# Tracking the origin of faecal pollution in surface water: an ongoing project within the European Union research programme

Anicet R. Blanch, Luis Belanche-Muñoz, Xavier Bonjoch, James Ebdon, Christophe Gantzer, Francisco Lucena, Jakob Ottoson, Christos Kourtis, Aina Iversen, Inger Kühn, Laura Moce, Maite Muniesa, Janine Schwartzbrod, Sylvain Skraber, George Papageorgiou, Huw D. Taylor, Jessica Wallis and Joan Jofre

## ABSTRACT

The objectives of this study are to generate knowledge about methods to track the sources of faecal pollution in surface waters, with the aim of having one or a few easy procedures applicable to different geographic areas in Europe. For this, a first field study using already proposed methods (genotypes of F-specific RNA bacteriophages, bacteriophages infecting *Bacteroides fragilis*, phenotypes of faecal coliforms and enterococci, and sterols) has been done in five areas representing a wide array of conditions in Europe. The present faecal indicators (faecal coliforms, enterococci, sulfite reducing clostridia and somatic coliphages) have also been included in this first field study. At the same time some emerging methods have been settled or adapted to water samples and assayed in a limited number of samples. The results of this first field study indicate that no single parameter alone is able to discriminate the sources, human or non-human, of faecal pollution, but that a 'basket' of 4 or 5 parameters, which includes one of the present faecal indicators, will do so. In addition, numerical analysis of the data shows that this 'basket' will allow the successful building of predictive models. Both the statistical analyses and the studied predictive models indicate that genotype II of F-specific RNA bacteriophages, the coprostanol and the ratio coprostanol: coprostanol+epicoprostanol are, out of the studied parameters, those with a greater discriminating power. Either because unsuccessful adaptation of the methods to water samples or because the preliminary assays in water samples indicated low discriminating capability, only three (sorbitol-fermenting bifidobacteria, some species of bifidobacteria detected by PCR with specific primers and phages infecting *Bacteroides tethaiotaomicron*) of the newly assayed methods have been considered for a second field study, which is currently underway. Expectations are that these new tools will minimize the number of parameters in the 'basket', or at least minimize the difficulty in assaying them.

**Key words** | faecal pollution, microbial indicators, sources, tracking, water

**Anicet R. Blanch** (corresponding author)
**Xavier Bonjoch, Francisco Lucena**
**Laura Moce, Maite Muniesa**
**Joan Jofre**
Department of Microbiology,
University of Barcelona, Diagonal 645,
08028 Barcelona, Catalonia, Spain
Tel: +34 934 029012 Fax: +34 934 039047
E-mail: *ablanch@ub.edu*

**Christophe Gantzer, Janine Schwartzbrod**
**Sylvain Skraber**
Laboratoire de Virologie du Milieu Hydrique,
Faculté de Pharmacie, Université de Nancy I,
5 rue Albert Lebrun, 54000 Nancy, France

**Aina Iversen, Inger Kühn**
Microbiology and Tumor Biology Center,
Karolinska Institute, Box 280,
S-171 77, Stockholm, Sweden

**James Ebdon, Huw D. Taylor, Jessica Wallis**
Environment & Public Health Research Unit,
School of the Environment, University of Brighton,
Cockcroft Building, Lewes Road,
Brighton BN2 4GJ, UK

**Christos Kourtis, George Papageorgiou**
State General Laboratory, Microbiological Section,
Kimonos 44, 1451 Nicosia, Cyprus

**Jakob Ottoson**
Water and Environmental Microbiology, SMI,
Swedish Institute for Infectious Disease Control,
SE 171, 82 Solna, Sweden

**Luis Belanche-Muñoz**
Departamento de Lenguajes y Sistemas
 Informáticos,
Universitat Politècnica de Catalunya,
Jordi Girona 1–3, Mòdul C6 (Campus Nord),
08034 Barcelona, Spain

## INTRODUCTION

Faecal pollution caused by human and animal waste can degrade aquatic environments for uses such as drinking water supply, contact recreation, shellfish harvesting or shellfish culturing and irrigation. Management and mitigation of faecal pollution would be more cost-effective if actual sources (human and/or non-human) could be

identified, apportioned and perhaps corrected at the source. The advantages of controlling the pollution as near to the source as possible seem unquestionable. In addition, the control of water quality based on the hazard analysis and critical control points (HACCP) principles requires the capability of tracking the origins of faecal pollution.

Microbes and chemicals have been investigated as potential tools for faecal source identification (Scott *et al.* 2002; Simpson *et al.* 2002; Field *et al.* 2003). Unfortunately, the most commonly used faecal indicator micro-organisms such as faecal coliforms, *Escherichia coli*, enterococci and sulfite reducing clostridia are found in both human and animal faeces.

Investigations into the use of microbes for faecal source identification have tended to involve four basic approaches: (i) speciation, based on findings that particular species may be indicative of human or animal sources; (ii) assemblage and ratios between different micro-organisms; (iii) phenotyping of bacterial isolates and (iv) genotyping of microorganisms.

A number of microbiological approaches have been proposed. Some of them are methods based in bacteria: the ratio faecal coliforms: faecal streptococci (Geldreich 1976), the detection of some *Bifidobacterium* spp. (Lynch *et al.* 2002) or their phenotypes (Gavini *et al.* 1991), speciation or genotyping of *Bacteroides* (Kreader 1995; Bernhard & Field 2000), the detection of *Rhodococcus coprophilus* (Mara & Oragui 1981), the biochemical phenotyping of *E. coli* (Hagedorn *et al.* 2003) or *Enterococcus* spp. (Manero *et al.* 2002), the ribotyping of *E. coli* (Carson *et al.* 2001; Parveen *et al.* 1999), the study of the repetitive DNA sequences of *E. coli* (Dombek *et al.* 2000) and the discriminant analysis of antibiotic resistance patterns for faecal enterococci (Wiggins 1996), *E. coli* (Parveen *et al.* 1997) and faecal coliforms (Whitlock *et al.* 2002). Other methods are based on bacteriophages: phages infecting some strains of *Bacteroides fragilis* (Puig *et al.* 1999; Tartera *et al.* 1989) and some serotypes (Osawa 1981) or genotypes (Hsu *et al.* 1995; Beelwilder *et al.* 1996) of F-specific RNA bacteriophages have been suggested. Other viral targets such as human viruses may also be useful (Jagals *et al.* 1995; Pina *et al.* 1998) although better concentration and quantification procedures need to be developed. Addition-

ally, the detection of given species of protozoa, such as *Giardia* (Brown 1993) and *Cryptosporidium* (Awad-El-Kariem *et al.* 1995), has been investigated. Some of the proposed methods allow a direct determination on a given sample, whereas others need the isolation of a number of isolates per sample followed by a characterization of the isolates.

Studies on chemicals had mainly concentrated on the study of the distribution pattern of different potential markers: caffeine, fragrance substances, fluorescent whitening and faecal sterol isomers (Leeming *et al.* 1996; Kerfoot & Skinner 1981).

At present, no single microorganism or chemical method has been demonstrated on its own to reliably distinguish human from animal faecal pollution in water (Sinton *et al.* 1998; Simpson *et al.* 2002). Moreover, most available studies on the topic are only partial and data comparing the densities of the different proposed faecal pollution markers in the same samples are lacking. More information comparing the different analytical data in a wide variety of samples, considering spatial (geographical distribution) and perhaps temporal factors and based on multidisciplinary approaches is needed. In addition to the factors which render the microbial and chemical indicators specific to human or animal origin, it has to be considered that their utility is obviously strongly dependent on the same set of factors which determine their value as general faecal indicators (i.e. irrespective of source): ease of assay, densities in faeces and sewage, and rates of disappearance in receiving waters.

Taking into consideration all this background, three main objectives were considered for the present study.

First, to compare the values of some of the potential microbiological or chemical tracers of the faecal source (presence of phages of *B. fragilis*, distribution of genotypes of F-specific RNA bacteriophages, phenotype distribution of faecal coliforms and enterococci, and distribution of faecal sterols) with the values of faecal indicators (*E. coli*, enterococci, clostridia and somatic coliphages) in waters heavily polluted with various well identified faecal sources in different European countries. The density of faecal indicators in the samples is, in our opinion. indispensable in order to characterize the sample and compare the results of the samples studied. The different sampling areas

represent most of the possible geographical, climatic and perhaps feeding habit differences found in Europe. Second, to improve or adapt to water samples emerging techniques based on DNA sequence recognition (human virus, protozoa and *Bifidobacterium*) and in phenotyping methods (antibiotic resistance analysis for enterococci) and explore their potential utility for faecal source tracking. For this, besides improving or adapting the techniques, they were assayed in a fraction of the samples in parallel to the methods indicated in the previous paragraph. Third, to evaluate the suitability of a multivariate approach that includes microbiological and chemical parameters.

## METHODOLOGY

### Establishment of operating principles

In order to establish a set of operating principles for data quality, a training session for staff from all the participant laboratories was done. Non-certified reference materials (bacterial strains and bacteriophages) were prepared and used during the training session. These reference materials were provided to the partners at the end of the session for quality control of the implementation of the methods in the participating laboratories. Additionally, sampling, delivery conditions and pre-treatment of samples were also fixed.

### Samples and sampling

The value of methods to distinguish between faecal pollution of human and animal origin was also supported by analysing hospital or municipal sewages, predominantly of human origin, and abattoir effluents or animal wastes (slurries), predominantly of animal (cattle, sheep, swine and poultry) origin. At least 12 samples of human wastewater and 12 samples of animal wastewater were analysed by each participant. Sampling, delivery conditions and pre-treatment of samples were performed according to standardized ISO protocols (Anonymous 1980, 1982, 1983, 1985, 1992a).

### Determination of microbial indicators

Four faecal indicators were measured: faecal coliforms, faecal enterococci, spores of the sulphite-reducing anaerobes and somatic coliphages. Standardized methods (Anonymous 1984, 1986, 1992b, 2000) were used for the enumeration of these indicators.

### Enumeration of bacteriophages potentially useful for faecal source tracking

F-specific RNA bacteriophages and phages infecting *Bacteroides fragilis* were enumerated following the ISO standardized methods (Anonymous 1995, 2001). The distribution of serotypes of F-specific RNA bacteriophages was done by studying the distribution of genotypes that was previously shown to parallel the distribution of serotypes (Hsu *et al.* 1995; Beelwilder *et al.* 1996). The distribution of genotypes was done by plaque hybridization as described elsewhere (Schaper & Jofre 2000) using the probes previously described by Hsu *et al* (1995).

### Phenotyping of faecal coliforms and faecal streptococci

A biochemical phenotyping was performed for the population of coliforms and enterococci using the microplates PhP-RS and PhP-RF according to the manufacturer (PhP-Plate Microplates Techniques AB, Sweden) and described procedures (Kühn *et al.* 1997). The sampling size of bacterial strains for diversity measurement was statistically defined as previously described (Bianchi & Bianchi 1982). The phenotypic diversity and the similarity between the bacterial populations in different samples will be measured by using Simpson's index and the population similarity coefficient $Sp$, respectively (Kühn *et al.* 1991). Additionally, the distribution of species of *Enterococcus* was analysed using a modification of a previously described matrix (Manero & Blanch 1999). All isolates within each population were compared to this reference file, and similarities above 0.90 were displayed. Similarly, the percentage of *E. coli* in the faecal coliforms was determined by comparing isolates with *E. coli* phenotypes related to this species.

## Determination of faecal sterols

The procedure for the analysis of sterols in waters, and particularly in wastewater with high concentrations of solid fractions, was performed as described (Leeming *et al.* 1996). First, the separation of the solid fraction of the samples was done by filtration through glass filters. Aliquots of 100 ml from each sample were filtered. Then the membranes were weighed and frozen at $-70°C$ until analysis. Later, chromatographic analyses for four main faecal sterols were performed: coprostanol (5β-cholestan-3β-ol), stigmastanol or 24-ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol), epicoprostanol (5β-cholestan-3α-ol) and cholestanol (5-α-colestan-3β-ol).

## Integrated data analysis

Data from all the laboratories were harmonized and revised to consolidate a matrix of results. Descriptive statistics were calculated for each of the studied variables. Correlation between variables and principal component analysis was also performed, as well as three classical regression analysis techniques: best subsets regression, binary logistic regression and linear regression (Johnson & Wichem 1992). Additionally, linear and quadratic discriminant analyses were conducted (Duda *et al.* 2001). These steps were carried out in order to get a first step for the interaction between variables and their discriminatory power. To study the practical possibility of building a predictive model, three different state-of-the-art machine learning methods were tested: an artificial neural network (Hertz *et al.* 1991), a support vector machine (Christianini & Shawe-Taylor 2000) and a decision tree (Quinlan 1993).

## Development of new methods or improvement of existing methods

### Bifidobacterium

Some *Bifidobacterium* species have been proposed as specific indicators of human faecal pollution of environmental water (Jagals & Grabow 1996), since some *Bifidobacterium* species have been associated with human origin (Biavati *et al.* 1992; Scardovi & Crociani 1974). The aim

was to evaluate the potential utility of using the HBSA medium (Mara & Oragui 1983) for the determination of human or animal faecal pollution based on distinguishing the sorbitol-fermenting strains. Additionally, the presence of some species of *Bifidobacterium* in wastewaters using previously defined PCR species-specific primers (Matsuki *et al.* 1999) was analysed in order to ascertain their utility in differentiating human from non-human faecal pollution.

## Phenotyping of *Bifidobacterium*

The biochemical phenotyping (Kühn *et al.* 1997) Phene System (PhP-Plate Microplates Techniques AB, Sweden) was modified to work with anaerobic bacteria by using suitable growing media and cultivation conditions. Different biochemical tests (L-arabinose, xylose, galactose, maltose, cellobiose, trehalose, palatinose, sucrose, lactose, melbiose, melezitose, inosine, mannitol, arbutin, sorbitol, galactolacton, sorbose, rhamnose, tagatose, amygdalin, sodium gluconate and salicin) were evaluated for the phenotyping of species of *Bifidobacterium* based on previous biochemical and physiological studies (Biavati *et al.* 1992). The selected tests were used to define a new Phene System microplate for bifidobacteria. The feasibility of this microplate was evaluated with *Bifidobacterium* type strains.

## Genotyping *Giardia*

On the basis of the information available at present, *Giardia lamblia* was chosen as the parasite protozoon with more potential for distinguishing human from non-human pollution (Paintlia *et al.* 1999; Homan *et al.* 1999; Hopkins *et al.* 1999). On the basis of the available sequences in Genbank a quantitative PCR procedure for detecting *Giardia lamblia* was settled and the sensitivity and specificity of the method were tested on purified *Giardia* cysts suspensions.

To detect *G. lamblia* in sewage by this procedure, a previous concentration–purification step was performed as described elsewhere (Versey *et al.* 1993; Hashimoto *et al.* 2001).

## Human viruses

Considering the available information and taking into consideration abundance and potential discriminatory capability (Puig *et al.* 1994, Pina *et al.* 1998), only adenoviruses and enteroviruses were included in the study. The selected protocol was by direct amplification of viral nucleic acids of viruses present in sewage. For this, they were extracted using commercial kits (QIAamp DNA Blood Mini Kit and QIAamp RNA extraction Kit. QIAGEN, GmbH, Hilden, Germany) directly from sewage (0.2 ml) or after concentration by centrifugal ultrafiltration of 10 ml previously decontaminated sewage. One set of primers for adenoviruses described by Pina *et al.* (1998), and two sets of primers described by Gow *et al.* (1991) and Pina *et al.* (1998), were used for PCR and RT-PCR nucleic acid amplification. Assays were performed by only two of the partners. Urban and hospital sewage were used as the source of human faecal pollutants and wastewater from abattoirs was used as the source of animal faecal pollutants.

## Antibiotic resistance patterns

Antibiotic resistance profiles in faecal streptococci have been reported, with variable success, to determine sources of faecal pollution (Hagedorn *et al.* 1999; Wiggins 1996). The determination of a suitable set of antibiotics and concentrations for resistance profiles in enterococci was done by testing them using a previously described replica plating method (Hagedorn *et al.* 1999). Later, the antibiotic resistance patterns in enterococci were performed according to established protocols (Wiggins 1996). Water samples from different origins (domestic wastewater, hospital and abattoir) were analyzed to validate the selected antibiotics and concentrations, and later statistical discrimination and clustering analysis were done using the procedure DISCRIM and Ward's method, respectively (Wiggins 1996).

## Other *Bacteroides* host strains

Phages detected by strain HSP40 of *Bact. fragilis* were described to discriminate human from animal pollution, since they were mostly found in faecal wastes of human origin (Tartera *et al.* 1989; Grabow *et al.* 1995; Armon 1993). But later it has been reported that they are found in very low numbers in municipal sewage from certain areas of the globe (Puig *et al.* 1999). In contrast, phages detected by strain RYC2056 are detected in similar numbers everywhere, but their discriminating power is much lower compared to that of phages detected by HSP40 (Puig *et al.* 1999). Both strains have a clinical origin. Bradley *et al.* (1999) reported that some strain of *Bacteroides* isolated from polluted waters detected high numbers of phages, but we had never access to such strains. To explore the chances of finding suitable *Bacteroides* strains isolated from non-clinical samples, *Bacteroides* strains were isolated from sewage using *Bacteroides* bile esculin agar supplemented with 10 μg/ml of gentamycin. Their capability to recover bacteriophages from sewage was tested following the standard method (Anonymous 2001).

## RESULTS AND DISCUSSION

### Occurrence and values of the potential microbiological or chemical tracers and microbial faecal indicators

A total of 184 samples from different wastewater were analyzed. A summary of the results are shown on Table 1. Only the samples that were analysed by all the microbial and chemical parameters have been used for the multivariate analysis.

### Faecal indicators

The density of microorganisms usually used as faecal indicators (faecal coliforms, enterococci, spores of sulfite-reducing clostridia and somatic coliphages) are very similar to those described in the literature for wastewater receiving human (Grabow *et al.* 1993; Armon 1993; Chung *et al.* 1998; Lucena *et al.* 2003) or animal faecal wastes (Havelaar *et al.* 1990; Tartera *et al.* 1989; Hill & Sobsey 1998) in various areas of the globe. No differences were observed between the wastewater samples from the different geographical areas studied.

**Table 1** │ Data from municipal or hospital sewage (human) and slaughterhouse wastewater (animal). Results are the average of log CFU/100 ml or log PFU/100 ml for faecal coliforms (FC), enterococci (FE), clostridia (CL), somatic coliphages (SOMCPH), total F bacteriophages (FTOTAL), F-RNA bacteriophages (FRNAPH), *Bact. fragilis* bacteriophages (RYC2056). Faecal sterols are in mg/g: coprostanol (COP), stigmastanol or 24-ethylcoprostanol (ETHYLCOP), epicoprostanol (EPICOP) and cholestanol (CHOL). The percentage of the rest of the values is also the average of samples: diversity of enterococci (DiE), *Ent. faecium* plus *Ent. faecalis* (FM-FS), *Ent. hirae* (Hir), *E. coli* Phene System patterns (ECP), *E. coli* confirmed (ECT). *N*: number of samples

| | Human | | | Animals | | |
|---|---|---|---|---|---|---|
| | Average | SD | *N* | Average | SD | *N* |
| FC | 7.05 | 0.58 | 89 | 7.60 | 0.94 | 95 |
| FE | 6.00 | 0.81 | 89 | 6.52 | 0.83 | 95 |
| CL | 5.20 | 0.69 | 89 | 5.29 | 1.26 | 95 |
| SOMCPH | 6.07 | 0.93 | 89 | 6.77 | 1.10 | 94 |
| FTOTAL | 5.48 | 0.76 | 87 | 5.43 | 1.52 | 85 |
| FRNAPH | 5.23 | 1.01 | 87 | 5.31 | 1.58 | 81 |
| Percentage of genotypes of F-RNA phages | | | | | | |
| I | 7 | – | 83 | 34 | – | 71 |
| II | 40 | – | 83 | 12 | – | 71 |
| III | 48 | – | 83 | 28 | – | 71 |
| IV | 5 | – | 83 | 26 | – | 71 |
| RYC205 6 | 3.82 | 1.01 | 85 | 3.76 | 1.23 | 85 |
| COP | 1042.73 | 3436.66 | 70 | 252.92 | 1147.86 | 69 |
| ETHYLC OP | 657.96 | 3151.15 | 70 | 2840.69 | 10210.25 | 69 |
| EPICOP | 643.79 | 3316.43 | 70 | 2033.00 | 10347.60 | 69 |
| CHOL | 1361.60 | 6351.94 | 70 | 441.26 | 1351.80 | 69 |
| DiE | 0.85 | 0.19 | 84 | 0.76 | 0.25 | 95 |
| FM-FS | 66.10 | 20.69 | 84 | 49.03 | 30.63 | 94 |
| Hir | 17.15 | 18.48 | 84 | 28.69 | 31.12 | 94 |
| DiC | 0.89 | 0.16 | 88 | 0.88 | 0.09 | 95 |
| ECP | 79.10 | 22.65 | 77 | 97.11 | 5.37 | 84 |
| ECT | 72.13 | 24.29 | 77 | 93.30 | 9.10 | 84 |

## F-specific RNA bacteriophages

F-specific RNA bacteriophages were detected in 100% of municipal and hospital sewage samples, whereas they had <50 PFU/100 ml in 30% of the abattoir samples. In all the positive samples the values ranged from $10^5$ to $10^6$ PFU/100 ml. Regarding genotype distribution, all the samples with human origin complied with the assumption that genotypes II and III are predominant. However, 27% of the samples with an animal origin failed to fulfil the assumption that genotypes I and IV predominate in animal faeces. The results indicate that there are not geographic differences. Results are in agreement with results reported previously in studies made in more limited geographical areas (Schaper *et al.* 2002).

## *Bacteroides fragilis* bacteriophages

Phages detected by strain RYC2056 of *Bact. fragilis* were found in all samples of sewage with human origin, with an average value of 3.9 $\log_{10}$ units per 100 ml. In wastewater samples of animal origin, they were below 50 PFU/100 ml in 17% of the samples and the average value was around 3.7 $\log_{10}$ units. The ratios between the numbers of any of the faecal indicators and the numbers of phages infecting strain RYC2056 were ten times higher in samples with a human origin than in samples with non-human origin. However, these differences are not enough to track the origin of the sample as the multivariate analysis indicates (see below).

## Phenotyping of faecal coliforms and enterococci

The biochemical phenotyping has been performed for the faecal population of coliforms and enterococci. Over 5000 faecal coliforms (FC) and 5000 enterococci (FE) strains were phenotyped. Statistical analyses of various parameters have been performed. It was found that high diversities were generally associated with urban sewage and slaughterhouses, both for coliforms and enterococci. Lower diversities were obtained from hospital sewage, and the lowest from farm animal waste. This pattern was similar for all countries studied (data not shown).

The faecal coliforms were identified as *E. coli* or non *E. coli* using a special control test included in the PhP-RE plate. The samples of urban sewage also showed a lower incidence of *E. coli* among the coliforms.

The enterococci were identified to species level by comparing them to a reference database as described above. The PhPWin software was modified to allow for these calculations in a simple and automated way. Also the species composition showed differences: among samples of human sources, *Ent. faecalis* and *Ent. faecium* dominated, followed by *Ent. hirae*. This is in agreement with other previous studies (Sinton & Donnison 1994; Manero *et al.* 2002; Blanch *et al.* 2003).

Population similarities based on biochemical fingerprinting and obtained from comparisons of related samples showed generally higher values than those from non-related samples. The analysis of phenotypic diversity and phenotype composition provides some complementary information for discriminating between samples of human and animal origin.

## Sterols

It was observed that sterols are associated with the solid fraction and no detectable levels were found in the liquid fraction of samples. The association of sterols with the solid fraction allows simplification of the extraction procedure after the separation of this fraction by filtration on the membranes and storing it frozen. Therefore the results refer to sterols associated with the solid fraction.

One-hundred and thirty-nine samples have been completely analyzed and 4 sterols have been determined for each sample: coprostanol, stigmastanol or 24-ethylcoprostanol, epicoprostanol and cholestanol. Usually, higher concentrations of coprostanol were found in human sewage samples than in any animal wastewater. However, stigmastanol and epicoprostanol were usually higher in animals (cows, pigs and poultry) than in human wastewaters. These results are in agreement with a similar analysis performed in other world regions (Leeming *et al.* 1996; Gilpin *et al.* 2002). However, none of the studied faecal sterols is exclusively related to humans or a specific animal species. Therefore, the exclusive use of these chemical indicators is not enough for ascertaining the origin of faecal pollution.

## Numerical analyses

A set of 121 samples, which come from a unique source of faecal pollution (animal or human) and with results available for all the tested parameters, were selected to perform a preliminary statistical analysis in order to discern the most determinant parameters as well as the possibility of building a predictive model.

All the numerical tests performed indicate that none of the parameters reported above is able alone to discriminate between faecal pollution of human and non-human origin in all situations. However, they also indicate that an appropriate 'basket' of as few as 4 or 5 microbial and chemical indicators will offer the possibility of identifying human and animal faecal inputs to surface waters with a high level of success.

In addition, the analyses of the results indicate that building predictive models (artificial neural networks, support vector machine and decision tree) will also be possible with a limited number of parameters.

Both the statistical analysis and the predictive models indicate that genotype II F-specific RNA phages, coprostanol and the ratio coprostanol: coprostanol + epicoprostanol are those parameters with the greater discriminant power. Results also indicate clearly that the introduction of at least one of the present faecal indicators, preferably sulfite reducing clostridia or somatic coliphages, is necessary for the 'basket' of parameters.

## New tools

### Bifidobacterium

It was confirmed that sorbitol-fermenting bifidobacteria showed yellow colonies on HBSA as already described (Mara & Oragui 1983) and that the ratio sorbitol-fermenting bifidobacteria: total bifidobacteria was significantly higher in samples of human origin than in samples of animal origin. Results also support recently suggested approaches that propose the detection of *Bif. adolescentis* (Lynch *et al.* 2002) and *Bif. dentium* (Nebra *et al.* 2003) by PCR as a tool for detecting human faecal pollution.

### Phenotyping of bifidobacteria

An improved medium for growing anaerobic bacteria in the PhenePlate system was developed and a specific bifidobacteria microplate developed. However, the bifidobacteria microplate needs to be improved for reproducibility before testing for feasibility on biotyping strains of this bacterial group isolated from water samples.

### Protozoa

The settled method of real time PCR allowed us to detect about 6–60 cysts of *G. lamblia* per reaction. However, its application to wastewater failed to give a positive reaction in 100% of hospital sewage samples. Further investigation of the potential application of PCR to detect sequences of *G. lamblia* for faecal source tracking requires more information on the numbers of humans needed to guarantee the presence in a sample and also improvements in the sensitivity of the method.

### Human viruses

Adenoviruses were detected only in samples with human origin. The percentage of positive samples detected in municipal sewage were similar to those reported previously (Pina *et al.* 1998), but most hospital sewage samples failed to give positive results. This may be due to the fact that the number of contributors to hospital sewage is smaller than the number of contributors to municipal sewage. In contrast, enteroviruses were found in both kinds of samples, with 31% of samples with a positive for animal origin. This discards enteroviruses, at least those detected with the primers used herein, as potential tools to track the origin of faecal pollution. In contrasst, the methods based on adenoviruses look promising, but require more information on the numbers of humans needed to guarantee the presence in a sample, and an improvement of the method.

### Antibiotic resistance patterns

Limitations in the existing agar technique for antibiotic resistant profiles in enterococci were overcome. Twenty

one antibiotics at up to four concentrations each were chosen to represent classes of both broad-spectrum and narrow-spectrum agents used in either human and/or animal populations. These were (in µg/ml): amoxycillin (4, 8, 16, 32), ampicillin (4, 16, 32, 64), apramycin (4, 8, 16, 32), cephalothin (8, 16, 32, 64), clindamycin (1, 4, 8, 16), erythromycin (8, 16, 32, 64), gentamicin (4, 8, 16, 128), kanamycin (4, 8, 16, 32), neomycin (8, 16, 32, 128), nitrofurantoin (4, 16, 32, 64), nitrofurazone (8, 16, 64, 128), ofloxacin (2, 8, 16, 32), oxytetracycline (16, 32, 64, 128), penicillin (2, 4, 8, 16), rifampicin (2, 4, 8, 32), streptomycin (8, 16, 32, 64), sulfathiazole (16, 32, 64, 128), tetracycline (2, 8, 32, 64), tylosin (2, 4, 16, 32), vancomycin (4, 8, 16, 32) and virginiamycin (2). The average rate of correct classification (ARCC) carried out by cross-validation was 72% for the studied samples which all came from the same geographical origin (UK samples). The ARCC in enterococci alone are not sufficient for a reliable differentiation of the origin of human and animal samples, though preliminary observations showed they could provide some information for discriminating faecal pollution between certain animal species in a local dimension.

### Other *Bacteroides* host strains

A strain, GA17, classified as *Bacteroides tethaiotao-micron*, was identified to detect values ranging from $5 \times 10^4$ PFUs to $5 \times 10^5$ PFUs per 100 ml in the great majority of samples of municipal sewage at one of the sites. At the moment the enumeration of phages infecting this strain can be done using the standardized method available for detecting phages of *Bacteroides fragilis*. A first survey made in Spain shows that they may be useful to track the faecal sources, since their numbers in animal faecal sources is very low compared to the other faecal microorganisms. To exemplify this, the ratio somatic coliphages/phages infecting strain GA17 in human sources averages $10^2$, whereas this ratio in animal sources is, on average, greater than $10^5$. These ratios are maintained in samples from hospital wastewater and from animal origin in France. The results regarding the densities of phages infecting *Bacteroides tethaiotaomicron* GA17 in

various kinds of water contaminated with faecal wastes of human and animal origin are very promising.

In summary, among the tested new potential tools reported above, only the enumeration of bacteriophages of *Bacteroides tethaiotaomicron*, the enumeration of sorbitol-fermenting bifidobacteria and the specific molecular detection of human *Bifidobacterium* spp. have been considered for the second phase of this study. Indeed, a second field study using all the variables already used during the first field study plus those indicated above is underway at this moment. Then, a complete set of numerical analyses will be performed.

## CONCLUSIONS

Information available so far seems to indicate that it is unlikely that any single parameter will be useful in all situations, but data presented here indicate that statistical analysis of appropriate 'baskets' of a few microbial and chemical indicators will offer the possibility of identifying and apportioning human and animal faecal inputs to surface waters. Data from investigations during the course of the framework of this project will hopefully provide enough information to define a feasible method based either on a single microbiological or chemical indicator, or on a ratio between parameters or on an appropriate 'basket' of microbiological and chemical indicators that will allow identifying and apportioning human and animal faecal inputs to waters.

The capability to determine accurately faecal sources would assist in the management of microbial water quality and in the surveillance and warning of pollution sources. Preventive or corrective action could then be taken in order to control the faecal source or at least to lower the faecal pollution levels.

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