BACTERIAL COAGULATION BY A CHLORINATED SOLVENT

by

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Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute
in candidacy for the degree of
MASTER OF SCIENCE
in
Sanitary Engineering

August, 1965

Blacksburg, Virginia
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I. INTRODUCTION

Almost all industrial waste effluents and all domestic sewage effluents contain microorganisms and organic matter which when released to the natural waterways impose a demand for oxygen during their decomposition. With the ever increasing volumes of effluents being discharged to the watercourses, there is a definite need for a more thorough treatment. Improper operation of some treatment processes such as sludge bulking from the activated sludge operation can cause considerable pollution. Sewage lagoons and oxidation ponds may cause trouble when algae blooms occur by permitting large quantities of algal growth to be emptied into the receiving streams. As waste water becomes an increasing fraction of supplies for drinking water, it will be necessary to have sewage effluents of a very high quality.

Dispersed growth aeration is a high-rate modification of the activated sludge process, but unfortunately flocculent growths do not develop. High B.O.D. loadings are required to provide enough food to keep the bacteria in the log growth phase. A greater percentage of the organic material could be removed in the dispersed growth process than in the conventional activated sludge process. However, since the bacteria are not settleable and many escape with the effluent, the actual B.O.D. removal is usually only about fifty percent.
If an economical method could be found that would prevent bacteria from escaping in the effluent, the overall B.O.D. removal and effluent quality could be greatly improved.

Coagulation with solvents may be a feasible method of removing the bacteria from effluents. In solvent coagulation, the interfacial tension between the water phase and the organic phase is high, thus causing the bacteria in the water phase to migrate to the liquid-liquid interface and remain there unless work is done on the bacteria to remove them. This phenomenon has been put to good use in the past by removing proteins from complex polysaccharides and bacteria from fermentation media. It is possible that a solvent coagulation process could be developed either to supplement dispersed growth aeration or to improve conventional waste treatment by superior removal of microorganisms at a key point in the treatment cycle.

Previous investigations in our laboratories with domestic sewage containing large populations of mixed microbial cultures gave erratic yields with the solvent coagulation process. The present study used pure cultures to determine to what degree different types of bacteria are removed by solvent coagulation and why the degree of removal varies from one bacterium to another. The goal is sufficient understanding to permit the design of a commercially feasible process.
II. REVIEW OF LITERATURE

Solvent extraction is used for the removal of grease and other organics from water in industrial processes and has been proposed for treating of industrial waste. The common processes using solvent extraction have been both batch and countercurrent with better results from the countercurrent methods (7).

Some processes of interest to waste treatment are: the extraction of grease from wool with a chlorinated solvent (4); the use of solvents in the analysis of grease described by Standard Methods (19); solvent extraction used by Hennigan and Nemerow to shorten the time required to make B.O.D. analysis on waste (8); and the desalination of water by solvent extraction (5). There has been scattered mention of scums and coagulation, but the various authors seem unaware of bacterial coagulation with chlorinated solvents. Sevag (17) developed a process based on this principle for the removal of proteins from biological fluids in 1940, but Dawson (6) in 1963 seems to be the first to mention it for treating waste.

The accumulation of bacteria at the solvent-water interface was noted by Mudd and Mudd in 1924 (11) and (12). They observed the interfacial forces exerted on different bacteria with a darkfield microscope and concluded that once bacteria
get in the interface between the organic liquid and the water they will remain unless work is done on the bacteria to remove them from the interface. Their work was conducted using motile, non-motile, and acid-fast bacteria of which some were gram positive and others gram negative. Surface active agents were indicated to be of importance in the coagulation of bacteria with solvents. They also concluded that temperature would affect the coagulation of bacteria with solvents. Dawson (6), however, concluded that temperature did not appear to affect the efficiency of coagulation of particles of a settled sewage using chlorinated solvents.

Dawson attempted to determine the applicability of a solvent coagulation process to domestic sewage. However, his results were variable. For example, the sample range of his suspended solids test on uncoagulated settled sewage was 107 mg/l with the standard deviation 32.5 mg/l. For coagulated settled sewage, the range of the suspended solids test results was 31 mg/l with the standard deviation 9.4 mg/l. Some variability of the results was undoubtedly a result of variations from the treatment plant, but the wide range of yields points out the limited understanding of the phenomenon. Dawson observed that pH had very little effect on coagulation of microorganisms from sewage with chlorinated solvents.
A problem not considered here is the possibility that the solvents may produce a greater contamination problem than the bacteria being removed. Since chloroform is slightly soluble in water, another less soluble solvent may have to be used to remove excess solvent. Rudolfs (16) also reports that solvents will affect sludge digestion by retarding or inhibiting the sludge digestion process. In some cases with solvent concentrations of only ten parts per million, the gas production has been reduced fifty percent in the sludge digestion process.

The coagulation of viruses and cell fragments at the interface of various solvents is the topic of a book by Albertsson (1), however, the same principles apply to the coagulation of bacteria.
III. METHODS AND MATERIALS

Reagents

Chloroform, (CHCl₃), (Certified), (Fisher). Used as an immiscible solvent.

Nutrient Agar, (Difco). Used as media for plate counts.

Nutrient Broth, (Difco). Used as media for bacteria.

Phosphoric Acid, (H₃PO₄), (Certified), (Fisher). Used to adjust pH.

Purifloc 501, (Dow). Used as an anionic surface active agent.

Purifloc 601, (Dow). Used as a cationic surface active agent.

Sodium Chloride, (NaCl), (Certified), (Fisher). Used to make conductive saline for Coulter counting.

Sodium Hydroxide, (NaOH), (Certified), (Fisher). Used to adjust pH.

7X, (Linbro). Used as a non-ionic surface active agent.

Apparatus and Materials

Autoclave, (Will). Used to sterilize media for samples and plate counts.

Beam Balance, (Ohaus), (Will). Used to determine weights of reagents used in mixtures.

Coulter Counter, (Model A), (Coulter). Used to count microorganisms.
Darkfield Quebec Colony Counter, (American). Used in making colony counts of microorganisms plated on nutrient agar.

Hot Air Oven, (Despatch). Used to sterilize glassware for bacteriological investigation.

Incubator (10°C), (Precision). Used to control settling temperature.

Incubator (25°C), (Precision). Used to control settling temperature.

Incubator (35°C), (Precision). Used to incubate agar plates in bacteriological investigation.

Incubator (37°C), (Precision). Used to control settling temperature.

Incubator (55°C), (Precision). Used to control settling temperature.

25 ml. Nessler Tubes. Used as mixing and settling chambers.

pH Meter, (glass electrode, Model N), (Beckman). Used to measure pH of samples.

Reciprocating Shaker, (Brunswick). Used to shake bacteria in medium during growth.

Vacuum Pump, (Wills). Used to filter medium.
Key to Suppliers and Manufacturers of
Reagents, Materials, and Apparatus


(Beckman). Beckman Instruments, Inc.; South Pasadena, California.

(Coulter). Coulter Electronics; Hialeah, Florida.

(Despatch). Despatch Oven Company; Minneapolis, Minnesota.

(Difco). Difco Laboratories, Inc.; Detroit, Michigan.

(Dow). The Dow Chemical Company; Midland, Michigan.


(Linbro). Linbro Chemical Company; New Haven, Connecticut.

(Brunswick). New Brunswick Scientific Company; New Brunswick, New Jersey.

(Precision). Precision Scientific Company; Chicago, Illinois.

(Will). Will Corporation; Rochester, New York.
Analytical Methods

This investigation was conducted with pure culture bacteria obtained from the Biology Department at Virginia Polytechnic Institute. These cultures were preserved during the time of investigation in cystine tryptic agar at room temperature. For each experiment, the bacteria to be tested were inoculated into a 250 ml. flask containing 100 ml. of sterile Bifco nutrient broth. The flask was placed on a reciprocating shaker at the optimum growth temperature required by that particular bacterium.

An extra 50 ml. of distilled water was used in each 1000 ml. batch of nutrient broth to compensate for a five percent loss of liquid during sterilization in the autoclave. The medium was made suitable for Coulter counting by filtration before autoclaving to remove any particles, and 105 ml. were dispensed into each of ten 250 ml. flasks sealed with aluminum foil.

Bacterial samples after sufficient time for growth were pipetted into 25 ml. Nessler tubes and shaken with chloroform. The volume of chloroform in most experiments was five percent by volume of the total 10 ml. mixture. Mixing was obtained by placing a stopper in the tube and shaking vigorously by hand for five seconds. After mixing, the mixture was settled in the Nessler tube under quiescent conditions for a specified length of time, which was usually 30 minutes.
A blank consisting of the bacterial sample without solvent was processed along with the bacterial sample containing the solvent. After settling, the supernatant was removed with a pipette. Care was exercised in withdrawing the supernatant so that the liquid was removed without disturbing the settled residue. One ml. of liquid was placed in 100 ml. of 0.9 percent saline for counting by the Coulter Counter. A description of Coulter counting is given in Appendix A.

The procedure for the standard plate count followed Standard Methods for the Examination of Water, Sewage and Industrial Waste (2) with the modification that nutrient agar was used as the growth medium.
IV. RESULTS

The purpose of this investigation was to obtain data on solvent coagulation of certain common bacteria to provide the foundations for a process for the removal of bacteria from waste effluents.

**Pure Cultures**

The bacteria chosen were all aerobic and normally non-pathogenic. They were all common to domestic sewage to some degree so that results are meaningful to real situations. The characteristics vary greatly among the various bacteria studied as shown by Table 1. This particular group of bacteria is cultured with relative ease in nutrient broth.

**Solvent Coagulation Under Normal Conditions**

The different bacteria were tested as described under Analytical Methods. These tests were conducted with as many standardized conditions as possible. The pH that the bacteria had produced at the time tested was not changed, and this series of samples was at room temperature. A coagulation time of 30 minutes and a five percent by volume concentration of chloroform were used. The results are presented in Table 2.

Although the nutrient broth and the saline electrolyte are membrane filtered, there are still particles of the size
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Shape</th>
<th>Size (microns)</th>
<th>Order of Growth</th>
<th>Motile</th>
<th>Gram</th>
<th>Common to Sewage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aerobacter aerogenes</em></td>
<td>Rods</td>
<td>0.7 by 1.5</td>
<td>Singly</td>
<td>No</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>Rods</td>
<td>2.5 by 4.5</td>
<td>Pairs, packets and short chains</td>
<td>Yes</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Rods</td>
<td>1.0 by 4.0</td>
<td>Short to long chains</td>
<td>Usually Positive</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Rods</td>
<td>0.5 by 2.0</td>
<td>Singly, pairs and short chains</td>
<td>Some</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>Rods</td>
<td>1.0 by 2.0</td>
<td>Short and long chains</td>
<td>No</td>
<td>Positive</td>
<td>Maybe</td>
</tr>
<tr>
<td><em>Micrococcus flavus</em></td>
<td>Spheres</td>
<td>0.8</td>
<td>Singly, clumps and groups</td>
<td>Not</td>
<td>Variable</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>Rods</td>
<td>0.7 by 2.0</td>
<td>Singly, pairs and chains</td>
<td>Yes</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Sarcina lutea</em></td>
<td>Spheres</td>
<td>1.0 to 1.5</td>
<td>Packets</td>
<td>No</td>
<td>Positive</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>Short Rods</td>
<td>0.5 by 0.3</td>
<td>Singly or short chains</td>
<td>Yes</td>
<td>Negative</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>Spheres</td>
<td>0.5</td>
<td>Singly, pairs, and groups</td>
<td>No</td>
<td>Positive</td>
<td>Maybe</td>
</tr>
</tbody>
</table>
Table 2: Solvent Coagulation of Various Bacteria

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>pH</th>
<th>Percent Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenes</td>
<td>7.9</td>
<td>93.5</td>
</tr>
<tr>
<td>Azotobacter chroococcum</td>
<td>7.2</td>
<td>97.3</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>7.0</td>
<td>37.5</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8.5</td>
<td>34.4</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>7.3</td>
<td>69.4</td>
</tr>
<tr>
<td>Micrococcus flavus</td>
<td>7.0</td>
<td>40.1</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>8.6</td>
<td>92.7</td>
</tr>
<tr>
<td>Sarcina lutea</td>
<td>7.1</td>
<td>65.1</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td></td>
<td>97.6</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>7.4</td>
<td>86.8</td>
</tr>
</tbody>
</table>
of bacteria that remain throughout filtering. To account for these particles, two broth samples, one a blank and the other with five percent chloroform, were processed the same as the bacterial samples except they had not been inoculated with bacteria. The average counts of the broth samples were subtracted from the counts of the respective blank and five percent chloroform bacterial samples so that only the count of the microorganisms would be used to determine the percentage of removal of the various bacteria.

It can be noted that with *Serratia marcescens* and *Azotobacter chroococcum* removals were above 97 percent. Coagulation of *Micrococcus flavus* gave only 40 percent removal. All of the bacteria investigated except *Sarcina lutea*, *Lactobacillus casei* and *Micrococcus flavus* had removals greater than 84 percent. This indicates that the process seems to remove bacteria quite effectively, but poor yields could result if certain strains were present.

**Effect of Settling Time on Solvent Coagulation**

Variations from the standard 30 minute settling time were studied with the results shown in Table 3.

From Table 3 it can be seen that there is a definite increase in removal of bacteria as the settling time is increased. There is a greater increase for *Sarcina lutea* perhaps because the percent removal at 0.5 hours is less than
Table 3: Effect of Settling Time on Solvent Coagulation of Bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Percentage Removal 0.5 hours</th>
<th>Percentage Removal 1.0 hours</th>
<th>Percentage Removal 1.5 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobacter aerogenes</td>
<td>93.5</td>
<td>-</td>
<td>99.4</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>87.9</td>
<td>91.1</td>
<td>94.7</td>
</tr>
<tr>
<td>Sarcina lutea</td>
<td>65.1</td>
<td>74.3</td>
<td>-</td>
</tr>
</tbody>
</table>

for the other two. *Aerobacter aerogenes* was removed greater than 99 percent in 1.5 hours of settling and it appears that *Bacillus cereus* would reach such a percentage in another hour.

**Effect of pH on Solvent Coagulation**

It is known that pH will affect the charges on bacteria at pH ranges of 2.5 to 3.5 and 12 to 14; however, it was desirable to know what effect if any pH would play at ranges of 6.0 to 10.0 which would be reasonable working ranges of treatment plants. The pH of the bacteria-broth mixture was recorded just before the test to confirm that the pH was the same as during the test under normal conditions. The pH of each bacterial sample was lowered to pH 6.0 with phosphoric acid; all had an initial pH greater than 6.0. *Proteus vulgaris* and *Escherichia coli* had initial values above pH 8.0; therefore they were also tested at pH 7.0. *Bacillus cereus*,

Micrococcus flavus, Sarcina lutea and Staphylococcus epidermidis had an initial pH around 7.0. These were tested at pH 8.0 and all six were tested at pH 10.0. The results in Table 4 indicate that removals are usually better in the normal to slightly higher than normal pH ranges for each except Bacillus cereus which was slightly better at a lower pH. Micrococcus flavus, Sarcina lutea, and Staphylococcus epidermidis showed best removals at their normal pH range. At pH 6.0, the Staphylococcus epidermidis control sample cleared up very quickly and the addition of chloroform only increased the removal by 2.7 percent.

Effect of Temperature on Solvent Coagulation

Mudd and Mudd (12) state that heating of the bacterial sample-chloroform mixture will change the interfacial tension, causing movement of the trapped bacteria. Typical cultures were tested at temperatures of 10°C, 25°C, 37°C, and 55°C and the results are in Table 5.

Each bacterial sample was grown at its normal temperature, then held until a constant temperature was obtained for the coagulation test.

Tests conducted at 25°C produced the best results with no exceptions. Samples tested at a temperature of 37°C produced the next best results except for the Escherichia coli sample which had its second best removal at 55°C.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Normal pH</th>
<th>Normal pH</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
<th>pH 10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>7.0</td>
<td>87.5</td>
<td>89.6</td>
<td>-</td>
<td>86.5</td>
<td>66.9</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8.5</td>
<td>84.4</td>
<td>55.6</td>
<td>74.9</td>
<td>-</td>
<td>89.3</td>
</tr>
<tr>
<td>Micrococcus flavus</td>
<td>7.0</td>
<td>40.1</td>
<td>24.9</td>
<td>-</td>
<td>10.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>8.6</td>
<td>92.7</td>
<td>20.3</td>
<td>77.3</td>
<td>-</td>
<td>94.3</td>
</tr>
<tr>
<td>Sarcina lutea</td>
<td>7.1</td>
<td>65.1</td>
<td>48.7</td>
<td>-</td>
<td>60.1</td>
<td>62.6</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>7.4</td>
<td>96.8</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>79.4</td>
</tr>
</tbody>
</table>
Table 5: Effect of Temperature on Solvent Coagulation

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>% Removal at 10°C</th>
<th>% Removal at 25°C</th>
<th>% Removal at 37°C</th>
<th>% Removal at 55°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>30.5</td>
<td>86.3</td>
<td>77.3</td>
<td>38.8</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>39.5</td>
<td>85.3</td>
<td>65.1</td>
<td>75.9</td>
</tr>
<tr>
<td>Micrococcus flavus</td>
<td>3.1</td>
<td>16.2</td>
<td>6.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>63.7</td>
<td>90.8</td>
<td>79.0</td>
<td>77.1</td>
</tr>
<tr>
<td>Sarcina lutea</td>
<td>22.7</td>
<td>65.0</td>
<td>46.0</td>
<td>32.8</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>27.1</td>
<td>85.1</td>
<td>74.2</td>
<td>34.2</td>
</tr>
</tbody>
</table>

Effect of Surface Active Agents on Solvent Coagulation

Most bacteria have surface charges which are produced by chemical groups on the surface. These functional groups are polar and usually give a net negative charge to bacteria. Therefore it seems that a cationic surface active agent might reduce the polar charges on the bacteria tested and cause a greater coagulation. The results of an investigation using *Bacillus cereus* indicates this to be true. Purifloc 601, a cationic surface active flocculant, was dissolved in distilled water at the recommended concentrations (14) of 10 percent by weight and 0.2 ml. was added to the 10 ml. bacterial sample in the Nessler tubes. The coagulation test
then showed removals of 96.1 percent as compared to 87.5 percent removal of *Bacillus cereus* in the controls. When an anionic surface active agent, Purifloc 501, was used, removal was only 33.5 percent, much less than the controls. A non-ionic agent, 7X, played no part in reducing or increasing coagulation of the bacteria by the solvent. The percent removal with the non-ionic surface active agent was 96.2 which was almost the same as with the controls.

**Effect of a Flocculating Agent on Solvent Coagulation**

Alum, which is used to great benefit in the sanitary engineering field as a flocculating agent, was used to determine if it would aid chloroform in coagulation of bacteria. From the results obtained, it seems that it does not hinder or help the solvent coagulation process. When alum is used in large enough quantities to produce a floc, then a high percentage of removal is obtained; however, this removal occurs with or without the addition of chloroform. Because this investigation seemingly had no relationship to bacterial coagulation with solvents, it was discontinued.

**Effect of Different Concentrations of Solvent**

Five samples using *Bacillus cereus* were set up, each with a different concentration of chloroform. The results in Table 6 show that the best removal was recorded with the five percent chloroform mixture.
Table 6: Effect of Different Concentrations of Solvent

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>10%</th>
<th>5%</th>
<th>2%</th>
<th>1%</th>
<th>0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>72.5</td>
<td>86.8</td>
<td>81.2</td>
<td>70.4</td>
<td>50.9</td>
</tr>
</tbody>
</table>

Reproducibility of Experiments

To determine what degree of reliability could be placed on the investigations conducted, six replicates of Bacillus cereus were run with five percent by volume mixture of chloroform at normal growth conditions. Table 7 shows that there is only a 3.4 percent difference between the maximum and minimum removal. The average is 87.2 and the standard deviation is 1.63. These results compare favorably with those of Bacillus cereus listed previously.

Solvent Coagulation Effect on Bacteria

An experiment was run to determine what percentage of the bacteria not coagulated were viable. Dawson (6) concluded that using domestic sewage samples many bacteria that were not coagulated also were not killed by the chlorinated solvents. It was thought that possibly with a pure culture of bacteria results might not be the same. However, after determining the total bacterial count remaining after a sample had been coagulated and plating out dilutions of this sample,
Table 7. Replication of Results with Tests on \textit{B. cereus}

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percent Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88.4</td>
</tr>
<tr>
<td>2</td>
<td>86.7</td>
</tr>
<tr>
<td>3</td>
<td>85.1</td>
</tr>
<tr>
<td>4</td>
<td>86.7</td>
</tr>
<tr>
<td>5</td>
<td>88.4</td>
</tr>
<tr>
<td>6</td>
<td>87.8</td>
</tr>
</tbody>
</table>

The results strongly indicate that a large percent of the \textit{Bacillus cereus} used in the experiment were not viable. The plate count at a dilution of 1:10 was 60 and the count at a dilution of 1:10^2 was 5. The actual count was 2.8 \times 10^6 microorganisms per ml. and only 600 microorganisms per ml. were viable. This indicates that the chloroform that is soluble in water is killing almost all of the bacteria that are not coagulated.

\textbf{Mixed Cultures}

A small unit was set up to obtain dispersed growth bacteria using 1.0 gm. dry milk and 1.0 gm. dextrose per liter
of water as feed, but a proper culture could not be obtained. Sugg (3) using an activated sludge unit very similar to the unit used by this investigator developed dispersed growth twice during his operations with raw domestic sewage as feed. His unit consisted of a plastic waste basket as the operation tank. The tank contents were aerated with a diffuser and a baffle was used to keep the settleable material from escaping with the effluent. The container had a capacity of 8.0 liters, and a feed rate of about 8.0 liters per day was being used when the dispersed growth occurred.

When these dispersed growths occurred, this investigator obtained some of the effluent and conducted solvent coagulation tests. The results of the tests on the first batch of dispersed growth effluent were encouraging in that with the normal five percent mixture of chloroform the percentage of removal of dispersed particles was 90.6, and when a one percent mixture of chloroform was used, the removal was 72.9 percent. With the second batch, two separate tests were run under normal conditions. The percent removals for the tests were 89.9 and 90.3. The pH was 7.8 and the tests were conducted at room temperature.

Other Experiments

To determine if the chloroform formed any droplets during the vigorous shaking which might be counted by the
Coulter Counter as bacterial particles, a test was conducted in the normal manner. However, two separate saline-bacterial supernatant samples were prepared for counting. Into one of the two samples, 1.0 ml. of distilled water was added, and into the other, 1.0 ml. of acetone was added. Blanks were run with distilled water and saline and acetone and saline. Chloroform is completely soluble in acetone; therefore, the count produced by the sample with acetone present minus the count of the acetone blank would be strictly counts of microorganisms. The count of the regular sample minus the regular blank was the same as the count of the sample with acetone minus the acetone blank. This indicated that no chloroform droplets were being counted.
V. GENERAL DISCUSSION

The solvent coagulation process definitely works on many pure cultures of bacteria. It works better on some than others, but no clear explanation was uncovered. The removals by coagulation for the pure cultures tested were, except for a few, all in excess of 84 percent at normal process conditions which were five percent by volume of chloroform and settling times of 30 minutes.

An increase in settling time shows an increase in percentage removal of the bacteria tested. However, settling times of much over 30 minutes might not be economical in an actual process.

The effect of pH seemed to differ to a slight degree with each bacterium tested. However, all tested showed best removals at or near their normal growth pH. Adjusting the pH seemed to have no marked improvement on the percentage of removal by solvent coagulation unless the change in pH was very great. However, at the extreme pH ranges, corrosion and other problems would require consideration.

Of the bacteria investigated, all were coagulated best at a temperature of about 25°C. Tests were conducted at temperatures of 10°C, 25°C, 37°C, and 55°C. As the temperature was varied from 25°C, the percentage of removals decreased. This can possibly be explained by the fact that in
order to have coagulation at the interface between the aqueous and organic phases the interfacial tension between these phases must be very large as compared to the interfacial tensions between the water and bacterium and the solvent and bacterium. However, interfacial tension of the liquid-liquid boundary is related to the mutual solubility of the two phases, with the largest interfacial tension occurring when the two liquids are completely insoluble.

When chloroform and water are heated, chloroform becomes more soluble in water and therefore will reduce the interfacial tension between the phases. This would be expected to produce less coagulation at the interface. It would seem that as the temperature was lowered to 100°C there would be a greater percentage of removals. However, this did not occur, possibly because the viscosity greatly increases as the temperature decreases. Better coagulation possibly occurred, but due to the increased viscosity the settling of the collected bacteria was adversely affected -- resulting in lower performance for the overall process. A longer settling time at the lower temperature might have produced greater percent removals.

Reducing the charge on the surface of the bacteria with the aid of a proper surface active agent proved to be of great benefit in the experiment conducted. Reducing the magnitude of the charge of the bacteria would reduce the
repulsion force of one bacterium on another, thus providing less chance of bacteria forcing each other out of the floc or interface. It is suggested that further investigations pay particular attention to the use of surface active agents.

The concentration of solvent used produced interesting results. Best removals were recorded at a five percent by volume concentration of solvent. Half or double this amount gave inferior yields. The results clearly indicate a reduction in percentage of removals as the concentrations of solvent are changed much from five percent. It is suggested that future investigations better delineate the optimum concentration of solvent.

The investigation to determine the viability of the bacteria remaining in suspension after the coagulation process proved that many of the bacteria in suspension were not killed, probably because they were either not exposed or exposed for only a short time to the chloroform during the shaking of the liquids.

Chloroform droplets were not remaining in suspension and being counted as microorganisms.

Upon testing a sample similar to that of an actual dispersed growth effluent containing large populations of mixed cultures, it was found that removal of bacteria by the solvent coagulation process was about 90 percent. This percentage of removal approaches good results and indicates that further refinement may produce a commercially feasible process.
VI. CONCLUSIONS

This investigation has led to the following conclusions:

1. Good removals of most bacterial species were observed.
2. Increased length of settling time increased the percent removal of pure cultures.
3. The solvent coagulation process worked best at a pH very near the pH produced by the bacteria during growth.
4. There was no advantage in changing from room temperature.
5. Proper surface active agents aided in the coagulation of bacteria in the solvent coagulation process.
6. Almost all of the bacteria in suspension after the coagulation process were not viable.
7. Good removals were observed using the solvent coagulation process on mixed cultures.
8. The chlorinated solvent coagulation process shows promise for commercial operations.
VII. SUMMARY

Chlorinated solvents cause the coagulation of bacteria at the interface between the solvent and the aqueous phase. Since dispersed bacteria are contained in the effluents of many sewage and industrial waste processes, a process based on solvent coagulation has practical importance.

With tests conducted on both pure and mixed culture bacteria, it was found that good removals of most pure culture bacterial species and of the mixed culture bacteria occurred using the chlorinated solvent coagulation process. Best percentages of removals were recorded when the bacterial samples were tested at room temperature and at the pH produced during incubation of the particular bacteria tested. Certain surface active agents aided in the coagulation of bacteria in the solvent coagulation process. An increase in settling time provided an increase in percentage of removal of pure culture bacteria. Many of the bacteria in suspension after the coagulation process remained viable.

This investigation provided a basis for future work and provided several leads to be studied.
VIII. ACKNOWLEDGEMENTS

The author would like to express his appreciation to his thesis advisor, Dr. Henry R. Bungay III, for his constant encouragement, prudent guidance, and constructive criticism.

The author also wishes to express his appreciation to Dr. William A. Parsons for helpful criticism and encouragement throughout his entire Master's program.

He would also like to thank the Public Health Service for supporting his work through a Research Fellowship.

The assistance given by Mr. E. G. Willard in the laboratory accelerated the completion of this thesis and is greatly appreciated by the author.

The encouragement and moral support given the author by his wife is also greatly appreciated.
IX. BIBLIOGRAPHY


Explanation of the Coulter Counter

The Coulter Counter determines the number of particles suspended in an electrically conductive liquid by forcing the suspension to flow through a small aperture having an immersed electrode on either side. As a particle passes through the aperture, it changes the resistance between the electrodes, thus producing a voltage pulse of short duration having a magnitude proportional to the particle volume. The series of pulses is then electronically counted by means of a gating circuit. The volume of sample flowing through the aperture is determined by either a mercury manometer switch linked hydraulically to the interior chamber or by a timed sampling period used primarily for small apertures which pass liquid too slowly for the manometer method.

Figure 1 shows a schematic diagram of the Coulter Counter. When the vacuum stopcock is opened, a controlled external vacuum initiates flow from the beaker through the aperture. An electric timer may be used to fix the flow interval, or the mercury column can be used to activate the switching circuits that control counting.

The voltage pulses are amplified and fed to a gating circuit having an adjustable threshold level. If this level is reached or exceeded by a pulse, the counter driver is
Figure 1: Schematic of Coulter Counter

Diagram showing the flow of signals and components.

- To vacuum pump
- Pulse amplifier
- Threshold circuit
- Oscilloscope display
- Start Gate Unit
- Digital display
- Mercury Suspension
- Aperture

Legend:
- Arrows indicating flow or connection
activated and the pulse is counted. The threshold level is also indicated on an oscilloscope screen by a brightening of the pulse segments above the threshold setting. The oscilloscope is only for operator convenience in adjusting the instrument and plays no part in the counting circuitry.

In the counting for this project, the aperture used was 50 microns in diameter, and the threshold setting was just below the bacterial size range. The settings were 3, 4, and 7 and were based on bacterial size distribution curves of other investigators in our laboratory.
XI. VITA

The author was born in Richmond, Virginia on November 22, 1941. In June, 1959, he graduated from Tappahannock High School in Tappahannock, Virginia. The following September he entered Virginia Military Institute in Lexington, Virginia. After completing the first year at VMI, he was married and resigned from school to work for a year. In September, 1961, the author entered Virginia Polytechnic Institute at Blacksburg, Virginia and received a B.S. degree in Civil Engineering in June, 1964.

A Public Health Service Research Fellowship was granted to the author in July, 1964, and starting in September of that year, he enrolled in the Sanitary Engineering graduate program of Virginia Polytechnic Institute.

Richard L. Blackwell, Jr.
BACTERIAL COAGULATION BY A CHLORINATED SOLVENT

by

Richard Lee Blackwell, Jr.

Abstract

When an aqueous solution containing bacteria is mixed with a chlorinated solvent, many bacteria are collected at the interface between the organic and aqueous phases. This phenomenon has been used for the removal of proteins from biological fluids (2), and also there have been attempts made to apply this principle to the removal of microorganisms and proteins from sewage and industrial waste (1).

The purpose of this investigation was to obtain data on the solvent coagulation of certain common bacteria to provide the foundations for a process for the removal of bacteria from waste effluents.

This investigation showed that removals of pure and dispersed mixed culture bacteria were roughly 90 percent when tests were conducted at room temperature with no adjustment of pH. A five percent by volume concentration of chloroform


and a coagulation time of 30 minutes were employed. As the coagulation time was extended from 30 to 90 minutes, increases in percent removal of pure culture bacteria were noted. However, adjustment of pH and temperature to values other than normal growth pH and room temperature adversely affected the percentage of removals. A cationic surface active agent produced a pronounced increase in the percent removal of a pure culture bacterium tested. Many of the bacteria in suspension after the coagulation process remained viable.

The investigation concluded that the chlorinated solvent coagulation process shows promise for commercial operations.