Activity: WT1 – Impact of liquid hog manure and irrigation management on broccoli safety: field experiment.

Objectives:
The aim of this study was to evaluate the prevalence of pathogenic and indicator microorganisms on broccoli fertilized with liquid hog manure or mineral fertilizer and irrigated 0, 1 or 2 times with contaminated water.

Actions completed
Field operations, sampling, and data analysis and interpretation are completed.

Methods
Experimental design
Twenty-four broccoli experimental plots were set on the Research and Development Institute for the Agri-Environment (IRDA) research farm located in St-Hyacinthe (Quebec) in 2011 and 2012. Plots were disposed in a split-plot factorial design including 6 treatments repeated 4 times. The fertilizer (mineral or liquid hog manure) was the main plot factor and the number of irrigations (0, 1 or 2) was the subplot factor. Treatments are presented in Table 1. Irrigation was done one day before the first broccoli sampling day in 2011 and in 2012 for treatments involving one irrigation. For treatments including 2 irrigations, they were done 17 days and one day before broccoli sampling in 2011, and 19 days and one day before broccoli sampling in 2012. Plots were 4 m long and 4 m wide, and contained 6 rows of Everest broccoli. Plants were set 30 cm apart in the row, and rows were 76 cm apart, for a total of 84 plants per plot. Replicates were set 8 m apart, and plots were 8 m apart.

Table 1. Treatments evaluated in broccoli experiment in 2011 and 2012.

Treatment	Number of irrigation applications	Fertilizer
1	0	Mineral fertilizer
2	1	Mineral fertilizer
3	2	Mineral fertilizer
4	0	Liquid hog manure
5	1	Liquid hog manure
6	2	Liquid hog manure

Soil was sampled before fertilization in order to apply adequate doses of liquid hog manure and mineral fertilizers. This sample was composed of fifteen subsamples (0-15 cm layer) taken in the whole field. Fertilizers application was based on soil analysis and plants requirements according to the "Centre de référence en agriculture et agroalimentaire du Québec" usually used by Quebec's agronomists and growers (CRAAQ, 2010). Soil and liquid hog manure physico-chemical analyses are presented in Tables 2 and 3, respectively. Urea (46-0-0), a balanced fertilizer (19-19-19) and potassium chloride (0-0-60) were used as source of mineral nitrogen (N), phosphorus (P_2O_5) and potassium (K_2O).

Table 2. Soil's physico-chemical properties of the experimental site in 2011 and 2012 [1]

	pH	Organic matter	P	K	Ca	Mg	Al	B	Cu	Fe	Mn	Zn	Na	texture
		(%)	Meh III (mg / kg)											
2011	6.38	0.98	107	81.6	751	55	531	0.149	1.06	19	13.2	2.93	3.7	sandy loam
2012	6.23	1.36	122	78.5	812	57	564	0.194	1.19	20	14.5	3.25	3.5	sandy loam

[1] samples were from the 0-15 cm surface layer.

Table 3. Physico-chemical properties of liquid hog manure spread in 2011 and 2012

	Total N	$N\text{-}NH_4$	P	K	Ca	Mg	Na
	mg/ kg						
2011	3486	2074	679	1633	705	306	685
2012	3342	1741	752	1342	897	342	693

Twelve plots (3 per replicates) were fertilized one week before broccoli planting with liquid hog manure and the others with mineral fertilizers applied at the same time. Liquid manure was pumped directly in the underground holding tank of a growing-finishing hog production. Doses of manure applied were based on P_2O_5 recommendations in order to avoid

phosphorus excess in environment. Quantities needed to provide 30 kg/ ha of P_2O_5 were 24 m^3/ha in 2011 and 22 m^3/ha in 2012. Nitrogen and potassium requirements not provided by manure were completed with mineral fertilizers. All fertilizers were soil-incorporated in the hour following spreading, paying a particular attention to avoid cross contamination between plots.

Irrigation water was taken from a stone quarry and transferred into two 10 000 litres tanks beside broccoli experimental site. For both seasons, liquid bovine manure from a dairy farm was added to tanks to contaminate water. In order to raise the *E. coli* levels in irrigation water without clogging the irrigation system, liquid hog manure was added to water in addition to bovine manure in 2012. This liquid hog manure came from a different farm from manure used as fertilizer. Liquid manures were used to contaminate water because it contains many strains of indicator microorganisms like *E. coli*, which have different survival capacity in the environment. Also, the ratio of pathogenic and indicator microorganisms populations is representative of a faecal contamination. Irrigation was performed using the Rain Jet system (Harnois industries) in order to spray-irrigate each plot individually. A water height of 25 mm was applied during each irrigation, with a flow of 4.1 US gallons per minute (gpm).

Sampling

Liquid manures used to contaminate irrigation water was sampled two days before irrigation to determine required volumes for water contamination. Bovine manure samples were taken in sterile 1-liter polypropylene bottles near the pump installed in the storage tank while mixing. Liquid hog manure samples were taken in sterile 1-liter polypropylene bottles directly in the underground holding tank.

Irrigation water samples were taken 3 times during irrigation in sterile 1-liter polypropylene bottles. While water was mixed in tanks using pumps, water samples were taken at the exit of the pump bringing water to plots.

Broccoli samples were taken 1, 3 and 5 days following the last irrigation, and will be referred as “sampling day 1”, “sampling day 3” and “sampling day 5” in this document. Each broccoli sample, consisting of three broccoli heads, was aseptically taken in each plot paying particular attention to leave external rows and a 50-cm buffer zone at the end of each row untouched to minimize edge effect. Disposable gloves and boots were changed before entering each plot. Broccolis were cut into one gram pieces in the laboratory and homogenized to form a composite sample. These composite samples were used for microbiological analyses.

Soil samples were taken in each plot on sampling day 5. Each soil sample consisted of ten homogenized 15 cm-depth subsamples taken from each plot to form composite samples. Subsamples were taken with an open ended soil probe. In order to reduce cross contamination between plots, probes were changed between each plot and new disposable plastic boots were also worn before entering a new plot.

All samples were kept at 4^{o}C until analyses which were performed less than 48 hours after sampling.

Microbiological analyses

Samples were analyzed to determine:

1- generic *Escherichia coli* counts in manure, broccoli and soil samples using Petrifilm *E. coli* / coliforms count plate according to manufacturer's instructions and Health Canada MFHPB-34 method. *E. coli* counts in water were determined by the membrane filtration method using the modified mTEC agar according to the “Centre d’expertise en analyse environnementale du Québec” (CEAEQ) MA.700-Ec-mTEC 1.0 protocol.

An enrichment procedure was also performed on broccoli and soil samples with the Colilert medium. Briefly, peptone water 0,1 % (225 ml) was added to 25 g of sample and incubated overnight at 35^{o} C. One millilitre of the culture was transferred in 9 ml of Colilert medium and tubes were bath-incubated 24 h at 35 ºC. Yellow and fluorescent tubes under a 6-watt, 365-nm UV light were considered as *E. coli*-positive. For each sample lot, negative (peptone water and colilert) and positive controls for *E. coli* (*E. coli* ATCC 25922) and total coliforms (*Klebsiella pneumoniae* ATCC 31488) were processed.

2- presence of *rfbE* gene (associated to *Escherichia coli* O157), and *stx1, stx2* and *eae* genes (associated to pathogenic *E. coli)* using PCR procedures as previously described (Wang G. et al. 2002, J. Clin Microbiol 40:3613-3619; Beaudry et al. 1996 J Clin Microbiol 34:144-148; Woodward et al. 1992 Vet Micro 31:251-261) and following EcL laboratory procedures (http://www.apzec.ca/en/Protocols#). This analysis was performed in EcL laboratory at the veterinary medicine faculty from Université de Montréal, in St-Hyacinthe (Quebec), by Dr John. M. Fairbrother’s team. Briefly, 25 g of sample were added to 225 ml of modified tryptic soy broth (mTSB) and incubated 24 h at 37 °C. After incubation, 1 ml of each enrichment was pelleted by centrifugation, washed twice with phosphate-buffered saline (PBS), resuspended in 0.5 ml of sterile deionized water and boiled for 10 minutes. The boiled cell suspensions were centrifuged and the resulting cell lysates were used as DNA template for PCR.

3- *Salmonella* spp. presence in manure, produce and soil samples using Health Canada MFLP-75 method and CEAEQ MA.700-Sal-PA 1.0 method for irrigation water samples. Briefly, an enrichment procedure was performed by adding 25 g of samples in 225 ml of tryptic soy broth (TSB) that was incubated at 35 °C for 18-24 h. For the irrigation water samples, 6 volumes of 50 or 100 ml, according to the turbidity of the samples, were filtrated, and then the membranes were placed in 75 ml of TSB. Following this pre-enrichment step, 6 drops of 30 µl of all TSB were transferred on a Modified Semi-solid Rappaport-Vassiliadis (MSRV) plate agar; and incubated 24h at 42°C. If no growth was observed after 24h, incubation was pursued up to 72h. When the migration zone was higher than 2 cm, result was presumed to be positive. For all positive samples, inoculation of a Brilliance Salmonella chromogenic agar plate was performed an incubated 24h at 35°C. After incubation, purple colonies were considered as *Salmonella* spp. All the positive strains were confirmed by further biochemical testing, using the RapID ONE system and the serotype analysis of the sample with a polyvalent serum, *Salmonella* O Antiserum Poly A-1& vi. Positive samples were sent to the Laboratory for Foodborne Zoonoses of the Public Health Agency of Canada (PHAC) in Guelph (Ontario) for serotyping.

4- *Listeria monocytogenes* presence using Health Canada MFHPB-30 slightly modified method. Briefly, 25 g of samples were aseptically transferred into 250 ml of UVM Modified *Listeria* Enrichment Broth (UVM) and incubated for 24 h at 30 °C. For irrigation water samples, 6 volumes of 50 or 100 ml of water, according to the turbidity, were filtrated and membranes were placed into 50 ml of UVM. After 24 h, 200 µl of all UVM enrichment broths were transferred into 10 ml of Fraser Broth and incubated for 24-48 h at 37 °C. Fraser selective enrichment was streaked onto Rapid 'L mono (RLM) plates when positive (brown-black color). RLM plates were incubated for 24-48 h at 37 °C. For each sample, *L. monocytogenes* presumptive blue without a yellow halo colonies were isolated and their identities were confirmed by PCR and PFGE procedures. PCR serovar identification procedure was performed in the laboratory of Dr Philippe Fravalo at the veterinary medicine faculty from Université de Montréal, in St-Hyacinthe (Quebec). PFGE pulsovars were determined in the « Laboratoire de santé publique du Québec » (LSPQ) in Quebec city.

For each analytical method, negative, positive and matrix controls were properly performed at each sample lot.

Statistical analysis

A generalized linear mixed model using SAS GLIMMIX procedure was performed to evaluate the impact of the treatments on *E. coli* prevalence. This model takes into account the Poisson distribution of the observations and the random effects of years, plots, subplots and subsamples within subplots, as described in Littell *et al.* (2006). Fixed effects of irrigation, date, fertilizer and interaction irrigation×fertilization were tested for significance with F tests and treatment mean counts were calculated and compared using contrasts.

Results

Liquid hog manure used for spreading

Generic *E. coli* content in liquid hog manure was 370 000 colony-forming units per gram (CFU/g) and 150 000 CFU/g in 2011 and 2012, respectively. *Salmonella* spp. was detected in liquid hog manure analyzed in 2011, but not in 2012. The serovar detected for the 2011's isolate was Typhimurium, phagotype 104 (antigens 4,5:i:1,2). No *Listeria monocytogenes* were detected in liquid hog manure samples. *rfbE, stx1, stx2* and *eae* genes were not found in 2011 and in 2012.

Liquid manures used for water contamination and irrigation water

Generic *E. coli* contents in manures used for water contamination and irrigation water samples are shown in Table 4. In 2011, *Salmonella* spp. was found in bovine manure used for irrigation 2, as well as in irrigation water from this irrigation. However, genetic characterization results showed different serovars. *Salmonella* Schwarzengrund (antigens 4:d:1,7) was detected in bovine manure, and *Salmonella* Derby (antigens 4:f,g:-) was found in irrigation water. Two hypotheses can explain the difference. First, manures can harbour many serovars that were not all recovered during analysis. Also, water from the stone quarry could have been contaminated by environmental sources and wildlife. Analysis of water before contamination showed generic *E. coli* counts under 10 CFU/ 100 ml. *L. monocytogenes* and *rfbE, stx1, stx2* and *eae* genes were not detected in manures and irrigation water sampled in 2011.

In 2012, *Salmonella* spp. and *rfbE, stx1, stx2* and *eae* genes were not detected in manures and irrigation water. *Listeria monocytogenes* was found in bovine manure from both irrigations, and irrigation water from irrigation 2. *Listeria* serovars are presented in Table 5. All detected pulsovars could be matched with previously described isolates recovered in human and food samples.

Table 4. Generic *E. coli* content in liquid manures used to contaminate water and irrigation water in 2011 and 2012.

	2011		2012	
	Irrigation 1	**Irrigation 2**	**Irrigation 1**	**Irrigation 2**
Liquid bovine manure (CFU/g)	8 875	3 125	67 000	83 000
Liquid hog manure (CFU/g)	not used	not used	5 300	78 000
Irrigation water (CFU/100 ml)	393	538	3 000	2 987

Table 5. *Listeria monocytogenes* serovars and pulsovars in 2012.

Isolate origin	Serovar	PFGE Pulsovar
Liquid bovine manure (both irrigations)	IVb	196
Irrigation water (irrigation 2)	IIa	188

Broccoli

All broccoli samples were under the method detection limit (10 CFU/ g) for generic *E. coli* (n = 144), even in 2012 where *E. coli* levels in irrigation water reached 3000 CFU/100 ml.

Enrichment procedure revealed an *E. coli* prevalence of 15 % and 51 % on broccoli samples taken in 2011 and 2012, respectively. Higher *E. coli* content in irrigation water applied in 2012 may explain difference between seasons. The impact of fertilizer, number of irrigations and sampling dates on *E. coli* prevalence in broccoli are shown in Table 6. Seasons 2011 and 2012 were statistically analyzed separately because *E. coli* contents in irrigations water were different.

Table 6. Impact of fertilizer, number of irrigations and sampling day on *E. coli* prevalence in broccoli in 2011 and 2012.

Parameter	Year			
	2011		2012	
	D.F.[1]	Pr>F	D.F.	Pr>F
Fertilizer	1	0.1818	1	0.2797
Number of irrigations	2	0.8909	2	**0.0499**
Sampling day	2	**0.0236**	2	0.2124

[1] degrees of freedom.

In 2011, the fertilizers (*P*= 0.1818) and the number of irrigation (*P*= 0.8909) showed no statistically significant impact on *E. coli* prevalence in broccoli. However, the sampling day had a significant impact on *E. coli* prevalence on broccoli (*P*= 0.0236). In fact, a fast decline of *E. coli* prevalence was observed on broccoli and reach 0 % 5 days after irrigation (Figure 1). Contrasts revealed a statistically significant difference between sampling day 1 and sampling day 3 (*P*= 0.0064). No pathogenic bacteria were recovered in broccoli in 2011.

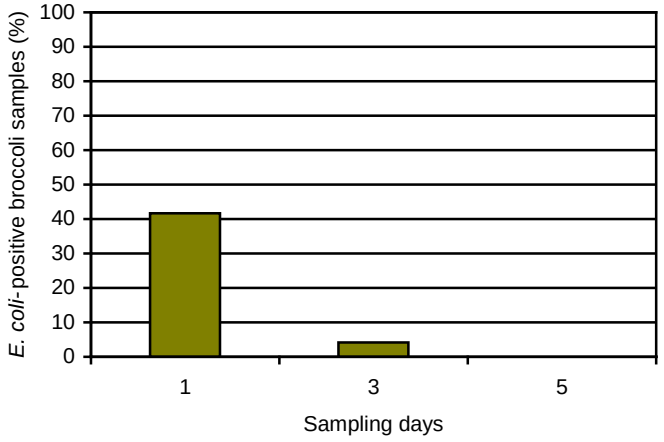

Figure 1. *E. coli* prevalence in broccoli samples according to sampling day in 2011.

In 2012, the number of irrigations had a statistically significant impact on *E. coli* prevalence (*P*= 0.0499) (Figure 2). The high *E .coli* content in irrigation water may explain this observation. The risk to recover *E. coli* on broccoli was 5.4-fold higher in plots that received 2 irrigations compared to no irrigation (*P*=0.0195). The risk was 3.7-fold higher in broccolis irrigated once instead of not irrigated (*P*=0.0611).

No *Salmonella* spp. and *rfbE, stx1, stx2* and *eae* genes were detected in broccoli samples in 2012. One broccoli sample was found positive to *Listeria monocytogenes* in 2012 (serovar IIa and PFGE pulsovar 394). Genetic characterization made by the LSPQ showed a new profile that could not be associated to previously described food or human isolates. This sample was taken on sampling day 1 in a manure-fertilized plot which received one irrigation. Irrigation water for this irrigation had also been found positive to this microorganism, but a different pulsovar was observed (serovar IIa, pulsovar 188). Therefore, no link could be established between irrigation and broccoli contamination.

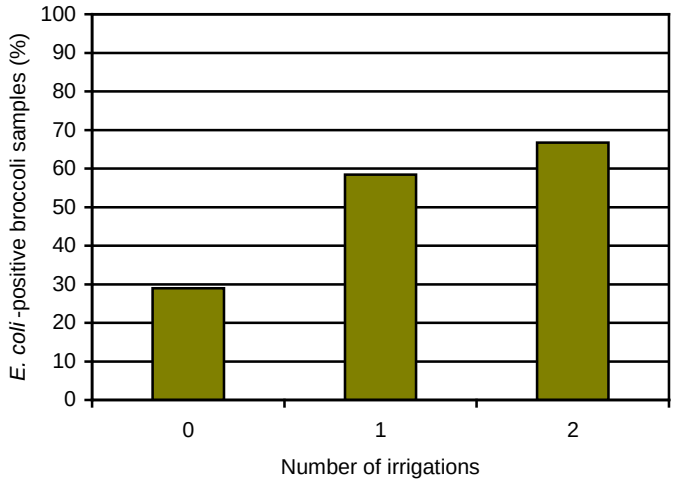

Figure 2. *E. coli* prevalence in broccoli samples according to the number of irrigations in 2012.

Soil

In 2011, *E. coli* counts could be determined in one soil sample (10 CFU / g) taken in a mineral-fertilized plot that received one irrigation. Enrichment procedure revealed a 100 % of *E. coli*-prevalence of in manure-fertilized soil samples and 75 % in mineral-fertilized plots (n=24). No link could be established between bacterial prevalence in soil samples and the number of irrigations.

In 2012, 10 samples presented countable *E. coli* populations. The higher bacterial content in 2012 irrigation water could explain this observation. Nine samples were from manure-fertilized plots that received no (3 samples), one (3 samples) or 2 irrigations (3 samples). The other positive sample came from a mineral-fertilized plot that received one irrigation. Statistical analysis was performed only on data in 2012, because the number of positive samples was too low in 2011. Statistically significant difference was observed between fertilizer treatments using enumeration procedure (*P*=0.0006). Average *E. coli* counts in soil samples taken in manure-fertilized plots (36.07 CFU/g) were statistically different from those in plots that have received mineral fertilizers (1.02 CFU/g). Enrichment procedure revealed a high *E. coli*-prevalence in soil samples that were from both manure-fertilized (100 % prevalent) and mineral-fertilized (92 %) plots (n=24). No link could be established between bacterial prevalence in soil samples and the number of irrigations.

Six soil samples presented positive results for *L. monocytogenes.* All samples came from plots that received 2 irrigations and presented the same genetic characterization than isolates recovered from irrigation water, i.e. serogroup IIa and PFGE pulsovar 188. This confirms that *Listeria monocytogenes* recovered in soil was from irrigation water. No *Salmonella* and *rfbE, stx1, stx2* and *eae* genes were detected in soil samples in 2011 and 2012.

Summary:

Experimental plots were set in order to evaluate the prevalence of pathogenic and indicator microorganisms on broccoli fertilized with liquid hog manure or mineral fertilizers and irrigated 0, 1 or 2 times with contaminated water. In 2011, results showed that *E. coli* prevalence in broccoli was affected by the sampling date (*P*= 0.0236), with a statistically significant difference between day 1 and day 3 following the last irrigation (*P*=0.0064). In 2012, the number of irrigations had a statistically significant impact on *E. coli* prevalence in broccoli (*P*=0.00499). In 2012, *E. coli* counts in soil were statistically influenced by the type of fertilizer (*P*= 0.0006). *Listeria monocytogenes* was recovered in 1 broccoli sample, but no link

could be established between the isolate recovered in broccoli to isolates in manure and irrigation water. *L. monocytogenes* serovar IIa PFGE pulsovar 188 was found in 6 soil samples and could be linked to irrigation water applied in all plots 5 days before these samples were taken. This study emphasizes the link between *E. coli* levels in irrigation water and the number of irrigations or the delay between irrigation and harvest on produce contamination. It also demonstrates that irrigation water may introduce *L. monocytogenes* in soil.