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FATE OF BACTERIAL AND VIRAL BIO-WARFARE AGENTS IN DISINFECTED WATERS

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Water	Chlorine	Bromine	<i>Yersinia pestis</i>		
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Fate	Vaeccinia	Venezuelan equine encephalitis (VEE)			
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PREFACE

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FATE OF BACTERIAL AND VIRAL BIO-WARFARE AGENTS IN DISINFECTED WATERS

1. INTRODUCTION

Since September 11, 2001 and the subsequent dispersal of anthrax contaminated letters through the US Postal Service, the threat of bio-terrorism has increased concerns about the safety and protection of the nation's critical infrastructure including water distribution systems. Currently, chlorination is the most common method used to disinfect potable water in the United States. However, bromination is also used by certain members of the armed forces for disinfection. Despite the use of chlorine or bromine to disinfect water, there is very limited data available with regard to the fate of bio-threat agents in such matrices. Therefore, the Joint Chemical Biological Radiological Agent Water Monitor (JCBRAWM) program was interested in examining the fate of selected pathogens in disinfected waters to ascertain whether these agents remain a significant threat after exposure to such water. Agents of interest are *Brucella* spp., *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, Vaccinia virus, and Venezuelan Equine Encephalitis (VEE) virus. Fate studies for these agents are discussed herein.

2. MATERIALS AND METHODS

2.1 Water Matrices.

Fate of selected pathogens was evaluated in three water matrices representative of waters used by different branches of the armed forces. The three water matrices were:

- a. **Formulated Tap Water** - Tap water was prepared from a recipe including multiple inorganic salts and acids and two organic acids. Refer to Appendix A for synthetic tap water preparation. This water matrix included the greatest number of possible interferents and lacked disinfectant capable of destroying the agent, thereby serving as a control.
- b. **Formulated Tap Water with Chlorine** - Tap water was prepared as described in Appendix B with the addition of calcium hypochlorite (HTH) at a final concentration of 1 part per million (ppm) or 1 mg/L free available chlorine (FAC). Free chlorine at 5 ppm was also included for spores only. This water matrix represented water purchased in bulk by Joint Forces. Refer to Appendix B for preparation of chlorine stock and chlorinated water.
- c. **Formulated Tap Water with Bromine** - Tap water was prepared as described in Appendix A with the addition of bromine at a final concentration of 2 ppm. This water matrix represented water produced onboard Navy ships. Refer to Appendix C for addition of bromine using bromine supplied by the Navy.

2.2 Agents and Culture Conditions.

Four bacterial agents were included in the present study: *B. anthracis* spores, *Y. pestis*, *F. tularensis*, and *Brucella spp.* Virulent strains were used for each of the bacterial agents and were handled under BSL-3 conditions. Two viral agents were included: Vaccinia virus and VEE. Viruses were handled under BSL-2 conditions.

For bacterial agents, vegetative cells were used for each agent except for *B. anthracis* Ames in which only spores were used (refer to Appendix D for spore production protocol). Overnight liquid cultures of *Y. pestis* CO92, *F. tularensis* Schu4, and *Brucella melitensis* 16M were grown in brain heart infusion at 28 °C, brain heart infusion plus 1% cysteine at 37 °C, and Brucella broth at 35 °C, respectively. Viable cell densities of the overnight cultures were determined by measuring absorbance at 600 nm (OD₆₀₀) and using turbidometric data previously obtained for each agent (data not shown) relating OD to CFU/mL to calculate cell densities. All densities were confirmed by serial dilution and plating onto tryptic soy agar plus 5% sheep blood, chocolate agar, and Brucella agar for *Y. pestis*, *F. tularensis*, and *Brucella*, respectively. Plates were allowed to incubate at 28 °C for 48 h for *Y. pestis*, 37 °C for 5-7 days for *F. tularensis*, and 35 °C for 24-48 h for *Brucella*, prior to counting colonies.

Viral strains used were Vaccinia virus strain WR and VEE strain TC-83, both of which are surrogate strains and handled under BSL-2 conditions. Vaccinia virus and VEE were propagated in BHK-21 and Vero monolayer cultures, respectively.

2.3 Test Procedure.

Prior to use, bacterial overnight cultures were pelleted via centrifugation (1,400 x g), washed once in cold phosphate buffered saline (PBS), and then resuspended in an equal volume of the formulated tap water. Immediately following resuspension in formulated tap water, the appropriate amount of overnight culture was added to a 10 mL water sample to achieve approximately 1×10^6 CFU/mL. Agent was allowed to incubate in each water sample at 25 °C for 0, 2, 4, 6, 24, and 30 h. An 8 h time point could not be performed due to time constraints in the BL3 laboratory. At each time point, 1 mL of sample was removed and sterile sodium thiosulfate was added to a final concentration of 0.005%. Samples were serially diluted and plated on appropriate agar as stated above. All plates were incubated under appropriate conditions stated above and then colonies were counted to calculate total CFU/mL counts for each agent at each time point for each water matrix tested. Water matrices were spiked in a similar manner with viral agents and plaque assay was performed to determine the amount of viable virus over time in each water matrix tested using BSC-40 or Vero cells and agar overlay.

3. RESULTS

Bacterial and Viral Agent Fate in Chlorinated and Brominated Water.

All data obtained for bacterial and viral agents is summarized below in graph format in Figures 1-5. Each figure shows the concentration (CFU/mL or PFU/mL) of each agent (i.e., number of viable organisms) at a specific time point in each water.

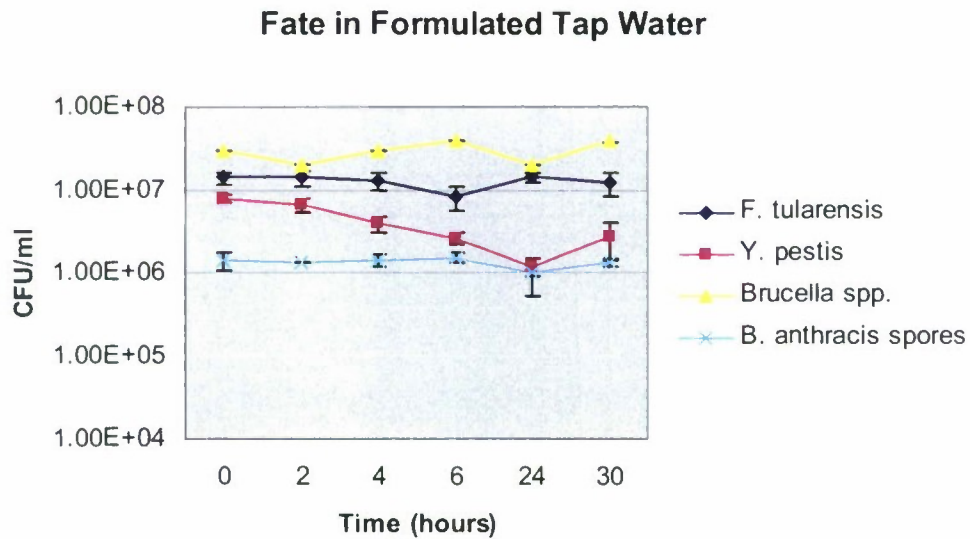


Figure 1. Fate in Formulated Tap Water.

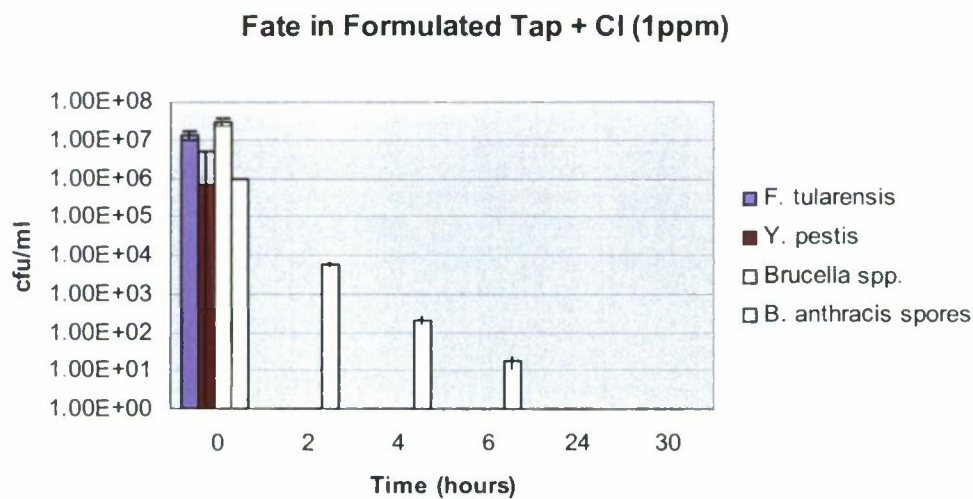


Figure 2. Fate in Formulated Tap Water with 1 ppm Chlorine.

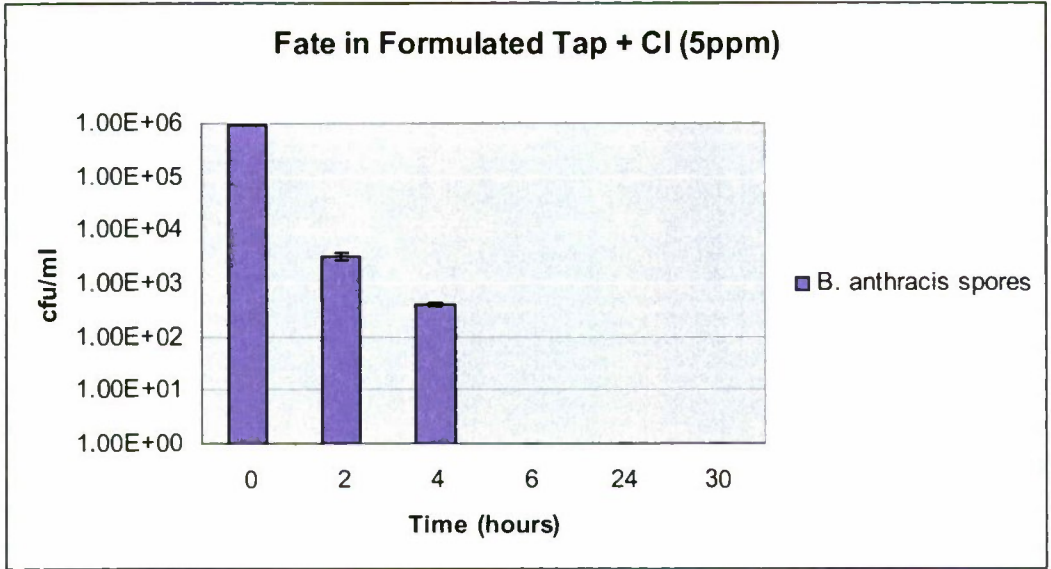


Figure 3. Fate in Formulated Tap Water with 5 ppm Chlorine.

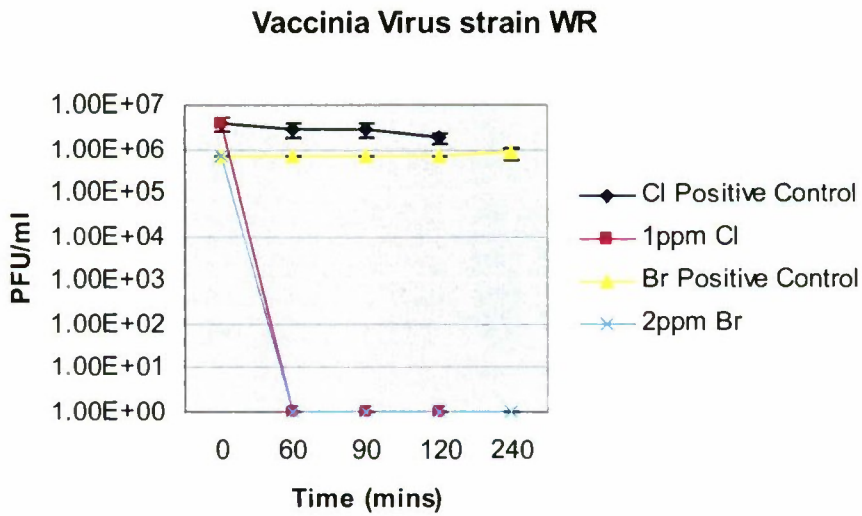


Figure 4. Fate of Vaccinia Virus in Disinfected Waters.

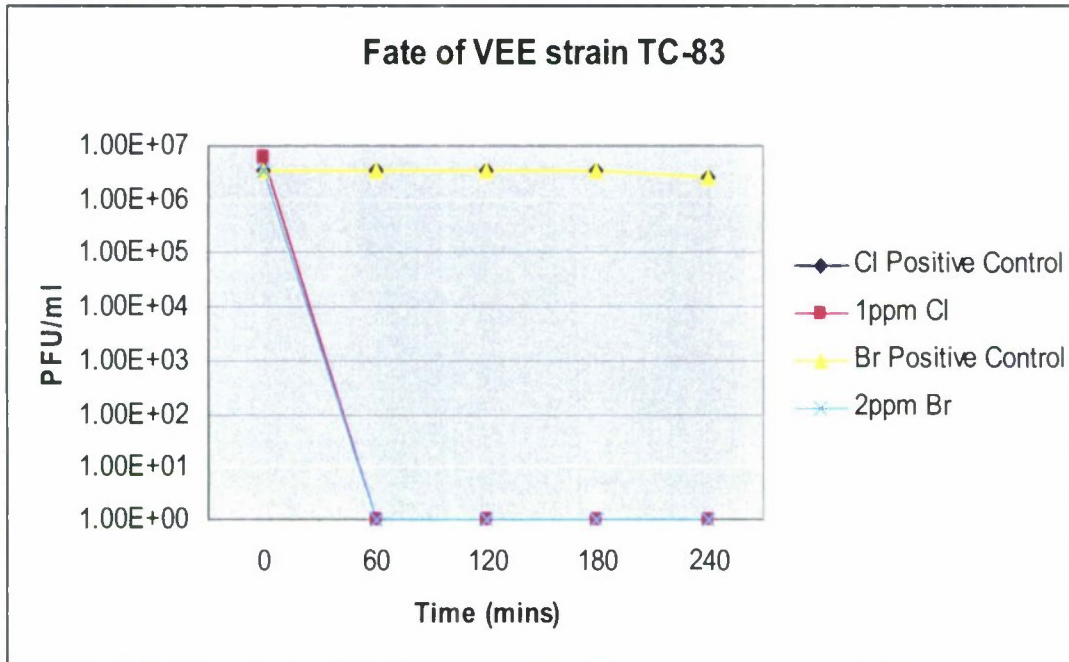


Figure 5. Fate of VEE TC-83 in Disinfected Waters.

4. CONCLUSIONS

Although disinfection of water supplies is common practice in the United States, limited data is available with regard to the length of time bio-threat agents can survive in those waters. Chlorination and bromination of water is practiced by branches of the military, and therefore, both were included in the present study. Fate of six selected agents in formulated tap water with bromine or chlorine was monitored over time by either serially diluting and plating or by performing plaque assays at various time points.

As shown above, chlorination proved effective at killing all agents tested. For bacterial agents, all vegetative organisms were killed in minutes, while spores required a longer incubation time in chlorinated water before they were no longer recoverable (approximately 6 h in 1 ppm chlorine and 4 h in 5 ppm chlorine). Viral agents were also inactivated within minutes in 1 ppm chlorinated water.

Current testing also evaluated the fate of viral agents in brominated water. Results showed that both viral agents were quickly inactivated by 2 ppm bromine.

Therefore, to summarize:

- Chlorine is an effective disinfectant against all agents tested.
- Bromine was effective at eliminating viral agents.

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APPENDIX A

PREPARATION OF SYNTHETIC TAP WATER

- Unless otherwise indicated, all chemicals were laboratory reagent grade.
- The concentrated stock solutions were prepared with ASTM Type I deionized water, were stored in opaque HDPE bottles at 4 °C, and had a shelf life of 6 months.
 - The prepared synthetic tap water was stored in a polycarbonate carboy at 4 °C and had a shelf life of 1 week.
 - The pH of the tap water should be between 7.6 and 7.8. If adjustment is required, use 1% HCl or NaOH (NOTE: In the batches we have prepared, no adjustment was necessary).
 - The order of addition has not been systematically evaluated, but the additions were always in the order outlined in the table below.
 - The recipe summarized below makes 1 L of synthetic tap water. Add approximately 500 mL of ASTM Type I deionized water to a 1 L Class A volumetric flask, add the indicated stock solutions (in the order listed), and then bring to the mark with deionized water. Wait 15-20 min, then check pH. Adjust (with minimal volume change) pH to 7.6-7.8 if required.

Chemical	Concentration of Stock (mg/L)	Amount of Stock Added (mL)	Final Concentration (mg/L)
NaHCO ₃	10,000	10.0	100
MgSO ₄ •7H ₂ O	1,000	13.4	13.4
K ₂ HPO ₄	1,000	0.7	0.700
KH ₂ PO ₄	1,000	0.3	0.300
(NH ₄) ₂ SO ₄	100	0.1	0.0100
NaCl	100	0.1	0.0100
FeSO ₄ •7H ₂ O	10.0	0.1	0.001
NaNO ₃	1,000	1.0	1.00
CaSO ₄	1,000	27.0	27.0
Humic Acid ^a	1,000	1.0	1.00
Fulvic Acid ^b	1,000	1.0	1.00

a). IHSS Suwannee River Humic Acid Standard, Cat. No. 1S101H.

b). IHSS Suwannee River Fulvic Acid Standard, Cat. No. 1S101F.

This recipe was provided courtesy by Dr. Mark LeChevalier of the American Water Works Association.

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APPENDIX B

ADDITION OF CHLORINE

Preparation of Chlorine Stock Solution

Weigh 1.00 g HTH (Part name DISINFECTANT CALCIUM 6OZ, Logistics NSN# 6840-00-255-0471, containing 65% available chlorine) into a 22 mL glass vial. Add 20.0 mL of ASTM Type 1 deionized water, cap, and shake vigorously for 1-2 min. Let the solids settle for 5-10 min before using. NOTE: This stock solution is not stable, and is made up just prior to preparing the water matrix.

Preparation of Water Matrix

Using a 100 mL graduated cylinder, add 100 mL of either reverse osmosis water or formulated tap water to an 125 mL amber, glass bottle.¹ Add 15 μ L of HTH stock solution,² cap, and swirl to mix. Remove the cap, and check the free residual chlorine level with a test strip.³ The free residual chlorine should be 1-3 ppm for reverse osmosis water and 0.5-1.0 ppm for formulated tap water. NOTE: This water matrix is not stable, and is only to be used for 1 work day (8-10 h).

¹The amber bottle should be conditioned with the chlorinated water prior to the first use (let it soak overnight). In addition, the old chlorinated water is left in the bottle until a new batch is made. This will reduce the chlorine demand of the bottle surfaces.

²Pipette the supernatant. Do not resuspend the solids prior to removing the 15 μ L.

³Hach spectrophotometer is currently used to measure the amount of free chlorine.

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APPENDIX C

ADDITION OF BROMINE

Background: Brominating feeders operate by proportioning a small stream of the water to be treated through the brominating cartridge (a packed bed of polymer beads that have bromine bonded to them), then blending that small stream back into the main stream of water to give the required dilution of bromine.

NOTE OF CAUTION: As the resin releases bromine/bromide it WILL SWELL ~ 15% OF ITS ORIGINAL VOLUME. Therefore, do not enclose a brominated resin bed in a rigid test vessel that does not account for this volume change and which may burst.

- Weighing and handling the dry resin is best done in a fume hood.

1. Set up a 1" diameter glass column (with a stopcock for flow control) with a glass frit or glass wool plug. Also obtain a squirt bottle with deionized water and a measuring vessel, (ex: 100 mL graduated cylinder).

2. Open brominating cartridge (EVERPURE, P/N 255340-416, 1.25 kg of brominated resin, 30% bromine).¹ No special safety precautions are necessary to open the cartridge.

3. Measure approximately 25 mL of resin. Adding deionized water will aid in transferring the resin from one container to another. Add this slurry to the glass column.

4. Pass either formulated tap water or reverse osmosis water through the resin at a chosen flow rate.²

5. Collect the brominated water and check total bromine residual using an ion-selective electrode.³

6. Dilute with formulated tap water or reverse osmosis water to the level of free bromine required in test. Verify by checking total bromine residual with and ion-selective electrode.

¹Over time, trace bromine vapors can be very corrosive. Weighing and handling the dry resin is best done in a fume hood. Store the dry resin in a double-bagged container in a well ventilated, cool area away from critical instruments or other items that might be damaged by corrosion. For long term storage, control the bromine vapor release by placing all of the resin in a glass or plastic jar and cover the resin with deionized water and seal.

²The level of bromine released from the resin will depend on the flow rate, water temperature, pH and the total dissolved solids (TDS) of the feed water.

³Electrode currently used is ORION 4-Star Portable pH/ISE meter (waterproof pH/conductivity system) with corresponding bromine ion selective electrode measuring total residual bromine.

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APPENDIX D

PROTOCOL FOR SPORE PREPARATION

Inoculum Preparation

Bacterial cells, stored at -80°C in Tryptic Soy Broth (TSB) supplemented with glycerol to a final concentration of 15% (v/v), are streaked for isolated single colonies onto solidified medium and incubated at appropriate growth temperature. Following the appearance of distinct colonies, a single colony is inoculated into 10 mL of TSB and grown to early stationary phase (0.7 - 0.8 at 600 nm; approximately 4 h). An aliquot of 0.4 mL bacterial culture is spread onto the surface of Sporulation Media A Medium (SMA) and incubated at appropriate growth temperature.

1.0 MATERIALS REQUIRED:

1.1 Reagents:

10 mL tube of Tryptic Soy Broth
Tryptic Soy Agar plates
150 mm Petri dishes containing Sporulation Medium
Sterile distilled water at 4°C

70% EtOH

Crystal violet Stain

1.2 Sporulation media A:

Nutrient broth 8g/L
KCl 2g/L
MgSO₄·7H₂O 0.5g/L
Agar 17g/L

Weigh and add the above ingredients one at a time to distilled water and autoclave for 15 min at 121°C . Once the media has cooled to 48°C or below, filter sterilize and add the following ingredients prior to pouring the plates.

	Final Concentration	Stock Conc.	Amount Added
Ca(NO ₃) ₂	5mM	1M	5ml/L
FeCl ₂	1μM	1mM	1ml/L
MnCl ₂	0.01mM	10mM	1ml/L
Glucose	0.1%	50%	2ml/L

After solidifying, plates are stored inverted at 4°C until needed.

1.3 Lab Supplies:

37 $^{\circ}\text{C}$ incubator
50 mL centrifuge tubes
Tube rotator for 15 mL tubes

Test tube racks for 15 and 50 mL tubes
Petri dish spreaders
Microscope slides
Disposable transfer pipettes
Bunsen Burner
Foreeps
10 mL pipettes
Pipetting device
Centrifuge
Hazardous waste disposal bag with support
Compound Light Microscope
Bibulous paper
Immersion Oil
Platform Shaker

2.0 DETAILED STEPS

2.1 Preparing the Inoculum

1. Pipette 10 mL sterile TSB into a sterile test tube.
2. Inoculate the TSB using a single colony of the strain of choice, from a fresh streaked plate (< 14 day after inoculation).
3. Allow the culture to incubate at 37 °C, while rotating until early stationary phase (approximately 4 h).

2.2 Seeding the Sporulation Plates

1. Label 150 mm Petri dishes of SMA Medium.
2. Inoculate, by aseptically spreading an aliquot of 0.4 mL of the early stationary phase culture onto each of the Petri dishes. Allow the dishes to dry upright.
3. Seal the Petri dishes *en mass* with parafilm individually allowing for one plate to be sampled daily assessing the production of spores.
4. Incubate the plates overnight, inverted, at 37 °C.

2.3 Microscopic Examination to Monitor for Spore Production

1. Clean a microscope slide with 70% EtOH. Label the slide to indicate the strain.
2. Place a large drop of sterile distilled H₂O on the slide.
3. Using a sterile loop, transfer a small amount of bacteria from the single Petri dish and suspend the cells in the water. Place the slide in the laminar flow hood atop the mesh grate at the front of the hood. Allow the slide to dry.
4. Heat-fix the bacteria to the slide by passing the slide through the flame of a Bunsen burner.
5. Apply crystal violet stain for approximately 1 min and then rinse and blot dry with bibulous paper

6. Examine the preparation under oil immersion (100X using a light microscope) for the production of spores. The spores will all be the same size and shape (one micron coccobacilli) and are readily distinguished from vegetative cells. Make a digital image of the preparation.
7. If spores comprise more than 95% of the culture, proceed to Step 2.4; otherwise, return the five plates to the incubator for an additional 24 hr of incubation.
8. Note: Alternatively, bacterial spore suspensions may be examined using Phase Contrast Microscopy.

2.4 Harvesting the Spores

1. Remove the Sporulation Plates from the incubator and place at 4°C. Wait 1.5 - 2 hr.
2. Pipette approximately 15 mL of sterile distilled H₂O into each Sporulation Plate and place the plates on the platform shaker. Set the shaker to rotate such that the water does not spill over the sides of the plates. Place two 50 mL centrifuge tubes in a rack
3. After ten minutes of rotation, use a sterile smooth plastic spreader and gently run the spreader over the surface of the Sporulation Plates to begin loosening them. Discard the spreader in the waste bag to be autoclaved. Cover with the top and continue rotating the plates for an additional 30 min.
4. Remove the plates from the platform shaker. Using a sterile 10 mL pipette, collect all of the cell suspension from each plate and pool it in the 50 mL sterile centrifuge tubes (from step 2.4.2).
5. Using a smooth sterile spreader, loosen the remaining cells from each dish.
6. Apply 10 mL of sterile distilled H₂O to each plate. Using a pipette, wash the surface of the plate with the water while tilting the plate to collect the cell suspension. Loosen all remaining material and pipette all of the material collected into the centrifuge tubes used in Step 4, above.
7. Balance the centrifuge tubes through the addition of sterile distilled H₂O.
8. Harvest spores by centrifugation at 3300 x g (3.8K) for 15 min., wash three times in 10 mL of cold sterile distilled water and re-suspend in a minimal amount of sterile distilled water. Discard the supernatant as biohazardous waste.
9. Aliquot the spore preparation to sterile microfuge tubes (1mL each) and store at -80 °C.