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Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe

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Abstract

Somatic coliphages, F-specific RNA bacteriophages, bacteriophages infecting *Bacteroides fragilis*, *Escherichia coli* and enterococci were counted in bathing waters in the late spring and summer. We tested fresh and marine bathing waters from North, South, East and West Europe expected to contain between 100 and 500 *E. coli* per 100 ml, although wider ranges were sometimes found. Bacteriophages were counted after concentration, since a preliminary study proved that this step was necessary to obtain positive counts. During monitoring, a first-line quality control with reference materials for bacteria and bacteriophages was performed by all the laboratories participating in the study. The same microbes were also counted in raw sewage samples from various areas in Europe, where the bacterial indicators and the three groups of bacteriophages were detected in roughly the same numbers. All groups of bacteriophages were detected in both fresh and marine bathing waters throughout Europe. Reliable and complete results from 147 samples showed that for log-transformed values, *E. coli* and bacteriophages were slightly correlated. However, the slope of the regression line changed according to *E. coli* concentration and the correlation diminished when this concentration was close to zero per 100 ml. The ratios between *E. coli* and phages in bathing waters differed significantly from those in sewage. The relative amounts of bacteriophages, mainly somatic coliphages and phages infecting *Bact. fragilis* RYC2056, increased in bathing waters with low *E. coli* concentration, especially in seawater samples containing <100 *E. coli* per 100 ml. The relationship of bacteriophages with respect to enterococci paralleled that of bacteriophages with respect to *E. coli*. Somatic coliphages and bacteriophages infecting *Bact. fragilis* are useful to predict the presence of some pathogens with the same origin as present bacterial indicators but with higher survival rates.

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1. Introduction

Major efforts are being made in many countries to improve the microbiological quality of bathing and shellfish-growing waters. Concerns about water quality have generated extensive discussion about the microbiological parameters to be included in water quality assessment. There is, for example, great controversy on the present standard for enteroviruses in the European Union regulations, Directive 76/610/EEC, which stipulates a standard of zero enterovirus for 10 l. The inclusion of a bacteriophage parameter has been proposed, but there is no general agreement as to the most appropriate group of bacteriophages or limit values.

Somatic coliphages [1,2], F-specific RNA bacteriophages [2–4] and bacteriophages infecting *Bacteroides fragilis* [2,5] have been proposed as potential indicators of viruses and/or persistent faecal microorganisms. Moreover, they have been reported in both sea and fresh waters, which may be used for bathing [6–12]. However, many of these studies were carried out either on only some of the phage groups, not all, or with methods not yet standardised. This clearly hinders the comparison of data on the occurrence of the above-mentioned groups of bacteriophages in different kinds of bathing waters.

The aim of this study was to evaluate the feasibility of bacteriophages for determining the microbiological quality of bathing waters. To this end, the occurrence and numbers of *Escherichia coli*, enterococci, somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bact. fragilis* in bathing waters were determined by 13 laboratories. All laboratories used standardised methods and carried out a first-line quality assurance with reference materials during the monitoring period. The waters tested are representative of all types of fresh and marine bathing waters, and include waters from different climatic conditions throughout the whole of Europe.

2. Materials and methods

2.1. Samples, sampling and sampling sites

Bathing waters in very distinct areas of Europe (Fig. 1) were sampled during the bathing season, from May to September of 1998 and 1999. Laboratories from each of the sampling locations had previously participated in two collaborative studies; thus all of them were considered to have successfully implemented the methods for detecting and counting the three groups of bacteriophages under study [13,14]. Eighty-nine sampling sites were from seawater locations and 54 were fresh water. For the 1998–1999 monitoring period, the sampling sites expected to contain <1000 *E. coli* per 100 ml were selected in each area on the basis of previous data [15]. Finally, 143 samples complying with the requirement of containing fewer than 1000 *E. coli* per 100 ml were analysed.

Fig. 1. Geographical distribution of bathing water sampling sites.

In the summer 1997, 136 samples with a very wide range of *E. coli* content ($0–1.3 \times 10^5$/100 ml) had been tested in three of the laboratories to determine the water volumes to be tested to find positive or significant results for all parameters. This procedure was used to determine whether phages should be concentrated.

For the final monitoring, the following volumes were analysed: 9.6 ml for *E. coli*; 9.6 ml for enterococci and 10 ml for somatic coliphages. Concentrated equivalents were 100 ml for somatic coliphages, 300 ml for phages infecting host strain *Salmonella typhimurium* WG49 (F-total), 300 ml for RNase resistant bacteriophages and 300 ml for bacteriophages infecting *Bact. fragilis*.

Microorganisms were also enumerated in a number of urban sewage samples in six of the sampling locations (Ireland, The Netherlands, Finland, France, Greece and Spain). From four to eight samples per laboratory were analysed.

2.2. Bacteriophage concentration

Bacteriophages were concentrated from bathing waters by the magnesium chloride flocculation method first described by Schultze and Lenk [16], but with some modifications, as described elsewhere [17]. Briefly, 10 ml of 1 mol l^{-1} magnesium chloride solution was added to 1 l of the water sample. 3.5 ml of 1 mol l^{-1} dipotassium hydrogen phosphate solution was then added dropwise to the sample while stirring, followed by the dropwise addition while stirring sodium hydroxide 2.0 mol l^{-1} until the appearance of turbidity (maximum final pH = 8.6). The mixture was then slowly stirred for 15 min to obtain a regular distribution of the flocks to allow the incorporation of phage particles into the floccules. Finally, the supernatant fluid was siphoned off and the loose sediment concentrated by low speed centrifugation (ca. 1000*g*) for 15 min. The supernatant was discarded and the sediment resuspended with 30 ml of peptone saline solution.

2.3. Bacteriophage enumeration

Plaque forming units (PFU) of somatic coliphages were counted by the double agar layer technique following the ISO 10705-2 standard [18]. F-specific RNA bacteriophages PFU numbers were determined as described in ISO10705-1 [19]. Bacteriophages plaquing on the host *Salmonella typhimurium* WG49 were counted as F-total bacteriophages and the difference between the F-total and the number of plaques counted on plates with 40 μg of RNase per ml into the assay medium was attributed to F-specific RNA bacteriophages [19,20]. Plaque forming units of bacteriophages infecting *Bact. fragilis* RYC2056 were also determined by the double agar layer method as described elsewhere [21], with some modifications introduced by Araujo et al. [22]. The method has been approved as an ISO standard [23] after the finalisation of the research described here. Strain RYC2056 detects similar numbers of phages in raw sewage samples from all over the world, although it does not differentiate human from animal faecal pollution [24]. In contrast, host *Bact. fragilis* HSP40, often used in the past, differentiates human from animal faecal pollution [25] but regularly detects low numbers of phages in some parts of the globe [24].

2.4. Bacteria enumeration

E. coli and enterococci were enumerated following the standard microtiter plate method [26,27] or in a few sampling locations according to the standardized national methods.

2.5. Quality assurance

A first-line quality control using reference material for both bacteria and bacteriophages was performed during monitoring. The reference material for *E. coli* and enterococci was produced, validated and used in the framework of a European collaborative study on bathing waters [28]. Pure cultures of bacteriophages ϕX174, MS2 and B56-1, prepared as described elsewhere [29], were used as reference material for somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bact. fragilis*, respectively. Thus, the data obtained in this study should be regarded as data of known and defensible quality.

2.6. Data computation and statistics

Statistical computations and tests were performed with the SAS Statistic Program [30]. Log-transformed values had been used for all computations and tests. Zero values were assigned as one. Log-transformed data had been plotted in some figures as boxes and whiskers. This plotting provides a summary statistics using five numbers: the minimum, the maximum, the median, the 25th and the 75th percentile. Differences were considered significant at $P < 0.05$ as determined by the appropriate comparative test.

3. Results

3.1. Numbers of bacteriophages and bacterial indicators in raw sewage

The box and whisker plots of values of indicator bacteria, *E. coli* and enterococci, somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bact. fragilis* RYC2056 in raw sewage are shown in Fig. 2. Most samples showed the same trend. *E. coli* is the most abundant microorganism, with concentrations ranging from 5×10^6 to 1×10^7 bacteria per 100 ml. Numbers of *E. coli* were 2–10 times higher than somatic coliphages, 5–20 times higher than enterococci, 15–80 times higher than F-specific RNA bacteriophages and 100–1000 times higher than *Bact. fragilis* bacteriophages. All the microorganisms were detected in all sewage samples tested.

3.2. Bacteriophage concentration from natural samples

The concentration efficiencies obtained for naturally occurring somatic coliphages in 1-l-water samples obtained by the different laboratories averaged 46.9% ($\pm$6.7 for the 95% confidence interval). These values do not differ significantly from those obtained for somatic coliphages (57.7%), F-specific RNA bacteriophages

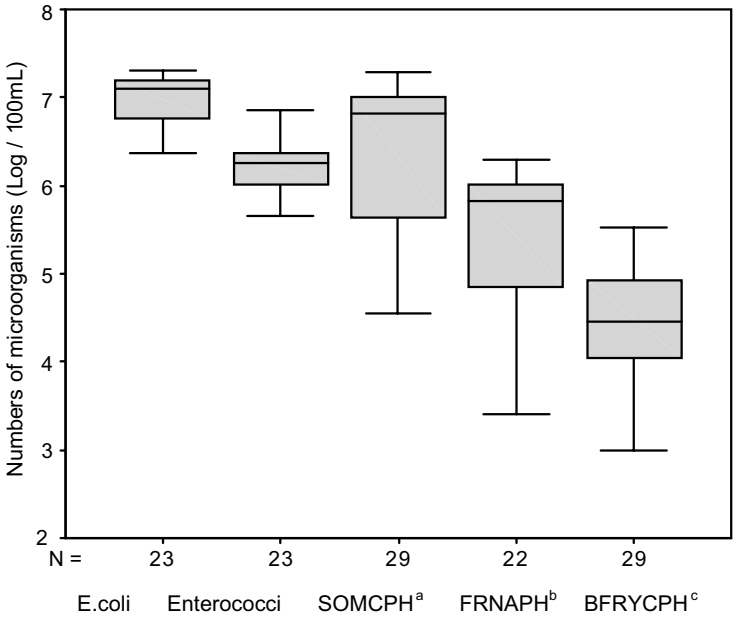

Fig. 2. Box and whisker plots of numbers of microorganisms in raw sewage from several European countries. [a]SOMCPH indicates somatic coliphages. [b]F-RNAPH indicates F-specific RNA bacteriophages. [c]BFRYCPH indicates bacteriophages infecting strain RYC 2056 of *Bact. fragilis*.

Table 1
Percentage of positive samples for bacteriophages in surface waters with various levels of faecal pollution

E. coli range[a]	n[b]	% of positive samples in 10 ml			
		Somatic coliphages	F-total	F-specific RNA phages	Phages of *Bact. fragilis* RYC2056
<100	58 (25)	71.5	9.5	1.9	8.0
100–1000	46 (22)	91.0	38.0	21.7	22.7
>1000	32 (26)	100	93.0	84.6	78.0

[a]Colony forming units per 100 ml of sample.
[b]Numbers of samples tested. In brackets the number of samples tested for bacteriophages infecting *Bact. fragilis* RYC2056.

(52.7%) and phages infecting *Bact. fragilis* (60.3%), as reported elsewhere with either spiked samples or highly polluted surface waters [17].

3.3. Results of preliminary monitoring of bathing waters

F-specific RNA bacteriophages and bacteriophages infecting *Bact. fragilis* were detected in a low percentage of 10 ml aliquots of bathing water samples containing <1000 *E. coli* per 100 ml (Table 1). Ten millilitre was the maximum volume of sample considered feasible by the plaque assay method. Consequently, it was considered necessary either to concentrate the sample or to study greater volumes of sample to find positive values for these phages.

Fig. 3 shows the box and whisker plots of the numbers of bacterial indicators and somatic coliphages in fresh and seawater samples containing more than 10^4 *E. coli* per 100 ml. These samples show a ratio between *E. coli* and somatic coliphages, which does not differ significantly from values in sewage, but differs significantly (one way ANOVA, $P<0.05$) from values in bathing waters with low *E. coli* content (Fig. 7). A similar trend can be observed for the ratio between enterococci and somatic coliphages (Fig. 8).

3.4. Occurrence of bacterial indicators and bacteriophages in bathing waters

Table 2 shows the percentage of samples in which microorganisms were detected during the

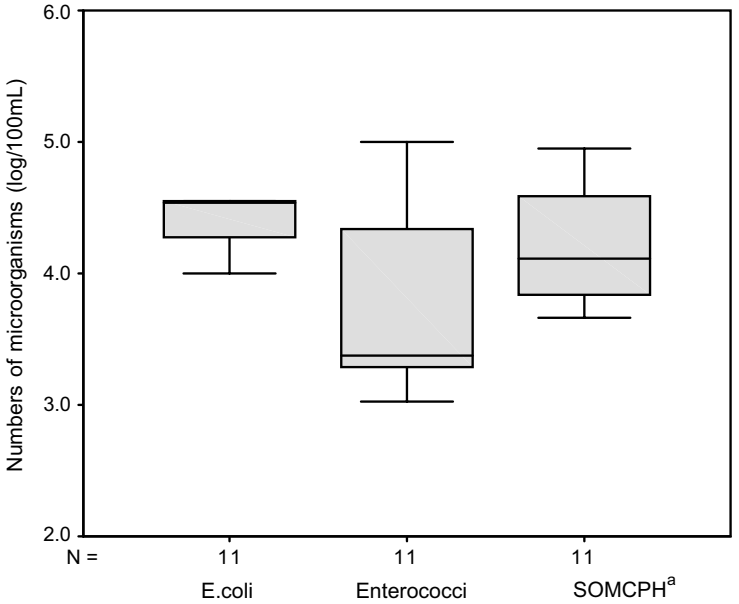

Fig. 3. Box and whisker plots of numbers of microorganisms in surface water samples with more than 10^4 *E. coli* per 100 ml. [a]SOMCPH indicates somatic coliphages.

Table 2
Percentage of samples containing the indicator microorganisms in bathing water

Sampling locations[a]	Type of water[b]	Number of samples	*E. coli*[c] (%)	Enterococci[c] (%)	SOMCPH[d] (%)	FRNAPH[e] (%)	BFRYCPH[e] (%)
Bilthoven[1]	FW	10	100	90	100	44	30
Tubingen[2]	FW	14	93	71	71	50	58
Vienna[3]	FW	10	100	100	100	100	75
Kiel[4]	FW	10	100	100	100	30	40
Dublin[5]	SW	12	100	92	75	25	67
Exeter[6]	SW	10	100	50	100	30	40
Lille[7]	SW	13	100	84	100	54	92
Athens[8]	SE	6	100	100	100	67	100
Montpellier[9]	SW FW	10	90	80	100	90	80
S.Sebastián[10]	SW	15	80	80	100	47	100
Ferrara[11]	FW	6	100	100	100	83	100
Barcelona[12]	SW	20	80	70	100	47	95
Mallorca[13]	SW	7	43	71	71	14	28

[a]The sampling locations were in: (1) The Netherlands; (2) Southern Germany; (3) Austria; (4) Northern Germany; (5) Ireland; (6) Southern England; (7) Northern France; (8) Greece; (9) Southern France; (10) Northern Spain (Atlantic); (11) Northern Italy; (12) Northern Spain (Mediterranean); (13) Northwestern Mediterranean.

[b]SW stands for seawater and FW stands for fresh water.

[c]In 9.6 ml.

[d]In a concentrate equivalent to 100 ml of sample.

[e]In a concentrate equivalent to 300 ml of sample.

1998 and 1999 monitoring period. From these data and also from data from sewage, it can be concluded that the methods for counting somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bact. fragilis* RYC2056 are applicable to a wide range of situations encountered in Europe, including fresh and salt water, and cold and warm climates.

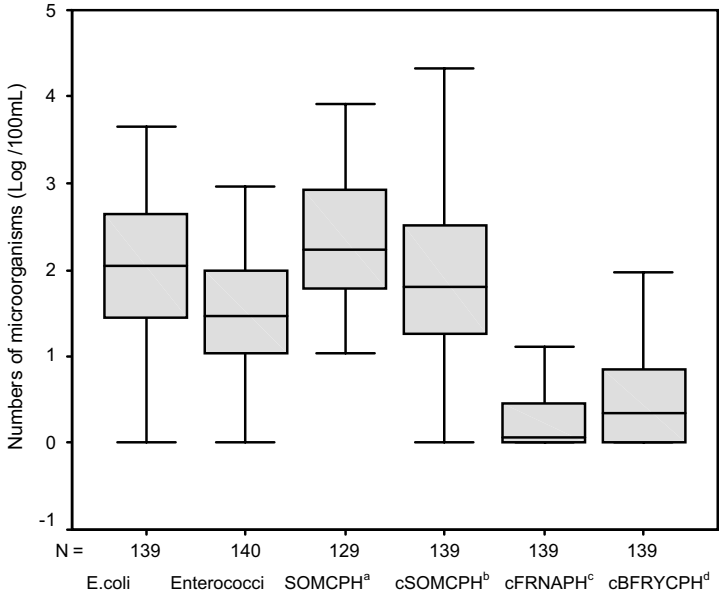

Fig. 4. Box and whisker plots of numbers of microorganisms in all bathing water samples tested. [a]SOMCPH stands for somatic coliphages counted without concentration. Since the tested volume was 10 ml, there were no values within 10 or 0 PFU per 100 ml, and since they were a few zeros, the computing program has considered them as outlayers; this is the reason why the minimum value is 10. [b]cSOMCPH indicates somatic coliphages counted after concentration. [c]cF-RNAPH stands for F-specific RNA bacteriophages counted after concentration. [d]cBFRYCPH stands for bacteriophages infecting strain RYC2056 of *Bact. fragilis* counted after concentration.

3.5. Numbers of bacterial indicators and bacteriophages in bathing waters

Fig. 4 shows the box and whisker plots numbers of microorganisms in all the bathing waters tested in 1998–1999, which clearly differs from the values in sewage (Fig. 2). Grouping data according to the levels of *E. coli* and water characteristics, fresh or seawater, indicates that this difference is more evident in the samples with <100 *E. coli* per 100 ml, especially in seawater samples containing <100 *E. coli* per 100 ml (Fig. 5).

Fig. 6 shows the correlation between the log-transformed values of the microorganisms counted without concentration, i.e., *E. coli*, enterococci and somatic coliphages. The correlation is significant for all parameters (Pearson, $P<0.05$). The highest correlation is between *E. coli* and enterococci, followed by that between *E. coli* and somatic coliphages. Second-order regression lines show a clear change of slope for low *E. coli* values, indicating a relative increase in the numbers of somatic coliphages.

The ratios between *E. coli* and somatic coliphages (Fig. 7) show that the relative proportions between these two organisms vary significantly from sewage to bathing waters. Indeed, in samples with low levels of *E. coli*, somatic coliphages clearly outnumbered *E. coli* both in fresh and sea bathing waters. This was significant (one-way ANOVA, $P<0.05$) considering all bathing water samples together and especially so in bathing water samples containing <100 *E. coli* per 100 ml.

A similar trend is observed regarding enterococci and somatic coliphages (Fig. 8). In this case, the differences are slighter but still significant (one-way ANOVA, $P<0.05$).

The ratios between the numbers of each group of bacteriophages, calculated from the numbers in sewage and from the numbers after concentration in marine bathing waters, are shown in Fig. 9. Since, as mentioned above, the concentration method performs similarly for the three groups of bacteriophages, the ratios between values obtained after concentration can be considered as unaffected by concentration and thus equivalent to those values obtained without concentration. The ratios between the pairs of bacteriophages vary significantly (one-way ANOVA, $P<0.05$) from raw sewage to bathing waters with *E. coli* numbers between 10 and 100 per 100 ml. The same trend, although less marked, was observed in freshwater samples. The relative numbers of somatic coliphages and bacteriophages infecting *Bact. fragilis* increased in all bathing waters.

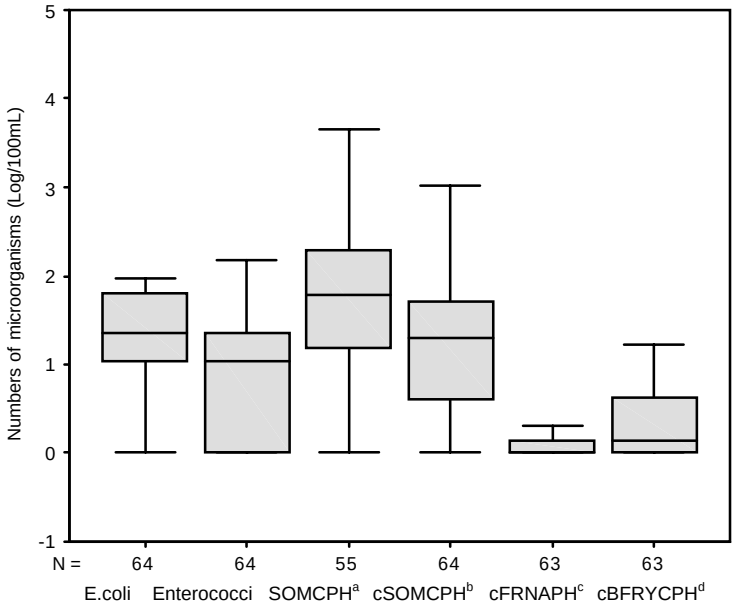

Fig. 5. Box and whisker plots of numbers of microorganisms in bathing seawater samples with <100 *E. coli* 100 ml. [a]SOMCPH stands for somatic coliphages counted without concentration. [b]cSOMCPH indicates somatic coliphages counted after concentration. [c]cFRNAPH stands for F-specific RNA bacteriophages counted after concentration. [d]cBFRYCPH stands for bacteriophages infecting strain RYC2056 of *Bact. fragilis* counted after concentration.

4. Discussion and conclusions

The first conclusion of this collaborative research is that standardised methods detect the same order of abundance for the bacterial indicators and the three groups of bacteriophages, somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting strain RYC2056 of *Bact. fragilis* in all the European sites studied. This was not the case with bacteriophages infecting strain HSP40 of *Bact. fragilis* [24]. Similar results, but including only part of the parameters measured in this study, have been reported elsewhere [8, 31–33]. Moreover, the three groups of phages were detected in bathing waters in all the locations studied. They may therefore be used as indicators in the different bathing water situations encountered in Europe.

The log-transformed numbers of somatic coliphages and *E. coli* are correlated but not strongly so. The correlation between coliphages and *E. coli* was lower than that between *E. coli* and enterococci. This suggests that bacteriophages provide information different from that provided by bacterial indicators regarding the residence time and sensitivity to inactivation factors. These two factors are relevant to the prediction of pathogenic pollutants in water.

The relative proportion of somatic coliphages and *E. coli* clearly changed from sewage to bathing waters when phages were counted without concentration. There is indeed a significant difference between the ratios in sewage or in very polluted bathing waters (*E. coli* $>10^4$ per 100 ml) and the ratios in bathing waters, where *E. coli* values ranged from 10 to 2000 per 100 ml. This change clearly favours the numbers of somatic coliphages in all samples with low numbers of *E. coli*. A similar change has been reported between faecal coliforms and somatic coliphages in bathing waters with various levels of faecal pollution in the United Kingdom [7]. A significant change in proportions, although of lower magnitude, was observed between somatic coliphages and enterococci.

The difference between the relative proportion of the three groups of bacteriophages after concentration in marine bathing waters and without concentration in sewage indicates that bacteriophages infecting *Bact. fragilis* RYC2056 increase their relative proportion with respect to somatic coliphages and, consequently, with respect to *E. coli*. In contrast, the relative numbers of F-specific RNA bacteriophages are lower than those of somatic coliphages, and thus their relative increase with respect to *E. coli* is not as marked as that of the other bacteriophages. Similar results have been reported elsewhere for various surface waters [9,12,34].

Such changes in relative numbers may result from phage replication, inputs of faecal contamination other than urban sewage and from a greater proportion of faecal bacteria affected by environmental stresses.

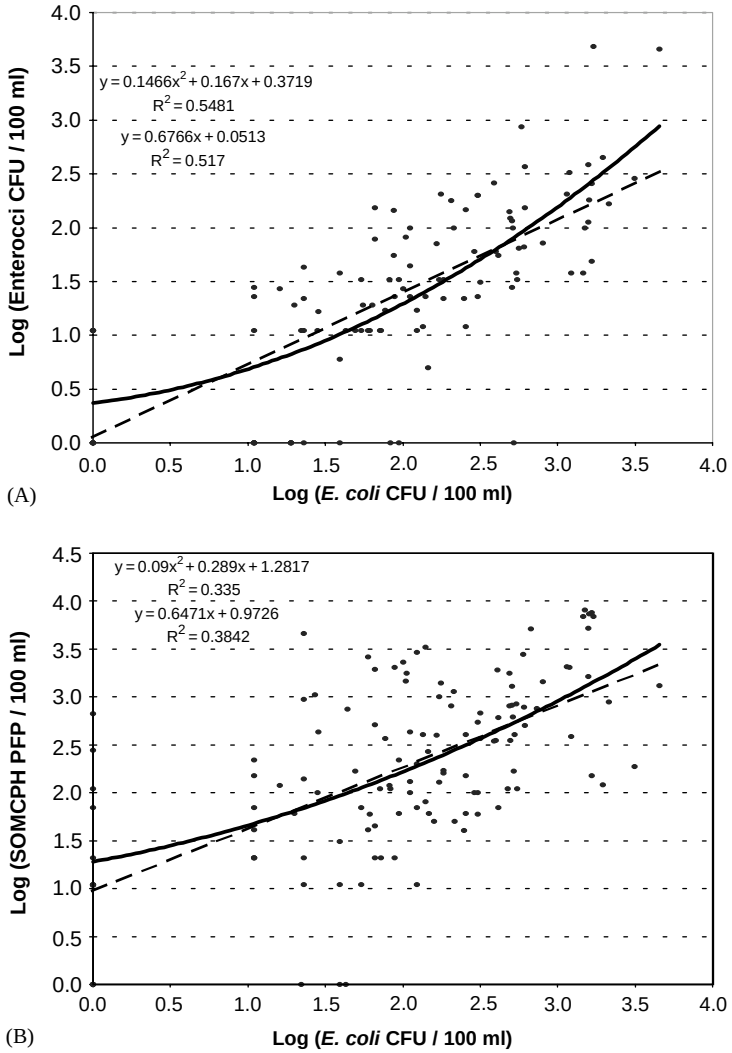

Fig. 6. First (- - - -) and second (—) order regression lines and correlations between *E. coli* and enterococci (A) and *E. coli* and somatic coliphages (B) in all bathing water samples analyzed.

Somatic coliphages may replicate in the environment, outside the human and animal gut [35,36]. However, there is some disagreement on the conditions necessary for replication, such as the host and phage concentrations required and the physiological state of the host bacteria [37,38]. The fact that inversion only occurred in waters with low numbers of all microbial contaminants and did not occur in the water samples with a high level of faecal pollution (which would have more adequate densities for replication) seems to minimize the importance of coliphage replication in the observed inversion of the ratios. Replication in the studied environments of F-specific RNA bacteriophages is unlikely [39]; that of bacteriophages infecting *Bact. fragilis* is even less so [25].

Coliphages and, to a lesser extent, F-specific-RNA bacteriophages and bacteriophages infecting *Bact. fragilis* RYC2056 have been described in animal faeces, animal slurries and abattoir sewage. However, in all these cases *E. coli* clearly outnumbers bacteriophages [3,24,25,31,40–44]. Therefore, fecal contamination other than urban sewage is unlikely to explain the observed changes.

The third possible explanation is the higher resistance of bacteriophages to wastewater treatments and, more importantly, to inactivation by natural factors, as supported by model experiments [45–49]. These studies explain the differences in the proportions between bacteriophages and that between bacteriophages and

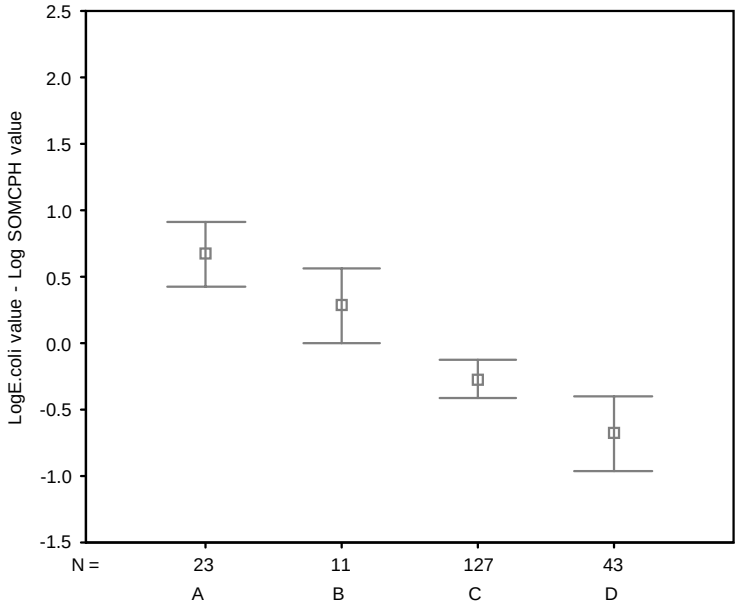

Fig. 7. Ratios between *E. coli* and somatic coliphages in various samples. Plots of the average (□) and the 95% confidence interval (bars). Type of water: (A) sewage; (B) surface water with more than 10^4 *E. coli* per 100 ml; (C) all samples from the 1998–1999 bathing water monitoring; (D) samples from the 1998–1999 bathing water monitoring containing less than 10^2 *E. coli* per 100 ml, which is the guide value in the European Guidelines.

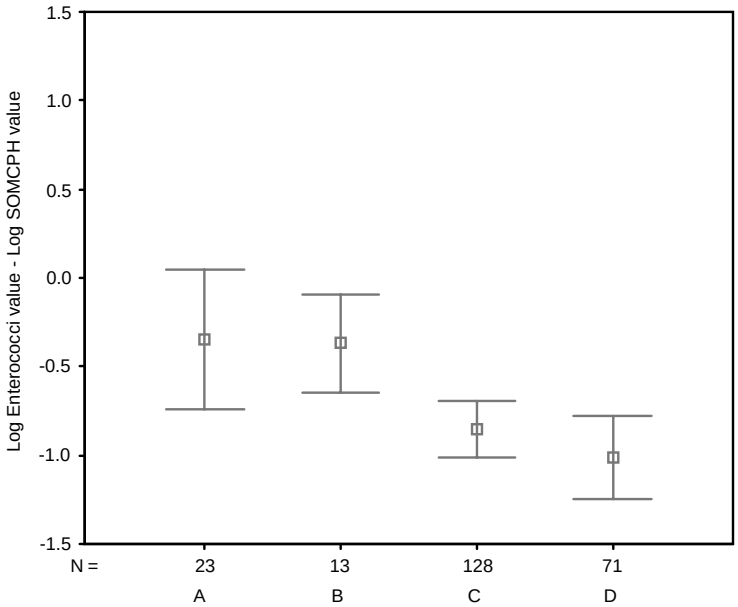

Fig. 8. Ratios between enterococci and somatic coliphages in various samples. Plots of the average (□) and the 95% confidence interval (bars). Type of water: (A) sewage; (B) surface water with more than 10^3 enterococci per 100 ml; (C) all samples from the 1998–1999 bathing water monitoring; (D) seawater samples from the 1998–1999 bathing water monitoring containing <33 enterococci per 100 ml, which is the guide value in the USA Guidelines.

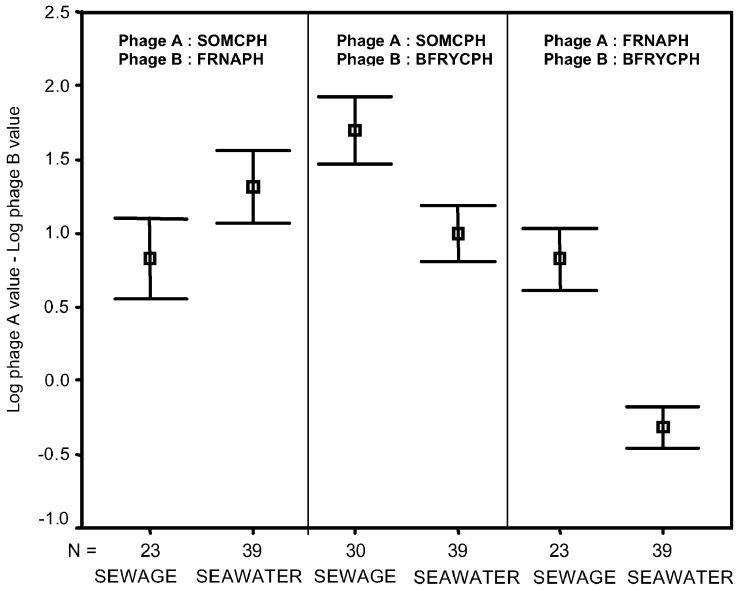

Fig. 9. Ratios between the groups of bacteriophages in sewage and bathing seawater samples containing <100 *E. coli* per 100 ml. Plots of the average (□) and the 95% confidence interval (bars).

the bacterial indicators studied. Since some pathogens, including enteric viruses, show higher survival rates in fresh water and marine environments than bacterial indicators, other types of indicators are needed.

The implementation of regulations has significantly improved bathing water quality and the annual monitoring campaign provides water managers with relevant background information for remedial actions to be taken. The addition of viruses into the guidelines (Directive 76/160/EEC on the Quality of Bathing Water) is probably useful to predict the presence of some pathogens with the same origin as current bacterial indicators but with higher survival rates. Our results show that some of the bacteriophages studied might supply this function. Bearing this in mind, at least two of the three groups of bacteriophages, namely somatic coliphages and bacteriophages infecting *Bact. fragilis* are clearly more persistent than *E. coli* and enterococci. In our opinion, the ratio between *E. coli* and somatic coliphages or *E. coli* and bacteriophages infecting *Bact. fragilis* provides more information on the presence of persistent pathogens such as viruses than do bacteria alone.

Somatic coliphages have the advantage of being very abundant. Moreover, they are easily quantified, although the contribution of their potential replication in the environment should be further quantified and apportioned.

Bacteriophages infecting *Bact. fragilis* are the most persistent. Moreover, they do not replicate in environmental conditions, but their numbers are low and so their detection and enumeration need concentration. Further research on more efficient host strains may be appropriate. In this respect, Bradley et al. [50] have reported *Bact. fragilis* host strains that are more efficient than the host strains used in the present study.

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