Rapid quantification of viable bacteria in water using an ATP assay

JiYoung Lee and Rolf A. Deininger

Information on the total number of bacteria in water has played a key role in assessing changes during water treatment. Based on the results of bacteriological tests, water treatment works can optimize treatment processes. If the treatment process is not adjusted in time, there may be insufficient treatment or breakthough, resulting in contamination of the entire water distribution system. Thus, rapid determination of the total bacterial count in drinking water is critical to the operators of treatment plants and distribution systems since it allows corrective measures to be taken immediately. Such measures include increasing the disinfectant dose or flushing and disposing of water with high bacterial counts. Operators can also detect whether a water supply system has been intentionally contaminated with biological agents if bacterial count is determined.

Heterotrophic plate count (HPC) is a procedure used to estimate the number of heterotrophic bacteria that form colonies on agar plates. The HPC analysis presently employed takes seven days and is not amenable to operator intervention. Techniques available to rapidly detect bacteria include fluorescence microscopic methods, detection of specific metabolites, antibody methods, and DNA-based methods.[1] However, many of these methods are expensive; require an enrichment step, sophisticated equipment, and expertise; are not able to determine viability; and are not suitable for routine analysis.

The determination of adenosine triphosphate (ATP) with a bioluminescence assay may solve the problems encountered in the detection of viable bacteria. The main function of the assay is to quantitate ATP, the important compound in metabolism that is found within all living cells. The assay is based on the reaction between the luciferase (enzyme), luciferin (substrate), and ATP. Light is emitted during the reaction,[2] and can be measured quantitatively and correlated with the ATP quantity extracted from bacteria.

This applications note describes the ability of the ATP assay to determine the total bacterial count in water in minutes with a handheld luminometer. For quality control purposes and to test the accuracy of the ATP and HPC tests, enumeration of the bacteria in a water sample was done using two epifluorescence microscopic methods: acridine orange direct count (AODC) and direct viable count (DVC). Water samples came from local, national, and international locations, which were selected based on their proximity to the laboratory, cooperating water utilities, and the authors. The ATP assay was also used to determine whether the method is applicable for ascertaining the bacterial levels in swimming pools and whirlpools. Decisions regarding the closing and reopening of a pool can be made promptly because bacteriological data are obtained in minutes. Pool safety can be increased since disinfectants can be added in a timely manner.

Experimental

Filtration of water samples

More than 120 drinking water samples were collected from taps in distribution systems and drinking fountains in the United States and abroad. For the swimming pool and whirlpool samples, 40 samples were collected in Washtenaw County (Michigan) during the summer when the pools were in use. A sterile syringe was used for drawing the water samples. The testing volumes of the water were between 0.1 and 20 mL, based on the expected number of bacteria in the sample. A Filtravette™ (**New Horizons Diagnostic Corp.** [**NHD**], Columbia, MD), which is a combination of a filter and a cuvette with a pore size of 0.45 µm, was placed into a 13-mm Swinex filter holder **(Millipore Corp.,** Bedford, MA). The filter holder was screwed onto the syringe and the water sample was pushed through the filter (*Figure 1*).

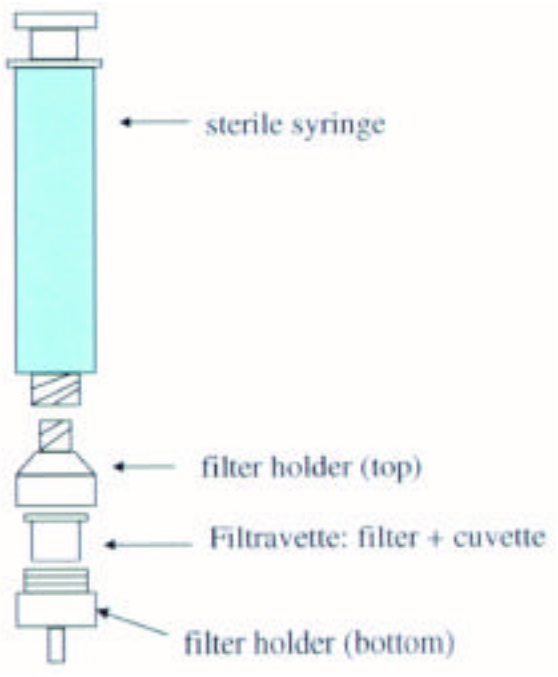

Figure 1 *Filtration of a water sample. The water sample is pressed through a Filtravette, which is inserted into a filter holder.*

ATP bioluminescence

The Filtravette was removed from the filter holder after filtration of the water sample and was placed onto a sterile blotting paper. A somatic cell releasing agent (**NHD**) was used according to the manufacturer's instructions to lyse all nonbacterial cells and to release ATP. With a specially converted 3-mL syringe, air pressure was applied to remove the nonbacterial ATP through the filter. At this stage, the Filtravette retained bacteria on top of the membrane filter, and the bacterial ATP remained within the bacterial cell membranes throughout this step of the procedure. The Filtravette was inserted into the microluminometer (model 3550, **NHD**) and the bacterial cell releasing agent was then added to lyse the bacterial cells retained on the surface of the filter. The released bacterial ATP was mixed with 50 µL of luciferin–luciferase (**NHD**). The light emission was recorded after a 10-sec integration of the light impulses; the unit was called a relative light unit (RLU).

The result was expressed as RLU/mL by dividing the RLU values by the filtered water volume. The detection limit and sensitivity of the luminometer was tested with a serially diluted ATP solution (**NHD**). Distilled deionized water was used for the dilution. The activity of the luciferin–luciferase was checked by using an ATP standard (**NHD**). The RLUs are proportional to the amount of ATP, and the amount of ATP is proportional to the number of viable bacteria.

Conventional methods

The total number of nonviable and viable bacterial cells was determined from formaldehyde-fixed (2%, vol/vol, final concentration) samples using the AODC method.[3] The bacterial cells were stained with acridine orange (0.01%, wt/vol, **Fluka,** Buchs, Switzerland) after filtration onto a 0.2-µm pore size black polycarbonate membrane filter (**Poretics,** Livermore, CA). Cells were enumerated at a magnification of 1000× with a Provis epifluorescence microscope (**Olympus Optical Co.,** Japan) equipped with a mercury arc lamp and 460–490 nm excitation filter. The number of bacteria was counted in 10 microscopic fields using three subsamples and was then averaged. The number of bacteria per milliliter of sample was calculated using the equation in Ref. 3.

The DVC method was performed by incubating the water samples with an antibiotic (nalidixic acid, 10 mg/L, **Sigma,** St. Louis, MO) and nutrients (yeast extract, 0.005% [wt/vol], **Difco,** Detroit, MI) for 24 hr at 20 °C. Nalidixic acid suppresses cell division without affecting other cellular metabolic activities. Thus, after incubation with nalidixic acid, the viable cells continued to metabolize nutrients and become elongated. After incubation, the fixation, counting, and calculation of elongated bacteria were via the AODC method.

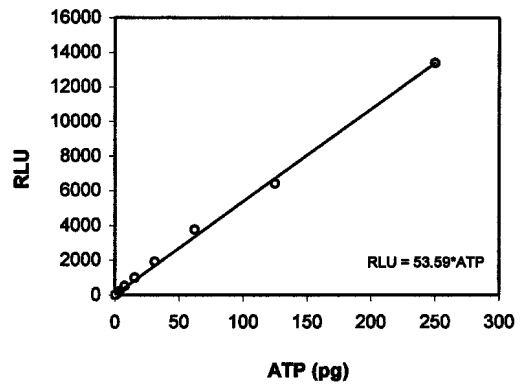

Figure 2 *The detection limit and sensitivity of the ATP assay with a microluminometer. The range of ATP concentration was between 0.2 pg and 250 pg (r = 0.999). (Copyright 1999 by Food and Nutrition Press, Inc., Trumbull, CT.)*

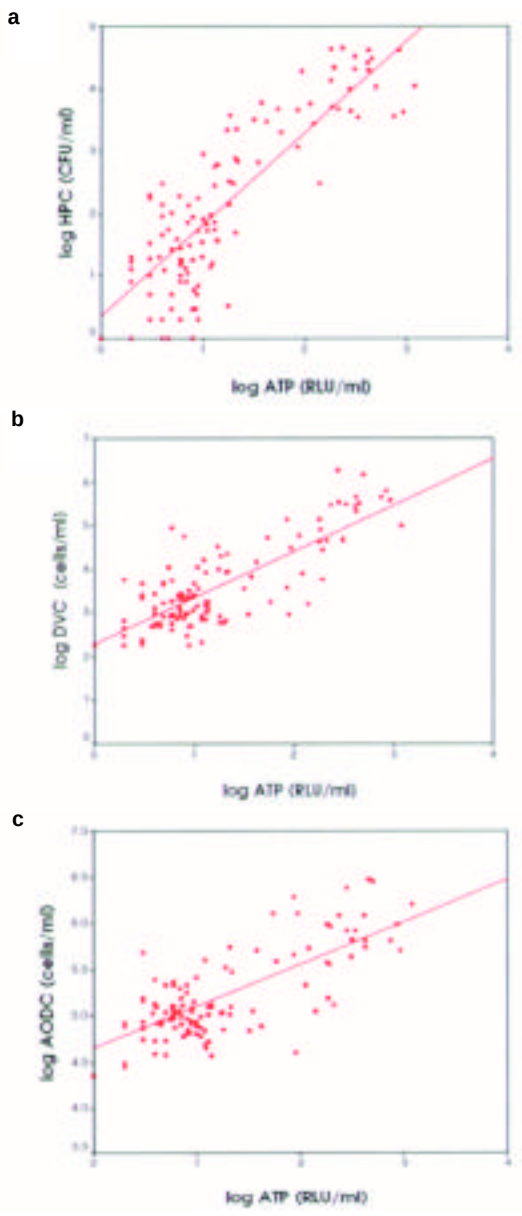

Figure 3 *The relationship between ATP and other conventional methods. The data were obtained from drinking water samples. a) ATP vs. HPC. The regression equation is: log HPC = 0.35 + 1.47 * log ATP (n = 114, r = 0.84). b) ATP vs. DVC. The regression equation is: log DVC = 2.29 + 1.06 * log ATP (n = 120, r = 0.804). c) ATP vs AODC. The regression equation is: log AODC = 4.6 + 0.46 * log ATP (n = 120, r = 0.68). (Copyright 1999 by Food and Nutrition Press, Inc., Trumbull, CT.)*

The HPC was determined for each water sample in triplicate using R2A medium (**Difco**). The bacterial colonies were counted after an incubation period of seven days at 28 °C. All of the testing methods (ATP bioluminescence, AODC, DVC, and HPC) were per-

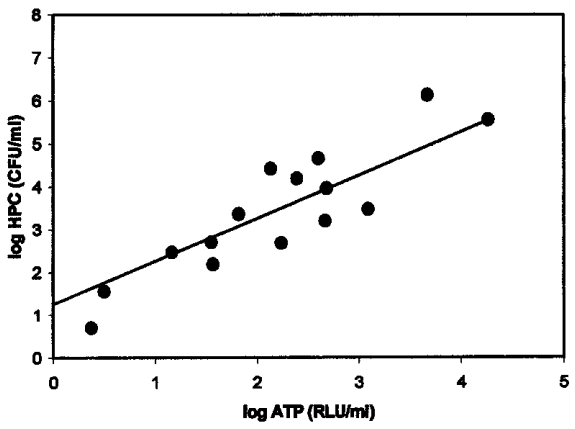

Figure 4 *The relationship between ATP and HPC from swimming pool and whirlpool samples (n = 40, r = 0.92, log HPC = 1.25 + 1.01 * log ATP). The data points less than 0 (log value) are not shown in this graph.*

formed three times. For the swimming pool and whirlpool samples, bacterial quantification was done by the ATP bioluminescence and HPC methods.

Results and discussion

Drinking water samples

To prove the applicability of the ATP bioluminescence method, the data obtained from the ATP bioluminescence, AODC, DVC, and HPC methods were compared.

The detection limit of ATP was determined with high accuracy (r = 0.999) (*Figure 2*). It showed that the microluminometer was able to determine ATP as low as 0.2 pg. It is known that the average ATP content in one bacterial cell is about 10^{-15} g (1 fg).[4] Thus, 0.2 pg corresponds to about 200 bacterial cells, which is the sensitivity of the ATP assay in terms of bacterial cell numbers, and is approximately equivalent to 4 CFU/mL. In drinking water, the bacterial level of concern is above 500 CFU/mL, according to the U.S. EPA. Therefore, the level of bacteria can be accurately detected using the ATP bioluminescence method.

The data demonstrated that there are high correlation coefficients between ATP, HPC, DVC, and AODC and, among those, the highest correlation coefficients were found between ATP and HPC (0.84) and ATP and DVC (0.80) (*Table 1*).

The relationship between ATP and HPC is shown in *Figure 3a*. In a number of cases in which a cooperating utility sent water samples, the authors transmitted the expected HPC results by e-mail or fax on the day the water samples were received and analyzed. After the incubation period, the expected HPC and the measured HPC were compared. The expected HPC was calculated with the regression equation based on the average ATP (RLU/mL). For instance, if the average value is 10 RLU/mL, the estimated HPC is 47 CFU/mL with a confidence interval (CI) between 32 (lower bound) and 71 (upper bound). In all but a few cases, the prediction and the measured HPC by the utility agreed. The regression equation is log HPC = 0.35 + 1.470 * log ATP (n = 114).

A factor that may affect the linear relationship between ATP and HPC (CFU) is the presence of injured bacteria, which cannot grow on agar plates. In treated drinking water, bacteria are exposed to disinfectants, and a large proportion of the bacterial population became injured.[5] Injured bacteria are viable but are not able to form colonies on agar plates. However, the injury does not directly affect the presence of intracellular ATP.[6] The relationship between ATP and DVC is shown in Figure 3*b*. Theoretically, when ATP is zero with no detectable light emission, the DVC should also be zero. The intercept of the regression equation of 2.29 (log scale) (i.e., 193 cells) indicates the detection limit of the procedure; it cannot be detected when the cell number is less than 193. This value is very close to 200, which was obtained from the determination of the detection limit with ATP solution. The regression equation in Figure 3*c* implies that when no detectable ATP occurs in the sample, a relatively large number of nonviable and viable cells are present and viewable using the acridine orange direct count method.

Pool samples

With the swimming pool and whirlpool samples, the results from the ATP assay correlated well with the results from the conventional plate count method (*Figure 4*).

Conclusions

The results show that ATP bioluminescence assays provide a rapid means of enumerating total numbers of viable bacterial cells. The miniaturized ATP bioluminescence method has been validated against the conventional plate method, direct viable count method, and acridine orange direct count method for the determination of heterotrophic bacteria in drinking water samples. The bacterial level of swimming pools can be also determined in minutes using the ATP bioluminescence method. The ATP assay was found to be rapid, sensitive, and simple, and can be done on-site with a portable power supply. At $3 per sample, it is also less expensive than other existing methods. The volume of water needed for filtration is small (0.1–20 mL).

In addition, the technique can be applied in many other fields, such as the bottled water industry; the food industry, including the beverage and brewing industries; and in wastewater treatment plants. Other applications include testing water for military troops when they are deployed to an area in which the potability of the available water is in question. The method can also be used to examine ultrapurified water employed in the pharmaceutical, biotechnology, and electronics industries. Due to the small size of the luminometer and the high sensitivity of the assay, the assay can also be used as a tool to monitor the biological conditions of water in a spacecraft.

Table 1
Correlation coefficients between ATP, HPC, DVC, and AODC methods for detecting bacterial levels in drinking water*

	ATP	HPC	DVC	AODC
ATP	1	—	—	—
HPC	0.84	1	—	—
DV C	0.80	0.75	1	—
AODC	0.68	0.66	0.73	1

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Dr. Lee is Research Investigator, and Dr. Deininger is Professor, Department of Environmental Health Sciences, University of Michigan, 109 S. Observatory, Ann Arbor, MI 48109, U.S.A.; tel.: 734-736-4133; fax: 734-764-9242; e-mail: jylee@umich.edu, rad@umich.edu.