FINAL REPORT

Field-grown salads: quantifying the risk of pathogen contamination through irrigation water.

FV 292

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Project title:	Field-grown salads: quantifying the risk of pathogen contamination through irrigation water
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Project leader:	Dr Jim Monaghan Harper Adams University College Newport Shropshire TF10 8NB
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Key workers:	Dr Jim Monaghan (Project Leader) Dr Mike Hutchison (Research Leader)
Location:	Harper Adams University College, Shropshire, TF10 8NB
Project co-ordinators:	Mr David Kennedy, Bakkavor
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Grower Summary

Headlines

- Even at the highest level of contamination the amount of pathogen remaining on the growing leaves of spinach and iceberg lettuce after one week of good growing weather was below the level that could be measured (<10 cfu g^{-1}) and was only sporadically detected by the second week and was absent in all leaf samples after three weeks.
- Consideration must be given to the time interval between irrigating and harvesting when using a potentially contaminated water source but that ideal crop growing conditions are the same as those that most rapidly degrade pathogens i.e. strong sunlight, dry and warm.
- A water source contaminated with a human pathogen poses a lower risk in the mid summer (when irrigation is most common).
- In some circumstances, i.e. poor drainage, soils retain potential pathogens for significantly longer than the surface of crops.

Background and commercial objectives

There is increased focus on the microbiological risks associated with the consumption of ready to eat produce within the retail sector. These concerns are being driven by two main factors, namely increased government (i.e. FSA and EU) scrutiny of food production practices and the legislative implementation of process controls. In addition, retailer's protocols are becoming more and more stringent to minimise the risk of 'bad press' and damaged brand resulting from food poisoning which is traced to their produce.

Previous HDC funded work (FV248) established that almost three quarters of salad crops were irrigated through direct abstraction of surface water, the most vulnerable to contamination with faecal pathogens from agricultural activities. In addition, the majority of salad crops (60-85%) were irrigated with overhead booms – directly applying water to the leaf surfaces. Sampling of water sources demonstrated that irrigation water quality was variable, at times exceeding the WHO guideline for coliform bacteria (although it should be noted that the WHO guideline is for drinking water). Nevertheless, the lack of data obtained from scientifically-sound studies which describes the real risk of pathogens entering the food chain from contaminated irrigation water has led to a situation where *any theoretical risk*, no matter how significant, has to be minimised. Consequently, growers are now being encouraged into investing in water disinfection systems (UV, ozonation etc) which are expensive to buy and to operate if anything other than a potable water source is used.

This project demonstrates the true risk to salad vegetables from irrigation water introducing pathogens to the soil (Year 1) or to the surface of the produce (Year 2) and the interaction with UV (sunlight) and temperature on the seasonal persistence of the pathogens.

The following questions (each being an experimental objective) were addressed in Year 1:

1. What is the persistence in the soil of pathogens introduced through irrigation at different times in a growing season?
2. Does persistence in the soil of pathogens introduced through irrigation differ with soil type?

The following questions (each being an experimental objective) were addressed in Year 2:

3. How are pathogens distributed within the soil after irrigation?
4. What is the persistence on the surface of crops of pathogens introduced through irrigation at different times in a growing season*.

*Heavy rain prevented completion of this objective – this will be completed in an extension to this project (FV 292a).

Summary of the project and main conclusions

Field trials were carried out at Harper Adams University College, Shropshire, to study persistence of added pathogens in the soil (2006) and on spinach and lettuce crops (2007-8).

Treatments

- 3 levels of pathogen* zero, low (1×10^{2} cfu ml^{-1}) and high (1×10^{5} cfu ml^{-1})
- 3 times through the year: May, July and September
- 2 soils: mineral, peat (2006)
- 2 crops: spinach and lettuce (2007-8)**

*Pathogens were applied as a composite mix of *Salmonella* Enteriditis, *Campylobacter jejuni* and *E. coli* O157(a non-pathogenic strain) each at a similar concentration.
**It was not possible to study persistence for the mid summer crop due to the heavy rain in 2007 but this planting will be repeated in 2008.

Irrigation treatments were applied at one event at the start of each experiment. Surface soil (2006) and crop foliage (2007-8) were sampled weekly and tested for the added pathogens.

What is the persistence in the soil of pathogens introduced through irrigation at different times in a growing season (Objective 1)?

Season had a marked effect on persistence of introduced bacteria. The hot, dry and high sunlight conditions of experiment 2 led to a marked reduction in the levels of bacteria recovered from the surface of the soil, with *Campylobacter* declining most rapidly. All three pathogens were at the level of enumeration after 2 weeks. In contrast the cooler, wetter and lower light levels experienced in experiment 1 and experiment 3 were associated with a greater persistence of pathogens. This was particularly marked in experiment 3 where *E. coli* O157, *Salmonella* and *Campylobacter* were still present in the surface of all plots after 6 weeks.

Summary Objective 1:

- Under conditions where there is the greatest requirement for irrigation i.e. hot and dry with strong sunlight the decline of pathogens introduced to the soil through irrigation is much more rapid and a moderate level of contamination would be undetectable after approximately 3 weeks.
- There is the possibility that pathogens introduced to the soil at high levels through irrigation water can persist for the duration of a lettuce or spinach crop, particularly with the environmental conditions commonly experienced at the start and end of the season (i.e. lower temperatures and light levels).
- We have so far been unable to find literature reporting pathogen persistence in soil from field experiments in summer conditions. Thus, the results of this study are novel, and can be used to properly inform regulators as to the realistic risks of contamination of fresh produce with contaminated irrigation water.

Does persistence in the soil of pathogens introduced through irrigation differ with soil type (Objective 2)?

Soil type had a significant effect on the persistence of introduced zoonoses particularly at the high level. There was a more rapid decline in all three pathogens in the peaty soil with the higher organic matter content compared to the mineral soil in experiments 1 and 3. It is suggested that the higher organic matter of the peaty soil was associated with a higher indigenous bacterial population i.e. it was a more biologically active soil, and that antagonistic interactions with indigenous microbial populations were influencing soil survival.

Summary Objective 2:

- Soil type had a significant effect on the persistence of introduced zoonotic agents particularly at the high level of contamination. Organic matter content and hence biological activity of the soil is associated with a more rapid decline in all three pathogens.

How are pathogens distributed within the soil after irrigation (Objective 3)?

Using the same soil columns that had been left *in situ* to weather for ~12 months it was observed that bacteria added in irrigation water do not significantly accumulate at the surface of soils but that soil properties influenced the distribution of bacteria through the column. Of the two soils studied the mineral soil had an even distribution of marker bacteria through the 30 cm column depth after 1 hour. In contrast, the peaty soil had a lower concentration of marker bacteria at the surface than 10 and 20cm depth. The more rapid transport of water away from the surface of the peaty soil could be explained by soil physical properties as the lower proportion of silt and higher proportion of sand would increase soil permeability. It is likely that the marked accumulation of bacteria in Year 1 was to some extent due to the surface of the columns being packed in the process of filling the buckets. The more weathered soil columns, along with a simulated 'tilled surface' will have increased the flow of water and hence bacteria away from the soil surface in Year 2.

Permeability may also explain why we did not see the same surface persistence of pathogens in the field soils sampled at the end of the crop experiments. Salmonella was present in the soil surface at the end of the late season experiment (Experiment 5) only. All other soil samples were free of the added pathogens. The crops were grown in a free draining sandy loam, and the soil surface was regularly observed to be dry.

Summary Objective 3:

- Soil that drains freely away from the surface may help to move pathogenic bacteria away from the soil surface and will minimise the potential for pathogenic bacteria to contaminate crop through soil splash dispersal.

What is the persistence on the surface of crops of pathogens introduced through irrigation at different times in a growing season (Objective 4)?

In contrast to soil persistence studied in Year 1, the persistence of pathogens on the surface of lettuce and spinach was much shorter. All three bacteria studied declined rapidly to absence in 14-21 days. It is likely that the persistence of pathogens will be even less in mid summer UK conditions (to be confirmed in extension work). This does not agree with the data reported from

the USA. Field trials carried out in Georgia, USA have demonstrated that *E. coli* O157:H7 applied through irrigation can persist on the surface of lettuce for 77 days after contamination (Islam *et al*., 2004a). The same workers have also reported persistence of *Salmonella enterica* Typhimurium as persisting on leaves of lettuce for 63 days (Islam *et al*., 2004b), i.e. pathogens are persisting for 3 to 4 times longer in the US work.

However, climate and season would have been very different between the two growing sites. The University of Georgia Horticulture Farm is located roughly on the same latitude as northern Morocco. The US work took place from October, over winter and, in marked contrast, the work at Harper Adams took place from early May to the end of September, meaning that crops will have experienced higher temperatures than the US crops and higher levels of sunlight, and hence UV; the US work would have received day lengths less than 11 hours whereas the UK work received day lengths exceeding 13-14 hours.

The climate has a marked effect on leaf borne pathogens – pathogens on dry leaves exposed to high levels of UV rapidly degrade The US data is a worse case scenario in conditions similar to North Africa in the winter (with greater rainfall!), *not leafy salad production conditions in the UK*.

Summary Objective 4:

- Moderate to high levels of leaf contamination through irrigation water will pose little or no risk after a maximum of three weeks in an average UK growing environment. It is likely that this persistence will be shorter for mid-summer production.
- Care is needed to prevent irrigation of crops with contaminated water within 3 weeks of harvest.
- Guidance for growers should be derived from work undertaken in a UK growing environment – it can be misleading to extrapolate international studies to the UK growing environment.

Financial benefits

This work is not well suited to estimating financial benefit. However, knowledge that prevents food illness associated with leafy salads can save the sector a lot of money. The *E. coli* O157 outbreak in the USA associated with spinach led to 1/3rd of the US spinach crop being lost.

Action Points for growers

A number of clear action points can be drawn from this work

1. Minimise the level of contamination of irrigation water.
2. Monitor irrigation water quality at the point of application.
3. Take particular care to use ‘clean’ water within three weeks of harvest of leafy salad crops
4. Maintain free draining surface soils through bed preparation to limit the accumulation of contaminated water near the soil surface.
5. Minimise soil contamination of crops through choice of irrigation system.

Science Section

Introduction

There is increased focus on the microbiological risks associated with the consumption of ready to eat produce within the retail sector. These concerns are being driven by two main factors, namely increased government (i.e. FSA and EU) scrutiny of food production practices and the implementation of process controls; and also retailers seeking to minimise the risk of 'bad press' and damaged brand resulting from food poisoning traced to their produce.

Previous HDC funded work (FV248) established that almost three quarters of salad crops were irrigated through direct abstraction of surface water, the most vulnerable to contamination with faecal pathogens from agricultural activities. In addition, the majority of salad crops (60-85%) were irrigated with overhead booms – directly applying water to the leaf surfaces. Sampling of water sources demonstrated that irrigation water quality was variable, at times exceeding the WHO guideline for coliform bacteria (although it should be noted that the WHO guideline is for drinking water).

It is not commercially viable, using available technology, to remove microbial contamination from those products consumed raw, such as salad vegetables. The only widely-accepted approach to minimise the risk of microbial contamination of produce is to monitor and regulate the potential sources of contamination e.g. Irrigation water, manure, worker hygiene.

There is a body of data on the persistence of faecal pathogens on hands and well-proven best-practice hand wash procedures to limit the spread of faecal pathogens. Similarly there is considerable data on pathogen reduction through the composting process. However, there is a very conspicuous lack of data on the persistence of pathogens introduced to cropping areas through contaminated irrigation water.

It is clear from published HPA and FSA data that there is only a relatively low incidence of food poisoning associated with consumption of fresh produce grown in the UK. Tyrrel (2004) suggested that one reason for the low level of observed food illness correlated with lower quality irrigation water applied to salad crops could be due to pathogen die-off between irrigation and consumption. Another more plausible explanation is that microbiological testing identifies only whether a potential pathogen is present in water. Pathogens evolve to become

suited to particular hosts and thus those encountered in agricultural environments are more likely to have the ability to infect livestock rather than humans. Thus, even if "pathogens" are present in irrigation water they may not represent a credible threat to human health.

Whilst there is a body of work on the persistence of faecal pathogens in soil incorporated manures and slurries in UK conditions (e.g. Hutchison *et al*. 2005), work to date on persistence of pathogens between irrigation and harvest has not been reported in the literature for UK (or European) growing conditions.

A number of recent studies in the USA, in response to a relatively high rate of food illness outbreaks associated with fresh produce, have looked at routes of transfer for faecal pathogens onto salad vegetable and other crops. Field work carried out in Georgia, USA has demonstrated that *E. coli* O157:H7 applied through irrigation can persist in soils for up to 200 days and on the surface of lettuce and coriander for 77 and 177 days after contamination respectively (Islam *et al*., 2004a & 2005). The same workers have also reported persistence of *Salmonella enterica* Typhimurium as persisting on leaves of lettuce for 63 days and parsley for 231 days; and in soils for 161 days (Islam *et al*. 2004b). Relative to *Salmonella* and *E. coli* O157:H7, *Campylobacter* is much less persistent in the environment. *Campylobacter* causes ten times more foodborne illness in the UK than *Salmonella* and *Campylobacter* combined (Adak *et al*., 2005). A controlled environment study reported that *C. jejuni* only survives on Spinach leaves for 5 days at 10°C although soil persistence was five times longer (Brandl *et al*. 2004). However, the direct relevance of these data to UK production systems is limited. The role of soil microflora, temperature and sunlight are known to influence degradation rates of these pathogens (Palacios *et al*., 2001; Brandl *et al*., 2004; Stine *et al*., 2005), although the exact mechanism of this influence is unknown. This proposal aims to address this knowledge gap.

This project aims to quantify the true risk to salad vegetables from irrigation water introducing pathogens to the soil (Year 1 & 2) or to the surface of the produce (Year 2) and the interaction with UV (sunlight) and temperature on the seasonal persistence of the pathogen.

Overall aim of the project

To quantify the seasonal persistence of pathogens introduced to soil and produce surfaces through contaminated irrigation water.

Specific objectives

- Establish the persistence in the soil of pathogens introduced through irrigation at different times in a growing season. (Year 1)
- Evaluate the effect of soil type on persistence in the soil of pathogens introduced through irrigation. (Year 1)
- Establish pathogen distribution within the soil after irrigation (Year 2)
- Establish the persistence on the surface of crops of pathogens introduced through irrigation at different times in a growing season. (Year 2)

Materials and Methods (Year 2)

Details of Year 1 experiments are presented in the first year report.

Soil experiments

The soil distribution of bacteria added through irrigation water was studied using the soil columns used in Year 1. Two soil types were sourced from Harper Adams University College farm: both were defined as silty loams but with differing organic matter contents. A representative sample of each soil type was sent to an external testing laboratory (Eurofins Ltd, Wolverhampton) for physical and chemical characterisation and results are presented in Table 1. For this report they are defined as peaty soil and mineral soil.

Table 1: Physicochemical profile of the (pre-irrigated) soil used for these studies

Analysis	Peaty Soil Mean result (n=2)	Mineral Soil Mean result (n=2)
Total Nitrogen (% m/m)	0.48	0.16
Organic matter (% m/m)	8.98	1.81
Particle size distribution (%):		
2000-600 µm – Coarse Sand	0.48	0.16
600-212 µm – Med Sand	8.98	1.81
212-63 µm – Fine Sand	6.5	7
63-20 µm – Coarse Silt	32	60.5
20-2 µm – Fine Silt	27	18
<2 µm – Clay	5	2.5

Columns of the two defined soils were held in large plastic buckets (60 cm diameter, 45 cm depth). The soils used were the original soils used in Year 1: the soil had been left in the buckets undisturbed for 12 months, allowing soil structure to form. Sample access holes were drilled into the side of the bucket at 10, 20, 30 cm from the surface and covered with duct tape.

Microorganisms used and their culture conditions

Escherichia coli K12 isolate EQ1, resistant to 40 µg ml^{-1} nalidixic acid, was stored in Protect beads (Technical Services Consultants, Heywood, UK) at 70°C, and resuscitated by removing one bead, inoculated by streaking on a Columbia Blood Agar (Oxoid, Basingstoke, UK) plate to obtain isolated colonies. Luria Bertani Broth supplemented with 40 µg ml^{-1} nalidixic acid (LBN; 30 ml) was equilibrated to 37°C, then inoculated with one colony of *E. coli* K12 EQ1 and incubated at 37°C overnight without shaking.

Plot inoculation

The surface of the soil columns was 'tilled' to a depth of 5 cm immediately before wetting using a hand hoe. 1 litre of borehole water, inoculated with 1 $x10^5$ cfu ml^{-1} marker, was poured carefully into the centre of the column and the soil was left for 1 hour before sampling. Samples were taken from the surface then progressively at 10, 20 and 30cm. A flame sterilised auger was used to take samples. The auger was sterilised initially and between core samples. Cores were collected into sterile bags and samples were processed within 2 hours.

Microbiological testing methods

Escherichia coli K12 EQ1 in soil samples were enumerated by preparing suitable serial dilutions in maximum recovery diluent (MRD; Oxoid) and spread plating 0.1 ml volumes onto Violet Red Bile agar containing 40 µg ml^{-1} nalidixic acid. Plates were incubated at 37°C overnight and typical colonies (red, with precipitate) were counted. Values were calculated as per g of wet weight of soil.

Crop experiments

Heavy rain prevented planting for the mid season experiment – this will be completed in an extension to this project (2008).

Treatments

- 2 experiments: early and late season timing
- 2 crops: Spinach, Wholehead lettuce
- 3 levels of pathogen* zero, low ($1×10^2$ cfu ml^{-1}) and high ($1×10^5$ cfu ml^{-1})
- 3 replications for each treatment

*Pathogens were applied as a composite mix of *Salmonella*, *Campylobacter* and *E. coli* each at a similar concentration.

Irrigation treatments were applied using designated watering cans at one event at the start of each experiment (Experiment 4 – 16 May 2007, Experiment 5 - 04 September 2007). Leaf tissue was sampled for pathogen recovery initially and at weekly intervals; soil was sampled at the end of the experiment.

Plot preparation

The experiments were carried out at Harper Adams University College, Shropshire in Birds Nest Field. The soil was a deep permeable sandy loam over loamy sand at 60 cm depth classified as Wick series (Beard, 1988). The trial area was under oats drilled the previous autumn. These were sprayed off and the land ploughed and power harrowed before a bed former was used. Iceberg lettuce transplants (*Lactuca sativa* cv Robinson) and spinach seed (*Spinacia oleracea* cv Toscane F1) were provided by PDM Ltd. Lettuces were transplanted by hand 1 week before treatments and spinach was drilled using a spider drill 10 days before treatments commenced. Plots were randomly distributed along a 1.4m wide bed. Each plot was 4 m long with a guard plot of 3 m between treatment plots. The lettuce and spinach beds were separated by 6 m of topped rye grass to prevent the risk of soil splash contaminating the adjacent crops. A sonic bird scarer was placed in the centre of the trial and cotton thread was suspended across the plots to deter birds. Electrified rabbit fencing surrounded the experimental area.

Water for irrigation treatments was sourced from the irrigation lagoon filled from a borehole at Harper Adams University College.

Environmental measurements

A portable weather station (Mini-Met; Skye Instruments, Llandrindod Wells, UK) was installed in the buffer strip surrounding the field plots. Precipitation was continuously collected in a rain gauge (Skye Instruments). All other parameters were recorded every 10 minutes. Soil temperature was recorded at a depth of 5 cm. Air temperature and air humidity at 20cm above ground, daylight hours and the intensity of solar radiation were recorded for the duration of the experiments at 35 cm above ground. All data were saved on a DataHog 2 device (Skye Instruments) which was downloaded weekly.

Microorganisms used and their culture conditions

The pathogens studied were a *Salmonella* Enteriditis, a *Campylobacter jejuni* and an E. coli O157 (which does not contain the genes for verocytoxin). The zoonotic agents used for these studies were all isolated originally from UK livestock. Because there are differences between

human and livestock isolates of these pathogens, it is far more likely that agricultural environments would be contaminated with livestock rather than human isolates of pathogens. The organisms used were *Salmonella enterica* serotype Enteriditis (strain S8167/99), *Campylobacter jejuni* (strain 20001424) and a non-verotoxin-producing *Escherichia coli* O157 (strain 20001383). *E. coli* O157 and *Salmonella* were propagated in Buffered Peptone Water (Oxoid, Basingstoke, UK), *Campylobacter* was grown in modified Exeter Broth (mEB; Nutrient Broth (Oxoid) supplemented with 1% (v/v) water-lysed fresh horse blood, 250 mg l^{-1} of sodium metabisulphate, 250 mg l^{-1} of sodium pyruvate and 250 mg l^{-1} of ferrous sulphate). No media supplements were inhibitory to the bacteria used. Cultures were grown without agitation or aeration at 37˚C (*E. coli* and *Salmonella*) or 42˚C (*Campylobacter*). *Campylobacter* incubators were filled using a custom formulated mixture of 10% (v/v) carbon dioxide, 5% (v/v) oxygen, and 85% (v/v) nitrogen (British Oxygen Company, Guilford, UK). *Campylobacter* media was equilibrated in the modified atmosphere for 6 hours before use for bacterial propagation.

Plot inoculation

Cultures of bacterial pathogens were introduced into irrigation water sourced from a farm borehole typical of that used by commercial salad growers in the UK. Bacteria were distributed through the water by gentle agitation taking care not to excessively oxygenate the liquid. The pathogens were applied at levels commonly found in the environment rather than artificially high 'spiked' levels. Initial levels of each individual bacterial pathogen in the contaminated waters were either 1×10^5 CFU ml^{-1} (high application) or 1×10^2 CFU ml^{-1} (low application). Negative control plots were watered with borehole water which did not contain any zoonotic agents. The mass of water used to irrigate each 5.6 m^2 field plot was 28 litres applied using a 5 litre watering can to give the equivalent of 5 mm overhead irrigation. The contaminated water was applied as a single treatment at the beginning of each experiment. All subsequent irrigation of all plots was according to standard commercial practices using fresh borehole water that did not contain pathogens through solid set irrigation. After each irrigation event, the water was left undisturbed to soak into the soil. Three replicate field plots were generated for each treatment and control. Declines in the numbers of each of the zoonotic agents were followed over a 6 week period or until no zoonotic agents could be detected. Two trials were run between May and October which is typical for the lettuce growing season in the UK. Heavy rain prevented planting for the mid season experiment – this will be completed in an extension to this project (2008).

Sample collection from field plots and transit to the laboratory

Samples for analysis were collected from each replicated field plot each week over a 6 week period or until no zoonotic agents could be detected. Each sample comprised a minimum of 10g of leaf material collected across the plot and collected using sanitised metal scissors. Soil samples were taken the week after no zoonotic agents were detected on the crops. Each sample comprised a minimum of 25 combined sub-samples taken from diverse areas of the plot and collected to a depth of 5cm using sanitised metal spoons. Soil and crop samples were kept cool (<15°C) for transport from the farm site to the laboratory. All microbiological testing commenced within 4h of sample collection.

Microbiological testing methods

Bacteria were initially enumerated from all of the samples taken. For the field samples, after bacterial numbers declined below the threshold for reliable enumeration, a switch to simple presence/absence detection using enrichment was made.

Enumeration of *Campylobacter* was by suspending 10g of sample in 10 volumes of mEB which had been pre-warmed to 42°C and pre-equilibrated in an atmosphere containing 10% CO_2. Decimal dilutions of suspended sample were undertaken in mEB before plating onto dried modified charcoal cefoperazone desoxycholate agar (mCCDA, Oxoid). For determination of the presence of *Campylobacter*, enrichment for 24h at 42°C in Exeter broth was undertaken before plating onto mCCDA. Campylobacters were incubated under microaerophilic conditions at 42°C. Confirmation of presumptive campylobacters for both tests was by corkscrew motility after microscopic examination of a loopful of bacteria in MRD and positive testing for Oxidase activity.

Numbers of *E. coli* O157 were determined by suspending either 10g (field experiments) or 1g (laboratory drainage experiments) of sample in Modified Tryptone Soya Broth (mTSB, Oxoid) undertaking decimal dilutions in mTSB and plating onto Modified Sorbitol MacConkey Agar (CT SMAC, Oxoid), supplemented with 2.5 µg ml^{-1} potassium tellurite and 0.2 µg ml^{-1}cefixamine). Presence of *E. coli* O157 was by enrichment in mTSB for 48h at 42°C. Confirmation of presumptive *E. coli* O157 for field samples was by agglutination with latex-mounted anti-O157 polyclonal antibody (Oxoid, Dryspot). Isolates from laboratory drainage experiments were not confirmed.

Salmonella numbers were determined by initial suspension and decimal dilution of the sample in 9 volumes of Rappaport Vassiliadis Soya enrichment broth (RVS, Oxoid). Plating was onto xylose lysine deoxycholate agar (XLDA, Oxoid). Presence of *Salmonella* was by pre-enrichment

in BPW at 37°C for 16 h. Enrichment was by transfer of 0.1 ml of the pre-enriched sample into 10 ml RVS medium and incubation at 42°C for 24h. Detection was by streaking onto XLDA. Confirmation for both *Salmonella* tests was by lack of oxidase activity and biochemical profiling (API20E; bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions.
Up to 5 presumptive colonies per plate were confirmed. Colony counts were converted to CFU g^{-1} waste according to the criteria specified by the International Standards Organisation.

Data Analysis

Log averages and associated standard deviations from each set of three replicates were calculated for each sample. R^2-values were determined by the least squares method and coefficients of variation (CV) calculated by dividing the means by the SD for each sample time. Groups of CVs were compared using the Mann-Whitney U-test for non-parametric data ($P<0.05$; SPSS 11.5, SPSS Inc., Chicago, USA).

Results (Year 2)

Pathogen distribution within the soil after irrigation

Following irrigation with labelled water, the marker bacteria were distributed evenly throughout the 30 cm soil profile in the mineral soil column (Table 2). However, the pattern of distribution was different in the peaty soil. There was a lower concentration of marker bacteria at the surface of the soil and at 30cm, with a 1 Log greater accumulation of marker bacteria in the 10 and 20cm samples.

Table 2: Numbers of nalidixic acid-resistant *E. coli* K12 marker recovered from different depths of soil wetted with marker-inoculated borehole water. Mean values are derived from three replicates undertaken on different days.

Soil type and position where the core was taken	Mean $\log_{10}$ numbers of marker (CFU g^{-1} wet weight soil)	Standard deviation associated with $\log_{10}$ numbers of the mean (CFU g^{-1} wet weight soil)
Mineral soil		
Surface	4.60	0.61
10 cm	4.66	0.69
20 cm	4.96	0.36
30 cm	4.22	0.61
Peaty soil		
Surface	3.84	0.36
10 cm	5.69	0.48
20 cm	5.05	0.61
30 cm	4.01	0.16

Pathogen persistence on crop tissue

Environmental conditions

The two crop experiments experienced different environmental conditions. Experiment 4 experienced variable moderate air temperature and light level and sporadic rainfall for the first three weeks (Appendix - Figure I). In contrast, Experiment 5 experienced declining air temperatures and low light levels, half the accumulated light energy of Experiment 4, with moderate rainfall towards the end of the experiment (Appendix - Figure II). The overall environmental conditions for both years are summarised in Table 2. The mid summer trial was postponed for a year due to heavy rainfall - July received 126 mm in 2007, 120 mm more than 2006.

Table 3: A summary of the environmental conditions experienced for each of the experiments in 2006 and 2007.

Measured environmental parameter (units)	*Early season*		*Mid season*		*Late season*	
	2006	2007	2006	2007	2006	2007
Accumulated rainfall (mm)*	82.0	39.8	6.0	(126)	106.8	21.6
Average daily temperature (°C)**	16.5	12.8	23.8	-	14.6	13.3
Average daily accumulated sunlight (Wm^{-2})	5436.8	5276.6	6506.6	-	2422.8	2535.6

* Crops were irrigated in addition to rainfall

** Temperature measured at 5 cm soil depth (2006); 20 cm height (2007)

In both experiments, the difference between the concentration of zoonotic agents applied were much greater than the concentrations recovered from leaf tissue. The treatments were applied to small plants and it is likely that the available surface binding sites were being saturated or nearly saturated by the low treatment. In all cases, the concentration of zoonotic agent recovered from the high treatment was as great or greater than that recovered from the low treatment, and the difference was more pronounced in lettuce.

Salmonella Enteriditis

A low level of contamination was detected in the control treatments of both experiments. This was observed in Experiment 4 at the initial analysis of spinach but at a low level (120 cfu g^{-1}) and on one sample only. Interestingly, in Experiment 5, both spinach and lettuce showed a level of contamination at week one that was not present at the initial sample. Only one lettuce

plant was contaminated at a low level (320 cfu g^{-1}), but all three spinach samples were contaminated at a range from 10 – 10^4 cfu g^{-1}. The high value may be due to contamination in the laboratory, but the background levels can be explained as coming from the irrigation source at HAUC, where heavy summer rains had washed some contamination from livestock production into the water source.

a) Lettuce

The decline of Salmonella on the surface of lettuce leaves was rapid in Experiment 4 with both treatments having declined to below the level of enumeration (<10 cfu g^{-1}) after one week (Figure 1). No Salmonella was detected on some plots in Experiment 4 after two weeks and all plots after three weeks (Table 4). The initial declines were similar in Experiment 5. One plot of the high level of contamination had a level of salmonella >10 cfu g^{-1} after one week. This may have been due to the contamination observed in the control treatment. However, in contrast to Experiment 4, no Salmonella was detected on leaf tissue by week two.

b) Spinach

Both treatments in Experiment 4 gave similar levels of recovery of Salmonella at the initial analysis ~1x10^4 cfu g^{-1}. This had declined to below an enumerable level after one week (Figure 2). Salmonella was detected in all plots after two weeks but was not detected after a further week (Table 4). In the late season experiment, recovered levels of salmonella declined rapidly after one week but a number of plots had low levels of salmonella present on the leaves at levels between 5 and 30 cfu g^{-1}. By the following week no salmonella was detected on the low treatment crop but all three plots at the high treatment had salmonella detected. After three weeks this contamination could no longer be detected.

c) Soil

No Salmonella was detected in the surface soil samples taken after three weeks in Experiment 4 but salmonella was present in the surface soil of 2 plots for each treatment after 4 weeks in Experiment 5 (Table 4)

Table 4. Number of plots (n=3) where *Salmonella* Enteriditis was detected on leaf tissue and surface soil following inoculation with Low and High levels of *Salmonella* Enteriditis applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5

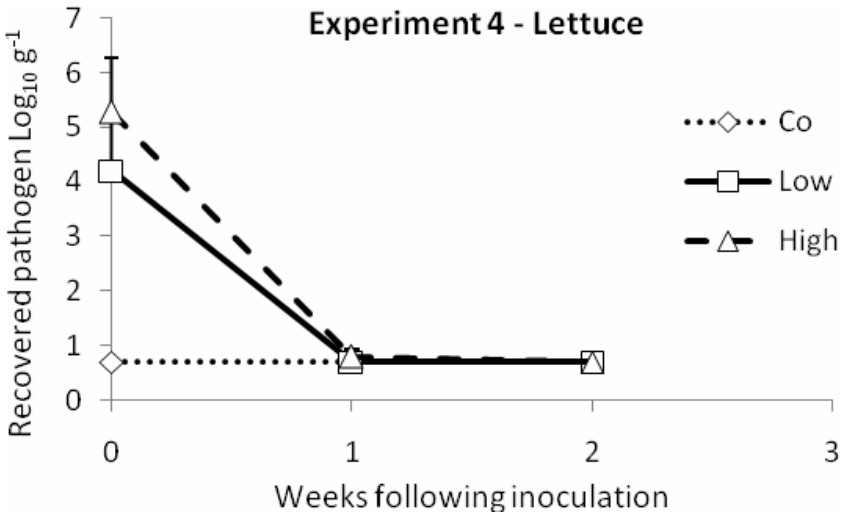
a)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	3	3
+2	2	1	3	3
+3	0	0	0	0
+3 (Soil)	0	0	0	0

b)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	3	3
+2	0	0	0	3
+3	0	0	0	0
+4 (Soil)	2	2	2	2

Figure 1. Recovered pathogen from leaf surface of lettuce following inoculation with Control, Low and High levels of *Salmonella* Enteriditis applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).



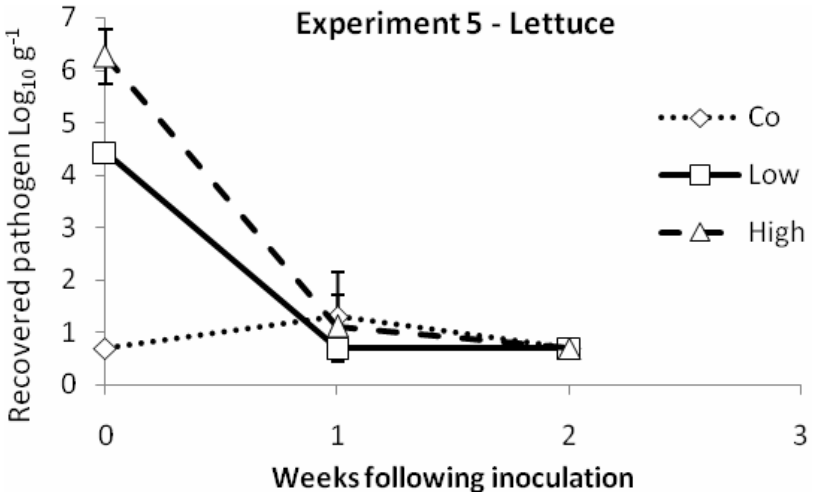
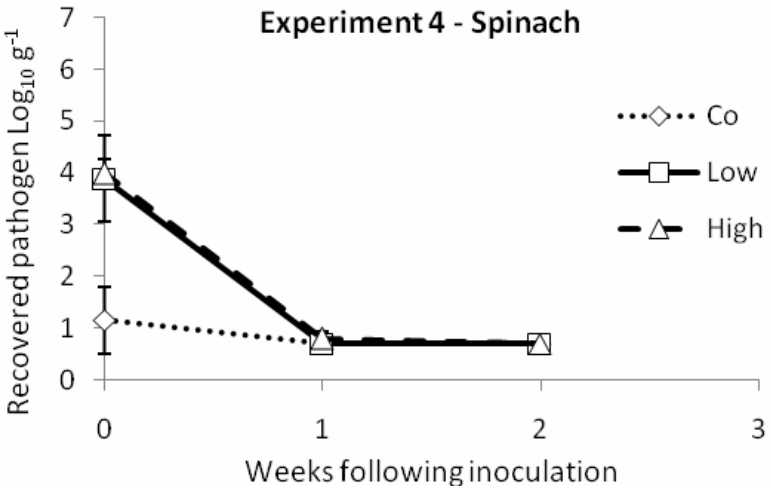
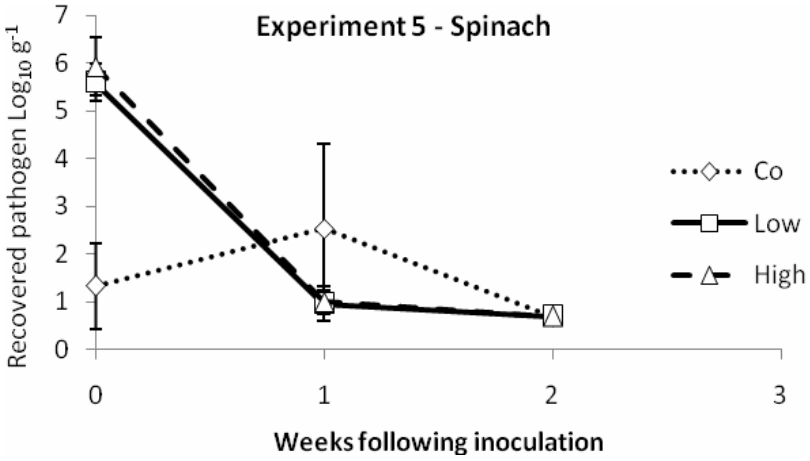


Figure 2. Recovered pathogen from leaf surface of spinach following inoculation with Control, Low and High levels of *Salmonella* Enteriditis applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).





E. coli O157

No *E. coli* O157 was detected in the control treatments in Experiment 4. A low level of contamination (40 cfu g^{-1}) was observed for one spinach plot after 1 week in Experiment 5.

a) Lettuce

E. coli O157 declined rapidly on lettuce leaves in both experiments. The low treatment in Experiment 4 and 5 and the high treatment in Experiment 5 had all declined to levels below 10 cfu g^{-1} after 1 week although a low level of E .coli > 10 cfu g^{-1} was observed for the high treatment in Experiment 4 (Figure 3). No *E. coli* O157 was detected on lettuce leaves 2 weeks after inoculation at the high and low level in the early season experiment. However, the high level treatment was more persistent in the late season experiment being detected for two week s after inoculation (Table 5).

b) Spinach

The early season experiment showed rapid declines of *E. coli* O157 on the leaves of spinach in one week. The level of *E. coli* O157 could not be enumerated from week 1 samples and was not detected on spinach leaves of both low and high treatments after 2 weeks (Fig 4 & Table 5). The late season experiment gave a more variable response. The high treatment had declined from $1x10^6$ Log cfu g^{-1} *E. coli* O157 on the surface following inoculation to <10 cfu g^{-1} after 1 week. In contrast the low treatment took two weeks to decline to <10 cfu g^{-1} (Figure 4). Nevertheless, *E. coli* O157 was not detectable on spinach leaves of either the low and high treatments in Experiment 5 after three weeks (Table 5).

c) Soil

E. coli O157 was not detected in the surface soil samples taken after three weeks in the early season experiment and four weeks in the late season experiment (Table 5).

Table 5. Number of plots (n=3) where *E. coli* O157 was detected on leaf tissue and surface soil following inoculation with Low and High levels of *E. coli* O157 applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5

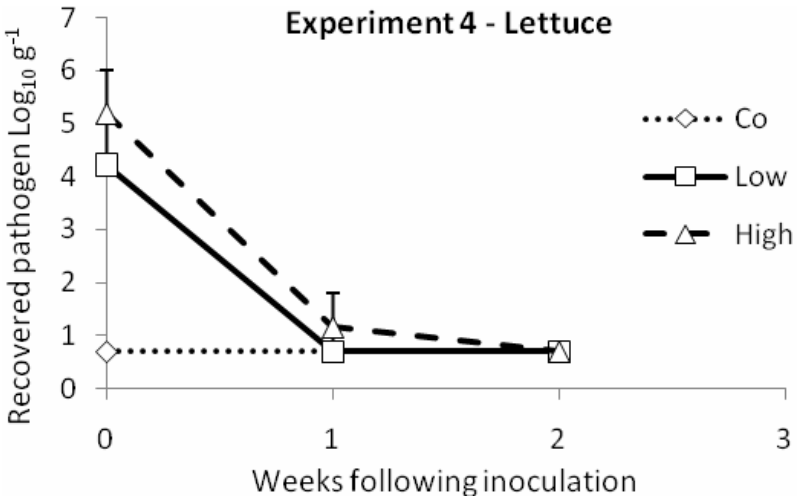
a)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	3	3
+2	0	0	0	0
+3 (Soil)	0	0	0	0

b)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	3	3
+2	0	2	1	1
+3	0	0	0	0
+4 (Soil)	0	0	0	0

Figure 3. Recovered pathogen from leaf surface of lettuce following inoculation with Control, Low and High levels of *E. coli* O157 applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).



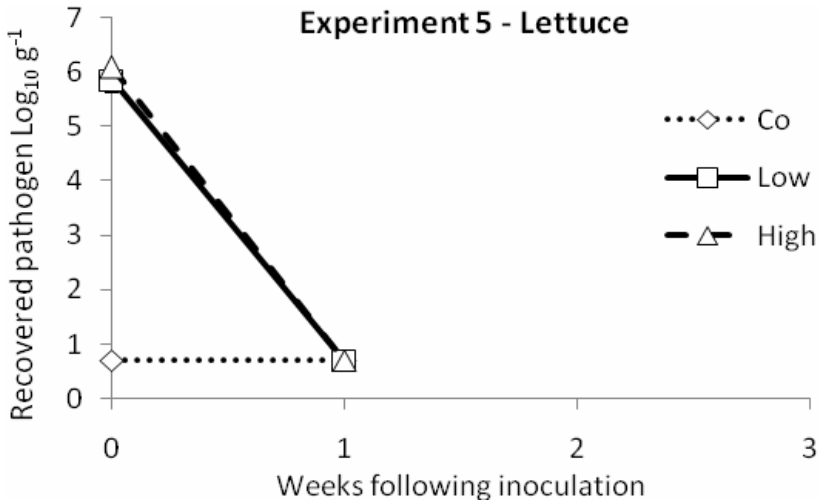
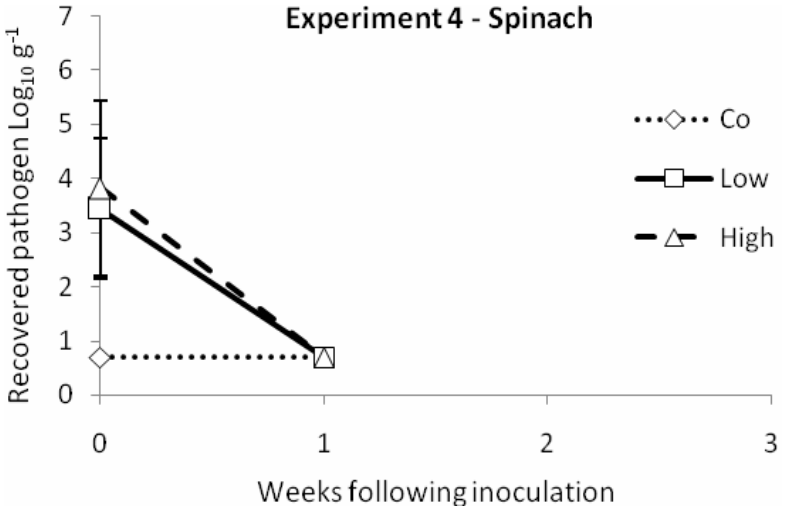
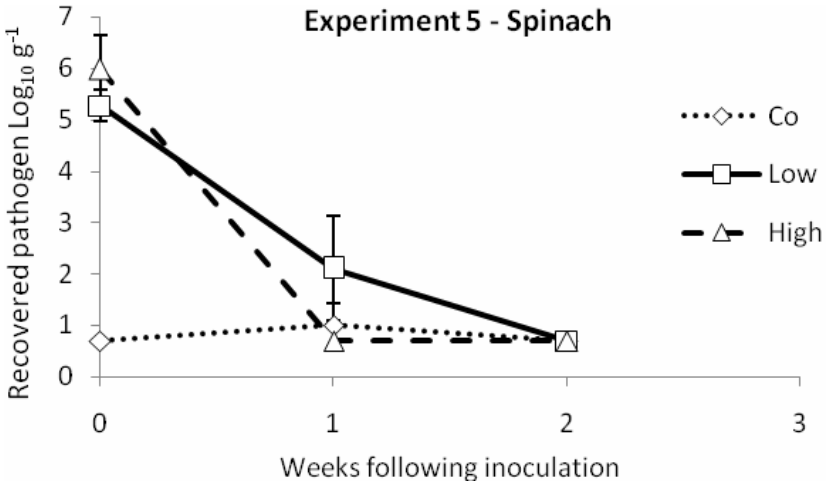


Figure 4. Recovered pathogen from leaf surface of spinach following inoculation with Control, Low and High levels of *E. coli* O157 applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).





Campylobacter jejuni

No contamination was observed in the control treatments of either experiment.

a) Lettuce

The high treatments of both experiments gave similar levels of initial Campylobacter recovery ~1x$10^{3.5}$ cfu g^{-1} which had declined to <10 cfu g^{-1} after one week (Figure 5). The low levels of inocula gave intermediate levels of initial recovery in both experiments but had also declined to <10 cfu g^{-1} after one week. Interestingly, the experiments differed in persistence of Campylobacter. In the early season experiment no Campylobacter was detected after 2 weeks, but it took an extra week before Campylobacter was absent from the crop in the late season experiment (Table 6).

b) Spinach

Campylobacter declined in spinach in a similar pattern as observed for lettuce. Both treatments had declined to levels below that which could be enumerated (<10 cfu g^{-1}) after one week. Campylobacter was absent from leaf material after 2 weeks in Experiment 4, and after 3 weeks in Experiment 5 (Table 6).

c) Soil

Campylobacter jejuni was not detected in any of the surface soil samples taken from the treated plots at the end of either experiment (Table 6).

Table 6. Number of plots (n=3) where *Campylobacter jejuni* was detected on leaf tissue and surface soil following inoculation with Low and High levels of *Campylobacter jejuni* applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5

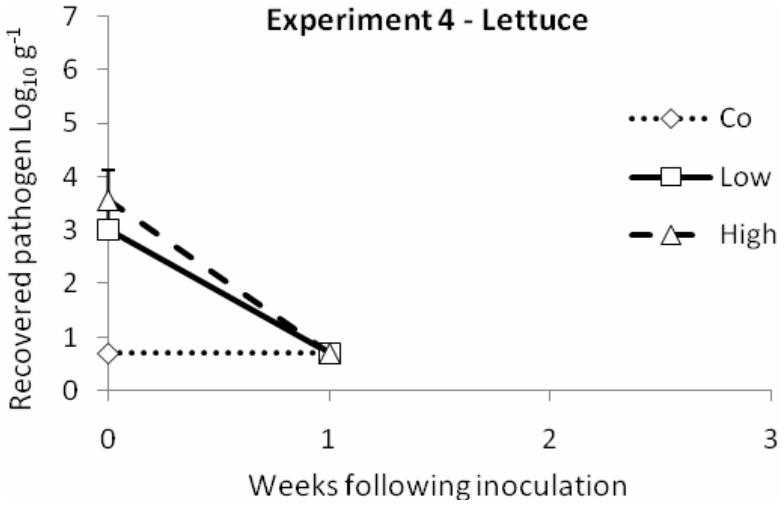
a)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	3	3
+2	0	0	0	0
+3 (Soil)	0	0	0	0

b)

	Lettuce		Spinach	
Week	Low	High	Low	High
+1	3	3	1	1
+2	1	3	1	1
+3	0	0	0	0
+4 (Soil)	0	0	0	0

Figure 5. Recovered pathogen from leaf surface of lettuce following inoculation with Control, Low and High levels of *Campylobacter jejuni* applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).



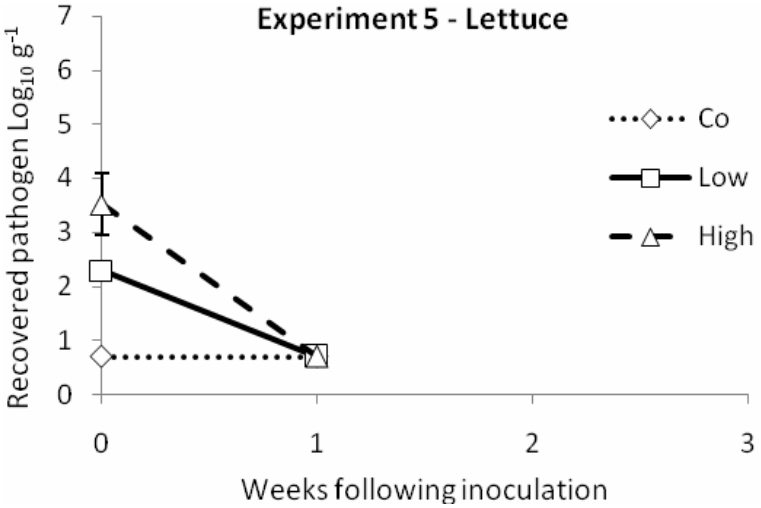
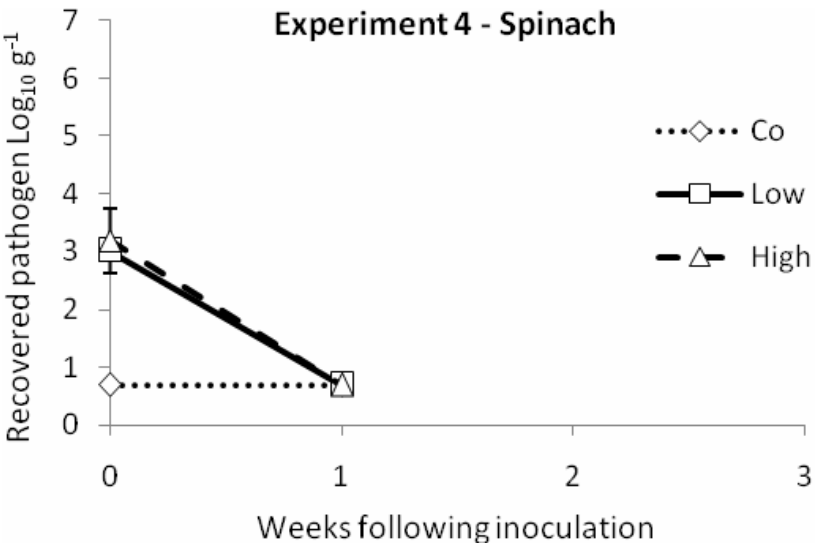
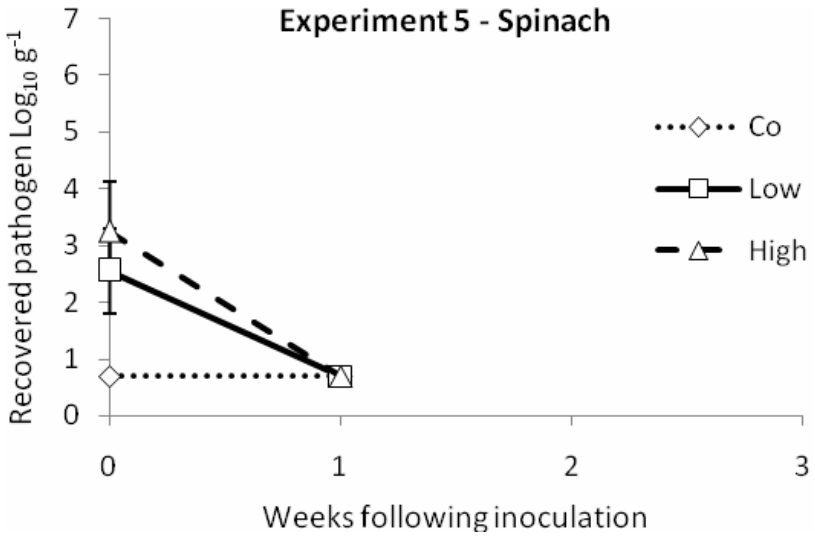


Figure 6. Recovered pathogen from leaf surface of spinach following inoculation with Control, Low and High levels of *Campylobacter jejuni* applied to the crop through overhead irrigation: a) Experiment 4; b) Experiment 5 (Bars represent +/- SD n=3).





Discussion (Years 1 & 2)

The weather conditions during the experiments provided contrasting conditions typical of early, mid and late season field production (see Table 3): experiment 1 started cold and wet and developed to be warm and dry with moderate sunlight levels; experiment 2 was hot and dry with high levels of sunlight; experiment 3 was wet with increasingly low light levels and temperatures as it progressed. Experiments 4 and 5 gave comparable conditions for the early and late season in Year 2, although the heavy rains in mid summer prevented the planting of crops and this experiment will be undertaken in 2008.

Does season have an effect on pathogen persistence in soil?

Season had a marked effect on persistence of introduced bacteria. The hot, dry and high sunlight conditions of experiment 2 were associated with the fastest observed reduction in bacterial numbers recovered from the surface of the soil. *Campylobacter* declined most rapidly. All three potential human pathogens were at or below the threshold for reliable enumeration after 2 weeks. In contrast the cooler, wetter and lower light levels experienced in experiment 1 and experiment 3 were associated with a greater and more prolonged persistence of pathogens. Persistence was particularly marked in experiment 3 where *E. coli* O157, *Salmonella* and *Campylobacter* were still present in the surface of all plots after 6 weeks. The increased persistence towards the end of season was also observed in the field soil samples taken at the end of Experiment 5 where Salmonella was still detected in the majority of plots after 4 weeks.

Field work carried out in Georgia, USA has shown that following application through irrigation water in October *E. coli* O157:H7 can persist in soils for up to 200 days (Islam *et al*., 2004a & 2005) and *Salmonella enterica* Typhimurium for 161 days (Islam *et al*. 2004b). The same workers reported that 6 weeks after the application of contaminated irrigation water, comparable to the high treatment studied in experiment 3, *E. coli* and *Salmonella* were being recovered from soil at levels of $1x10^3$ and $1x10^2$ CFU g^{-1} respectively. These levels are in general agreement with the data from experiment 3 in the mineral soil with low organic matter; although climatic conditions in GA are markedly different from those encountered in the UK (see below).

- Under conditions where there is the greatest requirement for irrigation i.e. hot and dry with strong sunlight the decline of pathogens introduced to the soil through irrigation is much more rapid and a moderate level of contamination would be undetectable after approximately 3 weeks.

- There is the possibility that pathogens introduced to the soil at high levels through irrigation water can persist for the duration of a lettuce or spinach crop, particularly with the environmental conditions commonly experienced at the start and end of the season (i.e. lower temperatures and light levels).
- We have so far been unable to find literature reporting pathogen persistence in soil from field experiments in summer conditions. Thus the results of this study are novel, and can be used to properly inform regulators as to the realistic risks of contamination of fresh produce with contaminated irrigation water.

Does soil type have an effect on pathogen persistence in soil?

It is well known that the key factor effecting pathogen survival in soils is moisture (e.g. Jamieson *et al.* 2002) and limited soil moisture reduces persistence of enteric pathogens including *Salmonella typhimurium* and *E. coli* in dry soils (Chandler and Craven, 1980). However, the trial plots in Year 1 were not irrigated and received the same rainfall, so any differences in persistence related to moisture would be down to water retention by the soils. Although both soils were silty soils, of the two soils the peaty soil would be expected to retain more water as it had a higher organic matter content (9% versus 2%) suggesting that the pathogens should have had the greater survival in this soil, contrary to observation. An alternative explanation, fitting the observed response, is that the higher organic matter of the peaty soil was associated with a higher indigenous microbial population i.e. a more biologically active soil, and that antagonistic interactions with indigenous microbial populations, and predation by amoebic organisms were influencing soil survival. Support for this comes from work showing that *E. coli* O157 (Jiang *et al.* 2002) and *Salmonella enterica* Newport (You *et al.* 2006) both persisted longer in autoclaved (sterilised) soils compared to unautoclaved soils. Further soil studies are needed to establish the role of soil type on pathogen survival and whether it is of practical importance in field production of ready to eat crops.

- Soil type had a significant effect on the persistence of introduced zoonotic agents particularly at the high level of contamination. Organic matter content and hence biological activity of the soil is associated with a more rapid decline in all three pathogens.

How are pathogens distributed within the soil after irrigation?

It was notable that the initial levels of pathogen recovered from soil in Year 1 were higher than expected. Plots of soil were contaminated with water containing approximately 1×10^{2} and $1x10^{5}$ CFU ml^{-1} for the low and high treatments respectively, but the levels recovered per g were

in the region of 2 logs higher for the low treatments and 1-2 logs higher for the high treatments. This effect was more marked in the mineral soils with higher initial levels of Salmonella and *E. coli* recovered compared to the peaty soil.

It was unclear whether this accumulation of target bacteria was due to some binding of bacteria to soil components, with water draining from the surface to leave a higher concentration of bacteria, or a pooling of water in the surface layer of soil. Using the same soil columns that had been left *in situ* to weather for ~12 months before the marker bacteria were applied, it was observed that bacteria added in irrigation water do not significantly accumulate at the surface of soils but that soil properties influenced the distribution of bacteria through the column. It is known that a certain amount of physical filtration of bacteria and adsorption to soil particles occurs in soils (Gerba *et al*., 1975) but also that preferential flow (i.e. water movement) can lead to rapid movement of bacteria through soil (Geohring *et al*., 1999). Of the two soils studied the mineral soil had an even distribution of marker bacteria through the column depth after 1 hour. In contrast, the peaty soil had a lower concentration of marker bacteria at the surface than 10 and 20cm depth. The more rapid transport of water away from the surface of the peaty soil would be expected as the lower proportion of silt and higher proportion of sand would increase soil permeability. It is likely that the marked accumulation of bacteria in Year 1 was to some extent due to the surface of the columns being packed in the process of filling the buckets and limiting water diffusion, similar results in soil column experiments were reported by Smith *et al*., (1985). The more weathered soil columns, along with a simulated 'tilled surface' will have increased the flow of water and hence bacteria away from the soil surface in Year 2.

Permeability may also explain why we did not see the same surface persistence of pathogens in the field soils sampled at the end of the crop experiments. Salmonella was present in the soil surface in the late season experiment (Experiment 5) only. All other soil samples were free of the added pathogens. The crops were grown in a free draining sandy loam, and the soil surface was regularly observed to be dry.

- Soil that drains freely away from the surface may help to move pathogenic bacteria away from the soil surface and will minimise the potential for pathogenic bacteria to contaminate crop through soil splash dispersal.

What is the persistence on the surface of crops of pathogens introduced through irrigation at different times in a growing season?

In contrast to soil persistence studied in Year 1, the persistence of pathogens on the surface of lettuce and spinach was much shorter. All three bacteria studied declined rapidly to absence in 14-21 days. It is likely that the persistence of pathogens will be even less in mid summer UK conditions (to be confirmed in extension work). This does not agree with the data reported from the USA. Field trials carried out in Georgia, USA have demonstrated that *E. coli* O157:H7 applied through irrigation can persist on the surface of lettuce for 77 days after contamination (Islam *et al*., 2004a). The same workers have also reported persistence of *Salmonella enterica* Typhimurium as persisting on leaves of lettuce for 63 days (Islam *et al*., 2004b), i.e. pathogens are persisting for 3 to 4 times longer in the US work. Both the US work and the work reported here took place in a field environment, with young plant material being inoculated with similar levels of pathogen. However, climate and season would have been very different between the two growing sites. The University of Georgia Horticulture Farm is located in Tifton, GA (32.0852 lat, -84.1830 lon), roughly on the same latitude as northern Morocco. The US work took place from October, over winter and, although met data was not presented in the papers, the temperature ranges over winter in Georgia are moderate, rarely approaching freezing with a range that is not too different to the UK in spring, but with higher night temperatures. In marked contrast the work at Harper Adams took place from early May to the end of September, meaning that crops will have experienced higher temperatures than the US crops and higher levels of sunlight, and hence UV; the US work would have received day lengths less than 11 hours whereas the UK work received day lengths exceeding 13-14 hours.

The climate has a marked effect on leaf borne pathogens – pathogens on dry leaves exposed to high levels of UV rapidly degrade The US data is a worse case scenario in conditions similar to North Africa in the winter (with greater rainfall!), <u>not leafy salad production conditions in the UK</u>.

- Even high levels of leaf contamination will pose no risk after a maximum of three weeks in a UK growing environment. It is likely that this persistence will be shorter in high summer production.
- Particular care is needed with short season crops such as babyleaf spinach
- Guidance for growers should be derived from work undertaken in a UK growing environment – it can be misleading to extrapolate international studies to the UK growing environment.

Why are pathogens more persistent in soils compared to leaf surfaces?

Over the two years we have observed that pathogens persist for a greater period of time in the soil than on the surface of crops. This can be explained by the very different environments experienced by the pathogens. As discussed previously the key factor effecting pathogen survival in soils is moisture (e.g. Jamieson *et al.* 2002). The soil offers a relatively moist and cool environment with exposure to direct sunlight (UV) only at the surface of the soil, whereas the leaf surface will be at times, dry, warm and exposed to UV light. The difference that the two environments have on pathogens can be seen clearly in the following table that summarises persistence of pathogens in the early and late season trial in Year 1 and 2.

Table 7. Persistence of pathogens in the soil and on the surface of crops measured as weeks to pathogen recovery below the level of enumeration (<10 cfug^{-1}) and weeks to absence of pathogen.

a) Salmonella Enteriditis

		Early season				Late season			
		Low		High		Low		High	
		<10 cfu	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*
Soil	Peat	3	>5	3	>5	3	>6	3	>6
	Mineral	3	>5	3	>5	3	>6	6	>6
Leaf	Lettuce	1	3	1	3	1	2	2	2
	Spinach	1	3	1	3	2	2	2	3

b) *E. coli* O157

		Early season				Late season			
		Low		High		Low		High	
		<10 cfu	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*
Soil	Peat	2	>5	>5	>5	2	>6	3	>6
	Mineral	4	>5	>5	>5	3	>6	>6	>6
Leaf	Lettuce	1	2	2	2	1	2	1	3
	Spinach	1	2	1	2	2	3	1	3

c) Campylobacter jejuni

		Early season				Late season			
		Low		High		Low		High	
		<10 cfu	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*	*<10 cfu*	*Absent*
Soil	Peat	-	-	-	-	1	>6	3	>6
	Mineral	-	-	-	-	2	>6	>6	>6
Leaf	Lettuce	1	2	1	2	1	3	1	3
	Spinach	1	2	1	2	1	3	1	3

What is the risk of introducing a reservoir of viable pathogen to the soil that may contaminate produce at harvest?

The experiments demonstrated that applying contaminated irrigation water to crops and soils introduces a risk to ready to eat produce grown in that soil. The persistence of the bacterial contamination in the soil particularly was related to the level of initial contamination, with the high level of contamination persisting longer than the low level in all cases. However, pathogen persistence was much lower on the surface of the crops – could the soil then pose a risk of re-contamination of the crop before harvest?

It appears that soil preparation has a potential large effect on the persistence of pathogens on the soil surface, and distribution into the soil profile. But clearly there is at least a hypothetical risk of infecting crops by soil contamination – either through rain/irrigation splash or harvesting - and this work would suggest that the risk is greatest at the start and end of the season. Further work will be carried out in an extension trial in 2008 to evaluate the risks from soil splash in spreading soil contamination to the crop.

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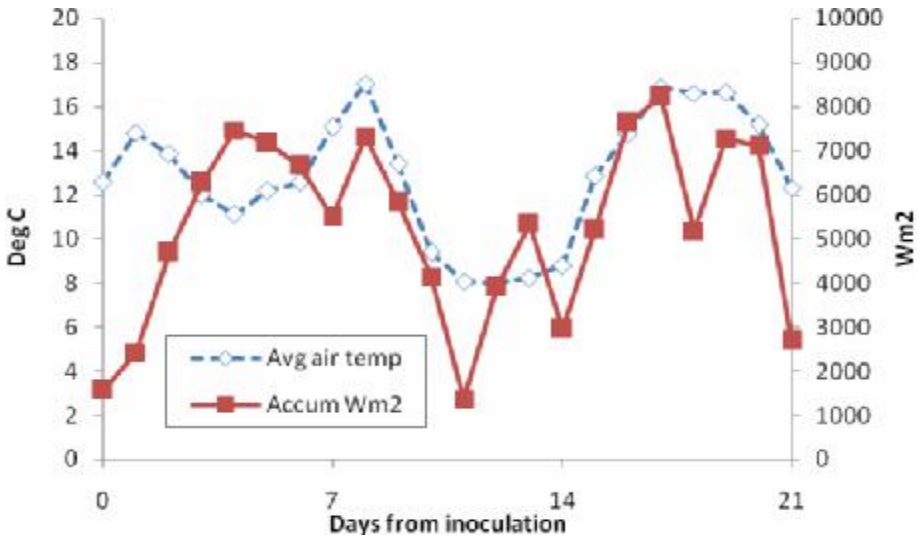
Appendix

Figure I. Weather data measured at the experimental site after the inoculation of soils (day 0 = 16 May 2007): a) Daily average air temperature (20 cm) and accumulated sun light; b) Daily rainfall. Experiment 4, Year 2.

a.



b.

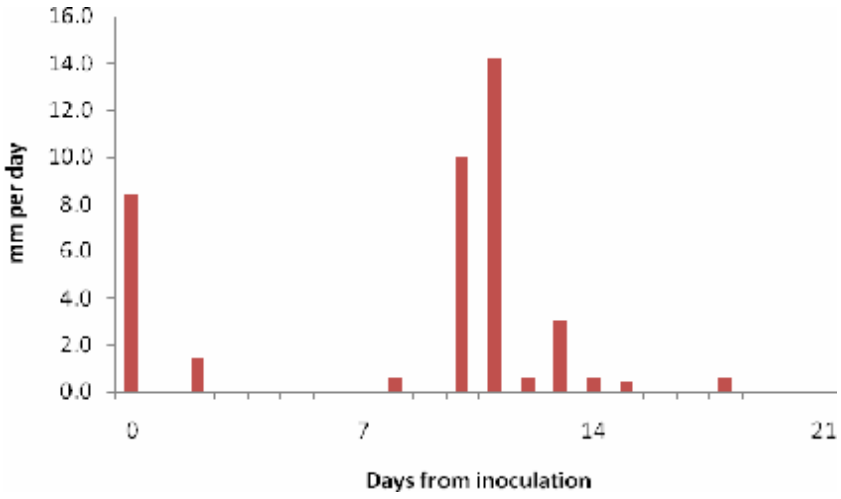
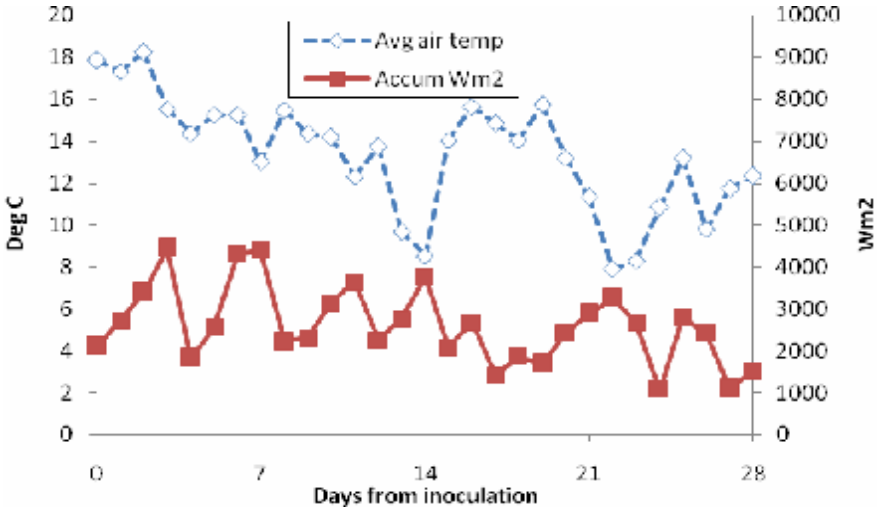


Figure II. Weather data measured at the experimental site after the inoculation of soils (day 0 = 5 September 2007): a) Daily average air temperature (20 cm) and accumulated sun light; b) Daily rainfall. Experiment 5, Year 2.

a.



b.