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Second collaborative study on analysis of bacteriophages in bathing waters

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Abstract

A second international collaborative study on bacteriophages in bathing waters was organised in March 1998. Fifteen European laboratories (including the organising laboratory at the National Institute of Public Health and the Environment, the Netherlands) participated in the study. The study consisted of two parts: (1) Analysis of naturally polluted standard samples for the enumeration of somatic coliphages (SOMCPH), F-specific phages (including the total number of F-specific phages: FTOTPH and F-specific DNA phages: FDNAPH) and phages of *Bacteroides fragilis* (BFRPH); (2) Application of a concentration technique (based on flocculation) to a mixture of phage reference materials (ΦX174 for SOMCPH, MS2 for FTOTPH and B40-8 for BFRPH). In agreement with the participating laboratories some of the data were excluded for further analysis because of technical problems in several laboratories. Analysis of the remaining data for the part of the study with the naturally polluted standard samples resulted in values for the repeatability (r) varying from 1.63 - 2.34 and for the reproducibility (R) varying from 3.10 - 5.72 for the different groups of bacteriophages. There was a greater variation in these results than those in which reference materials (with pure cultures of standard phages) were analysed. This was probably caused by a combination of extra Poisson variation in phage numbers in naturally polluted standard samples and the difficulties of interpreting plates of natural samples. Analysis of the results from the concentration technique showed low recovery of phage ΦX174 (2.2 % - 16.4 %) and variable recovery of phages MS2 (12.7 % - 99.4 %) and B40-8 (42.5 % - 142.9 %).

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Abbreviations and symbols

ABBREVIATIONS

BFRPH	phages of *Bacteroides fragilis*
(M)BPRMA	(Modified) Bacteroides Phage Repair Medium Agar
(M)BPRMB	(Modified) Bacteroides Phage Repair Medium Broth
B40-8	Phage of *Bacteroides fragilis*
cfp	colony forming particle
DAL	Double Agar Layer method
FDNAPH	F-specific DNA bacteriophages
FRNAPH	F-specific RNA bacteriophages (=FTOTPH - FDNAPH)
FTOTPH	Total number of F-specific bacteriophages (= FRNAPH + FDNAPH)
HSP40	*Bacteroides fragilis* (host for phages of *B.fragilis*)
MSA	Modified Scholtens' Agar
MSB	Modified Scholtens' Broth
MS2	F-specific RNA phage
Nal	Nalidixic acid
pfp	plaque forming particle
ps	peptone saline solution
RM	Reference material
rpm	rotations per minute
SAL	Single Agar Layer method
SOMCPH	Somatic coliphages
SOP	Standard Operating Procedure
ss(M)BPRMA	semi-solid (Modified) Bacteroides Phage Repair Medium Agar
ssMSA	semi-solid Modified Scholtens' Agar
ssTYGA	semi-solid Trypton Yeast Glucose Agar
TYGA	Trypton Yeast Glucose Agar
TYGB	Trypton Yeast Glucose Broth
WG5	*Escherichia coli* Nal^r (host for somatic coliphages)
WG49	*Salmonella typhimurium* (F^+ strain, host for FRNAPH)
ΦX174	Somatic coliphage

SYMBOLS

χ^2	Chi- square distribution
I	Number of vials
J	Number of replicates per vial
r	Repeatability (within laboratory precision)
R	Reproducibility (between laboratory precision)
s	Standard deviation
T_1	Cochran's dispersion test statistic to determine the variation in pfp within one vial of reference material (replicate variation)
T_2	Cochran's dispersion test statistic to determine the variation in pfp between different vials of one batch of reference materials
$\bar{x}$	Mean

Samenvatting

In maart 1998 werd het tweede internationale ringonderzoek voor analyse van bacteriofagen in zwemwater georganiseerd. Tijdens de studie werden faag referentiematerialen (RM's) en natuurlijk besmette standaard monsters geanalyseerd. Twee batches van de RM's waren ook tijdens het eerste ringonderzoek (Mooijman *et al.*, 1998) gebruikt: RM's met ΦX174 (voor somatische colifagen) en RM's met B40-8 (voor fagen van *Bacteroides fragilis*). Voor MS2 (voor F-specifieke fagen) was een nieuwe batch RM's bereid. Alle batches RM's bleken opgeslagen bij -70 °C, minimaal 1.5 jaar stabiel. De homogeniteit van de RM's voldeed aan de vooraf gestelde criteria. De natuurlijk besmette standaard monsters werden bereid door rioolwater te verdunnen met pepton fysiologische zoutoplossing. De verdunningen werden gemengd met glycerol (tot 5 % (v/v) in het monster), uitgevuld in kleine plastic vaatjes en opgeslagen bij (-70 ± 10) °C. De natuurlijk besmette standaard monsters vertoonden veel variatie in de tellingen van fagen tussen de vaatjes. Een verklaring voor deze grote variatie was waarschijnlijk de aanwezigheid van aggregaten in de monsters. Daarom werd besloten, alvorens de faag analyses uit te voeren, de standaard monsters te filtreren door een laag eiwit bindend filter (met 0.22 µm porie grootte).

Vijftien Europese laboratoria (inclusief het organiserende laboratorium) namen deel aan het ringonderzoek. De doelstellingen van het ringonderzoek waren:

- Evaluatie van de implementatie van de methoden voor de bepaling van de drie groepen van bacteriofagen in natuurlijk besmette standaard (water) monsters in verschillende EU-laboratoria: somatische colifagen (SOMCPH), F-specifieke fagen (bevattende het totaal aantal F-specifieke fagen (FTOTPH) en F-specifieke DNA fagen (FDNAPH)) en fagen van *Bacteroides fragilis* (BFRPH);
- Evaluatie van de implementatie van een concentreringstechniek (gebaseerd op flocculatie) voor het bepalen van de drie groepen bacteriofagen in een mengsel van faag-RM's in verschillende EU-laboratoria;
- Identificatie van de redenen voor afwijkende resultaten in individuele laboratoria, welke mogelijk kunnen leiden tot aanpassing van de protocollen.

De RM's en de standaard monsters werden, verpakt in droogijs, per koerier verzonden. De materialen kwamen bij alle deelnemende laboratoria in bevroren toestand aan.
Vanwege technische problemen, zoals problemen met het aflezen van de platen en problemen met de gastheer (in geval van BFRPH), werden resultaten van sommige laboratoria niet gebruikt voor verdere analyse. De belangrijkste conclusies van de studie waren:
- Resultaten van de kwaliteitscontroles met de faag RM's toonden vergelijkbare kwaliteit van analyse voor de drie groepen van bacteriofagen bij de meerderheid van de deelnemers;
- Meer variatie in resultaten binnen en tussen laboratoria bij analyse van de natuurlijk besmette standaard monsters dan in het geval van de analyse van faag RM's. Dit werd waarschijnlijk veroorzaakt door een combinatie van relatief grote variatie in resultaten van de natuurlijk besmette standaard monsters en de moeilijkheden (van relatief onervaren laboratoria) bij het interpreteren van platen van natuurlijke monsters;
- Lage recovery (2.2 % - 16.4 %) van faag ΦX174 (SOMCPH), na concentrering;
- Variabele recovery van fagen MS2 (FTOTPH; 12.7 % - 99.4 %) en B40-8 (BFRPH; 42.5 % - 142.9 %), slechts gedeeltelijk veroorzaakt door random variatie in faag concentraties.

Summary

In March 1998 the second international collaborative study on analysis of bacteriophages in bathing waters was organised. For this study phage reference materials (RMs) and naturally polluted standard samples were analysed. For the phage RMs two batches were used which were also used during the first collaborative study (Mooijman *et al.*, 1998): RMs containing ΦX174 (for somatic coliphages) and RMs containing B40-8 (for phages of *Bacteroides fragilis*). The batch of RMs containing MS2 (for F-specific phages) was newly prepared. All batches of RMs were stable for at least 1.5 years when stored at -70 °C. The homogeneity of the RMs fulfilled the pre-set criteria.
The naturally polluted standard samples were prepared by making different dilutions of sewage samples in peptone saline solution. The dilutions were mixed with glycerol (until 5 % (v/v) in the sample), distributed into small vials and stored at (-70 ± 10) °C. The analyses of these naturally polluted standard samples showed much variation in phage counts between the vials. An explanation for this large variation was probably the existence of aggregates in the samples. Therefore it was decided to filter the standard samples through a low protein binding filter (0.22 μm pore size), before performing phage analysis.

Fifteen European laboratories (including the organising laboratory) participated in the collaborative study. The objectives of the study were:
- Evaluation of the implementation of the methods for enumeration of the three groups of bacteriophages in naturally polluted standard (water) samples, in different EU laboratories: somatic coliphages (SOMCPH); F-specific phages, including the total number of F-specific phages (FTOTPH) and F-specific DNA phages (FDNAPH) and phages of *Bacteroides fragilis* (BFRPH);
- Evaluation of the implementation of a concentration method (based on flocculation) for enumeration of the three groups of bacteriophages in a mixture of phage RMs in different EU-laboratories;
- Identification of reasons for deviating results in individual laboratories, possibly leading to modifications of the test protocols.

The phage RMs and the standard samples were sent by courier service, packed in dry ice. The materials arrived frozen in all participating laboratories.
Due to technical problems, like difficulties with reading of the plates and problems with the host culture (in case of BFRPH), results of some laboratories were not used for further analysis.
The main conclusions of the study were:
- Results of the quality controls with the phage RMs showed comparable quality of analysis for the three groups of phages for the majority of participating laboratories;
- More variation in results within and between laboratories when analysing the naturally polluted standard samples than in case of analysing phage RMs. This was probably caused by a combination of relatively high variation in results of the naturally polluted standard samples and the difficulties (of relatively inexperienced laboratories) of interpreting plates of natural samples;
- Low recovery (2.2 % - 16.4 %) of phage ΦX174 (SOMCPH), after concentration;
- Variable recovery of phages MS2 (FTOTPH; 12.7 % - 99.4 %) and B40-8 (BFRPH; 42.5 % - 142.9 %), only partly caused by random variation in phage concentrations.

1. Introduction

In spring 1997 a first training session and a first collaborative study have been organised on the analysis of bacteriophages in bathing waters. In both sessions the phage methods for three groups of bacteriophages were introduced with mainly pure phage cultures. The three groups of bacteriophages were:
- Somatic coliphages (SOMCPH)
- F-specific RNA phages (FRNAPH)
- Phages of *Bacteroides fragilis* (BFRPH)

In the first training session the participants had a first acquaintance with the methods in a central laboratory at the Institute Pasteur in Lille. In the first collaborative study they applied these methods in their own laboratories. The results of the first training session and of the first collaborative study were very satisfactory. In the first collaborative study the largest variation in results was found with the BFRPH method. However, still a very acceptable Reproducibility value (representing the between laboratory precision) of (R=) 2.04 (back transformed from the log scale) was found. For FRNAPH, R was 1.73 and for SOMCPH, R was 1.52 (Mooijman *et al.*, 1998).
The next step in the learning process was applying the methods to naturally polluted samples. A second training session on this aspect was organised in December 1997. The second collaborative study with naturally polluted samples was organised by the Microbiological Laboratory for Health Protection of the National Institute of Public Health and the Environment (the Netherlands) in March 1998. Beside the analyses of naturally polluted samples, also a concentration technique was applied (introduced during the second training session in December 1997).
In case of naturally polluted samples, a total number of F-specific phages (FTOTPH) will be detected with the method applied. The number of FTOTPH will include mainly F-specific RNA phages (FRNAPH), but also (a small part) of F-specific DNA phages (FDNAPH). By applying the method in the presence of RNase, the F-specific DNA phages are detected. The difference between FTOTPH and FDNAPH will give the number of FRNAPH.

The main objectives of the second collaborative study were:
- Evaluation of the implementation of the methods for enumeration of the three groups of bacteriophages in naturally polluted standard (water) samples, in different EU laboratories: somatic coliphages (SOMCPH); F-specific phages, including the total number of F-specific phages (FTOTPH) and F-specific DNA phages (FDNAPH) and phages of *Bacteroides fragilis* (BFRPH);
- Evaluation of the implementation of a concentration method (based on flocculation) for enumeration of the three groups of bacteriophages in a mixture of phage RMs in different EU-laboratories;

- Identification of reasons for deviating results in individual laboratories, possibly leading to modifications of the test protocols.

This report describes the organisation and the results of the second collaborative study on bacteriophages in bathing waters. Chapter 2 deals with the characteristics of the phage reference materials and naturally polluted standard samples used in the study. The results of the study will be presented in chapter 3, and discussed in chapter 4.

2. Phage reference materials and naturally polluted (standard) samples

2.1 Materials and methods

2.1.1 Preparation and control

Phage reference materials (RMs)
The phages, the bacterial host strains and the preparation of the phage reference materials (RMs) were the same as described for the first collaborative study (Mooijman *et al.*, 1998; Ch.2).
The standard methods used for enumeration of the different phages were Amended ISO/CD 10705-2 of February 1997 (mainly DAL method) for enumeration of SOMCPH (Mooijman *et al.*, 1998; Annex 6), Amended ISO 10705-1 of February 1997 for enumeration of F-specific phages (including the total number of F-specific phages (FTOTPH) and F-specific DNA phages (FDNAPH); Mooijman *et al.*, 1998; Annex 7) and the procedure described in Annex 2 of this report for enumeration of BFRPH.

- The criteria for each batch of RMs were also the same as described for the first collaborative study:
  - Mean phage concentration: between *ca* 30 pfp/ml and *ca* 150 pfp/ml;
  - T_1 (variation within one vial): not significantly different from a χ^2 - distribution, at 95% confidence level and I(J-1) degrees of freedom. Where I is the number of vials and J is the number of replicates.
  - T_2 (variation between different vials) : For a homogeneous batch $T_2/(I-1)$ should be ≤ 2.

T_1 and T_2 results will give information on the homogeneity of the RMs. The formulas of T_1 and T_2 are described in Chapter 2 of Mooijman *et al.* (1998).

The batches of phage RMs used for the second collaborative study were the following:
For SOMCPH: RMs containing ΦX174, batch 040696 (same batch as used in trial 1);
For FRNAPH: RMs containing MS2, batch 220597 (different batch as used in trial 1);
For BFRPH: RMs containing B40-8, batch 260397 (same batch as used in trial 1).

Of all the phage RM batches, control charts were prepared. These charts will show whether a batch of RMs is stable (results are "in control") and it is also a tool for internal quality control. The preparation of the control charts is described in Chapter 2 of Mooijman *et al.* (1998).

Naturally polluted (standard) samples

In the period September-December 1997 sewage samples were collected in the Netherlands and in Spain. For each type of phage different dilutions of sewage samples were prepared, mixed with glycerol (until 5 % (v/v) in the sample) and stored at (-70 ± 10) °C. The sewage samples were diluted to obtain a "countable" number of plaques for each phage type (preferably 30 - 150 pfp/ml). It was decided in the September meeting of 1997 with the contractors to prepare the dilutions at the organising laboratory and not in the participating laboratories of the second collaborative study. This, to prevent variation due to dilution steps. For SOMCPH, 8 ml sewage of "The Bilt" (The Netherlands) of 281097 (ddmmyy) was mixed with 1900 ml sterile peptone saline solution (ps: Mooijman *et al.*, 1998; Annex 7) and 100 ml sterile glycerol. The mixture was distributed into vials in 2.5-3 ml aliquots and stored at (-70 ± 10) °C. In total 520 vials were prepared.
For F-specific phages, 44 ml sewage of "The Bilt" of 071197 was mixed with 2850 ml ps and 150 ml glycerol. The mixture was distributed into vials in 4.5 ml aliquots and stored at (-70 ± 10) °C. In total 540 vials were prepared.
For BFRPH, 2 litre sewage "Barcelona" (Spain) of 311097 was mixed with 100 ml glycerol. The mixture was distributed into vials in 3 ml aliquots and stored at (-70 ± 10) °C. In total 500 vials were prepared.

The mean number of pfp/ml and the homogeneity of the vials were checked before and after freezing of the vials, by checking 5 vials of each batch in duplicate. Thawing of the vials was performed by placing the vials at room temperature. Thawing time was dependent on the volume in the vial, and varied from 30 to 60 min. To check whether the mean level in the materials did not change too much, the batches were regularly controlled by analysing 5 vials in duplicate until the date of the second collaborative study (in total *ca* 4 months of storage at - 70 °C).

When analysing naturally polluted samples, a total number of F-specific phages (FTOTPH) will be detected with the standard method. The number of FTOTPH will include mainly F-specific RNA phages (FRNAPH), but also (a small part) of F-specific DNA phages (FDNAPH). By applying the method in the presence of RNase, the F-specific DNA phages are detected. The difference between FTOTPH and FDNAPH gives the number of FRNAPH.

In the standard method for the analysis of BFRPH filtering of the sample through a low protein binding filter (Millipore Millex GV, 0.22 µm pore size) is advised to eliminate background flora. The vials for the analysis of SOMCPH and F-specific phages were for later analysis (after *ca* one month of storage) also pre-filtered with the same type of filter. This was done to try to exclude large variations between vials, probably due to the existence of aggregates. The variation in results between the filtered and non-filtered vials of each batch of naturally polluted standard samples were checked by analysing the T_1 and T_2 values. The same criteria as described for the phage RMs (see above) were aimed at.

2.1.2 Concentration technique

The concentration technique introduced during the second collaborative study was based on a flocculation method described by Schulze and Lenk (1983). Details of the technique are given in Annex 3.
To check the recovery of the "standard" phages (used for preparing the RMs) in the concentration method an experiment was carried out prior to the collaborative study.
For this purpose the following batches of phage RMs were used:
For SOMCPH: RMs containing ΦX174, batch 270597 (mean level *ca* 35 pfp/ml);
For FRNAPH: RMs containing MS2, batch 220597 (mean level *ca* 65 pfp/ml);
For BFRPH: RMs containing B40-8, batch 260397 (mean level *ca* 90 pfp/ml).
A mixture of 18 ml suspension A was prepared by mixing 8 ml of ΦX174, 6 ml of MS2 and 4 ml of B40-8. Seven ml of suspension A was used for performing phage analysis before concentrating. The methods used for the phage analysis is described in 2.1.1. For each phage type, 1 ml of suspension A was analysed in duplicate except for FDNAPH which was analysed in singular. Ten ml of suspension A was added to 1 L of synthetic sea salt solution (Annex 4; 6.1). The solution was mixed and next the concentration method was applied as described in Annex 3. The concentrate (suspension B) was checked for the number of bacteriophages by analysing 10 times 1 ml of suspension B for SOMCPH, 10 times 1 ml of suspension B for FTOTPH, 10 times 1 ml of suspension B for BFRPH and 5 times 1 ml of suspension B for FDNAPH. The percentage of recovery of each phage by using this concentration technique was calculated.

2.2 Results

2.2.1 Somatic coliphages (SOMCPH)

Phage reference materials (RMs)
The batch of RMs containing ΦX174 was prepared on 040696 (ddmmyy) and was the same batch as used for the first collaborative study. Results of this batch were presented in Mooijman *et al* (1998). The batch fulfilled the criteria for the mean number of plaque forming particles (pfp) and for homogeneity. Three days after freezing at (-70 ± 10) °C the geometric mean number of pfp/ml of 5 vials (I=5) was 90.7. T_1 was not significantly different from a χ^2-distribution (2.71) and $T_2/(I-1)$ was ≤ 2 (0.16). The first control chart prepared of this batch showed a small initial decrease in the mean phage counts. The control chart was recalculated and then showed stable results. Since the first collaborative study new results were obtained and introduced into the (recalculated) control chart. This chart is shown in Figure 1. The values of the mean count and the control limits are given in Table 1. The last results in Figure 1 were analysed in August 1998, showing that this batch of RMs containing ΦX174 is stable for at least 2 years. For the outlying value in the chart (test number 39) no explanation was found.

Table 1 *Control chart values of ΦX174, batch 040696*

	pfp/ml
$\bar{x}$ - 3s	45.6
$\bar{x}$ - 2s	52.1
$\bar{x}$	68.0
$\bar{x}$ + 2s	88.7
$\bar{x}$ + 3s	101.4

$\bar{x}$: Geometric mean; $\bar{x} \pm 2s$: warning limits; $\bar{x} \pm 3s$: action limits

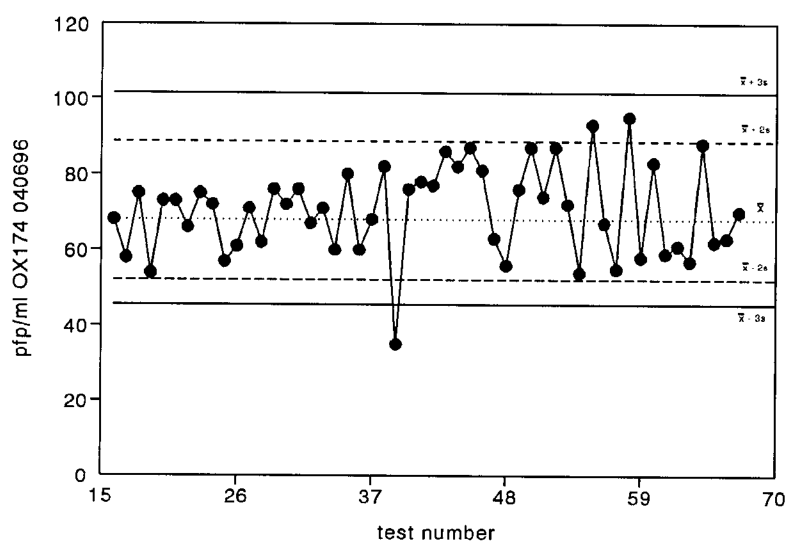

Figure 1 *Control chart of RMs containing ΦX174, batch 040696, on MSA with host strain WG5 Escherichia coli.*

Naturally polluted standard samples

The results for the somatic coliphages (SOMCPH) of the naturally polluted standard samples "The Bilt 281097" are given in Table 2. In this table the non-filtered samples show much variation in results. It occurred regularly that a vial of the non-filtered samples resulted in a very high amount of plaques which were in fact not countable. These results were not taken into account in this table, so that the real values of T_2/ (I-1) should even be higher than given here. When each vial was filtered separately through a low protein binding filter (with 0.22 µm pore size) the extreme counts were no longer detected. Filtering probably removed some aggregates which might exist in sewage. The geometric mean values of the filtered samples were somewhat lower than of the non-filtered samples, but still fulfilled the criteria. As filtering of the samples decreased the variation between vials (and also improved the variation in results within vials (T_1)), it was decided to prescribe filtering in the protocol of the second collaborative study.

Table 2 *Results naturally polluted standard samples "The Bilt 281097", SOMCPH*

Day	Non-filtered				Filtered (0.22 µm)			
	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)
0[1]	5	60.0	6.73	2.03	-	-	-	-
2	4[2]	84.7	19.8[3]	3.68	-	-	-	-
27	4[2]	130.8	4.44	1.31	-	-	-	-
76	5	123.6	3.06	3.09	5	109.0	5.65	1.32
83	4[2]	91.9	8.71	3.87	5	90.2	10.6	0.41
105	-	-	-	-	5	74.9	13.9[3]	4.23
123	-	-	-	-	5	90.2	6.57	2.33

I: number of vials (all calculated in duplicate)
[1]: Before freezing; [2]: Vial 5 has very high number of plaques (non-countable)
[3]: Significantly different from a χ^2- distribution:

I	Lower limit	Upper limit
4	0.48	11.14
5	0.83	12.83

2.2.2 F-specific phages

Phage reference materials (RMs)
A new batch of RMs containing MS2 was prepared on 220597 (ddmmyy). *Ca* 1000 vials were prepared and stored as described in 2.1.1. Before freezing and a few days after freezing, 9 respectively 10 vials of this batch were checked (each in duplicate) for the criteria mentioned in 2.1.1. The results are given in Table 3. The batch fulfilled the criteria stated in 2.1.1. Immediately after preparation and storage of this batch of RMs, vials were analysed for preparation of a control chart. This completed chart is presented in Figure 2. The values of the mean count and the control limits are given in Table 4. The last results in Figure 2 were analysed in December 1998, showing that this batch of RMs containing MS2 is stable for at least 1.5 years.

Table 3 *Results of RMs containing MS2, batch 220597*

	Before freezing	4 days after freezing at (-70 ± 10) °C
Geometric mean (pfp/ml)	66.5	65.2
T_1	10.3	9.6
T_2/ (I-1)	0.74	1.10
I	9	10

I: Number of vials; Critical values of χ^2-distribution (which T_1 should fulfil) at 9 or 10 degrees of freedom and 95% confidence: lower limit 2.70 respectively 3.25, upper limit 19.02 respectively 20.48.

Table 4 *Control chart values of MS2, batch 220597*

	pfp/ml
$\bar{x}$ - 3s	31.3
$\bar{x}$ - 2s	39.8
$\bar{x}$	64.5
$\bar{x}$ + 2s	104.4
$\bar{x}$ + 3s	132.9

$\bar{x}$: Geometric mean; $\bar{x} \pm 2s$: warning limits; $\bar{x} \pm 3s$: action limits

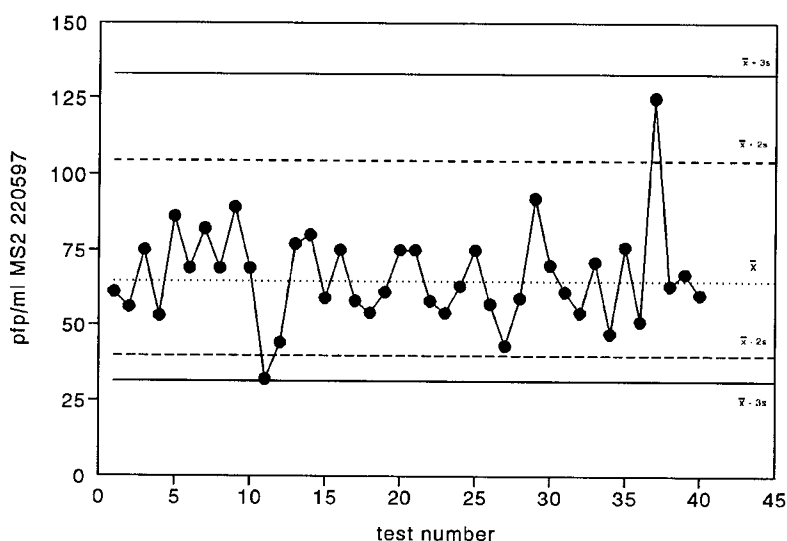

Figure 2 *Control chart of RMs containing MS2, batch 220597, on TYGA with host strain WG49 Salmonella typhimurium.*

Naturally polluted standard samples

The results for the F-specific phages of the naturally polluted standard samples "The Bilt 071197" are given in Tables 5 and 6. In Table 5 the results of the total number of F-specific phages (FTOTPH) are given. In Table 6 the results of the same vials for the number of F-specific DNA phages (FDNAPH) are given. Like for the determination of the somatic coliphages, the number of F-specific phages show much variation between the non-filtered vials. Filtering of the vials here also decreased the variation (lower value for T_2/ (I-1)). This procedure was therefore also introduced in the protocol of the second collaborative study for the determination of the F-specific phages in the naturally polluted standard samples.

Table 5 Results naturally polluted standard samples "The Bilt 071197", FTOTPH

Day	Non-filtered				Filtered (0.22 µm)			
	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)
0[1]	4[2]	198.5	1.97	28.6	-	-	-	-
3	5	163.3	2.93	2.68	-	-	-	-
18	5	90.6	37.3[3]	3.25	-	-	-	-
67	5	118.1	45.1[3]	7.20	5	106.1	26.4[3]	1.48
74	5	132.1	7.01	4.50	5	187.7	2.13	4.10
95	-	-	-	-	5	163.9	3.60	2.96
113	-	-	-	-	5	123.9	8.43	2.43

Explanations see Table 2

Table 6 Results naturally polluted standard samples "The Bilt 071197", FDNAPH

Day	Non-filtered				Filtered (0.22 µm)			
	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)
0[1]	5	10.4	5.0	0.75	-	-	-	-
3	5	11.5	1.09	0.40	-	-	-	-
18	5	6.4	4.81	3.97	-	-	-	-
67	5	9.3	2.58	3.40	5	5.4	0.48	1.30
74	5	7.8	8.64	0.38	5	6.1	4.21	1.23
95	-	-	-	-	5	12.5	3.39	0.64
113	-	-	-	-	5	8.7	4.59	3.18

Explanations see Table 2

2.2.3 Phages of *Bacteroides fragilis*

Phage reference materials (RMs)

The batch of RMs containing B40-8 was prepared on 260397 (ddmmyy) and was the same batch as used for the first collaborative study. Results of this batch were presented in Mooijman *et al.* (1998). The batch fulfilled the criteria for the mean number of plaque forming particles (pfp) and for homogeneity. One day after freezing at (-70 ± 10) °C the geometric mean number of pfp/ml of 10 vials (I=10) was 105.9. T_1 was not significantly different from a χ^2-distribution (10.6) and $T_2/(I-1)$ was ≤ 2 (0.56). A preliminary control chart was prepared with 10 data (Mooijman *et al.*, 1998). Since the first collaborative study new results were obtained and the mean and the limits of the chart were recalculated using 20 data.

This latter chart is shown in Figure 3. The values of the mean count and the control limits are given in Table 7. The last results in Figure 3 were analysed in September1998, showing that this batch of RMs containing B40-8 is stable for at least 1.5 years. Two outlying values are visible in the chart (test numbers 21 and 22). An explanation for these low values was the use of a poor batch of medium (the same batch of medium was used in both cases).
During the use of this batch of phage RMs and its control chart, switches were made from medium (BPRM) without the addition of bile (as used for the first collaborative study), to medium (MBPRM) with the addition of bile (test numbers 12 and 13), to again medium (BPRM) without bile (test number 14 and further).

Table 7 *Control chart values of B40-8, batch 260397*

	pfp/ml
$\bar{x}$ - 3s	69.9
$\bar{x}$ - 2s	78.1
$\bar{x}$	97.3
$\bar{x}$ + 2s	121.2
$\bar{x}$ + 3s	135.3

$\bar{x}$: Geometric mean; $\bar{x} \pm$ 2s: warning limits; $\bar{x} \pm$ 3s: action limits

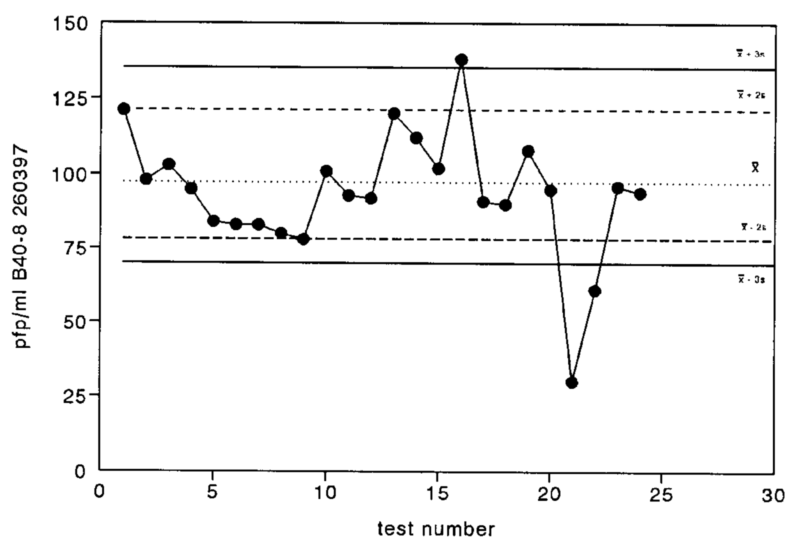

Figure 3 *Control chart of RMs containing B40-8, batch 260397, on (M)BPRMA with host strain HSP40 Bacteroides fragilis.*

Naturally polluted standard samples

The results for the phages of *Bacteroides fragilis* of the naturally polluted standard samples of Barcelona 311097 are given in Table 8. In the method for detection and enumeration of BFRPH in naturally polluted samples, the prefiltering of the samples through a low protein binding filter is advised. Therefore only results of filtered vials are given in Table 8. The mean values are relatively low (lower than the criterion of 30 pfp/ml). However, this was the highest value which was possible with the naturally polluted sample available. The sewage from Barcelona was used as such without making dilutions.

In Table 8 a differentiation is made for the results obtained from medium without bile (BPRM) and from medium with bile added (MBPRM). The results from MBRPM are somewhat higher than the results obtained from BPRM. The variation within vials (T_1) and between vials (T_2/ (I-1) fulfil the criteria as set in 2.1.1.

Table 8 Results naturally polluted standard samples "Barcelona 311097", BFRPH

Day	Filtered, cultured on BPRMA				Filtered , cultured on MBRMA			
	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)
0[1]	5	14.2	1.16	1.07	-	-	-	-
4	5	25.5	4.04	2.20	-	-	-	-
25	5	24.4	5.79	0.53	-	-	-	-
47	5	23.9	0.75[3]	0.99	5	29.8	2.70	1.40
103	-	-	-	-	5	27.4	1.78	1.48

Explanations see Table 2

2.2.4 Concentration technique

The results of the concentration technique with RMs are given in Table 9.

Table 9 Results concentration technique with RMs containing ΦX174 (batch 270597), MS2 (batch 220597) and B40-8 (batch 260397).

	Suspension A[1] (mean pfp/ml)	Suspension B[1] (mean pfp/ml)	% recovery
SOMCPH (ΦX174)	16	0.2	5
FTOTPH (MS2)	27.5	3.3	48
BFRPH (B40-8)	12	2.2	73

[1]: Suspension A is before concentrating, suspension B is after concentrating.

The total volume of suspension A added to the 1 litre synthetic sea salt solution was 10 ml. The total volume of the concentrate, suspension B was 40 ml.
In Table 9 no results are given for the determination of FDNAPH, because here only zero counts were found (which was expected when dealing with MS2). The recovery results for ΦX174 are low (5 %), which is probably a property of this standard phage. Earlier experiments carried out by the Department of Microbiology of the University of Barcelona showed a mean recovery of *ca* 50 % for SOMCPH in naturally polluted water samples (personal communication).
Unfortunately the low recovery of ΦX174 was detected only a few weeks before the collaborative study would be performed. At that moment no other phage RM for SOMCPH was available. It was therefore decided to continue the collaborative study with the concentration technique with ΦX174 as standard phage for SOMCPH with the remark to the participating laboratories that low recovery could be expected.

3. Collaborative study

3.1 Materials and methods

3.1.1 Design of the trial

The second collaborative study on bacteriophages was carried out in March 1998, according to the instructions described in the protocol (see Annex 4). Each participating laboratory received, a few weeks before the date of the trial a (big) parcel (by courier service) containing:

- Dry ice;
- 5 Vials containing naturally polluted samples “De Bilt 281097”, for analyses on SOMCPH;
- 5 Vials containing naturally polluted samples “De Bilt 071197”, for analyses on F-specific phages;
- 5 Vials containing naturally polluted samples “UB 311097”, for analyses on BFRPH;
- 8 Vials of reference materials containing phage ΦX174, batch 040696;
- 6 Vials of reference materials containing phage MS2, batch 220597;
- 4 Vials of reference materials containing phage B40-8, batch 260397.

After receipt all vials had to be stored immediately at (-70 ± 10) °C.

In a fixed period, the vials were thawn at room temperature and analysed in the following way:

Day 1 Enumeration of SOMCPH, FTOTPH, FDNAPH (in the presence of RNase) and BFRPH in naturally polluted “standard samples”.
Day 2 Reading of the plates of day 1
Day 3 Concentration technique with phage RMs and enumeration of SOMCPH, FTOTPH, FDNAPH (in the presence of RNase) and BFRPH.
Day 4 Reading of the plates of day 3.

The phage enumeration methods used during the study are described in 2.1.1 (DAL-methods).

For the analysis of the naturally polluted standard samples, the content of 5 vials were mixed per phage type. The mixture was “decontaminated” by filtration through low protein binding filters with 0.22 μm pore size. The filtrate was homogenised and analysed for the phage type mentioned for the specific naturally polluted standard sample. Each phage type was analysed in 10-fold. Beside the naturally polluted standard samples, quality control was performed by analysing one vial of an RM in duplicate for the phage concerned.
The design for the concentration technique was similar to the experiment described in 2.1.2. For the collaborative study, suspension A (before concentration) existed of a mixture of 12 ml

ΦX174, 6 ml MS2 and 4 ml B40-8 (batches of RMs mentioned above). Seven ml of suspension A was used for performing phage analysis before concentrating. For each phage type, 1 ml of suspension A was analysed in duplicate except for FDNAPH which was analysed in singular. Fourteen ml of suspension A was added to 1 L of synthetic sea salt solution (Annex 4; 6.1). The solution was mixed and next the concentration method was applied as described in Annex 3. The concentrate (suspension B) was checked for the number of bacteriophages by analysing 10 times 1 ml of suspension B for SOMCPH, 10 times 1 ml of suspension B for FTOTPH, 10 times 1 ml of suspension B for BFRPH and 5 times 1 ml of suspension B for FDNAPH.

Before counting, the plates were randomly labelled (separate labelling on day 2 and day 4) to eliminate the effects of extraneous factors. More details about the design of the second collaborative study are given in Annex 4. All results were recorded on a reporting form (Annex 5) and sent to the organising laboratory. The Standard Operating Procedures (SOPs) used during the trial were the same as the ones used during the first collaborative study (Mooijman *et al.*, 1998).

3.1.2 Analysis of the data

Naturally polluted standard samples
Like for the first collaborative study, the analysis of the data of the naturally polluted samples can be divided in two parts: the *data screening* and the *statistical analysis*.

* The *data screening* can again be subdivided into screening by the participating laboratories and statistical screening.

  Screening by the participants took place by means of an overview made by tabulating the raw results per laboratory. The raw data were back transformed from random numbers to the results found per phage method. The participants were asked to check the overview for any errors in the computer data for their own laboratory. Errors were reported to the organising laboratory for correction of the data set.
  Furthermore all technical details on the methods (as specified in the reporting form; Annex 5) were listed and discussed with the participating laboratories. Deviations from the protocol were discussed for possible effect on the results and decisions were taken about further analysis of results obtained under deviating technical conditions.

  The statistical screening concerns the analysis of variation between counts, performed per laboratory and per method. Due to the design of the trial, no variation between vials could be determined, only variation between replicates (of a mixture of the content of 5 vials). The replicate variation was determined by calculating T_1 values per laboratory and per method. The formula for T_1 is given in Mooijman *et al.* (1998). If the replicates were taken out of an (theoretically) optimal mixed suspension, the number of plaque forming particles

in the replicates should follow a Poisson distribution. In this latter case the T_1 statistic (for each laboratory) should follow a χ^2 - distribution with J-1 degrees of freedom (J is the number of replicates). A one-sided test with 95% upper critical value was performed to detect large variances between replicates, indicating a poor repeatability of counts. The T_1 value was also one-side tested with a 99% lower critical value, to detect small values of T_1, which may point to an unusually good repeatability of replicates. Largely deviating results for the T_1 values were also discussed in a meeting with the participating laboratories. In this meeting decisions were taken about further analysis of deviating results.

* The *statistical analysis* was performed using the statistical software SAS (6.12) for Windows. The data were transformed to logarithmic scale (^{10}log). An analysis of variance (Wardlaw, 1993), closely following ISO-guide 35 (Anonymous, 1989), was performed to detect (significant) differences in results between laboratories and methods. Outlying laboratories were detected by applying the Grubbs' test to the mean counts from each laboratory (Anonymous, 1988). The repeatability (r) and reproducibility (R) values were calculated per method (Anonymous, 1986). Because the log scale was used the definitions of r and R on the original scale are:
  - repeatability (r): the value below which the ratio between two geometric means of two vials in one laboratory may be expected to lie with a probability of 95% (within laboratory precision);
  - Reproducibility (R): the value below which the ratio between two geometric means of two vials from different laboratories may be expected to lie with a probability of 95% (between laboratory precision).

Concentration technique

The analysis of the data of the concentration technique also started with a *data screening*, which here only existed of the visual screening and the screening by the participating laboratories (see above).

The *statistical analysis* performed on the data of the concentration technique existed of a calculation of the percentage recovery per laboratory and phage type.

Furthermore a Monte Carlo simulation was performed using the statistical software @Risk (version 3.5.1; Palisade Cooperation Newfield/New York, USA). This simulation was performed to check whether random variation in phage concentrations could explain the observed variation in recovery results. For this simulation the following was taken into account:

- Design of the concentration experiment (preparation of the samples) as described in 3.1.1;
- Volume of suspension B is the mean of the volumes used in all participating laboratories (41.9 ml);
- The only variation is a Poisson distribution in the phage numbers, for which the arithmetic mean value of suspension A of all participating laboratories per phage type is used.

To reproduce the collaborative study, the simulation was performed with 15 replicates (for SOMCPH and FTOTPH) or 10 replicates (for BFRPH). The replicates here mean the number of 'simulated laboratories'. Because these low number do not lead to reliable estimates of the residual variation, the simulation was also performed on 900 replicates (for all phage types)

3.2 Results

3.2.1 Technical results

The main observations of the participating laboratories are summarised in Tables 10 to 19. The criteria described in the protocols, SOP's or ISO's are printed below each table. Deviating observations from these criteria are printed in bold type. Where deviations from the protocol were observed, the participants discussed the possible effects on the results.
The main subjects discussed are indicated below.

- Table 10:
  - The shipment time of the parcel of laboratory 12 was 3 days (in stead of 1 or 2). However, the samples were still frozen. Therefore no effect on the phage counts were expected.
  - Laboratories who performed the trial on more than one day, divided the work so that one phage method was done completely on one day. The sheets with random numbers were cut into parts, one for each phage group, and could still be used effectively. However, for the concentration technique dividing of the work over more than one day was not possible as the suspensions A and B had to be analysed on the same day for all phages. Laboratory 11 stored suspensions A and B at 5 °C and performed analysis of BFRPH one day later than the other phages. It was expected that this would influence the results and therefore the results of the trial concerning the concentration technique for BFRPH of laboratory 11 were excluded from further analysis.
  - Laboratory 12 performed the trial later than indicated in the protocol. However, very little effect was expected as the samples were stored at -70 °C.
  - Storage temperatures (of the samples) below -80 °C were considered as acceptable. Storage at -22/-27 °C was considered acceptable for a limited amount of time and was not expected to have influenced the counts.
- Table(s) 11 (a and b):
  - For the first collaborative study it was already discussed that a range of ± 0.1 or 0.2 pH unit for the media is unnecessarily strict and impractical. A range of ± 0.5 pH units was considered more realistic. In the (new) prescription of the medium for the determination of phages of *Bacteroides fragilis* (MBPRM) this range of ± 0.5 pH units was introduced (6.8 ± 0.5). If the range of ± 0.5 pH units is adopted for the interpretation of the data (for SOMCPH and FTOTPH: 7.2 ± 0.5) most of the pH values of the media are within the limits. A few pH values are still too low or too high. Another problem to this is the temperature of the medium when the pH is measured. Not measuring at room temperature

but e.g. at 45-50 °C (in the molten agar) can give a different pH value. Furthermore, very little is known of the effect of small pH deviations. It was therefore decided not to exclude any data because of deviating pH values.
- In some laboratories no Nalidixic acid (Nal) was added to the ssMSA and/or ssTYGA. Addition of Nal is important to suppress background flora. Not adding Nal to the medium can in case of naturally polluted samples lead to overcrowded plates. However, the naturally polluted samples used during the trial were filtered before use. With the used type of filter background flora is removed. Therefore very little problems were expected with counting of plates without Nal added.

- Table 12:
  - The pH of the buffer was set at 6 in the protocol. This to obtain a clear concentrate, in which the plaques would be easier to read than with a buffer of pH 7. However, two laboratories (laboratories 3 and 15) used a buffer with pH 7-7.4. No special problems with the detection of the plaques were reported by these laboratories.
  - It was discussed whether the use of more than 1 centrifuge tube for concentrating the final suspension would influence the recovery. It was agreed that the recovery results of the trial would be compared with the number of centrifuge tubes used.
  - In the protocol concerning the concentration technique, the temperature of centrifugation is set at (5 ± 3) °C. Four laboratories used higher temperatures (laboratories 3, 8, 10 and 15). It was not known whether these higher temperatures would effect the results. Therefore no data were excluded because of deviating centrifugation temperature.
- Tables 13-15:
  - No incubation temperatures (for incubation of inoculum and working cultures) are given in the tables because the temperatures were in all laboratories within the specified range of (36 ± 2) °C.
  - From the results of the first collaborative study (Mooijman *et al.,* 1998) it was already concluded that a minimum of shaking (e.g. manually every 30 min: laboratory 12) while incubating the host strains WG5 *Escherichia coli* and WG49 *Salmonella typhimurium* would be sufficient.
  - For the phage enumerations it is important to have a host strain in growing (log) phase. Practically the inoculum culture should contain *ca* 10^8 cfp/ml. For host strain WG5 all laboratories obtained a good inoculum culture in a reasonable period of time. Two laboratories reported a not very dense inoculum culture of WG49 (laboratory 10 for the trial with naturally polluted samples and laboratory 11 for the trial with the concentration technique). However, both laboratories did not mention problems with counting of the plaques, so that excluding of data did not seem to be necessary. For culturing host strain HSP40 *Bacteroides fragilis* the protocol was changed when compared to the protocol of the first collaborative study. According to the new protocol (Annex 2) it is possible to freeze a working culture at -70 °C. The inoculum culture is then prepared from the frozen working culture (in general the same procedure as for the other two phage types). In the old protocol (Mooijman *et al.,* 1998, Annex 8) the working culture was prepared by

making an "overnight" culture in broth. Most laboratories were not able to apply the new procedure during the trial. The time span for testing the new procedure had been too short. Therefore almost all laboratories (except laboratories 7 and 15) used the old protocol for preparing the working culture of HSP40 ("overnight culture"). The inoculum cultures were not in all laboratories sufficiently dense. Laboratories 4, 5, 7, 8 and 10 (trial with naturally polluted samples) and laboratories 5, 8 and 9 (trial with concentration technique) reported low viable counts. Laboratory 4 performed viable counts by membrane filtration, which might have given an underestimation of the viable count of the inoculum culture. Laboratories 7 and 8 mentioned, for the trial with naturally polluted samples, no problems in counting of the plaques. Laboratory 10 reported problems of half growth of the host culture on the plates for phage enumeration of the naturally polluted samples. For this trial the plates were not dried. However, for the trial with the concentration technique the MBPRMA plates were dried before use and no problems were detected here. It was agreed that the results of the trial concerning the naturally polluted samples for BFRPH of laboratories 5 and 10 and for the trial concerning the concentration technique for BFRPH of laboratories 5, 8 and 9 would be excluded from further analysis.
- Laboratory 3 reported, for the trial with naturally polluted samples, the use of an "overnight" culture as inoculum culture for the BFRPH method. However, no problems with the counting of the plaques were reported. Therefore no exclusion of data were made here.

- Table 16:
  - According to ISO 10705-1, the inoculum culture of WG49 should not longer be stored in melting ice than 2 hours. Laboratory 1 reported a much longer storage times of the culture on ice (180 and 250 min, instead of 120 min). As it was not sure whether this would influence the results, no data were excluded from further analysis because of this. However, it was agreed that more research should be done to test the influence of storage time of the inoculum culture of WG49 in ice.
- Table 17:
  - No time limit was set how long the suspension A and B should be kept at maximum at room temperature. In several laboratories (laboratories 5, 8, 9, 10, 11 and 13) both suspensions were longer than 2 hours at room temperature before using them. The influence of this was not known. It was discussed that it might be advisable to mention in the protocol for concentration, to store the concentrate in melting ice (instead of leaving it at room temperature), until phage enumeration is performed.
- Table 18:
  - The total time necessary to perform the phage enumerations was in most cases *ca* 15 min per phage type. This is probably sufficient short to prevent the phages from the (negative) influence of the temperature of the waterbath (45 °C) to keep the semi-solid agar molten.
- Table 19:
  - Laboratory 1 reported a long incubation time of the BFRPH plates of the trial concerning the naturally polluted samples. However, the laboratory did not report problems with

reading of the plaques. It was discussed that a long incubation time does not have influence on phage counts in samples with low background flora. However, in samples with high background flora, a long incubation time could disturb the reading of the plates.

- General remarks:
  - Laboratories 3 and 14 mentioned many difficulties with reading of the plates, concerning the trial with naturally polluted samples, for SOMCPH. Many plates contained air bubbles, so that it was difficult to differentiate bubbles from plaques. Both laboratories doubted their counts and decided to withdrawn their results of the SOMCPH, trial with naturally polluted samples.
  - Several laboratories mentioned some difficulties in reading of the plates, in most cases because of the presence of high number of plaques. However, some laboratories "double-checked" their counts by asking a second person to count the same plates as well. Therefore most of these laboratories trusted their counts, except for laboratory 8 for FTOTPH of the trial with naturally polluted samples. This laboratory decided to withdraw their results.
  - Laboratory 3 did not count any plaques in the concentrate (suspension B of the trial with the concentration technique) and was therefore excluded from further analysis.

A summary of the data excluded from further analysis is given in Table 20.

Table 10 Observations of participating laboratories: General questions

Lab-code	Shipment time parcel (days)[a]	Samples still frozen after arrival?	Date trial (ddmmyy)[b]		Temp. freezer (°C)
			Nat. poll.	Concentr.	max/min[c]
1	1	yes	230398	250398	-70 / -77
2	1	yes	240398	260398	**-22 / -25**
3	1	yes	300398	010498	-70 / -71
4	1	yes	3003&310398	010498	-65.6 / -70.6
5	2	yes	240398	260398	-68 / -71
7	1	yes	290398	310398	-68 / -72
8	1	yes	2303&010498	020498	-80
9	1	yes	230398	250398	-70
10	1	yes	300398	010498	**-22 / -27**
11	2	yes	2303&310398	**3003&310398**	-70 / **-84**
12	**3**	yes	**0604&080498**	**080498**	-60 / -75
13	1	yes	300398	300398	-74 / -78
14	1	yes	26&27&300398	010498	-80
15	2	yes	230398	240398	no info
16	organiser	yes	2303&240398	270398	-72 / -79

a: Date of mailing was 090398 (ddmmyy) by courier; shipment time 1-2 days at maximum
b: Date trial according to protocol: 16 March - 3 April 1998
Nat.poll.: Naturally polluted samples; Concentr.: Concentration technique
Lab 4, 8, 11, 12, 16: first date SOMCPH & FTOTPH, FDNAPH; second date BFRPH
Lab 14: Each phage type on a different date (for naturally polluted samples)
c: Storage temperature during receipt samples and date of the trial: (-70 ± 10) °C

Table 11a Observations of participating laboratories: pH media for full trial

Lab-code	pH media on day of the trial [a]					ssMSA + Nal? [b]
	PS	MSB	MSA		ssMSA	
			plates	bottles		
1	7.0	7.1	7.1	7.2	7.1	no
2	7.0	7.2	7.2[d]	7.0[d]	7.0[d]	yes
3	7.1	7.1	7.2[d]	6.9[d]	7.1[d]	no
4	7.0	7.0	7.0	no info	6.9	yes
5	6.6&**6.0**[c]	7.4&7.3	7.2&7.2	7.2&7.3	7.0&7.2	yes
7	7.1	7.3	7.4	7.3	7.2	no
8	6.5	7.2	7.0	7.0	7.0	no
9	6.9	7.5	7.2	7.4	7.2	yes
10	7.0	7.4	7.3	7.3	7.4	yes
11	7.1	7.2	7.1	7.2	7.2	yes
12	7.0	7.4	7.4	7.4	7.4	yes
13	6.8	7.5	**7.6**	**7.8**	**7.8**[d]	no
14	7.1	7.2	7.2	7.2	7.2	no
15	7.4	7.2	7.2	7.1[d]	7.2[d]	yes
16	7.0	7.4	7.3	7.4	7.2	yes

a: According to ISO 10705, pH values at room temperature (*ca* 20 - 25 °C): PS (peptone saline solution): 7.0 ± 0.5; MSB, MSA and ssMSA: 7.2 ± 0.2; b: According to ISO 10705-2, addition of Nal to ssMSA is advised in case of samples with high bacterial background flora; c: First value for trial with naturally polluted samples, second value for trial with concentration technique; d: pH measured at 45 - 50 °C

Table 11b Observations of participating laboratories: pH media for full trial

Lab-code	pH media on day of the trial [a]							ss-TYGA +Nal? [b]
	TYGB	TYGA		ss-TYGA	MBPRMB	MBPRMA	ss-MBPRMA	
		plates	bottles					
1	7.1	7.2	7.2	7.2	6.6	7.3	6.5	no
2	7.2	7.2[d]	7.2	7.2[d]	7.0	7.0[d]	7.2[d]	yes
3	**6.4**	7.2[d]	**6.4**[d]	**6.4**[d]	7.3	7.1[d]	6.9[d]	yes
4	7.1	7.1	no info	7.3	6.8	6.9	7.0	yes
5	**7.0**	**6.8**	**6.8**	7.1	6.6&6.9[c]	**7.6**&7.3	7.2	yes
7	7.1	7.1	7.1	7.1	7.0	7.0	7.1	no
8	**6.5**	**6.4**	**6.4**[d]	**6.6**[d]	7.2[e]	7.2[d]	7.2[d]	no
9	**7.0**	**6.6**	6.9	**7.0**	7.3	7.3	7.0	yes
10	7.1	7.1	7.1	7.1	7.1	7.3	7.1	yes
11	7.2	7.2	no info	7.2	7.2	7.1	7.2	yes
12	**7.4**	7.1	7.2[e]	7.2[e]	7.1	7.3	7.0[e]	yes
13	**6.8**	**7.0**	**6.8**[d]	**6.9**[d]	7.3[e]	7.0	6.5[d]	yes
14	7.2	7.2	7.2	7.2	7.0	7.0	7.0	no
15	7.1	7.2	7.2[d]	7.1[d]	7.2	7.1[d]	7.2[d]	yes
16	**7.5**	7.0	**6.9**	**7.0**	7.0	**8.0**	6.8	yes

a: According to ISO 10705 and to the BFRPH protocol, pH values at room temperature (*ca* 20 - 25 °C): TYGB, TYGA, ssTYGA: 7.2 ± 0.1 (for basal medium); MBPRMB, MBPRMA, ssBPRMA: 6.8 ± 0.5 (for complete medium); b: According to ISO 10705-1, addition of Nal to ssTYGA is advised in case of samples with high bacterial background flora; c: First value for trial with naturally polluted samples, second value for trial with concentration technique; d: pH measured at 45 - 50 °C; e: pH measured at 30 - 40 °C

Table12 *Observations of participating laboratories: Information on pH of buffer and synthetic sea salt solution and centrifugation process concerning the trial with concentration technique.*

Labcode	pH on day of the trial[a]		Centrifugation[b]		
	Buffer	Synth.sea salt	size (ml) / no. of tubes	temperature (°C)	time (min)
1	6.0	no info	750 / 1	5	15
2	6.0	7.0	250 / 1	4	15
3	**7.0**	7.4	600 / 1	**room temp.**	20
4	6.1	no info	300 / 2	4	23
5	5.9	6.8	500 / 1	4	15
7	6.0	8.6	250 / 1	4	15
8	6.0	6.4	50 / 4	**26**	15
9	6.0	8.2	500 / 1	5	28
10	6.0	no info	250 / 1	**23**	15
11	6.0	no info	250 / 1	7 ± 1	20
12	6.1	no info	200 / 2	5	20
13	6.2	7.9	30 / 6	5 ± 3	15
14	6.0	no info	*ca* 70 / 4	4	15
15	**7.4**[c]	no info	25 / 2	**10**	30
16	5.9	7.0	250 / 1	4	15

a: According to protocol of concentration, pH values at room temperature (*ca* 20 - 25 °C): Buffer for phages: 6.0 ± 0.2; Synthetic sea salt solution: no pH indication; b: According to protocol of concentration: 15 min. at (5 ± 3) °C; c: pH measured at 4 °C

Table 13 *Observations of participating laboratories: Inoculum culture of host strain WG5 Escherichia coli, for full trial*

Labcode	Type incubator / speed (min^{-1}) of ic[a]	Incubation time (min)[b]		Viable count WG5 x10^8 cfp/ml[b]	
		Nat. poll	Concentration	Nat. poll	Concentration
1	inc+r / 100	255	255	2.0	2.5
2	inc+r / 100	190	195	2.0	3.2
3	w+r / 100	160	150	4.0	6.2
4	inc+r / 100	205	180	2.2[c]	3.2[c]
5	w+r / 100	145	150	3.3	3.0
7	w+re / 196	135	135	2.0	2.5
8	inc+r / 100	190	203	1.8	2.9
9	w+ro / 100	121	130	1.6	1.6
10	inc+r / 100	180	180	0.6	0.8
11	w+r / 100	157	152	1.0	0.9
12	inc / shaken	190	140	1.6	1.9
13	w+b/f / 100	120	120	1.8	1.8
14	w+r / 60	175	135	3.0	4.0
15	inc+r / 100	310	300	4.7	4.4
16	inc+r / 100	195	195	1.5	2.4

a: ic: inoculum culture; inc+r: incubator with rotating platform; shaken: flasks were shaken every 30 min (by hand); w+r: waterbath with rotating platform; w+re: waterbath with "reciprocating" platform; w+ro: waterbath with rocking platform; w+ b/f: waterbath back and forth. According to ISO 10705: shaking speed of ic: (100 ± 10) min^{-1}. b: Nat. poll: trial with naturally polluted samples, Concentration: trial with concentration technique; Viable count measured by pour plates in MSA. Viable count is calculated from counts yielding between 30 and 300 colonies per plate; aimed values is *ca* 10^8 cfp/ml; c: by membrane filtration

Table 14 Observations of participating laboratories: Inoculum culture of host strain WG49 Salmonella typhimurium, for full trial

Labcode	Incubation time (min)[a]		Viable count WG49 x10^8 cfp/ml[a]	
	Nat. poll	Concentration	Nat. poll	Concentration
1	180	255	1.1	1.8
2	175	195	4.4	2.7
3	170	180	1.9	2.1
4	150	210	1.3[b]	1.4[b]
5	145	150	1.1	1.2
7	135	150	1.1	1.6
8	290	253	1.9	2.7
9	138	131	1.1	1.1
10	150	165	**0.4**	0.6
11	166	164	1.1	**0.5**
12	180	185	2.2	2.0
13	170	170	5.1	5.1
14	170	180	5.3	5.0
15	310	300	2.3	2.9
16	195	195	2.4	3.0

Type of incubators used for culturing the inoculum culture of WG49 are the same as given in Table 13.
a: Nat. poll: trial with naturally polluted samples, Concentration: trial with concentration technique; Viable count measured by pour plates in TYGA. Viable count is calculated from counts yielding between 30 and 300 colonies per plate; aimed values is *ca* 10^8 cfp/ml; b: by membrane filtration

Table 15 Observations of participating laboratories: Working culture and inoculum culture of host strain HSP40 Bacteroides fragilis, for full trial

Lab-code	wc frozen or o.n.[a]	Incubation time o.n. wc (h;min)[b]		Incubation time ic (min)		Viable count ic HSP40 x10^8 cfp/ml[c]	
		Nat.poll.	Conc.	Nat.poll.	Conc.	Nat.poll.	Conc.
1	o.n.	17;00	16;00	315	325	1.2	1.1
2	o.n.	17;30	17;30	180	180	1.3	3.3
3	o.n.	18;30	16;30	no info	185	5.5	1.6
4	o.n.	18;00	19;10	210	210	**0.06**[d]	1.2[d]
5	o.n.	19;45	21;55	315	235	**0.01**	**0**
7	frozen	-	-	240	240	**0.5**	1.9
8	o.n.	16;45	16;30	270	383	**0.5**	**0.2**
9	o.n.	18;00	17;05	94	199	2.2	**0.06**
10	o.n.	20;30	18;45	300	360	**0.4**	0.7
11	o.n.	15;43	15;45	323	323	1.3	0.7
12	o.n.	22;15	22;15	235	235	2.3	2.3
13	o.n.	17;00	17;00	360	360	0.6	0.6
14	o.n.	18;30	20;00	250	300	no info	0.7
15	frozen+on	18;30	16;30	375	240	1.9	1.0
16	o.n.	18;00	18;15	240	195	2.9	0.6

a: wc: working culture, o.n: "overnight culture"; b: According to the BFRPH protocol: use frozen working culture, or culture overnight. Nat.poll.: trial with naturally polluted samples, Conc.: trial with concentration technique; c: ic: inoculum culture, Viable count measured by "DAL" plates in ssMBPRMA. Viable count is calculated from counts yielding between 30 and 300 colonies per plate; aimed values is *ca* 10^8 cfp/ml; d: by membrane filtration

Table 16 *Observations of participating laboratories: Time inoculum cultures were stored on melting ice before performing phage analysis, full trial*

Lab-code	Time ic WG5 in ice (min)[a]		Time ic WG49 in ice (min)[b]		Time ic HSP40 in ice (min)[c]	
	Nat.poll.	Conc.	Nat.poll.	Conc.	Nat.poll	Conc.
1	160	180	**250**	**180**	-	-
2	5	9	4	34	-	-
3	25	60	20	30	-	30
4	20	14	25	14	20	14
5	22	15	12	25	-	-
7	114	135	49	73	85	26
8	95	60	75	60	20	30
9	24	73	13	106	15	41
10	15	15	15	35	15	15
11	32	37	22	40	49	80
12	80	115	60	**135**	105	105
13	50	50	55	55	-	-
14	15	10	10	15	-	-
15	-	-	-	-	-	-
16	25	70	25	70	60	70

ic: inoculum culture; -: ic not placed on ice; Nat.poll.: trial with naturally polluted samples, Conc.: trial with concentration technique; a: According to ISO 10705: use the same working day; b: According to ISO 10705-1: use within 2 hours; c: According to BFRPH protocol: use within 6 hours.

Table17 *Observations of participating laboratories: Information on suspensions A (before concentration) and B (after concentration), concerning the trial with concentration technique.*

Labcode	Max. time (min) suspensions at room temp.[a]		Total volume suspension B (ml)
	Suspension A	Suspension B	
1	60	30	48.5
2	115	115	43.0
3	50	50	42.5
4	90	82	39.5
5	130	190	38.5
7	98	123	42.0[c]
8	40	210	40.0
9	160	190	42.4
10	370	270	40.0
11	53[b]	53[b]	49.4
12	no info	no info	40.5
13	kept in ice	275	39.0
14	90	90	42.5
15	60	120	40.0
16	65	55	40.3

a: According to protocol: place suspension A *ca* 30 in prior to the phage enumeration at room temperature. Place suspension B on the laboratory bench and perform phage enumerations as soon as possible; b: Suspensions A and B were stored in refrigerator for one night, BFRPH enumeration was performed on the next day; c: Of suspension A, 26.8 ml was used for concentration instead of 14 ml.

Table 18 *Observations of participating laboratories: Total time necessary for phage enumerations, concerning the trial with naturally polluted samples.*

Labcode	Total time necessary for phage enumerations (min)		
	SOMCPH	F-specific[a]	BFRPH
1	8	12	7
2	15	20	15
3	8	13	5
4	25	15	25
5	10	20	10
7	9	30	12
8	9	30	8
9	13	31	15
10	30	30	15
11	11	15	11
12	10	20	15
13	10	15	15
14	40	55	25
15	10	20	25
16	8	13	8

a: F-specific: Total time for enumeration of FTOTPH and FDNAPH.

Table 19 *Observations of participating laboratories: Incubation of phage plates, full trial*

Lab-code	Inc. time (h;min) SOMCPH[a]		Inc. time (h;min) F-spec[a]		Inc. time (h;min) BFRPH[a]	
	Nat.poll.	Conc.	Nat.poll.	Conc.	Nat.poll.	Conc.
1	16;40	17;35	16;25	17;35	**36;00**	17;35
2	18;30	19;00	18;30	19;00	18;30	19;00
3	20;45	**21;35**	20;45	**21;30**	19;45	**22;05**
4	19;05	18;50	19;15	19;10	18;50	18;50
5	17;10	19;40	16;55	19;40	19;00	18;45
7	18;10	18;15	18;10	18;15	17;55	18;05
8	17;52	16;35	16;35	16;35	17;00	16;25
9	19;24	18;00	18;00	18;00	18;15	18;01
10	19;45	19;30	20;00	19;00	20;00	18;00
11	19;55	19;55	17;20	19;50	16;40	17;15
12	20;05	19;15	20;20	19;05	18;55	19;20
13	16;00	16;00	16;00	16;00	16;00	16;00
14	19;00	20;30	19;00	20;00	19;45	18;00
15	16;30	16;15	16;30	16;30	17;00	16;30
16	19;40	16;00	18;40	16;00	19;45	16;00

a: According to ISO 10705 and protocol of BFRPH: (18 ± 2) hours (BFRPH under anaerobic conditions). F-specific: FTOTPH and FDNAPH.

Table 20 Data excluded from further analysis

Lab-code	SOMCPH		FTOTPH		FDNAPH		BFRPH	
	Nat.poll.	Conc.	Nat.poll.	Conc.	Nat.poll.	Conc.	Nat.poll.	Conc.
1	rep.10[a]							
2								
3	all[b]			all[d]		all[d]		
4								
5							all[e]	all[e]
7								
8			all[c]					all[e]
9								all[e]
10							all[e]	
11								all[f]
12								
13								
14	all[b]							
15								
16								

Nat.poll.: trial with naturally polluted samples, Conc.: trial with concentration technique;
a: zero count;
b: air bubbles in medium, plates difficult to read;
c: problems with counting of the plaques;
d: no counts from suspension B;
e: problems with growth of host culture;
f: storage of suspension A and B at (5 ± 3) °C before enumeration

3.2.2 Statistical results

3.2.2.1 Naturally polluted standard samples

The geometric mean results of all participating laboratories for all phage methods before any exclusion of data are summarised in Figure 4.

After exclusion of the data mentioned in Table 20, box and whisker plots were prepared per phage method which are presented in Figures 5-8. In these figures, the dash in the middle of the box represents the median or 50th percentile of the data. The box extends from the 25th percentile to the 75th percentile (interquartile range). The box and the whiskers include the 99th percentile of the data. Circles include the values outside the 99th percentile.

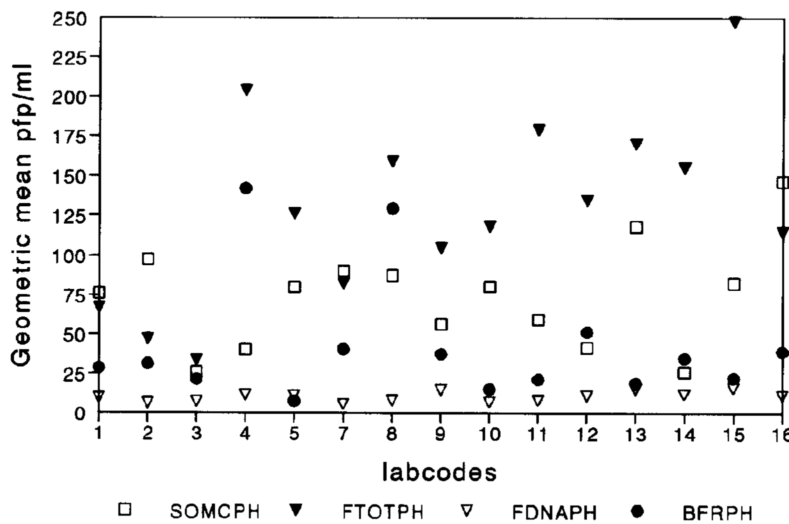

Figure 4 *Results of all participating laboratories for all phage types (trial with naturally polluted samples), before exclusion of data.*

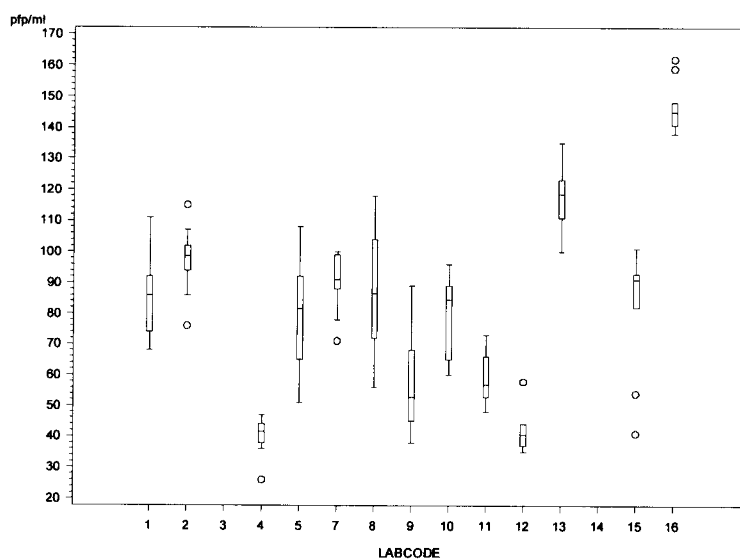

Figure 5 *Results of all participating laboratories for SOMCPH (trial with naturally polluted samples), after exclusion of data.*

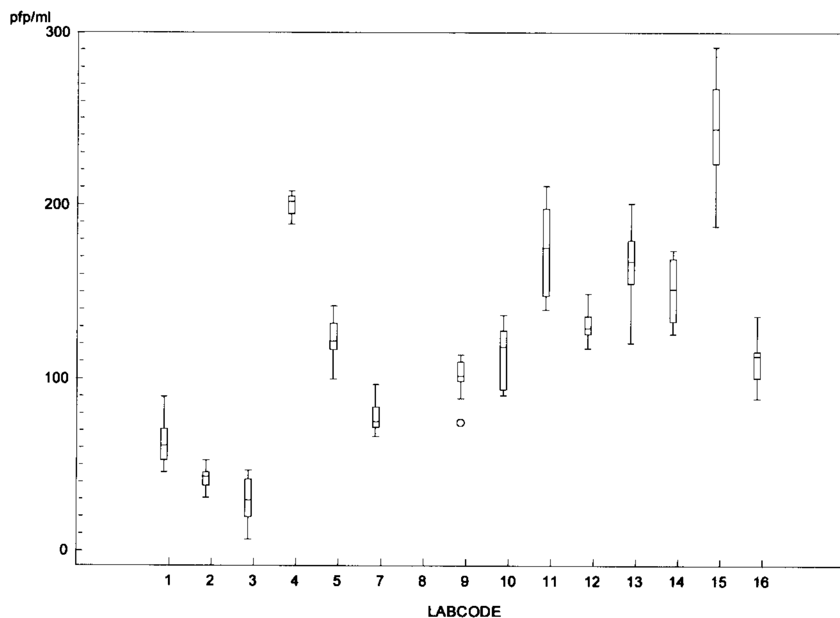

Figure 6 *Results of all participating laboratories for FTOTPH (trial with naturally polluted samples), after exclusion of data.*

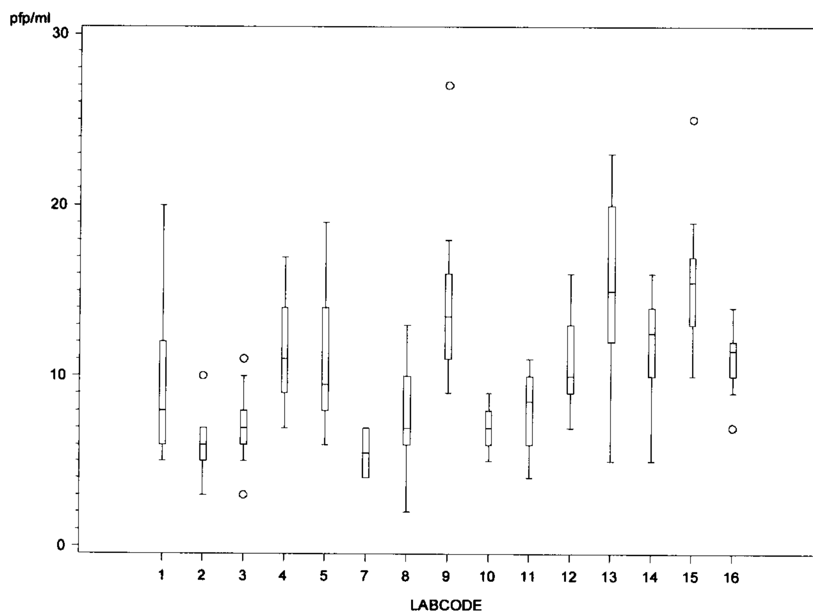

Figure 7 *Results of all participating laboratories for FDNAPH (trial with naturally polluted samples), after exclusion of data.*

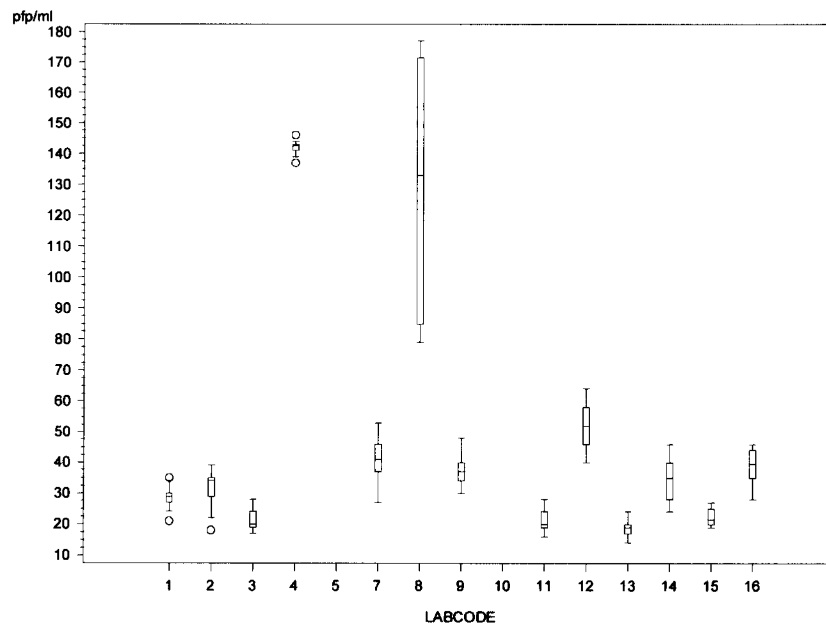

Figure 8 *Results of all participating laboratories for BFRPH (trial with naturally polluted samples), after exclusion of data.*

Arithmetic mean results and the T_1 values per laboratory and per phage method (after exclusion of data) are given in Tables 21-24. Beside the naturally polluted standard samples also phage reference materials (RMs) were analysed. For each phage type 1 vial of phage RM was analysed in duplicate, using the same method as used for the naturally polluted standard samples. To have an indication of the quality of the results, both results of each phage RM (per laboratory) were drawn in the control charts for each phage RM (see 2.2). These control charts with laboratory results are given in Figures 9-11. For the FDNAPH, no control chart was prepared as no positive control was used here. To check the FDNAPH method the phage RM containing the F-specific RNA phage MS2 was analysed in the presence of RNase. The results should be zero, which would indicate that a sufficient amount of RNase was added to the medium. Four laboratories found positive results with this negative control (laboratories 2, 7, 8 and 15). The number of plaques counted were for laboratory 2: 3 and 4; laboratory 7: 1 and 0; laboratory 8: 1 and 1; laboratory 15: 60 and 0. Laboratory 2 indicated that they had added less RNase to the medium than was indicated. They added 100 µl RNase to a bottle containing 50 ml ssTYGA, instead of 100 µl RNase to a tube containing 2.5 ml ssTYGA. This can explain the positive results. Laboratory 15 probably mixed the control results of the MS2 phage RMs. This laboratory reported for the positive control of MS2 in the FTOTPH method 60 and 0 plaques. For the "negative" control of MS2 in the FDNAPH method they reported 57 and 0 plaques. For the positive results of laboratories 7 and 8 no explanations were found.

Table 21 *Arithmetic mean, standard deviation and T_1 values per laboratory for **SOMCPH** (trial with naturally polluted samples), after exclusion of data.*

labcode	J	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T1
1	9	84.7	13.64	17.57[a]
2	10	97.4	10.71	10.60
4	10	40.4	6.17	8.48
5	10	80.2	18.62	38.90[a]
7	10	90.0	9.32	8.69
8	10	87.5	20.00	41.15[a]
9	10	56.6	15.45	37.96[a]
10	10	80.6	12.78	18.24[a]
11	10	59.5	8.55	11.07
12	10	41.7	6.60	9.40
13	10	118.2	10.40	8.24
15	10	82.6	19.68	42.18[a]
16	10	146.6	7.96	3.89

J : Number of replicates used for calculations.

a: Significantly different from a χ^2-distribution with J-1 degrees of freedom; χ^2 critical values :

J-1	Lower limit at 99% confidence	Upper limit at 95% confidence
9	2.088	16.919
8	1.646	15.507

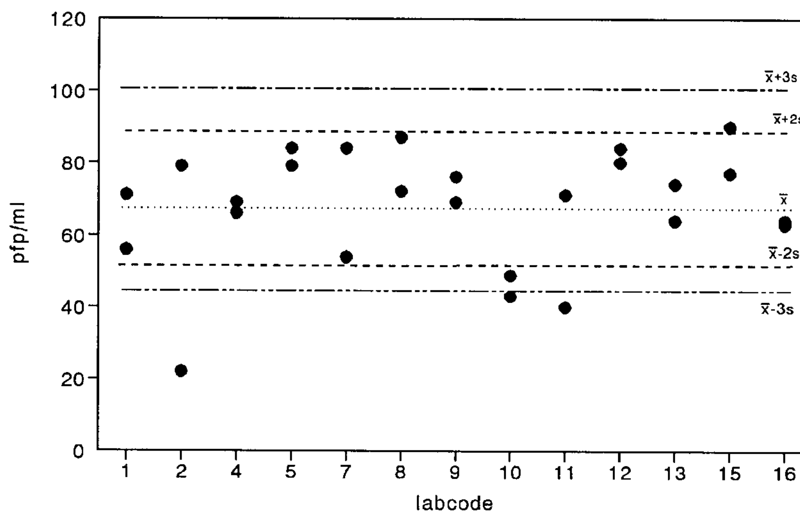

Figure 9 *Results of all participating laboratories (after exclusion of data), of phage RM containing ΦX174, batch 040696, drawn in the relevant control chart*

Table 22 *Arithmetic mean, standard deviation and T_1 values per laboratory for* ***FTOTPH*** *(trial with naturally polluted samples), after exclusion of data.*

labcode	J	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T1
1	10	67.1	13.25	23.56[a]
2	10	46.8	7.05	9.56
3	10	33.7	13.71	50.21[a]
4	10	203.7	6.82	2.05[a]
5	10	126.1	13.36	12.74
7	10	82.3	9.23	9.31
9	10	104.8	11.73	11.81
10	10	118.2	17.66	23.74[a]
11	10	178.8	26.61	35.65[a]
12	10	134.7	9.29	5.76
13	10	170.1	23.53	29.29[a]
14	10	155.1	18.21	19.25[a]
15	10	247.6	30.89	34.69[a]
16	10	114.9	13.96	15.26

Explanations see Table 21.

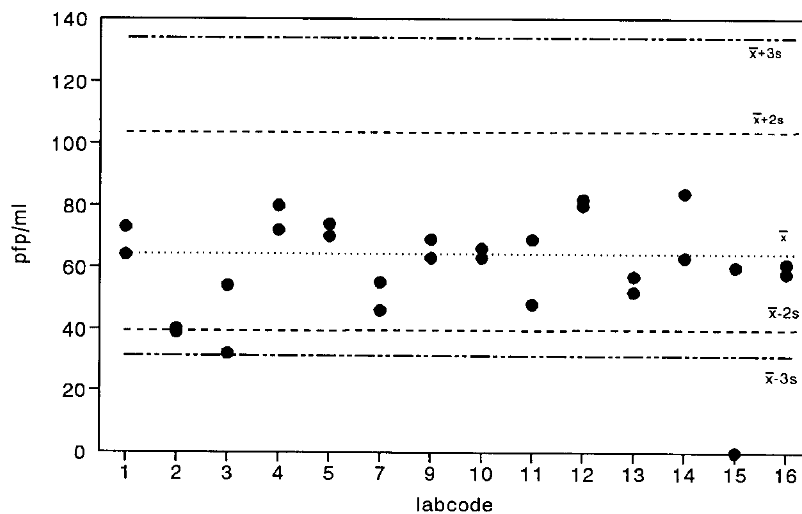

Figure 10 *Results of all participating laboratories (after exclusion of data), of phage RM containing MS2, batch 220597, drawn in the relevant control chart*

Table 23 *Arithmetic mean, standard deviation and T_1 values per laboratory for* ***FDNAPH*** *(trial with naturally polluted samples), after exclusion of data.*

labcode	J	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T1
1	10	9.5	4.72	21.11[a]
2	10	5.9	2.08	6.59
3	10	7.1	2.33	6.89
4	10	11.3	3.16	7.97
5	10	11.0	3.92	12.55
7	10	5.5	1.27	2.64
8	10	7.9	3.14	11.25
9	10	14.5	5.10	16.17
10	10	6.9	1.20	1.87[a]
11	10	8.0	2.31	6.00
12	10	10.8	2.97	7.37
13	10	15.0	6.24	23.33[a]
14	10	11.8	3.19	7.76
15	10	15.8	4.18	9.97
16	10	11.1	2.02	3.32

Explanations see Table 21.

Table 24 *Arithmetic mean, standard deviation and T_1 values per laboratory for* ***BFRPH*** *(trial with naturally polluted samples), after exclusion of data.*

labcode	J	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T1
1	10	28.6	4.20	5.54
2	10	31.4	6.69	12.82
3	10	21.5	3.37	4.77
4	10	142.0	2.54	0.41[a]
7	10	40.6	7.29	11.78
8	8	129.4	45.14	110.24[a]
9	10	37.5	5.34	6.84
11	10	21.4	4.12	7.12
12	10	51.5	7.44	9.68
13	10	19.0	2.79	3.68
14	10	34.9	7.68	15.21
15	10	22.3	3.02	3.68
16	10	39.1	6.06	8.46

Explanations see Table 21.

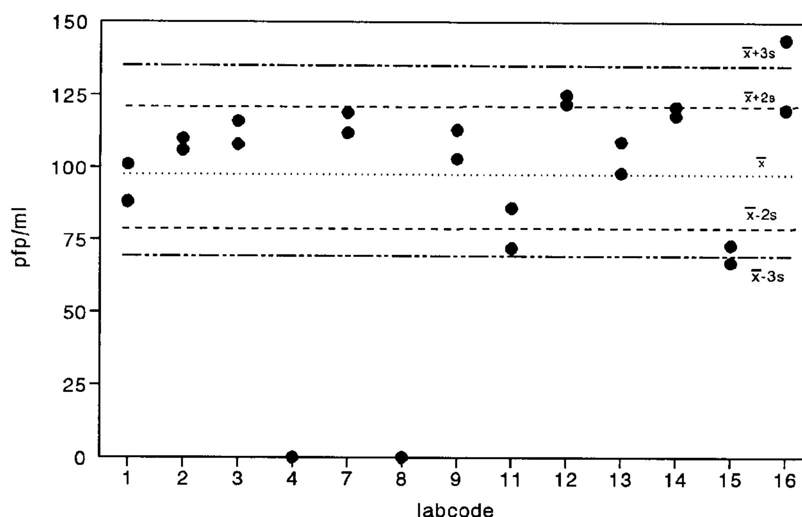

Figure 11 *Results of all participating laboratories (after exclusion of data), of phage RM containing B40-8, batch 260397, drawn in the relevant control chart*

Several laboratories found for T_1 significantly higher values than the χ^2 - distribution. In a few cases significantly lower values were found. No technical problems could be indicated causing these significant values, therefore the results were accepted to be used for further analysis.

After the analyses per laboratory, the means of all laboratories were compared (per procedure and after exclusion of data mentioned in Table 20). This was performed by using analysis of variance on the ^{10}log-transformed results. Although the analysis of variance showed the existence of significant differences between laboratories, the Grubbs' test did not detect any outliers.
Finally the repeatability (r) and reproducibility (R) were calculated per method. The results are shown in Table 25.

Table 25 *Repeatability (r) and reproducibility (R) values per method, for naturally polluted standard samples*

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

3.2.2.2 Concentration technique

After exclusion of the data mentioned in Table 20, the (arithmetic) mean values of suspension A (before concentration) and suspension B (after concentration) were calculated per phage type. The recovery percentages per laboratory and phage type were also calculated. For this purpose the volumes of the suspensions needed to be known. Of suspension A (the RM-mixture) 14 ml was added to 1 litre "water" before concentration. However, laboratory 7 used for this purpose 26.8 ml of suspension A. The total volume of suspension B for each laboratory is given in Table 17.
The results of each laboratory of the trial with the concentration technique, are given in Tables 26-28. In Figure 12 the percentage recoveries of all participating laboratories for the three phage methods (after exclusion of data) are shown.
In Table 29 the results of the Monte Carlo simulation are presented. The simulation was performed on the same number of laboratories which participated in the study (n=15, or n=10) and on a much larger number of replicates (n=900) to obtain stable estimates. In both cases it was found for all three phage types that the mean recovery was comparable to the mean recovery found during the collaborative study. However, the standard deviation (sd) and the coefficient of variation (cv) for the simulation were in all cases smaller than those found during the collaborative study. This indicates that the variation in recovery results found during the collaborative study could not only be explained by the random variation in phage numbers.

Table 26 Arithmetic mean, standard deviation and percentage recovery per laboratory for ***SOMCPH*** *(trial with concentration technique), after exclusion of data.*

labcode	Arithmetic mean (pfp/ml)		Standard deviation (pfp/ml)		%
	suspension A[a]	suspension B[b]	suspension A[a]	suspension B[b]	recovery
1	33.5	0.7	3.54	0.67	7.2
2	31.0	0.7	5.66	0.95	6.9
3	35.5	0.4	2.12	0.97	3.4
4	49.5	1.0	3.54	0.82	5.7
5	36.0	0.5	5.66	0.71	3.8
7	32.0	1.4	5.66	0.70	6.9
8	45.5	0.3	13.44	0.95	1.9
9	40.5	0.4	4.95	0.52	3.0
10	30.0	1.1	8.49	0.74	10.5
11	43.0	2.0[c]	2.83	1.73[c]	16.4
12	38.5	0.6	2.12	0.84	4.5
13	37.5	0.3	7.78	0.48	2.2
14	40.0	0.4	7.07	0.70	3.0
15	50.0	1.2	2.83	1.03	6.9
16	37.0	1.1	2.83	0.88	8.5

a: calculated of 2 replicates (before concentration); b: calculated of 10 replicates (after concentration); c: calculated of 3 replicates.

Table 27 *Arithmetic mean, standard deviation and percentage recovery per laboratory for **FTOTPH** (trial with concentration technique), after exclusion of data.*

labcode	Arithmetic mean (pfp/ml)		Standard deviation (pfp/ml)		%
	suspension A[a]	suspension B[b]	suspension A[a]	suspension B[b]	recovery
1	10.5	0.8	2.12	0.92	26.4
2	7.0	1.5	1.41	1.84	65.8
4	24.0	3.7	2.83	1.34	43.5
5	20.0	3.3	8.49	2.63	45.4
7	13.5	1.7	0.71	1.42	19.7
8	13.0	1.3	1.41	1.77	28.6
9	14.0	2.4	2.83	1.26	51.9
10	11.5	2.2	3.54	1.55	54.7
11	17.5	2.3[c]	2.12	1.98[c]	46.1
12	16.0	5.5	2.83	4.33	99.4
13	17.5	0.8	4.95	1.23	12.7
14	18.5	2.4	2.12	2.37	39.4
15	15.0	1.1	1.41	0.74	21.0
16	21.5	3.1	3.54	2.08	41.2

a: calculated of 2 replicates (before concentration); b: calculated of 10 replicates (after concentration); c: calculated of 7 replicates

Table 28 *Arithmetic mean, standard deviation and percentage recovery per laboratory for **BFRPH** (trial with concentration technique), after exclusion of data.*

labcode	Arithmetic mean (pfp/ml)		Standard deviation (pfp/ml)		%
	suspension A[a]	suspension B[b]	suspension A[a]	suspension B[b]	recovery
1	16.0	4.2	0.00	1.62	90.9
2	13.5	5.7	3.54	3.06	129.7
3	12.5	5.0	3.54	1.76	121.4
4	0.0	2.8	0.00	1.14	-[d]
7	23.5	7.1	3.54	2.77	47.3
10	9.5	3.9	0.71	2.85	117.3
12	24.0	5.3	1.41	1.70	63.9
13	19.0	2.9	8.49	1.45	42.5
14	18.0	5.0	2.83	1.76	84.3
15	7.0	3.5	1.41	2.51	142.9
16	21.0	5.0[c]	4.24	2.35[c]	68.0

a: calculated of 2 replicates (before concentration); b: calculated of 10 replicates (after concentration); c: calculated of 9 replicates; d: could not be calculated as the counts before concentration are zero

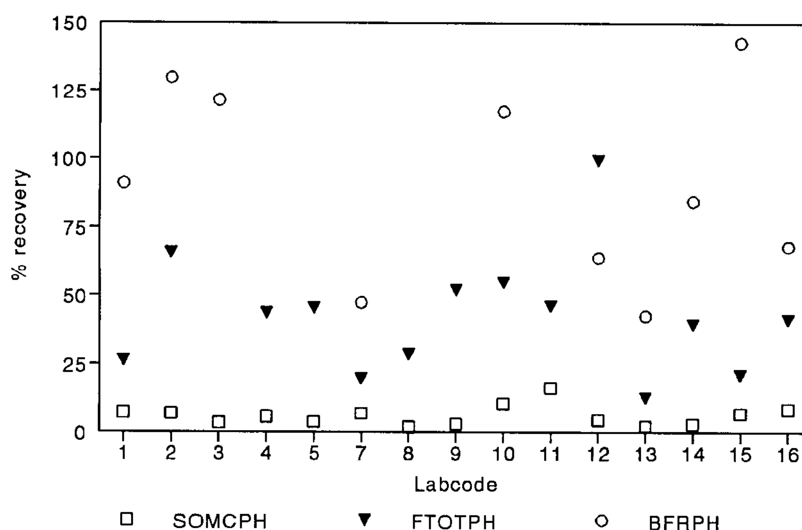

Figure 12 *Recovery results of the concentration technique of all laboratories for all three phage methods, after exclusion of data*

Table 29 *Mean results and variation of the recovery of the concentration technique obtained from the collaborative study and the Monte Carlo simulation*

	SOMCPH			FTOTPH			BFRPH		
	coll. study	simulation		coll. study	simulation		coll. study	simulation	
	n=15	n=15	n=900	n=15	n=15	n=900	n=10	n=10	n=900
Mean recovery (%)	6.1	5.9	6.3	42.6	50.3	44.5	90.8	99.7	94.7
sd recovery (%)	3.8	2.2	2.3	22.1	18.3	14.2	35.6	15.1	24.8
cv recovery	0.63	0.37	0.37	0.52	0.36	0.32	0.39	0.15	0.26

sd: standard deviation; cv: coefficient of variation (= sd/mean); n: number of replicates (participating laboratories or simulated laboratories)

During the discussion of the results with the participating laboratories questions were made whether the use of more than 1 centrifuge tube for concentrating the final suspension would influence the recovery. An attempt was made to compare the number of centrifuge tubes used during the trial (see Table 12) with the percentages of recovery. Six laboratories used more than 1 centrifuge tube (laboratories 4, 8, 12, 13, 14 and 15). Three of these six laboratories used two tubes (laboratories 4, 12 and 15), the others used 4 tubes (laboratories 8 and 14) or 6 tubes (laboratory 13). The numbers were too few to perform statistical analysis. The laboratory who used 6 centrifuge tubes indeed found somewhat lower recoveries when compared to the other laboratories. However, this result could as well be attributed to chance.

4. Discussion and conclusions

The phage reference materials (RMs) containing single phages fulfilled the criteria for homogeneity and showed a stable mean level, when stored at -70 °C for a sufficient period of time (at least 1.5 years). The standard samples containing a mixture of (naturally) phages showed more variation 'within' and 'between' vials when compared to the reference materials. An explanation for this high variation might be the existence of aggregates in the samples. By filtering the contents of the vials through a low protein binding filter (with 0.22 μm pore size) the 'extreme' variation disappeared. However, the variation between vials remained larger than in case of the phage RMs. In case of the RMs one is dealing with only one type of plaque, without the disturbance of plaques of different sizes and interference of background flora. In case of the naturally polluted (standard) samples these latter two points influence the ease of reading of the plates. The combination of large variation in the naturally polluted standard samples and the difficulties of interpreting some plates might have resulted in more variation in the results when compared with the single phage RMs. However, the naturally polluted standard samples represented the 'problems' which might occur when analysing samples in daily practice.

The naturally polluted standard samples for analysing SOMCPH and for analysing BFRPH showed higher mean counts after freezing of the samples when compared with the mean count on day 0 (before freezing). An explanation might be the existence of larger aggregates before freezing which separate into smaller aggregates after freezing. Furthermore, for BFRPH the switch of culturing on MBPRMA (bile added to the BPRMA) resulted in somewhat higher plaque counts in comparison with the counts found on BPRMA. Although bile added to the medium resulted in somewhat bigger plaques and higher plaque counts of BFRPH, the method became less 'robust' when bile was added. Many participating laboratories reported some problems with performing the BFRPH-method during the second collaborative study, specially with culturing the host strain. During a discussion with all participating laboratories it was decided to change again the composition of the medium for the detection of BFRPH by excluding bile.

The stability of the naturally polluted standard samples was sufficient (after 3-4 months of storage at -70 °C) for the purpose of the trial.

The first-line quality control of the analyses of the naturally polluted standard samples during the trial was performed by analysing pure culture phage RMs in duplicate. The results of each laboratory were indicated in the control chart of the phage concerned. In case of 'normal' day-to-day first-line quality control each laboratory prepares its own control chart. The control chart presented in Figures 9-11 were made from data obtained in only one laboratory

(the organising laboratory). Still the majority of the results of the phage RMs found in most of the participating laboratories were 'in control' in the relevant control charts, showing good quality in performing the phage methods. Unexplained results were found in laboratories 4 and 8 when analysing BFRPH. Both laboratories found high mean counts (higher than other participating laboratories) of BFRPH in the naturally polluted standard samples, but zero counts for the phage RMs (Figures 8 and 11 and Table 24). Furthermore, laboratory 8 found very high variation in counts between replicates. The negative results for the first-line quality controls raises some doubts in the results of the naturally polluted standard samples. However, during the discussion with the two laboratories, they both did not see reasons to doubt their results. They both were sure that what they had counted for the naturally polluted samples were all real plaques.

The values of repeatability (r) and reproducibility (R) found with the naturally polluted standard samples in this second collaborative study were higher than the values of r and R found with the phage RMs in the first collaborative study. This was not very surprising, bearing in mind that the variation in phage counts of the naturally polluted standard samples were higher than for the phage RMs. Also the earlier mentioned difficulties in reading the plates of naturally polluted samples might have lead to more variation in results. Still the repeatability (r) values found in the second collaborative study (= *ca* 1.6 for SOMCPH, FTOTPH and BFRPH) were not very different from the first study (=*ca* 1.4 for these three phage methods). This indicates a good within laboratory precision. The variation in results between laboratories was obviously higher in the second collaborative study when compared with the first collaborative study. Specially for BFRPH the highest value for the reproducibility (R= 5.72) was found with the naturally polluted standard samples. The earlier mentioned explanations for high variation in counts are probably also valid here. Furthermore, performing the BFRPH method caused some laboratories more problems than analysing samples for SOMCPH or F-specific phages.

It can be concluded that interpreting results of naturally polluted samples might need more experience than interpreting results of samples containing a pure phage culture.

The concentration technique showed low recovery of the chosen standard phage for SOMCPH ΦX174 (2.2 % - 16.4 %). The reason for this poor recovery was not found. According to the Department of Microbiology of the University of Barcelona it is possible to find a mean recovery of 50% for SOMCPH in naturally polluted water samples (personal communication). Despite the large variation in recovery, the mean recovery of ΦX174 is only 6.1%. This might indicate phage ΦX174 is not very representative for natural occurring somatic coliphages with respect to the recovery of the concentration (flocculation) method. For future experiments with the concentration technique it might be advisable test the use of another standard phage for SOMCPH.

Much variation was found in the percentage recovery of FTOTPH and BFRPH. The recovery of MS2 (FTOTPH) varied from 12.7 % to 99.4 %. For B40-8 (BFRPH) the recovery varied from 42.5 % to 142.9 %. The results of the Monte Carlo simulation showed that the variation in recovery for all three phages was higher than could be explained by the random distribution only. For all three phage RMs (ΦX174, MS2 and B40-8) it was shown that the variation in count results was at maximum a Poisson distribution ($T_2 /(I-1) \leq 1$; see 2.2). Therefore the variation in the percentage recovery found during the collaborative study was not only a result of the variation in results of the phage RMs. Other explanations need to be found, which may be linked to (performing) the concentration technique. The different steps in the concentration method can all cause some losses in the recovery. These losses may be influenced by the way of performing the method, like the fact whether a laboratory is experienced with the method or not. From the results (and discussion) of the collaborative study it is not possible to indicate which are the most critical steps in the concentration technique.

The question whether the use of more than one centrifuge tube for concentrating the final suspension would influence the recovery could not be answered from the results of this study. Further research might be necessary to this aspect.

Further research might also be necessary for analysing the influence of storage of the inoculum culture of WG49 *Salmonella typhimurium* in ice. From the results of the second collaborative study it was not clear whether longer storage than 2 hours of the inoculum culture of WG49 in ice would influence the phage counts.

Overall conclusions referring to the objectives of the second collaborative study:

- The quality control results by analysing phage RMs showed good quality in performing the three phage methods (SOMCPH, FTOTPH and BFRPH) in the majority of participating laboratories.
- A good within laboratory precision (r) for the three phage methods when analysing naturally polluted standard samples.
- More variation in results between laboratories for the three phage methods when analysing naturally polluted samples than when analysing phage RMs. Possible causes:
  - Large variation in phage counts in the naturally polluted standard samples;
  - Laboratories inexperienced in reading of plates from naturally polluted samples;
  - Problems with culturing the inoculum culture of HSP40 *Bacteroides fragilis*, probably caused by changes in the medium in the protocol of this study (addition of bile).
- All participating laboratories were able to apply the concentration method (based on flocculation). However, the results showed large variation in recovery for the three phages tested (ΦX174, MS2 and B40-8).

- Small modifications in the methods will be necessary:
  - Removal of bile in the prescription of the medium for the enumeration of BFRPH;
  - Storage of the concentrate, obtained from the concentration method, in melting ice (instead of storage at room temperature) until phage enumeration is performed.
- Laboratories can train themselves with the present methods for concentration and enumeration of the three types of bacteriophages (SOMCPH, FTOTPH and BFRPH) by analysing naturally polluted samples like sewage samples.

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Annex 2

Protocol for determination of *Bacteroides fragilis* phages

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PROTOCOL FOR DETERMINATION OF *BACTEROIDES FRAGILIS* PHAGES

General: For the preparation of all media, use the information in Annex A and Table 1.

1 Preparation of host strain cultures

1.1 Preparation of stock cultures

Rehydrate the content of a lyophilized ampoule of the reference culture of the host strain in 1 ml of MBPRMB (A.1), using a Pasteur pipette. Inoculate the suspension in 10 ml of MBPRMB (A.1) and incubate at (36 ± 2) °C for (18 ± 2) hours. Streak on a plate of MBPRMA (A.2). Incubate in an anaerobic jar or bag at (36 ± 2) °C for (36 ± 2) hours.

Alternatively if a culture in a slant is available, streak directly on a plate of MBPRMA (A.2). Incubate in an anaerobic jar or bag at (36 ± 2) °C for (36 ± 2) hours.

Inoculate cells (mass inoculation)[1] from the plate into 10 ml of MBPRMB (A.1) in a screw-capped glass tube. Incubate at (36 ± 2) °C for (18 ± 2) hours.

Mix culture and cryoprotector (A.6) in a ratio of 1:1 (vol:vol) Mix well avoiding bubble formation. Distribute into plastic vials in aliquots of ca 0,5 ml and store at (-70 ± 10) °C or in liquid nitrogen.

[1] Depending on the growth of the host strain on MBPRMA, inoculate 1/8 or more of the slant grown on the surface of MBPRMA, using a sterile cotton swab (e.g. in case of dense growth use 1/8 to inoculate 10 ml MBPRMB, in case of poor growth use 1/2 or the full slant).

NOTE

The first passage of the host strain should be stored as a reference in the laboratory. Purity of the culture should be checked before storage by Gram staining and by testing sensitivity to phage B40-8.

1.2 Preparation of working cultures

Thaw one vial of stock culture (1.1) at room temperature and streak on a plate of MBPRMA (A.2). Incubate in an anaerobic jar or bag at (36 ± 2) °C for (36 ± 2) hours. Fill a screw capped tube with prewarmed MBPRMB (A.1). Inoculate cell material (mass inoculation)[2], from the plate and incubate overnight at (36 ± 2) °C for (18 ± 2) hours.

Transfer this culture in MBPRMB (A.1) in a ratio of respectively 1,5:10 (vol:vol), into a screw-capped tube. Incubate at (36 ± 2) °C to reach approximately 10^9 cfp/ml. (**Practical approach**: *Incubate to reach the OD corresponding to approximately 2.10^8 cfp/ml according to your calibration curve (see calibration of turbidity measurements). Incubate for five hours more. At this time OD should begin to stabilize and viable counts should be approximately10^9 cfp/ml).*
Mix working culture and cryoprotector (A.6) in a ratio of 1:1 (vol:vol) avoiding bubble formation. Distribute into plastic (glass) vials in aliquotes of *ca* 1,5 ml and store at (-70 ± 10) °C for a maximum of 5 months[3].

---

[2] Depending on the growth of the host strain on MBPRMA, inoculate 1/8 or more of the slant grown on the surface of MBPRMA, using a sterile cotton swab (e.g. in case of dense growth use 1/8 to inoculate 10 ml MBPRMB, in case of poor growth use 1/2 or the full slant).

[3] This is the maximum time elapsed since the initiation of this procedure.

1.3 Calibration of turbidity measurements (for counts of micro-organisms)

Take one vial of working culture (1.2) from the freezer and thaw at room temperature. Add MBPRMB (A.1) to a tube for anaerobic cultures (e.g. Hungate tubes with butyl rubber stopper and screw cap) and warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Before inoculation, adjust the spectrophotometer to 0 (*for this purpose, take a tube as the one used for the calibration, fill it with the cryoprotector (A.6) used for freezing the working culture and MBPRMB (A.1) in a ratio of respectively 0,5:10 (vol:vol) and adjust the spectrophotometer reading to 0 on the tube).* Transfer the working culture into MBPRMB (A.1) in a ratio of respectively 1:10 (vol:vol). Tubes for anaerobic cultures may be inoculated/sampled by puncture. Incubate at (36 ± 2) °C. Every 30 minutes measure turbidity and withdraw by puncture a 0,2 ml sample for viable cell counts. Ensure that the tube is taken from the incubator for as short a time as possible.

Melt bottles of 50 ml ssMBPRMA (A.3) (basal agar) in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add hemin, Na_2CO_3 and antibiotics and adjust pH (see Table 1). Distribute 2,5 ml aliquotes into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.

Dilute samples to 10^{-8}, and add 1 ml volumes of the 10^{-6}, 10^{-7} and 10^{-8} dilutions to each tube of 2,5 ml of melted ssMBPRMA in duplicate. Pour on a layer of MBPRMA in a 90 mm Petri dish (A.2). Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside down in an anaerobic jar at (36 ± 2) °C for (36 ± 2) hours. Ensure that the process is performed in a period of time as short as possible and that the diluents have been autoclaved just before use (to have them free of oxigen). Count the total number of colonies in each plate yielding between 30 and 300 colonies and calculate the number of cfp/ml (consult ISO 8199 if necessary).

NOTE

This procedure should be carried out several times (approx. 2-3 times) to establish the relationship between absorbance measurements and colony counts. If sufficient data have been obtained, further work can then be based only on absorbance measurements.

1.4 Preparation of inoculum cultures

Take one vial of working culture (1.2) from the freezer and thaw at room temperature. Add MBPRMB (A.1) to a tube for anaerobic cultures (e.g. Hungate tubes with butyl rubber stopper and screw cap) and warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Before inoculation, adjust the spectrophotometer to 0 (*for this purpose, take a tube as the one used for the calibration, fill it with the cryoprotector (A.6) used for freezing the working culture and MBPRMB (A.1) in a ratio of respectively 0,5:10 (vol:vol) and adjust the spectrophotometer reading to 0 on the tube).* Transfer the working culture into MBPRMB (A.1) in a ratio of respectively 1:10 (vol:vol). Incubate at (36 ± 2) °C. After 2 hours measure turbidity every 30 minutes. At an absorbance corresponding to a cell density of approx 10^8 cfp/ml (based on data obtained on calibration of turbidity measurements, see 1.3), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use within 6 hours.

2 Standard procedure

Prepare an inoculum culture as described in 1.4.
Prewarm the sample to room temperature.
Melt bottles of 50 ml ssMBPRMA (A.3) (basal agar) in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add hemin, Na_2CO_3 and antibiotics and adjust pH (see Table 1). Distribute 2,5 ml aliquotes into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.

To each tube add 1 ml of sample, or dilution or concentrate. Examine each volume or dilution step at least in duplicate.
Add 1 ml of inoculum culture, mix carefully avoiding the formation of air bubbles and pour the contents on a layer of MBPRMA in a 90 mm Petri dish (A.2). Distribute evently, allow to solidify on a horizontal, cool surface and incubate the plates upside down in an anaerobic jar at (36 ± 1) °C for (18 ± 2) hours. After incubation, count the number of plaques on each plate. If it is not possible to count the plates after finishing incubation, keep the plates at 4 °C until reading.

NOTES
For samples containing high background flora it is recommended to decontaminate by filtration through low protein binding membranes, as for example those of polyvinylidene difluoride 0,22 µm pore size, or to add to the ssMBPRMA 300 µg/ml of kanamycin sulfate instead of 100 µg/ml.

Bacteroides fragilis is an anaerobic bacteria. Therefore, distribute inoculated tubes as fast as possible and introduce the plates into the anaerobic jars as soon as possible, once dried.

ANNEX A

CULTURE MEDIA AND DILUENTS

A.1 Modified Bacteroides Phage Repair Medium broth (MBPRMB)

Basal broth

Ingredient		Amount
Meat peptone		10 g
Casein peptone		10 g
Yeast extract		2 g
NaCl		5 g
Monohydrated L-cystein		0,5 g
Glucose		1,8 g
$MgSO_4.7H_2O$		0,12 g
(Prepared by ADSA-micro)		
Bile		2,5 g
$CaCl_2$ solution (0,05 g/ml, see below)		1 ml
Distilled water	(up to)	1000 ml

Dissolve the ingredients in the water. Add the $CaCl_2$ solution, mix well and distribute the medium in bottles in volumes of e.g. 200 ml. Sterilize in the autoclave at (121 ± 1)°C for 15 minutes. Store in the dark at (5 ± 3) °C for not longer than 1 week.

Calcium chloride solution (0,05 g/ml)

Ingredient	Amount
$CaCl_2.2H_2O$	5 g
Distilled water	100 ml

Dissolve the calcium chloride in the water while heating gently. Cool to room temperature and filter sterilize through an 0,22 µm pore size membrane filter. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Hemin solution

Ingredient	Amount
Hemin	0,1 g
NaOH	0,02 g
Distilled water	100 ml

Dissolve the ingredients in the water by magnetic stirring (may last 30 - 60 min). 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 6 months.

Disodium carbonate solution (1 mol/l)

Na_2CO_3		10,6 g
Distilled water	up to	100 ml

Dissolve disodium carbonate in the water. Filter-sterilize through an 0,22 µm pore size membrane filter. Store at room temperature for not longer than 6 months.

Complete broth

Basal broth	200 ml
Hemin solution	2 ml
Disodium carbonate solution	5 ml

Aseptically add the additives (directly before use) to the basal broth and mix well. Adjust to pH 6,8 ± 0,5 by aseptically adding HCl (e.g. 0,5 ml HCL 35%). Use immediate.

Note:
To prevent contamination it is recommended to add always Kanamycin monosulfate (final concentration of 100 µg/ml) and Nalidixic acid (final concentration of 100 µg/ml) to the medium. For preparation of the antibiotic solutions see A.4 and A.5.
Add 0,2 ml Kanamycin monosulfate (A.4) and 0,8 ml Nalidixic acid (A.5) to 200 ml complete medium.

A.2 Modified Bacteroides Phage Repair Medium agar (MBPRMA)

Basal agar

Basal broth (A.1; not-sterilized)	1000 ml
Agar	12 - 20 g*

*: Depending on the gel strength of the agar

Mix the basal broth and the agar while heating. Distribute the medium in bottles in volumes of e.g. 200 ml and sterilize in the autoclave at (121 ± 1)°C for 15 minutes. Cool to between 45 and 50 °C and add the additives (see below).

Complete agar

Basal agar (molten at 45 - 50 °C)	200 ml
Hemin solution (A.1)	2 ml
Disodium carbonate solution (A.1)	5 ml

Aseptically add the additives, mix well. Adjust to pH 6,8 ± 0,5 by aseptically adding HCl (e.g. 0,5 ml HCL 35%). Pour into Petri dishes (20 ml in dishes of 9 cm diameter). Allow to solidify and store in the dark at (5 ± 3) °C for not longer than two months. Place the plates at room temperature 1 - 2 hours before use.

Note:
To prevent conatmination it is recommended to add always Kanamycin monosulfate (final concentration of 100 µg/ml) and Nalidixic acid (final concentration of 100 µg/ml) to the medium. For preparation see A.4 and A.5.
Add 0,2 ml Kanamycin monosulfate (A.4) and 0,8 ml Nalidixic acid (A.5) to 200 ml complete medium.

A.3 Semi-solid Modified Bacteroides Phage Repair Medium agar (ssMBPRMA)

Prepare basal agar according to A.2 but use half of the mass of the agar (6 g - 10 g), depending on gel strength. The gel strength of ssMBPRMA is critical to obtain good results and if possible different concentrations should be tested. Choose the agar concentration that produces highest plaque counts but also controls plaque-size to reduce confluence. Distribute into bottles in volumes of 50 ml. Allow to solidify and store at (5 ± 3) °C during no longer than two months.
Before use, melt bottles of ssMBPRMA in a boiling waterbath, cool to between 45 and 50 °C. Aseptically add hemin, Na_2CO_3, antibiotics and adjust pH to 6,8 ± 0,5 (see A.2).

A.4 Kanamycin monosulfate

Note: Some containers will contain less than 100% active Kanamycin base. In case of 0,8 µg Kanamycin base per mg:

Kanamycin monosulfate	1,25 g
Distilled water	10 ml

Dissolve the ingredient in the water and mix well. Filter sterilize through an 0,22 µm pore size filter. Store at (5 ± 3) °C for not longer than 8 hours or at (-20 ± 5) °C for not longer than six months.

A.5 Nalidixic solution

Nalidixic acid	250 mg
NaOH-solution (1 mol/l)	2 ml
Distilled water	8 ml

Dissolve the Nalidixic acid in the NaOH solution, add distilled water and mix well. Filter sterilize through an 0,22 µm pore size filter. Store at (5 ± 3) °C for not longer than 8 hours or at (-20 ± 5) °C for not longer than six months.

A.6 Cryoprotector (Carrier)- BSA+Sucrose

Bovine serum albumine fraction V (BSA)		10 g
Sucrose		20 g
Distilled water	up to	100 ml

Dissolve the ingredients in the water by magnetic stirring during ca 1 hour. Filter sterilize through an 0,22 µm pore size filter (cellulose ester). Filtration could be difficult. Use immediate.

Note:
Prepare a sufficient amount of cryoprotector (fresh) for freezing the working cultures of *Bacteroides fragilis*. Store extra cryoprotector at (5 ± 3) °C. This latter can (only) be used as blank control for measurements in the spectrophotometer (to adjust to zero).

Table 1

MBPRM (Modified Bacteroides Phage Repair Medium) Preparation BFRPH Method

		Broth			ssAgar			Agar		
Volume	Unit	50 ml	200 ml	1 L	50 ml	200 ml	1 L	50 ml	200 ml	1 L
BPRM Medium	g	1.47	5.88	29.42	1.47	5.88	29.42	1.47	5.88	29.42
Bile bovine	g	0.125	0.50	2.50	0.125	0.50	2.50	0.125	0.50	2.50
$CaCl_2$ 0,05 g/ml	ml	0.05	0.2	1	0.05	0.2	1	0.05	0.2	1
Agar	g	-	-	-	0.3-0.5	1.2-2	6-10	0.6-1	2.4-4	12-20
Autoclave 15 minutes at 121°C										
Hemin 0,1 % in NaOH 0,02 %	ml	0.5	2	10	0.5	2	10	0.5	2	10
Na_2CO_3 1 M	ml	1.25	5	25	1.25	5	25	1.25	5	25
HCl 35 %	ml	0.125	0.5	2.5	0.125	0.5	2.5	0.125	0.5	2.5
Nalidixic Acid (25 mg/ml)	ml	0.2	0.8	4	0.2	0.8	4	0.2	0.8	4
Kanamycin (100 mg/ml)	ml	0.05	0.2	1	0.05	0.2	1	0.05	0.2	1
pH checking : pH = 6,3-7,3										

Annex 3

Concentration of Bacteriophages from water: $Mg(OH)_2$ flocculation

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Department of Microbiology
University of Barcelona
16/02/98

Concentration of Bacteriophages from water:
$Mg(OH)_2$ Flocculation

Procedure

Natural samples analysis.

1.-After homogenizing the sample by mixing, measure a 1000 ml volume and place it in a sterile bottle with a stir bar. Take another aliquote (45 ml) to count phages in the initial sample. (NOTE: Sample sould be prewarmed to room temperature).

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. Turbidity occurs.

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 fluffy sediment (approximately 250 ml of volume) by centrifugation (3000 rpm, 15 minutes, 5°C ± 3 °C).

9.-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 since no flocculs were observed.

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

ANNEX A

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.

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16/02/98

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 4

Protocol for the second collaborative study

pages 65 - 88

NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT
(RIVM)

MICROBIOLOGICAL LABORATORY OF HEALTH PROTECTION (MGB)

3 March 1998

Bacteriophages in Bathing waters

European Community contract no. SMT4-CT95-1603 (DG12-RSMT)

Protocol for the second collaborative study on bacteriophages in water March 1998

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BACTERIOPHAGES IN BATHING WATERS
PROTOCOL TRIAL II, MARCH 1998

Please read all instructions and the reporting form before starting the trial. Fill in the reporting form during the work as much as possible.

1. INTRODUCTION

In spring 1997 a first training session and a first collaborative study have been organised on bacteriophages in bathing waters. In both sessions the phage methods for three groups of bacteriophages were introduced with mainly pure phage cultures. The three groups of bacteriophages were:
- Somatic coliphages (SOMCPH)
- F-specific RNA phages (FRNAPH)
- Phages of *Bacteroides fragilis* (BFRPH)

In the first training session the participants had a first acquaintance with the methods in a central laboratory at the Institute Pasteur in Lille. In the first collaborative study they applied these methods in their own laboratories. The results of the first training session and of the first collaborative study were very satisfactory. In the first collaborative study the largest variation in results was found with the BFRPH method. However, still a very acceptable Reproducibility value of (R=) 2.04 (back transformed from the log scale) was found. For FRNAPH, R was 1.73 and for SOMCPH, R was 1.52.

The next step in the learning process is applying the methods to natural polluted samples. A second training session on this aspect has been organised in December 1997. The second collaborative study with natural polluted samples will be organised in March 1998. Beside the analyses of natural polluted samples, also a concentration technique will be applied (introduced during the second training session in December 1997).

In case of natural polluted samples, a total number of F-specific phages (FTOTPH) will be detected with the method applied. The number of FTOTPH will include mainly F-specific RNA phages (FRNAPH), but also (a small part) of F-specific DNA phages (FDNAPH). By applying the method in the presence of RNase, the F-specific DNA phages are detected. The difference between FTOTPH and FDNAPH gives the number of FRNAPH.

2. OBJECTIVES

The main objectives of of the second collaborative study are:

- Introduction of the enumeration of the three groups of bacteriophages (somatic coliphages (SOMCPH), F-specific RNA-phages (FRNAPH), and phages of *Bacteroides fragilis* (BFRPH) in natural polluted samples in participating laboratories.
- Introduction of a concentration method for enumeration of the three groups of bacteriophages in participating laboratories.

3. PRIOR WORK FOR EACH PARTICIPANT

Half February each participant received some information on the necessary work to be carried out prior to the study. Summarizing, this consists of:

- Checking the presence of the following documents:
  * Amended ISO 10705-1, February 1997 (method for F-specific phages, same as used in trial 1);
  * Amended ISO/CD10705-2, February 1997 (SOMCPH-mehod, same as used in trial 1);
  * SOP BCR-water/003 (930514): Temperature control of incubators for water microbiology (same as used in trial 1);
  * SOP BCR-water/004 (930514): pH measurement of bacteriological culture media (same as used in trial 1);
  * Protocol for determination of *Bacteroides fragilis* phages of UB 160298 (sent to the participants on 170298).
  * Concentration of bacteriophages from water: $Mg(OH)_2$ flocculation of UB 160298 (sent to the participants on 170298).
- If necessary, preparing (frozen) working cultures of:
  * WG5 *Escherichia coli* (host SOMCPH);
  * WG49 *Salmonella typhimurium* (host for F-specific phages), including QC check according to ISO 10705-1, 10.3;
  * HSP40 *Bacteroides fragilis* (host BFRPH), according to the last protocol of UB 160298.
- If necessary performing calibration curves of inoculum cultures for each method, to determine the relation between optical density and viable count for each batch of working culture that have been prepared (WG5 *Escherichia coli* for SOMCPH, WG49 *Salmonella typhimurium* for F-specific phages and HSP40 *Bacteroides fragilis* for BFRPH). When reproducible results are obtained, the relation between optical density and viable count (10^8 cfp/ml) can be used to prepare the inoculum cultures of each strain.
- If necessary ordering media, reagents and chemicals.
- Preparing media and solutions.

In case of any problems with host strains etc., please contact Kirsten Mooijman.

4. OUTLINE OF THE STUDY

Each participating laboratory will receive a few weeks before the date of the trial a (big) parcel (by courier service DHL) containing:

- Dry ice;
- 5 Vials containing natural polluted samples "De Bilt 281097", for analyses on SOMCPH (coded with red caps);
- 5 Vials containing natural polluted samples "De Bilt 071197", for analyses on F-specific phages (coded with yellow caps);
- 5 Vials containing natural polluted samples "UB 311097", for analyses on BFRPH (coded with green caps);
- 8 Vials of reference materials containing phage ΦX174, batch 040696 (coded with brown caps);
- 6 Vials of reference materials containing phage MS2, batch 220597 (coded with white caps);
- 4 Vials of reference materials containing phage B40-8, batch 260397 (coded with blue caps).

N.b.: The number of vials of the phage RM's contain (each) one extra vial to be used e.g. in case of loss or for extra QC checks.

In a fixed period, the vials are thawn at room temperature and analysed in the following way:

Day 1 Enumeration of SOMCPH, FTOTPH, FDNAPH (in the presence of RNase) and BFRPH in natural polluted "standard samples".
Day 2 Reading of the plates of day 1
Day 3 Concentration technique with phage RM's and enumeration of SOMCPH, FTOTPH, FDNAPH (in the presence of RNase) and BFRPH.
Day 4 Reading of the plates of day 3.

The methods to be used for this study are:

SOMCPH: DAL procedure (9 cm plates) according to AMENDED ISO/CD 10705-2, February 1997.
F-specific phages (FTOTPH & FDNAPH): DAL procedure (9 cm plates) according to AMENDED ISO 10705-1, February 1997.
BFRPH: DAL procedure (9 cm plates) according to "Protocol for determination of *Bacteroides fragilis* phages", UB 160298.
Concentration technique: "Concentration of bacteriophages from water: $Mg(OH)_2$ flocculation", UB 160298.

5. CHRONOLOGICAL DESCRIPTION OF THE TRIAL

Date (1998)

Feb. - March	Prepare working cultures of host strains, specially for HSP40 *Bacteroides fragilis*
2 - 9 March	Mailing, by MGB/RIVM, of the final protocols, reporting form, SOP's, two sheets of random labels and a label with the address of the RIVM to the participating laboratories. Furthermore, a box containing 6 sterile syringes with a nominal volume of 5 ml, 18 Millipore Millex GV membrane filters, with 0.22 µm pore size (lot no.: R7PM43482) and *ca* 35 g of synthetic sea salt will also be sent to the participants.
9 March	Mailing, by MGB/RIVM, of the reference materials and "standard natural polluted samples" in dry ice by courier service. When this parcel arrives at the participating laboratory, *record the date of arrival on the reporting form*. Inspect the contents of the parcel for completeness. In case the parcel is damaged, please contact Kirsten Mooijman. **Store the materials immediately at (-70 ± 10) °C.** Acknowledge the receipt of the parcel by sending a fax or an e-mail to Kirsten Mooijman.
10 - 23 March	Control every (working) day the temperature of the freezer and *record on the reporting form* (at minimum twice a day; morning and evening). Adjust the temperature setting of the 37 °C incubator when necessary, using a calibrated thermometer immersed in glycerol in a closed bottle (SOP BCR-water/003 of 930514). If necessary adjust a waterbath to (45 ± 1) °C.
16 - 23 March	Prepare glassware and media. Label plates, tubes, etc.
23 - 27 March	Week of second collaborative study
23 March	Day 1, Natural polluted samples: - Prepararation of inoculum cultures (WG5, WG49 and HSP40); - Checking viable counts of the inoculum cultures; - Filtering natural polluted samples through Millipore filters and collecting of filtrate, per phage type, in one bottle/tube; - Enumeration of phages in filtrates: 10 x 1 ml for all phages (SOMCPH, FTOTPH, FDNAPH and BFRPH); - Enumeration of phages in 1 RM per phage type in duplicate (SOMCPH, FTOTPH, FDNAPH and BFRPH).

24 March	Day 2, Natural polluted samples: - Reading of the plates of the viable counts (WG5 and WG49); - Random labeling and reading of all plates of the phage enumerations.
25 March	- Reading of the plates of the viable counts (HSP40) Day3, Concentration technique: - Prepararation of inoculum cultures (WG5, WG49 and HSP40); - Checking viable counts of the inoculum cultures; - Mixing of RM's (12 ml ΦX174, 6 ml MS2 and 4 ml B40-8) to suspension A; - Adding 14 ml of suspension A in 1 litre synthetic sea salt solution. Performing concentration technique resulting in ca 40 ml concentrate (suspension B); - Enumeration of phages in suspension A: 2 x 1 ml for SOMCPH, FTOTPH and BFRPH and 1 ml for FDNAPH; - Enumeration of phages in suspension B: 10 x 1 ml for SOMCPH, FTOTPH and BFRPH and 5 x 1 ml for FDNAPH.
26 March	Day 4, Concentration technique: - Reading of the plates of the viable counts (WG5 and WG49); - Random labeling of the plates of SOMCPH, FTOTPH and BFRPH, and reading of all plates of the phage enumerations.
27 March	- Reading of the plates of the viable counts (HSP40)
27 March - 3 April	Participants mail results (only the data, pages 19 and 30) to MGB/RIVM by telefax (+31 30 274 4434) and original reporting form by mail
April/May	Statistical analyses of the results at the RIVM
End April	First report will be sent to the participants, to check for completeness and correctness.
May	Second (draft) report send to the participants prior to the meeting or distributed during the meeting.
14, 15 May	Discussion of the results with the participants
June-August	Preparation of the report at the RIVM

6. DETAILED DESCRIPTION OF THE TRIAL

General: Unless otherwise stated, the tolerance of any measured value in this protocol is: stated value ± 5%.

6.1 Preparatory work

10 - 23 March 1998

- Adjust an incubator to (37 ± 1) °C (according to SOP BCR-water/003 of 930514).
- Adjust a waterbatch to (45 ± 1) °C
- Prepare glassware media and reagents described in the tables below. The mentioned figures are sufficient for performing the collaborative study on 23 - 27 March. The figures do not include the necessary media for the prior work (see 3).

For SOMCPH (amended ISO/CD 10705-2 of February 1997):

Media:
- 2 x 50 ml MSB (A.1) (4 x 50 ml in case separate blanks are needed for the spectrophotometer)
- 30 Petri dishes (of 9 cm), containing MSA (A.2)
- 2 bottles containing each 50 ml of ssMSA (A.3)
- 10 ml calcium chloride solution
- 100 ml peptone saline solution (ps)
- *ca* 500 ml (in a bottle) of MSA (A.2, complete medium)

Glassware and disposables:
- 2 sterile conical flask of 250-300 ml capacity with side-arm, or a plain sterile conical flask and cuvettes (4 flasks in case separate blanks are needed for the spectrophotometer)
- Sterile pipettes of 1 ml nominal capacity
- 18 sterile (empty) Petri dishes (of 9 cm)
- Sterile glass tubes, with caps, of nominal capacity of *ca* 10 ml
- Sterile bottle or tube with a nominal capacity of *ca* 25 ml

Apparatus:
- Spectrophotometer (for measuring absorbance)
- Incubator or waterbath thermostatically controlled at (37 ± 1) °C, and supplied with a rotating platform at (100 ± 10) min^{-1}
- Incubator or waterbath, thermostatically controlled at (37 ± 1) °C
- Waterbath, thermostatically controlled at (45 ± 1) °C
- Waterbath or equivalent device for melting of agar media
- Counting apparatus with indirect, oblique light.
- Whirlmixer

For FTOTPH and FDNAPH (amended ISO 10705-1 of February 1997):

Media and reagents:
- 2 x 50 ml TYGB (A.1) (4 x 50 ml in case separate blanks are needed for the spectrophotometer)
- 50 Petri dishes (of 9 cm) containing TYGA (A.2)
- 4 Bottles containing each 50 ml of ssTYGA (A.3)
- 20 ml calcium-glucose solution
- 100 ml peptone saline solution (ps; A.8)
- *ca* 500 ml (in a bottle) of TYGA (A.2, complete medium)
- 10 ml RNase solution (A.5)

Glassware and disposables:
- 2 sterile conical flask of 250-300 ml capacity with side-arm, or a plain sterile conical flask and cuvettes (4 flasks in case separate blanks are needed for the spectrophotometer)
- Sterile pipettes of 1 ml nominal capacity
- Sterile pipettes of 0.1 ml nominal capacity
- 18 sterile (empty) Petri dishes (of 9 cm)
- Sterile glass tubes, with caps, of nominal capacity of *ca* 10 ml
- Sterile bottle or tube with a nominal capacity of *ca* 25 ml

Apparatus:
- Spectrophotometer (for measuring absorbance)
- Incubator or waterbath thermostatically controlled at (37 ± 1) °C, and supplied with a rotating platform at (100 ± 10) min^{-1}
- Incubator or waterbath, thermostatically controlled at (37 ± 1) °C
- Waterbath, thermostatically controlled at (45 ± 1) °C
- Waterbath or equivalent device for melting of agar media
- Counting apparatus with indirect, oblique light.
- Whirlmixer

For BFRPH (Protocol for determination of *Bacteroides fragilis* phages, UB 160298):

Media and reagents:
- 100 ml of MBPRMB (A.1) with Nal and Km
- 50 Petri dishes (of 9 cm) containing MBPRMA (A.2; with Nal and Km)
- 4 Bottles containing each 50 ml of ssMBPRMA (A.3; with Nal and Km)
- 100 ml peptone saline solution (ps)
- *ca* 20 ml Hemin solution (A.1)
- *ca* 35 ml disodium carbonate solution (A.1)

Glassware and disposables:
- 2 sterile screw-caped glass tubes with nominal capacity of 30 ml (or more tubes in case of a smaller size)
- sterile pipettes of 1 ml nominal capacity

- 2 sterile (empty) Petri dishes (of 9 cm)
- sterile glass tubes with caps of nominal capacity of *ca* 10 ml.
- Sterile bottle or tube with a nominal capacity of *ca* 25 ml
- Sterile swabs

Apparatus:
- Spectrophotometer (for measuring absorbance)
- Incubator or waterbath thermostatically controlled at (37 ± 1) °C
- Waterbath, thermostatically controlled at (45 ± 1) °C
- Waterbath or equivalent device for melting of agar media
- Anaerobic jars and anaerogen bags + indicator (or equivalent for creating anaerobiosis)
- Counting apparatus with indirect, oblique light
- Whirlmixer
- pH meter (or paper)

For concentration technique (Concentration of bacteriophages from water: $Mg(OH)_2$ flocculation, 160298):

Reagents and diluents:
- 15 ml $MgCl_2$ - solution (1 mol/l; A.1)
- 10 ml K_2HPO_4 - solution (1 mol/l; A.2)
- 10 ml NaOH - solution (2 mol/l; A.3)
- 50 ml Buffer for phages (A.4)
- 1 litre of synthetic sea salt solution (see below)

Glassware and disposables:
- 1 sterile magnetic stirring bar
- Sterile pipettes of 10 ml nominal capacity
- Sterile centrifuge tubes with nominal volume of (preferably) 250 ml
- Sterile bottle or tube with a nominal capacity of *ca* 30 ml

Apparatus:
- Magnetic stirring apparatus
- Vacuum installation
- Centrifuge (for 3000 rpm, at 5 °C ± 3 °C)

Synthetic sea salt solution

Synthetic sea salt (batch 7J043Z, sent by MGB/RIVM)	22.5 g
Distilled water	1000 ml

Dissolve the synthetic sea salt in the water and sterilize in the autoclave at (121 ± 1) °C for 15 - 20 min. If not for immediate use, store at (5 ± 3) °C for not longer than 1 month.

Make sure that you have available:

- Working culture of host strain WG5 *Escherichia coli* (frozen vials at (-70 ± 10) °C; see amended ISO/CD 10705-2; 10.1.2);
- Working culture of host strain WG49 *Salmonella typhimiurium* (frozen vials at (-70 ± 10) °C; see amended ISO 10705-1; 10.1.2);
- Working culture of host strain HSP40 *Bacteroides fragilis* (frozen vials at (-70 ± 10) °C; see "Protocol for determination of *Bacteroides fragilis* phages", UB 160298; 1.2);
- Information per host strain about which absorbance of the inoculum culture corresponds to ca. 10^8 cfp/ml of the host strain.

20 March 1998

Note: Labeling of plates etc. can as well be done on the day of enumeration of the phages (e.g. during culturing of the inoculum cultures). Each participant should decide what is most convienent.

For natural polluted samples

- Label 2 Petri dishes with MSA as follows: ΦX174-1; ΦX174-2;
- Label 10 Petri dishes with MSA as follows: MSA 1; MSA 2 etc., up to and including MSA 10;
- Label one dish with MSA; blank ssMSA;
- Label one dish with MSA; blank WG5;
- Label 8 sterile empty Petri dishes as follows: WG5 10^{-5}-1; WG5 10^{-5}-2; WG5 10^{-6}-1; WG5 10^{-6}-2; WG5 10^{-7}-1; WG5 10^{-7}-2; MSA blank and MSA-ps blank;
- Label a sterile empty bottle or tube with a nominal capacity of *ca* 25 ml: SOMCPH.

- Label 4 Petri dishes with TYGA as follows: MS2 1.1; MS2 1.2; MS2 2.1+ (RNase); MS2 2.2+ (RNase);
- Label 10 Petri dishes with TYGA as follows: TYGA 1; TYGA 2 etc., up to and including TYGA 10;
- Label 10 Petri dishes with TYGA as follows: TYGA 1+ (RNase); TYGA 2+ (RNase) etc., up to and including TYGA 10+ (RNase);
- Label one dish with TYGA: blank ssTYGA;
- Label one dish with TYGA: blank WG49;
- Label 8 sterile empty Petri dishes as follows: WG49 10^{-5}-1; WG49 10^{-5}-2; WG49 10^{-6}-1; WG49 10^{-6}-2; WG49 10^{-7}-1; WG49 10^{-7}-2; TYGA blank and TYGA-ps blank
- Label a sterile empty bottle or tube with a nominal capacity of *ca* 25 ml: FTOTPH.

- Label 2 Petri dishes with MBPRMA as follows: B40-8 1; B40-8 2;
- Label 10 Petri dishes with MBPRMA as follows: MBPRMA 1; MBPRMA 2 etc., up to and including MBPRMA 10;
- Label one dish with MBPRMA: blank ssMBPRMA;
- Label one dish with MBPRMA: blank HSP40 ;
- Label 8 Petri dishes with MBPRMA as follows: HSP40 10^{-5}-1; HSP40 10^{-5}-2; HSP40 10^{-6}-1; HSP40 10^{-6}-2; HSP40 10^{-7}-1; HSP40 10^{-7}-2; ssMBPRMA blank and ssMBPRMA-ps blank
- Label a sterile empty bottle or tube with a nominal capacity of *ca* 25 ml: BFRPH.

For the concentration technique

- Label 2 Petri dishes with MSA as follows: MSA A1; MSA A2;
- Label 10 Petri dishes with MSA as follows: MSA B1; MSA B2 etc., up to and including MSA B10;
- Label one dish with MSA; blank ssMSA;
- Label one dish with MSA; blank WG5;
- Label 8 sterile empty Petri dishes as follows: WG5 10^{-5}-1; WG5 10^{-5}-2; WG5 10^{-6}-1; WG5 10^{-6}-2; WG5 10^{-7}-1; WG5 10^{-7}-2; MSA blank and MSA-ps blank;

- Label 3 Petri dishes with TYGA as follows: TYGA A1; TYGA A2; TYGA A3+ (RNase);
- Label 10 Petri dishes with TYGA as follows: TYGA B1; TYGA B2 etc., up to and TYGA B10;
- Label 5 Petri dishes with TYGA as follows: TYGA B1+ (RNase); TYGA B2+ (RNase) etc., up to and including TYGA B5+ (RNase);
- Label one dish with TYGA: blank ssTYGA;
- Label one dish with TYGA: blank WG49;
- Label 8 sterile empty Petri dishes as follows: WG49 10^{-5}-1; WG49 10^{-5}-2; WG49 10^{-6}-1; WG49 10^{-6}-2; WG49 10^{-7}-1; WG49 10^{-7}-2; TYGA blank and TYGA-ps blank

- Label 2 Petri dishes with MBPRMA as follows: MBPRMA A1; MBPRMA A2;
- Label 10 Petri dishes with MBPRMA as follows: MBPRMA B1; MBPRMA B2 etc., up to and including MBPRMA B10;
- Label one dish with MBPRMA: blank ssMBPRMA;
- Label one dish with MBPRMA: blank HSP40 ;
- Label 8 Petri dishes with MBPRMA as follows: HSP40 10^{-5}-1; HSP40 10^{-5}-2; HSP40 10^{-6}-1; HSP40 10^{-6}-2; HSP40 10^{-7}-1; HSP40 10^{-7}-2; ssMBPRMA blank and ssMBPRMA-ps blank

Or use any other labelling which can make distinction between the different plates.

6.2 Analytical work of the second collaborative study

References to the methods are:
For SOMCPH: amended ISO/CD 10705-2, Feburary 1997
For F-specific phages: amended ISO 10705-1, February 1997
For BFRPH: "Protocol for the determination of *Bacteroides fragilis* phages", UB 160298
For concentration technique: "Concentration of bacteriophages from water: $Mg(OH)_2$ flocculation", UB 160298.

6.2.1 Natural polluted samples

23 March 1998 (Day 1)

Prewarm all plates to room temperature. If necessary, dry the plates before use.

Inoculum cultures

Note: If the preparation of a frozen working culture of HSP40 *Bacteroides fragilis* was not succesful, it is also possible to use an "overnight" working culture of HSP40. Consult for this purpose the protocol for detection of bacteriophages of *Bacteroides fragilis* of February 1997 (first collaborative study), but use the modified medium. When it is necessary to prepare an overnight culture, all other work will be postponed with one day.

Prepare inoculum cultures for the following host strains:
- WG5 *Escherichia coli* (SOMCPH; 11.1, *ca* 20 ml);
- WG49 *Salmonella typhimurium* (F-specific phages; 11.1, *ca* 40 ml)
- HSP40 *Bacteroides fragilis* (BFRPH; 1.4, *ca* 20 ml)

Prewarm the broths (at ca. 37°C) before bringing the working cultures into the broths.
When an inoculum culture reaches a cell density of approximately 10^8 cfp/ml (based on earlier data), take this culture from the incubator. Quickly cool the inoculum cultures by placing them in melting ice. Use them within *ca* 4 hours. Enumerate of each inoculum culture the number of cfp/ml directly after placing the culture in melting ice, as follows:
Withdraw a 1 ml sample of each inoculum culture and prepare 10 fold dilutions in peptone saline solution (of 2 °C - 8 °C) until 10^{-7} dilution. Melt a sufficient amount of agar for each host strain (MSA for WG5; TYGA for WG49 and ssMBPRMA for HSP40). Cool the bottles with molten agar to (45 ± 1)°C. Prepare for each host strain pour plates of 1 ml volumes of 10^{-5}, 10^{-6} and 10^{-7} dilutions with the appropriate molten agar. For HSP40, follow the protocol of BFRPH described in 1.3 ("DAL" procedure). Analyse each dilution in duplicate. Use the labeled Petri dishes.
Prepare blanks in the following way: prepare a pour plate with 1 ml of sterile ps (in the same way as done for the viable counts). Use the Petri dish labeled (medium name)-ps blank. Pour (a similar amount of medium as for the viable counts) molten agar into the sterile empty Petri dish labeled (medium name)-

blank. Leave the plates to solidify. Incubate the plates of WG5 and WG49 aerobic at (36 $\pm$ 2) °C for (20 $\pm$ 4) hours. Incubate the plates of HSP40 anaerobic at (36 $\pm$ 2) °C for (36 $\pm$ 2) hours.

Enumeration of phages

The order in which the phage enumerations for the different phage types are performed is not prescribed.

SOMCPH amended ISO/CD 10705-2 11.2.2 DAL-Procedure

- Take inoculum culture WG5 *Escherichia coli* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Take the 5 vials of "De Bilt 281097" (red caps) from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Bring the contents of the 5 vials together in an empty sterile Petri dish. Mix carefully.
- "Decontaminate" the sample by filtration through the low protein binding membrane filters with 0.22 µm pore size. Use for this purpose one or two of the syringes sent to you and 1-5 of the (yellow) Millipore Millex GV filters. As soon as a filter clogs, use a new filter. Collect the filtrate in a sterile tube or bottle labeled SOMCPH.
- Take 1 vial of reference materials containing ΦX174 from the -70 °C freezer and place it at room temperature. Thawing of the vial will take *ca* 30 minutes.
- Melt 1 bottle of 50 ml ssMSA in a boiling waterbath and place in a waterbath at (45 $\pm$ 1) °C. Aseptically add calciumchloride solution (300 µl/50 ml) and distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 $\pm$ 1) °C.
- If necessary label the tubes 1, 2, 3 etc., up to and including 10, ΦX174-1, ΦX174-2 and blank ssMSA and blank WG5.
- Mix the tube/bottle containing the filtrate (labeled SOMCPH).
- Take 1 ml from the filtrate and add to the tube with molten ssMSA labeled 1.
- Take another 1 ml from the filtrate and add this to the tube with molten ssMSA labeled 2.
- Repeat 8 times, up to and including tube 10.
- Mix the vial containing ΦX174 on a whirlmixer or by turning the closed vial 5 times.
- Take 1 ml from the vial containing ΦX174 and add this to the tube labeled ΦX174-1.
- Take another 1 ml from the vial containing ΦX174 and add this to the tube labeled ΦX174-2.
- Add to each tube, except the tube labeled blank ssMSA, 1 ml of inoculum culture of WG5. Mix carefully, avoiding the formation of air bubbles and

pour the content of each tube on a layer of MSA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) hours.

Mind:
- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.
- Do not stack more than 4 plates.

F-specific phages (FTOTPH and FDNAPH), amended ISO 10705-1 11.1 (DAL procedure)

- Take inoculum culture WG49 *Salmonella typhimurium* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Take the 5 vials of "De Bilt 071197" (yellow caps) from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Bring the contents of the 5 vials together in an empty sterile Petri dish. Mix carefully.
- "Decontaminate" the sample by filtration through the low protein binding membrane filters with 0.22 µm pore size. Use for this purpose one or two of the syringes sent to you and 5-10 of the (yellow) Millipore Millex GV filters. As soon as a filter clogs, use a new filter. Collect the filtrate in a sterile tube or bottle labeled FTOTPH.
- Take 2 vials of reference materials containing MS2 from the -70 °C freezer and place it at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Melt 2 bottles of 50 ml ssTYGA in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add calcium-glucose solution (0.5 ml/50 ml) and distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.
- If necessary label the tubes 1, 2, 3 etc., up to and including 10, 1+(RNase), 2+(RNase) etc., up to and including 10+(RNase), MS2-1, MS2-2, MS2-1+(RNase), MS2-2+(RNase) and blank ssTYGA and blank WG49.
- Add 100 µl RNase solution to the ssTYGA in the tubes labeled (number)+.
- Mix the tube/bottle containing the filtrate (labeled FTOTPH).
- Take 1 ml from the filtrate and add to the tube with molten ssTYGA labeled 1.
- Take another 1 ml from the filtrate and add this to the tube with molten ssTYGA labeled 2.
- Repeat 8 times, up to and including tube 10.
- Take 1 ml from the filtrate and add to the tube with molten ssTYGA labeled 1+.
- Take another 1 ml from the filtrate and add this to the tube with molten ssTYGA labeled 2+.

- Repeat 8 times, up to and including tube 10+.
- Mix a vial containing MS2 on a whirlmixer or by turning the closed vial 5 times.
- Take 1 ml from this vial and add to the tube labeled MS2-1.
- Take another 1 ml from this vial and add to the tube labeled MS2-2.
- Mix the other vial containing MS2 on a whirlmixer or by turning the closed vial 5 times.
- Take 1 ml from this vial and add to the tube labeled MS2-1+.
- Take another 1 ml from this vial and add to the tube labeled MS2-2+.
- Add to each tube, except the tube labeled blank ssTYGA, 1 ml of inoculum culture of WG49. Mix carefully, avoiding the formation of air bubbles and pour the content of each tube on a layer of TYGA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 $\pm$ 2) °C for (18 $\pm$ 2) hours.

Mind:
- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.
- Do not stack more than 4 plates.

BFRPH method for *Bacteroides fragilis* phages (DAL procedure)

- Take inoculum culture HSP40 *Bacteroides fragilis* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration. If more than one tube of inoculum culture with HSP40 will be used, mix them together in one tube/bottle just before use.
- Take the 5 vials of "UB311097" (green caps) from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Bring the contents of the 5 vials together in an empty sterile Petri dish. Mix carefully.
- "Decontaminate" the sample by filtration through the low protein binding membrane filters with 0.22 µm pore size. Use for this purpose one or two of the syringes sent to you and 1-5 of the (yellow) Millipore Millex GV filters. As soon as a filter clogs, use a new filter. Collect the filtrate in a sterile tube or bottle labeled BFRPH.
- Take 1 vial of reference materials containing B40-8 from the -70 °C freezer and place it at room temperature. Thawing of the vial will take *ca* 30 minutes.
- Melt 1 bottle of 50 ml ssMBPRMA in a boiling waterbath and place in a waterbath at (45 $\pm$ 1) °C. Aseptically add Hemin solution (0.5 ml/50 ml), disodium carbonate solution (1.25 ml/50 ml) and antibiotics and adjust pH to 6.3 - 7.3. Distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 $\pm$ 1) °C.
- If necessary label the tubes 1, 2, 3 etc., up to and including 10, B40-8 1, B40-8 2 and blank ssMBPRMA and blank HSP40.
- Mix the tube/bottle containing the filtrate (labeled BFRPH).

- Take 1 ml from the filtrate and add to the tube with molten ssMBPRMA labeled 1.
- Take another 1 ml from the filtrate and add this to the tube with molten ssMBPRMA labeled 2.
- Repeat 8 times, up to and including tube 10.
- Mix the vial containing B40-8 on a whirlmixer or by turning the closed vial 5 times.
- Take 1 ml from the vial containing B40-8 and add this to the tube labeled B40-8 1.
- Take another 1 ml from the vial containing B40-8 and add this to the tube labeled B40-8 2.
- Add to each tube, except the tube labeled blank ssMBPRMA, 1 ml of inoculum culture of HSP40. Mix carefully, avoiding the formation of air bubbles and pour the content of each tube on a layer of MBPRMA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down in an anaerobic jar at (36 $\pm$ 2) ^{o}C for (18 $\pm$ 2) hours.

Mind: - Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.

24 March 1998 (Day 2)

Viable count results

Read the plates of the viable counts of host strain WG5 *Escherichia coli* and WG49 *Salmonella typhimurium* and note on the reporting form.

Phage enumerations

After the total incubation time all Petri dishes are taken out of the incubator (and out of the anaerobic jars). *Record the time and the temperature of the incubator and record whether anaerobic conditions were good for the BFRPH (note the colour of the indicator).* Place the Petri dishes, except the blanks, on a laboratory bench in 12 rows of 4 dishes as described in the table below.

Note: In case of loss of one (or more) sample(s), use a dummy for this sample, so that the labelling will be carried out correctly.

Each set of instructions contains a sheet of self-adhesive labels in the same lay-out as the table. The labels have the indication: "natural" (of natural polluted samples). Take care to use the right set of labels! The blank labels are not used. Recode the Petri dishes with these labels by transferring each label to the dish corresponding to the position of the label on the provided sheet. Make sure that the original labelling of the dishes is not visible

anymore. If the labels troubles you with the reading of the plates, you can also place the labels on the caps of the Petri dishes. Take care not to mix the caps!

Labeling plates of natural polluted samples:

sample	method			
	SOMCPH	FTOTPH	FDNAPH	BFRPH
RM-1	O	O	O	O
RM-2	O	O	O	O
1	O	O	O	O
2	O	O	O	O
3	O	O	O	O
4	O	O	O	O
5	O	O	O	O
6	O	O	O	O
7	O	O	O	O
8	O	O	O	O
9	O	O	O	O
10	O	O	O	O

O = 1 Petri dish

Restack the Petri dishes in the order of the random numbers. Hand over the set of dishes and the reporting form to another laboratory worker, who should not be aware of the original incubation conditions etc. The second worker counts all plaques (visible to the bare eye) on each plate and *records the number of plaques on the reporting form for natural polluted samples behind the random number corresponding to that on the dish.* The second worker should also examine the blanks and *record the results on the reporting form. Indicate on the reporting form also data of which you are not sure because of technical problems.*

Note: If a laboratory has too few qualified laboratory workers, the random labelling can be carried out alternatively. In this latter case a second worker should recode the Petri dishes with the random labels and restack the dishes in the order of the random numbers. The first laboratory worker can then do the counting. Note on the reporting form.

25 March 1998

Read the plates of the viable counts of HSP40 *Bacteroides fragilis* and note on the reporting form.

6.2.2 Concentration technique

25 March 1998 (Day 3)

Prewarm all plates, the synthetic sea salt solution and the solutions for concentration to room temperature. If necessary, dry the plates before use.

Inoculum cultures

Prepare inoculum cultures for the following host strains:
- WG5 *Escherichia coli* (SOMCPH; 11.1, *ca* 20 ml);
- WG49 *Salmonella typhimurium* (F-specific phages; 11.1, *ca* 40 ml)
- HSP40 *Bacteroides fragilis* (BFRPH; 1.4, *ca* 20 ml)

For the preparation and control of the inoculum cultures, follow the instructions given in 6.2.1. Also perform viable counts of each inoculum culture.

Concentration technique

- Take 6 vials containing ΦX174 (brown caps), 3 vials containing MS2 (white caps) and 2 vials containing B40-8 (blue caps) from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Bring 12 ml (6 x 2 ml) of ΦX174, 6 ml (3 x 2 ml) of MS2 and 4 ml (2 x 2 ml) of B40-8 together in an empty sterile tube or bottle (=suspension A).
- Mix carefully and place in melting ice.
- Add 14 ml of suspension A to 1 litre of synthetic sea salt solution (prewarmed to room temperature).
- Add a (sterile) magnetic stirring bar to this 1 litre solution and perform the concentration method according to the protocol " Concentration of bacteriophages from water: $Mg(OH)_2$ flocculation (UB 160298)".
- Place the final (*ca* 40 ml) suspension (= suspension B) on the laboratory bench and perform enumeration of phages as soon as possible.

Enumeration of phages

The order in which the phage enumerations for the different phage types are performed is not prescribed.

SOMCPH amended ISO/CD 10705-2 11.2.2 DAL-Procedure

- Take inoculum culture WG5 *Escherichia coli* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Take phage suspension A from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.

- Melt 1 bottle of 50 ml ssMSA in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add calciumchloride solution (300 µl/50 ml) and distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.
- If necessary label the tubes A1 and A2, B1, B2, B3 etc., up to and including B10 and blank ssMSA and blank WG5.
- Mix the phage suspension A on a whirlmixer or by turning the closed tube/bottle 5 times.
- Take 1 ml of suspension A and add to the tube with molten ssMSA labeled A1.
- Take another 1 ml of suspension A and add this to the tube with molten ssMSA labeled A2.
- Take 1 ml of suspension B and add this to the tube with molten ssMSA labeled B2.
- Take another 1 ml of suspension B and add this to the tube with molten ssMSA labeled B2.
- Repeat 8 times, up to and including tube B10.
- Add to each tube, <u>except the tube labeled blank ssMSA</u>, 1 ml of inoculum culture of WG5. Mix carefully, avoiding the formation of air bubbles and pour the content of each tube on a layer of MSA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) hours.

Mind:
- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.
- Do not stack more than 4 plates.

<u>F-specific phages (FTOTPH and FDNAPH), amended ISO 10705-1 11.1 (DAL procedure)</u>

- Take inoculum culture WG49 *Salmonella typhimurium* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Take phage suspension A from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Melt 2 bottles of 50 ml ssTYGA in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add calcium-glucose solution (0.5 ml/50 ml) and distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.
- If necessary label the tubes A1, A2 and A3+(RNase), B1, B2, B3 etc., up to and including B10, B1+(RNase), B2+(RNase) etc., up to and including B5+(RNase) and blank ssTYGA and blank WG49.
- Add 100 µl RNase solution to the ssTYGA in the tubes labeled (number)+.
- Mix the phage suspension A on a whirlmixer or by turning the closed tube/bottle 5 times.

- Take 1 ml of suspension A and add to the tube with molten ssTYGA labeled A1.
- Take another 1 ml of suspension A and add this to the tube with molten ssTYGA labeled A2.
- Take another 1 ml of suspension A and add this to the tube with molten ssTYGA labeled A3+.
- Take 1 ml of suspension B and add this to the tube with molten ssTYGA labeled B2.
- Take another 1 ml of suspension B and add this to the tube with molten ssTYGA labeled B2.
- Repeat 8 times, up to and including tube B10.
- Take 1 ml of suspension B and add to the tube with molten ssTYGA labeled B1+.
- Take another 1 ml of suspnsion B and add this to the tube with molten ssTYGA labeled B2+.
- Repeat 3 times, up to and including tube B5+.
- Add to each tube, except the tube labeled blank ssTYGA, 1 ml of inoculum culture of WG49. Mix carefully, avoiding the formation of air bubbles and pour the content of each tube on a layer of TYGA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 $\pm$ 2) °C for (18 $\pm$ 2) hours.

Mind:
- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.
- Do not stack more than 4 plates.

BFRPH method for *Bacteroides fragilis* phages (DAL procedure)

- Take inoculum culture HSP40 *Bacteroides fragilis* from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Take phage suspension A from melting ice and place it at room temperature, *ca* 30 min prior to the start of the phage enumeration.
- Melt 1 bottle of 50 ml ssMBPRMA in a boiling waterbath and place in a waterbath at (45 $\pm$ 1) °C. Aseptically add Hemin solution (0.5 ml/50 ml), disodium carbonate solution (1.25 ml/50 ml) and antibiotics and adjust pH to 6.3 - 7.3. Distribute 2.5 ml into culture tubes with caps, placed in a waterbath at (45 $\pm$ 1) °C.
- If necessary label the tubes A1 and A2, B1, B2, B3 etc., up to and including B10 and blank ssMBPRMA and blank HSP40.
- Mix the phage suspension A on a whirlmixer or by turning the closed tube/bottle 5 times.
- Take 1 ml of suspension A and add to the tube with molten ssMBPRMA labeled A1.
- Take another 1 ml of suspension A and add this to the tube with molten ssMBPRMA labeled A2.

- Take 1 ml of suspension B and add this to the tube with molten ssMBPRMA labeled B2.
- Take another 1 ml of suspension B and add this to the tube with molten ssMBPRMA labeled B2.
- Repeat 8 times, up to and including tube 10.
- Add to each tube, except the tube labeled blank ssMBPRMA, 1 ml of inoculum culture of HSP40. Mix carefully, avoiding the formation of air bubbles and pour the content of each tube on a layer of MBPRMA in the corresponding labeled Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down in an anaerobic jar at (36 $\pm$ 2) °C for (18 $\pm$ 2) hours.

Mind: - Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes.

26 March 1998 (Day 4)

Viable count results

Read the plates of the viable counts of host strain WG5 *Escherichia coli* and WG49 *Salmonella typhimurium* and note on the reporting form.

Phage enumerations

After the total incubation time all Petri dishes are taken out of the incubator (and out of the anaerobic jars). *Record the time and the temperature of the incubator and record whether anaerobic conditions were good for the BFRPH (note the colour of the indicator).* Place the Petri dishes, except the blanks and the plates of the F specific phage counts in the presence of RNase (FDNAPH) on a laboratory bench in 12 rows of 3 dishes as described in the table below.

Note: In case of loss of one (or more) sample(s), use a dummy for this sample, so that the labelling will be carried out correctly.

Each set of instructions contains a sheet of self-adhesive labels in the same lay-out as the table. The labels have the indication: "concentration" (of concentration technique). Take care to use the right set of labels! The blank labels are not used. Recode the Petri dishes with these labels by transferring each label to the dish corresponding to the position of the label on the provided sheet. Make sure that the original labelling of the dishes is not visible anymore. If the labels troubles you with the reading of the plates, you can also place the labels on the caps of the Petri dishes. Take care not to mix the caps!

Labeling plates of concentration technique:

sample	method		
	SOMCPH	FTOTPH	BFRPH
A1	O	O	O
A2	O	O	O
B1	O	O	O
B2	O	O	O
B3	O	O	O
B4	O	O	O
B5	O	O	O
B6	O	O	O
B7	O	O	O
B8	O	O	O
B9	O	O	O
B10	O	O	O

O = 1 Petri dish

Restack the Petri dishes in the order of the random numbers. Hand over the set of dishes and the reporting form to another laboratory worker, who should not be aware of the original incubation conditions etc. The second worker counts all plaques (visible to the bare eye) on each plate and *records the number of plaques on the reporting form for the concentration technique behind the random number corresponding to that on the dish*. The second worker should also examine the blanks and *record the results on the reporting form. Indicate on the reporting form also data of which you are not sure because of technical problems.*

Note: If a laboratory has too few qualified laboratory workers, the random labelling can be carried out alternatively. In this latter case a second worker should recode the Petri dishes with the random labels and restack the dishes in the order of the random numbers. The first laboratory worker can then do the counting. Note on the reporting form.

27 March 1998

Read the plates of the viable counts of HSP40 *Bacteroides fragilis* and note on the reporting form.

The reporting form is checked for completeness by the head of the laboratory, signed and the data (pages 19 and 30) mailed by telefax between 27 March and 3 April 1998 to Kirsten Mooijman. The original (complete) reporting form should be mailed by (normal) mail to the RIVM, using the enclosed self-adhesive label.

Abbreviations and where to find what

BFRPH	Phages of *Bacteroides fragilis* (BFRPH-method)
cfp	colony forming particle
DAL	Double Agar Layer method
FDNAPH	F-specific DNA bacteriophages (ISO 10705-1)
FRNAPH	F-specific RNA bacteriophages (ISO 10705-1)
FTOTPH	Total of F-specific bacteriophages (FRNAPH + FDNAPH; ISO 10705-1)
HSP40	*Bacteroides fragilis* (host for phages of *B.fragilis*; BFRPH method)
IPL	Institute Pasteur of Lille
Km	Kanamycine (ISO 10705-1,10.3 and BFRPH-method)
MBPRMA	Modified *Bacteroides fragilis* phage repair medium agar (BFRPH-method, A.2)
MBPRMB	Modified *Bacteroides fragilis* phage repair medium broth (BFRPH-method, A.1)
MGB	Microbiological Laboratory for Health Protection
MSA	Modified Scholtens' Agar (ISO/CD 10705-2, A2)
MSB	Modified Scholtens' Broth (ISO/CD 10705-2, A1)
Nal	Nalidixic acid (ISO 10705-1,A.4; ISO/CD 10705-2, A.4 and BFRPH-method, A.5)
pfp	plaque forming particle
ps	peptone saline solution (ISO 10705-1, A8)
RIVM	National Institute of Public Health and the Environment
RM	Reference material
SOMCPH	Somatic coliphages (ISO/CD 10705-2)
SOP	Standard Operating Procedure
ssMBPRMA	semi-solid MBPRM agar (BFRPH-method, A.3)
ssMSA	semi-solid Modified Scholtens' Agar (ISO/CD 10705, A3)
ssTYGA	semi-solid Tryptone-Yeast extract-Glucose Agar (ISO 10705-1 A3)
TYGA	Tryptone-Yeast extract-Glucose Agar (ISO 10705-1, A2)
TYGB:	Tryptone-Yeast extract Glucose Broth (ISO 10705-1, A1)
UB	University of Barcelona
WG5	*Escherichia coli* Nal^r (host for somatic coliphages; ISO/CD 10705-2, 8)
WG49	*Salmonella typhimurium* (F^+ strain, host for FRNAPH; ISO 10705-1, 8)

Annex 5

Reporting form of the second collaborative study

pages 89 - 120

3 March 1998

REPORTING FORM

BACTERIOPHAGES IN BATHING WATER TRIAL 2
MARCH 1998

FILL IN COMPLETELY (please in English)

Laboratory code (indicated on the random labels):..

Laboratory name: ....

Contact person : ....

Date of arrival of the parcel with materials: .... - .... - 1998

Was there still dry ice in the parcel?	☐ yes	☐ no
Were the vials still frozen?	☐ yes	☐ no
Was the parcel damaged?	☐ yes	☐ no

Please confirm the number of vials of each batch of materials:
(The numbers between brackets are the number of vials sent by MGB/RIVM)

Natural pol. samples for SOMCPH (De Bilt 281097; red caps):	.... vials (5)
Natural pol. samples for FTOTPH (De Bilt 071197; yellow caps):	.... vials (5)
Natural pol. samples for BFRPH (UB 311097; green caps):	.... vials (5)
ΦX174 (batch 040696; brown caps):	.... vials (8)
MS2 (batch 220597; white caps):	.... vials (6)
B40-8 (batch 260397; blue caps):	.... vials (4)

Date of the study with natural polluted samples: .... - .... - 1998

Date of the study with the concentration technique: .... - .... - 1998

General questions

1. What was the temperature of the freezer during the period the reference materials were stored in it? Please give a list with dates, times and temperatures. If the temperature is recorded continuously, please enclose a print-out of the period concerned.

2. What kind of water has been used for preparation of the media?
   - ☐ deionized
   - ☐ distilled in all-glass apparatus
   - ☐ distilled in an apparatus with metal parts
   - ☐ ultrafiltration/reverse osmosis
   - ☐ other, please specify ....

3. What kind and size of Petri dishes did you use for the media (if different Petri dishes were used for different media, please indicate)?
   ☐ Glass ☐ Plastic
   ☐ Vented ☐ Non-vented
   size:...mm

4. What kind of pipettes did you use and for which purpose?
   - ☐ Disposable (plastic), for:..
   - ☐ Glass, for:...
   - ☐ Automatic with "tips", where the tips were
   - ☐ with filter, for:..
   - ☐ without filter, for:...

   Remarks:...

5. Did you use a counting apparatus for reading the plates?
   ☐ yes ☐ no

   If yes, was this a
   - ☐ Light box and counting "by hand"
   - ☐ Light box, combined with a "counting pen"
   - ☐ Other, namely,...

6. How did you prevent possible contamination during working? If this is different for the different phage enumerations, please indicate.

☐ Work quickly on the laboratory bench
☐ Work near the flame
☐ Work in a Laminar air flow cabinet
☐ Other, namely ....

Remarks..

Materials SOMCPH

7. When did you prepare the media? How did you store the media? What was the pH of the media on that date and on the day of the trial (measured with a pH-meter)? Give on the day of the trial the pH of the complete medium (including additives). Also give the temperature of the solution at which the pH was measured. For pH measurement, see SOP BCR-water/004 (930514).

Medium	Date of preparation	storage temperature/°C	pH /temperature (°C)	
			day prep.	day trial
PS			/ °C	/ °C
MSB			/ °C	/ °C
MSA (plates)			/ °C	/ °C
MSA (bottle)			/ °C	/ °C
ssMSA			/ °C	/ °C
$CaCl_2$-solution				
NaI (if applicable)				

8. Did you add Nalidixic acid solution (NaI) to ssMSA? ☐ yes ☐ no
If yes, was NaI added:
☐ Before autoclaving the medium ☐ After autoclaving the medium

9. Did you dry the dishes with MSA before use?
☐ yes ☐ no
If yes, what procedure did you use?
- Drying temperature: .... °C
- Drying time: .... (give time in hours or minutes)
- Dried: ☐ in incubator
☐ in Laminar flow cabinet
☐ on laboratory bench
- During drying - dishes: ☐ open ☐ closed
- agar layer: ☐ upwards ☐ downwards

Materials FTOTPH and FDNAPH

10. When did you prepare the media? How did you store the media? What was the pH of the media on that date and on the day of the trial (measured with pH-meter)? Give on the day of the trial the pH of the complete medium (including additives). Also give the temperature of the solution at which the pH was measured. For pH measurement, see SOP BCR-water/004 (930514).

Medium	Date of preparation	storage temperature/°C	pH /temperature (°C)	
			day prep.	day trial
PS			/ °C	/ °C
TYGB			/ °C	/ °C
TYGA (plates)			/ °C	/ °C
TYGA (bottle)			/ °C	/ °C
ssTYGA			/ °C	/ °C
Ca-glucose				
RNase-sol.				
NaI (if applicable)				

11. Did you add Nalidixic acid solution (NaI) to ssTYGA? ☐ yes ☐ no
If yes, was NaI added:
☐ Before autoclaving the medium ☐ After autoclaving the medium

12. Did you dry the dishes with TYGA before use?
☐ yes ☐ no
If yes, what procedure did you use?
- Drying temperature: .... °C
- Drying time: .... (give time in hours or minutes)
- Dried: ☐ in incubator
☐ in Laminar flow cabinet
☐ on laboratory bench
- During drying - dishes: ☐ open ☐ closed
- agar layer: ☐ upwards ☐ downwards

Materials BFRPH

13. When did you prepare the media? How did you store the media? What was the pH of the media on that date and on the day of the trial (measured with a pH-meter)? Give on the day of the trial the pH of the complete medium (including additives). Also give the temperature of the solution at which the pH was measured. For pH measurement, see SOP BCR-water/004 (930514).

Medium	Date of preparation	storage temperature/°C	pH /temperature (°C)	
			day prep.	day trial
PS			/ °C	/ °C
MBPRMB			/ °C	/ °C
MBPRMA			/ °C	/ °C
ssMBPRMA			/ °C	/ °C
Hemin				
Na_2CO_3				
Kanamycin				
Nalidixic ac.				

14. Did you add Nalidixic acid solution and Kanamycin solution to ssMBPRMA?
☐ yes
☐ no

15. Did you dry the dishes with MBPRMA before use?
☐ yes ☐ no
If yes, what procedure did you use?
- Drying temperature: .... °C
- Drying time: .... (give time in hours or minutes)
- Dried: ☐ in incubator
☐ in Laminar flow cabinet
☐ on laboratory bench
- During drying - dishes: ☐ open ☐ closed
- agar layer: ☐ upwards ☐ downwards

Materials and general information concentration technique

16. When did you prepare the solutions? How did you store the solutions? What was the pH of the solutions on that date and on the day of the trial (measured with a pH-meter)? Also give the temperature of the solution at which the pH was measured. For pH measurement, see SOP BCR-water/004 (930514).

solution	Date of preparation	storage temperature/°C	pH /temperature (°C)	
			day prep.	day trial
Buffer for phages			/ °C	/ °C
synthetic sea salt			/ °C	/ °C
$MgCl_2$				
K_2HPO_4				
NaOH				

17. What size and how many centrifuge tubes were used?

Size: .... ml
Number: ....

18. Did you use a temperature regulated centrifuge?
☐ yes ☐ no

19. What was the temperature during centrifugation of the concentrate?

.... °C

20. What time did you start the centrifugation and at what time did you finish?

Start: .... h .... min
Finish: .... h .... min

21. Did you use a brake at the end of the centrifugation?
☐ yes ☐ no

NATURAL POLLUTED SAMPLES

SOMATIC COLIPHAGES (SOMCPH)

Inoculum culture(s) SOMCPH

22. How did you incubate your inoculum culture of WG5 *Escherichia coli*?
    - ☐ Incubator with rotating platform
    - ☐ Waterbath with rotating platform
    - ☐ Other, namely...

23. What was the temperature during incubation of the inoculum culture and at what speed was it shaken?

    Start incubation: time: ....h........................... min
    temperature:...................................... °C
    shaking speed: .... min^{-1}

    End incubation: time: ....h........................... min
    temperature:...................................... °C
    shaking speed: .... min^{-1}

    If continuous reading is used, please enclose a print out of the period concerned.

24. How did you measure absorbance?
    - ☐ In a conical flask with side-arm
    - ☐ In a cuvet
    - ☐ Other, namely...

25. At what filter range did you measure absorbance (between 500 and 650 nm)?

    .... nm

26. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice:
time: .... h .... min
absorbance: ....

Other times: ....
....

27. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of WG5?
.... h ....min

28. Did you follow the protocol for the viable counts of WG5?
☐ yes: pour plate with MSA
☐ no, namely ....

Phage enumeration SOMCPH

29. How did you melt your ssMSA and what was the time needed?
☐ In a boiling waterbath, for .... min
☐ In a microwave oven, for .... min, at .... Watt
☐ Other, namely ....

30. What was the temperature of the waterbath/incubator in which the molten ssMSA was placed? .... °C
How long did you keep your molten ssMSA in the waterbath/incubator between melting ssMSA and start of use?
.... (give time in min or hours)

31. At what time did you place the vials of standard natural polluted samples and reference material at room temperature (also give the temperature)?

Time vials at room temperature: .... h .... min
Room temperature at that moment: .... °C

32. At what time did you start and at what time did you finish the phage enumeration for SOMCPH (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

Start: .... h .... min

Finish: .... h .... min

33. What incubator did you use and what were the temperatures? Also note the start time and finish time of the incubation.
Note the temperature reading at the shelf where the plates are incubated.

Incubation at 37 °C in: ☐ fan assisted incubator
☐ standard (non fan assisted) incubator

Date: .... start time: .... h .... min
temperature: .... °C

Date: .... finish time: .... h .... min
temperature: .... °C

If continuous temperature reading is used, please enclose a print-out of the period concerned.

F-SPECIFIC PHAGES (FTOTPH and FDNAPH)

Inoculum culture(s) F-specific phages

34. How did you incubate your inoculum culture of WG49 *Salmonella typhimurium*?

☐ Incubator with rotating platform
☐ Waterbath with rotating platform
☐ Other, namely..

35. What was the temperature during incubation of the inoculum culture and at what speed was it shaken?

Start incubation: time: ....h........................... min
temperature:...................................... °C
shaking speed: .... min^{-1}

End incubation: time: ....h........................... min
temperature:...................................... °C
shaking speed: .... min^{-1}

If continuous reading is used, please enclose a print out of the period concerned.

36. How did you measure absorbance?

☐ In a conical flask with side-arm
☐ In a cuvet
☐ Other, namely..

37. At what filter range did you measure absorbance (between 500 and 650 nm)?

.... nm

38. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice:
time: .... h .... min
absorbance: ....

Other times: ....
....

39. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of WG49?
.... h ....min

40. Did you follow the protocol for the viable counts of WG49?
☐ yes: pour plate with TYGA
☐ no, namely ....

Phage enumeration F-specific phages

41. How did you melt your ssTYGA and what was the time needed?
☐ In a boiling waterbath, for .... min
☐ In a microwave oven, for .... min, at .... Watt
☐ Other, namely ....

42. What was the temperature of the waterbath/incubator in which the molten ssTYGA was placed? .... °C
How long did you keep your molten ssTYGA in the waterbath/incubator between melting ssTYGA and start of use?
.... (give time in min or hours)

43. At what time did you place the vials of standard natural polluted samples and reference materials at room temperature (also give the temperature)?

Time vials at room temperature: .... h .... min
Room temperature at that moment: .... °C

44. At what time did you start and at what time did you finish the phage enumeration for F-specific phages (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

Start: .... h .... min

Finish: .... h .... min

45. What incubator did you use and what were the temperatures? Also note the start time and finish time of the incubation.
Note the temperature reading at the shelf where the plates are incubated.

Incubation at 37 °C in: ☐ fan assisted incubator
☐ standard (non fan assisted) incubator

Date: .... start time: .... h .... min
temperature: .... °C

Date: .... finish time: .... h .... min
temperature: .... °C

If continuous temperature reading is used, please enclose a print-out of the period concerned.

PHAGES OF *BACTEROIDES FRAGILIS* (BFRPH)

Inoculum culture(s) BFRPH

46. Did you use a frozen working culture or an “overnight” working culture of HSP40 *Bacteroides fragilis* for preparing the inoculum culture?
    - ☐ frozen working culture
    - ☐ “overnight” working culture

47. If an “overnight” working culture of HSP40 was used, what was the temperature during incubation of this “overnight” working culture? Also note the start time and finish time of the incubation.

    Date: .... start time: .... h .... min
    temperature: .... °C

    Date: .... finish time: .... h .... min
    temperature: .... °C

    If continuous reading is used, please enclose a print out of the period concerned.

48. What kind of tubes did you use for culturing the inoculum culture of HSP40 *Bacteroides fragilis*?

    ☐ glass ☐ plastic
    ☐ with screw-caps ☐ other caps, namely...

    size: diameter: .... mm
    length: .... mm
    volume: .... ml

49. How many tubes did you inoculate for preparing the inoculum culture of HSP40? How many were well grown? How many were used (mixed)?

    No. of tubes inoculated: ....
    No. of tubes well grown: ....
    No. of tubes used: ....

50. What was the temperature during incubation of the inoculum culture of HSP40?

Start incubation: time: ....h........................... min
temperature:....................................... °C

End incubation: time: ....h........................... min
temperature:....................................... °C

If continuous reading is used, please enclose a print out of the period concerned.

51. How did you measure absorbance?

- ☐ In a screw-caped tube
- ☐ In a cuvet
- ☐ Other, namely...

52. At what filter range did you measure absorbance (between 500 and 650 nm)?

.... nm

53. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice
time: .... h .... min
absorbance: ....

Other times: ....
....

54. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of HSP40?
.... h ....min

55. Did you follow the protocol for the viable counts of HSP40?
☐ yes: "DAL" procedure with ssMBPRMA
☐ no, namely ....

56. What procedure did you use for culturing in anaerobic conditions?
☐ jar + gas (give the composition)..
☐ jar + commercial system, namely ....
☐ anaerobic cabinet
☐ other, namely...
How did you control the anaerobiosis during incubation?
....

<u>Phage enumeration BFRPH</u>

57. How did you melt your ssMBPRMA and what was the time needed?

☐ In a boiling waterbath, for .... min
☐ In a microwave oven, for .... min, at .... Watt
☐ Other, namely ....

58. What was the temperature of the waterbath/incubator in which the molten ssMBPRMA was placed? .... °C
How long did you keep your molten ssMBPRMA in the waterbath/incubator between melting ssMBPRMA and start of use?
.... (give time in min or hours)

59. At what time did you place the vials of standard natural polluted samples and reference material at room temperature (also give the temperature)?

Time vials at room temperature: .... h .... min
Room temperature at that moment: .... °C

60. At what time did you start and at what time did you finish the phage enumeration for BFRPH (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

Start: .... h .... min
Finish:....................... h .... min

61. What procedure did you use for culturing in anaerobic conditions?
- ☐ jar + gas (give the composition)..
- ☐ jar + commercial system, namely ....
- ☐ anaerobic cabinet
- ☐ other, namely...

How did you control the anaerobiosis during incubation?
....

62. What were the temperatures during incubation? Also note the start time and finish time of the incubation.
Note the temperature reading at the shelf where the plates are incubated.

Date: .... start time: .... h .... min
temperature: .... °C
Date: .... finish time: .... h .... min
temperature: .... °C
If continuous temperature reading is used, please enclose a print-out of the period concerned.

DATA NATURAL POLLUTED SAMPLES

VIABLE COUNTS

Note the number of colonies counted per plate:

	Dilution		
	10^{-5}	10^{-6}	10^{-7}
WG5	-	-	-
WG49	-	-	-
HSP40	-	-	-

MSA blank: ....

TYGA blank: ....

ssMBPRMA blank:.............................

MSA-ps blank: ....

TYGA-ps blank: ....

ssMBPRMA-ps blank:..............................

Remarks: ....

PHAGE ENUMERATION CONTROLS

SOMCPH: blank ssMSA:...

blank WG5:..

FTOTPH: blank ssTYGA:..

blank WG49:..

BFPRH: blank ssMBPRMA:...

blank HSP40:...

Remarks: ....

PHAGE COUNTS NATURAL POLLUTED SAMPLES

Laboratory code:..............................

Please record the total number of plaque forming particles (pfp) behind the appropriate random number. The random numbers also appear on the self-adhesive labels. Indicate data of which you are not sure because of technical problems.

Random number	pfp	Random number	pfp	Random number	pfp	Random number	pfp
1		2		3		4	
5		6		7		8	
9		10		11		12	
13		14		15		16	
17		18		19		20	
21		22		23		24	
25		26		27		28	
29		30		31		32	
33		34		35		36	
37		38		39		40	
41		42		43		44	
45		46		47		48	

Remarks: ....
....
....
....
....

CONCENTRATION TECHNIQUE

GENERAL

63. At what time did you take suspension B from the centrifuge and placed it at room temperature? At what time did you take suspension A from melting ice and placed it at room temperature? Also give the temperature.

Time suspension B at room temperature: .... h .... min
Time suspension A at room temperature: .... h .... min
Room temperature at that moment: .... °C

SOMATIC COLIPHAGES (SOMCPH)

Questions 22, 24, 25 and 28 are also of importance here. Please control whether the answers you have given to these questions correspond with the situation for the concentration technique. If not, please indicate in an annex.

Inoculum culture(s) SOMCPH

64. What was the temperature during incubation of the inoculum culture and at what speed was it shaken?

Start incubation: time: ....h........................... min
temperature:....................................... °C
shaking speed: .... min^{-1}

End incubation: time: ....h........................... min
temperature:....................................... °C
shaking speed: .... min^{-1}

If continuous reading is used, please enclose a print out of the period concerned.

65. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice:
time: .... h .... min
absorbance: ....

Other times: ....

66. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of WG5?
.... h ....min

<u>Phage enumeration SOMCPH</u>

67. How did you melt your ssMSA and what was the time needed?
- ☐ In a boiling waterbath, for .... min
- ☐ In a microwave oven, for .... min, at .... Watt
- ☐ Other, namely ....

68. What was the temperature of the waterbath/incubator in which the molten ssMSA was placed? .... °C
How long did you keep your molten ssMSA in the waterbath/incubator between melting ssMSA and start of use?
.... (give time in min or hours)

69. At what time did you start and at what time did you finish the phage enumeration for SOMCPH (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

Start: .... h .... min

Finish: .... h .... min

70. What incubator did you use and what were the temperatures? Also note the start time and finish time of the incubation.
Note the temperature reading at the shelf where the plates are incubated.

Incubation at 37 °C in:	☐ fan assisted incubator ☐ standard (non fan assisted) incubator
Date:	start time: .... h .... min temperature: .... °C
Date:	finish time: .... h .... min temperature: .... °C

If continuous temperature reading is used, please enclose a print-out of the period concerned.

F-SPECIFIC PHAGES (FTOTPH and FDNAPH)

Questions 34, 36, 37 and 40 are also of importance here. Please control whether the answers you have given to these questions correspond with the situation for the concentration technique. If not, please indicate in an annex.

Inoculum culture(s) F-specific phages

71. What was the temperature during incubation of the inoculum culture and at what speed was it shaken?

Start incubation: time: ....h........................... min
temperature:...................................... °C
shaking speed: .... min^{-1}

End incubation: time: ....h........................... min
temperature:...................................... °C
shaking speed: .... min^{-1}

If continuous reading is used, please enclose a print out of the period concerned.

72. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice:
time: .... h .... min
absorbance: ....

Other times: ....
....

73. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of WG49?
.... h ....min

Phage enumeration F-specific phages

74. How did you melt your ssTYGA and what was the time needed?
☐ In a boiling waterbath, for .... min
☐ In a microwave oven, for .... min, at .... Watt
☐ Other, namely ....

75. What was the temperature of the waterbath/incubator in which the molten ssTYGA was placed? .... °C
How long did you keep your molten ssTYGA in the waterbath/incubator between melting ssTYGA and start of use?
.... (give time in min or hours)

76. At what time did you start and at what time did you finish the phage enumeration for F-specific phages (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

Start: .... h .... min

Finish: .... h .... min

77. What incubator did you use and what were the temperatures? Also note the start time and finish time of the incubation.
Note the temperature reading at the shelf where the plates are incubated.

Incubation at 37 °C in: ☐ fan assisted incubator
☐ standard (non fan assisted) incubator

Date: .... start time: .... h .... min

temperature: .... °C

Date: .... finish time: .... h .... min

temperature: .... °C

If continuous temperature reading is used, please enclose a print-out of the period concerned.

PHAGES OF *BACTEROIDES FRAGILIS* (BFRPH)

Questions 48, 51, 52, 55, 56 and 61 are also of importance here. Please control whether the answers you have given to these questions correspond with the situation for the concentration technique. If not, please indicate in an annex.

Inoculum culture(s) BFRPH

78. Did you use a frozen working culture or an "overnight" working culture of HSP40 *Bacteroides fragilis* for preparing the inoculum culture?
    - ☐ frozen working culture
    - ☐ "overnight" working culture

79. If an "overnight" working culture of HSP40 was used, what was the temperature during incubation of this "overnight" working culture? Also note the start time and finish time of the incubation.

    Date: .... start time: .... h .... min
    temperature: .... °C

    Date: .... finish time: .... h .... min
    temperature: .... °C

    If continuous reading is used, please enclose a print out of the period concerned.

80. How many tubes did you inoculate for preparing the inoculum culture of HSP40? How many were well grown? How many were used (mixed)?

    No. of tubes inoculated: ....
    No. of tubes well grown: ....
    No. of tubes used: ....

81. What was the temperature during incubation of the inoculum culture of HSP40?

Start incubation: time: ....h........................... min
temperature:...................................... °C

End incubation: time: ....h........................... min
temperature:...................................... °C

If continuous reading is used, please enclose a print out of the period concerned.

82. What was the absorbance at the different measuring times?

t = 0: time: .... h .... min
absorbance: ....

t = the time just before placing the inoculum culture in ice
time: .... h .... min
absorbance: ....

Other times: ....
....

83. Give information about times:

At what time did you place the inoculum culture in melting ice?
.... h ....min

Did you keep the inoculum culture in melting ice during the phage enumeration? ☐ yes ☐ no

If no, at what time did you place the inoculum culture at room temperature?
.... h ....min
What was the room temperature at that moment? .... °C

At what time did you perform the viable counts of HSP40?
.... h ....min

Phage enumeration BFRPH

84. How did you melt your ssMBPRMA and what was the time needed?
   ☐ In a boiling waterbath, for .... min
   ☐ In a microwave oven, for .... min, at .... Watt
   ☐ Other, namely ....

85. What was the temperature of the waterbath/incubator in which the molten ssMBPRMA was placed? .... °C
   How long did you keep your molten ssMBPRMA in the waterbath/incubator between melting ssMBPRMA and start of use?
   .... (give time in min or hours)

86. At what time did you start and at what time did you finish the phage enumeration for BFRPH (this includes addition of sample and of inoculum culture to the tubes and pouring into plates)?

   Start: .... h .... min

   Finish:...................... h .... min

87. What were the temperatures during incubation? Also note the start time and finish time of the incubation.
   Note the temperature reading at the shelf where the plates are incubated.

   Date: .... start time: .... h .... min
   temperature: .... °C

   Date: .... finish time: .... h .... min
   temperature: .... °C

   If continuous temperature reading is used, please enclose a print-out of the period concerned.

DATA CONCENTRATION TECHNIQUE

VIABLE COUNTS

Note the number of colonies counted per plate:

	Dilution		
	10^{-5}	10^{-6}	10^{-7}
WG5	-	-	-
WG49	-	-	-
HSP40	-	-	-

MSA blank: ....

TYGA blank: ....

ssMBPRMA blank:.............................

MSA-ps blank: ....

TYGA-ps blank: ....

ssMBPRMA-ps blank:..............................

Remarks: ....

PHAGE ENUMERATION CONTROLS

SOMCPH: blank ssMSA:...

blank WG5:..

FTOTPH: blank ssTYGA:...

blank WG49:..

BFPRH: blank ssMBPRMA:..

blank HSP40:...

Remarks: ....

PHAGE COUNTS CONCENTRATION TECHNIQUE

Laboratory code:.............................

Please record the total number of plaque forming particles (pfp) behind the appropriate random number. The random numbers also appear on the self-adhesive labels. Indicate data of which you are not sure because of technical problems.

Random number	pfp	Random number	pfp	Random number	pfp
1		2		3	
4		5		6	
7		8		9	
10		11		12	
13		14		15	
16		17		18	
19		20		21	
22		23		24	
25		26		27	
28		29		30	
31		32		33	
34		35		36	

Remarks: ....
....
....
....
....

Name of laboratory worker doing the phage enumeration:

....

Date: . . . - . . . - 1998 Signature: ....

Name of laboratory worker doing the counting:

....

Date: . . . - . . . - 1998 Signature: ....

Name of the head of the laboratory

....

Date: . . . - . . . - 1998 Signature: ....

Remarks: ....

....

....

Fax the data (pages 19 and 30) to Kirsten Mooijman: +31 30 274 4434;
Mail the completed form to Kirsten Mooijman, RIVM (use the enclosed label).
Keep a copy for your own use.