AMRL-TR-68-173

BACTERIA SENSOR FOR REPROCESSED WATER—MICROBIOLOGICAL RESEARCH, DESIGN, AND FABRICATION

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FOREWORD

The research and development program described in this report was conducted at the Life Support Engineering Laboratories, Bioastronautics Section of General Electric's Space Systems Organization, Valley Forge Space Center, King of Prussia, Pennsylvania, for the Aerospace Medical Research Laboratory at Wright-Patterson Air Force Base, Ohio, under Contract F33615-67-C-1564 and in support of Project No. 6373, "Equipment for Life Support in Aerospace," and Task No. 637304, "Waste Recovery and Utilization." The technical monitor was Mr. A. B. Hearld, Biotechnology Branch, Life Support Division, Biomedical Laboratory.* This report covers work performed during the period 1 May 1967 through 31 August 1968.

The authors wish to acknowledge the valuable contribution of Mr. Joseph W. Boyd, who served as program manager through the initial design phase of the program, and to Messrs. F. W. Thomae and M. H. Bengson, who provided technical assistance throughout the program. The authors also wish to express their appreciation for the assistance provided by Mr. A. B. Hearld.

This technical report has been reviewed and is approved.

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*The Biotechnology Branch, Life Support Division, and Biomedical Laboratory were abolished during a reorganization in December 1968.

SUMMARY

The results of the developmental research leading to the design and fabrication of a short-time, electronic sensor to monitor the bacteriological quality of reprocessed water aboard spacecraft are reported. In the General Electric concept, the basic sensing capability is furnished by a Coulter Counter that selectively detects and counts particles of bacterial size. Detection is accomplished by comparing the number of bacteria-size particles in the reprocessed water sample at some point in time (t_0) with a particle count at some future point in time (t_f), i.e., following the establishment of conditions necessary to allow growth and multiplication of bacteria. A significant difference between the two counts strongly implies bacterial replication, and therefore the presence of viable organisms in the raw reprocessed water.

The microbiological research phase was geared to evaluating the relationships between a number of biological variables and their effect on sensor performance. Therefore, design requirements for the instrument were defined through the experimental manipulation of media, incubation time, and temperature on five selected strains of bacteria (probable contaminants or test organisms) of varying concentrations. Final criteria for the Bacteria Sensor incorporated a minimum of 4 hours incubation at 32 C. The medium developed employed dilute Nutrient Broth plus micromolar amounts of calcium, manganese and ferrous salts as necessary growth factors for the growth and multiplication of certain organisms from low levels of inocula. Sodium chloride and polysorbate 80 are also included to enhance the electrolytic quality of the fluid and to inhibit bacterial clumping. Medium supply for the instrument originates as a two-part concentrate: Nutrient Broth in one part and the remaining ingredients, described above, in the other. Contaminating particles of bacterial size in the medium or within the instrument are reduced by multiple filtration through a 0.22 μ pore size screen filters or treatment with particulate-free water.

Instrument operability tests demonstrated the capability of the sensor to carry out the basic design goals, i.e., to provide the conditions necessary for growth and multiplication of low level inocula of bacteria under short-time conditions, and to detect this increase in the particulate count by means of the electronics supplied. Experimental laboratory results with the test organisms indicate the theoretical sensitivity of the instrument to be in the neighborhood of one viable organism per milliliter of reprocessed water. However, qualification and standardization testing remains to be done to firmly establish the lowest concentration detectable for each possible contaminating organism.

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INTRODUCTION

BACKGROUND

Maintenance of human life requires water, oxygen, and food. Of these, water represents the greatest proportion, by weight, of the total of water, oxygen, and food needed per day. Accordingly, this need will place a premium on recovery and reuse of water consumed by space crews during extended flights. Reprocessed water from each of the experimental units currently undergoing testing must be judged on its purity and palatability. A complete potability analysis, both chemical and microbiological, is of prime importance. Indicators of chemical purity (e.g., pH and specific conductivity) are straightforward measurements and can provide a good indication of chemical quality. Microbiological acceptability, however, is more difficult to determine. The standard laboratory procedure involves adding appropriate dilutions of the water to a nutrient agar, incubating 24 to 48 hours, and then examining the culture visually for the presence or absence of bacterial growth. The long incubation time is required for the growth and multiplication of any viable bacteria present to macroscopic proportions.

Adaptation of these methods to space flight needs is obviously impractical. For useful operation aboard a spacecraft, a bacteria sensor should operate on a short-time basis (i.e., approximately 4 hours) with an automatic "Yes-No" output signal. This means that detection and enumeration must take place after a minimum incubation time on essentially single-cell bacterial units. Three possible techniques for accomplishing this are available: electronic, photo, or sonic. Of the three, the electronic technique displays sufficient sensitivity to both numbers and size of particles for useful application to this problem.

Counting Concept

A commercially available particle counter,[1] whose operation is dependent upon the nonconductive characteristic of living cell membranes, was considered adequate to provide the

1. Coulter Counter, Model B.Product of Coulter Electronics, Inc. General Office: 590 West 20th Street, Hialeah, Florida.

sensing, counting and display functions within the bacteria sensor system. By supplying a solution with good electrical conductivity (e.g., <200,000 ohms), an individual organism would appear as a small, nonconducting particle suspended in a conducting medium. The basis of this detection system for these particles is displayed schematically in figure 1 and described briefly in the following paragraph.

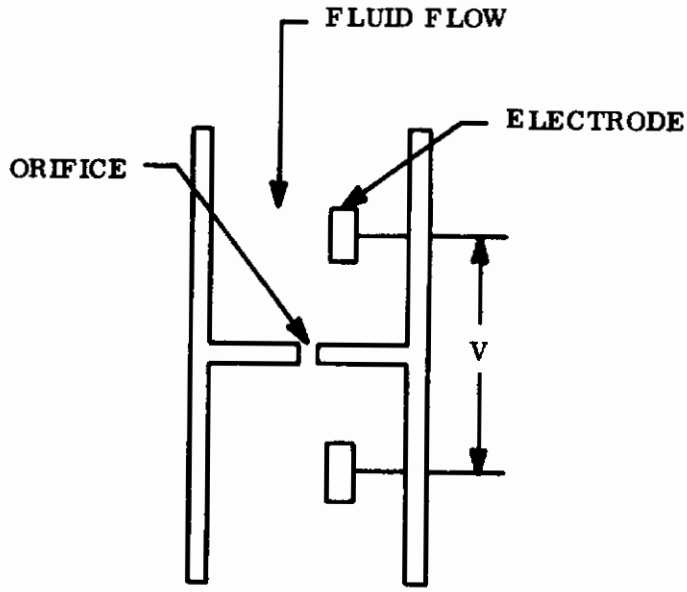

FIGURE 1. TYPICAL DETECTOR ORIFICE AND ELECTRODES

The sample liquid is forced to flow through an orifice made of nonconducting material, which has a diameter of between 2 and 20 times the diameter of the microorganisms being counted. A direct current also flows through the orifice between two electrodes. The lines of the current-flow crowd together at the orifice, resulting in a critical volume of high current flow, and any nonconductor contained in this volume will crowd the lines still further. This impediment to current flow (i.e., resistance change) is measured as a voltage drop across the electrodes. Because of the rapid passage of the microorganisms through the orifice, this voltage drop appears as a pulse, each pulse sensed by a digital counter which totals the number of microorganisms (or nonbacterial particles) that pass through the orifice. The magnitude of this pulse is proportional to the volume of the particle. By careful calibration, controls on the Coulter Counter can be preset to register only pulses that correspond to particles of bacterial size. An upper limit to the particle size can also be set to permit only a narrow size range of particles to be counted. This enables the system to count similar sized nonconducting particles and ignore all others.

Biological Basis

The use of this electronic particle-counting technique to monitor the microbiological quality of drinking water implies the capacity to discriminate between viable and non-viable particulates. The method proposed to provide this capability involves determining the number of particulates of bacterial size in the water to establish a baseline, followed by flow through an environment suitable for bacterial growth and multiplication, i.e., adequate nutrition and appropriate incubation time and temperature. Comparison of the initial count with the particle count on the water sample following this incubation period should indicate the presence or absence of viable organisms. A significant differential between particulate counts strongly implies bacterial multiplication, which in turn implies the presence of bacterial contaminants in the original water supply. The ultimate sensitivity of the total instrument then is determined by its ability to provide significant differences between the particulates present in the raw reprocessed water and the incubated reprocessed water.

Growth and multiplication of these bacteria is dependent upon a number of variables which include:

- Average physiological age of the microorganism
- Genetic character of the microorganism
- Nutritional constitution of the medium
- Time and temperature of incubation
- Number of microorganisms present
- Presence or absence of other competing elements such as phage, colchicine, or other inhibitory or biocidal factors.

The microbiological research phase of this program was geared to evaluating the relationships between these factors and their effect on sensor performance. Typical of the considerations is the fact that random bacterial contaminants in the water must be regarded as physiologically and genetically mixed populations. Since these factors, along with inoculum

size, cannot be predicted, the instrument sensitivity will be mediated by the interaction of all those variables listed above. Therefore, the design requirements for the instrument were defined through the experimental manipulation of media, incubation time, and temperature on selected strains of bacteria (probable contaminants) of varying concentrations.

SYSTEM CONCEPT

The major functional characteristics of the system are shown schematically in figure 2. Typically, a sample is drawn continuously from a holding tank or process water line. A small quantity of concentrated electrolyte solution is added to the sample to increase solution conductivity, and the sample stream is then split into two volumetrically equal streams. One half of the original sample is immediately filtered to remove large particulates and is then passed through a 25-micron orifice in the detector cell where the particle count is accomplished. This particle count represents the baseline or normal particulate background.

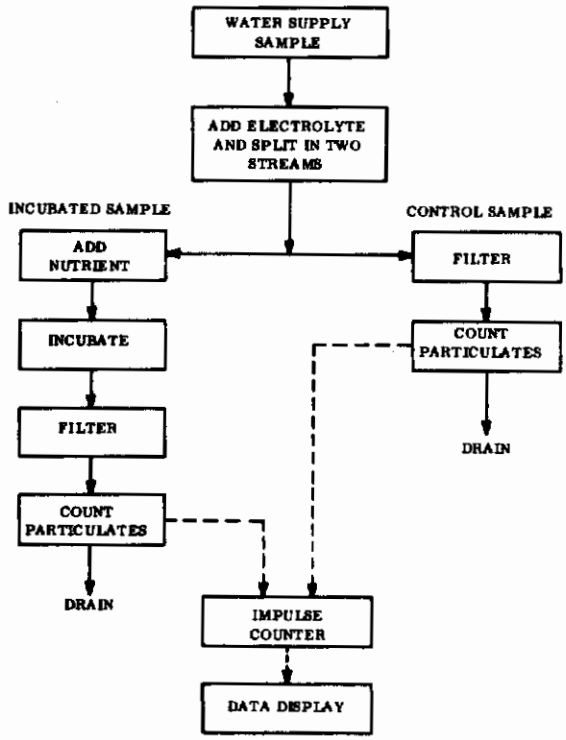

FIGURE 2. FUNCTIONAL BLOCK DIAGRAM OF BACTERIA SENSOR

The remaining half of the constantly flowing original sample has a liquid nutrient medium metered into it. The water-electrolyte-nutrient mixture then passes through an incubator coil which is maintained at 32 C. The small diameter tubing used in the coil preserves the "plug flow" characteristics of the sample. Therefore, any given bacteria in the sample are incubated for a specific period of time (4 hours in this unit) by virtue of the fact that little or no mixing occurs within the coil. Incubator effluent is filtered to remove large particulates (i.e., greater than bacteria size) and the incubated sample then passes through the orifice in the second detector cell. Comparison of the equivalent volume-counts of the incubated and nonincubated streams indicates either equal particle concentrations (hence no viable bacteria), or a significant difference signifying multiplication of viable bacteria in the reprocessed water sample.

The arrangement of these detectors in parallel was selected over a series arrangement in order to prevent reduction of bacterial viability from exposure to the strong electrical field in the orifice, and from any possible oxidants generated at the detector anode. The two detectors are monitored alternately by manually switching each sensor into the counter circuitry.

Of critical importance to the satisfactory operation of the instrument are two mutually dependent factors:

- The number of viable organisms per unit volume present in the initial sample which can grow and multiply to sufficient numbers and thus provide a significant differential (sensitivity), and
- The number of nonviable and/or nonorganic particles of bacteria size present (background).

In practical terms, the electronic sensor provides the ability to count individual bacterial cells immediately after minimum incubation. Conventional culture technqiues require a plate count, which includes a 24- to 48-hour incubation period in order to allow each viable cell to produce colonies of macroscopic proportions for visual counting. In other words, the Bacteria Sensor is designed to detect and count bacteria suspensions at a significantly earlier time than is possible in normal practice (by a factor of 6 to 12 times faster).

SECTION II

MATERIALS AND METHODS

MARKER ORGANISMS

Strains Employed

A list of those organisms[2] considered representative of bacterial contaminants likely to be found in reservoirs or other potable water sources is presented in table I.

TABLE I

LIST OF BACTERIA USED AS MARKER ORGANISMS IN DEVELOPMENT OF THE BACTERIA SENSOR

Organism	Gram Stain Reaction* and Typical Cell Size
1. Escherichia coli ATCC 10536	Gram-negative, rods 0.5 x 1-3 microns
2. Pseudomonas aeruginosa ATCC 17423	Gram-negative, rods, 0.5 x 1.5 microns
3. Xanthomonas translucens ATCC 10771	Gram-negative, rods 0.5=0.8 x 1-2.5 microns
4. Mima polymorpha ATCC 14291	Gram-variable, rods or cocci (depending upon nutrient medium) 0.5 x 8-16 microns
5. Bacillus subtilis ATCC 6633	Gram-positive, rods, 0.6-1 x 1.3-6 microns

* As per Hucker modification (New York Agricultural Experiment Station Technical Bulletin, 1927, 128).

2. Recommended for use as test cultures in contract requirements: Aerospace Medical Research Laboratories, Wright-Patterson Air Forse Base, Dayton, Ohio.

These bacteria were designated as markers and used as prototype contaminants or test organisms in the developmental biological investigations. Pure cultures of each of these organisms were obtained from the American Type Culture Collection (ATCC) in Rockville, Maryland, and maintained as pure cultures for the duration of this study.

Production of Experimental Lots of Test Organisms

All marker organisms were routinely cultured in Nutrient Broth[3], prepared as directed by the manufacturer. Experimental tests employed fresh, 16-hour (overnight) static growth cultures incubated at 32 C in 500 ml flasks containing 125 ml of sterile culture medium. Inocula for these fresh cultures were obtained from previous 16-hour cultures kept at refrigerator temperatures (4 C) for up to 6 weeks. The number of viable cells per ml was established by accumulated experience with each of these organisms under the conditions described. A guide to approximate inoculum size for the desired level of each test organism is presented in table II.

TABLE II

AVERAGE TITERS AND SUGGESTED INOCULA VOLUMES FOR "MARKER" ORGANSIMS

Organism	Suggested Inoculum* Volume To Be Added To 125 ml	Average 16-Hour Titer** Following Suggested Inoculum
E. coli	0.2 ml	6.5×10^8
P. aeruginosa	0.2 ml	1.5×10^9
X. translucens	1.0 ml	1.1×10^8
M. polymorpha	0.5 ml	8.6×10^7
B. subtilis	1.0 ml	1.1×10^7

* Obtained from previous 16-hour cultures held at 4 C.

** Based on a minimum of 11 separate determinations per organism.

3. Obtained from Bioquest (formerly BBL), Division of Becton, Dickinson and Company, Baltimore, Maryland.

Maintenance

All stock marker cultures were maintained on Nutrient Agar (BBL) slants after incubation for 24 hours at 32 C. The cultures were then stored at refrigerator temperatures (4 C) for up to 6 months before reculture.

ASSAY TECHNIQUES

Bacteriological determinations were performed in 20 by 150 mm screw-cap test tubes containing 25 ml of the test medium under study. The air-tight tube, which displayed little air/liquid interface, was used to emulate the growth environment roughly similar to that expected in the closed, small-bore tubing of the actual instrument.

Bacteria Enumeration - Determination of the growth and replication of the test organisms under experimental conditions was done in replicate Petri plates using the pour plate technique with Nutrient Agar.[4] Incubation of all plates was carried out at 32 C for 48 hours before enumeration of the colony-forming units was made with the aid of a New Brunswick colony counter.

MEDIUM PREPARATION

Experimental Growth Medium

Composition of the final growth medium, derived from experimental testing, is shown below (chemicals are reagent grade):

Ingredient	Amount Per Liter
Nutrient Broth (BBL)	2 g
Polysorbate (Tween) 80[5]	0.05% W/W
NaCl	4.38 g (0.075 M)
$Ca(NO_3)_2 \cdot 4H_2O$	288 mg (1.22 μ M)
$MnSO_4 \cdot H_2O$	11 mg (0.066 μ M)

4. American Public Health Association, 1955. Standard Methods for the Examination of Water, Sewage, and Industrial Wastes. 10th Ed. Am. Pub. Health Assoc., Inc., New York, N.Y. pp. 373-4

5. Product of Atlas Chemical Company, Wilmington, Delaware

*$FeSO_4 . H_2O$	10 mg (0.066 μ M)
*$NaC_6H_5O_7 . 2H_2O$	155.3 mg (0.53 μ M)
Distilled H_2O	To Volume

(*Ferrous sulfate and sodium citrate are mixed separately in distilled H_2O and added to remainder of medium.)

Concentrated Two-Part Medium

Precipitation of some components of the growth medium at high concentrations precluded the use of a single concentrated medium supply for the Sensor. Separation of the medium reservoir into two parts was found to be the most practical solution. Detailed instructions for the preparation of this medium are presented in Appendix I.

PREPARATION OF PARTICULATE-FREE WATER

Instrument sensitivity is directly affected by the amount of background particulate contamination. It is necessary to reduce these particulate contributions from containers, medium, tubing, etc. during operation of the Sensor. Therefore, rinse water must be prepared which contains particles less than the size of the expected bacterial contaminants ($\sim 0.5 \mu$). Specific details for the production of this high quality water are given in Appendix II.

PREPARATION OF GLASSWARE

Instructions for the preparation of glassware associated with the operation of the Sensor are shown in Appendix III.

SECTION III

MICROBIOLOGICAL INVESTIGATIONS - DEFINITION OF THE SENSOR BIOLOGICAL DESIGN REQUIREMENTS

GENERAL

Design and fabrication of the Bacteria Sensor required definition of some microbiological limits and restrictions of the test organisms under study: E. coli, P. aeruginosa, M. polymorpha, X. translucens and B. subtilis. Some factors which were considered critical in light of the design concepts described in Section I are listed below:

- Minimum incubation time - A practical design goal of approximately 4 hours was considered desirable.
- Optimum incubation temperature - The temperature employed will be dependent upon the incubation time. It should be noted that at the start of the investigation, 32 C was chosen as a compromise between optimum incubation temperatures of probable bacterial contaminants (Marker Organisms) and the need for minimum electrical power requirements.
- Nutritional requirements - The medium must be capable of supporting the rapid growth of all test organisms as well as being storable in concentrated form in order to meet minimum space and weight requirements aboard a spacecraft.
- Sensitivity - Minimum number of organisms required in order to produce detectable numbers of each test organism within the minimum incubation time should be on the order of 1 organism per milliliter.

The interdependency of these requirements necessitated careful structuring of the laboratory investigations within the scope of this program to provide an optimum permutation of these factors. The experimental phase of the program was therefore resolved into a study of generation time, effects of low inocula, and the development of a single sensor medium.

GENERATION-TIME STUDIES

Early experimental efforts were directed toward determination of growth curves for each test organism in an attempt to estimate the minimum incubation time. If the generation time[6] for each organism could be established, the minimum incubation period required to obtain a significant viable count might be derived. Consequently, the results of plate counts at hourly intervals during the exponential growth period were plotted on semi-logarithmic paper, i.e., bacterial count on a geometric scale (ordinate) versus incubation time on an arithmetic scale (abscissa). The time interval required for one doubling of the test population was calculated directly from the straight-line portion of the curve, i.e., the time required for one bacterium to divide during optimum growth (see data in Appendix IV).

The inoculum size obviously affected not only the slope of the log-growth curve, and therefore the generation time, but also the length of the lag phase, or the time from inoculation to start of geometric multiplication. Since short-time incubation was a desired objective for the most practical application of the Sensor, the lag phase would be the more critical element involved, and not the generation time. Since, during normal sensor operation, the inoculum size is an uncontrollable variable, it was decided to discontinue growth curve studies in favor of low-inocula experiments.

Specific details and data on the generation-time studies are given in Appendix IV; a more detailed discussion on the factors involved in bacterial lag-phase is given in Appendix V.

LOW INOCULA STUDIES

As previously indicated, a critical factor in demonstrating the ability of the Sensor to provide significant bacterial growth following a minimum inoculum was the lag phase or the time required for the cellular machinery to initiate geometric multiplication. Experiments

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6. As used in this discussion, generation time is defined as the time required for one organism to divide into two daughter cells during the exponential or log growth period. (See: Davis, D. B., Dulbecco, R., et al., Microbiology, pp. 142-6, Harper and Row, Inc., N.Y., 1967.)

were designed to employ a growth environment similar to that expected during Sensor operation. The major characteristics and/or restrictions of these experiments included the following:

- Experimental growth tubes - 20 by 150 mm screw-cap test tubes
- Medium - 25 ml Nutrient Broth (NB) per test tube. This volume provided a minimum of air/liquid interface.
- No aeration (agitation)
- Maximum 4 hours incubation at 32 C
- Inoculum - Derived from 16-hour cultures of each test organism grown at 32 C in NB. Appropriate dilutions were made in 9 ml NB blanks to provide a final concentration of approximately one organism per ml in duplicate test tubes.
- Assay - Bacterial growth in each tube was assayed immediately after incubation using Nutrient Agar and the pour-plate technique. Since relatively low numbers of bacteria were expected, triplicate plates of duplicate samples of 5 ml, 1 ml, and 0.1 ml per tube were assayed to insure reliable counts.

The results of a typical experiment are summarized in table III. The fold increase, or the arithmetic rate of inoculum multiplication within the 4-hour incubation period, was calculated to provide a common basis of comparison from culture to culture. As shown, all organisms exhibited many fold-increases from inocula of theoretically less than one bacterium per ml. Except for M. polymorpha, the fold increases were relatively constant regardless of inoculum size. With the tests performed using X. translucens, we were unable to detect any increase with the lowest inoculum employed. It is not known why M. polymorpha exhibited an increasing multiplication rate with each decreasing ten-fold inocula. The meaning of this apparent phenomenon could provide an interesting investigation within the field of bacterial physiology/nutrition.

TABLE III. THE INCREASE IN CONCENTRATION OF TEST ORGANISMS FOLLOWING LOW INOCULA AND 4-HOUR INCUBATION AT 32 C IN NUTRIENT BROTH

Organism	Inoculum Concentration (Cells/ml)*	Concentration After 4 Hours Incubation (Colonies/ml)*	Fold Increase**
B. subtilis	8.0	270	34
	0.8	15	20
	0.08	2	25
X. translucens	5.6	57	10
	0.56	5	9
	0.056	0	< 1
P. aeruginosa	5.0	240	48
	0.5	24	48
	0.05	1.5	30
E. coli	21.0	250	25
	2.1	40	20
	0.21	2	10
M. polymorpha	8.0	160	20
	0.8	58	72
	0.08	37	437

*Method of Assay - Conventional Plate Count Procedure; average of duplicate samples.

**Fold Increase is determined by dividing the 4-hour count by the initial count.

DEVELOPMENT OF SENSOR MEDIUM SUPPLY

Medium Preference Studies

The previous experiments demonstrated the theoretical possibility of detecting bacterial growth (multiplication) after 4 hours incubation at 32 C from inocula of approximately one organism per ml. This fulfilled the short-time incubation requirement of the Sensor. However, this apparent success immediately raises two questions: (1) can another medium provide higher fold-increases; and (2) can a shorter incubation time be substituted?

The first question, "Can a more satisfactory medium be found"? was tentatively answered by the results of the following experiment. Four additional commercially available synthetic media, known to support the growth of a wide variety of bacteria, were tested with the same low level inocula. Use of chemically defined media was rejected because the developmental time required to obtain the needed nutritional versatility was considered outside the practical scope of this investigation. All media were prepared, sterilized, and dispensed in 25 ml amounts into 20 by 150 mm growth tubes. Appropriate dilutions of each 16-hour culture were used to inoculate duplicate tubes with approximately one organism per ml, followed immediately by incubation at 32 C for 4 hours. Results obtained from this experiment are presented in table IV.

As shown, Brain Heart Infusion Broth (BHI) at normal concentration appeared to produce fold-increases as high as or higher than the other four media tested, especially with the E. coli culture. Since E. coli is considered indicative of fecal contamination and therefore epidemiologically important, this preferential quality warranted further study of BHI as a singular medium of choice for use in the Bacteria Sensor. (Subsequent data overruled this possibility.)

TABLE IV. COMPARISON OF GROWTH RATES (FOLD INCREASE)* FROM A LOW LEVEL INOCULUM OF EACH MARKER ORGANISM INTO 5 SELECTED SYNTHETIC MEDIA AFTER 4 HOURS INCUBATION AT 32 C**

Organism	Initial Inoculum Cells/ml	Fold Increase After 4 Hours Incubation at 32 C In:				
		Nutrient Broth	Nutrient Broth +5% Yeast Extract	Trypticase Soy Broth	Thio-glycolate Broth	Brain-Heart Infusion Broth
E. coli	1.3	8	8	17	22	108
P. aeruginosa	1.2	17	30	23	18	38
M. polymorpha	1.5	19	55	61	29	75
B. subtilis	3.4	15	26	21	5	26
X. translucens	1.1	10	16	18	9	14

*Fold increase was determined by dividing the final 4-hour count by the initial count.

**Method of assay - standard plate count - average of duplicate samples.

The second question, "Can a shorter incubation time be substituted?" was investigated in the next experiment. BHI and NB were compared as nutrient sources using minimal inocula for each organism as well as 2- and 4-hour incubation at 32 C in duplicate test tubes. The results, shown in table V, indicate that, contrary to the previous experiment, the fold increases at 4 hours with either BHI or NB were equivalent, except for E. coli where BHI appears to be superior. The bacterial counts at 2 hours were too low to be considered significant with either medium. From the results of these two experiments, it was provisionally concluded that:

- BHI medium appears to be as good a nutrient source as NB with low inocula of P. aeruginosa, X. translucens, M. polymorpha and B. subtilis cultures.
- With E. coli, BHI displayed higher fold increases than NB after 4 hours incubation at 32 C.
- Comparing the total counts per ml produced with low-level inocula after 4 hours incubation, and the minimum counts at 2 hours, it was felt that no real advantage would be obtained in terms of significant increase in counts per ml with an incubation time of less than 4 hours.

Electronic (Instrument) and Physical Requirements

Background. An essential factor for the successful application of the Bacteria Sensor will be near-optimum operation of the Coulter Counter electronics. Since the counting pulses are generated by proportional constriction of the electrical lines of flow through the aperture, it follows that the medium containing these particles must possess electrolytic properties of sufficient value to conduct an adequate current flow ($< 200,000$ ohms). (NB and BHI, at normal concentration, show about the same resistance as water.) In addition, nonbacterial particulates must be reduced to an absolute minimum if the instrument is to have sufficient sensitivity to detect low numbers of bacterial contaminants. The experimental investigations of these requirements are described in the following two sections.

TABLE V. SUMMARY OF INCREASES* OF LOW LEVEL INOCULA OF TEST-ORGANISMS WHEN INCUBATED FOR 2- AND 4-HOURS AT 32 C** IN BRAIN HEART INFUSION BROTH AND NUTRIENT BROTH

Organism	Initial Inoculum Cells/ml	Average Fold Increase/ml Culture			
		2-Hour Results		4-Hour Results	
		BHI Broth	Nutrient Broth	BHI Broth	Nutrient Broth
E. coli	1.4	~1	~1	25	9
	0.14	<1	<1	30	7
M. polymorpha	1.6	7	5	272	240
	0.16	4	6	120	360
X. translucens	2.0	2	2	12	12
	0.20	3	1	6	15
P. aeruginosa	1.8	2	3	37	37
	0.18	3	5	17	61
B. subtilis	4.8	5	5	57	48
	0.48	6	2	27	44

*Fold increase was determined by diving the 2- or 4-hour counts by the initial count.

**Method of assay - standard plate count - average of duplicate samples.

Electrolyte - Polysorbate (Tween)[7] 80 Studies. Sodium chloride at 0.85% (saline) provides excellent electrolytic properties, and is known to be physiologically compatible with the growth of bacteria. Duplicate flasks of NB and BHI medium, prepared with and without 0.85% NaCl, were inoculated with each of the test organisms and allowed to incubate 24 hours at 32 C. Twenty-four hours incubation was chosen to provide a more objective evaluation of the gross appearances by macroscopic examination as well as by viable counts. Results showed that both E. coli and M. polymorpha produced uniform, turbid cultures and essentially identical titers following the incubation period. With B. subtilis, X. translucens and P. aeruginosa, the 24-hour cultures exhibited heavy clumping and formation of long stringy mats, especially with the BHI medium containing the NaCl. The

7. Trade name for polysorbate 80, manufactured by Atlas Chemical Co., Wilmington, Delaware.

plate counts from each of these three cultures showed higher counts without NaCl; however, the NB flasks of B. subtilis, with or without NaCl, showed counts at least 1 log higher than the corresponding BHI flasks of B. subtilis, indicating possible preference for the NB medium.

In an attempt to resolve the need for adequate electrolyte properties without concomitant clumping following bacterial growth, other electrolytes were tested. Ammonium phosphate ($NH_2H_2PO_4$) and potassium phosphate (KH_2PO_4) at concentrations between 0.5 M and 0.0001 M, and sodium chloride (NaCl) at concentrations lower than 0.85% (i.e. < 0.15 M) were added to the BHI medium. The pH of each salt/medium concentration was measured, and the corresponding electrolytic properties were determined in a Standard Coulter Counter apparatus by measuring the resistance across the electrodes.

Results indicated that concentrations of $NH_2H_2PO_4$ and KH_2PO_4 above 0.001 M caused unacceptable pH changes in the medium (normal = 7.2 ± 0.1), but concentrations of 0.001 and 0.0001 M provided excessive electrical resistance properties ($\sim$240,000 ohms). Sodium chloride at 0.15 M and lower produced no detectable effects on the pH and demonstrated acceptable resistance readings. Twenty-four hour incubation of B. subtilis, X. translucens, and P. aeruginosa in duplicate flasks of BHI medium containing 0.001 or 0.0001 M amounts of each of the three salts was carried out at 32 C as described previously. Following the incubation period, visible strands and clumps of bacterial growth were evidenced in all flasks. Further experiments with NaCl and other salts were suspended.

As an alternate approach, the introduction of a surface-active agent as reported by Bernheim (1962a) and Brown (1964) was investigated. Polysorbate 80, a non-ionic complex mixture of polyoxyethylene ethers of mixed partial oleic esters of sorbital anhydrides, was added to the BHI medium in an effort to reduce or inhibit clumping during the growth period. The following experimental media were prepared:

- BHI medium - normal concentration (control)
- BHI medium + polysorbate 80 at 0.1 and 0.05% (polysorbate control)
- BHI medium + polysorbate 80 at 0.1 and 0.05% + NaCl at 0.01 M

Duplicate 20 by 150 mm growth tubes of each of the above medium were inoculated with B. subtilis and incubated at 32 C. After 24 hours, a plate count assay using Nutrient Agar was performed. Results indicate that the viable counts averaged better than a half-log higher in the test tubes containing either concentration of polysorbate 80 plus 0.01 M NaCl. Another experiment was conducted with the same media, but with lower levels of inocula of each of the test organisms: B. subtilis, P. aeruginosa, and X. translucens. Incubation was at 32 C for 4 hours.

As seen from the results summarized in table VI, polysorbate 80 at either concentration appeared to effect a definite increase in the viable count of P. aeruginosa. This was more apparent in the experimental tubes receiving the lower inoculum. Polysorbate 80 demonstrated no discernible adverse effect on B. subtilis or X. translucens. No clumping was visually observed following the 4-hour incubation period.

Particulate Contamination Control. Accumulated experience with the standard Coulter instrument fitted with a 30-micron diameter aperture strongly indicated the need to eliminate nonbacterial particulate matter from the suspending medium. Size-distribution plots using sterile saline (0.85% NaCl in distilled water) alone indicated high background counts which overlapped the anticipated size ranges of the bacteria suspensions. Finally, standard calibration techniques with latex spheres of known diameter established the fact that nonbacterial-sized particulates were indeed present in the sterile growth medium.

Experimentation showed that mulitple filtration of fluids through Millipore filters of 0.22 μ pore diameters provided water with an acceptable level of interfering particulates. A detailed description of the process ultimately adopted is presented in Appendix II of this report.

TABLE VI. EFFECT OF POLYSORBATE 80 ON BHI MEDIUM WITH 0.01M NaCl ON GROWTH OF X. TRANSLUCENS, B. SUBTILIS AND P. AERUGINOSA FOLLOWING MINIMAL INOCULA AND 4 HOURS INCUBATION AT 32 C

Organism	Inoculum Cells/ml In Growth Tube	Additions to BHI Medium*- Results of 4-Hour Plate Count							
		0.01% Polysorbate 0.01M Saline		0.05% Polysorbate 0.01M Saline		No Polysorbate 0.01M Saline		No Polysorbate 0.01M Saline	
		Average Cells/ml	Fold** Increase	Average Cells/ml	Fold Increase	Average Cells/ml	Fold Increase	Average Cells/ml	Fold Increase
X. translucens	1.3	66	51	63	48	73	56	67	51
	0.13	7	54	7	54	9	64	7	54
B. subtilis	80.0	TNTC***		TNTC		TNTC		TNTC	
	8.0	81	10	99	12	90	11	74	9
P. aeruginosa	0.8	81	42	33	41	19	24	20	24
	0.08	5	62	2	25	1	1	1	1

*Base medium = Brain Heart Infusion Broth at standard concentration.

**Fold increase is determined by dividing the 4-hour count/ml by the initial count/ml.

***TNTC = Too numerous to count; inoculum higher than anticipated.

Medium Concentration. Space flight hardware design must conform to limitations imposed by space and weight considerations. An obvious component of the Bacteria Sensor is the medium reservoir which must be large enough to supply flight demands of from 6 to 12 months duration. This means that medium ingredients should be stored in as concentrated form as possible. In order to determine the highest practical medium concentration, 100 ml quantities of BHI and NB were prepared at 10X, 25X, 50X and 100X concentrations, plus the proportional amounts of NaCl and polysorbate 80. After autoclaving, the gross appearance of each preparation was judged as to appearance and possible usefulness. Results are summarized in table VII.

From these data, it was concluded that:

- Suspensions of BHI concentrated greater than 10X are probably not practical although 15X concentrations might be possible with careful preparation.
- Concentrations of NB up to 50X and perhaps slightly beyond are practical for use in the medium reservoir.

TABLE VII. SOLUBILITY OF VARIOUS CONCENTRATION OF BHI AND NB MEDIA WITH CORRESPONDING LEVELS OF NaCl AND POLYSORBATE 80

Concentration Level	Gross Appearance Following Autoclaving	
	BHI Medium	NM Medium
10X*	Completely suspended - slightly viscous - usable	Clear, completely suspended, usable
25X	Heavy, viscous sludge - residue on rim - unusable	Clear, completely suspended, usable
50X	Heavy almost solid sludge - quite unusable	Increased viscosity, some evidence of sludge - usable
100X	Insoluble	Heavy and viscous, sludge, residue - not usable
*Corresponding concentrations of NaCl and polysorbate 80 were calculated from 0.01 M and 0.05% respectively.		

Dilute Medium Studies

Additional medium storage capacity without concomitant increase in volume requirements could be achieved by employing more dilute growth medium than that used in previous experiments. Since a Sensor design objective was the detection of the lowest possible numbers of viable bacteria with a 4-hour incubation period, nutritional demands should be significantly less than a system with large numbers of bacteria and longer incubation periods. Therefore, medium levels of 0.5X or even 0.1X that normally employed could provide an additional 2 to 10-fold concentration factor.

Duplicate 20 by 150 mm test tubes containing 1X, 0.75X, 0.5X, 0.25X, or 0.1X the standard concentration of NB and BHI medium were prepared. All tubes contained the same amount of NaCl (0.01M) and polysorbate 80 (0.05%). Two low levels of inocula were prepared from 16-hour cultures of E. coli grown in either BHI or NB medium. Separate inocula for each medium were prepared in order to preclude the possibility of physiological shock when the bacteria were transferred from one medium to the other. E. coli, which had previously displayed the most obvious differences in growth rate between the BHI and NB media, was chosen as the initial test organism.

Results displayed in table VIII show that although the inocula for the NB tubes were lower than anticipated, it is apparent that the NB medium displayed a more consistent effect on the growth rate of E. coli, even in concentrations as low as 0.1X. Increases of E. coli cells were higher in the more concentrated solutions of BHI, but dropped off abruptly when the concentration was below 0.5X. Consideration of the 5-fold difference in the inoculum size between the two media plus the consistent effect on growth rate prompted the decision to investigate the response of all five test organisms in NB alone.

Results of this experiment summarized in table IX show that NB at one-tenth the normal concentration was sufficient to effect a detectable increase in the growth rates of all organisms tested except P. aeruginosa. However, fold increases with X. translucens and B. subtilis were considered to be marginal at this medium concentration with either inoculum level. It was concluded that with NB medium at 0.25X concentration, E. coli,

TABLE VIII. SUMMARY OF RESULTS FROM 4 HOURS INCUBATION OF E. COLI AT 32 C FOLLOWING TWO MINIMAL INOCULA LEVELS IN DECREASING CONCENTRATIONS OF NB AND BHI MEDIUM

Medium* Concentration	Fold Increase** In NB At:		Fold Increase** In BHI At:	
	Inoculum = 0.15 Cells/ml	Inoculum = 0.015 Cells/ml	Inoculum = 0.5 Cells/ml	Inoculum = 0.05 Cells/ml
1X	33	(100)***	78	<1
0.75X	27	<1	96	26
0.5X	33	<1	68	18
0.25X	20	<1	4	<1
0.1X	27	<1	0	<1

*Each medium concentration contained 0.05% polysorbate 80 and 0.05 M NaCl.

**Fold increase is determined from the average count per ml at 4 hours divided by the average count per ml at 0 time - average of duplicate samples.

***Not considered significant.

TABLE IX. EFFECT OF DECREASING CONCENTRATIONS OF NUTRIENT BROTH (WITH 0.05% POLYSORBATE 80 AND 0.05M NaCl) ON GROWTH RATES (FOLD INCREASE) OF TEST ORGANISMS FOLLOWING MINIMAL INOCULA AND 4-HOUR INCUBATION AT 32 C

Test Organism	Initial Inoculum Cells/ml	Fold Increase* of Organism in Medium Concentration			
		1X	0.5X	0.25X	0.1X
E. coli	1.6	57	38	34	16
	0.16	38	75	25	38
P. aeruginosa	0.64	25	19	8	<1
	0.064	31	31	<1	<1
M. polymorpha	1.5	120	110	160	43
	0.15	47	73	67	33
B. subtilis	4.0	36	32	30	9
	0.4	47	80	50	7
X. translucens	4.2	19	15	12	5
	0.42	14	12	5	5

*Fold increase is determined by dividing final count per ml by initial count per ml - average of duplicate samples.

M. polymorpha and and B. subtilis would multiply to significant levels to provide detection of inocula, (i.e. bacterial contaminants) of approximately one organism per ml. Further, it was hypothesized that nutritional factor(s) necessary for initiation of cellular growth of P. aeruginosa and X. translucens were either absent or in subminimal quantities at this medium concentration (0.25X).

From these and other data discussed earlier, NB medium was chosen over BHI as the standard medium for the Bacteria Sensor. A summary of the principal reasons for this decision is given below:

- Greater solubility of NB
- Lower incidence of clumping or agglomeration with the test organisms
- More consistent effect on growth rate of test bacteria at lower medium concentrations

Growth Factor Studies

General. With the exception of P. aeruginosa, all test organisms appeared to multiply satisfactorily in dilute NB following the low inocula and four hours incubation at 32 C. It seemed likely, as discussed previously, that certain essential nutrient(s) required by Pseudomonas were either missing or in subminimal concentration under these conditions. If sensitivity was to be maintained at ~ 1 organism per ml, then an additional growth factor(s) would be needed. A search of the pertinent literature was followed by experimental investigation of likely possibilities.

Addition of Lactose, Ammonium Acetate, Potassium Phosphate. Gronlund (1963, 1966) indicated the need of Pseudomonas for ammonium ion (NH_4^+), and examination of commercial preparations recommended for the growth of Pseudomonas showed the presence of lactose as a common ingredient. Arbitrary concentrations of either lactose or ammonium acetate ($NH_4C_2H_3O_2$) were added to 1.0X, 0.5X, or 0.1X NB. Continuing experience with the Coulter Counter strongly suggested an increase in the electrolyte concentrations in order

to accommodate higher electronic sensitivity. Therefore, the NaCl content of each media preparation was increased from 0.01M to 0.075M; the concentration of polysorbate 80 remained at 0.05%. Duplicate 20 by 150 mm test tubes were inoculated with P. aeruginosa at approximately one organism per ml and incubated at the standard time and temperature. The results of this experiment are presented in table X.

TABLE X. SUMMARY OF RESULTS - FOLD INCREASE OF P. AERUGINOSA FOLLOWING MINIMAL INOCULUM OF DILUTE NB MEDIUM PLUS FOUR CONCENTRATIONS OF EITHER AMMONIUM ACETATE OR LACTOSE

Nutrient/ Concentration	Fold Increase* of P. Aeruginosa - Inoculum = 0.72 Cells/ml		
	1.0X NB**	0.5X NB**	0.1X NB**
Control	16	16	<1
Lactose			
0.3 M	6	4	1
0.06 M	19	20	3
0.03 M	22	22	3
0.003 M	22	14	<1
Ammonium Phosphate			
0.1 M	1	1	<1
0.05 M	3	1	<1
0.01 M	14	17	1
0.005 M	30	18	1

Experimental conditions: 4 hours incubation at 32 C - average of duplicate samples.

*Fold increase is determined by dividing final count per ml by initial count per ml.
**Prepared with 0.075 M NaCl and 0.05% polysorbate 80.

As shown in table X, neither the lactose nor the ammonium phosphate, at the concentrations tested, had any apparent effect on the multiplication rate of P. aeruginosa following incubation in the 0.1X NB medium. Lactose at 0.3 M, and the ammonium phosphate at 0.1 and 0.05 M, appeared to be somewhat toxic or inhibitory at standard NB concentrations (i.e., 1X NB). Fold increase with 0.005 M ammonium phosphate was almost double with IX NB, but showed no effect in the 0.5X or 0.1X NB medium.

Beruheim (1963b) and Thimann (1965) reported that ammonium ions in the presence of potassium and phosphate ions were essential for growth of P. aeruginosa. Since the previous experiment pointed to the possible beneficial effect of ammonium phosphate at 0.05 or lower, NB/10 (0.1X NB) was prepared with $NH_4C_2H_3O_2$ at 0.05 and 0.001 M, and with and without the presence of potassium phosphate (K_2HPO_4) at 0.004 M. Low inocula of each of the test organisms were used to seed duplicate tubes of each NB/10 medium variation followed by 4 hours incubation at 32 C. The results of this experiment are shown in table XI.

As suggested in table XI, all permutations of $HN_4C_2H_3O_2$ and K_2HPO_4 tested displayed either lower or equivalent fold increases than with the NB/10 medium control alone. No further investigation seemed warranted.

Addition of Metal Salts. A recent publication on the Bdellovibrio bacteriovirus by Shila and Brof (1964) described a minimal medium for the growth of P. aeruginosa and other bacteria as host organisms. The authors indicated that certain metal salts were essential as nutritional ingredients. Therefore, an experiment was designed to test the effect of these metals on the growth of P. aeruginosa and other test organisms from low inoculum and short incubation time. Base NB/10 medium (0.1X NB plus 0.075 M NaCl and 0.05% polysorbate 80) was prepared with and without the following three metal salts:

- $Ca(NO_3)_2 \cdot 4H_2O$ - 1.22 μM
- $FeSO_4$ - 0.066 μM
- $MnSO_4 \cdot H_2O$ - 0.066 μM

Tubes employing low inocula of each test organism incubated as before were used to investigate the effect of these salts on the multiplication rate or fold increase. A summary of the results obtained are presented in table XII.

As shown, micro-molar quantities of calcium, ferrous, and manganese salts appeared to significantly increase the multiplication rate of each of the five organisms tested, with the greatest effect on P. aeruginosa and X. translucens. This experiment was repeated for

TABLE XI. SUMMARY OF RESULTS - FOLD INCREASES FOLLOWING LOW INOCULA OF TEST ORGANISMS IN NB/10 PLUS AMMONIUM ACETATE AND WITH AND WITHOUT POTASSIUM PHOSPHATE

Marker Organism	Inoculum - Cells/ml	Fold Increases In: NB/10** Alone	NB/10** + 0.005M $NH_4C_2H_3O_2$	NB/10** + 0.001M $NH_4C_2H_3O_2$	NB/** + 0.005M $NH_4C_2H_3O_2$ +0.004 K_2HPO_4	NB/10** + 0.001M $NH_4C_2H_3O_2$ +0.004 K_2HPO_4
E. coli	0.4	17	12	12	10	17
P. aeruginosa	0.56	2	2	1	2	2
X. translucens	0.4	5	5	3	5	5
M. polymorpha	0.56	29	16	29	20	23
B. subtilis	0.52	38	13	12	12	15

Experimental Conditions: 4 hours incubation at 32 C - average of duplicate samples.

*Fold increase is determined by dividing the final count per ml by the initial count per ml.
**NB/10 = 0.1X NB plus 0.075 M NaCl and 0.05% polysorbate 80.

TABLE XII. SUMMARY OF RESULTS - FOLD INCREASES OF DILUTE INOCULA IN NB/10 MEDIUM WITH AND WITHOUT METAL SALTS FOLLOWING 4 HOURS INCUBATION AT 32 C

Organism	Inoculum - Cells/ml	Fold Increase* In: NB/10** Alone	NB/10** + Metal Salts***
E. coli	0.26	35	50
P. aeruginosa	0.52	<1	22
X. translucens	0.31	3	16
M. polymorpha	0.44	27	55
B. subtilis	0.76	18	35

* Fold increase - determined by dividing final count per ml by initial count per ml.

** NB/10 = 0.1X NB + 0.075 M NaCl + 0.05% polysorbate 80.

*** Metal Salts = $Ca(NO_3)_2 \cdot 4H_2O$ @ 1.22 μM

$FeSO_4$ @ 0.066 μM

$MnSO_4 \cdot H_2O$ @ 0.066 μM

confirmation and the results are summarized in table XIII. The data from this experiment are somewhat equivocal in that P. aeruginosa shows only a marginal increase in multiplication rate (fold increase) while X. translucens exhibits no discernible response. A possible explanation was revealed after examination of the stock $FeSO_4$ solution: A slight precipitate was observed in the preparation, indicating the possibility that oxidation of the ferrous ion (Fe^{++}) to ferric (Fe^{+++}) had occurred followed by formation of the insoluble hydroxide. Therefore, it was hypothesized that the ferrous ion was required before initiation of optimum growth could occur under the conditions of this experiment.

The next experiment was designed to investigate whether the ferrous ion was indeed required to initiate optimum growth. NB/4 medium (0.25X NB plus NaCl and polysorbate) was prepared with and without calcium and manganese salts, but no ferrous ion. Duplicate tubes of each medium preparation was seeded with low inocula of the test organisms followed by 4 hours incubation at 32 C.

TABLE XIII. SUMMARY OF RESULTS - REPEAT OF EXPERIMENT DESCRIBED IN TABLE XII

Organism	Inoculum - Cells/ml	Fold Increase* In:	
		NB/10** Alone	NB/10 + Metal Salts***
E. coli	0.21	24	71
P. aeruginosa	0.44	<1	5
X. translucens	0.28	4	4
M. polymorpha	0.24	21	83
B. subtilis	0.22	18	14

*Fold increase - determined by dividing final count per ml by initial count per ml.

**NB/10 = 0.1X NB + 0.075 M NaCl + 0.05% polysorbate 80.

***Metal Salts = $Ca(NO_3)_2 \cdot 4H_2O$ @ 1.22 μM
$FeSO_4$ @ 0.066 μM
$MnSO_4 \cdot H_2O$ @ 0.066 μM

The results of this experiment are presented in table XIV. As shown, the fold increase with E. coli was reduced in the NB medium containing the calcium and manganese salts, while X. translucens and B. subtilis were essentially unaffected. P. aeruginosa demonstrated an appreciable increase in the multiplication rate, and M. polymorpha showed a slight increase. The data are somewhat contradictory and difficult to interpret. The effect of the increased NB concentration (from 0.1X to 0.25X) is also difficult to assess.

The increase in the NB concentration described above was dictated by engineering considerations. Original plans called for a 500:1 dilution ratio between the medium reservoir and Sensor operation (50X medium concentrate by 0.1X operational dilution), but interaction of pump characteristics, flow rates, and through-put time necessitated a compromise involving a 128:1 ratio (32X concentrate by 0.25X operational dilution). Although this reduction decreased the reservoir capacity, it was felt that the additional nutrients could only aid the biological cause.

TABLE XIV. SUMMARY OF RESULTS - FOLD INCREASES OF LOW INOCULA OF TEST ORGANISMS IN NB/4 MEDIUM ALONE AND NB/4 PLUS METAL SALTS MINUS Fe^{++}

Organism	Inoculum - Cells/ml	Fold Increase* In:	
		NB/4** Alone	NB/4** + Ca^{++} & Mn^{++} ***
E. coli	0.24	96	58
P. aeruginosa	0.64	8	20
X. translucens	0.64	9	11
M. polymorpha	0.48	214	256
B. subtilis	0.56	16	16

Test Conditions: 4 hours incubation at 32 C - average of duplicate samples

*Fold increase is determined by dividing the final count per ml by the initial count per ml

**NB/4 = 0.25X NB Medium + 0.075M NaCl and 0.05% polysorbate 80.

***NB/4 + Ca^{++} & Mn^{++} = As above + $Ca(NO_3)_2 \cdot 4H_2O$ - 1.22 μM

$MnSO_4 \cdot H_2O$ - 0.066 μM

Another experiment was designed to investigate the effects of these metal salts on the growth of minimal inocula in the dilute NB medium, and to provide better definition to the questions raised by the previous experiments. If, as originally hypothesized, the ferrous ion is required for growth of P. aeruginosa and X. translucens from low inoculum, three possible avenues of investigation were considered.

- Addition of a chelating or sequestering agent to prevent oxidation of ferrous ion to ferric hydroxide
- Addition of a reducing agent to prevent oxidation by lowering the redox potential
- Addition of a substitute for Fe^{++} which would not be subject to formation of insoluble hydroxides

The addition of a reducing agent was rejected bacause of the probable toxic or inhibitory effects on the growth and metabolism of the test organisms. Examination of the periodic chart indicated that Co^{++} or Ni^{++}, which appear in the same period as iron, might be substituted for the Fe^{++}. Of the chelating agents, sodium citrate was chosen on the basis of its performance in similar biological situations. Information supplied by the manufacturer[8] indicated that constant amounts of unbound iron as Fe^{++} would be available. Sodium citrate only, plus Ca^{++} and Mn^{++}, was added to the NB/4[9] medium as a control for the citrate/ferrous complex and the $FeSO_4$ alone. The ferric salt, $Fe_2(SO_4)\cdot NH_2O$, plus the NB/4 medium with calcium and manganese salts was also tested as a possible replacement for $FeSO_4$. Low inoculum of either P. aeruginosa or X. translucens was used to seed duplicate tubes of each experimental medium described.

8. Pfizer Laboratories, Division of Charles Pfizer & Co., Inc., 235 E. 42nd Street, New York, N.Y. 10017

9. The abbreviation NB/4 hereafter designates 0.25X NB plus 0.075 M NaCl and 0.05% polysorbate 80.

The conclusions drawn from the results of the experiments summarized in table XV were as follows:

- Comparison with the controls indicated definite toxicity by the Ni^{++} and Co^{++} cations on the growth of P. aeruginosa and X. translucens.
- Substitution of the Fe^{+++} salt for Fe^{++} showed equivocal results - P. aeruginosa demonstrated a slight increase in the multiplication rate while X. translucens appeared to be inhibited.
- Addition of sodium citrate alone reduced the multiplication rate of X. translucens but promoted a slight increase with P. aeruginosa. Addition of the citrate/ferrous complex showed a definite enhancement of the multiplication rate of both of these organisms indicating the probable need for iron in the ferrous form. This was further confirmed by the positive results from the addition of freshly prepared $FeSO_4$ to the medium immediately before inoculation with test organisms.

The overall conclusion reached from this experiment was that ferrous iron was essential to the growth of low inocula of P. aeruginosa and X. translucens under the conditions of dilute medium and short incubation time.

To confirm the use of these metal salts in the Sensor standard medium, NB/4 plus the calcium and manganese salts, and with and without the citrate-iron complex, was repeated with low inoculum of each test organism.

The results, summarized in table XVI, confirm the positive effect of these salts on the growth-rate from low inocula, especially with the P. aeruginosa and X. translucens.

Additional experimentation may have been desirable to further establish the optimum formula for the Sensor medium, but program considerations dictated that firm decisions be made to allow unit fabrication to proceed.

TABLE XV. SUMMARY OF RESULTS - FOLD INCREASES FOLLOWING DILUTE INOCULUM OF P. AERUGINOSA AND X. TRANSLUCENS IN STANDARD NB/4 MEDIUM PLUS EXPERIMENTAL NUTRITIONAL ADDITIONS

Medium Variation	Fold Increases With:	
	P. aeruginosa Inoculum = 0.56 Cells/ml	X. translucens Inoculum = 0.4 Cells/ml
Standard NB/4* Medium (Control)	4	15
Standard NB/4 + S** (Control)	7	5
Standard NB/4 + S + $FeSO_4$ (0.066 μM)	14	17
Standard NB/4 + S + $Fe_2(SO_4)\cdot NH_2O$ (0.059 μM)	7	10
Standard NB/4 + S + $Na_3C_6H_5O_7\cdot 2H_2O$ (0.53 μM)	7	7
Standard NB/4 + S $Na_3C_6H_5O_7\cdot 2H_2O$ - $FeSO_4$ *** Complex	9	20
Standard NB/4 + S + $NiSO_4$ (0.066 μM)	2	2
Standard NB/4 + S + $CoSO_4$ (0.066 μM)	1	1

Experimental Conditions: 4 hours incubation at 32 C - average of duplicate samples

*Standard NB/4 Medium = 0.25X NB + 0.075M NaCl + 0.05% polysorbate 80

**S = $Ca(NO_3)_2\cdot 4H_2O$ @ 1.22 μM; $MnSO_4\cdot H_2O$ @ 0.066 μM.

***Complex = [$Na_3C_6H_5O_7\cdot 2H_2O$ @ 0.53 μM + $FeSO_4$ @ 0.066 μM] final medium concentration.

TABLE XVI. SUMMARY OF RESULTS - FOLD INCREASES FOLLOWING LOW INOCULUM OF TEST ORGANISMS INTO STANDARD NB/4 MEDIUM PLUS Ca^{++} AND Mn^{++} AND WITH AND WITHOUT FERROUS - CITRATE COMPLEX

Organism	Inoculum - Cells/ml	Fold Increase In:	
		NB/4[a] + S[b]	NB/4[a] + S[b] + Complex[c]
E. coli	0.4	55	65
P. aeruginosa	0.8	5	11
X. translucens	0.4	25	23
M. polymorpha	0.5	334	296
B. subtilis	0.14	50	93

Experimental Conditions: Four hours incubation at 32 C - average of duplicate samples.

a. NB/4 = 0.25X NB + 0.075M NaCl + 0.05% polysorbate 80.
b. S = $Ca(NO_3)_2\cdot 4H_2O$ @ 1.22 μM; $MnSO_4\cdot H_2O$ @ 0.066 μM.
c. Complex = Na [$C_6H_5O_7\cdot 2H_2O$ @ 0.53 μM + $FeSO_4$ @ 0.066 μM] final medium concentration:

SUMMARY - BIOLOGICAL REQUIREMENTS

From the data presented in the preceding section, the following biological requirements were defined:

- Incubation - An incubation temperature at 32 C ±1 for 4 hours was considered a minimum requirement.

- Medium - Preparation of the concentrated medium is described in detail in Appendix IV. Precipitation of certain components within the complete concentrated medium necessitated the adoption of a two-part medium: part one contained NB medium at a 32X concentration; part two contained the metal salts, electrolyte, and polysorbate 80, all at a 25X concentration. Proper adjustment of pump ratios provided final concentrations of suitable proportions.

- Background Concentration - Experience with the standard Coulter Counter showed that many particulates of the size of bacteria were normally present in distilled water and in a carefully prepared medium. Obviously, these contaminants would contribute to the background count, making subtle differences resulting from growth and replication of low inocula extremely difficult to detect. In addition, all internal surfaces of the instrument (tubing, filter, sensor housing, etc.) contribute particulates to the test or sample streams. The only practical solution to this problem involved two courses of action:

  - Choice of materials which inherently provide a minimum of particulate contamination, and

  - Rinsing or preparation of all material for the Sensor with water which has been rendered as free of particulates as possible.

- Sensitivity - It appears that, if operated under the conditions defined in this section, the instrument is capable of detecting viable bacterial contaminants from an initial concentration of approximately 1 to 10 organisms per milliliter of liquid to be monitored. This is contingent upon the reduction of electronic and particulate background contamination to an absolute minimum.

SECTION IV
SYSTEM DESCRIPTION

GENERAL

The Bacteria-Sensor will automatically assess the microbiological quality of drinking water on a go-no-go basis within 5 hours from sample admission to the unit. Sampling of water is continuous, as is the subsequent processing to develop the necessary conditions for microbiological growth and reproduction. Laboratory studies have been performed on water samples inoculated with the following select organisms at concentrations down to one organism per ml. The organisms tested were: B. subtilis, P. aeruginosa, M. polymorpha, X. translucens and E. coli. More detailed information regarding sensor performance is presented in a later section of this report.

The laboratory unit consists of two major subsystems, namely an Electronics Subsystem and a Circulation and Incubation Subsystem. These are mounted in a standard instrument rack, as shown in figure 3, which is a front view of the unit, and figure 4, which is a view from the rear. The numbered items in the photographs denote the major identifiable components in each subsystem. These include the following:

1. Coulter Counter, Model B Research Counter
2. Timer/Sequencer
3. Detector Selector Switch
4. Pump/Incubator Power Switches
5. Coulter Counter (rear view)
6. Liquid Storage Tanks
7. Circulation/Incubation Equipment Shelf

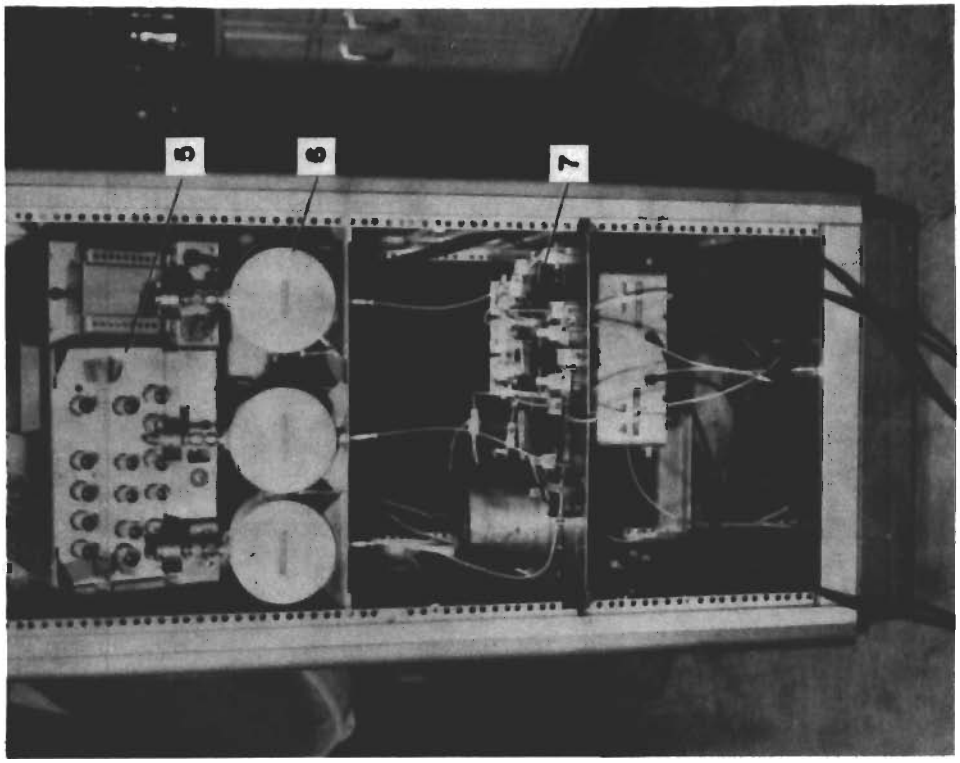

FIGURE 4.
BACTERIA SENSOR - REAR VIEW

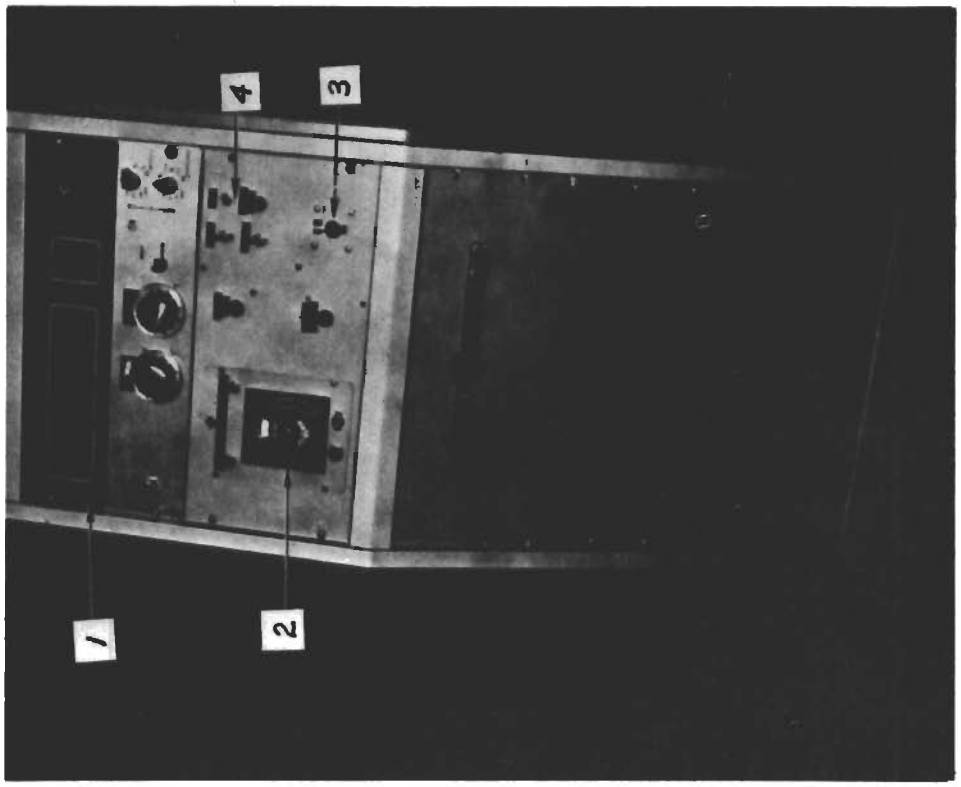

FIGURE 3.
BACTERIA SENSOR - FRONT VIEW

Not shown in either of these photographs is the AC Line Voltage Stabilizer used to power the unit. The transformer is used to provide a highly stable ac voltage to obtain optimum sensor sensitivity and minimize electrical interference.

ELECTRONICS SUBSYSTEM

The Model B Coulter Counter shown in the close-up photograph of figure 5 represents the major operational component in the Electronics Subsystem. This instrument provides the capability of detecting and counting individual particulates as each passes through the 25 micron orifice in each detector. The voltage pulse generated by the relatively nonconductive particle passing through the orifice while suspended in the electrically conductive water/ electrolyte fluid are electronically scaled by the Counter and subsequently displayed on the glow type decade counter, labeled 1A in the photograph. This counter has the capability of counting up to 100,000 particles in a 15-second period. Relative particle size (or size distribution, as labeled on the instrument) is continuously displayed as individual spikes on the oscilloscope built into the counter.

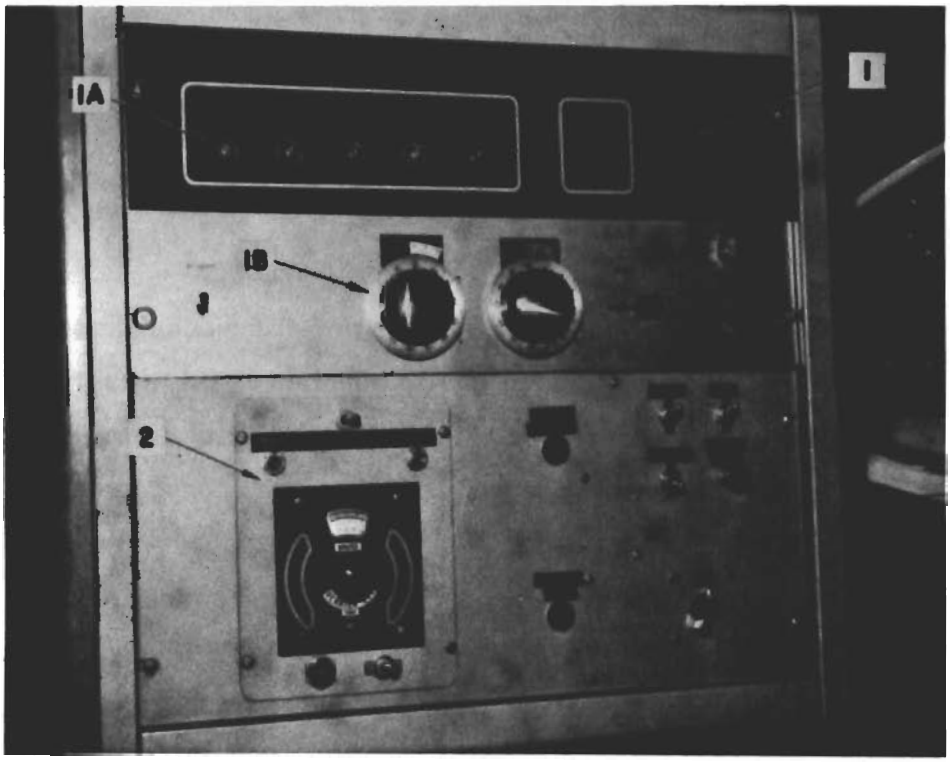

FIGURE 5. BACTERIA SENSOR - INSTRUMENT CONTROLS AND COULTER COUNTER

Range of particle sizes to be counted is preset at the counter with the threshold controls (1B). Both a lower threshold limit and an upper threshold limit may be programmed in if desired. During tests of this unit, the lower threshold limit was preset to disregard particles of less than 0.5 micron (nominal) while the upper limit was set at maximum so that all particles above the minimum size would be counted.

As will be shown later, the sample flow through the system is maintained at a constant rate. Therefore, judicious selection of time over which a given sample is counted provides a count rate/volume relationship. The sampling or counting time is controlled with a simple interval timer which can be preset for from 10 seconds (lower practical limit) to 30 minutes (maximum timer capability). The Timer/Sequencer will permit the count to proceed for the selected time period and then discontinue accumulation of particle counts on the decade counter until manually reset. The orifice selector switch (No. 3 in figure 3) puts the proper orifice into the counting circuit, number 1 being designated as the control (or non-incubated sample) and number 2 as the incubated sample.

For future units, the orifice selection, sample time selection, data acquisition and display can be electronically integrated to fully automate the system. Comparison of particle counts between the control and incubated samples can also be performed electronically and result in the sought for go-no-go signal.

Pump power switch is simply an on-off control of ac power to the constant speed pumps. A pump reversal push-button (hold to actuate) is also supplied to aid in operation of the unit. Incubator heater power switch and indicator lights similarly control ac power to the heating elements. Not shown in this photograph are two ac transformers used to control incubator temperature. One transformer is supplied for the preheater; the second is the main incubator heater power. (Transformers were installed during the test program).

CIRCULATION/INCUBATION SUBSYSTEM

Figure 6 is a top view of the major portion of the Circulation/Incubation Subsystem, showing

the pumps (7A and 7B), the filter housings (7C), incubator (7D), and the orifice flushing network (7E). Figure 7 is a bottom view of equipment shelf, showing the detector housings (8) and the associated valving and piping. By referring back to figure 4 (the rear view of the unit), the relationship between this equipment shelf and the storage tanks and electronics can be seen.

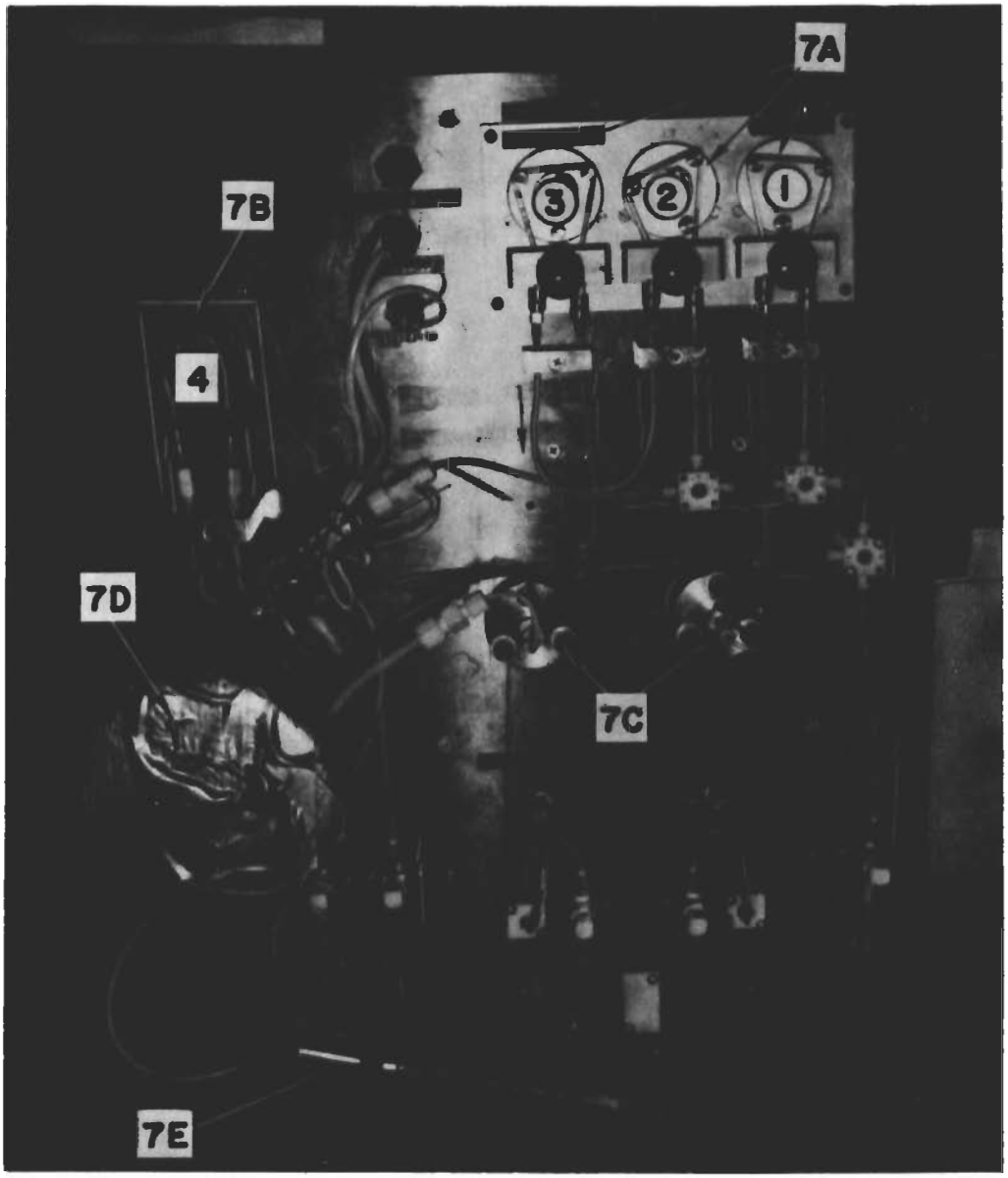

FIGURE 6. BACTERIA SENSOR - CIRCULATION/INCUBATION SUBSYSTEM

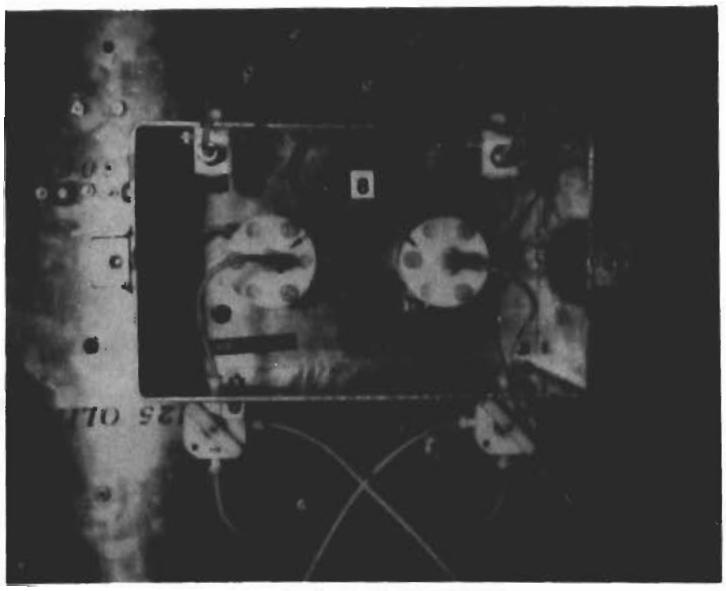

FIGURE 7. BACTERIA SENSOR - DETECTOR HOUSINGS AND ORIFICE FLUSHING NETWORK - BOTTOM VIEW

The pumps are medical type peristaltic pumps manufactured by the Holter Company of Bridgeport, Pennsylvania. They are designed to provide accurate metering of fluids at low flows for long periods of time. Use of this type of positive displacement pump also minimizes fluid contamination, since the pump tubes are of silicone rubber and reported to be heat sterilizable. Counting from the upper right hand corner of Figure 6, the pump functions are as follows: No. 1 Control Sample, No. 2 Incubated Sample, No. 3 Nutrient Additive, and No. 4 (not shown) labeled 7B is the Electrolyte Pump. The process flow is more readily followed in the schematic of figure 8.

Briefly, sample processing follows this general procedure. Water is continuously sampled at a nominal 12.8 ml per hour from the storage tank. The valve immediately downstream of the tank is a Teflon plug valve for isolation of the feed tanks. Electrolyte is added to the water at the first tee. The electrolyte is metered in at a nominal 0.5 ml per hour by an adjustable peristaltic pump.

The sample stream is then split into two equal streams. The first proceeds to pump No. 1, is then filtered and passes through detector No. 1 where the particle count is determined. This is the background or control count, there being no appreciable incubation beyond what

occurs at room temperature in the 40-60 minutes it takes the water to traverse this part of the system. The second portion of the water (now with electrolyte) has nutrient medium metered into it (from the nutrient pump No. 3) before it is pumped into the incubator. Line sizes are purposely kept small to minimize diffusion of a given slug of fluid either forward or backward. The attempt here is to achieve an approximation of "plug flow" through the system.

The incubator is a coil of small diameter (0.25 inch) tubing around a heater, oriented so that flow approximates a slug of fluid. Time through the incubator, which is maintained at 32 C, is approximately 4 hours. Filtration of large particles (> 14 micron) follows before passage through the No. 2 detector where the particulate count is determined.

If bacteria were present in the initial sample, the growth and reproduction achieved in the incubator would result in a significant increase in the number of particulates counted over that seen 4 to 5 hours earlier in the control or non-incubated sample. Without bacteria, count rates for each sample should be reasonably close to each other.

Since a 25-micron orifice is employed in each detector, it is not surprising that a method of back-flushing the detector housing and its orifice is required. A manual system for accomplishing this is shown schematically in figure 9. Simply described, it consists of a syringe connected to a fluid reservoir by suitably check-valved lines and manifolded to provide the capability of flushing either side of the orifice. The 3-way plug valve employed at the sample side of the orifice is ported to permit flushing without seriously disturbing the upcoming sample. This system has been satisfactory in keeping the unit operable over the periods of test. Automation by substitution of a small, high flow, low pressure pump and solenoid-operated Teflon plug valves appears to be an easily accomplished task to upgrade the unit design.

The necessity for use of small orifices in this system require that extreme care be used in the preparation of solutions and design and selection of materials and components in contact with the process fluids. Tubing runs, fittings, valves, and the detector housings are

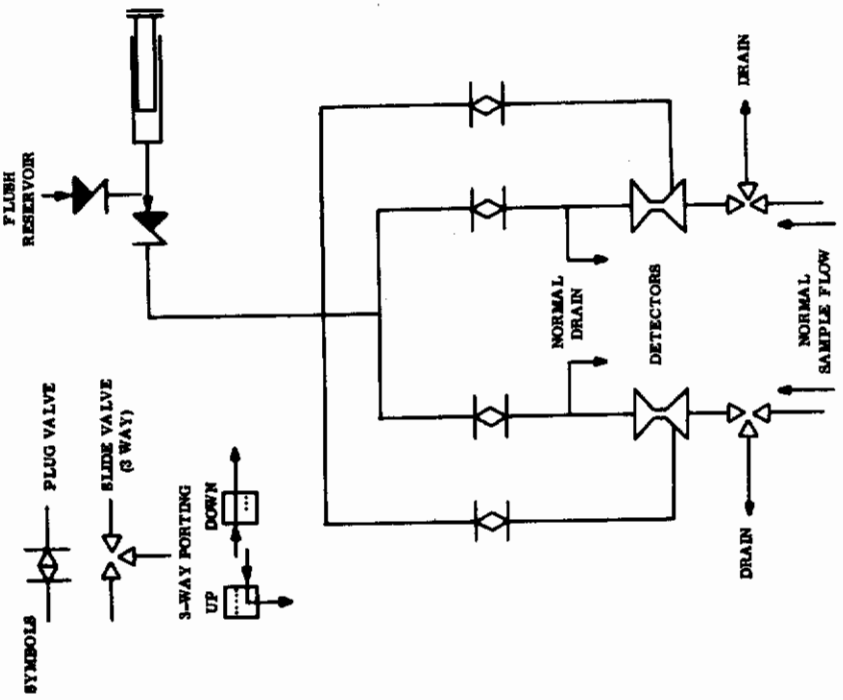

FIGURE 9. ORIFICE FLUSHING NETWORK

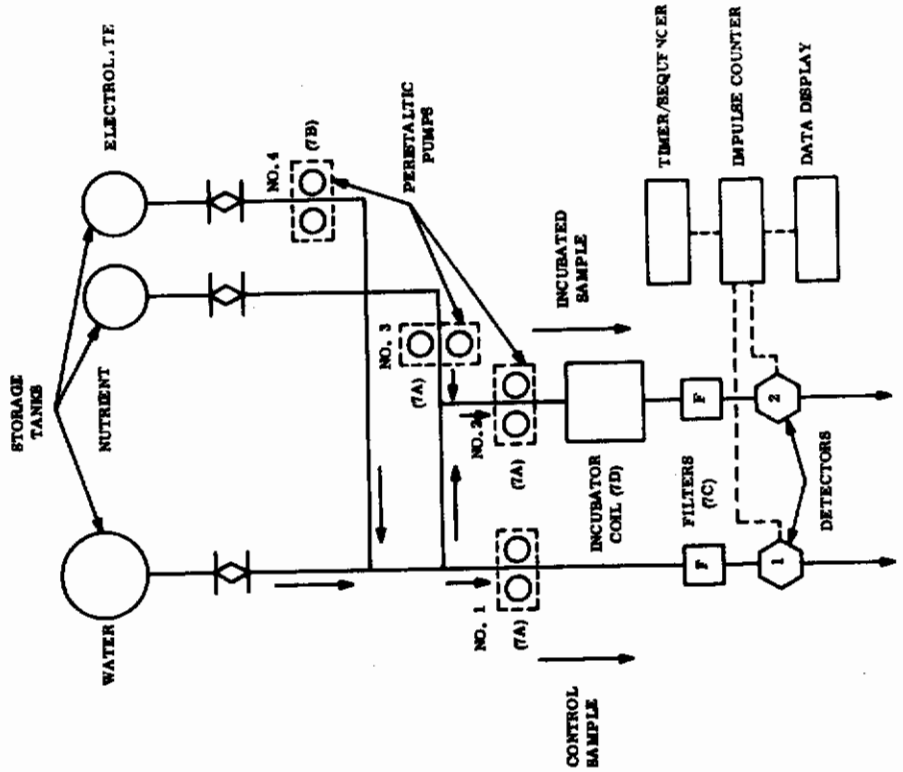

FIGURE 8. SCHEMATIC OF SENSOR SYSTEM

made of Teflon to minimize contamination. Fittings and valves are specially selected to provide smooth bores to eliminate places where bacteria might collect and inadvertently add to a given particulate count by reproducing and adding to the system. As previously mentioned, the pump tubes are medical grade silicone rubber. Pump connector fittings are nickel plated brass. The tanks are aluminum pipe, but have been coated after fabrication with an FDA approved epoxy phenolic coating.

Once started, the unit is self-operable, requiring only manual selection of orifice and time, manual data recording, and counter reset. Two runs have been completed in this manner with a minimum of operational difficulty. The unit, in its present form, is obviously built for laboratory use. The major components, however, have been used in spacecraft systems (e.g., the pumps slightly modified have been used in Biosatellite) or appear to be readily adaptable (e.g., electronics subsystem) with suitable redesign.

SECTION V

SYSTEM OPERABILITY TESTS

BIOLOGICAL RESULTS

Two experimental runs were completed on the Bacteria Sensor using low concentrations of E. coli as the test organism. Although certain mechanical and electronic elements within the instrument exhibited some unpredictable responses, the overall conclusion drawn from the data was that the Sensor was capable of performing the basic job for which it was designed, i.e., providing suitable conditions for the growth and increase of viable organisms and detecting that increase by means of the electronics provided.

In the first experiment, the tubing and particle-detectors were rinsed with sterile, particulate-free water for 24 hours. Then the two-part concentrated medium supply was inserted into the system and the instrument was allowed to operate continuously for another 24 hours. Flow rates on both orifices were monitored for consistent volume output, and 15-minute particulate counts were recorded throughout. The flow rate for the sample (incubated) line remained fairly consistent at approximately 6.2 ml per hour, but the control line appeared to be plagued with partial blockages and displayed irregular flow. The particle counts from both detectors (particularly the sample detector) were acceptably consistent during operation with the medium and sterile water alone, indicating either that the system was not contaminated with a chance microorganism or that there was no growth over the 24 hour period.

A 16-hour culture of E. coli was diluted in sterile, particulate-free water to 40 organisms per ml as determined by plate count. The sterile water was replaced with the E. coli suspension and particle counts were made alternately on each detector over the next 10 hours of operation. Inconsistent flow rates through the control detector (unincubated line) produced variable counts, making conclusive estimation of the background count difficult. At 5.7 hours after insertion of the bacterial suspension into the system, the average background count in the sample detector (incubated line) rose from 155 per ml to 7,600 per ml between two successive counting periods. The sudden and drastic increase in particulate count was considered to be strong presumptive evidence of bacterial multiplication, verifying the presence of viable microorganisms in the original test-water.

This data was plotted on semilog paper and is displayed in figure 10. The variable flow rate of the control line is depicted by a spread at each data point rather than a single point. The high control count at 7.7 hours is difficult to interpret, but in view of the readings obtained for the sample (incubated) line previous to 5.7 hours and the fact that nutrient and incubation temperatures were not available to the control line over the one hour through-put time, our considered recommendation would be to disregard this data point as spurious.

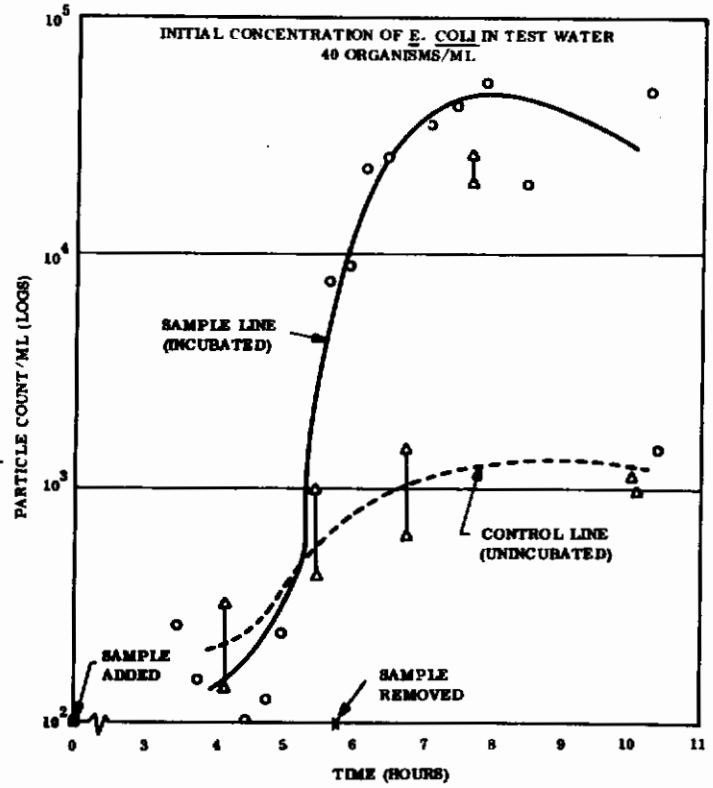

FIGURE 10. SENSOR OPERABILITY TEST I - DETECTION OF LOW LEVEL E. COLI CONTAMINANTS

Other Sensor elements which displayed less than satisfactory operation and therefore were responsible for additional variables to the test results are listed below:

- High variability of the resistance readings between the detector electrodes from measurements at each polarity. These readings determine the matching of the preamplifier in the Coulter Counter to the electrolytic capacity of the fluid being monitored.
- Extreme sensitivity of the electronics to vibration and/or voltage surges. This added an undetermined number of extraneous counts to the data.
- Inconvenient operation of the timer/sequencer. Manual operation required frequent attention for alternating between detectors; voltage surges produced by the relays during switching also added spurious counts to the total.
- Effects of changes in the aperture current and amplification switch-settings on the final results are not fully understood.

The second test, conducted at Wright-Patterson Air Force Base, also confirmed the ability of the Sensor to operate within the basic context for which it was designed. Variations to the experimental operation not present in the first test should be noted: rinsing of the Sensor, prior to addition of the test organisms, was carried out with particulate-free water in the absence of medium (especially the electrolyte); therefore, particle counts could not be obtained before adequate electrolyte concentrations reached the two detectors, i.e., about 1 hour for the control line and 5 hours for the sample line. Also, technical difficulties prevented the determination of the final concentration of E. coli organisms in the test inoculum.

The data from this experiment are plotted as before and are shown in figure 11. Control (unincubated) counts were first recorded about 1.5 hours after start of the test, or at the time the bacterial test suspension was replaced with sterile, particulate-free water. Since background counts could not be obtained with the sample (incubated) line, it was assumed that the precipitous increase in particle counts at 5.2 hours originated from approximately the same level as the control. It is difficult to interpret the 3 to 4 log difference in the count rate as anything other than the result of growth and multiplication of the bacteria from the test suspension.

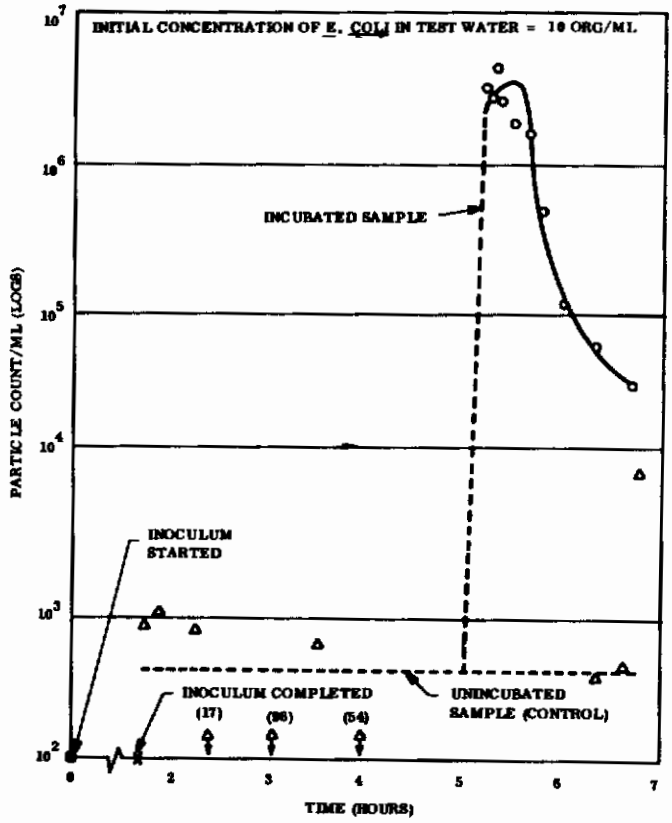

FIGURE 11. SENSOR OPERABILITY TEST II - DETECTION OF E. COLI CONTAMINANTS

SECTION VI

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Results of the experimental research, testing, and fabrication of the Bacteria Sensor indicate adoption of the following conclusions:

- Definition of biological requirements and limitations has been adequately realized from the developmental research efforts with the "Marker" organisms employed.
- Preliminary operational results with the Sensor has shown that the instrument fulfills the basic design goals: to automatically supply suitable conditions for the short-time growth and multiplication of low levels (1 to 10 organisms per ml) of contaminating organisms present in raw, reprocessed water; and to detect the resulting increase in particulate count with the electronics provided.
- It follows then that a basic design concept, fundamental to the operation of the Bacteria Sensor, has been demonstrated: bacteria-sized particles can be routinely detected by the electronic subsystem employed and the viable particulates quantitatively separated from nonviable ones through use of the parallel detector system.

RECOMMENDATIONS

It should be recognized that the basic concept and purpose of the Bacteria Sensor represents a significant departure from the standard, classical bacteriological procedures. This means that the diverse elements of the mechanical, electronic, and biological interfaces must be made to mesh completely in order to achieve a truly efficient and reliable instrument. The following recommendations are made with this purpose in mind:

- Standardization Techniques for the Coulter electronics and the circulation/incubation subsystem must be definitively established by a rigorous test program.
- The inherent instrument sensitivity range must be quantitated by careful testing with various inoculum levels of contaminating organisms both as single and mixed cultures.

- The timer/sequencer must be redesigned to operate automatically, i.e., automatic switching from one detector to the other, recording of particulate totals, and a programmed discriminator to provide a go-no-go signal.

- Unplugging of orifice blockage must be simplified and activated by automatic detectors.

APPENDIX I

PREPARATION OF CONCENTRATED TWO-PART MEDIUM FOR SENSOR RESERVOIRS

PART I - 32X NUTRIENT BROTH CONCENTRATE

a. Nutrient Broth - 256 g

b. Add 1 liter of particulate-free water (see Appendix II).

c. Stir with slight heat to aid in dissolving.

d. Filter twice through membrane filter of 0.45 μ pore size.

e. Filter twice through a 0.22 μ pore size membrane filter.

f. Dispense into sterile, 1 liter flask and cover with a rinsed aluminum foil cap.

g. Sterilize in autoclave for 15 minutes at 121 C (15 psi).

h. Remove from autoclave and allow to cool at room temperature.

i. When cool, seal aluminum cap with pressure tape to prevent evaporation before use.

PART II - 25X CONCENTRATED SALTS SOLUTION

All chemicals are reagent-grade quality:

a.	$FeSo_4$ (anhydrous)	250 mg
	$Na_3C_6H_5O_3 \cdot 2H_2O$	475 mg
b.	Particulate-free Distilled Water	950 ml

c. Dissolve and mix well at room temperature.

d. Add the following chemicals to the solution above and swirl until dissolved:

NaCl	112 g
$Ca(NO_3)_2 \cdot 4H_2O$	7.2 g

$MnSO_4 \cdot H_2O$	275 mg
Polysorbate 80 (25% Susp. W/W)	50 ml

e. Filter twice through a membrane filter of 0.45 μ pore size.

f. Filter twice through a 0.22 μ pore size membrane filter.

g. Dispense asceptically into appropriate size sterile flasks, then cover with sterile aluminum foil cap and seal with pressure tape to prevent evaporation. (Note: Concentrated salts solution cannot be autoclaved; polysorbate 80 will separate out of solution and layer at the top.)

APPENDIX II

PARTICULATE-FREE WATER

PREPARATION OF PARTICULATE-FREE WATER

High quality water can be produced by continuous recycling of distilled water through a high capacity membrane filter of 0.22 μ pore diameter[II-1]:

- A reasonably large reservoir of about 4 to 5 gallons is required to ensure an adequate supply of particulate-free water.
- Since distilled water routinely contains a considerable quantity of particulate debris between 0.22 to 3 μ diameter, the filtration process is begun with a 0.45 μ pore size filter. By observing flow rates (or pressure drops), it is possible to determine when the filter pores are becoming clogged and when fresh filters should be inserted.
- Generally, it is necessary to use two to three 0.45 μ filters (about 20 minutes per filter is average) before switching to the 0.22 μ pore size. When the flow rate appears to be steady with this filter, the water may be considered, for practical purposes, as particulate-free water.

DISPENSING WATER

A Gelman pressure can[II-2] is used to dispense the water produced by the pump/filter system:

- Using water from the effluent side of the filter housing, the pressure can, which has been thoroughly rinsed previously with distilled water, is rinsed again with five fillings of the particulate-free water.
- A high-pressure tank of dry nitrogen gas is used to charge the Gelman rinsing can for use. To prevent contaminants within the nitrogen tank from adding particulate matter to the rinse water, the downstream line from the tank is fitted with a high-pressure filter holder[II-3] containing a 0.22 μ filter. A tank gauge pressure of at least 50 psi is needed to charge the Gelman rinser.
- Finally, the outlet end of the Gelman rinser is fitted with a 0.45 μ pore size filter before using.

II-1. A useful pump and filter apparatus for this purpose is made by the Millipore Corporation of Bedford, Massachusetts, (Catalog No. XX41 142 20).

II-2. Manufactured by the Gelman Instrument Company, Ann Arbor, Michigan, (Model No. 7074).

II-3. As supplied by Millipore Corporation (Catalog No. XX45 047 00).

APPENDIX III

PREPARATION OF GLASSWARE

The following procedure is recommended:

- Wash in hot water with good detergent; do not use dichromate solutions.
- Rinse 5 times in hot tap water.
- Rinse 5 times in cool distilled water.
- Rinse 3 times in particulate-free distilled water.
- Air dry glassware on a lint free surface contained within a laminar flow cabinet.
- When dry, cover openings with aluminum foil caps rinsed with particulate-free water.
- Sterilize in dry heat oven for three hours at 250-260^{o}C.

APPENDIX IV

GROWTH CURVE STUDIES ON MARKER ORGANISMS FOR BACTERIA SENSOR

SUMMARY

Definition of minimum incubation requirements for the Sensor was attempted by growth curve studies on the following tests organisms [IV-1]. Escherichia coli (ATCC 10536), Mima polymorpha (ATCC 14291), Bacillus subtilis (ATCC 6633), and Xanthomonas translucens (ATCC 10771). Generation times for these organisms were determined from the exponential growth period of each curve. It was apparent that the inoculm size (number of viable organisms seeded) was a critical factor in the determination of this generation time. Since during normal Sensor operation the inoculum size would be unpredictable, the minimum incubation time necessary to produce a significant numbers of bacteria from low level inocula could not be inferred from the generation time.

EXPERIMENTAL

The following conditions were employed:

- Incubation temperature - 32 C $\pm$ 1 C
- Incubation time - 8 to 24 hours, as indicated
- Medium -

  Broth - Nutrient Broth, prepared as directed by the manufacturer; Trypticase Soy Broth plus 5% glycerol (one experiment)

  Assay - Nutrient Agar

- Growth Flasks - One liter flasks with cotton stoppers containing 250 ml of the sterile medium
- Inoculum - Duplicate growth flasks were seeded with one ml each of an overnight culture of the organism grown at 32 C in 9 ml of Nutrient Broth in a 16 by 150 mm test tube.

IV-1. Original culture of Pseudomonas received from ATCC (14671) required a salt-free medium and 20 C incubation. Replacement culture (ATCC 17423) was received too late for growth curve studies.

- Aeration - Static

- Assay methods - Each of the duplicate flasks was sampled hourly, beginning immediately after inoculation (except for one experiment each for E. coli and M polymorpha) for up to 12 hours. Thereafter, samples were taken every 2 hours in the studies of longer duration. Samples were diluted in sterile, physiological saline and duplicate plates were made from each of four appropriate dilutions using either the pour-plate or spread-plate method. Plates were counted with the aid of a Quebec colony counter after approximately 40 hours incubation at 32 C.

RESULTS

Data were plotted on semi-logarithmetic graph paper and are presented in figures IV-1 through IV-9. As illustrated, both E. coli and M. polymorpha showed rapid growth and multiplication within the first six hours following incubation. Generation times of 43, 61 and 15 minutes were calculated for E. coli (figures IV-1, IV-2 and IV-3 respectively) and 33 and 25 minutes for M. polymorpha (figures IV-4 and IV-5). Determination of the final inoculum concentration (zero count) was not done with the initial growth curves of these two organisms (figures IV-1 and IV-4). However, as shown in figures IV-2 and IV-3, the two-log difference in the inoculum with the E. coli culture had a pronounced effect on the lag phase, or the time required for the cellular machinery to begin logarithmetic growth. With a final inoculum size of 10^6 cells per ml, exponential growth began somewhere between zero and one hour after inoculation and incubation; with 10^4 cells per ml, logarithmetic growth was delayed 2 to 3 hours (lag phase).

The effect of inoculum size is also apparent with the B. subtilis curves (figure IV-6 and IV-7) where generation times of 17 and 45 minutes were calculated from inocula of 10^4 and 10^8 organisms per ml respectively. With the X. translucens, one log difference in the initial concentration had only a nominal effect on the generation times calculated (48 and 42 minutes respectively) as shown in figures IV-8 and IV-9.

CONCLUSIONS

Review, both of this data and the expected design criteria for the Sensor, dictated the following conclusions:

- Difference in inoculum size affects both the length of the logarithmetic growth phase and the time it is initiated.

- Estimation of minimum incubation time for the Sensor will depend upon the length of the lag phase and not the generation time during exponential growth. Because Sensor capabilities will be directed toward the detection of a minimum number of contaminating organisms, it is unlikely that this low level inoculum could be expected to reach exponential growth within a short-time incubation period.

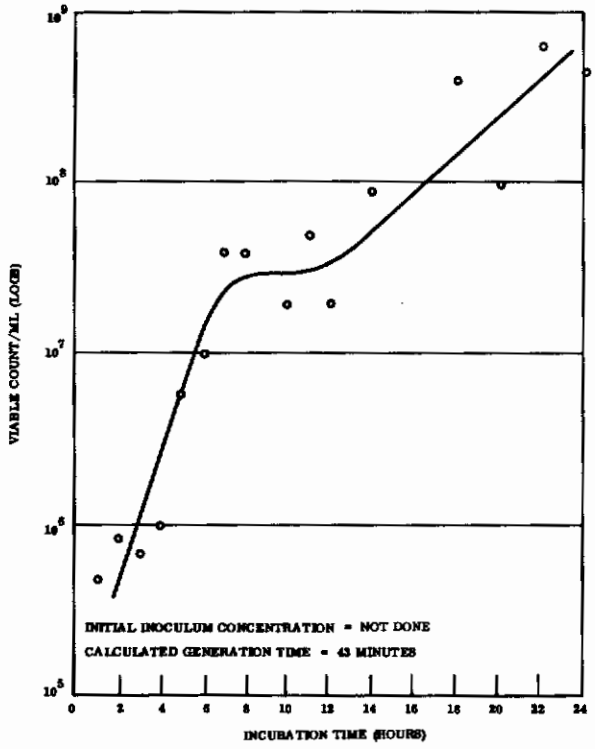

FIGURE IV-1.
GROWTH CURVE - ESCHERICHIA COLI

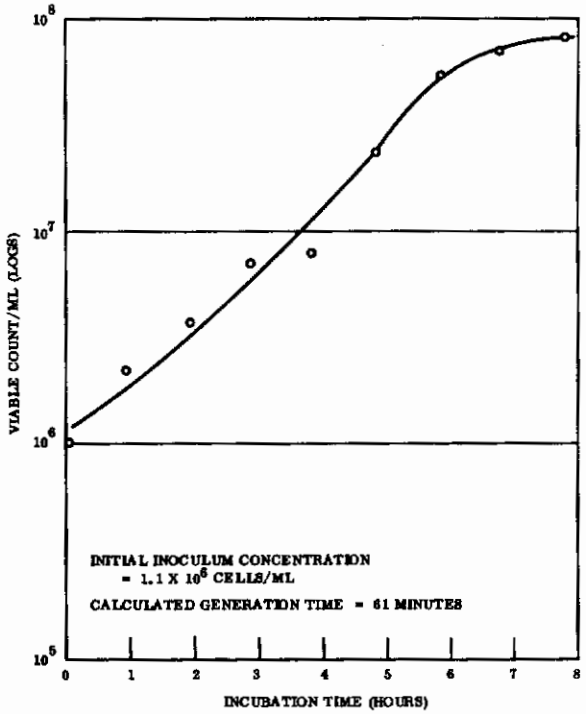

FIGURE IV-2.
GROWTH CURVE - ESCHERICHIA COLI

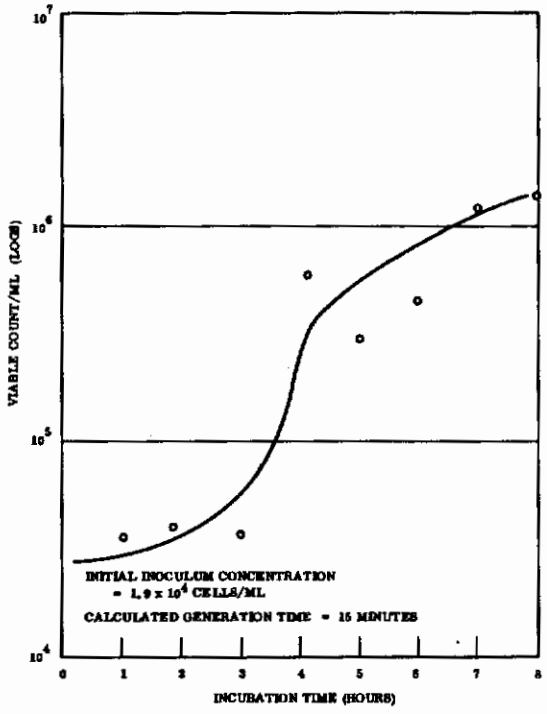

FIGURE IV-3.
GROWTH CURVE - ESCHERICHIA COLI

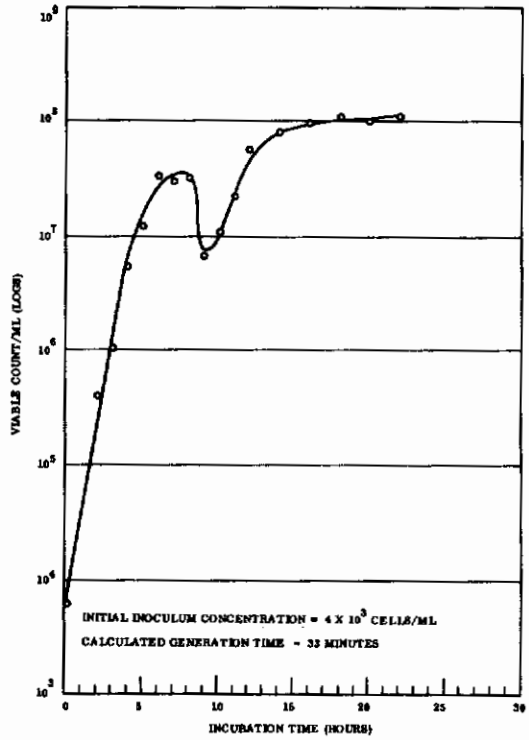

FIGURE IV-4.
GROWTH CURVE - MIMA POLYMORPHA

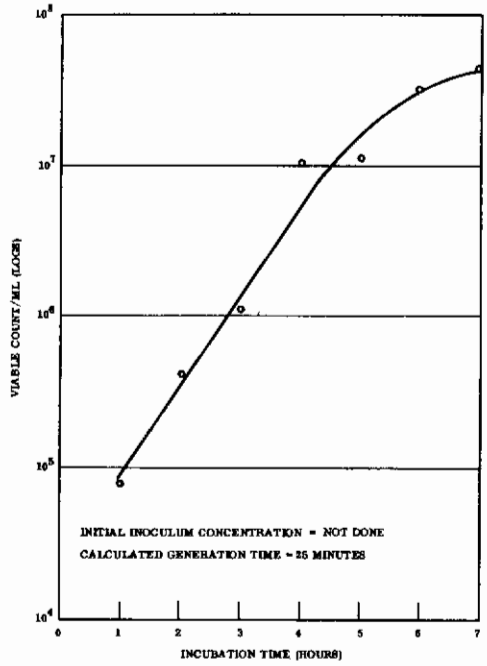

FIGURE IV-5.
GROWTH CURVE - MIMA POLYMORPHA

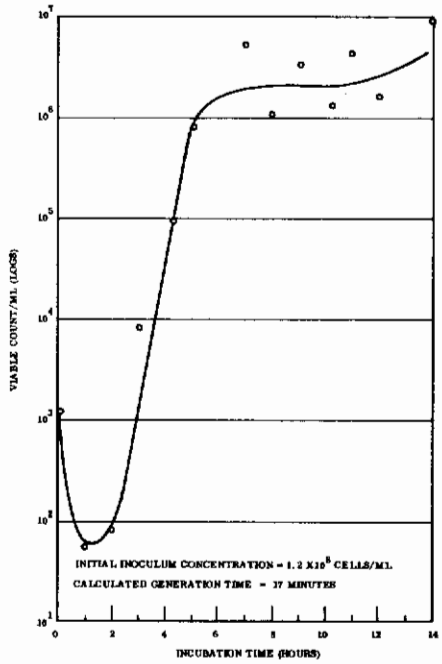

FIGURE IV-6.
GROWTH CURVE - BACILLUS SUBTILIS

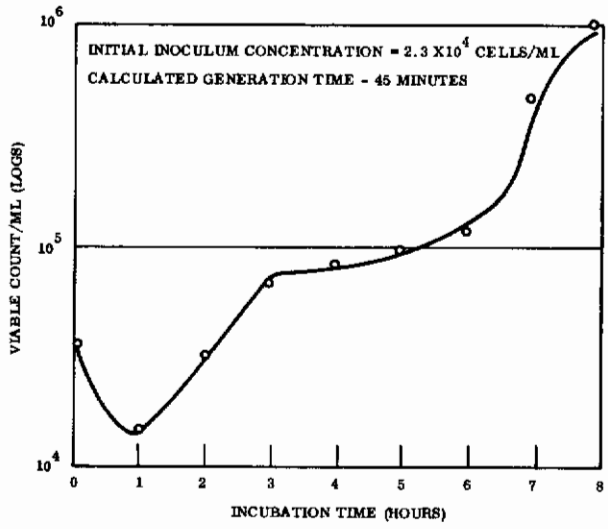

FIGURE IV-7.
GROWTH CURVE - BACILLUS SUBTILIS

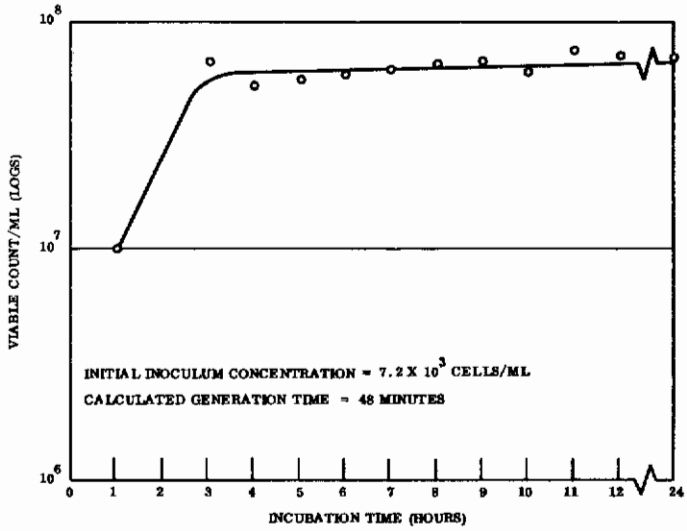

FIGURE IV-8.
GROWTH CURVE -
XANTHAMONAS TRANSLUCENS

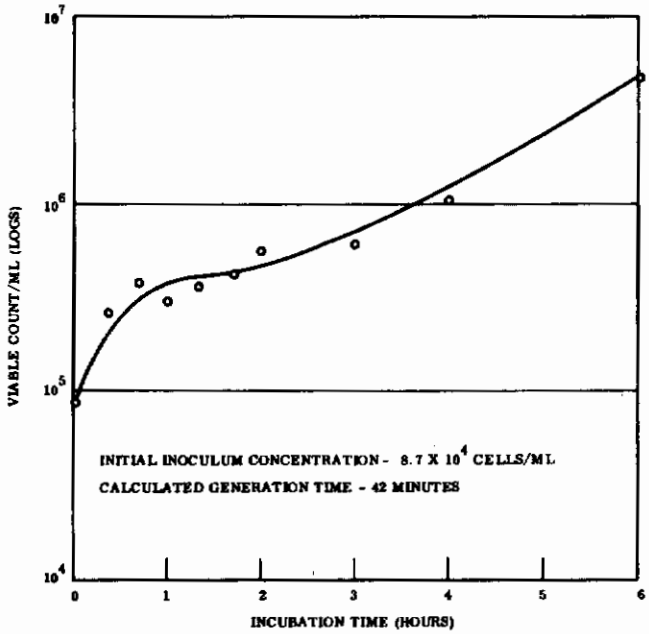

FIGURE IV-9. GROWTH CURVE - XANTHAMONAS TRANSLUCENS

APPENDIX V

BACTERIAL LAG PHASE

SUMMARY

The lag phase or period of adjustment in a freshly inoculated culture of bacteria can vary from culture to culture and is dependent upon several variables. The number of organisms inoculated, together with the average physiological age and the genetic characteristics of the species, affects the length of time required for initiation of cellular reproduction. Factors such as the nutritional constitution of the medium, temperature of incubation, and the effect of carbon dioxide also play a part. Random inocula represent mixed physiological populations of cells, all of which do not complete the lag phase simultantously; this, together with the factors mentioned above, accounts for the variability among cultures. Expected low numbers of bacterial contaminants, if any, within the on-board water storage tanks make consideration of the bacterial lag phase of prime importance.

INTRODUCTION

The addition of bacterial cells to a favorable growth medium is not followed immediately by a doubling of the population involved. Indeed, the cells tend to remain relatively unchanged and the viable number of bacteria may even decrease somewhat before beginning a gradual increase. This period of adjustment or lag phase is dependent upon a number of variables which are discussed in the following paragraphs. No attempt will be made to explore these factors at length, but merely to present the various conditions which affect the ability of a given bacterium to initiate growth and reproduction.

FACTORS WHICH INFLUENCE THE ADJUSTMENT OR LAG PHASE

Inoculum

- Size - The absolute number of viable organisms introduced to the fresh culture medium affects the length of the lag phase. There is some evidence that indicates a direct relationship between size of inoculum and the length of the adjustment period, but other factors within the inoculum itself are probably more important.

- Age of the Inoculum or Stage of Growth - Any bacterial inoculum represents a mixture ranging from physiologically young cells to those on the verge of death. However, any given inoculum will contain a preponderance of organisms representing a particular stage in the life cycle of the bacteria. Thus, the average age of these cells used as inoculum will have a direct bearing on the time needed for adjustment. For example, an inoculum derived from a culture growing at the maximum rate exhibits a very short lag phase; conversely, cells from old cultures will require an exceptionally long period of adjustment.

  It should be stated that spore-formers, such as Bacillus subtilis, represent a somewhat special case. A spore can lie dormant for several hours or up to several weeks before germination occurs, even under the most favorable conditions. Also, in any given inoculum involving a spore-former, depending upon the conditions under which it was obtained, the population will contain a percentage of cells already committed to sporulation which are not immediately affected by the transfer to a favorable medium.

- Genetic Character of the Inoculum - Various bacteria show inherent differences in their ability to initiate active growth and to develop at a maximum rate of speed in a favorable medium. E. coli requires a relatively short period of time to make the adjustment, while organisms such as Corynebacterium and Mycobacterium develop more slowly.

It can be seen from the discussion above that the number of organisms inoculated, together with the average physiological age and the genetic character of the species, affects the length of time required for initiation of cellular reproduction.

Medium

It is obvious that a favorable or a nutritionally adequate medium must be present before bacterial growth can be expected. However, it is possible to demonstrate a prolonged lag period in a medium that is not optimum for growth, even though some growth does occur. Often, the addition of small amounts of such nutrients as glucose or asparagine will reduce the length of this phase.

Temperature of Incubation

There are data which indicate that lowering the temperature of incubation increases the period of adjustment necessary for optimum growth and reproduction. Conversely, there

is also some evidence to show that, to a point, increased temperature beyond that required for the eventual maximum number of bacteria will decrease the lag time. The physiological and chemical events which occur during this period are undoubtedly temperature-dependent, and must be considered in any appraisal of the lag phase.

Effect of CO_2

The role played by carbon dioxide is not clearly defined. It has been speculated that:

- It acts as a stimulant to cell division - apart from the growth process.
- It is a factor in protoplasmic synthesis.
- It is actually used by some organisms directly in their metabolism.

Data have been presented which show that the removal of CO_2 from an environment which was otherwise favorable for the development of bacteria resulted in complete cessation of growth.

DISCUSSION

The lag phase in any one culture of bacteria is dependent upon more than just one of the factors mentioned above, and may even involve all of them. Cells are not quiescent or dormant during this period, but are active physiologically, synthesizing new protoplasm and increasing in size beyond their normal dimensions. Bacteria may be deficient in enzymes or coenzymes which must first by synthesized in amounts necessary for optimal operation of the chemical machinery. Since most inocula consist of mixed physiological populations, all organisms do not complete the lag period simultaneously, and there is a gradual increase in numbers until all cells are capable of dividing regularly.

In relation to the biological problems associated with the work on the bacterial sensor, it is expected that bacterial contamination of the water to be analyzed will be minimal. Therefore, priority must be given to a consideration of the lag time of these contaminants and the problem of detecting them within a maximum of incubation time of 4 hours.

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Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) General Electric Company Valley Forge Space Center King of Prussia, Pennsylvania 19406	2a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED 2b. GROUP N/A

3. REPORT TITLE

BACTERIA SENSOR FOR REPROCESSED WATER—MICROBIOLOGICAL RESEARCH, DESIGN, AND FABRICATION

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final Report, May 1967-August 1968

5. AUTHOR(S) (First name, middle initial, last name)

John A. Geating
Fred P. Rudek

6. REPORT DATE February 1969	7a. TOTAL NO. OF PAGES 60	7b. NO. OF REFS 7 Refs. 3 Bib. Citations
8a. CONTRACT OR GRANT NO. F33615-67-C-1564 b. PROJECT NO. 6373 c. Task No. 637304 d. Work Unit No. 637304004	9a. ORIGINATOR'S REPORT NUMBER(S) 9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report) AMRL-TR-68-173	

10. DISTRIBUTION STATEMENT

This document has been approved for public release and sale; its distribution is unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY Aerospace Medical Research Laboratory, Aerospace Medical Div., Air Force Systems Command, Wright-Patterson AFB, OH 45433

13. ABSTRACT

The results of the developmental research leading to the design and fabrication of a short-time, electronic sensor to monitor the bacteriological quality of reprocessed water aboard spacecraft are reported. The basic sensing capability is furnished by a Coulter Counter that selectively detects and counts particles of bacterial size. Detection is accomplished by comparing the number of bacteria-size particles in the reprocessed water sample at some point in time with a particle count at some future point in time, i.e., following the establishment of conditions necessary to allow growth and multiplication of bacteria. A significant difference between the two counts strongly implies bacterial replication, and therefore the presence of viable organisms in the raw reprocessed water. The microbiological research phase was geared to evaluating the relationships between a number of biological variables and their effect on sensor performance. Therefore, design requirements for the instrument were defined through the experimental manipulation of media, incubation time, and temperature on five selected strains of bacteria of varying concentrations. Instrument operability tests demonstrated the capability of the sensor to carry out the basic design goals. Experimental laboratory results with the test organisms indicate the theoretical sensitivity of the instrument to be in the neighborhood of one viable organism per milliliter of reprocessed water. However, qualification and standardization testing remains to be done to firmly establish the lowest concentration detectable for each possible contaminating organism.

DD FORM 1 NOV 65 1473

Security Classification

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Bacteria counter Aerospace water system Microbiological instrumentation						

Security Classification