APPLICATIONS OF CHEMILUMINESCENCE TO BACTERIAL ANALYSIS

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A rapid chemical method for determining bacteria populations would have obvious advantages over the 2-day microbiological plate count method in many areas of biological testing. Therefore a study was undertaken to investigate the use of the luminol chemiluminescence method for rapid detection of bacteria in a variety of applications. Previous studies of the method (Oleniacz et al., 1970; Marts and Wilkins, 1970) indicated that it should have greater potential than the adenosine triphosphate (ATP) bioluminescence method (Chappelle and Levin, 1968; McElroy et al., 1969) for monitoring bacteria levels in field applications where adequate laboratory facilities are not available.

The principle of the luminol chemiluminescence method for detecting bacteria is based on microbial activation of the oxidation of the luminol monoanion by hydrogen peroxide. The general reaction mechanism is shown in figure 1. In an aqueous alkaline solution, luminol (3-aminophthalhydrazide) is reported (Hodgson and Fridovich, 1973) to be in the form of the monoanion, which can be rapidly and energetically oxidized by hydrogen peroxide in the presence of the iron porphyrins contained in microorganisms (Erley et al., 1962; White and Roswell, 1972; Nikokavouras and Vassilopoulos, 1971). The intermediate products of oxidation could include free radicals such as hydroxyl, luminol, and O_2^- as well as luminol endoperoxide (Hodgson and Fridovich, 1973; Erley et al., 1962; White and Roswell, 1972; Nikokavouras and Vassilopoulos, 1971; Lee and Seliger, 1965). A final reaction product, the aminophthalate dianion, is formed in an electronically excited state which decomposes rapidly to the ground electronic state with loss of the excess energy as blue (430 nm) light. When the reaction takes place in the presence of excess reagents, the intensity of the light has been shown to be proportional to the concentration of bacterial porphyrins.

The choice of the chemiluminescence over the bioluminescence method was based on the advantages listed below, most of which are concerned with the greater adaptability of the chemiluminescence method to field applications. For example, the reagents are inexpensive, stable chemicals which do not require refrigeration or deep-freeze storage. Also, the preparation of the

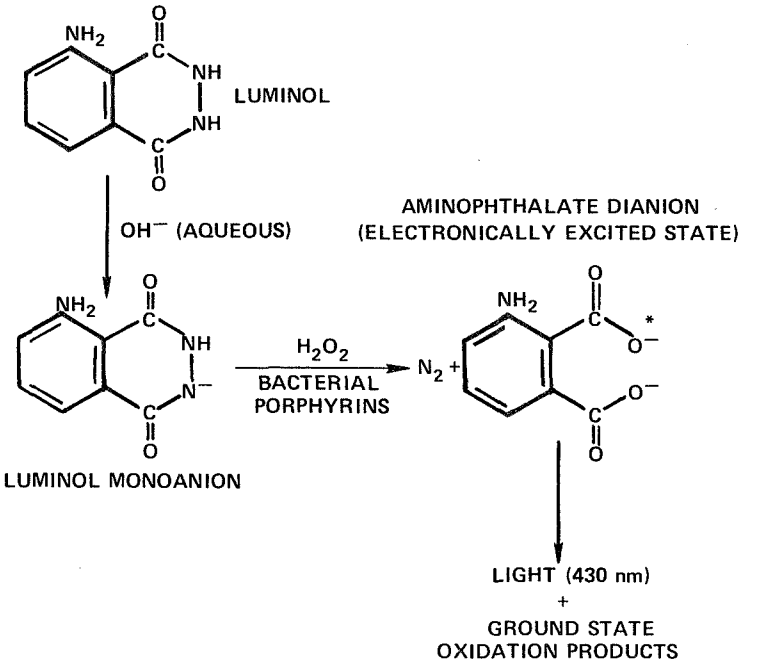

Figure 1. Detection of bacteria via chemiluminescence of luminol.

reagent and the analytical technique do not require the degree of care and manipulation that is needed in the bioluminescence method. Since the reagent is an alkaline solution which lyses the cells as rapidly as they are introduced, the separate lysing and extraction step is not required. Elimination of the prior lysing step, previously used in the chemiluminescence technique, was shown in this study to improve considerably the reproducibility and accuracy of the method in addition to simplifying it. When the cells were lysed with NaOH prior to analysis, very accurate timing between lysing and measurement was required for good reproducibility because the released porphyrins deteriorate rapidly in the presence of the lysed cells. In contrast, direct injection of the sample into the reagent eliminates the need for accurate timing.

The Aminco Chem-Glow Photometer (4-7441A), shown in figure 2, was used in all of these studies. It is an inexpensive, portable photomultiplier detector which gives a meter output showing the maximum light intensity produced when the sample is added to the reagent. In practice, one milliliter of the mixed reagent is introduced into the glass vial which is placed under the injection port opposite the photomultiplier tube in the light-tight rotatable reaction chamber. A volume of sample between 10 and 200 μl is injected into the reagent with the use of a mechanical injector attached to the syringe to ensure reproducible speed and sufficient force of injection for adequate mixing. The reproducibility is generally better than ± 5 percent.

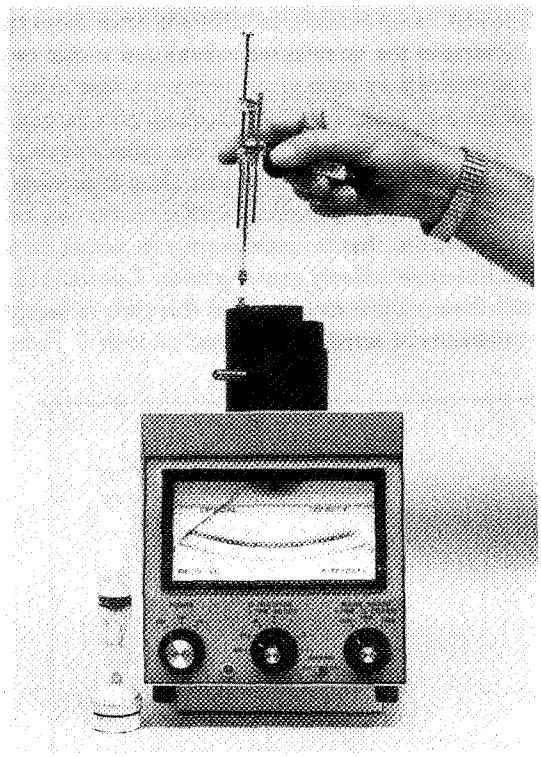

Figure 2. Aminco Chem-Glow Photometer, Shandon Mechanical injector, and filtering equipment.

Since activation of the chemiluminescence reaction of luminol is not limited to bacterial porphyrins, but can be promoted by metallic salts and some organic compounds, the reaction, as such, is not specific for bacteria. However, by separating the bacteria from the nonbacterial portion of the sample and by measuring and correcting for the light produced by the latter, the method used is specific for bacteria. This is accomplished simply with the use of a plastic syringe and bacterial membrane holder such as that shown to the left of the instrument in figure 2. A few milliliters of the sample are manually pushed through the bacterial membrane having a pore size of 0.20 to 0.45 μm. A volume of bacteria-free filtrate, equal to that used for the total sample, is injected into a fresh portion of reagent. The chemiluminescence intensity produced by the filtrate is then subtracted from that given by the total sample to yield the intensity due to the bacteria alone.

Results of a comparative study of the chemiluminescence and the plate count techniques on samples of cooling tower water are shown in figure 3. The six points represent samples taken at different times from two different cooling towers. Several of the samples were measured within two days after sampling, and others were aged in the bottle for several months at room temperature prior to measurement. Excellent correlation was found between the two tests in spite of the fact that the types of cells and stages of growth probably differed among the samples. It is expected that these factors would affect the porphyrin content similar to the way they affect the ATP content of the cells, that is, more porphyrin would be present in the larger cells and the more actively growing ones. Contrary to the expected interference from soluble metal salts in this type of sample, the emission due to nonbacterial activation was negligible in all these samples.

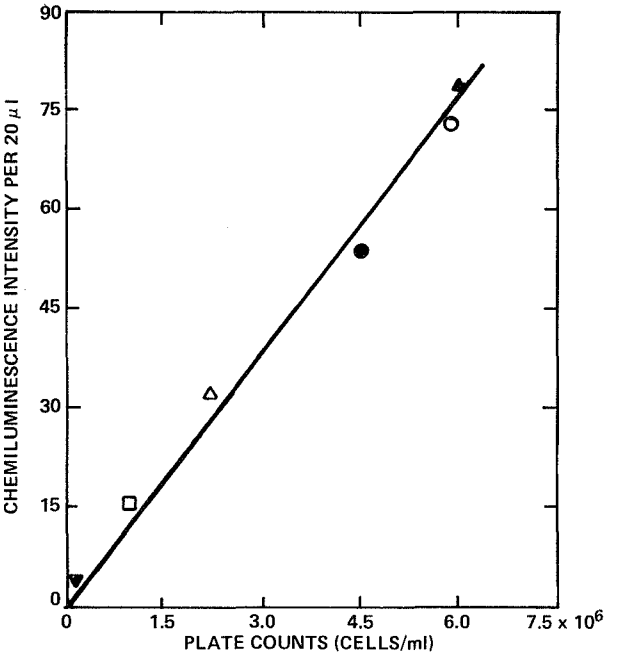

Figure 3. Cooling tower (CT) water.

Figure 4 illustrates the use of the chemiluminescence method in monitoring the effect of chlorine treatment in one of the cooling towers. Chlorine treatment was started at 9:30 a.m. and discontinued at about 3 p.m. Samples were collected every hour during treatment and every three hours after treatment was stopped. The decrease in cell population resulting from the treatment and the subsequent rise, beginning several hours after the treatment was stopped, is shown similarly by the plate counts and chemiluminescence measurements. In the initial study, an arbitrary correction was made for the chemiluminescence intensity due to the hypochlorite (ClO^-)

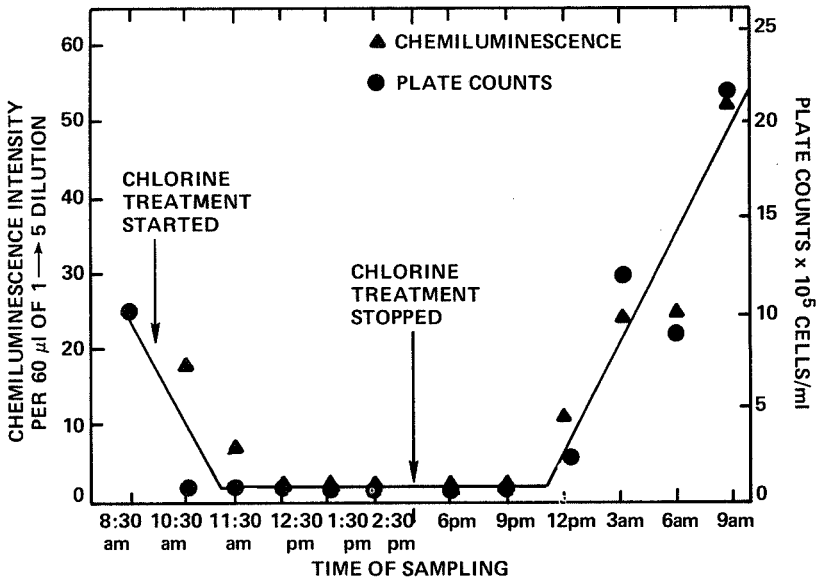

Figure 4. Chlorine treatment of cooling tower water.

present because the filtrate technique could not correct for it satisfactorily. In later studies, it was shown that interference by ClO^- could be eliminated completely by the addition of sodium thiosulfate to the sample.

The studies of cooling tower water show that the luminol chemiluminescence technique can be used to monitor changes in viable cell population both under normal conditions and during chlorine treatment. The limit of detection of bacteria by direct analysis, that is, without concentration of the cells, is estimated to be about 50,000 cells per milliliter for these samples based on plate-count analysis of the cell populations.

Good correlation between chemiluminescence and plate counts was also obtained in the analysis of process water used in paper mills. The results are shown in figure 5. In these samples, a considerable amount of paper pulp is present in the water. Since the microbial cells appear to be closely associated with the pulp, correlation between chemiluminescence and plate counts is dependent on identical sampling for both techniques so that the amount of pulp is the same. The range of replicates is shown by the horizontal bars for the plate counts and by the vertical bars for the chemiluminescence. Reproducibility was found to be considerably better for the latter. The limit of detection by the direct chemiluminescence method is estimated to be about an order of magnitude more for these samples than for the cooling tower, that is, about 500,000 cells per milliliter.

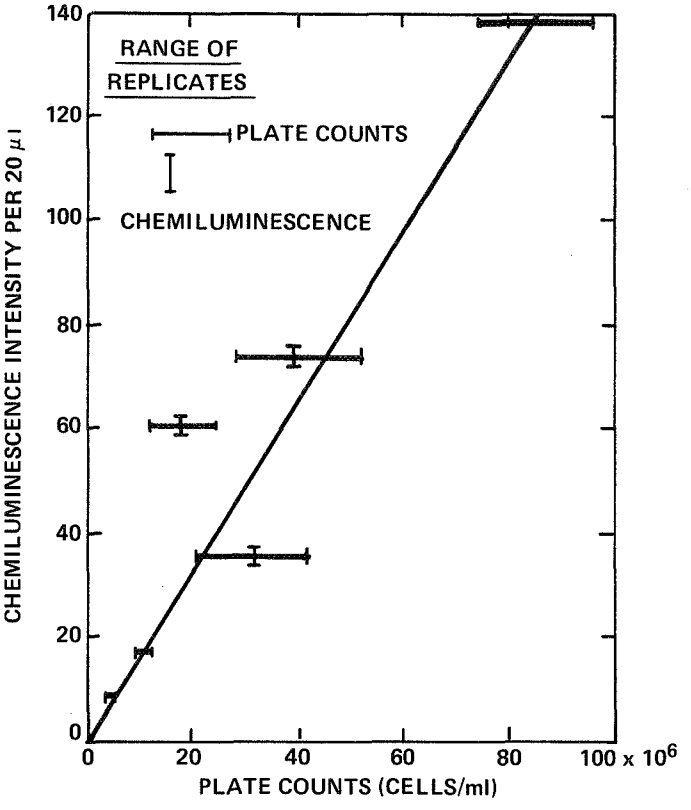

Figure 5. Process water containing paper pulp.

The chemiluminescence method has also shown good potential for monitoring the viable bacteria populations in activated sludge used in waste treatment plants to digest organic matter. Figure 6 shows the results of comparative tests on samples from the basin effluent of an industrial waste treatment plant taken over a period of several months. The amount of mixed liquor volatile suspended solids (MLVSS) is assumed to be indicative of the viability of the sludge. However, the fraction of the total MLVSS due to living microorganisms is variable, and it has been reported (McKinney, 1962; Patterson, 1970) that in some waste treatment plants it can often be as low as 25 percent. Considering the nonspecificity of the MLVSS test, correlation with the chemiluminescence intensities was better than expected. Considerably better correlation should be obtained when comparison is made with oxygen uptake, a test which gives a measure of the metabolic activity of the cells.

In addition to application to cooling tower water, paper mill water, and activated sludge, the method also has potential for applications such as:

- Bacterial cultures,
- Fermentation processes,
- Antibiotic efficacies,
- Process water, and
- Airborne bacteria.

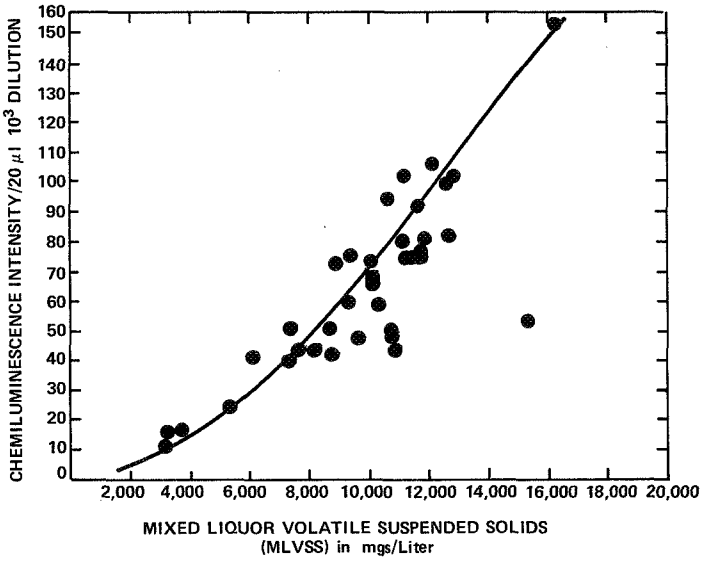

Figure 6. Chemiluminescence analysis of bacteria versus MLVSS of activated sludge.

These include standardization of bacterial cultures, monitoring of fermentation processes, determination of the efficacy of antibiotic levels, testing for purity of process water, and determination of levels of airborne bacteria. In the latter two applications, the bacteria levels are expected to be below that which can be measured by direct analysis. Therefore, a concentration technique is required. It has been shown that by collecting the cells contained in a large volume of water on a bacterial filter and extracting the active components with a 90:10 DMSO:H_2O solution, lower levels of bacteria can be measured.

In the technique used for low levels of bacteria, a volume of water ranging from 50 milliliters to several liters, depending on the level of bacteria, is filtered through a 47-mm, 0.2-μm, or 0.45-μm Acropor AN Gelman membrane by suction using a Sartorius Membranfilter plastic filtration system or equivalent apparatus. The membrane is then transferred to a 47-mm SWINNEX® Millipore filter holder. Exactly 2 ml of the DMSO (dimethylsulfoxide) solution are added directly with a pipet through the top opening

of the filter holder. After several minutes of contact with the filter, the DMSO solution is manually forced through the membrane by means of a plastic syringe. The procedure is repeated with a second 2-ml portion of DMSO solution and finally with 1 ml of sterile water. The total filtrate is then adequately mixed, and an aliquot is injected directly into the reagent. Sterilization of the membrane and filter system is not required. However, at least 5 ml of sterile water should be filtered through the membrane before it is used in order to remove any interfering substances. The reagent blank is obtained by passing 4 ml of DMSO solution and 1 ml of H_2O through the clean filter using the same technique as for the sample.

Comparative analyses of different samples of distilled water by chemiluminescence using the concentration technique described above and by plate counts are shown in figure 7. A volume of 500 ml of water was filtered for each sample, and 50 μl of the 5-ml extract was injected for the chemiluminescence measurement. The microorganism levels based on plate counts ranged from 6×10^3 to 140×10^3 cells/ml and good linearity was shown between the two techniques over this range.

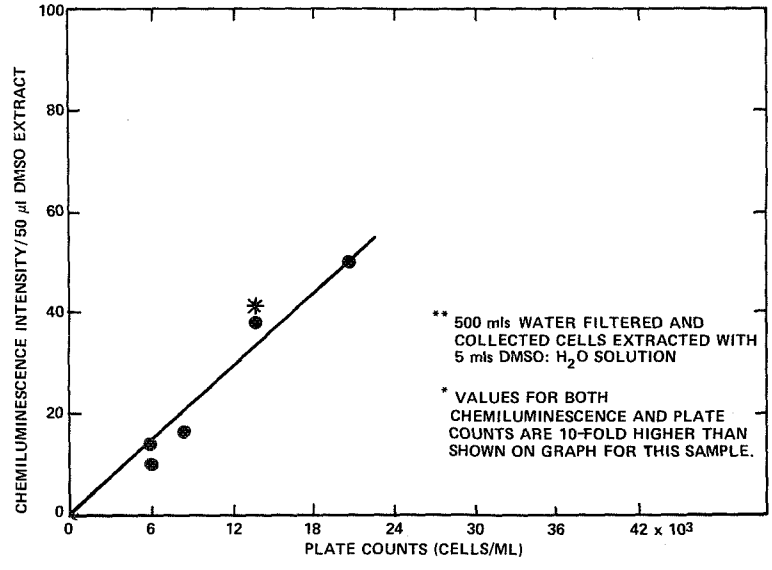

Figure 7. Distilled water: chemiluminescence** versus plate counts.

Testing for airborne bacteria could be accomplished by an impinger-type collection in water followed by concentration and extraction as described for distilled water. The lowest limit of detection of the cells in distilled water was 100 cells/ml, measured by concentrating the cells present in several liters of water. Modification of the method for application to lower

levels of bacteria by increasing the sensitivity of the reagent and improving the extraction procedure is being investigated.

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