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Preparation and use of reference materials containing bacteriophages

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Abstract

During the EU-project ‘Bacteriophages in bathing water’ (January 1996 - June 1999) research was carried out on the development of reference materials for the implementation of methods for the determination of bacteriophages in water. Several batches of reference materials (RMs) containing pure phage cultures, as well as naturally polluted standard samples, containing a mixture of (naturally occurring) phages and background flora, were prepared. Homogeneous and stable RMs containing pure phage cultures could be prepared for all three types of bacteriophages considered during the project: somatic coliphages (SOMCPH), F-specific RNA phages (FRNAPH) and phages of *Bacteroides fragilis* (BFRPH). The RMs were prepared by mixing phage suspensions with glycerol (to a final concentration of 5% v/v) and storing of the final materials at (-70 ± 10) °C. The standard phages used were ΦX174 for SOMCPH, MS2 for FRNAPH, B40-8 for BFRPH with host HSP40 and B56-1 for BFRPH with host RYC 2056. The phage RMs were shown to be useful for quality control purposes in the preparation of control charts. The naturally polluted standard samples were prepared by mixing sewage with peptone saline solution and glycerol. These materials were less homogeneous than the pure culture phage RMs. The standard samples were stable when stored at (-70 ± 10) °C for at least 4 months and also at (-20 ± 5) °C for a period of 1-2 months. At temperatures above 0 °C the stability was poor.

Contents

Abbreviations and symbols

ABBREVIATIONS

ATCC	American Type Culture Collection; 10801 University Boulevard; Manassas, VA 20110-2209; USA (http://www.atcc.org)
BFRPH	phages of *Bacteroides fragilis*
(M)BPRM	(Modified) Bacteroides Phage Repair Medium
B40-8	Phage of *Bacteroides fragilis*
DAL	Double Agar Layer method
FDNAPH	F-specific DNA bacteriophages
FRNAPH	F-specific RNA bacteriophages (=FTOTPH - FDNAPH)
FTOTPH	Total number of F-specific bacteriophages (= FRNAPH + FDNAPH)
HSP40	*Bacteroides fragilis* (host for phages (like B40-8) of *B.fragilis*); culture collection number: ATCC 51477.
IPL	Institute Pasteur of Lille
MGB	Microbiological Laboratory for Health Protection
MSA	Modified Scholtens' Agar
MS2	F-specific RNA phage; culture collection numbers: ATCC 15597-B1; NCTC 12487
NCTC	National Collection of Type Cultures; PHLS Central Public Health Laboratory; 61 Colindale Avenue; London NW9 5HT; UK
pfp	plaque forming particle
ps	peptone saline solution
RIVM	National Institute of Public Health and the Environment
RM	Reference material
RYC2056	*Bacteroides fragilis* (host for phages (like 56-1) of *B.fragilis*)
SAL	Single Agar Layer method
SOMCPH	Somatic coliphages
ssMSA	semi-solid Modified Scholtens' Agar
ssTYGA	semi-solid Tryptone Yeast-extract Glucose Agar
UB	University of Barcelona
WG5	*Escherichia coli* Nal^r (host for somatic coliphages); culture collection number: ATCC 700078.
WG49	*Salmonella typhimurium* (F^+ strain, host for FRNAPH); culture collection numbers: ATCC 700730; NCTC 12484.
ΦX174	Somatic coliphage; culture collection number: ATCC 13706-B1

SYMBOLS

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) $T_1 = \sum_i \sum_j [(z_{ij} - z_{i+} / J)^2 / (z_{i+} / J)]$
T_2	Cochran's dispersion test statistic to determine the variation in pfp between different vials of one batch of reference materials $T_2 = \sum_i [(z_{i+} - z_{++} / I)^2 / (z_{++} / I)]$
$\bar{x}$	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

Samenvatting

Tijdens het EU-project ‘Bacteriofagen in zwemwater’ (januari 1996 - juni 1999) werd onderzoek uitgevoerd naar de ontwikkeling van referentie materialen voor de implementatie van methoden voor de bepaling van bacteriofagen in water. Verschillende partijen referentie materialen (RMs) met reincultures van fagen, alsmede ook natuurlijk besmette standaard monsters met mengsels van (natuurlijk voorkomende) fagen en achtergrond flora werden bereid.
Voor alle drie de typen bacteriofagen welke tijdens dit project van belang waren, werden homogene en stabiele referentie materialen met reincultures van fagen bereid: somatische colifagen (SOMCPH), F-specifieke RNA fagen (FRNAPH; welke het verschil is tussen het totaal aantal F-specifieke fagen (FTOTPH) en het aantal F-specifieke DNA fagen (FDNAPH)) en fagen van *Bacteroides fragilis* (BFRPH). De faag RMs werden bereid door faag suspensies met glycerol te mengen (tot een eindconcentratie van 5% v/v) waarna de materialen bij -70 °C werden opgeslagen. De gebruikte standaard fagen waren: ΦX174 voor SOMCPH, MS2 voor FRNAPH, B40-8 voor BFRPH met gastheer HSP40 en B56-1 voor BFRPH met gastheer RYC2056. Faag B56-1, gebruikt als standaard faag voor de analyse van BFRPH met gastheer RYC2056, produceerde zeer kleine (< 1 mm) plaques welke moeilijk te tellen waren. Aangezien dit niet was gevonden door de Universiteit van Barcelona, waar de orginele isolaat van deze faag vandaan kwam, is mogelijk een mutant van faag B56-1 geïsoleerd, welke alleen kleine plaques gaf. Voor toekomstig werk is het verstandig om een nieuwe standaard faag te selecteren voor de kwaliteitscontrole van BFRPH met gastheer RYC2056.
De natuurlijk besmette standaard monsters werden bereid door rioolwater te mengen met pepton fysiologische zoutoplossing en glycerol. Deze natuurlijk besmette standaard monsters vertoonden echter meer variatie in de tellingen dan de RMs met faag reincultures, mogelijk door de aanwezigheid van aggregaten, verstoring door bacteriële achtergrond flora en dien ten gevolge moeilijkheden bij het lezen van de platen.
De faag RMs, alsmede ook de natuurlijk besmette standaard monsters, vertoonden goede lange termijn stabiliteit bij opslag bij (-70 ± 10) °C en bij (-20 ± 5) °C voor een korte periode (*ca* 1-2 maanden). Wanneer de materialen opgeslagen werden bij temperaturen boven 0 °C (+5 °C, of bij kamertemperatuur, zoals 25 °C), was een snelle daling in het gemiddeld aantal plaques zichtbaar bij alle geteste fagen. Deze resultaten tonen aan dat het nodig is om de faag RMs en ook de natuurlijk besmette standaard monsters, in bevroren toestand te houden om herhaalbare resultaten te kunnen krijgen. Ook tijdens verzending dienen de materialen bevroren te blijven (bijvoorbeeld door middel van verpakking in droogijs en verzending met een koerier).
De faag RMs hebben hun nut bewezen voor kwaliteitscontrole doeleinden door middel van bereiding van controlekaarten.

Summary

During the EU-project 'Bacteriophages in bathing water' (January 1996 - June 1999) research was carried out on the development of reference materials for the implementation of methods for the determination of bacteriophages in water. Several batches of reference materials (RMs) containing pure phage cultures, as well as naturally polluted standard samples, containing a mixture of (naturally occurring) phages and background flora were prepared.
It was shown that homogeneous and stable reference materials (RMs) containing pure phage cultures could be prepared for all three types of bacteriophages considered during the project: somatic coliphages (SOMCPH), F-specific RNA phages (FRNAPH; which is the difference between the total number of F-specific phages (FTOTPH) and the number of F-specific DNA phages (FDNAPH)) and phages of *Bacteroides fragilis* (BFRPH). The RMs were prepared by mixing phage suspensions with glycerol (to a final concentration of 5% v/v) and storing of the final materials at -70 °C. The standard phages used were ΦX174 for SOMCPH, MS2 for FRNAPH, B40-8 for BFRPH with host HSP40 and B56-1 for BFRPH with host RYC 2056. Phage B56-1, used as standard phage for the analysis of BFRPH with host strain RYC2056, produced very small (< 1 mm) plaques that were difficult to count. As this was not discovered at the University of Barcelona, where the original isolate of this phage was obtained, probably a mutant of phage B56-1 was isolated which only gave small plaques. For future work it is advisable to select a new standard phage for the quality control of the enumeration of BFRPH with host strain RYC2056.
The naturally polluted standard samples were prepared by mixing sewage with peptone saline solution and glycerol. However, these naturally polluted standard samples showed more variation in counts than the pure phage culture RMs, probably because of the presence of aggregates, interference by bacterial background flora and corresponding difficulties with reading of the plates.
The phage RMs, as well as the naturally polluted standard samples, showed good long-term stability when stored at (-70 ± 10) °C and also when stored at (-20 ± 5) °C for a short period of time (*ca* 1-2 months). However, when the materials were stored at temperatures above 0 °C (+5 °C, or at room temperature like 25 °C) a rapid decrease in the mean number of plaques was found for all phages tested. These results showed that it is necessary to store the phage RMs, and also the naturally polluted standard samples, frozen to get repeatable results. Also mailing of the materials should be performed while keeping the vials frozen (e.g. packed in dry ice and transported by courier services).
The phage RMs were shown to be useful for quality control purposes in the preparation control charts.

1. Introduction

From January 1996 to June 1999 an EU-project was carried out entitled: 'Feasibility study on the development of a method for the determination of bacteriophages for monitoring the microbiological quality of bathing waters' (Bacteriophages in bathing waters; SMT4-CT95-1603). Objectives of this project are summarised in Mooijman *et al.* (1998). One of the objectives was: 'Development of reference materials for the implementation of the methods'. During the project several batches of reference materials (RMs) containing bacteriophages were prepared. For the preparation of these RMs the information of Schijven *et al.* (1995) was used. Reference materials containing pure phage cultures were prepared, but also naturally polluted standard samples, containing a mixture of (naturally occurring) phages and background flora were prepared. The RMs and the standard samples were used as samples during the two training sessions and the two collaborative studies (Mooijman *et al*., 1998 and 1999a) of the project. The main aim of these training sessions and collaborative studies was to evaluate the implementation of the methods for enumeration of the three groups of bacteriophages in naturally polluted standard (water) samples, in different EU laboratories: somatic coliphages (SOMCPH); F-specific RNA phages (FRNAPH; which is the difference between the total number of F-specific phages (FTOTPH) and the number of F-specific DNA phages (FDNAPH)) and phages of *Bacteroides fragilis* (BFRPH). The RMs were used for quality control purposes during these studies, and also during monitoring phases of the project in different EU-laboratories. The standard phages for the RMs were: ΦX174 for SOMCPH, MS2 for FRNAPH, B40-8 for BFRPH with host strain HSP40 and B56-1 for BFRPH with host strain RYC2056.

Information about the preparation and use of the phage RMs and the naturally polluted standard samples has already been given in earlier reports (Mooijman *et al*., 1998 and 1999a). However, to have a complete overview on all batches that were used during the project, all available information has been summarised in this report. For detailed information concerning the results found with the phage RMs and the naturally polluted standard samples during the training sessions and collaborative studies, reference is made to the relevant reports (training sessions: Pierzo *et al*., 1999 and Pierzo and Demarquilly, 1999; collaborative studies: Mooijman *et al*., 1998 and 1999a).

2. Materials and methods

2.1 General

The standard methods used for enumeration of the different phages were Amended ISO/CD 10705-2 of February 1997 (single agar layer (SAL) and double agar layer (DAL) method) for enumeration of somatic coliphages (SOMCPH; Mooijman *et al*., 1998; Annex 6) and Amended ISO 10705-1 of February 1997 for enumeration of F-specific RNA phages (FRNAPH; Mooijman *et al*., 1998; Annex 7). The method for the enumeration of phages of *Bacteroides fragilis* (BFRPH) was continuously improved during the project. For the early experiments (until *ca* January 1998) the procedure as described in Annex 8 of Mooijman *et al*. (1998) was applied. From January 1998 until *ca* May 1998 the procedure as described in Annex 2 of Mooijman *et al*. (1999a) was used. After May 1998, bile was omitted from the medium.
Plaques produced on WG49 *Salmonella typhimurium* can originate from different phage groups: F-specific RNA phages (FRNAPH), F-specific DNA phages (FDNAPH) and somatic Salmonella phages. By analysing the naturally polluted samples with and without the presence of RNase, respectively the total number of F-specific bacteriophages (FTOTPH) and the number of F-specific DNA phages (FDNAPH) are enumerated. The difference between FTOTPH and FDNAPH gives the number of FRNAPH.

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, B40-8 (Tartera and Jofre, 1987) as standard phage for host strain HSP40 *Bacteroides fragilis* and phage B56-1 as standard phage for host strain RYC2056 *Bacteroides fragilis* (Puig *et al.*, 1997).
The bacterial host strains used for enumeration of the bacteriophages were:
WG5 *Escherichia coli*, ATCC 700078 for SOMCPH; WG49 *Salmonella typhimurium*, NCTC 12484 (or ATCC 700730) for FRNAPH, HSP40 *Bacteroides fragilis*, ATCC 51477 for BFRPH and RYC2056 *Bacteroides fragilis* (Puig *et al.*, 1997) for BFRPH.

2.2 Phage reference materials

2.2.1 Preparation

The preparation of the phage RMs was as follows:

- A high titre phage suspension was prepared (following Annex C of amended ISO 10705-1: Annex 7 of Mooijman *et al.,* 1998) 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 of Mooijman *et al.,* 1998, A8). Phage counts (" plaque tests") were performed on the dilutions expected to give a countable number of plaques (each dilution in duplicate). All dilutions were stored at (5 ± 3) °C;
- Guided by these 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 plaque forming particles per millilitre (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.

Each batch of phage RMs obtained a batch number, which existed of the date of preparation in ddmmyy.
The following batches of phage RMs were used during the project:

For SOMCPH:
RMs containing ΦX174, batches: 030195 (*ca* 100 vials), 130396 (*ca* 360 vials), 040696 (*ca* 1200 vials) and 270597 (*ca* 1000 vials).
Batch 030195 was prepared before the start of the project and was prepared in a slightly different way from the procedure described above. For the preparation of this batch no glycerol was added to the ps and the vials were stored at (-20 ± 5) °C instead of at (-70 ± 10) °C. As the mean phage concentration of this batch was > 200 pfp/ml it was decided to analyse only 0.5 ml per vial.
Batch 0406096 was used during the first and the second collaborative study.

For FRNAPH:
RMs containing MS2, batches 071293 (*ca* 1250 vials) and 220597 (*ca* 1000 vials).
Batch 071293 was already prepared in 1993 by Schijven *et al* (1995). In 1996 still a sufficient number of vials was remained in stock for further use during the project. This batch was also used during the first collaborative study. Batch 220597 was used during the second collaborative study.

For BFRPH:
RMs containing B40-8, batches 271196 (*ca* 360 vials) and 260397 (*ca* 1000 vials)
RMs containing B56-1, batch 090698 (*ca* 400 vials).
Batch 260397 (containing B40-8) was used during the first and the second collaborative study. The number of phages in batch 090698 (containing B56-1) appeared to be very high. This was noticed only a few months after the preparation of the batch when it was found out that the host strain used at that moment was of poor quality. Therefore it was decided to analyse only 0.2 ml per vial to get countable phage numbers.

2.2.2 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 (Heisterkamp *et al.*, 1993).

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.

2.2.3 Thawing studies

It was tested whether slow thawing (by placing at room temperature) or quick thawing (by placing in a waterbath of 37 °C) of the frozen vials (at -70 °C) containing the pure phage suspensions would influence the phage counts. The following experiment was carried out using phage RMs containing ΦX174 (batch 040696) and MS2 (batch 071293):
- Twenty vials of one batch of reference materials were taken from the -70 °C freezer;
- Ten vials were thawed (slowly) at room temperature and phage enumerations of the vials were made (in duplicate) as soon as the vials were completely thawed. Necessary time for thawing was noted. For analysis of ΦX174 the SAL method and for MS2 the DAL method (see 2.1) was used;
- The remaining ten vials were thawed quickly by placing them in a waterbath of (37 ± 1) °C for *ca* 2 min. Necessary time for thawing was noted. Each vial was enumerated in duplicate;
- Student t-test (Wardlaw 1993) was performed per batch on the geometric means of the duplicates.

2.2.4 Determination of stability

2.2.4.1 Control charts

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. Of some batches the number of available data was limited. In these cases the control limits were calculated with only 10 results. 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 (or 10) 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 construction and use of control charts is described by Dommelen (1995).

2.2.4.2 Challenge tests

To find out the influence of elevated temperatures to the stability of the phage reference materials a so-called challenge test was carried out. For this purpose phage RMs were stored at different temperatures and the number of phages were counted regularly.
The following experiment was carried out using phage RMs containing ΦX174 (batch 040696) and MS2 (batch 071293):
- At day = 0, 60 vials of each batch of RMs were stored at -70 °C (control), at -20 °C, at 5 °C and at 25 °C (making a total amount of 240 vials per batch);

- At day = 0 another 20 vials of the same batch was taken from the stock stored at -70 °C for the first control (day=0). The vials containing ΦX174 were quickly thawed by placing them in a waterbath of (37 ± 1) °C for 2.5 min. The vials containing MS2 were slowly thawed by placing them at room temperature. Each vial was enumerated in duplicate. The RMs containing ΦX174 were analysed by using semi-solid Modified Scholtens' Agar (ssMSA) with Nalidixic acid and the SAL method (see 2.1). The RMs containing MS2 were analysed by using semi-solid Tryptone glucose yeast extract agar (ssTYGA) with Nalidixic acid;
- Twice a week, 5 vials in duplicate of each batch and of each storage temperature were measured for in total 6 weeks. The thawing method and the enumeration procedure as described for day=0 were used (with as much as possible the same batches of media etc.).

The results (per batch) were log-transformed (natural logarithm) and analysed with linear regression (Wardlaw, 1993).

2.3 Naturally polluted standard samples

2.3.1 Preparation

A sample of *ca* 5 litre was taken on 170696 from a primary effluent of Santa Adrià (near Barcelona) by the Department of Microbiology of the University of Barcelona (UB). The sample was filtered through a 12 µm pore size filter at the UB. *Ca* 2.5 litre of the filtered sample was sent to the Microbiological Laboratory for Health Protection (MGB) of the National Institute of Public Health and the Environment (RIVM) by courier service (cooled with cooling elements). On 180696 the sample arrived at MGB. Here it was filtered (again) through a membrane filter with 12 µm pore size. The sample was called UB180696. At the day of arrival, the sample was analysed for SOMCPH (with host WG5) and F-specific bacteriophages (with host WG49; with and without the presence of RNase). The remaining sample was mixed with glycerol to a final concentration of 5% (v/v) and distributed into vials in 2.5 ml aliquots and stored at (-70 ± 10) °C.

For use during the second collaborative study (Mooijman *et al.,* 1999a) 3 batches of naturally polluted standard samples were prepared in the following way:
In the period September-December 1997 sewage samples were collected in the Netherlands and in Spain. For each type of phage different dilutions of sewage samples were prepared, mixed with glycerol (until 5 % (v/v) in the sample) and stored at (-70 ± 10) °C. The sewage samples were diluted with peptone saline solution (ps) to obtain a 'countable' number of plaques for each phage type (preferably 30 - 150 pfp/ml).
For SOMCPH, 8 ml sewage of 'De Bilt' (The Netherlands) of 281097 (ddmmyy) was mixed with 1900 ml sterile peptone saline solution (ps: Mooijman *et al*., 1998; Annex 7) and 100

ml sterile glycerol. The mixture was distributed into vials in 2.5-3 ml aliquots and stored at (-70 ± 10) °C. In total 520 vials of batch De Bilt 281097 were prepared.
For F-specific phages, 44 ml sewage of 'De Bilt' of 071197 was mixed with 2850 ml ps and 150 ml glycerol. The mixture was distributed into vials in 4.5 ml aliquots and stored at (-70 ± 10) °C. In total 540 vials of batch De Bilt 071197 were prepared.
For BFRPH, 2 litre sewage 'Barcelona' (Spain) of 311097 was mixed with 100 ml glycerol. The mixture was distributed into vials in 3 ml aliquots and stored at (-70 ± 10) °C. In total 500 vials of batch Barcelona 311097 were prepared.

2.3.2 Determination of homogeneity

Of batch UB180696 no separate homogeneity study was done, only in parallel with the thawing studies (see 2.3.3).
Of the other three batches the mean number of pfp/ml for the three phage types (SOMCPH, F-specific and BFRPH) and the homogeneity of the vials were checked before and after freezing of the vials, by checking 5 vials of each batch in duplicate. Thawing of the vials was performed by placing the vials at room temperature. Thawing time was dependent on the volume in the vial, and varied from 30 to 60 min.
In the standard method for the analysis of BFRPH filtering of the sample through a low protein binding filter (Millipore Millex GV, 0.22 µm pore size) is advised to eliminate background flora. The vials for the analysis of SOMCPH and F-specific phages were for later analysis (after *ca* one month of storage) also pre-filtered with the same type of filter. This was done to try to exclude large variations between vials, probably due to the existence of aggregates. The variation in results between the filtered and non-filtered vials of each batch of naturally polluted standard samples was checked by analysing the T_1 and T_2 values. The same criteria as described for the phage RMs (see 2.2.1) were aimed at.

2.3.3 Thawing study

A thawing study as described in 2.2.3 was performed with the naturally polluted standard samples UB180696. The samples were analysed for SOMCPH and for F-specific bacteriophages (FTOTPH and FDNAPH). For analysis of both phage types the DAL method (see 2.1) was used.

2.3.4 Determination of stability

2.3.4.1 Stability studies

A rough estimation of the stability of the naturally polluted samples of batch UB180696 was made by analysing, at the day of arrival (180696), the sample for SOMCPH (with host WG5) and F-specific bacteriophages (with host WG49; with and without the presence of RNase). On 010796 (14 days after sampling) and on 011096 (*ca* 3.5 months after sampling) again SOMCPH (with host WG5) were analysed. On 031096 (*ca* 3.5 months after sampling) also again F-specific bacteriophages (with host strain WG49) were analysed.

Batches De Bilt 281097 (for analysing SOMCPH), De Bilt 071197 (for analysing F-specific bacteriophages) and Barcelona 311097 (for analysing BFRPH) were regularly controlled for their stability by analysing 5 vials in duplicate until the date of the second collaborative study (in total *ca* 4 months of storage at - 70 °C).

2.3.4.2 Challenge test

A challenge test as described in 2.2.4.2 was performed with the naturally polluted standard samples UB180696. The vials were thawed by placing them at room temperature and analysed for SOMCPH and for F-specific bacteriophages (FTOTPH and FDNAPH). For analysis of both phage types the DAL method (see 2.1) was used. The vials were stored at the different temperatures for almost 10 weeks (analysis for SOMCPH stopped after almost 8 weeks of storage).

3. Results

3.1 Thawing studies

3.1.1 Phage reference materials

In Table 1 the results of the thawing studies performed with the RMs containing ΦX174 and MS2 are summarised. The results showed no (significant) differences between slow thawing of the vials at room temperature and quick thawing of the vials at 37 °C for these batches of RMs.

Table 1 *Results of thawing studies (at room temperature and at 37 °C), with RMs containing ΦX174 (batch 040696) and RMs containing MS2 (batch 071293).*

	ΦX174 batch 040696		MS2 batch 071293	
	Thawing at room temp.[1]	Thawing at 37 °C	Thawing at room temp.[1]	Thawing at 37 °C
Thawing time (min)	25	2.5	30	2
Geom. mean pfp/ml	81.4	78.4	80.1	74.3
T_1[2]	4.67	8.84	16.48	5.69
T_2/ (I-1) [2]	0.58	0.95	1.13	1.39
p (t-test)	0.254		0.082	

[1]: Room temperature was *ca* 24 °C; [2]: At I (no. of vials) = 10 the χ^2 - distribution, 2-sided at 95% confidence has a lower limit of 3.25 and an upper limit of 20.48.

3.1.2 Naturally polluted standard samples

In Table 2 the results of the thawing study performed on the naturally polluted standard (sewage) samples UB180696 are summarised. For SOMCPH, a significant difference in the geometric mean number of plaques between the two thawing methods was found. Slow thawing of the vials at room temperature (18 - 25 °C) resulted in a higher mean phage count when compared to the results obtained from vials which were quickly thawed at 37 °C. The T_1 value of the room temperature results was somewhat high. This was caused mainly by a large duplicate variation of one vial. When the results of this vial were not used in the calculation, the geometric mean phage count became 48.2 (was 49.8), T_1 became 9.41 (was

25.08) and T_2/ (I-1) became 1.26 (was 2.92). Skipping the results of this vial had very little effect on the geometric mean pfp/ml. Therefore it was concluded that the large duplicate variation found in this vial might have been caused by poor mixing of the vial.

For the FTOTPH and FDNAPH (and consequently also for the FRNAPH) no significant differences were found in the geometric mean number of plaques with the two thawing methods. For FDNAPH, the T_1 value of the results found with the vials thawed at room temperature also happened to be high. Here again this was caused by only one vial. Not using this vial for the calculations resulted in a geometric mean phage count of 72.9 (was 72.1), a T_1 of 11.78 (was 27.67) and a T_2/ (I-1) of 0.38 (was 0.38).

Table 2 *Results of thawing studies (at room temperature and at 37 °C) with naturally polluted standard samples of batch UB180696.*

	SOMCPH		FTOTPH		FDNAPH	
	Thawing at roomtemp[1]	Thawing at 37 °C	Thawing at roomtemp[1]	Thawing at 37 °C	Thawing at roomtemp[1]	Thawing at 37 °C
Thawing time (min)	30	2.5	30	2.5	30	2.5
Geom.mean pfp/ml [2]	49.8	38.6	158.6	160.2	72.1	69.9
T_1 [3]	25.08	4.25	10.85	9.84	27.67	3.00
T_2/ (I-1) [3]	2.92	1.78	2.05	1.85	0.38	0.88
p (t-test)	0.002		0.788		0.354	

[1]: Room temperature was 18-25 °C; [2] : SOMCPH: counts are pfp/ml of 10^{-3} dilution, FTOTPH: counts are pfp/ml of 0.5 ml of 10^{-1} dilution, FDNAPH: counts are pfp/ml of 1 ml of 10^{-1} dilution;
[3]: At I (no. of vials) = 10 the χ^2 - distribution, 2-sided at 95% confidence has a lower limit of 3.25 and an upper limit of 20.48.

3.2 Somatic coliphages

3.2.1 Phage reference materials

During the project 4 different batches of reference materials containing a pure culture of phage ΦX174 were used. Each batch was checked for the criteria as stated in 2.2.1. The results of each batch before and a few days after freezing are summarised in Table 3.

Table 3 *Results of 4 batches of phage reference materials containing ΦX174, before and a few days after freezing.*

Batch number	Before freezing			After freezing at (-70 ± 10) °C		
	$\bar{x}$	T_1	T_2 / (I-1)	$\bar{x}$	T_1	T_2 / (I-1)
030195[1]	unknown	unknown	unknown	69.3[2]	4.35[2]	4.14[2]
040696	101.5	1.14	1.99	90.7[3]	2.71[3]	0.16[3]
130396	101.9	4.65	2.07	71.9[4]	3.03[4]	1.02[4]
270597	43.8	5.05	0.25	42.2[4]	7.84[4]	1.73[4]

$\bar{x}$: Geometric mean pfp/ml; I: number of vials, I=5 except for batch 270597 where I=10; [1]: Batch was frozen at (-20 ± 5) °C, without glycerol added; [2]: Analysis *ca* 1 year after freezing at (-20 ± 5) °C, values are in pfp/0.5 ml; [3]: Analysis 3 days after freezing; [4]: Analysis 2 days after freezing

All batches fulfilled the criteria of the mean level (between *ca* 30 and *ca* 150 pfp/ml) and of the criterion for the variation within vials (T_1 not significantly different from a χ^2 - distribution at 95% confidence level). The criterion for homogeneity between different vials (T_2 / (I-1) ≤ 2) was also fulfilled, except for batch 030195. This batch was prepared (no glycerol added) and stored (at -20 °C instead of -70 °C) in a different way than the other batches.

Control charts were prepared of each batch. The geometric mean values and the values of the warning and action limits of all batches are given in Table 4. The filled charts are shown in Figure 1 (batch 030195), Figure 2 and 3 (both for batch 040696) and in Figure 4 (batch 270597). Of batch 130396 no control chart is shown as this batch was used in a very short period of time, mainly for experiments to improve the method for detection and enumeration of somatic coliphages (Mooijman *et al.*, 1999b). Batch 030195 (Figure 1) showed very variable results. It was also shown with the T_2/(I-1) value (see Table 3) that this batch was not very homogeneous. Batch 040696 was immediate after preparation and storage analysed for the preparation of a control chart. This completed chart is presented in Figure 2. The 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 a 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 3 and showed stable results for the period measured (September 1996 - September 1998). Vials of batch 270597 were also immediate after preparation and storage analysed for the preparation of the control chart. This batch showed stable results, no initial decrease was visible here. The results of the chart shown in Figure 4 were obtained in an almost 2 years period (May 1997 - March 1999). One result (test number 13) of the chart was indicated as ‘out of control’. It was found

out that for the analysis on that day expired agar plates of Modified Scholtens' Agar (MSA) were used.

Table 4 Control chart values in pfp/ml of 4 batches of phage reference materials containing ΦX174

Batch	$\bar{x}$ - 3s	$\bar{x}$ - 2s	$\bar{x}$	$\bar{x}$ + 3s	$\bar{x}$ + 2s
030195[1,2]	54.9	66.4	97.1	141.9	171.6
040696[3]	49.3	57.3	77.4	104.4	121.3
040696[3] after recalculation	45.6	52.1	68.0	88.7	101.4
130396[1]	34.4	41.4	60.0	87.0	104.8
270597[4]	22.2	26.4	37.4	52.8	62.8

$\bar{x}$: Geometric mean; $\bar{x} \pm 2s$: warning limits; $\bar{x} \pm 3s$: action limits; [1]: Measured from 10 data; [2]: pfp/0.5 ml; [3]: Measured from 20 data; [4]: Measured from 19 data.

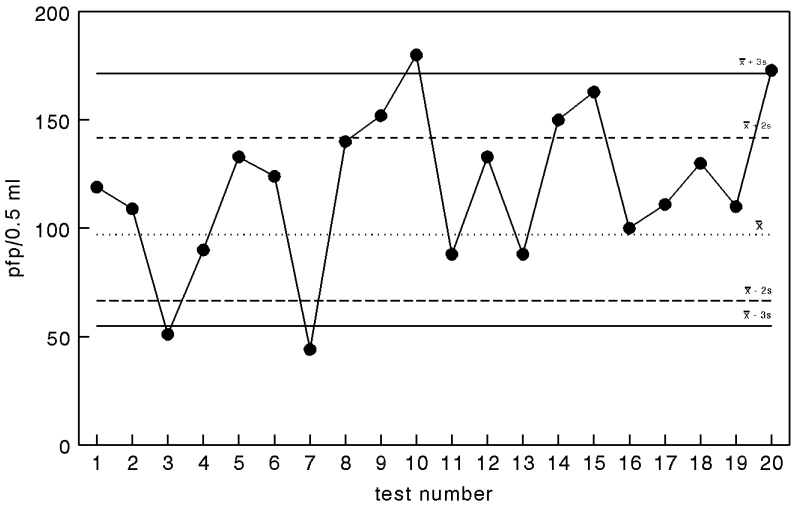

Figure 1 *Control chart of RMs containing ΦX174, batch 030195, with host strain WG5 Escherichia coli*

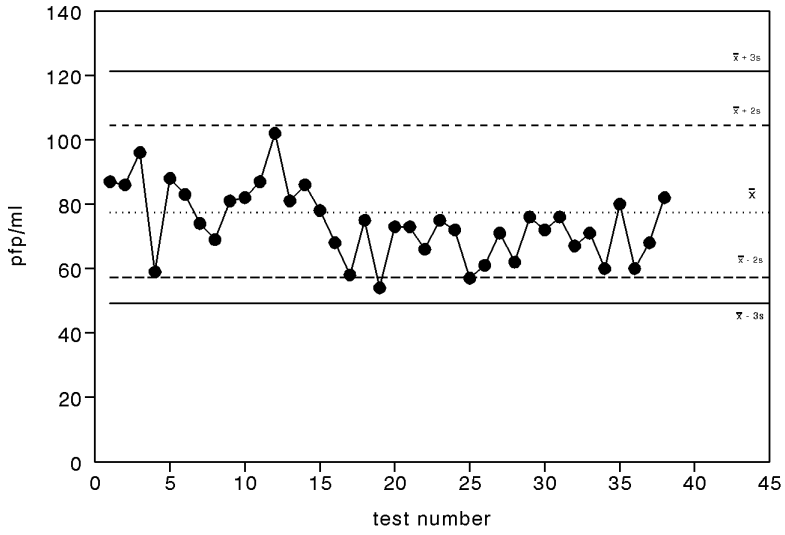

Figure 2 *Control chart of RMs containing ΦX174, batch 040696, with host strain WG5 Escherichia coli*

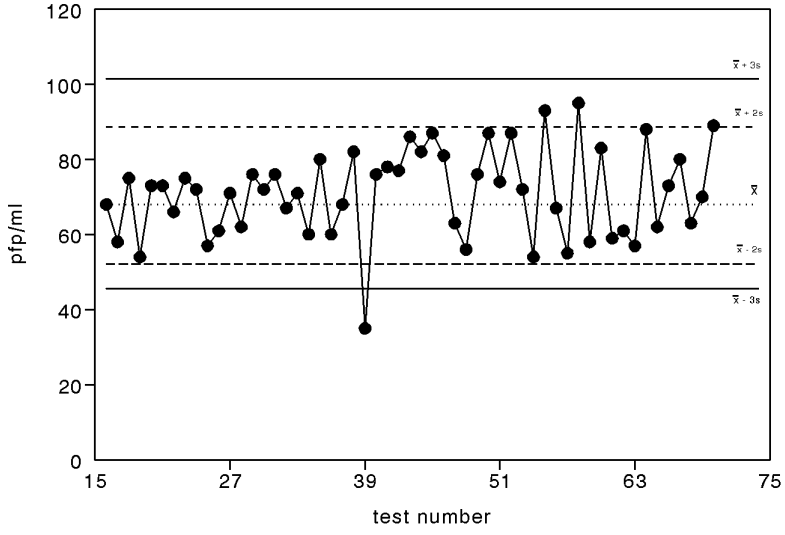

Figure 3 *Control chart of RMs containing ΦX174, batch 040696, with host strain WG5 Escherichia coli, after recalculation*

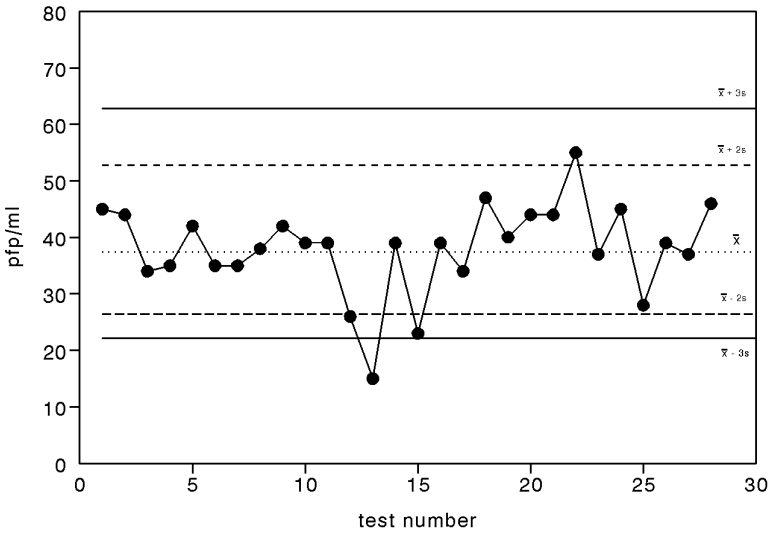

Figure 4 *Control chart of RMs containing ΦX174, batch 270597, with host strain WG5 Escherichia coli*

In Figure 5 the results of the challenge test of the batch of RMs containing ΦX174, batch 040696 are given. The test started on 040996 and finished on 171096. The results of the regression analysis showed no significant decrease (or increase) in the results of the vials stored at -20 °C and at -70 °C, in the 43 days of measurement. Storage of the vials at +5 °C or at +25 °C resulted in a significant decrease in the geometric mean number of pfp/ml. This decrease was most obvious if the materials were stored at 25 °C. In Table 5 the regression coefficients (on ln-scale) for all temperatures are given.

Table 5 *Regression coefficients (on ln-scale) of the challenge tests of RMs containing ΦX174, batch 040696.*

Storage temperature (°C)	Regression coefficients (b, on ln-scale)	95% confidence interval (min; max)
+25	-0.0715	-0.0694 ; -0.0736
+5	-0.0103	-0.0082 ; -0.0123
-20	-0.0013	+0.0008 ; -0.0034
-70	-0.0021	-0.0000 ; -0.0042

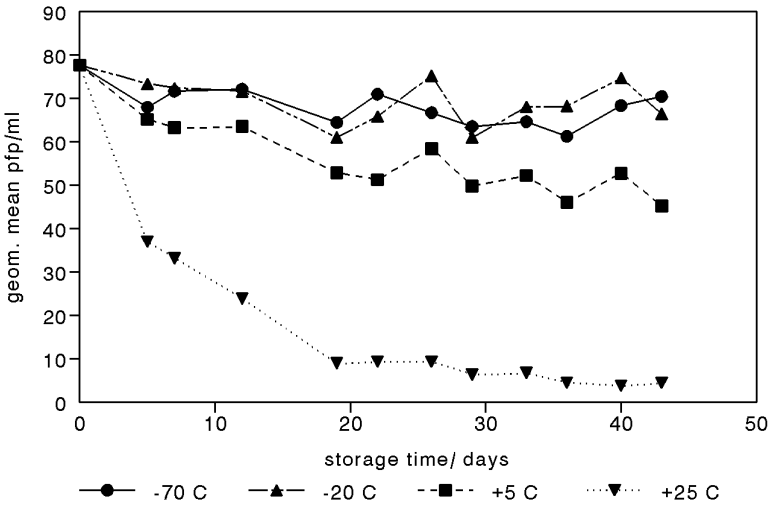

Figure 5 Results challenge test of RMs containing ΦX174, batch 040696 (start test at 040996)

3.2.2 Naturally polluted standard samples

The results for the somatic coliphages (SOMCPH) of the naturally polluted standard samples "De Bilt 281097" are given in Table 6. In this table the non-filtered samples show much variation in results. It occurred regularly that a vial of the non-filtered samples resulted in a very high number of plaques, which were in fact not countable. These results were not taken into account in this table, so that the real values of T_2/ (I-1) are even higher than given here. When each vial was filtered separately through a low protein-binding filter (with 0.22 µm pore size) the extreme counts were no longer detected. Filtering probably removed some aggregates that might exist in sewage. The geometric mean values of the filtered samples were somewhat lower than of the non-filtered samples, but still fulfilled the criteria. As filtering of the samples decreased the variation between vials (and also improved the variation in results within vials (T_1)), it was decided to prescribe filtering in the protocol of the second collaborative study (Mooijman *et al.*, 1999a).

Table 6 Results of naturally polluted standard samples "De Bilt 281097", SOMCPH

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

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

I	Lower limit	Upper limit
4	0.48	11.14
5	0.83	12.83

In Figure 6 the results of the challenge test of the counts of SOMCPH of the naturally polluted standard samples of batch UB180696 are given. The test started on 311096 and finished on 231296. In Table 7 the regression coefficients (on ln-scale) for all temperatures are given. The regression analysis showed for SOMCPH no significant decrease for the vials stored at -20 °C or at -70 °C. Like for the pure phage culture RMs, a significant decrease was found for the vials stored at 5 °C and 25 °C.

Table 7 Regression coefficients (on ln-scale) of the challenge tests of naturally polluted standard samples UB180696 for the counts of SOMCPH

Storage temperature (°C)	Regression coefficients (b, on ln-scale)	95% confidence interval (min; max)
+25	-0.1609	-0.1518 ; -0.1700
+5	-0.0205	-0.0174 ; -0.0236
-20	+0.0012	+0.0043 ; -0.0019
-70	+0.0005	+0.0036 ; -0.0026

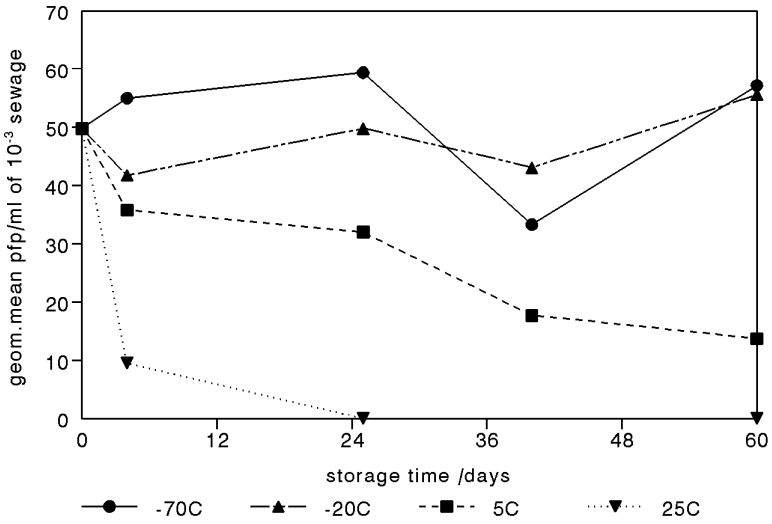

Figure 6 *Results challenge test of the counts of SOMCPH of the naturally polluted standard samples UB180696 (start test 311096)*

3.3 F-specific bacteriophages

3.3.1 Phage reference materials

During the project 2 different batches of reference materials containing a pure culture of phage MS2 were used. Each batch was checked for the criteria as stated in 2.2.1. The results of each batch before and a few days after freezing are summarised in Table 8. The batches fulfilled all stated criteria.

Table 8 *Results of 2 batches of phage reference materials containing MS2, before and a few days after freezing.*

Batch number	Before freezing			After freezing at (-70 ± 10) °C		
	$\bar{x}$	T_1	T_2 / (I-1)	$\bar{x}$	T_1	T_2 / (I-1)
071293	unknown	unknown	unknown	88.4[1]	7.80[1]	1.08[1]
220597	66.5	10.3	0.74	65.2[2]	9.60[2]	1.10[2]

$\bar{x}$: Geometric mean pfp/ml; I: number of vials, I=5 for batch 071293 and I=9 before freezing, I=10 after freezing for batch 220597; [1]: Analysis 1 day after freezing; [2]: Analysis 4 days after freezing

Of each batch control charts were prepared. The geometric mean values and the values of the warning and action limits of both batches are given in Table 9. The filled charts are shown in Figure 7 and 8 (both for batch 071293) and in Figure 9 (batch 220597). Immediate after preparation and storage of batch 071293, the vials were analysed for preparation of the control chart. This chart is presented in Figure 7. As for the batch of RMs containing ΦX174 (batch 040696), this batch, containing MS2, also showed some decrease in the (geometric) mean phage counts of MS2 in the first few months after freezing. Therefore the chart as presented in Figure 7 was also recalculated, using the data from the start of the project in January 1996. The recalculated chart is presented in Figure 8. This latter chart showed stable and 'in control' results for the period measured (January 1996 - January 1998). The vials of batch 220597 were also immediate after preparation and storage analysed for the preparation of a control chart. The results of this batch are shown in Figure 9. Here no initial decrease was seen and the batch showed to be stable (and results were 'in control') for the period of time measured (May 1997 - June 1999).

Table 9 Control chart values in pfp/ml of 2 batches of phage reference materials containing MS2

Batch	$\bar{x}$ - 3s	$\bar{x}$ - 2s	$\bar{x}$	$\bar{x}$ + 3s	$\bar{x}$ + 2s
071293	57.3	66.4	89.2	120.0	139.1
071293 after recalculation	49.0	57.7	80.0	111.1	130.9
220597	31.3	39.8	64.5	104.4	132.9

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

In Figure 10 the results of the challenge test of the batch of RMs containing MS2, batch 071293 are given. The test started on 040996 and finished on 171096. The results of the regression analysis showed no significant decrease (or increase) in the results of the vials stored at -20 °C and at -70 °C, in the 43 days of measurement. Storage of the vials at +5 °C or at +25 °C resulted in a significant decrease in the geometric mean number of pfp/ml. This decrease was most obvious if the materials were stored at 25 °C. In Table 10 the regression coefficients (on ln-scale) for all temperatures are given.

Table 10 Regression coefficients (on ln-scale) of the challenge tests of RMs containing MS2, batch 071293.

Storage temperature (°C)	Regression coefficients (b, on ln-scale)	95% confidence interval (min: max)
+25	-0.1260	-0.1193 ; -0.1327
+5	-0.0290	-0.0223 ; -0.0357
-20	+0.0107	+0.0174 ; +0.0040
-70	+0.0119	+0.0186 ; +0.0052

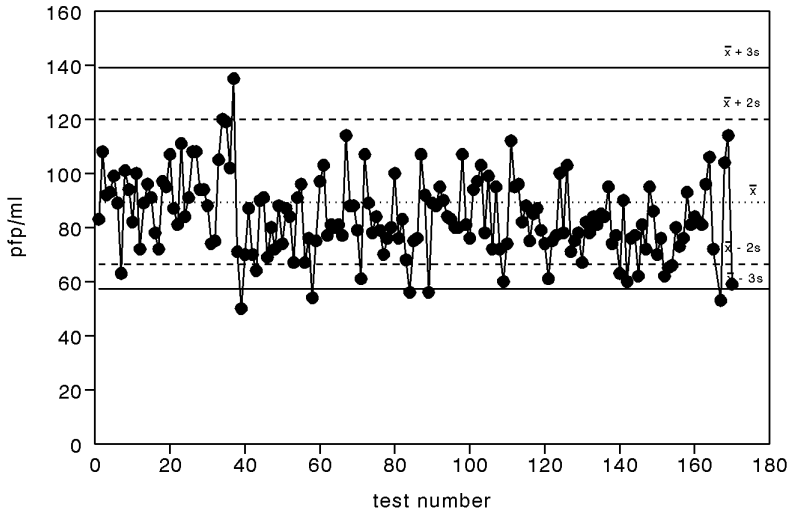

Figure 7 *Control chart of RMs containing MS2, batch 071293, with host strain WG49 Salmonella typhimurium*

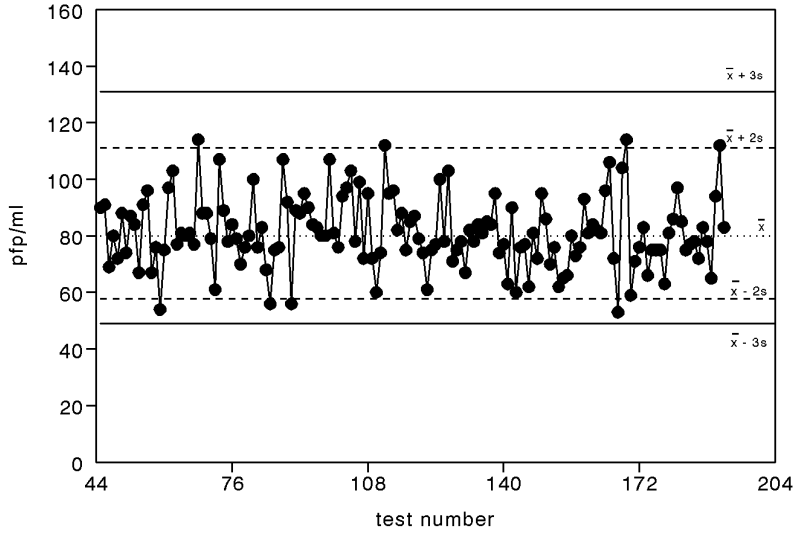

Figure 8 *Control chart of RMs containing MS2, batch 071293, with host strain WG49 Salmonella typhimurium, after recalculation*

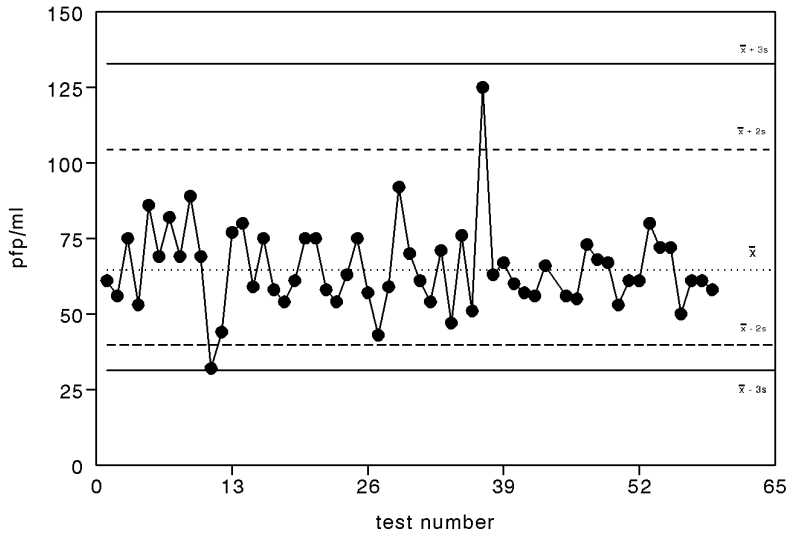

Figure 9 *Control chart of RMs containing MS2, batch 220597, with host strain WG49 Salmonella typhimurium*

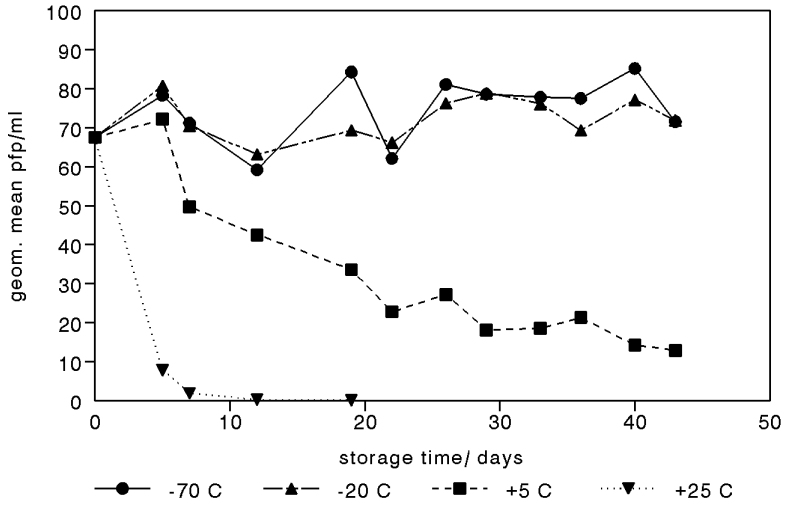

Figure 10 *Results challenge test of RMs containing MS2, batch 071293 (start test 040996)*

3.3.2 Naturally polluted standard samples

The results for the F-specific phages of the naturally polluted standard samples 'De Bilt 071197' are given in Tables 11 and 12. In Table 11 the results of the total number of F-specific phages (FTOTPH) are given. In Table 12 the results of the same vials for the number of F-specific DNA phages (FDNAPH) are given. Like for the determination of the somatic coliphages, the number of F-specific phages shows much variation between the non-filtered vials. Filtering of the vials here also decreased the variation (lower value for T_2/ (I-1)). This procedure was therefore also introduced in the protocol of the second collaborative study for the determination of the F-specific phages in the naturally polluted standard samples (Mooijman *et al.*, 1999a).

Table 11 Results of naturally polluted standard samples "De Bilt 071197", FTOTPH

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

Table 12 Results of naturally polluted standard samples "De Bilt 071197", FDNAPH

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

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

I	Lower limit	Upper limit
5	0.83	12.83

In Figures 11 and 12 the results of the challenge test of the counts of FTOTPH respectively of FDNAPH of the naturally polluted standard samples of batch UB180696 are given. The test started on 311096 and finished on 070197. No results for the materials stored at 25 °C are given, as the results were very variable at this temperature. On day=4 no plaques were detected of FTOTPH on the plates of the vials stored at 25 °C. However, discussing this result afterwards it might as well have been complete lysis of the host strain. On this day the counts of the FDNAPH varied between 9 and more than 300 plaques. On day=25 the counts of FTOTPH of the vials stored at 25 °C varied between 0 (or complete lysis) and more than 300 plaques. On this day the counts of the FDNAPH were decreased and varied between 0 and 3 plaques. If the mentioned zero counts found for FTOTPH were not zero, but in fact complete lysis of the host strain, this meant that many more phages were present than expected. Possible explanations could be:
- Growth of the phages or;
- Disintegration of aggregates.
Concerning the idea of 'growth' of the phages in the samples: The sewage samples were not filtered or treated with e.g. chloroform to remove bacteria before distribution and storage into vials. However, if host strains were present (like *E.coli*) in the sewage, they are not able to form F-pili at temperatures below 30 °C. Thus F-specific phages could only cause lysis to present host strains which had earlier formed F-pili and were able to grow at 25 °C. To check this theory, tenfold dilutions were made of some vials stored at 25 °C, on day=35. However, no plaques were counted in any of the dilutions (10^{-3}, 10^{-2}, 10^{-1} dilutions and non-diluted). This could be explained by the fact that all present phages were not longer viable after such a long time of storage at 25 °C. When looking at the results of the RMs containing MS2, a very quick die off occurred in the vials stored at 25 °C.
Another explanation might be the existence of aggregates of F-specific phages in the naturally polluted samples. At day=0 (before storage at elevated temperature), each aggregate was counted as one pfp. Under certain circumstances, like elevated temperatures, these aggregates might have disintegrated, resulting in very high plaque counts. However, long storage time of the samples at this elevated temperature might have a negative effect on the viability of the F-specific phages, finally resulting in very low (to zero) plaque counts.

In Table 13 the regression coefficients (on ln-scale) for the results of FTOTPH and FDNAPH of all temperatures are given. No separate calculations were made for the FRNAPH, as these counts are in fact the result of the counts of FTOTPH minus the counts of FDNAPH. The regression analysis showed for the FDNAPH no significant decrease for the vials stored at -20 °C or at -70 °C. Both FTOTPH and FDNAPH showed a significant decrease in the number of plaques when the vials were stored at +5 °C. FTOTPH also showed a significant decrease when the materials were stored at -20 °C as well as at -70 °C for the period measured (75 days). However, there was also a lot of variation between the results of one measurement day, but also between the different days (see Figure 11), which very much influenced the results.

Table 13 *Regression coefficients (on ln-scale) of the challenge tests of naturally polluted standard samples UB180696 for the counts of F-specific bacteriophages (FTOTPH and FDNAPH)*

Storage temperature (°C)	FTOTPH		FDNAPH	
	Regr. coeff.[1]	95% c.i. (min; max)[2]	Regr. coeff.[1]	95% c.i. (min; max)[2]
+25	n.r.	n.r.	n.r.	n.r.
+5	-0.0863	-0.0826 ; -0.0900	-0.0802	-0.0763 ; -0.0841
-20	-0.0080	-0.0053 ; -0.0107	-0.0023	+0.0005 ; -0.0051
-70	-0.0057	-0.0030 ; -0.0084	-0.0004	+0.0024 ; -0.0032

n.r.: no results; [1]: Regression coefficients (b), calculated on ln-scale; [2]: 95% confidence interval of b

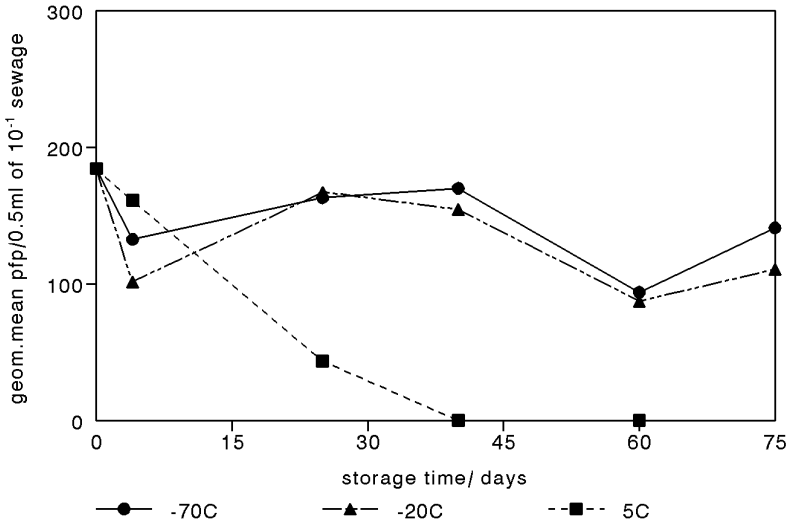

Figure 11 *Results challenge test of the counts of FTOTPH of the naturally polluted standard samples UB180696 (start test 311096)*

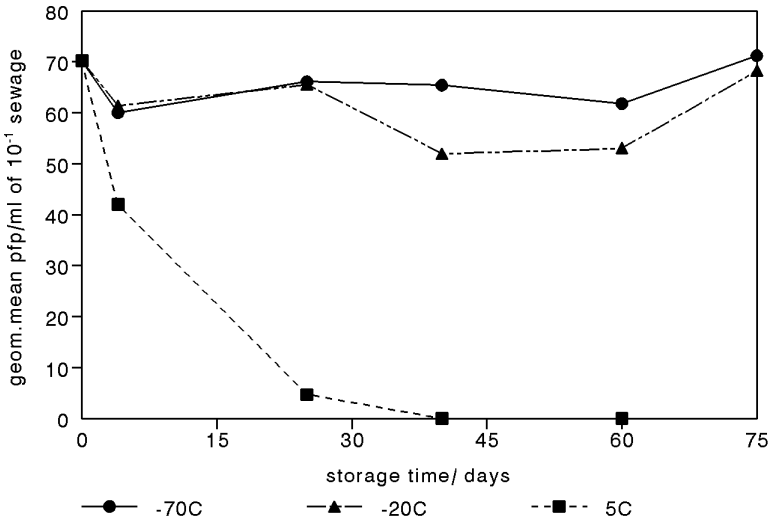

Figure 12 *Results challenge test of the counts of FDNAPH of the naturally polluted standard samples UB180696 (start test 311096)*

3.4 Phages of *Bacteroides fragilis*

3.4.1 Phage reference materials

During the project 2 different batches of reference materials containing a pure culture of phage B40-8 and one batch of reference materials containing a pure culture of phage B56-1 were used. B40-8 was used a standard phage for analysis of phages of *Bacteroides fragilis* (BFRPH) with host strain HSP40. B56-1 was used as standard phage for analysis of BFRPH with host strain RYC2056. Each batch was checked for the criteria as stated in 2.2.1. The results of each batch before and (a few days) after freezing are summarised in Table 14. They all fulfilled the pre-set criteria. However, the first analyses of B56-1 were made with a host culture of RYC2056 of poor quality. After this was detected, a new culture of this host strain was prepared and the phage counts appeared to become much higher. It was decided to analyse 0.2 ml of the vials of this batch of RMs instead of 1 ml. Unfortunately, the plaques of B56-1 were very tiny and therefore very difficult to count.

Table 14 Results of 2 batches of phage reference materials containing B40-8 and of 1 batch of phage reference materials containing B56-1, before and (a few days) after freezing.

Batch number	Before freezing			After freezing at (-70 ± 10) °C		
	$\bar{x}$	T_1	T_2 / (I-1)	$\bar{x}$	T_1	T_2 / (I-1)
B40-8, 271196	132.2[1]	10.8[1]	1.31[1]	127.1[1,2]	3.62[1,2]	1.09[1,2]
B40-8, 260397	93.3[3]	13.6[3]	1.53[3]	105.9[2,3]	10.6[2,3]	0.56[2,3]
B56-1, 090698, old host[4]	91[5]	no info	no info	80.1[1]	4.60[1]	1.19[1]
B56-1, 090698, new host[6]	no info	no info	no info	78.3[7]	1.12[5]	0.03[5]

$\bar{x}$: Geometric mean pfp/ml; I: number of vials; [1]: I=5; [2]: Analysis 1 day after freezing; [3]: I=10; [4]: Host was of poor quality; [5]: Mean of 3 replicates of one suspension; [6]: Host was of good quality, plaques were very small (pinpoints); [7]: I=2; pfp/0.2 ml; Analysis 2 months after freezing

Control charts were prepared of each batch. The geometric mean values and the values of the warning and action limits of the batches are given in Table15. The filled charts are shown in Figure 13 (batch 271196 of B40-8), Figure 14 (batch 260397 of B40-8) and Figure 15 (batch 090698 of B56-1). The chart of batch 271196 (phage B40-8) showed much variation in results during the period of measurement (November 1996 - April 1998; see Figure 13). Explanations for this high variation could be the fact that at the start of the use of these RMs little experiences with the method for the enumeration of BFRPH was present at RIVM/MGB. Furthermore, the method for the enumeration of BFRPH was regularly improved, and thus changed, during the course of the project. With the new batch of RMs containing phage B40-8 (batch 260397) more stable results were found during the period of measurement (March 1997 - September 1998; Figure 14). Two outlying values are visible in this latter chart (test numbers 21 and 22). An explanation for these low values was the use of a poor batch of medium (the same batch of medium was used in both cases).
Because of the problems with the host strain RYC2056, only few data were generated with the batch of RMs containing B56-1 (batch 090698). Therefore also results from the Departments of Microbiology of the University of Barcelona (UB) and of Institute Pasteur in Lille (IPL) were used to prepare the control chart. The results in the chart in Figure 15 show a stable batch (August 1998 - March 1999). However, the warning and action limits are very broad. This was probably caused by a combination of difficulties with counting of the plaques and combining results of three laboratories.

Table 15 *Control chart values in pfp/ml of 2 batches of phage reference materials containing B40-8 and 1 batch of phage reference materials containing B56-1*

Batch	$\bar{x}$ - 3s	$\bar{x}$ - 2s	$\bar{x}$	$\bar{x}$ + 3s	$\bar{x}$ + 2s
B40-8, 271196	89.7	101.7	130.9	168.5	191.1
B40-8, 260397	69.9	78.1	97.3	121.2	135.3
B56-1, 090698 [1]	18.7	27.5	59.1	127.0	186.1

$\bar{x}$: Geometric mean; $\bar{x} \pm 2s$: warning limits; $\bar{x} \pm 3s$: action limits; [1]: pfp/0.2 ml;

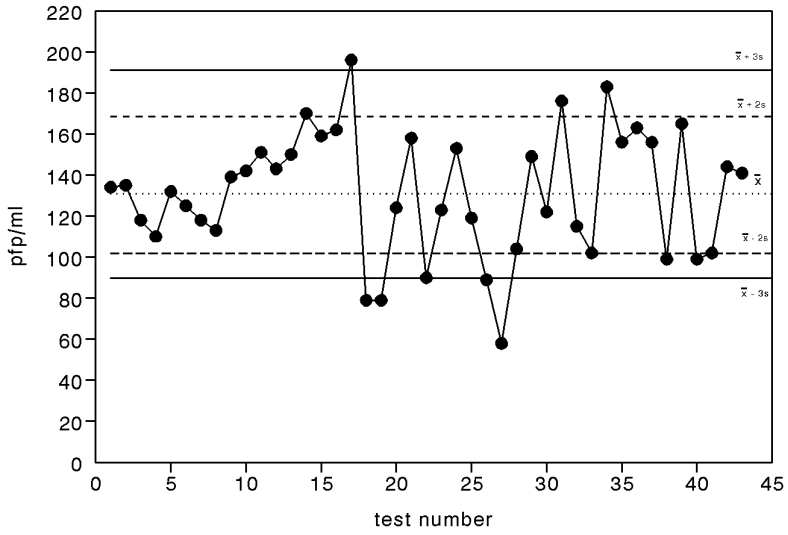

Figure 13 *Control chart of RMs containing B40-8, batch 271196, with host strain HSP40 Bacteroides fragilis*

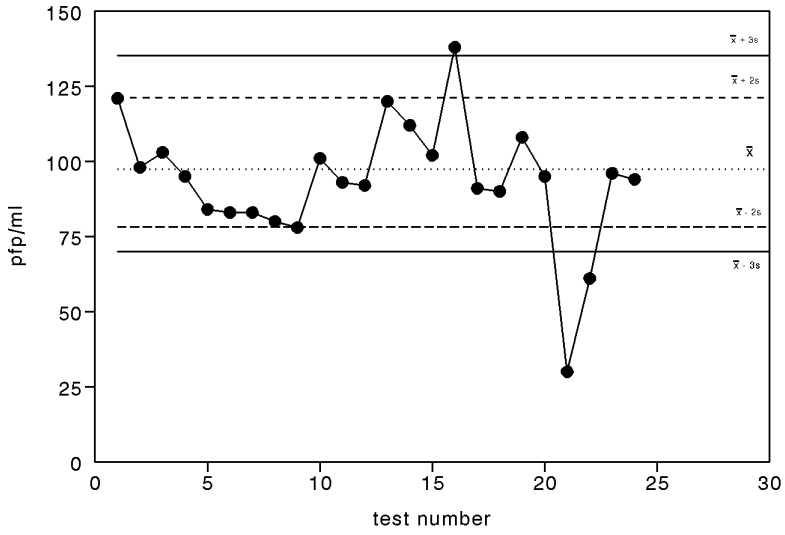

Figure 14 *Control chart of RMs containing B40-8, batch 260397, with host strain HSP40 Bacteroides fragilis*

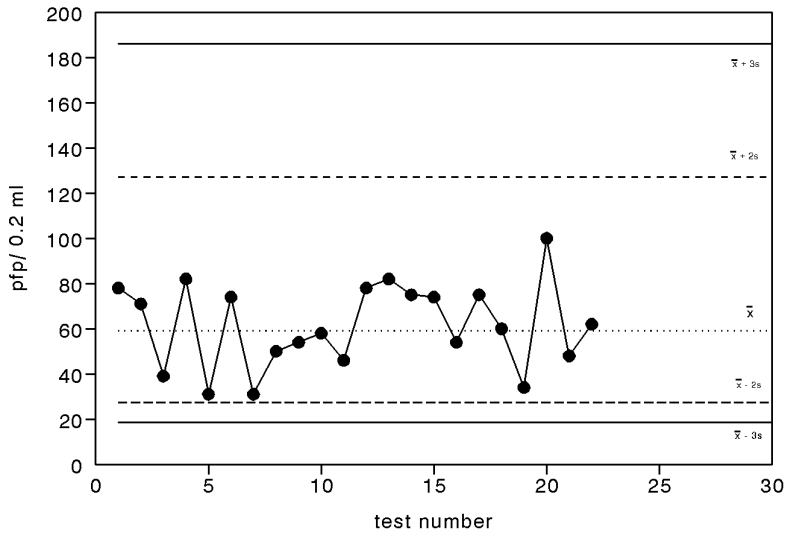

Figure 15 *Control chart of RMs containing B56-1, batch 090698, with host strain RYC2056 Bacteroides fragilis*

3.4.2 Naturally polluted standard samples

The results for the phages of *Bacteroides fragilis* of the naturally polluted standard samples of Barcelona 311097 are given in Table 16. For these phage analysis only host strain HSP40 *Bacteroides fragilis* was used. In the method for detection and enumeration of BFRPH in naturally polluted samples, the prefiltering of the samples through a low protein-binding filter is advised. Therefore only results of filtered vials are given in Table 16. The mean values are relatively low (lower than the criterion of 30 pfp/ml). However, this was the highest value that could be obtained with host HSP40 with the naturally polluted sample available. The sewage from Barcelona was used as such without making dilutions.
In Table 16 a differentiation is made for the results obtained from medium without bile (Bacteroides Phage Repair Medium: BPRM) and from medium with bile added (modified Bacteroides Phage Repair Medium: MBPRM). The results from MBRPM are somewhat higher than the results obtained from BPRM. The variation within vials (T_1) and between vials (T_2/ (I-1)) fulfilled the criteria as set in 2.2.1.

Table 16 *Results of naturally polluted standard samples 'Barcelona 311097', BFRPH enumerated with host strain HSP40 Bacteroides fragilis*

Day	Filtered, cultured on BPRMA				Filtered , cultured on MBRMA			
	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)	I	Geom. mean (pfp/ml)	T_1	T_2/ (I-1)
0[1]	5	14.2	1.16	1.07	-	-	-	-
4	5	25.5	4.04	2.20	-	-	-	-
25	5	24.4	5.79	0.53	-	-	-	-
47	5	23.9	0.75[2]	0.99	5	29.8	2.70	1.40
103	-	-	-	-	5	27.4	1.78	1.48

I: number of vials (all calculated in duplicate); [1]: Before freezing; [2]: Significantly different from a χ^2-distribution:

I	Lower limit	Upper limit
5	0.83	12.83

4. Discussion and conclusions

Homogeneous and stable reference materials containing pure phage cultures could be prepared for all three types of bacteriophages (SOMCPH, FRNAPH and BFRPH) by mixing phage suspensions with glycerol and storage of the final materials at -70 °C. In a similar way it was also possible to prepare naturally polluted standard samples (mixing sewage with peptone saline solution and glycerol). However, these latter samples showed more variation in counts than the pure culture phage RMs. Causes for these increased variations could be (a combination of):
- Aggregates in the natural samples;
- Plates are more difficult to read because of the presence of different types of plaques;
- Disturbance by (bacterial) background flora.
It was shown that when the samples were filtered through a low protein binding filter with 0.2 µm pore size the (extreme) variation was reduced, although the variation still remained higher than of the RMs containing pure phage cultures.

For the preparation of stable frozen bacterial RMs it was necessary to quickly freeze the samples by placing them in dry ice/ethanol and next store them at -70 °C (Schijven *et al.*, 1994). These bacterial samples also needed to be quickly thawed at 37 °C to obtain the best results. For the preparation of stable phage reference materials it was not necessary to freeze (and thaw) the samples quickly. It was even preferred to freeze and thaw these phage RMs slowly be simply placing them at -70 °C, respectively at room temperature (Schijven *et al.*, 1995). The results in this report also confirmed the preference for thawing the frozen vials slowly at room temperature. The number of somatic coliphages in slowly thawed vials of naturally polluted standard samples was significantly higher than the results obtained with vials thawed quickly at 37 °C. For reference materials containing pure phage cultures of ΦX174 or MS2 slowly thawing of the vials at room temperature or quickly at 37 °C did not show differences in phage count results.

Freezing of the phage reference materials at -20 °C, without the addition of glycerol is not preferred. A batch of RMs containing ΦX174 was stored in that way (batch 030195) and showed very variable results, while other batches stored at -70 °C, with glycerol added (to a final concentration of 5 % v/v) were more homogeneous. The inhomogeneity in the RMs stored at -20 ° could have been caused by differential die off of the phages in the vials.

The stability of the phage reference materials and of the naturally polluted standard samples was dependent on the storage temperature. The challenge tests showed that the stability of all materials for the enumeration of SOMCPH and for F-specific phages was best at -70 °C. For a ‘short’ period of storage (<50 days), -20 °C also is an alternative temperature for RMs containing ΦX174 or MS2. However for RMs containing B40-8 the stability at -20 °C was

poor (Jofre *et al*., 1997) and only storage at -70 °C seems to give sufficient stability to this type of materials. For the naturally polluted standard samples, similar results were found when analysing SOMCPH as for the phage RMs: good stability of the phages when stored at -70 °C and at -20 °C (for the time measured: *ca* 60 days) and poor stability at storage temperatures of +5 °C or +25 °C. Jofre and co-workers (1997) found similar results for naturally polluted standard samples for analysing BFRPH.
Some deviating results were found for the F-specific phages of the naturally polluted standard samples when stored at 25 °C. The high phage count (>300 pfp/ml) found after 25 days of storage, raised the presumption that the zero counts found after 4 days of storage were in fact complete lysis of the host strain. Growth of the phages in the sample does not seem to be likely. If a host strain would have been present in the sample, it could not have formed F-pili at 25 °C and thus no receptor place for the F-specific bacteriophages. If growth of the phages indeed would have happened in the samples stored at 25 °C, a similar effect would have been expected for the somatic coliphages, but this was not noticed in the test materials. Therefore the idea of possible disintegration of aggregates at 25 °C seem to be more likely as explanation for the possible increase in phage counts.
It would be informative if the challenge test with naturally polluted samples for the analysis of F-specific bacteriophages is repeated. If doing so, it is advisable also to analyse different dilutions of the samples stored at 25 °C. From the results of the present challenge test for the F-specific bacteriophages, not much can be concluded. A significant decrease in the mean number of plaque forming particles of FTOTPH in the vials stored at -20 °C as well as at -70 °C was found. However, the variation between vials of one measurement day and between vials of different days was large. This high variation in results was also found in the naturally polluted standard samples of De Bilt 071197 stored at -70 °C (Table 11). If, for instance, only the mean results of this latter material on day=3 and on day=95 (Table 11) are taken into account, the results are the same (163 pfp/ml). However, a lot of variation in the mean results were found in between day=3 and day=95 (varying from 91 to 188 pfp/ml).

The results of the different challenge tests confirm that it is necessary to freeze the phage RMs or naturally polluted samples for use as quality control samples. No guarantee of the number of phages can be given once the materials have been stored for more than one day at temperatures above 0 °C. Hence, mailing of the materials should also be in the frozen state. The best way of mailing is therefore packing of the samples in dry ice and short transport times (e.g. by using courier services).

The phage reference materials could be used for quality control of the phage enumeration methods. The control charts of the different batches of RMs showed whether the materials were stable, but also whether the analyses were repeatable on different days. In some control charts ‘out of control’ results (below the lower action limit) were detected. A few of these out of control results could be explained by the use of poor and/or old batches of media. Here the use of RMs in combination with a control chart had proven their value.

Phage B56-1, used as standard phage for the analysis of BFRPH with host strain RYC2056, produced very small (< 1 mm) plaques that were difficult to count. This phage was isolated by UB from naturally polluted samples. At isolation, the plaques were of well visible size (*ca* 2-3 mm). However, mailing of the phage from Spain to the Netherlands and next freezing at -70 °C, probably resulted in a mutation in the phage giving only small plaques. For future work it is advisable to prepare new reference materials with another standard phage for the quality control of the analysis of BFRPH with host strain RYC2056.

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