Assessment of drinking water quality using indicator bacteria and bacteriophages

Javier Méndez, Ana Audicana, Mercedes Cancer, Anna Isern, Julian Llaneza, Belén Moreno, Mercedes Navarro, Mª Lluisa Tarancón, Fernando Valero, Ferran Ribas, Juan Jofre and Francisco Lucena

ABSTRACT

Bacterial indicators and bacteriophages suggested as potential indicators of water quality were determined by public laboratories in water from springs, household water wells, and rural and metropolitan water supplies in north-eastern Spain. Indicator bacteria were detected more frequently than bacteriophages in springs, household water wells and rural water supplies. In contrast, positive bacteriophage detections were more numerous than those of bacteria in metropolitan water supplies. Most of the metropolitan water supply samples containing indicators had concentrations of chlorine below 0.1 mg l^{-1}, their indicator loads resembling more closely those of rural water supplies than any other samples taken from metropolitan water supplies. The number of samples from metropolitan water supplies containing more than 0.1 mg l^{-1} of chlorine that contained phages clearly outnumbered those containing indicator bacteria. Some association was observed between rainfall and the presence of indicators. Sediments from service reservoirs and water from dead ends in the distribution network of one of the metropolitan water supplies were also tested. Bacterial indicators and phages were detected in a higher percentage than in samples of tap water from the same network. Additionally, indicator bacteria were detected more frequently than bacteriophages in sediments of service reservoirs and water from dead end samples. We conclude that naturally occurring indicator bacteria and bacteriophages respond differently to chlorination and behave differently in drinking water distribution networks. Moreover, this study has shown that testing for the three groups of phages in routine laboratories is easy to implement and feasible without the requirement for additional material resources for the laboratories.

Key words | bacteria, bacteriophages, chlorine, drinking water, indicators

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INTRODUCTION

Water, it is thought, will be the single most important factor in terms of supporting human health and environmental sustainability in the 21st century. Among many other factors related to human health, water is implicated in the transmission of infectious diseases. Globally, approximately 5,000 deaths in the world are caused daily

by infectious waterborne microorganisms (Catley-Carlson 1993; Prüss & Havelaar 2001). The microbiological safety of drinking water is assessed using surrogate indicator microorganisms. However, it has been suggested that over 30% of cases of waterborne gastroenteritis have their origin in drinking water that fulfils the legislated quality requirements based on bacterial indicators. In this situation the disease is caused by viruses and protozoa (Craun 1996; Payment & Hunter 2001; Anderson & Bohan 2001). Since bacterial indicators do not give information relating to viruses and protozoa, and the methodologies applied to the direct detection of enteric viruses and protozoa are difficult and expensive, other indicators for these microorganisms are necessary.

To evaluate the virological quality of water, the use of bacteriophages as indicators has been proposed. Three groups of phages have been suggested: somatic coliphages, F-specific RNA bacteriophages and *Bacteroides fragilis* bacteriophages (IAWPRC 1991; Grabow 2001; Jofre 2002). Nowadays standardised methods are available for the detection and enumeration of each of these groups (ISO 1995, 2000, 2001; EPA 2000). Data obtained with the standardised methods on their occurrence and densities in different types of water, including drinking water, are needed to evaluate the usefulness of phages for assessing water quality.

Spain has a population of approximately 40,000,000 and a Mediterranean climate resulting in water scarcity and rainfall distributed very irregularly through the year, with sudden, heavy rainstorms mainly at the end of the dry season. According to data from the Spanish Ministry of Health the number of recorded waterborne outbreaks approaches 100 each year. For example, in 1998, there were 86 outbreaks that affected approximately 3,000 individuals (Binefa & Hernandez 2002). Outbreaks were distributed as follows: 23% bacterial, 17% viral, 2% parasitic and 57% of unknown origin. It may well be that infections caused by noroviruses account for 90 to 96% of outbreaks without identified causal agent (Binefa & Hernandez 2002).

Water from springs and household water wells accounted for 32.9% of the outbreaks, a high proportion compared with the proportion of water consumed from these sources; 65.9% of the outbreaks corresponded to water from water supply networks and 1.2% to bottled water. Rural water supplies with deficient treatment or no treatment accounted for the 47% of outbreaks attributed to water networks or rural areas, while 3% were linked to heavy rain episodes and the remainder to accidental deficiencies. The number of yearly outbreaks and cases per inhabitant is relatively high compared with other European countries (Anderson & Bohan 2001; Stanwell-Smith *et al*. 2003) and the United States (Craun 1996). In Spain, the majority of cities are served by water utilities that supply water that has received complex treatments and that follow harmonised monitoring strategies, whereas small rural communities rely upon water supplies that provide mostly groundwater; chlorination, if applied, is the sole treatment. On the other hand the regional public health authorities and the metropolitan water supply companies have accredited microbiology laboratories that perform numerous routine surveys of water quality.

This study was undertaken to assess the feasibility of methods based on bacteriophages in routine laboratories and at the same time to obtain information regarding the presence and densities of bacteriophages and bacterial indicators in drinking water in urban and metropolitan areas of north-eastern Spain. The study included springs, household shallow water wells, tap water from rural areas and tap water from metropolitan water supplies that have received complex multi-step treatments. Sediments that accumulate in service reservoirs and water sampled in dead ends of one of the distribution networks studied were also tested.

MATERIALS AND METHODS

Samples

The different laboratories participating in the study tested the water samples that they receive for routine analysis. Only the sediments of the service reservoirs and the water from dead ends were sampled on a non-routine basis. The bacterial indicators were tested according to the standardised methods being applied in each laboratory. The choice

of phages tested by each laboratory was determined by their financial and manpower capabilities.

Water from springs and household shallow water wells

One hundred and twenty-six samples were collected from springs and household shallow water wells that correspond to unprotected aquifers. Most of the fountains studied spring up through a man-made spout and historically were used for the villagers to obtain drinking water. These fountains are found mostly in upland areas, but the increasing urbanisation of the surrounding land has impaired the quality of the water that springs up in these fountains. Sometimes, but not always, it is announced that the water is not drinkable.

Water from rural drinking water supplies

One hundred and twenty-nine tap water samples were collected in rural communities that rely upon water supplies obtained mostly from groundwater sources. The physical nature of the water sources, together with the method of supply, the chlorination applied and the potential risk of contamination is expected to vary widely. Only 15% of the analysed samples contained free chlorine with concentrations ranging from 0.05 to 1.1 mg l^{-1}. Theoretically they should be chlorinated, but in practice it was not possible to determine the causes of the absence of free chlorine in the samples.

Immediately after sampling, water was dechlorinated by the addition of 1 ml of a 3% solution of sodium thiosulphate per litre before microbial analysis; 100 ml volumes of the samples were retained for chlorine determination.

Water from metropolitan drinking water supplies

Five hundred and twenty-two tap water samples were collected in five different cities served by complex multistep treatments including chlorination, with residual chlorine in the network. Of the analysed samples 4.5% did not contain free chlorine. The remainder had values of free chlorine ranging from 0.05 to 2.0 mg l^{-1}.

One of the water supplies studied, referred to as network A, was sampled for tap water (254 samples, which are included in the 522 samples of metropolitan tap water studied), water from dead ends (95 samples) and sediments from service reservoirs (124 samples). All of the water samples were collected in sterile containers, transported to the laboratory in a cold box, stored at 5 ± 3°C and processed within 24 h of sampling.

Water from dead ends of a metropolitan drinking water supply

Ninety-five water samples from dead ends of network A were collected and analysed as for the tap water samples.

Sediments from service reservoirs of a metropolitan drinking water supply

Sediments accumulating in the bottom of service reservoirs in network A were collected when the tanks were drained to remove the deposit. The physical characteristics of these samples varied. One hundred and twenty-four samples were analysed. After water removal, wet sediment remaining in the bottom of the reservoirs was collected in sterile containers. The samples were dechlorinated by the addition of 1 ml of a 3% solution of sodium thiosulphate. Each bacteriological parameter was analysed in 100 ml sediment volumes; 1,500 ml were processed to extract bacteriophages as indicated below.

Bacteriophage concentration

Bacteriophages in 1-litre water samples were concentrated by adsorption to mixed nitrate-acetate cellulose ester membrane filters (MF, Millipore, Bedford, Massachusetts) followed by elution according to the method first described by Sobsey *et al.* (1990). Briefly, $MgCl_2$ was added to the water sample to a final concentration of 0.05 M. The amended sample was then filtered through an acetate-nitrate cellulose ester membrane filter, 0.22 μm pore size and 47 mm diameter, at a rate of filtration of approximately 2 litres per h (33 ml per min). Thereafter, the membrane was cut into eight fragments and placed into a glass flask containing 5 ml of eluting solution (1% beef extract, 0.05 M NaCl and 3% Tween 80). The flask

was placed in an ultrasound-cleaning bath for 4 min. The eluted bacteriophages were counted as described below. The bacteriophages retained in the membrane fragments were counted by placing them face down on to a bacterial host monolayer.

Processing of sediments for microbiological analysis

One hundred ml of wet sediments were tested, without any extraction procedure, for the presence of each of the various bacterial indicators. In contrast, phages were extracted from sediments before phage determination. Here, sediments were centrifuged for 15 min at 3,000 rpm. The pellet was resuspended in 0.25 M glycine buffer, pH 10.5, at a ratio of 1:3 (w/v) as described previously for the extraction of phages and viruses from river and sea sediments and sludges (Araujo *et al*. 1997). After 10 min of magnetic stirring, volumes corresponding to 500 ml of sediment were tested for each one of the phage groups studied.

Chlorine determination

The free chlorine in the samples was determined by the DPD (N, N-diethyl-*p*-phenylenediamine) protocol according to Standard Methods (1998).

Bacterial determinations

Total coliforms (TC), faecal coliforms (FC), *E. coli* and enterococci (ENT) in water samples were detected according to the legislation (Anonymous 1990) ruling in Spain at the time that the study was performed and which was in agreement with the European Commission Directive (80/778/CEE). Spores of sulphite reducing clostridia (SRC) were enumerated according to the ISO standard procedure (ISO 1986). The laboratories participating in the study had been accredited for the use of these methods.

Some laboratories provided data on FC and others on *E. coli*. In the results section we have joined the data and taken the highest value included under the heading FC/*E. coli*. Values reported for bacteria in water samples always refer to 100 ml volumes.

For the sediments, the presence of each of the various bacterial indicators studied in 100 ml was tested as recommended by Standard Methods (1998) for the analysis of sediments.

Bacteriophage determinations

Bacteriophages were quantified by the double agar layer technique following the ISO 10705-2 standard (ISO 2000) for enumeration of somatic coliphages (SOMCPH), the ISO10705-1 (ISO 1995) for enumeration of F-specific RNA bacteriophages (FRNAPH) and the ISO 10705-4 (ISO 2001) for the enumeration of bacteriophages infecting *Bacteroides fragilis* (BFRPH). Data relating to the occurrence of bacteriophages in water samples refer to 1-litre volumes and data on densities refer to 100 ml.

For the sediment samples, presence/absence tests of volumes of eluate equivalent to 500 ml of wet sediment were performed as indicated in the corresponding standardised methods (ISO 1995, 2000, 2001).

Quality assurance

All bacteriological data were obtained by accredited laboratories that regularly monitor bacterial indicators. For the analysis of bacteriophages, each laboratory participated in a training session in which staff learnt to detect and concentrate bacteriophages by the methods used here. Subsequently, an inter-laboratory study was performed using reference phage suspensions, both reference bacteriophages and naturally occurring bacteriophages prepared as indicated in Méndez *et al*. (2002). Finally a first-line quality control using reference materials was performed during the monitoring. Thus, the data obtained in this study should be regarded as being of known and defensible quality.

Data computation and statistics

To perform the descriptive statistics and the Pearson's correlation test, the Statistical Package for Social Sciences

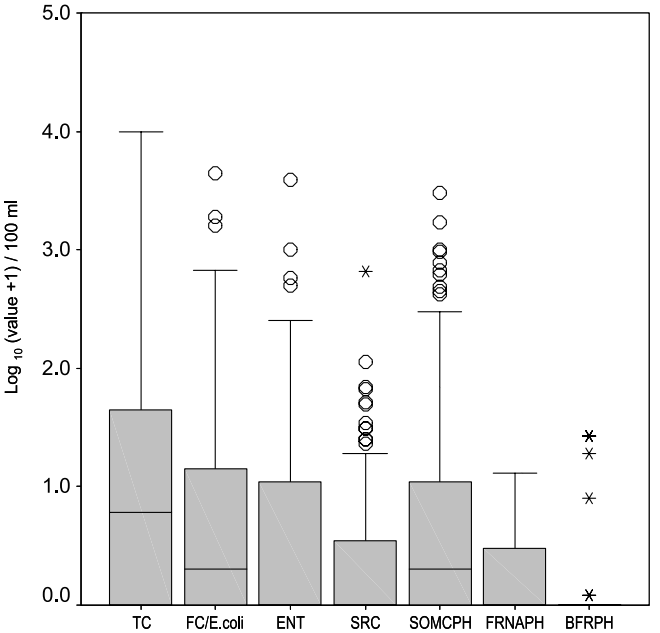


Figure 1 | Box and whisker plots of counts of the various indicators analysed in springs and household wells.

(SPSS 1999) was used. Some data were plotted as boxes and whiskers. This plotting provides summary statistics using five numbers: the minimum, the maximum, the median, the 25th and the 75th percentiles. The outlier (O) and extreme outlier (*) values are also plotted.

To determine the significance of the differences between the percentages of detection, the confidence limits for percentages test (Rohlf & Sokal 1969) was applied.

RESULTS

Microbiological status of springs and household shallow drinking water wells

A summary of the numbers of the different bacterial and bacteriophage indicators is shown in Figure 1. This dataset can be plotted in boxes and whiskers with a low number of outlier values and a very low number of extreme outlier values. The median values of total and faecal coliforms and somatic coliphages were greater than 1, whereas the median values of the remaining indicators were 0. The low number of samples tested for F-RNA specific bacteriophages does not allow statistical comparison of its numbers with those of the other parameters.

The densities of total coliforms, faecal coliforms/*E. coli*, enterococci, spores of sulphite reducing clostridia and somatic coliphages showed a significant correlation (Pearson's correlation test, $P<0.01$), whereas phages infecting *B. fragilis* did not correlate with any of the other indicators (Table 1).

In this dataset, the bacterial and viral indicators were detected in a considerable percentage of samples, ranging from 65.5% for total coliforms to 6.2% for phages infecting *B. fragilis* (Table 2).

Samples were commonly observed to contain several indicators (66.7%). As an example of the relationship between the presence or absence of more than one indicator, Table 3 shows the percentages of the different possible combinations of presence/absence of FC/*E. coli* and somatic coliphages. In this set of samples the combination + / + was the most frequent (40%), and the percentage of samples containing either only FC/*E.coli* or only somatic coliphages was similar.

Effects of rain

Data were available from a spring, coinciding with a rainy period that included an exceptional rain event of more than 200 mm in less than 24 h. Data plotted in Figure 2a and b shows the marked increases in density of indicator bacteria and phages after the heavy rain episode. The numbers of *E. coli*, enterococci and somatic coliphages increased by more than 2 $\log_{10}$ units, whereas those of spores of sulphite reducing clostridia and F-specific RNA phages increased by 1 $\log_{10}$ unit. Phages infecting *B. fragilis* were never detected in this spring.

Microbiological status of rural water supplies

A summary of the numbers of the different bacterial and phage indicators is shown in Figure 3. With the exception of F-specific RNA phages and phages infecting *B. fragilis*, data were plotted in boxes and whiskers, but with a notable number of outlier and also extreme outlier values.

Table 1 | Significant correlations (Pearson's correlations were significant at $P<0.01$) among the various indicators in the different drinking waters studied

Type of sample	TC	FC/*E. coli*	ENT	SRC	SOMCPH	FRNAPH	BFRPH
Springs and household wells ($n^a = 125$)	FC/*E. coli*	TC	TC	TC	TC	ND[b]	NC[c]
	ENT	ENT	FC/*E. coli*	FC/*E. coli*	FC/*E. coli*		
	SRC	SRC	SRC	ENT	ENT		
	SOMCPH	SOMCPH	SOMCPH	SOMCPH	SRC		
Rural water supplies ($n = 129$)	FC/*E. coli*	TC	C	TC	TC	ND	ND
	ENT	ENT	FC/*E. coli*	FC/*E. coli*	FC/*E. coli*		
	SRC	SRC	SRC	ENT	ENT*		
	SOMCPH	SOMCPH	SOMCPH*				
Metropolitan water supplies							
All samples ($n = 522$)	FC/*E. coli*	TC	TC	TC	TC	SRC*	NC
	ENT	ENT	FC/*E. coli*	FC/*E. coli*	FC/*E. coli*		
	SRC	SRC	SRC	ENT	ENT		
	SOMCPH	SOMCPH	SOMCPH	SOMCPH	SRC		
				FRNAPH*			
Samples containing <0.1 mg l^{-1} of chlorine ($n = 53$)	FC/*E. coli*	TC	TC	TC	TC	NC	NC
	ENT	ENT	FC/*E. coli*	FC/*E. coli*	FC/*E. coli*		
	SRC	SRC	SRC	ENT	ENT		
	SOMCPH	SOMCPH	SOMCPH				
Samples containing >0.1 mg l^{-1} of chlorine ($n = 426$)	NC	NC	NC2	NC	NC	NC	NC

[a] Number of samples tested.
[b]Not enough data available.
[c]No correlation.
*Significant only at $P<0.05$.

The median values of all the indicators were 0. The low number of samples tested for F-RNA specific bacteriophages and phages infecting *B. fragilis* does not allow statistical comparison of its numbers with those of the other parameters.

The densities of total coliforms, faecal coliforms/*E. coli*, enterococci, spores of sulphite reducing clostridia and somatic coliphages showed a significant correlation (Pearson's correlation test, $P<0.01$); however densities of enterococci and somatic coliphages showed a

Table 2 | Percentages of detection of the various indicators tested in the different drinking waters studied. Bacteria refer to 100 ml and phages to 1 l

Type of sample	TC	FC/*E. coli*	ENT	SRC	SOMCPH	FRNAPH	BFRPH
Springs and household wells ($n = 125$[a])	65.5	55.1	47.2	35.8	53.6	36.0[b]	6.2
Rural water supplies ($n = 129$)	38.6	28.1	35.7	37.5	34.1	0.0[c]	6.4[c]
Metropolitan water supplies All samples[d] ($n = 522$)	6.3	3.5	4.7	9.2	10.9	9.4	8.0
Samples containing < 0.1 mg l^{-1} of chlorine ($n = 53$)	28.6	24.0	22.0	47.5	26.5	20.0	18.5
Samples containing > 0.1 mg l^{-1} of chlorine ($n = 426$)	1.7	0.0	1.4	2.2	8.5	10.0	4.5

[a]Number of samples tested.
[b]Only 25 samples were tested for FRNAPH.
[c]Only 30 samples were tested for FRNAPH and BFRPH.
[d]Data on chlorine concentration of 43 samples was not available.

lower correlation (Pearson's correlation test, $P<0.05$) (Table 1).

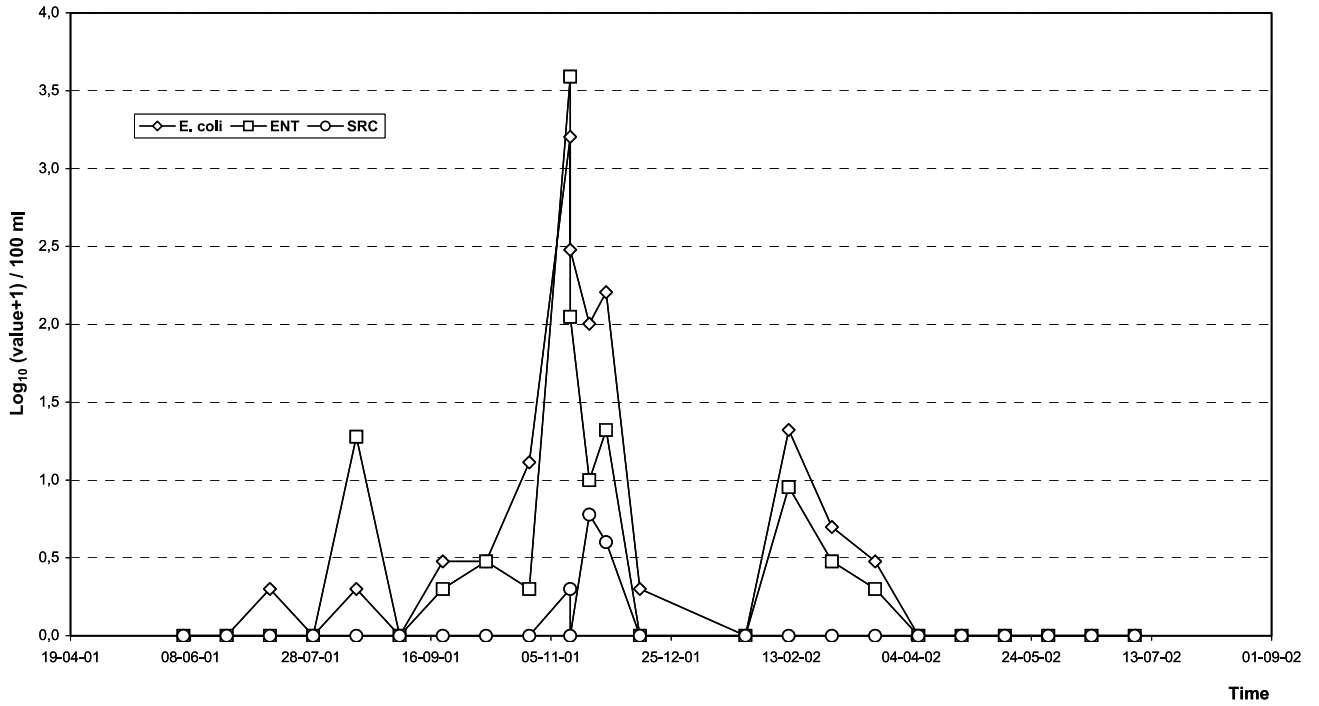
In this dataset, the various faecal indicators were detected in an important percentage of samples, ranging from 38.6% for total coliforms to 28.1% for FC/*E. coli* (Table 2). Samples containing two or more indicators were also frequent (43%), but less frequent than in the set of samples from springs and household wells. As an example of the relationship between presence or absence of more than one indicator, Table 3 shows the percentages of the different possible combinations of presence/absence of FC/*E. coli* and somatic coliphages. In this set of samples the combination $-/-$ was the most frequent (53.5%), and the percentage of samples containing somatic coliphages but not FC/*E. coli* was double the percentage of samples containing FC/*E. coli* but not somatic coliphages. Nevertheless, 9.3% of the samples contained both FC/*E. coli* and somatic coliphages.

Table 3 | Percentages of samples in which FC/*E. coli* and somatic coliphages were detected, in the different drinking waters studied

			Metropolitan water supplies		
	Springs and household wells	Rural water supplies	All samples	Samples with <0.1 mg l^{-1} of chlorine	Samples with 0.1 mg l^{-1} of chlorine
n[a]	(125)	(129)	(522)	(53)	(426)
FC/*E. coli* – ve[b]/SOMCPH – ve[b]	31.2	53.5	92.5	62.8	97
FC/*E. coli* – ve/SOMCPH + ve	13.6	18.4	4.1	9.8	3.0
FC/*E. coli* + ve/SOMCPH – ve	15.2	8.8	0.9	3.9	0.0
FC/*E. coli* + ve/SOMCPH + ve	40.0	19.3	2.5	23.5	0.0

[a]Number of samples tested.
[b]–ve=negative; +ve=positive.

(a)



(b)

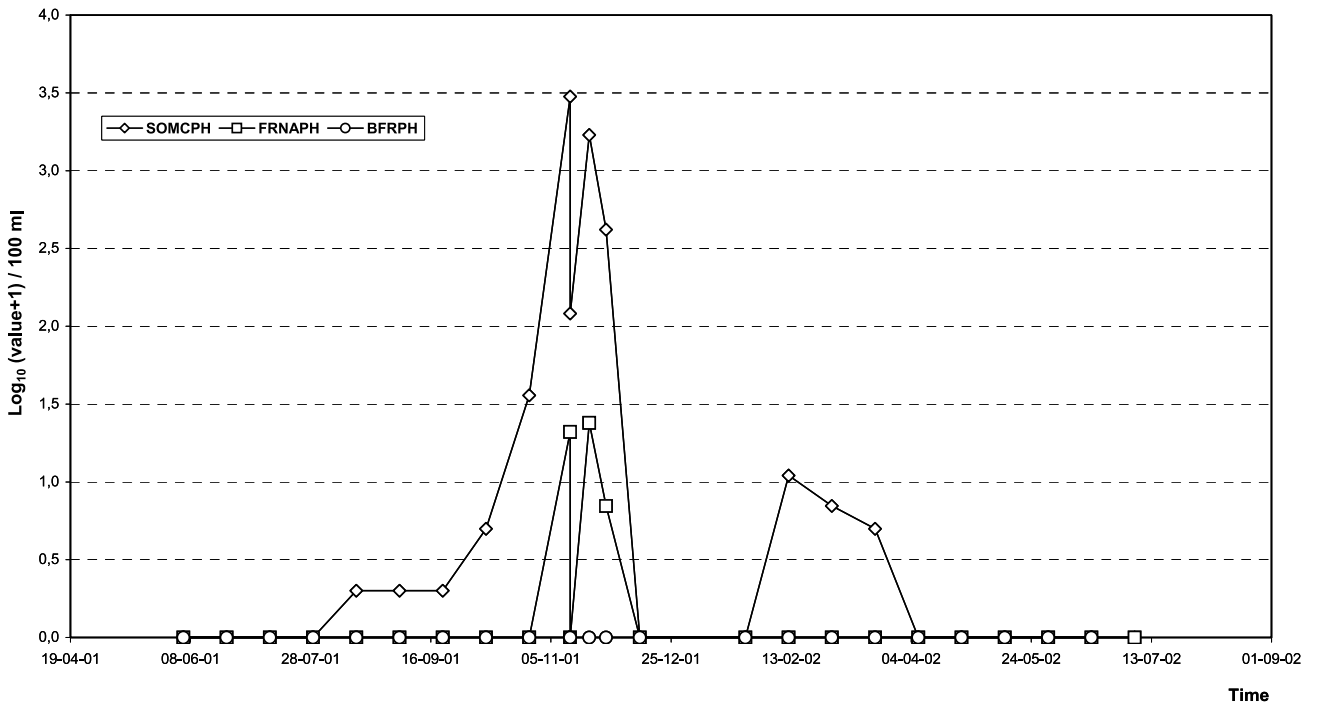


Figure 2 | Counts of bacterial indicators (a) and bacteriophages (b) in water samples from a spring during a heavy rain episode; the heavy rain episode was the week before the peak.

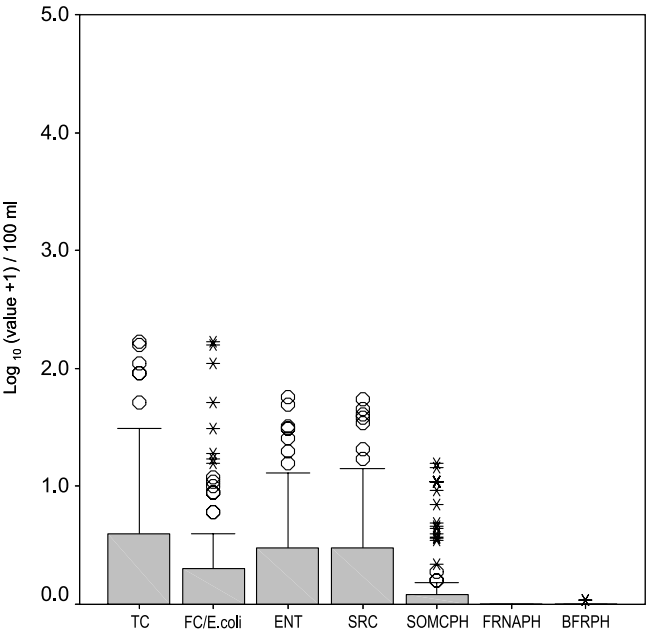


Figure 3 | Box and whisker plots of counts of the various indicators studied in rural water supplies.

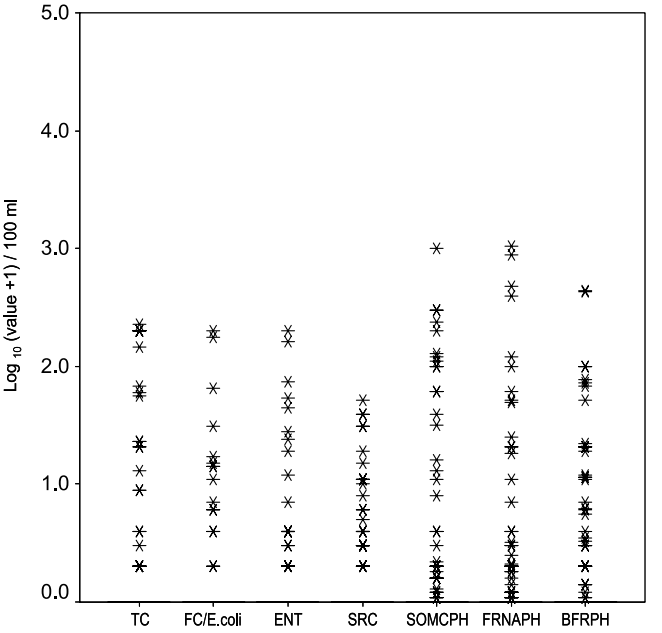


Figure 4 | Box and whisker plots of counts of the various indicators analysed in metropolitan water supplies.

Microbiological status of metropolitan water supplies

Water

A summary of the numbers of the different bacterial and phage indicators is shown in Figure 4. This set of data did not allow for plotting in boxes and all data are extreme outliers. The median values of all the indicators were 0.

The densities of total coliforms, faecal coliforms/*E. coli*, enterococci, spores of sulphite reducing clostridia and somatic coliphages correlated significantly (Pearson's correlation test, $P<0.01$). Densities of F-RNA specific bacteriophages showed some correlation with spores of sulphite reducing clostridia (Pearson's correlation test, $P<0.05$), whereas those of phages infecting *B. fragilis* did not correlate with those of any of the other indicators (Table 1).

The various faecal indicators were detected in less than 11% of the samples, ranging from 10.9% for somatic coliphages to 3.5% for FC/*E. coli* (Table 2). The percentages of detection of phages and spores of sulphite reducing clostridia were twice those for the detection of the classical bacterial indicators.

Samples containing several indicators were infrequent (6.1%). As an example of the relationship between the presence or absence of more than one indicator, Table 3 shows the percentages of the different possible combinations of presence/absence of FC/*E. coli* and somatic coliphages. In this set of samples the combination $-/-$ was the most frequent (92.5%), and the percentage of samples containing somatic coliphages but not FC/*E. coli* was four times the percentage of samples containing FC/*E. coli* but not somatic coliphages. Only 2.5% of the samples contained both FC/*E. coli* and somatic coliphages.

Role of chlorine

Data on chlorine concentration was available in 479 of the 522 samples of metropolitan water supplies tested. FC/*E. coli* were never detected in samples with more than 0.1 mg l^{-1} of chlorine (Table 1). The dataset of samples (426) with chlorine concentration higher than 0.1 mg l^{-1} and the dataset of samples (53) with chlorine concentration lower than 0.1 mg l^{-1} were therefore treated separately.

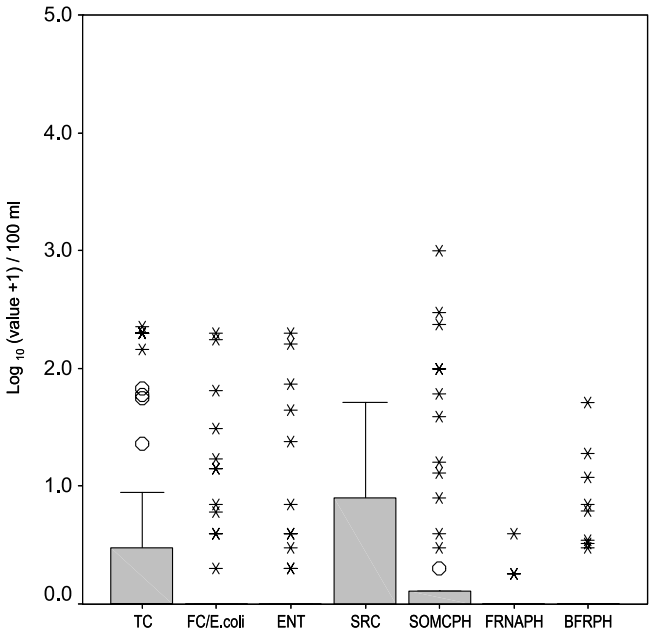


Figure 5 | Box and whisker plots of counts of the various indicators analysed in water samples from metropolitan water supplies containing less than 0.1 mg l^{-1} of chlorine.

The set of data corresponding to the samples with chlorine concentration lower than 0.1 mg l^{-1} allowed the data for some of the indicators to be plotted in boxes and whiskers, but with a significant number of outlier values and also extreme outliers (Figure 5), whereas the set of samples with a chorine concentration higher than 0.1 mg l^{-1}. did not allow the data to be plotted in boxes and all data were extreme outliers. The median values of all the indicators in both sets of samples were 0.

The densities of total coliforms, faecal coliforms/*E. coli*, enterococci and spores of sulphite reducing clostridia showed a significant correlation (Pearson's correlation test, $P<0.01$) and somatic coliphages correlated significantly with total coliforms, faecal coliforms/*E. coli* and enterococci (Pearson's correlation test, $P<0.01$), but failed to correlate with spores of sulphite reducing clostridia in the set of samples with less than 0.1 mg l^{-1} of chlorine (Table 1). Densities of the various indicators did not show any correlation in the set of samples with more than 0.1 mg l^{-1} of chlorine (Table 1).

The various faecal indicators were detected in an important percentage of the samples with chlorine concentration lower than 0.1 mg l^{-1}, ranging from 47.5% for spores of sulphite reducing clostridia to 18.5% for phages infecting *B. fragilis* (Table 2), whereas this percentage decreased significantly in the samples with a chlorine concentration higher than 0.1 mg l^{-1}. Indeed, the percentages of detection for all the bacterial indicators and phages in samples with chlorine concentration <0.1 mg l^{-1} and those with chlorine concentration >0.1 mg l^{-1} differed significantly (confidence interval limits of percentages test, $P<0.05$). The bacterial indicators, including spores of sulphite reducing clostridia, showed a greater decrease than phages in the percentage of detections in samples with free chlorine concentrations greater than 0.1 mg l^{-1} (Table 1).

Samples containing two or more indicators were still frequent (37%) among the samples with a chlorine concentration <0.1 mg l^{-1}, whereas they were 0 in samples with a chlorine concentration greater than 0.1 mg l^{-1}. As an example of the relationship between the presence or absence of more than one indicator, Table 3 shows the percentages of the different possible combinations of presence/absence of FC/*E. coli* and somatic coliphages. In the samples with chlorine concentration lower than 0.1 mg l^{-1}, the combination $-/-$ was the most frequent (62.8%) and the percentage of samples containing somatic coliphages but not FC/*E. coli* was almost three times that of samples containing FC/*E. coli* but not somatic coliphages. Nevertheless, 23.5% of the samples contained FC/*E. coli* and somatic coliphages. In the set of samples with chlorine concentration greater than 0.1 mg l^{-1}, the combination $-/-$ was 97% and the percentage of samples containing somatic coliphages but not FC/*E. coli* accounted for the remaining 3%.

Considering the indicator densities, the percentages of positive detections and the percentages of samples containing more than one indicator, the samples from the metropolitan water supply with chlorine concentration lower than 0.1 mg l^{-1} resemble more closely the set of samples from the rural water supplies than any of the other samples from the metropolitan water supplies.

Effect of rain

We were able to study the relationships between the detection of the various microorganisms in 254 samples

Table 4 | Percentages of detection of the various bacterial indicators and phages in different sample types in the metropolitan water supply network

Type of sample	TC	FC/*E. coli*	ENT	SRC	SOMCPH	FRNAPH	BFRPH
Tap water ($n = 254$)[a]	0.8	0.0	0.8	0.8	0.8	1.2	1.6
Water from dead ends ($n = 95$)	43.4	11.5	40.8	59.3	1.2	2.3	4.3
Sediments from reservoirs ($n = 129$)	37.9	2.4	ND[b]	50.0	6.5	4.1	7.3

[a]Number of samples tested.
[b]ND, not determined.

from water supply A and the occurrence of rain in a catchment area from which a substantial fraction of the surface water arises. The rainfall during the 7 days prior to sampling showed a significant correlation (Pearson's two-tailed test, $P<0.05$) with numbers of phages infecting *B. fragilis* in the sample. The association between rainfall and the presence of other phages or bacterial indicators was not significant. All samples from this group contained chlorine concentrations greater than 0.1 mg l^{-1}.

Microbiological status of water collected from dead ends in a metropolitan water supply

The percentage of samples collected from dead ends in a metropolitan water supply in which total coliforms, *E. coli*, enterococci and spores of sulphite reducing clostridia were detected were significantly higher (confidence limits for percentages test, $P<0.05$) than the percentages of detection found in tap water (Table 4). In contrast, the percentages of detections of phages were not different from those in tap water (confidence limits for percentages test, $P<0.05$). In these samples phages were detected less frequently than bacteria, the opposite of what occurs in flowing water.

Microbiological status of sediments from service reservoirs in a metropolitan water supply

Total coliforms and spores of sulphite reducing bacteria were present in a considerable number of samples of sediments (Table 4), significantly higher (confidence limits for percentages test, $P<0.05$) than the percentages found in tap water; whereas the percentages of detections of *E. coli* and phages were not significantly different from those found in tap water (confidence limits for percentages test, $P<0.05$). The percentages of detection of all bacterial indicators and phages were similar (confidence limits for percentages test, $P>0.05$) to the values found in dead ends. As observed for the dead ends the percentages of detection of phages were much lower than those for bacteria.

DISCUSSION

The microbial status of the water from springs and household water wells proves that groundwater from a shallow origin is particularly susceptible to contamination, probably due to a combination of point and diffuse sources. Comparing the burden of the various microorganisms of faecal origin found in these water samples with their loads in faecal wastes (Havelaar *et al*. 1986; Grabow *et al*. 1993; Chung *et al*. 1998; Jagals 2000; Contreras-Coll *et al*. 2002; Lucena *et al*. 2003) it seems clear that on their way from faeces to groundwater they undergo a differential reduction, since there is a change in proportions. Faecal coliforms and *E. coli* are those that undergo the greatest reduction. However, the diversity of factors affecting the reduction in the indicator numbers and the lack of knowledge of the geology of the areas studied do not allow us to make a detailed study or to derive clear conclusions.

The percentage of samples from rural water supplies failing to comply with drinking water quality standards was high, ranging from 20% for enterococci to 46% for faecal coliforms. However, similar ranges of failure have been described in different geographical areas of Europe (Humphrey & Cruickshank 1985; Reid *et al*. 2003). The percentages of positive detections and the numbers detected for the various indicators studied were lower than those for springs and household shallow water wells. Only the positive detections, but not the densities, of spores of sulphite reducing clostridia were higher in this sample set. This is probably due to the low susceptibility of sulphite reducing clostridia to low doses of chlorine. The absence of free chlorine in the samples studied indicates significant chlorination deficiencies in the rural water supplies studied.

The microbiological status of the metropolitan water supplies is much better than those of rural areas. This contradicts popular beliefs, but agrees with the reported distribution of waterborne outbreaks in Spain (Binefa & Hernandez 2002). It is interesting that most samples failing to comply with drinking water quality standards for bacterial indicators were those with a chlorine concentration lower than 0.1 mg l^{-1}, which show a similar microbiological quality to the rural water supplies. The presence of bacterial indicators in samples with a chlorine concentration greater than 0.1 mg l^{-1} decreased significantly. In contrast, reductions in the presence of the three groups of bacteriophages studied were less evident. This agrees with published observations relating to the presence of somatic coliphages in disinfected waters that fulfil the bacteriological criteria but still contain bacteriophages (El-Abagy *et al*. 1988; Ratto *et al*. 1989; Dutka *et al*. 1990; Dee & Fogelman 1992; Armon 1993; Armon *et al*. 1997) and enteroviruses (Schaffer *et al*. 1980). This particular observation confirms laboratory studies that indicate that the various groups of phages studied are more resistant to chlorination than are the bacterial indicators (IAWPRC 1991; Durán 2001; Durán *et al*. 2002; Jofre 2002).

However, in sediments and water samples from dead ends of a supply network, bacteria, with the exception of FC/*E. coli*, accumulated significantly more than bacteriophages. This may be because particles to which phages adsorb are smaller (Payment *et al*. 1988) than those to which bacteria bind (Geldreich 1996). Whatever the reason for this apparent contradiction it is clear that bacteriophages and indicator bacteria behave differently in water supply networks.

When analysis has been possible, a relationship between bacterial indicators and bacteriophages and rainfall has been observed. A similar trend for coliforms was reported by Rutter *et al*. (2000) and Reid *et al*. (2003), who described an increased number of faecal coliforms in water supplies during the rainy seasons. The concentrations of bacteria, viruses and bacteriophages are also known to increase in both surface fresh water and marine waters following rain (Paul *et al*. 1997; Loge *et al*. 2002; Dwight *et al*. 2002).

Considering the changes in correlation among densities, the changes in percentages of detection and the percentages of simultaneous detection of bacteria and bacteriophages in the various water supplies studied, we can conclude that bacteriophages and bacterial indicators exhibit different resistances to the removal and inactivation processes that they find on their way from faeces to drinking water.

Our observations of higher resistance of phages to disinfection treatments (IAWPRC 1991; Jofre 2002) and their different behaviour in water distribution networks compared with bacteria are in agreement with observations of high percentages of viral gastroenteritis with an origin in drinking water that fulfilled the quality criteria based on bacterial indicators (Craun 1996; Payment & Hunter 2001; Anderson & Bohan 2001). Our calculations suggest that, of the three groups of bacteriophages, those infecting *B. fragilis* are the ones that differ most from bacteria in their persistence from faeces to drinking water. Numbers available in sewages of human and animal origin as well as in faeces, indicate that the relative abundances of the three groups of phages are approximately 100SOMCPH:10FRNAPH:1BFRPH (Havelaar *et al*. 1986; Grabow *et al*. 1993; Chung *et al*. 1998; Jagals 2000; Contreras-Coll *et al*. 2002; Lucena *et al*. 2003).

Although it is difficult to know with precision the numbers of phages in a set of samples like the one corresponding to metropolitan water supplies because of the many negative results, applying the Thomas formula for the calculation of the most probable number for long

series of data (de Man 1975), the average concentration of the three groups of phages will be around 0.3 infectious units per litre. Thus, results indicate that phages infecting *B. fragilis* are those that survive better en route from faeces to drinking water, followed by F-specific RNA bacteriophages and finally somatic coliphages. However, determining somatic coliphages is the easiest and least time-consuming of all three groups of phages. In our opinion then, further studies are needed to clearly recommend one of the three as a candidate for routine testing.

Van der Kooij *et al*. (1995) demonstrated the importance of using multiple faecal indicator bacteria for monitoring the safety of water treatment. In our opinion the introduction of a bacteriophage indicator, any one of the groups studied here, will significantly increase the information regarding the effects of treatments and the quality of treated water. Analysis of a bacterial indicator plus phages of any of the groups studied here will not provide information about the presence of human viruses in the tested sample, but will give a clear indication of the probability of viruses escaping the treatment. Data obtained during this study show that testing for the three groups of phages in routine laboratories is easy to implement and feasible without requiring additional material resources in the laboratories.

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