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Project Summary

Firefly Luciferase ATP Assay Development for Monitoring Bacterial Concentrations in Water Supplies

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This research program was initiated to develop a rapid, automatable system for measuring total viable microorganisms in potable drinking water supplies using the firefly luciferase adenosine triphosphate (ATP) assay.

The ATP assay was adapted to an automatable flow system that, in less than 2 minutes, provided assays with sensitivity comparable with established methodology (10^5 bacteria/mL). Quality controls for required reagents were established. To achieve the sensitivity necessary for bacterial measurements in water, the sample must be concentrated before assay. Filtration systems were evaluated for ability to concentrate bacteria from large volume samples rapidly, efficiently, and without damage to the organisms. Results indicated most filtration systems tested had a limited capability to meet project criteria. Promising results (200- to 600-fold concentration and up to 88% recovery of bacteria) were obtained using hollow-fiber concentration systems modified to incorporate repeated backwash steps.

This Project Summary was developed by EPA's Municipal Environmental Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Current methods for examining bacteriological quality of potable water supplies depend on time-consuming culture procedures. Efficient control of contamination breakthroughs into finished waters or detection of water quality deterioration in distribution networks is delayed until results from the coliform test and standard plate counts become available. A method for rapid measurement of bacterial concentration in potable water would greatly enhance water quality maintenance and control. Criteria for an automated assay include sensitivity comparable to established methodology, minimum mechanical manipulations, real-time analysis, and quality controls for required reagents.

Intracellular adenosine triphosphate (ATP) extracted from microorganisms can be detected and quantified with the use of the firefly luciferase ATP assay. This assay is a rapid, "wet chemical" reaction that is easily automated. Relative ATP concentrations could be used to monitor changes in the levels of microbial populations as well as detect contamination breakthroughs into finished water supplies or water quality deterioration in network distribution systems in "real time." If the majority of microbes in the water supply are assumed to be bacteria, ATP levels could be

further used to estimate the total number of bacteria present. Experimental data indicate that the deviation of ATP content of bacteria, accounting for differences between species and variation during the growth cycle, does not exceed one order of magnitude.

The sensitivity limit of the ATP assay with pure cultures of bacterial strains is about 10^5 bacteria/mL. Assuming 500 bacteria/mL as an acceptable upper limit for the standard plate count population in drinking water, contaminating levels of bacteria must be concentrated by a factor of at least 200 to permit detection by an ATP assay. For example, a 20-L sample volume containing 500 bacteria/mL would have to be reduced to 100 mL with complete retention of bacteria to provide a concentration equal to the sensitivity limit of the assay. To ensure operation above the minimum sensitivity limit, bacterial concentration greater than 10^5 cells/mL would be desirable.

This study focused on development of an automated ATP assay and a suitable technique for concentrating low levels of bacteria from large sample volumes. The concentration technique had to: (1) be compatible with automation, (2) provide a high concentration factor, (3) have a rapid concentration rate, and (4) yield complete recovery of intact, undamaged bacterial cells in a reduced volume that could be diverted to an automated ATP assay system.

Automated ATP Assay

The established method for bacterial ATP assay generally includes adding ATP extractant by pipet to the bacterial sample in a polypropylene test tube, diluting by pipet, mixing, distributing the enzyme mixture into individual cuvettes, and finally, injecting 0.1 mL extracted, diluted sample into a luciferase-containing cuvette positioned in front of a photomultiplier tube.

The firefly luciferase ATP assay was adapted to an automated flow system that greatly reduced the mechanical manipulations required (Figure 1). Buchler peristaltic pumps move both sample and reagents through interconnected tubes. Relative flow rates shown in Figure 1 provide the optimal concentration of nitric acid extractant (0.1 N), with minimal sample dilution (50%), and 0.2 mL of luciferase enzyme solution. The luciferase enzyme is pulsed into the flow system only as the final processed sample reaches the glass coil positioned next to the photomultiplier tube. A Chem Glow photometer* (American Instrument Company) equipped with a coiled flow cell was used to measure maximum light output. Total light production was measured by coupling an Aminco Integrator-Timer to the photometer. Sample ATP concentrations were compared with ATP standards (0.1 μg ATP/mL); the latter were prepared by diluting purified ATP (Sigma Chemical Company) in sterile, deionized water.

ATP concentrations were converted to bacterial numbers using the conversion factor of 2.5×10^{-10} μg ATP/cell, which represents the average ATP content of 19 bacterial species. For application of the flow ATP assay to potable water analysis, the ATP conversion factor should be determined using bacteria isolated from actual water samples.

Figure 2 shows typical ATP concentration curves determined for *Escherichia coli* in saline and in tap water using the flow ATP assay.

Nitric acid (0.6 N) extraction was most suitable for bacterial ATP and was optimized for use in the flow system. Use of acid extraction requires that the final sample be buffered to pH 7.75 to optimize the luciferin-luciferase enzyme reaction.

Luciferin-luciferase extracted from dessicated firefly lanterns was obtained in purified form from E. I. Dupont de Nemours and Company, Inc., or was prepared in the laboratory. Use of highly purified luciferase requires adding synthetic luciferin and results in 100-fold increase in test sensitivity.

Purified luciferase is rehydrated in 0.25 M TRIS buffer containing 0.01 M $MgSO_4$ and 0.001 M Cleland's reagent (dithiothreitol). When Cleland's reagent is included, stability of rehydrated luciferase increases from 4 hours at 10°C to 8 hours at 10°C. At least 60% of functional activity can be preserved at 10°C in the dark for 48 hours by adding 0.001 M EDTA. Since luciferase is an enzyme, precaution must be taken to avoid denatuation due to overheating or exposure to marked temperature fluctuations. For ATP assay, the enzyme preparation should be brought to 20°C. To minimize inherent light from the luciferase mixture, rehydrated enzyme should be incubated at room temperature for 30 minutes before assay. Remaining inherent light should be less

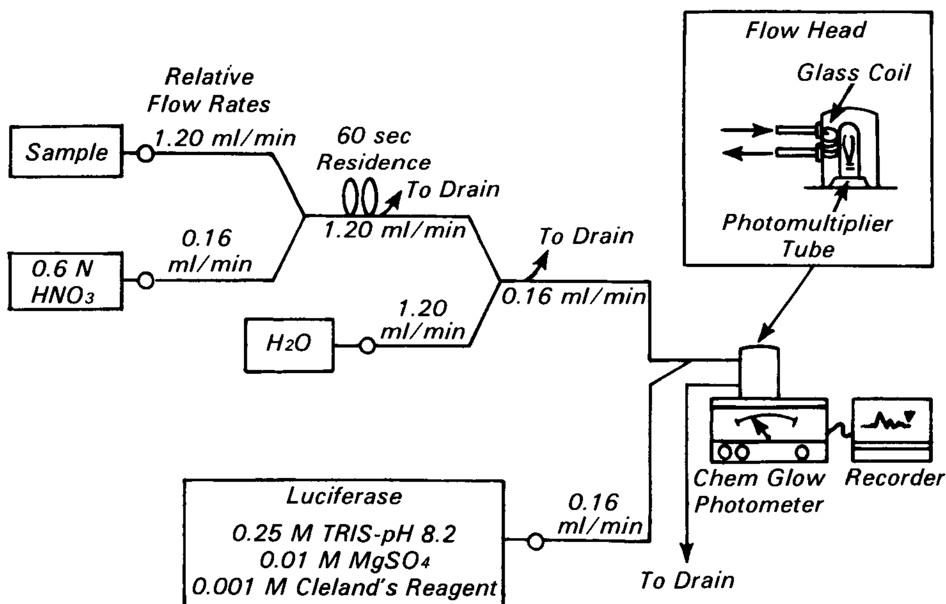


Figure 1. *Schematic of automated firefly luciferase flow system for detecting bacterial ATP including nitric acid extraction and subsequent dilution.*

*Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

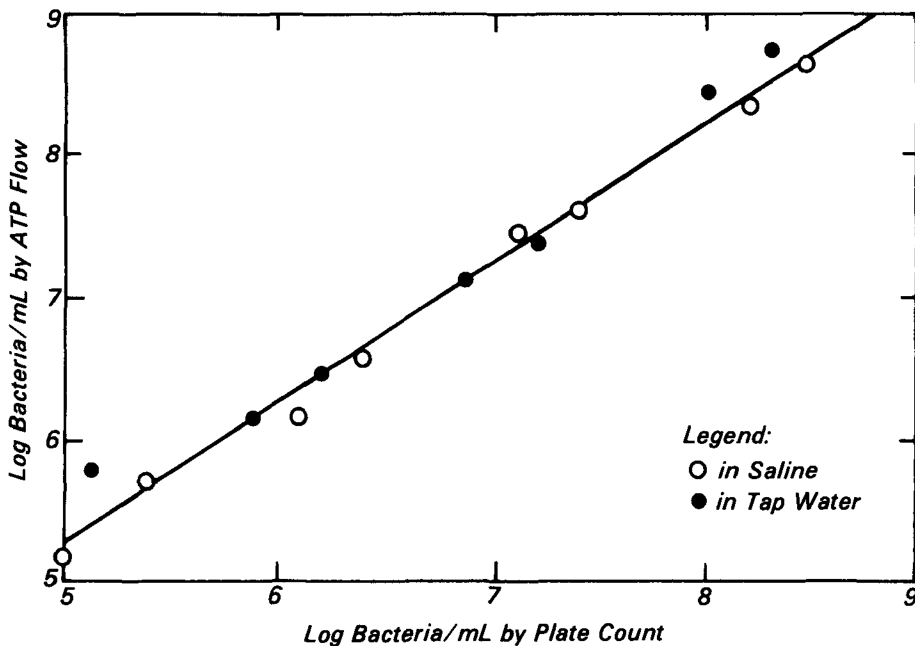


Figure 2. *Concentration curve of E. coli in saline and in tap water; bacteria/mL by ATP flow vs. plate count.*

than 10% after 30 minutes, and an appropriate control cuvette will indicate the inherent light correction needed.

ATP standards were prepared with the use of purified chemical ATP (Sigma Chemical Co.) in 0.001 M EDTA and 0.01 M $MgSO_4$. The purified ATP is used for daily quality control and for preparing ATP standard curves for determining sample ATP concentrations.

Loss of sample bacterial ATP due to hydrolysis by nitric acid extractant was minimized by 50% dilution after 60-second extraction, followed immediately by reaction with the luciferase enzyme system.

Bacterial Concentration Procedures

To detect contaminating levels of microorganisms in potable water using the firefly luciferase ATP assay, the organisms must first be concentrated to achieve at least the minimum sensitivity cell concentration of 10^5 cells/mL. For samples containing 500/mL, 200-fold or greater concentration must be achieved. If concentrated by membrane filtration, the cells must be recovered intact and undamaged in a small volume of water for subsequent assay using the ATP flow system. Recovery in a small volume of water rather than on a membrane surface would permit measurement of the bacterial population by standard plate count, Coulter count, or other method to confirm ATP assay results.

Additionally, the concentration procedure must be able to process large volume samples (at least 10 L) in a time period compatible with the desired sampling frequency without damaging the cells or significantly altering the cell ATP content.

Potential concentration techniques were tested for concentration of known densities of *E. coli* from sterile, deionized water. After a minimum of three test runs, if a concentration factor of 10 could not be achieved with 50% recovery of bacteria, further work with the technique was abandoned. The percentage of bacteria recovered in the final concentrate was determined by enumerating the bacteria before and after concentration. Standard plate counts, ATP assays, Coulter counts, luminol iron porphyrin assays, or a combination of these methods was used to determine cell concentration.

Four basic concentration techniques were tested; for some techniques, more than one type of unit was evaluated. The techniques tested were: centrifugation; direct in-line filtration, flat-surface membrane filtration, and hollow fiber filtration.

Centrifugation

Continuous flow centrifugation provided large sample volume processing with about 75% bacterial recovery, but concentration factors were very low. Mechanical manipulations involved in collecting the bacterial concentrates were not suitable for automation, so work on this procedure was discontinued.

Direct In-Line Filtration

Sample under pressure was passed through a 0.45 μm cellulose acetate filter (Swinnex filter) unit to concentrate bacteria. After sample filtration, attempts to backwash with small volumes of deionized water to resuspend cells for the ATP assay were unsuccessful. Adequate seals to prevent leakage could not be made using Nucleopore polycarbonate filters.

With 10-fold concentration of bacterial cells, attempts to assay ATP using direct extraction of ATP after filtration resulted in an average of only 18% recovery (Table 1).

Table 1. *Test Results Using Millipore In-Line Filter Concentration with In-Line Extraction*

Sample	In-Line Extraction (μg ATP/mL)	External Extraction (μg ATP/mL)	% Recovery with In-Line Extraction
1	3.3×10^{-4}	1.9×10^{-3}	17
2	3.5×10^{-4}	1.85×10^{-3}	19
3	2.5×10^{-4}	1.8×10^{-3}	14
4	8.1×10^{-5}	1.08×10^{-3}	7
5	1.0×10^{-4}	3.3×10^{-4}	30

Flat-Surface Membrane Filtration

Sample concentration by membrane filtration systems that use flat surfaces and high sample flow velocities parallel to the membrane filter surface was explored. The sample is concentrated by volume loss through the filter, but particles are retained in a decreased sample volume (the retentate) (Figure 3). Sample flow parallel to the membrane surface reduces particle buildup on the membrane surface. Three systems utilizing this principle were tested; these were Uni-pore Radial and Stirred Flow Cells (Biorad Laboratories), Sartorius Ultrafiltration System (Sartorius Corporation), and the Pellicon Cassette Molecular Filter (Millipore Corporation).

All flat-surface systems yielded poor results when tested with bacterial suspensions, and recoveries were less than 50%. A backwash step was added in an attempt to improve bacterial recoveries from such systems. Backwashing alone proved to be insufficient to recover the bacteria, and the volume of backwash required often negated any concentration effect. Adding Triton X-100 (a nonionic detergent) or 0.1% Rhozyme (proteases and glucosidases mixture isolated from *Aspergillus oryzae*) to samples as a filtration aid did not consistently improve bacterial recovery with any of the flat-plate systems, and concentration factors greater than 10 could not be achieved (Table 2).

Hollow-Fiber Membrane Filtration

Hollow-fiber membrane filters also incorporate sample flow parallel to the membrane surface, but the membrane configuration is tubular and has pores of controlled dimensions (Figure 4). The sample solution is pumped through a bundle of the hollow fibers, and particles larger than the pore size of the particular hollow-fiber filter in use are retained. Particle concentration occurs when low molecular weight solutes and water pass through the membranes into the filtrate. The sample volume is recirculated to achieve further volume reduction and consequent sample concentration. Backwashing was also necessary with the hollow-fiber membrane filter systems. Two hollow-fiber member filter systems, the Bio-Fiber 80 Mini-plant (Biorad Laboratories) and the Diaflow Hollow Fiber Concentrator (Amicon Corporation), were tested.

The Bio-Fiber 80 Mini-plant yielded up to 83% bacterial recovery at concentration factors of 15 to 250 with the use of repeated backwashing and reconcentration steps. However, the unit was removed from the market and is no longer available. The Amicon Diaflow Hollow Fiber unit, with a surface area of 1,000 cm^2 and 100,000-molecular weight (m.w.) cutoff, yielded nearly 100% bacterial recovery at 10-fold concentration without backwashing (Table 3). When the concentration factor was raised to 100 fold, recoveries dropped to only 32%. A dual cartridge unit with 50,000-m.w. cutoff cartridges, operated to provide 100-fold concentration without backwashing, gave inconsistent recoveries that ranged from 43% to 100%. After modification to permit backwashing, a 10,000-cm^2 cartridge yielded recoveries of 53% (single backwash) to 88% (three backwashes).

Hollow-fiber concentrators modified for backwash capability proved to be the only concentration systems tested that allowed greater than 200-fold concentration of bacteria with adequate recovery of the organisms (88%). The hollow fibers of the Amicon Diaflow unit are made of noncellulosic polymers that should be durable for a period of months. For extended use, cleanup steps may be necessary to prevent the buildup of bacteria within the fibers. Although a 30-minute rinsing procedure using sterile deionized water was effective between samples during testing, use of 0.1 NaOH followed by sterile deionized water rinse may be necessary during long-term use.

Manpower constraints forced Goddard Space Flight Center to discontinue projects not directly related to the space mission. They were, therefore, unable to evaluate and develop a satisfactory concentration system to go with the flow system for the ATP assay. Although preliminary results appeared promising, extended testing of the hollow-fiber concentrator (backwash modified) using

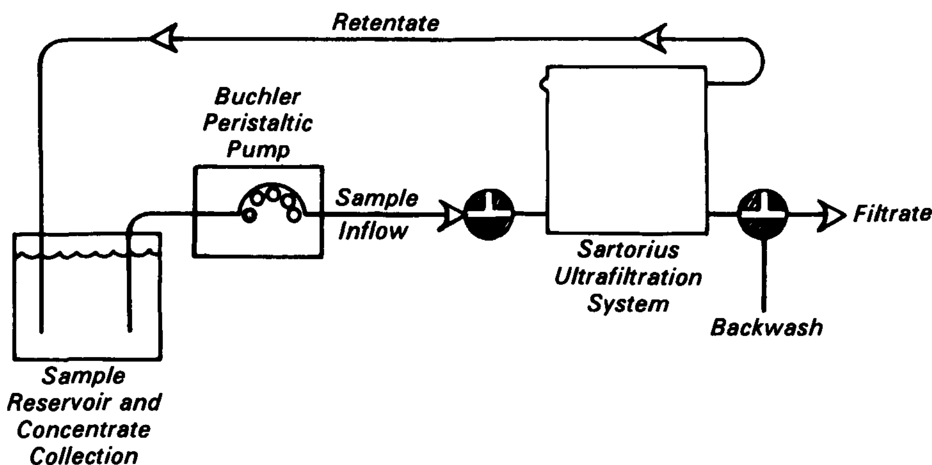


Figure 3. *Schematic of Ultrafiltration System using tangential flow and backwash. System is diagrammed in concentrating mode; valves are rotated 90° for backwashing provided by pressurized tap source of sterile, deionized water.*

Table 2. *Test Results Using Sartorius Ultrafiltration System with Tangential Flow and Backwash*

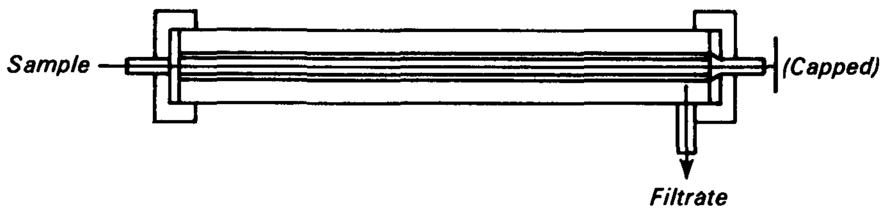
*Concentration Factor**	*% Recovery*	*Filtration Aid*
2	79	----
4	63	----
10	21	----
10	90	With TX
10	92	With Rhozyme
5	50	Cellulose Acetate Filter, TX
5	80	Polycarbonate Filter, TX

**Each factor represents a separate test.*

drinking water samples is needed before final recommendation can be made. If performance of the concentrator proved satisfactory with potable water samples, additional testing of the system, including the automated flow ATP assay would be necessary to establish overall system performance, sensitivity, reproducibility, and reliability.

The full report was submitted in fulfillment of Interagency Agreement No. EPA-IAG-D6-0982 by NASA/Goddard Space Flight Center under the sponsorship of the U.S. Environmental Protection Agency

Concentration Mode:



Backwash and Collection Mode:

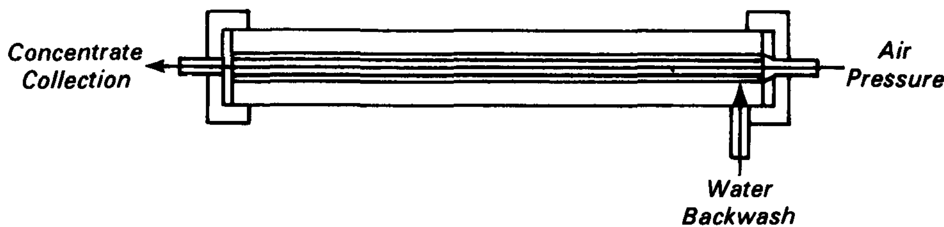


Figure 4. *Schematic of Amicon hollow fiber cartridge modified for backwash capability.*

Table 3. *Test Results with the Use of Amicon Hollow Fiber Cartridge with Backwash*

Cartridge Size Filter Area (cm^2)	*Maximum Concentration Factor*	*Test Procedure*	*% Recovery + σ**
1,000	*60*	*Backwash*	*95 ± 5*
10,000	*600*	*Backwash*	*53 ± 21*
		Backwash, Refilter, Backwash	*83 ± 14*
		Backwash, Refilter, Backwash, Refilter, Backwash	*88 ± 12*

**Standard deviations calculated on the basis of test runs.*

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***Donald Reasoner** is the EPA Project Officer (see below).*
The complete report, entitled "Firefly Luciferase ATP Assay Development for Monitoring Bacterial Concentrations in Water Supplies," (Order No. PB 81-163 271; Cost: $8.00, subject to change) will be available only from:
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