Somatic coliphages and bacterial indicators of bathing water quality in the beaches of Gipuzkoa, Spain

Jesús M. Ibarluzea, Belén Moreno, Elena Serrano, Karmele Larburu, María J. Maiztegi, Asunción Yarzabal and Loreto Santa Marina

ABSTRACT

Monitoring the quality of the bathing waters of Gipuzkoa (the Basque Country, Spain) makes it possible to assess the suitability of its 15 beaches for bathing throughout each season. In 1998, the parameters *E. coli*, somatic coliphages (SOMCPH) and F-specific RNA bacteriophages (FRNAPH) were incorporated into the bathing water quality monitoring system. This enabled the study of the link between bacterial and viral indicators as well as the analysis of the ratios between both types of indicators in waters with different levels of pollution. Although bacterial indicators (total coliforms (TC) and faecal coliforms (FC)) and enterococci showed a strong correlation between them, the correlations between the viral indicators and between the viral and bacterial indicators were weaker, though significant in all cases. The ratio between SOMCPH and *E. coli* indicates that at low levels of bacterial pollution (*E coli* $<$ 100 MPN/100 ml) SOMCPH outnumber *E coli*. In contrast, at higher levels of pollution (*E coli* $>$ 100 MPN/100 ml), SOMCPH numbers are lower than those of *E-coli*. The data reveal the presence of viral indicators in waters classified as suitable for bathing by the European Directive and alert us to their suitability.

Key words | bacteriophages, bathing waters, coliphages, *E. coli*, enterococci, indicators

Jesús M. Ibarluzea (corresponding author)
Loreto Santa Marina
Subdirección de Salud Pública de Gipuzkoa,
The Basque Government's Health Department,
Avda. de Navarra 24, 20013 San Sebastian,
Spain
Tel.:+34 943 022 749
Fax: +34 943 022 710
E-mail: *mambien3-san@ej-gv.es*

Belén Moreno
Public Health Laboratory of Gipuzkoa,
The Basque Government's Health Department,
Avda. de Navarra 24, 20013 San Sebastian,
Spain

Elena Serrano
Public Health Centre of Urola,
C/Salegui 14, 20800 Zarautz,
Spain

Karmele Larburu
Public Health Centre of Bidasoa,
Avda. Navarra 41, 20302 Irun,
Spain

María J. Maiztegi
Public Health Centre of Deba,
Jardines de Argatxa 1, 20600 Eibar,
Spain

Asunción Yarzabal
Department of Health, Environment and Consumption,
San Sebastian City Council,
C/Manuel Lecuona 7, 20013, San Sebastian,
Spain

INTRODUCTION

The presence of enteric viruses such as enterovirus, hepatitis A virus, adenovirus, reovirus and Norwalk-type virus has been documented in marine waters (Girones *et al.* 1993; Puig *et al.* 1994; Enriquez & Gerba, 1995; Muscillo *et al.* 2001; Pusch *et al.* 2005). These viruses can cause a number of waterborne diseases such as gastroenteritis, hepatitis, respiratory diseases and conjunctivitis, among others (Melnick 1984). It is also known that enteric viruses survive longer than bacterial indicators and there are suspicions that bacterial indicators of faecal pollution may not be enough to assess faecal pollution or to be used as indicators of human viruses (Scientific Committee on Toxicity, Ecotoxicity, & Environment 2004; Ballester *et al.* 2005).

The preamble to the new European bathing water directive (2006/7/EEC) (EEC 2006) states that although the global quality of bathing waters has improved significantly since Directive 76/160/EEC (EEC 1976) came into effect, it was desirable to update scientific and technical knowledge to a situation which has evolved over the last three decades. In addition, the new directive stresses the urge to conduct

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studies in order to incorporate more reliable indicators which will make it possible to foresee microbiological health risks and, consequently, better protect the population's health. Following the general tendency, the beaches of Gipuzkoa have also experienced a remarkable improvement from the middle of the 1980 s to the end of the 1990 s, mainly in locations where the impact of sewage has been greatly reduced by the implementation of sewage treatment plants (Bald *et al.* 2004).

Concerns about bathing water quality have generated extensive discussion on the parameters to be included in water quality assessment. The discussion has mainly focused on the feasibility of including a viral indicator of sewage pollution. Both somatic coliphages (SOMCPH) and F-specific RNA bacteriophages (FRNAPH) or bacteriophages infecting *Bacteroides fragilis* have been proposed as viral indicators and as persistent faecal pollution microorganisms (Tartera & Jofre 1987; IAWPRC 1991; Havelaar *et al.* 1993; Grabow 2001). The presence of such indicators has been reported in both sea and fresh bathing waters. However, many of these studies were carried out either on one or a group of phages, or with methods not yet standardised. This clearly hinders the comparison of the results provided by the studies (Contreras-Coll *et al.* 2002). Finally, the new directive has not incorporated any viral indicator into the list of parameters to be used in the assessment of recreational water quality.

The aim of this study was threefold: (1) to assess the feasibility of including somatic coliphages and F-specific RNA bacteriophages among the parameters of a bathing water monitoring system, (2) to determine and quantify the presence of bacterial indicators (total coliforms (TC), *E. coli* and enterococci and bacteriophages (SOMCPH and FRNAPH) in the beaches of Gipuzkoa, and (3) to analyse the relationship between bacterial and viral indicators in beaches of differing water quality, using the counts of bacterial indicators (FC), enterococci and *E. coli* as classification criteria. The information required to achieve the aforementioned aims was obtained through the bathing water quality monitoring system of the Basque Government's Health Department (Ibarluzea *et al.* 2000). The enumeration of *E. coli* and bacteriophages was incorporated into the monitoring system in 1998. The determination of FRNAPH ceased in 2001, whereas that of *E. coli* and SOMCPH has continued to the present. All the microbiological determinations were carried out at the Public Health Laboratory of Gipuzkoa.

MATERIAL AND METHODS

Beaches, sampling sites and sampling

The Gipuzkoan coastline comprises 15 beaches (Figure 1) and a total of 23 sampling sites. Three beaches (Zurriola,

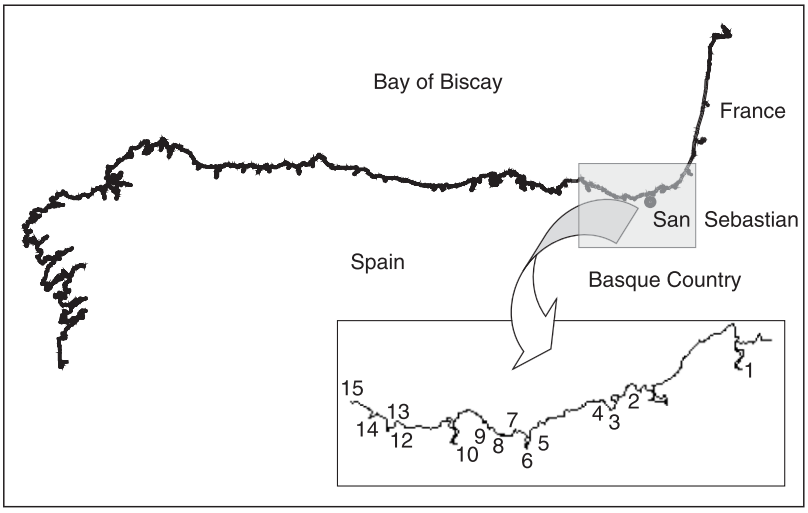

Figure 1 | Location of the sampling sites of the Bathing Water Quality Monitoring System of Gipuzkoa. 1, Hondarribia; 2, Zurriola; 3, La Concha; 4, Ondarreta; 5, Orio; 6, Oribarzar; 7, Zarautz; 8, Malkorbe; 9, Gaztetape; 10, Santiago; 11, Itzurin; 12, Deba; 13, Odarbeltz; 14, Mutriku; 15, Saturraran.

La Concha and Zarautz) have 3 sampling sites, and a fourth one (Ondarreta) has 2 sites. The reason for having more than one sampling site in these 4 beaches is related to their size, the influx of bathers and/or the beaches having areas with differing levels of microbiological pollution. All the mentioned beaches are included in the Bathing Water Monitoring Programme of the Basque Govenment's Health Department. The activities to control bathing water quality began in the early 1980s, although the Monitoring Programme was adapted in 1989 to suit the assessment criteria of the European Directive.

The official bathing season extends approximately from 15 May to 15 September, although in the 3 beaches of San Sebastian, the provincial capital and major city of Gipuzkoa, the season lasts until around 30 September. A sampling schedule for every beach is prepared prior to the opening of each season. Samples are generally taken every fortnight during May, June and September, and every week during July and August. The number of samples per site and season has ranged between 12 and 14 during the study period. The sampling sites are fixed and were selected according to their capacity to represent an area with a maximum influx of bathers or their capacity to pose a specific pollution risk. Water samples for microbiological analysis were taken at a depth of approximately 1 m and 30 cm below the surface. The containers, sterilisation, sample volume and sampling technique, as well as the refrigerated transport and storage of the samples, met the criteria established by Directive 2006/77EEC. The analysis of each sample began on the same day of the sampling at the Laboratory of the Public Health Subdirectorate of Gipuzkoa.

Assessment of bathing water quality

The Bathing Water Monitoring Programme of the Basque Govenment's Health Department has provided us with the water quality time series data of each sampling site for the period 1985–2005. The information on the quality of bathing waters includes the counts of the routine parameters: total coliforms (TC), faecal coliforms (FC) and enterococci, as well as the presence/absence of *Salmonella* in the areas considered the most polluted (Ibarluzea *et al.* 2000). Three additional parameters were included in 1998: SOMCPH, FRNAPH and *E. coli*, in a monthly sampling by which 4 or 5 samples are taken annually at each sampling site, providing information about both the routine parameters and those included "ad hoc". Therefore, the results shown in this study cover the period 1998–2005.

Bacterial determinations

Total coliforms (TC), faecal coliforms/termotolerant coliforms (FC) and enterococci were enumerated by membrane filtration methods: total coliforms on Coli ID agar (bio Merieux) at 36 ± 2°C for 20 ± 4 h; termotolerant coliforms according to Standard Methods (APHA 1998) and enterococci on oxolinic acid aesculin azide agar (Audicana *et al.* 1995), incubated at 36 ± 2°C for 44 ± 4 h. Enterococci confirmation was performed following ISO 7899-2 (ISO 2000). *E. coli* were enumerated following ISO 9308-3 (ISO 1998) by a miniaturised method (MPN). Values reported for bacteria refer to 100 ml volumes.

Bacteriophage determinations

Bacteriophages were quantified by a double agar layer technique following the ISO 10705-2 standard (ISO 2000) for enumeration of somatic coliphages (SOMCPH) and ISO 10705-1 (ISO 1995) for enumeration of F-specific RNA bacteriophages (FRNAPH). The volume of water tested for each phage amounted to 10 ml. Values reported for bacteriophages refer to 100 ml volumes. The detection limit (DL) for both phages was 10 PFU/100 ml. Values amounting to half of the detection limit were assigned to the bacteriophages in all samples with analytical results below the DL.

Quality assurance

All bacteriological methods are accredited by ISO 17025 (ISO 2005). For the analysis of bacteriophages pure cultures of ΦX174 and MS2 were used for a first-line quality control as reference material for SOMCPH and FRNAPH, respectively.

Data computation and statistics

The median and percentiles of the microbiological parameters for which Directive 76/160/EE establishes compulsory and guideline standards were used to assess bathing

water quality (Table 1). Values of the enumeration of bacteriological and viral indicators were transformed into their decimal logarithms in order to obtain log-normal distributions. Zero values were assigned as one. The descriptive statistics, Pearson's correlation test and the comparison of means (ANOVA one-way and Tukey's test) were made using the Statistical Package for Social Sciences, version 12.0 (SPSS, 1999). Some data were plotted as boxes and whiskers. The plotting provides summary statistics using five numbers; the minimum, the maximum, the median, the 25th and the 75th percentiles. P value ≤ 0.05 was considered significant for the different tests.

RESULTS

Table 2 describes the bathing water quality of each of the sampling sites, according to the values of the median and the 80th, 90th or 95th percentile of the counts of the microbiological parameters obtained during the study period (1998–2005). Out of the 23 sampling sites, 15 comply with both the compulsory and the guideline values for TC, FC and enterococci of Directive 76/160/EE. Three sampling sites (5A, 7A and 15A) do not comply with the compulsory values for TC and/or FC and another 5 (6A, 7B, 10A, 13A and 14A) breach the guideline values for TC and FC and/or enterococci.

Figure 2 shows the values of the median and the 25th and 75th percentiles of the bacterial and viral indicators of the samples where both types of indicators were analysed. The percentage of positive samples for the indicator microorganisms amounted to 96.9% for TC, 93.1% for FC, 97.6% for *E. coli*, 92.6% for enterococci, 72.6% for SOMCPH and only 25.5% for FRNAPH. All the parameters show a very significant range of values, of 3 orders of magnitude or above, except in the case of FRNAPH. The values of the median and the mean of TC (61.0 and 80.9) are between 2 and 3 times higher than those of FC (20 and 24.1), *E. coli* (23 and 30.1) and SOMCPH (21 and 32.8), more than 5 times higher than those of enterococci (11 and 13) and about one order of magnitude those of FRNAPH ($<$ DL and 8). The high percentage of samples with values of FRNAPH below the DL entails that the values of the median and mean of FRNAPH must be considered with caution.

Table 1 | Compulsory and guideline standards for microbiological indicators in bathing waters according to the 76/160/EEC Directive

Parameter	Compulsory standard*	Guideline standard**
Total coliforms/100 ml	10 000	500
Faecal coliforms/100 ml	2000	100
Enterococci/100 ml	–	100

*95% of the samples must meet the standard.
** 80% of the samples for TC and FC and 90% for enterococci must meet the standard.

Table 3 shows the correlation between the log-transformed values of the various microorganisms counted. The correlation between all the pairs of microorganisms was positive and significant ($p < 0.001$). TC and FC showed the strongest correlation ($r = 0.90$), and the rest of bacterial indicators showed moderately good correlations ($0.72 < r < 0.83$). SOMCPH showed a moderately good correlation with TC, FC, *E. coli* ($0.69 < r < 0.71$) and moderate correlations with enterococci ($r = 0.57$) and FRNAPH ($r = 0.49$).

Finally, Table 4 shows the relationship between the counted levels of FC, *E. coli* and enterococci with SOMCPH. As levels of *E. coli* increase, levels of SOMCPH increase too. Nevertheless, their relationship, measured in terms of the logarithm of the ratio between the counts of both indicators, shows that when *E. coli* counts are high ($>$500 MPN/100 ml) the values of that indicator exceed those of SOMCPH, whereas when *E. coli* counts are low ($<$100 MPN/100 ml) SOMCPH counts are higher. That is, the ratio turns from positive (0.48) to negative (-0.10) as *E. coli* counts change from high to low. The ratio between these indicators shows significant differences among the three *E. coli* levels studied (ANOVA one-way: $p < 0.001$), as well as between the pairs of levels (Tukey test: $p < 0.01$). The ratio between enterococci and SOMCPH also shows a relationship similar to that described for *E. coli*, since the ratio turns from slightly positive (0.07) to negative (-0.45) as enterococci counts change from high to low. The ratio between enterococci and SOMCPH shows significant differences among the three enterococci levels (ANOVA one-way: $p < 0.01$). The ratio between TC, FC or enterococci with FRNAPH was always positive, regardless of the cut-point of the reference bacterial indicator used.

Table 2 | Descriptive values of the bacteriological and viral indicators at the sampling sites of the beaches of Gipuzkoa

Beach:		TC				FC			FE		*E. coli*[e]				SOMCPH[e]		FRNAPH[e]		
Sampling site		*n*	P50	P80[c]	P95[a]	P50	P80[d]	P95[b]	P50	P90[d]	*n*	P50	P80	P95	P50	P95	*n*	P50	P95
1	A	111	20	170	1900	6	43	398	5	63	32	7	32	271	30	292	19	< DL	20
1	B	111	17	109	1965	9	37	337	6	74	32	5	32	287	30	440	19	< DL	10
2	A	197	35	255	1525	10	85	440	5	72	35	10	88	225	40	262	19	< DL	80
2	B	197	35	258	1242	13	83	425	6	80	35	10	87	349	20	260	19	< DL	20
2	C	195	30	250	1262	12	79	368	5	65	34	10	89	240	25	202	19	< DL	50
3	A	201	15	78	240	8	35	144	5	60	35	10	36	175	10	66	19	< DL	10
3	B	196	25	61	233	10	33	118	6	50	35	10	50	178	10	42	19	< DL	10
3	C	200	18	77	348	8	31	129	5	48	35	10	69	173	10	96	19	< DL	10
4	A	201	20	81	299	10	41	139	5	60	35	10	50	101	10	108	19	< DL	40
4	B	196	25	143	504	11	68	276	7	55	35	10	32	565	10	84	19	< DL	30
5	A	99	783	3200	10010	250	750	7400	50	600	33	162	464	2897	370	4440	15	20	230
6	A	108	97	565	2185	34	126	839	10	56	37	57	139	453	40	500	19	< DL	910
7	A	109	537	3600	12700	112	1250	6800	20	275	37	110	454	9096	40	2151	19	< DL	120
7	B	109	125	800	6900	28	175	1850	10	120	37	26	193	1999	10	810	19	< DL	70
7	C	109	32	225	1100	7	65	390	5	60	37	5	38	717	< DL	240	19	< DL	40
8	A	109	25	115	470	12	42	150	9	48	37	12	49	239	10	60	19	< DL	< DL
9	A	110	25	84	404	8	29	166	2	29	36	10	32	316	20	163	18	< DL	20
10	A	109	350	1925	9050	100	600	1925	30	270	37	140	637	1823	130	2024	19	< DL	210
11	A	109	8	30	142	2	8	16	2	12	37	5	10	53	< DL	31	19	< DL	< DL
12	A	106	28	221	1337	7	48	386	4	29	31	10	53	436	40	846	18	< DL	10
13	A	103	200	1190	3480	37	187	1370	13	180	35	54	282	930	205	2187	17	10	60

Table 2 | (*continued*)

Beach:		TC			FC			FE		*E. coli*[e]			SOMCPH[e]			FRNAPH[e]	
Sampling site	*n*	P50	P80[c]	P95[a]	P50	P80[d]	P95[b]	P50	P90[d]	*n*	P50	P80	P95	P50	P95	*n*	P50	P95
14	A 109	355	660	2430	135	343	580	23	150	34	104	302	637	190	1384	19	< DL	10
15	A 109	1004	2400	8085	195	480	2610	50	401	35	182	390	2254	250	2246	19	< DL	30

According to the 76/160/EEC Directive: [a] Compulsory standard (10000/100 ml); [b] Compulsory standard (2000/100 ml); [c] Guideline standard (500/100 ml); [d] Guideline standard (100/100 ml); [e] Non-standard for *E. coli*, SOMCPH and FRNAPH.

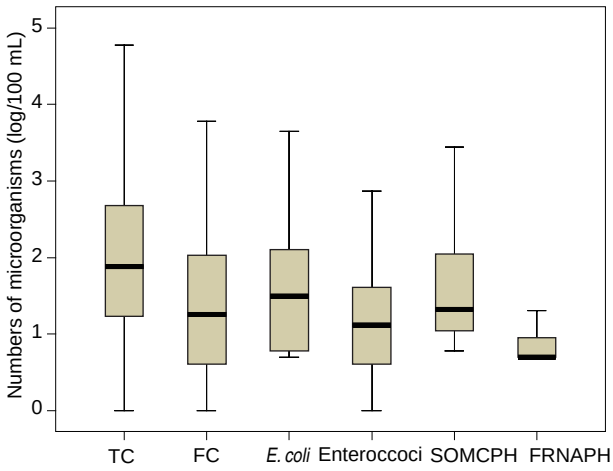

Figure 2 | Box and whiskers plots of counts of various indicators analysed in the bathing waters of Gipuzkoa 1998–2005. $N = 806$ for TC, FC, *E. coli*, enterococci and SOMCPH. $N = 427$ for FRNAPH.

The ratio between *E. coli* and SOMCPH was calculated for each of the sampling sites included in the bathing water quality monitoring system. The logarithm of the quotient of the counts was negative; that is, SOMCPH counts exceeded those of *E. coli* in 12 out of the 23 sampling sites (52%). Likewise, the ratio between enterococci and SOMCPH shows that in 22 out of the 23 sampling sites SOMCPH counts exceeded those of enterococci.

DISCUSSION

In 1998, the Bathing Water Monitoring Programme of the Basque Govenment's Health Department included the determination of bacteriophages, SOMCPH and FRNAPH

Table 3 | Pearson correlation coefficient (*r*) between different log-transformed numbers of indicator microorganisms in bathing waters*

	FRNAPH	SOMCPH	Enterococci	*E. coli*	FC
TC	0.41	0.71	0.74	0.82	0.90
FC	0.36	0.69	0.76	0.83	
E. coli	0.39	0.69	0.72		
Enterococci	0.31	0.57			
SOMCPH	0.49				

*806 samples, except in the case of FRNAPH where the number of samples is 429.

Table 4 | Mean values and ratios between *E. coli,* FC or enterococci and somatic coliphages in bathing waters with different levels of faecal pollution

E. coli*	***n***	***E. coli* GM****	**SOMCPH GM***	**Ratio***** : *E. coli* ÷ SOMCPH (95% CI)**
<100	624	15	19	−0.10 (−0.14: −0,05)
100–500	141	211	160	0.14 (0.03: 0.24)
>500	41	1270	361	0.48 (0.23: 0.71)
TOTAL	806	28	33	−0.03 (−0.07: 0.01)
Faecal coliforms*		***FC* GM****	**SOMCPH GM****	**Ratio***** : FC ÷ SOMCPH (95% CI)**
<100	610	10	18	−0.17 (−0.22: −0.12)
100–500	147	205	138	0.05 (−0.13: 0.23)
>500	49	1273	498	0.26 (0.05: 0.47)
TOTAL	806	24	33	−0.14 (−0.18: −0.09)
Enterococci*		**Enterococci GM****	**SOMCPH GM****	**Ratio***** : enterococci ÷ SOMCPH (95% CI)**
<100	699	9	25	−0.45 (−0.42: −0.47)
100–250	58	162	229	−0.15 (−0.05: −0.24)
>250	49	421	369	0.07 (−0.05: 0.18)
TOTAL	806	13	33	−0.39 (−0.37: −0.42)

*Enumeration in 100 ml of bathing water sample.
** GM: geometric mean.
*** Ratio between *E. coli* and SOMCPH: Log *E. coli* - Log SOMCPH; ratio between FC and SOMCPH: Log FC - Log SOMCPH; ratio between enterococci and SOMCPH: Log enterococci - Log SOMCPH.

and *E. coli*, in the list of parameters routinely monitored according to Directive 76/160/EEC. The use of standardised techniques enables us to easily obtain information on viral indicators. According to the time series data, the counts of bacterial indicators and SOMCPH are of the same order of magnitude in the bathing waters of Gipuzkoa, but FRNAPH counts are lower. Both bacteriophages have been found in all the beaches and sampling sites studied, and their presence extends to the different levels of sewage pollution found in European beaches (Contreras-Coll *et al.* 2002), which are also observed in the beaches of Gipuzkoa.

The correlations between the bacteriological indicators were strong or moderately strong, while the correlations between TC, FC, *E. coli* and enterococci with SOMCPH and FRNAPH were significant but lower than the former. Weaker correlation values suggest that the information provided by bacteriophages is different from that provided by bacterial indicators. This may be attributable to their differing level of sensitivity to environmental factors. The low correlation observed between SOMCPH and FRNAPH is, to a large extent, provoked by the significant percentage of negative samples for the latter viral indicator, compared to the high percentage of positive samples for SOMCPH. The differing percentages of positive samples for viral indicators may be caused by the capacity of SOMCPH to replicate in the water, outside the human and animal gut (Seeley & Primrose 1980; Vaughn & Metcalf 1975), against the slim chances of this happening in the case of FRNAPH (Novotny & Lavin 1971).

The negative ratio between FC or *E. coli* and SOMCPH (the numbers of coliphages exceed those of bacterial indicators in 6 out of the 15 sampling sites of the Gipuzkoan coast meeting Directive 76/160/EEC, and in 5 out of the 8 sampling sites not complying with it), along with the fact that the ratio between enterococci and SOMCPH is negative in 22 out of the 23 sampling sites, shows the predominance of that indicator in many of the samples, and also in beaches with differing levels of bathing water quality.

The ratio between FC, *E. coli* and enterococci with SOMCPH changes sign: from a positive ratio in waters with higher numbers of bacterial indicators, i.e. higher bacterial counts than SOMCPH counts, to a negative ratio in waters with low numbers of bacterial indicators, i.e. lower bacterial counts than SOMCPH counts. Therefore, waters which are best for bathing tend to have higher numbers of bacteriophages than bacterial indicators. This change has been described previously for FC in British beaches with different levels of sewage pollution (O' Keefe & Green 1989) and also for E. *coli* and enterococci in beaches of the Atlantic and Mediterranean coast of Europe (Contreras-Coll *et al.* 2002). The capacity of SOMCPH to replicate is unlikely to be related to this phenomenon, since the highest numbers of viral indicators are found precisely in waters with low levels of microbiological pollution.

The higher resistance of bacteriophages to sewage treatments, as well as to the effect of environmental factors (Gironés *et al.* 1989; Chung & Sobsey 1993; Hill & Sobsey 1998; Sinton *et al.* 1999), may explain the differences in the presence of bacteriophages and between these and the bacterial indicators studied. Thus, considering that: (1) bacteriophages may be more abundant in water with a good bacteriological quality, (2) the correlation between bacterial indicators and bacteriophages is moderate, (3) the pathogenic microorganisms which have been attributed the capacity to be waterborne are mainly viruses, and (4) the capacity of such viruses to resist in the environment is stronger than that of bacterial indicators, it would be appropriate to incorporate viral indicators of bathing water quality into the monitoring systems, for instance the determination of SOMCPH.

The main drawback of using a viral indicator to monitor bathing water quality is that the correlation between the pathogenic viruses and bacteriophages found in the water is not consistent. Thus, while some studies emphasise the good correlation between enteric viruses and coliphages and the absence between the latter and bacterial indicators (Jiang *et al.* 2001; Ballester *et al.* 2005; Mocé-Llivina *et al.* 2005), other studies have not found such a relationship (Jiang & Chu 2004; Vantarakis *et al.* 2005; Dizer *et al.* 2005). Moreover, the lack of epidemiological information to prove a clear link between levels of pathogenic viruses or viral indicators and the type of morbidity most frequently associated with bathing, either gastrointestinal (Kay *et al.* 1994, 2001; Prüss 1998) or respiratory (Fleisher *et al.* 1996), makes it difficult to determine which is the best indicator to be used and which are its reference values. In this respect, some information has been provided recently on the possible reference values for somatic coliphages based on the no-observed-adverse-effect levels obtained in randomised controlled trials conducted in freshwater bathing areas of Germany (Wiedenmann *et al.* 2006). This adds a further element to the discussion on the feasibility of using viral indicators to control bathing water quality.

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