DANMARKS OG GRØNLANDS GEOLOGISKE UNDERSØGELSE RAPPORT 2013/18

# Survival and Tranport of Faecal Bacteria in Agricultural soils

- Influenced by slurry application method

Ph.D. Thesis 2013

Tina Bundgaard Bech

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AND NATURAL RESOUCE MANAGEMENT
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G E U S

# PREFACE

This thesis has been submitted as partial fulfilment of the requirements for the degree of Philosophiae Doctor (Ph.D.) at the Department of Geosciences and Natural Resource Management, Copenhagen University, Denmark.

This study was performed at the Department of Geochemistry, The National Geological Survey of Denmark and Greenland (GEUS), from April 2008 to March 2013. A part of this work was done at UC Davis, California, USA.

The work has been funded by three sources, a Geocenter grant (number 11-2007), the Ph.D. programme International research school of water resources (FIVA) and University of Copenhagen, Department of Geosciences and Natural Resource Management.

This thesis includes a synopsis that describes important factors influencing the survival of introduced faecal bacteria in soil and factors influencing their transport. Relevant literature and below listed papers are discussed throughout the synopsis.

**Bech, T. B.**, A. Dalsgaard, O. S. Jacobsen & C. S. Jacobsen (2011). Leaching of *Salmonella enterica* in clay columns comparing two manure application methods. Ground Water 49: 32-42.

C. S. Jacobsen & **T. B. Bech** (2012). Soil survival of *Salmonella* and transfer to freshwater and fresh produce. Food Research International 45: 557-566.

**Bech, T. B.**, M. G. M. Amin, J. Kjaer, P. Olsen & C. S. Jacobsen. Field leaching of tetracycline resistant bacteria and *Escherichia coli* from injected pig manure. Submitted to Journal of Environmental Quality.

**Bech, T. B.**, A. Sbodio, C. S. Jacobsen & Trevor Suslow. Adhesion of *E. coli* and Salmonella sp. in runoff water under influence of polyacrylamide. Ready for submission to Journal of Environmental Quality.

**Bech. T. B.** & L. Fredslund. Survival of *E. coli* in soil comparing two pig manure application methods influenced by protozoan predation. Ready for submission to Applied and Environmental Microbiology.

**Bech, T. B.**, A. Rosenbom, P. Engesgaard & C. S. Jacobsen. The effect of bromide on pesticide mineralization. Paper in preparation for Vadose Zone Journal (Appendix A1)

My work in the past five years has involved the help from several people and I would like to give a special appreciation to my principal supervisor Carsten Suhr Jacobsen, for giving me the freedom to play in the laboratory and always having an open door when I needed help; and my internal supervisor Peter Engesgaard for introducing me to the different world of modelling. Due to my own spontaneity and the unforeseen this collaboration has been limited to manuscript 6.

Furthermore, Jeanne Kjær helped extract and analyze data from the VAP field sites with an always positive approach. Both Pia Jacobsen and Christina Lynge have provided skilled technical assistance and many valuable talks. During the ups and downs throughout the Ph.D. a special thanks go to both Annette Rosenbom and Line Fredslund for continuous encouragement, inspiration and numerous conversations. Also I would like to thank the rest of my colleagues at GEUS, in particular other Ph.D. students and master students, for creating a warm and friendly work environment.

Next, I would like thank Kate Scow for providing the contact to Trevor Suslow that had the initial idea behind the experiments presented in manuscript 4. The six month in the Suslow laboratory was intense but also great thanks to all the students and Eduardo Gutiérrez-Rodríguez, Gabriela Lopez-Velasco and Adrian Sbodio.

Last by not least family and friends, in particular Nadia Glæsner and my dad for proof reading the synopsis and Justin for taking care of our children and giving me the time to finish.

Copenhagen, March 2013

Tina Bundgaard Bech

# ABSTRACT

Today, there is yearly applied 34 million tonnes of animal waste to arable land in Denmark. This waste may contain pathogenic zoonotic bacteria and/or antibiotic resistant bacteria, and when applied to arable land there is a risk of contaminating groundwater, surface water, feeding animals or fresh produce. Prediction of faecal bacterial survival and transport in the soil environment will help minimize the risk of contamination, as best management practices can be adapted to this knowledge.

The aim of this Ph.D. is to study factors influencing faecal bacteria survival and transport in soil – it is based on both field scale and lab scale experiments. The influence of application method and slurry properties has been tested on both survival and transport.

## MANUSCRIPT I

Manuscript I addresses the effect of slurry application methods in clay cores in an outdoor multi-column lysimeter. The average proportion of leached *S. enterica* was 6.1% after injection and 0.6% after surface application. This large difference was not significant due to high variability among replicates. In addition, comparison of enumerations by selective plating and real-time PCR yielded similar concentrations of *S. enterica*, indicating that mainly viable and culturable cells were leached from the columns. Fluorescent dye Acid Yellow was applied to four selected columns and the distribution and size of active (dye-stained) pores was quantified. The profiles showed that the area covered by active pores ranged from 0.1% to 3.6%. The relatively small fraction of active pores in the soil profile was consistent with the evidence of rapid transport of *S. enterica* and chloride in the columns.

## MANUSCRIPT II

Manuscript II is a review that summarizes recent literature of the ecology of *Samonella* sp. in the soil environment including sources, survival, transport, and crop contamination. Furthermore, detection of *Salmonella* in environmental samples is discussed.

## MANUSCRIPT III

Manuscript III addresses the survival and leaching of tetracycline-resistant bacteria in comparison with faecal-indicator *E. coli* at two field sites. The bacterial survival did not differ

significantly between tetracycline-resistant bacteria and *E. coli* at both sites. Leaching of faecal bacteria was only detected at the Estrup site, where leaching of tetracycline-resistant bacteria reached 130 CFU $mL^{-1}$ in the drainage water. Preferential flow appears to be the dominant pathway. Even though tetracycline-resistant *E. coli* was seen in the manure it was not found among the tetracycline-resistant bacteria in the drainage water. Findings from this research suggest that faecal bacteria are leached under unsaturated conditions and that *E. coli* may not be suited as indicator bacteria for the leaching of tetracycline-resistant bacteria.

## MANUSCRIPT IV

Manuscript IV describes the effect of polyacrylamide (PAM) on bacterial adhesion and surface runoff from clay, clay loam and sandy loam soil. Four bacteria with different motility and hydrofobicity were tested: *E. coli*, *E. coli* O157:H7, *S*. Newport and *S*. Poona. PAM reduced bacterial adhesion with the largest decrease in the sand loam soil followed by clay and clay loam. The polyacrylamide effect depended on soil type, and from the clay soil polyacrylamide significantly increased both adhered and planktonic cells as compared to the control whereas a decrease was observed with the other two soils with the PAM treatment. Comparison among the four bacteria during runoff studies showed that *E. coli* was leached the most whereas *S*. Poona was retained in the soil. Motility and hydrophobicity could not explain this observation. The results of this laboratory-based study question the capability of PAM to protect surface water from pathogen contamination.

## MANUSCRIPT V

Manuscript V addressed differences in *E. coli* survival influenced by slurry type and slurry application method. Incorporation of slurry and liquid slurry increased *E. coli* decay rate in the soil compared to injection. *E. coli* survived for a longer period of time with liquid slurry than with slurry. This was possibly explained by an initial larger increase in protozoan numbers with the slurry treatment.

## MANUSCRIPT VI

Manuscript VI addresses a different topic and is a “lucky” spin off from testing the effect of bromide on the culturability of faecal bacteria. Bromide is often used as a conservative tracer in soil transport studies; however hitherto the effect of bromide on the soil microbial community has not been tested. Therefore, in the present study the effect of bromide on mineralization of glucose and the three pesticides: glyphosate, MCPA and metribuzin were tested in four

agricultural A-horizon soils representing the majority of soil types found in Denmark. A planned modelling of the bromide inhibition needs to be included before processing the data. The final discussion of the results has therefore not been possible before the deadline of the thesis. As a preliminary conclusion we recommend not to exceed an overall concentration 1 g Br $L^{-1}$. Manuscript VI is placed in appendix 1 because of the different topic as well as still being in progress.

# RÉSUMÉ

I Danmark bliver der årligt udbragt 34 millioner tons gylle på landbrugsjord, som kan indeholde patogene zoonotiske bakterier og/eller antibiotika resistente bakterier. Når gyllen udbringes på landbrugsjord er der risiko for at kontaminere grundvand, ferskvand, dyr og afgrøder. Viden om fækale bakteries overlevelse og transport i landbrugsjord kan medvirke til at minimere risikoen for kontaminering, eftersom "godt landmandsskab", kan tilpasses denne viden.

Formålet med denne Ph.D. afhandling er at uddybe vores viden om fækale bakteriers overlevelse og transport i jord – afhandlingen er baseret på både laboratorium og markskala forsøg. Betydningen af udbringningsmetoden samt gyllens tørstofindhold er blevet undersøgt for både overlevelse og transport.

## MANUSKRIPT I

Manuskript I adresserer effekten af gylle-udbringningsmetoden i ler søjler i et multi-kolonne lysimter. Den gennemsnitlige andel af udvaskede *S. enterica* var 6,1 % efter injektion og 0,6 % efter overfladeudbringning. På trods af den store forskel, var der ikke signifikant forskel på de to udbringningsmetoder, grundet en stor variation mellem søjlerne. Sammenligning af dyrkbare og ikke dyrkbare (real-time PCR) bakterier viste, at hovedsageligt dyrkbare bakterier blev udvasket. Distribution og størrelsen af aktive porer blev undersøgt med farvestoffet Acid Yellow. De forskellige jordsnit viste, at aktive porer udgjorde 0,1-3,6 % af det totale areal. Den relative lille andel af aktive porer stemmer overens med hurtig præferentiel udvaskning af *S. enterica* og chlorid.

## MANUSKRIPT II

Manuskript II er et review, som opsummerer nyere litteratur omhandlende *Salmonella* i jordmiljøet med fokus på kilder, overlevelse, transport og kontaminering af afgrøder. Yderligere bliver detektionsmetoder af *Salmonella* i miljøprøver diskuteret.

## MANUSKRIPT III

Manuskript III er baseret på et markskale forsøg, hvor overlevelse og transport af tetracyclin-resistente bakterier i forhold til fækal indikator bakterie *E. coli*, er undersøgt. Der var ikke signifikant forskel på overlevelsen af de to bakterier på hverken Silstrup eller Estrup lokaliteten. Udvaskning af fækale bakterier blev kun fundet på Estrup lokaliteten, hvor den højeste målte koncentration af tetracyclin-resistante bakterier var 130 CFU $mL^{-1}$ i drænvandet. Præferential

strømning var den dominerende rute for udvaskning af fækale bakterier. På trods af, at der blev fundet tetracyclin resistent *E. coli* i gyllen, blev disse ikke genfundet i drænprøverne. Resultaterne fra dette forsøg viser, at fækale bakterier blev udvasket under umættede forhold, samt at *E. coli* ikke er den bedst egnede fækale indikator for udvaskningen af tetracyklin-resistante bakterier.

## MANUSKRIPT IV

Manuskript IV omhandler effekten af polyacrylamid (PAM) på adhering af bakterier i overfladeafstrømning fra tre jorde. Fire bakterier med forskellig motilitet og hydrofobicitet blev undersøgt: *E. coli*, *E. coli* O157:H7, *S*. Newport and *S*. Poona. PAM reducerede den bakterielle adhering til de tre jorde med aftagende effekt for sandblandet lerjord, svær lerjord og lerjord. Effekten af PAM ved overfladeafstrømning afhænger af jordtypen. Fra den svære ler jord blev der udvasket flere adherede og planktoniske bakterier med PAM behandlingen i forhold til kontrol jorden, hvorimod PAM behandlingen reducerede udvaskningen af bakterier fra lerjorden og den sandblandede lerjord. Ved sammenligning af de fire bakterier blev *E. coli* udvasket i størst grad, hvorimod *S*. Poona blev kraftigst tilbageholdt i jorden. Motilitet og hydrofobicitet kunne ikke forklare denne forskel. Resultaterne fra dette forsøg sætter spørgsmålstegn ved hvorvidt en PAM behandlet jord yder ekstra beskyttelse mod kontaminering af ferskvand.

## MANUSKRIPT V

Manuskript V omhandler overlevelsen af *E. coli*, hvor de to gylle udbringningsmetoder nedfældning og pløjning sammenlignes for to forskellige gyller. Ved nedfældning var henfaldsraten af *E. coli* mindre sammenlignet med pløjning. *E. coli* overlevede længst med den gylle, som havde det laveste tørstofindhold. Dette kan muligvis forklares med en initial større opblomstring af protozoer med gyllen med højeste tørstofindhold.

## MANUSKRIPT VI

Manuskript VI omhandler nedbrydning af pesticider i jord, og udspringer fra om bromid har en indvirkning på dyrkbarheden af fækale bakterier. Bromid bliver ofte brugt som konservativ tracer i transport studier i jord. Til dags dato er det dog ikke blevet testet om bromid har en indvirkning på det mikrobielle samfund i jorden. Derfor testede vi, om bromid har en effekt på mineraliseringen af glukose samt de tre pesticider glyphosat, MCPA og metribuzin i fire A-horisont landbrugsjorde. Disse jorde repræsentere hovedparten af danske jorde. For at kunne

analysere data færdigt er der planlagt en modellering af data, med formål at kvantificere en bromid inhiberings-faktor. Som foreløbig konklusion anbefaler vi, at koncentrationen af bromid ikke overstiger 1 g $L^{-1}$. Manuskript VI er placeret i Appendix 1, fordi emnet afstikker fra de andre fem artikler og stadig er under udarbejdelse.

# CONFERENCE CONTRIBUTIONS

**Bech, T.B**., Sbodio, A., Jacobsen, C.S. & Suslow, T (2012) Attachment and run-off of *E.coli* sp. and *Salmonella* sp. under influence of polyacrylamide. International society of Microbial Ecology (ISME) 2012. Copenhagen, Denmark. (Poster)

**Bech, T.B.,** Amin, M., Olsen, P., Kjær, J. & Jacobsen, C.S**.** (2012) Leaching and survival of tetracycline resistant bacteria in field scale experiments. International society of Microbial Ecology (ISME) 2012. Copenhagen, Denmark. (Poster)

Lægdsmand, M. Amin, M., Kjær, J. **Bech, T. B**., Forslund, A., Hansen, M., Fredslund, L., Petersen, S. O. & Jacobsen, C. S. (2012) Præsentation af smitstoffer og steriod-østrogener, og om hvorvidt de kan udvaskes fra marker gødet med gylle, og om risikoen ændres ved separering af gyllen. Præsenteret ved den årlige Plantekongres 2012.

**Bech, T.B.,** Amin, M., Olsen, P., Kjær, J. & Jacobsen, C.S**.** (2012) Leaching and survival of tetracycline resistant bacteria in field scale experiments. SETAC, Milano, Italy. 15-19 May 2011 (Poster)

**Bech, T.B.,** Amin, M., Olsen, P., Kjær, J. & Jacobsen, C.S**.** (2012) Leaching and survival of tetracycline resistant bacteria in field scale experiments. ASM general meeting. San Diego, USA 23-27 May 2010 (Poster)

Amin, M. G. M., **Bech, T**., Forslund, A., Petersen, S. O. & Lægdsmand, M. (2010): Redistribution of contaminants from pig slurry after direct injection into soil. Agricultural Engineering Conference, France. 6-8 September, 2010.

**Bech, T. B**., Forslund, A., Jacobsen, O. S., Dalsgaard, A., Lægdsmand, M. & Jacobsen, C. S. (2009): Comparing CFU data with RealTime PCR targeting invA DNA in leachate from intact clay columns. Bacterial Genetics and Ecology - coexisting on a changing planet. Uppsala, Sweden 2009.

Forslund, A., Tønner-Klank, L., **Bech, T**., Jacobsen, C. S. & Dalsgaard, A. (2009): Transport and survival of *Salmonella* Typhimurium bacteriophage 28B and Cryptosporidium parvum from slurry applied to intact clay soil cores. FEMS 2009 - 3rd Congress of European Microbiologists, Gothenburg, Sweden. June 28 - July 2, 2009. Abstract and Poster presentation no.: 180, page 149 in Abstract Book.

**Bech, T. B**., A. Dalsgaard, O. S. Jacobsen & C. S. Jacobsen (2008) Leaching of *Salmonella* Senftenberg and Cryptosporidium Parvum in intact clay columns. AGU San Francisco 15-19 December (oral).

## List of Peer-reviewed papers not included in this Ph.D. thesis

M.G. Mostofa Amin, **Tina B. Bech**, Anita Forslund, Martin Hansen, Søren O. Petersen, and Mette Lægdsmand (2013) Redistribution and Persistence of Microorganisms and Steroid Hormones after Soil-Injection of Slurry. Submitted to Vadoze zone Journal

Forslund, A., Markussen, B., Toenner-Klank, L., **Bech, T.B.**, Jacobsen, O. S., Dalsgaard, A. (2011) Leaching of Cryptosporidium parvum Oocysts, *Escherichia coli*, and a Salmonella enterica Serovar Typhimurium Bacteriophage through Intact Soil Cores following Surface Application and Injection of Slurry. Applied and Environmental Microbiology 77: 8129-8138.

**Bech, T.B**., Johnsen, K., Dalsgaard, A., Laegdsmand, M., Jacobsen, O.H. and Jacobsen C.S. (2010) Transport and distribution of *Salmonella enterica* serovar Typhimurium in loamy and sandy soil monoliths with applied liquid manure. Applied and Environmental Microbiology 76:710-714.

# TABLE OF CONTENTS

# 1 INTRODUCTION

Animal waste[1] has been applied as a fertiliser in agriculture for centuries because it contains nutrients that are essential for plant growth, and when applied correctly result in enhanced crop yield due to increased soil fertility and soil quality (Diacono & Montemurro 2010). Today, 34 million tonnes of animal waste is yearly produced in Denmark, which has a substantial impact on the surrounding environment when applied to arable land. This has led to regulations regarding animal waste application to arable land with focus on elevated nitrate concentrations in groundwater and nitrogen and phosphorus in surface water. Animal waste application is regulated by volume and time of the year for application. Current regulations allow waste application to arable land from 1st of February or after frost to harvest, however this may be extended to 15/11 depending on crop cover (Danish Ministry of the Environment 2012).

Unfortunately, much less attention has been given to zoonotic bacterial pathogens in animal waste – bacteria capable of causing disease in both animals and humans. These have been associated with drinking water in Denmark (Ethelberg *et al.* 2005; Brüsch & Rosenberg 2008; Gubbels *et al.* 2012), and have had major impact on global human health during the last century. Jones *et al.* (2008) analysed approximately 300 outbreaks of human disease associated with new species or variant of an infectious agent from 1940 to 2004 and concluded that 60 % of the events were caused by zoonoses and 20 % of the events were associated with drug-resistance.

Zoonotic pathogens can be transported from animal to humans when animal waste is applied as fertiliser on crops eaten raw, by storm water runoff to recreational waters or by its percolation to drinking water reservoirs. Contamination of the surrounding environment may originate as either point or non-point sources. Point sources of faecal contamination include leaking storage tanks and manure piles. Opposite, non-point sources are the spreading of animal waste to arable land, which most likely is the dominant source and the most difficult to control. In order to assess the risk of pathogen contamination of food and water resources it is necessary to understand factors influencing pathogen fate in the agricultural environment. Despite extensive prior research, many gaps still remain in our knowledge of pathogen fate due to a diverse array of processes that influences pathogen survival in agricultural environments as well as complicated transport patterns by either runoff water or vadoze zone processes in which pathogens may migrate (Unc & Goss 2004; Pachepsky *et al.* 2006; Foppen & Schijven 2006; Bradford & Torkzaban 2008; Bradford *et al.* 2012). Decay studies in soil consistently suggest

[1] General term not differencing between animal source nor dry matter content

that outside the host animal, faecal bacteria do not survive well (Franz *et al.* 2005; Sinton *et al.* 2007; Semenov *et al.* 2009). Therefore, land management options may be a relevant approach to accelerate cell death by avoiding management practices likely to prolong survival. Avery *et al.* (2004) concluded that *E. coli* O157 persisted longer with subsurface injection compared to soil surface application. Traditionally, incorporation of animal waste has been achieved by soil tillage following surface application of manure. Injection of animal waste is an alternative that ensures immediate slurry incorporation. Injection can be performed in established crop and is increasingly used by Danish farmers to minimize odour and ammonia emissions (Nyord *et al.* 2008). However, there is scarce information comparing injection and incorporation of animal waste.

Therefore, this thesis focuses on how injection of animal waste into the soil matrix influences the survival and transport of faecal bacteria in agricultural soil compared to incorporation. It is important to identify how injection of animal waste influences the fate of pathogens in agricultural settings compared to more traditional application methods in order to minimise contamination of the surrounding environment. In order to address how the shift towards injection influences the fate of faecal bacteria this thesis consists of a synopsis in which current state of knowledge is reviewed. It consists of four main sections: **Section I** presents a brief overview of pathogen sources and Danish groundwater contamination. **Section II** reviews the current understanding of pathogen survival in soil. **Section III** discusses adhesion in soil, and **Section IV** discusses factors that influence transport of pathogens in soil.

## 1.1 OBJECTIVES

The main objective of this thesis was to address the following research questions:

I. How does slurry injection influence leaching of faecal bacteria (MS 1) (MS 3)

II. How does slurry injection influence survival of faecal bacteria (MS 3; MS 5)

III. How does polyacrylamide influence surface run-off of faecal bacteria (MS 4)

In addition to the objectives presented above, an additional study was carried out examining the addition of bromide, as a conservative tracer, and how this influences soil bacteria:

IV. Does the conservative tracer bromide effect indigenous soil bacteria (MS 6)

# 2 NON-POINT PATHOGEN OUTBREAKS

The presence of faecal bacteria in drinking water may originate from point or non-point sources. There is hitherto no clear evidence that contamination of drinking water may originate from non-point faecal sources. Non-point contamination is much more difficult to characterize because of temporal variability in pathogen occurrence and concentration. In addition transport is influenced by the hydrological pathways. It is though generally accepted that catchment source of faecal pollution occurs as a result of rainfall (Curriero *et al.* 2001; Gentry *et al.* 2007).

Catchment sources of faecal contamination may include: sewage sludge and animal wastes applied to land; animal feces deposited on land by grazing animals; discharges from septic tank systems; and indigenous fauna. The probability for pathogens to be available for transport is most likely influenced by duration and conditions of storage prior to land application. See section 2.1.1 (MS 2) for discussion of manure storage and effect on pathogen survival.

## 2.1 TRANSMISSION

Outbreaks originating from the land application of animal waste may occur through various routes (see Figure 2 in MS 2). Below are listed dominant routes:

- **Drinking water/surface water** may be contaminated through infiltrating water and surface water by surface run-off.
- **Fresh produce** may be contaminated through irrigation water or bacterial strains that have persisted in the soil.
- **Grazing animals** may be contaminated through drinking water or bacterial strains that have persisted in the soil.

Fresh produce in the US is responsible for the second highest number of outbreaks and infects the highest number of people (see Figure 1 in MS 2). As reviewed in MS 2 contaminated irrigation water was often the source of the contaminated fresh produce. The US Environmental Protection Agency (EPA) assessed 33% of US water in 2000 and found that of these 40% of streams, 45% of lakes, and 50% of estuaries were not clean enough to support fishing and swimming (US EPA 2000). More recently the EPA ranked “Agriculture” as the most probable source of threatened or impaired rivers and streams (US EPA 2013).

## 2.3 CONTAMINATION OF DANISH WATER

Groundwater is an important source of drinking water in many countries. The water supply in Denmark consists almost exclusively of untreated groundwater. For this reason, Danish drinking water is often considered free of pathogens. However, as seen in Figure 2.1, *E. coli* has been found in Danish groundwater during the last 30 years. *E. coli* was found in approximately 5% of the tested groundwater samples in concentrations exceeding the threshold limit of 1 CFU $100mL^{-1}$. By visual inspection there is a clear correlation between clay soil properties and positive findings of *E. coli* in groundwater wells.

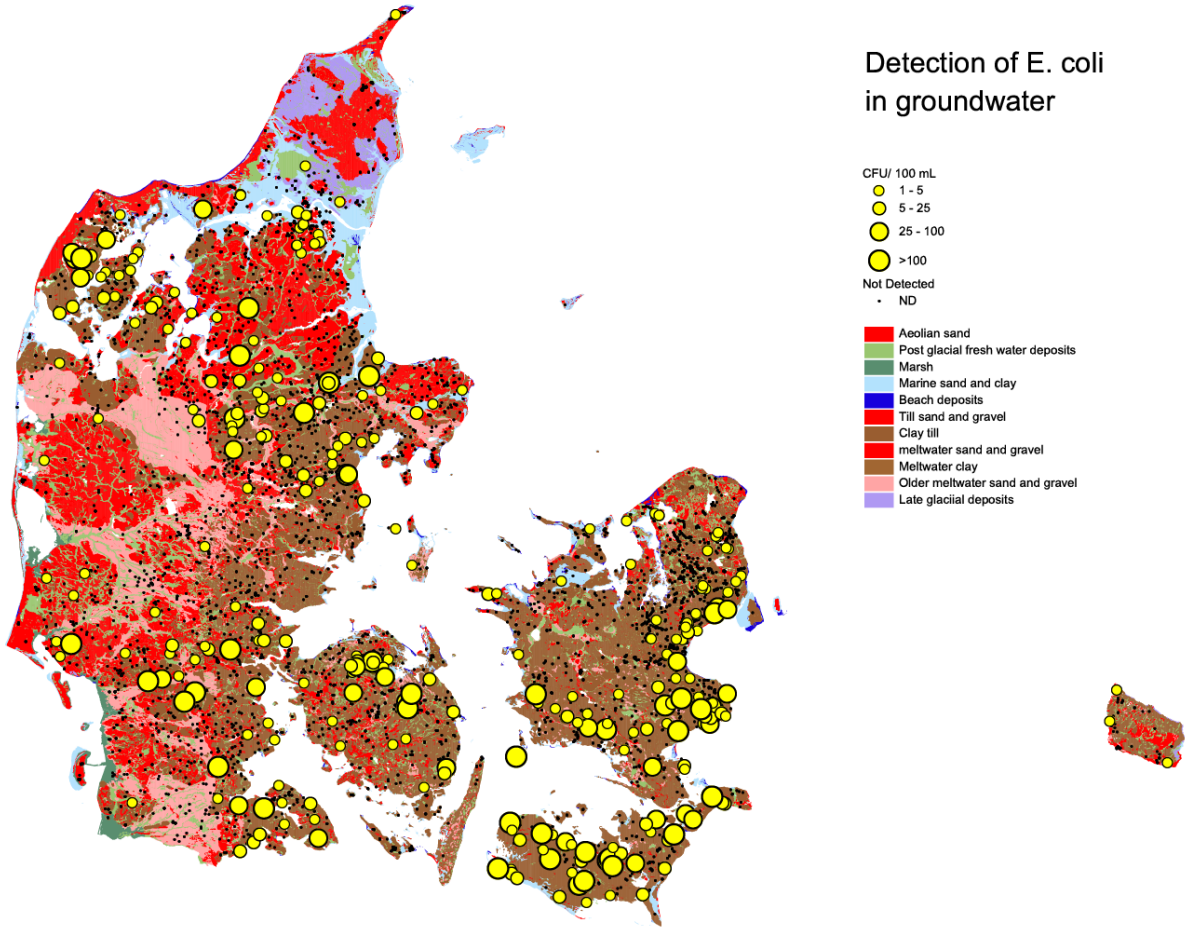


**Figure 2.1** Approximately 5000 groundwater samples have been tested for the presence of *E. coli* from 1982 to 2012. This data have been extracted from the Jupiter database on 04/02-2013 by Walter Brüsch.

Nevertheless, when comparing average monthly precipitation, number of contaminated groundwater samples and average *E. coli* concentration per month, there is no clear trend between precipitation and groundwater contamination (Figure 2.2). However, a detailed study

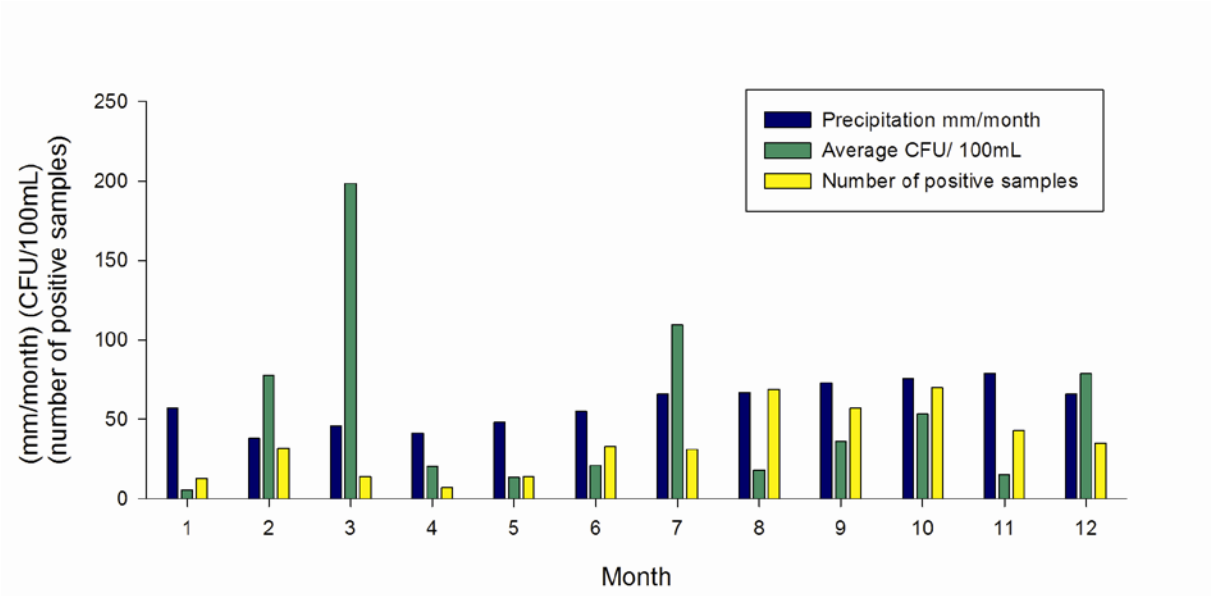


**Figure 2.2** Monthly average precipitations during the last 50 years (DMI); numbers of *E. coli* findings per month and max *E. coli* concentration per month.

of local precipitation intensities on a daily basis may show a better correlation with the contamination events. Considering the large volumes of animal waste applied to arable land in Denmark there are relatively few findings of faecal pollution in groundwater. Listed below are relevant factors that all influence the likelihood for a contamination event to take place:

- Sufficient high concentration of the pathogen in the animal waste.
- Survival time in the soil environment.
- Time between waste application and rainfall.
- Rainfall must be sufficient to induce a rapid preferential flow.
- Contamination of groundwater must remain in a sufficient high concentration.

These factors may all have been fulfilled during the outbreak of *Campylobacter Jejuni* in the city of Køge, Denmark, from 14–20 May 2010. Heavy rainfall on the 12$^{th}$ of May, and a *C. Jejuni* incubation time of 2-5 days, makes it more than likely that the contamination originates from a non-point source. The soil type in the Køge area is clay till that facilitates rapid preferential flow. However, Gubbels *et al.* (2012) argued that a non-point source was not the original source because previous heavy rainfall has not lead to contamination; still the source remains unknown.

# 3 PATHOGEN SURVIVAL IN SOIL

Prediction of faecal bacterial survival in the soil environment could minimize the risk of contaminating surface and groundwater resources if best management practices could be adapted to this knowledge. Since the survival is complex and influenced by several factors (table 3.1), a universal model describing decay has hitherto not been successful. Section 3.1 discusses important parameters influencing survival in soil, and 3.2 present theoretical modelling of bacterial decay.

## 3.1 FAECAL BACTERIAL DIE-OFF

Pathogens can, to a varying extent, persist in different environments such as manure, soil and water (Sandvang *et al.* 2000; Islam *et al.* 2004; Franz *et al.* 2005; Semenov *et al.* 2009; Semenov *et al.* 2011). When resource availability and important abiotic conditions are favourable populations of for example *E. coli* can survive and even grow in the soil (Gagliardi & Karns 2000; Berry & Miller 2005). However, with the fluctuating conditions seen in the soil environment growth may be limited and a gross bacterial death will take place in the soil environment (Semenov *et al.* 2007). Growth and death rates are determined by environmental conditions as well as adaption strategy of bacteria. In general, any of the factors in Table 3.1 can cause reduction of faecal bacterial population when the factor becomes limiting or excessive in the soil environment. The dominating factor may vary over time as a result of for example changes in soil water content due to precipitation. This dynamic system is most likely the reason for the contradictory results found in the literature.

### 3.1.1 PREDATION

In arable soil the protozoan population range from l0,000 to 100,000 per gram of soil decreasing with depth. Protozoa play an important role in soil, as predation of bacteria influences the soil microbial dynamics, contribute to organic matter degradation and nutrient release. Among soil size fractions protozoa are most abundant in the silt fraction. The silt fraction also contains the highest number of viable bacteria as well as the highest carbon content (Postma & vanVeen 1990; Winding *et al.* 1997). Interaction between indigenous soil predators and introduced bacteria has a profound impact on pathogen survival in soil. Important soil predators include bacteria feeding nematodes and protozoa. Jiang *et al.* (2002) found that in an autoclaved soil there was a greater survival of *E. coli* O157:H7 compared to a non-treated soil. Other studies have studied more specific inhibition of predators by using e.g. cycloheximide (inhibition of

protein synthesis in eukaryotic organism) which has confirmed correlations between microbial population size and grazing by protozoa (Heynen *et al.* 1988; Wright *et al.* 1993; Manzano *et al.* 2007; Badawi *et al.* 2012). This correlation between abundance of protozoa and bacterial decline in soil has also been shown for individual strains, such as *Rhizobium Leguminosarum* (Heynen *et al.* 1988), *Pseudomonas fluorescens* (Wright *et al.* 1993) and *E. coli* (Manzano *et al.* 2007).

**Table 3.1** Factors influencing the survival of enteric bacterial soils (Crane & Moore 1986; vanVeen *et al.* 1997).

| *Origin* | *Factor* | *Effect* |
|---|---|---|
| Biotic | Predation | Decrease population size |
| | Competition/diversity | Decrease population size |
| Abiotic | Soil texture | Fine texture protects from predation |
| | Soil water content | Low water content: water shortage, high osmolarity; High water content: anaerob conditions, increased nutrient availability by diffusion |
| | Organic carbon | Limited organic carbon results in starvation and reduction in activity |
| | Inorganic nutrients | Limitation result in starvation |
| | Temperature | Low temperature increase survival |
| Management | Application method | Survival is influenced by application method, however currently there is no clear trend |
| | | A high frequency may increase survival |
| | | High bacterial density increase survival time |

In the study by Manzano *et al.* (2007) the survival of *E. coli* was furthermore compared with survival of soil bacteria *Cupriavidus necator*, where complete protection only was found for *C. necator*, indicating that other factors than protozoan predation influenced survival of *E. coli* in soil. This was confirmed in a study by Recorbet *et al.* (1992) where a delayed die-off of *E. coli* in the absence of protozoa was observed.

Studies combining the effect of animal waste, soil predation and faecal bacterial survival are limited. Application of animal waste or organic matter to soil has been shown to increase the microbial biomass including protozoa and nematodes (Peacock *et al.* 2001; Treonis *et al.* 2010).

A recent study by (Garcia *et al.* 2010) found that survival of *S*. Typhimurium was significant less in the presence of manure compared to a soil without manure. In contrast Oliver *et al.* (2006) found faster *E. coli* decay when applied through water compared to application through slurry or manure[2]. Garcia *et al.* (2010) explained the decreased survival in the presence of manure with a larger increase in protozoan numbers. In MS 5 a greater increase in protozoan numbers was seen with the slurry with the higher dry matter content, which might explain the shorter survival time compared to the liquid slurry treatment.

### 3.1.2 COMPETITION

The general decrease of introduced bacterial strains into the soil may be explained by the old paradigm that most ecosystems are microbiostatic. This means that ecological niches are filled and difficult to invade. The precise influence of the native microbial diversity and community structure on survival of introduced bacteria is unclear, both factors are believed to be important (vanVeen *et al.* 1997; Semenov *et al.* 2008; van Elsas *et al.* 2012).

Animal waste application to agricultural land increases soil microbial biomass (Peacock *et al*. 2001; Bittman *et al*. 2005). The soil microbial changes are influenced by the volume and degradability of the waste product. Easily degradable compounds such as organic acids in insoluble or soluble organic matter can be degraded by several organisms. The presence of these compounds may result in a rapid increase in biomass and favours the growth of copiotrophic compared to oligotrophic organisms (Fierer et al. 2007). Copiotrophs have high nutritional requirements, and may exhibit high growth rates when resource conditions are abundant. In contrast, oligotrophs exhibit slower growth rates and are likely to outcompete copiotrophs in conditions of low nutrient availability due to their higher substrate affinities (Fierer *et al.* 2007).

Manure with high dry matter content will generally have a high C/N-ratio whereas slurry with less dry matter will have a low C/N-ratio. Therefore, the use of slurry tends to favour copiotrophs such as faecal bacteria. Van Elsas *et al.* (2012) found that the utilization of 10 different carbon types resulted in a *E. coli* O157:H7 growth rate that were approximately three times slower compared with 40 different soil bacteria. The difference in consumption rate would result in greater survival of the faster soil species because there is decreased probability of less-competitive strains to increase.

[2] Manure< 90% liquid; Slurry 90–96% liquid; Liquid slurry>96% liquid.

Influence of slurry or manure properties on faecal bacteria decay was studied in MS 3, where the survival of both *E. coli* and tetracycline resistant bacteria were shorter from the Silstrup slurry with a dry matter content of 6.3% compared to Estrup liquid slurry with a dry matter content of 0.8%. A similar result was seen in MS 5, where *E. coli* survived for a shorter period of time with the slurry treatment compared to the liquid slurry treatment. Other studies have also seen a decreased survival of *E. coli* O157:H7 with manure (Semenov et al 2009; Franz *et al.*, 2005). However, Semenov *et al.* (2009) observed the opposite result for *S*. Typhimurium where a longer survival in a manure amended soil was observed. Oliver *et al.* (2006) found that the *E. coli* decay rate decreased when applied to soil in the following order water>slurry>manure. The longer survival with manure was explained by *E. coli* remaining associated with manure aggregates that would provide protection and nutrients. Strain/serovar specific adaptation strategies may explain these contradictions along with differences in experimental setup.

The competitiveness of the native soil population is influenced by soil type and management history. Franz *et al.* (2008) found that the survival of *E. coli* O157:H7 tended to increase when the soil had a history of low quality manure (artificial fertilizer and slurry) compared to high quality manure (farm yard manure and compost). Organically managed soils show a higher diversity of bacteria and higher microbial biomass than conventionally managed soils (Bittman *et al.* 2005). Such characteristics make them less susceptible to introduced faecal bacteria (van Bruggen & Semenov 2000). Van Elsas *et al.* (2012) found a negative correlation between soil microbial diversity and survival of the introduced *E. coli* O157:H7. Furthermore, the amounts of applied animal waste influence the native population. Wong *et al.* (1998) tested the effect of different sewage application amounts on microbial activity in sandy soil, and concluded that application amounts in the range 50–150 g $kg^{-1}$ soil resulted in optimal soil microbial activity whereas higher concentrations impaired the microbial activity due to increased salinity. Jiang *et al.* (2002) found that *E. coli* O157:H7 cells were inactivated more rapidly in soils amended with cow manure at a ratio of 1 part manure to 10 parts (100 g $kg^{-1}$) soil at 15 and 21°C than in more dilute manure soil samples. This increased inactivation may be explained by an optimal soil microbial activity as concluded by Wong *et al.* (1998). Contradictorily, Berry and Miller (2005) compared how different amounts of manure added to a silt loam affected the survival of *E. coli* O157:H7. They found that increasing concentrations of manure in the soil increased survival time and under certain moisture levels growth was even seen.

The temporary increase in salinity around the animal waste impairs the native soil bacteria compared to faecal bacteria that are adapted to this level. As dispersion and dilution takes place the native bacteria will dominate again. One could speculate that this effect would be larger

when slurry is injected compared to incorporated due to a larger waste volume in a concentrated area.

### 3.1.3 SOIL TEXTURE

Soil texture influences faecal bacterial survival. Some have found positive correlations between fine textured soils and bacterial survival (Nicholson *et al.* 2005; Unc & Goss 2006a), while others have found no correlation between soil type and faecal bacteria survival (Natsch *et al.* 1996; Franz *et al.* 2005). Heijnen *et al.* (1991) found that adding kaolinite and bentonite improved the survival of Rhizobium, where the survival was significantly higher with 10% bentonite than 5% bentonite. In a review by England *et al.* (1993) it was concluded that clay type and clay content are important factors affecting survival of both indigenous and introduced bacteria in soil. Soils with a finer texture consist of smaller porespaces which influence faecal bacterial survival in relation to water holding capacity of the soil as well as protection from predation. Figure 3.1 depicts a schematic presentation of the total soil pore space; pores < 0.8 µm are non accessible due to size exclusion of bacteria, the protective pore space is from 0.8 to 3 µm where the bacteria are protected from predation, and the habitable pore space is from 0.8 µm to the maximum size of water holding pores. Pores with necks < 3 µm and between 3 and 6 µm have shown to positively affect the survival of introduced rhizobia whereas pores with necks > 6 µm had a negative effect due to predation (Postma & vanVeen 1990).

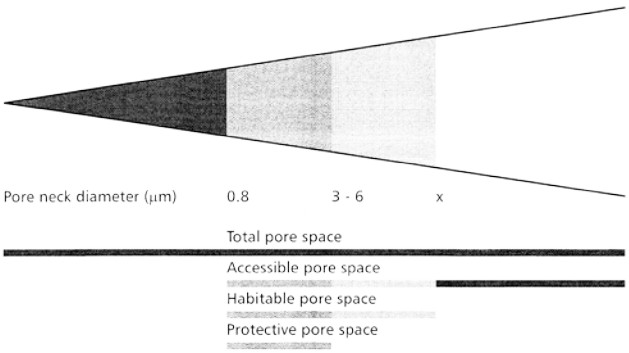


**Figure 3.1** Schematic presentation of total, accessible, habitable and protective porespace. X indicated the maximum diameter of water filled pores and will therefore depend on the soil water content (Postma & vanVeen 1990).

### 3.1.4 SOIL WATER CONTENT

Soil water content is of high important for microorganisms as they require water activity (>0.95) for active metabolism (Maier & Pepper 2000). Water is a continuous source of ions, nutrients, dissolved gasses, heat as well as bacteria and their predators (Standing & Killham 2007). A dry soil will induce water stress around the cell, whereas a saturated soil will induce anoxic conditions. Both extremes have severe consequences for survival of faecal bacteria in soil as dry conditions induce cell death, and anoxic conditions will shift cellular metabolisms to anaerobic processes.

Berry and Miller (2005) found that saturated conditions affected *E. coli* O157:H7 survival negatively. Oliver *et al.* (2006) found that *E. coli* declined more rapidly in a wet (50% moisture w/w) than dry soil (25%). Rothrock *et al.* (2012) found that *E. coli* O157:H7 survived better at 25 % VWC (volumetric water content) compared to 45% and explained this difference by a more aerobic environment at the lower water content. However, the observed difference at the two water contents was not found for the number of total bacteria (16S) and therefore the decreased survival at 45 % VWC may also be explained by increased competition from the indigenous microbial population. Opposite, Sinegani and Maghsoudi (2011) showed that increasing water content increased *E. coli* survival when applied through cow manure. However this trend was not observed when poultry manure and sewage sludge was applied to the soil. Oliver *et al.* (2006) found that the *E. coli* decay rate was more sensitive to increased water content when applied through slurry than manure. A more physical explanation is that soils with a lower matrix potential will hold cells within the protective porespace and thereby provide predation protection (Figure 3.1).

### 3.1.5 TEMPERATURE

Temperature is often an important factor influencing survival and possible growth of bacteria. Within the host animal there is a constant temperature, whereas fluctuation occurs in the soil environment. Most studies have been carried out at constant temperature, where it is generally observed that higher temperatures increase die-off when comparing within the range of 5◦C to 25◦C (Holley *et al.* 2006; Garcia *et al.* 2010). The effect of fluctuations in temperature was tested by Semenov *et al.* (2007) that found a decreased survival compared with constant temperature. It was furthermore shown, that the decreased survival increased with larger fluctuations in temperature.

The combined effect of die-off and growth has led to several different survival behaviours in soil (Figure 3.2) as proposed by Crane and Moore (1986). Faecal bacteria are known to exhibit a wide variety of behaviour in soil, and in general a simple first-order die-off should not be assumed. This is in particular important in the heterogeneous soil environment, where long-term survival of persistent subpopulations and regrowth may occur. This was found by Topp *et al.* (2003) where the die-off for an *E. coli* population showed "strain"-dependent behaviour.

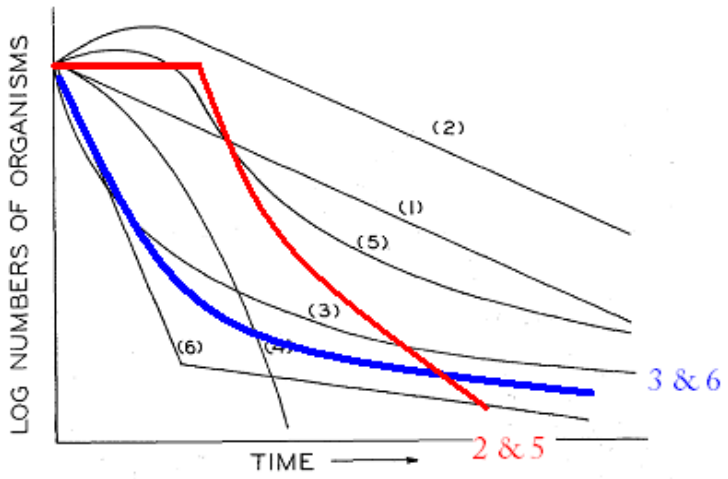


**Figure 3.2** Illustrative survival curves for microorganisms in soil based on literature information. Modified from Crane and Moore (1986)

The six different survival curves depicted in Figure 3.2 describe the following scenarios: 1) Bacterial decay is immediate and steady until the entire population is depleted and simply described by a first order reaction. This decay takes place in a totally unsuited environment for the faecal bacteria. 2) There is an initial lag phase that may be due to factors such as reduction of environmental stress on the bacteria due to dilution, increased nutrient supply, fewer antagonistic effects and/or change in oxygen level. 3) A constant reduction in the death rate is seen. This situation may occur in a larger bacterial population that consist of different small groups with different susceptibility to death and as such consist of different die-off rate constants. 4) This curve is opposite to curve 3 with an increase in death rate with time. This may occur when a toxic compound enters the bacterial environment with an increased concentration. 5) An initial stationary or growth is followed by a constant reduction in the death rate with time. 6) This curve consists of two first-order equations with an initial fast death rate followed by a slower one. This situation may occur upon introduction into a new environment such as the soil

where the first initial shock result in a fast decay followed by a more adapted decay. This situation is expected when a strong inhibiting factor is removed from the bacterial environment.

### 3.2.1 MODELLING FAECAL BACTERIA IN SOIL

The theoretical curves depicted in figure 3.2 seem to match exciting survival studies in soil. However, the decay of faecal bacteria in soil may be simplified further. By visual inspection of faecal bacteria survival curves in the literature, there appears to be two dominant patterns, the blue curve which is combining number 2 and 5, and the red curve that combines 3 and 6. Upon introduction into the soil environment faecal bacteria may initiate with lag phase or immediate decay. This decay should not be assumed as a simple first-order die-off, and is rarely observed even though this model is often fitted to decay data.

It is important to emphasis that most studies have been made with different *E. coli* serovars, which most likely have biased these observations. For example, in the study by Sinton *et al.* (2007) *Salmonella* and *E. coli* followed decay number 2 whereas *Campylobacter* followed decay number 1. Others have found that *Salmonella* decay follow number 3 (You *et al.* 2006; Garcia *et al.* 2010).

Decay initiated with a lag phase tends to dominate at normal soil temperature in the range 5–10°C. At higher temperatures there is a shift towards immediate decay (Cools *et al.* 2001; Jiang *et al.* 2002; Garcia *et al.* 2010; VanderZaag *et al.* 2010). When soil texture changes from fine to coarse texture the decay rate increases (Fenlon *et al.* 2000; Nicholson *et al.* 2005). Manure properties also influence the bacterial decay in soil. Generally, manure with dry matter content higher than ~5% tends to favour a lag phase or even growth in the beginning of the survival experiment, whereas with slurry die-off is immediate (Fenlon *et al.* 2000; Scott *et al.* 2006; Unc *et al.* 2006). Influence of manure properties on faecal bacteria survival in soil was studied in MS 3, where slurry with a higher dry matter content resulted in an initial lag phase whereas the liquid slurry resulted in an immediate decay. However, this difference was not seen in MS 5, where the decay was instant despite difference in dry matter. This lack of difference may be explained by a smaller volume setup in MS 5.

When faecal bacteria are injected through liquid slurry there will be a better spread into the soil environment compared with slurry (Appendix A3). This larger spread will induce immediate stress that initiates the decay. Whereas faecal bacteria injected through slurry remain more concentrated in the slurry injection slit (Appendix A3). This will provide protection from the soil

environment and delay the decay. This is in accordance with MS 5, where the incorporation led to faster decay compared to injection.

Relatively few have studied how injection influences the survival of faecal bacteria in soil. Semenov *et al.* (2009) compared the survival of *E. coli* O157:H7 for injection and incorporation and found a longer survival when slurry was injected. Whereas, when slurry or manure was surface applied there is generally seen a decreased *E. coli* survival compared to injection (Avery *et al.* 2004; Semenov *et al.* 2009). However, further research is needed to conclude on the influence of application method on faecal bacteria survival because animal waste properties most likely influences this effect.

# 4 PATHOGEN ADHESION TO ENVIRONMENTAL SURFACES

The adhesion of faecal pathogens to environmental surfaces greatly influences the survival and transport through processes such as retention, release, and aggregation. These processes are influenced by bacterial surface properties, particle surfaces and the soil solution. The physical and chemical factors governing the adhesion of bacterial cells in soil have been studied extensively. These include cell type, hydrophobic interactions, motility, surface charge and surface macromolecules (Redman *et al.* 2004; Walker *et al.* 2005a; Gargiulo *et al.* 2007; Long *et al.* 2009; Haznedaroglu *et al.* 2010). Nonetheless, the mechanisms controlling the adhesion of a bacterial cell are not fully understood. Most likely because bacteria are living organisms that exhibit metabolic and physiological changes, which affect their adhesive properties (Walker *et al.* 2005b).

## 4.1 DERJAGUIN-LANDAU-VERWEY-OVERBEEK THEORY

The adhesion of bacterial cells to soil particles has often been explained by the DLVO (Derjaguin-Landau-Verwey-Overbeek) theory of colloid stability. The DLVO theory simplifies the thermodynamic surface interaction and provides a conceptual understanding of bacterial adhesion in soil. The net interaction between the cell and the substrate surface is balanced by two additive forces, the van der Waals interactions (normally attractive) and repulsive electric double-layer interactions between the cell and substrate. Calculated DLVO interaction energy levels are used to determine if the adhesion conditions are *unfavorable* ($+V_T$) or *favorable* ($-V_T$). In figure 4 van der Waals and electric double-layer potentials are plotted as a function of separation distance between the particles. Summing these two curves ($V_T$) demonstrates that particles/cells have a net attraction in the primary or secondary minimum (well). The adhesion between the cell and soil particle is often *unfavorable* because both surfaces are negatively charged at natural pH levels in the soil.

The pioneering work by Marshall *et al.* (1971) suggested that the adhesion of bacterial cells to a surface consist of two stages, an initial reversible adhesion followed by a slower surface dependent irreversible adhesion. The initial adhesion is believed to be influenced by physical and chemical interactions between the planktonic cell and the surface and detachment may occur spontaneously. In the second step biological processes like growth and bacterial phenotype as well as removal of interfacial water, unfolding surface structures or rotation will result in a more irreversible adhesion (Busscher *et al.* 2010). The initial reversible adhesion is

located in the secondary minimum (figure 4.1), whereas the irreversible adhesion is in the primary minimum (Hermansson 1999; Palmer & Stoodley 2007).

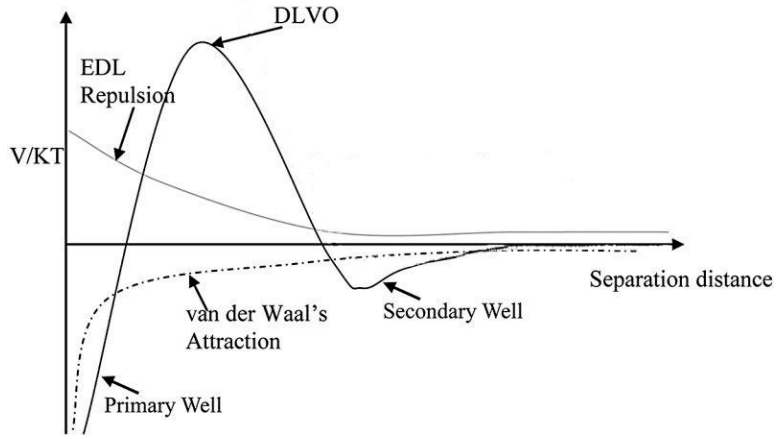


**Figure 4.1** van der Waals force (dashed line), electrostatic double layer (EDL) force (grey line), total DLVO forces, and elastic force plotted together to find total potential as a function of separation distance. V/KT, potential energy divided by Boltzmann´s constant and absolute temperature. Modified from (Hotze *et al.* 2010)

Redman et al (2004) found that the majority of *E. coli* cells were adhered in the secondary minimum, and that adhesion in this minimum was sensitive to changes in the ionic strength. In the primary minimum cells were not as sensitive to changes in the ionic strength. Redman *et al.* (2004) found that an increase in ionic strength resulted in an increase in adhered *E. coli*. Bacterial deposition is most likely occurring in the secondary minimum, which DLVO calculations indicate increases with ionic strength. A decrease in the ionic strength, that thereby eliminates the secondary energy minimum, results in a release of the previously adhered bacteria.

### 4.1.1 SOIL SOLUTION

The change between favourable and unfavourable conditions is most common induced by changes in the soil solution. A decrease in the ionic strength will expand the double layer thickness (Redman *et al.* 2004). Divalent ions will increase the ionic strength and decrease the thickness of the diffusive double layer compared to similar concentrations of monovalent ions. In figure 4.2 the amount of planktonic *E. coli* is shown, after an adhesion experiment to a sandy loam soil, comparing the effect of NaCl and $CaCl_2$ at two different ionic strength. The difference in ionic strength does not change the adhesion, whereas an increase in the presence $Ca^{2+}$ is observed. Haznedaroglu *et al.* (2009) compared the transport of *E. coli* O157:H7 and *Salmonella* Pullorum, and found that the *E. coli* was not influenced by changes in ionic strength ranging from

1–100mM whereas where as the adhesion of *S.* Pullorum increased with higher ionic strength. The authors explained this by the *E. coli* surface being insensitive to changes in ionic strength based on hydrophobicity and electrophoretic mobility.

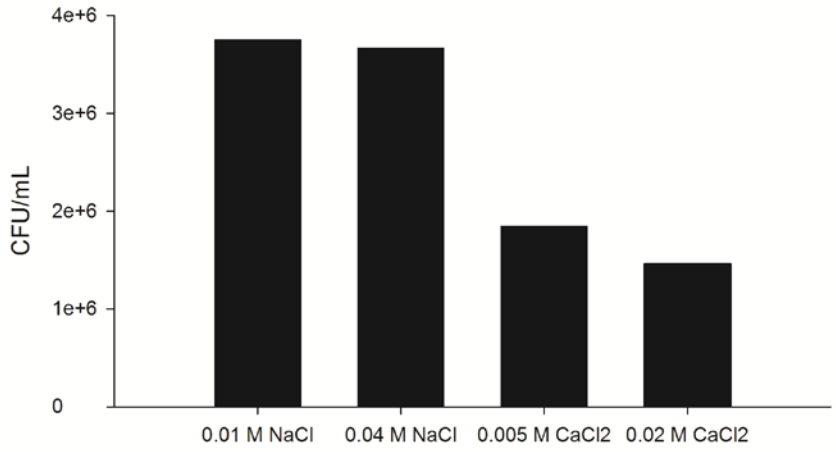


**Figure 4.2** Adhesion of *E. coli* to a sand soil in four different suspensions: 0.01 M NaCl, 0.04 M NaCl, 0.005 M $CaCl_2$ and 0.02 M $CaCl_2$. Experiment is made as the adhesion experiment from MS 4.

### 4.1.2 LIMITATIONS OF THE DLVO THEORY TO PREDICT BACTERIAL ADHESION

It remains a limitation for the prediction of bacterial cell adhesion that a living cell is not similar to a particle. Interfacial properties of the bacterial cell are important in understanding the microbial adhesion. In the DLVO theory a smooth surface is assumed, which is often not the case in reality. Czarnecki and Warszynski (1989) showed that surface roughness from 0 um to 0.05 um created differences in the interaction energy when comparing to the calculated one by the DLVO theory. Surface heterogeneity may create a local energy minimum that allows adhesion in situations where the mean total interaction should be repulsive (Warszynski & Czarnecki 1989). Bhattacharjee *et al.* (1998) found that both the repulsive DLVO interaction energy and the primary energy minimum, normally seen for smooth surfaces, appear to be considerably lower for rough particles. Duval and Gaboriaud (2010) states in a recent review that the electrophoretic behaviour of soft particles (permeable) differs substantially from hard particles (impermeable) because:

1) the distribution of charges in the ion-permeable layer characterizing the bacterial cell may be larger than the Debye length, and therefore the electric potential distribution may differ from the hard surface calculated model and

2) electroosmotic flow within the permeable layer may lead to differences between observed electrokinetic response and the expected one based on classical electrokinetic models for hard surfaces.

In order to describe or predict the adhesion of a bacterial cell the surface properties such as: cell radius, hydrophobicity, charge density and acidity are often measured. Besides the bacterial size the remaining factors are determined as an average value, and do not allow for more details regarding cell surface heterogeneity. Therefore, application of present physio-chemical mechanisms is not enough to explain bacterial adhesion. Walker *et al.* (2005a) compared stationary *E. coli* cells with mid-exponential phase and found that cell surface heterogeneity in surface charge resulted in less repulsion; and therefore increased adhesion.

Hydrophobic interactions are not accounted for in the DLVO even though they are usually the strongest of all long-range non-covalent interactions (vanOss 1995). Attraction between hydrophobic surfaces has been measured directly and can be as long as 80 nm (Christenson & Claesson 1988). Jacobs *et al.* (2007) found that hydrophobic strains showed a greater adhesion to sand. In the adhesion experiment in MS 4 there was no correlation between the hydrophobicity of four bacteria and three soils.

## 4.2 IMPACT OF MANURE ON ADHESION

Soil adhesion of faecal bacteria following slurry application is influenced by complex processes and interactions occurring between the soil particle and the bacterium, as well as the soil particle and the organic compound where the faecal bacteria is adhered. Adhesion of faecal bacteria have typically been tested by using pure cultures in batch experiments, and often described by liniar, non-liniar (Mankin *et al.* 2007) or by the DLVO theory (Redman *et al.* 2004; Torkzaban *et al.* 2008). Besides a conceptual understanding of the bacteria and soil particle interaction these studies may not mimic soil solution interactions driven by changes in the soil solution pH and ionic strength commonly found when animal waste is applied to soil (Husted *et al.* 1991; Safley *et al.* 1992). Furthermore, many of the cited references previous in this chapter have used quartz particles or glass beds (Walker *et al.* 2005a; Walker *et al.* 2005b; Haznedaroglu *et al.* 2009; Haznedaroglu *et al.* 2010) that does not represent a heterogeneous reactive soil. Relatively few studies have looked at the adhesion and fate of faecal bacteria in soils treated with animal waste.

When animal waste is applied to soil there is an increase in pH and the electric conductivity (Guber *et al.* 2007). This results in opposite effects on the adhesion because a higher pH

decreases adhesion in contrast to a higher electric conductivity that compromises the electric double layer. Still, there is a clear trend of a decreased bacterial adhesion in the presence of animal waste (Guber *et al.* 2005; Guber *et al.* 2007; Guzman *et al.* 2012). Guber *et al.* (2005) suggested that the decreased adhesion is due to:

- Modification of soil surface properties by soluble manure organic or inorganic constituents.
- Adhesion of faecal bacteria on manure particles.
- Competition between dissolved organic matter and bacteria for soil sorption sites.
- Modification of bacterial surfaces by dissolved organic matter.

In MS 4 the adhesion between four bacteria and three soil types was tested under the influence of polyacrylamide (PAM). A decreased adhesion in the presence of PAM was seen and this may be due to increased competition for sorption sites as well as changed surface properties of the soil.

An important factor that influences adhesion is whether the bacteria are planktonic or adhered to organic particles in the animal waste. Guzman *et al.* (2012) estimated that only 10 % of *E. coli* in swine slurry was planktonic and that 60 % was adhered in the size fraction from 8-50µm. Leung and Topp (2001) found that in an anaerobic swine slurry that the majority of bacteria was adhered whereas in an aerated swine slurry that aerobic planktonic populations (copithrophic) rapidly outnumbered the more stable anaerobic bacteria populations (oligohrophic). Therefore, the change from the anaerobic storage tank to the aerobic soil environment may change the proportion of adhered/planktonic cells.

# 5 TRANSPORT OF WATER AND FAECAL BACTERIA FROM MANURE

Even though it is well known that faecal bacteria enters water resources (US EPA 2000; US EPA 2013) many gaps still remain in our knowledge of pathogen fate due to diverse array of processes that influence faecal bacteria transport. Faecal bacteria are transported through/over the soil via flowing water and the degree of release depends on precipitation intensity (Schijven *et al.* 2004), manure composition, application method and hydraulic properties of the soil (Hutchison *et al.* 2004; Unc & Goss 2006b; Amin *et al.* 2013).

## 5.1 FLOW CHARACTERISTICS

Transport of bacteria is mostly passive and controlled by the presence of water fluxes. The partitioning of precipitation at the soil surface determines the direction of bacterial movement from the manure. Precipitation may infiltrate through the soil or via runoff determined by the soil hydraulic properties, soil cover, slope and characteristics of the precipitation. Therefore, this partitioning determines the risk of either groundwater contamination or surface water contamination. Surface run-off can generate extremely high pathogen loads to surface waters, and have been reported to be the dominant mechanism for pathogen export from arable land (Tyrrel & Quinton 2003; Gentry *et al.* 2007).

### 5.1.1 PRECIPITATION

The soil water content and intensity of precipitation greatly influences the number of active soil pores. At lower intensities, water will infiltrate into the soil matrix according to the hydraulic potential gradients. Javis (2007) stated in a review that 60 % of 115 cited articles concerning leaching experiments used irrigation intensities greater than 10 mm $h^{-1}$. Therefore, some precautions should be taken when reading the excising literature as manure application to agricultural land is rarely followed by artificial irrigation or rainfalls at such high intensity. Table 5.1 gives an overview over recent experiments including studies with natural precipitation or high irrigation intensities. Noteworthy is the lack of correlation between intensity and bacterial recovery.

### 5.1.2 INFILTRATION

Infiltration is the movement of water into the soil layers. A homogeneous sandy soil with many large pores will provide easy pathways through the soil matrix as opposed to a clayey soil with smaller pore spaces. The rate of infiltration is determined by the water-holding capacity of the soil, easiness of entry, soil hydraulic properties, vegetation types and vegetation cover. Faecal bacteria are transported within the infiltrating water and may be removed from the soil solution by filtration, straining or adhesion. Retention of faecal bacteria in soil is highest when matrix flow dominates (Abu Ashour *et al.* 1994; Aislabie *et al.* 2001; Unc & Goss 2003). Soils with high clay contents are generally considered to be dominated by preferential flow (i.e. only a few pores contribute to water flow). Lin *et al.* (1997) found that 3% of soil porosity contributed to 77% of the water transport in a clayey soils. Active pores in MS 1 ranged from 0.1–3.6 % of the total surface area.

**Table 5.1** Examples of bacterial recovery from experiments with different precipitation intensities.

| ***Bacterial strain*** | ***Soil*** | ***Precipitation*** | ***% recovery*** | ***Reference*** |
|---|---|---|---|---|
| ***E. coli*** | Sandy clay loam | Natural precipitation (3.1 mm $day^{-1}$) | 0.11 | (Brennan *et al.* 2010) |
| ***E. coli*** | | Natural precipitation (1.68 mm $day^{-1}$) | N. D | (Nyberg *et al.* 2010) |
| **Tetracycline-resistant bacteria** | Clay loam | Natural precipitation | 0.005 | (MS 3) |
| ***S.* Senftenberg** | Silt loam | Natural precipitation (2.6 mm $day^{-1}$) | 0.08 to 13.8 | (MS 1) |
| ***E. coli*** | Loamy sand | 10 mm $h^{-1}$ | 0.13–0.21 | (Amin *et al.* 2013) |
| ***Enterococcus* sp.** | | | 0.11–0.17 | |
| ***E. coli*** | Clay soil | Natural precipitation | 2.4–11.7 | (Aislabie *et al.* 2011) |
| ***E. coli*** | Clay loam | 11.3–46.4 mm $d^{-1}$ | 6.4–19.3 | (Guber *et al.* 2005) |
| ***E. coli*** | Loamy soil | 12.7 mm $h^{-1}$ | 1–13 | (Saini *et al.* 2003) |
| **Faecal coliform** | Silt loam | 10 mm $h^{-1}$ | 1–10 | (McMurry *et al.* 1998) |

### 5.1.3 PREFERENTIAL FLOW

Preferential flow commonly generates high-speed, high volume flow with minimal exchange with the soil matrix (Lin *et al.* 1997) and as such has a dominant influence on infiltration and contamination of receiving waters (McMurry *et al.* 1998; Artz *et al.* 2005; Bech *et al.* 2010). Preferential flow is associated with the macropores of the soil and is generally considered to occur in pores larger than 0.3–0.5 mm (Jarvis 2007). Bradford *et al.* (2013) concluded in a review that the general consensus from transport studies is that a decreased water content increase colloid (bacteria) retention when steady state conditions are reached because of increases in the air water interface (AWI) and changes in the flow field. Mosaddeghi *et al.* (2010) observed that saturated flow facilitated the bacterial transport at greater rates than the unsaturated flow. Greater filtration during unsaturated conditions was again explained by the presence of AWI. Along the same lines Javis (2007) concluded that soil matrix should be close to water saturation (>-10 cm) in order for preferential flow to become dominant. When non-equilibrium flow occurs infiltrating water does not have sufficient time to equilibrate with slowly moving antecedent water in the immobile or less mobile pore regimes of the soil matrix (Jarvis 2007). Nimmo (2012) stated in a recent review that:

> "Published case studies and observations of preferential flow make clear that preferential flow sometimes occurs in media much drier than saturation; can occur in pores incompletely filled; can occur in macropores before the onset of ponding, and even delay or prevent ponding; and can, in several ways, be *inhibited* by higher water content."

Tallon *et al.* (2007) found that increase in the initial soil water content from 0.19 to 0.33 made little difference in the leaching of *E. coli*, and that if anything, dry conditions led to highest leaching. The Principal Component Analysis (Appendix 4) shows a negative correlation between leaching of faecal bacteria and soil water content in MS 3. In MS 1 and MS 3, we found, that leaching of bacteria took place during non-saturated conditions and that relatively low precipitation intensities was enough to generate bacterial transport. This challenges some of the existing literature, as saturation may not be as an important factor as currently believed. Additional research is needed to study the impact of soil water content on leaching potentials of faecal bacteria – with normal precipitation intensities rather than using unrealistic high intensities.

### 5.1.4 SURFACE RUN-OFF

Surface run-off occurs when the rainfall intensity exceeds the infiltration rate of the soil and/or when the soil is water-saturated. Once surface runoff has been generated there is a potential for rapid transport of faecal bacteria to surface waters.

Bacterial transport is often simplified by assuming similar properties to soil particles. However, the more complex system in the soil manure environment makes it possible for bacteria to appear in three stages: adhered to soil particles, adheres to manure particles or in a planktonic form (Tyrrel & Quinton 2003). Runoff may be described by the classical movement of soil particles, detachment, transport and deposition. Detachment is a result of the impact of raindrop and velocity of flowing water, and it seems reasonable that these physical impacts will influence a cell and a particle in similar ways. Tyrrel and Quinton (2003) suggested that the rate at which bacteria are detached include number of bacteria, degree of protection from overlying soil or manure and strength of the adhesion to soil or manure particles. After detachment faecal bacteria may be transported either as planktonic cells in the soil solution, adhered to suspended soil or manure particle. Pathogen removal from flowing water is expected to be enhanced when bacteria are associated with soil aggregates due to increased density (Muirhead *et al.*, 2006).

In MS 4, we found that the distribution between planktonic and adhered cells was influenced by soil type. From the clay and clay loam soil approximately 80% of leached cells were adhered whereas for the sandy loam only 20% were associated with particles. This could be a result of higher proportion of smaller particles that becomes suspended from the clay soils and remain suspended. Characklis *et al.* (2005) found that 20–35 % of *E. coli* were associated with settleable particles in background watersamples and 30–55 % in stormwater samples at a low density residual land use area (soil type unknown). In a laboratory controlled experiment Muirhead *et al.* (2006) compared transport of attached and planktonic *E. coli* in a saturated soil and found that planktonic cells were easier transported across the soil and thereby to the receiving waters. Furthermore, when *E. coli* was pre-attached to soil particles (>45 µm) a significant reduction was found compared with inoculation of unattached cells. This is due to deposition of the particles during transport. Soupir *et al.* (2010) found that the percentage of *E. coli* and enterococci adhered to soil particles in runoff ranged from 28 to 49 %, this percentage increased in time when comparing runoff after 10 min and 30 min. However, there was not observed a difference in the leaching when comparing *E. coli* and enterococci. In contrast to the MS 4 experiment, Soupir *et al.* (2010) applied faecal bacteria through manure, and they concluded that due to lack of difference between soil types that most of leached bacteria were adhered to

manure particles. This is in accordance with Guzman *et al.* (2012), who found that 10% of *E. coli* was planktonic in swine slurry.

The distribution between the four bacteria in run-off water in MS 4 confirms that differences between serovars influence the adhesion to soil particles and thereby the likelihood for surface transport. Unfortunately, the tested motility and hydrophobicity could not explain this observed difference in runoff. Gannon *et al.* (1991) compared leaching of different bacteria and concluded that cell size was the only factor that explained differences between different leaching potential of the bacteria. Where size may be an obvious factor with infiltrating water due to filtration this may be of less importance in surface runoff.

## 5.2 SLURRY APPLICATION METHOD

Relatively scarce literature comparing how different types of animal waste and application method influence hydraulic properties is available even though the influence of slurry application on leaching of bacteria has been studied increasingly within recent years. Application method might influence the risk of leaching. Higher recovery of *S*. Senftenberg from injected clay columns was observed compared to surface applied columns, however due to large variation between replicates this difference was not significant (MS 1). Amin *et al.* (2013) found in a loamy sand column experiment that injection enhanced the leaching potential of *E. coli* and *Enterococcus* compared with surface application. These results are similar to the results from MS 1 and from both experiments it was speculated that a shorter leaching path could explain enhanced leaching from the injection treatment. Semenov *et al.* (2009) concluded that surface application of manure reduced the risk of groundwater contamination compared with injection of slurry in a sandy soil. However, the manure of that study had a dry matter content of 35% compared with 8.8% (MS 1) and 3–5.5% (Amin *et al.* 2013).

Long-term application of manure is known to improve soil structure due to increased organic matter content (Diacono & Montemurro 2010) and hence improves infiltration rates. Unc and Goss (2006b) compared the effect of liquid slurry and manure on the saturated hydraulic conductivity. Surface application of liquid slurry reduced the matrix flow by blocking smaller surface pores and thus accelerated flow through macropores. When exceeding the infiltration capacity of the soil application of liquid slurry additionally favoured surface runoff. Opposite, solid manure favoured matrix flow over macropore flow, and thereby tended to reduce the likelihood of runoff.

Effect of application method on leaching is influenced by both manure properties and by soil structure. In fine-textured soil with pronounced preferential flow, injection has shown to retain slurry (6.6 % dry matter) solutes and particles within the soil matrix compared with surface application of slurry, whereas no effect of application method was found in coarse-textured soils (Glaesner *et al.* 2011). The observed effect by Glaesner *et al.* (2011) may have been more profound with increasing dry matter content, as manure with high dry matter content remain more concentrated in a small soil volume in contrast to a thinner slurry which spread more into the soil environment (Petersen *et al.* 2003). Distribution of tetracycline resistant bacteria around the slurry injection slit was influenced by dry matter content of 0.8% and 6.4% (Appendix A3). The better spread with the liquid slurry into the surrounding soil environment may explain the findings of faecal bacteria in drainage water in MS 3, because of increased likelihood of reaching preferential flow paths in the soil.

# 6 CONCLUSIONS AND PERSPECTIVES

The focus of the thesis was survival and transport of faecal bacteria in agricultural soils influenced by slurry application method. The survival of faecal bacteria in soil is complex and affected by several interacting factors. The survival time and shape of the decay curve is among others influenced by slurry properties. Slurry with a higher dry matter content seems to result in a lag-phase followed by a rapid decline, whereas slurry with less dry matter results in immediate decay. When comparing the effect of injection versus incorporation it seems that:

- Liquid slurry will spread more into the soil environment than a slurry with a higher dry matter content. The greater spread may result in a rapid die-off due to the stress induced from the shift from the slurry tank to the soil environment (MS 3/A 3). Counteracting this effect is that the greater spread may increase the likelihood of faecal bacteria reaching protective porespaces – and thereby be protected from protozoan predation.
- Incorporation led to a more rapid decay compared to injection of slurry (MS 5).

Transport of bacteria follows water flow and at the soil surface it will partition between surface runoff and infiltration.

- Bacterial transport in surface runoff is influenced by soil type. A sandy soil will lead to a greater bacterial transport due to less sorption sites. Proportion of adhered and planktonic cells is also influenced by soil type where a sandy soil will have a larger proportion of planktonic cells in runoff water compared to a clay and clay loam soil (MS 4).
- Polyacrylamide reduced bacterial adhesion, and in runoff water may not always reduce bacterial concentrations (MS 4).
- Soil type influences the leaching of bacteria. A well structures soil with rapid preferential flow is required to generate faecal contamination of drain- or groundwater (MS 3).
- Leaching of *E. coli* and faecal tetracycline resistant bacteria correlated positively with 24 h antecedent precipitation, and correlated negatively with days since slurry application and soil water content (MS 3).
- It is questioned if faecal indicator bacteria *E. coli* is the best suited as it was not leached along with other tetracycline resistant-bacteria.

- Higher recovery of *S*. Senftenberg from injected clay columns was observed compared to surface applied columns, however due to large variation between replicates this difference was not significant (MS 1).

## 6.1 PERSPECTIVES

An increasing volume of literature has been concerned with non-point faecal contamination of surface and groundwater. Understanding the mechanisms behind findings of faecal bacteria in water resources is of paramount importance, as this knowledge will help preventing future outbreaks. With continuous emerging pathogens or the development of multi-drug resistant bacteria human health may be endangered.

Currently, water is tested for faecal pollution by the faecal indicator bacteria *E. coli*. However, this bacteria may not be the best suited when comparing its survival and transport with other pathogens such as *Salmonella* and *Campylobacter*. Furthermore, large variation within the *E. coli* strain has been documented, which makes it difficult to compare different research results, and as such also explain often conflicting results found in the literature. As a minimum future experiments should be conducted with best fit *E. coli* serovers – and thereby work with worst-case scenarios.

A comprehensive understanding of faecal bacteria survival and transport in soil is still lacking. The literature contains a number of contradicting and largely incomparable studies, from which it is difficult to extract a unifying hypothesis. A coherent series of experiments, where 1) the method of spiking the slurry has been defined (culture conditions, stressing the culture and initial concentration), 2) description of the test strain by surface properties, 3) description of the slurry properties regarding for example dry matter content and C/N-ratio, 4) and description of the soil including texture and microbial diversity. A series of defined experiments may provide the scientific base for designing a decay model of faecal introduced bacteria into the soil environment. This model could be incorporated into models, such as MACRO, and help predict faecal pollutions. In addition best management practices could be adapted to this knowledge.

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# MANUSCRIPT I

*Ground Water (2011)*

# Leaching of Salmonella enterica in Clay Columns Comparing Two Manure Application Methods

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ground water

# Leaching of *Salmonella enterica* in Clay Columns Comparing Two Manure Application Methods

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## Abstract

Transfer of zoonotic bacterial pathogens through intact soil columns was monitored in an outdoor lysimeter over 36 d. Manure spiked with *Salmonella enterica* serovar Senftenberg was applied to either the soil surface or injected 0.08 m into the soil to compare leaching associated with the two manure application methods. The highest concentrations of *S. enterica* (up to 60,000 *S. enterica* CFU/mL) were detected on Day 1 in the first drainage samples, with measurable but declining concentrations persisting for 10 to 36 d depending on replicate columns. The total recovery of leached *S. enterica* in drainage samples ranged from 0.08% to 13.8%. When comparing the two application methods, there was no statistically significant difference in the leaching concentration of *S. enterica* at each sampling time during the study period. In addition, comparison of enumerations by selective plating and real-time polymerase chain reaction yielded similar concentrations of *S. enterica*, indicating that mainly viable and culturable cells were leached from the columns. When the experiment was terminated, the fluorescent dye Acid Yellow was applied to four selected columns and the distribution of dye and size of active (dye-stained) pores were measured with a digital camera and visualization software. The profiles showed that the area covered by active pores ranged from 0.1% to 3.6%. The relatively small fraction of active pores in the soil profile was consistent with the evidence of rapid transport of *S. enterica* and chloride in the columns.


## Introduction

Water borne diseases often affect a large number of people, and have been reported in both Europe and North America (Blackburn et al. 2004; Smith et al. 2006; Beaudeau et al. 2008). In the United States, microbial pollution has been recognized as a major threat to water sources. The Environmental Protection Agency (EPA) assessed 33% of U.S. waters in 2000 and found that 40% of streams, 45% of lakes, and 50% of estuaries were not clean enough to support fishing and swimming (EPA 2000). Microbial contamination of groundwater in the United States is also widespread, especially in shallow aquifers where rapid recharge or mixing with surface water can occur (EPA 2006). Microbial pollution of groundwater is also common in Europe, as indicated by a recent study in Denmark, which found that about 40% of private wells contained *Escherichia coli* (Brüsch and Rosenberg 2008).

An important source of pathogens in the environment is animal husbandry, which can release pathogens into the environment by applying manure to agricultural land. Examples of this are shown in various field-scale studies that found pathogens in drainage water following manure application (Faust 1982; Patni et al. 1985; Vinten et al. 2002; Unc et al. 2003). In Walkerton, Canada, the municipal water system was contaminated with *E. coli* O157:H7 and *Campylobacter jejuni* in May 2000, which resulted in 2300 people requiring medical attention and the death of 7 people. The fecal contamination leading to this

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Received May 2009, accepted September 2010.

doi: 10.1111/j.1745-6584.2010.00766.x

outbreak was believed to have originated from manure by either infiltration or surface runoff into unprotected wells (Unc et al. 2004).

When manure is applied to agricultural land, there are three important factors that could influence the risk of contaminating the receiving water: (1) agricultural practices for manure application; (2) pathogen survival; and (3) pathogen transport. Important agricultural practices that can influence the contamination risk include the manure application method, volume of manure, frequency of application, and season. Injection of manure into the soil, typically at depths of 0.05 to 0.1 m, is widely used in Europe and North America. One of the advantages of this technique is reduced aerial transmission of $NH_3$, thereby avoiding nitrogen contamination of the surrounding ecosystem (Petersen et al. 2003). Other advantages of subsurface injection are outlined by Warner and Godwin (1988) and include reduction of offensive odors and reduced risk of crop contamination by pathogens. Unfortunately, survival of bacterial contaminants in the soil may increase when manure is injected into the soil. Hutchison et al. (2004) found longer survival for *Salmonella typhimurium* when pig slurry was incorporated into the soil immediately (<2 h) as compared to when incorporation into the soil was delayed (7 d) or unincorporated. Semenov et al. (2009) compared the survival of *S. typhimurium* in soil applied with slurry either by surface application or injection and found longer survival times for *S. typhimurium* when manure was injected into the soil.

*Salmonella* spp. has been reported to survive from a few days up to 332 d in manure-amended soils (Islam et al. 2004; Holley et al. 2006; You et al. 2006). The survival of introduced bacteria into the soil is determined by various factors such as temperature, moisture, soil type, texture, exposure to sun (ultraviolet [UV]) light, protozoan predation, and the initial number of organisms present. Cools et al. (2001) compared the survival of both *E. coli* and *Enterococcus* spp. derived from pig slurry in soil and found a longer survival at 5 °C compared to 25 °C. Furthermore, they compared three water contents from 60% to 100% of field capacity and found a longer survival of these bacterial fecal indicators with increased water content, although the effect was greatest at 5 °C.

When analyzing water samples for fecal pollution, there is a risk of underestimating the contamination when using traditional plate counts. Cultivation-independent methods have found higher concentrations of *Salmonella* spp. and *E. coli* as compared to plate counts for water samples (Domingo et al. 2000; Bjergbaek and Roslev 2005). The decrease in culturability on selective agar media compared to cultivation-independent observations has been explained by bacterial cells going into a so-called viable but nonculturable (VBNC) state. Turpin et al. (1993) found that intact *S. typhimurium* cells had a rapid die-off in nonsterile soil according to viable counts on selective agar. However, total direct counting showed that the cell number remained constant, whereas UV-killed cells prior to inoculation declined at a steady rate, indicating that viable cells can persist at cell numbers close to the initial inoculum. Marsh et al. (1998) found a high correlation between cell numbers determined by quantitative polymerase chain reaction (PCR) and direct microscopic counts for *S. typhimurium* during 54 d of exposure in soil, whereas the cell number declined rapidly when enumerated by plate counts.

Transport of bacteria through the soil depends on several factors such as soil type, precipitation, manure properties, and vegetation (McMurry et al. 1998; Entry et al. 2000). About 40% of the area of Denmark is covered by clayey till, which until recently was believed to provide effective protection from groundwater contamination due to the low hydraulic conductivity in the till matrix. Within the past few decades, however, several studies in clayey till have found that macropores, such as biopores and fractures, have a profound impact by increasing water flow and transport (Fredericia 1990; Villholth et al. 1998). Previous studies have shown that colloidal tracers may rapidly be transported several meters below the soil surface and often faster than solutes (McKay et al. 1993, 1999; Hinsby et al. 1996). The importance of preferential flow paths for bacterial transport in soil has also been documented (bu-Ashour et al. 1998; McMurry et al. 1998; Artz et al. 2005).

The present study was conducted in a silt loam, which has been shown in previous studies to be vulnerable to rapid transport of contaminants along fractures and biopores (Fredericia 1990; Villholth et al. 1998). A comparison of culture and direct detection methods was included, as we expected that the two manure application methods would provide different environments that may influence the culturability of *Salmonella enterica*. Surface-applied manure was spread over a larger area, whereas injected manure was concentrated 8 cm below the soil surface. The objectives of the present study were (1) to determine the differences in rate of pathogen transport following surface and subsurface (injection) application of pig manure inoculated with *S. enterica* (nal+) 775W; (2) to determine if manure application methods have an effect on the culturability of *S. enterica* (nal+) 775W; and (3) to determine if *S. enterica* (nal+) 775W remains culturable following transmission through the soil, at least for the duration of the experiment. These last two objectives were assessed by comparing bacterial concentrations obtained using direct plate counts and real-time PCR targeting the *inv*A gene.

## Materials and Methods

### Field Site and Column Excavation

The experiment was carried out in intact soil columns excavated from a field site near the city of Roskilde, Denmark. The soil is a silt loam developed from glacier deposits (Table 1) and is classified as Typic Argiudoll.

Soil columns were taken from a 4 × 16 m plot. A total of 15 samples were collected in stainless steel cylinders (0.15 m in diameter, 0.3 m in length). The cylinders were

**Table 1**
**Soil Texture, % Distribution**

| | 100% | | | | | | | 14.2% |
|---|---|---|---|---|---|---|---|---|
| Organic Matter | <0.002 mm | 0.002–0.02 mm | 0.02–0.63 mm | 0.63–0.125 mm | 0.125–0.2 mm | 0.2–0.5 mm | 0.5–2 mm | >2 mm |
| 2.4 | 20.1 | 19.9 | 12.6 | 17.8 | 13.0 | 11.2 | 3.0 | 14.2 |

pushed into the ground by a steady-state force with the weight of a car. Once the cylinder was in the soil, the surrounding area was carefully excavated to avoid soil structure disturbance. The excavated columns ranged from 0.22 to 0.29 m height due to difficulties collecting the soil columns. The average soil height was 0.24 m. The measured water content was 15.7% on a weight basis, which corresponded to 24% on a volume basis. The air-filled volume was about 26%.

### Lysimeter Setup and Sample Collection

Before the experimental onset, acid-washed filter sand and a stainless steel net were placed at the bottom of the columns. Columns used for the surface manure treatment remained untouched on the top of the columns. The top of the columns used for the injected treatment were prepared by removing a triangular-shaped trench from the center that was 0.08 m deep, 0.08 m long, and 0.05 m wide. The soil columns were then placed in an outdoor multicolumn lysimeter to acclimatize. The background concentration for *S. enterica* was below the detection limit and for chloride it was 10.4 parts per million (ppm) in water samples collected before manure application.

The experiment was based on natural precipitation except on Day 3, when 5 mm of distilled water was added to all soil columns. Drainage was obtained by applying a negative hydraulic head to the bottom of each soil column using a 0.7-m hanging water column and samples were stored in refrigerated (5 °C) 500-mL clean glass bottles. Effluent samples were collected and analyzed on Days 0, 1, 4, 6, 9, 13, 20, 27, and 36.

Data for precipitation and temperature were collected by a climate station placed at the multilysimeter with data logging every 30 min. The logger system was TGPR-1201 (Gemini, Chichester, UK) connected to a RAIN-O-MATIC professional (Pronamic, Silkeborg, Denmark) self-emptying and frost-proof electronic rain gauge and TGP-4520 with two PT-100 temperature probes for measuring aerial and refrigerator temperatures.

### Culture Conditions and Preparation of Inocula

The *S. enterica* serotype Senftenberg 775W strain was kept at −80 °C in 25% glycerol. The culture to be used for the experiments was prepared by incubating a loop of frozen culture overnight at 37 °C at 100 revolutions per minute (rpm) in Mueller Hinton broth (Oxoid Limited, Hampshire, UK). The inoculum was washed by centrifugation at 8000 g for 2 min and the pellet dissolved in 1 mL of 0.01 M phosphate buffer (5.7 mL of 1 M $NaH_2PO_4 \times H_2O$ + 4.2 mL of 1 M $Na_2HPO_4 \times 2H_2O$ in 1000 mL water, pH = 7.4).

Swine manure (8.8% dry matter) was taken from a manure storage tank and stored for 3 weeks at 2 °C before the experimental onset. Eight manure samples were prepared, each approximately with a weight of 0.044 kg; for the surface area of each column, this corresponds to a normal Danish agricultural application of 25 t manure/ha. To each 0.044-kg manure sample, 0.4 mL of *S. enterica* suspension was added, equaling a total number of $5 \times 10^7$ CFU. In addition, 1 mL of 3.9 M NaCl was added to each manure sample, corresponding to a concentration in the manure of 3.14 mg Cl/g manure. The manure thickness in the soil trench used to simulate injection was approximately 4 cm immediately after manure application. The manure was left uncovered to simulate the most commonly used manure application machines in agriculture.

### Culture of *S. enterica* in Effluent Samples

Drainage samples were analyzed for *S. enterica* by enumeration on selective MacConkey agar plates (Oxoid Limited, Hampshire, UK) with a nalidixic acid concentration of 40 ppm. Bacterial counts were performed by drop plating (five replicates of 10 μL) 10-fold dilution series and subsequent incubation overnight at 37 °C. Selected colonies were verified by agglutination with *Salmonella* polyvalent O-antisera (Statens Serum Institut, Copenhagen, Denmark).

### DNA Extraction

Drainage samples ranging from 0.04 to 0.18 L were centrifuged at 3900 g for 15 min at 5 °C. Pellets were dissolved in 1 mL of 0.010 M phosphate buffer and stored at −80 °C. DNA extraction was performed with the commercial FastDNA Spin Kit for Soil (BIO101, Vista, California). With the exception of the beating step and an additional freeze-thaw step, the protocol recommended by the manufacturer was followed. The beating step was changed from 1 × 30 s at speed 5.5 to 4 × 30 s at speed 4.0 in the FastPrep FP120. The freeze-thaw step was 1 h at −80 °C followed by 30 min at 37 °C. DNA was dissolved in 100 μL of DNase/RNase-free water and kept at −80 °C.

### Real-Time PCR Assay

The primers used for detection of *S. enterica* were targeting the *inv* A using forward: 5′-ACAGTGCTCGTTT ACGACC-3′ and *inv* A reverse: 5′-ACTGGTACTGATCG

ATAAT-3′ primers (Jacobsen and Holben 2007). The *inv*A gene is an essential gene for the invasion of epithelial cells by *Salmonella* and is found in most *Salmonella* serotypes (Malorny et al. 2003). The 20-µL reaction mixtures contained 0.08 µL of 0.4 µM forward primer, 0.08 µL of 0.4 µM reverse primer, 10 µL mastermix (DyNAmo ™HS SYBR® Green qPCR kit, Finnzymes, Finland), 2 µL bovine serum albumin (New England BioLabs Inc., Ipswich, Massachusetts), 6.34 µL $dH_2O$, and 1.5 µL DNA sample.

All PCR reactions were carried out as real-time PCR in iCycler iQ™ (BioRad, Hercules, California). Samples were set up in triplicates, and for each PCR setup three negative controls were included. The following protocol was used: 12 min at 95 °C for enzyme activation, 40 cycles of 30 s at 95 °C for denaturation, 30 s at 55 °C for primer annealing, 30 s at 72 °C for elongation, and 15 s at 77 °C for quantification of the *inv*A product. The protocol was finished with 1 cycle of 6 min at 72 °C for elongation. The threshold cycle ($C_T$) values were determined using the iCycler software. Quantification was based on an internal standard curve. Melting curves for the PCR products were determined to verify that the quantification was not based on unspecific amplifications. Randomly chosen PCR products were also analyzed by separation in 1.5% agarose gels followed by ethidium bromide staining for validation of size.

### Dye Experiment

For the dye tracer experiment with Acid Yellow 7 (AY7; $C_{19}H_{13}N_2NaO_5S$; CAS: 2391-30-2), columns S-1 and S-3 were selected as examples of soils with low recovery of *S. enterica* and columns I-3 and I-4 as examples of soils with high microbial recovery of *S. enterica*. This tracer was chosen due to its fluorescent properties and ability to dissolve in water (AY7: 22 g/L at 20 °C) (Käss 1998).

Columns were saturated to field capacity by applying eight doses of 100 mL water in 6-h intervals. AY7 was applied in two 200-mL portions of 10 g AY7/L to the soil surface. A peristaltic pump with a speed of 1 mL/min was used to apply the AY7 through three hoses equaling a total flow of 3 mL/min. Each column was then sliced into seven to eight slices.

### Tracer Distribution Image Procedure

The spatial distribution of reemitted light from AY7 was captured in a dark room with light from a UV lamp placed 0.5 m above the soil slice. Pictures were taken with a Canon EOS 40D and saved as a raw file with a picture resolution of 0.0036 $mm^2$/pixel. Pictures were analyzed with the ImageJ 1.41 freeware (http://rsbweb.nih.gov/ij/). The function Analyse Particles was used to describe the fraction of the soil surface area that was covered by the fluorescent dye as well as to describe the size distribution of the pores.

### Statistics

The leaching experiment of *S. enterica* through intact soil columns was performed by repeated measurements on the same nine soil columns during the 36-d study. Manure inoculated with *S. enterica* was applied to the soil column in two different ways on Day 0 and each soil column received only one treatment. Four soil columns received the injection treatment, while five columns were applied with manure on the surface of the soil column. Three control columns did not receive manure. Drainage water was analyzed at eight times during the 36 d of study. The outcome of the experiment was the concentration of *S. enterica* in the drainage water. Data were tested for normality by log transformation and equality of variance within treatment using a level of significance of 5%. This was performed with the PROC UNIVARIATE, PROC GLM and PROC PLOT procedures. Owing to repeated measurement on the same columns for an extended period of time, the Diggle model for repeated measurements was chosen, as it takes into account that observations from the same column tend to be positively correlated as well as observations taken close in time tend to be more correlated than observations far apart. The Diggle model was tested by the PROC MIXED function. All model testing was performed by the statistic computer programme SAS®, version 9.1 (SAS Institute Inc., Cary, North Carolina).

The comparison of culturable cell counts and quantification by real-time PCR were compared with a paired Student's test in SigmaStat, version 3.11 (SYSTAT Software Inc., San Jose, California).

## Results

Air temperature and precipitation data for the experimental site are shown in Figure 1. The temperature ranged from 5 °C to 10 °C, except for the first 3 d where temperatures were below 0 °C. A total of 95 mm of precipitation was measured during the 36-d duration of the experiment. On 7 d during the experiments, more than 7 mm rain was measured. On average, 68 mm of water percolated through each column, corresponding to 77% of the total precipitation (Table 2). The amount of percolated water ranged from 0.95 to 1.60 of the total soil water content over the 36-d period of the experiment.

Great variation in recovered *S. enterica* in the effluent water samples was seen, with the lowest value of 0.08% and the highest value of 13.8% of the amount of *S. enterica* applied to each column (Table 2). When comparing the amount of leached *S. enterica* between the two manure application methods, it can be seen that the injected treatment leached on average 6.1%, whereas the surface-applied treatment only leached an average of 0.6%. However, because of the high degree of variability observed in the concentration of leached *S. enterica*, this difference was not significant when comparing the two manure application methods during the study period ($P > 0.05$). Less variation was seen for the amount of leached chloride, which ranged from 47% to 84% for the columns with surface application of manure and 52% to 87% for the columns with injected manure. The average chloride leaching values from the columns with surface application

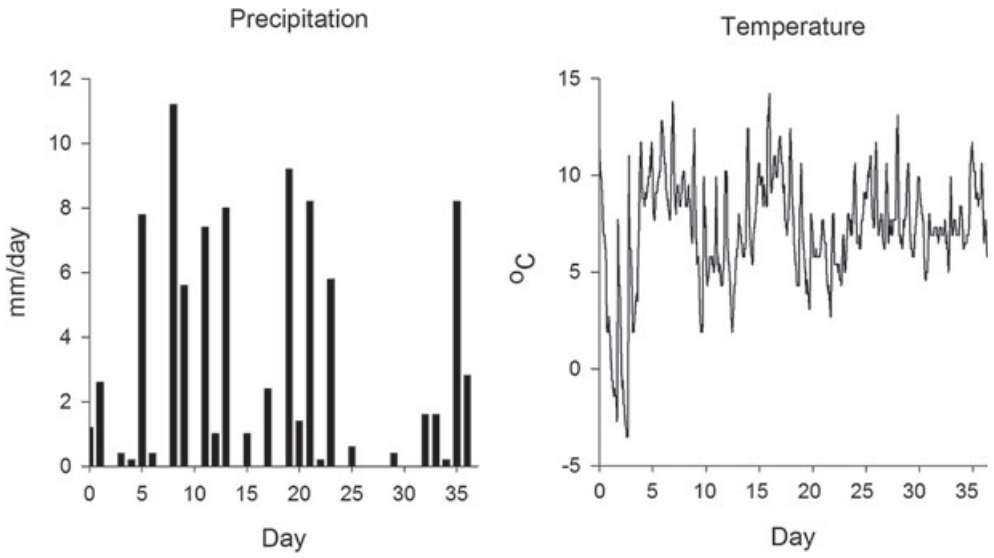


**Figure 1. Daily precipitation in mm/day and hourly temperature variations in degree Celsius.**

of manure and injection of manure were 62% and 65%, respectively.

The relative concentration of leached chloride and *S. enterica* and the amount of replaced water volume (WV) during the 36 d of the experiment are shown in Figure 2. The WV is calculated as an average value of the soil water content based on field site measurements with water content of 24%. All columns had close to one or more than one WV replaced. Both *S. enterica* and chloride had a rapid breakthrough, with each tracer appearing in the first effluent sample, collected 1 d after the addition of the manure. This corresponds to a first arrival WV of approximately 0.03 to 0.15 for the individual columns. For chloride, most leaching profiles show a small initial peak at Day 3 followed by a larger peak at Day 13. This corresponds with the precipitation, where there was a small precipitation event in the beginning of the experiment followed by a larger precipitation event between Days 8 and 13. For *S. enterica*, the highest concentrations were detected in the first or second water

**Table 2**
**Column Height, Percolated Water as a Soil Water Content, Percentage Recovery for *S. enterica* and Chloride, Highest Detected Concentration of *S. enterica***

| | Column Height (m) | Percolated Water/Soil Water Content (WV) | Total *S. enterica* Recovery (%) | Highest Concentration of *S. enterica* Found in Drainage Water (CFU/mL) | Total Chloride Recovery (%) |
|---|---|---|---|---|---|
| Surface-applied manure | | | | | |
| S-1 | 0.24 | 1.22 | 0.11 | 280 | 47 |
| S-2 | 0.29 | 1.10 | 1.97 | 855 | 55 |
| S-3 | 0.24 | 1.17 | 0.17 | 580 | 66 |
| S-4 | 0.23 | 1.44 | 0.31 | 1240 | 84 |
| S-5 | 0.22 | 1.12 | 0.60 | 2000 | 60 |
| Injected manure | | | | | |
| I-1 | 0.23 | 1.21 | 0.08 | 340 | 54 |
| I-2 | 0.25 | 1.06 | 1.84 | 12,000 | 52 |
| I-3 | 0.22 | 1.46 | 8.84 | 60,000 | 68 |
| I-4 | 0.21 | 1.60 | 13.8 | 50,900 | 87 |
| Control columns | | | | | |
| C-1 | 0.26 | 1.12 | 0.00 | 0 | 61 |
| C-2 | 0.24 | 0.89 | 0.00 | 0 | 47 |
| C-3 | 0.25 | 1.51 | 0.00 | 0 | 93 |
| Average surface-applied manure | 0.24 | 1.21 | 0.63 | 991 | 62 |
| Average injected manure | 0.24 | 1.33 | 6.14 | 30,800 | 65 |
| Average control | 0.25 | 1.12 | 0.00 | 0 | 67 |

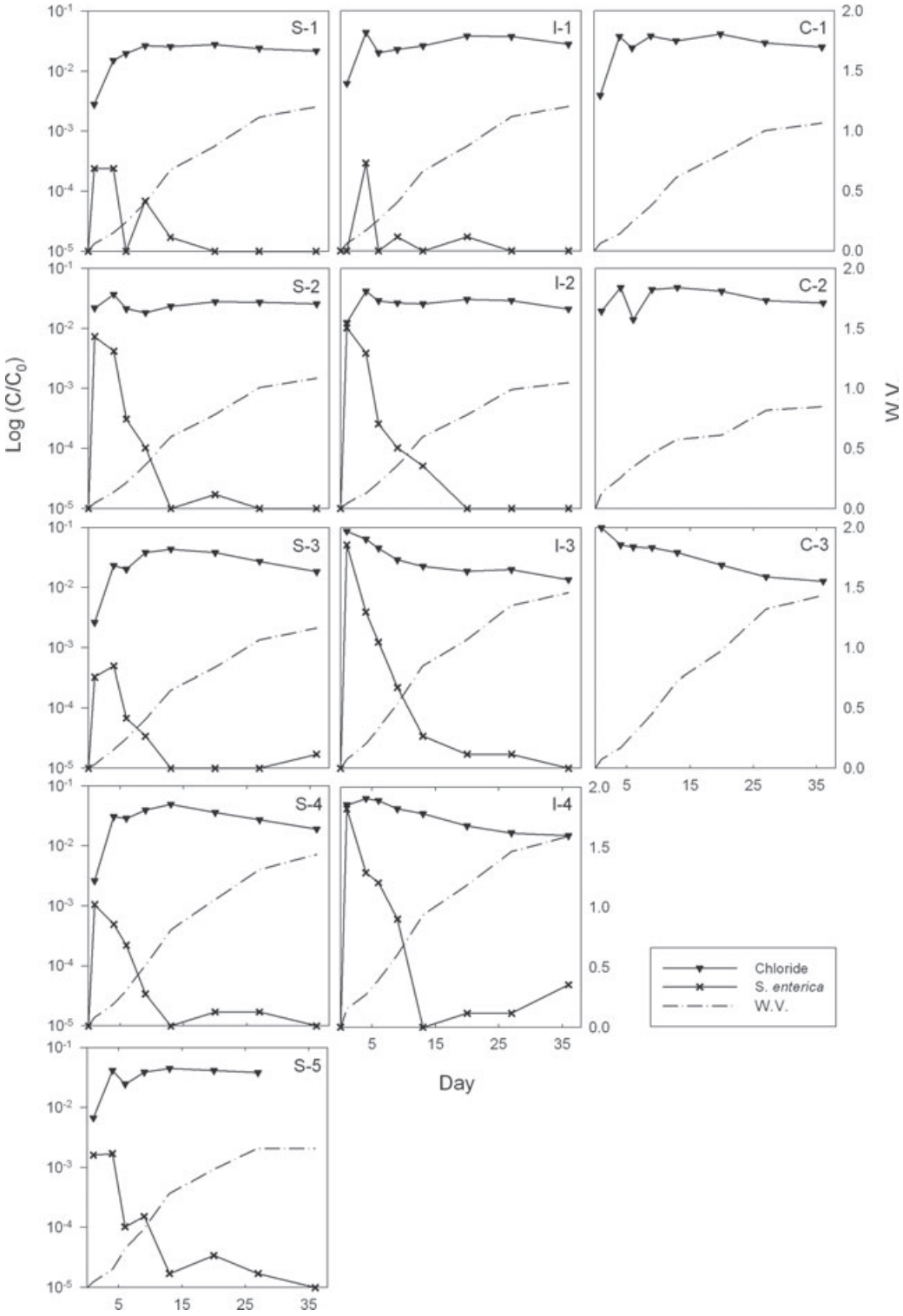


**Figure 2. Leaching data from 12 columns. Chloride and *S. enterica* are shown on the left *y*-axis as a relative concentration calculated as the concentration of the tracer in a water sample as a function of the tracer concentration in the manure. In S-5 the chloride data are missing from 36. Samples where *S. enterica* could not be detected are marked on the figure at a relative concentration of $10^{-5}$. The second *y*-axis shows the amount of water percolated given as WV replaced.**

sample, generally corresponding to the first small peak of the chloride. In the first water flow through the soil profile, *S. enterica* was found in concentrations up to $6.0 \times 10^4$ CFU/mL. For both manure treatments, there was a rapid decrease in *S. enterica* in the leachate over time. After 20 d, the measured concentrations of *S. enterica* were close to the detection limit of 100 CFU/mL. However, *S. enterica* was found sporadically in drainage samples up to 4 months after the experimental onset (data not shown).

Figure 3 shows the size distribution of pores down through the soil profile for soil columns: S-1, S-3, I-3, and I-4, as well as area fraction (percentage) covered by active pores. For all profiles, the proportion of the smallest fraction (0.01 to 0.04 cm in diameter) covered the largest area of active pores down through the soil profile. Only a few slices contained pores in the largest size range with pore diameters from 0.36 to 1.13 cm. Column S-3 differed from the others, as this column has a larger area of active pores. However, this did not correlate with leaching data given in Table 2. For *S. enterica*, the total recovery is low although the column is covered by the largest area of active pores. Sixty-six percent of chloride was leached from S-3 column, which is close to the average value for all columns.

The culture-based quantification of *S. enterica* was compared to real-time PCR quantification to determine if the test strain had entered a VBNC form during the first 9 d of the experiment (Figure 4). Each point in time shows the concentration of *S. enterica* determined by plate count, real-time PCR quantification as well as the standard deviation for both methods. When comparing the two detection methods, similar concentrations of *S. enterica* were found. On Day 1, the viable agar-plated-based counts are higher compared to the real-time PCR quantification; however, this difference is not significant (paired $t$-test; $P = 0.155$). The difference between the two detection methods remains nonsignificant with P values of 0.09, 0.172, and 0.055 on Days 4, 6, and 9, respectively.

## Discussion

The breakthrough curve for chloride in Figure 2 shows large variation between the different columns. The general trend of an asymmetric curve with an early breakthrough and pronounced tailing indicates that chloride is initially transported rapidly by macropores through the soil replacing a small fraction of the soil water, followed by slower transport through smaller pores. *S. enterica* is also detected in the first water sample indicating macropore flow, but generally declined very rapidly in subsequent samples. The differences in the breakthrough curves for chloride and *S. enterica* are likely due to a combination of factors. One of the factors is size exclusion of *S. enterica* from many of the smaller pores, which chloride can enter by advection and diffusion. This was attributed as an important factor in other tracer studies involving conservative solutes and microbial tracers in clay-rich soils, including studies by McKay et al. (1999) and McCarthy et al. (2002). Other factors that influence transport of *S. enterica*, but not chloride, include electrostatic attachment to soil grains, filtration,

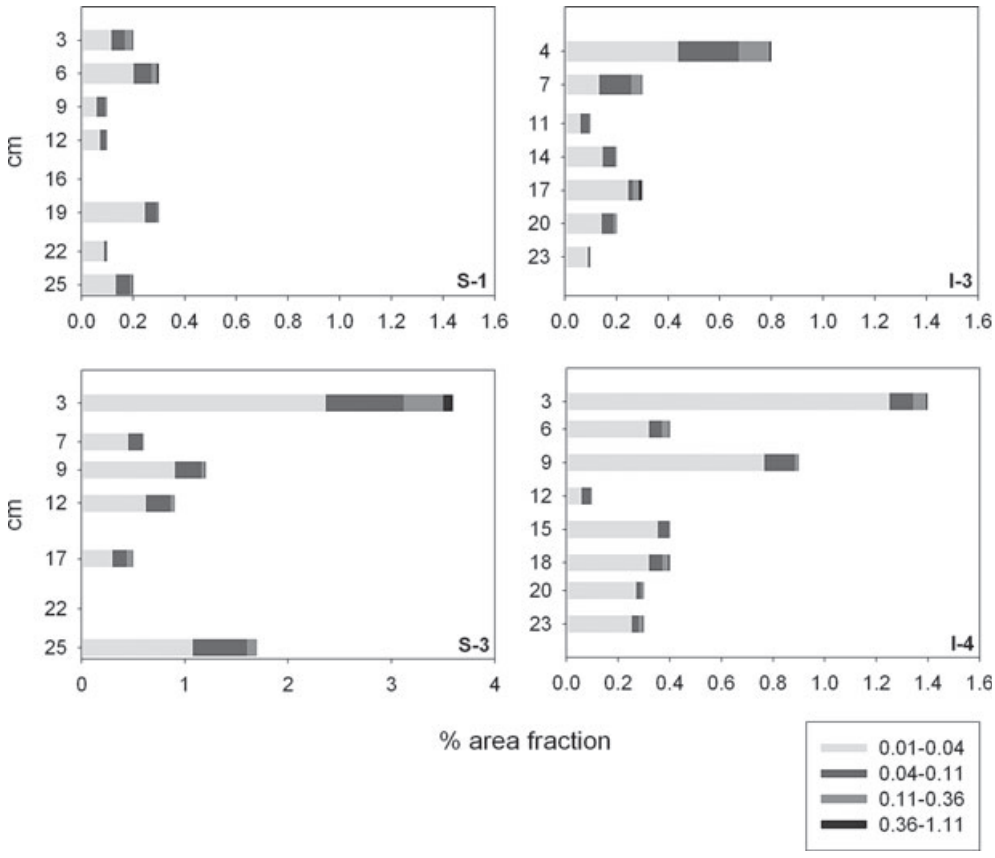


**Figure 3. The area fraction of preferential flow paths down through the soil profile quantified in seven to eight layers. The area fraction is divided into four diameter size fractions: 0.01 to 0.04 cm, 0.04 to 0.11 cm, 0.11 to 0.36 cm$^2$, and 0.36 to 1.11 cm. Data from the layer at 0.22 m are missing for column S-3 as this slice broke. The $x$-axis for column S-1 goes to 4%, whereas it is 1.6% for columns S-1, I-3, and I-4.**

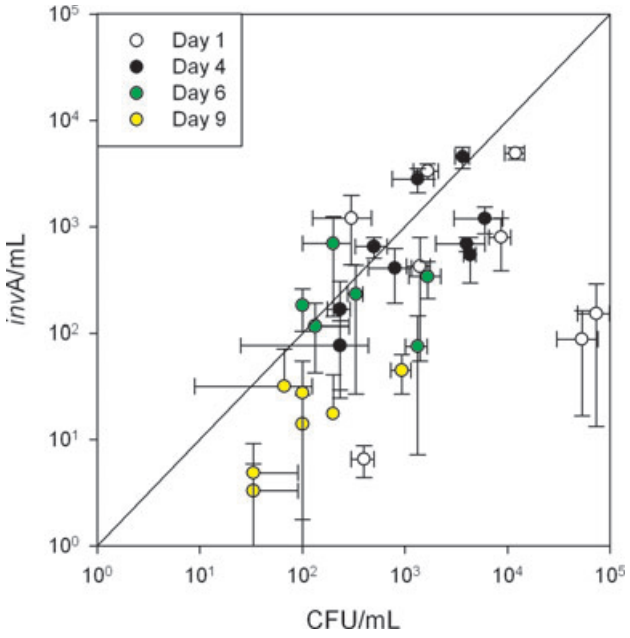


**Figure 4. Comparison of culture-based *S. enterica* counts with real time-PCR counts in leachate samples on Days 1, 4, 6, and 9.**

and die-off of the microorganisms, which are discussed later in this paper. For most columns, there is a second chloride peak just before one WV has been replaced. This second peak is more clearly evident when the data are plotted on arithmetic breakthrough curves (not shown) and indicated that smaller pores are also playing a role in downward flow of water and transport of chloride. The dye experiment with fluorescent AY7 showed that for pores larger than 0.01 cm in diameter, only up to 3.6% of the soil area was covered by active pores. On average, a total of 64.6% of chloride was recovered in the drainage water during the experiment. As chloride is neither degraded nor adsorbed to the soil particles, the remaining chloride would most likely have diffused into the soil matrix.

The soil columns were excavated from a well-structured grass field that has not been subject to mechanical disturbance for more than 10 years. The variation between the breakthrough curves can be explained by the soil heterogeneity. The soil type used in the present experiment was a silt loam with a clay content of 20.1%. Kjaergaard et al. (2004) found that soils with a clay content higher than 18% exhibited asymmetric breakthrough curves with a rapid breakthrough followed by the tailing as well as an increased amount of immobile water. The soil structure at the experimental site had previously been described by Lindhardt et al. (2001). Macropores made by burrowing organisms or roots occurred at the site down to 1.3 m b.g.s., and the largest vertical fractures intersect the whole clay till and reach the groundwater 4 m b.g.s. The dye experiment showed that S-3 differed from the other three columns by having larger area fractions covered by the fluorescent dye down through the soil profile. This may to some extent be explained by a particularly large number of roots in this column. When excluding column S-3, there is a correlation between total average area fraction from each column and total leached chloride ($R = 0.93$) as well as total water leached ($R = 0.73$). The large proportions of plant roots in S-3 may indicate that this pathway is of secondary importance as the amount of leached tracers was low or average from this column. Alternatively, disturbance of the soil structure when sampling as well as smearing at one of the ends would both have a greater impact on S. *enterica* as compared to chloride leaching.

The leaching of *S. enterica* showed large variations between all columns. Based on the initial added numbers of *S. enterica* to the manure, suspension between 0.08% and 13.8% was found in the drainage water during the experiment. Artz et al. (2005) found variation from 0.01% to 24% for intact replicate soil columns in the leaching of *E. coli* O157:H7 within 72 h. Smith et al. (1985) found that 90% of applied *E. coli* to 0.28-m high-silt soil columns was leached within 17 min. In the same experiment, about 70% of applied chloride was leached. We saw that up to 87% of the added chloride was leached, whereas only up to 13.8% of applied *S. enterica* was leached. The low recovery in the present experiment may be explained by the fact that this study was based on natural outdoor conditions, internal flow velocities, die-off, sorption, and filtration (Huysman and Verstraete 1993; bu-Ashour et al. 1994). Furthermore, the present experiment is based on a laboratory-grown culture that may have led to a more rapid die-off as compared to naturally occurring bacteria in the manure. Scott et al. (2006) compared the survival of a laboratory-grown *E. coli* O157:H7 vs. manure from infected cattle and found no difference in survival in the cattle manure. However, in water, the survival was 10 weeks shorter for the laboratory-grown culture.

The highest concentration of *S. enterica* was found in the drainage water during the first 3 d, although precipitation increased in amount and intensity after Day 3. This may be explained by attachment. Nola et al. (2005) found a rapid attachment within 30 min for both *E. coli* and *Salmonella* spp. For *Salmonella* spp., a high proportion of cells attached to the soil particles with a $K_f$ of 506 and 847 in two soil types. In the present experiment, the added test strain was applied with manure. Guber et al. (2005) found that *E. coli* sorbed stronger to the soil in the absence of manure. This could indicate that in the beginning where manure and bacteria are in the same vicinity, there is less attachment of *S. enterica* to the soil particles. As the manure is diluted due to precipitation, attachment becomes a more important factor in the retention of *S. enterica*. In contrast, the opposite effect of the added tracer chloride may be observed. Gannon et al. (1991) compared the effect of 0.01 M NaCl and distilled water on leaching of *Pseudomonas* sp. strain KL2 through a sand aquifer and found that NaCl lowered the leaching of the added strain. This was explained by increased sorption as a result of a decrease in the electric double layer around the soil particles. Drainage water

concentrations of $Cl^-$ in the present experiment were up to 260 ppm (≈0.007 M $Cl^-$). Gannon et al. (1991) also found that NaCl did not have an effect on bacterial aggregation as compared to distilled water.

For soil columns in which manure was applied by the same method, there was a large degree of variation for both leached chloride and *S. enterica*. These variations are most likely caused by the heterogeneity of the soil. In a previous study, Petersen et al. (1996) concluded that the variation in distribution of injected slurry component was caused by the soil heterogeneity. Gagliardi and Karns (2000) compared the transport of *E. coli* O157:H7 for tilled and no tilled clay loam and found that the difference in drainage concentration of *E. coli* O157:H7 was not significant for the two application methods. Stoddard et al. (1998) found that soils without tillage did not accelerate water contamination by fecal coliforms as compared to tilled soils.

The survival of *S. enterica* would be expected to be lower in the surface-applied manure as the bacterial cells are more exposed to sunlight, temperature, and moisture fluctuations as well as atmospheric drying (Natvig et al. 2002; Gessel et al. 2004; Hutchison et al. 2004; Holley et al. 2006). Semenov et al. (2009) compared the survival of *E. coli* O157:H7 and *S. typhimurium* in soil applied with slurry either by surface application or injection and found longer survival times for *S. typhimurium* when manure was injected. However, for *E. coli* O157:H7, there was no difference in survival. Avery et al. (2004) found that *E. coli* O157:H7 survived for 8 weeks when injected, compared to 6 weeks when applied to the soil surface.

During the first 9 d of the experiments, no significant difference was seen between CFU counts and real-time PCR quantification of *S. enterica*. This was of some surprise, as it has previously been reported that when *Salmonella* spp. enter an oligotrophic environment, like as soil (Turpin et al. 1993; Marsh et al. 1998) and water (Domingo et al. 2000), the VBNC state can be induced. Soil columns remained moist during the experiment due to frequent precipitation in combination with low temperatures (<10 °C). The average discharge from all columns was 77% of the total precipitation. Pedersen and Jacobsen (1993) found clear differences in the number of *Enterobacter cloacae* and *Alcaligenes eutrophus* after 14 d when comparing selective plating, direct viable counts (DVCs), and DNA hybridization in a field moist soil. In the same study, nonviable and VBNC cells were found in air-dried soil. Although other survival studies have reported that when *Salmonella* spp. are outside a host as in soil (Turpin et al. 1993; Marsh et al. 1998) and water (Domingo et al. 2000), the VBNC state may be induced. Baudart et al. (2002) found that proportions of VBNC cells in drinking water of enteric bacteria were between 85.2% and 100% of the total count using fluorescence in situ hybridization (FISH). Bjergbaek and Roslev (2005) found that cultivation-independent methods [DVCs, FISH, and green fluorescent protein (GFP)-marked cells] resulted in 10 to 20 times higher *E. coli* concentration compared to traditional culture-based enumeration methods.

A limitation in the present experiment was the use of relatively small soil columns, which may not represent field conditions mainly because of the edge effect on water movement. For all columns, a similar amount of water was found to leach through the columns. However, the leaching of chloride and *S. enterica* showed a high degree of variation, indicating that soil structure had a greater effect than the edge effect on leaching. As the columns were excavated in a relatively dry soil and saturated with water to field capacity before the experimental onset, the swelling of the soil would furthermore prevent any edge effect on leaching. When collecting an intact soil core sample, there is always the risk of destroying the internal structure of the soil. Our way of excavating the soil cores would to some degree have impacted the soil structure. However, since a rapid breakthrough of tracer and test organism was seen this damage seemed to be of minor importance.

## Conclusion

The study showed that *S. enterica* leached through intact clay soil columns. Based on the results from the A horizon, it is concluded that variation in soil structure influenced the leaching of *S. enterica*, where the total recovery ranged from 0.08% to 13.8% of the initial numbers applied. Concentrations up to 60,000 *S. enterica* CFU/mL were found in leachates. There was no statistical significant difference between *S. enterica* findings following surface application or subsurface injection of manure. In addition, the comparison of real-time PCR and plate counts yielded similar values, indicating that the leached cells were mainly viable cells.

## Acknowledgments

We thank Anita Forslund for the model testing and statistical analysis in SAS comparing the concentration of *S. enterica* in the leachate for the two manure application methods as well as providing the *S. enterica* 775W strain. We thank Szymon Kopalski, Lasse Gudmondsson, and Jens Bisgaard for skilled technical assistance with collection of intact soil cores. This work was supported by the Pathos Centre (ENV 2104-07-0015) funded by the Danish Strategic Research Council.

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# MANUSCRIPT II

*Food Research International (2012)*

# Soil survival of *Salmonella* and transfer to freshwater and fresh produce

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Food Research International 45 (2012) 557–566

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Review

# Soil survival of *Salmonella* and transfer to freshwater and fresh produce

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ARTICLE INFO

*Article history:*
Received 15 April 2011
Accepted 14 July 2011

*Keywords:*
*Salmonella*
Fresh produce
Survival
Transport
Farm environment

ABSTRACT

An increase in human outbreaks caused by *Salmonella* sp. has been recognised in many parts of the world. Outbreaks are increasingly related to consumption of fresh produce and an understanding of soil survival of *Salmonella* and transfer to water and to fresh produce are needed to control salmonellosis. The increase in outbreaks is most likely associated with better surveillance, greater consumer demand and a change in production and distribution. This review summarises the recent literature of the ecology of *Salmonella* sp. in soil environment including sources, survival, transport and crop contamination. Areas with a high density of animal production are recognised as a high risk for contaminating fresh produce, however the specific path of contamination remains unconfirmed. Suspected sources include improperly composted manure/wastewater, the use of irrigation water in the vicinity of animal production and wild animals.

Once introduced to the soil the survival of *Salmonella* sp. has been shown to be influenced by method of introduction, temperature and predation by soil protozoa amongst others. Detection of *Salmonella* in environmental samples is traditionally measured by classical growth dependent methods — these methods are discussed and although the new molecular based techniques still lack sensitivity compared to the classic techniques new possibilities are constantly emerging such as mRNA of the Salmonella invA gene directly in soil.

*Salmonella* sp. can be transferred to fresh water either through the preferential flow paths of the soil or in the surface run-off water. In addition to soil properties this transport is influenced cell properties such as size, electric charge and hydrophobicity.

The contamination of fresh produce is finally shown to follow several different routes including sprinkling and water splashes during rain events, the fresh produce can be contaminated by cells attached to the surface or by internal colonisation of the plant cells.



**Contents**



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0963-9969/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.foodres.2011.07.026

## 1. Introduction

Fresh fruit and vegetables are increasingly recognised as sources of *Salmonella* outbreaks. During the last three decades, the number of documented infections associated with the consumption of fresh produce has increased in the United States (Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). The increase in documented outbreaks (Franz & van Bruggen, 2008) may be related to a change in consumer demand towards a higher consumption of fresh produce. In addition more intensive farming methods in the production of fresh produce will affect a larger number of people. This is seen in Fig. 1 which shows that outbreaks in fresh produce result in a higher number of infection cases than outbreaks in any of the other food sources sampled.

From 1973 to 1997 *Salmonella* sp. were associated with outbreaks in seed sprout, melon, apple/orange juice, lettuce, tomatoes, precut celery and mixed fruit (Sivapalasingam et al., 2004). In 2008, a large outbreak of *Salmonella* Saintpaul occurred in the United States and was associated with the consumption of jalapeño and Serrano peppers. The identified strain was found in agricultural water and on the Serrano peppers at the farm in Mexico (Behravesh et al., 2011). A small survey of Mexican farms growing chilli peppers found *Salmonella* sp. in 43% of the irrigation water samples and in 60% of the tested pepper rinse (Gallegos-Robles et al., 2008). A 2005 outbreak in the United States of *Salmonella* Newport in tomatoes was tracked to irrigation ponds (Greene et al., 2008).

In a review, Lynch, Tauxe, and Hedberg (2009) stated three stages in the handling of fresh produce where contamination is most likely to occur: (1) in the field, (2) during the initial processing and (3) during the final preparation in the kitchen. This review focuses on the contamination in the field. It is considered that manure and irrigation water are the major sources of contamination of fresh produce in the field. Agricultural animals are widely recognised as carriers of *Salmonella* sp. When *Salmonella* finds its way to agricultural areas, the contamination risk will largely depend on its survival capabilities in manure, soil and in/on plants. The goal of this review is to give an overview of recent literature on sources/occurrences of Salmonella in agricultural production, survival in soil, transport to fresh water supplies and to fresh produce.

## 2. Sources of *Salmonella*

The spreading of animal waste and biosolids from wastewater treatment on agricultural land is an economic and practical solution for improving soil quality. Despite large quantities applied to agricultural land, approximately 100 times more animal manure is produced than biosolids in the United States (Gerba & Smith, 2005). Animal manure frequently contains zoonotic, pathogenic bacteria such as certain *Escherichia coli*, *Salmonella* sp., and *Campylobacter* sp. (Gerba & Smith, 2005; Guan & Holley, 2003; Oliver, Clegg, Haygarth, & Heathwaite, 2005; WHO Global Salm-Sur, 2006). Wastewater may contain bacterial pathogens such as *Salmonella* sp., *Shigella* sp., *Yersinia* sp., *Vibroe cholerae*, *Campylobacter jejuni* and pathogenic *E. coli* (Gerba & Smith, 2005; Sidhu & Toze, 2009; US EPA, 2003).

### 2.1. Salmonella recrudescence in the farm environment

*Salmonella* can persist in the farm environment for extended periods of time due to circulation within the farm between different

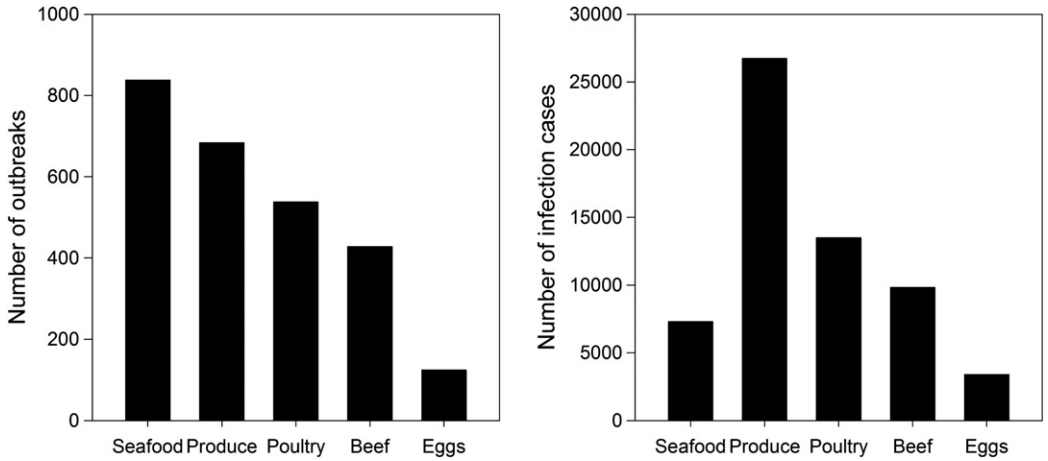


**Fig. 1.** Outbreaks in the most common single articles of food in the United States from 1998 to 2007 were compared to the number of infections caused by the same single articles (data from http://cspinet.org/new/pdf/outbreakalertreport09.pdf).

pools such as animals, excrement, soil and plants (Kupriyanov, Semenov, & van Bruggen, 2010). This can be explained by contamination of surrounding sources such as: feed, water troughs, barnyards and feeding equipment. Several studies have isolated *Salmonella* sp. both in animals and environmental samples (Baloda, Christensen, & Trajcevska, 2001; Dorr et al., 2009; Sandvang, Jensen, Baggesen, & Baloda, 2000; Weigel et al., 2007). Both Baloda et al. (2001) and Sandvang et al. (2000) found that a *Salmonella* Typhimurium clone persisted and survived in a Danish pig farm environment or in pigs with asymptomatic infection, explaining recrudescence of infection in the herd up to approximately 300 days.

### 2.2. Animal waste

The nature and content of *Salmonella* sp. in the farm environment will vary depending on several factors including: species of animal, herd health, herd size, age and housing environment (Ojha & Kostrzynska, 2007; Payne, Osborne, Jenkins, & Sheldon, 2007; Rajic et al., 2007). Often *Salmonella* is characterised by episodic outbreaks and therefore time of detection and spatial distribution will vary from farm to farm (Hoelzel & Bauer, 2008; Van Donkersgoed et al., 2009). Table 1 summarises recent larger occurrence studies of *Salmonella* sp. found in animal manure from pigs, cattle and poultry ranging from 1 to 31.5% occurrence.

#### 2.2.1. Animal waste storage

The most common practise for storing animal waste is as slurry in storage tanks in contrast to the previous method of storing farm-yard manure. Semenov, van Overbeek, Termorshuizen, and van Bruggen (2011) compared the two waste types and found that slurry differs from manure by having less dry matter, lower pH, less nitrogen, more dissolved organic matter and less diversity in microbial community. Pathogens have been shown to survive longer in the slurry than farm yard manure (Nicholson, Groves, & Chambers, 2005). This may be explained by the higher temperature in the farm-yard manure that has been shown to have a negative correlation with *Salmonella* survival (Franz, van Diepeningen, de Vos, & van Bruggen, 2005; Nicholson et al., 2005; Semenov, van Bruggen, van Overbeek, Termorshuizen, & Semenov, 2007).

Furthermore, the anaerobic conditions in the slurry have shown to increase survival for *E. coli* O157:H7 (Kudva, Blanch, & Hovde, 1998; Semenov et al., 2011). However, for *S.* Typhimurium identical survival in aerobic and anaerobic slurry was seen (Semenov et al., 2011). *S.* Typhimurium has been shown to be more resistant to environmental stresses and survive for longer periods of time in natural substrates compared to *E. coli* O157:H7 (Franz et al., 2005; Sinton, Braithwaite, Hall, & Mackenzie, 2007).

### 2.3. Biosolids from human wastewater

Prior to land application biosolids from waste treatment must be treated to reduce the levels of pathogens by: air-drying, anaerobic digestion, aerobic digestion, lime stabilisation or composting (Fig. 2) (US EPA, 2003). If the level of *Salmonella* is below the detection level of 3 MPN/4 g total solids, the biosolid is defined as Class A (US EPA, 2003). Class B biosolids may contain variable concentrations of bacterial pathogens including *Salmonella* sp. This classification determines how the biosolids are used. When it is used with fresh produce or comes in contact with the public the biosolids must be treated in a way that reduces the level of pathogens to below the detection limit (Class A).

During a period of 5 years the concentration of *Salmonella* in raw sludge ranged from 100 to $3.4\times10^4$ MPN (Most Probably Number)/g, whereas the concentration in anaerobically digested biosolids had decreased to an average value of 105 MPN/g (Gerba, Campo, Brooks, & Pepper, 2008). Pourcher et al. (2005) found that after one month composted sewage sludge had concentrations of *Salmonella* below detections limits. However, in a study by Castro-del Campo, Pepper, and Gerba (2007) a regrowth of *Salmonella* to $10^6$ CFU/g was seen when Class A biosolids were stored under anaerobically conditions prior to land application. Zaleski, Josephson, Gerba, and Pepper (2005) found regrowth of *Salmonella* up to $10^5$ CFU/g in anaerobically digested Class B biosolids (Table 2).

### 2.4. Contaminated irrigation water

Limited availability of good-quality water increases the need for the use of low-quality water for various purposes. Irrigation with low quality water may affect crop quality, crop production and the environment. In the United States, microbial pollution has been recognised as a major threat to water sources. The Environmental Protection Agency (EPA) assessed 33% of US water in 2000 and found that 40% of streams, 45% of lakes, and 50% of estuaries were not clean enough to support fishing and swimming (US EPA, 2000). Microbial contamination of groundwater in the United States is also widespread, especially in shallow aquifers where rapid recharge or mixing with surface water can occur (US EPA, 2006). Van Donkersgoed et al. (2009) found *Salmonella* in the water of the feedlot catch basin in 1 out of 21 samples in the spring. Warnick, Crofton, Pelzer, and Hawkins (2001) found *Salmonella* in 2% of tested water samples. The fate of *Salmonella* sp. in river water was tested by (Domingo, Harmon, & Bennett, 2000). After 45 days, a log 3 reduction of *Salmonella* was seen at room temperature. However, when the viable but non culturable (VBNC) cells were counted less than 1 log reduction was observed.

**Table 1**
*Salmonella* presence in various types of manure in different larger studies within the last 10 years.

| | Occurrence % | Isolates | Concentration | Country | Reference |
|---|---|---|---|---|---|
| Pig manure | 1.6 | *S.* Typhimurium, *S.* Derby, *S.* Infantis | N.D. | Germany | (Hoelzel & Bauer, 2008) |
| Cattle manure | 7.7 (10)[a] | N.D. | $2.1\times10^3$ ($2.5\times10^3$)[a] | UK | (Hutchison, Walters, Avery, Synge, & Moore, 2004) |
| Pig manure | 7.9 (5.2)[a] | N.D. | $6\times10^2$ ($6.1\times10^2$)[a] | UK | (Hutchison, Walters, Moore et al., 2004) |
| Poultry manure | 17.9 (11.5)[a] | N.D. | $2.2\times10^2$ ($4.0\times10^3$)[a] | UK | (Hutchison, Walters, Moore et al., 2004) |
| Cattle manure | 21 | Most frequent *S.* Typhimurium | N.D. | US | (Warnick et al., 2001) |
| Cattle faecal sample | 4.9 | N.D. | N.D. | US | (Fossler et al., 2005) |
| Pig manure | 31.5 | *S.* Typhimurium, *S.* Derby, *S.* Agona | N.D. | Canada | (Farzan, Friendship, Cook, & Pollari, 2010) |
| Cattle manure | 1 | *S.*Rubislaw<br>*S.* Saintpual<br>*S.* Mbandaka<br>*S.* Enteritidis<br>*S.* Typhimurium | N.D. | Canada | (Van Donkersgoed et al., 2009) |

[a] Fresh manure occurrence followed by stored manure occurrence in brackets.

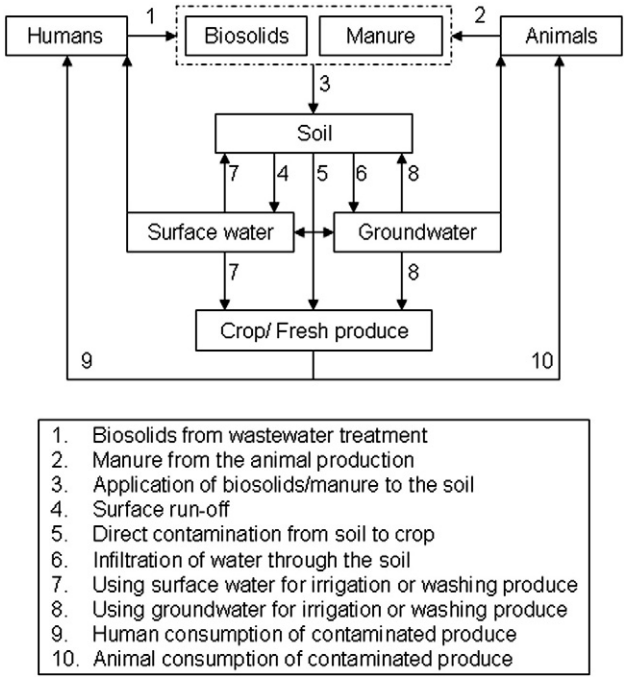


**Fig. 2.** Simplified routes of transfer of *Salmonella* from manure/biosolids through soil to water and/or crop/fresh produce and back to humans or animals. Note that for reasons for simplicity, no arrows for transfer from groundwater and surface water to respective humans and animals have been drawn.

## 3. Survival of *Salmonella* in soil

The survival of *Salmonella* sp. in soil is determined by various factors such as: temperature, moisture, soil type, presence of plants, exposure to sun (UV) light, protozoan predation and the initial number of organisms present. *Salmonella* sp. has been reported to survive from a few days up to 332 days in manure-amended soils (Holley, Arrus, Ominski, Tenuta, & Blank, 2006; Islam et al., 2004; You et al., 2006). It is important to underline that a complex soil environment makes the comparison of survival data of *Salmonella* sp. difficult and highly dependent on where in the soil environment the samples are taken.

A resent paper by Semenov, van Overbeek, and van Bruggen (2009) found that the presence of a newly developed plant root increased survival of *S.* Typhimurium in soil. This was however only found if the manure was injected into soil, whilst no influence of plant roots on survival of *Salmonella* was seen if the manure was applied to the soil surface.

### 3.1. Survival of salmonella in soil according to introduction method

As described in Section 2, *Salmonella* sp. may enter the soil environment from various sources, however manure remains the most common source. Manure has been shown to significantly decrease the survival of *S.* Typhimurium as compared to survival of *S.* Typhimurium in non-amended soil (Garcia, Baelum, Fredslund, Santorum, & Jacobsen, 2010). The higher nutrient level due to the manure addition may increase the activity of the indigenous soil microbial community including bacterial predators (Garcia et al., 2010). In addition, Nyberg, Vinneras, Ottoson, Aronsson, and Albihn (2010) found that the survival (CFU) of *S.* Typhimurium was significantly influenced by the application of cattle, poultry or urine in a sandy soil. The fastest reduction of 1 log unit (T90) was found in urine-amended soil (2.0 days) whilst the same reduction took 8.7 days in cattle-manure amended soil, and 15.3 days in poultry-manure amended soil.

Surface spreading of manure from pig and poultry production is leading to air pollution problems and thus injection of manure into the soil has been required. However, this application method might increase the survival of *Salmonella* sp. Hutchison, Walters, Moore, Crookes, and Avery (2004) found longer survival for *S.* Typhimurium when pig slurry was ploughed into the soil immediately (<2 h) as compared to ploughed into the soil after 7 days or unincorporated. Semenov et al. (2009) compared the survival of *S.* Typhimurium in soil applied with slurry either by surface application or injection and found longer survival times for *S.* Typhimurium when manure was injected into soil. In the same study they compared the survival of *E. coli* O157:H7 and *S.* Typhimurium and found longer survival times for *S.* Typhimurium when manure was injected, whereas there was no difference for *E. coli* O157:H7.

### 3.2. The influence of temperature on Salmonella survival

Temperature has been shown to be an important factor for the survival of *Salmonella* sp. in environmental samples with the general observation that higher temperatures result in increased die-off of *Salmonella* sp. (Arrus, Holley, Ominski, Tenuta, & Blank, 2006; Garcia et al., 2010; Guan & Holley, 2003). The largest decline in levels of *S.* Typhimurium was observed at 25 °C compared to at 15 °C and 5 °C (Garcia et al., 2010). Semenov et al. (2009) showed that survival of *S.* Typhimurium cells in cow manure-amended soil decreased faster at 23 °C than at 7 °C, and similar results were shown by Holley et al. (2006). Garcia et al. (2010) found that temperature cross-interacted with other factors determining survival, thus it was proven that manure addition decreased *Salmonella* survival at 5 °C and 15 °C whilst no differences was found at 25 °C most likely because the die-off was fast in both soil with an without manure. In a soil from an almond orchard the *Salmonella* CFU die-off was 5 log units at 35 °C after 30 days compared to 1 log unit at 20 °C (Danyluk, Zhao, & Doyle, 2007).

### 3.3. The influence of competition and predation on Salmonella survival in soil

The microbial populations in soil can be expected to influence the survival of *Salmonella* sp. upon its introduction to the soil. Increasing the general competition between bacteria could lead to a decreased survival of introduced *Salmonella enterica* (Franz et al., 2005). Another important factor influencing survival of *Salmonella* in soil is predation by protozoa. Brandl, Rosenthal, Haxo, and Berk (2005) investigated the viable form of *Salmonella enterica* in vesicles of the protozoa *Tetrahymena*. They observed that the protozoan ciliate *Tetrahymena* contained intracellular feeding vesicles with high densities of ingested *Salmonella enterica*. The subsequent release of vesicles containing high densities of *Salmonella enterica,* seemed to prolong survival in natural environments at sites contaminated with manure. In a study by Lacharme-Lora, Perkins, Humphrey, Hudson, and Salisbury (2009) several different bacteria were isolated from helminths in cattle and sheep faecal samples, including *Salmonella* gp 3B. The presence of *Salmonella* within intracellular protozoan vesicles likely leads to an underestimation of the actual population of the pathogen because they cannot be detected by plate counting when the soil suspension is plated (Brandl et al., 2005; Gourabathini, Brandl, Redding, Gunderson, & Berk, 2008).

Garcia et al. (2010) found that the decrease in *S.* Typhimurium CFU levels was related to growth of protozoa in the soil. After inoculation of *S.* Typhimurium at 5 °C and 15 °C, blooms in numbers of protozoa occurred within 24 days. Initial numbers of protozoa in manure-amended soils were shown to be 1.5 log higher than in soil samples without manure, indicating that a significant number of protozoa were added along with the manure that could explain

the decreased survival of *Salmonella* sp. in soil receiving manure as discussed above.

### 3.4. The influence of detection method on estimating Salmonella survival in soil

When analysing environmental samples for *Salmonella* sp., there is a risk of underestimating the contamination when using culture-based methods because bacterial cells may enter a viable but non-culturable (VBNC) state (Colwell, 2009). Therefore, cultivation-independent methods have found higher concentrations of *Salmonella* sp. compared to plate counts for water samples (Bjergbaek & Roslev, 2005; Domingo et al., 2000). Turpin, Maycroft, Rowlands, and Wellington (1993) found that intact *S.* Typhimurium cells had a rapid die-off in non-sterile soil according to viable counts on selective agar, whilst total direct counting showed that the cell number remained constant. The authors further compared the survival of inoculated intact cells and UV-killed cells and found that the later declined at a steady rate in soil in contrast to the intact cells.

Marsh, Morris, and Wellington (1998) found a high correlation between cell numbers determined by quantitative PCR and direct microscopic counts for *S.* Typhimurium during 54 days exposure in soil, whereas the cell number declined rapidly when enumerated by plate counts. The importance of the VBNC state is most likely influenced by the moisture and temperature conditions of the soil. In contrast Bech, Dalsgaard, Jacobsen, and Jacobsen (2011) found initially no significant difference between CFU counts and real-time PCR quantification of *S. enterica* serovar Senftenberg in drainage water after introduction into soil with low temperatures (<10 °C).

The introduction of molecular techniques is an important advance to reduce the time required for detection of *Salmonella* and to detect active bacteria in environmental samples through their DNA and RNA (Chiu & Ou, 1996; Cohen, Martin, Simpson, Wallis, & Neibergs, 1996). Numerous PCR based detection methods have been developed for detection and quantification of *Salmonella* in different matrices (Gonzalez-Escalona et al., 2009).

Chiu and Ou (1996) found that the *invA* gene can be used as a target for detection of *Salmonella* nucleic acids in soil samples because it is highly conserved in almost all *Salmonella* serotypes (Boyd, Wang, Whittam, & Selander, 1996; Rahn et al., 1992). *Salmonella* is an invasive organism requiring the expression of the *invA* gene to enter cultured epithelial cells (Galan, Ginocchio, & Costeas, 1992). Detection of *Salmonella* based on the presence of *invA* genes has previously been reported Cortez, Carvalho, Ikuno, Burger, and Vidal-Martins (2006) and validated as a target gene based on a multilab comparison study (Malorny et al., 2004).

*inv*A mRNA has been used as a biomarker for active cells (D'Souza, Critzer, & Golden, 2009; Gonzalez-Escalona et al., 2009; Jacobsen & Holben, 2007). Jacobsen and Holben (2007) showed that *inv*A mRNA prevented the detection of heat-killed cells that DNA based quantification still detected. The transcription of the *invA* gene may differ during different physiological states of the cell, which will affect assay specificity (D'Souza et al., 2009). Comparing *invA* mRNA and DNA quantification with CFU quantification Garcia et al. (2010) showed that the fast decline in mRNA numbers resulted in apparent underrepresentation of the presence of the strain in soil, whilst DNA and CFU gave the best correlation. What remains an unsolved question is if the use of an *inv*A-based reverse transcriptase PCR assays can be used to specifically identify infective *Salmonella* sp. cells in environmental samples.

## 4. Transport of *Salmonella* sp. to freshwater resources

The transport of bacteria in soil is recognised as being very complex due to interactions between processes such as water flow, physical filtration and retention of bacterial cells by attachment to soil particles. In addition, bacterial strain and physiological state will have an influence on the transport potential. Pathogens introduced to the soil environment with waste are either attached to waste particles or planktonic cells that may attach to soil particles upon introduction to the soil environment.

### 4.1. Contamination of water by Salmonella

Recent outbreaks of disease caused by *Salmonella* in water and produce prove that *Salmonella* strains in environmental sources influence human health (Corby et al., 2005; Safranek et al., 2009; Van Houten et al., 1998). The widespread occurrence of *Salmonella* in freshwater testifies to the fact that for many years water has been a *Salmonella* disease carrier (Kramer, Herwaldt, Craun, Calderon, & Juranek, 1996). Jenkins, Endale, and Fisher (2008) monitored a pond in a watershed consisting of grazed pasture, cropped fields applied with poultry manure and a wooded riparian zone from which animals are excluded. From August 2006 to May 2007 the inflow and outflow were tested five times, and naturally occurring *Salmonella* in concentrations from 0.1 to 3.4 MPN/L were found. During one year Haley, Cole, and Lipp (2009) investigated the level of *Salmonella* in an area in Georgia (United States). The bacterium was found in 79.2% of

**Table 2**
Leaching of *Salmonella* sp. from manure or sewage sludge to groundwater. The scaling of experiments is from experimental soil cores over large monoliths to field experiments.

| Pathogen | Experimental condition | Experimental outcome | | Reference |
|---|---|---|---|---|
| | | Soil | Drainage water | |
| *Salmonella enterica* serovar Typhimurium | Pig manure spiked with $8\times10^7$ CFU/mL was applied to clay and sand cores followed by artificial irrigation. | Highest recovery of 3.8% of total applied strain was found in the top 0.2 m decreasing with depth 28 days after the experimental onset. | Highest drainage concentration of $1.3\times10^5$ CFU/mL | Bech et al. (2010) |
| *Salmonella enterica* serovar Senftenberg | Pig manure spiked with $8\times10^7$ CFU/mL was applied to clay cores followed by natural precipitation | Not measured | Highest drainage concentration of $6\times10^4$ CFU/mL. Total recovery ranged from 0.08 to 13.8% | Bech et al. (2011) |
| *Salmonella enterica* serovar Typhimurium | Cattle manure and slurry applied to clay columns. $10^8$ CFU/g dry weight soil | Concentrations in soil decreased with depth from $10^7$ to $10^3$ CFU/g dry weight soil after one day | Not measured | Semenov et al. (2009) |
| *Salmonella enterica* serovar Typhimurium | Sewage sludge applied to intact clay cores. $6.5\times10^6$ CFU/g dry weight sludge fertilised soil | Increased levels of sewage sludge increased distribution down through the soil profile as well as survival times | 25–30% was recovered in the drainage water | Horswell et al. (2010)) |
| *Salmonella enterica* serovar Typhimurium | $1.5\times10^9$ CFU/core was applied with either poultry or cattle manure or human urine | Survival for 90 days in the top 5 cm with poultry manure. By enrichment survival for 180 days regardless of treatment | Strain were not detected in drainage water | (Nyberg et al. (2010) |
| *Salmonella* spp. | Water was sampled from the Little River Watershed. | | Concentration in surface water from 2.5 to 36.3 MPN/L | Haley et al. (2009) |

all samples, ranging from 2.5 to 36.3 MPN/L. The highest concentrations were detected in the summer months and showed correlation with rainfall intensities 1 and 2 days prior to the sampling ($r = 0.77$ and 0.68, respectively). During a 5-year period Setti et al. (2009) tested seawater off the southern Moroccan coast and found *Salmonella* spp. in 4.1% of the samples. *S. enterica* serotype Senftenberg and *S. enterica* serotype Blockley were detected. Setti et al. (2009) found a positive linear correlation between rainfall and presence of *Salmonella* in the seawater samples.

### 4.2. Water flow

Water flow through the soil system is complex and, for instance, determined by soil texture, soil structure, hydraulic properties, slope and soil cover. Rainwater and water from irrigation will either infiltrate into the soil or run off it. In a 49.3 $km^2$ catchment Gentry et al. (2007) found correlations between *E. coli*, turbidity, precipitation and total faecal load. Transport through the catchment was dominated by run-off, although there was also a potential for groundwater-dominated transport.

Transport of bacteria through the soil is mostly determined by the presence of water and its flow paths. Therefore, the path of the water flow will determine the direction of the bacterial transport. Faecal bacteria have been shown to enter both groundwater and surface water in field scale experiments (Gentry et al., 2007; Goss & Richards, 2008; Joy, Lee, Reaume, Whiteley, & Zelin, 1998; Stoddard, Coyne, & Grove, 1998; Unc & Goss, 2003; Vinten et al., 2002).

#### 4.2.1. Infiltration

The rate of infiltration is determined by the water-holding capacity of the soil, easiness of entry, soil hydraulic properties, vegetation types and vegetation cover. A sandy soil with larger pores will provide easy pathways through the soil matrix as opposed to a clayey soil with smaller pore spaces. However, clayey soils are often heterogeneous and contain macropores. Macropores provide a path where water velocity is fast when the soil is close to saturation; for review see (Jarvis, 2007).

The importance of preferential flow in macropores is determined by both length and connectivity. Several studies have shown that any soil that receives sufficient rates of water will lead to a rapid transport of bacteria through the soil (McMurry, Coyne, & Perfect, 1998; Unc & Goss, 2003). The importance of macropores for the transport of *Salmonella* sp. has been verified by outdoor lysimeters, both as regards natural precipitation (Bech et al. (2011) and irrigation (Bech et al., 2010).

#### 4.2.2. Overland flow

Overland flow of water occurs when the rainfall intensity exceeds the infiltration rate of the soil or when the soil is water-saturated. In general, the movement of a bacterial cell can be compared to that of a soil particle. In surface run-off, the classical movement of soil particles is: detachment, transport and deposition. To our knowledge, these processes have been given little attention regarding *Salmonella*. Therefore, this section is focuses on the transport of *E. coli* since the surface properties of this Gram-negative strain are expected to be more or less similar to those of *Salmonella*. However, *Salmonella* has been shown to have better survival strategies than *E. coli* in the natural environment; for review see (Winfield & Groisman, 2003).

The density and size of the particles determine the settling velocity in the run-off water. Small low-density particles will settle more slowly than large high-density particles. Paul and Clark (1996) estimated that microorganisms have a low density of 1.1 g/$cm^3$ and a size of 1–2 μm in diameter. In contrast, soil particle density is considered to be 2.65 g/$cm^3$, and such particles differ in size, the smallest being the clay fraction which is defined as <2 μm. McKergow and Davies-Colley (2010) monitored stormflow dynamics and *E. coli* in a 2180 $km^2$ large catchment and found that 98% of detected *E. coli* in the surface water occurred during storm events. Furthermore, they found that concentrations of *E. coli* exceeded the turbidity which most likely is explained by a smaller likelihood of deposition of *E. coli* compared to soil particles. In a laboratory controlled experiment Muirhead, Collins, and Bremer (2006) compared the transport of attached and planktonic *E. coli* in a saturated soil and found that planktonic cells were more easily transported across the soil and thus to the recipient waters. Guber, Pachepsky, Shelton, and Yu (2007) found that manure decreases the attachment of faecal coliforms to clay and silt fractions and therefore increases the proportion of planktonic cells in run-off water.

### 4.3. The effect of cell properties on transport

#### 4.3.1. Cell size

Filtration is a physical removal of bacteria from a soil solution. Factors that influence filtration are bacterial cell size and shape, particle size of the porous media, degree of water saturation and clogging of the soil (Stevik, Aa, Ausland, & Hanssen, 2004). When the size of a bacterial cell is greater than 5% of the mean interstitial pore diameter, filtration becomes an important factor (Herzig, Leclerc, & Legoff, 1970). Statistically, straining of bacterial cells has been shown to correlate with bacterial size (Gannon, Manilal, & Alexander, 1991).

The physiological state of a microbial cell is related to its size. For *Salmonella enterica* serovars the radius ranged from 0.42 to 0.56 μm in the mid-exponential phase and 0.38–0.49 μm in the late-exponential phase (Haznedaroglu, Zorlu, Hill, & Walker, 2010).

Haznedaroglu et al. (2010) also compared motile and non-motile *Salmonella enterica* serovars and found that the non-flagellated strains showed 10% retention whereas the two motile strains showed 25 and 45% retention. This was explained by the ability of the motile strains to swim towards pore spaces and surface irregularities that would otherwise not be accessible. Liu and Papadopoulos (1995) suggested that when the diameter of the pores is smaller than the length of the flagella, unidirectional motility occurs for *E. coli*. In pores larger than the flagella, the random tumbling will move bacteria in various directions.

#### 4.3.2. Cell charge

Attachment of bacterial cells to soil particles has been explained by the double layer theory (DLVO) (Derjaguin & Landau, 1941; Verwey, 1947). The theory assumes that particle attraction occurs over a short distance, termed primary minimum (<1 nm), and over a long distance, termed secondary minimum (5–10 nm). Between these two minima is a zone of maximum electrostatic repulsion which decreases with an increase in ionic strength of the aqueous solution (Marshall, Stout, & Mitchell, 1971). The retention of *Salmonella enterica* serovar Pullorum increased from 27% to 69% when the ionic strength increased from 1 to 100 mM through quartz packed columns (Haznedaroglu, Kim, Bradford, & Walker, 2009). In addition, the retention of *S.* Pullorum was compared to that of *Escherichia coli* O157:H7, and it was found that the retention of *E. coli* O157:H7 was higher because *S.* Pullorum was more negatively charged. Horswell et al. (2010) found that more *S.* Typhimurium cells were leached in the presence of sewage sludge and explains this by the presence of organic matter that will compete for the sorption sites on the soil surface.

#### 4.3.3. Cell hydrophobicity

Adhesion of *Salmonella* cells to soil particles has been shown to correlate with cell surface hydrophobicity (Stenstrom, 1989). Huysman and Verstraete (1993) found that hydrophobic strains were 2–3 times slower to percolate through soil columns. Haznedaroglu et al. (2010) found that the hydrophobicity of *Salmonella* enterica serovars was quite hydrophilic at ionic strength from 1 to 100 mM in both the mid- and late-exponential phase.

#### 4.3.4. Biofilm

The formation of biofilm has been shown to enhance retention of *E. coli* in soil (Abu-Lail & Camesano, 2003; Brombacher, Dorel, Zehnder,

& Landini, 2003). In a small-scale experiment, the leaching of *Salmonella* sp. was compared to that of *E. coli* as regards the ability of cells to produce biofilm. Correlations were found between biofilm formation and retention in the sandy columns. However, *Salmonella* spp. showed a smaller retention even though the ability to produce biofilm was similar to that of *E. coli* (Salvucci et al., 2009). This indicates that despite the ability of *Salmonella* spp. to produce biofilm, there is a higher risk of infiltration through the soil and thereby contaminating the groundwater.

## 5. Transfer of *Salmonella* sp. to fresh produce

Fresh produce contaminated with *Salmonella* sp. in the agricultural environment might be the primary route infection of people. The increased focus on raw vegetables as a healthy food source is highlighting the need to ensure that the produce is not contaminated with *Salmonella* sp. The use of organic sources for plant nutrients, as dictated for organic farming practise, will unavoidably lead to increased exposure. Since 2008 five reviews Franz and van Bruggen (2008) Holden, Pritchard, and Toth (2009) Teplitski, Barak, and Schneider (2009) Berger et al. (2010) Critzer and Doyle (2010) have been published with focus on pathogens in the agricultural production and the potential for fresh produce contamination.

These five reviews within the last three years represent a source of knowledge on the transfer of *Salmonella* sp. to fresh produce and as such we have decided to only include discussions on very recent publications that are not already covered in the five reviews.

### 5.1. Transfer from infected soil to plant surfaces

It is generally believed that the risk of contaminating produce in the field is influenced by two factors: (1) concentration of the pathogen in the soil which amongst others depend on soil and manure type as discussed in Section 3, and (2) distance from the soil to the edible part of the plant.

One of the main routes of transfer of *Salmonella* sp. to fresh produce is germinating seeds in manure-amended soil. Cress and oat seedlings were shown to be colonised by *S.* Typhimurium both above-ground parts and roots after the seeds germinated in an experimental pot with mixed soil and dung spiked with *Salmonella* sp. (Semenov, Kuprianov, & van Bruggen, 2010). The densities of *S.* Typhimurium in the soil influenced the relative distribution of *S.* Typhimurium on root and leaves. Whilst comparable densities were present on roots and leaves on seedlings grown in soil/dung mixtures with high numbers of *S.* Typhimurium, the numbers on the roots were lower than on the leaves when the seedlings were grown in soil/dung mixtures with low numbers of *S.* Typhimurium.

The infection of tomato seedlings with *S. enterica* was shown to continue several weeks after the *S. enterica* was introduced to the soil (Barak & Liang, 2008). It was seen that the population of *S. enterica* in the soil declined significantly whilst the number of infected tomato leaves was steady for the first five weeks. *S. enterica* was shown to be able to colonise tomato plants both from seedling germinating in *S. enterica* infested soil and upon irrigation with *S. enterica* infected water, but the highest number of the pathogen was found when exposed to irrigation water (Barak, Gorski, Liang, & Narm, 2009).

Interestingly, Semenov et al. (2010) showed that feeding the infested leaves to grape snails resulted in colonisation of the snails, and they found up to $5\times10^5$ *S. enterica* Typhimurium CFU per gramme of dry snail excrements. This route is interesting since the snails can move, and thus spread *S. enterica* Typhimurium from seedlings on the infested ground to trees.

*S. enterica* serovar Weltevreden has been shown to be able to colonise spinach rhizosphere and pyllosphere, and it was found, that whilst the population declined in the pyllosphere it actually increased over time in the rhizoshere (Arthurson, Sessitsch, & Jaderlund, 2011). They used an *inv*A-based PCR assay and the limit of detection was 10,000 cells per gramme of soil/rhizoshere/pyllosphere explaining why they found only *S. enterica* serovar Weltevreden in systems that initially received high numbers ($1\times10^6$) of the strain. Detection techniques aiming at fast and sensitive enumeration of *Salmonella* sp. in fresh produce have been the aim of many studies, but recently a paper by Miller, Davidson, and D'Souza (2011) focused on the detection of mRNA of *inv*A gene from *S.* Typhimurium directly on lettuce and tomato. The use of mRNA-based detection will focus on the viable cells, whilst DNA-based technique also detects dead cells. However the detection limit was as high as $10^6$–$10^7$ inoculated *S. enteric* Typhimurium on 25 g lettuce or 100 g tomatoes without pre-enrichment, such high detection levels make the technology non-feasible from a regulatory point of view.

The role of competitors and predators are likely to have a large effect on *Salmonella* sp. colonisation of the roots and leaves of fresh produce. In a study of the colonisation of cabbage grown in *S. enterica* contaminated soil, high temperature and thus microbial activity was suggested to explain why only very few *S. enterica* could be found on the leaves (Ongeng, Muyanja, Geeraerd, Springael, & Ryckeboer, 2011). Several factors can be expected but a clear route is the predation of *Salmonella* sp. by leaf- or soil-living protozoa. The influence of protozoa on the survival of *S. enterica* was tested using predators freshly isolated from lettuce from the supermarket as well as one previously isolated (Gourabathini et al., 2008). It was found that only one of the four tested protozoa (the previously isolated) was able to produce vesicles that contained *S. enterica.* Interestingly, an analysis of eleven randomly chosen bacteria isolated from the internal of a helmint from cattle manure showed the presence of *Salmonella* sp. (Lacharme-Lora et al., 2009).

### 5.2. Attachment and internalizsation of Salmonella sp. to plants

Plant leaves are colonised by a multitude of different bacteria and protozoa, but the general understanding is that the microbial community composition of leaves is not extensive (Raymond, Wyres, Sheppard, Ellis, & Bonsall, 2010). The use of new generation sequencing opens a new possibility for understanding the microbial ecology of leaves (Redford, Bowers, Knight, Linhart, & Fierer, 2010). The attachment and internalisation of *Salmonella* sp. to plant surfaces can be expected to depend on a several parameters including the plant surface characteristics. By testing the different cultivars of tomato, Barak, Kramer, and Hao (2011) found that they have different numbers of speck lesions and further that the numbers of *S. enterica* in the pylosphere correlated with the numbers of specks.

Swarming genes have been shown to be involved in *S. enterica* colonisation of alfalfa seedlings in soil (Barak et al., 2009). Mutants in two genes, coupled to swamping, colonised 3-days old alfalfa seedling at significantly lower levels 4 h, after inoculation. After 24 h the number of *S. enterica* did decline in all cases but the difference between the swarming negative mutants and the wild-type was still significant (Barak & Liang, 2008). The genes expressed during colonisation of tomatoes differ with plant genotype and age, and no specific functional gene traits have yet been found, but it can be seen that the genes normally involved in *S. enterica* colonisation of animal cells are not expressed, whilst *S. enterica* colonise plant cells (Noel, Arrach, Alagely, McClelland, & Teplitski, 2010).

Different *S. enterica* serovars have shown to be different in their ability to attach to the surface of lettuce and cabbage, and that the serovars showing best attachment were also those able to form biofilm in a standard microbial growth medium (Patel & Sharma, 2010).

## 6. Conclusion

The occurrence of *Salmonella* sp. in the farm environment is dependent on the health of the farm animals and management practises. Sub-clinical animals may be a continuous source of *Salmonella* either directly through the manure or indirectly through farm equipment.

Once manure is spread on the soil, *Salmonella* sp. can persist for several months. Direct contamination of seedling in manure amended soils is a common path for produce contamination. Alternatively, rainfall-induced splashes may contaminate the edible part of the plant. Also, run-off from manure amended soil results in contamination of surface water, which is often used to irrigate produce. There seems to be a tendency for a higher occurrence of *Salmonella* in the spring/summer due to intensive rainfall. This is also the time of the year where the demand for irrigation of produce is high.

A better understanding of the influences between the different pools (such as animals, excrement, soil and plants) in farm environment for *Salmonella* sp. is still required. Furthermore, the concentration of *Salmonella* sp. on the produce seems to be important to develop tools for fast and reliable test of *Salmonella* sp. occurrence. Here is it important to take into account that the VBNC fraction of *Salmonella*,sp. and how infectious *Salmonella* sp. remain outside the host for prolonged periods of time. This may be addressed by molecular methods using the combinations of directly extracted mRNA and DNA, but the detection limits are today still too high to be used outside scientific experiments with often unrealistic high starting concentrations of *Salmonella* sp.

## Acknowledgement

This study was supported by the Pathos project funded by the Strategic Research Council of Denmark ENV 2104-07-0015.

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# MANUSCRIPT III

*Journal of Environmental Quality, submitted*

# Field-scale leaching and survival of tetracycline-resistant bacteria and Escherichia coli from injected pig slurry

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# Field-scale leaching and survival of tetracycline-resistant bacteria and *Escherichia coli* from injected pig slurry

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KEYWORDS: Leaching, preferential flow, field scale, tetracycline, *E. coli*, pig slurry, survival, 16S rRNA sequencing

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**Abstract.**
In this research, the survival and leaching of tetracycline-resistant bacteria were compared with the fecal-indicator bacteria *E. coli*. Pig manure, with a natural content of both tetracycline-resistant bacteria and *E. coli*, were injected at two field sites with clay soil properties. This research had three goals 1) to compare survival of tetracycline-resistant bacteria and *E. coli* from injected manure in soil, 2) to compare the risk of contaminating the aquatic environment with tetracycline-resistant bacteria and *E. coli* due to root-zone leaching and 3) to determine differences in isolated tetracycline-resistant bacteria by 16S rRNA sequencing from manure, soil and drainage water. The bacterial survival did not differ significantly between tetracycline-resistant bacteria and *E. coli* at both sites. Leaching of fecal bacteria was only detected from the one field site, where leaching of tetracycline-resistant bacteria reached 130 CFU $mL^{-1}$ in the drainage water. The leaching of tetracycline-resistant bacteria was only significantly higher as compared to *E. coli* in some of the drainage samples. Preferential flow appeared to be the dominant pathway for the leaching of bacteria and was seen at both near-saturation and unsaturated conditions. Leaching of fecal bacteria were positively correlated with 24 h antecedent precipitation negatively correlated to days after slurry application.Isolated tetracycline-resistant bacteria belonged to the four phyla of Actinobacteria, Bacteroidetes, Proteobacteria and Firmicutes. Even though tetracycline-resistant *E. coli* was seen in the manure it was not found among the tetracycline-resistant bacteria in the drainage water. Findings from this research suggest that fecal bacteria are leached under unsaturated conditions and that *E. coli* may not be suited as indicator bacteria for the leaching of tetracycline-resistant bacteria.

**Introduction**
The spread of antibiotic-resistant bacteria in the environment undermines our ability to treat microbial infections and has become a growing public health challenge. Today antibiotic resistance is a global health problem that involves all major pathogens and antimicrobial drugs (Capita and Alonso-Calleja 2013) and is today classified as a global pandemic (EASAC, 2007). Furthermore, the future value of antibiotic therapies has been questioned by experts (Rosenblatt-Farrell, 2009). For decades, antibiotics have been commonly used on animal farms at therapeutic levels to treat diseases and at sub-therapeutic levels for growth promotion. The intensive use of antibiotics within primary production is the main reason for the spread of antimicrobial resistance throughout the food chain (Capita and Alonso-Calleja, 2013). Tetracycline is one of the most used antimicrobial agents within the agricultural production in the EU (Kools et al., 2008). The use of antibiotics in the agricultural production has resulted in a high level of antibiotic-resistant bacteria in animal waste (Sapkota et al., 2007; Parveen et al., 2006). Tetracycline resistance was seen in 80% of isolated *E. coli* from 90 Canadian farms (Varga et al., 2008) and in the range of 20-50% of isolated *E. coli* from farms in Florida (Parveen et al., 2006). As a consequence large-scale application of animal waste onto agricultural land releases large quantities of antibiotics, resistance genes, resistant bacteria as well as pathogenic bacteria into the soil environment (Chee-Sanford et al., 2001; Chee-Sanford et al., 2009; Kjaer et al., 2007; Vinten et al., 2002), counteracting the positive effects of spreading animal waste on agricultural land. Knapp et al. (2010) found that the level of tetracycline resistance genes in soils was approximately eight times higher in 2008 than the level in 1970-1979. Agerso et al. (2006) compared the persistence of tetracycline-resistant bacteria and the *tet* (M) resistance gene in soil and found that bacteria were recovered during the first 45 days whereas the *tet* (M) was found during the whole experimental period of 152 days. This difference was explained by viable but non-culturable bacteria, horizontal gene transfer of *tet* (M) to indigenous soil bacteria or the presence of plasmid DNA. Sengelov et al. (2003) observed that tetracycline-resistant bacteria decreased to observed levels prior to slurry application within 5 months. Despite a relative short

survival of tetracycline-resistant bacteria in the soil, resistant bacteria have been detected in surface waters and associated with agricultural activity (Sapkota et al., 2007; Lugo-Melchor et al., 2010; Yang and Carlson, 2003). Slurry applied to soils receiving intensive precipitation in the range 10–25 mm $h^{-1}$ has been shown to lead to a rapid transport of bacteria (McMurry et al., 1998; Unc and Goss, 2003). Chee-Sandford et al. (2001) demonstrated that a broad range of tetracycline resistance genes occurred in two swine-waste lagoons and that upon release into the environment, these genes could potentially mobilise and persist. A recent study by Walczak et al. (2011) even found that tetracycline-resistant *E. coli* was mobilized more rapidly through sand columns than tetracycline-susceptible *E. coli*. The authors speculated that the tetracycline resistance altered the bacterial surface properties and thereby enhanced the transport potential. Most transport studies of fecal bacteria have been conducted in soil columns (Guber et al., 2005; Nyberg et al., 2010), influenced by artificial irrigation (Bech et al., 2010; Unc and Goss, 2003; Semenov et al., 2009) or where the slurry is spiked with laboratory-grown strains (Franz et al., 2005; Gagliardi and Karns, 2000; Guber et al., 2005; Semenov et al., 2009). Small-scale experiments have the advantage of reproducibility providing knowledge on a specific problem. However, they provide little, if any, information on the inherent variability of the soil parameters affecting leaching at the larger scale provided by fields. This is of particular importance for structured soils, where preferential flow have a major impact on bacterial leaching (Aislabie et al., 2001; Bech et al., 2010; Safadoust et al., 2012).
In the present experiment the survival and transport of naturally occurring *E. coli* and tetracycline-resistant bacteria following field application of pig slurry were examined at two agricultural sites. Upon injection of pig slurry the survival was followed for 46 days and leaching to drainage water and groundwater was followed for approximately 9 months. Leachate was collected for every 2 mm precipitation and analysed for *E. coli*, tetracycline-resistant bacteria, bromide and turbidity. In addition DNA was extracted from tetracycline-resistant bacteria isolated from slurry, soil and drainage water in order to differentiate bacteria retained in the soil from leached tetracycline-resistant bacteria.
We believe this is the first study to combine survival and detailed transport of tetracycline-resistant bacteria at field scale and provide information of bacterial species detected in both soil and drainage water. Such information is needed to assess health risks associated with antibiotic-resistant bacteria contaminating waterways.

## Material and Methods

### Site description

Two systematically tile-drained sites located at Silstup and Estrup, Denmark were used in the present experiment. The two sites are part of the Danish Leaching Assessment Programme, which focuses on pesticides used in crop production, and intensively monitors leaching from different agricultural research fields representative of Danish conditions (Lindhardt et al., 2001; Kjaer et al., 2011).
The two sites are well described with respect to soil properties, geological setting, and important hydrological parameters such as precipitation, soil water content, groundwater table and drainage runoff are monitored continuously. Several groundwater monitoring wells are installed in the shallow groundwater (1.5–5.5 meters depth). Drainage samples were taken flow-proportional, which is a well-proven sampling strategy for analysing leaching processes of contaminants (Kjaer et al., 2005; Kjaer et al., 2007; Kjar et al., 2011; Juhler et al., 2010). Precipitation was measured using a tipping-bucket rain gauge system and soil water content was measured with a CR10X-controlled Time Domain Reflectometry (TDR) system.

Estrup

The Estrup site is a 1.26 ha cultivated field. The soil is a sandy, loamy soil located on glacial till, with tile drains installed at an average depth of 1.1 m. The ground water table is shallow, 1–3 m below the ground surface (b.g.s.). The upper meter of the soil is heavily fractured and bioturbated containing 100–1,000 biopores $m^{-2}$ (Lindhardt et al., 2001). Three pedological profiles were classified as Aquic Argiudoll, Abruptic Argiudoll and Fragiaquic Glossudalf according to the USDA Soil Taxonomy.

Silstrup

The Silstrup site is a 1.69 ha cultivated field. The soil is a sandy clay loam located on glacial till, with tile drains installed at an average depth of 1.1 m. The soil is heavily fractured and bioturbated. At 0.6 m b.g.s. there has been estimated 400 biopores $m^{-2}$ (Lindhardt et al., 2001). Two pedological profiles were classified as Alfic Argiudoll and Typic Hapludoll.

**Table 1** Physical and chemical properties to 1 m depth at the Silstrup and Estrup field sites, for detailed site description see (Lindhardt et al., 2001).

| **Horizon** | **Depth** | **OM†** | **Soil texture (mm) %** | | | **pH‡** | **CEC§** |
|---|---|---|---|---|---|---|---|
| | cm | % | <0.002 | 0.002-0.063 | >0.063 | | meq/100g |
| **Estrup** | | | | | | | |
| **Ap** | 10-20 | 4.1 | 20.0 | 26.6 | 49.3 | 7.75 | 20.5 |
| **Bt (g)** | 35-45 | 2.9 | 19.8 | 25.9 | 51.4 | 6.36 | 16.4 |
| **Bt(g)2** | 51-61 | 0.5 | 11.8 | 11.2 | 76,5 | 6.32 | 10.5 |
| **Cc** | 85-95 | 0.3 | 50.2 | 19 | 30.5 | 6.35 | 27.1 |
| **Silstrup** | | | | | | | |
| **Ap** | 10-20 | 3.4 | 18.3 | 29.8 | 48,5 | 6.66 | 20.7 |
| **Bv** | 45-55 | 0.5 | 30.1 | 24.8 | 44.6 | 6.67 | 19.8 |
| **Bt(g)** | 115-125 | 0.3 | 43.4 | 21.1 | 35.2 | 7.39 | 17.6 |

†: Organic matter determined by 1.72× total organic carbon by dry combusting.
‡: pH determined in a $CaCl_2$ solution.
§:Cation exchange capacity.

Slurry characterization

Pig (*Sus scrofa domesticus*) slurry from two individual farms near the two field sites was used. Background information on both slurries is given in table 2, where a substantial difference between dry matter content and bacterial concentrations is seen. Both slurries were randomly picked, and the concentration of both *E. coli* and tetracycline-resistant bacteria most likely represents normal concentrations found in Danish piggeries.

Slurry application

A tractor-mounted harrow tine injector was used to apply pig slurry directly into the pre-ploughed soil with nozzles 25 cm apart. Prior to the present experiment slurry was last applied at Estrup in April 2005 and at Silstrup in April 2006.

Both fields received pig slurry twice. At Silstrup 41 t slurry $ha^{-1}$ was injected to a depth of 10 cm on 2 April 2009. This application of slurry with a concentration of 300,000 CFU tetracycline-resistant bacteria $mL^{-1}$ led to an application of $12.3\times10^{8}$ CFU $m^{-2}$ (Table 2). One week later spring barley (Hordeum vulgare L.) was sown. A second application of slurry (13 t $ha^{-1}$ at 4–5 cm depth) was done on 15 September 2009 in a crop of red fescue (Festuca rubra L.).

At Estrup 55 t slurry $ha^{-1}$ was injected to a depth of 11 cm on 6 April 2009. The concentration of tetracycline-resistant bacteria in the slurry was 35,000 CFU $mL^{-1}$ resulting in a concentration of $1.9\times10^{8}$ CFU $m^{-2}$ (Table 2). Spring barley was sown 2 days later. During the second application, 17 t slurry was injected to a depth of 7–11 cm $ha^{-1}$ on 20 August 2009. On 24 August 2009 the field was ploughed and sown with winter rape (Brassica napus L.).

**Table 2** Analysis and quantity of pig slurry from the two applications at Estrup and Silstrup.

| | | **Manure application** | **Dry matter** | **Tetracycline-resistant bacteria** | | ***E. coli*** | |
|---|---|---|---|---|---|---|---|
| | | t/ha | % | CFU/mL slurry | CFU/$m^2$ | CFU/mL slurry | CFU/$m^2$ |
| **Estrup** | 4 April | 55 | 0.79 | 35,000 | $1.9\times10^{8}$ | 20,000 | $1.1\times10^{8}$ |
| | 20 August | 17 | 1.64 | 10,600 | $3.8\times10^{7}$ | 1950 | $1.1\times10^{7}$ |
| **Silstrup** | 2 April | 41 | 6.34 | 300,000 | $12.3\times10^{8}$ | 100,000 | $4.2\times10^{8}$ |
| | 15 September | 13 | 5.17 | 200,000 | $8.4\times10^{8}$ | 13,000 | $5.5\times10^{7}$ |

Enumeration of tetracycline-resistant bacteria and *E. coli*

Two gram of freshly sieved soil was mixed with 18 mL of 0.010 M phosphate buffer (5.7 mL 1M $NaH_2PO_4\times H_2O$ + 4.2 mL 1M $Na_2HPO_4\times 2H_2O$ in 1000 mL water, pH=7.4) and sonicated in a glass tube for 20 s. Tetracycline-resistant bacteria were enumerated in tenfold-dilution series prepared from 1 mL aliquots of the extract. Triplicate 100 µL samples were spread on Müeller Hinton broth (Oxoid Limited, Hampshire, United Kingdom) mixed with 1.5 w/w Bacto Agar (Becton Dickinson, Franklin Lakes, New Jersey) containing 25 ppm tetracycline followed by incubation at 37°C for 18–24 hours. CFU were counted and converted to CFU $g^{-1}$ soil. Drainage samples were vortexed for 5 to 10 seconds followed by plating on Müeller Hinton plates as described above. Colonies were counted and given as an average value from three plates as CFU $mL^{-1}$ drainage water with standard deviations. Viable *E. coli* was enumerated as described above by selected *E. coli* Petrifilm (3M a/s, Denmark).

16S rRNA sequencing

Tetracycline-resistant bacteria were stored in 96 well microtitter plates containing 100 µL Müeller Hinton broth with 25 ppm tetracycline and 50 µL glycerol. The plates were incubated at 37°C for 3–5 hours prior to storage at -80°C. The 96 colonies were randomly picked from slurry, whereas all colonies were picked from both soil and drainage water. Frozen colonies were sequenced to detect possible differences in the dominant strains in the slurry, the soil and the drainage water. As bacteria were only leached from the Estrup site, sequencing has only been done for this site. Genomic DNA was extracted from 96 colonies from slurry and from 23 colonies isolated from soil on day 18. From the drainage water genomic DNA was isolated from 85 colonies leached in the second event (Figure 2). A small aliquot of the frozen colonies were transferred to 2 mL microtitter plates and incubated in Müeller Hinton broth containing 25 ppm tetracycline overnight at 37°C. The UltraClean Microbial DNA isolation kit (Mobio Laboratories, Carlsbad, CA) was used to extract DNA followed by storage at -20°C. Bacterial 16S rRNA genes were amplified from the DNA extracts by PCR with universal primers 27F and 1492R. The reaction mixture (20 µl) contained 2.5 µL DreamTaq mastermix, 0.2 µL DreamTaq, 0.4 µL dNTP, 13.9 $H_2O$, 1 µL of each primer and 1 µL template DNA.

The PCR reaction conditions were as follows: 2 min at 95°C, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, and 90 s at 72°C. To ensure complete elongation, a final step of 72°C for 7 min was performed. The PCR products were purified at the Macrogen facilities (Amsterdam, the Netherlands). To minimise the possibility of sequencing errors, the PCR products were sequenced

bidirectional. The forward and reverse sequences were aligned in BioEdit 7 (Ibis Biosciences) in order to create a consensus alignment with a general length larger than 1350 bp. The nearest culturable relative (>97% similarity) of each strain was found by using the individual 16S rRNA gene sequences as query in the Ribosomal database project (http://rdp.cme.msu.edu/).

Survival in the soil

Triplicate samples profiles were taken on day 0, 1, 6, 18 and 46/49 after slurry application in 20 m sections of three selected slurry injection slits. The sample profile was 20×15×4 cm at Silstrup and 20×17×4 cm at the Estrup site with the slurry slit in the centre of each sample profile. Each soil profile was spilt into six fractions and 2 g soil was taken from each fraction for bacterial quantification. An average value for CFU $g^{-1}$ soil was calculated for each profile. Each survival time point represents an average value of CFU $g^{-1}$ soil from three soil profiles with calculated standard deviations. All soil samples were kept cool and analysed within 24 h.

Despite 3–4 years without slurry application at both sites there was seen a background concentration of 3.3 CFU *E. coli* $g^{-1}$ soil and no tetracycline-resistant bacteria at Estrup. At Silstrup 3.3 CFU *E. coli* $g^{-1}$ soil and 5.4 CFU tetracycline-resistant bacteria $g^{-1}$ soil was seen.

Drainage water samples and groundwater samples

One year prior to slurry application weekly samples of drainage water were analysed for the presence of tetracycline-resistant bacteria and *E. coli*. Out of the 45 drainage samples taken flow proportionally one sample tested positive with a concentration of 6 tetracycline-resistant bacteria $mL^{-1}$. After slurry application drainage water was sampled flow proportionally during 9 months. The sampler was activated by precipitation events and for every 2 mm drainage runoff a subsample of 200 mL were collected. Each drainage sample contained up to nine pooled subsamples. Total of 139 drainage samples were collected at Estrup whereas only 68 drainage samples were collected from Silstrup. Each bottle was analysed for turbidity, bromide, *E. coli* and tetracycline-resistant bacteria. Bromide was measured on an ion chromatograph (Dionex) and turbidity on PhotoFlex (WTW) according to the DIN EN ISO 7027 standard. Groundwater samples were collected monthly from two groundwater monitoring wells and tested for the same parameters as the drainage water.

Statistics

The number of colonies per plate was expressed as CFU $g^{-1}$ soil, and standard deviations were calculated from each sample. The decline in CFU with time was fitted to a logistic function by non-linear regression for each replication in SigmaPlot 11 (Systat Software, Inc): $C_t = a \times e^{(-b \times t)} + c \times e^{(-d \times t)}$, where $C_t$ is the CFU $g^{-1}$ soil at time t (days), $a$ is the initial CFU $g^{-1}$ soil, $b$ and $d$ are the slope parameter for the rate of change ($day^{-1}$) and $c$ is the CFU $g^{-1}$ soil when the decay rate change from the fast to the slower. This model was used by Rogers et al. (2011) and showed the best correlations with the present experiment compared to other tested models (linear, logistic or exponential). T-test was performed to identify differences in the best-fit decay rate coefficients for tetracycline-resistant bacteria and *E. coli*. The level of significance ($\alpha$) was taken as 0.05. Principal Component Analysis (PCA) was used to analyze relationships among all observed variables. Data were arranged in a matrix, where each column corresponds to one of the total of 16 variables, each characterising one of the 28 samples. The data matrix was analysed by The Unscrambler X 10.1 software package (CAMO Software, Norway).

**Results and Discussion**

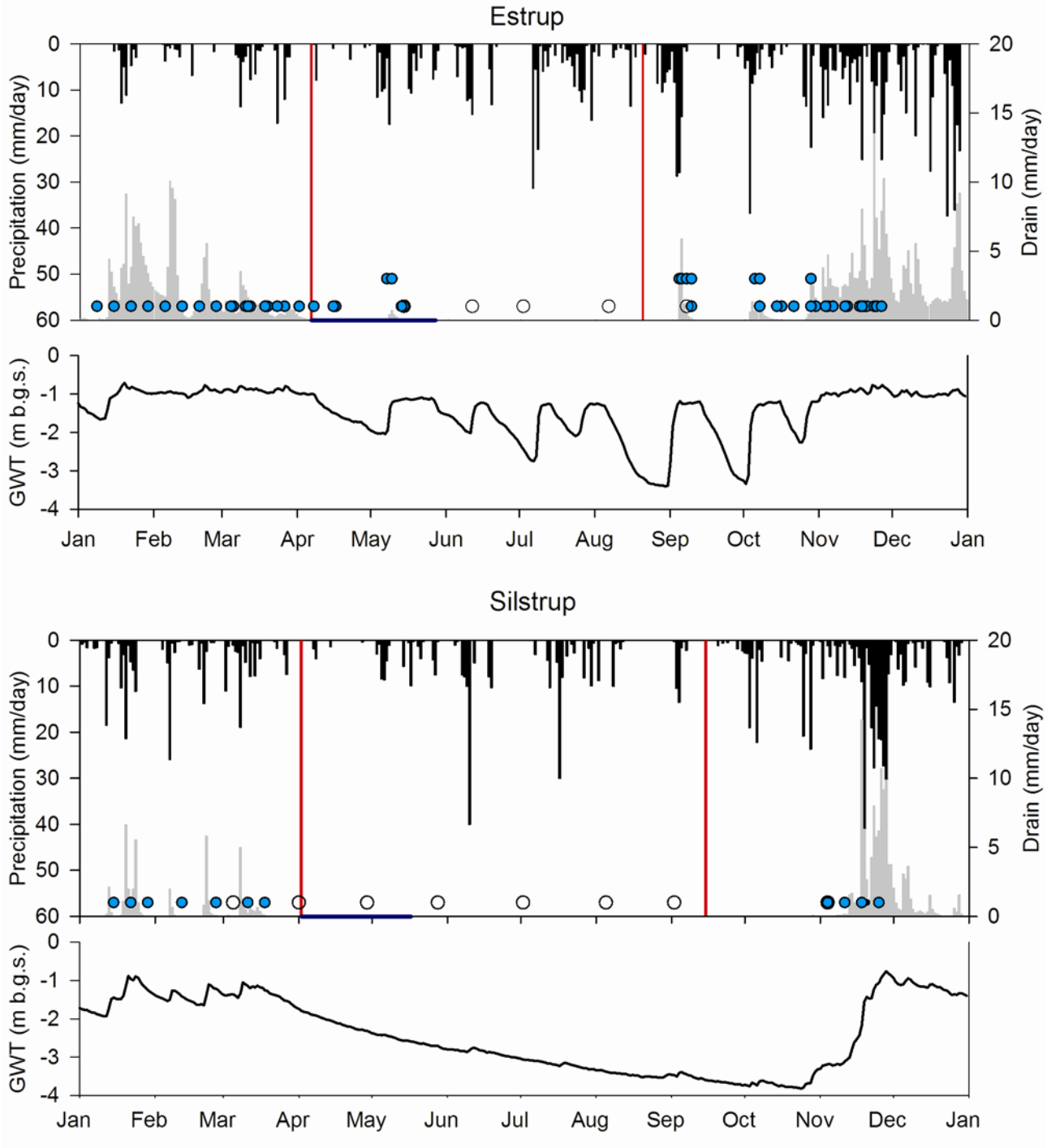


**Figure 1.** Precipitation and drainage runoff in mm/day from Estrup and Silstrup. The vertical red lines indicate slurry application day. The time of the survival experiment is indicated by the horizontal blue line. The round open circles indicate groundwater samples tested and small blue circles indicate drainage water samples. Drainage samples that contained viable bacteria are located higher. The lower figure from both sites shows the location of the groundwater table as metres below ground surface (m b.g.s.).

Simultaneously with the survival experiment water from drains and groundwater monitoring wells were tested for *E. coli* and tetracycline-resistant bacteria. Figure 1 gives an overview of the sampling period as regards precipitation, drainage, groundwater table, slurry application day and

time of the survival experiment. The survival experiment was conducted after the first slurry application in April as indicated by the horizontal blue line.

Survival in soil

There was a rapid decay for both tetracycline-resistant bacteria and *E. coli* at both field sites (Figure 2). After 46/49 days the bacterial concentrations were near the detections limit of 0.3 and 3 CFU $g^{-1}$ soil for *E. coli* and tetracycline-resistant bacteria, respectively. Other studies have found survival times of viable *E. coli* ranging from 17 to 70 days (Franz et al., 2011; Nyberg et al., 2010; Semenov et al., 2009) and for viable tetracycline-resistant bacteria ranging from 45 to 150 days (Agerso et al., 2006; Sengelov et al., 2003). The shorter survival found in the present experiment may be explained the natural fluctuations in temperature and soil moisture occurring at a field scale experiment. This is in contrast to laboratory experiments, where the soil is typically exposed to constant temperature, moisture content and steady-state conditions. Semenov et al (2009) found that fluctuations in temperature decreased the survival time of both S. Typhimurium and *E. coli* O157:H7. In addition, leaching of the fecal bacteria below the 20cm zone in the present experiment would have reduced the bacterial concentration in the soil.

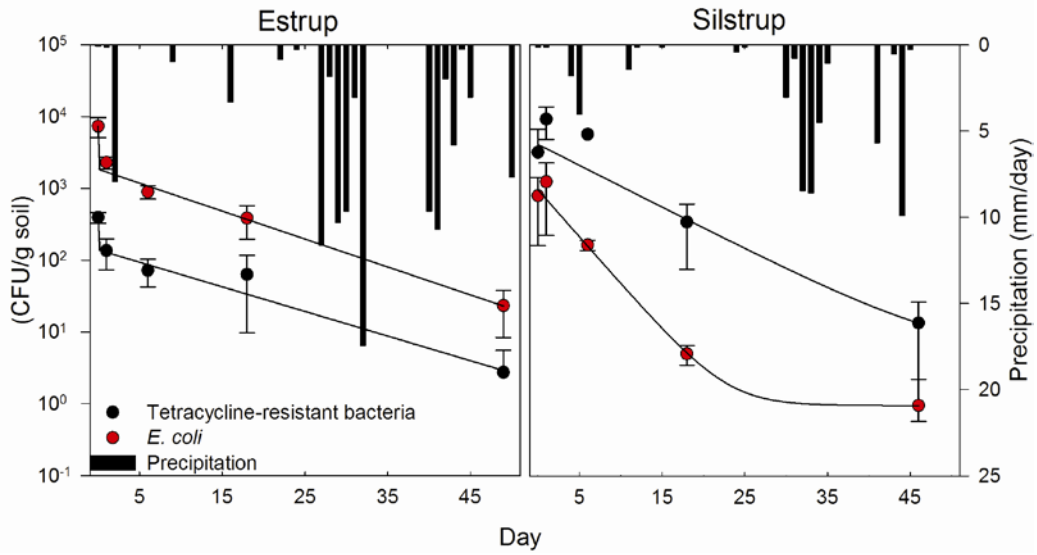


**Figure 2.** Average concentration of *E. coli* and tetracycline-resistant bacteria with standard deviations. The solid lines are the best fit of the non-linear model.

The two experiments, which differed with respect to soil type, precipitation and slurry composition, represented two different environments regarding the slurry soil interaction. As a result there were seen differences in the bacterial die-off curves when comparing sites. At Estrup an immediate fast decay was followed by a slower decay rate from day six and onwards. At Silstrup there was an initial stationary phase or even growth during the first day for *E. coli* and until day six for tetracycline-resistant bacteria. After the initial phase *E. coli* showed a rapid decay whereas a slower decay for tetracycline-resistant bacteria was seen. Due to these differences some precaution should be taken when comparing the estimated decay rates (table 3). Statistically, there was no significant difference between decay rate coefficients (T-test). However, there was a general trend of a faster decay rate after the stationary phase at Silstrup for both tetracycline-resistant bacteria and *E. coli* than at Estrup. The slightly faster decay rates at Silstrup may be explained by a higher dry matter content in the pig slurry at Silstrup (6.34%) as compared to Estrup (0.79%) which resulted in an approximate application of 6 ton dry matter $ha^{-1}$ more at Silstrup.

The initial lag phase at Silstrup may be explained by the pig slurry remaining more concentrated in the injection slit due to higher dry matter content, and protect the fecal bacteria from the initial shock that the soil environment induces. It is generally accepted that slurry properties influence the survival of fecal bacterial in soil. Several have found that slurry with a high content of nutrients increase the population of the indigenous soil community and there through increase competition, and as a consequence decrease survival of introduced fecal bacteria as compared to a less nutrient-rich slurry (Franz et al., 2008; Jiang et al., 2002; Semenov et al., 2009). The initial stationary phase seen at Silstrup was also seen by Scott et al. (2006), that explained the initial growth of *E. coli* in a pig slurry applied loamy soil or sandy loam soil by the volatile fatty acid content of the slurry. However, the ability to grow was soil dependent and not seen in a silt loam soil.

**Table 3.** Best fit non-liniar regression values of $R^2$, decay rate coefficients for viable *E. coli* and tetracycline-resistant bacteria from Estrup and Silstrup.

| | **Estrup** | | **Silstrup** | |
|---|---|---|---|---|
| | *E. coli* | Tetracycline-resistant bacteria | *E. coli* | Tetracycline-resistant bacteria |
| **$R^2$** | 0.97 | 0.93 | 0.94 | 0.76 |
| **a** | 5516 | 256.4 | 960.7 | 4124 |
| **b** | 284.8 | 55.5 | 0.30 | 0.13 |
| **c** | 1853 | 139.4 | 0.95 | 4.9 |
| **d** | 0.090 | 0.079 | ~0 ($1.9\times10^{-11}$) | ~0 ($5.65\times10^{-10}$) |

When comparing the survival of *E. coli* and tetracycline-resistant bacteria at each site, the higher concentration of *E. coli* from the Estrup soil was surprising because the concentration of *E. coli* in the manure was approximately half of the tetracycline-resistant population. The opposite was seen at Silstrup where the higher concentration of tetracycline-resistant bacteria in the soil correlated with the initial concentrations found in the pig slurry. An initial rapid die-off in the tetracycline-resistant bacteria population at Estrup may be explained by a better spread into the soil environment that would result in greater bacterial stress due to fewer nutrients. Bacterial strains have different survival strategies that may explain these differences in survival (Guan and Holley, 2003; Scott et al., 2006; Topp et al., 2003).

Leaching

Leaching of both tetracycline-resistant bacteria and *E. coli* was only seen at the Estrup site (Figure 1), where precipitation led to leaching on several occasions. Figure 1 shows how each event of bacterial detection in drainage water is correlated with a groundwater table near 1 m b.g.s. compared to Silstup where fluctuations in the groundwater table is less sensitive to precipitation. The first drainage event was 33 days after the April application of slurry. Recovery in the drainage water was $0.3\times10^{-4}$ % of tetracycline-resistant bacteria and $0.2\times10^{-5}$ % of *E. coli*. The recovery was calculated as the total amount found in the drainage water as a proportion of what was applied to the field site with slurry. The low recovery is most likely a combined effect of the die-off in the soil and one precipitation event with a total of 17.8 mm during 47 hours with the highest intensity of 4.7 mm $h^{-1}$. Following the slurry application in August bacteria were detected in several drainage events. Overall, as seen in figure 1, there is more precipitation after the August application. During two days there was 64 mm precipitation with intensities up to 10 mm $h^{-1}$ (2nd event, Figure 3). 14 days after slurry application concentrations up to 130 CFU tetracycline-resistant bacteria $mL^{-1}$ were seen, whereas for *E. coli* the concentration was only 3 CFU $mL^{-1}$. For tetracycline-resistant bacteria the recovery was $0.5\times10^{-2}$ % and for *E. coli* $0.3\times10^{-2}$ %. Leaching of fecal bacteria lasted for up to two months after the August slurry application.

At Silstrup there was no drainage run-off after the first slurry application, and after the second slurry application, it took approximately 50 days for the first drainage run-off to take place. All water samples tested after slurry application at Silstrup contained neither tetracycline-resistant bacteria nor *E. coli*. The decline in the introduced bacterial population resulted in a decreased risk of leaching with time. Despite precipitation events with intensities ranging from 1–2 mm $h^{-1}$ within the first weeks after slurry application the drainage system remained dry because of the low-lying groundwater table (GWT) (Figure 1). The drainage system first became active when the GWT was in the vicinity of 1.1m b.g.s. by the end of November. Saini et al. (2003) found that the concentration of *E. coli* decreased with increasing time between slurry application and the first precipitation event, and that highest concentrations of *E. coli* were found in the first leachate sample after slurry application. This correlates with the present study where the highest concentration of tetracycline-resistant bacteria was found in the first drainage event after slurry application with 6.7 CFU $mL^{-1}$ in May and 130 CFU $mL^{-1}$ in September. For *E. coli* the highest concentration in the drainage was lower with concentrations in the spring of 0.7 CFU $mL^{-1}$ and 3.3 CFU$mL^{-1}$ in the fall. Significantly higher concentrations of tetracycline-resistant bacteria were leached compared to *E. coli* in some of the drainage samples. However, the overall difference was not significant (two-way ANOVA).

In addition to the soil hydraulic parameters and survival time of the bacteria, manure properties would also have influenced the leaching potential of the bacterial strains. The presence of manure components have been shown to decrease the filtration through the soil because of competition for sorption sites (Gagliardi and Karns, 2000; Johnson and Logan, 1996; Unc and Goss, 2003). Mosaddeghi et al. (2009) found when comparing different manures that the filtration of *E. coli* was inversely correlated to the DOC concentration of the three manures. However, the higher proportion of dry matter applied to the Silstrup soil was not enough to generate bacterial leaching to the drainage water. An important difference between the two sites as that after the second slurry application the soil was ploughed four days later at Estrup whereas at the Silstrup site the soil was not disturbed after the slurry injection. The ploughing would have led to an increased spread of the slurry and possible increased the risk of fecal bacteria reaching the preferential flow system. In addition, the thinner slurry at Estrup would have spread better into the surrounding soil.

When comparing leached bacterial concentrations to precipitation volumes and intensities in a PCA loading plot there was observed a positive correlation with 24 h antecedent precipitation and a clear negative correlation between leached faecal bacteria and days after slurry application.

Detailed information about precipitation and drainage (mm $h^{-1}$) is combined with inorganic measurements and bacterial concentrations from the events at Estrup (figure 3). The drainage water was very sensitive to precipitation, responding within a few hours to even small precipitation events (Figure 3). This fast travel time indicates the importance of preferential flow. Kjaer et al. (2007) estimated that the time for water to reach the drain with piston flow through the soil matrix would be 98 days. Bacteria were detected 14-33 days after slurry application, which proves that preferential flow rather than piston flow took place. The first event was 33 days after the slurry

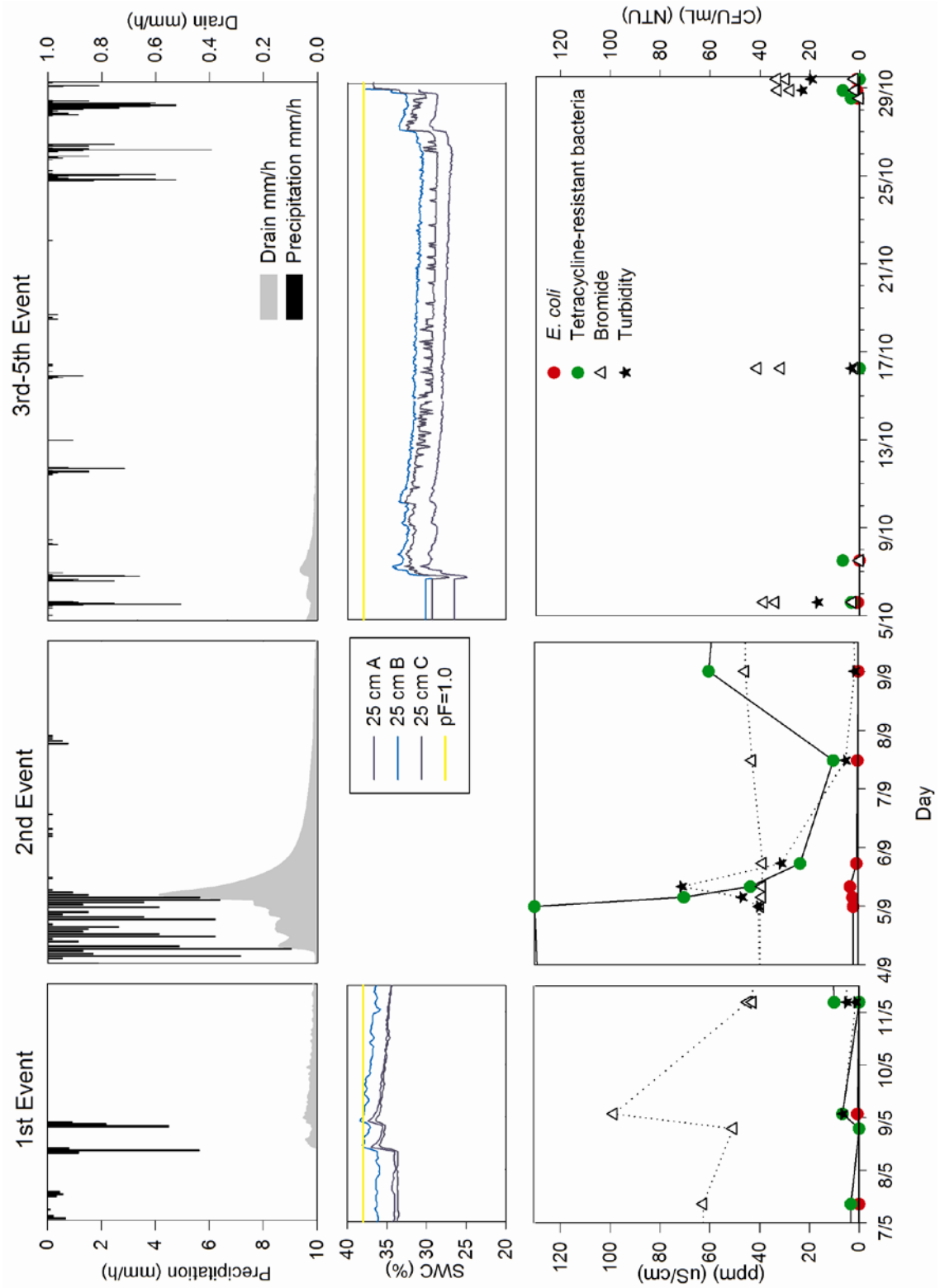


**Figure 3.** Detailed overview of the precipitation events leading to leaching of bacteria. Precipitation and drainage is given as mm/hour in the uppermost figures. In the middle, the soil water content is shown as three separate measurements every hour; the yellow line indicates the matrix potential of 10 cm. The dynamics of *E. coli*, tetracycline-resistant bacteria, bromide and turbidity in collected drainage samples are plotted together in the lowermost figures.

application in April, where only a small peak of bacteria is seen. However, in this event the highest peak of bromide was seen, which most likely was a result of the first precipitation event after field application of the inorganic tracer. The rapid appearance of bromide in the drainage system again proves the importance of the rapid preferential flow systems at Estrup.
The second event was the largest event regarding the concentration of leached fecal bacteria. Along with the increase of bacteria in the drainage water there was seen an increase in turbidity. This increase was a result of particle release due to the rapid flush through the macropores. The importance of preferential flow for downward movement of bacteria has previously been documented (Aislabie et al., 2011; Bech et al., 2010; bu-Ashour et al., 1998; McMurry et al., 1998; Safadoust et al., 2011; Safadoust et al., 2012). Events 3–5 were a result of smaller precipitation events that led to some drainage runoff. The bacterial concentrations were lower compared to the second event, but viable fecal bacteria were found approximately 2 months after slurry application. At the last three events bromide was still found at concentrations similar to the first two events. However, turbidity was lower than the second event and is most likely explained by smaller precipitations intensities.
Preferential flow with bacteria occurred at variable saturated conditions; however the highest bacterial concentrations were found after the August application where the saturation was smallest. The soil water content (SWC) during the events of bacterial transport is seen in Figure 3. Due to monitoring problems the SWC for the second event is unfortunately not available. Javis (2007) states in a review that water pressures must be close to saturation (>-10cm) to generate non-equilibrium flow and transport in macropores. At Estrup the soil matric potential of -10cm equalled SWC of 0.38$cm^3$ $cm^{-3}$. In May the SWC was close to 0.38$cm^3$ $cm^{-3}$ whereas in October the average SWC was approximately 0.30$cm^3$ $cm^{-3}$. Leaching of bacteria at unsaturated conditions has previously been shown with high irrigation intensities ranging from 8–80mm $h^{-1}$ (bu-Ashour et al., 1998; Safadoust et al., 2011). However, in the present experiment the preferential flow through the vadose zone occurred with the highest precipitation intensity of 9mm $h^{-1}$. These findings contradict other studies where it has been concluded that unsaturated transport supports greater cell adsorption and filtration (Jewett et al., 1999; Mosaddeghi et al., 2010; Schafer et al., 1998; Wan et al., 1994). However, Powelson and Mills (2001) found that in columns with variable unsaturated flow, the cell concentration in outflow increased simultaneously with a decrease in volumetric water content. Nimmo (2012) recently stated in a review that preferential flow in some cases is more effective in dry than wet soils as a result of for example clay swelling in the moist soil or the fact that a soil with a lower soil water content may be a result of a high connectivity within the preferential flow system. This was revealed by Rosenbom et al. (2008) where the dye-tracers Acid yellow 7 and Sulforhodamine B infiltrated to a greater depth with SWC of 0.26$cm^3$ $cm^{-3}$ as compared to 0.30$cm^3$ $cm^{-3}$, where ~0.36$cm^3$ $cm^{-3}$ approached saturation. Tallon et al. (2007) found that the initial soil wetness had no effect on the leaching of *E. coli* and if anything the driest treatment resulted in the deepest transport. Greater knowledge on factors controlling preferential flow is still needed in order to model the risk of contaminating receiving waters. This study has shown that bacteria were still leached at soil matrix potentials less than -10cm which is the common threshold limits in models to generate preferential flow.

Tetracycline-resistant strains

From the Estrup site, DNA was extracted from colonies isolated from the slurry, soil and drainage water samples. Table 3 gives an overview of the isolated strains from each environment. From the slurry all isolated strains belonged to the Escherichia/Shigella genus. Most likely this result was biased by the cultivation approach on a rich media where a fast growing bacterium such as *E. coli* would dominate the plate. It has been estimated that about 1% of the total bacterial community from pig slurry is found by plating (Leung and Topp, 2001). However, we believe that cultivation-based

approaches despite their known drawbacks are the best way to access the spread of tetracycline resistance in this environment. Isolated soil colonies showed greater variation where 53.8% belonged to Proteobacteria, 30.8% Bacteriodes and 15.4% Actinobacteria. A similar pattern in isolated strains from a slurry-treated soil was found by Ghosh and LaPara (2007), where the four phyla of Proteobacteria, Bacteroides, Firmicutes and Actinobacteria were found. Due to the decline in viable tetracycline-resistant bacteria in the soil, these strains were most likely introduced to the soil rather than a result of transfer of resistance genes to indigenous soil bacteria. The background concentration of tetracycline-resistant bacteria prior to slurry application was 0 CFU $g^{-1}$ soil at Estrup. This correlates with a study by Götz and Smalla (1997) who concluded that the presence of slurry had a positive effect on plasmid mobilisation as a result of an increase in available nutrients. Still, the occurrence of transfer was low and it was not possible to determine if the plasmid with resistance originated from the slurry or the soil.
The drainage water was tested one year before slurry application where only one sample tested positive for the presence of tetracycline-resistant bacteria. Therefore, all leached tetracycline-resistant bacteria most likely originated from the pig slurry. As for the soil, there was seen variation in leached strains where 88.7% of the isolates belonged to Proteobacteria, 4.8% to Bacteriodes and 6.5% to Firmicutes (Table 4). The lack of tetracycline-resistant *E. coli* in the drainage water was expected as the leaching of tetracycline-resistant bacteria was significant higher than *E. coli* (Figure 3). Similar survival in the soil for *E. coli* and tetracycline-resistant bacteria indicates that the difference in the drainage is most likely due to different transport properties in between strains. Known factors to influence transport include cell size, flagella, surface charge and hydrophobicity (Gannon et al., 1991; Haznedaroglu et al., 2009; Haznedaroglu et al., 2010; Huysman and Verstraete, 1993; Stenstrom, 1989).

**Table 4.** Number of isolated tetracycline-resistant bacteria from manure, Estrup soil on day 18 and drainage water after the manure application on 20 August.

| **Bacterial division** | **Best phylogenetic match** | **Drainage water** | **Soil** | **Manure** |
|---|---|---|---|---|
| **Actinobacteria** | *Arthrobacter luteolus* | | 1 | |
| | *Streptomyces* sp. | | 1 | |
| **Bacteroidetes** | *Myroides odoratimimus* | 2 | | |
| | *Myroides odoratus* | 1 | | |
| | *Enterococcus faecium* | | 2 | |
| | *Enterococcus hirae* | | 1 | |
| | *Sporosarcina* sp. | | 1 | |
| **Firmicutes** | *Lactococcus lactis* | 1 | | |
| | *Staphylococcus epidermidis* | 1 | | |
| | *Vagococcus* sp. | 2 | | |
| **Proteobacteria** | *Acinetobacter* sp. | 26 | | |
| | *Alcaligenes* sp. | 1 | 1 | |
| | Alcaligenaceae bacterium | | 2 | |
| | *Enterobacter amnigenus* | 1 | | |
| | *Escherichia* sp. | | | 7 |
| | *Escherichia coli* | | 3 | 69 |
| | *Escherichia fergusonii* | | | 2 |
| | *Morganella morganii* | 11 | | |
| | *Providencia* sp. | 7 | | |

| | | | |
|---|---|---|---|
| *Providencia alcalifaciens* | 3 | | |
| *Providencia heimbachae* | 3 | | |
| *Providencia rettger* | 1 | | |
| *Raoultella* sp. | 1 | | |
| *Raoultella terrigena* | 1 | | |
| *Shigella* sp. | | | 8 |
| *Shigella boydii* | | | 1 |
| *Shigella sonnei* | | 1 | 6 |

**Conclusion**

Similar survivals of *E. coli* and tetracycline-resistant bacteria were seen at the two field sites after injection of pig slurry, and within 46 days the bacterial concentration in the soil was near the detection limit of 3 CFU $g^{-1}$ soil for *E. coli* and 0.3 CFU $g^{-1}$ soil for tetracycline-resistant bacteria. Leaching of fecal bacteria was detected from the sandy loam soil with concentrations up to 130 tetracycline-resistant bacteria $mL^{-1}$. Two months after slurry application tetracycline-resistant bacteria and *E. coli* were no longer detectable in the drainage water. Leaching of fecal bacteria were positively correlated with 24 h antecedent precipitation negatively correlated to days after slurry application. Preferential flow was the dominant pathway for the leaching of bacteria and was seen at both near-saturation and unsaturated conditions.

Despite the presence of tetracycline-resistant *E. coli* in the slurry the strain was not found among the tetracycline-resistant bacteria in the drainage water. *E. coli* is often used as a fecal indicator in slurry-applied experiments due to its low background concentration in soil. However, our finding questions the use of *E. coli* as a fecal indicator bacteria.

**Acknowledgments**

This study was funded by the PATHOS centre (ENV 2104-07-0015). We thank the PLAP-programme which made the present study possible as well as Carl H. Hansen, Lasse Gudmundsson, Poul L. Bjerg, Jens Molbo (ongoing field monitoring and data preparation), Christina Lynge and Pia Jacobsen (laboratory technician).

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# MANUSCRIPT IV

*Ready for submission to Journal of Environmental Quality*

# Adhesion of *Escherichia coli* and *Salmonella enterica* in runoff influenced by polyacrylamide

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# Adhesion of *Escherichia coli* and *Salmonella enterica* in runoff influenced by polyacrylamide.

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Key words: Polyacrylamide, runoff, surface water quality, adhesion, bacterial pathogens,

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**Abstract**

Anionic polyacrylamide (PAM) is used in irrigation for erosion control and has improved runoff water quality by for example reduced turbidity, nutrients and pollutants.
In the present study the effect of polyacrylamide on bacterial adhesion was tested to a clay, clay loam and sandy loam soil. Four bacteria with different motility and hydrophobicity were tested: *E. coli*, *E. coli* O157:H7, *S.* Newport and *S.* Poona. A lab-scale runoff model was used to evaluate the effect of polyacrylamide on adhered and planktonic bacterial transport, total suspended solids and total dissolved solids. PAM reduced bacterial adhesion to the three soils with the largest decrease in the sandy loam soil followed by clay and clay loam. In the runoff experiment there was significant decrease in total dissolved solids with the PAM treatment in the clay loam and clay soil as compared to the control whereas this difference was not found in the sandy loam. Bacterial leaching from the clay soil was significantly increased by the PAM treatment for both adhered and planktonic cells as compared to the control. From sandy loam there was a significant decrease of adhered cells with the PAM treatment, whereas there was no difference for planktonic bacteria. PAM treatment significantly decreased the leaching of planktonic cells whereas adhered cells were unaffected from the clay loam. Comparison among strains during runoff studies showed that *E. coli* was leached the most whereas *S.* Poona was retained in the soil. Motility and hydrophobicity could not explain this observation.
The results of this laboratory-based study question the capability of PAM to protect surface water from pathogen contamination.

**Introduction**

Contamination of surface waters with pathogens or fecal indicator organisms originating from stockpile run-off or land-applied waste solids and slurries is well documented (Haley et al., 2009; Jenkins et al., 2008; Van Donkersgoed et al., 2009; Warnick et al., 2001). Heavy rainfall can result in acute surface runoff or lead to infiltration and preferential flow through soil and, ultimately leading to the contamination of the receiving water (Muirhead et al., 2006a; Semenov et al., 2009; Tyrrel and Quinton, 2003). Curriero et al. (2001) found correlation between heavy rainfall and disease outbreaks from 1948 to 1994 in the US. The Environmental Protection Agency (EPA) assessed 33% of U.S. waters in 2000 and found that 40% of streams, 45% of lakes, and 50% of estuaries were impaired and not suitable for recreational uses such as fishing and swimming (US EPA, 2000). Human exposure to recreational waters containing pathogens is a recognized risk for infection; the probability increasing with the extent of bodily contact and pathogen loading. Therefore, it is imperative to find strategies to prevent or minimize contamination of surface waters.

This has led to implementation of best management strategies influencing pathogen spreading in the environment including manure storage time, manure application rate, manure application method and manure application time related to crop cover. In addition, riparian filter strips may be constructed to increase settling and filter surface runoff before entering vulnerable surface waters. A more alternative or synergistic approach is the application of polymers such as the anionic polyacrylamide (PAM) to soil, which has been shown to reduce soil erosion by up to 84% (Lentz and Sojka, 2009; Tumsavas and Kara, 2011; Lentz and Sojka, 1994b; Lentz et al., 1998; Lentz and Sojka, 2000; Sepaskhah and Shahabizad, 2010; Zhang and Miller, 1996). In addition, PAM has reduced the environmental impact of agricultural activities by reducing particulate phosphorus (Goodson et al., 2006), total phosphorus and fecal coliform bacteria (Sojka and Entry, 2000),

biochemical oxygen demand (BOD) and nitrate (Lentz and Sojka, 1994a; Lentz and Sojka, 2000; Goodson et al., 2006; Aase et al., 1998) in runoff water.

PAM has been used since 1995 and is recommended by the Natural Resources Conservation Service (NRCS) for agricultural purposes. It is a high weight polymer containing >150,000 acrylamide monomers. The molecule is long, linear and negatively charged. The degradation of the polymer is approximately 10% $year^{-1}$ (Barvenik, 1994) whereas the more toxic monomers is completely degraded within 5 days in the soil (Shanker et al., 1990).

The application of PAM to soil may also reduce the bacterial load in the effluent water and thereby reduce pathogen contamination of surface and groundwater. Sojka and Entry (2000) found that a PAM treatment reduced the total bacterial number in surface runoff as compared to non treated soils. Entry and Sojka (2000) found that PAM+$Al(SO_4)_3$ and PAM+CaO reduced total coliform bacteria, fecal coliform bacteria and fecal streptococci by 10- to 1000-fold in water flowing 1 and 27 m downstream. Entry et al. (2003) reported a 30-50% reduction of total coliform bacteria and fecal coliform bacteria as a result of PAM treatment. Previous studies have focused on coliform bacteria, total bacteria and total bacterial biomass, fecal coliform bacteria and fecal streptococci as major indicator microorganisms. However, recent studies and review have detailed the lack of correlation between indicator microorganisms and the presence of pathogenic bacteria in surface water (Duris et al., 2009; Shelton et al., 2011; Pachepsky et al., 2011).

The existing literature may as such provide an inaccurate picture of the efficacy of indicator retention in agricultural runoff as a measure of reduced risk or mobility of specific pathogenic bacteria in PAM treated soils. Therefore the aim of this study was to evaluate the effect of PAM treated soils on the adhesion properties and runoff water carriage of enteric pathogenic bacteria with different hydrophobicity and motility.

## Material and Methods

### *Soil*

Surface soil (A-horizon) was collected from major agricultural facilities in Salinas, California US. A total of three soil types were used in this study (Table 1). Soils were collected one month prior to the experimental onset and were stored at 4 °C.

**Table 1** Soil characteristics

| Soil | pH | Sand % | Silt % | Clay % | SOM % | CEC meq $100g^{-1}$ | Fe ppm | Al ppm |
|---|---|---|---|---|---|---|---|---|
| Clay loam | 6.83 | 40 | 25 | 35 | 2.37 | 33.4 | 23400 | 22100 |
| Clay | 7.39 | 34 | 20 | 46 | 3.48 | 43.3 | 38200 | 29100 |
| Sandy loam | 6.34 | 74 | 13 | 13 | 1.46 | 13.5 | 15700 | 12900 |

***Bacterial Strains and inoculum preparation***

Strains of *E. coli* (TVS354), *Salmonella enterica* Newport (PTVS73), *Salmonella enterica* Poona (PTVS124) and *E. coli* O157:H7 (PTVS46) were used in this study. All strains were rifampicin-resistant (80 mg $L^{-1}$), which facilitated detection with negligible interference from soil background bacteria recovery and enumeration.

Bacterial strains were cultured at 37 °C for up to 18 h on tryptic soy agar (TSA, BD Diagnostics, Sparks MD, USA), supplemented with 80 mg $L^{-1}$ of rifampicin (rif, Fisher Scientific). One colony was transferred to 5 mL TSB with 80 mg $L^{-1}$ rif for 18 hours at 37 °C. A total of 100 µL of cell suspension (stationary phase) was transferred to fresh 5 mL TSB rif. Cells were harvested after 6 hours at the end of the exponential phase. The bacterial suspension was centrifuged at 1500×*g* for 10 min. The pellet was washed twice in Butterfield's phosphate buffer (BPB) (Whatman Inc. Piscataway, NJ, USA) and re-suspended in BPB. Cultures were stored at 10 °C for 18 hours followed by adjustment of optical density to an $OD_{620}$ value of 0.700 which corresponds to approximately $10^8$ CFU $mL^{-1}$. Cultures were serially diluted and plated to determine the best estimate of cell concentration in the bacterial suspension.

***Hydrophobicity and motility of selected strains***

The hydrophobicity of the strains was measured using an assay based on the bacterial adherence to hydrocarbons using octane as the organic liquid as previously described (Rosenberg et al., 1980). The cells were grown as described above and re-suspended in a buffer containing 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g $KH_2PO_4$, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ in 1 L of nanopure water adjusted to pH 7.1. One mL of a washed cell suspension with an optical density of 0.700 $OD_{620}$ and 0.5 mL of octane were vortexed for 60 s in a 2 mL Eppendorf test tube. The difference in $OD_{620}$ was measured after 30 min. Hydrophobicity was determined as: $(100 \times (OD_A - OD_B)/OD_A\}$ where $OD_A$ and $OD_B$ represent the optical density of the aqueous phase before and after the hydrophobicity test, respectively.

The motility was tested by a swimming assay on 10% TSB plates with 0.30% agar. An aliquot of 5 µL $10^8$ CFU $mL^{-1}$ culture was carefully placed in the center of the plate. The plates were incubated at 29 °C overnight in a humid environment. Swarm radii were measured with a caliper and made in triplicate.

***Adhesion experiment***

One g of soil and 9 mL of M9 mineral salts 5X (Difco, Lawrence, USA) buffer adjusted to an electric conductivity of 1.3 dS $m^{-1}$ were mixed in a 15 mL Falcon tube (BD Biosciences, San Jose, California, USA). 74 µL of a PAM (SoilFloc E300, Hydrosorb, inc, Orange, Ca, USA) stock solution (0.37 %) was added to achieve a final concentration of 0.9 µg $mL^{-1}$ (0.09 µg PAM g $soil^{-1}$). The soil buffer suspensions were mixed for 36 hours at 150 rpm at 10°C. A total of 50 µL of inoculum ($OD_{620}$ 0.700) was added to the soil suspension resulting in a concentration of $10^6$-$10^7$ CFU $mL^{-1}$ in soil suspension. Samples were analyzed after five hours at 150 rpm at 10°C to allow adhesion between strain and soil.

A buoyant-density separation procedure was used to separate attached cells from planktonic cells. One mL of Histodenz (Sigma Alldrich, St. Louis, USA) at a density of 1.319 was added below the soil suspension followed by centrifugation for 20 min at 3,000 *G* in a swing-out rotor centrifuge

(Termo CL3R). The two layers were separated by decanting the fractions into a sterile 15 mL falcon test tube and the pellet was resuspended in 5 mL BPB. Cell concentrations were determined in both supernatant and soil pellet by preparing ten-fold dilutions and plating 100 µL on TSA rif. The percentage of attached cells was calculated based on the bacterial concentration from both fractions. The effect of PAM on bacterial flocculation was tested in the range 0–10 mg PAM $L^{-1}$, and comparable levels of enumeration were indistinguishable among the treatments (data not shown).

***Soil size distribution***

A total of 250 g of fresh soil and 25 mL sterile nanopure water were mixed with a spatula in 1 L plastic bottles. PAM was added to 25 mL of sterile nanopure to achieve a final concentration of 0.09 µg PAM g $soil^{-1}$. Soils were stored for 1 week at 10°C and sieved through an 8 mm sieve and air dried at 25°C. Samples were sieved using a wet sieving method as previously described by Elliot (1986). Briefly, an 80 g air dried subsample was submerged for 5 min on a 2000 µm sieve. Aggregates were separated by moving the sieve up and down 3 cm with 50 repetitions during 2 min. The following size fractions: >2000 µm, 2000-250 µm, 250-53 µm and <53 µm were collected and oven-dried (50°C). The soil size distribution was determined in triplicates.

***Runoff experiment***

Prior to the runoff experiment the fresh soil was sieved through an 8 mm sieve and allowed to air dry at 25°C. The runoff trays were packed with dry soil at a density of 1.3 g $cm^{-3}$ in boxes (40 cm long, 4 cm deep, and 11 cm wide). One day before the onset of the runoff 500 mL M9 solution was added to the soil resulting in 25 % soil water content. All runoff experiments were conducted on a 5% slope and were repeated in triplicate. The bacterial strains were grown as previous described and a cocktail consisting of 0.25 mL of each strain was diluted into 9 mL M9 solution and pipette evenly on the soil surface two hours prior to the onset of the runoff to allow adhesion. The total amount of applied strains was $10^8$ CFU giving an average concentration in the soil of $5\times10^4$ CFU $g^{-1}$ soil. M9 solution was pumped using a Rainin peristaltic pump onto the top of the soil at a flow rate of 31 mL $min^{-1}$ to generate runoff. In the PAM treatment there was a concentration of 10 µg PAM $mL^{-1}$ whereas in the control experiment a M9 solution without PAM was used. From each runoff experiment 10 independent water-samples of 50 mL aliquots were collected for analysis. Attached and unattached cells were determined by the buoyant-density separation procedure as described above from a 10mL subsample.

A second runoff assessment from each tray was done 48 hours later where one water sample was collected, and 32 randomly collected colonies from the attached and planktonic fraction from each runoff was isolated. Collected colonies were streaked on TSA rif and incubated overnight at 37°C. Two to three colonies were picked and resuspended in BPB to yield an $OD_{620}$ of 0.3-0.5. The cell suspension was subsequently centrifuged at 13.000x*g* for 1 min and suspended in 200 µL of 1X Tris EDTA buffer. Cell lysates were obtained by heating cells suspensions for 10 min at 95°C. DNA from isolated colonies were analyzed through repetitive extragenic palindromic elements (REP) (Versalovic et al., 1991) to identify their unique band patterns and to determine the distribution of leached strains within the cocktail.

Each PCR reaction contained 5 µL 5X colorless GoTaq® buffer (Promega) supplemented with 2.5 µL 25 mM MgCl (Invitrogen), 0.5 µL 10 mM dNTP (Promega), 2.5 µL 10 µM REP1R-I (IIIICGICGICATCIGGC), 2.5 µL 10 µM REP2-I (ICGICTTATCIGGCCTAC), 2.5 µL 10% DSMO (Sigma), 1.25 unit GoTaq® (Promega, Madison WI), 7.75 µL $H_2O$ and 1.5 µL DNA sample. In each PCR well 15 µL mineral oil was placed on top to avoid evaporation.

PCR amplifications were done in an Applied Biosystems cycler with an initial denaturation (4 min, 94°C) followed by 30 cycles of denaturation (94°C, 1 min), annealing (40°C, 1.5 min) and extension (6 5°C , 8 min), followed by a single final extension (65°C, 16 min). DNA fragments were separated in a 1.2% agarose gel for 3 h at 120 V. Isolates showing indistinguishable band patterns were grouped manually and compared with the band patterns of matched inoculum-source pure colonies from the culture collection.

The remaining 40 mL were centrifuged in a centrifuge with a swing out-rotor to determine the solid matter content by centrifugation for 10 min at 2352 rpm (910 g). The settling time for the fraction >0.45 µm was determined as described by Gimbert et al. (2005). The supernatant was decanted, and both fractions were dried at 60°C to determine total suspended solids (TSS) (>0.45 µm) and total dissolved solids (TDS) (<0.45 µm)

***Statistics***

Counted colonies were expressed as CFU g soil$^{-1}$ or CFU mL$^{-1}$ with calculated standard deviations. Data were compared with ANOVA in SigmaPlot 11 (Systat Software, Inc). If normality test failed data was analysed with the Mann-Whitney Rank Sum test. Statistical significant difference among treatments was established when p-value was lower to 0.05.

***Results***

Bacterial surface properties

The swimming assay showed that the motility of *E. coli* O157:H7 was impaired as compared to the other three strains (Table 2). Even though the diameter varied between *S. Poona*, *S.* Newport and *E. coli* these differences should be cautiously used, and therefore we simply conclude that these strains were motile without differentiating or ranking among them. The hydrophobicity showed some variation (Table 2), whereby the two *Salmonella* serovars were characterized as having the lowest hydrophobicity values followed by *E. coli* O157:H7 (6.5 %) and *E. coli* (15.1%).

**Table 2** Cell surface properties. Mean value and standard deviations are based on triplicate.

| | Motility (cm) | Hydrophobicity (%) |
|---|---|---|
| PTVS142 *S.* Poona | 1.6 ± 0.66 | 0.9 ± 0.6 |
| PTVS73 *S.* Newport | 3.4 ± 1.4 | 1.4 ± 1.2 |
| TVS354 *E. coli* | 2.9 ± 0.17 | 15.1 ± 7.3 |
| PTVS46 *E. coli* O157:H7 | 0.5 ± 0 | 6.5 ± 2.0 |

Soil size distribution

PAM changed the distribution of soil size fractions (Figure 1). The PAM treated clay soil had a non significant decrease in the <53 µm and 53-250 µm fractions and an increase in two larger fractions. The PAM treated clay loam and sandy loam had a decrease in the three smallest fractions and an increase in the fraction larger than 2 mm. Statistically, the difference between mean values of PAM treated soils and controls were not significant. The greatest effect of PAM was seen for the clay loam followed by sandy loam and clay, when comparing the mean for each treatment.

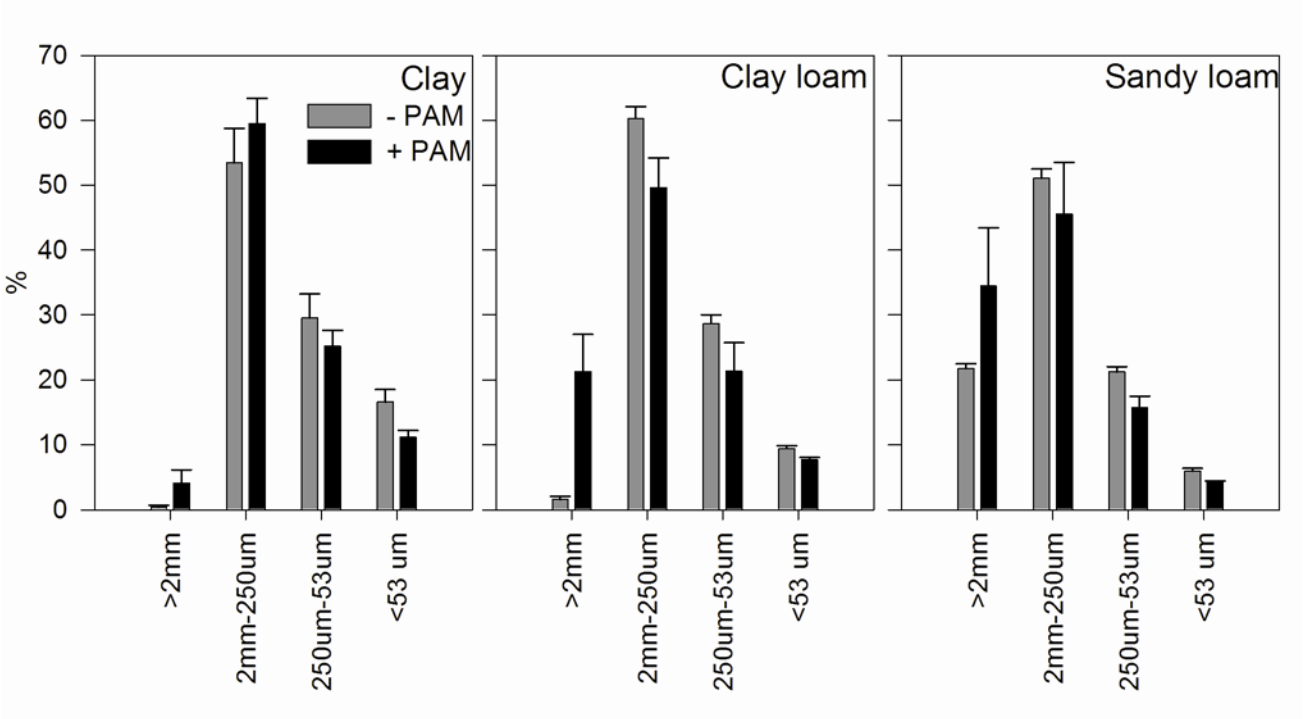
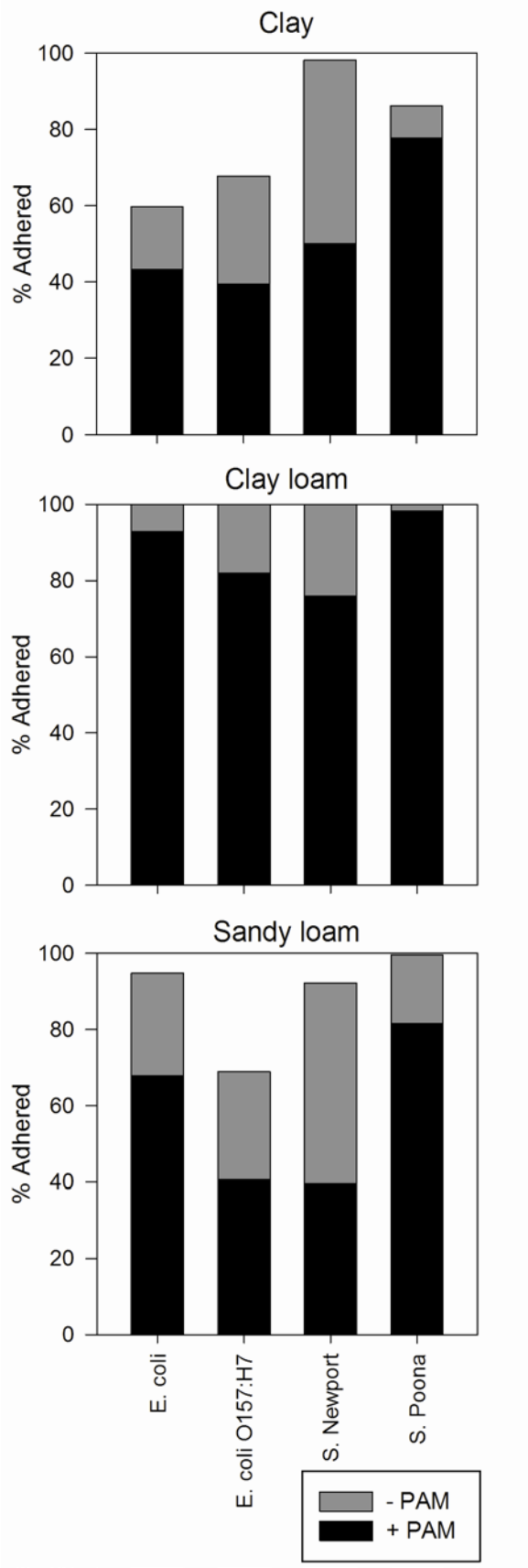



**Figure 1** Difference in soil size distribution comparing a control with PAM treated soil for the three soils clay, clay loam and sandy loam. Average values and standard deviations are based on triplicate samples.

Adhesion

PAM treated soils showed decreased bacterial adhesion as compared to the controls (Figure 2). The effect of the PAM treatment on bacterial adhesion differed between soil types and decreased in the following order: sandy loam>clay>clay loam. The effect of the PAM treatment on species level was highest for the sandy loam soil, where only the adhesion of *S.* Poona was not significantly different. *S.* Poona showed the strongest adhesion affinity compared to the other strains regardless of the PAM treatment. A decrease in bacterial adhesion due to the PAM treatment was significant for the following combinations: Clay/*S.* Newport, Clay loam/*E. coli* O157:H7, Sandy loam/*E. coli*, Sandy loam/ *E. coli* O157:H7 and Sandy loam/*S.* Newport.




Runoff experiment

The unplanted soil boxes with three different soils represented a scenario where a large amount of erosion is expected with heavy rainfall. The soil erosion was greatest from the clay and clay loam soil with a decreasing trend, whereas the erosion was both lower and constant from the sandy loam soil (Figure 3).

In the clay soil PAM significantly decreased TDS in the runoff water compared to the control ($P=0.002$). However, PAM significantly increased the bacterial concentration from the applied bacterial cocktail in the runoff water for both adhered ($P=0.048$) and planktonic cells ($P=0.014$). The runoff from the PAM treatment in the clay loam soil showed a significant decrease in TDS ($P=0.001$), planktonic cells ($P<0.001$) and adhered cells ($P=0.008$). For the sandy loam soil the PAM treatment did not have a significant effect on the amount of TDS in the runoff water ($P=0.07$). There was a significant decrease in the adhered cells with the PAM treatment in the runoff water ($P<0.001$). However, for the planktonic cells the difference between the PAM treatment and control was not significant ($P=0.824$). In table 3 is summarized the average value for TSS, TDS, planktonic cells and adhered cells from the different runoff experiments. Total suspended solids were averaged among the first 1 to 5 water samples in the runoff due to uncertainty in the quality of the measurements. The recovery of bacteria during the PAM treated runoff were ~40% for the clay loam soil, ~100% for the clay soil and ~100% for the sandy loam soil, and ~50%, ~80% and ~100% for the control soils, respectively.

**Figure 2** The percentage of adhered bacterial strains for the three soils clay, clay loam and sandy loam comparing a control with a PAM treated soil. Values are based on triplicates adhesion experiments, where the percentage of adhered cells is calculated as the fraction of the sum of adhered and planktonic cells.

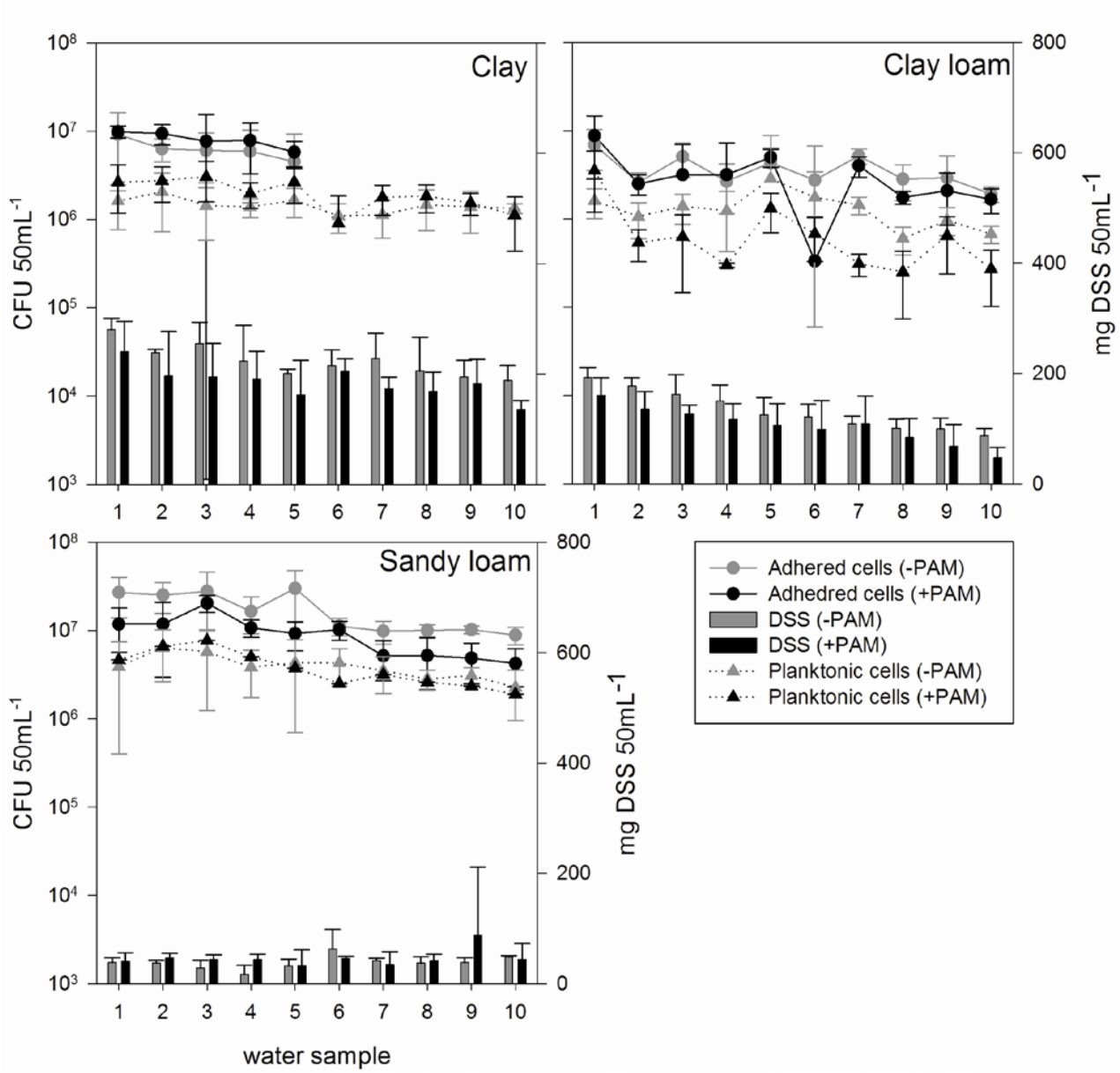



**Figure 3** Runoff data for the three soils based on triplicate experiments. Bars represent dissolved suspended solids (<0.45um) comparing a control (grey) with a PAM treatment (black). The number of planktonic cells are given as CFU $50mL^{-1}$ with (triangles) and the number of adhered cells are given as CFU 50mL-1 (circles). For both adhered and planktonic cells the PAM treatment is colored black and the treatment without PAM is colored grey.

## Distribution of bacterial strains collected from runoff

Genotyping of colonies collected from the last runoff sample were utilized to determine the distribution of the four bacterial strains in the collected runoff with REP-PCR as a fingerprinting technique (Table 4). *S.* Poona was retained the most whereas *E. coli* was the most abundant in the runoff water. The PAM treatment did not change the distribution between the four bacteria in runoff.

**Table 3** total suspended solids (TSS), total dissolved solids (TDS) and bacterial counts given as an average value of the 10 runoff samples comparing the control with the PAM treatment. All samples have been calculated as mg $50mL^{-1}$ or CFU $50mL^{-1}$. Standard deviations are based on triplicates.

| | TSS (>0.45 µm) mg | | DSS (<0.45 µm) mg | | Attached cells (CFU $50mL^{-1}$) | | Planktonic cells (CFU $50mL^{-1}$) | |
|---|---|---|---|---|---|---|---|---|
| | +PAM | -PAM | +PAM | -PAM | +PAM | -PAM | +PAM | -PAM |
| Clay loam | 35.6 | 35.4 | 105.1 ±31.7 | 132.3 ±20.8 | $32.4\times10^5$ $\pm75.2\times10^4$ | $37.1\times10^5$ $\pm90.6\times10^4$ | $8.4\times10^5$ $\pm27.4\times10^4$ | $13.6\times10^5$ $\pm63.7\times10^4$ |
| Clay | 3.7 | 8.1 | 185.0 ± 29.7 | 222.8 ± 9.4 | $81.2\times10^5$ $\pm15.7\times10^5$ | $64.0\times10^5$ $\pm18.3\times10^5$ | $20.3\times10^5$ $\pm82.1\times10^4$ | $14.5\times10^5$ $\pm61.1\times10^4$ |
| Sandy loam | 14.7 | 38.3 | 46.2 ± 17.0 | 38.1 ± 6.0 | $9.4\times10^6$ $\pm30.0\times10^5$ | $17.7\times10^6$ $\pm28.8\times10^5$ | $40.3\times10^6$ $\pm16.9\times10^5$ | $40.2\times10^6$ $\pm20.5\times10^5$ |

***Discussion***

Previously published studies on PAM have mainly focused on soil erosion. Few studies have looked at the impact on the soil microbial community (Sojka et al., 2006), survival of introduced bacteria (Spackman et al., 2003) and bacterial surface runoff (Entry et al., 2003; Sojka and Entry, 2000; Spackman et al., 2003). Common for these studies was a focus on fecal indicator organisms concluding that PAM decreased bacterial concentration in runoff water and that it was indiscriminate to the type of microorganisms. The present experiment contradicts some of these findings as we found that effect of PAM was influenced by both soil type and bacterial strain, within the experimental design parameters and isolates utilized.

PAM altered aggregate size in the three soils with a decreased effect in the following order clay>clay loam>sandy loam (Figure 1) which positively correlates with the pH, clay content and CEC value (Table 1). Others have found correlation between aggregate stability influenced by PAM and the clay content, electrolyte concentration, pH and the exchangeable cations (Mamedov et al., 2010; Nadler et al., 1996).

There was a general trend of decreased bacterial adhesion in the presence of PAM in the adhesion experiment (Figure 2). This may be explained by a physically reduction in the surface area due to agglomeration of soil particles (Lu et al., 2002a). In addition, the lack of divalent cations in the soil suspension would prevent cation bridging between the repulsive negative charges of the bacterial surface and the PAM molecule. In a preliminary adhesion experiment for *E. coli*, we found a larger adhesion in 0.005 M $CaCl_2$ solution compared to a 0.01 M NaCl solution. However, for both solutions a decrease in bacterial adhesion was observed in the presence of PAM. The greatest decrease in adhesion was seen in the sandy loam. This is most likely explained by fewer sorption sites in the sandy loam soil as compared to the clay and clay loam. Limited sorption sites would create competition between PAM molecules and bacterial cells as both contain carboxyl groups. When comparing the clay and clay loam soil, a similar effect of the PAM treatment was seen; this

may be explained by similar soil properties such as texture, SOM and CEC (Table 1). Huysman and Verstraete (1993) attributed a higher bacterial adhesion in a clay loam soil to greater cation exchange capacity.

**Table 4** Distribution of the four bacterial strains from the 18 runoff experiments. 32 colonies were isolated from the last runoff water sample.

| | Planktonic/ attached | PAM treatment | *E. coli* | *E. coli* O157:H7 | *S.* Newport | *S.* Poona |
|---|---|---|---|---|---|---|
| Clay | Planktonic | +PAM (n=32) | 10 | 13 | 8 | 1 |
| | | - PAM (n=32) | 13 | 11 | 8 | 0 |
| | Adhered | + PAM (n=32) | 16 | 10 | 6 | 0 |
| | | - PAM (n=32) | 10 | 16 | 4 | 2 |
| Clay loam | Planktonic | + PAM (n=32) | 21 | 4 | 7 | 0 |
| | | - PAM (n=32) | 16 | 6 | 10 | 0 |
| | Adhered | + PAM (n=xx) | N.D. | N.D. | N.D. | N.D. |
| | | - PAM (n=XX) | N.D. | N.D. | N.D. | N.D. |
| Sandy loam | Planktonic | + PAM (n=32) | 25 | 3 | 3 | 1 |
| | | - PAM (n=32) | 18 | 7 | 4 | 3 |
| | Planktonic | + PAM (n=32) | 27 | 3 | 2 | 0 |
| | | - PAM (n=21) | 15 | 3 | 2 | 1 |

When comparing the adhesion of the four strains the effect of the PAM treatment varied. The adhesion of *S.* Poona was not significantly changed in the presence of PAM. *S.* Poona was also the bacterial strain with the strongest adhesion to all three soil types. Compared to the cell surface properties (Table 2) *S.* Poona was the most hydrophilic strain. However, in the current literature it is generally accepted that there is a positive correlation between adhesion and hydrophobicity. Stenstrom (1989) measured the adhesion of *S.* Typhimurium to soil particles and found that the negative surface charge was of minor importance compared to hydrophobicity. Huysman and Verstraete(1993) found that hydrophobicity was the most important factor for adhering in a sandy soil. This may be explained by hydrophobic interactions usually are the strongest of all long-range non-covalent interactions (van Oss, 1995). In contrast, Gannon et al. (1991) found no correlation between hydrophobicity and transport for 19 strains in a loamy soil. The adhesion trait characteristic of the *S.* Poona isolate may be influenced by different factors. Further testing of, for example, cell size, surface charge density and extracellular polymeric substance content may explain this observation. A study by Lu et al. (2002b) found that sorption of nonionic herbicides

was unaffected by PAM whereas a decrease in the sorption of anionic herbicides was observed. Letey (1994) concluded in a review that the order of adsorption of PAM is cationic>nonionic>anionic. Therefore, the bacterial surface charge may have influenced the effect of the PAM treatment.

The results from the runoff experiments showed that the bacterial retention depended on the soil type and that the recovery of bacterial strains correlated with the adhesion experiment as the soils with high adhesion properties lead to a larger degree of retained bacteria. Whether or not the bacteria were leached in the adhered or planktonic form were mostly influenced by the soil type. Soils containing a high proportion particles >0.45um will have a higher proportion of adhered cells in runoff whereas a soil with a smaller content will have a higher proportion of planktonic cells in runoff. From the clay and clay loam soil the approximately 80% of leached cells were adhered whereas the opposite was seen for the sandy loam where 20% were associated with particles. PAM did not significantly influence this partitioning. Characklis et al. (2005) found that 20-35 % of *E. coli* were associated with settle able particles in storm water. In a laboratory controlled experiment Muirhead et al. (2006b) compared the transport of attached and planktonic *E. coli* in a saturated soil and found that planktonic cells were easier transported across the soil and thereby to the receiving waters. Furthermore, when *E. coli* was preattached to soil particles (>45 μm) a significant reduction was seen compared to inoculation of unattached cells. Soupir et al. (2010) found that the percentage of *E. coli* and enterococci adhered to soil particles in runoff ranged from 28 to 49 % and that this proportion increased in time when comparing runoff at 10 min and 30 min. As bacterial suspensions were applied to the soil surface two hours prior to the runoff this could facilitate a higher proportion of adhesion in this experiment.
The leaching of both TSS and TDS generally decreased in the presence of PAM, which means that adsorption between both TSS and TDS to the PAM molecule occurred (Table 4). The runoff from the sandy loam and clay loam soil correlates with other studies as there is seen decrease in bacterial cells. However, from the clay soil there was seen an increase in bacterial concentration with the PAM treatment which is in contrast with existing literature (Entry and Sojka, 2000; Entry et al., 2003; Sojka and Entry, 2000). At the same field site Entry and Sojka (2000) and Sojka and Entry (2000) found a decrease in total bacteria and microbial biomass in a silt loam, whereas Spackman et al. (2003) found lack of difference of total coliform in the surface water regardless of PAM treatment. The soil properties at this site differs from the soils used in the present experiment by having a silt content of 60–75%, whereas the clay loam had the highest silt content of 25%. The effect of PAM on the soil size distribution experiment was highest for the clay loam, and may be an indication of the silt fraction playing an important role. Entry et al. (2003) compared the leaching in three soils and found no difference for fecal coliforms. In the adhesion experiment the four bacterial strains showed a smaller affinity towards the clay soil compared to the clay loam and sandy loam (Figure 2). Unfortunately, our experiments do not explain these observations.

The effect of PAM in the runoff experiment did not vary between strains (Table 5). When comparing the leaching of the four strains it was clearly seen that *S.* Poona was retained the most and *E. coli* was leached the most from the three soils. As *S.* Poona adhered the most to the soils it is most likely this adhesion to soil particles that prevented leaching. *E. coli* differed from the other bacteria by having the highest hydrophobicity. This observation is difficult to reconcile with other reports, as hydrophobicity is known to increase adhesion. We assume other parameters to influence the outcome that resulted in this apparently anomalous observation. In order to describe this complex system more research is needed to provide the basis for any conclusive statements.

### *Conclusion*

The present study showed that the effect of PAM on the complex interaction between soil particles and bacterial cells is not as simple as previous articles have concluded. We have shown that in the presence of PAM there is a decrease in bacterial adhesion for pathogens *S.* Newport, *S.* Poona, *E coli* O157:H7 and non pathogenic *E. coli*. The degree of this change is influenced by soil type and bacterial strain.

Runoff from sandy loam and clay loam decreased bacterial concentration in leachate in the presence of PAM whereas an increase from the PAM treated clay soil was seen. The PAM treatment did not change the adhered/planktonic distribution between bacteria in the leachate. However, there was seen a strong retention of *S.* Poona whereas *E. coli* was recovered in greatest proportion in leachate. Hydrophobicity and bacterial motility could not explain this difference.

More research is needed to investigate interactions between PAM and different soils in order to minimize the agricultural impact on the surrounding environment. In addition it is very important to include different relevant pathogens as we have shown different leaching potential.

### *Acknowledgement*

This study was funded by the PATHOS centre (ENV 2104-07-0015). Contributions to the funding resources were provided by the California Leafy Greens Research Program.

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# MANUSCRIPT V

*Ready for submission to Journal of Applied and Environmental Microbiology*

# Slurry application method influences *Escherichia coli* decay in clay soil

Tina B. Bech & Line Fredslund

# Slurry application method influences *Escherichia coli* decay in clay soil

Running title: Decay of *Escherichia coli* in soil

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**Abstract**
Incorporation of either liquid slurry or slurry resulted in a faster *E. coli* decay rate compared to injection. This difference was not explained by protozoan numbers. Survival of *E. coli* was shorter with slurry compared to liquid slurry, and may be explained by an initial significant larger protozoan population with the slurry treatment.

Application of slurry to agricultural land is a source of nutrients and organic matter and when applied correctly improves crop yield (1). Inappropriate use of animal slurry may lead to unwanted environmental effects such as odour, ammonia emissions, as well as pollution and contamination of surface or groundwater with xenobiotics and/or zoonotic pathogenic organisms (2, 3, 4, 5, 6, 7). The survival time of slurry-borne bacteria in arable soil influences the risk of pathogen contamination of surface and groundwater. The pathogen mitigation is controlled by advective transport; therefore longer survival will increase the likelihood of a sufficient heavy rainfall event to occur. Survival of fecal indicator *E. coli* in arable soil has been shown from a few weeks to months and is influenced by both biotic and abiotic factors, for review see Bradford et al. (8), Guan and Holley (9), Pachepsky (10). Dominant factors include temperature (11), water content (12, 13, 14), soil type (15, 16) and indigenous soil microbial community (17, 18). Substantial research has improved our knowledge of pathogen decay in agricultural soil. Nevertheless, many gaps still remain as a result of the many processes that influence pathogen fate in agricultural soil. Changes in agricultural management such as application methods further complicate this fate. Traditionally, incorporation of slurry has been achieved by soil tillage following surface application of slurry. Injection of slurry is an alternative that ensures immediate slurry incorporation. Injection is increasingly used by farmers to minimize odour and ammonia emissions (7). Therefore, it is important to identify how injection of slurry influences the fate of pathogens in agricultural soil compared to more traditional application methods. Little if any attention has been given to the interactions between slurry application method and survival of fecal bacteria. Therefore, in the present experiment we compared injection versus incorporation of pig slurry and followed both the decay of fecal indicator *E. coli* and the numbers of active protozoa.

The experiment was made with a clay soil which has been proved to elongate the survival of introduced fecal bacteria due to protection from predation compared to a more coarse soil (16). The soil texture was as follows; clay (49.3 %), silt (26.6 %), sand (20 %) and soil organic matter (4.1 %). The gravimetric soil water content was 18 % W/W. Two different pig slurries with a natural content of *E. coli* were used, a liquid slurry with a dry matter content of 1.64% and a slurry with a dry matter content of 5.17%; see table 1 for further details.

TABLE 1 Manure properties.

| | Dry matter | Total N | $NH_4^+$-N | *E. coli* | Protozoa |
|---|---|---|---|---|---|
| | % | Kg $ton^{-1}$ | Kg $ton^{-1}$ | CFU $mL^{-1}$ slurry | MPN $mL^{-1}$ slurry |
| Liquid slurry | 1.64 | 2.76 | 2.24 | 1800 | 273 |
| Slurry | 5.17 | 5.92 | 4.13 | 2600 | 122 |

The experiment was set up in sterile 50 mL test tubes. Prior to the experimental onset the soil was 2 mm sieved and carefully separated into 10 g subsamples. The injected treatment was made by having 5 g soil in the bottom of the tube, 1 mL pig slurry in the middle and 5 g soil on top. For the mixed treatment 10 g soil was applied and 1 mL slurry was added and then mixed with a plastic inoculation needle. Test tubes were stored in the dark at 10 °C and analyzed on day 0, 1, 3, 7, 13/14, 22/23, 30 and 44 by destructive sampling. From day 30 onwards only soil from the liquid slurry treatment was analyzed due to longer survival of *E. coli*. The destructive sampling was done by adding 20 mL 0.010 M phosphate buffer (5.7 mL 1M $NaH_2PO_4 \times H_2O$ + 4.2 mL 1M $Na_2HPO_4 \times 2H_2O$ in 1000 mL water, pH=7.4) to each test tube followed by shaking for 30 min at 150 rpm. 10 fold dilution series were prepared and *E. coli* were enumerated on Select *E. coli* Petrifilm (3M). *E. coli* was quantified after 24 hours at 37 °C. CFU were converted to CFU $g^{-1}$ and standard deviations were calculated based on triplicates.

For the quantification of protozoa a 3-fold dilution series of soil samples was prepared in 96-well microtiter plates (Nunc-Thermo Scientific, Roskilde, Denmark). Dilutions were made in modified Neff's amoeba saline buffer with 0.10 g tryptic soy broth $L^{-1}$ as described by Page (19). Plates were incubated at 10°C in the dark, and protozoan enumeration was done by visual inspection of single wells with an inverted microscope after 7 and 21 days of incubation. Most Probable Number (MPN) calculations were done with a MPN Assay analyzer software according to Hurley and Roscoe (20). MPN numbers were compared by *t*-tests. The *E. coli* data were fitted to a logistic function by non-linear regression for each replication in SigmaPlot 11 (Systat Software, Inc): $C_t = a \times e^{(-b \times t)} + c \times e^{(-d \times t)}$, where $C_t$ is the CFU $g^{-1}$ soil at time t (days), $a$ is the initial CFU $g^{-1}$ soil, $b$ and $d$ are the slope parameter for the rate of change ($day^{-1}$) and $c$ is the CFU $g^{-1}$ soil when the decay rate change from the fast to the slower rate. F-tests ($P<0.05$), as described by Robinson (21), along with an evaluation of how realistic the parameters were, was combined to deem which model best described *E. coli* decay. This model was used by Rogers et al. (22) in a pathogen soil survival study and described in a review by Crane and Moore (1986). T-test was performed to identify differences in the best-fit decay rate coefficients.

The decay was best described by combining two first-order equations consisting of an initial fast decay rate (b) followed by a slower rate (d). Easton et al. (23) suggested the following: "Microorganisms may die off at a rapid rate until the carrying capacity of the environment is approached. The slower, second phase of decay may occur when organisms are better supported by the nutrients present." In the four experiments a faster initial decay rate was seen (b) followed by a slower decay rate (d). When comparing application methods the experiment showed that the incorporated treatment resulted in a faster initial decay rate compared to the injection. This difference was significant for the slurry application ($P<0.001$) and almost significant for the liquid slurry ($P=0.067$). When comparing the influence of slurry properties on the decay of *E. coli* a nearly significant difference was seen for the incorporation ($P=0.056$) whereas the injection treatment showed no difference ($P=0.48$). A small dry matter difference of 3.5% may explain lack of difference. The slower decay rate (d) showed no significant difference between treatments. However, despite the lack of significant difference between the two slurry types a longer survival time was seen for the liquid slurry applications.

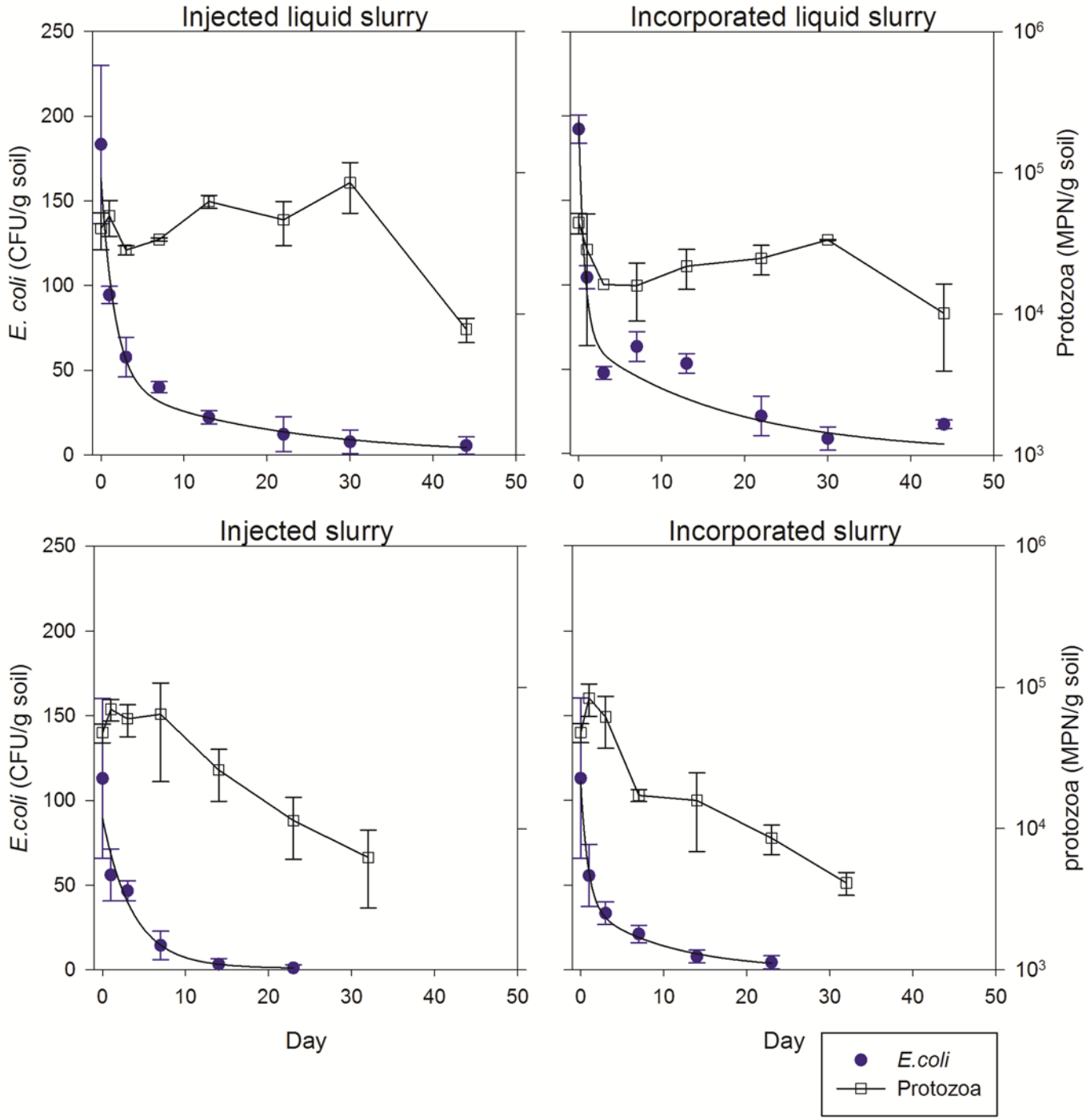


**FIG 1** Survival of *E. coli* in a clay soil comparing injection and incorporation for the liquid slurry and slurry with a detection limit of 1.1. *E. coli* survival data were fitted by non-linear regression to a logistic function (black line). The concentration of protozoa is given as a most probable number (MPN). Standard deviations are based on triplicates.

**TABLE 2** *E. coli* survival data were fitted by non-linear regression to the logistic function $C_t = a \times e^{(-b \times t)} + c \times e^{(-d \times t)}$, where $C_t$ is the CFU $g^{-1}$ soil at time t (days), *a* is the initial CFU $g^{-1}$ soil, *b* and *d* are the slope parameter for the rate of change ($day^{-1}$) and *c* is the CFU $g^{-1}$ soil when the decay rate change from the fast to the slower.

| | Liquid slurry | | Slurry | |
|---|---|---|---|---|
| | Injected | Incorporated | Injected | Incorporated |
| $R^2$ | 0.96 | 0.76 | 0.97 | 0.97 |
| *A* | 120.30 | 128.90 | 86.1 | 72.56 |
| *B* | 0.60 | 1.44 | 0.28 | 1.15 |
| *C* | 42.86 | 68.32 | 3.47 | 38.4 |
| *D* | 0.052 | 0.051 | 0.055 | 0.10 |

Addition of both slurry and liquid slurry resulted in a rapid increase in the number of protozoa regardless of application method. From the liquid slurry treatment a 4 fold increase was seen within the first 24 hours followed by a slow increase lasting to day 30. From the slurry treatment a rapid 10 fold increase was seen for the first 3 days with the incorporated treatment and the first 7 days for the injected application. The rapid protozoan response is in accordance with current literature where it is found that the number of protozoa in soil may show abrupt changes, caused by factors such as hotspots (like slurry), rhizosphere effects or rainfall (24, 25, 26). Griffiths et al. (27) found an increase in protozoan biomass to reach maximum within 4 days for pig slurry and within 8 days for cattle slurry, and explained this difference by the carbon contained in the pig slurry was more readily available for microbial utilization than in the cattle slurry. The initial larger protozoan increase seen with the slurry treatments may explain the shorter *E. coli* survival time. Amin et al. (28) found higher recovery of both *E. coli* and *Enterococcus* with injected liquid slurry (3.14% total solids) compared to injected raw slurry (5.65% total solids).

For both slurry types a faster decay was observed when the slurry was incorporated. Physically, the slurry was either concentrated within a confined area with the injection method or spread into a greater soil volume when incorporated. These different soil-slurry interactions influenced the survival of *E. coli*, where the liquid slurry would have spread more into the surrounding soil. Petersen et al. (29) found that cattle manure remained more concentrated around an injection slit compared to a more liquid pig slurry. This greater spread may explain why the liquid slurry injection had a faster decay than injected slurry. A larger infiltration with the liquid slurry would bring the bacteria in better contact with the soil environment and thereby increase the likelihood of protozoan predation compared to the slurry-saturated injection slit. However, protozoan predation could not explain the rapid die-off. Instead abiotic factors such as osmotic stress, temperature, anaerob/aerob may have resulted in this rapid die-off, followed by a slower decay upon adaption the soil environment. Overall, this experiment has shown that incorporation of slurry resulted in a faster *E. coli* decay rate compared to injection. This difference could not be explained by protozoan predation; therefore we propose that physical soil-slurry interactions influenced *E. coli* decay - however, further research is needed to conclude. A larger increase in protozoa with the slurry treatment may explain the shorter *E. coli* survival time.

**Aknowledgement**

This study was funded by the PATHOS centre (ENV 2104-07-0015), Geocenter Denmark (11-2007), The International research school of water resources (FIVA) and University of Copenhagen.

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# APPENDICES

# MANUSCRIPT VI

*In progess for Vadose Zone Journal*

## The effect of Bromide on Pesticide Mineralization

Tina B. Bech, Peter Engesgaard, Annette Rosenbom and Carsten S. Jacobsen

## The effect of bromide on pesticide mineralization in soil


### *Abstract*

Traditionally bromide is considered and used as an almost ideal conservative tracer for the study of water transport through soils. The anion is often applied together with non-conservative solutes such as pesticides to estimate the retardation (sorption behaviour and degradation processes) of these in the soil. In this context bromide is often applied in concentrations ranging from 1–200 g $Br^- L^{-1}$ a range, in which single cell organisms such as soil bacteria can be inhibited in their growth. Bromide may, hence, affect the biodegradation of no-conservative solutes in soil.

In the present study we investigated the effect of bromide on soil bacterial mineralization of glucose and the three pesticides: glyphosate, MCPA, and metribuzin. Four agricultural A-horizon soils representing the majority of soil types found in Denmark were chosen. Bromide was added to microcosms containing 2.5 g of soil to give a soil solution concentration of 0, 1, 5, and 10 g $Br^- L^{-1}$.

Our study concluded that bromide has a negative effect on pesticide mineralization. The inhibitory effect varied depending on the bromide concentration, type of pesticide, and soil.

We recommend using the minimum needed concentration and not exceeding a concentration of 1 g $Br^- L^{-1}$.

### *Introduction*

The almost conservative tracer bromide has through decades been used to study leaching patterns through a representative range of soils and has highlighted distinct differences in their hydrology and their leaching potential for different solutes her among pesticides. Compared to other conservative tracers the advantages of bromide are: i) it is present in low background concentrations in groundwater and soil (Flury and Papritz, 1993); ii) Rarely sorbs to soil particles (Levy and Chambers, 1987) and iii) it is stable towards microbiological activities (Davis et al., 1985). Käss (1998) later stated that a conservative tracer must be harmless to man, animals, and plants.

Flury and Papritz (1993) reviewed the effect of bromide in natural environments, and concluded that the effect concentrations on growth for single cell aquatic organisms range from 0.11 to 4.6 g $Br^- L^{-1}$ and a groundwater quality criterion of 1 mg $Br^- L^{-1}$ should not be exceeded, respectively. To our knowledge the effect of bromide on soil bacterial functionality has not been investigated. Still, several studies have used bromide as the conservative tracer in concentrations ranging from 0.14 to 260 g $Br^- L^{-1}$ (Boesten and van der Pas, 2000; Brown et al., 2000; Fomsgaard et al., 2003; Junior et al., 2004; Nielsen et al., 2011).

When bromide is used in transport studies, it is either applied as a pulse or at a constant concentration in the application water; and often exceeding the above recommended concentration of 1 mg $Br^- L^{-1}$. Few studies have measured the concentration of bromide in the soil after application. Habekost et al. (2011) found, when irrigating with 50 mm containing bromide at a concentration of 0.14 g $Br^- L^{-1}$, concentrations up to 62 μg $Br^- g^{-1}$ soil with an average concentration of 40 μg $Br^- g^{-1}$. The highest concentrations were located near fractures/biopores. In a well structured soil there will be bromide hot spots as found by Habekost et al (2011), whereas in a sandy soil bromide infiltration will be dominated by

matrix flow. In the slower matrix flow bromide will be diluted as a result of diffusion and dispersion processes with the soil matrix water.

Pesticide degradation follows a chemical or microbial pathway or a combination of both. The degradation of most pesticides in soil is enzymatically catalyzed by microorganisms and generally follows zero-order or first-order kinetics (Topp et al., 1997). This implies that pesticides are degraded by co-metabolism along with the general metabolic activities of the soil community and provide little or no energy to the bacteria involved. Degradation of a pesticide is normally a stepwise process leading to various end products. When the pesticide is totally mineralised the end product is $CO_2$, and some of the pesticide carbon may be build into the soil microorganisms or into the soil organic matter. The ability of different soils to degrade pesticides depends to a large extend on soil physical and biological properties, with sorption characteristics and the capacity for microbial degradation being two key factors (Alexander, 2000).

The objective of the present experiment was to investigate if the conservative tracer bromide influenced the mineralisation of pesticides in different soils. Three model pesticides with different sorption and biological degradation were chosen to represent a span of herbicide characteristics: MCPA, metribuzin, and glyphosate. MCPA is expected to be degraded fast and to show limited sorption. Metribuzin is expected to be slowly degraded and persistent in soil (Kjaer et al, 2005). Glyphosate is expected to show strong sorption to the inorganic fraction and be slowly degraded. In addition glucose was tested, because it represents an energy source used by a broad variety of bacteria. Furthermore, it will be attempted to determine a “bromide inhibition factor” based on 1st order degradation kinetics (**this work has not been finished**).

### *Material and methods*

## Soil

The four chosen agricultural sites represents the majority of soil types found in Denmark. The soils at Faardrup and Slaeggerup are clayey till and the Jyndevad and Tylstrup soil are sand. Soil characteristics are given in Table 1. Soil from the A-horizon were collected as small subsamples within a few square meters and mixed thoroughly. All soils were sieved (2 mm) after sampling and stored at 10°C for 3 days prior to the experimental onset of the mineralisation experiment. The $K_D$-value (dissociation constant) was estimated in the soils for glyphosate and MCPA at a concentration of 1 μg pesticide $g^{-1}$ soil (for method see appendix A2).

**Tabel 1** Physical and chemical properties of the selected soils (Faardrup, Slaeggerup, Tylstrup, and Jyndevad; (Lindhardt et al., 2001) her among the estimated sorption coefficients of glyphosate and MCPA for all four soils.

| Field site | TOC [%] | Clay [%] | Silt [%] | Sand [%] | pH | Soil type | $K_{D,Glyphosate}$ [L $kg^{-1}$] | $K_{D,MCPA}$ [L $kg^{-1}$] |
|---|---|---|---|---|---|---|---|---|
| **Faardrup** | 1.4 | 14-15 | 25 | 57 | 5,95 | Sandy loam | 16.45 | 0.35 |
| **Slaeggerup** | 1.4 | 20-24 | 25-33 | 41-54 | 5,71 | Clayey loam | 25.83 | 0.42 |
| **Tylstrup** | 2 | 6 | 13 | 78 | 5,49 | Fine sand | 20.51 | 0.58 |
| **Jyndevad** | 1.8 | 5 | 4 | 88 | 5,81 | Coarse sand | 18.14 | 0.49 |

## Mineralisation

Mineralisation of glucose, glyphosate, MCPA, and metribuzin was studied in microcosms by weighing 2.5 g of moist soil (moisture content similar to field content) into a flask and adding 125 μL 20 ppm pesticide solutions with a radioactivity of approximately 10,000 DPM in each microcosm. The pesticide solutions were mixed well into the soil. This gave an initial concentration of 1 μg pesticide $g^{-1}$ soil. KBr solutions of 2 mg $mL^{-1}$, 10 mg $mL^{-1}$ and 20 mg $mL^{-1}$ prepared and 125 μL was added to the microcosm to give a concentration of 0.1, 0.5 and 1.0 mg Br $g^{-1}$ soil.

A small test tube with 1 mL 0.5M NaOH was placed in the flask to trap any $^{14}C$-$CO_2$ formed by mineralization. The flask was sealed and placed in the dark at 10°C. The NaOH trap was replaced on day 5, 9, 14, 19, 23 and 30 for glucose, glyphosate, MCPA and metribuzin. The $^{14}C$-$CO_2$ content was determined using a Wallac 1409 liquid scintillation counter after mixing with 10mL Optiphase HiSafe 2 scintillation cocktail (Wallac, Finland). Radioactivity was converted to percentage mineralization of the pesticide in the microcosms.

**Modelling (in working progress)**

The mineralization of glucose, glyphosate, MCPA and metribuzin was modelled by a first order model:

$$P = S_0\left[1 - e^{(-kt)}\right]$$

Where, $P$ is product formation ($CO_2$), $S_0$ is the initial substrate concentration, and $k$ is the mineralization rate constant.

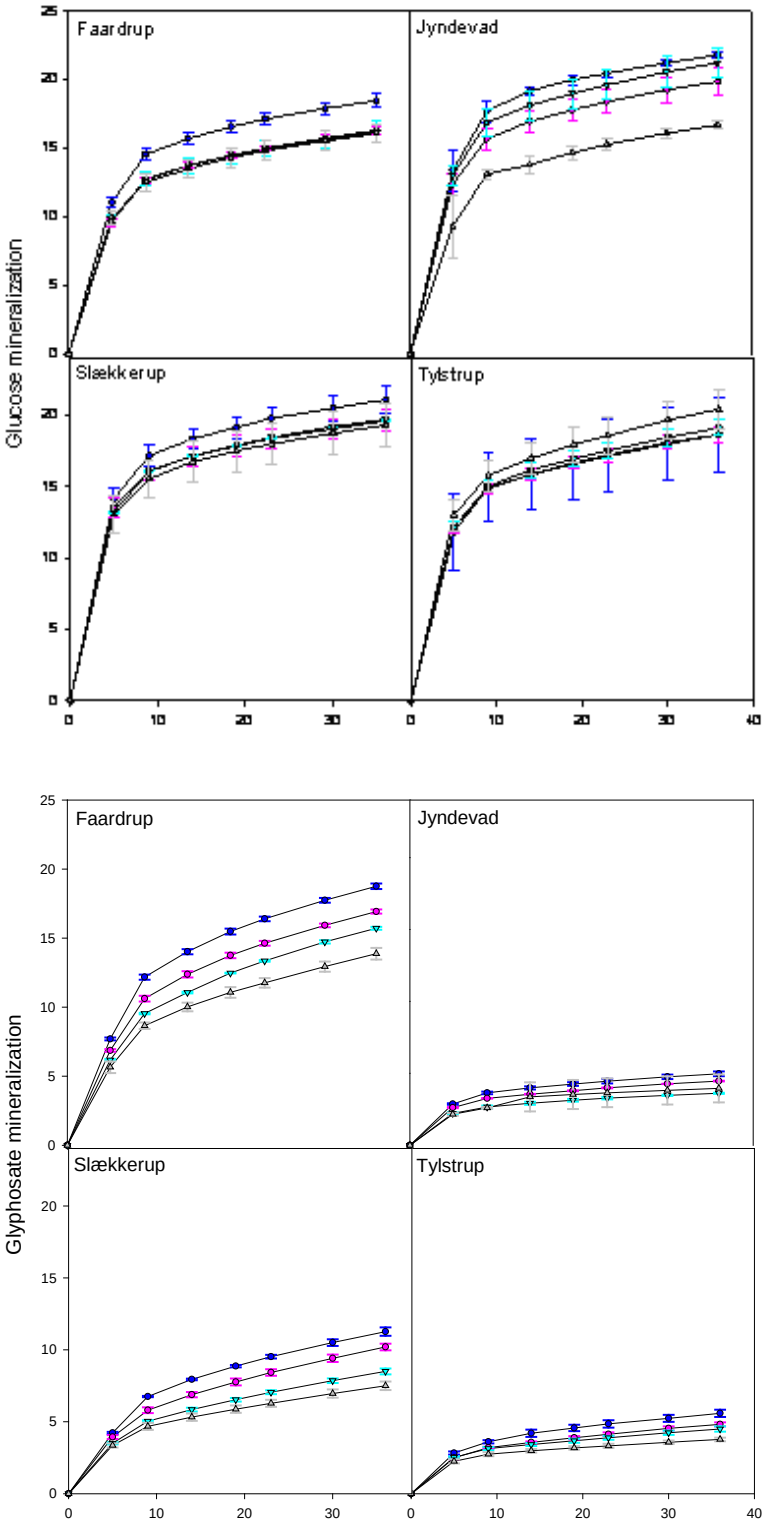
**Statistics**

The effect of bromide will be tested by comparing calculated mineralization rate constants by two-way ANOVA ($P<0.05$) using triplicate values. Initial comparison between bromide treatments has been done by one-way ANOVA where the calculated cumulative mineralization value at day 36 was compared with the control soil.

## *Results and discussion*

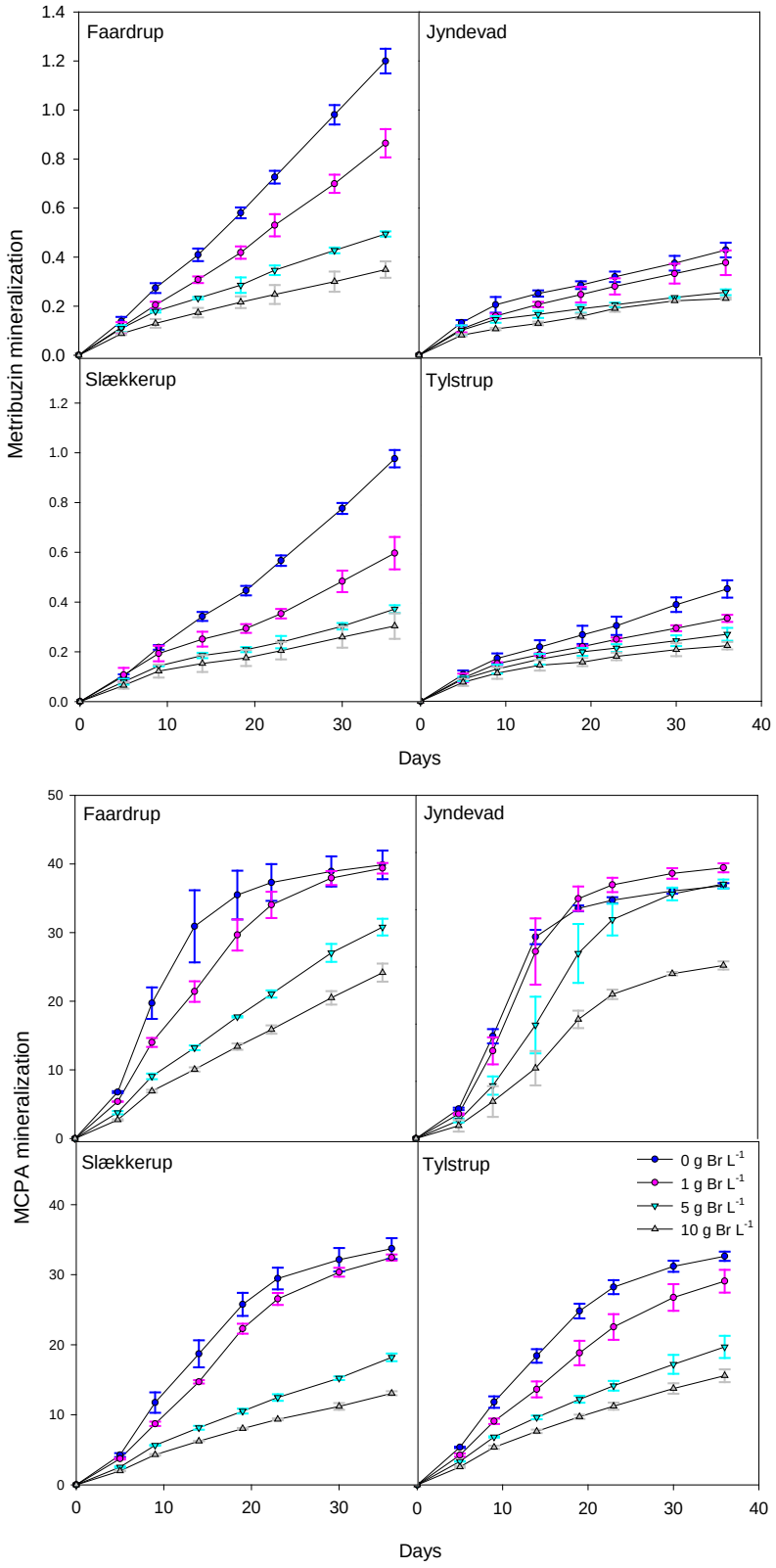
### Mineralization influenced by soil type

All substrates were mineralized without a lag-phase in all four soils. The amount of total accumulated $CO_2$ after a period of 36 days varied between soil type and substrate.




Glucose is mineralized rapidly in all four soils. By the end of the experiment 18–20% is mineralized. Similar mineralization curves indicate that soil sorption properties are of minor importance. The relatively small proportion of mineralized glucose indicates that a large proportion of added pesticide carbon is build into the soil microorganisms. By the end of the experiment 5–18% of glyphosate is mineralized. At Jyndevad and Tylstrup only 5% is mineralized and this is most likely explained by strong sorption, because sorption.

**Figure 1** Accumulated mineralization during the 36 days incubation period. Each sample was determined in triplicates with standard deviations being indicated by vertical bars. The mineralization of glucose and glyphosate was measured for the two clayey soils (Faardrup and Slaeggerup) and two sandy soils (Jyndevad and Tylstrup) at four bromide levels (0, 1, 5, and 10 g $Br^- L^{-1}$).




characteristics is a key factor in the capacity for microbial degradation (Alexander, 2000)

Glyphosate degradation shows a decreasing mineralization rate during the experiment. This indicates that the mineralization does not support microbial growth as in accordance with Gimsing et al. (2004).

The metribuzin degradation is smallest and by the end of the experiment only 0.4–1.2% has been mineralized. Similar small mineralization was observed by Mortensen and Jacobsen (2004).

**Figure 2** Accumulated mineralization during the 36 days incubation period. Each sample was determined in triplicates with standard deviations being indicated by vertical bars. The mineralization of metribuzin, and MCPA was measured for the two clayey soils (Faardrup and Slaeggerup) and two sandy soils (Jyndevad and Tylstrup) at four bromide levels (0, 1, 5, and 10 g $Br^{-}$ $L^{-1}$).

Metribuzin has ring structures that are not easily mineralized and are co-metabolically degraded by soil organisms. Kjaer et al. (2005) found that after 4 years 8% of added metribuzin was still recovered in the top soil at the Tylstrup site, despite a low $K_D$-value of 0.94.

The mineralization curve is linear at Faardrup and Slaeggerup whereas a tendency to a first order is seen for Jyndevad and Tylstrup. This difference between sites may be explained by the size of the microbial population (Moorman and Harper, 1989; Mortensen and Jacobsen, 2004). However, as seen in appendix 2 figure 2, similar concentrations of total CFU and pseudomonas populations are observed when comparing soil type.

MCPA degradation differs by following a sigmoidal curve, consisting of a short lag phase, a steep segment with rapid MCPA mineralization, and a plateau after mineralization had ceased. By the end of the experiment 31–43% had mineralized depending on the soil type. MCPA has a $K_D$- value under 1 (table 2) and is therefore less influenced by soil sorption properties.

## Mineralization influenced by bromide concentration

There are for most combinations of soils and pesticides seen a negative correlation between the concentration of bromide and the mineralization. In Table 3 is summarised t-test comparison of cumulative mineralization value at day 36 with the control soil. Mineralization of glucose differs from the pesticides by being less affected by bromide. At both Tylstrup and Slaeggerup all three concentrations of bromide significantly reduces the mineralization of the three pesticides. At Faardrup the low concentration of bromide does not significantly decrease mineralization, whereas the remaining mineralization is significantly reduced in the presence of bromide. At Jyndevad the concentration of 1 mg $Br^- L^{-1}$ does not significantly reduce the mineralization whereas the higher concentrations reduce the mineralization.

**Tabel 3** Calculated cumulative mineralization values were compared to control mineralization with student *t*-test comparing triplicates. P values indicate if the accumulated mineralization at day 36 is significantly (α=0.05) different from control.

| | mg $Br^-$ $L^{-1}$ | Faardrup | Jyndevad | Slaeggerup | Tylstrup |
|---|---|---|---|---|---|
| **Glucose** | 1 | P>0.001 | 0.014 | | |
| | 5 | P>0.001 | 0.383 | 0.183 | 0.474 |
| | 10 | P>0.001 | P>0.001 | | |
| **Glyphosate** | 1 | P>0.001 | 0.25 | P>0.001 | P>0.001 |
| | 5 | P>0.001 | 0.001 | P>0.001 | P>0.001 |
| | 10 | P>0.001 | 0.037 | P>0.001 | P>0.001 |
| **MCPA** | 1 | 0.682 | 0.602 | P>0.001 | P>0.001 |
| | 5 | P>0.001 | P>0.001 | P>0.001 | P>0.001 |
| | 10 | P>0.001 | P>0.001 | P>0.001 | P>0.001 |
| **Metribuzin** | 1 | P>0.001 | 0.067 | P>0.001 | P>0.001 |
| | 5 | P>0.001 | P>0.001 | P>0.001 | P>0.001 |
| | 10 | P>0.001 | P>0.001 | P>0.001 | P>0.001 |

Potential reasons for decreased mineralization

A typical ionic strength (IS) in soil solution is ~0.03. The added concentrations of bromide range from 0.008–0.08 IS and are therefore within the normal range. Changes in ionic strength influences sorption, and it is generally accepted that an increase in ionic strength decrease the electric double layer and thereby increase the likelihood of sorption (Martins and Mermoud, 1998). Opposite, Iglesias et al. (2010) found that the amount of adsorbed MCPA on the surface of goethite and of humic acid-coated goethite increased as the ionic strength decreased.

For glyphosate, de Jonge and de Jonge (1999) concluded that changes in ionic strength from 0.002–0.2 and from 0.001–0.1 for KCl and $CaCl_2$, respectively, did not influence the sorption. Little information is available on the effect of bromide on pesticide sorption. We therefore believe the changes in ionic strength within this experiment did not directly influence sorption of pesticides. This is important as it has been shown that sorbed pesticides are degraded more slowly than when in soil solution.

Chemicals and nutrients in the vadose zone are very heterogeneous distributed and therefore greatly influences microbial activity and distribution in the soil. The unsaturated zone is

dominated by surface-attached microcolonies whereas planktonic cells are limited to short episodes of high saturation (Or et al., 2007). The initial high proportion of adhered cells would result in a limited effect of an increased ionic strength. It is though noteworthy, that increase in ionic strength increase bacterial adhesion due to a decrease in electric repulsion between surfaces (Redman et al., 2004).

In an additional experiment the viability of *Pseudomonas* and total bacteria were counted comparing the control soils with soils containing a bromide concentration of 10 g $L^{-1}$. During two weeks bromide did not influence the number of viable soil bacteria. Information on the effect of bromide on soil bacteria is limited, whereas several studies have looked at increased salinity and how this effect different substrate degradation. Tam et al. (2002) observed inhibition of growth and biodegradation of phenenthrene of a bacterial isolate at high salinity level (IS=0.6) compared to 0.008–0.08 IS in the present experiment. Chandra et al. (2002) found that addition of KCl up to a certain level stimulated C mineralization whereas a decline was noticed at higher concentrations. Azam and Müller (2003) found decreased $CO^2$ production in the range from 1-6 mg NaCl $g^{-1}$ soil when $NH_4^+$ was the nitrogen source in soil whereas when the nitrogen source was $NO3^-$ there was an increase in $CO_2$ production when comparing the control soil with 1 mg NaCl $g^{-1}$ soil. Compared to these salinity studies the present experiment was conducted with much lower concentrations.

***Conclusion***

When designing an experiment it is important to minimize the anthropogenic effect and mimic natural conditions. The use of bromide in high concentrations may have lead to false positive pesticide leaching results. This effect on false positive is of great importance in clay

soils due to bromide “hot-spots” (elevated concentrations) and preferential flow may be accelerated by gravitation flow due to bromide content.

Further research is needed to conclude why the degradation of pesticides is impaired in the presence of bromide in concentrations often used in tracer studies. We recommend using the minimum needed concentration and not exceeding a concentration of 1 g $Br^- L^{-1}$.

***Acknowledgement***

The study was funded by This study was funded by the PATHOS centre (ENV 2104-07-0015). Moreover we are grateful to the Danish Pesticide Risk Assessment Programme for supplying soil samples and additional material.

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# ADDITIONAL DATA TO MS 6

In MS 6 it was seen that bromide had a negative influence on the mineralization of three pesticides and glucose. Two additional experiments were conducted in order to conclude on the results:

1. In order to minimize the number of unknown parameters in the model used, we tested the kinetics of the sorption of both glyphosate and MCPA.
2. Did bromide influence the number of viable cells in the soil, testing both the total number as well as the more sensitive Pseudomonas.

**Sorption kinetics**

The kinetics studies were conducted in order to mimic the mineralisation experiment for glyphosate and MCPA. Triplicate samples of 2.5 g dried soil were mixed 125 µL 20 ppm pesticide and 125 µL 2 % Natriumazid. The added concentration of Natriumazid inhibited the mineralization of pesticides. 2250 µL water was added to give a 1:1 ratio between soil and liquid. Samples were stored at 10°C and concentration in the liquid fraction was determined after 1 hour, 4 hours, 1 day, 3 days, 7 days and 14 days. Samples were rotated for 10 min followed by centrifugation for 10 min at 2000 rpm. 1 mL of the supernatant was then transferred to a 2 mL safelock eppendorf tube and given a second centrifugation for 10 min at 13000 rpm to remove remaining soil particles from the suspension. The supernatant was mixed with 10 mL scintillation cocktail (Optiphase HiSafe 2, Wallac, Finland) before radioactivity was measured on a scintillation counter for 10 min.

**Results**

For both MCPA and glyphosate a rapid adsorption to the solid phase is observed. For MCPA a difference from 25% to 45% adsorption is seen, whereas for glyphosate it varies from 93% to 97%.

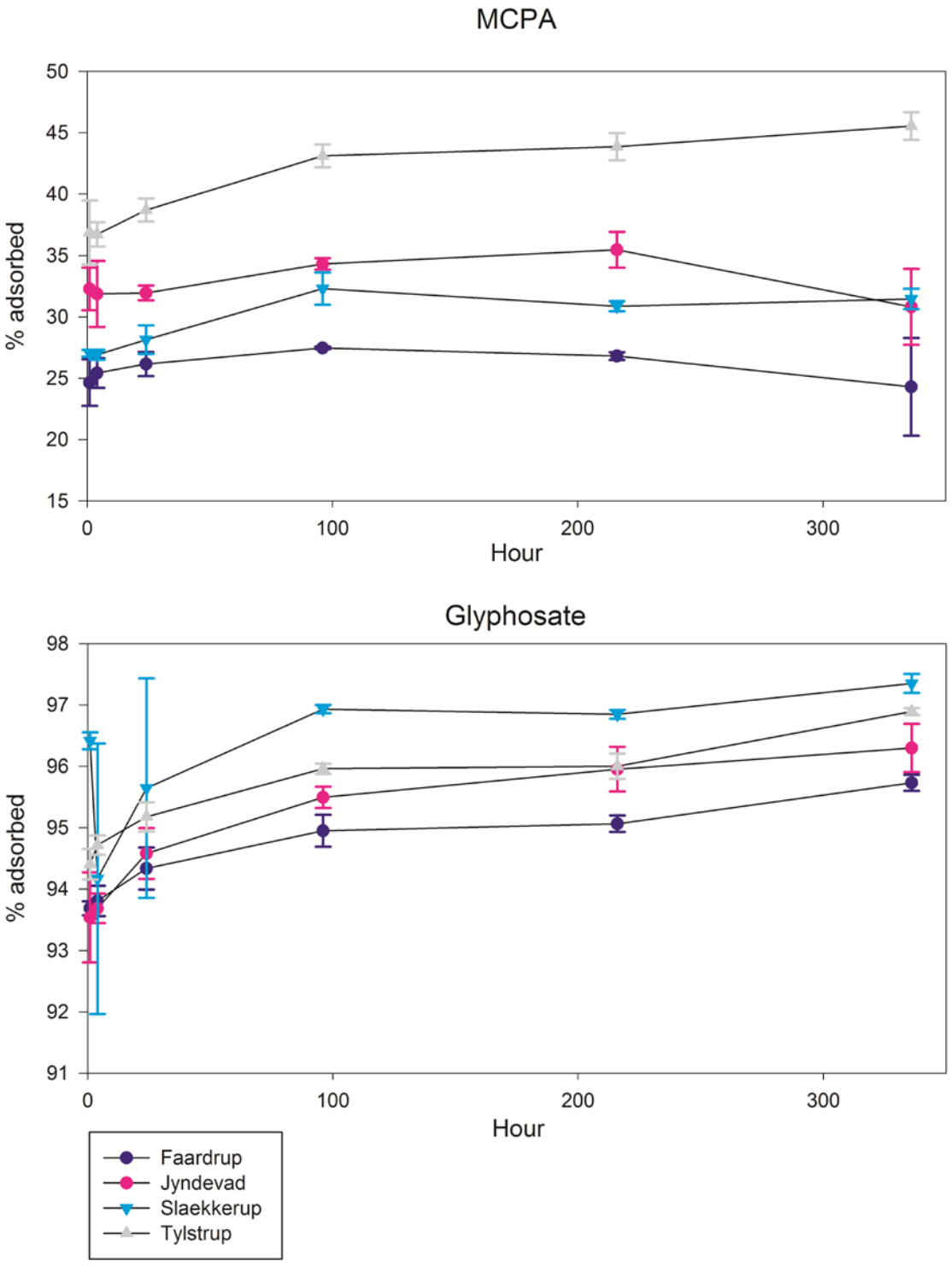


**Figure 1** Sorption kinetics for MCPA and glyphosate in the four soils Faardrup, Jyndevad, Slaekkerup and Tylstrup. Standard deviations are based on triplicate.

**Viable cells in soil under influence of bromide**

The effects of bromide on culturable bacteria were tested by comparing the untreated soil with the spiked soil containing a bromide concentration of 1 mg Br $g^{-1}$ soil. The viability experiment was conducted in 50 mL plastic test tubes with 2.5 g soil. A control soil without bromide was compared to a soil with a bromide content of 1 mg/g soil. The experiment was stored at 10 C and the concentrations of viable cells were tested on day 0, 3, 5, 8 and 14. Cells were quantified by adding 22.5 mL water to the test tube and shaking for 30 min at 150 rpm followed by 10 fold dilution series. Pseudomonas was quantified on Gould S1, where CFU was counted after 48 hours. Total viable cell numbers in the soil were quantified on 1/300 TSA and counted after 3 weeks. This experiment is not based on triplicates.

**Results**

There was no significant effect of bromide on the viable pseudomonas or total bacterial number in the four soils.

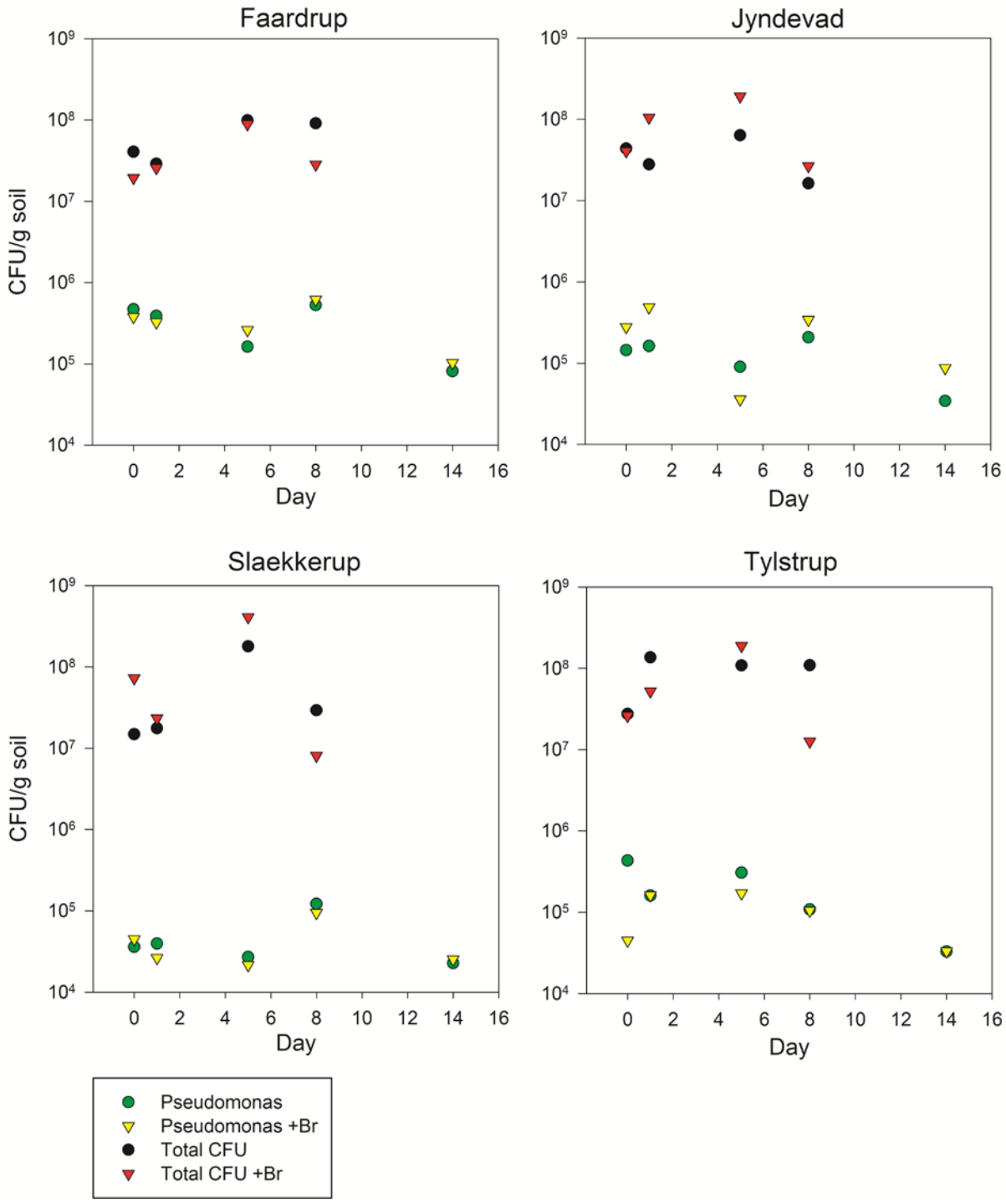


**Figure 2** Concentration of total CFU and pseudomonas in the four soils Faardrup, Jyndevad, Slaekkerup and Tylstrup with and without bromide at 1 mg Br $g^{-1}$ soil. Average value is based on triplicate.

# DISTRIBUTION OF TETRACYCLINE RESISTANT BACTERIA AROUND SLURRY INJECTION SLIT.

Additional data to MS 3

## Soil Sampling Around Injection Slit

Soil samples were collected after 1 hour, 1, 6, 18 and 46 (Silstrup) or 49 (Estrup) days after slurry application. Each sampling day three intact samples with dimensions (width×depth×length) of 20×15×4 $cm^3$ (Silstrup) or 20×17×4 $cm^3$ (Estrup) were taken perpendicularly to the slit with a custom-made stainless steel template. Each sample was subdivided as indicated in figure 1 to isolate six different sections representing 12% (A and B), 18% (C and D), 16% (E), and 24% (F) of the total volume. The depth of the upper layer corresponded to the slurry injection depth. Each section (A–F) was mixed thoroughly after weighing before further analysis.

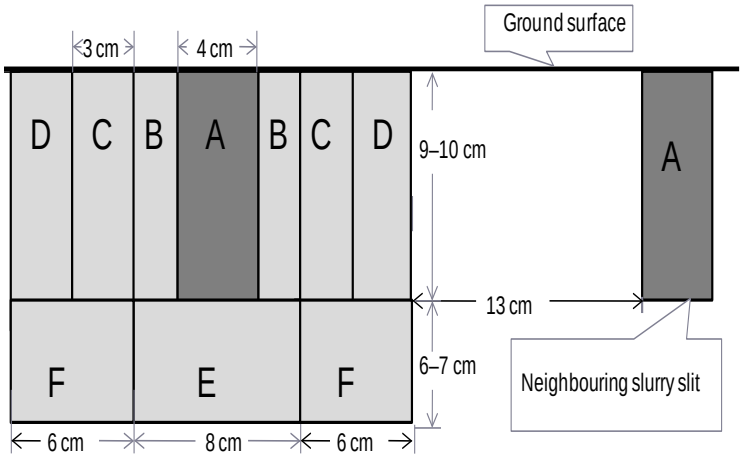


**Figure 1** Scematic presentation of the soil sampling (Amin et al., 2013).

## Result

The spreading of tetracycline resistant bacteria differed at the two sites. At Estrup liquid slurry with dry matter content of 0.8% spread rapidly into the surrounding soil. Similar concentrations in section A, B and C were seen after 1 hour. At Silstrup the concentration remained highest in section A throughout the experiment. The higher dry matter content of 6.8% would have prevented a spread into the surrounding soil environment.

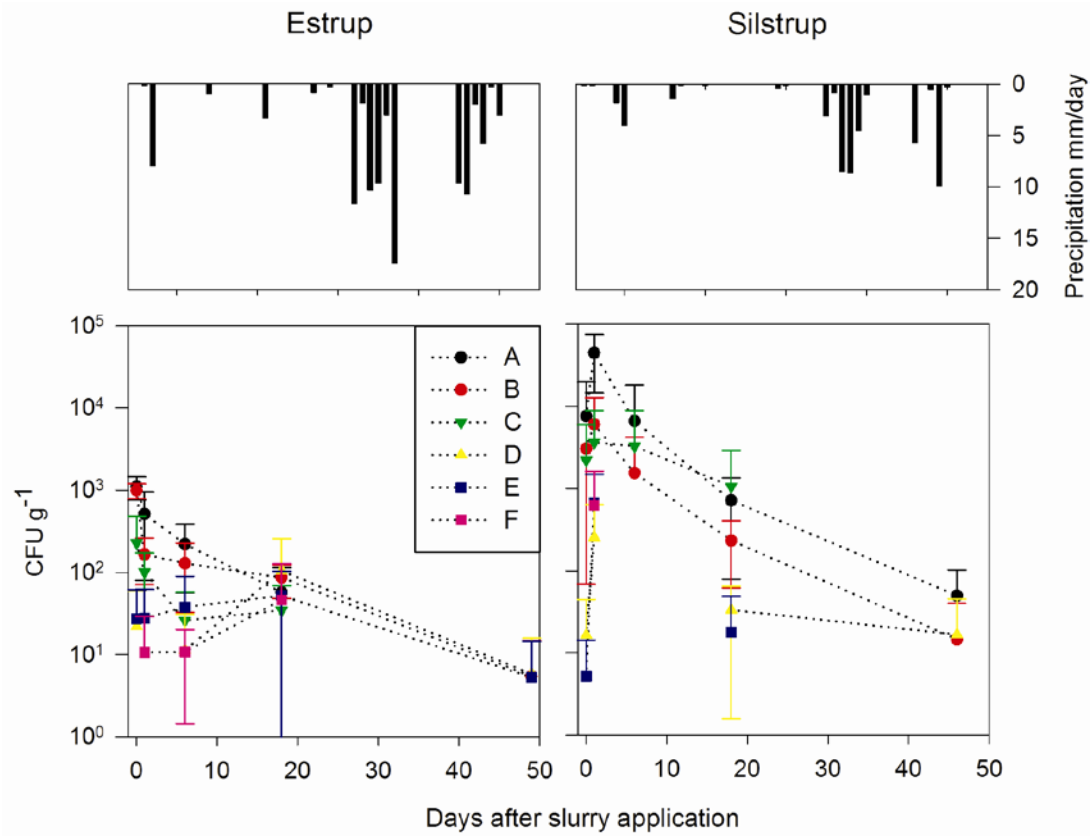


**Figure 2** Distribution of tetracycline resistant bacteria at Estrup and Silstrup. Standard deviations are based on triplicates.

# PRINCIPAL COMPONENT ANALYSIS

Additional data to MS 3

From the leaching experiment at Estrup (MS 3) data have been analysed by PCA. Drainage samples were analysed for Bromide (ppm), Chloride (ppm), Conductivity and the concentration of tetracycline resistant bacteria (CFU $mL^{-1}$) and *E. coli* (CFU $mL^{-1}$). In the sampling time interval max intensity in precipitation (M Precipitation), drainage runoff (M drainage), average precipitation (A Precipitation) and drainage runoff (A drainage) was also recorded, based on hourly average values. 'Day after slurry' refers to the number of days between manure application and drainage runoff; mL water is the sample volume; SWC is soil water content at depth 25cm, 40cm, 60 cm and 90cm respectively.

**Data analysis:**

Principal Component Analysis (PCA) was used to analyze relationships among all observed variables. Data were arranged in a matrix, where each column corresponds to one of the total of 16 variables, each characterising one of the 28 samples. The data matrix was analysed by The Unscrambler X 10.1 software package (CAMO Software, Norway).

After deleting outliers, the PCA *loading* plot $p_1$-$p_2$ explains 50% of the total variance in the dataset; $PC_3$ and $PC_4$ only explained an additional 5% and 4% respectively, and did not show identifiable systematic (and therefore not shown here).

To verify if the negative correlation between precipitation and drainage runoff was due to a time difference from precipitation to drainage collection, a second PCA with 24 h antecedent precipitation was also carried out. This gave a PCA *loading* plot $p_1$-$p_2$ explaining 52% of the total variance in the dataset, with $PC_3$ and $PC_4$ again only explaining additional 7% and 9%

respectively (in this alternative analysis $PC_3$ and $PC_4$ again did not show identifiable systematic and was therefore not considered further).

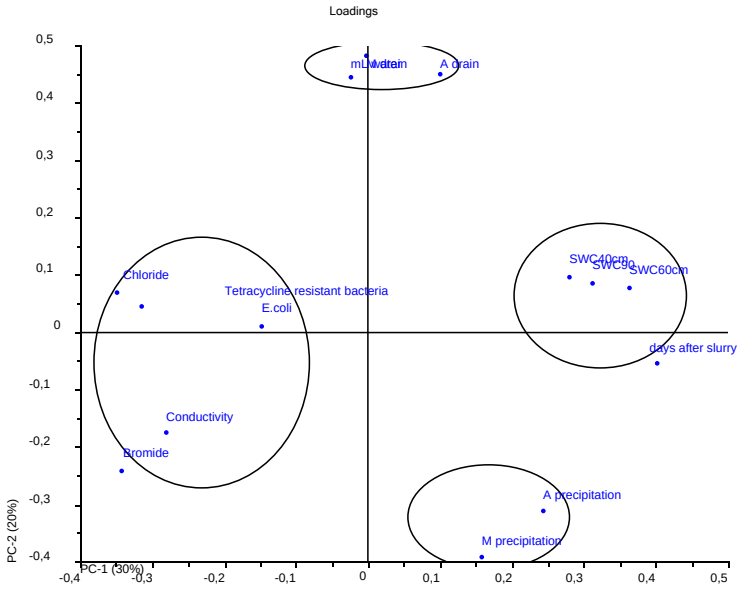


**Figure 1** *Loading* plot PC1 vs. PC2 illustrating the correlation relationships between variables in the Estrup leaching data set (MS 3).

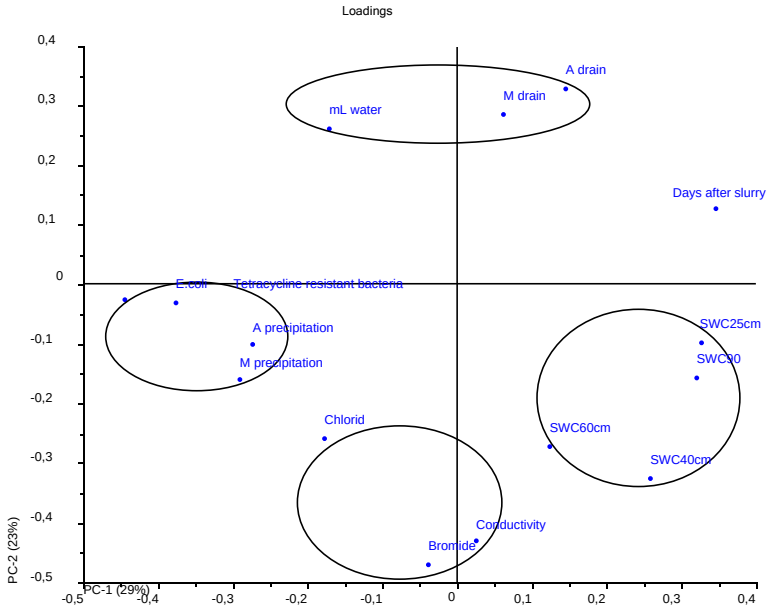


**Figure 2** *Loading* plot PC1 vs. PC2 illustrating the correlation relationships between variables in the Estrup leaching data set (MS 3) (24 h antecedent precipitation).

## Interpretation

At the level of a total of 50–52% variance, the grouping in both loading plots reflects a clear negative correlation between leached faecal bacteria and days after slurry application which is a direct result of bacterial die-off, filtration or adhesion to non-mobile particles. There can also be observed a negative correlation between soil water content and leaching of faecal bacteria. In figure 1 a positive correlation between precipitation and soil water content is observed substantiating that precipitation induces higher soil water content. However, when precipitation is 24 h antecedent this correlation turns negative. More importantly, a positive correlation between precipitation and the leaching of faecal bacterial is observed. The lack of correlation between average and max drainage runoff surprised, as bacteria are transported with the flowing water. This may be explained by the bacterial die-off and the continuous drainage runoff.

Along PC2 a negative correlation is observed between bromide, chloride and conductivity (negative $PC_2$ scores) and drainage (positive $PC_2$ scores). Fast drainage runoff is strongly influenced by preferential flow and therefore consists of precipitation with a low conductivity compared to soil matrix infiltration.

## Conclusions

- Despite similar grouping in both PCA, I believe that the second analysis, where precipitation data have been altered is the correct one to analyse, even though the groupings are slightly more spread.
- Leaching of faecal bacteria correlates with precipitation that is 24 h antecedent.
- Leaching of faecal bacteria is negatively correlated to soil water content. More research is needed to conclude on the importance of the soil water content and the leaching potential of faecal bacteria.