Chemiluminescent Method for Detecting Microorganisms in Water

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■ Two automated systems were devised for the detection of microorganisms contained in water samples. The detection principle was based upon the measurement of light emission resulting from the interaction of microbial cells with alkaline luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in the presence of sodium perborate or sodium pyrophosphate peroxide. Each automated system detected 10^3 to 10^5 viable bacterial cells per ml. of water seeded with either *Aerobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, or *Serratia marcescens*. Maximum light emission occurred within 1 minute of the admixture of bacterial cells with the luminol reagent. The automated systems displayed linearity in response to varying cell numbers of each test organism. The results suggest that luminol chemiluminescence may have potential application for the microbial examination of water.

The importance of water as a potential mode for the transmission of pathogenic microorganisms to man is well known. As a possible consequence of the fecal contamination of water, certain enteric pathogens—e.g., *Salmonella paratyphi B*, *Salmonella typhimurium*, *Salmonella typhosa*, *Shigella flexneri*, and *Shigella sonnei*—may be found in polluted waters (Heukelekian, 1953). However, in view of the limited numbers of enteric pathogens present, as compared to the quantities of nonpathogenic coliforms and enterococci found in contaminated water, the examination of water for microbial pollution involves the use of methods designed to detect the latter types of organisms (Kabler, 1962). Of the techniques available, the coliform density method is the one recommended officially (American Public Health Association, 1965). Although the coliform density method is used to establish the probable fecal contamination of water, plate counts are employed frequently to determine the total number of viable cells (pathogens and nonpathogens) contained in water. Both methods involve the cultivation of microorganisms on a suitable growth medium for a given period of time. Consequently, these methods are cumbersome with respect to the handling and processing of numerous water samples and do not provide immediate information regarding the presence of microorganisms in water.

Since water is a constantly changing system, a need exists for automated instrumentation capable of the routine monitoring of its quality (Elving, 1967). A sensitive and rapid automated technique for the detection of microorganisms in aqueous suspensions was described recently (Oleniacz, Pisano, *et al.*, 1967). The detection principle was based upon the measurement of light emission resulting from the microbial activation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) chemiluminescence in the presence of sodium perborate. Although the system was designed initially for the detection of biological aerosols, the method may be equally adaptable to the determination of the microbial content of water. In the present study, improvements were made in the automated luminol-perborate system and an additional luminol system, containing sodium pyrophosphate peroxide, was automated for the detection of microorganisms.

Experimental

Apparatus. The automated luminol-peroxide system was composed of the following instruments: AutoAnalyzer Sampler II and proportioning pump (Technicon Corp., Ardsley, N. Y.); light-tight reaction chamber containing a spiral-type glass flow cell (flow capacity, 4.0 ml. per minute) mounted adjacent to an RCA 4473 photomultiplier tube; line-operated photometer (Model 520 M, Photovolt Corp., New York), having a photometric scale calibrated in arbitrary light units; X-Y recorder (Moseley Model 7030AM, Hewlett-Packard, New York), operated at a sweep time of 50 seconds per cm. on the x-axis.

The instrumentation employed for the automation of the luminol-perborate system included: AutoAnalyzer Sampler II and proportioning pump; light-tight reaction chamber containing a glass cylindrical flow cell (previously described by Oleniacz, Pisano, *et al.*, 1967). The flow cell has a capacity of approximately 3.0 ml. and was mounted adjacent to an RCA 1P28 photomultiplier tube; line-operated photometer (Model 520 SP, Photovolt Corp.); microvolt-ammeter (Model 425 A, Hewlett-Packard); strip-chart recorder (Technicon-Bristol, Technicon Corp.), modified by the addition of a Bristol Model D reference voltage source. The paper drive of the recorder was at the rate of 0.62 cm. per minute.

Reagents. LUMINOL-PEROXIDE SYSTEM. The stock luminol solution contained 0.37 gram of luminol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per liter of 0.12*N* NaOH. To insure reproducible light intensities, the solution requires aging for 1 hour and then must be used within 5 hours. The stock peroxide solution contained 20 grams of $Na_4P_2O_7 \cdot 2H_2O_2$ and 0.25 gram of disodium ethylenediaminetetraacetate (EDTA) per liter of distilled water.

LUMINOL-PERBORATE SYSTEM. Luminol reagent was prepared by dissolving 0.106 gram of luminol and 3.6 grams of anhydrous dextrose in 20 ml. of 0.2*N* NaOH. The solution was then diluted to 1 liter with distilled water. The perborate stock solution consisted of a 1.5% (w./v.) solution of $NaBO_3 \cdot 4H_2O$ in distilled water.

Microorganisms. *Escherichia coli* is known to be the principal nonpathogenic coliform found in water contaminated with fecal material. Members of the *Pseudomonas* and aerogenes-like groups of bacteria can also be found in water (Bonde, 1966; Clark, Geldreich, *et al.*, 1957; Hoadley and

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McCoy, 1965; Ringen and Drake, 1952), although their presence does not necessarily signify gross fecal pollution of water. In addition to *E. coli* and the enteric pathogen *Salmonella typhimurium*, the following gram-negative bacteria were therefore used as test organisms in the present study: *Aerobacter aerogenes*, *Proteus mirabilis*, *Pseudomonas fluorescens*, and *Serratia marcescens*.

Trypticase Soy Broth (Baltimore Biological Laboratories, Md.) cultures of each organism were incubated for 24 hours at their respective optimal growth temperatures. Following the growth period, the bacterial cells were collected by centrifugation, 12,000 G for 15 minutes at 2° C. The harvested cells were washed three times with sterile distilled water and resuspended in water to original volume.

Water Samples. Seeded water samples were prepared by making appropriate dilutions of each of the test organisms in distilled water. The amount of viable cells present in the samples was determined by plating each organism on Trypticase Soy Agar.

Procedure. LUMINOL-PEROXIDE SYSTEM. Figure 1 is a schematic diagram of the automated system devised for the detection of microorganisms. The sampler cups, in sequence, contained seeded water sample, control (sterile distilled water), seeded water sample, and control. The control functioned as a wash for the removal of the seeded water sample from the reaction flow cell, and provided a base line of the background luminescence of the luminol reagent. The sampler was operated at the rate of 30 samples per hour; the seeded water samples were thus assayed at the rate of 15 per hour. The sample (either seeded water or control) was drawn from the sampler cup into a pump tube at a flow rate of 0.6 ml. per minute. The latter line was joined to an air pump tube (flow rate, 0.32 ml. per minute) by means of a D1 cactus connector. Luminol reagent (1.2 ml. per minute) was introduced into the system, and pyrophosphate peroxide added through pump tubing at the rate of 0.42 ml. per minute. The luminol and peroxide lines were connected by an HO cactus, and the combined reagent segmented by air pumped at the rate of 0.32 ml. per minute. Blending of the luminol and peroxide reagents occurred in a single mixing coil (1 minute mixing time). The mixed reagent and the sample were then led separately into the light-tight reaction chamber where they were connected by a "t" (size Al) and immediately conducted to the flow cell. The interaction of the bacterial sample with the luminol reagent resulted in instantaneous light emission. The photomultiplier, coupled to the photometer, detected the light emitted from the reaction. Tracings of the light intensities were made on the recorder.

LUMINOL-PERBORATE SYSTEM. A block diagram of the automated luminol-perborate system is shown in Figure 2. Sterile distilled water (control), 3.9 ml. per minute, was pumped

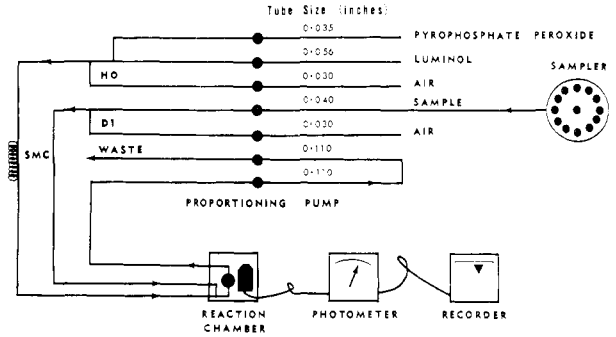

Figure 1. Schematic diagram of automated luminol-peroxide system for detection of microorganisms in water

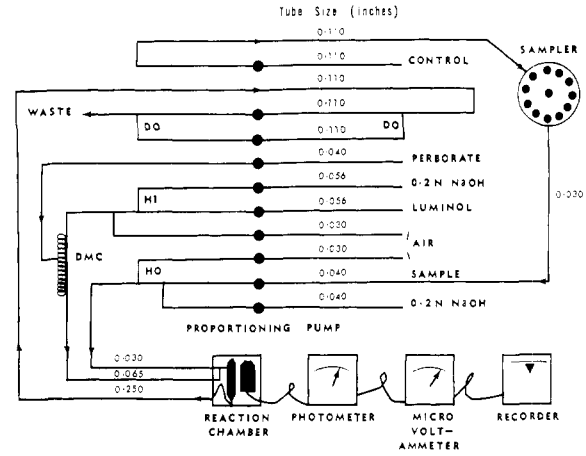

Figure 2. Schematic diagram of automated luminol-perborate system for detection of microorganisms in water

into the sampler wash chamber. The control water was then drawn into alternate sampler cups, and seeded water samples placed in the remaining cups. The operation of the sampler and flow rate of water samples into the manifold were the same as those described for the luminol-peroxide system. When the water sample was introduced into the manifold, it was diluted 1 to 1 with 0.2*N* NaOH and air-segmented (air rate, 0.32 ml. per minute). The sample was next conducted to the light-tight reaction chamber via transmission tubing at a rate of 0.32 ml. per minute. Active luminol reagent was prepared in situ in the following manner. The stock luminol solution was pumped into the system at a rate of 1.2 ml. per minute. This solution was then diluted 1 to 1 with 0.2*N* NaOH and air-segmented (air rate, 0.32 ml. per minute). The luminol, NaOH, and air lines were joined via an HI cactus and led to a glass double-mixing coil. In addition to entry and exit ports at opposite ends, the mixing coil had an entry port of capillary bore midway along the coil length. Sodium perborate, 0.6 ml. per minute, was added through the latter port. The mixed luminol-perborate reagent and the water sample were led separately into the light-tight reaction chamber. Immediately prior to entrance into the flow cell, the two lines were connected by a "t" (size Al). The light emitted from the chemiluminescent reaction was detected by the photomultiplier. The signal from the photomultiplier was fed to the photometer, passed through the microvolt-ammeter, and traced on the recorder. Results were quantitated by subtracting the background luminescence of the luminol reagent from the peak light units obtained for each of the seeded water samples.

As compared with the luminol-perborate system previously described (Oleniacz, Pisano, *et al.*, 1967), and the luminol-peroxide system reported here, the present luminol-perborate system had certain desirable features. Improvements were made which included the addition of NaOH to the test sample and the in situ preparation of working luminol reagent. With the latter improvement, the need for aging of the luminol solution, and the time restriction for its use, were eliminated. The addition of NaOH to the test sample represented a significant change in the system. Kenny and Kurtz (1951) reported that the reaction of an acid with alkaline luminol will result in light emission. Consequently, the luminol reacton may conceivably give false positive signals if acidic water is used as the test sample. Using the present luminol-perborate system, the latter possibility is unlikely since the water sample is made alkaline with NaOH prior to its reaction with luminol.

Results and Discussion

The criteria used for the evaluation of the applicability of analytical techniques to the measurement of water quality have been reviewed recently (Elving, 1967). The prime factors requiring consideration include speed of response, sensitivity, and selectivity. Speed of response is an essential characteristic for automated methods since remedial measures are frequently required for the maintenance of water quality. Automated instruments, therefore, should have a minimal time lag between the sampling step and the information-output steps (Elving, 1967). In the present study, approximately 4 minutes elapsed from the time of introduction of reagents and seeded water samples into the proportioning pump to the resulting reaction in the flow cell. With both of the automated luminol systems, maximum light emission occurred within 1 minute of the admixture of bacterial cells with the luminol reagent. A typical recording of the bacterial activation of luminol chemiluminescence, in the presence of perborate, is shown in Figure 3. Similar recordings were obtained with the luminol-peroxide system. Figure 3 shows that the chemiluminescence method was capable of excellent resolution; different degrees of light intensity were observed when small changes were made in the numbers of viable cells activating luminol.

The automated methods described here are nonspecific—i.e., identification of the bacteria responsible for activating luminol chemiluminescence is not provided, and the luminescence technique cannot differentiate quantitatively one organism in the presence of a mixed population. Each system exhibited linearity, however, in response to varying cell numbers of a given organism when a single bacterial species was used as the test organism (Figures 4 and 5). The curves obtained were reproducible, within experimental error. Examination of the linear plots revealed that, in general, peak light emission varied from organism to organism when equivalent cell concentrations of the different bacterial species were tested. Figures 4 and 5 also show that each system displayed good sensitivity for the detection of the bacteria contained in the seeded water samples.

Typical sensitivities of the two automated methods for bacterial detection are given in Table I. Although gram-negative bacteria were employed in the present study, the sensitivities of the automated systems for two gram-positive bacteria, *B. subtilis* and *S. aureus*, were included in the table for comparative purposes. Each of the automated systems detected approximately equivalent numbers (2.3 to 2.6 $\times$ 10^3 cells) of *B. subtilis*, whereas the luminol-perborate method was the more sensitive of the two techniques for the detection of *S. aureus*. In the case of gram-negative bacteria, fewer cells of the enteric coliform *E. coli* were required for the activation of light emission in the luminol-peroxide system as compared to the luminol-perborate system. Each method, however, detected less than 10^4 viable *E. coli* cells. When the enteric pathogen *S. typhimurium* was used as the test organism, the luminol-perborate system responded to 4.2 $\times$ 10^4 cells whereas 2.1 $\times$ 10^5 cells were required for luminol activation in the presence of peroxide. The two systems were equally sensitive towards either *A. aerogenes* or *S. marcescens*, detecting 7.2 $\times$ 10^4 and 1.9 $\times$ 10^4 cells, respectively. In comparison to the luminol-peroxide system, the perborate system displayed greater sensitivity for the detection of *P. mirabilis* and *P. fluorescens*.

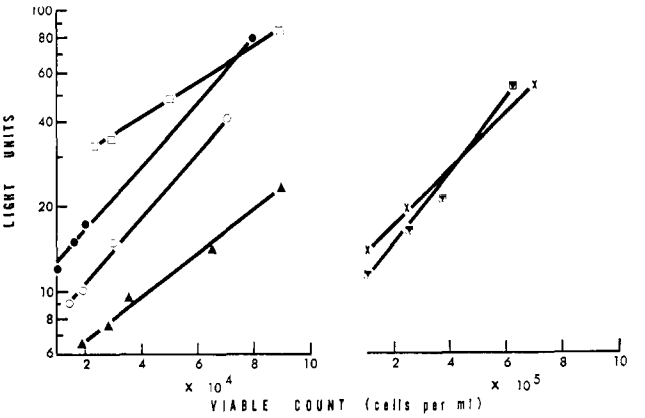

Figure 4. Linear response of luminol-perborate system to various bacterial species

Cell numbers shown were those contained per milliliter of seeded water. Symbols: □, *P. fluorescens*; ●, *E. coli*; ○, *P. mirabilis*; ▲, *S. marcescens*; X, *S. typhimurium*; ▼, *A. aerogenes*

The lower limit of sensitivity of both automated systems is dependent upon the magnitude of the background luminescence of the luminol reagent. Although the systems were capable of detecting 10^3 to 10^5 bacterial cells per ml. of water, it may be possible to increase the sensitivity by using appropriate techniques for concentrating bacteria in water samples.

Preliminary experiments revealed that the luminol chemiluminescence method was equally responsive to heat-killed bacterial cells, as compared to viable cells. Other organisms capable of activating luminol chemiluminescence include fungi (Oleniacz, Pisano, *et al.*, 1967) and protozoa (Rosenfeld, 1967).

Certain transition metals, such as iron, are known to be inducers of luminol chemiluminescence (White, 1961). An evaluation of the possible effects of metallic salts on the bacterial activation of luminol chemiluminescence was therefore

Table I. Comparison of the Minimal Number[a] of Various Bacterial Species Detected in the Two Chemiluminescence Systems Employed

Organism	Luminol + Peroxide[b]	Luminol + Perborate[c]
A. aerogenes	7.2 $\times$ 10^4	7.2 $\times$ 10^4
B. subtilis	2.3 $\times$ 10^3	2.6 $\times$ 10^3
E. coli	3.4 $\times$ 10^3	8.0 $\times$ 10^3
P. mirabilis	9.2 $\times$ 10^4	7.1 $\times$ 10^3
P. fluorescens	1.4 $\times$ 10^4	9.3 $\times$ 10^3
S. typhimurium	2.1 $\times$ 10^5	4.2 $\times$ 10^4
S. marcescens	1.9 $\times$ 10^4	1.9 $\times$ 10^4
S. aureus	2.6 $\times$ 10^5	8.0 $\times$ 10^4

[a] Viable cells per milliliter of seeded water.
[b] $Na_4P_2O_7 \cdot 2H_2O_2$.
[c] $NaBO_3 \cdot 4H_2O$.

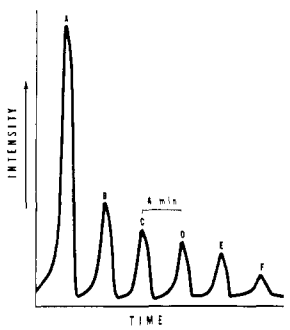

Figure 3. Typical recording of bacterial activation of luminol chemiluminescence in presence of sodium perborate

Symbols: A = 8.0; B = 2.6; C = 2.0; D = 1.6; E = 1.0; F = 0.8 $\times$ 10^4 viable *E. coli* cells per milliliter of water

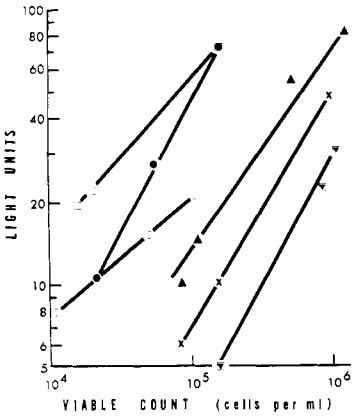

Figure 5. Linear response of luminol-peroxide system to various bacterial species

Cell numbers shown were those contained per milliliter of seeded water. Symbols: □, *S. marcescens*; ●, *E. coli*; ○, *P. fluorescens*; ▲, *A. aerogenes*; X, *P. mirabilis*; ▼, *S. typhimurium*

required. The luminol-perborate system was selected as the analytical method, and *E. coli* was chosen as the test organism for this phase of the investigation. The inclusion of ferric chloride in *E. coli* seeded water resulted in light emission greater than that observed when *E. coli* and ferric chloride were tested separately (Figure 6). Emitted light increased with increasing ferric chloride concentrations. However, the ferric chloride induction of chemiluminescence in the absence of bacteria suggests that false positive results may be obtained when monitoring natural waters for microbial content. Additional experimentation revealed that this difficulty could be overcome if a portion of the water sample was passed through a bacterial filter, and its ability to activate luminol chemiluminescence determined. The value obtained was subtracted from the value for the identical unfiltered water sample. It was therefore possible to assess the presence of bacteria in water samples containing significant amounts of iron. Ferrous chloride, to concentrations of $9 \times 10^{-4}M$, also enhanced the bacterial activation of luminol chemiluminescence (Figure 6). When used at a concentration of $9 \times 10^{-3}M$, ferrous chloride exerted a quenching effect on the bacterially activated light emission process. The light emitted was only slightly greater than that obtained with ferrous chloride alone. Calcium chloride, which was incapable of activating light emission, intensified the bacterial activation of luminol when

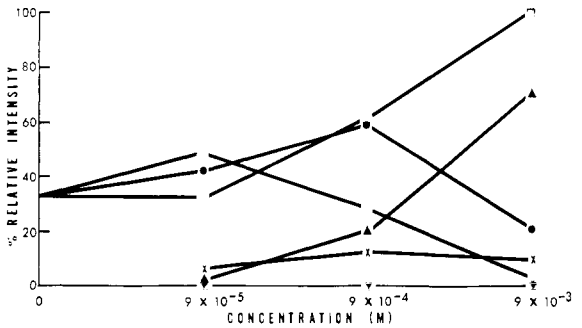

Figure 6. Effect of metallic salts on bacterial activation of luminol chemiluminescence in perborate system

Symbols: ○, $CaCl_2$ + *E. coli*; ●, $FeCl_2.4H_2O$ + *E. coli*; □, $FeCl_3.6H_2O$ + *E. coli*; X, $FeCl_2.4H_2O$; ▲, $FeCl_3.6H_2O$; ▼, $CaCl_2$. Salt concentrations were those contained in seeded water. *E. coli* cell count was 3.2×10^6 viable cells per milliliter of seeded water

present in the seeded water in a concentration of $9 \times 10^{-5}M$. Light emission then decreased and complete quenching occurred at a calcium chloride concentration of $9 \times 10^{-3}M$ (Figure 6). Although two of the metallic salts tested had a quenching effect on the bacterially activated light emission process, the latter findings do not necessarily present an insurmountable obstacle for the use of the chemiluminescence method in water pollution studies. Conceivably, the sensitivity of the system may be enhanced by diluting the test sample with distilled water, followed by concentration of the microbial cells through filtration techniques.

In many instances, the choice of an analytical method for water pollution studies is dependent upon the type of water to be examined—i.e., raw water reserves, drinking water, or rivers and streams. The analytical technique reported here might be useful for monitoring water samples, or other aqueous solutions, in situations where a rapid indication of the possible presence of microorganisms is required. The results of the present study suggest that luminol chemiluminescence may have value as an adjunct or alternative to the plating techniques presently used in the examination of water for microbial content.

It is interesting to note that luminol chemiluminescence has also been employed for the detection of cyanide (Bianchi, Demichelli, *et al.*, 1961; Musha, Ito, *et al.*, 1959), organophosphorus nerve gases (Goldenson, 1957; Matkovic and Weber, 1964), and organophosphorus pesticides (Weber and Matkovic, 1964). Thus, it may be possible to apply automated luminol chemiluminescence to the detection of toxic materials, such as these, in water or air. Additional studies would be desirable to ascertain further the value of luminol chemiluminescence in the environmental sciences.

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