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# Chemical Inactivation of Organisms in Liquids

**Guidelines for the chemical inactivation of organisms in liquid cultures or supernatants with proof of efficacy and safe disposal**

**For the FOPH,**
**Federal Office of Public Health**

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**Legal Notice**

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# 1 Introduction

## 1.1 Initial situation

In establishments and laboratories that are subject to the Containment Ordinance ContainO[1], organisms in liquids are often chemically inactivated. The inactivated liquids can then be disposed of via waste water. However, in very few cases is it clear that these liquid wastes are quite free of organisms. In a laboratory, the same inactivation conditions have generally been used for years. Questions on the efficacy or on the degree of inactivation of the employed methods may often not be convincingly answered, and it is not clear whether the requirements of the Containment Ordinance are always met.

## 1.2 Legal bases and enforcement

The ContainO basically stipulates that in activities in Class 1, organisms have to be safely disposed of, and in activities in Classes 2-4 have to be inactivated (Annex 4, (Art. 12), let. 2, safety measures 23 and 33). According to the purpose laid down in the ContainO, human beings, animals and the environment are to be protected from damage and impairment by infectious waste.

In the scope of the ContainO, *liquids with organisms* are understood primarily to mean waste that is contaminated with organisms. These are e.g. liquid cultures and used culture media or (cell-) culture supernatants.[2]

These guidelines are a guide for implementing ContainO. Consequently, the focus on liquids contaminated with organisms, as treated in these guidelines, is very limited. However, the fundamental reflections and process schemes can be adapted to all types of contaminated liquids.

In the comments on ContainO[3] of 1 June 2012 (German version), we note from the middle of page 37:

> *Therefore, in justified cases the autoclave may now be omitted, e.g. when a chemical inactivation can be carried out to an equivalent level.*

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1 Ordinance on Handling Organisms in Contained Systems (Containment Ordinance, ContainO, May 2012; SR 814.912)

2 See ContainO, Annex 4, (Art. 12), no. 2, Special safety measure no. 33: Inactivation of microorganisms in contaminated material and waste, and on contaminated equipment, from animals and plants and of process fluid in the case of "P" production activities

3 Containment Ordinance (ContainO) of 1 June 2012 – Comments (267/2006-01825/06/50/03/F165-0482)
http://www.bafu.admin.ch/biotechnologie/01744/01749/index.html?lang=de&download=NHzLpZeg7t,lnp6I0NTU042l2Z6ln1acy4Zn4Z2qZpnO2Yuq2Z6gpJCHdYF7fWym162epYbg2c_JjKbNoKSn6A--

The Containment Ordinance ContainO also allows, besides the thermal inactivation by steam (autoclaving), the inactivation of organisms in liquids by antimicrobial chemicals. In such cases the substitution of the autoclave is always subject to authorisation.

The remarks on page 40 of the comment to the ContainO in the second paragraph further state, in regard to Safety measure 23:

> *The autoclave may be omitted for safety levels 1 to 3 if cultures and enrichments of micro-organisms as well as any possible contaminated waste can be inactivated (Class 2 and 3) by other inactivation methods on-site with a validated and comparable effect or safely disposed of (Class 1). For notifiable activities this requires an authorisation from the competent federal office (Art. 17).*

On page 41 – in regard to safety measure 33 – it is stipulated:

> *Professional autoclaving is deemed to be the method of choice for inactivating waste. In general, alternative inactivation methods are permissible if they can be deemed to be equivalent and have been validated.*

On the basis of these comments on the ContainO, the present guidelines clarify:

1. what a *chemical inactivation to an equivalent degree to autoclaving* is
2. what a "*validated and comparable effect*" is
3. what the meaning of "*inactivated*" is.

Disposal of chemically inactivated liquids via waste water is conditional on having a certificate of proof of efficacy that an inactivation achieved with chemicals is comparable to that achieved by autoclaving. Should this not be the case and the chemical inactivation is not (verifiably) complete, then the reduction rate has to be determined under worst-case conditions[4], and in the risk assessment reported that the safe disposal is guaranteed in the sense of the ContainO.

On the grounds of efficacy and environmental impact, priority is also basically given to autoclaving. Compared with the time and effort spent for the selection of a suitable disinfectant, including evidence of efficacy of the chemical inactivation method, autoclaving is generally the simpler alternative.

## 1.3 Objectives and structure of the guidelines

The guidelines serve as the basis for all establishments that want to replace autoclaving contaminated liquid waste by adopting chemical inactivation. Replacing autoclaving is subject to authorisation. Chemically inactivated liquids may be disposed of via waste water if the reduction

[4] See chapter 5.3

rate of the inactivation fulfils certain conditions (see Chapter 6). These guidelines explain the theoretical background and point out the necessary experimental steps.

At the same time the document serves as an aid to the cantonal biosafety specialist agencies in their enforcement activities to assess and evaluate the methods used in practice. The final objective is to ensure the efficacy of a chemical inactivation method used in an establishment, and to be able to clearly demonstrate this to the authorisation and enforcement authorities. These guidelines are subdivided into 10 main chapters.

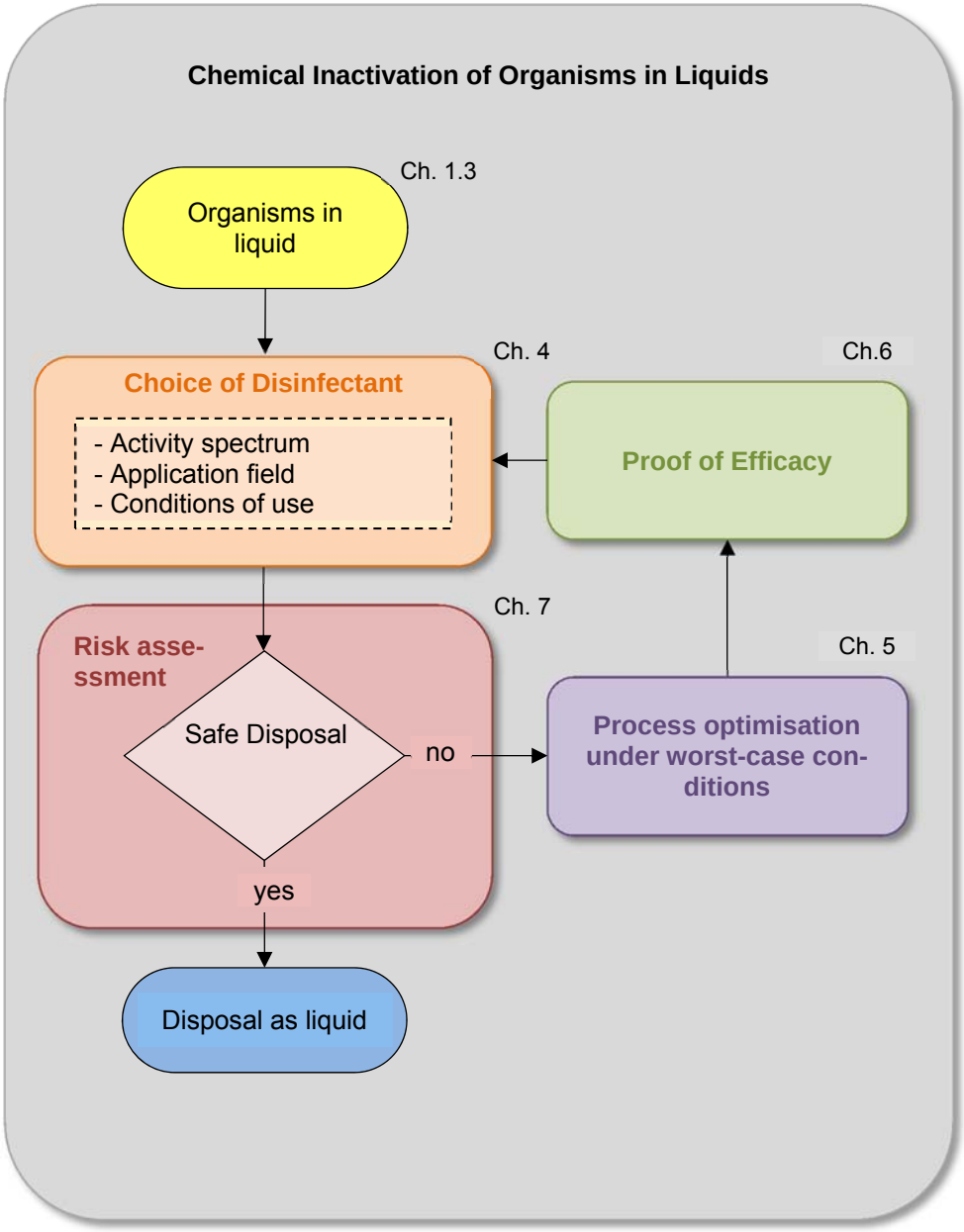


**Figure 1: Chemical Inactivation of Organisms in Liquids**

*Chapter 2* lists aspects that can be used as a checklist by establishments and by the authorisation and enforcement authorities in order to test the efficacy of a chemical inactivation process for organisms in liquids.

*Chapter 3* presents an overview of the various terms and definitions for inactivation and to the related objectives.

*Chapter 4* illustrates the criteria – in regard to the organism (spectrum of activity), the field of application and the conditions of application (environmental parameters) – that are to be taken into account for the optimal choice of the disinfectant for a chemical inactivation.

*Chapter 5* describes how the efficacy of the inactivation of organisms in liquids is influenced by a series of parameters and how these parameters have to be varied in order to test the methods and the proof of efficacy.

*Chapter 6* describes how the efficacy can be quantified.

*Chapter 7* illustrates the manner in which the risk assessment has to disclose that human beings, animals and the environment are protected from damage and impairment by the potentially remaining amounts and concentrations of the organisms.

*Chapter 8* describes other aspects of the safety of the application that also need to be considered besides the inactivation of organisms.

*Chapter 9* summarises the most important points and *inter alia* compares chemical inactivation with sterilisation by autoclaving. In addition, the importance of the proof of efficacy is established for chemical inactivation methods.

In *Chapter 10* the important terms and definitions are defined, together with a list of selected Standards, references and sources for further information.

# 2 Checklist for establishments and authorities

Establishments have to be able to comprehensibly justify, both internally and to the authorisation and enforcement authorities, that the release of organisms into the environment is prevented by the use of the inactivation methods.

The checklist comprises thirteen aspects or measurable parameters, which assist an establishment to compile an inactivation method, and thereby enable the efficacy of the proposed chemical inactivation methods to be assessed either internally or by the authorisation and enforcement authorities.

1. Does the establishment use a uniform standard inactivation process?
2. Is the inactivation carried out according to a process published in the technical literature, and under identical conditions? Does the publication exist?
3. Is the inactivation process defined in an internal company Standard Operating Procedure SOP?
4. Can it be justified why the inactivation process in question has been selected? *(Chapter 4)*
5. Is the composition of the organisms in the liquid to be inactivated known?
6. Is the maximum number of organisms in the liquid to be inactivated known (absolutely and as a titre)? *(Chapters 5.4 and 6.4)*
7. Are the relevant influence factors known that may inhibit the inactivation in the particular laboratory setting? *(Chapter 5.2)*
8. Has the efficacy of the inactivation process been tested under the worst-case conditions (maximum organic loading of the liquids with organisms and media components, as well as the presence of all inhibiting influence factors)? *(Chapter 5.3)*
9. How long is the contact time of the agent for the chemical inactivation until extrapolated zero growth of the organisms (extrapolation based on the initial number and the detection limit of the organisms)? *(Chapters 5.4 and 6.3)*
10. In the case of mathematically indescribable or unknown inactivation kinetics, does the adopted contact time correspond to four times the duration of the inactivation time for extrapolated zero growth? *(Chapter 6.2)*
11. With imprecise knowledge of the overkill rate of the process, is it possible to specify in a risk assessment that no hazards and impairments for humans, animals, plants and the environment emanate from possible incompletely inactivated organisms? *(Chapter 6.2)*
12. Is the inactivation method regularly checked for its efficacy? *(Chapter 6.3)*
13. Can it be satisfactorily justified that by employing the inactivation methods the treated liquids can be safely (from the microbiological viewpoint) disposed of into the environment? *(Chapters 6.2 and 7)*

# 3 Terms, definitions and different inactivation objectives

Fundamentally, organisms in liquids can be inactivated either by autoclaving or by chemical disinfection. The choice of autoclaving or chemical inactivation methods depends on the initial situation and the inactivation objective. Terms pertaining to inactivation and inactivation objectives are defined below.

## 3.1 Inactivation

According to the definition of Standard EN 1270: 1999, inactivation relating to micro-organisms means the *partial or total destruction* of a given activity up to the destruction of the microbiological system.
Because the definition of inactivation does not include the total elimination of organisms and transmissible genetic material, one always speaks of *total* inactivation, where this is needed as the objective of the inactivation. Only a *total* inactivation guarantees the absence of infectious and transmissible genetic material (organisms, plasmid, RNA).[5]

## 3.2 Disinfection

Disinfection is defined as a process for reducing the number of viable micro-organisms.
For commercial disinfectants an agent is approved if a log 4 reduction, i.e. 99.99%, is achieved in the organism count within a prescribed and practicable contact time and under specified conditions. According to the guidelines of the Robert Koch Institute, a disinfectant is deemed to be effective if the organism count is reduced by 4 to 5 log stages under pre-defined conditions.[6]
The Federal Office of Public Health FOPH specifies as follows: 5 log stages for bacteria and 4 log stages for fungi or viruses.[7] 4 Log stages means a titre reduction of 99.99%, or that 0.01% of the viruses that were originally present may still be infectious.

According to ContainO, this definition of efficacy for disinfectants is not sufficient for the inactivation of organisms in liquids. In order to meet the ContainO requirements, a higher reduction objective is needed in a chemical inactivation, depending on the initial titre, in order to afford a reduction of the organism count that is required for a safe disposal of liquids.

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5 In situations in which the primary concern is the destruction of DNA or RNA, chemical inactivation (e.g. with NaOH) is probably more effective – in the sense of total destruction – than autoclaving.

6 In the suspension test those concentrations of a disinfectant are deemed to be effective against viruses if the titre of infectious viruses is reduced by at least 4 powers of ten (≥ 4 log reduction) over the exposure time in question. (Guidelines of the Deutschen Vereinigung zur Bekämpfung der Viruskrankheiten (DVV) and of the Robert Koch-Institute (RKI) on the testing of chemical disinfectants for efficacy against viruses in human medicine; edition of 1 August 2008; page 941; DOI 10.1007/s00103-008-0615-5)

7 http://www.bag.admin.ch/anmeldestelle/13604/13869/13880/14043/; BAG Startseite > Biozidprodukt > Desinfektionsmittel > Wirksamkeitsdossier: *Durchführung der Tests*

In microbiological and molecular biological laboratories when the titres of liquids that contain viruses, depending on the experiment, are higher by an order of magnitude than in liquids that contain bacteria, the inactivation rates or the required log stages clearly differ for bacteria and viruses.
This is also apparent, for example, when using the definition of efficacy for norovirus disinfectants. The infective dose of noroviruses at 10 to 100 viruses is very low and the excreted amount of infectious viruses (up to $10^{11}$ viruses/gram of stool) is very high. In addition, the infection may also result from contact with contaminated surfaces because the tenacity of these viruses, i.e. the resistance to environmental influences, is very high (page 179 in Schwebke and Rabenau, 2012[8]). A disinfectant that is effective against noroviruses must therefore, under pre-defined standard conditions, possess a significantly higher inactivation rate than 4 log stages[9] in order to be able to meet the requirements in practice of a hygienic disinfection.

## 3.3 Decontamination

According to the definition in the Standard EN 12740:1999, the term *Decontamination* stands for the elimination of microbial contamination or its reduction to an *acceptable* level.
The “acceptable” level is not further defined in the Standard.

## 3.4 Sterilisation

Theoretically, sterilisation means the killing of all micro-organisms. In practice, this means that a total sterilisation succeeds indeed approximately, but not with 100% security (see the definition of the terms, Chapter **Fehler! Verweisquelle konnte nicht gefunden werden.**).

Autoclaving under standard conditions[10] meets the requirements of a sterilisation, and for disposal of solid and liquid wastes serves as the reference for the total inactivation and consequently for a safe disposal (see Chapter 6.2).

## 3.5 Validating and proving the efficacy

Validation according to the EN Standard 12740:1999[11] is a documented procedure for recording and evaluating results that are used to prove that a process continuously yields a product that complies with the stipulated properties.

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8 Ingeborg Schwebke; Holger F. Rabenau (2012) Aktueller Stand zur Viruzidieprüfung – ein Überblick Hygiene & Medizin 37 (7/8) http://nbn-resolving.de/urn:nbn:de:0257-10026059 oder http://www.rki.de/DE/Content/Infekt/Krankenhaushygiene/Desinfektionsmittel/Virusinaktivierung/Viruzidiepruefung.pdf?__blob=publicationFile

9 See footnote 7

10 See chapter 10.1

11 EN Standard 12740:1999: This Standard gives guidance on methods for handling, inactivating and testing of waste containing organisms arising from biotechnology laboratory activities and processes. It is concerned with methods to reduce the risks arising from exposure to waste derived from laboratory-scale activities which contains organisms hazardous or potentially hazardous to humans,

The difference between validating and proving the efficacy is not clear but gradual: the validation must demonstrate *that a process continuously yields a product*. In order to satisfy the term "continuously", the proof of the efficacy has to be performed repeatedly or statistically substantiated. Whether the proof of efficacy for a validation has to be performed three or even five times, remains a matter of opinion.

It is important that the proof of efficacy satisfies the principles for a scientific procedure and is meaningful and verifiable. To prove the efficacy a method should have been tested at least three times.

animals, plants or the environment. Such waste may include organisms whether as solid, liquid or gaseous by-products or effluent, together with items or equipment required to be disposed of and which may be contaminated with organisms. Wastes may be generated by biotechnology, clinical, molecular biology, microbiology and other laboratories in activities where organisms are handled, genetically modified organisms are created or used or by laboratory processes involving material of human, animal or plant origin. This European Standard does not apply to other types of waste from human healthcare or other medical treatment activities.

# 4 Criteria for choosing the agent for the chemical inactivation

The choice of an agent for chemical inactivation fundamentally requires that: the disinfectant should have the highest possible efficacy for inactivating the organisms.
Besides the reliability, the irreversibility of the effect is also important and, depending on the application purpose, also the material compatibility, hard water stability, safety of use, dosability and cost effectiveness.
In the choice of the disinfectant for the chemical inactivation of organisms, three major aspects are to be taken into account as a precondition for optimum use:

1. Spectrum of activity (with high specificity for the organism(s) to be inactivated in the liquid)
2. Field of application (for example application in liquids) and
3. Application conditions (includes *inter alia* the contact time and concentration of the inactivating agent and takes into account the significant influencing factors).

## 4.1 Spectrum of activity

The spectrum of activity of a disinfectant is defined by its effect on certain families of organisms and is tested on selected bacteria and viruses, mainly of medical significance. With these pathogens the spectrum of activity as well as the optimum concentration of active substance and contact time can be determined under defined conditions.
Each disinfectant acts in varying degrees as either a bactericide, fungicide, tuberculocide (mycobacteria), virucide[12] or sporicide. No disinfectant exists that kills off all types of microorganisms with the same efficiency; consequently, products with supplementary activity spectra would potentially have to be used for mixtures of organisms (e.g. bacteria and viruses). In this regard, the mutual compatibility of the various agents would need to be clarified.
In the list of disinfectants and disinfection processes[13] tested and recognised by the Robert Koch Institute, four categories of differently resistant organisms are differentiated by the spectrum of activity. With the data on the spectrum of the sensitive organisms those disinfectants that inactivated organisms most efficiently in the laboratory can be selected for the chemical inactivation (see chapter 5.7).

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12 With virus inactivation, sometimes a differentiation is made between the efficacy on “only enveloped viruses” or on “enveloped and non-enveloped viruses”. Enveloped viruses tend to be more sensitive to inactivating substances.

13 Online source: http://www.rki.de/DE/Content/Infekt/Krankenhaushygiene/Desinfektionsmittel/Desinfektionsmittelliste.pdf?__blob=publicationFile

## 4.2 Field of application

The application purpose “inactivation of organisms in liquids” is not indicated for the majority of commercially available inactivation agents. Common uses for commercially available products and their suitability for inactivating organisms in liquids are listed[14] in the following Table.

**Table 1: Field of application and suitability for inactivating organisms in liquids**

| | Application fields | Suitability for inactivating organisms in liquids |
|---|---|---|
| 1. | Disinfecting hands and body | *unsuitable* |
| 2. | Disinfecting surfaces and rooms | |
| 3. | Disinfecting equipment and instruments | *Possibly suitable as these agents are designed for immersion bath methods and thus for longer service lives. They retain their action even for higher protein concentrations.*<br>*It is therefore obvious to also use the corresponding products in the laboratory to inactivate organisms in liquids.* |
| 4. | Disinfection of laundry. | |

The practical conditions for the use of a commercially available product in the laboratory generally differ from the manufacturer’s instructions.

In particular:

As soon as the conditions of application (field and conditions of application) differ from the instructions for use, the users themselves are responsible for the proof of efficacy.

The user has to be able to demonstrate - for example to the enforcement authorities - the efficacy of a disinfectant, either by exactly following the instructions for use under the described factors of influence and provide proof of this, or by demonstrating in a methodologically correct manner the efficacy under the practical, given conditions. The reference to a published and analogous method of use of an inactivating agent likewise suffices.
Therefore, the efficacy under the practical given conditions is to be demonstrated for each non-standardised application of a disinfectant for inactivating organisms.

## 4.3 Conditions of use

In addition to the spectrum of activity and the field of application, the conditions of use are a crucial factor for the achievable degree of inactivation (see chapter 5.1). The conditions of use include parameters such as exposure time, concentration of the inactivating agent and possibly the optimum pH. In addition, the level of inactivation - depending on the type of the inactivating

[14] In all four application fields there is also the “disinfectant cleaner”. This is understood to mean disinfectants that also act as cleaning and optionally as care products.

agent - depends to varying degrees on factors such as temperature, stability (lifetime), possibly on humidity and surface conditions as well as the presence of proteins, dirt, and possibly surfactants and catalysts. In order to determine the efficacy of the inactivation, the various influencing factors need to be identified and their effect on the efficiency of the inactivation assessed. The influencing factors and their significance in regard to the achievable inactivation degree are described in more detail in chapter 5.2.

# 5 Procedure and worst-case conditions

The process optimisation includes various steps, as shown schematically in Figure 2, and discussed in more detail in the following sub-chapters.

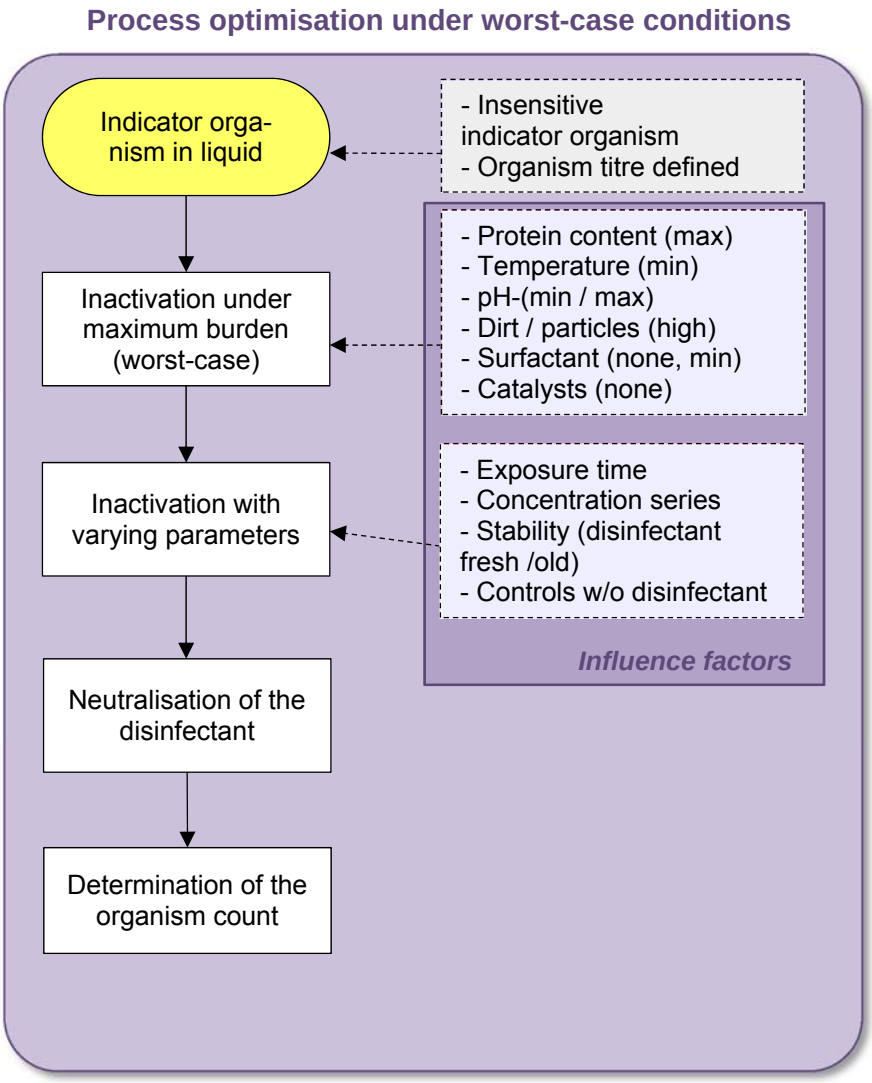


**Figure 2: Process optimisation under worst-case conditions**

## 5.1 Indicator organisms

The liquid to be inactivated generally contains not only one (single) type of organism, but rather a variety of organisms or a mixture of organisms. In such cases an indicator organism has to be defined and its inactivation rate determined. An indicator organism has to fulfil the following requirements:

1. Insensitivity (resistance) towards the selected procedure and as high as possible in comparison to that of the existing spectrum of organisms.

2. Type of organism that corresponds to the highest risk level with the lowest infectious dose, or that represents such an organism.
3. The titre of the organism should be easily determined.

Here, two categories of indicator organisms can be differentiated:

A) The indicator organism corresponds to one of the cultivated organisms or is already present in the mixture of organisms, and as a “bioindicator” represents a mixture of organisms and fulfils the above described conditions.

B) In the second case the organism, because it can be easily detected, is supplied externally (spiked) as the indicator test organism.

**Table 2: Two categories of indicator organisms**

| **Bioindicators** | **Indicator-test organisms** |
|---|---|
| The organism is **already present** in an existing mixture of organisms (or is the sole representative). | The organism is **supplied externally** to a mixture of organisms. |
| Its elimination signals the inactivation of the other organisms. (Specific case: only a single type of organism exists in the liquid.) | In a second priority the indicator-test organism should be as weakly pathogenic as possible. |

If the inactivation with the indicator organism corresponds to the desired reduction rate, then it can be assumed that the reduction rate has also been achieved for the other organisms.

For a mixture of organisms, the following applies to both the bioindicator as well as to the indicator-test organism:

The indicator organism has to be as insensitive (resistant) as possible to the selected procedure. It must be present in the highest concentration (titre) or in the greatest amount (total count) and simultaneously represent the highest risk group with the lowest infection dose.
If the inactivation rate for this indicator organism permits a safe disposal, then the inactivation conditions apply representatively to the other organisms in the mixture.

Due to the different resistance or sensitivity of organisms against a specific inactivation procedure, one must differentiate between various categories of organisms.

They are:

- Spore formers
- Bacteria
- Mycobacteria

- Enveloped viruses
- Non-enveloped viruses
- Single-cell parasites
- Fungi
- Prions[15]

If, for example, spore formers are not present in a mixture of organisms, then the efficacy of an agent does not need to be tested against spore formers.

## 5.2 Influence factors

In chapter 4, the spectrum of activity, field of application and conditions of use were cited as criteria for selecting a disinfectant, because they are the most important aspects for the efficacy of a disinfectant. Not only the choice of procedure but also its optimisation are strongly dependent on the influence factors (see Figure 2). Understanding the relevance of these influence factors is essential for proving the efficacy[16] and for optimising the procedure.

The conditions of use in the chemical inactivation can be differentiated into:

1. Exposure time or contact time[17] (example in Figure 3)
2. Concentration of the disinfectant[18] (example in Figure 4)
3. Temperature[19] (example in Figure 5)
4. Proteins[20] (example in Figure 6)
5. Optimum pH[21]
6. Age of the inactivating agent, i.e. storage time since its initial use (example in Figure 7)
7. Positive or negative effect of surfactants[22]

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15 Prions are mentioned here for the sake of completeness. The inactivation of prions in liquids is a specific case that we do not intend to discuss here. Prion-containing liquids absorbed in absorbent material and their subsequent incineration as hazardous waste is an alternative safe disposal path.

16 The difference between spectrum of activity, efficacy, effect and efficiency is discussed in the guidelines. See chapter 6.2

17 The exposure period is determined by the ingress of the disinfectant into the micro-organism, the interaction with the micro-organism and the evaporation time of the solvent.

18 The frequent assumption that a high concentration brings about a better efficacy is only partly true. In disinfection water often plays a decisive role. For example, absolute alcohol does not disinfect hands; in contrast 60 to 80% alcohol solutions have a good disinfecting action. It should be noted that only distilled water is to be used for dilution.

19 In analogy with chemical reactions, the disinfecting action increases with increasing temperature.

20 Proteins (serum, casein) often reduce the efficacy of disinfectants. In such cases the concentration has to be increased or another active substance has to be selected.

21 Many agents for chemical inactivation are optimally effective only at a certain pH. Take note of the manufacturer's data!

8. Dirt[23]
9. Stability (longevity of the disinfectant)[24]
10. Catalysts

Each of these parameters may influence the efficacy and efficiency of a chemical inactivation. The optimisation of an inactivation requires that the relevant parameters have either been optimised or shown to have no influence because they are irrelevant or constant or do not vary. Exclusion criteria for the procedure may optionally be drawn from this work, for example “inactivation must not be carried out outside the temperature range of 22 °C to 30 °C” or “inactivation must not be carried out outside the pH range of 5 to 7”, because the inactivation is no longer ensured outside these ranges.

The publication, *Guidelines for the validation and application of alternative inactivation methods to heat inactivation using an autoclave*[25] presents inactivation curves and critical points based on three exemplary inactivation procedures: sodium hydroxide NaOH, active chlorine[26] and ultraviolet radiation. Individual experiments from these procedural developments are illustrated in this and the following chapter. This publication also provides an overview of further inactivation procedures, their functional basis and their advantages and disadvantages.

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22 Residues of cleaning agents and other substances may render the disinfectant inactive. For example, cationic/quaternary disinfectants are inactivated by anionic cleaning agents. Different disinfectants may also have an antagonistic effect when mixed together.
Surfactants (surface-active substances) may also increase the efficiency of a disinfectant by enabling the disinfectant to reach the organisms.

23 Coatings of dirt or grease may impede or prevent the destruction of micro-organisms, and the destruction is often impossible, in cases when the coatings have dried on.

24 Disinfectants diluted in-house with distilled (deionised) water are to be freshly prepared on a daily/weekly basis.

25 Guidelines for the validation and application of alternative inactivation methods to heat inactivation using an autoclave; 2016; Kantonales Laboratorium Basel-Stadt; contracting body: Federal Office of Public Health FOPH

26 e.g. household bleach

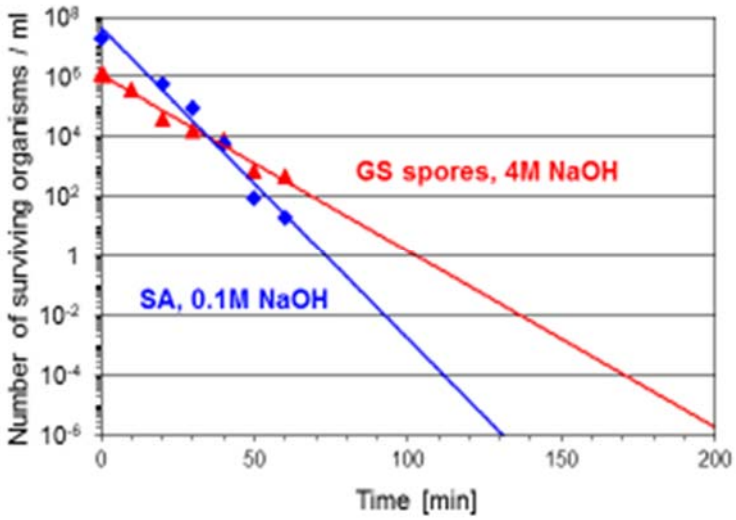


**Figure 3: Exposure time**[27]

Inactivation of *S. aureus* (SA, blue) with 0.1M NaOH and *G. stearothermophilus* (GS, red) with 4N NaOH for a period of 360 minutes. Samples were taken at various times and tested for replicable bacteria (SA) and germinable spores (GS). In the log scale presentation depicted here of the number of surviving organisms as a function of time, both of the death rates are linear and can be extrapolated to a SAL value (here $10^{-6}$).

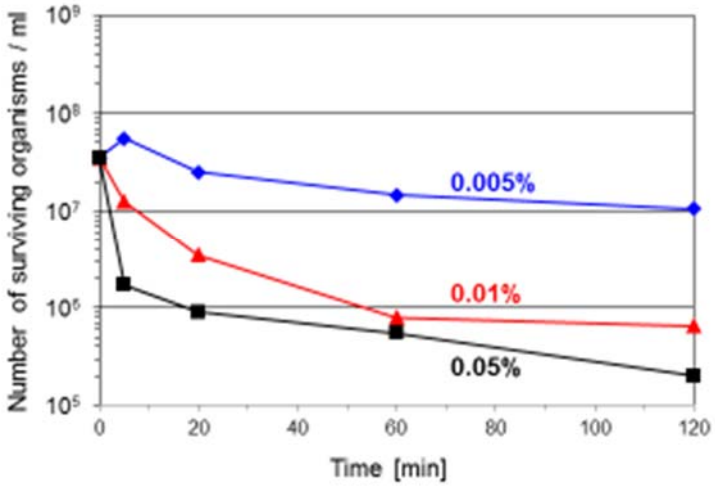


**Figure 4: Concentration of the Disinfectant**

Inactivation of MS2 bacteriophages with various concentrations of Haz-Tabs (product based on an active chlorine compound; 0.005%, 0.01%, 0.05%). Samples were taken at various times and tested for plaque-forming units. In this example active MS2 bacteriophages were found

[27] See footnote 25. Also applies to Figures 4 to 10.

during the entire period. The death rate is not linear but flattens out, such that a safe inactivation cannot be guaranteed.[28]

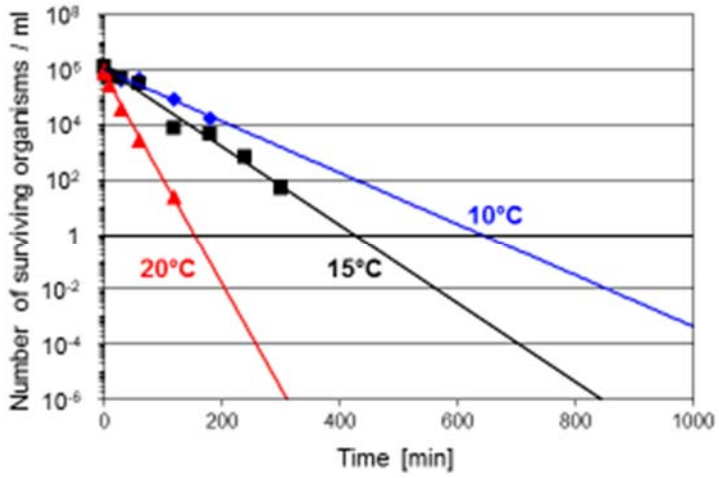


**Figure 5: Temperature**

Inactivation of *G. stearothermophilus* with 2M NaOH at different temperatures (10 °C, 15 °C, 20 °C). Samples were taken at various times and tested for germinable spores (GS). Reducing the incubation temperature from 20 °C to 15 °C in this example requires an approximate tripling of the exposure time to achieve a SAL value of $10^{-6}$.

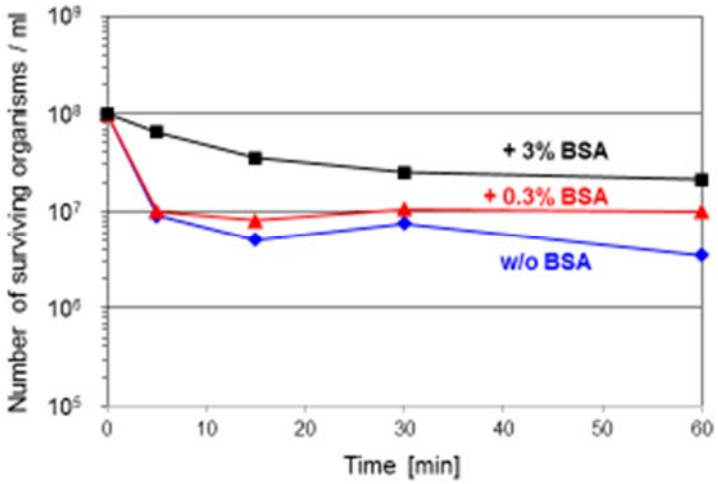


**Figure 6: Organic loading with proteins**

The inactivation of MS2 bacteriophages with 0.1% Haz-Tabs (product based on an active chlorine compound) in the absence (blue) and presence of an organic load at different concentrations (0.3% (red) and 3% (black) BSA, bovine serum albumin). The protein concentration of liquid cell culture waste is normally about 0.3%. Samples were taken at various times and test-

[28] In practice a more concentrated solution would have to be used in order to obtain an adequate inactivation efficacy. In the example, the concentration was chosen in order to make the effects visible.

ed for plaque-forming units. The presence of BSA in the inactivation procedure lowers the killed quantity of MS2 phages in the example by a factor of approximately five to ten[29].

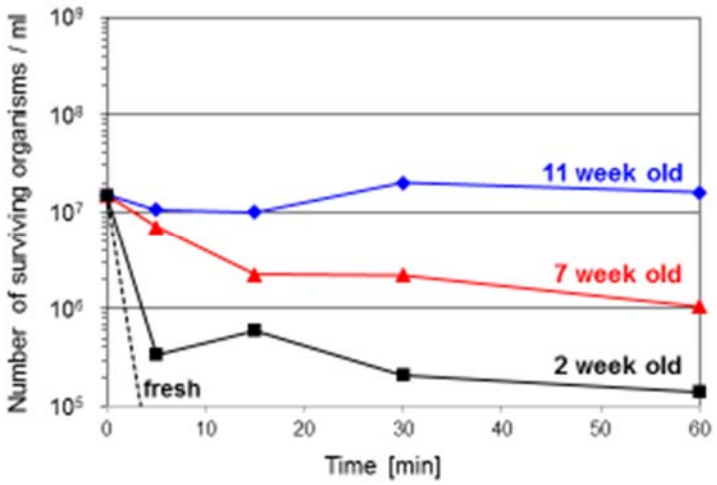


**Figure 7: Age of the inactivation agent, corresponding to its storage time**
The inactivation of MS2 bacteriophages with 0.1% Haz-Tabs (product based on an active chlorine compound), wherein differently fresh/old solutions were used: (dashed line) freshly made up on the day of the experiment, (black, solid line) 2 weeks old, (red) 7 weeks old, (blue) 11 weeks old. Samples were taken at various times and tested for plaque-forming units. With the freshly made solution, no bacteriophages were detected after 5 minutes, whereas the efficacy decreased with increasing age of the solution and was even absent with the 11 week old solution.[30]

## 5.3 Inactivation at maximum loading (worst-case)

Optimisation of the procedure (also) means that at a maximum load of organisms and in the most adverse chemical and physical conditions (worst-case), the pre-defined reduction in the organism count is guaranteed and the safe disposal into the environment is ensured.

The maximum loading (worst-case) consists of:

1. all possible negative conditions, such as the highest possible loading of organisms with the most resistant microbe (which is addressed by the choice of the indicator organism) and
2. all factors of influence in their maximum extent and which are relevant to the chosen inactivation procedure (apart from those that are so limited by exclusion criteria that their influence on inhibition can be excluded; examples in Figure 7 and Figure 8).

29 See footnote 28

30 See footnote 29

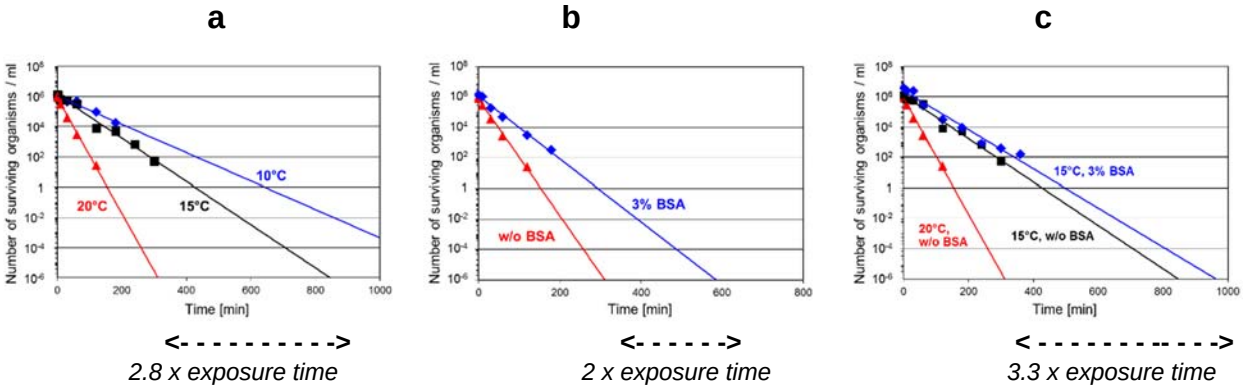


**Figure 8: Worst-case 1**

Inactivation of *G. stearothermophilus* with 2M NaOH under worst-case conditions (Figure c, blue line (15 °C, 3% BSA). The combination of two conditions that lengthen the exposure time required for the SAL $10^{-6}$. low temperature (Figure a, 15 °C versus 20 °C; black versus red lines; 2.8 times exposure time) and the presence of 3% BSA (Figure b, red versus blue line; twice the exposure time), when combined (= worst-case), results in a 3.3 times exposure time (Figure c, red versus blue line).

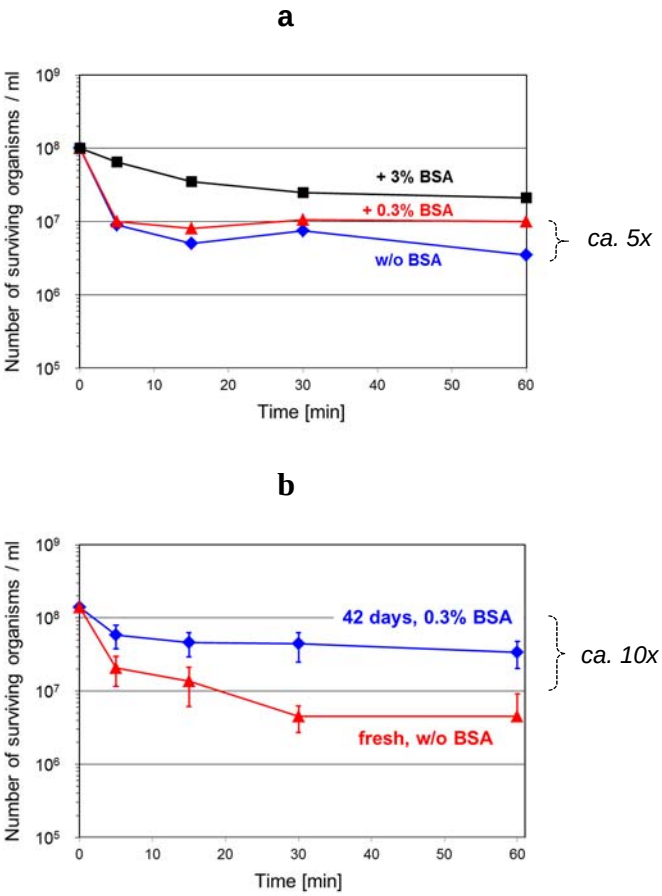


**Figure 9: Worst-case 2**

Inactivation of MS2 bacteriophages with 0.01% Haz-Tabs under worst-case conditions (Figure b, blue curve: 42 day-old solution, 0.3% BSA). The combination of two conditions that reduce

the killing efficacy: Presence of BSA (Figure a, none versus 0.3% BSA, efficacy reduced by ca. factor 5) and age of the Haz-Tab solution (not shown, see Figure 7), when combined (= worst-case), results in a killing efficacy reduced by a factor of ca. 10.[31]

The factors of influence listed in Table 3 tend to have either a positive or negative effect on the inactivation efficacy.

**Table 3: Status of influence factors which result in a negative influence on the inactivation efficacy**

| Influence factors | Condition in the worst-case |
|---|---|
| **Protein content** | high |
| **Temperature** | low |
| **pH** | *case-by-case* |
| **Dirt/Particles** | many |
| **Surfactants** | *case-by-case* |
| **Catalysts** | none |
| **Exposure time** | short |
| **Concentration** | low |

## 5.4 Inactivation with varying parameters

In order to determine the inactivation kinetics the following parameters can be tested against each other.

1. Length of exposure or exposure time
2. Concentration of the disinfectant
3. Organism count (titre)
4. Temperature
5. Inhibitor load (e.g. proteins)
6. Stability of the disinfectant (fresh/old)

Experimentally, only one of the parameters is varied, the others remain constant. In the majority of cases the exposure time or the concentration of the agent for chemical inactivation is varied.

The temperature is generally room temperature RT. It should be noted that the efficacy of the inactivation can be significantly increased at higher temperatures (chemo-thermal disinfection, e.g. with NaOH).

[31] See footnote 29

For the organism count, a concentration should be chosen which is in the maximum range of the typical laboratory situation. The same applies to the concentration of inhibitors which should reflect a high loading (worst-case).
The stability of the disinfectant becomes a significant parameter when the agent decomposes chemically and may become inactive within the period of use or when stored in the laboratory. With a series of experiments on a disinfectant prepared with different freshness, it can be shown that the agent does not lose its inactivation potential during typical storage times in the laboratory.

**Table 4: Examples of possible parameters**

| | Range | Notes |
|---|---|---|
| **Exposure time** | *Time series:*<br>• some hours,<br>• 15 hours (overnight)<br>• some minutes | In general, the inactivation of organisms in liquids requires periods of some hours. |
| **Concentration of the Disinfectant** | Undiluted,<br>Dilution factors (1:5; 1:10, 1:100 etc.) | The final ratio of disinfectant to the sample volume is relevant. |
| **Organism count (titre)** | Bacteria, fungi e.g. $10^6$/ml,<br>Viruses e.g. $10^5$ to $10^6$/ml,<br>Parasites e.g. $10^3$/ml | May also be higher; take account of the actual titre. |
| **Temperature** | 15° C (worst-case),<br>20 °C room temperature RT,<br>40 to 60 °C* | * at higher temperatures bear in mind harmful vapours. |
| **Inhibitor load (e.g. proteins)** | Estimate concentration | Casein, albumin, serum proteins etc. |
| **Stability of the disinfectant (fresh/old)** | Hours to months | Agent-dependent and whether diluted or not |

## 5.5 Neutralisation of the disinfectant for the detection procedure

The degree of inactivation is determined after the inactivation from the count of the surviving organisms. For this the organisms from the inactivation solution have to be added to a growth medium (e.g. for bacteria) or to cell cultures (e.g. for viruses). In order not to obtain false-negative results the inactivation agent has to be neutralised, i.e. the inactivating effect has to be disabled. For experimental series with variable exposure times the quenching of the inactivation is an experimental challenge that has to be solved in some way for each application. Examples of possible methods include:

- Molecular sieve processes (i.e. binding, for example by means of Sephadex columns[32],
  Figure 10: Molecular sieve process for neutralising the disinfectant)
- Centrifuging
- Chemically (e.g. pH shift / neutralisation[33])
- Diluting the inactivation solution and separating the organisms (e.g. by centrifugation)
- Filtration and rinsing.

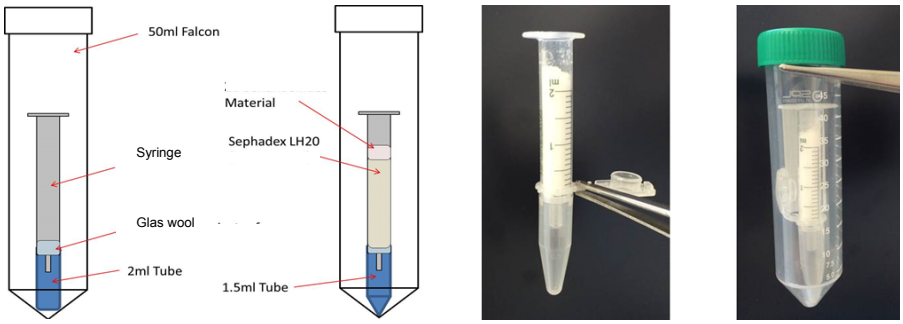


**Figure 10: Molecular sieve process for neutralising the disinfectant**
Elimination of the toxicity of the disinfectant (e.g. active chlorine compounds) by means of purification by molecular sieve processes (Sephadex).

## 5.6 Determining the organism count

Normally, known methods for the quantitative determination or the titre of the organisms used in the laboratory are available there. The quantitative measurement of bacteria is simpler than for viruses or certain parasites. Detection by cultivation is the method of choice and is easily carried out for bacteria and bacteriophages. Viruses can be detected with the help of cell cultures. In this regard, prior neutralisation of the disinfectant (elimination of the toxic action) is of particular importance.
The polymerase chain reaction PCR may represent an alternative to cultivation only in exceptional cases, wherein here the count of viable organisms can only be indirectly inferred. The PCR analysis shows only the remaining DNA/RNA, and does not differentiate between the presence of still infectious organisms and transformable DNA.

32 See Standard: EN13610 (Chemical disinfectants – Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas)

33 See Standard: EN13727 (Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity in the medical area)

Important: Only evidence that shows that DNA/RNA is *no longer* present can be accepted as proof of the absence of infectious units. This evidence is methodologically challenging and is only possible with positive controls and time series.

Instead of organisms that are usually used in the laboratory, indicator-test organisms may also be used if they are easier to detect or to cultivate (e.g. MS2 bacteriophages[34]). See also chapter 5.1.

## 5.7 Optimisation and efficiency

Optimisation means efficiency and is the yardstick for efficacy or the cost-benefit ratio. Besides lower economic costs for the agent, it is worthwhile to strive for an optimised relationship between **high efficacy** and **shorter exposure time** and higher **safety of application** as well as **low environmental impact**.

Consequently:

> The efficient, optimised use of an agent for chemical inactivation means primarily an application with little active substance, at the lowest possible concentration, in the shortest time and with good material compatibility with minimum stress for the user and the environment.

High activity, high safety of application and low environmental impact are three aspects that also partially contradict each other, and therefore often have to be weighed against each other. See Chapter 8. The meanings of activity and efficacy are described below in Chapter 6.

[34] ssRNA virus with four genes from the family of the *Leviviridae.* See also the publication referred to in footnote 25.

# 6 Proof of efficacy

## 6.1 What does proof of efficacy mean?

In order to be able to prove the efficacy of an inactivation method for organisms, firstly the aim of the inactivation has to be defined, against which the degree of inactivation can be measured. For autoclaving this is unambiguous; as a validated method, autoclaving offers – when the conditions are respected – an exactly pre-defined degree of inactivation or of killing the organisms.[35]

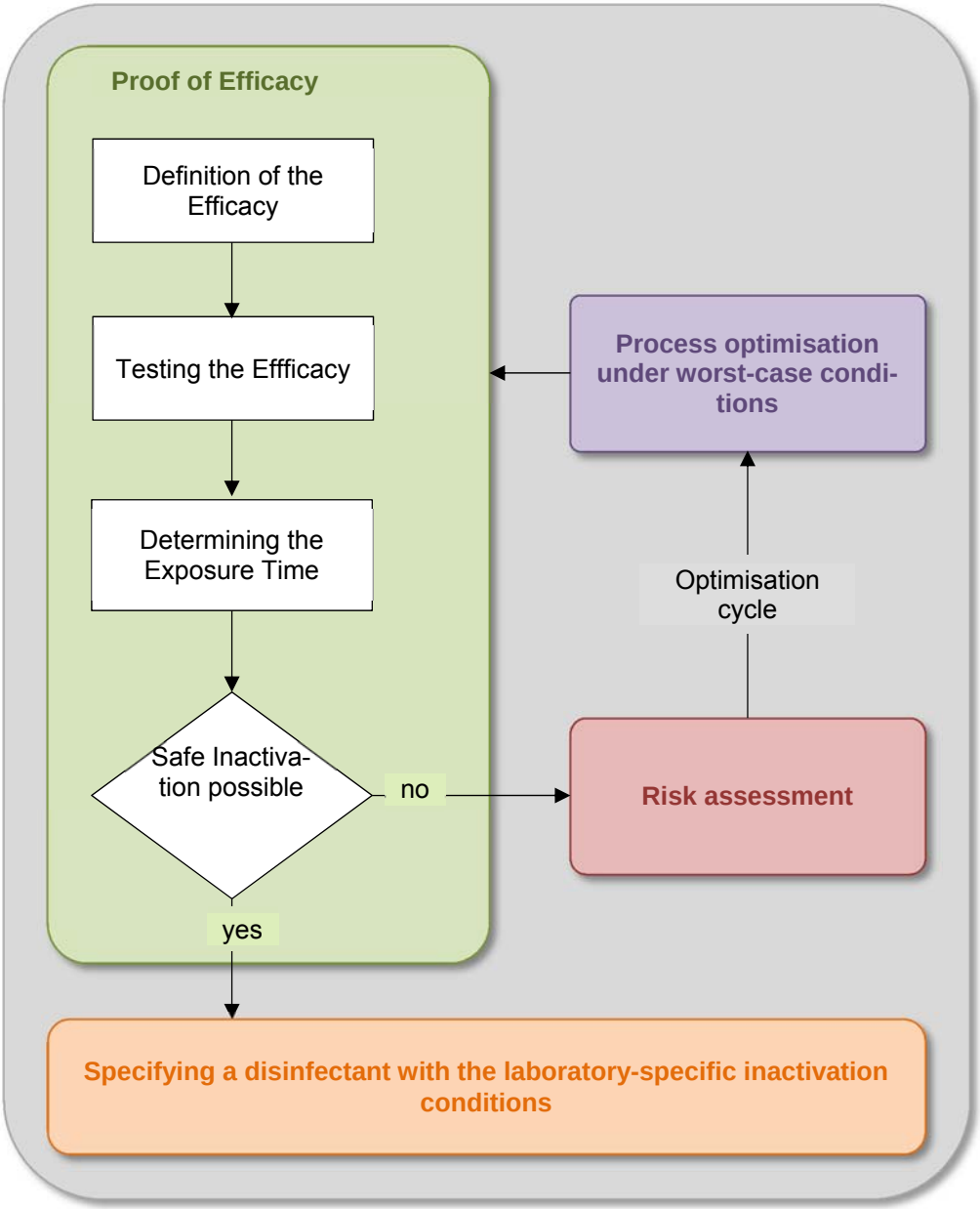


**Figure 11: Proof of efficacy**

The inactivation conditions for autoclaving are clearly defined. Compared to this, the inactivation conditions for each disinfectant in a varying composition of liquids (with various types of organ-

[35] See Chapters 3.4 and 10.1.

ism and different ingredients) have first to be established, and the efficacy of the employed method is to be determined for the organism to be inactivated.

However, how can the *efficacy* be determined? In order to do justice to the title, *Chemical Inactivation of Organisms in Liquids - Guidelines for the chemical inactivation of organisms in liquid cultures or supernatants with proof of efficacy and safe disposal*, the term *inactivation efficacy* has to be clarified.
The proof of efficacy should satisfy the principles of a scientific procedure and be meaningful and verifiable. Compared to a validation (see the definition in chapters 3.5 and 10.1), the proof of efficacy requires fewer claims to multiple repetitions and standardisation (see chapter 3.5).

## 6.2 Reduction rate as a measure of the efficacy and safe chemical inactivation

Chemical inactivation should enable the safe disposal of liquids, such that the inactivated liquids can neither impair nor endanger humans, animals or the environment (see chapter 7.3).
In order to take a decision on whether a safe disposal is possible, there is a need for definitions of the terms *efficacy of the inactivation* or *inactivated liquids.* For this, efficacy and efficiency of the inactivation process would have to be ascertainable and quantifiable.

The **efficacy** (or the effectiveness) is a measure of the achievement of the objective (**action, effect**) and stands here for a specified degree of inactivation.[36] The degree of inactivation depends on the reduction rate that is further described below.

For a sterilisation process a sterility assurance level (SAL, Figure 4) of $10^{-6}$ is internationally uniformly demanded. A SAL of $10^{-6}$ means that in one million identically treated units of the bioburden, there may remain a maximum of one micro-organism capable of reproduction, or in other words, the theoretical residual content of micro-organisms capable of reproduction in one unit of the bioburden is at most $10^{-6}$ colony-forming units. For a titre of $10^{6}$ organisms, sterilisation therefore means an overall reduction of $10^{12}$, i.e. a theoretical, i.e. mathematically calculated 12-log reduction of the organism count.[37]

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36 A disinfectant has an effect on an organism if said organism belongs to the spectrum of activity of the disinfectant. The efficacy takes into account not only the effect itself, but also the rate, the required concentration etc. and therefore has a broader meaning.

37 The D-value (decimal reduction time D) denotes the time needed for a 1-log reduction, i.e. the time in which nine tenths of a population of organisms die off and hence the number of organisms is reduced to one tenth of the original value. This reduction by a power of ten corresponds to a kill rate of 90%. As this time D is strongly dependent on the temperature and also on other conditions, typically the temperature is given as an index, e.g. $D_{121}$. Further information on sterilisation can be found in Wikipedia under https://de.wikipedia.org/wiki/Sterilisation

A SAL of $10^{-6}$ for the typical exponential inactivation kinetics of thermal inactivation by autoclaving means a doubling of the (measurable) inactivation time that is required to theoretically reduce a titre of $10^6$ organisms down to one entity (cell) of an organism (6-log reduction).

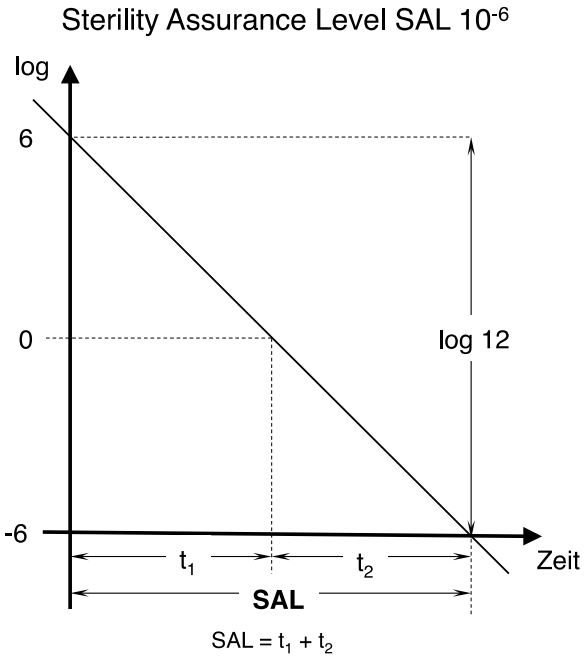


**Figure 12: Sterility Assurance Level SAL (Organism titre as a function of exposure time)**[38]

A SAL value[39] is determined by extrapolating the exponential inactivation kinetics, or the first-order inactivation kinetics, which can be displayed as a straight line.
The SAL value (e.g. $10^{-6}$) for disinfection by chemical inactivation may only be ascertained in the case of first-order inactivation kinetics. In all other cases, the SAL value cannot be determined.

The value $t_1$ can be determined only approximately, because the detection limit is theoretically one unit (cell) of an organism; in practice, however, this detection cannot be proven.[40] In this example of the inactivation kinetics $t_1$ and $t_2$ are identical. $t_1 + t_2$ correspond to the SAL-value of $10^{-6}$ (as in Figure 12).

The SAL value of $10^{-6}$ is an accurate indication for an inactivation that in practice is also called an overkill.

38 Own presentation

39 A Sterility Assurance Level of $10^{-6}$ is required for the definition of “sterile”, and means the probability that a maximum of one micro-organism capable of reproduction is contained in one million equally treated entities of the bioburden.

40 From the biological viewpoint each value <1 corresponds to zero; a biological entity is dead if it meets the condition <1.

An overkill can also be achieved with chemical inactivation but can be proved neither theoretically nor practically. The procedure for determining an exposure time for the safe inactivation with chemicals is therefore as follows:

In order to determine the exposure time for a sufficiently safe inactivation with a chemical inactivation agent at a given concentration and under given conditions, one has to determine the initial time (t): the inactivation time required to achieve an extrapolated zero growth of the organisms.

The exposure time for safe inactivation is four-times this time t.

This may be expressed by the formula:

$t_{SI}$ (exposure time for safe inactivation) $= 4\, t_{exN}$ (exposure time for extrapolated zero growth)

The exposure time for extrapolated zero growth ($t_{exN}$) is derived from the inactivation process starting from the initial organism count down to the detection limit of the organisms.

The uncertainty that results from the fact that the chemical inactivation (mainly with low concentrations of the chemical) does not follow orderly inactivation kinetics, is compensated in that the exposure time for the complete inactivation is quadrupled and not 'only' doubled as with the SAL of $10^{-6}$. The experimental derivation for this requirement is illustrated graphically in Table 8 in Chapter 6.4.

## 6.3 Testing the efficacy

The inactivation kinetics must be determined in order to test the efficacy of an agent for chemical inactivation. In order to enable a well-founded statement on the efficacy of the chemical inactivation, a series of controls is required as reference parameters (see Table 5).
They are:

1. Control with organisms without disinfectant
2. Control without organisms with disinfectant
3. Negative control without organisms without disinfectant (sterility testing)

**Table 5: Framework parameters for the determination of the inactivation kinetics**

| Framework parameters for the determination of the inactivation kinetics | | |
|---|---|---|
| **inactivation kinetics** | Inactivation with disinfectant as a function of duration of exposure, concentration and possibly storage life of the disinfectant under worst-case conditions | a |
| **Negative controls** (Controls should show no growth) | Sterility testing for the neutralisation step (solutions etc.) | b |
| | Sterility testing of the inactivation diluent (control relating to inactivation with disinfectant) | c |
| | Sterility testing of the organism diluent (control relating to the addition of test organisms) | d |
| **Positive controls** (Controls should show growth) | Eliminating an inactivating property of the liquid waste and control for the titre determination of the test organism | e |
| | Confirming the complete neutralisation of the disinfectant, control for the titre determination of the test organism | f |
| | Recovery rate: control for the titre determination of the test organism | g |
| | Recovery rate for organisms in the liquid waste | h |
| **Titre determination** | Organism count in the contaminated liquid waste | i |
| | Test organisms (e.g. $10^6$ cfu) | k |

All the theoretically possible controls are listed in Table 5. Depending on the experimental approach some controls may be dispensed with, for example, if the addition of the test organisms is negligible with respect to the liquid volume, hence making the control with the corresponding volume of a diluent irrelevant.

The organism count or the titre of the organisms (i) in the liquid waste and with the added test organisms (for spiking) is to be determined as a control. In addition, it shall be demonstrated in the controls that the sterility of the individual experimental steps is ensured. It is particularly important that the control (f), with which the disinfectant is shown to have been effectively neutralised prior to the cultivation, and hence that the detection of the organisms is not falsified due to residual disinfectant. This has particular significance for the detection of viruses in cell cultures, where the cells (for the detection of the viruses) are relatively sensitive to the disinfectant. Also, when inactivating bacterial spores, due consideration must be given to neutralising (i.e. washing out) the disinfectant in the spore envelope prior to plating out the spores.

Figure 13 shows how controls (e and f) are carried out after the disinfectant neutralisation step by adding a low concentration of test organisms (the detection can then be made without dilution steps).

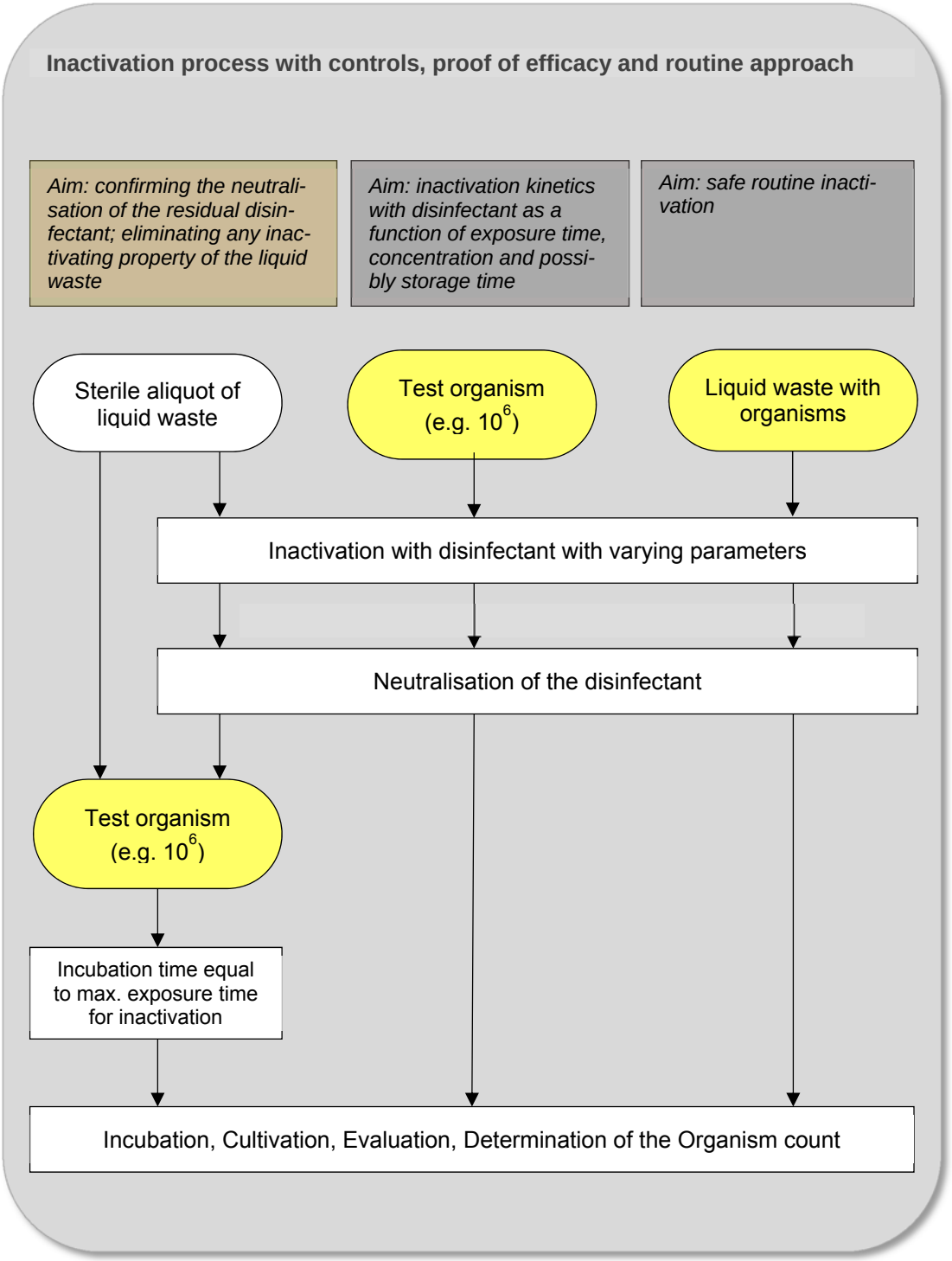


**Figure 13: Inactivation procedures with controls, proof of efficacy and routine approach**

Those steps or added components listed in the following table show schematically how the inactivation kinetics can be determined.

**Table 6: Experimental steps or the addition of components**

This Table discusses the columns of the subsequent Table 7.

| | Experimental steps or the addition of components | Notes |
|---|---|---|
| 1. | Aliquot containing sterilised liquid waste FL | *This serves to exclude an inactivating property inherent to the liquid waste* |
| 2. | Corresponding volume of diluent (as 1) | *Compensation for the volume of the aliquot added in point 1 to the sterile liquid waste* |
| 3. | Aliquot containing liquid waste with organisms | *Inactivation series* |
| 4. | Addition of test organisms ($10^6$ cfu) | *Determination of the inactivation kinetics* |
| **5.** | **Addition of the agent for the chemical inactivation** | *Carrying out the inactivation with time and concentration series* |
| 6. | Corresponding volume of diluent (as 5) | *Compensation of the volume of agent added under point 5 for the chemical inactivation* |
| 7. | Neutralisation step (nullifying the action of the disinfectant) | *Eliminating or excluding the effect of the disinfectant* |
| 8. | Addition of test organisms ($10^2$ cfu) | *Controlling whether the disinfectant has been effectively neutralised* |
| 9 | Adding the corresponding volume of diluent | *Compensation for the volume of the aliquot with test organisms added under point 8* |
| 10. | Maximum exposure time as for the inactivation | *Requirements for selected positive controls* |
| 11. | Analysis | *Incubating, cultivating, / cfu determination* |

The addition of “corresponding volumes of diluents” (steps 2, 6 and 9) may be left out if these volumes are so small that the titre determination would not be compromised.

Table 7 summarises Tables 5 and 6 and shows how, in addition to the determination of the inactivation kinetics, the required controls and titre determinations are compiled. The Table should be read from left to right. Each of the required steps or the components to be added (buffer, indicator organisms etc.) are indicated (black points) in the columns.

**Table 7: Testing the efficacy**

| **Testing the efficacy** | | ***Experimental steps / components*** *(for comments see Table 6)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Determining the*** | ***Objective*** | *1. Aliquot of liquid waste FL (sterile)* | *2. corresponding vol. diluent* | *3. liquid waste with organisms* | *4. test organism ($10^6$ cfu)* | *5. addition of disinfectant* | *6. corresponding vol. diluent* | *7. neutralisation of disinfectant* | *8. addition of test organisms (e.g. $10^6$ cfu)* | *9. corresponding vol. diluent* | *10 max. exposure time as for inactivation* | *11. analysis of growth* |
| Inactivation kinetics | *Inactivation with disinfectant as a function of exposure time, concentration and possibly storage time* | | | ● | | ● | | ● | | | | |
| | | | | | ● | ● | | ● | | | | |
| Negative controls | *Sterility check for neutralisation step* | ● | | | | | | ● | | | | – |
| | *Sterility check inactivation diluent* | ● | | | | | ● | ● | | | | – |
| | *Sterility check organism diluent* | ● | | | | | | ● | | ● | | – |
| Positive controls | *Excluding an inactivating property of the liquid waste* | ● | | | | | | | ● | | ● | + |
| | *Confirming the complete neutralisation of the disinfectant* | ● | | | | ● | | ● | ● | | ● | + |
| | *Recovery rate for test organism* | | | | ● | | ● | ● | | | | + |
| | *Recovery rate for organisms in the liquid waste* | | | ● | | | ● | ● | | | | + |
| Titre determination | *Organism count in the contaminated liquid waste* | | | ● | | | ● | | | | | + |
| | *Test organisms (e.g. $10^6$ cfu)* | | | | ● | | ● | | | | | + |

## 6.4 Determination of the exposure time for safe inactivation

This chapter contains an example of how the exposure time, concentration and when necessary the storage time of the disinfectant are to be varied in order to determine the inactivation kinetics.

For autoclaving, it is generally accepted that theoretically 100% inactivation does not occur. Theoretically, because on purely practical grounds a total inactivation can simply not be proved. However, with autoclaving, on the basis of the exponential inactivation kinetics, the overkill rate

can be described, at least theoretically, and expressed numerically. With chemical inactivation the situation is less simple, because here in most cases the inactivation kinetics cannot be represented in a simple manner (or only in certain concentration ranges) and therefore no theoretical inactivation target can be extrapolated. Nevertheless, exposure times can be determined which fulfil the aim of an overkill rate. The determination of the parameters for the chemical inactivation in the form of a defined and quantifiable reduction rate requires that measurement series to extrapolated zero-growth are carried out under standardised conditions with *only one* disinfectant and *only one* indicator organism[41].

In practice, the course of the inactivation could proceed according to the scheme in Table 8.

***In the experimental approach:***

- The agent for the chemical inactivation is tested in at least three different concentrations (disinfectant-conc. A, B and C)
- The exposure time is varied as a time series ($t_x$, $2t_x$, $4t_x$, $8t_x$ etc.).
- As one of the possibly relevant influence factors the storage time ($t_y$) of the disinfectant is varied ($t_y$, $2t_y$, $3t_y$ etc.)

***With the assumptions that***

- The indicator organism is representative for the mixture of organisms to be inactivated and the concentration is shifted in the tested range of $10^6$ cfu/ml or higher, up to e.g. $10^9$ cfu/ml.
- **(+/-)** means a still only barely detectable growth in an undiluted sample (detection limit) and therefore the corresponding exposure time is considered to be the time needed for a log 6 reduction (orange field). The determination of the total inactivation can be established only indirectly, because the reduction rate can be shown only as far as the detection limit or to the directly detectable growth of organisms.

41 A known example for an indicator organism for validating autoclaving processes is *Geobacillus stearothermophilus*. In chemical inactivation processes there are some test organisms that are regularly used for efficacy testing of disinfectants; the organisms principally originate from the spectrum relevant to hospitals (HIV, HBV, mycobacteria).

**Table 8: Determination of the exposure time for safe inactivation as a function of concentration and storage time of the disinfectant**

| **Determination of the exposure time for safe inactivation** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Indicator organism** | For example $10^6$ to $10^9$ cfu/ml | | | | | | | | | | | | |
| **Influence factors (e.g. storage time)** | **$t_y$** | | | | **$2t_y$** | | | | **$3t_y$** | | | | etc. |
| **Exposure time** | **$t_x$** | **$2t_x$** | **$4t_x$** | **$8t_x$** | **$t_x$** | **$2t_x$** | **$4t_x$** | **$8t_x$** | **$t_x$** | **$2t_x$** | **$4t_x$** | **$8t_x$** | etc. |
| Disinfectant conc. A | +/+ | +/- | -/- | -/- | +/+ | +/+ | +/- | -/- | +/+ | +/+ | +/- | +/- | |
| Disinfectant conc. B | +/- | -/- | -/- | -/- | +/+ | +/- | -/- | -/- | +/+ | +/+ | +/- | -/- | |
| Disinfectant conc. C | -/- | -/- | -/- | -/- | +/- | -/- | -/- | -/- | +/+ | +/- | -/- | -/- | |
| Positive control | + | + | + | + | + | + | + | + | + | + | + | + | |
| Negative control | – | | | | – | | | | – | | | | |

**Table 9: Interpretation of the growth and colour code:**

| **Growth** (duplicate determination) | | **Colour** | **Exposure time** | **Interpretation** |
|---|---|---|---|---|
| **+/+** | Clear growth in both duplicates | +/+ | | Insufficient exposure time at the given concentration |
| **+/-** | Scarce growth (detection limit) in both duplicates or no growth in one duplicate | +/- | **$t_{exN}$** (exposure time to extrapolated zero growth) | |
| **-/-** | No growth in either duplicate | -/- | | |
| **-/-** | No growth in either duplicate | -/- | **$t_{SI}$** (exposure time for safe inactivation) | Acceptable exposure time: guaranteed overkill |

***Interpretation examples based on the results postulated in*** Table 7***:***

- The inactivating agent has a limited storage life: the efficacy decreases over time ($t_y$, $2t_y$ and $3t_y$).
- In order to be able to guarantee a total inactivation, the disinfection has to be carried out under the following conditions[42] with a disinfectant having a storage time $t_y$:
  a. Disinfectant conc. A: Safe inactivation after an exposure time of at least $8t_x$.
  b. Disinfectant conc. B: inactivation after an exposure time of at least $4t_x$.
  c. Disinfectant conc. C: inactivation after an exposure time of at least $2t_x$.

42 These fields are marked dark green in the above table and denote the acceptable exposure time with a practically guaranteed overkill (see Chapter 6.2).

# 7 Risk assessment

The proof for the safe disposal is the focus of the risk assessment in Chapter 7.

## 7.1 Parameters and possible criteria

Required parameters for the risk identification[43] are:

1. Absolute quantity (total count) of the organisms released into the environment
2. Concentration (titre)
3. Properties, such as propagation, dissemination, persistence as well as tenacity (inactivation kinetics in the environment, particularly in waste water)
4. Infectious dose for existing pathogens

Possible criteria for assessing the risk are:

1. Can the propagation, dissemination and persistence in the environment be excluded in practice?
2. Is the infectious dose for the pathogens present in the environment exceeded?
3. etc.

Also, take note of ContainO, Annex 2.

## 7.2 Risk assessment and notes on the flow chart

The risk assessment process is presented as a flow chart in the Figure. The individual steps are numbered and subsequently discussed.

1. For the risk assessment, it must be clear in which risk group the organisms in the liquid to be inactivated are classified (see Chapter 1.2) and the most suitable chemical inactivation procedure has been identified (see Chapters 4 and 5).
2. For the risk assessment the usual procedure (in the establishment or laboratory) for the chemical inactivation is chosen as the methodological basis. This presupposes that the corresponding parameters are not only definable but also specified.
3. The inactivation or reduction rate that is achieved by (correctly implemented) autoclaving is deemed to be the reference value for the *total* inactivation (see Chapter 6.2).
4. If a reduction by chemical inactivation has been determined to be comparable with that obtained by autoclaving, then no further risk assessment is required. The organisms are deemed to be totally inactivated; consequently a release of organisms into the environment is prevented and a safe disposal is ensured.

[43] The situation-specific risk assessment and evaluation can be guided by the provisions of ContainO (Annex 2.1; Art. 6 and 26).

5. In the case where the reduction by chemical inactivation does not correspond to the rate that can be achieved by correctly implemented autoclaving, then this rate has to be experimentally determined according to the procedure in Chapter 5.

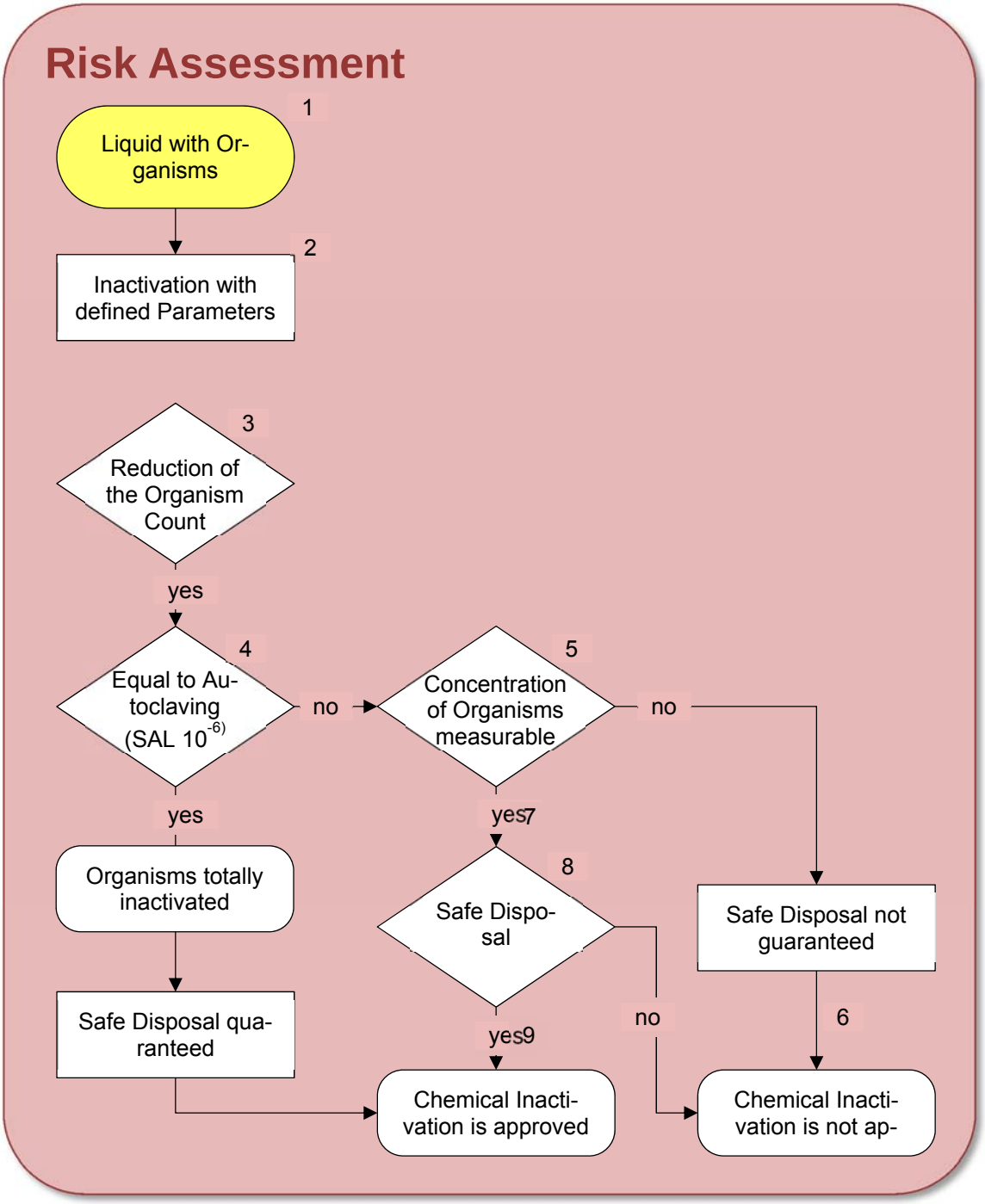


**Figure 14: Flow chart for the risk assessment**

6. If neither the absolute quantity nor the concentration of the organisms can be determined after the inactivation process, then no well-founded statement is possible of whether a safe disposal is guaranteed. Therefore the chemical inactivation is not allowed.

7. In order to carry out the risk assessment, the absolute organism count as well as the concentration (titre) in the inactivated liquids must be measurable and known.
8. The risk assessment must show that human beings, animals, plants and the environment are protected from damage and impairment resulting from the disposal via waste water.
9. A disposal by chemical inactivation is deemed safe if the exposure time of the agent for the chemical inactivation guarantees an overkill. See Chapters 6.2 and 6.4. Even with the overkill inactivation, it cannot theoretically be excluded that no organism reaches the environment via waste water. Besides the overkill inactivation, the following argument also applies to the minimisation of risk: Subsequent to the inactivation, even if a small number of organisms were to reach the environment but can neither proliferate, disseminate nor survive there, then a safe disposal is possible and the chemical inactivation carried out under proven overkill conditions is allowed.

## 7.3 Criteria for a safe disposal of biological agents

To be able to safely use the chemical inactivation of organisms for the disposal of liquid waste into the environment, requirements to meet the desired end conditions have to be stipulated.

For the risk assessment the requirements focus on the inactivated liquids that permit a safe disposal into the environment. Based on the theoretically possible, remaining organism count (absolute and/or based on the concentration), the safe disposal is conditional on the reasonable assumption that human beings, animals, plants and the environment are protected from damage and impairment.

For the decision on whether a safe disposal is possible, the organism count after the inactivation must be quantifiable and be measured. The methodological approaches for determining the efficacy are presented in Chapters 4 and 5.

Consequently:

From the microbiological viewpoint, chemically inactivated liquids are safely disposable into the environment if in the disposal - according to ContainO[44] - human beings, animals, plants and the environment are protected from damage and impairment.
The exposure to chemicals must meet the legal requirements of the Waters Protection Act.

## 7.4 Harmless disposal as waste water

The disposal of liquid waste must comply with the provisions of the Waters Protection Act[45] and the Waters Protection Ordinance[46]. This concerns – besides the exposure to chemicals – principally two aspects:

1. Dilution
2. Exceeding the pH.

To 1.) Polluted waste water must neither be diluted nor mixed with other waste water in order to comply with the requirements. Dilution or mixing is permitted if this is appropriate for the treatment of the waste water and as a result no more potential water pollutants are discharged than would be the case if the waste waters were treated separately (WPO, Annex 3.2, no. 1, para. 2, let. b).

To 2.) For an inactivation with NaOH the pH of the resulting waste water must not exceed 9.0 when discharged into the public sewers. See WPO, Annex 3.2, no. 2, no. 1.

In regard to the chemical load, the two following special cases are to be considered in regard to the disposal of chemically inactivated liquids containing organisms:

1. Antibiotic residues in media supernatants
2. Chlorinated organic waste or chlorination of organic substances with chlorine bleach[47]
3. Residual amounts of active chlorine.

In specific cases of chemical inactivation, it has to be checked whether the disposal of the liquids is required as chemical hazardous waste according to the provisions of the OMW (OMW Art. 4 to 7 and Annex 1).

---

44 ContainO, Art. 1 Aim: This Ordinance is intended to protect human beings, animals and the environment, as well as biological diversity and its sustainable use, from hazards or harm caused by handling organisms, their metabolic products and wastes in contained systems.

45 Federal Act of 24 January 1991 on the Protection of Waters; Waters Protection Act; (WPA) SR 814.20

46 Waters Protection Ordinance (WPO) of 28 October 1998; SR 814.201

47 Aqueous solution of sodium hypochlorite (NaClO)

# 8 Safety of use

The safety in use involves the following aspects:

1. Occupational safety and health protection
2. Environmental protection
3. Protection against fire and explosion
4. Safety labels
5. Critical factors (storage life).

Fundamentally, only approved disinfectants may be used in Switzerland.[48] This also applies to products imported from abroad.

The inactivating substances in disinfectants come from quite different chemical substance groups. They are: aldehydes, aldehyde cleavers, alcohols, alkylamines or alkylamine derivatives, amphoteric surfactants, chlorine-, bromine-, iodine-cleaving compounds, chloramines, glycol derivatives, guanidine or guanidine derivatives, caustic solutions, peroxide compounds, phenols, phenol derivatives, phenol ethers, pyridine derivatives, quaternary compounds[49], mineral acids, organic acids and heavy metal compounds.[50] Some substances from these material groups are strongly damaging to health and/or the environment and can be hazardous to humans and the environment. When used incorrectly, alcohols present a risk of fire and explosion. If at all possible, disinfectants that contain chlorine and phenols should be avoided for reasons of health and environmental protection. For the same reasons, aldehydes should only be used when there are no equivalent alternatives.

The safety label[51] of a product offers some guidance for a preliminary evaluation when selecting an agent for chemical inactivation. In this respect, it should be borne in mind that the label refers to the product in the container. This means that a disinfectant without hazardous substance labelling and used undiluted may, in certain circumstances, be more harmful to health and/or to the environment than an agent that exhibits a hazardous substance label, but is used when highly diluted.[52] An in-depth evaluation requires an understanding of both human- and eco-toxicology.

Depending on the field of application, the substances need to be tested for skin and mucous membrane compatibility. They should possess no or a low acute oral, dermal or inhalation toxicity.

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48 http://www.bag.admin.ch/anmeldestelle/13604/13869/13880/index.html?lang=de; BAG Startseite > Biozidprodukt > Desinfektionsmittel

49 N.B. Quaternary compounds in disinfectants may contribute to the development of resistance to antibiotics.

50 Verbund für Angewandte Hygiene (VAH) List of Disinfectants, status: 1. April 2014; ©mhp-Verlag 2015; http://www.vah-online.de/uploads/PDF/vorwort_deutsch_mhp.pdf

51 The safety symbols are described in detail in the SUVA leaflet “Safety labelling” (order number 44007.D). (PDF)

52 Indications on safe application: when diluting – particularly with large volumes - ensure that the solution is thoroughly mixed.

# 9 Conclusions

Laboratories or establishments which want to chemically inactivate liquid waste and then dispose of it via waste water need to fulfil the following conditions:

1. Contaminated liquid waste may be chemically inactivated and disposed of via waste water if the efficacy of the inactivation is comparable with the result obtained by autoclaving.
2. The standardised inactivation conditions for worst-case situations are defined and documented in a standard operating procedure (SOP).
3. The order of magnitude of the maximum possible organism count (quantitative upper limit and concentration) must be known *before* and *after* the inactivation.
4. A risk assessment shall demonstrate that a discharge of possibly incompletely inactivated organisms via waste water would not endanger and impair human beings, animals and the environment.

Total inactivation, as is achieved by definition with autoclave sterilisation, may certainly be envisaged for the chemical inactivation, but is ultimately not practically provable. This can be explained by the fact that an overkill rate, as can be extrapolated for the steam inactivation (see Figure 12), cannot or hardly be calculated for the chemical disinfection, because in most cases no mathematically derivable inactivation kinetics exist. Consequently, in practice, the objective of a total inactivation with chemical agents can only be indirectly proven.

The overkill rate in the inactivation with chemicals may be defined in such a way that a safe exposure time is accepted to be four-times the time that is needed for the inactivation to reach the extrapolated zero growth.

As a formula:

$t_{SI}$ (exposure time for safe inactivation) $= 4\, t_{exN}$ (exposure time to extrapolated zero growth)

The exposure time to the extrapolated zero growth ($t_{exN}$) is derived, based on the inactivation progress, from the initial organism count, down to the detection limit of the organisms.

Of great significance to the context-sensitive risk assessment is the demonstration that the disposal of liquid waste after chemical inactivation is possible without endangering humans, animals and the environment. Enforcement provisions of the WPO require that a proof of efficacy for the applied chemical inactivation of liquid waste be submitted and has to demonstrate that human beings, animals and the environment are protected from damage and impairment resulting from the possible remaining amount and concentration of the organisms. Autoclaving under standard conditions (see Chapters 1.2 and 6.2) serves as a yardstick for the total inactivation for the safe disposal of waste.

In the case where one is unwilling or unable to spend the required time and effort to provide the proof of efficacy and the risk assessment, then the method of choice for the inactivation of organisms in liquids would continue to be autoclaving. On the grounds of efficacy and environmental impact, priority is to be given to autoclaving. When comparing the time and effort spent to choose the disinfectant as well as for the proof of efficacy for a chemical inactivation process, it is generally the case that the autoclaving process is the simpler alternative.

# 10 Definitions, Standards and supplementary documents

## 10.1 Definitions

Regarding the definitions in the Standard EN 12740:1999

| | Term | Definition |
|---|---|---|
| 1. | Autoclaving | Conditions of a temperature of ≥121 °C and ≥1.05 barG pressure for ≥15 minutes meet the SAL of $10^{-6}$ for the vast majority of organisms. |
| 2. | Decontamination (EN-Standard*) | Elimination of microbial contamination or reduction to an *acceptable level*. |
| | *Comment* | *The term "acceptable level" requires a more precise definition.* |
| 3. | Disinfectant (EN-Standard*) | Chemical agent that is capable *of reducing* the number of viable micro-organisms. |
| 4. | Disinfection (EN-Standard*) | Process for reducing the number of viable micro-organisms with the aid of various physical and chemical methods. |
| | *Comment* | *In the field of hygiene disinfection means the killing or inactivation of pathogenic micro-organisms, such that they no longer present any danger. It is a means for the targeted reduction of the bacterial count which does not normally lead to sterility.* |
| 5. | Decimal reduction value (D-value) | The decimal reduction value states the required time in minutes at a given temperature to lower the bacterial count by one power of ten, which corresponds to a kill rate of 90%. |
| 6. | Inactivation (EN-Standard*) | *Partial or total* destruction of a given activity until the destruction of the microbiological system. |
| | *Comment* | *Only "**total inactivation**" means the absence of infectious and transferable genetic material (plasmids, RNA)* |
| 7. | Micro-organism (EN-Standard*) | Cellular and non-cellular microbiological entity capable of replication or of transferring genetic material. |
| 8. | Micro-organism WPO, Art 3, let. b) | Microbiological entities, in particular bacteria, algae, fungi, protozoa, viruses and viroids; cell cultures, parasites, prions and biologically active genetic material are also regarded as micro-organisms. |
| 9. | Sterile (EN-Standard*) | A state free of viable micro-organisms. |
| | *Comment* | *See REMARK 1[53] and REMARK 2[54]* |
| 10. | Sterilisation | Process to reach a sterile state. |

[53] *REMARK 1: in practice such a requirement of absolute absence of viable micro-organisms cannot be met. However, the "sterile" state may be considered proven by the use of approved or generally recognised procedures for sterilisation.*

[54] *REMARK 2: The process of inactivating viable micro-organisms during the course of sterilisation is usually described by an empirical mathematical function, usually by an exponential function. Mathematically, such functions can be reduced to very small values, but never to zero. However, these empirical functions can be used to monitor or to evaluate the process parameters of a sterilisation procedure in order to establish a target level of inactivation of viable micro-organisms.*

| | Term | Definition |
|---|---|---|
| | (EN-Standard*) | |
| | *Comment* | *Sterilisation refers to viable organisms, whereas inactivation also includes non-cellular biological agents.* |
| 11. | Sterility Assurance Level (SAL) | A Sterility Assurance Level of $10^{-6}$ is required for the definition of "sterile", and means the probability that a maximum of one micro-organism capable of reproduction is contained in one million equally treated entities of the bioburden. |
| 12. | Validation (EN-Standard*) | Documented procedure for recording and evaluating results that are used to prove that a process continuously yields a product that complies with the stipulated properties. |
| 13. | | Validation is a documented procedure for providing, recording and interpreting results that are required in order to establish the statement that a process always affords products that comply with the stipulated specifications (SN EN ISO 17665-1, 3.60). |

* Definitions according to Standard EN 12740:1999-10[55]

## 10.2 Selected Standards in the field of disinfection

Biotechnology - Laboratories for research, development and analysis - Guidance for handling, inactivating and testing of waste; German version

DIN EN 13610: Chemical disinfectants - Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas. Test method and requirements (phase 2, step 1); European Committee for Standardization (CEN) 2003

DIN EN 14476: Chemical disinfectants and antiseptics - Virucidal quantitative suspension test for chemical disinfectants and antiseptics used in human medicine - Test method and requirements (phase 2, step 1). European Committee for Standardization (CEN) 2005

DIN EN 14675: Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 2, step 2). European Committee for Standardization (CEN) 2006

55 EN Standard 12740:1999: This Standard gives guidance on methods for handling, inactivating and testing of waste containing organisms arising from biotechnology laboratory activities and processes. It is concerned with methods to reduce the risks arising from exposure to waste derived from laboratory-scale activities which contains organisms hazardous or potentially hazardous to humans, animals, plants or the environment. Such waste may include organisms whether as solid, liquid or gaseous by-products or effluent, together with items or equipment required to be disposed of and which may be contaminated with organisms. Wastes may be generated by biotechnology, clinical, molecular biology, microbiology and other laboratories in activities where organisms are handled, genetically modified organisms are created or used or by laboratory processes involving material of human, animal or plant origin. This European Standard does not apply to other types of waste from human healthcare or other medical treatment activities.

DIN EN 14885: Application of European Standards for chemical disinfectants and antiseptics 2007-01. European Committee for Standardization (CEN) 2006

DVG. Guidelines for testing chemical disinfectants. In: e. V. DVG, ed. Verlag der Deutschen Veterinärmedizinischen Gesellschaft e. V. Gießen; 2007

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*Aktueller Stand zur Viruzidieprüfung – ein Überblick Hygiene & Medizin 37 (7/8)*; Ingeborg Schwebke; Holger F. Rabenau (2012) http://nbn-resolving.de/urn:nbn:de:0257-10026059 oder http://www.rki.de/DE/Content/Infekt/Krankenhaushygiene/Desinfektionsmittel/Virusinaktivierung/Viruzidiepruefung.pdf?__blob=publicationFile

*Desinfektionsmittelliste des Verbund für Angewandte Hygiene (VAH)*, Stand: 1. April 2014; ©mhp-Verlag 2015; http://www.vah-online.de/uploads/PDF/vorwort_deutsch_mhp.pdf

*Leitlinie der Deutschen Vereinigung zur Bekämpfung der Viruskrankheiten (DVV) und des Robert Koch-Instituts (RKI) zur Prüfung von chemischen Desinfektionsmitteln auf Wirksamkeit gegen Viren in der Humanmedizin*; Fassung vom 1. August 2008; Seite 941; DOI 10.1007/s00103-008-0615-5)

*„Liste der vom Robert Koch-Institut geprüften und anerkannten Desinfektionsmittel und -verfahren";* Online Bezugsquelle: http://www.rki.de/DE/Content/Infekt/Krankenhaushygiene/Desinfektionsmittel/Desinfektionsmittelliste.pdf?__blob=publicationFile

# Annex

## Guidelines for the validation and application of alternative inactivation methods to heat inactivation using an autoclave

(01/07/2016/v21); 2016; Kantonales Laboratorium Basel-Stadt;

Contracting body: Federal Office of Public Health FOPH

# Guidelines for the validation and application of alternative inactivation methods to heat inactivation using an autoclave

*Auftraggeber*:
Bundesamt für Gesundheit BAG

*Auftragnehmer*:
Kantonales Laboratorium Basel-Stadt (Vertrag: "Nachweis von gefährlichen Organismen im Zusammenhang mit ESV-relevantenTätigkeiten und B-Ereignissen", Vertrag-Nr. 14.008571 /604.0001 /-398)

*Datum/Version:*
01/07/2016/v21

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# 2. Introduction

## 2.1 Context

When handling organisms in a laboratory, liquid and solid cultures containing living organisms are generated. According to the Containment Ordinance (CO[1]), disposal of these cultures has to be performed in such a way that they do not represent a hazard to the population or the environment after being released from the 'contained' system into the sewage or the public waste disposal system. The preferred method of liquid and solid culture inactivation is heat inactivation using an autoclave. However, the CO allows the inactivation and disposal of solid cultures by external companies. Liquid cultures may be inactivated using alternative inactivation processes. Nevertheless, the commentaries on the CO state that the alternative process needs to be tested and validated by the user and must be approved by the federal authorities. Federal authorities are receiving increasing numbers of applications for approval, since autoclaves are expensive and alternative inactivation processes at a first glance can appear easier and more cost effective. However, there are many aspects and parameters to consider for a proper validation and the routine application of these methods.

## 2.2 Aim of this manual

In this manual, we aim to provide guidelines for the validation and (routine) application of alternative inactivation methods. This is essential, because various factors may determine the success of both the validation and the application of alternative inactivation methods. This manual will outline challenges during the validation process and highlight specific minimal requirements that should be fulfilled. The manual is intended to support laboratory users and the cantonal and federal authorities in deciding whether certain minimal requirements are met.

## 2.3 Definition of important terms and fundamentals

In order to understand the basics of inactivation, we will first provide an overview of important terms used in combination with the inactivation of organisms if not provided in the CO[1]. The overall aim of inactivation is to obtain a sterile product, which can be disposed of safely.

**Inactivation:** Partial or full destruction of a given activity up to destruction of the microbiological system[2].

**Sterile:** State of being free from viable microorganisms[2]. (See also section of sterility assurance level: SAL).

**Sterilization:** Process used to reach a sterile product[2].

**Disinfection:** Process of reducing the number of viable microorganisms by various physical and chemical methods. Disinfection usually does not lead to sterility[3].

**Decontamination:** Removal of microbiological contamination or reduction to an acceptable level[3].

[1] Ordinance on Handling Organisms in Contained Systems (Containment Ordinance); legally binding version: Verordnung über den Umgang mit Organismen in geschlossenen Systemen vom 9. Mai 2012 (Stand am 1. Juni 2015)

[2] Biotechnology – Laboratories for research, development and analysis. Guidance for handling, inactivation and testing of waste (EN12740:1999)

[3] See footnote 2

**Validation**: Documented procedure for obtaining, recording and interpreting the results needed to show that a process will constantly yield a product complying with pre-determined specifications[3].

**Killing kinetic**

A killing kinetic curve results from the connection of data points obtained by measuring the number of surviving organisms after a specific incubation time or radiation dose exposed to a specific inactivation method.

The example shows surviving *Geobacillus stearothermophilus* spores and vegetative *Staphylococcus aureus* after autoclaving for specific time intervals. In these cases, the killing kinetic curves are linear and therefore the D-value can be defined as the time to reduce the number of a specific microorganism by 1 log cycle i.e. $N_0/N_t = 10$. The D-value is specific to a temperature. When indicating D-values for an organism, the temperature at which the organism was tested must also be stated. This is usually done as a subscript of 'D' (Figure 1).

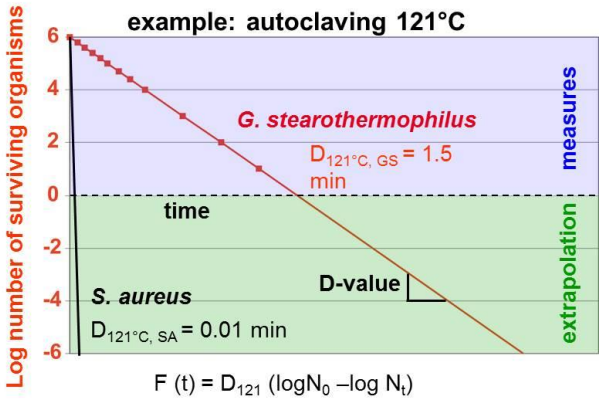


Figure 1: Killing kinetic of *G. stearothermophilus* spores and vegetative *S. aureus* inactivated by autoclaving at 121°C. Over time, samples are taken and cultured to monitor the log number of surviving organisms. The D-value is defined as the time it takes to reduce the number of organisms by 1 log cycle. The figure also shows that spores of *G. stearothermophilus* are more resistant to autoclaving than *S. aureus*, as indicated by a less steep killing kinetic curve.

**Sterility assurance level (SAL)**

Sterility means the absence of all viable microorganisms including viruses (Sharp 1995). At present, a sterility assurance level (SAL) of $10^{-6}$ is desired for sterilization procedures, i.e., a probability of not more than one viable microorganism in an amount of one million sterilized items of the final product. Since a SAL of $10^{-6}$ is difficult to measure experimentally, model situations need to be created, with the help of which conclusions can be drawn regarding the treatment conditions necessary to attain sterility meeting the SAL of $10^{-6}$ (see below). These are done using the reduction rate (D-value: Figure 1).

By extrapolating the reduction after sterilization, a theoretical overall performance of the procedure of 12 log increments (overkill conditions) is demanded to verify an SAL of $10^{-6}$ (see Figure 1). By comparison, other recommendations for thermal sterilization procedures demand only evidence that the difference between the initial contamination and the number of test organisms at the end of the process amount to more than six orders of magnitude. A practical proof of the required level of sterility assurance of $10^{-6}$ is not possible. For more details on sterility assurance levels, see von Woedtke and Kramer (2008).

**Deviations from the sterility concept**

Theoretically, when using non-thermic inactivation methods (anything other than the autoclave), killing kinetic curves are not linear and result in a so called 'tailing' (Lambert and Johnston 2000; Kramer and Assadian 2008). There are many hypotheses for this, but one

reasonable explanation for the tailing effect of killing kinetic curves with chemical disinfectants is 'quenching' i.e. the depletion of the disinfectant resulting in a reduced concentration (Johnston et al. 2000). In some cases, e.g. when excess chemical is used, nearly linear killing kinetic curves can be achieved. Nevertheless, it is important to note that a SAL of $10^{-6}$ cannot be guaranteed for the inactivation methods with non-linear killing kinetic curves.

**Biological indicators**

A biological indicator is a characterized preparation of a specific microorganism with a defined concentration that provides a defined and stable resistance to a specific inactivation process. If the inactivation resistance to a specific process is not known, it has to be demonstrated that it is more resistant than the organisms present in the unit of material to be sterilized. Biological indicators are usually viewed as a model for biological load to simulate the most resistant biological indicator organism[4]. Spore-forming microorganisms (e.g. *G. stearothermophilus, Bacillus atrophaeus* and *B. pumilus*) are widely used as biological indicators, because these microorganisms are highly resistant to inactivation compared to other microorganisms (Figure 2). The inactivation resistance hierarchy of microorganisms depicted below represents a rough guide and may also depend on the inactivation method.

There are three conditions for the use of an organism as biological indicator:

1. The inactivation resistance of the specific microorganism should be as high as possible towards the inactivation method. The inactivation of such highly resistant microorganisms encompasses all less resistant organisms, including most pathogens.
2. The specific microorganism ideally should not be pathogenic.
3. The specific microorganism needs to be culturable without great effort.

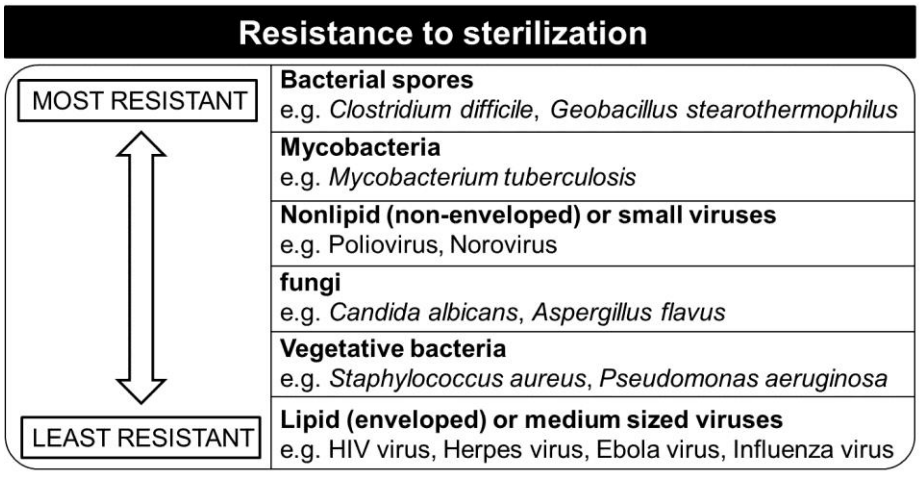


Figure 2: Order of inactivation resistance. This hierarchy considers broad classifications of microbial categories and it is considered a rough guide to general susceptibility patterns of microorganisms to sterilization. The hierarchy may depend on the inactivation method. Figure adapted from Favero and Bond (1991).

A biological indicator can be used to assist the process development and process validation steps of an alternative inactivation method (Figure 3) or to assess the performance of the inactivation equipment during routine monitoring.

[4] For more information on requirements for biological indicators, see EN 11138: Sterilization of health care products - Biological indicators - Part 1: General requirements (ISO 11138-1:2006)

**Types of biological indicators**
There are three types of biological indicators. Each type incorporates a known species of a microorganism of a known inactivation resistance.
***Type 1***: Spores that are already added to a carrier (disk, strip, filter paper, glass, plastic or other material), packaged to maintain integrity and viability of the inoculated carrier. Carriers should not be degraded by the inactivation process. This type of biological indicator mimics a surface and can be used to validate surface sterilization processes.
***Type 2***: Spore suspensions consist of a known concentration and are directly inoculated on or into a unit of material to be sterilized. The stock spore suspension contains dormant (non-germinating) spores and is held in a non-nutritive liquid, e.g. 40% ethanol. Spore suspensions are probably the best option for validating the efficacy of chemical disinfectants.
***Type 3***: Self-contained indicators consist of a spore suspension in its own medium in ampules. They often contain a dye, which indicates positive or negative growth following incubation. This type is not suitable for testing chemical inactivation methods, because the chemical disinfectant cannot penetrate the ampules (no contact with the biological indicator).

*G. stearothermophilus, B. atrophaeus* and *B. pumilus* spores can be purchased from different companies, e.g. BAG Healthcare[5].

**Chemical indicators and physical sensors[6,7]**
Chemical indicators and physical sensors are used to monitor the inactivation process. Chemical indicators are designed to respond with a characteristic chemical change to one or more of the physical conditions during the inactivation process. In contrast, physical sensors are usually a piece of equipment (e.g. pH meter) indicating that that a certain condition has been fulfilled. Consequently, the chemical indicator/physical sensor is process-specific and each inactivation method requires the use of different chemical indicators or physical sensors. For example, when using an acid for inactivation, it is not possible to use the same chemical indicator for acids as for bases (Table 1). Chemical indicators are designed to detect potential inactivation failures, but they do not test for sterility.

Chemical indicators have been classified by the Food and Drug Administration (FDA) into six classes depending on their action.

***Class I:*** process indicators. They are intended to be used with individual items to demonstrate that the items have been exposed to the inactivation process and to distinguish between processed and unprocessed items. An example is autoclave tape.
***Class II:*** dynamic air removal test (formerly called the Bowie-Dick test)
***Class III:*** single parameter indicators (measure only one of the parameters of the inactivation process)
***Class IV***: multi-parameter indicators. These indicators reveal a change in one or more predefined process parameters based on a chemical or physical change resulting from exposure to a process. They provide much more information about the inactivation cycle than a Class III indicator.
***Class V:*** chemical indicators. These are designed to react to all of the critical parameters over a specified range of inactivation cycles. Their performance has been correlated to the performance of the relevant biological indicator under the labeled conditions of use. This means that chemical integrators closely resemble biological indicators, but cannot be used to replace them.

[5] http://www.bag-healthcare.com/hygiene-monitoring/sterilisationsindikatoren/biologische-indikatoren
http://www.spdceus.com/monitoring_sterilization_process.htm
http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1035.html

[6] For a good overview of chemical indicators, see: http://www.sterislifesciences.com/Products/Process-Indicators/Chemical-Indicators.aspx

[7] For more information on requirements for chemical indicators, see EN 11140: Sterilization of health care products - Chemical indicators - Part 1: General requirements (ISO 11140-1:2005)

***Class VI:*** Emulating Indicators. These are designed to confirm the presence or absence of specific time and temperature parameters during a cycle, and integrate all the critical parameters of the inactivation cycle (temperature, saturated steam and exposure time).

**Interfering factors**

Several physical and chemical factors influence the efficacy of an inactivation method. These include, for example external factors such as temperature, humidity or light exposure, and internal factors such as pH, water hardness or organic load (Cremieux 1986). Depending on the inactivation process, there is a specific set of interfering factors which applies. In general, the activity of inactivation methods increases as the temperature increases, but there is a point at which e.g. chemical inactivation agents can degrade, if heated too much. The temperature at which chemical inactivation agents degrade can be defined as an exclusion factor. Concerning pH, there are different mechanisms. For example, an increased pH improves the antimicrobial activity of agents containing aldehydes (Table 1), but decreases the activity of others such as hypochlorites. Relative humidity influences the activity of gaseous agents such as ethylene oxide. Water hardness reduces the reduction rate of some chemical inactivation processes, because divalent cations (e.g. $Mg^{2+}$ and $Ca^{2+}$) interact with soap to form insoluble precipitates.

Organic load (proteins) such as serum, blood or fecal material may interfere with the activity of the inactivation process in at least two ways: First, a chemical reaction between the disinfectant and the organic load may result in a complex that is less germicidal or non-germicidal leaving less of the active agent to attack the microorganisms. Secondly, organic material may protect microorganisms from attack by acting as a physical barrier. For the same reason, another interfering factor can be biofilm formation. Biofilms are formed by bacteria and fungi and their presence make it more difficult disinfectants to penetrate the microbial matrix.

# 3. Steps during the validation process of alternative inactivation methods

## 3.1 Work flow for validation and routine application

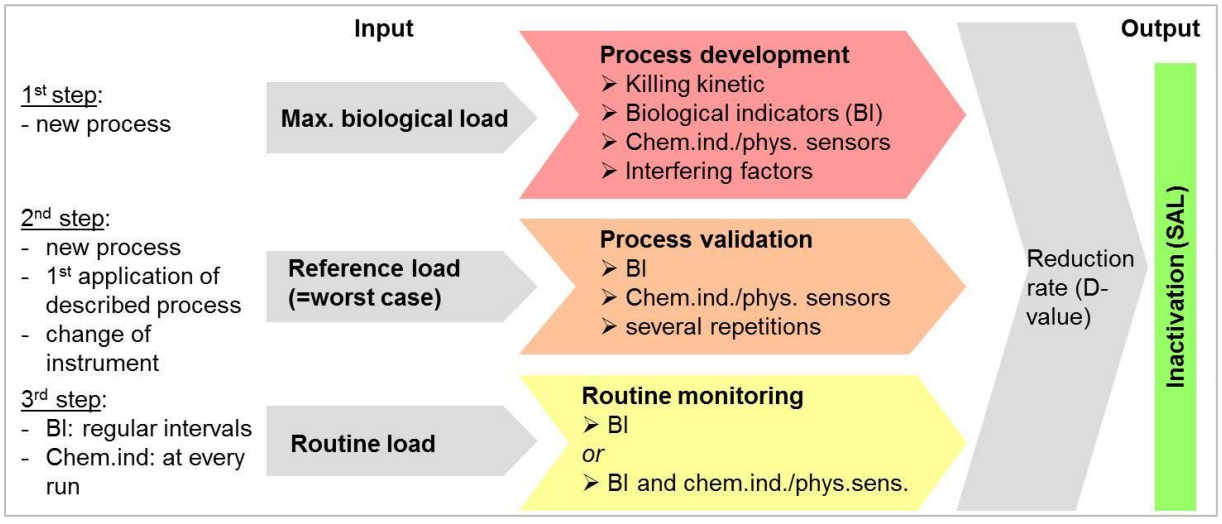


Figure 3: Workflow and involved factors during process development, process validation and routine application of inactivation methods.

**1st step: Process development**
During the process development of a new process phase, it is important to assess the killing kinetic curve (Figure 1) of the inactivation method using the maximal biological load. Suitable biological indicators (see 1.2) should be used to demonstrate that the process is leading to the desired reduction rate (e.g. SAL of $10^{-6}$). Suitable chemical indicators and/or physical sensors should be used to monitor the chemical/physical inactivation conditions. Different interfering factors (see 1.2, 5.1.2 and 5.4.2) need to be assessed. Process development only needs to be done once for an entirely new inactivation method. The result of the process development phase is a definition of the worst case scenario which is based on the worst possible combination of interfering factors. Therefore, all interfering factors first need to be identified during this step, although discovering all possible interfering factors is not always feasible. Using this worst case scenario, the validation process can be performed for new processes.

**2nd step: Process validation**
During the process validation, a reference load (=worst case scenario) is tested with biological indicators. The physical and chemical conditions are also monitored. Process validation needs to be done for new processes or first-time applications of a previously described (published) process. It is especially done when a new setup or a new instrument has been purchased.

**3rd step: Routine monitoring**
During routine monitoring of an inactivation method, a routine load is inactivated and biological and chemical indicators are used to assure functionality of the equipment and verify the physical and chemical conditions. Especially chemical indicators or physical sensors are used at every inactivation cycle. Inactivation of biological indicators needs to be verified at regular intervals.

## 3.2 Factors affecting the validation process

During all steps of process development and validation, the success of the inactivation method depends on whether **liquids or solids** are being inactivated. Therefore, different systems and different requirements are valid for solid and liquid wastes and these parameters need to be verified. The biological indicators and chemical indicators

Since microorganisms also differ in their inactivation resistance (Figure 2), it is necessary to keep in mind **what types of organisms** are being inactivated: **bacteria, spores, viruses or fungi.** The parameters used during process validation will differ depending on the organism. It is also important to keep in mind that published D-values are always organism and inactivation method specific and results are not transferable. Therefore, D-values must be determined for each species and at varying conditions considering interfering factors.

During all validation steps, the following treatment parameters may need to be monitored:
**For most inactivation methods:**

- Exposure time
- Volume of unit

**For some inactivation methods:**

- Concentration: important for chemical inactivation agents
- Temperature: important for chemical inactivation agents, but not so important for e.g. UV
- Pressure: important for gaseous agents
- Surface texture: important when sterilizing surfaces

# 4. Alternative inactivation methods

The selection of an appropriate alternative inactivation method may be determined by a number of criteria. These include:

1. Effectiveness of the alternative inactivation method
2. Applicability of the method to different organisms and media (solids or liquids)
3. Detoxification requirements and lack of toxic byproduct formation
4. Occupational and environmental hazards associated with the alternative inactivation method
5. Ease of handling and application
6. Operational costs

In this chapter, we provide a catalogue of different alternative inactivation methods considering the criteria mentioned above.

Table 1: Different inactivation methods and their mode of action, cost and advantage/disadvantage.

| | Method | Active component | Spec-trum[8] | Activity range | Mechanism | Known inter-fering factors | Combination options | costs | Comments<br>Environmental/safety concerns |
|---|---|---|---|---|---|---|---|---|---|
| Physical approaches | **Autoclave** | Hot water vapor | Ba, Vi, Fu, Sp, * | 100% rF | Denaturation | Not suitable for large volumes | not necessary | $$$ | High energy costs, not suitable for large volumes |
| | **Ultraviolet Light (UV)** | Energy-rich photons | Ba, Vi, Fu, Sp, * | 250-300nm, 400J/m2 | Physical DNA damage | Turbidity (tailing effect) | all | $$ | High energy costs, not effective against Listeria and Ameba |
| | **Micro-/Ultra-Filtration** | Sand filter, plastic filter membranes | Ba, Vi, Fu, Sp, * | NA | Mechanic separation | Turbidity, filters can clog | all | $$ | Disposal of filter necessary, spectrum depends on pore size of filter. Spores are very small, only recommended when com-bined with other approaches, only small volumes |
| | **Ultrasound** | Low frequency Ultra-sound (20-35kHz) | Ba, Vi, Fu, Sp, * | 500-5000 J/L | Cavitation, mechanic shearing forces on cells | | all | $$ | High energy costs, only for combined use |

[8] Abbreviations: Ba: bacteria, Vi: viruses, Sp: spores, Fu: fungi, *: reduced or no efficacy towards Prions

| | Method | Active component | Spectrum[8] | Activity range | Mechanism | Known interfering factors | Combination options | costs | Comments<br>Environmental/safety concerns |
|---|---|---|---|---|---|---|---|---|---|
| | **Ionized radiation** | β- and γ-radiation ($^{60}Co$, $^{137}Cs$) | Ba, Vi, Fu, Sp, * | $10^4$ J/m$^2$ | Protein and DNA damage | | NA | $$$ | Radiation emitters are very expensive and process generates radioactive waste |
| Chemical approaches | **Ozone** | $O_3$, $H_2O_2$ | Ba, Vi, Fu, Sp, * | 0.1mg/L | Oxidation | Organic compounds | UV | $$$ | Requires a lot of energy, generates toxic compounds, formation of hxdroxyl-radicals in water, no storage possible, residual $O_3$ has to be removed |
| | **Chlorine** (halogens) | $Cl_2$, NaClO, $Ca(ClO)_2$, $ClO_2$ | Ba, Vi, Fu, Sp, * | 1-5% | Oxidation | Organic and reactive compounds, light | filtration, Ultrasound, UV | $ | unstable, not biodegradable, corrosive |
| | **Bases** | NaOH | Ba, Vi, Fu, Sp, Prions | >1 Molar | Protein denaturation | Acids (neutralization) | heat, UV, filtration, chem. | $ | Not effective against Mycobacteria, low pollution potential, can be neutralized using acids, significant safety concern for user when handling product |
| | **Acids** | $H_2SO_4$, $C_6H_8O_7$, | Ba, Vi, Fu, Sp, * | 1 Molar | Protein denaturation | Bases (neutralization) | heat, UV, filtration, chem. | $ | Low pollution potential, can be neutralized using bases, significant safety concern for user when handling product |
| | **Aldehyde** | Formaldehyde, Glutaraldehyde, Glyoxal | Ba, Vi, Fu, Sp, * | 0.5-5% | Protein denaturation | Development of resistance | heat, UV, filtration, chem. | $ | Stable, non-polluting, some compounds carcinogenic, irritating to mucosa |
| | **Per-compounds** | $H_2O_2$, Peracetic acid (PAA), Potassium-peroxomonosulfate | Ba, Vi, Fu, Sp, * | 0.02-35% | Oxidation | Unstable compounds, activity reduced by catalases and peroxidases | heat, UV, filtration | $ | Biodegradable, broad spectrum of activity |
| | **Phenol-derivatives** | P-Chlor-m-Kresol, p-Chlor-m-xylenol, o-Phenyl-phenol | Ba, Vi, Fu, * | 0.1-5% | Protein denaturation | Organic compounds | Heat, UV, filtration, chem | $ | Safety hazard (neurotoxicity, carcinogenic), not biodegradable and toxic, not recommended. |
| | **quaternary ammonium compounds** | Benzalkonium chloride, Cetylpyridinium chloride, Didecyl-dimethyl-ammonium chloride | Ba, Vi, Fu, * | 2-7% | Surface active, partially denaturing | Organic compounds, anionic soaps, $CaCO_3$, iron | UV filtration, chem. Ultrasound | $ | Not biodegradable, "esterquats" are more easily biodegradable, especially the fatty acids |

| | Method | Active component | Spectrum[8] | Activity range | Mechanism | Known interfering factors | Combination costs options | Comments<br>Environmental/safety concerns |
|---|---|---|---|---|---|---|---|---|
| Combined approaches | **Advanced Oxidation** | UV (182nm), Ozone, $H_2O_2$, catalysators | Ba, Vi, Fu, Sp, * | | Cleavage of $H_2O_2$ to hxdroxyl-radicals | See individual approaches | Is combined | Superior to individual approaches, optimization of energy and chemical input, reduction of chlorine concentration possible |
| | **Ultrasound + chlorine** | Ultrasound first or simultaneously | Ba, Vi, Sp | | See individual approaches | See individual approaches | Is combined | Superior to individual approaches, optimization of energy and chemical input |
| | **Ultrasound + UV** | Ultrasound first for reduction of turbidity, UV penetration more effective | Ba, Vi, Fu, Sp | | See individual approaches | See individual approaches | Is combined | Superior to individual approaches, optimization of energy and chemical input, tailing-effect reduced. UV dose or exposure time can be reduced |
| | **Combination of different chemical approaches** | Different combinations possible | Improved spectrum | | See individual approaches | See individual approaches | Is combined | Additive, synergistic and antagonistic interactions possible, broader spectrum of activity towards different organisms |
| | **UV + chemical approaches** | UV first or simultaneously | Improved spectrum | | See individual approaches | See individual approaches | Is combined | Superior to individual approaches, optimization of energy and chemical input |
| | **Chemo-thermic disinfection** | NaOH, heat and chlorine | Improved spectrum | | See above (individual approaches) | See above (individual approaches) | Is combined | Chemical doses can be reduced by heat. |

# 5. Conclusion

## 5.1 Minimal requirements

- Determination of a worst-case scenario for the unit to be inactivated (BI or suitable test organism with high inactivation resistance, interfering factors present in the unit).
- Process validation (Figure 3, step 2) needs to be performed for all alternative inactivation methods and for new equipment using the worst-case scenario. Step 1 can be omitted for published killing kinetic curves for BI using known chemical indicators.
- If killing kinetic curves of an inactivation method are not linear, it has to be demonstrated that no organism of a $10^8$ CFU/PFU suspension of a BI survives (this is just a suggestion…).
- If a waste contains several different microorganisms, inactivation data need to be obtained for each of the biological agents of concern using the identical conditions.

## 5.2 Critical factors for process validation

- Non-linear killing kinetic curves $\rightarrow$ SAL cannot be guaranteed
- Assessment of all possible interfering factors
- Availability of biological indicators for a given inactivation method
- Availability of chemical indicators for a given inactivation method

# 6. Examples for the validation of alternative inactivation methods

In order to outline in more detail the different steps of process validation and to highlight some challenges to be encountered when developing and validating a new alternative inactivation method, we provide experimental data for three alternative inactivation methods,
two chemical methods:

- sodium hydroxide (NaOH, see Chapter 6.1),
- free chlorine as in sodium hypochlorite (Javel) or sodium dichloroisocyanurate (NaDCC, see Chapter 6.4),

and one physical method:

- Ultraviolet light ($UVC_{254nm}$, see Chapter 6.2),
-

and one combination of two methods (NaOH and $UVC_{254nm}$, see Chapter 6.3).

We outline some strategies for process development and validation of these three inactivation methods.

Nevertheless, if you plan to use one of the same inactivation methods, our data do not replace process development or validation in your laboratory. Interfering factors and test organisms may vary leading to a different worst-case scenario in your laboratory.

## 6.1 NaOH for the inactivation of bacteria

Sodium hydroxide (NaOH, base, see Table 1) was used to inactivate two different organisms: bacterial spores of *G. stearothermophilus* (GS) and vegetative cultures of *Staphylococcus aureus* (SA). *G. stearothermophilus* spores were chosen, because they are also used as biological indicators and are very difficult to inactivate by autoclaving.

### 6.1.1 Inactivation resistance and killing kinetic

In a first step of process development, it is essential to assess the killing kinetic and inactivation resistance of both the biological indicator and the test organism to demonstrate that the biological indicator is more resistant to the inactivation process than the test organism. In our example with NaOH as chemical disinfectant, we first assessed the inactivation resistance of GS spores and vegetative SA. Different concentrations of NaOH were incubated with GS spores or vegetative SA for different time intervals, i.e. 1, 10, 20, 30, 40, 50, 60 and 120 minutes and tested for the number of surviving organisms. By 120 minutes, the number of surviving organisms of GS and SA was below the detection limit for the chosen sodium hydroxide concentrations (Figure 4). After incubation, the chemical disinfectant has to be neutralized in order to eliminate residual antimicrobial activity[9]. Since sodium hydroxide is a base, it can be neutralized with the acid HCl-HEPES. Neutralization (pH=7) was verified using pH-indicator strips.

For the validation of chemical disinfectants, a catalogue of potentially suitable neutralization media can be found in the corresponding European Norms (EN, see Chapter 7).

[9] **EN13727** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area.

After neutralization, the inactivated bacterial suspension is serially diluted and plated on the appropriate medium and enumerated.

Using this approach, we found that GS spores and vegetative SA exhibited a different inactivation resistance to sodium hydroxide (Figure 4). GS spores were more resistant than vegetative SA. A 0.1M solution was sufficient to inactivate vegetative SA cultures, but the GS spores required at least a 4M solution and a longer incubation time to reach the desired SAL of $10^{-6}$. Nevertheless, for the chosen organisms and within the tested range of sodium hydroxide concentrations, inactivation of GS and SA follows a linear killing kinetic. This means that an extrapolation is possible and the desired SAL of $10^{-6}$ is likely eventually reached. For GS this is after around 205 minutes and for SA around 130 minutes (Figure 4).

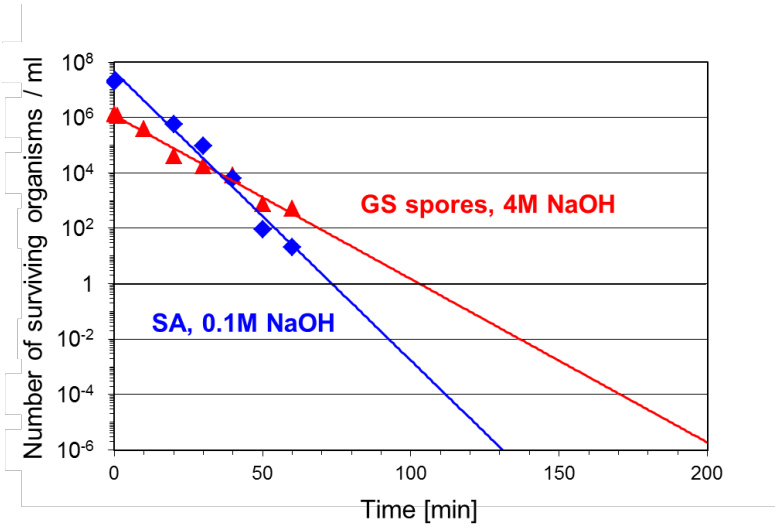


Figure 4: Linear killing kinetic.
Killing kinetic of the inactivation of *G. stearothermophilus* (GS, red) spores using 4M NaOH and vegetative *S. aureus* (SA, blue) using 0.1M NaOH. At the indicated time points a sample was removed and analysed for surviving bacterial entities. Plotting the number of surviving organisms in a logarithmic way, the killing kinetics are linear and can be extrapolated up to a SAL-value of $10^{-6}$.

### 6.1.2 Interfering factors of sodium hydroxide inactivation

In a second step of process development, different interfering factors for the inactivation with NaOH were tested. The first one was inactivation **temperature** (Figure 5). Depending on where the inactivation is performed (at room temperature in a laboratory or in a colder room of 10°C or 15°C), other killing kinetic curves are monitored. In the case of 2M NaOH solution, we showed that decreasing temperatures reduce the efficacy of the disinfectant as chemical inactivation method against GS spores and much longer incubation times (>800 minutes compared to around 300 minutes) are required to achieve the desired SAL of $10^{-6}$.

**Organic load** is another interfering factor. We simulated the presence of organic load in a test solution by the addition of bovine serum albumin (BSA) to a 2M NaOH solution (Figure 6). By the addition of 0.3% BSA, the incubation time is doubled (300 minutes compared to around 600 minutes), meaning that organic load also reduces the efficacy of sodium hydroxide inactivation.

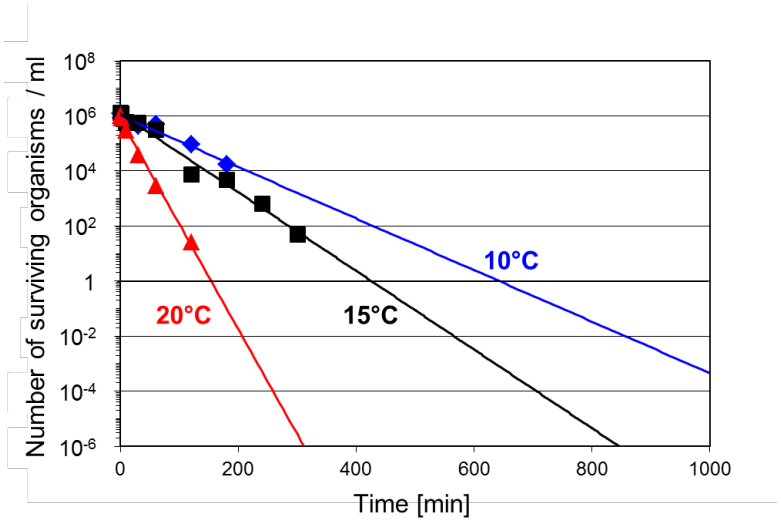


Figure 5: Influence factor "temperature".
Inactivation of GS spores using 2M NaOH at different temperatures as interfering factors. At the indicated time points a sample was removed and analysed for surviving bacterial entities. At the lower temperature of 15°C (black), an increased incubation time of just under threefold was needed to reach the SAL-value of $10^{-6}$ as compared to the higher temperature of 20°C (red).

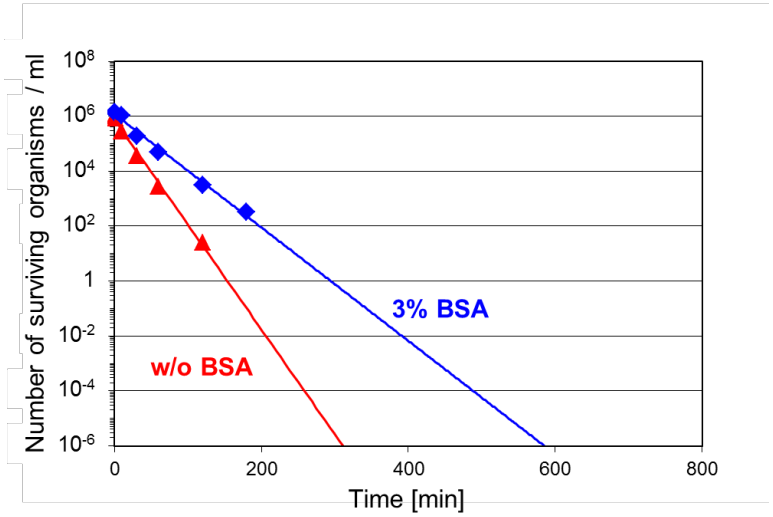


Figure 6: Influence factor "organic load".
Inactivation of GS spores using 2M NaOH (at 20°C) in the absence (red) or presence of organic load (3% BSA, blue). At the indicated time points a sample was removed and analysed for surviving bacterial entities. The reduction rate is reduced thus leading to a twofold increase in the required time to reach a SAL of $10^{-6}$.

### 6.1.3 Testing a worst-case scenario using GS spores

A worst-case scenario most likely consists of a combination of interfering factors. Below, we provide an experimental example for the cumulative effect of interfering factors (Figure 7). We tested organic load combined with a reduced temperature (15°C). Combined interfering factors (0.3% BSA and reduced temperature) slow down the inactivation process even more than when only one interfering factor such as temperature is present.

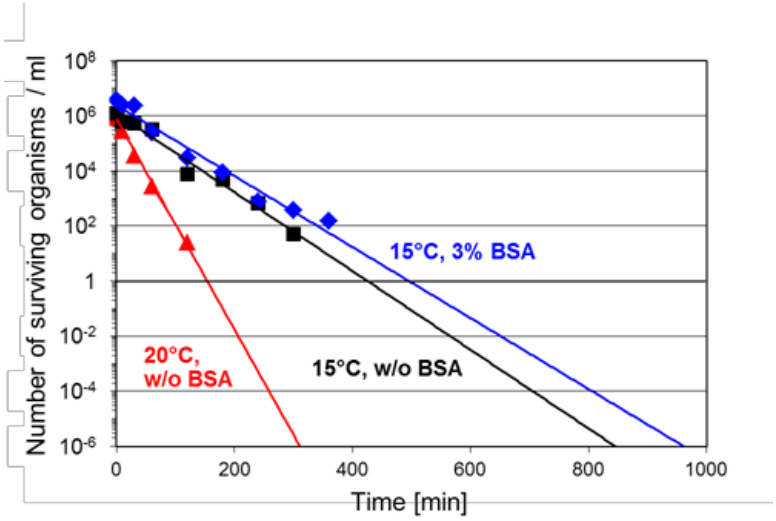


Figure 7: Worst-case.
Effect of combined interfering factors (lower temperature, 15°C and organic load, 3% BSA, blue) on the inactivation of GS spores with 2M sodium hydroxide (NaOH). At the indicated time points a sample was removed and analysed for surviving bacterial entities. The reduction rate is reduced thus leading to a 3.5-fold increase in the required time to reach a SAL of $10^{-6}$ (as compared with the higher temperature of 20°C and without organic load, red). Interestingly, the effect of organic load was less pronounced on the inactivation if it was performed at lower temperature of 15°C (blue vs black; as compared with blue vs red in Figure 6).

It is important to note that the interfering factors presented here are only examples of the most obvious interfering factors. Not all interfering factors have been determined for the inactivation of GS spores by sodium hydroxide. Therefore, a final worst-case scenario applicable for all lab situations cannot be defined yet based on these data.

## 6.2 Ultraviolet light ($UVC_{254nm}$) for the inactivation of GS spores

UV light ($UVC_{254nm}$) kills by inducing DNA damage. This DNA damage is different in vegetative bacteria compared to bacterial spores, thus affecting inactivation resistance. When vegetative bacteria are exposed to UV light, thymine dimers are formed between adjacent thymine molecules in the same DNA strand ultimately leading to DNA damage. This DNA damage is very difficult to repair, but some bacteria are capable of it, e.g. *Deinococcus radiodurans*. In contrast, bacterial spores form a photoproduct called 5-thyminyl-5,6-dihydrothymine (TDHT) upon UV light exposure and resistance to UV light has been shown to be linked to TDHT removal (Munakata and Rupert 1972) or the presence of DNA coating small, acid-soluble spore proteins, for example present in *B. subtilis* (Setlow 1992). Therefore, bacterial spores are far more resistant to UV light compared to vegetative bacteria.

We exposed GS spores to different doses of UV light and the number of surviving GS spores was monitored. When combining the measurements of the number of surviving organisms and UV dose, there are two different extrapolation possibilities resulting in different reduction rates (Figure 8). Compared to sodium hydroxide inactivation, the $UVC_{254nm}$ killing kinetic curve is not linear.

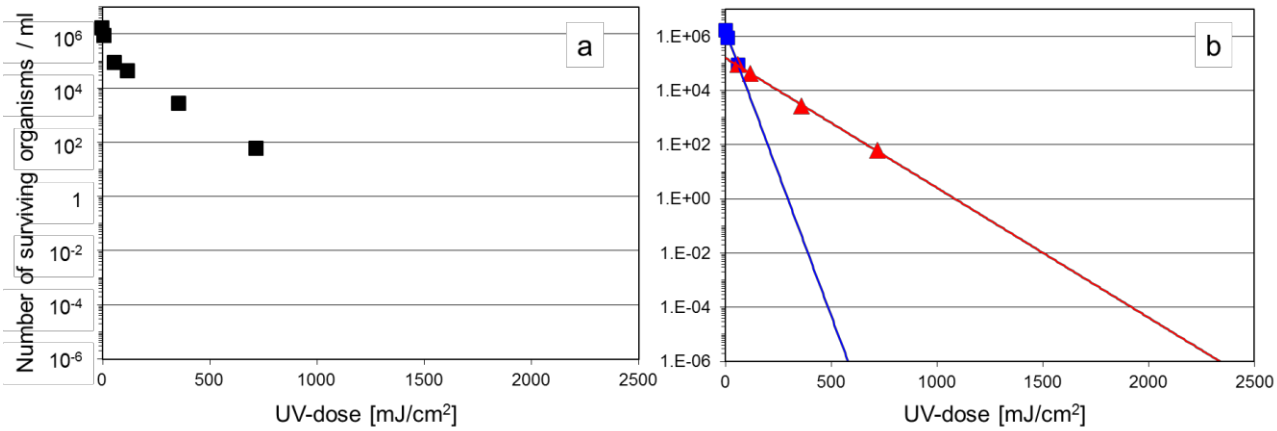


Figure 8: Ambiguous killing curve.
Inactivation of GS spores using different doses of $UVC_{254nm}$. At the indicated time points a sample was removed and analysed for surviving bacterial entities resulting in an ambiguous killing kinetic (a). There are two possibilities to extrapolate the killing kinetic curve (b; blue and red) resulting in different reduction rates.

## 6.3 Combined approach: NaOH and $UVC_{254nm}$

Another promising alternative inactivation approach is the combination of two different inactivation methods (Table 1). Especially radiation-based inactivation approaches are easily combined with e.g. chemical inactivation agents. Therefore, we also tested the two former inactivation methods, NaOH and $UVC_{254nm}$, together (Figure 9). The combination of a 0.1M NaOH solution with 0.2mW/cm² $UVC_{254nm}$ resulted in a much steeper killing kinetic curve demonstrating that the combination of methods can be very effective.

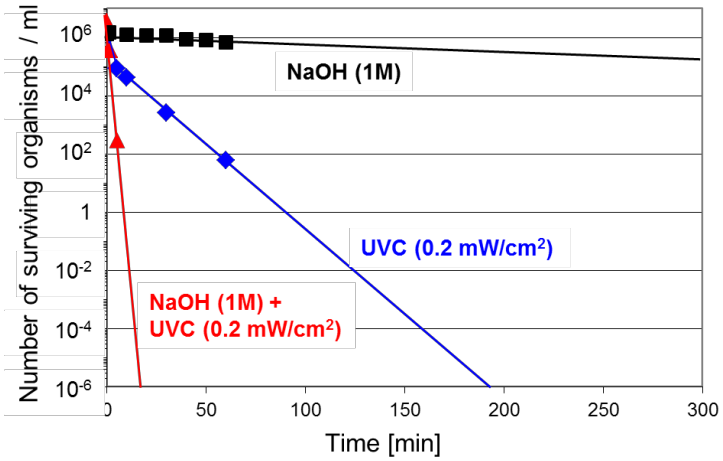


Figure 9: Increase of efficacy by combined methods.
Killing kinetic curves of GS spores using $UVC_{254nm}$ combined with NaOH (red) compared to both inactivation methods alone (black and blue). At the indicated time points a sample was removed and analysed for surviving bacterial entities. The resulting killing kinetic curve is much steeper for the combined method compared to UVC alone (blue), i.e. the combined treatment is more effective thus needing an incubation time 10-times shorter to reach the SAL-value of $10^{-6}$.

## 6.4 Chlorine to inactivate viruses and bacteriophages

### 6.4.1 Inactivation resistance and killing kinetic

The third tested inactivation method was based on the action of free chlorine ions, as in sodium dichloroisocyanurate (NaDCC, Haztab, Guest Medical). Other widely used products that act via free chlorine are sodium hypochlorite (household bleach or Javel), chlorine dioxine and chloramines-T. Sodium hypochlorite was compared with NaDCC solutions and performed equally well for the inactivation of bacteriophages. Nevertheless, the advantage of NaDCC tablets over hypochlorite solutions is that they can be stored for a longer time without losing activity. However, once in solution, NaDCC loses free chlorine faster (Coates 1985). We confirmed this by measuring the amount of free available chlorine in NaDCC and sodium hypochlorite solutions. Chlorine products have a broad spectrum of antimicrobial activity, but the exact mechanism by which free chlorine destroys microorganisms has not been elucidated in detail. It may result from a number of factors including: oxidation of sulfydral enzymes and amino acids, ring chlorination of amino acids, loss of intracellular contents, inhibition of protein synthesis, oxidation of respiratory components, decreased adenosine triphosphate production, breaks in DNA and depressed DNA synthesis (Dychdala 2001).
The aim of these experiments was to test the efficacy of virus inactivation using a chlorine releasing product. Since experiments with tissue cultures and viruses (e.g. adenovirus) are very laborious and quantification relies on quantitative PCR, we chose the *E. coli* bacteriophage MS2 as a surrogate test organism. Bacteriophage MS2 was ideal, because it is easy to propagate, handle and count, and it exposes a high inactivation resistance to chemical disinfectants compared to other viruses and bacteriophages (Lehmann and Bansemir 1987). Nevertheless, bacterial spores were shown to be more resistant to chlorine inactivation than MS2 bacteriophage (Clevenger et al. 2007; Oie et al. 2011).

The efficacy of NaDCC was tested using a similar experimental setup compared to GS spore inactivation using sodium hydroxide. NaDCC solutions were incubated with MS2 bacteriophage suspensions for 5 to 120 minutes and neutralized using a sodium-thiosulphate based neutralization medium (Woolwine and Gerberding 1995). The number of plaque forming units (PFU) was enumerated on an *E. coli* soft agar overlay at different time intervals after addition of NaDCC. First, different concentrations of NaDCC were tested (Figure 10).

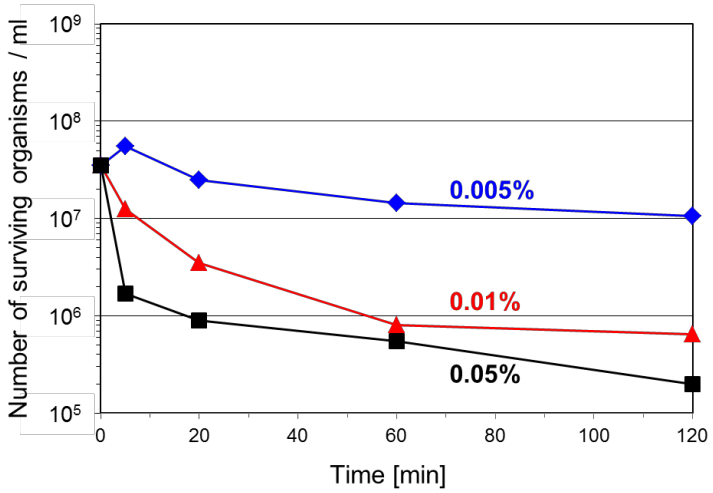


Figure 10: Non-linear or ambiguous killing kinetic.
Killing kinetic of MS2 bacteriophage in response to three different concentrations of sodium dichloroisocyanurate (NaDCC, 0.05% black, 0.01% red, 0.005% blue). Higher concentrations reduced the number of surviving organisms below the detection limit ($2.5\times10^5$ PFU/ml). At the indicated time points a sample was removed and analysed for surviving phage entities resulting in a non-linear or ambiguous killing kinetic and no sufficient inactivation.

From these experiments, we conclude that chlorine inactivation of bacteriophage MS2 does not follow a linear killing kinetic, meaning a linear extrapolation is not possible. Therefore, reaching the desired SAL of $10^{-6}$ cannot be guaranteed for NaDCC, but to achieve satisfactory results, a much higher concentration of NaDCC needs to be used. This, however, can also be problematic especially considering the release of chlorinated products into the environment, which are highly corrosive and not biodegradable.

### 6.4.2 Interfering factors of chlorine inactivation

Four potential interfering factors of chlorine inactivation were tested. The first one was **organic load** – again simulated by the addition of BSA (Figure 11). Two different concentrations were tested (0.03% and 0.3%). We found that an addition of 0.3% BSA reduces the efficacy of chlorine for bacteriophage inactivation drastically resulting in nearly flat killing kinetic curves (=no killing). Therefore, the addition of 0.3% BSA represents a worst-case scenario in terms of organic load. As a reference, we determined the expected organic load in a typical laboratory waste originating from tissue culture using a Bradford Assay. We found that this typical laboratory waste contains an organic load of around 0.2-0.3% protein. Nevertheless, when validating chlorine releasing products, we recommend either to assume a worst-case scenario (=0.3% organic load) or to measure the organic load of a typical unit to be inactivated using a Bradford or Lowry Assay.

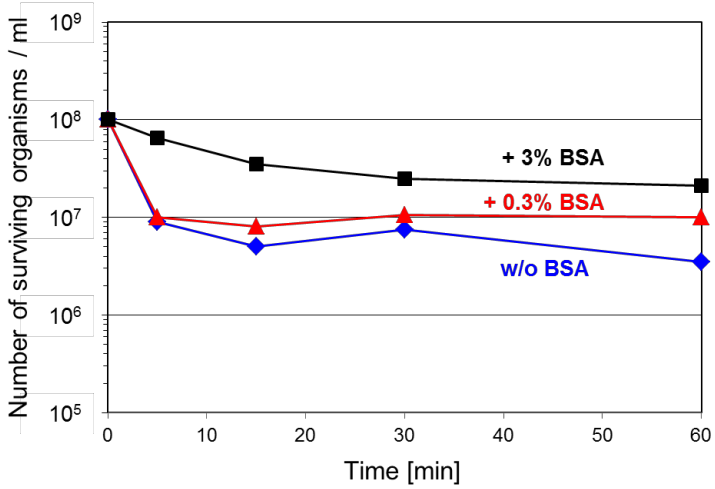


Figure 11: Influence factor "organic load".
Inactivation of MS2 bacteriophage with sodium dichloroisocyanurate (NaDCC 0.05%) in the absence (blue) or presence of organic load (0.3% red and 3% BSA black). At the indicated time points a sample was removed and analysed for surviving phage entities resulting in a non-linear or ambiguous killing kinetic and no sufficient inactivation. The presence of organic load reduced the efficacy of chlorine inactivation even more, especially at 3% BSA.

We then tested the inactivation **temperature** as second interfering factor. We defined that at our laboratory a room temperature of 18°C would represent lowest possible temperature. Comparing room temperatures of 18°C and 25°C, we did not observe any difference in inactivation efficacy of NaDCC and bacteriophage MS2 (data not shown). In a next step, we experimentally showed that a critical interfering factor for NaDCC is **age** (alias **shelf life**). We tested four differentially aged NaDCC solutions (fresh, 2, 7 and 11 week old solutions; Figure 12). While the 7-week old solution still slightly reduced the titer of an MS2 bacteriophage solution, an 11-week old solution did not show any inactivation. We therefore recommend using solutions which are not older than 3-4 weeks and to test for the amount of free available chlorine (FAC) in the solution before and after the experiment using e.g. the Dimethyl-4-phenylenediamine (DPD)-method (see 5.4.3).

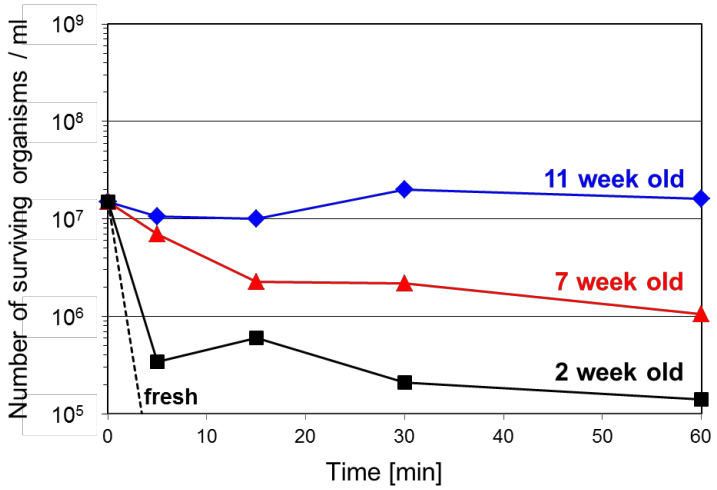


Figure 12: Influence factor "age of solution/shelf life".
Inactivation of MS2 bacteriophage with 0.1% NaDCC of different age (fresh: black stippled; 2, 7 and 11 week old: black, red and blue solid, respectively). At the indicated time points a sample was removed and analysed for surviving phage entities. With the exception of the freshly prepared solution, all older NaDCC resulted in an insufficient inactivation exhibiting non-linear or ambiguous killing kinetics.

At the same time, we tested **light exposure** of these differentially aged solutions. We had stored an aliquot of each solution either exposed to light or in the dark (protected with foil). We found that in the containers used (50ml Falcon tubes), light exposure did not affect the disinfection efficacy of NaDCC (data not shown). Further, we tested whether light protected solutions of NaDCC still lost the same amount of free available chlorine over time and found that they did (data not shown). However, this might be different for other inactivation agents acting via free chlorine. For example, Rutala et al. (1998) found that storage of sodium hypochlorite in brown closed bottles significantly reduced free chlorine loss compared to solutions stored in clear containers. Another factor affecting free available chlorine loss over time is the concentration at which a solution is stored (Rutala et al. 1998). More concentrated solutions (1%) of sodium hypochlorite lose chlorine faster than more diluted ones (0.1%). Therefore, we recommend storing solutions at higher concentrations.

It is important to note that the interfering factors presented here are only examples of the most obvious interfering factors. Not all interfering factors have been determined for the inactivation of bacteriophages by free chlorine based products. Therefore, a final worst-case scenario applicable for all lab situations cannot be defined yet based on these data.

### 6.4.3 Testing of a worst case scenario using MS2 Bacteriophages

A worst-case scenario was defined as using a 6 week old NaDCC solution that contains organic load (0.3% BSA). We could clearly show that the efficiency of inactivation of MS2 bacteriophages for the old NaDCC solution were lower in the presence of organic load when compared to a freshly made NaDCC solution without organic load (Figure 13).

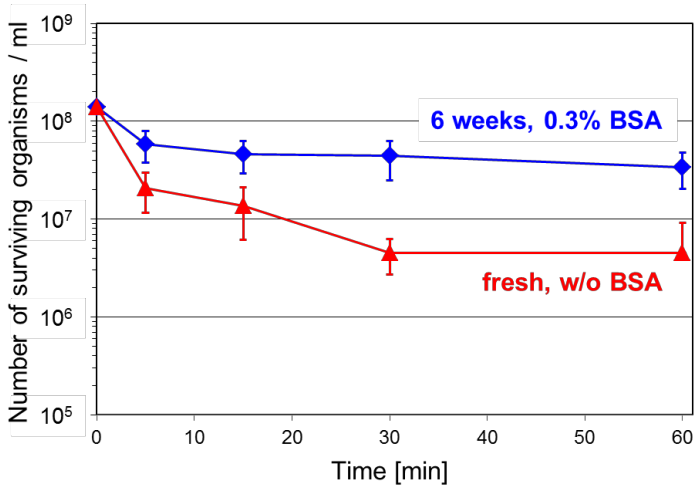


Figure 13: worst-case.
Inactivation of MS2 bacteriophage with 0.05% NaDCC freshly prepared without organic load ("best case") compared to 0.05% NaDCC 6 week old in the presence of 0.3% BSA. At the indicated time points a sample was removed and analysed for surviving phage entities. A more substantial loss in inactivation efficacy for the aged NaDCC solution with organic load (blue) compared to the "best case" (red) was observed as in the case of only organic loaded NaDCC (Figure 11, red curve). However, both cases NaDCC resulted in an insufficient inactivation exhibiting non-linear or ambiguous killing kinetics.

### 6.4.4 Testing of the worst-case scenario using Adenovirus in a cell culture system

The same worst-case scenario as described in 5.4.3 was used in an adenovirus (Ad5) infection experiment. HEK293 cells were infected with Ad5, which were differentially inactivated using fresh or NaDCC solutions aged in the presence of an organic load at different concentrations. The free chlorine activity was neutralized by cleaning the solution over a Sepharose column (Sephadex LH20; Figure 14). The increase in absolute numbers measured by qPCR of adenovirus genomes for each condition in the supernatant of the cell culture well indicates the presence of infectious adenovirus. For the experiment, we used different times of exposure [5 / 15 / 30 / 60 min], different NaDCC concentrations [1% / 0.1% / 0.01% / 0.001%], fresh vs. aged NaDCC solutions and presence or absence of organic load (0.3% BSA). In addition, several controls were added to test the functionality of the bioassay (Figure 15).

As shown in Figures 15, freshly made solutions are sufficient to completely inactivate the adenovirus at all tested concentrations, while NaDCC solutions aged in the presence of an organic load exhibited a significantly lower inactivation efficacy.

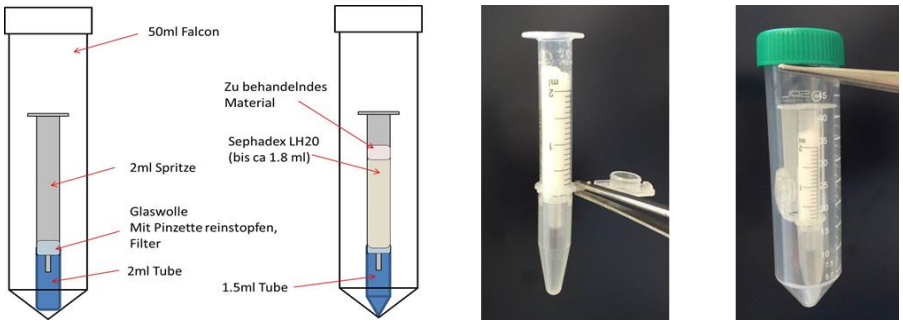


Figure 14: Neutralisation of the toxic effect of the inactivation solution using a Sepharose column.

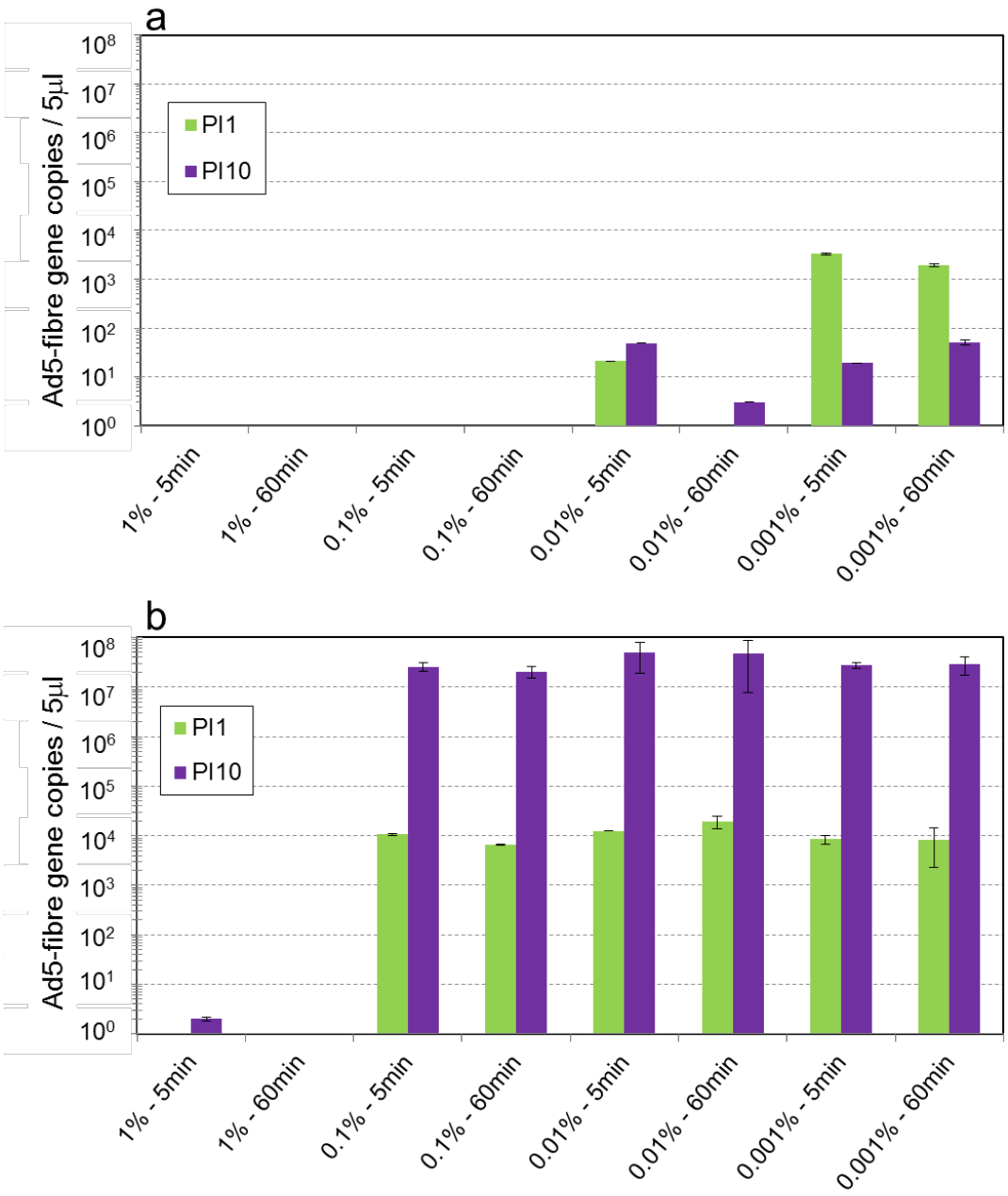


Figure 15: Worst-case.
Inactivation of Ad5 with (a) freshly made NaDCC solutions of different concentrations (1%, 0.1%, 0.01%, 0.001%) without additional organic load in comparison with (b) NaDCC solutions of different concentrations (1%, 0.1%, 0.01%, 0.001%) aged 36 days in the presence of organic load (0.3% BSA). At the indicated time points a sample was removed and analysed for Ad5 infectivity. Green and violet bars indicate the total amount of Ad5 genomes 1 day (1PI) and 10 days after infection (PI10), respectively. In the worst-case scenario (b), except for the most concentrated solution (1% NaDCC), all concentrations failed to completely inactivate Ad5 exposed to the respective solution whereas in the "best case" (a) all NaDCC concentrations were sufficient to completely inactivate Ad5.

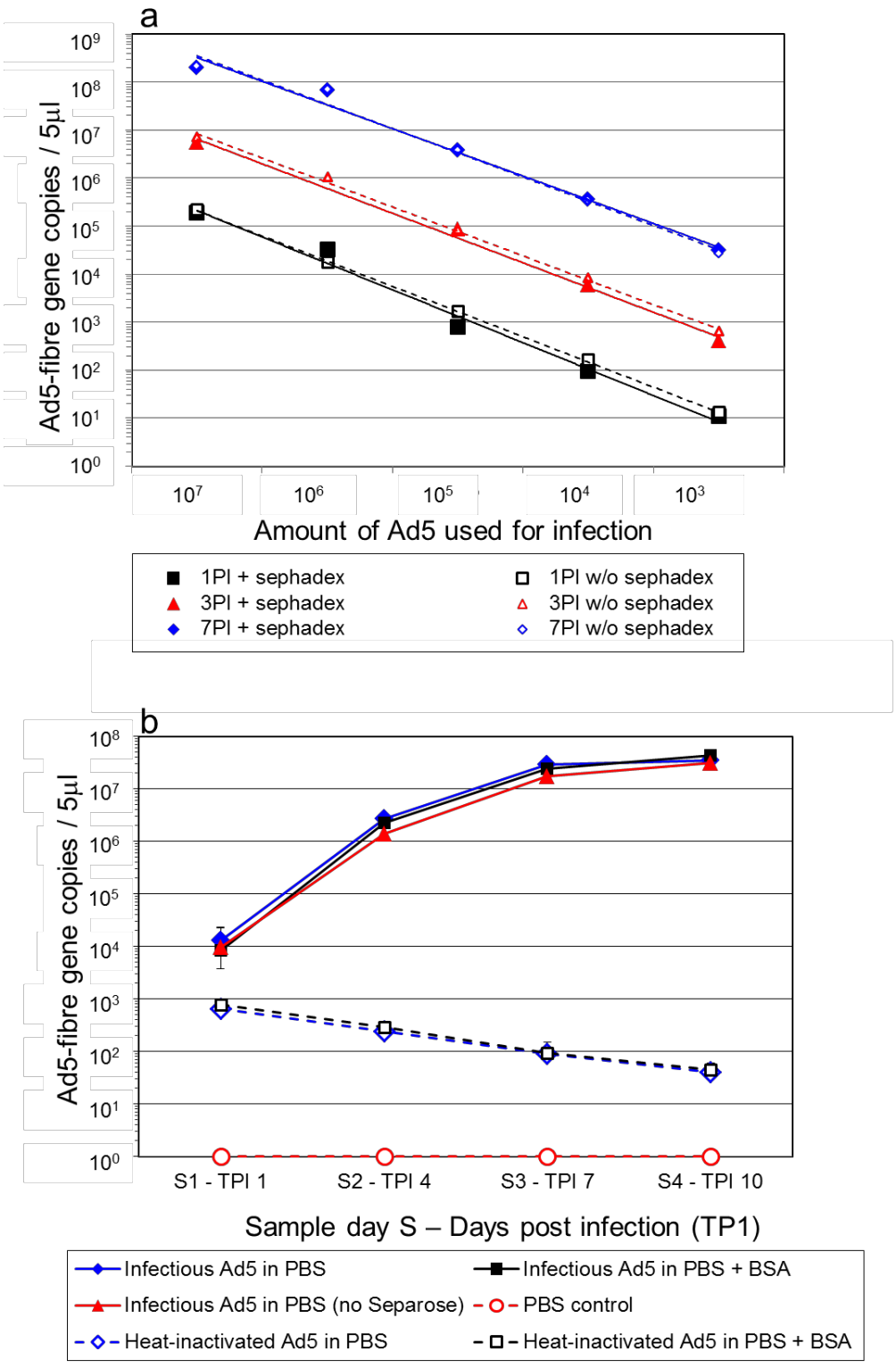


Figure 16: Controls.
(a) To test the linearity of the Ad5 reproduction in the infectivity assay (i.e. Ad5 genome copies in the supernatant) with or without prior Sepharose cleaning, different amounts of Ad5 ($10^3$ to $10^7$) were added to infect HEK293 cells and the Ad5-genome copies in the cell culture supernatant were measured at days 1, 3 and 7 post infection.
(b) To test the functionality of the infectivity assay using infectious Ad5 in PBS (with or without additional organic load, 0.3% BSA) as well as heat-inactivated Ad5 samples or PBS itself were added to HEK293 and the cell culture supernatant was analysed for Ad5 genome copies.

## 6.5 Testing chemical indicators for $UVC_{254nm}$ and chlorine inactivation

### 6.5.1 Potential for using erythrosine B as chemical indicator for $UVC_{254nm}$ inactivation

$UVC_{254nm}$ dosage is continuously measured with a radiometer. This radiometer represents an important physical sensor and should be used at every inactivation cycle using $UVC_{254nm}$. However, the measured dosage may not be the same in the entire load. Therefore, the use of additional indicators is recommended. Erythrosine B was tested as chemical indicator for $UVC_{254nm}$ inactivation. Erythrosine B is traditionally used in the food industry as food coloring, and its absorbance peaks at 525nm (Figure 17 a). This absorbance decreases largely following an exponential function to around 700 mJ/cm$^2$ (Figure 17 b; Putt et al. 2012). With higher dosages than 700 mJ/cm$^2$, the decrease in percentage of remaining absorbance is not linear anymore. During inactivation, cuvettes containing erythrosine B are placed within the unit. After inactivation with a specific UV dosage, the absorbance at 525 nm is measured using absorptiometry. The color change from red to colorless can also be seen by the naked eye (Figure 17 c).

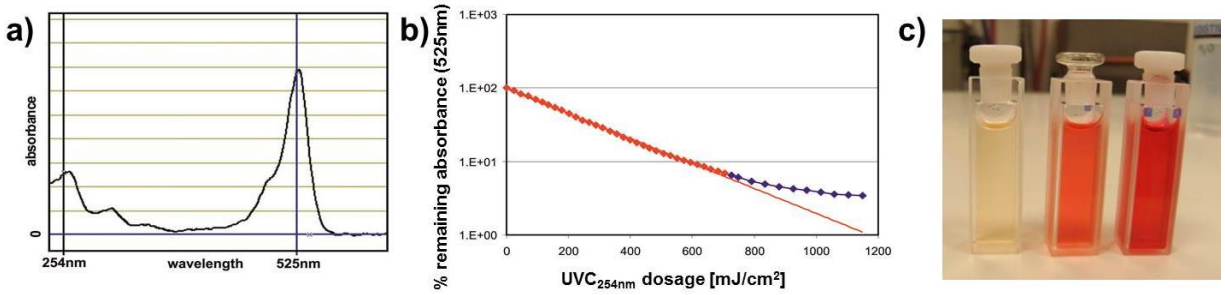


Figure 17: (a) Absorbance spectrum of erythrosine B reaching a maximum at 525nm. (b) Percent of remaining absorbance of erythrosine B exposed to increasing dosages of $UVC_{254nm}$. The absorbance drops exponentially until to approximately 700 mJ/cm$^2$. (c) Color change of erythrosine B upon exposure to $UVC_{254nm}$.

A major advantage of this system is that different cuvettes can be placed at different positions within a unit yielding values for the emitted $UVC_{254nm}$ dosage at different locations in varying distances to the $UVC_{254nm}$ source. In conclusion, we found that erythrosine B has potential to be used as chemical indicator for $UVC_{254nm}$ inactivation, but still needs to be verified further on a larger scale more closely resembling the laboratory setting (e.g. testing with larger volumes or liquids and solids).

### 6.5.2 Potential for using DPD as chemical indicator for chlorine inactivation

We evaluated the potential of DPD (N, N-diethyl-p-phenylenediamine sulfate) as chemical indicator to measure the amount of free available chlorine (FAC) in NaDCC or sodium hypochlorite solutions. The amount of FAC is the amount of chlorine which is not bound to nitrogenous compounds (=organic load) and which is able to react with microorganisms. FAC is the determining factor for inactivation. Chlorine reacts with DPD (Oxycon DPD, Figure 18 a) in the presence of a suitable buffer (Oxycon Start, Figure 18 a) to produce a pink color (Figure 18 b, c, d). Depending on the concentration of FAC in a solution, the pink color is more intense, which can then be measured at 520 nm, using an LED-based spectrophotometer (Figure 18 e) yielding the amount of FAC in mg/L. Besides the amount of FAC, the DPD method can also be used to determine the amounts of combined chlorine (i.e. the amount of chlorine bound to a nitrogenous compound) and total chlorine using the Oxycon 2 buffer.

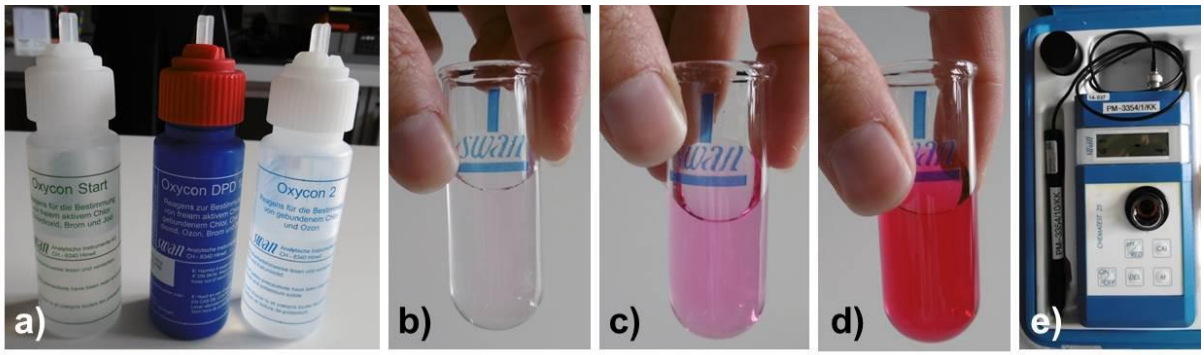


Figure 18: (a) Reagents used for chlorine measurements – Oxycon Start: buffer, Oxycon DPD: DPD for FAC measurements, Oxycon 2: for total and bound chlorine measurements (b) chlorine solution containing small amounts of FAC (<1000 mg/L in a 1% solution) (c) chlorine solution containing intermediate amounts of FAC (4000 mg/L) (d) chlorine solution containing high amounts of FAC (>6000 mg/L) (e) spectrophotometer for FAC measurements.

It has been shown previously that chlorine solutions cannot be stored indefinitely (Rutala et al. 1998), but some compounds lose FAC faster than others. In order to address this question in our NaDCC (Haztab) solutions, we monitored the concentration of FAC over time (for 36 days). We demonstrate that NaDCC solutions lose FAC over time and interestingly, the decline is nearly linear (Figure 19). We additionally monitored the concentration of FAC in NaDCC solutionsin the presence of an organic load (0.3% BSA). This simulates the scenario where an inactivation solution is added to a container prior to the input of contaminated liquid waste. With the contaminated liquid waste, the organic load is introduced, and therefore the chlorine solution ages with the organic load. When aged together, a much faster (4x) decrease in FAC can be observed compared to just aging without organic load (Figure 19).

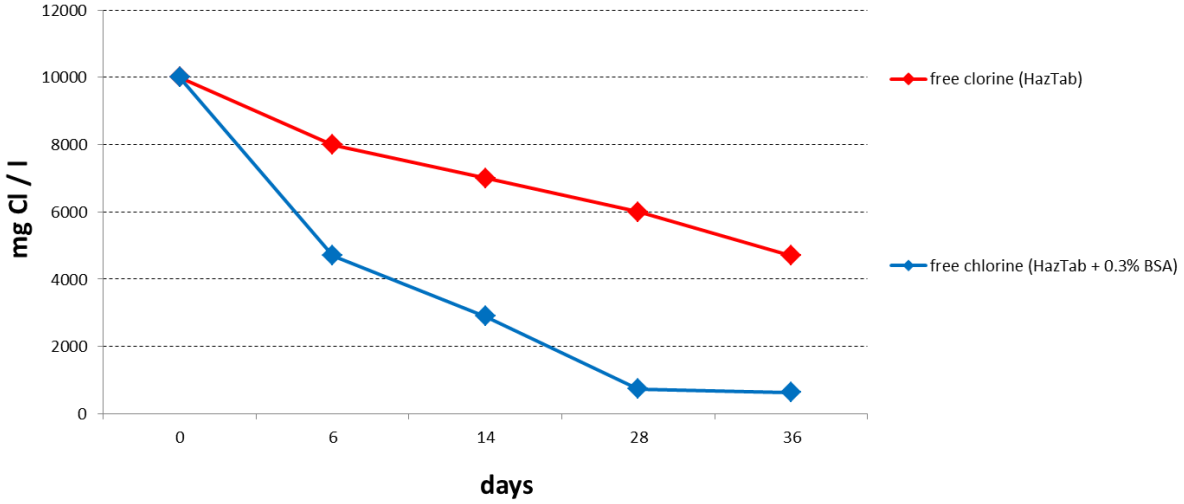


Figure 19: Process monitoring.
Free available chlorine in NaDCC (HazTab) solutions over time. The red line represents aging of a NaDCC solution without organic load added. The blue line indicates aging of a NaDCC solution in the presence of organic load (0.3% BSA).

NaDCC used here is only one example of chlorine solutions, normal household bleach (aka Javel, sodium hypochlorite) is frequently used in laboratories for the inactivation of contaminated liquid waste. To compare NaDCC with sodium hypochlorite solutions, we evaluated the amount of FAC and their inactivation efficacy. We measured equal starting concentrations of FAC in NaDCC and sodium hypochlorite solutions, but did not observe a decay of FAC (aged sodium hypochlorite solutions still had the same concentration of FAC after 36 days, data not shown). Nevertheless, they differed slightly in their inactivation efficacy (Figure 20).

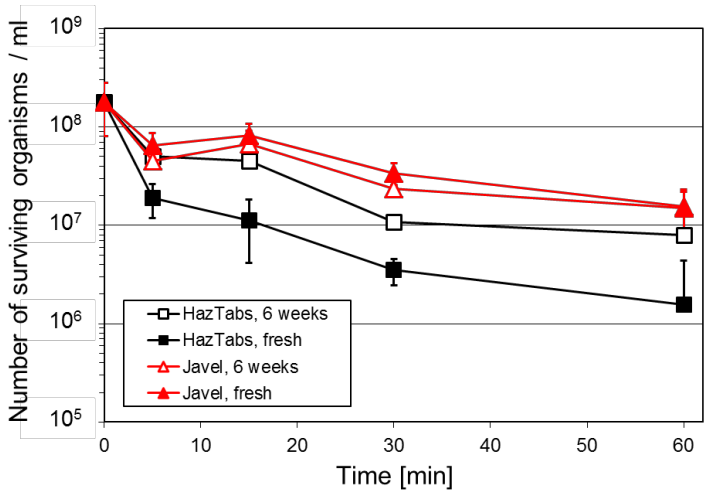


Figure 20: Inactivation efficiency of a NaDCC solution and sodium hypochlorite solution (Axxel Javel: purchased from pharmacy) both diluted to a working concentration of 0.01%.

In conclusion, the DPD method can be used to quantify the amount of FAC in a solution. It is recommended to measure the amount of FAC in a solution prior to inactivation. When diluting chlorine solutions, the final concentration can be adjusted with the measurements obtained for FAC. Nevertheless, a disadvantage of the DPD method is that swimming pools contain far less FAC and thus, chlorine solutions need to be diluted. Depending on the amount of FAC, this can be up to $10^{-5}$. Creating a dilution series can introduce errors in FAC measurements and subsequent calculation of FAC amounts.

# 7. Resources for process validation protocols

There are European Norms (EN), i.e. certified standard procedures which can assist during the validation process. They provide detailed protocols and represent helpful guidelines for the validation of different chemical disinfection methods and different organisms. They can be purchased for around CHF 150.00 from the publisher "Beuth" (www.beuth.de).

List of potentially useful European norms:
**EN12740** – Biotechnology – Laboratories for research, development and analysis - Guidance for handling, inactivating and testing of waste (EN 12740:1999)

**EN 11138** – Sterilization of health care products – Biological indicators – Part1: General requirements (ISO 11138-1:2006)

**EN 11140** – Sterilization of health care products – Chemical indicators – Part 1: General requirements (ISO 11140-1:2005)

**EN 1040** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics-Test method and requirements (phase1)

**EN 1275** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics – Test method and requirements (phase 1)

**EN 1650 –** Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2, step 1)

**EN 1656** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in veterinary field-Test method and requirements (phase 2, step 1)

**EN 1657** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity of chemical disinfectants and antiseptics used in veterinary area – Test method and requirements (phase 2, step 1)

**EN13610** – Chemical disinfectants – Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas (phase 2/step 1)[10]

**EN 13624** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants for instruments used in medical area – Test method requirements (phase 2, step 1)

**EN 13697** – Chemical disinfectants and antiseptics – Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food and industrial, domestic and institutional areas – Test method and requirements without mechanical action(phase 2,step 2)

**EN 13704** – Chemical disinfectants – Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas – Test method and requirements (phase 2, step 1)

[10] This EN was used as a guideline for the validation of Javelle as chemical disinfectant.

**EN13727** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidal activity in the medical area (phase 2/step 1)[11]

**EN 14204** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants and antiseptics used in veterinary area – Test method and requirements (phase 2, step 1)

**EN 14347** – Chemical disinfectants and antiseptics – Basic sporicidal activity - Test method and requirements (phase 1)

**EN 14348** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of mycobactericidal activity of chemical disinfectants and antiseptics in the medical area including instrument disinfectants – Test method and requirements (phase 2, step 1)

**EN14476** – Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of virucidal activity in the medical area – Test methods and requirements (phase 2/step 1)

**EN 14562** – Chemical disinfectants and antiseptics – Quantitative carrier test for the evaluation of fungicidal or yeasticidal activity for instruments used in medical area – Test method requirements (phase 2, step 2)

**EN 14885** – Chemical disinfectants and antiseptics – Application of European Standards for chemical disinfectants and antiseptics

[11] This EN was used as a guideline for the validation of NaOH as chemical disinfectant.

# 8. FAQs

**Q:** If data concerning killing kinetic and reduction rate of my desired inactivation method with my desired organism is already published, do I have to redo these experiments?

**A:** As long as the same organisms, the same experimental conditions and the same interfering factors were present in the published dataset, the process development step can be skipped and directly proceeded to the process validation step to demonstrate validity at your laboratory.

**Q:** What do I have to do, if the killing kinetic of my desired inactivation method is not linear?

**A:** In this case, a much higher concentration of disinfectant and/or a much longer incubation time need to be followed in order to assure sterility. A SAL of $10^{-6}$ can never be guaranteed in this case, but absence of viable microorganisms needs to be demonstrated using biological indicators and validated assuming a worst-case scenario.

**Q:** How do I determine the organic load in my liquid waste, which I want to inactivate?

**A:** A Bradford Assay can be done to determine the protein quantity in your waste. A typical tissue culture waste contains around 0.2-0.3% protein.

**Q:** What is important when choosing biological indicators?

**A:** First you have to show that the biological indicator is more resistant than the organisms you want to inactivate. We would recommend using bacterial spores and comparing these results with the organisms most likely present in your inactivation material. This will show whether you can use commercially available biological indicators.

**Q:** What do I do, if there are no biological indicators for my desired inactivation method?

**A:** You cannot use this method.

**Q:** What do I do, if there are no chemical indicators for my desired inactivation method?

**A:** This is definitely not an ideal situation. Either you have to show that it is a validated product stored under the correct condition (always containing a concentration of XYZ). In addition, you have to use biological indicators at every inactivation cycle.

**Q:** How long do process development and process validation (as shown in Figure 2) take?

**A:** You should anticipate 6 months of experimental work to assess killing kinetic, test the interfering factors and test the chemical and biological indicators. Make sure to start with the latter two, so you do not waste time with the first two and then figure out that there are no chemical/biological indicators for your desired inactivation method.

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