates limit its detection, compared to the high titer capability virus. Attempts at calculating mass balances based on the 8 h plume viral distribution were unsuccessful due to the lack of detailed viral concentration data at critical locations.

.

Conclusions from the natural gradient multi-virus natural gradient study:
Observations from this experiment suggest the following: 1) average transport rates for a portion of the virus are similar to the average groundwater flow velocity as defined by the conservative tracer bromide (i.e. advective transport); 2) aqueous virus concentrations were significantly reduced during transport by apparent attachment to aquifer material; 3) attenuated polio virus concentrations versus time and distance declined at a faster rate than did the three bacteriophages (i.e. poliovirus may have a higher collision efficiency); 4) long tails on virus breakthrough curves imply that some of the attached viruses are released back into the groundwater over time (i.e., adsorption/desorption results in retarded transport); 5) poliovirus breakthrough may be truncated by high rates of attachment with negligible desorption while, unattached poliovirus travels at rates similar to bromide.

These findings imply that virus-impacted water in a highly conductive aquifer creates two problems with regard to water quality. The first is a high concentration of virus moving with the average velocity of the groundwater. This work showed that high

37

concentrations of virus reach and pass the commonly used 30.5 m setback distances between wells and septic systems that is implemented in most states. The second is that attached viruses appear to be released back into the aqueous phase creating a long-term low-level viral source, and are subsequently transported downgradient to wells.

References Cited

Bales, R.C., Gerba C.P., Grondi G.H., and Jensen S.L 1989. bacteriophage transport in sandy soil and fractured tuff. *Appl. Environ. Microbiol.* 55: 2061-2067.

Bales, R.C., Li S., Maguire K.M., Yahya M.T, Gerba C.P., and Harvey R.W. 1995. Virus and bacteria transport in a sandy aquifer, Cape Cod, MA. *Ground Water.* 33: 653-661.

DeBorde, D.C., Woessner, W.W., Lauerman, B., and Ball, P. 1998a. Virus occurrence and transport in a school septic system and unconfined aquifer. *Ground Water.* 36: 825-834.

DeBorde, D.C., Woessner, W.W., Lauerman, B., and Ball, P. 1998b. Coliphage prevalence in high school septic effluent and associated groundwater. *Water Research.* 32: 3781-3785.

Harvey, R. W., and Garabedian S. P. 1991. Use of colloid filtration theory in modeling movement of bacteria through a contaminated sandy aquifer. *Environ. Sci. Technol.* 25: 178-185.

Noonan, M.J. and McNabb, J.F. 1979. Movement of bacteriophages in ground water in New Zealand, Ann Mtg. Amer. Soc. Microbiol, 221.

Pieper, A.P., Ryan J.N., Harvey R. W., Amy G. L, Illangasekare T.H. and Metge D.W. 1997. Tranport and recovery of bacteriophage PRD1 in a sand and gravel aquifer: effect of sewage-derived organic matter. *Environ. Sci. Technol.* 31: 1163-1170.

Rossi, P., Carvalho-Dill A. De, Müller I., and Aragno M. 1994. Comparative tracing experiments in a porous aquifer using bacteriophages and fluorescent dye on a test field located at Wilerwald (Switzerland) and simultaneously surveyed in detail on a local scale by radio-magneto-tellury (12-240 kHz). *Environmental Geology.* 23: 192-200.

Chapter 4

NOTE: This work was submitted for publication in *Environmental Science and Technology*.

Viral Attachment During Groundwater Transport Under Field Pumping Conditions: Mass Balance Approach

Patrick N. Ball, William W. Woessner, Dan C. DeBorde and Thomas Troy

ABSTRACT

Groundwater supplies contaminated with microbes cause over half of the water-borne disease outbreaks in the United States. However, models necessary to predict pathogen transport require additional field scale calibration data. The bacteriophages MS2, ΦX174 and PRD1, attenuated poliovirus type-1 (CHAT strain), and bromide were seeded into an instrumented flow field as a slug 21.5 m from a well pumping at a steady rate of 408 L/min to produce a forced gradient. The well is located in sand and gravel with cobbles, and is within a rapid flowing (27 m/d), cold (10° C), neutral (pH 7.2), unconfined groundwater system. Over the 47 h duration of the test, 77% of the bromide, 55 % of the PRD1, 17% of the MS2, 7% of the ΦX174 and 0.12% of the poliovirus injected masses were recovered. In general, the percentage of virus recovery appears correlated with reported viral isoelectric point (pI) values, with the possible exception of PRD1. Relative breakthrough (RB) analyses at the pumping well overestimated the mass of PRD1 and MS2 collected, and more closely represented the captured masses of ΦX174 and poliovirus. Analyses of MS2 and bromide data show that the majority of attachment occurred within the first few meters and hours of transport.

INTRODUCTION

Approximately 50% of the potable water supplying over 100 million people throughout the United States originates as groundwater (1). During the past few decades, the occurrence of disease attributed to consumption of contaminated groundwater has steadily risen (2, 3). Over 900,000 groundwater-related disease episodes are reported annually in the United States (1) and it is estimated that, worldwide, approximately 7 million disease episodes are associated with use or consumption of contaminated groundwater (2-4).

The contamination of groundwater may occur from a variety of sources including broken sewer lines, land application of sewage sludge or septic systems prevalent in many suburban and rural areas (5-14). These effluents may contain pathogenic human viruses. Of particular interest are enteric viruses that are shed in fecal matter during bouts of infection and may be present at 10^8 to 10^{10} virions per gram of feces (15, 16). These pathogens are of particular concern because their infectious dose can be exceedingly low (16), and they are generally quite robust, being able to withstand low pH and organic solvents. These characteristics allow virus survival in groundwater for up to 9 months (10, 13 and data unpublished), and depending on groundwater flow rates and aquifer sediment characteristics, transported tens to hundreds of meters (17-20).

In an effort to protect the public from water-borne diseases, Congress passed the Safe Drinking Water Act in 1986. Current proposed revisions to that act include the Ground Water Disinfection Rule (21), where, under one provision, public water supplies would be exempt from treatment for pathogens if it could be demonstrated that acceptable levels of pathogens could be achieved by "natural disinfection" (21). Natural disinfection is the reduction of contaminant levels by physical, mechanical, chemical and

41

biological processes occurring along the groundwater flow path, and represents an attractive alternative to conventional water treatment.

The main processes controlling viral attenuation in groundwater systems include mechanical dispersion, inactivation, and irreversible sorption (23-26). Additionally, survival can be dependent on both viral and site characteristics (10, 27, 28). Many of these characteristics can be quantified in the laboratory. However, only a limited number of controlled field experiments have been conducted that allow transfer of laboratory results to field scales and conditions. Also, most field efforts have focused on observing the transport of a single virus type, usually a surrogate for a disease-causing virus, and usually over only a few meters under natural gradient conditions (18, 29, 30, 31). Natural-gradient, multi-virus transport studies are rare (19, 22) and single- or multi-virus transport studies involving forced gradient flow to a pumping well are even more uncommon (32).

A major limitation of field-scale virus tracer tests found in the literature is the general absence of virus mass balance calculations. This occurs because lack of solid phase data, limited sampling frequency, detection limits of analytical techniques, and analytical error introduce unacceptable uncertainty in mass balance calculations (19).

A proposed method for granting disinfection variances for viruses is based on the development of one or more models that would predict virus concentrations at a supply well (23, 33 -35). However, models employed to date generally under-predict concentrations and over-predict travel times of viruses, making them inadequate predictors of virus fate and transport in the field (12, 22).

42

Our research was designed to quantify, under field conditions, attachment and transport characteristics of four viruses using mass balance and breakthrough analysis techniques. Four seeded viruses rapidly moved through over 20 m of aquifer within the capture zone of a pumping well. The results obtained allowed quantification of virus attachment, evaluation of relative breakthrough (RB) calculations (as suggested by Harvey and Garabedian (36)), and an examination of the possible factors controlling attachment.

Field Methods

The multi-virus injection experiment was conducted within a remote grassland portion of the active floodplain of the Clark Fork River at the Erskine Fishing Access Study Site located approximately 24 km west of Missoula, Montana (Fig. 1). Prior to the experiment, the viruses chosen for use were approved by the University of Montana Biohazards Committee, Missoula City-County Health Department, Montana Department of Environmental Quality, and Region 8 EPA. Also, an Environmental Assessment (Montana Environmental Policy Act) was submitted at the request of the Montana Department of Fish, Wildlife, and Parks.

The aquifer underlying this area is shallow and unconfined, dominated by cobbles and gravel, with lenses of medium- to coarse-grained sands extending to a depth of 6 m. Below this depth the aquifer is predominately finer sands. The sediments are essentially free of iron coatings and are composed of regional lithology of argillaceous meta-sedimentary and granitic source rocks. The high-energy fluvial depositional environment at this site has resulted in a highly heterogeneous sediment package. Hydraulic conductivities derived from standard aquifer testing using four pumping wells and tracer

43
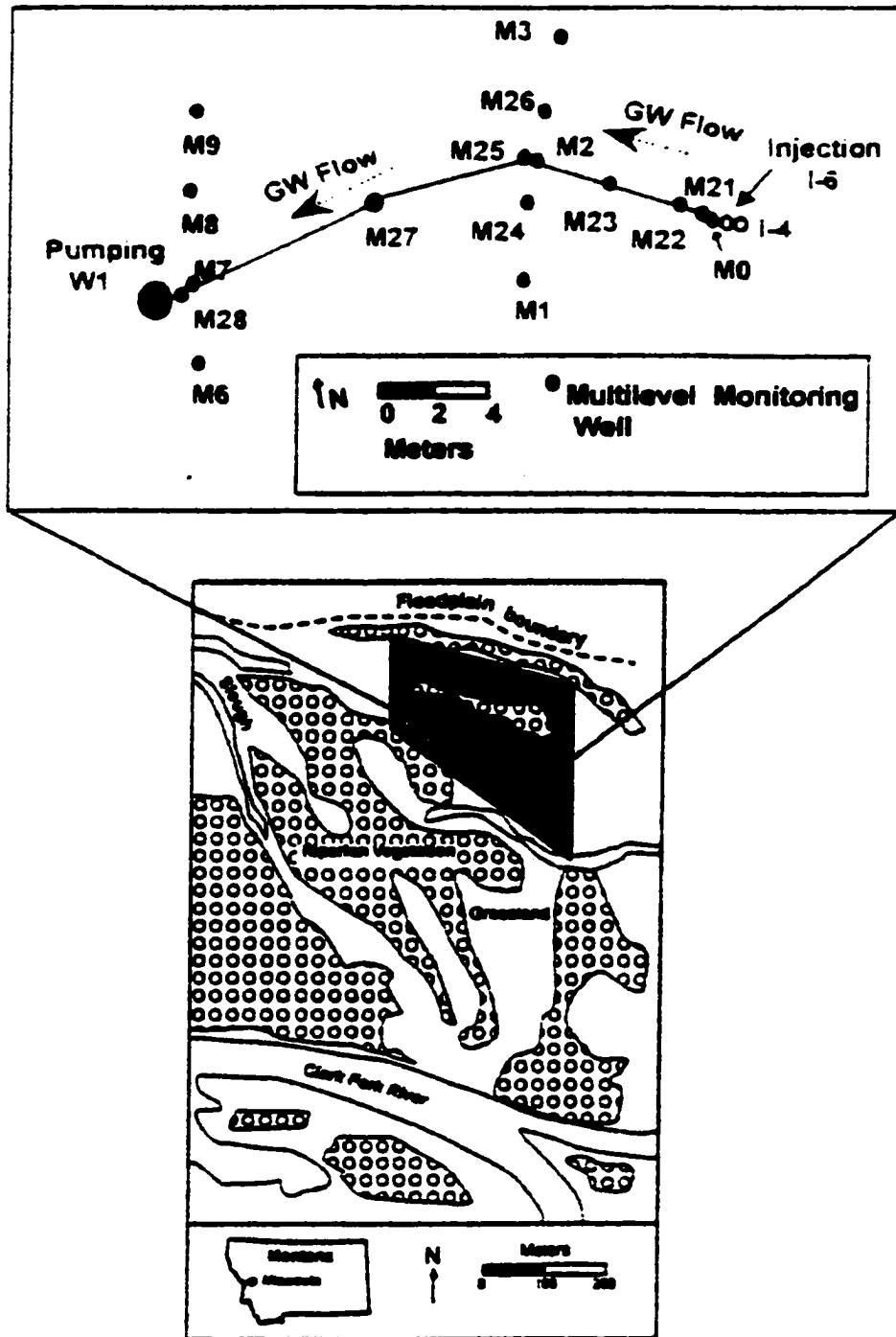
Figure 1. Location of Erskine tracer site. The insert map shows location of the injection well (I6), multilevel monitoring wells (M) and pumping well (W1). The arrows indicate groundwater flow direction. Black line shows the location of a verticle profile longitudinal to flow.

44

breakthrough curve analysis range from 800 to 36,000 m/d. The water table is typically

2.1 to 2.5 m below ground surface (19). A portion of the aquifer contains a zone of

preferential flow (K=13,000 m/d) extending from the water table to approximately 2.7 m;

it is within this zone that the majority of the tracer network is located (22). Additional

hydrologic properties and water chemistry data is contained in Table 1.

Table 1. Aquifer characteristics and water chemistry

Hydrologic Properties	
Porosity	0.15
Gradient	0.00043
K (m/d)	800-36,000
GW Velocity (m/d)	27
Water Chemistry	
Water Type	Calcium, Bicarbonate
Conductivity	288 μS/cm
DO	3.5 mg/l
pH	7.2
Temp. (C)	10.3

*DeBorde, et al. (25)

A tracer well network was developed previously for natural gradient experiments

(19). The well network contains injection and multi-level observation wells containing

three to four 0.5 cm diameter polyethylene ports bundled to a 1.3 cm diameter PVC tube

perforated over 5 cm at its bottom. For this work a new tracer injection well, I6 and

additional multi-level samplers were added resulting in the network shown in Fig 1. The

site was instrumented with over 100 small diameter and multi-level wells that allowed

water level monitoring and sampling at three to four depths within 2.5 m of the water

table. The injection well (I6) located 21.5 m from the pumping well (W1) was completed

45

as a 2.54 cm diameter PVC tube perforated to allow release of the tracer over an interval

extending from the water table to a depth of 0.45 m. Well W1 was constructed of 10.2 cm

diameter steel casing with 3 m of wire wrapped screen (0.12 cm wide slots) extending

from 1.6 to 4.6 m below land surface (22). An identical second well (W4) located 180 m

down groundwater-gradient (west) from W1, was used for re-injection of the pumped

water. Spacing of pumping and injection wells was designed and tested to assure minimal

pumping flow-field disruption and to prevent re-circulation of re-injected water. Well W1

was pumped at a constant rate of 408 L/min for 1.3 h to establish a steady state flow field

and then continued at this constant rate for an additional 47 h. Once the steady state was

established, 37.8 L of site groundwater containing bromide and the four viruses was

injected by gravity in I6 over a 15 min period (Table 2).

Samples were collected from W1 over the next 47 h at half-hour intervals for the

first 7 h, at 1 h intervals from 8 to 32 h, 2 h intervals to 40 h, and then at 43 and 47 h.

Sample volumes and frequencies were based on a prior pilot test using bromide and the

coliphage MS2 (22).

Viral and bromide samples were collected in both 50 and 250-ml sterile

containers from a valve located at the well head and immediately placed on ice. Samples

were transported to the University of Montana, Division of Biological Sciences

laboratory and held at 4oC until analyzed, generally within 24 - 48 h. Controls held at

similar temperatures had no detectable loss in infectivity over a month. Bromide samples

were collected at the well head, placed on ice and transported to the University of

Montana, Murdock Environmental Geochemistry Laboratory. Virus and bromide samples

46

Table 2: Virus and Bromide Concentrations values at W1.

Tracers					
Time	MS2**	PHIX174**	PRD1**	Polio***	Br****
(hours)	(PFU/mL)	(PFU/mL)	(PFU/mL)	(PFU/mL)	mg/L
0	ND*	ND	ND	ND	ND
0.5	ND	ND	ND	ND	ND
1	ND	ND	ND	ND	ND
1.5	ND	ND	ND	ND	ND
2	1.67E+04	1.82E+01	1.55E+03	1.04E-01	7.50E-04
2.5	4.08E+05	3.85E+02	2.69E+04	8.35E-01	5.58E-02
3	9.65E+05	5.50E+02	6.84E+04	1.25E+00	1.85E-01
3.5	1.33E+06	8.47E+02	7.48E+04	6.30E-01	2.67E-01
4	7.70E+05	5.06E+02	8.03E+04	1.04E-01	2.89E-01
4.5	8.91E+05	4.73E+02	9.90E+04	1.04E-01	2.73E-01
5	7.28E+05	3.30E+02	4.93E+04	1.04E-01	2.45E-01
5.5	5.94E+05	2.25E+02	6.31E+04	ND	2.12E-01
6	4.20E+05	1.65E+02	3.04E+04	ND	1.83E-01
6.5	4.18E+05	1.26E+02	4.54E+04	ND	1.56E-01
7	2.44E+05	1.14E+02	3.24E+04	ND	1.36E-01
8	1.77E+05	7.81E+01	2.60E+04	ND	1.04E-01
9	1.00E+05	6.16E+01	1.95E+04	ND	7.88E-02
10	1.08E+05	5.17E+01	1.56E+04	ND	6.78E-02
11	5.72E+04	2.64E+01	1.23E+04	ND	5.78E-02
12	4.29E+04	3.41E+01	1.11E+04	ND	4.88E-02
13	4.73E+04	2.55E+01	6.82E+03	ND	4.58E-02
14	4.62E+04	2.77E+01	1.03E+04	ND	4.18E-02
15	6.16E+04	1.54E+01	6.91E+03	ND	4.08E-02
16	5.17E+04	2.18E+01	7.83E+03	ND	3.78E-02
17	4.84E+04	1.67E+01	7.77E+03	ND	3.88E-02
18	6.49E+04	2.09E+01	5.17E+03	ND	3.68E-02
19	3.74E+04	2.04E+01	5.41E+03	ND	3.58E-02
20	5.06E+04	1.85E+01	6.80E+03	ND	3.48E-02
21	3.19E+04	1.38E+01	7.26E+03	ND	3.38E-02
22	4.51E+04	1.93E+01	6.03E+03	ND	3.38E-02
23	2.80E+04	1.22E+01	5.55E+03	ND	3.08E-02
24	2.19E+04	1.46E+01	4.76E+03	ND	3.08E-02
26	2.53E+04	1.47E+01	5.37E+03	ND	2.88E-02
28	2.10E+04	1.59E+01	4.04E+03	ND	2.68E-02
30	2.13E+04	1.92E+01	4.33E+03	ND	2.48E-02
32	2.09E+04	1.43E+01	4.14E+03	ND	2.18E-02
34	1.41E+04	1.83E+01	2.45E+03	ND	2.18E-02
36	1.37E+04	2.34E+01	2.44E+03	ND	1.88E-02
40	1.04E+04	2.87E+01	1.76E+03	ND	1.58E-02
44	8.11E+03	3.13E+01	1.76E+03	ND	1.38E-02
47	8.07E+03	3.20E+01	1.04E+03	ND	8.75E-03

corrected for background
*Sampled, Not Detectable
** +/-15%, 95% confidence limits
*** +/- 60%, 95% confidence limits
**** +/-14%, 95% confidence limits

47

were collected less frequently from the injection well and selected multilevel ports of monitoring wells located within the tracer study area.

Analytical Techniques

Bromide was determined using standard ion chromatography methods (37). Analytical detection limit (PQL) was 0.01 mg/l. Combined analytical and sampling precision were determined to be 14% (95% confidence limits) based on replicate analysis.

.

Strains, media and growth conditions: The following viruses and their host cells were used for enterovirus and coliphage assays, as well as virus stock propagation: Poliovirus type 1, Chat attenuated vaccine strain, ATCC VR-192 and its host, Buffalo Green Monkey Kidney (BGM) cells; somatic coliphage ΦX174, ATCC 13706-B1 and its host *Escherichia coli* C, ATCC 13706; male-specific coliphage MS2, ATCC 15597-B1 and its host *E. coli* C3000 ATCC 15597 and coliphage PRD-1 with its host *Salmonella typhimurium*. The latter were a generous gift from Dr. Suresh Pillai of Texas A&M University. The BGM cells were originally obtained as a gift from USEPA, Cincinnati, Ohio.

BGM and ELAH media were filter sterilized through a 0.22 micron filter and stored at 4° C. These solutions were tested for sterility by monitoring 100 ml aliquots, held at 37° C, for contaminant growth. Before use, a 5 ml aliquot of PSA (penicillin (100units/ml), streptomycin (0.1mg/ml), amphotericin B (25 μg/ml)) and 0.25 ml of tetracycline was added to each media. Both PSA (Sigma) and tetracycline (Sigma) were prepared as per directions in the USEPA Manual of Virological Methods (47).

48

A frozen stock of BGM cells was used as a starting culture and propagated weekly or biweekly with a 1:3 or 1:4 split, following the procedures as given by the USEPA Manual of Virological Methods (47). Stock BGM cells were grown in 175 cm^2 tissue culture flasks in BGM media amended with 5 or 10% heat inactivated fetal calf serum (Gibco). BGM media contains a 1:1 mixture of L-15 (Leibovitz, Sigma) and MEM (Minimal Essential Eagle with Hanks salts and L-glutamine without NaHCo$_3$, Sigma) supplemented with 0.75g/l NaHCo$_3$.

To build a large stock for injection, poliovirus was amplified on the BGM cells. To accomplish this, 5 ml of poliovirus with an approximate titer of 7.5x10^4 PFU/ml was used to inoculate confluent BGM cells into 50 175cm^2 tissue culture flasks. The cells were prepared for the inoculum by removing BGM maintenance media and rinsing briefly with 10 ml of ELAH media (Earles lactalbumin hydrosyslate (Sigma) supplemented with 2.2g/l NaHCO$_3$) without serum. ELAH was also used for cell maintenance after virus addition. The inoculated cells were incubated for 90-120 minutes at room temperature to allow virus adsorption. After this period, 40-50 ml of ELAH containing 2% IgG-free calf serum (Gibco) was added. Flasks were incubated statically at 37° C and virus propagation was monitored based on microscopic analysis of cell pathogenicity.

Large stocks of the three bacteriophages were attained through propagation on proper bacterial hosts. To achieve this single bacterial colonies were picked from fresh tryptone-yeast agar (1% w/v tryptone (Difco), 0.1% w/v yeast and dextrose (Difco), 0.022% w/v CaCl$_2$ and 0.8% w/v NaCl with 1.5% agar) plates grown at 37° C, for each respective bacterial host. The colonies were used to inoculate 10 ml of tryptone-yeast

49

(TY) broth, which was incubated at 37° C on a shaker overnight. The overnight cultures were then transferred into polypropylene centrifuge tubes and stored at -70° C for future use as inoculum. Klett flasks (Bellco) containing sterile TY broth, were inoculated 1:20 with either fresh or frozen overnight cultures. These cultures were monitored at 510 nm until they reached mid-log phase of growth, between 0.500 and 0.600 OD units. These cultures were then inoculated with their respective bacteriophage and allowed to grow overnight at 37° C with shaking.

The bacteriophage and poliovirus were harvested by precipitation after a initial large-volume, low-speed centrifugation to remove cell debris. The resulting viral suspensions were brought to a final concentration of 0.5 M NaCl and 7.5% polyethylene glycol (PEG) 8000, and gently stirred overnight at 4° C. The viral precipitates were pelleted by centrifugation at 3400 rpm at 4° C for 25 min then, resuspended in phosphate buffer saline (PBS) (Add 5.38 g/l sodium phosphate, monobasic, 8.66 g/l sodium phosphate, dibasic and 8.77 g/l of NaCl. Adjust pH to 7.4 with NaOH). This solution was then dialyzed against 2.5 liters of stirring PBS for 24 h at 4° C with three buffer changes. Control experiments indicated that this method was essentially quantitative for virus recovery. For poliovirus, the final volume of concentrate was mixed 1:1 in Earle's minimal media (Sigma) supplemented with lactalbumin hydrolysate (Sigma). These samples were stored at −80° C until used in field injection experiment.

Coliphage assay: A single agar plaquing method was employed to assay all three bacteriophage because of its relative simplicity and efficiency. The single agar procedure was performed as follows: bacterial host cultures were grown to mid-log phase, as described previously and placed on ice to halt growth. One ml of host bacteria was added

50

to 10 ml of ground water sample and placed in a 37° C water bath for 3 to 5 m. Next, 11 ml of soft agar (2x TY broth with 1.4% w/v agar (Difco) held at 51° C) was added to the mixture. Ten ml of the mixture was immediately poured onto each of two 100 mm petri dishes. After the agar hardened, the dishes were inverted in a 37° incubator. The phage titer in PFU/ml was then determined by counting the number of plaques that formed on the plates.

Poliovirus assay: The poliovirus in groundwater samples was assayed on 3-day old BGM cells that were grown in 25 cm^2 tissue culture flasks. The cells are prepared for the assay as described previously with minor modifications made to compensate for the volume difference of the tissue culture flasks. Briefly, 1 ml of sample, diluted one to one with ELAH media containing antibiotics without calf serum, was added to the BGM cells in tissue culture flasks. The sample was exposed to the BGM host cells for 90 min at room temperature to initiate viral attachment. The inoculum was then removed and 10 ml of an agar-medium overlay was added to the flasks. The agar-medium overlay is comprised of equal volumes of a 3.0% autoclaved agar water solution and a solution, of: 80% v/v 2X 199 medium (Sigma); 4% v/v Gamma Globulin-free calf serum (Gibco); 6% v/v of a 7.5% NaHCo3 solution; 2% v/v of a 1% $MgCl_2$ solution; 4% v/v 1M HEPES buffer (Sigma) plus 2% v/v PSA; and 0.1% v/v tetracycline. This solution was supplemented with 2% v/v of sterile skim milk (Difco) just prior to addition of the 3% agar solution. The agar-medium overlay was held in a 41° C water bath during use. After the overlay was added, the flasks were covered to protect them from light and allowed to harden before they were inverted and put in a 37° C incubator. The flasks were monitored for 5 days, with plaques counted on a daily basis. The titer was then determined when the

51

number of plaques counted remained constant. To visualize plaques on the BGM cells, 2 ml of 0.1% crystal violet in 50% ethanol was applied after the agar overlay was carefully removed. The stain helped differentiate living cells from dead cells and thus aided in counting cells. To increase detection levels of the poliovirus assay, a similar PEG precipitation procedure was employed to the groundwater samples described above. The final volume was mixed 1:1 in Earle's minimal media supplemented with lactalbumin hydrolysate and filtered through a 0.45 μm filter to remove indigenous bacteria. These samples were stored at -70° C until host cells were ready.

Sampling and analysis errors were calculated at +/-15% at 95 % confidence limits for the bacteriophage assays. The poliovirus was found at lower concentrations in the groundwater and errors were correspondingly higher (+/-60%) increased as expected.

Data Analyses

Distribution bromide and MS2 were mapped in plain and cross sectional view for the 3 h sampling time point (Figs 2 and 3). Data were reviewed to assess the location of the center of mass to assure sampling network captured the bromide and MS2.
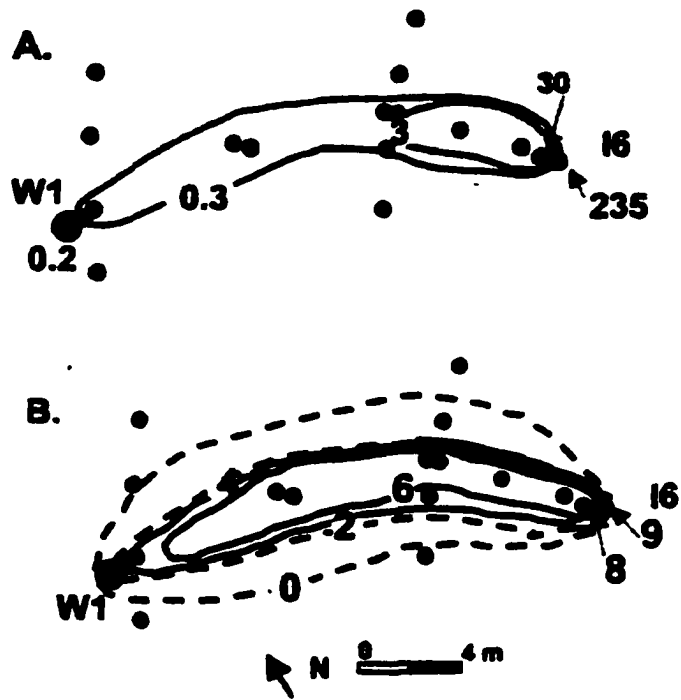
Figure 2. Plain-view maps of bromide (A) and MS2 (B) concentrations at 3 h sampling time point. Concentrations of bromide are in mg/l and MS2 in log PFU/ml. The highest concentrations measured at 3 h sampling are shown.
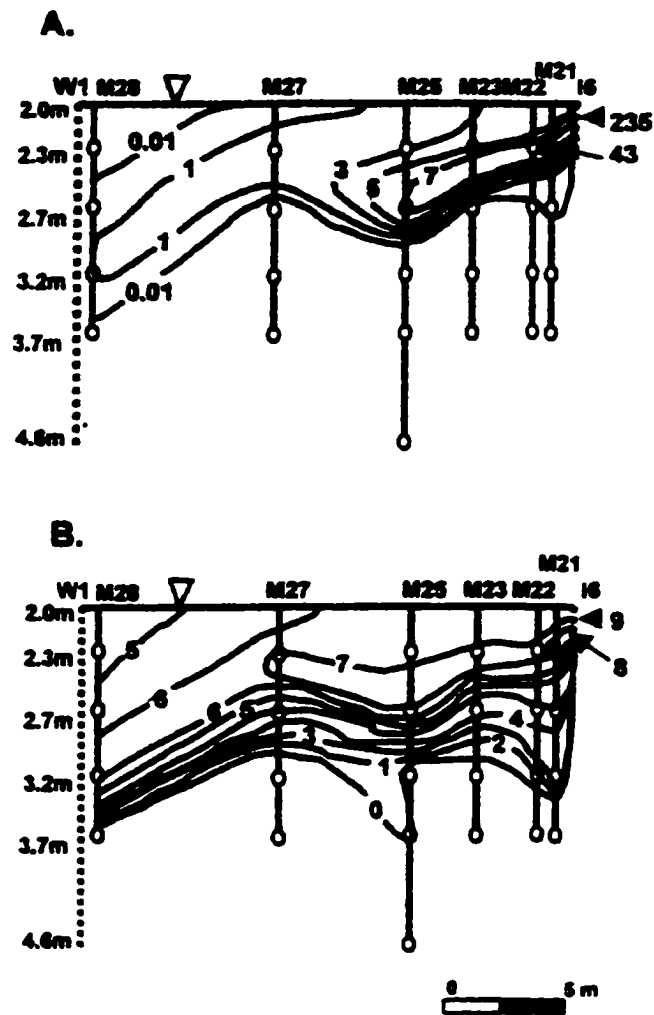
Figure 3. Longitudinal profiles of bromide (A) and MS2 (B) concentrations at 3 h sampling. Concentrations of bromide are in mg/l and MS2 in log PFU/ml. The highest concentrations measured at 3 h sampling are shown. Profile location is shown in Figure 1.

54

Breakthrough curves for the four viruses and bromide were constructed and peak arrival times were compared at W1. W1 breakthrough data were used to compute relative breakthrough (RB) as described by Harvey and Garabedian (36). The RB is calculated using concentration versus time data from a sampling point centered in the tracer plume. For these analyses, the breakthrough data at W1 were used to compare the ratio of the measured and source virus concentrations to a similar ratio of the conservative tracer (bromide):

$$RB = \int_{t_0}^{t_f} \frac{C_t}{C_0} \, dt \Big/ \int_{t_0}^{t_f} \frac{Br_t}{Br_0} \, dt \quad (1)$$

where C_0 and Br_0 are the initial respective virus and bromide concentrations at the injection well (in PFU/ml and mg/L, respectively), C_t and Br_t are the concentrations at W1 at some time t after the tracer injection, and t_0 and t_f are the times representing the beginning and end of the breakthrough curve. Relative attachment (RA) was computed as $1 - RB$.

Mass balances were calculated for bromide and each virus by integrating the measured virus concentrations pumped from W1 over 47 h. Concentrations between sampling periods were averaged and then multiplied by the volume of water extracted between sampling periods to compute the 47 h mass balance for bromide and the viruses. These values were then compared to relative breakthrough computations (RB) as described above (36).

To examine the behavior of the viruses at the injection well and at select multi-level samplers and W1, plots of the normalized difference in the bromide and virus

55

concentration ratios relative to the bromide ratio were generated by applying the following equation:

$$A = (Br_{C/C_0} - Virus_{C/C_0})/ Br_{C/C_0} \quad (2)$$

where Br_{C/C_0} is the normalized bromide concentration at time t and $Virus_{C/C_0}$ is the normalized virus concentration at t, and A is dimensionless and ≤ 1.0.

RESULTS

Breakthrough of bromide and the viruses was detectable at W1 2 h after injection (Table 2; Fig. 4). Bromide and coliphage concentrations remained above detection limits during the remainder of the 47 h test, while the poliovirus concentration fell below detection limits (0.1 PFU/ml) after the 5 h sampling time. Breakthrough peak concentrations occurred at 3.5 to 4 h for bromide and the bacteriophages. The poliovirus peak occurred earlier at 3 h. Previous work at this site has suggested that the observed poliovirus peak may be the result of truncation of the breakthrough curve by the attachment process and/or related to the more rapid transport of this virus, though the latter is considered less likely (19). Bromide, MS2 and PRD1 breakthrough curve tailing behavior is similar, continuing to decline slowly over time. However, after leveling off, ΦX174 concentrations increased over the last 15 h (Fig. 4; Table 2).

56
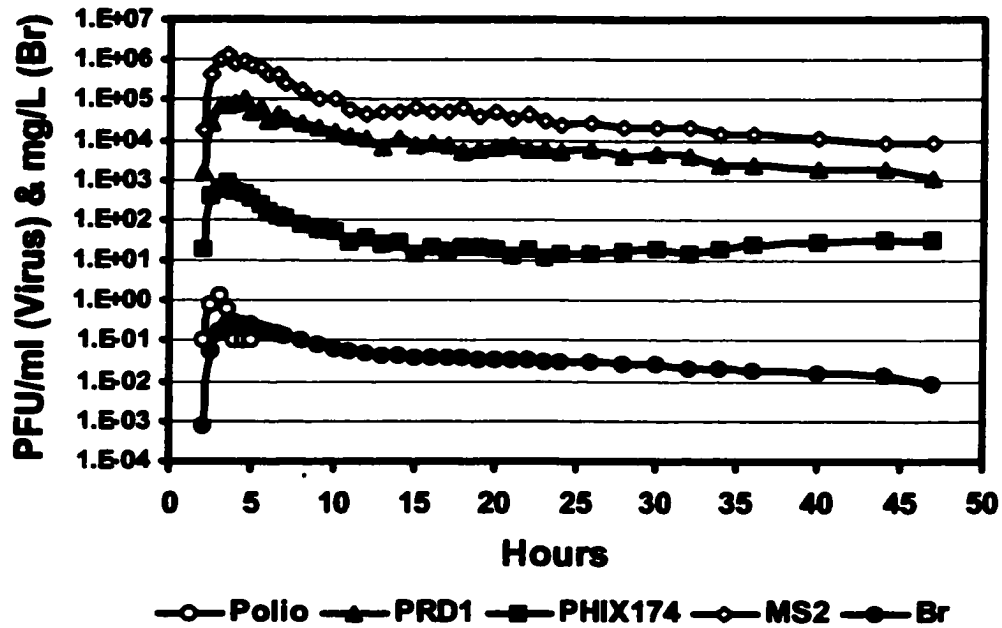
Figure 4. Bromide and virus breakthrough curves at W1.

Comparison of the cumulative mass of tracers collected at W1 with the initial injection mass shows a recovery of 77%, 55%, 17%, 7% and 0.12% of bromide, PRD1, MS2, ΦX174 and poliovirus respectively (Fig. 5). Assuming the bromide recovery reflects conservative transport in the heterogeneous flow field, about 45% of the PRD1, 83% of the MS2, 93% of the ΦX174 and >99% of the poliovirus remained in the aquifer system at the end of the test period. Analysis of the breakthrough curves for RB yielded values of 80%, 27%, 10% and 0.05% for PRD1, MS2, ΦX174 and poliovirus, respectively. A summary of the mass balance data and RB analysis is presented in Table 3.

57
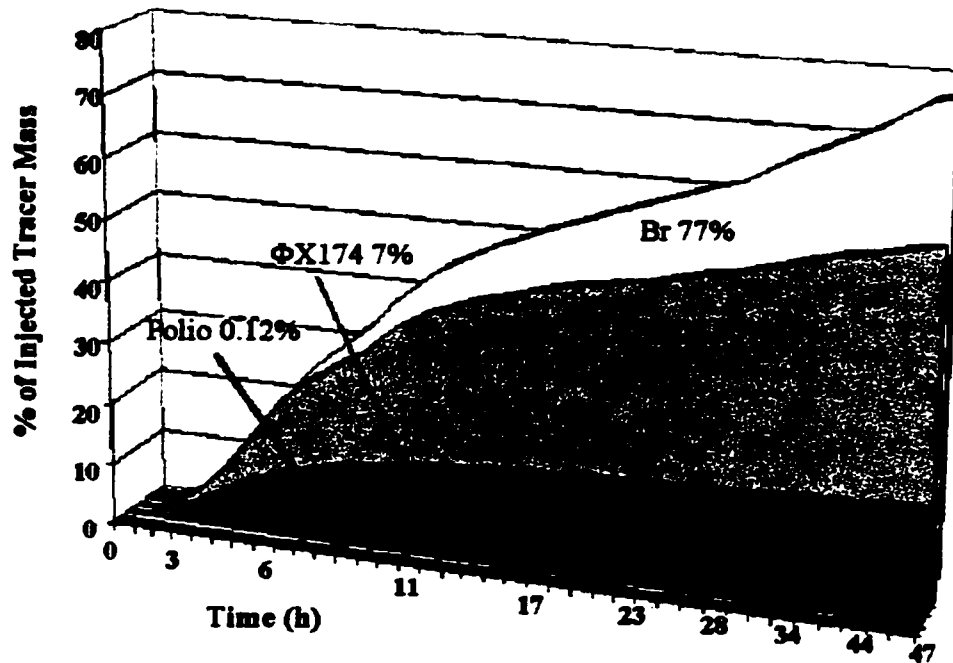
Figure 5. The cumulative percent of bromide and virus masses recovered at W1 during 47 h of pumping at 408 l/min.

Table 3. Results of mass balance computations and RB analyses at W1.

Tracer	% Mass Recovered	%RB	% Mass Remaining in the Aquifer	%RA (100%-%RB
Bromide	77	N/A	23	N/A
PRD1	55	80	45	20
MS2	17	27	83	83
ØX174	7	10	93	90
polio	0.12	0.05	>99	>99

The transport process was examined by plotting the normalized difference in C/Co ratios (Equation 2) verses time at W1 (Figure 6A and 6B). The negative values at 2 h indicate that the C/Co value for the viruses was greater than that for bromide. However, after the initial sampling MS2, ΦX174 and poliovirus ratios were less than the bromide ratio (i.e. positive values). Poliovirus ratios were continuously positive. The PRD1 ratio was more variable, becoming less than the bromide ratio after 3.5 h, then negative again at the 4.5 h sampling interval. Virus data at the injection point and multilevel-sampling wells were less complete than at W1. Data from the less frequently sampled injection well (no additional poliovirus sampling after the initial injection sampling) show ΦX174 normalized ratios were initially positive (time zero and 1 h) while PRD1 at the 2 and 3 h samplings and MS2 at 2 h sampling were negative (Fig. 6C). The normalized ratios for these two bacteriophages then remained positive for the duration of the experiment, increasing to 0.80 to 0.90, respectively (Fig. 6C). The ΦX174 ratio became negative by the 24 h sampling and remained negative for the duration of the experiment. Groundwater samples collected at multilevel sampling ports were analyzed primarily for MS2 (Fig. 7). The MS2 normalized difference ratio at the injection well and sampling points within 2 m of I6 were at 0.40 to 0.50 within 1.5 h of the injection. At 7.5 m from the injection point the first detectable virus and bromide arrival (1 h) resulted in a negative ratio value, as did the sampling at W1 (2 h) (Figure 6B and 7). The normalized ratio then increased to a plateau of 0.7 to 0.8.
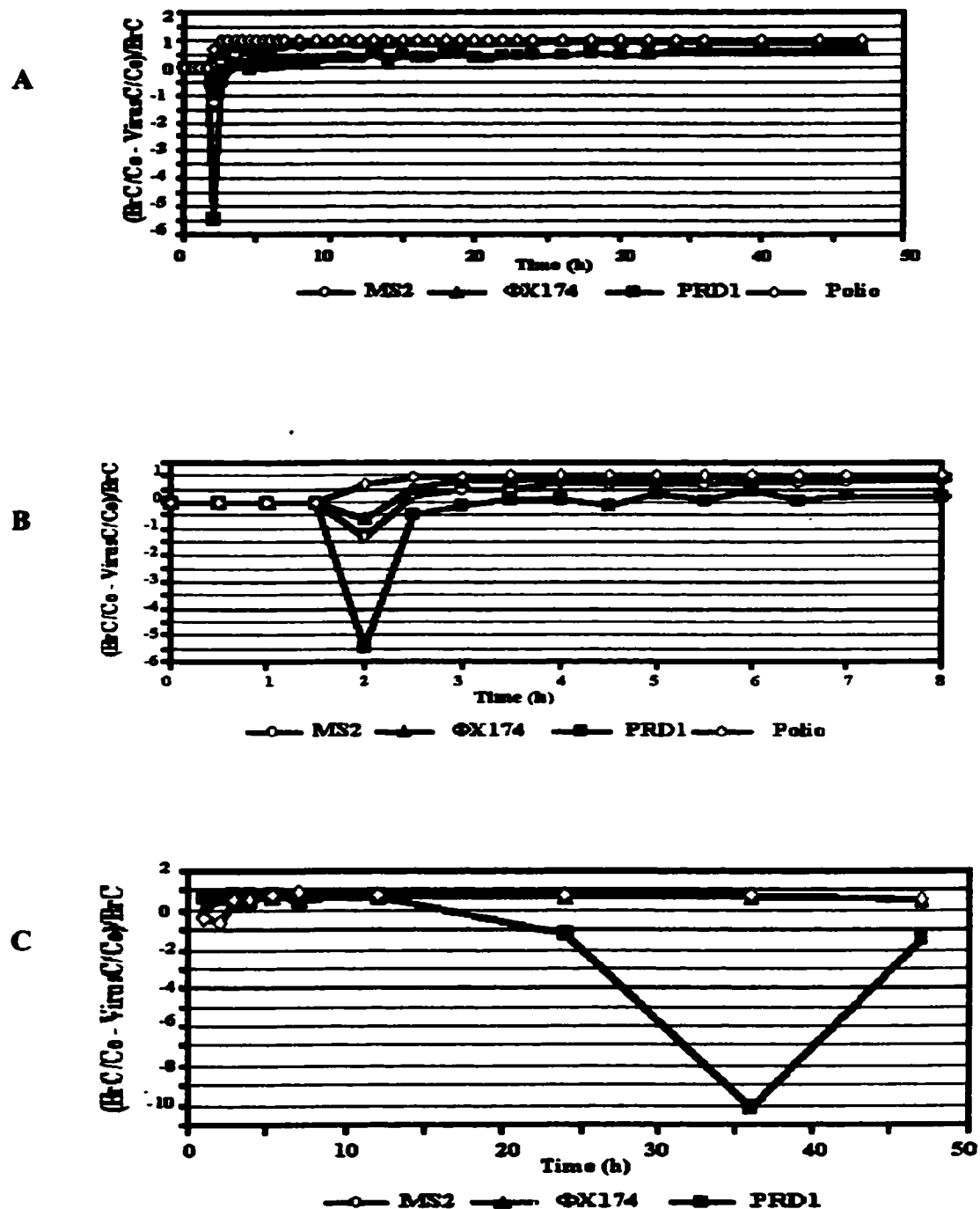
Figure 6. (A) The difference between the C/Co ratio for bromide and each for virus normalized to the C/Co ratio for bromide plotted at W1 for the first 47 h of sampling. (B) Details of the first 8 h of sampling at W1 from Figure 6A. (C) The difference between the C/Co ratio for bromide and each virus normalized to the C/Co ratio for bromide plotted at I6 for each sampling time.

60
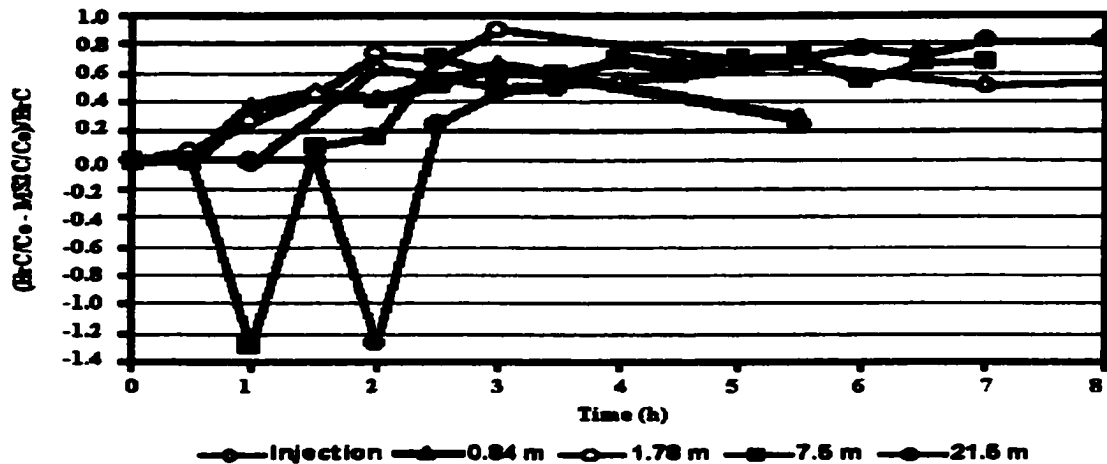
Figure 7. The difference between the C/Co ratio for bromide and MS2 normalized to the C/Co for the bromide plotted at the injection and monitoring wells.

DISCUSSION

Virus Breakthrough at W1

Transport of bacteriophage within the coarse-grained heterogeneous floodplain deposit during both natural gradient conditions (19) and a preliminary pumping experiment (22) revealed that peak virus concentrations at all monitoring points occurred at times coincident with the bromide peak. Based on peak arrivals during this experiment (3.5 to 4 h) bacteriophage and bromide were being transported at an average rate of 129 to 147 m/d along the 21.5 m flow path. However, actual velocities increase from about 95 m/d at I6 to over 240 m/d within 1 m of W1.

The poliovirus peak appears to have arrived at W1 at about 3 h. We have previously proposed that this apparently more rapid transport of poliovirus at this coarse-grained site is an artifact of breakthrough truncation caused by its high attachment rate (19). Restated here, the attachment process causes a shift to the left of the poliovirus

61

peak with respect to the bromide and coliphage peaks. The bromide peak could be adjusted to match the polio virus peak as follows:

$$Br_{tADJ} = [Br_t - (KtBr_t)] \qquad (3)$$

where, Br_t = bromide concentration (C/C_0) at time t (h); t = time of arrival at an observation point (h); K = net virus attachment value/h; and Br_{tADJ} = the attachment-transformed Br data. K was calculated from a set of ionic tracer and virus field data by substituting virus concentrations for Br_{ADJ}. This attenuation hypothesis is also supported by the more asymmetric shape (steeper leading edges) of the breakthrough curves for all bacteriophages as compared to the Br breakthrough curve (i.e. the proposed attachment process has resulted in the different symmetry of the poliovirus curve compared to that of the bacteriophage curves).

Comparing the mass balance for the viruses to the bromide tracer allows quantification of the attachment process throughout the duration of the experiment (assuming that all unaccounted for virus are attached to the aquifer sediment and inactivation is negligible over the short duration of the experiment) (29). Using the range of measured average flow velocities, the groundwater flowing between I6 and W1 was exchanged 12 to 13.4 times during the 47 hr experiment. However, 23 % of the bromide, and higher percentages of each virus remained in the aquifer at the end of the test (Table 3). This suggests that the aquifer is highly heterogeneous. RB analyses preformed as suggested by Harvey and Garabedian (36) generally overestimated the mass of PRD1 (80% v 55%) and MS2 (27% v 17%) that was collected at W1 (Table 3). The predicted

62

RB of ΦX174 and poliovirus more closely approximated the measured values (Table 3). Apparently, the behavior of PRD1 and MS2 was poorly represented by the RB analysis in this aquifer. Part of this discrepancy may be due to the way the RB analysis equally weights sampling intervals even though they vary in length. Further, the velocity was not constant in this flowfield which may affect predicted RB. Averaging of the tracer concentrations for intervals of pumping time during mass balance calculations may have also made comparison of the two methods less appropriate.

Table 4. Virus and Sediment Characteristics

Virus	Diameter (nm)	pI*
PRD-1	65	4.2 (>3.2**)
MS2	24	3.9
ØX174	32	6.6
poliovirus(type 1, chat strain)	28	7.5 & 4.5
Aquifer Sediment		
Quartz dominated		2-3.5

*Gerba (23) and Dowd et al. (32)   **Ryan et al. (28)

The ordering of virus attachment (from less to more) in this near neutral pH groundwater system is approximately coincident with the commonly reported isoelectric point values of the viruses (Table 4). PRD1 (pI=4.2), however, is an exception to this general observation as its reported pI value is slightly higher than that of MS2 (pI=3.9), yet it sorbed to a lesser extent (more mass arrived at W1). Because all of the viruses in

63

this experiment were subjected to identical conditions, it appears that some other inherent property of PRD1 mitigates its sorption behavior.

Several factors that may contribute to virus sorption in groundwater systems include electrostatic interactions, ionic interactions, london forces (van der Waals) and hydrophobic effects (24, 38-41). However, depending on the pH of the system and the viral isoelectric points, the resulting forces may be either attractive or repulsive. Because the outer surface (capsid) of the virion is composed of proteins, functional group ionization may lead to a variety of charged groups (both positive and negative) being present on the virus surface. In general, the overall surface charge of a virus will be positive if pH conditions are below its pI and negative if above it. However, localized areas on the virus surface may be dominated by either positive or negative charges, and may influence viral sorption (42). Generally, mineral surfaces found in sediments are predominately negatively charged at near-neutral pH. Thus, viruses with the highest pI values (polio and ΦX174) would likely attach to the sediments to a greater extent compared to those with lower pI values (MS2 and PRD1). This is consistent with our observations and those of prior studies (43).

That MS2 and PRD1 adsorb at all may seem like an anomaly. In theory, these bacteriophage should be experiencing a net repulsive force due to their overall net negative charge. A model proposed by Bohn et al. describes how forces between two particles of similar charge can attract one another (26, 44). Briefly, two layers of counter-ions are associated with the colloidal particle. The inner layer, or Stern layer, is held tightly to the molecule, neutralizing the charge on its surface. The outer (Gouy) layer is more diffuse but contains enough oppositely charged ions to attract particles of like

64

charge. The conditions within the Gouy layer may control the attractive forces acting in the vicinity of the aquifer sediment particle. These forces, along with van der Waals attractions, are believed to control colloid particle interactions (e.g. a virus) as described by the DLVO (Derjaguin-Landau-Verway-Overbeek) theory (26, 45). Loveland et al. (39) point out that reversible attachment of like-charged particles may occur in the secondary minimum of a DLVO potential energy profile. It is suggested that this may be due to comparable thermal and potential energy existing between the objects (virus and sediment).

Since van der Waals interactions are generally considered the primary attractive force for colloidal particles, particle diameter may also influence the sorption process (41, 43). The propensity of larger diameter viruses, like PRD1, for attachment to a fixed aquifer particle may be restricted because of the dominance of more like charges (resulting in net repulsion). Small changes in the diameter of spherical particles translate into significant changes in surface area. Further, infinitesimal change in distance between objects may lead to a substantial decrease in energy between them as van der Waals interactions act over only very short distances (26). In this situation, the potential energy between the virus and sediment is reduced leading to less attachment (39). PRD1 may not be able to overcome the primary maximum energy barrier as is required to become irreversibly adsorbed. Instead it would stay in a minimum energy state and be only transiently adsorbed, thus more likely to undergo subsequent detachment. Consequently, although pI value is probably the primary property controlling sorption, in the case of MS2 and PRD1, diameter appears to also be an important factor (41, 43).

This explanation assumes the commonly reported pI value of 4.2 for PRD1 is representative of conditions at our site. Ryan et al. (31), conducting virus transport studies at Cape Cod, MA recently determined the pI of PRD1 to be approximately 3.2, considerably lower than the commonly reported value in Table 5 and lower than that of MS2. If conditions at our site are similar to those reported by Ryan et al. (31), the degree of virus attachment at our site would correlate very well with increasing viral pI values.

The behavior of PRD1, MS2 and ΦX174 (initial negative normalized ratios) indicate that viruses are being preferentially transported through more direct flowpaths to W1 than was bromide during the initial phase of the experiment (Figure 6A and 6B). Under forced gradient conditions, more water preferentially flows through the larger pore channels. The C/Co ratio for PRD1 also remains higher than the equivalent C/Co ratio for bromide at W1 for a longer period of time than the other viruses. Possibly, pore exclusion and the resulting transport through only larger pores at this field site facilitates transport of the larger diameter PRD1 virus and results in a greater mass arriving at W1. Thus, although pI value is probably the primary property controlling virus sorption, in the case of PRD1, diameter also appears to be an important controlling factor (32, 36).

Virus Detachment

PRD1, MS2 and attenuated poliovirus are detaching during the experiment, as indicated by the long tails on the breakthrough curve (Fig. 4). The rate of detachment of ΦX174 increased after 20 to 25 h as indicated by the change in slope in this region of the breakthrough curve (Fig. 4), and the negative values shown in Figure 6. A similar detachment trend was observed at the injection well, occurring between the 14 and 24 h

sampling times. Apparently, ΦX174 attachment is only partially reversible under our test conditions. This is in contrast to lab studies which report that contact time with sediments is the most important factor controlling attenuation, and further, that ΦX174 transport is not retarded (2, 40). Our field observations for ΦX174 may reflect the rapid transport velocities and/or large number of pore volumes that were exchanged during our experiment.

Additional Observations

Plots of normalized C/Co ratio vs. time (equation 2), and the frequent sampling intervals, revealed two additional aspects relative to virus transport under forced gradient conditions. The occurrence of negative normalized ratios at W1 that indicate preferential transport of the bacteriophage have been described previously. In contrast, bromide, as an non-reactive ion, enters all available pores, which results in a lower normalized bromide value at its initial detection at W1. A similar response was observed for MS2 at a sampling point 7.5 m from the injection point (Fig. 7). In this field setting, the apparent pore exclusion process appears to dominate transport only temporarily. The attachment process then becomes the primary factor.

Another notable observation is that normalized ratios for MS2 are 0.30 to 0.40 during the first 1 h sampling within 0.84 and 1.78 m of I6 (Fig. 7). Limited sampling for other viruses showed similar trends with plateaus at 0.70 to 0.80 within 4 to 6 h of injection. This suggests the attachment process occurs rapidly and in the immediate vicinity of I6 with only an additional 0.20 to 0.30 increase in the normalized ratio recorded at wells 7.5 - 21.5 m down-gradient of W1. Others have also reported a high

degree of attachment occurring close to a virus source (29). Only ΦX174 appeared to show a reversal in this process, suggesting its attachment is less stable with time.

Based on our experimental results, a concentration vs. time relationship for virus travelling to, and arriving at, a pumping well located 21.5 m from a slug virus source in a similar groundwater system could be estimated using the following equation:

$$\text{Virus}_{C_t} = (\text{Br}_{C_t/C_o} * (1-A) * \text{Virus}_{C_o}) \quad (4)$$

where a sampling location at time t, Virus_{C_t} is the virus concentration, Br_{C_t/C_o} is the normalized bromide concentration, A is the solution to equation 2, and Virus_{C_o} is the initial concentration of the injected virus. Though specific for our site and experimental design, this relationship may prove useful for estimating peak virus concentrations at a down-gradient well and/or to plan initial concentrations for field seeding experiments. Using this strategy, a preliminary bromide tracer experiment would be required to obtain Br_{C_t/C_o} data for a site. Approximate values of A (0.7 to 0.8) derived from MS2 and PRD1 results could be used to estimate Virus_{C_t}. Analysis of the behavior of attenuated poliovirus would require an A value of about 0.99. Before generating a more robust bromide-virus relationship (as for equation 4), additional field experiments conducted under differing conditions with the same and other viruses would be needed to evaluate whether A can be reasonably estimated without the need to seed indicator virus at a particular site.

Optimal modeling of virus transport will require additional development of time-distance and virus attachment-detachment relationships. Results to date indicate that

such factors could be derived at our field site only by conducting additional virus seed experiments under a variety of pumping conditions, combined with more comprehensive sampling of multilevel monitoring wells within the plume flow path. Specific attachment and detachment rates could then be derived and used to calibrate virus transport models.

ACKNOWLEDGMENTS

References

1. Rose, J.B.; Altas, R.M.; Gerba, C.p,; Gilchrist, M. J.; LeChevallier, M. W.; Sobsey, M. D.; Yates, M.V. In Microbial Pollutants in our Nation's Water; Environmental and Public Health Issues. American Society of Microbiology. Washington, D.C.

2. Jin, Y.; Yates, M.V.; Thompson, S.S.; Jury, W. *Environ. Sci. Technol.* 1997, 31, 548-555.

3. Kramer, M. N.; Herwaldt, B. L.; Craun, G.F.; Calderone, R.L.; Juranek, D.D. *J. Am. Water Works.* 1996, 88, 66-80.

4. Keswick, B.H.; Gerba, C.P. *Environ. Sci. Technol*, 1980, 14, 1290-1297.

5. Wellings, F.M.; Lewis, A.L.; Mountain, C.W.; Pierce, L,V Yates, M.V., Gerba, C.P., and Kelley, L.M., *Appl. Environ. Microbiol.* 1975, 29, 751 – 757.

6. Mathess, G.; Pekdeger, A. *Survival and Transport of Pathogenic Bacteria and Viruses in Ground Water.* Ground Water Quality, John Wiley & Sons, New York, 1985.

7. Pekdeger, A.; Mathess, G. *Environ. Geology.* 1983, 5, 49 – 52.

8. Bitton, G.; Farrah, R.; Rukin, J.; Butner, J.; Chou. Y. *Groundwater.* 1983, 21, 401 – 405.

9. Gerba, C.; Powelson, D.; Yahya, M.; Wilson, L.; Amy G. *Water Sci. Tech.* 1991, 24, 95-102.

10. Yates, M.; Gerba, C.; Kelley, L.; 1985. *Appl. Environ. Microbiol.* 1985, 49, 778-781.

11. Yates, M.; Yates, S. *Groundwater*, 1989, 27, 202-208.

12. Yates, M.; Jury, W. *J. Eviron. Qual.* 1995, 24, 1051-1055.

13. Jansons, J.; Edmonds, L.; Speight, B; Bucens, M. *Water Resources.* 1989a, 23, 301-306.

14. Jansons, J.; Edmonds, L.; Speight, B; Bucens, M. *Water Resources.* 1989b, 23, 293-299.

15. Rao, C.V.; Melnick, J.L. *Human Viruses in Sediments, Sledges, and Soils.* CRC Press: Boca Raton, Fl. 1987.

16. Abbaszadegan, M.; Stewart, P.; LeChevallier, M. *Appl. Environ. Microbiol.* 1999, 65, 444 – 449.

17. Bales, R.; Li, S.; Maguire, K.; Yahya, M.; Gerba, C.; Harvey, R. *Groundwater* 1995, 33, 653-661.

18. Pieper, A.; Ryan, J.; Harvey, R.; Amy, G.; Illangasekare, T.; Metge, D. *Environ. Sci. Technol.* 1997, 31, 1163-1170.

19. DeBorde, D.; Woessner, W.; Kiley, Q.; Ball P. *WATER RES.* 1999, Vol. 33: 2229-2238.

20. Noonan, M.J.; McNabb, J.F. *Ann. Mtg. Amer. Soc. Microbiol.* 1979, 221.

21. United States Environmental Protection Agency. *Fed. Reg.* 1992, 52.

22. Woessner, W.; DeBorde, D. *In Headwater Control IV: Hydrology, Water Resources and Ecology in Headwaters.* 1998 Meran/Merano, Italy.

23. Yates, M.; Yates, S.; Wagner, J.; Gerba, C. *J. of Contaminant Hydrology,* 1987, 1, 329-345.

24. Goyal, S.; Gerba, C. *Appl. Environ. Microbiol.* 1979, 38, 241-247.

25. Bales, R.; Li, S.; Maguire, K.; Yahya, M.; Gerba, C. *Water Resources Res.* 1993, 29, 957-963.

26. Gerba, C. *Ad. App. Microbiol,* 1984, 30, 113-168.

27. Gerba, C.; Goyal, S.; Cech, I.; Bogdan, G. *Environ. Sci. Technol* 1981, 5, 940-944.

28. Hurst, C.; Gerba, C.; Cech, I. *Appl. Environ. Microbiol.* 1980, 40, 1067-1079.

29. DeBorde, D.; Woessner, W.; Lauerman, B.; Ball P. *Groundwater* 1998, 36, 825-834.

30. DeBorde, D.; Woessner, W.; Lauerman, B.; Ball P. *Water Res.* 1998, 32, 3781-3785.

31. Ryan, J.N.; Elimelech, M.; Ard, R.A.; Harvey, R.W; Johnson, P.R. *Environ. Sci. Technol.* 1999, 33, 63-73.

32. Bales, R.; Li, S.; Yeh, T.C.J.; Lenczewski, M.E.; Gerba, C.P. *Water Resources Res.* 1997, 33, 639-648.

33. Mathess, G.; Pekdeger, A.; Schroeter, J. *Environ. Geology.* 1988, 2, 171 – 188.

34. Corapcioglu, M.Y.; Hardias, A. *J. Hydrol.* 1984, 72, 149 – 169.

35. Corapcioglu, M.Y.; Hardias, A. *Adv. Water Resour.* 1985, 8, 188 – 200.

36. Harvey, R; Garabedian, S. *Environ. Sci. Technol.* 1991, 25, 178-185.

37. Pfaff, J. Office of Research and Development, USEPA 1993

38. Burge, W.; Enkiri, N. *J. Environ. Qual.* 1978, 7, 536-542.

39. Loveland, J.; Ryan, J.; Amy, G.; Harvey, R. *Colliods Surf. A: Physiochem. Eng Asp.* 1996, 107, 205.

40. Murray, J.P.; Parks, G.A. In *Particles in "Water: Characterization, Fate, effects and Removal;* Kavanaugh, M.C., Leckie, J.O., Eds.; ACS Advances in Chemistry Series 189: American Chemical Society: Washington, D.C. 1980. 97-103.

41. Bales, R.; Hinkle, S.R.; Kroeger, T.W.; Stocking, K.; Gerba, C.P. *Environ. Sci. Technol.* 1991, 25, 2088-2095.

42. Penrod, S.L.; Olson, T.M.; Grant, S.B. *Lanmuir* 1996, 12, 5576-5587.

43. Dowd, S.E.; Pillai, S.D.; Wang,S.; Corapcioglu, M.Y. *Appl. Environ. Microbiol.* 1998, 64, 405-410.

44. Bohn, H,; McNeal, H,N,; O'Conner, G. *Soil Chemistry* 1979, Wiley, New York, New York.

45. Harvey, R.W., Kinner, N.E., Bunn, A., MacDonald, D., and Metge, D. *Appl. Environ. Microbiol.* 1995, 61, 209-217.

46. Funderburg, S.W.; Moore, B.E.; Sagik, B.P.; Sorber, C.A. *Water Res.* 1981, 15, 703-711.

47. USEPA, 1995. Virus Monitoring Protocol for the Information Collection Requirements Rule. EPA/814-B-95-002. USEPA, Cincinnati, OH.

Chapter 5

NOTE: This work to be submitted for publication.

Partitioning of MS2 During Transport in a Septic Effluent Impacted Unconfined Aquifer

Patrick N. Ball, William W. Woessner, Jeffery Fink, Dan C. DeBorde and William Holben

Abstract

Equitable rules to protect public health and safety can be developed only by taking into account the presence of both unbound and bound viruses in aquifer systems. Few field studies have attempted mass balance analysis (i.e., total numbers of virus or bacteria added = fraction bound + fraction free + fraction inactivated). Without such field-scale mass balance data, regulatory agencies lack sufficient information to assess risks accurately and formulate appropriate protective regulations. The bacteriophage MS2 and a bromide tracer were seeded as a slug source into the water table a well (IW1) located 3.24 m upfield from a well (EW1) pumped at a constant rate. The breakthrough and partitioning behavior was observed and quantified. For the aqueous phase, groundwater samples were collected at predetermined intervals over a 48 h period. The portion of the seeded virus collected at the EW1 was calculated by integrating the mass collected over averaged time intervals throughout the duration of the 48 h test. The mass collected during this time was 45.3% of the total input virus at the injection well. For the solid phase analyses, sediment cores were extracted from the flowfield along a transect between the injection well and the pumping well. The bacteriophage was extracted from

the sediment sections and the data presented as virus/gram. The predicted dimensions of the plume were determined by flow models and bacteriophage samples and each sediment section was appropriately weighted by width, depth and length to approximate the plume shape at that particular location. After analysis of the solid phase samples, the total corrected mass contribution was 5.5%. The total mass contributed by both the aqueous and solid phase was 51% of the original input mass of MS2 indicating that approximately 49% of the input mass eluded detection. In addition, the relative breakthrough (RB), relative attenuation (RA) and collision efficiency (α) were calculated. The RB calculation was comparable to the actual mass captured in the well. However the RA calculation overestimated the mass contributed by the solid phase. This suggests that irreversible adsorption may account for less attenuation then was previously hypothesized.

Introduction

The 1986 Congress recognized the inadequacy of existing regulations governing groundwater supplies to protect public health from water-borne diseases and amended the Safe Drinking Water Act to allow inclusion of additional contaminants on a three-year cycle. Although agencies are aware that human enteric viruses cause groundwater contamination, at the present time most public water supplies relying on groundwater are not required to monitor for viruses. However, efforts to revise current rules and formulate new regulations that would protect groundwater from viral pathogens are hampered by a limited understanding of the processes affecting movement and fate of viral pathogens in various groundwater systems (Yates and Jury, 1995; Macler, 1995). Attachment of viruses during transport, inactivation, and hydrodynamic dispersion are recognized as primary processes controlling aqueous phase viral concentrations measured in groundwater (Goyal and Gerba, 1979; Noonan and McNabb, 1979; Gerba, 1984; Yates et al., 1987; Bales et al., 1993, 1995; Rossi et al., 1994; Pieper et al., 1997; DeBorde et al., 1998a,b). The key factors influencing rates of attachment and detailed quantification of this process under field conditions have been assessed only indirectly at a few field sites (Alhajjar et al., 1987; Jansons et al., 1989a,b; Bales et al., 1989, 1995, 1997; Rossi et al., 1994; Pieper et al., 1997; Ball et al., 1999; Schijven et al., 1999).

Virus inactivation has been studied in lab settings and at a limited number of field sites (Hurst et al., 1980; and Yates et al., 1985; Yates and Yates, 1987; Jansons et al., 1989a; Snowdon et al., 1989; Yahya et al, 1993; Pieper et al., 1997; Ball et al., 1999; Schijven et al., 1999). Literature to date generally indicates that inactivation rates are directly proportional to groundwater temperature (Hurst et al., 1980; Yates et al., 1985;

Yates and Yates, 1987; Jansons et al., 1989a; Snowdon et al., 1989; Powelson et al., 1990; Yahya et al., 1993). It has also been suggested that sorbed virus may have slower inactivation rates than those in the aqueous phase (Moore et al., 1975, Liew and Gerba, 1980, Grant et al., 1993).

The potential for survival of virus in groundwater systems for periods of months to years increases the chances of virus reaching groundwater supplies (Yahya et al., 1993; Yates and Yates, 1987; DeBorde et al. 1999; data unpublished). In column and field experiments, it has been observed that a portion of the attached virus detach from the bound phase over time. This process is poorly quantified, yet it represents a potential source of persistent viral contamination. This process can be enhanced when systems are chemically or hydrologically perturbed (Bales et al., 1993; Loveland et al., 1996). Thus, the sorbed portion may represent a persistent risk to public health, especially considering the low infectious dose of some viruses (Abbaszadegan et al., 1999).

Development of equitable rules that will protect public health and safety requires understanding processes governing the transport and survival of both the aqueous phase and bound phase virus. The work described here was designed to quantitatively examine the partitioning of a virus between the aqueous phase and bound phase during transport at the field scale. These data describe processes within a sand and gravel dominated unconfined aquifer that is receiving septic system effluent. The partitioning process was primarily observed by seeding the bacteriophage MS2 into the capture zone of a pumping well, analyzing aqueous and solid phase samples, and computing an aqueous and bound phase mass balance. Various factors controlling virus binding and survival were examined by column experiments and elution of virus from field sediment cores.

76

Materials and Methods

Bacteriophage: The bacteriophage MS2 was used as a model organism for the seeding experiment. MS2, a virus of *Escherichia coli* and innocuous to humans, was injected as a slug input source at 1.27×10^{14} total virus in 2 l of virus-free site groundwater. This bacertiophage is well characterized and has been used previously in numerous field investigations (Bales et al., 1991; Jin et al., 1997; DeBorde et al., 1998a,b, 1999; Ball et al., 1999; Schijven et al., 1999). Physically similar to most human enteroviruses, MS2 is a small (27-30 nm dia.), naked icosahedral RNA virus with a reported isoelectric point (pI) of 3.9 (Bales et al., 1991). MS2 has generally been accepted as a surrogate for human pathogens in many studies, and is under consideration by the USEPA as a marker organism for monitoring water quality (IAWPRC Study Group, 1991).

Field site: The Frenchtown High School Site is located about 25 km west of the University of Montana, Missoula Campus (Fig. 1). This rural high school disposes of approximately 12,000 L/d of sewage and wastewater in a 1860 m^2 drainfield south of the school. This system and the underlying unconfined aquifer were described previously by DeBorde et al. (1998a,b). At the study site, septic effluent discharges in the vadose zone and flows through 1.8 to 2.4 m of fine to medium sand to the water table located 3.1 - 3.2 m below land surface (DeBorde et al. 1998; Lauerman, 1999). Groundwater in the 5 m thick sand and gravel aquifer flows through the site from the northeast to the southwest at about 3.6 m/d. The study site was located approximately 6 m downfield from the southern edge of the septic-drainfield (Fig. 1). Physical and chemical properties of the study site are summarized in Table 1.
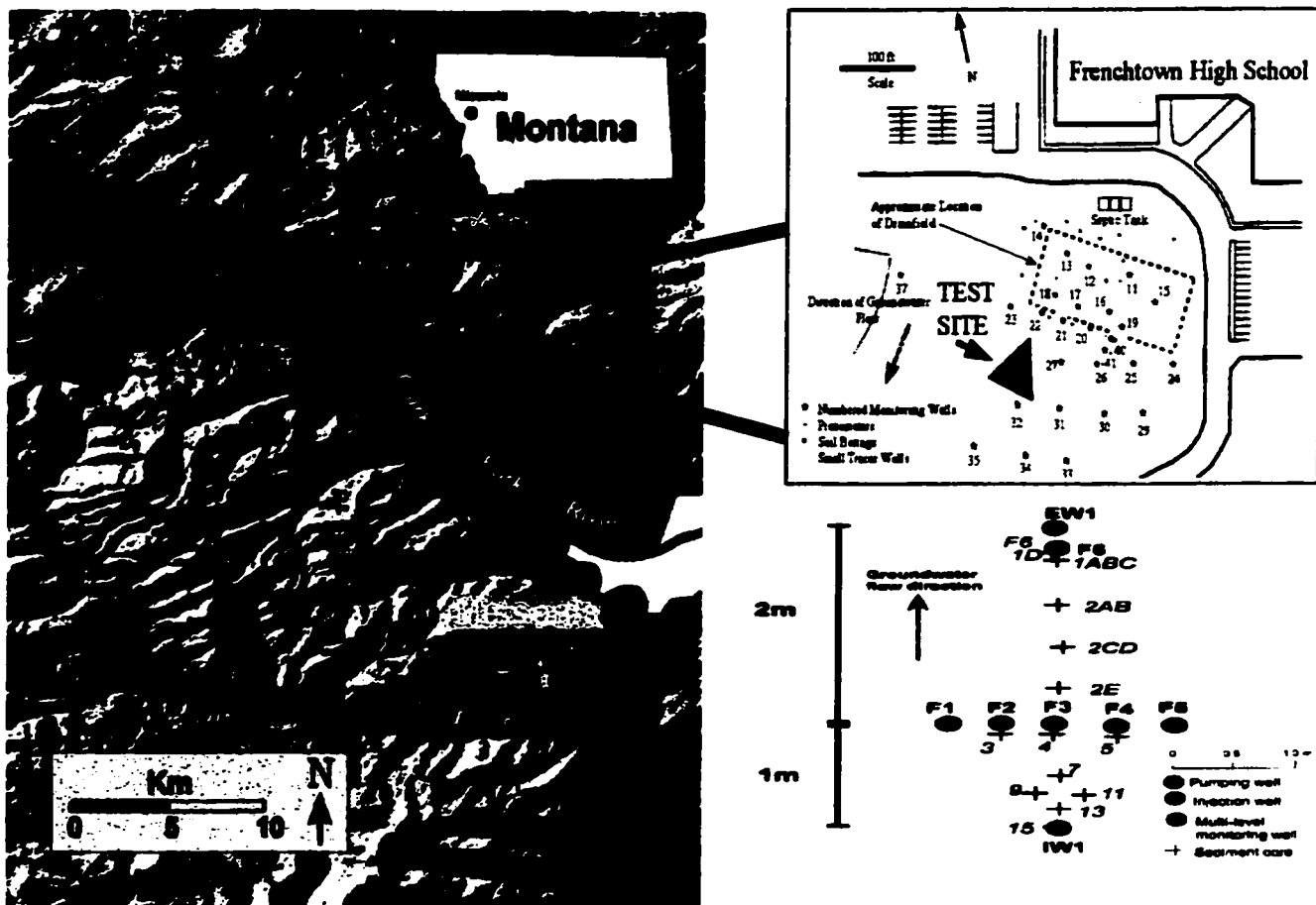
Figure 1. Location of Frenchtown High School field site with inset of septic system and locations of injection well, monitoring well and pumping well indicated. Also indicated are the locations of the sediment cores extracted from the site.

Field Methods:

(i) Well construction: A GEOPROBE™ 5400 was used to drive a flush-coupled steel casing (3.2 cm ID) with an expendable point to a depth of 4.6 m. Piezometers for sampling and the injection well were installed by inserting the screened PVC well pipe into the casing and allowing the hole to collapse around the wells during casing extraction. Coarse

78

Table 1. Hydrological and water quality data for Frenchtown High School field site. Information gathered from previous work by Lauerman (1999) as well as current research.

Hydrologic Properties	
Porosity	0.25
Gradient	0.002
K (m/d)	240 – 300
GW Velocity (m/d)	3.6
Water Chemistry	
Water Type	Calcium, Bicarbonate
˙Conductivity	325 μS/cm
Dissolved oxygen (DO)	4.7 mg/l
pH	6.6
Ave. Temperature (C°)	9.6
Ca (mg/l)	63
Mg (mg/l)	18
Na (mg/l)	17
K (mg/l)	2.1
Cl (mg/l)	6.9
No$_3$-N (mg/l)	4.3
HCO$_3$ (mg/l)	200

Colorado silica sand was used to fill any un-collapsed areas of the borehole above the water table (20 to 40 cm) then the remaining hole was backfilled with sand from the site and surface-sealed with bentonite. Bundle piezometers (multilevel samplers) were constructed using a center tube of 1.3 cm OD PVC pipe perforated over an interval of 7.6 cm from its base (4.8 m) and wrapped with a fine mesh nylon screen (Fig. 2). To the outside of this PVC tube a series of 0.95 cm OD polyethylene tubes perforated over the terminal 5 cm, and wrapped with fine nylon screen were located at intervals 2.9, 3.1, 3.2, 3.4, 3.5, 3.6, 3.8, 4.0, and 4.2 m below land surface (Fig. 2). The 2.54 cm diameter PVC injection well (IW) was constructed by perforating the terminal 25 cm and wrapping the perforations

with a fine mesh nylon screen. This well was installed with the perforations extending 25 cm below the water table (Fig. 2). The 7.6 cm diameter production well (EW1) consisted of a sand point with 1.5 m of #30 slot (0.76 cm) wire wrapped screen installed from 3.3 to 4.8 m below land surface (Fig. 2). The well was installed by augering a pilot hole to the water table, then driving the well into place with the GEOPROBE™.
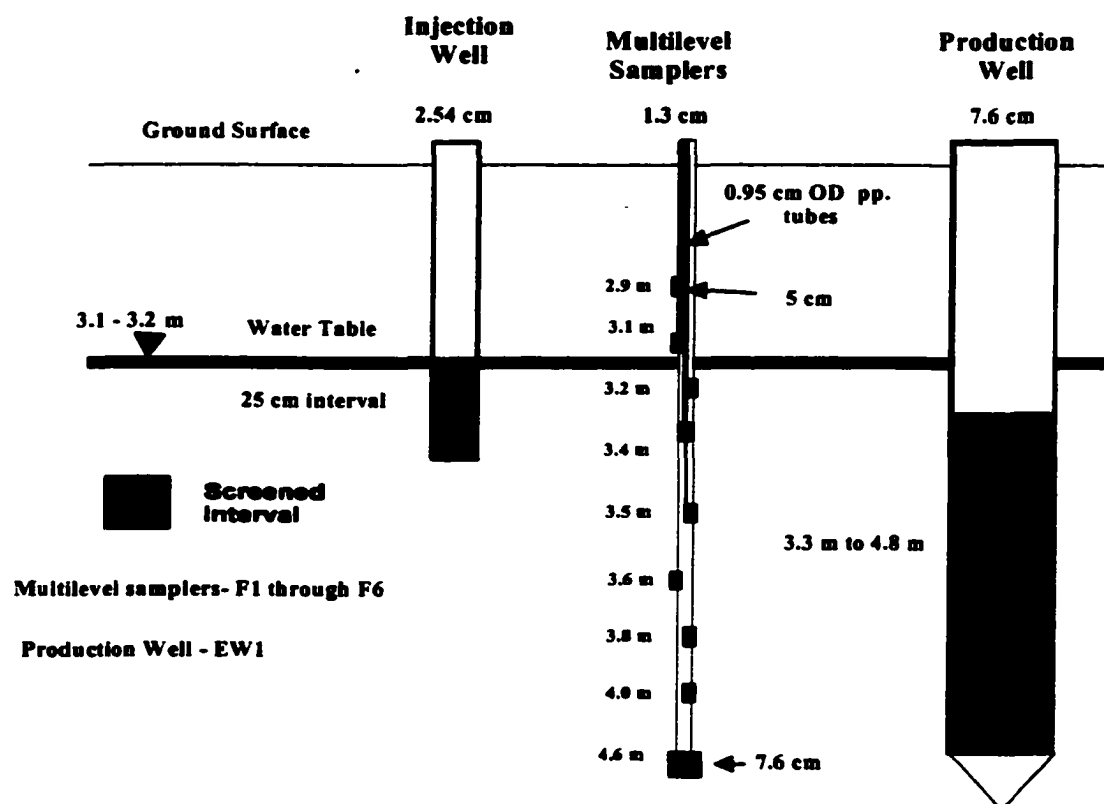
Figure 2. Details of the arrangement and construction of multilevel sampling wells, injection and production well

(ii) Conservative Tracer Tests: Well EW1 was pumping at a constant rate of 45.4 L/m until steady state flow conditions were established (approximately 1.3 h). As the discharge rate was held constant, 2 l of bromide tracer (1458 mg/L) was injected at IW1 as a slug over a 15 m interval. The multilevel wells were sampled using a peristaltic

pump. The production well (EW1) was also sampled over a 24 h period by obtaining samples directly from the well head.

After reviewing test results, it appeared bromide concentrations in the production well were below detection limits of 0.01 mg/l. To derive a dilution factor occurring at EW1, a separate 70 minute test was performed by injection of a 1 L slug of bromide with a concentration of 1025 mg/l of bromide at F6-3.2 located less than 0.5 m from EW1 which was being pumped at a 45.4 L/m. These results and the bromide data collected at F6 ports 3.2 and 3.4 were used to develop a synthetic bromide breakthrough curve for the 24 h bromide test at EW1.

(iii) Groundwater Sampling: A steady state flow field was created by maintaining discharge at well EW1 at 45.4 L/m during the 48 hour test (Fig. 1). Aqueous samples were obtained from the multilevel samplers F1 through F6 using peristaltic pumps equiped with Masterflex™ tubing (Cole Parmer Instrument Co., Vernon Hills, IL) to collect 50 to 100 ml of groundwater per sample. The tubing was autoclaved and installed to each multilevel sampling port (0.25 OD polyethylene tubing) where it remained dedicated to that port for the duration of the experiment. To avoid contaminating hourly samples, prior to sample collection a minimum number of bore volumes were purged and deposited into a waste container. Samples were also collected from the pumping well EW1 at predetermined intervals (0.5 to 1.0 h) based on prior tracer studies. All samples were collected in sterile polypropylene containers, and immediately placed on ice for transport to the laboratory at The University of Montana. During the experiment, samples were taken at upgradient wells, to monitor the background concentration of native male-specific bacteriophage entering the network system from the drainfield.

81

(iv) Solid Phase Sampling: The coring of the saturated zone was concentrated on centers of the viral plumes within the first 3 m of the injection well. Using the GEOPROBE™ (Fig.1 and 3) unit a borehole was augered to the approximate location of the water table (3.1 to 3.2 m below land surface). Because of the difficulty in obtaining intact cores from the sand and gravel unit, 0.9 m by 7.6 cm sections of new Shelby coring tubes were used to take multiple sediment cores at and below the water table. Cores were extracted in the field and samples representing approximately 15 cm intervals were placed in individual plastic Zip-loc™ bags, labeled and immediately placed on ice. Where complete undisturbed intact cores were obtained they were wrapped with sterile foil and placed horizontally on ice. All samples were transported directly to the laboratory for analysis.

Laboratory Methods:

(i) Sample Processing for MS2 Analysis: Samples collected from the liquid phase were assayed by the single layer plaque method with appropriate dilutions. The bacteriophage propagation and assay methods were described in detail previously in chapter 4 and in DeBorde et al. (1998a).

Sediment samples were mixed by hand until homogeneous, carefully emptied out of bags onto clean surfaces, visually examined for anomalies, photographed and archived. Large cobbles were removed from the homogenized mixture and samples of the mixed sediments, gravels and sand were placed in a pre-weighed sterile 50 ml polypropylene centrifuge tubes. Tubes were re-weighed to obtain true sample weights (75 - 87 g). These samples were considered to represent of the homogenized core section corresponding to individual locations within the field site.

82
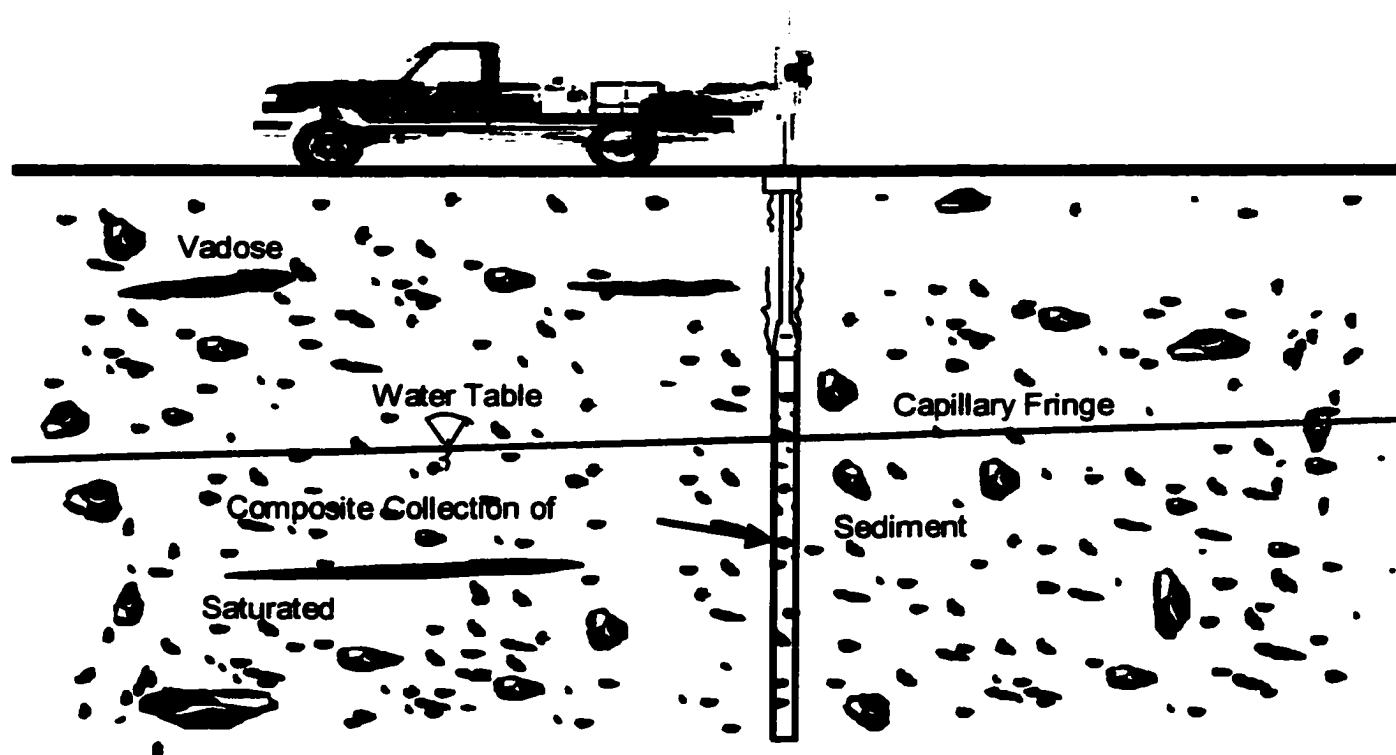
Figure 3. Depiction of the GEOPROBE™ unit extracting sediment from field site.

Virus were extracted from core sections by a modified version of the Berg method

(Hurst, 1997). Briefly, 50 ml of 5% beef extract solution (50 g beef extract, 6.7 g

$Na_2HPO_4 * 7H_2O$ and 0.6 g citric acid) was added to each solid phase sample (Wt/Vol

ranged from 57 to 68%). The mixture was shaken on high for 30 min on a tabletop-

oscillating shaker (Eberbach Corp. Ann Arbor, MI) to thoroughly suspend the virus. The

virus suspensions were then subjected to centrifugation for 20 min of 8000 x g at 4° C to

remove sediment debris. Ten ml of cleared virus suspension was mixed with 10 ml of 1X

tryptone-yeast broth (TYB) (1% w/v tryptone, 0.1% w/v yeast, 1% w/v dextrose, 0.022%

w/v CaCl$_2$ and 0.8% w/v NaCl) and assayed following the single-agar layer technique as described by DeBorde et al. (1998 a).

Background levels of MS2: Both aqueous and solid phase background levels of MS2 were assayed prior to injection to determine the level of resident MS2 within the study site. The installation of the multi-level wells (F1-F6) provided cores and coring sediment for determination of solid phase background virus levels. Levels of MS2 in all core sections assayed were determined to be below the detection limit (0.1 PFU/ml). Upon completion of their installation, these same wells were assayed for aqueous phase MS2 and all wells were below detection limits at every sampling port. To insure that there was no detectable background MS2, a complete sampling was conducted just prior to the slug injection. These samples were also all below detection limits.

MS2 inactivation: MS2 survival was determined for both laboratory and field setting temperatures. The rate of laboratory inactivation was tested by assaying a stock of MS2 diluted in site groundwater held at 4^0 C. The temperature field samples were maintained at prior to assays. Previously, the rate of field inactivation was determined by holding field injectate (virus in site groundwater) at temperatures that were equivalent to the site groundwater (DeBorde et al., 1998a). This was accomplished by suspending an Oakridge Tube (Nalgene, Nalge Nunc International, Rochester, NY) containing the groundwater/virus solution below the water table in a well located at the Frenchtown High School field site. Samples were periodically taken and brought back to the laboratory where they were assayed. The results of this control experiment can be seen in Figure 4. The graph displays a natural log transformation of the change in MS2 titer (as infective particles) as a function of time.
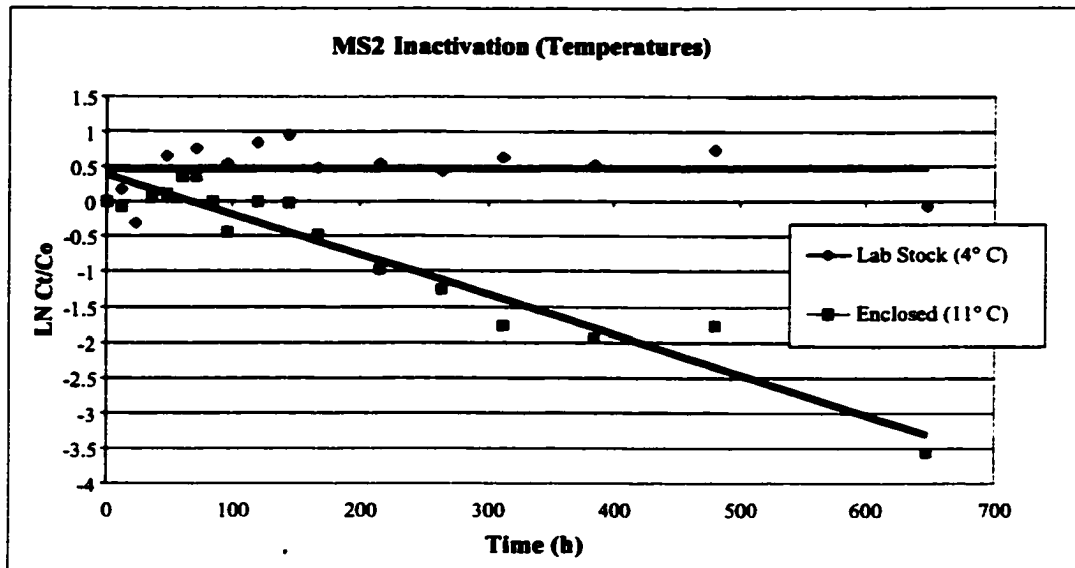
Figure 4. Plot of temperature dependent inactivation rates determined for MS2 under laboratory storage conditions (4° C) and at field conditions (11°). The graph displays a natural log transformation of the change in MS2 titer as a function of time.
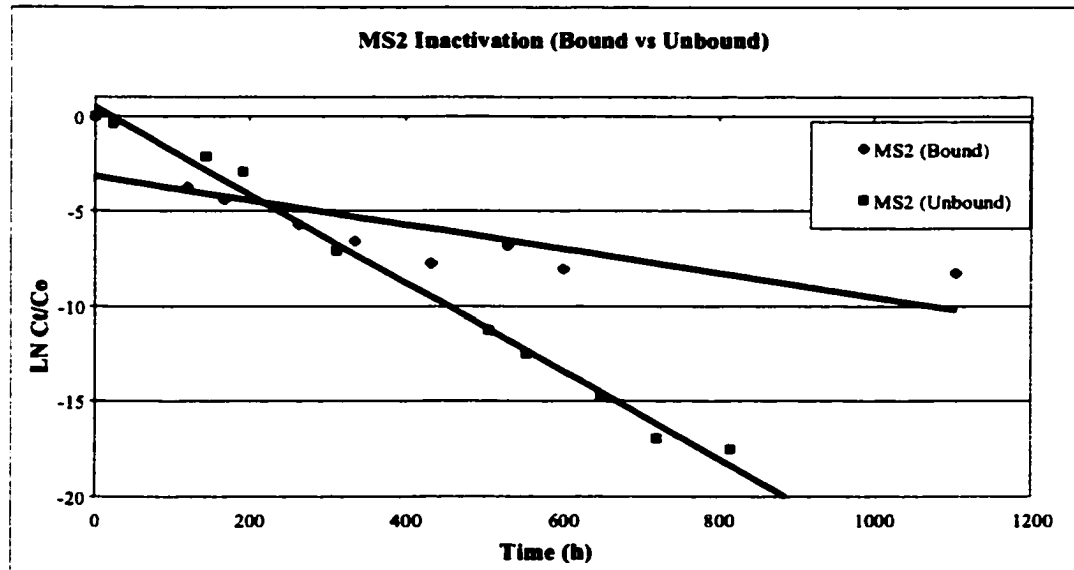
Figure 5. Plot of the inactivation rates of bound and unbound MS2 at a temperature range of 12° to 17° C. The graph displays a natural log transformation of the change in MS2 titer as a function of time.

85

To compare inactivation between bound and unbound virus, a groundwater solution of known titer was incubated with saturated sediment. The viral solution and solution/sediment slurry were then incubated at approximately 15° C and assayed during the next 46 days (Fig. 5). Figure 5 also represents a natural log transformation of change in MS2 titer as a function of time. The objective of the experiment was to determine rates of inactivation based on virus titer end points. The nature of the experiment does not allow for absolute quantification of bound virus, however the goal of the experiment did not necessitate this.

Typically inactivation rates are expressed in terms of (hours^{-1}) based on the following rate equation:

$$C_t = C_o * e^{-Kt}$$

where C_t is virus concentration at time t, C_o is initial concentration and K is the inactivation rate. The inactivation rate was determined for each of the experiments described above and the data are presented in Table 4.

Virus elution efficiency: Extraction efficiency from sediment was determined by a series of experiments in which a known amount of virus diluted in site groundwater was added to homogenized sub-samples of background cores. To allow for adsorption, this mixture was incubated at 4° C for approximately one hour. The inoculated sediment samples were then subjected to the solid phase extraction procedure described above. An average extraction efficiency of 73% (+/- 7.7%) was determined by averaging the results of the six trials. In addition, a control experiment determined viral inactivation during shaking in the beef extract extraction buffer to be 17%, or 83% survival rate. Thompson et al. (1998) have shown that MS2 inactivation during batch sorption experiments

86

occurred during agitation and correlated it to forces associated with air-water/air-solid interface on polypropylene surfaces. The combined effects of these two factors were considered in the final determination of MS2 mass contained in the solid phase.

Site sorption: To determine whether site sediment and/or groundwater may have an effect on virus adsorption, an additional control experiment was performed. Small columns were constructed which contained sand-sized sediment from either Frenchtown High School or the Erskine fishing access sites (DeBorde et al., 1998a,b, 1999). Triplicate columns contained equal amounts of coarse sands and fine gravel representing sediments typical of the sites and in contact with the viruses. After the columns were conditioned with their respective site groundwater 24 to 36 hours at 4° C, MS2 diluted in the relevant site groundwater was applied to the top of the column in 0.5 pore volume and allowed to flow through at equal rates (calibrated by drops collected per unit time interval). Groundwater was then applied continuously in 1 pore volume increments until six fractions containing a total volume of approximately 70 ml were collected. Each fraction was analyzed by the single layer plaque assay and total PFU/volume was determined. Total PFU was determined by summing the fractions. The percent MS2 unabsorbed was determined and is displayed graphically in Fig. 6a.

At the conclusion of the sorption assays, four of the six columns were analyzed to ensure that preferential flow was not responsible for the differences in sorption. The experimental design was similar to that of the virus assay except that bromide was used as a conservative tracer, less volume was used, and a greater number of fractions were collected. To accomplish this, bromide (50 ppm) contained in 0.5 pore volume was flushed through the column and chased by site groundwater. 2 ml fractions were collected

87

and analyzed using a bromide probe (Cole-Parmer, Vernon Hills, IL). The millivolt

readings obtained were converted to bromide concentrations by log transformation

regression analysis based on standardized bromide concentrations. The results, shown in

Fig. 6b, are C/Co bromide values versus fraction number and indicate that the bromide

tracer behaved similarly during transport through the columns. These data would suggest

that MS2 was not transported under preferential flow conditions through these columns.

Percent MS2 Unadsorbed

Bromide C/Co
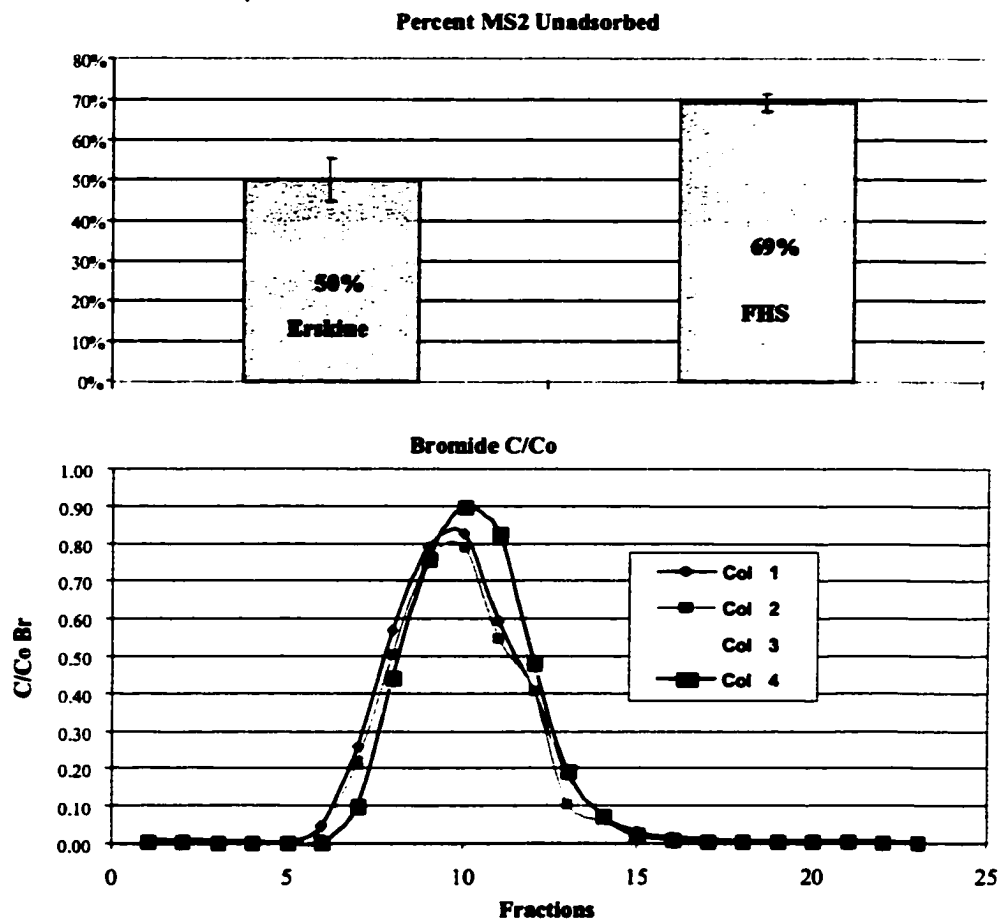
Figure 6. 6A - Bar graph comparing the percent of unabsorbed MS2 from small-scale columns using site specific sediment and groundwater (Erskine vs Frenchtown High School). 6B – Graph comparing the behavior of bromide during transport through columns 1 through 4 used in experiment 6A.

88

Column construction: Both the inactivation and sorption experiments were performed in 60 CC syringe (Becton Dickinson, Franklin NJ) columns. To construct the columns, the bottom of the syringes were fitted with fine polypropylene mesh, to hold in sediment material, and PVC stopcocks (Cole-Parmer, Vernon Hills, IL) to control flow rates. The syringes and mesh were sterilized by autoclaving prior to use. Each stopcock was assumed to be sterile based on lack of contamination in pre-flush fractions from the column. The column structure is shown schematically in Fig. 7.

Syringe Column

Site specific groundwater

Site specific sediment

Polypropylene mesh

Stopcock
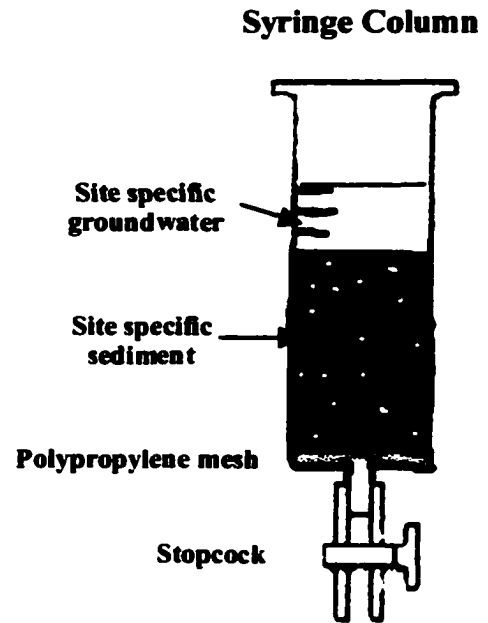
Figure 7. Schematic representation of small column construction for sorption studies of sediments.

Data analysis:

Mass Balance Calculations:

(i) Aqueous phase: To calculate the contribution to the total mass from the aqueous phase, samples collected at EW1 were assayed and the data integrated over the 48 h time course of the experiment. Data were integrated by averaging the sum of the PFU/ml between consecutive sampling time intervals, multiplying this value by the average rate of groundwater pumped between the intervals, and then multiplying this product by the actual time between sampling intervals.

(ii) Solid phase: To calculate the contribution to the total mass balance from the solid phase, the viral plume shape was estimated based on the results of multi-level well samples (defining the plume edges and depth) and modeling of the capture zone of the pumping well. Virus mass values were based on the results of extractions from cores taken along the centerline of the plume. Eluted virus concentrations were converted to numbers of virus per gram sediment, then differentiated into intervals representing the plume area associated with the location of each core sample. Sediment volumes were then computed by multiplying the area by the identified plume thickness. This number was in turn multiplied by $1.5g/cm^3$, the average density of unconsolidated sediment (Fetter, 1994), to provide the total grams of sediment contained in each respective flow-field sector. Finally an estimate of the total viral mass contained in this volume was obtained by multiplying the virus/gram value for the appropriate core sample by the mass of the interval. The total quantity of virus attached to sediment was determined by summing the values for all intervals within each sampling area.

Additional Calculations:

Mattess and Pekdeger (1981) suggested that it was appropriate to employ filtration theory to describe the removal of colloidal particles during viral transport. Harvey and Garabedian (1991) extended this by proposal incorporating a colloidal filtration model into a simple, one-dimensional transport model describing viral transport in subsurface environments. Colloidal filtration allows particle removal by irreversible attachment to be incorporated into a transport model.

90

(i) Relative Breakthrough (RB) and Relative Attenuation (RA): Relative virus breakthrough and relative virus attenuation were calculated for the monitoring well F3-3.4 (1 m from the injection point) using the procedure of Harvey and Garabedian (1991). RB is a measurement that compares the ratio of source virus concentration and measured values to those of bromide at the same points.

$$RB = \int_{t_0}^{t_f} \frac{C_t}{C_0}\, dt \,/\, \int_{t_0}^{t_f} \frac{Br_t}{Br_0}\, dt \qquad (1)$$

Where C_0 is the initial concentration of virus (PFU/ml)
Br_0 is the initial concentration of bromide (mg/L)
C_t virus concentration at wells at time t after injection
Br_t bromide concentration at wells at time t after injection
t_0 time representing the beginning point of the breakthrough curve
t_f time representing the ending point of the breakthrough curve

Essentially one integrates the area under the breakthrough curve for both virus and bromide. The value derived for RB may then be converted to a percentage (RB x 100) and subtracted from 100 to establish a new precentage value designated as relative attenuation (RA).

(ii) Collision efficiency: An additional parameter that can be calculated is the collision efficiency, α (Harvey and Garabedian, 1991; Pieper et al, 1997). In porous media, α is the ratio of collisions resulting in attachment to the total collisions between virus and aquifer grains. α is thought to be an indicator of the intersurface forces (controlled by physicochemical factors) experienced by the virus as they contact aquifer grains and may be a useful expression that allows for virus attenuation to be compared between sites with varying hydrogeological properties. α values are calculated by the following set of equations:

91

$$\alpha = d[(1 - 2\ (\alpha_L/x)\ \ln\ (RB)^2) - 1] / 6(1-\theta)\eta\ \alpha_L \quad (2)$$

where d is the average grain diameter (mm)
        α_L is longitudinal dispersivity (m)
        x is transport distance (m)
        RB is relative breakthrough as described before
        θ is the porosity
        η is the single collector efficiency (for Brownian diffusion contribution)

$$\eta = 0.93A_s^{1/3}(k_B T/\mu d_p dv)^{2/3} \quad (3)$$

where k_B is the Boltzmann constant $(1.38 \times 10^{-23}\ J/mol \times K)$
        T is the absolute temperature
        μ is the dynamic viscosity of the fluid $(1.139 \times 10^{-3}\ kg/m \times s)$
        d_p is the particle (virus) diameter (m)
        d is the average grain size
        v is the fluid velocity
        A_s is the Happel sphere-in-cell model correction factor

$$A_s = (1 - \varepsilon^5) / (1 - 1.5\varepsilon + 1.5\varepsilon^5 - \varepsilon^5) \quad (4)$$

where $\varepsilon = (1 - \theta)^{1/3}$

The single collector efficiency term, η is the rate at which particles strike a single porous media grain divided by the rate at which particles move toward the grain. It represents the physical factors that determine particle collisions. The variables used for this calculation were obtained or derived from data at F3-3.4 and are given in Table 2.

(iii) Virus Transport Behavior: Behavior of the virus during transport was examined at the monitoring well F3-3.4. Plots of the normalized difference in the bromide and virus concentration ratios relative to the bromide ratio versus time were generated using the following quation:

$$A = (Br_{C/Co} - Virus_{C/Co})/\ Br_{C/Co} \quad (5)$$

where $Br_{C/Co}$ is the normalized bromide concentration at time t
        $Virus_{C/Co}$ is the normalized virus concentration at t
        A is dimensionless and ≤ 1.0.

Plots of A versus time are positive when the C/Co value of bromide is greater than the

C/C_0 value of the virus.

Table 2. Parameters used to calculate collision efficiencies (α) during transport a
Frenchtown High School.

Parameters	Values	Comment
Velocity	0.15 m/h	Measured by virus peak arrival at F3
*Average Grain Diameter	0.0024 m	Sieve analysis-measurement reflecting the sand and gravel fraction
	0.00014 m	Sieve analysis-measurement reflecting the finer sand fraction
Longitudinal Dispersivity	0.003	Obtained via type curve fitting Using a Peclet number of 40
Distance	1 m	Distance between IW1 and F3
**Porosity	0.25	Determined by estimation based on sediment type and consistency
Relative Breakthrough	0.50	Calculated (see materials and methods)
Relative Attenuation	0.50	Calculated (see materials and methods)
Collision Efficiency (α)	0.035	Calculated using parameters form this table with .0024 m grain dia.
	0.0003	Calculated using parameters form this table with .00014 m grain dia.

* and ** were determined in a previous study at this site (DeBorde 1998)

Results:

Bromide was injected as a slug at the water table in IW1 at the Frenchtown High

School field site on June 23, 1998. A follow-up Br tracer test was conducted on July 7,

1998 between monitoring well F6 and the pumping well EW1 to determine a dilution

constant occurring at EW1. The duration of the sampling for this test was 70 minutes.

After the completion of the bromide dilution test, bacteriophage MS2 was injected as a

slug at IW1. Groundwater samples were collected over the next 48 h from F3-4, F6-3.2

and the pumping well EW1. Samples were taken less frequently from selected ports of

wells F1, F2, F4 and F5, and all ports of all wells at the 48 h time point. On July 9 and

10, 1998 saturated sediment cores and sediment material were extracted between the

93

pumping well, EW1, and the injection well IW1 (refer to Fig. 1). Coring was successfully completed to a depth of approximately 1.5 m below the water table.

Breakthrough Curves: Breakthrough curves were constructed using data from wells F3 and F6 at 3.4 m and 3.20 m depths intervals respectively (Fig. 8A and 8B). Preliminary bromide tracer tests revealed that these wells and depths exhibited the greatest mass passing through them. In addition, a breakthrough curve was also constructed for the samples collected at well EW1 (Figure 8C). The viral breakthrough curves for wells F3 and F6 are typical of MS2 breakthrough curves observed in other field experiments (Ball et al., 1999, DeBorde et al., 1998a,b). The breakthrough curves exhibit a steep leading edge and a long trailing tail. The peak MS2 mass arrived at F3 at 6 h with a concentration of 1.02×10^9 PFU/ml (C/C_o of .016). Peak MS2 mass arrived at F6 at 24 h with a concentration of 1.30×10^9 PFU/ml (C/C_o of .021). In contrast to these wells, the EW1 breakthrough curve geometry was different, exhibiting two distinct peaks (at 4 h and 20 h). The 4 h peak mass was 2.12×10^4 PFU/ml (C/C_o of 3.36E-07) and the 20 h peak mass was 1.85×10^6 PFU/ml (C/C_o of 2.92E-05). The shape of the second and larger peak had fairly typical geometry in that the leading edge is quite steep and the trailing edge is long and flat.

It appears that the MS2 peak arrived slightly ahead of the bromide peak at F3-3.4 (Fig.8A). Such behavior has been reported in previous field experiments (Rossi et al., 1994, DeBorde et al., 1998 a & b). The peak concentration of MS2 arrived at 6 h, while the bromide had concentration peaks at 6.5 and 7 h. In contrast, the simulated bromide curve for F6-3.20 (generated from data obtained at F6-3.4 and the dilution test conducted between F6 and EW1) indicates that the bromide peak may have arrived prior

94
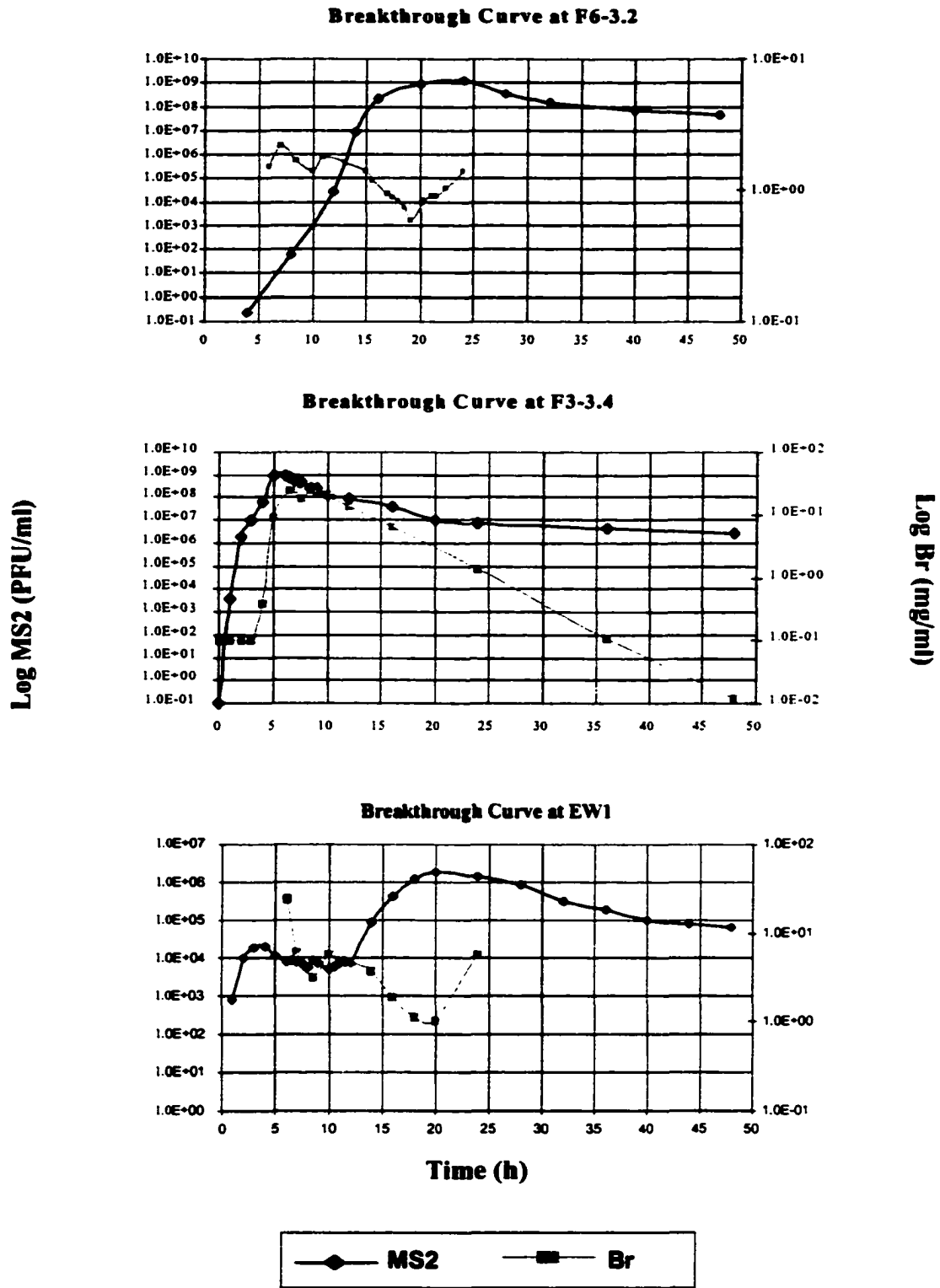
Figure 8. Breakhrough curves for bacteriophage MS2 and bromide: A) F3 – 3.4 m, B) F6 – 3.2 m and C) EW1. Note that the scale on the right is for bromide and that on the left is for MS2.

95

to the MS2 peak. It also appears that there is the beginning of a second peak between 18 and 24 h. Unfortunately the bromide tracer tests did not extend beyond 24 h, so the data for the second peak are incomplete. As for EW1, the data set was incomplete and again is simulated from bromide data derived from F6. The first sampling point at EW1 (6 h) contained the greatest concentration of bromide. However, there is no way to determine the actual relative position of the peak, nor can the magnitude be determined from the incomplete data set. The last sampling point at EW1 (24 h) indicates that a second peak may be starting. If that is the case, this bi-phasic curve is similar to the MS2 breakthrough curve that clearly has two distinct peaks. The second and most significant peak of MS2 arrived prior to that of the bromide (Fig. 8C).

Relative Breakthrough (RB), Relative Attenuation (RA) and Collision Efficiency (α): Due to the incomplete bromide data obtained from F6 and EW1, RB and RA were only calculated at F3.4. For MS2, at F3-3.4, the RB was 51% and the RA was 49%. The latter value is a comparison of the time-integrated mass of MS2 relative to that of bromide (Harvey et al., 1989).

In addition, the RB calculation was used to calculate the collision efficiency (α) at F3-3.4. For F3-3.4 α values ranged from 0.0003 to 0.035.

Viral Transport Behavior: The viral transport process was examined by plotting the normalized difference in C/Co ratios (Equation 5) verses time at F3-3.4 (Fig. 9). The negative values at the 4 h and 5 h sampling points indicate that the C/Co value for the viruses is greater than that for bromide. Bromide was below detection limits for sampling times prior to this, even though MS2 was detectable 3 h prior. After the 5 h sampling point, normalized ratios were positive values and remained positive for the remainder of

96

the sampling times. Interestingly, the ratios reached a fairly static level (0.77) by 8.5 h, and then fluctuated only slightly through the remaining time periods, gradually increasing to 0.88 (Fig.9). These positive values indicate that the C/Co values for the virus were less than for bromide indicated the possible occurrence of preferential flow.
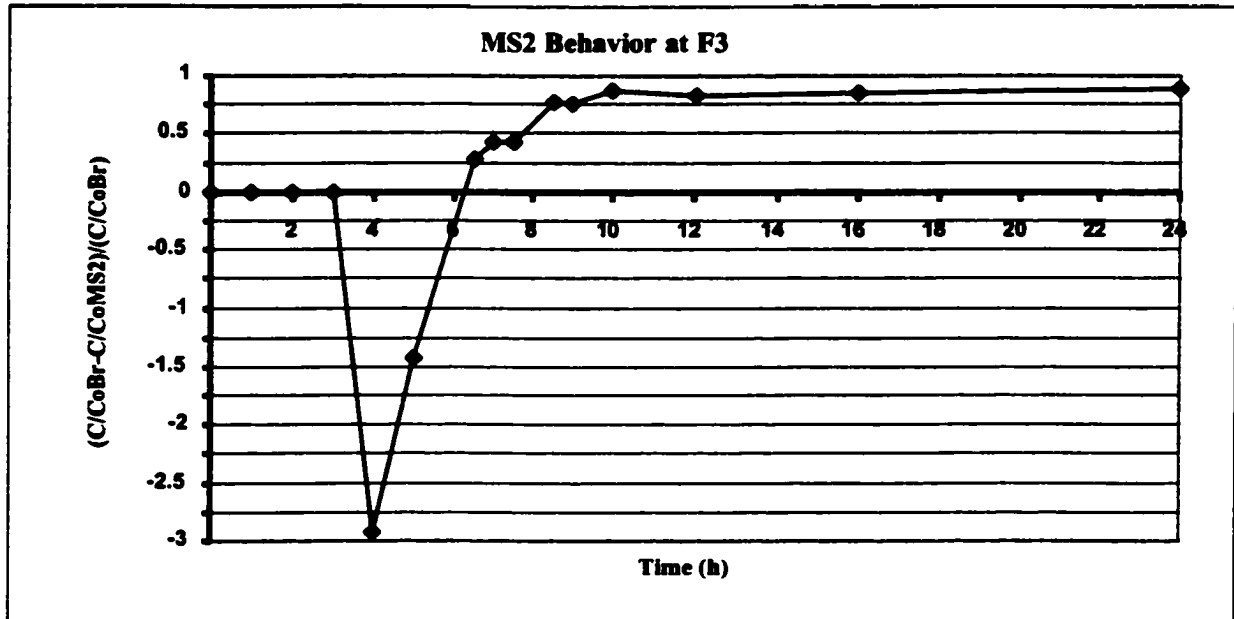
Figure 9. Plot of the differences in the C/Co ratio for bromide and MS2 normalized to the C/Co ratio for bromide plotted at F3 for the first 24 hours of the experiment.

Mass Balance (Aqueous Phase): Data from groundwater samples collected at EW1 were integrated over the 48 h time course of experiment. This integrated value revealed that 45.3% (5.74 X 10^{13} total PFU) of the MS2 injected at IW1 was recovered in groundwater samples drawn from EW1 (Fig. 10). The C/C$_o$ value for MS2 at the 48 h sampling time period was greater than all of the C/C$_o$ values prior to the 14 h sampling period. This suggests that a significant amount of viral mass remained within the aqueous portion of the system after the 48 h period. Although this phenomenon is difficult to envision based on Figure 10, it is much clearer in Figure 8C which shows the tail of the

97

breakthrough curve at EW1 at 48 h is only approximately 1.5 log units below its peak

value at 20 h (6.46 x 10^4 vs. 1.85 x 10^6). Using the simulated data for Br collected at

EW1, an apparent total of < 5% of the injected bromide was captured during the 24 h

bromide tracer test conducted compared to the first 24 h of the MS2 injection test in

which about 26% of the injected mass was collected.
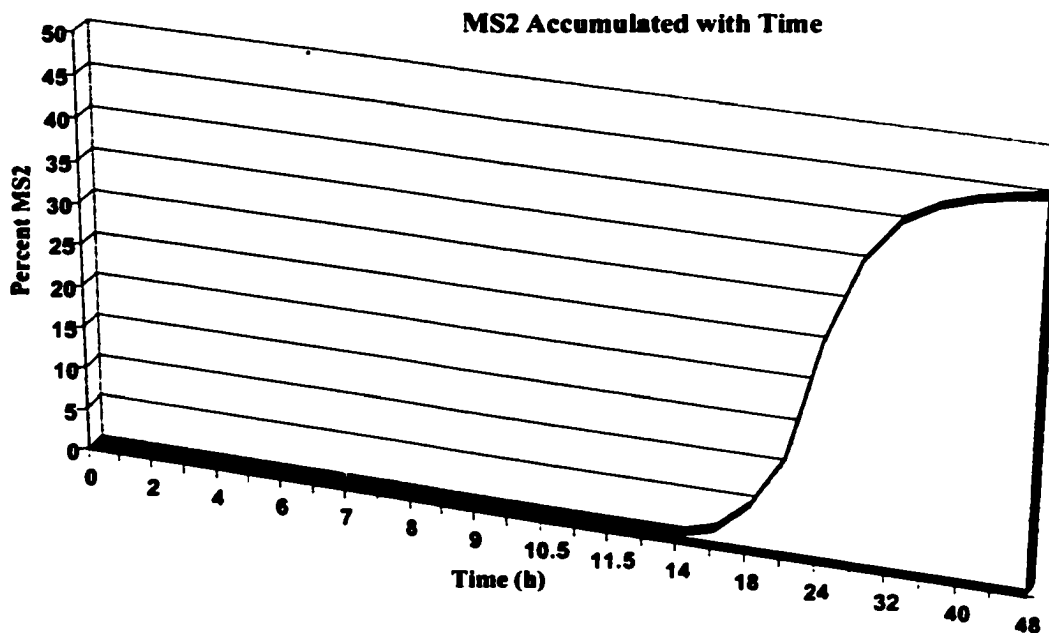
Figure 10. Aqueous phase samples collected at EW1 and integrated over the 48 hour
time course of experiment. Analysis revealed that 45.3% (5.74 X 10^{13} total PFU) of the
MS2 injected at IW1 was recovered in the groundwater samples drawn from EW1 during
the pumping test.

The 48 h "snapshot" sampling of the entire flowfield for MS2 allowed for

analysis of the distribution of bacteriophage. Figure 11 provides a graphic representation

of MS2 distribution in the multi-level sampling wells. From this figure, it is clear that the

majority of MS2 was dispersed throughout the system in a fairly even fashion, from IW

through F3, F6 and finally into EW1 in both the horizontal and vertical planes.

98
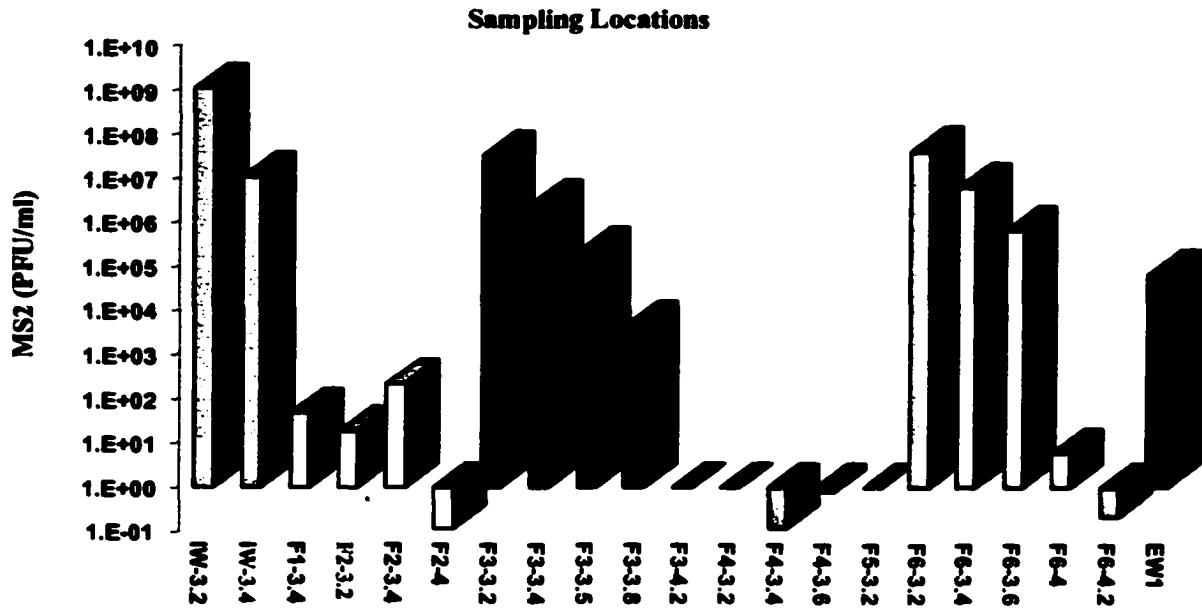
Figure 11. 48 h "snapshot" sampling of MS2 in the multi-level monitoring wells illustrating the distribution of bacteriophage throughout the flow-field (refer to Fig. 1)

Mass Balance (Solid Phase): Analysis of the cores extracted from the field in the approximate center of the path of the viral plume, indicated that approximately 5.5% (7.0 X 10^{12} total PFU) of the input virus mass was attached to the solid phase within the flow-field system (Fig. 12 and Table 3). The majority (> 97%) of the solid phase viral mass was located within 0.5 m of the injection well (cores 7, 13 and 15) and >78% was located between the 3.4 and 3.5 m depth intervals.

99

Table 3. Relative and total percent of MS2 extracted from Frenchtown High School field-site. Columns and rows correspond to core number and depth intervals respectively (refer to Fig. 1).

Depth (m)	Core								Total MS2	Total %
	15	**13**	**7**	**4**	**2E**	**2CD**	**2AB**	**F6**		
2.7 - 2.9	5.11E+09	ND	2.43E+11	5.00E+08	ND	ND	ND	ND	2.48E+11	0.20
2.9 - 3.1	2.11E+09	1.31E+09	9.17E+10	7.56E+07	ND	2.53E+08	1.37E+09	1.60E+08	9.70E+10	0.08
3.1 - 3.2	6.42E+10	1.58E+11	7.87E+10	9.27E+07	ND	ND	ND	5.86E+08	3.02E+11	0.24
3.2 - 3.4	2.73E+11	5.36E+11	2.85E+12	2.17E+09	5.71E+10	ND	7.41E+09	7.24E+08	3.73E+12	2.94
3.4 - 3.5	3.71E+11	1.06E+12	2.34E+11	2.99E+10	6.92E+10	3.56E+09	4.88E+09	2.57E+08	1.77E+12	1.39
3.5 - 3.7	6.05E+10	7.06E+11	6.54E+10	2.70E+09	ND	ND	ND	8.66E+07	8.35E+11	0.66
3.7 - 3.8	ND	1.16E+10	ND	3.29E+08	ND	ND	ND	8.92E+07	1.20E+10	0.01
Total MS2	7.76E+11	2.47E+12	3.56E+12	3.57E+10	1.26E+11	3.81E+09	1.37E+10	1.90E+09	7.0 E+12	
Total %	0.61	1.95	2.81	0.03	0.10	0.00	0.01	0.00		5.50

Groundwater flow direction

Depth	%
2.7 - 2.9	0.20%
2.9 - 3.1	0.08%
3.1 - 3.2	0.24%
3.2 - 3.4	2.94%
3.4 - 3.5	1.39%
3.5 - 3.7	0.66%
3.7 +	0.01%
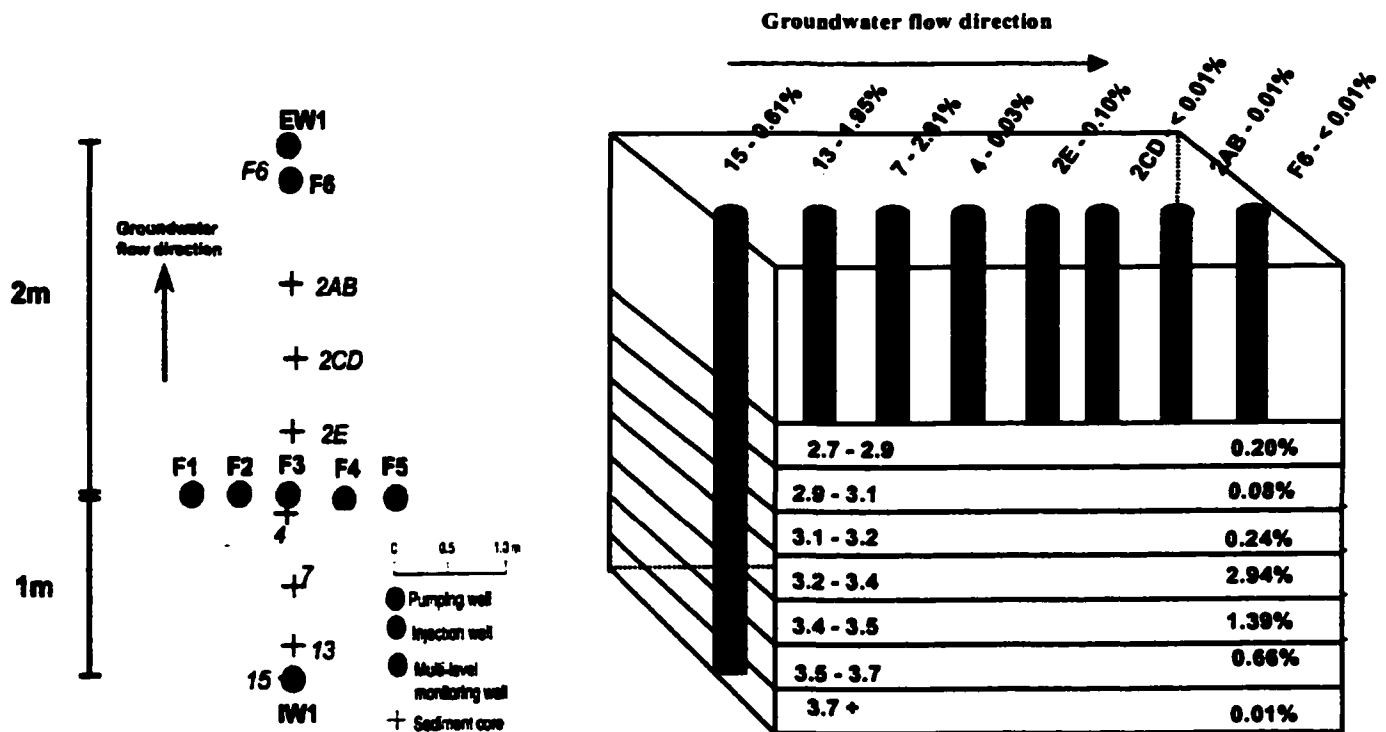
Figure 12. Relative contribution (as % of total) of MS2 from each depth and core interval within the flowfield.

100

Discussion:

The viral injection experiment undertaken at the Frenchtown High School field site was designed to generate a mass balance of the input bacteriophage MS2. To complete this computation, quantification of fate, and the mass of bacteriophage in the aqueous and solid phase must be considered. The portion of the seeded virus collected at the pumping well was 45.3% of the total input virus at the injection well, while the bacteriophage extracted from the sediment sections accounted for 5.5% of the seeded mass.

The total mass contributed by both the aqueous and solid phase was ~ 51% of the original input mass of MS2. This means that approximately 49% of the input mass eluded detection. Since such extensive loss of virus by inactivation is not supported by results of control experiments, the virus are most likely within the interstitial fluids located between the injection point and the pumping well.

Breakthrough Curve Observations: Although the peak virus concentration at F6 arrived 18 h later (24 h) than at F3 (6 h), the viral concentration was 1.27 times greater in F6 (1.02×10^9 vs. 1.30×10^9 PFU/ml). There are a number of possible explanations for this observation. The first, and possibly most logical, explanation is that the peak mass was not captured during any of the scheduled sampling periods at F3. For example, the peak may have passed F3 sometime between hour 5 and 6 or between hour 6 and 6.5. Examination of viral concentrations at hours 5 and 6 indicates that the peak concentration was closer to the 6 h sampling time point.

Another possibility is that the position of the F3 sampling well was not in the center of the plume, but rather located to one side. However, at the 48 h comprehensive sampling F2 and F4, which bracket F3 approximately 30 cm to the East and West,

101

respectively, indicated that the mass at F3 was at least 4 orders of magnitude greater then either F2 or F4 at similar depth intervals (Fig. 11). It is still possible, and in fact likely, that F3 was not exactly in the center of the viral mass.

Alternatively, F3 and F6 were both located in very nearly the same position relative to the center of the observed viral density, then it would appear that the pumping of EW1 might be responsible for this phenomenon. Pumping of EW1 (located approximately 20 cm behind F6) may have concentrated the virus from the up-gradient aqueous phase into a smaller volume of groundwater around F6 as it converged at EW1. However, if this were the case, then the bromide concentrations should have confirmed this observation by showing the same trend. Unfortunately, this can not be confirmed or discounted because of the incomplete bromide data gathered at F6. However, it does appear that bromide may be increasing as indicated by the final sampling time point.

Another possible explanation of the observed behavior is an effect of groundwater flow velocity on virus adsorption. At lower flow rates, the virus have increased chances to contact soil particles and increased contact time with those particles, thus allowing for increased adsorption probability. Lance and Gerba (1980) observed that doubling flow rates through soil columns increased viral breakthrough significantly. Gerba et al. (1981) reported similar findings, showing a negative correlation between flow rate and adsorption. Jin et al. (1997) argued that residence time for ΦX174 and MS2 within a column had a direct effect on the degree of attentuation. Because the groundwater velocity increases in a logarithmic manner as it approaches the extraction well in our system, this remains as a possible explanation for the observed transport behavior.

102

Finally, it is conceivable that the observed behavior is a consequence of preferential flow paths in the system. Perhaps some geological structures provided a more favorable conduit through the aquifer between IW1 and F6 as opposed to IW1 and F3. There may exist a zone (a lense of coarser material) within the aquifer that contains a greater hydraulic conductivity (K) that allows transport to the extraction well to be enhanced. This type of heterogeneous geological distribution is not unprecedented in this formation. Woessner et al. (1998) reported that a zone of preferential flow created an area with increased hydraulic conductivity (6.5 times the average K of the site) that was responsible for a greater concentration of MS2 arriving at a sampler located in a coarse-grained flood plain aquifer. However, this was not verified by observation of sediment material from cores.

Further examination of F3 and F6 breakthrough curves shows differences in tailing behavior. For F3, from the peak at 6 h to 24 h there was an overall 2.2 log reduction or a 1.22×10^{-1} log reduction/hour. For F6, from the peak at 24 h to 48 h, there was only a 1.5 log reduction or a 6.25×10^{-2} log reduction/hour. This trend may indicate that detachment in the vicinity of the extraction well (near F6) may be increased as flow velocity increases. Harton and Wilson (1998), observed periods of increased virus detachment when flow rates were substantially elevated.

Some substantiation of the argument that detachment affects down-gradient virus concentration comes from comparison of behavior between the virus and bromide breakthrough curves at F3 (Fig. 13). During the final 24 hours of the experiment the C/Co value for bromide at F3 was reduced by approximately 2 log units.

103
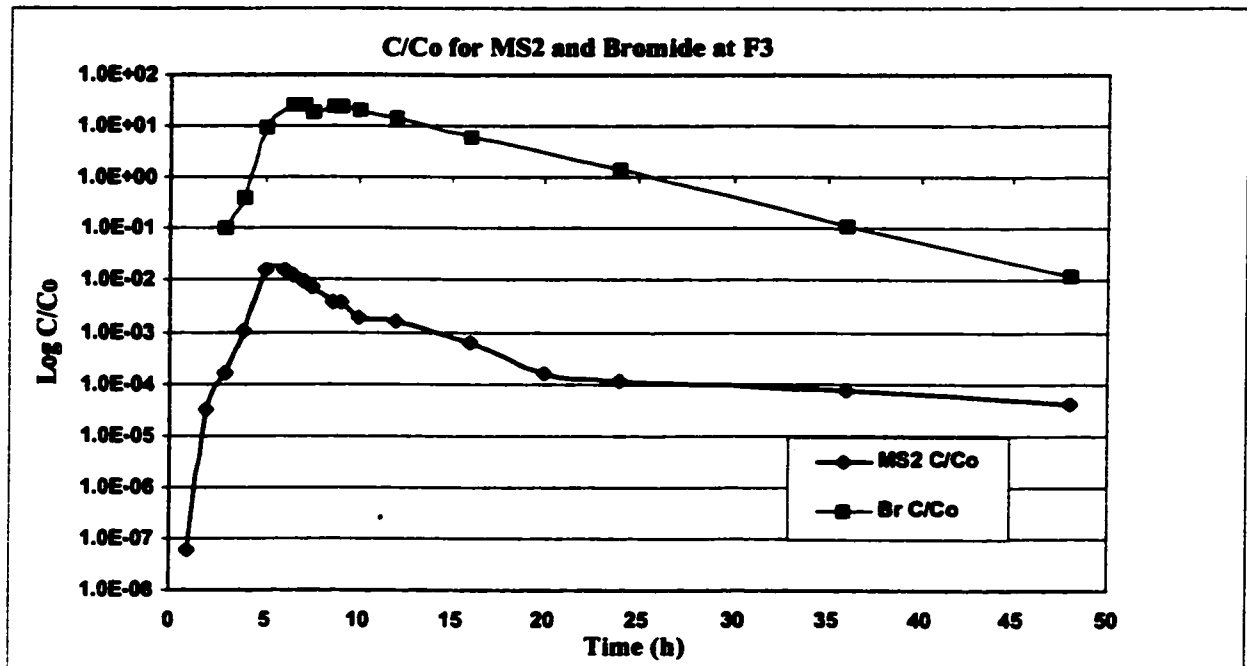
Figure 13. Breakthrough curves (as C/Co) for MS2 and bromide at F3. Note that during the final 24 h C/Co for MS2 decreases by approximately 0.5 log units while bromide decreases by approximately 2 log units.

During this same time period, the C/Co value of virus was reduced by approximately 0.5 log units. If we assume that the loss in the non-reactive bromide is due to dispersion and that the virus would conform to this sort of dispersive behavior, then one would assume that the loss of virus would be equal. That scenario neglects to take into account the adsorptive nature of the virus, which would increase its attenuation. To reconcile this discrepancy, it is possible that detachment of virus from up-gradient locations contributes to the moderate slope of the virus breakthrough curve tail. This implies that the observed differences between the normalized bromide and virus curves may be a result of behaviors inherent in the virus and not occurring in non-reactive tracers, i.e. retardation. Schijven et al. (1999), using a one-site kinetic model to approximate parameter values that included attachment and detachment coefficients, were able to

104

simulate maximum breakthrough values for MS2 from a field experiment adequately.

However, they were unable to use the model to fit estimated data to the trailing edge of

the observed data that showed higher than predicted numbers of viruses. They concluded

that there must be an unknown removal/release process taking place that affected trailing

edge behavior. They also suggested that the attachment process must be reversible.

Peak arrival times for bromide and virus at F3 appeared to differ slightly. For

MS2 the peak arrival time was 6 h, while the bromide peak apparently arrived between

6.5 and 7 h (bromide concentrations were equal at these two times). However, this

phenomenon is not unprecedented as similar findings from field experiments have been

reported by Powelson et al. (1993), Rossi et al. (1994) and DeBorde et al. (1998 a,b).

Bales et al. (1989) using column experiments, calculated that transport rates for

the bacteriophages MS2 and f2 were 1.5 to 2.0 times that of bromide. Kretzschmar et al.

(1999), studying deposition rates in sediment columns, reported significantly faster

breakthrough for latex beads compared to bromide. In both cases, breakthrough rate was

considered a function of a pore exclusion process. However DeBorde et al. (1998 b)

proposed that the high collision efficiencies of virus could result in a truncation of

breakthrough curves. This would shift the peak arrival of the viral breakthrough curve to

the left (earlier in time) producing a peak arrival that is earlier than the non-truncated

bromide plume. While MS2 does not have as high a collision efficiency as poliovirus, its

estimated α value is significant relative to bromide.

Finally, the MS2 breakthrough curve at EW1 was examined in detail. As

mentioned previously, it appears to contain two separate and unequal peaks. The most

logical explanation for this behavior is the existence of a preferential flow path(s)

105

between IW1 and EW1. In this scenario, the mass of MS2 entered the groundwater, some portion was immediately drawn into conduits of higher (preferential) flow. These high flow zones apparently bypassed F6 as indicated by a comparison of MS2 concentrations in F3 and EW1 at similar times (compare Figs. 8A-C). For example, the 8 h sampling reveals that F6 contains 6.15×10^1 pfu/ml while EW1 contains 5.73×10^3 pfu/ml. However, this may simply be a function of the larger screened interval at EW1 collecting over a greater area in the aquifer than the sample ports of F6.

RB, RA and α Observations: The degree of virus attenuation by attachment was examined using the procedure described by Harvey and Garabedian, (1991). At F3 the calculated RB of 51% was very similar to that measured for MS2 by DeBorde et al. (1999) in a natural gradient field experiment at this site. There was a substantial difference in the transport distances between the two studies (1 m and 7.5 m), yet the α values were still quite similar. This may indicate that the majority of the attenuation occurs quickly and within close proximity of the injection site, a hypothesis supported by the solid phase data discussed previously.

The RA value indicates that 49% of the mass was attenuated in this region, presumably by adsorption to aquifer material. This was unexpected considering that the groundwater chemistry in the area is impacted by the septic tank effluent. Both Pieper et al. (1997) and Ryan et al. (1999) found variation in the calculated RB using the bacteriophage PRD1 during transport studies in a sand dominated aquifer containing both uncontaminated and sewage-impacted zones. In both studies the sewage-impacted zone had a significantly higher RB with respect to the uncontaminated zone. While the

106

bacteriophage was different in those experiments, and site chemistry not identical, one would have expected to see the same general trends apply to the current experiments.

The α values measured for MS2 transport at F3 range from 0.0003 to 0.035. The range is a reflection of the two predominant grain diameter values of the sediment; 0.00014 m (finer sand fraction) and 0.0024 m (coarser sands and gravels). This range of values is consistent with those reported from other field transport tests. DeBorde et al. (1999) reported α values ranging from 0.004 to 0.182 for MS2 in a sand and gravel dominated aquifer. Schijven et al. (1998) estimated the α value of F-specific RNA bacteriophages during dune infiltration to be 0.002 for a well located 2 m from the input source. However at a second well location, 4 m from the source, the α value dropped to approximately 0.0007. In the sand-dominated Cape Cod aquifer, Pieper et al. (1997) and Ryan et al. (1999), using PRD1 estimated an α value of 0.013 for the uncontaminated portion and 0.0014 in the contaminated zone. At the same site, Harvey et al. (1989, 1995), studying transport of bacteria and protozoa calculated α value ranges of 0.0054 - 0.0097 and 0.026 - 0.069 respectively. Interestingly, the α values calculated for PRD1 in column studies using Cape Cod sands were much higher (ranging from 0.59 to 0.94) than those reported by both Pieper and Ryan (Kinoshita et al., 1993). The observed differences in calculated α values were attributed to organic matter content of the sediments.

Ryan et al. (1999) suggest that charge environment as a function of pH (zeta potential) on the biocolloid and aquifer grain geochemical heterogeneities are the most important factor when considering α interactions. In a complex environment such as the

flow fields used here there are a number of factors which contribute to these parameters, and determining one controlling factor is unlikely.

Virus Transport Observations: To examine the transport behavior of MS2 at F3, a plot of the normalized difference in bromide and virus concentration ratios relative to bromide concentration versus time were generated using equation 5. The initial negative normalized ratios (at the 4 and 5 h sampling times) indicate that detectable MS2 is reaching F3 prior to detectable bromide (Fig. 9). A similar approach produced comparable results during an earlier field experiment preformed by Ball et al. (1999). It was suspected that pumping conditions caused increased volumes of groundwater to be channeled into the larger pore diameters with liquid velocity being proportional to the square of the pore radius. This created a preferential flow condition that may have led to or exacerbated a pore exclusion process. Under these conditions, the viruses do not interact with a large portion of the sediments, decreasing the adsorption probability (Yates and Yates, 1989). This would also appear to be a valid explanation for the negative values in the current case. After the 5 h sampling time there was a steady increase of values (all positive, indicating C/Co values for virus being less than bromide.) until the 8.5 h sampling where an apparent plateau was encountered (Fig. 9). This behavior may be the result of two concurrent factors: attachment and detachment. The majority of the attachment takes place quickly and in the immediate vicinity of the injection well. At early time periods after injection, dynamic adsorption equilibrium occurs between the virus and sediment (Grant et al., 1993). This is reversible, and equilibrium is established when the rate of adsorption equals the rate of desorption. In a dynamic aquifer system the unabsorbed portion is likely to be transported under the

108

influence of advection, and may be affected by a pore exclusion process. The latter likely accounts for the early peak arrival and the initial negative normalized ratios of virus at downgradient monitoring wells. With time, equilibrium is established with a somewhat steady state of aqueous virus concentration, and these viruses are transported downgradient by advection. This temporal steady state declines over time as the adsorbed virus source is continually diminished. This situation may explain the plateau of the normalized ratios and long tailing in breakthrough that is eventually established. This scenario is also supported by the observation of slow detachment of virus from sediments reported by Schijven et al. (1999).

Virus Partition Analysis Observations: An attempt to quantify viruses in the aqueous and solid phases was undertaken to test the validity of the RB and RA analysis.

Aqueous Phase: The 45% of the input MS2 collected at EW1, in theory, represents the %RB of MS2 as calculated by the method of Harvey et al. (1989, 1991). To test the validity of this computation, one may compare the calculated %RB value with that of the viral mass of injected MS2 recovered from EW1. Previously Ball et al. (1999) performed this analysis on data collected from the Erskine Fishing Access research site and determined that the RB analysis slightly overestimated the true mass of MS2 collected at a pumping well (27% vs. 17%). For the present study, the only valid RB data available are from F3 and not EW1. To extrapolate the data for estimates at EW1 we must make the following assumptions: 1) Since the majority of the adsorption occurs quickly and in the immediate vicinity of the injection point, there is a relatively small difference in the virus mass reaching EW1 compared to F3. Although the peak masses differ by roughly 1000 fold, this difference is likely due to the 1000 fold dilution at EW1

109

due to pumping. Data from solid phase analysis also support this notion. 2) The pumping effect allows for minimal dispersion of the bromide so the mass of bromide at EW1 is comparable to that at F3, disregarding the 1000 fold dilution. 3) The conservative nature of the bromide results in no significant attenuation between F3 and EW1. 4) Prior data from a natural gradient experiment (Ball et al. 1999) suggests that RB declined approximately 3% per meter during transport. This may be a maximum value since dispersion was likely a major cause of this decline under those conditions.

Using 3% per meter reduction, the % RB at EW1 would be approximately 45% (51% at F3 - 6% (2 m travel to EW1)). It would thus appear that the RB of 51% computed for F3 is quite reasonable based on the 45% mass of MS2 that was collected at EW1.

Solid Phase: The MS2 sorbed to the solid phase of the aquifer is, in theory, equal to the RA. We can thus compare directly measured sorbed virus to the calculated RA. We were able to recover 5.5% of the total input MS2 from the solid phase of the aquifer. This is a somewhat unsatisfactory result, considering the calculated RA, and the difference in observed mass indicated that 49% of the total mass of virus remained in the aquifer (presumably adsorbed to sediments). Therefore, a discrepancy of about 43% exists.

There are a few factors that may help explain this outcome. First, there may have been a greater amount of MS2 that remained in the aqueous phase than anticipated. The 48 h sampling interval contributed ~ 1.4% of the total MS2 collected. This extended tailing continues over time as the later sampling periods typically showed little change in slope over time. Further, the solid phase coring required two days to complete, with most core retrieval occurring on day two. By extrapolation based on the predicted slope

110

of the EW1 breakthrough curve tail section, the anticipated aqueous phase total mass would be approximately 49% through an additional 48 h period. Although this is a crude approximation, this mass (presumably originating from an sorbed solid phase source) could have passed through the site. However, this still does not balance the mass.

Another possibility is that viral inactivation may reduce values for both the aqueous and solid phases in our activity based assays. However, evidence from the inactivation control experiments does not support that claim in the context of this experiment. Inactivation rates (-K) were determined by the equation presented in Materials and Methods (Laboratory methods – (ii) controls – survival). Table 4 below displays these for both laboratory and simulated field conditions.

Table 4 – Inactivation rates determined under various conditions. Inactivation rates are expressed as a positive constant value but are calculated as – K values and determined by the equation $C_t = C_o * e^{-Kt}$ for both laboratory and simulated field conditions.

Sample	Experimental Conditions	Inactivation Rate (- K) (hours^{-1})
Lab Stock (4^0 C)	MS2 in groundwater held at 4^{0}C. Test sample storage effects.	8.75 x 10^{-5}
Enclosed (11^0 C)	MS2 in groundwater held at 11^{0}C. Test field inactivation rates.	5.52 x 10^{-3}
MS2 liquid (15^0 C)	MS2 in groundwater held at 15^{0}C. Compare inactivation rates to bound.	2.14 x 10^{-2}
MS2 bound (15^0 C)	MS2 in groundwater and sediment held at 15^{0}C. Compare inactivation rates to unbound.	1.34 x 10^{-2}

Using these K values, it would take over 417 hours (17 days +) at the temperatures in the field to produce a – log reduction of MS2 in the aqueous phase. In addition, it was also apparent that the aqueous phase MS2 was inactivated more quickly than the bound

111

phase. The temperature at which this experiment was conducted is not representative of that in the field but likely represents an overestimation of in-situ inactivation rates.

It is possible that the conditions or method in which the elution control experiments were conducted may not have simulated in-situ processes such as, sorption sufficiently. For example, the hour incubation period may not have been sufficient for complete adsorption resulting in an over estimation of elution efficiency. Loveland et al. (1996) in viral attachment – detachment experiments reported that the majority of attachment occurs within an hour, but that reaching equilibrium may take up to 5 hours.

Another possibility is that viruses were trapped in unconnected pore spaces or dead-end pores. In this situation, the virus would only appear to be sorbed to sediment but in reality are in the aqueous phase. During some phase of the coring procedure these viruses were drained out of the system, essentially lost, and therefore not accounted for by either aqueous or solid phase assays. This situation could be enhanced by physical perturbations occurring during the coring process and core recovery. Coring may have mobilized large numbers of previously sorbed viruses.

It should also be noted that a full solid phase sample set (all depth intervals) was not obtained (see Table 3). However, the missing data are almost exclusively from cores and depths predicted to be less significant in terms of viral mass. We estimate that the missing data would likely account for only an additional 2%.

It is difficult to explain the results from the solid phase analysis. It is likely a combination of the noted factors that resulted in the observed solid phase values. However, the data indicate that a significant portion of the virus mass may remain in the aqueous phase of the aquifer and is not necessarily adsorbed to sediment. This is an area

112

that requires further investigation, both in terms of the techniques employed and in interpretation of results.

Additional Observations

As noted previously, most (97%) of the measured solid phase mass was located within 0.5 m of the injection well. These data support the prior observations of DeBorde et al. (1998b) and Schijven et al. (1999) who determined that relative bacteriophage removal declined with distance from the input source. The data also bolster the hypothesis that viral detachment is the primary process maintaining virus concentrations after the main mass has passed a monitoring point, accounting for the long gradual declining tail typically observed on breakthrough curves (Bales et al. 1995).

That the fact the majority (>78%) of MS2 was found between the 3.20 and 3.50 m depth intervals indicates little vertical dispersion and that the majority of virus remained at the level of injection. It thus seems unlikely that any significant virus mass passed EW1.

There is strong evidence in the literature that sewage impacted groundwater or sediments reduce virus adsorption (Bales et al. 1989,1991,1995, 1997; Kinoshita et al. 1993; Powelson et al. 1993, 1994; Jin et al. 1997; Pieper et al. 1997; Ryan et al. 1999; Schijven et al. 1999). Sobsey et al. (1995) found that viral reduction was greater in sandy soil columns perfused with groundwater then in those perfused with wastewater effluent. They also noted appreciable numbers of virus in effluents from coarse sandy columns. One might predict that coarse sandy and gravel sediments, especially saturated

113

with septic impacted groundwater, may be very inefficient at virus adsorption. The results of our field experiments seem to confirm those predictions.

To further examine the effect of septic effluent small-scale column experiments were conducted (see Materials and Methods – Laboratory methods- (ii) controls- site sorption). Frenchtown High School site sediment was compared to sediment from the Erskine site which has similar sediment textures, but is not under the influence of septic waste. The results showed that the fraction of unabsorbed MS2 for Frenchtown High School sediments was 19% greater than that of Erskine sediments (69% versus 50%). The difference is also reflected in the lower α values calculated for Frenchtown High School, 0.0003, versus the lowest value for Erskine 0.004. This points to the importance of α calculations for specific viruses and sites. Determining α on a case-by-case basis may aid in predicting the degree of attenuation expected for a suite of viruses within a variety of sediment conditions and types.

Conclusions

Our attempts to quantify the partitioning behavior of the bacteriophage MS2 during transport through a large septic system drain field met with mixed results. Overall, it would appear that the RB calculation was quite useful for predicting the mass of injected MS2 that reached the extraction well. On the other hand, due to a combination of factors, validating the RA value by direct observation was less successful. However, this initial and novel effort was valuable for laying the conceptual framework for future

114

experiments to address issues concerning partitioning behavior. Below are some useful recommendations for further experiments:

1) A comprehensive knowledge of the site hydrogeological and chemical properties is required for comparison to other studies.

2) Thorough site characterization attained by conservative tracer experiments is required for appropriate and complete virus (or other) reactive tracer injection experiments (for example; to determine length of experiment and appropriate injection concentrations).

3) For solid phase analysis, recovery of intact cores can be difficult, depending on the matrix composition. An improved method for core recovery would be beneficial. To this end, perhaps prefabricated-screened cores constructed of site material and placed in-situ in predetermined locations in the flow field may provide a viable alternative that would produce more reliable data.

4) Further investigation is required to fully understand what factors (hydrological, physicochemical, viral properties) control attenuation of virus concentrations in aquifer systems.

Abbaszadegan, M., Stewart, P. and LeChevallier, M. 1999. A strategy for detection of viruses in groundwater by PCR. *Appl. Environ. Microbiol.* 65: 444-449.

Alhajjar, B. J., Stramer, S. L., Cliver, D. O. and Harkin, J. M.. 1988. Modeling of biological tracers from septic systems. *Water Res.* 22: 907-915.

Bales, R. C., Gerba, C. P., Grondin, G. H. and Jensen, S. L. 1989. Bacteriophage transport in sandy soil and fractured tuff. *Appl. Environ. Microbiol.* 55: 2061-2067.

Bales, R., Hinkle, S.R., Kroeger, T.W., Stocking, K. and Gerba, C.P. 1991. Bacteriophage adsorption during transport through porous media: chemical perturbations and reversibility. *Environ. Sci. Technol.*, 25: 2088-2095.

Bales, R., Li, S., Maguire, K., Yahya, M., and Gerba, C. 1993. MS2 and poliovirus transport in porous media: hydrophobic effects and chemical perturbations. *Water Resources Res.* 29: 957-963.

Bales, R.C., Li, S., Maguire, K.M., Yahya, M.T., Gerba, C.P. and Harvey, R.W. 1995. Virus and bacteria transport in a sandy aquifer, Cape Cod, MA. *Ground Water.* 33: 653-661.

Bales, R.C., Li, S., Yeh, T., Lenczewski, M.E. and Gerba, C.P. 1997. Bacteriophage and microsphere transport in saturated porous media: forced-gradient experiment at Borden, Ontario. *Water Resources Res.* 33: 639-648.

Ball, P. N., Woessner, W. W., DeBorde, D. C. and Troy, T. 1999. Viral attachment during groundwater transport under field pumping conditions: mass balance approach. Submitted to *Environmental Science and Technology*.

DeBorde, D., Woessner, W., Lauerman, B. and Ball P. 1998a. Virus occurrence and transport in a school septic system and unconfined aquifer. *Groundwater* 36: 825-834.

DeBorde, D., Woessner, W., Lauerman, B., Ball P. 1998b. Coliphage prevalence in high school septic effluent and associated groundwater. *Water Res.* 32: 3781-3785.

DeBorde, D. C., Woessner, W. W., Kiley, Q.T. and P. Ball. 1999. rapid transport of virus in a floodplain aquifer. *Water Res.* Vol. 33. 2229-2238.

Fetter, C.W. 1994. Applied Hydrogeology, Third Edition: Prentice Hall, Upper Saddle River N.J.

Gerba, C.P., Wang, D.S. and Lance, J.C. 1981. Effect of soil permeability on virus removal through soil columns. *Appl. Environ. Microbiol.* Vol. 42, pp. 83-88.

Goyal, S. M. and Gerba, C. P. 1979. Comparative adsorption of human enteroviruses, simian rotavirus, and selected bacteriophages to soils. *Appl. Environ. Microbiol.* 38: 241-247.

Grant, S.B., List, E.J. and Lidstrom, M.E. 1993. Kinetic analysis of virus adsorption and inactivation in batch experiments. *Water Resources Res.* 29: 2067-2085.

Harton, J. and Wilson, G.V. 1998. Influence of flow rate on bacteriophage in weathered and fractured scale. *Water Resources Res.*

Harvey, R. W., George, L.H., Smith, R. L. and LeBlanc, D. R. 1989. Transport of microspheres and indigenous bacteria through a sandy aquifer: results of forced-gradient tracer experjmnts. *Environ. Sci. Technol.* 23: 51-56.

Harvey, R.W., and Garabedian, S.P. 1991. Use of Colloid Filtration Theory in Modeling Movement of Bacteria Through a Contaminated Sandy Aquifer. *Environ. Sci. Technol.* 25: 178-185.

Harvey, R.W., Kinner, N.E., Bunn, A., MacDonald, D., and Metge, D., 1995, Transport behavior of groundwater protozoa and protozoa-sized microspheres in sandy aquifer sediments. *Appl. Environ. Microbiol.*, 61: 209-217.

Hurst, C.J., Gerba, C.P. and Cech, I. 1980. Effects of environmental variables and soil characteristics on virus survival in soil. *Appl. Environ. Microbiol.* 40: 1067-1079.

Hurst, C.J. 1997. Sampling viruses from soil. pp. 400-405. In Manual of Environmental Microbiology, ed. Hurst, C.J., G.R. Knudsen, M.J. McInerney, L.D. Stetzenbach and M.V. Walter. ASM Press, Washington, D.C.

IAWPRC study group on health related water microbiology, bacteriophages as model viruses in water quality control. 1991. *Water Res.* 25: 529-545.

Jansons, J., Edmonds, L.W., Speight, B. and Bucens M.R. 1989a. Movement of viruses after artificial recharge. *Water Research.* 23: 293-299.

Jansons, J., Edmonds, L.W., Speight, B. and Bucens M.R. 1989b. Survival of viruses in groundwater. *Water Research.* 23: 301-306.

Jin, Y., Yates, M.V., Thompson, S.S. and Jury, W. 1997. Sorption of viruses during flow through saturated sand columns. *Environ. Sci. Technol.* 31: 548-555.

Kretzschmar, R., Barmettler, K., Grolimund, D., Yan, Y., Borkovec, M. and Sticher, H. 1997. Experimental determination of colloid deposition rate and efficiencies in natural porous media. *Water Res. Research.* 33: 1129 – 1137.

Kinoshita, T.R., Bales, R.C., Maguire, K.M. and Gerba, C.P. 1993. Effecto of pH on bacteriophage transport through sandy soils. *J. Contam. Hydrol.* 14: 55-70.

Lance, J. C. and Gerba C. P. 1980. Effect of ionic composition of suspending solution on virus adsorption by a soil column. *Appl. Environ. Microbiol.* 47: 484-488.

Lauerman, B.C., MS Thesis, Department of Geology, The University of Montana, Missoula, 1999.

Liew, P. and Gerba, C.P. 1980. Thermostabilization of enteroviruses by estuarine sediment. *Appl. Environ. Microbiol.* 40: 305-308.

Loveland, J., Ryan, J., Amy, G. and Harvey, R. 1996. The reversibility of virus attachment to mineral surfaces. *Colliods Surf. A: Physiochem. Eng Asp.* 107: 205-221.

Macler, B. A. 1995. Developing a National Drinking Water Regulation for Disinfection of Ground water. *GMWR*. Fall. 77-84.

Matthess, G and Pekdeger, A. 1981. Concepts of a survival and transport model of pathogenic bacteria and viruses in groundwater. *Science of Tot. Environ* 21: 149-159.

Moore, B.E., Sagik, B.P. and Malina, J.F. 1975. Viral association with suspended solids. *Water Res.* 9: 197-203.

Noonan, M.J. and McNabb, J.F. 1979. Movement of bacteriophages in groundwater in new zealand. *Ann Mtg. Amer. Soc. Microbiol*, 221.

Pieper, A.P., Ryan, J.N., Harvey, R. W., Amy, G.L., Illangasekare, T.H. and Metge, D.W. 1997. Tranport and recovery of bacteriophage prd1 in a sand and gravel aquifer: effect of sewage-derived organic matter. *Environ. Sci. Technol.* 31: 1163-1170.

Powelson, D.K., Simpson, J.R. and Gerba, C.P. 1990. Virus transport and survival in saturated and unsaturated flow through soil columns. *J. Environ. Qual.* 19: 396-401.

Powelson, D.K., Gerba, C.P. and Yahya, M.T. 1993. Virus transport and removal in wastewater during aquifer recharge. *Water Res.* 27: 583-590.

Powelson, D.K., and Gerba, C.P. 1994. Virus removal from sewage effluent during saturated and unsaturated flow through soil columns. Water Res. 28: 2175 - 2181.

Rossi, P., Carvalho-Dill A. De, Müller I., and Aragno M. 1994. Comparative tracing experiments in a porous aquifer using bacteriophages and fluorescent dye on a test field located at Wilerwald (Switzerland) and simultaneously surveyed in detail on a local scale by radio-magneto-tellury (12-240 kHz), *Environ. Geol.* 23. 192-200.

Ryan, J.N., Elimelech, M., Ard, R.A., Harvey, R.W. and Johnson, P.R. 1999. Bacteriophage prd1 and silica colloid transport and recovery in an iron oxide-coated sand aquifer. *Environ. Sci. Technol.* 33: 63-73.

Schijven, J.F., Hoogenboezem, W. and Stakelbeek, A. 1998. Reduction of FRNA-bacteriophages and faecal indicator bacteria by dune infiltration and estimation of sticking efficiencies. *Water Sci. and Technol.* 38: 127 – 131.

Schijven, J.F., Hoogenboezem, W., Hassanizadeh, S.M. and Peters, J.H. 1999. Modeling removal of bacteriophages ms2 and prd1 by dune recharge at Castricum, Netherlands. *Water Resources Res.* 35: 1101-1111.

Snowdon, J.A., Cliver, D.O. and Converse, J.C. 1989. Inactivation of poliovirus 1, as a function of temperature, in mixed human and dairy animal wastes. *Water Management & Research.* 7: 135-142.

Sobsey, M.D., Hall, R.M. and Hazard, R.L. 1995. Comparative Reductions of Hepatitis A virus, enteroviruses and coliphage ms2 in miniature soil columns. *Water Sci. and Technol.* 31: 203 – 209.

Thompson S. S. and Yates M. V. 1999. Bacteriophage inactivation at the air-water-solid interface in dynamic batch systems . *Appl. Envir. Microbiol.* 65: 1186-1190.

Woessner, W. W., Troy, T., Ball P.N. and DeBorde D. C. 1998. Virus transport in the capture zone of a well penetrating a high hydraulic conductivity aquifer containing a preferential flow zone: challenges to natural disinfection. *Proceeding of Source Water Protection, International 98.*

Yahya, M., L. Galsiomes, C. P. Gerba, and R. C. Bales. 1993. Survival of bacteriophages MS-2 and PRD-1 in ground water. *Water Sci. and Tech.,* 27: 409-412.

Yates, M.V., Gerba, C.P. and Kelley, L.M. 1985. Virus persistence in groundwater. *App. and Env. Microb.* 49: 778-781.

Yates, M.V. and Jury, W.A. 1995. On the use of virus transport modeling for determining regulatory compliance. *J. Environ. Qual.* 25: 1051-1055.

Yates, M.V. and Yates, S.R. 1987, A comparison of geostatistical methods for estimating virus inactivation rates in ground water, *Water Res.* 21: 1119-1125.

Yates, M.V. and Yates, S.R. 1989, Septic tank setback distances: A way to minimize virus contamination of drinking water. *Ground Water.* 27: 202-208.

119

Chapter 6

Concluding Remarks

Why is groundwater so important and why do we study subsurface viral transport? As much as 40% of the water we use in the United States is derived from groundwater sources. Over the last decade, the incidence of water-borne illnesses attributed to groundwater has increased by 50%, bringing the total reported illnesses to more than seven million per year. In addition, groundwater can be difficult and expensive to treat or remediate. Microorganisms such as viruses, protozoa and bacteria are quite different in terms of size, survival mechanisms and transport characteristics. For these reasons, protection of groundwater resources and assessments of pathogen risk demand the study of subsurface microbial transport. Such studies are complex and often necessitate a multidisciplinary approach. The studies presented herein concerning viral transport in the subsurface are important in developing a more complete understanding and provide insights for appropriate strategies for protecting the health and safety of groundwater supplies.

There have been a number of previous studies illustrating the presence of viral pathogens in water supplies, yet our understanding of their movement and fate in subsurface environments remains limited. The majority of the current preceptions comes from laboratory experiments preformed on a limited spatial and temporal scale (e.g., in small columns). To further our current level of understanding, we performed virus injection and recovery experiments to assess the movement and fate of viruses in the subsurface. Some additional and unique qualities of these studies were inherent and

120

others planned including: the nature of the aquifer (coarse, cold and highly conductive); the use of multi-component tracers (bromide, three different bacteriophages and an attenuated human virus); attempting mass balance calculations that quantify partitioning behavior between the aqueous and solid phases.

The work presented in chapter three discribed the results of a multi-virus natural gradient experiment conducted in a coarse-grained cold aquifer. Based on the results of this study, it was concluded that high concentrations of virus are capable of moving with the average velocity of the groundwater and are capable of surpassing commonly used setback distances between source wells and septic systems. Virus adsorption appears to be a significant factor in viral attenuation, however adsorbed virus were capable of subsequent release representing a continuing source of viral contamination (Fig. 1).
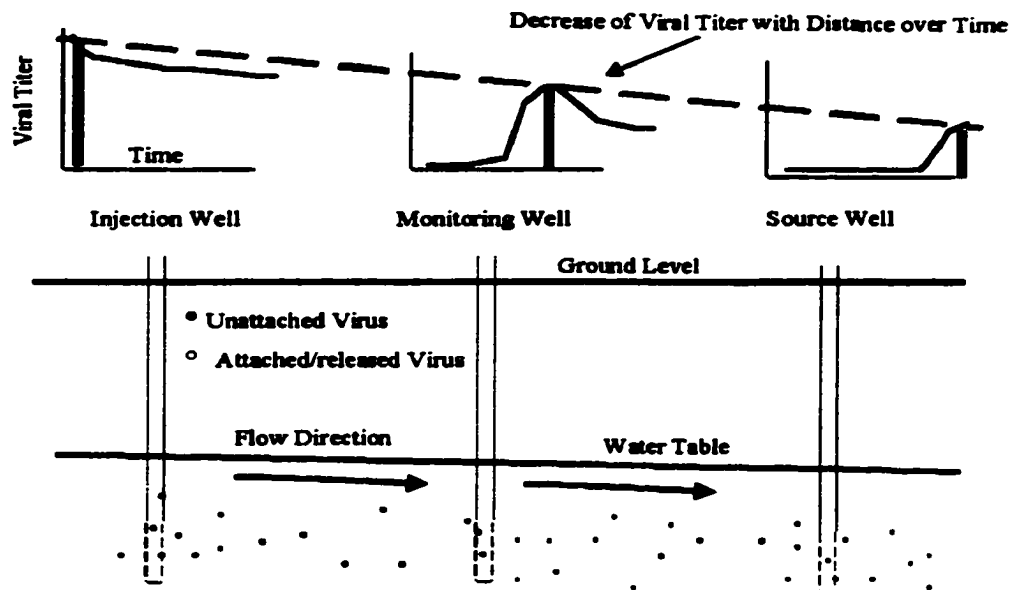
Figure 1. Depiction of virus (breakthrough curves) as they enter groundwater and travel downgradient.

121

This is particularly significant considering that viruses within the portion of the aquifer impacted by the seeding remain viable for more than nine months. It also appears that high collision efficiencies might mask true transport rates of some viruses. This seems to be the case for poliovirus. These initial data provided the framework for follow-up studies presented in Chapters four and five.

Chapter four presented data gathered from the same site at which the natural gradient experiment was conducted. From this study it was determined, through a mass balance experiment performed under a forced gradient, that viral attenuation was most likely affected by inherent viral properties such as isoelectric points and possibly size. That work also suggested that the majority of adsorption occurred rapidly and in close proximity of the injection point. In addition, in this particular hydrogeological setting, the relative breakthrough (RB) calculation did not satisfactorily determine PRD1 and MS2 concentrations arriving at the extraction well. However, RB calculations more closely approximated the observed behavior of ΦX174 and poliovirus. These results suggest that viral properties are an important factor contributing to their fate and transport in subsurface environments and, in particular, affecting adsorption/desorption behavior. This experiment also indicated that pumping conditions might induce a pore exclusion transport phenomenon.

Although it is difficult to make direct comparisons between the natural gradient (M7) and forced gradient (W1) studies because different wells were involved in sampling, some general trends can be examined. Table 1 illustrates the calculated RB for the natural and forced gradient experiments. Table 1 also contains the measured mass recovered during the forced gradient experiment to facilitate comparison of the data. In

122

general, it appears that active pumping significantly increases the mass of bacteriophage arriving at an extraction well. Curiously, the forced gradient appeared to have the opposite effect on poliovirus transport.

Table 1. Comparison of relative breakthrough data between natural and forced gradient experiments

Experiment	Forced Gradient		Natural Gradient
Tracer	% Mass Recovered	%RB	%RB
PRD1	55	80	12
MS2	17	27	15
ØX174	7	10	6
polio	0.12	0.05	0.2

This observation is further supported by the data contained in Fig 2. which illustrates the influence the pumping well has on the peak relative concentrations (C/Co) values of the viruses at approximately 7.5 m from the injection site.
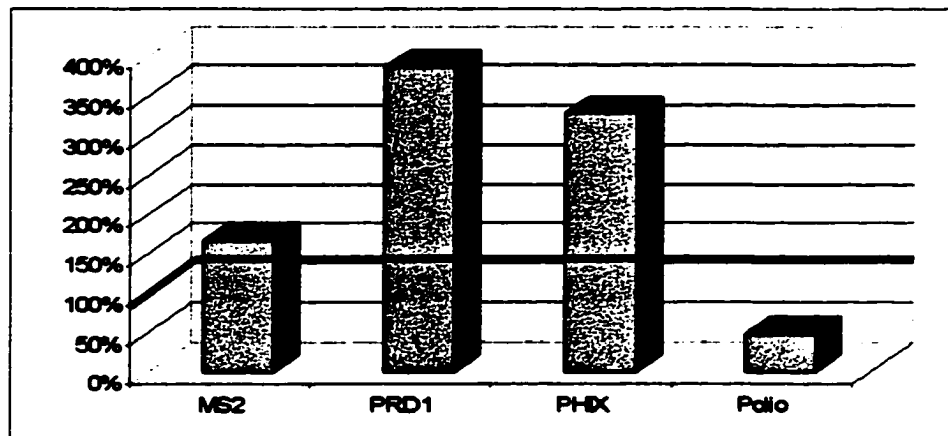
Figure 2. Forced Gradient C/Co / Natural Gradient C/Co of each virus at approximately 7.5 m. At 100% (dark line) the relative concentrations would be equal.

Collectively, for the three bacteriophages, the influence of the forced gradient dramatically increased the peak C/Co values over those of the natural gradient. However, poliovirus exhibited a decreased C/Co value 53% under forced gradient flow compared to the natural gradient by 53%. This result is possibly due to the high collision efficiency (α) of poliovirus which, in turn, can be correlated to its relatively high isoelectric point. Over the same distance, transport rates (m/d) were enhanced under forced gradient conditions by 3 to 4 times for the bacteriophage and approximately 2.5 times for poliovirus as compared to rates under natural gradient conditions. This indicates that, within this particular hydrogeologic setting, the pumping at the extraction well did not increase viral dispersion along the flow path but instead increased transport in a more uniform fashion. In summary, under these experimental conditions the influence of an enhanced gradient: 1) increased aqueous phase concentrations; 2) decreased the log reduction of virus per meter (i.e., attenuation); and 3) decreased time of travel for bacteriophages along the flow path. However this was not true for poliovirus which showed only a decrease in travel time. All indications suggest that inherent viral properties play a key role in subsurface transport behavior.

While we were able to calculate a mass balance for the aqueous portion of the virus during transport, we are aware that a significant portion of the viruses (the sorbed fraction) was accounted for only by inference. Understanding the significance of this adsorbed portion of the viral mass led us to realize the importance of conducting a two-phase mass balance experiment (aqueous and solid phases).

Chapter five presents data from a study in which we attempted to calculate a mass balance by determining virus-partitioning behavior between the aqueous and solid phases

during transport. The results from this combined forced-gradient/coring study allowed us to account for a total of 51% of the input virus concentration. This approach also allowed us to test the validity of the theoretical parameters, RB, RA and α. Although these calculated values were consistent with the measured values of virus concentrations through time, it was assumed that the attenuated portion was adsorbed to the aquifer. However our findings revealed that the missing viral mass may not necessarily be tightly sorbed, instead they are entrained in lower velocity portions of the aquifer or dead end pores or only tenuously adsorbed to the sediments. The coring and solid phase analyses helped confirm what had been suspected regarding the location of the sorbed viruses since the majority (97%) of the virus recovered from the solid phase was within the immediate vicinity (0.5 m) of the injection site.

Policy Implications:

The data collected from our studies on viral transport in the subsurface suggest a need to address the following issues. 1) The use of bacteriophage as indicators of viral contamination may not accurately reflect the behavior of enterioviruses, such as poliovirus. 2) Under similar "worst case" conditions as in cold, highly transmissive subsurface environments set-back distances between potable water wells and potential virus sources must exceed the commonly used 30.5 m. 3) Viruses in aquifers may remain a threat to public health for periods beyond expected due to prolonged periods of slow detachment. 4) Predicting the fate and transport of virus requires detailed data concerning physical and chemical characterization of the aquifer properties. 5) Current theoretical calculations regarding virus breakthrough and attenuation may not be a satisfactory method of determining virus concentrations arriving at groundwater sources. They lack

the proper input parameters that account for attachment and detachment. Log reduction in virus concentrations is site and virus dependent.

Suggested Research:

The results of these studies and others indicate that the complex nature of natural aquifer systems make analysis of viral subsurface transport and fate difficult. However, field studies are necessary for testing theoretical applications and more importantly, observing and measuring real phenomena related to virus transport. With that in mind, the following issues should be addressed.

1) Continued field injection experiments that include mass balance analysis of both aqueous and solid phases with improved coring and viral recovery techniques. 2) Long term in-situ survival experiments using live assays with appropriate virus species should be performed. 3) We must determine the effects of groundwater velocity on viral attenuation. 4) There is a need to develop attachment and detachment rates for viruses that would allow more robust simulation of virus transport in relation to the surface properties of both viruses and sediment.

126

Chapter 7

Assessment of Microbial Community Structure in Landfill Material by Bisbenzimidazole-Cesium Chloride Equilibrium Density Gradients, Denaturing Gradient Gel Electrophoresis (DGGE) and Sequencing of 16S rDNA Genes.

Patrick N Ball and William Holben. Division of Biological Sciences, The University of Montana

Abstract

Assessing the structural complexity of microbial communities in environmental samples is critical to understanding how they function. Detecting and characterizing native microbial species is paramount to this. However, this can be a daunting task considering that some estimates place bacterial density between 10^8 and 10^9 cells per gram of surface soil, possibly representing several thousand different populations (Torsvik et al. 1990a,b). Recognizing the shortcomings of culture based methods, which typically recover only 0.1 – 1.0% of the organisms in a sample, recent years have seen a proliferation of molecular techniques utilized to investigate these questions. In this study we employed and combined three molecular techniques in an attempt to determine the community structure in two samples representing different, but common, sanitary landfill practice, namely direct emplacement, and landfilling of incinerated municipal solid waste (MSW). Using bisbenzimidazole-cesium chloride equilibrium density gradients,

127

total community DNA directly extracted from landfill samples was fractionated based on its %G+C content. To increase the resolution of this coarse examination of total community structure, we employed denaturing gradient gel electrophoresis (DGGE) to examine fractions of the total community. Finally, a more traditional clone and sequence technique was applied to the community DNA fractions in an attempt to identify community members and assess the microbial diversity of these landfill ecosystems.

By using this combination of methods we were able to demonstrate microbial community differences between the two types of landfill samples. More importantly, we were able to identify some pertinent microbial community members, and show the existence of a diverse community structure that may include some species that are unique to or dominant in landfill ecosystems.

128

Introduction

Landfills are important constructed systems for managing societal waste, yet they remain a relatively unexplored complex terrestrial environment. One of the major barriers to the study of landfill environments is their intrinsic heterogeneity (Barlaz 1996a, 1997; Gerba et al. 1992; Palmisano and Barlaz 1996). Carefully planned laboratory studies have shed some light on certain events that occur during organic decomposition. However in-situ studies are most desired for determining the true nature of a landfill's operation (Barlaz et al. 1989). The objective of this study was to investigate the total microbial community structure of two differing landfill ecosystems. To do so, we employed three various approaches to compliment and enhance the investigation. G+C content-based fractionation of the total microbial community DNA provided a broad scale view of the two populations. Coupling this with comprehensive and selective DGGE analysis provided a more complete means of viewing and comparing community structures. Finally, to identify members of the landfill communities, clone libraries were generated from select fractions of the total communities. All three methods indicated that there was a diverse community present. Additionally, sequence analysis suggests the possibility for the existence of some unique organisms.

Municipal solid waste (MSW) typically consists of articles such as wrapping and packaging materials, discarded furniture and appliances, clothing, food

scraps, cans, bottles, newspapers, wood and yard trimmings and clippings (US EPA 1999a). Other more toxic materials and synthetic chemicals may also be included, such as paints, paint thinners and batteries. Data from 1996 show that the U.S. produced more than 209 million tons of MSW, equivalent to roughly 4.3 pounds of waste per person per day. Of that, 57% was buried in landfills (US EPA 1999a). It is likely that this trend will mandate landfilling as a significant means of managing MSW in to the future. This is of particular concern for areas of limited size and space. To ease this crisis, incineration of material prior to burial is considered a viable option. Incineration can reduce the overall amount of combustible material in landfill-bound waste by 90% volume and 60% weight (US EPA 1999a). This option also has potential detrimental consequences, most of which are concerned with air emission quality. These issues are beyond the scope of this investigation. Nevertheless, there is still a need to dispose of post incineration material, which typically consists of fly ash mixed with noncombustible and incompletely combusted materials (US EPA 1999a). Regardless of management strategy, landfills remain the preferred terminal disposal method of MSW.

Although MSW may be diverse in nature, cellulose (a natural polymer composed of repeat units of the monomer glucose) and hemicellulose (a natural polymer composed of several different sugars, mostly pentoses) comprise 45-60% of the dry weight of MSW and represent the major biodegradable constituents of MSW (Barlaz 1996a; 1997; and Gerba et al. 1992). Lignin is

another common organic compound found in landfills and is usually associated

with cellulose possibly as degradation intermediates. Structurally, lignin is a

complex of cross-linked aromatic rings, and under anaerobic conditions is

considered extremely recalcitrant and has been implicated as interfering with

cellulose degradation (Barlaz, 1996a; 1997; and Gerba et al. 1992).

The decomposition of these abundant compounds in landfills usually

culminates in the production of methane (CH_4) and carbon dioxide (CO_2) (Barlaz

et al. 1990). This process can be extremely slow, and depending on conditions,

decomposition rates can be essentially negligible (Barlaz, 1996a,b; 1997).

Although chemical and physical processes can affect decomposition directly, the

process is controlled mainly through microbially mediated interactions. Microbial

degradation involves the biological reduction (in complexity) of chemical

compounds catalyzed and kinetically controlled through enzymatic activities.

There are many additional factors that contribute to this overall process, but our

concern for the purposes of this investigation are the microbial consortia

involved.

There is a paucity of information in the literature on the representative

microbial community members found in landfills. The majority of the available

information comes from culture-based techniques that are typically concerned

with isolating populations of organisms that carry out specific activities such as

cellulose degradation (Westlake et al. 1995), anaerobic or aerobic fermentation

(Palmisano et al. 1993) or anaerobic decomposition (Qian and Barlaz 1996).

Others infer the nature of microbial landfill residents by characterizing

microorganisms in aquifers underlying the landfill (Ludvigsen et al. 1999; Roling

et al. 2000).

More recently a few studies using molecular approaches have attempted

to describe landfill microbial communities. Lloyd-Jones and Lau (1998) cloned

16S rDNA sequences from a landfill site in Quebec to investigate the microbial

diversity of a polycyclic aromatic hydrocarbon (PAH) and polychlorinated biphenyl

(PCB) contaminated environment. Their results indicated that the vast majority

of the cloned sequences (>90%) showed little similarity to known sequences

contained within the Ribosomal Database Program (RDP). Based on the

clustering of the sequences in a phylogenetic tree construct, the majority of the

clones matched taxonomic divisions (Low G+C Gram-positive and

α, δ, γ- Proteobacteria) indicated by a blast search conducted on the sequences

through the National Center for Biotechnology Information (NCBI). In another

study, van Verseveld et. al. (1999) used Biolog plates and denaturing gradient

gel electrophoresis (DGGE) to profile the physiology and members of a Dutch

landfill site under investigation for its in-situ bioremediation potential. The DGGE

pattern clearly showed that different consortia are distributed spatially in the

landfill. Although they did not publish any information regarding the identity of

community members, the DGGE approach they employed provides a convenient

way of monitoring the community as a whole in various locations throughout the

landfill. Further, this investigation claimed to have inventoried approximately 111

132

clone sequences representing, as yet, unidentified organisms. Another recent study by Wise et. al. (1999) used dilution-based culture analysis and DGGE analysis to isolate novel members of methanotrophic bacteria in a former landfill site located in Georgia.

Microbial communities in soils, sediments and other terrestrial environments can be extremely complex (Borneman et al. 1996; Liestack and Stackebrandt 1992; Stackebrandt et al. 1993; Torsvik et al. 1990a,b; Ward et al. 1990). DNA renaturation experiments suggest that there are 10^3-10^4 genome equivalents per gram of soil (Torsvik et al. 1990a), representing possibly up to 10^4 individual species (Torsvik et al. 1990b). Characterizing the phenotypic and genotypic diversity in complex microbial communities has become easier with the emergence of modern molecular techniques in microbial ecology. These techniques offer many advantages over the more traditional methods to assess these parameters (Head et al. 1998; Lane et al. 1985; Olsen et al. 1986; Pace et al. 1986), and have allowed for initial identification of many presumptively new microorganisms (Barns et al. 1994,1999; Dojka et al. 1998,2000; Hugenhultz et al. 1998a,b; Kuske et al. 1997; Liesack and Stackenbrandt 1992; Stackenbrandt et al. 1993; Ward et al. 1990; Pace 1997), as well as many that appear to be cosmopolitan (Kuske et al. 1997).

Perhaps the greatest drawback to the more traditional, culture-based methods such as plate counts, colony morphology analysis and biochemical assays, is their inability to reliably capture all of the microbial types present in

133

any give sample (Amann et al. 1995; Hugenhultz and Pace 1996; Pace et al.

1986). This is capitulated in the notion of viable but unculturable microbes

(Colwell et al. 1985; Roszak and Colwell 1987) and, as a consequence, typically

only 0.1 to 1.0% of all organisms in a given environmental sample are culturable,

leaving >99% unculturable (Staley and Konopka 1985). Further it is suggested

that only 20% of the prokaryotic organisms occurring in nature have been

identified (Muyzer et al. 1993).

A more direct method of assessing microbial numbers is by microscopy,

particularly when used in conjunction with fluorescent dyes, such as 4',6-

Diamidino-2-phenylindole (DAPI) or acridine orange (Hobbie et al. 1977).

Although superior to plate counts, in terms of total number of organisms, these

also have their shortcomings. Since many bacteria share similar physical

attributes, such as size and general morphology the ability to characterize

genetically or metabolically distinct populations is severely limited. Further,

background interference caused by indiscriminant dye binding in many cases can

give erroneous results.

Despite these limitations, the importance of traditional microbiological

methods cannot be overlooked. Based on the establishment and analysis of pure

cultures of microorganisms a systematic scheme based on physical, metabolic

and biochemical analysis of characteristics has been established. Without this

framework of established identities, there would be no anchoring point for

determining or interpreting the importance of DNA or protein sequence

relatedness of newly identified organisms. Indeed, bacterial relatedness, such as indicating that two isolates are similar species, must not only share no less than 70% DNA–DNA homology (97% for 16S rDNA molecules), but also phenotypic and ecological relevance (Stackebrandt and Goebel 1994). Discarding these traditional methodologies and established systematics for the identification of microorganisms in favor of sole reliance on molecular phylogenetic techniques would be a mistake. The traditional methods provide a crucial framework of relevant phenotypic attributes (i.e. activities) rather then relying solely on analysis of sequence divergence to characterize relatedness of microbial populations.

More recent techniques based on 16S rRNA, rRNA genes (rDNA) or functional gene PCR amplification using specific or universal primers are now commonplace in many environmental microbial ecology laboratories. In principle, similar techniques may be applied to a number of genes, usually tailored to answer a particular question. However, for identifying a specific microbial population at least to the genus level, the 16S subunit of the ribosome (16S rRNA) is generally considered the best choice. This gene sequence, which encodes a critical cellular function, has maintained its overall functional and structural integrity throughout millions of years of evolution. For 16S rDNA sequence analysis, the basis for the approach is anchored in both the relative similarities (conserved regions) and slight differences in sequences (variable regions) of the 16S rDNA molecule. Certain sequences within the 16S rDNA are

highly conserved from organism to organism (signature sequences) while other sequences are specific only among distinct groups ("phylogenetic cohorts") of organisms, typically at the domain or division level of taxonomy. However, some short stretches of sequence have been identified as group, genus or species-specific (Amann et al. 1995). Based on the distribution of these conserved and variable regions, PCR primers can be designed to bind the conserved regions and span the variable regions within the 16S rDNA gene. By examining these unique areas (via sequencing analysis or hybridization probes) one can determine the likely identity of the organism or at least determine its closest match to other known organisms. This can best be achieved through software-based matching of similar sequences by way of electronic databases and is only as reliable as the database itself. The existence of extensive databases (e.g. Ribosomal Database Project [RDP]) is another reason why the 16S rDNA gene is a good tool for molecular analysis.

Molecular phylogenetic techniques have made us realize that life can be genetically designated into three primary domains; *Bacteria, Eucarya* and *Archaea* (Woese 1987) as opposed to plants and animals as previously thought. Although these approaches may suffer from their own deficiencies, such as lack of definitive species and sub-species resolution and inability to indicate function and activity, they can nicely demonstrate the enormous microbial diversity existing in terrestrial and other environments. In fact, there are currently 36 to 38 divisions (containing ca. 400 named genera and 4,200 species) recognized

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

within the *Bacteria* domain, representing a three-fold increase over the 12

"relatedness" groups (known as divisions) within the domain *Bacteria* which

Woese described in 1987 (Woese 1987). About one third of these divisions

contain only environmentally derived sequences (i.e. uncultured organisms) and

many of the remaining divisions are only poorly represented by cultured

organisms and are dominated by sequence data alone (Hugenholtz et al. 1998).

It is therefore difficult to predict the breadth of the diversity within these groups.

Besides detection of non-culturable populations, direct molecular

approaches allow for the monitoring of specific populations in a complex

assemblage of microorganisms, and the ability to determine community

responses (of both general and specific community members depending on the

design of the experiment) to environmental changes or perturbations.

While there is no doubt that molecular techniques have made major

contributions to our understanding of microbial ecology, we must remain

cognizant of some precautions when using these methods to describe residents

of environmental habitats. As pointed out by some researchers, uncertainties

related to rRNA gene copy numbers (Cole and Girons, 1994; Farrelly et al. 1995),

sequence domination by select members of a microbial community (Ward et al.

1992), quantitative recovery of nucleic acids from environmental samples

(Holben 1997), PCR conditions (Head et al. 1998), and chimera and heteroduplex

formation (Liesack et al. 1991; Kopczyski et al. 1994; Wang and Wang 1997)

may hamper interpretation and confound results. Further, Hugenholtz et al.

137

(1998) point out that analyzing sequences <500 nucleotides (nt) long may be insufficient for phylogenetic determinations of novel organisms.

In this study we used a multi-tiered approach to examine the variations and complexities in the microbial community structures in two samples. One sample was identified as typical landfill material, and consisted of a mixture of trash and soil recovered from an established landfill in Japan. The other sample, also from Japan, consisted of the residual ash from incinerated trash that was subsequently mixed with soil and buried. Although these samples are different in their composition, they are representative of two common methods for disposing of municipal solid waste (MSW).

Using a combination of contemporary molecular techniques, identified typical members of two different landfill microbial communities. In doing so, we hope that this will serve as a pilot study that may lead to further investigations into the practice of landfilling MSW.

Ultimately an understanding of the ecology, physiology and biochemistry of microorganisms and their influences on microbial processes, is important in determining and enhancing their role in waste decomposition and mineralization. Eventually, this type of information may aid in design and management of landfills. For example, landfill amendments or perturbations that may enhance or inhibit certain microbial activities may be a desirable management strategy. Customized microbial inocula, depending on circumstance, may also prove be a beneficial management tool.

138

Approach: The first method used to examine these landfill microbial communities employs fractionation of total community DNA based on the characteristic percent guanine and cytosine (%G+C) content of microbial populations. Purified total community DNA was fractionated by bisbenzimidazole-cesium chloride equilibrium density gradients as described by Holben and Harris (1995). Essentially, this is accomplished by inducing changes in the buoyant density of DNA based on its %G+C content by interactions with the DNA binding dye bisbenzimidazole. Because DNA from different organisms contains characteristic and different relative amounts of G and C (hence A and T), different molar amounts of bisbenzimidazole (Hoeseht 33258) bind to the adenine and thymidine bases inducing differences in the bouyant density of the DNA which fractionates at distinct locations in cesium chloride equilibrium density gradients. The utility of this approach is based in its comprehensiveness and not necessarily in its level of taxonomic resolution. However, chromosomal %G+C content is characteristic for some phylogenetic groups of prokaryotes at some coarse level of taxonomy, generally at the genus level. This technique generates a profile of the entire microbial community in terms of relative abundance versus %G+C content. The relative abundance of various phylogenetic groups may be estimated by the relative abundance of DNA at some particular %G+C. This is an excellent technique for resolving a highly diverse community into a more manageable level of complexity and thereby allowing previously intractable questions to be addressed.

The %G+C fractionation approach has been successfully used to characterize and monitor microbial communities in a number of environments. Holben and Harris (1995) originally used this methodology to follow changes in soil bacterial communities after carbon and water amendments. Nusslien and Tiedje (1998) examined bacterial community diversity in chronologically young tropical soil. They also employed it as a method to compare bacterial communities in similar soils following changes in vegetative cover (Nusslien and Tiedje 1999). Gsell et al. (1997) used it to examine the seasonal relationship between the sediment bacterial community structure and physicochemical parameters in groundwater upwelling zones within an alkaline fen. Holben et al. (1998) investigated microbial populations in various compartments within a multi-compartment activated sludge system for nitrogen removal. Apajalahti et al. (1998) initially characterized the total bacterial community in the gastrointestinal (GI) tract of chickens and Santo Domingo et al. (1998) recently used the approach to describe how the diet of crickets influences their hindgut bacterial community. This approach also allows one to fractionate total community DNA into components that contain only portions of the community structure. Fractionation of the %G+C profile permits one to selectively investigate areas of particular interest, such as those areas that contain varying biomass quantities in response to a treatment.

A second approach utilized in these experiments is denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993). Using this approach we examined

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the diversity within specific bisbenzimidazole gradient fractions of particular

interest to provide additional resolution of diversity. In addition, we compared

the total community diversity as determined by this method as well. DGGE

analysis operates on the principle that double stranded DNA fragments of similar

length can be separated by polyacrylamide gel electrophoresis through gels cast

with a gradient of denaturant based on sequence differences (Myers et al. 1987;

Muyzer et al. 1993). This is achieved through electrophoresis of PCR ampiified

16S rDNA amplicons in polyacrylamide gels that contain uniformly increasing

amounts of denaturing agents (urea and formamide) run under a constant

elevated temperature (typically 60-65° C). Sequence variation within the

fragments gives rise to randomly dispersed melting domains having different

melting temperatures. It is within these melting domains where a transition from

helical to open strands occurs, impairing the electrophoretic mobility of the DNA

fragment (Muyzer et al. 1993). At some point in the gel gradient, a given

fragment reaches a position where migration dramatically slows or ceases due to

an effective change in the shape of the denatured DNA molecule. Thus 16S rDNA

fragments of differing sequence will, by virtue of their inherent physical

properties, stop moving in the gel at a specific point providing a gel band

characteristic of the organism that encodes that particular sequence. To increase

the resolving power of DGGE, a series of guanine and cytosine residues called a

GC-clamp (generally 30 - 50 nucleotides long) is typically added to the 5′ end of

one of the PCR primers (Sheffield et al. 1989). This GC-rich stretch is a non-

melting region that keeps the two strands from separating completely into two detached strands that would have increased mobility (Myers et al. 1985,1987; sheffield et al. 1989).

DGGE has been used in a variety of applications and environments. Some of the applications include the following. Muyzer et al. 1993 first described the use of DGGE for environmental applications to analyze complex microbial communities in mats and biofilms, and subsequently for examining hydrothermal vent communities (Muyzer et al. 1995). The biodiversity and activity of sulfate-reducing bacteria in stratified water columns from Danish fjords was studied by Teske et al. (1996) by means of DGGE analysis of both 16S rDNA and rRNA. Felske et al. (1996) tried a similar approach to analyze microbial communities in soil samples. A number of researchers have applied DGGE to analyze soils of various types and origins for microbial community diversity and structure with success (Gelsomino et al. 1999; Kowalchuk et al. 1997; Kuske et al. 1997; McGraig et al. 1999a; Nüsslein and Tiedje 1998,1999; Ovreas and Torsvik 1998). Additionally, a variety of aqueous environments have been studied using DGGE to profile community diversity (McGraig et al. 1999b; Ovreas et al. 1997; Schafer et al. 2000; Teske et al. 1996). One aquatic system that has illuminated our view of microbial diversity and proven to be a goldmine of unique and invaluable products are hot spring environments. Ward et al. (1998) provide an excellent review of work on characterizing cyanobacteria communities in hot springs. Ferris et al. (1996) define the hot spring community as determined by a DGGE

142

profile, and Ferris and Ward (1997) chronicled the seasonal dynamics of the community by DGGE. Muyzer and Smalla (1998) provide an excellent review of applications of DGGE in microbial ecology.

Another method that can be used to reduce the overall complexity of a community or focus on a particular population or guild is to use functional-gene or group-specific primers (Brinkhoff and Muyzer 1997; Holben et al. 1998). DGGE is a valuable tool for assessing microbial community structure in a variety of environments. This approach offers a means of viewing a complex assemblage of microorganisms in quick and efficient manner but of itself, provides no information regarding the identity of the organisms analyzed.

The third approach by which we examined landfill community structure provides the most detailed analysis and highest resolution of bacterial populations based on DNA sequence analysis and comparison. While numerous other studies have employed 16S rDNA sequence analysis, they generally used a "shotgun" approach where 16S sequences were amplified from total community DNA and cloned randomly. Thus, the most abundant organisms are most readily detected. However, this "shotgun" approach is not well suited to assessing total community diversity.

To better assess diversity in our landfill samples, the same fractionated samples that we preformed DGGE analysis on, were used to construct clone libraries of 16S rDNA fragments PCR amplified using a set of universal primers. Clones were screened for proper-sized inserts by restriction digest analysis. Five

143

clones of proper insert size representing each of the five fractions from the two individual landfill samples were prepared and subjected to DNA sequence analysis. To identify these organisms, the sequences were matched to similar sequences available in the RDP.

.

144

Materials and Methods:

Landfill sample preparation:

The two landfill samples were provided by the National Institute for Resources and Environment in Tsukuba, Japan. One sample was identified as typical landfill material, consisting of a mixture of unprocessed municipal trash and soil (hereafter Raw). The other sample consisted of residual ash from incinerated trash that was mixed with soil (hereafter Ash). These two samples represent current technologies for sanitary landfills in Japan and elsewhere.

Portions of each sample were made into a slurry by combining 80 g of either landfill material with 40 ml of sterile deionized water in a Waring blender. The slurry was made homogeneous by blending for 30 s at full speed. Large portions of solid material were removed prior to homogenization.

Isolation and purification of total community DNA:

Isolation: Total community DNA was extracted from the landfill slurries by the direct lysis method of Holben (1997) with minor modifications. Briefly, extraction buffer was composed of 200mM sodium phosphate buffer ($NaPO_4$), 100mM ethylenediamine tetraacetate (EDTA) and 1.5% sodium dodecyl sulfate (SDS). The pH was adjusted to 8.0 by the addition of phosphoric acid, and the solution sterilized by autoclaving. Triplicate samples were prepared by combining 20 g of landfill slurry with 20 ml of extraction buffer in sterile Oak Ridge tubes containing

145

10 g of sterile glass (5 g of 0.2 mm and 5 g of 1 mm diameter; Sigma Chemical Co., St. Louis, MO.). The samples were then placed in a 70° C water bath and mixed every 5 min by vigorous vortexing. The slurries were then placed on a reciprocal platform shaker and shaken on the high setting (approximately 100 oscillations/minute) for 30 min at room temperature. Large particulate sand soil and cell debris were removed by centrifugation at 10,000 RPM (7,796 x g) for 10 min at 10° C.

The supernatant was transferred to clean Oak Ridge tubes and incubated on ice for 30 min to precipitate the SDS. The supernatant was centrifuged (Sorvall RC 5B Plus with SS34 rotor) at 10,000 RPM (7,796 x g) for 10 min at 10° C. The supernatants were transferred to clean Oak Ridge tubes and brought to a final volume of 25 ml by addition of dH_2O. To this, 24.7 g of finely ground cesium chloride (CsCl; Cabot Corp., Revere, Pa.) was added, mixed by gentle inversion until completely dissolved and allowed to stand at room temperature for 15 min to precipitate any residual proteins. The mixture was then subjected to low speed centrifugation at 5,000 RPM (1,949 x g) for 10 min at 10° C. The precipitate formed a floating layer that was gently decanted. The remaining solution was subsequently transferred to 36.2 ml OptiSeal^{tm} polyallomer ultracentrifuge tubes (Beckman Instruments Inc., Palo Alto, Ca.) containing 2 ml of 10mg/ml ethidium bromide (EtBr). After mixing by gentle inversion, the refractive index (R_f) of the solution was adjusted to 1.3870 by addition of finely

146

ground CsCl (to increase R$_f$) or deionized water (to increase R$_f$) using by a refractometer (Milton Roy Company, Rochester, N.Y.).

The tubes were carefully balanced by addition of CsCl balance solution (R$_f$ = 1.3870, 1 mg/ml EtBr), properly sealed and placed in a Beckman VTi50 rotor. The EtBr-CsCl gradients were subjected to centrifugation in a Beckman L7 ultracentrifuge at 45,000 RPM (90,000 x g) for 16 h at 18° C.

CsCl Gradient extraction: As a result of CsCl – EtBr equilibrium density gradients established by ultracentrifugation, the DNA from the landfill samples was purified and formed a discrete band within the gradient. The DNA band was visualized under UV illumination and extracted by piercing the side of the ultracentrifuge tubes, below the DNA band, with a 16-gauge needle attached to a 5 ml syringe (Sambrook et al. 1989).

Triplicate samples from each landfill were pooled and a second CsCl – EtBr equilibrium density gradient purification process was conducted using the protocol described above. The final volume of the extracted DNA was approximately 5 ml. This second round of ultracentrifugation produced a more concentrated and purified form of the total community DNA.

Isopropanol extraction of the Ethidium bromide: To remove the ethidium bromide from the purified DNA, a series of isopropanol extractions were performed. The final 5 ml volume of DNA (from the above steps) was transferred to a 14 ml polypropylene Falcon^{tm} tube (Becton Dickenson Labware, Lincoln Park, New Jersey). An equal volume of isopropanol (saturated with 5 M NaCl) was

added, mixed by gentle inversion and allowed to sit until discrete phases formed. The top organic isopropanol layer was then carefully removed with a Pasteur pipette and discarded. This process was repeated a total of six times, until the pink color was no longer visible.

DNA concentration: The DNA solution (from the previous steps) was transferred into 30 ml Corex^{tm} tubes. Two volumes of deionized water and then one total volume of cold isopropanol were added. The tube was covered with parafilm, mixed by vortexing, and incubated overnight at −20° C. The resulting DNA precipitate was pelleted by centrifugation at 7,500 RPM (4,385 x g) for 1 h at 4° C. Following centrifugation, the supernatant was decanted and the tubes were inverted and allowed to completely air dry. The resulting DNA pellet was resuspended in 400 µl of sterile deionized water by vigorously rinsing the side of the tube. The final solution was then transferred to a 1.5 ml eppendorf microfuge tube.

The DNA was further concentrated by the addition of 1/10 volume (40 µl) of 3M sodium acetate (pH 5.2) and 2 times the total volume (880 µl) cold 100% ethanol (EtOH). After overnight incubation at −20° C, the DNA precipitate was collected by centrifugation in a table-top microfuge (Eppendorf model 5415C) at 14,000 RPM (16,000 x g) for 30 min at 4° C. The precipitated DNA formed a small white pellet at the bottom of the microfuge tube. The supernatant was decanted and the pellet rinsed with 1 ml of cold 70% ethanol, then subjected to centrifugation at 14,000 RPM for 5 min after which the supernatant was

decanted. This washing process was repeated and the pellet vacuum dried at 45° C with centrifugation in a Labconco Centrivap Concentrator (Labconco Corp., Kansas City, Missouri) for 15 min. The DNA pellet was then resuspended in 100 μl of sterile deionized water.

DNA quantification: The total community DNA was quantified by spectroscopy in a Hewlett Packard (Hewlett Packard, Waldbronn, Germany) 8453 UV-visible spectrophotometer using the UV-visible ChemStation software package.

DNA visualization: The total community DNA was visualized by gel electrophoresis to confirm that the average fragment size was >20 Kb to facilitate the %G+C gradient analysis. Briefly, 1.5% agarose (SeaKem GTG, FMC Bioproducts, Rockland, ME) gels were prepared by adding 1.5 g of agarose per 100 ml of 1x Tris-acetate-EDTA (TAE) buffer (4.84 g/l Tris, 1.14 ml/l glacial acetic acid, 2 ml/l 0.5M EDTA (pH 8.0)). The agarose solution was brought to a final concentration of 0.125μg/μl EtBr from a 125μg/μl EtBr stock solution prior ti casting the gels. DNA samples were prepared for loading on to the gel by combining the appropriate volume of 5x loading dye (100 mM EDTA, 50% glycerol, 0.15% bromophenol blue, 0.15% xylene cyanole) to a sample of DNA for a final loading dye concentration of 1x. Typically, 4 to 8 μl of DNA sample with 1 to 2 μl dye were loaded on gels. Gels were run on a horizontal gel apparatus (Mupid 2 Mini-Gel Electrophoresis System, Japan) in 1x TAE buffer containing a final concentration of 0.125μg/μl EtBr.

149

Total community profile analysis by bisbenzimidazole gradients:

Preparation of gradient components: The purified total community DNA samples were fractionated by bisbenzimidazole-cesium chloride equilibrium density gradients as described previously by Holben and Harris (1995). The bisbenzimidazole-cesium chloride equilibrium density gradients were prepared by the following method: A solution of cesium chloride (CsCl) was prepared by the adding 231.8 g of finally ground CsCl into 250 ml deionized water. Adjustments to the CsCl solution was made so that a refractive index (R_f) of 1.3980 was achieved. The solutions were sterilized by passing through a 0.22 μm syringe Acrodisc[tm] filter (Gelman Sciences, Ann Arbor, MI). A 1 mg/ml stock solution of bisbenzimidazole (Hoechst No. 33258, Sigma Chemical Co.) was prepared and 30 μl was combined with 25 ml of CsCl solution in 36.2 ml OptiSeal[tm] polyallomer ultracentrifuge tubes. Total community DNA solutions containing either 50 μg or 75 μg from the Raw and Ash landfill samples in a total of 200 μl were prepared by dilution into sterile deionized water. Additionally, control DNA solutions were prepared by dilution of stocks from pure culture DNA solutions (Sigma Chemical Co.). Four total control DNA solutions were prepared, each containing 10 μg of DNA from each of the following bacterial species (total of 30 μg of DNA per control solution): *Micrococcus lysodeikticus* (72% G+C), *Escherichia coli* (50% G+C) and *Clostridium perfringens* (27% G+C) in a total of 200 μl. These latter

150

DNA stock solutions were used as controls for constructing standard curves of %

G+C content by regression analysis.

Table 1: Sample concentrations prepared for analysis by bisbenzimidazole
gradients.

Sample	Content/Concentration
A1	ASH landfill 50 μg
A2	ASH landfill 75 μg
B1	RAW landfill 50 μg
B2	RAW landfill 75 μg
C1	Control 30 μg total
C2	Control 30 μg total
C3	Control 30 μg total
C4	Control 30 μg total

Both landfill and control DNA samples were added to the

bisbenzimidazole-cesium chloride solutions and vortexed thoroughly. The

remaining volume of the tubes was filled with the CsCl solution (R_f 1.3980)

sealed and balanced.

Fractionation of community DNA based on % G+C content: The DNA

samples were subjected to ultracentrifugation at 33,000 RPM (90,000 X g) for 72

h at 18° C in a Vti-50 rotor (Beckman). Following centrifugation the equilibrated

gradients were fractionated by displacement with Fluorinert (Sigma Chemical

Co.) injected into the bottom of the ultracentrifuge tubes using a syringe pump

at 1.5 ml/min. The displaced gradients were passed through a flow-through UV

absorbance detector (Econo - UV, Bio-Rad, Hercules, CA.) and absorbance at 280

nm was continuously recorded during fractionation. The selection of detection at

280 nm is based on much lower levels of background absorbance even though

the measured absorbance of DNA is at 260 nm. A fraction collector (Model 2128

Fraction Collector, Bio-Rad) was used to collect the fractionated DNA in equal

volumes of approximately 1 ml.

Denaturing Gradient Gel Electrophoresis (DGGE):

PCR amplification of 16S rDNA: Partial 16s rDNA sequences of the

component populations in total community DNA or bisbenzimidazole fractions

were PCR amplified using a universal primer set which targeted the region

between nucleotides 536 and 907. The sequence of 536 forward

(5′ CAGCACGCCGCGGTAATA 3′) and 907 reverse (5′ TTTGAGTTTCCTTAACTGCCC

3′) primers are published in Ferris et al. (1997) and Devereux and Mundfrom

(1994) respectively. The forward primer (536fc) contained a GC clamp composed

of an additional run of 40 nucleotides (5′

CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC 3′). Community analysis

by denaturing gradient gel electrophoresis (DGGE) was performed essentially as

described by Muyzer et al. (1993). PCR amplification was performed in 50 µl

reactions containing: 0.2 mM dNTPs (Promega, Madison, WI); 20 pmoles of each

primer; 5 µl of 10x PCR Buffer, (Boehringer Mannheim, GmbH, Germany) and 2.5

units of *Taq* DNA polymerase (Boehringer Mannheim). The PCR amplification

reaction was performed with a PTC–100 thermocycler (MJ Research, Watertown,

MA.) under conditions suggested by Muyzer et al. (1993), with minor

modifications. Their touchdown procedure was varied by increasing the final 55°

primer annealing cycle to 10 cycles and increasing the final 72° extension period

to 15 min. Thus, the entire cycle consisted of an initial 5 min 95° strand

disassociation cycle; a one minute primer annealing cycle with an initial

temperature of 65° C. The primer annealing temperature was then decreased by

1° increments every other cycle until 55° C (for a total of 20 cycles). A this

temperature (55° C) a total of 10 cycles was conducted a; a 3 min 72° C primer

extension cycle (except for the final 15 min period at the end of the 30 cycles);

with a one min 95° C strand disassociation cycle. The thermocycler was

programmed with a 4° final holding temperature. The PCR products were

analyzed on 1.5% agarose gels and quantified by comparison with low DNA

mass ladder (Gibco BRL, Life Tecnologies, Gaithersburg, MD) through

densitometric analysis using the Gel – Doc 1000 (Bio-Rad) transilluminator

system integrated with Molecular Analyst Software (Bio-Rad) package.

It appeared that co-precipitation of contaminating substrates that

inhibited subsequent enzymatic amplification occurred. However, this was for the

most part overcome by dilution of starting template DNA in sterile deionized

water. Under some circumstances, re-amplification of amplicons was necessary

to obtain enough DNA (250 to 500 ng) to visualize by DGGE analysis. When this

was the case, re-amplification was conducted under similar PCR conditions.

Typically pooled PCR products were concentrated by ethanol precipitation as

described above. All PCR reaction sets included appropriate positive and negative controls.

The mixtures of PCR products representing partial 16S rDNA sequences amplified from total community DNA were separated by (DGGE) using the Bio-Rad D GENE System (Bio-Rad). The denaturing gradient gel was prepared from equal volumes of a 45% denaturant/5% acrylamide solution (18% formamide (v/v) and 3.14 M urea in 5% N,N'-Methlenbisacrylamide) and a 60% denaturant/10% acrylamide solution (24% formamide (v/l) and 4.19 M urea in 10% N,N'-Methlenbisacrylamide). The 18 x 20-cm vertical gradient gels were formed by using a linear gradient former (Hoeffer Scientific Instruments, San Francisco, CA). This gel was allowed to polymerize for 30-60 minutes. A 16-well 5% acrylamide (5% N,N'-Methlenbisacrylamide) stacking gel was poured on top of the denaturing gel. The polymerized DGGE gel was placed in the D GENE apparatus that contained 6 l of 1x TAE buffer described above. PCR product (250 to 500 ng) was mixed with loading buffer described above and loaded directly into the formed wells. The gel was run at 100 V for 14 to 16 hours at 60° C. As a positive control, 50 to 100 ng PCR products amplified from of purified DNA of *Clostridium perfringens* was applied to one lane in the gel.

Banding patterns were visualized with a 5X solution of SyberGreen I (FMC Products, Rockland, ME) in 1x TAE. The gel was covered with 7 ml of SyberGreen I solution, gently rubbed to insure complete coverage and incubated

154

at 37° C for approximately 1 h for complete staining. Banding patterns in the gels were viewed and photographed with a UV transilluminator.

Bands of interest were excised from the DGGE gel, placed in 1.5 ml eppendorf microfuge tubes and macerated. 50 μl of elution buffer (50mM KCl; 10 mM Tris-HCl (pH 9.0); 0.1% Triton X-100) was added and the mixture incubated at 37° C for 4 hours to elute the DNA from the gel as described elsewhere (Muyzer et al. 1996). This DNA was stored at –20° C for future cloning experiments.

Cloning of PCR products.

PCR Amplification: The PCR amplification of the partial 16S rDNA sequences was performed on bisbenzimidazole gradient fractions by methods described previously, with the exception that the 536 forward primer did not contain the clamp sequence.

Cloning of PCR Products: PCR products from total community DNA or bisbenzimidazole gradient fractions were purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Santa Clarita, CA) according to the manufacture's specifications. Cloning of purified PCR products was conducted using the T7Blue-3 Perfectly Cloning Blunt Kit (Novagen, Inc. Madison, WI). This procedure involves an initial end conversion reaction that remove 3' A overhangs typically generated by Taq polymerase. This was carried out by the use of the end conversion mix contained in the cloning kit.

155

The amounts of DNA used in the end conversion reactions varied from sample to sample, ranging from 15 ng to 85 ng, contained in a final volume 10 µl. The end-converted PCR products were then ligated into blunt ended pT7blue-3 vector that was prepared by digestion with *EcoR* V (GibcoBRL, Life Technologies™) and treatment with calf intestinal alkaline phosphatase (GibcoBRL, Life Technologies™) according to manufacture's specifications. The pT7blue-3 plasmid carries an ampicillin resistance marker and the α-complementation fragment of *lac Z* containing an in-frame polylinker region. This polylinker contains the previously mentioned blunt end (*EcoR* V) cloning site that is flanked by *EcoR* I sites. Ligations were done in 12 µl reactions containing approximately 50 ng (50 ng/µl) of blunt end vector, between 15 and 85 ng of blunt end PCR product (representing no less then a 3:1 molar ratio of insert to vector), and 1µl of buffered T4 DNA ligase (4 units). The reactions were incubated at 22° C for 2 - 3 h. Transformation of competent *E. coli* Nova-blue strain cells (Novagen, Inc. Madison, WI) was accomplished by the method of Chung et al. (1989). Briefly, 20 ml of Luria Broth (LB) (1% w/v tryptone; 0.5% w/v yeast extract; 0.5% NaCl) containing 300 µg/ml amplicillin and 12.5 µg/ml tetracycline was inoculated with one ml of an overnight culture of Nova-blue cells and incubated at 37° C for 2 h with shaking. The Nova-blue strain carries tetracycline resistance marker (Tn:10) on the F′ episome that facilitates the blue-white detection strategy to ease selection. The culture was transferred to a 50 ml conical tube and centrifuged at 6,000 RPM for 5 min. The cell pellet was then

156

resuspended in approximately 1 ml of TSS (LB supplemented with 10% w/v polyethylene glycol 8000 (PEG) 5% v/v dimethyl sulfoxide (DMSO) 50 mM $MgCl_2$), incubated on ice, and aliquoted in 100 μl volumes into 1.5 ml eppendorf tubes. The entire volume of ligated plasmid (12 μl) was added to the competent cells. This mixture was then incubated on ice for 45 min. Following the incubation, 900 μl of TSS was added and the cells were allowed to grow for 75 to 90 min wiht shaking at 37° C. Subsequently, 100 μl of the transformed cells was spread plated onto LB plates containing 12.5 μg/ml tetracycline, 300 μg/ml ampicillin, 40 μl of X-gal stock solution (20 mg/ml in dimethylformamide) and 4 μl of IPTG stock solution (200 mg/ml). The remaining volume of cells was pelleted by centrifugation (30 sec at 10,000 RPM in a microfuge) and then resuspended in 100 μl of TSS and plated as described above.

Screening of clones: Clones were selected based on blue/white colony formation where white colonies indicate that target DNA has been ligated into the cloning vector. White colonies were selected and grown overnight in 5 ml LB cultures containing 12.5 μg/ml tetracycline and 300 μg/ml ampicillin.

Confirmation of insert DNA: Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN Inc.) according to manufacturer's specifications. The presence of properly sized inserts was confirmed using restriction digestion analysis with *EcoRI* (GibcoBRL, Life Technologies™). For positive confirmation, 8 μl of prepared plasmid DNA was digested with *EcoRI* (GibcoBRL, Life Technologies™) according to manufacturer's specifications. Putative clones were

157

analyzed for inserts of approximately 400 base pairs (bp) by gel electrophoresis. If the digestion product(s) totaled ~400 bp they were subjected to DNA sequence analysis described below.

DNA sequence analysis: Each confirmed clone was subjected to DNA sequence analysis using 536F as the sequencing primer. Sequencing was performed at either of two different facilities; The University of Montana Murdock Molecular Biology Facility, or MWG-Biotech (High Point, N.C.)

Phylogenetic analysis: DNA sequence chromatograms were confirmed by visual analysis using EditView ABI automated sequence-viewing software, version 1.0.1 (http://www2.perkin-elmer.com/ga/editview/editview.htm). Corrections and edits were made manually as necessary using the raw data chromatograms as references. The sequences were then submitted to the Ribosomal Database Project II (RDP-II) worldwide web site (http://www.cme.msu.edu/RDP/). Detection of potential chimeric PCR products was facilitated using the Chimera Check feature of the RDP site, and likely chimeras were not considered further. To assess the uniqueness of all the sequences, a similarity matrix was generated using the distance similarity matrix option of the RDP II site. Sequences with high similarity values were grouped. These common groups were manually aligned by editing sequences with additions of gaps and deletions using Seqpup™ available as a shareware program at (http://iubio.bio.indiana.edu/soft/molbio/seqpup/). Corrected and aligned sequences were then analyzed using Sequence Match option in the RDP II site.

158

SAB values obtained were used as a measure of sequence relatedness to those

currently held in the RDP II database. Sequences with particularly low SAB

scores (~ < 0.5) were submitted to BLAST searching at

(http://www.ncbi.nlm.nih.gov/BLAST/). The results were reported as %

homology to the sequences returned from the search of these databases. For

this purpose, percent homology is defined as the "similarity attributed to descent

from a common ancestor" (Altschul et al. 1990).

159

Results

Microbial Community analysis. Total microbial community diversity and structure from two distinct landfill samples were examined by three complimentary methods. Overall the data indicate the existence of complex microbial communities that appear to contain great diversity. In addition, data from fractionation based on % G+C content and DGGE profiles suggested that predominant members of the communities had a high G+C content.

Teasing apart the microbial assemblage of an environment is challenging. Although landfill environments have a diverse nutrient load, the majority of the nutrients are stable over time, a situation suited for the establishment of microbial climax communities (Harvey et al, 1997; Palmisano and Barlaz, 1996). Over time the composition of these communities is thought to remain similar (Hurst, 1997). However, at any given time the plausibility for the existence of transient, microbial populations may develop via niche enhancement due to environmental perturbances (Harvey et al, 1997; Hurst, 1997). These less prominent community members may be masked by numerically dominant established populations. Under these conditions, the less prominent members are not well represented in any survey based on a "shotgun" cloning approach. To facilitate recovery of DNA sequences from "minority" members of the community, we combined multiple approaches to produce ordered sub-samples of the community, each of which contained reduced complexity. This was achieved through the combination of three different methods of DNA-based

160

analysis. We first employed microbial community fractionation based on its %G+C content to each of the two landfill samples. Secondly, we examined total sample and fractionated sample communities by DGGE analysis. Lastly, DNA from fractionated samples was cloned and sequenced to specifically identify microbial community members and evaluate the microbial diversity within landfill ecosystems. Based on the results obtained, no single population was dominant in any sample and enhanced detection of species diversity was realized. When posing purposeful questions in regards to microbial community composition, it is desirable to seek a more meaningful and tractable level of complexity. To accomplish this, we propose that this general strategy should prove useful for phylogenetic surveys of complex microbial communities.

There was also strong evidence that community differences exist between the two samples. It appears that differing landfill practices may support differing microbial communities. In terms of management strategies, this may be important information. Understanding the interactions between the various existing microorganisms could lead to amendment protocols that may enhance a landfill's longevity, emission control, decomposition rates and magnitude and mitigation of harmful leachate.
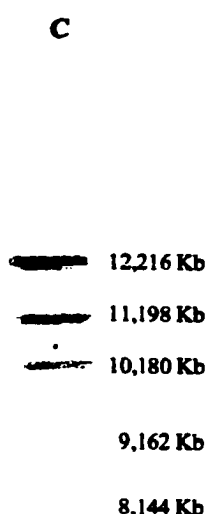
Total DNA extraction from landfill environments:

The direct lysis protocol for the extraction of total community DNA yielded DNA molecules of predicted proper size (Fig. 1) and quantities of DNA expected from

161

environments abundant in microorganisms (Table 2). Estimates of bacterial density in landfill soils are typically 1×10^9 to 1×10^{10} cells/gram (Barlaz, 1996 and Palmisano and Barlaz, 1996). If the average bacterium contains 9×10^{-15} g of DNA/cell (Holben, 1997) then the estimated quantity of DNA extracted would range between 9 μg and 90 μg/gram of soil. Based on these approximations, our recovery of DNA from landfill environments (refer to table 2) was reasonable.

A   B   C

12,216 Kb
11,198 Kb
10,180 Kb

9,162 Kb

8,144 Kb

A - Raw sample
B - Ash sample
C - High Molecular Weight marker

Figure 1. Total community DNA visualized on a 1.5% agarose gel. Lane A Raw total community sample; Lane B Ash total community sample; Land C High Molecular Weight marker.

Table 2. Total community DNA yields from landfill samples.

Sample	Calculated amount DNA based on spectrometry	Projected DNA recovery gram^{-1} landfill matrix	Estimated efficiency of DNA recovery
Raw landfill	3.3 μg/μl	7.7 μg/g	7.9% to 79%
Ash landfill	2.0 μg/μl	5.1 μg/g	5.6% to 56%

Recoveries may have been greater if relatively large items from the MSW samples would not have been removed. Observations made by Suflita et al. 1992, suggest that the majority of landfill microorganisms reside on surfaces of the relatively large constituents of MSW, so by their removal a significant biomass may have been absent for DNA extraction.

162

Community analysis by Bisbenzimidazole-Cesium Chloride Equilibrium Density Gradients:

Aqueous mixtures of 10 µg of DNA from three bacteria species: *Clostridium perfringens* (27% G+C), *Escherichia coli* (50% G+C) and *Micrococcus lysodeikticus* (72% G+C), were used to generate a relative abundance vs. % G+C control profile representing a range of % G+C content typically found in bacteria (Fig 2).

% G+C of Control DNA
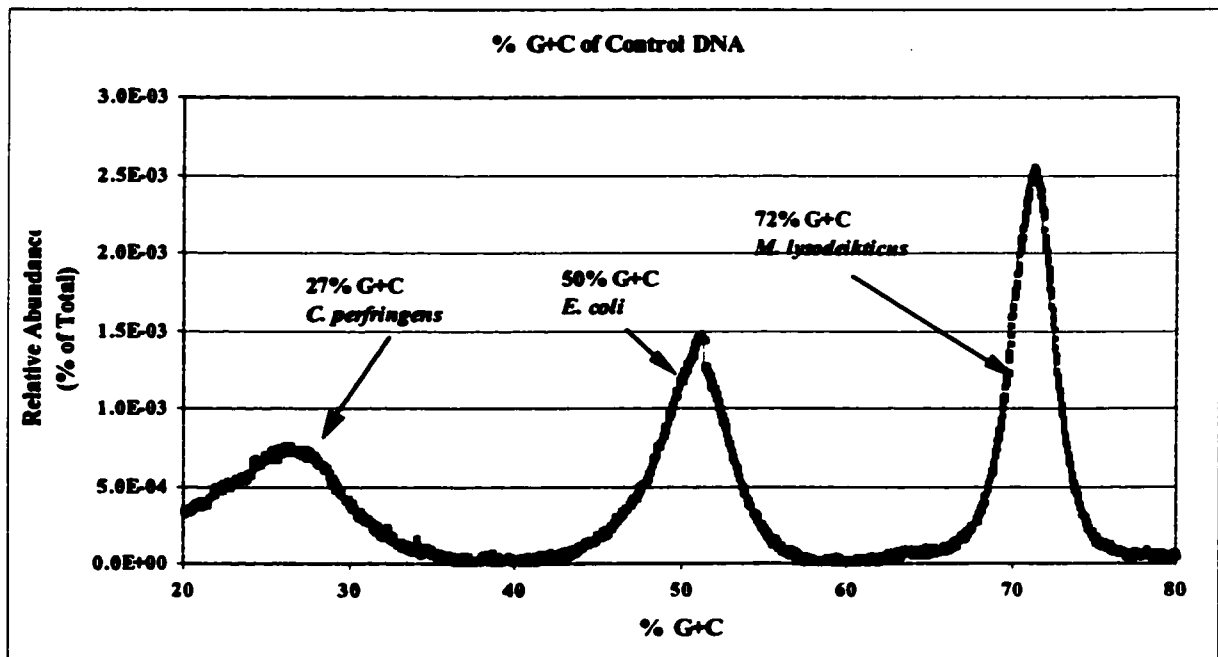
Figure 2. Profiles of bisbenzimidazole-cesium chloride gradient-separated DNA from control organisms indicating Relative Abundance vs % G+C content of DNA.

This approach can also be applied to samples containing unknown and mixed populations of bacteria. The total community profile analysis by bisbenzimidazole gradients revealed differences in community composition between the two

163

landfill communities (Fig. 3). Both communities produced relatively small peaks

of roughly the same magnitude representing populations having approximately

39% G+C content. However, the raw landfill sample had a significant peak at
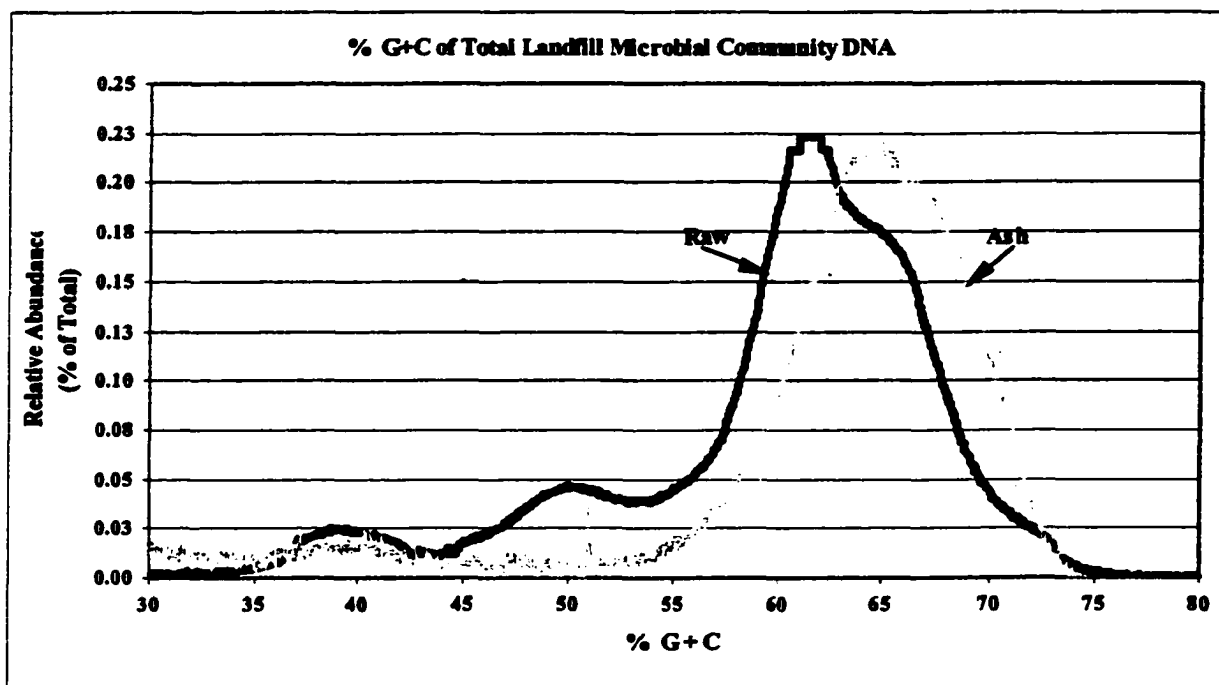
Figure 3. Total community DNA profiles of ash and raw landfill samples based on Bisbenzimidazole-Cesium Chloride Gradient fractionatation.

approximately 50% G+C, whereas the ash landfill profile has no discernable peak

in this area. The raw landfill sample had its greatest abundance of total DNA

centering around 61% G+C content, with an additional considerable shoulder of

DNA centered around 65%. The largest peak for the ash community centered at

65%, corresponding to the shoulder on the lagging edge of the raw sample

described above. Further, the ash sample had a significant shoulder peak at about 68% G+C content.

Denaturing Gel Gradient Electrophoresis (DGGE):

From the bisbenzimidazole gradient analysis it was suggested that the two landfill communities contained detectable differences. However, due to the limited resolution of that approach, it was difficult to say anything meaningful regarding the overall diversity within each sample. By coupling the DNA fractionation approach with that of the DGGE analysis of PCR amplified partial 16s rDNA genes, we obtained enhanced resolution and additional information regarding microbial diversity. Results from DGGE analysis of the total community indicated that there was substantial diversity in each community sample, and substantial differences between each community (figure 4).
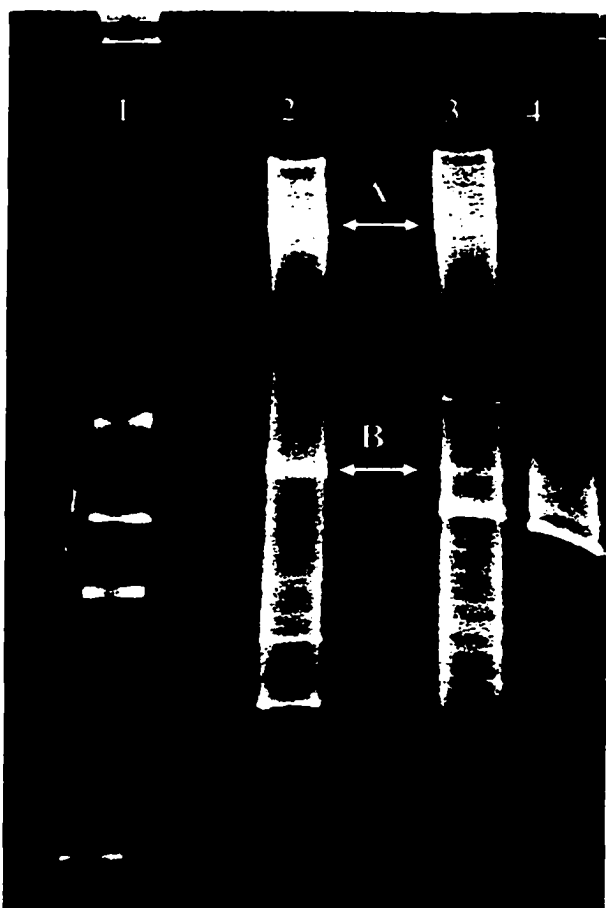
Figure 4. DGGE analysis of PCR amplified partial 16s genes of total community DNA from Raw and Ash landfill samples. Lane 1, 1 Kb marker; Lane 2, Raw landfill sample; Lane 3, Ash landfill sample; Lane 4, *Clostridium perfringens* DNA as control.

The DGGE banding patterns of the two communities indicate that there is richer microbial diversity in the ash landfill sample versus that of the raw based on the total number of bands present. However, it is possible that a significant portion of the microbial community may not have been resolved in this gel, as evidenced by the abundance of poorly-resolved bands in both community samples as indicated by the arrow A in Fig. 4. Interestingly, the two communities appear to share few dominant phylotypes. However, there is at least one common band between the communities, as indicated by arrow B on Fig. 4.

To obtain additional information on the populations present in each sample, we divided the fractionated total community DNA from each microbial landfill community into five fractions that were subsequently analyzed by DGGE analysis of partial 16s rDNA amplicons. Each sub-sample from each community represented a range of % G + C content as indicated in Table 3.

Table 3. Fractionated total community DNA from each sample was divided into 5 sub-samples representing ranges of % G + C content.

Fraction	1	2	3	4	5
% G+C	38 - 40%	49 - 51%	60 - 62%	64 – 66%	69 - 71%

Each gradient fraction was PCR amplified with a set of universal primers as described previously. The amplicons were then subjected to DGGE analysis along side their total community counterpart (Fig. 5). Lanes 7 and 13 of Fig. 5

represent the pattern obtained from the total community DNA of the raw and ash samples, respectively. These samples are equivalent to those in Fig. 4 and the observations regarding their comparison were stated previously.

Lanes 2 – 6, and 8 - 12 of Fig. 5 represent the analyzed sub-samples of the total raw and ash landfill communities respectively.
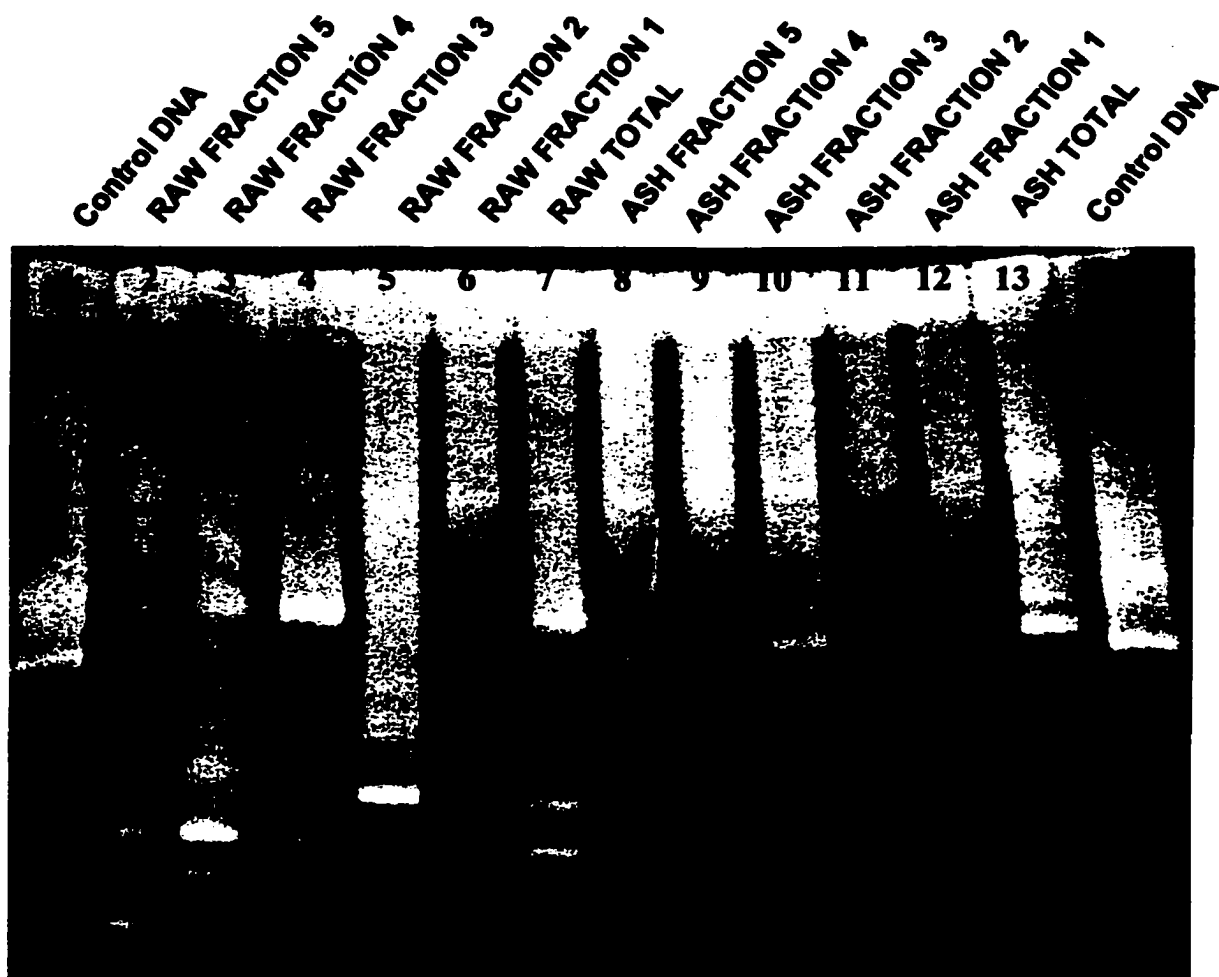

Figure 5. DGGE analysis of raw and ash samples. Lanes 2 through 6 are fractions of the composite raw sample shown in lane 7. Lanes 8 through 12 are fractions of the composite ash sample shown in lane 13. The fractions correspond to the same %G+C for each of the raw and ash samples. Refer to table 3 for %G+C of fractions.

167

A comparison between the corresponding fractions of the different samples was made and provides some interesting insights. In fraction 1 (lanes 6 and 12) which corresponds to 38 - 40% G+C content, there is no evident banding pattern for the ash sample. However, there appears to be a band in the corresponding raw sample. This is unexpected because, based on the % G+C profile, each community had a slight peak indicating that there was DNA in that fraction. The lack of PCR product in fraction 1 of the ash sample may result from products present only in the poorly resolved portion of the gel, or possibly from an inefficient or inhibited PCR reaction.

Fraction 2 (lanes 5 and 11), which corresponds to 49 - 51% G+C content, shows major differences between the two samples. The ash sample (lane 11) has no obvious bands, which was predicted from the % G+C profile. However, the raw sample displays a distinct pattern of one dominant band and two slightly visible bands (lane 5). This pattern can also be distinguished in lane 7, which represents the entire community of that sample.

Fraction 3 (lanes 4 and 10), which corresponds to a 60 - 62% G+C content, also reveals distinct differences between samples. Both indicate significant diversity based on the number of bands in this fraction, but different microbial populations are clearly present. This was expected based on the total % G+C profile of each sample. For the raw sample, this fraction represented the greatest % of total (lane 4). By comparison, this fraction represented only about half the abundance in the ash sample, yet, it appears to contain an equal or

greater amount of diversity then its counterpart (lane 10 vs lane 4). Not only does there appear to be substantial diversity within this fraction, but there are also a number of dominant bands within the overall-banding pattern.

Fraction 4 (lanes 3 and 9), corresponding to around 65% G+C content also shows major differences in banding patterns between samples. Lane 4 appears to show the greatest diversity while at the same time having the greatest number of major bands. Curiously, lane 9 has only a few minor bands, yet based on the total % G+C profile of the entire community, it represents the fraction with the greatest % of total for the ash sample. This may be another case where the amplicons are poorly resolved or the PCR reaction was inhibited.

Finally, fraction 5 (lanes 2 and 8), representing about 70% G+C content, also shows differences between samples. Lane 2 appears much like lane 3 in general banding pattern, but the intensity of the major bands has diminished. By contrast, lane 8, the last fraction of the ash sample, appears devoid of visible bands.

The relative position of 16s rDNA amplicons in the DGGE gel does not correspond to the overall % G+C content of the total DNA. Presumably this indicates that small stretches of DNA (in this case ~ 400 bp) may show localized differences in % G+C content or that the functional constraints on rDNA molecules prevent them from acquiring the general % G+C content of the genome at large.

169

Overall the results from DGGE analysis indicate that there is a diverse microbial community structure both within and between landfill ecosystems.

Community analysis by sequencing partial 16s rDNA genes.

To identify the phylogenetic affiliation of members of the ash and raw landfill microbial communities, random clone libraries were generated from respective sub-samples. Five clones from libraries of each fraction, for a total of 50 clones, were sequenced. Of the 50 sequences obtained, 29 (Table 4) contained usable sequence data. The others were rejected based on inadequacies in sequence quality, or if they were possible chimeras.

Of the 29 clones sequenced, no identical sequence matches to known sequences in the Ribosomal Database (RDP) were obtained. In fact, only one clone achieved a Sab score (indicating degree of similarity) > 0.950.

The majority of the clones had Sab values between 0.500 and 0.800 and the identities of their closest match are reported in Table 4. Clones with Sab scores below 0.600 were also subjected to Blast searches to access additional rDNA sequences to aid in identifying their phylogenetic affiliations (table 4).

Comparative analysis across the landfill samples to confirmed sequences from established databases revealed a range of bacterial diversity. Only one sequence was putatively identified as other than Eubacterial origin. Most of the best match identities were organisms that might be envisioned as members of a heterogeneous terrestrial environment such as a landfill.

170

Table 4. Most closely related organisms from RDP with a description of their relevance in context to a landfill environment. Clones in category R1 through R5 are from raw landfill sample fractions 1 through 5. Clones in category A1 through A5 are from ash landfill sample fractions 1 through 5.

G+C	CLONE	Best Match RDP	Relevant Information	SAB	RDP REF	Own Best Match	SAB	Best Result From Sequence	Hamming
30-35	R1A	Smithella propionica sr.	anaerobic propionate-degrading syntroph important in methane production	0.72	1297	A1C	0.54		
35	R1B	Flavobacterium ferrense	ubiquitous isolated from soil and freshwater, hydrolytic capabilities	0.741	1178	NA			
50-63	R1C	Desulfuromonas palmitatis str. SOBY1	anaerobic oxides organic acids, Fe II reducer	0.581	1362	NA			
NA	R2A	Unidentified marine eubacterium 16S rRNA gene	unidentified soil bacteria	0.547	327	R3C	0.63	Desulfuromonas acetoxidans 16S ribosomal RNA gene	90%
30-40	R2B	Amenitinus maritimus str PL12FS	marine origin related to Cytophagales	0.544	1303	NA		Uncultured eubacterium WCHB1-32 16S rRNA gene	91%
NA	R2C	Clone M13	16S Eukaryotic partial sequence	0.5	897	NA		Unidentified eubacterium of Amazon 16S rRNA gene	92%
61	R2D	Devosia riboflavina str Foster 4R3337	gram negative aerobe was pseudomonas proteobacteria alpha	0.829	1365	R3D	0.73		
63	R3A	Verrucomicrobium sp str VeGlc2	ferments glucose to organic acids, found in soils and sediments	0.377	1420	R4B	0.81	Uncultured marine eubacterium Hstpl53 16S rRNA gene	91%
63	R3B	Rhodomicrobium kindenclarsticus str RP5557	aerobic findane (chlorinated aliphatic compound) degrader	0.698	1407	NA			
NA	R3C	Unidentified marine eubacterium 16S rRNA gene	unidentified soil bacteria	0.905	327	R2A	0.63		
61	R3D	Devosia riboflavina str Foster 4R3337	gram negative aerobe was pseudomonas proteobacteria alpha	0.777	1365	R4C	0.97		
65	R4A	Pirococcus versatus	oxidize sulfur compounds, isolated from contaminated soil and wastewater	0.637	1313	NA			
63	R4B	Verrucomicrobium sp str VeGlc2	ferments glucose to organic acids, found in soils and sediments	0.511	1420	R3A	0.81	Unidentified eubacterium of anoxic soil 16S rRNA gene	89%
61	R4C	Devosia riboflavina str Foster 4R3337	gram negative aerobe was pseudomonas proteobacteria alpha	0.769	1365	R3D	0.97		
70	R5A	Azospirillum largemobile	nitrogen fixer, isolated from soil environments in association with roots	0.5	1309	NA		Olsenia tricae endosymbiont 16S rRNA gene	89%
>62	R5B	Actinomadura citrea	Actinomycetes related (thermophilic genus) from composted manure (lignin degrader)	0.916	1320	NA			
NA	A1A	Unidentified eubacterium 16S rRNA gene	unidentified soil bacteria	0.538	1332	NA		Uncultured bacterium #0319-7G21 16S rRNA gene	95%
41-45	A1B	Macrococcus carouselicus 16S rRNA gene	isolated from animal skins (horses) aerobic gram positive	0.527	1473	A3B	0.91	Unidentified eubacterium RFLP24 16S rRNA gene	90%
42-44	A1C	Syntrophus pentunae str. HOGoe1	grows in cocultures with methanogens degrades benzoate to methane and acetate	0.579	1437	R1A	0.55	Syntrophus sp. partial 16S rRNA gene	90%
50-52	A2A	Desulfobulbus elongatus str FP	propionate-oxidizing, sulfate reducer	0.596	1363	NA		Desulfobulbus rhabdoformis 16S rRNA gene	90%
50-60	A2B	Geobacter sp JW-3 16S rRNA gene	widespread in anaerobic environments, oxidize organics to CO2, Fe II reducer	0.942	1342	NA			
51	A2C	Desulfocapsa thiozymogenes str Bra2	new gram negative, strict anaerobe, sulfate or sulfur-reducer, dissimilatory bacteria	0.723	1390	NA			
NA	A3A	Phenylobacterium immobile str E	gram-negative bacterium, degrades chlorinated aromatics	0.723	1384	NA			
41-45	A3B	Macrococcus carouselicus 16S rRNA gene	isolated from animal skins (horses) aerobic gram positive	0.552	1473	A1B	0.91	Unidentified eubacterium RFLP24 16S rRNA gene	90%
65-73	A4A	Cellulomonas fermentans	aerobic cellulose degrader	0.826	1404	NA			
61-64	A4B	Mesorhizobium hanshanense str A-1BS	isolated from chinese soils, N fixer, acid producer	0.942	1392	NA			
67	A5A	Ralstonia pickettii str K-288	toluene degrader, isolated from low oxygen aquifers	0.957	1257	NA			
65	A5B	Burkholdena sp strain RP007	aerobic PAH degrading	0.925	1381	NA			
34	A5C	Deferribacter thermophilus str BMA	thermophile, Mn and Fe reducer, found in hydrocarbon rich environment	0.625	1444	NA			

171

Many of these "best-match" organisms are known to degrade recalcitrant anthropogenic, but common, environmental contaminants. Others have metabolic pathways that produce substrates, either directly or in cooperation with other microorganisms, involved in methane production, a hallmark by-product of biological landfill decomposition. Overall, the numerically dominant sequence type was *Devosia riboflavina* (str. Foster 4R3337), which was recently transferred from the genus *Pseudomonas*. It occurred only in the raw landfill sample, and was present in fractions 2, 3 and 4 (Table 4).

Fraction 3 also produced a clone common to fraction 2 and another with fraction 4. This inter-fraction similarity confirmed what was unveiled in DGGE banding patterns. These three fractions contained 38% of all the sequences, of which 64% of them were different. Fractions 1 and 5 of the raw sample contained fewer overall sequences, but all were dissimilar. The apparent sequence diversity obtained from this analysis of the ash sample exceeded that of the raw sample. The greater phylotype richness of the ash sample was predicted from a comparison of the DGGE patterns (Fig. 4 and 5). Of the 13 sequences considered from these fractions, only two were identified as being from the same organism. Fractions 1 and 3 contained a sequence identified as *Macrococcus carouselicus*, a bacterium typically associated with composted manure. The matches to the other sequences obtained also were consistent with being members of a microbial landfill community, demonstrating abilities consistent with environmental microorganisms.

172

Of the sequences analyzed, 75% were different for one another in the raw

sample, while 92% of the ash sample were distinct from one another. Of the

number of clones analyzed, 83% of the sequences occurred only once. There

were no common sequences shared between the raw and ash landfill microbial

communities in spite of the evidence indicated by Fig. 4, arrow B. However,

many of the best-match identities were consistent with organisms having

common metabolic characteristics, and likely occupying similar niches in their

respective habitats.

The success of this polyphasic approach to analyzing complex microbial

community structures was confirmed by the consistency with which the G+C

content of the genomic DNA (Table 4; column 1) of the closest matched

organisms coincided with the G+C content of the landfill sample's fractions. The

majority of organisms fractionated in accordance G+C content reported in the

literature (Table 4; column 1).

173

Discussion

Our current understanding of MSW decomposition has been developed based on laboratory observations and experiments, and validated to some extent through limited field observations (Barlaz et al. 1989; Barlaz 1996a,b, 1997; and Gerba et al. 1992). Barlaz et al. (1989) described the process of refuse decomposition as occurring in a series of phases. Even these early studies recognized that the onset of the decomposition process could occur efficiently only after the proper microbial assemblages are established. Briefly, he identified these phases as:

1) Aerobic decomposition – Oxygen entrained in refuse at burial and associated with moisture is depleted through aerobic metabolism and large quantities of CO_2 are produced. Due to high levels of microbial activity, temperature increases and the system moves towards a more anaerobic condition.

2) Anaerobic acid decomposition – With the rapid depletion of oxygen, microbial activities shift accordingly and more organic acids are produced. Under anaerobic conditions CO_2 increases, pH drops and acidic conditions prevail, inhibiting further aerobic decomposition and most hydrolysis of solids.

3) Methane production - Significant methane and carbon dioxide production begins. This shift in microbial activity leads to acid consumption and increasing pH conditions. An increase in cellulose and hemicellulose degradation begins.

174

4) Decelerated methane production - Carboxylic acids are depleted, pH

increases and the rate of methane production becomes increasingly more

dependent on the rate of cellulose and hemicellulose decomposition directly.

Christensen et. al. (1995) extended these descriptions by pointing out that in

theory, a landfill could return to an aerobic state through an additional series of

steps. Most importantly, this would allow for microbially mediated oxidation of

methane to carbon dioxide to occur.

Although our current knowledge is limited, an increased understanding of

the microorganisms and processes involved in MSW decomposition would come

from knowledge of the microorganisms involved, and their metabolism and

requirements. Unfortunately, a paucity of direct field identification data exists

concerning this information.

Our study represents an early and important step towards accomplishing

that. Based on the observations by researchers, such as Palmisano et. al.(1993)

who were able to culture comparable numbers of anaerobic and aerobic bacteria,

and in light of the model proposed by Barlaz, predictions of microorganisms

present in landfill ecosystems can be made. Using the anaerobic decomposition

pathway designed by Brock et al. (1994), as a preliminary guide, modifications

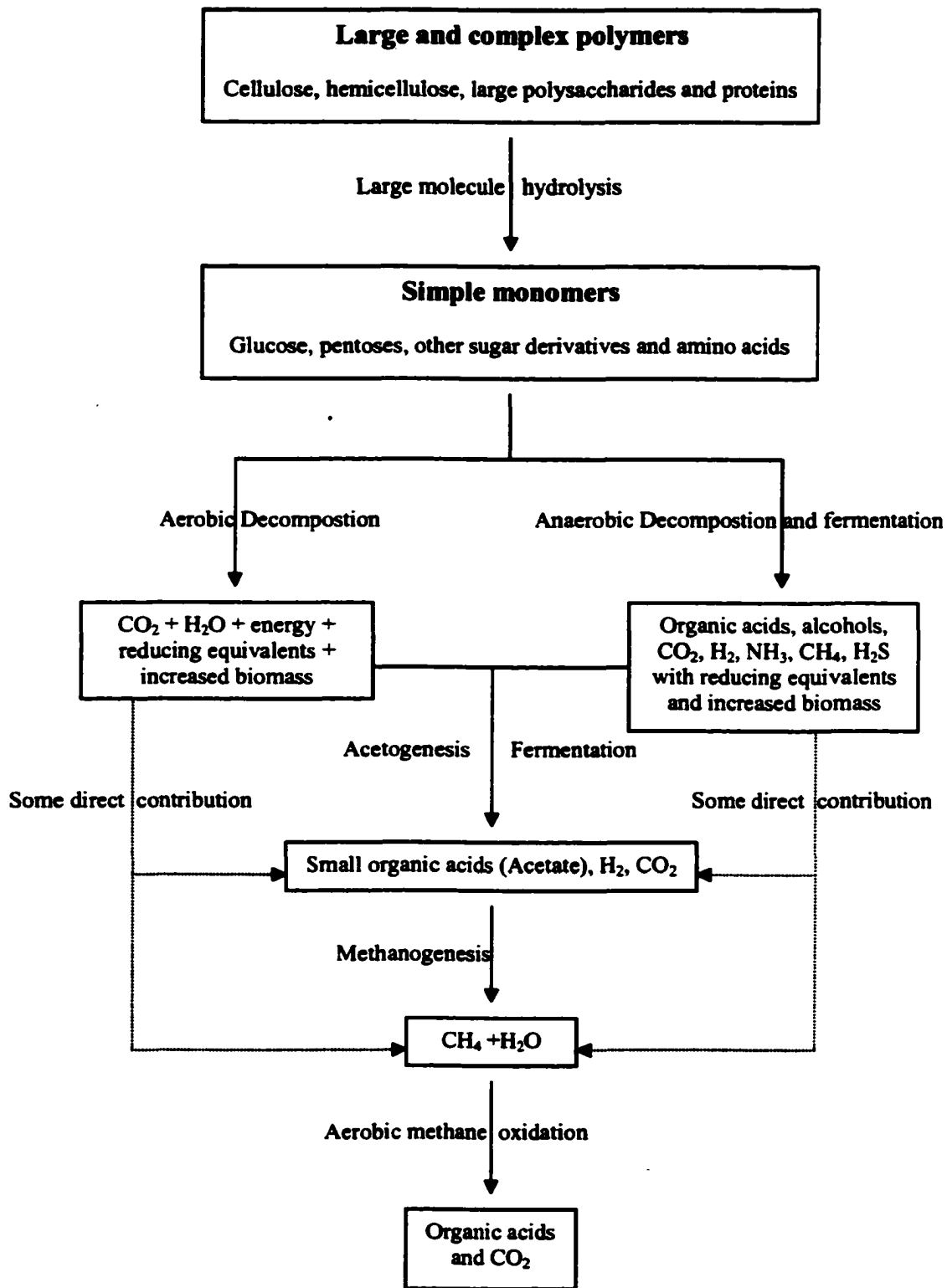were made to include aerobic decomposition and methane oxidation (Fig. 6).

175

Figure 6. Proposed overall process of decomposition of landfill material.

This diagram considers only the major carbon sinks as a source for microbial growth and succession. It should be realized that the nature of carbon sources and electron donors and acceptors varies greatly in a landfill environment, and this heterogeneity is likely to lead to the development of many metabolically diverse populations of microorganisms.

Predictions of likely types of microorganisms to be present based on Fig. 6 are cited below.

- Hydrolytic and cellulolytic bacteria: low GC gram positives (genera *Clostridium, Bacillus*), high GC gram positives (genera *Corynebacterium, Actinomyces, Cellulomonas, Eubacterium* and propionic acid bacteria), and some gram negative bacteria (genera *Cytophaga* and *Bacteroides*).

- Aerobic decomposers: *Proteobacteria* (genera *Pseudomonas, Arthrobacter* and *Azotobacter*) and other heterotrophic gram negative organisms (genera *Rhizobium* and *Bradyrhizobium*).

- Anaerobic and facultative decomposers: low GC gram positives (genera *Clostridium, Bacillus*), sulfate and sulfur reducing bacteria (genera *Desulfovibrio* and *Desulfuromonas*), and facultative gram negative bacteria (genera *Enterobacter* and *Serratia*).

- Fermentative and acidogenic bacteria (genera *Clostridium* and *Acetobacter*).

- H₂ producing bacteria (genera *Syntrophobacter* and *Syntrophomonos*).

- Methane producers (any of the 17 genera of methanogenic Archea).

- Methane consumers (genera *Methylococcus* and *Methylomonas*).

- In addition, fungi such as *Trichoderma* and *Chaetomium* are common cellulose degrading microorganisms and probably account for a large portion of the cellulose degradation occurring in landfills (Palmisano and Barlaz 1996).

The use of bisbenzimidazole gradients provided a convenient method to determine the structure of complex microbial communities by fractionating them based on % G+C content of the genomic DNA of the component populations. This approach generates a profile of relative abundance of related phylogenetic groups that may be present in these types of environments, and is particularly powerful when information on which populations are present is obtained.

The DNA fractionation approach also proved to be a useful and informative way to compare gross microbial community differences between different sites. However, this technique has limitations. Because of its broad level of resolution, identifying specific populations of bacteria is difficult (Holben and Harris, 1995). Many bacteria, particularly those of the same genus, have DNA with similar % G+C content. As a consequence, their absence at one site may be masked by the presence of one or several closely related organisms at another. Their loss at one site may also go unnoticed if a related genus or species becomes dominant.

This study coupled bisbenzimidazole gradients analysis with DGGE analysis and partial 16s rDNA sequence analysis. With the DGGE method, assessing genetic diversity becomes more feasible because it is based on smaller scale sequence variations found between more conserved genetic regions of the rDNA

genes. As with most methods, certain caveats must be applied when using DGGE. Though it can distinguish groups of phylogenetically related populations without sequence information, it can only indicate relative similarities or differences between banding patterns. Thus, one cannot conclusively describe which populations comprise these communities without further analysis such as cloning and sequencing of relevant genes.

Despite the limitations of each approach, used in conjunction, these techniques provide a complementary suite of microbial community analytic tools. For the purposes of our investigation, the combination of these methods provided relevant information to allow comparison of resident populations in the two communities. This approach also provided insight to the complex nature of landfill microbial community structures in general. As individual methods, they revealed that there were distinct differences in microbial community structure between the raw and ash landfill samples and shed some light as to the identity of some residents.

Generally speaking, the bisbenzimidazole gradients analysis indicated that there were apparent differences in the two landfill communities and that there was greater diversity in % G+C content within the raw landfill sample than in the ash landfill sample. However, as pointed previously, it said little about the diversity that may be contained within each community. From this fractionating process we inferred the types of organisms that may comprise each community. Based on this % G+C analysis (refer to Fig. 3), it's assumed both samples

179

contain a microbial population of *Clostridium, Streptococcus* and *Bacillus* species (spanning the lower % G+C region), along with a significant population of high GC gram positive and *Proteobacteria* present in both communities. These classifications of microorganisms include a number of the common and dominant soil bacteria genera such as: *Agrobacterium* (55 to 65% G+C), *Alcaligenes* (55 to 65% G+C), *Arthobacter* (60 to 70% G+C), *Pseudomonas* (55 to 68% G+C), *Rhizobium* (59 to 65% G+C) and *Bradyrhizobium* (60 to 65% G+C). The raw sample has an additional substantial population of organisms with a G+C content in the 50% range, such as the gamma group of the *Proteobacteria*. It is possible that incineration altered some component within the waste that is critical for success of a certain population of microorganisms. It is also conceivable that the components that comprise the raw MSW sample contained an inoculum that have 50% G+C content.

The % G+C general fractionation profile of the total community structures from these landfill samples displays similar trends to those % G+C fractionation profiles of the total community structures from other terrestrial environments, such as those explored by Holben and Harris (1995) and Nusslein and Tiedje (1998 and 1999). That is, the majority of the DNA has a % G+C content in the 50 to 70% range, underscoring the notion that soil microbial communities contain organisms capable of performing similar functions, and occupying similar niches in varying environments. These guilds or ecotypes contain a comparable

180

genomic % G+C, thus are likely to co-fractionate in a bisbenzimidazole gradient profile.

Though we could infer that the landfill samples contained a substantial microbial diversity, based on bisbenzimidazole gradients analysis of the total community structure, it was confirmed by DGGE analysis of the total community (refer to Fig. 4). From the number of visible bands detected on the gel, it was evident that the landfill communities contained a tremendous amount of microbial diversity.

Further, examination of the total community DGGE profiles of the two samples (Fig. 4) revealed additional information that could not be gleaned from the bisbenzimidazole gradient analysis. In contrast to the bisbenzimidazole gradient analysis, the DGGE banding patterns suggested that the ash landfill sample contains a richer microbial diversity than that of the raw landfill. However, because a significant portion of the microbial community may not have been completely resolved in this gel we must use caution when making definitive observations about overall community diversity.

Further, it appears that the two communities share few dominant phylotypes, and relative to the number of bands, the raw sample has more dominant bands than the ash sample. This situation conforms to the theory that ecosystems with greater diversity have fewer dominant species (Ward et al. 1998). In these environments competition aids in the establishment of diversity and limits the establishment of dominance by select phylotypes.

181

To gather additional information on the diversity of the total community structure contained within the landfill samples, the bisbenzimidazole gradients were sub-fractionated into portions based on G+C content which appeared to possess the most relevant biomass (refer to Table 3, Fig. 3). By doing so we hoped to be able to PCR amplify 16S rDNA genes of those community members that may not have been represented well in a total community "shotgun" analysis approach.

In general, results appear to be consistent with expectations. The diversity of the entire community (Fig. 5 lanes 7 and 13) reflects the diversity contained in the five sub-fractions of the raw and ash landfill samples (Fig. 5 lanes 2 – 6, and 7 – 12). For the raw sample it is clear that individual fractions, as a whole, displayed more intense banding patterns than that of the total community fraction. This suggests that certain fractions possess dominant microbial community members that are not necessarily as prevalent in the DGGE analysis of the total community. This is particularly evident for bands near the bottom of lanes 2 and 3 in Fig 5. This may imply that DGGE diversity analyses of complex total microbial communities are not necessarily accurate indicators of the overall structure of microbial assemblages.

This situation was not nearly as evident in the ash sample. The DGGE analysis of the total ash sample community displayed as intense a banding pattern as any single sub-fraction (lane 13 versus lanes 8 – 12) displayed. In fact, lanes 8 and 12 appear devoid of any bands, yet we know from cloning

182

experiments that they indeed contained bacterial 16S sequences. This is a difficult circumstance to reconcile. Because of the tremendous diversity contained within the total microbial community of the ash sample, one would have predicted that the individual fractions would have displayed more intense banding patterns dispersed more evenly throughout the sub-fractions. Instead, fractions 3 and 4 (lanes 9 and 10 in Fig 5) hold most of the DGGE bands. There is some logic and satisfaction in this circumstance. In both the raw and ash sample the majority of the DGGE bands reside in fractions 3 and 4, which correlates well with what one would expect based on the amount of DNA profiled in the bisbenzimidazole gradients (refer to Fig. 3).

Comparing the ash to the raw samples' sub-fractions DGGE banding patterns substantiated the differences in their total community structure that was predicted from bisbenzimidazole gradient and DGGE analysis of the total microbial community. It is apparent that the two differing MSW management strategies sustain varying microbial communities.

To reveal the identity of the community members, a phylogenetic analysis was conducted by sequencing 16S DNA clones generated from the sub-fractionated ash and raw landfill samples (refer to Table 4).

As predicted from previous data, the ash sample contained greater phylogenetic richness than the raw sample. Further, the two sites shared no common species, but some have close phylogenetic affiliations and most importantly, similar metabolic characteristics and capabilities (refer to Table 4).

183

The diverse potential metabolic capabilities contained within the microbial community should come as no surprise. The heterogeneity of carbon sources and electron donors and acceptors in a landfill ecosystem offers an environment that would facilitate colonization by a diverse microbial assemblage. Although the microbial communities appear different they are likely to function similarly. From a more practical aspect, it would be beneficial to study the communities' functionality and efficacy based on turnover rates of components from their environments.

Our prediction for the presence of microorganisms based on the model outline in Fig. 6 was reasonably accurate. The majority of the "best match" microorganisms present have the metabolic capabilities to conform to this model and lend sound support to the "putative" identification of the two landfill community members. Others possess metabolic characteristics that allow them to obtain nutritional requirements from unique sources, which may be likely present in landfills. Examples of this include *Rhodanobacter lindaniclasticus*, capable of degrading lindane (chlorinated aliphatic compound) and *Ralstonia picketii*, capable of degrading toulene.

Because the majority of the SAB scores were low, qualifiers such as, "best match" and "putative" must be used. However, since landfill environments provide unique environments and their total microbial community surveys remain relatively uncharted, it is conceivable that these low SAB scores suggest the presence of yet unidentified bacterial species or sub-species. This would certainly

not be unprecedented in unusual and unexplored environments such as landfills (Lloyd-Jones and Lau 1998; van Verseveld et al. 1999; Westlake et al. 1995; Wise et al. 1999).

Initially we were surprised by the absence of Methanogens. Particularly with the presence *Smithella propionica* (fraction 1 in raw sample) and *Syntrophus gentianae* (fraction 1 in ash sample), two bacteria that have been isolated in co-cultures with Methanogens. However, we also identified bacteria in both samples whose closest matches were *Desulfuromonas palmitatis* (fraction 1 in raw sample), *Desulfobulus elongatus* (fraction 2 in ash sample) and *Desulfocapsa thiozymogenes* (fraction 2 in ash sample), all of which are capable of sulfate reduction. It is possible that the competition between Methanogens and Sulfate-reducing bacteria (SRBs) for H_2 as an electron donor is to the advantage of SRBs and that Methanogens are present, but their activity is mitigated to the extent that their growth is limited. If this is true, it is likely temporary.

Another possibility for the paucity of Methanogens is because they are *Archaea*, and the universal primer set used was not stringent enough for proper binding to their 16S rDNA genes. As a consequence, they are not amplified during PCR.

Studies such as this one, which use a multi-tiered approach to query complex microbial communities in relatively unexplored environments, are excellent approaches for comparison and survey purposes. In our study, we

determined that two different management strategies likely engender different microbial community structures, each containing diverse microbial species with seemingly similar metabolic potentials. Through phylogenetic analysis of limited magnitude we identified community assemblage members whose "best match" in established databases distinguished them as likely candidates in our proposed model of landfill microbial community members. Further, we suggest that due to the physical and chemical environments of landfills and their relative unexplored nature, some of the "best match" identities could be undescribed bacterial species. Based on our results, we recommend that a more comprehensive study be undertaken, which includes more intensive phylogenetic analysis coupled with *in-situ* decomposition rate experiments. This would be an excellent means of determining what strategies, amendments or inocula may used for managing MSW in the future.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143-169.

Apajalahti, J. H., Laura K., Särkilahti, Brita R. E., Mäki, J. Pekka, Heikkinen, Päivi H. Nurminen, and W. E. Holben. 1998. Effective Recovery of Bacterial DNA and Percent-Guanine-Plus-Cytosine-Based Analysis of Community Structure in the Gastrointestinal Tract of Broiler Chickens. *Appl. Envir. Microbiol.* 64: 4084-4088.

Bagnara, C., Toci, R., Gaudin, C., Belaich, J.P. 1985. Isolation and characterization of a cellulolytic microorganism, *Cellulomonas fermentans* sp. nov. *Int. J. Syst. Bacteriol.,* , 35, 502-507

Barlaz, M. A., Schaefer, D. M. and R. K. Ham, 1989. Bacterial population development and chemical characteristics of refuse decomposition in a simulated sanitary landfill. *Appl. Env. Microbiol.,* 55, 1, 55 - 65.

Barlaz, M. A., 1996a. "The Microbiology of Municipal Solid Waste Landfills," in *Solid Waste Microbiology,* A. C. Palmisano and M. A. Barlaz, ed., CRC Press, Boca Raton, FL, p. 31 - 70.

Barlaz, M. A. 1996b. Microbial studies of landfills and anaerobic refuse decomposition. In *Manual for Environmental Microbiology.* American Society for Microbiology, Washington, D.C.

Barlaz, M. A., 1997, "Microbial Studies of Landfills and Anaerobic Refuse Decomposition," *Manual for Environmental Microbiology,* Am. Soc. for Microbiol., Washington, D.C.

Barns, S.M., Fundyga, R.E., Jefferies, M.W. and Pace, N.R. 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc. Natl. Acad. Sci. USA.* 91: 1609-1613.

Barns, S. M., Takala, S. L., Kuske, C. R. 1999. Wide distribution and diversity of members of the bacterial kingdom acidobacterium in the environment. *Appl. Environ. Microbiol.* 65: 1731-1737

Ben Dekhil, S., Cahill, M., Stackebrandt, E., Sly, L.I. 1997. Transfer of *Conglomeromonas largomobilis* subsp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* comb. nov., and elevation of *Conglomeromonas largomobilis* subsp. *parooensis* to the new type species of *Conglomeromonas*, *Conglomeromonas parooensis* sp. nov. Syst. Appl. Microbiol., 20, 72-77.

Brinkhoff, T. and Muyzer, G. 1997. Increased species diversity and extended habitat range of sulfur-oxidizing Thiomicrospira spp. *Appl Environ Microbiol.* 63: 3789-96.

Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek NG, Jansen JL, Nienhuis J, Triplett EW. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl Environ Microbiol.* 62: 1935-1943.

Brock, T.D., Madigan, M.T.. Martinko, J.M., Parker J. 1996. (Eds.) Bioiogy of Micro-organisms. Prentice Hall International, 8th Ed.

Chung, C.T., Niemela, S.L., Miller, R.H. 1989. One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci.* U S A. Apr. 86(7):2172-5.

Christensen, T. H., Kjeldsen, P., Bjerg, P. L., Jensen, D. L., Christensen, J. B., Baun, A., Albrechtsen, H., Heron, G. (accepted) "Biogeochemistry of Landfill Leachate Plumes" *Applied Geochemistry.*

Coates, J.D., Lonergan, D.J., Phillips, E.J.P., Jenter, H., Lovley,D.R. 1995. Desulfuromonas palmitatis sp. nov., a Marine Dissimilatory Fe(III) Reducer That Can Oxidize Long Chain Fatty Acids. *Arch. Microbiol.* 164, 406-413

Cole, S.T. and Girons, I.S. 1994. Bacterial Genomics. *FEMS Microbiol. Rev.* 14: 139-160.

Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. B., Huq, S. A. and Palmer, L. M. 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. *Bio/Technology* 3: 817-820.

Devereux, R. and Mundfrom, G.W. 1994. A Phylogenetic Tree of 16s rRNA Sequences. *Appl. Environ. Microbiol.* 60: 2203-2210.

Dojka, M. A., Harris, J.K. and Pace, N. R. 2000. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of bacteria. *Appl Environ Microbiol.* 66: 1617-21.

Dojka, M. A., Hugenholtz, P., Haack, S. K. and N. R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Environ. Microbiol.* 64: 3869-3877.

Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucl. Acids. Res.* 19: 4008.

Egan, S., Wiener, P., Kallifidis, D., Wellington, E., 1998. Transfer of Streptomycin Biosynthesis Gene Cluster within Streptomycetes Isolated from Soils. *Appl. Environ. Microbiol.* 64: 5061-5063

Farrelly, V., Rainey, F.A., Stackebrandt, E. 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16s rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol.* 61: 2798-2801.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E., Akkermans, A.D. 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology.* 143:2983-2989.

Ferris, M.J., Muyzer, G., Ward, D.M. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring community. *Appl. Environ. Microbiol.* 62: 340-345.

Ferris, M.J., Nold, S.C., Revsbech, N.P., Ward, D.M. 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. *Appl. Environ. Microbiol.* 63:1367-1374.

Ferris, M.J. and Ward, D.M. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 63:1375-1381.

Fischer, A., Kroppenstedt, R. M., Stackebrandt, E. 1983. Molecular-genetic and chemotaxonomic studies on *Actinomadura* and *Nocardiopsis. J. Gen. Microbiol.* 129:3433-3446.

Gelsomino, A., Keijzer-Wolters, A.C., Cacco, G. and van Elsas, J.D. 1999. Assesment of bacterial community structure in soil by polymerase chain reaction and denaturing gel electrophoresis. *J. Microbiol. Meth.* 38: 1 – 15.

Gerba, C.P., Ham, R.K., Palmisano, A.C. . Rathje, W.J., Robinson, J.A., Suflita, J. M. 1992. The World's Largest Landfill: A Multidisciplinary Investigation, *Environmental Science and Technology*, v. 26, n. 8, p 1487.

Goodman,R.M., Bintrim,S.B., Ireland,J.S., Joseph,D.A., Donohue,T.J., Handelsman,J. and Roberts,G.P. Molecular diversity of Bacteria from soil as determined by analysis of amplified small subunit ribosomal RNA gene sequences Unpublished

Greene, A.C., Patel, B.K.C., Sheehy, A.J. 1997. Deferribacter thermophilus gen. nov., sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *Int. J. Syst. Bacteriol.* 47, 505-509.

Gsell, T.C., Holben, W.E., and Ventullo, R.M. 1997. Characterization of the sediment bacterial community in groundwater discharge zones of an alkaline fen: a seasonal study. *Appl. Envir. Microbiol.* 63: 3111-3118.

Harvey, R. W., Suflita, J. M. and McInerney, M. J. 1997. Overview of Issues in Subsurface and Landfill Microbiology. In: *Manual of Environmental Microbiology*. (Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., and Walter, M.V., Eds.) Amer. Soc. Microbiol. Press. p.523-525.

Head, I.M., Saunders, J.R., Pickup, R.W. 1998. Microbial evolution, diversity and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35: 1-21.

Hobbie, J. E., Daley, R. J. and Jasper, S. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 3: 1225–1228.

Holben, W.E., 1997. Isolation and Purification of Bacterial Community DNA from Environmental Samples. *Manual of Environmental Microbiology*. American Society for Microbiology Press, Washington, D.C.

Holben, W.E., and D. Harris. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. *Mole. Ecol.* 4:627-631.

Holben, W.E., Noto, K., Sumino, T., Suwa, Y. 1998. Molecular Analysis of Bacterial Communities in a Three-Compartment Granular Activated Sludge System Indicates Community-Level Control by Incompatible Nitrification Processes. *Appl. Environ. Microbiol.* 64:2528-2532.

Hugenhultz, P., Goebel, B.M., Pace, N.R. 1998a. Impact of culture-independent studies of the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180:4765-4774.

Hugenholtz, P. and Pace, N.R. 1996. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends Biotech.* 14: 190-197.

Hugenholtz, P., Pitulle, C., Hershberger, K.L. and Pace, N.R. 1998b. Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* 180: 366-76.

Hurst, C.J., 1997. Introduction to Environmental Microbiology. *Manual of Environmental Microbiology.* American Society for Microbiology Press, Washington, D.C.

Jackson, C. R., Churchill, P. R. and Roden, E. E. 2000. Successional changes in bacterial structure during epilithic biofilm development. *Ecology.* 82: 212-224.

Janssen, P. H., Schuhmann, A., Bak, F., Liesack W. 1996. Disproportionation of inorganic sulfur compounds by the sulfate-reducing bacterium Desulfocapsa thiozymogenes gen. nov., sp. nov. *Arch Microbiol.* 166: 3, 184-192.

Janssen, P.H., Schuhmann, A., Morschel, E., Rainey, F.A. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl Environ Microbiol.* 63(4):1382-8.

Kjeldsen, P., Christophersen, M. (accepted) "Composition of leachate from old landfills in Denmark" Accepted to *Waste Management & Research.*

Kopczynski, E.D., Bateson, M.M.,Ward, D.M. 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Appl. Envir. Microbiol.* 60: 746-748.

Kowalchuk, G.A., Stephen, J.R., De Boer ,W., Prosser, J.I., Embley, T.M., Woldendorp, J.W. 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol.* 63: 1489-1497.

Kuske, C.R., Barns, S.M., Busch, J.D. 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. *Appl Environ Microbiol.* 63: 3614-3621.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L. and Pace, N.R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A.* 82: 6955-9.

Laurie, A.D., Lloyd-Jones, G. 1999. The phn genes of Burkholderia sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J Bacteriol.* 181(2):531-40.

Liesack, W., Weyland, H., Stackebrandt, E. 1991. Potential risks of gene amplification by PCR as determined by 16s rDNA analysis of a mixed-culture of strict barophilic bacteria. *Microb. Ecol.* 21: 191-198.

Liesack, W. and Stackebrandt, E. 1992. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.* 174: 5072-5078.

Lingens, F., Blecher, R., Blecher, H., Blobel, F., Eberspächer, J., Fröhner, C., Görisch, H., Görisch, H., Layh, G. 1985. *Phenylobacterium immobile* gen. nov., sp. nov., a gram-negative bacterium that degrades the herbicide chloridazon. *Int. J. Syst. Bacteriol.*, 35, 26-39.

Lipski, A., Reichert, K., Reuter, B., Spröer, C., Altendorf, K. 1998 Identification of bacterial isolates from biofilters as *Paracoccus alkenifer* sp. nov. and *Paracoccus solventivorans* with emended description of *Paracoccus solventivorans. Int. J. Syst. Bacteriol.*, 48, 529-536.

Liu, Y., Balkwill, D.L., Aldrich, H.C., Drake, G.R. Boone, D.R. 1999. Characterization of the anaerobic propionate-degrading syntrophs *Smithella propionica* gen. nov., sp. nov. and *Syntrophobacter wolinii. Int. J. Syst. Bacteriol.*, 49, 545-556.

Lloyd-Jones, G. and Lau, P.C. 1998. A molecular view of microbial diversity in a dynamic landfill in Quebec. *FEMS Microbiol Lett.* 162:219-26.

Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A. and Goodwin, S. 1993. *Geobacter metallireducens* gen. nov., sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch. Microbiol.*, 159, 336-344.

Ludvigsen, L., Albrechtsen, H., Ringelberg, D.B., Ekelund, F., Christensen, T.H. 1999. Distribution and Composition of Microbial Populations in a Landfill Leachate Contaminated Aquifer (Grindsted, Denmark). *Microb Ecol.* 37:197-207

Mccammon, S.A. and Bowman, J.P. 2000. Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov. and *Flavobacterium xanthum* sp. nov., nom. rev., and reclassification of [*Flavobacterium*] *salegens* as *Salegentibacter salegens* gen. nov., comb. nov. *Int. J. Syst. Evol. Microbiol.*, 50, 1055-1063.

Muyzer, G., De Waal, E., Uitterlinden, G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700.

Muyzer, G., Hottentrager, S., Teske, A. and Wawer, C. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – A new molecular approach to analyze the genetic diversity of mixed microbial communities. In A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn ed. Molecular microbial ecology manual 3.4.43 Kluwer Academic Publishers, Dordrecht, The Netherlands. 1-23.

Muyzer, G., Teske, A., Wirsen, C.O. and Jannasch, H.W. 1995. Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol.* 164: 165-72.

Muyzer, G., and Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis in microbial ecology. *Antonie Leeuwenhoek* 73:127–141.

Myers, R.M., Fischer, S.G., Lerman, L.S. and Maniatis, T. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* 13: 3131-3145.

Myers, R.M., Maniatis, T. and Lerman, L.S. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol.* 155: 501-527.

McCaig, A. E., Glover, L. A., Prosser, J. I. 1999a. Molecular Analysis of Bacterial Community Structure and Diversity in Unimproved and Improved Upland Grass Pastures. *Appl. Environ. Microbiol.* 65: 1721-1730.

McCaig, A.E., Phillips, C.J., Stephen, J.R., Kowalchuk, G.A., Harvey, S.M., Herbert, R.A., Embley, T.M. and Prosser, J.I. 1999b. Nitrogen cycling and community structure of proteobacterial beta-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. *Appl Environ Microbiol.* 65: 213-20.

Nakagawa, Y., Sakane, T., Yokota, A. 1996. Transfer of "*Pseudomonas riboflavina*" (Foster 1944), a gram-negative, motile rod with long-chain 3-hydroxy fatty acids, to *Devosia riboflavina* gen. nov., sp. nov., nom. rev. *Int. J. Syst. Bacteriol.*, 46, 16-22.

Nalin, R., Simonet, P., Vogel, T.M. Normand, P. 1999. *Rhodanobacter lindaniclasticus* gen. nov., sp. nov., a lindane-degrading bacterium. *Int. J. Syst. Bacteriol.*, 49, 19-23.

Nüsslein, K. and Tiedje, J. 1998. Characterization of the Dominant and Rare Members of a Young Hawaiian Soil Bacterial Community with Small-Subunit Ribosomal DNA Amplified from DNA Fractionated on the Basis of Its Guanine and Cytosine Composition. *Appl. Envir. Microbiol.* 64: 1283-1289.

Nüsslein, K. and Tiedje, J. 1999. Soil Bacterial Community Shift with Change from Forest to Pasture Vegetation in a Tropical Soil. *Appl. Envir. Microbiol.* 65: 3622-3626.

Olsen, G.J., Lane, D.J., Giovannoni, S.J., Pace, N.R. and Stahl, D.A. 1986. Microbial ecology and evolution: a ribosomal RNA approach. *Annu. Rev. Microbiol.* 40:337-65.

Ovreas, L., Forney, L., Daae, F.L. and Torsvik, V. 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol.* 63: 3367-73.

Ovreas, L., and Torsvik, V. V. 1998. Microbial Diversity and Community Structure in Two Different Agricultural Soil Communities. *Microb. Ecol.* 36: 303-315.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science.* 276: 734–740.

Pace, N.R., Stahl, D.A., Lane, D.J. and Olsen, G.J. 1986. The analysis of natural microbial populations by ribosomal RNA sequences. *Adv. Microbiol. Ecol.* 91: 1-55.

Palmisano, A.C., Maruscik, D.A. and Schwab, B.S. 1993. Enumeration of fermentative and hydrolytic micro-organisms from three sanitary landfills. *Journ. Gen. Microbiol.* 139: 387-391.

Palmisano, A. C. and Barlaz, M. A. 1996. "Introduction," in Solid Waste Microbiology, A. C. Palmisano and M. A. Barlaz, ed., CRC Press, Boca Raton, FL, p. 1 - 30.

Qian, X. and Barlaz, M.A. 1996. Enumeration of Anaerobic Refuse-Decomposing Microorganisms on Refuse Constituents. *Waste Manage. Res.*14:151-161.

Roling, W.F., van Breukelen, B.M., Braster, M., Goeltom, M.T., Groen, J. and van Verseveld, H.W. 2000. Analysis of microbial communities in a landfill leachate polluted aquifer using a new method for anaerobic physiological profiling and 16S rDNA based fingerprinting. *Microb Ecol.* 40: 177-188.

Roszak, D.B. and Colwell, R.R.. 1987. Survival Strategies of Bacteria in the Natural Environment. *Micro. Rev.* 51: 365-379.

Samain, E., Dubourguier, H.C., Albagnac, G. 1984. Isolation and characterisation of *Desulfobulbus elongatus* sp. nov. from a mesophilic industrial digester. *Syst. Appl. Microbiol.*, 5, 391-401.

Sambrook, J., Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Springs Harbor Laboratory, Cold Springs Harbor, N.Y.

Santo Domingo, J.W., Kaufman, M.G., Klug, M.J., Holben, W.E., Harris, D., Tiedje, J.M. 1998. Influence of diet on the structure and function of the bacterial hindgut community of crickets. *Mole. Ecology.* 7: 761-767.

Schafer, H., Servais, P. and Muyzer, G. 2000. Successional changes in the genetic diversity of a marine bacterial assemblage during confinement. *Arch Microbiol.* 173: 138-45.

Schöcke, L. and Schink, B. 1997. Energetics of methanogenic benzoate degradation by *Syntrophus gentianae* in syntrophic coculture. *Microbiology* 143: 2345-2351

Sheffield, V.C., Cox, D.R., Lerman, L.S. and Myers, R.M. 1989. Attachment of a 40-base pair G+C-rich sequence (GC clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci U S A.* 86: 232-236.

Stackebrandt, E. and Goebel, B.M. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. System. Bacteriol.* 44: 846-849.

Stackebrandt, E, Liesack ,W., Goebel, B.M. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* 7: 232-236.

Staley, J.T. and Konopka, A.E. 1985. Measurement of in situ activities of heterotrophic microorganisms in aquatic and terrestrial habitats. *Ann. Rev. Microbiol.*, 39: 321-46.

Torsvik ,V., Goksoyr, J., Daae, F.L. 1990a. High diversity in DNA of soil bacteria. *Appl Environ Microbiol.* 56:782-787.

Torsvik, V., Salte, K., Sorheim, R., Goksoyr, J. 1990b. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Appl Environ Microbiol.* 56: 776-781.

Teske, A,, Sigalevich, P., Cohen, Y., Muyzer, G. 1996. Molecular identification of bacteria from a co-culture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Appl Environ Microbiol.* 62: 4210-4215.

U. S. Environmental Protection Agency, Characterization of Municipal Solid Waste in the United States: 1999 Update: Executive Summary, Office of Solid Waste.

van Verseveld, H. W., van Breukelen, B. M., Röling, W. F. M., Braster, M., Soeteman, A. A. , Groen, J. 1999. In-Situ Bioremediation at Old Dutch Landfills: Plume Delineation, Intrinsic Degradation Capacity and Determination of Microbial Community Structure. Remediation of Polluted sites Conference, Fermantle West Australia. March 21-25, 1999.

Wang, G. and Wang. C.-Y. 1997. Frequency of formation of chimeric molecules as a consequence of PCR coamplification of 16s rRNA genes form mixed bacterial genomes. *Appl Environ Microbiol.* 63: 4645-4650.

Wang, E.T., van Berkum, P., Sui, X.H., Beyene, D., Chen, W.X. Martinez-Romero, E. 1999. Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. Int. J. Syst. Bacteriol. 49, 51-65.

Ward, D.M., Bateson, M.M., Ferris, M.J., Nold, S.C. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol. Mol. Biol. Rev.* 62:1353-1370.

Ward, D.M., Bateson, M.M., Weller, R., Ruff-Roberts, A.L. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. p. 219-286 In K.C. Marshall (ed), *Advances in Microbial Ecology.* Vol. 12. Plenum Press, New York, N.Y.

Westlake, K., Archer, D.B., Boone, D.R. 1995. Diversity of Cellulolytic Bacteria in Landfill. *Journ. of Appl. Bacter.* 79:73-79.

Wise, M. G., Vaun Mcarthur, J., Shimkets L. J. 1999. Methanotroph Diversity in Landfill Soil: Isolation of Novel Type I and Type II Methanotrophs Whose Presence Was Suggested by Culture-Independent 16S Ribosomal DNA Analysis. *Appl. Environ. Microbiol.* 65: 4887–4897.

Woese, C.R. 1987. Bacterial Evolution. *Microbiol. Rev.* 51: 221-271.

Woese, C.R. 1993 "*Anaeroflexus maritimus*" str. PL12FS DSM 2831. Unpublished

Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., Nishiuchi, Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol. Immunol.*, 39, 897-904.