

Minimal Requirements for Parameters to be Used in the Development of Predictive Models for Microbial Source Tracking: Somatic Coliphages and Phages Infecting *Bacteroides* as Examples

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Abstract (Scientific)

*Several methods and approaches for measuring parameters to determine fecal sources of pollution in water have been developed in recent years. No single microbial or chemical parameter has proved sufficient to determine the source of fecal pollution. Combinations of parameters involving at least one discriminating indicator and one universal fecal indicator offer the most promising solutions for qualitative and quantitative analyses. The universal (non-discriminating) fecal indicator provides quantitative information regarding the fecal load. The discriminating indicator contributes to the identification of a specific source. The relative values of the parameters derived from both kinds of indicators could provide information regarding the contribution to the total fecal load from each origin. It is also essential that both parameters characteristically persist in the environment for similar periods. Numerical analysis, such as inductive learning methods, could be used to select the most suitable and the lowest number of parameters to develop predictive models. These combinations of parameters provide information on factors affecting the models, such as dilution, specific types of animal source, persistence of microbial tracers, and complex mixtures from different sources. The combined use of the enumeration of somatic coliphages and the enumeration of *Bacteroides*-phages using different host specific strains (one from humans and another from pigs), both selected using the suggested approach, provides a feasible model for quantitative and qualitative analyses of fecal source identification.*

Abstract (Dissemination)

*The determination of fecal pollution sources (MST or microbial source tracking) in surface waters has become an essential issue in the management of catchments. Though efforts have been made during the last few years on the identification of MST indicators and on the development of standardized techniques for their analysis, no single microbial or chemical parameter has proved sufficient to determine the source(s) of fecal pollution. Recent MST predictive models, based on combinations of the most suitable and the lowest number of indicators, have been defined by using inductive learning methods. A feasible model for quantitative and qualitative analyses of MST is based on the combined use of the enumeration of somatic coliphages and the enumeration of *Bacteroides*-phages using different host specific strains (one from humans and another from pigs). This experimental approach allowed identification of the minimal requirements for parameters used in MST predictive models.*

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Introduction

The disposal in watersheds of fecal waste derived mainly from different human activities is a current practice world-wide. Wastewater treatment plants contribute to eradicating or diminishing the most serious problems, at least in developed countries. However, further efforts are necessary to determine the maximum fecal load that watersheds can receive while guaranteeing proper management of resources characterized by appropriate microbial quality and lowered health risk.

Fecal pollution of surface waters comes from different sources: municipal sewage, slaughterhouse wastewaters, manure and biosolid disposal, wildlife, and undetermined runoff. Microbial source tracking (MST) denotes procedures using different microbial indicators to establish the origin of fecal pollution in water. The microbial indicators presently used to determine water quality do not provide information about the origin of fecal pollution. This has led to a search for microbial and chemical indicators which can provide information on the origins of fecal pollution. Most potential indicators require the development of feasible techniques for their detection.

MST has a short history, becoming a defined knowledge topic in the early 1980s as a result of social and legal pressures. Since then it has rapidly grown into an established research field. Within this development we can identify three distinct stages, each of which provided the knowledge and technological base necessary for the subsequent stages.

The Development of MST

Stage 1: Proposing indicators and developing detection techniques

Starting in the 1970s, there were studies trying to find host-specific indicators of fecal pollution in water (Geldreich, 1976; Mara & Oragui, 1981; Osawa *et al.*, 1981). There has not been consensus among researchers regarding the best indicators for MST. Since microbial indicators normally used to measure water quality fail to distinguish the origins of fecal pollution, at first most MST research was aimed at defining new indicators and appropriate detection methods. Many different approaches are still published today in order to provide new methods (Layton *et al.*, 2006; Reischer *et al.*, 2007) or to suggest new indicators for fecal source detection (Martellini *et al.*, 2005; Payán *et al.*, 2005). MST methods have been described and discussed in detail in several technical reviews (Field *et al.*, 2003a; Simpson *et al.*, 2002; Scott *et al.*, 2005; Stoeckel & Harwood, 2007), and the Stage 1 approaches were mainly based on the requirements of cultivating the microorganisms and the dependence of a prior developed library for reference. Based on these characteristics, two kinds of MST methodologies can be distinguished.

Library-dependent methods

Two MST subgroups can be differentiated, those based in phenotypic characteristics and those based in genotypic characteristics. Among the first subgroup antibiotic resistance analysis of fecal streptococci (Hagedorn *et al.*, 1999; Wiggins, 1996), fecal coliforms and *E. coli*

(Parveen *et al.*, 1997; Whitlock *et al.*, 2002), or carbon source utilization for fecal streptococci, enterococci and *E. coli* (Hagedorn *et al.*, 1999; Harwood *et al.*, 2000; Manero *et al.*, 2002; Wallis & Taylor, 2003) have all been extensively evaluated. These approaches are limited because a library constructed for one study in a particular geographical area cannot usually be used in other regions. Waterborne microorganisms can vary widely according to geographical area, season, diet of the human population, and even the sampling time of day. Increasing the number of entries in the library, or merging similar libraries, reduces success rates and increases costs.

Several approaches based in genotypic characterisation have been studied. Though genotypic methods predict the source material more successfully than phenotypic methods (Griffith *et al.*, 2003), high false positive rates have been found in interlaboratory studies comparing most common genotyping -library-dependent methods (Myoda *et al.*, 2003). These results highlight the need to modify and optimize these genotyping library-dependent methods.

Applications of these phenotypic and genomic assays both depend on cultivation, as pure cultivated strains should be obtained before application for the best results.

Library-independent methods

These do not require the prior development of representative libraries and offer a wide range of applications. Some of the methods require culture of microorganisms, whereas others do not. Many different indicators and detection and/or enumeration techniques

have been performed as part of approaches based in culture: fecal coliforms / streptococci ratio (Geldreich, 1976), detection of some bifidobacterial populations or species on selective media (Mara & Oragui, 1983; Bonjoch *et al.*, 2005), specific primers and probes for the detection of host-associated bifidobacteria species (Lynch *et al.*, 2002; Nebra *et al.*, 2003), detection of the enterococcal surface protein *esp* (Scott *et al.*, 2005), phages infecting some strains of *Bacteroides fragilis* (Puig *et al.*, 1999; Tartera *et al.*, 1989), serotypes (Osawa *et al.*, 1981) and genotypes of F-specific RNA bacteriophages (Hsu *et al.*, 1995), and FAME of fecal coliforms (Duran *et al.*, 2006). The detection of some protozoa such as *Giardia* (Brown, 1993) and *Cryptosporidium* (Awad-EI-Kariem *et al.*, 1995) have also been considered.

Culture-independent and library-independent methods have some potential advantages, such as not requiring the prior development of libraries, but they are compromised by the abundance of cosmopolitan isolates. Furthermore, they are faster and can detect uncultivated microorganisms which are numerically dominant in feces. Different indicators and methods have also been suggested and developed, for instance: T-RFLP and *E. coli* toxin genes (Field *et al.*, 2003b), specific PCR primers for the selective detection of host related *Bacteroidetes-Prevotella* group (Bernhard & Field, 2000; Field *et al.*, 2003a; Reischer *et al.*, 2007), or the use of specific primers for real-time PCR among *Bacteroides spp.* (Layton *et al.*, 2006), species-specific detection of *Bifidobacterium spp.* (Bonjoch *et al.*, 2004), bovine enteroviruses (Ley *et al.*, 2002), human, porcine and bovine adenovirus (Maluquer de Motes *et al.*, 2004; Pina *et al.*, 1998), porcine teschoviruses (Jimenez-

Clavero *et al.*, 2003) or polyomaviruses (McQuaig *et al.* (2006).

Besides microbiological indicators, other types of indicators had been proposed. Chemical studies have mainly concentrated on the distribution pattern of different potential markers: caffeine, fragrance substances, fluorescent whitening, and fecal sterol isomers (Leeming *et al.*, 1996; Standley *et al.*, 2000). Recently, new approaches using eukaryotic mitochondrial DNA to differentiate sources in fecally contaminated surface water have been explored (Caldwel *et al.*, 2007). However, field studies using most of the numerous chemical and microbiological methods available for MST show that no single method succeeds in pinpointing the origin of fecal pollution (Malakoff, 2002; Simpson *et al.*, 2002; Stewart *et al.*, 2003).

At this stage, several limitations to source identification approaches were identified, including the assay of inappropriate samples, the use of approaches that are not spatially stable, over-emphasis on methods rather than on identifying appropriate source indicators (tracers), or trying to determine tracer and source tracking methods at the same time. Different research groups working independently on the development of MST agreed that more than one method would be needed to achieve feasible solutions for MST. They observed that the standardization of methods and data sharing were essential to progress, as there were usually geographical, climate or diet limitations for the different indicators suggested. The comparison of approaches and results from different researchers covering wider geographical areas was considered essential, leading to method comparison studies.

Stage 2: Collaborative and comparative studies

A U.S. comparative MST study was organized by the Southern California Coastal Water Research Project (SCCWRP), with support from the U.S. Environmental Protection Agency (EPA), to test, compare, and improve MST methods (Malakoff, 2002). The common *experimental* design was based on analyzing water matrix samples spiked with fecal material from known sources. Several laboratories used library-dependent phenotypic techniques (Harwood *et al.*, 2003; Griffith *et al.*, 2003), and it was observed that the internal accuracy of the libraries did not correspond to the accuracy of source prediction. Moreover, no method predicted the source material in the blind samples perfectly. Library-based methods frequently produced false positives and incorrectly identified the fecal source. However, genotypic methods, among library-based methods, performed better than phenotypic methods, although the genotype-based and library-independent methods (Myoda *et al.*, 2003) showed a high false positive rate and were not quantitative. Culture-independent and library-independent genotypic methods were also assayed (Field *et al.*, 2003b; Noble *et al.*, 2003). High rates of false positives and negatives for the selected methods were obtained, indicating the need of improving these methods as well. Additionally, performance in real scenarios using environmental water samples was not tested in the SCCWRP study, as no method performed well enough to proceed to that stage. Consequently, the effects of the load of fecal material, persistence of indicators in the environment, dilution effects of waterbodies, or a mixture of fecal origins were not considered. These effects could

challenge the feasibility of each method examined.

A comparison of the statistical methods used in library-based approaches to MST (Ritter *et al.*, 2003) in the SCCWRP study showed no widespread agreement as to which method was most appropriate. A high degree of variability across assayed statistical methods was detected, and no single statistical method emerged as superior. The authors recommended careful examination of libraries, removal of nuisance variables, and searching for more discriminating numerical methods.

Almost at the same time, an international and multidisciplinary European study supported by the European Commission was planned using a different experimental design and approach from that used in the U.S. (Blanch *et al.*, 2004, Blanch *et al.*, 2006). A simple differentiation between human and non-human sources was the primary goal, and the examination of highly polluted wastewaters or slurries was planned to avoid the failures reported in previous studies (often related to the use of dilute samples that gave values below experimental thresholds). The European collaborative study also included both a wide range of geographical areas and several indicators of fecal pollution from both human and non-human sources, necessary to define ratios between discriminating and non-discriminating indicators and to define the persistence of the values of fecal contaminants in the environment. Furthermore, identification of statistical or inductive learning methods to develop appropriate predictive models was also included.

First, training sessions and reference materials for quality control were used

during the initial study. This was the first reported MST collaborative study with such quality control measures. Then the study compared potential microbiological or chemical MST tracers using techniques already well established with the values of conventional indicators. At the same time emerging methods were further developed or adapted for water samples. Some of these newly developed, improved, or adapted methods proved to be feasible for the following stage of the project (Blanch *et al.*, 2004). At this point, all the research groups performed the following determinations: enumeration of fecal coliform bacteria, enterococci, *Clostridium perfringens*, somatic coliphages, F-specific RNA phages and bacteriophages infecting *Bacteroides fragilis* RYC2056, genotyping of F-specific RNA phages, biochemical phenotyping of fecal coliform and enterococci, enumeration of four main fecal sterols (coprostanol, ethylcoprostanol, epicoprostanol, and cholestanol), enumeration of total and sorbitol-fermenting bifidobacteria, detection (presence or absence) of *Bif. adolescentis* and *Bif. dentium*, and enumeration of bacteriophages infecting the *Bacteroides thetaiotaomicron* GA17 strain.

The results demonstrated that fecal coliform, enterococci, clostridia, total bifidobacteria, somatic coliphages, F-specific RNA phages and phages infecting the RYC2056 strain of *Bact. fragilis* have similar relative densities in municipal or human-derived wastewaters in the different geographical areas studied; consequently all of them can potentially be used as indicators of either human or non-human fecal loads. In contrast, concentrations of other parameters were different depending on the origin of fecal contamination. No significant differences related to the size of

the human communities contributing fecal pollution were observed (from a few hundred to 1.5 million inhabitants). Thus samples polluted by communities of around 100 inhabitants were representative of human fecal pollution.

Stage 3: Development of predictive models by integrated analysis

As indicated above one of the objectives of the European project was to develop appropriate predictive models. A number of statistical methods (discriminant analysis, nearest neighbour technique (maximum similarity), and artificial neural networks) were tested. Because there is no consensus regarding the most appropriate statistical analysis to determine the optimal set of variables for developing MST predictive models, other inductive learning methods which have been used successfully in many disciplines were studied (Blanch *et al.*, 2006). Three objectives were pursued (Table 1) leading to the formulation of predictive models: i) to determine the most discriminating tracers that show broad and consistent geographical stability; ii) to identify subsets of variables derived from the parameters measured with the highest discriminative capacity; and iii) to evaluate and compare statistical or inductive learning methods to develop MST predictive models using the minimum number of variables.

Once again, no single microbial or chemical parameter was able to determine the source of fecal pollution. It was concluded that combinations of the most promising tracers could be used to determine the lowest number of parameters needed to define variables for the development of predictive models with the highest possible fecal source discrimination rate (Blanch *et al.*, 2006). This integrated approach also

observed variations between statistical analyses, in agreement with previous studies (Ritter *et al.*, 2003; Wiggins, 1996). Those studies also demonstrated that none of the typically used statistical techniques emerged as superior. However, the European study results showed that some combinations of library-independent methods with a consistently high degree of discriminatory power successfully distinguished the origin of fecal pollution but not at a quantitative level.

Several statistical and inductive learning methods (Euclidean k-nearest-neighbour, linear Bayesian classifier, quadratic Bayesian classifier, and Support Vector Machine) were used to define predictive models with combinations of variables that provided different degrees of correct classification (Blanch *et al.*, 2006). Some of the predictive models achieved 100% success in distinguishing between pollution of human and non-human origin of wastewaters. Moreover, no differences were found between the various European geographical locations in the prediction models developed. The optimum predictive model consisted of the pair of variables (ratio of numbers of somatic coliphages and phages infecting *Bact. thetaiotaomicron* GA17 strain, and numbers of somatic coliphages). This model allowed successful classification of fecal source in all cases for all the inductive learning methods tested (Table 1). Alternative predictive models were also found that showed marginally lower rates of correct classification and/or required more parameters (Table 2).

The experimental approach and numerical methods proved to be extremely accurate and very simple. Models using only two variables and a linear separation that discriminates between human and non-

human fecal pollution in wastewaters were defined. Combinations of variables based on a discriminating indicator and a universal fecal indicator offered the best results. The discriminating indicator identifies the source, and the universal (non-discriminating) fecal indicator provides information on the fecal load. This distinction is a novelty with respect to the pairs of methods described in other studies, such as combinations of sterols (Leeming *et al.*, 1996) or ratios between traditional bacterial indicators (Geldreich, 1976). The combination may also offer advantages when analyzing other samples (such as diluted, aged, and mixed samples). These results, together with those emerging from method comparison studies in the U.S., made it clear that new approaches for MST were required and that novel approaches need to solve, step by step, the different factors that could influence successful identification of fecal pollution sources. These factors are the nature of the dominant fecal pollution contributors (anthropogenic or non-anthropogenic pollution), persistence of indicators and their measured parameters, effects of dilution in watersheds, presence of complex mixtures from several distinct animal species, and the selection of

appropriate and consistent numerical methods for the development of models.

Searching for Feasible MST Predictive Models: The Case for the Somatic Coliphages-Bacteriophages of *Bacteroides*

The discriminative capacity of phages infecting *Bacteroides* (Tartera & Jofre, 1987; Tartera *et al.*, 1989) is in agreement with the reported discriminative capacity of *Bacteroidetes* (Bernhard & Field, 2000). Somatic coliphages and bacteriophages infecting *Bacteroides* *spp.* are non-pathogenic, easily and quickly detectable, and quantifiable. These characteristics make them highly suitable for tracking fecal sources. Furthermore, standardized methods for detecting and enumerating both already exist (ISO, 2000; ISO 2002; USEPA, 2000).

The results of the European study were very promising regarding the suitability of using only somatic coliphages and phages infecting *Bacteroides* as parameters. However, the results do raise several questions that should be addressed: i) How does dilution of fecal contaminants affect the feasibility and suitability of the approach? ii) How does



Guadalquivir River scene, Seville, Spain

Table 1: Percentage of Correct Classification by the Different Methods for Developing Predictive Models*

FRNAPH II+III, BTHPH	# of Variables	NN	LBC	QBC	SVM
SOMCPH, SOMCPH / BTHPH	2	100.0	100.0	100.0	100.0
FC, FC/BTHPH	2	100.0	99.0	99.0	100.0
FRNAPH II+III, BTHPH	3	97.1	84.5	99.0	-

*using the lowest number of the 38 simple and derived variables. The methods are:

NN: Euclidean 1-nearest-neighbour classifier
 LBC: linear (LBC) and Bayesian classifiers
 QBC: quadratic Bayesian classifiers
 SVM: Support Vector Machine (SVM).
 SOMCPH: Enumeration of somatic coliphages
 SOMCPH/BTHPH: Ratio of enumerations of somatic coliphages and phages infecting *Bact. thetaiotaomicron* GA17

FC: Fecal coliforms
 FC/BTHPH: Ratio of enumeration of fecal coliforms and the new host strain *Bact. thetaiotaomicron* GA17
 FRNAPH II + III: Sum of the percentages of genotypes II and III of F-specific RNA bacteriophages
 BTHPH: Enumeration of bacteriophages infecting the host strain *Bact. thetaiotaomicron* GA17

(Blanch et al., 2006)

Table 2. Alternative Predictive Models*

Variables	# of Variables	% Correct Classification
ECP, FRNAPH II	2	97.1 (1NN)
BA, FRNAPH II	2	97.1 (LBC)
FRNAPH I, FRNAPH II, FC	3	97.1 (QBC)
BA, FRNAPH II, ECP	3	97.1 (LBC)
SOMCPH, FRNAPH II, FRNAPH I	3	97.1 (QBC)
BA, FRNAPH II, FC	3	97.1 (LBC)
FRNAPH I, FRNAPH II, ECP	3	99.0 (1NN)
ECP, FRNAPH II, BA, SFBIF	4	99.0 (QBC)
SOMCPH, FRNAPH II, BA, SFBIF	4	100.0 (1NN)

*other than those using the variable BTHPH (enumeration of bacteriophages infecting the host strain *Bact. thetaiotaomicron* GA17). The most successful classification rate is given and the numerical method indicated.

1NN: Euclidean 1-nearest-neighbour classifier
 LBC: Linear Bayesian Classifier
 QBC: Quadratic Bayesian Classifiers.
 ECP: Percentage of *E. coli*/Ph-Plate phenotypes

FRNAPH II: Percentage of genotype II of F-specific RNA bacteriophages
 BA: Presence or absence of *Bif. Adolescentis*
 FRNAPH I: Percentage of genotype I F-specific RNA bacteriophages
 FC: Fecal coliforms
 SOMCPH: Enumeration of somatic coliphages
 SFBIF: Enumeration of sorbitol-fermenting bifidobacteria
 (Blanch et al., 2006)

the persistence of the parameters in the environment, or their resistance to inactivation, affect the numerical relationship between the selected indicators? iii) Can geographical differences regarding the suitable *Bacteroides* host strains be accounted for with this approach? iv) Is it possible to detect bacterial hosts for non-human hosts (that is, to differentiate different animal sources, not just human from non-human sources?) v) Is it possible to detect them by molecular methods? In the remainder of this section, these questions are addressed one by one.

Abundance in fecal sources

For dilution not to affect the validity of the approach being evaluated, both phages must be copious in samples with high fecal loads. Somatic coliphages are present in raw municipal, hospital and military camp sewage in concentrations ranging from 10^6 to 10^7 PFU /100 ml, usually less than one order of magnitude lower than the concentration of fecal coliforms wherever it was measured (Blanch *et al.*, 2006; Chung *et al.*, 1998; Contreras-Coll *et al.*, 2002; Grabow *et al.*, 1993; Lodder & de Roda, 2005;). Deborde *et al.* (1998) detected somatic phages in all 43 samples of septic effluents tested, with an average value of 2.3×10^4 PFU per 100 ml; and Lucena *et al.* (2003) reported detection of somatic coliphages in all samples of septic effluents tested, with values similar to those reported for sewage in the same area. High counts of somatic coliphages are also detected in raw and treated sludge (Lasobras *et al.*, 2002; Mingote-Cardiergues *et al.*, 1999). Somatic coliphages are also very abundant in abattoir wastewater, with

values that represent the same ratio to fecal coliforms as in municipal wastewater (Blanch *et al.*, 2006; Grabow *et al.*, 1993; Havelaar and Hogeboom, 1984; Tartera *et al.*, 1989). Reported densities of somatic coliphages in slurries vary, probably because of the vagueness of the term “slurry,” but they still are present in the same proportion to fecal coliforms that was found in abattoir wastewaters (Blanch *et al.*, 2006; Cole *et al.*, 2003; Hill & Sobsey, 1998).

Data on phages detected by the GA17 strain of *Bacteroides tethaiotaomicron* correspond, to date, only to a geographical area that includes most of Europe (Blanch *et al.*, 2006; Payán, 2006). More than 10^5 PFU per 100 ml is consistently detected in municipal sewage, sewage from an approximately 100-bed hospital, and sewage from a military camp hosting about 200 individuals in Southern Europe (Blanch *et al.*, 2006). Values for phages detected by strain GA17 of *Bacteroides tethaiotaomicron* in raw sludge also maintain the same relationship to fecal coliform bacteria and to somatic coliphages found in sewage (Payán, 2006). Values in non-human fecal wastes are either zero or very low; at least 3 \log_{10} units lower, compared to the values for human fecal wastes (Table 3).

Enumeration of somatic coliphages is feasible in most fecally contaminated sources after 10^4 - 10^5 dilution. In the case of phages detected by the GA17 strain of *Bacteroides tethaiotaomicron*, acceptable dilution (without need of concentration) ranges between 10^{-2} and 10^{-3} . In both cases, testing a 10 ml

sample is feasible. Moreover, both types of phages can be concentrated with similar efficiency using a very simple method for concentrations up to one litre of water (Mendez *et al.*, 2004). These detectable concentrations of phages account for most of the situations in which fecal source tracking is needed.

No seasonal variations are detected in either the concentrations of somatic coliphages or those of bacteriophages detected by strain GA17 in sewage (Payán, 2006). Furthermore, no variation has been observed in the numbers of phages detected in raw sewage over the three year period since GA17 was first isolated and tested (Payán *et al.*, 2005; Payán, 2006). Neither have variations in the numbers of bacteriophages in raw sewage in the area of Barcelona been noticed in the time elapsed since RYC2056 and HSP40 were first used to count bacteriophages in raw sewage, ten and 20 years ago, respectively (unpublished data). Sudden changes should not be expected.

Persistence in the environment and resistance to inactivating treatments

Short persistence in the environment, as in the cases of the anaerobic bacteria *Bifidobacterium* (Carrillo *et al.*, 1985) and those belonging to the *Bacteroides* (*Bacteroidetes*) group (Fisdal *et al.*, 1985), might hinder their use as tracers of fecal sources. Persistence in the environment and resistance to inactivating treatments may also affect the performance of the phages, based on proportions between indicators. For example, the ratio between numbers of somatic coliphages and numbers of

phages infecting *Bacteroides thetaiotaomicron* GA17 must be consistent over many different environmental variables.

Copious available data indicates that somatic coliphages and bacteriophages infecting strains HSP40 and RYC2056 of *Bacteroides fragilis* persist similarly in the water environment and that they are reasonably persistent (Contreras-Coll, 2000; Contreras-Coll *et al.*, 2002; Duran *et al.*, 2002). Both persist much longer than fecal coliforms, whose concentrations in fecal matter also make them suitable as the non-discriminating parameter. There is not as much information available about phages infecting strain GA17 of *Bacteroides thetaiotaomicron*, which have the same morphology as those infecting strains HSP40 and RYC2056 of *Bacteroides fragilis* (Payán, 2006; Queralt *et al.*, 2003). However, the existing data indicate that they behave similarly to the phages infecting other *Bacteroides* host strains; they show similar inactivation in river water (Payán, 2006) and seawater (Mocé *et al.*, 2005), and they are found in the same proportions in river and sea water as in raw sewage (Mocé *et al.*, 2005; Payán, 2006).

As indicated above, the fate of microbial parameters in wastewater treatments will affect their suitability for tracking fecal sources, since secondary effluents from wastewater treatment plants are, at least in developed countries, the most important contributor of fecal contamination of human origin in receiving water bodies. The ratios of numbers of somatic coliphages to numbers of bacteriophages infecting strain GA17 in secondary effluents

appear to be similar to those in raw sewage (Payán, 2006).

In tertiary treatments, which today include a variety of removal and

disinfecting procedures, the elimination of various potential source tracking microbes might be different. Preliminary data once again indicate that somatic

Table 3. Average of Log Number per 100 mL and Standard Deviation (SD) of Fecal Coliforms (FC), Somatic Coliphages (SOMCPH), and Phages Infecting *Bacteroides tethaiotaomicron* GA17 (BTHPH) in Waters with Fecal Contaminants of Different Origins

Contaminants of Different Origins	FC (log CFU/100ml)		SOMCPH (log PFU/100ml)		BTHPH (log PFU/100ml)	
	Average	SD	Average	SD	Average	SD
Raw urban sewage	7.31	0.14	6.80	0.30	5.00	0.37
Secondary effluent	5.18	0.82	5.29	0.82	3.14	1.00
Tertiary effluent	0.83	1.12	3.53	0.80	1.72	1.11
Raw sludge	ND	ND	5.87	0.30	4.51	0.15
Pig abattoir wastewater	7.73	0.78	7.57	0.54	1.85	0.36
Cattle abattoir wastewater	6.81	0.33	6.60	0.47	<1.70	-
Poultry abattoir wastewater	7.67	0.50	5.81	1.11	<1.70	-
River ²	3.52	0.30	3.80	0.26	2.63	0.37
Sea ^{2,3}	1.00 ¹	0.93 ¹	1.86	0.57	0.44	0.35

¹ Values corresponded to *E. coli*

² Mostly influence by disposal of treated urban sewage

³ Data extracted from Mocé-Llivina *et al.*, 2005

ND: Not determined

coliphages and bacteriophages infecting strains RYC2056 of *Bacteroides fragilis* and GA17 of *Bacteroides tethaiotaomicron* resist various treatments more robustly than fecal coliform bacteria do. The data also show that they are eliminated at similar rates. The ratios of somatic coliphages and bacteriophages infecting *Bacteroides tethaiotaomicron* GA17 in a set of samples of effluents from very different tertiary treatment facilities are still of the same order of magnitude as those found in sewage. The possible exceptions to this are oxidation ponds and wetlands, which are both long-

term processes and susceptible to new contamination by feral animals, mostly birds. In this case spurious results are sometimes found (Payán, 2006).

The differences in persistence in water environments and resistance to water treatments shown by fecal coliform bacteria and phages infecting strain GA17 of *Bacteroides tethaiotaomicron* reduces the suitability of this pair of parameters for fecal source tracking despite performing excellently in recently contaminated waters (Blanch *et al.*, 2006). The genotypes of F-specific RNA bacteriophages also show

differences in persistence and resistance, with genotype I being more persistent and resistant than genotype II, which in turn is more persistent and resistant than genotypes III and IV (Cole *et al.*, 2003; Schaper *et al.*, 2002). This leads to a predominance of genotypes I and II in rivers and seawaters independent of the origin (Cole *et al.*, 2003; Mocé *et al.*, 2005). This is a serious limitation, especially in open waters where pollution may not be from recent events.

Thus, in some cases (for example, anaerobic bacteria), low levels of persistence and of resistance, and in other cases (such as the pair coliform bacteria-bacteriophages infecting strain GA17 or the proportions of genotypes of F-specific RNA bacteriophages), mismatched persistence and resistance, means that some of the tracers of fecal sources being considered are unsuitable. Limitations on the use of fecal sterols are also associated with their low levels of persistence (Szucs *et al.*, 2006).

Suitable *Bacteroides* hosts for different geographical areas

Different strains of *Bacteroides fragilis* recover different numbers of phages from sewage or sewage polluted waters. They also differ in their capability to discriminate human from animal fecal contamination. Thus whereas strain HSP40 is better at detecting phages in human fecal wastes (Tartera *et al.*, 1989), strain RYC2056 detects phages both in human and non-human fecal wastes (Puig *et al.*, 1999; Blanch *et al.*, 2006). Also, whereas RYC2056 detects similar numbers of phages in sewage around the globe (Lucena *et al.*, 2003; Puig *et al.*, 1999), strain HSP40 shows geographic

differences, detecting useful concentrations, from 10^3 to 10^4 PFU per 100 ml, in the Mediterranean area (Armon, 1993; Tartera *et al.*, 1989) and South Africa (Grabow *et al.*, 1993) but failing to detect such significant concentrations in Northern Europe (Puig *et al.*, 1999) and the U.S. (Chung *et al.*, 1998).

Similar behaviour seems likely in other species of *Bacteroides*. *Bacteroides thetaiotaomicron* GA17 also exhibits geographic variations (Blanch *et al.*, 2006; Payán *et al.*, 2005). In fact, the most significant differences were related to the characteristics of the plaques. These were mostly clear with only a small percentage of turbid ones in Spain, France, and Cyprus, but in the United Kingdom most of the plaques were very turbid. These turbid plaques could make data collection more difficult. In Sweden, an intermediate situation was observed. However, in spite of small differences in concentrations, the pair somatic coliphages-bacteriophages infecting strain GA17 fit the predictive model described above both in the United Kingdom and Sweden. A feasible method was designed and used to isolate strain GA17 from raw municipal sewage. In order to assess whether the isolation of strain GA17 was a fortuitous event or a generally applicable method, it was decided to apply the method again in North Eastern Spain, the area where GA17 was isolated, and in Great Britain (Payán *et al.*, 2005). Moreover, the screening and isolation technique has low development costs compared to library-dependent source tracking techniques (Malakoff, 2002). The strain GB-124 isolated from sewage in Southern Great Britain, which is closely analogous to *Bacteroides ovatus* (Payán *et al.*, 2005) has been applied to a field study in the Southeast of England. Results suggest that

the host strain GB-124 is specific to human feces and is therefore a potential indicator of anthropogenic sources of fecal contamination in river catchments (Ebdon *et al.*, 2007). The GA17 strain covers a wide geographical area that extends to the countries of the Northern Mediterranean shore, which is also covered by HSP40. No data are available for Southern Mediterranean coastal areas. The exact area that might be covered by strain GB-124, suitable for Great Britain, requires further investigation.

Other authors have also indicated geographical differences for other procedures, for example, antibiotic resistance analysis and ribotyping of *E. coli* (Whitlock *et al.*, 2002; Parveen *et al.*, 2006). The extension of the geographical areas covered by different procedures as well as the cost of adapting them to different areas needs to be further investigated and evaluated.

Suitable *Bacteroides* hosts for tracking non-human hosts

The next question is whether it will be possible to find *Bacteroides* hosts that can differentiate non-human fecal sources with a reasonable effort, for example, isolating potential *Bacteroides* hosts from matrixes contaminated with feces of a given animal species, as was done to isolate strains GA17 and GB-124. Some progress has been made applying the previously described isolation technique and using wastewater from a pig slaughterhouse. Two strains of *Bacteroides*, PG76 and PG1226, which are closely analogous to *Bacteroides fragilis*, have been isolated. They might well fulfil the requirements of a tracer of porcine fecal contamination (Payán, 2006). In preliminary studies performed in north-

eastern Spain, more than 10^5 per 100 ml were detected in porcine abattoir wastewaters and significantly lower numbers in municipal sewage and wastewater from abattoirs slaughtering either cattle or poultry (Payán, 2006). These results should be interpreted cautiously but are very encouraging.

Molecular methods for the quantitative detection of specific phages in predictive models

Detecting and enumerating bacteriophages by culture methods have the advantages of being simple and economical. However, in the future molecular methods based on genomic differentiation will emerge (Soule *et al.* (2006; Reischer *et al.* (2007). For instance, the development of microarrays containing DNA fragments with specific sequences of the bacteriophages detected by bacterial hosts that discriminate between animal hosts is one possibility. These sequences could be sufficient to develop reliable DNA microarrays or could be combined with other discriminating sequences to improve source tracking. Once the bacteriophages detected by various discriminating host bacteria are known, it should not be difficult to make genomic characterizations, to detect these different phages, and to design either specific probes or primers. In fact, it was possible to design genomic methods to specifically detect the majority of phages infecting strain HSP40 of *Bacteroides fragilis*, which, as mentioned earlier, is a discriminating host (Puig *et al.*, 2000). Genomic characterization studies of these bacteriophages are needed to make progress with DNA-based quantification of these tracers.

Conclusions

The developments described in this paper, tracers and methods, and the challenges for finding feasible predictive models are illustrated by the case of using bacteriophages to define the requirements for a suitable MST indicator. Minimal performance requirements for an indicator in the development of feasible predictive models are summarized in Table 4.

At present, there are several predictive models, for certain types of water that fulfil most of these requirements. Among these, the predictive model based on the enumeration of somatic coliphages (non-discriminating parameter) and their ratio to bacteriophages infecting *Bacteroides* host-specific strains has emerged as a feasible MST model when distinguishing human from non-human pollution sources in wastewater samples. The recent isolation of *Bacteroides* host-specific strains from polluted water from animal sources and the persistence in the environment and resistance to water treatments of bacteriophages infecting *Bacteroides* spp. are encouraging. Such results will help develop feasible predictive MST models by inductive learning methods for their future application to different types of water samples polluted by fecal contamination.

Table 4. Minimal Performance Requirements to be Fulfilled by MST Indicators for Their Use in Predictive Models

Requirement	Comments
More than one indicator	No single indicator can determine the origin of fecal sources in all cases. At least two parameters, one which discriminates sources and one which does not, are required to create predictive models capable of distinguishing fecal pollution sources.
Combination of indicators to resolve mixture of fecal sources	In predictive models, combining several discriminating indicators for different fecal sources could provide the relative contribution to the total fecal load from each source.
Prevalence in water	The concentrations (densities) of the selected indicators should be detectable by the respective method of measurement for any matrix of water analyzed.
Persistence and resistance	The persistence in the environment and the resistance to water treatments of the different indicators used in predictive models should be similar.
Suitable for numerical methods	Numerical analyses (inductive learning methods) other than traditional statistical methods are reliable tools for the selection of variables (indicators and their parameters) and the development of predictive models.
Universal feasibility	The parameters selected should be consistent with the development of MST predictive models and independent of geography, climate, or dietary habits.
Low cost and easy performance	The indicators and their parameters should be accessible without incurring large economic or logistic costs.

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