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# Application of the ATP Assay for Monitoring the Disinfection of Wastewater Streams

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## Abstract

Plate count tests and ATP (adenosine triphosphate) assays were conducted on the following: water from waste treatment plant effluents chlorinated in the laboratory, water from waste treatment plants chlorinated by the plant facilities, and bacterial suspensions (prepared from laboratory cultures isolated from waste treatment plants) chlorinated in the laboratory. Both ATP levels and plate counts decreased in proportion to the amount of chlorine added to the water. However, the response of ATP and plate counts to the application of chlorine differed by several orders of magnitude. Despite reductions in the plate count parameters in excess of five logs (>99.999%), a significant amount of the ATP was recovered in the chlorinated samples. Furthermore, the relatively insensitive response of the ATP parameter to chlorine occurred even when all the species in the sample should have been capable of growing on the plate count media before chlorination. According to their response to nutrients and metabolic poisons, chlorinated cells which contain ATP, but are not measured by plate counts, can be metabolically active. Experiments in which chlorinated and unchlorinated cells were treated with proflavine suggest that the lethal action of chlorine, at the dosages studied, can be at a site affecting DNA synthesis. An assay based on ATP techniques to measure the ability of microorganisms to attach surfaces was developed. This assay responded more sensitively to chlorination than assays of the total ATP levels of the sample. It is concluded that the ATP assay can be a useful tool in monitoring and controlling the disinfection of biological systems. However, care must be exercised in its application and its interpretation.

# Résumé

On a procédé à des numérations des bactéries en milieu solide et titré l'ATP (adénosine triphosphate) d'échantillons d'effluents d'épuration, chlorés en laboratoire ou sur place, et de suspensions de bactéries (préparées en laboratoire à partir de souches isolées dans des installations d'épuration), chlorées en laboratoire. La teneur en ATP et le nombre de bactéries ont diminué à mesure qu'a augmenté la quantité de chlore ajoutée à l'eau. Toutefois, les effets de cet ajout sur les deux paramètres ont différé de plusieurs ordres de grandeur. Malgré une réduction de plus de 99,999 % du nombre de bactéries, on a en effet continué à déceler beaucoup d'ATP dans les échantillons chlorés. De plus, la résistance relative de l'ATP au chlore a été observée même quand toutes les espèces de l'échantillon auraient dû pouvoir croître dans le milieu de culture avant la chloration. Leurs réactions face aux substances nutritives et aux poisons métaboliques montrent que les cellules chlorées, qui contiennent de l'ATP mais qui ne sont pas comptées, peuvent présenter une activité métabolique. Les résultats du traitement des cellules chlorées ou non à la proflavine portent à croire que l'effet toxique du chlore, à la concentration utilisée, intervient à un site qui détermine la synthèse de l'ADN. On a mis au point une méthode qui s'inspire des méthodes d'analyse de l'ATP pour mesurer la capacité des micro-organismes à se fixer aux surfaces. Cette méthode permet de mesurer les effets de la chloration de façon plus précise que le titrage de l'ATP totale. On conclut donc que le titrage de l'ATP peut servir à contrôler et à régler la désinfection des systèmes biologiques. Il est toutefois important d'être prudent dans l'application de cette méthode et dans l'interprétation de ses résultats.

# Acknowledgments

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# Application of the ATP Assay for Monitoring the Disinfection of Wastewater Streams

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INTRODUCTION:

Effluents from many biological waste treatment plants such as municipal sewage plants and meat and poultry packing plants are disinfected by chlorination to combat the spread of water borne diseases. One problem in the disinfection process is the lack of an appropriate process control parameter. The need to regulate the process carefully is clear. It is important to apply sufficient chlorine to kill pathogens in the wastewater. On the other hand, applying too much chlorine is uneconomical and it is desirable to minimize the adverse effect that chlorine and its by-products have on the environment.

A useful process control parameter not only has to be capable of measuring the effectiveness of the disinfection process but the analytical methods involved must yield results within an hour. This time restriction is based on the typical maximum time that wastewater effluent can be held before it has to be released into the environment. Thus, parameters such as total plate counts and fecal coliform and total coliform counts fail to meet this criterion.

Free available chlorine has been used as a process control parameter for disinfection. However, this parameter has many shortcomings. The length of contact time of the chlorine with the effluent must be well defined in order for this parameter to have any meaning. Free available chlorine levels provide no information about the survival of bacteria in the centre of clumps which chlorine may have failed to penetrate. Furthermore, free available chlorine measurements are not directly related to the killing of pathogens. The use of free available chlorine is entirely based on the assumption that a certain minimum residual applied for a certain period of time will inactivate the pathogens in the water. Therefore, a process control parameter more directly related to viable organisms is desirable.

ATP is a parameter which can be assayed well within the time requirements for use in the control of the disinfection process. There is considerable evidence that ATP is related to the mass of viable organisms (1,2,3,4,5,6). In addition, the

ATP assay would be capable of demonstrating the presence of living cells in a clump of cells or debris which had not been penetrated by chlorine.

The use of ATP assay to measure a variety of types of microbicidal agents and processes has been reported in the literature. For example, the U.S. Environmental Protection Agency has used the assay to monitor the mortality of plankton caused by hot water discharges from thermal generating plants (7). The toxicity of heavy metals has been measured by the ATP technique in both algal bio-assay systems (8) and in activated sludge (9). Some value has also been demonstrated in applying the ATP assay in algal dye toxicity studies (10). In addition, medical investigators have been successful in applying the ATP assay to measure the effectiveness of antibiotics and anti-bacterial drugs (11,12,13,14). In industry, the application of ATP to measure the effectiveness of biocides in treating subsurface oil injection waters has been reported (15).

O'Brien and Gere Engineers Inc. (16,17) are the only investigators who have published data concerning the use of the ATP assay in monitoring disinfection processes. Much of their studies involved a combination treatment with both chlorine and chlorine dioxide and were performed on combined sewer overflows. They found that decreases in ATP levels during disinfection paralleled decreases in total coliform and fecal coliform counts. Also, the linear correlation coefficient of ATP with fecal coliform counts increased with disinfection of the samples. It was suggested that fecal coliforms were more resistent to disinfection than many of the other species in the untreated sample and were contributing an increased amount of the ATP in the chlorinated samples. These investigators concluded that it was feasible to use ATP as a reliable and rapid indicator to control the disinfection process.

Therefore, providing that the lethal dosage of chlorine on the total biomass in the waste treatment effluent is related to the lethal dosage of chlorine for pathogens in a reasonably consistent manner, it would appear that ATP has potential as a control parameter for the disinfection of wastewater streams.

ANALYTICAL METHODS:

(i) ATP

All reagents were prepared in autoclaved double deionized water (conductivity <1 microhmos/cm). This water produced a negligible response when assayed for ATP. All glassware was cleaned by overnight soaking in 4% Contrad 70 (Canlab), rinsing three times with deionized water and once with double deionized water. Prior to use, the glassware was incubated at least 6 hours at 100°C. The extraction method employed was that described by Tobin et al (19) and Afghan et al (20).

Five mls of effluent sample were filtered through a 24 mm Reeve Angel 984 H glass fibre filter, using a vacuum of 250 mm of mercury. The filter was immersed in 5 mls of boiling glycine - MgEDTA buffer pH 10.0 ± 1, ($10^{-2}$ M glycine (Sigma Chemical Co.), 5 x $10^{-3}$ M magnesium ethylenediaminetetraacetate (Fisher Scientific Co.)). The glycine-MgEDTA buffer was heated in glass scintillation vials (Amersham/Searle) held in a Pierce Reacti-Therm heating block set at 110-115°C.

After insertion of the filter, the vial was capped and agitated periodically for 5 minutes. The vial was then placed in an ice bath to cool. The cooled extract was transferred to a sterile plastic tube (Falcon) and centrifuged in an MSE Model GT-2 centrifuge for 5 minutes at 3800 rpm. The supernatant was stored in sterile plastic tubes at 4°C.

ATP extracts were assayed with crude firefly extract mixtures. Crude firefly extract was prepared by rehydrating each vial of firefly extract (Sigma Chemical Co.) with 15 mls of 2.5 x $10^{5}$ M HEPES buffer with a pH of 7.50 (Sigma Chemical Co.). After the rehydrated extract was allowed to stand for 30 minutes, it was filtered through a 0.45 micron membrane filter (Millipore, type HA).

Following an incubation period at room temperature of 18 hours, 0.33 mg

of D-luciferin (Sigma Chemical Co.) was mixed with the extract. This mixture was distributed into plastic scintillation vials (Fisher Scientific Co.) in 0.5 ml aliquots and frozen at - 18°C. The extract was thawed at a room temperature water bath for 7 to 15 minutes before use. ATP assays were performed by mixing 0.4 ml of ATP solution in glycine-MgEDTA buffer with the firefly extract aliquot and inserting the scintillation vial into an ATP Photometer (SAI Technology Co.) according to the manufacturer's instructions. The 60 second integration mode was used.

It was found that neutralizing the glycine buffer was unnecessary. The buffering capacity of the HEPES buffer in the firefly extract was sufficient to maintain the pH of the reaction mixture within an optimal range for the enzymatic reaction.

The crude luciferin-luciferase reagent was calibrated daily with ATP standards prepared by dissolving crystalline disodium ATP (Sigma Chemical Co.) in glycine - EDTA buffer.

(ii) Fecal coliform counts were determined according to APHA Standard Methods (14th edition) by membrane filter procedures. The 47 mm white gridded membrane filters (Millipore Type HC) were incubated on Bacto m-FC agar (Difco Laboratories) in a 44.5°C water bath (Blue M Electric Company) for 20 $\pm$ 2 hours. Colonies were counted with the aid of a low power binocular microscope.

(iii) Total coliform counts were also determined according to APHA Standard Methods by membrane filter procedure. The procedure is similar to the fecal coliform procedure except that m ENDO-LES agar (Difco Laboratories) was used. Incubations were performed at 35°C in a Thelco incubator for 24 hours.

(iv) To obtain the data shown in Fig. 1, total plate counts were performed by a membrane filter procedure. The filtration procedure was performed in the same manner as that used for the fecal coliform count except that 0.45 micron, 47 mm diameter black-gridded membrane filters (Millipore Type HA) were employed. The filters were placed on Bacto Tryptone Glucose Extract (TGE) Agar (Difco Laboratories) and incubated at room temperature (22-24$^{o}$C) for 3 days. Colonies were counted with the aid of a low-power binocular microscope.

In all the other cases, total plate counts were performed by a spread plate procedure in which 100 $\mu$ls of sample or diluted sample were spotted onto a TGE agar plate from the sterile tip of an Eppendorf microlitre syringe. The spot was then spread over the agar surface with a sterile glass rod. Following an incubation period of five days at room temperature, the colonies were counted with the aid of a Quebec colony counter.

RESULTS AND DISCUSSION:

Investigations began by comparing the results of the ATP assay with the traditional bacteriological parameters used in evaluating the disinfection process, such as coliform counts, fecal coliform counts and total plate counts.

Laboratory Bench Scale Studies on Sewage Treatment Plant Effluents.

A sample of effluent from a sewage treatment plant prior to its entry to the chlorine chamber was brought to the laboratory where it was divided into aliquots. Chlorine was added at various dosages to the aliquots and at various time intervals samples were removed. At the end of a time interval the chlorine was neutralized by adding excess sodium thiosulphate and the sample was assayed for the plate count parameters and ATP.

An example of the results obtained from these laboratory studies in shown in Figure 1. All three plate count parameters decreased by several logs while the ATP level decreased by less than one log. This type of experiment was repeated nine times with similar results. Similar results were obtained when ATP and plate count parameters were compared in samples taken before and after a sewage treatment plant chlorination chamber and also in another bench scale study.

Studies with Laboratory Cultures.

To investigate why ATP levels remain relatively high in chlorinated samples another experiment was conducted using a bacterial suspension which contained species which should be capable of growing on a plate count assay. This suspension was prepared by inoculating a TGE agar plate with a diluted aliquot of wastewater and incubating the plate 2 to 3 days at room temperature. A plate with 300 to 500 colonies was selected and the bacterial colonies were washed off the surface, suspended and diluted in deionized water.

Results of chlorinating one of these suspensions which was produced from a plate inoculated with the effluent of a poultry packing plant are shown in Figure 2. Plate counts were performed by the spread plate technique on same type of media on which the bacteria had been grown. Again, ATP was several orders of magnitude less

sensitive to chlorine than the plate count.

This type of experiment demonstrates that the lack of sensitivity of ATP to chlorine occurs even when all microbial species in the sample are known to be capable of growing on the plate count medium.

Thus, it appears that chlorinated cells may contain ATP but may be incapable of dividing to produce a colony in a plate count.

Metabolic Activity in Chlorinated Microorganisms.

Chappelle et al (21) have reported other evidence that ATP may be associated with cells not capable of producing a colony on a plate count. This group has presented evidence that a method involving metabolic poisons may distinguish between normal cells and intact cells not capable of division. The rationale for this method was that a metabolic poison such as carbon monoxide, sodium azide, or arsenate should have no effect on a cell which contains ATP but is metabolically inert. A normal cell responds by a marked decreased in ATP levels.

The results of an experiment designed to test the usefulness of this procedure for measuring the efficacy of chlorination are shown in Table 1.

A sample of secondary effluent from a municipal waste treatment plant was divided into two portions, one of which was treated with 4 mg/l of chlorine for 30 minutes. At the end of this time, any residual chlorine was neutralized by adding an excess of sodium thiosulphate and the samples were assayed for ATP and for total plate count by the spread plate procedure. Following these assays both of the samples were treated with 100 ppm of methylene bis-thiocyanate, a metabolic poison. After an incubation period of 45 minutes, the samples were assayed for ATP.

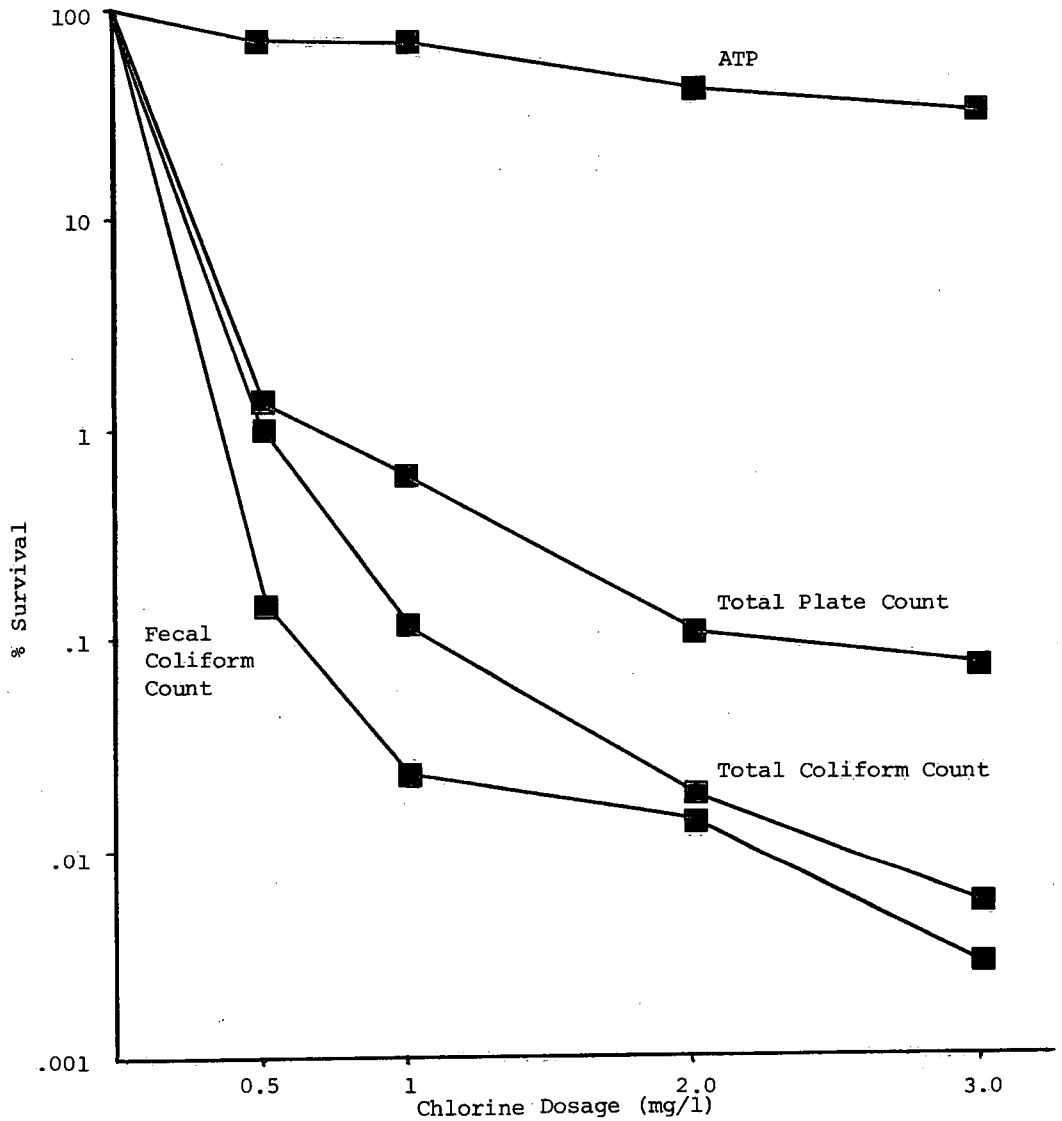


FIGURE 1: DISINFECTION EFFICIENCY IN CHLORINATION OF SECONDARY EFFLUENT ACCORDING TO ATP, TOTAL PLATE COUNT, TOTAL COLIFORM COUNT AND FECAL COLIFORM COUNT

Initial ATP concentration = 5.24 ng/ml
Initial Total Plate Count = $2.5 \times 10^6$/ml
Initial Total Coliform Count = $6.9 \times 10^5$/100 ml
Initial Fecal Coliform Count - $3.7 \times 10^5$/100 ml

Residual chlorine was neutralized by adding excess thiosulphate after 60 minutes.

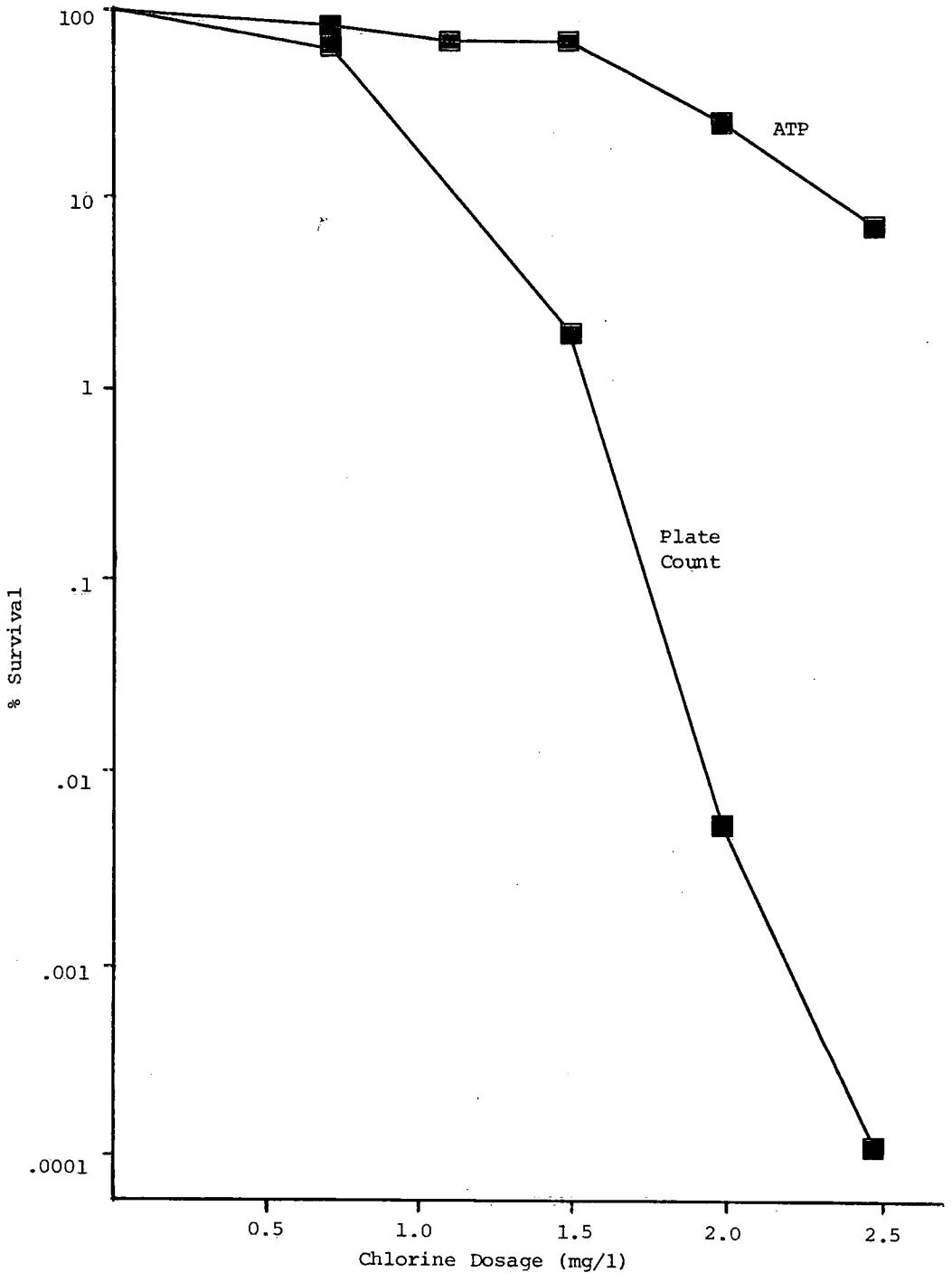


FIGURE 2: DISINFECTION EFFICIENCY IN CHLORINATION OF A LABORATORY CULTURE OF A MIXED BACTERIA POPULATION ISOLATED FROM THE SECONDARY EFFLUENT OF AN ONTARIO POULTRY PACKING PLANT

Initial ATP Concentration = 45 ng/ml
Initial Plate Count = $7.9 \times 10^7$/ml

TABLE 1

Effect of a Metabolic Poison (Methylene Bis-thiocyanate) on Chlorinated and Unchlorinated Cells

| | ATP (ng/ml) Before Treatment | | ATP (ng/ml) After Treatment With Methylene Bis-thiocyanate | Reduction By Poison |
|---|---|---|---|---|
| Control | 8.3 | (30,000) | 2.5 | 82% |
| 4 ppm Chlorine Added | 1.8 | ( 62) | 0.6 | 67% |

The numbers in brackets are the results of total plate counts per ml.

The results shown in Table 1 demonstrate that the chlorination causes a large decrease in plate count but only a relatively small decrease in ATP. However, the decrease in ATP following the treatment with the metabolic poison was similar in both samples. An experiment with a different metabolic poison, sodium azide, added to samples of chlorinated and unchlorinated secondary effluent showed similar results.

A different approach to distinguishing chlorinated cells from unchlorinated ones on the basis of metabolic activity was attempted. Instead of adding poisons to the cells which interfere with metabolic activity, nutrients were added to stimulate their activity. If cellular metabolism were destroyed by chlorination, one would expect no increase in ATP when a nutrient was added to these cells, while the ATP levels should increase when a nutrient is added to unchlorinated cells.

The results of two experiments performed with a suspension of mixed bacteria populations obtained by washing off colonies from a TGE agar plate are shown in Table 2. Nutrient broth was added after the chlorine treatment which was terminated with excess sodium thiosulphate. All incubations in broth were performed at room temperature (21-22$^{o}$C).

Even in the samples where the bacterial population was severely reduced according to the plate count, the ATP levels increased after incubation with 10% nutrient broth.

Two experiments performed on secondary effluent from a municipal sewage plant showed similar results.

TABLE 2

Response of Chlorine-Treated Bacteria to Nutrient Broth

| Dosage of Chlorine (mg/l) | ATP (ng/ml) Hours of Incubation in 10% Nutrient Broth | | | | |
|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 Hrs. |
| 0 ppm | ($1 \times 10^8$) | 50 | 130 | 120 | 90 |
| 1 ppm | ($2.4 \times 10^4$) | 38 | 90 | 80 | 100 |
| 5 ppm | (60) | 12 | 22 | 19 | 10 |

| Dosage of Chlorine (mg/l) | ATP (ng/ml) | | |
|---|---|---|---|
| | | No Incubation | 0.5 Hr. Incubation with 10% Nutrient Broth |
| 0 | ($2.2 \times 10^7$) | 30 | Not Done |
| 1 | ($2.1 \times 10^4$) | 8.4 | 17 |
| 5 | ( 20) | 0.049 | 0.11 |
| 50 | ( 20) | 0.038 | 0.068 |

The number in brackets are plate counts.

These experiments and the ones with the metabolic poisons indicate that chlorinated cells which are not producing colonies, may still have some metabolic activity. Therefore, an approach based on metabolic activity to evaluate the effectiveness of chlorination which relates to results of plate count tests does not appear promising.

Effect of an Inhibitor of DNA Synthesis on Chlorinated Cells.

Chlorine may act primarily on a cellular function other than general energy metabolism, such as DNA synthesis. An example of a chemical which interferes with DNA

replication is proflavine. It has been reported to cause incorrect base pairing in DNA replication and to inhibit DNA synthesis itself (22). If the primary site of action of chlorine is on DNA synthesis, chlorinated cells would not react to proflavine while untreated cells would be affected by this chemical. Another way of expressing this reduction would be to say that the percent survival of a chlorinated sample's ATP levels should be directly proportional to the percent kill in the sample according to the plate count.

Table 3 shows the results of three experiments conducted to determine whether this approach has potential use. These experiments were conducted by adding 200 mg/l of proflavine hemisulphate and measuring ATP and plate count in the samples before the addition of proflavine and then measuring ATP after the incubation period. The ATP levels were insensitive to proflavine in the samples which had received sufficient chlorine to achieve kills of approximately two logs or greater.

Thus, the effect of chlorine in these samples appears to be related to a cell function with which proflavine reacts, possibly DNA synthesis. However, a considerable amount of experimentation would be required to support this idea. Furthermore, additional testing is required to define the consistency of the behaviour of chlorinated effluents to proflavine treatment. For example, in two experiments conducted after those shown in Table 3, the reduction of ATP by proflavine in the chlorinated samples was similar to the reduction in the untreated samples. Yet, according to the plate counts, chlorination caused a kill of greater than 90%.

TABLE 3

Effect of Proflavine on Chlorinated Cells

| SAMPLE | Length of Proflavine Treatment (minutes) | ATP Before Proflavine Treatment (ng/ml) | ATP After Proflavine Treatment (ng/ml) | Percent Survival of ATP After Proflavine | Percent Kill Total Plate Count |
|---|---|---|---|---|---|
| EXP. 1 | | | | | |
| Secondary Effluent | 45 | 11.8 | 4.5 | 38 | Not Done |
| Effluent + 10 mg/l Chloride | 45 | 3.42 | 3.5 | 100 | Not Done |
| EXP. 2 | | | | | |
| Secondary Effluent | 60 | 3.35 (290,000) | 0.32 | 9.6 | - |
| " " + 2 mg/l $Cl_2$ | 60 | 0.55 ( 15,000) | 0.14 | 25 | 95 |
| " " + 5 mg/l $Cl_2$ | 60 | 0.10 ( 80) | 0.14 | 100 | 99.97 |
| " " + 10 mg/l $Cl_2$ | 60 | 0.068 ( 40) | 0.086 | 100 | 99.99 |
| EXP. 3 Secondary Effluent Spiked with Mixed Liquor Suspended Solids | 5 | 110 (116,000) | 35.5 | 32 | |
| " " + 2 mg/l $Cl_2$ | | 83 (106,000) | 35.0 | 42 | 9 |
| " " + 4 mg/l $Cl_2$ | | 46 ( 28,000) | 21 | 46 | 76 |
| " " + 6 mg/l $Cl_2$ | | 33 ( 9,200) | 31 | 94 | 92 |
| " " + 10 mg/l $Cl_2$ | | 16 ( 3,674) | 19 | 100 | 97 |

Numbers in brackets are plate counts.

Attempt to Predict the Disinfection Action of Chlorine by Means of its Antifoulant Activity.

Chlorine is also used extensively as an agent to control the undesirable attachment of microbial growths (23, 24). Experiments with an attachment assay, based on the

ATP technique, were conducted to assess the potential value of attempting to relate the antifoulant activity of chlorination with its disinfection action.

The assay consisted essentially of immersing a membrane filter in a wastewater sample for the desired incubation period. Then the filter was removed from the sample and the ATP from the attached biomass was extracted from the filter and measured.

The results of an experiment with a bacterial suspension prepared by washing colonies from a TGE agar plate inoculated with sewage effluent are shown in Table 4. The period allotted for attachment was two hours. This attachment period followed the chlorine treatment which was terminated by adding excess sodium thiosulphate.

TABLE 4

Relationship of an Attachment Assay to Disinfection by Chlorine

| | Total Plate Count | ng ATP in 0.067 ml of Water | ng ATP per Filter |
|---|---|---|---|
| Control (no chlorine added) | $7.8 \times 10^8$ (100%) | 2.5 (100%) | 28 (100%) |
| 3 ppm Chlorine added | $5.8 \times 10^6$ (0.73%) | 1.7 (67%) | 6.4 (23%) |
| 5 ppm Chlorine added | $1.18 \times 10^5$ (0.015%) | 0.43 (17%) | 1.6 (5.9%) |

The number in brackets are the percent survival of the microorganism in the sample after chlorination according to total plate count, ATP assay, and the attachment assay.

The ATP levels of the water surrounding the filter and the ATP levels accumulated on the filter can be compared by contrasting the ATP from the filter with amount of ATP found in the same volume of water that the filter occupies. By weighing filters before and after immersion in water it was found that the filter occupied a water volume of 0.067 ml.

The results in the table demonstrate that after two hours there was more than ten times more biomass attached to the filter than there was in the same volume of water. Furthermore, the amount of ATP associated with the filter decreased in accordance with the chlorine treatment in a slightly more sensitive way than the ATP level in the water.

Thus, this attachment assay may have applications in assessing the disinfection of wastewater effluents. It may also be useful for predicting biological fouling such as in drinking water lines. However, considerable development work is required. For example, the incubation period and the type of surfact required for attachment require optimization.

CONCLUSIONS:

The ATP assay may have potential as a process control parameter in disinfection. For example, ATP levels were related to the amount of chlorine applied to a sample. However, there is a major difference in the sensitivity to chlorine of ATP and the traditional plate count parameters. Therefore, on the basis of results of a single ATP assay on a sample of wastewater effluent, it would be difficult to relate this with much certainty to a plate count parameter. The ATP level itself may not be the most valuable process control parameter; perhaps a dose-response curve of chlorine and ATP may be the most informative.

Considerable advances are also being made in the plate count technology whereby significant increases in recoveries especially in chlorinated samples are reported. When this technology has been further developed, it may be interesting to compare the new methods to the ATP parameter.

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