ASSESSING DIFFERENTIAL TRANSPORT OF CHEMICAL AND BIOLOGICAL CONSTITUENTS IN GROUNDWATER IMPACTED BY A MUNICIPAL WASTE WATER TREATMENT SYSTEM

by

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ABSTRACT

An increasing number of homes and subdivisions rely on septic systems and decentralized wastewater systems to treat domestic sewage. These treatment systems can release poorly treated wastewater if regular maintenance is not performed or maximum loading capabilities are exceeded. To better quantify signs of wastewater impacts on groundwater, this study sampled groundwater down-gradient of an aerated sewage lagoon which discharges high concentrations of waste to groundwater. The intent is to better understand how chemical and biological constituents originating from wastewater behave and move through groundwater. Groundwater samples were collected in four locations down-gradient from the infiltration beds every other week from June – August 2012. Samples were analyzed for chloride, boron, nitrate-N, total coliforms, *Escherichia coli,* and *Bacteroides* species using AllBac and HF183 primers. The *Bacteroides* genus is abundant in the gut and specific to the guts of warm blooded mammals, the species targeted by AllBac is present in all mammals and HF183 is specific to humans. It was hypothesized that chloride and boron would behave similarly and conservatively moving down-gradient and nitrate-N would behave non-conservatively due to its ability to denitrify under reduced conditions. It was also hypothesized that *Bacterodies* species (AllBac and HF183), would be a better microbiological indicator of wastewater. Findings from this study indicated strong relationships between chloride and boron as they move down-gradient from the source (R^2=0.9901, p = 0.003) and weaker relationships between chloride and nitrate-N (R^2=0.4371, p = 3.8e-6). Additional evidence indicated nitrate-N may be behaving non-conservatively as it moves down-gradient due to denitrification in areas with dissolved oxygen levels <0.5 mg/L, but it may be behaving conservatively further down-gradient after denitrification has occurred. The microbiological analyses indicated that *Bacteroides* may not be a better indicator organism in areas with low *Bacteroides* presence. However, *Bacteroides* (AllBac) had a strong relationship with total coliforms (R^2=0.785, p=1.25e-4) and *E. coli* (R^2=0.750, p= 6.57e-10) indicating similar behavior in groundwater. Findings from this study allow for a better understanding of the transport of these biological and chemical constituents in groundwater, and further elucidation would be possible through development of a groundwater model.

CHAPTER ONE

INTRODUCTION

Purpose of Study

The extent and severity of groundwater contamination from domestic wastewater disposal across Montana and globally is on the rise. Over 50% of the U.S. population relies on groundwater as their source of domestic water and 15% of the U.S. population relies on a private well system (USEPA 2002a). With this large number of households relying on groundwater as a source of drinking water, contamination issues need to be better understood and addressed. Globally, groundwater pollutants from wastewater are on the rise and include nitrate-N, chloride, biological constituents such as *Escherichia coli* (*E. coli*) O157:H7*, Cryptosporidium, Giardia, Legionella,* and Hepatitis A; all of which pose risks to human health (Kelly 2008; Drake and Bauder 2005; Moolenaar 2011; Brunkard et al. 2011). In many areas, this increase can be attributed to developments with a high density of septic systems (Yates 1985). It has been estimated that 750,000 to 5.9 million people contract illness from contaminated groundwater every year (Macler and Merkle 2000). The Center for Disease Control reports the majority of waterborne disease outbreaks in the United States from 1971-2006 originate from groundwater sources (Moolenaar 2011).

According to the current U.S. Census, the population in Montana has increased 9.7% from 2000 – 2010 and the population in the Gallatin Valley has increased more than

35% in the same time frame (Census 2010). The number of new wells drilled during 2000-2010 was the largest increase for any decade to date (Figure 1.1).

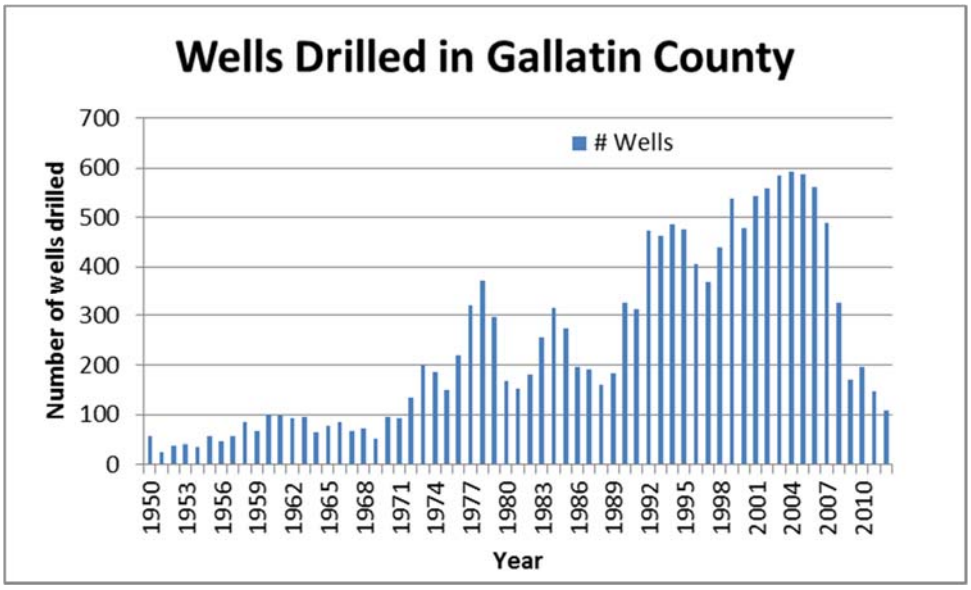

Figure 1.1: Number of wells drilled in Gallatin County from 1950-2012. Accessed from the Montana Bureau of Mines and Geology website on Sept 29, 2012.

In Montana, a 2005 study assessed the long-term trends of nitrate-N in Helena and found an increase of 28.7% in nitrate-N values from 1990-2000 (Drake and Bauder 2005). This notable nitrate-N increase in groundwater in the decade preceding the 2000-2010 boom in onsite water systems in Montana punctuates the need for timely attention to understand and address domestic wastewater groundwater contamination sources. The work presented in this thesis focuses on evaluation of analytical tools for quantifying and understanding behavior of domestic wastewater constituents in groundwater. While this study was conducted down-gradient from a wastewater lagoon, the intent is that the findings will also be relevant for assessing nonpoint source groundwater pollution from private onsite wastewater systems (septic systems).

Research Questions

The study area for this project is located immediately down-gradient of two aerated sewage lagoons, which provide primary treatment of wastewater. Effluent is then transferred to infiltration beds where it is allowed to seep into groundwater. Loading of sewage to the treatment lagoons exceeds the designed capacity of the system to properly treat the waste. Thus, the infiltration beds provide a continuous and relatively high concentration contaminant source, which allows for the evaluation of chemical and biological constituent behavior in groundwater moving down-gradient from the source. Specifically, chloride, boron, nitrate-N, dissolved oxygen, specific conductivity, total coliforms, *E. coli* and *Bacteroides* were assessed. Broader implications from this project allow for a better understanding of how biological and physical processes affect groundwater impacted by wastewater. This may provide insight on how to recognize signs of a wastewater contaminated well.

Hypothesis and Predictions

The combination of conservative and non-conservative chemical parameters (chloride, boron, nitrate-N) will allow for a better understanding of the dynamics and flow path of the sewage plume down-gradient from the source. Monitoring for biological constituents *Bacteroides* and *E. coli* in groundwater will help assess whether *Bacteroides* may be a more sensitive indicator organism than *E. coli*. Data collection in this project is structured to address the following hypotheses and predictions.

H1: Chloride, as a conservative indicator of wastewater, will have the greatest concentrations in the wells closest to the infiltration beds. Therefore, the greatest

concentrations will be in sequentially numbered Well 1 followed by Well 2, Well 3, and Well 4.

P1.1: Chloride concentrations will be greatest in wells closest to the infiltration beds (Wells 1 and 2) and have the lowest concentrations in the wells furthest from the infiltration beds (Wells 3 and 4).

P1.2: Chloride concentrations will not be related to how close the well is to the infiltration beds.

H2: Due to the relatively conservative nature of chloride and boron, a correlation will exist between the concentrations of the two parameters for all samples.

P2.1: Both chloride and boron will act conservatively as they move down-gradient and will hence display similar trends spatially and temporally.

P2.2: Chloride will act conservatively, demonstrating a consistent decrease in concentration moving down-gradient, but boron will not act conservatiely and will not be correlated to chloride concentration across spatial and temporal coordinate space.

H3: Due to the non-conservative nature of nitrate-N, concentrations will decrease more rapidly with distance from the source than chloride and boron.

P3.1: Nitrate-N is not acting consevatively in the contamination plume and exhibits a greater loss in concentration moving down-gradient than chloride and boron.

P3.2: Nitrate-N is acting conservatively, displaying a loss of nitrate-N concentrations similar to chloride and boron.

H4: *Bacteroides* analysis using AllBac and HF183 qPCR primers will be a more sensitive indicator of groundwater contamination than *E. coli* and total coliforms analysis using the IDEXX method and will be present at detectible levels when *E. coli* is absent.

P4.1: *Bacteroides* will not be detected in the water.

P4.2: *Bacteroides* species AllBac and HF183, will be detected in wells that are negative for *E. coli* and total coliforms.

P4.3: *E. coli* and total coliforms will be detected in wells that are negative for AllBac and HF183 *Bacteroides.*

H5: There will be no spatial or temporal correlations between any of the parameters.

P5: Water down-gradient of the infiltration beds is not being impacted by wastewater.

If results support H1 , then a simple two dimensional assessment of well proximity and position relative to a contaminant source and groundwater flow may be sufficient predict contamination levels. If H2 is sustained, then it is suggestive that boron is acting as conservately as chloride as it moves down-gradient. This relationship may indicate that boron is not influenced by any adsorption complexation or biological activity. This would support the assertion that the main influence on boron and chloride concentration is physical (dilution and dispersion). If results support H3 and low oxygen concentrations are recorded, it is suggestive that nitrate-N is acting non-conservately and denitrification may be occuring in the groundwater as contaminants move down-gradient within the plume. In order for denitrification to occur, a sufficient source of dissolved organic carbon (DOC) and low dissolved oxygen levels must be present. If results

support H4, it is redolent that *Bacteroides* may be a better bacterial indicator than total coliforms and *E. coli*. If results support H5 it may mean that the wells down-gradient of the infiltration beds are not being influenced by wastewater and that the conditions recorded at those wells are representative of background conditions.

CHAPTER TWO

LITERATURE REVIEW

Nationally Emerging Issues in Groundwater Quality

Although 70% of the Earth's surface is covered with water, 96.5% is saline and unusable (Shiklomanov 1993). Of the small percent that is fresh, about half is locked up in glaciers and inaccessible, leaving less than 1.8% available for human use (Shiklomanov 1993). The global demand for water is increasing at double the rate of the world population growth and groundwater levels are drastically decreasing (Ground Water Protection Council 2007). In the United States, groundwater levels have declined approximately 200 feet in Louisiana, 400 feet in Houston, Texas, 100 feet in the High Plains aquifer, more than 100 feet in some areas of the Pacific Northwest, and between 300-500 feet in the Desert Southwest (Ground-Water Depletion Across the Nation 2003; Taylor 2001; McGuire 2003; Burns 1997).

Unfortunately, this precious resource continues to be compromised and the increase in elevated contaminants in groundwater can be associated with the overuse of water resources, the lack of proper management, and anthropogenic sources such as: fertilizers, pesticides, oxides from automobiles, fixation of nitrogen in croplands, and animal manure (Rupert 2008; Puckett 2011; Murgulet and Tick 2009). The most prevalent chemical contamination issue in groundwater, globally, is elevated nitrate-N concentrations (Spalding 1993). Elevated nitrate-N concentrations, above 10 mg/L, are a health concern for pregnant women or infants as elevated levels can cause

methemoglobinemia (blue baby syndrome) which can be fatal to infants, and chronic consumption of elevated levels can lead to an increased risk of cancer (Ward 2005).

On-Site Wastewater Treatment – Individual Systems

On site wastewater treatment systems (OWTS) are commonly the only option for small developments or communities to dispose of wastewater when access to a centralized municipal system is not feasible. Conventional wastewater treatment systems can be an effective method of treating sewage if properly designed, maintained, and installed in appropriate soils (USEPA 1997). Across the nation, OWTS provide septic disposal for over 26 million households and businesses; this is equivalent to one in four households in the United States (USEPA 2002b). In Montana, approximately 39% of residents rely on OWTS and OWTS are the third largest contributor to groundwater contamination in the country (Katz, Eberts, and Kauffman 2011; USEPA 2002b). Nationally, 10-20% of OWTS fail but that percent varies by state (Katz, Eberts, and Kauffman 2011; USEPA 2002b) and over 500 communities have identified health issues due to improperly treated wastewater impacting groundwater that is used for drinking (USEPA 1996, 1997; Bremer and Harter 2012). Estimated failure rates in Montana have not been determined.

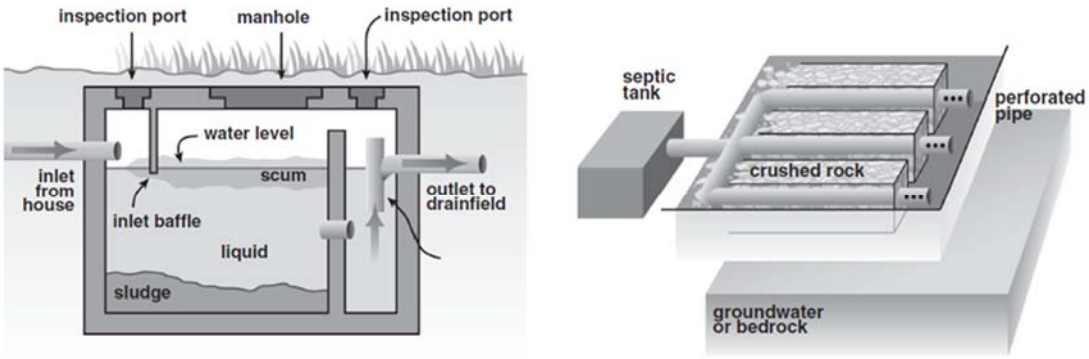

Figure 2.1: Septic System Impact on Surface Waters: A Review for the Inland Northwest 2005 Tri-State Water Quality Council, p. 4

Septic tanks are not designed to remove anything other than solids, grease and oils (Figure 2.1). The tank acts as an aerobic bioreactor by allowing the natural bacteria found in wastewater to partially digest the organic solids and the remaining effluent passes through the tank and into a soil treatment area (drainfield) where additional treatment occurs as the water moves down through the soil (Panno 2007; USEPA 2002b). If installed at proper depths, in appropriate soils, the effluent is treated in the drainfield through adsorption, filtration and other biogeochemical processes occurring in the soil (USEPA 2002b). Wastewater constituents still in the water when it reaches groundwater are diluted in the aquifer mixing zone. Conventional septic systems discharge approximately 30-95 mg/L of total nitrogen from partially treated wastewater that passes through the drainfield (Nicklin 2010; Alhajjar 1989; Walker 1973).

Decentralized Wastewater Management

Decentralized wastewater systems are defined as collecting sewage from multiple homes, a small community, or industry for treatment and disposal of wastes near the

collection site (Tchobanoglous 1995). These types of systems are considered to be public sewage systems and are defined in Montana as a system that collects, transports, treats and disposes of sewage that serves 15 or more connections or 25 or more individuals daily for at least 60 or more days in a year, and can be owned by either public or private entities (ARM 17.38.101). Decentralized wastewater systems service over 60 million people in the United States and are often the only choice in areas where it is not feasible, economically or geographically, to hook up to a central wastewater treatment plant (Crites 1998). Decentralized wastewater systems come in many forms including; lagoon systems, wetland and aquatic systems, and land treatment systems (Crites 1998). There are four main types of lagoon treatment systems, aerobic and facultative (which are commonly used in the West) and partial-mix aerated and anaerobic systems (Crites 1998). The main differences between these systems depend on the depth and the amount oxygen introduced into the system. Aerobic and facultative systems allow for bacterial and photosynthetic activity at different depths in the system whereas partial-mixed aerated and anaerobic lagoons are much deeper, contain greater anaerobic regions, and hold more sludge with longer holding times (Crites 1998).

Facilitation of different biological processes is the fundamental treatment approach behind all types of decentralized wastewater systems. Aerobic lagoons, the type of system being used at the study area for this project, are designed to maximize the photosynthetic activity of algae to increase the oxygen levels which increases the efficiency of the microorganisms to degrade the organics (Crites 1998). Despite the treatment processes in lagoons, pathogens, nitrogen, phosphorus, ammonium, trace

metals, and suspended solids are commonly present in conventional treatment lagoon effluent (Cameron 2003; Katz, Eberts, and Kauffman 2011).

Typical concentrations of untreated wastewater range from 20-85 mg/L for nitrate-N, 30-100mg/L for chloride, and 10^3-10^7 cells/100mL of fecal coliforms and 10^5-10^8 *E. coli* cells/100mL (Tchobanoglous 1991; McCray 2009). Properly treated effluent from an aerated lagoon should produce nitrate-N levels ranging from 7.2 mg/L to 10.1 mg/L but these levels depend on numerous factors such as loading quantities and concentrations, hold times in the lagoons, weather, algal growth, and type of aeration (Reed 1995; Crites 1998). The levels of BOD (biological oxygen demand) from aerobic lagoon effluent generally range from 20-40 mg/L and total suspended solids range from 80-140 mg/L (Crites 1998; Reed 1995).

Improperly treated wastewater can impact groundwater in numerous ways. Wastewater can contribute pathogens, excess nutrients such as nitrogen or phosphorus, household chemicals from cleaners and detergents, and pharmaceuticals to groundwater, compromising groundwater quality (Müller et al. 2012; Walker 1973; Vengosh 1994). Impairments from groundwater have the potential to impact surface water too, as groundwater and surface water are inherently linked as groundwater feeds many seeps, creeks and streams (Hackett et al. 1960; Kendall 1998; Ground Water Protection Council 2007; Winter 1998).

Groundwater contamination from sewage is rarely evident though simple human observation of the water. However, in extreme instances, strong odors, changes in color, or an oil film on the surface can be immediate indicators of heavy contamination. In

most instances, sewage contamination is difficult to detect especially in cases where sewage is impacting but not heavily contaminating water quality. Risks associated with contamination can be present even when water quality issues are not readily detected. For instance, viral pathogens, which often go undetected, accounted for 5% and potentially up to 28% of the waterborne outbreaks during 1987-1996 in the United States (Laws 2000). In Delhi, India in 1955, a public drinking water supply treated with chlorine had levels of fecal indicator bacteria in the water below applicable human health thresholds, but over 20,000 cases of hepatitis A were recorded from users of this water supply (Laws 2000). This situation highlights the fact that even when traditional water quality parameters are at acceptable levels, risk to human health from contamination can exist.

Parameters for Evaluating Wastewater Contamination

Identifying the source of contamination is crucial for informing management decisions and implementing best management practices to address a pollution issue (Section (2012). Knowing the source of the contamination is beneficial because it can provide a better indication of the health risk associated with the parameter (Kinzelman 2012). For instance, consumption of groundwater with high nitrate-N concentrations originating from fertilizers may carry a higher risk of exposure to pesticides and not pathogens, while high nitrate-N concentrations from wastewater may carry a greater risk of exposure to pathogens but with little risk of exposure to pesticides (Puckett 2011; Yan and Sadowsky 2007). Numerous studies across the nation and around the globe have assessed which parameters are useful indicators of wastewater contamination. Some

examples of these parameters include: nitrate-N, chloride, nitrogen and oxygen isotopes, bromide, boron, pathogens, dissolved organic carbon (DOC) and other chemicals that originate primarily from anthropogenic sources such as pharmaceuticals, caffeine, and sucrose (Kendy 2001; Mullaney 2009; Quast 2006; Goldberg 1985; Cao, Griffith, and Weisberg 2009; Daughton 1999). To overcome the limitations of interpretation of each parameter, multiple parameters are often assessed together to gain greater insight on the source of the contamination (Seiler 1999).

Nitrate-N is one of the most commonly used parameters. Aerated sewage lagoons and individual septic systems are not built to remove nitrate-N or chloride (Crites 1998). Permitting of septic systems is based on the concept that nitrate is diluted by groundwater within a designated mixing zone and theoretically does not impact water sources down gradient, but heavily contaminated plumes in groundwater can contain elevated levels of nitrate-N for up to 130 meters (Robertson 1991). However, because nitrate-N can also come from agricultural sources, it is not a reliable indicator of wastewater contamination (Rupert 2008; Puckett 1994).

Chloride is a highly soluble, conservative tracer that is not lost due to biogeochemical processes, is present in foods, liquids, a major component in soft water systems (magnesium and calcium are exchanged for sodium chloride) and becomes concentrated in wastewater (Crites 1998). In 2005, 3.1% of the salt use in the United States was for the treatment of hard water (Kostick, Milanovich, and Coleman 2007). Elevated concentrations of chloride can be indicative of wastewater influence but other sources of chloride do exist (Mullaney 2009; Brown 2009; Kendy 2001). Other sources

of chloride include atmospheric deposition, road salt, agriculture fertilizers (potassium chloride), and leaching of chloride-containing rocks (Liao et al. 2012; Mullaney 2009; Crites 1998). Due to the fact that nitrate-N and chloride can originate from multiple sources, other parameters are used in conjunction to help decipher if the source is from wastewater effluent.

Boron can be another conservative parameter to assess wastewater impacts (Quast 2006; Vengosh 1994). Boron, which is highly soluble, can originate from anthropogenic sources such as sodium perborate or boric acid which is found in many detergents and cleaners (Vengosh 1994; Bassett 1995). Similar to chloride and nitrate-N, boron is not removed in the treatment of wastewater and is present when wastewater percolates into the ground (Bassett 1995). Boron is highly mobile but adsorption onto soil particles has been found in clay soils where iron, aluminum, oxides and hydroxides are present with maximum adsorption occurring between a pH of 8-10 (Bundschuh 1993; Goldberg 1985).

Isotope analysis is commonly used in conjunction with chemical analyses to assess sources of nutrients or chemicals in groundwater. Some of the most common isotopes used for analysis of wastewater contamination in groundwater are nitrate-N($\delta^{15}N/\delta^{14}N$), oxygen ($\delta^{18}O/\delta^{16}O$), and boron ($\delta^{11}B/\delta^{10}B$) isotopes (Aravena 1993; Fogg 1998; Kendy 2001; Katz, Eberts, and Kauffman 2011; Quast 2006; Vengosh 1994). Isotopes are atoms with slight differences in their atomic mass due to differences in the number of neutrons in the element (Aravena 1993). Differences in concentrations of isotopes of the same element can be produced by biological, physical and chemical processes where one isotope is selectively used over another causing a depletion of one

and an enrichment of another (Fogg 1998; Xue et al. 2009). Distinctions in the isotopic composition of various pools of elements in nature can provide a useful tool for understanding sources and pathways of elements through the hydrologic cycle (Kendall 1998). Atmospheric nitrogen, fertilizers, sewage, animal waste, soils and organic matter all have characteristic isotopic signatures (Kreitler 1979; Fogg 1998). However, in the case of stable nitrogen isotopes, many of the isotopic signatures may overlap making it difficult to discern the exact source of the nitrogen (Xue et al. 2009). Another limitation with nitrogen isotopes is that soil nitrogen cycling can change the original $\delta^{15}N$ and $\delta^{18}O$ values leading to biased results (Xue et al. 2009; Kendall 1998; Kellman 2005). Employing isotopic ratios of multiple elements can help resolve source and flux questions, but these investigations must be done in the context of the important pathways and fluxes in the study environment (Xue et al. 2009).

Other wastewater indicator parameters include anthropogenic constituents that are not found naturally in the environment such as pharmaceuticals, optical brighteners, chemicals in personal-care products and cleaning products, caffeine, sucralose and volatile organic compounds (VOCs) (Cao, Griffith, and Weisberg 2009; Seiler 1999; Squillace 2004; Daughton 1999; Oppenheimer et al. 2011). If any of these parameters are detected, it would imply that anthropogenic sources are the cause of the contamination. Research in this realm is relatively new and with the increasing sensitivity of analytical instruments, it is possible to detect trace amounts (nanograms per liter) of these compounds (Seiler 1999). Testing for these anthropogenic constituents in

conjunction with traditional parameters, like nitrate, can be especially effective at determining the source of contamination (Seiler 1999).

Microbiological Indicators in Groundwater

Traditionally, indicator organisms such as total coliforms, *Escherichia coli*, fecal streptococci, and enterococci are used to determine if groundwater is contaminated by wastewater (Crites 1998). These indicator organisms are often cultured and quantified to determine which organisms are present; but unfortunately, these culturing techniques do not indicate the source of the bacteria and there are limitations with culturing (Balleste et al. 2010; Bower et al. 2005; Bernhard and Field 2000). Culturing techniques can be biased, under representing or over representing the bacteria being cultured, with the accuracy of culturing bacteria often in question (Bernhard and Field 2000). An alternative to culturing that is also helpful in source determination is to use molecular techniques such as microbial source tracking (MST). MST in this context uses library independent, culture free techniques to analyze the 16S rDNA sequence of bacteria to determine molecularly 'who is there'(Hagedorn, Blanch, and Harwood 2011).

In 2000, Oregon State University researchers, Anne Bernhard and Katherine Field, set out to determine which sources of fecal contamination were polluting Tillimook Bay, causing shellfish closures and huge economic losses. Knowing the source of contamination is beneficial to assist management decisions to help remediate the situation (Harwood et al. 2009). Bernhard and Field developed a MST method that used the 16S rDNA gene of *Bacteroides. Bacteroides* is a taxonomic class, within the phylum *Bacteroidetes* and is a gram negative, non-spore forming anaerobic bacterium present in

all mammalian microbiomes (Wexler 2007). Bernhard and Field developed primers for *Bacteroides* species that were specific to humans and the rumen. *Bacteroides* was chosen due its host-specific distributions, its predominance in the guts (~25% of the bacteria found in the colon are *Bacteroides)*, and the high amount of genomic sequencing data for this specific gene that is available in online databases (Dick and Field 2004; Wexler 2007).

Since the original study in 2000, numerous related studies have tested the effectiveness of these primers in surface water spatially in the US and throughout the world and other primers (pig, cat, dog, waterfowl, fish) have been developed (Balleste et al. 2010; Bower et al. 2005; Field and Samadpour 2007; Shanks et al. 2011; Fremaux et al. 2009; Fogarty 2005; Layton et al. 2006; Walters 2009). In 2011, two separate studies were the first to confirm the presence of *Bacteroides* in groundwater that was suspected to be influenced by septic effluent (Johnson et al. 2011; Knappett et al. 2011). The two studies searched for the presence of *Bacteroides*, but the researchers did not specifically use *Bacteroides* as an indicator organism to determine wastewater impacts (Johnson et al. 2011; Knappett et al. 2011). The work conducted in the current project is one of the first efforts to use *Batceroides* as an indicator organism of wastewater contamination in groundwater.

River Rock Study Site

River Rock Subdivision, in Belgrade, Montana has over 1200 homes, town homes, and condos and is one of the largest subdivisions in Belgrade. The subdivision

was originally approved in 1978 and in 1999 the subdivision development began (Morrison-Maierle 2010). River Rock was allowed to build in accordance with the 1978 regulations for wastewater permiting which constisted of primary and a secondary aerated sewage lagoons and seven infiltration beds where effluent seeps into the ground (District 2012) . The initial approvals contained a clause to maintain land to dispose of septage by spray irrigation, but this requirement was removed in 2003 (Morrison-Maierle 2010). In its place, the Montana Department of Environmental Quality requested that River Rock voluntarily submit an application for a Montana Groundwater Pollution Control System (MGWPCS) permit (Morrison-Maierle 2010). After 2003, monitoring wells down-gradient from the treatment lagoons were periodically monitored and revealed nitrate-N levels exceeding 10mg/L, which is the EPA maximum contaminant level (MCL) set for drinking water human health standards.

Effluent concentrations of nitrogen, fecal coliforms, biological oxygen demand, and total suspended solids continually increased from 1999- 2007 and eventually surpassed influent concentrations; this occurred when the subdivision was completely built out (which increased loads), and the retention times were reduced, which led to increasing effluent concentrations (Regensburger 2009). Nitrogen concentrations over 60 mg/L were recorded and fecal coliforms counts surpassed 7,000 organisms/100mL during testing in 2007 (Regensburger 2009). Although monitoring was supposed to continue, nitrogen and total coliforms were not sampled after October of 2007.

In August of 2007, homeowners downgradient from the River Rock treatment lagoons and infiltration beds became suspicious of their water quality after the water

started smelling of sewage and a child had become sick and was hospitalized (personal communication with homeowner). After initial investigations and water testing, it was suspected that the River Rock wastewater treatment lagoons and infiltration beds were not adequately treating the current loads. It is speculated that after heavy rain storms in early June of 2007, storm water was releaesed into the lagoons which overwhelmed the treatment systems releasing scarcely treated sewage into the ground (Report 2008). Water quality results from private wells down-gradient of the lagoon indicated influence from wastewater having undergone little or no treatment.

In 2008, half a dozen homeowners down-gradient of the sewage lagoons sued the River Rock Water and Sewer District for contaminating their wells ("Homeowners sue River Rock over pollution" 2008). This lawsuit prompted the Gallatin Local Water Quality District (GLWQD) to study water quality conditions near River Rock. During 2008, GLWQD tested multiple homes and monitoring wells throughout the year down-gradient from the treatment lagoons as well as areas surrounding the River Rock Subdivision to better understand the background levels of parameters. The GLWQD found nitrate-N values over 40 mg/L at the monitoring wells down-gradient of the infiltration beds and over 20 mg/L of nitrate-N in a shallow well of a homeowner directly down-gradient from the lagoons (‘Well 2’ in this report). Chloride levels over 30 mg/L and boron levels over 100 µg/L were also noted, both of which far exceeded the reported background levels.

CHAPTER 3

STUDY AREA AND METHODS

Study Area

One of the first and most comprehensive groundwater studies performed in the Gallatin Valley was a USGS study performed in the 1950s by O.M. Hackett. The three year study broke down the Gallatin Valley into four areas and ten subareas in which comprehensive descriptions of geology, groundwater availabilty, movement, recharge, storage, discharge rates, and water quality were assessed (Hackett et al. 1960). Years later, Dunn (1978), S.E. Slagle (1995) and Elosie Kendy (2001) performed simliar groundwater studies in the Gallatin Valley and generally confirmed the findings from the Hackett study. Dunn reassessed some of the same wells used in the Hackett study as well as some new wells and concluded that there was no significant change in the groundwater levels and groundwater quality since the Hackett study 23 years prior (Dunn 1978). In 1995, Slagle slightly altered the groundwater flow contours in the Gallatin Valley, but today the Hackett and Slagle groundwater contours are both used in groundwater studies.

Gallatin County is the fastest growing county in Southwestern Montana. The county encompasses 6,817 km² (2,632 square miles) and resides on the northern end of the Rocky Mountains, east of the Continental Divide (Kendy 2001). Gallatin County is home to mountainous regions and wide alluvial valleys, and is the fifth most populous county in Montana. The largest city centers in the county include Bozeman, Belgrade, Three Forks, and Big Sky.

The Gallatin Valley is an intermontane basin that is almost entirely contained within Gallatin County (Figure 3.1). Gallatin Valley is about 40 km long by 32 km wide with an area of approximately 1,400 sq km (Hackett et al. 1960). It is flanked by the Bridger Mountain Range on the northeast, the Madison Plateau on the west, and the Gallatin Range to the south. Elevations within the watershed range from 1,250 meters at the mouth to 3,048 meters at the highest elevation in the Gallatin Range, with the Gallatin Valley at approximately 1,920 meters (Kendy 2001). The Gallatin Valley is drained by various tributaries flowing predominantly north to confluence with the East Gallatin and subsequently the Gallatin River (Kendy 2001).

Climate in the Gallatin Valley is typical of mid-altitude montane regions in the Rocky Mountains. Cold, snowy winters, and mild, dry summers are characteristic in this region. The 112 year average daily temperature ranges from -5.73°C in January and up to 18.92°C in July with annual precipitation of 46.5 cm and 215.4 cm of snowfall (*Western Regional Climate Center* 2012).

Data from this study was collected in Belgrade, Montana. Belgrade is located in the Gallatin Valley at 45.78°N 111.19°W, at an elevation of 1,357 meters. Belgrade is a fast growing community and has seen a 54% population increase since 1990 (*Belgrade Chamber of Commerce* 2012). The 2010 US Census estimates the population of Belgrade to be 7,389 individuals. A large portion of this growth has occurred in high density subidivisions that house hundreds of residents.

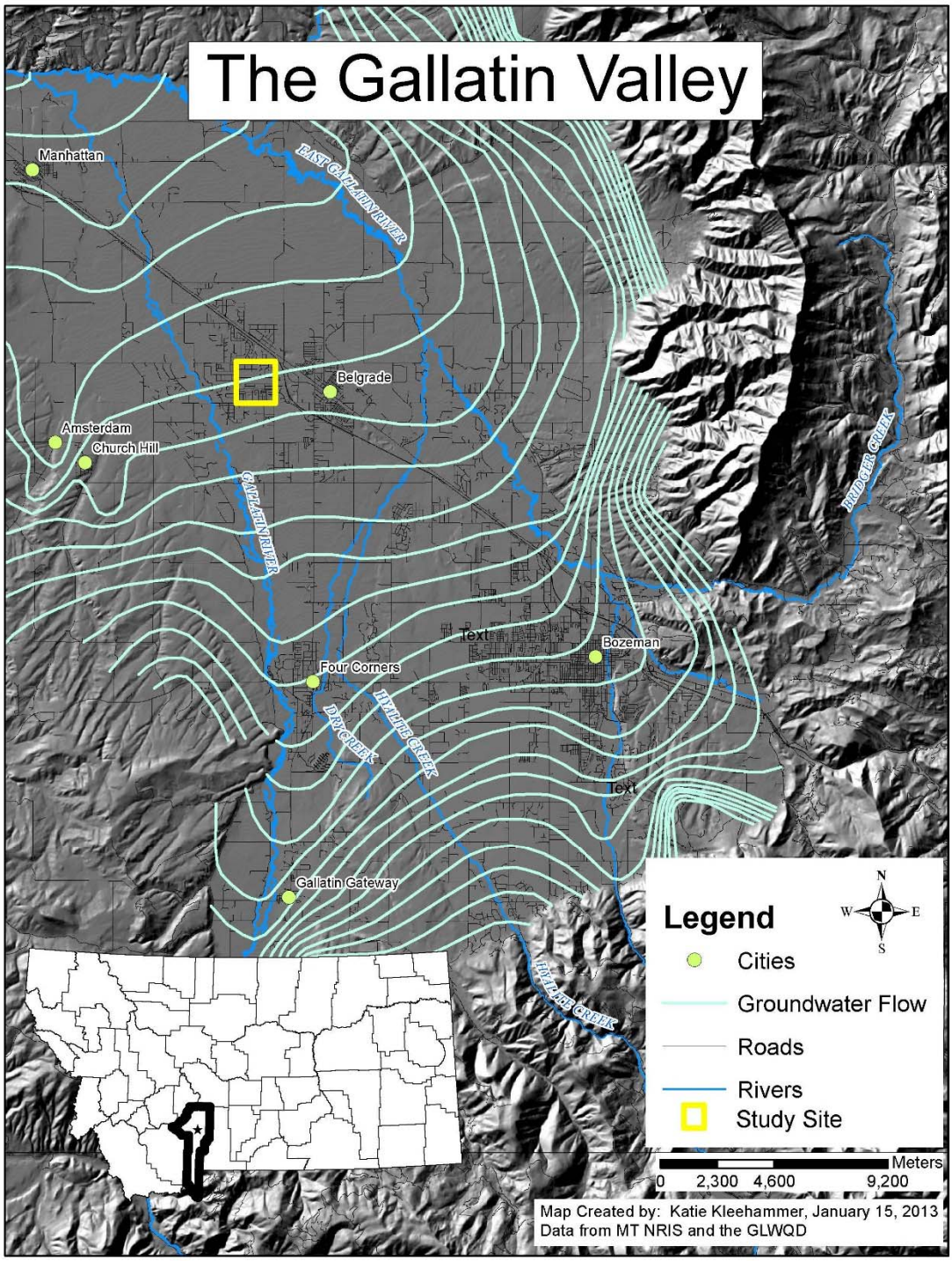

Figure 3.1: Map of the Gallatin Valley (groundwater contours from Hackett study and digitized in ArcMap by the Gallatin Water Quality District)

Geology of the Belgrade Area

The geology in Belgrade is predominatley Quaternary alluvium deposits from the Gallatin River and consists of large, coarse bed material such as cobbles, gravel, and sand (Hackett et al. 1960; Kendy 2001). The Quaternary allumvium in this area ranges from 21 meters deep a few miles south of Belgrade to over 122 meters deep a few miles north of Belgrade (Hackett et al. 1960; English 2007). These basin deposits usually result in unconfined aquifers that are highly productive. Pump tests used in the Belgrade area during the Hackett study found coefficients of transmissitvity to average about 70,000 gpd per foot (Hackett et al. 1960). The groundwater in the Quaternary alluvium deposits underneath Belgrade is the most reliable groundwater resouce for the Gallatin Valley (Kendy 2001).

Groundwater in the Belgrade Area

Groundwater generally flows from southeast to northwest across the Gallatin Valley, following the slope of the valley (English 2007). Near Belgrade, the Gallatin River becomes a losing stretch which recharges groundwater and alters the groundwater flow direction to a more northeasterly direction than the northwest flow that dominates the majority of the valley (English 2007). Groundwater in the Gallatin Valley is recharged from the Gallatin River, mountain front stream recharge from various tributaries, subsurface flow from bedrock, and infiltration of precipitation (Covino and McGlynn 2007; Kendy 2001). Recharge also occurs from a network of irrigation conveyances, the extent of which is currently being investigated by the Montana Bureau of Mines and Geology Ground Water Investigations Project (GWIP).

Depth to watertable varies considerably in the Gallatin Valley. In areas near the Gallatin and East Gallatin Rivers the watertable is approximately 3 meters below the surface resulting in subirrigation of agricultural fields in some areas. In some of the mountaineous areas, the water table can be over 30 meters below surface and experience fluctuations of more than 12 meters. Areas of Belgrade have seen high fluctuations in watertable depth due to irrigation influences; over a 12 meter change in groundwater levels have been reported during the irrigation season, especially during times when flows are low in the Gallatin River (Dunn 1978). Continuous static water levels from a well nearby the River Rock subdivision (Cobblestone) showed minimual fluxuation in static water level (< 2 meters) from June 2011 through 2012 (data from the Gallatin Local Water Quality District).

Sample Site

The study area for this project is located down-gradient of the River Rock subdivision in Belgrade, MT. The area is characterized by continuous loading of wastewater effluent to groundwater with elevated nitrate-N and chloride. Water quality sampling for this study was conducted at Wells 1-4 while Wells 5-7 were only used to assess groundwater flow direction (Figure 3.2). Wells used in this study pull water from the same aquifer (GWIC well logs). Well 1 pulls water from 2.7 meters below the water table and is screened 3 meters from the bottom, Well 2 (the deepest well sampled) pulls water from approximately 15.8 meters below the water table. Wells 3 and 4 pull water from approximately 10.7 meters and 13 meters below the water table (Figure 3.3).

Figure 3.2: Study site map

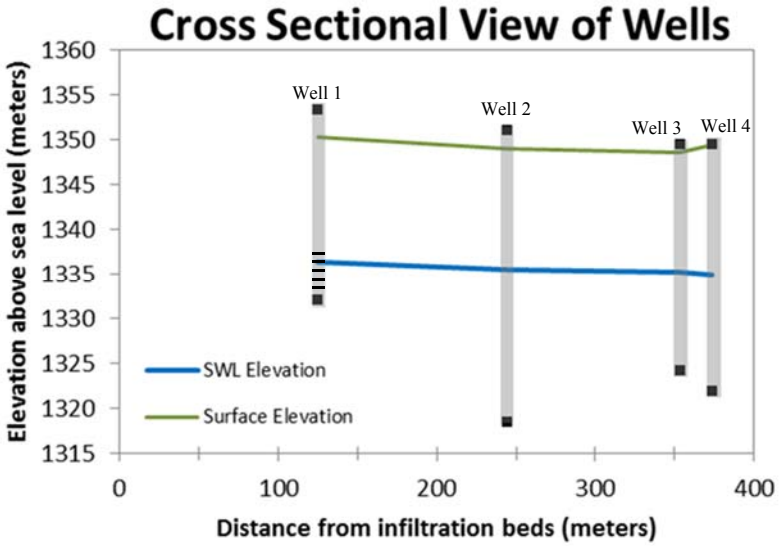

Figure 3.3: Cross sectional view of wells sampled for water quality at study site

All wells were installed before initiation of this study and are down-gradient of the infiltration beds (Figure 3.2). Well 1 is the closest well to the infiltration beds (125 m) and was drilled by homeowners as a monitoring well in response to concerns about water quality impacts from the infiltration beds. Well 2, approximately 244 m away from the infiltration beds, is a domestic well originally drilled in 2000 to serve the private residence on the property. After the suspected contamination in 2007, use of the well was discontinued. For the purpose of this project, a temporary submersible pump was installed in Well 2 to facilitate water sample collection. Well 3, approximately 353 m away from the infiltration beds, is also a domestic well that serves a household across the street from Well 2. Water from this well was used for all purposes until 2007 and is currently used for basic household needs with the exception of drinking and cooking. Well 4, approximately 374 m from the infiltration beds, is a domestic well producing water used for household consumption. The well is believed to be on the fringe of the contamination plume and residents at this site treat their water with a reverse osmosis system before consumption.

Groundwater Characteristics

Hydraulic head (head) is the sum of the elevation head plus pressure head which is a measurement of the total pressure at a certain point above a datum (Schwartz & Zhang, 2003). Water flows from high pressure to low pressure (or high head to low head), and by determining the hydraulic head at each well, flow direction can be estimated. For this study, hydraulic head was calculated by subtracting the static water level (distance from well head to water level) in the well from the elevation of the well

head using sea level as the datum. Well head elevations were measured with a survey grade GPS and static water levels were recorded with an electric tape on July 2, 2012.

The simplest methods for assessing flow direction can be visualized with a plan view and accounts for the two horizontal dimensions. Flow in the vertical direction can also be important and must be evaluated with nested wells (wells in close proximity or in the same bore hole) completed at different depths in the aquifer. Two of the wells (Well 2 and Well 5) at the study site are in close proximity to one another (about 7 meters apart). Well 2 is completed at 30.5 meters depth and Well 5 is completed at 91.5 meters. Well 5 has a lower hydraulic head than Well 2, indicating a downward vertical gradient of 0.011 meters of head per meter of depth (Figure 3.4.a).

Presence of a vertical gradient would introduce error into an assessment of water table elevation based on measurement of static water level in wells completed at different depths below the water table. This error would increase as a function of depth and be greater at wells completed deeper in the aquifer. To remove the error, it is necessary to adjust the measured static water levels based on the completion depth of the well below the water table. The 0.11 m/m correction factor derived from Wells 2 and 5 was multiplied by the depth below the uncorrected water table height for Wells 2-5 and 7 (Wells 1 and 6 are screened throughout the water table and do not require adjustment) . The pre and post correction water level elevations are depicted in Figures 3.4.a and b.

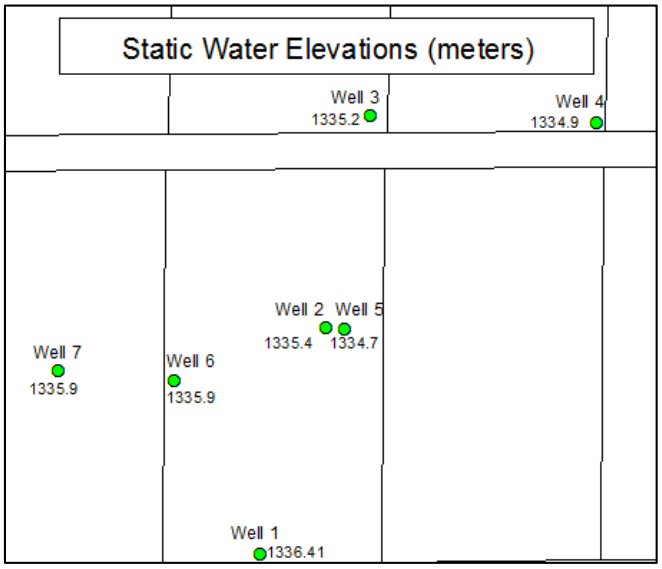

Figure 3.4.a: Uncorrected water table elevation estimates from direct measurement of static water elevations

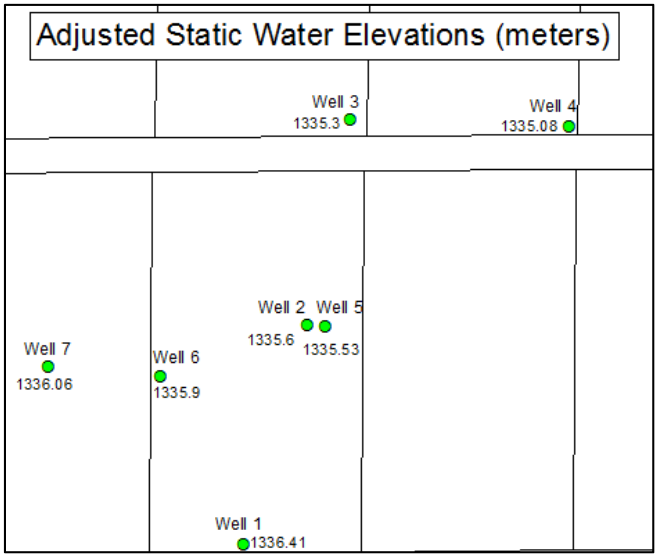

Figure 3.4.b: Water table elevations adjusted for presence of downward vertical gradient

Once hydraulic head is established at each well, the groundwater flow direction can be assessed. Groundwater flow direction is found by triangulation, a simple method that uses three wells to define a plane and assumes constant gradation between points. An equipotential line is drawn from the well with intermediate water elevation to the

point with the same elevation on the line between the high and low wells.) A perpendicular line is drawn through the equipotential line indicating flow direction. Triangulation was performed between multiple sets of wells before and after the water elevation adjustments (Figure 3.5.a and Figure 3.5.b)

In general, groundwater flow vectors before water elevation adjustment show inconsistent flow directions. Flow vectors using Well 1 and nearest neighbors range from due north to northeast, while one vector between Wells 2, 3, and 4 faces almost due east (Figure 3.5.a). Flow vectors using adjusted water elevations were not dramatically different but were better aligned and more intuitive (Figure 3.5.b). In both cases, it appears that when Well 1 is included in the vector analysis, the flow is more northerly. Groundwater movement in the Belgrade area has been mapped in the past as moving to the northeast. The influence of Well 1 indicating a more northerly flow may suggest groundwater mounding resulting from loading of wastewater from the infiltration beds.

Well 1 had the highest water level elevations in the study and showed greater fluctuation in water level elevations than the other wells. The water level in Well 1 varied 0.53 meters, Well 2 varied 0.36 meters, Well 3 varied 0.3 meters and Well 4 varied 0.19 meters. The greater fluctuation in water elevation in Well 1 could be further support for presence of groundwater mounding due to close proximity to the infiltration beds. Groundwater mounding would affect local groundwater gradient and impact the movement and direction of the contaminant plume. Groundwater mounding may be causing groundwater closer to Well 1 to move more north, as seen in the flow vectors.

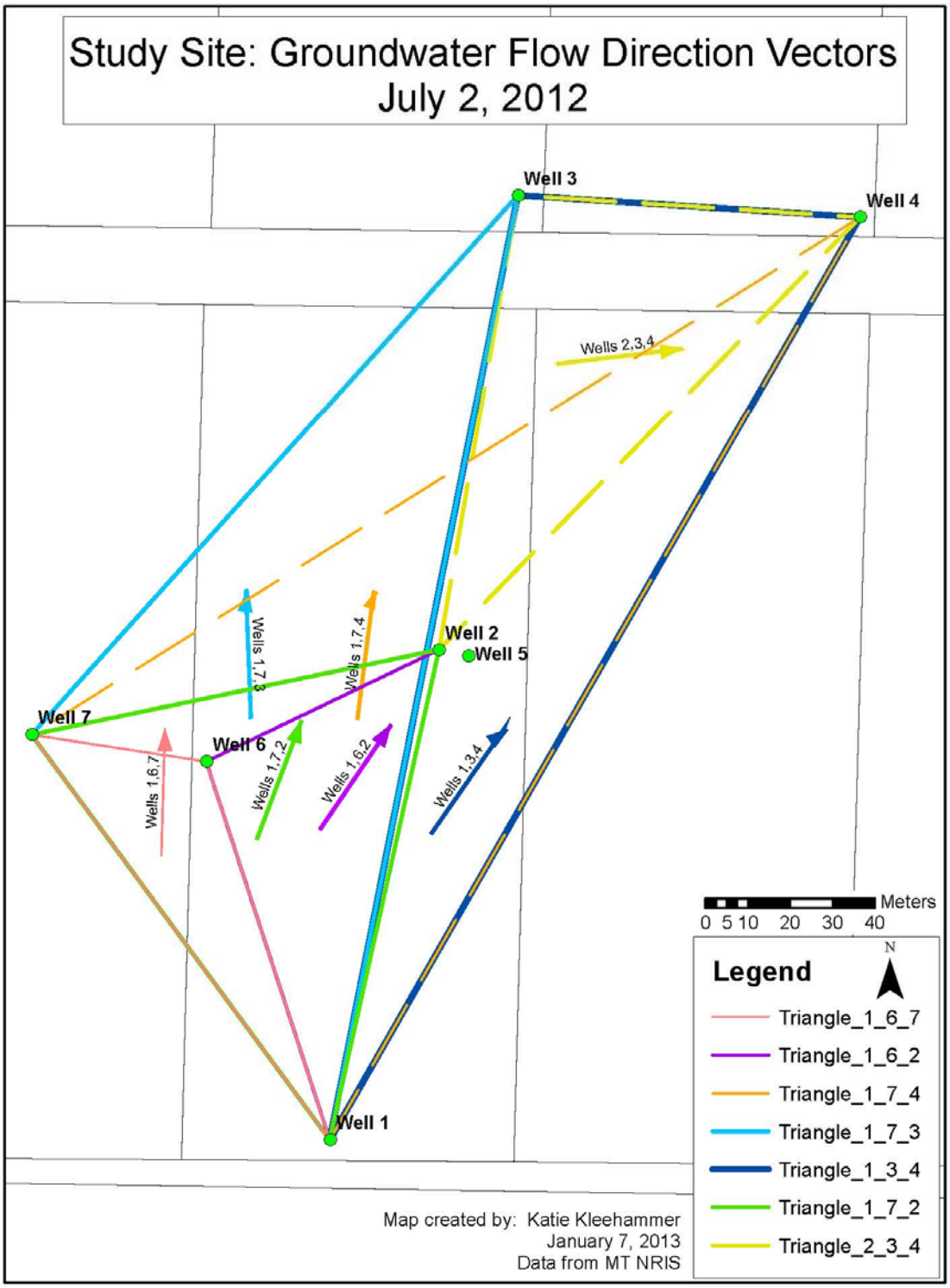

Figure 3.5.a: Groundwater flow vectors *before* adjustment of water level elevations for presence of a vertical gradient

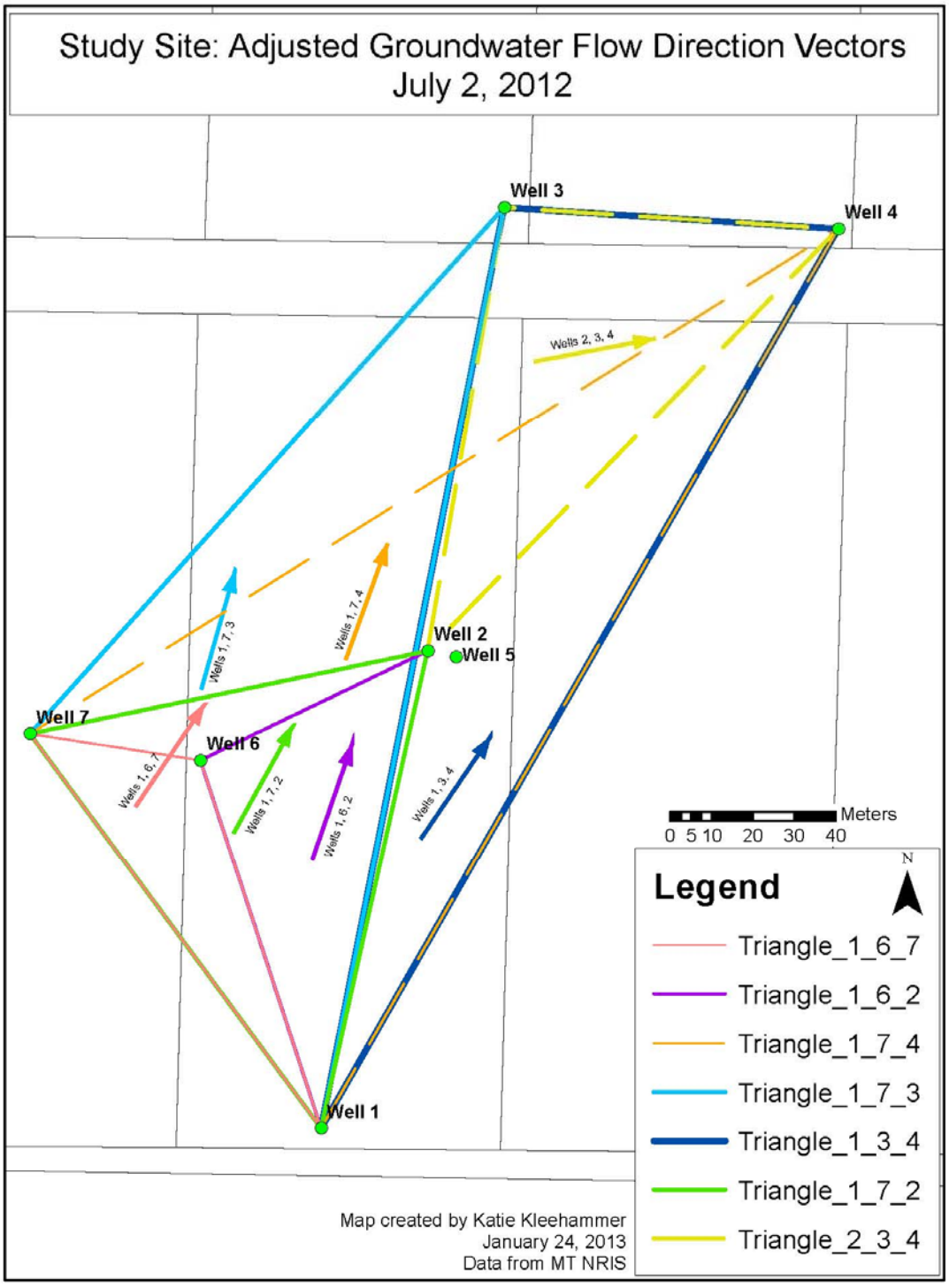

Figure 3.5.b: Groundwater flow vectors *after* water level elevation adjustments for presence of a vertical gradient

Once the water moves farther down-gradient from the influence of groundwater mounding, it would return to its natural northeasterly flow, which is depicted in the flow vector for Wells 2, 3, and 4.

Estimated Groundwater Velocity

Groundwater velocity (v) is the hydraulic conductivity (K) over the effective porosity (n_e) (which takes into consideration the pores size, material, and retention), multiplied by the hydraulic gradient (dh/dl) (Basic Hydrogeology 2010).

$$\textbf{Velocity (v)} = \left(\frac{K * dh}{n_e * dl}\right)$$

It was not possible to perform pump tests for this study, so aquifer characteristics were estimated from previous pump tests in the area along with substrate information from well logs. Well logs for each of the study wells were obtained from the Montana Bureau of Mines and Geology online Groundwater Information Center database (*Groundwater Information Center* 2012). In all four wells, the majority of the stratum that was drilled through consisted of fine gravels, sands, cobbles and some silt and clay. Effective porosity (n_e) values from literature are 0.22 for sand and 0.19 for gravel (Basic Hydrogeology 2010).

Hydraulic conductivity quantifies the ease with which water can move through a media and has units of length per time (Schwartz and Hubao 2003). Hydraulic conductivity is assessed through pump tests or aquifer tests which draw down the water table for a period of time while monitoring the pumping rate and/or by monitoring the recovery rate after pumping stops. Although the hydraulic conductivity has not been determined for this specific study site, pump tests and aquifer tests have been performed

by previous studies in the Belgrade area. The hydraulic conductivity from these tests range from 45.4 m/day (Morrison-Maierle 2010) to 54.86 m/day (Custer 2008) to 182.9m/day (Hackett et al. 1960). For the purposes of this study, the low and high values of hydraulic conductivity were used to assess the range of likely groundwater travel times through the site. Table 3.1 shows the range of travel times between Well 1 and each of the other 3 wells using low and high effective porosity and hydraulic conductivity levels.

Table 3.1: High and low flow rate estimates between wells

			K= 45.4 m/day		K=182.9 m/day	
			Low ne (sandy) 0.22	High ne (gravels) 0.19	Low ne (sandy) 0.22	High ne (gravels) 0.19
Distances between Wells	**Meters**	**I (Hydraulic Gradient)**	**Travel Time (days)**	**Travel Time (days)**	**Travel Time (days)**	**Travel Time (days)**
Wells 1 & 2	118.6	0.0068	69	60	17	15
Wells 1 & 3	228.1	0.0048	133	116	33	29
Wells 1 & 4	251.8	0.0052	147	128	37	32

The shortest travel times between wells ranges from 18 to 50 days with the longest time frames spanning 85 to 235 days. Because hydraulic conductivity and effective porosity were not quantified in this study, a range of high and low values must be used and the assumption made that the flow times will fall within this range. Sampling started on May 3 and ended on Aug 28, 2012, covering a 117 day span. If the true travel times are closer to the longer estimates, the water collected from Well 1 at the beginning of the sample period would have reached Well 2 during the sample period, but not Wells 3 and 4. If true travel times are closer to the shorter travel estimates, significant overlap exists in the water sampled at Well 1 and that collected later in time at the down-gradient wells. Anecdotal evidence of groundwater travel time derived from

time elapsed between the contamination event and noticed water quality issues in Well 2 (244meters) suggests a groundwater velocity of approximately 4 meters/day.

Methods – Field

Prior to sample collection, each well was purged long enough to evacuate at least three well volumes and field parameters (conductivity, dissolved oxygen, pH and temperature) were monitored for stabilization according to EPA protocols (Hunter 2011). Well 1 was purged and sampled using a permanently installed pump which required attachment to a generator on site. Well 2 required installation of a temporary pump that was powered using a generator. Wells 3 and 4 are currently in use for domestic water and were purged and sampled with permanently installed pumps. At each well, a hose was connected to the well or faucet with a flow splitter to direct a portion of water into a flow-through cell where a YSI 556 multi-meter measured water quality parameters. The remaining portion of flow was directed through a garden hose and discharged away from the well head. Flow rate was determined by timing how long it took to fill a five gallon bucket. Field water quality parameters (temperature, dissolved oxygen, specific conductance, and pH) were recorded every few minutes to assess when the parameters had stabilized indicating water was coming from the aquifer rather than the well. Parameter stabilization was based on information in Table 3.2 (Crone and English 2009). Parameter stabilization invariably took place after 3 well volumes had been purged, so the standard three well volume purging guideline was always exceeded.

Table 3.2: Parameter stabilization criteria, measured every five minutes, from the Gallatin Local Water Quality District Standard Operating Procedures for groundwater sampling

Parameter	Stabilization Criteria	Reference
pH	+/- 0.1	Puls and Barcelona, 1996; Wilde et al., 1998
Specific Conductance	+/- 3%	Puls and Barcelona, 1996
Dissolved Oxygen	+/- 0.3 mg/L	Wilde et al., 1998

Before the first site and between each of the sites, the first two feet of the electric tape (used to measure static water level) was disinfected with 10% bleach and rinsed with deionized (DI) water. The tubing that discharged water to the YSI was rinsed three times with 10% bleach and three times with DI water. Once water quality parameters were stabilized, gloves were used to handle sample containers. Water was collected in two 700 mL, sterile, Whirl-Pak bags (Nasco, Modesto, Cali.). The bags were tightly sealed and placed in a cooler along with the primary samples for biological and chemical analysis. One additional 125 mL bottle was filled, immediately preserved with nitric acid, and placed in the cooler for boron analysis. Sample collection started at Well 4 (lowest concentrations) and ended at Well 1 (highest concentrations) to help reduce potential for cross contamination of samples.

Field equipment blanks were collected on days when all four wells were sampled, after Well 1 (the last well). For the equipment blank, 10% bleach was run through the tubing three times. Tubing was then rinsed three times with DI water, followed by another triple rinse of ultra-pure, autoclaved, Millipore water. Once disinfection was complete, the ultra-pure, autoclaved, Millipore water was poured through the tubing and collected in a Whirl-Pak bag to mimic the sample collection process.

Methods – Lab

Once sampling was completed for the day, samples were transported directly to the lab for microbiological and chemical analysis processing. Sample processing always followed the same order that samples were collected, starting with Well 4. All equipment used for sample processing of *Bacteroides* was autoclaved to ensure sterilization and the lab bench and area was sterilized with 95% ethanol. To start, 100 mL of a sample was filtered through a .22 μM filter using a magnetic filter apparatus. The filter was removed with flame sterilized tweezers and placed into a 15 mL falcon tube. The filtrate was used to triple rinse four 20 mL scintillation vials. The process was repeated a second time with an additional 100 mL of filtered water. Again, the second filter was placed in another 15 mL falcon tube and this time the filtrate was used to fill the four 20 mL scintillation vials just rinsed with the above filtrate. Once this was completed, the water and filters were frozen at -80°C.

Between samples, the magnetic filter was sterilized with 95% ethanol, triple rinsed with DI water and triple rinsed with ultra-pure autoclaved Millipore water. Tweezers were flame sterilized between samples and the lab bench was disinfected with 95% ethanol. The collected filtrate water in the scintillation vials was immediately frozen at -80°C until they were sent to Energy Laboratories Inc. in Billings, Montana for analysis of chloride and nitrate+nitrite as N using EPA method E353.2 (USEPA 1993). The 125 mL bottle of water for dissolved boron analysis was placed in a 3°C refrigerator upon return to the lab. At the end of the field season, the samples were sent to Energy

Laboratories Inc. within the six month hold time for dissolved boron analysis using standard method E200.8 (International 2012).

Microbiology Methods

For the microbiological analysis, the IDEXX method, an EPA approved method for total coliforms and *E. coli* analysis was used. Sample water from the Whirl-Pak bag was transferred to a sterile 100 mL IDEXX bottle (IDEXX Laboratories). A nutrient packet was added to the sample and once the nutrients were dissolved, the water was transferred to an IDEXX quanti-tray. The IDEXX quanti-tray splits the sample into 97 wells which acts as a serial dilution (IDEXX Laboratories Inc., Westbrook, Maine). The tray is sealed and incubated at 35°C for 24 hours. Wells that turn yellow after the incubation period are positive for total coliforms and wells that fluoresce under a black light are positive for *E. coli.* Results are given as most probable number (MPN) with a detection range from one to 2,419 viable cells/100 mL.

Molecular Microbiology Methods

Once all of the samples were collected for the project, the frozen filters were processed and analyzed for *Bacteroides* gene abundance. First, DNA was extracted from the filters by cutting the filters in half and processing the half filter with the MP Biomedicals FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA). Following all steps in the manufacturer's instructions, the end product resulted in DNA eluted in 50 µL of DNAse free water. The eluted water was tested using a NanoDrop spectrophotometer to ensure adequate concentrations and purity of DNA in the eluted

DNAse free water. Once the DNA was extracted, quantitative polymerase chain reaction (qPCR) was performed to assess the number of copies of *Bacteroides* in each sample. Each sample was analyzed once in a run; replication was accomplished across batches by running the single samples in three different runs.

Two different *Bacteroides* specific qPCR primers were used in the analysis; AllBac, a general *Bacteroides* primer and HF183, a human specific primer (Layton et al. 2006; Seurinck et al. 2005). AllBac is less specific than HF183 and should encompass HF183 allowing AllBac to detect greater gene copy numbers than HF183. Both primers have been used extensively in surface water microbial source tracking studies (Layton et al. 2006; Seurinck et al. 2005; Dick and Field 2004). Samples were run in 0.1 mL strip tubes in 25 µL volumes with 12.5 µL of SYBR Green Master Mix, 1 µL of forward and reverse primer at 10µM concentration, 2.5 µL bovine serum albumin (BSA), 6 µL DNAse free water and 2 µL of target DNA (sample). All reagents were pipetted with low retention barrier tips and were always changed between samples. The qPCR sample preparation was conducted under a laminar flow hood that was disinfected with ethanol and UV light to reduce the risk of extraneous DNA in the air being amplified in the sample. Once samples were completed and capped, they were run on a 72 –well Rotor-Gene 6000 Real-Time Analyzer machine. The temperature profile for AllBac was initiated at 50°C for 2 minutes followed by 95°C for 10 minutes and 40 cycles of 95°C for 30 seconds and 60°C for 45 seconds (Layton et al. 2006). The temperature profile for HF183 was initiated at 50°C for 2 minutes followed by 95°C for 10 minutes and 40 cycles of 95°C for 30 seconds, 53°C for 60 seconds, and 60°C for 60 seconds (Seurinck et

al. 2005). Both qPCR runs (AllBac and HF183) ended with a melt curve analysis with the temperature increasing 1°C from 60-95°C.

Standards Development: Quantitation assays using a series of samples with known concentrations were used to assess DNA copy numbers. Results from known samples were used to create a standard curve and unknown samples were extrapolated from this relationship. For this project, ten-fold dilution standards for each primer were created ranging from 30 copies up to 3×10^5 copies.

To create the standards, PCR products with AllBac and HF183 primers were processed with the Lucigen GC Cloning and Amplification Kit with pSMART® GC Vectors (Lucigen Corporation, Middleton, WI). The PCR products were ligated into the pSMART® GC Vectors and then transformed into the provided E.cloni® cells following the manufacturer's instructions. The E.cloni® cells were plated on kanamycin plates and incubated overnight at 37°C. Colonies were picked and placed in test tubes containing LB broth with 30µg/ml of kanamycin and incubated in a water bath at 37°C overnight to grow the picked colonies. The next day, the Qiagen Mini Prep Kit was used to extract the plasmids from the E.cloni® cells (Qiagen, Valencia, Cali.). Plasmid concentrations were measured using the Nano Drop and used to develop the standards.

CHAPTER 4

RESULTS

Static Water Levels

While past studies in the Belgrade area have found fluctuations in annual water table elevation of up to 12 m (Dunn 1978), the greatest fluctuation measured within this study was 2 m and a nearby well (Cobblestone) monitored with a pressured transducer by the Gallatin Local Water District showed a 1.4 meter fluctuation during the same period (Figure 4.1). Static water levels at Well 1 were on average 0.25 meters higher than Wells 2-4 and 0.3 meters higher than the Cobblestone well which is approximately 450 meters northwest of Well 1 and is more representative of natural background water elevations. Greater fluctuation in Well 1 may indicate that varying recharge rates from the infiltration beds is affecting groundwater levels in Well 1. Well 1 saw increasing groundwater levels through the majority of the study. Wells 2-4 showed a slight increase in groundwater levels. All wells showed a decrease in water level height during the last sampling event. Wells 3 and 4 are in use for domestic supply, so water levels are subject to influence from pumping. The dips in static water elevation at Well 4 may be due to increased water use for lawn irrigation.

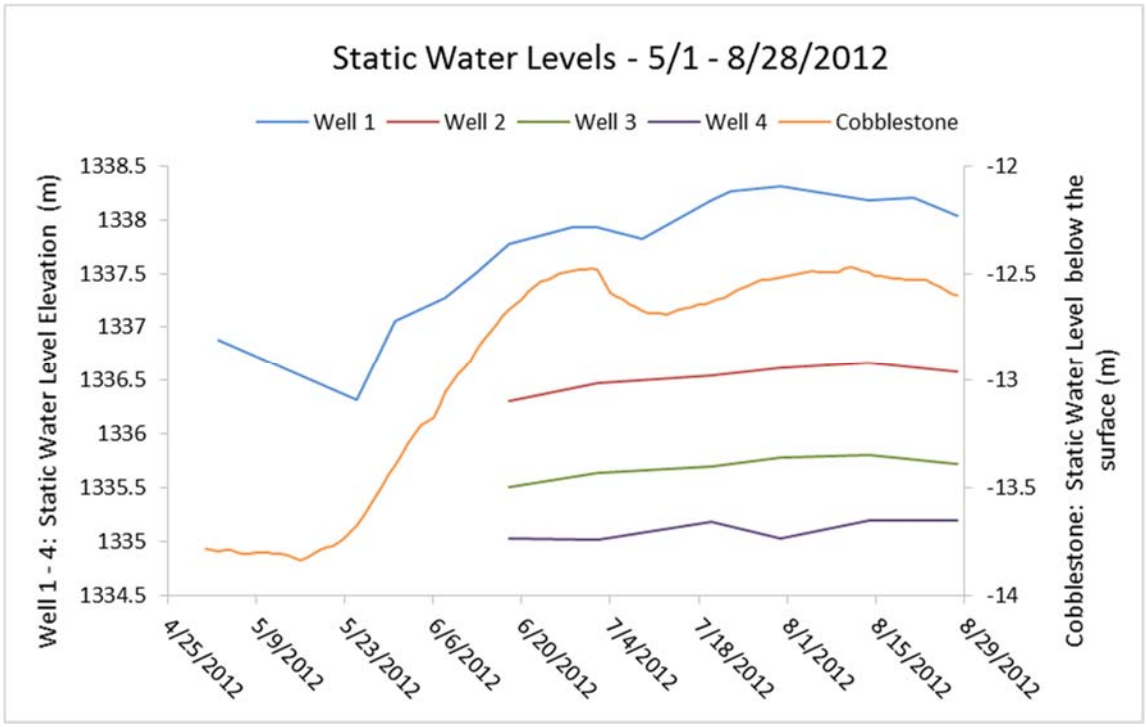

Figure 4.1: Static water levels at each well during the study period. Cobblestone data from the Gallatin Local Water Quality District

Field Parameters

Field parameters (pH, specific conductance, dissolved oxygen) measured at each site revealed Well 1 was strongly impacted by the infiltration beds resulting in slightly acidic water and high levels of specific conductivity. Well 3, followed by Well 2 and Well 4 showed decreasing signs of wastewater impacts. Well 3 was the only well where low dissolved oxygen levels less than 0.5 mg/L were recorded. At these low levels, it is possible for denitrification to occur (Puckett 2011). A statistical table below summarizes the differences in the field parameters amongst the four wells (Table 4.1).

Table 4.1: Summary statistics (max, mean, median, minimum, standard deviation) of each field parameter at every well

pH						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	6.87	6.67	6.69	6.29	0.12	15
Well 2	7.11	7.08	7.10	7.04	0.03	5
Well 3	7.09	7.04	7.04	7.01	0.03	6
Well 4	7.23	7.21	7.21	7.18	0.02	6
Specific Conductivity (µS/cm)						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	1088	986	968	920	51	15
Well 2	642	628	624	616	11	5
Well 3	761	748	747	737	7	6
Well 4	558	540	537	528	12	6
Dissolved Oxygen (mg/L)						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	6.41	2.97	2.40	1.65	1.34	15
Well 2	2.35	2.16	2.19	1.88	0.15	5
Well 3	0.51	0.40	0.39	0.27	0.07	6
Well 4	7.05	6.49	6.40	6.03	0.43	6

Chemical Analyses

Chloride and Boron Relationships

Figure 4.2 depicts the range of chloride and boron concentrations detected in each well during the study. Background concentrations were collected from wells surrounding the study area thought not to be affected by wastewater. A t-test performed between each well for both chloride and boron confirmed all sites are significantly different from each other. A statistical table of chloride and boron (Table 4.2) shows the mean concentration of background levels for chloride was 4.6 mg/L and 11.6 µg/L for boron. Both chloride and boron concentrations in all four wells are significantly greater than mean background concentrations (Figure 4.2).

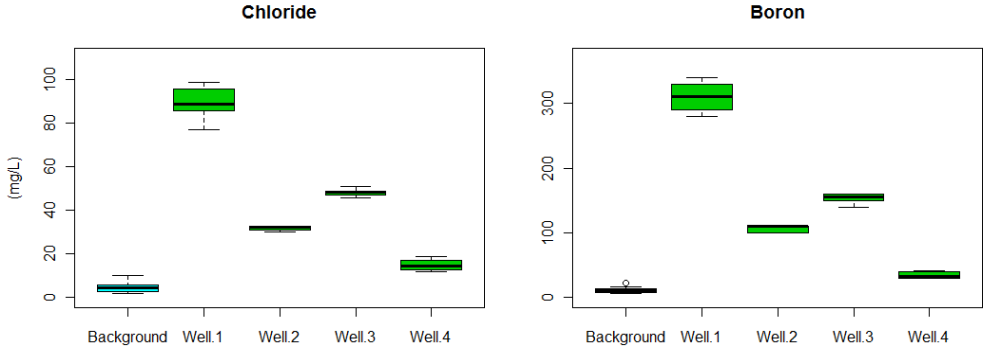

Figure 4.2: Boxplot of chloride (mg/L) and boron concentrations (µg/L) across all wells

Table 4.2: Summary statistics for chloride and boron at each site

Chloride (mg/L)						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	99	90.3	89	77	6.6	15
Well 2	33	32.0	32.5	30	1.2	6
Well 3	51	48.3	48.5	46	1.6	6
Well 4	19	15.0	14.5	12	2.4	6
Background	10	4.6	4.35	2	1.9	72
Boron (µg/L)						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	340	310.0	310	280	21.6	12
Well 2	110	106.0	110	100	4.9	5
Well 3	160	153.3	155	140	7.5	6
Well 4	41	34.5	33	30	4.6	6
Background	22.7	11.6	11	7	4.4	12

For both chloride and boron, Well 1 has the greatest concentrations followed by Well 3, Well 2, and Well 4. Well 2 produced lower chloride and boron concentrations than Well 3, even though Well 2 is closer to the infiltration beds.

When chloride and boron are assessed temporally (Figure 4.3), the two parameters have similar concentration patterns at each well throughout the sampling period. At Well 1, chloride and boron both increased throughout the study with chloride showing slightly greater weekly variation. The chloride concentrations at Well 1 ranged from 77 to 99 mg/L. Boron concentrations ranged from 280 to 340 µg/L. At Well 2 and Well 3, the chloride and boron concentrations remained relatively static. In Well 2 chloride ranged from 30 to 33 mg/L and boron ranged from 100 to 110 µg/L. At Well 3 chloride ranged from 47 to 51 mg/L and boron ranged from 140 to 160 µg/L. At Well 4 chloride ranged from 12 to 19 mg/L with a slight decrease through time and boron ranged from 30 to 40 µg/L with a slight increase through the first half of the study and a decrease in the second half of the study.

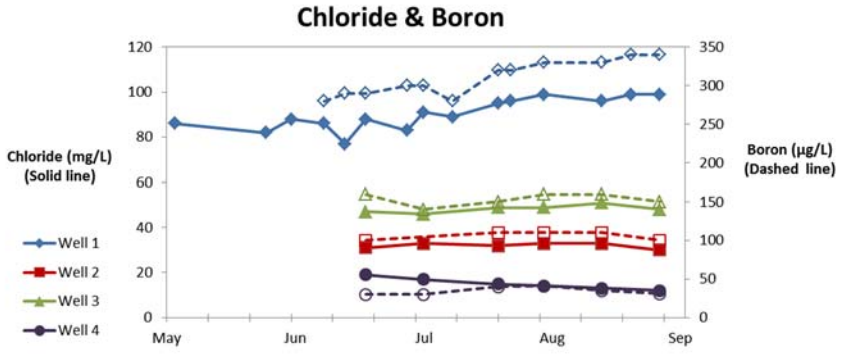

Figure 4.3: Temporal chloride and boron concentrations at each well (chloride is solid line, boron is dashed line)

The covariation of chloride and boron concentrations observed in the time series plots was quantified using linear regressions. Chloride is plotted on the x-axis because it is assumed to be a conservative tracer and is hence the independent variable. Chloride

and boron samples from all wells are included in the regression in Figure 4.4.A which illustrates a strong correlation (R^2=0.9901, y=3.5331x-14.023, p-value = 0.003). Removing Well 1 from this relationship also shows a strong relationship between parameters (R^2=0.967, y=3.521x-14.164, p-value=1.63e-12). When each well is assessed individually, greater variability among the relationships is present which is indicated by smaller R^2 values ranging from 0.135 (p= 0.4737) at Well 4 up to 0.8284 (p=0.0318) at Well 2, with Well 1 presenting the most significant relationship with a p-value of 0.0008. The regression in Well 4 indicates an inverse correlation between chloride and boron, but the concentrations observed are very low and the relationship is not statistically significant (p=0.4737).

Chloride and Nitrate-N Relationships

Figure 4.5 shows nitrate-N concentrations above background levels (1.45 mg/L) at each site. A t-test of the nitrate-N concentrations between each site indicate all wells are significantly different than background concentrations using a 95% confidence level. The concentrations between Well 2 and Well 3 were not significantly different (p-value of 0.564). The general pattern of nitrate-N concentrations among wells reveals some notable differences from chloride. The range of nitrate-N values in Well 1 is greater than the range in chloride values which may have implications for reduction of nitrate in wastewater either during treatment or subsequently in groundwater before reaching the sampling well.

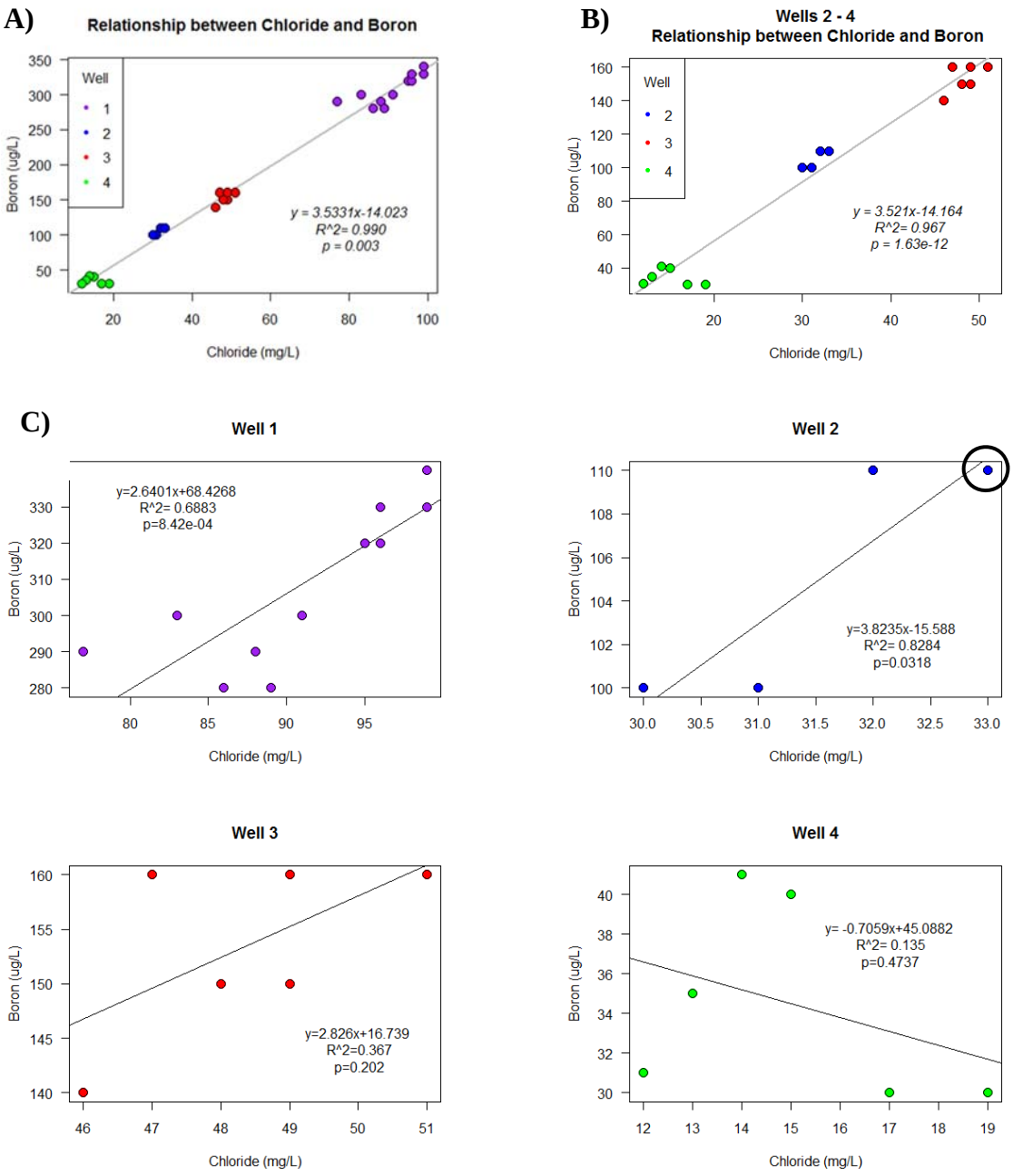

Figure 4.4: A) Chloride and boron regression relationships for all data points at all wells B) Chloride and boron regression relationships at Wells 2-4. C) Chloride and boron regressions displayed at each well. Circled point in Well 2 graph represents two data points.

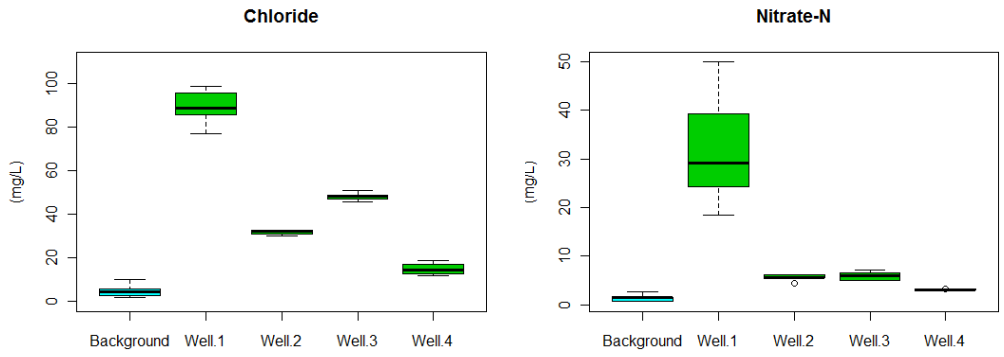

Figure 4.5: Chloride and nitrate-N boxplots for all samples for all wells.

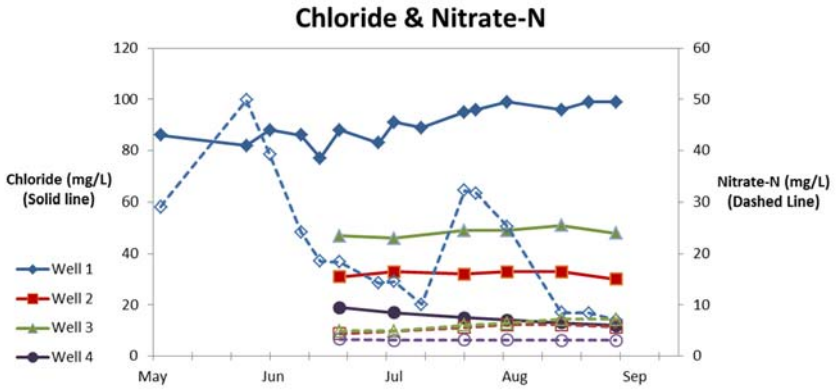

Figure 4.6: Temporal chloride and nitrate-N concentrations at all wells. Chloride concentrations are solid lines, nitrate-N are dashed lines

Table 4.3: Summary statistics for nitrate-N at each site

Nitrate-N (mg/L)						
	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	49.9	22.10	18.5	7.06	12.1	15
Well 2	6.16	5.57	5.65	4.34	0.7	5
Well 3	7.18	6.11	6.24	4.92	0.9	6
Well 4	3.32	3.15	3.12	3.07	0.1	6
Background	3.89	1.65	1.45	0.06	1.1	86

Assessment of chloride and nitrate concentrations through time does not reveal the parallel behavior observed for chloride and boron (Figure 4.6). Notable differences in nitrate-N/chloride concentration patterns occurred in Well 1 as chloride concentrations steadily climbed and nitrate-N concentration fluctuated greatly, with two pronounced peaks during the study period. Nitrate-N values in Well 1 ranged from 49.9 mg/L down to 7.06 mg/L, potentially indicating how responsive nitrate-N concentrations in this well are to loading from the infiltration beds (Table 4.3). Nitrate-N concentrations at Wells 2-4 had little variation between max and min values and are more similar among wells than those for chloride (Table 4.3 and Figure 4.5).

The regression between all chloride and nitrate-N data points is not as strong as the regression between chloride and boron with a R^2 of 0.437. As was evident from the nitrate-N time series (Figure 4.7), there is a sharp decrease in nitrate-N concentrations down-gradient from Well 1. Due to the close proximity of Well 1 to the infiltration beds, nitrate-N levels at this well were not characteristic of equilibrated groundwater conditions. To assess conditions more representative of natural groundwater attenuation, Well 1 was removed from the regression, and Wells 2-4 had a stronger relationship with an R^2 value of 0.753 ($p=6.36e-6$) (Figure 4.7.B). When the relationship between chloride and nitrate-N is assessed at each individual well, there is variability in the R^2 values ranging from 0.100 up to 0.610 (Figure 4.7.C). The individual chloride, nitrate-N relationships are strongest in Wells 3 and 4 which are furthest from the source of contamination.

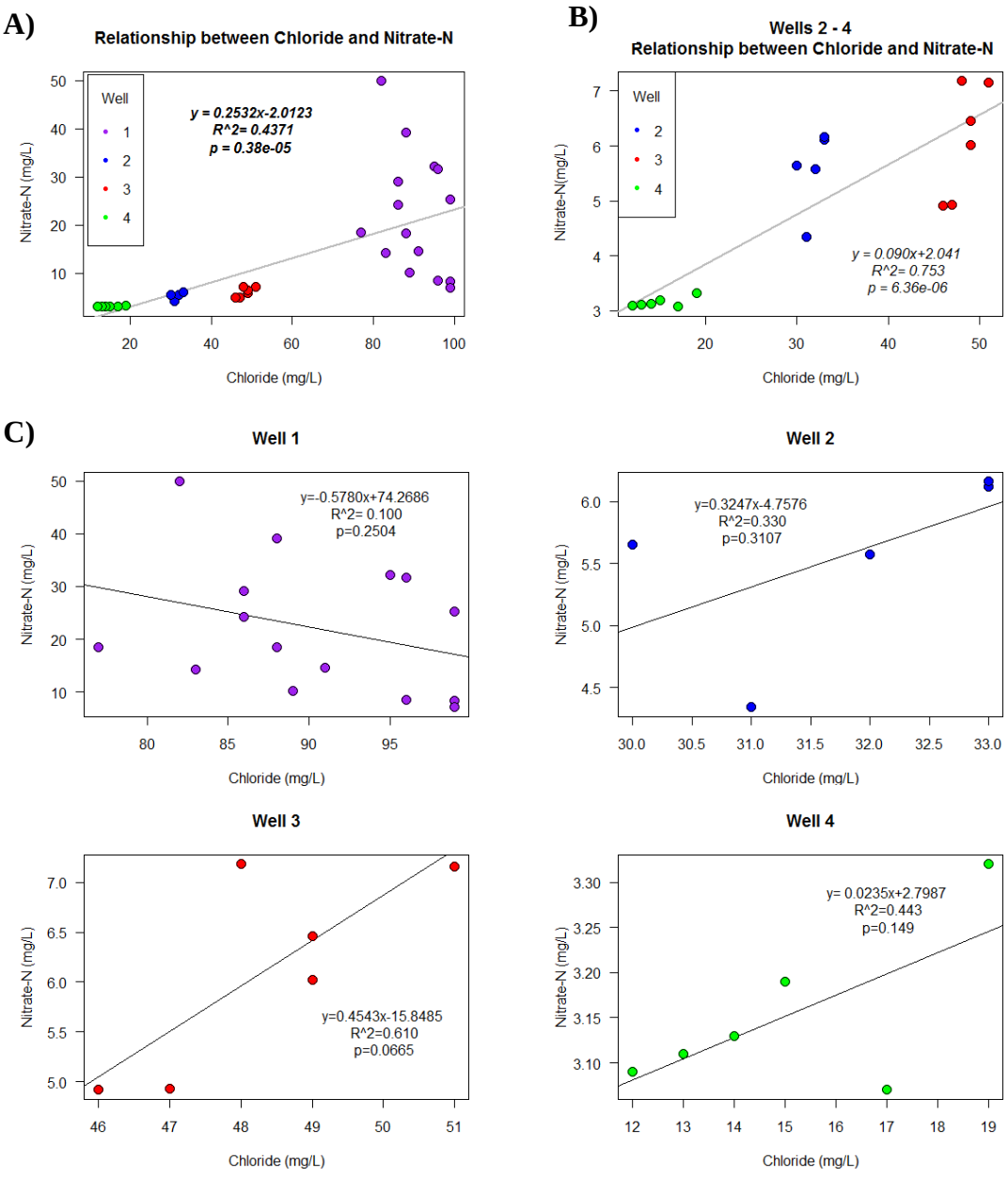

Figure 4.7: A) Chloride and nitrate-N regression relationships for all data points at all wells B) Chloride and nitrate-N regression relationships at Wells 2-4. C) Chloride and nitrate-N regressions at each well.

Biological Analyses

Table 4.4 depicts summary statistics for total coliforms and *E. coli*, two traditional biological indicators, at the four wells. Total coliforms were only detected in Wells 1 and 2. Well 1 counts ranged from 22 up to over 2,419 cfu/100 mL and Well 2 counts ranged from 2 to 27 cfu/100 mL. *E. coli* was only detected in Well 1 with counts ranging from 2 to 24 cfu/100 mL. *E. coli* was not detected in Well 2 although total coliforms were detected.

Table 4.4: Summary statistics of total coliforms and *E. coli* at each well

Total Coliforms

	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	2420	657	243	22	840	12
Well 2	27	8	2	0	10	6
Well 3	0	0	0	0	0	6
Well 4	0	0	0	0	0	6

E.coli

	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	24	7	2	0	8	12
Well 2	0	0	0	0	0	6
Well 3	0	0	0	0	0	6
Well 4	0	0	0	0	0	6

Bacteroides AllBac analysis should encompass all HF183, as HF183 is a more specific subset of AllBac. Well 1 had the highest concentrations of both *Bacteroides* parameters. AllBac concentrations were greater than HF183 concentrations at Well 1, but at Wells 2-4 the HF183 concentrations were higher than AllBac. *Bacteroides* concentrations in Wells 2-4 for both AllBac and HF183 were not significantly different from each other or from background levels with p-values greater than 0.05. Well 1

concentrations of AllBac and HF183 were significantly different from blank concentrations (p-values= 0.004 and 0.0011, respectively). The median AllBac copy numbers in the blank samples were slightly below the means for Wells 2-4 (Table 4.5). HF183 median blank concentrations exceeded the median concentrations at Wells 2-4.

Table 4.5: Summary statistics for AllBac and HF183 at all four wells

***Bacteroides* - AllBac**

	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	1506	480	233	92	462	14
Well 2	48	41	39	34	5	6
Well 3	57	42	39	37	7	6
Well 4	91	45	39	30	21	6
Blank	44	37	37	33	3	6

***Bacteroides* - HF183**

	Max	Mean	Median	Minimum	Standard Deviation	Sample Size
Well 1	579	274	217	147	138	14
Well 2	176	112	101	82	31	6
Well 3	129	101	98	83	15	6
Well 4	186	119	108	88	33	6
Blank	133	115	117	90	14	6

Boxplots of AllBac and HF183 concentrations indicate Well 1 has the greatest concentration and range of *Bacteroides* copies with a range of 92 to 1506 copies/100 mL of AllBac and 147 to 579 copies/100mL of HF183 (Figure 4.8).

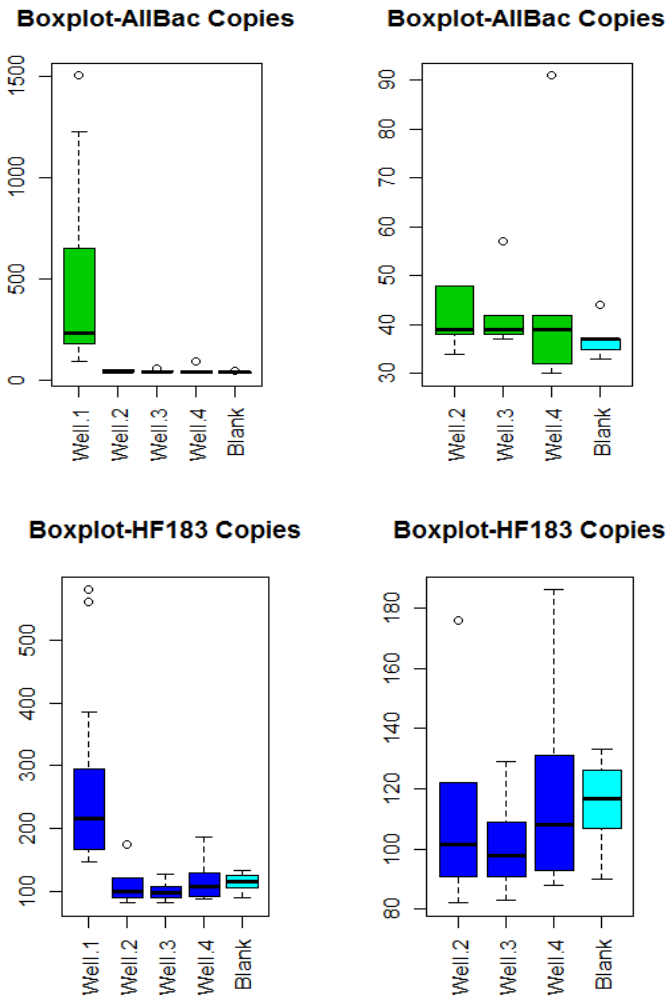

Figure 4.8: Boxplots of AllBac and HF183 copy numbers at all four wells (boxplots on the right are the same dataset plotted without Well 1).

For AllBac copies, the boxplot indicates that concentration distributions trend slightly downward moving from Well 2 to Well 4. The boxplot of HF183 is the inverse of the AllBac boxplot, with distribution concentrations increasing from Well 2 and 4. HF183 also shows a greater variability in concentrations at Wells 2-4 than is found for AllBac.

Chloride and Biological Relationships

The lack of detection of *E. coli* and total coliforms in most wells precluded a regression of their concentrations against chloride as was conducted with other parameters (Figure 4.9). Well 1 regressions with chloride concentrations indicated no relationship between total coliforms and *E. coli,* Well 2 chloride and total coliforms displayed a relationship of R^2=0.515 but was insignificant as p-values were >0.05 (p-value= 0.172) (Figure 4.10)

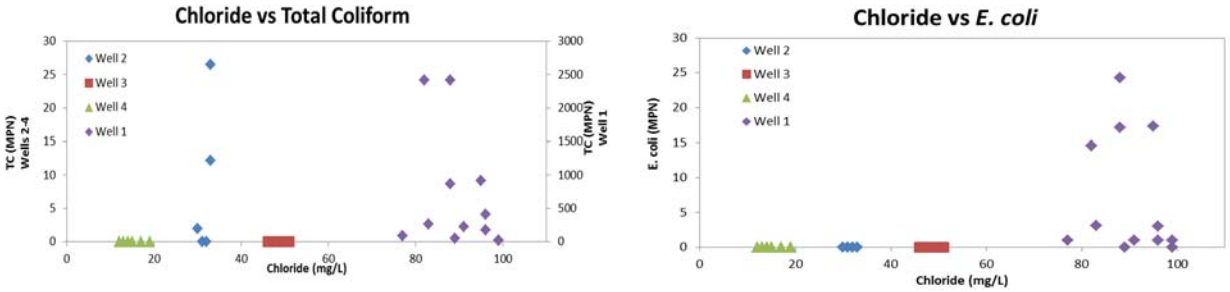

Figure 4.9: Regressions between chloride and total coliforms and *E. coli*

Chloride and *Bacteroides* relationships were assessed to determine how conservatively *Bacteroides* may be moving through the groundwater. The relationships between both chloride and AllBac and chloride and HF183 copy numbers were relatively poor, with R^2 values of 0.247 and 0.348, respectively (Figure 4.11.A). The relationship did not improve with the exclusion of Well 1 (Figure 4.11.B) as it did for the chloride vs. nitrate regression (Figure 4.7.B)

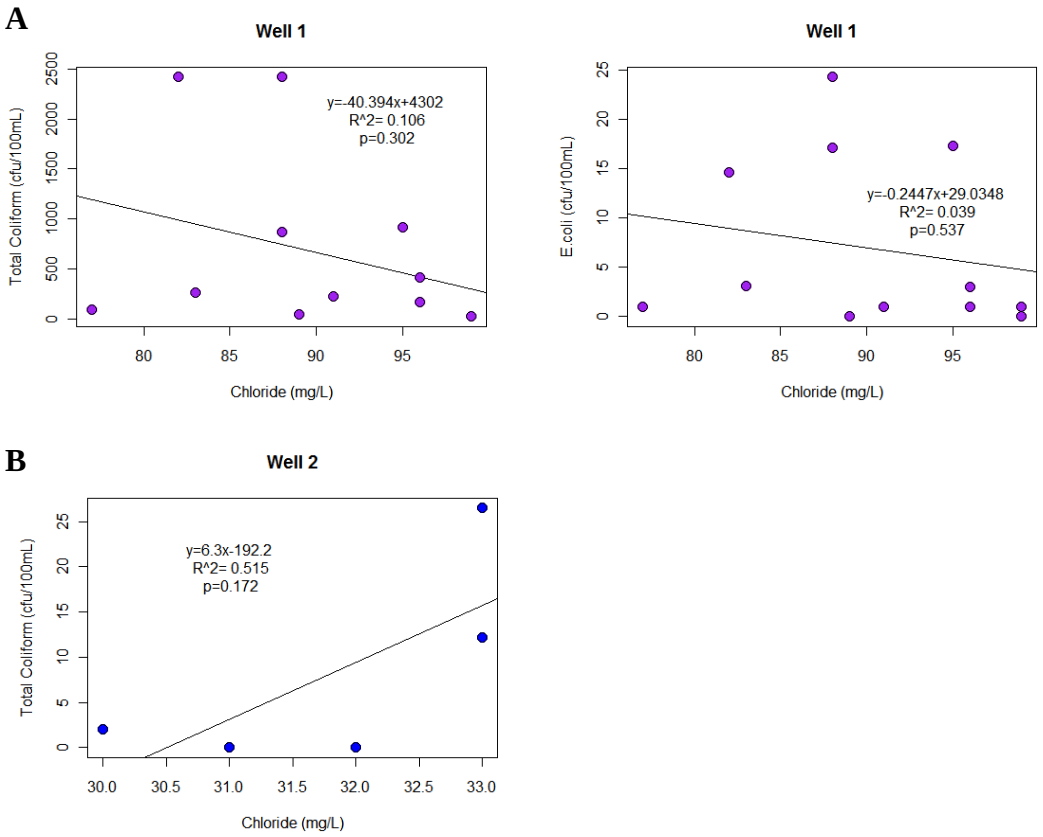

Figure 4.10: A) Chloride relationships between total coliforms and *E. coli* at Well 1. B) Chloride and total coliforms relationship at Well 1.

Total Coliforms, *E. coli, Bacteroides* Relationships

Assessment of AllBac copy numbers and total coliforms and AllBac and *E. coli* shows the parameters behaving very similarly through time (Figure 4.12.A). The largest peaks and valleys in concentration are in alignment while a few of the smaller peaks and valleys in the second half of the time series are slightly out of alignment. The concentration of HF183 through time does not follow the pattern of total coliforms or *E. coli* as closely as AllBac. While the largest peaks and valleys in HF183 concentrations

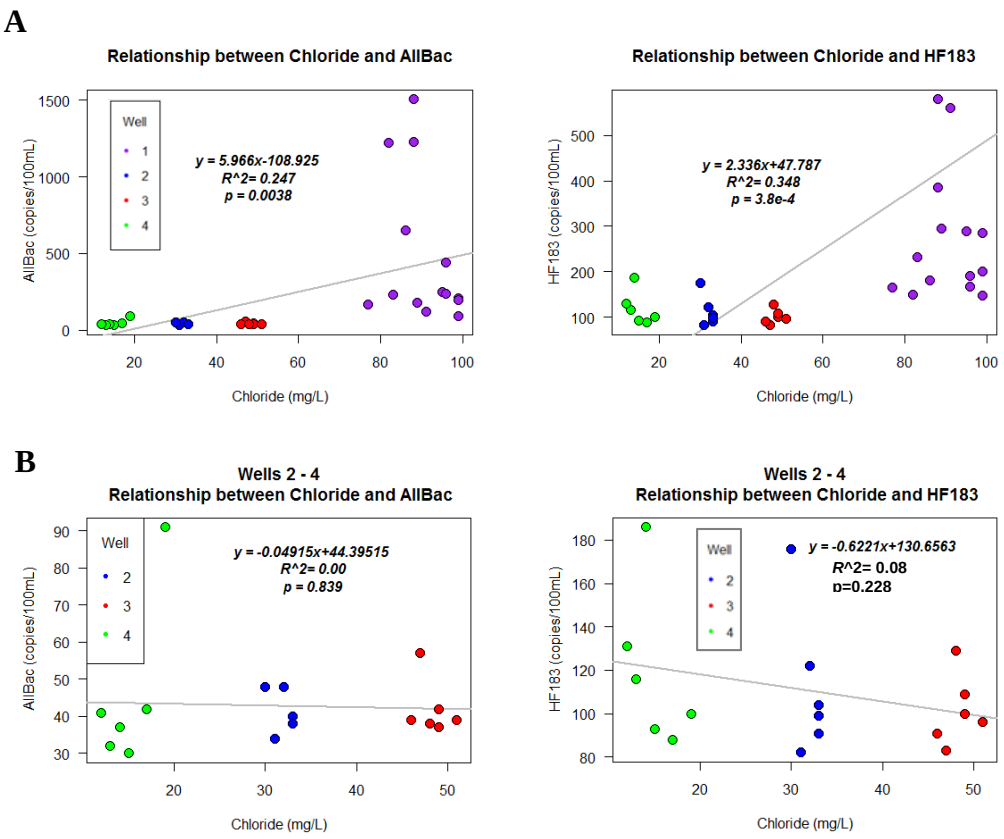

Figure 4.11: A) Relationships of chloride and AllBac copy numbers at all wells and chloride and HF183 copies at all wells. B) Relationship of chloride and AllBac copies at Wells 2-4, relationship of chloride and HF183 copies at Wells 2-4

are similar to total coliforms and *E. coli*, the HF183 analysis is much more variable and does not track as well as AllBac with traditional indicator organisms (Figure 4.12.B). *E. coli* is a subset of total coliforms; therefore, the two parameters should be closely related and this was confirmed with a strong R^2=0.88 (p-value=2.22e-6). The y-intercept of this relationship is -1.417 which indicates a greater presence of total coliforms in a sample (Figure 4.13).

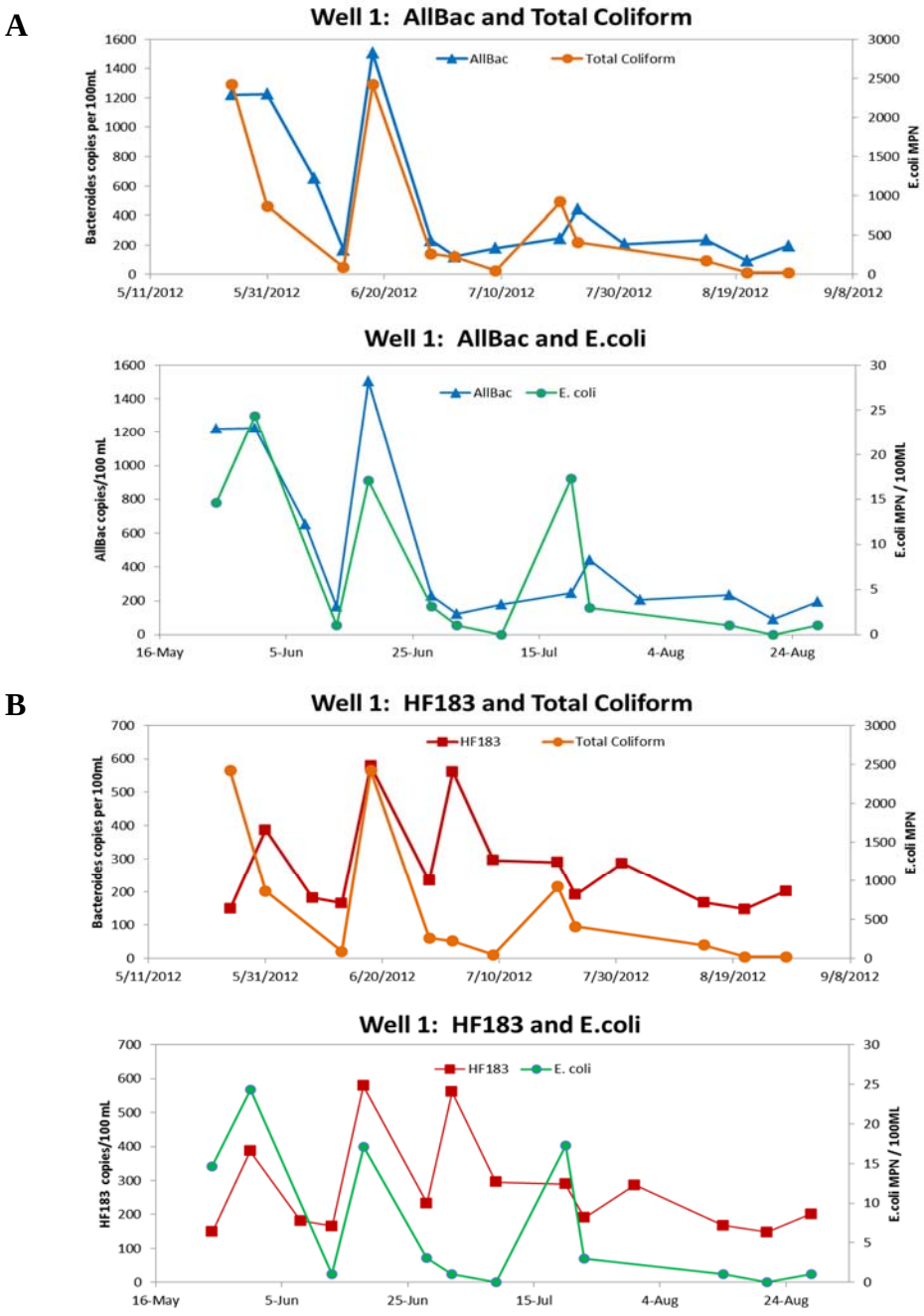

Figure 4.12: A) AllBac copy numbers, total coliforms and *E. coli* plotted through time at Well 1 B) HF 183 copy numbers, total coliforms and *E. coli* plotted through time at Well 1

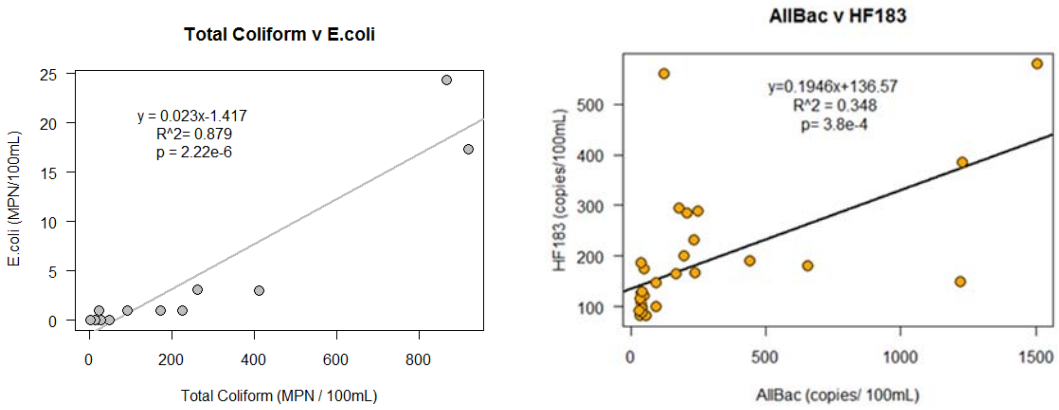

Figure 4.13: Regressions between total coliforms and *E. coli* and AllBac and HF183 copy numbers

A regression between AllBac and HF183 copy numbers does not indicate a strong relationship between the two parameters (R^2=0.348, p-value=0.00038). The intercept is 137, indicating the presence of higher quantities of HF183 than AllBac, which is counter intuitive.

To quantify how well *Bacteroides* correlates with total coliforms and *E. coli*, AllBac and HF183 copy numbers were plotted against these parameters to create regression relationships (Figures 4.14 and 4.15). Analyses were only performed using Well 1 as this was the only site where *E. coli* was detected and the only site with *Bacteroides* concentrations significantly above background levels.

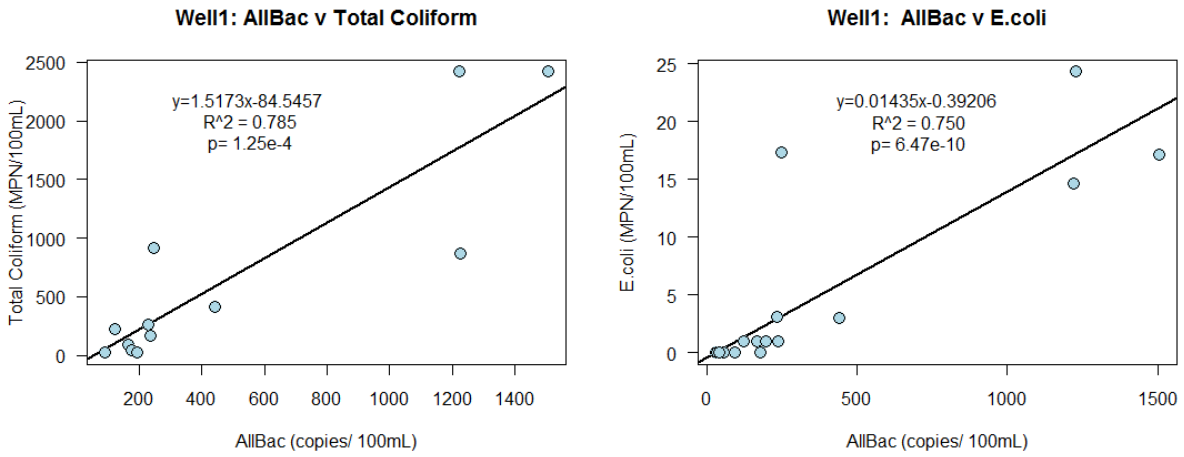

Figure 4.14: AllBac copy number relationships with total coliforms and *E. coli*

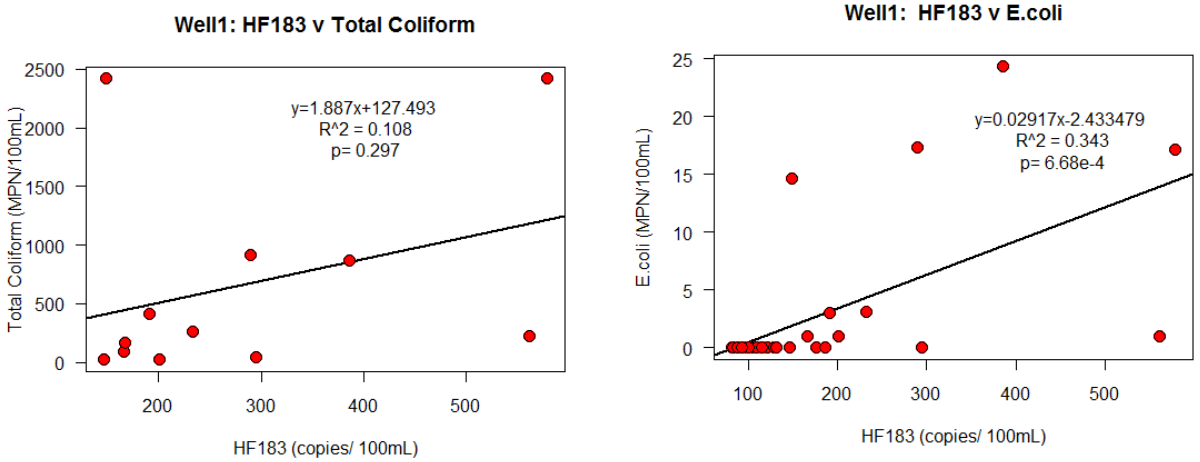

Figure 4.15: HF183 copy number relationships with total coliforms and *E. coli*

Regression relationships between AllBac copy numbers, total coliforms and *E. coli* had higher correlations (R^2 of 0.785 and 0.651 respectively) than HF183 (R^2 of 0.1076 and 0.139 respectively). Although HF183 had consistently higher copy numbers than AllBac, which could suggest greater sensitivity, it did not have a strong linear relationship with total coliforms or *E. coli.*

CHAPTER 5

DISCUSSION

Hypothesis 1: Were Chloride Concentrations Greatest at Wells Closest to the Infiltration Beds?

Chloride behaves conservatively in groundwater (Mullaney 2009) and is assumed to be a reliable quantitative tool for assessing wastewater influence on a given well at a given time. As such, it was hypothesized that chloride concentrations would be greatest in wells closest to the infiltration beds before dilution and dispersion had reduced the levels. The data collected for this project does not support this hypothesis; Well 2 exhibited lower chloride concentrations than Well 3. This finding implies that a simple two-dimensional model that only accounts for flow direction and distance from the source is not sufficient to predict chloride concentrations.

Lower chloride values in Well 2 than Well 3 suggests existence of a vertical concentration gradient with lower chloride concentrations deeper in the contamination plume (Well 2 pulled water from lower in the aquifer). The stability of elevated chloride concentrations through time at Wells 2 and 3 supports the assertion that these wells are influenced by the wastewater plume and that chloride is a useful tool for assessing the influence. Chloride concentrations at Wells 2 and 3 varied over a constrained range of 3mg/L and 5 mg/L respectively while Well 1 saw a range of 22 mg/L over the duration of the project (Figure 4.3). This indicates that Well 1 responds much more dynamically to chloride concentration changes from the source while the additional travel distance to

Wells 2 and 3 allows for damping of changes in concentration. The damping effect might also be a factor of well depth as Wells 2 and 3 are pulling water from deeper in the aquifer than Well 1. Well 4 displays a range of chloride concentrations (7 mg/L) intermediate to the other wells suggesting variable influence of wastewater. This suggests that wastewater influence on Well 4 may be affected by the degree of groundwater mounding produced by the infiltration beds. During periods of relatively high recharge from the beds, mounding produces a gradient that pushes more wastewater toward Well 4. When recharge from infiltration beds is reduced, less mounding occurs and the natural northeast flow direction prevails which results in only modest wastewater influence on Well 4. This interpretation is consistent with the assertion that Well 4 is on the fringe of the plume.

The variability in chloride concentrations at each well and among sites signifies that a three-dimensional consideration of the contamination plume is necessary to predict chloride concentrations down-gradient of the source. A three dimensional model is needed to incorporate vertical flow, groundwater mounding, concentration gradients, aquifer depths, aquifer characteristics, dilution and dispersion and flow rates.

Hypothesis 2: Did Chloride and Boron Concentrations Behave Similarly Across Space and Time?

Boron is thought to be equivalently conservative to chloride in soils that are free of clay deposits that contain iron, aluminum, oxides and hydroxides (Bundschuh 1993; Goldberg 1985). Data from this project suggests boron acts conservatively in

groundwater, but that it may be somewhat less conservative and display somewhat different behavior than chloride.

Chloride and boron relationships were assessed between all sample locations and revealed a tight correlation with an R^2 value of 0.9901 and a p-value of 0.003. In addition, both chloride and boron displayed greater concentrations at Well 3 than Well 2 (Figure 4.2). This is additional supporting evidence that the two constituents behave similarly. This also supports the notion that a vertical gradient is present in the wastewater plume as discussed under Hypothesis 1.

At Well 1, chloride and boron concentrations trend slightly upward through the study with chloride displaying greater variation in concentration. Increased chloride variation is suggestive that chloride is more responsive to wastewater pulses in groundwater from infiltration bed recharge. Boron's reduced variation through time at Well 1 indicates its travel is dampened, resulting in less divergence from the trend (Figure 4.3). Wells 2 and 3 are directly in the center of the contamination flow path and chloride and boron track closely at these sites. Well 4, on the outskirts of the contamination plume, highlights the differential transport of boron and chloride as they move further from the contamination source. Throughout the study, chloride at Well 4 decreases, whereas boron increases until midway through the study when it begins to decrease. These differences in concentration behavior may represent a lag in transport of boron compared to chloride, indicating boron is moving through the aquifer more slowly than chloride. Boron, neutrally charged, can be slowed in the presence of negatively charged soil particles, such as clay and organic matter. Chloride, being negatively

charged, would move freely in a negatively charged environment. Another consideration is that boron concentrations may have been more subject to noise because boron levels were close to the detection limit (10 μg/L).

When assessing chloride-boron relationships for individual wells, the strongest relationships appeared in wells closer to the contamination source (Table 5.1). This decreasing correlation between chloride and boron with distance from the source supports the assertion that transport of boron is retarded relative to chloride.

Table 5.1: Chloride and boron regression relationships at each well

	Regression Equation	R squared	p-value	Sample Size
Well 1	y = 2.6401x + 68.4268	0.688	0.000842	14
Well 2	y = 3.8235x - 15.588	0.8284	0.0318	5
Well 3	y = 2.826x + 16.739	0.3674	0.202	6
Well 4	y = -0.7059x + 45.0882	0.135	0.4737	6

While the parallel behavior of chloride and boron across all wells indicates potential utility of boron as a tool for assessing wastewater influence, there are apparent limitations to its use. If boron were equally conservative as chloride, then the ratio of the two parameters tracked through time at an individual well would be a very useful tool for quantifying wastewater influence. However, the variability in the chloride-boron relationship in individual wells makes it apparent that this relationship cannot be used alone as a reliable indicator of wastewater influence. Instead, it may be necessary to revert to a simple assessment of the magnitude of the two parameters in individual wells with an understanding that there is a difference in the travel speeds of the two indicators that can result in concentrations not tracking together perfectly. In the case of this study

site, a groundwater contaminant transport model is needed to better assess and understand three-dimensional flow and different retardation coefficients for transport of different parameters.

Hypothesis 3: Does Nitrate-N Act Non-Conservatively and Decrease More Quickly than Chloride and Boron?

Nitrate-N, the most frequently found water contaminant in the world, is a complex parameter due to its non-conservative nature, extreme solubility, mobility, and the ability to change species due to biological transformation of nitrate-N (Spalding 1993; Kendy 2001). Data from this project supports the hypothesis that nitrate-N concentration loss was greater than chloride and boron and that nitrate-N was acting non-conservatively. Swift changes in nitrate-N concentrations at Well 1 highlight its mobility in groundwater. Mean concentrations of nitrate-N in Wells 2-4 were much closer to one another than mean concentrations of chloride and boron in those wells (Table 4.2 and 4.3). Nitrate-N values at Wells 2-4 were also significantly lower than the concentrations found at Well 1. This large loss of nitrate-N concentration moving from Well 1 down-gradient indicates denitrification may be occurring in the contamination plume.

Nitrate-N can be converted by microbial activity via denitrification, reducing nitrate-N concentrations by transforming nitrate-N to N_2 gas, in the presence of low dissolved oxygen (<0.5mg/L) and high dissolved organic carbon (DOC) (Puckett 2011). Well 1 did not exhibit clear signs of denitrification as nitrate-N levels were high and variable. In fact, low pH values at Well 1 could be the result of hydrogen ions released during the nitrification of ammonium to form nitrate in the vicinity of the well. Nitrate-N

concentrations decreased dramatically from Well 1 to Well 2, and although dissolved oxygen levels <0.5mg/L were not recorded at Well 2, the significantly lower nitrate-N levels at Well 2 as well as their small variation suggest a large loss of nitrate between the two wells. Dissolved oxygen levels <0.5mg/L were recorded at Well 3 during five sampling events with a range of 0.51mg/L to 0.27 mg/L, which is a range likely conducive for denitrification (Puckett 2011). Wells 2-4 showed small variation of nitrate-N throughout the study indicating it is acting more conservatively down-gradient.

When comparing nitrate-N concentrations to chloride, it is apparent the two constituents behave quite differently. Well 1 exhibited the greatest variation in nitrate-N concentration with values ranging from 7 mg/L to over 49 mg/L within a four month time span. This extreme fluctuation, coupled with relatively stable chloride concentrations at Well 1, indicates consistent loading from the infiltration beds, but inconsistent treatment efficiencies for nitrate-N. Regressing nitrate versus chloride to assess their relationship among all wells produced an R^2 value of 0.4 and p-value=0.38e-5. However, removing Well 1 from the regression resulted in a stronger correlation (R^2=0.8, p-value=6.36e-6). Well 1 is not as representative of attenuated nitrate concentrations in groundwater due to its close proximity to the infiltration beds. The relationship for each well between nitrate-N and chloride was strongest at Well 3 and weakest a Well 1 (Table 5.2). This is inverse of the relationship between chloride and boron where the relationships were strongest in wells closer to the contamination plume. The stronger regressions between nitrate-N and chloride in Wells 3 and 4 may be due to a more conservative behavior of nitrate-N in close proximity to these wells, down gradient from the denitrification zone.

Table 5.2: Chloride and nitrate-N regression relationships at each well

	Regression Equation	R squared	p-value
Well 1	y = 0.5780x + 74.2688	0.100	0.250
Well 2	y = 0.3247x - 4.7576	0.330	0.311
Well 3	y = 0.4543x - 15.8485	0.610	0.067
Well 4	y = 0.0235x + 2.7987	0.443	0.149

The large loss of nitrate-N after Well 1 appears to be due to denitrification; after which nitrate-N behaves more conservatively and is primarily impacted by dilution and dispersion.

Hypothesis 4: Is *Bacteroides* a More Sensitive Biological Indicator of Wastewater Contamination than *E. coli* or Total Coliforms?

Biological analyses evaluated the utility of traditional biological indicators (total coliforms and *E. coli*) and new indicators (*Bacteroides*) for wastewater contamination assessment. There are many biological indicators that can be used to assess the impacts of wastewater; however, not all of the indicators (including total coliforms and *E. coli*) are specific to humans. This lack of specificity can limit interpretations about the contamination source. This project will be one of the first to assess the applicability of using *Bacteroides* as a wastewater indicator in groundwater; dozens of studies have used *Bacteroides* primers in surface water microbial source tracking, but to date, only a few studies have searched for *Bacteroides* in groundwater (Johnson et al. 2011; Knappett et al. 2011). These studies confirmed *Bacteroides* presence in water thought to be influenced by wastewater, but did not assess the utility of using *Bacteroides* as a quantitative indicator organism (Johnson et al. 2011; Knappett et al. 2011).

Due to sensitivity differences based on the qPCR analysis, *Bacteroides* was detected at all of the wells and was present in samples that were negative for *E. coli* and total coliforms. The *Bacteroides* 16S rRNA genes that should be amplifiable by the HF183 qPCR primer has never been isolated and it is uncertain how many copies of the 16S rRNA operon are present in each cell, but studies have suggested that there are 5 copies per cell (Seurinck et al. 2005; Bernhard and Field 2000). This makes it difficult to make a direct comparison between *E. coli* and total coliforms cell counts and *Bacteroides* qPCR results.

Bacteroides Results

Bacteroides was detected at every well, but the highest abundances were recorded at Well 1. In Wells 2-4, the amount of *Bacteroides* fell close to or within the range that was detected in the blank samples. A blank equipment sample with autoclaved, ultra-pure, Millipore water and analyzed for *Bacteroides* for each day samples were collected. Every equipment blank sample during the course of the project tested positive for both AllBac and HF183. This is likely due to non-optimized qPCR analysis where primer dimers caused amplification of DNA and artificially elevated counts, but it is also possible that field equipment may not have been adequately disinfected between sample sites causing contamination. Because equipment blanks reported relatively high concentrations of *Bacteroides* copies, any copy number that is lower than the equipment blank cannot be assumed to be reliable.

In AllBac copy number analyses, blank sample variation is most likely due to noise at low detection limits, indicating concentrations of AllBac were too low to produce

a confident result. The standard curve regression between AllBac concentration and cycle threshold (CT), the cycle at which a sample fluoresces above background, produced R^2 greater than 0.97 for all three trials. This suggests developed standards were a good representation of copy numbers. The standard curve regression was not as strong for HF183 trial runs with R^2 values around 0.9, which could indicate that the standards developed for use in the standard curve were not optimal.

As with prior parameters of boron and nitrate-N, *Bacteroides* was also compared against chloride do determine its conservative nature. Biological agents are larger in size than their chemical counterparts and are more susceptible to filtration and adsorption to soil particles. Chloride relationships with *Bacteroides* were poor, reinforcing the view that biological parameters are not conservative and that they behave differently from chemical parameters (Figures 4.10 and 4.11).

Bacteroides Versus Traditional Indicators

Relationships varied between *Bacteroides,* total coliforms, and *E. coli*. Regressions between total coliforms and *E. coli* showed a strong correlation (R^2=0.88 and p-value=2.22e-6) which indicated a greater presence of total coliforms than *E. coli* (Figure 4.13). The regression between AllBac and HF183 was poor (R^2=0.348, p-value=0.00038) and showed greater presence of HF183 copy numbers, which is counter intuitive as AllBac, being less specific, should encompass HF 183 based estimates (Figure 4.13).

Time series plots of AllBac copy numbers and total coliforms and AllBac copy numbers and *E. coli* showed fairly similar patterns. AllBac did not always track with

total coliforms and *E. coli*. The large peaks and valleys were similar but some of the smaller peaks and valleys were offset by a week which could be due to differential transport of the organism as a result of size or morphology, or it could be a function of random variability in concentrations of organisms moving through the system. Relationships between AllBac copies and total coliforms and AllBac copies and *E. coli* were strong in Well 1 with R^2 values of 0.8, indicating the different bacteria are behaving similarly in groundwater.

Table 5.3: Regression relationships between AllBac and HF183 copy numbers with total coliforms and *E. coli*

	Regression Equation	R squared	p-value
AllBac v total coliforms	y = 1.5173x – 84.5457	0.785	0.000125
AllBac v *E. coli*	y = 0.01435x – 0.39206	0.750	6.47e-10
HF183 v total coliforms	y = 1.887x + 127.493	0.108	0.297
HF183 v *E. coli*	y = 0.02917x – 2.43348	0.343	0.000668

When assessing the regression relationship between AllBac copy numbers and total coliforms, 56 copies of AllBac were present for every one cell of total coliforms. Similar findings occurred with AllBac and *E. coli*; for every one *E. coli* cell, there are approximately 27 copies of AllBac present. AllBac's strong correlation with both total coliforms and *E. coli* indicates it is behaving similar to these parameters.

Time series plots of HF183 copy numbers did not track well with total coliforms or *E. coli*. Again, poor relationships with HF183 show it may not be as good of an indicator as total coliforms or *E. coli* (Table 5.3). HF183 did not have as strong of regression relationship with AllBac and *E. coli*. This could be due to issues in qPCR assays and run optimization. Although HF183 was present at higher levels than AllBac

in most samples, HF183 had a greater variability in the amount of noise found in the data. This could indicate that HF183 was more sensitive than AllBac leading to increased copy numbers due to extraneous amplifications of DNA in the sample, such as primer dimers, and not necessarily representative of the true amount of HF183 present in the sample.

Due to low concentrations of AllBac and HF183 at Wells 2-4, it cannot be assumed that these organisms were present as the concentrations were too close to or below the blank sample concentrations. It appears that *Bacteroides* may not be a useful quantitative indicator organism in areas with low *Bacteroides* concentrations due to the difficultly of detecting low copy numbers.

CHAPTER 6

CONCLUSIONS

This study aimed to better understand the transport of biological and chemical constituents found in groundwater impacted by wastewater. By elucidating the behavior of these parameters across time and space, results provide insight on how to determine if a well is being impacted. The study was also one of the first to use *Bacteroides* as an indicator organism of wastewater contamination in groundwater. The findings presented in this thesis did not support Hypothesis 1 (chloride concentrations would be greatest at wells closest to the infiltrations beds) but it did present evidence of a vertical concentration gradient in the contamination plume. Findings supported Hypothesis 2 which assessed if chloride and boron concentrations would behave similarly. Boron concentrations behaved relatively conservatively when compared against chloride in groundwater and both are useful indicators of wastewater contamination. Hypothesis 3 questioned if nitrate-N would act non-conservatively and decrease more quickly than chloride and boron. There was evidence supporting the assertion that nitrate-N was behaving non-conservatively as denitrification may be occurring between Well 1 and 2, but nitrate-N may be behaving more conservatively in Wells 3 and 4 once initial denitrification has occurred. Hypothesis 4 investigated if *Bacteroides* would be a better wastewater indicator organism than *E. coli* and total coliforms. Analysis of *Bacteroides* revealed that AllBac may be a feasible alternative biological indicator in areas that are close to a contamination source (Well 1). In areas further from a contamination source

(Wells 2-4), AllBac concentrations were too low to determine copy numbers. The human associated *Bacteroides* species (HF183) does not appear to be a good biological indicator in groundwater as low levels of HF183 were difficult to quantify.

Data from this project indicates further research is needed to develop a groundwater model to better understand the physical transport of constituents vertically and horizontally through groundwater at this location. The development of a groundwater model for predicting parameter concentrations moving down-gradient would allow for comparisons among collected samples and predicated concentrations to better understand the relationship between loss of each parameter and distance from the contamination source. Additional analyses such as isotopes may support a new hypothesis that δ15N would be greater at Wells 2-4 than at Well 1 if denitrification is occurring. Future qPCR work on *Bacteroides* in groundwater would be beneficial to determine what quantity of *Bacteroides* presence would indicate a contamination issue. Additional work on optimizing the detection of HF183 at low concentrations would also be valuable.

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