BILE ACIDS AS SPECIFIC FAECAL POLLUTION INDICATORS IN WATER AND SEDIMENTS

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Abstract

Microbiological indicators such as *Escherichia coli* (E. coli) have been extensively applied to monitor sewage contamination in waters and sediments. However, it has been accomplished by many researchers that microorganism indicators of faecal pollution in aquatic environments have limited applicability, owing to their lack of specificity and variable life span induced by environmental factors such as sunlight and chlorination. This review highlights the use of chemical indicators for faecal or sewage pollution monitoring. It highlights that the differences in bile acid distributions in animal faeces could be utilized to differentiate inputs in an environment. Furthermore, the high resistance to degradation by some of the bile acids would make them better suited for long standing pollution compared to coprostanol, as it is more readily degraded. Bile acid data could be used in conjunction with other available evidence, be it ethnographic or as part of a multi-biomarker approach, employing 5 -stanols and bacterial indicators, such as *C. perfringens*, to distinguish amongst the different environmental inputs.

Keywords: Bile acids, faecal pollution, bacteria, chemical indicators, sterols

Introduction

Mammalian species are major sources of pollution in the natural environment, specifically from their faecal matter, if methods of proper disposal are not adhered to. When faecal matter (50 % bacteria and the rest fatty acids, steroids, undigested foodstuff) are excreted and introduced into the environment, the presence of pathogens poses a serious threat to the health of living organisms. This may result in many parasitic infections and water borne diseases. Although almost all the earth's surface is contaminated to some extent,

it is those substances that cause acute cases of contamination that pose the most risk to living organisms and to the ecosystem as a whole that need to be determined.

Wild and pastoral (including urban e.g. dogs) animals are thought to be major contributors to faecal pollution in storm drainage water. Agriculture is another major contributor to environmental damage because it involves enormous changes to the natural environment. With the increased interest in livestock production, farm effluents have become a particular problem, especially if the animals are not confined to buildings. Similarly, when sewage sludge or manure is applied to agricultural land as a means of increasing crop yields and or as a practical method to solve a disposal problem, it poses some serious environmental problems. Furthermore, pathogens and other persistent toxic organic compounds may be introduced.

The aims of this review are to assess the usefulness of chemical compounds as indicators of faecal pollution in water and sediments; highlighting bile acids as species specific biomarkers of faecal pollution. The characteristics of chromatographic techniques employed for determination of bile acids in faecal impacted samples are compared and discussed.

Faecal bacterial indicators of faecal pollution in aqueous and solid samples

Bacteria are a part of the normal faecal microbiota. Their presence in the receiving environment indicates faecal contamination, even though they may not directly reflect the actual health risk. Consequently, the traditional biological methods for detecting sewage pollution in water bodies have relied on bacterial indicators that are faecal in origin. Most commonly used are the *Escherichia coli (E. coli*), total coliforms and faecal streptococci (Park *et al*, 2006; Isobe *et al*, 2002). Grazing animals mostly wild, contribute high background counts of faecal coliforms and faecal streptococci to waterways. Against this background, of unknown health risk, the influence of point source discharge must be determined. It is generally believed that microorganisms in animal faeces are less likely to cause human diseases than those that are contained in the sewage. However, Brock (1979) demonstrated that some microbial diseases could be transmitted from animals to humans. It is worth mentioning that measuring concentrations of the faecal indicator bacteria that are required by law does not help to characterize the input from multiple pollution sources. Commonly used bacterial (*E. coli* and faecal coliforms) markers are present in similar numbers in both human and animal faeces (Sinton and Doninson, 1994). An attempt to distinguish between human input in the form of sewage discharge effluents, and non-point sources such as runoff from farms was carried out in a number of streams in the USA

(Sorenson *et al*, 1989). The results obtained with the traditional microorganisms namely, faecal coliforms, and faecal streptococci fluctuated widely, giving no insight to the possible inputs. This was also accomplished in a similar study in Turkey (Kacar, 2011).

Distinguishing inputs can be possible with specific bacteria found in different animal faeces (Jaffrezic *et al*, 2012). For example, human faeces generally contain only enterococci, while ruminants have *Streptococcus bovis* (*S. bovis*) and horses have *Streptococcus equinus* (*S. equinus*) (Oragui and Mara, 1984). The limitations to this approach are that other animals also contain enterococci, which may lead to unreliable identification of the source of pollution. Additionally, *S. bovis* and *S. equinus* survive poorly in the environment (Sinton and Doninson, 1994; McFeters *et al*, 1974) compared to the enterococcus group. Several permutations have been suggested. For example using a ratio of faecal Coliforms to streptococci (FC to FS) as a distinguishing measure of whether a faecal pollution source is human or animal (Feachem, 1975). The reported ratio of < 0.7 failed when the ratios were applied to environmental wastes from meat works. This was attributed to the rapid inactivation of the *S. bovis* and *S. equinus*. However, the shift in FC to FS approach could be exploited to distinguish between human and animal faecal inputs (Feachem, 1975). Yet, Sinton and Donnison (1994) investigated a range of faecal, effluents and water samples and found that the ratios were very similar in all, but very fresh faecal samples. The study showed that the ratio could only be useful as a tracer of recent pollution.

The application of the microorganisms to studying faecal contamination in solid samples such as sediments and soils is limited. Their application is hampered by several factors such as lack of specificity e.g. coliform bacteria are derived not only from faeces of warm blooded animals, but also from plants and soils and also that they possess variable survival rates. Bacterial densities fluctuate due to mortality induced by sunlight (Chevremont *et al*, 2012) and predators at a rate that may be quite different to the pathogens die-off rate. Highly sensitive markers which are unique to the source of input have been used to trace sewage pollution.

Chemical biomarkers of faecal pollution in the environment

Chemical biomarkers associated with anthropogenic waste include non-ionic surfactants, specifically linear alkylbenzenes, polycyclic aromatic hydrocarbons, caffeine and faecal sterols. However many of these substances are not specific to the faecal input. The ones that offer specificity towards faecal pollution are faecal sterols (5 -stanols and bile acids). The primary pathway for the elimination of cholesterol**,** the dominant mammalian sterol, is by transformation into bile acids and other stanol/sterols, which are then excreted

and can be detected in the faeces. The 5 -stanols have received attention in the past thirty years, and more recently, bile acids as useful biomarkers for sewage/faecal pollution. The presence or absence may indicate whether faecal pollution is prevalent or not, which indirectly may indicate the health hazards e.g. whether viruses and pathogens could be present.

Faecal sterols

Coprostanol and other sterols have been suggested as better indicators of modern sewage pollution (Furtula *et al*, 2012; Ayebo *et al*, 2006; Isobe *et al*, 2002).

The source specificity of sterols in animal faeces has been suggested to be controlled by the sterols in their specific diets, the biota resident in the animal's digestive tract and that some animals can biosynthesize sterols (Leeming *et al*, 1994). Leeming *et al* (1994) suggested that the sterol fingerprint portrayed by animal faeces might be utilized for distinguishing between the human and animal sources of faecal pollution. For example, human, seal, pig and cat produce coprostanol as their major product from microbial hydrogenation of cholesterol, while 5 -cholestanol can be attributed to sedimentary bacteria. Faecal sterols from ruminant animals such as cows and sheep contain a higher relative proportion of 5 -campestanol and 5 -stigmastanol due to the high amount of campesterol and sitosterol from their vegetable diet. Such studies have been undertaken in modern and archaeological samples. For example, Venkatesan and Santiago (1989) used the sterols to differentiate sewage pollution from pollution by marine mammals in Santa Monica Basin sediments. The sterol profiles were similar to sewage sludge, indicating that marine mammals' contribution was not sufficient to cause its recognition in the sediments. In another study by Furtula *et al* (2012) the efficiency of sewage treatment plants (STPs) in removing sterols was undertaken. Variable sterol removal was observed to be influenced by sample location and treatment type, with lower concentrations observed in the effluent, however the sterol signature was preserved with cholesterol and coprostanol prevalent, indicating human faecal pollution

Faecal sterols from various animals such as humans and 14 other species of animals common in urban or rural areas have been examined, and the findings indicated that many sterols were not unique to a certain type of animal species. Venkatesan and Santiago (1989) have proposed the epicoprostanol : coprostanol ratio as a useful index to distinguish between inputs of domestic, urban sewage and material of non-human origin. In various Antarctic sediments, a ratio of 1 to 5 was observed and thought to indicate marine mammal inputs, more specifically, from whales (Venkatesan and Santiago, 1989; Venkatesan *et al*, 1983)

Grimalt *et al* (1990) used the ratio of the relative concentration of coprostanol to the corresponding 5 -isomer, [5 □/(5□ + 5□
urban pollution, whereas ratios between 0.1 and 0.3 corresponded to sediments collected from unpolluted areas. The ratio was applied to water particulate and sediments and faecal matter was positively identified. This ratio was further modified to take account of epicoprostanol that may be formed from the degradation of coprostanol in archaeological samples. However, in environments that have high algal production, the increased production of 5 -cholestanol would affect the ratio. In this case, [5 -3/(5 -ratio)] cholestan
be could used instead (Grimalt *et al*, 1990). As already mentioned, ruminants produce more 5 -stigmastanol than coprostanol Evershed and Bethell (1996) proposed the use of coprostanol : 5 -stigmastanol ratio as a useful parameter by which human and ruminant faecal inputs can be differentiated. Values greater than 1.5 were considered indicative of a human derived faecal source, while lower values indicated higher production of the 5 - stigmastanol, reflecting ruminant sources. However, the use of such compounds is limited because they have been found to be common to different animal species (Noblet *et al*, 2004). To accurately quantify the contribution of sources other than human effluents, biomarkers unique to other animals or groups of animals need to be identified.

Bile acids have been proposed as potential sewage pollution indicators, especially if complemented by sterols (Elhmmali *et al*, 1997).

Origin and significance of bile acids

Elimination of cholesterol from the human body takes place primarily by the faecal route as bile acids and neutral sterols. The bile salts are produced in the liver. The two primary bile acids formed are chenodeoxycholic (3 -dihydroxy-5 -cholanoic, and cholic (3 -trihydroxy-5 -cholanoic) acids.

The bile accumulates in the gall bladder for subsequent discharge into the small intestine.

In the lower part of the intestine, microorganisms hydrolyze amide linkages of conjugates to form free bile acids, and remove C_7 hydroxyl groups to become secondary bile acids and their epimers and keto bile acids. The primary bile acids thus formed are further metabolized by intestinal flora, and the products are partially reabsorbed and transported to the liver. They then may undergo further structural modifications to yield free deoxycholic and lithocholic acids from cholic and chenodeoxycholic acids, respectively. The system produces a great variety of bile salts among and within species and individuals. The

individual bile acids may differ from each other in the number, position and stereo configuration (□□ or □□) of the hydroxyl groups.

The vast number of bile acids present in different animal faeces confers some specificity of the occurrence of certain bile acids in certain animal species. Bile acids are excreted mainly in the faeces and a small amount (< 5 %) in the urine (Alme *et al*, 1977). Qualitative and quantitative composition of bile acids excreted in the faeces is influenced by the health of the animal, its diet and the intestinal microflora.

In mammals, the principal bile acids are 5 -C_{24} cholanoic acids. Chenodeoxycholic and cholic acids are the main primary bile acids formed in the bile. The pig bile has a unique bile acid, hyocholic acid which is produced by 6 -hydroxylation of chenodeoxycholic acid, and is converted by intestinal microorganisms during enterohepatic circulation to hyodeoxycholic (3 -dihydroxy-5 □ cholanoic) their faeces (Costa *et al*, 1994). The work by Setchell and co-workers (1983; 1987) revealed the different bile acids that could be isolated in the faeces of healthy human faeces as well as in patients. Quantitative bile acid excretion has been shown to exhibit wide day–to-day variations. In humans, it ranges from 100 to 1000 mg day^{-1} (Setchell *et al*, 1987).

The variability of bile acid composition in fresh faeces of cow, sheep and pig were examined following the procedures in Elhmali *et al* (1997). The bile acids identified in cattle faeces of different age groups were similar, with deoxycholic acid being the principal component, and isolithocholic, lithocholic, allodeoxycholic, and chenodeoxycholic existing as minor components. The calf and the cow were found to produce more deoxycholic acid relative to the other two bile acids. Notably, deoxycholic acid exists in much lower concentration in the analyzed adult male (bull) compared to the calf and cow. It is suspected that the probable causes of this difference could be related to the age, diet and sex of the individual animal. The hydroxy acid distribution in sheep faeces parallel those obtained in cattle faeces with deoxycholic acid being the major component with -and -hydroxy acids being present in substantial amounts (Obuseng, 2002).

In faeces of pigs of different ages throughout the age groups, hyodeoxycholic acid was the principal bile acid, with its 3 - and 3 -epimers. The distribution pattern of bile acids was similar for one, two and five year olds, namely: hyodeoxycholic > lithocholic > isolithocholic > 3 -epimer of hyodeoxycholic > 3 -epimer of hyodeoxycholic acid.

There is a large inter- and intra-individual variation in bile acid concentration in faeces of different animals, even between animals of the same age. These subtle differences would probably be insignificant when studying faecal input from these animals in

environmental samples. Most significantly, similar bile acid profiles were identified in the faeces of animals of all ages.

The vast number of bile acids present in different animal faeces confers some specificity of the occurrence of certain bile acids in certain animal species. Deoxycholic acid is a major component in most farm animals (cow, sheep) as well as in humans, but is absent in pig faeces. Porcine faeces can be distinguished from that of humans (or other omnivores) or ruminant based on the detection of 6-hydroxylated bile acids.

Bile acids as markers of faecal inputs

A study by Elhmmali *et al* (1997) highlighted the usefulness of faecal bile acids and sterols as a means to distinguish inputs by various animals, and demonstrated their potential for survival in archaeological environments. The differences in the distribution of these biomarkers have been utilized to identify the major inputs into environmental pollution (Leeming *et al*, 1994; Venkatesan and Santiago, 1989; Elhmmali *et al*, 2000; Bull *et al*, 2002)

Bile acids have been found to be highly resistant to microbial degradation processes, and persist in contrasting environments. Lithocholic, deoxycholic and ketonic bile acids were detected in 2000-year-old human coprolites discovered in Lovelock cave, Nevada. These compounds were present at similar relative abundances but at lower concentrations than in the fresh faeces (Lin *et al*, 1978; Knights *et al*, *1983*) analyzed soils from a Roman ditch at the Antoine Wall in Bearsden, Scotland. Even though the coprolites were not visible due to the wet and acidic conditions, chemical analysis revealed high concentrations of deoxycholic acid (and 5 -stanols), traces of lithocholic and cholic acid in the topmost horizon. Although the data alone could not be used solely to indicate the faecal source, consultation with historical evidence suggested that the ditch had been used as a drainage channel for a latrine.

Quantitative chromatographic analysis of bile acids

Although the specificity of these biomarkers outweighs those of the previously mentioned biomarkers, there are some complications associated with the analysis of bile acids in soil/sediments. Generally, one may find the following: (i) bile acids in faeces are present in higher concentrations than in environmental samples; (ii) bile acids may bind to particulate material by either adsorption or other unknown mechanisms and (iii) the soils and sediments contain compounds that elute in the same fraction as bile acids, which co-elute with some of the bile acids during GC analyses. Application of analytical techniques to study bile acid distributions in faecal material and environmental (soil, sediment etc) samples involves appropriate extraction of the total lipids. The lipid fraction is subjected to various purification steps followed by chromatographic separation by GC or HPLC in combination

with various detection methods. Some of the chromatographic techniques used for bile acid analysis have been reviewed (Leeming and Nichols, 1996; Scalia, 1995). However, the choice of a technique depends on the task, nature of matrix, expected concentration of bile acids in the sample and the precision required.

Gas chromatography coupled with mass spectrometry remains a very useful tool for elucidation of the complex mixture of bile acids that exist in different environmental samples (Kumar *et al*, 2011; Setchell and Vestal, 1989; Suh – Jen *et al*, 2011). Capillary GC/MS offers greater potential, for both identification and quantification of many bile acids. However, bile acids lack the volatility required by GC. Bile acids have been routinely analysed as the methyl ester-trimethylsilyl ether derivatives because of the ease of preparation and the good resolution attained on most capillary columns (Elhmmali *et al*, 1997). Steps such as hydrolysis, acid-neutral separations need to be carried out beforehand, which makes the procedure lengthy.

A method for the characterization and quantification of bile acids based on use of gas chromatography and gas chromatography/mass spectrometry (GC/MS) in the selected ion-monitoring (SIM) mode was developed (Obuseng, 2002). The analytical protocol was adopted and modified from Elhmmali (Elhmmali *et al*, 1997; Elhmmali *et al*, 2000). This method was based on selecting specific *m/z* values (ions) from the full scan electron ionization (EI) spectrum of an individual bile acid. A quadrupole instrument was used because it was simple to operate in the SIM mode and easy to automate. The applicability of the developed procedure was tested using sediment samples collected from the River Avon, United Kingdom. GC/MS full scan analysis of the acidic fraction of the total lipid extract (TLE) revealed the presence of long chain hydroxy acids in the same fraction as the bile acids. The study revealed that lithocholic, deoxycholic and chenodeoxycholic acids are major biomarkers for faecal pollution along the Avon River, especially from sewage inputs. The bile acids were quantified by both GC-flame ionization detector (FID) and GC/MS in the SIM mode. Although the precisions of the two methods were comparable, it was observed that quantification of bile acids using the GC-FID method artificially overestimated the amounts of lithocholic acid by a factor of four, this was due to the co-elution of the C_{26} □-hydroxy acid with the lithocholic acid. However, deoxycholic and chenodeoxycholic acids were not affected.

Upon employing SIM-GC/MS, enhanced selectivity was attained due to the elimination of interference from ions due to co-eluting peaks. Another important feature of the technique was the increased sensitivity, i.e. dwell time per ion is increased, and hence,

components of low abundance can be detected. This was illustrated by one sample collected upstream well away from the sewage treatment works. Bile acid distributions similar to the sediments collected nearer to the sewage treatment works were obtained, but the concentrations of the various components were dramatically lower. These were attributed to animal faeces from surrounding farms.

High performance liquid chromatography (HPLC) has an advantage over GC as direct analysis of the naturally occurring different classes of bile acids can be performed without the need for deconjugation (Roda *et al*, 1992; Scalia, 1988; Scalia, 1995; Perwaiz *et al*, 2003). A mass spectrometer (MS) combined with the HPLC column offers both the selectivity and specificity in the determination of free and conjugated bile acids. Perwaiz *et al* (2003) reported a method using HPLC-MS in electrospray tandem mass spectrometry (ES-MS/MS) in the negative scan mode. The results obtained indicated the state of conjugation and the concentrations of the different bile acids in the samples. The ES-MS/MS spectra were simple and showed only the molecular ion peaks.

A modified HPLC method developed by Scalia (1988) was used in our laboratory for the determination of free and conjugated bile acids using both diode Array and ESI-mass spectrometer as detectors (Moshoeshoe, 2005). Optimization of the HPLC-MS method gave the best results when elution was carried out using acetonitrile : sodium acetate (7:3 v/v). The prominent (base) peak for all the compounds was the $(M-H)^-$ ion and this enabled the identification of the different bile acids. The spectra obtained were simple and showed few fragmentation.

Cholic acid, lithocholic acid and taurocholic acid were the major bile acids present in sewage impacted water collected from a constructed wetland used to treat sewage from a local college, Tlokweng, Botswana. The bile acid profile was similar to that found in human faecal material. A similar bile acid profile was identified in treated water, although the concentrations were reduced.

Analysis of fresh cow, goat and sheep faeces showed the presence of deoxycholic acid, lithocholic acid and cholic acid as major bile acids in the faeces of these animals. The profiles were similar in all the herbivore faecal material analysed.

Advantages of chemical biomarkers over micro-organisms as faecal indicators

Although the steroidal compounds occur at low levels in environmental samples, improvements in the analytical methods used for sterol analysis such as GC/MS offers higher sensitivities such that sterols could also prove to be better indicators of faecal pollution than the bacterial indicators (Evershed and Bethell, 1996; Nichols *et al*, 1996). Limits of detection

and quantification of coprostanol in bulk water samples have been evaluated by Eganhouse *et al* (1988) using high resolution GC/MS. The results from their experiments showed that coprostanol could be detected and quantified at high dilutions prevalent in the oceans, even though background contamination needs to be reduced. This has been made much easier by the availability of newly developed sensitive techniques such as GC/MS in the tandem mode whereby low levels can be detected (Evershed and Bethell, 1996).

It is worth noting that the behavior of chemical and bacterial sewage indicators is different (Jeanneau *et al*, 2012). The environmental conditions determine the stability of both, for example, under aerobic conditions, chemical biomarkers degrades quickly, but it is better preserved in anoxic conditions. Steroidal (including bile acids) compounds and microorganisms are strongly associated with particulate matter (Elhmmali *et al*, 2000; Hatcher and McGillivary, 1979), although they may be bound differently to them. The two classes of markers were correlated using sediments and water sample obtained from the Sydney inner-shore region. Good correlations were observed for coprostanol versus *C. perfringens* spores both in water ($r^2 = 0.97$) and in sediments ($r^2 = 0.96$). Poor correlation was observed for the faecal coliforms in water ($r^2 = 0.8$), while a reasonable correlation existed in sediments ($r^2 = 0.91$) (Nichols *et al*, 1993). A similar study was undertaken by Leeming and Nichols (1996) in the same study area. They correlated coprostanol to enterococci ($r^2 = 0.77$-0.96), and coprostanol to thermo tolerant coliforms ($r^2 = 0.52$). The inability to establish a close relationship between bacterial densities and concentrations of coprostanol was attributed to the fluctuations of bacterial populations in the water due to chlorination and or contributions of coliform of non faecal origin, although the bacterial indicators were preserved in sediments. The methods that are used for determining coliform bacteria sometimes yield confusing and variable results, influenced most importantly by environmental conditions, even though they are said to be more sensitive.

Chemical biomarkers appear to be more resistant to environmental stress than the faecal bacteria, as the faecal coliforms are killed by exposure to sunlight, temperature variations and by chlorination (Devane *et al*, 2006; Duereth *et al*, 1986; Gourmelon *et al*, 2010; Litton *et al*, 2010)

Conclusions

It can be concluded that bile acids are diagnostic biomarkers of faecal inputs into modern and ancient soils and sediments. To accurately quantify the contribution of sources other than human effluents, biomarkers unique to other animals or groups of animals need to be identified. A more versatile approach in distinguishing mixed inputs has been the use of a

combination of different biomarkers such as host specific bacterial indicators, sterols and bile acids data (Evershed and Bethell, 1996) used in conjunction with sterol data and ethnographic evidence.

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