

EFFICACY TESTING OF POTENTIAL ISAV DISINFECTANTS

FINAL REPORT

PREPARED FOR

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EXECUTIVE SUMMARY

The objective of the current study was to design and execute a series of prescribed experiments to test the following seventeen disinfectant products/procedures for their ability to inactivate ISAV both in the absence and presence of blood/mucus material:

1. Germkill (150 ppm, 15 min, 15°C)
2. Mikroklene (150 ppm, 15 min, 15°C)
3. Microdyne (150 ppm, 15 min, 15°C)
4. Adage (150 ppm, 15 min, 15°C)
5. Ovadine (150 ppm, 15 min, 15°C)
6. Ovadine (108 ppm, 15 min, 15°C)
7. Buffodine (108 ppm, 15 min, 15°C)
8. Hibitane (27 ml/L, 15 min, 15°C)
9. Hyperox (8 ml/L, 15 min, 15°C)
10. Virkon (1:100 dilution, 20 min, 15°C)
11. Formic acid (pH 3.5, 8 hr, 15°C)
12. Bleach (40 ml/L, 15 min, 15°C)
13. Heat (60°C, 10 min)
14. Heat (100°C, 30 s)
15. Sodium dodecyl sulphate (SDS) (500 µM, 1 hr, 15°C)
16. Sodium dodecyl sulphate (SDS) (100 µM, 1 hr, 15°C)
17. Savlon (1:30, ~1/2 hr (no blood/mucus), ~1 hr (blood/mucus), 15°C for 15 min then 4°C for remainder of incubation)*

* incubation period adjusted from original proposal; see attached report for explanation

For the testing in the absence of blood/mucus material, only 3 treatments did not show at least a 3 log reduction of ISAV beyond cytotoxic levels:

- 1) Heat (100°C, 30 s)
- 2) SDS (500 µM, 1 hr, 15°C)
- 3) SDS (100 µM, 1hr, 15°C)

For the testing in the presence of blood/mucus material, 8 treatments did not show at least a 3 log reduction of ISAV beyond cytotoxic levels:

- | | |
|--|-------------------------------------|
| 1) Ovadine (108 ppm, 15 min, 15°C) | 5) Ovadine (150 ppm, 15 min, 15°C) |
| 2) SDS (100 µM, 1 hr, 15oc) | 6) Hibitane (27 ml/L, 15 min, 15°C) |
| ** 3) Mikroklene (150 ppm, 15 min, 15°C) | 7) SDS (500 µM, 1 hr, 15°C) |
| 4) Buffodine (108 ppm, 15 min, 15°C) | **8) Savlon (1:30, ~1 hr, 15°C) |

** further tests might show Mikroklene and Savlon capable of producing at least a 3 log reduction of ISAV beyond cytotoxic levels; see attached report for explanation

1.0 INTRODUCTION

One of the key factors in achieving containment of infectious salmon anemia virus (ISAV) is the need to ensure that there are effective disinfectant products and procedures available to salmon growers. Such products are needed not only to disinfect surfaces with which potentially infected fish material may come into contact, but also to disinfect the surfaces of eggs to minimize the potential for vertical transmission of the virus to succeeding generations.

Within the industry there are several products utilized for disinfection, most of which have not been tested for the ability to kill ISAV. As such, in order to have confidence in disinfection procedures, the industry requires data on the subject. After discussion amongst NBSGA, NBDAFA and RPC, seventeen disinfectants/treatments were selected for testing.

2.0 OBJECTIVE

The objective of the current study was to design and execute a series of prescribed experiments to test the following seventeen disinfectant products/procedures for their ability to inactivate ISAV both in the presence and absence of blood/mucus material:

1. Germkill (150 ppm, 15 min, 15°C)
2. Mikroklene (150 ppm, 15 min, 15°C)
3. Microdyne (150 ppm, 15 min, 15°C)
4. Adage (150 ppm, 15 min, 15°C)
5. Ovadine (150 ppm, 15 min, 15°C)
6. Ovadine (108 ppm, 15 min, 15°C)
7. Buffodine (108 ppm, 15 min, 15°C)
8. Hibitane (27 ml/L, 15 min, 15°C)
9. Hyperox (8 ml/L, 15 min, 15°C)
10. Virkon (1:100 dilution, 20 min, 15°C)
11. Formic acid (pH 3.5, 8 hr, 15°C)
12. Bleach (40 ml/L, 15 min, 15°C)
13. Heat (60°C, 10 min)
14. Heat (100°C, 30 s)
15. Sodium dodecyl sulphate (SDS) (500 µM, 1 hr, 15°C)
16. Sodium dodecyl sulphate (SDS) (100 µM, 1 hr, 15°C)
17. Savlon (1:30, ~1/2 hr (no blood/mucus), ~1 hr (blood/mucus), 15°C for 15 min then 4°C for remainder of incubation)*

*Originally the incubation period for Savlon was to be for 15 min. Due to problems with cytotoxic effects, methodology had to be adjusted and this affected incubation times.

3.0 MATERIALS AND METHODS

3.1 Generation of ISAV

ISAV for use in efficacy testing in the absence of blood/mucus was generated by inoculating Bay of Fundy isolates onto salmon head kidney (SHK) cell monolayers grown in Leibovitz's L-15 media (with glutamine) (Gibco BRL) supplemented with 5% fetal bovine serum (FBS), 0.07% β -mercaptoethanol and 1 x antibiotic-antimycotic (Gibco BRL). Infection was allowed to proceed at 15°C until a strong cytopathic effect (CPE) had developed. ISAV-infected cell lysate was harvested and stored at -80°C. Lysate from uninfected cells was also stored for use in negative controls. ISAV was titered by TCID₅₀ determination using the Spearman-Kärber method (1,2).

3.2 Generation of Blood/Mucus Material

Approximately 200 certified disease-free salmon of 10 g average size were acclimated for 10 days at 8-10°C in 300 L tanks, approximately 100 fish per tank, using a flow rate of 5 L/min. One tank of fish was then injected intraperitoneally with 0.2 ml of ISAV generated from SHK cells showing a high degree of cytopathology. The other tank of approximately 100 fish was left untouched. Infection was allowed to proceed in the injected fish until mortalities began to appear (approximately 10 days post injection). Immunofluorescent antibody tests (IFAT) for ISAV (6) were done on kidney imprints from the morts. On day 11 post-injection surviving fish were sacrificed and blood and mucus material was collected and stored at -80°C. The same was done for the tank of fish which received no ISAV injection. ISAV was titered from the infected blood/mucus by TCID₅₀ determination using the Spearman-Kärber method (1,2). The blood/mucus material from the unchallenged fish was applied to the SHK cell line and analyzed by ISAV RT-PCR.

3.3 Neutralization and Toxicity Testing of Disinfectants

Three approaches were tested for their ability to remove cytotoxic reactions produced by test mixtures on the SHK cell line: i) secondary chemicals (eg. sodium thiosulphate for iodophor-containing compounds) as recommended by chemists, suppliers, etc. were added to test mixtures in an effort to neutralize their toxic components; ii) test mixtures were spun through Centricon 100 or 300 filtration columns (Millipore), designed to remove unwanted compounds (eg. detergents) dictated by the pore size and chemistry of the filter; iii) test mixtures were diluted in Leibovitz's L-15 media (with glutamine) supplemented with 5% FBS and 1 x antibiotic-antimycotic. For all of these approaches duplicate 0.1 ml samples were simultaneously inoculated into 1 ml of SHK cells, at a concentration allowing 80-95% confluency, in wells of a 24-well cell culture plate. Ten-fold dilutions of the test mixtures were also made and applied to SHK cells to determine at what point cytotoxic reactions could be diluted out. Cells were incubated at 15°C and monitored for cytotoxic effects for approximately 1 week.

3.4 Efficacy Testing of Disinfectants/Procedures

Note: for the testing in the presence of blood/mucus, ISAV-infected blood/mucus was substituted for ISAV below and uninfected blood/mucus for ISAV-negative culture material below.

3.4.1 Disinfectant/Procedure Testing (performed in duplicate)

Frozen stocks of ISAV were removed from -80°C, thawed and suspended in Hanks' salt solution. Disinfectants were prepared at double the test concentration in distilled water. Equal volumes of the ISAV mixture (in Hanks' salt solution) and the double-concentrated disinfectants were mixed and incubated at 15°C for the test incubation period. Immediately thereafter 8 serial ten-fold dilutions were made in Lebovitz's L-15 media (with L-glutamine) supplemented with 5% FBS and 1 x antibiotic-antimycotic. In the case of Savlon, reaction mixtures were spun through Centricon 300 columns after the 15°C incubation and then serially ten-fold diluted. Note that spins were at 4°C for ~15 min (no blood/mucus) or ~45 min (presence of blood/mucus). The amount of contact time with ISAV is approximate as it was dependent on how quickly the mixture spun through the filter column. From each dilution 0.1 ml was inoculated in quadruplicate into SHK-containing wells. Inoculated wells were incubated at 15°C for 31 days and monitored for CPE.

For the heat treatments, ISAV suspended in Hanks' salt solution was added to an equal volume of water and incubated at 60°C or 100°C. The mixtures were cooled and eight serial ten-fold dilutions were then made and plated as above. The undiluted treated sample was also plated.

3.4.2 Cytotoxicity Control (performed one replicate)

The same was done as above (3.4.1) except frozen ISAV-negative culture material was used and only 3 serial ten-fold dilutions were made and plated in quadruplicate.

3.4.3 ISAV Positive Control (performed one replicate)

The same was done as above (3.4.1) except that the ISAV suspended in Hanks' salt solution was mixed with an equal volume of water and not double the test concentration of disinfectant. For the heat treatments, this positive control was not incubated at the test temperatures of 60°C and 100°C.

3.4.4 Negative Media Control (performed one replicate)

The same was done as above (3.4.1) except ISAV-negative culture material suspended in Hanks' salt solution was used and mixed with an equal volume of water and not double the test concentration of disinfectant. No serial ten-fold dilutions were made. The mixture was plated directly into SHK-containing wells in quadruplicate.

3.4.5 Neutralized (by dilution) Disinfectant Control (performed one replicate)

Frozen stocks of ISAV were removed from -80°C, thawed and suspended in Hanks' salt solution. Disinfectants were prepared at double the test concentration in distilled water. Equal volumes of the ISAV mixture (in Hanks' salt solution) and water were mixed and incubated at 15°C for the test incubation period. Equal volumes of double the test concentration of disinfectant and Hanks' salt solution were mixed and incubated at 15°C for the test incubation period. Immediately thereafter 0.1 ml from each mixture was added to 0.8 ml of Lebovitz's L-15 media (with L-glutamine) supplemented with 5% FBS and 1 x antibiotic-antimycotic. Seven more serial ten-fold dilutions were made in the same media. For Savlon, after the 15°C incubation period, mixtures were spun through Centricon 300 columns prior to dilution as above. For heat treatments samples were allowed to cool prior to dilution. From each dilution 0.1 ml was inoculated in quadruplicate into SHK-containing wells. Inoculated wells were incubated at 15°C for 31 days and monitored for CPE.

4.0 RESULTS

4.1 Generation of ISAV

The generation of ISAV stock for use in efficacy tests in the absence of blood/mucus was achieved through inoculation of ISAV isolates from the Bay of Fundy onto the SHK cell line. A high titer was achieved allowing approximately 10^6 TCID₅₀/0.1 ml to be used for experiments in the absence of blood/mucus.

4.2 Generation of Blood/Mucus Material

The generation of infected blood/mucus material was achieved by challenging Atlantic salmon with 10^7 TCID₅₀ ISAV and sacrificing the population once infection had been established. This required approximately 10 days. Establishment of infection was confirmed by examining the first few morts for pathology and identifying ISAV in kidney imprints by IFAT. The blood/mucus material collected from the sacrificed fish allowed for ISAV titers of approximately 10^5 TCID₅₀/0.1 ml to be used for efficacy testing in the presence of blood mucus.

Blood/mucus material was also collected from Atlantic salmon not injected with ISAV. The material was confirmed to be negative for the pathogen by the lack of CPE development on the SHK cell line and the lack of ISAV nucleic acid amplification in reverse transcriptase polymerase chain reaction (RT-PCR) on RNA extracted from the blood/mucus material. RT-PCR was as in Griffiths *et al* (3) only primers FA3 and RA3 were used (5).

4.3 Neutralization and Toxicity Testing of Disinfectants

Attempts to neutralize disinfectants had two objectives in mind: i) to control the exposure time of ISAV to the active ingredients of the disinfectants; ii) to limit the toxicity of the disinfectants to the SHK cell line. Toxicity on the cell line masks any CPE development by ISAV itself and is therefore undesirable. Attempts were made to neutralize and detoxify treatment mixtures by utilizing Centricon 100 or 300 columns (Millipore) with the objective of retaining ISAV particles while filtering away active disinfectant ingredients. Although this method showed some promise in some instances for reducing cytotoxicity, it was also shown that sometimes enough chemical was retained to continue killing ISAV beyond the test incubation period. Other attempts to neutralize treatment mixtures included the addition of neutralizing chemicals as recommended by chemists, suppliers, etc. In most cases the resulting mixtures remained very toxic to SHK cells when 0.1 ml were plated. In these cases cytotoxicity could be removed by diluting the mixtures ten to one thousand-fold depending on the disinfectant. It was also determined that this same dilution of test mixtures, to which no neutralizing chemicals had been added, could achieve comparable cytotoxicity results. This latter approach had the benefit of reduced risk of introducing any secondary compounds to the mixture potentially created between the neutralizing chemicals and the disinfectants, which might ultimately affect the viability of ISAV. Therefore, this latter approach was adopted along with controls during the efficacy testing to ensure that the disinfectants themselves were no longer active in their diluted state. Note that for Savlon, prior to performing dilution of treatment mixtures, it was necessary to employ Centricon 300 columns to remove some of the cytotoxic compounds. The dilution at which the reaction mixture could be plated without cytotoxic effects represented the point at which CPE's would be compared against positive controls. This dictated the maximum log reduction value that could be viewed for each chemical. Due to differences in the cytotoxicity limits and neutralization strategy of each chemical, it was possible to confirm higher log reduction values for some chemicals than for others.

4.4 Efficacy Testing of Disinfectants/Procedures

As stated above due to differences in the cytotoxicity limits and neutralization strategy for each chemical, it was possible to confirm higher log reduction values for some chemicals than for others. As well slight differences existed between testing days in the positive control titer allowing for a slightly higher or lower degree of difference between the positive control and the treated ISAV. Therefore, any comparisons made between chemicals/procedures should, in fairness, consider the limit at which each chemical could maximally show effectiveness based on methods adopted in this study. Could cytotoxicity issues be equally resolved for all chemicals, variation amongst log reduction values would be based solely on efficacy and not on a combination of efficacy, starting ISAV titers, and cytotoxicity differences as they existed in this study. **When cytotoxicity exists, virucidal data is acceptable when at least a 3 log reduction in titer can be demonstrated beyond the cytotoxic level (4).** It was this standard that was considered when evaluating efficacy results.

4.4.1 Absence of Blood/Mucus

Table 1 outlines the data obtained for testing in the absence of blood/mucus material. Raw data can be seen in attached appendices. Note that TCID₅₀ values/0.1 ml for the positive controls were approximately 10⁶, but did vary some from test day to test day (column 2). Cytotoxicity control values (column 3) varied simply because some chemicals/treatments were more toxic to the cells than others (eg. bleach (TCLD₅₀ = 1.50) produced greater toxic effects on SHK than formic acid (TCLD₅₀ = 0.50)). Replicate treatments for each chemical (columns 4 and 5) agreed well and in those instances where slight differences existed, an average value was calculated and used to determine inactivation. Little variation occurred between post neutralization control values (column 6) and positive control values (column 2) indicating that the dilution done of the treatment mixtures immediately following their incubation periods (or the use of Centricon 300 columns plus dilution in the case of Savlon) successfully inactivated the chemicals against ISAV. Log₁₀ virus inactivation values (column 7) were calculated as follows:

$$\text{inactivation} = (\text{positive control} - \text{cytotoxicity control}) - (\text{treatment} - \text{cytotoxicity control})$$

Based on EPA (Environmental Protection Agency) standards (4), **chemicals were considered to be efficient in inactivating ISAV when they demonstrated at least a 3 log reduction beyond the cytotoxic level. Of the 17 chemical/treatments tested only 3 did not meet this requirement: heat (100°C, 30s), SDS (500 µM, 1 hr, 15°C), SDS (100 µM, 1 hr, 15°C).**

4.4.2 Presence of Blood/Mucus

Table 2 outlines the data obtained for testing in the presence of blood/mucus material. Raw data can be seen in attached appendices. Note that TCID₅₀ values/0.1 ml for the positive controls were approximately 10⁵, but did vary some from test day to test day (column 2). Cytotoxicity control values (column 3) varied simply because some chemicals/treatments were more toxic to the cells than others (eg. Hyperox (TCLD₅₀ = 1.50) produced greater toxic effects on SHK than Virkon (TCLD₅₀ = 0.75)). Replicate treatments for each chemical (columns 4 and 5) agreed well in most cases and in those instances where slight differences existed, an average value was calculated and used to determine inactivation. The only replicate treatment values which did not agree well were those of Mikroklene (ie. treatment values = 10^{3.75} and 10^{1.50}). More replicate values would have been useful here to give a more confident value for efficacy. Little variation occurred between post neutralization control values (column 6) and positive control values (column 2) indicating that the dilution done of the treatment mixtures immediately following their incubation periods (or the use of Centricon 300 columns plus dilution in the case of Savlon) successfully inactivated the chemicals against ISAV. Log₁₀ virus inactivation values (column 7) were calculated as follows:

$$\text{inactivation} = (\text{positive control} - \text{cytotoxicity control}) - (\text{treatment} - \text{cytotoxicity control})$$

Based on EPA standards (4), **chemicals were considered to be efficient in inactivating ISAV when they demonstrated at least a 3 log reduction beyond the cytotoxic level. Of the 17 chemical/treatments tested 8 did not meet this requirement: Ovadine (108 ppm, 15 min, 15°C), SDS (100 µM, 1 hr, 15°C), Mikroklene (150 ppm, 15 min, 15°C), Buffodine (108 ppm, 15 min, 15°C), Ovadine (150 ppm, 15 min, 15°C), Hibitane (27 ml/L, 15 min, 15°C), SDS (500 µM, 1 hr, 15°C), and Savlon (1:30 dilution, ~1 hr (ie.15°C for 15min, 4°C for ~45 min))**. Note however, that Savlon performed well for the window of opportunity which existed for log reduction to be definitively observed. Slightly lower initial ISAV titers and the difficulties encountered detoxifying Savlon made it difficult to observe reduction. Centricon 300 columns followed by dilution improved toxicity levels but not quite enough. Should the level of toxicity be further resolved testing might show this chemical to meet the 3 log reduction criteria set for the others. As well, further treatment replicates for Mikroklene, which showed more severe variability as compared to replicates for other chemicals, might also confirm Mikroklene capable of producing at least a 3 log reduction in ISAV titer.

Table 1. Data for efficacy testing in the **absence** of blood/mucus

Chemical or Treatment	Positive Control TCID ₅₀ /0.1ml	Cytotoxicity Control TCLD ₅₀ /0.1ml	Treatment Replicate 1 TCID ₅₀ /0.1ml ¹	Treatment Replicate 2 TCID ₅₀ /0.1ml ¹	Post Neutralization TCID ₅₀ /0.1 ml ¹	Virus Inactivation log ₁₀
60°C, 10 min	10 ^{6.50}	10 ^{-0.50}	10 ^{-0.50}	10 ^{-0.50}	10 ^{6.25}	10 ^{7.00}
100°C, 30 s	10 ^{6.50}	10 ^{-0.50}	10 ^{3.75} (Avg. 10 ^{3.62})	10 ^{3.50} (Avg. 10 ^{3.62})	10 ^{6.00}	10 ^{2.88}
Formic Acid	10 ^{5.75}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{6.00}	10 ^{5.25}
SDS (500 µM)	10 ^{5.75}	10 ^{0.50}	10 ^{3.00}	10 ^{3.00}	10 ^{5.50}	10 ^{2.75}
SDS (100 µM)	10 ^{5.75}	10 ^{0.50}	10 ^{6.00} (Avg. 10 ^{5.75})	10 ^{5.50} (Avg. 10 ^{5.75})	10 ^{5.75}	10 ^{0.00}
Ovadine (108 ppm)	10 ^{6.25}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{6.50}	10 ^{5.75}
Virkon	10 ^{6.25}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{6.00}	10 ^{5.75}
Ovadine (150 ppm)	10 ^{6.00}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.75}	10 ^{5.50}
Microdyne	10 ^{6.25}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.75}	10 ^{5.75}
Mikroklene	10 ^{5.75}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.75}	10 ^{5.25}
Adage	10 ^{6.25}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.50}	10 ^{5.75}
Germkill	10 ^{5.75}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{6.00}	10 ^{4.25}
Bleach	10 ^{6.25}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{6.00}	10 ^{4.75}
Buffodine	10 ^{6.25}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{6.00}	10 ^{4.75}
Hyperox	10 ^{6.25}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.75}	10 ^{4.75}
Hibitane	10 ^{6.00}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.25}	10 ^{4.50}
Savlon	10 ^{5.50}	10 ^{2.50}	10 ^{2.50}	10 ^{2.50}	10 ^{5.50}	10 ^{3.00}

Notes: ¹ TCID₅₀ here includes CPE from ISAV infection and cytotoxicity reaction.

Table 2. Data for efficacy testing in the **presence** of blood/mucus

Chemical or Treatment	Positive Control TCID ₅₀ /0.1ml	Cytotoxicity Control TCLD ₅₀ /0.1ml ²	Treatment Replicate 1 TCID ₅₀ /0.1ml ¹	Treatment Replicate 2 TCID ₅₀ /0.1ml ¹	Post Neutralization TCID ₅₀ /0.1 ml ¹	Virus Inactivation log ₁₀
100°C, 30 s	10 ^{5.00}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.25}	10 ^{4.50}
60°C, 10 min	10 ^{5.00}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{5.25}	10 ^{4.50}
Bleach	10 ^{5.25}	10 ^{0.50}	10 ^{0.50}	10 ^{0.50}	10 ^{4.75}	10 ^{4.75}
Virkon	10 ^{5.25}	10 ^{0.75}	10 ^{0.75} (Avg.10 ^{0.62})	10 ^{0.50} (Avg.10 ^{0.62})	10 ^{5.00}	10 ^{4.63}
Formic Acid	10 ^{5.50}	10 ^{0.75}	10 ^{0.50} (Avg.10 ^{0.62})	10 ^{0.75} (Avg.10 ^{0.62})	10 ^{5.25}	10 ^{4.88}
Ovadine (108 ppm)	10 ^{4.75}	10 ^{1.25}	10 ^{4.75} (Avg.10 ^{5.00})	10 ^{5.25} (Avg.10 ^{5.00})	10 ^{5.25}	10 ^{-0.25}
SDS (100 µM)	10 ^{5.25}	10 ^{1.25}	10 ^{5.00} (Avg.10 ^{5.12})	10 ^{5.25} (Avg.10 ^{5.12})	10 ^{5.25}	10 ^{0.13}
Mikroklene	10 ^{5.00}	10 ^{1.25}	10 ^{3.75} (Avg.10 ^{2.62})	10 ^{1.50} (Avg.10 ^{2.62})	10 ^{5.00}	10 ^{2.38}
Buffodine	10 ^{4.75}	10 ^{1.38}	10 ^{4.75} (Avg.10 ^{4.62})	10 ^{4.50} (Avg.10 ^{4.62})	10 ^{5.25}	10 ^{0.13}
Ovadine (150 ppm)	10 ^{5.25}	10 ^{1.50}	10 ^{4.75}	10 ^{4.75}	10 ^{5.50}	10 ^{0.50}
Microdyne	10 ^{5.25}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.75}	10 ^{3.75}
Adage	10 ^{4.50}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.25}	10 ^{3.00}
Germkill	10 ^{5.00}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.25}	10 ^{3.50}
Hyperox	10 ^{5.25}	10 ^{1.50}	10 ^{1.50}	10 ^{1.50}	10 ^{5.25}	10 ^{3.75}
Hibitane	10 ^{5.25}	10 ^{1.50}	10 ^{2.75} (Avg.10 ^{2.62})	10 ^{2.50} (Avg.10 ^{2.62})	10 ^{5.25}	10 ^{2.63}
SDS (500 µM)	10 ^{5.25}	10 ^{1.88}	10 ^{5.25} (Avg.10 ^{5.50})	10 ^{5.75} (Avg.10 ^{5.50})	10 ^{5.50}	10 ^{-0.25}
Savlon	10 ^{4.25}	10 ^{2.50}	10 ^{2.50}	10 ^{2.50}	10 ^{3.50}	10 ^{1.75}

Notes: ¹ TCID50 here includes CPE from ISAV infection and cytotoxicity reaction.

² See Appendix B for notes on the calculation of this value.

Table 3. Chemicals/procedures which showed at least a 3 log reduction in ISAV titer

Chemical/Procedure	Absence of Blood/Mucus	Presence of Blood/Mucus
Heat (100°C, 30 s)		✓
Heat (60°C, 10 min)	✓	✓
Bleach (40 ml/L, 15 min, 15°C)	✓	✓
Virkon (1:100, 20 min, 15°C)	✓	✓
Formic Acid (pH 3.5, 8hr, 15°C)	✓	✓
Ovadine (108 ppm, 15 min, 15°C)	✓	
SDS (100 µM, 1 hr, 15°C)		
Mikroklene (150 ppm, 15 min, 15°C)	✓	see note
Buffodine (108 ppm, 15 min, 15°C)	✓	
Ovadine (150 ppm, 15 min, 15°C)	✓	
Microdyne (150 ppm, 15 min, 15°C)	✓	✓
Adage (150 ppm, 15 min, 15°C)	✓	✓
Germkill (150 ppm, 15 min, 15°C)	✓	✓
Hyperox (8 ml/L, 15 min, 15°C)	✓	✓
Hibitane (27 ml/L, 15 min, 15°C)	✓	
SDS (500 µM, 1 hr, 15°C)		
Savlon (1:30, ~1/2 hr (no blood/mucus), ~1 hr (blood/mucus), 15°C for 15 min then 4°C for remainder of incubation)	✓	see note

✓ = showed at least a 3 log reduction; no ✓ = did not show at least a 3 log reduction

Note: further studies recommended; see results and discussion

5.0 DISCUSSION

Within the aquaculture industry, disinfection plays an important role in the control of disease transmission. There are a number of disinfectants which exist within the industry and the conditions under which they are used varies. Disinfectants may be used to treat equipment, work surfaces, slaughter areas, etc. that have been potentially exposed to pathogens. The effectiveness of disinfectants can be dependent on a number of factors including concentration used, time of exposure, temperature of the surroundings, amount of organic matter being treated, etc. Chemicals used at identical concentrations and exposure time at one temperature (eg. room temperature) may not perform the same at another temperature (eg. 4°C). The presence of organic matter, such as blood and mucus, may inactivate the disinfectant reducing or eliminating its ability to act against the pathogen of interest.

With the recent emergence of ISAV within the local aquaculture industry the goal of this study was to look at a limited number of disinfectants, concentrations, temperatures, and organic matter loads to gain at least some insight on what treatments and conditions have the ability to kill ISAV. Two extremes were chosen with regards to organic load in an effort to mimic best and worst case scenarios that might exist within industry. One set of tests looked at the ability of disinfectants or heat to inactivate ISAV-infected blood/mucus material while the other tested against ISAV suspended in a salt solution with no blood/mucus material present. Rather than seed blood/mucus with ISAV, fish were challenged and sacrificed to generate infected ISAV blood/mucus to more closely correlate with levels and type of infection that might exist within industry.

One of the technical hurdles of doing such tests is to work out a method that can neutralize chemicals post-treatment to control exposure time and eliminate any toxic effects that the treatment mixtures might have on the cell line used to detect viral viability. Cytotoxic effects can mask or prevent CPE development by viral particles. Direct plating of treatment mixtures is desired but often not possible. Although a number of secondary chemicals and filtering column approaches were tried in this study, they generally did not eliminate toxic effects on the SHK cell line. When cytotoxicity exists a 3 log reduction in viral titer beyond the cytotoxic level is considered acceptable (4). The dilution approach which was adopted proved sufficient for titrating out cytotoxicity and inactivating further action of the chemicals after the test incubation time. The small amount of variability that did exist between post neutralization titers and positive control titers was likely due to varying distribution of ISAV particularly in the blood/mucus material. ISAV adheres to organic debris and hence could concomitantly be slightly unequally distributed giving rise to the differences seen between post neutralization and positive control titers. This was likely also the reason for slight variation amongst positive control titers from test day to test day.

For the testing in the absence of blood/mucus material, only 3 treatments did not show at least a 3 log reduction beyond cytotoxic levels:

- 1) Heat (100°C, 30 s)
- 2) SDS (500 µM, 1 hr, 15°C)
- 3) SDS (100 µM, 1hr, 15°C)

All other treatments produced at least a 3 log reduction under the conditions which were tested. Had cytotoxic levels been able to have been further reduced, some treatments may have been confirmed for even higher reduction values, but unfortunately this was a limitation of the testing methodology.

For the testing in the presence of blood/mucus material, 8 treatments did not show at least a 3 log reduction beyond cytotoxic levels:

- 1) Ovadine (108 ppm, 15 min, 15°C)
- 2) SDS (100 µM, 1 hr, 15°C)
- * 3) Mikroklene (150 ppm, 15 min, 15°C)
- 4) Buffodine (108 ppm, 15 min, 15°C)
- 5) Ovadine (150 ppm, 15 min, 15°C)
- 6) Hibitane (27 ml/L, 15 min, 15°C)
- 7) SDS (500 µM, 1 hr, 15°C)
- * 8) Savlon (1:30, ~1 hr, 15°C)

A special note should be made with regards to Mikroklene and Savlon above (*). Table 2 shows that Savlon performed well for the window of opportunity which existed for log reduction to be definitively observed (compare positive control titer, level of cytotoxicity and treatment titer). Slightly lower initial ISAV titers and the problems encountered detoxifying Savlon made it difficult to observe reduction. Centricon 300 columns followed by dilution improved cytotoxic levels but not adequate enough to observe a possible 3 log drop given the starting positive control titer. Should the level of cytotoxicity be resolved further testing might show this chemical to meet the 3 log reduction criteria set for the others. Only an approximation for incubation time can be given for Savlon due to the use of Centricon columns which only gradually filters the treatment mixture through. This filtration was more gradual for the blood/mucus material than for that without blood/mucus material and hence the different testing times are observed between the 2 scenarios (ie. ~1 hr vs. ~1/2 hr). As well, further treatment replicates for Mikroklene, which showed more severe variability (ie. replicate 1 = $10^{3.75}$ vs. replicate 2 = $10^{1.50}$) as compared to replicates for other chemicals, might also confirm Mikroklene capable of producing at least a 3 log reduction in ISAV titer. Previous studies at RPC with Mikroklene showed good reduction of ISAV in the presence of blood/mucus. There were differences between this former study and the current one, however, which might produce different results. In the former experiments ISAV was seeded into filtered blood/mucus material; seeding and filtration inevitably diluted the amount of organic matter and hence this mixture would not have been as likely to have inactivated the disinfectant before it could target ISAV. Also, the level of cytotoxicity for

Mikroklene was less in the former study than in the current allowing the possibility to observe a larger log reduction. Cytotoxic levels were sometimes increased in this study due to the presence of fungal and bacterial contaminants in some wells (see Appendix B). It was undesirable to filter sterilize the blood/mucus in this study because the goal was to mimic as closely as possible a blood/mucus scenario within industry. Filtration would have removed organic matter and reduced ISAV titer.

All other treatments done in the presence of blood/mucus material met the 3 log reduction value standard. Again had it been possible to further reduce cytotoxic levels, some treatments may have been confirmed for even higher reduction values, but unfortunately this was a limitation of the testing methodology.

In general all chemicals which performed well in the presence of blood/mucus, performed well in the absence of blood/mucus as might be expected, with the one exception being the heat treatment at 100°C for 30 s. One explanation as to why this treatment may not have proved as effective in the absence of blood/mucus was due to the starting ISAV titer. Tests in the absence of blood/mucus had higher starting treatment titers, TCID₅₀ ~10⁶/0.1 ml as compared to those in the presence of blood/mucus where TCID₅₀ titers of only ~10⁵/0.1 ml had been generated from the fish challenge. The titer in the absence of blood/mucus study was not adjusted to match that of the presence of blood/mucus study because this would have reduced and limited the window of opportunity to view log reductions.

Table 3 summarizes which chemicals/ procedures produced at least a 3 log reduction of ISAV in the presence and absence of blood/mucus material. Although only a limited number of concentration/ temperature/ organic matter/exposure time scenarios were tested, the data in this study does provide increased insight on what conditions can reduce ISAV activity. This new knowledge should aid in decisions surrounding ISAV disinfection procedures.

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Appendix A - Raw Data - Absence of Blood/Mucus

Note: + (CPE), 0 (no CPE), T (cytotoxicity), np (not plated)

Heat 60°C, 10 min

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁰	np	0000	0000	0000	np
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	+++0
10 ⁻⁶	++00		0000	0000	++++
10 ⁻⁷	++00		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Heat 100°C, 30 s

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁰	np	0000	++++	++++	np
10 ⁻¹	++++	0000	++++	++++	++++
10 ⁻²	++++	0000	++++	++++	++++
10 ⁻³	++++	0000	++++	+++0	++++
10 ⁻⁴	++++		+000	+000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	++00		0000	0000	++00
10 ⁻⁷	++00		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Formic Acid

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10^{-1}	++++	0000	0000	0000	++++
10^{-2}	++++	0000	0000	0000	++++
10^{-3}	++++	0000	0000	0000	++++
10^{-4}	++++		0000	0000	++++
10^{-5}	++++		0000	0000	++++
10^{-6}	+000		0000	0000	++00
10^{-7}	0000		0000	0000	0000
10^{-8}	0000		0000	0000	0000

SDS (500 μ M)

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10^{-1}	++++	0000	++++	++++	++++
10^{-2}	++++	0000	++++	++++	++++
10^{-3}	++++	0000	+000	++00	++++
10^{-4}	++++		+000	0000	++++
10^{-5}	++++		0000	0000	++++
10^{-6}	+000		0000	0000	0000
10^{-7}	0000		0000	0000	0000
10^{-8}	0000		0000	0000	0000

SDS (100 μ M)

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10^{-1}	++++	0000	++++	++++	++++
10^{-2}	++++	0000	++++	++++	++++
10^{-3}	++++	0000	++++	++++	++++
10^{-4}	++++		++++	++++	++++
10^{-5}	++++		++++	++++	++++
10^{-6}	+000		++00	0000	+000
10^{-7}	0000		0000	0000	0000
10^{-8}	0000		0000	0000	0000

Ovadine (108 ppm)

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10^{-1}	++++	0000	0000	0000	++++
10^{-2}	++++	0000	0000	0000	++++
10^{-3}	++++	0000	0000	0000	++++
10^{-4}	++++		0000	0000	++++
10^{-5}	++++		0000	0000	++++
10^{-6}	+++0		0000	0000	++++
10^{-7}	0000		0000	0000	0000
10^{-8}	0000		0000	0000	0000

Virkon

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+++0		0000	0000	+000
10 ⁻⁷	0000		0000	0000	+000
10 ⁻⁸	0000		0000	0000	0000

Ovadine (150 ppm)

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+000		0000	0000	+000
10 ⁻⁷	+000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Microdyne

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+++0		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Mikroklene

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+000		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Adage

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	0000	0000	0000	++++
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+++0		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Germkill

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+000		0000	0000	++00
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Bleach

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+++0		0000	0000	++00
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Buffodine

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	+++0
10 ⁻⁶	+++0		0000	0000	+++0
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Hyperox

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	++++
10 ⁻⁶	+++0		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Hibitane

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++++		0000	0000	+++0
10 ⁻⁶	+000		0000	0000	0000
10 ⁻⁷	+000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Savlon

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	+++0		0000	0000	++00
10 ⁻⁶	+000		0000	0000	++00
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Appendix B - Raw Data - Presence of Blood/Mucus

Note: + (CPE), 0 (no CPE), np (not plated)

Important Note: In the case of blood/mucus results, wells were considered “cytotoxic” if they were unreadable for ISAV infection due to chemical cytotoxicity (T) or the presence of bacteria (B) or fungus (F). The cytotoxicity caused by each of these is distinct from that of ISAV CPE. It was undesirable to filter sterilize the blood/mucus in this study because the goal was to mimic as closely as possible a blood/mucus scenario within industry. Filter sterilization would have removed organic matter and reduced ISAV titer. The existence of bacteria and fungus in the blood/mucus material and their sporadic and varied infection of wells sometimes produced inconsistency between cytotoxicity controls and treatment samples in terms of how many wells were “unreadable”. Therefore, in these instances, the $TCLD_{50}$ value used for cytotoxicity control in Table 2 was calculated by looking at the 2 treatment replicates and considering the degree of chemical, bacterial and fungal “cytotoxicity”. Instances where the cytotoxicity controls did not accurately represent the toxicity in the treatment samples are noted below.

Heat 100°C, 30 s

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10^0	np	BBBB	TTTT	TTTT	np
10^{-1}	BBBB	0000	0000	0000	BBB+
10^{-2}	B+++	0000	0000	0000	++++
10^{-3}	++++	0000	0000	0000	++++
10^{-4}	++++		0000	0000	++++
10^{-5}	++00		0000	0000	+000
10^{-6}	0000		0000	0000	++00
10^{-7}	0000		0000	0000	0000
10^{-8}	0000		0000	0000	0000

Heat 60°C, 10 min

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁰	np	TTTT	TTTT	TTTT	np
10 ⁻¹	BBBB	0000	0000	0000	BBB+
10 ⁻²	B+++	0000	0000	0000	B+++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++00		0000	0000	+++0
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Bleach

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BB++	B000	0000	0000	BBB+
10 ⁻²	++++	0000	0000	0000	B+++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	+++0		0000	0000	+000
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Virkon

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	B000	F000	0000	BBBB
10 ⁻²	BB++	0000	0000	0000	B+++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++00		0000	0000	++00
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	+000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Formic Acid

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBBB	0000	0000	F000	BBBB
10 ⁻²	++++	0000	0000	0000	BB++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++00		0000	0000	++00
10 ⁻⁶	++00		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Ovadine (108 ppm)

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	FFF0	BBB+	BBB+	BB++
10 ⁻²	B+++	0000	++++	++++	++++
10 ⁻³	++++	0000	++++	++++	++++
10 ⁻⁴	++++		++++	++++	++++
10 ⁻⁵	+000		+000	++00	+++0
10 ⁻⁶	0000		0000	+000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

SDS (100 µM)

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBBB	B000	BBB+	B+++	BBBB
10 ⁻²	B+++	0000	B+++	B+++	++++
10 ⁻³	++++	0000	++++	++++	++++
10 ⁻⁴	++++		++++	++++	B+++
10 ⁻⁵	+++0		++00	+++0	++00
10 ⁻⁶	0000		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Mikroklene

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	F000	BBBB	FF++	BBBB
10 ⁻²	B+++	B000	++++	0000	B+++
10 ⁻³	++++	0000	+++0	0000	++++
10 ⁻⁴	++++		++00	0000	++++
10 ⁻⁵	+000		0000	0000	++00
10 ⁻⁶	+000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Buffodine

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	BBFT	BBB+	BBB+	BBBB
10 ⁻²	B+++	B000	B+++	++++	B+++
10 ⁻³	++++	0000	++++	++++	B+++
10 ⁻⁴	++++		++++	++++	++++
10 ⁻⁵	+000		+000	0000	++00
10 ⁻⁶	0000		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Ovadine (150 ppm)

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	F000	BBBB	BBB+	BBB+
10 ⁻²	++++	0000	B+++	++++	BBB+
10 ⁻³	++++	0000	++++	++++	B+++
10 ⁻⁴	++++		++++	++++	++++
10 ⁻⁵	++00		+000	+000	++++
10 ⁻⁶	+000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Microdyne

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BB++	TTTT	TTTT	TTTT	BBBB
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	+++0		0000	0000	++++
10 ⁻⁶	0000		0000	0000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Adage

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	TTTT	TTTT	TTTT	BBBF
10 ⁻²	++++	0000	0000	0000	++++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	0000		0000	0000	+++0
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Germkill

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	TTTT	TTTT	TTTT	BBBB
10 ⁻²	B+++	0000	0000	0000	BB++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	+000		0000	0000	+++0
10 ⁻⁶	+000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Hyperox

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	TTTB	TTTT	TTTT	BBBB
10 ⁻²	BB++	0000	0000	0000	BB++
10 ⁻³	++++	0000	0000	0000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++00		0000	0000	+++0
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	+000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

Hibitane

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBB+	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	0000	++++	+++0	++++
10 ⁻³	++++	0000	+000	+000	++++
10 ⁻⁴	++++		0000	0000	++++
10 ⁻⁵	++00		0000	0000	+++0
10 ⁻⁶	+000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

SDS (500 μ M)

Dilution	Positive Control	Cytotoxicity ¹ Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	BBBB	BB00	BBBB	BBBB	BBBB
10 ⁻²	B+++	0000	B+++	BB++	B+++
10 ⁻³	++++	0000	++++	++++	B+++
10 ⁻⁴	++++		++++	++++	++++
10 ⁻⁵	+++0		++00	++++	+++0
10 ⁻⁶	0000		+000	+000	+000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000

¹ not used to calculate values for cytotoxicity controls in Table 2.

Savlon

Dilution	Positive Control	Cytotoxicity Control	Treatment - Replicate 1	Treatment - Replicate 2	Post Neutralization Control
10 ⁻¹	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻²	++++	TTTT	TTTT	TTTT	TTTT
10 ⁻³	++++	0000	0000	0000	+++0
10 ⁻⁴	+++0		0000	0000	+000
10 ⁻⁵	0000		0000	0000	0000
10 ⁻⁶	0000		0000	0000	0000
10 ⁻⁷	0000		0000	0000	0000
10 ⁻⁸	0000		0000	0000	0000