University of Alberta

Transport of Viable but Non-Culturable *Escherichia coli* O157:H7 in Soil and Groundwater

by

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Dedication

To my parents, Brian and Karma Kartz, for everything you have done and everything you continue to do to ensure I succeed. Thank you for all your love, support, and encouragement.

Abstract

The influence of the viable but non-culturable (VBNC) state on specific phenotypic traits of *Escherichia coli* O157:H7 as well as its transport behaviour in porous media was examined in this study. *E.coli* O157:H7 is a human pathogen capable of entering a VBNC state following exposure to sublethal stress. In the VBNC state, *E.coli* O157:H7 is not detectable by culture assays; yet, is able to retain its ability to cause human illness. This study examined specific transport-related properties of culturable and VBNC *E.coli* O157:H7 cells. As well, transport behaviors of the two cellular states were compared using sand-packed columns under steady-state flow. When *E.coli* O157:H7 cells entered a VBNC state, significant decreases in the hydrophobicity and lengths/widths of the cells, and a significant increase in extracellular polymeric substances on the cell surfaces were measured. Transport experiments indicated significantly ($p<0.05$) greater mass transport of VBNC cells through unwashed sand compared to culturable cells. This research contributes to the current knowledge describing VBNC *E.coli* O157:H7 cells, raises questions concerning the accuracy of culture-based *E.*coli O157:H7 identification protocols, and suggests that bacteria transport in the subsurface is a truly dynamic process.

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List of Symbols, Nomenclature, and Abbreviations

BMP	Beneficial Management Practice
BTC	Breakthrough Curve
C	Concentration
CFU	Colony Forming Unit
$CuSO_4$ - $5H_2O$	Copper (II) Sulfate Pentahydrate
DAPI	4', 6-Diamidino-2-Phenylindole
DIC	Differential Interference Contrast
EC	Electrical Conductivity
E.coli	*Escherichia coli*
EPS	Extracellular Polymeric Substances
E[t]	Mean Residence Time
K_s	Saturated Conductivity
L	Spatial Measurement
LPS	Lipopolysaccharide
M	Mass
MATH	Microbial Attachment to Hydrocarbon
N	Molar
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
$Na_2C_4H_4O_6$	Sodium Tartrate
NaOH	Sodium Hydroxide
OD	Optical Density
q	Volumetric Flow Rate
RPM	Revolutions per Minute
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
UV	Ultra-Violet
V	Velocity
VBNC	Viable but Non-Culturable
W	Watts
ΔH_T	Change in Total Head
Δz	Column Height
θ	Volumetric Water Content

1. General Introduction

In recent years, safe and secure drinking water has come to the forefront as an issue of concern for public health in Canada. In May 2000, a waterborne disease outbreak occurred in Walkerton, Ontario, Canada, as a result of land application of manure contaminated with pathogenic bacteria (e.g. *Escherichia coli* O157:H7). The bacteria were able to contaminate one of the town's groundwater drinking wells. This outbreak was responsible for the deaths of seven people and caused an additional 2300 to become severely ill (O'Connor, 2002). This caught the attention of people across Canada and forced the nation to question the safety of their drinking water; water which everybody assumed to be healthy and safe. This tragic event reiterated the risk that pathogenic organisms pose against human health if they are allowed to contaminate drinking water sources.

It has been estimated by Statistics Canada that 30% of the Canadian population, mainly those living in rural areas, relies on groundwater for domestic use. In Alberta, this number is closer to 23% (Statistics Canada, 1996b). Along with domestic use, groundwater is used for a wide variety of industrial and commercial activities, including oilfield injection practices and oil sands operations (Alberta Environment, 2010). Groundwater can also contribute significant discharge to surface water bodies, such as rivers and wetlands, thus supporting aquatic ecosystems (Alberta Environment, 2009). Consequently, groundwater is considered a valuable resource, one which requires appropriate protection. This is especially true when one considers that the land application of

potential pathogen-bearing animal and human wastes is widely-used, particularly in rural settings, as an economical waste disposal option.

To put these potential risks into perspective, in 2006, it was estimated that Canadian livestock produced approximately 500,000 tonnes of manure daily, which translates to over 180 million tonnes annually (Statistics Canada, 2008). Moreover, in a study on waterborne disease outbreaks in the United States from 1986 to 1998, it was found that for all those instances where the causative agent could be identified, the microbes generally originated from farm animal sources (Centres for Disease Control and Prevention, 1998). In addition to animal waste pollution, in the United States, decentralized waste water treatment systems release approximately 15 billion litres of treated human effluent every day from 26 million homes, businesses, and recreation facilities (United States Census Bureau, 1999; Gerba and Smith, 2005). In Canada, it has been estimated that 15% of households, mainly in rural areas, utilize septic tank systems (Statistics Canada, 1996a), which may be vulnerable to leaking and can result in indirect contamination of groundwater resources.

1.1. Microbes in water and soil

It has been known for more than a century that the land application of animal and human feces and untreated wastes can lead to infiltration of pathogens to soil and groundwater systems (Gerba and Smith, 2005). To address this threat, various management practices have been developed concerning the land-application of potential pathogen-bearing wastes in the hope of protecting soil and groundwater resources (Agriculture and Agri-Food Canada, 2000). However,

despite these beneficial management practices (BMPs), percolation of microbes through the soil profile and into groundwater resources still occurs. Conboy and Goss (1999) studied 300 drinking water wells in southern Ontario and found that 48% of the wells were considered unsafe as drinking water supply as they exceeded the Ontario Drinking Water Objectives (Ontario Ministry of the Environment, 1999) for either total coliform bacteria (≤1 colonies/100mL in consecutive samples) or fecal coliform bacteria (zero detectable bacteria/100mL). The authors conclude by stating that a significant proportion of the bacteria present in the wells were of fecal origin with its likely source being animal manure. The probable reason for the contamination of soil and groundwater sources is that >40% of the manure produced annually in Canada is on farms where the farm operators were unfamiliar with BMPs for manure in their region. These manure BMPs are designed to prevent manure runoff, protect groundwater and surface water, and minimize manure nutrient losses and odour emissions into the environment (Statistics Canada, 2004).

Numerous pathogens have been implicated in causing waterborne disease outbreaks as a result of their ability to leach from agricultural and domestic waste sources, run-off overland, or infiltrate the subsurface and subsequently contaminate surface and groundwater. Examples of pathogens that have caused documented waterborne disease outbreaks include *Campylobacter* spp., *Salmonella* spp., *Listeria monocytogenes*, *E.coli* O157:H7, *Cryptosporidium parvum*, and *Giardia lamblia* (e.g. Gerba and Smith, 2005).

Time and costs required often prevent the detection of individual pathogens; consequently, indicator organisms of enteric origin are often used to determine the presence and fate of other pathogenic enteric organisms (e.g. Crane et al., 1981). Fecal coliforms, a group of Gram-negative, non-spore forming, facultatively anaerobic bacilli bacteria that are passed through the fecal excrement of humans, livestock and wildlife, are the most commonly used indicator organisms. Statistics Canada (1996a) has determined that Canadian livestock produce approximately 1.3×10^{18} colonies of fecal coliform bacteria, including *Escherichia coli* (*E.coli*) species, daily in their manure. *E.coli* is the most common fecal coliform and although most strains are non-pathogenic, some strains (e.g. *E. coli* O157:H7) pose a serious health risk to humans (Jamieson et al., 2002) as seen in the Walkerton incident. Therefore, the presence of fecal indicator organisms in an environmental sample indicates the prospect of fecal contamination and the possible presence of pathogenic fecal bacteria.

1.2. *Escherichia coli* O157:H7

Escherichia coli O157:H7 was first recognized as a pathogen in 1982 following two outbreaks of a then unknown gastrointestinal illness that affected at least 47 people in Oregon and Michigan, USA (Riley et al., 1983).

This bacterium is a Gram-negative facultative anaerobe, capable of adhering within the intestinal tract of humans following ingestion (Griffin and Tauxe, 1991). Infection with this organism can result in abdominal cramping, bloody or non-bloody diarrhea, as well as the more serious hemolytic uremic

syndrome, which can cause acute kidney failure in the immuno-compromised such as the elderly and infant children (Boyce et al., 1995).

The pathology of *E.coli* O157:H7 is a result of the production of Shiga-like toxins (O'Brien and Holmes, 1987) which ultimately prevent protein synthesis in cells expressing the appropriate receptor for the toxins, leading to cell death (Rabinowitz and Donnenberg, 1996). It is reported that an estimated 73500 illnesses are caused by *E.coli* O157:H7 infection annually in the United States resulting in approximately 2000 hospitalizations and 61 deaths (Mead et al., 1999). Along with the Walkerton incident, numerous other reports have implicated *E.coli* O157:H7 as being the causative agent of outbreaks, which resulted from fecal contamination of a drinking water source (Swerdlow et al., 1992; Licence et al., 2001; Olsen et al., 2002). In a particularly devastating incident, Bopp et al. (2003) describes the largest outbreak of diarrhea associated with waterborne *E. coli* O157:H7 ever reported in the United States. This incident in August 1999 in upstate New York, affected 775 people, including two deaths, and was caused by the contamination of a drinking water well either from a septic system or a manure storage site.

Considering studies have shown that a significant proportion of livestock herds in Canada and the USA contain individuals which are actively shedding *E.coli* O157:H7 in their feces (e.g. Faith et al., 1996; Chapman et al., 1997; Shere et al., 1998; Laegreid et al., 1999; Van Donkersgoed et al., 1999; Galland et al., 2001; Dodd et al., 2003; Vidovic and Korber, 2006), it should be expected that water resources are at risk of becoming contaminated, especially in rural areas.

As a result, it is imperative that protocols be established so that appropriate detection methods can be employed to seek out sources of contamination accurately and efficiently. This will help ensure preventative measures can be taken to avert future outbreaks.

1.2.1. Viable but non-culturable *Escherichia coli* O157:H7

Detection of *E.coli* O157:H7 in the environment may not always be as simple as a straight-forward culture test on standard media, as is routinely done for fecal indicator organisms (e.g. Raj et al., 1961; Hanninen et al., 2003; Bower et al., 2005; Sinclair et al., 2009). Recent microbiological studies have demonstrated that *E.coli* O157:H7 may become non-culturable, but remain viable, when exposed to sublethal stresses (e.g. Liu et al., 2008). This includes changes in temperatures, elevated or reduced osmotic concentrations, nutrient starvation, levels of oxygen (Oliver, 1993) and/or exposure to chloramines in tap water (Liu et al., 2008). Bacteria in the viable but non-culturable (VBNC) state fail to grow on the routine bacteriological media on which they would normally develop into colonies, but are still alive (Oliver, 2000). However, despite their typically low levels of metabolic activity, they may again become culturable upon resuscitation as their environment becomes more favorable (Oliver, 2009).

The VBNC state is considered to be a survival strategy adopted by bacteria in order to endure imposed stress' (Oliver, 1993). This behavior can increase the likelihood of false negative results for culture-based risk assessment tests (Liu et al., 2008). This is significant considering the most widely used and economical indicator organism enumeration techniques identify only culturable

bacterial cells. Thus, it is possible that these techniques are underestimating actual bacterial populations considerably (Jamieson et al., 2002), which, in the case of human pathogens such as *E.coli* O157:H7, has important implications for public health.

1.3. *Escherichia coli* O157:H7 transport in porous media

Pathogen fate and transport in porous media is a complex issue as it is influenced by the properties of the soil system, the suspending solution, and the bacterial cells. The distribution of, and interactions with, soil particles will greatly determine the fate and transport of bacterial cells through porous media. Two processes which have been shown to greatly limit bacteria transport (Jamieson et al., 2002) are the ability of soil particles to strain or filter cells (Scholl et al., 1990; Bradford et al., 2006; Tallon et al., 2007) and the capacity for cells to attach to soil particles via electrostatic interactions (Pringle and Fletcher, 1983; Gannon et al., 1991b; Jacobs et al., 2007). These processes depend greatly on the physical and morphological characteristics of the soil system, including texture, structure, and porosity (Abu-Ashour et al., 1992), to name a few, as well as the bacterial cells themselves. Cell characteristics such as length and width (Bradford et al., 2002; Bradford et al., 2006; Gannon et al., 1991a), surface charge (Bolster et al., 2006; Jacobs et al., 2007), hydrophobicity (Williams and Fletcher, 1996; DeFlaun et al., 1999), and cell-surface coating (Rijnaarts et al., 1996; DeFlaun et al., 1990; Williams and Fletcher, 1996; Simoni et al., 1998) have all been shown to influence bacterial filtration, attachment, and overall transport in soil systems. Therefore, knowledge regarding the phenotypic and morphological

attributes of bacterial cells is of importance if one hopes to understand their unique transport characteristics in soil.

In previous studies, bacterial cells entering the VBNC state have been shown to undergo morphological changes and phenotypic alterations. For instance, Oda et al. (2004) noted *E.coli* O157:H7 cells miniaturize and transition from a rod shape into a coccoid-shape; Signoretto et al. (2000) found that *Enterococcus faecalis* VBNC cells were twice as resistant to mechanical disruption as exponentially growing, stationary, or UV-killed cells; and Lleo et al. (2007) showed that some species of VBNC enterococci exhibit decreased adhesion-ability compared to actively growing cells and were incapable of forming complete biofilms. The latter study attributed these differences to reduced metabolism and altered surface characteristics resultant from the VBNC state transition.

1.4. Objectives

Thus far, numerous studies have been published detailing the transport of culturable *E.coli* O157:H7 through various porous media under both laboratory (e.g. Gagliardi and Karns, 2000; Artz et al., 2005; Bradford et al., 2006; Semenov et al., 2009) and field conditions (e.g. Fenlon et al., 2000; Ogden et al., 2001; Vinten et al., 2002). However, to date, there has been no examination of the transport of viable but non-culturable (VBNC) *E.coli* O157:H7 cells in soil systems. It is hypothesized that upon transition into a VBNC state, *E.coli* O157:H7 cell morphological and phenotypic characteristics are altered to such an extent that their transport properties in soil and groundwater will differ

considerably from culturable cells. Understanding and quantifying phenotypic and transport properties of VBNC cells, however, is hampered by the lack of methodology to produce consistent large batch suspensions of VBNC cells for use in standardized assays. Motivated by these queries, the objectives of this thesis are to:

- Develop a method to induce large quantities of *E.coli* O157:H7 cells into a VBNC state with high efficiency and viability in a relatively short time period
- Determine whether significant changes to important transport-influencing cell properties including the length/width, hydrophobicity, and extracellular polymeric substance composition of *E.*coli O157:H7 cells occur when induced into a VBNC state
- Determine if differences exist in transport parameters of VBNC and culturable *E.coli* O157:H7 cells under simulated groundwater flow conditions using column transport assays

This research will advance the current knowledge base describing *E.coli* O157:H7 phenotypic properties. It will also determine whether culturable fecal indicator organisms and, most notably culturable *E.coli* O157:H7 cells, are appropriate for gauging the transport of VBNC *E.coli* O157:H7 cells in soil and groundwater systems. Overall, this thesis will contribute to the development of better water quality indicators and assessments of risk for contamination of water resources.

1.5. References

Abu-Ashour, J., Joy, D.M., Lee, H., Whiteley, H.R., and Zelin, S. 1992. Transport of microorganisms through soil. *Water, Air, and Soil Pollution*. 75(1-2): 141-158.

Agriculture and Agri-Food Canada. Prairie Farm Rehabilitation Administration. 2000. Water quality matters: Agricultural best management practices. Hilliard, C. and Reedyk, S.

Alberta Environment. 2009. Alberta tier 1 soil and remediation guidelines. 978-0-7785-7591-7 (Printed edition).

Alberta Environment. 2010. Water used for oilfield injection purposes. Accessed on 12 Oct. 2010. <http://environment.alberta.ca/01729.html>.

Artz, R.R.E., Toenend, J., Brown, K., Towers, W., and Killham, K. 2005. Soil macropores and compaction control the leaching potential of *Escherichia coli* O157:H7. *Environmental Microbiology*. 7(2): 241-248.

Bolster, C.H., Walker, S.L., and Cook, K.L. 2006. Comparison of *Escherichia coli* and *Campylobacter jejuni* transport in saturated porous media. *Journal of Environmental Quality*. 35(4): 1018-1025.

Bopp, D.J., Sauders, B.D., Waring, A.L., Ackelsberg, J., Dumas, N., Braun-Howland, E., Dziewulski, D., Wallace, B.J., Kelly, M., Halse, T., Musser, K.A., Smith, P.F., Morse, D.L., and Limberger, R.J. 2003. Detection, isolation, and molecular subtyping of *Escherichia coli* O157:H7 and *Campylobacter jejuni* associated with a large waterborne outbreak. *Journal of Clinical Microbiology*. 41(1): 174-180.

Bower, P.A., Scopel, C.O., Jensen, E.T., Depas, M.M., and McLellan, S.L. 2005. Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. *Applied and Environmental Microbiology*. 71(12): 8305-8313.

Boyce, T.G., Swerdlow, D.L., and Griffin, P.M. 1995. *Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. *The New England Journal of Medicine*. 33(6): 364-368.

Bradford, S.A., Yates, S.R., Bettahar, M., and Simunek, J. 2002. Physical factors affecting the transport and fate of colloids in saturated porous media. *Water Resources Research*. 38(12): 63-1 – 63-12.

Bradford, S.A., Simunek, J., and Walker, S.L. 2006. Transport and straining of *E. coli* O157:H7 in saturated porous media. *Water Resources Research*. 42(W12S12): 1-12.

Centers for Disease Control and Prevention. 1998. Surveys of applied waterborne disease outbreaks compiled by the Center for Disease Control and Prevention from 1986 to 1998. *Morbidity and Mortality Weekly Reports*. CDC, Atlanta.

Chapman, P.A., Siddons, C.A., Cerdan Malo, A.T., and Harkin, M.A. 1997. A 1-year study of *Escherichia coli* O157 in cattle, sheep, pigs and poultry. *Epidemiology and Infection*. 119(2): 245-250.

Conboy, M.J. and Goss, M.J. 1999. Contamination of rural drinking water wells by fecal origin bacteria—survey findings. *Water Quality Research Journal of Canada.* 34(2): 281-303.

Crane, S.R., Westerman, P.W. and Overcash, M.R. 1981. Die-off of fecal indicator organisms following land application of poultry manure. *Journal of Environmental Quality.* 9(3): 531-537.

DeFlaun, M.F., Tanzer, A.S., McAteer, A.L., Marshall, B., and Levy, S.B. 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. *Applied and Environmental Microbiology*. 56(1): 112-119.

DeFlaun, M.F., Oppenheimer, S.R., Streger, S., Condee, C.W., and Fletcher, M. 1999. Alterations in adhesion, transport, and membrane characteristics in an adhesion-deficient pseudomonad. *Applied and Environmental Microbiology*. 65(2): 759-765.

Dodd, C.C., Sanderson, M.W., Sargeant, J.M., Nagaraja, T.G., Oberst, R.D., Smith, R.A., and Griffin, D.D. 2003. Prevalence of *Escherichia coli* O157 in cattle feeds in midwestern feedlots. *Applied and Environmental Microbiology*. 69(9): 5243-5247.

Faith, N.G., Shere, J.A., Brosch, R., Arnold, K.W., Ansay, S.E., Lee, M-S., Luchansky, J.B., and Kaspar, C.W. 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology*. 62(5): 1519-1525.

Fenlon, D.R., Ogden, I.D., Vinten, A., and Svoboda, I. 2000. The fate of *Escherichia coli* and *E. coli* O157 in cattle slurry after application to land. *Journal of Applied Microbiology*. 29: 149S-156S.

Gagliardi, J. and Karns, J. 2000. Leaching of *Escherichia coli* O157:H7 in diverse soils under various agricultural management practices. *Applied and Environmental Microbiology*. 66(3): 877-883.

Galland, J.C., Hyatt, D.R., Crupper, S.S., and Acheson, D.W. 2001. Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. *Applied and Environmental Microbiology*. 67(4): 1619-1627.

Gannon, J.T., Manilal, V.B., and Alexander, M. 1991a. Relationship between cell surface properties and transport of bacteria through soil. *Applied and Environmental Microbiology*. 57(1): 190-193.

Gannon, J., Tan, Y., Baveye, P., and Alexander, M. 1991b. Effect of sodium chloride on transport of bacteria in a saturated aquifer material. *Applied and Environmental Microbiology*. 57(9): 2497-2501.

Gerba, C.P. and Smith, Jr., J.E. 2005. Sources of pathogenic microorganisms and their fate during land application of wastes. *Journal of Environmental Quality*. 34(1): 42-48.

Griffin, P.M., and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E.coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*. 13: 60-98.

Hanninen, M-L., Haajanen, H., Pummi, T., Wermundsen, K., Katila, M-L., Sarkkinen, H., Miettinen, I. and Rautelin, H. 2003. Detection and typing of *Campylobacter jejuni* and *Campylobacter coli* and analysis of indicator organisms in three waterborne outbreaks in Finland. *Applied and Environmental Microbiology*. 69(3): 1391-1396.

Jacobs, A., Lafolie, F., Herry, J.M., and Debroux, M. 2007. Kinetic adhesion of bacterial cells to sand: Cell surface properties and adhesion rate. *Colloids and Surfaces B: Interfaces*. 59(1):35-45.

Jamieson, R.C., Gordon, R.J., Sharples, K.E., Stratton, G.W. and Madani, A. 2002. Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*. 44: 1.1-1.9.

Laegreid, W.W., Elder, R.O., and Keen, J.E. 1999. Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiology and Infection*. 123(2): 291-298.

Licence, K., Oates, K.R., Synge, B.A., and Reid, T.M.S. 2001. An outbreak of *E. coli* O157 infection with evidence of spread from animals to man through contamination of a private water supply. *Epidemiology and Infection*. 126(1): 135-138.

Liu, Y., Gilchrist, A., Zhang, J., and Li, X. 2008. Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Applied and Environmental Microbiology*. 74(5): 1502-1507.

Lleo, M., Bonato, B., Tafi, M.C., Caburlotto, G., Benedetti, D., and Canepari, P. 2007. Adhesion to medical device materials and biofilm formation capability of some species of enterococci in different physiological states. *FEMS Microbiology Letters.* 274(2): 232-237.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C. 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases.* 5(5): 607–625.

O'Brien, A.D. and Holmes, R.K. 1987. Shiga and Shiga-like toxins. *Microbiology and Molecular Biology Reviews*. 51(2): 206-220.

O'Connor, D. 2002. A summary report of the Walkerton inquiry: The events of May 2000 and related issues, Queen's Printer for Ontario, Toronto, Ontario, Canada.

Oda, M., Morita, M., Unno, H., and Tanji, Y. 2004. Rapid detection of *Escherichia coli* O157:H7 by using green fluorescent protein-labeled PP01 bacteriophage. *Applied and Environmental Microbiology*. 70(1): 527-534.

Ogden, I.D., Fenlon, D.R., Vinten, A.J.A., Lewis, D. 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. *International Journal of Food Microbiology*. 66(1-2): 111-117.

Oliver, J.D. Formation of viable but nonculturable cells. In: Kjelleberg, S. (ed.) Starvation in Bacteria, Plenum Press, New York, 1993. p. 239-272.

Oliver, J.D. The public health significance of viable but nonculturable bacteria. In: Colwell, R.R. and Grimes, D.J. (eds.) Nonculturable Microorganisms in the Environment, American Society of Microbiology Press, Washington, DC, 2000. p. 277-299.

Oliver, J.D. 2009. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews* "Accepted Article"; doi: 10.1111/j.1574-6976.2009.00200.x.

Olsen, S.J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., and Mead, P. 2002. A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: Implications for rural water systems. *Emerging Infectious Diseases*. 8(4): 370-375.

Ontario Ministry of the Environment. Ontario Drinking Water Objectives, Revised 1994. ISBN 0-7743-8985-0. Queens Printer for Ontario. 1999.

Pringle, J.H. and Fletcher, M. 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Applied and Environmental Microbiology*. 45(3): 811-817.

Rabinowitz, R.P. and Donnenberg, M.S. *Escherichia coli*. In: Paradise, L.J., Bendinelli, M., and Friedman, H., (eds.) Enteric Infections and Immunity, Plenum Publishing Corporation, New York, 1996. p. 101-121.

Raj, H., Wiebe, W.J., and Liston, J. 1961. Detection and enumeration of fecal indicator organisms in frozen sea foods II. *Enterococci*. *Applied Microbiology*. 9(2): 171-174.

Rijnaarts, H.H., Norde, W., Bouwer, E.J., Lyklema, J., and Zehnder, A.J.B. 1996. Bacterial deposition in porous media: Effects of cell-coating, substratum hydrophobicity, and electrolyte concentration. *Environmental Science and Technology*. 30(10): 2877-2883.

Riley, L. W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine*. 308(12): 681–685.

Scholl, M.A., Mills, A.L., Herman, J.S., and Hornberger, G.M. 1990. The influence of mineralogy and solution chemistry on the attachment of bacteria to representative aquifer materials. *Journal of Contaminant Hydrology*. 6(4): 321-336.

Semenov, A.V., van Overbeek, L., and van Bruggen, A.H.C. 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Applied and Environmental Microbiology*. 75(10): 3206-3215.

Shere, J.A., Bartlett, K.J., and Kaspar, C.W. 1998. Longitudinal study of *Escherichia coli* O157:H7 dissemination on four dairy farms in Wisconsin. *Applied and Environmental Microbiology*. 64(4): 1390-1399.

Signoretto, C., del Mar Lleo`, M., Tafi, M.C., and Canepari, P. 2000. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Applied and Environmental Microbiology*. 66(5): 1953-1959.

Simoni, S.F., Harms, H., Bosma, T.N.P., Zehnder, A.J.B. 1998. Population heterogeneity affects transport of bacteria through sand columns at low flow rates. *Environmental Science and Technology*. 32(14): 2100-2105.

Sinclair, A., Hebb, D., Jamieson, R., Gordon, R., Benedict, K., Fuller, K., Stratton, G.W., and Madani, A. 2009. Growing season surface water loading of fecal indicator organisms within a rural watershed. *Water Research*. 43(5): 1199-1206.

Statistics Canada. 1996a. Environment Accounts and Statistics Division and Agriculture Division. Estimated fecal coliform bacteria production in livestock manure by sub-sub-basin, 1996. Catalogue No. 16F0025XIB. Accessed on Sept 28 2010. <http://www.statcan.gc.ca/pub/16f0025x/fecal-fecaux/4225577-eng.pdf>

Statistics Canada. 1996b. Quarterly estimates of the population of Canada, the provinces and the territories. 11-3, Catalogue no. 91-001, Ottawa.

Statistics Canada. 2004. Manure management in Canada. Beaulieu, M.S. 21-021-MIE. 1(2): 1-52.

Statistics Canada. 2008. EnviroStats. Catalogue no. 16-002-X. 2(4): 1-25. Accessed on Sept 28 2010. < http://www.statcan.gc.ca/pub/16-002-x/16-002-x2008004-eng.pdf>

Swerdlow, D.L., Woodruff, B.A., Brady, R.C., Griffin, P.M., Tippen, S., Donnell,Jr., H.D., Geldreich, E., Payne, B.J., Meyer, Jr., A., Wells, J.G., Greene, K.D., Bright, M., Bean, N.H., and Blake, P.A. 1992. A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. *Annals of Internal Medicine*. 117(10): 812-819.

Tallon, L. K., Si, B. C., Korber, D. and Guo, X. 2007. Soil wetting state and preferential transport of *Escherichia coli* in clay soils. *Canadian Journal of Soil Science.* 87(1): 61–72.

United States Census Bureau. 1999. National data chart for total occupied housing units. U.S. Census Bureau, Washington, DC. Accessed on Sept 28 2010. <www.census.gov/hhes/www/housing/ahs/03dtchrt/tab2-1.html>.

Van Donkersgoed, J., Graham, T., and Gannon, V. 1999. The prevalence of verotoxins, *Escherichia coli* O157:H7, and *Salmonella* in the feces and rumen of cattle at processing. *Canadian Veterinary Journal*. 40(5): 332-338.

Vidovic, S. and Korber, D.R. 2006. Prevalence of *Escherichia coli* O157 in Saskatchewan cattle: Characterization of isolates by using random amplified polymorphic DNA PCR, antibiotic resistance profiles, and pathogenicity determinants. *Applied and Environmental Microbiology*. 72(6): 4347-4355.

Vinten, A.J.A., Lewis, D.R., Fenlon, D.R., Leach, K.A., Howard, R., Svoboda, I., and Ogden, I. 2002. Fate of *Escherichia coli* and *Escherichia coli* O157 in soils and drainage water following cattle slurry application at 3 sites in southern Scotland. *Soil Use and Management*. 18(3): 223-231.

Williams, V. and Fletcher, M. 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Applied and Environmental Microbiology*. 62(1): 100-104.

2. Induction of culturable *E.coli* O157:H7 cells into a viable but non-culturable (VBNC) state and analysis of culturable and VBNC *E.coli* O157:H7 cell properties

2.1. Introduction

Escherichia coli O157:H7 is a Gram-negative facultative anaerobe capable of causing disease in humans at an infective dose of less than 100 cells (Yousef and Carlstrom, 2003). Through the production of Shiga-like toxins, invading *E.coli* O157:H7 is able to disrupt and ultimately prevent protein synthesis in cells expressing the appropriate receptor for the toxins, leading to cell death. Human infections from *E.coli* O157:H7 may manifest as mild to severe bloody diarrhea, fever, vomiting, and in rare cases, hemolytic uremic syndrome (Rabinowitz and Donnenberg, 1996) which has substantial effects on the kidneys. The occurrence of hemolytic uremic syndrome is particularly problematic for the immunocompromised, including young children and the elderly (Rigsbee et al., 1997).

E.coli O157:H7 was first recognized as a pathogen in 1982 following a multi-state outbreak in the United States as a result of patrons consuming inadequately cooked beef patties at a restaurant chain (Riley et al., 1983). Along with ground beef, implicated in the transmission of *Escherichia coli* O157:H7 are, but not limited to, a variety of foods (Griffin and Tauxe, 1991) including dairy, produce, and meat products, person-to-person contact, animal contact (Rangel et al., 2005), as well as waterborne infections due to the land application of animal and human wastes (Jackson et al., 1998; O'Connor, 2002; Hrudey et al., 2003). It is

reported that an estimated 73500 illnesses are caused by *E.coli* O157:H7 infection annually in the United States resulting in over 2000 hospitalizations and 61 deaths (Mead et al., 1999).

The fate of *E.coli* O157:H7 in the environment has significant implications for public health. Detection of *E.coli* O157:H7 in environmental samples, however, may not always be as simple as a straight-forward culture test on standard media as has been relied upon in numerous past studies including Mechie et al. (1997), Jackson et al. (1998), Vinten et al. (2002), and Johnson et al. (2003), to name a few. Recent microbiological studies have demonstrated that many species of bacteria, including *E.coli* O157:H7, may become viable but non-culturable (VBNC) when exposed to sublethal stresses (Oliver, 2005) such as elevated or reduced temperatures, elevated or reduced osmotic concentrations, nutrient starvation, levels of oxygen (Oliver, 1993) or exposure to chloramines in tap water (Liu, 2008). These stresses may cause the cells to enter a state that is still metabolically, but not reproductively active; that is, they do not undergo cellular division on conventional media and, therefore, will not produce colonies (Rigsbee et al., 1997). This behavior increases the likelihood of false negative tests which has important implications for public health since the cells retain the ability to produce toxins while in the VBNC state (Liu et al., 2009).

Justifiably, the necessity to study the VBNC state of bacterial species is one of importance and intrigue and is littered with hurdles including the requirement to produce consistent stock cultures of cells in the VBNC state. Methods currently used to induce cells into the VBNC state include decreased

incubation temperatures (Gupte et al., 2003; Oliver and Day, 2004), starvation (Muela et al., 2008), exposure to hydrogen peroxide (Asakura et al., 2007), and exposure to chloramines (Liu et al., 2008), among others. Research to understand the changes that occur within and on the surfaces of bacterial cells as they transition from a culturable to a viable but non-culturable state is also an ongoing task. Previous work, for instance, has shown that culturable, rod-shaped gram-negative cells undergo a dramatic reduction in size and transition to a coccus-form when in the VBNC state (Catenrich and Makin, 1991; Jiang and Chai, 1996; Signoretto et al., 2002). It has also been shown that the transition from the exponential growth phase of *E. coli* KN126 to the VBNC state is accompanied by concomitant modification of the peptidoglycan, which becomes more cross-linked and richer in covalently bound lipoprotein, as well as undergoing a marked reduction in glycan strand length (Signoretto et al., 2002). The growth of polymer-like extensions, speculated to be of an exopolysaccharide nature, on the surfaces of *Vibrio parahaemalyticus* cells in the VBNC state has also been noted (Jiang and Chai, 1996).

The potential for *E.coli* O157:H7 cells to alter specific phenotypic and morphological characteristics when induced into a VBNC state could have significant effects on how this bacterium behaves in the environment. For instance, previous studies have shown that cell shape, hydrophobicity, and extracellular polymeric substance composition, among others, influence the ability of bacterial cells to be transported through soil and groundwater systems. Weiss et al. (1995) showed that smaller, rounder cells will often experience less

filtering than larger cells due to their ability to negotiate smaller pore spaces. Jacobs et al. (2007) showed that hydrophobicity plays an important role in determining how well cells will incorporate into the bulk flow solution and attach to substrates in a groundwater setting, and Tsuneda et al. (2003) showed that extracellular polymeric substance composition and length will influence the attachment capability of cells to solid substrates, such as soil particles. The transport behaviour of VBNC *E.coli* O157:H7 cells in soil and groundwater systems have even greater public health significance when one considers that the most widely-used and economical enumeration techniques for determining *E.coli* O157:H7 contamination identifies only culturable bacterial cells.

Though it has already been shown that *E.coli* O157:H7 cells are capable of entering a VBNC state (Rigsbee et al., 1997; Liu et al., 2008), few studies discuss the phenotypic or behavioral changes this bacterial species undergoes when in the non-culturable state. This could be, in part, due to the relatively large quantities of cells required to perform the respective tests, such as soil transport assays, as well as the time required to induce the VBNC state. Consequently, the first objective of this study is to develop a modified protocol, based on Liu et al. (2009), to produce consistent stock cultures of viable but non-culturable *E.coli* O157:H7 cells in quantities great enough to perform analytical tests in a timely manner while ensuring cell viability. Second, VBNC *E.coli* O157:H7 cells will be analyzed for their hydrophobicity, lengths and widths, as well as extracellular polymeric substance composition (EPS), properties shown to influence their behaviour in the environment, to add to the current knowledge base regarding the

transition of *E.coli* O157:H7 cells into the VBNC state in response to environmental stress.

2.2. Materials and Methods

2.2.1. Culturable *E.coli* O157:H7 preparation

E.coli O157:H7 (strain ADRI V241) stock culture (kept at -80°C) was initially streaked onto a Trypticase soy agar (TSA) plate (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C. After 24 hours, a single colony was used to streak a second TSA plate. Following incubation (37°C, 24 h) of this second streaked plate, a single colony was utilized to inoculate 1 mL of Trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ) which was then incubated overnight (12-14 h, 37°C, 125 rpm). The following morning, a fresh culture was produced by inoculating the overnight culture into TSB at a 50:1 (vol./vol.) ratio. This fresh culture was then incubated (37°C, 125 rpm, 3.5 h).

Following incubation, *E.coli* O157:H7 cells in the fresh culture were harvested using a Micromax RF centrifuge (Thermo IEC, Waltham, MA) at 4000 rpm for 25 min. Following harvesting, the cell pellet was washed three times in sterile, deionized water in order to remove any remaining growth medium using identical centrifuge settings as harvesting. Centrifuge settings were chosen to correspond with settings used in the subsequent VBNC *E.coli* O157:H7 preparation procedure to ensure consistency. Following the final wash, the culturable cell pellet was resuspended in 0.85% NaCl and incubated at 4°C until

further analysis. This cell preparation procedure was followed for the culturable *E.*coli O157:H7 cell characterization assays.

2.2.2. VBNC *E.coli* O157:H7 preparation

In this study, the procedure developed by Liu et al. (2009) was used as a springboard to develop a new method to produce VBNC *E.coli* O157:H7 cells in large quantities with high viability in a relatively short period of time. Based on the principle that chloraminated tap water is generally characterized as being highly oligotrophic and is oxidatively stressful to bacterial cells, and upon exposure will cause the bacteria to enter a VBNC state (Liu et al., 2008), we experimented with total chloramine concentrations in tap water samples, tap water sample volumes, cell incubation temperatures and times, quantities of cells, and washing procedures in order to generate reproducible, large quantity batches of viable but non-culturable *E.coli* O157:H7 cells. The development of the following procedure was an iterative process and took four consecutive months of effort to complete.

Initial steps focused upon determining the ideal tap water volume, incubation time and temperature, and chloramine concentration range to induce the *E.coli* O157:H7 cells into a non-culturable state. Next, to increase the viability of induced samples to an acceptable level on par with Liu et al. (2009), cell washing procedures were explored. This involved altering centrifuge settings and cell suspending procedures. It was eventually determined that by decreasing the centrifuge speeds and limiting the stress to cells while resuspending by using a simple inversion technique, it was possible to achieve induction of *E.coli*

O157:H7 cells into a non-culturable state while retaining a high viability. The new method is described as follows:

E.coli O157:H7 (strain ADRI V241) stock culture (kept at -80°C) was initially streaked onto a Trypticase soy agar (TSA) plate (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C. After 24 hours, a single colony was used to streak a second TSA plate. Following incubation (37°C, 24 h) of this second streaked plate, a single colony was utilized to inoculate 1 mL of Trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ) which was then incubated overnight (12-14 h, 37°C, 125 rpm). The following morning, a fresh culture was produced by inoculating the overnight culture into TSB at a 50:1 (vol./vol.) ratio. This fresh culture was then incubated at (37°C, 125 rpm, 3.5 h).

Following incubation, *E.coli* O157:H7 cells in 1 mL (~10^9 cells/mL) of the fresh culture were harvested in 20 mL of deionized water using a Sorvall Biofuge Primo centrifuge (Mandell Scientific Co., Guelph, ON) at 4000 rpm for 25 min. Following harvesting, the cell pellet was washed three times in 20 mL of sterile, deionized water in order to remove any remaining growth medium using identical centrifuge settings as harvesting.

After the third wash, the supernatant was removed and the cells were resuspended in 30 mL of 4ºC filter-sterilized chloraminated tap water (Edmonton, Alberta) with a concentration ranging from 1.5-2.0 mg /L total chloramine as determined by amperometric back titration using an Autocat 9000 Chlorine Amperometric Titrator (Hach, Loveland, CO). The chloramine concentration

range was determined by using Liu et al. (2009) as an initial reference, then through a number of induction trials, an optimum range was determined which produced desired results. VBNC induction attempts using total chloramine concentrations above 2.0 mg/L were found to result in marked decreases in cell viability. The cell suspensions were incubated at 4ºC for 30 min. (As a side note, it was often necessary, particularly during colder months, to allow tap water samples to incubate at room temperature (up to 24 h) in Erlenmeyer flasks prior to filtration in order to decrease the total chloramine concentrations to within a usable range via volatilization).

Following induction into the VBNC state, the cells were washed three times using 20 mL of deionized water, utilizing an identical protocol as seen prior to induction. This was done to remove any lingering tap water which may affect cell viability. Following the final wash, the cell pellet was resuspended in 1 mL of 0.85% NaCl and a culturability test performed on the cell suspension. This test was performed by removing a 250 µl aliquot from the VBNC cell suspension and placing it into an empty, sterile plate. Warm TSA was then poured into the plate, allowed to solidify, then incubated overnight at 37°C. The remaining stock VBNC cell suspension was incubated at 4ºC overnight. The following morning (~18 h later), the plates were viewed to see if any colony growth had taken place. If present, the number of colonies was recorded, and a viability test on the suspension was subsequently completed.

2.2.3. Determination of VBNC viabilty

The viability test was completed using a Live/Dead BacLight bacterium viability kit (Molecular Probes Europe, Leiden, The Netherlands) as per the manufacturer's instructions. It uses SYTO 9 stain and propidium iodide to measure membrane integrity to distinguish live cells (intact cell membrane) from dead cells (lacking an intact cell membrane) (Liu et al. 2008). Briefly, a 50 µl aliquot of the VBNC cell suspension was added to 0.85% NaCl to a final volume of 1 mL. To this suspension, 2.5 µl of the propidium iodide and Syto 9 mixture was added. This was allowed to incubate at room temperature for 30 minutes in the dark. Following this time, 0.85% NaCl was added to a final volume of 2 mL. Samples stained with these reagents were filtered through 0.22-µm-pore-size nitrocellulose membrane filters (Millipore, Billerica, MA). An epifluorescence microscope (Zeiss Axioskop 2 FS) along with a DAPI filter and a 100x objective was used to view live and dead cells. A series of 10 images were taken from each different sample (e.g. Image 2-1). The bacteria were then counted and calibrated using Adobe Photoshop CS5 (Adobe; San Jose, California, USA) to give the percent of living cells in the suspension as well as providing a count of the total number of cells in the suspension tested. This procedure was repeated with ten different *E.coli* O157:H7 colonies in sample sizes ranging from 5 to 20 mL.

This induction procedure was used to produce the stock VBNC suspensions of *E.coli* O157:H7 utilized in the subsequent cell property characterization assays.

2.2.4. Cell property characterization

2.2.4.1. Cell hydrophobicity

To determine the relative hydrophobicity of the culturable or VBNC *E.coli* O157:H7 cells, the microbial attachment to hydrocarbon (MATH) assay was utilized (Rosenberg, 1980) which measures the partitioning of cells between an aqueous phase and a hydrophobic phase. This test was performed by first determining the optical density of a 5 mL cell suspension at 546 nm using a Lambda 35 UV/Vis spectrometer (PerkinElmers, Waltham, MA). Next, 4 mL of the cell suspension (OD_{546} ~ 0.29) was combined with 1 mL of a hydrocarbon, in this case dodecane (ReagentPlus, Sigma-Aldrich) in a 17x100 mm test tube. The suspensions were vortexed (Fisher Vortex Genie 2, Fisher Scientific) on high speed continuously for two minutes then allowed to stand for 30 minutes to allow separation of the aqueous and hydrocarbon phases. After this time, a 1 mL sample of the aqueous phase was removed and its optical density measured at 546 nm. After acquiring this second optical density reading, the relative hydrophobicity of the initial cell suspension could be determined using,

$$\text{Hydrophobicity} = \frac{(C_o - C_{30})}{C_o} \times 100 \qquad [2.1]$$

Where C_o is the initial optical density of the aqueous phase prior to hydrocarbon addition and C_{30} is the optical density of the aqueous phase following hydrocarbon addition, mixing, and allowing phase separation for the time previously stated. Equation [2.1] provides a measure of the percentage of cells which have moved from the aqueous electrolyte solution into the hydrocarbon phase. The MATH assay was performed for three different colony suspensions in duplicate (n=6) for both culturable and VBNC *E.coli* O157:H7 cells.

2.2.4.2. Cell length and width

Lengths and widths of VBNC and culturable *E.coli* O157:H7 cells were collected by utilizing a Axioplan II microscope (Carl Zeiss) in differential interference contrast (DIC) mode equipped with an CoolSnapHQ digital cooled CCD Camera (Photometrics, Tuscon, USA). Prior to microscopic examination, TSA-coated glass slides were utilized to immobilize the cells for viewing. These slides were prepared by pouring warm 2% Trypticase soy agar over the slides, allowing to solidify, then cutting excess agar from the slides using a razor blade. Cells suspended in 0.85% NaCl were then applied (20 µl) to the coated slides and a cover glass was sealed over the cells using clear nail polish. This assay was performed in triplicate for both the VBNC and culturable cellular states with 100 images gathered from each replication for a total of 300 cells processed for each cell state. Metamorph software (Molecular Devices) was used to capture the DIC images. The images were then analyzed and individual cell lengths and widths were determined using the ImageJ image processing program (Rasband, W.S, U. S. National Institutes of Health).

2.2.4.3. Extracellular polymeric substance purification and analysis

2.2.4.3.1. Extracellular polymeric substance purification

The extracellular polymeric substance (EPS) purification technique utilized was previously described by Gong et al. (2009) and utilizes formaldehyde-sonication exposure combined with ethanol precipitation to isolate and purify the tightly-bound cellular EPS structures from the loosely-bound and free EPS.

Initially, cell pellets of either VBNC or culturable *E.coli* O157:H7 cells were resuspended in 12 mL of 0.85% NaCl and their optical density adjusted to approximately 0.35 at 546 nm using a Lambda 35 UV/Vis spectrometer (PerkinElmers, Waltham, MA). Next, 50 µl of culture suspension was combined with 950 µl of 0.85% NaCl and stained using a Live/Dead BacLight bacterium viability kit (Molecular Probes Europe, Leiden, The Netherlands) as per the manufacturer's instructions. Stained samples were filtered through 0.22-µm-pore-size nitrocellulose membrane filters (Millipore, Billerica, MA) and 10 cell images were taken for each sample using an epifluorescence microscope (Zeiss Axioskop 2 FS) along with a DAPI filter and an inverted camera. These images were processed manually and the viability and number of cells/mL determined for each suspension analyzed.

Following image analysis, 5 mL of the culture suspension was centrifuged using a Sorvall Biofuge Primo centrifuge (Mandell Scientific Co., Guelph, ON) at 4000 rpm for 25 min. The supernatant was removed and the cell pellet was resuspended in 10 mL of 0.22% formaldehyde (ACS grade, Fisher Scientific) in 8.5% NaCl and incubated at 4°C for 2 hours. Following incubation, the suspension was aliqouted into five 2 mL centrifuge tubes and centrifuged (3700xg, 4ºC, 15 min) using a Micromax RF centrifuge (Thermo IEC, Waltham, MA). The supernatant was removed from each tube and the pellets were resuspended in 2 mL of deionized water and centrifuged again to rinse away any remaining formaldehyde solution and non-EPS cellular material. The pellets were then collected and 50 mL of deionized water was added to each tube for every

gram of pellet (~1 mL of water/tube). The resuspended cell suspensions were then sonicated (Sonicor S-50, Sonicor INC., Wallingford, CT) at 50 W for 2 minutes to detach any loosely-bound EPS and purify the EPS (Gong et al., 2009). The suspensions were then centrifuged (3700xg, 4ºC, 15 min) once more to collect the purified EPS.

The final step of the EPS purification assay was to precipitate the EPS. This was facilitated by combining the pellets into a 15 mL centrifuge tube in 5 mL 0.85% NaCl and 10 mL ice-cold ethanol and incubating overnight (~16 h) at 4°C. The following day, the precipitated EPS was collected by centrifugation (4000 rpm, 30 min) using a Sorvall Biofuge Primo centrifuge and resuspended in 10 mL deionized water for further analysis.

2.2.4.3.2. Extracellular polymeric substance analysis

The purified EPS was analyzed for total protein using the Lowry method (Lowry et al, 1951) and total sugars using the phenol-sulfuric acid assay (Dubois et al., 1956) in a manner similar to Gong et al. (2009).

The Lowry method involved aliqouting 0.3 mL of EPS suspension into a 17x100 mm sterile culture tube followed by 1.5 mL of Lowry reagent (24.5 mL of 2% Na_2CO_3 in 0.1 M NaOH, 0.25 mL of 2% $Na_2C_4H_4O_6$, and 0.25 mL of 1% $CuSO_4$ - $5H_2O$). The mixture is then vortexed (Fisher Vortex Genie 2, Fisher Scientific) and allowed to incubate for 10 min at room temperature in the dark. After 10 minutes, 0.075 mL of 1 N Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) is added to the culture tube, mixed by vortexing, and incubated again at room temperature in the dark for 30 minutes. The absorbance of the mixture was

then determined at 500 nm using a Lambda 35 UV/Vis spectrometer (PerkinElmers, Waltham, MA) and the total protein in the sample calculated from a standard curve prepared from Bovine serum albumin (Sigma-Aldrich) (2 mg/mL) (Appendix A).

The phenol-sulfuric acid method involved adding 2 mL of EPS suspension to a clean, sterile glass vial followed by the addition of 1 mL of 5% phenol solution (ReagentPlus, Sigma-Aldrich) and 5 mL of concentrated sulfuric acid 95.5% (ACS Pure, Fisher Scientific). The mixture was then incubated at room temperature for 10 minutes followed by incubation in a water bath (IsoTemp 210, Fisher Scientific) at 28ºC for 20 minutes. The solution color was subsequently allowed to stabilize at room temperature for an additional hour. The absorbance of the mixture was then determined at 480 nm using a Lambda 35 UV/Vis spectrometer (PerkinElmers, Waltham, MA) and the total sugar in the sample calculated from a standard curve prepared from Xanthan gum (Sigma-Aldrich) (2 mg/mL) (Appendix B).

2.2.5. Statistical Analysis

Paired T-tests were used to elucidate any statistically significant differences between cell properties. All statistical analyses were conducted using R statistical software (R Development Core Team; Vienna, Austria) and differences were considered significant at $p<0.05$.

2.3. Results

2.3.1. Development of an *E.coli* O157:H7 VBNC induction protocol

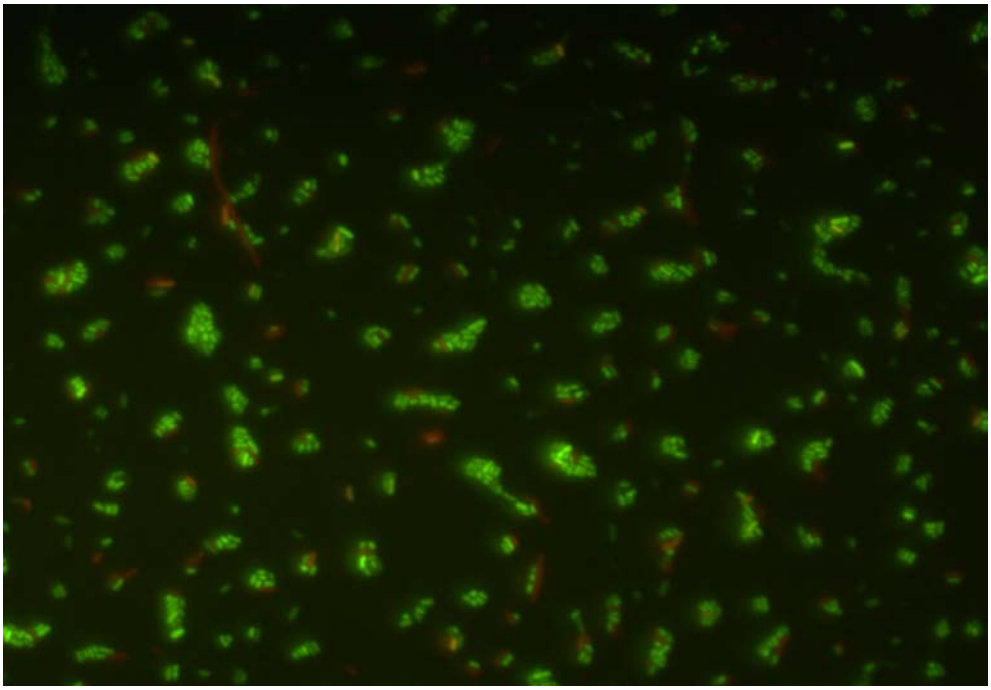

Image 2-1. Example of a Live/Dead BacLight image of viable but non-culturable *E.coli* O157:H7 cells used to calculate the percent viability as well as total cell count using the previously described induction procedure (Green cells=viable, red cells=non-viable).

Using the VBNC induction procedure previously described in the methodology section, culturability tests revealed a range of 0 CFU/mL to 480 CFU/mL with a mean of 49.8 CFU/mL and a standard deviation of 151.2 CFU/mL for the ten replications. Seven of ten culturability tests revealed zero colony formation, two tests revealed colony numbers amounting to less than 20, and one outlying test showed 480 colonies develop. The test with 480 colonies is the reason behind the large standard deviation in relation to the mean. Despite having what appears to be a large number of colony forming units in this particular test, the 480 colonies account for less than 0.01% of the total number of cells/mL in that particular sample (Table 2-1). Due to this fact, the reliability of this procedure to induce a non-culturable state, despite the misleading standard

deviation, is not in question. Following the culturability tests, viability tests were conducted and used to produce ten images for each replication, which were then utilized to reveal an average total cell number (viable and non-viable cells), percent of cells in a non-culturable state, and percent viability for each sample replicate (Table 2-1). From the data, the total number of *E.coli* O157:H7 cells attempted to be induced into a VBNC state ranged from $1.93x10^7$ cells/mL to $36.01x10^7$ cells/mL with an average total cell count of $19.87x10^7$ cells/mL and standard deviation of $15.71x10^7$ cells/mL over the ten replications. The percent of cells that entered a non-culturable state was consistently above 99.99% for the ten samples. Finally, the induction procedure developed was able to achieve a range in percent cell viability that varied from 79% to 92% with an average percent cell viability of 86% with a standard deviation of 4.57%. In summary, the induction procedure produced large quantities of viable but non-culturable (VBNC) *E.coli* O157:H7 cells.

Table 2-1. Total count, colony forming units per mL, percent non-culturable cells, and percent viability of VBNC *E.coli* O157:H7 cells from ten sample replications using a modified VBNC induction procedure (standard deviations are in parentheses) .

Replication	Cell Numbers (x 10^7 cells/mL)	Colony Forming Units (per mL)	Non-Culturable Cells (%)	Viability (%)
1	2.21	0	100	92
2	2.60	0	100	90
3	2.50	15	>99.99	84
4	1.93	3	>99.99	87
5	39.82	0	100	82
6	33.26	480	>99.99	79
7	36.01	0	100	88
8	27.93	0	100	85
9	27.91	0	100	81
10	24.47	0	100	92
Average	**19.87 (15.71)**	**49.8 (151.2)**	**>99.99**	**86 (4.57)**

2.3.2. Comparison of select culturable and VBNC *E.coli* O157:H7 properties

The assays utilized to characterize specific properties of the *E.coli* O157:H7 cells indicate that significant ($p<0.05$) differences exist between the VBNC and culturable cells (Table 2-2).

The cell morphology tests showed cell lengths and widths varied significantly ($p<0.05$) between the *E.coli* O157:H7 cell culturable and VBNC states. The lengths of the culturable cells varied from 3.93 μm to 1.26 μm, with a mean length and standard deviation of 2.3 μm and 0.5 μm, respectively. The VBNC cell lengths were significantly ($p<0.05$) smaller, they varied from 3.21 μm to 0.82 μm, with a mean length and standard deviation of 1.7 μm and 0.4 μm, respectively. Cell widths from the culturable cells had a range of 1.53 μm to 0.72 μm with a mean width and standard deviation of 1.1 μm and 0.15 μm, respectively. By contrast, VBNC cells had a smaller width with a range of 1.70 μm to 0.54 μm and an overall decrease in mean width and standard deviation to 0.98 μm and 0.19 μm, respectively. The results demonstrate that cells, upon entry into a VBNC state, miniaturize as well as transition from a rod-shape to a coccoid-shape.

Lowry assays and the Phenol-sulfuric acid assays revealed that, following a transition into the VBNC state, *E.coli* O157:H7 cells undergo a significant increase ($p<0.05$) in the amount of tightly bound proteins and carbohydrates. The total amount of tightly bound proteins on the culturable cell surfaces ranged from 28.7 $\mu g/10^8$ cells to 37.1 $\mu g/10^8$ cells, with a mean and standard deviation of 33.9 $\mu g/10^8$ cells and 4.0 $\mu g/10^8$ cells, respectively, while the total amount of tightly

bound proteins on the VBNC cell surfaces ranged from 32.9 μg/10^8 cells to 53.8 μg/10^8 cells, with a mean and standard deviation of 44.7μg/10^8 cells and 7.2 μg/10^8 cells, respectively.

The Phenol-sulfuric acid assay demonstrated that total, tightly-bound sugars ranged from 9.6 μg/10^8 cells to 13.1 μg/10^8 cells with a mean and standard deviation of 11.2 μg/10^8 cells and 1.1 μg/10^8 cells, respectively, for the culturable cells and a range of 16.2 μg/10^8 cells to 26.8 μg/10^8 cells with a mean and standard deviation of 20.0 μg/10^8 cells and 4.0 μg/10^8 cells, respectively, for the VBNC *E.coli* O157:H7 cells.

Looking at the ratio of protein-to-sugar content prior to and after induction, culturable *E.coli* O157:H7 cells had a ratio of 3.03:1 (protein:sugar), while VBNC *E.coli* O157:H7 cells had a ratio of 2.2:1 (protein:sugar) indicating a proportionally greater increase in surface sugars compared to surface proteins following entry into the VBNC state for *E.coli* O157:H7 cells.

Finally, the results from the microbial attachment to hydrocarbon (MATH) assay, an indication of the relative hydrophobicities of the VBNC and culturable cell suspensions, revealed a significant difference ($p<0.05$) between the two cellular states. The culturable cells showed a range from 22% to 40%, with a mean relative hydrophobicity of 32.5% and standard deviation of 6.8% compared with the VBNC *E.coli* O157:H7 cells which illustrated a range from 14% to 23%, with a mean relative hydrophobicity of 16.9% and a standard deviation of 3.4%.

Table 2-2. Measured values of selected cell properties for culturable and VBNC *E.coli* O157:H7 (standard deviations are in parentheses).

Cell Type	Hydrophobicity (%)	Cell Dimensions		EPS Composition	
		Length (µm)	Width (µm)	EPS Protein (µg/10^8 cells)	EPS Sugar (µg/ 10^8 cells)
Culturable	32.5 (6.8) a	2.3 (0.5) a	1.1 (0.15) a	33.9 (4.0) a	11.2 (1.1) a
VBNC	16.9 (3.4) b	1.7 (0.4) b	0.98 (0.19) b	44.7 (7.2) b	20.0 (4.0) b

*Within each column, values with different letters are significantly different at the $p<0.05$ level

2.4. Discussion

Viable but non-culturable denotes a state in which bacterial cells cannot be detected by standard culture on enriched agar media, although remaining viable and capable of resuscitation under favorable conditions. Employed mainly by Gram-negative bacteria, this cell state has been proposed as a strategy for survival by bacteria in the natural environment (Gupte et al., 2003) in response to such chemical and natural stresses as decreased incubation temperatures (Gupte et al., 2003; Oliver and Day, 2004), starvation (Muela et al., 2008; Lindbäck et al., 2009), exposure to hydrogen peroxide (Asakura et al., 2007), exposure to heavy metals (Ordax et al., 2006) and exposure to chloramines (Liu et al., 2008), among others. It has been shown bacteria in the VBNC state can retain their ability to cause infection in hosts (Jones et al., 1991; Oliver and Bockian, 1995; Liu et al., 2009) which has significant implications on public health and renders the acquisition of knowledge regarding the VBNC state one of great importance.

In this study, we have developed a new induction procedure, using chloramine exposure, to induce *Escherichia coli* O157:H7 cells into a VBNC

state. The procedure, modified from Liu et al. (2009), was shown to induce a relatively large quantity of cells (>10^8 cells/mL) with a high viability (86% on average) and very low culturability (>99.99% of total cells becoming non-culturable). The new procedure retains a short incubation time as outlined in the original publication. Using the procedure, we were able to demonstrate that the transition from a culturable to a VBNC state for *E.coli* O157:H7 cells is accompanied by significant changes in the lengths and widths, extracellular polymeric substance compositions, and hydrophobicities of the induced cells.

There have been numerous studies completed outlining the induction of bacterial species into a viable but non-culturable state, including *E.coli* O157:H7 cells. The problem with the majority of induction procedures, however, are two-fold: First, many of the published methods for stimulating a VBNC state require lengthy incubation periods which becomes inconvenient and inefficient for the researcher. For instance, Oda et al. (2004) utilized an incubation period of one week under starvation conditions in phosphate buffered saline and a reduced temperature to induce *E.coli* O157:H7 cells into a VBNC state. In a study by Muela et al. (2008), *Escherichia coli* strain 416 was induced into a VBNC state under varying levels of stress including starvation alone, photooxidative stress caused by illumination, and finally starvation combined with illumination and incubation in natural sea water. The results showed that cells entered a VBNC state in 6 days, 3 days, and 1 day, respectively. Even exposure to 0.05% hydrogen peroxide under starvation conditions required 48 hours to provoke *E.coli* O157:H7 strain F2 cells into a VBNC state (Asakura et al., 2007).

Procedures utilized by Signoretto et al. (2000), Pruzzo et al. (2003), and Lai et al. (2009) also required prolonged incubation periods of six weeks in minimal media to induce *Vibrio parahaemolyticus* cells, 3 weeks in artificial sea water at reduced temperature to induce *Vibrio cholerae* O1 cells, and 16 days in sterilized lake water at reduced temperature to induce *Enterococcus faecalis* 56R cells, respectively.

Second, many of the reviewed procedures do not attempt to induce a large quantity of cells per volume. This is an issue if a large quantity of cells in a VBNC state is required to study a particular cell property. For instance, Lleo et al. (2007) utilized 10^7 CFU/mL when inducing enterococcal species into the VBNC state, Signoretto et al. (2000) used cell quantities of $1\text{-}2x10^6$ CFU/mL in their procedure, and Liu et al. (2009) induced *E.coli* O157:H7 cells into the VBNC state using chloraminated tap water within 15 minutes, however, only at a cell density of 10^6 CFU/mL.

Following optimization, it was determined that the new method described in this study could be used to induce approximately $10^8\text{-}10^9$ *E.coli* O157:H7 cells into a VBNC state in 30 minutes. This was accomplished by increasing the volume of deionized water the cells are washed with pre- and post-incubation, increasing the incubation period slightly, and using low-speed centrifugation and tube inversions during the cell washes, in contrast with Liu et al. (2009). These necessary changes are most likely explained by the increase in *E.coli* O157:H7 cells employed in our protocol. The increase in cell numbers will thus require a greater volume of water to wash away growth media or excess tap water, as well

as a lengthier incubation period to ensure complete cellular interaction with the chloramines in the tap water to illicit the desired response. Lowering the centrifugation speeds was necessary to maintain high viability. It is unclear why high centrifuge speeds decrease viability, but it may be due to the finding that Gram-negative VBNC cells, as demonstrated using *E.coli* KN126, have been shown to have an autolytic capability higher than that of exponentially growing cells as well as no increase in mechanical resistance of the cell wall (Signoretto et al., 2002). This could lead to a scenario where the synergistic effects of the chloramine exposure combined with the high-speed centrifugation can lead to cell autolysis. Signoretto et al. (2000), however, showed that Gram-positive *E.faecalis* cells in the VBNC state had a cell wall that was more resistant to mechanical disruption than exponentially growing cells. Similarly, Wong and Wang (2004) demonstrated that VBNC *V. parahaemolyticus* cells were more resistant to thermal inactivation, low salinity, and mild acid stresses than the exponential phase cells possibly due to the upregulation of specific factors or regulons.

The results from the cell length/width study revealed a miniaturization as well as a transition from a rod-shape to a coccus-form for *E.coli* O157:H7 cells in the VBNC state. This finding has been substantiated in numerous other studies (Costa et al., 1999; Signoretto et al., 2002; Pruzzo et al., 2003; Oda et al., 2004) whom all noted a similar shrinking and shift in shape when studying their respective VBNC Gram-negative bacterial species. It has been suggested that these changes occur in order to increase the surface area-to-volume ratio of the

cells to minimize the nutritional requirements of the cells while maintaining a large surface area with the external surroundings to facilitate nutrient uptake (Jiang and Chai, 1996; Abdallah et al., 2008) when the cells are in the VBNC state.

The second cell property to be looked at was the bound proteins and sugars on the surface of the culturable and VBNC cells, which make up the majority of the extracellular polymeric substances (EPS) but also includes humus-like substances, uronic acids, and DNA in smaller quantities (Liu and Fang, 2003). Utilizing an EPS purification technique previously described by Gong et al. (2009), which isolates only the tightly bound EPS on the surface of the cells, the results showed a significant upregulation of both bound sugars and proteins on the surfaces of VBNC *E.coli* O157:H7 cells when compared with culturable cells. The reasoning behind using this EPS purification technique was primarily the desire to explore only tightly-bound EPS which forms a type of capsule around the cell surface, whereas the loosely-bound or free EPS is exuded from the cell and released into the surrounding media (Nielsen and Jahn, 1999) and can easily be washed away. Long et al. (2009) showed that it is the tightly-bound EPS, namely the proteins and sugars, which will have the greatest influence on adhesion to environmental substrates. In their study, the bound EPS from the cell surfaces of four bacterial strains were removed which resulted in a decrease in ability to bind to silica surfaces. Similar findings were also shown by Li and Yang (2007) who demonstrated that an increase in the amount of loosely-bound or free EPS will worsen the bioflocculation and weaken the structure of sludge

flocs due to cells being more slippery and having a higher bound water content. Liu et al. (2007) also observed a decrease of deposition to surfaces by removal of EPS from cell surfaces and postulated that EPS enhancement of cell deposition is possibly due to the formation of attractive polymeric interaction between silica surfaces and major EPS components like polysaccharides and proteins.

Initially, it was speculated that EPS production would decrease for *E.coli* O157:H7 cells upon entry into the VBNC state in an effort to conserve energy to maintain intracellular activity; however, this was not the case. The upregulation of EPS that was found in this study might be a defense mechanism for the VBNC cells in response to the chloramine stress. This phenomenon was demonstrated by Ryu and Beuchat (2005) who found that a strain of *Escherichia coli* O157:H7 which overproduces EPS had increased resistance to chlorine exposure compared with other strains producing lower levels of EPS. Chen et al. (2004) described the upregulation of colonic acid-containing EPS on the surfaces of *E.coli* O157:H7 cells following exposure to environmental stress and went on to show that *E.coli* O157:H7 mutants deficient in colonic acid production were more susceptible to oxidative and osmotic stresses than their wild-type counterparts. Similarly, Roberson et al. (1992), Weiner et al. (1995), Ordax et al. (2006), and Henriques and Love (2007) all noted similar EPS upregulation in studies examining bacterial responses to environmental stresses.

The final cell property explored and compared was hydrophobicity using the microbial attachment to hydrocarbon (MATH) assay. Our results show that *E.coli* O157:H7 cells stimulated into a VBNC state through exposure to

chloramines undergo a significant decrease in their relative hydrophobicity, compared with culturable cells. Similar findings were reported by Hedge et al. (2008) who observed that oxidative stress by exposure to hydrogen peroxide had an inhibitory effect on the cell surface hydrophobicity and adherence of *E. coli*. Similarly, Uberos et al. (2001) found that incubating *Escherichia coli* in Mueller Hinton broth containing the oxidizing agents melatonin and vitamin E, also resulted in decreased surface hydrophobicity. Both of these papers support the view that oxidative stress plays an important role in modifying the surface characteristics of *E. coli*, which could affect the micro-organisms capacity to adhere to substrates. The molecular affect of the stress response on *Escherichia coli* was also examined by Zhang et al. (2007) who were able to show that a gene called *ycf*R, which is upregulated 10-fold under chlorine treatment (Wang et al., 2009), can modulate *E. coli* K-12 biofilm formation through various pathways including decreasing surface hydrophobicity by altering surface membrane protein expression.

To make sense of these relative hydrophobicity results we must consider what is occurring on the surface of the *E.coli* O157:H7 cells in the VBNC versus the culturable states. The hydrophobicity of a cell is a reflection of the presence of both hydrophobic and hydrophilic groups in the EPS, and is represented as an average of the hydrophobicity of its components. Different components with different physical and chemical characteristics should play different roles in cellular surface properties (Wang et al., 2006). Neu and Marshall (1990) indicated that that the main type of polymers found on the surface of bacteria are

proteins and polysaccharides and these components can either be hydrophobic or hydrophilic depending on their composition. From our results, it appears that an increase in surface proteins and sugars has subsequently led to a decrease in the hydrophobicity of *E.coli* O157:H7 cells in the VBNC state.

Numerous studies have shown the dominant surface carbohydrate of Gram-negative bacteria as being lipopolysaccharide (LPS) molecules. The portion of the LPS which extends out from the cell, known as the O-antigen portion, is made up of repeating charged sugar units which renders them hydrophilic (Bower and Rosenthal, 2006; Raszka et al., 2006; Liu, H., 2008; Matthysse et al., 2008). It seems logical then that an increase in the quantity of LPS molecules on the surface of the VBNC *E.coli* O157:H7 cells will render the cell more hydrophilic. This is consistent with the increase in surface sugars and decrease in hydrophobicity seen in this study. Past studies, however, have shown that an increase in surface protein content is associated with an increase in cell hydrophobicity (Jorand et al., 1998; Raszka et al., 2006). For this study, it would seem then that the upregulation of surface carbohydrates had a greater influence on the hydrophobicity of the VBNC *E.*coli O157:H7 cells than the upregulated surface proteins. This finding can possibly be explained by the ratio of surface protein-to-surface sugar prior to and following induction into a VBNC state for *E.coli* O157:H7 cells. The data (Table 2-2) indicates that there was a proportionally greater increase in surface carbohydrates following entry into the VBNC state than surface proteins. Liao et al. (2001) isolated the EPS from filamentous microorganism in sewage sludge. They suggested that the proportions

of EPS components (i.e. proteins:carbohydrates) were more important than the quantities of individual EPS components in controlling hydrophobicity. It is possible then, that the results of this study were caused by the shielding nature of the upregulated LPS hydrophilic O-antigen branches extending from the surface of the VBNC cells forming a hydrophilic layer around the VBNC *E.coli* O157:H7 cells. This would effectively mask the influence of the hydrophobic surface proteins, causing a subsequent lowering of VBNC cell hydrophobicity.

2.5. Conclusions

It is abundantly evident that *Escherichia coli* O157:H7 has considerable clinical significance as a human pathogen. It is also well documented that this pathogen is capable of becoming viable but non-culturable (VBNC) when exposed to environmental and chemical stresses, while still maintaining its capacity to produce disease-causing factors, making it a considerate threat to public health. Our research outlines the development of a novel procedure by which *E.coli* O157:H7 cells can be induced into a VBNC state using chloraminated tap water in a relatively short time period and in large densities while still maintaining high viability. This procedure can allow for increased user efficiency as well as widening the assay options for VBNC *E.coli* O157:H7 cells which otherwise may not have been possible. Using the new induction procedure, we were also able to show that *E.coli* O157:H7 cells when stimulated into a VBNC state undergo a significant change in their size and morphology, their surface EPS composition, and their hydrophobicity. It is suggested that the decrease in cell size and change in shape of the VBNC *E.coli* O157:H7 cells is to

minimize the nutritional needs of the cell while still increasing the surface area-to-volume ratio; while the increase in surface proteins and carbohydrates are a defense mechanism in response to the environmental stress which, as a result, leads to a decrease in VBNC cell hydrophobicity. These findings will become important when analyzing differences between culturable and VBNC bacteria cells in terms of adhesive abilities, flocculation capacities, and cell resistance capabilities, for instance. Overall, this study suggests that the VBNC state is an adaptive response by bacteria to stress and the changes the cells undergo are significant and will likely influence how the cells interact with their environment.

2.6. References

Abdallah, F.B., Lagha, R., and Bakhrouf, A. 2008. Resuscitation and morphological alterations of *Salmonella bovismorbificans* cells under starvation in soil. *World Journal of Microbiology and Biotechnology*. 24(8): 1507-1512.

Asakura, H., Panutdaporn, N., Kawamoto, K., Igimi, S., Yamamoto, S., and Makino, S. 2007. Proteomic characterization of enterohemorrhagic *Escherichia coli* O157:H7 in the oxidation-induced viable but non-culturable state. *Microbiology and Immunology*. 51(9): 875-881.

Bower, S. and Rosenthal, K.S. 2006. The bacterial cell wall: The armor, artillery, and achilles heel. *Infectious Diseases in Clinical Practice*. 14(5): 309-317.

Catenrich, C.E. and Makin, K.M. 1991. Characterization of the morphologic conversion of *Helicobacter pylori* from bacillar to coccoid forms. *Scandinavian Journal of Gastroenterology*. 181:58–64.

Chen, J., Mei Lee, S., and Mao, Y. 2004. Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *International Journal of Food Microbiology*. 93(3):281– 286.

Costa, K., Bacher, G., Allmaier, G., Dominguez-Bello, M.G., Engstrand, L., Falk, P., DE Pedro, M.A., and Garcia-DEL Portillo, F. 1999. The morphological transition of *Helicobacter pylori* cells from spiral to coccoid is preceded by a substantial modification of the cell wall. *Journal of Bacteriology*. 181(12): 3710-3715.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28(3): 350-356.

Gong, A.S., Bolster, C.H., Benavides, M., and Walker, S.L. 2009. Extraction and analysis of extracellular polymeric substances: Comparison of methods and extracellular polymeric substance levels in *Salmonella pullorum* SA 1685. *Environmental Engineering Science.* 26(10): 1523-1532.

Griffin, P.M. and Tauxe, R.V. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E.coli*, and the associated hemolytic uremic syndrome. *Epidemiologic Reviews*. 13: 60-98.

Gupte, A.R., de Rezende, C.L.E., and Joseph, S.W. 2003. Induction and resuscitation of viable but nonculturable *Salmonella enterica* serovar Typhimurium DT104†. *Applied and Environmental Microbiology.* 69(11): 6669-6675.

Hedge, A., Bhat, G.K., and Mallya, S. 2008. Effect of exposure to hydrogen peroxide on the virulence of *Escherichia coli*. *Indian Journal of Medical Microbiology*. 26(1): 25-28.

Henriques, I.D.S. and Love, N.G. 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. *Water Research*. 41(18): 4177-4185.

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., and Hrudey, E.J. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Science and Technology*. 47(3): 7-14.

Jackson, S.G., Goodbrand, R.B., Johnson, R.P., Odorico, V.G., Alves, D., Rahn, K., Wilson, J.B., Welch, M.K., and Khakhria, K. 1998. *Escherichia coli* O157:H7 diarrhoea associated with well water and infected cattle on an Ontario farm. *Epidemiology and Infection*. 120(1): 17-20.

Jacobs, A., Lafolie, F., Herry, J.M., and Debroux, M. 2007. Kinetic adhesion of bacterial cells to sand: Cell surface properties and adhesion rate. *Colloids and Surfaces B: Interfaces*. 59(1): 35-45.

Jiang, X. and Chai, T-J. 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. *Applied Environmental Microbiology*. 62(4): 1300–1305.

Johnson, J.Y.M., Thomas, J.E., Graham, T.A., Townshend, I., Byrne, J., Selinger, L.B. and Gannon, V.P.J. 2003. Prevalence of *Escherichia coli* O157:H7 and *Salmonella* spp. in surface waters of southern Alberta and its relation to manure sources. *Canadian Journal of Microbiology*. 49(5): 326-335.

Jones, D.M., Sutcliffe, E.M., and Curry, A. 1991. Recovery of viable but non-culturable *Campylobacter jejuni*. *Journal of General Microbiology*. 137(10): 2477-2482.

Jorand, F., Boue-Bigne, F., Block, J. C., Urbain, V. 1998. Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Water Science and Technology*. 37(4 –5): 307–315.

Lai, C-J., Chen, S-Y., Lin, I-H., Chang, C-H., and Wong, H-C. 2009. Change of protein profiles in the induction of the viable but nonculturable state of *Vibrio parahaemolyticus*. *International Journal of Food Microbiology*. 135(2): 118-124.

Li, X.Y. and Yang, S.F. 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Research*. 41(5): 1022-1030.

Liao, B.Q., Allen, D.G., Droppo, I.G., Leppard, G.G., and Liss, S.N. 2001. Surface properties of sludge and their role in bioflocculation and settleability. *Water Research*. 35(2): 339-350.

Lindbäck, T., Rottenberg, M.E., Roche, S.M., and Rørvik, L.M. 2009. The ability to enter into an avirulent viable but non-culturable (VBNC) form is widespread among *Listeria monocytogenes* isolates from salmon, patients and environment. *Veterinary Research*. 41(1): 08.

Liu, H. Pathogenic bacterial sensors based on carbohydrates as sensing elements. In: Zourob, M., Elwary, S., and Turner, A. (eds.), Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems, Springer Science and Business Media, New York, 2008. pp. 660-681.

Liu, Y. and Fang, H.H. 2003. Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. *Critical Reviews in Environmental Science and Technology*. 33(3): 237-273.

Liu, Y., Yang, C-H., and Li, J. 2007. Influence of extracellular polymeric substances on *Pseudomonas aeruginosa* transport and deposition profiles in porous media. *Environmental Science and Technology*. 41(1): 198–205.

Liu, Y., Gilchrist, A., Zhang, J., and Li, X. 2008. Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Applied and Environmental Microbiology*. 74(5): 1502-1507.

Liu, Y., Wang, C., Tyrrell, G., Hrudey, S.E., and Li, X-F. 2009. Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environmental Microbiology Reports.* 1(2): 155-161.

Lleo, M., Bonato, B., Tafi, M.C., Caburlotto, G., Benedetti, D., and Canepari, P. 2007. Adhesion to medical device materials and biofilm formation capability of some species of enterococci in different physiological states. *FEMS Microbiology Letters.* 274(2): 232-237.

Long, G., Zhu, P., Shen, Y., and Tong, M. 2009. Influence of extracellular polymeric substances (EPS) on deposition kinetics of bacteria. *Environmental Science and Technology*. 43(7): 2308-2314.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*. 193:265-275.

Matthysse, A.G., Deora, R., Mishra, M., and Torres, A.G. 2008. Polysaccharides cellulose, poly-1,6-N-acetyl-D-glucosamine, and colanic acid are required for optimal binding of *Escherichia coli* O157:H7 strains to alfalfa sprouts and K-12 strains to plastic but not for binding to epithelial cells. *Applied and Environmental Microbiology*. 74(8): 2394-2390.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C. 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases.* 5(5): 607–625.

Mechie, S.C., Chapman, P.A., and Siddons, C.A. 1997. A fifteen-month study of *Escherichia coli* O157:H7 in a dairy herd. *Epidemiology and Infection*. 118(1): 17-25.

Muela, A., Seco, C., Camafeita, E., Arana, I., Orruno, M., Lopez, J.A., and Barcina, I. 2008. Changes in *Escherichia coli* outer membrane subproteome under environmental conditions inducing the viable but nonculturable state. *FEMS Microbiology Ecology.* 64(1): 28-36.

Neu, T.R. and Marshall, K.C. 1990. Bacterial polymers: Physicochemical aspects of their interactions at interfaces. *Journal of Biomaterial Applications*. 5(2):107-133.

Nielsen, P.H. and Jahn, A. Extraction of EPS. In: Wingender, J., Neu, T.R., and Flemming, H-C. (eds.), Microbial extracellular polymeric substances: characterization, structure, and function, Springer-Verlag Berlin Heidelberg, Germnay, 1999. 49-73.

O'Connor, D.R. Report of the Walkerton inquiry part 1: the events of May 2000 and related issues. Toronto: The Walkerton Inquiry, 2002:1–504.

Oda, M., Morita, M., Unno, H., and Tanji, Y. 2004. Rapid detection of *Escherichia coli* O157:H7 by using green fluorescent protein-labeled PP01 bacteriophage. *Applied and Environmental Microbiology*. 70(1): 527-534.

Oliver, J.D. Formation of viable but nonculturable cells. In: Kjelleberg, S. (ed.) Starvation in Bacteria, Plenum Press, New York, 1993; pp. 239-272.

Oliver, J.D. and Bockian, R. 1995. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. *Applied and Environmental Microbiology*. 61(7): 2620-2623.

Oliver, J.D. and Day, A.P. 2004. Changes in membrane fatty acid composition during entry of *Vibrio vulnificus* into the viable but nonculturable state. *The Journal of Microbiology*. 42(2): 69-73.

Oliver, J.D. 2005. The viable but nonculturable state in bacteria. *The Journal of Microbiology*. 43: 93-100.

Ordax, M., Marco-Noales, E., López, M.M., and Biosca, E.G. 2006. Survival strategy of *Erwinia amylovora* against copper: Induction of the viable-but-nonculturable state. *Applied and Environmental Microbiology*. 72(5): 3482-3488.

Pruzzo, C., Tarsi, R., del Mar Lleò, M., Signoretto, C., Zampini, M., Pane., L., Colwell., R.R., and Canepari, P. 2003. Persistence of adhesive properties in *Vibrio cholerae* after long-term exposure to sea water. *Environmental Microbiology*. 5(10): 50-858.

Rabinowitz, R.P. and Donnenberg, M.S. *Escherichia coli*. In Paradise, L.J., Bendinelli, M., and Friedman, H., (eds.) Enteric Infections and Immunity, Plenum Publishing Corporation: New York, 1996; pp. 101-121.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., and Swerdlow, D.L. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*. 11(4): 603-609.

Raszka, A., Chorvatova, M., and Wanner, J. 2006. The role and significance of extracellular polymers in activated sludge. Part I: Literature review. *Acta hydrochimica et hydrobiologica*. 34(5): 411-424.

Rigsbee, W., Simspon, L.M., and Oliver, J.D. 1997. Detection of the viable but nonculturable state in *Escherichia coli* O157:H7. *Journal of Food Safety*. 16(4): 255-262.

Riley, L. W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine*. 308(12): 681–685.

Roberson, E.B. and Firestone, M.K. 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Applied and Environmental Microbiology*. 58(4): 1284-1291.

Rosenberg, M., Gutnick, D., and Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology letters*. 9(1): 29-33.

Ryu, J-H. and Beuchat, L.R. 2005. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: Effect of exopolysaccharide and curli production on its resistance to chlorine. *Applied and Environmental Microbiology*. 71(1): 247-254.

Signoretto, C., del Mar Lleo`, M., Tafi, M.C., and Canepari, P. 2000. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Applied and Environmental Microbiology*. 66(5): 1953-1959.

Signoretto, C., del Mar Lleo`, M., and Canepari, P. 2002. Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. *Current Microbiology*. 44(2): 125-131.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS: Microbiology Letters*. 223(2): 287-292.

Uberos, J., Augustin, C., Leibana, J., and Munoz-Hoyos, A. 2001. Comparative study of the influence of melatonin and vitamin E on the surface characteristics of *Escherichia coli*. *Letters in Applied Microbiology*. 32(5): 303-306.

Vinten, A.J.A., Lewis, D.R., Fenlon, D.R., Leach, K.A., Howard, R., Svoboda, I., and Ogden, I. 2002. Fate of *Escherichia coli* and *Escherichia coli* O157 in soils and drainage water following cattle slurry application at 3 sites in southern Scotland. *Soil Use and Management*. 18(3): 223-231.

Wang, Z., Liu, L.L., Yao, J., and Cai, W. 2006. Effects of extracellular polymeric substances on aerobic granulation in sequencing batch reactors. *Chemosphere*. 63(10): 1728-1735.

Wang, S., Deng, K., Zaremba, S., Deng, X., Lin, C., Wang, Q., Tortorello, M.L., and Zhang, W. 2009. Transcriptomic Response of *Escherichia coli* O157:H7 to Oxidative Stress. *Applied and Environmental Microbiology*. 75(19): 6110-6123.

Weiner, R., Langille, S., and Quintero, E. 1995. Structure, function and immunochemistry of bacterial exopolysaccharides. *Journal of Industrial Microbiology*. 15(4): 339-346.

Weiss, T.H., Mills, A.L., Hornberger, G.M., and Herman, J.S. 1995. Effect of bacterial cell shape on transport of bacteria in porous media. *Environmental Science and Technology*. 29(7): 1737-1740.

Wong, H.C. and Wang, P. 2004. Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of Applied Microbiology*. 96(2): 359-366.

Yousef, A.E. and Carlstrom, C. Food Microbiology: A Laboratory Manual. Hoboken, New Jersey. John Wiley & Sons, Inc. 2003.

Zhang, X. S., Garcia-Contreras, R., and Wood, T.K. 2007. YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. *Journal of bacteriology*. 189(8): 3051–3062.

3. Transport of culturable and VBNC *E.coli* O157:H7 cells through columns packed with washed or unwashed sand

3.1. Introduction

Escherichia coli O157:H7 is a pathogenic bacterium which can cause serious disease symptoms in humans following ingestion. Symptoms can manifest within hours of and up to 10 days following ingestion of the bacteria, and can range from severe abdominal cramping and bloody diarrhea (hemorrhagic colitis) to hemolytic uremic syndrome, an unusual type of kidney failure which can ultimately prove fatal. Though detrimental in humans, this bacterium is able to form commensal relationships within the intestinal tract of cattle, poultry, and other animals (Canadian Food Inspection Agency, 2009). The preference of *E.coli* O157:H7 to inhabit the intestinal tract of animal hosts, however, exposes the bacteria to risk of excretion. Epidemiologic studies that have been conducted throughout Canada and the USA have shown that a significant proportion of cattle herds contain individuals which are actively shedding *E.coli* O157:H7 in their feces (Faith et al., 1996; Shere et al., 1998; Laegreid et al., 1999; Van Donkersgoed et al., 1999; Galland et al., 2001; Dodd et al., 2003; Vidovic and Korber, 2006).

The application of animal manures to soil as crop fertilizers is an important means for recycling the nitrogen and phosphorus which the manures contain (Gagliardi and Karns, 2000). In scenarios where livestock density is very high, such as for feedlots, most manure is land applied close to source at high application rates for economical reasons. This diminishes its traditional role as a

soil amendment and it is often viewed as a disposal problem rather than the utilization of a valuable nutrient source. This high density of manure application can ultimately lead to the degradation of soil and water resources (Larney and Hao, 2007).

Animal manure application to agricultural land, though widely practiced, has been cited as a major source of pathogenic microorganisms in surface and groundwater systems (Reddy et al., 1981; Jamieson et al., 2002). Land application of contaminated manure may result in the infiltration of the pathogenic organisms into the subsurface and ultimately lead to a public health risk if drinking water supplies become contaminated (Unc and Goss, 2004). For instance, in May 2000, Walkerton, Ontario, Canada's drinking water system became contaminated with deadly bacteria, primarily *Escherichia coli* O157:H7, following the land-application of contaminated manure. Despite the fact that the manure had been applied using accepted farm practices, the bacteria were able to move through the soil profile and into one of the towns drinking water wells, ultimately leading to the deaths of 7 people and causing illness in an additional 2300 (O'Connor, 2002). Contamination of drinking water sources with *E.coli* O157:H7 have been documented throughout the developed world including Canada (Jackson et al., 1998; Hrudey et al., 2003), the United States of America (Swerdlow et al., 1992; Olsen et al., 2002), and Scotland (Licence et al., 2001). The common link between the majority of these case studies was human infection through the consumption of untreated water from a private well or from a well which had been improperly treated. Due to the health risks associated with

human infection, it becomes imperative that protocols be established utilizing detection methods that seek out sources of contamination accurately and efficiently so preventative measures may be taken to avert future outbreaks.

Detection of *E.coli* O157:H7 in the environment, however, may not always be as simple as a straight-forward culture test on standard media. Recent microbiological studies have demonstrated that *E.coli* O157:H7 may enter a viable but non-culturable state (VBNC) when exposed to sublethal stresses (Oliver, 2005) such as elevated or reduced temperatures, elevated or reduced osmotic concentrations, nutrient starvation, levels of oxygen (Oliver, 1993) or exposure to chloramines (Liu, 2008). In the stress-induced VBNC state, the cells are still metabolically active, but not reproductively active; that is, they do not undergo cellular division on conventional media and, therefore, will not produce colonies (Rigsbee et al., 1997). This behavior increases the likelihood of false negative tests which has important implications for public health since the number of viable *E.coli* O157:H7 bacteria may be systematically underestimated by traditional culture-based methods.

Numerous studies have been published detailing the transport of culturable *E.coli* O157:H7 in varying soil systems under both laboratory and field conditions (e.g. Gagliardi and Karns, 2000; Ogden et al., 2001; Vinten et al., 2002; Bradford et al., 2006; Semenov et al., 2009). However, to date, the transport characteristics of VBNC *E.coli* O157:H7 cells in these systems has not been examined. Studies have shown that bacterial cells entering the VBNC state undergo morphological changes. For instance, Oda et al. (2004) noted *E.coli* O157:H7 cells transition

from a rod to a coccoid-shape; Signoretto et al. (2000) found that *Enterococcus faecalis* VBNC cells were twice as resistant to mechanical disruption as exponentially growing, stationary, or UV-killed cells; and Lleo et al. (2007) showed that some species of VBNC enterococci exhibit decreased adhesion-ability compared to actively growing cells and were incapable of forming biofilms completely, differences which they attributed to reduced metabolism and altered surface characteristics resultant from the VBNC state transition. In addition, our own findings suggest *E.coli* O157:H7 cells induced into a VBNC state undergo a decrease in their relative hydrophobicity, an increase in tightly-bound surface protein and sugar expression, as well as miniaturization and shortening to a coccus-like form, all in relation to their exponentially-growing, culturable form (Section 2.3.). These findings are important as the morphological and surface characteristics of bacterial cells have been shown to strongly influence their transport and adhesive properties. Characteristics such as length and width (Gannon et al., 1991; Bradford et al., 2002; Bradford et al., 2006), surface charge (Bolster et al., 2006; Jacobs et al., 2007), hydrophobicity (Williams and Fletcher, 1996; DeFlaun et al., 1999), and cell-surface coating (DeFlaun et al., 1990; Rijnaarts et al., 1996; Williams and Fletcher, 1996; Simoni et al., 1998) have all exhibited influence over bacterial attachment and transport in soil systems.

Past studies have utilized bacterial breakthrough curves (BTCs), derived from either field (Pang et al., 1998) or laboratory (i.e. Mallants et al., 1994) experiments, along with time moment analysis to estimate bacterial transport parameters through porous media. Valocchi (1985) described time moments as

being physically meaningful descriptors of the mass concentration breakthrough of a transport assay. Time moments, for instance, can be used to gauge the mass flux, the mean travel time, and the degree of spreading (dispersion) of a bacterial breakthrough curve. Given the findings that many species of bacteria are capable of undergoing morphological changes upon entering the VBNC state, it becomes important to determine whether the transport properties of *E.coli* O157:H7 within soil and contaminated groundwater are also altered upon entering a VBNC state**.** Therefore, the objective of this study is to use packed porous medium column transport experiments to quantify transport properties of these two cellular states. In particular, cumulative mass flux will be measured in a controlled laboratory setting and time moment analysis applied to the resulting breakthrough curves to elucidate any potential transport differences which may exist when *E.coli* O157:H7 cells transition from a culturable to a VBNC state. The results from this research will contribute to the development of better water quality indicators and assessments of risk for contamination of water resources.

3.2. Methods

3.2.1. Culturable *E.coli* O157:H7 preparation

E.coli O157:H7 (strain ADRI V241) stock culture (kept at -80°C) was initially streaked onto a Trypticase soy agar (TSA) plate (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37°C. After 24 hours, a single colony was used to streak a second TSA plate. Following incubation (37°C, 24 h) of this second streaked plate, a single colony was utilized to inoculate 1 mL of Trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes,

NJ) which was then incubated overnight (12-14 h, 37°C, 125 rpm). The following morning, a fresh culture was produced by inoculating the overnight culture into TSB at a 50:1 (vol. /vol.) ratio. This fresh culture was then incubated (37°C, 125 rpm, 3.5 h).

Following incubation, *E.coli* O157:H7 cells in the fresh culture were harvested using a Micromax RF centrifuge (Thermo IEC, Waltham, MA) at 4000 rpm for 25 min. Following harvesting, the cell pellet was washed three times in sterile, deionized water in order to remove any remaining growth medium using identical centrifuge settings as harvesting. After the final wash, the culturable cell pellet was resuspended in 0.85% NaCl and incubated at 4°C until needed.

3.2.2. VBNC *E.coli* O157:H7 preparation

The novel method utilized to prepare *E.coli* O157:H7 cells in the VBNC state was derived from Liu et al. (2009) and is described in detail in Section 2.

Briefly, *E.coli* O157:H7 cells were initially prepared as described for culturable *E.coli* O157:H7 cells (Section 3.2.1.). Following the final wash, the supernatant was removed and the cells were resuspended in 30 mL of 4ºC filter-sterilized chloraminated tap water (Edmonton, Alberta) with a concentration ranging from 1.5-2.0 mg /L total chloramine as determined by amperometric back titration using an Autocat 9000 Chlorine Amperometric Titrator (Hach, Loveland, CO). The cell suspensions were incubated at 4ºC for 30 min. (As a side note, it was often necessary, particularly during colder months, to allow tap water samples to incubate at room temperature (up to 24 h) in Erlenmeyer flasks prior to

filtration in order to decrease the total chloramine concentrations to within a usable range via volatilization).

Following induction into the VBNC state, the cells were washed three times using deionized water, utilizing an identical protocol as seen prior to induction, in order to remove any lingering tap water which may affect cell viability. Following the final wash, the cell pellet was resuspended in 0.85% NaCl and a culturability test performed on the cell suspension. This test was performed by removing a 250 µl aliquot from the VBNC cell suspension and placing it into an empty, sterile plate. Warm TSA was then poured into the plate, allowed to solidify, then incubated overnight at 37°C. The remaining stock VBNC cell suspension was incubated at 4°C overnight. The following morning (~18 h later), the plates were viewed to see if any colony growth had taken place.

The viability test was completed using a Live/Dead BacLight bacterium viability kit (Molecular Probes Europe, Leiden, The Netherlands) as per the manufacturer's instructions. It uses SYTO 9 stain and propidium iodide to measure membrane integrity to distinguish live cells (intact cell membrane) from dead cells (lacking an intact cell membrane) (Liu et al. 2008). Briefly, a 50 µl aliquot of the VBNC cell suspension was added to 0.85% NaCl to a final volume of 1 mL. To this suspension, 2.5 µl of the propidium iodide and Syto 9 mixture was added. This was allowed to incubate at room temperature for 30 minutes. Following this time, 0.85% NaCl was added to a final volume of 2 mL. Samples stained with these reagents were filtered through 0.22-µm-pore-size nitrocellulose membrane filters (Millipore, Billerica, MA). An epifluorescence microscope

(Zeiss Axioskop 2 FS) along with a DAPI filter and a 100x objective was used to view live and dead cells. A series of 10-15 images was taken from each sample. The bacteria were then counted and calibrated to give the percent of living cells in the suspension a well as providing a count of the total number of cells in the suspension per milliliter. This procedure was found to induce >99.99% of *E.coli* O157:H7 cells at a density of $>10^8$ cells/mL into a VBNC state while retaining a mean cell viability of 86% (see Section 2).

3.2.3. Column transport experiments

Columns for the transport experiments were set up in a manner similar to Bolster et al. (2009).

3.2.3.1. Sand preparation

Sieved quartz sand was used for the transport experiments. The sieved quartz sand had a particle size range between 250 and 500 µm. Prior to use in the column, the sand was washed in boiling 1 M hydrochloric acid for 2 hours to remove metal oxides, then rinsed with deionized water until the rinse water pH reached a level very near the pH of the water prior to rinsing. The sand was then autoclaved and dried for 24 hours in an oven at 110°C. Unwashed, autoclaved sand was also utilized in transport experiments. Prior studies (e.g. Scholl and Harvey, 1992; Bolster et al., 2001) using acid-washed sand and metal-oxide coated sand particles have shown to have a significant influence on the transport characteristics of bacterial cells through soil columns. In both cases previously referenced, bacterial retention in columns utilizing metal-oxide coated sand particles was greater than columns packed with acid-washed sand. Previous work

(Section 2.3) has shown that phenotypical and surface chemical variations exist between culturable and VBNC *E.coli* O157:H7 cells. It is in our interests then to utilize columns packed with both sand types, either acid-washed or unwashed (presence of metal-oxides). This will help elucidate any transport and retention differences between culturable and VBNC *E.coli* O157:H7 cells due to possible adsorptive and surface interaction heterogeneities brought on by cell induction into a VBNC state. Utilizing both sand types, according to past studies, will provide an environment more supportive of bacterial mass flux (acid-washed sand) and one more conducive for cell-particle interaction and bacteria retention (unwashed sand).

3.2.3.2. Saturated conductivity determination

The average saturated hydraulic conductivity of the sand was 186.1 cm/h. The saturated hydraulic conductivity of the sand was determined by steady-state flow measurements in saturated cores under a constant hydraulic head gradient. First, cheese cloth was attached to the open bottom of an aluminum core (radius = 2.35 cm, height = 7.5cm) followed by the incremental packing of sand to a constant bulk density at a final height of 5 cm within the core. The sand-packed core was saturated from the bottom up through capillary action by submerging the bottom portion of the core in deionized water. This minimized any air bubbles from being trapped within the core, ensuring all pores were fully saturated. Next, the core was attached to a constant head water supply device (water trough) and water applied at a rate to maintain a constant 2 cm pond of water on the sand surface while the water was allowed to pass through the column. Saturated

conductivity was determined by measuring the mass of water that passed through the column per unit time and subsequently employing Darcy's flow equation [3.1]

$$q = K_s * (\Delta H_T/\Delta z) \qquad [3.1]$$

where q ($L^3 L^{-2} t^{-1}$) is the volumetric flux of water per unit area per unit time leaving the column, K_s ($L\ t^{-1}$) is the saturated hydraulic conductivity of the soil, ΔH_T (L) is the difference in total soil water potential at the top and bottom of the column, and Δz (L) is the height of soil in the column. This scenario was repeated for ten trials of differing length of time and run in duplicate to generate an average measure of the saturated conductivity for this sand.

Considering the high conductivity of the quartz sand, a horizontal column was used to control the magnitude of the flux of the flow solution through the columns. A vertical column would have resulted in very fast bacterial transport, preventing efficient sampling. By employing horizontal column assays, we were able to maintain saturated conditions and reduce the solution flux to a desired rate to enhance bacterial interaction with the matrix and extend the time interval so that reliable samples could be taken.

3.2.3.3. Column setup

Acrylic cores (Soil Measurement Systems, Tucson, USA) used for the transport assays were 2.5 cm in diameter and either 5.0 cm or 10.0 cm in length for a total core volume of 24.5 cm^3 or 49.1 cm^3, respectively. One end of the core was covered by a 210 μm polypropylene mesh membrane filter (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) to allow flow through of solution while preventing sand escape when the column is in the vertical position

during initial saturation steps. The biologically-inert mesh chosen also discourages bacterial adsorption. Both ends of the columns were flanked by perforated brass plates which facilitates even dispersal of applied solution across the entire cross-sectional area of the sand. The ends were then sealed by acrylic plates with a single hole bored into the center of each to be used as either an inlet or an outlet. A schematic of the flow apparatus is shown in Image 3-1. The column is entirely sealed to prevent fluid leaking or outside contamination. The pump that feeds the flow solution, as well as the solution containing bacteria into the column was a syringe-type pump (PHD 2000 Programmable, Harvard Apparatus). For this setup, we utilized two 10 mL syringes feeding either the bacteria-free solution or the bacteria suspension into the column. The choice of source solution was facilitated by two T-valves located between the one way valve and the column for each syringe pathway. This allows fluid from one syringe to enter the column while fluid from the other syringe circulates back into its reservoir (Images 3-1 and 3-2). The valves allow for quick changes from bacteria-free solution to bacteria flow solution or vice versa, as well as eliminating any excess air that may exist in the lines prior to injection into the column. The fluid pathways were made of Tygon B-44-4X tubing (Fisher Scientific) which is also designed to prevent bacterial entrapment or adsorption (Saint-Gobain Performance Plastics, 2007). The acrylic core, tubing, syringes, and valves were sterilized prior to each use by either autoclaving or cleaning with 70% ethanol.

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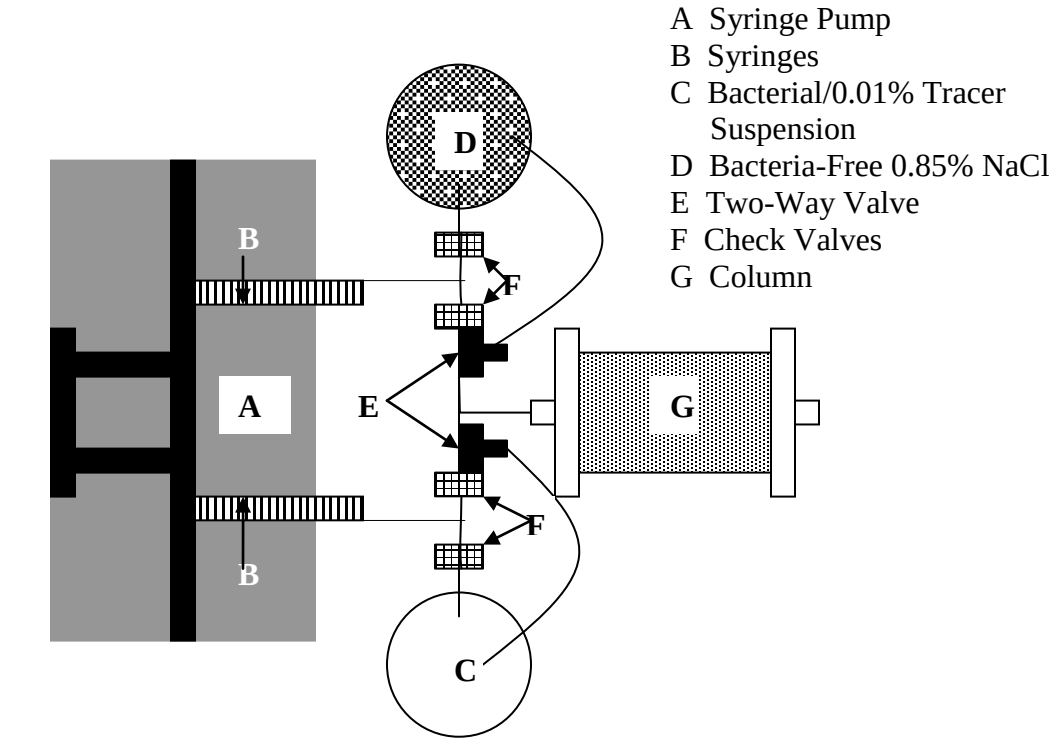


Image 3-1. Overhead schematic of column setup used in transport assays.

Image 3-2. Image of column and pump setup used in transport assays. Pictured is a 10 cm column containing washed sand.

3.2.3.4. Column packing

Initially, a column was wet packed using deionized water with the column orientated in a vertical direction. This was done by adding sand in increments, saturating the sand using deionized water, stirring the sand to release trapped air, and finally, packing. The sand was added to the top of the column and then the end plate was attached. The final packed average bulk density of the columns was determined to be 1.67 (0.02) g/cm^3 and the estimated average porosity of the sand was determined to be 0.37 (0.08) cm^3/ cm^3, considering a particle density of 2.65 g/ cm^3. Pore volumes of 9.04 cm^3 and 18.30 cm^3 were calculated for the 5 cm and 10 cm columns, respectively. Column packing was performed in a biosafety cabinet to maintain sterility of sand pack.

3.2.3.5. Transport of 0.01% NaCl, VBNC, and culturable *E.coli* O157:H7

From previous experimentation, it was determined that an ideal flow solution to maintain VBNC viability was 0.85% NaCl (Section 2.3.). Therefore this flow solution was applied to the column via the bottom inlet using the syringe pump set at a rate of 0.89 (0.004) mL/min. This method of saturation allowed for any air within the pores to escape and be replaced by the flow solution. Following saturation, the column was re-orientated in a horizontal position and flow solution was added (5 pore volumes) to obtain steady-state flow within the sand pack at a rate of 0.89 (0.004) mL/min.

Once steady-state flow was achieved in each column using 0.85% NaCl, a pulse of reduced concentration NaCl (0.01%) was added for 1.0 pore volume while maintaining flow at a rate of 0.89 (0.004) mL/min. The 0.01% NaCl pulse

was added as a conservative tracer to characterize the transport characteristics of the sand flow column. After pulse application, the effluent from the column was sampled in two minute increments for 3 pore volumes. The electrical conductivity, EC, of each sample was then analyzed using an Orion 5-Star pH/RDO/Conductivity Portable Multiparameter Meter (ThermoScientific) coupled with an Orion DuraProbe 4-Electrode Conductivity Cells probe (ThermoScientific). The EC data was used to construct a breakthrough curve of the conservative tracer to determine the transport characteristics of the sand. For each flow column/experiment the conservative pulse was carried out and the graphs compared to determine the consistency of flow column set-up and sand packing.

Following the conservative tracer application, bacteria-free flow solution (0.85% NaCl) was pumped through the sand pack at a rate of 0.89 (0.004) mL/min for a total of 5 pore volumes to re-establish consistent pore water concentration. Next, the flow of 0.85% NaCl was diverted from the column and a pulse of 0.85% NaCl flow solution containing either VBNC or culturable *E.coli* O157:H7 was added for 1 pore volume. The flow solution with bacteria had an optical density of approximately 0.225 at 546 nm (for the 5.0 cm column) or approximately 0.2 at 546 nm (for the 10.0 cm column) and was applied at the steady-state flow rate of 0.89 (0.004) mL/min. These optical densities are approximately equivalent to a concentration of 10^7-10^8 cells/mL. After bacterial pulse applications, sampling of effluent occurred in two minute increments beginning two minutes after the bacterial pulse application and continued for 5

pore volumes. Following sampling, 1 mL aliquots from each effluent sample were used to determine the optical density of the sample at 546 nm using a Lambda 35 UV/Vis spectrometer (PerkinElmers, Waltham, MA). These results were then related to the original optical density of the flow solution (C/Co) and graphed in relation to time. A standard curve was developed to ensure that a linear relationship exists between optical density and cell concentrations used in the column assays (Appendix C). This was done by preparing three suspensions of culturable *E.coli* O157:H7 cells in an identical manner as previously discussed (Section 3.2.1.) and the optical densities of the suspensions adjusted to approximately 0.220. Sample dilutions of the suspensions were then prepared and ranged from zero dilution (pure culture) and incrementally increased linearly to complete dilution (pure 0.85% NaCl). The optical densities of the samples were determined using the spectrophotometer and subsequently graphed. Following analysis, the adjusted R^2-value was found to be 0.999 indicating a strong linear relationship between cell concentration and dilution within the approximate range of cell densities utilized in the transport assays thereby validating the cell quantity detection method.

Column experiments were run in triplicate for each cell state in the 5 cm columns (Appendix D, E) using washed sand, five runs were done for each cell state in the 10 cm columns using washed sand (Appendix F, G), and two runs were completed for each cell state in the 10 cm columns using unwashed sand (Appendix H, I).

3.2.3.6. 0.01% NaCl tracer travel time determination

Originally described by Aris (1958), time moment analysis is a statistical technique for characterizing breakthrough curves without bias towards a specific transport model (Skopp, 1985; Lennartz and Kamra, 1998). In time moment analysis, hydrodynamic properties of the system are investigated using time moments calculated from BTCs. The zeroth time moment of a BTC is a measure of the tracer mass recovered from the system; the first time moment is a measure of the average travel time through the system; and the second time moment is a measure of the variance about the mean travel time (Brooks and Wise, 2005). For the purposes of this study, the zeroth and first moments of the collected conservative tracer breakthrough curves were determined. First, equation [3.2] was used to estimate the area underneath each breakthrough curve (Englert et al., 2009)

$$t_o(t) = \int_0^t C(L,t) * dt \qquad [3.2]$$

where t_o is a measure of the area underneath each breakthrough curve up to time (t) (min) and C(L, t) (M L^{-3}) is the tracer concentration in the effluent collected at time (t) from the column (L) as measured spectrophotometrically.

Once the area beneath the tracer breakthrough curves is determined, it is possible to apply an accumulated mass concept (Harvey and Gorelick, 1995), which is an integrated measure of mass flux over time at an initial location. Using this approach, the accumulated mass which has exited the column within a sampling interval can be calculated by taking the product of the zeroth temporal moment beneath the breakthrough curve and the applied volumetric flow rate of the pore solution within the column (Englert et al., 2009), creating equation [3.3]

$$M_o(L, t) = \int_0^t q * C(L,t) * dt \qquad [3.3]$$

where M_o ($M\ L^{-2}$) equals the total mass flux per pore area of bacteria eluted from the column (L) up to time t, q refers to the volumetric flow rate ($L^3\ L^{-2}\ t^{-1}$) of the flow solution within the column, and C(L, t) ($M\ L^{-3}$) is the concentration of conservative tracer in the effluent collected at time (t) from the column (L) as measured spectrophotometrically. Equation [3.3] was used to calculate the mass recovery percentage of 0.01% NaCl from the 5 and 10 cm columns.

Finally, to calculate the mean residence time (Berglund and Cvetkovic, 1998) of the conservative tracer in the 5 and 10 cm columns, the first moment is calculated using equation [3.4]

$$E[t] = \frac{\int_0^\infty t * q * C(L,t) * dt}{M_T\ (L)} \qquad [3.4]$$

where M_T ($M\ L^{-2}$) equals the total mass per pore area of bacteria eluted from the column (L) over all sampling intervals, q refers to the volumetric flow rate ($L^3\ L^{-2}\ t^{-1}$) of the flow solution within the column, C(L, t) ($M\ L^{-3}$) is the concentration of conservative tracer in the effluent collected at time (t) from the column (L) as measured spectrophotometrically, and E[t] is the mean residence time (t) of the conservative tracer in the columns.

Using the first temporal moment, a mean residence time of 19.10 (0.4) minutes and 33.52 (0.6) minutes was calculated for the 5 and 10 cm columns, respectively.

To further characterize the conservative pulse, we can estimate the convective velocity of the 0.01% NaCl through the 5 and 10 cm columns using equation [3.5]

$$V = \frac{q}{\theta} \qquad [3.5]$$

where V ($L\ t^{-1}$) is equal to the estimated velocity of the conservative pulse through the column, q is the applied volumetric flow rate ($L^3\ L^{-2}\ t^{-1}$), and θ is the volumetric water content ($L^3\ L^{-3}$). Given the estimated convection velocity of the 0.01% NaCl pulse, the estimated travel time can be calculated using equation [3.6]

$$t = \frac{x*\theta}{q} \qquad [3.6]$$

where t is equal to the estimated travel time of the conservative pulse through the column (t), x is the length of the column (L), θ is the volumetric water content ($L^3\ L^{-3}$), and q is the applied volumetric flow rate ($L^3\ L^{-2}\ t^1$). Equation [3.6] simplifies to equation [3.7]

$$t = \frac{\text{pore volume}}{q} \qquad [3.7]$$

where t is equal to the estimated travel time of the conservative pulse through the column (t), pore volume is the volume of liquid-filled porous space in a packed column conducting water ($L^3\ L^{-2}$) per area, and q is the applied volumetric flow rate ($L^3\ L^{-2}\ t^1$).

When piston flow is considered, it is assumed that mean solute breakthrough will occur approximately 1.5 pore volumes following the pulse input (considering the 1 pore volume solute pulse input used in these assays).

When this consideration is applied to the results from equation [3.7], then the estimated mean travel times of the 0.01% NaCl conservative tracers through the 5 and 10 cm columns would be 15.24 and 30.84 minutes, respectively.

When the measured mean solute travel times from equation [3.4] are used to calculate the number of pore volumes required (equation [3.7]) for mean conservative tracer breakthrough seen in the column assays, pore volumes of 1.87 (0.05) and 1.62 (0.04) were required for the 5 and 10 cm columns, respectively. These values are slightly higher than the 1.5 pore volumes expected in both cases. Reasons for this could be retardation of the tracer, errors in calculating the first time moments of the conservative tracer breakthrough curves, errors in estimating porosity (which assumes a particle density of 2.65 g cm^{-3}), and the extra distance the tracer must travel through the tubing and core end caps.

The basis behind characterizing the transport of the conservative tracer pulses and comparing with estimated values is to ensure that column packing was consistent and there is no by-passing of pore space. The low standard deviations from the measured mean solute travel times and measured pore volumes for both column lengths, along with the relatively small differences observed between expected mean solute breakthrough pore volumes and measured values indicates columns were consistently packed and preferential flow is unlikely.

3.2.3.7. Bacterial mass flux determination

Similar to the conservative tracer characterization, determining the mass flux of the culturable and VBNC cells through the 5 and 10 cm columns began by determining the zeroth temporal moment for the respective breakthrough curves

using equation [3.2]. Once the area beneath the tracer breakthrough curves was determined, the accumulated mass concept (Harvey and Gorelick, 1995) could be applied using equation [3.3] to determine cumulative mass flux and percent recovery of bacteria from 5 and 10 cm columns.

These equations ([3.2], [3.3]) were used to quantify and compare culturable and VBNC *E.coli* O157:H7 mass transport under varying conditions through a direct numerical integration technique (Brooks and Wise, 2005) using the trapezoidal rule by interpreting the area underneath each breakthrough curve, within specific sampling time intervals. The first temporal moment, or mean residence time, of the bacterial pulses was not determined because of the tailing seen in the BTCs. This tailing acts to skew the mean travel times making them an ineffective representation of the actual travel time of the primary VBNC and culturable *E.coli* O157:H7 breakthrough pulses.

Once the cumulative mass flux data was determined (equation [3.3]) for each cell type using the various sand-column combinations, the results were plotted against time. Next, this data was transformed by taking the natural logarithm of each set of data points using SigmaPlot v.11 (Systat Software, Inc., San Jose, CA, USA). Using this software program, curves were fit to each set of data and 95% confidence and prediction bands applied to the fitted curves using the standard errors of the fitted function parameters. The SigmaPlot v.11 software program defines confidence bands as the region of uncertainties in the predicted values over a range of values for the independent variable, while prediction band refers to the region of uncertainties in predicting the response for a single

additional observation at each point within a range of independent variable values Using these fitted curves and bands, it was possible to compare the transport of VBNC and culturable *E.coli* O157:H7 cells through a common sand type and column length and say, with 95% confidence, whether or not the cumulative mass flux of the cells is different by looking for overlapping confidence and prediction band intervals. Additionally, if differences were suspected, linear portions of the raw data curves were fitted and slopes compared to determine variations in proportional mean mass accumulation.

3.2.4. Statistical Analysis

Student's T-tests and Multi-factor ANOVA were used to elucidate any statistically significant differences between masses of bacteria transported through 5 cm and 10 cm columns, respectively. These statistical analyses were conducted using R statistical software (R Development Core Team; Vienna, Austria) and differences were considered significant at $p<0.05$. Standard errors, adjusted R^2 values, and slope parameters of cumulative mass flux fitted curves were determined using SigmaPlot (Systat Software, Inc., San Jose, CA, USA). Significance of slopes ($p<0.05$) was determined using Student's T-tests in SigmaPlot.

3.3. Results

3.3.1. 5 cm columns

Initially, the transport of VBNC and culturable *E.coli* O157:H7 cells through acid-washed sand packed in 5 cm columns was measured. These preliminary transport assays were completed in order to ensure that bacterial

breakthrough was occurring under the applied conditions as well as to validate the sensitivity of our detection method used to measure the concentration of bacteria cells in an effluent sample. The average breakthrough curves for each tracer applied to the 5 cm column—VBNC *E.coli* O157:H7 cells, culturable *E.coli* O157:H7 cells, and a conservative NaCl tracer are given in Figure 3-1. The breakthrough curves are depicted as the concentration of tracer (bacterial or conservative) in the effluent sample in relation to the original input concentration (C/C_o) as a function of time. The NaCl tracer curve was included as a visual aid to estimate and compare the travel times and mass transport capabilities of the bacterial tracers with a known conservative tracer. Since the concentration of the 0.01% NaCl tracer was less than the 0.85% NaCl flow solution, it was necessary to represent the points of the tracer breakthrough curves as ($1-C/C_o$).

All three breakthrough curves exhibit a slight log normal distribution shape indicated by the shorter time period to go from baseline concentration to peak concentration than from peak concentration back to baseline concentration. Both the VBNC and culturable *E.coli* O157:H7 breakthrough curves begin to emerge in the effluent earlier, at the 3 to 4 minute mark, versus the conservative tracer which first emerges at the 9 minute mark. This was likely due to background detection error using the spectrophotometer while detecting cells at early sampling times. Following initial detection, the concentration of the conservative tracer in the effluent increases more quickly and to a greater maximum than both states of *E.coli* O157:H7. The maximum C/C_o reached by the NaCl tracer is 0.85 at a time of 18 min while the maximum reached by both

the VBNC and culturable cells is 0.19 at a time of 17 min. The conservative tracer then took longer to decrease to baseline levels than the bacterial tracers. When exploring the breakthrough curves of the VBNC and culturable *E.coli* O157:H7 cells, they do not appear to be considerably different. Overall, the transport of the culturable and VBNC cells through the column was similar with both initially appearing in the effluent at a similar time point, both peaked at approximately the same time and at the same concentration, and both decreased to nearly baseline levels in the tail-end of their respective curves. One noticeable difference, however, occurs during the decrease to baseline levels where the concentration of VBNC *E.coli* O157:H7 cells in effluent samples decreases at a greater rate than their culturable counterparts.

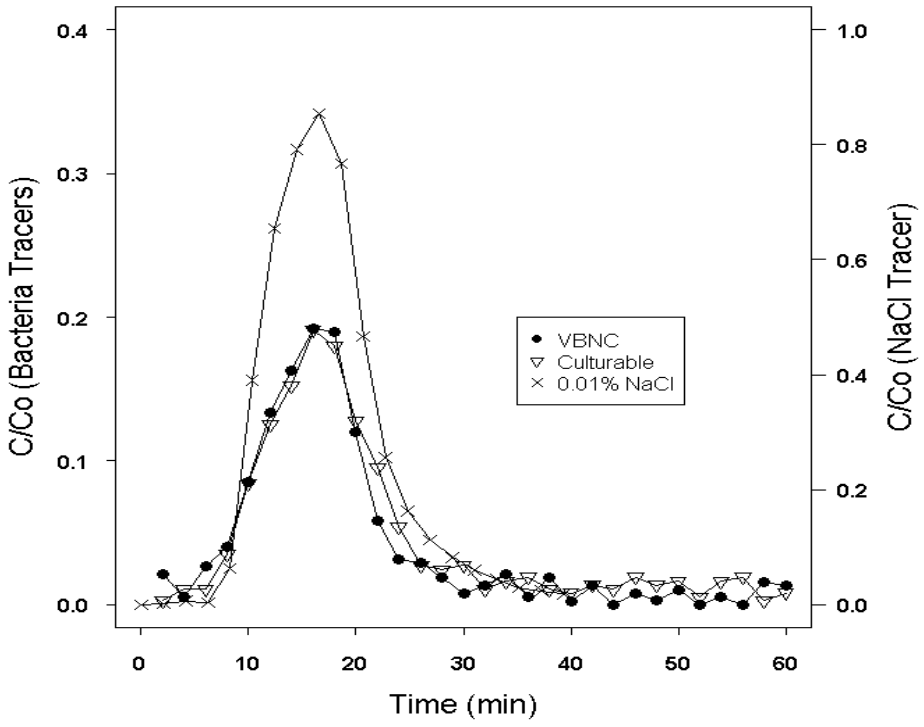


Figure 3-1. Observed breakthrough data for culturable and VBNC *E.coli* O157:H7 cells, as well as a conservative 0.01% NaCl tracer, using washed sand in a 5 cm column. The left y-axis is used to gauge bacteria breakthrough while the right y-axis is used to gauge conservative tracer breakthrough.

Using Equation [3.3], it was possible to determine the mean accumulated mass recovery of the VBNC and culturable E.coli O157:H7 tracers from the 5 cm column data (Table 3-1). It was decided to determine the mass recovery in sampling intervals based on the estimated mean travel time of the 0.01% NaCl tracer, which was found to be 15.24 min using equation [3.7]. Therefore, the sampling intervals were set to be approximately 1x the mean travel time of the conservative tracer (0-16 min), 2x the mean travel time of the conservative tracer (0-32 min), 3x the mean travel time of the conservative tracer (0-48 min), and the entire length of time column effluent was sampled (0-60 min). For the initial sampling period of 0-16 min, the VBNC *E.coli* O157:H7 cells had a slightly greater mean mass flux of 1.34 M L^{-2} compared with a mean mass flux of 1.12 M L^{-2} for the culturable *E.coli* O157:H7 cells. In the following sampling interval (0-32 min), these mean mass fluxes increased to 2.13 M L^{-2} and 2.11 M L^{-2} for the VBNC and culturable *E.coli* O157:H7 cells, respectively. The third sampling interval (0-48 min) showed a change with culturable cells showing a slightly higher mass recovery than VBNC cells, 2.33 M L^{-2} versus 2.29 M L^{-2}, respectively. The final sampling interval (0-60 min) continued this observation with the mean mass flux of the culturable cells slightly exceeding that of the VBNC cells with values of 2.46 M L^{-2} and 2.43 M L^{-2}, respectively. In all cases, it was determined that the mean mass flux of VBNC and culturable *E.coli* O157:H7 through the 5 cm column were not statistically ($p<0.05$) different.

Examining the ratio of cell mass input versus mass output detected, VBNC *E.coli* O157:H7 cells showed a total mass recovery of 26.9 % while the culturable

cells exhibited a total mass recovery of 27.0 % (Table 3-1). These values were found to be not significantly different ($p<0.05$). In comparison, the 0.01% NaCl conservative tracer had a total mass recovery of 96.7 % with a standard deviation of 1.6 %, slightly less than the expected 100% recovery.

Table 3-1. Mass recoveries for VBNC and culturable *E.coli* O157:H7 cells using washed sand in 5 cm columns (standard deviations are in parentheses)

	Mass Recovery ($M\ L^{-2}$)	
Sampling Interval (min)	**VBNC *E.coli* O157:H7**	**Culturable *E.coli* O157:H7**
0-16	1.34 (0.17) a	1.12 (0.14) a
0-32	2.13 (0.12) a	2.11 (0.29) a
0-48	2.29 (0.17) a	2.33 (0.25) a
0-60	2.43 (0.21) a	2.46 (0.35) a
Total Mass Recovery (%)	26.9 (2.4) a	27.0 (3.7) a

**Within each sampling interval, values with the same letter are not considered significantly different ($p<0.05$)

3.3.2. 10 cm columns

Transport assays through washed and unwashed sand were then completed for both culturable and VBNC *E.coli* O157:H7 cells using 10cm columns. Trials involving washed sand and unwashed sand were replicated five and two times, respectively, for both *E.*coli O157:H7 cell states. The 0.01% NaCl and *E.coli* O157:H7 breakthrough data were expressed in terms of the relative concentration. The average breakthrough curves are depicted in Figure 3-2 (washed sand) and 3-3 (unwashed sand).

From Figure 3-2, the shape of the conservative tracer curve appears symmetrical which differs from the 0.01% NaCl curve found using the 5 cm column (Figure 3-1) which demonstrated a slight log normal distribution; however, the maximum concentration of 0.95 using the longer column exceeds

what was reached using the shorter column. The maximum concentration was reached at 34 to 36 minutes which is approximately double the 18 minutes required for the conservative tracer to reach its breakthrough curve peak using the 5 cm column.

Examination of the curve constructed using culturable cell passage through washed sand displays a maximum concentration of 0.071 occurring at the 32nd minute followed by a steep decline. The tail of the culturable-washed curve remains at a concentration ranging from 0.006 to 0.03 following the initial decrease from the primary breakthrough causing the appearance of a log normal distribution. The VBNC *E.coli* O157:H7-washed sand curve displays a maximum concentration of 0.082 which occurs at the 30th minute. Similar to the culturable-washed curve, the tail of this curve continues to show breakthrough of VBNC cells ranging from 0.014 to 0.03 following the primary breakthrough.

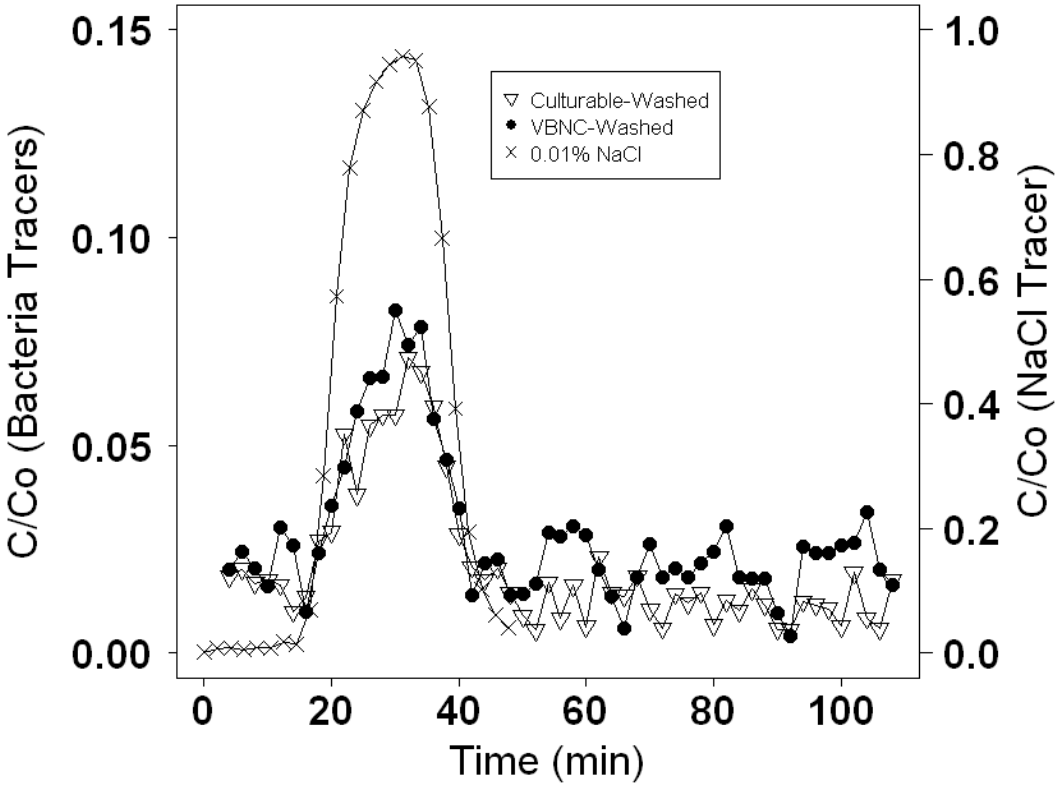


Figure 3-2. Observed breakthrough data for culturable and VBNC *E.coli* O157:H7 cells, as well as a conservative 0.01% NaCl tracer, using washed sand in a 10 cm column. The left y-axis is used to gauge bacteria breakthrough while the right y-axis is used to gauge conservative tracer breakthrough.

The average breakthrough curves derived from VBNC or culturable cell transport through unwashed sand exhibited breakthrough peaks which appear to occur concurrently with the conservative tracer (Figure 3-3). The breakthrough curve of culturable *E.coli* O157:H7 cells show a maximum breakthrough concentration of 0.025 occurring at a time of 32 minutes. The curve also displays an asymmetrical shape depicted by a gradual increase to the peak followed by a rapid decrease to baseline levels where the concentration readings fluctuate slightly for the remaining sampling times. The curve which represents the transport of VBNC *E.coli* O157:H7 cells through the 10 cm column packed with

unwashed sand has a primary peak of 0.033 which occurs at 26 to 30 minutes followed by second peak of 0.34 which occurs at the 38th minute. The overall shape of the curve is more normally distributed than the unwashed-culturable breakthrough curve. The tail of the culturable curve developed using unwashed sand appears to stay near the baseline following the primary breakthrough pulse; however, the VBNC curve shows frequent spikes above baseline levels between the 40th and 80th minutes. Interestingly, the unwashed VBNC *E.coli* O157:H7 breakthrough curve exhibits a large spike in the tail section at the 88th minute which was observed in both replicates.

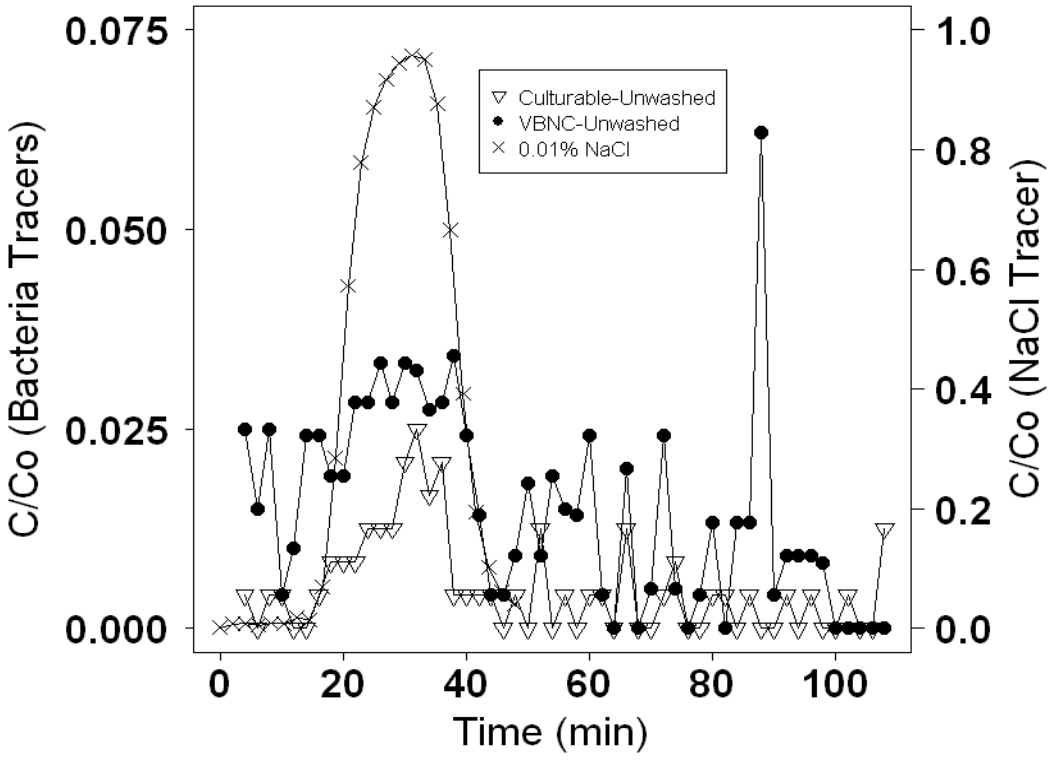


Figure 3-3. Observed breakthrough data for culturable and VBNC *E.coli* O157:H7 cells, as well as a conservative 0.01% NaCl tracer, using unwashed sand in a 10 cm column. The left y-axis is used to gauge bacteria breakthrough while the right y-axis is used to gauge conservative tracer breakthrough.

Overall, comparing Figures 3-2 and 3-3, the mass accumulation of VBNC and culturable *E.coli* O157:H7 cells in the effluent of columns packed with unwashed sand appears to be less than what is seen in columns packed with washed sand. This is suggested by the visibly smaller area beneath the individual unwashed sand breakthrough curves. However, observed breakthrough peaks appeared to occur at approximately the same time regardless of the sand type or the physiological state of the bacteria.

The mean accumulated mass in column effluents was also determined for the bacterial tracers transported through 10 cm columns packed with washed or unwashed sand (Table 3-2). Employing Multi-Factor ANOVA, it was determined that for all sampling intervals except 0-16 minutes, there was significantly ($p<0.05$) greater mass transport of either VBNC or culturable *E.coli* O157:H7 cells through washed sand when compared with the mass transport of culturable cells through unwashed sand. Though this finding was the only one deemed significant (Table 3-3), there were trends which seemed to develop. The most prominent one being consistently high levels of VBNC *E.coli* O157:H7 cell mass transport through a sand type, regardless of sampling interval, compared to culturable cells. In general, the data showed VBNC *E.coli* O157:H7 transport through washed sand to produce the greatest mass in the effluent, followed by culturable *E.coli* O157:H7 transport through washed sand, then VBNC *E.coli* O157:H7 transport through unwashed sand; finally, culturable VBNC *E.coli* O157:H7 transport through unwashed sand consistently demonstrated the least mass flux across all sampling intervals. This trend was apparent for all sampling

intervals except 0-16 min where there was greater mass flux of VBNC cells through unwashed sand than culturable cells through washed sand.

The greater relative concentrations seen in the tails of the washed sand BTC's (Figure 3-2) than in the unwashed sand BTC's (Figure 3-3) are also substantiated in Table 3-2. As an example, these "fatter" tails are apparent when the 0-48 min sampling interval for the two sand types is compared with their respective 0-108 min sampling interval. It can be seen that there is an overall greater mass flux of cells from 48 to 108 min for cells transported through washed sand, regardless of cell type, than through unwashed sand. The washed sand data showed that VBNC *E.coli* O157:H7 cells had greater mass flux through the 48-108 min period, a total of 1.12 M L^{-2} or 41% of the total effluent mass versus 0.63 M L^{-2} or 31% of the total culturable *E.coli* O157:H7 mass transported for the same time period. Through the unwashed sand, the VBNC cells had 0.53 M L^{-2} or 37% of their mean cumulative mass breakthrough during the 48 min to 108 min interval compared with 0.16 M L^{-2} or 33% of the mean cumulative mass for the culturable cells during the same time period. This cumulative mass flux data, in addition to demonstrating that cells have greater transport potential through washed sand, reveals that there is greater mass transport of VBNC cells during the 48 min to 108 min period than culturable cells, regardless of sand type.

The results also exposed that the variability in the transport of VBNC cells through the 10 cm columns, regardless of sand type or sampling interval, is greater than what was found for culturable *E.coli* O157:H7 cells as indicated by the consistently greater standard deviations.

When the total mass recovery of bacterial cells was compared against the total pulse mass inputs, VBNC *E.coli* O157:H7 cells showed a total mass recovery of 17.1 % and 9.2 % through washed and unwashed sand, respectively. Culturable *E.coli* O157:H7 cells exhibited a total mass recovery of 12.8 % and 3.1 %, through washed and unwashed sand, respectively (Table 3-2). The percent recovery of VBNC and culturable cells from columns packed with washed sand was found to be significantly greater ($p<0.05$) than the recovery of culturable cells from columns packed with unwashed sand. From the 10 cm column experiments, the 0.01% NaCl tracer exhibited a mean percent recovery of 98.1 % with a standard deviation of 2.3 %. This mean percent was slightly higher than what was seen in the 5 cm column assays, and very near the 100 % recovery theoretically-assumed for a conservative tracer.

Table 3-2. Mass recoveries for VBNC and culturable *E.coli* O157:H7 cells using unwashed and washed sand in 10 cm columns (standard deviations are in parentheses)

		Mass Recovery (M L^{-2})	
Sampling Interval (min)	**Sand Type**	**VBNC *E.coli* O157:H7**	**Culturable *E.coli* O157:H7**
0-16	Unwashed	0.27 (0.32) a	0.04 (0.02) a
	Washed	0.30 (0.15) a	0.23 (0.08) a
0-32	Unwashed	0.66 (0.56) a,b	0.23 (0.04) b
	Washed	1.10 (0.14) a	0.92 (0.08) a
0-48	Unwashed	0.92 (0.69) a,b	0.33 (0.13) b
	Washed	1.61 (0.25) a	1.40 (0.18) a
0-64	Unwashed	1.10 (0.81) a,b	0.38 (0.19) b
	Washed	1.93 (0.33) a	1.59 (0.26) a
0-80	Unwashed	1.23 (0.90) a,b	0.43 (0.18) b
	Washed	2.20 (0.45) a	1.76 (0.31) a
0-96	Unwashed	1.44 (0.85) a,b	0.46 (0.22) b
	Washed	2.47 (0.60) a	1.91 (0.32) a
0-108	Unwashed	1.45 (0.83) a,b	0.49 (0.22) b
	Washed	2.73 (0.68) a	2.03 (0.39) a
Total Mass Recovery (%)	Unwashed	9.2 (5.3) a,b	3.1 (1.4) b
	Washed	17.1 (4.6) a	12.8 (2.3) a

**Within each sampling interval, values with the same letter are not considered significantly different ($p<0.05$)

Table 3-3. Tukey comparisons and P-values for all treatment interactions in 10 cm column transport assays.

Sampling Interval (min)	Tukey Comparisons	P-value
0-16	Washed-Culturable:Unwashed-Culturable	0.443
	Unwashed-VBNC: Unwashed-Culturable	0.452
	Washed-VBNC: Unwashed-Culturable	0.226
	Unwashed-VBNC: Washed-Culturable	0.992
	Washed-VBNC: Washed-Culturable	0.901
	Washed-VBNC: Unwashed-VBNC	0.995
0-32	Washed-Culturable:Unwashed-Culturable	0.011
	Unwashed-VBNC: Unwashed-Culturable	0.220
	Washed-VBNC: Unwashed-Culturable	0.002
	Unwashed-VBNC: Washed-Culturable	0.476
	Washed-VBNC: Washed-Culturable	0.509
	Washed-VBNC: Unwashed-VBNC	0.107
0-48	Washed-Culturable:Unwashed-Culturable	0.007
	Unwashed-VBNC: Unwashed-Culturable	0.266
	Washed-VBNC: Unwashed-Culturable	0.002
	Unwashed-VBNC: Washed-Culturable	0.275
	Washed-VBNC: Washed-Culturable	0.683
	Washed-VBNC: Unwashed-VBNC	0.077
0-64	Washed-Culturable:Unwashed-Culturable	0.014
	Unwashed-VBNC: Unwashed-Culturable	0.275
	Washed-VBNC: Unwashed-Culturable	0.003
	Unwashed-VBNC: Washed-Culturable	0.434
	Washed-VBNC: Washed-Culturable	0.508
	Washed-VBNC: Unwashed-VBNC	0.094
0-80	Washed-Culturable:Unwashed-Culturable	0.024
	Unwashed-VBNC: Unwashed-Culturable	0.3458
	Washed-VBNC: Unwashed-Culturable	0.004
	Unwashed-VBNC: Washed-Culturable	0.519
	Washed-VBNC: Washed-Culturable	0.452
	Washed-VBNC: Unwashed-VBNC	0.107
0-96	Washed-Culturable:Unwashed-Culturable	0.029
	Unwashed-VBNC: Unwashed-Culturable	0.281
	Washed-VBNC: Unwashed-Culturable	0.004
	Unwashed-VBNC: Washed-Culturable	0.696
	Washed-VBNC: Washed-Culturable	0.369
	Washed-VBNC: Unwashed-VBNC	0.141
0-108	Washed-Culturable:Unwashed-Culturable	0.036
	Unwashed-VBNC: Unwashed-Culturable	0.370
	Washed-VBNC: Unwashed-Culturable	0.004
	Unwashed-VBNC: Washed-Culturable	0.629
	Washed-VBNC: Washed-Culturable	0.275

	Washed-VBNC: Unwashed-VBNC	0.091
Total Mass Recovery (%)	Washed-Culturable:Unwashed-Culturable	0.043
	Unwashed-VBNC: Unwashed-Culturable	0.400
	Washed-VBNC: Unwashed-Culturable	0.005
	Unwashed-VBNC: Washed-Culturable	0.647
	Washed-VBNC: Washed-Culturable	0.317
	Washed-VBNC: Unwashed-VBNC	0.108

When the natural logarithm of the mean cumulative mass flux data was plotted against time for VBNC and culturable cells transported through 5 cm columns packed with washed sand (Figure 3-4) it can be seen that there is an apparent overlap between the two curves, particularly during later sampling times. This is in accordance with what was seen in Figure 3-1 and Table 3-1. When the curves were fitted to an exponential function and 95% confidence and prediction bands applied to the fitted curves, it is apparent there is overlap of the bands. From Table 3-4, the fitted VBNC and culturable curves both had adjusted R^2-values of 0.99. The calculated exponential equation coefficients and coefficient standard errors are represented in Table 3-4.

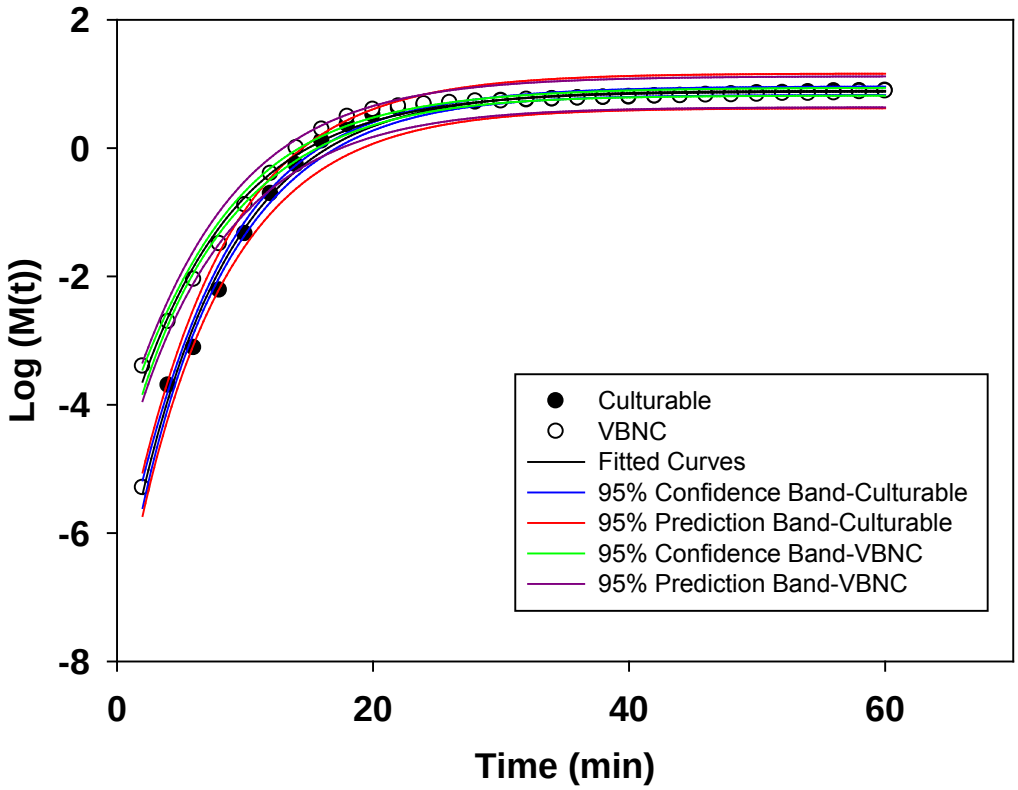


Figure 3-4. Log cumulative mass flux data and fitted exponential curves for culturable and VBNC *E.coli* O157:H7 cells using washed sand in a 5 cm column. Fitted curves are flanked by 95% confidence bands and 95% prediction bands. Based on equation f=y0+a*(1-exp(-b*x)).

From Figure 3-5, both the VBNC and culturable log data curves exhibit characteristics of an exponential rise to maximum function with the VBNC data appearing to have a greater mean mass accumulation throughout all sampling times. This is in agreement with the findings from Table 3-2. However, similar to what was apparent from Figure 3-4, when culturable and VBNC cells were passed through the 10 cm columns packed with washed sand, it is visible that there is high overlap of the fitted curve confidence and prediction bands. The fitted curve adjusted R^2-values, calculated exponential equation coefficients, and coefficient standard errors are represented in Table 3-4.

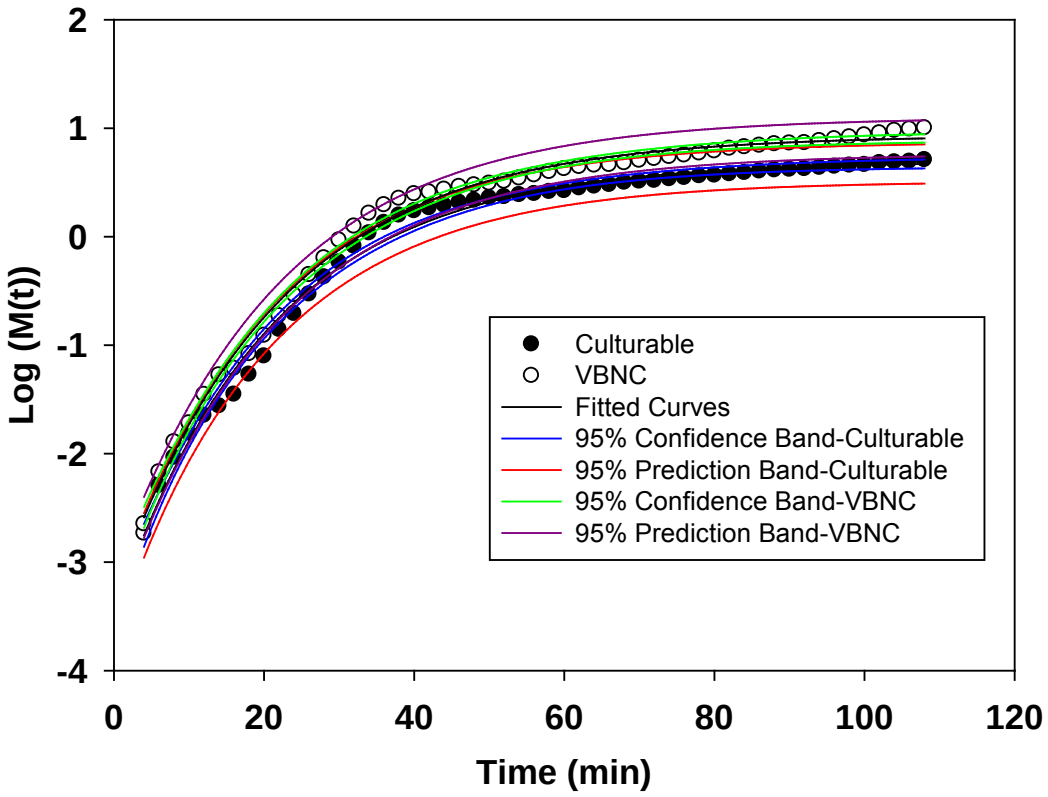


Figure 3-5. Log cumulative mass flux data and fitted exponential curves for culturable and VBNC *E.coli* O157:H7 cells using washed sand in a 10 cm column. Fitted curves are flanked by 95% confidence bands and 95% prediction bands. Based on equation f=y0+a*(1-exp(-b*x)).

The final set of mean log cumulative mass flux data (Figure 3-6) displays the transport of VBNC and culturable *E.coli* O157:H7 cells through 10 cm columns packed with unwashed sand. Similar to Figures 3-4 and 3-5, both log data curves exhibit an exponential rise to maximum shape. However, for this assay configuration, the raw data curves show VBNC mean cumulative mass flux as being greater than culturable mean mass flux throughout all sampling times. When curves are fitted to each set of data and confidence and prediction bands applied, there is no overlap of either band type. The fitted curve adjusted R^2-values were found to be 0.98 and 0.99 for the culturable and VBNC data,

respectively. The calculated exponential equation coefficients and coefficient standard errors are represented in Table 3-4.

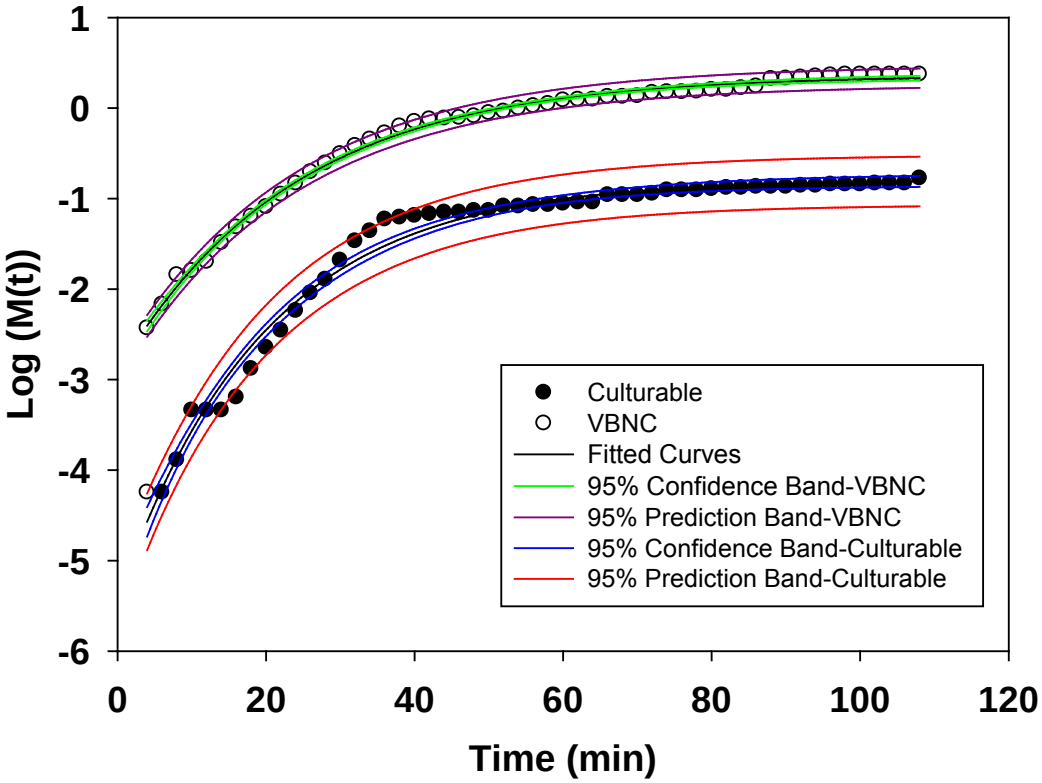


Figure 3-6. Log cumulative mass flux data and fitted exponential curves for culturable and VBNC *E.coli* O157:H7 cells using unwashed sand in a 10 cm column. Fitted curves are flanked by 95% confidence bands and 95% prediction bands. Based on equation f=y0+a*(1-exp(-b*x)).

Table 3-4. Parameters and adjusted R^2 -values from the non-linear regression of log cumulative mass flux data for culturable and VBNC *E.coli* O157:H7 cells used in the column transport assays based on the exponential rise to maximum equation $y=y_o+a*(1-e^{-b*x})$.

Column Length (cm)	Sand Type	Cell type	Parameters				
			Coefficients		Coefficient Standard Error		Adjusted R^2
			a	b	a	b	
5	Washed	Culturable	8.23	0.13	0.18	0.004	0.99
		VBNC	5.82	0.13	0.15	0.005	0.99
10	Washed	Culturable	4.18	0.05	0.07	0.002	0.99
		VBNC	4.24	0.05	0.07	0.001	0.99
10	Unwashed	Culturable	4.64	0.05	0.12	0.002	0.98
		VBNC	3.28	0.04	0.04	0.001	0.99

Applying linear regression to the linear portions (40-108 minutes) of the mean log cumulative mass flux data sets displayed in Figure 3-6, we arrive at Figure 3-7. From this figure it can be seen that the slope of the VBNC data is slightly greater than the culturable data. This finding is verified when linear curves are fit to the log transformed data. From Table 3-5, the adjusted R^2 and slope values of the fitted curve for the culturable data are 0.94 and 0.0058, respectively, and the adjusted R^2 and slope values for the VBNC data are 0.0082 and 0.97, respectively. These adjusted R^2-values suggest a strong goodness of fit between the measured culturable and VBNC data curves and their respective fitted model curves. In terms of the slope parameter, the higher coefficient value depicted by the VBNC fitted curve indicates a greater proportional increase in mean mass accumulation of the non-culturable cells in column effluent in comparison to culturable cells for the same sampling period. It was determined that the slopes of these two lines are significantly different ($p<0.05$) using a Student's T-test. This finding is in agreement with what was seen in Table 3-2

where the mean cumulative mass flux and percent recovery from the 48 min to 108 min sampling period was found to be greater for the VBNC trials compared to the culturable trials.

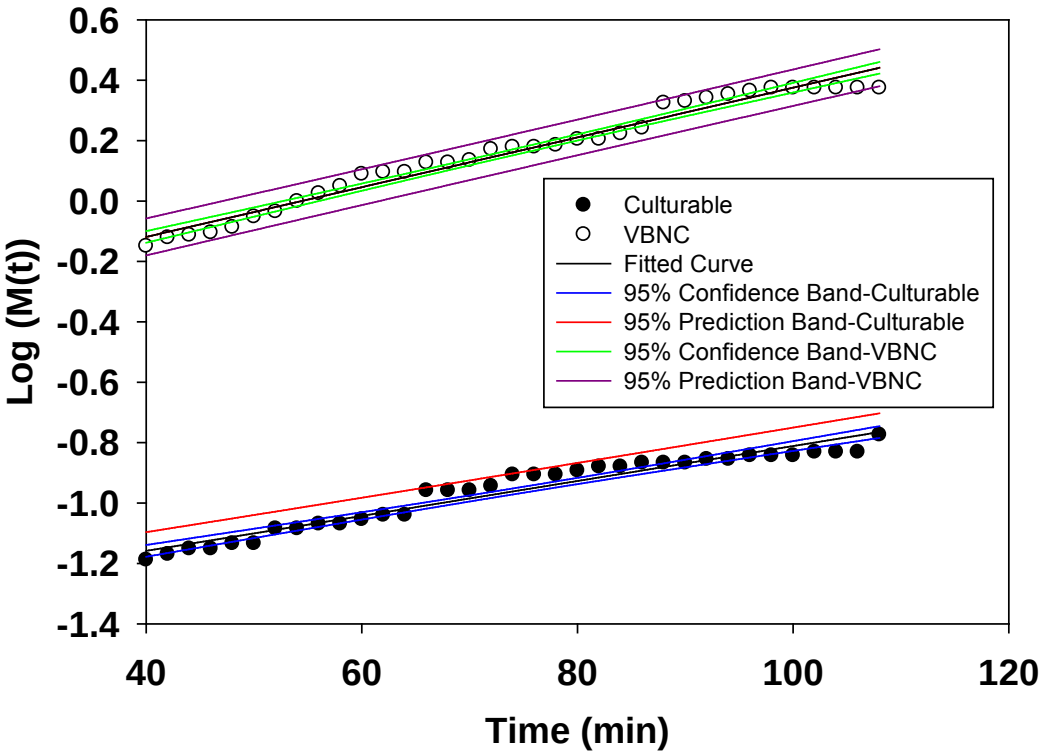


Figure 3-7. Log cumulative mass flux data and fitted linear curves for culturable and VBNC *E.coli* O157:H7 cells using unwashed sand in a 10 cm column from 40 min to 108 min. Fitted curves are flanked by 95% confidence bands and 95% prediction bands. Based on equation f = y0+a*x.

Table 3-5. Slope parameters and adjusted R^2-values from the linear regression of log cumulative mass flux data of culturable and VBNC *E.coli* O157:H7 cells from 40 min to 108 min using 10 cm columns packed with unwashed sand based on the linear polynomial equation $y=y_o+ax$.

Column Length (cm)	Sand Type	Cell type	Parameters		
			Slope	Slope Standard Error	Adjusted R^2
10	Unwashed	Culturable	0.0058 a	0.0002	0.94
		VBNC	0.0082 b	0.0002	0.97

** Values with the same letter are not considered significantly different ($p<0.05$)

In this study, we used breakthrough curves, temporal moment analysis, applied mass accumulation, and curve fitting to decipher transport differences between culturable and VBNC *E.coli* O157:H7 cells through packed-sand columns. From initial transport assays using 5 cm columns packed with washed sand, it was observed that both VBNC and culturable cells transported similarly. This conclusion was drawn through their similar breakthrough curves, insignificantly different mass accumulation data regardless of sampling interval, and overlapping confidence and prediction bands following curve-fitting.

Following these initial tests, we then utilized 10 cm columns to increase the time and distance over which the VBNC or culturable cells were allowed to interact with the solid substrate. In addition to increased column length, we employed unwashed sand particles, still bearing naturally-adsorbed metal oxides, along with the acid-washed clean sand, free of metal oxides, to determine any potential differences between transport of VBNC and culturable *E.coli* O157:H7 cells in the 10 cm columns. It was after these changes were made that transport discrepancies between VBNC and culturable cells became noticeable. Breakthrough curves along with mass accumulation data acquired from the use of 10 cm columns displayed four observable trends: i) The total mass of VBNC *E.coli* O157:H7 cells transported through the column generally exceeded that of culturable cells, regardless of sampling interval or sand type, ii) Greater mass transport was exhibited in columns packed with washed sand than unwashed sand, regardless of cell type, iii) Greater "tailing" of breakthrough curves was exhibited in columns using washed sand than unwashed sand, regardless of cell type, iv)

Breakthrough peaks of primary bacterial breakthrough curves coincided with peak breakthrough of conservative tracer breakthrough curves regardless of sand type or column length.

In addition, non-linear curve fitting revealed consistently greater mass accumulation of VBNC cells compared to culturable cells when utilizing 10 cm columns packed with unwashed sand, regardless of sampling time, as well as zero overlapping of confidence or prediction bands. Similarly, linear regression showed columns packed with unwashed sand had significantly ($p<0.05$) greater mean mass accumulation of VBNC cells than culturable cells in the effluent during the later stages of the transport assays as indicated by the slopes of the fitted curves. These regression studies strongly suggest that there is significantly greater bacterial mass accumulation in unwashed sand column effluents when bacterial pulses consist of VBNC cells versus culturable cells.

3.4. Discussion

3.4.1. VBNC and culturable *E.coli* O157:H7 mass flux differences

The observation that VBNC *E.coli* O157:H7 cells had a generally greater mass flux through 10 cm columns packed with washed sand as well as the significantly greater mass transport of VBNC cells through unwashed sand compared to culturable cells can possibly be explained by considering key phenotypic differences between the cell types, as well as the porous medium itself. It has been suggested that *E.coli* O157:H7 cells undergo significant changes in certain morphological and phenotypical traits when induced into a viable but non-culturable state. These changes have been shown to include a

decrease in their relative hydrophobicity, an increase in tightly-bound surface proteins and sugars, as well as miniaturization and shortening to a coccus-like form, all in relation to their exponentially-growing, culturable form (See Table 2-2). Transport and retention of particulates, such as bacterial cells, in the soil is recognized as being a very complex physical and chemical phenomenon dependent on the interaction of the various complex properties of soil, cells, and suspending solutions. Four main factors which influence bacteria transport through soil include (1) soil characteristics such as grain size and soil structure which control the active porosity, (2) filtration/size exclusion due to micropores, (3) straining within organic materials pads formed on soil surface, and (4) retention of bacterial cells on soil mineral and organic particles by adsorption and adhesion (Unc and Goss, 2004).

The observed differences in mass flux between the culturable and VBNC *E.coli* O157:H7 cells could be a result of their miniaturization and transition from a rod-shape to a coccoid-shape upon entry into a VBNC state. A decrease in size and shift to a spherical shape could increase the potential for a microbial cell to negotiate smaller pore spaces effectively decreasing the straining or filtration capacity of a soil. Bolster et al. (2009) found increased bacterial recovery and lower deposition rate to be correlated with smaller cell width when they utilized sand-packed columns to elucidate transport differences between *E.coli* isolates. Gannon et al. (1991) observed a significant relationship between cell length and transport capabilities, with shorter cells having a higher transport percentage. Cell shape has also been shown to influence bacterial transport through soil.

Weiss et al. (1995), using 14 strains of bacteria, noted the preferential transport of bacterial cells with a spherical shape through columns packed with clean quartz sand. Though cell dimensions have been shown to affect their transport through sand-packed columns, in our study, we do not consider cell size and shape to be the only determining factor behind the observed differences in transport characters of VBNC and culturable cells. The reasoning behind this is the particle size distribution of sand utilized in our column assays. Xu et al. (2008) suggests that when the ratio of the colloid diameter to the mean grain diameter is less than approximately 0.008, straining will be insignificant. In our case, utilizing sand particles with a size range of 250 to 500 μm and considering the culturable and VBNC cell sizes (See Table 2-2), it is possible subtle differences in physical straining does exist, however, it is unlikely that straining was the major contributing factor behind the observed retention patterns of the VBNC and culturable cells.

The hydrophobicity of bacteria has also been previously reported as having a strong influence on attachment and subsequently a strong impact on retention and transport of bacteria through soil. Stenstrom (1989), for instance, noted that *Salmonella typhimurium* attachment to quartz, albite, feldspar, and magnetite was positively correlated with hydrophobicity. As well, Jacobs et al. (2007), in a study examining 21 different bacterial isolates, determined that the hydrophobic strains were better retained by the sand matrix than the hydrophilic strains. In our previous study, we noted a significant decrease in the hydrophobicity of *E.coli* O157:H7 cells when they enter a VBNC state.

Therefore, it is possible that hydrophobicity has played a role in the findings from this study where we found the less hydrophilic culturable *E.coli* O157:H7 cells had a lower mass flux than the more hydrophilic VBNC *E.coli* O157:H7 cells through both sand types. This trend could be a result of the less hydrophilic culturable cells being "expelled" from the bulk of water due to the strong tension at the cell–water interface (water surface tension) leading to increased rates of bacterial adsorption at the solid–liquid interface (Unc and Goss, 2004). Van Loosdrecht et al. (1987) hypothesized this phenomenon as being a defence mechanism generated by the terrestrial, lacustrine, or near-shore microorganisms to encourage spreading during times of stress, such as starvation. They postulated that bacterial cells can decrease their hydrophobicity in order to detach from a substrate in order to move to a new geographical position where, presumably, adequate nutrition will exist. On the other hand, Kjelleberg and Hermansson (1984) suggested that copiotrophic bacteria may increase their hydrophobic character under starvation conditions in order to be relocated to the solid-liquid interface in order to scavenge surface materials. Nevertheless, the difference in hydrophobicity of the culturable and VBNC *E.coli* O157:H7 cells utilized in our column assays may be considered as an influencing property determining the transport properties of the cells through the sand-packed columns, particularly through unwashed sand. The decrease in the VBNC cell hydrophobicty, compared to their culturable counterparts, potentially could have allowed the VBNC cells to better incorporate into the bulk flow solution and travel predominantly down pore centre lines. This would prevent them from interacting

with the positively-charged metal-oxide coated sand particles which would have otherwise undoubtedly increased their retention in the columns.

The presence of proteins and sugars on the surfaces of bacterial cells has also been examined in order to determine their importance in bacterial attachment to solid surfaces. Lutterodt et al. (2009) determined that Ag43 expression, an *E.coli* surface membrane protein, along with bacterial motility had a significant effect on *E.coli* retention in clean quartz sand columns. In addition, Tsuneda et al. (2003), using 27 heterotrophic bacterial strains isolated from a wastewater treatment reactor, found that if the EPS amount is relatively small, cell adhesion onto solid surfaces is inhibited by electrostatic interaction, and if it is relatively large, cell adhesion is enhanced by polymeric interaction. In our previous study (See Table 2-2.), we found that VBNC *E.coli* O157:H7 cells exhibit an increase in cell surface sugars and proteins in relation to their culturable counterparts. In our column assays, we did not see increased retention of VBNC cells compared to culturable cells which suggests, in this case, that the increase in extracellular polymeric substance composition did not enhance cellular retention. This observation may have been due to size exclusion, where microbes tend to be carried near pore centerlines and cannot enter slow flow zones next to pore walls due to their large size (Buchan and Flury, 2008) effectively decreasing the ability of the surface molecules to make contact and attach to solid substrate. The interruption of DLVO-AB interactions may have also played a role. The DLVO-AB model describes the interactions of smooth particles with solid surfaces above separation distances of 2 nm. According to this concept, cells adhere as they

approach a surface close enough to be attracted and to be pulled into a sufficiently deep energy minimum (Jucker et al., 1998). It has been postulated that surface polymers hinder the cells to reach the energy minimum, since the approach to the surface requires their compression (Rijnaarts et al., 1996; van Loosdrecht et al., 1990; Jucker et al., 1998) resulting in steric hindrance.

Considering this previous work, it is possible that, along with being less hydrophobic, that the increase in surface EPS expression has lead to the significant differences seen when VBNC and culturable *E.coli* O157:H7 cells were transported through columns packed with unwashed sand. The increase in the quantity of structures protruding from the surfaces of the VBNC cells, compared to the culturable cells, would thus require greater compression in order for the negatively-charged cell surface to come close enough to enter a sufficiently deep energy minimum with the positively-charged metal-oxide coated sand particles. Bolster et al. (2009) also did not notice a significant correlation between EPS production and bacterial retention in their study on the transport of 12 different *E.coli* isolates through washed sand.

Though previous literature supports our findings that a decrease in hydrophobicity or cell size, a transition to a spherical shape, or an increase in EPS can increase a microbes ability to transport through aquifer-like material, we cannot say that one or more of these were the definitive property(s) that led to our observed trends. There is the possibility that other cell or soil properties not tested for were the cause of the increased relative mass transport of VBNC *E.coli* O157:H7 cells in comparison to their culturable counterparts. What can be said is

that there does appear to be difference in the transport properties of VBNC and culturable *E.coli* O157:H7 cells through the washed or unwashed sand utilized in our laboratory assays.

3.4.2. VBNC and culturable *E.coli* O157:H7 mass flux through washed and unwashed sand

When using 10 cm columns, it became apparent from the breakthrough curve and mass accumulation data that the *E.coli* O157:H7 cells, regardless of reproductive state, were able to transport more efficiently through columns packed with acid-washed sand than through unwashed sand. At pH levels normally found in the environment, most natural surfaces, including clean quartz sand and bacteria, are negatively charged. As a result of these negative charges, repulsion between clean quartz sand and bacteria would be expected due to their associated double layers of counterions (Richardson et al., 2000). By washing the sand in acid, we were effectively able to remove any metal-oxide coatings that were associated with the surfaces of the natural quartz particles. This is significant because metal oxide coatings provide a positively-charged surface that can significantly increase bacterial deposition at the mineral surface (Ryan et al., 1999).

As a result, one would expect greater retention of negatively-charged *E.coli* cells on the surfaces of metal-oxide coated sand particles, due to attractive forces conferred by the oppositely-charged bacteria and aquifer material, than on acid-washed sand surfaces where electrostatic repulsion between the like-charged bacteria and sand would hinder retention and deposition. This outcome was seen

by Bolster et al. (2006) whom demonstrated lower relative recoveries of both *E.coli* O157:H7 and *Campylobacter jejuni* cells when transported through columns packed with Fe- or Al-coated sand particles than in columns packed with uncoated sand. Scholl et al. (1990) showed that the degree of attachment of cells to mineral surfaces was correlated with the sign and magnitude of the charge at the solid-liquid interface. Specifically, they showed that attachment of a negatively-charged *Arthrobacter sp*. of bacteria was much greater to the positively-charged surfaces of limestone, Fe-hydroxide coated quartz, and Fe-hydroxide coated muscovite than to the negatively-charged surfaces of clean quartz and clean muscovite. Similarly, Shani et al. (2008) used negatively-charged fluorescent microspheres to show that colloid retention was much higher in natural sand than in its acid-washed counterparts Taking an alternate approach, Sharma et al. (1985) showed that by chemically-increasing the negative charge on the surface of *B.subtilis* and *P.flourescens* cells using heparin, lignosulfate, polyacrylic acid, and sodium pyrophosphate they were able to increase their transportability through sand packs.

Our breakthrough curve and mass accumulation data, which show greater recovery of negatively-charged VBNC and culturable *E.coli* O157:H7 cells from acid-washed sand than unwashed sand, are likely a result of electrostatic forces that take place at the bacteria surface-mineral surface. These forces will positively influence deposition onto metal-oxide coated unwashed sand and hinder deposition onto clean, negatively-charged washed sand.

3.4.3. "Tailing" of breakthrough curves in washed sand

More pronounced tailing was found to occur when culturable or VBNC *E.coli* O157:H7 cells were transported through 10 cm columns packed with acid-washed sand, than through unwashed sand. In columns packed with unwashed sand, it is likely that the electrostatic attraction between the oppositely-charged sand and *E.coli* cells is causing their strong retention; therefore, following a primary breakthrough, the adsorbed cells are able to better withstand the drag forces applied by the moving flow solution and remain bound to the solid substrate. It is important to note, however, that the breakthrough curves of VBNC or culturable *E.coli* O157:H7 cells through unwashed sand were not completely free of tailing, particularly for the VBNC cells. The tailing that was demonstrated indicates that even despite the attractive forces between the cells and the unwashed quartz, their likely was reversible adsorption and cell aggregate release from pores occurring.

In the washed-sand columns it is likely cell aggregation in narrow pore-passages and subsequent cell release at a time following the primary breakthrough which is leading to the breakthrough tailing seen in the VBNC and culturable curves. Fontes et al. (1991) noticed tailing in breakthrough curves constructed following transport of sediment bacteria through columns packed with acid-washed quartz sand. They attributed their findings to desorption of cells from mineral surfaces or deflocculation of aggregated cells in pore throats too narrow to prevent the flow of amassed cells. In our case, considering the negative charge on the surface of the clean quartz particles as well as on the surface of the VBNC and culturable *E.coli* O157:H7 cells, it is unlikely the tailing is a result of

reversible adsorption. Instead, we postulate that cells become trapped in small pores and are released over a period of time due to shear forces from the flowing solution through the pore space resulting in relative cell concentrations greater than zero following the primary breakthrough. In a study done by Wollum and Cassel (1977) describing the transport of a *Streptomyces sp.* through sterile sand they describe considerable tailing nearly 3 pore volumes following a primary breakthrough peak. They hypothesized that the tailing was a result of cells becoming trapped in narrow pores and subsequently releasing over time into water flowing through the porous medium. The ionic strength of the flow solution has been implicated in facilitating pore clogging. Fontes et al. (1991) suggested that higher ionic strength solutions can enhance the aggregation of bacterial cells, leading to increased pore clogging and, therefore, decreased recovery. In our columns we utilized 0.85% NaCl as a flow solution, therefore it is possible that the positively-charged sodium molecules were able to compress the diffuse-double layers of the cells which facilitated their aggregation which lead to pore clogging, regardless of sand type. The aggregated VBNC or culturable *E.coli* O157:H7 cells in the unwashed sand were then better able to resist the shear forces of the flowing solution due to attractive forces between the cells and the metal oxide coated sand surfaces creating secondary or reversible adhesion (van Loosdrecht et al., 1989), whereas cells in washed sand columns were more susceptible to pore clogging and subsequent release due to repulsive forces between clean sand and the negatively charged cellular surfaces.

3.4.4. Bacterial breakthrough peaks coinciding with conservative tracer breakthrough peaks

Upon examination of breakthrough curves developed from our transport assays using columns of length 5 or 10 cm, it is apparent that the peak breakthrough of *E.coli* O157:H7 cells, regardless of cell state or sand type, coincide with the conservative tracer peak breakthrough. The ability of microbes to travel as quickly or faster than the mean-pore water velocity has been attributed to two differential flow effects (1) size exclusion which, as previously mentioned, occurs when microbes tend to be carried near pore centerlines and due to their relatively large size, cannot enter slow-moving zones alongside pore wall where flow is slowed due to drag, and (2) detouring effects, where, due to their large size, microbes are preferentially transported through larger pores, avoiding smaller pores (Ginn et al., 2002; Unc and Goss, 2004; Buchan and Flury, 2008). Thus, microbes, and other larger colloids, can experience the higher velocities near pore centerlines, yielding an average velocity that is higher than that of a dissolved tracer (Murphy and Ginn, 2000). These flow effects can ultimately lead to microbial peak breakthrough occurring concurrently or even sooner than a dissolved conservative tracer. The simultaneous breakthrough of microbial and conservative tracers has been seen in previous studies. Bradford et al. (2002), using latex fluorescent colloids, observed that bromide and colloidal breakthrough occurred quite similarly for a given sand. Using long glass capillary tubes packed with sand, Shonnard et al. (1994) showed suspensions of *M.trichosporium* OB3b were detected in the effluents significantly earlier and in higher relative

concentrations than the inert tracer utilized, phenol red. Under field conditions, Harvey and George (1989) observed earlier breakthrough of stained groundwater bacteria relative to bromide in a forced-gradient experiment which they later attributed to size exclusion. Considering the previous work then, it is likely that we are observing similar breakthrough of conservative and microbial tracers due to the ability of the relatively large microbial colloids to take advantage of the greater flow rate at the centre of pores as well as being able to bypass smaller pores. Conservative tracer molecules, on the other hand, are often small enough to advect or diffuse into smaller fine fractures connected to preferential flow pathways, which overall contribute little to the bulk flow of the groundwater, and can effectively hinder their transport velocity and peak breakthrough in comparison with microbial tracers (Harvey, 1997).

3.5. Conclusions

From the breakthrough curves, mass accumulation, and curve fitting data, this study suggests that the transport properties of *E.coli* O157:H7 cells change when they transition into a VBNC state. Using 10 cm columns packed with acid-washed or unwashed quartz sand, we were able to see that VBNC cells consistently had greater mass accumulation in effluent sampling intervals, regardless of sand type. In particular, the use of columns packed with unwashed sand revealed significantly greater mean cumulative mass flux of VBNC cells compared with culturable cells. These findings may possibly be attributed to changes in morphological and surface characteristics of *E.coli* O157:H7 cells when they enter into a VBNC state, including decreased hydrophobicity,

increased surface sugar and protein expression, as well as smaller, more spherical cell shapes. We also noticed greater mass transport through 10 cm columns packed with acid-washed sand than unwashed sand, regardless of cell type, which was attributed to greater bacterial adsorption to metal-oxide coated sand particles found in unwashed sand columns. Greater "tailing" of BTC's was observed through columns of washed sand which was attributed to cells aggregating in narrow pores and subsequently releasing over time, while "tailing" in unwashed sand columns was attributed to reversible adhesion along with pore clogging and subsequent release. Finally, the concurrent breakthrough of conservative and microbial tracers was determined to be a result of size exclusion. Overall, from the results, it can be determined that bacterial transport through porous media is a truly dynamic process which will vary according to bacterial traits, the composition of the flow solution, the characteristics of the soil, as well as the population structure of VBNC and culturable cells. Future work will be needed to further explore the transport of VBNC bacteria through soil to further elucidate the effects that various soil forms and flow solutions will have on their mass transport and velocity. Overall, our research has shown that there exist potential differences in the transport of VBNC and culturable *E.coli* O157:H7 cells through porous media. This finding will be important when considering contamination of aquifer-like materials as well as opening dialogue into whether or not currently-used culture-based protocols are reliable enough to accurately determine contamination sources and pathways.

3.6. References

Aris, R. 1958. On the dispersion of linear kinematic waves. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences*. 245(1241): 268-277.

Berglund, S. and Cvetkovic, V. 1998. Temporal moment analysis of contaminant extraction: effects of flow heterogeneity and spatially variable source concentrations. *Groundwater Quality: Remediation and Protection* (Proceedings of the GQ'98 Conference held at Tubingen, Germany, September 1998). IAHS Publ. no. 250.

Bolster, C.H., Mills, A.L., Hornberger, G.M., and Herman, J.S. 2001. Effect of surface coatings, grain size, and ionic strength on the maximum attainable coverage of bacteria on sand surfaces. *Journal of Contaminant Hydrology*. 50(3-4): 287-305.

Bolster, C.H., Walker, S.L., and Cook, K.L. 2006. Comparison of *Escherichia coli* and *Campylobacter jejuni* transport in saturated porous media. *Journal of Environmental Quality*. 35(4): 1018-1025.

Bolster, C.H., Haznedaroglu, B.Z., and Walker, S.L. 2009. Diversity in cell properties and transport behavior among 12 different environmental *Escherichia coli* isolates. *Journal of Environmental Quality*. 38(2): 465-472.

Bradford, S.A., Yates, S.R., Bettahar, M., and Simunek, J. 2002. Physical factors affecting the transport and fate of colloids in saturated porous media. *Water Resources Research*. 38(12): 63-1 – 63-12.

Bradford, S.A., Simunek, J., and Walker, S.L. 2006. Transport and straining of *E. coli* O157:H7 in saturated porous media. *Water Resources Research*. 42(W12S12): 1-12.

Brooks, M.C. and Wise, W.R. 2005. Quantifying uncertainty due to random errors for moment analyses of breakthrough curves. *Journal of Hydrology*. 303(1-4): 165-175.

Buchan, G.D. and Flury, M. 2008. 'Pathogens: Transport by Water', Encyclopedia of Water Science, Second Edition, 1: 1, 808 — 811

Canadian Food Inspection Agency. 2009. *E. coli* O157:H7 food safety facts preventing foodborne illness. <*http://www.inspection.gc.ca/english/fssa/concen/cause/ecolie.shtml*>. Accessed on September 13, 2010.

DeFlaun, M.F., Tanzer, A.S., McAteer, A.L., Marshall, B., and Levy, S.B. 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. *Applied and Environmental Microbiology*. 56(1): 112-119.

DeFlaun, M.F., Oppenheimer, S.R., Streger, S., Condee, C.W., and Fletcher, M. 1999. Alterations in adhesion, transport, and membrane characteristics in an adhesion-deficient pseudomonad. *Applied and Environmental Microbiology*. 65(2): 759-765.

Dodd, C.C., Sanderson, M.W., Sargeant, J.M., Nagaraja, T.G., Oberst, R.D., Smith, R.A., and Griffin, D.D. 2003. Prevalence of *Escherichia coli* O157 in cattle feeds in midwestern feedlots. *Applied and Environmental Microbiology*. 69(9): 5243-5247.

Englert, A., Hubbard, S.S., Williams, K.H., Li, L., and Steefel, C.I. 2009. Feedbacks between hydrological heterogeneity and bioremediation induced biogeochemical transformations. *Environmental Science and Technology*. 43(14): 5197-5204.

Faith, N.G., Shere, J.A., Brosch, R., Arnold, K.W., Ansay, S.E., Lee, M-S., Luchansky, J.B., and Kaspar, C.W. 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology*. 62(5): 1519-1525.

Fontes, D.E., Mills, A.L., Hornberger, G.M., and Herman, J.S. 1991. Physical and chemical factors influencing transport of microorganisms through porous media. *Applied and Environmental Microbiology*. 57(9): 2473-2481.

Gagliardi, J. and Karns, J. 2000. Leaching of *Escherichia coli* O157:H7 in diverse soils under various agricultural management practices. *Applied and Environmental Microbiology*. 66(3): 877-883.

Galland, J.C., Hyatt, D.R., Crupper, S.S., and Acheson, D.W. 2001. Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. *Applied and Environmental Microbiology*. 67(4): 1619-1627.

Gannon, J.T., Manilal, V.B., and Alexander, M. 1991. Relationship between cell surface properties and transport of bacteria through soil. *Applied and Environmental Microbiology*. 57(1): 190-193.

Ginn, T.R., Wood, B.D., Nelson, K.E., Schebe, T.D., Murphy, E.M., and Clement, T.P. 2002. Processes in microbial transport in the natural subsurface. *Advances in Water Resources*. 25(8-12): 1017-1042.

Harvey, R.W. and George, L.H. 1989. Transport of microspheres and indigenous bacteria through a sandy aquifer: results of natural- and forced-gradient tracer experiments. *Environmental Science and Technology*. 23(1): 51-56.

Harvey, C. F. and Gorelick, S. M. 1995. Temporal moment-generating equations - modeling transport and mass-transfer in heterogeneous aquifers. *Water Resources Research.* 31(8): 1895-1911.

Harvey, R.W. 1997. Microorganisms as tracers in groundwater injection and recovery experiments: a review. *FEMS Microbiology Reviews*. 20(3-4): 461-472.

Hrudey, S.E., Payment, P., Huck, P.M., Gillham, R.W., and Hrudey, E.J. 2003. A fatal waterborne disease epidemic in Walkerton, Ontario: comparison with other waterborne outbreaks in the developed world. *Water Science and Technology*. 47(3): 7-14.

Jackson, S.G., Goodbrand, R.B., Johnson, R.P., Odorico, V.G., Alves, D., Rahn, K., Wilson, J.B., Welch, M.K., and Khakhria, R. 1998. *Escherichia coli* O157:H7 diarrhoea associated with well water and infected cattle on an Ontario farm. *Epidemiology and Infection.* 120(1): 17-20.

Jacobs, A., Lafolie, F., Herry, J.M., and Debroux, M. 2007. Kinetic adhesion of bacterial cells to sand: Cell surface properties and adhesion rate. *Colloids and Surfaces B: Interfaces*. 59(1): 35-45.

Jamieson, R.C., Gordon, R.J., Sharples, K.E., Stratton, G.W., and Madani, A. 2002. Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*. 44: 1.1-1.9.

Jucker, B.A., Zehnder, A.J.B., and Harms, H. 1998. Quantification of polymer interactions in bacterial adhesion. *Environmental Science and Technology*. 32(19): 2909-2915.

Kjelleberg, S. and Hermansson, M. 1984. Starvation-induced effects on bacterial surface characteristics. *Applied and Environmental Microbiology*. 48(3): 497-503.

Laegreid, W.W., Elder, R.O., and Keen, J.E. 1999. Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiology and Infection*. 123(2): 291-298.

Larney, F.J. and Hao, X. 2007. A review of composting as a management alternative for beef cattle feedlot manure in southern Alberta, Canada. *Bioresource Technology*. 98(17): 3221-3227.

Lennartz, B. and Kamra, S.K. 1998. Temporal variability of solute transport under vadose zone conditions. *Hydrological Processes*. 12(12): 1929-1949.

Licence, K., Oates, K.R., Synge, B.A., and Reid, T.M.S. 2001. An outbreak of *E. coli* O157 infection with evidence of spread from animals to man through contamination of a private water supply. *Epidemiology and Infection*. 126(1): 135-138.

Liu, Y., Gilchrist, A., Zhang, J., and Li, X. 2008. Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Applied and Environmental Microbiology*. 74(5): 1502-1507.

Liu, Y., Wang, C., Tyrrell, G., Hrudey, S.E., and Li, X-F. 2009. Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environmental Microbiology Reports.* 1(2): 155-161.

Lleo, M., Bonato, B., Tafi, M.C., Caburlotto, G., Benedetti, D., and Canepari, P. 2007. Adhesion to medical device materials and biofilm formation capability of some species of enterococci in different physiological states. *FEMS Microbiology Letters.* 274(2): 232-237.

Lutterodt, G., Basnet, M., Foppen, J.W.A., and Uhlenbrook, S. 2009. The effect of surface characteristics on the transport of multiple *Escherichia coli* isolates in large scale columns of quartz sand. *Water Research*. 43(3): 595-604.

Mallants, D., Vanclooster, M., Meddahi, M., and Feyen, J. 1994. Estimating solute transport in undisturbed soil columns using time-domain reflectometry. *Journal of Contaminant Hydrology*. 17(2): 91-109.

Murphy, E.M. and Ginn, T.R. 2000. Modeling microbial processes in porous media. *Hydrogeology Journal*. 8: 142-158.

O'Connor, D. 2002. A summary report of the Walkerton inquiry: The events of May 2000 and related issues, Queen's Printer for Ontario, Toronto, Ontario, Canada.

Oda, M., Morita, M., Unno, H., and Tanji, Y. 2004. Rapid detection of *Escherichia coli* O157:H7 by using green fluorescent protein-labeled PP01

bacteriophage. *Applied and Environmental Microbiology*. 70(1): 527-534.

Ogden, I.D., Fenlon, D.R., Vinten, A.J.A., Lewis, D. 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. *International Journal of Food Microbiology*. 66(1-2): 111-117.

Oliver, J.D. Formation of viable but nonculturable cells. In: Kjelleberg S (ed.) Starvation in Bacteria, Plenum Press, New York, 1993. pp 239-272.

Oliver, J.D. 2005. The Viable but Nonculturable State in Bacteria. *The Journal of Microbiology*. 43: 93-100.

Olsen, S.J., Miller, G., Breuer, T., Kennedy, M., Higgins, C., Walford, J., McKee, G., Fox, K., Bibb, W., and Mead, P. 2002. A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: Implications for rural water systems. *Emerging Infectious Diseases*. 8(4): 370-375.

Pang, L., Close, M., and Noonan, M. 1998. Rhodamine WT and *Bacillus subtilis* transport through an alluvial gravel aquifer. *Groundwater*. 36(1): 112-122.

Reddy, K.R., Khaleel, R., and Overcash, M.R. 1981. Behavior and transport of microbial pathogens and indicator organisms in soils treated with organic wastes. *Journal of Environmental Quality*. 10(3):255-266.

Richardson, R.L., Mills, A.L., Herman, J.S., and Hornberger, G.M. 2000. Effect of humic material on interactions between bacterial cells and mineral surfaces. *Laboratory of Microbial Ecology Department of Environmental Sciences*. Clark Hall, University of Virginia. Charlottesville, VA. 22904-4123.

Rigsbee, W., Simspon, L.M., and Oliver, J.D. 1997. Detection of the viable but nonculturable state in *Escherichia coli* O157:H7. *Journal of Food Safety*. 16(4): 255-262.

Rijnaarts, H.H., Norde, W., Bouwer, E.J., Lyklema, J., and Zehnder, A.J.B. 1996. Bacterial deposition in porous media: Effects of cell-coating, substratum hydrophobicity, and electrolyte concentration. *Environmental Science and Technology*. 30(10): 2877-2883.

Ryan, J.N., Elimelech, M., Ard, R.A., Harvey, R.W., and Johnson, P.R. 1999. Bacteriophage PRD1 and silica colloid transport and recovery in an iron oxide-coated sand aquifer. *Environmental Science and Technology*. 33(1): 63-73.

Saint-Gobain Performance Plastics. 2007. Tygon® Food, Milk, and Dairy Tubing. Accessed on 21 Dec. 2010. <http://www.tygon.com/uploadedFiles/SGtygon/Documents/Tygon_Tubing/Tygon-B444X-Food-Tubing.pdf>

Scholl, M.A., Mills, A.L., Herman, J.S., and Hornberger, G.M. 1990. The influence of mineralogy and solution chemistry on the attachment of bacteria to representative aquifer materials. *Journal of Contaminant Hydrology*. 6(4): 321-336.

Scholl, M.A. and Harvey, R.W. 1992. Laboratory investigations on the role of sediment surface and groundwater chemistry in transport of bacteria through a contaminated sandy aquifer. *Environmental Science and Technology*. 26(7): 1410–1417.

Semenov, A.V., van Overbeek, L., and van Bruggen, A.H.C. 2009. Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Applied and Environmental Microbiology*. 75(10): 3206-3215.

Shani, C., Weisbrod, N., and Yakirevich, A. 2008. Colloid transport through saturated sand columns: Influence of physical and chemical surface properties on deposition. *Colloids and Surfaces: Physiochemical and Engineering Aspects A.* 316(1-3): 142-150.

Sharma, M.M., Chang, Y.I., and Yen, T.F. 1985. Reversible and irreversible surface charge modification of bacteria for facilitating transport through porous media. *Colloids and Surfaces*. 16(2): 193-206.

Shere, J.A., Bartlett, K.J., and Kaspar, C.W. 1998. Longitudinal study of *Escherichia coli* O157:H7 dissemination on four dairy farms in Wisconsin. *Applied and Environmental Microbiology*. 64(4): 1390-1399.

Shonnard, D.R., Taylor, R.T., and Hanna, M.L. 1994. Injection-attachment of *Methylosinus trichosporium* OB3b in a two-dimensional miniature sand-filled aquifer simulator. *Water Resources Research*. 30(1): 25-35.

Signoretto, C., del Mar Lleo`, M., Tafi, M.C., and Canepari, P. 2000. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Applied and Environmental Microbiology*. 66(5): 1953-1959.

Simoni, S.F., Harms, H., Bosma, T.N.P., Zehnder, A.J.B. 1998. Population heterogeneity affects transport of bacteria through sand columns at low flow rates. *Environmental Science and Technology*. 32(14): 2100-2105.

Skopp, J. 1985. Analysis of solute movement in structured soils, in Bouma, J. and Raats, P. A. C. (eds.), Proc. ISSS Symposium on Water and Solute Movement in Heavy Clay Soils. ILRI, Wageningen, The Netherlands. pp. 220-228.

Stenstrom, T.A. 1989. Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. *Applied and Environmental Microbiology*. 55(1): 142-147.

Swerdlow, D.L., Woodruff, B.A., Brady, R.C., Griffin, P.M., Tippen, S., Donnell,Jr., H.D., Geldreich, E., Payne, B.J., Meyer, Jr., A., Wells, J.G., Greene, K.D., Bright, M., Bean, N.H., Blake, P.A. 1992. A waterborne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhea and death. *Annals of Internal Medicine*. 117(10): 812-819.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS: Microbiology Letters*. 223(2): 287-292.

Unc, A. and Goss, M.J. 2004. Transport of bacteria from manure and protection of water resources. *Applied Soil Ecology*. 25(1): 1-18.

Valocchi, A.J. 1985. Validity of the local equilibrium assumption for modeling sorbing solute transport through homogeneous soils. *Water Resources Research*. 21(6): 808-820.

Van Donkersgoed, J., Graham, T., and Gannon, V. 1999. The prevalence of verotoxins, *Escherichia coli* O157:H7, and *Salmonella* in the feces and rumen of cattle at processing. *Canadian Veterinary Journal*. 40(5): 332-338.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G., and Zehnder, A.J.B. 1987. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Applied and Environmental Microbiology*. 53(8): 1898-1901.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., and Zehnder, A.J.B. 1989. Bacterial adhesion: A physiochemical approach. *Microbial Ecology*. 17(1): 1-15.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W., and Zehnder, A.J.B. 1990. Influence of interfaces on microbial activity. *Microbiological Reviews*. 54(1): 75-87.

Vidovic, S. and Korber, D.R. 2006. Prevalence of *Escherichia coli* O157 in Saskatchewan cattle: Characterization of isolates by using random amplified polymorphic DNA PCR, antibiotic resistance profiles, and pathogenicity determinants. *Applied and Environmental Microbiology*. 72(6): 4347-4355.

Vinten, A.J.A., Lewis, D.R., Fenlon, D.R., Leach, K.A., Howard, R., Svoboda, I., and Ogden, I. 2002. Fate of *Escherichia coli* and *Escherichia coli* O157 in soils and drainage water following cattle slurry application at 3 sites in southern Scotland. *Soil Use and Management*. 18(3): 223-231.

Weiss, T.H., Mills, A.L., Hornberger, G.M., and Herman, J.S. 1995. Effect of bacterial cell shape on transport of bacteria in porous media. *Environmental Science and Technology*. 29(7): 1737-1740.

Williams, V. and Fletcher, M. 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Applied and Environmental Microbiology*. 62(1): 100-104.

Wollum II, A.G. and Cassel, D.K. 1977. Transport of microorganisms in sand columns. *Soil Science Society of America Journal*. 42(1): 72-76.

Xu, S., Liao, Q., and Saiers, J.E. 2008. Straining of nonspherical colloids in saturated porous media. *Environmental Science and Technology*. 42(3): 771-778.

4. Summary

The viable but non-culturable state of bacteria has been described as a physiological response by the cell to an imposed external stress causing them to lose the ability to form cultures on standard bacteriological media. Cells in the VBNC state typically demonstrate very low levels of metabolic activity, but upon resuscitation are again culturable (Oliver, 2005). Bacterial cells induced into a VBNC state have been shown to undergo morphological and phenotypical changes (Catenrich and Makin, 1991; Jiang and Chai, 1996; Signoretto et al., 2002). Pathogenic *E.coli* O157:H7, responsible for numerous groundwater-borne disease outbreaks (e.g. O'Connor, 2002) is one such species capable of entering a VBNC state (Rigsbee et al., 1997). This is of considerable significance to public health as the most widely-used and economical enumeration techniques for determining *E.coli* O157:H7 contamination in the environment identifies only culturable bacterial cells; therefore, it is possible that they are considerably underestimating actual bacterial populations (Jamieson et al., 2002).

This research explores whether the transport characteristics of VBNC *E.coli* O157:H7 cells in a groundwater system differ from those of culturable *E.coli* O157:H7 cells. The results will help determine if culturable *E.coli* O157:H7-based risk assessment indicators are accurate in qualitatively describing VBNC *E.coli* O157:H7 transport and contamination.

A new procedure was developed for inducing *E.coli* O157:H7 cells into a VBNC state. To provoke *E.coli* O157:H7 to become viable but non-culturable, cells were incubated in microcosms of tap water containing stress-inducing

chloramines. The development of the induction procedure was an iterative process which first required optimizing tap water chloramine concentrations, tap water volumes, and incubation lengths and temperatures, to stimulate the cells into a non-culturable state. Next, the procedure was further manipulated in order to increase the viability of the non-culturable cells. Decreasing the imposed stress on the cells during washing procedures facilitated an increase in cell viability following induction. The reduction in stress experienced by cells during washing was accomplished by lowering centrifuge speeds and utilizing tube inversion resuspension techniques. The end product was an induction procedure which reliably induces culturable *E.coli* O157:H7 cells into a VBNC state in a relatively short period of time while maintaining high viability.

Using culturable *E.coli* O157:H7 cells as well as the VBNC cells produced from the induction procedure, specific phenotypic and morphological cell properties were analyzed and compared. Images of VBNC and culturable *E.coli* O157:H7 cells were captured using DIC microscopy and evaluated for individual cell lengths and widths using software image analysis. When *E.coli* O157:H7 cells enter a VBNC state they undergo a significant ($p<0.05$) decrease in both their length and width dimensions and transition from a rod-shape to a coccus-shape.

Microbial attachment to hydrocarbon assays were utilized to determine the hydrophobicity of VBNC and culturable cells. The partitioning of cells between a hydrocarbon and an aqueous phase indicated that VBNC *E.coli* O157:H7 cells are significantly ($p<0.05$) less hydrophobic than culturable cells.

The extracellular polymeric substance composition of cells was analyzed for tightly-bound surface sugars using Phenol-sulfuric acid assays and tightly-bound surface proteins using Lowry assays. The results of these experiments revealed that VBNC *E.coli* O157:H7 cells express a significant ($p<0.05$) upregulation of both surface-bound proteins and sugars compared with culturable *E.coli* O157:H7 cells.

These cell properties were chosen because they can influence the transport of bacteria through soil and groundwater (e.g. Bolster et al., 2009). The comparison of cell properties suggests that the physical and surface chemical changes that *E.coli* O157:H7 cells undergo when induced into a VBNC state will influence their behavior in the environment. Compared to culturable cells, the VBNC cells will be less likely to attach to solid substrates, will more easily be incorporated into the liquid-water phase, and their smaller size will allow them to pass through a wider range of pore sizes.

To test whether differences in transport parameters exist between culturable and VBNC *E.coli* O157:H7 cells a series of column transport experiments were carried out. Under conditions of steady-state flow, pulses of VBNC and culturable *E.coli* O157:H7 cells were added to horizontal transport columns packed with sand to simulate transport through a sandy aquifer. Effluents from the column were quantified for cells and subsequently used to construct breakthrough curves and develop mass flux accumulation data. Using a 5 cm column packed with washed sand showed no obvious visual differences between breakthrough curves of VBNC and culturable cells. Furthermore, mass

accumulation data and curve fitting of log cumulative mass flux data for the two cell states was not statistically different. However, breakthrough curves and mass accumulation data from longer columns (10 cm), utilizing acid-washed and unwashed sand, displayed apparent differences between the transportation of these two cell types. Following exponential curve-fitting of the mean log cumulative mass flux data, it was found that VBNC cell mass transport through unwashed sand was statistically greater ($p<0.05$) than the mass accumulation of culturable *E.*coli O157:H7 cells through unwashed sand, regardless of sampling time. Along with this significant finding, four general trends developed from the 10 cm column breakthrough curves, mass accumulation data, and curve-fitting assays. These trends include:

- The total mass of VBNC *E.coli* O157:H7 cells transported through the column exceeded that of culturable cells, regardless of sampling interval or sand type
- Greater mass transport was exhibited in columns packed with washed sand than unwashed sand, regardless of cell type
- Greater "tailing" of breakthrough curves was exhibited in columns using washed sand than unwashed sand, regardless of cell type
- Breakthrough peaks of primary bacterial breakthrough curves coincided with peak breakthrough of conservative tracer breakthrough curves regardless of sand type or column length

4.1. Conclusions and Recommendations

Traditionally, the most widely-used and economical techniques for determining and quantifying bacterial contamination of environmental resources, including soil and groundwater, relies on the ability of bacteria to form colonies (Maukonen and Saarela, 2009). Thus, if culturable *E.coli* O157:H7 is used as a representative transport indicator of an entire O157:H7 population in a groundwater or soil contamination case, then the transport characteristics of the total *E.coli* O157:H7 population of cells, including those in a VBNC state, must be understood.

The observed trends from the breakthrough curves and mass accumulation data, the significant findings from the exponential and linear curve-fitting, along with the significant changes in the phenotypic and morphological properties analyzed, suggests that transport differences between the cellular states is bound to occur in subsurface environments.

Future research will need to explore how the VBNC cells behave under altered soil system parameters where their physical alterations may result in greater transport efficiency compared to culturable cells. Ultimately, by running transport studies using soil systems that are better suited to identify attachment and filtration differences between the two cell types, one could better understand whether the significant changes in the VBNC cell characteristics equates to significant changes in their transport properties. Changes to the soil system can include: e.g. the use of finer textured material, alterations in pH and ionic strength of the flow solution, and organic matter amendments.

Along with utilizing laboratory systems, field studies using potentially contaminated sites may be done to explore whether VBNC *E.coli* O157:H7 cells are present and if so, explore their lateral and vertical positions in relation to culturable cells. A field study like this could add extremely potent real-world information regarding the overland and subsurface transport of VBNC cells in comparison to culturable *E.coli* O157:H7 cells.

Overall, considering the importance that previous studies have placed on colloidal morphology (e.g. Bradford et al., 2002), surface coatings (e.g. Tsuneda et al., 2003), and hydrophobicity (e.g. Williams and Fletcher, 1996), generalizations about the transport of VBNC *E.coli* O157:H7 based on results from their culturable counterparts should be made with vigilance. With the development and increasing popularity of culture-independent bacteria quantification techniques, including flow cytometry, epifluorescence microscopy (Paulse et al., 2007), and quantitative polymerase chain reaction (Liu et al., 2008), one must question whether the risks associated with the potential errors from culture-based techniques doesn't outweigh the higher costs and technicality of the culture-independent techniques. Basically, it becomes a simple question of whether the reduction in risks associated with more sophisticated detection methods are deemed to be worth the extra costs associated with these methods (whether one would rather be "safe than sorry"). This is a decision that society will have to make. Regardless, this is the first known study into the transport of VBNC bacteria in a groundwater system. This thesis will hopefully reinforce the notion that bacterial transport in subsurface systems should be considered from a

continuously dynamic viewpoint and will be heavily influenced by not only the physical, chemical, and biological properties of the soil system, but by the physical characteristics of the microbes as well.

4.2. References

Bolster, C.H., Haznedaroglu, B.Z., and Walker, S.L. 2009. Diversity in cell properties and transport behavior among 12 different environmental *Escherichia coli* isolates. *Journal of Environmental Quality*. 38(2): 465-472.

Bradford, S.A., Yates, S.R., Bettahar, M., and Simunek, J. 2002. Physical factors affecting the transport and fate of colloids in saturated porous media. *Water Resources Research*. 38(12): 63-1 – 63-12.

Catenrich, C.E. and Makin, K.M. 1991. Characterization of the morphologic conversion of *Helicobacter pylori* from bacillar to coccoid forms. *Scandinavian Journal of Gastroenterology.* 26: 58–64.

Jamieson, R.C., Gordon, R.J., Sharples, K.E., Stratton, G.W., and Madani, A. 2002. Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*. 44: 1.1-1.9.

Jiang, X. and Chai, T-J. 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. *Applied Environmental Microbiology.* 62(4): 1300–1305.

Liu, Y., Gilchrist, A., Zhang, J., and Li, X. 2008. Detection of viable but nonculturable *Escherichia coli* O157:H7 bacteria in drinking water and river water. *Applied and Environmental Microbiology*. 74(5): 1502-1507.

Maukonen, J. and Saarela, M. 2009. Microbial communities in industrial environment. *Current Opinion in Microbiology*. 12(3): 238-243.

O'Connor, D. 2002. A summary report of the Walkerton inquiry: The events of May 2000 and related issues, Queen's Printer for Ontario, Toronto, Ontario, Canada

Oliver, J.D. 2005. The viable but nonculturable state in bacteria. *The Journal of Microbiology.* 43: 93-100.

Paulse, A.N., Jackson, V.A., and Khan, W. 2007. Comparison of enumeration techniques for the investigation of bacterial pollution in the Berg River, Western Cape, South Africa. *Water SA.* 33(2): 165-174.

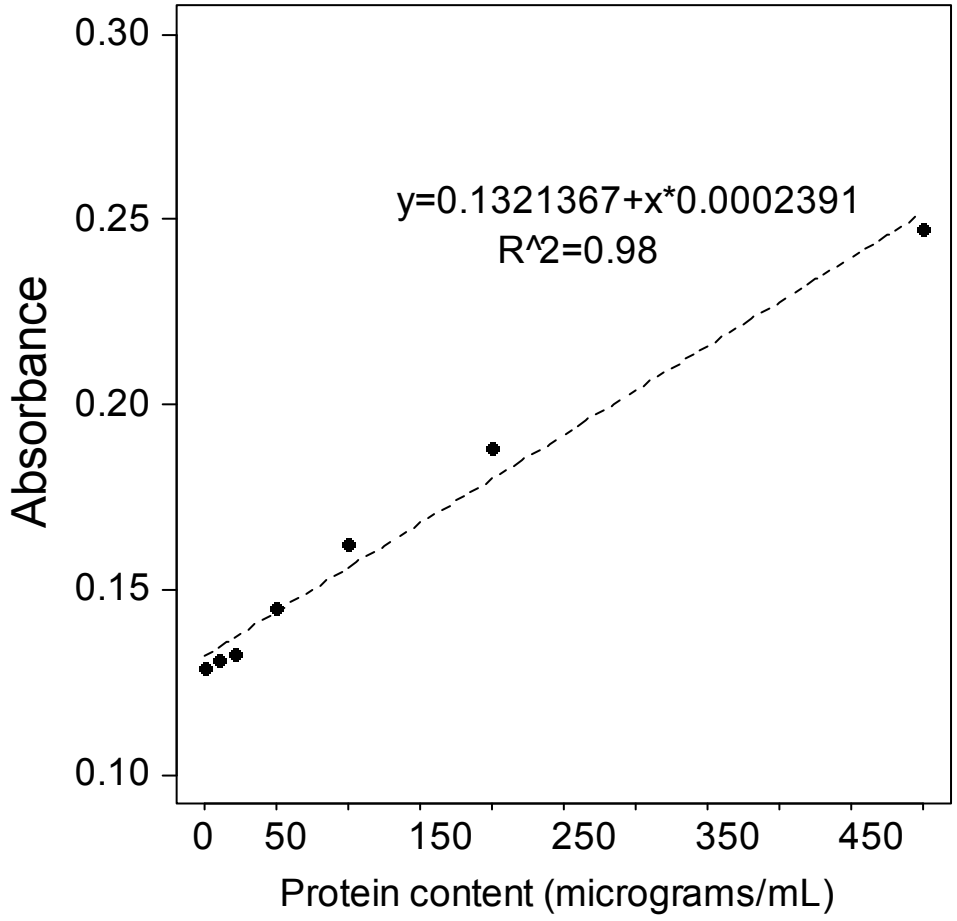
Rigsbee, W., Simspon, L.M., and Oliver, J.D. 1997. Detection of the viable but nonculturable state in *Escherichia coli* O157:H7. *Journal of Food Safety*. 16(4): 255-262.

Signoretto, C., del Mar Lleo`, M., and Canepari, P. 2002. Modification of the peptidoglycan of *Escherichia coli* in the viable but nonculturable state. *Current Microbiology*. 44(2): 125-131.

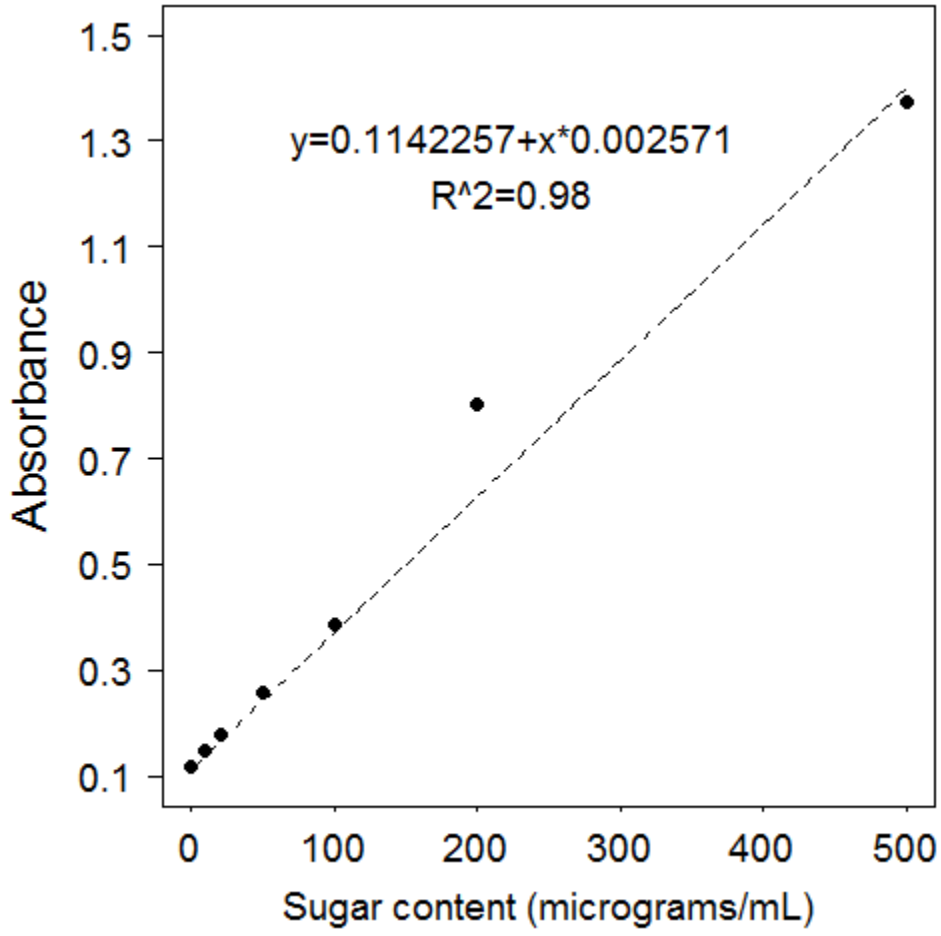
Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A., and Hirata, A. 2003. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS: Microbiology Letters*. 223(2): 287-292.

Williams, V. and Fletcher, M. 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. *Applied and Environmental Microbiology*. 62(1): 100-104.

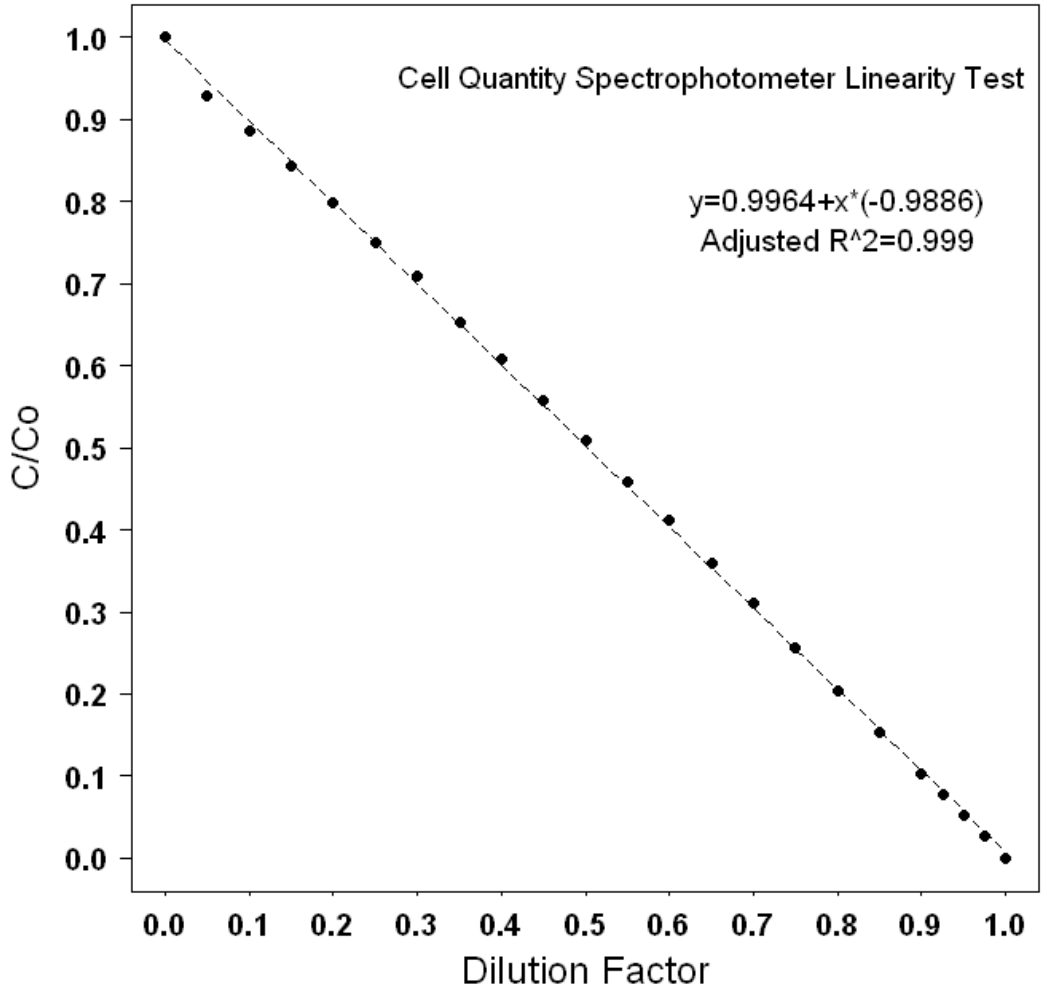
Appendix A: Standard curve for Lowry assays



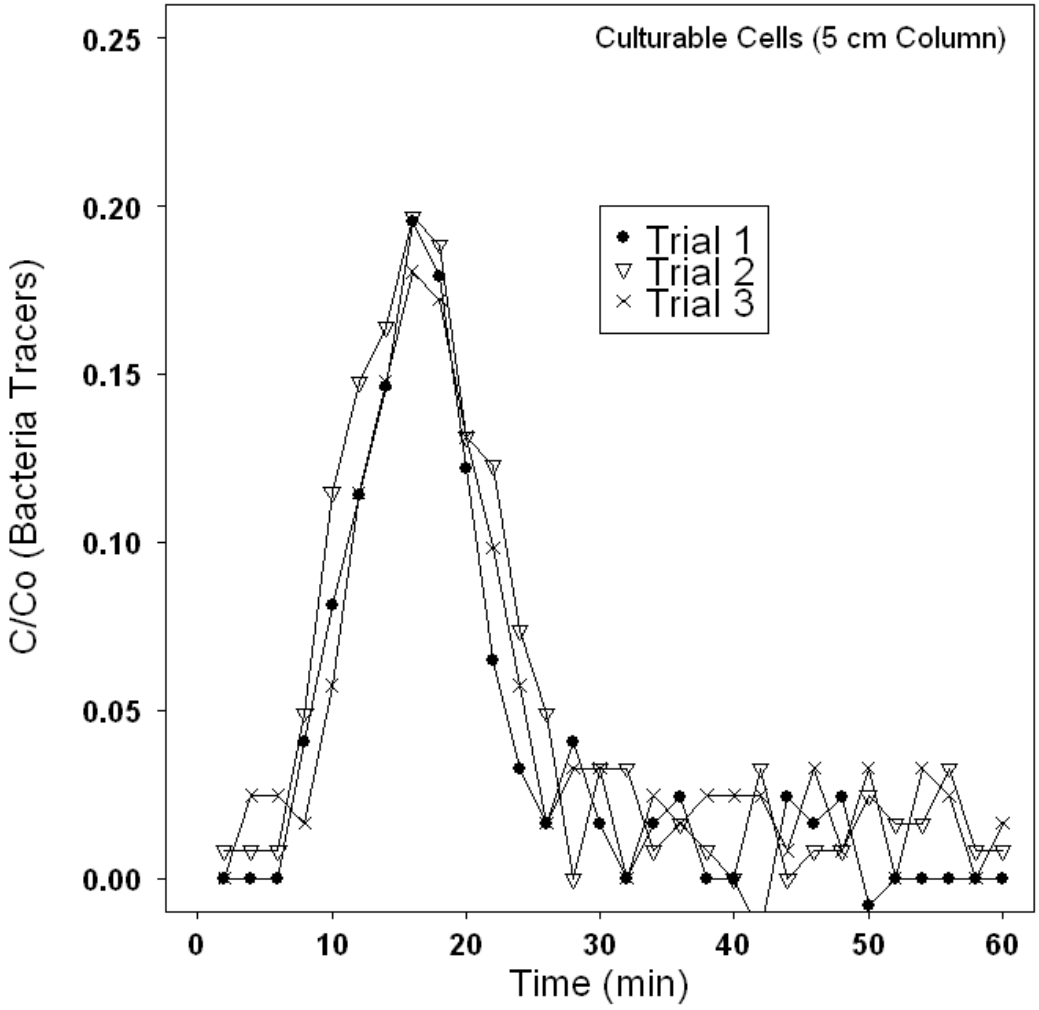
Appendix B: Standard curve for Phenol-sulfuric acid assays



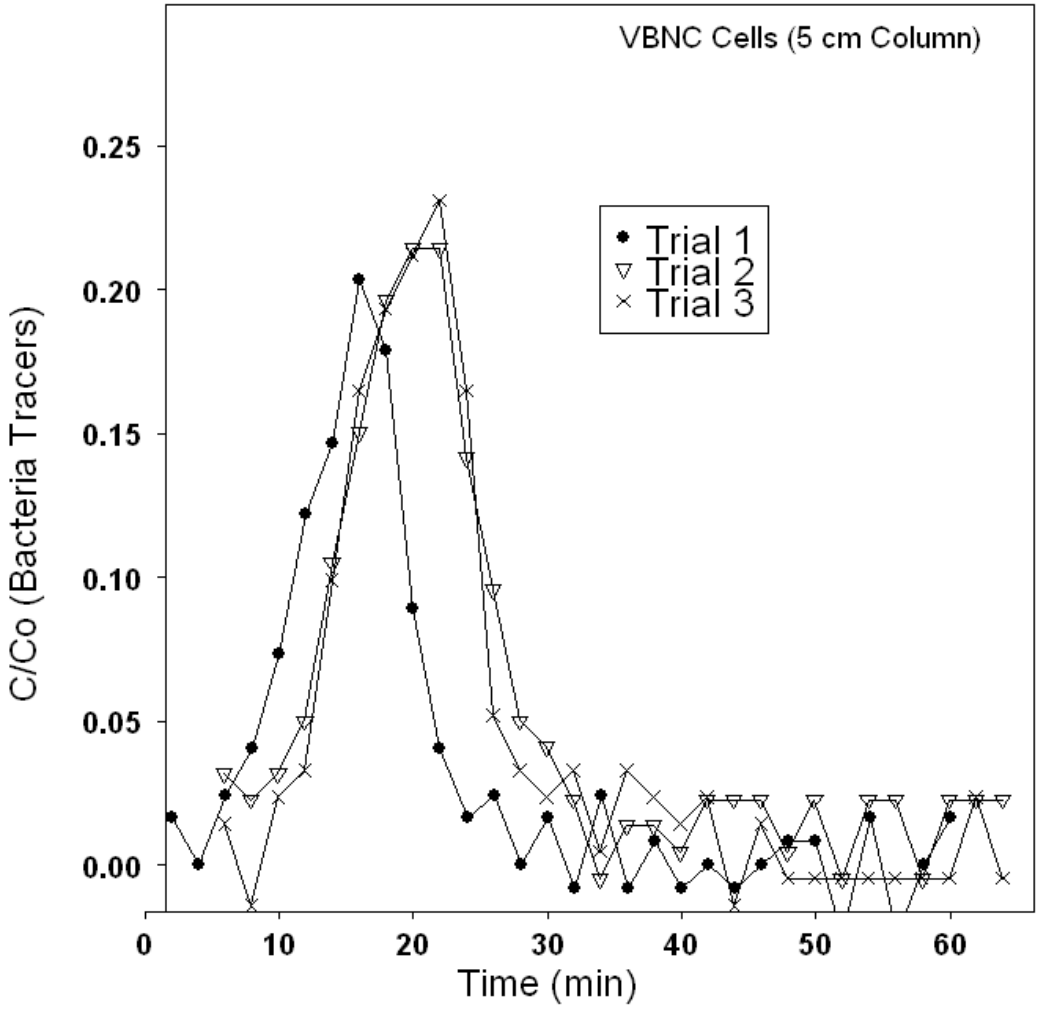
Appendix C: Standard curve demonstrating the linear relationship between cell quantity and absorbance as determined spectrophotometrically



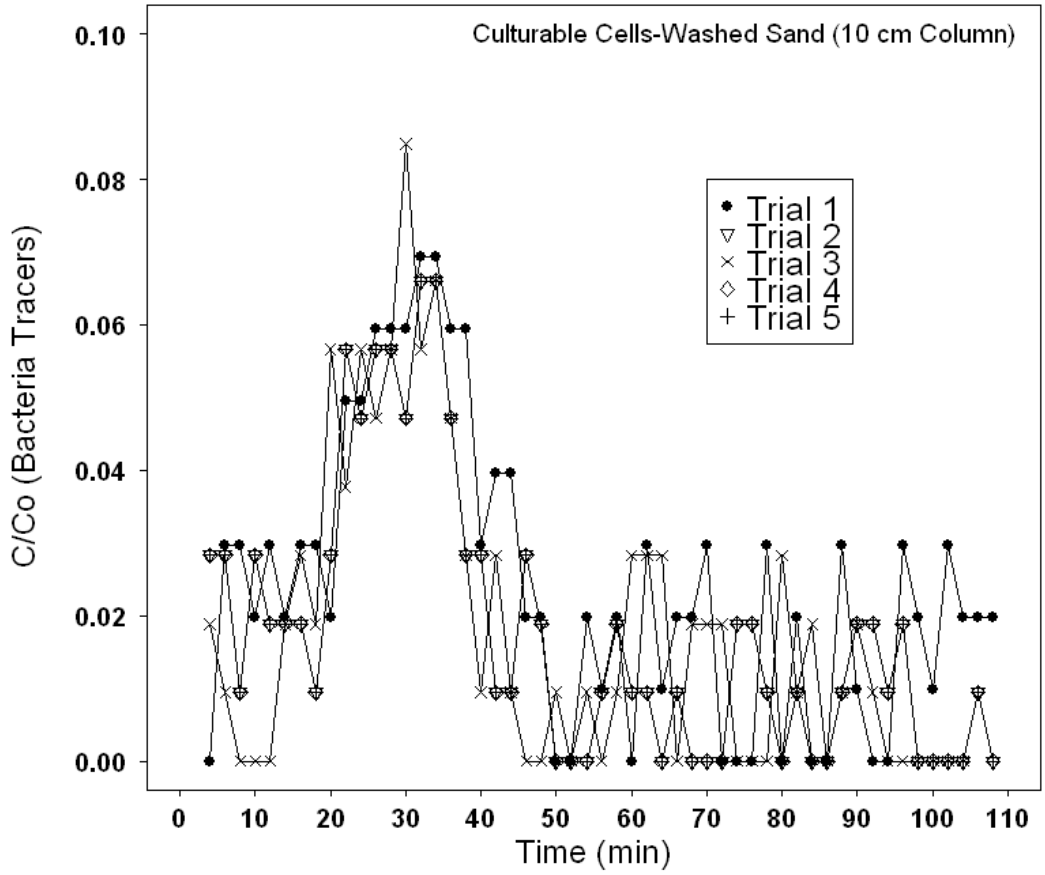
Appendix D: Breakthrough curves of culturable *E.coli* O157:H7 cells through washed sand using 5 cm columns



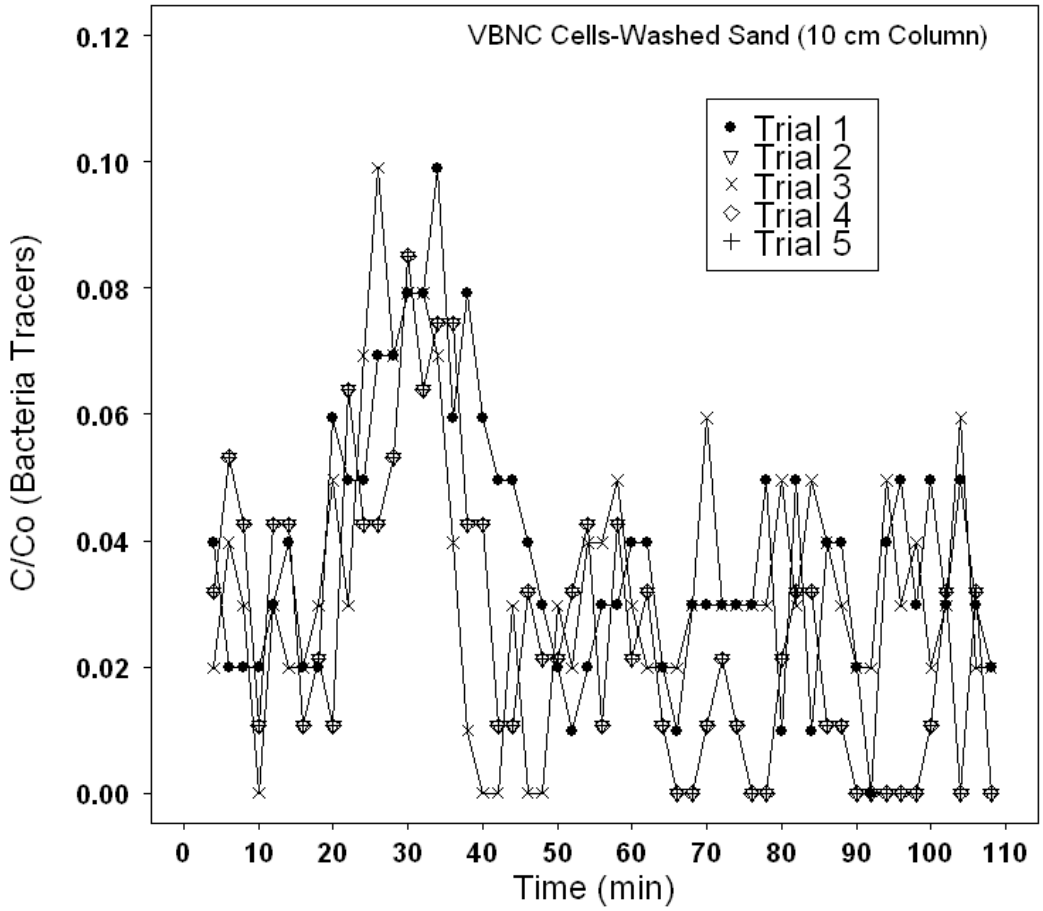
Appendix E: Breakthrough curves of VBNC *E.coli* O157:H7 cells through washed sand using 5 cm columns



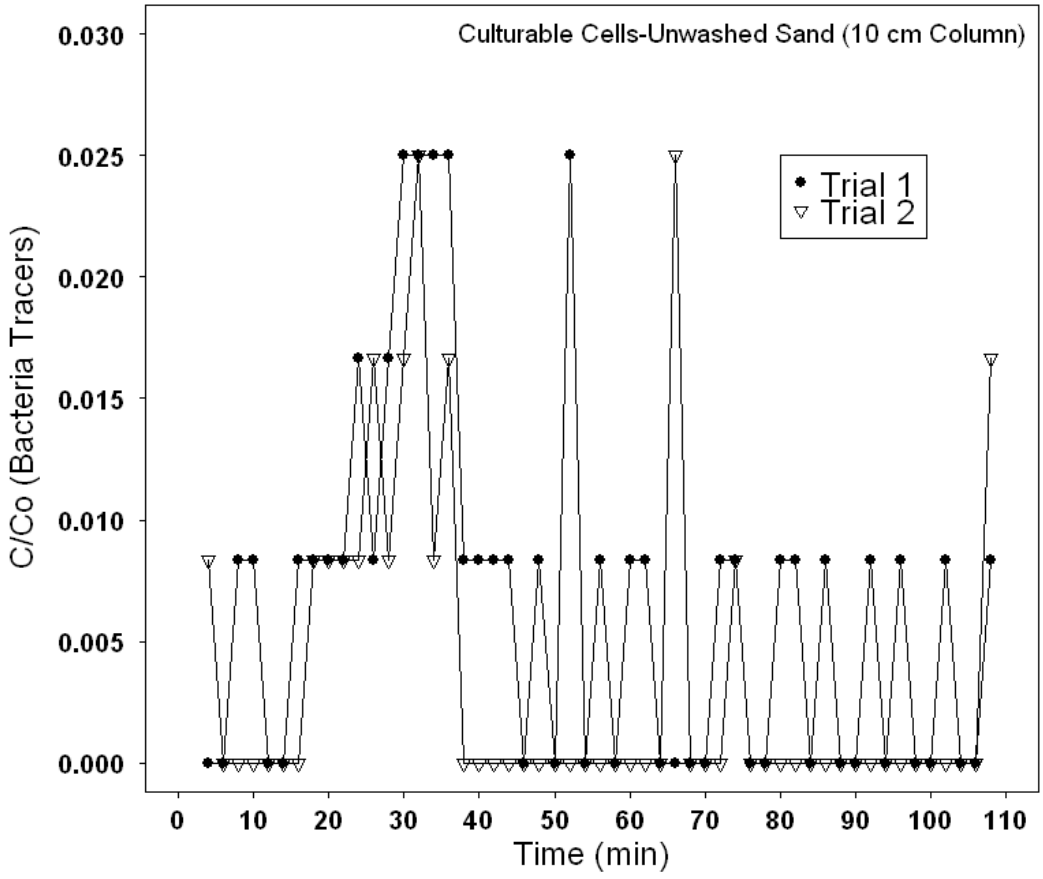
Appendix F: Breakthrough curves of culturable *E.coli* O157:H7 cells through washed sand using 10 cm columns



Appendix G: Breakthrough curves of VBNC *E.coli* O157:H7 cells through washed sand using 10 cm columns



Appendix H: Breakthrough curves of culturable *E.coli* O157:H7 cells through unwashed sand using 10 cm columns



Appendix I: Breakthrough curves of VBNC *E.coli* O157:H7 cells through unwashed sand using 10 cm columns