Alternative methods in tracking sources of microbial contamination in waters

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Abstract

A key factor in the management and remediation of impaired ground- and surface water is the ability to distinguish the sources of faecal contamination. Several approaches have been adopted as microbial source tracking methods (MST), which are generally classified as culturing, phenotypic, genetic, and chemical MST. None of the techniques used thus far can be considered a standard; important factors, such as the statistical correlation between the source and the faecal indicator and the understanding of the environmental fate of the faecal pollutants, still need attention.

The most promising MST methods available today are based on the genetic fingerprinting of faecal micro-organisms. However, research is very active also in the investigation of pharmaceuticals and personal care products discharged in the environment together with faecal waste.

An updated overview of MST methods to distinguish human from animal sources of faecal pollution is presented here, focusing particularly on the potentialities of new chemical tracers.

Keywords: faecal contamination, microbial source tracking methods, bacterial source tracking methods, pharmaceuticals and personal care products

Introduction

As a consequence of the serious health threats posed by water-borne pathogens, faecal contamination is one of the main quality factors in drinking water, in aquaculture and in recreational water. Traditionally, the evaluation of the health risk for waters contaminated by faeces is obtained through the quantification of certain indicators, and only rarely by the direct measurement of the real hazard, which is the actual concentration of the pathogens. The most commonly used faecal indicators are micro-organisms that are always present in faeces, and are unable to reproduce outside the intestinal tract. In fact, enteric micro-organisms and pathogens should disappear from the water body after a finite period from the contamination event. In order to obtain a reliable estimate of the health risks, faecal indicators must satisfy certain criteria defined by Gerba (Maier et al., 2000; Table 1). These organisms are not necessarily source-specific, they can be hosted indistinctively by humans, farm animals or wildlife. Consequently, the typical indicators (e.g. faecal coliforms, *E. coli* and enterococci) give a good estimate of the health risks only in the case of drinking water, for which there is zero tolerance to faecal contamination (James and Evison, 1979; Maier et al., 2000). On the other hand, the use of non-source-specific faecal indicators often results in a vague estimate of health risks in aquaculture and recreational waters, where the presence of these contaminants is tolerated within specific limits. In these circumstances, the detection of faecal contaminants should be obtained simultaneously to the identification of the sources of pollution.

TABLE 1
Characteristics of the ideal faecal indicator, adapted from Maier et al. (2000)

1	The indicator must be present whenever faecal contamination is present. In case of a micro-organism, it should be a member of the microflora of warm-blooded animals; in case of chemical substance it should be associated solely to faecal discharges.
2	The indicator should not be present in the environment other than when there is faecal contamination. In the case of a micro-organism, it should not grow in the environment.
3	The indicator should be good for all types of environment (surface, marine and ground waters).
4	The concentration of indicator should be greater or at least equal to that of the pathogen.
5	The indicator must have a reasonably longer 'survival time' (persistence) if compared to the most resilient pathogen.
6	The quantification of the indicator (including sampling and measurement) should be faster, easier to perform and more sensitive than that of the pathogen.
7	The quantification of the indicator (including sampling and measurement) should be less expensive than that of the pathogen.

The techniques to identify the sources of faecal contamination in water have been defined as microbial source tracking (MST) methods, or bacterial source tracking methods. MST methods are based on the detection of a 'tracer' that can be used as a fingerprint to obtain a complete characterisation of the contamination (i.e. type of pollution, source, timing, severity, etc.). The tracer can be either a faecal micro-organism or a chemical

TABLE 2	
Classification of MST methods	
Direct monitoring of pathogens	Human enteric viruses (Enterovirus, Adenovirus, Norwalk virus)
	Eggs of helminths (intestinal worms)
Culturing methods	Faecal coliforms/faecal streptococci (FC-FS ratio)
	Faecal streptococci species identification
	FC-FS ratio shift
	Bifidobacteria spp. (sorbitol-fermenting *Bifidobacteria*)
	Rhodococcus coprophilus
	Bacteroides spp.
	Pseudomonas aeruginosa
	Phages of *Bacteroides fragilis*
	F-RNA phage subgroup
	Streptococcus bovis
Phenotypic methods	Antibiotic resistance analysis (ARA) or multiple antibiotics resistance analysis (MAR)
	Serogrouping
	Carbon utilisation profile
Genetic methods	Ribotyping
	Amplified fragment length polymorphism (AFLP)
	Pulse-field gel electrophoresis (PFGE)
	Repetitive PCR (rep-PCR)
	Denaturating gradient gel electrophoresis (DGGE)
	Host-specific molecular markers (LH-PCR; T-RFLP)
Chemical methods	Faecal stanols
	Fluorescent whitening agents
	Sodium tripolyphosphate
	Long-chain alkylbenzenes
	Caffeine
	Musk fragrances
	Estrogens
	Human pharmaceuticals
	Animal growth promoters

substance discharged with the faecal waste. In the first case, the microbe should reproduce only in the intestinal tract of a specific host (the source) in addition to satisfying the same basic criteria mentioned above for a generic faecal indicator organism (Table 1). In the latter case, the chemical should be found only in the diet of a specific host or should be discharged only with faecal waste from a specific source. Another fundamental requisite is the environmental fate of the tracer, which should be similar to that of the water-borne pathogens. Nevertheless, it can be convenient to use an indicator with a longer lifespan, particularly when it is required to define the vulnerability to faecal pollution of an aquifer.

MST methods are a fundamental tool in water management. For instance, the effective remediation of impaired waters requires the quantification of the contaminant loads (and accordingly of the contamination source) that the water body can bear without negative effects on quality. Once these loads, also called total maximum daily load (TMDL), have been obtained for each stressor, the administrator of the water body can decide which action should be taken in order to effectively reduce the impact. Environmental agencies are showing increasing interest in implementing protocols for monitoring water quality, including MST, to define the TMDL (US-EPA, 2001; Bernstein, 2002; USGS, 2004).

MST methods play an important role also in relation to the emerging problem of zoonoses. In fact, although the attention of health organisations is presently focused on food-borne zoonoses, a number of pathogens transmitted by animals is already

on the list of major water pollutants (e.g. *Giardia lamblia; Cryptosporidium parvum*), and there is increasing concern for other zoonotic waterborne pathogens (Slifko et al., 2000), particularly in relation to the intensive farming operations, commonly defined as concentrated animal feedlot operations (CAFOs). As a matter of fact, CAFOs constitute a significant environmental stressor because of the very high load of faecal waste produced, and simultaneously because they are an ideal incubator for the development of antibiotic-resistant organisms, due to the intensive use of drugs as therapeutics or growth promoters.

Accordingly, CAFOs are the ideal environment for the selection of new viruses, which are only rarely infective for humans, but could be spread through groundwater. MST methods play a key role also in epidemiological studies for the identification of the limits of tolerance to faecal contaminants with the various final uses of water. In surface waters the quality targets are related to uses such as drinking, fish-farming and recreational waters. The typical stressors for surface water are industrial and residential sewer systems, septic tanks, urban waste-water treatment plants, animal farms, CAFOs, urban waste dumps, and wild animals. The choice of the appropriate MST method should be finalised to distinguish among these sources.

Coastal or estuarine waters also present a diversified set of faecal sources. It is commonly believed that marine water is an unsuitable environment for endogen microbes, since the survival of pathogens and other intestinal micro-organisms is highly reduced. Nevertheless, it has been observed that the persistence of enteric bacteria can be significantly increased by other environmental factors such as turbidity, concentration of suspended solid, turbulence, etc. (Alkan et al., 1995; Yang et al., 2000). Therefore, the choice of MST methods needs specific attention in the marine environment. It is worth noting that in aquaculture other important factors such as toxins produced by algae (e.g. *Dinophysis* spp.; *Cyanobacteria* (Haider et al., 2003)) can also represent a serious health risk.

Groundwater is considered relatively less vulnerable to faecal pollution as a consequence of the filtrating effects of soil layers (Bitton and Gerba, 1984). The most common sources of faecal pollution are septic systems, sewers, cesspits from CAFOs, and wildlife droppings. Several studies have been published on the fate of micro-organisms in groundwater in relation to the hydrogeological and geological characteristics of the aquifers (Matthess and Pekdeger, 1981; Conboy et al., 2000; Gordon et al., 2003). The identification of faecal pollution sources in groundwater is often very difficult, although geographic and hydro-geological information on the surrounding area can provide valuable clues.

The first extensive review on MST methods was published by Sinton (1998). Other studies published recently by Scott et al. (2002), Simpson et al. (2002) and Meays et al. (2004) focus mainly on genetic and phenotypic methods. In this paper, an updated overview of MST methods is given, focusing particu-

larly on the potentialities of new chemical methods, such as the detection of drugs and personal care products to distinguish human from CAFO sources of faecal pollution.

Classification of microbial source tracking methods

Various approaches can be applied to distinguish sources of faecal contamination. The classification of MST is usually made according to the distinctive characteristic of the pollutant used as a marker. Four main groups are identified, namely culturing, phenotypic, genetic and chemical MST methods. Indeed, the direct detection of human or animal specific pathogens, such as enteric viruses and intestinal worms, should also be included. This approach, which avoids the use of indicators, is the most effective way of determining the health risks associated with a target waterborne pathogen. However, it is possible that a body of water polluted by faecal waste contains other pathogens. Furthermore, the detection is complex, time-consuming and expensive.

In the culturing methods a microbial species hosted uniquely by one of the stressor sources (humans, or farming animals, or wildlife) is detected by the use of a selective recovery medium. Alternatively, concentration ratios characteristic of a faecal source are obtained by the enumeration of non-specific indicators.

In the phenotypic methods a typical trait of the faecal indicator is used as distinctive characteristic. Several phenotypic characters can be used, but the method that seems more effective is the analysis of antibiotic resistance profile (ARA).

The group of genetic methods involves the use of DNA fingerprinting to identify a source-specific indicator or also to identify a non-specific faecal micro-organism that has developed peculiar genetic traits after adaptation to the intestinal conditions of a specific faecal source.

In chemical methods the tracer is a molecule that can be associated uniquely to one type of faecal pollution. Here the development of powerful analytical techniques plays a key role. The difficulty to correlate the environmental fate of the tracer to that of the pathogens is one of the limitations of these methods.

Direct detection

Because enteroviruses possess a high degree of host-specificity, their detection in water is a clear indication of human faecal contamination and gives the most direct assessment of the health risks. Nonetheless, water polluted by human faeces does not necessarily contain enteroviruses, and therefore this method is not always reliable to identify faecal sources. Jagals et al. (1995) investigated cytopathogenic viruses in a river exposed to faecal pollution produced by domestic animals and by humans. Other enteroviruses (echovirus, coxsackievirus B, coxsackievirus A, poliovirus, hepatitis A virus, Norwalk virus, reoviruses, and rotaviruses) have been used to monitor wastewater treatment plants and groundwater (Sedmak et al., 2003; Fout et al., 2003). Molecular techniques for the detection of enteric pathogens were reviewed by Toze (1999). As stated previously, the main disadvantage of this alternative is that the absence of enteroviruses in a water sample does not exclude faecal pollution.

Also helminth eggs (of host-specific intestinal worms) were considered as a faecal source indicator by Gaspard et al. (1995) and Malicki et al. (2001), but there is still not enough evidence to evaluate how effective this approach is.

Culturing methods

The culturing methods are based on the isolation of a micro-organism species (or of a group of species) from all the microbes polluting a body of water. This micro-organism should be exclusively a member of the microflora of one of the suspected sources. In the case of bacteria, the isolation is usually obtained by culturing a dilution of the water sample in a selective medium, or more often by membrane filtration technique. Sometimes the procedure comprises other steps to confirm the identity of the presumptive isolate. Total and faecal coliforms are the most commonly used indicators of faecal pollution in water but are not source-specific. When the indicator is a protozoan or virus, specific isolation techniques are used. The drawback of the culturing methods is that only few organisms are host specific and satisfy simultaneously the criteria for the faecal indicator (Maier et al., 2000); moreover, the culturing media are only rarely completely selective.

Faecal streptococci

The group of faecal streptococci comprises the enterococci species (*Enterococcus faecium, E. faecalis, E. durans, E. avium, E. gallinarum*) together with two non-enterococci (*Streptococcus equinus, S. bovis*). The concentration is obtained by membrane filtration, using m-Enterococcus or KF agar as a growth medium. Faecal streptococci have been studied extensively in the past, and most studies have revealed that they are more persistent than faecal coliforms (FC) in groundwater (Geldreich, 1976).

Three different methods have been proposed for faecal streptococci as indicators of faecal contamination sources:

- **Faecal coliforms vs. faecal streptococci ratio (FC/FS)**: According to Geldreich et al. (1969), a ratio of greater than 4 indicates human faeces (FC/FS > 4), while less than 0.7 indicates animal faeces (FC/FS < 0.7).
- **Species identification**: This method is based on the different ratio of enterococci and streptococci species in faeces determined statistically for different warm-blooded animals; according the statistical studies human faeces contain predominantly enterococci species, while animal faeces have a significant number of non-enterococci (Geldreich et al., 1969; Geldreich, 1976; Wheeler et al., 2003). In this case, the use of a specific growth medium for non-enterococci is required.
- **FC/FS ratio shift**: This approach is based on the different die-off coefficients for faecal coliforms and faecal streptococci in stored samples. Human sources, dominated by enterococci that are typically more persistent, should initially exhibit a high FC to FS ratio (> 4), which then decreases with time. Non-human sources, dominated by *S. bovis* and *S. equinus*, which are less persistent than faecal coliforms, should initially have a low FC to FS ratio (< 0.7) that increases with time (Geldreich et al., 1969; Geldreich, 1976).

These methods, although rapid, requiring minimal expertise and sometimes with satisfactory outcomes (Jagals et al., 1995; Jagals et al., 1996; Wheller et al., 2003), have proven unreliable in recent studies and they are now being opposed (Scott et al., 2002; Simpson et al., 2002).
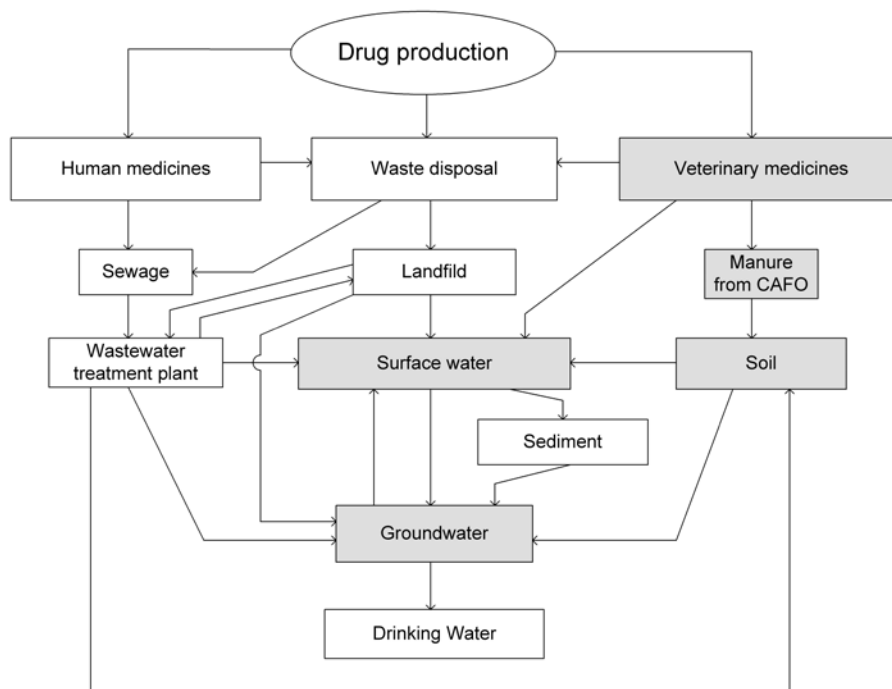
Figure 1
Pathways of human and veterinary drugs in the environment (adapted from Kümmerer, 2001)

Bifidobacteria species

Bifidobacteria (a genus in the family Actinomycetaceae) is a group of micro-organisms that are present in very high concentrations in human faeces, in particular *B. adolescentis* and *B. longum*. Certain species have been found also in animals but never in unpolluted environments. The *Bifidobacteria* hosted exclusively by humans have the ability to ferment sorbitol; this subgroup, called sorbitol-fermenting *Bifidobacteria* (SFB), is composed of *B. adolescentis* and *B. breve*, and can be used as an indicator of human source of faecal pollution.

Presently, only one growth medium has been formulated for the isolation of SFB: the human *Bifidobacteria* sorbitol-fermenting agar (HBSA), used after membrane filtration as described by Mara et al. (1983). This method proved to be reliable (Jagals et al., 1995; Jagals et al., 1996; Long et al., 2003) but there is still the need to improve selectivity and sensitivity of the growth medium.

Other *Bifidobacteria* species have been recently used as source-specific indicators: Lynch et al. (2003) used the Bifidobacterium medium (BFM), developed by Nebra et al. (1999), combined with colony hybridisation (digoxigenin (DIG-)-labelled oligonucleotide probe) to identify *B. adolescentis*. On the other hand, Nerba et al. (2003) proposed the use of *B. dentium* (human specific) as indicator organism. The distribution of *Bifidobacteria* in different environments has been described by Ventura et al. (2001) and by Gavini (2003).

Rhodococcus coprophilus

R. coprophilus is an actinomycete that can be found in herbivore dung and pasture runoff, but it is absent in human faeces. For this reason it can be used as a specific indicator of faecal contamination from grazing animals. Its persistence in waters and sediments is considerably longer than that of faecal streptococci and other commonly used faecal indicators. The method for the recovery and enumeration of this species is described by Oragui et al. (1983), and recently, molecular techniques have been applied to detect this species (Savill et al., 2001). *R. coprophilus* can be a very reliable indicator, as demonstrated in recent studies (Jagals et al., 1995; Long et al., 2003; Gilpin et al., 2002; Gilpin et al., 2003).

Bacteroides species

The *Bacteroides* genus is among the most abundant bacteria found in human faeces, 100 times greater in number than *E. coli*. Since they are almost absent in animal faeces, these species have a potential role as indicator of anthropogenic sources. In particular, *B. fragilis* has been found only in human faeces at very high concentrations. Only few methods for recovery and enumeration of *Bacteroides* species are available. The most common way to isolate *B. fragilis* is by use of *Bacteroides* bile esculin agar (BBE) as described by Livingston et al. (1978); another method involves the use of WCPG medium, after membrane filtration (Tartera et al., 1987). Despite their high potential as source-specific indicators, *Bacteroides* species have not attracted much attention recently because of their short persistence in the waters.

Phages of Bacteroides fragilis

As previously mentioned, some *Bacteroides* species are host-specific, in particular *B. fragilis,* but have only a short lifespan in the environment.

Tartera et al. (1987) used a bacteriophage of *B. fragilis*, a virus infecting this bacterial species, as a human specific faecal indicator. This bacteriophage is specific and significantly more persistent in the water environment than *B. fragilis* (the viral target). The method of enumerating *B. fragilis* bacteriophages is the double-layer agar technique (with plaque detection), using *Bacteroides* phage recovery medium (BPRM) (Pepper et al., 1995). Although this technique is not very complex, and *B. fragilis* bacteriophages are highly specific, there are still uncertainties about the reliability of this MST method (Maier et al., 2000; Sinton et al., 1998).

F-RNA phage subgroup

F-RNA phages are a group of icosahedral phages that attach specifically to the F-pili of bacteria (filamentous structures on the cell walls of 'm' bacterial strains). F-RNA coliphages infect coliform bacteria, and have been classified in three subgroups; Subgroups II and III have been isolated only in human faeces, while Subgroup I was found only in non-human mammals. There are several methods of detecting coliphages, and, once detected, the subgroups can be identified using immunological or genetic tests (Calci et al., 1998; Cole et al., 2003). What limits the use of this method is the complexity of detection.

Phenotypic methods

This set of MST methods is based on the detection of phenotypic characteristics developed by different lineages of the same bacterial species hosted in animals or humans. These phenotypic differences are caused by the different conditions to which the microbes are exposed in the intestinal tract of the hosting species. The drawback of phenotypic methods is that different species of enteric micro-organisms can show very similar biochemical responses, potentially causing a non-unique phenotypic fingerprint. However, the detection of multiple phenotypic characteristics increases the accuracy of the method.

Antibiotic resistance analysis

Antibiotic resistance analysis (ARA) is used to differentiate bacteria of the same species by their varying response to antibiotic treatment. In fact, the bacterial flora present in the human intestine is exposed to conditions different from that typical of domestic animals, because of the difference in the dietary uptake of antibiotics and other pharmaceuticals. This situation generates bacterial strains that respond differently to antibiotic treatment, giving a characteristic profile that can be used as a fingerprint to identify faecal sources.

The procedure, simple but time-consuming, involves the isolation and culturing of the target organism, followed by replica-plating of the isolates on media with increasing antibiotic concentrations. Typically, several antibiotics are considered singularly or in mixtures. After incubation, the plated colonies are observed and the susceptibilities are recorded for each antibiotic to generate an 'antibiotic resistance profile', which is compared to known profiles typical of the strain of the bacteria (Whitlock et al., 2002; Wiggins et al., 2003).

ARA has been successfully used for different indicator organisms: sulphate-reducing *Clostridia* as indicators of the practice of disposing pig manure to land (Huysman et al., 1993), faecal streptococci (Wiggins, 1996), enterococci (Booth et al., 2003; Graves et al., 2002). The main limitation of ARA is that an adequate database of profiles is needed.

Serogrouping

This method is based on the presence of different somatic (O) antigenic determinants in bacterial strains of the same species. Sero-grouping has been successfully used in a set of samples coming from different faecal sources, a good percentage of which has been successfully typed with an insignificant overlapping between the predominant serotypes (Praveen et al., 2001). Also this method necessitates an adequate databank of anti-sera profiles.

Other phenotypic methods

The use of the carbon utilisation profile to distinguish faecal sources was reported by Hagedorn et al. (2003). This technique is based on the utilisation of the BIOLOG system to determine the profile. The system can identify over 2 000 species of micro-organisms and it was used to differentiate among several *Enterococcus* species with good results. Because this method has been used only in one case, further study is needed to evaluate its effectiveness as an MST method.

Genetic methods

Genetic methods use the genotypic profile of intestinal bacteria to discriminate sources of faecal pollution. The indicator organisms for which the DNA is analysed can be host-specific – e.g. *Bifidobacterium dentium*, see Nebra et al. (2003) – or non-specific (e.g. *Escherichia coli*); in the latter case the adaptation to a host must result in a characteristic genetic profile. Several genetic techniques have been applied as MST methods, and because of their precise nature, they are currently preferred among all the other MST alternatives. However, genetic MST methods necessitate an adequate database of profiles, which can change with the geographic location and can also vary in time.

Since this paper is intended as a general overview of all the possible MST methods, only the main techniques are reviewed. For a more detailed description of the genetic method applied as MST the reader is referred to the works published by Simpson et al. (2002) and Meays et al. (2004).

Ribotyping

Ribotyping is a method of DNA fingerprinting that examines the rRNA genetic material in each bacterial isolate and produces a banding pattern image using oligonucleotide probes after treatment of genomic DNA with restriction endonucleases. This image is used to classify the indicator organism by strains, and is the basis for comparison of unknown to known sources. The procedure implies the DNA extraction and purification, followed by its digestion with restriction enzymes; then the DNA is separated via gel electrophoresis, denatured and blotted onto a membrane; it follows the hybridisation with specific-rRNA DNA probes and finally the membrane is exposed to a chemiluminescent substrate and digitally imaged (Scott et al., 2002).

The ribotyping technique was used in several studies. Parveen et al. (1999) were the first to successfully apply this method to discriminate human from animal species of *E. coli;* similar results were also obtained by Carson et al. (2001). Hartel et al. (2003) pointed out variations of genetic profiles in wild life, while Scott analysed the variations of genetic profiles with geographic location (Scott et al., 2003). The necessity of different databases for each geographic region can represent a limitation for this and all other methods based on the genetic profiles of non-specific bacteria.

Repetitive PCR DNA fingerprinting

This method uses interspersed repetitive DNA sequences located in different parts of the target indicator genome to generate specific fingerprints. The two alternative techniques that proved to be suitable as MST are the repetitive extragenic palindrome sequence PCR (rep-PCR) and the extragenic repeating elements PCR (Box-PCR) (Dombek et al., 2000; Baldy-Chdzik

et al., 2003; Borges et al., 2003; Albert et al., 2003; McLellan et al., 2003; Carson et al., 2003).

Genetic markers

These methods can distinguish the origin of faecal pollution through the identification of a labelled target gene sequence from the DNA of the indicator bacteria. The considerable advantage of using genetic markers is that culturing is not required. Bernhard et al. (2000a; 2000b) were the first to use this technique as MST method; in particular they used human and animal specific genetic markers by amplifying 16S ribosomal DNA fragments from *Bifidobacteria* species and from members of the *Bacteroides-Prevotella* group, on which they performed length heterogeneity PCR (LH-PCR) and terminal restriction fragment length polymorphism analyses (T-RFLP) (Field et al., 2003; Berhard et al., 2003). The same researchers recently applied these techniques on the genome of *Bacteroides* genus. Khatib et al. (2003) selected STII toxin gene from *E. coli* as the target sequence to identify pig faecal pollution. Recently, Simpson et al. (2004) used universal eubacterial primers and *Bacteroides-Prevotella* group-specific primers to identify equine sources.

Other fingerprinting methods

Other DNA fingerprinting techniques were tested as MST methods: Dicuonzo et al. (2001) used pulse-field gel electrophoresis (PFGE); Farnleiner and co-workers (2000) adopted the denaturating gradient gel electrophoresis (DGGE) technique; Gaun et al. (2002) and Leung et al. (2004) applied amplified fragment length polymorphism (AFLP) analysis; Seurinck et al. (2003) assessed 16S-23S rRNA intergenic spacer region (ISR)-PCR; Tsai et al. (2003) used magnetic capture hybridisation - polymerase chain reaction (MCH-PCR); El Fantroussi et al. (2003) selected oligonucleotide micro-arrays to characterise rRNA extracted from microbial populations without PCR amplification.

Most of these techniques were applied in single studies; therefore, more research is needed to access their potentialities as MST methods.

Chemical methods

Chemical methods are based on the detection of a substance (chemical tracer) that is related to a specific faecal source but is not found in unpolluted waters. In some cases the tracer is directly associated with faeces (it is released from the host's intestine), while in others it is simply discharged together with faeces in wastewaters.

These methods can only give limited information on the health risks because they are not easily correlated to the presence of waterborne pathogens. Nevertheless, their detection can give a certain indication of the origin of pollution and of the vulnerability of the body of water.

Digestion metabolites

Many substances produced in the digestive system of warm-blooded animals can be detected in faecal wastewaters. Ammonia (often measured in water samples as NH_3-N) is one of the main metabolites, but it cannot be considered a good index of faecal contamination because it is produced also by rotting vegetation, and it has been found also in unpolluted waters. Other human metabolites, like uric acid and urobilin, have been considered as MST but they do not seem specific enough (Sinton et al., 1998).

Only faecal sterols have been successfully used as source-specific faecal indicators. Faecal sterols are a group of cholestane-based sterols found in faeces. They are comprised of coprostanol, sitosterol, campestanol and 5-beta-stanol. Coprostanol is the principal human faecal sterol and can be used as a reliable tracer of faecal pollution (Sinton et al., 1998; Leeming et al., 1996a; Atherholt et al., 2003). The analysis of faecal sterols is based on high-resolution gas chromatography and mass spectrometry (Jayasinghe et al., 1998; Borjesson et al., 1999; Truong et al., 2003) and has been used effectively in studies by Leeming et al. (1996b), Maldonado et al. (1998), Leeming et al. (1998), Schonning et al. (2002), Suprihatin et al. (2003), and Isobe et al. (2004).

Sinton et al. (1998) proposed the direct detection of human or animal DNA sloughed off the intestinal tract as a chemical MST method, but no study of this kind has been done. Another approach that can give interesting results, but has not been tested yet, is the use of the isotopic partitioning ratios for elements in the major compound found in human or animal metabolites in analogy to studies done in forest ecosystems (Garten, 2006).

Detergents and brighteners

Chemicals contained in liquid and powder detergents are usually associated with discharges containing faecal material. Three main groups of substances have been investigated as faecal indicators: fluorescent whitening agents (FWAs), sodium tripolyphoshate (STPs) and long-chain alkylbenzenes (LBAs) (Sinton et al., 1998). FWAs are incorporated in powder detergents and can be easily detected using fluorometric measurements or thin layer chromatography (Gilpin et al., 2002; Close et al., 1989; Hayashi et al., 2002; Poiger et al., 1999). STPs are a major component of washing powders and can be measured by ion-exchange combined with colorimetric techniques. LBAs are a group of synthetic hydrocarbons intensively used as anionic surfactants in detergents, and their determination can be done using organic solvent extraction followed by gas chromatography (Holts et al., 1992; Martins et al., 2002). LBAs have also been investigated in relation to soil pollution (Jensen, 1999; Binetti et al., 2000; Carlsen et al., 2002).

Because detergents and brighteners can also be released with industrial wastewaters, their application as MST methods should be carefully considered in relation to the characteristics of the body of water.

Caffeine and fragrances

Caffeine was detected for the first time in wastewaters by Sievers and co-workers three decades ago (Sievers et al., 1977). Seiler et al. (1999) attempted for the first time to use caffeine as a faecal indicator in groundwater. In their study they were able to detect and quantify this substance in several samples using HPLC without the need of pretreatment extraction; however, they concluded that caffeine is not a good faecal indicator (and neither a good MST) because it was not detected in several samples of polluted water. New techniques have been proposed to improve the analytical resolution for trace contaminants (Burkhardt et al., 1999; Piocos et al., 2000), and recently caffeine has been reused as a tracer of human faecal sources by Standley et al. (2000), Weigel et al. (2002), and recently by Buerge et al. (2003a).

Synthetic musk fragrances, including polycyclic musks (Galaxolide – HHCB; Tonalide – AHTN; Traseolide – ATTI; Phantolide – AHMI; Celestolide – ADBI; and Cashmeran – DPMI)

TABLE 3		
List of the target emerging contaminants in waters, adapted from USGS (2003)		
Veterinary and human antibiotics		
Tetracyclines		**Sulphonamides**
Chlortetracycline		Sulfachlorpyridazine
Doxycycline		Sulfamerazine
Oxytetracycline		Sulfamethazine
Tetracycline		Sulfathiazole
Fluoroquinolones		Sulfadimethoxine
Ciprofloxacin		Sulfamethiazole
Enrofloxacin		Sulfamethoxazole
Norfloxacin		**Others**
Sarafloxacin		Lincomycin
Macrolides		Trimethoprim
Erythromycin-H_2O (metabolite)		Carbadox
Tylosin		Virginiamycin
Roxithromycin		
Human drugs		
Prescription		**Non-Prescription**
Metformin (antidiabetic agent)		Acetaminophen (analgesic)
Cimetidine (antacid)		Ibuprofen (anti-inflammatory, analgesic)
Ranitidine (antacid)		Codeine (analgesic)
Enalaprilat (antihypertensive)		Caffeine (stimulant)
Digoxin		1,7-Dimethylxanthine (caffeine metabolite)
Diltiazem (antihypertensive)		Cotinine (nicotine metabolite)
Fluoxetine (antidepressant)		
Paroxetine (antidepressant, anti-anxiety)		
Warfarin (anticoagulant)		
Salbutamol (antiasthmatic)		
Gemfibrozil (antihyperlipidemic)		
Dehydronifedipine (antianginal metabolite)		
Digoxigenin (digoxin metabolite)		
Sex and steroidal hormones		
Biogenics		**Pharmaceuticals**
	17*b*-Estradiol	17*a*-Ethynylestradiol (ovulation inhibitor)
	17*a*-Estradiol	Mestranol (ovulation inhibitor)
	Estrone	19-Norethisterone (ovulation inhibitor)
	Estriol	Equilenin (hormone replacement therapy)
	Testosterone	Equilin (hormone replacement therapy)
	Progesterone	**Sterols**
	cis-Androsterone	Cholesterol (faecal indicator)
		3*b*-Coprostanol (carnivore faecal indicator)
		Stigmastanol (plant sterol)

and nitro musks (musk xylene-MX; and musk ketone-MK), are chemicals widely used in cosmetics and in personal and household care products. Musk fragrances were studied for the first time as tracers of human faecal contamination by Standley et al. (2000), and are currently attracting the attention of the scientific community (Fromme et al., 2000; Buerge et al., 2003b; Ricking et al., 2003; Lee HB et al., 2003; Peck et al., 2004); their environmental fate is also the object of study (Heberer, 2003). Musk fragrances are detected by solid or supercritical-fluid extraction followed by gas chromatography/mass spectrometry.

Although the presence of caffeine and musk fragrances is an indication of human sources, it is not yet clear whether they can be suitable indicators of faecal pollution.

Pharmaceuticals and other drugs

The awareness of the potential risks brought about by pharma-ceuticals in the environment started growing in the mid 1990s, when scientists observed deleterious effects on fish and other freshwater fauna as a consequence of the presence of endocrine disrupting agents at trace levels in aquatic ecosystems (Halling-Sorensen et al., 1998; Daughton et al., 1999; Jorgensen et al., 2000; Sumpter, 2003; Petrovic et al., 2004). During the same period, a number of popular drugs were detected at concentrations ranging from nanograms to micrograms per litre in groundwater (Eckel et al., 1993; Holm et al., 1995), surface water, and in particular outlet streams from sewage treatment plants (Ternes, 1998).

Nowadays, the study of pharmaceuticals and personal care products (PPCPs) in the environment is an important topic in environmental studies, and research in this field is growing exponentially. A comprehensive review of the studies on PPCPs in the environment is given by Kümmerer (2001) and Daughton et al. (2001). The high level of attention given by environmental

TABLE 4
Veterinary medicines that have been monitored and detected in ground- and surface waters (adapted from Boxall, 2003)
Oxolinic acid
Oxytetracycline
Chlortetracycline
Ivermectin
Sarafloxacin
Cypermethrin
Tetracycline
Lincomycin
Diazinon
Trimethoprim
Tylosin
Benzoate

agencies to PPCPs gives evidence of the importance of these pollutants (Barnes et al., 2002; Kolpin et al., 2002; US-EPA, 2004), and a list of the 'emerging contaminants' has been prepared by USGS (2001).

The unfolding of this new dimension in environmental science is favoured mainly by the progress in the instrumental analysis of trace contaminants. Liquid and gas chromatography coupled with tandem-mass spectrometry (Marchese et al., 2003; Hilton et al., 2003; Loffler et al., 2003; Ferrer et al., 2003) are the prominent techniques among those developed in this field (Petrovic et al., 2003).

The drugs found most frequently in surface and wastewaters in North America are clofibric acid (cholesterol control drug), carbamazepine (antiepileptic drug) and salicylic acid (Weigel et al., 2002; Ternes, 1998, Lee et al., 2003b, Metcalfe et al., 2003, Boyd et al., 2003; Sacher et al., 2003; Bila et al., 2003). Clofibric acid has also recently been proposed as a marker for anthropogenic contamination (Clara et al., 2004). In order to evaluate the potentialities of these drugs to distinguish faecal sources it is necessary to understand their environmental fate better.

Antibiotics and other drugs used as growth promoters in CAFO are also capturing the attention of environmental scientists in Europe and North America (USGS, 2003; Boxall et al., 2003; Scribner et al., 2003; Boxall et al., 2004). As previously mentioned, the main concern in relation to these drugs is their environmental fate, and in particular the risk of favouring the development of antibiotic-resistant micro-organism (i.e. super-bugs). There is still uncertainty about the quantities of growth promoters currently used in CAFO (Blackwell, 2003), but some of the most persistent drugs have already been found in watersheds (Boxall et al., 2003) (Table 3) and for this reason they could be used as tracers of CAFO faecal pollution. There is no published study on the use of growth promoters as MST, but once again the feasibility of this approach is contingent on by the understanding of the environmental fate of these drugs.

Summary and concluding remarks

Microbial source tracking (MST) has recently become a relevant issue in water quality monitoring and management, and several different approaches have been adopted to distinguish faecal pollution sources. The enumeration of indicator micro-organisms, which is the traditional method in the assessment of health risks for waterborne pathogens, was also the first method applied in tracking sources of faecal pollution.

Nowadays, MST methods can be classified into four main groups: culturing, phenotypic, genetic and chemical. According to the recent literature (Scott et al., 2002; Simpson et al., 2002), the most promising and reliable MST methods available are ribotyping, host-specific genetic markers (genetic methods) and antibiotic resistance analysis (phenotypic method), but more research is needed for each of these techniques before they become the standard MST methods in water quality analysis.

Among the alternatives currently considered, the detection of pharmaceutical and personal care products (PPCPs) seems promising. The key factors in evaluating the reliability of PPCPs are their environmental fate and the correlation to a specific faecal source. Currently, only limited experimental evidence is available to access their applicability as MST methods.

Although the rapid development of genetic techniques and bio-sensors makes us infer that waterborne pathogens will be identified directly, quickly and inexpensively in the near future (Estes et al., 2003), it is likely that the use of traditional faecal indicator organisms will continue to play an important role. It is also unlikely that a unique MST method will be found effective in all possible situations. As recently suggested by Gilpin et al. (2003) and Pickup et al. (2003), the combination of microbial, genetic and chemical methods is probably going to be the optimal solution to distinguish sources of faecal contamination in ground- and surface waters.

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