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Water quality — Detection and enumeration of bacteriophages —

Part 3: Validation of methods for concentration of bacteriophages from water

Qualité de l'eau — Détection et dénombrement des bactériophages —

Partie 3: Validation des méthodes de concentration des bactériophages dans l'eau

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 10705-3 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

ISO 10705 consists of the following parts, under the general title *Water quality — Detection and enumeration of bacteriophages*:

— *Part 1: Enumeration of F-specific RNA bacteriophages*

— *Part 2: Enumeration of somatic coliphages*

— *Part 3: Validation of methods for concentration of bacteriophages from water*

— *Part 4: Enumeration of bacteriophages infecting* Bacteroides fragilis

Water quality — Detection and enumeration of bacteriophages —

Part 3: Validation of methods for concentration of bacteriophages from water

WARNING — Persons using this part of ISO 10705 should be familiar with normal laboratory practice. This part of ISO 10705 does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is imperative that personnel involved in validation of methods for concentration of bacteriophages from water have relevant experience with the methods of enumeration of bacteriophages (see ISO/TR 13843[1]).

1 Scope

This part of ISO 10705 specifies the general principles for assessing the performance of methods for the concentration of bacteriophages from water. Concentration is recommended for those water samples expected to contain < 3 pfp (plaque-forming particles) per millilitre. Concentration methods can be applied to all kinds of water provided that the amount and nature of suspended solids and/or dissolved matter do not interfere with the concentration procedure.

This part of ISO 10705 does not give specific details of concentration methods, but outlines the fundamental principles for evaluating the suitability of a particular method for a given type and volume of water. Annex A gives examples of methods that have been found satisfactory and their fields of application.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 8199, *Water quality — General guide to the enumeration of micro-organisms by culture*

ISO 10705-1, *Water quality — Detection and enumeration of bacteriophages — Part 1: Enumeration of F-specific RNA bacteriophages*

ISO 10705-2, *Water quality — Detection and enumeration of bacteriophages — Part 2: Enumeration of somatic coliphages*

ISO 10705-4, *Water quality — Detection and enumeration of bacteriophages — Part 4: Enumeration of bacteriophages infecting* Bacteroides fragilis

ISO/IEC Guide 2, *Standardization and related activities — General vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/IEC Guide 2 and the following apply:

3.1
bacteriophages
bacterial viruses which are capable of infecting selected host strains

NOTE Bacteriophages produce visible plaques (clearance zones) in a confluent lawn of the host strain grown under appropriate culture conditions.

4 Principle

The sample is treated according to a method of choice, by which the bacteriophages are concentrated from a relatively large volume of sample (100 ml up to several litres) to a smaller volume (typically from a few to 20 ml). The concentrated sample is then analysed for bacteriophages according to an International Standard method or other suitable protocol.

The concentration method to be evaluated should be carefully described in a protocol, following ISO standard layout as much as possible. The description should include the target group(s) of bacteriophages and their detection method(s), the types of water and ranges of volumes to be analysed, as well as exceptions to the field of application, e.g. turbidity.

The method is validated according to principles laid down in this part of ISO 10705. The validation procedure consists of determining the recovery of bacteriophages from a series of samples, seeded with naturally polluted water (raw or treated sewage). The recovery is studied in a range of volumes, and particular attention is paid to its reproducibility.

5 Reagents

Use ingredients of uniform quality and chemicals of analytical grade for the preparation of culture media. For information on storage see ISO 8199, except where indicated in this part of ISO 10705. Alternatively, use dehydrated complete media and follow strictly the manufacturer's instructions.

Other grades of chemicals may be used provided they can be shown to lead to the same results.

5.1 Water, for the preparation of media, glass-distilled water or de-ionized water free from substances that might inhibit bacterial growth under the conditions of the test, and at least Grade 3 as specified in ISO 3696.

5.2 Diluent, for making dilutions, peptone-saline solution or another suitable diluent in accordance with ISO 6887-1 or ISO 8199.

5.3 Culture media and reference cultures, as specified in the corresponding standard method of ISO 10705-1, ISO 10705-2 and ISO 10705-4 for the phage assay.

5.4 Glycerol (ρ = 870 g/l), autoclaved at (121 ± 3) ºC for 15 min and stored in the dark at room temperature for a period no longer than 1 year.

6 Apparatus and glassware

SAFETY PRECAUTIONS — Field apparatus should be disinfected before use. Apply safety precautions appropriate to the disinfectant solution used. Some stages of the concentration process may involve the application of hydrostatic or pneumatic pressure. Observe relevant safety precautions.

Use usual microbiological laboratory equipment as specified in the method for the phage assay (Clause 8), and the protocol for the concentration method.

7 Sampling

Samples up to 10 l can conveniently be transported to the laboratory. Take the samples and deliver them to the laboratory as specified in ISO 8199 (see also ISO 19458[2]). For larger samples, it is advisable to perform the first step of the concentration procedure in the field. This process may take up to several hours. If parallel examination for indicator bacteria or other micro-organisms is carried out, take a time-proportional sample for these analyses, preferably by filling a sample bottle with a side flow from the concentration apparatus. Filters, precipitates or other products from the first concentration step may be further treated in the field, or may be transported to the laboratory. Include the transport and storage conditions of intermediate stages of the process in the validation procedure.

8 Preparation of sewage samples for spiking

Obtain a sample of primary or secondary (biologically treated) sewage and centrifuge at 1 000 g for 20 min or filter through an 8 µm to 12 µm membrane filter. Store supernatant or filtrate on melting ice. Enumerate the target bacteriophages in 1 ml volumes according to the chosen method. If necessary, dilute the sample to obtain a concentration of 60 pfp to 200 pfp (plaque-forming particles) per millilitre. Add glycerol to obtain a final volume fraction of 5 %; mix well. Distribute 10-ml aliquots into glass or plastic bottles (or tubes, or vials) and freeze at (−20 ± 5) °C or (−70 ± 10) °C. Thaw two bottles at room temperature. From each bottle, examine two 0,5-ml aliquots for the target bacteriophages. The average counts should be within the limits as specified above (i.e. 30 pfp to 100 pfp per plate). Analyse the counts for within and between bottle homogeneity as follows:

$$T_1 = \sum_{i=1}^{I}\sum_{j=1}^{J}\left[\left(z_{ij} - \frac{z_{i+}}{J}\right)^2 \Big/ \left(\frac{z_{i+}}{J}\right)\right]$$

where

T_1 is Cochran's dispersion test statistic to determine the variation in pfp within one vial of reference material;

z_{i+} is the total count of plaques of the duplicates of one vial.

$$z_{i+} = \sum_{j=1}^{J} z_{ij}$$

I is the number of vials (in this case 2);

J is the number of duplicates (in this case 2);

The number of degrees of freedom for T_1 is equal to $I(J-1)$ and

$$T_2 = \sum_{j=1}^{J}\left[\left(z_{i+} - \frac{z_{++}}{I}\right)^2 \Big/ \left(\frac{z_{++}}{I}\right)\right]$$

where

T_2 is Cochran's dispersion test statistic to determine the variation in pfp within different vials of one batch of reference material;

z_{++} is the total count of plaques for all vials and duplicates $z_{++} = \sum_{i=1}^{I}\left(\sum z_{ij}\right)$.

The number of degrees of freedom for T_2 is equal to $I-1$.

If the phages are randomly distributed within and between the vials, T_1 and T_2 follow approximately a χ^2 distribution with respectively 2 and 1 degrees of freedom. Accept the samples if $0{,}01 < T_1 < 5{,}99$ and $T_2 < 3{,}84$.

NOTE Somatic coliphages, F-specific RNA bacteriophages and bacteriophages infecting *Bacteroides fragilis* naturally occurring in raw sewage partially purified as indicated above, do not suffer significant inactivation when frozen with a volume fraction of 5 % glycerol and they can be preserved frozen below (–20 ± 5) °C and preferably at (–70 ± 10) °C without significant decrease in numbers for at least one year.

9 Procedure

9.1 Preparation of spiked samples

9.1.1 Batch methods

Obtain samples from all types of water mentioned in the scope of the concentration procedure. Obtain the samples on different days, preferably representing different seasons and climatic conditions. Study a minimum of five samples for each sample water type. Let V_{max} be the maximum volume of sample to be treated by the concentration method under evaluation. The volume of sample to be obtained for the validation procedure shall then at least be $3 \times V_{max}$. Prepare containers with the following volumes of sample:

— $0{,}125 \times V_{max}$;

— $0{,}250 \times V_{max}$;

— $0{,}500 \times V_{max}$;

— V_{max}.

To each container, add 1 ml of spiking material (see Clause 8) pre-warmed at room temperature. Preserve the remainder of the spiking material on melting ice.

9.1.2 In-line concentration methods

Perform field studies for all types of water mentioned in the scope of the concentration procedure. Carry out the studies on different days, preferably representing different seasons and climatic conditions. Study a

minimum of five samples for each water type. Let V_{max} be the maximum volume of sample to be treated by the concentration method. Perform field studies with the following volumes of sample:

— 0,125 × V_{max};

— 0,250 × V_{max};

— 0,500 × V_{max};

— V_{max}.

Treat each volume as described in the protocol of the concentration procedure. Add 1 ml of spiking material (see Clause 8) pre-warmed to room temperature to approximately 10 ml of diluent (5.2). Allow the concentration apparatus to operate under stable conditions. Inject then the total volume of diluent plus spiking material in the inflow to the concentration apparatus (e.g. by piercing the needle of a syringe through a hose) in four similar portions, each after passage of approximately one-fifth of the water volume to be treated.

9.2 Evaluation of recovery

Treat the spiked samples as described in the protocol of the test concentration method, including all sample transport and conservation steps, imitating as much as possible the sample transport steps of natural samples. Assay the total volume of the final concentrate in 1 ml portions, or fractions if the final volume of concentrate is not a whole number of millilitre. Any additional bacteriophages remaining on the concentration surfaces should be assayed when possible, e.g. phages retained in the filters.

In parallel, assay two 0,5-ml aliquots of spiking material. The values obtained shall be used to calculate the concentration efficiency, which will allow the determination of the number of phages introduced in the different volumes to be concentrated and will also allow the calculation of T_1 and T_2 of each one of the bottles with regard to other bottles. If more than 20 % (1 in 5) of the spiking material samples do not comply with the acceptable values of T_1 and/or T_2, discard the spiking material. If $\leqslant$ 20 % of the spiking material samples do not comply with T_1 and/or T_2, discard the results of this assay and perform a new assay.

Anomalous or extreme results are characteristic of microbiological measurement. Occasionally it is acceptable to discard a result on the basis of simple observation of the data. However, it is preferable to apply an appropriate statistical test. Use the Dixon test to discard extreme values.

Perform a minimum of five experiments with results that have not been rejected before data analysis.

If the method is evaluated using a natural water suspected of containing phages detected by the same bacterial host as the test bacteriophages, then determine the background counts of phages. Plaque an aliquot or concentrate V_{max} and count the concentrate. If the sample contains a number of phages $>$ 20 % of the phages spiked into the sample, heat the water and keep it at 80 °C for 30 min and allow it to cool prior to use. If the number of naturally occurring bacteriophages is $<$ 20 % of the added phages, then enumerate them and take them into account in the data analysis (Clause 10).

10 Calculation

Calculate the recovery, η, expressed as a percentage, as follows:

$$\eta = N_c / N_s \times 100\ \%$$

or in the case that the sample is contaminated with naturally occurring bacteriophages:

$$\eta = (N_c - N_{no}) / N_s \times 100\ \%$$

where

N_c is the total number of plaques recovered from a concentrate;

N_s is the number of plaques from 1 ml of the spiking material;

N_{no} is the number of naturally occurring bacteriophages in the water sample.

For all assayed volumes, calculate the arithmetic average recovery $\bar{x}$, the standard deviation s and the confidence interval.

The empirical evidence indicates that the recovery values follow a normal distribution or are normally distributed. To obtain a better estimation of the confidence intervals, the following mathematical expression is recommended.

$$\left\{\bar{x} - ts/\sqrt{n} \;;\; \bar{x} + tx/\sqrt{n}\right\}$$

where

$\bar{x}$ is the arithmetic average recovery;

s is the standard deviation;

n is the number of η values;

t is the value of the t-distribution (t-values for different combinations of confidence intervals and degrees of freedom can be found in t-distribution tables). In case of n = 5 (degrees of freedom = 4) and a 95 % confidence interval t = 2,776.

Plot η plus the confidence intervals against the volume of sample and examine for linearity. If confidence intervals of η for the different volumes overlap, it can be considered that the response is linear and that consequently there are not volume effects. If there are volume effects, determine the maximum volume that can be concentrated.

Combine the results of all volumes for which the method can be recommended and calculate the arithmetic mean of η, $\bar{x}$, the standard deviation, s, and the relative standard deviation, $s/\bar{x}$.

The mean of η with all data will give a better estimation of the efficiency of recovery of the method.

The relative standard deviation, $s/\bar{x}$, will provide an indication of the reliability of the method. Consider the method reliable if $s/\bar{x} \leqslant 0{,}5$.

See Annex B for an example of the validation process described in Clause 9 and the expression of the results.

If the method is used regularly in a laboratory, users are recommended to plot the recovery data on a guidance chart.

11 Analytical quality control

Repeat the evaluation of recovery given in Clause 10 regularly if the method is in routine use. It is then sufficient to analyse the results at 0,5 × V_{max}. Repeat the procedure at least before the use of new batches of critical reagents, filters, etc.

12 Test report

The test report shall contain the following information:

a) a reference to this part of ISO 10705, i.e. ISO 10705-3;

b) a reference to the standard describing the bacteriophage assay method;

c) a reference to the protocol describing the concentration method;

d) all details necessary for complete identification of the sample;

e) any information on unusual sample characteristics that may have influenced recovery, turbidity, algal growth, surface scums, colour etc.;

f) any other information relevant to the method.

Annex A
(informative)

Recommended methods for concentration of bacteriophages from water depending on the volume, turbidity and particle content

A.1 Adsorption/elution method using electropositive filters

This method is based on that of Logan *et al.* [3]

The method can be used for different types of samples and different volumes of sample, using batch processing or in-line filtration, when appropriate. The method is recommended for volumes of water ranging from 10 l to 100 l.

When turbidity is high (as judged from blocking of the filter before the desired volume is filtered), use prefiltration through a series of filters, e.g. 10 µm, 5 µm and 1 µm. Elute the prefilters according to elution applied to the electropositive filters as indicated below.

Adjust the pH of the water to 5,5 to 6,0 by slow addition of 1 mol/l HCl or 1 mol/l NaOH with constant stirring or by in-line injection.

Adsorb the phages by passage through an electropositive filter [e.g. AMF-CUNO Zeta Plus series S[1)]]

Elute bound phages by passage of an appropriate volume of eluent solution. The method and volume depend on the filter used. For example, 50 mm flat sheet filters are eluted by applying 10 ml to 15 ml of eluent, passed by gravity and light vacuum after 10 min; cartridge filters are eluted by recirculation of 500 ml to 1 000 ml of eluent. Use one of the following eluents:

— 5 mmol/l arginine, 1 % to 3 % by mass of beef extract, pH 9,0;

— 0,5 mol/l NaCl, 4 % by mass of beef extract, pH 9,0;

— 1,5 % by mass of beef extract, 1 % by mass of polyoxyethylene sorbitan monooleate [Tween 80[2)]], pH 9,0.

Neutralize the eluate to pH 7.

Large volumes of eluate need to be reconcentrated by ultrafiltration using a membrane of 10 000 relative molecular mass cut-off. Apparatus with tangential flow filtration are recommended.

A.2 Membrane filtration

This method is based on Sobsey *et al.* [4] which is a simple membrane filter method to concentrate and enumerate male-specific RNA coliphages, with modifications to improve recovery after filtering volumes greater than 100 ml.

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1) AMF-CUNO Zeta Plus series S is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 10705 and does not constitute an endorsement by ISO of this product.

2) Tween 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 10705 and does not constitute an endorsement by ISO of this product.

This method is recommended for volumes ranging from 100 ml to 1 l of waters with turbidity < 2,0 NTU (nephelometric turbidity units).

For concentrating bacteriophages from 1 l of water, proceed as follows:

a) Add $MgCl_2$ to the water sample to reach a final concentration 0,05 mol/l.

b) Filter the sample through a membrane filter of mixed cellulose esters (cellulose nitrate and cellulose acetate), 0,22 µm pore size, 47 mm of diameter. Filter at a rate of approximately 1 l in 30 min.

c) Cut the membrane filter in 8 fragments and place them into a glass flask containing 5 ml of eluting solution (1 % by mass of beef extract, 0,5 mol/l NaCl and 3 % by mass of Tween 80). Place the flask into an ultrasound-cleaning bath for 4 min.

d) Count the eluted bacteriophages by the double layer agar method.

e) Count the bacteriophages retained in the membrane fragments, by placing the fragments face down onto a host monolayer.

A.3 Flocculation with magnesium hydroxide

This method is based on Schulze and Lenk[5]. It consists of concentrating coliphages from drinking water using $Mg(OH)_2$ flocculation with minor modifications to allow the concentration of phages sensitive to high pH (for example F-RNA bacteriophages).

This method is recommended for volumes ranging from 100 ml to 1 l of waters with turbidity > 2,0 NTU.

For concentrating phages from 1 l of water proceed as follows:

a) Add 10 ml of a magnesium chloride solution (1 mol/l) to 1 l of water sample.

b) Add to the sample dropwise while stirring 3,5 ml of a dipotassium hydrogen phosphate solution (1 mol/l).

c) Add dropwise while stirring sodium hydroxide solution (2 mol/l) until turbidity appears (maximum final pH = 8,6).

d) Slowly stir the mixture for 15 min to obtain a regular distribution of the flocs to allow the incorporation of the phage particles into the flocs.

e) Allow the mixture to stand for 30 min to allow the settling of the flocs.

f) Siphon off the supernatant and concentrate loose sediment by low speed centrifugation (average of 1 000 *g* to 1 500 *g*) for 15 min.

g) Carefully discard the supernatant and resuspend the sediment with 30 ml of a suitable diluent. Resuspension requires vigorous shaking until flocs disappear completely.

h) Assay directly or store at 4 °C for a maximum of 2 d.

Annex B
(informative)

Example of a validation process of a concentration method

B.1 Spiking material

Spiking material was prepared as indicated in Clause 8.

Phage counts as indicated in Clause 8 are given in Table B.1.

Table B.1 — Phage counts of spiking material

Vial number	Counts 1	Counts 2	Mean of vial $\frac{z_{i+}}{J}$	Sum of counts z_{i+}
Vial 1	76 (z_{11})	77 (z_{12})	76,5	153
Vial 2	72 (z_{21})	70 (z_{22})	71	142
Vial 1 + Vial 2	—	—	—	295 (z_{++})

Calculation of T_1

T_1 for Vial 1 $(76 - 76{,}5)^2/76{,}5 + (77 - 76{,}5)^2/76{,}5 = 0{,}006$

T_1 for Vial 2 $(72 - 71)^2/71 + (71 - 72)^2/71 = 0{,}028$

T_1 (Vial 1) + T_1 (Vial 2) = 0,006 + 0,028 = 0,034, which is $< 5{,}99$

Homogeneity within vials is then acceptable.

Calculation of T_2

$T_2 = (153 - 295/2)^2/295/2 + (142 - 295/2)^2/295/2 = 0{,}41$, which is $< 3{,}84$.

Homogeneity between vials is then acceptable.

B.2 Preparation of spiked samples

125 ml, 250 ml, 500 ml and 1 000 ml volumes of spring water were spiked with 1,0 ml taken from one of the vials of the spiking material prepared as indicated in Clause 8.

B.3 Concentration

The above indicated water samples and 1 000 ml of non-spiked water were concentrated using the selected method.