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FECAL COLIFORM SOURCE IDENTIFICATION USING CHEMICAL TRACERS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Engineering and Science

---

by
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August 2008

Accepted by:
Alan W. Elzerman, Committee Chair
John T. Coates
Cindy M. Lee

ABSTRACT

Fecal sterols and fluorinated whitening agents (FWA) were used as chemical tracers for fecal coliform source tracking in Cane and Little Cane creeks in Walhalla, SC. Fecal sterols were quantifiable in both stream sediments and suspended particulates using an extraction, clean-up, and analytical method modified from Isobe et al. (2002) with a N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization scheme. Only human sterol source identification ratios were able to be calculated using the chosen sterol suite. Human fecal signatures were seen in both the sediment and the water column at various points along both creeks, indicating human fecal pollution is contributing to the fecal coliform pollution. Because there was little deposition of sediment at sampling sites along the creeks and the sterol loading on suspended particulates was about the same or greater than that in the sediment, it is recommended that future studies on this system concentrate on quantifying fecal sterol loadings in the water column. Further, water column samples will provide an instantaneous picture of fecal loadings. Sediment samples are easier to process and may be useful for specific investigations. Results were compared to microbial source tracking (MST) methods by a collaborating researcher and showed consistencies for only some of the sample sites, which may have been due to false negatives or differences in sampling dates and matrices. FWA was never above presumptive sewage detection levels in the creeks even when sediment samples indicated some historical human fecal pollution at sites. Due to its specificity for human input and the apparent requirement of significant levels to give a positive signal, it is suggested that FWA analysis be discontinued unless a significant human fecal input is suspected.

An expanded study is recommended to compare fecal sterols in the water column during base and storm flow using the method developed here and an expanded sterol suite to correlate fecal loadings with different sources in the watershed. Using this information, BMPs could be implemented with a focus on addressing actual fecal loadings. The method could also be used to help quantify surface water quality improvements after BMP implementation.

DEDICATION

To Mom, Dad, Marisa, and Lauren.

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LIST OF SYMBOLS AND ABBREVIATIONS

Arabic Symbols

n	Number of samples used to determine statistical data

Greek Symbols

Σ	Sum of

Abbreviations

BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DCM	Dichloromethane (methylene chloride)
DDI	Double deionized water
DHEC	Department of Health and Environmental Control
FAME	Fatty acid methyl ester
FOLKS	Friends of Lake Keowee Society
FWA	Fluorinated whitening agent
GC	Gas chromatography
LOQ	Limit of quantitation
NOM	Natural organic matter
MS	Mass spectrometry
MST	Microbial source tracking
PPCP	Pharmaceuticals and person care products
RPD	Relative percent difference
%RSE	Percent relative standard error
SC	South Carolina

SID	Source identification
SP	Suspended particulate
TMDL	Total maximum daily load
TOC	Total organic carbon
TMS	Trimethylsilyl
WW	Wastewater
WWTP	Wastewater treatment plant

CHAPTER 1

BACKGROUND

Fecal coliform bacteria are used as indicator organisms of fecal pollution. While fecal coliforms themselves do not pose a health threat, their presence in water indicates fecal pollution and thus the possibility of other harmful bacteria and viruses. Because of their abundance and relative ease of measurement fecal coliforms remain a standard of regulatory compliance, despite rising concerns about their specificity and ability to correlate with pathogens (Scott et al., 2002). Of the more than 1000 water stations out of compliance on the proposed 2008 303d list of impaired waters for South Carolina, more than 300 were out of compliance due to elevated fecal coliform counts (DHEC, 2008). The high number of waters with elevated coliform levels has raised concerns about how to best identify and reduce fecal inputs.

1.1 Site Description

Little Cane Creek flows into Cane Creek, which empties into Lake Keowee in Oconee County, South Carolina, as shown in Figure 1.1. Both creeks have been on South Carolina's 303(d) list of impaired waters since 1998 for violating fecal coliform standards. Section 303(d) of the Clean Water Act requires that for surface waters in violation of water quality standards total maximum daily loads (TMDLs) be calculated that can be used to determine the maximum amount of contaminant a water body may receive (with a safety factor) and still be in compliance (Elshorbagy et al., 2005). The 303d list is generated every two years and the TMDLs are considered a first step toward

returning a water body to compliance. TMDLs have been calculated by the South Carolina Department of Health and Environmental Control (SC DHEC) for the creeks using fecal coliform loads for non-point sources (based on coliform loading during normal flow) plus a margin of safety, but no source tracking methods have been used to determine how different non-point sources are actually impacting the total coliform loading (DHEC, 2005).

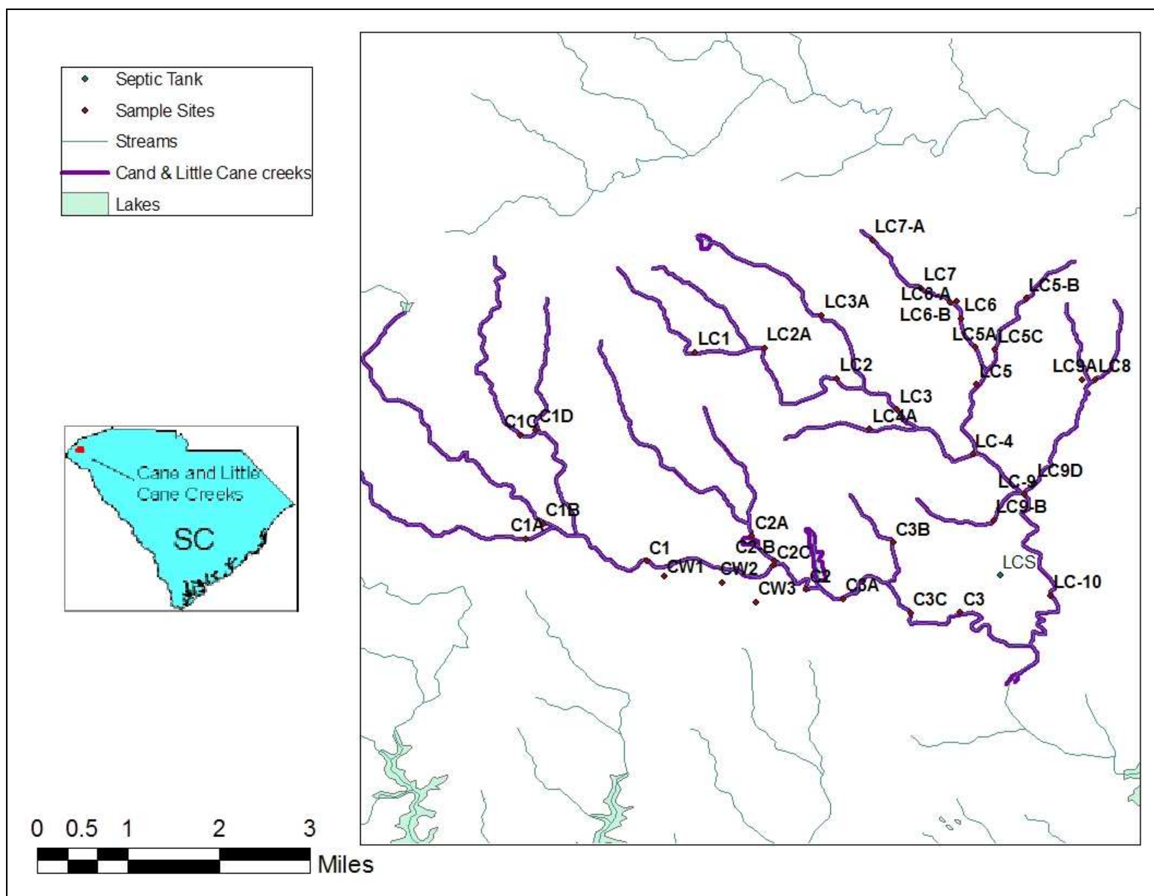


Figure 1.1 Map of Cane and Little Cane creeks with all FOLKS sampling sites labeled (shapefiles courtesy of Morris Warner, Clemson Extension Network).

Both watersheds are primarily forested (Cane 76%; Little Cane 90%) with small amounts of pasture land (6-7% in each). The Cane Creek watershed is the more developed of the two (10.9% urban versus 0.9%) and contains the towns of Walhalla and West Union. While the town of Walhalla has a sewer system many residences in the area have septic systems: according to the 2000 census 800 households (1800 people) in the Cane watershed and 650 households (1700 people) in the Little Cane watershed use septic systems. Neither Cane nor Little Cane creeks have any point sources of wastewater. A map of the watershed delineating land use is shown in Figure 1.2 and a map of roads and septic repairs is shown in Figure 1.3.

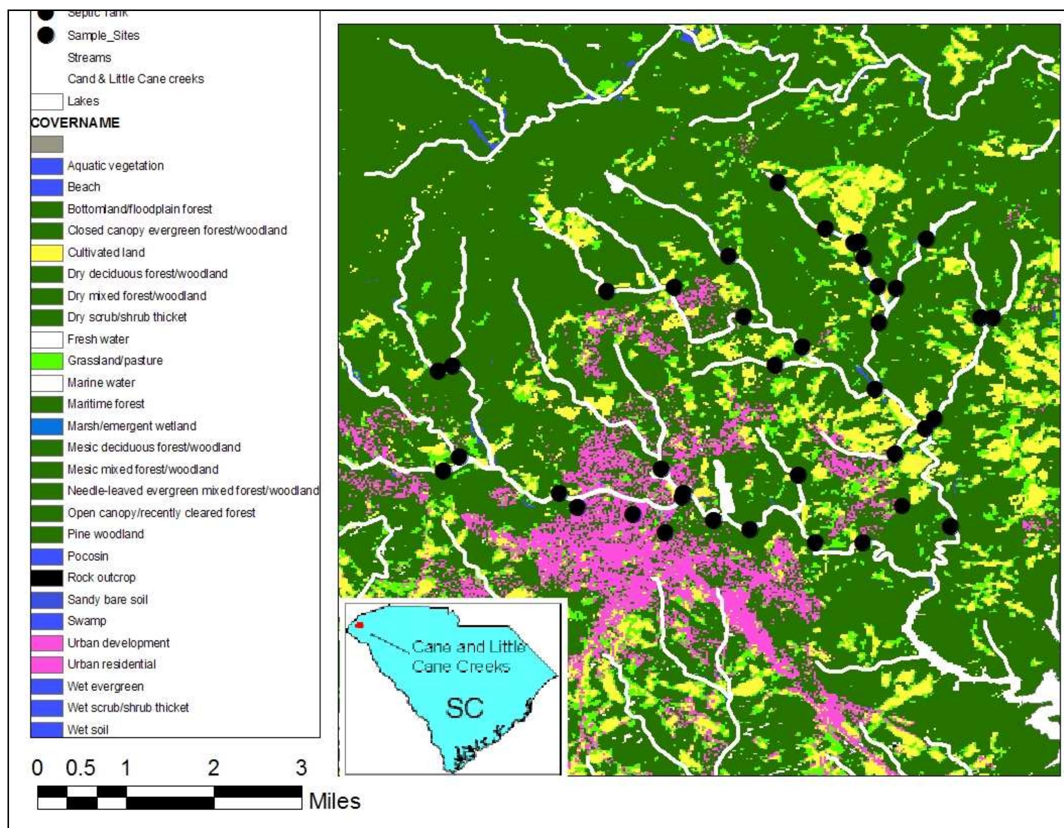


Figure 1.2 Land use data for Cane and Little Cane creeks. (data from SC DNR, available online http://www.dnr.sc.gov/GIS/gisdata.html)

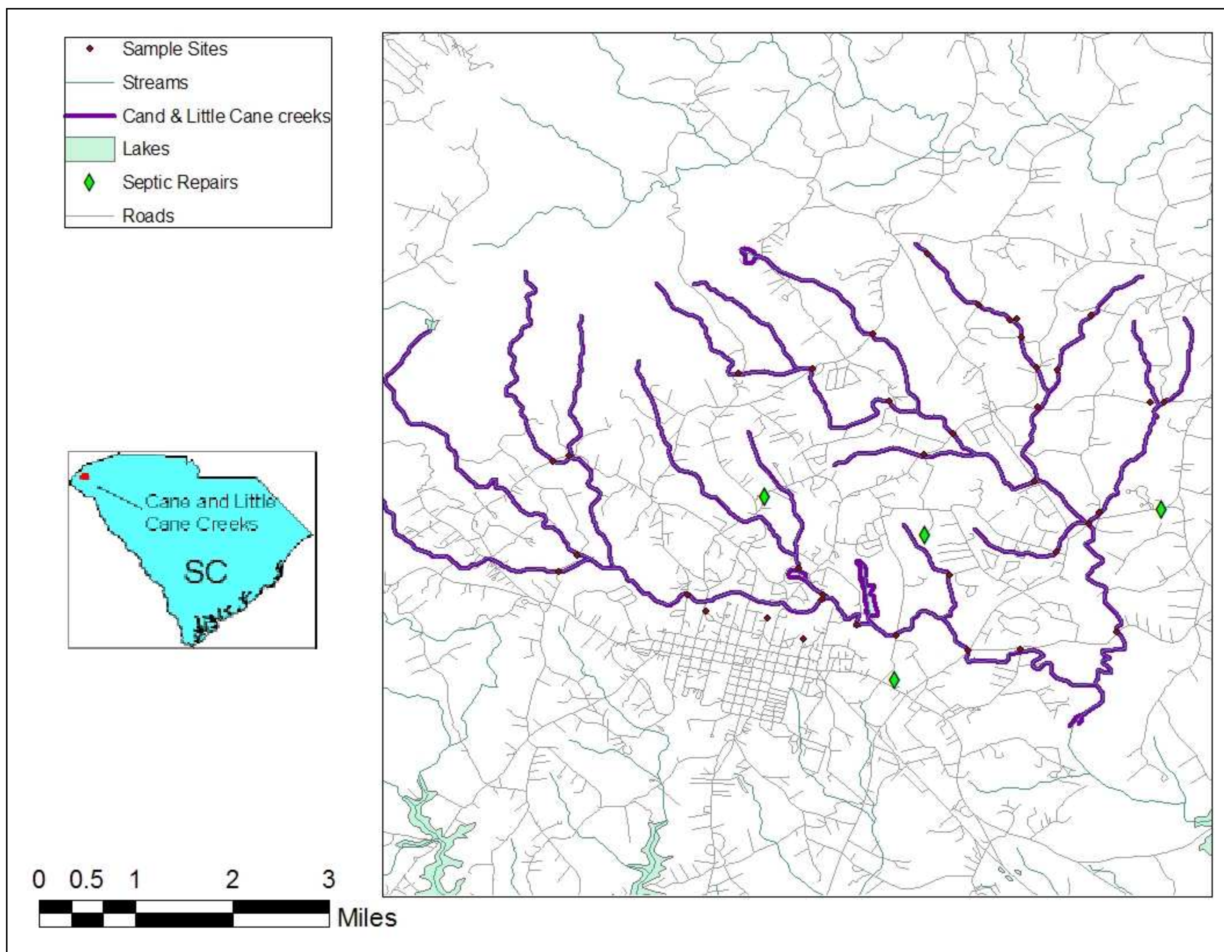


Figure 1.3 Roads and septic system repairs (made in 2007) (shapefiles courtesy of Morris Warner, Clemson Extension Network).

A review of land cover data conducted by DHEC enumerated the possible fecal coliform sources in the watershed, including failing septic systems, sewer overflows and leaks, agricultural runoff, cattle defecation directly in streams, urban run-off, and forest runoff (DHEC, 2005). Because many households in the watershed use aging septic systems it is likely that some are failing to properly treat their wastewater, thus allowing inadequately treated wastewater to reach the creeks through the groundwater or even by

contaminating surface runoff (see Figure 1.3 to see sites of septic system repairs made in 2007). Another possible source of untreated wastewater into the creeks is from overflows and leaks in the sewer system. The town of Walhalla uses a combined sewer system which has been reported to overflow into the creeks during storm events. Possible sources of nonhuman coliform include runoff from land with fecal matter and direct deposition of fecal matter into the creeks. Several farms have cattle which defecate on the land and directly into the streams and likewise fecal matter from wild animals, like beaver and deer, can be washed or deposited into the stream. Runoff and direct fecal deposit into the streams may also be an issue with uncurbed pets such as dogs and cats. Also, some fields in the watershed are subject to manure application and this runoff too could be contributing to the fecal pollution in the creeks.

To address these possible sources, Friends of Lake Keowee Society (FOLKS) has been working to educate people in the watershed by encouraging farmers to put up fences to keep cattle out of and away from the creeks, providing information about septic tank maintenance, and offering cost share opportunities for fence building and septic repairs. Several fences have been built and failed septic systems in the area have been identified, although their impact on water quality remains unproven.

1.2 Source Tracking

Knowing land cover uses and possible fecal coliform sources alone may not yield an accurate conclusion as to the sources responsible for the pollution in this complex system. This is because source contributions may not necessarily correlate with the area of land they cover (unfortunately, however, land cover data are all that is used for

calculating TMDLs). Using microbial or chemical tracers and markers allows for evaluation of the contributions of individual sources or at least can rule out or in certain sources. Many source tracking methods are available, each with its own limitations, and there is no clear simple, accurate, and standard test to determine the source of fecal coliforms (Scott et al., 2002; Stoeckel and Harwood, 2007). Source tracking methods are either microbial or chemical. Microbial methods are either phenotypic or genotypic, library based or library independent (Stoeckel and Harwood, 2007). Library based methods can be fairly accurate but require a large database and are susceptible to false positives, while library independent tests are simpler but tend to give false negatives (Stoeckel and Harwood, 2007). Microbial methods were not chosen for this work because while possibly more accurate, library based methods require creating a library, which was beyond the scope and timetable for this project and was already being pursued by other FOLKS collaborators (Clemson Extension and US EPA, Athens).

Marirosa Molina at the US EPA lab in Athens, GA performed Length Heterogeneity Polymerase Chain Reaction (LH-PCR) and Terminal Restriction Fragment Length Poly-morphism (T-RFLP) analyses for microbial source tracking (MST). These are genotypic methods that exploit Bacteroides-Prevotella, fecal indicator anaerobic bacteria, which has significant differences in DNA between source species (Simpson et al., 2002). Bacteroides-Prevotella are short lived in water (4-5 hours) and thus the results of these techniques can indicate only recent fecal pollution (Simpson et al., 2002). The advantages of these methods are that neither a library nor cultivating of bacteria is required but they are technically demanding and require expensive equipment (Simpson

et al., 2002). The methods are subject to false negatives results which are a function of detection limit (Simpson et al., 2002). One of the goals of the MST work was to help establish detection limits for the MST procedure. Lou Jolly in the Clemson Extension Network was using a carbon assimilation method to track fecal coliform sources, but no data were available for use or comparison by the time this study was complete.

Chemical methods include measuring fecal sterols, both human and veterinary pharmaceuticals and antibiotics, and other organic compounds such as detergents, disinfectants, and hormones that are common co-contaminants with fecal coliforms. Among these possibilities fecal sterols and detergents are the most promising chemical tracer methods due to their frequency of detection and relatively high concentrations in affected water, as well as their conservative nature (Glassmeyer et al., 2005; Kolpin et al., 2002). The utility of measuring fecal sterols and whitening agents will be discussed below as well as why other chemical tracers were inappropriate for this study.

1.3 Fecal Sterols

Fecal sterols are present in varying amounts in the feces of human and other animals. Sterols include cholesterol and its breakdown products (Nishimura and Koyama, 1977; Wilkins and Hackman, 1974). Table 1.1 lists sterols and their sources. Sterols have been used for source tracking due to their abundance in feces and their specificity to origin. Leeming et al. (1996) determined that humans and animals have significantly different amounts of various sterols in their feces (due to differences in diet

Table 1.1 Systematic (IUPAC), trivial name, major ion for MS monitoring (only included for sterols quantified in this study), and description of sterols for analyzed sterols and other commonly quantified sterols.

Trivial Name	IUPAC Name CAS No	Major ion (m/z)	Description
Coprostanol	5β-cholestan-3β-ol 360-68-9	215	Human fecal biomarker-high relative amounts indicate fresh human contamination
Coprostanone	5β-cholestan-3-one 601-53-6	231	Oxidation product of coprostanol
Cholesterol	cholest-5-en-3β-ol 57-88-5	368	Major ubiquitous sterol. C27 precursor to 5α and 5β-stanols.
3β-Cholestanol	5α-cholestan-3β-ol 80-97-7	215	Normal reduction product of cholesterol. Thermodynamically most stable isomer is ubiquitous- if the ratio of coprostanol/cholestanol is <0.3, origin of 5α-stanols may not be human fecal.
5α-Cholestanone	5α-Cholestan-3-one 15600-08-5	231	Oxidation product of cholestanol
Stigmasterol	24-ethylcholestan-5,22E-dien-3β-ol 83-48-7	394	Usually used as terrestrial sterol biomarker
β-Sitosterol	24-ethylcholest-5-en-3β-ol 83-46-5	396	C29 precursor to 5α and 5β- stanols. Usually used as a terrestrial sterol biomarker.
Stigmastanol	24-ethyl-5α-cholestan-22E-en-3β-ol 19466-47-8	215	Algal sterol biomarker, also found in reducing environments.
Epicoprostanol	5β-cholestan-3α-ol 516-92-7	n/a	Present in sewage sludges-high relative amounts to coprostanol suggest older fecal contamination
Campesterol	24-methylcholest-5en-3β-ol 474-62-4	n/a	Terrestrial, typical in higher plants.
Brassicasterol	24-methylcholestan-5,22E-diene-3β-ol 474-67-9	n/a	Algal sterol biomarker.
24-methylenecholesterol	24-methylcholesta-5,24(28)-dien-3βo 474-62-4l	n/a	Algal sterol biomarker and precursor to C29- higher plant sterols.
24-ethylcoprostanol	24-ethyl-5β-cholestan-3β-ol 4736-91-8	n/a	Herbivore fecal biomarker- high relative amounts to sitostanol indicate herbivore fecal contamination.
24-ethyl-epicoprostanol	24-ethyl-5β-cholestan-3α-ol	n/a	Also present in some herbivore feces.
Dinosterol	4,23,24-trimethyl-5α-cholest-22E-en-3β-ol 58670-63-6	n/a	Derived from dinoflagellates.

and metabolism), allowing a "sterol fingerprint" that can be used to distinguish between different sources of contamination. Despite the specificity of the sterol "fingerprints" there is no standard method yet for this analysis as it is a novel area of research. As discussed below, researchers often measure different suites of sterols.

Human feces contain primarily coprostanol, on average 200mg to 1g of coprostanol per day per person (Walker et al., 1982). Ruminant animals excrete large amounts of 24-ethylcoprostanol, the C_{29} homologue of coprostanol (Leeming et al., 1996). Sterol ratios are used for fecal source identification to reduce bias compared to looking at just total sterol concentrations, which are affected by the amount of organic matter and sediment particle size (Bull et al., 2002; Hawkins Writer et al., 1995). The fraction of coprostanol (coprostanol/Σsterols) and fraction of 24-ethylcoprostanol (24-ethylcoprostanol/Σsterols) have both been used by researchers to distinguish between sources (Chan et al., 1998; Grimalt et al., 1990; Isobe et al., 2004; Leeming et al., 1996; Maldonado et al., 1999; Noblet et al., 2004); Readman et al., 2005; Suprihatin et al., 2003). A high coprostanol/Σsterols ratio correlates with human fecal pollution, while a high 24-ethylcoprostanol/Σsterols ratio correlates with ruminant animal fecal pollution.

Several ratios have been explored and quantitative limits established which are useful for distinguishing between human and other fecal pollution source. Commonly used ratios to indicate human fecal pollution include the following:

Coprostanol/3β-Cholestanol (1)

Coprostanol/Cholesterol (2)

Coprostanol/(3β-Cholestanol+Cholesterol) (3)

Coprostanol/(Coprostanol+3β-Cholestanol) (4)

Coprostanone/(Coprostanone+5α-cholestanone) (5)

Epicoprostanol/Coprostanol (6)

The sterol signature for human fecal pollution is indicated when ratio (1)>0.3 (Grimalt et al., 1990). It is considered to be the most reliable of the sterol ratios (Pratt, 2005) but was developed for use in temperate waters and studies in known polluted tropical waters, suggesting it may not be reliable in all climates (Isobe et al., 2002). When ratio (2) >0.2 (Takada et al., 1994) human fecal pollution is indicated. Ratio (3) >0.06 indicates large point source inputs of human fecal pollution and =0.06 for smaller non-point source inputs (Hawkins-Writer et al., 1995). Ratio (4) >0.7 (Grimalt et al., 1990) indicates human fecal pollution and human fecal pollution is indicated when ratio (5) >0.7 (Grimalt et al., 1990). High values (>1.0) for Equation (6) indicate treated or older sewage inputs (Mudge and Duce, 2005). Epicoprostanol is thought to be produced in anoxic

environments like mud and sewage sludge by anaerobic bacterial populations (McCalley et al., 1981).

Commonly used ratios for herbivore fecal pollution include:

24-ethylcoprostanol/β-sitosterol (7)

24-ethylcoprstanol/sitostanol (8)

where high ratios indicate herbivore fecal pollution (Leeming and Nichols, 1996). Other ratios have been used to distinguish between human fecal pollution and other sterol sources like marine phytoplankton (Vankatesan and Kaplan, 1990) or to measure microbial cholesterol reduction in sediment (Patton and Reeves, 1999; Readman et al., 2005) that are not applicable to the fecal source identification goals of this work.

Sterols tend to associate with particulates (Brown and Wade, 1984) and are fairly persistent in anoxic sediment (Nishimura and Koyama, 1977). Although their utility as markers for life millions of years ago is being debated (Volkman, 2005), sterols have been used to determine historical fecal pollution loadings in sediment cores from the Kaoping River (Jeng et al., 1996), New York Bight (Hatcher and McGillivary, 1979), and other sites for shorter time periods on the order of decades.

Comparing the abundance of different sterols and their ratios to one another in surface water has allowed for the identification of fecal pollution sources without further chemical tracer studies in the Santa Monica Basin, California (Vankatesan and Kaplan,

1990), various urban and rural areas of Spain and Cuba (Grimalt et al., 1990), the Missouri River (Hawkins Writer et al., 1995), around Sydney, Australia (Leeming et al., 1996; Nichols et al., 1996), the southeastern waters of Hong Kong (Chan et al., 1998), Victoria Harbor, Canada (Mudge and Lintern, 1999), the Mediterranean and Black Sea (Maldonado et al., 1999), San Pedro shelf sediments, California (Maldonado et al., 2000), Torrens and Patawalonga catchment waters, South Australia (Suprihatin, 2003), the Lower Santa Ana River Watershed, California (Noblet et al., 2004), Western Malaysia and Mekong Delta, Vietnam (Isobe et al., 2004; Isobe et al., 2002), the Black Sea (Readman et al., 2005), Moreton Bay, Southeast Queensland, Australia (Pratt et al., 2007; Pratt, 2005), and along the north coast of New South Wales, Australia (Shah et al., 2007; Shah et al., 2007). In addition, sterols and their ratios have also been combined with other chemical tracers (e.g. whitening agents) to determine fecal pollution sources on the South Island of New Zealand (Gilpin et al., 2003; Gilpin et al., 2002), the Pearl River and South China Sea (Peng et al., 2005), Deal Lake, New Jersey (Sankararamakrishnan and Guo, 2005), along the Avon River, Bristol, U.K. (Elhmmali et al., 2000) and throughout North American WWTP effluent and runoff (Standley et al., 2000). In these studies, fecal sterols have been used both to rule out (i.e. Noblet et al., 2004) and implicate human waste problems (i.e. Gilpin et al., 2003; Readman et al., 2005). Sterol signatures have also been shown to respond to individual wastewater releases (Noblet et al., 2004; Pratt, 2005; Pratt et al., 2007).

1.4 Whitening Agents

Detergents and brighteners have been used as tracers of wastewater contamination, and include fluorescent whitening agents (FWA), sodium tripolyphosphates (STPs) and long-chain alkylbenzenes (LABs). Of these, FWA have received the most attention. FWA are diarylethenes that resemble structurally the dyes used on cotton cloth (Poiger et al., 1996). FWA make up 0.15% of detergents and are used to brighten clothing (Poiger et al., 1996). After washing, 5-80% of the FWA remain in the wash water (Poiger et al., 1996). FWA have been used as tracers of domestic wastewater from septic tanks (Close et al., 1989) as well as wastewater treatment plant (WWTP) effluent (Gilpin et al., 2003; Gilpin et al., 2002; Poiger et al., 1996; Sankararamakrishnan and Guo, 2005) and in places where both these and industrial sources were possible (Hartel et al., 2007; Hartel et al., 2007; Uchiyama, 1979). One disadvantage of FWA as tracers versus sterols is that FWA are an indirect indicator of the possibility of wastewater: they are byproducts from laundry whereas fecal sterols are excreted in fecal matter. Additionally, FWA are only markers of anthropogenic inputs, and thus do not aid in identification of animal sources. Also, certain FWA photodegrade in the environment (Canonica et al., 1997; Kramer et al., 1996). Hayashi et al. (2002) attributed the ~10-20% loss of FWA to photodegradation when analyzing the utility of FWA as molecular markers for anthropogenic pollution in Tokyo Bay and adjacent rivers. Another problem with using FWA is that the most rapid and simplest method of measurement, direct fluorimetric detection, is subject to interferences from natural organic matter (NOM) and non-wastewater pollution with chemicals that fluoresce at the

same excitation and emission wavelength as FWA, such as motor oil (Hartel et al., 2007b; Uchiyama, 1979). Additionally, FWA have a high affinity for binding to sediments and therefore may be removed from septic plumes before reaching streams (Kramer, 1992). Despite these problems, the ease of measuring FWA makes it potentially powerful in quickly identifying human wastewater pollution.

Mapping the FWA concentration along the course of streams will allow for comparisons of FWA concentrations up and downstream of possible sources, as determined by land cover data, and perhaps will be able to isolate human wastewater inputs. For example, if a spike in concentration is measured at a location where sewer overflows are possible or down gradient of homes with failing septic tanks, this would provide evidence of a source of human wastewater entering the stream. Further, determining whether or not fecal coliform counts correlate with FWA concentrations provides evidence as to whether human wastewater is likely contributing a significant amount of coliform to the creeks. If FWA concentrations and fecal coliform counts strongly correlate it is likely that human wastewater is the major contributor of fecal coliform for a given stretch of stream. A drawback of the FWA analysis is that it gives no information to differentiate between the other possible sources (cattle or wildlife) of fecal pollution.

1.5 Shortcomings of Other Chemical Tracers

Several other chemical tracers, like caffeine, fragrances, pharmaceuticals and personal care products (PPCPs), plasticizers, and flame retardants, have been used or suggested as indicators of human wastewater and fecal pollution, but all have

shortcomings in their applicability to source tracking (Scott et al., 2002). One challenge in using chemical methods is identifying chemicals that will be abundant enough for accurate and precise measurement. Given the small human population in the Cane and Little Cane creek watersheds it is likely that many chemical tracers will remain below detection limits due to their lack of use in large quantities, or will require more difficult techniques like extracting from large volumes of water. Another issue is that for many possible chemical tracers their fate in the environment is not well enough understood. For instance, research into PPCPs in the environment is a new and rapidly expanding field, but beyond their measurability in the field not much data are available, particularly as to their fate in the environment (Cimenti et al., 2007).

A final problem in identifying chemical tracers is finding chemicals that are suitably conservative in the environment. For example, while the presence of caffeine indicates anthropogenic pollution (Ferreira, 2005; Glassmeyer et al., 2005; Kolpin et al., 2002; Peeler et al., 2006; Sankararamakrishnan and Gou, 2005; Siegener and Chen, 2002) it has been criticized as a tracer because it is not conservative (Seiler et al., 1999). This leads to ambiguity in the interpretation of a negative result – the lack of caffeine either means there was no contamination, there was contamination but the caffeine already degraded, or there is contamination but it is low enough to make caffeine below detection.

CHAPTER 2

OBJECTIVES

The purpose of this study was to use chemical tracer methods to identify possible point and non-point sources of fecal coliform pollution in Cane and Little Cane Creeks.

Specifically, the objectives were to:

1. identify sampling locations that likely contain a range of sterol concentrations and fecal pollution sources,

2. measure chemical tracers (fecal sterols, FWA, and/or others) at selected sampling points on Cane and Little Cane creeks,

3. interpret results of chemical tracer measurements by comparing measured sterol ratios to known ratios for human or animal pollution, determining the magnitude of FWA pollution at sites, etc. and match results with identified sources,

4. compare results of different source tracking methods, and

5. provide suggestions as to which sources should be controlled to improve surface water quality and which sources do not affect surface water quality.

CHAPTER 3

MATERIALS & METHODS

3.1 Chemicals

Methanol (GC Grade, 99.9%) and isooctane (2,2,4-trimethylpentane) (GC Grade, 99.99%) were purchased from EMD Chemicals Inc., hexane (ACS grade, 98.5%) from BDH Chemicals Ltd., and acetone (HPLC grade, 99.9+%) and dichloromethane (DCM) (HPLC grade, 99.9+%) from Burdick and Jackson. All solvents were purchased in grades high enough to eliminate the need for further purification. Silica gel (100-200 mesh, Type 150A) was purchased from BioRad and Mallinckrodt Chemicals. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from TCI America.

Coprostanol, coprostanone, and 5α-cholestanone for standards were purchased from Sigma, stigmasterol and stigmastanol for standards from TCI America, β-sitosterol from CalbioChem, and 3β-choelstanol for standard from AlfaAesar. Surrogate standard cholesterol-d6 was purchased from Cambridge Isotope Laboratories and internal standard perylene-d12 from ChemService. FWA standard Tide (Proctor and Gamble) was purchased from a local grocery store.

3.2 Sampling

3.2.1 Sterols

About 50mL of sediment was collected from deposition zones in plastic syringes from up to seven selected sampling sites along the streams on four separate sampling trips (5/2/2008, 5/21/2008, 6/4/2008, and 6/23/2008). Specific sites are discussed in

Section 4.1.2. Syringes were used to scoop fine surface sediment, not for taking sediment cores. Samples were stored in a cooler on ice while in the field and during transport. After transport to the laboratory, samples were transferred to glass bottles and stored in glass jars at 4°C wet until they could be wet sieved and left overnight to dry in the hood. Typically wet-sieving was performed within 1-2 days but samples were never stored wet for more than a week. Then, samples were stored in dry glass jars at 4°C until they were extracted, derivatized, and analyzed. Analysis was performed as soon as possible (never storing for more than 30 days) as there is potential for sterol degradation as a function of time during storage, prior to extraction (Pratt, 2005). Whenever possible all three samples were analyzed for each site to help determine spatial variation at a given site, and always at least one of the triplicate samples from each sampling at each site was analyzed in replicate to determine variability in analytics.

For water column samples, 20L of water was collected in five 4L brown borosilicate jugs in duplicate (when possible) and stored at 4°C for no more than four days before filtering. Samples were filtered through three prebaked GF/F filters and the filters were then stored wet at -25°C until analysis. For analysis, filters were removed from the freezer and left to thaw and dry in the hood for several hours. Filters were stored for up to nine months before extraction. Water samples were collected on six separate sampling trips in the fall of 2007 (9/6/2007, 9/22/2007, 9/26/2007, 10/4/2007, 10/22/2007, and 6/23/2008) and once in the spring of 2008 (6/23/2008), collecting samples from up to three sites a trip. Sample sites included LC1, LC2, LC4, LC5, LC7, LC10, C1, C2C, and C3, labeled on the map in Figure 1. Sediment weights on the filters

were either determined by baking and weighing after extraction (9/6-10/22) or by air drying and weighing before extraction (6/23/08).

One method blank was run with each batch to ensure there were not sources of analytes in the solvents or added during the extraction and derivatization scheme. The method blank was also used to help in setting the analytical detection limit. Minimum quantitation limits were arbitrarily set at five times the background and were validated with standards.

3.2.2 Fluorinated Whitening Agents

Triplicate 10-mL water samples were taken from sites established by FOLKS along Cane and Little Cane Creeks, Walhalla, SC, in solvent rinsed 10mL to 1L borosilicate glass bottles. Samples from the Oconee County/Coneross Creek WWTP (WWTP), which receives the sewered water from Walhalla, were collected in triplicate (5/13/2008, 5/21/08). Samples were stored in the dark at 4°C and processed as soon as possible, always within 12 hours. Three sampling sweeps were made during the winter (2/19/2008) and spring (5/2/2208, 5/21/2008) of 2008 in different flow conditions (low and normal), collecting samples from five to 20 sites (see results for sites).

FWA samples were taken and split in triplicate during all rounds of sampling. One DDI water blank was included with each batch to ensure there was not interference from FWA contamination of glassware.

3.3 Preparation for Analysis

3.3.1 Sterols

3.3.1.1 Extraction

All glassware was solvent rinsed with methanol, acetone, and hexane. Sediment samples were homogenized and wet-sieved prior to analysis and aliquots were analyzed. Extraction and purification were based on Isobe et al. (2002). Approximately 5g of sediment was scooped into 50-mL centrifuge tubes and ultrasonically extracted in 30mL methanol, 30mL methanol:DCM (1:1, v/v), and 30mL DCM, consecutively, for 15 minutes each. For filters, all three filters were placed in a single 50-mL centrifuge tube and processed the same as the sediment samples. After each extraction, vials were centrifuged at 1200rpm for five minutes. The solvent supernatant was collected after each extraction and transferred to a 100-mL pear shaped flask. The combined solvent extracts were concentrated to dryness using a rotary evaporator at 35°C. Efficiency of the extraction schemes was evaluated by running blanks spiked with sterol standards and spiked sediment or filter samples and calculating recovery.

The dry weight of samples was determined for subsamples after baking at 105°C for 24 hours, cooling in a desiccator, weighing, and repeating until a constant weight was achieved.

3.3.1.2 Purification by silica gel chromatography

Samples were purified by silica gel chromatography, modified from the method used by Isobe et al. (2002). The silica gel was baked at 380°C for four hours to remove organic contamination, activated at 200°C for 5-6 hours, then deactivated by adding 5%

(w/w) distilled water and stored in an air-tight glass jar in a desiccator until use. Samples were dissolved in 1 mL of hexane/DCM (3:1, v/v) and pipetted on top of the silica gel column (1cm i.d. x 9cm; 100-200 mesh). Aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, linear alkylbenzenes and other components of similar polarity were eluted with 20 mL of hexane/DCM (3:1, v/v). Sterols were eluted with 40mL DCM. The 2nd fraction was collected in a 100 mL pear shaped flask and rotoevaporated to dryness at 30°C. The residue was dissolved in 1 mL of DCM and transferred to a GC vial and blown to dryness under N_2 stream.

3.3.1.3 Derivatization

The residue was dissolved in 100µl BSTFA and heated to 60°C for 24 hours in a sand bath to facilitate derivatization (Leeming et al., 1996; Pratt, 2005). During derivatization with BSTFA, the BSTFA trimethylsilates the alcohol functionalities making the sterols volatile and thermally stable for GC analysis. Samples were made up to a final volume of 500µL by adding 200µL isooctane and 200µL of a 5.0×10^{-5}g/mL perylene d-12 (internal standard) in isooctane for GC-MS analysis.

3.3.2 FWA

FWA was determined by direct fluorimetric detection and thus no sample preparation scheme was used, based on Hartel et al. (2007a). Water samples were transferred directly into plastic cuvettes for analysis using disposable glass pipettes.

3.4 Analysis

3.4.1 Sterols

GC analysis was performed on a Varian 3800 GC with a Varian 4000 MS fitted with a 60m DB-5ms column (0.25 ID, 0.25µm film thickness) with helium as the carrier gas. Flow was set to 1ml/min. Injections of 1µl were made into a 290°C injector in splitless mode with the split turned on at 0.75 minutes. The transfer line was set to 310°C and the ion trap to 220°C. The column oven was programmed to 50°C for 1.5 minutes, ramped at 16.5°C/minute to 180°C, ramped at 1.3°C/minute to 280°C, and finally ramped at 6.6°C/minute to 310 and held for 30 minutes. The MS was run in select ion monitoring mode (SIM) and quantitation was based on peak areas of major ions as listed in Table 1.1.

Calibration curves were generated using standard solutions of the following sterols: coprostanone, coprostanol, cholesterol, cholestanol, cholestanone, stigmasterol, β-sitosterol, and stigmastanol. The calibration curves were made to correct for any inconsistencies in the amount of sample injected via the autosampler by plotting the ratio of the mass of analyte to the mass of internal standard against the ratio of the peak area of the analyte to the peak area of the internal standard. Relative response factors were calculated based on the perylene d-12 internal standard (m/z 264) and used to determine whether the response was linear with respect to concentration within the chosen concentration range (~5-100ng/µL). Given the exploratory nature of this research, control charting was established at (at most) +/-25% of response factor for a calibration range between 5ng-100ng. With each batch, a check standard was measured at the

beginning of a batch analysis and if it was within the stated confidence interval (+/-25%) then the GC was not recalibrated. If the check standard was outside the confidence interval bounds another check standard was run and if it was also outside the confidence interval bounds then the GC was recalibrated. Check standards were run again at the end of each batch analysis period. Full calibrations were not run with each batch except as needed because of the extended length of time needed to analyze each sample (at 2 hours/sample, ten hours were required to create a calibration curve). In addition, an isooctane blank was run with each batch to establish an analysis baseline and to validate no analyte carryover was occurring during analysis.

3.4.2 FWA

Standards of commercial laundry detergent Tide (Procter and Gamble, contains optical brightener DAS1) plus double deionized (DDI) water were prepared (25mg/L, 50mg/L, 75mg/L, 100mg/L, 150mg/L, 200mg/L) at least 2 hours prior to analysis to allow initial degradation to occur (Center of Watershed Protection and Pitt, 2004). Tap water and DDI water blanks were analyzed with each sweep. Samples were analyzed at room temperature in discrete mode on a MolecularDevices M2 fluorimeter and read within 30 seconds to avoid any heating effects of the fluorimeter's UV lamp. The excitation wavelength was fixed at 360nm and emission wavelength at 410nm (Close et al., 1989). Equivalent detergent concentration of >100mg/L has been considered positive for optical brightener and, therefore, indicating likely contamination by human fecal pollution (Hagedorn et al., 2003).

Standard additions for FWA analysis were made by adding 200, 500, and 1000 mg of detergent per liter to 500mL of water from Little Cane Creek, Cane Creek, and CCWWTP in triplicate. Standard additions were analyzed the same as samples described above.

3.5 Data Handling

All data handling was performed in Microsoft Excel using standard statistical methods. Statistical significance was determined using student's t-test with a 95% confidence interval.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Sterols

4.1.1 Method Development and Quality Assurance and Control

4.1.1.1 Notes on Other Methods Tried

Two different extraction and two different derivatization methods were tried before choosing a technique for each step in the sample preparation. Extraction was attempted using a modified Bligh Dryer technique followed by saponification based on Pratt (2005), as well as the final method chosen based on Isobe et al. (2002). Derivatization was attempted via acetylation according to Isobe et al. (2002) in addition to the final method chosen, trimethylsilylation based on Pratt (2005) (note: variations using BSTFA in temperature, time, and presence of catalyst have been performed by all authors whose techniques are mentioned below except Isobe et al. (2002)). The results and reasons for selection of the final method are described below.

Several extraction methods have been used for sterol analysis. The most popular is a modified Bligh Dryer (Leeming et al., 1996; Leeming et al., 1998; Leeming and Nichols, 1996; Nichols et al., 1993; Nichols et al., 1996; Pratt, 2005; Pratt et al., 2007; Suprihatin et al., 2003) (described below). Solid-phase extraction followed by supercritical fluid extraction (Noblet et al., 2004), Soxhlet extraction (Elhmmali et al., 2000; Grimalt et al., 1990; Readman et al., 2005; Readman et al., 2004; Shah et al., 2007a; Shah et al., 2007b), and ultrasonic extraction (Isobe et al., 2002; Isobe et al., 2004; Maldonado et al., 1999; Mudge and Lintern, 1999) using different solvents have been

done as well. No known study has been performed comparing these methods except for Isobe et al. (2002) who compared a Soxhlet extraction to their ultrasonic extraction.

In this study, the extraction based on Pratt et al. (2007) was performed on sediment before the ultrasonic extraction based on Isobe et al. (2002) was chosen. It involved a modified Bligh Dryer extraction technique followed by saponification to remove saponifiable lipids before derivatization. The modified Bligh Dryer technique first required extracting sediment samples into 30 mL water, 75 mL methanol, and 37.5 mL chloroform (in a separatory funnel, shaking vigorously and leaving overnight) then adding 37.5 mL chloroform and 37.5 mL of water and back extracting into the solvent. The lower solvent layer was then collected and dried followed by saponification with 10% NaOH solution, extraction, and reduction to dryness for derivatization. This method was abandoned for several reasons, including because it was more time consuming, involved a more complex double extraction, required more glassware and solvent, and did not have a clean-up step. Further, Isobe et al. (2002) determined that saponification affected relative recoveries of sterols drastically enough that they recommended skipping that step. The method of extraction (Isobe et al., 2002) included a clean-up step and was faster, used less sediment and solvent, and required only one extraction (no back extraction) and thus was considered easier to implement and likely a better choice in terms of recovery.

Derivatization by both acetylation and trimethylsilation were tried before settling on trimethylsilation. The acetylation method was based on Isobe et al. (2002), acetylating the alcohol functionalities with acetic anhydride catalyzed by pyridine,

followed by removal of the acidic byproducts formed. Complete derivatization could not be achieved following the method used by Isobe et al. (2002), nor by modifying their scheme by adding more catalyst, adding more derivatizing agent, adding both more catalyst and more derivatizing agent, providing more time for derivatization to occur, or heating. Compounds showed double peaks where a smaller peak would rise from the tail of peaks identified by the NIST library as being the derivatized forms of the analyte making quantification of the peak area impossible. The chromatograms did not improve with any of the modifications listed above. Isobe et al. (2002) stated they chose this method because derivatization with BSTFA, which fails in the presence of moisture, was found to be incomplete in their humid climate because samples dissolved in BSTFA went crystalline. Aside from the problems with quantifying β-sitosterol (see Section 4.1.1.4) that could have been caused by incomplete derivatization, the BSTFA derivatization chosen was found to give more complete derivatization (see Figure 4.1). Problems with samples crystallizing only occurred when a waterbath was used to heat samples and were never observed when the mode of heating was switched to heating in a sand bath. In addition, the BSTFA derivatization is recommended because it is easier than the acetylation. To get trimethylsilyl (TMS) forms of the sterols, extracted sterols are dried and dissolved in BSTFA and left for 24 hours at 60°C, then are made up in solvent for analysis. Acetylation requires 24 hours to derivatize followed by an extraction into solvent, sodium sulfate chromatography to remove any inadvertently collected water, drying under N_2, and then dissolving in solvent for analysis. BSTFA derivatization also avoids the use of pyridine, the toxic and corrosive catalyst used in the acetylation scheme.

Additionally, BSTFA derivatization has been used (in varying schemes) by all researchers mentioned in this paper other than Isobe et al. (2002).

4.1.1.2 Chromatography

Example chromatograms in SIM and full scan mode are shown in Figures 4.1 and 4.2, respectively. Sterols were identified by comparing retention times for authentic standards. Some sterols were in the NIST MS library in their trimethylsilyl (TMS) ether form (i.e. derivatized cholesterol) and those are indicated in Figure 4.2. All sterols and the internal standard were quantified by integrating the peak area of their major ion as measured in SIM mode. Examination of the chromatograms indicates that other compounds with similar elution times were present in the samples. This is not surprising because the sterol suite chosen did not exhaust all possible sterols in the samples. Library searches indicated campesterol but without having standards for other sterols it is difficult to confirm the identity of the peaks and impossible to relate the peak area to concentration.

The sterol suite chosen for this study was selected for several reasons. The goal was to choose a sterol suite that maximized the number of calculable source identification (SID) ratios. As mentioned in the introduction different sterol suites have been perused by different researchers, therefore choosing a suite that overlapped with other work was a priority. A constraint on the chosen sterol suite was cost. SID using sterols is still not a standard method and sterol standards can be expensive because demand is not high. Notably, 24-ethylcoprostanol was not quantified in this study because of the price ($500/10mg). 24-Ethylcoprstanol is the herbivore fecal marker and could have provided

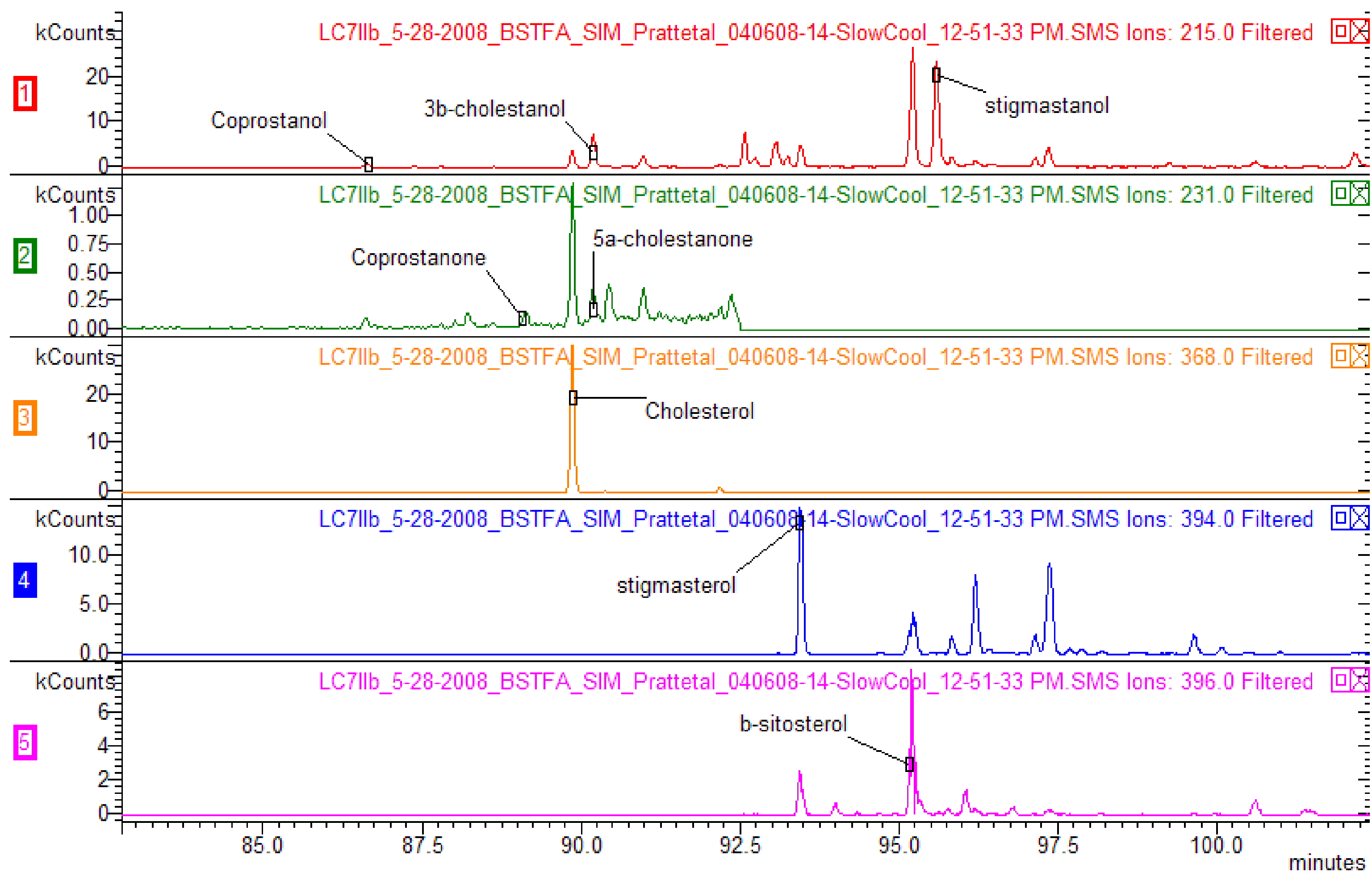


Figure 4.1. Chromatograms for LC7IIb (5/21/08) in SIM mode for (1) 215, (2) 231, (3) 368, (4) 394, and (5) 396 with analytes of interest labeled.

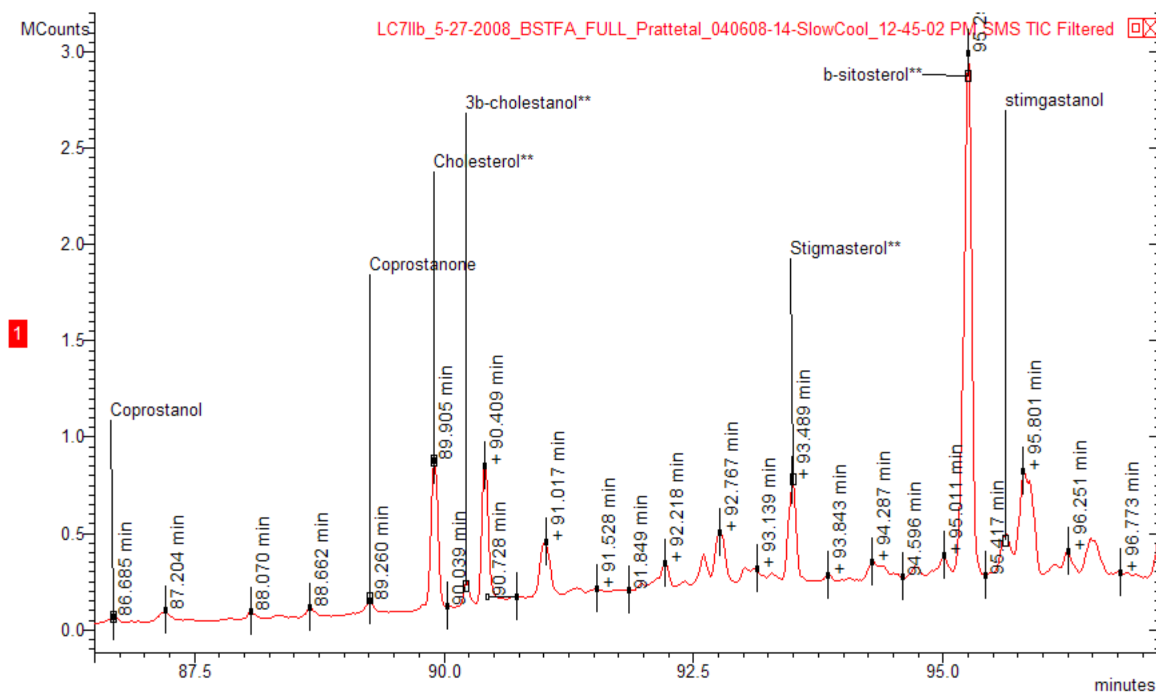


Figure 4.2. Full scan chromatogram for LC7IIIb (5/21/08) with analytes labeled. ** For compounds that were identified as library matches in addition to matching the retention time and major ions for each analyte. The others were not in the library.

valuable information about this stream system considering the large cattle population in the watershed. It is recommended that this sterol be purchased for future work as will be discussed in Chapter 5.

<u>4.1.1.3 Surrogate Standard</u>

Radio-labeled cholesterol (cholesterol-d6) (m/z 370) was purchased for use as a method internal standard when the derivatization scheme was via acetylation (see description of derivatization in Section 4.1.1.1). It was suggested as the method internal standard for derivatization via acetylation by Isobe et al. (2002) but it was found to

coelute with cholesterol when acetylation was being evaluated as the derivatization scheme. Despite the fact that it was purchased for use as the method internal standard for acetylation, cholesterol-d6 was evaluated for use as a method internal standard for this study again after the derivatization scheme was changed to trimethylsilylation. However, cholesterol-d6 and cholesterol did not have significantly different retention times when derivatized using BSTFA and again created a quantification problem by coeluting with cholesterol. Because it coeluted with an analyte of interest in the final derivatization scheme, cholesterol-d6 was abandoned as a method internal standard. Noblet et al. (2004) successfully used cholesterol-d6 as an internal standard (for quantifying inconsistencies in injection volume rather than as the method internal standard to quantify losses due to inconsistencies in extraction and quantitative transfer including spills) with the same derivatization scheme but used a different phase GC column (DB-XLB, (14%-Diphenyl)-Methylpolysiloxane, as opposed to DB-5ms, (5%-Phenyl)-methylpolysiloxane). Hawkins-Writer et al. (1995) reported using cholesterol-d7 as a surrogate standard and did not report problems with co-elution but also did not specify the column used for GC-MS analysis. Because it would have only accurately quantified efficiency of extraction for unlabeled cholesterol and would not have necessarily been extracted as efficiently as the rest of the compounds, there was no surrogate standard and perylene d-12 was used as the internal standard. Any spills that occurred during preparation for analysis were noted. Others have successfully used 5α-cholestane (Leeming et al., 1996; Leeming et al., 1998; Leeming and Nichols, 1996; Nichols et al., 1993; Nichols et al., 1996; Pratt, 2005; Pratt et al., 2007), 5α-androstan-3β-ol

(Maldonado et al., 1999; Noblet et al., 2004; Peng et al., 2005; Peng et al., 20002; Readman et al., 2005; Readman et al., 2004), and 5β-pregnol (Elhmmali et al., 2000) as a surrogate standard for BSTFA derivatization; and these compounds could be useful to purchase for future investigations. None of these three alternative surrogates would serve as perfect compounds for determining extraction efficiencies because of the structural differences between them and the analytes of interest, but each would help quantifying any losses due to spills.

4.1.1.4 Calibration and β-sitosterol Quantification Issues

Example calibration curves for sterols are included as Figures A 1a-8a with their corresponding plots of relative response in Figures A 1b-8b in Appendix A. R^2 for the calibration curves were all above 0.99 except for coprostanone with R^2=0.9772 and 5α-cholestanone with R^2=0.9884. Relative response was reasonably constant (+/-15%) for all analytes (see Figures A 1b, Figure A 3b, Figure A 4b, Figure A 6b, Figure A 7b, Figure A 8b in Appendix A) except coprostanone and 5α-cholestanone (Figures A 2b and 5b respectively in Appendix A). For the stanones, relative response increased with increasing mass. The response increased more in intervals between the lower masses (between 5ng and 20ng) than the higher masses: coprostanone's response at 100ng is about seven times that at 5ng and 5α-cholestanone's response at 100ng is about four times that at 5ng. Because the calibration curves R^2 values were above 0.95 and due to the exploratory nature of this study the relative responses for coprostanone and 5α-cholestanone were considered acceptable. It is thought that the ketone functional group on these compounds (absent from the other analytes which are alcohols) may have

affected derivatization as BSTFA trimethylsilates by undergoing nucleophilic attack on the silicon atom which displaces the active hydrogen proton in an alcohol group (Knapp, 1979). Quality control check standards were run at the start and end of each analysis batch and when they fell out of +/-25% range for any analyte (except β-sitosterol) the instrument was recalibrated as explained above. β-sitosterol was seen to have split peaks for some samples and occasionally in the quality control test standard making accurate quantification impossible. Since it was not needed in the ratios used for source identification, β-sitosterol data are not included, but possible reasons for the peak splitting are discussed below. A calibration curve for the internal standard, perylene-d12 is included in Appendix A as Figure A 10 to demonstrate that its response was linear with concentration as well. Perylene-d12 was always spiked into samples at the same concentration but because the purpose of adding it was to make sure that injection volume was constant it is important to check that it has a linear response with concentration in the expected concentration region.

Split peaks can be seen for a number of reasons, including poor injection technique, poor column installation, mixed sample solvent, detector overload, coeluting contaminant peaks, or sample degradation (Anal Chem, 1998). It is important to note that the chromatography of β-sitosterol was not consistently poor and occurred during analysis of check standards as well as samples. No specific pattern was observed, for example one check standard run immediately after being made could have split peaks for β-sitosterol while another would not. Most of the possible causes can be ruled out. Poor injection technique (i.e. too low injector temperature) was unlikely because if this were

the case one would expect the peaks to be consistently split, and the same goes for poor column installation. If the mixed solvent used for analysis (100mL BSTFA mixed with 400mL isooctane) was the cause, one would expect that to be a consistent problem, splitting peaks for β-sitosterol and likely other sterols during every analysis. Detector overload is unlikely because some test standards had split peaks and these standards were known to be at concentrations in the acceptable range for the MS detector. Again, because test standards showed peak splitting it is unlikely that a contaminant was coeluting since there was none in the solvent blank.

It is possible that β-sitosterol may not be stable in the derivatized form, that incomplete derivatization caused an underivatized compound to co-elute with β-sitosterol, or that TMS β-sitosterol was not stable in the mixed solvent. Pratt et al. (2007) noted that occasionally derivatization was incomplete using the same derivatization scheme and suggested that humid weather might have been the cause, but they did not specify how they knew derivatization was incomplete or whether it was for all compounds. Isobe et al. (2002) chose to derivatize with an acetylation scheme because humidity caused samples to become crystalline (BSTFA attacks alcohol functionalities and forms crystals when exposed to moisture), but this problem was never observed in this work. Perhaps a derivatization study singling out β-sitosterol could reveal the problem, but since it was not needed for any of the SID sterol ratios chosen for this project, the quantitation of β-sitosterol is not reported as the chromatography was not reliable. An example of a split β-sitosterol peak is shown in chromatogram in Figure 4.1.

4.1.1.5 Silica Gel Clean-up Modification

A clean-up step after extraction prior to derivatization and analysis was included to help remove organic interferences. Without the clean-up step, almost all samples were colored, which generally indicates a sample may not be pure enough for GC analysis. The concern is dirty samples can introduce contaminants that foul the injection port liner or the GC column and that contaminants may coelute with compounds of interest. Other researchers have used both silica gel (Isobe et al., 2004; Isobe et al., 2002) and combined alumnia-silica gel (Grimalt et al., 1990; Peng et al., 2005; Peng et al., 2002; Readman et al., 2005; Readman et al., 2004) chromatography columns to purify samples. The method chosen was based on Isobe et al. (2002). The original method proposed by Isobe et al. (2002) involved a silica gel clean-up column: after extraction, the solvents were reduced to dryness and the remaining dried residue was dissolved in DCM:hexane (1:3) and eluted from the column with successive aliquots of 20 mL DCM:hexane (1:3), 40 mL DCM, and 20 mL DCM:acetone (7:3). Isobe et al. (2002) combined the second and third fractions to obtain the sterol containing sample. It was noted in this study that after extraction but prior to clean-up, sample residues were yellow to brown in color. After collecting and combining the 2nd and 3rd fractions as Isobe et al. (2002) did, samples retained most of their original color, indicating the continuing presence of organic contaminants. As it was observed that the DCM:acetone fraction eluted most of the color from the columns, it was decided not to elute this fraction and not include it in the final sample.

To ensure that collecting only the DCM fraction as the sterol analysis fraction did not lead to significant losses, stock standard sterol solution (500 µL of 0.01g/100mL solution) was pipetted onto a clean-up column and all of the three fractions above described by Isobe et al. (2002) were eluted, and kept individually (uncombined) for derivatization and analysis. The purpose was to see what percentage of each sterol was eluted with each fraction. The test was performed in triplicate and results are shown in Table 4.1, as percentage of sterol recovered with standard deviation and percent relative standard error. The first fraction, which was discarded in both schemes, contained 0.0-0.4% of the initial sterol by weight. The second fraction contained from 76.3 to 91.7% depending on the sterol, and thus represented the majority of the initial amount added. The third fraction contained only 0.1 to 3.0% depending on the sterol. Thus it was

Table 4.1 Clean-up column method validation. Mean % recovery by fraction +/- Standard deviation with (%RSE).

	Fraction1 20mL Hexane:DCM (3:1, v/v)	**Fraction 2 40mL DCM**	**Fraction 3 30mL Acetone:DCM (3:7, v/v)**	**Unaccounted for**
Coprostanol	0.3 +/- 0.4 (122.1)	91.7 +/- 5.8 (6.4)	0.8 +/- 0.4 (54.7)	7.2 +/- 5.2 (72.4)
Coprostanone	0.0 +/- 0.1 (155.6)	84.6 +/- 0.8 (0.9)	0.1 +/- 0.1 (54.6)	15.3 +/- 0.8 (5.4)
Cholesterol	0.2 +/- 0.2 (127.0)	82.8 +/- 3.9 (4.7)	1.3 +/- 0.2 (15.4)	15.7 +/- 3.7 (23.7)
3b-cholestanol	0.3 +/- 0.4 (148.3)	80.6 +/- 2.6 (3.3)	2.9 +/- 0.4 (13.5)	16.2 +/- 2.0 (12.6)
5a-cholestanone	0.0 +/- 0.0 (146.3)	82.4 +/- 1.0 (1.2)	0.1 +/- 0.1 (57.9)	17.5 +/- 1.0 (5.4)
stigmasterol	0.2 +/- 0.2 (134.8)	81.0 +/- 0.6 (0.7)	1.4 +/- 0.2 (12.4)	17.4 +/- 0.5 (2.7)
b-sitosterol	0.4 +/- 0.4 (112.9)	77.0 +/- 5.4 (7.1)	2.7 +/- 0.3 (10.8)	19.9 +/- 6.0 (30.3)
stigmastanol	0.3 +/- 0.5 (145.8)	76.3 +/- 1.1 (1.4)	3.0 +/- 0.2 (6.8)	20.4 +/- 1.7 (8.4)

demonstrated that collecting only the second fraction recovered a majority of the sterols and the decision to not collect the third fraction did not significantly diminish recovery. Adding the percent recovery for all three fractions revealed that anywhere from 7.2 to 20.4% of the sterols were not accounted for by any of the three eluted fractions. Presumably, the sterols unaccounted for were retained on the silica gel column. It is possible that transfer was not quantitative, but that would have only introduced small losses. No spills were noted.

4.1.1.6 Recovery

Recovery for the method was determined by spiking blanks (either empty centrifuge tubes or fresh filters) with 500 μL of stock standard sterol solution (~0.01g/100mL) and running through the methods for extraction, clean-up and derivatization, and analysis in triplicate. Percent recoveries for each sterol are listed in Table 4.2. The average recovery ranged from 38.1 to 69.6% depending on the sterol of

Table 4.2 Percent recovery +/- Standard deviation with (%RSE) for spiked blanks.

	"Sediment"	Filters
	n=3	n=3
Coprostanol	69.6 +/- 2.5 (2.5)	59.6 +/-11.8 (19.7)
Coprostanone	46.8 +/- 6.4 (6.4)	38.1 +/- 13.0 (34.0)
Cholesterol	62.4 +/- 1.6 (1.6)	49.5 +/- 11.0 (22.3)
3b-cholestanol	57.8+/- 5.4 (5.4)	45.4 +/- 13.3 (29.2)
5a-cholestanone	57.4 +/- 4.3 (4.3)	40.7 +/- 9.5 (23.3)
stigmasterol	58.1 +/- 0.9 (0.9)	46.1 +/- 10.2 (22.1)
b-sitosterol		44.6 +/- 10.3 (23.2)
stigmastanol	53.8 +/- 4.5 (4.5)	43.1 +/- 11.9 (27.5)

interest. Recovery for the spiked sediment blank had smaller standard deviations than for the filters, with a relative standard deviation from 0.9-6.4% for sediment and 19.7-34.0% for filters. The standard deviations of the recovery for each compound represent precision of the recovery. Comparing standard deviation of the recoveries between the sediment and filter it can be seen that recovery for sediment was more precise. This result is not surprising as it is suspected that the microtip sonicator probe used did not emit enough energy for quantitative recovery from the filter samples, as evidenced by the fact that filters were not pulverized after sonication and the fact that extraction efficiency was low at low concentrations (see Section 4.1.1.7). However, despite this possible problem, there was not a significant difference in recovery between the different blanks except for 5α-cholestanone: significantly more was recovered from the sediment blank than the filter blank.

It is also necessary to compare each sterol to the other sterols in the analyzed suite in terms of recovery. Considering the ultimate purpose of measuring these sterols is for use in sterol ratios, comparing recovery from compound to compound is important. The goal is for recovery to not be significantly different to the point where the ratios become skewed. Thus, it is important to take a closer look at significant differences between recoveries by analyte before using them in ratios. In the spiked sediment blank there is a statistically significant difference in recovery between certain analytes. Recovery for coprostanol and cholesterol was significantly greater than for the other sterols, with recovery of coprostanol greater than that for cholesterol. Recovery for coprostanone was significantly smaller than for the other sterols, and stigmastanol recovery was

significantly greater than that for stigmasterol. For the spiked filter blanks a statistically significant difference in recovery between the analytes is seen only between coprostanol and the two quantified stanones: significantly more 5α-cholestanone was recovered than coprostanone. Possible ratio skewing due to differences in recovery will be discussed in Section 4.1.6.1 as it pertains to each ratio.

4.1.1.7 Extraction Efficiency

Sediment samples (one sample split and performed each in replicate for each of two sites) and a filter (one duplicate sample from a single site) were spiked with 500µL of the stock standard sterol solution and run through extraction, clean-up, derivatization and analysis. Aliquots of two sediment samples C1 (5/21/08) and LCS (5/4/08) were split and each of the splits was spiked and left to equilibrate for four days. One filter sample C3 (10/21/07) was spiked and left for one day to equilibrate. It is unknown if the time the spiked samples were left to equilibrate was enough for the spiked sterols to equilibrate with the sediment or the filters. In order to determine whether these times were sufficient one would need to perform an equilibration batch experiment spiking samples and leaving them to equilibrate for varying amounts of time (24, 36, 48 hours, etc) to determine how long it takes for equilibration to be achieved. Results are listed in Table 4.3. The majority (57.7-70.3%) of the spiked sterols from sediments was recovered and 60.0-67.3% of the spiked sterols from the filter were recovered. Comparing percent relative differences between the two sediment spikes reveals that reproducibility for the two ranged from 0.7% to 14.9% and showed variability from compound to compound and from one sample to the other. Only one filter spike was

performed due to the amount of labor required to collect and process a 20-L water sample in duplicate.

There were no significant differences in the amount of sterols recovered from one analyte to another in the spiked LCS sample. The C1 spiked sample however had significantly more cholesterol recovered than coprostanol and significantly less 3β-cholestanol than cholesterol. The filter sample C3 was done in singlet and thus no comment can be made as to whether the differences in amounts of sterols recovered was significant between compounds, but simply looking at the percentages suggests that no blatantly significant differences exist.

Table 4.3 Mean percent recovery +/- difference with (RPD) for sediment splits and mean percent recovery for filter spiked with 500uL of ~0.01g/mL stock solution. Sediment was left to equilibrate for 4 days and filters for 1 day.

	C1 Sediment n=2 4days	**LCS Sediment n=2 4days**	**C3 Filter n=1 1day**
	(5/21/08)	**(6/4/08)**	**(10/27/07)**
Coprostanol	65.6 +/- 1.6 (2.4)	62.6 +/- 1.2 (2.0)	65.3
Coprostanone	62.9 +/- 9.4 (14.9)	70.3 +/- 4.5 (6.4)	67.3
Cholesterol	69.2 +/- 1.0 (1.5)	65.8 +/- 3.1 (4.7)	63.0
3b-cholestanol	67.0 +/- 0.5 (0.7)	61.8 +/- 2.6 (4.2)	61.0
5a-cholestanone	67.2 +/- 5.8 (8.6)	61.5 +/- 2.2 (3.5)	63.8
stigmasterol	67.4 +/- 3.8 (5.7)	64.7 +/- 5.5 (8.4)	61.1
b-sitosterol			
stigmastanol	65.2 +/- 4.2 (6.4)	57.7 +/- 3.9 (6.7)	60.0

4.1.1.8 Derivatization Efficiency

Efficiency of derivatization was not calculated as the sterol standards were purchased in their un-trimethylsilylated form and derivatized with the same BSTFA scheme as used for the samples. TMS ether standards are not available for purchase for all the sterols measured. Therefore, efficiency of derivatization was built into the calibration. The calibration curve for any analyte related the known mass of sterol prior to derivatization with the peak area of the derivatized form of the sterol and because of this there was no motivation to determine its efficiency.

4.1.1.9 Method Blanks and Limits of Quantitation

Method blanks were performed for both the sediment and filter samples. Blanks for the sediment involved extracting without adding any sample and for the filters involved extracting from three fresh prebaked filters that had not filtered any samples. The initial method blank run indicated that some potentially significant cross contamination (0-19.9% error for cholesterol) was occurring. Because of this, effort was put in to better clean glassware (scrubbing during solvent rinsing) and the sonicator probe tip (wiping with methanol and hexane between samples). After implementation of better cleaning methods most sterols were no longer measurable in the blank and the two that were, cholesterol and stigmastanol, represented an added error of at most 0.4%.

Limit of quantitation (LOQ) was arbitrarily set at five times the background noise, which was determined by running solvent blanks. LOQ in sediment and water were calculated by taking the on-column limit of detection and assuming either 5 g of sediment or 20 L of water would be extracted. LOQs are listed by compound are included in Table

4.4. The LOQ was determined to range from 0.1ppb for coprostanol, 3β-cholestanol, 5α-choelstanone, and stigmastanol to 0.4ppb for coprostanone in sediment, and from 5.2 ppt for stigmastanol to 0.019 ppb for coprostanone in the water column. The detection limit

Table 4.4 Limit of quantitation by compound, arbitrarily set at 5 times the background. ng/g sediment was calculated assuming 5g of sediment and ng/L water assuming 20-L of water.

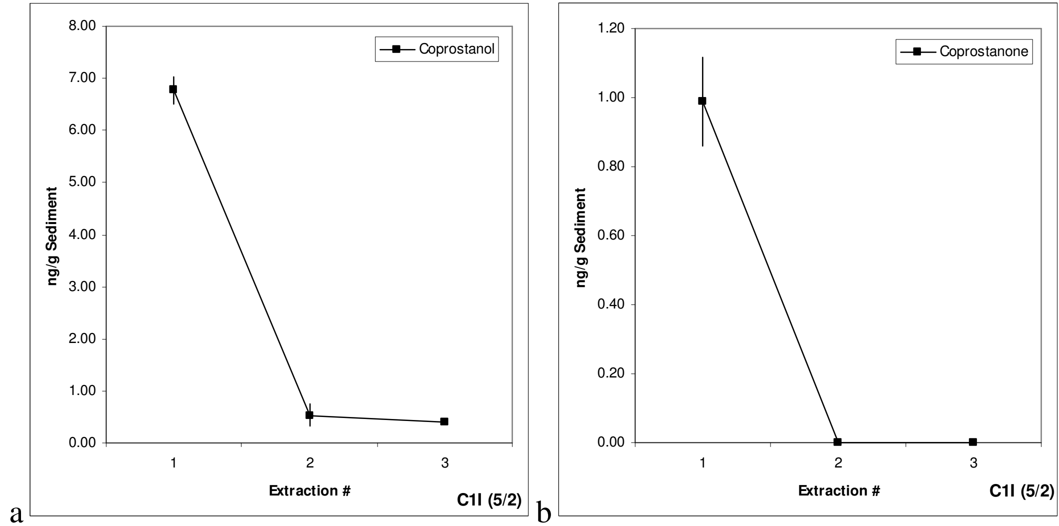
Compound	ng on column	ng/g Sed	ng/L Water
Coprostanol	$1.4x10^{-3}$	0.1	$7.2x10^{-3}$
Coprostanone	$3.8x10^{-3}$	0.4	$1.9x10^{-2}$
Cholesterol	$1.6x10^{-3}$	0.2	$8.1x10^{-3}$
3b-cholestanol	$1.1x10^{-3}$	0.1	$5.3x10^{-3}$
5a-cholestanone	$1.2x10^{-3}$	0.1	$5.9x10^{-3}$
Stigmasterol	$2.4x10^{-3}$	0.2	$1.2x10^{-2}$
b-Sitosterol	$1.6x10^{-2}$	1.6	$7.9x10^{-2}$
Stigmastanol	$1.0x10^{-3}$	0.1	$5.2x10^{-3}$

calculated here for sediment is similar to the 0.1ng/g for 5 g of sediment reported by Isobe et al. (2002), whose procedure this study's was based on. However, the detection limit for water samples in this study was 1-2 orders of magnitude lower in water than the 0.5ng/L in 1 L reported by Isobe et al. (2002). The lower detection limit in water for this study is due to the fact that a larger volume of water (20 L versus 1 L) was used for extraction.

4.1.1.10 Extraction in Triplicate

Extraction efficiency for samples was also evaluated by repeating the procedure from extraction to analysis for the same sample two times after the initial extraction. For sediment one sample from each of the first three sampling rounds (C1 5/2/2008, C2C 5/21/2008, C3 6/4/2008) was split and each split was extracted in triplicate. For filters,

one duplicate sample from fall 2007 (LC10 10/9/2007) was extracted in triplicate. Results are shown in Figures 4.3-4.5 for sediments and in Figure 4.6 for the filter.



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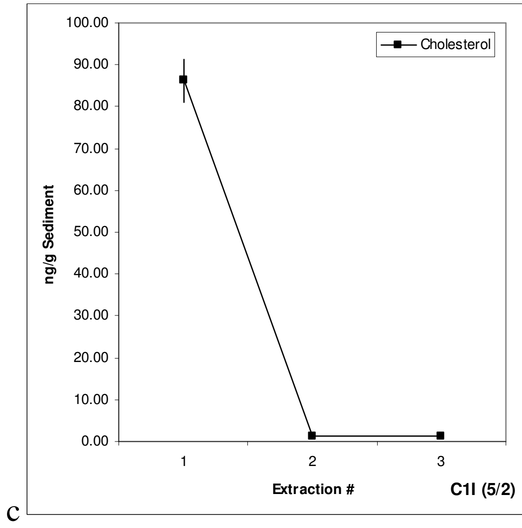

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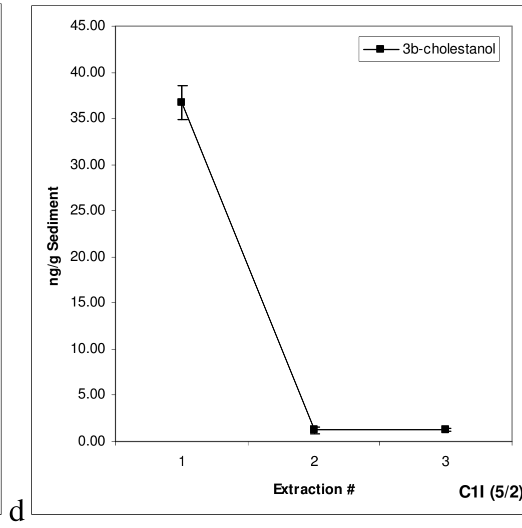

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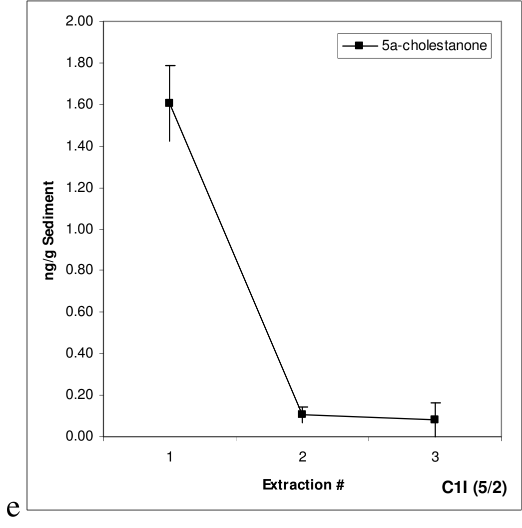

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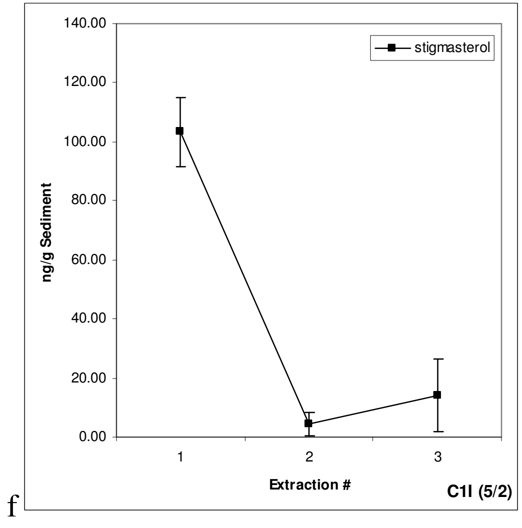


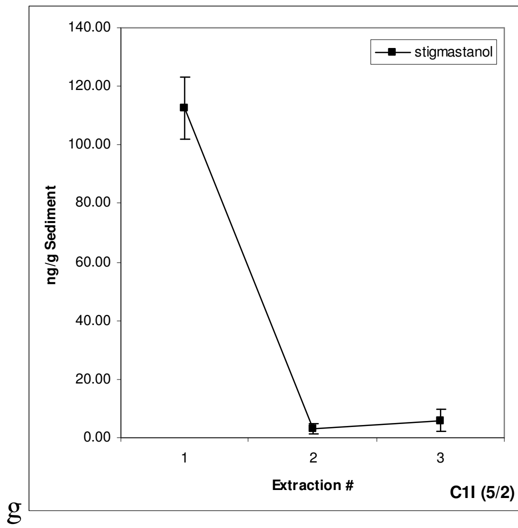
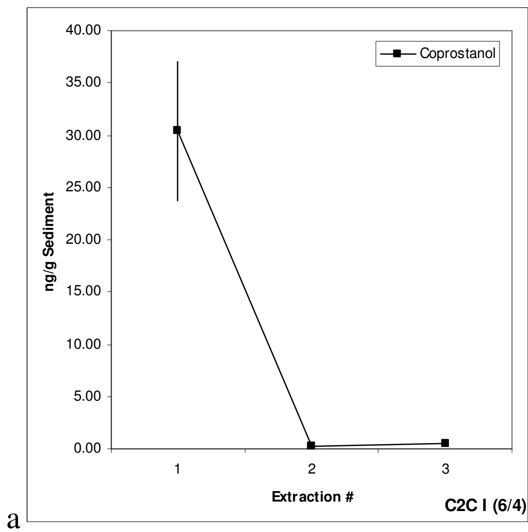
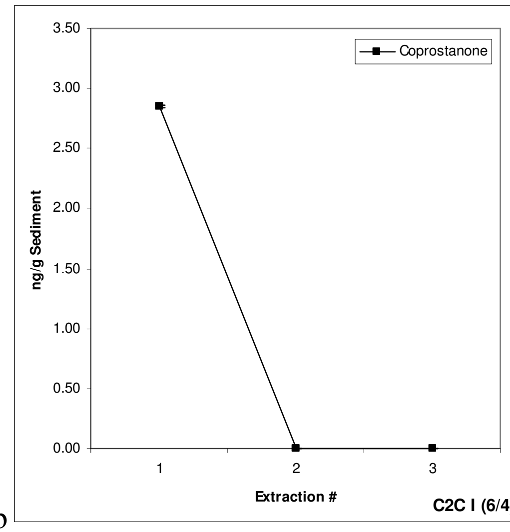
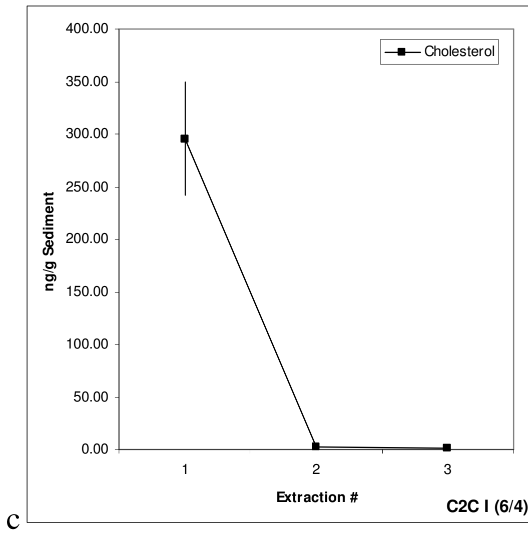
g 

Figure 4.3 Results of triple extraction of C1 I from 5/2/2008 performed in duplicate.

a 

b 



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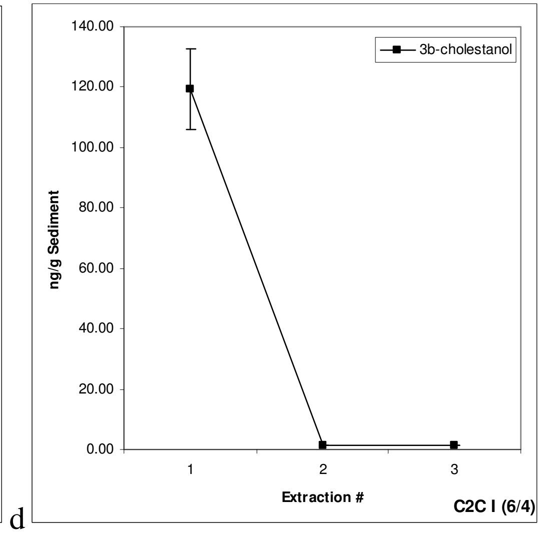

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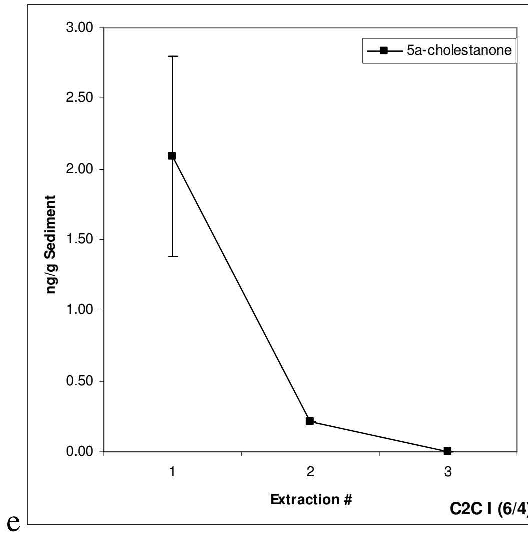

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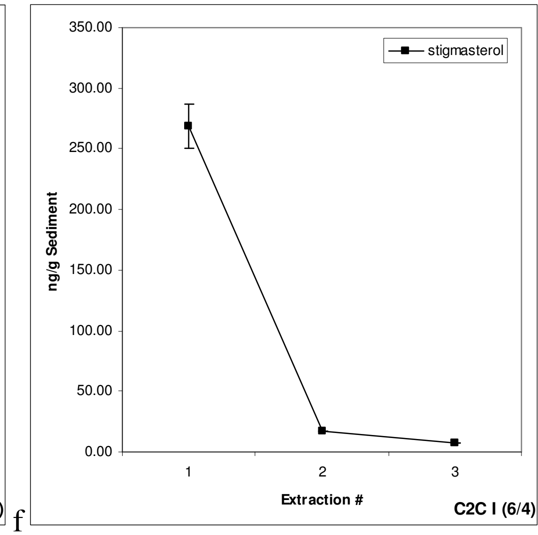

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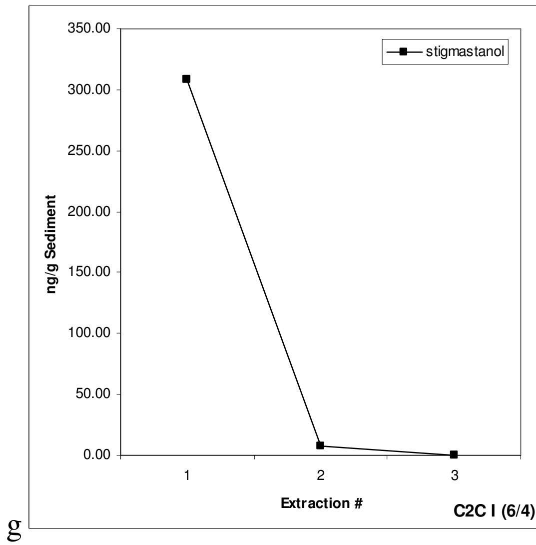
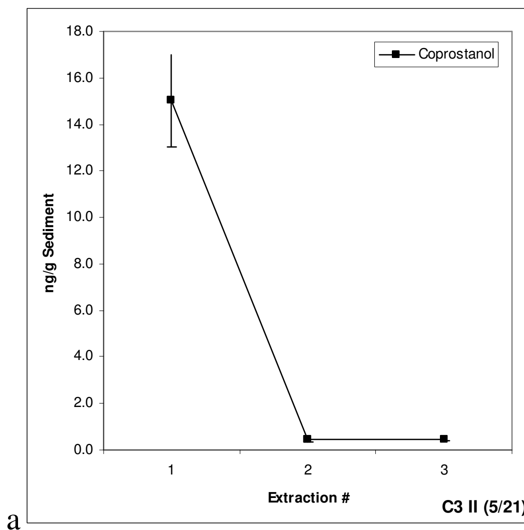
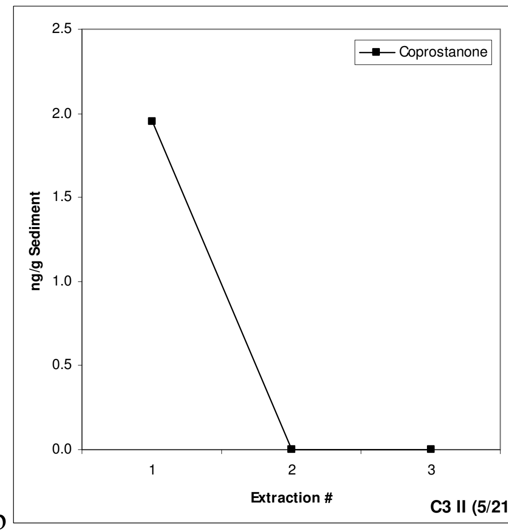
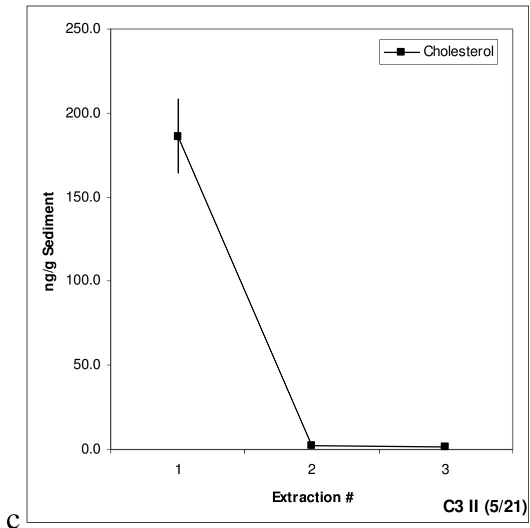
g 

Figure 4.4 Results of triple extraction of C2C I from 6/4/2008. The first extraction is the only one showing relative percent difference because the rest were performed in singlet.

a 

b 



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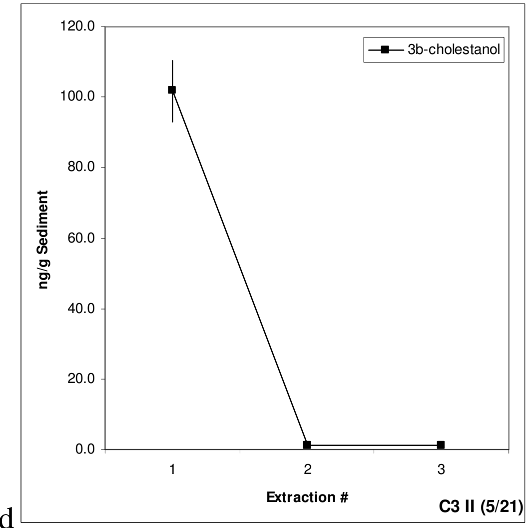

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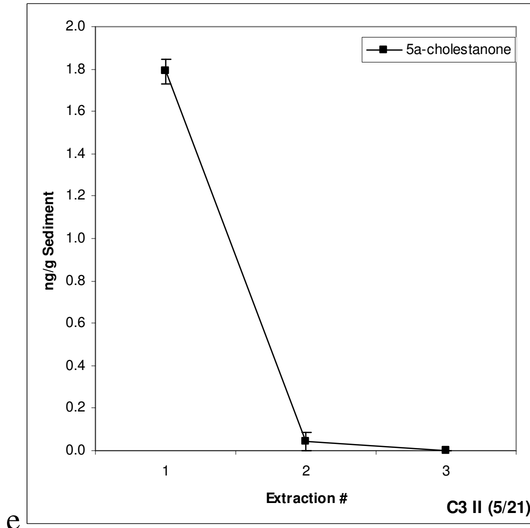

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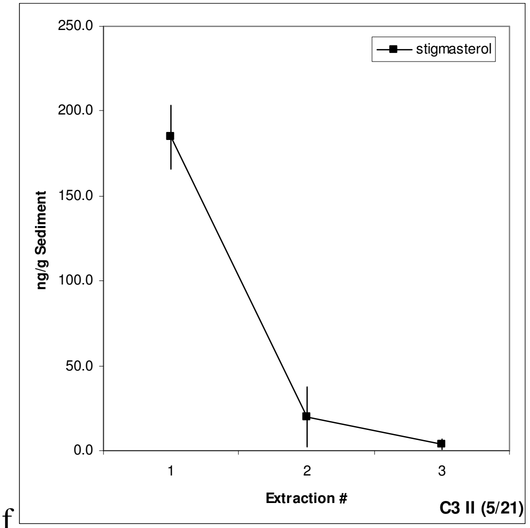

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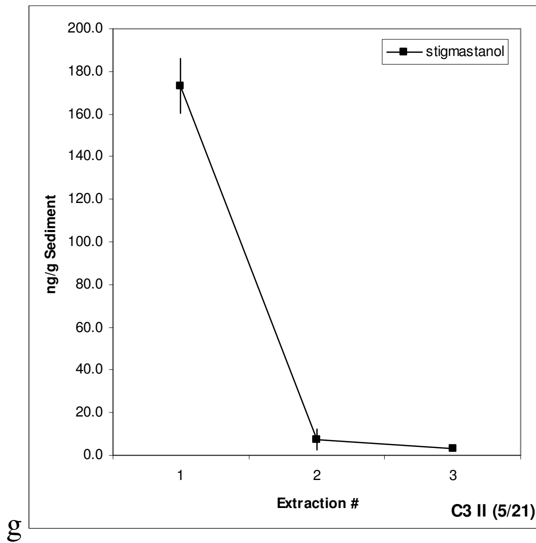

Figure 4.5 Results of triple extraction for C3 from 5/21/2008 performed in duplicate.

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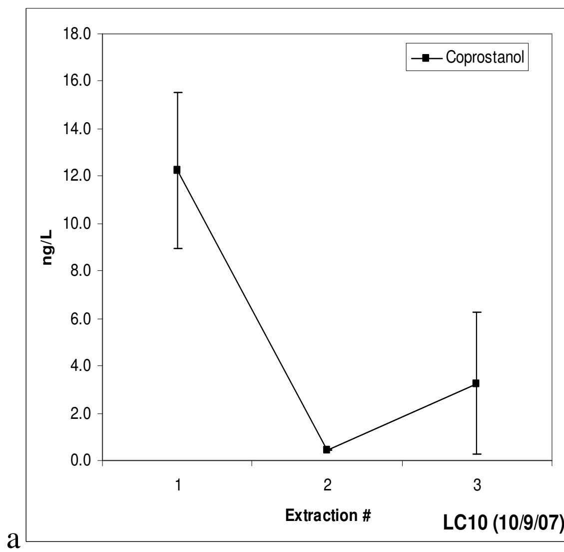

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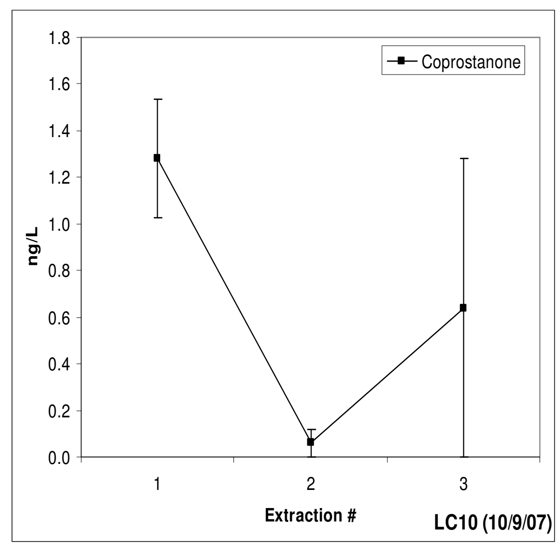

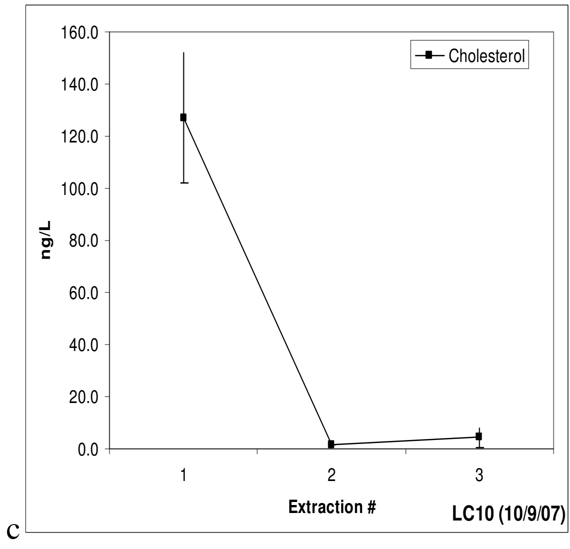

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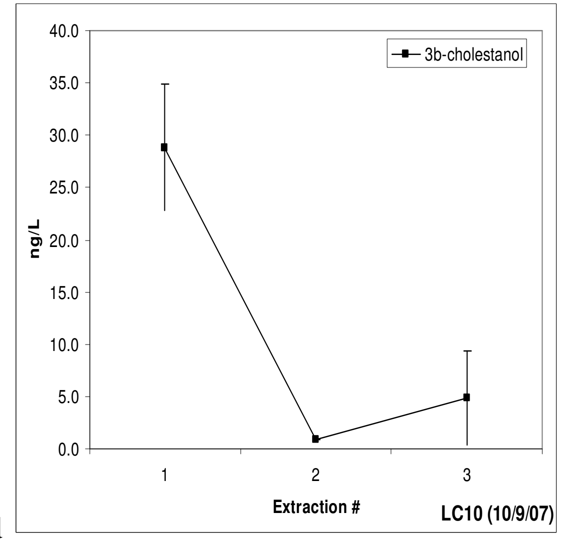

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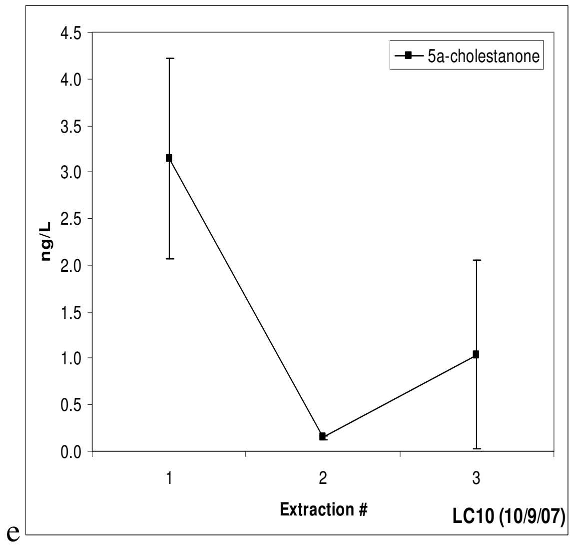

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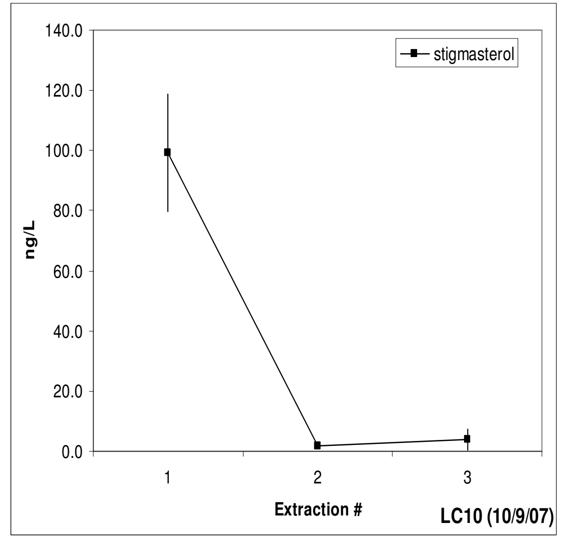

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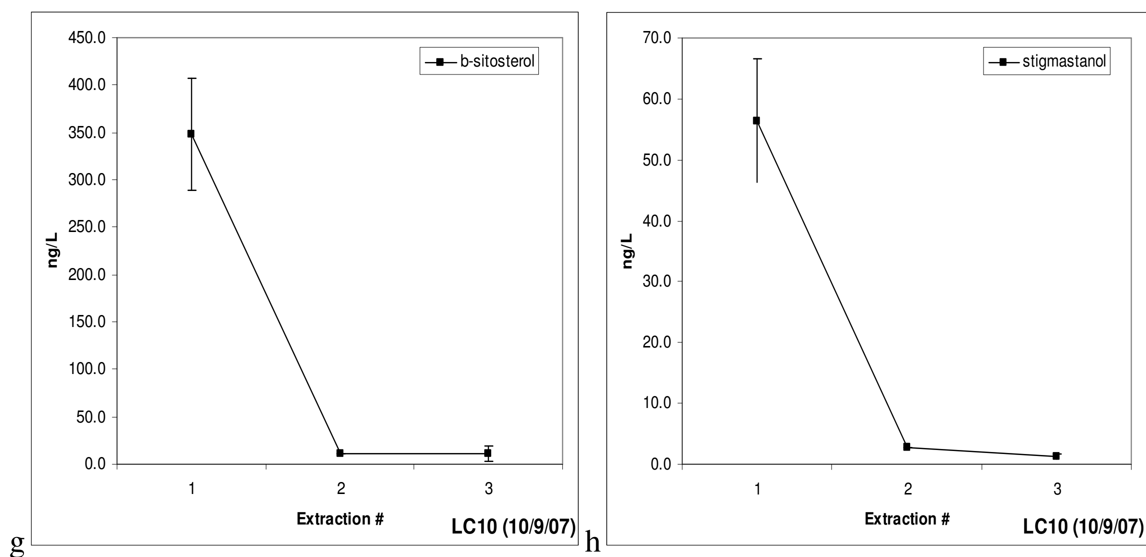


Figure 4.6 Triple extraction for LC10 filter sample from 10/9/2007 performed in duplicate.

Between the first and second extraction from sediments for all sites and analytes there was about a 97% reduction in the amount of sterols that could be extracted from the samples with a minimum observed drop of 89% and a maximum of 100%. Between the second and third extraction the amount of sterols extracted was generally about the same and either slightly increased or decreased, on average representing a 98% reduction from the original amount of sterols extracted ranging from 86-100%. The percentage decrease for each individual sterol between extractions varied across the samples except for coprostanone which was below detection after the first extraction for all samples. For C1 (5/2) and C3 (6/4), both of which had extraction was performed in duplicate, there was a statistically significant decrease in sterol concentration for all sterols between the 1st and 2nd as well as the 1st and 3rd extraction but no significant decrease between the 2nd and 3rd extractions. These results indicate that extraction from the sediment was efficient.

Extraction of a duplicate filter sample was also performed three times to determine extraction efficiency. Between the first and second extraction there was an average 97% decrease in sterol concentration ranging from 95.2-98.8%. Between the second and third extraction about 97% decrease in sterol concentration occurred again for cholesterol, stigmasterol, and stigmastanol and notably lower decreases (50.1-83.1%) were seen for coprostanol, coprostanone, 3β-choelstanol, and 5α-choelstanone. For 5α-cholestanone there was not a significant decrease in concentration between the 1st and either of the subsequent extractions. For coprostanol, coprostanone, and 3β-cholestanol there was a significant drop in sterol concentrations between the 1st and 2nd extractions but not the 1st and 3rd. It is thought the inefficiency in extraction that these results would imply is a function of large error in quantification at low concentrations. The analytes that did show statistically significant decreases between both the 1st and 2nd and the 1st and 3rd extractions were initially from one to two orders of magnitude greater in concentration than the analytes that did not show significant decreases. This was not a problem with the sediments (extraction efficiency was not affected by initial concentration), but for sediments there was a better reproducibility than the filters at low concentrations as can be seen by comparing the magnitude of the error.

4.1.2 Sampling Design

Different sampling schemes were pursued in terms of the number of sampling sites for sterols and FWA because of the difference in processing and analysis times required by the two methods. FWA analysis was rapid and simple (<1minute/sample) and required no sample preparation, making collection from more sampling sites

possible. A set of sterol samples took four days to prepare and another day to analyze one 6-sample batch and thus fewer sites were chosen to make more sampling trips feasible. Sampling locations were subject to change if analytes were found to be below detection or if other more promising spots arose.

FWA sampling was conducted during low (2/19/2008) and normal (5/2/2008 and 5/21/2008) flow. The first sampling trip was meant to be a small scoping study and when no signal was observed a broader sweep of sampling sites was made (see Section 4.2.1 for in depth discussion of results). When no signal was observed on the broader sweep, sampling was scaled back. Since FWA sampling required such small volumes, using sampling poles and rope made sampling from bridges possible, and thus steep slopes, fences, and lack of permission to sample from private property did not constrain site selection.

In contrast, sampling sites for sterols were constrained for several reasons, including access issues. The sediment and large water samples required sampling in the stream beds, therefore, steep hikes from the road (LC4), lack of homeowner permission (LC8), and fences (LC6a) made certain sites impossible to sample. Specifically for sediment, sites were constrained by lack of accessible deposition zones, which contain the fine sediment where sterols are expected to be more concentrated than in the coarse sediments, because either flow was too quick (LC3), stream beds were too rocky (LC6), or there were no areas likely to allow for deposition of suspended particles like abrupt changes in stream width or meanders. For the water samples, sampling required deep enough water in the streams to acquire sample, and several sites in the headwaters ran too

shallow to submerge 4L jugs (LC7 on occasions, C3b). Given these constraints, sites were selected to represent locations likely to contain a range of sterol concentrations based on fecal coliform counts and a variety of fecal pollution sources based on both land cover data and results from MST analysis (personal communication Marirosa Molina, US EPA, Athens), as listed in Table 4.5.

Table 4.5 Sampling sites for sterols and background information for each. *Sediment # Filters

Site ID	**Land Cover/ Anecdotal**	**CFU/100mL from Spring & Fall 2007**	**LH-PCR and T-RFLP Results**
C1 * #	Ag, septic, sewer, beaver	100, 2660, 2100, 3000, 50, 180, 200	no data
C2C * #	Sewer	800	Cattle, Human
C3 * #	Sewer	300, 2960, 3800, 1800, 300, 150, 300	Cattle, Bird1F
LC1 * #	Woods, cattle horses	1900, 2100, 900, 3400, 100, 160, 1900	Cattle
LC2 #	Septic, horses	400, 840, 6900, 2200, 250, 580, 400	Cattle
LC4 #	Horses, cattle	300, 140, 1100, 5300, 250, 390, 300	Cattle
LC5 #	Forest, horses, dairy, hog, septic, manure, beaver	4100, 1900, 6800, 6800, 900, 350, 4100	Cattle
LC7 * #	Septic, forest	1600, 300, 9400, 3500, 650, 90, 300	Cattle
LCS *	Known failed septic 200 yards from stream on steep slope	no data	no data
LC10 * #	Culmination of LC	1000, 260, 800, 3000, 50, 180, 1000	Cattle

4.1.3 Sediment Results

Sediment results are reported as average ng/g sediment (dry) with error, sorted by site and date in Tables 4.6-4.12. Error was calculated for duplicate samples by using the average of the differences from the mean and for triplicate samples by calculating the

standard deviation of the three results. Likewise, relative percent difference (RPD) and percent relative standard error (%RSE) are reported as appropriate given the sample size. Sterols were above detection limits in all sediment samples with the exception of coprostanone at LC7 on 6/4/08. Concentrations of coprostanone were generally low and the analyte had a higher detection limit than most of the other analytes, so it is not surprising that it was below detection in one sample. A "total sterols measured" value summing all sterols measured is not reported even though other researchers generally report such a number. It is excluded since reporting total sterols can be misleading because (1) there is no set suite of sterols that researchers have chosen to measure, (2) the sterols quantifiable in this study were limited by the sterol standards purchased and do not represent all sterols that were in the samples nor do they even exhaust the sterols others chose to measure, and (3) it is not needed for any of the proposed sterol ratios that are utilized in this work.

Surface sediment samples were scooped into plastic syringes because using the syringes to take sediment cores was not possible without hitting sand or rock due to shallow sediment depths at the study sites. In general, sediment samples can offer information on historical loadings of contaminants, depending on the inputs and dynamics of the system. Originally, sedimentary sampling was to be performed by taking sediment cores with the plastic syringes. However, once in the field it became apparent that such a sampling scheme would not work for two reasons: (1) in this stream system, as mentioned earlier, adequate deposition zones were not present at all sites, and (2) very little fine grained sediment was deposited even in deposition zones and when

driving the syringes into the stream bed they either hit sand or rock (with the exception of site C2C). Since sterols will preferentially associate with the fine grained sediment compared to sand and rocks (Brown and Wade, 1984), sediment cores were not taken but rather syringes were used to scoop fine grained sediment from the top of the stream bed. The presence of only a thin layer of fine grained sediment even at apparent deposition zones in the stream implies that these were not actually deposition zones in the true sense. It is unlikely that perfect deposition was taking place and the deposition that did occur was probably subject to losses by scour, causing deposited sediment layers to be thin. Thus, the sediment samples likely do not provide a true historical (nor a true instantaneous) view of the sterol loadings to the streams. While the signatures in the sediment are still worth discussing in terms of possible sources, they will likely not correlate with loadings and can not be seen as providing an accurate picture of the intensity of sterol loadings in the streams. Nonetheless, sterol data from the sediment measurements can still be somewhat useful in that they provide information about what sources may be playing or may have played a role in sterol and, therefore, fecal loadings to the streams. Despite the lack of confidence in sediment samples correlating with historical loads, sediment samples were still considered worth collecting for comparison to water samples and because they required less labor than water samples. Sediment samples were also helpful in providing samples for use in method development that required less labor than water samples.

Previous sterol studies that looked at sediments were conducted in areas more likely to have true deposition zones, like bays (Pratt et al., 2007; Grimalt et al., 1990),

Table 4.6 Sterol concentrations and ratios for sediment and filters from LC1.

LC1	**6/4/08**						**9/6/07**	
	Split			**Overall**			**Filter**	
	n=2			**n=3**			**n=1**	
	Avg ng/g	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**	**ng/L**	***ng/g SP****
Coprostanol	23.8	2.8	11.7	21.5	4.4	20.3	4.5	*488.9*
Coprostanone	2.1	0.8	38.4	1.8	0.7	40.6	0.5	*52.6*
Cholesterol	393.8	33.0	8.4	405.2	69.6	17.2	260.0	*28180.3*
3b-cholestanol	198.3	21.6	10.9	150.9	22.4	14.9	42.9	*4646.9*
5a-cholestanone	3.0	0.1	4.5	2.6	0.4	14.6	0.6	*64.7*
stigmasterol	508.2	56.3	11.1	517.5	57.7	11.2	150.9	*16354.3*
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	*nd*
stigmastanol	506.2	44.6	8.8	394.2	59.4	15.1	65.0	*7049.8*
Equation (1) >0.3	0.12	0.00	0.9	0.15	0.04	27.8	0.11	
Equation (2)>0.2	0.06	0.00	3.4	0.05	0.01	10.5	0.02	
Equation (3)>0.06	0.04	0.00	2.5	0.04	0.00	10.2	0.01	
Equation (4)>0.7	0.11	0.00	0.8	0.13	0.03	23.9	0.10	
Equation (5)>0.7	0.39	0.08	21.6	0.40	0.09	22.3	0.45	

*Weight of suspended particulates determined by weighing filters after extraction with solvent and drying at 105°C. nd=not determined

Table 4.7 Sterol concentrations and ratios for sediment and filters from LC7.

LC7	5/21/08						6/4/08						9/20/07	
	Split			**Overall**			**Split**			**Overall**			**Filter**	
	n=3			**n=2**			**n=2**			**n=3**			**n=1**	
	Avg ng/g	+/-	**%RSE**	**Avg ng/g**	+/-	**RPD**	**Avg ng/g**	+/-	**RPD**	**Avg ng/g**	+/-	**%RSE**	**ng/L**	***ng/g SP****
Coprostanol	17.5	19.9	113.7	4.6	1.4	30.7	1.6	0.1	4.7	2.1	1.3	62.4	1.8	*379.2*
Coprostanone	3.7	3.1	83.6	1.7	0.0	1.5	0.0	0.0	NA	0.2	0.3	173.2	0.7	*141.1*
Cholesterol	316.7	125.5	39.6	311.7	105.8	33.9	27.5	5.4	19.7	34.7	28.4	81.7	105.7	*21905.7*
3b-cholestanol	90.2	52.0	57.7	73.6	32.7	44.4	11.8	2.3	19.7	16.6	13.4	80.8	25.4	*5266.6*
5a-cholestanone	5.3	5.6	105.8	1.7	0.6	35.3	0.4	0.2	46.0	2.6	3.6	138.2	0.5	*104.7*
stigmasterol	364.5	148.5	40.7	401.0	111.3	27.8	26.8	5.1	19.1	55.0	76.8	139.6	62.1	*12874.0*
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	*nd*
stigmastanol	227.6	103.4	45.4	227.5	89.1	39.2	42.8	6.9	16.2	59.4	48.9	82.3	33.8	*7000.2*
Equation (1) >0.3	0.18	0.12	68.1	0.07	0.01	15.9	0.14	0.02	15.1	0.14	0.02	16.6	0.07	
Equation (2)>0.2	0.05	0.05	97.3	0.02	0.00	3.6	0.06	0.01	15.1	0.07	0.01	20.8	0.02	
Equation (3)>0.06	0.04	0.04	90.8	0.01	0.00	5.9	0.04	0.01	15.1	0.05	0.01	18.2	0.01	
Equation (4)>0.7	0.15	0.09	61.0	0.06	0.01	14.9	0.12	0.02	13.4	0.12	0.02	14.7	0.07	
Equation (5)>0.7	0.45	0.08	17.2	0.52	0.09	16.7	0.00	0.00	NA	0.12	0.21	173.2	0.57	

*Weight of suspended particulates determined by weighing filters after extraction with solvent and drying at 105°C. nd=not determined

Table 4.8 Sterol concentrations and ratios for sediment and filters from LC10.

LC10	5/21/08						6/4/08						9/25			10/9		
	Split			Over-all			Split			Over-all			Filters			Filters		
	n=2			n=2			n=2			n=3			n=2			n=2		
	Avg ng/g	+/-	RPD	Avg ng/g	+/-	RPD	Avg ng/g	+/-	RPD	Avg ng/g	+/-	%RSE	Avg ng/L	+/-	RPD	Avg ng/L	+/-	RPD
Coprostanol	10.7	3.4	32.0	54.7	47.5	86.7	33.7	6.4	19.0	214.1	310.5	145.0	4.7	1.5	31.6	12.2	3.3	26.8
Coprostanone	1.7	1.5	84.2	2.3	2.0	88.2	4.0	0.6	14.4	25.9	37.4	144.3	0.5	0.1	27.1	1.3	0.3	19.8
Cholesterol	229.7	70.3	30.6	420.3	260.9	62.1	569.3	98.0	17.2	1633.0	2010.3	123.1	60.1	16.0	26.7	127.0	25.0	19.7
3b-cholestanol	81.8	17.2	21.0	129.2	64.6	50.0	154.9	12.6	8.2	421.2	493.2	117.1	13.1	3.2	24.4	28.8	6.0	21.0
5a-cholestanone	2.3	0.8	34.5	3.2	1.7	51.9	3.6	0.5	12.6	19.4	29.8	153.8	1.3	0.3	25.2	3.1	1.1	34.3
stigmasterol	152.5	34.2	22.4	203.2	84.9	41.8	378.4	69.2	18.3	1003.8	1211.4	120.7	37.4	9.1	24.3	99.4	19.5	19.6
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
stigmastanol	165.4	18.0	10.9	186.0	38.6	20.8	258.5	21.0	8.1	632.6	665.8	105.3	22.4	5.1	22.8	56.4	10.1	18.0
Equation (1) >0.3	0.13	0.02	11.8	0.3	0.21	64.8	0.22	0.02	11.0	0.37	0.20	53.7	0.35	0.03	7.7	0.42	0.03	6.2
Equation (2)>0.2	0.05	0.00	1.5	0.1	0.05	53.4	0.06	0.00	1.9	0.10	0.04	45.0	0.08	0.00	5.3	0.09	0.01	7.5
Equation (3)>0.06	0.03	0.00	4.3	0.1	0.04	56.5	0.05	0.00	3.8	0.08	0.04	46.9	0.06	0.00	5.8	0.08	0.01	7.3
Equation (4)>0.7	0.11	0.01	10.5	0.2	0.12	54.7	0.18	0.02	9.1	0.26	0.10	40.0	0.26	0.01	5.7	0.30	0.01	4.4
Equation (5)>0.7	0.33	0.18	53.6	0.3	0.16	51.6	0.52	0.00	0.9	0.67	0.22	32.6	0.29	0.00	1.4	0.30	0.03	10.9

nd=not determined

Table 4.9 Sterol concentrations and ratios for sediment from LCS.

LCS	5/21/08									6/4/08					
	Split-1			Split-2			Over-all			Split			Over-all		
	n=2			n=2			n=3			n=2			n=3		
	Avg ng/g	+/-	RPD	Avg ng/g	+/-	RPD	Avg ng/g	+/-	%RSE	Avg ng/g	+/-	RPD	Avg ng/g	+/-	%RSE
Coprostanol	8.9	0.7	8.0	12.6	2.2	17.4	20.7	19.8	95.7	53.7	3.2	5.9	28.8	19.0	66.0
Coprostanone	0.9	0.4	50.6	1.2	0.1	11.0	3.7	5.1	138.6	7.6	0.6	7.8	5.1	2.7	53.8
Cholesterol	271.0	8.9	3.3	208.8	11.5	5.5	491.6	419.2	85.3	1186.8	13.4	1.1	720.4	392.3	54.5
3b-cholestanol	56.8	0.3	0.6	34.6	2.4	6.9	83.0	63.6	76.7	222.6	2.2	1.0	134.5	74.4	55.3
5a-cholestanone	2.0	0.9	44.4	1.7	0.5	31.4	3.7	3.0	81.4	7.4	1.1	14.6	4.7	3.3	69.5
stigmasterol	200.9	6.1	3.0	185.4	20.8	11.2	346.9	279.8	80.7	852.4	68.1	8.0	575.3	302.4	52.6
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
stigmastanol	139.0	1.9	1.3	135.2	20.2	15.0	219.3	123.5	56.3	577.9	14.7	2.6	370.1	192.8	52.1
Equation (1) >0.3	0.16	0.01	7.4	0.37	0.09	24.0	0.24	0.08	33.4	0.24	0.01	5.0	0.21	0.04	17.0
Equation (2)>0.2	0.03	0.00	11.3	0.06	0.01	22.7	0.04	0.01	24.3	0.05	0.00	4.8	0.04	0.01	18.0
Equation (3)>0.06	0.03	0.00	10.6	0.05	0.01	22.9	0.03	0.01	25.7	0.04	0.00	4.8	0.03	0.01	17.8
Equation (4)>0.7	0.14	0.01	6.4	0.27	0.05	17.8	0.19	0.05	28.5	0.19	0.01	4.0	0.17	0.02	14.3
Equation (5)>0.7	0.34	0.21	61.0	0.42	0.05	12.5	0.39	0.23	59.0	0.51	0.02	3.4	0.53	0.04	8.0

nd=not determined

Table 4.10 Sterol concentrations and ratios for sediment and filters from C1.

C1	**5/2/08**			**6/4/08**						**6/23/08**						**6/23/08**		
	Split			**Split**			**Over-all**			**Split**			**Over-all**			**Filter**		
	n=2			**n=2**			**n=3**			**n=2**			**n=3**			**n=1**		
	Avg ng/g	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**	**ng/L**	***ng/g SP****	***ng/g SP****
Coprostanol	5.2	0.0	0.2	20.7	0.9	4.4	21.8	16.6	76.2	63.3	0.6	0.9	37.9	21.5	56.8	33.3	*58.8*	*59.9*
Coprostanone	1.2	0.2	13.5	2.1	0.1	2.8	1.9	1.0	52.3	9.0	0.2	1.9	6.0	2.8	45.8	1.3	*2.4*	*2.4*
Cholesterol	75.7	4.5	5.9	361.2	36.4	10.1	321.8	152.4	47.4	920.7	16.6	1.8	636.5	280.3	44.0	685.7	*1211.5*	*1234.7*
3b-cholestanol	30.7	0.2	0.7	85.6	10.3	12.1	84.6	46.6	55.0	250.2	0.1	0.0	192.1	58.7	30.6	100.8	*178.0*	*181.4*
5a-cholestanone	0.7	0.0	1.0	1.4	0.1	5.6	2.1	0.7	34.3	8.8	0.5	5.5	5.1	2.8	53.9	2.4	*4.3*	*4.4*
stigmasterol	79.2	1.5	1.9	272.5	13.1	4.8	247.3	134.1	54.2	741.6	16.5	2.2	581.6	167.6	28.8	350.3	*618.9*	*630.7*
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	*nd*	*nd*
stigmastanol	92.5	5.2	5.6	206.3	10.8	5.2	207.8	89.6	43.1	537.6	16.3	3.0	451.7	94.4	20.9	209.8	*370.6*	*377.7*
Equation(1) >0.3	0.17	0.00	0.5	0.24	0.02	7.7	0.23	0.08	33.1	0.25	0.00	0.9	0.19	0.06	31.7	0.33		
Equation (2)>0.2	0.07	0.00	5.7	0.06	0.00	5.7	0.06	0.02	37.6	0.07	0.00	2.7	0.06	0.01	24.3	0.05		
Equation (3)>0.06	0.05	0.00	4.2	0.05	0.00	6.1	0.05	0.02	36.3	0.05	0.00	2.3	0.05	0.01	25.3	0.04		
Equation (4)>0.7	0.15	0.00	0.4	0.20	0.01	6.2	0.19	0.05	28.1	0.20	0.00	0.7	0.16	0.04	27.0	0.25		
Equation (5)>0.7	0.62	0.03	4.7	0.60	0.01	1.1	0.46	0.16	34.6	0.48	0.01	1.5	0.53	0.05	9.3	0.35		

*Weight of suspended particulates determined by weighing filters after extraction with solvent and drying at 105°C. **Weight of suspended particulates determined by weighing filters after air drying before extraction. nd=not determined

Table 4.11. Sterol concentrations and ratios for sediment and filters from C2C.

C2C	5/21/08						6/4/08						6/23/08						6/23/08		
	Split			**Over-all**			**Split**			**Over-all**			**Split**			**Over-all**			**Filter**		
	n=2			**n=3**			**n=2**			**n=3**			**n=2**			**n=3**			**n=1**		
	Avg ng/g	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**% RSE**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**% RSE**	**ng/L**	***ng/g SP*****	***ng/g SP****
Coprostanol	37.0	2.1	5.7	38.0	5.5	14.6	28.3	10.2	0.1	19.2	10.2	53.1	12.5	3.7	29.2	19.3	4.1	21.2	14.3	*56.5*	*57.3*
Coprostanone	2.6	0.0	1.6	2.9	0.9	30.4	2.1	0.9	7.2	1.2	0.9	71.8	1.2	0.2	15.3	3.6	3.0	82.7	2.3	*9.2*	*9.3*
Cholesterol	269.6	16.1	6.0	278.8	240.6	86.3	291.5	82.2	8.0	193.2	82.2	42.5	227.4	28.9	12.7	298.0	123.6	41.5	394.5	*1562.6*	*1585.8*
3b-cholestanol	112.9	4.1	3.7	87.7	66.5	75.9	115.7	40.3	5.9	66.7	40.3	60.4	55.9	2.8	5.0	93.8	31.2	33.2	50.3	*199.3*	*202.2*
5a-cholestanone	2.2	0.1	2.8	2.4	0.2	9.8	1.6	0.3	5.2	1.2	0.3	28.3	1.3	0.2	13.7	2.2	1.1	49.3	1.6	*6.5*	*6.6*
stigmasterol	254.6	19.1	7.5	218.6	160.2	73.3	247.4	83.7	5.5	145.7	83.7	57.5	151.7	16.8	11.1	349.9	236.4	67.5	203.6	*806.2*	*818.2*
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	*nd*	*nd*
stigmastanol	211.0	10.3	4.9	168.4	126.8	75.3	290.6	91.0	6.4	168.5	91.0	54.0	178.8	22.7	12.7	261.4	65.1	24.9	104.3	*413.1*	*419.2*
Equation (1) >0.3	0.33	0.01	2.0	1.1	1.42	123.8	0.25	0.04	5.8	0.29	0.04	13.8	0.22	0.05	24.5	0.22	0.05	24.8	0.28		
Equation (2)>0.2	0.14	0.00	0.3	0.4	0.47	123.3	0.10	0.02	7.8	0.10	0.02	16.3	0.06	0.02	40.4	0.07	0.01	19.4	0.04		
Equation (3)>0.06	0.10	0.00	0.4	0.3	0.35	123.8	0.07	0.01	7.3	0.07	0.01	13.3	0.05	0.02	37.4	0.05	0.01	19.5	0.03		
Equation (4)>0.7	0.25	0.00	1.5	0.4	0.28	69.0	0.20	0.02	4.7	0.23	0.02	10.6	0.18	0.04	20.3	0.18	0.04	20.2	0.22		
Equation (5)>0.7	0.54	0.00	0.6	0.5	0.06	11.8	0.56	0.11	5.4	0.47	0.11	22.9	0.49	0.07	14.7	0.59	0.09	14.6	0.58		

Table 4.12. a. Sterol concentrations and ratios for sediment from C3.

C3	**5/21/08**						**6/4/08**						**6/23/08**					
	Split			**Over-all**			**Split**			**Over-all**			**Split**			**Over-all**		
	n=2			**n=3**			**n=2**			**n=3**			**n=2**			**n=3**		
	Avg ng/g	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**% RSE**	**Avg ng/g**	**+/-**	**RPD**	**Avg ng/g**	**+/-**	**%RSE**
Coprostanol	13.8	0.4	3.2	59.9	73.2	122.2	42.0	7.1	16.8	42.7	12.9	30.3	200.4	156.5	78.1	158.9	174.9	110.1
Coprostanone	1.5	0.5	33.0	6.1	8.6	140.7	3.0	0.5	16.3	3.2	1.4	44.0	66.7	60.0	90.0	49.2	67.2	136.5
Cholesterol	161.4	6.2	3.8	751.4	971.9	129.3	440.0	62.0	14.1	499.3	270.0	54.1	829.7	156.5	18.9	798.0	342.8	43.0
3b-cholestanol	88.9	1.5	1.7	226.9	223.9	98.7	152.6	20.6	13.5	184.2	61.9	33.6	551.0	269.0	48.8	471.8	323.8	68.6
5a-cholestanone	1.4	0.2	16.1	13.2	20.1	152.1	3.6	0.6	16.4	4.0	2.4	59.2	187.4	178.9	95.4	130.9	204.0	155.9
stigmasterol	178.2	8.8	4.9	526.6	581.4	110.4	350.9	59.9	17.1	388.2	161.4	41.6	865.0	233.7	27.0	859.1	271.4	31.6
b-sitosterol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
stigmastanol	162.3	5.9	3.7	307.4	236.1	76.8	266.6	26.7	10.0	299.9	55.4	18.5	824.5	382.7	46.4	775.0	402.8	52.0
Equation (1) >0.3	0.16	0.00	1.5	0.22	0.07	33.6	0.27	0.01	3.4	0.23	0.03	11.6	0.30	0.14	47.4	0.27	0.15	56.3
Equation (2)>0.2	0.09	0.00	0.6	0.09	0.01	11.9	0.10	0.00	2.8	0.09	0.02	23.2	0.21	0.15	69.5	0.17	0.16	95.0
Equation (3)>0.06	0.06	0.00	0.1	0.06	0.01	9.1	0.07	0.00	2.9	0.07	0.01	15.6	0.12	0.08	62.3	0.10	0.08	81.0
Equation (4)>0.7	0.13	0.00	1.3	0.18	0.05	27.5	0.21	0.01	2.6	0.19	0.02	9.3	0.22	0.08	38.6	0.20	0.09	44.6
Equation (5)>0.7	0.51	0.12	24.3	0.38	0.07	18.5	0.45	0.00	0.1	0.46	0.04	8.8	0.35	0.09	26.1	0.41	0.15	37.7

nd=not determined

Table 4.12 b. Sterol concentrations and ratios for filters from C3.

C3	9/6	9/27				10/9	6/23/08		
	Filter	Filter				Filter	Filter		
	n=1	n=2				n=1	n=1		
	ng/L	Avg ng/L	+/-	RPD	*Avg ng/g SP**	ng/L	ng/L	*ng/g SP***	*ng/g SP**
Coprostanol	10.9	7.8	3.8	48.3	*1479.9*	1.1	11.2	*32.5*	*34.0*
Coprostanone	3.0	1.6	1.0	63.9	*307.65*	0.2	1.7	*5.1*	*5.3*
Cholesterol	241.6	166.4	11.4	6.9	*31008.9*	14.0	390.0	*1127.8*	*1179.3*
3b-cholestanol	47.1	26.1	5.6	21.4	*4893.4*	2.7	55.0	*159.1*	*166.4*
5a-cholestanone	3.4	2.0	0.9	45.1	*385.4*	0.2	2.4	*6.9*	*7.3*
stigmasterol	156.1	130.0	4.1	3.2	*24182.6*	7.8	242.4	*701.1*	*733.1*
b-sitosterol	nd	nd	nd	nd	*nd*	nd	nd	*nd*	*nd*
stigmastanol	55.6	38.2	7.2	18.8	*7160.7*	4.2	91.1	*263.5*	*275.6*
Equation (1) >0.3	0.23	0.28	0.08	30.0		0.42	0.20		
Equation (2)>0.2	0.05	0.05	0.02	42.9		0.08	0.03		
Equation (3)>0.06	0.04	0.04	0.02	41.3		0.07	0.03		
Equation (4)>0.7	0.19	0.22	0.05	23.9		0.29	0.17		
Equation (5)>0.7	0.46	0.41	0.06	16.0		0.49	0.42		

*Weight of suspended particulates determined by weighing filters after extraction with solvent and drying at 105°C. **Weight of suspended particulates determined by weighing filters after air drying before extraction. nd=not determined

seas (Maldonado et al., 1999; Readman et al., 2005; Peng et al., 2005; Gilpin et al., 2003; Gilpin et al., 2002), lakes (Sankararamakrishnan and Gou, 2005), harbors (Mudge and Lintern, 1999), continental shelves (Maldonado et al., 2000; Vankatesan and Kaplan, 1990), and river deltas (Isobe et al., 2002; Isobe et al., 2004; Grimalt et al., 1990). Studies conducted in rivers have been done upstream of locks and damns, known to be areas of deposition (Hawkins Writer et al., 1995) or using only water samples (Elhmmali et al., 2000; Standley et al., 2000). No known studies have quantified sterols in stream sediments.

4.1.3.1 Method precision

Method precision for the sediment samples was evaluated by splitting one of the (at most) three samples taken at each site. Reproducibility ranged from 0.0% to 84.2% relative difference, averaging around 15% relative difference for any given analyte between splits. Several ways to compare the reproducibility and sample variability will be discussed: (1) across all analytes in the sterol suite at a given site, (2) looking at one analyte at a given site measured on different days, (3) looking at one analyte across the different sites, and (4) across the entire analyte suite at different sites. (1) Reproducibility was not constant across analytes on any given split. For example, at LC10 on 5/21/08 the RPD ranged from 10.9% for stigmastanol to 84.2% for coprostanone. But some splits had smaller ranges between the most and least reproducibly quantifiable sterol, like C1 on 6/23/08 which had 0.0% RPD for 3β-cholestanol and only went up to 5.5% RPD for 5α-cholestanone. (2) There were also differences in reproducibility for a given sterol at the same sampling site on different

dates; at C2C RPD for the splits was 5.7%, 0.1%, and 29.2% relative difference for coprostanol on the different sampling dates. The range of RPDs for analytes between dates varied for both compounds and sites. (3) In addition, reproducibility was not consistent for any given analyte across sites on a given date; coprostanol quantified on 6/4/08 had RPDs ranging from 0.1% at C2C to 19.0% at LC10. (4) Finally, some sites had greater reproducibility between splits; C1 never had a RPD greater than 13.5% while at LC10 RPDs were generally between 20 and 30% but went as high as 84%. One sample split was notably less reproducible; LC7 (n=3, 5/21/08) had over 100% relative standard deviation, but this large error may be attributable to poor homogenization of the sediment prior to analysis.

From this data set one could conclude that precision was not necessarily constant across analytes for any given split. Nor was precision necessarily a function of analyte since there was no pattern in reproducibility of any analyte between sites or dates; although, often the stanones had worse reproducibility. This may mean precision was a function of compound for the stanones or that precision may have been varying with initial sterol concentration since it is notable that coprostanone and cholestanone were generally found at lower concentrations than the other sterols that often had higher RPDs. Any patterns pointing to precision as function of site may suggest that certain sites had sediment that was easier to homogenize or that was homogenized better. Also, given the limited number of samples used for this study it was impossible to get a precise measure of the method precision.

4.1.3.2 Spatial Variability

Spatial variation by site and date is represented by the overall concentration of each sterol with the data listed in Tables 4.6-4.12a. For C1 on 5/2/2008 no spatial variation data are available. Overall concentration of each sterol for all other sites and dates was determined by analyzing discrete samples from 2-3 different deposition areas at each site and taking the average of the results across the different areas. The number of discrete samples is not constant because originally the sampling plan (used 5/2/2008 and 5/21/2008) involved analyzing only two samples per site to represent spatial variation and when these results suggested there may be an apparent spatial variation greater than expected but actually from method precision alone, the third sample taken was analyzed if possible. Subsequent samplings planned for three samples per site to improve precision.

Generally, variation seen for any given sterol in the overall measurement (note: overall refers to spatial average value for one sterol, not the sum of all sterols measured) was greater than the variation expected from the method. This can be demonstrated by comparing the percent relative difference for the split sample to the percent relative error (or percent relative difference as appropriate given the number of spatial samples) for the overall data at a given site and on a given date. The difference between the split and overall average percentage errors varied site to site and day to day and was as small as 16% (LC10, 6/4/08) and as large as 100% (C3, 5/21/08). The greater error in the overall sterol concentrations suggests there may have been a spatial variation in deposited sterol concentration due to factors that cause spatial variation in sterol deposition such as

differences in residence times for eddies, flow rates, particle size and organic carbon content. There also could have been spatial variation in the amount of deposited sediment removed by scouring affecting the sterol profile, as mentioned earlier. Alternatively, supposed spatial variation could be an artifact of varying organic carbon contents as results reported have not been normalized for organic carbon (as will be discussed in detail below) or particle size, which varies inversely with organic carbon content. It is possible any of these caused the greater variability in overall sterol concentrations compared to the splits and it is possible that two or all three were working in concert. Given the data collected it is impossible to determine the cause. A study of deposition is well beyond the scope of this work, but measuring and correcting for TOC would help determine if spatial variation in organic carbon content was contributing to the spatial variations in sterol concentrations.

As mentioned in the Section 1.3, sterol concentration on sediment is a function of organic carbon content. Organic molecules in general are more likely to associate with the fine sediment than the sandy fraction (Brown and Wade, 1984). It is likely that organic carbon content varied from site to site or from area to area at a given site. However, it is possible that variation in organic carbon content from area to area at a given site was an artifact of the sample collection procedure. As described in Section 2.1.1, surface sediments were scooped using plastic syringes because cores would only collect 1-2 g of fine sediment and the rest would be sand or rock. Even though care was taken to scoop only surface sediment, inevitably some of the sand and rock fraction was collected and any sand fraction smaller than 500μm could pass through the sieve and add

to the weight of the analyzed aliquot without proportionately contributing to the sterol content. A sandy sample would have less mass of sterol per gram of sediment because it adds weight without adding appreciable amounts of sterols. Certainly, some samples taken were visibly sandier than others, but without TOC or other appropriate data this was unquantifiable.

Other researchers (e.g. - Pratt et al., 2007) who have normalized for TOC chose to measure TOC using a simple combustion technique. It was decided for this study not to include such a TOC analysis as it generally overestimates the amount of TOC and is quite inaccurate. Thus, rather than correct for organic carbon content using a method that introduces such large uncertainty it was decided to refrain from adjusting for organic carbon content. No TOC analysis was included in this study for simplicity as well. This, however, increases uncertainty for comparing magnitude of sterols from site to site and even across areas at a given site. Further study including and correcting for TOC would be needed to confirm whether differences in organic carbon content were causing spatial variability to be greater than the method precision. It is also possible, however, that the greater spatial variability may be an artifact of the small sample size used for this study. SID ratios, the ultimate purpose for measuring sterols, have the benefit of correcting for TOC.

While generally the variability in the overall sterol measurement was greater than the variability in the split sample, LC7 on 5/21/08 and LC1 on 6/4/08 are notable exceptions. LC7 showed greater error in the splits than the overall data and LC1 had similar error for both split and overall. As previously mentioned, the gross error in the

LC7 5/21/08 split was likely to have been attributable to poor homogenization. For LC1, the similarity in the method precision and the spatial variation may imply that (1) there really was less spatial variability in sterol deposition at LC1, (2) lack of scouring or scour occurring evenly across the streambed resulted in less spatial variability at LC1, or (3) organic carbon content between spatial samples at LC1 was more similar than at other sites. Again, adding in TOC analysis would be the easiest way to narrow these possibilities.

As mentioned above, the amount of spatial variation in the samples was different at different sites. For example, on 6/4/08 LC10 showed over 100% RSE for all sterols while LC1 had less than 20% RSE for almost all compounds. The possible reasons for this are similar to the reasons listed above for differences in area to area (spatial variability) at a site but simply applied from site to site (i.e. differences in TOC site to site). Again, TOC measurement could help determine if perhaps certain sites had more homogenous organic carbon content from samples collected spatially than other sites.

4.1.3.3 Temporal and Site to Site Comparisons

Comparing the sterol concentrations between sampling dates at a given site can give an idea of how inputs may have varied between dates but also, as explained above, how concentrations may have been influenced by differences in deposition and scouring of deposited sediment. Between sampling dates significant differences were only seen at LC7, C2C, C1, and C3. LC7 had significantly higher concentrations for all analytes except coprostanol on 5/21/08 compared to 6/4/08. C2C had significantly more coprostanol, coprostanone, and cholestanone on 5/21/08 than 6/4/08. C1 had a

significantly greater amount of 5α-cholestanone on 6/4/08 than 5/21/08 and significantly higher concentrations of coprostanone, 3β-choelstanol, stigmastanol, and stigmasterol on 6/23/08 than on 6/4/08. Finally, C3 had significantly more stigmasterol and stigmastanol on 6/23/08 than on 6/4/08. The rest of the sites showed no significant differences between any sterol concentrations between sampling dates. For LC1 sampling was only done on 6/4/08 so no comparison between sampling dates is possible. Differences in sites between dates could have been caused by differences in inputs or differences in losses due to scouring. No heavy rains occurred between sampling dates, but it was noted in the field that water was lower on 6/4/08 than 5/21/08 at LC7, which is closer to the head waters. No stream depth or width measurements were made at sites. Considering there are only two stream level monitoring sites in the watershed, measuring depth and width would be helpful for quantitative comparisons of flow between dates that may affect and, therefore, help explain temporal concentrations and depositions.

Comparing sterol concentrations at different sites taken on the same date technically should provide information about the difference in sterol loadings between sites. However, given the lack of confidence in the notion that sediments were consistently deposited with similar scouring and post-depositional changes such comparisons likely may not provide reliable information that would correlate with the inputs between sampling sites. Yet, it is worth mentioning that the only significant difference between sites sampled on 5/21/2008 was between LC7 and C2C for coprostanol and coprostanone, which were significantly higher at LC7. More site to site variability was seen for the 6/4/2008 sampling date than during the 5/21/08 sampling

date. Significant differences existed between LC1 and LC7, LCS, C1, C2C, C3 for one or more analytes, between LC7 and LCS, C1, C2C, and C3 for almost all analytes, between LCS and C2C for three analytes, and between C2C and C3 for almost all analytes. On 6/23/08 there were significant differences between C2C and the two other samples sites, C1 and C3.

It is difficult to say why there were more significant differences site to site on 6/4/08 than 5/21/08. It is possible there were differences in inputs and losses due to scouring plus the possible roles that differences in the TOC by site or introduced by inconsistent sampling technique. For these same reasons, comparing a site to an upstream and down stream point in terms of sediment may not indicate differences that are a function of fecal loadings to the creeks. Also, as mentioned earlier, the sedimentary concentrations of sterols are unlikely to necessarily correlate with nearby inputs. Therefore, comparison of sterol profiles between different sampling sites will be pursued solely using SID ratios, not on a direct concentration basis, as mentioned earlier.

Sterol ratios have the additional benefit of helping to correct for differences in organic content. However, ratios will not correct for differences in deposition rates and scouring between sites that cause changes in the sterol profiles. It is believed to be unlikely that deposited sediment with sterols would lose individual sterol compounds at significantly different rates from one another since sterols are structurally similar and, thus, their adsorption/desorption behavior should be similar. Differences in chemical or microbial weathering, however, could possibly affect the sedimentary sterol profile. If

sterols are affected in different ways and to different degrees, for example, due to differential microbial degradation rates, the SID ratios would be skewed.

4.1.4 Water Column Results

As described in Chapter 3, Materials and Methods, particulate matter was collected by filtration of water samples and analyzed for sterols as a way to investigate sterols in the stream water column. Filter, or water column, results are reported in average ng/L water with precision reported as RPDs whenever duplicates were analyzed, or simply ng/L for singlet samples in Tables 4.6-4.14. Estimates of the sterol concentrations in ng/g suspended particulates (SP) (dry weight basis) are included as well to facilitate comparison between concentrations on suspended and deposited particulates, which will be discussed along with the probable sources of error in these values in Section 4.1.5. Sterols were always above detection in filter samples with the exception on one occasion (i.e. LC7 6/4/08) for coprostanone, the oxidation product of coprostanol, which had a higher detection limit than almost all other analytes (LOQs are listed in Table 4.4). Sterols were only quantified from the SP phase of water samples as opposed to both the SP phase and filtrate of water samples because previous studies have found >95% of sterols were present in the SP fraction of water (Isobe et al., 2002 and references therein).

4.1.4.1 Method Precision

Method precision of the filter samples was explored by taking duplicate water samples at selected sites. Duplicates were not taken at all sites, only LC2, LC5, LC10 and C3, as mentioned earlier, due to the labor requirements associated with water

Table 4.13 Sterol concentrations and ratios for filters from LC2 and LC4.

	LC2	**9/20/07**		**10/4/07**			**10/22/07**			**LC4**	**9/20/07**	
		Filters		**Filters**			**Filters**				**Filter**	
		n=1		**n=2**			**n=2**				**n=1**	
		ng/L	***ng/g SP****	**Avg ng/L**	**+/-**	**RPD**	**Avg ng/L**	**+/-**	**RPD**		**ng/L**	***ng/g SP****
Coprostanol		5.7	*718.8*	5.1	0.5	10.6	7.3	2.5	34.0		8.1	*2260.5*
Coprostanone		0.2	*28.8*	0.4	0.1	27.6	0.6	0.1	12.9		1.4	*406.1*
Cholesterol		136.4	*17099.0*	162.6	2.1	1.3	185.3	48.0	25.9		99.3	*27816.3*
3b-cholestanol		31.9	*3997.2*	26.7	2.2	8.2	31.9	7.2	22.6		25.2	*7050.5*
5a-cholestanone		0.4	*56.0*	0.3	0.1	19.9	0.5	0.2	39.8		2.4	*668.6*
stigmasterol		91.9	*11510.3*	91.3	2.5	2.7	127.4	51.5	40.4		68.4	*19161.6*
b-sitosterol			*61654.2*									*72919.3*
stigmastanol		52.3	*6550.8*	41.7	2.1	5.1	52.1	14.6	27.9		43.5	*12193.8*
Equation (1) >0.3		0.18		0.19	0.00	2.4	0.22	0.03	12.3		0.32	
Equation (2)>0.2		0.04		0.03	0.00	11.9	0.04	0.00	8.8		0.08	
Equation (3)>0.06		0.03		0.03	0.00	10.5	0.03	0.00	9.4		0.06	
Equation (4)>0.7		0.15		0.16	0.00	2.1	0.18	0.02	10.1		0.24	
Equation (5)>0.7		0.34		0.58	0.02	3.4	0.53	0.07	13.6		0.38	

*Weight of suspended particulates determined by weighing filters after extraction with solvent and dried at 105°C.

Table 4.14 Sterol concentrations and ratios for filters from LC5.

LC5	**9/20/07**		**10/4/07**			
	Filter		**Filters**			
	n=1		**n=2**			
	ng/L	*ng/g SP**	**Avg ng/L**	**+/-**	**RPD**	*ng/g SP**
Coprostanol	18.2	*3921.4*	12.4	1.1	8.5	*2370.5*
Coprostanone	1.5	*312.5*	0.7	0.2	24.2	*135.9*
Cholesterol	163.3	*35189.1*	173.6	17.1	9.9	*33225.1*
3b-cholestanol	37.9	*8176.5*	33.7	4.5	13.2	*6453.5*
5a-cholestanone	0.9	*190.8*	0.3	0.1	16.8	*65.65*
stigmasterol	104.6	*22538.7*	125.4	8.9	7.1	*23974.5*
b-sitosterol		*60753.8*				*70355.1*
stigmastanol	61.0	*13137.1*	65.6	6.9	10.6	*12550.1*
Equation (1) >0.3	0.48		0.37	0.02	4.8	
Equation (2)>0.2	0.11		0.07	0.00	1.4	
Equation (3)>0.06	0.09		0.06	0.00	1.9	
Equation (4)>0.7	0.32		0.27	0.01	3.5	
Equation (5)>0.7	0.62		0.67	0.02	2.5	

*Weight of suspended particulates determined by weighing filters after extraction with solvent and dried at 105°C.

samples. These method precision values will include sampling error as well. Water column samples were taken from dynamic zones toward the middle of streams and required people collecting samples to wade into the stream. Care was taken to collect samples upstream of the sampler and to wait for re-suspended sediment to settle or wash away to minimize collecting any sediment re-suspended from the bottom by the samplers, but inclusion of bottom sediment can not be totally ruled out. RPDs ranged from 3.2-62.3% for all sites and compounds and were on average around 23%. Like the sediment samples, RPDs for the filters varied analyte to analyte for any given sample split and between any given analyte across sampling sites. There are limited data available for making comparisons between RPDs at the same site on different dates. At LC10 the average RPD of all analytes was quite similar: 26% on 9/25/07 and 23% on 10/9/07. At LC2, the only other site where filters had been collected and analyzed in duplicate more than once, there was a larger difference between the RPDs of duplicate samples taken on different dates: 11% on average for 10/4/08 and 29% on average for 10/22/08. More water samples would be needed to be analyzed in duplicate or more before any conclusions can be drawn about reproducibility at a given site on different dates. For the 6/23/08 water column sampling, samples were collected in singlet because (1) reproducibility was acceptable for samples taken in the fall, (2) to save time and labor.

4.1.4.2 Temporal and Site to Site Comparisons

Since so few samples were taken in duplicate it is hard to say whether there are actually significant differences between sampling dates for the majority of sites sampled. At LC10 there was a significantly higher concentration on 10/4/07 than 9/25/07 of each

sterol quantified except 5α-cholestanone, which was present at trace levels on both dates. The differences at LC10 were either due to differences in inputs, are an artifact of differences in stream water flow volume, or are a result of inadvertent re-suspension of sediment or any combination of the three. At LC2 there was not a significant difference between sterols quantified on 10/4/07 and 10/22/07. For 9/20/07 when duplicates were not taken at LC2 an estimate of whether the concentrations were significantly different can be made by checking to see if the concentrations on 9/20/07 were within the error of the other two sampling dates when duplicates were taken. Doing so, the 9/20/07 sampling at LC2 shows concentrations that are likely not significantly different from concentrations on 10/4/07 and 10/22/07. The lack of significant difference would imply either that water levels and inputs were the same or that inputs increased proportionally with water levels. At C3, sampling on 9/25/07 was performed in duplicate and on all other dates in singlet (9/6/07, 10/9/07, 6/23/08). Comparing between dates, samples from 10/9/07 appear to be significantly lower than on all other dates, which fell in or close to the range of error on the 9/25/07 sampling. Again the possible causes are lower sterol inputs reaching the streams or higher water levels in the streams diluting the sterol concentration on 10/4/07 as compared to the other dates, or a combination of the two. Finally, at LC5, samples were taken in duplicate on 10/4/07 and in singlet on 9/20/07. The concentrations of sterols on both dates seem similar in magnitude. None of the remaining sites where sterol concentrations in the water column were measured (LC1, LC7, C1, LC4) had samples taken on more than one date.

Again lack of duplicates for most of the sampling dates makes it impossible to make statements as to whether differences between sites sampled on the same date are significant. On 9/25/07, there was significantly more cholesterol, 3β-choelstanol, stigmastanol, and stigmasterol at LC10 than at C3. LC10 and C3 are both the furthest down stream sites for each creek before Little Cane joins Cane Creek. On 10/4/07 duplicates were taken at both LC2 and LC5. LC5 showed significantly more coprostanol, stigmastanol, and stigmasterol than LC2. There is a larger volume of water in the stream at LC5 than LC2 and thus the differences are probably due to differences in sterol inputs between the sites because otherwise one would have expected a decrease at LC5 due to dilution. For other sampling sites and dates comparisons will only be made in terms of the SID ratios.

4.1.5 Sediment and Water Column Comparison and Conclusions

The sediment and water column samples taken for this study each presented their own sampling, analysis, and interpretation challenges and provided different information. Sediment sampling was undertaken with two different motivations in mind: (1) water column samples, at 20L (5x4-L jugs) required more labor to collect and time to prepare for analysis as filtering could take up to nine hours per sample, (2) because sterols associate significantly with the suspended particulate phase (>95% according to Isobe et al. (2002)), it was thought that sediment samples might, therefore, show a greater sterol signal per gram of particulate matter. Thus, more data were collected in the form of sediment samples, which were also used for method development.

Several factors, however, suggest that future studies should be conducted with sampling focused on the water column. As explained above, sterols quantified in the sediment from this system did not provide a measure of intensity of loading nor did they correlate with fecal loading since they are a function not only of possible variations in deposition but also to losses due to scouring and sediment diagenesis. In contrast, water column samples give an instantaneous picture of sterol loading in the system that should be possible to correlate with the individual loadings, assuming no re-suspended solids are included. Further, the sterol loadings on the SP phase are about the same or possibly greater than in the sediment. Concentrations of sterols on the SP phase were estimated by dividing the determined sterol mass by the mass of the SP. SP mass was determined either by weighing filters after filtering water, extracting, and baking and/or comparing air dried weights after filtering but before extracting to the weight of the clean filters. The former provides an underestimate of the SP weight because it underestimates the final filter weight as there were some losses of particulates and pulverized filter during extraction; therefore, it overestimated the sterol concentration. The later provide an overestimate of SP weight because air drying at room temperature cannot remove all water left on the filter; therefore, the second method likely underestimated the sterol concentration. For the 6/23/08 sampling dates SP weight was estimated by both methods and the values, while following the pattern explained above, do not appear to be significantly different (see Tables 4.10, 4.11, and 4.12b).

Estimates of the SP concentration of sterols for the fall water samples are orders of magnitude higher than sediment concentrations taken in the spring. The results from

the only sampling date where sediment and filter samples were analyzed at the same sites (6/23/08), however, do not suggest such a large concentration difference between sterols on the SP and sediment. At C2C sterol concentrations are about double on the SP those on the sediment, but at C1 sterol SP concentrations fall within or closely around the error of the sedimentary concentrations and the same for C3. This suggests that perhaps there is a seasonal effect in the sterol loading in the water column compared to the sediment or that the difference is a result of differences between the specific dates sampled.

It is important to note the sediment samples contain varying amounts of sand and mica that were small enough to pass through the sieve and do not have a high enough organic content to sorb sterols, while the suspended particulate phase only contains clay sized particles that have a high organic content. Therefore, sediment weights are influenced by the sandy fraction making sterol concentrations appear lower. More sediment to SP comparisons would be necessary for confirmation, but the current data suggest that SP concentrations are about the same or may be greater than sediment sterol concentrations.

Withstanding any error in measuring the true mass of particulates in the samples, the water column sterol measurements will more closely reflect current source inputs and, therefore, will be more helpful in determining current or instantaneous fecal coliform sources. Water column samples also will be less vulnerable than sediment samples to the skewing by inclusion of sand fractions since sand will not generally be suspended in the water column, except during periods of elevated flow. Further, water column samples do not need to be corrected for TOC as they are expressed in ng/L not ng/g. Finally, this

decision is supported by the fact that water column sampling has not been pursued by researchers measuring sterols in rivers unless the researchers were confident that depositions zone were present. Based on this information, recommendations for future studies are elaborated in Chapter 5.

4.1.6 Source Tracking Results

Using sterol ratios rather than using the magnitude of any given sterol for SID is done because researchers noted that sterol concentrations were dependent on both sediment particle size as well as TOC. Thus, using ratios helps correct for the effect of these two sediment parameters, which may have been affecting the sterol concentrations on sediments in this study. The results of each of the sterol ratios calculated are discussed by site below. It is worth noting that if any of the calculated ratios discussed below meets the threshold criteria then the sample is thought to have tested positive for human fecal contamination. This does not necessarily mean that other fecal sources are not playing a role at any sites that test positive. Others have demonstrated samples testing positive for both human and herbivore pollution (i.e. Pratt, 2005). Likewise, a ratio below the selected cutoff does not preclude the presence of human inputs. The threshold values have been found to be useful for identifying sources, but they are still indicators, not absolute evidence. The results of the SID ratios for sediments will only be discussed in terms of the overall result for a site unless no overall value is available.

4.1.6.1 Ratios

Of the 31 total (sediment overall + filters) samples analyzed, 10 tested positive for human fecal input using Equation (1), including LC4, LC5, LC10, C1, C2C, and C3.

Before discussing the SID results site by site it is important to address how differences in extraction efficiency between sterols may have skewed the ratios. For Equation (1), as mentioned in Section 4.1.1.6, there was no significant difference in the amount of coprostanol recovered compared to cholestanol for either the spiked sediment or the spiked filter samples. Therefore, one may conclude that Equation (1) should not have a bias to being skewed towards or away from showing a positive signal for human fecal pollution.

Equation (2) only tested positive for humans for one out of the 31 total samples. It has been suggested that this ratio should be used with caution (Pratt, 2005) because cholesterol can be degraded in aerobic environments (Quirk et al., 1980). For the spiked sediment blank there was significantly better extraction for cholesterol than coprostanol; on average 3.2-3.6% greater extraction efficiency was seen for cholesterol than coprostanol. The water column samples showed no significant differences in recovery or extraction efficiency for either sterol. Therefore, one would not expect skewing of Equation (2) for the filters and perhaps a slight bias toward testing negative for human in the sediment. Considering this ratio only tested positive once, showing a negative human result on several occasions when Equation (1) and or (3) was positive, it seems possible that skewing due to extraction efficiencies may have played a role. Another possibility is that Equations (1) and or (3) were indeed overestimating the human fecal content of the creeks. Had more positive results for human input been seen it might have indicated that cholesterol was being degraded, thereby increasing the ratios value and skewing the results.

Equation (3) tested positive for humans the most times of any of the equations used for this study: 13 of the 31 total samples. Equation (3) is potentially especially helpful because Hawkins-Writer et al. (1995) suggested that a ratio of 0.06 corresponds with NPS inputs of human fecal pollution as opposed to point source inputs, which are not present in this system. In the sediment, extraction efficiency was evaluated by spiking two samples with sterol standards. The first spike showed no significantly different extraction efficiencies for any sterol and the second had significantly more cholesterol extracted than coprostanol and significantly less 3β-cholestanol than cholesterol. These results seem to suggest that no significant skewing should be affecting the results of Equation (3) in the sediment as a result of extraction efficiency, especially if the differences in cholestanol and cholesterol cancel. Again, extraction efficiencies in the filter were only performed once so no discussion can be presented about differences between sterols, although comparing sterol to sterol, all recoveries seemed relatively similar.

Equations (4) tested positive for human input only once and (5) never tested positive for human fecal input. This result is not surprising considering in recent work (Isobe et al., 2002) both equations failed to test positive in water known to have human fecal contamination. Isobe et al. (2002) suggested lowering the threshold to 0.5 for tropical water and perhaps it may need to be lowered for temperate waters with NPS pollution since the thresholds were designated by Grimalt et al. (1990) for point source pollution in temperate waters. There were no significant differences in the amount of extractable coprostanol and cholestanol in either sediment or filter samples, therefore,

this ratio is not likely skewed by differences in extraction efficiencies. If the threshold were lowered to 0.5 for Equation (4) there still would have been no positive hits for human contamination nor would any sites have had values within one standard deviation of having a positive hit. Equation (5) in contrast would have had 11 more positive hits if lowered to 0.5, significantly, making it essentially correlate with the results of Equations (1) and (3), and six instances where a positive hit was within one standard deviation of the meanIf one were to assume that Equations (1) and (3) were perfect at indicating human fecal inputs, lowering the threshold for Equation (5) to 0.5 would slightly overestimate human fecal contribution in comparison to the others. Perhaps an intermediate value should be considered.

4.1.6.2 LC1

As can be seen in Table 4.6, LC1 was sampled once for water (9/6/07) and once for sediment (6/4/08) and on neither date had any SID ratios testing positive for human fecal inputs. A review of land cover data suggested that woods, cattle, and horses are likely sources at LC1 and the SID ratios not indicating human input would match what was expected from land cover data. This result matches the SID performed by Marirosa Molina (personal communication), whose MST work found cattle to be the source of fecal pollution at LC1 and saw no signature for human input. LC1 has been found to have fecal counts out of compliance with DHEC regulations, and the results here suggest that human fecal input is not the source. LC1, however, is not considered a “hot spot” for FOLKS 2008 sampling but rather is planned for use as a “clean” head water sample.

4.1.6.3 LC2

LC2 was sampled on three separate dates (9/20/07, 10/4/07, and 10/22/07) for water only and the results are listed in Table 4.13. On no occasion did any of the SID ratios on average test positive for human fecal input nor did the human fecal threshold fall within the error of the value for any SID ratio. Land cover data suggested cattle, horses, or human septic tanks to be possible sources of fecal input at LC2. The results of this study suggest that human fecal pollution was not contributing at LC2, although sampling at different points in the unit hydrograph during a rain event may show human fecal pollution if effluent from leaking septic tanks is being mobilized by the rain. Considering the fecal coliform counts are measured from base flow water samples, human fecal pollution is likely not contributing to the high coliform counts at this site during base flow. The negative result for LC2 matches the SID work performed by Marirosa Molina (personal communication), during the same sampling season, which found cattle to be the source of fecal pollution at LC2 and no evidence of human inputs. LC2 is considered a "hot spot" for FOLKS 2008 sampling.

4.1.6.4 LC4

The only sampling data for LC4 is a water column sample taken on 9/20/07, listed in Table 4.13. Equation (1) and Equation (3) both tested positive for human fecal input on this date. Equation (3) had a value of 0.06, the suggested threshold for NPS human fecal pollution. Interestingly, LC4 is immediately surrounded by horse and cattle but is down stream from points on the north western reach of Little Cane Creek that have some human population with homes on septic tanks. LC4 tested positive for cattle fecal

pollution according to MST work (Marirosa Molina, personal communication) but not human fecal pollution. LC4 was out of compliance occasionally but is not being considered a “hot spot” for fecal pollution in the FOLKS 2008 sampling plan. The fact that the SID results from sterol analysis seem not to match the MST work may be because the sterol analysis results only mean that human fecal pollution is *contributing* to the sterol in the water column at this site. Had the SID ratio for herbivores been quantified it is possible that it would test positive as well. Only comparing the magnitudes of the two appropriate SID ratios would allow statements to be made about which source is contributing the most fecal pollution. Also, the MST analysis is susceptible to false negatives and perhaps the negative human result found was false. Combining the sterol and MST results suggests that human and cattle fecal pollution may both be playing a role in the fecal loading at LC4.

4.1.6.5 LC5

Sampling at LC5 was limited to water samples taken on 9/20/07 and 10/4/07, and results are listed in Table 4.14. On both dates LC5 tested positive for human fecal contamination using Equation (1) and Equation (3). Equation (3) was well above the NPS human input threshold (0.06) on 9/20/07 and at the NPS human input threshold on 10/4/07. On both dates, Equation (5) was close to testing positive for human input, the threshold was set at 0.7 and the values were 0.62 and 0.67 on 9/20/07 and 10/4/07, respectively. Also, it is worth noting that values of all SID ratios were higher on 9/20/07 than 10/4/07, as demonstrated by the fact that values for the first sampling are outside the error for the second. Land cover data suggest a variety of possible inputs in the area

immediately surrounding LC5: horses, cattle, manure spreading for fertilizing fields, beaver, septic tanks, and inputs from wild animals living in forested land. The water column results suggest that human fecal contamination is playing a role in the fecal loading at LC5. Any human fecal pollution at LC5 must have come from human fecal inputs further upstream, likely failed septic tanks. MST methods found cattle to be a source of fecal pollution but not humans (Marirosa Molina, personal communication). This result is similar to that seen at LC4 where sterol SID ratios tested positive for human inputs while MST work pointed to cattle as a source of fecal pollution but did not find evidence of human contribution. Again, combining these results suggests that perhaps both are playing a role, but again the herbivore SID ratio would need to be quantified in order for statements comparing the magnitude of the inputs to be made. LC5 is not part of the FOLKS 2008 sampling plan but points upstream of LC5 (LC5A and LC5C) are included.

4.1.6.6 LC7

LC7 was sampled for both water (9/20/07) and sediment (5/21/08, 6/4/08), and the results are listed in Table 4.7. None of the SID ratios ever tested positive for human fecal pollution, although the threshold for NPS human fecal pollution was within the range of error for sediment sampled on 6/4/08. LC7 had significantly different sterol SID ratio values between 5/21/08 and 6/4/08 for Equations (1), (2), (3), and (5). While these differences did not affect the conclusions (human vs no human) drawn from the SID ratios they do suggest that the sterol profiles changed at LC7 between sampling dates. Values were significantly higher on 6/4/08 than 5/21/08 and this is likely due to the fact

that the flow was much less on 6/4/08, either concentrating inputs or reducing losses due to scouring.

LC7 is immediately surrounded by forested land but is down stream from homes that use septic tanks and cultivated land. Sterol results suggest that human fecal pollution is not playing a large role at this site but again, sampling during the course of a rain event when leaking septic effluent is more likely to be mobilized would provide stronger evidence for the lack of human fecal inputs. MST results tested positive for cattle fecal pollution and not human, which is consistent with the lack of positive human fecal SID ratios. LC7 is not considered a "hot spot" by FOLKS for the 2008 sampling season.

4.1.6.7 LC10

LC10 was sampled for water column samples in duplicate on 9/25/07 and 10/9/07 and for sediment on 5/21/08 and 6/4/08. On all dates for both matrices LC10 tested positive for human fecal pollution using Equations (1) and (3). For the water column samples, LC10 showed a stronger human fecal loading on 10/9/07 than 9/25/07, as evidenced by significantly larger ratio values for both Equations (1) and (3) on 10/9/07. Further, on 10/9/07 Equation (3) was greater than the threshold for NPS human fecal pollution while on 9/25/07 the value was equal to the threshold for NPS human fecal pollution. The difference between the two water column samplings could be due to greater human inputs on 10/9/07 or due to similar human fecal inputs concentrated in a smaller water volume due to low water levels. For the sediment samples there was not a significant different in SID ratios between dates but it is worth mentioning that Equation (3) yielded values above the NPS human fecal input threshold on both sampling dates.

Also, for the 6/4/08 sediment sampling the human fecal threshold for Equation (5) was within the error of the measured value.

LC10 is the sampling site used to represent the culmination of Little Cane Creek and is included in the FOLKS 2008 “hot spot” sampling scheme. The three upper branches of the creek join upstream of LC10, and down stream of LC10 Little Cane flows into Cane creek. Therefore, all of the possible fecal sources upstream of LC10 could be contributing to the coliform pollution, including cattle, horses, failed septic tanks, beavers, cultivated land spread with manure, and wild animals living in the forested land such as deer. The sterol SID ratio results suggest that human fecal pollution is playing a role at LC10, indicating that failing septic tank effluent is reaching the stream. MST work (Marirosa Molina, personal communication) only found evidence of cattle fecal pollution at LC10. Since so many of the sterol samples tested positive for human input and in the case of Equation (3) strongly so, it is likely that MST was exhibiting a false negative for human input at this site or perhaps that there were differences in inputs between sampling dates used for the different techniques. It may be more likely that the MST was exhibiting a false negative; the MST technique is novel and the sensitivity of the technique is still under evaluation. Thus, there is an as of yet unknown amount of uncertainty for the MST technique that must be taken into consideration when interpreting any negative results. As for any possible comparisons that could be made in terms of the magnitude of the different possible fecal sources, again, sterol analysis with herbivore markers would be necessary to make such a determination.

4.1.6.8 LCS

LCS was sampled for sediment on 5/21/08 and 6/4/08 and results are listed in Table 4.9. No water column samples were taken because the water levels were too low to collect 20L samples. LCS never tested positive for human fecal input, although the human threshold for Equation (1) was within the error of the value for 5/21/08 sampling. LCS was chosen as a sampling site because it is located at the bottom of a steep slope about 200m away from a known failed septic tank in a neighborhood where other failed septic systems had already been identified and repaired. It is noteworthy that on neither of the sediment sampling dates was there overland flow from the failed tank, which was pooling in a hole in the home's backyard, to the stream. Sediment sterol measurements suggest that the failed septic tank was not affecting sterol loadings in the stream during either sampling event, but it is suspected that the site may test positive for human inputs via sterol SID ratios if water column samples were taken during a rain event. LCS was not sampled for MST analysis and thus no comparisons can be made between the two methods. No information about fecal coliform counts at LCS is available either, and the site will not be included in the 2008 FOLKS sampling. FOLKS will be helping the homeowner via a price share plan to repair the tank, hopefully in the summer of 2008.

4.1.6.9 C1

C1 was sampled for sediment three times (5/2/08, 6/4/08, and 6/23/08) and for water once (6/23/08), and results are listed in Table 4.10. The sediment samples never tested positive for human inputs, although the human threshold was within one standard deviation of the mean for Equation (1) on 6/4/08 and for Equation (2) on both 6/4/08 and

6/23/08. Significant differences are seen in the sterol SID ratios for sediment between 5/2/08 and 6/4/08 but not between 6/4/08 and 6/23/08. There was a significantly stronger human fecal signal on 6/4/08 than 5/2/08, shown by comparing values for Equations (1), (2), (4), and (5). The difference in values between days could signal differences in deposition due to fluctuations in stream levels or losses due to scouring. The water sample showed a positive human signal for Equation (1) only.

C1 according to land cover data could contain human fecal pollution from septic tanks or sewers, or non-human fecal pollution from cattle, agricultural fields spread with manure, beaver, or animals from forested lands. Sediment results suggest that human fecal pollution could be contributing to the fecal pollution at C1 and the water column results showed a stronger human signal. MST identified no sources at C1 (Marirosa Molina, personal communication). Apparently either the MST technique is showing false negatives or the fecal pollution is coming from a source it cannot detect (like beaver) at C1 since elevated fecal coliform counts have been measured. According to recent fecal counts, Cane Creek is being considered in compliance by FOLKS with the assumption that leaking sewer lines and popping sewer covers during storm events were causing the fecal coliform counts to be out of compliance in the 2007 sampling season. Coliform counts were within compliance for the 6/23/08 sampling date (122CUF/100mL, see Table 4.15) and the site is being included in the FOLKS 2008 sampling as a reference point. However, the positive human SID in the water column suggests that human fecal pollution is still reaching the stream at C1 even though the levels may not be high enough to be driving the coliform counts out of compliance. Coliform analysis was performed

using standard EPA approved methods by three separate DHEC certified labs: Goldie and Associates (Seneca, SC), Clemson Department of Forestry (Lou Jolly), and Greenville Water Laboratory (Greenville, SC).

Table 4.15 Coliform counts by site and lab in CFU/100mL for 6/23/08 sampling.

	Lab 1	Lab 2	Lab 3	Avg	+/-	%RSE
C1	105	151	110	122	25	20.5
C2C	183	136	400	240	141	58.8
C3	94	96	130	107	20	18.7

4.1.6.10 C2C

C2C was sampled three times for sediment (5/21/08, 6/4/08, and 6/23/08) and once for water (6/23/08). Results are listed in Table 4.11. On 5/21/08 Equations (1), (2), and (3) tested positive for human fecal contamination. On 6/4/08 Equation (3) tested positive for human fecal pollution and the human threshold was within one standard deviation of the mean value for Equation (1). On 6/23/08 no SID ratios tested positive for human fecal pollution, although the human input indicator threshold was within one standard deviation of the mean for Equation (3). A significant difference in sterol SID ratios occurred between 6/4/08 and 6/23/08 for Equations (2) and (3) for which both ratios on 6/4/08 indicated a significantly larger human signal. It is possible that the inputs were greater on 6/4/08 or that the losses due to scouring were greater on 6/23/08.

In the water column none of the SID ratios indicated human fecal contamination, but Equation (1) had a value of 0.28 which is quite close to the 0.3 threshold for the ratio. There was agreement in the lack of a positive signal for human fecal coliform in the sediment or the water column on 6/23/08. No water was collected other than on 6/23/08

so no comments as to whether there was a significant difference in SID ratios observed in the water column between dates can be made.

C2C could conceivably have fecal loadings from any source or combination of sources upstream, including septic tanks or sewers, cattle, agricultural fields spread with manure, beaver, or animals from forested lands. A sewer line crosses Cane Creek at C2C and popping sewer covers during storm events have been found within hundreds of yards of the sampling site. According to FOLKS, the town of Walhalla repaired the popping sewer caps at C2C and Cane Creek is considered in compliance. Coliform counts from the 6/23/08 sampling, however, were still high (240CFU/100mL, see Table 4.15). C2C is the only site that has a positive hit for human fecal pollution using the MST technique although MST results also indicated fecal pollution from cattle. The MST results, again, are from Fall 2007, before the sewer cover was fixed, and none are available for Spring 2008. Nonetheless, at C2C the sterol and MST techniques have both shown human input signals, suggesting that C2C was affected by human fecal contamination in fall 2007 and perhaps still may be. To catch a leaking sewer problem (or perhaps septic tank input) water sampling would need to be conducted during a rain event (although a popping sewer cover may be easily implicated by visual evidence).

4.1.6.11 C3

C3 was sampled for sediment on 5/21/08, 6/4/08, and 6/23/08, and results are listed in Table 4.12a. Water column samples were collected on 9/6/07 in singlet, 9/27/07 in duplicate, 10/9/07 in singlet, and 6/23/08 in singlet and are included in Table 4.12b. In the sediment, Equation (3) tested positive for human fecal input on all sampling dates and

Equation (1) tested positive within one standard deviation of the mean on 6/23/08. Equation (3) suggested NPS human fecal inputs on 5/21/08 and 6/4/08 and more direct inputs on 6/23/08. There was not a significant difference between the values of the sterol ratios for any of the dates sampled, implying either the deposited loadings were similar or that scour made the loadings appear similar.

The water column samples showed a positive human fecal signal only on 10/9/07 for Equation (1) and Equation (3), although the 9/27/07 sampling date was within one standard deviation of the mean for testing positive for human fecal input using Equations (1) and (3). Because only one sample was taken in duplicate it is impossible to state whether the differences in values between dates were statistically significant. However, comparing magnitudes suggests that the 10/9/07 sample had a higher value (Equation (1) equaled 0.42 on 10/9/07 compared to 0.23, 0.28+/-0.08, 0.2 on the other sampling dates) than the other sampling dates that strongly suggests human fecal pollution while the others did not or not as strongly. Since water column samples should correlate with inputs one could assume that there was a greater human input on 10/9/07 than the other sampling dates perhaps due to a storm event mobilizing human fecal input from septic or sewer systems.

Sediment samples suggest that human fecal pollution has played a role in the sterol loading at C3 while water samples only showed a positive signal on 10/9/07. Further, sediment taken on 6/23/08 showed a positive signal for human fecal pollution while the water column sample did not. This result suggests that the human signal in the sediment was showing there had been some historical human fecal input but not one

recently occurring in the water column. More paired sampling could track such differences between the deposited and suspended particulates, but due to the uncertainty in the sediment samples, pursuing water column samples would be a better choice since they will necessarily correlate with inputs. Coliform counts taken on 6/23/08 indicate that C3 is in compliance with DHEC regulations (107CFU/100mL, see Table 4.15).

C3 is the final culminating sampling point on Cane Creek and could be affected by any or all of the fecal sources that could enter upstream, including septic tanks, sewers, cattle, agricultural fields spread with manure, beaver, or animals from forested lands. Sediment samples suggest that human fecal pollution has played a role in the sterol loading at C3 while water samples only showed a positive signal on 10/9/07. MST found signals for cattle and bird fecal pollution at C3 but not human (Marirosa Molina, personal communication). Again, the fact that sterols showed a positive signal for human input and the MST did not may be because MST is subject to false negatives; MST was done on different sampling dates from the water column sampling and loads were a function of date; or the sterol analysis with the given suite used for this study provides no information about other fecal sources and the other sources are much greater than the human inputs. A better way to compare the methods would be to do a sampling for both sterols and MST in the water column on the same day. Again, FOLKS is considering Cane Creek as being in compliance now that upgrades to sewer caps have been made and the coliform counts from 6/23/08 are in compliance. More water results would be necessary before sterol SID could confirm this, but sediment results indicate that there has been some historical human fecal pollution reaching C3.

4.1.6.12 Comparing Coliform Counts to Human Inputs

Unfortunately, sampling could only be coordinated such that coliform counts (performed by Goldie & Associates, Seneca, SC, the Greenville Water Lab, and Lou Jolly, Clemson University) and sterols were measured on the same day once (6/23/08) and only for three sites (C1, C2C, and C3). Nonetheless, it is worthwhile to see whether even in this small data set there was any correlation between the human fecal sterol, coprostanol, and coliform counts as well as the human SID ratios and coliform counts. If coliform counts correlate with human input it would suggest that the human input is playing a large enough role in the fecal loadings to affect the coliform count. If coliform counts do not correlate with human input it would suggest that other fecal sources are having a greater effect on the fecal loadings to the creeks. Correlations for sterols and SID ratios for sediment are included as Figures B 1a-B 1l and for filters as Figure B 2a-B 2l in Appendix B.

For the sediment there was never a significant difference in the means for any of the sterol concentrations, nor for Equation (1), Equation (2), Equation (3), Equation (4), or Equation (5) between sampling sites C1, C2C, and C3 on 6/23/08. Nor was there a significant difference between coliform counts at the sites (see Table 4.15 for coliform results). Considering Labs 1 and 2 have a smaller difference in values from one another than either with Lab 3 suggests that sampling or handling in the field was creating the large error in coliform counts, if one assumes that analytical precision was not the cause because all were performed at DHEC approved lab facilities. However, such an assumption is not necessarily acceptable given the susceptibility of fecal coliform

quantitation to large uncertainty and bias (hence the quality assurance and control protocol calling for analysis by three separate labs). Because the uncertainties overlap, no correlation can be made between the sediment sterol concentrations and coliform counts. The inability to correlate could suggest several things. (1) Human fecal inputs may in fact correlate with coliform counts but sterol measurements in the sediments and the fecal counts are too imprecise to reveal the correlation. Perhaps if the variability was reduced in the sediment results by correcting for TOC and if, as suggested by Lou Jolly, turbidity was creating error in the coliform count results, more careful sampling could make coliform counts more precise and the error for both coliform counts and sterol concentrations could be reduced to reveal a correlation. (2) Human fecal inputs actually do not correlate with coliform counts, implying that human fecal inputs are not the major fecal source at these sites. (3) Sedimentary sterol loads do not correlate with water column fecal loads and, therefore, coliform counts. Of these (3) seems the most likely although (1) is probably playing a role as well. In some locations, (2) is likely applicable as well. Sediment sterol loads at best are a measure of historical fecal loadings and likely may not correlate with the instantaneous water column conditions. This is further supported by the fact that water column coprostanol and human SID ratio correlate with coliform counts, as will be discussed next. Others have found correlations between sediment and water column fecal sterol loads (Isobe et al., 2002) but these studies were conducted in areas that likely had more consistent deposition and were less subject to differential losses due to scouring. It is likely as well that uncertainty in sediment sterol concentrations and coliform counts is too great to reveal a correlation or lack thereof.

Perhaps including the recommendations above in future studies would help reduce uncertainty enough to elucidate these factors.

Because there was no significant difference in coliform counts between sites, no correlations can be made for the filter samples between sterol concentrations or any of the SID ratios. No error is included in the measurements for the filter (water particulates) samples because they were taken in singlet, but it is suspected, considering that the RPDs for filter duplicates were generally smaller than the %RSEs for sediment triplicates, that the differences between the sites for the filters are actually statistically significant. Inclusion of duplicates would be needed, however, to confirm this suspicion. The fact that correlations cannot be made between sterol measurements in the water column or SID ratios and coliform counts could result from several factors. (1) Human fecal inputs as evidenced by the presence of coprostanol and SID ratios in the water column do correlate with coliform counts but error in coliform counts is sufficient to mask correlations. (2) Human fecal inputs do not correlate with coliform counts, implying human fecal pollution is not the major source of fecal coliform at these sites. To figure out which of these is the case would require further study with improvement of the precision for coliform counts. Unlike the sediment, it is believed that fecal sterol concentrations in the water column should correlate with inputs. Other researchers have tried correlating coprostanol concentrations with coliform counts and found a logarithmic relationship between coprostanol and coliform counts where human fecal loadings were causing the majority of the fecal pollution (Isobe et al., 2002). Adding in 24-

ethylcoprostanol would be especially helpful here because if herbivore fecal pollution is the major fecal source 24-ethylcoprostanol should correlated with coliform counts.

4.1.6.13 Source Tracking along Streams

Comparing fecal signatures along sampling points on the streams can give insight into where fecal loadings are originating. For example, if an upstream site shows no human signature but a site does so further down stream one could assume that the human fecal contamination is entering between the two points. Withstanding the limited data set, the fact that sampling results represent data from two different seasons and several different dates within those seasons, and the different matrices explored, it is still worth discussing how fecal signatures change along the course of the streams. Of course, it is necessary to remember that while water column concentrations will correlate with inputs, sediments will not necessarily correlate with recent inputs or historical loadings but rather will give an inconsistent snapshot of the historical deposition subject to losses via scouring and decomposition.

Little Cane Creek has two main branches that meet just south of LC4, and they will be discussed in terms of the western and eastern branch. Looking at the map shown in Figure 1.1, the western branch includes LC1, LC2, LC2A, LC3, LC3A, LC4, and LC4A and the eastern includes LC5, LC5A, LC5B, LC5C, LC6, LC6A, LC6B, LC7 and LC7A. On the western branch, only including sties sampled for sterols in this study, water flows from LC1 to LC2 to LC4 before meeting with the eastern branch. On the eastern branch the water flows from LC7 to LC5 and then both branches combine before reaching LC10. On the western branch no human signal was found at LC1 or LC2,

suggesting that the community north of LC1 that is served by septic tanks may not be contributing to the fecal loadings in Little Cane Creek. By LC4 a positive human signal was measured, suggesting human inputs may have entered between LC2 and LC4, especially since both were sampled for water on the same date. Combining this with land cover data would suggest leaking septic tanks between the two sites were to blame. On the eastern branch no human signal was detected at LC7 but was detected at LC5, and one of the sampling dates for these data sets over lapped (water column samples taken 9/20/07). The fact that the SID result was different at LC7 and LC5 on a sampling date that overlapped suggests that temporal changes in inputs did not cause the different signatures at the two sites. Rather, it suggests that somewhere between LC7 and LC5 on the eastern branch there in an introduction of human fecal input and again probably from septic tanks. Flow from all LC points join by LC10, including LCS. No human signal was seen in the eastern branch, nor at LCS which joins the creek south of where the two branches combine, but human fecal pollution was detected in the western branch. At LC10 a human fecal signal was present as well, suggesting that by the end of Little Cane Creek there is human fecal contamination whether coming only from the eastern branch or coming from the eastern branch and other sites south of LC4 after which the two branches join.

Cane Creek will be discussed by dividing it into a northern and southern branch, where the northern branch includes C2A, C2B, and C2C and the southern includes C1, C1A, C1B, C1C, C1D, CW1, CW2, and CW3, which join before reaching C2 and eventually flow into C3. C1 tested positive for human fecal input in the water column

and in a few cases human fecal input was within a standard deviation of the mean for the sediment. This suggests that upstream of C1 there has been human fecal input, likely coming from leaking sewer lines in the town of Walhalla or possibly from homes with failed septic tanks. C2C tested positive for human input in the sediment or within one standard deviation of the mean for the sediment, and it also had ratios close to the threshold for indicating human input for the water column (measured in singlet), suggesting human fecal contamination may still be reaching Cane Creek upstream of or at C2C. Again, the source would likely be leaking sewer lines, but failing septic tanks could possibly be contributing. By C3 there was a smaller signal for human input in the sediment and water column, suggesting that perhaps by the time water reaches C3 some of the human sterol input has been degraded or perhaps diluted below detection limits or threshold ratio values. If dilution was the case, more human inputs could be occurring between C3 and the points upstream but likely not in large quantities. The low coliform counts measured on the 6/23/08 sampling date (see Table 4.15) when both water and sediment samples were taken for C1, C2C, and C3 indicate that any human fecal signal seen was not so large as to take the sites out of compliance.

A better way to determine how inputs along the stream play a role in the fecal inputs would be to take water column samples at sites at the same time on the same day and compare the instantaneous sterol fingerprints (i.e.- a synoptic survey). Doing so would not only allow for correlations between input concentrations (unlike in the sediment) but also remove the error inherent in any up/down stream comparisons made here when comparing between different combinations of matrices and dates. Once fecal

sources had been determined and BMPs put in place the water column sterol profiles could be used to track whether the BMPs actually affected the sterol profile in the water column.

4.2 FWA

An example calibration curve for FWA is included in Appendix A as Figure A 10. Calibration curves for FWA analysis were generated from fresh stock solutions on each analysis date. The R^2 for calibration curves ranged from 0.97-0.99. FWA analysis results for Little Cane Creek are shown in Figure 4.7, Cane Creek in Figure 4.8, and for the Coneross Creek Waste Water Treatment Plant (CCWWTP) and the failed septic system upstream of LCS in Figure 4.9. Results are expressed in equivalent mg detergent per liter, meaning that if all the fluorescence measured at the given emission and excitation wavelength was due to FWA it would have come from that many mg of formulated detergent. As mentioned earlier, since FWA is subject to interferences from NOM and non-wastewater pollution with chemicals, such as motor oil, that fluoresce at the same excitation and emission wavelength as FWA (Hartel et al., 2007a; Uchiyama, 1979), it is, therefore, unlikely that all fluorescence would be from FWA. However, the effect of these interferences was taken into account when the threshold for FWA pollution was suggested to be set at 100mg/L by Hagedorn et al. (2003).

4.2.1 Sampling Results

Samples were taken and split in triplicate. Multiple samples were not taken because the FWA, if present, was likely well mixed in the water and would show only temporal changes in FWA in water flowing through the streams.

Using the calibration curves to calculate equivalent mg detergent per liter the DDI water blanks had -1.35 to 1.59 equivalent mg of detergent per liter. In the creek water, the values were low and random and do not follow any pattern with land use. Results are shown for Little Cane Creek in Figure 4.7 and for Cane Creek in Figure 4.8. Concentrations were so low that the uncertainty was often several times larger than the value at a given site. In Little Cane Creek, equivalent mg detergent per liter ranged from -0.84 to 2.93 and in Cane Creek from -0.53 to 3.01. Creek water on 2/19/08 showed significantly greater FWA signal than blanks at C3, LC1, LC4, LC7, and LC8, but the values were well below the FWA pollution threshold suggested by Hagedorn et al. (2003). On 5/2/08 all sites showed significantly larger FWA signals than the blank except C3B, LC4A, and LC8, but again all had values significantly below the suggested FWA pollution threshold. Finally on 5/21/08, all sites showed significantly greater FWA signals than the blank except LC10 and LCS, and again all creek samples were significantly below the suggested FWA pollution threshold. The small signals at the sampling sites are likely due to fluorescent algae, NOM, or contaminants such as motor oil or brightener from paper trash rather than FWA from detergents. The lack of an FWA signal at LCS, downstream of a known failed septic tank that tested positive for FWA (see below) is not so surprising considering septic effluent was pooled in a hole in the home's backyard and not flowing overland into the stream at the time of sampling. If the failed septic tank had a plume reaching the stream it is likely that the FWA was removed due to its strong affinity for adsorption to soil before the plume reached the stream, thus

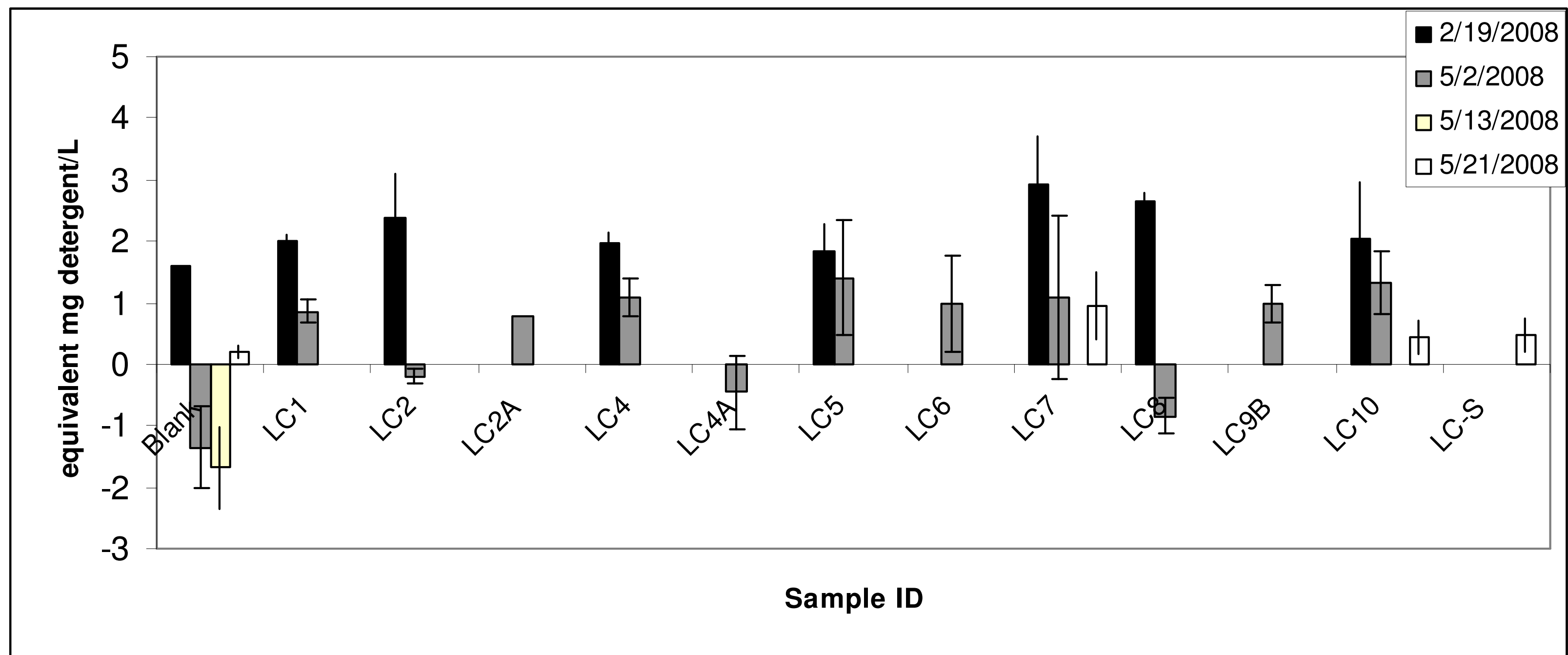


Figure 4.7 FWA results for Little Cane Creek.

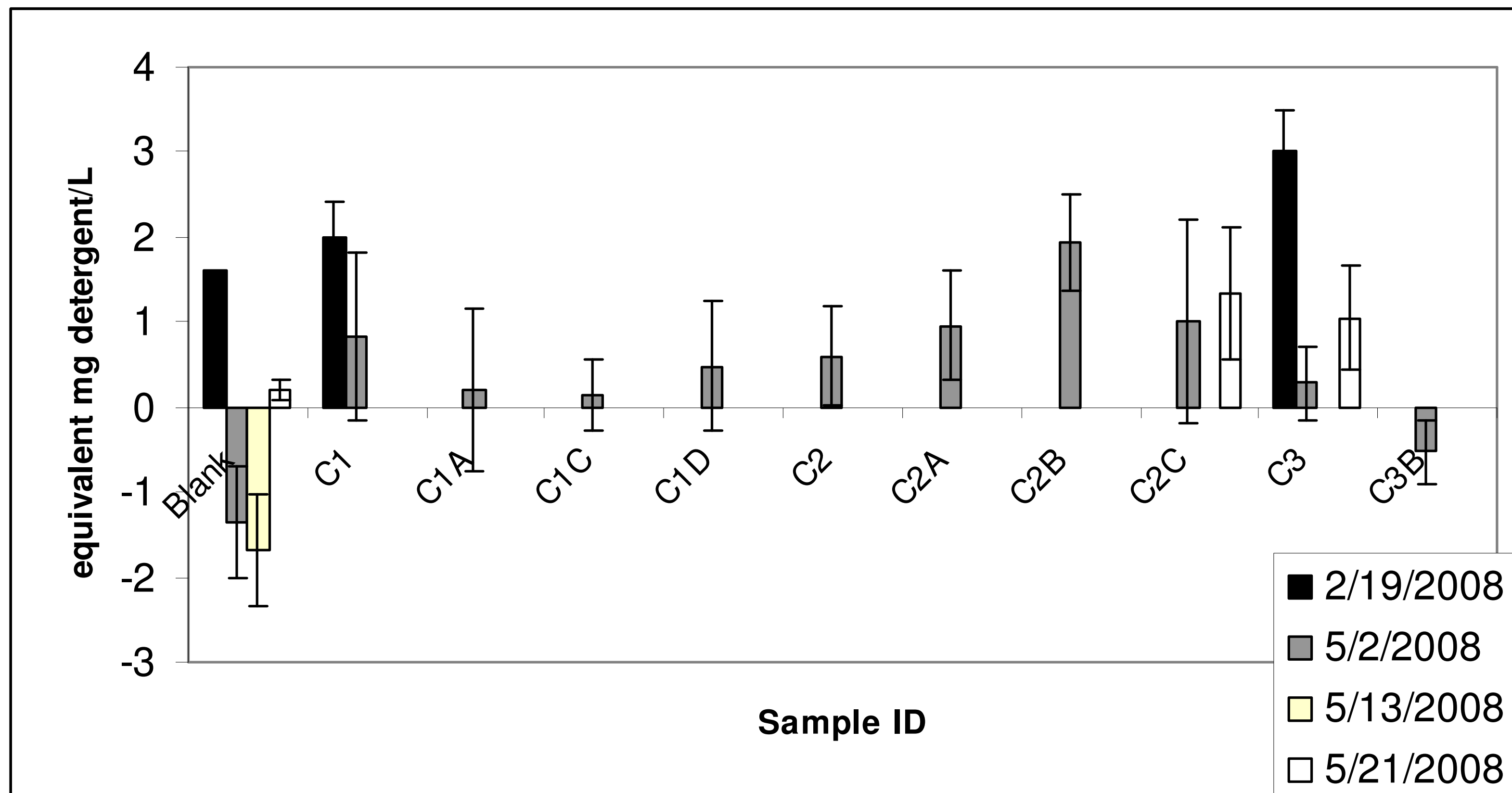


Figure 4.8 FWA results for Cane Creek.

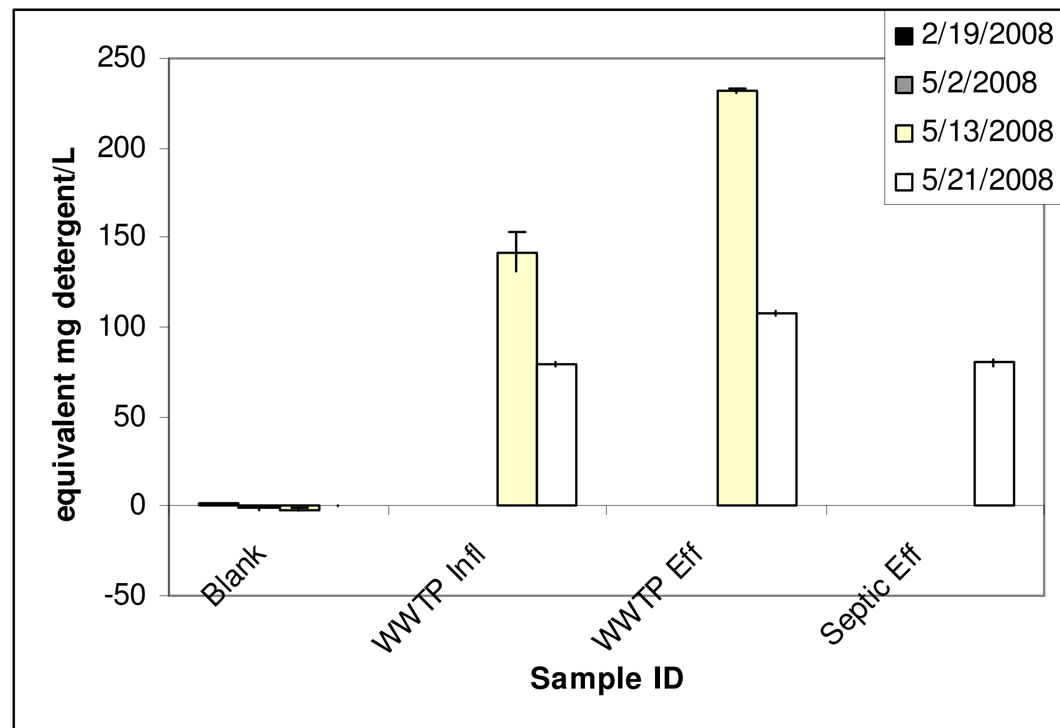


Figure 4.9 FWA results for the Coneross Creek WWTP influent and effluent and the failed septic upstream of LS.

in such a scenario providing a false negative.

Results for CCWWTP and the failing septic system are shown in Figure 4.9. CCWWTP influent had 79.0 to 141.9 equivalent mg of detergent per liter and the effluent had 107.2 to 237.1 equivalent mg detergent per liter. The water from the failed septic system had 80.5 equivalent mgdetergent per liter. WWTP influent and effluent and water from the failed septic system all showed significantly larger brightener signals than the blanks. While again, all of the fluorescence in these samples is likely not from brighteners, the high magnitude of the brightener signal and known composition of wastewater suggests that much of it is from FWA. To confirm that large FWA signal is indeed coming from brightener and not interferences would of course require more sophisticated analytical techniques, such as HPLC.

WWTPs have been shown to remove 30-98% of FWA depending on the type of treatment used (Kramer, 1992), and thus one would have expected the effluent to have a lower FWA concentration than the influent. But, the WWTP influent appeared to have a significantly smaller signal for brightener than the treated wastewater. It is important to note that by visual inspection, as would be expected, WWTP influent had higher turbidity and that the detection method here was direct fluorimetery without any clean-up for interferences. It is probable that if the influent was filtered to remove suspended particles blocking fluorescence it would have had a greater brightener signal than the effluent. Filtering both influent and effluent samples before detection would help confirm or deny this hypothesis. It is also possible that the effluent contained other fluorescing constituents, such as NOM from biological treatment, or that FWA in the influent was

lost due to sorption to particulate matter (Kramer, 1992). It is noteworthy that the effluent from the failed septic tank had an FWA signal that was not significantly different from the WWTP influent, which makes sense as the WWTP influent was composed mostly of raw untreated sewage from homes since samples were not taken during storm events.

Hagedorn et al. (2003) suggested using 100 equivalent mg/L as a threshold for indicating brightener pollution. The value was set to be high enough to ensure that stream background fluorescence from NOM or algae was not producing false positives. Using the 100mg/L cutoff neither the CCWWTP influent on 5/21/2008 nor the failed septic system effluent would have qualified as having brightener pollution. Since the purpose of the cutoff is that it be set low enough to detect wastewater sources, it is suggested that this limit be lowered for the current study system. Of course, before doing so a more thorough look at the temporal nature of brightener signals measured in wastewater would be appropriate. It is possible that the hours chosen for sampling (weekday mornings) do not coincide with heavy detergent use. Grab samples were taken on all occasions and no efforts were made to determine if the FWA concentrations had a temporal pattern. Samples from both the CCWWTP and failed septic system were taken in the morning hours on a weekday. It is thought that if samples were taken from the WWTP during hours when more laundry is likely to be done (evenings, weekends) the signal might be higher. Further study could provide confirmation that may or may not be helpful in this project. No values could be found in the literature for the concentration of FWA in mg detergent/L for wastewater influent, but Poiger et al. (1998) reported total

concentrations of DAS1 and DSBP, two FWA commonly used in detergents, to be from 12.7-29.9 μg/L in WWTP influent from Zurich-Glatt, Switzerland. Assuming 0.15% of detergent is composed of FWA by weight that would equal 1.91×10^{-3}-4.49×10^{-3} equivalent mg of detergent per liter. This is well below the suggested human wastewater value, which was deliberately set high to avoid false positives from organic interferences. Researchers have used the suggested threshold to indicate human fecal pollution from failing septic tanks (Hartel et al., 2007a). However, the results of Poiger et al. (1998) combined with the WWTP influent findings for this study suggest that lowering the threshold value would be reasonable.

The lack of any stream samples testing positive for FWA does not necessarily mean that human wastewater is not reaching the streams. FWA analysis can give false negatives. As stated earlier, FWA have shown potential to degrade in sunlight. Also, FWA sorbs strongly to sediment, thus it could be removed in subsurface flow from a failed septic plume before reaching the streams or by suspended particulates in the stream. It is also possible that brighteners are present but below detection. However, if that is the case brightener levels would not be high enough to indicate human fecal pollution anyway. Given that no positive signals were found, sampling plans were scaled back from attempts to do a synoptic survey of as many sites as possible on the creeks to hitting a smaller set of sites with a range of land use and possible fecal sources. Had a positive signal been seen, determination as to whether it represented FWA as opposed to NOM or other interferences would have been necessary. Since signals were so low, sampling for brighteners was suspended. FWA sampling might be worthwhile in a rain

event to catch potential septic system leakage, human “hot spots” were identified by other source tracking methods.

4.2.2 Standard Additions

As a check to determine if components in the sample matrix were dampening FWA signals, a series of standard additions was performed on a subset of samples from the 5/21/08 sampling trip. Results from the standard additions are shown in Figures 4.10, 4.11, and 4.12 for LC7, C3 and WWTP influent, respectively. The creek samples were chosen to represent a range of fecal sources. LC7 is close to the top of Little Cane Creek, immediately surrounded by woods while C3 is the final sampling site on Cane Creek and

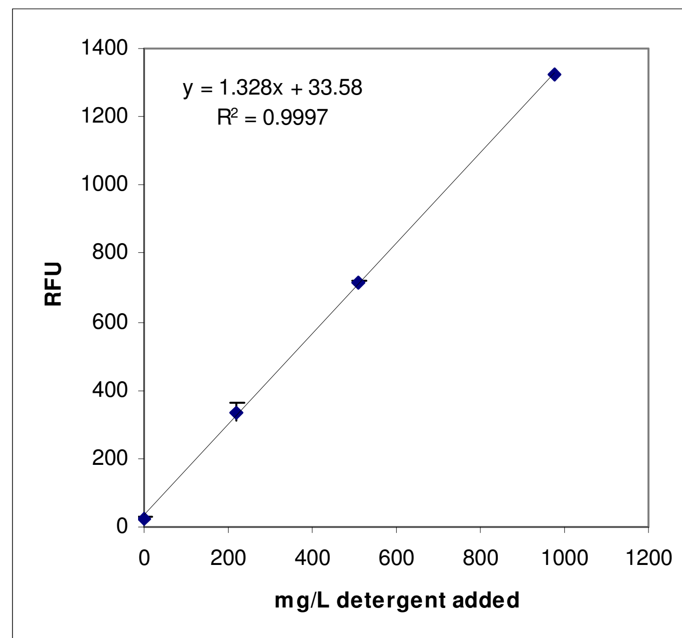


Figure 4.10 Standard addition for LC7.

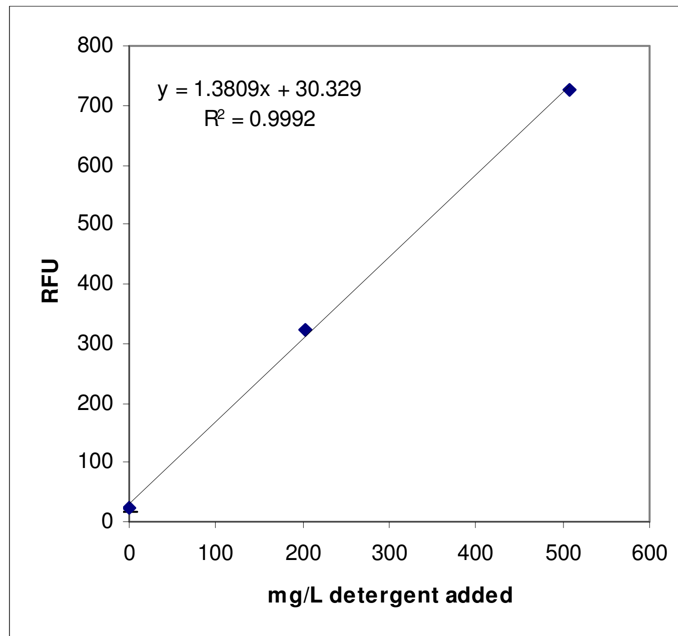


Figure 4.11 Standard addition for C3.

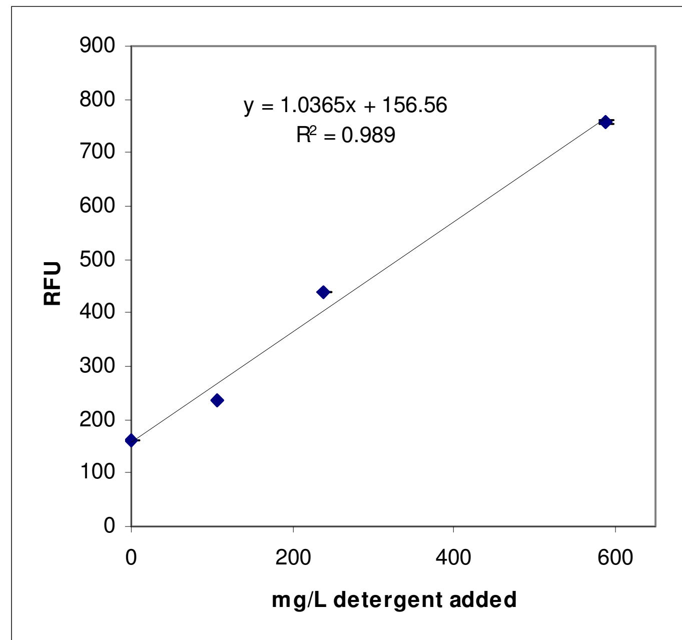


Figure 4.12 Standard addition for CCWWTP influent.

The standard additions show that if brightener is added to creek water or wastewater a larger FWA signal is observed. Comparing the slope for the plots for the creek standard additions reveals that the slopes, 1.305 for LC7 and 1.324 for C3, are similar but slightly smaller than the slope of the calibration curve for that day, 1.704. The difference is small and may not be significant. The slope for the plot of standard additions to the WWTP influent has a slope of 1.048, which again is smaller than that for the calibration. The difference between the slopes for the WWTP influent standard addition is greater than that for either of creek standard additions, which are about the same as each other. This greater difference may imply some of the fluorescence is being blocked by the WWTP influent, perhaps due to turbidity or FWA sorption to SP. Again, a check if turbidity was the cause would be to filter samples before analysis but after FWA spiking and see if the signals change. If it increases it is likely turbidity was dampening the FWA signal; if it decreases either sorbed FWA was removed or other compounds that fluoresce at the given excitation and emission wavelength were removed. To check if sorption to SP and settling of SP was causing the dampened signal water could be filtered prior to FWA spiking. If the signal was greater when samples were filtered before spiking than for not filtering before spiking it would imply that sorption to SP was in fact dampening the FWA signal and would reinforce the suspicion that the threshold value for FWA pollution should be lowered.

4.3 Comparing Methods

Two different chemical tracer methods were used for this study, and both gave information only about human inputs. Only two sampling events included both methods (5/2/08 and 5/21/08), and sterol analysis for both dates was confined to the sediment. FWA was never detectable at levels high enough to indicate human wastewater pollution in the streams. Sterol profiling, however, did produce positive signals for human fecal pollution. Site by site, LC7 tested negative for both, LC10 was positive for human fecal sterols and negative for FWA, LCS was within a standard deviation of testing positive for human and was negative for FWA, C1 was negative for both, C2C was positive for human fecal pollution and negative for FWA as was C3. Thus, the two methods provide conflicting results. This could be due to differences in the analyzed matrix: FWA was always measured in the water column and was susceptible to rapid degradation while sterols were measured in the sediments only (on overlapping dates) and thus it is possible that the sediments were pointing to historical human fecal inputs that would not have been instantaneously seen in the water column. Also, it is possible that the FWA was showing a false negative, as discussed above, or that the sterol analysis was overestimating human fecal inputs. Combined with the MST method (albeit across seasons), only C2C showed a positive human signal by MST and sterol analysis, which was consistent with some of the sterol profiling but not with FWA results. Combining all source tracking methods on the same sampling trip and performing all measurements in the same matrix would be the best next step to compare the methods and confirm whether

the observed differences were actually related to fecal inputs or were a function of different sampling dates, matrices, or technique biases.

CHAPTER 5

CONCLUSIONS AND RECCOMENDTATIONS

This work demonstrated that fecal sterols are present at quantifiable levels in Little Cane and Cane Creek and further developed a method that was capable of isolating and measuring fecal sterols both in the sediment and the water column. Using SID ratios capable of indicating human fecal pollution, several sampling sites were found to test positive for signatures of human fecal inputs. In contrast, FWA methods were unable to detect human fecal inputs at any site along Little Cane or Cane Creek, but could detect FWA indicating positive signals of human input in WWTP influent and effluent as well as septic tank effluent. Comparing results of the two techniques suggests either that the sterol ratios were overestimating human fecal pollution, FWA analysis was providing false negative signals probably due to the inability of the simple fluorescence method used to differentiate low levels of FWA against the background signal, or perhaps that the inconstancy was an artifact of different sampling dates and matrices. The later two are believed to be most likely. Comparing sterol results to MST techniques performed in the fall of 2007, one site tested positive according to both sterol and MST for human fecal input, but the MST did not match the sterol data for any other sites. This apparent discrepancy is either due to actual differences at the sites for different sampling dates, overestimation of the human fecal input using sterol profiles, susceptibility of MST methods to false negatives, or perhaps, again, differences between what is seen in the water column and bottom sediments.

For future study it is recommended that all SID sampling be performed on the same day to facilitate better comparison between methods. Also, due to the suspicion that sediment in the creeks does not represent true historical (nor instantaneous) fecal sterol loadings and, therefore, has a complicated correlation with inputs, it is recommended that future sampling focus on sampling the water column. This would also make it so all analyses (sterols, FWA and MST were performed on the same matrix and thus remove the matrix choice as a possible source of error. Sampling in the water column could be targeted to occur during base flow to establish baseline fecal sterol levels, and then sampling at different stages in the unit hydrograph could be used to gain information about how storm events affect fecal loadings. It is thought that storm events may affect fecal loadings through mobilizing failed septic effluent, sewer overflow, or increasing overland flow inputs such as from animals, farms or agricultural fields spread with manure. If sampling in the sediment is continued it should be expanded to include TOC analysis along with the sterol analyses. Doing so would help determine if the difference between method and spatial variation is due to actual spatial differences in sterol concentration or due to differences in organic carbon content of the sediment. Easier sampling, faster sample preparation, and the ability to collect samples even in very low flow, however, are benefits of sediment sampling that must be considered before sampling from the sediment is suspended. Likely, certain sites with very low flow may require sediment sampling to be able to do a sterol analysis.

In future studies the sterol suite should be expanded to include 24-ethylcoprostanol in order to allow for SID of herbivorous fecal pollution. It is possible

that both the sites that tested negative and positive for human fecal pollution are being affected by herbivore fecal pollution. Comparing the magnitude of the human to the herbivore signatures would allow conclusions about which (if either) is more important at a site. Such information could be used to better direct money earmarked for BMPs.

As for FWA analysis, it is recommended that a rain event be sampled. If no signal is seen it is recommended that analysis for FWA be stopped unless significant human input “hot spots” of pollution are identified. More specific techniques for FWA analysis are available, including HPLC, but considering that the methods are time consuming and the fact that sterol and MST can both detect human fecal pollution, pursuing more complex FWA analyses may be a waste of time and resources.

Limited information can be gleaned from this study as to the NPS inputs contributing to fecal pollution in Cane and Little Cane Creeks. It can be said, however, that human fecal pollution is likely playing a role in both Little Cane Creek in the eastern branch and down stream sections and in Cane Creek possibly throughout the creek. Future study would be needed to narrow down where the human input signal is coming from, and it would be best if it were expanded to include other fecal sterols to identify other possible fecal sources in the streams. Also, it would be best to coordinate so that coliform counts are performed when sterol samples are taken since fecal coliform counts are used for determining compliance.

This work is significant not only because it further developed a technique for measuring fecal sterols but also because it demonstrated that fecal sterols are quantifiable and potentially useful in SID for small watersheds. To the author’s knowledge it is the

first study done in such a small watershed. This work is also useful because it compares different emerging source tracking techniques. The information gained from this study can give FOLKS an outline of where human fecal pollution may be occurring and may be contributing to coliform counts in the creeks so future inquiries can be better focused. Designed as a scoping study, this work provides the required stage for a study that would provide FOLKS and DHEC with valuable SID information so BMP funds can be spent most efficiently. Ultimately, the sterol SID technique could even be used to monitor the impact of BMPs.

APPENDICES

Appendix A

Calibration Curves

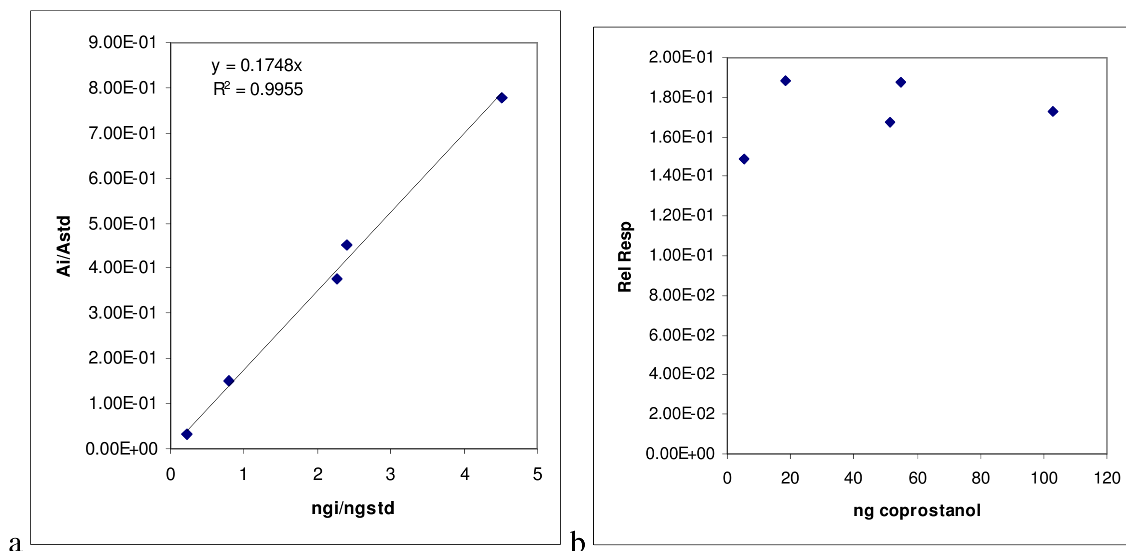


Figure A 1 a. Calibration curve for coprostanol. b. Relative response plot for coprostanol.

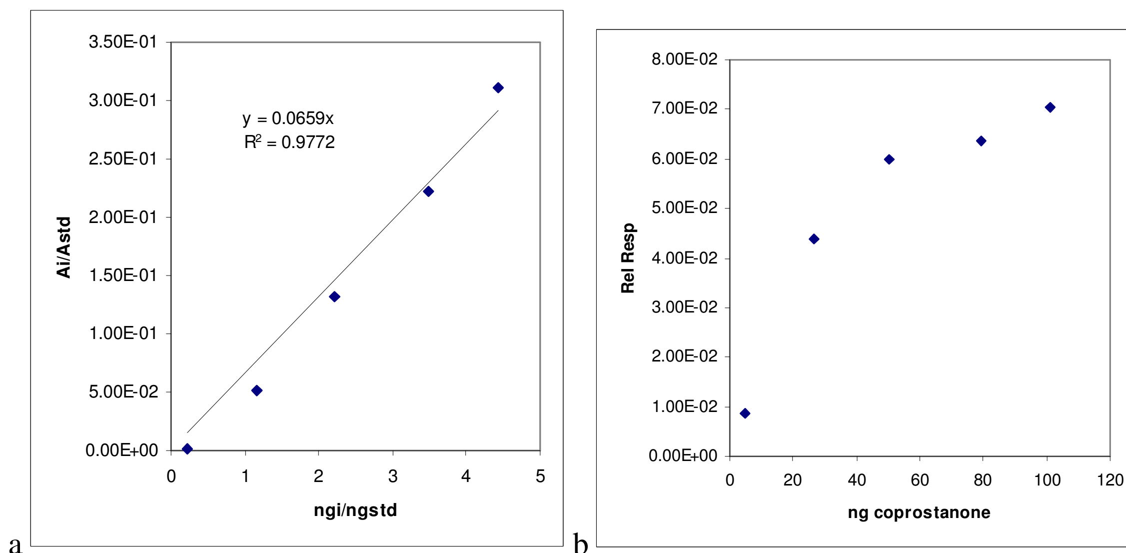


Figure A 2 a. Calibration curve for coprostanone. b. Relative response plot for coprostanone.

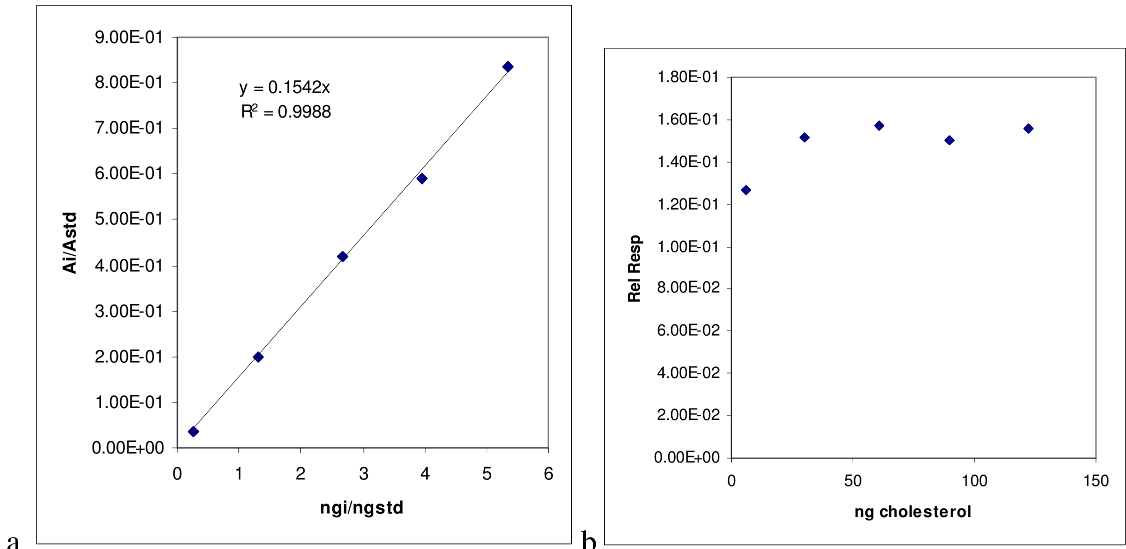


Figure A 3 a. Calibration curve for cholesterol. b. Relative response plot for cholesterol.

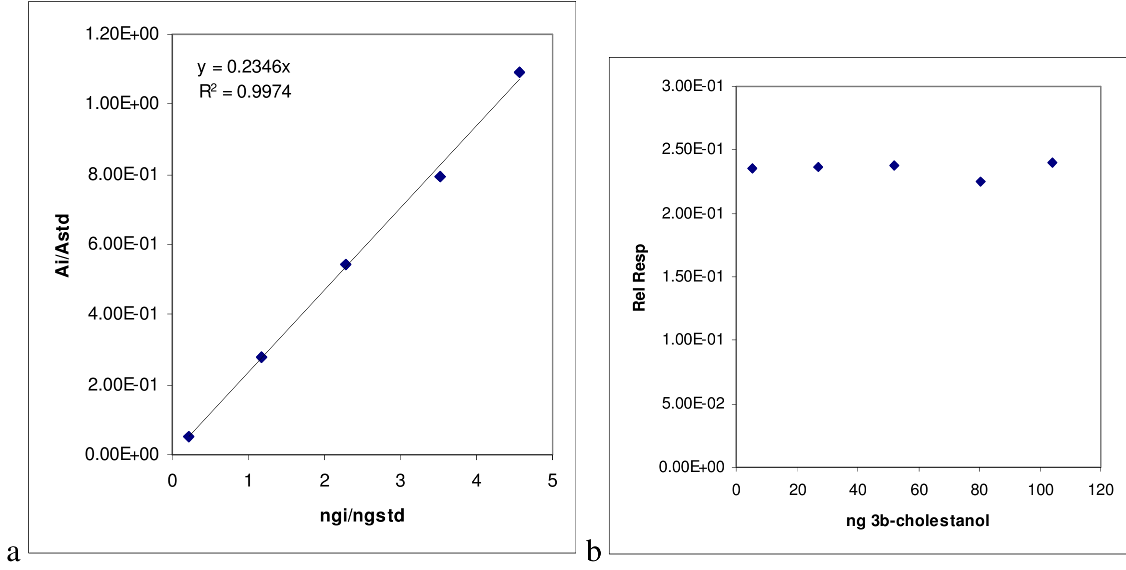


Figure A 4 a. Calibration curve for 3β-cholestanol. b. Relative response plot for 3β-cholestanol.

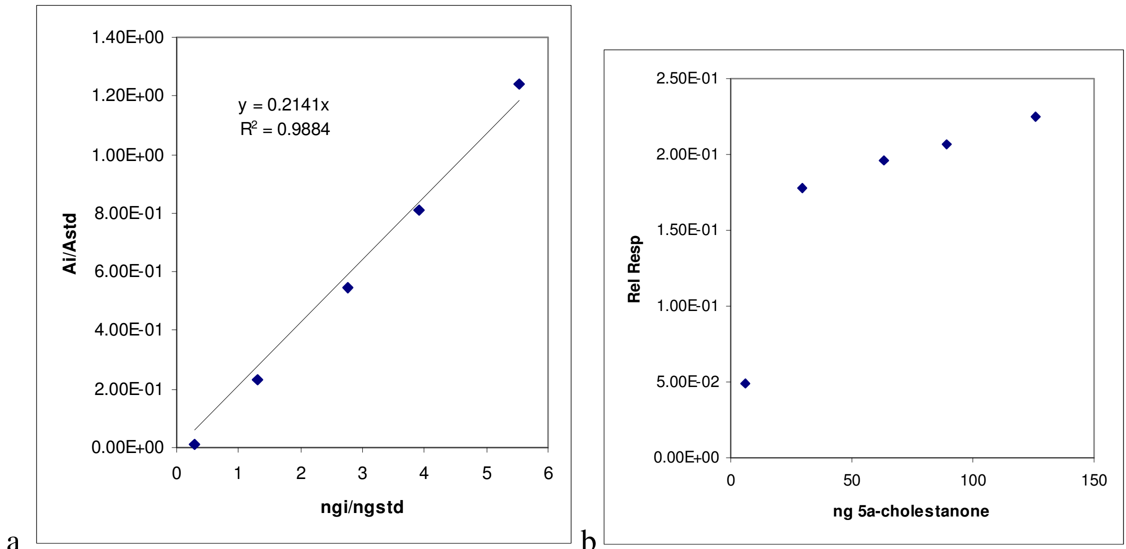


Figure A 5 a. Calibration curve for 5α-cholestanone. b. Relative response plot for 5α-cholestanone.

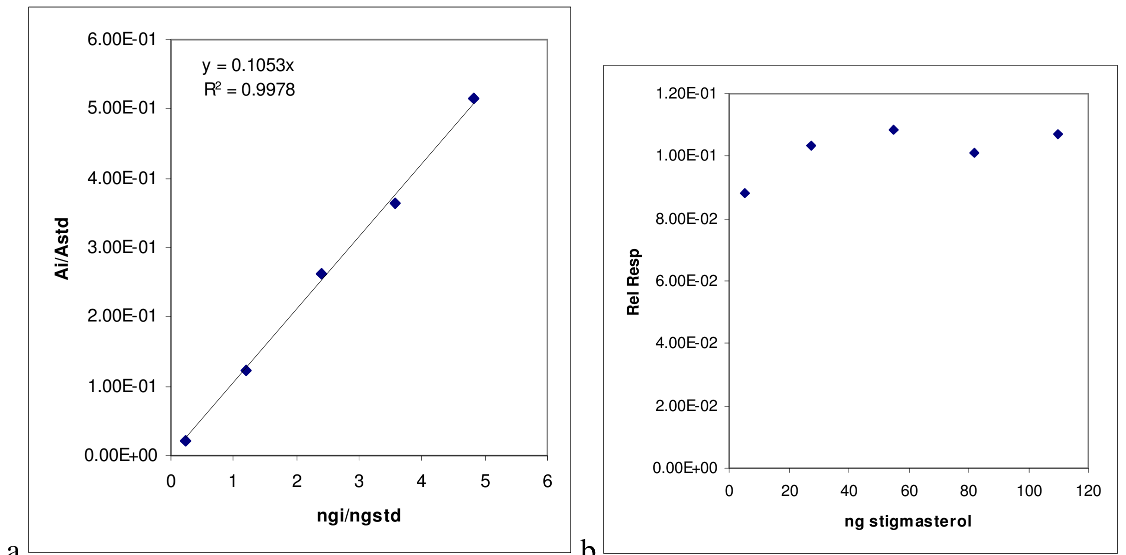


Figure A 6 a. Calibration curve for stigmasterol. b. Relative response for stigmasterol.

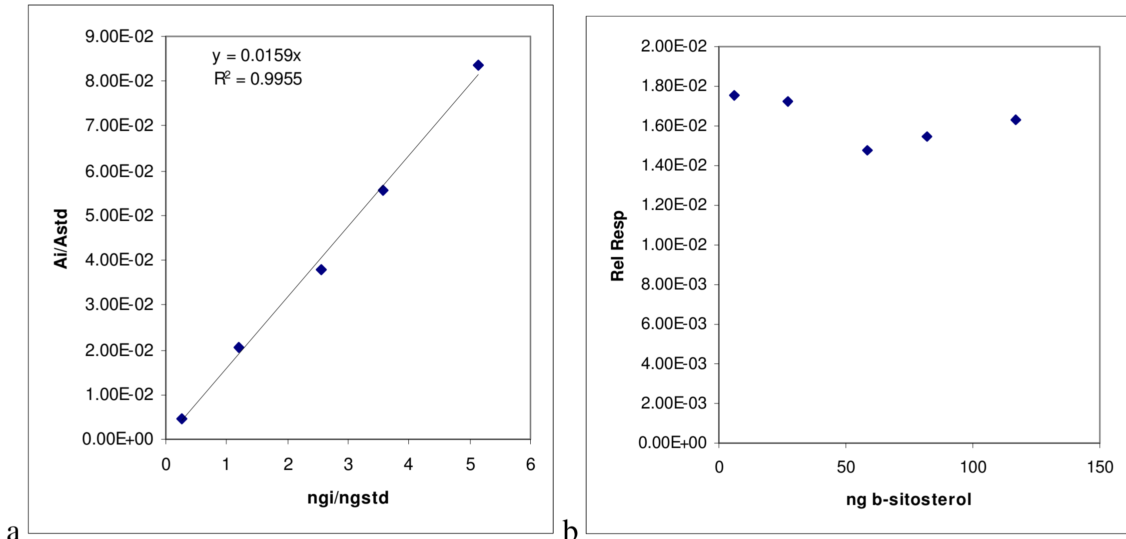


Figure A 7 a. Calibration curve for β-sitosterol. b. Calibration curve for β-sitosterol.

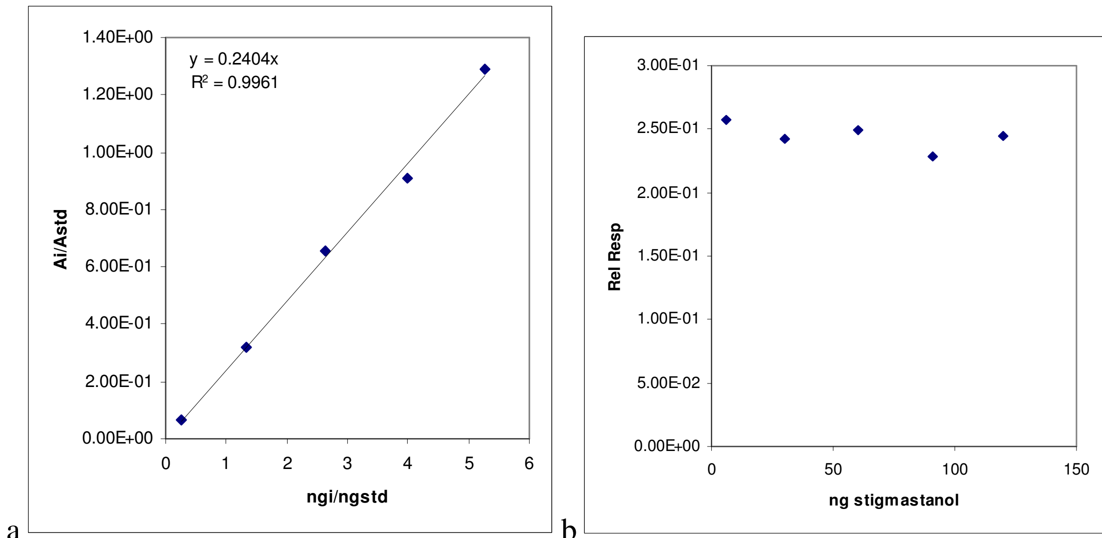


Figure A 8 a. Calibration curve for stigmastanol. b. Relative response plot for stigmastanol.

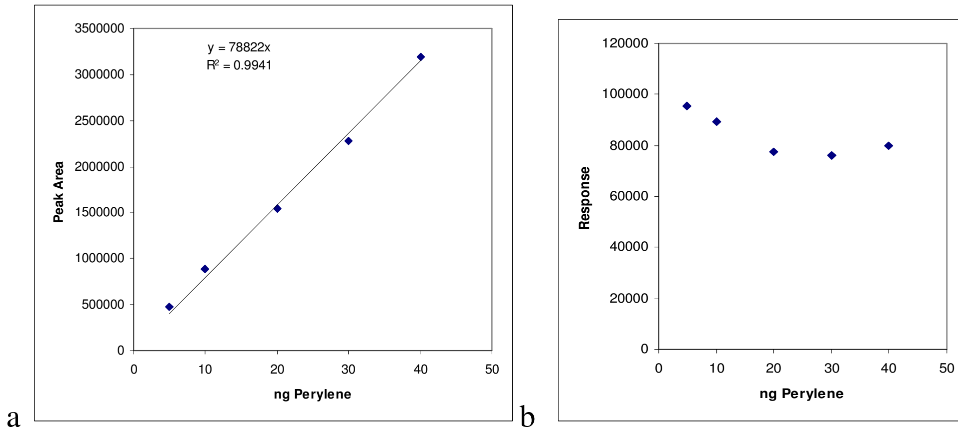


Figure A 9. a. Perylene d12 calibration b. Response for perylene.

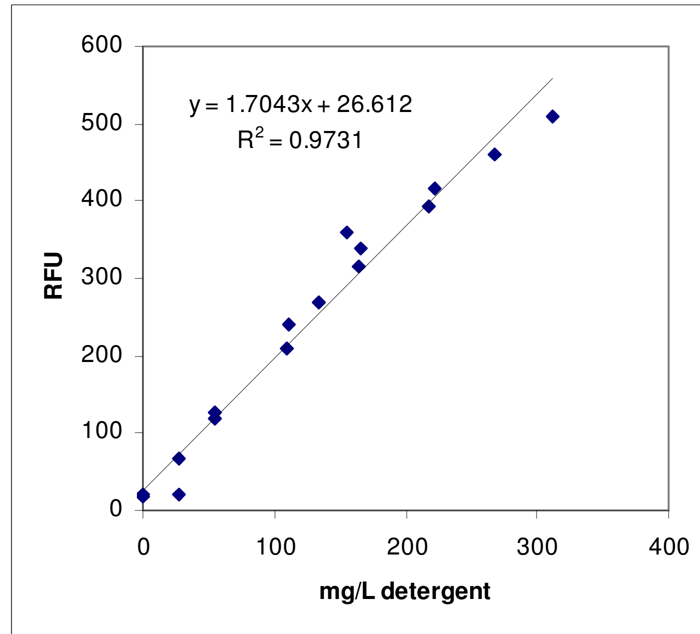
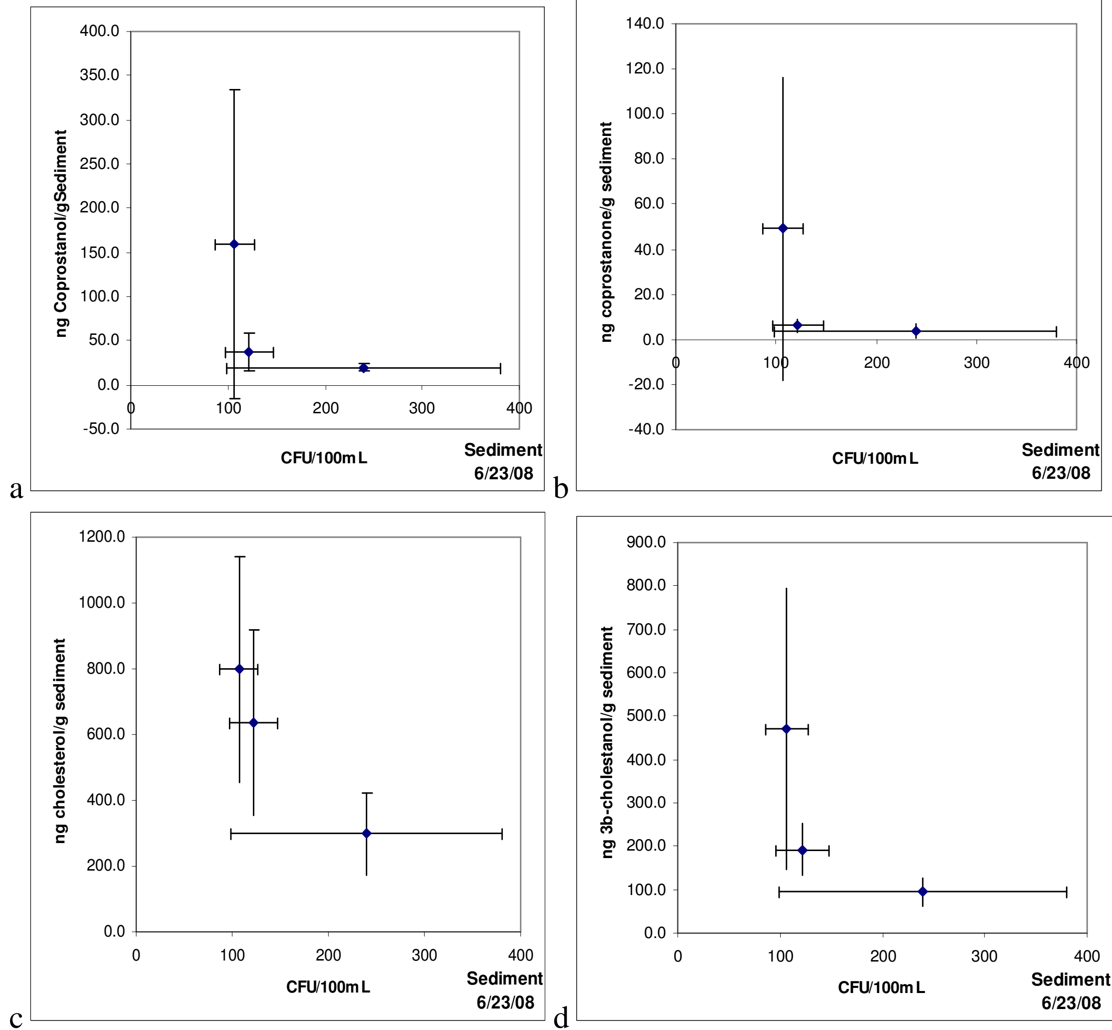


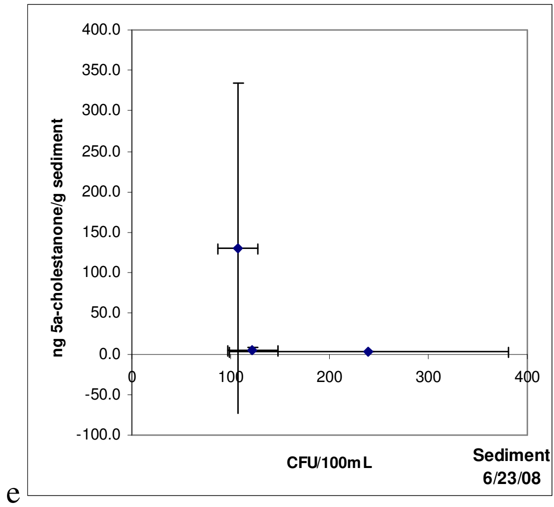
Figure A 10 Example calibration curve for FWA analysis. Created same day standard additions were performed.

Appendix B

Correlations between Sterols, SID ratios, and coliform counts

Correlations between sterols and SID ratios and coliform counts taken at C1, C2, and C3 on 6/23/08 are depicted below.





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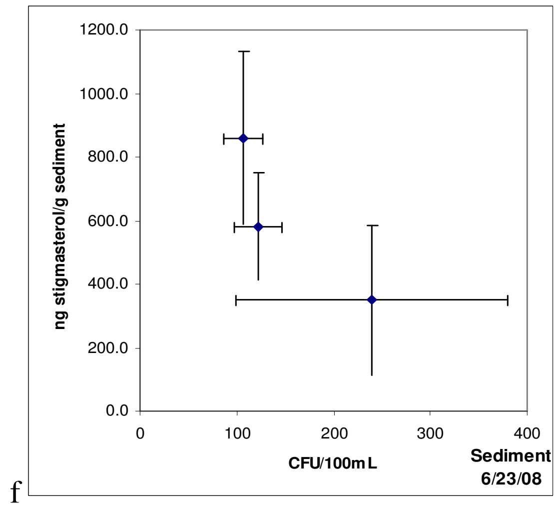

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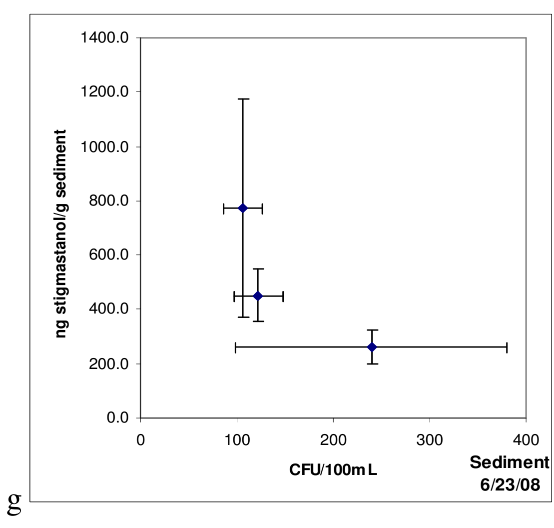

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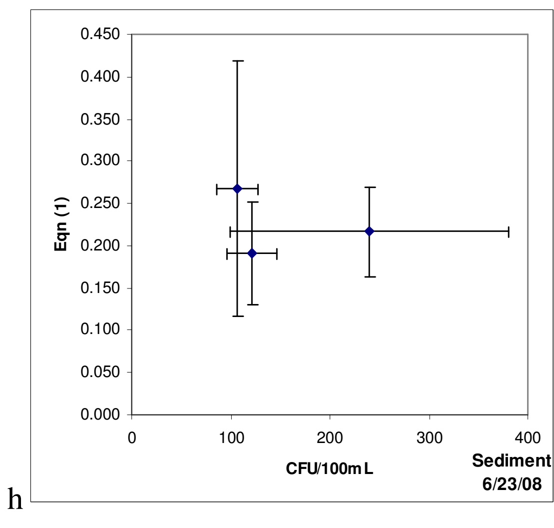

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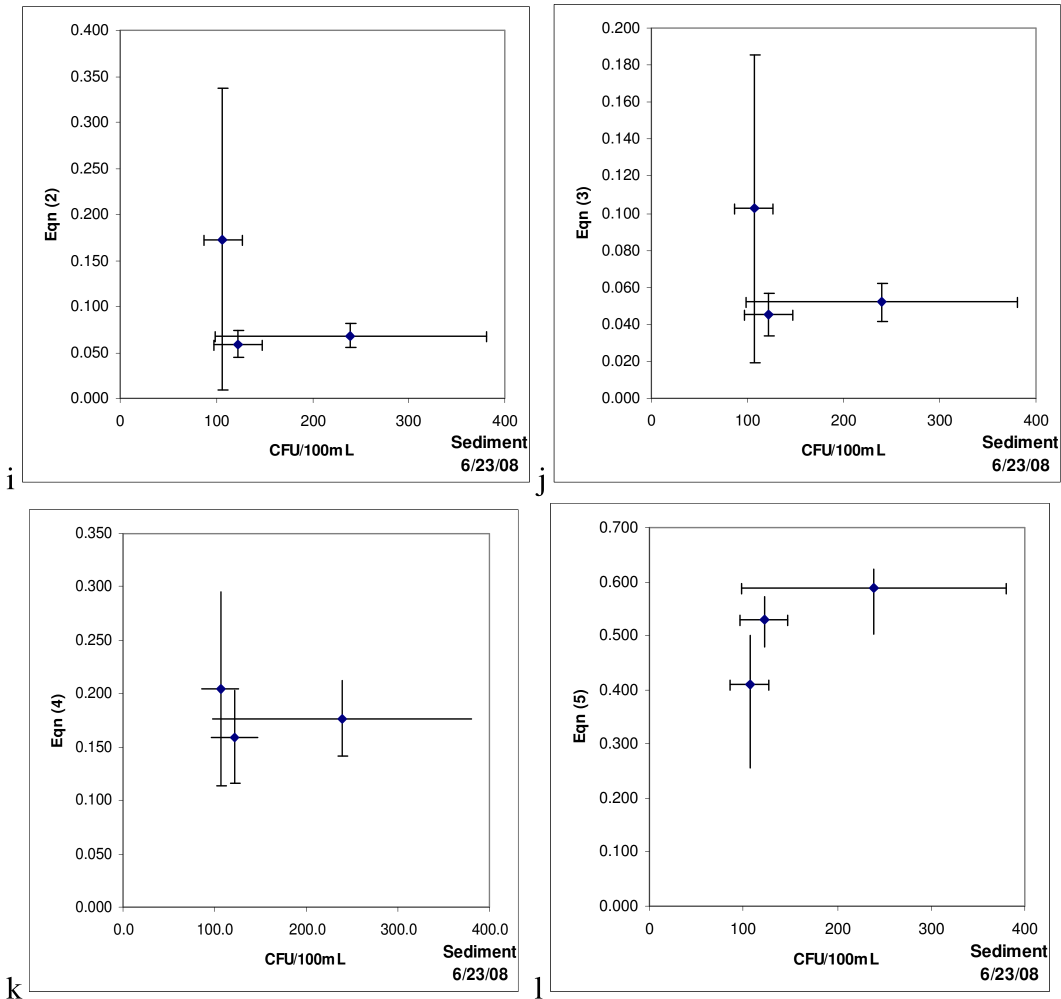
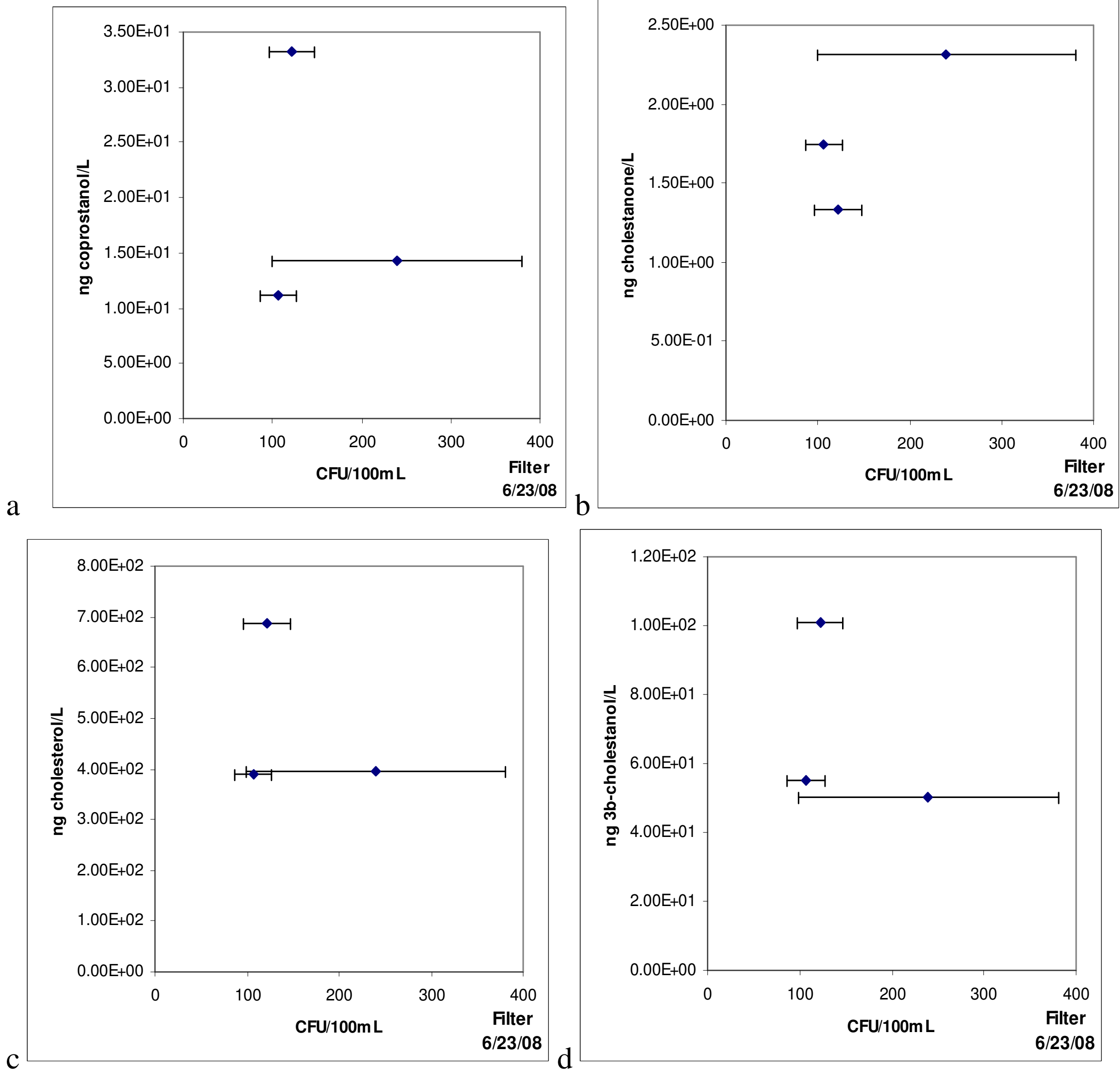


Figure B 1. Correlations between a. Coprostanol, b. Cholestanone, c. Cholesterol, d. 3b-cholestanol, e. 5a-cholestanone, f. stigmasterol, g. stigmastanol, h. Equation (1), i. Equation (2), j. Equation (3), k. Equation (4), and l. Equation (5) for sediment samples C1, C2C, and C3 and coliform counts taken 6/23/08.


3.50E+01
3.00E+01
2.50E+01
2.00E+01
1.50E+01
1.00E+01
5.00E+00
0.00E+00
ng coprostanol/L
0
100
200
300
400
CFU/100mL
Filter
6/23/08
a
2.50E+00
2.00E+00
1.50E+00
1.00E+00
5.00E-01
0.00E+00
ng cholestanone/L
0
100
200
300
400
CFU/100mL
Filter
6/23/08
b
8.00E+02
7.00E+02
6.00E+02
5.00E+02
4.00E+02
3.00E+02
2.00E+02
1.00E+02
0.00E+00
ng cholesterol/L
0
100
200
300
400
CFU/100mL
Filter
6/23/08
c
1.20E+02
1.00E+02
8.00E+01
6.00E+01
4.00E+01
2.00E+01
0.00E+00
ng 3b-cholestanol/L
0
100
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CFU/100mL
Filter
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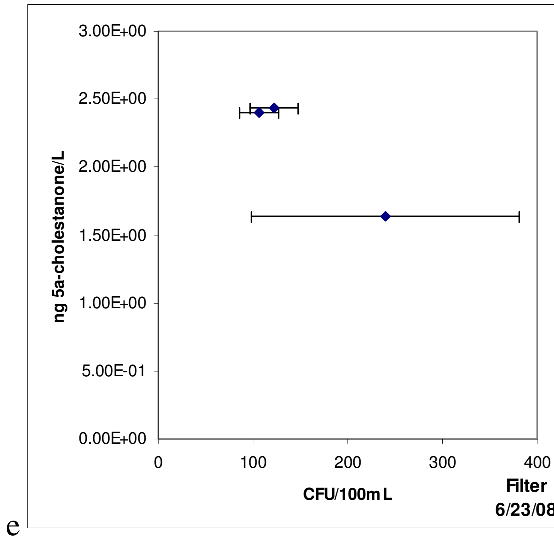

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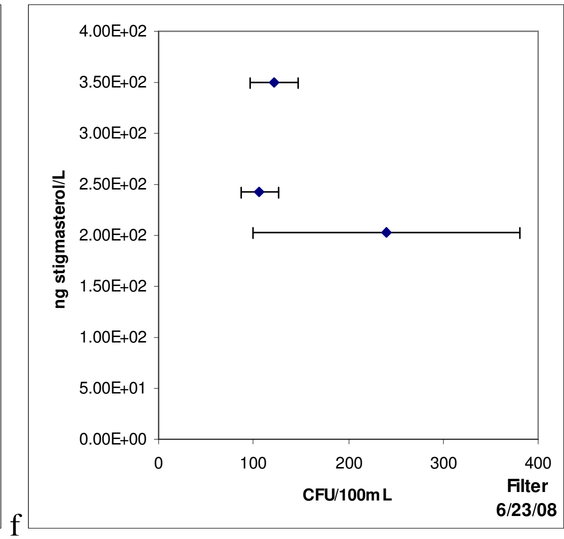

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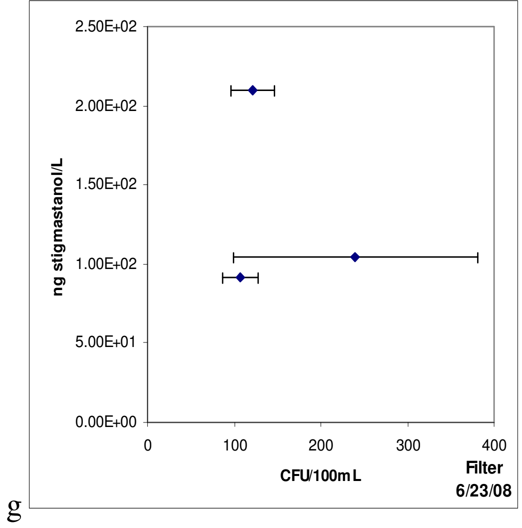

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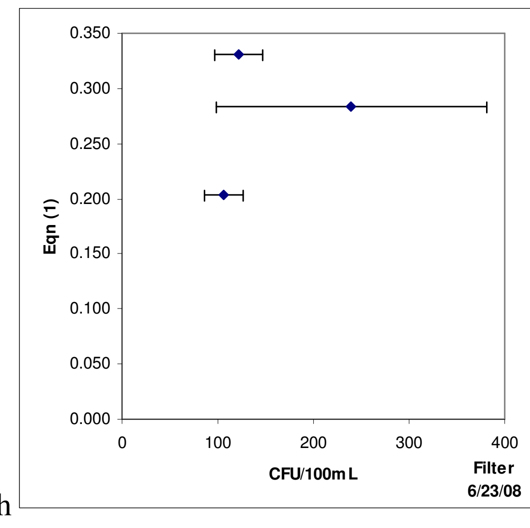

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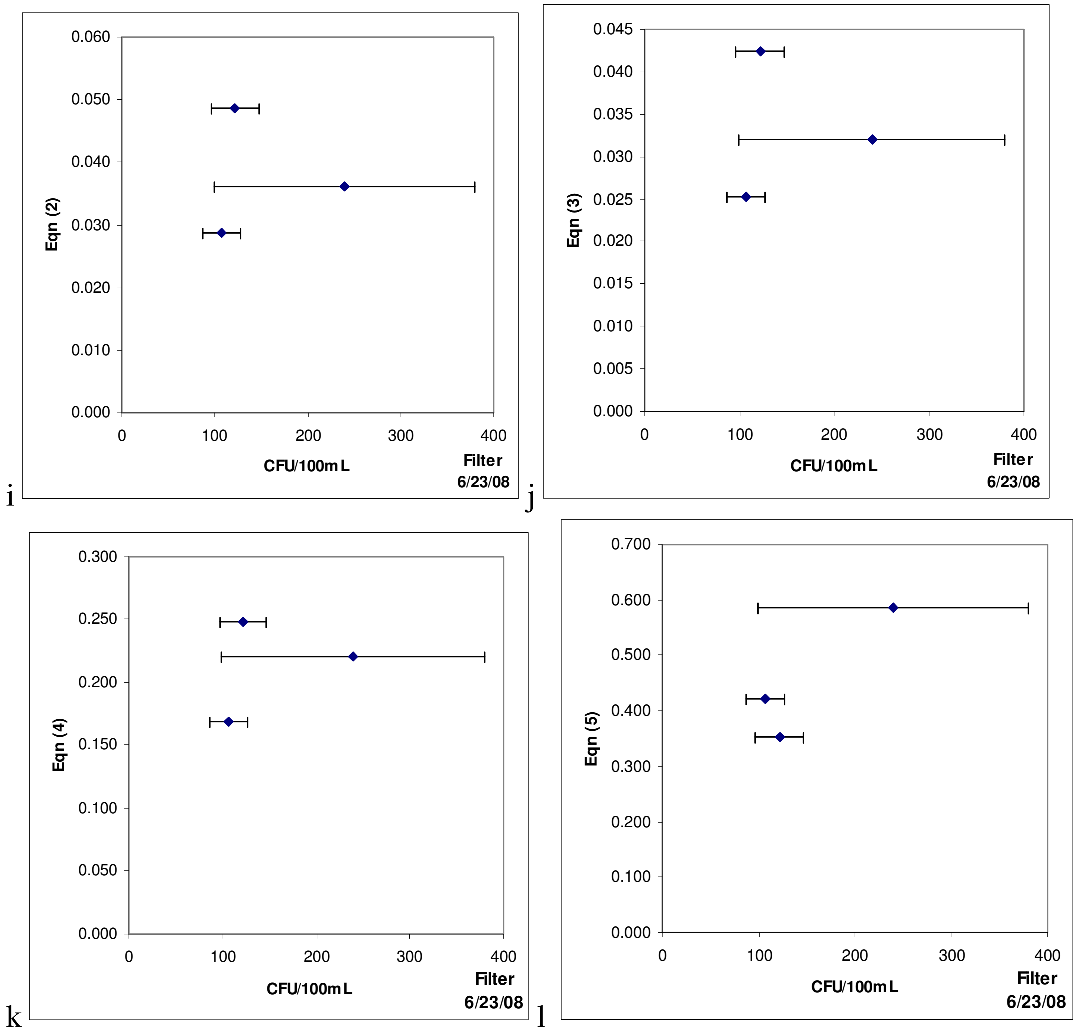

Figure B 2. Correlations between a. Coprostanol, b. Cholestanone, c. Cholesterol, d. 3b-cholestanol, e. 5a-cholestanone, f. stigmasterol, g. stigmastanol, h. Equation (1), i. Equation (2), j. Equation (3), k. Equation (4), and l. Equation (5) for water column samples C1, C2C, and C3 and coliform counts taken 6/23/08.

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