Umwelt Bundesamt

Quantitative determination of the efficacy of drinking water disinfectants

Contents

Foreword

The test procedure described in this paper was developed by staff of Section II 3.3, Drinking Water Resources and Water Treatment[1]), of the German Federal Environment Agency in collaboration with Section II 1.4, Microbiological Risks. The test procedure has been approved both by the Drinking Water Commission of the Federal Ministry of Health at the Federal Environment Agency and by the appropriate working group of the German Technical and Scientific Association for Gas and Water (DVGW). This is the first initiative to provide scientifically validated efficacy criteria for drinking water disinfectants suitable for regulation.

Introduction

Hygienic risks in drinking water and disinfection

Relevant pathogens which could be spread via drinking water are e.g. campylobacter, EHEC, noroviruses, adenoviruses, hepatitis viruses, giardia, or cryptosporidia. They belong to the groups of bacteria, viruses and parasites.

They enter the aquatic environment chiefly via animal or human faeces, which, in addition to pathogens, usually contain a large amount of *Escherichia coli* and other harmless bacteria. As a result, routine water sampling for the presence of these typical faecal organisms (i.e. *Escherichia coli* or enterococci) suffices during drinking water quality monitoring to indicate faecal pollution and thus the possibility that pathogens are present. Therefore, these bacteria are called ‘indicator organisms’. In addition to *Escherichia coli*, bacteriophages are discussed as indicators for viruses. The indicator concept is valuable since it is impossible to test for all potentially occurring pathogens at reasonable costs. The target for disinfectants, however, should be not only the inactivation of the (rather sensitive) indicator bacteria, but should preferably more broadly also include the inactivation of viruses (i.e. action as broad-band disinfectant).

The Drinking Water Directive DWD (98/83/EC) requires that pathogens should not be present in drinking water in concentrations constituting a potential danger to human health. Sufficient elimination of pathogens in the process of drinking water treatment has to be guaranteed. Disinfection is one such elimination step.

However, pathogens can enter drinking water after treatment, i.e. during distribution. Possible reasons for contaminations are insufficient hygiene at construction points along the water pipe system or their emergence from biofilms in water pipes. Where such contamination needs to be addressed, disinfectants with a long lasting capacity must be applied.

A basic paradigm for the use of substances to treat drinking water – including disinfectants – is that they should only be added for specific hygienic or technical reasons, which limits their application to the minimum volumes that are essential for achieving the targeted effect (principle of minimisation) and only under conditions optimizing their efficacy.

Currently, there is no disinfectant available to eliminate (oo-)cysts of cryptosporidium and giardia under concentrations commonly used for drinking water.

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Test method

This test guideline describes a simulation process to determine whether a disinfectant that has been suggested for the purposes described in Section 1 has a sufficient bactericidal and virucidal effect for safe application in drinking-water supplies. The test is a basic assay, classified as phase 2 step 2 test to examine efficacy against planktonic (i.e. suspended in water) microorganisms in water. To test for efficacy against biofilms (e.g. containing Legionella), other tests have to be applied. The determined efficacy of the tested disinfectant can differ due to water quality or other specific conditions. For example, efficacy will increase with increasing water temperature. Therefore, irrespectively of the generic assessment of the efficacy of a given disinfectant or disinfection procedure, it remains important to validate its reliability and efficacy on site for the respective specific purpose.

The efficacy criteria in this test are based on the efficacy of hypochlorite and chlorine dioxide as reference. The reference concentrations at 20 minutes after dosing are determined to be 0.1 mg/l for hypochlorite and 0.05 mg/l for chlorine dioxide at a dosage concentration of about 0.4 mg/l for hypochlorite and chlorine dioxide. The elimination target for the test organisms and test viruses is set at a reduction of log 4 (99.99 %) during the test procedure (lower reduction rates would not provide sufficiently clear quantitative results while higher reduction rates would require unrealizablelarge amounts of test organism suspensions for continuous dosing in the flow-through mode). The test water is discarded after passing the test pipe.

A flow-through mode was chosen for better reflection of conditions in drinking-water treatment as compared to experiments in batch assays (as are used for the assessment of disinfection efficacy for some other applications). The procedure is conducted as laboratory test in a semi-technical test rig; it simulates application conditions at waterworks in actual practice. If results show sufficient efficacy, the concentration of the tested disinfectant can be described as concentration necessary to achieve this effect. While this concentration applies only to the specified experimental conditions, the test as such can nonetheless determine whether or not a disinfectant is generally suitable for use in drinking water disinfection. In practical applications, dosing may deviate from the concentration determined to be sufficiently effective by this test, depending on indication, application conditions and legal regulations. Determining the adequate doses for different water matrices and application scenarios are a separate (next) step not addressed here.

The test does not include the analysis of disinfection by-products in order to prevent health risks. In general, toxicological risk should be assessed at the disinfectant concentration used in this test. It is important that the limits set in Annex I of the Drinking Water Directive DWD (98/83/EC) are met.

1 Scope

This test procedure defines minimum standards for sufficient bactericidal and virucidal effects of disinfectants that are introduced into water intended for drinking water production (e.g. in a waterworks), or into drinking water in the distribution network. The test covers efficacy against planktonic microorganisms and viruses in water. Other tests have to be used to evaluate efficacy against biofilms (e.g. containing Legionella). Moreover, though not the primary purpose of this test, it can be used to evaluate disinfectants for water for animal consumption.

The test is part of the Technical Guidance Document in support of the Biocidal Product Regulation (EU) No 528/2012 concerning the availability on the market and use of biocidal products. The test shouldbe used for active substances and for the assessment of product dossiers. If an active substance or product fails the test an authorization should not be granted. In case that the test is not applicable, other tests have to be used. An overview of EN tests for disinfectants can be found in EN14885. Annex V of this standard also includes tests for Product-type 5.

Furthermore, this test is also recommended to be used by member states as part of the assessment of drinking water disinfectants under the Drinking Water Directive DWD (98/83/EC).

A prerequisite for the determination of bactericidal or virucidal effects is that the substance is quantitatively measurable at reasonable costs and that its effect can be neutralised (inhibited) within a short period of time (Annexes C and D) in order to stop it from reacting during sampling and thus enable the determination of residual disinfectant in routine monitoring. If an active substance cannot be neutralised, this test must be adapted or other methods must be applied.

Under specific conditions (see section 5.7) additional test conditions may be required for a final assessment.

General information for the practical application of drinking water disinfectants:

As drinking water disinfection is complex, the efficacy of the disinfection procedure should be verified for each individual water supply. The scope of this test is therefore not to assess efficacy on site, but rather to assess the basic performance of the substance or procedure.

2 Normative References

This test guideline contains specifications from standards that are identified by means of dated and undated references. These normative references are quoted at the respective points in the text, and the respective standards are also detailed below. In the case of dated references, alterations or revisions of these standards are only part of this test guideline if they have been included in the guideline by means of alterations or revisions. With undated references, the latest edition of the respective standard applies.

EN ISO 7899-1. Water quality - Detection and enumeration of intestinal enterococci in surface and waste water - Part 1: Miniaturized method (most probable number) by inoculation in liquid medium (ISO 7899-1:1998)

EN ISO 7899-2. Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method (ISO 7899-2:2000)

EN ISO 9308-1. Water quality - Detection and enumeration of Escherichia coli and coliform bacteria - Part 1: Membrane filtration method (ISO 9308-1:2000)

EN ISO 9308-3. Water quality - Detection and enumeration of Escherichia coli and coliform bacteria in surface and waste water - Part 3: Miniaturized method (most probable number) by inoculation in liquid medium (ISO 9308-3:1998)

EN ISO 8199. Water quality - General guidance on the enumeration of microorganisms by culture

EN 12671. Products for the treatment of water for human consumption − chlorine dioxide.

EN ISO 7393-2. Water quality - Determination of free chlorine and total chlorine - Part 2: Colorimetric method using N,N-diethyl-1,4-phenylenediamine, for routine control purposes (ISO 7393-2:1985)

EN ISO 10705-1. Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages (ISO 10705-1:1995)

EN ISO 10705-2. Water quality - Detection and enumeration of bacteriophages. Enumeration of somatic coliphages

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products (Biocidal Products Regulation)

3 Definitions

The following definitions apply to the terms used in this test guideline:

3.1 Product

The definition of ‘biocidal product’ given in Article 2(1) of the Biocidal Products Regulation applies:
"- any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action,
- any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action.

A treated article that has a primary biocidal function shall be considered a biocidal product."

3.2 Active substance

The following definition of ‘active biocidal substance’ pursuant to the Biocidal Products Regulation applies:
"*[...] substance or a micro-organism that has an action on or against harmful organisms.*"

3.3 Disinfectant

A disinfectant is a product or substance that reduces the number of microorganisms (fungi, yeasts, viruses, algae, bacteria or spores) in or on an inanimate matrix - achieved by the irreversible action of a product, to a level judged to be appropriate for a defined purpose.

3.4 Reducing or reduction of the number/concentration of microorganisms

The disinfection during the test is measured by reducing or reduction of the number of microorganisms and is conducted using the detection methods described in this test guideline (see Section 5.5.1.2).

3.5 Test organism

Bacteria and bacteriophages (viruses) are used as test organisms in tests performed in accordance with this guideline.

3.6 Microorganisms

In this determination, the term **microorganisms** is used for bacteria, bacteriophages and viruses.

3.7 Contact time

The period of contact between disinfectant and test organism.

4 Requirements

The requirements set below apply only in connection with the underlying test procedure detailed in section 5.

4.1 Bactericidal requirements

An active substance or product can only be described as having sufficient bactericidal effect for application in drinking water if the following criterion is met:
Ability of a product or active substance to achieve a reduction in the concentration of living, vegetative bacterial cells of *Escherichia coli and Enterococcus faecium* reference strains (see Sections 3.4 and 5.2.1), under the conditions specified in this guideline, of at least 2 $\log_{10}$ steps after a contact time of 10 minutes and 4 $\log_{10}$ steps after 25 minutes.

Reference strains	Reduction | Contact time	Reduction | Contact time
Escherichia coli	2 $\log_{10}$ steps | 10 minutes	4 $\log_{10}$ steps | 25 minutes
Enterococcus faecium	2 $\log_{10}$ steps | 10 minutes	4 $\log_{10}$ steps | 25 minutes

4.2 Virucidal requirements

An active substance or product can only be described as having sufficient virucidal effect for application in drinking water if the following criterion is met:
Ability of a product or active substance to achieve a reduction in the concentration of bacteriophages (viruses) MS2 *and* PRD1 reference strains (see Sections 3.4 and 5.2.1), under the conditions specified in this guideline, of at least 2 $\log_{10}$ steps after a contact time of 10 minutes and 4 $\log_{10}$ steps after 25 minutes.

Reference strains	Reduction | Contact time	Reduction | Contact time
Bacteriophage MS2	2 $\log_{10}$ steps | 10 minutes	4 $\log_{10}$ steps | 25 minutes
Bacteriophage PRD1	2 $\log_{10}$ steps | 10 minutes	4 $\log_{10}$ steps | 25 minutes

5 Tests

5.1 Principle

The required test rig for this procedure operates in a flow-through mode.
Microorganisms and disinfectants are dosed continuously into the test water. The test water is discarded after passing through the test rig.
Test organisms (bacteria and viruses) are added with continuous water flow at the transfer point into a pipe through which a defined water sample (see Section 5.2.3.2) flows. Their concentrations in the water sample have to be determined. Subsequently, the disinfectant to be tested is injected into the volume flow. Test organisms and disinfectant have to be mixed completely with the water. Therefore, a pipe unit with a small diameter is needed to ensure turbulent flow. The point of injection marks the starting point of disinfection. Following three defined periods of contact between the test organism and the disinfectant, samples are taken at the sampling taps provided for this purpose. Immediately at sampling, the disinfection process in the sample is inhibited (neutralised) and the concentrations of test organisms are determined (see Section 5.2.3.6). The disinfectant concentrations at all taps are recorded. In order to satisfy the efficacy requirements for a disinfectant in this test procedure, the above specified reduction of test organisms has to be achieved after 10 and 25 minutes. The test rig is shown in Figure 1.
The test organisms used are non-infectious indicators of faecal contamination (see Introduction) and therefore also for hygienic risks from pathogens. The test implements two indicators for putative human pathogenic bacteria and two indicators for putative human pathogenic viruses.

Efficacy of the indicator *Bacillus subtilis*, intended as an indicator for spore-forming microorganisms, was not sufficient under the described test conditions.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal and virucidal effects are assessed using the following bacteria and viruses:

a) *Escherichia coli* A3, obtainable from the Federal Environment Agency[2].
b) *Enterococcus faecium,* obtainable from the Federal Environment Agency[2].
c) Bacteriophage MS2 (DSM[3] 13767, ATCC®[5] 15597-B1™)
d) Bacteriophage PRD1 (DSM[3] 19107)

Should other strains be used they should be cultivated under optimal growth conditions (temperature and atmosphere), similar to the reference bacteria and reference viruses. The precise process should be documented in the test report (see Section 5.8).

The test bacteria are common indicators of human faecal contamination of water. Bacteriophages are viruses that attack bacteria. They are similar in size and shape to human pathogenic viruses, but harmless for humans and relatively easy to analyse. The bacteriophage MS2 is an F-specific RNA virus, PRD1 is a somatic DNA bacteriophage with similarity in coat protein structures and genome organisation to adenoviruses. Growth of test viruses to high titers is required for this test. Caveat: a high titer can increase aggregations of viruses, thus reducing efficacy.

5.2.2 Host bacteria for bacteriophages

Salmonella typhimurium WG49 (NCTC[4] 12484) is used as host strain for the bacteriophages MS2 and PRD1.

5.2.3 Culture media and reagents

5.2.3.1 General

Reference is made in the test procedure to other standards. Equipment needed to perform tests in accordance with these other standards is not listed.
The reagents must be of "analytically pure" grade quality and be appropriate for microbiological purposes. Pre-prepared media of the same composition and produced as instructed by the manufacturer can be used for production of culture media.

5.2.3.2 Defined water sample

The following basic conditions must be met by the defined water sample:

- temperature: 15 °C ± 2 °C
- pH value: 7.5 ± 0.2
- dissolved organic carbon (DOC): 2.0 mg/l ± 0.3 mg/l

The pH value is regulated through the addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH). Only DOC that occurs naturally in water should be used (as artificial

[2] Umweltbundesamt, Federal Environment Agency, Section II 1.4 Microbiological Risks, Corrensplatz 1, 14195 Berlin, Germany
[3] DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7 B, 38124 Braunschweig, Germany
[4] Health Protection Agency Culture Collections, Health Protection Agency, Centre for Emergency Preparedness and Response, Porton Down, Salisbury, SP4 0JG, United Kingdom
[5] ATCC - American Type Culture Collection. www.atcc.org.

DOC does not show valid results). To adjust the DOC water can be diluted with reverse osmosis water. Production of specifically defined water samples is performed in a storage tank. Thorough mixing has to be ensured. The water should be free of biocides or disinfectant by-products.
Concentrations of ions in the test water (anion and kation) should be known before starting the test procedure. As it is a basic test; only test-water parameters which have the most influence on the disinfection procedure are adjusted. The temperature of the test water should be moderate (15°C ± 2 °C).
The choice of a standard pH value of 7.5 is based on the equilibrium of hypochlorous acid and hypochlorite at this pH, which is relevant when dosing chlorine (which is used as point of reference for efficacy; see above). The DOC concentration of 2 mg/l provides conditions which are moderately challenging for most oxidizing disinfectants . The inoculation of test organisms and their medium adds only insignificant amounts of DOC.

5.2.3.3 Lactose peptone bouillon

Lactose peptone bouillon, a liquid culture medium, is used for the propagation of *E. coli*.

Peptone obtained from casein	17.0 g/l
Peptone obtained from soy flour	3.0 g/l
Lactose	10.0 g/l
Sodium chloride	5.0 g/l
Demineralised water	1000 ml

Dissolve the substances in demineralised water in a flask (with a stir bar on a magnetic stirrer), adjust pH value to 7.2 ± 0.2 at 25 °C, portion into 100 ml flasks, seal with a cellulose stopper and aluminium foil, and autoclave for 15 minutes at 121 °C.

5.2.3.4 Glucose bouillon

Glucose bouillon, a liquid culture medium, is used for the propagation of *E. faecium.*

Peptone obtained from casein	15,0 g/l
Meat extract	4,8 g/l
D-glucose	7,5 g/l
Sodium chloride	7,5 g/l
Demineralised water	1000 ml

Dissolve the substances in demineralised water in a flask (with stir bar on a magnetic stirrer), adjust pH value to 7.2 ± 0.2 at 25 °C, portion into 100 ml flasks, seal with a cellulose stopper and aluminium foil, and autoclave for 15 minutes at 121 °C.

5.2.3.5 CASO-Agar (casein soy peptone agar)

Detection and enumeration of *Escherichia coli* and coliform bacteria (tryptone soy agar, *TSA*):

Casein (tryptically digested)	15g
Soy peptone	5g, NaCL 5g
Agar (in powder or flake form)	15g-25g depending on gel strength
Distilled water	1000ml

5.2.3.6 Neutralisation medium

A substance that halts the effect of a disinfectant, including active byproducts, is called a neutralisation medium or inhibitor. In order not to falsify measurements this reaction has to be concluded within a few seconds. The neutralisation medium must not have any negative influence on the test, in particular the test organisms.
The inhibitability of a disinfectant is one of the general acceptance criteria for active substances and products for drinking water disinfection. Only a fast inhibition of active substances guarantees safe water supplies in case of an overdose. For the test the neutralisation or removal of the active substance provides an efficacy analysis at a defined contact time. If a neutralisation of active substances is not possible this test must be modified or other methods must be applied. After validation the filtration method could be a suitable method. Any modification should be validated.
Sodium thiosulphate can be used to neutralise (Annex D) oxidative chlorine and chlorine dioxide compounds.

5.2.3.7 Chloroform

Chloroform ($CHCl_3$), 99 %, M = 119.38 g/mol is used for the production of phage suspensions (see Section 5.4.3).

5.3 Equipment

5.3.1 General

Reference is made in this test procedure to other standards. Here we list only equipment that is not within the scope of these other standards. The standard equipment of a microbiology laboratory is required. Special equipment and materials that are required to test the efficacy of disinfectants are listed in Annex A.

5.3.2 Centrifuge

Centrifugation with a minimum rotational speed of 6000 rpm and centrifuge tubes of a volume of 200 ml.

5.3.3 Incubator

Incubator temperature controlled at 44 °C ± 0.5 °C
Incubator temperature controlled at 37°C ± 2 °C

5.3.4 Autoclave

Operated at 121 °C + 3 °C and 115 °C + 3 °C
Photometer

5.4 Production of viral and bacterial suspension

5.4.1 General

Microbial strains have to be preserved in accordance with the requirements of EN 12353.

5.4.2 Bacterial suspension

5.4.2.1 *E. coli* A3

One strain *of E. coli* A3 from a working culture that has been frozen at -80° C ± 2 °C is streaked on CASO agar (*casein soy peptone agar*) (5.2.3.5) and incubated for 8 h ± 1 h at 36 °C ± 2 °C. The culture grown on the agar is retrieved with an inoculation loop and used for inoculation of 100 ml lactose peptone bouillon (5.2.3.3). The inoculated culture is incubated for 20 hours ± 4 hours at 36 °C ± 2 °C and retrieved through centrifugation (6000 rpm, 15 minutes). The supernatant is discarded after centrifugation and rinsed with a defined sterile water sample (6000 rpm, 15 minutes). The rinsed pellet is placed into a 100 ml sterile defined water sample. The bacterial suspension has to be preserved at 4 °C ± 2 C and inoculated after two hours at the latest into the prepared storage vessel of the test facility (5.5.1).

5.4.2.2 *E. faecium* Teltow 11

One strain of *E. faecium* Teltow 11 from a frozen working culture is smeared on CASO agar and incubated for 7 h ± 2 h at 36 °C ± 2 °C. The culture grown on the agar is retrieved with an inoculation loop and used for inoculation of 100 ml glucose bouillon (5.2.3.4). The inoculated culture is incubated for 20 hours ± 4 hours at 36 °C ± 2 °C and retrieved through centrifugation (6000 rpm, 15 minutes). The supernatant is discarded after centrifugation and rinsed with a defined sterile water sample (6000 rpm, 15 minutes) and the pellet is rinsed and placed into a 100 ml sterile defined water sample. The bacterial suspension has to be preserved at 4 °C ± 2 °C and inoculated after two hours at the latest into the prepared storage vessel of the test facility (5.5.1).

5.4.2.3 *Salmonella thyphimurium* strain WG49

The microbial working cultures are produced in accordance with EN ISO 10705-1. *Salmonella typhimurium* WG49 mutant has low pathogenicity and should be handled in accordance with national and international laboratory safety requirements (e.g. category 2).

5.4.3 Bacteriophage suspension

For the production of the bacteriophage suspensions for testing, the host-strain *Salmonella typhimurium WG49* is cultivated 15 h ± 2 h in a thermomixer (80 rpm; 20 h ± 4 h; 36 °C ± 2 °C. TYGB is used as liquid medium (5.4.2.3).
25 ml TYGB are preheated to ambient temperature in a 300 ml Erlenmeyer flask, inoculated with 0.25 ml for 15 h ± 2 h and incubated in the thermomixer for 90 minutes at 36 °C ± 2 °C.
Bacteriophage suspensions from reference stocks are adjusted to a final concentration of plaque-forming units (pfu) of about 10^6 - 10^8/ ml. Suspensions are subsequently incubated for 4 to 5 hours (see above). 2.5 ml of chloroform (5.2.3.7) are added under an extractor fan and thoroughly mixed. After sealing, the flask is stored overnight or at least for 4 hours at 5 °C ± 3 °C. The aqueous phase is transferred into a small pipe and centrifuged with 6000 rpm for 20 minutes. The supernatant is carefully decanted or pipetted off.
A decimal series of dilutions is produced and investigated in a plaque assay (5.5.1.2) to determine the virus titre. The produced bacteriophage lysate is preserved until the test at 5 °C ± 3 °C, or frozen in 5 ml portions in cryotubes at 80 °C ±10 °C. In order to minimise the input of dissolved organic carbon (DOC) into the storage container, not more than 10 ml of phage suspension should be added (5.4.3).

5.5 Procedure

5.5.1 Operation of the test facility

a) The storage container is filled with a defined water sample (5.2.3.2).
b) A volume flow of 400 litres per hour (± 20 l/h) is set.
c) The retention time of the defined water sample has to be constantly adjusted as follows:
    i. Sampling tap 1: 25 seconds ± 10 seconds (validating measurement 5.7.2)
    ii. Sampling tap 2: 10 minutes ± 30 seconds (test value 1)
    iii. Sampling tap 3: 25 minutes ± 60 seconds (test value 2)

   Determination of the retention time of the defined water sample in the disinfectant test rig is described in Annex B.
d) Test organisms are produced as described in Section 5.4 and placed into the storage container of the test rig. The bacterial suspensions (*E. coli, Enterococcus faecium*) are added together with the bacteriophage MS2. Analysis of bacteriophage PRD1 for testing is conducted in a separate test preparation (to allow differentiation of RNA und DNA test bacteriophages). In the bacterial suspension storage container the concentration of bacteriophages should be between (1×10^9 - 1×10^{11}) pfu /100 ml, and that of bacteria between (1×10^8 - 5×10^9) CFU/ 100 ml. The test organisms are added to the defined water sample in a ratio of 1:1000, so that the concentration in the test facility (at Tap 0) is between (1×10^5 - 5×10^6) CFU/ 100 ml or (1×10^6 – 1×10^8) pfu /100 ml.
e) The selected disinfectant concentration is added continuously. The required physico-chemical parameters (e.g. concentration of active substance, flow-through and temperature) in the test rig have to be satisfied for a time period of at least 60 minutes (stabilization phase). Then sampling can take place.
f) The disinfectant neutraliser (inhibitor) is initially put into the sterile sampling vessel. Chlorine and chlorine dioxide e.g. can be neutralised with sodium thiosulphate. Without modification this test is unsuitable for disinfectants which cannot be neutralised immediately (5.2.3.6). For each experiment full neutralisation of the disinfectant has to be checked.
g) Cleaning the test rig between different tests is unnecessary, since the test rig always runs in the flow through mode. Residual microorganisms will be diluted or flushed out. Analyses had shown that no positive samples were found between test cycles. The existing biofilm has only a minor impact on the efficacy of various disinfectants and is a component of any drinking water disinfection. Development of a biofilm has rather to be regarded as a desired effect to best simulate realistic conditions of disinfection.

For the determination of bactericidal and virucidal effects, 50 ml samples (or larger amounts, if necessary) are taken at taps 3 to 0 (Annex A). To ensure instant neutralisation of the disinfectant, the sampling bottles should be shaken well.

After filling, the vessel is quickly sealed, vigorously shaken two or three times and immediately placed onto a rapid mixer (fast ‘vortex’). Thorough mixing of the sample with the inhibitor should be ensured, which is evidenced by deep vortex formation. The sample is then once more vigorously shaken.

Should larger sample volumes be required, a 1 litre glass bottle is held under the respective tap and filled while swirling it, leaving free a largish volume of air. After sampling the bottle is immediately sealed and vigorously shaken. The samples are stored in the refrigerator at 5 °C ± 3 °C until analysis.

The disinfectant concentration is measured after every microbiological sampling of taps 1 to 3, so that the depletion of disinfectant can be measured.
The disinfectant concentration at time zero is calculated on the basis of the concentration of the storage vessel and the dilution factor arising in the volume flow of the defined water sample.

Before sampling, the flow rate from every sampling tap has to be measured. Based on flow measurement, the precise contact time between test organism and disinfectant has to be calculated, taking account of the correction factor (see Annex B). Should contact times not comply with specifications (5.5.1c) the flow rate has to be correspondingly adjusted.

Sampling is repeated three times at intervals of 30 to 60 minutes.

5.5.1.1 Test of complete neutralisation of the disinfectant

An additional sampling vessel with neutralisation medium (see Section 5.2.3.6) is prepared for each repeat. This sample is taken at Tap 1. After filling, the vessel is quickly sealed, vigorously shaken two or three times and immediately placed onto a rapid mixer (fast ‘vortex’). Thorough mixing of the sample with the inhibitor should be ensured, which is evidenced by deep vortex formation. Then the sample should once more be shaken vigorously. The sample is checked for reactive disinfectant residues. Neutralisation may be regarded as complete if no disinfectant is detected after this procedure.

5.5.1.2 Analysis of microbiological samples

The bacteriological parameters of the samples are checked as soon as possible, no longer than 4 hours after test, the virological parameters at latest after 36 hours. The samples must be stored in a cool place at 5 °C ± 3 °C.

The following detection methods have to be applied for test organisms:

a) *E. coli* A3 (EN ISO 9308-1 and -3)
b) *E. faecium* Teltow 11 (EN ISO 7899-1 and -2)
c) MS2 (ISO 10705-1:2001)
d) PRD1 (EN ISO 10705–2:2001)

For the detection of test bacteria, and depending on the expected concentration, the membrane filtration method (EN ISO 9308-1 or EN ISO 7899-2) or the MPN method (EN ISO 9308-3 or EN ISO 7899-1) is employed with corresponding dilution levels. With membrane filtration at least two discs (parallel analysis) per sample are prepared.

5.6 Calculation and presentation of results

5.6.1 Calculation of bacterial concentrations

With the MPN method the result is directly obtained as MPN/ml or MPN/100 ml.
In the case of membrane filtration, results are calculated in cfu/ml according to EN ISO 8199.

5.6.2 Calculation of bacteriophage concentration

If available, dishes with more than 30 well-separated plaques should be selected. Where the number of plaques is invariably less than 30 per dish, those plates with the largest sample volume should be selected. Dishes with fewer than 10 plaques or more than 200 plaques cannot be used for quantitative determination. On the basis of the number of enumerated plaques, the number X of plaque-forming units of somatic bacteriophages in 1 ml of the sample is calculated as follows:

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

X number of plaque-forming units of somatic coliphages per millilitre (pfu/ml)
N total number of enumerated plaques of all dishes
n_1, n_2 number of parallel determinations, related to each dilution F_1, F_2;
V_1, V_2 applied sample volumes in millilitres, related to F_1, F_2;
F_1, F_2 dilution or concentration factor, related to V_1, V_2
(F = 1 for an undiluted sample, F = 0.1 for tenfold dilution, F = 10 for tenfold concentration etc.).

When merely one dilution or concentration is enumerated, the formula is simplified to:

$$X = \frac{N}{nVF}$$

Example:
A sample was undiluted and diluted 1:10, and in each case two parallels investigated. Enumeration had the following result:

Dilution	Result pfu
undiluted	98; 91
-1	10; 5

Calculation of the result:
N = 98 + 91 + 10 + 5 = 204
Sample quantity V_1 = 1 ml, number of parallel determinations n_1 = 2, factor F_1 = 1, since undiluted,
Sample quantity V_2 = 1 ml, number of parallel determinations n_2 = 2, factor F_2 = 0.1, since diluted 1:10,

$$X = \frac{204}{(2 \cdot 1 \cdot 1) + (2 \cdot 1 \cdot 0.1)}$$

Result: 93 pfu/ml or $9.3 \cdot 10^2$ / 100 ml

5.6.3 Statement of results

The results of microbiological measurements are presented in tables and graphics. The graphical presentation encompasses the results of individual measurement series and the average value depending on contact time (Annex E). The detection limit has to be stated, and data recorded in the unit pfu per 100 ml or CFU per 100 ml.

Disinfection concentrations at the sampling taps 1 to 3 have to be presented in tabular form in mg/litre. In addition, disinfectant concentration in the storage container and computed concentration in volume flow have to be stated in mg/litre. Flow-rate measurement data and precise contact times (after correction) must also be stated (Annex B).

5.7 Evaluation

The interpretation of the results is described in Section 5.7.1.

If the requirements defined in Chapter 4.1 and 4.2 are met, the disinfectant in the applied concentration can be assumed to display sufficient efficacy for the described scope (Chapter 1).

It is important that the concentrations tested are within close range of those needed to meet the efficacy criteria of 2 logs in 10 minutes and 4 logs removal in 25 minutes. If the evaluation shows substantial over- or underdosing the test should be repeated in order to determine the concentration that meets these criteria.

Since the reference concentration that results from this test procedure corresponds to the described experimental conditions, the concentration of the disinfectant in waters with other physico-chemical properties (pH value, DOC, temperature) might have to be adjusted.

If the disinfectant is to be applied under conditions that deviate considerably (chemical composition of the defined water sample, temperature etc.) from test conditions, additional test conditions may be required to allow a definite conclusion to be drawn concerning sufficient efficacy.

If in applying this test procedure the requirements defined in Chapter 4 are not met, the disinfectant at the concentration applied is not suitable for use for the scope described in Chapter 1.

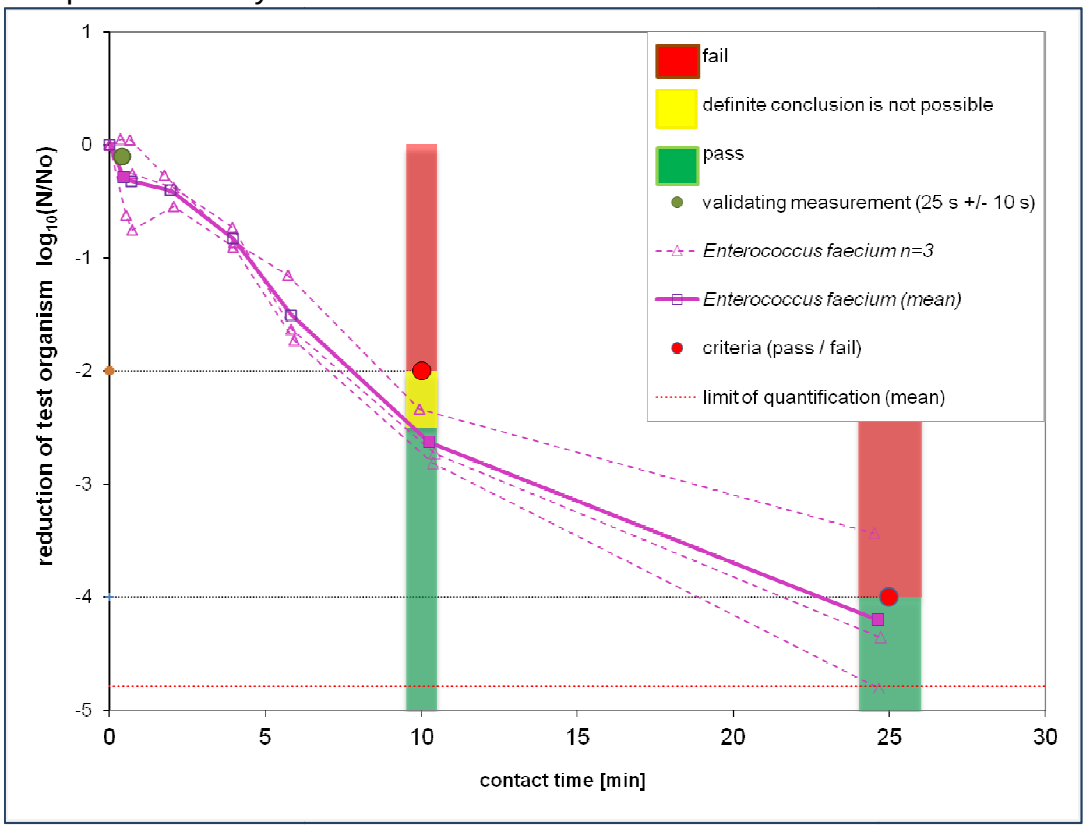
5.7.1 Interpretation of results

Depending on the variation of the test, results shall be interpreted as follows:
The test procedure (5.1) must be performed at least twice. One run of the test provides three reduction curves (three repeats) for each test organism / bacteriophage. For each repeat $\log_{10}(N/N_0)$ has to be calculated and plotted. Also the mean has to be calculated and added to the diagram. The mean defines whether the test is passed or failed (see table below and Annex E).

Contact time [min]	**Reduction of test bacteria and test bacteriophages** [mean $\log_{10}$ scale N/N_0]			
	0 – (-1.9)	-2.0 – (-2.4)	-2.5 – (-3.9)	≥ (-4)
10	☒	☒/✓ definite conclusion is not possible, further experiments needed (see Annex F)	✓	✓
25	☒	☒	☒	✓

☒ = fail
✓ = pass

Graphical plot of efficacy criteria:



An interpretation of variation is shown in Annex F page 31.

5.7.2 Validation measurement

The validation measurement (5.5.1c) provides additional information on the kinetics with which an active substance or product achieves its effect. It is measured after a contact time of 25 seconds ± 10 seconds on tap 1.
Very high efficacy in the test procedure at this point indicates that an excessively high concentration might have been applied. Measuring errors and uncertainties can also be more closely examined.

5.8 Test report

The test report must refer to this guideline.
The test report must at least include the following data:

a) Identification of the testing laboratory and the client.
b) Identification of the test conducted:
    1. Identification of product and active substance
    2. Batch number and use-by date (if available)
    3. Manufacturer, supplier, date of delivery (if available)
    4. Prescribed storage conditions
    5. Diluent recommended by the manufacturer for application of the product
    6. Active substance and its concentration
c) Test conditions:
    1. Date/dates of test (period of analysis)
    2. Physico-chemical data on the defined water sample (5.2.3.2)
    3. Concentration of active substance or product
    4. Formation of precipitates or flocculation is documented
    5. Incubation temperature
    6. Neutralisation medium or inhibitor applied (5.2.3.6)
    7. Characterisation of additionally used bacterial and viral strains, together with the propagation processes applied
d) Test results:
    1. Data for each run has to be presented.
    2. Reference concentration[5]
    3. Assessment of bactericidal and virucidal effects
    4. Number of reruns per test organism
    5. Specific comments
    6. Conclusion
    7. Place, date, signature
e) Concentraton needed to meet the efficacy criteria of 2 logs in 10 seconds and 4 logs reduction in 25 seconds

[5] If results show sufficient efficacy (see Section 4), the concentration of the tested disinfectant can be described as concentration necessary to achieve this sufficient effect or reference concentration.

Annex A: Facility for testing the efficacy of drinking water disinfectants

1 Introduction and facility setup

The disinfectant test rig required under the terms of this test instruction has to be operated in continuous flow mode (all four taps have a continuous flow out). Test water flows only once through the test rig and is discarded afterwards. It has to be demonstrated that the test rig has no negative effect on the test organisms. Therefore, a run without disinfectant clarifies whether or not the concentration of test organisms is the same at all taps. This analysis should be repeated from time to time. Moreover, the biosafety precautions and biosafety levels of the test organisms have to be strictly observed.
The test facility must allow water samples to be taken at four sampling taps (Tap 0 to Tap 3 (see Figure 1)). These taps are small additional pipes (see Figure 1b) designed in such a way that retention and contact times can be realized:

Tap 0 = test organisms without disinfectant (start of disinfection and negative control)
Tap 1 = 25 seconds ± 10 seconds (validating measurement, 5.7.2)
Tap 2 = 10 minutes ± 30 seconds (test value 1)
Tap 3 = 25 minutes ± 60 seconds (test value 2)

The precise calculation and experimental determination of contact times is described in Annex B. National or international laboratory safety requirements for biological hazard must be observed. The water sample has a high concentration of test organisms and system pressure is between 2 and 5 bars.
In phases in which the facility is not used, and at least three weeks before running a disinfectant test, a low volume flow of drinking water must be passed through the facility. Between the tests no cleaning steps are normally required, besides flushing for a period of some days. The biofilm in the water pipe has only a minor effect and renders the test conditions even more realistic (simulation of disinfection e.g. in water works).

The dosing of a defined water sample takes place without pressure at a transfer point, from which the water is pumped into the facility. Measurement of the physico-chemical parameters pH value, conductivity, redox potential and temperature is carried out at the transfer point, so that characterisation of the defined water quality is possible on inflow. Measurements of pressure and total flow must also be conducted in the test facility. Pressure in the facility should be around 3 bar (a range between 2 - 5 bars is acceptable).

a) Dosing of test organisms takes place directly at the base of the transfer-point pipe section by means of a peristaltic pump. The disinfectant itself is injected directly into the pipe system. Rapid and thorough blending is required. Before dosing, the test organism concentration is determined at Tap 0 (negative control without disinfectant). A concentration in the test rig between (1×10^5 - 5×10^6) CFU/ 100 ml or (1×10^6 – 1×10^8) pfu /100 ml should be detectable at tap 0.

The physico-chemical parameters pH value, conductivity, redox potential and temperature of the defined water sample in the outflow of the measuring section must also be recorded.
The physico-chemical parameters determined at inflow and outflow are continuously recorded (for example, by way of a memograph).

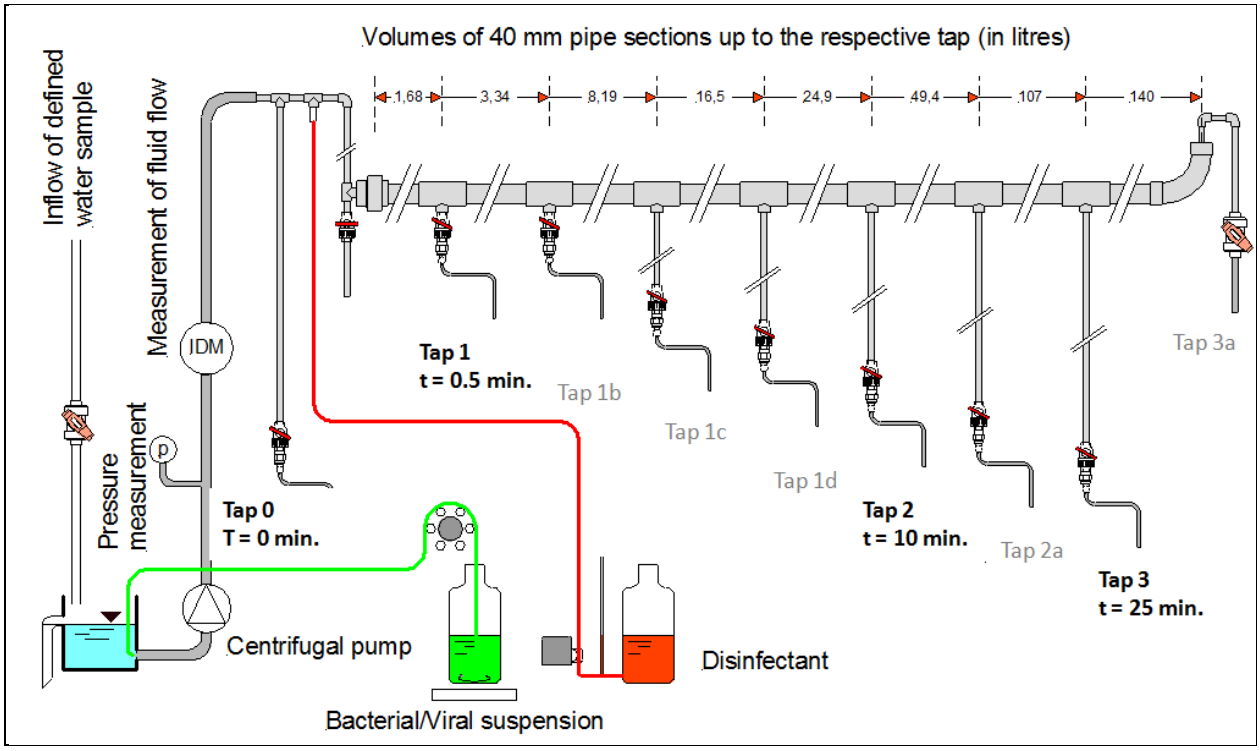


Figure 1) Schematic example of a disinfectant test rig. For the efficacy test stipulated in this guideline tap 0, tap 1, tap 2 and tap 3 are needed that satisfy the prescribed contact times (5.5.1 c). Further taps to describe efficacy kinetics more precisely are recommended. After passing the rig, test water is discarded (flow-through mode).

Figure 1b) Taps are small additional pipes. For the efficacy test stipulated in this guideline tap 0, tap 1, tap 2 and tap 3 are needed. Further taps to describe efficacy kinetics more precisely are recommended. After passing the rig, test water is discarded (flow-through mode).

1.1 Further technical requirements

- The pipe diameter is between 26 – 40 mm. The ideal diameter is 33 mm.
- A flow rate of 0.016 m/s and 0.097 m/s must be achieved.
- Concentrations of bacteriophages (viruses) and bacteria are shown in Section 5.5.1(d).
- Before the initial disinfectant test is carried out, it has to be confirmed that no reduction of test organisms occurs within the facility. For this purpose, samples are taken from all taps and analysed for the test organisms.
- The sample volume must be 50 ml, and it must be possible to draw it within 20 to 40 seconds.
- The applied water pressure within the facility should be between 2 and 5 bar. In this range the pressure has no significant influence on the test organisms or on the efficacy of the disinfectant. The pressure is adapted to common drinking water distributions systems.
- Only materials which are proven suitable for coming into contact with drinking water have to be used for the test rig. The disinfectant feed pipe has to be chemically inert.

Annex B: Determination of contact time between organisms / viruses and disinfectant

Retention time / contact time is a decisive parameter for the assessment of the efficacy of a disinfectant. The test rig runs in a flow-through mode with continuous dosing of disinfectant and test organisms. The contact time is defined as the time from the beginning of dosing to the time the sample is drawn from the tap.

Determination of retention time takes place in two separate procedures that supplement each other (Chapter 1). On the one hand contact time is calculated theoretically on the basis of flow rates and pipe diameters. The other procedure is its experimental determination by tracer experiments. These tracer experiments should be repeated from time to time, and during such tests no efficacy test can be run in parallel.

Examination of the consistency between both methods on the basis of an appropriate comparison criterion, as well as the testing of the possibility for quick and as simple as possible determination of retention time at each sampling tap are necessary during the experimental procedure (Chapter 2).

Index:

1 Determination of retention time (not during efficacy test)

1.1 Theoretical calculation of retention time / contact time

For theoretical calculation of contact time in the test facility, the individual pipe sections are measured. On the basis of a given flow (in line with requirements in this guideline), contact time $t_{c,cal}$ is calculated in accordance with Equation (1). This equation is based on indirect proportionality between contact time and flow rate. A reduction in flow rate

accordingly leads to longer contact time and *vice versa*. It is therefore possible to regulate contact time through a change in flow rate.

$$t_{C;calc} = \sum t_x + t_y + t_z = \sum \left(\frac{\left(r_x^{\,2} \cdot \pi\right) \cdot l_x}{Q_x} \right) + \frac{\left(r_y^{\,2} \cdot \pi\right) \cdot l_y}{Q_y} + \frac{\left(r_z^{\,2} \cdot \pi\right) \cdot l_z}{Q_z} \qquad (1)$$

$t_{C,calc}$: Contact time calculated [min]

t_x: Contact time in test section [min]

t_y: Contact time in subsection between test section and sampling tap [min]

t_z: Contact time in sampling tap [min]

r: Inner diameter of pipes [m]

l: Length of test section [m]

Q: Volume flow [l/h]; with $Q_y = Q_z$

1.2 Experimental determination of retention time / contact time by tracer experiments

This determination should be repeated every 12 months. During the procedure no efficacy test can be run. For determination of retention times by means of tracer tests pulses of a saline solution NaCl are dosed into the test facility and conductivity changes at the sampling taps measured in the out-flow. For statistical evaluation of the results at least 10 saline dosages are required for each sampling point (Tap 1 to Tap 3) in each case. Total flow prior to input into the test section has to be adapted to requirements in the test procedure. Individual flow rates of all taps should be set at between 5 l/h and 15 l/h.
A comparison between theoretical retention time and measured retention time (tracer tests) enables sound calculation of actual contact times.
In order to obtain reliable comparison criteria, the plotted tracer curves are defined as the times of maximum conductivity as well as of 50% of throughput (50th percentile).

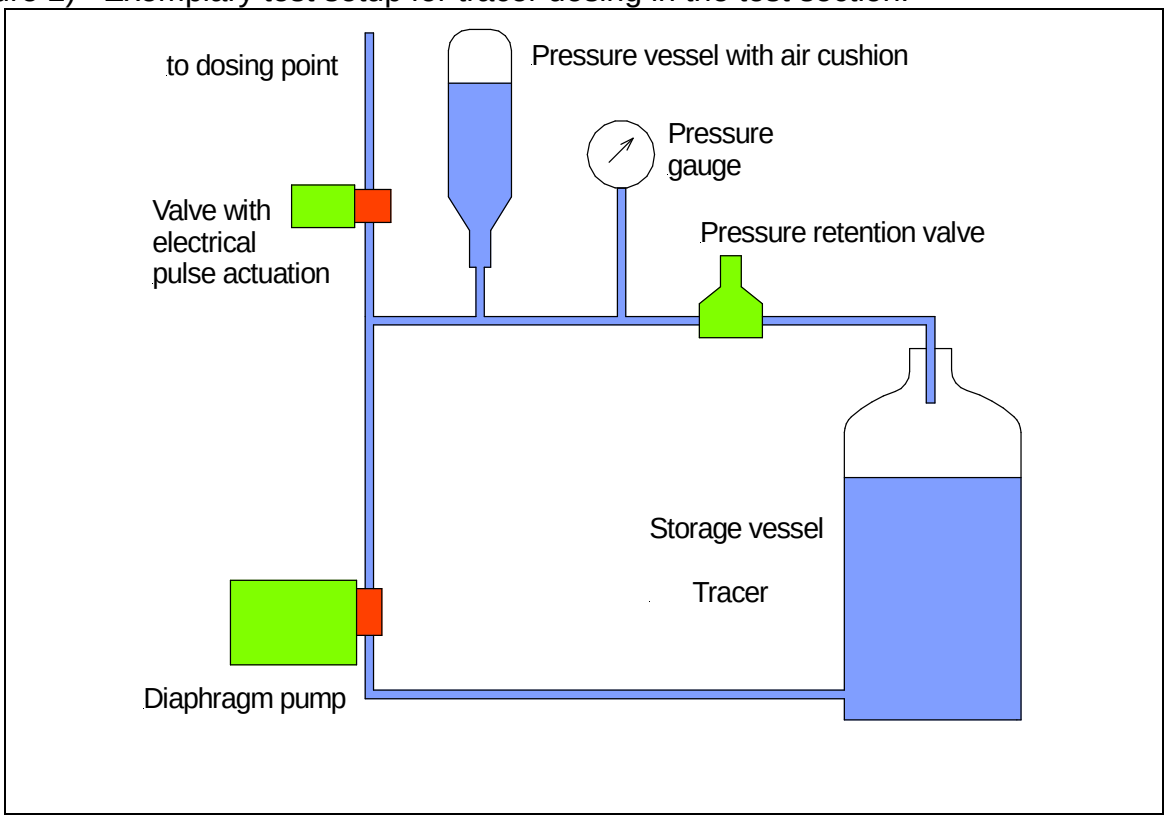
1.2.1 Test setup for tracer experiments

Dosing of the saline solution is carried out with the aid of a diaphragm pump from a storage vessel. A solenoid valve with electrical pulse actuation enables pulsative dosing. Pulse intervals and the time when the solenoid valve is open can be infinitely varied by a control system (Figure 2).
In order that the complete conductivity curve can be recorded, with an increase in retention time from tap to tap pulse intervals must also be increased up to the next dosing.
In order to obtain evaluable measuring signals also in the case of taps with longer retention times, the times are increased during which the solenoid valve is open.

Table 1) Duration and intervals of electrical pulse actuation of a solenoid valve

Measuring point	Duration of pulse signal [s]	Pulse interval [min]
Tap 1	0.02	0.5
Tap 2	0.2	10
Tap 3	1.0	25

Figure 2) Exemplary test setup for tracer dosing in the test section:



1.2.2 Measurement and data recording (tracer experiment)

Once the saline solution has been introduced into the test facility (see Figure 2) conductivity at selected sampling taps can be measured.

A change in conductivity is measured with two electrodes. Recording of the measuring signal takes place with the aid of a measuring transducer. A conductivity value has to be recorded for each second
Figure 3).

The recorded measuring signals are not calibrated, since for determination of the time values of respective comparison parameters (maximum, 50th percentile) merely the respective conductivity changes are evaluated related to a base signal, and as a result only the respective time values are required.

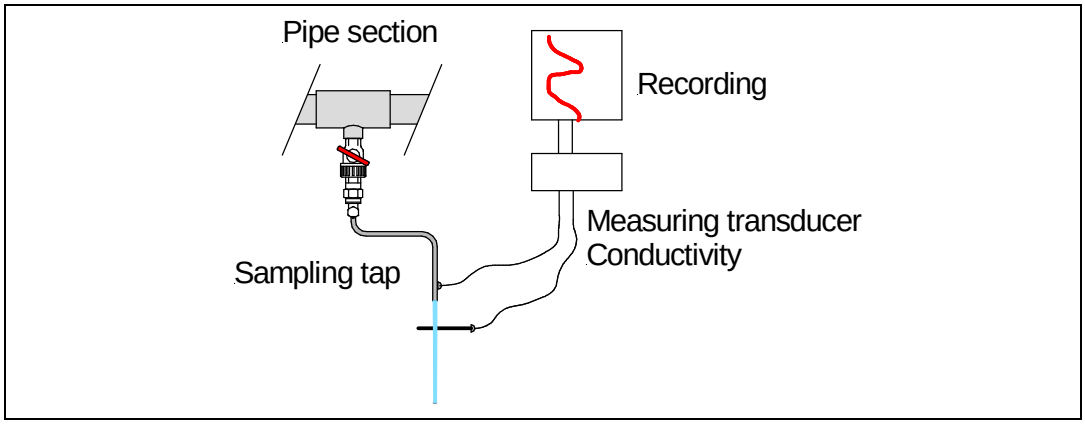


Figure 3) Measurement and data recording configuration

1.2.3 Data evaluation and visualisation

For the purpose of graphical illustration recorded conductivity values are plotted over a period of time. Start time is the moment of dosing the tracer into the test section. Figure 4 displays a conductivity distribution curve.

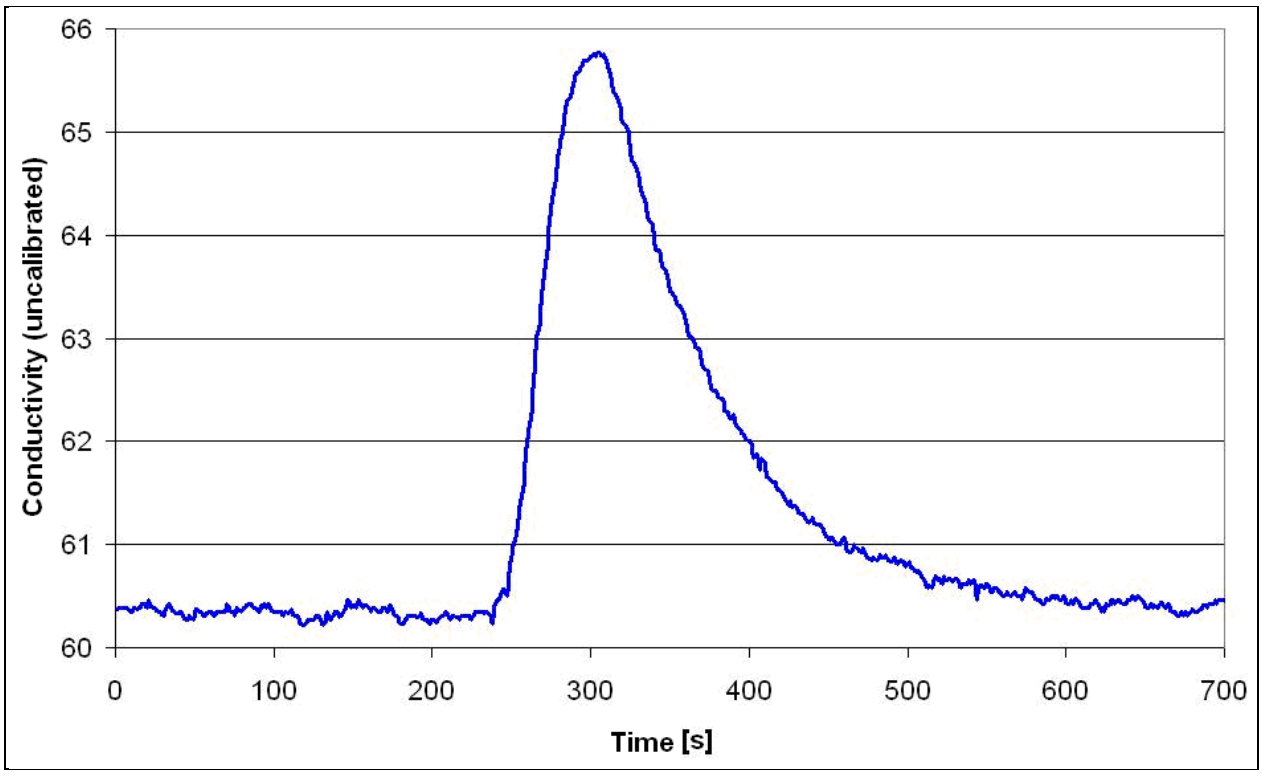


Figure 4) Exemplary graphical evaluation of tracer tests

This curve shows characteristic maximum conductivity, depending on the respective sampling taps, to which a precise time value can be allocated from raw data.
For further evaluation of this conductivity curve the zero baseline – that is background noise – must first be recorded. For this purpose, an average conductivity value is defined before the beginning of the peak.

With the aid of background noise, deviations of measured conductivity from this base signal are now determined.
The sum of all deviations gives the total peak area.
Dividing this value by two gives the value at which 50% of the saline solution has flowed through. A time value can also be allocated to this value from raw data.
The following figure illustrates the two parameters used for comparison.

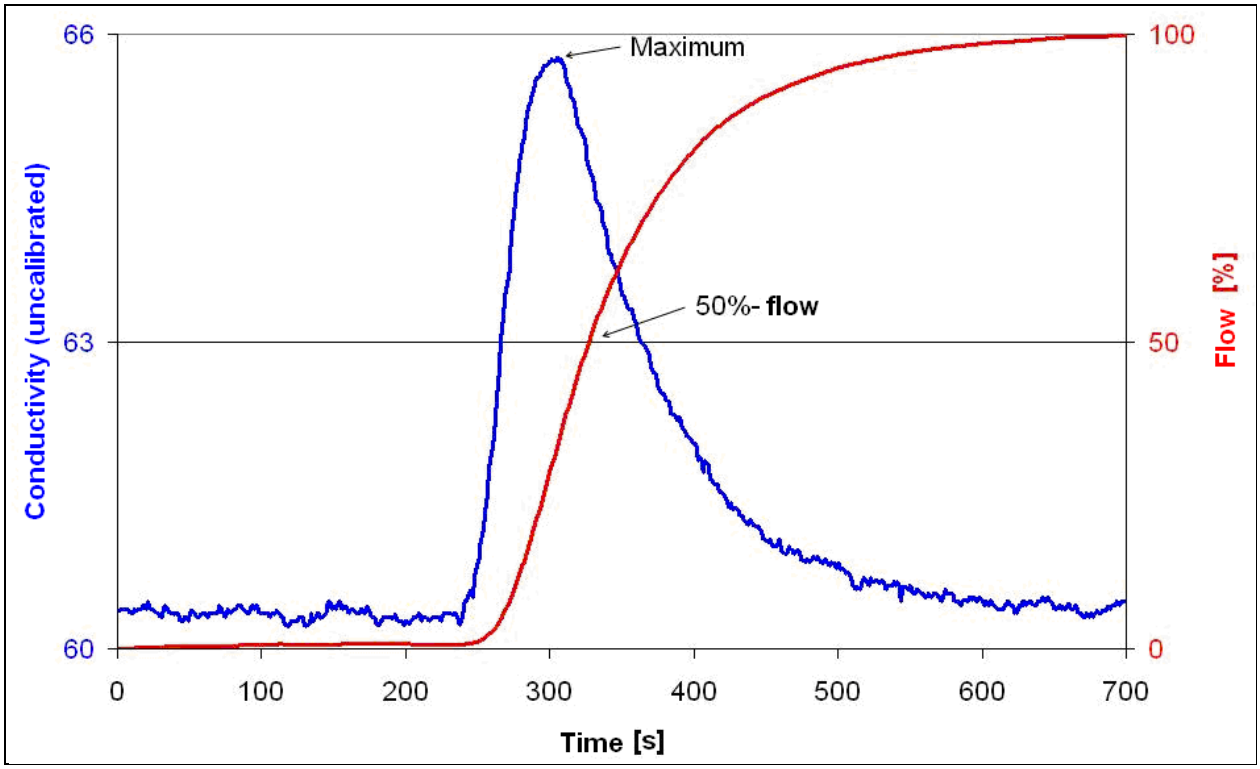


Figure 5) Exemplary graphical evaluation of tracer tests. Blue curve: Conductivity changes with maximum (left axis); red curve: cumulative curve with 50th percentile (right axis)

In order to be able to reliably evaluate respective conductivity changes, especially for the value of the 50th percentile and also in the case of long retention times, a trend line is added to the diagram (Figure 6, red line). This way, an individual conductivity value can be established for every point in time by means of a linear equation with a negative slope. At this step a trend of the background noise can be normalized.
On the basis of the method described above, the time value of the 50th percentile can then be calculated.

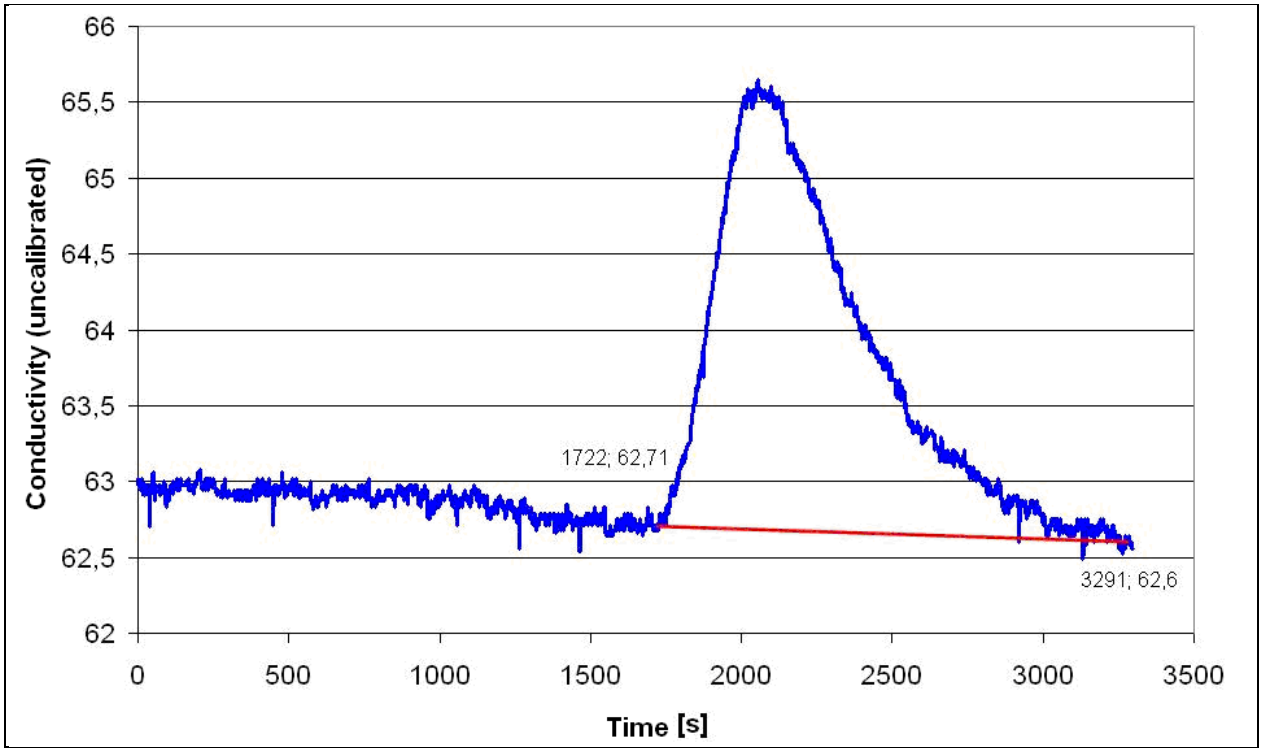


Figure 6) Exemplary graphical evaluation of tracer tests

Since every peak has to be individually evaluated, and at least 10 dosages of saline solution carried out at every tap, the average of all time values calculated at a tap and for a parameter is established. In order to be able to draw sound comparisons between the parameters, respective standard deviations must always be stated.

1.3 Comparison of theoretical and tracer experiments for determination of retention time / contact time (not during efficacy test)

Measurements:
Varied results for calculated ($t_{calculated}$) retention times and measured ($t_{measured\ 50}$) retention times by means of trace tests are shown for the comparison parameters of maximum and 50th percentile of the tracer in Table 2. Standard deviations (s) arise for measured time values from the 10 to 15 dosages of saline solution at each tap.

Table 2) Exemplary results of tracer test for both comparison parameters

			Maximum		50th percentile	
Measuring point	**Flow rate [l/h]**	**$T_{calculated}$ [1)] [min]**	**$T_{measured\ M}$ [2)] [min]**	**s [s]**	**$T_{measured\ 50}$ [3)] [min]**	**s [s]**
Tap 1	400	0.5	0.488	29.3	0.62	37.5
Tap 2	400	10.0	10.00	600.5	10.58	634.9
Tap 3	400	25.0	24.97	1498.3	26.17	1570.7

[1)] Theoretical retardation time see Section 1.1
[2)] maximum, see Figure 4 and Section 1.2.3
[3)] 50th percentile, see Figure 5 and Section 1.2.3

2 Interpretation and additional correction factors for retention time / contact time during efficacy test

In order to be able to make reliable statements on actual retention times in the test facility, without having to repeat tracer tests before each experiment, a correction factor c is applied (equation 2) for every sampling point, which, when multiplied with retention time calculated on the basis of volume flow, results in the actual retention time (t_{actual}) (Table 3).

$$C_{correlation\_factor} = \frac{t_{measured\_50(percentile)}}{t_{calculated}} \quad (2)$$

Correction factors for the 50th percentile are deemed to be the factors that, when multiplied with the theoretically calculated retention time, best reflect actual retention time for each sampling tap (maximum is not taken into account).

Table 3) Exemplary calculation of actual retention time for each sampling tap

Measuring point	Determination of actual retention time
tap n	$T_{actual\ time\ of\ tap\ n} = c_{correlation\ factor} \cdot t_{calculated,\ tap\ n}$
tap 1	$T_{actual,\ tap\ 1} = 1.24 \cdot t_{calculated,\ tap\ 1}$
tap 2	$T_{actual,\ tap\ 2} = 1.058 \cdot t_{calculated,\ tap\ 2}$
tap 3	$T_{actual,\ tap\ 3} = 0.047 \cdot t_{calculated,\ tap\ 3}$

$T_{actual\ time\ of\ tap\ n}$: actual contact time during each efficacy experiment
$c_{correlation\ factor}$: see equation (2) and Section 1.3
$t_{calculated,\ tap\ n}$: computed on the basis of flow rates during each experiment and pipe diameters (according to Section 1.1 with real flow rates)

Contact time is required as a parameter for assessment of efficacy in the test facility. It therefore has to be determined prior to each testing for each tap (Section 1). Since direct determination of contact time is <u>not</u> possible with an internal tracer during the efficacy test, it has to be computed on the basis of flow rates during each experiment and pipe diameters ($t_{calculated,\ tap\ n}$). As this may contain errors, contact time is determined experimentally before or after the actual efficacy test by tracer tests in order to derive a correction factor for the result calculated from flow rates and pipe diameters. These are used for quick and simple determination of retention times in the actual efficacy experiment.

Annex C: General acceptance criteria for drinking water disinfectants

Besides efficacy, drinking water disinfectants must meet further general criteria in order to be regarded as suitable for use in drinking-water supplies. The following acceptance criteria must be evaluated for authorization (see Section 1, Scope).

a) Inhibitability / neutralisation
b) On-site measurability
c) Carbon-free source
d) Disinfectant by-products of toxic relevance must be known
e) Simple dosing
f) Stable dosing solution
g) Safe for humans and environment

With the exception of inhibitability / neutralisation (a), general acceptance criteria are not components of this test (see Section 1, Scope).

Annex D: Example of a neutralisation compound

Use of sodium thiosulphate for neutralisation of oxidative chlorine compounds

With a sample volume of 50 ml, 40 µl of a 10% sodium thiosulphate solution is initially transferred into an adequate tube. The result is a final concentration of approximately 0.08 % sodium thiosulphate. The inhibitor/neutraliser is added in surplus. Before the inhibitor can be used it must be established that it has no effect on the cultivation of the test organisms (see Section 5.2.3.6).The full neutralisation of the active substance should be proven in an extra step (see Section 5.5.1.1).

With a sample volume of 1 litre, 160 µl of a 50% sodium thiosulphate solution is initially transferred into the sampling vessel.

Annex E: Exemplary presentation of results

Figure 7 demonstrates an example of a curve of an active substance or product that achieves the minimum efficacy for one of the four reference test organisms listed in Section 4. The substance passes the test if it achieves the efficacy criteria for all four test organisms. The measurement after ~30 seconds provides an additional validation (5.7.2).

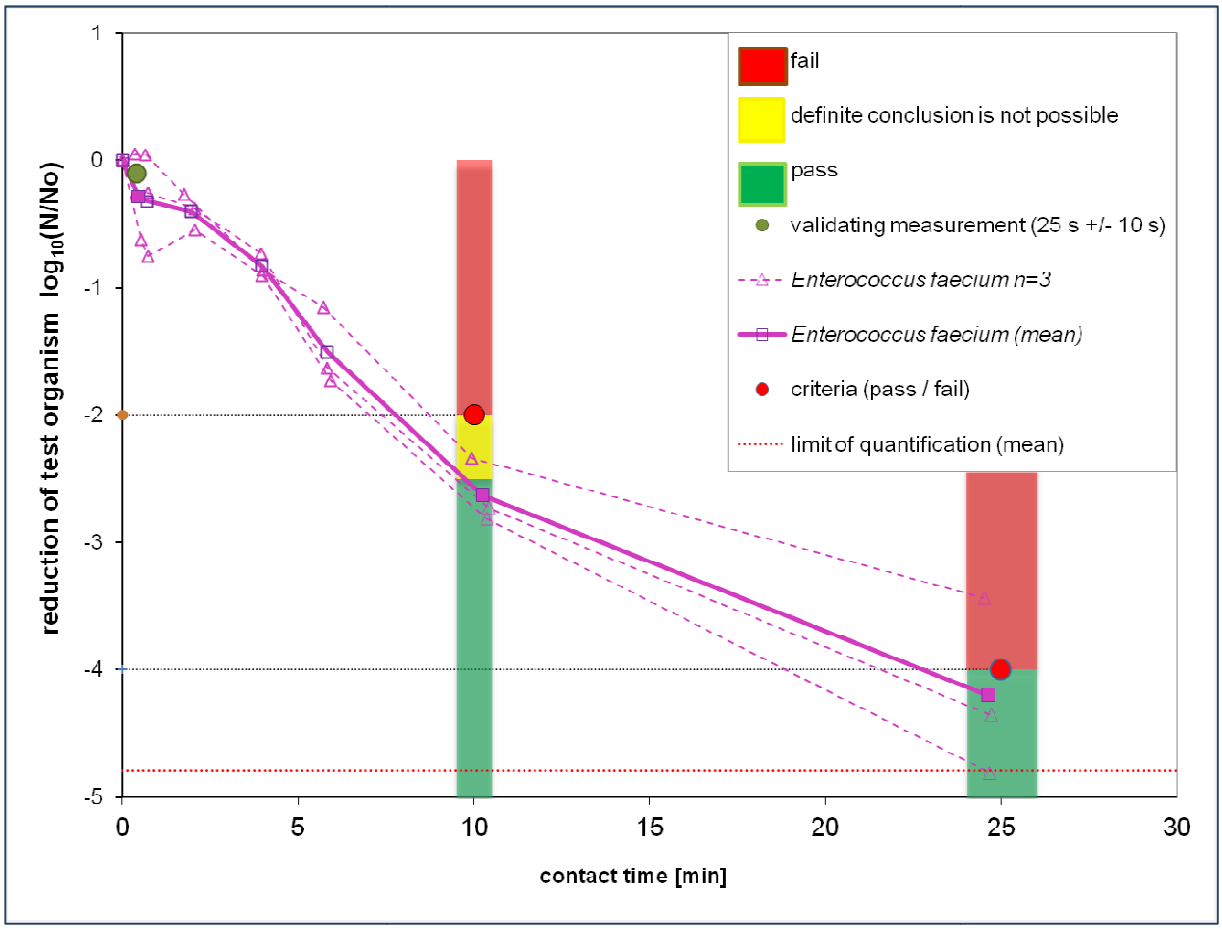


Figure 7) Exemplary reduction of *Enterococcus faecium*. The columns show the reduction range at which the substance passes or fails the test (5.7.1). The green point (close to the y-axis) shows the result of the validating measurement after 25 s. The curves show further measurements at different contact times between criteria points as sampled from the taps of the specific test rig shown in Figure 1 on page 19 which serve to characterise the reduction kinetics more closely but are not obligatory. The yellow column shows the reduction between 2 $\log_{10}$ and 2.5 $\log_{10}$ which does not provide valid results due to variability of the method (Annex F). Note that the pass/fail criterion is set at the upper end of this range of variability (and not in the middle) in order to ensure that the criterion is met. Also, after 25 minutes contact time, no variability range can be determined due to the low levels of test bacteriophages (close to the limit of quantification) achieved at this point. The limit of determination for bacteriophages in the test ranges between > 4 log10 and ≤ 5 log10 steps.

Annex F: Interpretation of variation

1 General efficacy of reference active substances

The efficacy criteria (see Section 4) in this test are based on the efficacy of sodium hypochlorite (chlorine) and chlorine dioxide (see Introduction). The efficacy of these two reference active substances was determined in experiments under different conditions. Figure 8 gives an overview of the reduction of test organisms between 9 and 11 minutes contact time. To find the most suitable bacteriophages (viruses) for the test, analyses were performed with MS2, PRD1, 241, 138 and PhiX174 (data not shown). The bacteriophages 138 and 241 (somatic phage) were isolated from the Teltow Channel in Berlin (Germany). These bacteriophages were not chosen for the test because they are non standard phages and because phage 241 shows a high variability. The titer of standard bacteriophage PhiX174 proved too low for the test. The results emphazise the high efficacy of standard active substances and prove the criterion of 2 $\log_{10}$ reduction after 10 minutes contact time, because also low concentrations of active substances between 0.12 and 0.20 mg/l show that this criterion is met by chlorine and chlorine dioxide.

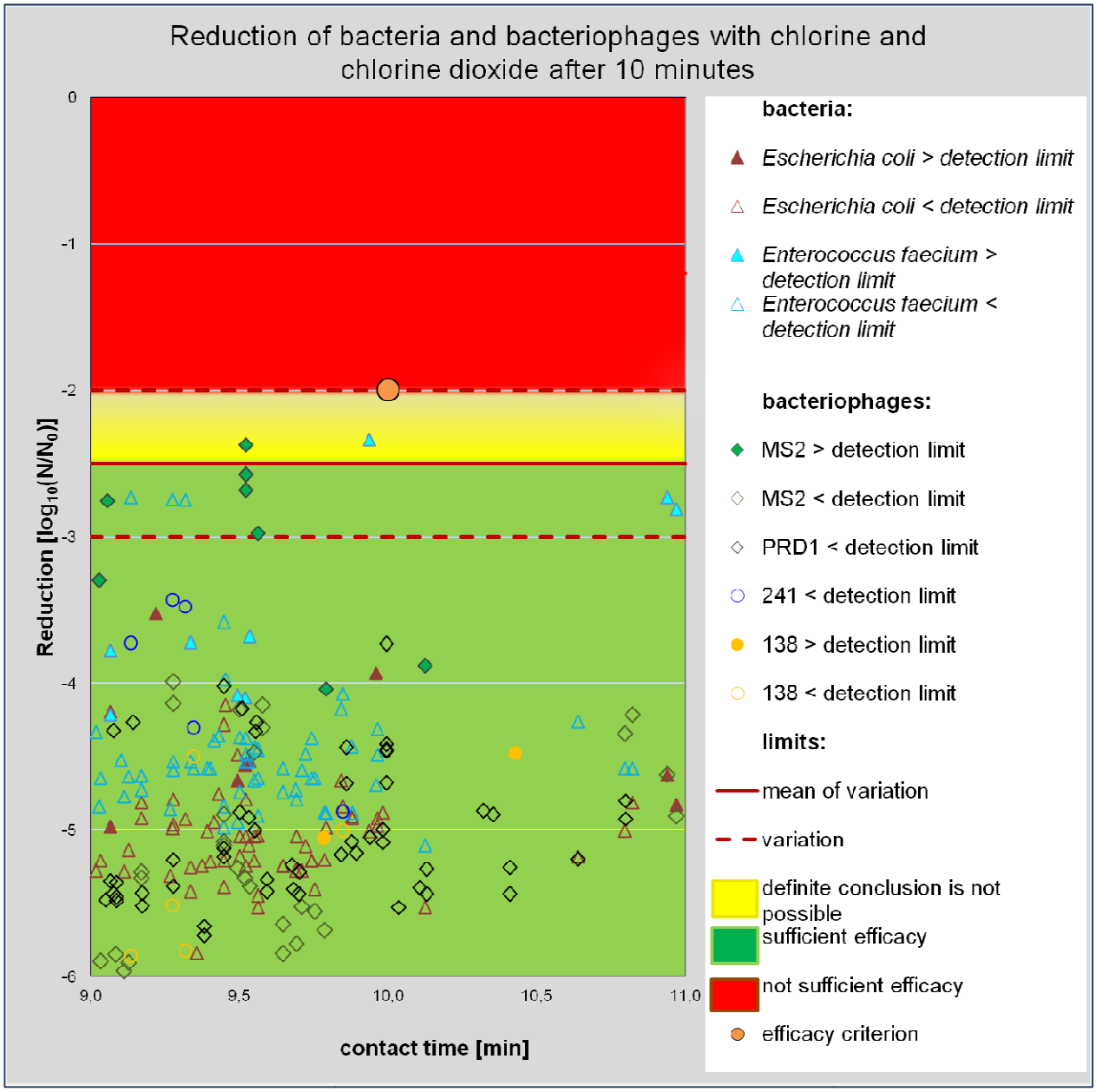


Figure 8) Reduction of test organisms after ~10 minutes contact time with chlorine (sodium hypochlorite) or chlorine dioxide (n=209). Due to the standard deviation (see Section 2) of a half-$\log_{10}$ step (dashed lines) a definitive conclusion cannot be drawn within a range of reduction of 2 $\log_{10}$ and 2.5 $\log_{10}$ (yellow) steps (uncertainty marked yellow). If the mean of three repeats is in the red area the active substances or product fails the test (see Section 5.7, Evaluation).

2 Interpretation of the test's standard deviation

To estimate the standard deviation of the test, experiments with similar conditions (active substances, DOC, temperature, concentration of active substance, pH value) were grouped. The standard deviation for every value from the mean of each group shows the precision of the test. Figure 9 shows the standard deviation of contact time in percent and deviation of reduction of test organisms in $\log_{10}$ scale after 10 minutes. It

can be assumed that the standard deviation after 25 seconds and 25 minutes is the same, because analytical methods and technical conditions are similar.
According to Figure 9 a deviation of a half-$\log_{10}$ step can be assumed to be the standard for the method. That means that a reduction after 10 minutes between 2 $\log_{10}$ and 2.5 $\log_{10}$ steps allows no definite conclusion to be drawn, because due to the deviation the reduction could be also lower than 2 $\log_{10}$. In that case the test should be repeated, while increasing the concentration of active substance.

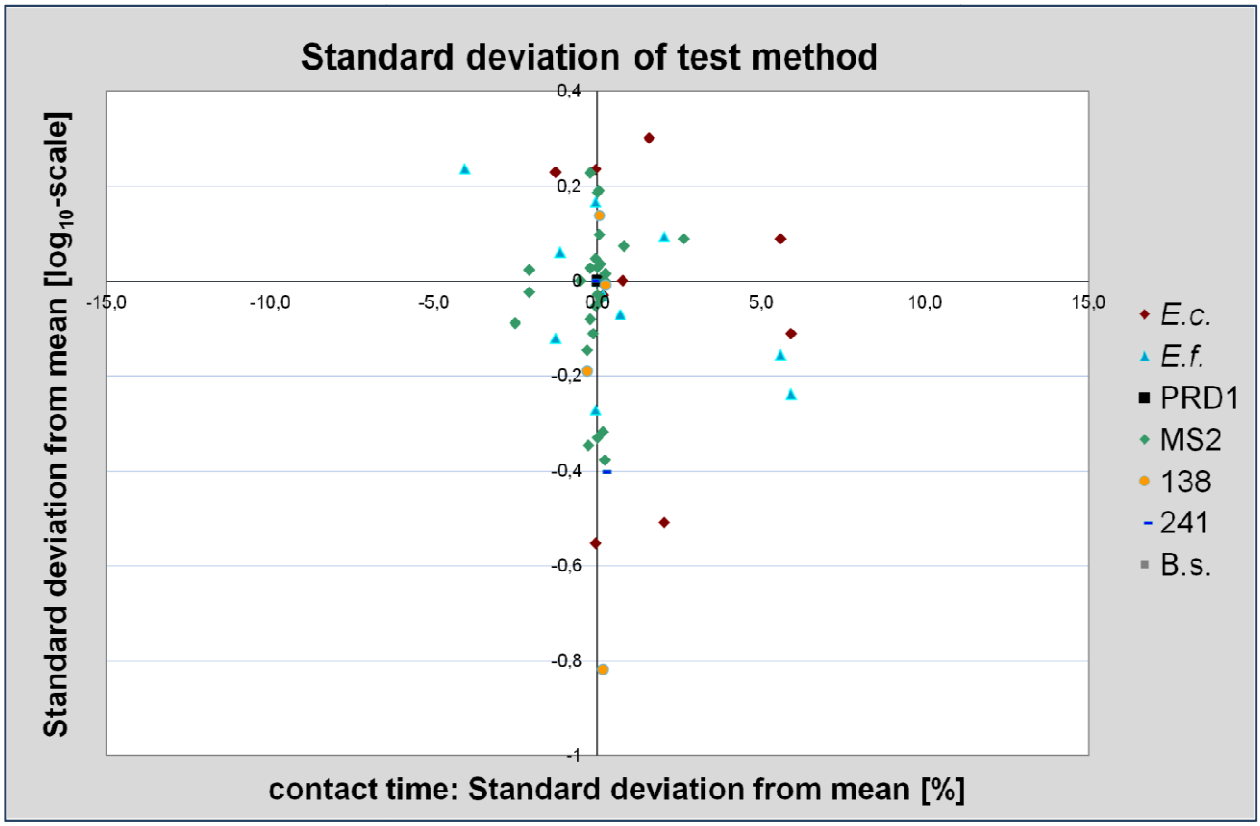


Figure 9) Standard deviations from the mean in $\log_{10}$ scale and percent. Experiments with similar conditions were grouped and the deviation of each group from the mean was calculated (n=53). Abbreviations: E.c. = *Escherichia coli*; E.f. = *Enterococcus faecium*; bacteriophages: PRD1, MS2, 138, 241; *Bacillus subtilis*.