CYTOTOXICITY OF POLYAMINOBENZENE SULFONIC ACID FUNCTIONALIZED SINGLE WALL CARBON NANOTUBES ON *ESCHERICHIA COLI* K12 CELLS IN SUSPENSION AND IN BIOFILM

Submitted by

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ABSTRACT

Carbon nanotubes (CNT's) are considered to be a very promising material and are widely used in various technological applications. But their widespread use also increases the risk of increased release of these particles into the environment. CNT's are used in various forms for various applications. To add to, microorganisms in the environment prefer to form biofilms and are found to be more resistant to chemical disinfection. This study aimed at studying the cytotoxicity of polyaminobenzene sulfonic acid functionalized CNT's (SWCNT PAB's) on *Escherichia coli* (*E. coli*) cells in biofilm and in suspension. An experimental setup with the CDC bioreactor as a CSTR was build to grow *E. coli* in biofilm and in suspension. Favorable conditions for *E. coli* growth were maintained throughout the study. A cell plate count test was conducted on the third day of the study to establish the cell count method for the exposure run and as well to check for sufficient growth of *E. coli* cells in biofilm. The SWCNT PAB has 5mg/mL solubility in water and can be added to the reactor through the inoculation port. The exposure run was planned to be conducted on day 5 of the experiment but not carried out due to time constraint.
1. INTRODUCTION

Carbon nanotubes (CNT's) are nano-sized, hollow, graphite cylinders, which were developed in 1991 by Iijima (1). Their unique physical and chemical properties have raised the expectation of their use in various fields such as medicine, chemistry, electronics, materials, etc (1). However their increased application and production increases the risk of their increased dispersion into the environment. Hence their potential toxic effects need to be studied before these compounds are in widespread use. CNT's could be either single walled or multi-walled and could be used in their pristine form or could be modified for specific usage. As the toxicity potential of nano particles depend on the specific physiochemical and environmental factors each of these nano particles need to be evaluated on their toxicity individually (2).

Bacteria can exist in biofilms by attaching to surfaces and living in groups. These are different from their planktonic counterpart and exhibit more tolerance to conventional chemical disinfection (3). Hence it could be expected that the effect on CNT's on cells in suspension and in biofilm could be different. This study aims at understanding the cytotoxicity of polyaminobenzene sulfonic acid functionalized carbon nanotubes on the *E. coli* K12 cells that are in biofilm and in suspension.
2. BACKGROUND

CNT's could be either single walled (SWCNT) or multi walled (MWCNT). CNT's in their pristine form are hydrophobic and hence they form into aggregates when discharged in water. However they might get dispersed in water given a longer duration of time. CNT's when functionalized with another group like amide, carboxylic group etc may become water soluble due to the hydrophilic nature of the functional group attached. Functionalized CNT's are widely used in biology and biomedical application for carrying drugs inside the human system (4).

Recent studies have indicated the toxicity of SWCNT's is more than that of MWCNT's (5). A study conducted by Elimelech et al. (2007), confirms the cytotoxicity of pristine SWCNT's on E. coli cells by the cell wall piercing mechanism. However oxidized MWCNT's are more toxic than pristine MWCNT's (1). Functionalized CNT's are found to be less or not toxic due to their hydrophilic nature. This was confirmed by the study on human T cells with functionalized CNT's (4). Functionalized CNT's are rapidly taken up by the human B and T lymphocytes but does not affect viability of the cells (4). However no literature was found relating to their effect on microorganisms.
3. METHODOLOGY

To test the cytotoxicity of SWCNT-PAB’s on *E. coli* K12 cells in suspension and in biofilm, *E. coli* was grown in a CDC bioreactor, which is a continuously stirred bioreactor. This reactor helps in growing the cells both in suspension and in film. The reactor was maintained under aerobic conditions and well fed with a continuous supply of fresh media to sustain the growth of *E. coli* K12. The effluent of dead cells was constantly removed by pumping. After approximately 3 days of inoculating the reactor with cells, samples (triplicates) were collected from both the biofilm and the cells in suspension and a plate count test was conducted to check for sufficient growth of the cells. After 5 days, the exposure test was planned, by conducting plate count on samples (triplicates) collected just before the application of the SWCNT’s and immediately after the application of the SWCNT’s. Another cell count test was planned to be conducted after 24 hours of application of the SWCNT’s. The detailed methodology is described in the following sections.
4. EXPERIMENTAL SETUP

Figure 4.1 shows the complete experimental setup. The experiment was setup in a fume hood.

![Figure 4.1. Experimental Setup showing the bioreactor with a continuous flow of media in the temperature bath on a stir plate.](image)

In this study, it was important to keep all experimental units sterile and sterilization was carried out either by heat (autoclaving) for glassware or by chemical (treatment with ethanol) for heat sensitive units (tubing). A CDC bioreactor (Biosurface technologies Ltd) was used to grow the *E. coli* K12 cells in biofilm and in suspension. The reactor consists of a 1L container with the polyethylene top housing 8 removal rods. Each of the rods has 3 removal HDPE coupons, for a total of 24 sampling opportunities. At the center of the
reactor there is a detachable magnetic stirrer with a baffle wall attached. This was not used in this experiment as the baffle wall did not rotate continuously and was replaced by a normal magnetic stir bar. There is an outlet for the effluent at just above the 400 mL mark of the container. However the effective volume of the liquid in the reactor was estimated to be 350 mL as the rods displaced approximately 50 mL of liquid. The reactor consists of three ports on the polyethylene top, one for media flow, one for inoculation and one for airflow.

A magnetic stirrer was placed at the bottom of the reactor. LB media was prepared as per the protocol in Appendix 2 and was transferred aseptically to the reactor up to the 410 mL mark (i.e. just up to the outlet). The reactor was closed with the polyethylene top along with the rods, and was sealed with autoclave tape. The effluent outlet and the three inlet ports on the top of the reactor were then closed with aluminum foil. The reactor was then sealed with autoclave tape autoclaved for 15 mins at slow speed.

Fresh media needs to be supplied continuously to the reactor to maintain growth of the *E. coli* in the reactor during the study period. A four-liter bottle with an outlet at the bottom was used as the reservoir of fresh media. The flow from reservoir bottle to the reactor was designed to be by gravity. The bottle was sealed at the top and at the bottom outlet by an aluminum foil. The aluminum foil is then taped with autoclave tape and autoclaved for 15 mins at slow speed. The sterilized bottle is then connected with sterile tubing to the media inlet of the reactor and a pinch unit was used to control the flow through the tube. The bottle is then filled with 4L of autoclaved media aseptically. The bottle is then kept at a suitable height from the reactor height to facilitate flow by gravity. However the media flow was not turned on for the first 24 hours.
The outlet of the reactor was connected to the ORCBS waste container using chemically sterilized tubing. The effluent outflow was controlled by a peristaltic pump maintained at a lower flow rate (20 rpm) to ensure a constant volume of liquid suspension in the reactor. The reactor was then placed in the temperature bath maintained at 37°C by a heating unit. The air supply of the fume hood was connected to a conical flask (with a side outlet) with sterile cotton balls, and the outlet of the conical flask was connected to the air inlet of the reactor. Tubing’s and connectors for connecting the various units (air inflow, media inflow, effluent outflow, tubing for the inoculation port along with the control value) were washed with ethanol before connecting to sterilize them.
5. EXPERIMENTAL PROCEDURE

5.1 GROWTH OF E. coli – CELL CULTURE AND INOCULATION

A 10 mL cell culture tube was inoculated aseptically a day before with E. coli K12. Yu Yang, a graduate student, Department of Plants and Soil Sciences, Michigan State University, provided the E. coli K12 agar culture. Few milliliters’ of the culture was then aseptically transferred to the reactor, through the inoculation port on the reactor. The temperature bath was placed over a magnetic stir plate, and the magnetic stirrer within the reactor was set to stir at a slow mixing rate (40-50 rpm approximately) to enable a continuously stirred tank reactor (CSTR) condition. The airflow to the reactor also was turned on to make the reactor aerobic. The media flow from the media reservoir was turned on a day after inoculation, and was set at a rate such that it would compensate the evaporation losses as well as to maintain sufficient media for the growth of E. coli cells. It was not set to a particular value and was later estimated during the experiment.

The system was allowed to run for 3 days continuously with periodic monitoring. The temperature bath, magnetic stirrer, media flow, effluent outflow were periodically monitored to ensure that everything was working fine throughout the three day period. Also the tubing’s and the media reservoir bottle were visually checked for cross contamination either by environmental microbes or by E. coli backward growth.

As E. coli might take 2-5 days to grow into at least a thin biofilm (6), a plate count test was planned on day 3 to check for sufficient growth of biofilms and to establish the sampling and plate count method. Also the CNT’s exposure was planned after 5 days of growth.
5.2 SAMPLING FOR INITIAL CELL COUNT TO CHECK FOR SUFFICIENT GROWTH

On the third day from inoculating the reactor, samples of the cells in biofilm and in suspension were collected to perform a cell count by the pour plate count method. Adequate amount of 1.5 mL centrifuge tubes were autoclaved and aseptically filled with 900 µL buffered dilution water. These tubes were arranged in a holder and the sampling and dilution series was performed under the fume hood. The fume hood was first sterilized by spraying ethanol and then the experiment was conducted near a flame to avoid contamination. The sampling was done in triplicates.

One of the rods from the reactor was removed along with the three coupons present on it and a sterile plug is placed in place of the rod in the reactor to prevent contamination. The coupons were removed and placed in separate sterile containers. The coupons were first washed in phosphate buffer solution (Appendix 1) to remove any unattached cells from the coupon. Then they were transferred to a 30 mL cell culture tube with 10 mL of phosphate buffer and sonicated for 2 mins (7). 100 µL of this solution was used for further dilution (10^{-2} dilution).

Around the same time when the rod was removed, three aliquots of 100µL samples of the suspended cells were also collected in a 1.5 mL vial containing 900µL of dilution water (10^{-1} dilution).
5.3 Plate count method

The plate count method is a direct cell count method, which helps in counting the viable cells only. It works on the assumption that each viable cell yields one colony on the plate after the incubation period. Also by the use of a specific medium to plate the plate count method can help in counting only the target species. For conducting plate count method, the sample has to be first diluted before plating. If the right dilution is not plated, there will be either too many colonies on the plate (grown into each other making counting difficult) or little/no cells (not being representative of the sample). Since the right dilution is not known more than one dilution was made.
Figure 5.3.1. Procedure for viable count by serial dilution of the sample of (a) cells in suspension, (b) cells in biofilm on the coupon.

Figure 5.3.1 (a) elaborates the procedure for viable count by serial dilution for the cells in suspension and figure 5.3.1 (b) elaborates the procedure for viable count by serial dilution for the cells in the biofilm on the coupon. 100µL of the 10 mL samples in the cell culture tube are diluted (decimal dilutions) further to obtain a dilution of $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$ and $10^{-8}$ of the initial sample. Similarly the 100µL triplicate samples of the cells in suspension were
also diluted to obtain a dilution of $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$ of the initial sample. Sterile pipette tips were used for dilution and the tips were changed between dilutions. All dilutions were made using the buffered dilution water (Appendix 2).

The plate count was performed by the spread plate method. 100µL of each dilution was poured using a pipette to the center (introduces another $10^{-1}$ dilution) of a sterile agar plate and spread using a sterile bend glass spreader. The glass spreader was sterilized by dipping in ethanol followed by heating in flame. The spreader was first cooled in the agar on the side of the plate and then was used to spread the cells by rotating the plate. To avoid contamination while plating, the lid of the plate was opened only slightly to allow the pipette tip.

The plates were then incubated at 37°C in an incubator. The number of colony forming units (CFU’s) was counted after 24 hours of plating using a Quebec colony counter (Appendix 3). The colonies counted (Appendix 1) were then multiplied by the dilution factor, which is the inverse of the dilution.
5.4 Exposure to SWCNT PAB’s

The impact of the SWCNT’s was to be studied on the fifth day of growth of the biofilm in the reactor. The SWCNT PAB’s are available in powder form (Carbon Solutions Inc.,) and have a water solubility of 5mg/mL. The concentration of the SWCNT’s planned to be exposed was 50µg/mL. Thus 17.5 mg of the SWCNT’s could be mixed with 3.5 mL of water and sonicated for 30 minutes in water (1).

Samples of cells in suspension and cells in biofilm should be collected as described in section 5.2 and are plated as described in section 5.3. These are the initial number of CFU’s before exposure. After collecting and plating samples, the SWCNT solution should be added to the reactor through the inoculation port. Samples of cells in suspension and cells in biofilm should again be collected to study the immediate impact of exposure to SWCNT PAB’s. These are again diluted and plated as described in sections 5.2 and 5.3. Another set of samples should be collected 24 hours after the dosage of SWCNT’s to study the long term impact of the CNT’s on the E. coli cells.
6. **RESULTS**

Table 6.1 gives the results of the cell count test conducted on day 3 of the experiment. Though all dilutions were plated, the less diluted samples plated had too many colonies and the highly diluted samples plated didn’t have any colonies on them. Hence only those plates that had countable colonies (between 25 to 250 colonies) are presented in this report (Appendix 1).

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm on coupon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log CFU's/mm²</td>
<td>5.358</td>
<td>5.057</td>
<td>5.233</td>
<td>5.216</td>
<td>0.15</td>
</tr>
<tr>
<td>Suspended cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log CFU's/mL</td>
<td>8.427</td>
<td>8.455</td>
<td>8.476</td>
<td>8.452</td>
<td>0.025</td>
</tr>
</tbody>
</table>

The media flow rate from the media reservoir to the bioreactor was estimated to be 0.083 mL/min. The height and the diameter of individual coupons were measured to be 3.9 mm and 12.5 mm using vernier calipers. The surface area exposed for biofilm formation was thus calculated to be 122.72 mm². The data obtained from the triplicate samples was averaged and the standard deviation was calculated. The results expressed as mean±standard deviation are 5.21 ± 0.15 log CFU/mm for the biofilm per coupon and 8.45 ± 0.02 log CFU/mL in suspension (Figure 6.3).
Figure 6.3 Plate count results expressed as mean±standard deviation for both cells in biofilm and in suspension.

The plate count of the samples collected before the application of the CNT's to the reactor and after the application of the CNT's can also be expressed in log CFU’s/mL for the cells in suspension or log CFU’s/mm² for the cells in biofilm. The difference in the number of viable cells after exposure to SWCNT-PAB’s will help in identifying the impact of the same.
7. FUTURE STUDY

The experimental run to study the exposure effects of SWCNT-PAB’s was not completed due to time constraint and could be completed as part of future study. However since some primary studies have found that functionalized SWCNT's are not toxic as that of pristine CNT's, it could be expected that the exposure run with SWCNT-PAB’s may not produce a significant decrease in the number of viable counts (4). Also as the standard deviation of the triplicate cell counts by the plate count method is only in the order of 0.15 log CFU’s/mL it is not expected to interfere with the decrease in cell counts due to SWCNT's. However if need may arise alternate methods like direct microbial count, or viewing the cells under a scanning electron microscope may help in studying the impact further.

Alternatively, this set up could also be used to study the impact of pristine CNT's on the *E. coli* cells in suspension and as biofilm. Though CNT’s are not very easily soluble in water, the study by Cheng et al. (1) has reported that a water solubility of 50 mg/L can be achieved by continuously stirring the CNT's in water for 30 mins. Thus, a similar procedure can be adopted to test the cytotoxicity of the CNT solution on the *E. coli* cells in suspension and in biofilm.
8. REFERENCES


### Table A.1 CFU’s counted on the agar plates plated with samples from the biofilm on the coupon

<table>
<thead>
<tr>
<th>Trial</th>
<th>Plate Name</th>
<th>CFU's counted on plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BF.Ta.1 (10^{-3} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Ta.2 (10^{-4} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Ta.3 (10^{-5} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Ta.4 (10^{-6} dilution)</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>BF.Ta.5 (10^{-7} dilution)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>BF.Ta.6 (10^{-8} dilution)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>BF.Tb.1 (10^{-3} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Tb.2 (10^{-4} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Tb.3 (10^{-5} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Tb.4 (10^{-6} dilution)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>BF.Tb.5 (10^{-7} dilution)</td>
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</tr>
<tr>
<td></td>
<td>BF.Tb.6 (10^{-8} dilution)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>BF.Tc.1 (10^{-3} dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>BF.Tc.2 (10^{-4} dilution)</td>
<td>Too many to count</td>
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<tr>
<td></td>
<td>BF.Tc.3 (10^{-5} dilution)</td>
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</tr>
<tr>
<td></td>
<td>BF.Tc.4 (10^{-6} dilution)</td>
<td>21</td>
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<tr>
<td></td>
<td>BF.Tc.5 (10^{-7} dilution)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BF.Tc.6 (10^{-8} dilution)</td>
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</tbody>
</table>
Table A.2 CFU's counted on the agar plates plated with samples from the biofilm on the coupon

<table>
<thead>
<tr>
<th>Trial</th>
<th>Plate Name</th>
<th>CFU's counted on plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S.Ta.1 (10-2 dilution)</td>
<td>Too many to count</td>
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<tr>
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<td>S.Ta.2 (10-3 dilution)</td>
<td>Too many to count</td>
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<tr>
<td></td>
<td>S.Ta.3 (10-4 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Ta.4 (10-5 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Ta.5 (10-6 dilution)</td>
<td>267</td>
</tr>
<tr>
<td></td>
<td>S.Ta.6 (10-7 dilution)</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>S.Tb.1 (10-2 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Tb.2 (10-3 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Tb.3 (10-4 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Tb.4 (10-5 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Tb.5 (10-6 dilution)</td>
<td>285</td>
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<tr>
<td></td>
<td>S.Tb.6 (10-7 dilution)</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>S.Tc.1 (10-2 dilution)</td>
<td>Too many to count</td>
</tr>
<tr>
<td></td>
<td>S.Tc.2 (10-3 dilution)</td>
<td>Too many to count</td>
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<td></td>
<td>S.Tc.3 (10-4 dilution)</td>
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<tr>
<td></td>
<td>S.Tc.4 (10-5 dilution)</td>
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<td></td>
<td>S.Tc.5 (10-6 dilution)</td>
<td>299</td>
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<tr>
<td></td>
<td>S.Tc.6 (10-7 dilution)</td>
<td>40</td>
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</tbody>
</table>
APPENDIX B

PROTOCOL FOR PREPARATION OF MEDIA AND OTHER SOLUTIONS

B.1 PROCEDURE FOR PREPARING LB MEDIA

Materials

1. Bacto-Tryptone 10 grams
2. Bacto-yeast extract 5 grams
3. Sodium chloride (NaCl) 10 grams

Method

Take a 1L conical flask and add the measured quantities of chemicals listed above. Add 1L of deionized water and mix well by means of a magnetic stirrer. Once the media turns into a clear liquid, remove and transfer it to a 1L screw cap bottle. Close the screw cap loosely to prevent pressure build up in the liquid while autoclaving. Put the autoclave tape and autoclave the bottle for 30 minutes. Remove from the autoclave and refrigerate at 4°C.

B.2 PROCEDURE FOR PREPARING LB AGAR

Materials

1. Bacto-Tryptone 10 grams
2. Bacto-yeast extract 5 grams
3. Sodium chloride (NaCl) 10 grams
4. Agar 15 grams
Method
Take a 1L conical flask and add the measured quantities of chemicals listed above. Add 1L of deionized water and mix well by means of a magnetic stirrer. Take a 1L conical flask and add the measured quantities of chemicals listed above. Add 1L of deionized water and mix well by means of a magnetic stirrer. Once it turns into a clear liquid, remove and transfer two 500 mL portions to two 1 L screw cap bottle. Close the screw cap loosely to prevent pressure build up in the liquid while autoclaving. Put the autoclave tape and autoclave the bottle for 30 minutes. Remove from the autoclave and pour out into plates before it gets cold.

B.3 Procedure for preparation of Buffered water for dilution during plate count

(as per section 9050 C 1.a, Water Standard Methods for the examination for Water and Wastewater 20\textsuperscript{th})

To prepare stock phosphate buffer solution, dissolve 34.0 g of Potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}) in 500 mL reagent-grade water, adjust to pH 7.2 ± 0.5 with sodium hydroxide (NaOH) and dilute to 1L with reagent-grade water. Discard turbid stock solutions.

1. Add 1.25 mL of stock phosphate buffer solution and 5.0 mL magnesium chloride solution (81.1g of MgCl\textsubscript{2}.H\textsubscript{2}O/L reagent-grade water) to 1L reagent grade water.

2. Autoclave the buffered dilution water prepared.
**B.4 Procedure for preparation of phosphate buffer**

(as per section 9216 B 3.a, Standard Methods for the examination for Water and Wastewater 20th)

1. Dissolve 13.6g KH$_2$PO$_4$ in water and dilute to 1L

2. Adjust to pH 7.2 if necessary, filter through 0.2μm membrane filter.
APPENDIX C

LIST OF FIGURES

Figure C.1. Setup 1 - with syringe pumps for fresh media flow. Failed due to inability to prevent leakage in system

Figure C.2. Setup 2 - with a 1 liter media bottle. Flow was by gravity. Failed due to difficulty in keeping the system sterile
Figure C.3. Plate count of cells in biofilm – Dilutions $10^{-4},10^{-5},10^{-6}$ are shown.

Figure C.4. Plate count of cells in biofilm – Dilutions $10^{-2},10^{-3},10^{-4},10^{-5},10^{-6}$ and $10^{-7}$ are shown.
Figure C.5. Quebec colony counter