Preparation of biological contaminated samples for chemical analysis

Hilde Teien, Else-Marie Fykse and Janet Martha Blatny

Norwegian Defence Research Establishment (FFI)

7 December 2009

FFI-rapport 2009/01946

109101

P: ISBN 978-82-464-1680-9
E: ISBN 978-82-464-1681-6

Keywords

Diklormetan

Bacillus athrophaeus (BG)

Inaktivering

Fjerning av BG sporer

Sterilfiltrering

Approved by

Janet Martha Blatny	Project Manager
Jan Ivar Botnan	Director

English summary

FFI has established laboratory facilities in order to receive and analyze samples containing a mixture of chemical, biological and radiological (CBR) agents, so-called “mixed samples”. Detailed procedures are needed to correctly handle a mixed sample, and care must be taken during opening of these samples. FFI is currently optimizing such procedures especially addressing the need to analyze simultaneously the sample for the presented C and B agents as well as reducing the risk of exposure to personnel. To make sure that a potential biological agent is not regarded an infectious, or may contaminate the laboratory, inactivation of such agents is essential before further analysis of the presence of chemical or radiological agents.

The aim of this study was to investigate if dichloromethane (DCM) used for extraction of chemical warfare agents may inactivate spores and vegetative cells using *Bacillus athrophaeus* as a model organism (also known as *Bacillus globigii*, BG-spores) present in aqua’s and soil samples. We further investigated if additional steps in the extraction procedure were able to remove/inactivate bacteria present in the sample. Growth analysis was used for this investigation (CFU).

Our results showed that BG spores were able to survive in DCM after an overnight incubation (21 hour). However, the viability (culturability) of vegetative BG cells was reduced (10.000 times) after a 30 minute incubation period.

A strong reduction (almost 100 %) of the growth of BG was obtained by first extracting the sample (soil) in DCM followed by a filtration (0.45 μm membrane filter) of the extraction solution at BG concentration levels < 10^8 CFU/ml. However, an extraction followed by a sterile filtration (0.22 μm pore size) of the sample (soil and liquid) at concentration levels at 10^6 and 10^9 CFU/ ml showed a complete reduction (100 %) of the growth of BG. Care must be taken during filtration as the filter may be overloaded. In samples containing a high start concentrations of BG spores, >10^9 CFU/ml, bacterial growth (< 0.1 %) was observed in some cases. To overcome this problem serial dilution of the samples using different pore sizes may be performed.

Sammendrag

FFI har etablert et laboratorium for håndtering og analyse av prøver for kjemiske (C) og biologiske (B) trusselstoffer, samt radioaktive (R) stoffer. En slik kapasitet krever rutiner og prosedyrer for preparering av prøver for analyse av CBR- midler. Ved mottak av en CBR-prøve er et av de første stegene i prosedyren å analysere prøven for innhold av biologiske trusselstoffer. Denne rapporten beskriver et ledd i en mer omfattende prosedyre for hvordan en CBR-kontaminert prøve kan prepareres for kjemisk analyse med minimal risiko for at analyseutøverne skal bli eksponert for biologiske trusselstoffer.

For kjemisk analyse er det nødvendig å benytte organiske løsningsmidler, f. eks. diklormetan (DCM), for ekstraksjon av kjemiske trusselstoffer fra en kontaminert prøve. Våre resultater viser at BG sporer (simulant for *Bacillus anthracis* som forårsaker miltbrann) overlever i DCM og at analysepersonell kan derfor bli utsatt for en eksponering mot biologiske trusselstoffer. Ved bruk av filtrering (0,22 µm porestørrelse) som et tilleggstrinn i prøveprepareringen etter ekstraksjon med DCM, var det mulig å fjerne 99,99 % av sporene. Vi anbefaler at det videre utføres kjemiske analyser for å teste om bruken av et slikt sterilfilter vil ha noen påvikning på kjemiske trusselstoffer og at det testes ut minimum mengde prøvevolum for å få nok prøvemateriale for ekstraksjon av slike trusselstoffer.

Contents

Preface

This work is a part of FFI project 1099 “Detection and identification of biological agents - a part of biological defence” were the main topic is to develop methods for establishment of identification preparedness to receive and analyze samples containing a mixture of chemical (C), biological (B) and radiological (R), CBR- agents, so-called “mixed samples”.

This report is a documentation of experiments investigating if dichloromethane (DCM) could inactivate *Bacillus athrophaeus* (BG) spores and vegetative cells. Further, the aim of these experiments was also to investigate if BG spores could be eliminated from samples using the procedure for extraction of chemical agents from soil or liquid samples.

1 Introduction

FFI has established laboratory facilities in order to receive and analyze samples containing a mixture of chemical, biological and radiological (CBR) agents, so-called “mixed samples”. The laboratory facilities at FFI are unique among the Northern nations. NATO has emphasized the need for such capability to increase the global preparedness against such threat agents, and FFI takes part in NATO’s annual trials for analyzing mixed samples.

Detailed procedures are needed to correctly handle a mixed sample, and care must be taken during opening of such a samples. FFI is currently optimizing such procedures especially addressing the need to analyze simultaneously the sample for C and B agents as well as reducing the exposure of these agents to the personnel. To make sure that a potential biological agent is not infectious for personnel, or contaminating the laboratory, inactivation of the biological agents is essential before analysis for chemical or radiological agents.

During the opening (removal of the packing) of a mixed sample the sample is first screened for radiological (R) agents by Auto mess (Alfa-/beta probe) and then for chemical agents (C) by CAM. Then the sample will be separated in four different parts, one to R, B and C and one for backup (Figure 1.1).

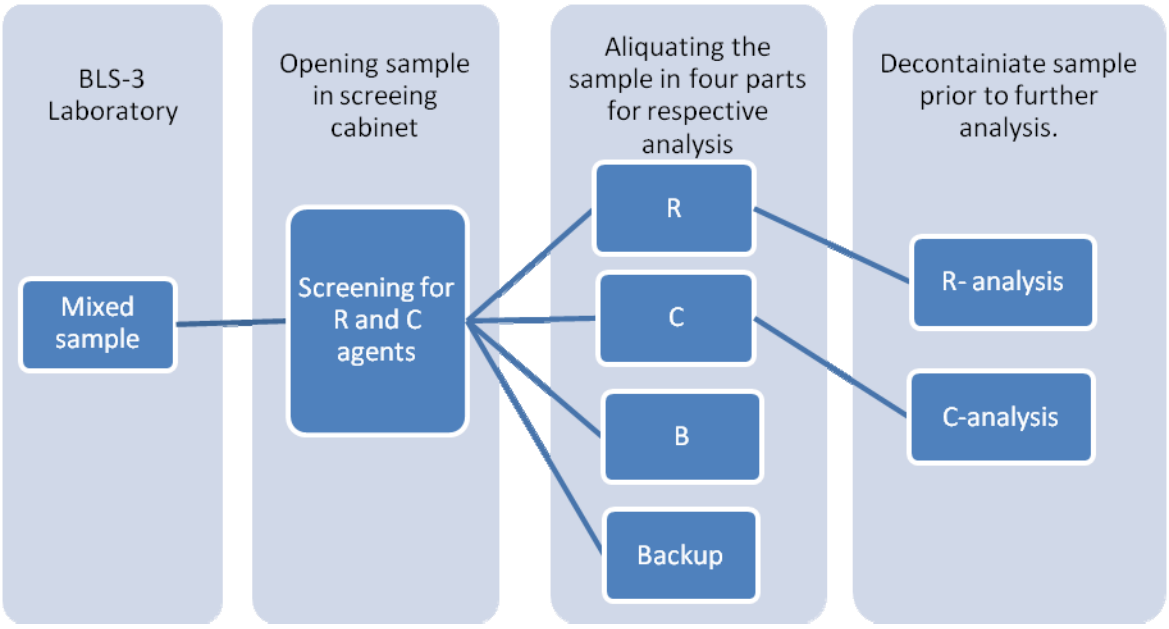

Figure 1.1 Illustration of handling a mixed sample potentially containing chemical (C), Biological (B) and radiological (R) agents.

Dichloromethane (DCM) is commonly used for extraction of chemical agents from an environmental sample and is recommended by OPCW (Organization for the Prohibition of Chemical Weapons) as an efficient extraction solvent [1]. Thus, DCM is frequently used at FFI.

However, DCM has been showed to promote growth of bacteria. From the literature it is known that DCM can be a substrate for several microorganisms involved in remediation among others *Methylobacterium* and *Dehalobacter* (belonging to the genera *Bacillus/Clostridium*) [2, 3]. In order to confirm that DCM did not have an impact on the growth of bacteria and spores in a mixed sample potentially containing both chemical and biological agents, we performed growth analysis of *Bacillus atrophaues*[a] *(*also known as *Bacillus globigii*, BG spores) vegetative cells and spores in the presence of DCM as well as in a soil samples to be extracted with DCM.

The extraction procedure for chemical warfare agents in soil sample includes, in addition to the extraction with DCM, sonication for 5-10 min, centrifugation for 3 min and filtration of the extracts with a 0.45 µm membrane filter [1] (Appendix D). We wanted to examine if some of these processes eliminated or had an inactivating effect on bacteria. Using one of the procedures already established for extraction of chemical agents would be time-saving, which is important when analyzing a potential dangerous sample. Spores of BG were used in all experiments.

2 Material and methods

2.1 Chemicals, microorganisms and growth conditions

The chemicals used are dichloromethane (CH_2CL_2) [4], CAS no 75-09-2, ultra gradient from Mallin krodt Baker B.V. and phosphate buffered saline (PBS).

The strain *Bacillus athrophaeus* (also known as *Bacillus globigii*, BG spores) provided by Dugway Proving Ground, Utah, Lot nr. #10-124 was used in the study. Dry BG spores were used in all analyses except from the test with vegetative cells (1 gram of dry BG spores corresponding to 10^{11} CFU). Germination of the spores was performed by growing in tryptic soy broth (TSB) and incubation at 37 °C overnight. The samples with BG were serially diluted, and CFU were enumerated by plating 100 µl of each dilution onto TSB agar plates (two or three parallels) and incubated at 37 °C overnight.

2.2 Inactivation of BG spores with DCM

Dry BG spores were dissolved in DCM using special centrifuge tubes in teflon with screw cap, 30 ml, Nalagene, that are suitable for DCM[b]. Immediately after the mixing of BG spores and DCM by handshaking, the lid of the tube was opened to release the gas pressure. The tubes were then continuously shaken and incubated for various times at room temperature (half an hour, three hours and overnight) in a Heidolph Multi Reax shaking table (Table 2.1). After incubation the tubes with BG spores and DCM were centrifuged (IEC centra CUR centrifuge) at room temperature for 10 min at 3000 rpm. The BG spores were thus concentrated in a loose pellet at the bottom of the tube. The density of DCM (1,325 gm/cm^3, [4]) and BG spores (1.3 gm/cm^3, [5]) are

[a] *Bacillus athrophaeus* spores are named BG spores in this report
[b] DCM can damage rubber and some plastic material [2]

nearly similar. Therefore, the pellet of BG was easily mixed with DCM during further handling and precaution was taken to avoid mixing. The BG pellet was washed twice with 2 ml PBS (phosphate buffered saline) to make sure that all DCM was removed before plating out on TSB agar plates. PBS was used as a negative control. After inactivation and washing (to remove DCM), 100 µl of serial diluted samples were plated on TSB agar plates (two or three parallels) and incubated at 37 °C overnight. The plates were then examined for growth and single colonies were counted and CFU/ml was calculated/ estimated.

Table 2.1 Quantities of dry BG spores and incubation time used in the initial inactivation experiment with DCM.

Quantity of dry BG, [mg]	Incubation time, [hours/ minutes]	Dichloromethane DCM [ml]	CFU/ml
50	30 min	1	10^9
10	30 min	2	10^8
	3 h		
	20 h 30 min[c]		
1	20 h 30 min[a]	5[d]	10^5

2.2.1 Inactivation of vegetative BG cells

BG spores were germinated as described above in section 2.1. 0.5 ml of BG spores (10 mg/ml in PBS corresponding to 10^9 CFU/ ml) was inoculated into 50 ml TSB medium. The liquid culture was incubated at 37 °C overnight. A sample from the liquid culture was examined under the microscope to make sure that all spores were germinated. One ml of the culture was centrifuged and the pellet of vegetative cells was dissolved in 2 ml DCM, and the experiments were further performed as described for BG spores (2.2).

2.3 Removal of BG spores from soil samples using filtration

The soil from Kjeller/FFI was autoclaved. Dry BG spores were added to the soil using two different procedures. I) BG spores were added directly to the soil or, II) 1 ml of BG spores was dissolved in PBS prior to adding to the soil. The extraction procedure for chemical agents in soil samples was used (Appendix D). The following steps are included in the DCM extraction procedure; extraction of the soil with DCM including sonication 5-10 min (Sonorex Super, Bandelin) and centrifugation for 3 min. This is repeated twice followed by filtration of the DCM extract using a 0.45 µm membrane filter (minisart RC25, Sartorius) as shown in Figure 2.1. During centrifugation two phases are separated; the sediment in the bottom of the tube and the DCM as a liquid phase on the top. The BG spores are present in the thin layer on top of the sediment phase. After each step in this procedure, 100 µl of serial diluted DCM solutions was

[c] Overnight
[d] 1 mg BG mixed with 5 ml DCM was diluted to give a start CFU/ml of 10^5.

plated on TSB agar plates (two to three parallels) and incubated overnight at 37 °C. The plates were then examined for growth and single colonies were counted and CFU/ml was estimated. For extraction 5 g soil and 5 x 2 ml DCM was used. For extraction of chemical agents 10 g of soil and 10 x 2 ml of DCM are used.

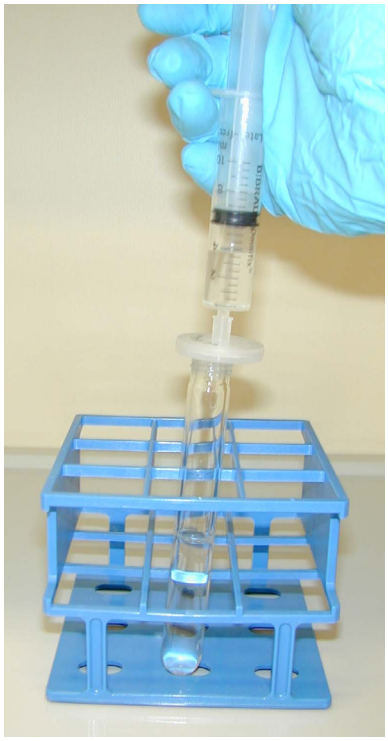

Figure 2.1 Illustration of the filtration procedure with a syringe membrane filter, (0.45 µm pore size).

2.3.1 Filtration of the upper half of DCM extract

As mentioned above, the centrifugation step in the extraction procedure separated BG from DCM. However, the density of BG and DCM is similar and BG easily was mixed with DCM during handling of the samples. In this experiment only the upper half of the DCM extract (2.5 ml) was pipetted of and filtrated (a total of 5 ml DCM). The procedure was otherwise performed as described before in 2.3.

2.3.2 Sterile filtration of BG samples

In these experiments the procedure described in section 2.3 was used except for using a 0.22 µm sterile filter (Millipore Millex-GV filter with a hydrophillic PVDF membrane ref: SLGV033NS), instead of the 0.45 µm filter. In the initial analyses PBS was used instead of DCM as results showed that there were no difference in bacterial growth (BG spores) when incubated in PBS compared to DCM. Two different concentrations of spores were used, 2.8 x 10^6 CFU/ml and 1.1 x 10^9 CFU/ml (Table 2.2). Different dilutions of the filtrated extract were plated out on TSB agar plates and bacterial growth measured as described before.

Table 2.2 The initial concentration of BG in soil samples in the filtration experiments.

Pore size of the syringe filter	0.22µm	
Start concentration [CFU/ml]	1.1×10^9	2.8×10^6
Total volume of DCM [ml]	10 (2 x 5 ml)	
Soil [g]	5	
Added BG solution [ml]	1	
Number of test [n=]	10	9

Dry BG spores were dissolved in PBS making a stock solution of 1.4×10^6 CFU/ml, 3.0×10^6 CFU/ml and 3.8×10^9 CFU/ml. Stock solutions with various concentrations (CFU/ml) and various volumes (ml) were filtrated using the 0.22 µm sterile filter and plated out as described above. See Table 2.3 for concentration and volume of the filtrated sample.

Table 2.3 The initial concentration of BG in PBS and the volume filtrated in the filtration experiments.

Pore size of the syringe filter	0.22 µm				
Start concentration [CFU/ml]	1.4×10^6	3.0×10^6		3.8×10^9	
Volume filtrated [ml]	2	1	5	5	1
Number of parallels [n=]	1	5	4	2	3

3 Results and discussions

As a part of establishing well-defined procedures to handle, prepare and analyze mixed CBR samples, one of the initial steps prior to chemical analysis of such samples is to minimize the personnel risk of exposure to biological agents. This can be done by inactivating the biological agents after the biological analysis has been performed. However, at the same time preparation of the sample for chemical analysis needs to be done, in which DCM is commonly added in order to obtain efficient extraction of the chemical agent from the environmental sample.

We have analyzed whether bacteria will survive during an extraction of chemical agents. Also, we propose a modified procedure to remove bacteria and spores from mixed samples to be prepared for chemical analysis and to minimize exposure of biological agents to the chemists.

3.1 Inactivation of BG spores with DCM

The aim of the initial experiments was to investigate if BG was inactivated in DCM/ or to confirm as described in the literature that DCM promote growth. The results showed that BG spores and vegetative cells were still culturable after incubation in DCM.

BG spores and vegetative cells were incubated for 30 minutes, 3 hours and overnight (21 hours) in DCM (see section 2.2). The number of CFU/ml of BG in PBS was nearly similar to that

obtained in the DCM samples (Figure 3.1 and Figure 3.2). The effect observed in lower CFU/ml (4.5 times and 128 times lower), especially on the vegetative cells (10.000 times lower), can be due to loss of cells during the washing procedure due to mixing of pellet and supernatant as described in section 2.2.

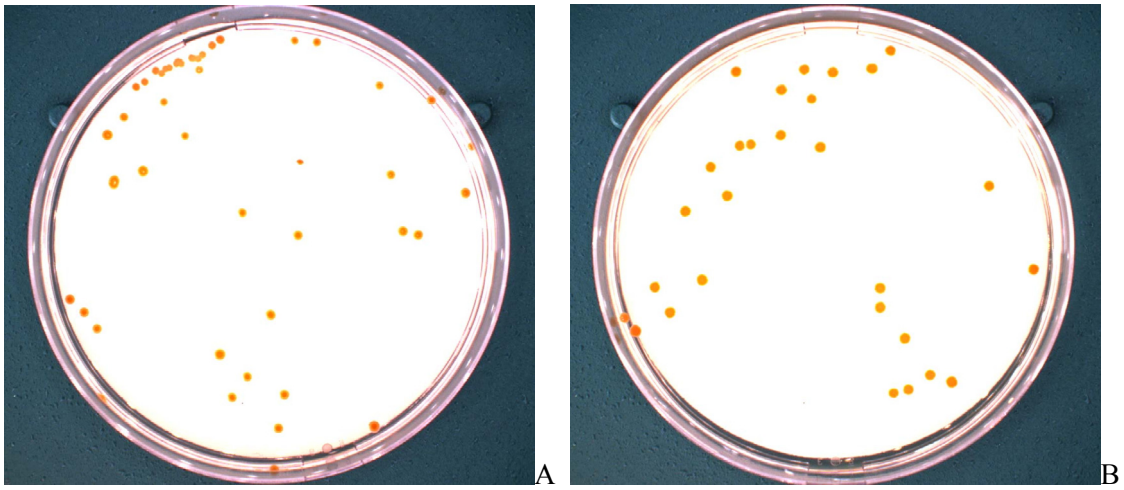

Figure 3.1 Bacterial growth of a 10^7 dilution of BG spores (10^9 CFU/ml) on TSB agar plates after incubation in DCM for 3 hours. A) Positive control – BG spores incubated in PBS. B) BG spores incubated in DCM.

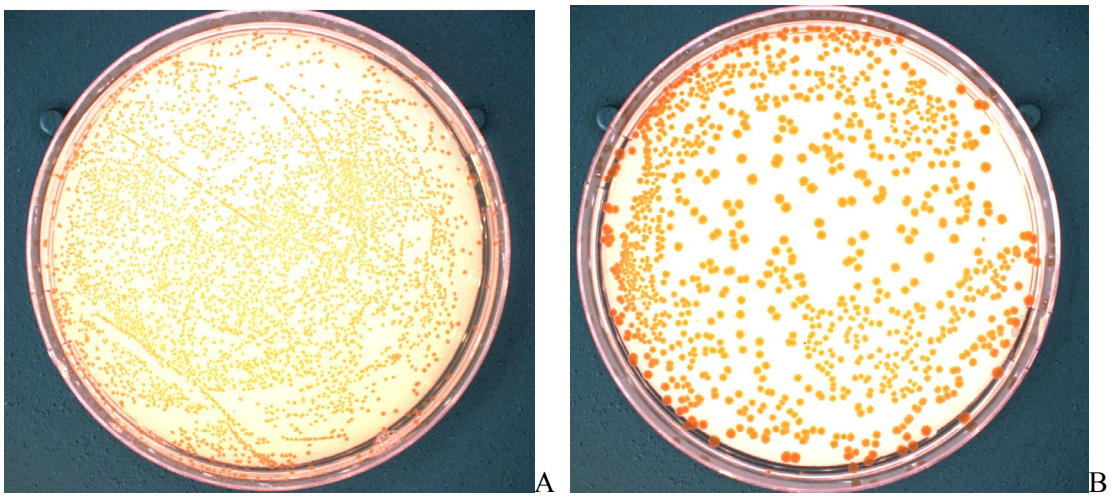

Figure 3.2 Bacterial growth of a dilution of BG spores (3.2 x 10^5 CFU/ml) on TSB agar plates after incubation overnight in DCM. A) Positive control – BG diluted with PBS. B) BG incubated with DCM overnight. The reduction in the number of colonies was probably due to loss during washing.

Since the results of these experiments showed that DCM had almost no effect on the viability (measured by culturability) of BG spores and vegetative cells, no further DCM inactivation experiments were performed. The results are summarized in Table 3.1 and Table 3.2.

As mention in section 1 it is known that DCM can be a substrate for several microorganisms involved in remediation among others *Methylobacterium* and *Dehalobacter* (belonging to the genera *Bacillus/Clostridium*) [2,3]. Therefore, it is not surprising that BG spores were found to be culturable in DCM.

Table 3.1 Bacterial growth of BG spores on TSB agar plates after DCM incubation. 100 µl of serial diluted samples were plated.

Start concentration [CFU/ml]	Sample	Incubation time [hours/ minutes]	100 µl of a dilution	Single colonies in mean average	Results on TSB Plates [CFU/ml]
50 mg BG/ 1ml DCM (5×10^9 CFU/ml)	BG/DCM	30 min	10^1	Dense growth	
			10^3	Dense growth	
10.9 mg BG/ 2 ml DCM (5.4×10^8 CFU/ml)	BG/DCM	30 min	10	Dense growth	
			10^3	Dense growth	
			10^5	123	1.2×10^8
			10^7	8	8×10^8
9.8 mg BG/ 2 ml DCM	BG/DCM	3 h	10^7	25	2.5×10^9
	Positive control (BG/PBS)			43	4.3×10^9
9.7 mg BG/ 2 ml DCM	BG/DCM	20 h 30 min (overnight)	10^7	50	5.0×10^9
	Positive control (BG/PBS)			55	5.5×10^9
3.2×10^5 CFU/ml	BG/DCM	20 h 30 min (overnight)	10^1	25	2.5×10^3
			10^2	2	2.0×10^3
	Positive control (BG/PBS)	0 min	10^3	32	3.2×10^5
		20 h 30 min	10^3	6	6.0×10^4

Table 3.2 Bacterial growth of vegetative BG cells on TSB agar plates after DCM incubation. 100 µl of serial diluted samples were plated.

Start concentration [CFU/ml]	Sample	Incubation time [hours/ minutes]	100 µl of a dilution	Single colonies in mean average	Results on TSB plates [CFU/ml]
10^9 CFU/ml	BG/DCM	30 min	undiluted	Dense growth	-
			10^3	84	$8.5x10^{5e}$
	Positive control (BG/PBS)		undiluted	Dense growth	-
			10^3	Dense growth	-

3.2 Removal of BG spores from soil samples using filtration

Since the results of the initial experiments showed that DCM had almost no effect on the viability (measured by culturability) of BG spores and vegetative cells we wanted to analyse if some of the other steps in the extraction procedure could eliminate or have an inactivating effect on the spores.

To imitate the extraction procedure of chemical agents from soil samples, BG spores (two different start concentrations of spores were used, 1.0 x 10^8 CFU/ml and 6.6 x 10^7 CFU/ml) were added to soil as described in the methods (2.3). The chemical agents were extracted from soil using DCM, this including sonication and centrifugation, and as a final step the extracted liquid was filtrated through a 0.45 µm filter as described in 2.3. The liquid phase with DCM was plated out after each step in the extraction procedure. The results of these experiments showed that only the filtration step had an effect on the bacterial growth. The bacterial growth was strongly reduced (< 99.99 %) after filtration using the 0.45 µm membrane filter but the extract was not completely sterile (610 CFU/ml and 40 CFU/ml) see Table 3.3. Therefore, we examined if we got a sterile DCM extract free for BG spores when only the upper half of the DCM extract phase was pipetted of (Figure 3.3), filtrated (0.45 µm pore size) and plated out on TSB agar plate. The result showed bacterial growth also in the two experiments where only the upper half of the DCM extract in the centrifugation tube was used and filtered see Table 3.4, indicating that the spores were distributed all over the DCM phase and not only in the thin layer between the pellet and DCM. For more details see Appendix B and C.

[e] The reduction in CFU/ml after DCM incubation was probably due to loss during the washing procedure.

Table 3.3 Bacterial growth after filtration of BG spores in DCM through a 0.45 µm filter.

Pore size of the syringe filter	0.45µm	
Start concentration [CFU/ml]	1.0×10^8	6.6×10^7
Bacterial growth [CFU/ml] in filtrate	610	40

Figure 3.3 Illustration of the DCM extract that was pippeted of when only the upper half of the extract was filtrated (0.45 µm pore size).

Table 3.4 Bacterial growth after filtration of the upper half of DCM liquid phase through a 0.45 µm filter.

Pore size of the syringe filter	0.45µm
Start concentration [CFU/ml]	1.0×10^8
Bacterial growth [CFU/ml] in filtrate	380
	1400

3.2.1 Removal of BG spores using sterile filtration

In order to completely reduce growth of bacteria/spores a 0.22 µm filter was used instead of a 0.45 µm as the experiments in section 0 showed that the latter filter was not successful (showed 0.01 % growth). The intention using sterile filtration is that the pore size of the filter (0.22 µm) is too small for biological materials to pass the filter. We performed sterile filtration with soil and liquid sample added BG spores. The results showed that sterile filtration (0.22 µm) effectively eliminated the spores from the solution. Sterile filtration reduced the bacterial growth 100 % in nearly all experiments. In soil and liquid samples, using a start concentration of BG of 10^6 CFU/ml, a sterile solution (totally 9 parallels of each) was obtained (Table 3.4). However, soil samples in which a high concentration (10^9 CFU/ml, i.e. a 1000-fold higher) of BG spores, bacterial growth was obtained in 6 of the 10 soil samples (Table 3.5).

Table 3.5 Bacterial growth after sterile filtration (0.22 µm) of BG spores extracted from soil.

Start concentration [CFU/ml]	1.1 x 10^9	2.8 x 10^6
Number of test [n=]	10	9
Bacterial growth [CFU/ml] after filtration	0	0
	0	0
	0	0
	14100	0
	140	0
	10	0
	0	0
	116000	0
	110	0
	3400	

The observation of bacterial growth when high concentration levels (i.e. 10^9 CFU/ml) of BG were added to the sample may be due to the high pressure needed to be used on the syringe necessary for efficient filtration. The filters become overloaded when the start concentration of BG was as high as 10^9CFU/ml. The problem with overloaded filter in soil sample can also be due to the humus from the soil in the extracted liquid. This problem can be solved by performing a serial filtration using a large pore size (e.g. 0.53 µm) first to remove humus particles, followed by a 0.45 µm filter to remove smaller humus particles and finally using a sterile filter (0.22 µm pore size) to remove all the spores or bacteria.

Filtration of 1 ml stock solution of BG spores in PBS, with a concentration of 10^9 CFU/ml, was not a problem, but filtration of 5 ml was difficult. The reason for this was most likely that the capacity of the filter was exceeded, as a total of 2 out of the 15 liquid sample analyzed showed bacterial growth, see Table 3.5. Minimum volumes needed for chemical analyses are not examined in this work. In the present experiment, 10 ml of DCM was used for extraction of

chemical agents from soil (normally 2x10 ml is used for extraction soil sample at FFI, Appendix D) and 1, 2 and 5 ml were used in the filtration of BG spores in PBS (Table 3.6).

Table 3.6 Bacterial growth after sterile filtration (0.22 µm) of BG spores in PBS.

Start concentration [CFU/ml]	1.4×10^6	3.0×10^6		3.8×10^9	
Volume filtrated [ml]	2	1	5	5	1
Number of parallels [n=]	1	5	4	2	3
Bacterial growth [CFU/ml]	210	0	0	30	0
		0	0	0	0
		0	0		0
		0	0		
		0			

4 Conclusion

DCM does not have an impact on bacterial growth in environmental "mixed samples" potentially containing CBR agents. As DCM is commonly used for extraction of chemical agents from samples, there is a risk that personnel may be exposed to infectious bacteria if these are not inactivated prior to chemical analysis. Thus, we show that additional steps in the preparation step for chemical analysis are needed.

A complete reduction of bacterial growth after DCM extraction of a mixed sample is obtained by sterile filtration (0.22 µm filter). However, care must be taken during filtration as the filter may become overloaded.

Further investigations may include serial filtration and autoclave. Serial filtration might prevent clogging and overloading of the filter. However, autoclaving is the most effective method to eliminate bacteria. Further analyses regarding extraction of chemical agents must be performed. Such analyses include testing the 0.22 µm filter and the extraction volume needed for the chemical agent analyses.

5 Acknowledgement

Thanks to engineer Aase Marie Opstad for the technical support with the introduction to the extraction procedure used for chemical warfare agents in soil and liquid sample.

Appendix A Instrumentation

Centrifuge, IEC centra CUR centrifuge
Centrifuge tubes in teflon with screw cap, 30 ml, Nalagene
Test tube with screw cap, 15 ml, Pyrex
Incubation cabinet, Edmund Bühler, KS-15 control
Heidolph Multi Reax shaking table
Ultrasonic bath, Sonorex Super, Bandelin
Filter, 0.45 µm membrane filter type minisart RC25, Sartorius
Filter, 0.22 µm sterile filter (Millipore Millex-GV filter with a hydrophillic PVDF membrane ref: SLGV033NS.
10 ml syringe, B/Braun Omnifix Latex-free
Weight, B310S, Sartorius
Weight, CP224S, Sartorius
Spreader T. shape irradiated/5, sterile, VWR
Spreader, T. shape, glass spattle
Glass plate, Schott 100x10 mm
Pipettes, Finnpipette, 100-1000 µl and 20-200 µl
Pipettes, glass pipette, 230 mm, Volac

Appendix B Results of soil samples

Table B.1 The table gives an overview of the process performed with each of the soil samples and the result.

Trial number	Prosess performed	Sample						Results bacterial growth		
		Solvent	Volum [ml]	Soil [g]	BG [mg]	Added 1 ml solution with BG [CFU/ml]	Plated out 100 µl	Single colonies		
								Counted	Mean average	CFU/ml
1A[f]	Handshaking	DCM	2X5	5.05			undiluted	0+0	0	0
	1x sonication									
	1x centrifugation									
	2x sonication and 2x centrifugation									
	Filtration, 0.45 µm filter		10							
1B[g]	Handshaking	PBS	2X5	4.95			undiluted	0+4	4	0
	1x sonication									
	1x centrifugation									
	2x sonication and 2x centrifugation									
	Filtration, 0.45 µm filter		10							
2A	Handshaking	PBS	2X5	6.353	10.2		undiluted	dense growth	-	-
	1x sonication and 1x centrifugation								-	-
	2x sonication and 2x centrifugation								-	-
	Filtration, 0.45 µm filter		10					3+61	32	320
							10^3	0+0	-	-
							10^5	0+0	-	-
2B	Handshaking	DCM	2X5	5.17	6.6		undiluted	dense growth		
	1x sonication and 1x centrifugation									
	2x sonication and 2x centrifugation									

[f] The first test is performed to examine the procedure.

[g] This second test is performed to analyse if the soil used in 1A naturally contained some bacteria.

	Filtration, 0.45 µm filter		10					4+4	4	40
							10^3	12+4[h]	-	-
							10^5	1+1	-	-
3A	2x sonication and 2x centrifugation	PBS	2X5	5.0	10		undiluted	dense growth	-	-
							10^3	826 +842	834	$8.3x10^6$
							10^5	6+16	11	$1.1x10^7$
	Filtration, 0.45 µm filter		10				undiluted	43+33	38	380
							10^3	0+0	-	-
							10^5	24+37	-	-
3B	2x sonication and 2x centrifugation	DCM	2X5	5.0	10		undiluted	dense growth	-	-
							10^3	22+58	40	$4x10^5$
							10^5	1+0	1	10
	Filtration, 0.45 µm filter		10				undiluted	140+0	140	1400
							10^3	2+43	-	-
							10^5	1+0	-	-
4A	3X filtration of 3A	PBS	5				undiluted	0+0+1	1	10
4B	2X filtration of 3B	DCM	5				undiluted	0+0	0	0
	3X filtration of 3B						undiluted	0	0	0
5A	Sterile filtration, 0.22 µm filter	PBS	10	5.031		1.1×10^9	undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
							10^3	0+0	0	0
5B	Sterile filtration, 0.22 µm filter. [i]	DCM					undiluted	0+0	0	0
							10^1	0+15	-	-
							10^2	0+18	-	-
							10^3	0+4	-	-
6A	Sterile filtration, 0.22 µm filter	PBS	10	5.043			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
6B				5.043			undiluted	dense growth	-	-
							10^1	139+ 143	141	14100

[h] Bacterial growth that we assume is being a result of contamination is marked with grey in the table.
[i] The membrane in the syringe filter decomposed, so we had a quick change of filter under the filtration. Another type of sterile filter was used.

							10^2	0+0	0	-
6C				5.043			undiluted	13+14	14	140
							10^1	2+3	3	-
							10^2	0+0	0	-
6D				5.043			undiluted	1+0	1	10
							10^1	0+0	0	-
							10^2	0+0	0	-
7A	Sterile filtration, 0.22 µm filter	PBS	10	5.022		2.8×10^6	undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
7B				5.066			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
7C				5.033			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
8A	Sterile filtration, Millex 0.22 µm filter	PBS	10	5.034		2.8×10^6	undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	1+0	0	0
8B				5.000			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
8C		DCM	10	5.007			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
8D				5.005			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
8E				5.019			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
8F				5.009			undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
9A	Sterile filtration, Millex 0.22 µm filter	PBS	10	5.023		1.0×10^9	undiluted	0+0	0	0
							10^1	0+0	0	0
							10^2	0+0	0	0
9B				5.035			undiluted	dense growth	-	-
							10^1	growth	-	-
							10^2	115+116	116	116000
9C				5.001			undiluted	0+11	11	110

							10^1	0+0	0	-
							10^2	0+0	0	-
9D				5.013			undiluted	342+ 337	340	3400
							10^1	0+0	0	-
							10^2	0+0	0	-

Appendix C Results on liquid samples

Table C.1 The table gives an overview of the process performed with each of the liquid samples.

Trial number	Prosess performed	Sample					Results bacterial growth		
		Solvent	Volum solvent [ml]	Volum stock solution (ml)	Concentration of stock solution [CFU/ml]	Plated out 100 µl	Single colonies		
							Counted	Mean average	CFU/ml
10	Sterile filtration, Millex 0.22 µm filter	PBS	1	1	2.8×10^6	undiluted	20+21	21	210
						10^1	4+6[j]	5	-
						10^2	3+5	4	-
11A	Sterile filtration, Millex 0.22 µm filter			1	3.0×10^6	undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
11B						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
11C						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
11D						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
11E						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
12A	Sterile filtration, Millex 0.22 µm filter			5	3.0×10^6	undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
12B						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0

[j] Bacterial growth that is a result of contamination is marked with grey in the table. (A test performed showed that the PBS used to make serial dilutions of the filtrate was contaminated).

12C						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
12D						undiluted	0+0	0	0
						10^1	0+0	0	0
						10^2	0+0	0	0
13A	Sterile filtration, Millex 0.22 µm filter. [k]			5	3.8×10^9	undiluted	0+3	3	30
						10^1	0+1	1	100
						10^2	0+0	0	-
13B						undiluted	0+0	0	0
14A	Sterile filtration, Millex 0.22 µm filter.			1		undiluted	0+0	0	0
						10^1	0+1	1	100
14B						undiluted	0+0	0	0
						10^1	0+1	1	100
14C						undiluted	0+0	0	0
						10^1	0+0	0	0

[k] The filter was changed under the filtration, because the pores in the filter got dense nearly in the beginning of the filtration

Appendix D Procedure for extraction of chemical agents from soil samples

PRØVEOPPARBEIDELSEPROTOKOLL

Sedimentprøver

Dato:	
Signatur:	

Ekstraksjonsprosedyre	Utstyr	Kommentar
Let the samples warm up to room-temperature slowly. **S I: Nonpolar CWA-related chemicals** Extract 1-10 g of the sample with two portions of dichloromethane (1:1 ml solvent/g sample) by sonication, shaking, tumbling or agitation for 5-10 min. If necessary centrifuge and filtrate the extract. Do that twice. Combined the extract and dry with anhydrous sodium sulphate by adding the sulphate to the extract. If necessary concentrate the sample to approximately 1 ml with mild nitrogen flow. Avoid dryness of the sample, because CWA-related chemicals are firmly adsorbed to glass surfaces from residues of organic extracts. This dichloromethane sample is analysed for nonpolar CWC-related chemicals. Store the samples in a freezer at –20 °C.	Vei inn prøve i 30 ml teflon sentrifugeglass med skrukork (ant desimaler??) Ekstraher 2 X ultra resi diklormetan fra Baker i ultralydbad i 10 min Sentrifuger i 3 min ved 2000 G (Type sentrifuge?) Filtreres 0,45µm membranfilter (type) Kimax reagensrør Autosamplerglass	

Acronyms

BG – *Bacillus globigii*
BSL-3 – Bio Safety level-3
CFU – Colony forming units
DCM – Dichloromethane
FFI – Forsvarets forskningsinstitutt, Norwegian Defence Research Establishment
OPCW – Organization for the Prohibition of Chemical Weapons
PBS – Phosphate-buffered saline
TSB – Tryptic Soy Broth

References

[1-5]

Bibliography

[1] The Ministry for Foreign Affairs of Finland, "Recommended operating procedures for sampling and analysis in the verification of chemical disarmament (ROP),"Helsinki, 1994.

[2] V. I. Krausova, F. T. Robb, and J. M. Gonzàlez, "Bacterial degradiation of dichloromethane in culturs and natural environments," *Jornual of Microbiological Methods, Volume 54, Issue 3, September 2003, Pages 419-422*, 2003.

[3] P. K. Van and S. Undersucht, "Biological dehalogenation and halogenation reactions," *Chemosphere 2003 July, 52 (2):299-312*, 2003.

[4] "HMS-DATABLAD, DIKLORMETAN," KPT Naturfag a.s, 2003.

[5] R. H. Doi, L. R. Brown, G. Rodgers, and Y. Hsu, "*Bacillus subtilis* Mutant Altered in spore Morphology and in RNA Polymerase Activity," Proceedings of the National Academy of Sciences, Vol 66, No.2, 404-410,1970.