Final Report

DNR Project # 13-CTP-03

Subsurface Fate and Transport of *Cryptosporidium* in Soils of Wisconsin's Carbonate Aquifer Region

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PROJECT SUMMARY

Title: Subsurface Fate and Transport of *Cryptosporidium* in Soils of Wisconsin's Carbonate Aquifer Region

Project I.D.: # 13-CTP-03

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Background/Need: *Cryptosporidium parvum* is a waterborne pathogen that has been demonstrated to have a significant reservoir in cattle manure. As agriculture in Wisconsin is famous for its dairy industry, there are approximately 1.3 million dairy cows in Wisconsin in addition to about 250,000 beef cattle. Due to storage and land spreading of animal manure, the risk exists for transport of pathogenic *Cryptosporidium* spp. to groundwater. Transport of waterborne pathogens to subsurface aquifers has been discounted in the past based on the assumption that physical straining would occur. However, disease outbreak statistics support the proposition that waterborne pathogens can be transported to groundwaters used for drinking water. Therefore, research is needed to understand the potential fate and transport pathways for pathogens such as *Cryptosporidium* oocysts to drinking water wells.

Objectives: This study has three main objectives: 1) determine whether irradiated *C. parvum* are effective surrogate soil surface-to-groundwater tracers for future field studies of *C. parvum* transport; 2) determine the *C. parvum* fate and transport potential for several Wisconsin soils which have developed overlying northeastern (NE) Wisconsin's vulnerable carbonate aquifer; and 3) relate the soil *C. parvum* transport capacity to soil texture, hydraulic conductivity, bulk density, porosity, and hydrophobicity.

Methods: The study was conducted in two stages. Stage I consisted of collecting intact soil cores and bulk soil samples from ten different soil series commonly found in NE Wisconsin's carbonate aquifer region. These soils were thoroughly characterized using standard methods to select a suite of soils with varying characteristics for use in subsequent *Cryptosporidium* transport column studies. The second stage involved monitoring the transport of *Cryptosporidium* oocysts through intact soil columns of different soil series under simulated rain conditions. Side-by-side comparisons of transport of live and irradiated oocysts were performed to assess the potential for using irradiated oocysts in future field studies. U.S. EPA Method 1623.1 was modified to enumerate the presence of oocysts in soil column leachate and at various depths within the soil cores.

Results, Discussion and Conclusions: Experimental results indicate transport of *Cryptosporidium* oocysts is possible in soils (especially with silt loam texture) from the carbonate aquifer region of Wisconsin. The majority of transport to groundwater is primarily influenced by the first flush of water infiltrating through soil macropores and fractures, as flow greatly outpaced the bromide tracer, presumably bypassing any ability the soil has to strain *Cryptosporidium* oocysts out of leachate. Soils with significant clay content in their B horizons may be capable of attenuating subsurface movement of waterborne pathogens from land applied manure. Results also support the assumption that a significant portion of *Cryptosporidium* oocysts will sorb or be physically entrapped in the soil, especially in soils with high clay content. Experimental results suggest similarity in subsurface transport behavior between irradiated oocysts and live oocysts.

Recommendations: Based on the findings discussed above, recommendations for preventing the migration of *Cryptosporidium* oocysts from land applied manure include: 1) perform tillage after the application of liquid or solid manure; 2) increase setback distances for soils of concern from fractured bedrock, drinking water wells, or other bodies of water; 3) determine a minimum depth of soil treatment zone above glacial till or bedrock for manure application. These recommendations are expected to reduce the likelihood of *C. parvum* transport to drinking water supplies by providing conditions for soils to sorb and attenuate the movement of oocysts prior to rainfall or snow melt events.

Key Words: *Cryptosporidium*, soil, transport potential, groundwater tracer

Funding: Wisconsin Groundwater Coordinating Council

I. INTRODUCTION

Cryptosporidium and *Giardia* are two parasitic pathogens that can be transported to source waters and pose a significant risk to human health. Contaminated drinking water is a known source of human infectivity that has resulted in both illness and death in recent history. In fact, an estimated 403,000 illnesses and one hundred deaths are attributed to an outbreak in Milwaukee in 1993 following exposures to drinking water contaminated with *Cryptosporidium* oocysts (MacKenzie *et al.*, 1994; Craun *et al*., 2006). Between 2010 and 2014, three of the ten documented waterborne outbreaks in Wisconsin were attributed to *Cryptosporidium* contamination (R. Klos, WI DHS, March 2, 2015). Outbreaks such as these are a major driving force behind recent U.S. Environmental Protection Agency's (U.S. EPA) revisions to regulations related to the Safe Drinking Water Act (PL 93-523, 1974).

There are numerous recognized species of *Cryptosporidium*, but *C. parvum* along with *C. hominis* are the species responsible for clinical illness in immunocompetent humans and *C. parvum* is recognized as the most important biological water contaminant in the U.S. (Rose *et al*., 1997). *Cryptosporidium* spp. infections cause acute and chronic diarrhea (Barwick *et al*., 2003; Ferguson *et al*., 2003) and human infections and disease outbreaks caused by *C. parvum* and *C. hominis* are universal (Himathongkham *et al*., 1999; Barwick *et al*., 2003; Fayer *et al*., 2004). The Centers for Disease Control and Prevention (CDC) estimate that there are approximately 748,000 cases of cryptosporidiosis each year in the United States, equivalent to approximately 0.2 percent endemic infection rate (Scallan *et al.*, 2011).

C. parvum is prevalent among cow herds and known to be present in high concentrations in dairy calf manure. Thus, *Cryptosporidium* oocysts are commonly found in manure (Himathongkham *et al*., 1999; Graczyk *et al*., 2000; Sischo *et al*., 2000; Ferguson *et al*., 2003; Fossler *et al.*, 2005) and are of particular concern for surface water and groundwater sources with exceptionally high or preferential transmissivities such as carbonate aquifers (Atteia and Kozel, 1997; Auckenthaler *et al*., 2002; UW Extension, 2007; Harvey *et al*., 2008; Nadine Göppert, 2008). A widely publicized watershed *Cryptosporidium* contamination by dairy cattle in the U.K. has raised awareness of dangers associated with non-bacterial pathogens. Concerns associated with environmental *C. parvum* can be attributed to several factors including: 1) *C. parvum* do not need to reproduce outside the host to remain infectious, 2) a very low infective dose (as little as 10 oocysts) is necessary to initiate an infection in humans (Dupont *et al*., 1995; Olson *et al*., 1999; Caccio, 2005), and 3) waterborne oocysts are relatively resistant to traditional chlorine disinfection (Sobsey, 1989; Sterling, 1990). *C. parvum* oocysts have been detected frequently, at low concentrations, in groundwater samples in the U.S. (Hancock *et al*., 1997) and Europe (Lisle and Rose, 1995) and studies by Harvey *et al*. (2008) suggested that *C. parvum* would move preferentially over several 100 m in carbonate aquifers without even a tenfold reduction in numbers. Field data are also available confirming deeper penetration of *C. parvum* in subsurface environments (Sinclair and Ghiorse, 1987) with vadose zone transport through macropores (Mawdsley *et al*., 1996a; 1996b; Darnault *et al*., 2004; Harter *et al*., 2008).

The physico-chemical characteristics of *C. parvum* facilitate their transfer between environmental compartments. *C. parvum* oocysts carry a negative charge under a wide range of environmentally relevant pH conditions (pH 5 to 8.5), with this negative charge decreasing with decreasing pH and becoming near-neutral around pH 2.5 (Drozd and Schwartzbrod, 1996; Brush *et al*., 1998). Oocysts are approximately spherical (4.5 to 5.5 µm in diameter (Medema *et al*., 1998)) with a density slightly higher than that of water (1.06 g/cm^3; sedimentation rate = 0.35 µm/s (Medema *et al*., 1998)) and do not exhibit strong hydrophobicity. Their low settling rate and polar characteristics increase potential for transport whereas their colloidal size could provide some opportunities for physical entrapment (*i.e*., straining). Oocyst negative surface charge is not conducive for attachment to the solid phase, since most environmental particles are negatively charged under environmentally relevant pH conditions.

Groundwater contamination by manure-borne microorganisms in NE Wisconsin is a significant and increasing problem, where thin soils overlie carbonate bedrock and manure storage and spreading by the region's many agricultural operators is a common practice (UW Extension, 2007). According to the Final Report of the Northeast Wisconsin Karst Task Force (UW Extension, 2007), "In Morrison Township, Brown County, Feb-March 2006, 86 wells tested unsafe for Coliform and/or *E. coli*," after a significant rain/snowmelt event and from over 1000 well tests between 2002 and 2005, 35% have returned positive results for coliform bacteria and 4.6% for *E. coli*. Although little well testing for *Cryptosporidium* has been conducted in NE Wisconsin, primarily because of prohibitive cost of analysis (approximately $500 per sample), it is likely to be present (Liz Heinen, 2011, WI DNR NE Region Drinking Water Systems Specialist). When three children, ages 18 months, 4.5 and 13 years, of the

Tracy Zimmerman family contracted cryptosporidiosis (the 18 month old's case was nearly fatal), *C. parvum* contamination of their farm's well, which draws from the carbonate aquifer in northern Dodge County, WI, was suspected.

There is clearly a need to improve our understanding of underlying processes controlling environmental fate and transport of *C. parvum* oocysts, particularly via soil to shallow carbonate aquifers that serve as drinking water supplies. Wisconsin has over 1 million private drinking wells, and groundwater is the drinking water source for roughly 70% of residents and 95% of communities (Kevin Masarik, 2014, Central Wisconsin Groundwater Center groundwater outreach specialist). Therefore, preservation of groundwater resources is critical for maintaining a safe drinking water supply. Land application of livestock manure is a regular component of current producers' land and nutrient management plans. Pathogens found in land applied manure can include bacteria, viruses and parasites (Gessel *et al*., 2004; Gerba and Smith, 2005; Pepper *et al*., 2006). Because most current dairies and livestock producers rely heavily on antibiotics to maintain the health of their herds and flocks, there has also been a significant increase in antibiotic resistance in fecal bacteria and antibiotic resistant strains are increasingly found in soil (Hamscher *et al*., 2002; Brooks *et al*., 2007) and groundwater (Chee-Sanford *et al*., 2001; Ozgumus *et al*., 2007), potentially elevating the risk of disease outbreaks in human populations.

A significant body of work has shown that *C. parvum* oocysts and colloids with similar physico-chemical properties can be significantly attenuated by sands and sand filtration systems (Yao *et al*., 1971; Tobiason and Omelia, 1988; Logan *et al*., 1995; Tufenkji *et al*., 2004; Bradford and Bettahar, 2005). Straining appears to be an important mechanism, with significant oocyst retention noted in most column experiments at shallow depths close to the injection point. However, data obtained by Mawdsley *et al*. (1996a; 1996b) from both undisturbed and disturbed soil column studies using a variety of soil textures point to significant downward migration of oocysts. Transport of oocysts could occur due to advection and hydrodynamic dispersion while attachment to solid surfaces and straining could attenuate their migration through porous media (Harter *et al*., 2000; Bradford and Bettahar, 2005). When macropore transport predominates, lack of filtration and contact time for interaction with sorptive surfaces could facilitate significant downward movement of oocysts (Mawdsley *et al*., 1996a; 1996b; Darnault *et al*., 2004; Pachepsky *et al*., 2006; Harter *et al*., 2008) and bacteria such as *E. coli* (Unc and Goss, 2003; 2004; Guber *et al*., 2005).

While a relatively large body of research exists for the occurrence of *Cryptosporidium* and the environmental factors that may affect their presence in, transport to, and detection in drinking water supplies (Robertson *et al.*, 1992; Fayer, 1994; Chauret *et al.*, 1995; and Boyer and Kuczynska, 2003), there are several conditions and situations that influence their movement through soils yet to be fully investigated. Some of these parameters include event rainfall, antecedent rainfall, streamflow and hydrology, land use characteristics, and correlations with indicator organisms and source tracking indicators. Differences in land use characteristics can also play a role in the permeability of soils, and presence and intensity of contaminant sources. Land characteristics such as vegetative cover and slope have also been demonstrated to be critical to include in models to predict transport of *Cryptosporidium* in overland flow (Bhattarai *et al.*, 2011) and risk for presence of *Cryptosporidium* contamination (Samadder *et al.*, 2010).

To minimize contact and risk from pathogenic *C. parvum*, irradiated *C. parvum* oocysts are used outside the U.S. as performance testing standards in certified laboratories to assess recovery efficiencies. Irradiated oocysts are prepared by passing the Iowa strain through infected calves. The oocysts are separated from other fecal matter via flotation. The final oocyst suspension is enumerated and gamma irradiated to inactivate the sporozoites. It is assumed that, surface characteristics of test oocysts are similar to nascent oocysts without the risk of infection. However, the validity of this assumption has not been well documented in the literature. Side-by-side comparison of live and irradiated oocysts will provide valuable information for future research on *C. parvum* fate and transport. A major challenge for research related to environmental dynamics of pathogens involves the use of live pathogens (especially for larger-scale and field-level experiments) and their analysis, which limits the ability to determine the influence of numerous chemical controls on fate and transport. The use of irradiated oocysts as surrogates in future field studies should help overcome these issues.

Therefore, the objectives of this study were to: 1) determine whether irradiated *C. parvum* are effective surrogate soil surface-to-groundwater tracers for future field studies of *C. parvum* transport; 2) determine the *C. parvum* fate and transport potential for several Wisconsin soils which have developed overlying NE Wisconsin's vulnerable carbonate aquifer; and 3) relate the soil *C. parvum* transport capacity to soil texture, hydraulic conductivity, bulk

density, porosity, and hydrophobicity. Experiments with viable oocysts allowed for assessment as to whether surface characteristics of the oocysts remained unaltered after exposure to γ-radiation. A primary motivation for this work was to gain insights on the influence of soil physical properties that would inform future field studies.

II. PROCEDURES AND METHODOLOGY

Site Selection:

Northeastern Wisconsin's vast carbonate aquifer region has a multitude of soils with vastly differing physical and textural characteristics. The soils of Wisconsin's NE region were objectively screened to identify the most predominant agricultural soils within the region, based on land area. Personnel in the Land and Water Conversation offices of Door, Brown, Calumet, and Kewaunee Counties, Liz Heinen (WDNR NE Region Water Quality Specialist), UW-Extension offices, and Dr. Fred Madison (Emeritus Professor of UW-Madison, Department of Soil Science) were consulted to identify willing private land owners whose land represents 10 soils with the broadest range of soil properties (*i.e.*, textures, series). The 10 soils selected for preliminary evaluation included: Emmet (EmA), Hochheim (HmB), Hortonville (HrB), Kewaunee (KnB), Lomira (LvB), Longrie (LoB), Onaway (OhB), Oshkosh (OnB), Theresa (ThB), and Waymor (WoB). (Note: the NRCS Soil Survey soil map unit is followed in parentheses by its abbreviation that denotes the map unit of the soil followed by a letter (A or B) indicating the slope of the land area where the soil was sampled).

Soil Sampling:

Soil sampling was conducted in two separate stages, with distinct objectives and methodology for each stage. To avoid issues associated with differential soil drainage and proximity to the water table, all soils were collected from the same landscape position (the summit) for both stages. The main objective of the first stage was to document the physical soil properties of all 10 study soils. Undisturbed cylindrical soil cores (7.3 cm I.D. x 7.5 cm length) were collected in triplicate using an AMS brand soil core sampler with plastic liners and loose soil samples were collected (also in triplicate) from both the A and B soil horizons with a shovel. The collected soil cores and loose samples were refrigerated at 4 °C until all soil analyses were completed.

The second stage involved collection of comparatively larger intact undisturbed soil columns (15 cm I.D. x 50 cm length) for the purpose of examining *Cryptosporidium parvum* (*C. parvum*) transport potential. During the second stage, three soils (LvB, HmB, and KnB) of the 10 for which physical properties were characterized, were selected as they represented a range of transport potentials. Soils EmA and LoB were excluded from consideration as their gravelly and rocky soil texture would have prevented collection of intact undisturbed soil columns. A minimum of four intact soil columns (for each soil series) were collected in 15 cm x 50 cm aluminum columns using a truck mounted vertical hydraulic press (Giddings Machine Company, Windsor, CO) to drive the aluminum column vertically into the ground. The columns were then manually retrieved by trenching along the sides and severing the interface between the soil and the bottom of the column. Prior to insertion of the aluminum column, the bottom edge was sharpened to reduce frictional forces and a thin layer of petroleum jelly was applied to the inside of the column to provide a good seal between the soil and the core wall. This step also served to prevent bypass flow of water via the interface between the soil and column wall during rainfall simulations. Within 24 h of retrieval, the columns were saturated from the bottom up with hot water (approximately 50 °C) to remove invertebrates, inspected for preferential flow via the column wall, and allowed to saturate for 48 h to ensure invertebrate removal or inactivation. The columns were then drained, capped, and stored at 4 °C prior to experimentation.

Soil Physical Properties:

Soil physical property analyses included soil texture, percentage of organic matter, hydraulic conductivity, macroporosity, hydrophobicity, and bulk density and all were conducted following standard published methodology. Soil texture analysis (% sand, silt, and clay) was performed with loose soil using a hydrometer based particle settling method (UW-SPAL, 2004). Gravel texture modifiers were determined gravimetrically as the percentage of rock fragments > 2 mm from the total dry weight of the soil sample (USDA-NRCS, 2013). The percentage of organic matter within the soil was quantified using a loss-on-ignition method (Schumacher, 2002) on loose soil. An exact amount of 105 ºC oven dry soil (< 5 g) was heated to approximately 440 °C for at least 8 h. The percentage of material ignited (volatilized) represented the percentage of organic matter within the loose soil. A constant head

hydraulic conductivity analysis developed by Klute and Dirksen (1986) was performed on the small soil cores (7.3 cm I.D. x 7.5 cm length). Prior to analysis, the soil cores were saturated for 24 h using deionized (DI) water containing 0.0025 *M* $CaSO_4$. A constant head of 7.5 cm of 0.0025 *M* $CaSO_4$ was applied to each core. Once the outflow from the soil core had stabilized, four replicate outflow volumes were sampled. Macroporosity was determined using porous ceramic plates and pressure chambers as outlined in ASTM D6836-02 (2008). Small soil cores were initially saturated for 24 h using degassed 0.0025 *M* $CaSO_4$. Soil cores were analyzed at four different pressures - fully saturated, 0 cm H_2O, 25 cm H_2O, and 50 cm H_2O. The "Water Drop Penetration Time" analysis used by Doerr (1998) was performed on air-dried soil cores to determine their hydrophobicity. Fifteen droplets of DI water with a volume of 30 µL were dropped from a height no greater than 5 mm. The time from droplet impact to complete penetration was recorded. Increased penetration times indicate a greater level of hydrophobicity. Soil bulk density was determined as the mass of oven dry (105 °C) soil contained within the volume of the small (7.3 cm I.D. x 7.5 cm length) soil cores (Burt, 2009).

Rainfall Simulator:

The rainfall simulator is a hybrid of two devices developed by Boyer *et al.* (2008) and Peterson *et al.* (2012). The part of the simulator that supplied simulated rainfall was designed and built to fit as a cap for the individual aluminum soil columns. As a whole, the rainfall simulator is comprised of the aluminum soil column, a sealed PVC base cap, vacuum pump, leachate collection system, peristaltic pump, and the rainfall simulator cap (Figure B.3). The PVC base cap contained a rigid PVC drain plate to support the column, a 75 µm (200-mesh) stainless steel screen laid atop the drain plate, and a tube fitted drain outlet. A screen size of 75 µm was selected to prevent soil aggregate migration at the bottom of the column, while still allowing *C. parvum* (4 - 6 µm O.D.) to migrate through the screen via matrix flow. To provide an air tight seal between the PVC base cap and column fitting, three rubber O-rings, coated with petroleum jelly, were installed. With the air tight seal, a small vacuum force of 127-mm of water was applied to the collection bottle system as well as the base of the soil column to simulate natural forces acting on the bottom of the soil column (Peterson *et al.*, 2012). Leachate that passed through the PVC base cap was collected in a 250 mL bottle under vacuum. Air expelled from the bottle passed through a GE brand HEPA-VENT filtration disk prior to being released to the open atmosphere to prevent airborne release of *C. parvum*.

The rainfall simulator cap contained a segmented coil of rigid plastic tubing pierced with 22 blunted needles (Figure B.3). The needles were supplied via a multi-channel peristaltic pump, wherein segments of 4 or 5 needles were independently supplied to prevent an uneven distrubtion of liquid across the coil due to the pressure drop associated with each needle. The cap was calibrated prior to the start of the simulation on a volume per pump rpm basis and modeled as a linear fit equation. To minimize the impact of water droplets, the height of the blunted needles was adjusted and set to an elevation no greater then 2.5 cm above the soil surface. The cap was also manually rotated throughout the duration of the simulation to randomize the location of rain drop impact. Together the rotating coil of tubing, 22 blunted needles, and peristaltic pump provided an evenly distributed and constant supply of droplets to the soil columns.

C. parvum and Irradiation Methodology:

Live *C. parvum* oocysts stocks were purchased from the Sterling Parasitology Laboratory located at the University of Arizona (Tucson, AZ). Sterling's Iowa isolate oocysts were purified from infected Holstein calves using discontinuous sucrose and cesium chloride centrifugation gradients and stored at 4 °C in an antibiotic solution containing 0.01% Tween 20, 100U of penicillin, 100 µg of gentamicin per mL (UA Sterling, 2015). The Wisconsin State Laboratory of Hygiene (WSLH, Madison, WI) personnel enumerated all subsequent Sterling oocysts inoculative doses and spikes for this study with flow cytometry. Oocysts were inspected for intactness via microscopy by WSLH personnel prior to enumeration and stored at 4 °C in a solution of purified MilliQ water and 0.01% Tween 20 post enumeration. The WSLH personnel maintained fresh stocks of *C. parvum* oocysts from Sterling and all stocks (inoculative doses and spikes) were discarded 60 days post shed date (time of collection from calf) of the oocysts. Stocks used in this study were discarded 50 days post shed date to ensure the viability of the oocysts throughout the duration of rainfall simulation and subsequent processing for analysis.

Irradiated *C. parvum* oocysts were produced from Sterling Parasitology stock oocysts solution. The stock live oocysts received a dose of 2.5 kGy of irradiation using a cesium irradiator (J. L. Shepherd Mark I Unit) available within the Department of Environmental, Health and Safety at UW-Madison. Post irradiation and prior to

enumeration via flow cytometry, the WSLH personnel again inspected the irradiated oocysts for intactness via microscopy. The irradiated enumerated oocysts were likewise stored at 4 °C in a solution of purified MilliQ water and 0.01% Tween 20 post enumeration and subject to the same post shed date expiration date as the live oocysts.

Inoculation of Manure:

The suspended solids (SS) and total solids (TS) content of liquid manure varies throughout the year and by producer. In addition, there is a known inverse relationship between SS & TS content and *C. parvum* transport potential (Kuczynska, 1999; Searcy *et al.*, 2005). Irrigation of liquid manure is rising in popularity, which requires TS contents between 1% and 4%. For our experiments, we determined liquid cattle manure with 1% TS at an application rate of 5000 gallons of manure per acre (50 ton/ha) to be appropriate to assess *C. parvum* transport.

Liquid cattle manure was obtained from the UW-Madison, Arlington Agricultural Research Station (AARS) in Arlington, WI from an agitated separated liquids pumping pit. Fresh manure was collected prior to the start of every rainfall simulation experiment, analyzed for TS using standard methods (APHA *et al.*, 2012), and diluted with DI water to 1% TS. Manure was prepared for the simulation by inoculating 85 mL of 1% liquid manure with $1.0x10^6$ *C. parvum* oocysts (live or irradiated).

C. parvum Transport Experimental Procedure:

Soil column rainfall simulations were performed in two separate phases. Both live and irradiated *C. parvum* were used in Phase I for duplicate rainfall simulations on a single soil series, Lomira (LvB). In Phase II, dupicate rainfall simulations were conducted on three soil series, LvB, Hochheim (HmB), & Kewaunee (KnB), using irradiated *C. parvum*. Each soil column rainfall simulation was conducted in two steps over the course of a two week period. Step one involved determining the pore volume and preliminary infiltration rate of the column, and conducting the required *C. parvum* blanks and spikes for leachate and soil samples from the column as required by U.S. EPA Method 1623.1 (Telliard, 2005). Step two consisted of the application of inoculated manure to the surface of the soil column and the collection of leachate and soil samples, and analysis using U.S. EPA Method 1623.1 (U.S. EPA, 2012).

Prior to step one, the top soil surface of each column was manually leveled by removing the aggregates. Unstable soil aggregates were removed from the bottom soil column surface. The resultant stable soil columns were then measured depth wise in length to determine the soil volume. Prior to the start of a rainfall simulation (steps one and two), the soil column was saturated with 0.0025 *M* $CaSO_4$ from the bottom up for 48 h via the PVC base cap and then drained of free flowing water. Immediately after drainage subsided (step one), the soil column apparatus was weighed and the wet weight of soil was computed (by subtracting the weight of the column apparatus). The pore volume of the soil contained within the column was approximated as:

Pore Volume (mL) = [Wet Soil g – (Volume soil mL * Bulk Density g/mL)] / Density H_2O g/mL (1)

The preliminary infiltration rate of the column was the maximum rate of simulated rainfall that did not produce ponding on the soil surface. To determine this infiltration rate in step one, rainfall (using D.I. water) was applied starting at a low application rate and slowly increased until stable ponding occurred, while under a vacuum of 127 mm H_2O. After the preliminary infiltration rate was determined, the simulation continued until 750 mL of leachate, when applicable, was collected for leachate blank and spike processing under U.S. EPA 1623.1. The soil collected during the top and bottom surface leveling and unstable aggregate removal process were retained and used as the matrix material for soil blank and spike processing under U.S. EPA 1623.1, and for measuring soil pH and percent moisture content (Telliard, 2005; U.S. EPA, 2004).

For step two of the rainfall simulation, a narrow band of petroleum jelly was initially applied to the soil surface where it was in direct contact with the aluminum column wall. This step further reduced the possibility of bypass flow. A vacuum force of 127 mm H_2O was then applied to the column prior to the application of the inoculated manure. The surface application of liquid inoculated manure (1% TS), containing $1.0x10^6$ *C. parvum* oocysts (live or irradiated), was performed by pouring the manure directly over the soil in small volumes, so as to prevent ponding. The emptied bottle containing the inoculated manure was rinsed 3 times, each with 5 mL of DI water, and the rinsate was then applied to the surface of the soil. After the manure and rinsate were allowed to completely

saturate into the soil, the application of simulated rainfall began with a solution of 0.1 M sodium bromide in DI water. The bromide solution was used as a conservative tracer to monitor the breakthrough response of the applied solution (Peng, 2011). The rainfall simulation continued until either one pore volume of leachate was recovered from the column or a maximum of 2.5 days had elapsed from the initiation of the simulation. Bromide concentration in the leachate was monitored every 250 mL and leachate samples for *C. parvum*, pH, and electrical conductivity (EC) analysis were collected at set intervals of 5, 25, 45, 65, 85, 100% of the column's estimated pore volume. At the end of rain simulation, the soil was manually removed from the aluminum column in 10-cm sections. The soil was removed from the bottom up, as the surface horizon was assumed to contain the highest concentration of *C. parvum* oocysts and could cross contaminate the other lower concentration soil horizons as they were removed. All leachate and soil samples were stored at 4 ºC until analyzed for *C. parvum*.

C. parvum Liquid and Soil Analysis U.S. EPA 1623.1:

The *Cryptosporidium* analysis was conducted at WSLH. Leachate and manure samples were subsampled as required by using 1/1, 1/4, 1/25, 1/100, 1/300, or 1/1000 dilutions to keep oocysts counts under a maximum of 1000 oocysts per subsample and analyzed in accordance with U.S. EPA Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMA/FA (Telliard, 2005; U.S. EPA, 2012). As leachate and manure samples were under 250 mL, Step 12.0 "Filtration and Elution" in U.S. EPA 1623.1 was skipped and samples proceeded directly to Step 13.0 "Sample Concentration and Separation (Purification)" (Telliard, 2005; U.S. EPA, 2012).

In the absence of a published U.S. EPA standard for the extraction of *C. parvum* specifically from a soil matrix, a method was developed and utilized by the research team in consultation with WSLH *Cryptosporidium* analysts. The extraction method that was developed included a discontinuous soil, sucrose, sodium hexametaphosphate, phosphate buffered extraction solution centrifugation gradient step, denoted as SPEG, prior to analysis by U.S. EPA 1623.1 (starting on step 13.0). The extraction of *C. parvum* from the soil required the dispersion of 5 grams (dry weight basis) of soil in 50 mL phosphate-buffered saline (PBS) stock solution (containing 0.5% 7x detergent and 5% sodium hexametaphosphate) in 250 mL centrifuge tubes. The solution was gently swirled until the soil was dispersed and then allowed to settle for at least 10 min. The solution was then vortexed on a medium-high setting for 30 s and allowed to settle again for 10 min before the floatation step. The discontinuous soil, sucrose, and extraction solution centrifugation gradient was created using a 20 mL syringe and a blunted stainless steel cannula to underlay the settled soil solution with 40 mL of 1.18 specific gravity (4 ºC) sucrose solution. The solution was then centrifuged at 1,050*g* to separate the soil, sucrose, and extraction solution into three discontinuous phases. The extraction solution and 20 mL of sucrose phase that interfaced with the extraction solution, which contained the captured *C. parvum*, were removed and placed into a 250 mL centrifuge tube containing 135 mL of R.O. water. The diluted extraction solution and sucrose interface were then analyzed in accordance with U.S. EPA method 1623.1 starting on step 13.0 (Telliard, 2005; U.S. EPA, 2012).

Given the potential to have high quantities of *C. parvum* oocysts (1,000) on the resultant slide, the positive determination of *C. parvum* was streamlined so microscopy could be achieved within a reasonable timeframe consistent with U.S. EPA 1623.1 guidelines. The positive determination of *C. parvum* oocysts was made at 400x magnification and required all of the following: 1) size 4-6 µm, 2) ovoid or round shape, 3) positive immunofluorescence assay (FA) florescence using a fluorescein isothiocyanate (FITC) stain, and 4) fluorescent outer shell wall of appropriate thickness. When the authenticity of the *C. parvum* was questioned, FA and differential interference contrast (DIC) examination at 1000x magnification was utilized to determine if the *C. parvum* met requirements 1 - 4 and contained the appropriate internal morphological characteristics.

Data Analysis:

The long duration of simulated rainfall events and the inherently lengthy duration to complete U.S. EPA 1623.1 for the analysis of *C. parvum*, ultimately reduced the number of experimental replicates that were possible. Given the low numbers of replicates, the data set has been analyzed for statistical sufficiency when possible; however, observational conclusions have also been made based on apparent strong trends, but lack statistical sufficiency primarily as a result of low replicability within the data set.

The dataset consists of *C. parvum* recoveries from the two phases of rainfall simulation and soil physical properties analysis on loose soil and the small 7.3 cm x 7.5 cm soil cores. The observed counts of *C. parvum* oocysts (C)

recovered from the initial dose of $1.0x10^6$ oocysts (C_{To}) from the two rainfall simulation phases were organized by six comparative simulation responses: *C. parvum* total recovery (C_{T1}/C_{To}), *C. parvum* fraction recovered in leachate (C_{TLec}/C_{To}), *C. parvum* fraction recovered in soil (C_{TSoil}/C_{To}), *C. parvum* fraction recovered in leachate first flush ($C_{TLec5\%}/C_{To}$), leachate *C. parvum* hydrograph profile, and soil *C. parvum* depth distribution profile. Statistical comparison of *C. parvum* recoveries and the effect of irradiation, as well as soil properties on recoveries was analyzed with a Welch's Two sample t-test in R statistical software ($\alpha = 0.05$) in combination with confidence intervals. Welch's t-test was chosen as it can statistically model a dataset with a low level of replication. Confidence intervals were also required as proof of a significant Welch's t-test finding, given the inherent variability associated with low replication. The *C. parvum* hydrograph and soil *C. parvum* depth distribution profiles were visually compared. A data set comprising 5 replicates of both live and irradiated soil spike *C. parvum* recoveries, from soil series LvB from Phase I, was analyzed using a 2-way ANOVA (Tukey) in SAS ($\alpha = 0.05$).

III. RESULTS AND DISCUSSION

Soil Properties:

The physical properties of the 10 soils investigated are summarized in Table 1. These soils are representative of the variety of NE Wisconsin soils. Soil textures were predominantly loam or silt loam in the A horizon. In the B horizon, clay loam or clay were predominant for all soils. Gravel modifiers were also required for two soil series EmA and LoB as they had a high percentage of rock fragments larger than 2 mm in diameter (Table 1).

Bulk densities for all A horizon soils were close to the value (1.33 g/cm^3) required for ideal plant and root development (Table 1; USDA-NRC, 2015b). This was expected as all fields were or had been in agricultural production, requiring some type of recent A horizon tillage. Soil bulk densities in the B horizon were also at or near optimal values for uncompacted subsoils; however, bulk density for LoB, KnB, and OnB were high enough to potentially restrict root growth given their texture, but not high enough to be considered a plow pan or compacted soil (USDA-NRC, 2008). These restrictive horizons could be a result of increased clay content in the KnB and OnB soils and high percentage of gravel in LoB soil, both of which increase soil density.

The majority of soils were observed to be hydrophilic, indicating that the soil surface allowed for unrestricted infiltration of water (Table 1). The clay textured A and B horizon soils of OnB & KnB, as well as the A horizon of the silt loam LvB were slightly hydrophobic as they exhibited a limited ability to repel water droplets. Increasing clay content imparts higher negative charge in soils, in general, which might enable those soils to more readily repel water molecules (CU Extension, 2007; NCDA, 1999). While particle charge is the most apparent factor driving water repellence in the clay soil, other factors such as the presence of residual fats or oils from manure may contribute in the A horizon of the LvB soil. Overall, soil porosity was inversely related to bulk density (Table 1). Porosities for loam textured soils were within normal ranges; however, porosities for clay textures and those containing gravel modifiers were below typical values (Rawls, 1982).

The saturated hydraulic conductivity (K_{sat}) of the soils (Table 1) covered the full extent of the USDA-NRCS soil textural class K_{sat} scale, from impermeable to very rapid conductivity (USDA-NRC, 2015a). The majority of soils (loam or silt loam) had measured K_{sat} rates classified as moderately rapid, which is above the classification typical of their textural class (Table 1). Clay textured soils exhibited the greatest variation in K_{sat} values. The A horizon clay soils were classified as moderate or moderately rapid, also above their typical textural K_{sat} class. The high K_{sat} rates are most likely the result of nonmatrix and interstructural macropores within the soil. While measuring the extent of macropores in these soils was beyond the scope of this project, based on visual evidence, the soils that exhibited higher than expected K_{sat} routinely contained nonmatrix and interstructural pores. Clay B horizon soils (OnB and KnB) were typically impermeable, which is lower than their textural K_{sat} classification attributable to elevated bulk densities and below normal porosities. As bulk density increases and porosity decreases, the soil contains less available pore space reducing nonmatrix and interstructural pores, and thus restricting the ability of water to migrate through the soil. The observed slight hydrophobicity of the clay soils would also inherently restrict water migration as the soil particles naturally repel water throughout the denser B horizon.

Table 1: Essential physical properties for the 10 investigated soils:
Soil series and map units are given according to NRCS soil survey records for the sample locations.

Soil Series	Map Unit	Soil Horizon	*Soil Textural Class	** K_{sat} µm/sec	USDA K_{sat} Class	*Porosity mL/mL	*Bulk Density g/cm^3	*% Organic Matter	*Hydrophobicity
Hochheim	HmB	Ap	SiL	63.7	R	0.40	1.3	4.5	H
		Bt	SCL	1.3	S	0.35	1.5	2.2	H
Lomira	LvB	Ap	SiL	158.7	VR	0.43	1.2	3.9	SH
		Bt	SiCL	3.4	MS	0.39	1.3	1.9	H
Theresa	ThB	Ap	SiL	30.0	MR	0.41	1.3	3.8	H
		Bt	SiCL	0.6	S	0.39	1.4	2.4	H
Onaway	OhB	Ap	L	14.3	MR	0.37	1.4	4.1	H
		Bt	CL	0.1	I	0.36	1.5	0.9	H
Waymor	WoB	Ap	SiL	92.8	R	0.47	1.1	4.7	H
		Bt	CL	3.1	MS	0.38	1.5	1.6	H
Oshkosh	OnB	Ap	CL	40.3	MR	0.39	1.3	3.5	SH
		Bt	C	0.1	I	0.40	1.4	1.9	SH
Hortonville	HrB	Ap	CL	145.1	VR	0.38	1.3	5.3	SH
		Bt	CL	0.1	I	0.41	1.4	3.0	H
Kewaunee	KnB	Ap	SiC	26.4	MR	0.40	1.4	3.8	SH
		Bt	C	0.1	I	0.37	1.5	2.4	SH
Emmet	EmA	Ap	L	17.6	MR	0.43	1.2	5.0	H
		Bs	LG	27.2	MR	0.34	1.5	1.5	H
Longrie	LoB	Ap	LG	161.1	VR	0.44	1.1	5.8	H
		Bs	SLVG	1.5	MS	0.27	1.8	0.8	H

*(n) = 3 **(n) = 12

Soil Textural Class Abbreviations: (C) clay; (CL) clay loam; (L) loam; (LG) loam gravelly; (S) sand; (SCL) sandy clay loam; (Si) silt; (SiC) silty clay; (SiL) silt loam; (SLVG) sandy loam very gravelly

USDA K_{sat} Abbreviations: (K_{sat}) saturated hydraulic conductivity; (VR) very rapid; (R) rapid; (MR) moderately rapid; (MS) moderately slow; (S) slow; (I) impermeable

Hydrophobicity Abbreviations: (H) Hydrophilic; (SH) Slightly Hydrophilic

Organic matter (OM) in the A horizon of Wisconsin soils generally ranges from 1 – 6% by dry mass (Cooperband, 2002) and soils containing at least 3.4% OM are generally considered to be fertile agricultural soils (Loveland, 2003). The percent OM for the soils in this study ranged from 3.5 to 5.8% (Table 1). While OM is known to affect *C. parvum* migration (Kuczynska, 1999; Searcy *et al.*, 2005), the observed range of OM was not expected to influence transport potential.

Soil Selection:

The selection of soils for *C. parvum* transport analysis was performed based both on the physical properties of the soils as well the practicality of retrieving a usable large intact soil column. Soil series EmA and LoB were excluded from consideration as their gravelly and rocky soil texture would have prevented the retrieval of intact undisturbed soil columns. Three soils, LvB, HmB, and KnB, were then selected from the remaining eight soil series for further evaluation.

While excluded from transport study, there is still potential risk for *C. parvum* transport through EmA and LoB soils; for example, LoB has similar physical soil properties as soils found, in this study, to have potential to transport *C. parvum*. These similarities included a low A horizon bulk density, high porosity, and K_{sat} classification of very rapid. The very rapid A horizon K_{sat} class (161.1 µm/sec) of LoB combined with its permeable B horizon indicate a high potential to transport water and associated *C. parvum* through the soil matrix, nonmatrix, and interstructural complex.

The three soils were selected to represent NE WI soils based on their physical properties. LvB and HmB represent the more common silt loam texture in the selected soils, yet their physical properties were different enough to expect varying abilities to transport *C. parvum*. LvB had the highest A horizon K_{sat} classification (very rapid), and lower bulk density and higher porosity compared to the HmB A horizon. Therefore, LvB was expected to represent soils

with a **high** potential for *C. parvum* transport. The silty clay KnB soil was expected to restrict *C. parvum* transport (**low** potential) due to increased clay content in the B horizon, restrictive bulk density & impermeability, slightly hydrophobic composition, and comparatively lower porosity of the KnB B horizon. HmB was selected to represent an **intermediate** level of *C. parvum* transport potential as its physical properties were in between those of LvB and KnB. (Note: the three transport potential categories specified above should be viewed within the context of the 10 soil series evaluated in this study).

C. parvum Transport: Soil Extraction Analysis

While an U.S. EPA-certified soil matrix *C. parvum* extraction method is not available, several soil extraction methodologies with various efficacies have been published for a variety of soil textures (Telliard, 2005; US EPA, 2012). Error associated with many of the published methods was a concern because *C. parvum* recoveries were reported below U.S. EPA minimum recovery standards for 1623.1 or methods were only applicable to certain soil textures (Kuczynska *et al*, 1999; Kato *et al.*, 2002; Zilberman *et al.*, 2009; Peterson *et al.*, 2012). Given this uncertainty, an unanticipated central outcome of this project was the development of a reliable soil extraction methodology for a variety of soil textures. The discontinuous soil, sucrose, sodium hexametaphosphate, PBS, extraction solution centrifugation gradient step (SPEG) developed by the research team (described in Section II) provided a critical analytical tool for the detection of *C. parvum* in soil matrix. Established as a preceding step to U.S. EPA 1623.1, the SPEG method utilized similar equipment, media, and centrifugation to 1623.1 to maximize efficiencies, with the primary goal of maintaining foregoing U.S. EPA requirements in the U.S. EPA Method 1623.1 after the completion of the SPEG step.

Central to the development of SPEG was the capacity to maintain a mean *C. parvum* spike recovery percentage at or above 32%, as required by section 9.6.3.2 in U.S. EPA 1623.1 (Telliard, 2005; U.S. EPA, 2012). A baseline mean *C. parvum* spike recovery percentage using R.O. water, initiated from step 13.0 to the conclusion of 1623.1, was first established. The baseline 1623.1 percentage of 75.3%, shown in Figure 1, served as a control to ensure the efficacy of analysis preceding the SPEG step. The baseline mean was next established for the SPEG methodology without soil (67.7%) and with soil (44.1%) to determine the effect of the SPEG method and the soil on the baseline 1623.1 recovery. Soil extraction methodologies published by Kato *et al.* (2002) and Peterson *et al.* (2012) were implemented by this study as a preceding step to U.S. EPA 1623.1, in the same manner as the SPEG method, to establish a comparison of methodologies. While Kato *et al.* (2002) and Peterson *et al.* (2012) may not have intended their methods to be preceding steps to U.S. EPA 1623.1, they are both published protocols which are able to produce an extraction fluid able to be analyzed via 1623.1. The average *C. parvum* soil extraction recovery obtained following the methods of Kato (21.7%) and Peterson (8.0%) were below U.S. EPA minimum recovery standards and comparatively lower than those of our SPEG method (Figure 1).

While the SPEG method does slightly reduce mean *C. parvum* spike recoveries in 1623.1, it was able to maintain a mean recovery above 32% during testing and throughout the duration of experimental trials (Figure 1). From Figure 1, it is clear that SPEG is the only soil extraction method able to produce a mean recovery percentage above U.S. EPA minimum requirements. The efficacy of any methodology used to obtain soil *C. parvum* concentrations should be held to a similar standard as data obtained from U.S. EPA 1623.1.

C. parvum Transport: Irradiated Surrogate Analysis

The experiments using live and irradiated *C. parvum*, in Phase I, provided evidence that irradiated *C. parvum* is an effective surrogate for live *C. parvum* for use in surface-to-groundwater transport studies. Observational similarities and trends, as well as statistical similarities from the six comparative simulation responses [1) C_{T1} / C_{To}, 2) C_{TLec} / C_{To}, 3) C_{TSoil} / C_{To}, 4) $C_{TLec5\%} / C_{To}$, 5) leachate *C. parvum* hydrograph profile, and 6) soil *C. parvum* depth distribution profile] were utilized in the decision process. For responses 1- 4, comparisons were performed using a Welch's t-test and their *p*-values, shown in Table 2, illustrate the similarity and statistical consistency between live and irradiated *C. parvum* recoveries. While similar in their *C. parvum* responses, corresponding confidence intervals indicate high variance for all factors. Although the variance is uniform, wide confidence interval distribution patterns diminish statistical significance (Table 2). The uniform distribution of variance does however indicate that a finding of statistical similarity between the live and irradiated *C. parvum* is still plausible. Given this plausibility, it would appear the primary cause for the high variance is inherent to the small data set that could be generated in this study due to constraints imposed by field sampling and pathogen analysis protocols.

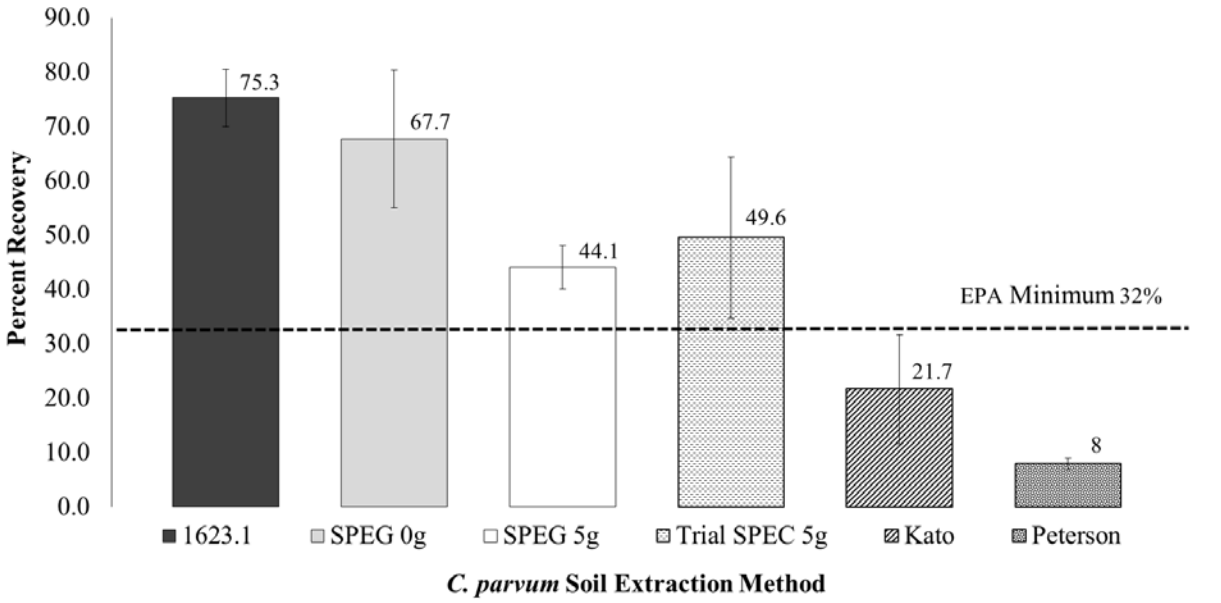

Figure 1: Comparison of percent recovery obtained with different *C. parvum* soil extraction methodologies: *Percent recovery as shown: U.S. EPA 1623.1 using RO Water, SPEG with 0 g of soil, SPEG with 5 g of soil, SPEG during study with 5 g soil, Kato et al. (2002) method with 5 g of soil, Peterson et al. (2012) method with 5 g of soil.*

Differences in soil extraction recoveries were also apparent between live and irradiated *C. parvum*. Using the SPEG soil extraction method, a data set comprising 5 replicates of both live and irradiated soil spike *C. parvum* recoveries for LvB soil were analyzed with a 2-way ANOVA (Tukey) in SAS ($\alpha = 0.05$). With a *p*-value = 0.0003, irradiated *C. parvum* was observed to have lower recoveries (35.3%) as compared to live oocysts (45.8%). While the difference between live and irradiated soil extraction was significant, it was compensated for by the spike recovery protocol in U.S. EPA 1623.1. As progressively lower spike recoveries in turn receive a correspondingly larger multiplication factor, a reduction in soil extracted irradiated oocysts would not be seen in the finalized data.

Evaluation of the leachate *C. parvum* hydrograph profile (response #5) and soil *C. parvum* depth distribution profile (6), further supports the conclusion that irradiated *C. parvum* are effective surrogates for live *C. parvum.* The presence of a first flush effect is clearly evident (Figure 2) denoted by the heavily left-skewed live and irradiated leachate hydrographs. The left-skewed pattern indicates a rapid migration of *C. parvum*, able to outpace the conservative bromide tracer and penetrate through the entirety of the soil profile (Figure B.1). Both the hydrographs also exhibit a rapid decline in *C. parvum* concentrations immediately after the first 5% of one pore volume of leachate has been collected.

Table 2: Comparison of simulation parameters from rainfall simulations using live and irradiated *C. parvum*: *C. parvum total recovery (C_{T1}/C_{To}), C. parvum fraction recovered in leachate (C_{TLec}/C_{To}), C. parvum fraction recovered in soil (C_{TSoil}/C_{To}), C. parvum fraction recovered in leachate first flush ($C_{TLec5\%}/C_{To}$) in Phase I.*

Reponse	Recovery		*p* -value	Confidence Interval	
	Live	Irradiated		(-)	(+)
C_{T1}/C_{To}	0.65	0.45	0.374	-99	138
C_{TLec}/C_{To}	0.18	0.05	0.576	-201	227
C_{TSoil}/C_{To}	0.47	0.40	0.759	-73	86
$C_{TLec5\%}/C_{To}$	0.91	0.89	0.845	-41	44

n = 2

The soil depth distribution profiles (Figure 3) provide additional evidence for the suitability of irradiated *C. parvum* to be used in transport studies. The live and irradiated *C. parvum* soil distributions have remarkable similarities in two main aspects. Firstly, both show an exponential decay type response, with a strong affinity to remain within the top 0 - 10 cm of the soil profile, paired with a dramatic decrease in concentration thereafter (Figure 3). Secondly, the soil distribution confirms that both live and irradiated *C. parvum* have the ability to migrate through the A and into the B soil horizons, as well as breakthrough the bottom of the soil profile.

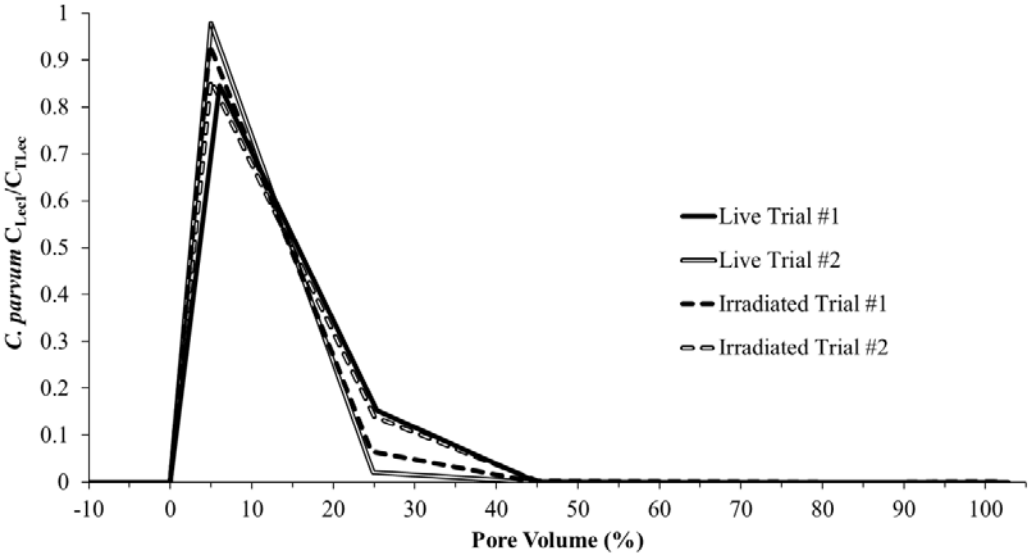

Figure 2: Fraction of *C. parvum* recovered in leachate (C_{Lec1}/C_{TLec}) for Phase I live and irradiated *C. parvum* tests: *The fraction of recovered irradiated C. parvum in leachate at incremental sampling intervals (C_{Lec1}) as a function of the total recovered C. parvum in leachate (C_{TLec})*

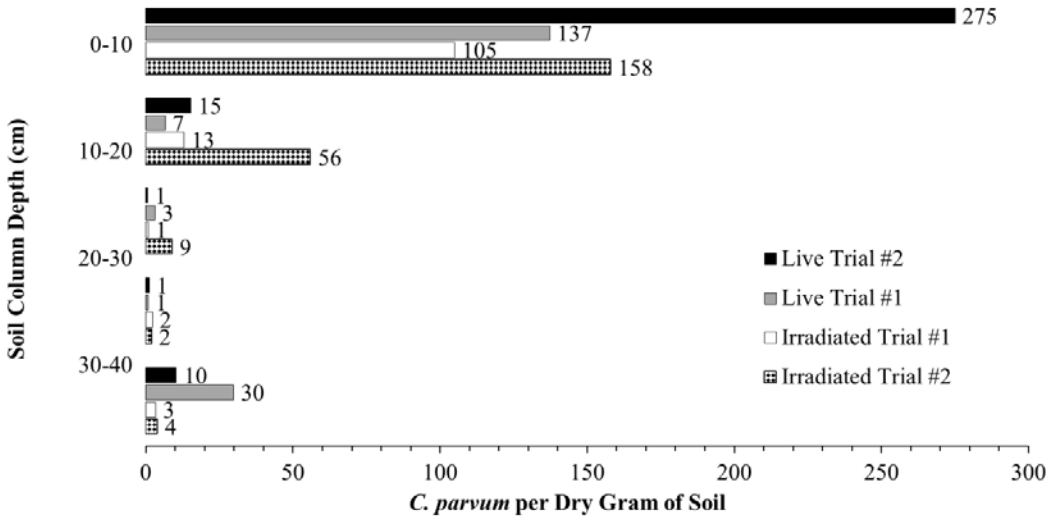

Figure 3: The depth-wise distribution of live and irradiated C. parvum in soil columns (Phase II tests):

<u>*C. parvum* Transport Potential of NE WI Soils:</u>

Transport of *C. parvum* in leachate through soil nonmatrix and interstructural pores is a complex process and influenced by a variety of *in-situ* soil characteristics. The soil column rainfall simulations conducted in Phase II on soil series LvB, HmB, and KnB revealed the influence of certain soil characteristics (specifically texture) on *C. parvum* subsurface transport. Similar to Phase I, conclusions from Phase II are based on observational similarities, trends, statistical comparisons based on six simulation responses, and soil characteristics.

Welch's t-test analysis indicated a statistically similar C_{T1}/C_{To} and C_{TSoil}/C_{To} simulation response for all soil series and highlighted two main trends. Uniformity in C_{T1}/C_{To} signifies that all of the soils tested yielded a high level of *C. parvum* recovery over the entire range of experimental conditions used. Likewise, uniformity in C_{TSoil}/C_{To} signifies that all study soils retained a comparable fraction of *C. parvum* in various incremental soil depth sections analyzed. Further analysis using these ratios was not expected to provide additional information.

Significant differences were observed in leachate *C. parvum* transport among the soils. A comparison of the *C. parvum* fraction recovered in leachate (C_{TLec}/C_{To}) indicated that soil LvB had a significantly higher fraction in the leachate matrix (0.05) as compared to KnB (0.0002) ($p = 0.025$). A broader examination by soil texture indicated that silt loam soils, both LvB & HmB, had a greater *C. parvum* fraction recovered in leachate (0.04) compared to the silty clay soil KnB (0.0002) ($p = 0.049$). The relative recovery values in leachate followed the order for the three soils originally hypothesized for transport potential differences: LvB (high) > HmB (intermediate) > KnB (low). The *C. parvum* fraction recovered in leachate first flush ($C_{T5\%}/C_{To}$), further expands on the differences in leachate transport potential. At a significance level of $\alpha = 0.1$, soil series KnB demonstrated a significantly lower $C_{T5\%}/C_{To}$ recovery compared to both LvB and HmB. The silty clay soil (KnB) again has a significantly lower $C_{T5\%}/C_{To}$ recovery compared to the silt loam soils (LvB & HmB) ($p = 0.033$).

The leachate *C. parvum* hydrograph profiles, lastly illustrate the differences in leachate transport and allow for broad conclusions to be made regarding subsurface transport potential. Figure 4 illustrates the strong dependency that soils LvB and HmB have on the first flush effect to drive leachate *C. parvum* transport. The severely left skewed hydrographs (observed only for silt loam soils) for both LvB and HmB are similar to the results presented in Phase I and again revealed the ability of rapid *C. parvum* migration to outpace the bromide tracer (Figure B.2). It, therefore, appears that the silt loam textured soils in NE WI could be susceptible to *C. parvum* transport primarily driven by the first flush effect.

In contrast, the silty clay soil series KnB exhibited minimum susceptibility for subsurface *C. parvum* transport with an average C_{TLec}/C_{To} recovery of 0.0002. The KnB leachate *C. parvum* hydrograph profiles, shown in Figure 4, illustrate the differences between the soils and how they transport *C. parvum* in leachates. In simulation # 2 for the KnB soil, the resistance offered to surface infiltration and subsequent leachate migration resulted in no leachate generation (Figure 4) and ponding of 99.5% of the applied liquid manure on the soil surface. Moreover, when leachate was transported through the KnB soil profile (simulation # 1), the peak *C. parvum* concentration was delayed and did not occur until 25% of a pore volume was collected (Figure 4). Since we evaluated only one silty clay soil series (i.e., KnB), broader conclusions for this textural class cannot be provided at this point. However, the ability of soils with high clay content to attenuate subsurface *C. parvum* transport is apparent from our results.

It is important to note that the leachate from the silt loam soils (LvB and HmB) contained well above the minimum inoculative dose (10 oocysts) of *C. parvum* for human infection (Dupont *et al*., 1995; Olson *et al*., 1999; Caccio, 2005). Soil LvB was observed to transport the highest quantity of *C. parvum* oocysts averaging approximately 50,000 oocysts in less than 5 L of leachate, with approximately 90% of that occurring in the first flush (250 mL). In contrast, the silty clay (KnB) soil transported far fewer oocysts in leachate (0 – 400 oocysts) and could be of far less concern with respect to sub surface transport. While oocyst concentrations would be further diluted upon reaching groundwater supplies, it would stand to reason that the silt loam soils pose a far greater risk of maintaining a minimal infective pathogen concentration in groundwater.

The *C. parvum* fraction recovered in soil (C_{TSoil}/C_{To}), as stated earlier, is on average similar for the three soils tested (~86%). However, the C_{TSoil}/C_{To} masks the *C. parvum* distribution patterns evident in the soil depth distribution profiles (Figure 5). LvB and HmB soil distribution profiles both displayed a similarly skewed exponential decay type response with significant retention in the top 0 – 10 cm of the soil column (Figure 5). Furthermore, *C. parvum* was recovered at depths below 40 cm (i.e., in the 40 – 50 cm section) from both LvB and HmB soils, indicating the ability of *C. parvum* to migrate through the A and B horizons.

The soil *C. parvum* depth distribution profiles of the silty clay KnB soil again expressed two dramatically different patterns compared to the silt loam soils. *C. parvum* appears to either: (1) display a bimodal distribution (i.e., discontinuity) with high *C. parvum* concentrations apparent at both the 0 – 10 cm and 40 – 50 cm soil sections or (2) remain solely in the top 0 – 10 cm of soil (Figure 5). In profile (1), *C. parvum* directly migrated from the surface horizon across the horizon interface into the B horizon where it concentrated in the lower 40 – 50 cm section.

Transport across the A horizon with little or no trace of *C. parvum* in between would indicate that this movement was facilitated by connected interstructural macropores. The sudden entrapment of *C. parvum* in the B horizon could possibly suggest abrupt macropore discontinuity in the B horizon, thus restricting the mode by which *C. parvum* traveled through the slower and more resistive soil matrix. In profile (2), *C. parvum* was unable to infiltrate below 0 – 10 cm, indicating an immediate resistance to infiltration and, thus, transport of *C. parvum* at the surface of KnB and absence of connected interstructural macropores.

Parallel to the leachate profiles, the soil *C. parvum* depth distribution profiles displayed strong dissimilarities driven by their textural classifications (Figure 5). The silt loam soils (LvB & HmB) overwhelmingly exhibited a strong affinity to retain the majority of *C. parvum* close to the soil surface (0 – 10 cm); however, breakthrough and transport in the first flush readily occurred. In contrast, the silty clay soil was able to either retain the vast majority of *C. parvum* in both the A and B horizon (when transport occurred), or solely restrict *C. parvum* to the soil surface (when transport was prevented). Similar to the leachate profiles, it is apparent that the silt loam texture has the potential to transport *C. parvum* throughout the entirety of the soil profile, while silty clay restrict or prevent transport at various stages in the soil profile.

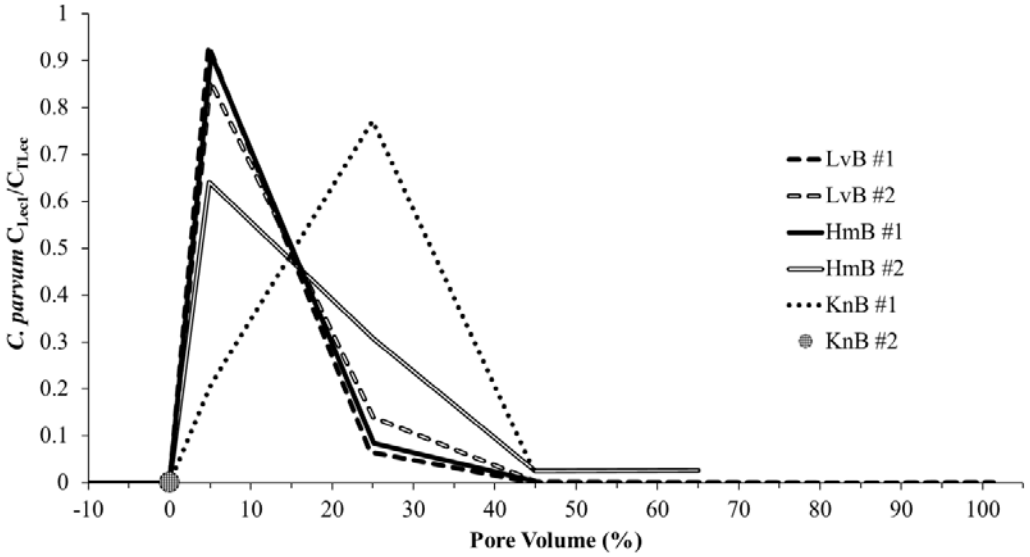

Figure 4: C_{Lec1}/C_{TLec} of Lomira, Hochheim, and Kewaunee soils in Phase II tests with irradiated *C. parvum*: *The fraction of recovered irradiated C. parvum in leachate at incremental sampling intervals (C_{Lec1}) as a function of the total recovered C. parvum in leachate (C_{TLec})*

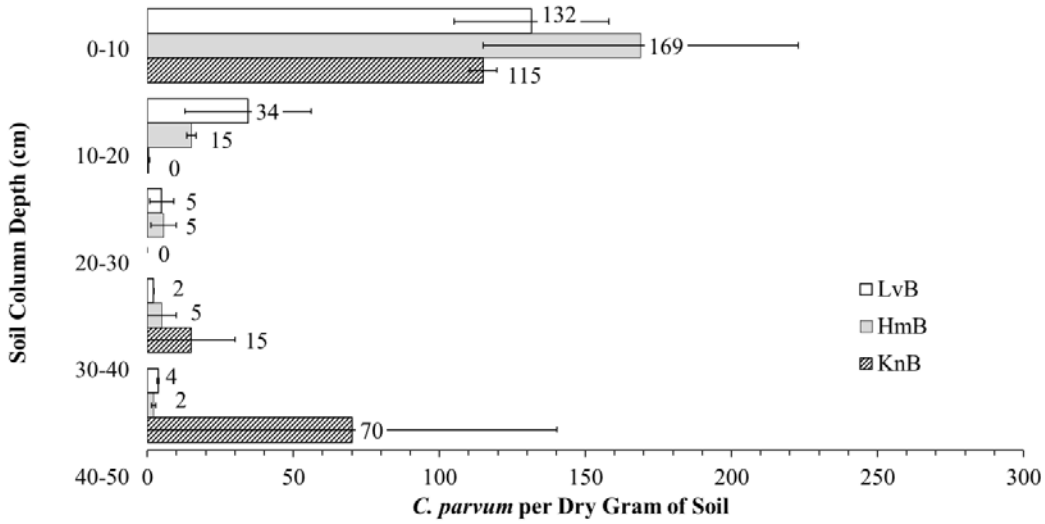

Figure 5: The depth-wise distribution of irradiated *C. parvum* in soil columns (Phase II tests):

C. parvum Transport: Influence of Soil Properties on Transport Potential

Soil physical properties determined in the study include bulk density, porosity, organic matter content, hydraulic conductivity, and hydrophobicity. Specific properties, such as, hydraulic conductivity and hydrophobicity were found to play a significant role in a soil's transport potential; however, statistical interaction was found between those aforementioned properties and their governing soil series or texture. Therefore, only general inferences can be made about the overall influence of specific soil physical properties on transport potential. General observations indicate that soils or textures with higher hydraulic conductivity (K_{sat}) rates appear to promote *C. parvum* transport, especially in conjunction with flow pathways created by cracks, root or invertebrate holes, and other various macropore formations. Conversely, soils that are slightly hydrophobic, contain bulk densities known to inhibit root development, or have below normal porosity lead to conditions that attenuate *C. parvum* transport.

IV. CONCLUSIONS AND RECOMMENDATIONS

The transport potential of *C. parvum* through the soil matrix and into groundwater supplies is dependent on several ambient *in-situ* and anthropogenic factors. This study investigated *C. parvum* transport potential through soils in the NE Wisconsin's carbonate aquifer region. The use of large intact soil cores in laboratory rainfall simulation experiments provided key missing sources of data to the *C. parvum* transport knowledge base. Information generated in this study can be used to guide future field studies across various landscapes.

The development and implementation of the discontinuous soil, sucrose, sodium hexametaphosphate, PBS, extraction solution centrifugation gradient step (SPEG) filled a critical missing analytical role in this study. Intended as a preceding step to U.S. EPA 1623.1, SPEG maintained a mean *C. parvum* spike recovery percentage at or above 32%, as required by section 9.6.3.2 in U.S. EPA 1623.1 (Telliard, 2005; U.S. EPA, 2012), both throughout initial development and implementation of the study. Further analysis on other soil textures is required to determine the robustness of SPEG as a soil extraction method across the full textural range. In this study, SPEG met U.S. EPA requirements for the extraction of *C. parvum* from loam and clay textured soils.

Irradiated *C. parvum* was determined to be an effective surrogate to live *C. parvum* for soil surface-to-groundwater transport studies. In all comparisons of *C. parvum* transport, irradiated *C. parvum* performed similarly to live *C. parvum*. Irradiated and live *C. parvum* in leachate both exhibited a first flush effect, a rapid flux of *C. parvum* oocysts able to outpace bromide, and the ability to "break through" the entire soil column. In the soil matrix, both irradiated and live *C. parvum* possess an affinity to retain close to the surface (0 - 10 cm), while maintaining the ability to consistently break through the A and B horizon interface, as well as penetrate through the bottom of the soil column. These similarities in transport behavior render irradiated *C. parvum* an effective tracer in leachate and soils for future field studies of *C. parvum* transport.

Irradiated *C. parvum* was used to assess the transport potential through several soils representative of NE Wisconsin's vulnerable carbonate aquifer region. Silt loam soils demonstrated an ability to transport *C. parvum* through the soil profile and potentially into groundwater supplies at potentially infectious concentrations. Of the soils studied, the silt loam soils had the highest ability for transport based on a first flush effect in leachate *C. parvum* hydrographs. This first flush effect was further exacerbated in the presence of intact connective interstructural macropores in the Lomira silt loam series. Following the first flush, an exponential decay response was observed in *C. parvum* leachate concentrations, indicating that silt loam possessed an ability to retain *C. parvum* within the surface soil horizon after the initial transport process concluded.

The Kewaunee series silty clay soil (KnB), on the other hand, appeared capable of attenuating the subsurface transport of *C. parvum*. When subsurface transport was not evident in this soil type, water ponded and *C. parvum* was not transported below the soil surface interface. When subsurface transport was present in KnB, the influence of nonmatrix and interstructural macropores appeared to be the driving transport mechanism. This effect was apparent through the discontinuity in KnB's soil depth-wise distribution profile, wherein *C. parvum* migrated between soil horizons with little or no trace of *C. parvum* in the intermediate soil horizons. Soil matrix transport, while a comparatively slower means of transport, would lower *C. parvum* transport potential through KnB columns and modulate the first flush effect. The sluggish movement via matrix flow would allow for more interaction and retention by soil particles resulting in observed lower leachate concentrations and a higher proportion of *C. parvum*

retained in the soil matrix. KnB was the only silty clay texture investigated in this study; however, it is reasonable to assume that similar clay textured soils may also resist *C. parvum* transport.

The *C. parvum* oocysts that were collected post rainfall simulation were intact for all soils studied. However, infectivity was not measured as the oocysts were previously inactivated by gamma irradiation. Given the rapid migration of *C. parvum* through the silt loams, it is likely that the oocysts would remain viable as the leachate progresses through the soil profile. The delayed or absence of detection of *C. parvum* in KnB (silty clay) leachate would presumably allow for more inactivation time at the surface or within the soil profile. Given the increased potential for *C. parvum* attenuation in KnB, the likelihood that *C. parvum* would remain viable until they reach a groundwater source is low.

Interaction between various soil physical characteristics limited our ability to determine statistical significance of the role of specific soil properties on *C. parvum* transport. However, soil textures with higher hydraulic conductivity (K_{sat}) rates and soil macropores appear to promote *C. parvum* transport while soils with lower K_{sat} (slightly hydrophobic, compacted bulk densities, low porosity) appear to inhibit *C. parvum* transport.

This study was able to provide substantial evidence to further our understanding of *C. parvum* transport and bolster the potential for beneficial outcomes of future research. To fully understand how environmental factors such as landscape position, soil depth, antecedent soil moisture, temperature, and soil properties affect *C. parvum* transport more research is required. Additional laboratory study can increase our understanding of controllable environmental factors such as temperature and soil moisture, while also substantiating SPEG as a broad spectrum soil extraction method. Ultimately, field study will be required to transfer the knowledge gained on transport mechanisms/pathways to develop and implement management practices.

Human modifications to the land and farming practices were not investigated, but will influence *C. parvum* transport potential. For example, no-till practices that utilize cover crops with large tap roots (e.g., radish) that leave large diameter (> 0.5cm) vertical macropores (30 - 50cm) (Tomasz & Kulig, 2008; ASA, 2009: Entry *et al.*, 2010;) would increase the potential for rapid transport of *C. parvum* past the soil A horizon and well into the B horizon before any soil based attenuation occurs. The application of liquid manure on bare soil surface without post tillage could increase the likelihood of *C. parvum* transport through intact macropores

The following recommendations to reduce *C. parvum* transport to groundwater are based on the findings of this study. The first recommendation is to advocate tillage after the land application of liquid or solid manure, especially prior to a rainfall or snow melt event. This practice would break interconnected soils macropores and would afford the soil matrix a greater opportunity to sorb and attenuate the transport of oocysts. The second recommendation is to encourage new management strategies for the land application of manure to soil textures with high transport potential. Soil series and textures with high hydraulic conductivity (K_{sat}) and those capable of supporting large macropores should be considered for new management strategies. Such strategies could include increased setback distances from fractured bedrock, drinking water wells, or other bodies of water, as well as establishing a minimum depth of soil treatment zone above glacial till or bedrock to reduce the likelihood of *C. parvum* transport to drinking water supplies.

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APPENDIX A: Presentations

Zopp, Z., Thompson, A., Karthikeyan, K., and Long, S. 2014. Comparison of Breakthrough and Downward Migration of Live and Irradiated *Cryptosporidium parvum* and Microspheres Under Simulated Rainfall. Annual Meeting of the American Water Resources Associate Wisconsin Section. Wisconsin Dells, WI, March 13-14.

Zopp, Z., Thompson, A., Karthikeyan, K., and Long, S. 2015. *Cryptosporidium* in Soil: Subsurface Transport of *Cryptosporidium* Oocysts in Soils of Wisconsin's Carbonate Aquifer Region. Wisconsin State Laboratory of Hygiene; Science Day. Madison, WI, March 3.

Zopp, Z., Thompson, A., Karthikeyan, K., and Long, S. 2015. Subsurface Transport of *Cryptosporidium* Oocysts in Soils of Wisconsin's Carbonate Aquifer Region. Annual Meeting of the American Water Resources Associate Wisconsin Section. Oconomowoc, WI, March 5-6.

APPENDIX B

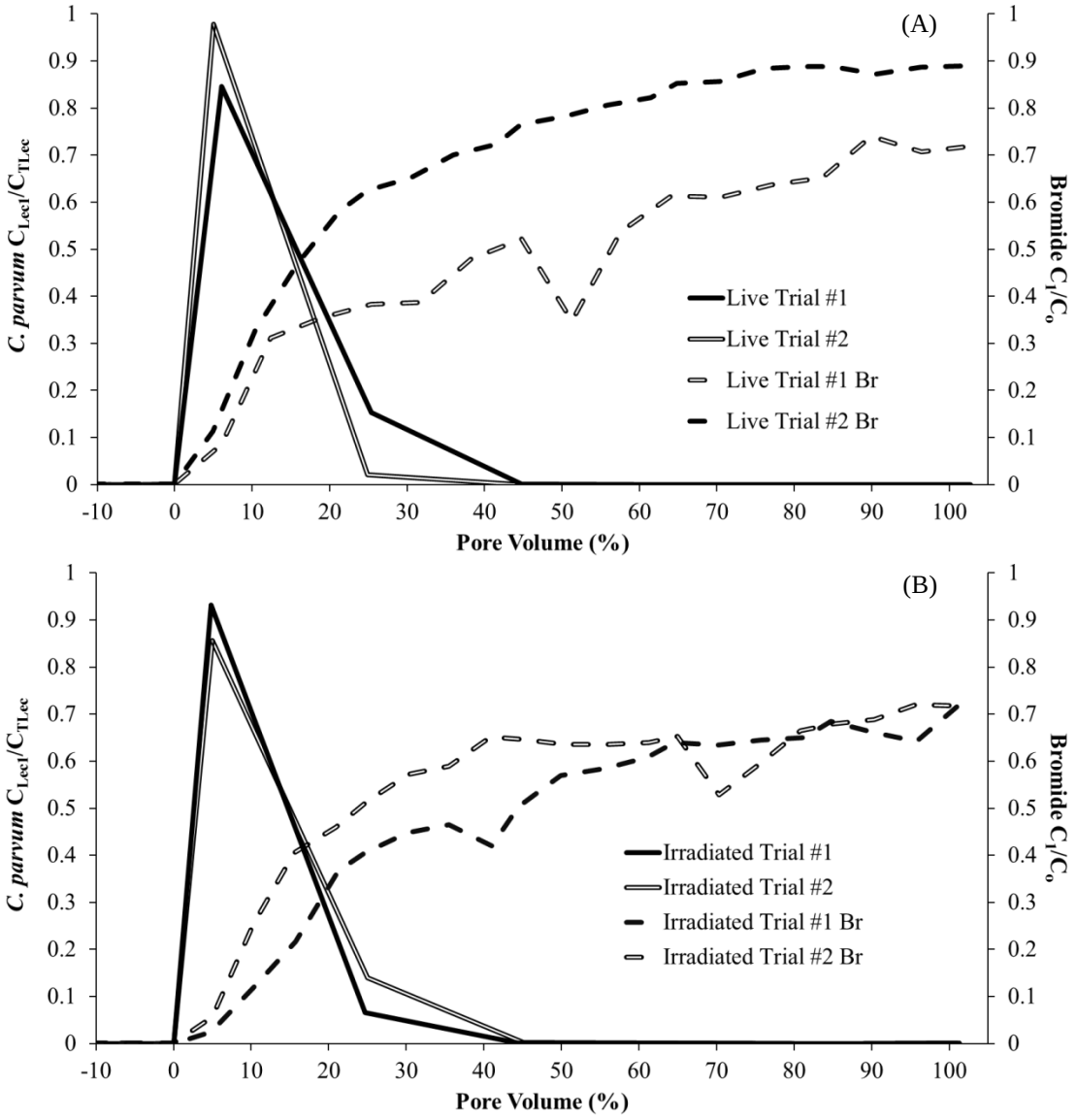

Figure B.1: C_{Lec1}/CT_{Lec} of Phase I live (A) and irradiated (B) *C. parvum* with bromide curves:
Live and irradiated C. parvum are shown in continuous lines corresponding to their bromide recovery curves (Br) shown in dashed lines, in Phase I. The fraction of recovered irradiated C. parvum in leachate at incremental sampling intervals (C_{Lec1}) as a function of the total recovered C. parvum in leachate (C_{TLec}).

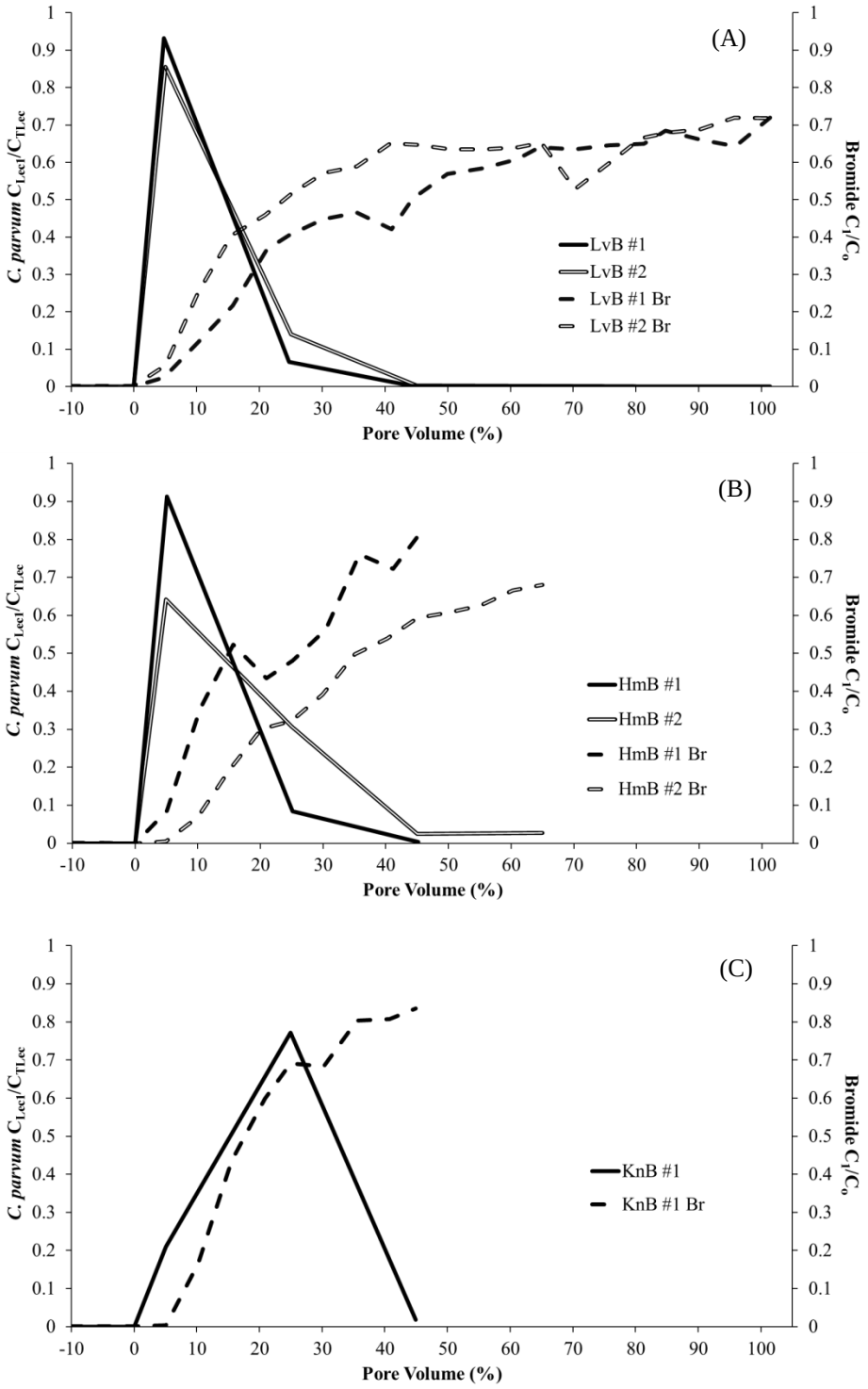

Figure B.2: C_{Lec1}/C_{TLec} of Lomira (A), Hochheim (B), and Kewaunee (C) soils with bromide curve in Phase II: *Lomira (LvB), Hochheim (HmB), and Kewaunee (KnB) are shown with continuous lines corresponding to their bromide recovery curves (Br) shown in dashed lines, in Phase II. The fraction of recovered irradiated C. parvum in leachate at incremental sampling intervals (C_{Lec1}) as a function of the total recovered C. parvum in leachate (C_{TLec}).*

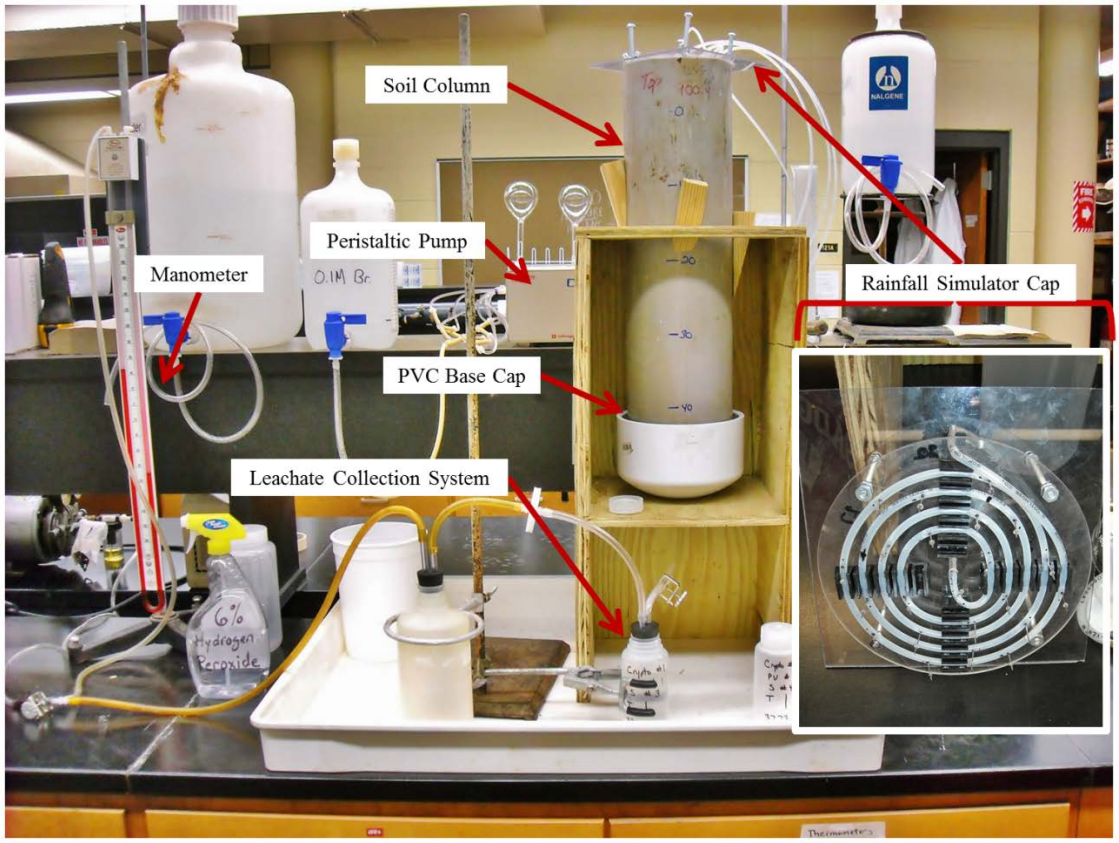

Figure B.3: Rainfall simulator apparatus:
Shown with aluminum soil column, sealed PVC base cap, manometer, leachate collection system, peristaltic pump, and the rainfall simulator cap.