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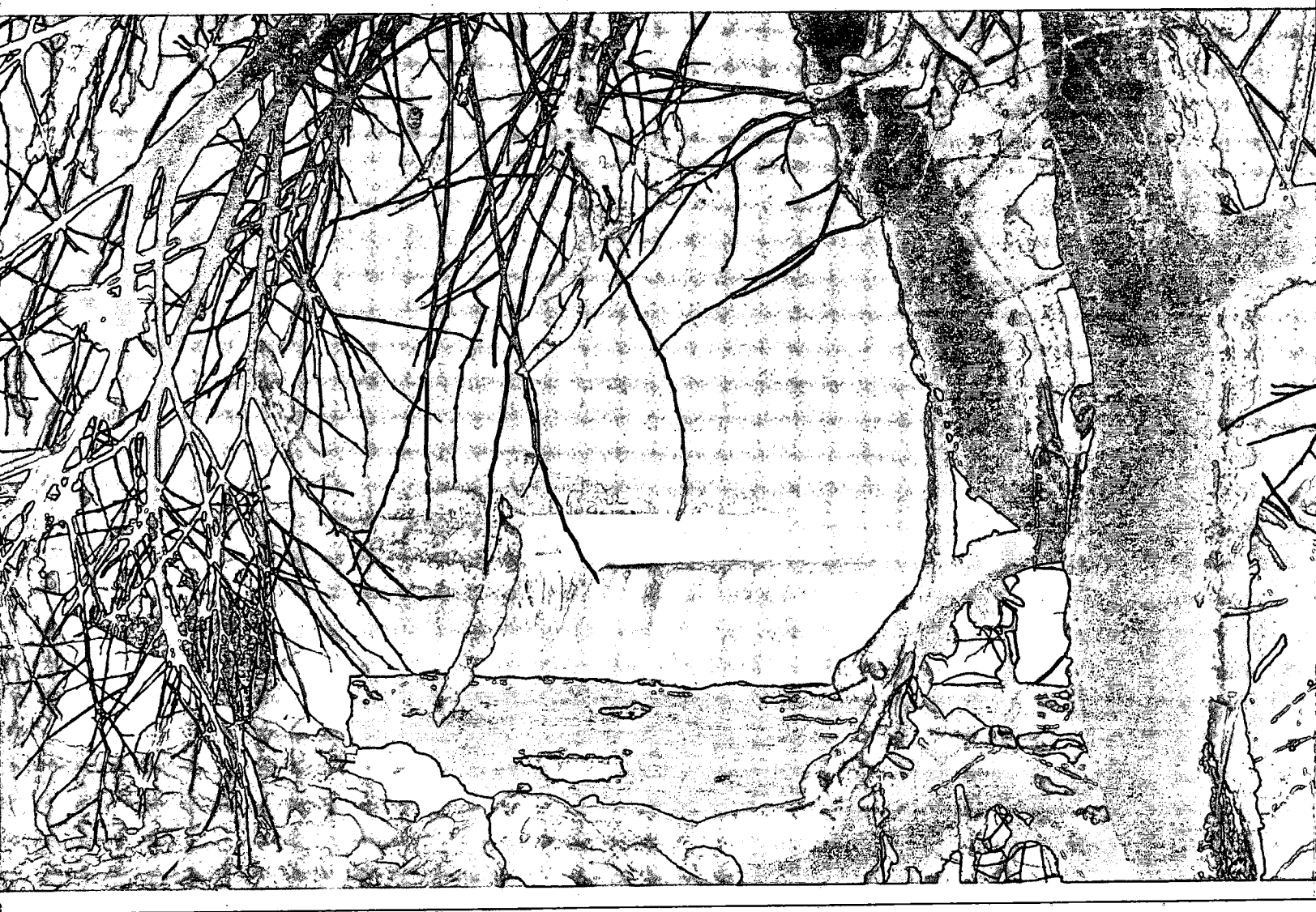
K.D. Switzer-Howse and B.J. Dutka



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(Résumé en français)

INLAND WATERS DIRECTORATE,
NATIONAL WATER RESEARCH INSTITUTE,
CANADA CENTRE FOR INLAND WATERS,
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Abstract

A study on the distribution of fecal sterols within a sample revealed that most sterols were particle bound and heterogeneously dispersed. This information could be used to monitor the efficiency of sewage treatment processes.

Biodegradation studies of fecal sterols by natural samples (sewage, effluent and lake water) and pure cultures indicate the requirement of sequential degradation by a variety of bacteria for rapid and complete degradation.

Résumé

Une étude de la distribution des stérols fécaux dans un échantillon a révélé que la majorité d'entre eux étaient liés à des particules et dispersés de façon hétérogène. Ces caractéristiques pourraient être mises à profit dans le contrôle de l'efficacité des méthodes de traitement des eaux d'égout.

Des études de la biodégradation des stérols fécaux dans des échantillons naturels (eaux d'égout, effluents et eaux lacustres) et dans des cultures pures démontrent que, pour être rapide et complète, la dégradation doit être séquentielle et effectuée par plusieurs espèces de bactéries.

Introduction

The necessity of developing and establishing safe, fast and reliable water quality tests has become more apparent with the increased need to re-use available water supplies. Most bacteriological water quality criteria are based on examining water samples for fecal pollution indicator bacteria, in particular total coliforms and fecal coliforms.

In recent years, however, the usefulness of these methods has been questioned (5). In the United States the National Technical Advisory Sub-Committee on Public Water has even gone so far as to state that more suitable means of testing water should be found.

What is needed is an indicator of fecal material which is non-pathogenic and which, when tested for, would consistently be found in high concentrations in raw sewage and polluted waters, but would be absent from non-fecally contaminated water. It would also have to be readily distinguishable from other pollutants. Many investigators have suggested the use of coprostanol (5β-cholestan-3β-ol), whose only well-documented source is the feces of humans and the higher animals (2, 6, 7, 8, 9, 14, 15, 17). Coprostanol is relatively stable and non-pathogenic and can be detected even in the presence of other lipid-like compounds in water (6, 15, 17). Coprostanol levels have been found to be highest in untreated raw sewage, and tend to decrease as the sewage proceeds through the treatment process. Similarly, coprostanol levels are found to be highest near sewage plant outfalls, with decreasing levels apparent as one moves farther upstream or downstream from the outfall (2, 6, 7, 8, 9, 14, 15, 16, 17).

Unlike biological indicators, fecal sterols do not appear to be affected by chemical disinfectants, toxic pollutants and heat treatments (2, 8, 9, 14, 17). Thus, their presence could indicate the existence of fecal pollution in situations where an industrial waste rendered commonly-used indicator tests useless.

The Microbiology Laboratories Section of NWRI has been studying the feasibility of using coprostanol and cholesterol as indicators of fecal pollution in water. In the course of these studies, several findings have been made which may have a direct bearing on the use of coprostanol as an indicator of fecal pollution.

Distribution of Fecal Sterols in Sewage and Water Samples

In most of the studies carried out in this and other laboratories, it was assumed that fecal sterols were dissolved and evenly dispersed throughout the sample. Thus, if a sample of water contained fecal sterols, one could assume that it also contained other fecal matter and that the level of fecal sterols present indicated the degree of fecal contamination. As our studies progressed, however, it was found that not only were fecal sterols difficult to dissolve in water, but also samples that contained large amounts of particulate matter invariably contained higher levels of fecal sterols. Furthermore, during some tests on preservation methods, an analysis was made on some raw sewage supernatants and only 4% of the coprostanol and 5% of the cholesterol were recovered. This indicated the need to re-examine the distribution of fecal sterols within a sample. If it was found that fecal sterols were not primarily in solution, would this alter the original premise concerning the feasibility of using fecal sterols to indicate fecal pollution. Further studies were conducted to answer the following two questions:

1. Are fecal sterols present in aqueous solutions or are they found attached to the particulate matter?
2. If fecal sterols do adhere to particulate matter, how will this affect sampling technique and storage, their biological degradation and subsequent removal from the environment, and will this fact alter the usefulness of fecal sterols as indicators of fecal contamination?

METHODS

Distribution of Fecal Sterols Within Sample

Samples of raw sewage and clarifier effluent were collected from the Burlington Skyway Sewage Treatment Plant, Burlington, Ontario. Each sample was thoroughly mixed before 1-litre aliquots were transferred to glass litre bottles. Each litre sample then had 1 ml/l of 1% $HgCl_2$ added as preservative. The 1-litre samples were then equally divided into two groups: (a) the first to be processed immediately; (b) the second to be analyzed after 7 days storage at 4°C. The fecal sterol analysis was that outlined by Dutka *et al.* (6). Each of the two sample groups (a and b) were divided randomly into three subgroups and labelled as to the pre-analysis treatment they would receive (Fig. 1). Set 1 samples were analyzed without further treatment. Set 2 samples were centrifuged for 15 min at 12,000 rpm. The supernatant was decanted, measured and placed in a clean bottle for analysis; the wet weight of the pellet was calculated, then the pellet was resuspended in 1 litre of sterile double-distilled water in a clean bottle and analyzed. Set 3 samples were subjected to 10 min sonic disruption, followed by 15 min centrifugation at 12,000 rpm. The volume of supernatant was determined and transferred to a clean bottle; the weight of the pellet was calculated, then it was redissolved in 1 litre sterile distilled water. The five resulting samples were extracted with hexane and cleaned up for analysis using the method of Dutka *et al.* (6). The above procedures were repeated with the second group of samples after storage at 4°C for 7 days.

A heterotroph spread plate count (6) was also done on the clarifier effluent and the preserved clarifier effluent at $\Delta T = 0$ and $\Delta T = 1$ week.

Adherence of Fecal Sterols to Container Walls

After the regular hexane extractions had been carried out on each of the stored samples, some of the empty bottles were washed three times with 50-ml aliquots of hexane, and the combined hexane washings of each bottle were evaporated to 1 ml under N_2 gas. Gas liquid chromatographic (GLC) analysis was performed on these samples as a check on the absorption properties of the glass bottles used for storage and the ability of the regular extraction method to remove all fecal sterols present.

RESULTS AND DISCUSSION

Table 1 indicates that the majority of fecal sterols, in most cases over 70%, are found in the centrifuged sediment pellet portion of the sample, regardless of whether or not the sample had first undergone sonication. In the raw sewage samples, there was a ten-fold difference between sedimented fecal sterols and those

Table 1. Distribution of fecal sterols in raw sewage and clarifier effluent after (1) centrifugation (2) sonication -- centrifugation

Sample and Treatment		TIME 0 CONTROL					1 WEEK INCUBATION 4°C				
		Quantity Analyzed	Tests	Total µg per Sample	% of Control Coprostanol	% of Control Cholesterol	Quantity Analyzed	Tests	Total µg per Sample	% of Control Coprostanol	% of Control Cholesterol
Untreated Raw Sewage		1 litre	Cop.	480			1 litre	Cop.	380	79.1 of 0 hour	
			Chol.	390				Chol.	320		82.1 of 0 hour
Centrifuged Raw Sewage	Supernatant	1 litre minus sediment	Cop.	23	4.8		1 litre minus sediment	Cop.	31	8.2	
			Chol.	25		6.4		Chol.	32.5		10.2
	Sediment Pellet	24.55 g	Cop.	336	70		14.54 g	Cop.	356.2	93.7	
			Chol.	270		69.3		Chol.	290.8		90.9
					74.8	75.7				101.9	101.1
Sonicated Raw Sewage Centrifuged	Supernatant	1 litre minus sediment	Cop.	35	7.3		1 litre minus sediment	Cop.	47	12.4	
			Chol.	40		10.3		Chol.	48		15.0
	Sediment Pellet	3.49 g	Cop.	335	67.8		7.43 g	Cop.	216	56.8	
			Chol.	265		67.9		Chol.	164		51.3
					77.1	78.2				69.2	66.3
Untreated Clarifier Effluent		1 litre	Cop.	1.3			1 litre	Cop.	2	153.8 of 0 hour	
			Chol.	1.7				Chol.	2		117.6 of 0 hour
Centrifuged Clarifier Effluent	Supernatant	1 litre minus sediment	Cop.	.4	30.8		1 litre minus sediment	Cop.	.9	45.0	
			Chol.	.4		23.5		Chol.	.9		45.0
	Sediment Pellet	1.8 g	Cop.	1.4	107.7		3.7 g	Cop.	2.3	115.0	
			Chol.	2.0		117.6		Chol.	2.4		120.0
					148.5	141.1				160.0	165.0
Sonicated Clarifier Effluent	Supernatant	1 litre minus sediment	Cop.	.5	38.5		1 litre minus sediment	Cop.	.6	30.0	
			Chol.	.6		35.3		Chol.	.6		30.0
	Sediment Pellet	1.55 g	Cop.	2.2	169.2		1.78 g	Cop.	1.4	70.0	
			Chol.	2.3		135.3		Chol.	1.5		75.0
					205.7	170.6				100.0	105.0

NOTE: Cop. = Coprostanol
Chol. = Cholesterol

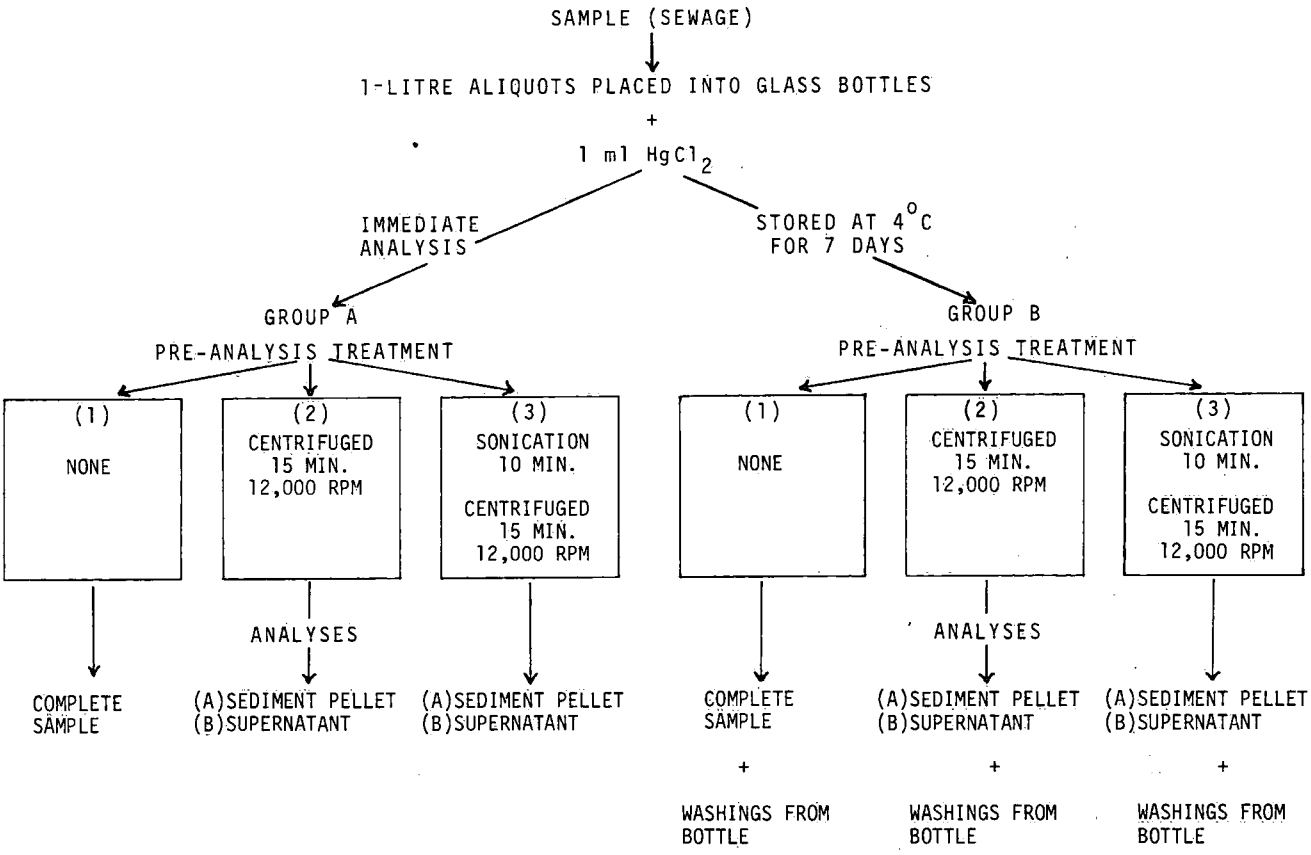

Figure 1. Sample treatment for determining distribution of fecal sterols.

found in the supernatant. Sonication produced no significant change in these figures. The difference, however, was not as marked in clarifier effluent samples, as there was only a four-fold difference in sediment fecal sterol levels compared with supernatant fecal sterol levels.

The apparent 25% loss of fecal sterols between the untreated raw sewage and the centrifuged sample (Table 1) could very well be related to the proportion of particulate matter in the original samples. Table 2 indicates that fecal sterol losses are not related to the container. It would appear from the data that fecal sterols are primarily bound to particulate matter and what at first appears as a ''loss'' is, in fact, an indication of the uneven distribution of particulate matter between measured aliquots. These results emphasize the difficulty in achieving true sample duplication, owing to the variety of particulate matter and its innate ability to bind fecal sterols to itself. This would explain the recovery variability seen in the data. Previous work by Dutka *et al.* (6) recently repeated (Table 3) illustrates that good reproducibility can be achieved. Therefore, even though most fecal sterols are organically bound rather than in solution, if sufficient care is taken when removing aliquot samples for analysis, one can expect the results to lie within two standard deviations of the mean, indicative of a high level of significance. One may assume that a single sample will exhibit the same proportion of fecal sterols as the whole if the sample has undergone a thorough mixing either in the sewage treatment plant or in open waters. This, of course, means that the type of sample collected could affect the outcome. One must decide whether the sample has an uncharacteristically high level of particulate matter due to the disturbance of the sediment layer, or whether the water is naturally laden with debris. Also, a sediment sample should be tested, as it would likely contain higher levels of fecal sterols if the water body was indeed receiving fecal wastes which were rapidly settling out.

The heterotroph plate counts performed on the clarifier effluent indicated that 1 ml of 1% $HgCl_2$ solution used for preserving the samples did not destroy all the bacteria in these samples. The original concentration of cells (24×10^4) at time 0 declined to 100 cells per millilitre immediately after mixing with 1 ml of

Table 2. Analyses of Hexane Washings for Coprostanol and Cholesterol from Bottles Used to Store Sewage Samples for One Week at 4°C

Bottle washing sample from:*	Test parameter	µg/l	% of total original 1-week sample†
Raw sewage, 1 week at 4°C, untreated	Coprostanol	0.10	$\frac{.10}{380} \times 100 = .03$
	Cholesterol	0.11	$\frac{.11}{320} \times 100 = .03$
Centrifuged raw sewage sample, 1 week at 4°C	Coprostanol	0.27	$\frac{.27}{380} \times 100 = .07$
	Cholesterol	0.34	$\frac{.34}{320} \times 100 = .11$
Centrifuged raw sewage sample (duplicate), 1 week at 4°C	Coprostanol	0.11	$\frac{.11}{380} \times 100 = .03$
	Cholesterol	0.31	$\frac{.31}{320} \times 100 = .10$
Sonicated centrifuged raw sewage sample, 1 week at 4°C	Coprostanol	0.11	$\frac{.11}{380} \times 100 = .03$
	Cholesterol	0.16	$\frac{.16}{320} \times 100 = .05$

* See Table 1, 1 week sewage sample.
† See Table 1, 1 week untreated sewage sample.
Note: All samples contained 1 ml of 1% $HgCl_2$ added as preservative at the time of collection.

Table 3. Replicate Analyses of Sewage and Clarifier Effluent Samples for Coprostanol and Cholesterol

	Sample	Coprostanol µg/litre	Cholesterol µg/litre
Raw Sewage	1	270	230
	2	250	210
	3	200	180
	4	200	160
	5	220	160
	Mean	228	188
	Standard deviation*	31.14	31.1
Clarifier Effluent	1	18	16
	2	21	16
	3	25	22
	4	31	31
	5	15	14
	Mean	22	20
	Standard deviation*	6.2	6.9

*All µg/l levels within two standard deviations of mean.

1% $HgCl_2$. Samples with 1 ml of $HgCl_2$/litre stored at 4°C for 1 week produced an average plate count of 44 colonies per millilitre. These results indicate that more than 1 ml of 1% $HgCl_2$ would be required to destroy all the bacteria to prevent regrowth and possible biodegradation of fecal sterols.

Biodegradation of Coprostanol and Cholesterol

Several studies have been carried out on cholesterol degradation (3, 10, 11, 13, 18) including reviews by Arundi (1) and Wettstein (19), however, little information is available on the biological degradation of coprostanol.

Turfitt (18) found that some soil species of *Proactinomyces* were capable of utilizing coprostanol as their sole carbon source. Since previous studies have indicated that many bacterial strains are able to degrade cholesterol, which is structurally similar to coprostanol, it seems reasonable to assume that there should be other bacteria capable of degrading coprostanol. Smith *et al.* (16) believed that the microbial degradation of fecal sterols in sewage treatment plants was a possibility. Kirchmer (18) also indicated that biological degradation was at least partially responsible for the disappearance of fecal sterols in sewage. A study was therefore undertaken to find bacteria which were able to degrade coprostanol and cholesterol and to evaluate their efficiency. The main objectives of this study were:

(i) To examine the possibility that under sterile conditions coprostanol or cholesterol might undergo a natural breakdown with time;

(ii) To isolate bacterial strains capable of growth in a chemically defined media containing coprostanol or cholesterol as the sole carbon source; and

(iii) To examine the degradation of coprostanol and cholesterol by selected bacterial strains (from (ii)) and by natural bacterial cultures taken from a sewage treatment plant, Hamilton Bay and Lake Ontario.

METHODS

Natural Degradation

Lake water and sewage samples were used to test for natural degradation of coprostanol and cholesterol. One-litre samples were placed into pre-cleaned glass bottles, which were covered with foil and autoclave sterilized at 120°C for 15 min. The samples were augmented with coprostanol and cholesterol to ensure a minimal final concentration of 50 ppb, and stored at 4°C. Coprostanol and cholesterol concentrations were tested at the following time periods: immediately after addition of coprostanol and cholesterol, 1 week storage after addition, 3 weeks storage after addition, and 5 weeks storage after addition.

Isolation of Coprostanol- and Cholesterol-Degrading Bacteria

A series of selective enrichment procedures were initiated to isolate coprostanol- and cholesterol-degrading bacteria. The basal medium used contained: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4$, 0.001 g $(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O$, 5.0 g $(NH_2)_2 SO_4$ per litre of double glass-distilled water (pH 6.9-7.2). The medium was filtered through Whatman No. 1 paper to remove the precipitate, dispensed in 200-ml aliquots in 500-ml Erlenmeyer flasks, and then autoclaved at 120°C for 15 min. All glassware was washed in chromic acid and rinsed three times in double glass-distilled water prior to use.

Six of the above prepared flasks were divided into three groups of two flasks (Fig. 2). One flask from each group had sufficient coprostanol added to ensure a level of 250 ppb. Cholesterol was added to the other flasks to also achieve levels of 250 ppb. For group 1, 1 ml of raw sewage was added to each flask; group 2 flasks received 1 ml of effluent and the third group, 1 ml of lake water. The flasks were capped with "Velcro" tops and put on a shaker at 20°C for 4 days, then 1 ml from each flask was transferred to another similar flask and incubated 4 days at 20°C on a shaker. This procedure was repeated a third time after which a loopful of inoculum from each flask was spread on purified agar plates (Oxoid purified agar) containing one of either 50 ppb coprostanol, 250 ppb coprostanol, 50 ppb cholesterol or 250 ppb cholesterol. All plates were incubated at 20°C for 4 days.

Single isolated colonies were picked from these plates and purified on similar agar plates. Fourteen of the largest purified colonies which appeared morphologically different were transferred to flasks containing the carbon-free media supplemented with either 100 ppb coprostanol or cholesterol and incubated for 2 days at 20°C. Three

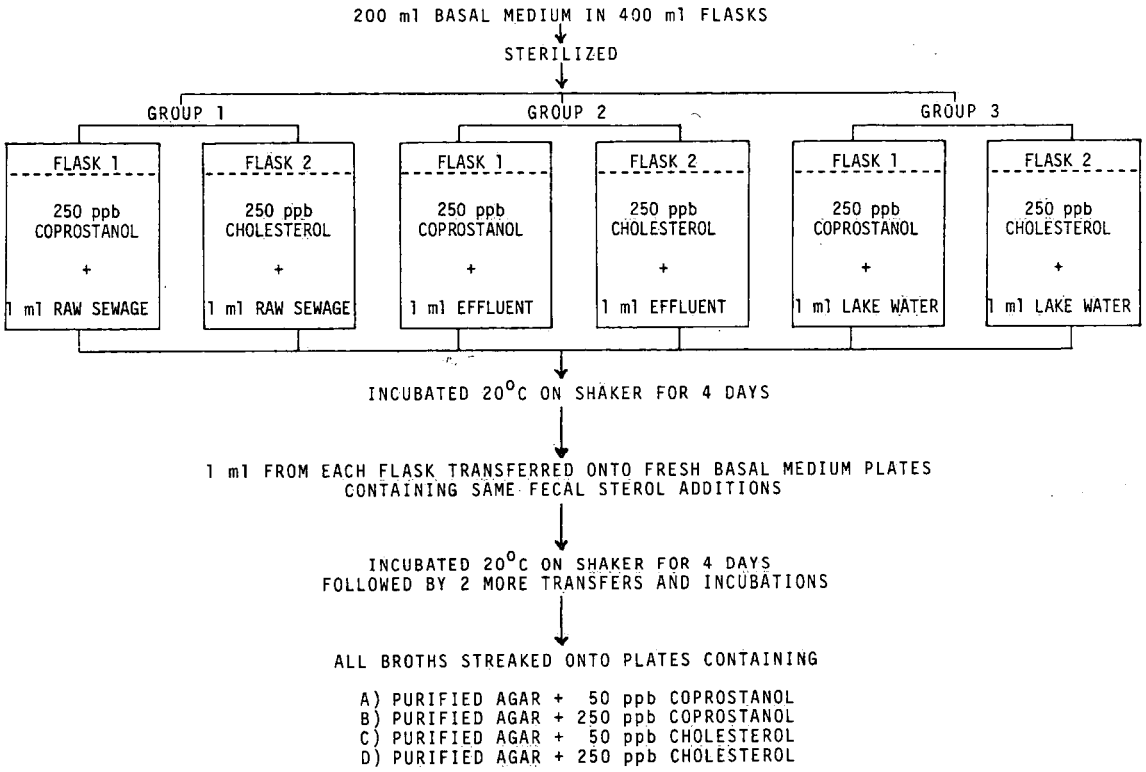

Figure 2. Flowchart for isolation of fecal sterol degrading bacteria.

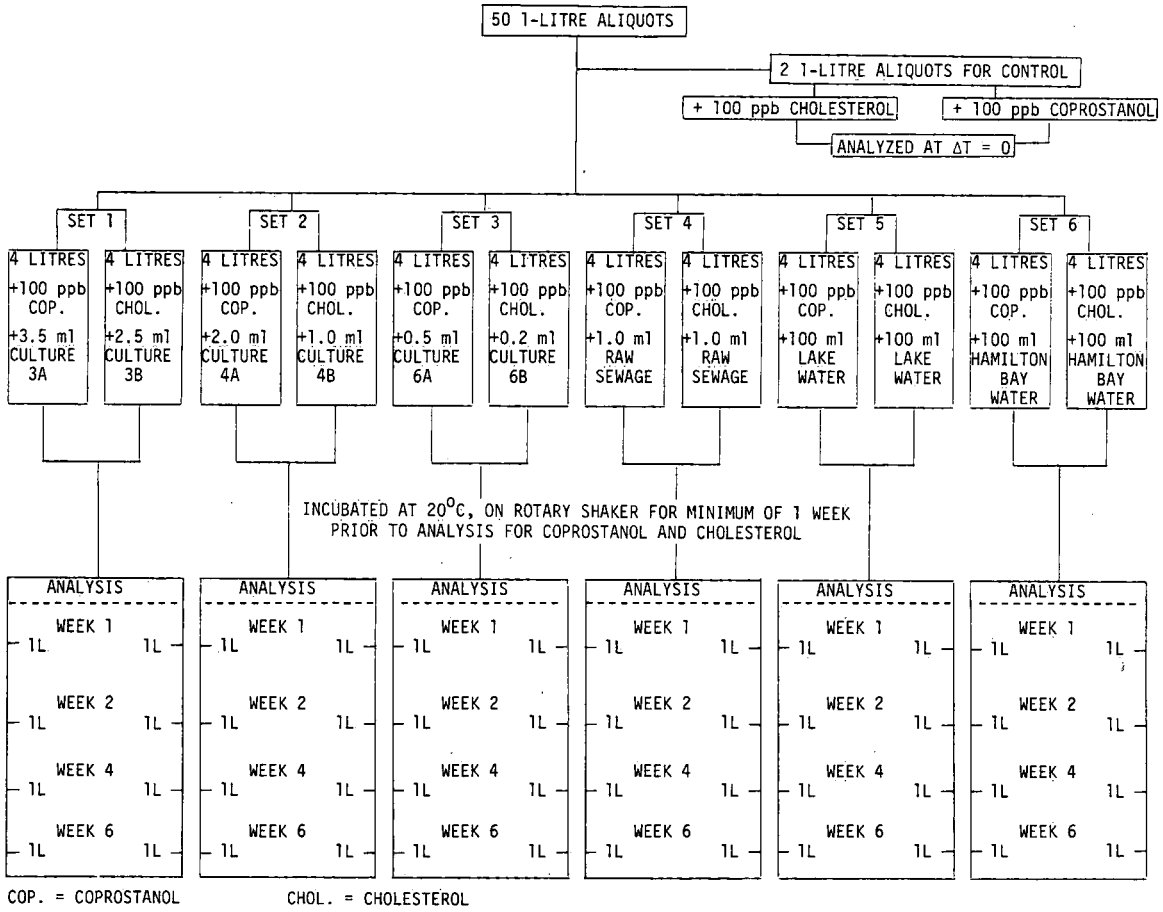

Figure 3. Flowchart for first biodegradation study.

transfers were made in the above media, after which transfers were made to nutrient agar.

Identification of Isolated Bacteria

From the 14 colonies originally selected as potential coprostanol and cholesterol degraders, three bacteria which grew well in coprostanol- and cholesterol-supplemented media were selected for identification and biodegradation studies. Tests used to identify these bacteria tentatively are shown in Table 4.

Biodegradation Tests

One-litre aliquots of the carbon-free basal medium were placed into 50 pre-cleaned glass bottles and sterilized. The bottles were divided into six sets of eight bottles, with two bottles being kept as controls (Fig. 3). In four bottles of each set coprostanol was added to the 100 ppb level and into the other four bottles, 100 ppb cholesterol. The control bottles had similar amounts of coprostanol and cholesterol added.

Plate counts were performed, using nutrient agar, on sewage, lake water, Hamilton Bay water samples and overnight nutrient broth cultures of organisms No. 3 (*Flavobacterium*), No. 4 (*Pseudomonas*) and No. 6 (*Flavobacterium*). Counts were performed to determine the amount of inoculum required so that each set of bottles would receive approximately the same number of bacteria (1.0×10^6).

At time 0, the six sets of coprostanol- and cholesterol-containing bottles received the following inocula (Fig. 3):

Set 1 Coprostanol bottles 3.5 ml of overnight culture No. 3A* *(Flavobacterium)*

* Letter "A" indicates bacteria grown in coprostanol-enriched medium;

Table 4. Identification Tests for Bacteria Used in Fecal Sterol Biodegradation Study

Culture No.	Colour	Gram's stain	Growth 35°C	Motility 20°C	Motility 35°C	MacConkey agar
3	Yellow	–	+	±	+	–
4	White	–	+	+	+	+
6	Yellow	–	+	–	–	–

Culture No.	Oxidase	Catalase	Glucose OF	Nitrate broth	Lactose broth	Gelatin liquefaction
3	±	+	0(F)	–	–	+
4	+	+	0	+	–	–
6	–	+	–	–	–	–

Culture No.	Identification tests						Identification
	Indol	M.R.	V.P.	Citrate	H_2S	Urease	
3	–	–	±	–	–	–	*Flavobacterium* spp.
4	–	–	–	+	–	–	*Pseudomonas* spp.
6	–	–	–	–	–	–	*Flavobacterium* spp.

Cholesterol bottles 2.5 ml of overnight culture No. 3B* *(Flavobacterium)*

Set 2 Coprostanol bottles 2.0 ml of overnight culture No. 4A (*Pseudomonas*)
Cholesterol bottles 1.0 ml of overnight culture No. 4B (*Pseudomonas*)

Set 3 Coprostanol bottles 0.5 ml of overnight culture No. 6A (*Flavobacterium*)
Cholesterol bottles 0.2 ml of overnight culture No. 6B (*Flavobacterium*)

Set 4 Coprostanol bottles 1.0 ml of raw sewage
Cholesterol bottles 1.0 ml of raw sewage

Set 5 Coprostanol bottles 100 ml of lake water
Cholesterol bottles 100 ml of lake water

Set 6 Coprostanol bottles 100 ml of Hamilton Bay water
Cholesterol bottles 100 ml of Hamilton Bay water

After inoculation all samples were incubated at 20°C for the duration of the experiment. Samples were analyzed after 1, 2, 4 and 6 weeks of incubation. Because of limitations on shaker space, samples to be analyzed after 1 and 2 weeks incubation were placed immediately on the shaker. When the 1-week samples were analyzed, the 4-week samples were placed on the shaker. Similarly, after the 2-week samples were analyzed, the 6-week samples were placed on the shaker.

Prior to analysis for coprostanol and cholesterol (6), all samples were filtered through 0.45 μm Gelman GN-6 membrane filters to remove bacteria so that coprostanol and cholesterol which might be bound to, or inside, the bacterial cells would not be included in the analysis.

The biodegradation experiment was then repeated with the following modifications:

(i) Only bacterial cultures No. 4 and No. 6 were used, along with sewage, lake water and Hamilton Bay water, to inoculate the samples;

(ii) Sufficient coprostanol and cholesterol were added to achieve 50 ppb levels in each flask;

(iii) Samples were tested after 1, 2 and 3 weeks incubation;

(iv) Sterile 1-, 2- and 3-week controls were prepared using carbon-free media and treated as samples.

* Letter "B" indicates bacteria grown in cholesterol-enriched medium.

Retention of Coprostanol and Cholesterol on Membrane Filters

One-, two- and three-week coprostanol and cholesterol sterile controls from the 50 ppb biodegradation study were filtered using Gelman GN-6 membrane filters.

Each membrane was homogenized in 15-20 ml of pesticide grade hexane for 5 min. The hexane was decanted and homogenization repeated twice more with 20 ml hexane each time. Two unused sterile membranes were homogenized, separately, in similar fashion and labelled control No. 1 and No. 2. The hexane homogenates were filtered through dried Na_2SO_4 into round-bottom flasks. Hexane washings of the homogenizer and Na_2SO_4 funnel were added to the respective flasks. The hexane washings were evaporated and treated as normal samples for coprostanol and cholesterol evaluation.

RESULTS AND DISCUSSION

One of the first problems encountered in these studies was the insolubility of coprostanol and cholesterol in water. Saad and Higuchi (12) reported the solubility of cholesterol to be 26 μg per litre at 30°C, while the C.R.C. *Handbook of Chemistry and Physics* (4) states that coprostanol is insoluble in water. After many attempts we decided to prepare a very concentrated standard in pesticide grade hexane, so that theoretically only a very small amount of this solution would have to be added to a litre of water to produce the required ppb level. Our studies revealed that this was not the case. Although appropriate amounts of pure compound were added to hexane and appropriate amounts of the hexane-sterol solution were added to the test samples, the desired concentrations were rarely achieved.

In our 5-week study on the occurrence and rate of natural degradation of fecal sterols in a sterile water solution it was found that there was less than a 1% decrease of coprostanol values and a maximum decrease of 20% in cholesterol values. However, because of the problems encountered with concentrated standard preparation, it is believed that the cholesterol decrease over the 5-week period may be due to solubility and recovery problems. The study results indicate, therefore, that there was little or no natural breakdown of coprostanol in water over a 5-week period. Cholesterol breakdown, if any, was less than 20% of the total input.

Fourteen different bacteria, based on colonial morphology, were initially selected for their ability to produce

large colonies on media containing coprostanol and cholesterol as sole carbon sources. From these 14 colonies, three were selected for identification and further studies. Table 4 indicates that two of the colonies were *Flavobacterium* spp. (No. 3, No. 6) and the other, a *Pseudomonas* spp. (No. 4).

In Table 5 the data from the first biodegradation study are presented. Within one week at least 95% of the cholesterol and 91% of the coprostanol were removed by the natural populations of bacteria present in sewage, Lake Ontario water and Hamilton Bay water. In pure culture degradation studies, No. 6, a *Flavobacterium* spp., was the only organism able to achieve greater than 90% reduction of coprostanol after 2 weeks incubation. Furthermore, pure culture inoculations never produced more than 95% degradation and they also showed considerable variation in biodegradation during the 6-week study period. These variations may have been due to initial problems with duplicating inoculum levels of coprostanol and cholesterol or may have been caused by the adsorption of coprostanol and cholesterol to the membrane filters.

Since it had been shown that coprostanol and cholesterol were bound to particles, it was decided to filter all samples which were subjected to degradation in order to remove the bacteria and any coprostanol or cholesterol

Table 5. Six-week microbiological degradation study of fecal sterols in carbon-free medium by natural bacterial populations and pure culture inocula

Sample and Inoculum	µg/litre and % Decrease from 0 hr Control							
	Week 1		Week 2		Week 4		Week 6	
	Cop.	Chol.	Cop.	Chol.	Cop.	Chol.	Cop.	Chol.
CONTROL								
Medium + Cop. 94.70 µg/l								
Medium + Chol. 73.45 µg/l								
Medium + Cop. + 1 ml of raw sewage	8.57 91%		.99 99%		.13 99%		.96 99%	
Medium + Chol. + 1 ml of raw sewage		3.8 95%		.94 98%		.48 99%		.30 99%
Medium + Cop. + 100 ml of Lake Ontario water	6.14 94%		5.82 94%		1.90 98%		.17 99%	
Medium + Chol. + 100 ml of Lake Ontario water		2.46 97%		1.56 98%		.33 99%		.55 99%
Medium + Cop. + 100 ml of Hamilton Bay water	4.67 95%		2.45 97%		1.87 98%		.27 99%	
Medium + Chol. + 100 ml of Hamilton Bay water		1.60 98%		2.80 96%		2.59 96%		LA
Medium + Cop. + .5 ml Bacteria No. 6A*	27.33 71%		5.29 94%		8.75 91%		8.13 91%	
Medium + Chol. + .2 ml Bacteria No. 6B*		25.33 66%		5.72 92%		6.88 91%		3.93 95%
Medium + Cop. + 2.0 ml Bacteria No. 4A	20.90 78%		11.86 87%		LA		LA	
Medium + Chol. + 1.0 ml Bacteria No. 4B		8.2 89%		6.26 91%		18.28 75%		7.3 90%
Medium + Cop. + 3.5 ml Bacteria No. 3A	67.37 39%		18.13 81%		64.75 31%		17.09 82%	
Medium + Chol. + 2.5 ml Bacteria No. 3B		14.32 81%		8.07 89%		12.25 83%		8.19 89%

*A Bacteria cultured in Coprostanol Broth.

*B Bacteria cultured in Cholesterol Broth.

Cop. = Coprostanol

Chol.= Cholesterol

LA = Lab Accident

bound intimately with these bacteria. In some of the samples, especially those inoculated with raw sewage, Hamilton Bay water and Lake Ontario water, there were varying amounts of debris which were removed by the filtration process. Based on the inoculum size, this debris would not be greater than 0.05% of that found in a litre of centrifuged sewage (Table 1) and thus would not account for more than 3 μg decrease in coprostanol or cholesterol levels. Thus, even though it has been established that coprostanol and cholesterol can be physically removed from solution by becoming particle bound, it is highly unlikely that the rapid reduction in fecal sterol levels within one week, as shown in Tables 5 and 6, is due to the pre-analysis filtering and subsequent particle removal.

In evaluating Table 5 data it soon became obvious that the membrane filter itself could also be a potential binding point for the fecal sterols. Therefore, in the following degradation study (Table 6) the sterile uninoculated controls were filtered in a similar fashion to the test samples. The results are shown in Table 7.

From Table 7 it can be seen that the Gelman GN-6 membranes bound an average of 8.6 μg coprostanol and 5.3 μg cholesterol. Thus, if one considers the fecal sterols bound to the membrane filter and the particles added by the inoculum and retained by the filter, Table 5 indicates that mixed cultures degraded at least 80% of the fecal sterols within one week and over 90% within 2 weeks.

Data presented in Table 6, the second modified degradation study, reveal some of the problems encountered in fecal sterol degradation studies. The filtered weeks 1-3 sterile control samples (no bacterial inoculum)

Table 6. Modified 3-week microbiological degradation study of fecal sterols in carbon-free medium by natural bacterial populations and pure culture inocula

Sample and Inoculum	µg/litre and % Decrease from 0 hr Control					
	Week 1		Week 2		Week 3	
	Cop.	Chol.	Cop.	Chol.	Cop.	Chol.
CONTROL						
Medium + Cop. 22.0 µg/l	17.0		11.0		14.0	
Medium + Chol. 10.0 µg/l		5.6		10.0		4.3
Medium + Cop. + 1 ml of raw sewage	6.5 30%		.61 97%		.84 96%	
Medium + Chol. + 1 ml of raw sewage		.43 96%		<.05 >99%		.34 97%
Medium + Cop. + 100 ml of Lake Ontario water	1.9 91%		.28 99%		.50 98%	
Medium + Chol. + 100 ml of Lake Ontario water		<.05 >99%		0.1 99%		<0.5 >99%
Medium + Cop. + 100 ml of Hamilton Bay Water	.20 99%		.96 96%		.36 98%	
Medium + Chol. + 100 ml of Hamilton Bay Water		<0.5 >99%		<0.5 >99%		<0.5 >99%
Medium + Cop. + 0.5 ml Bacteria No. 6A*	17.0 23%		4.5 80%		4.8 79%	
Medium + Chol. + 0.2 ml Bacteria No. 6B*		10.0 0%		.40 96%		3.0 70%
Medium + Cop. + 2.0 ml Bacteria No. 4A	8.2 63%		3.7 83%		3.5 84%	
Medium + Chol. + 1.0 ml Bacteria No. 4B		5.2 48%		2.8 72%		2.4 76%

*A Bacteria cultured in Coprostanol Broth.

*B Bacteria cultured in Cholesterol Broth.

Cop. = Coprostanol

Chol.= Cholesterol

appear to be very poor replicates of the unfiltered control. Comparison of Table 7 data with weeks 1-3 sterile control data (Table 6) indicates that by adding the amount of fecal sterol lost by the filtration process to weeks 1-3 control samples, all control fecal sterol levels are very similar and there is little or no coprostanol or cholesterol degradation under sterile conditions.

Table 7. Coprostanol and Cholesterol Levels in Membrane Filters Used for Biodegradation Control Samples (Table 6)

Membrane	Coprostanol μg/membrane	Cholesterol μg/membrane
Control filter (1) unused	< 0.1	< .01
Control filter (2) unused	< 0.1	< .01
Coprostanol 1-week control	5.8	
Cholesterol 1-week control		4.2
Coprostanol 2-week control	9.2	
Cholesterol 2-week control		0.6
Coprostanol 3-week control	11.0	
Cholesterol 3-week control		11.0
Mean	8.6	5.3
	(17% of total added)	(10% of total added)

Table 6, natural microbial population data, indicates that cholesterol was degraded slightly faster than coprostanol, while in the first study (Table 5) both were degraded to the same degree. Another anomaly that can be seen in Table 6 is the greater biodegradation by the lake water inoculum compared to the sewage inoculum. In the pure culture studies, very little biodegradation took place within the first 2 weeks compared to that observed during the first study (Table 5). This discrepancy may be due to (a) variations in original fecal sterol levels or (b) the metabolic state of the inoculated organisms.

In summary, the results presented in Tables 5 and 6 show that the most rapid biodegradations occurred when the microbial population present in natural water samples was used as the source of inoculum. This indicates that biodegradation proceeds sequentially and a variety of bacteria are required for fecal sterol degradation. Support of the sequential biodegradation process is provided by the mixed culture inoculum data where it can be seen that the decrease in coprostanol and cholesterol are parallel, while with pure culture inocula No. 4 of Tables 5 and 6 and No. 3 of Table 5, this parallelism is not noticeable.

Conclusions

1. The observation that fecal sterols become particle-bound enhances their usefulness in detecting fecal water pollution and monitoring sewage treatment efficiency. The level of fecal sterols found may be indicative of treatment efficiency, as the better treatment the sewage receives, the more the particulate matter is removed.
2. It is generally considered that indicator and pathogenic bacteria are associated with particulate matter. Thus, if the treatment process makes the use of bacterial indicators unfeasible, the presence of potentially dangerous fecal material could still be detected by using fecal sterol levels.
3. Since fecal sterols are primarily particle bound, caution must be exercised when sampling to ensure a valid representative sample. Furthermore, analytical results will be affected by the degree of mixing within the water body itself. In the laboratory a representative sample can only be achieved by thorough prolonged mixing.
4. Fecal sterol levels are insignificantly lowered by adherence to the walls of glass storage containers.
5. Fecal sterols can be removed by membrane filtration; where pre-analysis filtration is required, the membranes should be included as part of the test.
6. In natural samples, 90% of fecal sterols can be degraded within 2 weeks by the indigenous microbial population. Thus the detection of fecal sterols in natural waters would indicate recent or continuing influx of fecal pollution.

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