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Water quality — Sampling for microbiological analysis

Qualité de l'eau — Échantillonnage pour analyse microbiologique

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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 19458 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Introduction

Appropriate sampling is essential to provide representative samples to the laboratory in charge of testing. Sampling features depend on the objective of sampling, but also on the nature of the sample. Microorganisms are living organisms. In addition, when they are introduced into water, they do not form a perfect solution, but a suspension with an inherent degree of variability.

Sampling objectives may serve different purposes, which are described in the ISO 5667 series of standards (ISO 5667-1, ISO 5667-2 and ISO 5667-3):

a) determination of the compliance of a water with a regulatory quality specification;

b) characterization of any contamination, its level (mean) and its variations:

   1) what is its random variation?

   2) is there a trend?

   3) are there cycles?

c) identification of the sources of pollution.

Regarding the number or frequency of samples, it will vary according to the aim of the sampling.

The minimum number of samples will be low if the mean concentration differs greatly from the specification (much lower or much higher), and the minimum number of samples will be higher if the mean concentration and the specification are close to one another. Similarly, in case b), when looking for a trend: the less obvious the trend, the higher the frequency of sampling (see also Annex A).

Water quality — Sampling for microbiological analysis

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This standard 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 absolutely essential that tests conducted according to this standard be carried out by suitably trained staff.

1 Scope

This International Standard provides guidance on planning water sampling regimes, on sampling procedures for microbiological analysis and on transport, handling and storage of samples until analysis begins. It focuses on sampling for microbiological investigations.

General information in respect to the sampling from distinct water bodies is given in the respective parts of ISO 5667.

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 5667-1, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes and sampling techniques*

ISO 5667-2, *Water quality — Sampling — Part 2: Guidance on sampling techniques*

ISO 5667-3, *Water quality — Sampling — Part 3: Guidance on the preservation and handling of water samples*

3 Sampling point

The sampling site shall provide representative characteristics and account for any vertical, horizontal and temporal variations and shall be identified precisely following the general recommendations of ISO 5667-1 and ISO 5667-2, taking into account additional aspects specific to microbiology.

Sampling points where conditions are unstable should be avoided, and the heterogeneity of the hydraulic system shall be taken into consideration. In studies on the efficacy of disinfection, the sampling point shall be chosen to ensure that the reaction is complete.

EXAMPLE Examples of how the heterogeneity of the system may influence the results are given below.

— It is not equivalent to take a subsurface or a surface sample, or a subsurface sample “contaminated” during recovery through the surface film. In some instances (e.g. lakes, swimming pools), the concentration in the surface film can be 1 000 times higher than in the subsurface.

— All the points of a network are not equivalent, as there may be dead ends and sections where the flow is reduced, particularly if the network is fed from two sources.

— The quality at the outlet of a well-mixed tank is generally the same as in the body of water, but can be quite different from the inlet.

4 Sampling technique

4.1 Personnel

Formal training, training records and determination of competence shall be described for all those who sample, and this information shall be properly documented.

4.2 Sample containers

4.2.1 General

For routine samples (for example, sampling at taps, recreational waters, swimming pool waters), use clean, sterile bottles. The volume of the bottles should be adequate for analysis of all requested parameters.

For sampling by immersion in clean waters, use bottles that are sterile both inside and out and protected, for example, by kraft paper (to keep dry after autoclaving), aluminium foil or by plastic outer bags.

If not autoclavable, sterilization with gamma rays or by ethylene oxide may be used. The bag can then be opened just before sampling and can also serve as a glove to hold the bottle to provide maximum asepsis before being placed on a pole or other sterilizable sampling apparatus.

Alternatively, the outside of sample bottles may be disinfected immediately prior to immersion by a suitable disinfectant such as isopropanol (4.3.1.1) and allowed to dry before use. This is not suitable for analysis of spore-forming bacteria.

In most cases, 500 ml bottles are sufficient, as less than five categories of microorganisms are measured, each involving inoculation of a maximum of 100 ml.

In some cases, larger volumes are necessary, e.g.:

— for bottled water analysis (250 ml per parameter);

— for *Legionella* spp. or *Salmonella* spp. (up to 1 l);

— for viruses, *Giardia* cysts, *Cryptosporidium* oocysts, amoebae in clean waters, from 10 to several hundred litres or more are examined. Usually, a concentration step is made on site using a cartridge filter which is then transported to the laboratory.

Bottles can be made of glass or various plastics (polypropylene, polystyrene, polyethylene, polycarbonate). Usually glass is preferred for re-use, and polyethylene is used as disposable.

Adhesion to surfaces can lower the detection of microorganisms, and the critical tangential surface tension γ has to be considered if a non-standard material is used [13].

Closures can be a ground glass or plastic stopper for glass bottles, a plastic press-on lid for plastic bottles or jars, or a plastic or metal screw cap for either. Bottle openings closed with plastic or glass stoppers should be further protected from contamination by, e.g. aluminium foil.

When larger volumes are necessary for the assay of, for example, viruses, *Salmonella* spp., amoebae, *Cryptosporidium* oocysts, *Giardia* cysts, it is sometimes necessary to analyse tens of litres or hundreds of

litres. To avoid the difficulties of handling, refrigerating and agitating such volumes, a concentration step *in situ* (by flocculation, centrifugation or filtration) is recommended. Peristaltic pumps can be used with sterile tubing.

NOTE 1 Metal caps, especially aluminium, can produce toxicity when autoclaved. This can be prevented by incorporating a heat-resistant leak-proof liner.

NOTE 2 Certain materials can also give toxic by-products when heat sterilized, even in a dry oven, or induce pH changes.

NOTE 3 Some brands of cotton wool used to make plugs for glassware may become toxic if they are heated for too long at too high temperatures.

NOTE 4 Press-on plastic lids attached to the bottle or jar have several advantages in that they are as leak-proof as screw-caps, and the lids can stand open, which facilitates filling and pipetting. When open, the lid remains linked to the bottle, so bottles and closures are kept together, and the lid is also protected from contamination.

4.2.2 Sterilization of bottles

If re-used, clean glass bottles and their closures with a non-toxic, phosphorus-free detergent, followed by a thorough rinse with deionized or distilled water.

Autoclave bottles at (121 ± 3) °C for at least 15 min. Keep the closure of the bottles loose, to allow the steam to replace all the air during the temperature rise, and to prevent plastic bottles from collapsing when cooling. Tighten screw caps after sterilization. Autoclave glass stoppers separately from the bottle, or use a paper or aluminium separator to prevent the stopper sticking on cooling.

If necessary, sterilize bottles in a dry oven for at least 1 h at (170 ± 10) °C. Separate ground glass stoppers from the neck by a paper strip or a piece of string to avoid jamming during cooling. The bottles should be traceable to the sterilization date.

Control the effectiveness of the sterilization process by chemical or biological indicators.

When sterilization is not possible with any other means, disinfect by immersing open bottles in boiling water for at least 30 min. Immediately after boiling, empty the bottles and close them with boiled caps and wrapped in clean paper.

NOTE 1 Polyethylene bottles can be sterilized by exposure to ethylene oxide gas, but, because of its toxicity, the procedure is carried out in specialized facilities and time allowed for desorption of the ethylene oxide. It is therefore not used as a routine laboratory procedure.

NOTE 2 Exposure to gamma rays produced by a ^{60}Co or ^{137}Cs source or to accelerated electrons of sufficient energy (1×10^4 Gy to 2×10^4 Gy) is a very efficient sterilization technique, available in specialized installations. There is no residual antibacterial activity, but some materials may be altered by polymerization after repeated irradiation.

4.2.3 Inactivation of disinfectants

To assess the microbiological quality of water disinfected by an oxidant (e.g. chlorine, chloramine, bromine or ozone), stop the action of the oxidant as soon as the sample is taken. Add a reducing agent such as sodium thiosulfate to the sample bottles.

The theoretical mass of sodium thiosulfate (pentahydrate) necessary to inactivate 1 mg of chlorine is 7,1 mg. Thus, 0,1 ml of sodium thiosulfate pentahydrate solution (4.3.1.2) is added for each 100 ml of bottle capacity. This will inactivate at least 2 mg/l and up to 5 mg/l of free chlorine residual, depending on inactivation dynamics, which is sufficient for the majority of samples.

In certain circumstances, such as foot baths in swimming pools, disinfection measures (e.g. *Legionella* eradication in drinking water distribution systems), higher chlorine concentrations can be found and a proportionately higher dosage of sodium thiosulfate will be necessary.

Sodium thiosulfate is not destroyed by autoclaving or dry heat. Ensure that the pH of the sodium thiosulfate solution is around neutral (low pH can cause decomposition).

Sodium thiosulfate has no effect on the sample and can be used for non-chlorinated waters too.

NOTE It has been claimed that *Legionella* are sensitive to sodium and that potassium thiosulfate is preferable, but no adverse effect of sodium has been detected at the concentration used to inactivate usual chlorine concentrations.

For other disinfectants, corresponding inactivation measures need to be taken. If inactivation is not possible or feasible, it has to be reported.

Chelating agents have been recommended to protect bacteria from the toxic action of heavy metals such as copper or zinc. Ethylene dinitrilotetraacetic acid (EDTA) or sodium nitrilotriacetate (NTA) ($Na_3C_6H_6NO_6$) can be used as a filter-sterilized solution at a final concentration of about 50 mg per litre but should only be added when necessary (e.g. water treated with silver or copper). Silver can also be inactivated by sodium sulfide. Add 1 ml of a sodium sulfide solution (4.3.1.3) to 1 l of sample.

4.2.4 Quality control of sample bottles

4.2.4.1 Testing of sterility

The laboratory shall ensure the sterility of the sample bottles, whether they are prepared in-house or commercially, whether they are made of glass or of plastic. Commercially prepared bottles should be delivered with a certificate of sterilization as a condition for acceptance, and sterility tests are also advisable on the batch in use. This relates to the batch of bottles after labelling, addition of inactivation agents where relevant, and storage.

The sterility of bottles can usually be guaranteed by control of the sterilization process. If not, the sterility of the containers should be tested.

EXAMPLE The following are examples of testing procedures (usually performed a rate of 1 per 100 bottles):

a) "Roll bottle" method

This consists of introducing 20 ml or 50 ml of melted nutrient agar (plate count agar) into the test bottle and lining the walls with agar by rotating the bottle while cooling (under a trickle of water if necessary). Incubation at (22 ± 2) °C for five days should give no visible growth.

b) Liquid broth method

This consists of placing 20 ml to 50 ml of thioglycollate or other nutrient broth inside the bottle, rolling the bottle to wet the walls and incubating at (22 ± 2) °C for five days. No turbidity should appear if sterile.

4.2.4.2 Testing for the presence of inactivating agents

The presence of thiosulfate may be checked by an iodometric method:

$$I_2 + 2\ S_2O_3^{2-} \longrightarrow 2\ I^- + S_4O_6^{2-}$$

Add 10 ml distilled water to the bottle and titrate with iodine solution (4.3.1.4), using starch or thiophene as an end point titration agent.

4.2.4.3 Testing for residual toxicity in sample bottles

Residual toxicity in sample containers may result from the washing procedure of glassware, from the release of components or additives from plastic bottles and also from the sterilization process. Routine use of glass or polyethylene bottles does not require a regular check for toxicity, but if in any doubt, test according to Geldreich, 1975 [8] (for example).

4.3 Reagents, apparatus and materials

4.3.1 Reagents

4.3.1.1 Ethanol, volume fraction $\varphi(C_2H_5OH) = 70\ \%$, **isopropanol**, volume fraction $\varphi[(CH_3)_2\ CHOH] = 70\ \%$, or **hypochlorite solution**, $\rho(CLO^-) \approx 1$ g/l.

4.3.1.2 Sodium thiosulfate pentahydrate solution, $\rho(Na_2S_2O_3 \cdot 5H_2O) = 18$ mg/ml.

4.3.1.3 Sodium sulfide solution, $\rho(Na_2S) = 0{,}1$ mg/ml.

4.3.1.4 Iodine solution, $c(I_2) = 0{,}05$ mol/l.

4.3.2 Apparatus and materials

In addition to sample containers, the following items may be necessary.

4.3.2.1 Soap and towels.

4.3.2.2 Gas blow lamp and **refill**.

4.3.2.3 Jars or beakers, disinfecting wipes.

4.3.2.4 Lighter, matches.

4.3.2.5 Markers, pencils, labels.

4.3.2.6 Spanners, pliers, screwdrivers, knife.

4.3.2.7 Icebox and ice or ice packs, portable refrigerators or refrigerated compartments in vehicles.

4.3.2.8 Thermometer or temperature recorder.

4.3.2.9 Ballasted bottle-carrier or equivalent, with rope or chain (preferably stainless steel, at least the bottom part).

4.3.2.10 Pole or long forceps or samplers adapted to various depths.

4.3.2.11 Maps, list of sampling points, sampling forms.

4.3.2.12 Vehicle and papers, identity or authorization card.

4.3.2.13 Waterproof (safety) boots.

4.3.2.14 Apparatus to measure pH, chlorine, dissolved oxygen, conductivity.

4.3.2.15 Sterile gloves.

4.4 Filling procedure

4.4.1 Potable water from a tap

4.4.1.1 General

Sampling at a tap can have different purposes:

a) to determine the quality of the water in the distribution main (which is the responsibility of the distributor);

b) to know the quality of the water as it flows from the tap to be consumed — as it is delivered to the tap — (which can be altered by the service network inside the building);

c) to know the quality of the water as it is consumed, i.e. as it flows out of the (possibly contaminated) tap.

Samples to assess the quality in the main [case a)] are best taken at special taps (also in the distribution system) that are close to the main distribution, clean, without attachments and disinfectable by flaming or suitable equivalent.

Normal taps may be used to assess the quality in the mains [still case a)], if they are disinfectable by flaming but in case of unclear results, consider the service network as potential source of contamination.

The situation described in case b) is the method of choice to assess the quality of drinking water including the influence of the service network inside the building. In this case, taps disinfectable by flaming are not always available and other disinfection methods [application of hypochlorite solution, ethanol or isopropanol (4.3.1.1)] need to be considered.

The situation described in case c) is the method to assess the quality of drinking water in special situations, e.g. outbreaks.

Depending on the purpose, it is either necessary or incorrect to:

— remove attached devices and inserts;

— disinfect the tap;

— flush (see Table 1).

Table 1 — Sampling at a tap for different purposes

Purpose (see above)	**Water type**	**Remove attached devices and inserts**	**Disinfect**	**Flush**
a)	In the distribution main	Yes	Yes	Yes
b)	As it is delivered to the tap	Yes	Yes	No [a] (minimal)
c)	As it is consumed	No	No	No
[a] Flush briefly only to overcome influence of disinfection of the tap.				

Sampling from drinking water storage tanks is usually made from a tap on the outlet. Subsurface samples are sometimes taken from the tank itself, in which case, bottles that are sterile both inside and outside (see 4.2.1) are required.

Ensure samples are taken aseptically using clean hands or sterile gloves with protection of the sample from air drifts and splashing.

During filling, the inside of the bottle closure shall not come in contact with anything (fingers, ground, pocket, teeth).

Leave some air space in the bottle to allow for adequate shaking before analysis.

Close the bottle immediately. Do not use this water sample for the measurement of temperature or any other on-site tested parameter.

For details, see as well ISO 5667-5.

4.4.1.2 Water in treatment works and storage tanks

In water treatment works and storage tanks, dedicated sample taps should be provided on each outlet main and other sampling points. These should be capable of being sterilized by flaming, maintained in a clean state, labelled clearly and used exclusively for sampling. For details, see ISO 5667-5 and ISO 5667-13.

Flaming a metal tap intensely with a blowlamp ensures disinfection of the mouth if the temperature there reaches 80 °C or more. This is not the case if water remains in the heated portion.

NOTE Flaming with a lighter is only superficial (not sufficient).

4.4.1.3 Water in the distribution main

To determine the quality in the distribution main, sample in the distribution main or close to it (usually just after the water meter). Ensure that no contamination from the outer surface of the tap reaches the sample. Do not sample taps with leaking spindles and avoid mixer taps, if possible. Take out any faucet nozzle or other attachment or insert (spanners and pliers shall be available). Scrape off any dirty (scale, slime, grease or other extraneous matter) and fully open and close the tap repeatedly to rinse out the dirt from the tap. Disinfect the tap preferentially by flaming (after flaming and opening the tap, a sizzling noise should occur). Subsequently, open the tap to half-flow and flush until constant water temperature is reached (to overcome the water inside the building). Then place the open bottle in the water flow and fill it under aseptic conditions.

Only if flaming is not possible, disinfect the tap by other adequate methods. To disinfect the mouth of a plastic tap, after thorough cleaning, dip it for 2 min to 3 min in a beaker with hypochlorite solution, ethanol or isopropanol (4.3.1.1). Alternatively, a swab or a wash bottle or similar device may be used to disinfect the outside and as much of the inside as possible.

Subsequently, allow the water to flow long enough to minimize the influence of the network inside the building. It is necessary to know the detailed layout of the network (volume of tanks or softeners and retention time) to determine the flush time before sampling. Water temperature stabilization may be monitored to achieve the same effect for the same purpose. Many water microorganisms result from disruption of biofilm and resuspension of deposits from joints or knees in case of peak flows and pressure jars. To minimize these effects, open the tap at maximum flow for 5 s to 10 s, then reduce to half flow for the time needed, and place the bottle under the tap without closing and re-opening the tap.

4.4.1.4 Water as it is delivered to the consumers tap

To determine the quality as it is delivered to the consumer's tap, ensure that no contamination from the outer surface of the tap reaches the sample. Scrape off any dirt (scale, slime, grease or other extraneous matter) which could fall off, before filling the bottles. Do not sample taps with leaking spindles. Take out any faucet nozzle or other attachment or insert (spanners and pliers shall be available). Disinfect the tap preferentially by flaming or, if not possible, by other adequate methods (see above). Subsequently, allow the water to flow just long enough to ensure that the sample has no residual thermal or disinfectant effect. Place the bottle under the tap without closing and re-opening the tap.

4.4.1.5 Water as it is consumed

To determine the quality of the water as it is consumed (e.g. in outbreak situations), contamination of the water by bacteria from the outside of the tap and from any attachment or device should be taken into consideration. Therefore, attachments should be kept in place and the tap should not be disinfected prior to sampling.

4.4.2 Water from springs and wells

The analysis of well water can have different purposes:

1) to know the quality of the ground water;

2) to know the quality of the well water;

3) to know the quality of the water as it is used.

Depending on the purpose, different sampling types need to be chosen, where it has to be differentiated between boreholes or wells in which pumps are permanently installed and boreholes or wells without permanently installed pumps. Table 2 and Table 3 give an overview on the kind of sampling method which should be chosen depending on the purpose in wells with and without a permanently installed pumping device.

Table 2 — Sampling of well water for different purposes in wells with permanently installed pumping devices and a metal tap or outlet

Purpose	Water type	Pumping	Disinfection of the tap
1	Ground water	yes (extensive)	yes
2	Well water	no (minimal)	yes
3	Water as it is consumed	no	no

Table 3 — Sampling of well water for different purposes in wells without permanently installed pumping devices

Purpose	Water type	With submersible pump (clean)	With an inside and outside sterile bottle	From a bucket
1	Ground water	+ [a]	–	–
2	Well water	+ [b]	+	–
3	Water as it is consumed	–	–	+

[a] After extensive pumping.

[b] Only minimal pumping.

Boreholes or wells in which a pump is permanently installed, usually also have a metal tap or outlet. Depending on the purpose of sampling, extensive pumping is necessary or not, and disinfection of the tap, preferentially by flaming, is necessary or not, see Table 2.

Extensive pumping for purpose 1 means that the pumping should last until constant water temperature and electric conductivity are reached or at least a three times renewal of the well volume is guaranteed. For purpose 2 (to know the quality of the well water) only a short water exchange is necessary, just to overcome the influence of the disinfection of the tap. For purpose 3, neither pumping nor disinfection of the tap is necessary.

Boreholes or wells without a permanently installed pump (Table 3) are sampled with a submersible pump for purpose 1. This pump is used only for clean waters and again extensive pumping (see above) shall be guaranteed. For purpose 2, preferentially a sterile sampling device should be used which includes a ballasted carrier. Alternatively, a clean submersible pump may also be used after only minimal pumping in advance. For purpose 3, when water from wells or boreholes without pumping devices is used by consumers, for instance, by buckets, etc., fill the water from the bucket in a sterile sampling bottle.

4.4.3 Water from swimming pools

For sampling ("after") filters or on the pipes feeding pools, dedicated sampling taps shall be available, welded short onto the pipes to avoid stagnation. Fill the bottles in the same way as for taps in the distribution main (4.4.1.3).

For sampling the inflow of the water into the pool (after clarification, treatment and chlorine injection) take the sample at a distance from the injection point, where the disinfectant residual is stable.

Normal surveys of pool water involve subsurface sampling (−10 cm to −30 cm), using a sampling pole, opposite the inlet. Use clean, sterile bottles. In the absence of vertical water flow, attention should be taken to select the more appropriate and representative sampling point. The bottle is introduced horizontally to avoid losing thiosulfate, then turned upright until enough water has been collected.

NOTE On the surface of pool water, in quiet conditions, a microlayer forms with an accumulation of microorganisms like staphylococci inside floating skin scales. In some swimming pools, surface contamination can also be evaluated by sampling the overflow peripheral drain.

4.4.4 Surface waters

4.4.4.1 Bathing waters

Bathing waters (lakes, rivers, seaside) are usually classified after a series of measurements, over a season. The sampling points shall be strictly defined.

The sampling points should be representative of the water quality at the site used by the majority of the bathers, or where pollution is expected, depending on the purpose of the sampling.

Take subsurface samples (−20 cm to −30 cm) in a 1 m to 1,5 m deep water column. Introduce the bottle upside down in the water to the sampling depth. Subsequently, fill the bottle by turning it sidewards and upwards to avoid contaminations. Where a current exists, hold the bottle upstream.

At some beaches, a water column of 1 m is not achieved and the sample needs to be taken at a shallower depth. This should be noted and special attention paid to resuspension effects.

NOTE One of the major sources of variation in beach water quality is the resuspension of bacteria adsorbed on clay or organic silt. Coarse sand present in hydrodynamic zones does not adsorb many contaminants. Various natural and manmade causes of resuspension can increase sanitary risks and should be taken into account, for example, spring tides, storms, boating. But faulty sampling can have the same effect (e.g. filling too close to the bottom, agitating the sediment, own ship movement).

4.4.4.2 Sea, lakes, rivers: from a boat

Seasonal patterns and vertical stratification of lake and sea water and mixing of river water should be considered when selecting the exact sampling sites.

Many devices are available to take subsurface or deep samples offshore, but oceanographic bottles used for chemistry (Go-Flow, Nansen or Van Doorn bottles [12]) are not sterilizable and are not appropriate.

The J-Z system [12] consists of a sterile glass bottle, under vacuum. It is fitted with a rubber stopper and a glass tube, sealed and bent so that it is near the rope. When the bottle is at the desired depth, a messenger is sent on the rope, breaks the tube and the bottle fills. Piggybacking on a Nansen bottle is also possible.

Syringe systems and various other techniques have been used, the most complicated ones maintaining the water sample at the *in situ* pressure, for the study of barophilic bacteria.

When using such devices, or a stem pole in shallow waters, keep the disturbance of sediments by the operator, ship, anchor, etc. to a minimum.

From a ship adrift, take samples from the lee side, not to windward. From a ship lying at anchor, or being headed, take from the stem.

Any possible contamination from ropes carrying sterile instruments should be minimized, using, for example, stainless steel wire or a chain at the bottom of the line.

4.4.5 Waste waters

Use disposable gloves or sterilizable poles or forceps for subsurface sampling, to minimize the infection risk for the sampling personnel. Remove dirt from the bottle outer surfaces and/or put them in clean bags and transport them separately from drinking water samples. Procedures (sampling devices) mentioned in 4.4.4.2 (sea lakes, rivers) are applicable.

4.4.6 Surface-associated microorganisms

Take biofilm samples by mechanically scraping the surface with a sterile spatula, blade or swab. Suspend the biofilm sample in a sterile sample container and analyse after homogenization.

NOTE The spatial arrangement of the microorganisms will be destroyed by this qualitative sampling procedure.

Corrosion bacteria are sought in the sediment obtained from liquid samples through filtration, decantation or centrifugation. Particles of corroded metal can be obtained by scraping or inducing “water hammer” (sudden change of pressure in a water pipe).

Sulfate-reducing bacteria can sometimes be found in water, but their role in metal corrosion is best proven by swabbing inside tubercules which are still wet, rather than sampling the water only.

4.5 Sampling form

Uniquely identify and label the bottle, and fill in the sampling form (samplers log) before, or just after, sampling.

The form shall at least indicate the name and address of the client, the list of parameters to analyse, date, time and location of sampling as well as the name of the person who is taking the sample. The nature (origin) of the sample and the purpose of the analysis are also needed as they may help with the choice of methods. Other details may be necessary for the correct interpretation of the results (e.g. temperature, biocides, exact sampling point, any observations on phenomena which could affect the microbiological quality of the water).

5 Transport and storage

5.1 Transport

Keep the time between sampling and analysis in the laboratory as short as possible. For drinking waters, analysis should ideally be started within the same working day.

Cool samples — ideally (5 ± 3) °C — during transport (e.g. by using ice packs or melting ice), unless otherwise stated in specific standards. Take care not to freeze them (except for viruses). Protect samples from sunlight.

For samples transported for periods over 8 h, it is necessary to monitor and record the temperature.

Transport conditions shall be documented.

Nevertheless be careful:

— not to put ice-packs in direct contact with the sample, as this can result in freezing it [10];

— to adjust the number, volume and position of ice packs according to samples number, mass and initial temperature [9].

Document the procedure required for longer transport times ($>$ 8 h).

Warm and cold samples shall be transported separately.

For guidance on the transport and storage of water samples for physico-chemical, chemical, radiochemical and biological analyses, see ISO 5667-3.

Between 0 °C and 45 °C, bacterial reactions are proportional to the temperature. If a microflora is multiplying, the higher the temperature, the faster the multiplication. On the other hand, if it is dying, the reaction is also accelerated by warming. In bacteriology, a Q_{10} of 2 is usually assumed. This means that a temperature increase of 10 °C increases the speed by a factor of two, both in multiplication and mortality processes. It is thus important to cool samples during transport, but not to freeze them, as ice formation can be responsible for the death of the majority of cells ($>$ 99 %). Samples for virus analysis only, can be kept at -70 °C if appropriate cryoprotectant is added to the sample.

NOTE 1 In optimal conditions, one division of an *E. coli* cell takes 20 min and leads to 1×10^9 cells 10 h later. However, water contains other microorganisms and inadequate nutrients, so such multiplication cannot take place. On the other hand, the microflora can be halved in less than 20 min; the disinfectant action of chlorine without inactivation is a matter of seconds.

NOTE 2 Most experiments performed on the storage of water samples for bacteriological examination show a beneficial effect of refrigeration below 10 °C. The ideal temperature range of (5 $\pm$ 3) °C, is targeted by placing the sample in an ice-box, with ice (preferably artificial ice-packs). But, obviously the temperature of the water will not be (5 $\pm$ 3) °C as soon as the bottle is put into the box. There is a period of temperature equilibration during which the temperature within the box will be variable due to:

— the ice box (volume, insulating characteristics);

— the outer temperature;

— the mass of water samples and their initial temperature;

— the mass of ice.

NOTE 3 “Artificial ice” packs exert a greater cooling effect than true ice and will not melt, thus limiting the risks of labels becoming detached, ink marks becoming obliterated or samples contaminated. They are therefore preferable to true ice.

5.2 Time delay

The delay between sampling and analysis includes transport, registration and processing in the laboratory.

Time delay between sampling and analysis may reduce the reliability of the results. Consequently, samplers and analysts shall work together to keep to a minimum the number of samples analysed on the day after sampling. Time delays should be as short as possible and noted on laboratory reports.

NOTE 1 According to Reference [4], the maximum delay often mentioned of 8 h represents the delay of a round trip between the Royal Public Health Laboratory in London and the farthest point on the Thames, by horse-buggy in the last century. Despite modernization in transport systems, the present tendency is to allow longer delays. In reality, contradictory experimental observations exist in the literature.

NOTE 2 In disinfected waters as well as in sea or river waters, cells of bacterial faecal indicators seem to be in a mortality process, suggesting a weakened physiological state. In Reference [8], the author recommended a maximum delay of 8 h, according to earlier studies of these waters.

NOTE 3 For untreated drinking water or some eutrophic waters, longer delays are proposed by some authors. However, all microbiologists and specialized books recommend analysis of samples as soon as possible, as a clear majority of studies show significant changes even with refrigeration [5], [6], [7].

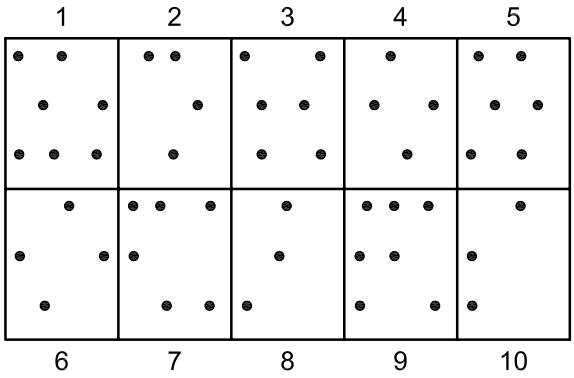
Table B.1 summarizes the recommendations for maximum delay extracted from the literature. The times mentioned are tentative: they depend on type of water, physiological state of the microorganisms (e.g. disinfection or not), and even the analytical method. However, values for maximum sample storage times including transport time and temperatures in the specific standards shall be followed.

Results obtained beyond the recommended time of storage should have a note stating results obtained after n hours.

Annex A
(informative)

A priori determination of the number of samples to analyse to determine the mean concentration of microbes in water with a given confidence, for quantitative determination derived by cultivation of microorganisms

A.1 General



None of the ten 100 ml-portions contains the theoretical mean of 5 microorganisms per 100 ml concentration.

Figure A.1 — Example of distribution of 50 microorganisms in a well-mixed volume of 1 l of water

It can be demonstrated that under ideal conditions of homogenization, the distribution of the number of bacteria approximates to Poisson law.

Characterized by a variance which is equal to the mean, this law is indeed observed for low densities ($<$ 12 colony-forming particles per analysed volume), e.g. for *Salmonella* or *E. coli* in drinking waters.

For higher concentrations($\geqslant$ 12 colony-forming particles per analysed volume), the variance, s^2, is often greater than expected:

$$s^2 = K \cdot m, \text{ where } K > 1.$$

The distribution is overdispersed or contagious; K is called the overdispersion coefficient. m is the arithmetic mean count.

A.2 Calculation

To calculate the number of samples to analyse, proceed as follows:

a) choose the tolerable deviance D on the results: e.g. $\pm$ 20 %, $\pm$ 50 %, expressing percent values as decimals (e.g. 20 % = 0,20);

b) having, if possible, an idea of the concentration level of the water to be analysed, estimate the expected average count m per test portion;

c) and having, if possible, a hypothesis of the value of the overdispersion coefficient K using Table A.1;

d) then determine the number of samples N from the equation given below:

$$N = \frac{K \cdot \chi_1^2}{m \cdot D^2}$$

where

N is the number of samples;

χ_1^2 is the value of the chi-squared distribution with one degree of freedom (the value for 95 % significance is 3,84.);

K is the variance-to-mean ratio, overdispersion coefficient [14];

D is the deviance tolerated, expressed as decimal fraction of the mean;

m is the arithmetic mean count.

Table A.1 — Estimation of the overdispersion coefficient K from the type of water and the number of microorganisms expected

Water turbidity	Concentration of microorganisms m (colony-forming particles per analysed volume)			
	< 12	12 to 30	30 to 50	> 50
Clear water	K = 1	K = 1,5	K = 3	K = 8
Turbid	K = 1	K = 2	K = 4	K = 12
Very turbid	K = 1	K = 2	K = 5	K = 16

A.3 Examples

A.3.1 Example A.3.1

For example, if the precision target is 20 % around an estimated mean of 5 colony-forming particles per analysed volume, the overdispersion coefficient is K = 1 (Table A.1), then the required number N of samples with a confidence level of 95 % is:

$$N = \frac{3,84}{(0,2)^2 \times 5} = 19,2$$

Thus, a number of N = 19 samples is needed in order to determine the microbial concentration of a water sample containing around 5 colony-forming particles per analysed volume, with a tolerable deviance of $\pm$ 20 %.

If only 5 samples are taken, the relative precision will not be better than 40 %.

If only one sample is analysed, the risk of a false negative is 70 %.

A.3.2 Example A.3.2

For a concentration around 30 colony-forming particles per analysed volume, Table A.1 gives an overdispersion coefficient of K = 4 (variance 4 times higher than the mean) for turbid waters.

At the confidence level of 95 % and for a tolerable deviance of 20 %, the calculation of the number of samples to analyse is:

$$N = \frac{4 \cdot 3,84}{(0,2)^2 \cdot 30} = 12,8$$

Therefore, 13 samples will have to be analysed in order to estimate the microbial concentration, with 20 % tolerable deviance and 95 % confidence, in turbid water containing around 30 colony-forming particles per analysed volume.

A.3.3 Example A.3.3

Turbid surface water with expected microbial concentration around 15 colony-forming particles per analysed volume K = 2 (Table A.1).

For a tolerable deviance of $\pm$ 20 %, the number of samples to analyse is:

$$N = \frac{2 \cdot 3,84}{(0,2)^2 \cdot 15} = 13$$

For a tolerable deviance of $\pm$ 50 %, the number of samples to analyse is:

$$N = \frac{2 \cdot 3,84}{(0,5)^2 \cdot 15} = 2$$

A.3.4 Example A.3.4

Tolerable deviance adopted: $\pm$ 20 %

Turbidity of the water analysed: clear water

Expected microbial concentration: 20 colony-forming particles per analysed volume

K = 1,5 (from Table A.1)

Number of samples to analyse:

$$N = \frac{1,5 \cdot 3,84}{(0,2)^2 \cdot 20} = 7$$

Seven samples are taken and analysed.

The results are: 13, 15, 7, 8, 19, 8, 13.

Then

$$m = \frac{13 + 15 + 7 + 8 + 19 + 8 + 13}{7} = 11,9$$

(considerably lower than expected)

and

$$s^2 = \frac{(13 - 11,9)^2 + (15 + 11,9)^2 + (7 - 11,9)^2 + (8 - 11,9)^2 + (19 - 11,9)^2 + (8 - 11,9)^2 + (13 - 11,9)^2}{7 - 1} = 19,5$$

$$K = \frac{19,5}{11,9} = 1,6$$

so, the number of samples was appropriate and can be used for further surveys.

Annex B
(informative)

Recommended (R) and acceptable (A) values for maximum sample storage times including transport time and temperatures unless otherwise specified in specific standards

Table B.1

	Maximum sample storage time (h) including transport		Storage water temperature °C		Observation [a]
	R	A	R	A	
General					
Culturable microorganisms (22 °C, 30 °C, or 36 °C)	8	12	5 ± 3		
Faecal indicators, vegetative bacteria					
E. coli (and coliform bacteria)	12	18	5 ± 3		
Enterococci	12	18	5 ± 3		
Clostridium perfringens (vegetative cells)	12	18	5 ± 3		
Spores					
Spores of sulfite-reducing bacteria *(Clostridium* spp.*)*	24	72	5 ± 3		Die-off observed in raw waters after 24 h
Viruses					
Bacteriophages	48	72	5 ± 3		
Faecal pathogens					
Salmonella spp. and other *Enterobacteriaceae*	12	18	5 ± 3		
Enteroviruses	48	72	5 ± 3		
	1 month		−70	−20	
Cryptosporidium oocysts	24	96	5 ± 3	ambient	
Giardia cysts	24	96	5 ± 3		
Other microorganisms					
Amoebae	24	96	5 ± 3		
Pseudomonas aeruginosa	8	12	ambient	5 ± 3	
Legionella spp.	24		5 ± 3	ambient	
		48	5 ± 3		
Cyanobacteria	48	72	5 ± 3		Lysis sometimes appears within a few hours
Campylobacter (thermophilic spp.*)*	24		3 ± 2		Oxygen-sensitive
Total bacteria for epifluorescence	1 year		ambient		Sample to be stabilized in dust-free vial, + formaldehyde (final concentration 3 %) in the dark
Helminth eggs	48	72	5 ± 3		
		1 week	5 ± 3		Sample stabilized at pH = 2

[a] See References [1], [2], [4], [5], [6], [7], [8], [9], [11] and [15] in the Bibliography.

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