Robustness of a Biological Indicator Resistance Test Rig

- a study of different parameters, methodologies and their impact on the measured D-value of the biological indicator

LUNDS TEKNISKA HÖGSKOLA
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Foreword

The purpose with this diploma work was to evaluate the robustness of the basic method and test rig used by Tetra Pak for testing the spores (biological indicators) resistance against liquid hydrogen peroxide. The bachelor thesis was preformed at Tetra Pak, R&D Aseptic Technology in Lund from January to June 2005.

We want to thank Tetra Pak for letting us do this work, for letting us use theirs premises and of course the use of the EWA-rig. We also want to thank our instructors at Tetra Pak, Kristina Eriksson and Berit Helmfrid for theirs support and guidance. Finally we want to thank our examiner at LTH Anita Tocaj for her support and angle of approach.

Abstract

The objective with the diploma work was to evaluate the robustness of the basic method and test rig used by Tetra Pak for testing the spores (biological indicators) resistance against liquid hydrogen peroxide. Tetra Pak uses liquid hydrogen peroxide as a sterilising agent in their filling machines to achieve an aseptic product. The resistance of the biological indicators is important to know when testing new filling machines and for validation of filling machines. The spore that was used in these tests was *Bacillus* spores. The assignment was to investigate the influence of different parameters. To compare the different testes a D-value is calculated. D-value is the time required to kill 90% of the viable spore population at a specified temperature.

The experiment is divided in tree steps, preparations, sterilisation and recovery and also plate reading and calculations. The first step is preparations of the carriers, which involves punching the carriers and inoculating them with spores. The carriers, i.e. the spores, are then exposed to hydrogen peroxide in the test rig. After exposure the carriers are placed on the stirrer for recovery of the surviving spores. The spores are plated and are allowed to grow on Plate Count Agar, PCA, in Petri dishes. The last step in the experiment is to read the Petri dishes (enumerating the colony forming units) and calculate a D-value.

The results of the different tests are:
Thickness – Tetra Pak's packaging material of different thickness (at least up to 30%) can be used interchangeable in the test rig.
Material: It is clear that different materials affect the D-value and is not interchangeable in the test rig.
Surface: There is an indication that different surface structure affects the D-value and therefore is not interchangeable in the test rig.
Load *B. subtilis*: There is a large spread (low R^2) in the results when using a low load, i.e. spore concentration, ($10^{3,3}$ spores/ml) and it is clear that it is not advisable to use low loads when a determination of D-value is performed in the test rig. There is also an indication that the lower load gives a lower D-value.
Drop: There is a difference in the D-value due to inoculation method (drop vs. spray). It is clear that the inoculation method has to be fixed (standardized). There is also a risk that different drop inoculations methods might give different results.
Outgrowth rate: No conclusions could be drawn about the impact of outgrowth rate.
Type of spores: It is important to know how the indicator organism behaves in comparison with other spores. This can be judge by both D-value and the appearance of the survival curve.

Stirrer's positions: The stirring of the bath might affect the D-value and it's important to keep it constant. In the used test rig the impact of the tested changes of stirring was not major but still noticeable.
Time from exposure to beaker: The time between exposures and immersing in "stop-solution" has a major impact on the repeatability of the D-value, with increased time the repeatability decreases.
Spores lost in bath: An increased exposure time in the bath don't cause more loss of spores in the bath.
Material used in recovery beaker: There is an indication that the material (plastic or glass) in the recovery beaker has an impact on the accuracy of the result. The plastic beakers decrease the R^2 (how well the regression-line fits the data) thus indicating a larger spread in the data.

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1. Introduction

This diploma work was done at Aseptic Technology R&D, Tetra Pak in Lund.

1.1 Tetra Pak

Tetra Pak was founded by Ruben Rausing (1895-1983), with purpose to manufacture package material and packaging machines for liquid provisions. In 1929, Ruben Rausing and Erik Åkerlund founded Scandinavia's first factory, Åkerlund & Rausing, for packaging in Sweden. This company became one of the largest packaging manufacturers in Europe and the first steps in development of a carton for milk began. Tetra Pak establishes 1951 in Lund.
Today, Tetra Pak has developed to a large international company with approximately 3000 employees only in Lund. Tetra Pak has several different cartons for example Tetra Classic, Tetra Rex, Tetra Brik and Tetra Top (see figure 1). [11]

Figure 1, Different packages

It is important that the packaging material and the machines do not contaminate the product. Tetra Pak are therefore testing their machines with biological indicators to see if sterile conditions can be achieved. To evaluate the level of microbiological reduction in the packaging machines, a test-organism (biological indicator) is used. The test-organism should have a well-defined and reproducible resistance against the sterilisation system in order to make it possible to compare the results. The test organisms that are used in this study are *Bacillus* spores principally *Bacillus subtilis*. The resistance of these spores is tested in a test rig called EWA-rig, which is developed by Tetra Pak.

1.2 Background to the assignment and problem formulation

The objective with the diploma work is to evaluate the robustness of the basic method and the test rig used by Tetra Pak for resistance testing of spores against liquid hydrogen peroxide. Tetra Pak has developed and used this methodology for several years. Tetra Pak uses liquid hydrogen peroxide as sterilising agent in theirs filling machines to achieve an aseptic product. The resistance of the biological indicators (test-organisms) is important to know when testing new filling machines and for validation of filling machines. The spores that are used

in these tests are *Bacillus* spores. The assignment is to investigate how the reduction of the test-organisms (spores) is affected by different methodology/parameters. The parameters to be investigated are:
Carriers: *i.e*., how different materials, material thickness and surfaces affects the sterilisation.
Spores: *i.e*., how load concentrations, different type of inoculation, different spores and outgrowth rate affect the sterilisation.
Test rig procedure: *i.e*., how different physical conditions and handling procedures affects the sterilisation.
The effectiveness of the sterilisation is given by the decimal reduction time (D) or D-value.

1.3 Definitions

Carrier – the object (*e.g.* package, packaging material etc,) the test organism are applied on

Load – spore concentrations that are inoculated on the carrier, normally given in log units

Biological indicator – test organism, in this study *Bacillus* spores.

D-value - the time required to inactivate 90% of the viable spore population at a specified temperature and hydrogen peroxide concentration.

Spores – the resting stage in the growth cycle of certain bacteria that is resistant to heat and chemicals.

Catalase - an enzyme that breaks down hydrogen peroxide to oxygen and water, thus making sure that no residues of hydrogen peroxide is left on the exposed sample.

2. Theory

2.1 Sterilization

The definition of sterilization is the process when living cells, spores and viruses are inactivated. There are physical and chemical methods available for sterilization. When sterilization is achieved by a chemical agent, the chemical is called a sterilant. The pattern of microbial reduction (see figure 2) is exponential, *i.e.* the reduction curve can never reach zero and therefore irrespective of the length of the sterilization cycle some fraction of the original population remains viable. [2].

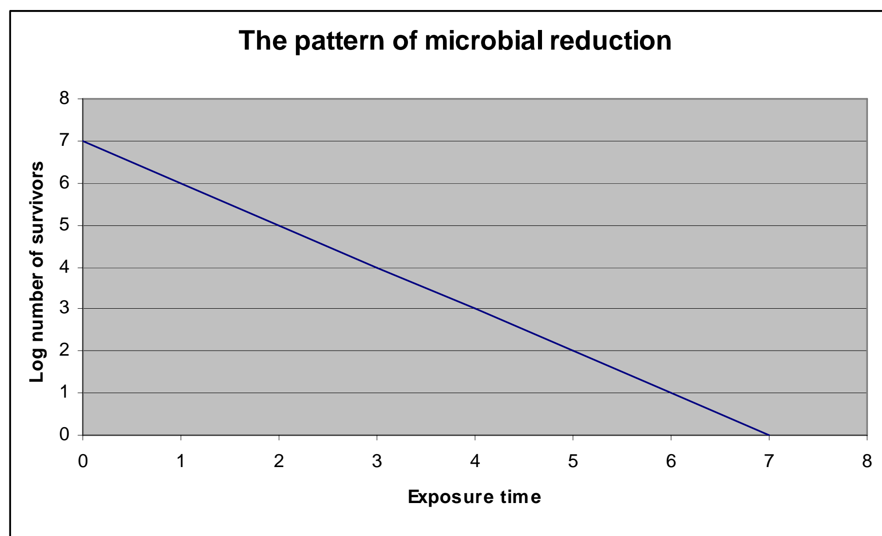


Figure 2, The pattern of microbial reduction

To estimate the resistance of a microorganism, different methods can be used. A very common method used in the food industry is to calculate a decimal reduction time, D-value.

The decimal reduction time is the time required to inactivate 90% (one log cycle reduction) of the viable microorganisms or spores at a specified set of parameters i.e. temperature and concentration. The log number of surviving cells/spores is plotted versus exposure time. When a straight-line response is obtained the D-value can be read easily from the graph by calculating the inverse of the slope.

The D-value is calculated from the formula: $D - value = \frac{\exp osure\ time}{\log N_0 - \log N_u}$,

where N_0 = the initial number of spores and N_u = the final number of spores. [1], [2]

If the curve is tailing or shouldering the D-value should be calculated from the straight portion of the graph and the shoulder and tail has to be taken into consideration (see figure 3).

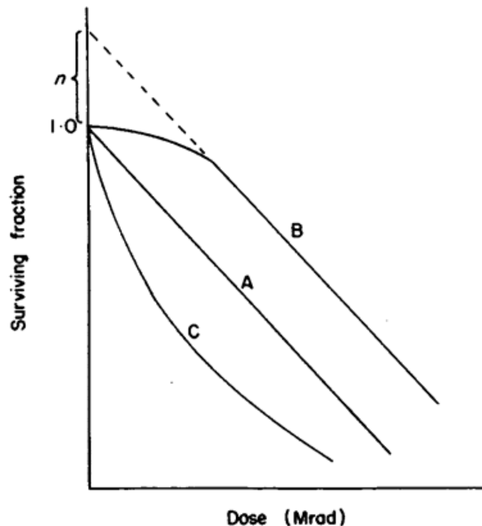


Figure 3, Type of inactivation curves: A Straight, B Shoulder and C tailing [1]

2.2 Hydrogen peroxide

Hydrogen peroxide has been used as a sporicidal and bactericidal agent for several years. Hydrogen peroxide, H_2O_2, is a colourless liquid with a distinct odour with a boiling point at 150 °C. It is highly soluble in water and ether. When hydrogen peroxide is dissolved in water it appears as a week acid and function as a very effective oxidizing agent. Hydrogen peroxide is safe and stable under recommended storage and handling conditions. [11]
To neutralize hydrogen peroxide after the sterilisation catalase can be added. Catalase is an enzyme that is used to remove residual of hydrogen peroxide. Catalase converts hydrogen peroxide to oxygen and water: $2H_2O_2 \rightarrow O_2 + 2H_2O$
That crystalline catalase could be used to disperse residual peroxide was discovered by Curran et. Al in 1940. [7], [10], [14].

2.3 Aseptic packaging

Microorganisms are a big problem in the food industry. To prevent microbial spoilage of the food product it is important that the packaging machines don't contaminate the package during filling. When sterility is maintained the package is aseptic. In aseptic packaging systems hydrogen peroxide is often used for the chemical sterilization of the packaging material. The task of aseptic packaging is to achieve sterility and thus maintain the composition of the product in regard to taste, consistence and nutrients. The sterilization agent could be applied in different ways for example spraying, steam or immersed bath due to which packaging machines that are used. [3]

2.4 Spores

A number of gram-positive bacteria can form a special resistant, dormant structure called an endospore. Endospores develop within vegetative bacterial cells of several genera: *Bacillus* and *Clostridium* (rods), *Sporosarcina* (cocci), and others. These structures are extraordinarily resistant to environmental stresses such as heat, ultraviolet radiation, gamma radiation, chemical disinfectants and desiccation (drying out). Spore formation commences when growth ceases due to lack of nutrients. [1], [2] Spores are often used as a test organism in the food industry to assure the production of a sterile product. The reason to use spores is that they are more resistant than bacteria and therefore if the spores don’t survive then nothing else has survived and the product is sterile.

2.4.1 *Bacillus*

The genus *Bacillus* contains gram-positive and endospore-forming rods. They use organic compounds as source of energy and they are usually motile. The genus is aerobic or sometimes facultative. The spores used in this diploma work are mainly from *Bacillus subtilis. B. subtilis** is a gram-positive bacterium that is rod shaped. The bacterium is aerobe and often spore forming. The spores are resistant to heat and desiccation. *B. subtilis* is neither pathogenic nor toxic. [2], [9]

Spores from two other *Bacillus* species were also studied in this diploma work, *Bacillus pumilus* and *Bacillus globigi (Bacillus atrophaeus).*

*According to Bergey’s manual of Systematic Bacteriology it is Bacillus subtilis, but Bacillus subtilus is frequently used in some publications.

2.4.2 Outgrowth rate

The outgrowth rate is the percentage of spores (colony forming units, CFU) that is able to grow out on the media or colony forming units (PCA 48h at 37°C) / number of spores estimated by Direct Microscopic Count in a Petroff Hausser counting chamber, *i.e.* a measurement of the fraction of spores that are viable respectively dormant. A dormant spore does not grow out, in this case on Plate Count Agar. A dormant spore can be activated for example by a heat chock. Earlier tests that was made on Tetra Pak indicates that *B. pumilius* has an outgrowth rate, which is higher, or equal to 90% while *B. subtilis* only have an outgrowth rate less or equal to 10%. If there are a large number of dormant spores this could affect the D-value when higher loads of spores are used. If there are a few spores scattered on a surface (i.e. viable load of log 3) the dormant spores (in *B. subtilis* case 90%) will make it more crowded. The area is already crowded if there are many spores (i.e. viable load of log 6), thus the dormant spores will make it more crowded (an actual load of log 7). It's likely that the spores will protect each other and this might make it more difficult for

the hydrogen peroxide to inactivate the spores, according to earlier tests that were made at Tetra Pak.

2.4.3 Biological indicators

A biological indicator is defined as a unit containing microorganisms of known concentration and resistance to the given sterilizing agent. It can be expected to follow a predicable reduction rate when exposed to certain physical or chemical agents. Biological indicators are used to document the efficacy of sterilization methods. The indicator serves only to demonstrate that conditions necessary for sterilization were achieved, it cannot independently validate product sterility. If biological indicators do not have proven predictability and reproducibility, they are little value in sterilization quality control. This is the first and primary consideration in selecting an appropriate biological indicator. [9]

2.5 Spore resistance to hydrogen peroxide

A number of factors influence the destruction of spores by hydrogen peroxide: [7]

- **Hydrogen peroxide concentration.** Hydrogen peroxide solutions are bactericidal but not highly sporicidal at low concentrations. The sporicidal efficiency increases with increasing concentration. To obtain rapid sporicidal action concentrations of approximately 35% are utilized to sterilize the packaging material.
- **Temperature.** The temperature of hydrogen peroxide solutions has an evident effect on the rate of spore destruction. At room temperature, hydrogen peroxide is not rapidly sporicidal. The temperature that is used during sterilization usually is between 70 and 90°C.
- **Ions.** Certain inorganic salts have been found to increase the bactericidal activity of hydrogen peroxide.
- **Ultrasonic waves.** Treatment with hydrogen peroxide and ultrasonic waves produces a synergistic effect on the destruction of bacterial spores.
- **Ultraviolet radiation** has the same effect as ultrasonic waves

In this diploma work was the hydrogen concentration and temperature constant during the tests. The concentration of hydrogen peroxide was 30 % and the temperature was 60° C.

3. Method

The standard procedure for resistance testing a spore batch is described in this section. In this diploma work several parameters has been changed and those are described along with the test description.

The experiment is divided in tree steps, preparations, sterilisation and recovery followed by plate reading and calculations. The first step is preparations of the carriers, which involves punching the carriers and inoculating the spores. The carriers, *i.e.* the spores, are then exposed to hydrogen peroxide in the test rig. After the exposure the carriers are placed on a stirrer for recovery of the surviving spores. The surviving spores (CFUs) are plated and allowed to grow on Plate Count Agar, PCA, in Petri dishes. The last step in the experiment is to read the Petri dishes (enumerate the CFU) and calculate the D-value.

3.1 Preparations

1. Carriers
2. Sterilization of the material

1. Carriers
    - Round pieces with a diameter of 45 mm are cut out from the packaging material (Tetra Brik Aseptic Juice 200 ml). The cutting is done with a cutting-equipment. These round pieces of packaging material are the carriers. It's important that there are no creases on the carriers, *i.e.* the surface is smooth. A crease is an indent in the packaging material that makes it possible to bend the material when the package is formed in the filling machine.
    - The carrier is placed in the spraying equipment (SAM) where the spores are sprayed (inoculated) on to the carrier's food contact surface (inside of the formed package) in a fixed appropriate concentration, $10^{5,6}$- $10^{6,0}$. The spore batches are stored, frozen in vials, in the Ultra freezer at -70°C.

2. Sterilization of the material
    - Before the EWA-rig experiment the following material should be autoclaved (120°C, 20 min): magnetic spin bars, dilution tubes, tweezers and phosphate buffer.

3.2 Sterilisation and recovery

1. Preparations of the test rig
2. Exposure of the carriers
3. Recovery of the spores

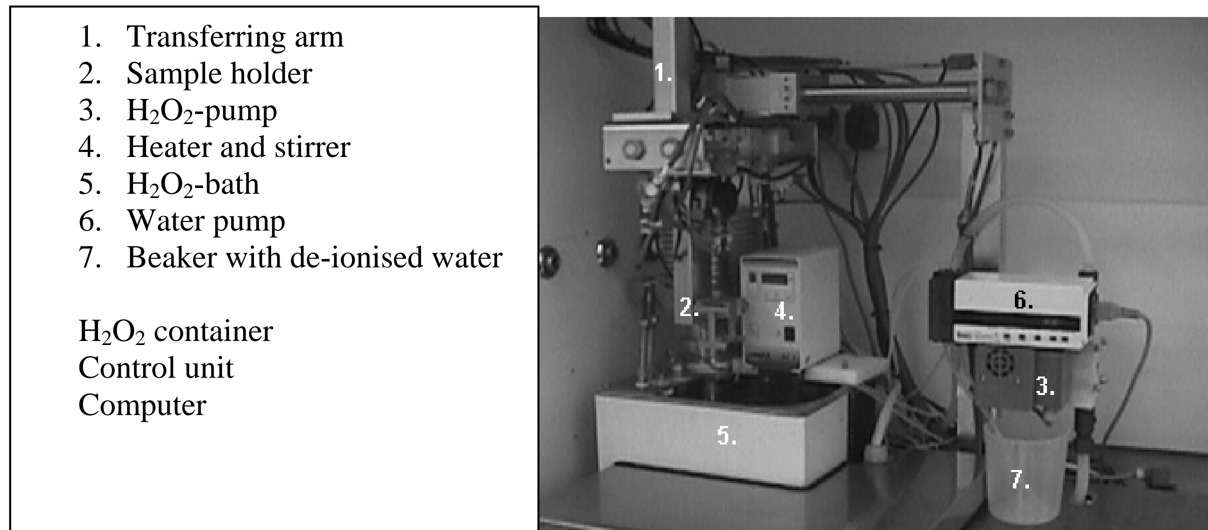


Figure 4, The EWA-rig

1. Preparations of the test rig
   - The bath (5) is filled up with 35% hydrogen peroxide and deionised water is added to dilute the concentration to 30 ± 0, 5 %. After that the stirring and heater (4) is turned on and the temperature in the bath is set to 60 ± 0, 2˚C (see figure 4).
   - Before the experiment is started a titration of hydrogen peroxide is made. This titration is made to see if the refractometer is working and showing the right concentration in the bath. The chemicals that are used are: 0,02 M potassium permanganate $KMnO_4$ and 10% sulphuric acid H_2SO_4.
   - The calculated amount of catalase (1000 units/ml phosphate buffer) is weight up and is mixed with 0,05 M phosphate buffer (pH = 7,0) 10 ml of this solution is transferred to the beakers.

2. Exposure of the carriers
   - The carriers are exposed to hydrogen peroxide by dipping in the hydrogen peroxide bath at different exposure times, 1 – 30 seconds, which are the standard exposures times. One experiment consists of 10 different exposures times (made in duplicate) and three references. The reference carriers are not exposed to hydrogen peroxide. The references are made for calculating the initial spore concentration on the carrier.

3. Recovery of the spores

- After the exposure in the bath the carrier is immediately transferred to the beaker with 10 ml of catalase buffer solution. The catalase solution breaks down the hydrogen peroxide remaining on the carrier.
- The beaker is placed on a magnetic stirrer (rotating at a fixed speed of 700 rpm) with a magnetic spin bar (on top of the carrier) for at least five minutes; this is done to remove the spores from the carriers into the sample solution.
- The sample solution is then diluted to different concentrations and 1 ml is pour plated into a Petri dish with plate count agar (double samples are made on every dilution).
- The Petri dishes are incubated in 30-32 ˚C for 4-5 days, (the references are incubated in 36-38 ˚C). [8]

Each resistance test is normally made up of three tests (performed separately) but due the short time period in this diploma work only two tests were made.

3.3 Reading plates and calculations

- The Petri dishes with 30-300 CFU (colony forming units) at each exposure time is counted. The plates with CFU fewer than 10 are excluded due to the uncertainty of such low count.
- The mean value is calculated for each exposure time and together with the dilution the log reduction is calculated according to the formula: log reduction = log ($CFU_{reference} – CFU_{exposure\ time}$) + dilution. Then the log cycle reduction is plotted against the exposure time. A linear regression analyse is applied to the graph. To calculate the D-value the inverse of the regression slope is used, see part 2.1. The R^2 value is also calculated and used to evaluate how well the regression-line fits the data. A value of R^2 close to 0 implies that the correlation between *x* and *y* is weak while a value close to 1 implies a strong correlation. The Tetra Pak standard requires an R^2 value larger than 0,95 for an approved test-organism.
- The calculations of the min and max D-values are based on a 95 % confidence level. The confidence interval shows the minimum and maximum slope of the regression line. The min- and max values are then used to create floating bar diagram, which show the interval between the min and max value of the D-value. The floating bars are done to compare the different tests and they also show how much spread there is in the data, *i.e.* how much the values of the log cycle reduction differ from the regression line.
- To compare the results from the tests a table (see the table below) is created. The contents of the table are the D-value, the min- and max-

values for the D-value and the R^2- value. In each test two test series (test 1 and test 2) and the D-value shows the results from these two tests. The summary of these two tests is compared to the reference to see if there is any difference. The min- and max-values are the minimum and maximum values of the confidence interval from the regression analyses of the D-value and shows how much the D-values are spread. The R^2- value shows how straight the line is in the graph. [8]

	D-value	**D-value min-max**	**R^2**
Summary TBA/J 1000	6	5-8	0,95
Test 1	6	5-8	0,95
Test 2	6	5-8	0,95
Reference (TBA/J 200)	6	5-8	0,95

4. Tests

In this chapter is the design of the different tests is described (the tests are not in the same order as they were done). The tests are:

1. **Carriers**
    - Thickness (0,32mm/0,45mm)
    - Material (packaging material/stainless steel/plastic)
    - Surface (rough/smooth)

2. **Spores**
    - Load (~log 6/~log 3)
    - Type (B. *subtilis*/B. *pumilus*/B. *atrophaeus*)
    - Inoculation method (spray/drop)
    - Load-influence of outgrowth rate (low/high)
    - Freezing of spore-suspension (frozen/re-frozen)
    - Dilution solution (water/40% Ethanol)

3. **Test rig procedure**
    - Stirring recovery
    - Stirring in bath
    - Time from exposure to beaker
    - Spores lost in bath
    - Beaker material

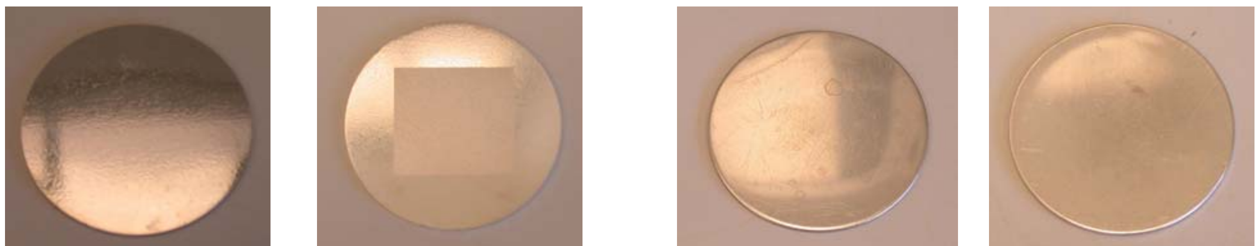
4.1 Carriers

4.1.1 Impact of carrier thickness on D-value

This test was made to evaluate if the thickness of the carriers might affect the D-value of the test organism. The carrier TBA/J 200 (Tetra Brik Aseptic/Juice 200ml) the standard carrier was used as reference in every test. In this test the standard TBA/J 200, 0,32 mm, was compared with a thicker material, TBA/J 1000 (Tetra Brik Aseptic/ Juice 1000 ml), 0,45 mm. Two testes were made on TBA/J 1000 and one on TBA/J 200.

4.1.2 Impact of carrier material on D-value

This test was made to see if the result for steel and plastic differed from packaging material. The three tested materials were the standard packaging material (TBA/J 200), stainless steel (polished) and a plastic transparent multilayer packaging material (TWA clear). Four testes were made on steel, two on plastic and one test was made on the standard carrier. Two tests with steel were made with a carrier having a rectangular blasted pattern on the back, giving a rough surface (see figure 5) that might trap excess hydrogen peroxide. Since this could affect the sterilization another steel carrier without a rough surface was used for the following two tests. Spores were in all cases sprayed on the polished side of the steel carriers.



Type 1 the inoculated side *Type 1 the back* *Type 2 the inoculated side* *Type 2 the back*

Figure 5, Carriers made of stainless steel

4.1.3 Impact of surface, structure, of the carrier on the D-value

This test was made to show if the structure of the surface affected the D-value of the tested organism. In the test of surface, the packaging material TBA/K (Tetra Brik Aseptic/K) was tested against TBA/J 1000. TBA/J 1000 was used as a reference in this test because the two materials have the same thickness. TBA/K has a smoother surface (blow moulded plastic film) than TBA/J 1000 (extruded plastic film).

4.2 Spores

4.2.1 Impact of load on D-value

This test was made to show if the spore concentration affected the D-value of the tested organism. The standard carriers were inoculated with two different spore loads,
$10^{3,3}$ spores/carrier and $10^{5,8}$ spores/carrier, the standard concentration for EWA-rig tests. To change the load to $10^{3,3}$ spores/carrier the spore solution was diluted before it was injected in the spray equipment. If the lower spore concentration $10^{3,3}$ spores/carrier could be used instead of the standard concentration this could be a solution when testing a very small amount of spores (*i.e.* spores detected in a package).

4.2.2 Impact of inoculation method on D-value

This test was made to evaluate if there was a difference in D-value between drop inoculation and spray inoculation. The spore suspension was inoculated as a 10 µl drop on the carriers and was compared to the standard spray inoculation. Drop inoculation is a widely used method in laboratories and therefore it is interesting to see if there is a difference between the methods. A possible difference between the results from the two types of inoculation is that the drop inoculation could result in a higher D-value due to that the spores are protecting each other or a lower D-value due to that they are washed of more easily in the bath.

4.2.3 Impact of outgrowth rate on D-value

B. subtilis and *B. pumilus* were used in this test. Two different spore loads, log 3,3 and log 5,8 (standard for *Bacillus subtilis*) were compared. These tests were made to see if the outgrowth rate affects the D-value. In comparison with *B. subtilis* the outgrowth rate might increase the D-value for *B. pumilus* because the outgrowth rate (*i.e.* more viable spores are growing out on Plate Count Agar) for this spore is much higher.

4.2.4 Test to illustrate different type of reduction curves, *Bacillus globigi* (*Bacillus atrophaeus*) vs. *Bacillus subtilis*

Two testes were made using *B. globigi*. This spore was tested to give an example of a spore, which exhibits a shoulder curve instead of the straight line of *Bacillus subtilis*.

4.2.5 Impact of freezing spore-suspension on D-value

Three different tests were made to see if there were any differences in D-value depending on how the spores were diluted. One test was done with spores directly from a original vial, one from spores that were diluted (the Tetra Pak standard and the standard in this diploma work), and one from spores that were

diluted and then refrozen. There might be a difference between the tests depending on that the spores could aggregate when they are refrozen.

4.2.6 Impact of dilution solution on D-value

In the first test the spores was diluted in 40% ethanol following to the standard procedure (same concentration as storage solution) and in the second test the spores were diluted in water. The used spore batch is kept frozen in 40% ethanol. To see if the water contents in the dilution media has an impact on the D-value (*i.e.* making the spores aggregate – an aggregated spore is more difficult to inactivate). When the spores are diluted in water they could aggregate and if the spraying equipment doesn't dissolve the aggregates, the carriers are sprayed with the aggregate. These might be easier to remove from the carrier during exposure or aggregated spores might protect each other and this could affect the D-value.

4.2.7 Test of standard spore batch

A test was made on another older well-documented *Bacillus subtilis* spore batch. This test was made in order to verify that the test-rig and procedures were mastered.
If the D-value corresponds with the Tetra Pak previous results any problem or faults with the test rig and the procedure could be excluded.

4.3 Test rig procedure

4.3.1 Stirring recovery test

In this experiment the stirring during the recovery procedure was examined. The standard procedure for this method is stirring with pattern (see figure 6), but this procedure requires attention during the complete process and is therefore not the most resource efficient.
The tested stirring methods were:

- Stirring with pattern (see figure 6), 20 sec at each position, in total 1 min.
- Stirring for 1, 2, 3 and 5 minutes (without pattern).
- As the stirring of the beakers often last for more than 5 minutes a second stirring test was made to compare stirring (without pattern) for 5 minutes and 10 minutes.

The expected results were that there was a trend that the release of spores increased with the increasing stirring time. (The inoculation load in this test was lower than standard to decrease the number of necessary dilutions.)

Figure 6, Magnetic stirrer with beakers on stirring

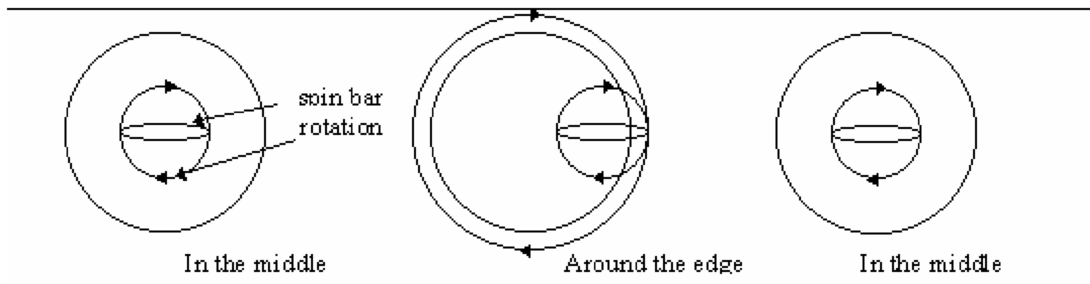


Figure 7, Stirring with pattern

4.3.2 Impact of the stirrer's position in the bath on D-value

This test was made to see if the position of the stirrer outlet affected the measured D-value. The stirrer's nozzle in the hydrogen peroxide bath is normally pointing straight down (0° angel). The stirrer's nozzle position in the hydrogen peroxide bath is turned to the left (60°) and right (65°). The angels represents that the nozzle is turned as much as possible without splashing from the bath occurring. The right and left positions are compared to the nozzle's original position in the bath (see figure 8). When the positions of the nozzle are changed this affects the stirring in the bath. The D-value could be affected due to that there are different streams where the carriers are dipped and this differs in the different positions.

Figure 8, The positions of the stirrer's nozzle

4.3.3 Impact of time from exposure to beaker on D-value

This test was made to evaluate if the time between exposure and transfer into the beaker would affect the results. Normally the carrier was transferred to the beaker immediately after exposure; this usually takes two seconds. Here the carrier was transferred to the beaker 10 seconds after exposure. During this time the hydrogen peroxide could affect the spores for a longer time than if the carriers are placed in the beaker immediately and this might decrease the D-value.

4.3.4 Impact of test organisms lost in bath on D-value

This test was made to see if spores are lost in the bath. This test was made according to standard procedure but the bath was filled with water instead for hydrogen peroxide. Spores that are lost in the bath have an effect on the calculated D-value. If the same fraction of spores is lost independent of exposure time this will only impact the Y-value in the graph (were the regression line crosses the Y-axis). If an increasing amount of spores are lost in the bath with increasing exposure time the D-value shows a lower value than the actual D-value because less spores has been killed than the D-value shows.

4.3.5 Impact of beaker material on D-value

This test was made to verify if glass beakers could be replaced by plastic beakers. The standard test is performed using glass beakers and if disposable plastic beaker could be used instead the tests would be less labour intense.

5. Results & Discussion

5.1 Carriers

5.1.1 Impact of carrier thickness on D-value

The different thicknesses don't affect the D-values. The results from the tests are shown in table 1 and figure 9,10 and 11. The curve (see figure 9) shows that there is no difference between the two tests of TBA/J 1000. The floating bars (see figure 11) indicate that there are no differences between the two thicknesses and the spread of the obtained data is small.

This test has a lower D-value than the following tests and therefore several hypotheses/explanation was evaluated.

1. Hypothesis: Spores were mistakenly diluted in water for these tests instead of ethanol (5.2.6), test indicated no difference between water and 40% ethanol
2. Hypothesis: The plastic beaker were introduced after the thickness test and thus might be the cause for a different D-value (5.3.5), test indicated that this didn't cause the difference
3. Hypothesis: The spores were not refrozen (after dilution and before spraying) in these first test and this might affect the D-value (5.2.5), test indicated that this didn't cause the difference
4. Also an old batch with known resistance were tested to verify that the procedure and methods were correctly performed (5.2.7), tests indicated that the performance was proper

As indicated none of the above tests could explain the deviating results. One possible explanation could be that the test batch used is not a Tetra Pak produced crop and there is no guarantee that the batch isn't made up of different fractions of spores with different resistance. Unfortunately there was no time left over to redo these first tests.

	D-value	**D-value min-max**	**R^2**
Summary TBA/J 1000	6,2	5,8-6,6	0,970
Test 1	6,2	5,6-6,8	0,971
Test 2	6,2	5,7-6,9	0,973
Reference (TBA/J 200) Not pooled with other references due to the D-value change	6,4	5,8-7,2	0,957

Table 1, Thickness

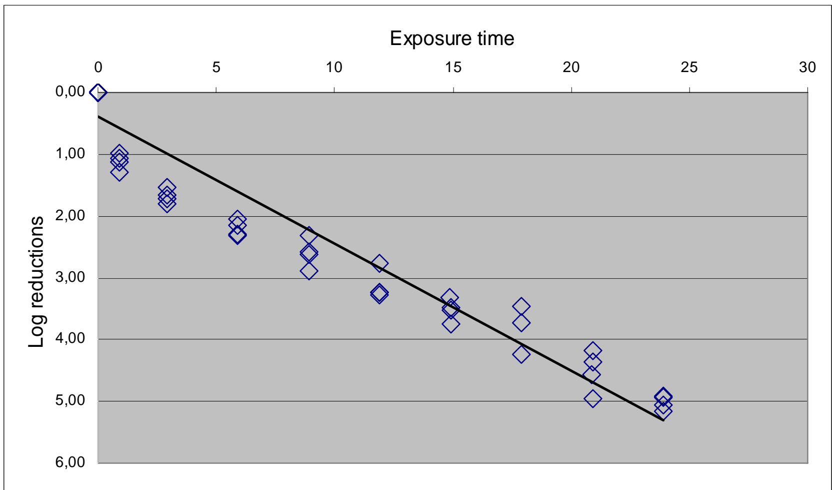


Figure 9, Summary of the two TBA/J 1000 tests

This is the graph representing the D-value of the two tests. The slope of the regression (1/a) line is the D-value. As can be seen in table 1 the R^2 is high indicating that the result is linear.

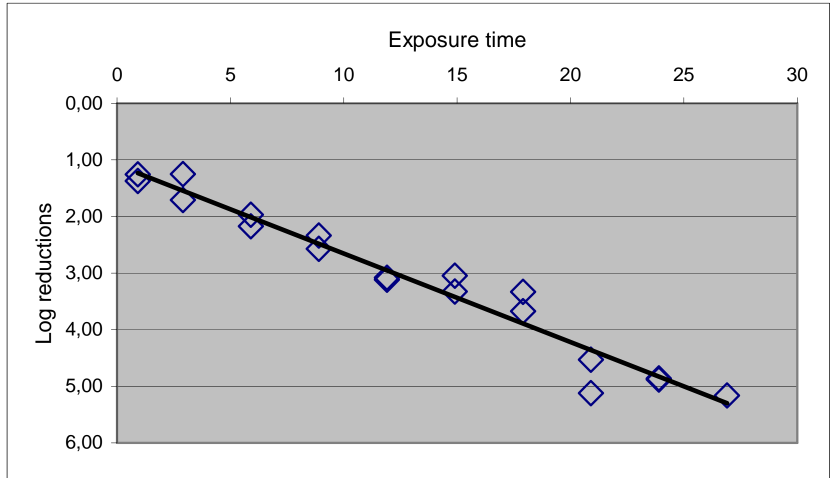


Figure 10, The reference TBA/J 200

Unfortunately since the D-value changed after this test the reference sample cannot be pooled with the rest of the references.

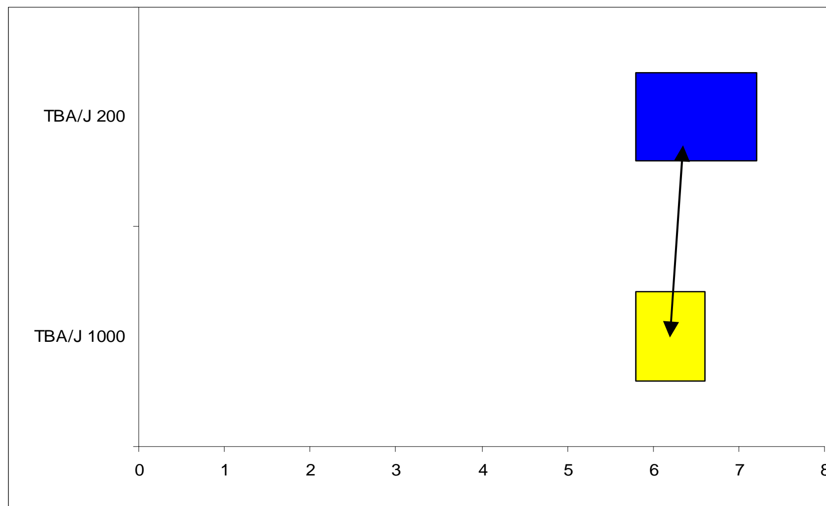


Figure 11, Floating bars for thickness

As can be seen the results overlap each other. This indicates that there is little or no difference between the D-values. The arrows indicate the mean D-value.

Summary

The test indicates that packaging material of different thickness (at least up to 30%) can be used interchangeable in the test rig.

5.1.2 Impact of material on D-value

The D-value for spores on polished stainless steel is higher than for the other materials; hence it takes longer time to reduce the number of test organisms on steel than on the standard packaging material or on plastic packaging material. It probably takes longer time to heat up the steel carrier because of its thickness (0.7 mm). As can been seen in figure 16 and from the calculated R^2 (see table 2), the results from steel tests are spread (not so linear).

The results from the carrier of steel type 1 (see figure 5) are excluded because they aren't representative due to the blasted area on the back. Extra hydrogen peroxide remained in the blasted area and there was not enough catalase in the buffer solution to neutralize the extra hydrogen peroxide.

The plastic carrier gave a lower D-value than the packaging material carrier. The graph (see figure 15) shows that longer exposure time gives more spread results. The plastic carrier has a smoother surface than the paperboard packaging material has (see figure 12 and 13), and that is probably the reason for the faster inactivation of spore. The spores can probably not be protected on this surface as much as it can in the paperboard packaging material (TBA/J is rough) surface and therefore are the spores probably easier to reach for the hydrogen peroxide.

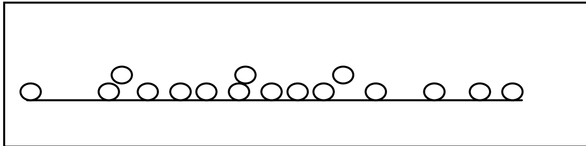


Figure 12, Surface of the tested Plastic material

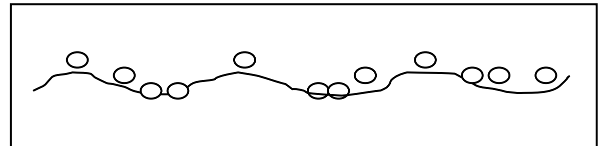


Figure 13, Surface of the TBA/J 200 packaging material

The floating bars in figure 16 from the material tests show that both the carrier of steel and plastic overlaps the reference, but they don't overlap each other. If they overlap each other it indicates that the difference between the D-values is small.

There were two problems with the material tests.

1. The plastic carriers slipped out of the sample holder on the way up from the bath. The solution was to expose extra carriers and to skip the first exposure time (1 second), because at this exposure time the carrier always slipped.
2. The steel carrier is thicker and therefore it was difficult to place and remove the carrier in the sample holder. This led to that it took longer time to place and remove the carrier in the sample holder (this could explain the less linear result of the stainless steel data, see 5.3.4).

	D-value	**D-value min-max**	**R^2**
Summary steel	10,6	9,1-12,6	0,812
Test 1 steel	11,1	9,1-14,2	0,836
Test 2 steel	10,0	8,1-13,3	0,804
Summary plastic	7,6	6,9-8,6	0,924
Test 1 plastic	7,4	6,5-8,4	0,947
Test 2 plastic	7,5	6,4-9,0	0,936
Pooled references (see 5.6)* All done with TBA/J 200	8,4	7,4-9,5	0,870

Table 2, Material

* The reference samples have not been run parallel with the other tests, but they are run in the vicinity of the other tests (not always in the same day). Since the reference tests for all tests are run under equal conditions only one test has been made in the vicinity of each test set-up. A D-value for the "pooled" reference samples has been calculated in 5.6.

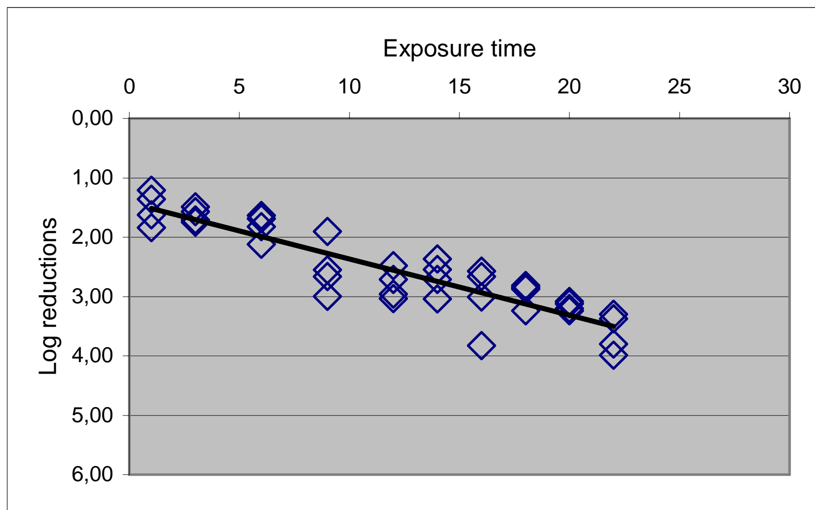


Figure 14, Summary of the two tests on the stainless steel carriers

The variation in result is probably due to the problems with handling the thick steel carriers.

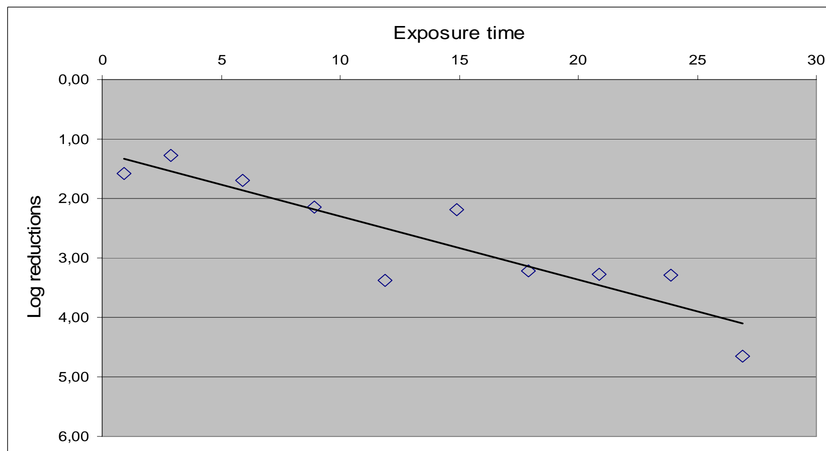


Figure 15, Summery of the two tests on plastic material

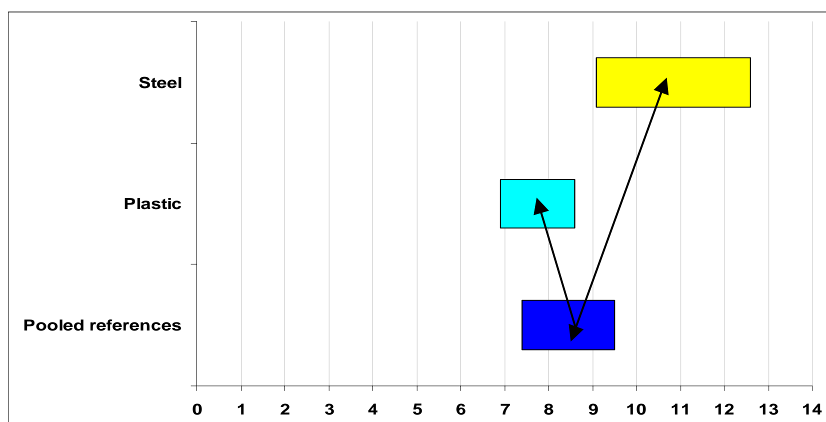


Figure 16, Floating bars for materials

Summary

It is clear that different materials affect the D-value and is not interchangeable in the test rig.

5.1.3 Impact of surface (structure) of the carrier on the D-value

The testes that were made on different surfaces indicate that the carrier with the smoothest surface has a lower D-value. This was the expected result according to earlier results at Tetra Pak. The conclusion is dimmed by the fact that the reference sample has a large spread. Figure 17 shows that the two tests with TBA/K don't differ from each other. The spread is greater for TBA/J 1000 than TBA/K (see figure 19). Since TBA/K is the smoothest carrier the spores are probably easier to reach with the hydrogen peroxide because the spores don't "hide" in the carrier's surface as much as in the reference material. This theory agrees with the results from the plastic carrier (5.1.2). Both plastic carrier and TBA/K have a D-value between 7,1-7,5. (See figure 12 and 13).

	D-value	D-value min-max	R^2
Summary (TBA/K)	7,3	6,7-8,1	0,850
Test 1 (TBA/K)	7,5	6,5-8,9	0,918
Test 2 (TBA/K)	7,1	6,5-8,0	0,958
Reference (TBA/J 1000)	8,6	7,2-10,8	0,868

Table 3, Surface (TBAJ/1000 was reference in order to have similar thickness as TBA/K)

Please note that the reference test cannot be pooled with the other reference samples.

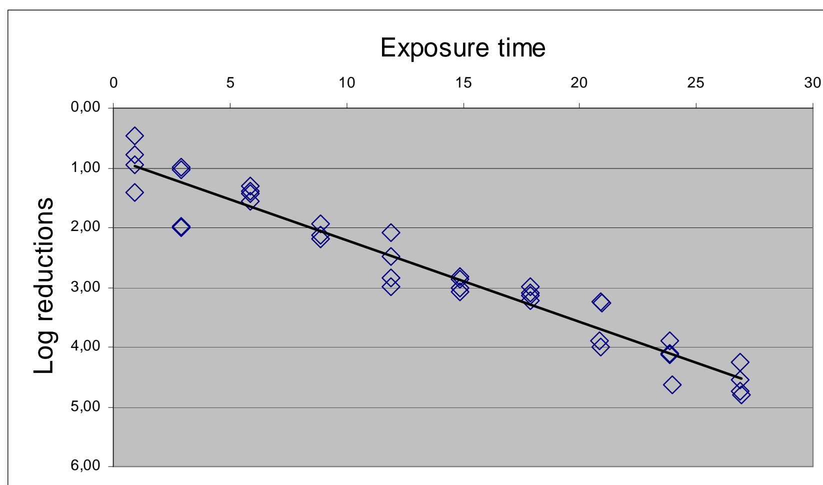


Figure 17, Summary of the two testes on the smooth surface (TBA/K)

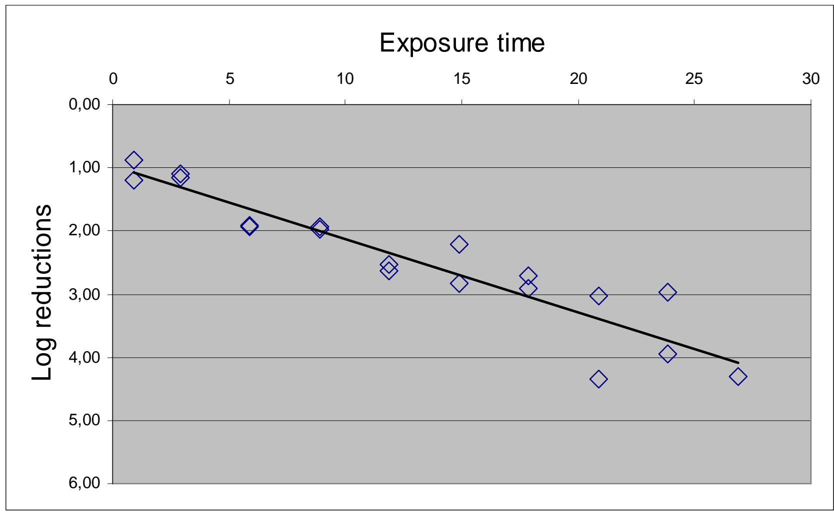


Figure 18, The reference TBA/J 1000

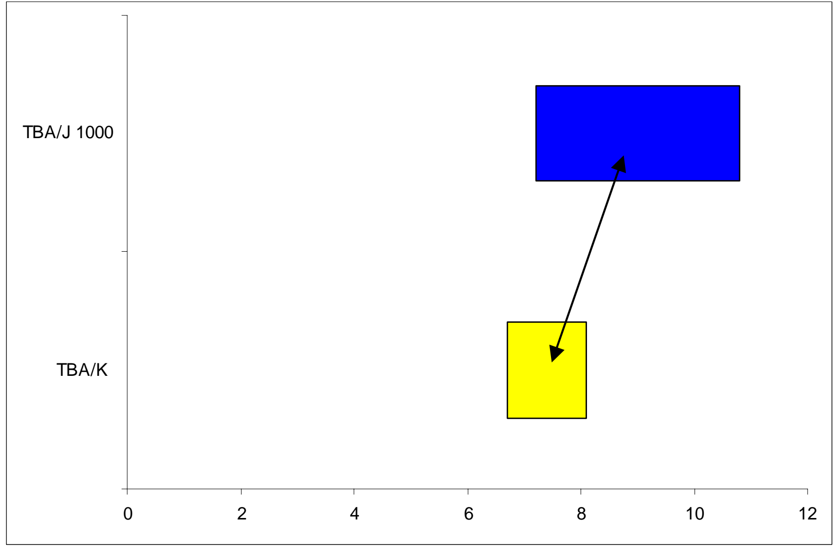


Figure 19, Floating bars for the surface test

Summary

Unfortunately only one reference test was made (can't be pooled with the other reference tests) which makes it difficult to compare the results. However there is an indication that different surface structure affects the D-value and therefore is not recommended to interchange different surfaces in the test rig.

5.2 Spores

5.2.1 Impact of load on D-value

The results for the test with the lower spore concentration $10^{3,3}$ spores/carrier are more spread (none-linear) and therefore a conclusion of the impact of D-value is difficult to draw. Two testes were made with the lower load (3,3) and after that result (4,9 and 7,5 respectively) two more testes were made to confirm the variation in D-value, as can be seen this is the case.

It is obvious that the spread and the D-value variation are bigger when there is a decrease in the load. The result is therefore that it isn't suitable to use low loads for D-value determinations. Both figure 20 and 21 shows the big spread in these tests.

	D-value	D-value min-max	R^2
Summary load 3,3	6,6	4,6-10,2	0,426
Test 1 load 3,3	4,9	3,9-6,6	0,812
Test 2 load 3,3	7,5	5,3-13,2	0,603
Test 3 load 3,3	5,6	3,5-13,7	0,508
Test 4 load 3,3	7,2	5,8-9,5	0,812
Pooled references (see 5.6) All with load 5.6-6.0	8,4	7,4-9,5	0,870

Table 4, Test of load

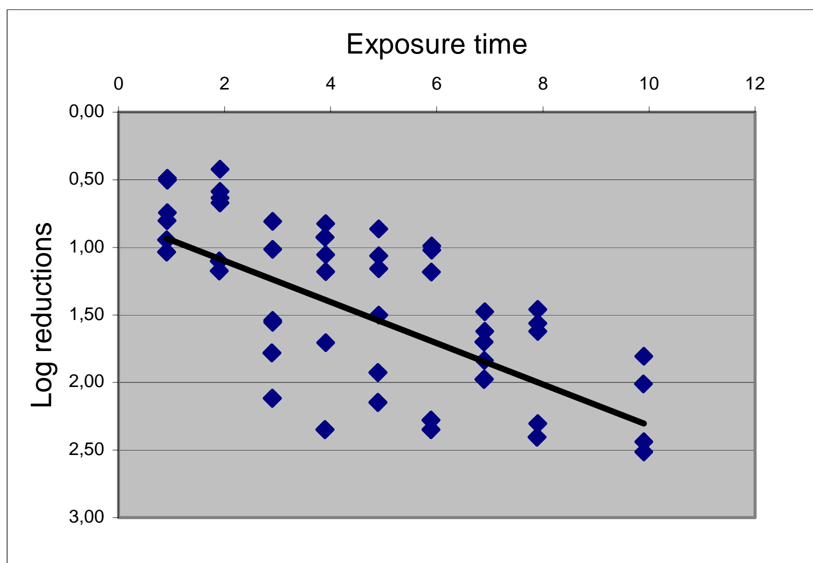


Figure 20, Summary load 3,3

Not only the variation in D-value but also the low R^2 indicates that a low load do not give repeatable results. The R^2 value should preferable be closer to 1 when as many as 4 tests have been preformed and the D-values should not differ too much from each other.

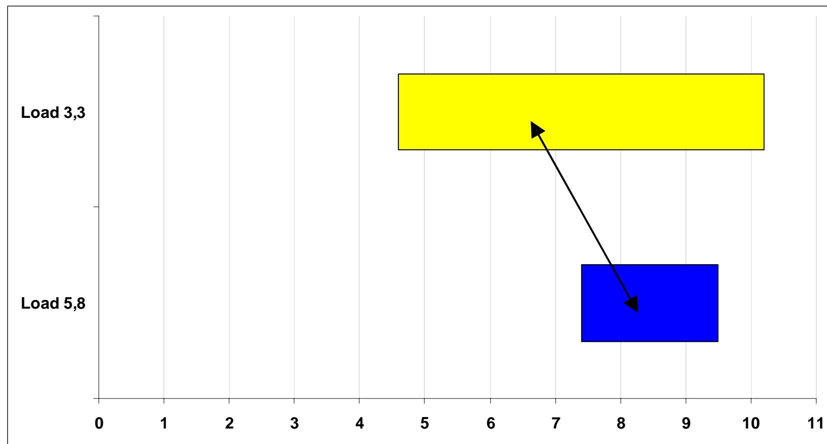


Figure 21, Floating bars for the load test

Again the large spread with the low load can be seen.

Summary

There is a large spread (none-linear) in the results when using a low load (3,3 logs) and it is clear that it should not be used in the test rig. The indication is also that the lower load might give a lower D-value (based on the mean D-value in each test).

5.2.2 Impact of inoculation method on D-value

There are two theories on how the D-value will be affected by using drop inoculation:

1. The D-value will increase as the spores are spread over a smaller area, and thus may protect each other (see figure 21). Figure 22 shows how the spores are distributed on a bigger area when they are sprayed.
2. The D-value will decrease as the spores might fall off in clusters by the stirring in the bath.

The two tests with drop inoculation of spores on the carrier resulted in the highest D-value. The floating bars in figure 24 show that there is a difference between the inoculation methods, as they don't overlap each other. If the D-values are almost the same the floating bars overlap each other and there is no difference proven between the two methods. There might also be a problem with this kind of inoculation (drop) because it's easy that the dry spores drop flakes off but this problem doesn't shows in this test. The drop can also flakes off during storage. To avoid this several small drops can be inoculated instead of one big drop.

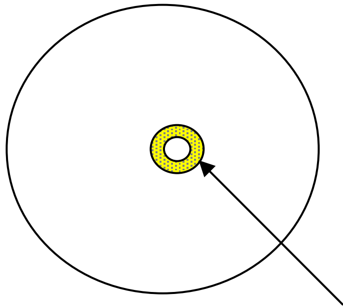


Figure 21, Drop inoculation
Most of the spores will be concentrated to the outer edges of the drop.

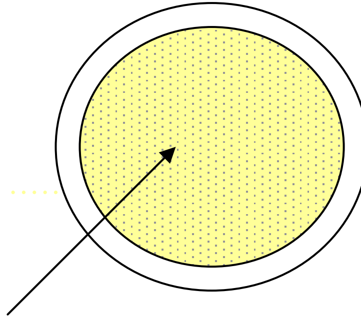


Figure 22, Spray inoculation
The spores are evenly spread over a large surface.

	D-value	D-value min-max	R^2
Summary	10,7	9,6-12,1	0,899
Test 1	10,4	8,9-12,7	0,889
Test 2	11,0	9,5-13,0	0,914
Pooled references (see 5.6) All spray inoculated	8,4	7,4-9,5	0,870

Table 5, Drop test

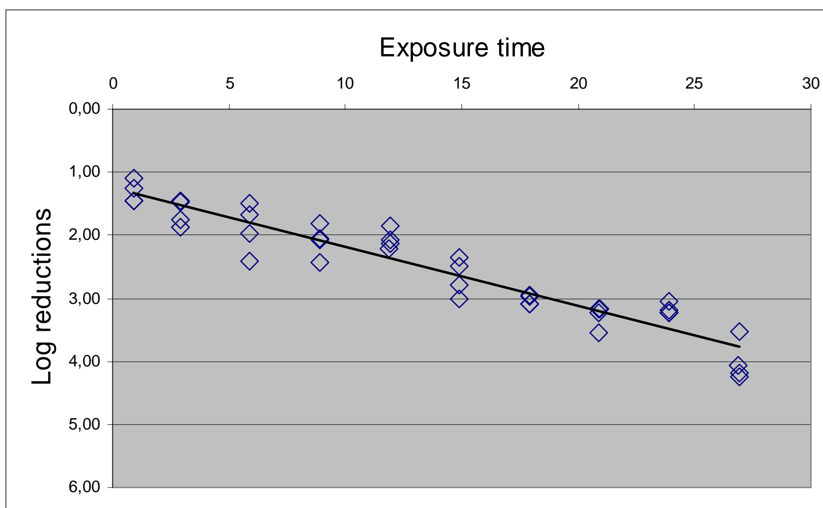


Figure 23, Summary of the two tests inoculated with drops

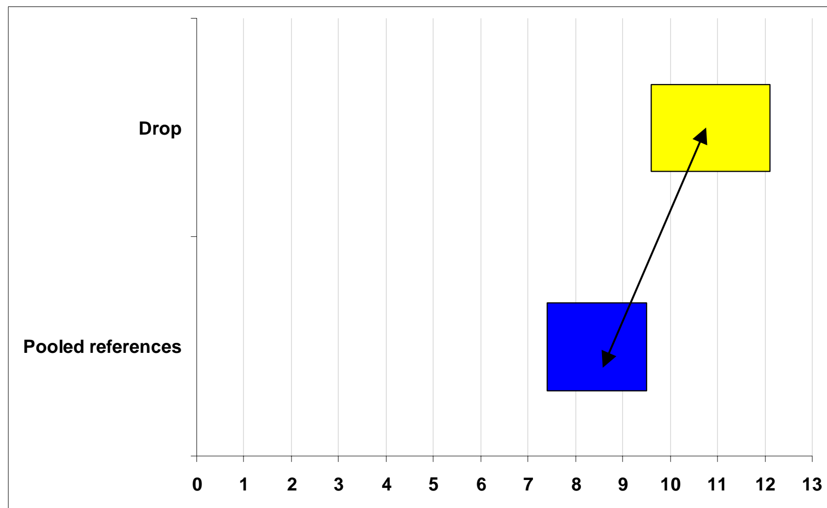


Figure 24, Floating bars for the test of inoculation

Summary

There is a difference in the D-value due to inoculation method. It is clear that the inoculation method has to be fixed (standardized). There is a risk that different drop inoculations methods might give different results. The spray inoculation method is probably a more stable method.

5.2.3 Impact of outgrowth rate on D-value

This results shows that *B. pumilus* (commonly used as test-organism for UV-light and irradiation) has a lower D-value than *B. subtilis* irrespective of the spore load. Spores tested with load 3,3 show slightly higher D-value than spores tested at load 5,8. Due to the spread in data (especially at 3.3) this conclusion is uncertain. This relationship between load concentrations and D-value is contrary compared to *B. subtilis* (5.2.1), see figure 27. Due to the spread in data (especially at 3.3) this conclusion is uncertain. There is no significant difference between the two loads for *B. pumilus*, which have a high outgrowth rate, and the conclusion is that there are only a few dormant spores that can protect each other from hydrogen peroxide.

For *B. subtilis*, which have a low outgrowth rate, there is a possible difference between the two spore concentrations; this could be due to that the dormant spores are protecting each other at the higher load.

Both spores show more spread (none-linear pattern) pattern at the lower load.

	D-value	D-value min-max	R^2
Summary load 3,3	3,9	2,8-6,4	0,497
Test 1 load 3,3	3,3	2,1-8,2	0,529
Test 2 load 3,3	4,0	2,8-7,1	0,634
Summary load 5,8	3,1	2,5-4,1	0,746
Test 1 5,8	3,4	2,4-5,8	0,717
Test 2 5,8	2,8	2,1-4,2	0,781

Table 6, outgrowth rate

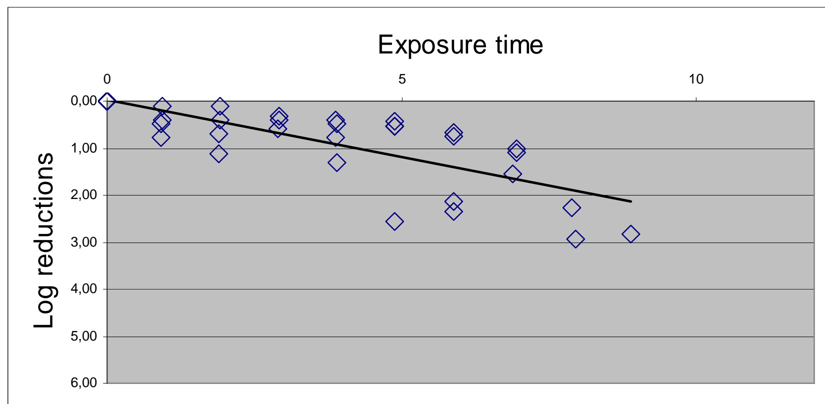


Figure 25, Load 3.3

Both the variation in D-value and the low R^2 indicates that a low load do not give repeatable results.

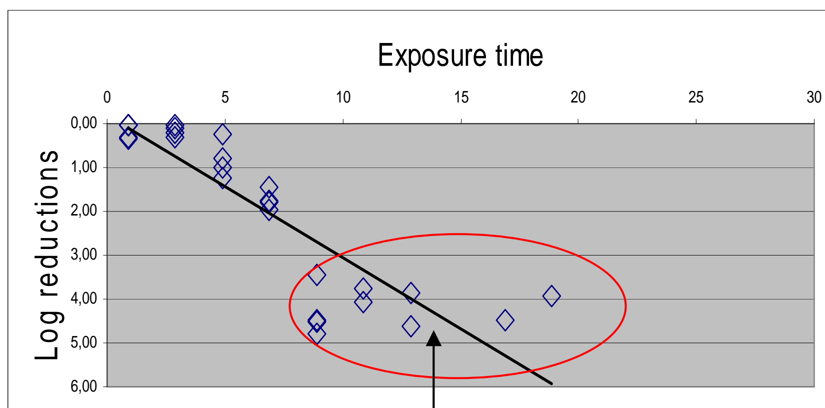


Figure 26, Load 5.8

There is evidence of a possible tail, i.e. the values obtained after 9 seconds exposure seems to be independent of the exposure time.

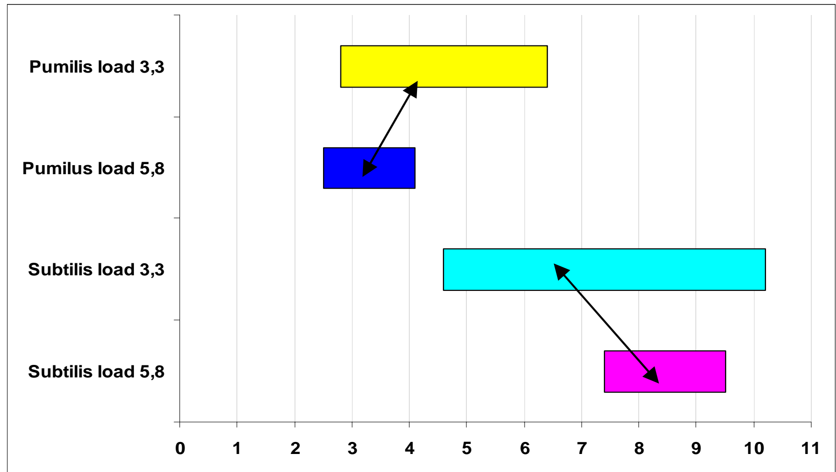


Figure 27, Floating bars for a comparison with B. pumilus and B. subtilis in different loads.
The large spread in result at the lower loads makes it difficult to draw any conclusions.

Summary
No conclusions could be drawn about the impact of outgrowth rate.

5.2.4 Test to illustrate different type of reduction curves, *Bacillus globigi (Bacillus atrophaeus)* vs. *Bacillus subtilis*

This is an example of a spore that has a non-linear pattern of inactivation (see figure 28). For this spores the curve is divided in two parts. The D-value is calculated individually for the two parts using linear regression. The D-value for the upper part is 8,7 seconds and for the lower part 2,6 seconds.

	D-value	R^2
Summary	3,4	0,904
Points 1-8 seconds	8,7	0,650
Points 10-18 seconds	2,6	0,837

Table 7, B. globigi

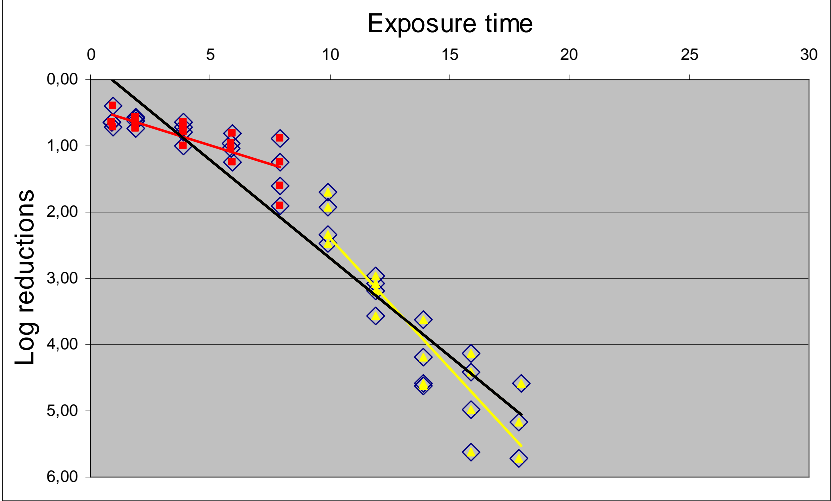


Figure 28, B. globigi (Bacillus atrophaeus)

This type of inactivation pattern makes *B. globigi (Bacillus atrophaeus)* more difficult to use as test-organism. If the total D-value (black line) is used and the exposure time is short the killing efficiency of a filling machine will be underestimated.

Summary
It is important to know how the indicator organism behaves.

5.2.5 Impact of freezing spore-suspension on D-value

In this test three different methods for spore handling before inoculation are tested and compared and the result is that the D-values are almost the same. The conclusion is that the D-value does not change when the spores are diluted, or if the spores are refrozen after being diluted *i.e.* the spores remains unchanged to these procedures. The tests are overlapping and there was a small spread for all of them (see figure 31).

	D-value	D-value min-max	R^2
Test 1 **Used direct from freezer**	8,2	7,0-9,8	0,902
Test 2 **Taken from freezer, diluted and refrozen**	8,0	6,8-9,6	0,897
Pooled references (see 5.6) Taken from freezer, diluted and used (not refrozen)	8,4	7,4-9,5	0,870

Table 8, Diluting/refrozen

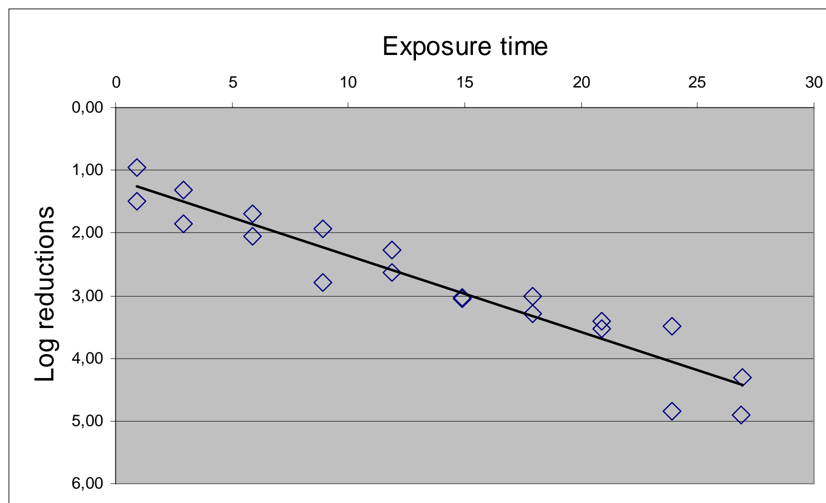


Figure 29, Direct from vial

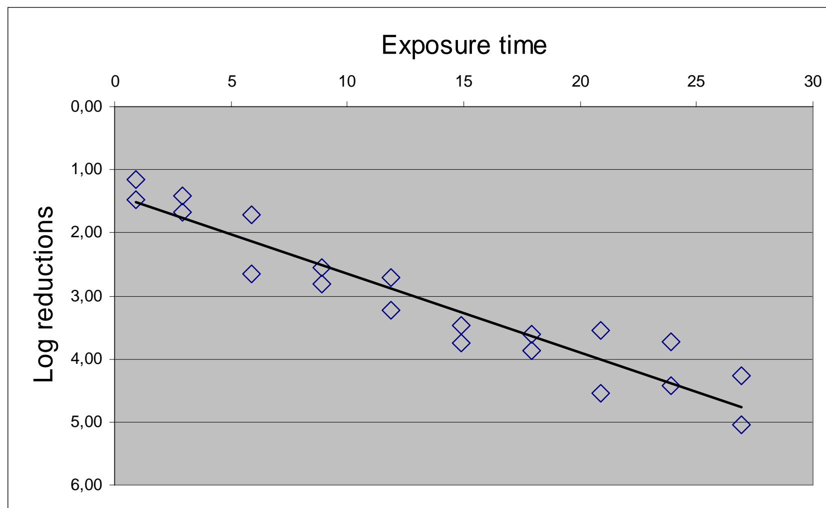


Figure 30, Diluted and refrozen

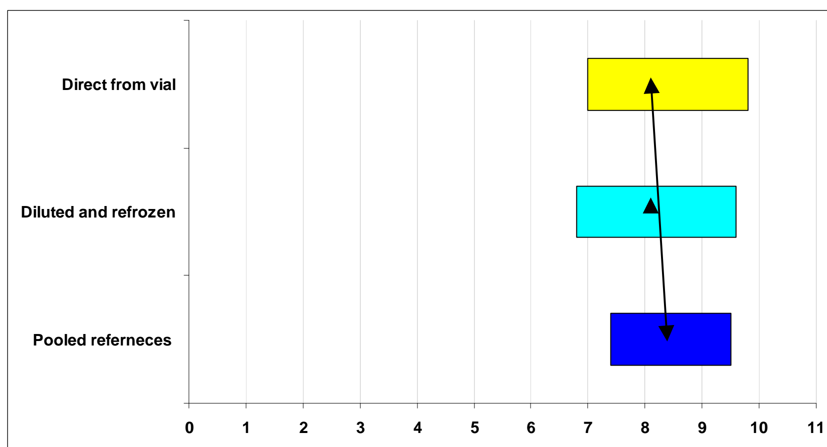


Figure 31, Floating bars for the test of diluting/refrozen

Summary

The refreezing of the test-organism before inoculation onto carrier seams not to have an impact on the D-value.

5.2.6 Impact of dilution solution on D-value

These tests show that there is no difference between the spores diluted in water or in 40% ethanol before spray inoculation. The D-values overlap each other (see figure 33). This is a confirmation of earlier testes that was made at Tetra Pak.

	D-value	D-value min-max	R^2
Test diluted in water	8,2	7,3-9,2	0,947
Pooled references (see 5.6) All diluted in ethanol	8,4	7,4-9,5	0,870

Table 9, Test of dilution solution

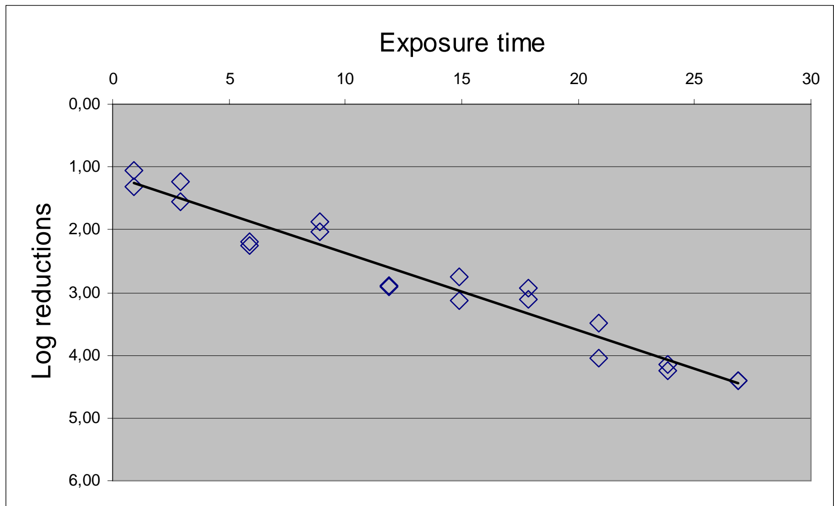


Figure 32, Diluted in water

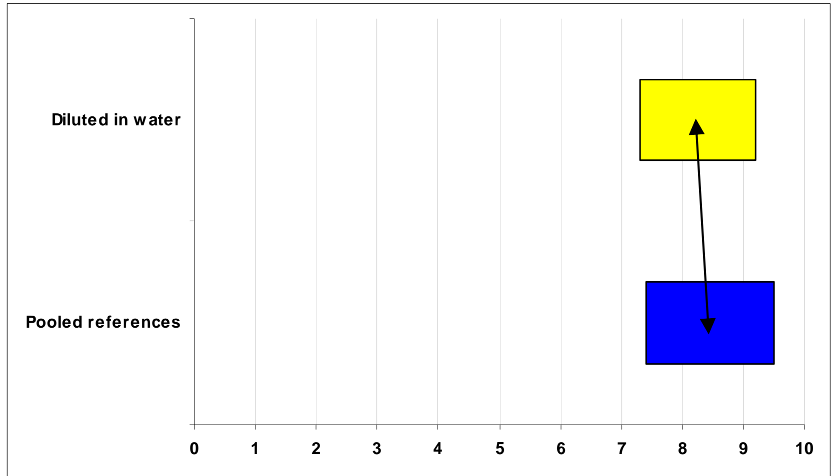


Figure 33, Floating bars for the test of dilution solution

Summary

The dilution solution seems not to have an impact on the D-value.

5.2.7 Test of old spore batch

The results from the test with a different batch of *B. subtilis* (960812) agree with results performed on this spore batch in 1996. This result proves that the procedures and methods are correctly performed. There is no difference between the tests because they overlap each other (see figure 35).

	D-value	D-value min-max	R^2
Summary	8,4	7,6-9,4	0,909
Test 1	8,4	7,1-10,2	0,888
Test 2	8,5	7,5-9,7	0,939
Tetra Pak test (Based on 3 tests)	8,1	7,7-8,5	0,985

Table 10, Batch test

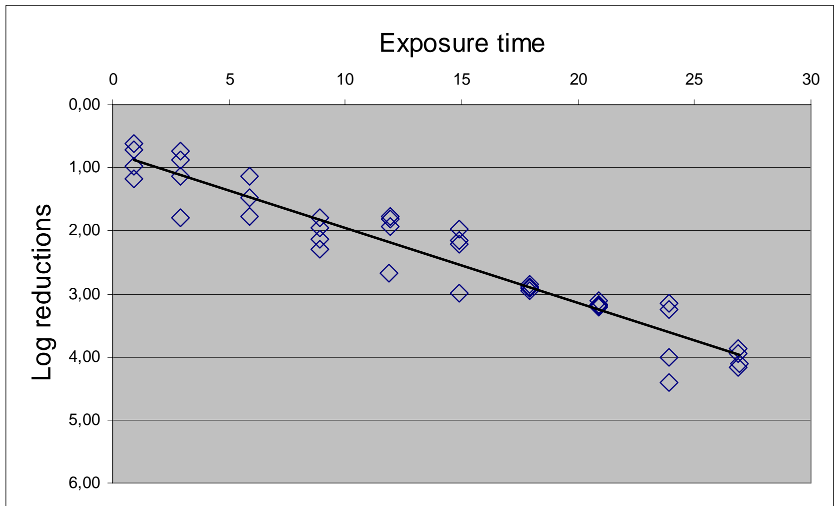


Figure 34, Summary of batch test

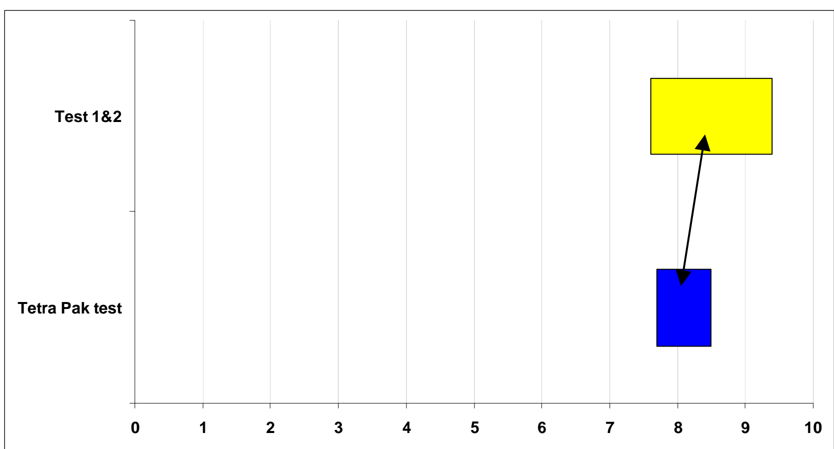


Figure 35, Floating bars for the test of batch

Summary

The test procedures are mastered since the same value was obtained. The low R^2 value might be due to the plastic beakers (see 5.3.2)

5.3 Test rig procedure

5.3.1 Stirring recovery test

Stirring	Number of samples	Mean value of recovered spores (log)	Standard deviation
1 min, pattern (Reference)	6	4,98	0,20
1 min	6	4,69	0,20
2 min	6	4,69	0,37
3 min	6	4,77	0,11
5 min	6	4,96	0,18

Table 11, Result from the first stirring test

Stirring with pattern in comparison with stirring without pattern; the amount of recovered spores points towards that stirring with pattern for 1 minute and stirring without pattern for 5 minutes gives the same result (see figure 36 and table 11).
There is also a trend that the recovery of spores increases with increasing stirring time. This result is logical and expected.

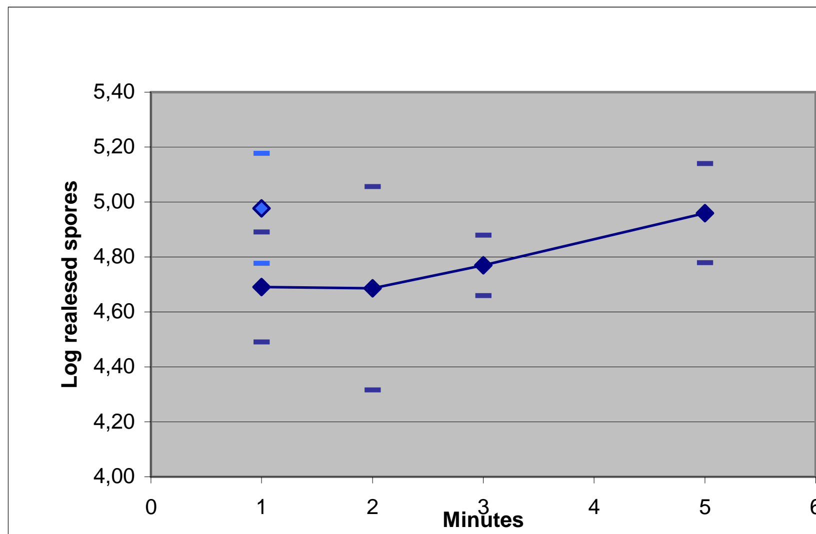


Figure 36, Stirring recovery

Even if the differences are within the allowed standard deviation (± 0,2 log units) there is a trend that the recovered spores increases with the increased time. 5 minutes seems to be equal with the standard pattern method. After the result from this first test it was interesting to continue this experiment and compare stirring without pattern for 5 and 10 minutes.

Stirring	**Number of samples**	**Mean value of released spores (log)**	**Standard deviation**
5 min	6	4,98	0,17
10 min	6	5,00	0,14

Table 12, Result from the second stirring test

The result from the second stirring test indicates that there is no difference between stirring for 5 and 10 minutes. There is a limit here when no more spores can be recovered with the standard load concentration (see table 12).

5.3.2 Impact of stirring in the bath on D-value

The results from these testes show if the positions of the stirrer affect the results or not. The obtained D-value is lower when the position of the stirrer is turned to the left or to the right in compared to the normal position. In comparison between right and left stirrer position there is no difference (see figure 39). This result indicates that the stirring in the bath have an impact, as indicated by

earlier tests made by Tetra Pak, a larger change in the stirring would probably have given a larger difference. If the turbulence in the bath increases a bigger amount of spores could rinse off.

	D-value	D-value min-max	R^2
Summary left	7,3	6,5-8,2	0,907
Test 1	7,6	6,6-9,0	0,917
Test 2	7,0	6,1-8,3	0,923
Summary right	7,0	6,1-8,1	0,858
Test 1	6,6	5,8-7,5	0,949
Test 2	7,1	6,3-8,2	0,935
Pooled references (see 5.6) All with stirrer nozzle in standard position	8,4	7,4-9,5	0,870

Table 13, Stirring in bath

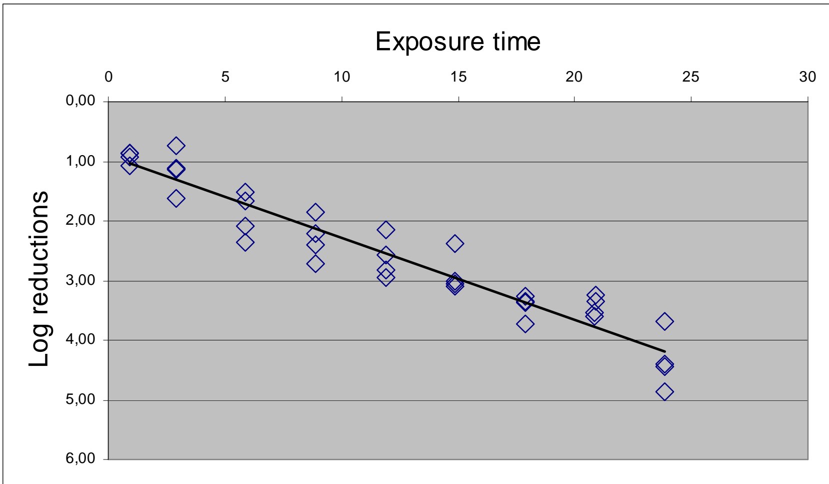


Figure 37, Summary of the tests of stirring left

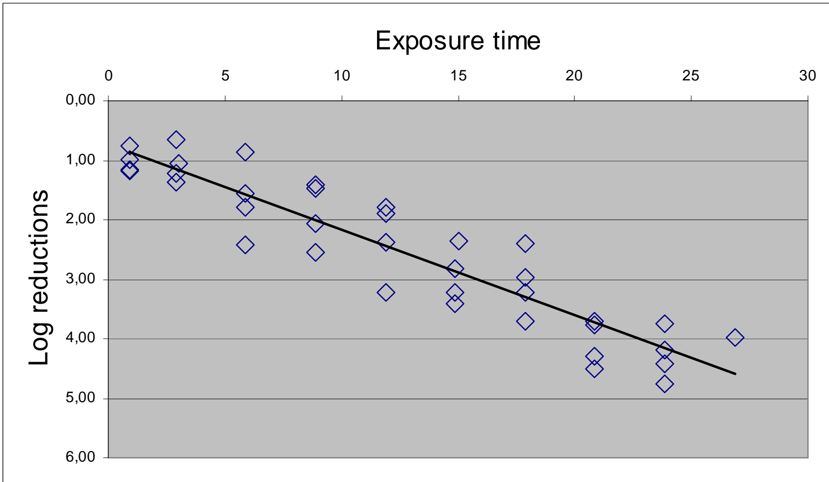


Figure 38, Summary of the tests of stirring right

As can be noted the spread in the result is larger when the stirrer nozzle is moved to the right. A possible explanation is that there is a shorter distance to the wall of the bath than when the nozzle is turned left.

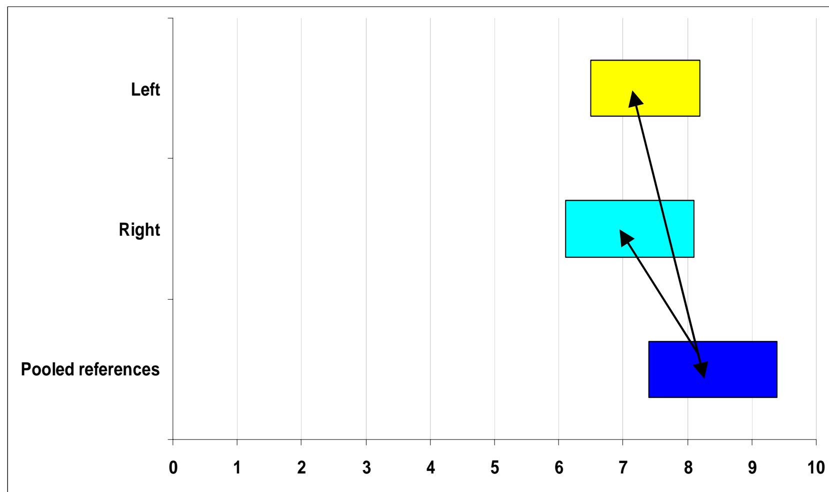


Figure 39, Floating bars for the test stirring in bath

Even if the difference between the tests is small, earlier tests by Tetra Pak have shown that a more extreme change in the stirring pattern will affect the D-value.

Summary

The stirring might affect the D-value and it's important to keep it constant. In the used test rig the impact of the tested changes of stirring was not major but still noticeable.

5.3.4 Impact of time from exposure to beaker on D-value

This test was done to see if the time between exposure and immersing in "stop-solution" affects the D-value. The results are spread but there is probably a small difference between these tests (10s before immersion) and reference (2s before immersing). The spread in the tests could depend on how much hydrogen peroxide that is shake-off after the dipping. Other factors that could affect the spread of results are the humidity and ambient temperature in the laboratory. The conclusion is that if the time from exposure to beaker takes 10 seconds that doesn't disturb the test remarkably (based on average D-value of tests), but the repeatability decreases.

	D-value	**D-value min-max**	**R^2**
Summary	8,5	7,7-9,6	0,856
Test 1	10,3	8,5-13,2	0,838
Test 2	8,4	7,5-9,6	0,938
Test 3	7,4	6,7-8,3	0,958
Pooled references (see 5.6) All with 2s to immersion	8,4	7,4-9,5	0,870

Table 14, Time from exposure to beaker

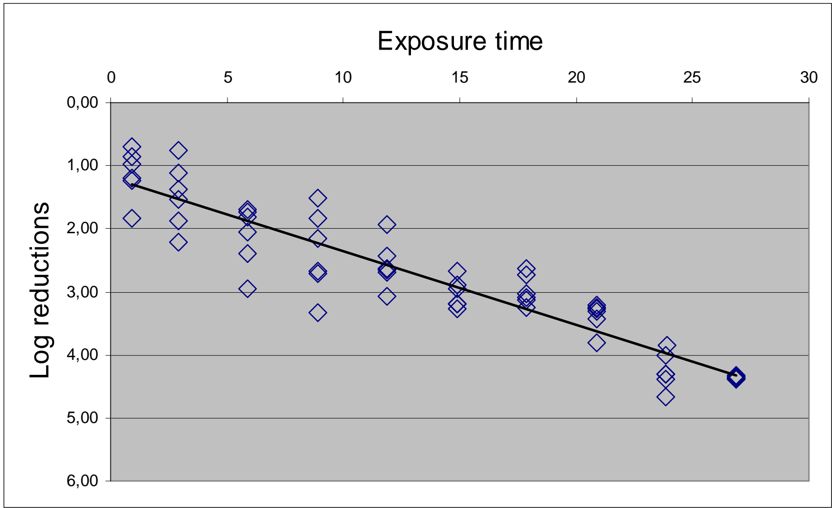


Figure 40, Summary of the tests on time from exposure to beaker

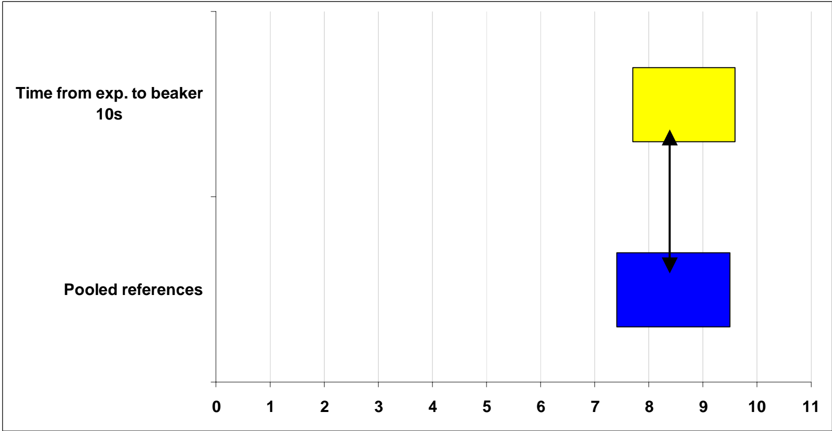


Figure 41, Floating bars for the test time from exposure to beaker

Summary

The time between exposure and immersing in "stop-solution" have a major impact on the repeatability of the result; with increased time the repeatability decreases.

5.3.4 Impact of test organisms lost in bath on D-value

These testes were made to see if there was a large amount of spores that was lost in the bath. The result shows (see figure 42) that the there is no large amount spores lost in the bath since the curve is almost constant and the spores that are lost in the bath doesn't disturb the "D-value".
If the amount of spores that are lost in the bath increases with increasing exposure time should the D-value be lower than the actual value.

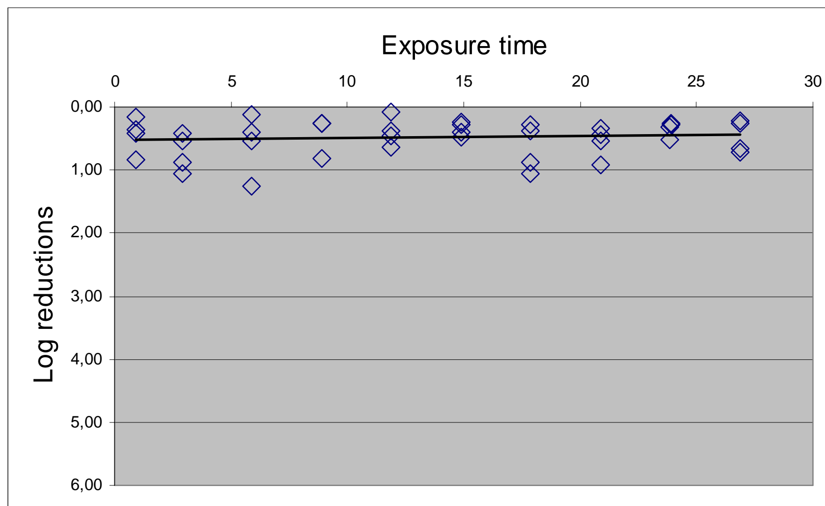


Figure 42, Spores lost in bath

Summary

The increased exposure time in the bath do not increase the loss in the bath.

5.3.5 Impact of beaker material on D-value

These tests were made at the same day and under the same conditions. The results show that using beakers of glass results in a lower D-value. The D-value in other tests has shown the D-value in plastic beaker to be around 8.4, which is the same as the glass beaker in this test.

The spread seems larger when using the plastic beaker if only these two tests are compared. However there are test done with plastic beaker that have just as good R^2 as the glass beakers show in this test (see 5.6). The plastic beakers were chosen to make the testing more rapid (no pre-sterilisation and no cleaning) and looking at the R^2 of the tests this might have been a bad choice. The discussion of the beaker material came up after the D-value change (see 5.1.1). It was decided to continue with the plastic beaker since the offer such an advantage in making the tests more rapid.

	D-value	D-value min-max	R^2
Test 1 beaker of glass	8,5	7,3-10,1	0,954
Test 2 beaker of plastic	9,4	6,9-14,6	0,810
Pooled references (see 5.6) All with plastic beakers (includes test 2)	8,4	7,4-9,5	0,870

Table 15, Beaker test

It can be noted that the R^2 of the reference tests have ranged from 0,81-0,96 (see 5.6).

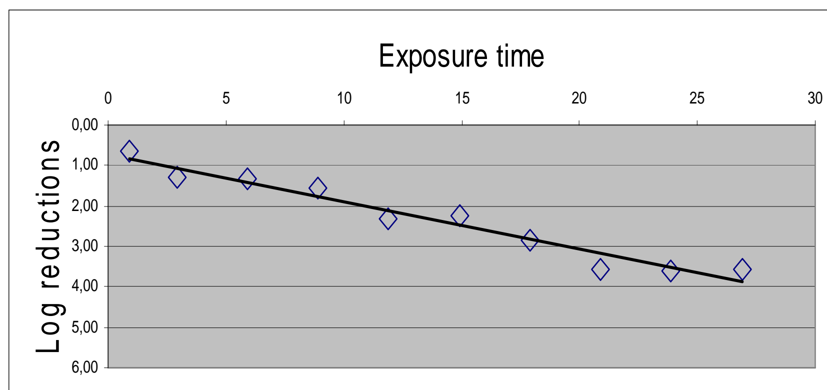


Figure 43, Beaker of glass

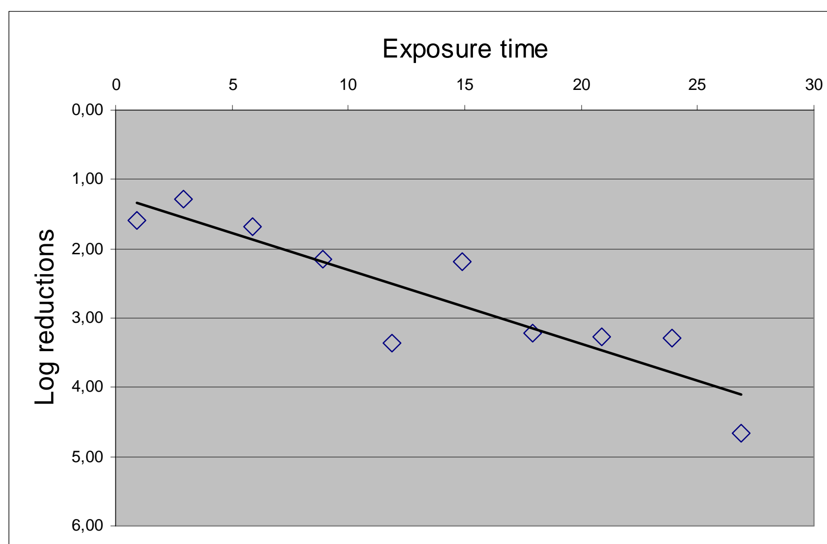


Figure 44, Beaker of plastic

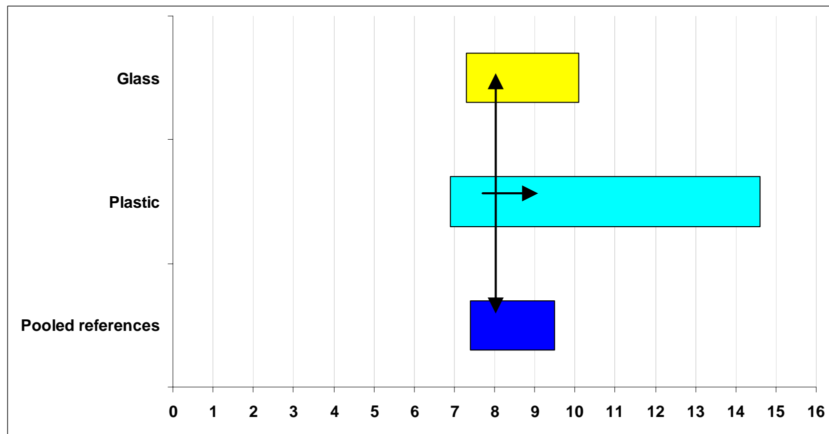


Figure 45, Floating bars for the test of beaker

Summary

The beaker material might have a large impact on the spread of the results

5.4 References (TBA/J 200)

This table shows the references to the tests that have been made. The reference samples have not been run parallel with the all tests, but they are run in the vicinity of the tests (not always in the same day). Since the reference tests for all tests are run under equal conditions only one test has been made in the vicinity of each test set-up. A D-value for the "pooled" reference samples has been calculated. Figure 46 – 53 shows the references from the different tests.

The references were run with:

- TBA/J 200 packaging material as carrier
- An load of 5,6-6,0 log BsA
- Spray inoculation of spores
- A diluted but not refrozen spore suspension before spraying
- A spore suspension kept and diluted in 40% ethanol before spraying
- The stirrer nozzle in a standard position
- A time from exposure to immersion in stop-solution of 2 seconds
- A recovery beaker made of plastic

The results from this test indicate stability and a natural variation. All tests are overlapping each other (see figure 56).

Test	D-value	Min-max	R^2
5.1.1. Thickness	Excluded due to D-value changes		
5.1.2. Material	9,2	7,7-11,4	0,865
5.1.3. Surface structure	Excluded since TBA/J 1000 was used		
5.2.1. Load	8,3	7,6-9,3	0,961
5.2.2. Inoculation	8,8	7,6-10,5	0,905
5.2.5. Diluted and refrozen/not refrozen	8,2	7,1-9,6	0,920
5.2.6. Dilution solution	8,2	7,3-9,2	0,947
5.3.2. Stirring in bath	8,1	7,0-9,7	0,909
5.3.4. Time from exposure to beaker	8,5	7,2-10,5	0,872
5.3.5. Beaker material	9,4	6,9-14,6	0,810
All reference tests pooled together	8,4	7,4-9,5	0,870

Table 16, D-value from the references

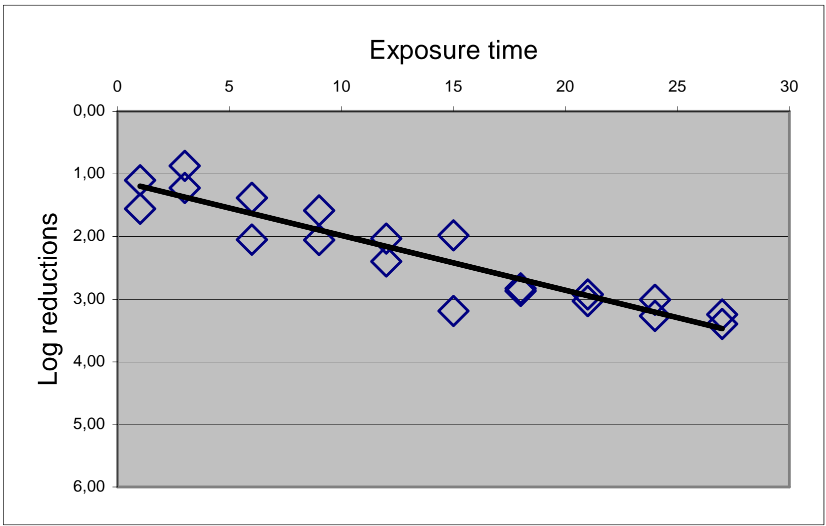


Figure 46, Material (5.1.2)

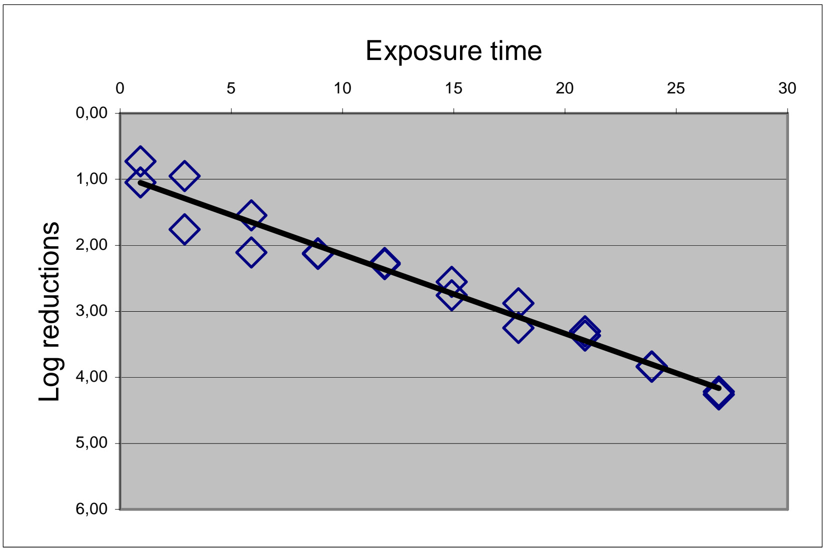


Figure 47, Load (5.2.1)

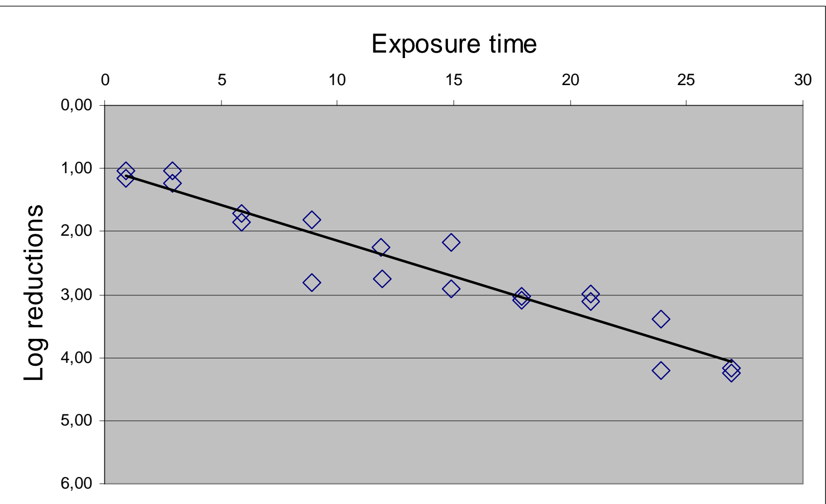


Figure 48, Inoculation (5.2.2)

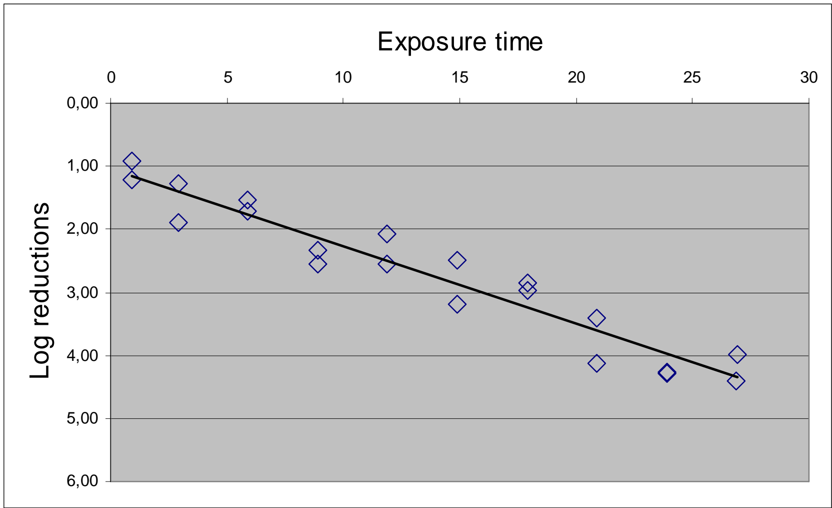


Figure 49, Diluted and not refrozen (5.2.5)

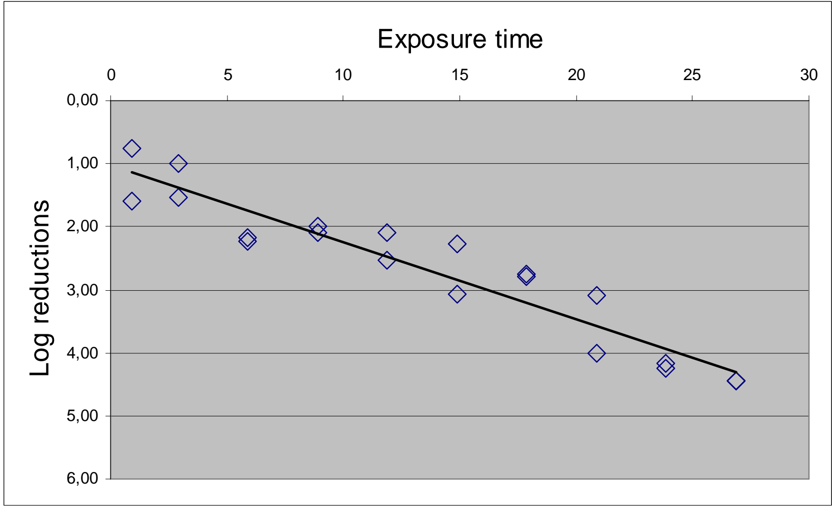


Figure 50, Dilution solution (5.2.6)

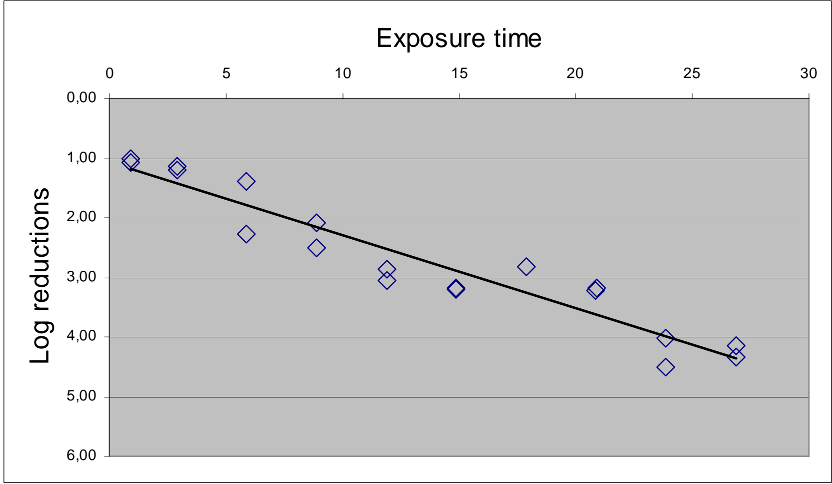


Figure 51, Stirring in bath (5.3.2)

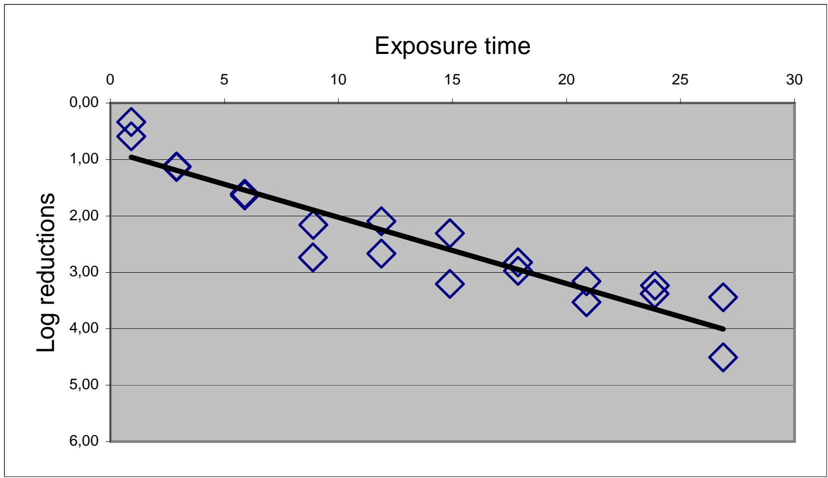


Figure 52, Time from exposure to beaker (5.3.4)

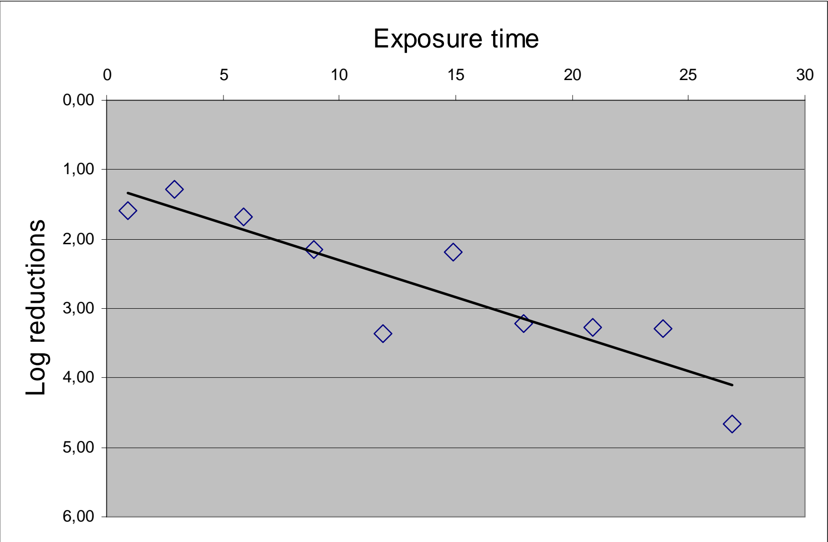


Figure 53, Beaker of plastic (5.3.5)

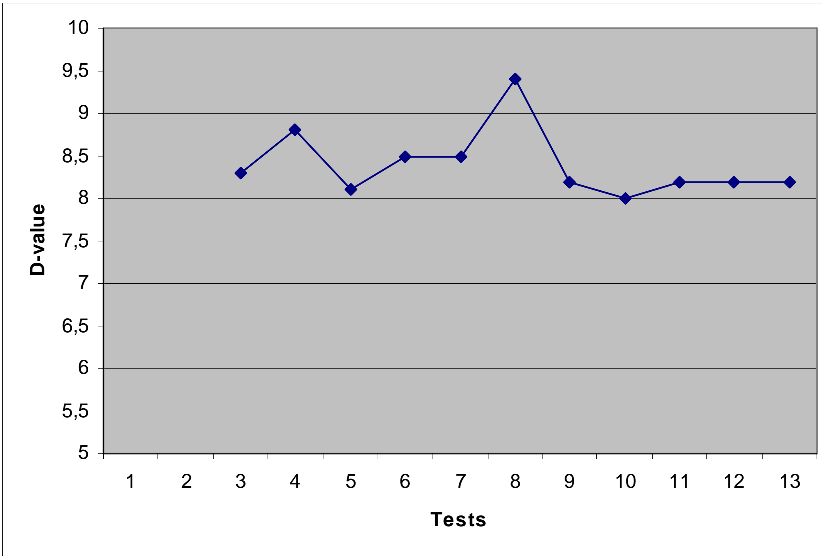


Figure 54, Curve on the references D-value

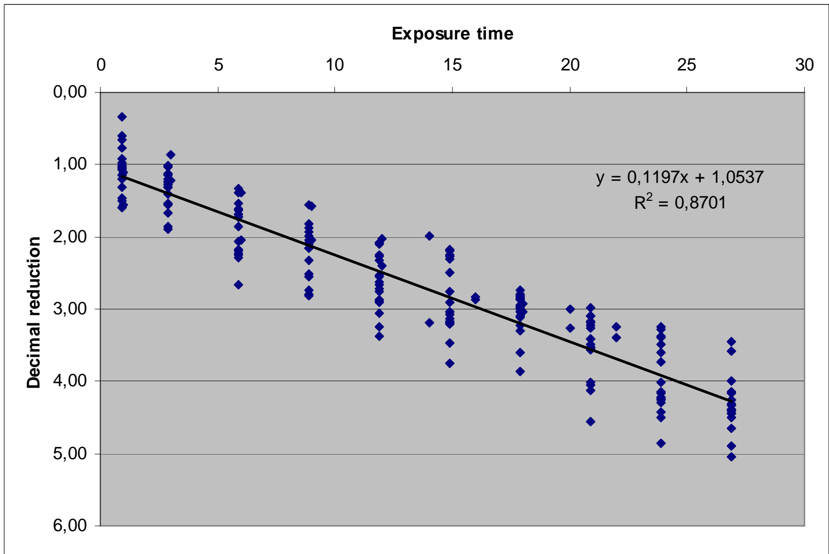


Figure 55, Summery of all the pooled references

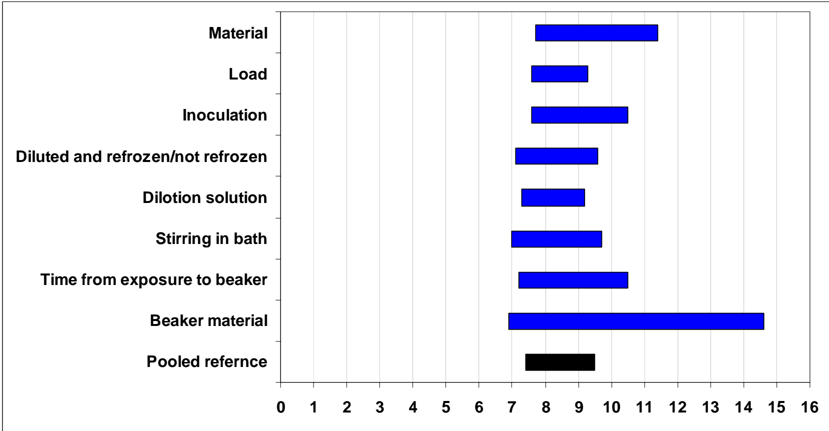


Figure 56, Floating bars for all the pooled references

6. Conclusions & Further investigation

6.1 Conclusions

Test	Results
Thickness	Packaging material of different thickness (at least up to 30%) can be used interchangeable in the test rig.
Material	It is clear that different materials affect the D-value and are not interchangeable in the test rig.
Surface	There is an indication that different surface structure affects the D-value and therefore is not interchangeable in the test rig.
Load *B. subtilis*	There is a large spread in the obtained results when using a low load (3,3 logs) and should therefore not be used when the D-value is determined. The indication is also that the lower load gives a lower D-value.
Inoculation methods	There is a difference in the D-value due to inoculation method. It is clear that the inoculation method has to be fixed (standardized). There is a risk that different drop inoculations methods might give different results.
Outgrowth rate	No conclusions could be drawn about the impact of outgrowth rate
Type of spore	It is important to know how the indicator organism behaves, if the log cycle reduction curve has a shoulder, a tail or is straight
Stirrers positions	The stirring might affect the D-value and it's important to keep it constant. In the test rig used the impact of the changes of stirring was not major but still noticeable.
Time from exposure to beaker	The time between exposure and immersing in "stop-solution" have a major impact on the repeatability of the D-value. With increased time the repeatability decreases.
Spores lost in bath	An increased exposure time in the bath does not increase the spore loss in the bath.
Beaker material	Impact repeatability/ spread

6.2 Further investigation

To further develop the robustness of the method the following tests are suggested:

- Stirring rate in the bath. Increase and decrease the stirring rate with the stirring arm in different positions.
- Time from the exposure to the beaker. Increase the time to 20 seconds from the exposure to the beaker to see how it affects the D-value.
- Load. To further investigate the effect of low load on the spread of the obtained result and the D-value. Maybe load of 4 logs should be used instead of 3,3 logs.

Following tests could also be investigated:

- Shape of carrier, a square formed carrier tests against the round standard carrier.
- The bath, the volume and form changed
- How deep the carrier dips, different deepness's test

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[13] http://www.tetrapak.com
[14] http://144.92.196.21/pdf/resources/whey/bleachingnumb.pdf

8. Appendix

8.1 Material

The materials that are used in the different tests are:

- Spores: *Bacillus subtilis* NCA 7252 batch: SGM 020307
  Bacillus subtilis NCA 7252 batch: 960812
  Bacillus pumilus ATCC 27142 batch: 940505
  Bacillus globigi (Bacillus atrophaeus) ATCC 9372 batch: TR 200412
- Cutting equipment
- Spraying equipment (SAM)
- Ewa-rig
- Control unit
- Computer
- Gloves
- Tweezers
- Carriers
- Beakers
- Magnetic stirrer
- Magnetic spin bar
- 0,05 M phosphate buffer solution with catalase (Morrison and Reitger buffer with 0,005 % Tween)
- 0,02 M potassium permanganate $KMnO_4$ and 10% sulphuric acid H_2SO_4.
- H_2O_2, 35 %
- Petri dishes
- PCA – Plate Count Agar
- Burner