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Use of *Enterococcus*, BST and Sterols for Poultry Pollution Source Tracking in Surface and Groundwater

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1. Introduction

Maintaining and preserving the quality of surface and ground waters involves many challenges, one of the most serious being bacteriological contamination caused by discharge of human and animal waste. Water resources may become contaminated with pathogens from human or animal feces as a result of malfunctioning wastewater operations (treatment plants or septic systems), stormwater or combined sewer overflows, poor management practices for storing or land-applying livestock manure, and defecation by livestock and wildlife in or near surface waters. Pollution source identification is crucial in order to improve best management practices and eliminate consequent health risks to the general public and aquatic ecosystems. Distinguishing between human and animal sources of fecal pollution in water has been a subject of many studies (Tyagi et al., 2009a). Microbial source tracking methods have employed a wide range of micro-organisms (e.g., fecal coliforms, total coliforms, bifidobacteria, *E. coli*, enterococci) for identifying sources of water pollution, but each has certain limitations (Tyagi et al., 2009a). Moreover, many microbes are not host-specific, making them ineffective for source identification. Chemical methods for fecal source tracking include analysis of sterols, bile acids, caffeine, whitening agents etc., with sterols being the most widely used indicator compound (Bull et al., 2002; Saim et al., 2009; Tyagi et al., 2009b). Both classes of methods have been somewhat successful in identifying pollution sources but not fully evaluated and accepted as established methods in environmental studies.

Enterococci are the second most studied group of bacteria in the field of microbial source tracking (following *E. coli*) due to their connection to humans and animals as well as their

recent significance as a clinical pathogen (Figueras et al., 2000; Aarestrup et al., 2002; Scott et al., 2005) and ability to persist in the environment (Harwood et al., 2000). Different strains of enterococci populate the digestive tracts of humans and animals, making them a good indicator of water contamination. A metabolic fingerprint database developed by Ahmed et al., (2005) for enterococci was able to distinguish between human and animal sources despite the fact that a number of biochemical phenotypes were found in multiple host groups. Bacterial Source Tracking (BST) uses unique genetic markers in *Bacteroides* (naturally occurring bacteria in the intestinal flora) to identify organisms responsible for fecal pollution in aquatic environments, and has been used for detection of bacteriological contamination from different sources such as humans, ruminant animals, dogs, pigs, horses, and elk (Bernhard et al., 2000a,b; Dick et al., 2005a,b). Unfortunately, primers for poultry and other birds are not available.

Sterols are organic molecules, a family of compounds that occur naturally in animals, plants and fungi. They have a steroid ring structure and varying functional groups that confer specific characteristics (such as polarity, bioactivity and lipophilicity) to the molecule. Coprostanol, the major compound in human faeces and a product of the microbial reduction of cholesterol in the higher animal guts, has been considered an indicator of faecal pollution. Other cholesterol congeners (campesterol, sitosterol and stigmasterol) are also degraded by bacteria in the intestinal tract of higher mammals to stanols. Higher concentrations of coprostanol have been found in human sewage than in animal wastewater and concentrations of stigmastanol and epicoprostanol were usually higher in animal (cows, pigs, and poultry) than in human wastewater (Blanch et al., 2004). Sterol analysis, a widely used chemical method for identifying fecal pollution sources is based on the fact that different sterol compounds are associated with human or animal waste and their presence/absence and relative concentrations and ratios can be used as an indication of the origin of water contamination (Chou & Liu, 2004; Devane et al., 2006; Gilpin et al., 2003; Bull et al., 2002; Jardé et al., 2007a, b; Saim et al., 2009).

Interpretation of findings from these and other markers can be improved by the application of chemometric techniques which are gaining ground in evaluation of environmental data (Brodnjak-Voncina et al., 2002; Mendiguchía et al., 2004; Singh et al., 2005; Terrado et al., 2011). The most common chemometric methods are cluster analysis (CA) and principal component analysis (PCA) with factor analysis (FA). The goal of CA is to identify groups of objects (such as sampling sites) that give similar, homogenous results with respect to extent or type of fecal pollution, whereas PCA enables a reduction in data and description of a given multidimensional system by a smaller number of new variables (Loska & Wiechula, 2003). Pollution sources and dischargers can also be identified using PCA (Einax et al., 1998; Loska & Wiechula, 2003).

The Fraser River valley of British Columbia is considered the poultry capital of Canada. The poultry waste generated from the industry is used as fertilizer and spread onto the fields, thus creating a non-point source run-off type of surface and ground water pollution. The objective of this study was to determine the extent and sources of fecal contamination in surface and ground water in this poultry dominated agricultural area. In particular, we tested *Enterococcus* isolates as source tracking indicators for poultry in combination with chemical indicators sterols, BST and chemometric analysis.

2. Materials and methods

2.1 Sample collection

Surface water was collected from 12 sites and ground water was sampled at 28 sites in the Abbotsford area of British Columbia, Canada, near poultry farms and berry farms that use poultry litter as fertilizer (Figure 1). Surface water was sampled for bacteriological water quality and for sterol analysis in December 2009; all samples were grab water collections. Ground water was sampled in April, August and December 2009 for microbial water quality and sterols. At each site, three full well volumes were pumped out of the piezometer (purged) using a submersive Hydrofit pump prior to sampling with low density polyethalene (LDPE) tubing (dedicated for each well) located close to the well screen. A minimum of three line volumes were purged from the sample tubing prior to sample collection.

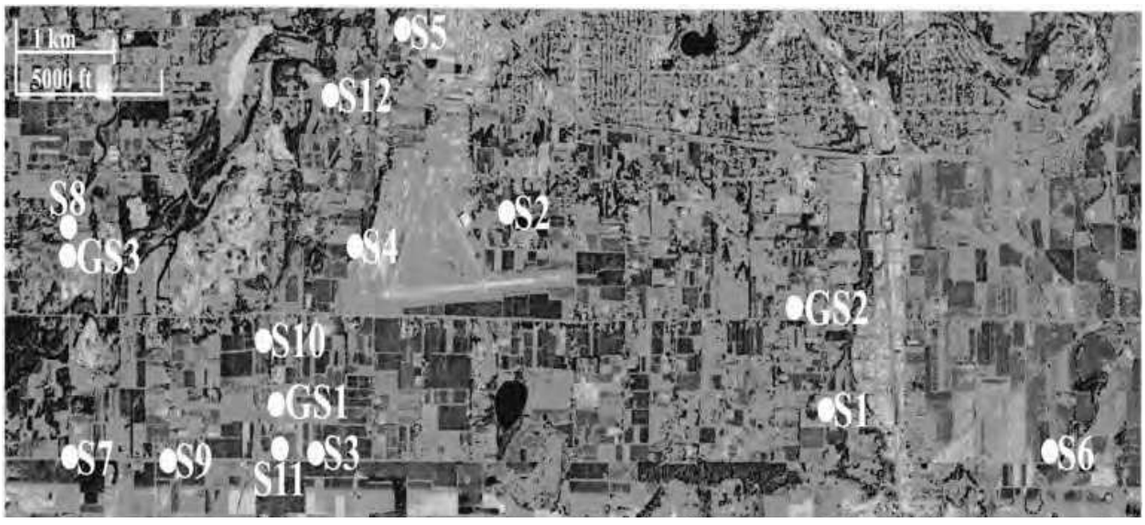

Fig. 1. Aerial map of the sampling area located in the Lower Fraser Valley region of British Columbia, Canada. "S" and "GS" indicate surface water and groundwater sampling sites, respectively. The map was generated using Google Maps

Water samples were collected in: (1) 250-mL sterile polypropylene bottles for bacteriological analyses (total and fecal coliform, *E. coli* and *Enterococcus*); (2) certified clean one liter amber glass bottles for sterol analysis and (3) one liter sterile polypropylene bottles for BST analysis. Samples were placed on ice packs in coolers (~ 4°C) and shipped to the laboratory where they were kept in a cold-room (≤4°C) until analyzed. Samples for BST analysis were filtered within 24 h of collection.

Litter samples were collected from two different poultry farms: a broiler farm and a layer farm, in at least three different locations in the poultry barn. Samples from broiler barns (total of 4) were collected at the beginning (day 3) and end of the production cycle (day 35). Samples were collected by an analyst wearing gloves and using a sterile scoop, and placed into sterile falcon tubes. The samples were kept on ice until analysis, which was performed within 24 h of collection (samples from broiler farm). Samples from layer farms were collected once, frozen after collection and analyzed at a later date.

2.2 Bacteriological analysis

Analysis of enterococci in water samples was performed using a membrane filtration technique whereby samples retained on filter paper were incubated on mE agar for 48 h at 41°C followed by incubation on Esculin Iron Agar (EIA) for 20 minutes at 41°C (USEPA, 2000). Colonies that appeared pink to red with dark precipitation on EIA were verified using Biolog Microbial ID system in combination with Biolog Gram Positive Aerobic Bacteria Database (Release 6.01, Biolog, Hayward, CA) Results are reported as colony-forming units (cfu) per unit volume.

For enterococci in poultry litter samples, 5-6 g of litter was weighed into 10-ml of 0.85% sterile saline in a sterile 50-mL falcon tube. The tube was vortexed on high for one minute and serial dilutions were plated on KF streptococcal agar (Difco, Detroit, MI). Red or pink colonies on the KF agar were verified using Biolog Microbial ID system in combination with Biolog Gram Positive Aerobic Bacteria Database (Release 6.01, Biolog). Isolated colonies of confirmed *Enterococcus* were inoculated into 5 ml of tryptic soy broth containing 6.5% NaCl and incubated for 5 – 12 hours at 35°C; one milliliter of this culture was then combined with 325 µL 80% glycerol (20% glycerol final concentration) and stored at -40°C until further analysis. Confirmed *Enterococcus* isolates were identified to species level using multiplex PCR (Jackson et al., 2004).

Total and fecal coliform and *E coli* analyses of water samples were performed using procedures based on "British Columbia Environmental Laboratory Manual for the Analysis of Water, Wastewater, Sediment and Biological Materials" (2005 Edition) (Horvath, 2009).

2.3 Sterol analysis

Analytical grade standards were purchased from Sigma–Aldrich (Oakville, ON) for 17 compounds (mestranol, norethindrone, equol, estrone, equilin, norgestrel, 17 α-ethinylestradiol, 17 α-estradiol, 17 β-estradiol, estriol, coprostanol, epicoprostanol (cholestanol), cholesterol, desmosterol, campesterol, stigmasterol and β-sitosterol); equol was purchased from Fluka (Oakville, ON). Primary standards were made in acetone at a concentration of 1 mg/ml and stored at -20°C. Acetylated mixture calibration standards of 0.02 to 0.5 µg/L were made every two months and stored at -20°C. Surrogate 17 β-estradiol-d3 and internal standard p-terphenyl-d14 were added to every sample. Solvents, sodium chloride and potassium carbonate were purchased from VWR (Edmonton, AL) and all chemical reagents were of analytical grade.

Sterol extraction and detection were conducted according to the sterol method used at the Pacific Environmental Science Centre, North Vancouver BC, Canada (Environment Canada, 2005). Briefly, 800 mL of unfiltered sample was acidified with sulfuric acid to pH ~ 3 and surrogate β-estradiol-d_3 was added. After stirring samples with 100 ml of dichloromethane for two hours, they were transferred into separatory funnels and the organic layers separated. Samples were then concentrated and derivatized with pyridine/acetic acid and re-extracted with petroleum ether in the presence of 10% potassium carbonate solution. The organic layers were concentrated to near dryness and reconstituted in 200 µl of internal standard (p-terphenyl-d_{14}).

Extracted samples for sterol analysis were injected into Agilent 5973 MS system (injector 280°C), carried by helium flow of 1.2 mL/min, separated on Rtx-5ms column (30 m x 0.25

mm x 0.25 µm film thickness) by the following temperature gradient: initial temperature 70˚C hold for 1 min, 30˚C/min to 180˚C, 5˚C/min to 310˚C and hold on 310˚C for 4 min. Eluting compounds were analyzed by mass spectrometer and ChemStation software (revision A.01.01, Palo Alto, CA) and sterols quantitated using internal standard method. List of sterols and their limits of quantification are presented in Table 1. Quality control blanks and spikes were run with each batch of samples. Various sterol ratios were calculated to determine the presence of fecal contamination and its likely source (Table 2).

2.4 Bacterial Source Tracking (BST)

BST analysis was conducted according to the BST method used at the Pacific Environmental Science Centre, North Vancouver BC, Canada (Environment Canada, 2006). One liter water samples were filtered through AP15 prefilters (Millipore Corporation, Billerica, MA) to remove large pieces of material. Prefiltrate was split into two aliquots (500 ml each), which were then filtered through 0.22 µm filters (Supor-200, PALL Corporation, Ann Arbour, MI). The filters were stored individually in 15 mL tubes containing 0.5 mL of GITC lysis buffer (5M guanidine isothiocyanate, 100 mM EDTA and 0.5% sarkosyl) at -20°C.

Sterol	Common names	Formula	LOQ (µg/L)
24*α*-Methyl-5-cholesten-3*β*-ol	Campesterol	$C_{28}H_{48}O$	0.005
Cholest-5-en-3 *β*-ol	Cholesterol	$C_{27}H_{46}O$	0.009
5*β*-Cholestan-3*β*-ol	Coprostanol	$C_{27}H_{48}O$	0.005
3*β*-cholesta-5,24-dien-3-ol	Desmosterol	$C_{27}H_{44}O$	0.008
3-*β*-5-*β*-cholestan-3-ol,	Dihydrocholesterol (cholestanol)	$C_{27}H_{48}O$	0.007
Cholest-5-en-3 *α*-ol	Epicoprostanol	$C_{27}H_{48}O$	0.005
1,3,5,7-Estratetraen-3-ol-17-one	Equilin	$C_{18}H_{20}O_2$	0.07
3,4-Dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol	Equol	$C_{15}H_{15}O_3$	0.1
	17α-Estradiol	$C_{18}H_{24}0_2$	0.01
	17β-Estradiol	$C_{18}H_{24}0_2$	0.01
1,3,5(10)-Estratriene-3,16α,17β-triol	Estriol	$C_{18}H_{24}O_3$	0.01
3-Hydroxyestra-1,3,5(10)-trien-17-one	Estrone	$C_{18}H_{22}O_2$	0.02
19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol	17α-Ethinylestradiol	C_{20}-H_{24}-O_2	0.1
17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol 3-methyl ether	Mestranol	$C_{21}H_{26}O_2$	0.01
13β-Ethyl-17α-ethynyl-17β-hydroxygon-4-en-3-one	Norgestrel	$C_{21}H_{28}O_2$	0.07
19-nor-17alpha-ethynyl-17beta-hydroxy-4-androsten-3-one	Norethindrone	$C_{20}H_{26}O_2$	0.08
5-Stigmasten-3β-ol	β-Sitosterol	$C_{29}H_{50}O$	0.007
24-Ethylcolesta-5,22E-dien-3*β*-ol	Stigmasterol	$C_{29}H_{48}O$	0.007

Table 1. Sterols and limits of quantification (LOQ)

For each sample, DNA was extracted from one AP15 pre-filter and one 0.22 µm filter. DNA extraction was performed with the Qiagen DNeasy kit (Mississauga, ON), and the manufacturer's instructions were followed with the following exception: for the first steps,

Buffer AL (provided with the kit) and 100% ethanol (Commercial Alcohols, Langley, BC) were added to the 15 ml tubes (containing filters and GITC buffer) in 1:1:1 ratios with 1 minute of vortexing after each addition of a liquid.

Ratio #	Sterol Compound Ratio	Human Fecal Contamination (a)			Literature Reference
		Yes	Unsure	No	
1	Coprostanol / (Coprostanol + Cholestanol) 5β/(5β+5α)	> 0.7	0.3 - 0.7	< 0.3	Devane 2006; Bull 2002; Fattore 1996; Chan 1998; Grimalt 1990; Carreira 2004; Froehner 2009; Marvin 2001; Patton 1999; Reeves 2005; Zhang 2008; de Castro Martin 2007
2	(Coprostanol + Epicoprostanol) / (Coprostanol + Epicoprostanol + Cholestanol)	> 0.7	0.3 - 0.7	< 0.3	Bull 2002; Reeves 2005
3	Epicoprostanol / Coprostanol	< 0.2	0.2 - 0.8	> 0.8	Froehner 2009; de Castro Martin 2007
4	Coprostanol / Cholesterol	> 0.5	-	< 0.5	Gilpin 2003; Fattore 1996; Carreira 2004; Patton 1999; Reeves 2005; Zhang 2008
5	Coprostanol / Cholestanol	> 0.5 >0.4	0.3 - 0.5	< 0.3	Devane 2006; Roser 2006 Shah 2007
6	Coprostanol / (Cholestanol + Cholesterol)	> 0.2	0.15 - 0.2	< 0.15	Chan 1998
7	Coprostanol / Epicoprostanol	> 1.5	-	< 1.5	Fattore 1996; Marvin 2001; Patton 1999; Reeves 2005; Zhang 2008

#	Ratio	Ratios for Differentiating Sources of Fecal Contamination (b) Value*	Source	
8	(Coprostanol + Epicoprostanol) / Cholesterol	>3.7 <0.7	pig chicken and/or cow	Jardé 2007
9	(Campesterol + Sitosterol) / Cholesterol	>1.5 <1	pig/chicken/cow human	Jardé 2007
10	Epicoprostanol / (Coprostanol + Cholestanol)	<0.01 >0.1	human cattle/horse/deer	Standley 2005

Table 2. Sterol ratios for identifying (a) human fecal contamination and (b) differentiating sources of fecal contamination

Three aliquots (600 µl each) were loaded onto the DNeasy columns and washed according to the manufacturer's protocol. The pure genomic DNA samples were stored at -20°C in sterile 1.5 ml tubes (Fisher Scientific, Ottawa, ON). Extracted DNA was amplified by Polymerase Chain Reaction (PCR) carried out with a DNA engine Tetrad 2 (Bio-Rad Laboratories Canada, Toronto, ON). The samples were tested with all *Bacteroides* primers available (Table 3), which identify feces from humans, ruminant animals, pigs, horses, dogs, elk, and general *Bacteroides*. After agarose gel electrophoresis of PCR samples, gels were visualized and scored in a bio-imaging system (Gene Genius, Fisher Scientific, Ottawa, ON) and the

program GeneSnap was used to capture the image from a CCD camera. Positive matches were made by correlating the bands with the DNA ladder and the known size of the positive bands as published (Bernhard et al., 2000a,b, Dick et al., 2005a,b). All negative controls (included at every stage) were blank and all positive controls worked appropriately.

Organism	Primer Set	reference
Human	HF134F / HF654R	Bernhard et al., 2000b
	HF183F / Bac708R	Bernhard et al., 2000b
Ruminant Animal	CF128F / Bac708R	Bernhard et al., 2000b
	CF193F / Bac708R	Bernhard et al., 2000b
Pig	PF134F / Bac708R	Donation from K. Field
	PF163F / Bac708R	Dick et al., 2005b
Horse	HoF597F / Bac708R	Dick et al., 2005b
Dog	DF475F / Bac708R	Dick at al., 2005a
Elk	EF447F / EF990R	Dick at al., 2005a
General *Bacteroides*	Bac32F / Bac708R	Bernhard et al., 2000a

Table 3. Bacterial Source Tracking (BST) primers

2.5 Chemometric approach

The goal of the chemometrics approach is to display the most significant patterns in the complex data sets. The most popular statistical methods are principal component analysis (PCA) which provides information on the most meaningful parameters to describe a large data set and cluster analysis (CA) which identifies natural groupings within a data set. Sterol data were used in both PCA and CA; the statistical analyses were performed by XLStat2009 statistical program.

Principal component analysis (PCA) generated principal components (PCs). Varimax rotation was applied on the PCs with eigenvalues greater than 1 (Kim & Mueller, 1987) in order to obtain new groups of variables called varimax factors (VFs) that better interpret the data set (Juahir et al., 2009). Cluster analysis applied on surface water samples data identified similarities in the sterol composition and grouped sampling sites accordingly.

3. Results

3.1 Bacterial contamination

Total coliform, fecal coliform and *E. coli* in surface water ranged from 100-17,000 cfu/100 mL, <1-700 cfu/100 ml and <1-690 cfu/100 mL, respectively (Table 4). Several groundwater locations also tested positive for total coliform ranging from 25-17000 cfu/100 ml, although the majority of groundwater samples showed no evidence of total coliform contamination (Table 5). Only one location, BC-008, consistently tested positive for total coliform.

Enterococcus was detected in all surface water samples and at 3 groundwater sites. *Enterococcus* counts ranged from 2 to 2100 cfu/100 mL for surface water samples (Table 4) and 1 to 5 cfu/100 ml for groundwater samples (Table 5). Seven enterococci were isolated

from groundwater, 85 were isolated from surface water samples and 163 were isolated from poultry litter, for a total of 255 isolates. In the August 2009 sampling, the groundwater site 94-SH-29 had one presumptive *Enterococcus* isolate but the confirmation test did not verify this result. Previous samplings of the 94-SH-26 site and 94-SH-29 site in November 2008 (data not presented) had similar issues, yielding 49 and 74 presumptive enterococci respectively, but none of the presumptive results were confirmed.

Sample location	Total Coliforms	Fecal Coliforms	E. coli	Enterococcus
S1	2700	26	7	9
S2	500	5	4	6
S3	17000	<1	<1	12
S4.	800	95	95	10
S5	560	2	1	2
S6	2500	56	22	52
S7	100	7	6	29
S8	2500	700	690	2100
S9	3200	2	2	71
S10	1300	13	6	2
S11	1200	6	6	7
S12	2800	5	5	11

Table 4. Bacterial counts for surface water sites

	Apr-09		Aug-09		Dec-09	
Field ID	Total Coliforms	*Enterococcus*	Total Coliforms	*Enterococcus*	Total Coliforms	*Enterococcus*
94Q14	<1	<1	2	<1	n/s*	n/s
94Q20	<1	<1	1	<1	n/s	n/s
94Q27	<1	<1	<1	<1	n/s	n/s
PC25	<1	<1	<1	<1	n/s	n/s
PC35	<1	<1	2	<1	n/s	n/s
PC55	<1	<1	440	<1	<1	<1
PC75	<1	<1	<1	<1	n/s	n/s
PB55	<1	<1	2	<1	n/s	n/s
PB75	<1	<1	<1	<1	n/s	n/s
91-11	<1	<1	240	1	n/s	n/s
91-12	<1	<1	30	<1	n/s	n/s
91-15	n/s	n/s	n/s	n/s	<1	<1
ABB5	<1	<1	390	<1	n/s	n/s
ABB3	60	<1	4	<1	n/s	n/s
ABB-10	n/s	n/s	n/s	n/s	8	<1
ABB-06	n/s	n/s	n/s	n/s	1500	<1
ABB-02	n/s	n/s	n/s	n/s	3	<1
91-3	7	<1	230	<1	n/s	n/s
91-1	<1	<1	<1	<1	<1	<1
94-SH-26	1400	<1	<1	<1	22	<1
94-SH-29	3100	<1	<1	1**	2200	<1
BC-008	25	<1	17000	5	4400, 4800	<1
BC349	<1	<1	5	<1	1	<1
US-04	n/s	n/s	n/s	n/s	1	<1
US-02	n/s	n/s	n/s	n/s	10	1
FT7-22	n/s	n/s	n/s	n/s	2500	<1
FT5-12	n/s	n/s	n/s	n/s	1	<1
FT1-24	n/s	n/s	n/s	n/s	14	<1

Table 5. Bacterial counts for groundwater (colony counts in cfu/100 mL).*n/s-not sampled, **not confirmed as *Enterococcus*

3.2 Identification of enterococci

From speciation analysis, *E. faecalis* accounted for the largest portion of the environmental isolates at 26.6% (n=25), but *E. faecium* was a close second at 24.5% (n=23). *E. mundtii* (n=12), *E. durans* (n=9), *E. casseliflavus* (n=8), *E. hirae* (n=4), *E. gallinarum* (n=2) and *E. raffinosus* (n=1) were also present. All 29 isolates from the litter samples from the layers farm were *E. faecium*. Broiler barns were tested for *Enterococcus* on Day 3 (close to beginning of production cycle) and Day 35 (end of the production cycle). Samples collected on Day 3 contained a fairly even mix of *E. gallinarum* (27.6%; n=29), *E. faecalis* (26.7%; n=28), *E. faecium* (16.2%; n=17) and *E. hirae* (25.7%; n=27) as well as smaller numbers of *E. cassiflavus* (n=1), *E. durans* (n=1), and two isolates that reacted positively with the *Enterococcus* genus primers but not with any of the species primers. For Day 35 samples, *E. faecium* was the dominant species at 72.4% (n=21) followed by *E. faecalis* at 20.7% (n=6), *E. durans* at 3.5% (n=1), and *E. hirae* at 3.5% (n=1).

3.3 Sterols in surface and groundwater

Surface water samples were tested for a total of 18 sterols (Table 1) but only 8 sterols were detected (Figure 2). The fecal sterols cholesterol, dihydrocholesterol (cholestanol) and desmosterol were detected in all 12 sampling sites ranging from 0.275-7.710 µg/L, 0.022-1.040 µg/L and 0.031-1.119 µg/L, respectively. Coprostanol was detected in all but two sites and ranged from 0.006-0.086 µg/L. Epicoprostanol was detected in six sampling sites ranging from 0.005-0.048 µg/L. The plant sterols campesterol, stigmasterol and β-sitosterol were detected in all 12 sampling sites ranging from 0.044~1.692 µg/L, 0.072~2.928 µg/L and 0.361-10.072 µg/L, respectively.

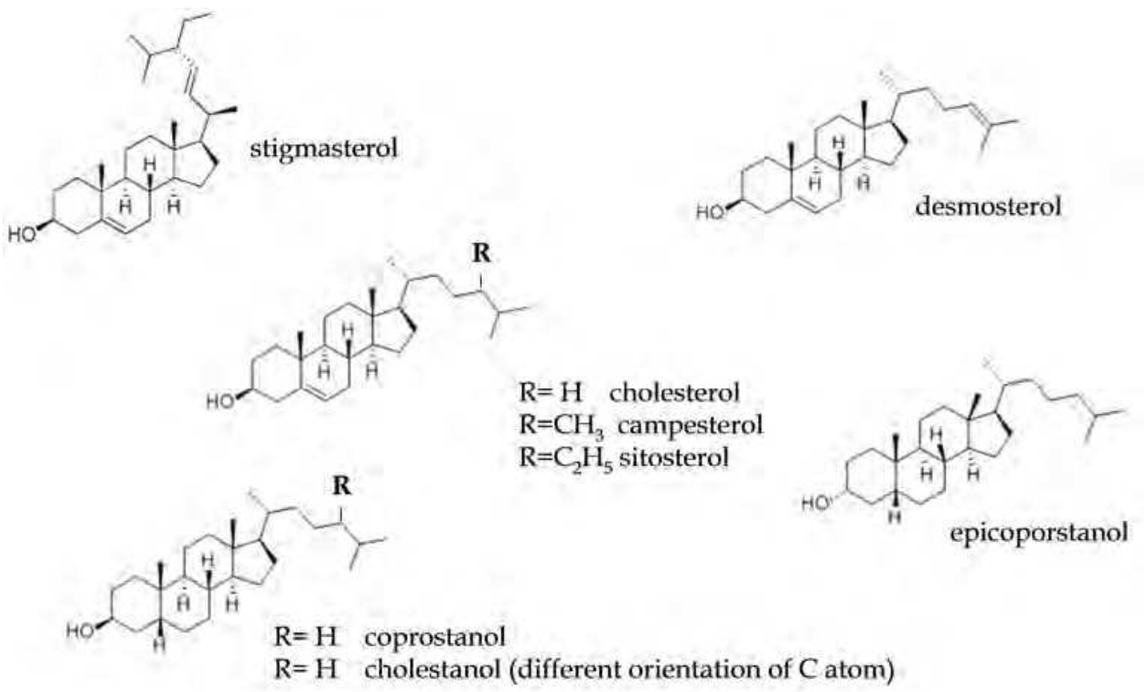

Fig. 2. Structure of major sterol compounds detected in this study

Cholesterol was present in all groundwater samples ranging from 0.022-0.480 µg/L, 0.023-0.590 µg/L and 0.009-0.155 µg/L in April 2009, August 2009 and December 2009 respectively. A few sites regularly tested positive for low concentrations of beta-sitosterol, campesterol, signmasterol, desmosterol, and dihydrochloresterol. Coprostanol was sporadically detected. In December 2009, 15 groundwater sites were sampled for sterols, and six sterols were detected. Cholesterol was detected in all 15 sampling sites ranging from 0.018~0.209 µg/L. Desmosterol was only detected in two sampling sites (FT7-22 and PC-55) ranging from 0.018~0.035 µg/L. Dihydrocholesterol was detected in four sampling sites (ABB-06, FT1-24, FT7-22 and PC-55) ranging from 0.011~0.030 µg/L. Plant sterol campesterol was detected in eight sampling sites (ABB-06, ABB-02, BC008, US-04, US-02, FT1-24, FT7-22 and PC-55) ranging from 0.005~0.032 µg/L. Stigmasterol was detected in all but five sampling sites (ABB-10, 94SH-29, BC349, 91-15 and 91-11) ranging from 0.015~0.668 µg/L. β-sitosterol was detected in all but one (91-11) sampling site ranging from 0.012~0.956 µg/L. Coprostanol was not detected in groundwater during this sampling period.

Based on sterol analyses, ten sterol ratios were calculated and presented in Table 6.

	Identifying Human Fecal Contamination							Differentiating Source of Contamination		
	sterol ratio#									
Field ID	1	2	3	4	5	6	7	8	9	10
Surface water										
S1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	**1.53**[c]	n/a
S2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	1.33	n/a
S3	0.046	0.089	1.000	0.002	0.049	0.002	1.00	0.003[b]	1.43	0.046
S4	0.413	0.526	0.579	0.091	0.703[a]	0.081	1.73[a]	0.144[b]	2.03[c]	0.239[d]
S5	0.415	0.529	0.588	0.092	0.708[a]	0.081	1.70[a]	0.145[b]	1.42	0.244[d]
S6	0.358	0.469	0.583	0.065	0.558[a]	0.058	1.71[a]	0.103[b]	1.49	0.209[d]
S7	0.135	n/a	n/a	0.014	0.156	0.013	n/a	n/a	2.79[c]	n/a
S8	0.077	0.114	0.556	0.016	0.083	0.014	1.80[a]	0.025[b]	0.889[a]	0.043
S9	0.047	0.083	0.833	0.001	0.049	0.001	1.20	0.002[b]	0.535[a]	0.039
S10	0.296	n/a	n/a	0.048	0.421[a]	0.043	n/a	n/a	1.64[c]	n/a
S11	0.280	n/a	n/a	0.039	0.389	0.036	n/a	n/a	1.64[c]	n/a
S11rep	0.280	n/a	n/a	0.040	0.389	0.036	n/a	n/a	1.50[c]	n/a
S12	0.250	n/a	n/a	0.038	0.333	0.034	n/a	n/a	2.31[c]	n/a
Groundwater										
91-11 (GS1)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
BC-008 (GS2)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.563[a]	n/a
BC-008 (GS2)-D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.613[a]	n/a
US-02 (GS3)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	0.527[a]	n/a

Table 6. Sterol ratios for surface and groundwater samples. Underlined values indicate contamination from (a) humans; (b) chicken and/or cow; (c) pig/chicken/cow; (d) cattle/horse/deer

3.4 Fecal pollution source identification using bacterial and chemical indicators

Sterol ratios (calculated from the sterol concentrations) showed that 6 of the 12 surface water samples coded for chicken or cow (ratio #8) contamination (Table 6). Six surface water samples coded for pig/chicken/or cow (ratio #9) contamination, five samples coded for human contamination (ratios #5 and #7), and 3 samples coded for cattle/horse/deer contamination (ratio #10). In the case of the three groundwater samples analyzed for sterol content, 2 samples showed evidence of human fecal contamination. BST analyses of surface water showed that 10 samples tested positive for markers for general fecal contamination while 5 of 12 samples tested positive for one of two markers for ruminant animals (consistently marker CF193F/Bac708R). Comparison of enteric isolates for environmental samples with isolates from poultry litter showed that 10 of 12 surface water samples and 2 of 3 groundwater samples, contained enterococci isolates that grouped with isolates from poultry litter (Table 7).

	Bacterial Source Tracking (BST)						
Field ID	Result from Sterol Ratio	human fecal contamination	pig fecal contamination	ruminant animal fecal contamination	Positive Fecal Contamination	*Enterococcus* isolates group with isolates from litter	Summary contribution to contamination
Surface Water							
S1	pig, chicken, or cow	absent	absent	absent	present	no	
S2	none	absent	absent	1 of 2 markers	present	yes	ruminant
S3	chicken or cow	absent	absent	absent	absent	yes	chicken
S4	human; human pig, chicken or cow chicken or cow cattle, horse or deer	absent	absent	absent	present	yes	chicken human
S5	human human chicken or cow chicken cattle, horse or deer	1 of 2 markers	absent	absent	present	yes	chicken human
S6	human human chicken or cow cattle, horse or deer	absent	absent	absent	present	yes	chicken human
S7	pig, chicken, or cow	absent	absent	absent	absent	yes	chicken
S8	human human chicken or cow	absent	absent	absent	present	no	human
S9	human chicken or cow	absent	absent	1 of 2 markers	present	yes	ruminant chicken
S10	pig, chicken, or cow	absent	absent	1 of 2 markers	present	yes	ruminant chicken
S11	pig, chicken, or cow	absent	absent	present	present	yes	ruminant chicken
S12	pig, chicken, or cow	absent	absent	1 of 2 markers	present	yes	ruminant chicken
Ground Water							
91-11 (GS1)	n/a	absent	absent	absent	absent	yes	
BC-008 (GS2)	human	absent	absent	absent	absent	yes	
US-02 (GS3)	human	absent	absent	absent	absent	no	human

Table 7. Summary of sterols, BST and *Enterococcus* results

Comparison amongst the indicators of fecal pollution showed that for site S5, two (#5 and #7) of seven sterol ratios indicative of human fecal contamination tested positive for human contamination and three ratios (#1, #2 and #3) were "unsure" (Table 6). For the same site, BST analysis indicated one of two markers present for human contamination. The same sterol ratios (#5 and #7) indicating human contamination were found for sites S4 and S6; however for these sites, sterols results were not confirmed by BST analysis. For site S8, two sterol ratios belonging to different groups [(coprostanol/episoprostanol) and (campesterol+sitosterol)/cholesterol)] identified a human pollution source; the epicoprostanol/coprostanol ratio gave a "unsure" result for human contamination (Table 6). Interestingly, site S8 also had the highest *Enterococcus* count but none of the environmental *Enterococcus* isolates grouped with isolates from poultry litter. Because BST analysis of S8 did not detect a ruminant contribution, all indicators point to human fecal contamination. For sites S3 and S7, sterol ratios indicated poultry contamination since BST did not detect any ruminant or pig contributions and some of the enterococci isolates from both locations grouped with the poultry litter isolates. For sites S10, S11 and S12, the sterol ratio for differentiating source of contamination indicated ruminant animals (cow) or chicken as a source. Ruminant contribution was confirmed by BST analysis (both markers present); some of the enterococci isolates for this location also grouped with isolates from poultry litter, indicating contribution from both animal sources. PCA showed that site S9 grouped with sites S3 and S7 (Figure 3): enterococci isolates grouped with the isolates from poultry litter and BST analyses showed one of two markers for ruminant fecal contamination, indicating ruminant and chicken sources contributed to pollution of these sites.

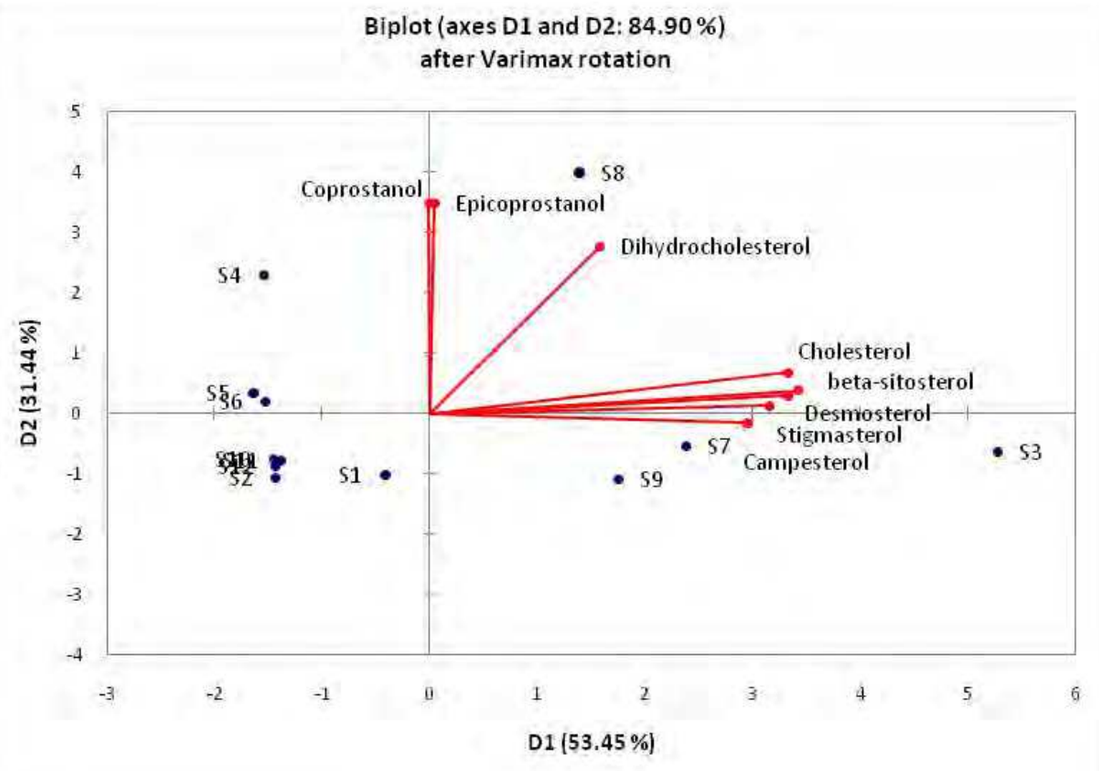

Fig. 3. Biplot (loadings and scores) on the first two principal components (sterol data)

3.5 Chemometric analysis

Most of the variation in the sterol data for surface water was explained by first two PCA factors (Table 8). The first factor (VF1) contributed 53.45% of the total variance and consisted of mostly plant sterols (cholesterol, desmosterol, campesterol, stigmasterol and β-sitosterol). The second factor, VF2 (31.44%), showed high positive loading of coprostanol, epicoprostanol and dihydrocholesterol (animal sterols). The strong loadings of coprostanol, episcoprostanols and dihydrocholesterol in VF2

(Table 8) suggested the possibility of sewage contamination since these compounds are widely used as chemical markers, especially coprostanol (Carreira et al., 2004; Isobe et al., 2004).

Parameters	VF1	VF2
Coprostanol	-0.002	**0.966**
Epicoprostanol	0.013	**0.967**
Cholesterol	**0.927**	0.188
Dihydrocholesterol	0.440	**0.769**
Desmosterol	**0.880**	0.034
Campesterol	**0.822**	-0.046
Stigmasterol	**0.929**	0.080
β-sitosterol	**0.955**	0.106
Eigenvalue	4.515	2.277
Variability (%)	53.45	31.44
Cumulative %	53.45	84.89

Table 8. Factor loadings (after varimax rotation) of sterols in surface water samples. Strong loadings (>0.75) are shown in bold

CA applied to the surface water sterol data grouped by site identified three clusters: cluster 1 consisted of samples from sites S1, S2, S4, S5, S6, S10, S11 and S12; cluster 2 consisted of samples from S3, S7 and S9; cluster 3 contained only site S8 (Figure 4). Analyses of cluster I showed that sites were grouped by sources of contamination: chicken and human (S4, S5, S6) versus ruminant and chicken (S10, S11, S12). For cluster 2 sorces of contamination were chicken whereas for cluster 3 human contamination is evident. The CA analysis on sterols generated three groups (Figure 5). Cluster 1 consisted of 3 sterols namely campesterol, β-sitosterol and stigmasterol (all plant sterols); cluster 2 consisted of cholesterol and desmosterol, while cluster 3 consisted of animal sterols (coprostanol, epicoprostanol and dihydrocholestrol). Cluster analysis supported the PCA analysis that suggested there are two potential sources of fecal contamination (human and animal) in surface water samples, which is in an agreement with the sterol ratio data.

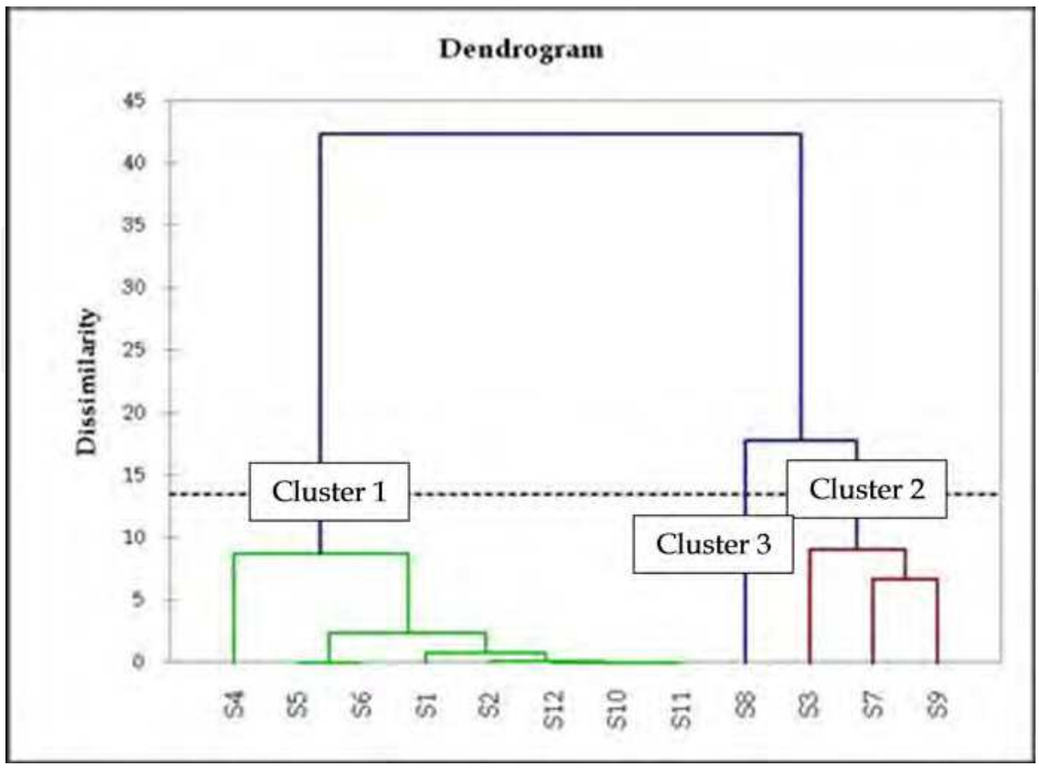

Fig. 4. Dendrogram showing the clustering of sampling sites based on sterols data

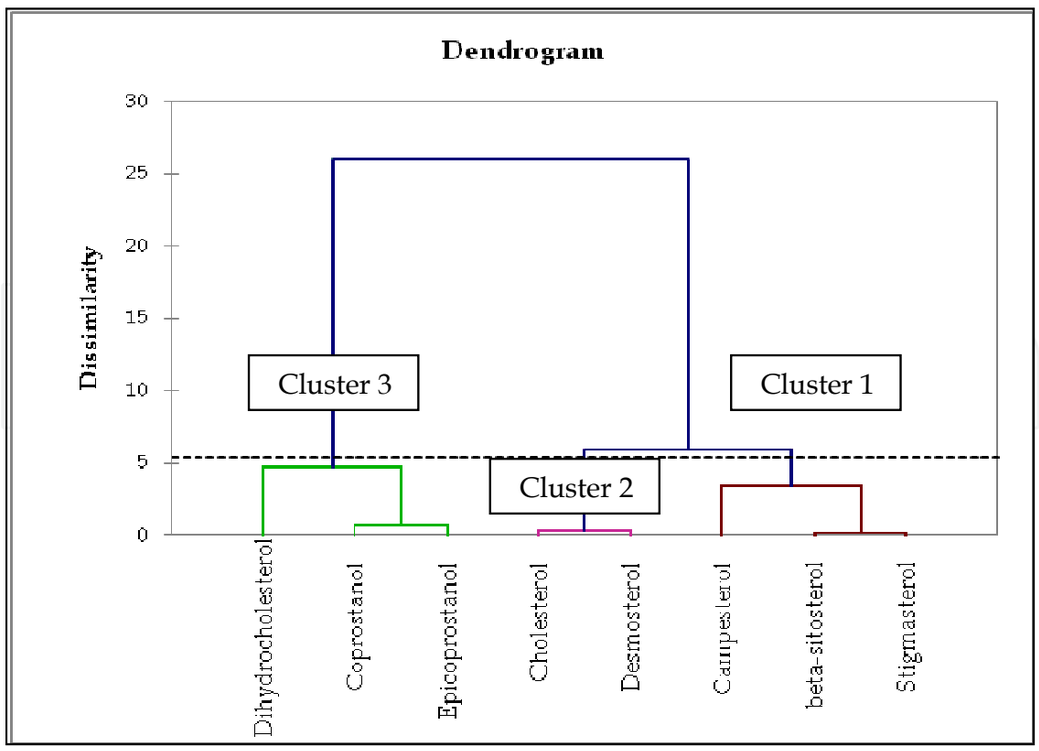

Fig. 5. Dendrogram showing clustering of sterols as variables

4. Discussion

Impacts of human and agricultural activities on surface and ground water quality are a fact of life around the globe as evidenced by studies in Australia, Europe, United States and Canada where results indicate that fecal pollution is a pervasive problem (Betcher et al., 1996; Blanch et al., 2004; O'Leary et al., 1999). Recent study in US indicated that human impacts on watershed hydrology affected 86% of assessed streams (Carlisle et al., 2010). Within the European Union, a project on tracking fecal pollution in surface water involved scientists from seven countries and a battery of microbial and chemical indicators with the objective to generate knowledge on the best methodologies for tracking sources of fecal pollution in surface water (Blanch et al., 2004). EU is committed to improve overall water quality through Integrated River Basin Management program which is a part of the European Water Framework Directive. Comparison of bacteriological water quality, sterol concentrations and ratios, and BST results for water samples collected from an agricultural (poultry-dominated) area in southern British Columbia, Canada showed that 100% (n=12) of surface water sites and 15% (n=20) groundwater sites had fecal contamination. Of the surface water sites, most (75%) showed evidence of poultry fecal contamination, 33% also showed evidence of human fecal contamination while 42% tested positive for ruminant contamination.

The broad extent of fecal pollution in surface and ground water has provided impetus for scientific studies and regulatory actions to identify fecal pollution sources and undertake corrective measures; however, such regulatory actions are only as effective as the methods employed to identify fecal pollution sources. As a result, microbiologists and organic chemists have explored the usefulness of many different markers for conclusively isolating sources of fecal pollution (Balleste et al., 2010; Blanch et al., 2004; Tyagi et al., 2009a). The over-arching conclusion of these studies was that a battery of markers is required to accurately ascribe fecal pollution source. Yet to our knowledge, few if any studies have examined a multi-metric approach for poultry pollution source tracking in surface and ground water. In our study, microbial markers, BST and enterococci were investigated for fecal source tracking.

4.1 Microbial markers for source tracking of poultry fecal pollution

Our results showed that BST was useful for detection of human and ruminant pollution in surface water; BST is the most specific of all microbial methods and can distinguish among human and animal fecal contamination, but the drawback is that primers are not available for many species, birds being one of them. A possible issue with the BST may be the method detection limit which is not easy to establish and needs to be addressed in the future.

Analysis of *Enterococcus* showed that isolates in water samples grouped with isolates from litter for 10 out of 12 surface water sites and for 2 out of three groundwater sites. *Enterococcus* has been explored as a microbial source tracking indicator for human fecal pollution specifically enterococcal surface protein (*esp*) in *Enterococcus faecium* (Scott et al., 2005). Primers developed by these authors were successful in 97% of sewage and septic samples and did not react with birds and different livestock samples. Fingerprinting or pattern matching of enterococcal isolates from poultry farms with ones found in the environment has been introduced by our group (unpublished data) and does not rely on

libraries as do some MST methodologies (Balleste et al., 2010 and references within). Our method is very sensitive with only one enterococcal CFU required as opposed to 58 enterococcal CFU necessary for *esp* gene for human detection (Scott et al., 2005). In our study, nine *Enterococcus* environmental isolates and two from day 35 from layers farm reacted positively with the *Enterococcus* genus marker but did not react with any of the species markers used in the multiplex PCR. These unknown isolates could either belong to a species not tested in the multiplex PCR, or they could belong to one of the tested species but have a mutation in the *sodA* gene such that the species-specific primers do not bind.

Other bacterial total coliform, fecal coliform and *E. coli* were also measured and are useful only as general indicators of the fecal pollution. Total and fecal coliform have been regulated as indicators for rapid sanitary quality of different water bodies but they are not useful for source tracking. There is no regulation in regards to *Enterococcus* bacteria for surface water in general in Canada, but the one surface water location was above the 350 cfu/L *Enterococcus* guideline for Canadian Recreational Water Quality (Health and Welfare Canada, 1992) although the guideline values is based on a geometric mean of at least five samples taken over 30 days.

Although different bacteria and combinations of bacteria have been used in the literature for fecal source tracking (Blanch et al., 2004; Tyagi et al., 2009 and references within), the approach used in this study represents a unique integration of BST and enterococci as indicators of fecal pollution.

4.2 Chemical markers for source tracking of fecal pollution

To provide further scientific support for our microbiological findings, BST analysis and *Enterococcus* results, sterol analyses were conducted on surface and groundwater. Sterols indicative of fecal source pollution (cholesterol, coprostanol epicoprostanol desmosterol and dihydrocholesterol) were detected in all surface water samples; phytosterols (campesterol, stigmasterol and b-sitosterol), an indication of herbivores pollution sources, were detected (Leming et al., 1996; Reeves & Patton, 2005). The presence of individual sterols is generally not sufficient for tracking of fecal pollution in the environment although, for example, coprostanol is considered a marker for sewage contamination (Leeming et al., 1996, Shah et al., 2007). Sterol ratios are more reliable and widely used in the literature for source pollution tracking (References in Table 2). Sterol ratio criteria for human fecal contamination have been developed based on experimental data mainly from analysis of sterols in sediment although they have been tested and applied in water environments in the connection with discharge from waste water treatment plant discharge (Chan et al., 1998; Chou & Liu, 2004). Although there are numerous studies using sterol ratios for tracking human fecal contamination, few studies have used these ratios for differentiating sources of fecal contamination. Sterol ratio criteria for differentiating sources of fecal contamination are derived mostly from studies of agricultural environment manures and slurries matrices (Jardé et al., 2007; Standley et al., 2000), with the later study showing that ratios #8 and #9 successfully discriminated pig slurry from poultry and dairy manure. These two ratios were also successful in this study. Ratio #10 was found to be the most useful ratio for tracking agricultural activities in 19 North American watersheds (Standley et al., 2000). In our study, 5 ratios (#5, 7, 8, 9 and 10) proved useful for fecal pollution source tracking. In cases of contamination from human and animal sources, ratios for identifying human fecal contamination that contain cholesterol

may be failing due to increased concentrations of cholesterol from animal sources. For sampling site 9, only one ratio for differentiating sources of contamination (namely campesterol+citosterol)/cholesterol) indicated human fecal contamination. In this case, increased concentration of cholesterol from animal sources is probably decreasing the ratio so this may be false positive, particularly as this result was not confirmed by a any other indicator. Thus, we recommend use of as many ratios as can be calculated based on the sterol analysis in suite in order to positively identify sources of contamination.

The combination of bacteriological and chemical indicators presented in this study represents a unique integration of indicators. To our knowledge, no similar study has been conducted and reported in the literature. Furthermore, this approach was able to indicate several sources of fecal contamination simultaneously.

4.3 Chemometric approach for source tracking of human and poultry fecal pollution

Although microbiological and chemical indicators were used in the combination for fecal pollution tracking, there are few studies where they were further supported by the chemometric analysis. A study of an Australian water supply system comprised of rivers, channels and drains monitored only two sterols (coprostanol and cholesterol) for a longer period of time (12 months) and used cluster analyses to detect similarities among the sampling sites (Hussain et al., 2010). In this study, clustering of sites by sterol profile suggested four clusters that differed by the source. Shah et al. (2007) showed that cluster analysis of fecal sterols of humans, herbivores, birds and carnivores could distinguish between birds and the other three categories, with humans and herbivores and also herbivores and birds being well separated whereas humans and carnivores were more closely associated. Saim et al. (2009) applied cluster analysis on their sterol data (only 5 sterols analyzed) from various point sources (sites included sewage treatment plants, chicken farms, quail farms and horse stables) and concluded that chicken and quail generated a separate cluster that can be distinguished. In this study, CA also grouped sites by sources of contamination: chicken and human, ruminant and chicken, human only and chicken only. Furthermore, CA of sterols resulted in three groups: plant sterols, human fecal sterols and animal sterols. Thus the CA results provide further corroboration of our predictions of fecal sources contamination based on BST, *Enterococcus* and sterol ratio results.

Principal Component Analysis is another chemometric technique for pattern recognition that is often used in the combination with CA. To improve interpretation of principal components, Varimax rotation is recommended. Resulting VF coefficients with values greater that 0.75 are considered to have strong correlation and in our data, all eight sterols (coprostanol, epicoprostanol, cholesterol, cholestanol-dihydrocholesterol, desmosterol, campesterol stigmasterol and β-sitosterol) were strongly correlated. Plot of discriminate functions in Saim et al. (2009) also showed clear separation of human and chicken sources. Similar PCA results presented by Leeming et al. (1996) showed human, hens and cow and sheep were clearly separated on the PCA plot. Results of PCA in this study further supported CA analyses results. Biplot on first two principal components clearly identified two groups of sterols. Moreover, sampling sites on the same biplot are grouped as per the CA clusters. For sites S3 and S7, sterol ratios indicated only poultry contamination; BST did not detect any ruminant or pig contributions and some of the enterococci isolates from both

locations grouped with the poultry litter isolates. These findings were further supported by the high loading of β-sitosterol from PCA analysis. Previous studies (Leeming et al 1996; Saim et al 2009) reported β-sitosterol as major sterol in chicken and other bird species. Combination of chemometric analyses with bacteriological and chemical indicators for site 8 clearly indicated only human contamination. Surprisingly, groundwater site GS3 is located close to site S8 (Figure 1) and for both locations enterococci did not group with chicken isolates. Because the entire area is serviced by septic tanks, human fecal contamination from sewage is a possibility. Hence, for other sites where there was more that one possible source of contamination, our multi-indicator approach was able to identify sources. To our knowledge, such a comprehensive approach has not been reported in the literature for fecal source tracking.

5. Conclusion

Our study showed that application of multi-indicator approach consisting of *Enterococcus*, bacterial source tracking (BST), sterol analysis and chemometric analyses could successfully identify sources of fecal contamination in agricultural areas dominated by poultry operations and associated human activities. An ability to ascribe sources when confronted with a complex pollution situation is essential for planning management actions and implementing best management practices. Our results will assist further efforts to protect and preserve surface and ground water quality from the impacts of human and agricultural activities.

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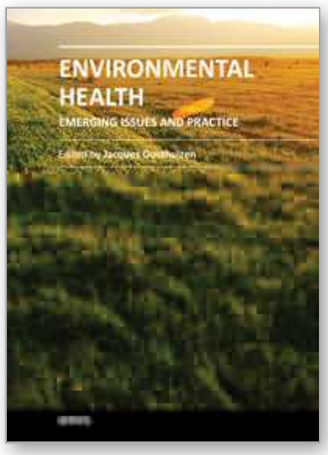

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Environmental health practitioners worldwide are frequently presented with issues that require further investigating and acting upon so that exposed populations can be protected from ill-health consequences. These environmental factors can be broadly classified according to their relation to air, water or food contamination. However, there are also work-related, occupational health exposures that need to be considered as a subset of this dynamic academic field. This book presents a review of the current practice and emerging research in the three broadly defined domains, but also provides reference for new emerging technologies, health effects associated with particular exposures and environmental justice issues. The contributing authors themselves display a range of backgrounds and they present a developing as well as a developed world perspective. This book will assist environmental health professionals to develop best practice protocols for monitoring a range of environmental exposure scenarios.

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