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Bacteriophages in bathing waters

"A feasibility study on the development of a method based on bacteriophages for the determination of microbiological quality of bathing waters"

COMPETITIVE AND SUSTAINABLE GROWTH

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CHEMICAL ANALYSIS

Bacteriophages in bathing waters

"A feasibility study on the development of a method based on bacteriophages for the determination of microbiological quality of bathing waters"

J. JOFRE(1), F. LUCENA(1), K. MOOIJMAN(2), V. PIERZO(3), R. ARAUJO(1), M. BAHAR(2), C. DEMARQUILLY(3) and A. HAVELAAR(2)

(1) Universidad de Barcelona
Departamento de Microbiología
Barcelona, Spain

(2) Rijksinstituut voor Volksgezondheid en Milieuhygiëne
Laboratory of Water and Food Microbiology
Bilthoven, The Netherlands

(3) Institut Pasteur de Lille
Services des Eaux-Environnement
Lille, France

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Contact: "Growth Infodesk" – European Commission, rue de la Loi, 200 (MO75 3/62), B-1049 Brussels
Fax: +32-2-296 67 57 – E-mail: growth@dg12.cec.be – UEL: http//www.cordis.lu/growth

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SUMMARY

Methods for the detection and enumeration of somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis* had been standardised and validated. Conditions for the preparation, transport and distribution of bacteriophage reference materials and preservation of samples had been defined. A method based on flocculation with $Mg(OH_2)$ with concentration efficiencies from about 40% was settled to concentrate phages from bathing waters. All methods were successfully implemented in routine laboratories all around the EU. Data on the occurrence of bacteriophages as compared to *E. coli* and Enterococci are available from diverse situations encountered in the EU. Results allow to conclude that the potential of phages for the determination of the microbiological quality of bathing waters merits to be considered since their determination is feasible and their behaviour in natural water differs from the behaviour of bacterial indicators and consequently they add valuable information.

1. Introduction

This project corresponds to a Dedicated Call for Proposals in Support of Community Policies entitled "A feasibility study on the development of a method based on bacteriophages for the determination of the microbiological quality of bathing waters".

The requests specified in the background information document produced by the European Commission as a supplement to the SMT (Standards, Measurements and Testing Programme) Dedicated Call (Annex 1) and consequently the objectives of the project can be summarized as follows:

✓ Making available standardised protocols for the concentration, detection and enumeration of bacteriophages (somatic coliphages, F-specific RNA bacteriophages and bacteriophages of *Bacteroides fragilis*) including collaborative studies for the validation of the methods.

✓ Implementation of the methods in a selection of laboratories representing diverse situations as encountered in the EU.

✓ Realization of field work to test the operability of the methods in diverse situations as encountered in the EU.

✓ Obtaining preliminary data on the occurrence of bacteriophages as compared to bacterial indicator organisms (*E. coli* and Enterococci).

Remark: Annexes 2, 3, 4 and 5 contain summarised basic information on bacteriophages.

2. Optimisation of methods for the enumeration of bacteriophages

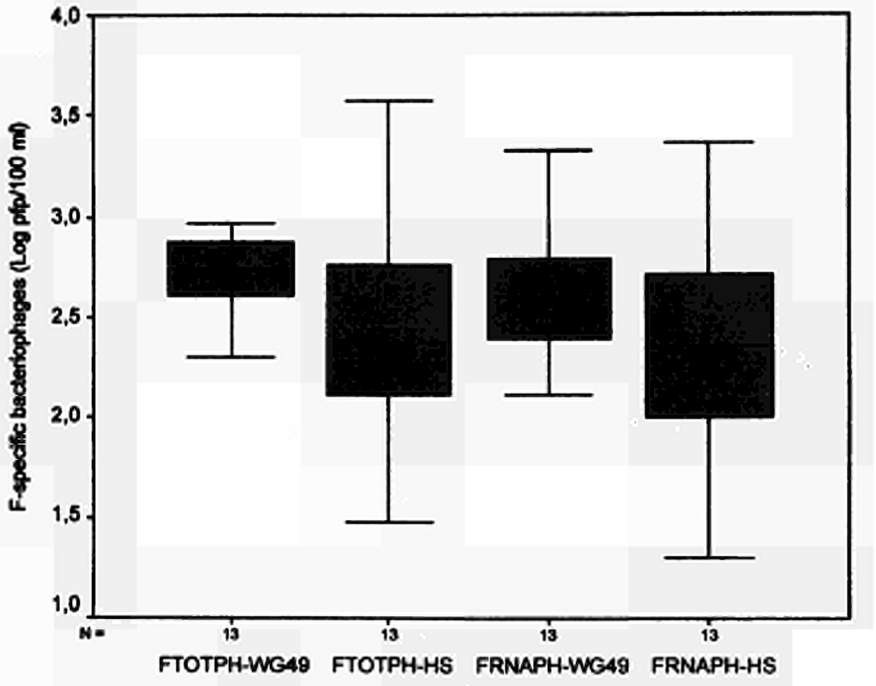
2.1 Somatic coliphages

At the start of the project, a methodology was available for the detection of somatic coliphages, both regarding the host bacteria and the procedures, and a draft ISO standard (ISO/CD 10705-2, August 1995) was discussed. There were some remaining methodological details to be defined for the final settling of the method. Most of these details were resolved for the account of this project (Mooijman *et al.*, 1999b). Probably the most important pending question was about the use of either the single agar layer method (SAL) or the double agar layer method (DAL) for counting plaque forming particles (pfp). Finally the chosen method was the DAL. The method to determine the viable counts of the host culture, the non-viability of using frozen inoculum cultures and the convenience of shaking the inoculum culture were other main contributions of the project to the final ISO standard (Mooijman *et al.*, 1999b) which was in the final stage of approval in the beginning of year 2000.

2.2. F-specific RNA bacteriophages

An ISO standard (Anonymous, 1995) was available when the project started. However, few problems still remained regarding the method, some concerning the host and some concerning the procedure. A number of experiments have been done for the account of this project to solve some of those problems (Mooijman *et. al.*, 1999c). The first question concerned the choice of the host strain. In the ISO standard, *Salmonella thyphimurium* WG49 is mentioned as host strain. However, another promising host strain (*Escherichia coli* HS Famp) was selected by Debartolomeis and Cabelli (1991). A number of water samples of different locations (Bilthoven and Barcelona) analysed during the exploratory monitoring of the summer of 1997 were tested for F-specific RNA phages using both host strains. Data summarised in Figure 1 show that strain WG49 recovers in the great majority of samples slightly higher counts than strain HS. Therefore, after these data, the recommended strain to be used as the host strain for the detection of F-specific RNA bacteriophages is WG49.

Figure 1 .- Comparison of two host strains (WG-49 *Salmonella typhimurium* and HS *Escherichia coli*) for the enumeration of F-specific bacteriophages in bathing waters.



Note 1.- Also, according to data obtained by Mèlanie Schapper (EU fellowship SMT4-CT97-9012 associated to this project), to study the distribution of serotypes/genotypes of F- specific RNA bacteriophages in environmental samples, WG49 is the recommended strain. The recommended method for studying such a distribution is plaque hybridisation and strain WG49 gives better transfer of phages and of RNA from the plaques to the membranes (N+ hybond membranes). Strain HS of *E. coli* produced monolayers which contains some mucous substance that impairs the transfer. Serotypes/genotypes of F-specific RNA bacteriophages may have a potential to distinguish between human and animal faecal pollution.

The second problem regarding the host was the genetic stability (maintenance of the F-plasmid) of strain WG49. Different approaches regarding the composition of the culture media and maintenance have been conducted with little success. Therefore, further research needs to be done on this topic, and it will be worthwhile trying to make WG49 more robust. However the experiments done to optimise this problem have reinforced the importance of following carefully the procedures described in the ISO standard, including the control of the strain used (regarding the percentage of lactose negative colonies acceptable in working cultures).

Some experiments have been performed regarding the test procedure. Changes in the growth media, and the addition of a few additives to the assay medium have not led to major improvements of the method. In contrast it has been shown that the incoculum culture can not be stored in melting ice for more than two hours before performing the assay.

From the experiments performed for the account of this project it could be concluded that the existing ISO version (Anonymous, 1995) for the detection and enumeration of F-specific RNA phages was already of good quality and small changes in the text would only be necessary to better clarify the existing information. Changes were included in an amended ISO standard (Annex 7 in Mooijman *et al.*, 1998) that was used in this project.

⊖ *Note 2.- In the ISO standards for both somatic coliphages and F-specific RNA bacteriophages an alternative plaque assay is recommended for samples with low phage counts, based on increased volumes of media, host cell culture and sample and the use of larger plates to perform the test. However a few data obtained in the project (Mooijman et al., 1999b) and data obtained by other authors (Grabow et al. 1997) during the period of development of this project show that this alternative method gives significantly lower counts (less than 50%) than the conventional method. Taking into consideration the concentration efficiencies described further on, we will recommend a concentration and a posterior check of the concentrate by the standard method for samples with low counts. This will give about the same percentage of recovery and is less expensive.*

2.3 Bacteriophages infecting *Bacteroides fragilis*

At the project start, the methodology for the detection and enumeration of *Bacteroides fragilis* bacteriophages was far less developed than the methodologies available for somatic coliphages and F-specific RNA bacteriophages. Consequently more extensive research was needed on methodology. The two major objectives were: first improving recovery and second making the method more user-friendly (for details Jofre *et al*, 1999a; Puig *et al.*, 1998; Puig *et al.*, 1999a; Puig *et al.*; 1999b).

2.3.1. Improvement of phage recovery

To improve phage recovery, two approaches were followed: First the search for an optimal strain, and second optimal assay conditions.

Search for an optimal strain.- By improving phage recovery with strain HSP40 and by testing other *Bacteroides fragilis* strains.

a) Improvement of phage recovery by strain HSP40

It was tried to improve genetically the strain. However, it was not possible to obtain hypersensitive mutants. It was however possible to identify that the receptor for the most abundant phages detected by *B. fragilis* HSP40 are proteins from the outer membrane, which opens for the future the possibility of obtaining either mutants or better growing conditions (as for example λ phages and growing the host cell on the presence of manose) which will count higher numbers of bacteriophages. However, this aim exceeded the frame and possibilities of the project.

The second approach investigated changes in the assay conditions. The most important changes were:

- ✓ Composition of the assay medium by testing different components: carbon source (various sugars and various concentrations); different salt concentration, addition of tensoactive substances and addition of different combinations of antibiotics.

- ✓ Manipulation of the cultures of the host in the absence of oxygen.

The only change that improved significantly recovery was the addition of 0.25 mol/L bile to the culture medium increased (approx. increase by a factor of 2). However, the addition of 0.25 mol/L bile gave a turbid broth, which made it very difficult to follow the optical density of liquid cultures and consequently to standardise the growth of the inoculum culture (Jofre *et al.*,1999a). After confirming this difficulty in the second training session (Pierzo and Demarquilly, 1998) and in the second collaborative study (Mooijman *et al.*, 1999a), it was decided not to introduce bile into the medium.

The recovery of bacteriophages infecting *B. fragilis* was improved by a factor greater than 2, when the test was performed inside an anaerobic cabinet (Jofre *et al.*, 1999a). However,

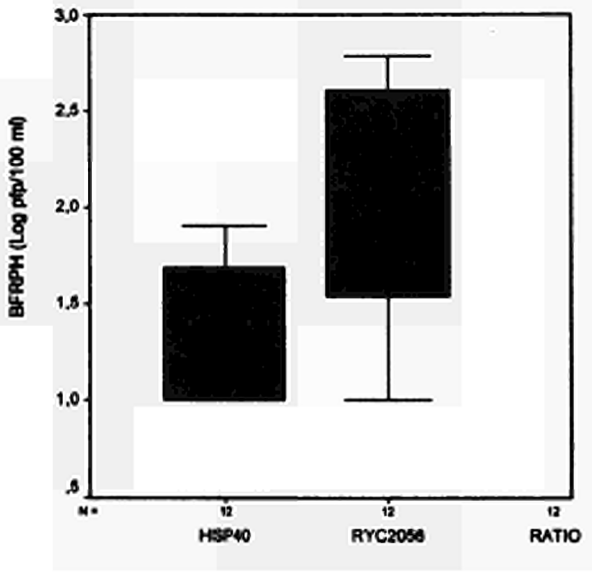
anaerobic cabinets are not a habitual facility in most routine laboratories, and consequently this approach was as well abandoned.

The use of an oxyrase enzyme (Oxyrase®), a complex of the membrane of *E. coli* that reacts with free oxygen rendering the environment anaerobic, was tested both to be used instead of anaerobic jars and to see whether its use improved the recovery of bacteriophages. The addition of Oxyrase® to the media and the application of a triple agar layer method to count pfp, allowed to achieve results with *Bacteroides fragils,* equivalent to those obtained on an aerobic bacteria (Puig *et. al.* 1998). The addition of Oxyrase® to the media and to the sample before the quantification assay, also improved clearly (by a factor of 2) the recovery of bacteriophages (Jofre *et al.*, 1999a). Oxyrase® is very promising, however the present commercial form (liquid that should be kept at low temperatures) makes its use difficult in routine. The potential availability of a dry Oxyrase® form would make its use recommendable.

b) New host strains.

B. fragilis HSP40, the available host strain at the beginning of the project, is considered to be able to detect around 10^4 phages per 100 ml in raw sewage from different geographical areas (Spain, Israel, South Africa, France). However several authors (non published data) reported that in other geographic areas, the number of phages detected by strain HSP40 was much lower. In order to find a more convenient strain, 115 new strains of *B. fragilis* were assayed for recovering phages from sewage. Among all of them, RYC2056 was selected because it allows to recover higher numbers of phages than strain HSP40 in the areas were strain HSP40 demonstrated good recovery performances, and moreover it allows to recover similar numbers of phages in sewage from all geographical areas tested in Europe. Data obtained in the preliminary monitoring showed that the ratio between the number of phages detected by and HSP40 in bathing waters was similar to the ratios obtained in sewage (Figure 2), thus indicating a similar behaviour in nature. In contrast RYC2056 detects also bacteriophages in faeces of animals and sewage from slaughterhouses. However, the ratio of phages detected by RYC2056 versus other faecal micro-organisms (faecal bacterial indicators, somatic coliphages and F-specific bacteriophages) is much lower in samples with faecal pollution of animal origin than of human origin (Jofre *et. al.* 1999a, Puig *et al.*, 1999).

Figure 2.- Comparison of numbers of phages detected by strains HSP40 and RYC2056 of *Bacteroides fragilis* in bathing waters.



2.3.2 Practicability of the method

The slow growth of the host strain, the frequent contamination of the cultures, probably caused by the richness of the medium and the low survival rate of the HSP40 cells when freezing with the cryoprotector (5% glycerol), made the method cumbersome. Therefore, efforts were made to make the method more user-friendly.

After having tested different combinations and concentrations of antibiotics, the addition of kanamycin and nalidixic acid to the culture medium allowed to prevent contamination of the host cultures and to prevent the growth of background flora when testing polluted samples (Jofre *et al.*, 1999a).

A number of cryoprotectors have been tested and a mixture of Bovine serum albumin factor V and sucrose proved to be an excellent carrier for both *B. fragilis* HSP40 and *B. fragilis* RYC2056. Frozen cultures of both strains, stored at -70°C, keep high numbers of viable cells after several months (at least 12) and cultures obtained by direct inoculation of inoculum cultures with small aliquots of frozen working cultures reach the necessary numbers of cells

to perform the assay in 2-3 hours. The same number of bacteriophages is recovered as for the inoculum cultures obtained by the usual way (Jofre *et al.*, 1999a).

Many details regarding the final procedure, (e.g. the enumeration of host strain, the possibility of storing for several hours the inoculum culture in melting ice, the time storage of the media, etc.), have been investigated during this project. They took into consideration the results of the specific research on the methods (Jofre *et al.*, 1999a) and observations made during the training sessions and the collaborative studies (Mooijman *et al.*, 1998; Mooijman *et al.*, 1999a; Pierzo *et al.*, 1998; Pierzo and Demarquilly, 1998). All these developments have allowed the settling of a test method similar in complexity to the methods described for the other bacteriophages. The only difference lays in the precautions that need to be taken because of the anaerobic nature of the host strains. An ISO standard benefiting from the research (Anonymous, 1999b) was sent as a DIS inquiry at the end of 1999. The method can be used to detect phages infecting strains HSP40 and RYC2056 of *B. fragilis*. However, because of the low number of phages detected by strain HSP40 in some European areas, the ISO standard will be established for strain RYC 2056.

Both *B. fragilis* RYC2056 (ATCC 700786) and reference phage B56-3 (ATCC 700786-B1) are available from ATCC.

3. Reference materials

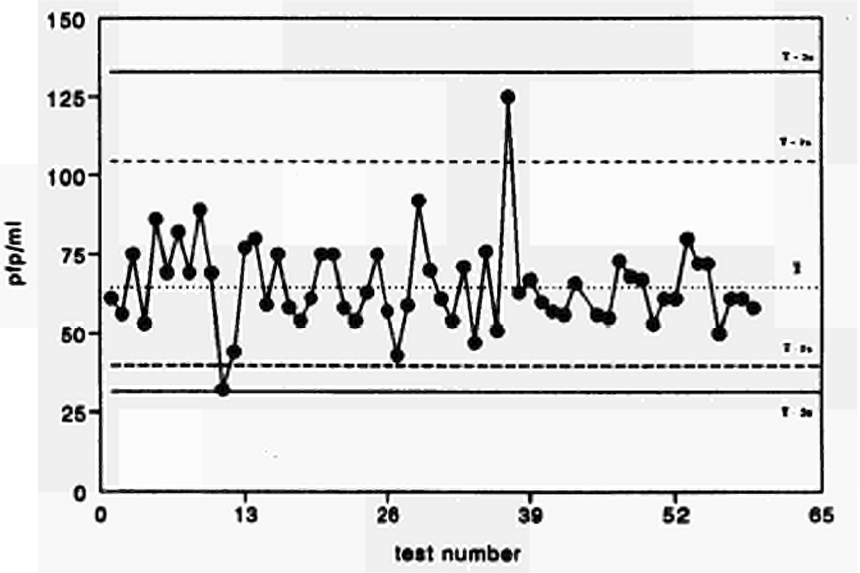
Research was carried out for the development of suitable reference materials for the validation of the methods as well as for quality control purposes during the monitoring campaigns.

Several batches of reference materials (RMs) containing pure phage cultures, as well as two kinds of naturally polluted standard samples, one containing a mixture of naturally occurring phages and background flora and the other containing a mixture of naturally occurring phages were prepared and tested for homogeneity and stability. Homogeneity was calculated according to T_1 (a measure for homogeneity within vials) and T_2 (a measure for homogeneity between vials) tests (Heisterkamp et al., 1995). Stability was determined by the preparation of

control charts (Dommelen, 1995). For an optimal homogeneous batch, T_1 is not significantly different from a χ^2-distribution with I (J-1) degrees of freedom where I is the number of vials and J the number of replicates. Furthermore $T_2/(I-1)$ should be $\leq$ 2.

It has been shown that homogeneous and stable reference materials (RMs) containing pure phage cultures could be prepared for the three types of bacteriophages analysed during the project: somatic coliphages, F-specific RNA bacteriophages and phages infecting *Bacteroides fragilis* (Mooijman et al., 1999d). The reference materials were prepared by mixing phage suspensions with glycerol (5% v/v) and storage of the final materials at -70° C. In Figure 3 an example of a control chart of a batch of RMs containing MS2 (FRNAPH) is given. This batch (batch 220597) fulfilled the criteria for homogeneity. At a geometric mean level of 66,5 pfp/ml, T1 was not significantly different from a χ^2 – distribution and T2/(I-1) was equal to 0,7.

Figure 3.- Characteristics and control chart of RMs containing MS2 (batch 220597), enumareted with host strain WG49 *Salmonella typhimurium*.



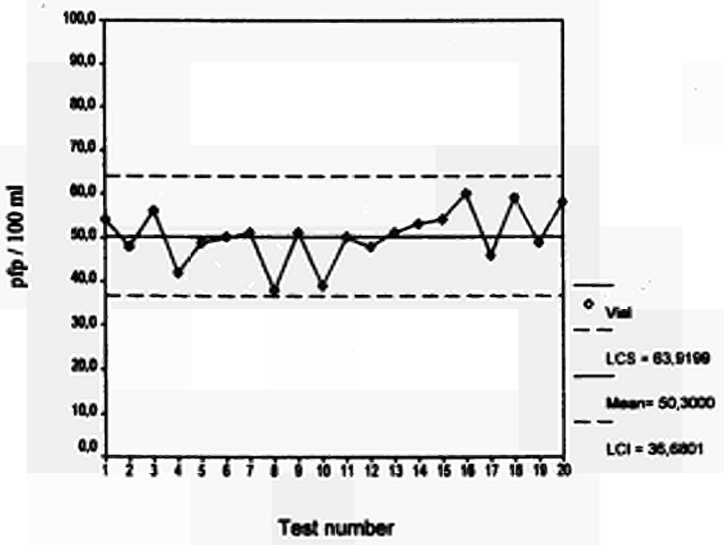
The standard phages used were ϕX174 for somatic coliphages, MS2 for F-specific RNA bacteriophages, B40-8 for *B. fragilis* HSP40, and B56-1 and B56-3 for *B. fragilis* RYC2056. B56-1 was first selected for RYC2056, but during the preparation of the reference material it

was shown that it presented a high frequency of mutants giving very small plates. During the last months of the project, a new standard phage, B56-3, has been chosen that shows a much more suitable behaviour than B56-1. B56-3 was used to prepare reference materials, which fulfilled both the homogeneity and the stability criteria, comparable to the RMs of the other standard phages (Jofre et al., 1999a).

The naturally polluted standard samples were prepared in a similar way as the standard phage RMs. For this purpose sewage was mixed with a peptone saline solution and glycerol. However, these naturally polluted standard samples showed more variation in results than the standard phage RMs. This may be due to the existence of aggregates, or variability in size and shape of plaques and/or disturbances because of bacterial background flora, or difficulties in reading the plates (Mooijman et al., 1999d).

The RMs containing a mixture of naturally occurring phages were prepared as follows. Sewage was centrifuged at low speed. The supernatant was thoroughly shaken and filtrated through a 0.22 µm filter. The filtrate was thoroughly shaken and then mixed with a peptone saline solution and glycerol to a final concentration of 5 % (v/v). The RMs prepared in this way were as homogeneous and stable as the standard phage suspensions (Jofre et al., 1999a) (see Figure 4).

Figure 4.- Characteristics and control chart of RMs, naturally occurring phages, enumerated with host strain RYC2056 *Bacteroides fragilis*.



- Results of RMs (naturally polluted standard samples) containing BFRPHRYC2056, batch 191096, Geometric mean (pfp/ml) 49,945; T_1 : 1,940; T_2: 0,230; I. 4; Critical values of X^2 - distribution (which T_1 should fulfil) at 2 or 1 degrees of freedom and 95% confidence: 5.99 and 3.84 respectively.

The stability of the reference materials at different temperatures is described in the following section. However to get repeatable and reproducible results the phage RMs should be kept frozen.

4. Conservation and transport of reference materials and samples

Conservation and transport conditions of reference materials and samples containing bacteriophages are similar for the three groups of bacteriophages studied in this project.

4.1 Conservation of samples, reference materials and concentrated samples

Samples stored at 5 °C for increasing periods of time, containing low numbers of phages, showed a faster decrease in the numbers of bacteriophages than samples with high contents. This effect was more significant for F-specific bacteriophages. However, it is recommended that samples should not be stored more than 48 hours before analysis at 5 °C (Jofre *et. al.*, 1999b). After 48 h a slow but steady decrease in the numbers of phages is detected. During the monitoring exercise, samples were never analysed more than 24 hours after sampling.

Naturally polluted samples (i.e. sewage) and bacteriophage reference materials have shown a good long-term stability (several months, up to 1.5 years have been observed for RMs used in the second collaborative study) when stored at (-70±10) °C (see Figures 3 and 4) and also when stored for a short period of time (*ca.* 1-2 months) at (-20±5) °C. However, when either samples or reference materials were stored at temperatures above 0 °C (5 °C or at room temperature) a rapid decrease in the mean number of plaques was visible for all phages tested (Mooijman *et al*, 1999d). The conservation of frozen RMs and samples requires the addition of cryoprotectors, i.e. 5% (v/v) glycerol can be used for the three groups of bacteriophages (Mooijman *et al*, 1999d; Jofre *et al.*, 1999b).

The stability at –70°C of the different groups of bacteriophage concentrates obtained by flocculation with magnesium chloride showed different survival rates (Jofre et al. 1999b). The F-specific bacteriophages seem to be more sensitive than the other two tested groups.

4.2 Thawing of frozen reference materials and naturally polluted samples

Effect of thawing conditions could not be demonstrated. The number of phages found after the sample was thawed for 30 minutes at room temperature or 2.5 minutes at 37 °C was negligible. For SOMCPM measured in naturally polluted samples, slowly thawing of the vials at room temperature resulted in a higher mean phage count when compared to the results obtained from vials which were quickly thawn at 37 °C (Mooijman, 1999d).

Some samples were submitted to successive temperature changes, including more than one freezing and thawing. Some of the phage suspensions, e.g. ΦX174, tolerated relatively well the operation but others, e.g. B40-8, did not (Jofre et al., 1999b).

4.3 Transport of samples and reference material

Transport, as well as conservation of samples, should be done at 5 °C. After 48 hours some of the bacteriophages, mainly F-specific RN bacteriophages, suffer a significant decrease in numbers (Jofre et al., 1999b).

The decrease in the mean number of plaques after storage exceeding 48 hours above 0 °C and the bad tolerance to repeated freeze-thawing, obliges to transport the samples and RMs frozen (e.g. mailing of the materials in dry ice).

The good survival of frozen phages has allowed to send reference materials and samples (sewage) to the different European laboratories participating in the project. The good survival of some phages under some storing conditions described above may give additional flexibility to monitoring studies.

5. Concentration methods

A number of methods potentially useful for concentrating bacteriophages were assayed with spiked samples. Two alternative methods were retained for further developments. These methods were chosen on the basis of the three following criteria:

a) it should concentrate somatic coliphages, F-specific RNA bacteriophages and phages *infecting B. fragilis*;

b) it should not require extra or sophisticated equipment (i.e. ultrafiltration was discarded)

c) it should be applicable to all sort of bathing waters (fresh water, sea water, clear water, turbid water).

Several methods based on filtration (adsorption-elution) on different supports and several methods based on flocculation with different flocculants were screened. Table 1 summarises the potential of the methods tested on spiked samples (Jofre et al., 1999c).

Table 1.- Comparison of concentration methods of bacteriophages in bathing water.

Method of concentration	SOMCPH	FRNAPH	BFRPH
Inorganic membranes[1]	+	-	++
Mixed cellulose acetate/nitrate filters[2]	++	++	++
Modified cellulose acetate filters [3]	-	-	-
Other membrane filters	-	-	-
Electropositve filters. Z-plus MK100 [4]	-	-	NT
Cellulose nitrate filters[5]	++	++	++
Organic flocculation [6]	++	+	+
Flocculation in beef extract with amonium sulfate [7]	++	++	++
Flocculation with magnesium hydroxide [8]	++	++	++

NT : non tested. ++ mean recoveries of 50±20% ; + mean recoveries < 30%; - recoveries < 10%

1.- ANODISC ® Lucena, F., et al. (1995); 2.- L.W. Sinton, et al. (1996); 3.- Farrah, S.R. and Preston, D.R.(1985); 4.- Sobsey M.D. and Glass B.L. (1980); 5.- Seeley N.D. and Primose S.B., (1988); 6.- Katzenelson, E., et al. (1976); 7.- Shields, P.A., and Farrah, S.R.(1985); 8.- E.Schulze and J. Lenk. (1983).

From all the methods screened, two based on adsorption-elution and two based on flocculation showed good potential as shown in Table 1. However, only two of these methods were chosen and studied in more detail since they were more simple and required either any or very little extra equipment or material.

The filtration in mixed cellulose acetate/nitrate filters and flocculation with magnesium chloride were further studied. The original methods (Sinton et al., 1996 and Schulze and Lenk, 1983) were slightly modified either to allow the concentration of the three groups of phages with similar efficiency or to improve efficiency. The efficiency of the two methods was extensively studied both with samples spiked with suspensions of pure cultures of bacteriophages representing the three groups of bacteriophages and with natural samples, both from fresh and sea water (Jofre et al., 1999c). Table 2 summarises the results of concentration efficiency for the three groups of bacteriophages in natural waters. Both methods were equally efficient for the three groups of phages and for both fresh and sea water. However, some problems of filtration of turbid waters, resulted in choosing the magnesium chloride flocculation methods through the rest of the project. Later, a prefiltration step for the filtration method has been developed that will allow its application to turbid waters (Jofre et al., 1999c).

Table 2.- Percentage of recovery of naturally occurring bacteriophages in bathing waters.

Method concentration	Bathing waters	SOMCPH % recovery Mean (sd)	FRNAPH % recovery Mean (sd)	BFRPH % recovery Mean (sd)
Flocculation	Seawater	49.0 (31.02)	36.0 (6.3)	42.5 (37.5)
	n[1]	*26*	*5*	*5*
	Freshwater	54.0 (27.1)	50.0 (24.5)	47.5 (40.3)
	n	*16*	*12*	*8*
Filtration	Seawater	42.0 (23.0)	67.5 (28.3)	44.5 (22.5)
	n	*35*	*24*	*10*
	Freshwater	54.0 (25.6)	49.0 (27.2)	35.5 (27.6)
	n	*15*	*16*	*6*

Note. Values with recoveries over 100% have been considered as 100%
1) number of samples tested

The flocculation method with magnesium chloride was slightly modified, and applied as indicated in Annex 6. The method was introduced in the second training session and in the second collaborative study. The results reported (Pierzo and Demarquilly, 1998; Mooijman et al., 1999a) did not differ substantially from data shown in Table 2. In the second collaborative study a very low concentration efficiency was noticed with phage ϕX174, the reference bacteriophage for somatic coliphages as shown in Table 2 and in the test performed during the second training session (Pierzo and Demarquilly, 1998). Phages like ϕX174 are only a very

small fraction of somatic coliphages (Muniesa, et al, 1999). Therefore, it was decided to use this concentration method for the monitoring. During the second collaborative study (Mooijman et al., 1999a) a large variability in recovery was observed. However, studies on recovery of viruses performed by experienced laboratories show also high variability (Melnick et al, 1984). An ISO standard for the validation of methods to concentrate bacteriophages (Anonymous, 1999c) is under development following the experience gained during the development of this project.

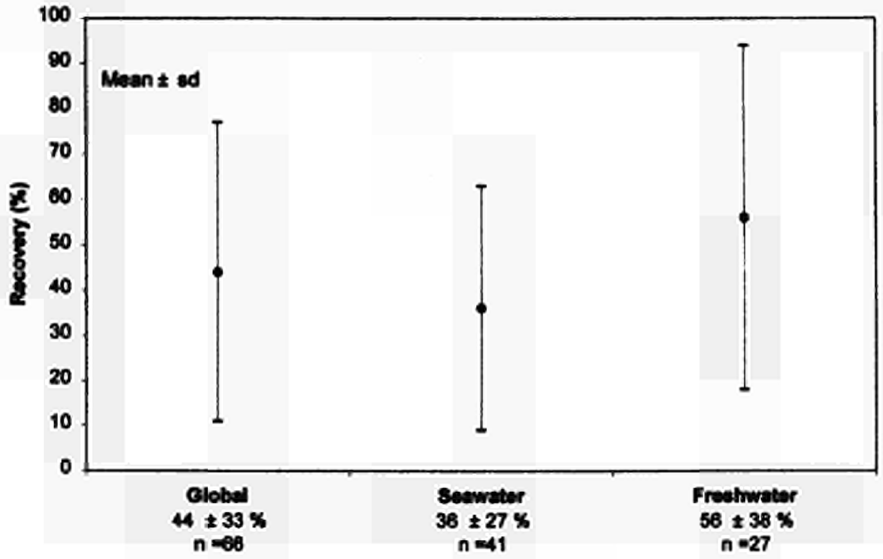
The recoveries of somatic coliphages obtained in the monitoring exercise performed by the partners in summer 1998 and by the participants in late spring 1999, are very similar and averaged 44 %. The recoveries were different according type of water, *ca* 60% (20%-80%) for fresh water and *ca* 35% (10 %-65%) for seawater, although the differences were statistically non-significant (Figure 5).

As a conclusion, a method based on flocculation with magnesium hydroxide was selected and adapted for this study, since it was:

easy to handle;
applicable to routine microbiology laboratories with no need of extra equipment;
applicable to different types of waters (seawater, freshwater, more or less polluted and/or turbid);
applicable to somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*.

The main disadvantages being that sometimes the plaques, mainly those of F-specific RNA bacteriophages, were difficult to read. The method is also time consuming, which makes it impractical for monitoring high numbers of samples.

Figure 5.- Evaluation of the recovery of naturally occurring somatic coliphages (results of 1999 monitoring).



Although the chosen method has been useful for the purpose of the project, further research on concentration methods for phages is recommended. Tests performed for the account of this project indicate that adsorption on cellulose nitrate filters may be a good alternative approach, but extra work regarding prefiltration needs to be done for analysing water samples with high turbidity.

6. Implementation of the methods in different laboratories and validation of the methods

One of the objectives of the project was to implement the methods in different laboratories representative of the various situations encountered in the EU. Besides the three partners, 15 laboratories, participated in the interlaboratory trials (Annexes 7 and 8). Many of the participants, with long experience in measuring bacterial indicators, had no or little experience in the determination of bacteriophages.

The implementation of the methods in these laboratories was programmed through training sessions and collaborative studies. Besides securing the right implementation of the methods

to provide data, the methods were validated regarding repeatability (r), reproducibility (R) and robustness (Anonymous, 1986).

6.1 Training of participants

Training of participants was done in two training sessions. They were organised by IPL. Detailed protocols were prepared and reference materials were provided by RIVM. A pretraining session was previously held with the three core partners.

6.1.1 Pretraining session

The 3 core partners IPL, RIVM and UB participated in the pretraining session in December 1996. (Pierzo and Hernandez, 1997). The objectives of this pretraining session were:

1. Evaluating the possible effect of some minor methodological changes on the methods for the detection of bacteriophages.

2. Checking the feasibility of the work-programmes tentatively designed for the first training session with all 15 partners. Two main points were evaluated:
   i) the quantity of daily work load without altering the quality of the work, taking into consideration that many of the participant, had no or little experience in the bacteriophages methods;
   ii) the feasibility of the logistics for the organisation of the training session.

Conclusion of the pretraining session:

- Methods:
  the results obtained in this session, allowed:
  i) to confirm strain *E. coli* WG5 as the suitable host for somatic coliphages;
  ii) to reinforce the choice of strain *Salmonella typhimurium* WG49 as the suitable host for F-specific RNA bacteriophages;
  iii) to obtain additional data reinforcing the choice of DAL instead of SAL method for the

detection of somatic coliphages;

iv) to become aware that special care should be taken in describing the procedure regarding the preparation of inocula for the preparation of cultures of *Bacteroides fragilis*.

- Workload:

  the experience gained allowed a good programming of the first training session. It was also decided to remove from the training session the presence/absence tests, which are included in the ISO standards. The reasons being to decrease on the one hand the quantity and diversity of work, and to reduce on the other hand the high risk of bacteriophage contamination during the amplification step and due to the high resistance of bacteriophages to dessication.

- This session allowed to fine tune the detailed protocols for the training session.

6.1.2 First training session

The objectives of this training session were:

1. Training of the participants in culturing and maintaining the bacterial host strains used for the three groups of bacteriophages (somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *B. fragilis* HSP40) using the enumeration methods as described in the ISO protocols

2. Training of the participants in enumeration methods for the three groups of bacteriophages on standard reference materials (suspensions of pure cultures of reference phages) and on natural samples (sewage samples).

Outcome of the training session:

- All participants learned to culture and maintain the host strains as well as to detect and enumerate bacteriophages. The method for *B. fragilis* was more difficult than those for the

other two groups of bacteriophages. This was likely due to the anaerobic nature of *B. fragilis* and to the fact that this method was still under development.

- The enumeration experiments were designed to obtain values for repeatability (r) and reproducibility (R')*, on all 3 types of bacteriophages (Table 3). Although, some repeatability (r) and reproducibility (R') problems were detected for F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis*, it could be concluded that non trained participants obtained very good results.

Table 2. - Repeatability (r) and reproducibility (R') values per method, using phage reference materials, obtained during the first training session.

	r	R'
SOMCPH	1.39	1.64
FRNAPH	1.53	1.71
BFRPH	1.41	2.04

* R' is the reproducibility obtained by different laboratories in assays performed in a central laboratory

- The enumeration of phages on sewage samples gave also good results. The values for somatic coliphages, F-specific RNA bacteriophages and *B. fragilis* HSP40 phages were similar to the values reported elsewhere (Jofre et al., 1999d).

It could be concluded that the performance of the 15 participants as regards to repeatability and reproducibility as well as the RMs and the organisation were quite satisfactory.

6.1.3 Second training session

The 15 participants plus one of the partners (RIVM) participated in the second training session, which was held in December 1997 (Pierzo and Demarquilly, 1999). The objectives of this training session were:

1. Training of participants on the method of concentration of bacteriophages from spiked and natural fresh and sea water samples.

2. Training of participants on the changes introduced into the method to enumerate phages infecting *B. Fragilis* e.g. media composition and host strain culture, maintenance, addition of bile and test of a new mixture of antibiotics in the media, the use of frozen working cultures in the procedure.

3. Enumeration of standard bacteriophage reference materials (RMs) as a first line quality control tool.

Outcome of the session:

- The concentration method for the enumeration of the natural bathing water samples did not pose technical problems. There were only few missing data due to technical problems and few participants showed outlying data. Results of phage enumeration on raw and concentrated spiked natural bathing waters gave additional information concerning the recovery of the concentration method. These data were similar both in mean values and variability to data reported elsewhere in this report (Jofre et. al, 1999c, Jofre et al., 1999d).

- The changes introduced into the enumeration method for bacteriophages infecting *Bacteroides fragilis* HSP40 caused difficulties to the participants and resulted in many failures in the tests. Because of these difficulties it was decided to wait for the results of the second collaborative study to decide whether or not to implement all or only some of the proposed modifications on this method.

- Finally, the repeatability (r) and the intralaboratory reproducibility (R') for the enumeration of RMs were determined from the positive controls performed each day. Results for somatic coliphages were as good as the ones of the first session. The values for r and R' for F-specific RNA bacteriophages were still good but higher than the ones of the first training session. For technical reasons the inoculum cultures were not checked for optical density, and this may be the cause of the increased variability. This confirms the necessity of controlling the optical density of the inoculum culture. The r and R' values for bacteriophages infecting *Bacteroides fragilis* were also higher than in the first session.

As indicated above, problems were detected in the implementation of methodological changes.

6.2 Collaborative studies

Two collaborative studies were organised and conducted by RIVM.

6.2.1 First collaborative study

The first collaborative study was organised in May 1997. Fifteen laboratories (plus the organising laboratory) participated in this study. Adapted protocols were prepared by the co-ordinator (Mooijman et. al. 1998). The objectives of this collaborative study were:

1. Testing the mailing of phage reference materials (RMs) to the different EU laboratories.

2. Testing the methods for detecting and enumerating somatic coliphages, F-specific RNA bacteriophages and phages infecting *B. fragilis* in different EU laboratories by using phage reference materials.

3. Identification of reasons for non-comparability of test results between laboratories, which may lead to possible modifications of the test protocols.

In spite of the little time between the training session and the collaborative study (problems to obtain materials, preparation of the media) all laboratories were able to participate successfully in the study.

Outcome of the study:

- The RMs can be successfully sent by courier service, packed in dry ice.

- Regarding the implementation of the methods it was concluded that except a few data relatively good results were obtained. After exclusion of some results due to technical problems, the analysis of variance indicated differences between laboratories, but no

outlyers were detected according to the Grubbs' test (Anonymous, 1988). Hence, it can be concluded that relatively good results can be obtained with the three assayed methods for the detection of phages in the reference materials. This was also shown in the values of the repeatability (r) and the reproducibility (R) (Table 3). The repeatability of all three methods was 1.35-1.38. This value is very near the theoretical lowest possible value. At a mean count of *ca.*70-90 pfp/ml and in the case of a Poisson distribution, this theoretical values is *ca.* 1.25 (Mooijman et al. 1992). The reproducibility (R) values are only slightly higher (1.52-2.04) than the values of r. It can therefore be concluded that the participating laboratories produced reproducible results. Consequently, the procedures described for the enumeration of the three types of bacteriophages were a good basis for further use in enumeration of phages in naturally polluted samples.

Table 3.- Repeatability (r) and reproducibility (R) values per method, using phage RMs during the first collaborative study

	r	R
SOMCPH	1.35	1.52
FRNAPH	1.38	1.73
BFRPH	1.38	2.04

The first collaborative study gave also useful information regarding the methods, the most remarkable being:

i) the possibility of introducing some flexibility at some stages of the somatic coliphages method (i.e. pH of media, or temperature);
ii) the preference of shaking the inoculum cultures of strains WG5 and WG49;
iii) the preference to calibrate in each laboratory the growth curve of host cultures in order to know when the inoculum culture is ready for the test, that protocols can not be based on incubation time only, and that the number of cells is important, in particular for F-RNA bacteriophages;
iv) some observations made during this study confirmed problems of robustness of strain WG49.

6.2.2 Second collaborative study

The second collaborative study was organized in March 1998 (Mooijman et. al. 1999a). The objectives of this study were:

1. The evaluation of the implementation of the methods for enumeration of the three groups of bacteriophages in naturally polluted (water) samples, in different EU laboratories: somatic coliphages (SOMCPH), F-specific phages, F-specific DNA phages (FDNA phages) and phages of *Bacteroides fragilis* (BFRPH).

2. The evaluation of the implementation of a concentration method (based on flocculation with magnesium chloride) for enumeration of the three groups of bacteriophages in a mixture of phage RMs in different EU-laboratories

3. The identification of reasons for deviating results in individual laboratories, possibly leading to modifications of the test protocols.

4. Like in the second training session, a first-line quality control of the analysis of the naturally polluted samples was set up. Pure culture phage RMs were analysed in duplicate.

Outcome of the second collaborative study:

The values of (r) and (R) found with the naturally polluted standard samples in the second collaborative study were higher than the values of (r) and (R) found with the standard phages RMs in the first collaborative study (Table 4). In particular, the (R) values were higher (e.g. *B. fragilis* phages) probably due to the problems reported by some laboratories for implementing the modified method. In addition, interpreting results of naturally polluted samples needs more experience than interpreting results of samples containing a pure phage culture.

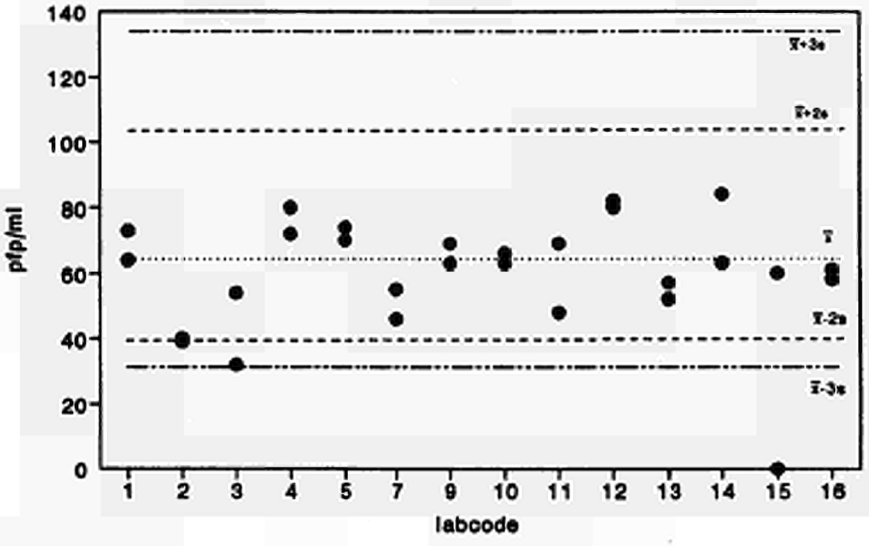
Table 4.- Repeatability (r) and reproducibility (R) values per method, using naturally polluted standard samples during the second collaborative study.

	r	R
SOMCPH	1.64	3.10
FTOTPH	1.63	5.19
FDNAPH	2.34	3.16
BFRPH	1.65	5.72

All the laboratories were able to apply the concentration method. The concentration technique showed low recoveries for the chosen standard bacteriophage, ϕX174, as explained earlier in this report. Large variations were found in the percentage of recovery of F-specific bacteriophages and phages infecting *B. fragilis*. They could not be explained by random distribution only. These results indicate that extreme care must be taken at each of the steps of the concentration procedure. Furthermore, it is preferable to store the concentrate in melting ice, instead of storage at room temperature, until phage enumeration is performed. As a consequence of the results of this study, the participants were advised to train themselves with the method before the bathing water monitoring exercise.

The results of the quality control for each laboratory were indicated in a control chart. These charts, for each phage, were made from data obtained by the organising laboratory (Figure 3). Still the majority of the results of the pure culture phages found in most of the participating laboratories were considered "in control" (see Figure 6 as an example and compare to Figure 3). This demonstrates a good quality in implementing the phage methods. However, some of the laboratories reported problems with the modified method for *B. fragilis* bacteriophages. This indicates that the modified method was less robust that the old method. Taking into consideration the quality control data and the data obtained during the second training session, it was decided that bile should not be added to the media, despite that this addition allowed higher recoveries of bacteriophages from water samples.

Figure 6.- Results of all participating laboratories (after exclusion of data), of phage RM containing MS2, batch 220597, drawn in the relevant control chart.



7. Field studies

A number of different kinds of natural samples were tested for the bacteriophage content and the presence of bacterial indicators, as requested by the dedicated call. These requests were:

- To test the operability of the method in diverse situations encountered in the EU
- To obtain preliminary data on the occurrence of bacteriophages as compared to bacterial indicator organisms.

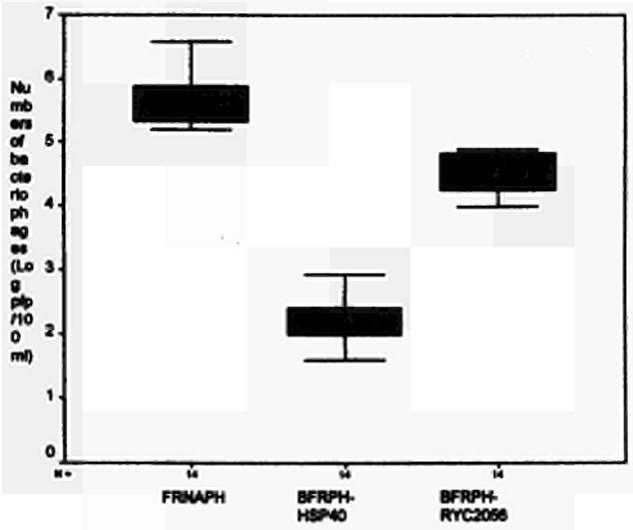
These requests were answered through the following studies:

1. Testing raw sewage for *B. fragilis* phages
2. Testing of widely distributed sewage for *E. coli*, Enterococci and phages
3. Exploratory monitoring of bathing waters
4. Monitoring of bathing waters performed by the partners during summer 1998
5. Monitoring of bathing waters performed by the participating laboratories during late spring 1999.

7.1 Operability of the method in diverse situations as encountered in the EU

When the project started, *B. fragilis* HSP40 was selected as host strain. However, personal communications of different researchers indicated that phages detected by this strain were very scarce in some geographical areas. Therefore, after the verification of the good performance of strain RYC2056 in the area of Barcelona (Jofre et al., 1999a, Puig et al. 1999a), its capability to detect bacteriophages everywhere in the EU was tested. Sewage samples from different sites in the E.U. (Austria, Denmark, France, Germany, The Netherlands, Ireland, Italy and Portugal) were frozen with 5 % glycerol and transported under dry ice to Barcelona. They were maintained frozen at -70 °C until testing. The results of the bacteriophage counts are summarised in Figure 7. They confirmed the great variability regarding the presence of *B. fragilis* HSP40 in sewage with very low numbers in some areas. Phages detected by RYC2056 were found in all sewage samples, in amounts ranging from 10^4 to 10^5 plaque forming particles per 100 ml. This is about one order of magnitude lower than the numbers of F-specific bacteriophages (Jofre et. al, 1999a).

Figure 7.- Box plots of numbers of bacteriophages infecting *Bacteroides fragilis* compared to F-RNA specific phages in sewage from different European countries.



E. coli, enterococci, somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *B. fragilis* RYC2056 were enumerated in sewage samples from Ireland, The Netherlands, Finland, France, Greece and Spain. The different micro-organisms are present in sewage from all these areas within similar proportions (Jofre et al. 1999d). Figure 8 shows the box plots of data of raw sewage from the indicated areas. Other authors (Araujo et al., 1997; Grabow et al, 1993, Havelaar et al., 1984, Nieuwstad et al., 1988) found results which were similar to the ones observed in this study.

Data summarised in Table 5 about the different microbial parameters studied during the monitoring of the bathing waters, indicate that all groups of bacteriophages are found in different areas of Europe, from the Baltic Sea to Spain and from Ireland to Greece, both in fresh and sea water.

Figure 8.- Box plots of numbers of microorganisms in sewage from different European countries.

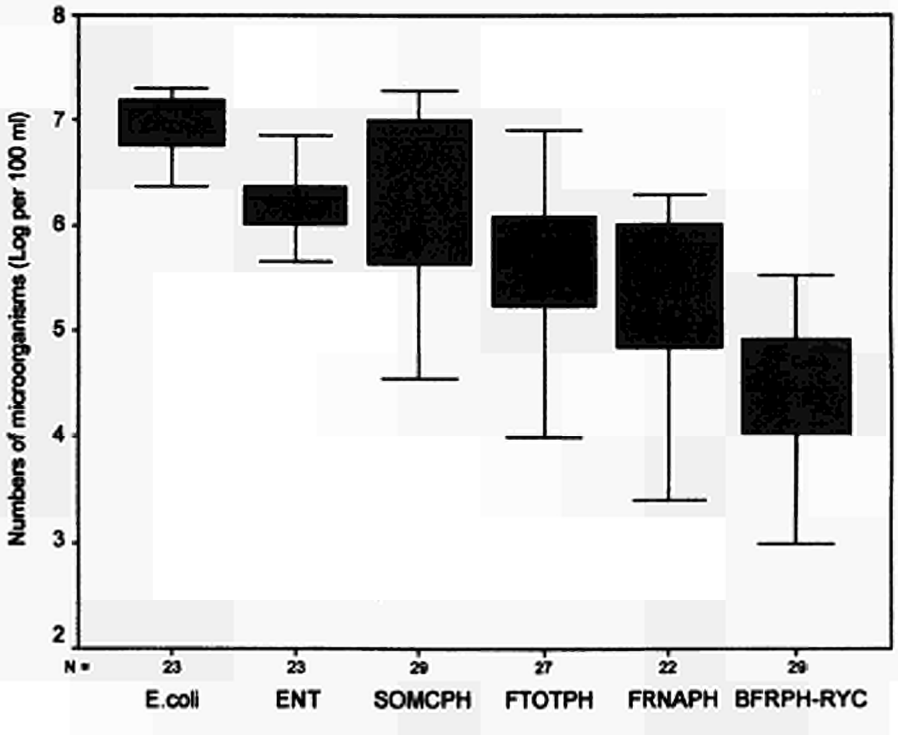


Table 5.- Percentage of samples containing the indicator microorganisms in bathing water.

	N (samples)	E.coli* (%)	Enterococci* (%)	SOMCPH** (%)	FTOTPH*** (%)	FRNAPH*** (%)	BFRRYC*** (%)
Bilthoven[1]	10	100	90	100	67	44	30
Tubingen[2]	14	93	71	71	50	50	58
Vienna[3]	10	100	100	100	100	100	75
Kiel[4]	10	100	100	100	60	30	40
Dublin[5]	12	100	92	75	33	25	67
Exeter[6]	10	100	50	100		30	40
Lille[7]	13	100	84	100	61	54	92
Athens[8]	6	100	100	100	100	67	100
Montpellier[9]	10	90	80	100	90	90	80
S.Sebastián[10]	15	80	80	100	47	47	100
Ferrara[11]	6	100	100	100	100	83	100
Barcelona[12]	20	79	70	100	95	47	95
Palma[13]	7	43	71	71	14	14	28

* In 9.6 ml;
** In a concentrate equivalent to 100 ml of sample;
*** In a concentrate equivalent to 300 ml of sample;

1) The Netherlands; 2) Central Germany; 3) Austria; 4) Northern Germany; 5) Ireland; 6) Souther England; 7) Northern France; 8) Greece; 9) Southern France; 10) Northern Spain (Atlantic) ; 11) Northern Italy; 12) Northern Spain (Mediterranean); 13) Northwestern Mediterranean.

Therefore, according to the data obtained on sewage and on bathing waters, it can be concluded that the methods for enumerating somatic coliphages, F-specific RNA and bacteriophages infecting *B. fragilis* RYC2056 are operational in diverse situations as encountered in the EU.

7.2 Occurrence of bacteriophages compared to bacterial indicators.

7.2.1 Preliminary monitoring

A preliminary monitoring was organised during summer 1997 by the partners (IPL, UB, RIVM). The main objectives were to obtain information about the levels of *E. coli*, enterococci, somatic coliphages, F-specific RNA bacteriophages and phages infecting strains HSP40 and RYC2056 of *Bacteroides fragilis* in a diverse set of bathing waters (fresh water, sea water, polluted, non polluted, turbid, clear) in order to design (methods, volumes to be

tested, etc.) and organise the monitoring of bathing waters to be done by all participants (Jofre et al., 1999d).

The preliminary monitoring allowed to:

i) establish the levels of the different groups of phages in bathing water samples compared to the levels *E. coli*;

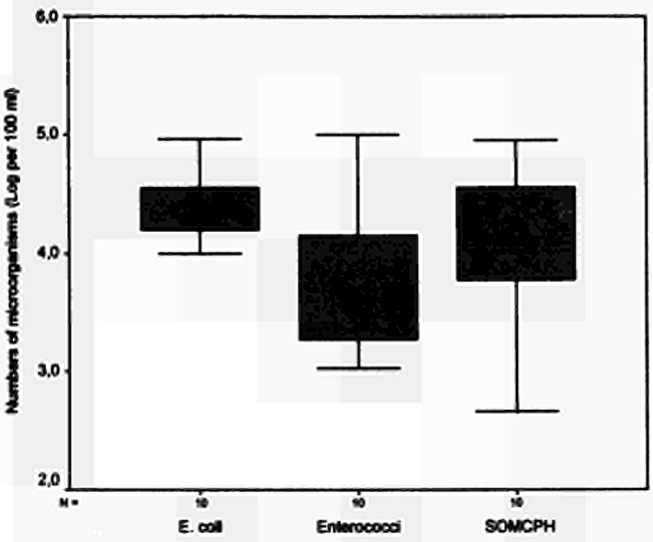
ii) detect the low presence of phages infecting *B. fragilis* HSP40 in the bathing waters of some of the areas;

iii) confirm the need of concentrating samples for the enumeration of F-specific RNA bacteriophages and phages infecting *B. fragilis* RYC2056;

iv) determine the volumes to be tested for each parameter during the monitoring to ensure a reasonable percentage of positive samples.

Additional information obtained from this study refers to data reported in the section on improvement of methods (comparison of recovery of phages with WG49 and HS, and the comparative recovery of phages by HSP40 and RYC2056 in the area of Barcelona). A valuable set of samples with *E. coli* numbers between about 10^4 and 10^5 cfu/ml (Fig 9) was obtained. They were not considered in the range of *E. coli* counts acceptable for water samples, but they show a proportion of *E. coli* and somatic coliphages similar to the proportions in sewage. This should be kept in mind for further studies on the potential effect of phage replication in the water environment on the somatic coliphage numbers.

Figure 9.– Numbers of *E. coli*, enterococci and somatic coliphages in fresh and seawater samples (*E. coli* between $10^4 - 10^5$ cfu/100 ml).



7.2.2 Monitoring of bathing waters

In order to provide preliminary information regarding amount of phages compared to bacterial indicators (*E. coli* and Enterococci) in diverse situations as encountered in the EU, a bathing water monitoring was scheduled for spring 1999.

Only few modifications were introduced into the monitoring programme as a consequence of the preliminary exercise of the partners (Jofre et al. 1999d). Protocols, data collection and analysis were mainly done by IPL and UB, whereas RIVM prepared and provided RMs for the first-line quality control during the monitoring.

- *Samples and sampling sites:*

Seawater from Barcelona, Lille, Dublin, San Sebastian, Palma de Mallorca, Montpellier, Athens and Exete

Freshwater from Bilthoven, Tübingen, Ferrara, Montpellier, Wien, Kiel.

Samples were recommended to contain, whenever possible, 50 and 2000 *E. coli* per 100 ml. To ensure as much as possible the desired level of *E. coli* in the samples, these were tested immediately after sampling for *E. coli* and stored overnight at 5 °C. If the numbers of *E. coli*

were in the desired range, the sample were processed for all parameters. In some areas it was rather difficult to find samples within the given contamination range.

- *Enumeration method:*

E. coli and Enterococci were mostly measured using the French standard microtiter plate methods (AFNOR NFT 90-433 and NFT 90-432 respectively). However, some of the participants used the standard methods of their respective countries.

Somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *B. fragilis* RYC2056 were tested according to the ISO draft methods indicated earlier. In the preliminary monitoring performed by the partners, bacteriophages infecting *B. fragilis* HSP40 were also measured. Because only low counts were found it was decided to delete the enumeration of these bacteriophages in the monitoring campaign. Bacteriophages were concentrated by the magnesium chloride flocculation method described in Annex 6.

Remark:

the Environmental Agency at Exeter (UK) participated in the monitoring, but applying a different monitoring scheme and methodology. They included phages in their routine sampling of bathing waters. Bacteria were analysed according to the British standards. Bacteriophages were concentrated by the British standard method for the concentration of enteroviruses (Seeley and Primrose, 1982). Aliquots of the concentrate corresponding to 100 ml of the bathing water sample were assayed for the three groups of bacteriophages, that were enumerated according to the ISO methods.

Physicochemical parameters as salinity, turbidity, water and air temperature were also measured and recorded.

- *Volumes of sample:*

The volumes tested were the following.

- For *E. coli* and Enterococci 9.6 ml
- Somatic coliphages were counted directly from 10 ml of sample and an aliquot of the concentrate equivalent to 100 ml of sample.
- F-specific-RNA bacteriophages were counted in the concentrate equivalent to 300 ml of sample. A total of concentrate equivalent to 600 ml of sample was

used to count DNA phages

- Phages infecting *B. fragilis* RYC2056 were counted in a concentrate equivalent to 300 ml of sample
- Phages infecting *B. fragilis* HSP40 were counted in a concentrate equivalent to 1000 ml of sample (only in the monitoring performed by partners).

- *Quality control:*

A first line quality control with RMs for bacteria and phages was followed during the entire monitoring by both partners and participants. The reference material for *E. coli* and Enterococci were PHLS lenticules (SMT4-CT96-2061). For bacteriophages pure cultures of ϕX174, MS2 and B56-1 were prepared as described above at the RIVM. The quality control results of each laboratory were plotted into a specific control chart made from data obtained by RIVM (Figure 10A). Still the majority of the results of the pure culture phages found in most of the laboratories were "in control" in the corresponding control charts (Figure 10B). Some participants reported problems with the small plaques given by B56-1. Precisely, these problems led to the choice of another phage (B56-3) as reference phage for *B. fragilis* RYC2056.

Figure 10A.- Control chart of RMs containing ϕX174, batch 270597, with host strain WG5 *Escherichia coli*.

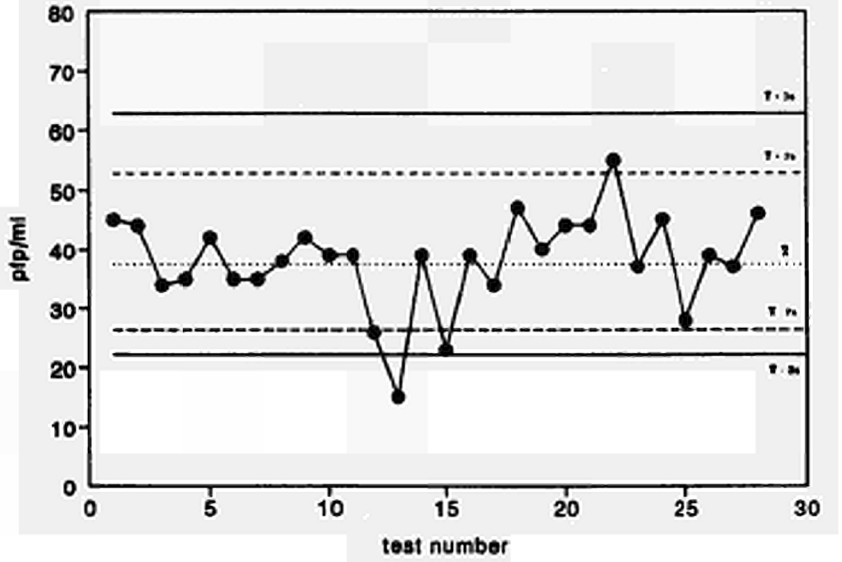
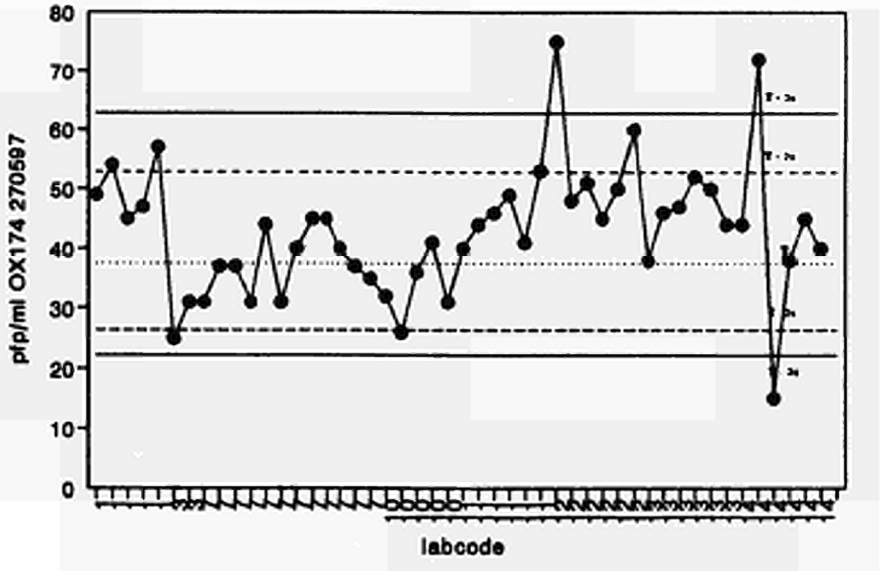


Figure 10B.- Control chart of RMs containing ϕX174, batch 270597, with host strain WG5 *Escherichia coli*.



Despite the fact that some laboratories used different methods to count *E. coli* and Enterococci, the great majority of values remained "under control" in the relevant control chart. The conclusion of this quality control study is that values obtained during the monitoring are fully reliable.

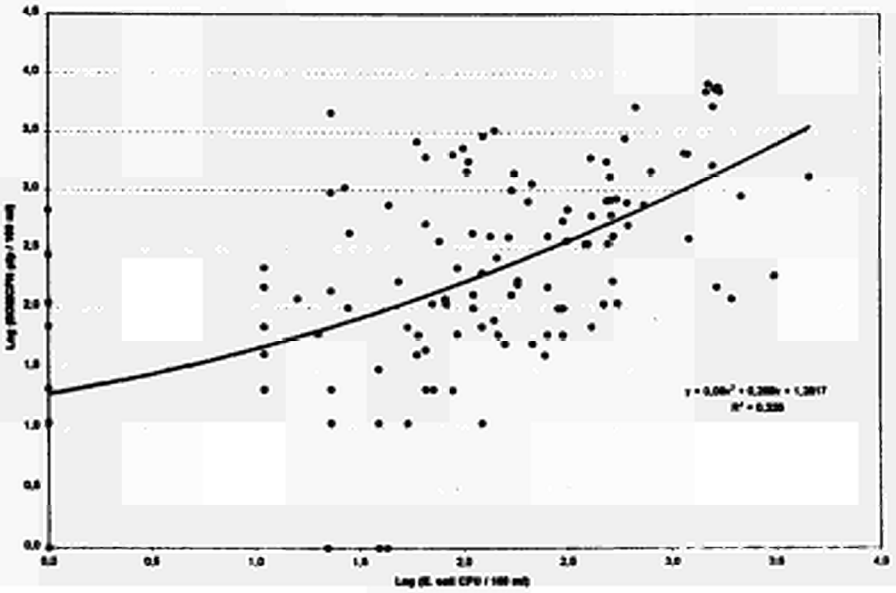
The monitoring exercise allowed to collect a large set of samples (147). Figures 11, 12 and 13 illustrate some of the results.

7.2.3 Conclusion

For log transformed values a correlation exists between *E. coli* and all the groups of phages.

However, the slope is changing according to *E. coli* numbers. The correlation diminishes clearly when the content of *E. coli* is close to zero. In the latter case, it seems that instead of a first order correlation, there is a second order correlation (Figure 11).

Figure 11.- Correlation of microorganisms in bathing waters.
E.coli versus somatic coliphages.



The statistical analysis indicates that more *E. coli* implies more phages. However, there is an indication that samples with lower levels of *E. coli* demonstrate other ratios; relatively more phages are detected.

The ratios between *E. coli* and phages in bathing waters differ from the ratios in sewage. The relative amounts of bacteriophages increases in bathing waters as shown in Figure 12, compared to Figure 9. This increase is much more evident in sea water samples containing low numbers (<100 CFUs per 100 ml) of *E. coli* (Figure 13). This observation is further illustrated by Figure 14. It shows a comparison for samples with ratios between low numbers of *E. coli* and numbers of somatic coliphages in sewage and in sea water. A comparison of the ratios *E. coli/phage* for the three groups of phages (Figure 15) in sewage and in concentrated sea water samples containing low numbers of E. coli seems to indicate that the relative increase is higher for phages infecting RYC2056. The most plausible explanation for the change in the ratios is that phages survive better than *E. coli* in the environment and that the inactivation factors seem to affect differently phages and bacteria. This change in ratios is more evident in the Southern seawater samples than in the Northern freshwater. Type of water, regional differences, distance between the sampling site and the pollution source, etc. seem to affect these ratios. However, with the available data it has not been possible to explain the changes by regression analysis taking in consideration a simple quantitative variable, as turbidity, or water temperature or salinity. The behaviour of phages with respect to Enteroccocci is similar to *E. coli*. More data based on a different sampling design are needed to obtain a better conclusion on the value of bacteriophages as indicator organisms.

Figure 12.- Box plots of numbers of microorganisms in bathing waters (includes all data of the monitoring).

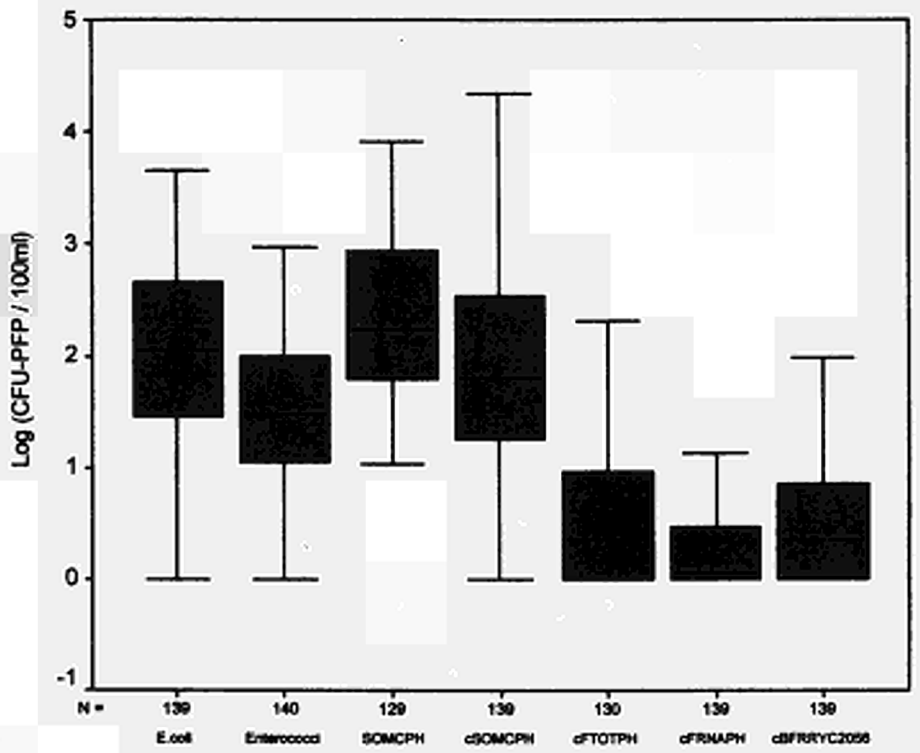


Figure 13.- Box plots of numbers of microorganisms in bathing water (includes seawater samples with *E.coli* < 100cfu/100 ml).

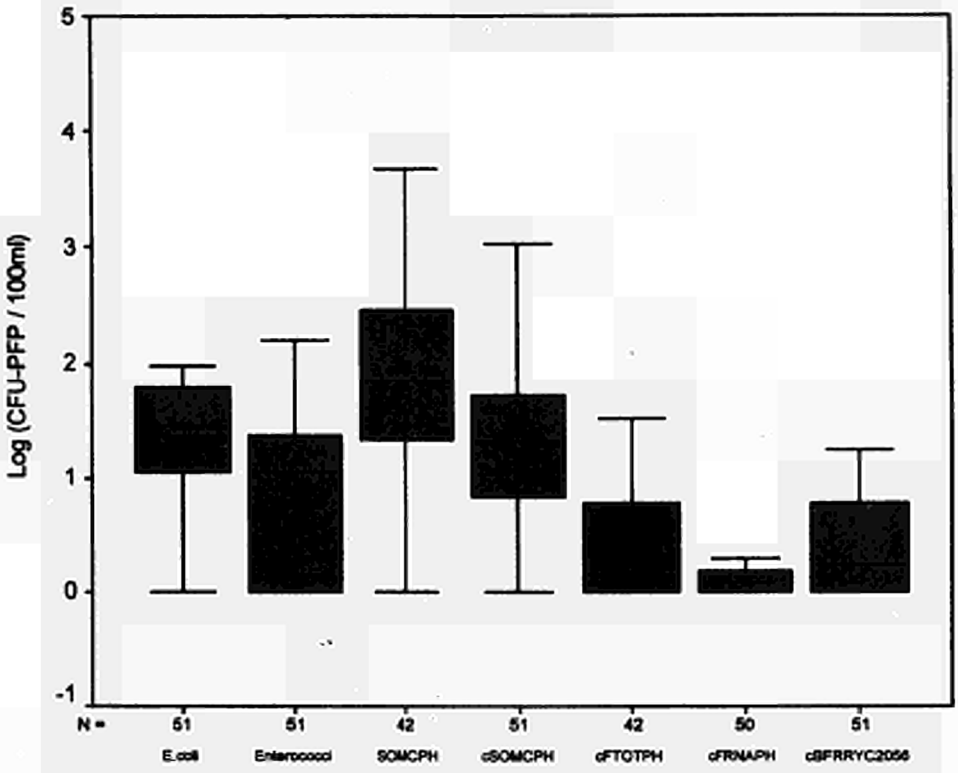


Figure 14.- Box plots of the ratios between numbers of *E. coli* and somatic coliphages in sewage and sea water samples containing 10-100 *E. coli* per 100 ml.

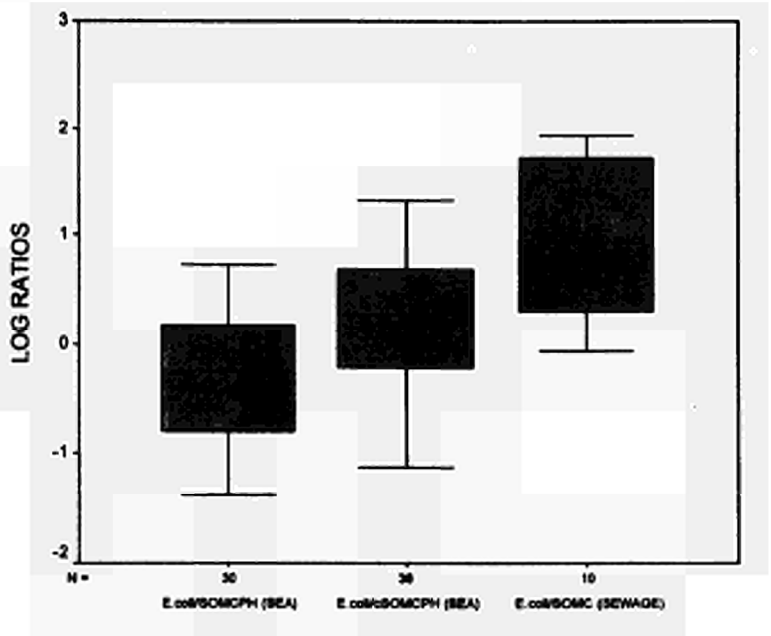
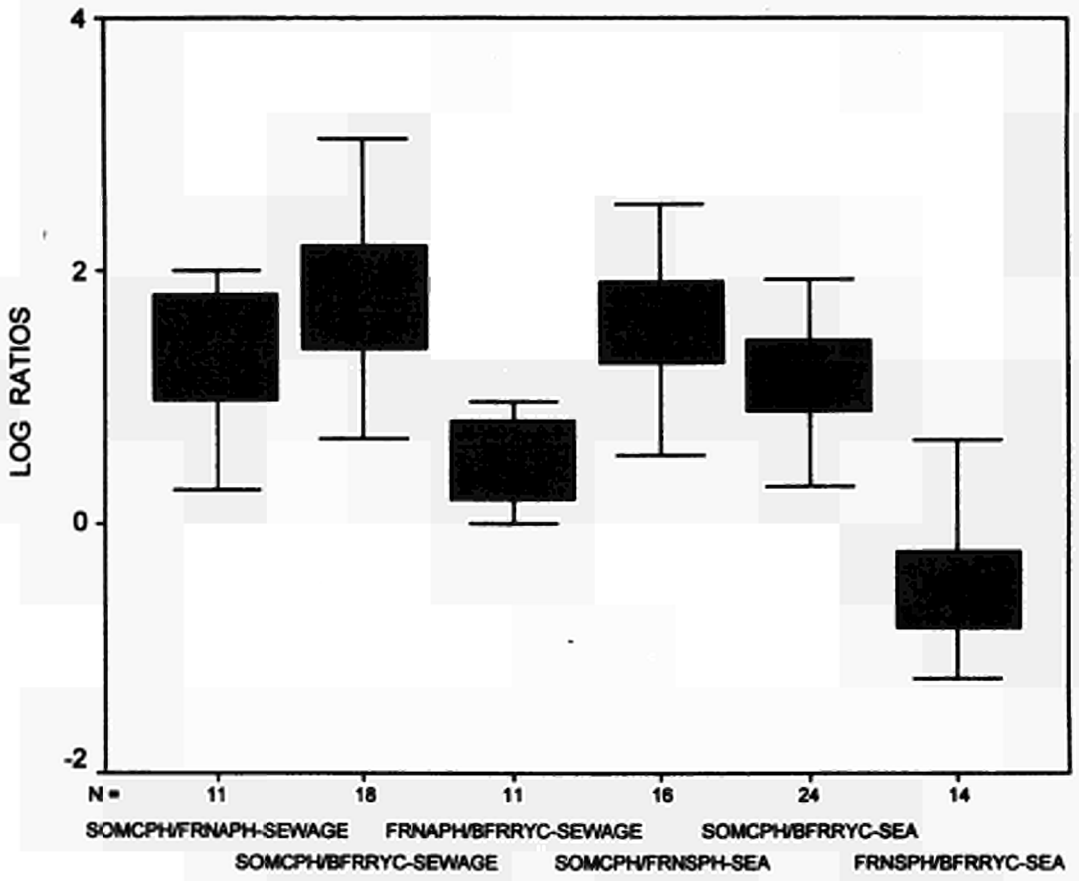


Figure 15.- Box plots of the ratios between numbers of the different groups of phages in sewage and in sea water containing 10-100 *E. coli* per 100 ml.



8. Candidate phage or phages for a possible standard

Although the choice of a candidate phage for a standard (maximum allowable level) was neither a requirement of the dedicated call nor an objective of the project, the results of this study allow to draw some preliminary conclusions.

The potential of phages to be used as additional reliable indicators for fecal contamination should be considered since their behaviour in natural water differ from the behaviour of bacterial indicators. It can not yet be decided which type of phages at which maximum acceptable amount should be retained. However, the data obtained in this project allow to make the following considerations regarding bacteriophages in bathing waters:

- ✓ The F-specific and consequently the F-RNA bacteriophages does not seem to provide much additional information compared to the present indicator bacteria regarding differential decay.

- ✓ Phages infecting *Bacteroides fragilis* seem to be the most informative due to their differential decay. It seems to be the most critical group of bacteriophages, mainly in seawater with low levels of *E. coli*. However, this group of phage exhibits low counts and needs a preliminary of concentration step.

- ✓ Somatic coliphages give also very interesting information regarding differential decay. According to the easy methodology and to the high numbers found, the somatic coliphages must be considered as potential indicators.

- ✓ The ratio number of bacteria versus phages are highly informative, and consequently they may be considered as a potential indicator (or indicators), but more information is needed before accurate quantified data can be given.

9. Final remarks

The main objectives of the project have been fulfilled.

✓ Validated methods and standardised protocols (ISO 10705-1, 1995; ISO/FDIS 10705-2 and ISO/DIS 10705-4) for the detection and enumeration of the three groups of bacteriophages are available. Phage reference materials have been prepared and used successfully for the validation of the methods and for quality control purposes.

Table 6 summarises the qualifications of the methods taking into consideration the results of the training session, the collaborative studies and the results of monitoring as well as the opinion of partners and participants. Table 6 was reached by consensus between partners and participants.

Conditions for phage reference materials and sample conservation and transport were determined. This allowed to send reference materials and samples (sewage) to the different laboratories participating in the project.

A concentration method based on flocculation with magnesium hydroxide was chosen and adapted for this study. Although this method was useful for the purposes of the project, future research on concentration methods for bacteriophages is recommended. Research done for the account of this project indicate that adsorption on cellulose nitrate filters may be a good alternative, but extra work needs to be done for samples with high turbidity.

✓ The methods were successfully transferred to the great majority of laboratories (3 partners plus 15 participants) which participated in this project.

✓ The methods are operational in diverse bathing water situations as encountered in Europe.

✓ Data on the occurrence of bacteriophages as compared to *E. coli* and Enterococci are now available from diverse situations encountered in Europe.

It can be concluded from these results that the potential of phages for the determination of the microbiological quality of bathing waters merits to be considered since their behaviour in

natural water differs from the behaviour of bacterial indicators and consequently they contribute valuable additional information. However, the data obtained from the monitoring did not give enough information to conclude about the use of a given group of bacteriophages as indicator for measuring water quality. This could be the aim of a future study, to be formulated according to the knowledge gained with this project.

Table 6.- Qualifications for the methods to detect the three groups of bacteriophages

	SOMCPH	F-RNA	BFRPH
Host strain [1]	+++	++	+
Background flora [2]	+(+)	++	+++
Plaque reading [3]	++	+	+++
Learning [4]	+++	++	+
Time results [5]	+++	++	++
Media preparation [6]	+++	++	+
Reference Materials (RMs) [7]	+++	+++	+++
r from RMs [8]	+++	+++	+++
r from Naturall samples [9]	+++	+++	+++
R from RMs [10]	+++	+++	+++
R from Naturall samples [11]	+++	+(+)	+
Robustness RMs [12]	+++	+++	+++
Robustness Naturall samples [13]	+++	+	++
Need to concentrate [14]	+++	+	+
Safety [15]	++	+	+++
Material Cost	+++	+(+)	+(+)
Personnel Cost	+++	+	++
Laboratory equipment [16]	+++	+++	+++

+: Worst ; ++: Intermediate ; +++: Best ;
Culturing of the host strain
Interference of background flora in phage assays of polluted samples
Easiness of detection of plaques in the host monolayer
Learning of the method
Time necessary to have results
Preparation and conservation of media
Preparation of reference materials
Repeatability with reference materials (pure cultures of bacteriophages)
Repeatability with naturally polluted samples
Reproducibility with reference materials (pure cultures of bacteriophages)
Reproducibility with naturally polluted samples
Robustness with reference materials (pure cultures of bacteriophages)
Robustness with naturally polluted samples
Need of concentrating bathing water samples to find significant numbers of phages.
Safety regarding potential health effects or contamination of the laboratory with bacteria affecting other tests performed in it.
Need of extra laboratory equipment.

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ANNEX 1

A FEASIBILITY STUDY ON THE DEVELOPMENT OF A METHOD BASED ON BACTERIOPHAGES FOR THE DETERMINATION OF MICROBIOLOGICAL QUALITY OF BATHING WATERS.

BACKGROUND

Directive 76/160/EEC on the Quality of Bathing Waters has a standard for enteroviruses in the form of an Imperative value of 0 per 10 litres, to be conformed in 90% of all samples taken. Measurements must be made "when an inspection of the bathing area shows that the substance may be present or that the quality of the water has deteriorated. Very few measurement are actually being made in Member States. This is related mainly to the specialized nature and relatively high cost of these analyses. If measurements are made, they are done according to non-standardized methodology, reflecting different histories in Member States. No attempts at standardization or method comparisons have yet been made. The description of the method in the Guideline is very general and allows many good or bad methods to be used.

In the proposed revision of the Guideline (94/C 112/03) the enterovirus parameter is retained, with the same I-value. Measurements must be made monthly, except when the water has met stringent bacteriological standards in the previous season, and if no chemically disinfected sewage sludge is discharged. In that case, only two measurement per bathing season are necessary. The retention of the enterovirus parameter in the revised Directive is discussed, one of the reasons being that the measurement process still is costly and has not adequately been standardized. The Commission has proposed a bacteriophage parameter as an alternative, but has not yet been able to choose which bacteriophage parameter is most appropriate, or to propose G- or I-values. Data on the relationships between bacteriophages and viruses in natural waters are limited. A literature review on behalf of DG XI (Havelaar, 1993) has identified F-specific RNA bacteriophages as an appropriate model for enteroviruses in bathing waters, with the possible exception of the Mediterraean. these waters, and possibly others, phages of *Bacteroides fragilis* might be more appropriate. It was also concluded that somatic coliphages should not be used in an EU Directive, and that faecal streptococci have many properties that make them attractive virus models, although probably less so than bacteriophages. Nevertheless, for all three groups of bacteriophages, field studies with

standardized protocols were recommended to choose one or more bacteriophage parameter(s), and to formulate limit values.

SCIENTIFIC AND TECHNICAL OBJECTIVE

The project should lead to the availability of standardized protocols for the concentration, detection and enumeration of bacteriophages. This includes the selection of representative groups of bacteriophages in particular F-specific RNA bacteriophages and phages of *B. fragilis*, if necessary somatic coliphages), in a format adoptable by ISO and CEN. The work should preferably be linked to the present work on bacteriophage methods in ISO/TC147/SC4/WG11.

The work will include in particular:

- ✓ a preliminary study on the target bacteriophages;
- ✓ research on the development and validation of methods to determine the selected target phages;
- ✓ interlaboratory studies with several EU laboratories to test the developed methods;
- ✓ evaluation of the methods on several bathing waters of various origins (fresh and sea waters);
- ✓ drafting of the methods in the requested format

The developed methods will include the following steps;

- ✓ sampling techniques and possibly strategies including preconcentration aspects;
- ✓ sample transport and conservation aspects including quality control of the sample integrity;
- ✓ methods of determination and the validation of the various steps of the methods;
- ✓ adequate expression and reporting of results

The interlaboratory method performance studies shall:

- ✓ be prepared with detailed protocols;

- ✓ comprise adequate samples and sample transport conditions;
- ✓ include meetings where participants can exchange results and views to improve the outcome of the methods;
- ✓ include adequate data treatment procedures in order to establish the most reliable performance criteria of the methods.

Note:

A particular attention will be given to performance criteria of the developed methods in particular in terms of intra- and interlaboratory reproducibility, traceability to established phage standards, quality control and quality assurance aspects for the validation of the methods within the testing laboratories.

Quality criteria shall be quantified. The methods should be developed and improved so that they can be used by monitoring laboratories throughout the EU, and the implementation of the method in a selection of laboratories should be organized. The project must also include field work to test the Operability of the methods in diverse situations as encountared in the EU, and to obtain preliminary data on the occurrence of bacteriophages as compared to bacterial indicator orgnisms (*E. coli* and faecal streptococci).

It is recommended that the project is formulated so that in a next phase comparative measurements between bacteriophages and viruses can be commissioned

TIMESCALE

The project will take three years.

FORMAT

The work should lead to the submission of validated methods in international standardization bodies and the development of protocols and reference materials for interlaboratory testing.

RESTRICTIONS

Participants should represent all climatic zones and water typologies in the EU. The methods should be applicable to fresh and marine waters.

Annex 2

GENERAL DESCRIPTION OF SOMATIC COLIPHAGES (SOMCPH)

Somatic coliphages are bacteriophages (bacterial viruses) which consist of a capsid containing single- or double-stranded DNA as the genome. The capsids may be of simple cubic symmetry or complex structures with heads, tails, tail-fiber, etc. They belong to the morphological groups A-D and are classified into the following families: *Myoviridae* (ds DNA, long contractile tails, capsids up tu 100 nm), *Siphoviridae* (dsDNA, long non-contractile tails, capsids 50 nm), *Podoviridae* (dsDNA, short non- contractile tails, capsids 50 nm) and *Microviridae* (ssDNA, no tail, capsid 30 nm). Somatic coliphages are virulent phages which attach to lypopolysaccharide or protein receptors in the bacterial cel wall of selected host strains and may lyse the host cell in 20-30 min under optimal conditions. They produce plaques of widely different size and morphology.

The presence of somatic coliphages in a water sample usually indicates pollution by human or animal faeces or by wastewaters containing these excreta. They thus provide rapid and simple method for detection of faecal pollution, and their resistance in water and food tends to resemble that of human enteric viruses more closely than faecal bacteria commonly used as quality indicators. Natural host strains of somatic coliphages include besides *Escherichia coli* other, closely related bacterial species, some of which may occur in pristine waters, so that exceptionally somatic coliphages may also multiply in these environments.

Escherichia coli WG5 is the selected strain for the detection of somatic coliphages. They produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions. For it the sample is mixed with a small volume of semi-solid nutrient medium. A culture of host-strain (inoculum culture-see Annex 5) is added and the mixture plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles (pfp)(or plaque-forming units(pfu)) per unit of volume.

Annex 3

GENERAL DESCRIPTION OF F-SPECIFIC RNA BACTERIOPHAGES (FRNAPH)

F-specific RNA bacteriophages (FRNAPH) are bacteriophages (bacterial viruses) which consist of a simple capsid of cubic symmetry of 21 - 30 nm in diameter and contain single stranded RNA as the genome. They belong to the morphological group E and are classified into the family *Leviviridae*. They are infectious for bacteria which posses the F-or sex plasmid originally detected in Escherichia coli K-12, and adsorption to the F- or sex-pili coded by this plasmid. The F-plasmid is transferable to a wide range of Gram-negative bacteria.

The presence of F-specific RNA bacteriophages in a water sample usually indicates pollution by human or animal faeces or by wastewaters containing these excreta. They thus provide rapid and simple method for detection of faecal pollution, and their resistance in water tends to resemble that of human enteric viruses more closely than faecal bacteria commonly used as quality indicators. Replication of F-specific bacteriophages is not possible in many environmental conditions since sex-pili are not sintetised at temperatures below 31-32 °C.

Salmonella typhimurium WG49 is the selected strain for the detection of F-specific RNA bacteriophages They produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration of 40 (occasionally 400) g of RNase in the plating medium.

The sample is mixed with a small volume of semi-solid nutrient medium. A culture of the host-strain (inoculum culture) is added and the mixture plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. These plaques (FTOTPH) are from F-specific RNA bacteriophages, plus F-specific DNA bacteriophages and some somatic phages of the host strain. Parallel plates with RNAase in the plating medium, allows to count the non F-specific RNA bacteriophages (FDNAPH). The difference between FTOTPH and FDNAPH are the FRNAPH. The results are expressed as the number of plaque-forming particles (pfp) (or plaque forming units(pfu)) per unit of volume.

Annex 4

GENERAL DESCRIPTION OF BACTERIOPHAGES INFECTING *Bacteroides fragilis* (BFRPH)

The most abundant bacteriophages infecting *Bacteroides fragilis* (BFRPH), one of the most abundant bacteria in the gut, belong to the family *Siphoviridae* with flexible tail (dsDNA, long non-contractile tails, capsides up to 60 nm). Phages infecting *Bacteroides fragilis* are virulent phages which attach to molecules in the cell wall of the host bacteria and may lyse the host cell in 30-40 minutes under optimal conditions. They produce clear plaques which do not differ very much in size and morphology.

The presence of phages infecting *Bacteroides fargilis* RYC 2056 in a water sample indicates pollution by human or animal faeces or by wastewaters containing these excreta. Bacteriophages infecting strain RYC2056 have been detected in similar concentrations in raw sewage samples from different parts of the world. In the great majority of analysed sewage samples the ratio numbers of somatic coliphages:numbers of bacteriophages infecting RYC2056 ranges from 100 to 200 and the ratio numbers of F-specific RNA bacteriophages :bacteriophages infecting RYC2056 ranges from 10 to 20. These ratios use to be one log $_{10}$ higher in abattoir sewage. Bacteriophages infecting *B. fragilis* have not been reported to be able to multiply under environmental conditions and they are quite resistant to natural inactivation as well as to most water disinfection procedures resembling that of human enteric viruses more closely than faecal bacteria commonly used as quality indicators

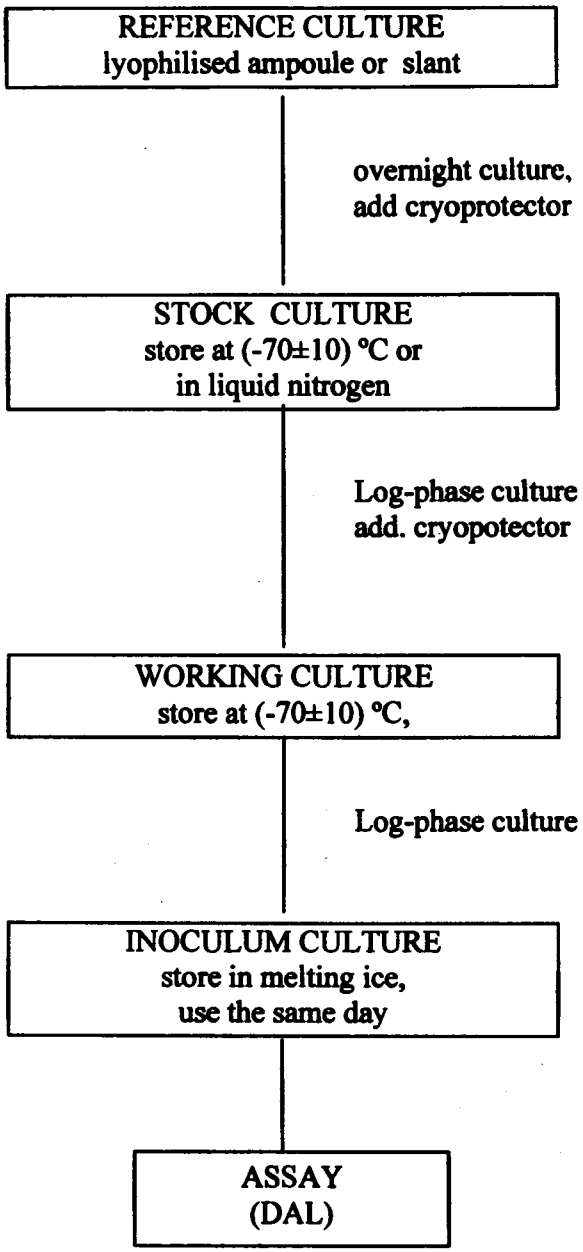
The method has proved to be applicable to other strains as for example *Bacteroides fragilis* HSP40 (ATCC51477) (Tartera C. and J. Jofre. 1987. Appl. Environ. Microbiol **53**:1632-1637). Bacteriophages infecting *B. fragilis* HSP 40 have been described to indicate preferably faecal pollution of human origin. However concentrations of phages infecting strain HSP40 are very low in sewages of some geographical areas.

Phages of *B. fragilis* produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions. The sample is mixed with a small volume of semi-solid nutrient medium. A culture of host-strain (inoculum culture- see annex

5) is added and the mixture plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles (pfp) (or plaque-forming units(pfu)) per unit of volume.

Annex 5

Scheme for culturing and maintenance of host strains for phage detection



Annex 6

Concentration of bacteriophages from water by flocculation with $Mg(OH)_2$

Procedure

Natural samples analysis.

1.- Prewarm the sample and homogenize by stirring. Take a 1000 ml aliquot and place it in a sterile bottle with a magnetic stir bar. Take another aliquot (45 ml) to count phages in the initial sample.

2.- Add 10 ml of 1M magnesium chloride (A.1) to the 1L water sample.

3.- Add 3.5 ml of 1M dipotassium hydrogen phosphate (A.2) in drops while magnetic stirring.

4.- Adjust pH to 8.5 ± 0.1 with 2N sodium hydroxide (NaOH) (A.3), add in drops while magnetic stirring at room temperature.

5.- Mixture is further magnetic stirred slowly for 15 minutes at room temperature.

6.- Flocs are then permitted to settle for 30-40 minutes at room temperature.

7.- Carefully siphon off the supernatant.

8.- Concentrate the loose sediment (approximately 250 ml of volume) by centrifugation (3000 rpm, 15 minutes, 5°C ± 3 °C).

9.- Carefully discard the supernatant.

10.- Resuspend the sediment with 30 ml of "Buffer for phages" (A.4) at pH 6.0 ± 0.2 at room temperature. Homogenize carefully until flocs dissapear completly.

11.- Count the number of bacteriophages in the concentrate by the double-agar layer technique.
12.- Analyse all the volume (aprox. 40 ml) for 1 or more bacteriophages methods, e.g.:

- 10 ml for SOMCPH
- 10 ml for BFRPH
- 10 ml for FRNAPH (with RNAse)
- 10 ml for FRNAPH (without RNAse).

REAGENTS AND DILUENTS

A.1 Magnesium chloride solution (1 mol/l)

$MgCl_2.6H_2O$		20.3 g
Distilled water	up to	100 ml

Dissolve the magnesium chloride in the water. Sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark at room temperature for not longer than 2 months.

A.2 Dipotassium hydrogen phosphate solution (1 mol/l)

K_2HPO_4		17.4 g
Distilled water	up to	100 ml

Dissolve the dipotassium hydrogen phosphate in the water. Sterilize in the autoclave at (121 ± 1) °C for 15 min. Store at room temperature for not longer than 2 months.

A.3 Sodium hydroxide solution (2 mol/l)

NaOH		8 g
Distilled water	up to	100 ml

Dissolve the sodium hydroxide in the water. Sterilize in the autoclave at (121 ± 1) °C for 15 min. Store at room temperature for not longer than 6 months

A.4 Buffer for Phages

Basal buffer

- Disodium phosphate (Na_2HPO_4)	7 g
- Potassium dihydrogen phosphate (KH_2PO_4)	3 g
- NaCl	5 g
- Distilled water	1000 ml

Dissolve the ingredients in the water. Adjust pH to 6.0 ± 0.2 with HCl 35%. Distribute in bottles in volumes of 200 ml or larger and sterilize in the autoclave at (121 ± 1) °C for, 15 minutes. Store at room temperature for not longer than 2 months.

Magnesium sulphate solution (0.1 mol/l)

$MgSO_4.7H_2O$		2.5 g
Distilled water	up to	100 ml

Dissolve the magnesium sulphate in the water. Filter sterilize through an 0.22 µm pore size membrane filter, or sterilize in the autoclave at (121 ± 1) °C for 15 min. Store at room temperature for not longer than 2 months.

Calcium chloride solution (0.01 mol/l)

$CaCl_2.2H_2O$	0.15 g
Distilled water	up to 100 ml

Dissolve the calcium chloride in the water while (if necessary) heating gently. Cool to room temperature and filter sterilize through an 0.22 µm pore size membrane filter. Store at room temperature for not longer than 2 months

Complete buffer

Basal buffer	1000 ml
Magnesium sulphate solution	10 ml
Calcium chloride solution	10 ml

Aseptically add magnesium sulphate solution and calcium chloride solution to basal buffer and mix well. If not for immediate use, at room temperature for not longer than 2 months.

ANNEX 7

List of participants

Albrecht WIEDENMANN / Petra KRÜGER
Abteilung für allgemeine Hygiene und Umweltmedizin
Universität Klinikum Tübingen
Wilhemst.31
D-72074 TÜBINGEN
DEUTSCHLAND

Montserrat BOQUE GENOVARD
Conselleria de Medi Ambient, Ordenacio del Territori Litoral
Laboratori de Medi Ambient
Avda. Gabriel Alomar i Villalonga, 33 bajos
07006 PALMA DE MALLORCA
SPAIN

Christiane HÖLLER
Institut für Hygiene und Umweltmedizin
Brunswiker Str. 4
D-24105 KIEL
DEUTSCHLAND

Hans RUITER
Dienst Binnenwateren
R.I.Z.A. afdeling Microbiologie
P.O. box, 17
8200 LELYSTAD
THE NETHERLANDS

Andrew GAWLER
The Environmental Agency - Exeter Laboratory
Manley House / Kestrel Way
EX2 7LQ EXETER
U.K.

Rene MOLLER MADSEN
Environmental and Food Agency
Microbiology Laboratory
Lille Tornbjerg vej 24
5220 ODENSE SO
Denmark

Vincent YOUNG
Eastern Health Board
Public Analyst's Laboratory, Sir Patrick Dun's
Lower Grand Canal St.
DUBLIN 2
REPUBLIC OF IRELAND

Conceiçao COSTA
Insituto Nacional de Saude
Dr Ricardo Jorge - Largo 1° Dezembro s/n
4300 PORTO
PORTUGAL

Maarit NIEMI
Finnish 1 Dezembro s/n°
P.O. Box 140
00251 HELSINKI
FINLAND

Bernadette VALENTIN
Parc Euromédecine
Institute Bouisson Bertrand
Rue De la Croix Verte-Route Ganges
F-34090 MONTPELLIER
FRANCE

Belen MORENO MONTOYA
Laboratorio de Saludad Publica - Laboratorio de Microbiologia
Delegacion Territorial de Sanidad de Gipuzkoa
Gobierno Vasco
Avenida de Navarra, 4
20013 SAN SEBASTIAN
SPAIN

Maria LAMBIRI
Bacteriology Department
National School of Public Health
196, Leofros Alexandras Avenue
11521 ATHENS
GREECE

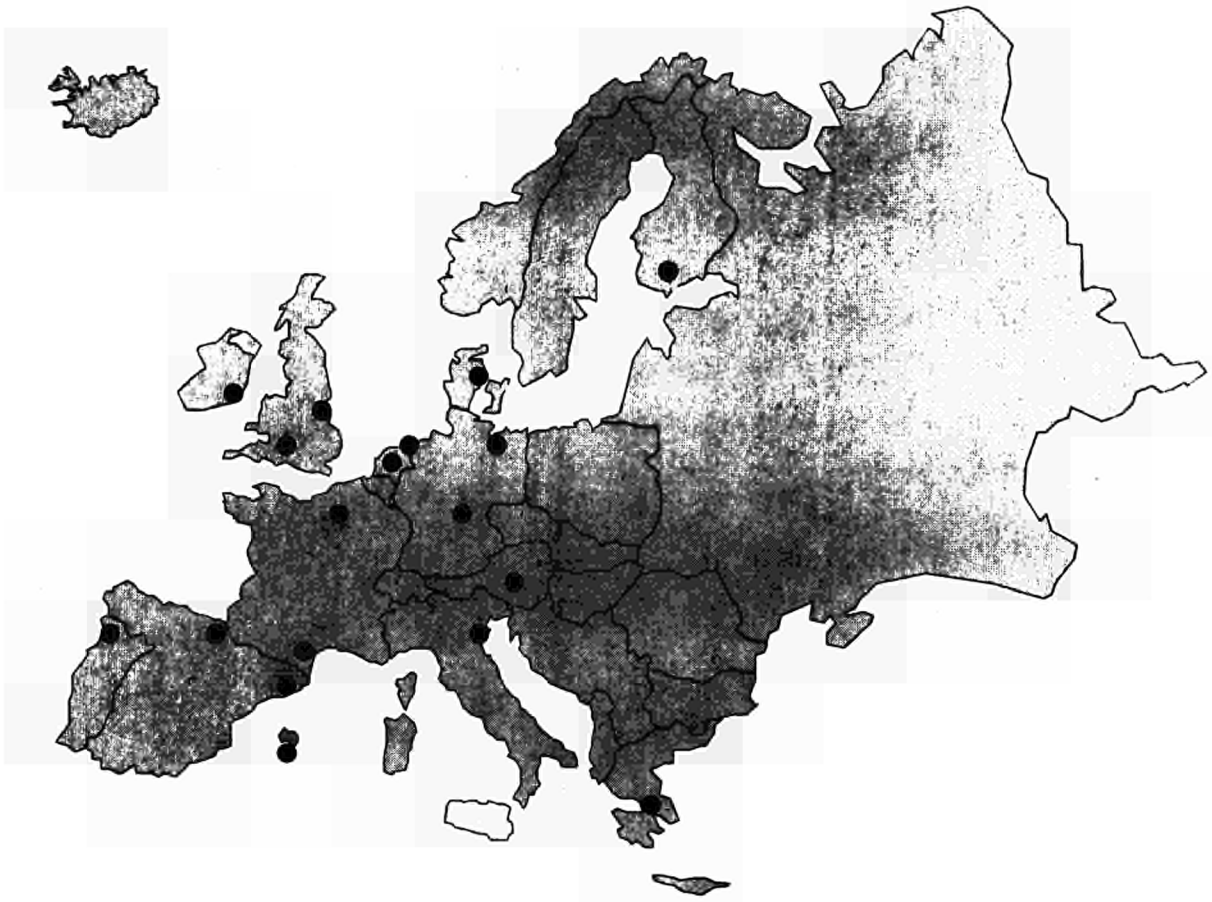
Regina SOMMER
Hygiene Institut
University of Vienna
Kinderspitalgasse, 15
A-1095 WIEN
OSTERREICH

Graziella MIROLO
ARPA - Sezione provinciale di Ferrara
Corso Giovecca, 169
I-44100 FERRARA
ITALY

Simon WARD
Environmental Microbiology Research Unit
PHLS (Public Health Laboratory Service)
University Hospital / Queen's Medical Centre
NG7 2UH NOTTINGHAM
U.K.

ANNEX 8

Geographical distribution of participants



ANNEX 9

Personnel that has contributed to the accomplishment of this project

For the partners

UBCN.DM

Jofre, J. Coordinator
Araujo, R.
Cerdá, M.
Contreras, N.
Lucena, F.
Mendez, J.
Monedero, M.
Monleon, T.
Muniesa, M.
Puig, A.

RIVM.LWFM

Mooijman, K.A. Scientist responsible
Bahar, M
Ghameshlou, Z
Havelaar, A.H.

IPASLI.SEE

Pierzo, V. Scientist responsible
Hernandez, J-F.
Simonart, T.

Collaboration with Statistical A3B Association of ILIS (Health engineering institute of Lille, University of Lille II):

Demarquilly, C.
Lemdani, M.

For the participant laboratories are indicated in Annex 7

To all of them we deeply aknowledge their efforts for fulfiling the aims of the project.

ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS:

ϕX174	Somatic coliphage
AFNOR	Association Française de Normalisation
AOAC	Association of Official Analytical Chemists
APPROX	Approximately
ATCC	American Type Culture Collection
B40-8	Bacteriophage of *Bacteroides fragilis*
B56-1	Bacteriophage of *Bacteroides fragilis*
B56-3	Bacteriophage of *Bacteroides fragilis*
BFRPH	Bacteriophages of *Bacteroides fragilis*
BFRRYC2056	Bacteriophages RYC2056 of *Bacteroides fragilis*
ca.	Approximately
CEN	European Commission of Normalisation
cfu	Colony forming unit
cBFRRYC2056	Concentrated bacteriophages infecting strain RYC2056 of *B. fragilis*
cSOMCPH	Concentrated somatic coliphages
cFRNAPH	Concentrated F-specific RNA bacteriophages
cFTOTPH	Concentrated total number of F-specific bacteriophages
DAL	Double Agar Layer method
DG XI	General Direction XI
Dpt.	Department
ds	Double stranded
E. coli	*Escherichia coli*
E. coli K12	Strain of *Escherichia coli*
EEC	European Comission
ENT	Enterococci
EU	European Union
FDNAPH	F-specific DNA bacteriophages
FRNAPH	F-specific RNA bacteriophages (= FTOTPH – FDNAPH)
FTOTPH	Total number of F-specific bacteriophages (= FRNAPH + FDNAPH)
HS	Strain of *Escherichia coli* HS (F Amp R)
HSP40	Strain of *Bacteroides fragilis*

IPASLI.SEE	Institut Pasteur de Lille. Service des Eaux-Environnement
IPL	Institut Pasteur de Lille
ISO	International Standarization Organization
ISO/CD	International Standarization Organization / Comitee Draft
ISO/DIS	International Standarization Organization / Draft International Standard
ISO/FDIS	International Standarization Organization / Final Draft International Standard
I-values	Impeartive values
MAR9	Somatic coliphage
MS2	F-specific RNA phage
NT	Not Tested
pfp	Plaque forming particle (equivalent to plaque forming unit, pfu)
RIVM	Rijksinstituut voor Volksgezondheid en Milieuhygiëne
RIVM.LWFM	Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Laboratory of Water and Food Microbiology
RM	Reference Material
RYC2056	Strain of *Bacteroides fragilis*
SAL	Single Agar Layer method
SOMCPH	Somatic coliphages
ss	Single stranded
STM	Standards, Measurements and Testing
TYGA	Trypton Yeast Glucose Agar
UB	Universidad de Barcelona
UBCN.DM	Universidad de Barcelona, Departamento de Microbiología
WG49	Strain of *Salmonella typhimurium* (F^+ strain, host for FRNAPH)
WG5	Strain of *Escherichia coli* Nal^r (host for somatic coliphages)

SYMBOLS:

X^2	Chi-square distribution
I	Number of vials
J	Number of replicates per vial
n	Number of samples

r	Repeatability
R	Reproducibility
Sd	Standard deviation
T_1	Cochran's dispersion statistic test to determine the variation in pfp within one vial of reference material (replicate variation)
T_2	Cochran's dispersion statistic test to determine the variation in pfp between different vials of one batch of reference material

European Commission

EUR 19506 Bacteriophages in bathing waters -"A feasibility study on the development of a method based on bacteriophages for the determination of microbiological quality of bathing waters."

J.Jofre, F. Lucena, K. Mooijman, V. Pierzo, R. Araujo, Mahdieh Bahar, Catherine Demarquilly and Arie Havelaar.

Luxembourg: Office for Official Publications of the European Communities

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Methods for the detection and enumeration of somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis* had been standardised and validated. Conditions for the preparation, transport and distribution of bacteriophage reference materials and preservation of samples had been defined. A method based on flocculation with $Mg(OH_2)$ with concentration efficiencies from about 40% was settled to concentrate phages from bathing waters. All methods were successfully implemented in routine laboratories all around the EU. Data on the occurrence of bacteriophages as compared to *E. coli* and Enterococci are available from diverse situations encountered in the EU. Results allow to conclude that the potential of phages for the determination of the microbiological quality of bathing waters merits to be considered since their determination is feasible and their behaviour in natural water differs from the behaviour of bacterial indicators and consequently they add valuable information.

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Centrum VTI SR
Nám. Slobody, 19
SK-81223 Bratislava
Tel. (421-7) 54 41 83 64
Fax (421-7) 54 41 83 64
E-mail: europ@tbb1.sltk.stuba.sk
URL: http://www.sltk.stuba.sk

SLOVENIJA

Gospodarski Vestnik
Dunajska cesta 5
SLO-1000 Ljubljana
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MALAYSIA

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MÉXICO

Mundi Prensa Mexico, SA de CV
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PHILIPPINES

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SOUTH AFRICA

Eurochamber of Commerce in South Africa
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SOUTH KOREA

The European Union Chamber of Commerce in Korea
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Tel. (82-2) 22 53-5631/4
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SRI LANKA

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