# Report Of Analyses

Prepared by:

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the MiL, Inc.

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## Summary Report of Analysis
**[MILB-6708]**

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**Description and Chain of Custody Record Information**:
**Friday, April 7, 2000 10:30 AM—12:30PM**: On-site experimentation was initiated and completed as described below under consultation with the client and Bruce C. Hemming, Ph.D., MiL inc. . Seven samples consisting of approximately 110ml each were obtained and returned to the laboratory for processing.

MiL, Inc. REPORT & Invoice No.: MILB-6708
Purchase Order No. -verbal
Project Name: Waterclave Test Unit Challenge

**Introduction and experimental design:**
The purpose of this investigation was to introduce experimentally a microbial challenge population of bacteria into an instrument designed for sterilization of dental unit water lines to determine the bacteriocidal capacity of the test model unit. The "waterclave" unit was installed as shown in the figures below. Parameters important in this test include temperature, pressure, and flowrate employed. The temperature of the sterilization column was observed by a manufacturer installed sensor which read 263°F [128°C and between 22.6-23 psi during the course of the experiment. Flowrate was controlled by a valve at the point of collection following a chiller unit design to reduce the temperature of the treated water. The flowrate was approximated by measurement of the volume in a catch basin over time. This ranged in the vacinity of 50 ml/min [1.0 L/20 min]. At this flowrate the water temperature exiting the chiller unit was found to be ca. 50°C. The estimated volume of the unit was cited at 4.0 L. The flowrate used represents 3 L./hr or 0.7926 gallon/hr. [.7926 gallon /hr x 128 oz / gallon = 101 oz/hr]. For dental unit water lines, this would represent between 25-26 patients/hr or 33 to 35 chairs with a retention time of 40 min as charted by the manufacturer of the unit.

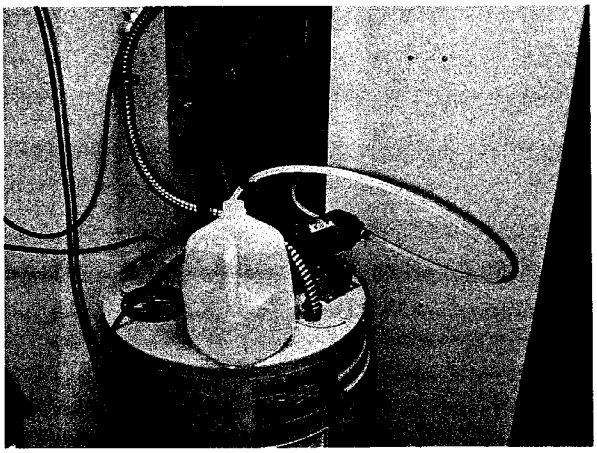

Figure 1. Inoculum introduction setup and method.

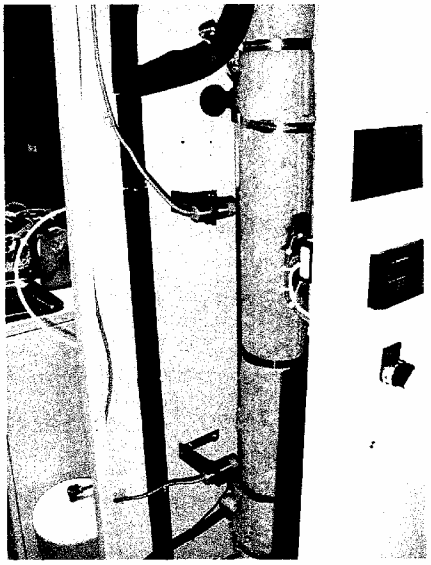

Figure 2. Waterclave unit showing sterilization column and sensor/monitor [at right].

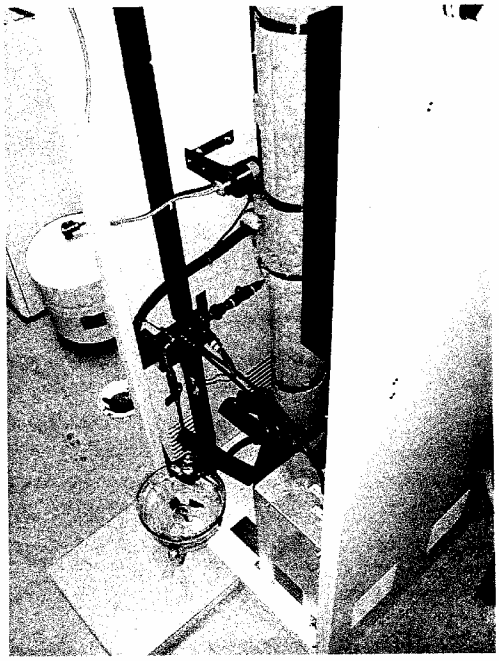

Figure 3. Glass collection basin shown at bottom of column
[chiller unit to the right of collection basin]

The bacterial isolate selected as the challenge test candidate was the recipient of the genetically engineered *lac*ZY gene marker system developed by Dr. Hemming. The strain used was identified as *Pseudomonas putida* LRO508. The engineered strain behaves identically as its wild type parent with the characteristic exceptions conferred on it by the expression of the *lac*ZY marker system. Expression of the lacZ gene provides within the cytoplasm of the cell the enzymatice protein product, beta-galactosidase, which functions to break the glycosidic bond of the disaccharide sugar, lactose, to form glucose and galactose. These simple sugars are then easily metabolized. The lacY gene product forms the loactose carrier protein or permease found as an integral membrane transport protein in the transfomred strain. These genes permit its growth on lactose as a sole carbon source and provide the ability to cleave a colorless substrate, a lactose analog termed Xgal, to produce a blue colored indolyl derivative and galactose, thereby providing a selectable chromogenic marker for this type of bacteria when introduced into the closed test system environment. This strain also produces a yellow fluorescent

pigment which services as a siderophore or iron-binding pigment for the cell. When grown on Pseudomonas F agar plates the bacterial colonies of this strain will fluoresce under 360 nm UV light. One liter of this strain was grown up in Trypticase Soybroth for the inoculum at 30°C for 24 hours using a Puritan-Bennett Oxygen Generator and selected antifoam agent as custom to Microbe Inotech Labs, Inc. to attain high density cell growth. The inoculum was diluted with commercial distilled water on-site, approximately 3.0L was introduced into this system. The approximate 700 ml remaining was transported back to the lab to determine accurately the introduced population density.

Figure 4. Example of strain streak plate for inoculum production under black light

**Processing:**

[Modified Membrane Filter Technique Bacterial Plate Count Method SOP: MIC –002-1, 9/1/97 and modified as describe below and using SOP MIC-006-1] Within 20 minutes of reception in the laboratory an aliquot from each sample was checked for volume and a 100ml aliquot was prepared for each sample. Nalgene analytical filter units [0.45$\mu$m pore size, Lot 352614, Exp. 10-2001] were used in conjunction with a vacuum manifold to process the samples. During processing the filter membrane and sides of the filter funnel were rinsed twice with sterile water to collect and redistribute the organisms. The membrane filters were removed from the united with tweezer sterilized by flame. The filters were aseptically transferred in a laminar flow biological cabinet and plated onto previously prepared and dried Pseudomonas F agar medium in Petri plates. Observations for colony forming units (CFU) are made after 24 and 48 hours of incubation at 28°C and specific observations were made for the marked fluorescent pseudomonad used as the inoculum.

**Results:**

**Total Heterotrophic Plate Count Results:**

| DATA: Direct Count: Colony Forming Units (CFU / membrane/ 100ml ) on Pseudomonas F agar medium | | | |
|---|---|---|---|
| **Sample** | **Description** | **24 Hours** | **48 Hours** |
| 1 | Inoculum As Introduced | $1.60 \times 10^{10}$ | $5.38 \times 10^{10}$ |
| 2 | T= 0 Sample taken prior to introduction of inoculum | Below detection <1/100ml | Below detection <1/100ml |
| 3 | T = 2.0 L elution volume after sample introduction | Below detection <1/100ml | Below detection <1/100ml |
| 4 | T = 2.5 L elution volume | Below detection <1/100ml | Below detection <1/100ml |
| 5 | T = 3.0 L elution volume | Below detection <1/100ml | Below detection <1/100ml |
| 6 | T = 4.0 L elution volume | Below detection <1/100ml | Below detection <1/100ml |
| 7 | T = 4.5 L elution volume | Below detection <1/100ml | Below detection <1/100ml |

**Observations, Comments and Conclusions:**

As demonstrated in the data above the unit is very effective at sterilization of the bacterial inoculum introduced under the stated conditions of temperature, pressure and flowrate. The species employed is typical of water-borne strains in municipal drinking waters, etc. Inorganic salts and other bacterial medium components from the inoculum were observed to make the water in the catch basin cloudy. This was first noticed in sample no. 4.

Sample 5 was taken after the time of switch over to introduction of distilled water rather than inoculum. The effluent water was clear in the 500ml before taking sample 7. The mixing in the serilization column shortens the observed elution time over the total void volume of the system. The unit is very effective at sterilization of the water for this organism and very probably all bacterial water flora. With the clarity of the water, the installment of a UV bactericidal light [ex. AquaFine Corporation] would be a secondary killing mechanism which would also be a very effective backup system; but, which is in fact rendered unnecessary by the waterclave unit's efficacy.

Thank you from the Staff on this Project:

Kirk M. Hartwein — Lab Manager

Bruce C. Hemming, Ph.D. — Pres./CEO