Methodology for Application of Adenosine Triphosphate Determination in Wastewater Treatment

Gilbert V. Levin,* J. Rudolph Schrot, and Walter C. Hess
Biospherics Inc., 4928 Wyaconda Road, Rockville, Md. 20852

■ Efficient operation of activated sludge sewage treatment plants requires a rapid, accurate method for determining sludge viable biomass. Bioluminescent assay of microbial adenosine triphosphate (ATP) has been adapted for this purpose. An ATP extraction technique in which diluted sludge was treated with boiling TRIS buffer for 1 min was found to be adequate. ATP, dissolved oxygen (DO) uptake rate, tyrosine, total microscopic cell count, and turbidity were determined in pure cultures. After these tests indicated the applicability of ATP monitoring to activated sludge control, studies were made in a 20-mgd treatment plant. ATP analysis was employed to control mixed liquor biomass through regulation of return sludge. A one-month test period showed good plant performance and revealed close coupling between ATP content and metabolic activity of the sludge.

The operation of an activated sludge wastewater treatment plant is based on the metabolic activity of the viable organisms present in the mixed liquor. Present control practice attempts to measure active biomass by the determination of total suspended solids (SS), or, sometimes, suspended volatile solids (SVS) in the mixed liquor. In recognition of the deficiencies of the suspended solids measurement as an index of viable organisms, several alternative assays have been proposed. Some of these are estimation of DNA, total protein, organic nitrogen, and enzyme activity.

Adenosine triphosphate (ATP) is a universal constituent of living cells which has the added advantage of being highly labile when the death of cells releases the ATP in the presence of living microorganisms. The measurement of this compound, thus, has the unique characteristic of distinguishing viable biomass (*1*); and, therefore, the concentration of ATP represents the viable fraction of sludge. Furthermore, the ATP/biomass ratio has proved to be relatively constant (*2*). The possibility of using ATP for control of the activated sludge process has been the subject of several earlier papers (*3–6*).

Materials and Methods

Luciferin-Luciferase Preparation. A commercial (Du Pont Chemical Co.) lyophilized preparation of the firefly reagent was used. It was stored at −10°C, and solutions were prepared daily, in morpholine buffer, as directed by the manufacturer.

ATP Standards. A standard stock solution of ATP (1 mg/l.) (Calbiochem) was prepared weekly in pH 7.75 TRIS buffer (0.025*M*). All water was prepared to contain less than 10^{-3} mg/l ATP by heating distilled water in the autoclave for 2 hr at 121°C. Serial dilutions of the stock solution were made daily with TRIS buffer to provide standard solutions ranging from 10–0.01 mg/l.

Analytical Procedures. Tyrosine was measured by the method of Folin and Ciocalteu (*7*). Total microscopic bacterial counts were made with a Zeiss phase contrast microscope. Dissolved oxygen (DO) levels were determined with a Beckman O_2 Analyzer (Model No. 777). The sensor was inserted through the side of the growth flask and uptake rate was measured as a decrease in DO when the air supply was stopped. Optical density (OD) of the sample was determined with a Bausch & Lomb Spectronic 20 Spectrophotometer set at a wavelength of 420 nm.

ATP Extraction. Several extractants were investigated including butanol (*8*) with sonication, perchloric acid (*9, 10*), trichloroacetic acid, and boiling TRIS buffer (*1*). Of these, the last was selected on the basis of simplicity of procedure and reproducibility of results for the extraction of ATP from sludge and mixed liquor. One milliliter of the appropriate diluted mixture was added to a 50-ml volumetric flask containing approximately 35 ml of boiling TRIS buffer held in a boiling water bath. The flask was then shaken a few seconds and transferred to an ice bath. Upon cooling, the final volume was brought to 50 ml and filtered.

ATP Assay Procedure. Aliquots of test solutions (10 µl) containing ATP were placed in cuvettes using a 50 µl syringe and disposable tips. The cuvette was then placed in the instrument (*8*). The enzyme mixture (50 µl) was then injected into the sample cuvette, and the light emission recorded. This order of addition (enzyme into sample), rather than sample into enzyme, significantly improved the precision of the assay. When this method was used, the entire sample volume was quantitatively delivered to the bottom of the cuvette. There was no chance for cross-contamination between samples since disposable pipet tips were used. Also, the vigorous injection of the excess and, therefore, noncritical, volume of enzyme provided better mixing of the reactants.

Another important aspect of development which improved accuracy and precision was the elimination of injection through a septum which became contaminated after repeated use. An adaptor, Figure 1, was fixed to the barrel

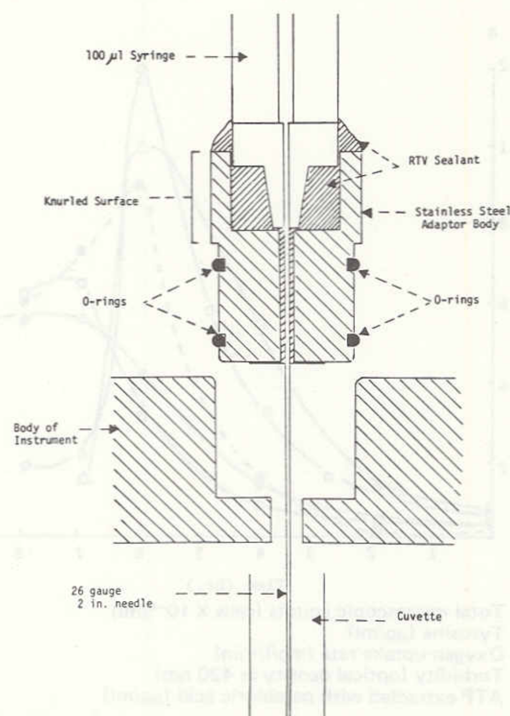


Figure 1. Syringe adaptor used to eliminate septum light seal

Reprinted from ENVIRONMENTAL SCIENCE & TECHNOLOGY, Vol. 9, Page 961, October 1975
Copyright 1975 by the American Chemical Society and reprinted by permission of the copyright owner

of the injection syringe. The syringe and adaptor formed the light seal when fitted into the recess in the top of the instrument and provided an entry hole so that the needle did not have to contact the septum during entry or withdrawal. Thus, subsequent injections were not contaminated by contact of the needle point with a septum that was wet on the bottom side with the splash droplets of earlier injections.

Results

Extraction of ATP from Activated Sludge. The effect of boiling time on ATP extraction from sludge is shown in Figure 2. There was no apparent advantage in using the longer boiling times of 5 or 10 min, recommended by other workers (*3, 11*). A boiling time of 30–60 sec was, therefore, adopted.

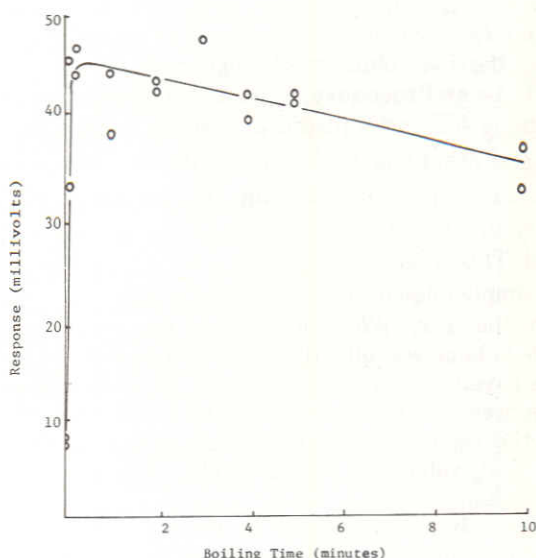
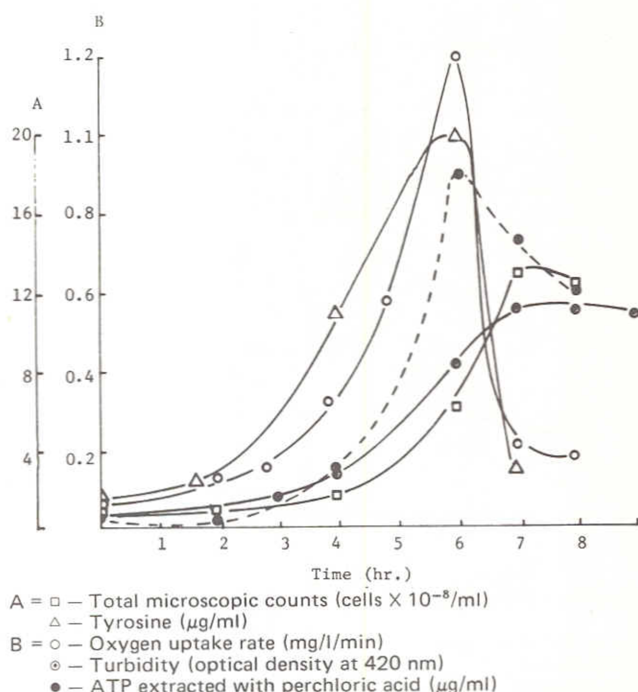


Figure 2. Effect of boiling time on extraction of ATP



A = □ — Total microscopic counts (cells X 10^{-8}/ml)
△ — Tyrosine (μg/ml)
B = ○ — Oxygen uptake rate (mg/l/min)
⊙ — Turbidity (optical density at 420 nm)
● — ATP extracted with perchloric acid (μg/ml)

Figure 3. ATP, tyrosine, oxygen uptake rate, total count, and turbidity of a growing *E. Coli* culture (nutrient broth, 25°C)

Sludge dilution may exaggerate assay differences because of the clumped, heterogenous nature of activated sludge. Blending was investigated as a means of obtaining more representative samples. Although blending has been reported to increase microbial plate counts, 2 min (high-speed with a Sears blender) decreased the ATP levels of four separate sludge samples by 10–28%. The possibility exists that less rigorous blending might produce better results; however, it was not investigated further. Blending only slightly improved precision of the assay.

The extraction efficiency of the boiling TRIS buffer method was found to be affected by the amount of sludge present. Dense sludge yielded inordinately low ATP results unless diluted sufficiently. Sludge containing concentrations of ATP greater than 2 μg/ml should be diluted prior to extraction. Best results were obtained when the sludge was diluted with ATP-free water to 0.1–1 mg/l ATP prior to extraction. For mixed liquor, the dilution found useful was 1:10, for return sludge, 1:20.

ATP added to sludge could be recovered quantitatively only when the ATP addition was made to heat-killed sludge. When ATP was added directly to viable sludge, the ATP began to disappear immediately, presumably as the cells incorporated or degraded it. This fact, however, eliminates the possibility that extracellular ATP might contribute significantly to the measured ATP level of sludge.

ATP Concentration in Pure Cultures. A series of experiments was conducted to compare ATP concentration with several other parameters indicative of cell population and metabolic state during the bacterial growth cycle. This was for the purpose of better understanding the ATP index and its applicability to monitoring and controlling the activated sludge process.

Pure cultures of *E. coli, Z. ramigera,* and a gram-positive spore-forming rod, designated *Bacillus sp.,* were grown in nutrient broth at 25°C for the *E. coli* and at 35°C for *Z. ramigera* and *Bacillus sp.* A 2-liter flask was equipped

Table I. Concentrations of ATP Per Cell, Based on Total Microscopic Count and ATP per Mg Dry Weight, Based on Tyrosine

Time, hr	ATP, μg/cell	ATP, μg,[a] per dry wt, mg, of cells
	E. coli	
0	3.9×10^{-10}	1.34
2	6.3×10^{-10}	1.79
4	17.0×10^{-10}	1.94
6	6.3×10^{-10}	1.49
8	6.3×10^{-10}	1.65
	Av = 8.0×10^{-10}	1.64
	Z. ramigera	
5.5	30.8×10^{-10}	8.36
25.5	3.1×10^{-10}	8.06
29.5	5.0×10^{-10}	6.71
	Av = 13.0×10^{-10}	7.71
	Bacillus sp.	
3.0	7.2×10^{-9}	2.83
4.5	5.3×10^{-9}	1.49
6.0	9.7×10^{-9}	4.78
7.0	7.6×10^{-9}	2.54
21.0	7.5×10^{-9}	3.43
24.0	7.1×10^{-9}	2.69
	Av = 7.4×10^{-9}	2.90

[a] Calculations based upon assumption that tyrosine is 2% of protein by weight and protein is 50% cell dry weight.

with a side port for the oxygen sensor. Influent air and effluent gas were passed through the stopper in tubes containing sterile glass wool filters. An air flow of approximately 150 cc/min was used. Oxygen uptake readings were made by stopping the air flow and recording the decrease in oxygen concentration with time. Sampling of the culture was performed by clamping the exhaust air tube and collecting the sample that was pneumatically pushed up and out of a sampling port. Samples were analyzed for total protein (tyrosine), ATP, and total microscopic count immediately after collection.

Figure 3 illustrates the results obtained from a culture of *E. coli.* Oxygen uptake rate, tyrosine, and ATP reached maxima at approximately 6 hr of growth and then markedly declined, whereas turbidity remained at a high level and total count declined only slightly. Similar results were obtained with cultures of *Z. ramigera* and *Bacillus sp.*

Table I shows the concentrations of ATP per cell based upon the total microscopic counts and the calculated μg ATP/mg dry wt of cells based upon the tyrosine determination.

These data show that the amount of ATP per dry weight of cells remains relatively constant for a given species of bacteria even under conditions of the various phases of growth. The amount of ATP per dry weight of cells fell within a narrow range for each species. *E. coli, Z. ramigera,* and *Bacillus sp.* showed average values of 1.64 ± 0.30, 7.71 ± 0.65 and 2.9 ±1.12 (μg ATP/mg dry wt of cells). Holm-Hansen (*12*) has shown that the ATP pools in 30 different species of algae during exponential growth all fell within relatively narrow limites—i.e., approximately 0.35 ± 0.05% of total cell carbon. He also showed that some algae display decreased ATP levels under conditions of nitrogen or phosphorus deficiency.

It is unlikely that nutrient deficiency played a role in these pure culture analyses; however, it is reasonable to suggest that the ratios of ATP/protein (tyrosine was used to compute dry weight), and ATP/total cell carbon (used by Holm-Hansen) are not directly related. The percentage of nonprotein cellular carbon would be expected to vary among species and for different physiological states to a greater extent than would protein. However, our results showed the greater variation. This may, in part, be explained by the fact that the two studies concerned unrelated organisms.

Table I permits calculation (dividing highest by lowest reported values) of the range of μg ATP/cell for *E. coli, Z. ramigera,* and *Bacillus sp.*—4.3, 9.9, and 1.8 fold, respectively. The corresponding calculated ranges for μg ATP/mg cells were 1.4, 1.2, and 3.2 fold, respectively. A better correlation of ATP with biomass is thus indicated than with numbers of viable cells. Where total metabolic activity is the function of concern, as in sewage treatment, this added aspect of the ATP assay would be an advantage. It might also be noted, from Figure 3, that the turbidity lagged behind the decrease in cell population while the ATP assay anticipated it—a valuable quality for a plant control sensor. The limited pure culture work confirmed the sensitivity of the method and its apparent applicability to activated sludge process monitoring and control. A test of that applicability was indicated.

ATP Measurements in a 20-Mgd Activated Sludge Sewage Treatment Plant. Studies were performed at the Baltimore City Back River Sewage Treatment Plant. This is a conventional plug flow, activated sludge plant with a constant 20-mgd flow and an average incoming BOD of 215 mg/l. A MLSS of 1500–2000 mg/l is generally maintained with a 25% sludge return. Aeration contact time is approximately 5 hr.

During a one-week period of measurements, the ATP level of the mixed liquor was 2.8–3.4 mg/l at the head of the aeration basin. Changes in the rate of sludge return were

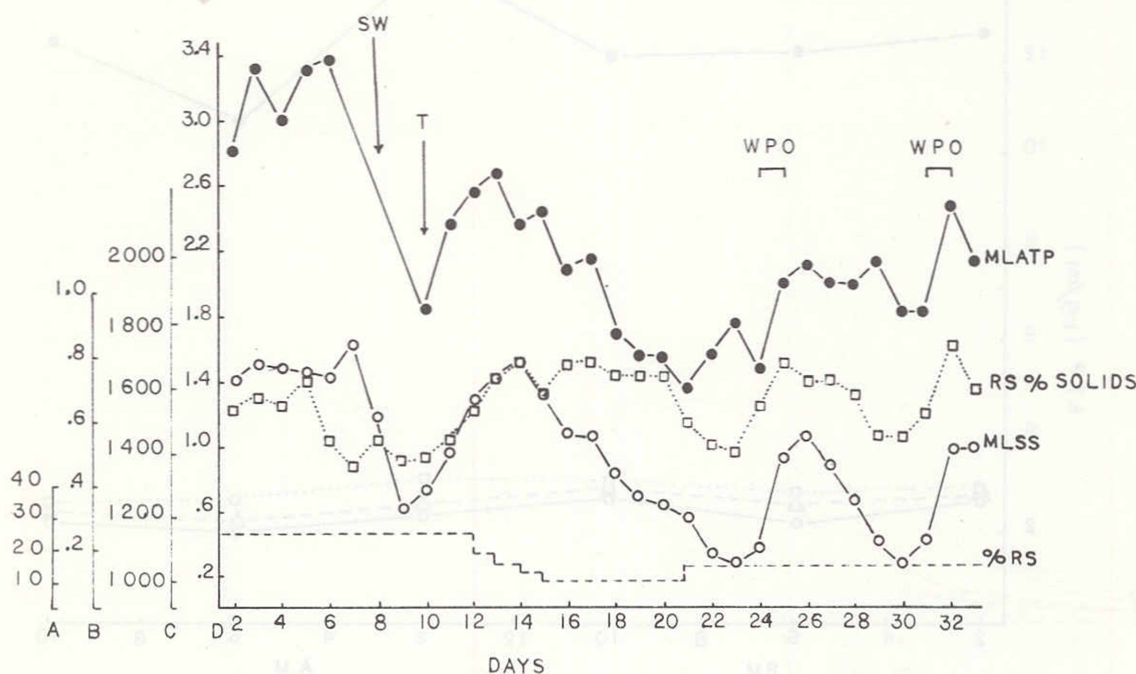


Figure 4. Parameters measured at Baltimore Back River Activated Sludge Plant
A - - -RS rate (%)[a]
B □ . . . □ Solids in RS (%)[a]
C ○ – ○ MLSS (mg/l)[a]
D ● – ● MLATO (μg/ml)
SW, large slug of sludge wasted
T, test period started
WPO, waste pump off
[a] Data supplied from treatment plant records.

imposed as shown in Figure 4. The levels of MLATP, MLSS, and percent solids in the return sludge as affected by these changes were monitored for a one-month period.

Beginning on Day 18, the return sludge rate was decreased stepwise until an 8% return rate was reached. It was evident after several days, however, that the 8% return was not sufficient to maintain a stable concentration of MLATP; therefore, on Day 21, the return sludge was increased to 12%. A comparison of MLATP and MLSS shows that these two parameters followed rather similar patterns with one significant difference. Changes in the return sludge rate caused changes in the MLATP level much sooner than changes were observed in the MLSS. Thus, the ATP measurement, but not the suspended solids measurement, would permit real-time control of return of sludge, based on metabolic events which determine treatment.

A 12% sludge return was sufficient to allow for sludge and ATP accumulation, whereas an 8% return continued to result in decreasing levels of ATP and solids. This would support the possibility that the optimum sludge return for this treatment plant, during the period of testing, was somewhere between 8 and 12%. The efficiency of treatment during this period of reduced sludge return as compared with a period immediately preceding the test, is given in Table II.

Table II. Efficiency of Treatment During ATP Measurement Tests

	Prior to test	During test
Return sludge, mgd	4.5	2.6
BOD reduction, %	96 (95)	95
TOC reduction, %	69	71
SS reduction, %	94 (91)	93
Waste sludge[a] produced, lb/day	27,000 (25,400)	17,000
SDI	0.88 (0.45)	0.89

N.B.: Values in parentheses are the average of data obtained during prior two months of operation. Other values obtained prior to test are the average of the data obtained in an approximately two-week period immediately preceding the reduction in return sludge.
[a] Determined by multiplying pump capacity (mgd) times percent solids in sludge.

As shown, the plant's high level of efficiency was maintained, but there was a significant reduction in the quantity of waste sludge produced. This latter effect may be partially explained by the greater contact time resulting from the reduced return flow, and perhaps increased oxygen transfer to microorganisms, thereby achieving greater biological oxidation.

Measurements of ATP were performed on return sludge and mixed liquor at the head, middle, and end of the aeration basin. As shown in Figure 5, analyses were made at 2-hr intervals during a 20-hr period. Despite fluctuations of incoming BOD loading (approximately twofold), the ATP level remained relatively constant in the mixed liquor. A slight increase in ATP was observed as sludge moved from the head, to the middle, to the end of the aeration basin. Values of 2.31 ± 0.35, 2.52 ± 0.24, and 2.73 ± 0.25 mg/l, respectively, were obtained. The ATP level of return sludge showed greater variability and averaged 12.31 ± 0.79 mg/l. A coefficient of variation of 1.1% was obtained for the duplicate analyses.

Discussion

The concept that ATP measurements of activated sludge may provide a rapid and logical method for controlling the

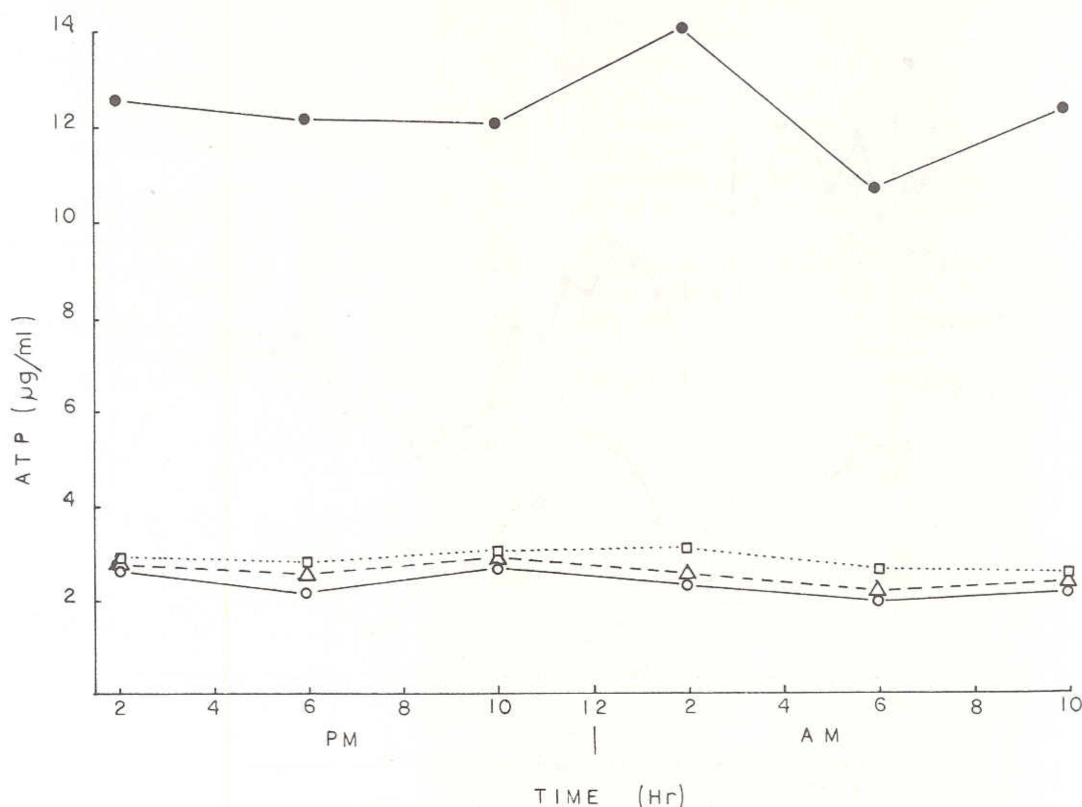


Figure 5. ATP in mixed liquor and return sludge at Baltimore Back River Activated Sludge Plant
● — ● Return sludge aeration basin sample point △- -△ Middle
○ — ○ Head □. . . End

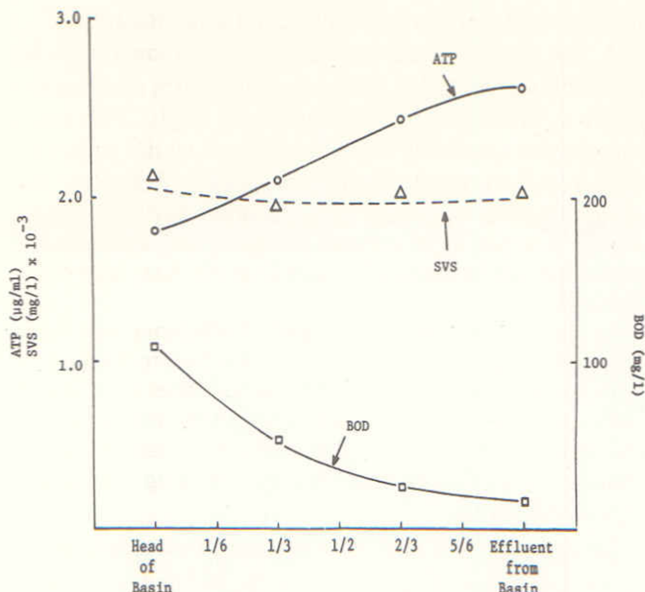


Figure 6. ATP, BOD (settled) and SVS at various points in aeration basin at Baltimore Back River Treatment Plant

food-to-microorganism ratio for maximum efficiency of the activated sludge process is based on the theory that ATP is more nearly indicative of active biomass than is MLSS or MLSVS. The latter two parameters do not correlate well with viable biomass. Figure 6 plots SVS, ATP, and BOD at various stages in the aeration basin of an activated sludge treatment plant. As shown, an increase in ATP throughout the aeration basin correlated with decreased BOD. The SVS showed no apparent change. Patterson, et al. (*3*) calculated that only 15–20% the MLSVS at the University of Florida plant was viable biomass. McKinney (*13*) has estimated that the ratio may range from 25–50%. Lesperance (*14*) suggests that 80% of the MLSVS in normal sludge is biological material, but cautions that this figure does not hold true for certain industrial wastes. Weddle and Jenkins (*4*) state that the viable content of conventional activated sludge is between 10 and 20%. These estimates indicate that the range in percent viable biomass of normal sludge may be in the neighborhood of fivefold. A fivefold ignorance factor in the active fraction of sludge would have a major influence on efficiency of the activated sludge process.

Too many or too few viable organisms with respect to the incoming organic load could give rise to unstable process conditions and lowered efficiency. Changes in the active fraction of the sludge could easily be monitored with the ATP technique and the plant process shifted to adjust to TOC influent levels that can also be monitored in real time. Improved quality and stability of the effluent would be expected. Return sludge pumping would be minimized, thereby reducing operating costs and effectively increasing plant capacity.

Acknowledgment

William A. Hasfurther, Chief, Division of Wastewater of Baltimore, Md., was most helpful in providing cooperation for the full-scale study at the Baltimore Back River Treatment Plant. The assistance of Jerald D. Wingeart, Terry Armbruster, and Hans Urtes, of that plant, is gratefully acknowledged.

Literature Cited

(1) Levin, G. V., Clendenning, J. R., Chappelle, E. W., Heim, A. H., Rocek, E. "A Rapid Method for the Detection of Microorganisms by ATP Assay; its Possible Application in Virus and Cancer Studies," *BioSci.*, 14, 37 (1964).
(2) D'Eustachio, A. J., Levin, G. V. "Levels of Adenosine Triphosphate During Bacterial Growth," *Bact. Proc.*, **121**, 1967.
(3) Patterson, J. W., Brezonik, P. L., Putnam, H. D. "Measurement and Significance of Adenosine Triphosphate in Activated Sludge," *Environ. Sci. Technol.*, **4**, 569 (1970).
(4) Weddle, Clark L., Jenkins, David, "The Viability and Activity of Activated Sludge," *Water Res.*, **5**, 621–40, Pergamon Press, Great Britain, 1971.
(5) Chiu, S. Y., Kao, I. C., Erickson, L. E., Fan, L. T. "ATP Pools in Activated Sludge," *J. Water Pollut. Control Fed.*, **45**, 1746 (1973).
(6) Kao, I. C., Chiu, S. Y., Fan, L. T., Erickson, L. E. "ATP Pools in Pure and Mixed Cultures," *ibid.*, **45** (5), 926–31, 1973.
(7) Folin, O., Ciocalteu, V., "Determination of Tyrosine," *J. Biol. Chem.*, **73**, 627 (1929).
(8) Chappelle, E. W., Levin, G. V., "Use of the Firefly Bioluminescent Reaction for Rapid Detection and Counting of Bacteria," *Biochem. Med.*, **2**, 41 (1968).
(9) Picciolo, G. L., Kelbough, B. N., Chappelle, E. W., Fleig, A. J., "An Automated Luciferase Assay of Bacteria in Urine," Goddard Space Flight Center, NASA TM X-65521, 1971.
(10) Knowles, C. J., Smith, L., "Measurements of ATP Levels of Intact Azotobacter Vinelandii Under Different Conditions," *Biochim. Biophys. Acta*, 152–60 (1970).
(11) Holm-Hansen, O., Booth, C. R., "The Measurement of Adenosine Triphosphate in the Ocean and Its Ecological Significance," *Limnol. Oceanogr.*, **11**, 510 (1966).
(12) Holm-Hansen, Osmund, "ATP Levels in Algal Cells as Influenced by Environmental Conditions," *Plant Cell Physiol.*, **11**, 689–700 (1970).
(13) McKinney, R. E., "Microbiology for Sanitary Engineers," McGraw-Hill, pp 167–9, New York, N.Y., 1962.
(14) Lesperance, Theodore W., "A Generalized Approach to Activated Sludge, Part I–Basic Biochemical Reaction," *Water Works Wastes Eng.*, 44–6 (1965).

Received for review March 14, 1974. Accepted June 13, 1975. Work supported by U.S. Environmental Protection Agency under Contract No. 14-12-419 and Contract No. 14-12-871.