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First collaborative study on bacteriophages in bathing waters

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Abstract

The first international collaborative study on bacteriophages in bathing waters was organised in May 1997. Sixteen European laboratories (including the organising laboratory) participated in the study. They analysed phage reference materials (RMs) for the enumeration of somatic coliphages (RMs containing ΦX174), F-specific RNA phages (RMs containing MS2) and phages of *Bacteroides fragilis* (RMs containing B40-8). The reference materials were found to fulfil the criteria for stability and homogeneity and were considered suitable for the collaborative study. During a discussion with the participating laboratories about the results of the study, the group concluded to exclude some data from further statistical analysis because of technical problems which occurred at the laboratorium level when analysing the samples. Analysis of the remaining data showed the following values of repeatability (r): 1.35 - 1.38 and reproducibility (R): 1.52 - 2.04. These values are close to the theoretical optimum. This indicates that the participating laboratories were able to apply the present methods for the enumeration of the three types of bacteriophages on phage reference materials.

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Samenvatting

In mei 1997 werd het eerste internationale ringonderzoek voor bacteriofagen in zwemwater georganiseerd. Voor dit doel werden referentiematerialen (RMs) voor drie typen bacteriofagen bereid. De materialen bestonden uit een batch van kleine plastic epjes, met in ieder epje *ca* 2.4 ml faagsupensie. Deze faagsuspensie werd bereid door een hoge titer faagsuspensie te verdunnen in pepton fysiologisch zout oplossing, tot een telbaar aantal (tussen 30 en 150 plaque vormende deeltjes per ml; pvd/ml). Bij deze oplossing werd glycerol toegevoegd (eindconcentratie 5% (v/v)), verdeeld over de epjes en opgeslagen bij (-70 ± 10) °C. De volgende fagen werden gebruikt: ΦX174 voor somatische colifagen; MS2 voor F-specifieke RNA fagen en B40-8 voor fagen van *Bacteroides fragilis*. De RMs werden gecontroleerd op homogeniteit en stabiliteit. Er kon geconcludeerd worden dat de RMs voldeden aan de (vooraf gestelde) criteria en bruikbaar waren voor het ringonderzoek. Zestien Europese laboratoria (inclusief het organiserende laboratorium) namen deel aan het ringonderzoek. De doelstellingen van het ringonderzoek waren:

- Het testen van de mogelijkheid om faag-RMs te versturen naar EU-laboratoria;
- Het testen van de implementatie van de drie hieronder genoemde methoden in verschillende EU-laboratoria, gebruik makend van faag-RMs:
  * Somatische colifagen in water (SOMCPH);
  * F-specific RNA fagen in water (FRNAPH);
  * Fagen van *Bacteroides fragilis* in water (BFRPH);
- Identificatie van de redenen voor afwijkende resultaten in individuele laboratoria, welke mogelijk kunnen leiden tot aanpassing van de protocollen.

Voor dit doel analyseerde ieder laboratorium (m.b.v. standaard protocollen):

- Tien epjes van RMs met ΦX174, voor de bepaling van SOMCPH;
- . Elf epjes van RMs met MS2, voor de bepaling van FRNAPH (10 zonder, 1 met RNase);
- Tien epjes van RMs met B40-8, voor de bepaling van BFRPH.

De RMs werden, verpakt in droogijs, per koerier verzonden. De materialen kwamen bij alle deelnemers in bevroren toestand aan (zoals ook gewenst was).
Vanwege de korte periode tussen de eerste trainingsessie en het eerste ringonderzoek, hadden enkele deelnemers problemen met het bereiden en de kwaliteit van hun media, wat resulteerde in afwijkende pH waarden. Na discussie met de deelnemers werd geconcludeerd dat de in de ISO aangegeven pH range erg strict is en zo mogelijk verbreed zou moeten worden. Echter, extreme pH waarden kunnen mogelijk wel de faagtellingen beïnvloeden. Daarom werden resultaten verkregen van media met "extreem" afwijkende pH waarden niet gebruikt bij verdere analyse. Enkele andere data werden niet gebruikt bij verdere analyse vanwege: calibratie problemen met pipetten, één replica van één epje afwezig, problemen met groei van de gastheer. Uiteindelijk bleven voldoende data over om de herhaalbaarheid (r) en de reproduceerbaarheid (R) te berekenen. De waarden van r en R (1.35 en 1.52 voor SOMCPH; 1.38 en 1.73 voor FRNAPH; 1.38 en 2.04 voor BFRPH respectievelijk) waren dichtbij het theoretisch optimum. Dit demonstreert dat de deelnemende laboratoria reproduceerbare resultaten produceerden. Verder zijn de beschreven procedures voor de faagbepalingen een goede basis voor verder gebruik bij de bepaling van fagen in natuurlijk besmette monsters.

Summary

In May 1997 the first international collaborative study on bacteriophages in bathing waters was organised. For this purpose reference materials for three types of bacteriophages were prepared. The materials consisted of a batch of small plastic vials, each containing *ca* 2.4 ml phage suspension. This phage suspension was prepared by diluting a high titre phage suspension in peptone saline solution until a "countable" level (between 30 and 150 plaque forming particles per ml; pfp/ml). To this solution glycerol was added (final concentration 5 % (v/v)), distributed into vials and stored at (-70 ± 10) °C. The following phages were used: ΦX174 for somatic coliphages; MS2 for F-specific RNA phages and B40-8 for phages of *Bacteroides fragilis*.
The reference materials were checked for stability and homogeneity. It was concluded that these reference materials (RMs) fulfilled the pre-set criteria and could be used for the collaborative study.
Sixteen European laboratories (including the organising laboratory) participated in the collaborative study. The objectives of this study were:
- Testing the feasibility of mailing phage reference materials to different EU laboratories;
- Testing of the implementation of the next three methods in different EU laboratories by using phage reference materials;
  * Somatic coliphages in water (SOMCPH);
  * F-specific RNA phages in water (FRNAPH);
  * Phages of *Bacteroides fragilis* in water (BFRPH).
- Identification of reasons for deviating results in individual laboratories, possibly leading to modifications of the test protocols.

For this purpose each participating laboratory analysed (using standardised protocols):
- Ten vials of RMs containing ΦX174 for enumeration of SOMCPH;
- Eleven vials of RMs containing MS2 for enumeration of FRNAPH (10 without, 1 with RNase);
- Ten vials of RMs containing B40-8 for enumeration of BFRPH.

The RMs were sent by courier service, packed in dry ice. All participants received the materials still frozen in the parcel (as intended).
Due to the short period between the first training session and the first collaborative study, some participants had problems with the preparation and the quality of their media, resulting in some deviating pH values. After discussion with the participants it was concluded that the pH range indicated in the ISO is very strict and should, if possible, be broadend. However, extreme pH values could still influence the phage counts. Therefore, results obtained from media with strongly deviating pH values were not used for further analysis. A few other data were excluded from further analysis because of: calibration problems with pipettes, one replicate count missing of one vial, problems with the growth of the host strain. Still sufficient data remained for calculation of repeatability (r) and reproducibility (R). The values found for r and R (1.35 and 1.52 for SOMCPH; 1.38 and 1.73 for FRNAPH; 1.38 and 2.04 for BFRPH respectively) were close to the theoretical optimum. This demonstrates that the participating laboratories produced reproducible results. Furthermore, the procedures described for the enumeration of the three types of phages are a good basis for further use in enumeration of phages in natural polluted samples.

1. Introduction

In January 1996 an EC-funded project on bacteriophages in bathing waters started. This project intends to lead:

- to the availability of standardised methods for the concentration, detection and enumeration of three groups of bacteriophages that can be used for the determination of the microbiological quality of bathing waters;
- to determine the operationality of these methods in diverse situations as encountered in the EU;
- to obtain preliminary data on the occurrence of bacteriophages as compared to bacterial indicator organisms, that can be the basis for the design of a future field work for the comparison of measurements between bacteriophages and viruses.

To reach these objectives the following steps are taken:

1. Standardisation and optimisation of the methods for detection and enumeration of:
   - Somatic coliphages in water (SOMCPH);
   - F-specific RNA phages in water (FRNAPH);
   - Phages of *Bacteroides fragilis* in water (BFRPH).
2. Selection, standardisation and optimisation of a concentration technique for the three types of bacteriophages in water.
3. Development of reference materials for the implementation of the methods.
4. Implementation of the methods in a number of laboratories of the EU to test the feasibility of the methods, by
   - 4.a First training session in a central laboratory to introduce the phage methods and phages to the participants with mainly pure phage cultures;
   - 4.b First collaborative study to introduce the phage methods into the laboratories of the participants, with mainly pure phage cultures;
   - 4.c Second training session in a central laboratory to introduce concentration technique(s) and use of phage methods on natural polluted samples to the participants;
   - 4.d Second collaborative study to introduce concentration technique(s) and the analysis on natural polluted samples into laboratories of the participants.
5. Testing the methods in field conditions and measure the levels of the different bacteriophages in comparison to bacterial standards in various bathing waters representing, as far as possible, all possible bathing water typologies in the EU.

This report describes the organisation and the results of the first collaborative study on bacteriophages in bathing waters (objective 4.b).
Chapter 2 of this report deals with the characteristics of the phage reference materials used in the study. The results of the collaborative study will be presented in chapter 3, and discussed in chapter 4.

2. Phage reference materials

2.1 Materials and methods

2.1.1 Preparation of the phage reference materials

For each group of bacteriophages reference materials were prepared. The (general) method of preparation was the same for all materials. The methods used for enumeration of the different phages were Amended ISO/CD 10705-2 of February 1997 (SAL and DAL method) for enumeration of SOMCPH (Annex 6), Amended ISO 10705-1 of February 1997 for enumeration of FRNAPH (Annex 7) and the procedure described in Annex 8 for enumeration of BFRPH.

The phages used for the preparation of the reference materials were:
ΦX174, ATCC 13706-B1 as "standard" somatic coliphage; MS2, NCTC 12487 (or ATCC 15597-B1) as "standard" F-specific RNA phage and B40-8 (Tartera and Jofre, 1987) as "standard" *Bacteroides fragilis* phage.
The bacterial host strains used for enumeration of the bacteriophages were:
WG5 *Escherichia coli*, ATCC 700078 for SOMCPH; WG49 *Salmonella typhimurium*, NCTC 12484 for FRNAPH and HSP40 *Bacteroides fragilis*, ATCC 51477 for BFRPH.

The preparation of the phage RMs was as follows:
- A high titre phage suspension was prepared (e.g. see annex C of amended ISO 10705-1: Annex 7) and stored in small aliquots (e.g. 0.5 ml or 1 ml) in vials at (5 ± 3) °C.
- One vial with high titre phage suspension was diluted (tenfold dilutions) in peptone saline solution (ps; Annex 7, A8). Phage counts (" plaque tests") were performed on the dilutions expecting to give a countable number of plaques (each dilution in duplicate). All dilutions were stored at (5 ± 3) °C.
- According to the phage count results a suspension was prepared (using the dilutions from the refrigerator) of at least 1 litre ps with 5% (v/v) glycerol (of (5 ± 3) °C) with a final phage concentration of *ca* 30 -150 pfp/ml. The suspension was well mixed and distributed into vials in *ca* 2.4 ml aliquots and stored at (-70 ± 10) °C.
- At least five vials were kept separate to check the phage concentration and the homogeneity before freezing (each vial in duplicate).

- The criteria for each batch of RMs were:
  - Mean phage concentration: between *ca* 30 pfp/ml and *ca* 150 pfp/ml;

- T_1 (see below): 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 (see below) : For a homogeneous batch T_2/ (I-1) should be ≤ 2.

2.1.2 Determination of homogeneity

For the determination of the homogeneity of the phage reference materials the variation within and between vials was calculated. For this purpose the T_1 and T_2 tests were applied.

T_1 is a measure for the variation within one vial of reference material (replicate variation). T_2 is a measure for the variation between different vials of one batch of reference materials.

$$T_1 = \sum_i \sum_j [(z_{ij} - z_{i+} / J)^2 / (z_{i+} / J)]$$

where z_{ij} is the number of pfp per analytical portion (j) of vial i, $z_{i+} = \sum_j z_{ij}$ (sum of numbers of pfp in all replicates of vial I) and J is the number of analytical portions (replicas) per vial.

$$T_2 = \sum_i [(z_{i+} - z_{++} / I)^2 / (z_{++} / I)]$$

where $z_{++} = \sum_i (\sum_j z_{ij})$ (sum of numbers of pfp in all vials of one batch) and I is the number of vials.

In the case of a Poisson distribution T_1 and T_2 follow approximately a χ^2-distribution with respectively I(J-1) and (I-1) degrees of freedom. In this case the expected values of T_1 and T_2 are the same as the number of degrees of freedom. Hence, T_1/ {I(J-1)} and T_2/ (I-1) are expected to be equal to one.
In practice it was shown that it is well possible to find a T_1 value which indeed follow a χ^2-distribution. However the T_2 value is in most cases larger than a χ^2-distribution. To have a measure for good homogeneity of the vials T_2/ (I-1) is calculated. If $T_2/(I-1) \leq 2$, the homogeneity of the vials is still acceptable.

2.1.3 Determination of stability

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"; see below) and it is also a tool for internal quality control.
The control charts for each batch of RMs were prepared in the way described below.
For determination of the (control) limits, 20 vials of each batch of RMs were analysed on different days and by different technicians. Each vial was analysed in singular. For calculation

of the control limits the counts were ^{10}log transformed. Based on the ^{10}log transformed counts, the mean ($\bar{x}$) and the standard deviation (s) were calculated. The standard deviation was calculated as follows:

$$s = 0.8865 * \bar{R} \qquad \bar{R} = \frac{1}{n-1} \sum_{i=2}^{n} |x_i - x_{i-1}|$$

Where n = number of observations and x_i = the i^{th} observation.

The calculation of $\bar{R}$ (the so-called moving range) is based on the sum of the differences between the first and the second count, the second and the third count and so on. This method of calculating the standard deviation is less influenced by 'extreme' counts (more robust) as sometimes found in microbiology.

The control limits were calculated (on the ^{10}log scale) as follows:

Warning limits: $\bar{x} \pm 2s$ Action limits: $\bar{x} \pm 3s$

The control chart was constructed after back transformation to the normal scale of the values for the mean ($\bar{x}$) and the upper and the lower action and warning limits. Back transformation leads to asymmetrical control charts. The results of the 20 vials analysed for the calculation of the limits were also indicated in a control chart. It was checked whether these counts met the criteria stated below (meaning that the analytical process is under control). If the results of one (or more) of these 20 vials were out of control, the cause for the erroneous result needed to be identified and a decision about the validity of the result had to be taken. If the result was not valid (i.e. the cause for the deviation could be found), it was not used for the calculation of the control limits.
Once the chart was constructed one vial was analysed in singular for each series of analyses and the result was indicated in the control chart. The result was out of control if:

- there was a single violation of the action limit ($\bar{x} \pm 3s$);
- two out of three observations in a row exceeded the same warning limit ($\bar{x} \pm 2s$);
- nine observations in a row were on the same side of the mean ($\bar{x}$);
- six observations in a row were steadily increasing or decreasing.

If the results were out of control, the cause for it had to be identified and a decision was taken about the validity of the results of the particular series of analysis.
A new control chart was constructed if necessary, for example when a shift in the mean level occurred (indicating some instability in the batch of RMs).
More information about costruction and use of control charts is described by Dommelen (1995).

2.2 Results

2.2.1 Reference materials for somatic coliphages (ΦX174)

The batch of RMs containing ΦX174 was prepared on 040696 (ddmmyy). *Ca* 1200 vials were prepared and stored as described in 2.1.1. Before freezing and a few days after freezing, 5 vials of this batch were checked (each in duplicate) for the criteria mentioned in 2.1.1. The results are given in Table 1. The batch fulfilled the criteria stated in 2.1.1.
Directly 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 1. This chart shows an initial (small) decrease in the mean phage counts of ΦX174 directly after freezing. After *ca* 3 months of storage of the batch at (-70 ± 10) °C stabilisation seemed to occur. Therefore the control chart was recalculated, using the data obtained after *ca* 3 months of storage of the batch. This recalculated chart is presented in Figure 2 and shows stable results.

Table 1 Results of RMs containing ΦX174, batch 040696

	Before freezing	3 days after freezing at (-70 ± 10) °C
Geometric mean (pfp/ml)	101.5	90.7
T_1	1.14	2.71
T_2/ (I-1)	1.99	0.16
I	5	5

I: Number of vials; Critical values of χ^2-distribution (which T_1 should fulfil) at 5 degrees of freedom and 95% confidence: lower limit 0.83, upper limit 12.83.

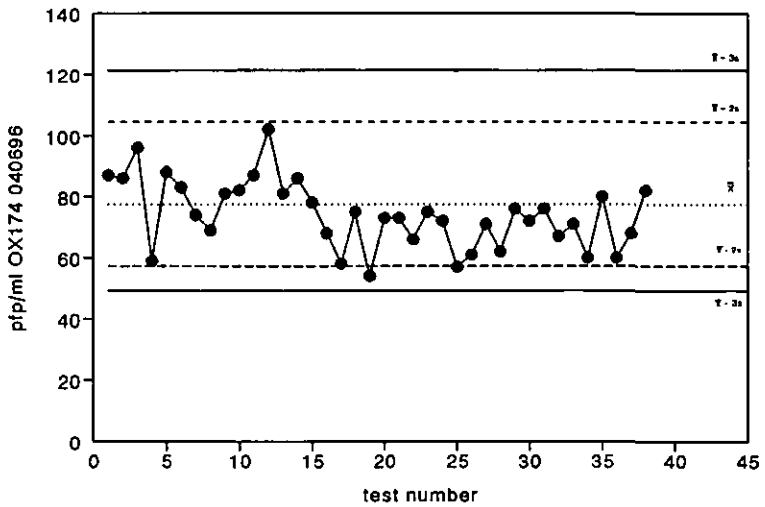

Figure 1 *Control chart of RMs containing ΦX174, batch 040696, on MSA with host strain WG5 Escherichia coli.*

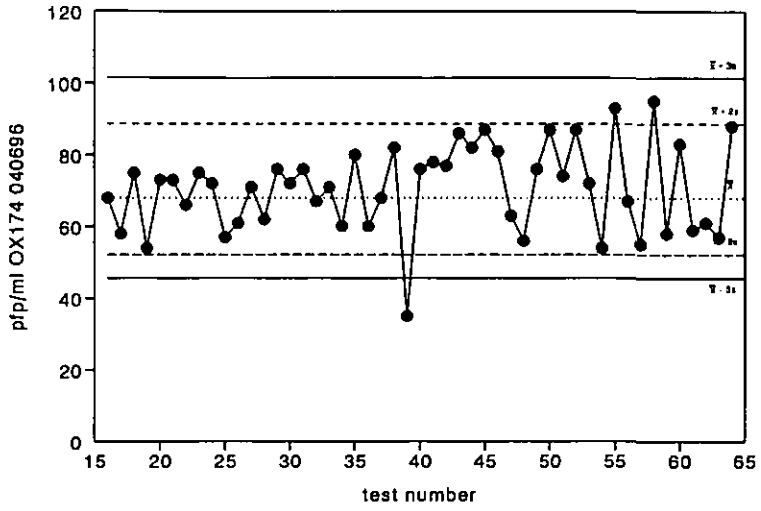

Figure 2 *Control chart of RMs containing ΦX174, batch 040696, on MSA with host strain WG5 Escherichia coli, after recalculation.*

2.2.2 Reference materials for F-specific RNA phages (MS2)

The batch of RMs containing MS2 was already prepared on 071293 (in the way as described in 2.1.1). One day after freezing (results before freezing were not available), the batch fulfilled the criteria (measured on 5 vials in duplicate). The results are given in Table 2.

Table 2 Results of RMs containing MS2, batch 071293

	1 day after freezing at (-70 ± 10) °C
Geometric mean (pfp/ml)	88.4
T_1	7.8
T_2/ (I-1)	1.08
I	5

Explanations see Table 1.

A control chart of this batch of RMs was prepared directly after preparation and storage of the batch in 1993. This chart is presented in Figure 3. As for the batch of RMs containing ΦX174, this batch also shows some decrease in the mean phage counts of MS2 in the first few months after freezing. Therefore this control chart was also recalculated, using data from the start of the project in January 1996. The recalculated chart is presented in Figure 4.

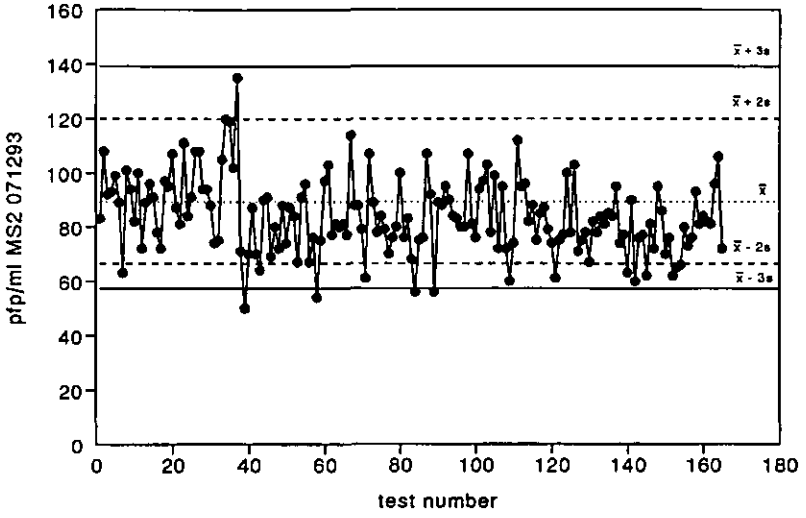

Figure 3 *Control chart of RMs containing MS2, batch 071293 on TYGA, with host strain WG49 Salmonella typhimurium.*

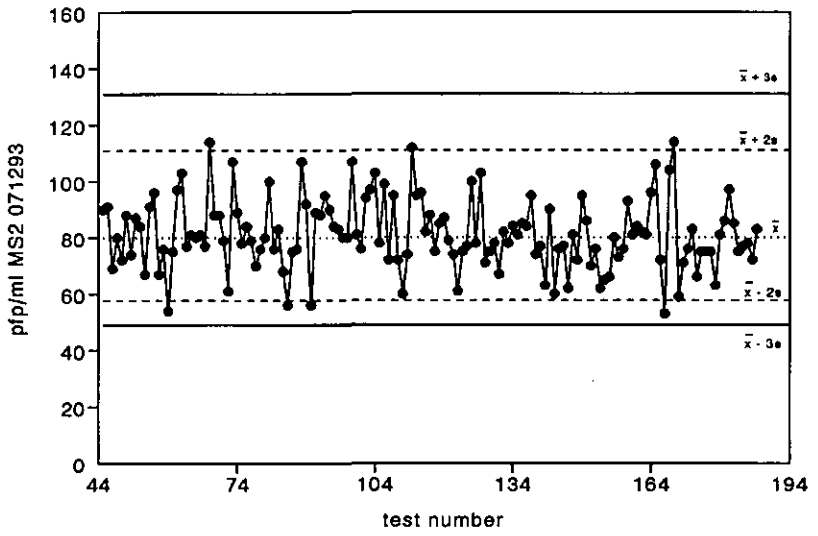

Figure 4 *Control chart of RMs containing MS2, batch 071293 on TYGA, with host strain WG49 Salmonella typhimurium, after recalculation.*

2.2.3 Reference materials for phages of *Bacteroides fragilis* (B40-8)

The batch of RMs containing B40-8 was prepared on 260397. Ca 1000 vials were prepared and stored as described in 2.1.1. Before and one day after freezing the batch fulfilled the criteria (measured on 10 vials in duplicate). The results are given in Table 3.

Table 3 Results of RMs containing B40-8, batch 260397

	Before freezing	1 day after freezing at (-70 ± 10) °C
Geometric mean (pfp/ml)	93.3	105.9
T_1	13.57	10.56
T_2/ (I-1)	1.53	0.56
I	10	10

Critical values of χ^2-distribution (which T_1 should fulfil) at 10 degrees of freedom and 95% confidence: lower limit 3.25, upper limit 20.48.

Soon after preparation and storage of this batch of RMs, vials were analysed for preparation of a control chart. The number of available results from this batch of RMs containing B40-8 is limited. Therefore the limits of the control chart were calculated from 10 measurements only. The chart is presented in Figure 5. The limited number of data show a relatively stable batch.

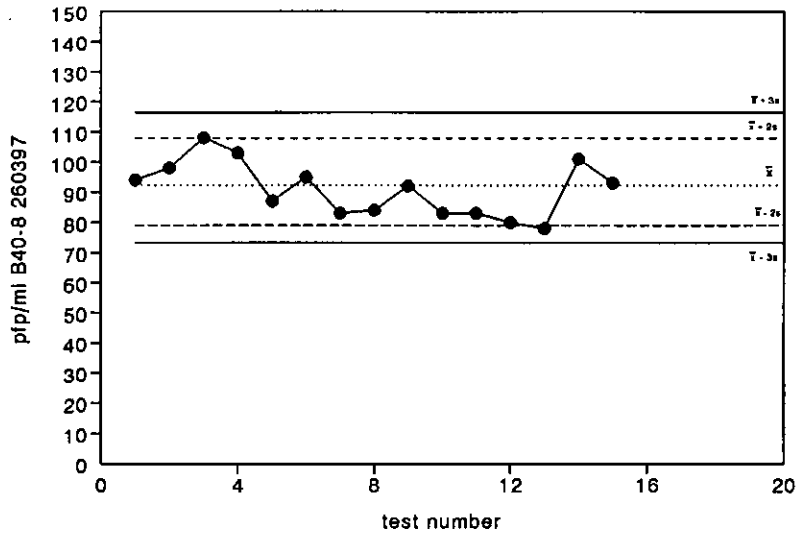

Figure 5 *Control chart of RMs containing B40-8, batch 260397, on BPRMA with host strain HSP40 Bacteroides fragilis.*

3. Collaborative study

3.1 Materials and methods

3.1.1 Design of the trial

The first collaborative study on bacteriophages was carried out in May 1997, according to the instructions described in the protocol (see Annex 2). Each participating laboratory received, a few weeks before the date of the trial a (big) parcel, by courier service, containing:

- Dry ice;
- A small box containing:
  - 10 vials of reference materials containing phage ΦX174, batch 040696;
  - 11 vials of reference materials containing phage MS2, batch 071293;
  - 10 vials of reference materials containing phage B40-8, batch 260397.

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

At a fixed date, the vials were thawn at room temperature and analysed (using the DAL procedure) for:

a. Number of somatic coliphages (SOMCPH), according to amended ISO/CD 10705-2 (February 1997; Annex 6);
b. Number of F-specific RNA-phages (FRNAPH), according to amended ISO 10705-1 (February 1997; Annex 7), of which 10 vials without the addition of RNase in duplicate, 1 vial with and without the addition of RNase.
c. Number of phages of *Bacteroides fragilis* (BFRPH), according to the method for detection of bacteriophages of *B.fragilis* (February 1997; Annex 8).

Before counting the plates were randomly labelled to eliminate the effects of extraneous factors. Further details about the design of the trial are given in Annex 2. All results were recorded on a reporting form (Annex 3) and sent to the organising laboratory. The Standard Operating Procedures (SOP's) used during the trial are described in Annexes 4 and 5.

3.1.2 Analysis of the data

The analysis of the data can be divided in two parts: the *data screening* and the *statistical analysis*.

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

  Visual screening means that the recoded counts from each laboratory were visually inspected for labelling errors and for counts that were reported as doubtful or as rough estimates. These results were excluded from further analysis.

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 phage methods. 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 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. The variation between duplicate counts within the vials was determined by calculating T_1 values per laboratory and per method. The variation in counts between vials was determined by calculating T_2 values per laboratory and per method. The formulas for T_1 and T_2 were given in 2.1.2. It was assumed that the number of plaque forming particles in the replicates follow a Poisson distribution, in which case the T_1 statistic (for each laboratory) should follow a χ^2 - distribution with I(J-1) degrees of freedom and a significance level $\alpha = 0.05$. If the T_1 value of a laboratory was significantly different from a χ^2 - distribution, the T_1 results of each vial were further considered. In this latter case T_1 should follow a χ^2 - distribution with J-1 degrees of freedom at $\alpha = 0.05/I$. As for the batch of RMs, the $T_2/(I-1)$ per laboratory and per method was also expected to be ≤ 2. Where I is the total number of vials analysed in one laboratory and J is the number of replicates per vial.
If one replicate count was missing, no T_1 could be calculated and it was decided not to use the (other) result of the vial concerned.
Deviating results for the T_1 and T_2 values were 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 (6.11) 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).

3.2 Results

3.2.1 Technical results

The main observations of the participating laboratories are summarised in Tables 4 to 11. The criteria described in the protocol, 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 following (main) subjects were discussed:

- Laboratory 8 received the RMs late on 29 April. The parcel was picked up by security and stored at room temperature until the next morning. There was no more dry ice in the box, but the materials were still frozen; the counts were probably not affected.
- Storage temperatures (of the RMs) below -80 °C were considered as acceptable. Storage at -20/-26 °C of the RMs was considered acceptable for a limited amount of time and was not expected to have influenced the counts.
- 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 three parts, one for each phage group, and could still be used effectively.
- A range of ± 0.2 pH unit (for the media of SOMCPH and FRNAPH) was considered to be unnecessarily strict and impractical by the participants. A range of ± 0.5 pH units was considered more realistic. This limit was adopted for the interpretation of the data. On the basis of these data, still results of some laboratories were excluded from further analysis (see Table 12) as it was expected that extreme pH values would influence the phage counts. For the media of BFRPH no range for the pH was set. As the temperature of the medium also influences the pH, it was not possible to give a uniform pH range for the best quality of the media. Therefore no data were excluded for the enumeration of BFRPH because of extreme pH values.
- It was considered necessary that the host cultures WG5 *Escherichia coli* (for SOMCPH) and WG49 *Salmonella typhimurium* (for FRNAPH) were incubated while gently shaking, without specifying a limit for the rotations per minute (rpm). Laboratory 12 had manually shaken the flasks every 30 min. This was considered to be minimal but no reason to eliminate the data. The final criterion should be the time needed to reach the right inoculum density.
- Some laboratories used temperatures of more than 46 °C to prevent semi-solid agars from solidifying after adding sample and host strain to the medium outside the waterbath. Because of cooling of the semi-solid agars after addition of the sample, the host strain was not expected to have been exposed to high temperatures. It was discussed whether there was a preferred way of inoculating the semi-solid agars: in the waterbath or outside. There was no clear preference, and it was decided not to prescribe any form.
- Four of the 16 participants reported problems with the maintenance or the preparation of host strain WG49 *Salmonella typhimurium*, which was considered to lack of robustness of

the strain. Laboratory 13 had so many problems with the strain that the results of this laboratory for this phage method (FRNAPH) were withdrawn.

- Laboratory 10 had low absorbance and colony count of HSP40 *Bacteroides fragilis* (for BFRPH) but they had a good host lawn on the plate, therefore the results were considered valid.
- Laboratory 6 reported that they had calibration problems with the pipettes while performing the BFRPH method. They had problems with pipetting the host strain, which was exposed to oxygen for a long time. They therefore decided to withdraw their results for the BFRPH method

A summary of the data excluded from further analysis is given in Table 12.

Table 4 Observations of participating laboratories: General questions

Lab-code	Shipment time parcel (days)[a]	RMs still frozen after arrival?	Date trial[b] (ddmmyy)	Temp. freezer (°C) max / min[c]
1	1	yes	130597	-62 / -66
2	1	yes	130597	-63 / -71
3	1	yes	130597&150597	-71
4	1	yes	140597&200597	-69.2 / -70.7
5	1	yes	130597&140597	-68 / -71
6	1	yes	130597	-80 / **-81**
7	1	yes	150597	-69 / -72
8	2	yes	130597	-80
9	1	yes	130597	-74 / -76
10	1	yes	130597	**-20 / -26**
11	1[d]	yes	130597&140597& 220597	-80 / **-86**
12	2	yes	200597&210597	-73 / -76
13	1	yes	110597	-70
14	1	yes	130597&140597	**ca -85**
15	1	yes	130597	**-53.1** / -67.4
16	organiser	yes	130597	-71.6 / -74.9

a: Date of mailing was 280497 by courier; shipment time 1-2 days at maximum
b: Date trial: 130597 ± 1 week (050597 - 230597)
c: Storage temperature during 290497 and date of the trial: (-70 ± 10) °C
d: New MS2 vials sent on 200597 (received on 210597)

Table 5a Observations of participating laboratories: pH media

Lab-code	pH media on day of the trial [a]				ssMSA + Nal? [b]
	PS	MSB	MSA	ssMSA	
1	6.8	7.2	7.3	7.1	yes
2	**6.0**[c]	**7.5**[d]	**8.0**[e]	**7.5**[e]	no
3	7.3	7.2	7.2	7.0	no
4	7.0	7.4	7.4	7.3	yes
5	6.7	**7.6**	7.2	7.3	no
6	7.1	**8.0**	**7.8**	**8.0**	no
7	7.0	7.1	7.2	7.0	no
8	no info	no info	no info	no info	no
9	6.8	7.4	7.2	7.1	yes
10	7.0	**8.6**	**8.5**	**8.3**	no
11	7.1	7.1	7.0	7.1	no
12	6.9	7.1	7.0	7.1	yes
13	6.5	**7.5**	**7.6**	**7.6**	yes
14	7.0	7.1	7.0	7.0	no
15	7.2	7.2	**8.0**	7.2	no
16	7.3	7.1	**7.6**	**7.5**	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: Addition of NaI to ssMSA optional.
c: pH measured at 4°C; d: pH measured at 37 °C; e: pH measured at 45 °C

Table 5b Observations of participating laboratories: pH media

Lab-code	pH media on day of the trial [a]						ssTYGA +Nal? [b]
	TYGB	TYGA	ssTYGA	BPRMB	BPRMA	ssBPRMA	
1	7.1	7.1	7.2	7.1	7.0	6.9	yes
2	**6.0**[c]	**6.0**[d]	**6.0**[d]	7.5[c]	7.5[d]	7.5[d]	no
3	7.2	7.2[d]	7.2[d]	7.0	7.0[d]	7.0[d]	no
4	7.2	7.1	7.1	7.2	7.0	6.8	yes
5	**7.0**	**7.7**[e]	**6.9**	7.3	**7.6**	7.1	no
6	7.3	7.2	7.2	7.0	6.8	7.0	no
7	7.2	7.1	7.2	7.3	7.1	7.3	no
8	no info	no info	no info	no info	no info	no info	no
9	7.1	7.3	**7.4**	7.1	7.1[d]	7.1[d]	yes
10	**8.5**	**8.3**	**8.0**	7.0	7.1	7.1	no
11	7.1	7.1	7.2	7.0[c]	7.0	7.0[d]	no
12	7.2	**7.0**	**7.0**	7.2	7.3	7.4	yes
13	**7.0**	**7.0**	**6.9**	7.0	7.0[d]	7.2[d]	yes
14	7.1	**7.0**	7.1	7	7	7	no
15	7.2	**7.6**	**6.9**	7.0	**8.7**	7.3[c]	no
16	**7.0**	7.1	7.2	**5.6**	**6.0**	**6.2**	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); BPRMB, BPRMA: 7.0 (for complete medium), ssBPRMA: 7.2 (for complete medium); b: Addition of NaI to ssTYGA optional.
c: pH measured at *ca* 37 °C; d: pH measured 45 - 50 °C; e: pH measured at *ca* 10 °C

Table 6 Observations of participating laboratories: Information on incubator type and incubation temperatures of working culture (of HSP40) and inoculum cultures (of WG5, WG49 and HSP40) and of all phage enumerations.

Lab-code	Type incubator / speed (min^{-1}) of ic WG5 and WG49 [a]	Temperature of incubation of wc of HSP40 and ic of WG5, WG49 and HSP40 min - max (°C) [b]	Type of incubator phage enumerations SOMCPH and FRNAPH [c]	Temperature of incubation of all phage enumerations min - max (°C) [d]
1	inc+r / 100	36.0	f	36.0
2	inc+r / 100	37 - 38	s	37 - 38
3	w+r / 100	37.0	s	37
4	inc+r / 100	36.2 - 37.3	f	34.5 - 37.4
5	w+r / 100	37.1 - 37.2	f	37.1 - 37.3
6	inc+r / 100	36.5 - 37.0	f	37.0
7	w+re / ***ca* 200**	36.8 - 37.0	f	36.5 - 36.9
8	inc+r / 100	37.0	s	37.0
9	w+ro / 100	36.7 - 37.0	f	36.8
10	inc+r / 100	36.5 - 37.0	s	36.5 - 37.0
11	w+r / *ca* 100	36.0 - 36.2	f	35.8 - 36.6
12	**inc / -**	36.5 - 37.0	f	36.4 - 37.0
13	**GFL 1083 / 40**	35.0 - 36.5	s	35.8 - 36.2
14	w+r / **60**	37.0	s	37
15	inc+r / 100	37.1 - 37.2	f	37.2
16	inc+r / 100	35.6 - 37.4	f	35.6 - 37.4

a: ic: inoculum culture; inc+r: incubator with rotating platform; w+r: waterbath with rotating platform; w+re: waterbath with "reciprocating" platform; w+ro: waterbath with rocking platform. According to ISO 10705: shaking speed of ic: (100 ± 10) min^{-1}.
b: wc: working culture. According to protocol BFRPH: incubation temperature of wc: (36 ± 2) °C. According to ISO 10705 and protocol of BFRPH: incubation temperature of ic: (36 ± 2) °C
c: f: fan-assisted; s: standard
d: According to ISO 10705 and protocol of BFRPH: incubation temperature of phage enumerations: (36 ± 2) °C

Table 7 Observations of participating laboratories: Inoculum culture of host strain WG5 Escherichia coli.

Lab-code	Incubation time (min)	Absorbance at t = 0	Absorbance at t = "end"	Viable count WG5 x 10^8 cfp/ml [a]	ic in melting ice (min) [b]
1	195	0.013	0.677	1.2	70
2	150	0.030	0.446	2.6	17
3	134	no data	0.280	1.8	20
4	165	0.126	0.875	1.0	40
5	145	0.000	0.250	3.0	10
6	130	0.011	0.382	0.9	10
7	135	0.022	0.384	1.9	38
8	180	no data	0.660	1.1	9
9	90	0.047	0.468	0.9	20
10	240	0.042	0.155	**0.1**	60
11	150	0.012	0.643	1.9	70
12	180	0.010	0.330	1.9	55
13	128	0.027	0.543	1.7	94
14	130	0.010	0.400	0.8	47
15	195	0.120	0.385	2.9	0
16	165	0.014	0.460	1.6	25

a: 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.
b: ic: Inoculum culture; According to ISO/CD 10705-2: place ic in melting ice and use the same working day.

Table 8 Observations of participating laboratories: Inoculum culture of host strain WG49 Salmonella typhimurium.

Lab-code	Incubation time (min)	Absorbance at t = 0	Absorbance at t = "end"	Viable count WG49 x 10^8 cfp/ml [a]	ic in melting ice (min) [b]
1	150	0.009	0.360	1.2	85
2	195	0.020	0.528	3.2	25
3	145	no data	0.250	1.8	20
4	165	0.015	0.485	1.3	61
5	135	0.000	0.150	1.1	20
6	213	0.007	0.394	1.3	10
7	135	0.014	0.285	1.3	20
8	240	no data	0.268	1.4	4
9	120	0.073	0.384	2.8	30
10	120	0.043	0.135	**0.5**	20
11	160	0.067	0.473	1.3	64
12	180	0.020	0.330	2.9	80
13	208	0.052	0.892	**0.4**	49
14	140	0.009	0.400	1.6	25
15	210	0.008	0.355	2.9	0
16	205	0.011	0.579	3.4	75

a: 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: ic: Inoculum culture; According to ISO 10705-1: place ic in melting ice and use within 2 hours.

Table 9 Observations of participating laboratories: Working culture and inoculum culture of host strain HSP40 Bacteroides fragilis.

Lab-code	Incubation time		Absorbance at		viable count HSP40	ic at roomtemp. before start use (min)
	wc (h;min)[a]	ic (min)	t = 0	t = "end"	x 10^8 cfp/ml[b]	/room temp. (°C) [c]
1	17 ; -	130	0.153	0.283	3.4	50 / 24
2	18 ; -	150	no data	0.415	6.1	50 / 21.5
3	17 ; 30	135	0.140	0.350	4.1	16 / 21
4	17 ; 15	165	no data	0.500	1.6	25 / 21.8
5	18 ; -	180	0.100	0.240	1.3	30 / 20.0
6	18 ; 15	200	0.117	0.358	2.0	148 / 20.5
7	18 ; 25	150	0.107	0.200	1.2	82 / 24
8	18 ; 15	360	no data	0.457	1.2	20 / 28
9	21 ; 40	120	0.203	0.415	3.0	35 / 24.4
10	18 ; -	120	0.077	0.143	**0.7**	60 / 24
11	19 ; -	182	no data	0.402	2.4	30 / 22
12	19 ; -	150	0.140	0.350	0.9	40 / 26
13	15 ; -	180	0.128	0.360	1.4	25 / 22
14	19 ; 30	130	0.155	0.350	1.0	22 / 22
15	17 ; -	165	0.210	0.430	1.7	50 / 22.6
16	20 ; -	240	0.140	0.430	5.0	50 / 22.6

a: wc: working culture; according to protocol BFRPH: incubation time "over night".
b: Measured by pour plates in BPRMA. Viable count is calculated from counts yielding between 30 and 300 colonies per plate; aimed values is *ca* 10^8 cfp/ml.
c: According to protocol BFRPH: use ic HSP40 immediate after taking it from the incubator.

Table 10 Observations of participating laboratories: Phage enumerations

Lab-code	temperature at which the ss-agars were kept (°C) [a]	Total time necessary for phage enumeration (min) [b]		
		SOMCPH	FRNAPH	BFRPH
1	45	15	20	25
2	45	25	25	20
3	45	10	15	10
4	46	30	39	20
5	46	15	20	25
6	45	23	23	17
7	46	30	36	44
8	45	36	47	50
9	45	25	35	25
10	45	30	40	30
11	**48**	22	19	30
12	46	15	15	25
13	45	22	27	20
14	**48**	25	30	25
15	44	20	12	20
16	45	15	21	11

a: According to ISO-10705 and protocol BFRPH: (45 ± 1) °C. b: Total time includes addition of sample and of inoculum culture to the tubes and pouring into plates.

Table 11 Observations of participating laboratories: Incubation of phage plates

Lab-code	Incubation time of phage plates (hours ; min) [a] SOMCPH	FRNAPH	BFRPH	Anaerobic conditions BFRPH [b]
1	18 ; 45	18 ; 45	19 ; -	j + cs
2	17 ; 45	17 ; 45	17 ; 45	j + cs
3	18 ; 40	18 ; 40	19 ; 15	j + cs
4	17 ; -	18 ; -	19 ; 20	j + cs
5	19 ; 15	17 ; -	17 ; -	j + cs
6	20 ; 25	19 ; 25	**43 ; 35**	j + cs
7	18 ; 12	18 ; 12	19 ; 22	j + cs
8	18 ; -	18 ; -	18 ; 30	j + cs
9	**14**	18 ; -	17 ; -	j + g & j + cs
10	16 ; -	19 ; 30	18 ; -	other
11	18 ; -	20 ; 05	19 ; 45	j + cs
12	18 ; 50	18 ; 50	20 ; 30	j + cs
13	19 ; -	19 ; -	20 ; 15	j + cs
14	19 ; 30	17 ; 10	**25 ; 30**	j + cs
15	18 ; -	20 ; -	20 ; 40	j + cs
16	20 ; 50	20 ; 50	20 ; 50	j + cs

a: According to ISO 10705 and protocol of BFRPH: (18 ± 2) hours. b: j+g: jar & gas; j+cs: jar & commercial system

Table 12 Data excluded from further analysis

labcode	SOMCPH (ΦX174)	FRNAPH (MS2)	BFRPH (B40-8)
1	vial 5 (a)	vial 5 (a)	
2	all data (b)	all data (b)	
3			vial 7 (c)
4			
5			
6	all data (b)		all data (d)
7			
8		vials 2, 3 (e)	
9		vial 5 (e)	
10	all data (b)	all data (b)	vial 10 (f)
11			
12			
13		all data (g)	
14			
15	all data (b)		
16			

(a): No reference material was added; (b): Problems with pH medium; (c): Reference material was added twice to two tubes; (d): Calibration problems with the pipettes;
(e): Count a or count b missing; (f): Count a=0 and count b=0;
(g): Problems with the growth of the host strain and segregation of lactose-negative variants.

3.2.2 Statistical results

The geometric mean results of all participating laboratories for the three phage methods before any exclusion of data are summarised in Figure 6.
After exclusion of the data mentioned in Table 12, box and whisker plots were prepared per phage method which are presented in Figures 7-9. 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.

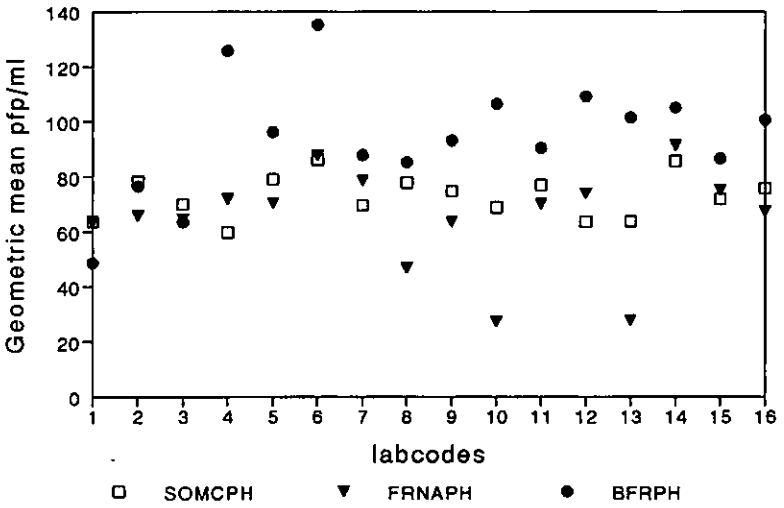

Figure 6 *Results of all participating laboratories for all three phage methods before exclusion of data.*

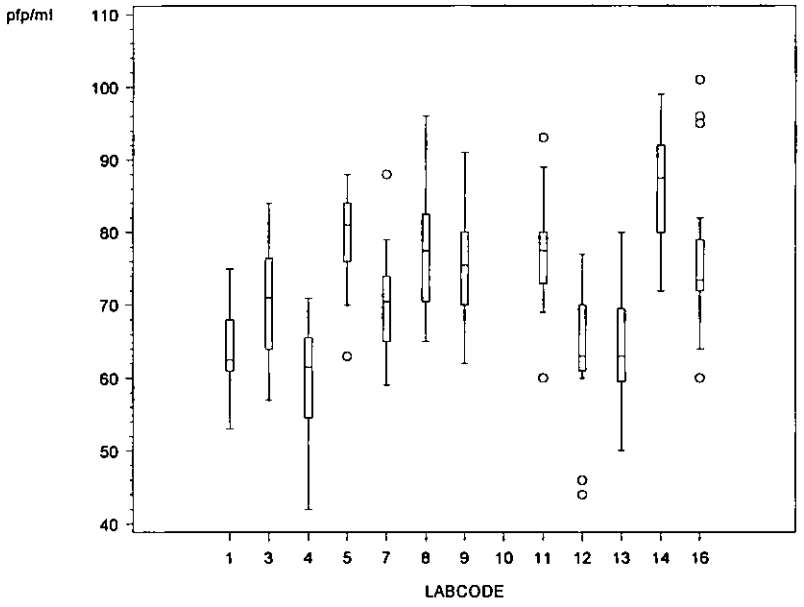

Figure 7 *Results of all participating laboratories for SOMCPH after exclusion of data.*

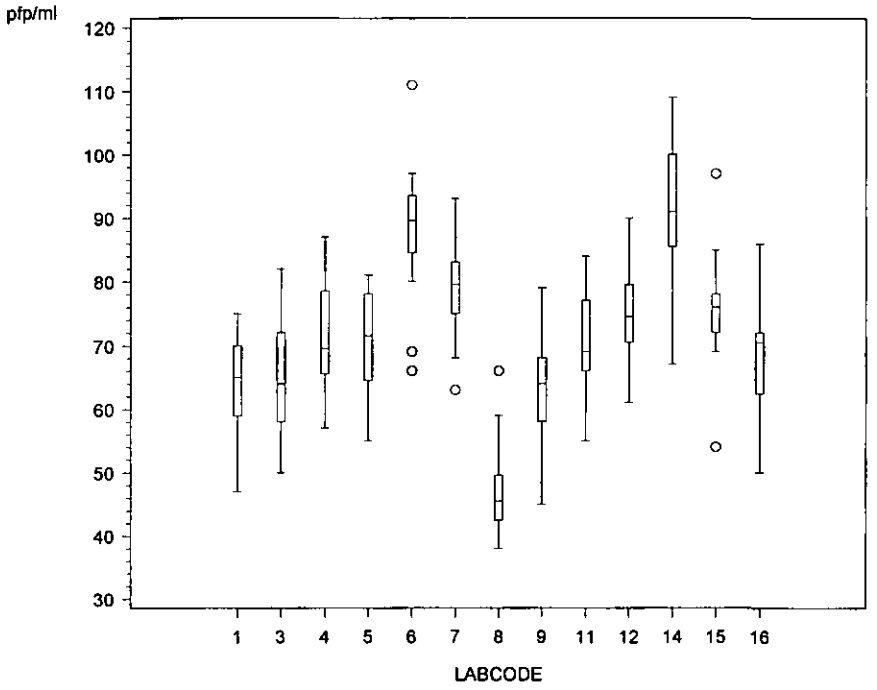

Figure 8 *Results of all participating laboratories for FRNAPH after exclusion of data.*

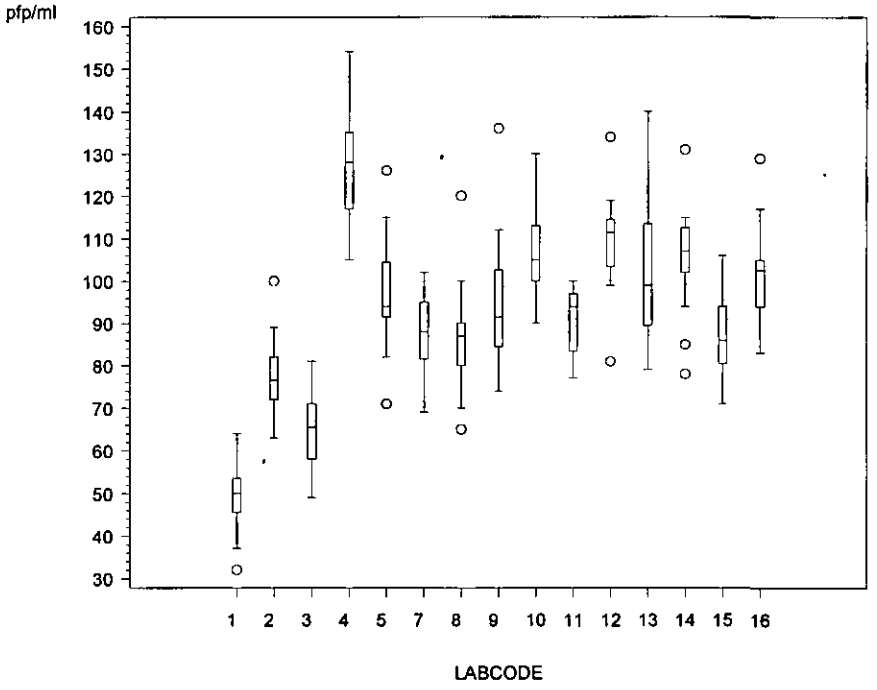

Figure 9 *Results of all participating laboratories for BFRPH after exclusion of data.*

Mean results and T_1 and T_2 values per phage method (after exclusion of data) are given in Tables 13-15. A one-sided test with a 95% upper critical value was performed to detect large variances within capsules (large T_1 value), indicating a poor repeatability of counts of duplicates. 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 counts of duplicates.

*Table 13 Arithmetic mean, standard deviation, T_1 and T_2 value per laboratory for **SOMCPH***

lab-code	I	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T_1	T_2	T_2/ (I-1)
1	9	64.0	6.00	4.68	4.83	0.60
3	10	70.5	8.22	4.93	13.4	1.49
4	10	60.2	7.54	10.9	7.61	0.85
5	10	79.4	6.92	6.82	4.90	0.54
7	10	70.0	7.10	8.51	4.95	0.55
8	10	78.2	8.32	9.16	7.39	0.82
9	10	75.2	7.04	4.77	7.57	0.84
11	10	77.3	7.14	8.44	4.12	0.46
12	10	64.3	8.67	9.15	13.9	1.54
13	10	64.2	7.25	8.11	7.50	0.83
14	10	86.2	8.50	6.40	9.61	1.07
16	10	76.5	10.4	15.8	10.0	1.12

I: Number of vials (used for calculations)

*Table 14 Arithmetic mean, standard deviation, T_1 and T_2 value per laboratory for **FRNAPH***

lab-code	I	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T_1	T_2	T_2/ (I-1)
1	9	63.7	7.78	5.48	10.9	1.36
3	10	65.0	8.86	14.0	8.71	0.97
4	10	72.2	8.64	10.2	9.35	1.04
5	10	70.8	7.83	6.09	10.3	1.15
6	10	88.2	9.77	10.3	10.7	1.19
7	10	78.9	7.50	6.62	7.02	0.78
8	8	47.2	7.27	10.0	5.97	0.85
9	9	63.8	9.01	15.3	6.54	0.82
11	10	70.6	7.88	6.11	10.8	1.20
12	10	74.4	7.44	6.20	7.93	0.88
14	10	92.0	10.2	6.52	15.0	1.67
15	10	75.6	7.87	10.8	4.83	0.54
16	10	67.9	8.20	10.5	8.70	0.97

I: Number of vials (used for calculations)

*Table 15 Arithmetic mean, standard deviation, T_1 and T_2 value per laboratory for **BFRPH***

lab-code	I	Arithmetic mean (pfp/ml)	Standard deviation s (pfp/ml)	T_1	T_2	$T_2/(I-1)$
1	10	49.4	7.73	11.9	11.5	1.27
2	10	77.0	8.48	3.39	14.3	1.59
3	9	64.6	9.79	7.84	17.5	2.19
4	10	126	12.9	14.0	11.0	1.22
5	10	96.8	11.9	11.4	16.3	1.81
7	10	88.2	8.37	9.50	5.79	0.64
8	10	86.0	11.7	12.3	17.3	1.92
9	10	94.2	14.6	19.4[a]	22.6	2.51
10	9	107	10.5	9.12	8.64	1.08
11	10	90.8	7.65	3.98	8.39	0.93
12	10	110	10.6	17.3	2.41	0.27
13	10	103	16.9	28.0[a]	23.8	2.65
14	10	106	11.2	9.88	13.1	1.46
15	10	87.2	10.5	12.7	11.2	1.24
16	10	101	10.7	10.6	10.6	1.18

I : Number of vials used for calculations.
a: Significantly different from a χ^2-distribution with 10 degrees of freedom, tested at the 99% confidence level for the lower limit (2.56) and at the 95% confidence level for the upper limit (18.31).

Laboratories 9 and 13 found for T_1 significantly higher values than the χ^2 -distribution (Table 15). For laboratory 9 this could not be attributed to one specific vial. For laboratory 13 only one vial had a significant T_1 value (vial number 5). However, 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 (T_1 and T_2), the means of all laboratories were compared (per procedure and after exclusion of data mentioned in Table 12). 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 16.

Table 16 Repeatability (r) and reproducibility (R) values per method.

	r	R
SOMCPH	1.35	1.52
FRNAPH	1.38	1.73
BFRPH	1.38	2.04

4. Discussion and conclusions

The phage reference materials have proven to be useful as standard samples for first-line quality control (control charts) and as standard samples for the collaborative study. After an initial decrease (after preparation) in the number of plaque forming particles per ml (pfp/ml), all three phage RMs stabilised well when stored at -70 °C. Even a short period (*ca* 2 weeks) of storage at -20/-26 °C did not effect the number of pfp/ml of all three phages.
Mailing of the materials was possible by packing the RMs in dry ice and shipping them by courier service. Materials mailed in this way arrived frozen in all participating laboratories.
The homogeneity of the materials fulfilled the pre-set criteria, so that the variation in results between vials of one batch was sufficiently small to make it possible to detect differences between laboratories.

The methods for the three phage enumerations were introduced during the first training session. The time span between this first training session (March 1997) and the first collaborative study (May 1997) was too short according to many participants. They had little time to prepare all necessary materials for the study. Furthermore, some products were hard to obtain from the manufacturers (not enough in stock, long delivery time), causing extra delay in the preparation of the media.

The accepted pH range of media is in many cases subject for discussion. For many media the pH range is arbitrarily chosen, not really funded on practical experience. In the (draft) ISO versions for the enumeration of SOMCPH and FRNAPH the pH ranges of the media is ± 0.2 pH unit. From the results of the collaborative study it can be concluded that this range may be unnecessarily strict. A range of ± 0.5 pH unit is more practical and still gave good results.
The pH range of the media for the enumeration of BFRPH was not yet set, as well as the measuring temperature, and needs to be further considered for improving this draft ISO version (ISO 10705-4).

Preparation of inoculum cultures of all three host strains (WG5 *Escherichia coli*, WG49 *Salmonella typhimurium* and HSP40 *Bacteroides fragilis*) did not cause problems in most of the participating laboratories. It was concluded that WG5 and WG49 should be incubated while gently shaking, however this can even be minimal (e.g. shaking by hand every 30 min). No limits are necessary for the rotations per minute. Most important is that the inoculum culture finally reaches the right cell density (ca 10^8 cfp/ml) within a limited period of time. The incubation times necessary to reach this cell density varied per laboratory and per host strain. For WG5 this varied between 90 and 195 min, for WG49 between 120 and 240 min and for HSP40 between 120 and 360 min. Thus it can be concluded that it is not possible to simply give an incubation time for the preparation of an inoculum culture. The procedure which is described in the draft ISO's for the preparation of inoculum cultures is: first: determine the relation between optical density and viable count (per host strain) and second: prepare an inoculum culture, by culturing the host strain up to an optical density which corresponds with a cell density of *ca* 10^8 cfp/ml. This procedure seems to be a bit complicated, but it is still the most reliable procedure to obtain an optimal inoculum culture.

The host strain for the enumeration of FRNAPH, WG49 *Salmonella typhimurium,* is not very robust. This is a well-known problem, but not easy to solve. This strain possesses a plasmid

from an *Escherichia coli*, which has the genetic information for the formation of the F-pili. As this plasmid is a "strange" plasmid for *Salmonella typhimurium*, it is not firmly present in the strain. It can be lost very suddenly, leading to the fact that no F-pili will be made anymore and thus no F-specific phages can be detected. More research is necessary to find procedures to optimise the conditions of the strain.

After the discussion with the participants on technical details it was concluded to exclude some data from further analysis. The main reason for excluding data of the results of SOMCPH and FRNAPH was because of (largely) deviating pH values of the media. This problem might be attributed to the short period of time between the training session and the collaborative study. The participants had too little time (and little experience) to be able to test all media extensively before use. Despite this problem, most of the remaining data were of high quality. For SOMCPH and for FRNAPH none of the T_1 values were significantly different from a χ^2 distribution, for BFRPH only two laboratories found a significant T_1 value. Thus, most of the laboratories did not have any problems with reproducing replicates. Furthermore, most of the T_2 values of all three phage methods were relatively small (in most cases $T_2 /(I-1) \leq 2$), indicating small variations between vials. The few (relatively) high values of T_2 were mainly caused by the significant high T_1 values.

After exclusion of the data as indicated in Table 12, the analysis of variance indicated differences between laboratories, but the Grubbs' test could not detect any outliers. Hence, it can be concluded that if the methods do not show technical deviations, relatively good results can be obtained with the three (present) phage methods for the detection of phages in the reference materials. This was also shown in the results of the repeatability (r) and of reproducibility (R). The repeatability of all three methods was 1.35 - 1.38. This value is very near the theoretical lowest possible value. At a mean count of *ca* 70-90 pfp/ml and in the case of a Poisson distribution, this theoretical value is *ca* 1.25 (Mooijman et al., 1992). The reproducibility (R) values are only slightly higher than the values of r (SOMCPH, R = 1.52; FRNAPH, R = 1.73 and BFRPH, R = 2.04). It can therefore be concluded that the participating laboratories produced reproducible results. The procedures described for the enumeration of the three types of phages are a good basis for further use in enumeration of phages in natural polluted samples.

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- Mrs. B.Moreno Montoya, Gobierno Vasco, Departemento de Sanidad, Lab. Microbiologia, Donostia-San Sebastian.

United Kingdom:
- Mr. A.Gawler, The Environment Agency, National Laboratory Service, Exeter Devon.
- Mr. S.Ward, Public Health Laboratory Service, Environmental Microbiology Research Unit, Nottingham.

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Abbreviations and symbols

ABBREVIATIONS

BFRPH	phages of *Bacteroides fragilis*
BPRMA	Bacteroides Phage Repair Medium Agar
BPRMB	Bacteroides Phage Repair Medium Broth
B40-8	Phage of *Bacteroides fragilis*
cfp	colony forming particle
DAL	Double Agar Layer method
FRNAPH	F-specific RNA bacteriophages (= no. of F^+-phages - no. of FDNAPH)
HSP40	*Bacteroides fragilis* (host for phages of *B.fragilis*)
MSA	Modified Scholtens' Agar
MSB	Modified Scholtens' Broth
MS2	F-specific RNA phage
pfp	plaque forming particle
ps	peptone saline solution
RM	Reference material
rpm	rotations per minute
SAL	Single Agar Layer method
SOMCPH	Somatic coliphages
ssBPRMA	semi-solid 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

α	Significance level
χ^2	Chi- square distribution
I	Number of vials
J	Number of replicates per vial
$\bar{R}$	moving range
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
$z_{i+} = \sum_j z_{ij}$	Sum of numbers of pfp in all replicates of vial I
$z_{++} = \sum_i (\sum_j z_{ij})$	Sum of numbers of pfp in all vials of one batch

Annex 1 Mailing list

1 EU/Measurements and Testing Programme, Dr. E.Maier
2 Directorate-General of RIVM
3 Director SVM, drs. G.J. Guijt MBA (SVM, Bilthoven)
4-5 Dr. J.Jofre, University of Barcelona, Spain
6 Mrs.dr. V.Pierzo, Institute Pasteur Lille, France
7 Depot Nederlandse Publicaties en Nederlandse Bibliografie
8 Director Sector II, Prof. Dr. Ir. D.Kromhout
9 Head Microbiological Laboratory for Health Protection, Dr. Ir. A.M.Henken
10 Dr. Ir. E.J.T.M. Leenen
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15-29 Participating laboratories
30 SBD/Voorlichting & Public Relations
31 Bureau Rapportenregistratie
32 Bibliotheek RIVM
33-45 Bureau Rapportenbeheer
46-60 Reserve

For information to:
61 Hoofdinspectie Milieuhygiene

Annex 2

Protocol for the first collaborative study

pages 33 - 48

NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT (RIVM)

MICROBIOLOGICAL LABORATORY OF HEALTH PROTECTION (MGB)
14 April 1997

Bacteriophages in Bathing waters

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

Protocol for the first collaborative study on bacteriophages in water of May 1997

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- Institute Pasteur of Lille (IPL), Water and Environment Department, 1, rue du Professeur Calmette, B.P. 245, 59019 LILLE CEDEX, France, Tel.: +33 3 20 87 7730, Fax: +33 3 20 87 7383.
- National Institute of Public Health and the Environment (RIVM), Microbiological Laboratory for Health Protection (MGB), P.O. Box 1, 3720 BA Bilthoven, The Netherlands, Tel.: 31 30 274 3537, Fax: 31 30 274 4434.

Organizer collaborative study:
RIVM, MGB
Contact persons:

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BACTERIOPHAGES IN BATHING WATERS
PROTOCOL TRIAL I, MAY 1997

Please read all instructions and the reporting form before starting the trial. Fill in the reporting form during the work and not afterwards.

1. INTRODUCTION

In January 1996 the work for this EC-project started and intends to lead:

- to the availability of standardized methods for the concentration, detection and enumeration of three groups of bacteriophages that can be used for the determination of the microbiological quality of bathing waters;
- to determine the operationality of these methods in diverse situations as encountered in the EU;
- to obtain preliminary data on the occurrence of bacteriophages as compared to bacterial indicator organisms, that can be the basis for the design of a future field work for the comparison of measurements between bacteriophages and viruses.

To reach these objectives the following steps were and will be done:

1. Standardization and optimization of the methods for detection and enumeration of:
   - Somatic coliphages in water (SOMCPH)
   - F-specific RNA phages in water (FRNAPH)
   - Phages of *Bacteroides fragilis* in water (BFRPH)

2. Selection, standardization and optimization of a concentration technic for the three types of bacteriophages in water.

3. Development of reference materials for the implementation of the methods.

4. Implementation of the methods in a number of laboratories of the EU to test the feasibility of the methods, by
   4.a First training session in Lille to introduce the phage methods and phages to the participants with mainly pure phage cultures;
   4.b First collaborative study to introduce the phage methods into the laboratories of the participants, with mainly pure phage cultures;
   4.c Second training session in Lille to introduce concentration technic(s) and use the phage methods on natural polluted samples to the participants;
   4.d Second collaborative study to introduce concentration technic(s) and the analysis on natural polluted samples into laboratories of the participants.

5. Testing the methods in the field conditions and measure the levels of the different bacteriophages in comparison to bacterial standards in various bathing waters representing, as far as possible, all possible bathing water typologies in the EU.

At this moment (April 1997) much has been done (and is still continued) on steps 1, 2 and 3 by the partners of the project. Step 4a (first training session) has just been finished, although the report will take somewhat more time. This protocol will describe the work for step 4b (first collaborative study).

Hopefully the Lille training session has made all participants enthousiastic about the phage methods, so that everybody could initiate the methods in their own laboratory.

2. OBJECTIVES

1. Testing the feasibiltity of mailing phage reference materials to different EU laboratories;
2. Implementation of the following methods in EU laboratories and testing their feasibility by using phage reference materials;
   * Somatic coliphages in water (SOMCPH);
   * F-specific RNA phages in water (FRNAPH);
   * Phages of *Bacteroides fragilis* in water (BFRPH).
3. Identification of reasons for deviating results in individual laboratories.

3. PRIOR WORK FOR EACH PARTICIPANT

For performing this collaborative study the following work prior to the study needs to be carried out in each laboratory:

- Checking the list of materials necessary to perform bacteriophage enumerations in water. This list was given to you at the training session in Lille. If necessary order the necessary materials.
- Check whether you have received (in Lille) the following documents:
  * Amended ISO 10705-1, February 1997 (FRNAPH-method);
  * Amended ISO/CD10705-2, February 1997 (SOMCPH-mehod);
  * Method for detection of bacteriophages of *Bacteroides fragilis*, February 1997.

  If you miss one of these documents please contact Kirsten Mooijman (address and numbers on the first page of this protocol).
- Culture the following host strains you received in Lille:
  * WG5 *Escherichia coli* (host SOMCPH)
  * WG49 *Salmonella typhimurium* (host FRNAPH)
  * HSP40 *Bacteroides fragilis* (host BFRPH)

prepare stock cultures and working cultures for each host (see the methods applied);
- Perform 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 you have prepared (WG5 *Escherichia coli* for SOMCPH, WG49 *Salmonella typhimurium* for FRNAPH and HSP40 *Bacteroides fragilis* for BFRPH). When you have obtained reproducible results, then use the relation between optical density and viable count (10^8 cfp/ml) to prepare the inoculum cultures of each strain.
- If possible also perform quality control of host strain WG49 *Salmonella typhimurium*. For this purpose a high titre suspension of phage MS2 and of host strain *Escherichia coli* K12 (NCTC 12486 or ATCC 23631) have been sent to you earlier.

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;

a. A small box containing 10 vials of reference materials containing phage ØX174;
b. A small box containing 11 vials of reference materials containing phage MS2;
c. A small box containing 10 vials of reference materials containing phage B40-8.

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

At a fixed date, the vials are thawn at room temperature and analysed (using the DAL procedure) for:

a. Number of somatic coliphages (SOMCPH), according to amended ISO/CD 10705-2 (February 1997);
b. Number of F-specific RNA-phages (FRNAPH), according to amended ISO 10705-1 (February 1997), of which 10 vials without the addition of RNase in duplicate, 1 vial with and without the addition of RNase.
c. Number of phages of *Bacteroides fragilis* (BFRPH), according to the method for detection of bacteriophages of *B.fragilis* (February 1997).

All number of plaques are counted. Data will be reported to the organizing laboratory on an enclosed reporting form for (statistical) analysis. The results will be reported to the participants. If individual laboratories have largely deviating results they can try to find a solution for these differences before the meeting in Brussels. The results will be discussed afterwards in a plenary meeting.

5. CHRONOLOGICAL DESCRIPTION OF THE TRIAL

Date (1997)

21 April	Mailing, by MGB/RIVM, of the final protocol, reporting form, SOP's, a sheet of random labels and a label with the address of the RIVM to the participating laboratories.
28 April	Mailing, by MGB/RIVM, of the reference materials 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 (see 4.). In case the parcel is badly damaged please contact Kirsten Mooijman. **Store the reference materials immediately at (-70 ± 10) °C.** Acknowledge the receipt of the parcel by sending a telefax or an e-mail to Kirsten Mooijman
29 April - 13 May	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.
5 - 12 May	Prepare glassware and media and working cultures of host strains
13 May	First day of the trial: - Preparation of inoculum cultures (WG5, WG49 and HSP40) - Checking viable counts of the inoculum cultures - Enumeration of phages (SOMCPH, FRNAPH and BFRPH)
14 May	Second day of the trial: - Reading of the plates of the viable counts (WG5 and WG49) - Reading of the plates of the phage enumerations
15 May	Last day of the trial: - Reading of the plates of the viable counts (HSP40)
15 - 23 May	Participants mail results (only the data) to MGB/RIVM by telefax (+31 30 274 4434) and original reporting form by mail
June/ July	Statistical analyses of the results at the RIVM

July/ August	The first report, which contains a table of raw data as they are fed into the computer and a table with data classified according to the factors of the experimental design (each laboratory will receive its own results only) will be sent to the participating laboratories, to check for completeness and correctness.
July/ August/ September	The second (draft) report, which contains the results of the statistical analysis (of all participating laboratories) will be sent to the participants. Individual laboratories which have largely deviating results will try to find a solution for these differences (e.g. by extra tests).
2 - 3 October	Discussion of the results with the participants in Brussels.
February '98	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%.

29 April - 12 May 1997

- Adjust an incubator to (37 ± 1) °C (according to SOP BCR-water 1003 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 13 May. 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:
- 50 ml Modified Scholtens' Broth (MSB: A1)
- 25 Petri dishes (of 9 cm), containing Modified Scholtens' Agar (MSA; A2)
- 2 bottles containing each 50 ml of semi-solid Modified Scholtens' Agar (ssMSA; A3)
- 10 ml calcium chloride solution
- 100 ml peptone saline solution (ps)
- 250 ml (in a bottle) of MSA or another appropriate nutrient agar

Glassware (/plastic):
- 1 sterile conical flask of 250-300 ml capacity with side-arm, or a plain sterile conical flask and cuvettes.
- Sterile pipettes of 1 ml nominal capacity
- 8 sterile (empty) Petri dishes (of 9 cm)
- Sterile glass tubes, with caps, of nominal capacity of *ca* 10 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 FRNAPH (amended ISO 10705-1 of February 1997):

Media and reagents:
- 50 ml Trypton-Yeast extract-Glucose Broth (TYGB; A1)
- 25 Petri dishes (of 9 cm) containing Trypton-Yeast extract-Glucose Agar (TYGA; A2)
- 2 Bottles containing each 50 ml of semi-solid Trypton-Yeast extract Glucose Agar (SS TYGA; A3)
- 15 ml calcium-glucose solution
- 100 ml peptone saline solution (ps; A8)
- 250 ml (in a bottle) of TYGA
- 100 µl RNase

Glassware (/plastic):
- 1 sterile conical flask of 250-300 ml capacity with side-arm, or a plain sterile conical flask and cuvettes.
- Sterile pipettes of 1 ml nominal capacity
- Sterile pipettes of 0.1 ml nominal capacity
- 8 sterile (empty) Petri dishes (of 9 cm)
- Sterile glass tubes, with caps, of nominal capacity of *ca* 10 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 (Detection of bacteriophages of *Bacteroides fragilis* of February 1997):

Media and reagents:
- 150 ml of *Bacteroides fragilis* repair medium broth[1], with NaI and Km (BPRMB)
- 25 Petri dishes (of 9 cm) containing BPRMA (with NaI and Km)
- ca 100 ml of ssBPRMA (with NaI and Km)
- 100 ml peptone saline solution (ps)
- 250 ml (in a bottle) of BPRMA (with NaI and Km)
- Material for Gram staining

Glassware (/plastic):
- 5 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
- 8 sterile (empty) Petri dishes (of 9 cm)
- sterile glass tubes with caps of nominal capacity of *ca* 10 ml.

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
- Sterile swabs
- Whirlmixer

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* (on BPRMA, at least 1 plate (well grown), see detection of bacteriophages of *Bacteroides fragilis*).
- Information per host strain about which absorbance of the inoculum culture corresponds to ca. 10^8 cfp/ml of the host strain.

[1] If BPRM-broth is stored at (5 ± 3) °C, it may become "turbid". This will largely disappear after storage of the sterile broth at 37 °C for *ca* one night.

12 May 1997

Prewarm *ca* 100 ml BPRMB to *ca* 37°C. Add to 3 screw-caped tubes with a nominal capacity of 30 ml (or another suitable volume), 30 ml (prewarmed) BPRMB. Inoculate each tube with cell material of host-strain HSP40 of ¼ of a well grown agar plate (use a swab). Incubate overnight at (36 ± 2) °C.

NB: Only one tube is sufficient for preparing an inoculum culture on 13 May. However strain HSP40 does not always grow equally. The chance of obtaining at least one well grown tube is increased by using 3 tubes here.

- Label 20 Petri dishes (of 9 cm) with MSA as follows: MSA 1.1; MSA 1.2; MSA 2.1; MSA 2.2 etc., up to and including MSA 10.2
- 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 20 Petri dishes (of 9 cm) with TYGA as follows: TYGA 1.1; TYGA 1.2; TYGA 2.1; TYGA 2.2 etc. up to and including TYGA 10.2
- Label one dish with TYGA; TYGA 11 - RNase
- Label one dish with TYGA: TYGA 11 + 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 20 Petri dishes (of 9 cm) with BPRMA as follows: BPRMA 1.1; BPRMA 1.2; BPRMA 2.1, BPRMA 2.2 up to and including BPRMA 10.2
- Label one dish with BPRMA: blank ssBPRMA
- Label one dish with BPRMA: blank HSP40
- Label 8 sterile empty Petri dishes 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; BPRMA blank and BPRMA-ps blank

Or use any other labelling which can make distinction between the different plates. Leave all plates at room temperature for one night.

13 May 1997 (first day of the trial)

References to the methods are:
For SOMCPH: amended ISO/CD 10705-2, Feburary 1997
For FRNAPH: amended ISO 10705-1, February 1997
For BFRPH: detection of bacteriophages of *Bacteroides fragilis*, February 1997

Inoculum cultures

Prepare inoculum cultures for the following host strains:
- WG5 *Escherichia coli* (SOMCPH; 11.1);
- WG49 *Salmonella typhimurium* (FRNAPH; 11.1)
- HSP40 *Bacteroides fragilis* (BFRPH)

N.b.: Before inoculating the inoculum culture of HSP40, check the (well) grown overnight (working) culture of HSP40 by performing Gram staining. The microscopic result should show Gram negative (red) rods.

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 of WG5 and WG49 by placing them in melting ice. The inoculum culture of HSP40 should not be cooled in melting ice, but placed on the laboratory bench as soon as the right cell density (of *ca* 10^8 cfp/ml) is reached. Start the phage enumeration for BFRPH directly after taking the inoculum culture of HSP40 from the incubator.
Enumerate of each inoculum culture the number of cfp/ml directly after placing the culture in melting ice (or on the laboratory bench), 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 BPRMA 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. Each dilution in duplicate. Use the labeled Petri dishes. Prepare blanks in the following way: add 1 ml of sterile ps to molten agar and pour into the sterile empty 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 ± 2) °C for (20 ± 4) hours. Incubate the plates of HSP40 anaerobic at (36 ± 2) °C for (36 ± 2) hours.

Enumeration of phages

The order in which the phage enumerations for the three different phage types are performed is not prescribed. It might be possible that it would be more suitable to start phage enumerations of BFRPH (because of the need of quickly using the inoculum culture).

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 10 vials of reference materials containing ØX174 from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Melt 2 bottles of (each) 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 1.1, 1.2, 2.1 etc., up to and including 10.2 and blank ssMSA and blank WG5.
- Mix vial 1 (randomly chosen, the vials are not numbered) containing ØX174 on a whirlmixer or by turning the closed vials 5 times.
- Take 1 ml from vial 1 and add this to tube 1.1.
- Take another 1 ml from vial 1 and add this to tube 1.2
- Repeat for the other 9 vials.
- 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. If necessary add the inoculum culture in two steps: e.g. starting with 10 tubes, mixing and adding to a plate of MSA. Next the remaining tubes. Note the exact procedure.
- Do not stack more than 4 plates.

<u>FRNAPH, 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 the 11 vials of reference materials containing MS2 from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Melt 2 bottles of (each) 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 aliquots into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.
- If necessary label the tubes 1.1, 1.2; 2.1 etc., up to and including 10.2 and blank ssTYGA and blank WG49 and 11 and 11 RNase.
- Add 100 µl RNase solution to the ssTYGA in tube "11 RNase".
- Mix vial 1 (randomly chosen, the vials are not numbered) containing MS2 on a whirlmixer or by turning the closed vials 5 times.
- Take 1 ml from vial 1 and add this to tube 1.1.

- Take another 1 ml from vial 1 and add this to tube 1.2
- Repeat for the other 10 vials (where the last tube is labeled 11 RNase).
- 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 ± 2) °C for (18 ± 2) hours.

Mind:

- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes. If necessary add the inoculum culture in two steps: e.g. starting with 10 tubes, mixing and adding to a plate of TYGA. Next the remaining tubes. Note the exact procedure.
- Do not stack more than 4 plates.

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

- Take the 10 vials of reference materials containing B40-8 from the -70 °C freezer and place them at room temperature. Thawing of the vials will take *ca* 30 minutes.
- Melt the ssBPRMA in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Make sure that all additives are present in the medium (Hemin, Na_2CO_3, NaI and Km) and that the pH of the medium is correct (pH=7). Distribute 2.5 ml aliquots into culture tubes with caps, placed in a waterbath at (45 ± 1) °C.
- If necessary label the tubes 1.1, 1.2, 2.1 etc., up to and including 10.2 and blank ssBPRMA and blank HSP40.
- Mix vial 1 (randomly chosen, the vials are not numbered) containing B40-8 on a whirlmixer or by turning the closed vials 5 times.
- Take 1 ml from vial 1 and add this to tube 1.1.
- Take another 1 ml from vial 1 and add this to tube 1.2
- Repeat for the other 9 vials.
- Add to each tube, except the tube labeled blank ssBPRMA, 0,5 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 BPRMA 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 ± 2) °C for (18 ± 2) hours.

Mind:

- Make sure that inoculated tubes remain in the waterbath for not more than 10 minutes. If necessary add the inoculum culture in two steps: e.g. starting with 10 tubes, mixing and adding to a plate of BPRMA. Next the remaining tubes. Note the exact procedure.
- Do not stack more than 4 plates.

14 May 1997

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

Relabelling and counting

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 vial 11 of FRNAPH) on a laboratory bench in 10 rows of 6 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.

sample	method		
	SOMCPH	FRNAPH	BFRPH
1	O O	O O	O O
2	O O	O O	O O
3	O O	O O	O O
4	O O	O O	O O
5	O O	O O	O O
6	O O	O O	O O
7	O O	O O	O O
8	O O	O O	O O
9	O O	O O	O O
10	O O	O O	O O

O = 1 petri dish

Each set of instructions contains a sheet of self-adhesive labels in the same lay-out as the table. 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!

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 behind the random number corresponding to that on the dish*. The second worker should also examine the blanks and the results of vial 11 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.

15 May 1997

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 mailed by telefax between 15 and 23 May 1997 to MGB/RIVM (Kirsten Mooijman/Mahdieh Bahar). 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)
BPRMA	*Bacteroides fragilis* phages repair medium agar (BFRPH-method)
BPRMB	*Bacteroides fragilis* phages repair medium broth (BFRPH-method)
cfp	colony forming particle
DAL	Double Agar Layer method
FRNAPH	F-specific RNA bacteriophages (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)
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 10705-1,10.3 and BFRPH-method)
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
SOP	Standard Operating Procedure
ssBPRMA	semi-solid BPRM agar (BFRPH-method)
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 3

Reporting form of the first collaborative study

pages 49 - 70

REPORTING FORM

BACTERIOPHAGES IN BATHING WATER TRIAL1

FILL IN COMPLETELY (please in English)

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

Laboratory name: .....

Contact person : .....

Date of arrival of the parcel with reference materials: ..... - ..... - 1997

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

Please confirm the number of vials of each batch of reference materials:

ΦX174 (batch 040696): ..... vials

MS2 (batch 071293): ..... vials

B40-8 (batch 260397): ..... vials

Date of the trial: ..... - ..... - 1997

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 of officially calibrated thermometers did you use?
   - range: .....
   - scale division: .....
   - maximum error (systematic & random): .....
   - certification date of calibration: .....
   - number of certification report: .....

4. Did you also use not officially calibrated thermometers?
   ☐ yes
   ☐ no

   If yes, how did you perform the calibration of these thermometers?
   ☐ According to SOP BCR-water/003 of 930514
   ☐ Other, please specify .....
   .....

   For which purpose(s) did you use these thermometers during the trial?
   .....

SOMATIC COLIPHAGES (SOMCPH)

Materials SOMCPH

5. 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)? 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			/	°C	/	°C
ssMSA			/	°C	/	°C
$CaCl_2$-solution						
NaI (if applicable)						

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

7. What kind and size of Petri dishes did you use for the MSA medium?
☐ Glass ☐ Plastic
☐ Vented ☐ Non-vented
size:.....mm

8. 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

9. What kind of pipettes did you use?
   - ☐ Disposable (plastic)
   - ☐ Glass
   - ☐ Automatic with "tips"; tips ☐ with filter or ☐ without filter

   In case different kind of pipettes were used, please specify the experiment for which each kind of pipette was used:

   .....

Inoculum culture(s) SOMCPH

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

11. 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.

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

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

14. 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: .....
.....

Please include with this reporting form, the calibration curve for the used batch of working culture.

15. 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
At what time did you start the phage enumeration (for SOMCPH)?
..... h .....min
At what time did you finish the phage enumeration (for SOMCPH)?
..... h .....min

16. Which method did you use for viable counts of WG5?
☐ Pour plate
☐ Membrane filtration
☐ Spread plate
☐ Other, namely .....

17. Which medium did you use for viable counts of WG5?
- ☐ MSA
- ☐ Other, namely .....

Phage enumeration SOMCPH

18. 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 .....

19. 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)

20. At what time did you place the vials of reference materials at room temperature (also give the temperature)? At what time did you start with (and when did you finish) the addition of RM to ssMSA?
Time RM's at room temperature: ..... h ..... min
Room temperature at that moment: ..... °C
Time start addition of first RM to ssMSA: ..... h ..... min
Time addition of last RM to ssMSA: ..... h ..... min

21. How did you mix the vials of RM's?
- ☐ On a whirlmixer
- ☐ By hand (shaking 5 times)
- ☐ Other, namely .....

22. At what time did you add the inoculum culture to the first tube and when to the last tube?
Start time addition inoculum culture to first tube: ..... h ..... min
Finish time addition inoculum culture to last tube: ..... h ..... min

23. How did you perform the procedure?
☐ Add RM to each tube, next add inoculum culture to each tube and next pour into Petri dishes on a layer of MSA.
☐ Add RM to each tube, next add inoculum culture to tubes 1-10, pour tubes 1-10 into Petri dishes on a layer of MSA and continue for the other tubes.
☐ Other, namely.....
.....

24. How did you prevent possible contamination during working?
☐ Work quickly on the laboratory bench
☐ Work near the flame
☐ Work in a Laminar air flow cabinet
☐ Other, namely .....

25. 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
Temperature 13 May :	start time: ..... h ..... min temperature: ..... °C
Temperature 14 May:	finish time: ..... h ..... min temperature: ..... °C

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

26. 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,.....

F-SPECIFIC RNA-PHAGES (FRNAPH)

Materials FRNAPH

27. 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)? 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			/	°C	/	°C
ssTYGA			/	°C	/	°C
Ca-glucose						
RNase-sol.						
Nalidixic ac. (if applicable)						

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

29. What kind and size of Petri dishes did you use for the TYGA medium?
☐ Glass ☐ Plastic
☐ Vented ☐ Non-vented
size:.....mm

30. 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

31. What kind of pipettes did you use?

☐ Disposable (plastic)

☐ Glass

☐ Automatic with "tips"; tips ☐ with filter or ☐ without filter

In case different kind of pipettes were used, please specify the experiment for which each kind of pipette was used:

.....

Inoculum culture(s) FRNAPH

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

☐ Incubator with rotating platform

☐ Waterbath with rotating platform

☐ Other, namely.....

33. 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.

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

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

36. 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: .....
.....
Please include with this reporting form, the calibration curve for the used batch of working culture.

37. 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
At what time did you start the phage enumeration (for FRNAPH)?
..... h .....min
At what time did you finish the phage enumeration (for FRNAPH)?
..... h .....min

38. Which method did you use for viable counts of WG49?
- ☐ Pour plate
- ☐ Membrane filtration
- ☐ Spread plate
- ☐ Other, namely .....

39. Which medium did you use for viable counts of WG49?
- ☐ TYGA
- ☐ Other, namely .....

Phage enumeration FRNAPH

40. 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 .....

41. 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)

42. At what time did you place the vials of reference materials at room temperature (also give the temperature)? At what time did you start with (and when did you finish) the addition of RM to ssTYGA?
Time RM's at room temperature: ..... h ..... min
Room temperature at that moment: ..... °C
Time start addition of first RM to ssTYGA: ..... h ..... min
Time addition of last RM to ssTYGA: ..... h ..... min

43. How did you mix the vials of RM's?
- ☐ On a whirlmixer
- ☐ By hand (shaking 5 times)
- ☐ Other, namely .....

44. At what time did you add the inoculum culture to the first tube and when to the last tube?
Start time addition inoculum culture to first tube: ..... h ..... min
Finish time addition inoculum culture to last tube: ..... h ..... min

45. How did you perform the procedure?
☐ Add RM to each tube, next add inoculum culture to each tube and next pour into Petri dishes on a layer of TYGA.
☐ Add RM to each tube, next add inoculum culture to tubes 1-10, pour tubes 1-10 into Petri dishes on a layer of TYGA and continue for the other tubes.
☐ Other, namely .....
.....

46. How did you prevent possible contamination during working?
☐ Work quickly on the laboratory bench
☐ Work near the flame
☐ Work in a Laminar air flow cabinet
☐ Other, namely .....

47. 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
Temperature 13 May :	start time: ..... h ..... min
	temperature: ..... °C
Temperature 14 May:	finish time: ..... h ..... min
	temperature: ..... °C

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

48. 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,.....

PHAGES OF *BACTEROIDES FRAGILIS* (BFRPH)

Materials BFRPH

49. 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)? 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
BPRMB			/	°C	/	°C
BPRMA			/	°C	/	°C
ssBPRMA			/	°C	/	°C
Kanamycin						
Nalidixic ac.						

If additives to the BPRM medium are not added on the date of preparation, please give appropriate information in the next table.

Medium	Date and time of adding additives	storage temperature/°C	pH /temperature (°C) day addition		day trial	
BPRMB			/	°C	/	°C
BPRMA			/	°C	/	°C
ssBPRMA			/	°C	/	°C

50. Did you add Nalidixic acid solution and Kanamycin solution to ssBPRMA?
- ☐ yes
- ☐ no

51. What kind and size of Petri dishes did you use for the BPRMA?
- ☐ Glass ☐ Plastic
- ☐ Vented ☐ Non-vented

size:.....mm

52. Did you dry the dishes with BPRMA 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

53. What kind of pipettes did you use?

☐ Disposable (plastic)

☐ Glass

☐ Automatic with "tips"; tips ☐ with filter or ☐ without filter

In case different kind of pipettes were used, please specify the experiment for which each kind of pipette was used:

.....

<u>Inoculum culture(s) BFRPH</u>

54. What kind of tubes did you use for culturing the (overnight) working culture and for culturing the inoculum culture of HSP40 *Bacteroides fragilis*?

☐ glass ☐ plastic

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

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

55. How many tubes did you inoculate for preparing the (overnight) working culture of HSP40 (inoculated on 12 May)? How many were well grown?

No. of tubes inoculated: .....

No. of tubes well grown: .....

56. What was the temperature during incubation of the (overnight) working culture of HSP40?

Start incubation:	time: .....h..... min
(12 May)	temperature:..... °C
End incubation:	time: .....h..... min
(13 May)	temperature:..... °C

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

57. What was the result of the Gram staining of the (overnight) working culture?
.....

58. How many tubes did you inoculate for preparing the inoculum culture of HSP40? How many were well grown?
No. of tubes inoculated: .....
No. of tubes well grown: .....

59. 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.

60. How did you measure absorbance?
- ☐ In a screw-caped tube
- ☐ In a cuvet
- ☐ Other, namely.....

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

..... nm

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

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

absorbance: .....

t = the time just before placing the inoculum culture on the laboratory bench

time: ..... h ..... min

absorbance: .....

Other times: .....

Please include with this reporting form, the calibration curve for the used batch of working culture.

63. Give information about times:

At what time did you place the inoculum culture on the laboratory bench?

..... h .....min

What was the room temperature at that moment? ..... °C

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

..... h .....min

At what time did you start the phage enumeration (for BFRPH)?

..... h .....min

At what time did you finish the phage enumeration (for BFRPH)?

..... h .....min

64. Which method did you use for viable counts of HSP40?

- ☐ Pour plate
- ☐ Membrane filtration
- ☐ Spread plate
- ☐ Other, namely .....

65. Which medium did you use for viable counts of HSP40?

- ☐ BPRMA
- ☐ Other, namely .....

66. 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?

.....

Phage enumeration BFRPH

67. How did you melt your ssBPRMA 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 ssBPRMA was placed? ..... °C
How long did you keep your molten ssBPRMA in the waterbath/incubator between melting ssBPRMA and start of use?
..... (give time in min or hours)

69. At what time did you place the vials of reference materials at room temperature (also give the temperature)? At what time did you start with (and when did you finish) the addition of RM to ssBPRMA?
Time RM's at room temperature: ..... h ..... min
Room temperature at that moment: ..... °C
Time start addition of first RM to ssBPRMA: ..... h ..... min
Time addition of last RM to ssBPRMA: ..... h ..... min

70. How did you mix the vials of RM's?
☐ On a whirlmixer
☐ By hand (shaking 5 times)
☐ Other, namely .....

71. At what time did you add the inoculum culture to the first tube and when to the last tube?
Start time addition inoculum culture to first tube: ..... h ..... min
Finish time addition inoculum culture to last tube: ..... h ..... min

72. How did you perform the procedure?
☐ Add RM to each tube, next add inoculum culture to each tube and next pour into Petri dishes on a layer of BPRMA.
☐ Add RM to each tube, next add inoculum culture to tubes 1-10, pour tubes 1-10 into Petri dishes on a layer of BPRMA and continue for the other tubes.
☐ Other, namely .....

73. How did you prevent possible contamination during working?
- ☐ Work quickly on the laboratory bench
- ☐ Work near the flame
- ☐ Work in a Laminar air flow cabinet
- ☐ Other, namely .....

74. 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?

.....

75. 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.

Temperature 13 May :	start time: ..... h ..... min
	temperature: ..... °C
Temperature 14 May:	finish time: ..... h ..... min
	temperature: ..... °C

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

76. 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,.....

DATA

VIABLE COUNTS

Note the number of colonies counted per plate:

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

MSA blank: ..... MSA-ps blank: .....
TYGA blank: ..... TYGA-ps blank: .....
BPRMA blank:..... BPRMA-ps blank:.....

Remarks: .....

CONTROLS

SOMCPH: blank ssMSA:.....

blank WG5:.....

FRNAPH: blank ssTYGA:.....

blank WG49:.....

BFPRH: blank ssBPRMA:.....

blank HSP40:.....

Remarks: .....

PHAGE COUNTS

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	
49		50		51		52	
53		54		55		56	
57		58		59		60	

Remarks: .....

.....

.....

.....

.....

Name of laboratory worker doing the phage enumeration:

.....

Date: . . . - . . . - 1997 Signature: .....

Name of laboratory worker doing the counting:

.....

Date: . . . - . . . - 1997 Signature: .....

Name of director:

.....

Date: . . . - . . . - 1997 Signature: .....

Remarks: .....

.....

.....

Fax the data to Kirsten Mooijman: +31 30 274 4434;
Mail the completed form to Kirsten Mooijman, RIVM (use the enclosed label).

Annex 4

SOP BCR-water/003: Temperature control

pages 71 - 73

FINAL version,
SOP BCR-water/003
930514

TEMPERATURE CONTROL OF INCUBATORS FOR WATER MICROBIOLOGY.

1. INTRODUCTION

Incubation temperature is often an important differentiating characteristic in water microbiology. For example, the discrimination of total and thermotolerant coliform bacteria relies entirely on the ability of the latter group to grow and ferment lactose at 44 °C. It is therefore important that unambiguous procedures are followed in the maintenance of incubator temperatures at the desired level. This is especially so if round-robin tests are organized, in which different laboratories analyse the same materials. This document gives a general guidance, which is by no means intended as a complete set of instructions, but rather as a minimum to be followed in international (certification) trials of reference materials for water microbiology.

2. SCOPE AND FIELD OF APPLICATION

This Standard Operating Procedure (SOP) describes a general method for measuring and recording the temperature in incubators, used for work with reference materials for water microbiology, as supplied by the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

3. PRINCIPLES

The temperature in an incubator is checked at least twice daily using calibrated thermometers immersed in glycerol.

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

4. APPARATUS AND GLASSWARE

4.1 Apparatus

4.1.1 Incubators, thermostatically controlled with specifications according to the work to be done.

4.1.2 Calibrated mercury thermometer, traceable to primary standards, range 0-60 °C or another appropriate range, scale division 0.1 °C.
In addition to thermometers which have been calibrated by a certified organization, in-house calibrated thermometers can also be used. The calibration procedure should by preference be carried out by qualified personnel using a reliable procedure. The procedure described under 5 can be used as a guidance.

4.2 Glassware

4.2.1 Glass serum bottle of 30 ml nominal capacity, with screwcap with rubber septum. In this septum a hole should be punched which exactly accomodates the stem of the thermometer (4.1.2). Any similar combination of bottle and cap will be satisfactory.

5. PROCEDURE

Place a bottle (4.2.1), filled with ca 25 ml of glycerol and fitted with a thermometer, on the top shelf of the incubator. Place a similar bottle on the lower shelf. Make sure that the bulb of the thermometer is well immersed in the glycerol. For incubators with an air-volume of less than 100 l, and equipped with a mechanical ventilation system, one bottle in the centre will be satisfactory. Read the temperatures at least twice daily; in the morning when the incubator has been closed all night, and in the afternoon just before the end of the day's work. Record the readings in a log-book. Adjust the thermostatical control when necessary. Note adjustment in a (log)book.

A procedure to calibrate a thermometer to an officially calibrated thermometer is described below.

- Use a calibrated and a not-calibrated thermometer with similar ranges and scale divisions.
- Adjust a waterbath, in which clear water is circulated at approximately 30 °C. Control the temperature of the water with the calibrated thermometer. Start the procedure only if the water is at a constant temperature.
- Take the end of the two thermometers, one in each hand, and immerse both thermometers completely in the water. Keep the thermometers close together, without touching one another.
- Read the temperature when they are constant. If necessary stop the circulation of the water for a short period (eventually, ask a second person) to read the thermometers.
- Record the eventual deviation of the not-calibrated thermometer.
- Repeat the procedure in total 3 times at 30 °C. The noted deviations should correspond in all cases. If not, repeat the procedure until in total 3 similar deviations are noticed.
- Repeat the same procedure at approximately 37 °C and at 44 °C.
- Record all deviations in a (log)book.
- Use appropriate corrections, if necessary, when using the thermometer.

6. TEST REPORT

The test report shall specify the temperature readings done in the period that a particular series of reference materials was examined, Any further observations relating to the accuracy of the temperature control in the incubators should also be recorded.

7. REFERENCES

Speck ML (ed.). Compendium of methods for the microbiological examination of foods. 1976 American Public Health Association, Washington DC. pp. 11-12.

Annex 5

SOP BCR-water/004: pH measurement

pages 74 - 80

FINAL version,
SOP BCR-water/004
930514

PH MEASUREMENT OF BACTERIOLOGICAL CULTURE MEDIA.

1. INTRODUCTION

The pH of culture media may have an important effect on the growth of microorganisms. Most microorganisms have an optimum pH around neutrality (7.0). However, some microorganisms have an optimum pH below or above this level, so that changing the pH of the culture medium may also change the selectivity of the medium. Although the pH of the culture medium is a very important factor there is not yet an internationally standardized procedure available for its measurement. Some manufacturers of commercial dehydrated media prescribe pH measurement of the media at 50 °C, others require 25 °C. Problems arise particularly when the pH of solid culture media has to be measured. This document gives one possibility of measuring the pH of liquid and solid culture media and is an arbitrary choice from the many available options. It should be used particularly in trials with reference materials for water microbiology. It is of great importance that each participating laboratory uses the same method, so that differences between laboratories in the measured pH of the culture media can be attributed to differences in preparation of media etc. and not to differences related to the way of measuring the pH.

2. SCOPE AND FIELD OF APPLICATION

This Standard Operating Procedure (SOP) describes a general method for measuring the pH of liquid and solid culture media, especially the media used for the enumeration of bacteria in reference materials for water microbiology, as supplied by the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

3. REFERENCES

Anonymous. Determination of the pH value. ISO/TC 147/SC 2/WG31. Draft version, February 1988. Geneva; International Organization for Standardization. Document no. N113.

Anonymous. Water, bepaling van de pH. Nederlandse Norm NEN 6411. Eerste druk, november 1981. Nederlands Normalisatie Instituut, Delft, The Netherlands (in Dutch).

Linnet, N. (ed); pH measurements in theory and practice. Radiometer A/S, Copenhagen 1970, first edition.

4. DEFINITIONS

The pH is the negative logarithm of the hydronium ion activity (dimension mol/l).

5. PRINCIPLE

The pH measurement of culture media involves the following stages:

- adjustment of the pH-meter;
- for liquid culture media: pH measurement in (a part of) the medium in a test tube;
- for solid culture media: first: pulverizing the solidified medium and transferring to a test tube;
  second: addition of distilled water and measurement of the pH.

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

6. APPARATUS AND GLASSWARE

6.1 Apparatus

6.1.1 A pH-meter with slope factor adjustment and temperature compensation device allowing a read out.
6.1.2 A glass electrode combined with a reference electrode filled with an appropriate electrolyte solution as supplied by the manufacturer.
6.1.3 Calibrated mercury thermometer, traceable to primary standards, range 0-60 °C or another appropriate range, scale division 0.1 °C.
6.1.4 Drying oven for temperatures of 110-130 °C.
6.1.5 Spatula.
6.1.6 Magnetic stirring device.
6.1.7 Teflon magnetic stirring bars of ca 2 cm length.

6.2 Glassware

6.2.1 Erlenmeyer flask of 250 ml nominal capacity.
6.2.2 Volumetric flasks of 1000 ml nominal capacity
6.2.3 Test tubes 17-18 mm x 150 mm.
6.2.4 Beakers of 50 ml nominal capacity.

7. CHEMICALS

Only freshly (glass) distilled water (pH ca 7) should be used.
The reagents should be of analytical grade.
Use by preference commercial buffer solutions for the pH measurements.
Use always fresh solutions, taken directly from the stock, in case the buffer has been supplied as a solution; freshly dissolved, in case the buffer has been supplied as a mixture of salts. If no commercial solutions are available, the following buffer solutions can be used for the calibration of the pH-meter.

7.1 Electrolyte solution

For the filling of the glass electrode only an electrolyte solution as supplied by the manufacturers should be used.

7.2 Standard buffer solution with pH-value 6.857 (25 °C)
Dry some (p.a.) potassium dihydrogen phosphate (KH_2PO_4) and (p.a.) disodium hydrogen phosphate (Na_2HPO_4), both free from crystal water, for 2 h at 110-130 °C before weighing out the sample. After cooling in a desiccator over blue silicagel, dissolve 3.388 g KH_2PO_4 and 3.533 g Na_2HPO_4 in about 800 ml water in a volumetric flask (6.2.2) at the temperature at which the flask was calibrated. Bring to 1000 ml with water of the same temperature at which the volumetric flask has been calibrated.

7.3 Standard buffer solution with pH-value 4.005 (25 °C)
Dry some (p.a.) potassium hydrogen phtalate ($KHC_6H_4(COO)_2$) for 2 h at 110-130 °C before weighing the sample. After cooling in a desiccator over blue silicagel, dissolve 10.12 g $KHC_6H_4(COO)_2$ in about 800 ml water in a volumetric flask (6.2.2) at the temperature of the flask calibration. Bring to 1000 ml with water of the same temperature at which the volumetric flask has been calibrated.

7.4 Standard buffer solution with pH-value 9.179 (25 °C)
Dissolve 3.80 g of disodium tetraborate deca-hydrate ($Na_2B_4O_7.10H_2O$) in about 800 ml water in a volumetric flask (6.2.2) at the temperature of the flask calibration. Bring to 1000 ml with water of the same temperature at which the volumetric flask has been calibrated.

7.5 Cleaning solution of the glass electrode
Dissolve 5 g pepsin in 100 ml hydrochloric acid (HCl) of 0.1 mol/l.

8. PROCEDURE

8.1 Calibration of the pH-meter
A new glass electrode has to be conditioned for at least 8 h in a buffer solution or the electrolyte solution (7.1), unless the manufacturer prescribes differently.
Keep the glass electrode always in a buffer solution with pH-value 4 (7.3), unless the manufacturer prescribes differently. Replace this solution at least every two weeks.
Measure standard buffer solutions and samples at room temperature (20-25 °C).
Use every day fresh buffer solutions (7).
Check whether the glass electrode is connected with the pH-meter, and whether there is sufficient electrolyte solution in the glass electrode. The level of the electrolyte solution should be 1 cm below the filling gap of the electrode, fill up with electrolyte solution (7.1) when necessary. Follow the manufacturers instructions concerning the type of electrolyte solution.
1. Rinse the electrode with water.
2. Remove superfluous water carefully with a tissue.
3. Bring ca 40 ml of the standard buffer solution with pH-value around 7 (7.2) into a beaker (6.2.4) with a magnetic stirring bar (6.1.7). Place the beaker on a magnetic stirring device (6.1.6) and stir with moderate speed.
   Measure the temperature of the standard buffer solution, using a calibrated thermometer (6.1.3).
   Adjust the pH-meter to the measured temperature (this should be between 20-

30 °C).
Remove the cap of the filling gap of the electrode.
Place the electrode in the standard buffer solution. Take care that the electrode is well immersed in the buffer solution (the porous grid has to be below the surface of the solution), without making contact with the magnetic stirring bar or the glass wall of the beaker. Add more buffer solution when necessary.
Bring the zero point of the meter on the pH of the standard buffer solution, taking into account the temperature of the solution. Use table 1 (or a table of the manufacturer) if the temperature of the solution is not 25 °C.

4. Set the pH-meter at stand-by and rinse the electrode with water.
5. Remove superfluous water carefully with a tissue.
6. For samples with pH > 7 use a second standard buffer solution with pH-value around 9 (7.4). For samples with pH < 7 use a second standard buffer solution with pH-value around 4 (7.3).
   Bring ca 40 ml of the standard buffer solution into a beaker (6.2.4) with a magnetic stirring bar (6.1.7). Place the beaker on a magnetic stirring device (6.1.6) and stir with moderate speed. Measure the temperature of the solution using a calibrated thermometer (6.1.3).
   Adjust the pH-meter at the measured temperature.
   Place the electrode in one of these two buffer solutions. Take care that the electrode is well immersed in the buffer solution.
   Adjust the slope of the pH-meter until the pH of the second standard buffer solution is reached, taking into account the temperature of the solution; use table 1 (or a table of the manufacturer) if the solution is not 25 °C.
7. Repeat 1-6 until further repeat gives no further improvement.

8.2 pH measurement of liquid culture media

Measure the pH of the medium on the same day as the medium is used for microbiological analysis.
Bring ca 40 ml of the liquid medium into a beaker (6.2.4) with a magnetic stirring bar (6.1.7). Place the beaker on a magnetic stirring device (6.1.6) and stir with moderate speed.
Calibrate the pH-meter with the relevant standard buffer solutions (mostly buffer solutions with pH-values of ca 7 and 9) (8.1).
Measure the temperature of the liquid medium in the beaker using a calibrated thermometer (6.1.3). The temperature of the medium should be between 20 and 30 °C.
Adjust the pH-meter at the measured temperature.
Rinse the glass electrode with water.
Remove superfluous water carefully with a tissue.
Place the electrode in the liquid medium. Take care that the electrode is well immersed in the medium (the porous pin has to be below the surface of the solution), without making contact with the magnetic stirring bar or the glass wall of the beaker. Add more medium when necessary. Measure the pH, read out after the signal has become stable. Record on the appropriate data sheet.
Rinse the electrode carefully with water.

8.3 pH measurement of solid culture media

Measure the pH of the medium on the same day as the medium is used for microbiological analysis.

For measuring the pH of an agar-medium, the medium first has to be poured in Petri dishes. Allow the medium to solidify at room temperature. Cut the agar-medium of one Petri dish into pieces with a spatula (6.1.5) and bring it into a test tube (6.2.3). About half of the contents of the test tube should be filled with pieces of the agar medium. Add ca 3 ml freshly distilled water (pH ca 7) to the test tube to improve the contact with the electrode. Allow to equilibrate for ca 5 minutes. Calibrate the pH-meter with the relevant standard buffer solutions (mostly buffer solutions with pH-values of ca 7 and 9) (8.1).

Measure the temperature of the medium mixture in the test tube using a calibrated thermometer (6.1.3).

Adjust the pH-meter to the measured temperature.

Rinse the glass electrode with water.

Remove superfluous water carefully with a tissue.

Place the electrode in the test tube and mix the medium by moving the electrode up and down, still taking care that the electrode is well immersed in the medium. Measure the pH, read out after the signal has become stable. Record on the appropriate data sheet.

Rinse the electrode carefully with water (and a tissue). Use by preference hot tap water of ca 80°C for the rinsing, unless the manufacturer prescribes differently, to protect the glass electrode from clogging with the agar.

8.4 Maintenance of the instrument

As long as the following requirements are fulfilled for an electrode, it may be deemed to be in perfect working order:

1. Fast response (In calibrating, the indication should reach the final pH of the buffer, ± 0.03 pH, within 1 min);
2. Stable response during stirring (± 0.03 pH);
3. Sensitivity better than 95%;
4. Buffer adjustment is possible.

After extensive measuring series the necessity to clean the measuring equipment becomes obvious for instance because of the fact that the pH of a standard solution requires a longer setting time of the instrument's display. Therefore place the glass electrode in the cleaning solution (7.5) for at least 2 h. Rinse with water afterwards. The glass electrode should be cleaned at least once a week or more frequent when necessary.

The manufacturer's instructions regarding storage and maintenance must be observed.

9. TEST REPORT

The test report should contain all information on operational details, not mentioned or specified in this SOP, that might influence the test result. The pH-value shall be reported to at least one decimal place. The temperature at which the measurement is carried out must also be reported. Furthermore a precise identification of the sample has to be given.

Tabel 1

pH's of standard buffer solutions at temperatures of 20-30 °C.

Temperature (°C)	KH_2PO_4 + Na_2HPO_4 pH = 6.857 (25 °C)	$KHC_6H_4(COO)_2$ pH = 4.005 (25 °C)	$Na_2B_4O_7.10H_2O$ pH = 9.179 (25 °C)
20	6.873	4.000	9.225
25	6.857	4.005	9.179
30	6.843	4.011	9.138

Annex 6

Amended ISO/CD 10705-2, Feb. 1997: Enumeration of somatic coliphages

pages 81 - 98

WATER QUALITY - DETECTION AND ENUMERATION OF BACTERIOPHAGES

PART 2: ENUMERATION OF SOMATIC COLIPHAGES

1 SCOPE

This standard specifies a method for the detection and enumeration of somatic coliphages by incubating the sample with an appropriate host-strain. The method is applicable to all kinds of water, sediments and sludge extracts, where necessary after dilution. In the case of low phage numbers, a preconcentration step may be necessary for which a separate International Standard will be developed. The method is also applicable to shellfish extracts.

2 NORMATIVE REFERENCES

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 10705. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 31-0:1992 (third edition) *Quantities and units - part 0: General principles.*

ISO 3696: 1987 *Water for analytical laboratory use - Specification and test methods.*

ISO 5667-1:1980 *Water quality - Sampling - Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1982 *Water Quality - Sampling - Part 2: Guidance on sampling techniques.*

ISO 5667-3:1985 *Water quality - Sampling - Part 3: Guidance on the preservation and handling of samples.*

ISO 6887:1983 *Microbiology - General guidance for the preparation of dilutions for microbiological examination.*

ISO 8199:1988 *Water quality - General guide to the enumeration of micro-organisms by culture.*

3 DEFINITIONS

For the purpose of this part of ISO 10705, the following definition applies.
Somatic coliphages are bacterial viruses which are capable of infecting selected *Escherichia coli* host strains (and related strains) by attachment to the bacterial cell wall as the first step

of the infectious process. They produce visible plaques (clearance zones) in a confluent lawn of host bacteria grown under appropriate culture conditions.

4 PRINCIPLE

The sample is mixed with a small volume of semi-solid nutrient medium. A culture of host-strain is added and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. The results are expressed as the number of plaque-forming particles per unit of volume (pfp/ml, pfp/l, etc.).

5 SAFETY PRECAUTIONS

The host strain used in this standard is non-pathogenic to man or animals and should be handled in accordance with the normal (national or international) safety procedures for bacteriological laboratories. Somatic coliphages are non-pathogenic for man and animals, but some types are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling cultures of high titre or when inoculating cultures of the host strains. Such procedures shall be carried out in a biohazard cabinet or a separate area of the laboratory. Chloroform is a carcinogenic substance. Observe relevant safety precautions or use an alternative of equal efficacy.

6 DILUENT, CULTURE MEDIA AND REAGENTS

6.1 Basic materials

Use ingredients of uniform quality and chemicals of analytical grade (or alternative grade if proved suitable for the test) for the preparation of culture media and reagents and follow the instructions given in annex A. For information on storage see ISO 8199, except where indicated in this standard. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.
For the preparation of media, use glass-distilled water or deionized water free from substances which might inhibit bacterial growth under the conditions of the test, and complying with ISO 3696.

6.2 Diluent

For making sample dilutions, use peptone-saline solution or another diluent copmlying with ISO 6887.

7 APPARATUS AND GLASSWARE

Usual microbiological laboratory equipment, including

7.1 **Hot-air oven for dry-heat sterilization and an autoclave**. Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

7.2 **Incubator or water bath**, thermostatically controlled at (36 ± 2) °C.

7.3 **Incubator or water bath**, thermostatically controlled at (36 ± 2) °C and equipped with a rotating platform at (100 ± 10) min^{-1}.

7.4 **Water bath**, thermostatically controlled at (45 ± 1) °C.

7.5 **Water bath or equivalent device** for melting of agar media.

7.6 **pH meter**.

7.7 **Counting apparatus** with indirect, oblique light.

7.8 **Deep freezer**, thermostatically controlled at (-20 ± 5) °C.

7.9 **Deep freezer**, thermostatically controlled at (-70 ± 10) °C or liquid nitrogen storage vessel.

7.10 **Spectrophotometer**, capable of holding cuvettes of 1 cm optical path length or side-arm of nephelometric flasks (7.17) and equipped with a filter for the range of 500 - 650 nm with a maximum bandwidth of ± 10 nm.

Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including

7.11 **Petri dishes** of 9 cm or 14 - 15 cm diameter, vented.

7.12 **Graduated pipettes** of 0,1, 1, 5 and 10 ml capacity and **Pasteur pipettes**.

7.13 **Glass bottles** of suitable volumes.

7.14 **Culture tubes** with caps or suitable alternatives.

7.15 **Measuring cylinders** of suitable capacity.

7.16 **Conical flasks** of 250 - 300 ml capacity, with cotton wool plugs or suitable alternatives.

7.17a **Cuvettes**, of optical path length 10 mm or

7.17b **Nephelometric conical flasks** with cylindrical side-arms fitting in the spectrophotometer (7.10) see Figure 1; capacity 250 - 300 ml with cotton wool plugs or suitable alternatives.

7.18 **Membrane filter units** for sterilization, pore size 0,2 µm.

7.19 **Plastics vials**, lidded, of 1,5 - 3 ml capacity.

7.20 **Refrigerator**, temperature set at (5 ± 3) °C.

8 MICROBIOLOGICAL REFERENCE CULTURES

For samples with low bacterial background flora *Escherichia coli* strain C, ATCC 13706. For samples with high bacterial background flora its nalidixic acid resistant mutant *E. coli* strain CN (Havelaar AH and Hogeboom WM, Antonie van Leeuwenhoek 1983;49:387-397), also called WG5 (Grabow WOK, Cobrough P, Appl Environ Microbiol 1986;52:430-433). The ATCC number of this latter strain is ATCC 700078.

Bacteriophage ΦX174, ATCC 13706-B1.

NOTE 1
The ATCC strains are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockvile, Maryland, USA.
Strain WG5 (CN) will be deposited with ATCC.

9 SAMPLING

Take samples and deliver them to the laboratory in accordance with ISO 8199, ISO 5667-1, ISO 5667-2 and ISO 5667-3.

10 PREPARATION OF TEST MATERIALS

10.1 Culturing and maintenance of host strains

The culturing and maintenance of host strains involves several stages which are summarized in Figure 2.

NOTE 2
For the culturing of the host strains in the several stages it is prescribed to shake the cultures at (100 ± 10) min^{-1}. Not shaking of the culture is also possible, but not advisable as the time needed to reach the same culture density will be longer than compared to a shaken culture.

10.1.1 Preparation of stock cultures.

Rehydrate the contents of a lyophilized ampoule of the reference culture of the host-strains in a small volume of MSB (A.1) using a Pasteur pipette (7.12). Transfer the suspension to 50 ml of MSB in a 300 ml conical flask (7.16). Incubate for (20 ± 4) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Add 10 ml (i.e. a final concentration of 15-20% v/v) of glycerol (A5) and mix well. Distribute into plastics vials (7.19) in *ca* 0,5 ml aliquots and store at (-70 ± 10) °C or in liquid nitrogen.

NOTE 3
This first passage of the host strains should be stored as a reference in the laboratory.

10.1.2 Preparation of working cultures.

Thaw one vial of stock culture (10.1.1) at room temperature and inoculate on a plate of McConkey-agar (A.6) or another lactose containing medium in such a way that single colonies will be obtained. Incubate at (36 ± 2) °C for (20 ± 4) h. The remaining content of the vial of stock culture can be used to inoculate more plates on the same working day (if necessary), otherwise it should be treated as contaminated waste.
Add 50 ml of MSB to a conical flask of 300 ml (7.16) and warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Select 3 - 5 lactose-positive colonies from the McConkey-agar and inoculate material from each of these colonies in the flask with MSB. Incubate for (5 ± 1) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Add 10 ml of glycerol (A5) and mix well. Distribute over plastic vials (7.19) in 1,2 ml aliquots and store at (-70 ± 10) °C for a maximum of 2 years.

NOTE 4
If a great number of tests is anticipated, several conical flasks can be inoculated in parallel.

10.2 Calibration of absorbance measurements for counts of viable micro-organisms.

Take one vial of working culture of the host strain from the freezer and thaw at room temperature. Add 50 ml of MSB to a nephelometric conical flask (7.17b), warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Adjust spectrophotometer reading to 0 on filled side-arm. Alternatively, use plain conical flask (7.16) and adjust spectrophotometer reading to 0 on broth transferred to cuvette (7.17a). Inoculate 0,5 ml of working culture. Incubate at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3) for up to 3,5 h. Every 30 min measure absorbance and withdraw a 1 ml sample for viable counts, assuring that the flask is taken from the incubator as short as possible.
Dilute samples to 10^{-7} and prepare pour plates of 1 ml volumes of the 10^{-5}, 10^{-6} and 10^{-7} dilutions with molten nutrient agar, in duplicate. Alternatively perform membrane filtration with 1 ml volumes of the same dilutions. Incubate at (36 ± 2) °C for (20 ± 4) h (using 7.2). Count the total number of colonies on/in each plate yielding between 30 and 300 colonies and calculate the number of cfp/ml (consult ISO 8199 if necessary).

NOTES
5. 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.
6. If the cell density of approx. 10^8 cfp/ml is not reached within 3,5 h of incubation, it is also possible to inoculate 1 ml of working culture instead of 0,5 ml.

11 PROCEDURE

11.1 Preparation of inoculum cultures

Take one vial of working culture from the freezer and thaw at room temperature. Add 50 ml of MSB to a nephelometric conical flask (7.17b), or plain conical flask (7.16) and prewarm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Adjust the spectrophotometer reading to 0 as described in 10.2. Inoculate 0,5 ml of workingculture into MSB. Incubate at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Measure absorbance every 30 min. At an absorbance corresponding to a cell-density of approx. 10^8 cfp/ml (based on data obtained in 10.2), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use the same working day.

11.2 Standard procedure

11.2.1 Single Agar Layer (SAL) procedure

Prepare an inoculum culture as described in 11.1.
Melt tubes of 10 ml ssMSA (A.3) in a boiling water bath (7.5) and place in a water bath at (45 ± 1) °C, aseptically add 60 µl of calcium chloride solution (A.2). 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 (*E. coli* C), mix carefully avoiding the formation of air bubbles and pour the contents in one 14 - 15 cm or two 9 cm Petri dishes (7.11). Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h.

11.2.2 Double Agar Layer (DAL) procedure

Prepare an inoculum culture as described in 11.1.
Melt bottles of 50 ml ssMSA (A.3) in a boiling waterbath (7.5) and place in a waterbath at (45 ± 1) °C. Aseptically add calcium chloride solution (A2) (300 µl/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath 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 MSA in a 9 cm Petri dish (A.2). Distribute evenly, allow to solidify on a horizontal, cool surface and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h.

NOTES
7. If a great number of tests is anticipated, several conical flasks can be inoculated *in parallel.*
8. If necessary, plates can be read after 6 h of incubation. This may be useful if a preliminary count is required.

9. Triphenyltetrazoliumchloride (0,1 ml per plate of a freshly prepared solution of 0,1 g in 10 ml ethanol 96%) can be added to enhance contrast for counting plaques.

11.2.3 SAL and DAL

The addition of ice-cold sample and host-culture to the semi-solid agar may lead to a sharp drop in temperature and solidification of the medium. Assure a sufficient time interval between these two steps to allow reheating. However, make sure that inoculated tubes remain in the water bath for not more than 10 minutes.
Dry the plates by incubating with partially opened lids, if necessary. Then cover and invert for final incubation.
Do not stack more than 6 (preferably 4) plates.
Count the number of plaques on each plate within 4 hours after finishing incubation, using indirect oblique light.

11.3 Method for samples with high bacterial background flora

Proceed according to 11.2.
Add nalidixic acid to ssMSA (A.3) to give a final concentration of 250 µg/ml. Use *E. coli* WG5 as the inoculum culture.

NOTE 10
Nalidixic acid is heat-stable. It can either be added from a filter-sterilized solution after melting of soft agar, or can be added before autoclaving.

11.4 Samples with low phage counts

Proceed according to 11.2.2 but use the following modifications:

- 10 ml of ssMSA, 60 µl of calcium chloride solution, 1 ml of host culture and 5 ml of sample in duplicate per dilution step.
- Pour over 50 ml of MSA in a 14 - 15 cm Petri dish (or use two 9 cm Petri dishes, each containing 20 ml of MSA).

NOTE 11
This procedure will be able to detect one plaque forming particle in 50 ml or 100 ml, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

11.5 Presence/absence test

Mind that this procedure will result in high titre phage suspensions. Take appropriate precautions, like working in a biohazard cabinet or in a separate area of the laboratory.

Add 25 ml of MSB (A.1) to a plain conical flask (7.16) and prewarm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Add 150 µl of calcium chloride solution (A.2) and 0,25 ml of working culture (10.1.2), incubate at (36 ± 2) °C, while shaking at (100 ± 10) min^{-1} for approx. 3 h. Add 1 ml of sample or a dilution thereof (prewarmed to room temperature) and continue incubation for (18 ± 2) h.
Transfer 1 ml of the culture to a centrifuge tube, add 0,4 ml of chloroform, mix well and centrifuge at 3000 *g* for 5 min.
Prepare an inoculum culture as described in 11.1. Melt bottles of 50 ml ssMSA (A.3) in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add calcium chloride solution (A2) (300 µl/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath at (45 ± 1) °C. To each tube, add 1 ml of inoculum culture, mix carefully avoiding the formation of air bubbles and pour the contents onto a layer of MSA (A.2) in a 9 cm Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and dry in a laminar flow cabinet or in a (36 ± 2) °C incubator for 30 min, while the plates are inverted with the lids off.
Place one drop of the chloroform-treated culture on the inoculated plate using a fine capillary or pipette. Do not damage the top agar layer. Leave the spot to dry and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h.
Examine the plate for a clear zone in the spotted area, which is indicative of the presence of somatic coliphages in the original sample.

NOTES

12. This procedure can also be used in an MPN format (ISO 8199) or to examine larger samples. In the latter case, use double strength MSB (double the amounts of ingredients in the same amount of water as used for single strength MSB; add a proportional volume of calcium chloride solution) in equal volumes as the sample. To obtain sufficient aeration during enrichment, make sure that the volume of sample and broth is not greater than 20% of the nominal capacity of the conical flask.
13. More than one spot can be placed on the surface of an inoculated plate.

11.6 Quality assurance

11.6.1 Plaque count procedures (11.2 - 11.4)

With each series of samples, examine a procedural-blank using sterile diluent as the sample and a reference control of ΦX174, prepared as follows:
From a high titre phage culture (e.g. as described in Annex C), prepare a decimal dilution series and plate out according to 11.2. Store the dilution series in a refrigerator overnight. Count the number of plaques, from the dilution series, and prepare 100 - 1000 ml of a suspension with an expected concentration of plaque-forming particles of ΦX174 of approx. 100 ml^{-1}. Add 5 % (v/v) of glycerol (A5). Distribute into plastics vials in 2,4 ml aliquots and store at (-70 ± 10) °C. Thaw vials of the reference control of ΦX174 before use and plate out according to the procedure used (11.2 or 11.4). Plot the results on a control chart. Discard the reference control samples if the mean number of pfp/ml decreases.
Optionally, use in addition a naturally polluted reference control sample, using sewage or surface water, diluted to a concentration of plaque forming particles of approx. 100 ml^{-1} in peptone-saline and 5 % (v/v) glycerol and stored at (-70 ± 10) °C. Discard the reference

control samples if the concentration of somatic coliphages decreases and is still decreased following a re-test.

11.6.2 Presence/absence test (11.5)

Prepare a control sample as described in 11.6.1 with a concentration of plaque-forming particles of ΦX174 of approx. 5 ml^{-1}. Examine at least one control sample in parallel with each series of samples tested, expecting to obtain a positive test-result. To examine possible interfering effects from the samples, consider also adding the control sample to a second enrichment culture containing the actual sample.

NOTE 14
In the absence of easily available standardized reference materials, any exchange programme of reference samples between laboratories or other interlaboratory tests should be encouraged.

12 EXPRESSION OF RESULTS

12.1 Plaque count procedures (11.2 - 11.4)

Select plates with well separated, and preferably more than 30 plaques, whenever present. If only counts below 30 per plate are found, select plates inoculated with the largest volume of sample. From the number of plaques counted, calculate the number of plaque-forming particles of somatic coliphages in 1 ml of the sample as follows:

$$pfp/ml = \frac{N}{(n_1 V_1 F_1) + (n_2 V_2 F_2)}$$

pfp/ml	is the confirmed number of plaque forming particles of somatic coliphages per ml;
N	is the total number of plaques counted on plates according to 11.2, 11.3 or 11.4;
n_1, n_2	is the number of replicates counted for dilution F_1, F_2;
V_1, V_2	is the test volume used with dilution F_1, F_2;
F_1, F_2	is the dilution or concentration factor used for the test portion V_1, V_2 (F = 1 for an undiluted sample, F = 0,1 for a ten-fold dilution, F = 10 for a ten-fold concentrate, etc.).

If only one dilution/concentrate is counted, simplify the formula to:

$$pfp/ml = \frac{N}{n V F}$$

Refer to ISO 8199 for further details.

12.2 Presence/absence test (11.5)

Express the results as "somatic coliphages (not) detected in *V* ml", *V* being the volume of sample examined.

13 TEST REPORT

The test report shall contain the following information:
a) a reference to this standard;
b) all details necessary for complete identification of the sample;
c) the inoculation procedure used;
d) the incubation time if different from the standard time in chapter 11;
e) the results expressed in accordance with chapter 12;
f) any other information relevant to the method.

ANNEX A
(NORMATIVE)

CULTURE MEDIA, REAGENTS AND DILUENTS

A.1 Modified Scholtens' Broth (MSB)

Peptone	10	g
Yeast extract	3	g
Meat extract	12	g
NaCl	3	g
Na_2CO_3-solution (150 g/l)	5	ml
Mg solution (100 g $MgCl_2.6H_2O$ in 50 ml water)	0,3	ml
Distilled water	1000	ml

Dissolve the ingredients in hot water. Adjust the pH so that after sterilization it will be 7,2 ± 0,2. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Preparation of $MgCl_2$-solution
$MgCl_2.6H_2O$ is very hygroscopic and shall not be stored in the crystalline form once the container has been opened. Therefore, use the total contents of a container and dissolve in the appropriate amount of water, e.g. dissolve 100 g of $MgCl_2.6H_2O$ in 50 ml water. The final concentration of Mg^{2+} in this solution will be 4,14 mol/l. Sterilize by autoclaving and store at room temperature in the dark.

A.2 Modified Scholtens' Agar (MSA)

Basal medium

Peptone	10	g
Yeast extract	3	g
Meat extract	12	g
NaCl	3	g
Na_2CO_3-solution (150 g/l)	5	ml
Mg solution (100 g $MgCl_2.6H_2O$ in 50 ml water)	0,3	ml
Agar	12-20	g*
Distilled water	1000	ml

*) Depending on the gel strength of the agar

Dissolve the ingredients in boiling water. Adjust the pH so that after sterilization it will be 7,2 ± 0,2. Distribute the medium in bottles in volumes of 200 ml and sterilize in the

autoclave at (121 ± 1) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Calcium chloride solution (1 mol/l)

$CaCl_2.2H_2O$	14,6 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.

Complete medium

Basal medium	200 ml
Calcium chloride solution	1,2 ml

Melt the basal medium and cool to between 45 and 50 °C. Aseptically add the calcium chloride solution, mix well and pour into Petri dishes as follows:
- 20 ml in dishes of 9 cm diameter
- 50 ml in dishes of 14 cm diameter

Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 1 month if well protected against desiccation.

A.3 Semi-solid Modified Scholtens' Agar (ssMSA)

Prepare basal medium according to A.2 but use half of the mass of the agar (6g - 10 g), depending on gel strength; the gel-strength of ssMSA 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 tubes in volumes of 10 ml for the SAL-procedure, or into bottles in volumes of 50 ml for the DAL-procedure.

NOTE
Triphenyltetrazoliumchloride (1 ml of a solution of 1 g in 100 ml ethanol 96% per 100 ml of ssMSA) can be added to improve contrast for counting plaques.

A.4 Nalidixic acid 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 membrane filter, or sterilize in the autoclave ar (121 ± 1) °C for 15 min. Store at (5 ± 3) °C for not longer than 8 hours or at (-20 ± 5) °C for not longer than six months.

A.5 Glycerol (sterile)

Glycerol (870 g/l)	100 ml

Distribute into bottles in 20 ml volumes and sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark for not longer than 1 year.

A.6 McConkey agar

Pepton	20 g
Lactose	10 g
Bile salts	5 g
Neutral red	75 mg
Agar	12 - 20 g
Distilled water	1000 ml

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7,4 ± 0,1 at 25 °C. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 1) °C for 15 min. Cool to between 45 °C and 50 °C and pour 20 ml in Petri dishes of 9 cm diameter. Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months.

ANNEX B
(INFORMATIVE)

GENERAL DESCRIPTION OF SOMATIC BACTERIOPHAGES

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

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

ANNEX C
(INFORMATIVE)

CULTURING OF BACTERIOPHAGE ΦX174

Use normal procedures for phage propagation as described in the open literature. The following is an example of a procedure which has given good results.
Introduce 25 ml of MSB into a conical flask of 300 ml volume and inoculate with *E. coli* or a nalidixic acid resistant mutant (see 8). Incubate for (20 ± 4) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1}.
Prewarm 25 ml of MSB in a conical flask of 300 ml volume to room temperature and inoculate with 0,25 ml of the host culture.
Incubate as above for 90 min. Add ΦX174 from a stock solution to give a final concentration of plaque-forming particles of approx. 10^7 ml^{-1}.
Incubate as above for 4 - 5 h. Add 2,5 ml of chloroform ($CHCl_3$), mix well and place overnight at (5 ± 3) °C.
Decant the aqueous phase in centrifuge tubes and centrifuge at a minimum of 3000 *g* for 20 min.
Pipette the supernatant carefully and store at (5 ± 3) °C.

NOTES
1. The titre of the phage-suspension should be above 10^9 ml^{-1}. In some cases it may be necessary to repeat the cycle to obtain sufficiently high titres, higher phage inputs may then be used.
2. The titre of the phage stock suspension will slowly decrease in time.

Figure 1. Nephelometric conical flasks for culturing the host strain

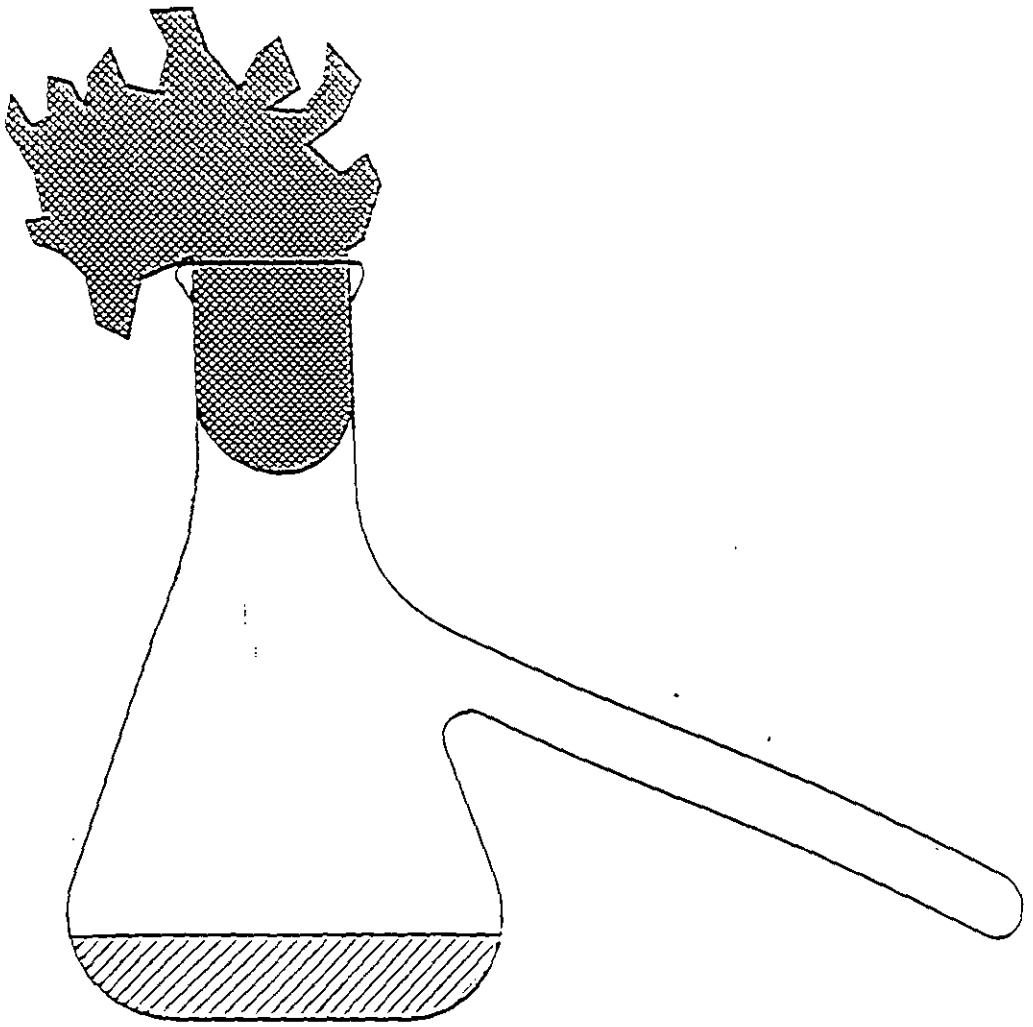
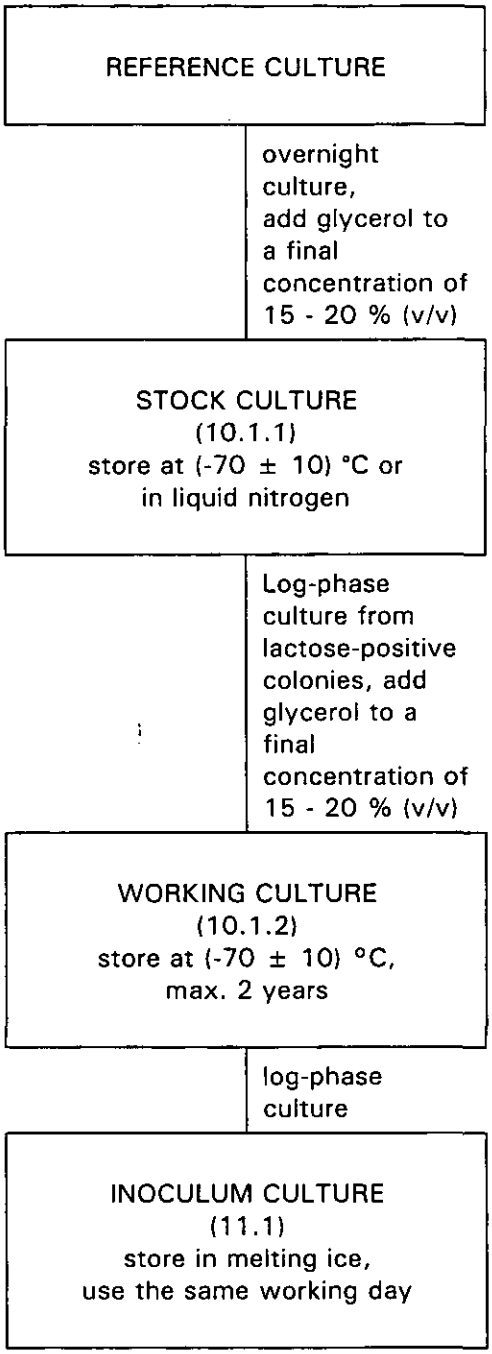


Figure 2. Scheme for culturing and maintenance of host strains



Annex 7

Amended ISO 10705-1, Feb. 1997: Enumeration of F-specific RNA bacteriophages

pages 99 - 116

WATER QUALITY - DETECTION AND ENUMERATION OF BACTERIOPHAGES

PART 1: ENUMERATION OF F-SPECIFIC RNA BACTERIOPHAGES

1 SCOPE

This standard specifies a method for the detection and enumeration of F-specific RNA bacteriophages by incubating the sample with an appropriate host-strain. The method can be applied to all kinds of water, sediments and sludges, where necessary after dilution. In the case of low numbers, a preconcentration step may be necessary for which a separate International Standard will be developed. The method can also be applied to shellfish extracts. Depending on the relative abundance of F-specific RNA bacteriophages to background organisms, additional confirmatory tests may be necessary and are also specified in this standard.
The presence of F-specific RNA bacteriophages in a water sample generally indicates pollution by wastewater contaminated by human or animal faeces. Their survival in the environment, removal by widely used water treatment processes and concentration or retention by shellfish resembles that of food- and waterborne human enteric viruses, for example the entero-, hepatitis A, and rotaviruses.

2 NORMATIVE REFERENCES

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 10705. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Member of IEC and ISO maintain registers of currently valid International Standards.

ISO 3696:1987 *Water for analytical laboratory use - Specification and test methods.*

ISO 5667-1:1980 *Water quality - Sampling - Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1982 *Water Quality - Sampling - Part 2: Guidance on sampling techniques.*

ISO 5667-3:1985 *Water quality - Sampling - Part 3: Guidance on the preservation and handling of samples.*

ISO 6887:1983 *Microbiology - General guidance for the preparation of dilutions for microbiological examination.*

ISO 8199:1988 *Water quality - General guide to the enumeration of micro-organisms by culture.*

3 DEFINITIONS

F-specific RNA bacteriophages are bacterial viruses which are capable of infecting a specified host strain with F- or sex-pili to produce visible plaques (clearance zones) in a confluent lawn grown under appropriate culture conditions, whereas the infectious process is inhibited in the presence of a concentration of 40 (occasionally 400) µg/ml of RNase in the plating medium.

4 PRINCIPLE

The sample is mixed with a small volume of semi-solid nutrient medium. A culture of host-strain is added and plated on a solid nutrient medium. After this, incubation and reading of plates for visible plaques takes place. Where necessary, simultaneous examination of parallel plates with added RNase for confirmation by differential counts is carried out. The results are expressed as the number of plaque-forming particles per unit of volume (pfp/ml, pfp/l, etc.).

5 SAFETY PRECAUTIONS

The host strain used is a *Salmonella typhimurium* mutant of low pathogenicity and should be handled in accordance with the appropriate (national or international) safety procedures for this bacterial species. F-specific RNA bacteriophages are non-pathogenic for man and animals, but are very resistant to drying. Appropriate precautions should therefore be taken to prevent cross-contamination of test materials, particularly when examining or handling cultures of high titre or when inoculating cultures of the host strains. Such procedures shall be carried out in a biohazard cabinet or a separate area of the laboratory. Chloroform is a carcinogenic substance. Observe relevant precautions or use an alternative of equal efficacy.

6 DILUENT, CULTURE MEDIA AND REAGENTS

6.1 Basic materials

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media and reagents and follow the instructions given in annex A. For information on storage see ISO 8199, except where indicated in this standard. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.
For the preparation of media, use glass-distilled water or de-ionized water free from substances which might inhibit bacterial growth under the conditions of the test, and in complying with ISO 3696.

6.2 Diluent

For making sample dilutions, use peptone-saline solution as indicated in annex A.8.

6.3 **Reagents**

6.3.1 **RNase from bovine pancreas**, specific activity approx. 50 units/mg (Kunitz).

6.3.2 **Antibiotic discs** for susceptibility testing with nalidixic acid (130 μg; 9 mm) and kanamycin (100 μg; 9 mm).

6.3.3 **Glycerol**, 870 g/l.

7 APPARATUS AND GLASSWARE

Usual microbiological laboratory equipment, including

7.1 **Hot-air oven for dry-heat sterilization and an autoclave.** Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

7.2 **Incubator or water bath**, thermostatically controlled at (36 ± 2) °C.

7.3 **Incubator or water bath**, thermostatically controlled at (36 ± 2) °C and equipped with a rotating platform at (100 ± 10) min^{-1}.

7.4 **Water bath**, thermostatically controlled at (45 ± 1) °C.

7.5 **Water bath or equivalent device** for melting of agar media.

7.6 **pH meter.**

7.7 **Counting apparatus** with indirect, oblique light.

7.8 **Deep freezer**, thermostatically controlled at (-20 ± 5) °C.

7.9 **Deep freezer**, thermostatically controlled at (-70 ± 10) °C.

7.10 **Spectrophotometer**, capable of holding 1-cm cuvettes or side-arm of nephelometric flasks (7.17b) and equipped with a filter in the range of 500 - 650 nm with a maximum band-width of ± 10 nm.

Usual sterile, microbiological laboratory glassware or disposable plastics ware according to ISO 8199 and including

7.11 **Petri dishes** of 9 cm or 15 cm diameter, vented.

7.12 **Graduated pipettes** of 0,1, 1, 5 and 10 ml capacity.

7.13 **Glass bottles** of suitable volumes.

7.14 **Culture tubes** with caps or suitable alternatives.

7.15 **Measuring cylinders** of suitable capacity.

7.16 **Conical flasks** of 250 - 300 ml capacity, with cotton wool plugs or suitable alternatives.

7.17a **Cuvettes**, 1 cm optical path length or

7.17b **Nephelometric conical flasks** with cylindrical side-arms fitting in the spectrophotometer (7.9) see Figure 1; capacity 250 - 300 ml with cotton wool plugs or suitable alternatives.

7.18 **Membrane filter units** for sterilization, pore-size 0,2 μm.

7.19 **Plastic vials**, lidded, of 1,5 - 3 ml capacity.

8 MICROBIOLOGICAL REFERENCE CULTURES

Salmonella typhimurium strain WG49, phage type 3 Nal^r (F' 42 *lac*::Tn5), NCTC 12484.
Bacteriophage MS2, NCTC 12487 or ATCC 15597-B1.
Escherichia coli K-12 Hfr from appropriate culture collection, e.g. NCTC 12486 or ATCC 23631.
N.b.: Another reported promissing host strain for the detection of F-specific RNA phages is *Escherichia coli* HS(pFamp)R (Debartolomeis and Cabelli, 1991)[1]. This strain is resistant for ampicillin (coded on Famp plasmid), streptomycin and nalidixic acid (both coded on chromosome) and lactose negative. In this AMENDED ISO the procedures are described for host strain WG49, as the authors have little experiences with host strain Famp. However, other laboratories have shown that for the detection or enumeration of F-specific RNA phages the same materials and procedures described here for host strain Famp can be used.

NOTE 1
The NCTC strains are available from the National Collection of Type Cultures, 61 Colindale Avenue, London NW9 6HT, England.
The ATCC strains are available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A.

9 SAMPLING

Take samples and deliver them to the laboratory in accordance with ISO 8199, ISO 5667-1, ISO 5667-2 and ISO 5667-3.

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[1] Debartolomeis, J. and Cabelli, V.J. (1991). Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. Applied and Environmental Microbiology, 57, 5, 1301-1305.

10 PREPARATION OF TEST MATERIALS

10.1 Culturing and maintenance of host strains WG49 and *E. Coli* K12 Hfr

The culturing and maintenance of host strains involves several stages which are summarized in Figure 2. The figure also indicates the stages where quality control of the host culture is performed.

10.1.1 Preparation of stock cultures.

Rehydrate the contents of a lyophilized ampoule of the reference culture of the host-strains in a small volume of TYGB (A.1) using a Pasteur pipette. Transfer the suspension to 50 ml TYGB in a 300 ml conical flask (7.16). Incubate for (20 ± 4) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Add 10 ml of glycerol (A.6) and mix well. Distribute into plastic vials (7.19) in *ca* 0,5 ml aliquots and store at (-70 ± 10) °C or in liquid nitrogen.

NOTE 2
This first passage of the host strains should be stored as a reference in the laboratory.

10.1.2 Preparation of working cultures.

Thaw one vial of stock culture (10.1.1) at room temperature and inoculate on a plate of McConkey-agar (A7) in such a way that single colonies will be obtained. Incubate at (36 ± 2) °C for (20 ± 4) h. The remaining content of the vial of stock culture can be used to inoculate more plates on the same working day (if necessary), otherwise it should be treated as contaminated waste.
Add 50 ml of TYGB to a conical flask of 300 ml (7.16) and warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Select 3 - 5 lactose-positive colonies from the McConkey-agar and inoculate material from each of these colonies in the flask with TYGB. Incubate for (5 ± 1) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Add 10 ml of glycerol and mix well. Distribute over plastic vials (7.19) in 1,2 ml aliquots and store at (-70 ± 10) °C for a maximum of 2 years. Control the quality of the working culture according to 10.3.

NOTES
3. If a great number of tests is anticipated, several conical flasks can be inoculated in parallel.
4. If quality control fails, prepare new inocula from the stock culture. After repeated failures, or if the stock culture is depleted, obtain a new lyophilized ampoule of reference culture. Do not subculture repeatedly in the laboratory.

10.2 Calibration of absorbance measurements for counts of viable micro-organisms

Take one vial of working culture of host strain WG49 from the freezer and thaw at room temperature. Add 50 ml of TYGB to a nephelometric conical flask (7.17b), warm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Adjust spectrophotometer reading to 0 on filled side-arm. Alternatively, use plain conical flask

(7.16) and adjust spectrophotometer reading to 0 on broth transferred to cuvette (7.17a). Inoculate 0,5 ml of working culture. Incubate at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3) for up to 3,5 h. Every 30 min measure absorbance and withdraw a 1 ml sample for viable counts, assuring that the flask is taken from the incubator as short as possible.
Dilute samples to 10^{-7} and prepare pour plates of 1 ml volumes of the 10^{-5}, 10^{-6} and 10^{-7} dilutions with molten TYGA (A2). Alternatively perform membrane filtration of 1 ml volumes of the same dilutions. Incubate at (36 ± 2) °C for (20 ± 4) h (using 7.2). Count the total number of colonies on/in each plate yielding between 30 and 300 colonies and calculate the number of cfp/ml (consult ISO 8199 if necessary).

NOTES

5. 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.
6. If the cell density of approx. 10^8 cfp/ml is not reached within 3,5 h of incubation, it is also possible to inoculate 1 ml of working culture instead of 0,5 ml.

10.3 Quality control of host strain WG49

Use a culture as prepared in 10.2.
At t = 0 h and t = (time necessary to reach approx. 10^8 cfp/ml) , spread 0,1 ml volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions on McConkey agar (A7) or another suitable lactose containing medium, in duplicate. Incubate at (36 ± 2) °C for (20 ± 4) h. From plates yielding between 30 and 300 colonies, count the number of lactose-positive and -negative colonies and calculate the percentage of lactose-negative colonies.
At time t = (time necessary to reach approx. 10^8 cfp/ml), spread 0,1 ml of the 10^{-1} dilution on a plate of McConkey agar (A7), place one disk with nalidixic acid (Nal) and one disk with kanamycin (Km) on the plates and incubate for (20 ± 4) h at (36 ± 2) °C.
Measure inhibition zones around the antibiotic disks.

The host-strain is acceptable if the following criteria are met, at t = (time necessary to reach approx. 10^8 cfp/ml):

Plate count on TYGA (9.2):	7 - 40 x 10^7 cfp/ml
Lactose-negative colonies (plasmid segregation):	< 8 %
Inhibition zone around Nal disk:	absent
Km disk:	< 25 mm diameter

NOTE 7
Antibiotic disks with a different diameter or concentration can be used; another criterion for the maximum inhibition zone around the Km disk should be set.

Check the host strain for sensitivity for F-specific RNA bacteriophages as follows: Prepare reference control samples of bacteriophage MS2 as follows:
From a high titre phage culture (e.g. as described in Annex C), prepare a decimal dilution series and plate out according to 11.1 but use the *E. Coli* K-12 Hfr host-strain. Store the

dilution series at (5 ± 3) °C overnight. Count the number of plaques, from the dilution series, and prepare 100 - 1000 ml of a suspension of MS2 in peptone-saline which is expected to contain approx. 100 pfp/ml. Add 5 % (v/v) of glycerol. Distribute into plastic vials (7.19) in 2,4 ml aliquots and store at (-20 ± 5) °C or (-70 ± 10) °C.
Thaw 2 vials at room temperature, combine in one tube and plate out 1 ml volumes in duplicate on the *E. Coli* K-12 Hfr strain and on WG49 according to 11.1. Count the number of plaques on each plate and calculate the recovery on WG49 relative to the *E. Coli* strain. Accept WG49 if the recovery is >.80 %.

11 PROCEDURE

11.1 Standard procedure

Take one vial of working culture from the freezer and thaw at room temperature. Add 50 ml of TYGB to a nephelometric conical flask (7.17b), or plain conical flask (7.16) and prewarm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Adjust the spectrophotometer reading to 0 as described in 10.2. Inoculate 0,5 ml of workingculture into TYGB. Incubate at (36 ± 2) °C while shaking at (100 ± 10) min^{-1} (using 7.3). Measure absorbance every 30 min. At an absorbance corresponding to a cell-density of approx. 10^8 cfp/ml (based on data obtained in 10.2), take the inoculum culture from the incubator and quickly cool the culture by placing it in melting ice. Use within 2 h. It is essential that the culture is quickly cooled to prevent loss of F-pili by the cells, which will negatively influence recovery.

Melt bottles of ssTYGA (A.3) in a boiling waterbath (7.5) and place in a waterbath at (45 ± 1) °C. Aseptically add calcium-glucose solution (0,5 ml/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a water bath 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 and pour the contents over the surface of a 9 cm TYGA plate. Distribute evenly, allow to solidify on a perfectly horizontal, cool surface and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h. Do not stack more than 6 (preferably 4) plates.

Mind: The addition of ice-cold sample and host-culture to the top-agar may lead to a sharp drop in temperature and solidification of the medium. Assure a sufficient time interval between these two steps to allow reheating. However, make sure that inoculated tubes remain in the water bath for not more than 10 minutes.

Count the number of plaques on each plate within 4 hours after finishing incubation, using indirect oblique light.

11.2 Method for samples with high bacterial background flora

Add nalidixic acid to ssTYGA to a final concentration of 100 µg/ml.

NOTE 8
Nalidixic acid is heat-stable. It can either be added from a filter-sterilized solution (A.4) (0,2 ml/50 ml) after melting of ssTYGA, or can be added to TYGA before autoclaving.

11.3 Confirmatory test

In parallel to the series of plates described under 11.1, prepare a similar series with RNase-solution, (A.5) added to the tubes of ssTYGA to a final concentration of 40 µg/ml (i.e. 100 µl of RNase solution to 2,5 ml ssTYGA in a tube).
Confirmatory tests should at least be carried out
* when examining new sampling points.
* regularly at fixed sampling points when N_{RNase}/N (see 12) is usually less than 10 %.
* always at fixed sampling points when N_{RNase}/N is usually > 10 %.
* if large, circular, clear plaques with smooth edges (probably somatic *Salmonella* phages) are regularly seen.

NOTE 9
In rare cases RNA-phages may not be inhibited by RNase at 40 µg/ml and it may be necessary to increase the concentration of RNase to 400 µg/ml.

11.4 Samples with low phage counts

Proceed according to 11.1 but use the following modifications:
- 10 ml of ssTYGA, 1 ml of host culture and 5 ml of sample in duplicate per dilution step.
- pour over 50 ml of TYGA in a 14 cm Petri dish.

NOTE 10
This procedure will be able to detect up to 1 pfp/50 or 100 ml, if 10 or 20 plates are inoculated in parallel. Due to the high consumption of culture media, it may be advisable to use concentration methods which will also be necessary for even lower counts.

11.5 Presence/absence test

Mind that this procedure will result in high titre phage suspensions. Take appropriate precautions, like working in a biohazard cabinet or in a separate area of the laboratory.

Add 25 ml of TYGB (A.1) to a plain conical flask (7.16) and prewarm to at least room temperature (faster grow will occur if the broth is prewarmed to 37 °C). Add 250 µl of calciumglucose solution (A.1) and 0,25 ml of working culture (10.1.2), incubate at (36 ± 2) °C, while shaking at (100 ± 10) min^{-1} (using 7.3) for approx. 3 h. Add 1 ml of sample or a dilution thereof (prewarmed to room temperature) and continue incubation for (18 ± 2) h. Transfer 1 ml of the culture to a centrifuge tube, add 0,4 ml of chloroform, mix well and centrifuge at 3000 *g* for 5 min.
Prepare an inoculum culture as described in 11.1. Melt bottles of ssTYGA (A.3) in a boiling waterbath and place in a waterbath at (45 ± 1) °C. Aseptically add calciumglucose solution (A1) (500 µl/50 ml) and distribute 2,5 ml aliquots into culture tubes with caps, placed in a

water bath at (45 ± 1) °C. To each tube, add 1 ml of inoculum culture, mix carefully avoiding the formation of air bubbles and pour the contents onto a layer of TYGA (A.2) in a 9 cm Petri dish. Distribute evenly, allow to solidify on a horizontal, cool surface and dry in a laminar flow cabinet or in a (36 ± 2) °C incubator for 30 min, while the plates are inverted with the lids off.
Place one drop of the chloroform-treated culture on the inoculated plate using a fine capillary or pipette. Do not damage the top agar layer. Leave the spot to dry and incubate the plates upside-down at (36 ± 2) °C for (18 ± 2) h.
Examine the plate for a clear zone in the spotted area, which is indicative of the presence of F-specific RNA phages in the original sample.

NOTES
11. This procedure can also be used in an MPN format (ISO 8199) or to examine larger samples. In the latter case, use double strength TYGB (double the amounts of ingredients in the same amount of water as used for single strength TYGB; add a proportional volume of calcium glucose solution) in equal volumes as the sample. To obtain sufficient aeration during enrichment, make sure that the volume of sample and broth is not greater than 20% of the nominal capacity of the conical flask.
12. More than one spot can be placed on the surface of an inoculated plate.

11.6 Quality assurance

11.6.1 Plaque count procedures (11.1 - 11.4)

With each series of samples, examine a procedural-blank using sterile diluent as the sample and a reference control sample of MS2 (10.3). Plot the results on a control chart.
Optionally, use in addition a naturally polluted reference control sample, using sewage or surface water, diluted to approx. 100 pfp/ml in peptone-saline and 5 % (w/v) glycerol and stored at (-20 ± 5) °C or (-70 ± 10) °C. Discard the reference control samples if the concentration of RNA-phages decreases.

11.6.2 Presence/absence test (11.5)

Prepare a reference control sample as described in 10.3 with a concentration of plaque-forming particles of MS2 of approx. 5 ml^{-1}. Examine at least one control sample in parallel with each series of samples tested, expecting to obtain a positive test-result. To examine possible interfering effects from the samples, consider also adding the reference control sample to a second enrichment culture containing the actual sample.

NOTE 13
In the absence of easily available standardized reference materials, any exchange programme of reference samples between laboratories should be encouraged.

If sensitivity to phages is lost (not usual but may happen very sudden and complete), prepare a new set of inocula according to 10.1.2.

12 EXPRESSION OF RESULTS

12.1 Plaque count procedures (11.1 - 11.4)

Select plates with 30 - 300 plaques whenever present. From the number of plaques counted, and taking into account the results of previous confirmatory tests, calculate the number of plaque-forming particles of F-specific RNA bacteriophages in 1 ml of the sample as follows:

$$pfp / ml = \frac{N - N_{RNase}}{n} \times F$$

pfp/ml	confirmed number of plaque forming particles of F-specific RNA bacteriophages per ml.
N	total number of plaques counted on WG49 plates according to 11.1, 11.2 or 11.4
N_{RNase}	total number of plaques counted on WG49 plates with RNase according to 11.3
n	the number of replicates
F	the dilution (or concentration) factor (1/5 in the case of 11.4)

12.2 Presence/absence test (11.5)

Express the results as "F-specific RNA phages (not) detected in V ml", V being the volume of sample examined.

13 TEST REPORT

The test report shall contain the following information:

a) a reference to this standard;
b) all details necessary for complete identification of the sample;
c) if a confirmatory test was used and the ratio of N_{RNase} to *N* (as a percentage);
d) the results expressed in accordance with article 12;
e) any other information relevant to the method.

ANNEX A
(NORMATIVE)
CULTURE MEDIA, REAGENTS AND DILUENTS

A.1 Tryptone-yeast extract-glucose broth (TYGB)

Basal medium

Trypticase peptone	10 g
Yeast extract	1 g
NaCl	8 g
Distilled water	1000 ml

Dissolve the ingredients in hot water. Adjust pH so that after sterilization it will be 7,2 ± 0,1 at 25 °C. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Calcium-glucose solution

$CaCl_2.2H_2O$	3 g
Glucose	10 g
Distilled water	100 ml

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

Complete medium

Basal medium	200 ml
Calcium-glucose solution	2 ml

Aseptically add calcium-glucose solution to basal medium and mix well. If not for immediate use, store in the dark at (5 ± 3) °C for not longer than 6 months.

A.2 Tryptone-yeast extract-glucose agar (TYGA)

Basal medium

Trypticase peptone	10 g
Yeast extract	1 g
NaCl	8 g
Agar	12 - 20 g*
Distilled water	1000 ml

*) Depending on the gel strength of the agar

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7,2 ± 0,1 at 25 °C. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark at (5 ± 3) °C for not longer than 6 months.

Complete medium

Basal medium	200 ml
Calcium-glucose solution (A1)	2 ml

Melt the basal medium and cool to between 45 °C and 50 °C. Aseptically add calcium-glucose solution, mix well and pour in Petri dishes:
- 20 ml in dishes of 9 cm diameter
- 50 ml in dishes of 14 cm diameter

Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months if well protected against desiccation.

A.3 Semi-solid tryptone-yeast extract-glucose agar (ssTYGA)

Prepare basal medium according to A.2 but use half of the weight of agar (6 - 10 g), depending on gel strength; the gel strength of ssTYGA is critical to obtain good results and if possible different concentrations should be tested. Distribute over bottles in 50 ml volumes.

A.4 Nalidixic acid solution

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

Dissolve nalidixic acid in NaOH-solution, add distilled water and mix well. Filter-sterilize through an 0,22 μm membrane filter. Store at (5 ± 3) °C for not longer than 8 hours or at (-20 ± 5) °C for not longer than 6 months.

A.5 RNase-solution

RNase	100 mg
Distilled water	100 ml

Dissolve RNase in water while heating for 10 min at 100 °C. Distribute over plastic cups in 0,5 ml volumes and store at (-20 ± 5) °C for not longer than 1 year. Thaw at room temperature before use.

A.6 Glycerol (sterile)

Glycerol (870 g/l)	100 ml

Distribute over bottles in 20 ml volumes and sterilize in the autoclave at (121 ± 1) °C for 15 min. Store in the dark for not longer than 1 year.

A.7 McConkey agar

Pepton	20 g
Lactose	10 g
Bile salts	5 g
Neutral red	75 mg
Agar	12 - 20 g
Distilled water	1000 ml

Dissolve the ingredients in boiling water. Adjust pH so that after sterilization it will be 7,4 ± 0,1 at 25 °C. Distribute the medium in bottles in volumes of 200 ml and sterilize in the autoclave at (121 ± 1) °C for 15 min. Cool to between 45 °C and 50 °C and pour 20 ml in Petri dishes of 9 cm diameter. Allow to solidify and store in the dark at (5 ± 3) °C for not longer than 6 months.

A.8 Peptone saline solution

Peptone	1,0 g
Sodium chloride	8,5 g
Distilled water	1000 ml

Dissolve the ingredients in about 950 ml of the water if necessary by heating. Make up to 1000 ml with the water, dispense in convenient volumes and autoclave at (121 ± 1) °C for 15 min. The final pH should be 7,0 ± 0,5. Store in the dark for not longer than 6 months.

ANNEX B
(INFORMATIVE)

GENERAL DESCRIPTION OF F-SPECIFIC RNA BACTERIOPHAGES

F-specific RNA bacteriophages are bacteriophages (bacterial viruses) which consist of a simple capsid of cubic symmetry of 21 - 30 nm in diameter and contain single-stranded RNA as the genome. They belong to the morphological group E and are classified into the family *Leviviridae.* The family presently consists of two genera; *Levivirus,* for which phage MS2 is the type species and *Allolevivirus,* for which phage Qß is the type species. They are infectious for bacteria which possess the F- or sex plasmid orginally detected in *Escherichia coli* K-12, and adsorption to the F- or sex-pili coded by this plasmid. The F plasmid is transferable to a wide range of Gram-negative bacteria. The infectious process is inhibited by the presence of RNase in the growth medium, which can be used to distinguish between the F-specific RNA bacteriophages and the rod-shaped F-specific DNA bacteriophages of the *Inoviridae* family.

ANNEX C
(INFORMATIVE)
CULTURING OF BACTERIOPHAGE MS2

Use standard procedures for phage propagation as described in the open literature. The following is an example of a procedure which has proven to give good results.
Introduce 25 ml TYGB into a conical flask of 300 ml and inoculate with an appropriate host strain (e.g. *E. Coli* K-12 Hfr, NCTC 12486). Incubate for (20 ± 4) h at (36 ± 2) °C while shaking at (100 ± 10) min^{-1}.
Prewarm 25 ml TYGB in a conical flask of 300 ml to 35 - 37 °C and inoculate with 0,25 ml of the overnight culture.
Incubate as above for 90 min. Add MS2 from a stock solution to a final concentration of approx. 10^7 pfp/ml.
Incubate as above for 4 - 5 h. Add 2,5 ml of chloroform ($CHCl_3$), mix well and place overnight at (5 ± 3) °C.
Decant the aqueous phase in centrifuge tubes and centrifuge at a minimum of 3000 x *g* for 20 min.
Pipet the supernatant carefully and store at (5 ± 3) °C.

NOTES
14. The titer of the phage-suspension should be above 10^{10} pfp/ml and may reach up to 10^{13} pfp/ml. In some cases it may be necessary to repeat the cycle to obtain sufficiently high titres, higher phage inputs may then be used.
15. The titre of the phage-stock suspension will slowly decrease in time.

Figure 1. Nephelometric conical flasks for culturing the host strain

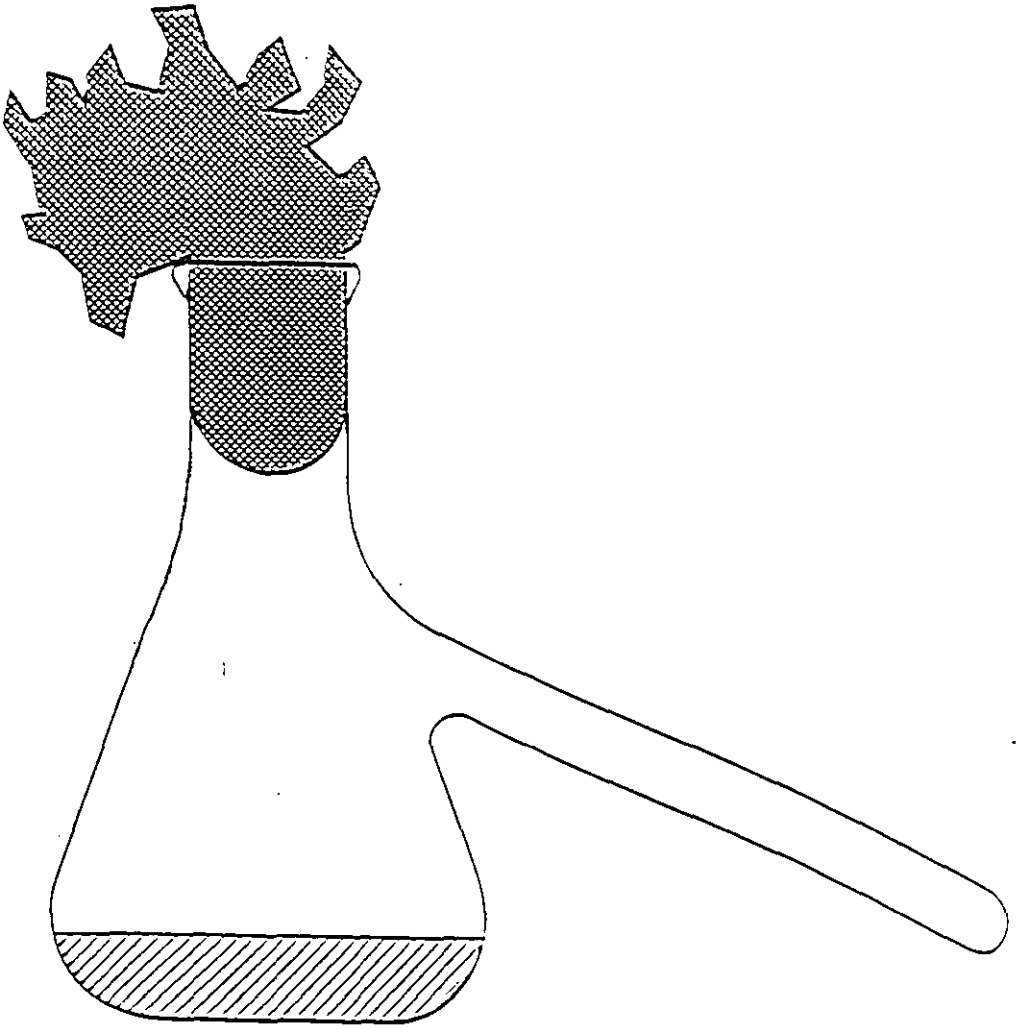
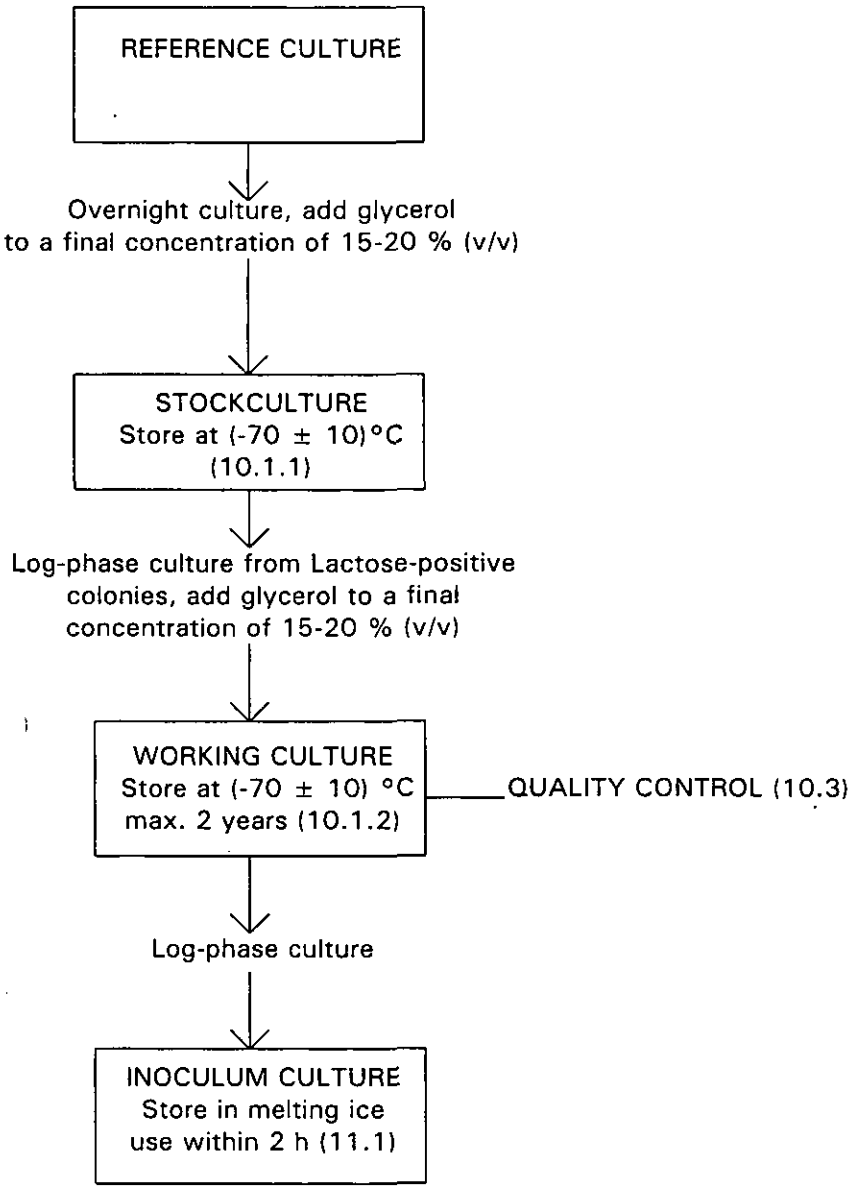


Figure 2. Scheme for culturing, maintenance and quality control of host strain WG49



Annex 8

Detection of bacteriophages of *Bacteroides fragilis*

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DETECTION of BACTERIOPHAGES of *Bacteroides fragilis*

Recommended strains. The recommennded strain is *Bacteroides fragilis* HSP40 (ATCC 51477) (Tartera and Jofre, 1987). Bacteriophage B40-8 (Tartera and Jofre, 1987).

Culturing and maintenance of host strains

* **Culture conditions.** *Bacteroides fragilis* is an anaerobic bacteria, therefore it should be manipulated as a such. However, *B. fragilis* does not require very rigurous anaerobic conditions either for manipulation or for growing. However, incubations of cultures in agar medium have to be performed in anaerobic jars. In contrast, if the cultures are prepared in liquid medium, it is sufficient to fill completely with medium the tubes, flasks and bottles and close with screwable caps.

* **Preparation of stock cultures.** Rehydrate the content of a lyophilized ampole of the reference culture of the host strain with 3 ml of BPRM-broth using a Pasteur pipete in a 3 ml screw-capped vial. Incubate for 4-5 hour at (36 ± 2) °C. Add the suspension to 30 ml of BPRM-broth in a 30 ml screw-caped tube. Incubate overnight (18 ± 2) hours at (36 ± 2) °C . Alternatively if a culture in a slant is available, streak on a plate of BPRM agar. Incubate on an anaerobic jar or bag for (36 ± 2) hours at (36 ± 2) °C . Inoculate cells from the slant into 30 ml of BPRM-broth in a 30 ml screw-caped tube. Incubate overnight at (36 ± 2) °C.

Add 20 % (v/v) steril glycerol to a liquid culture of the host strain at the end of the logarithmic growth phase. Mix well. Distribute into vials in 1 ml aliquotes and store at (- 70 ± 10) °C.

NOTE. 1) This first passage of the host strain should be strored as a standard in the laboratory. 2) Purity of the culture should be checked before storage. 3) Sensitivity to B40-8 should be tested before storage.

* **Preparation of working cultures.** Thaw one vial of stock culture at room temperature and streak on a plate of BPRM agar. Incubate on an anaerobic jar or bag for (36 ± 2) hours at (36 ± 2) °C. If necessary store at (5 ± 2) °C for not longer than 2 weeks on an anaerobic jar or bag. Use one slant (*) to prepare inoculum cultures and one slant to prepare fresh subcultures. Do not use more than 10 - 12 serial passages from one stock culture.
(*) The slant grown on the surface of the Petri dish.

* **Preparation of inoculum cultures.** Add 30 ml of BPRM broth to a 30 ml.screw-caped tubes. Inoculate cell material from a slant (**) of working culture. Incubate overnight at (36 ± 2) °C.

Add 27 ml of BPRM broth to a 30 ml screw-caped tubes. Adjust spectrophotometer reading to 0. Add to each tube 3 ml of the previous culture.

Incubate at (36 ± 2) °C. After 1 hour measure turbidity every 30 minutes. For calibration of turbidity measurements follow the normal procedures as described in the open literature, taking all the precautions derived from the anaerobic characteristics of *B. fragilis*. At the turbidities corresponding to the concentrations of colony forming particles (CFP) recommended for the different detection methods, the culture is ready for performing tests (DAL, presence/absence or MPN adaptation of the presence/absence test). For DAL is recommended an $O.D._{620\ nm}$ = 0,3 - 0,5. For presence/absence tests (enrichments) an $O.D._{620\ nm}$ = 0,6 - 0,8.

(**)The slant should occupy the whole surface of the plate. It is recommended to use ¼ of the plate per tube

*** Calibration of turbidity measurements (alternatively).** Add 30 ml of BPRM broth to a screw-caped 30 ml tube. Inoculate cell material from a slant on BPRM-agar. Incubate overnight at (36 ± 2) °C. Transfer 2 ml of this culture to a 30 ml tube for anaerobic cultures (Hungate tubes with butyl rubber stopper and screw cap; Bellco Glass, Inc.) containing 28 ml of BPRM broth. Before inoculation, adjust the spectophotometer reading to 0 on the tube. 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 1 ml sample for viable cell counts. Ensure that the tube is taken from the incubator for as short a time as possible. For viable counts use standard procedures, ensuring that the process is performed in a period of time as short as possible, that the diluents had been previously reduced and that the plates are incubated on an anaerobic jar.

*** Standard procedures for quantifying bacteriophages**

 # Double Agar Layer (DAL) procedure.

Prewarm sample at room temperature. Melt tubes of 2,5 ml of ssBPRM-agar in a boiling water bath and place in a water bath at (45 ± 1) °C. Before to add the sample and the host strain to ssBPRM-agar take out the tubes from the bath (the best is to take two tubes each time to avoid agar solidification).

To each tube add 1 ml sample, or dilution or concentrate. Examine each volume or dilution step at least in duplicate.

Add 0,5 ml of inoculum culture, mix carefully avoiding the formation of airbubbles and pour the contents onto a layer of bottom BPRM-agar in a 90 mm Petridish. Distribute evently, allow to solidify on a horizontal, cool surface and incubate the plates upside down in an anaerobic jar at (36 ± 2) °C for (18 ± 2) hours. After incubation, count the number of paques on each plate.

With each series of samples examine a procedural-blank using sterile diluent and standard prepatation of B40-8. Stocks of B40-8 prepared as described below maintain the numbers for at least 6 months when stored at 4°C.

For samples containing high background flora it is recommended to decontaminate by filtration through low protein binding membranes, as for example those of GV Millex from Millipore, 0,22 μm pore size (Araujo et al, 1993).

NOTE: 1) *B. fragilis* is an anaerobic bacteria. Therefore: distribute inoculated tubes as fast as possible; introduce the plates into the anaerobic jars as soon as possible, once dried.

 # MPN-procedure

Add 10 ml of double concentrated BPRM broth to 30 ml screw-caped tubes.

Inoculate series of 5 tubes with 10 ml of consecutive decimal dilutions prewarmed at room temperature and reduced of the samples. For instance:

- 5 tubes with 10 ml of the sample
- 5 tubes with 10 ml of the dilution 1/10
- 5 tubes with 10 ml of the dilution 1/100

The number of dilutions to inoculate is estimated from the presumed phage concentration of the sample.

Add to each tube 10 ml of a host culture in the exponential phase containing 10^8 cell per ml ($O.D._{620\ nm}$ = 0,3). Tubes should be completely full when incubated under non anaerobic conditions.

Incubate at (36 ± 2) °C for 24 hours.

Transfer 1 ml of the culture to a centrifuge tube, add 0,4 ml of chloroform, mix well and centrifuge at 3000 g for 5 minutes.

Melt sufficient tubes containing 2,5 ml of ssBPRM-agar in a boiling water bath and place in a water bath at (45 ± 1) °C. To each tube add 0,5 ml of inoculum culture, host culture in the logarithmic grow phase containing around 10^8 bacteria per ml ($O.D._{620\ nm}$ = 0,3 - 0,5). Mix carefully avoiding the formation of airbubbles and pour the contents onto a layer of bottom BPRM-agar in a 90 mm Petridish. Distribute evently, allow to solidify on a horizontal, cool surface.

Place one drop of each one of the chloroform-treated cultures on the inoculated plates using a fine capillary or a pipette. Do not damage the top agar layer. Leave the spot to dry and incubate the plates upside down in an anaerobic jar at (36 ± 2) °C for (18 ± 2) hours.

Examine the plate for clear zone in the spotted area, which is indicative of the presence of *B. fragilis* phages in the original sample.

Refer the results to the MPN tables (APHA, 1985).

For quality assurance, prepare a control sample with a concentration of plaque forming particles of B40-8 of approximately 10 ml^{-1}. Examine at least one control sample in parallel with each series of samples tested, expecting to obtain a positive test-result.

 # Presence/absence test in medium volumes

The precedent method can also be used to examine the presence of bacteriophages in large (100 ml or 500 ml) samples. For instance, for presence/absence in 100 ml, proceded as follows:

Prepare recipients of inoculum culture, 250 ml screw-caped steril bottles, containing 100 ml of double concentrated BPRM broth. Add 100 ml of the sample prewarmed at room temperature. Add 30 ml of a host culture in the exponential phase containing 10^8 cell per ml ($O.D._{620\ nm}$ = 0,6 - 0,8). Bottles should be completely full with BPRM broth double concentrated and caps screwed when incubated under non anaerobic conditions. Incubate for 24 hours at (36 ± 2) °C.

Remove 1 ml and proceed as for the MPN test.

NOTE: To avoid the noxious effect of oxigen disolved in the sample on the host cells, mainly for the MPN and the presence/absence test, it is recommended to treat the sample for the removal of oxigen. This can be done through the bubbling of nitrogen for 5 minutes at a rate of 5 L. min^{-1} or for addition of a reducing solution, as for example Na_2S (final concentration 0,04 % w/v). By adding a resarzurine solution (0,5 ml . 100 ml^{-1} of a solution of 0,025 g.100 ml^{-1}), anaerobiosis is marked by the change of color from blue to straw.

Culturing of bacteriophage B40-8

Use normal procedures for phage propagation as described in the open literature. The following is an example of a procedure which has given good results.

Incubate a culture of *B. fragilis* as for a inoculum culture as described above. When the culture reaches $O.D._{620\ nm}$ = 0,1 - 0,2 (about 10^8 cell per ml) add B40-8 from a stock to give a final concentration of plaque forming particles of approximately 10^8 ml^{-1} (rate 1:1 CFU:PFU).

ncubate overnight. Mix with chloroform.. Shake thorougly with a mixotube for 3 minutes and centrifuge at a minimum of 3000 g for 10 minutes.
Pipette the supernatant carefully into a sterile tube and store at (5 ± 2) °C. It is recommended to filtrate the supernatant through GV Millex filters from Millipore, pore size 0,22 μm.

The titre of the phage suspensions should be above 10^9 ml^{-1} and frequently reaches values over 10^{10} ml^{-1}. The titre of the phage stock suspension will decrease very slowly in time.

References

Tartera C. and J. Jofre. 1987. Bacteriophages active against *Bacteroides fragilis* in sewage polluted waters. Applied and Environmental Microbiology. **53**: 1632-1637.

Tartera C., R. Araujo, T. Michel and J. Jofre. 1992. Culture and Decontamination Methods Affecting Enumeration of Phages Infecting *Bacteroides fragilis* in Sewage. Applied and Environmental Microbiology **58**: 2670-2673.

APHA. 1985. Standard methods for the examination of water and wastewater, !6th ed. American Public Health Association. Washington D.C. USA.

MEDIA

The recommended medium is BPRM (Tartera et al, 1991).

BPRM-broth

* Meat peptone	10 g
* Casein peptone	10 g
* Yeast extract	2 g
* NaCl	5 g
* Monohydrated L-cysteine	0,5 g
* Glucose	1,8 g
* $MgSO_4 . 7 H_2O$	0,12 g
* Distilled water	999 ml
* $CaCl_2$ in solution (0,05 g/ml)	1 ml

Sterilize at 121°C for 15 minutes. Then asseptically add the indicated amounts of the following filter-sterilized solutions:

* Hemin, 0,1 % (w/v) in NaOH 0,02 % (w/v)	10 ml
* Na_2CO_3 1 M	25 ml

Asseptically adjust to pH 7 with HCl 35 %.

It is recommended to add always Kanamycine monosulfate (100 $\mu g\ ml^{-1}$) and Nalidixic acid (*) (100 $\mu g\ ml^{-1}$) to the medium to prevent contamination.

BPRM-broth is recommended storage at 37°C no longer than one week.

(*) Disolve Nalidixic acid according to ISO 10705-2.

BPRM-agar for plates

* BPRM-broth
* 1,5 % agar-agar

Keep the plates at 4°C during long time but take them at room temperature 1h-2h before use.

ssBPRM-agar

* BPRM-broth
* 0,7 % agar-agar

ssBPRM-agar is recommended to be used within 1 week after preparation.

It can be kept melt at 55°C or aliquoted in 2,5 ml per tube solidified and just before use, melt it in a boiling water bath.