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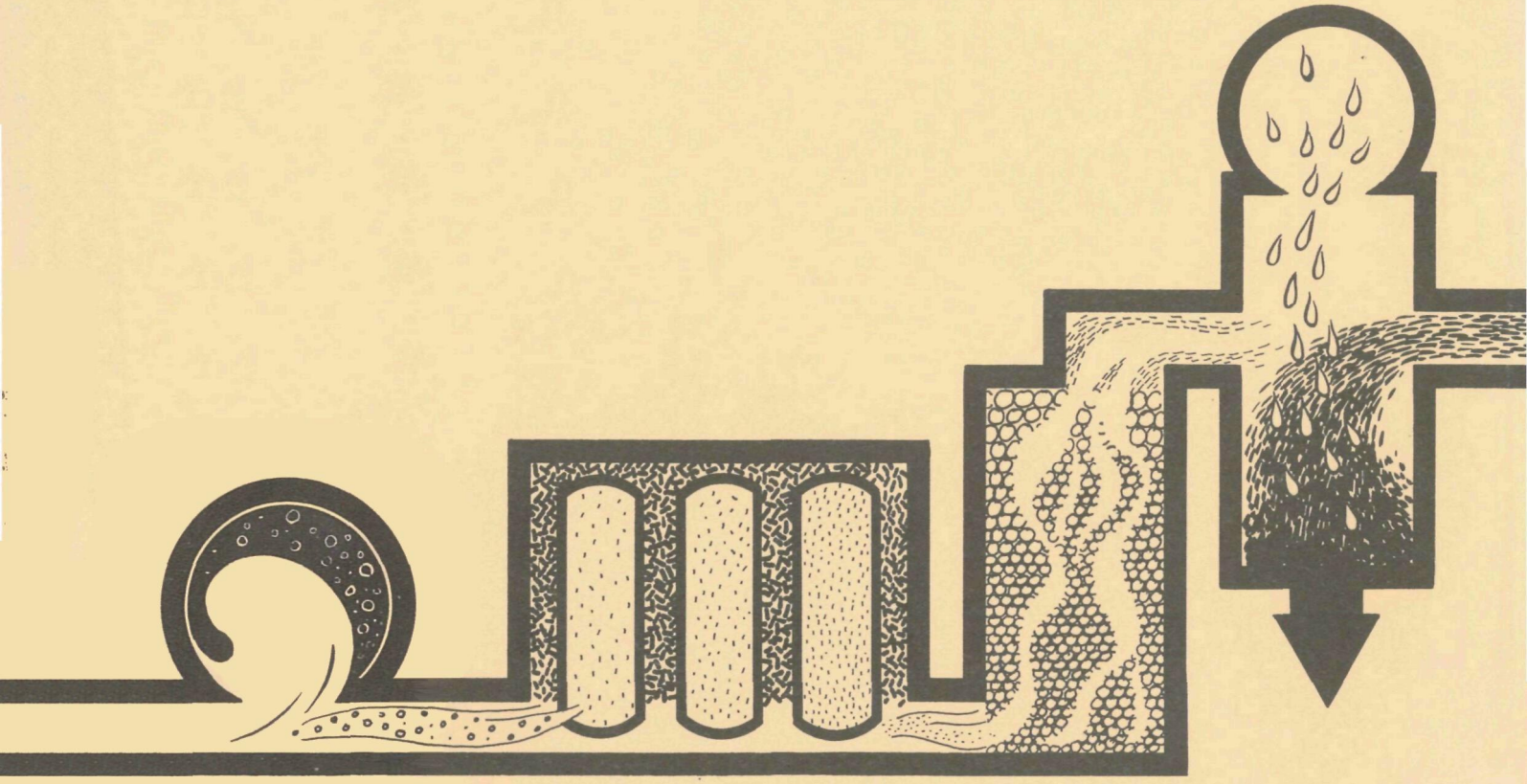
Biomass Determination - A New Technique for Activated Sludge Control



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BIOMASS DETERMINATION - A NEW TECHNIQUE FOR ACTIVATED SLUDGE CONTROL

by

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for the

Office of Research and Monitoring

ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

Research was conducted to determine the feasibility of using ATP as a measure of viable biomass in activated sludge. Methods were developed for the extraction of ATP from sludge and mixed liquor, and for the determination of ATP using the firefly bioluminescent procedure. Measurements of ATP were conducted on various pure cultures, pilot plant and full-scale activated sludge treatment plants. Additional parameters including BOD, TOC, oxygen uptake rate, and suspended solids were measured to provide comparative and supportive information. Preliminary tests in which ATP measurements of biomass were used to control the percent sludge return were conducted at two full-scale municipal sewage treatment plants. Lowered return sludge rates were found to produce effective treatment and increase the biological activity of the sludge. Changes in the rate of return sludge resulted in changes in ATP concentration of mixed liquor which preceded changes in suspended solids by as much as 24 hours. The assay was found to be reproducible and rapid. Results can be obtained within approximately ten minutes. This report was submitted in fulfillment of Project Number 17090 EEM, Contract Number 14-12-419, and Project Number 17050 EOY, Contract Number 14-12-871, under the sponsorship of the Environmental Protection Agency.

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FIGURES

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SECTION I

CONCLUSIONS

1. Adenosine triphosphate can be extracted from sludge and mixed liquor using boiling Tris buffer, perchloric acid or trichloroacetic acid; however, the use of boiling Tris buffer had advantages over the other methods.

2. Methods based upon the firefly bioluminescent reaction were developed for the rapid quantitative assay of solutions containing ATP.

3. Pilot plant studies were conducted to test the use of ATP for control of return sludge rates. A concentration of 2 μg/ml ATP in the first aeration tank was found to be most effective for treatment of waste of 240 mg/l BOD_5.

4. Preliminary studies using ATP measurements for control of aeration sewage treatment have been performed in full-scale treatment plants. The assay was performed without difficulty and gave indications of possible utility.

SECTION II

RECOMMENDATIONS

Studies in both the Baltimore and Arlington plants were designed as preliminary investigations of the feasibility of operational control by means of ATP assay of biomass. These initial goals have been reached. Studies should now be conducted for longer periods of time throughout seasonal changes. If possible, the plants under study should be divided into test and control sections.

In addition to the influence of weather and climate conditions, in-plant ATP measurements should be made during bulking, shock loading, severe hydraulic overloading, introduction of toxic materials, heavy silting, and foam formation.

Pilot plant studies should be continued to support full-scale plant operations when these types of problems arise. Many of the operational stresses mentioned above can be duplicated in the pilot plant. Information gained by pilot plant operation can be rapidly converted into useful action in a full-scale plant.

Further studies to determine the optimum concentration of ATP should be conducted. The influence of sewage strength, temperature, contact time, and sludge activity should all be determined and integrated into a workable operational procedure.

A manual for the plant operator should be prepared providing information on methods and procedures to be followed in actual plant operation. This should apply to several types of activated sludge plants and contain precise information to permit effective control under all types of foreseeable conditions.

SECTION III

INTRODUCTION

The operation of an activated sludge sewage treatment plant depends upon microorganisms in the return sludge. The basic assumption is that the recycled sludge is composed of living cells which absorb and metabolize components of the incoming waste. The effectiveness of the return sludge is related directly to the number and physiological state of these cells. The parameter currently used for return sludge control is total suspended solids. McKinney (1) points out that living microorganisms may constitute as little as 25% of the suspended volatile solids. Patterson, et al. (2) came to a similar conclusion and state that "a significant portion of the suspended volatile solids is nonviable organic material not associated with the oxidative degradation of the substrate."

Since the functional portions of any biological treatment plant are the living microorganisms which comprise the sludge, a means of measuring this active fraction is highly desirable. Conventional methods of microbial enumeration are difficult to apply because of the mixed and clumped nature of the flora present, and the time required for culturing techniques. Conventional plating methods do not produce results for at least one day - too late to be of use for control of the plant.

Adenosine triphosphate (ATP) is universally present in living microorganisms and its measurement by the firefly bioluminescent reaction is rapid. This study was therefore undertaken to determine the feasibility of using the measurement of ATP in sludge as a workable parameter for control of aeration sewage treatment. The task involved the fabrication of an instrument suitable for measurement of light emitted in the firefly bioluminescent reaction, establishment of suitable controls and standards, selection of an extraction procedure, establishment of a methodology which would yield useable levels of precision and accuracy, correlation of ATP measurements with other parameters of cellular activity and plant operation, and a preliminary test of the basic principle in full-scale plant operations.

SECTION IV

PRELIMINARY STUDIES

The application of the firefly bioluminescent assay for ATP to the control of operation of sewage treatment plants was begun by Biospherics Incorporated in July 1968. At that time there was no assurance that a commercial bioluminescence instrument would become available soon enough for us. Accordingly, a portion of the contract called for the design and fabrication of an ATP instrument. The ATP assay instrument fabricated under the contract is shown in Figure 1. It integrates a photomultiplier light sensing device with a biochemical reaction chemaber as a composite structure. In the detection of very low levels of light, it is necessary to place the light source as close as possible to the detector, and ambient light from an external source must be excluded from contact with the sensor.

FIGURE 1

ATP Assay Instrument

Figures 2 and 3 provide details on the design of the reaction drum. The drum, with a milled recess for the test cuvette, rotates from a front loading position to that directly in front of the sensor window. Optimum coupling is achieved, and extraneous light eliminated by the long, blackened path around the drum. Injection is through a replaceable rubber seal directly over the cuvette. Figure 4 shows a block diagram of the complete instrument.

Procedures for the quantitative extraction of the ATP from biological materials were examined. Two extractants, butanol and dimethylsulfoxide (DMSO) were used under various conditions. While difficulty in achieving quantitative extraction was encountered with all extractants, a procedure employing 100% butanol as the extractant, coupled with sonication for ten minutes was used.

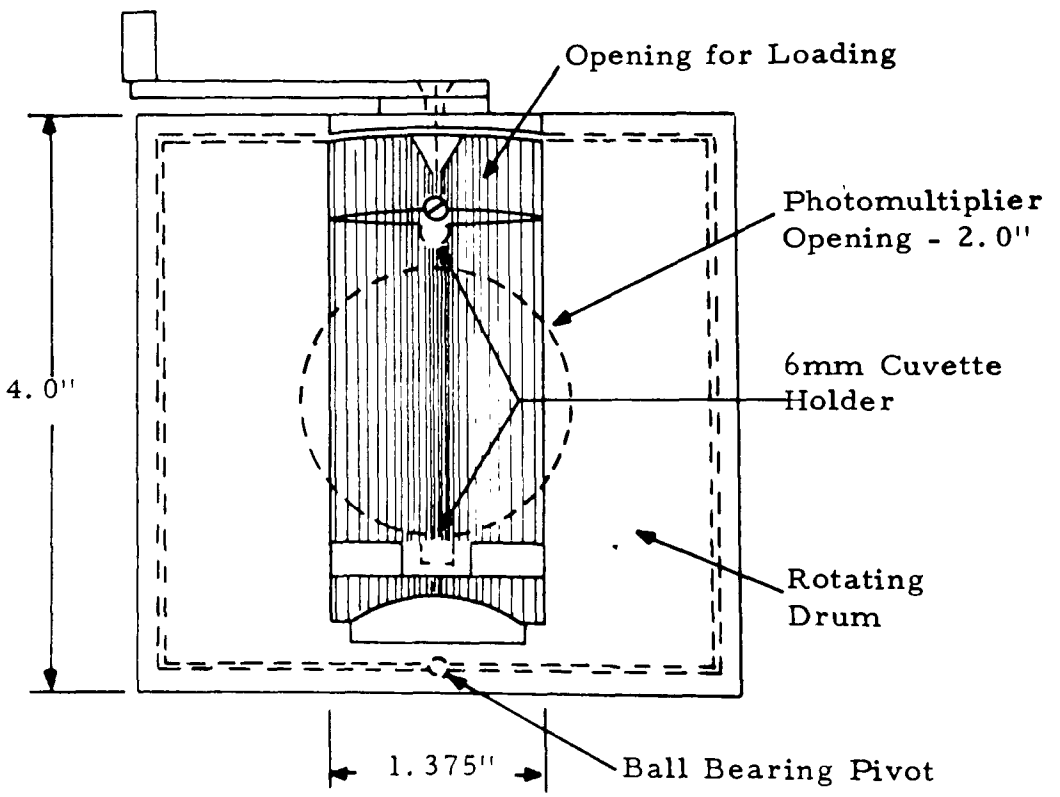


FIGURE 2

Cuvette Turette

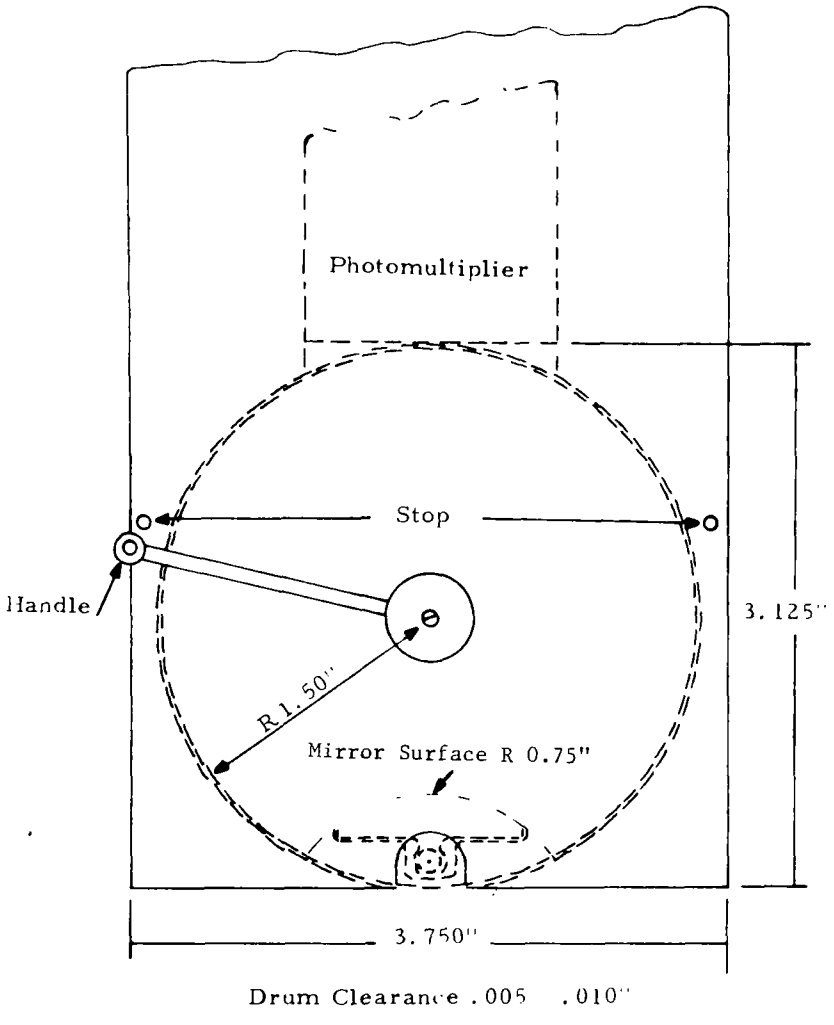


FIGURE 3

Photomultiplier Mount and Reaction Chamber

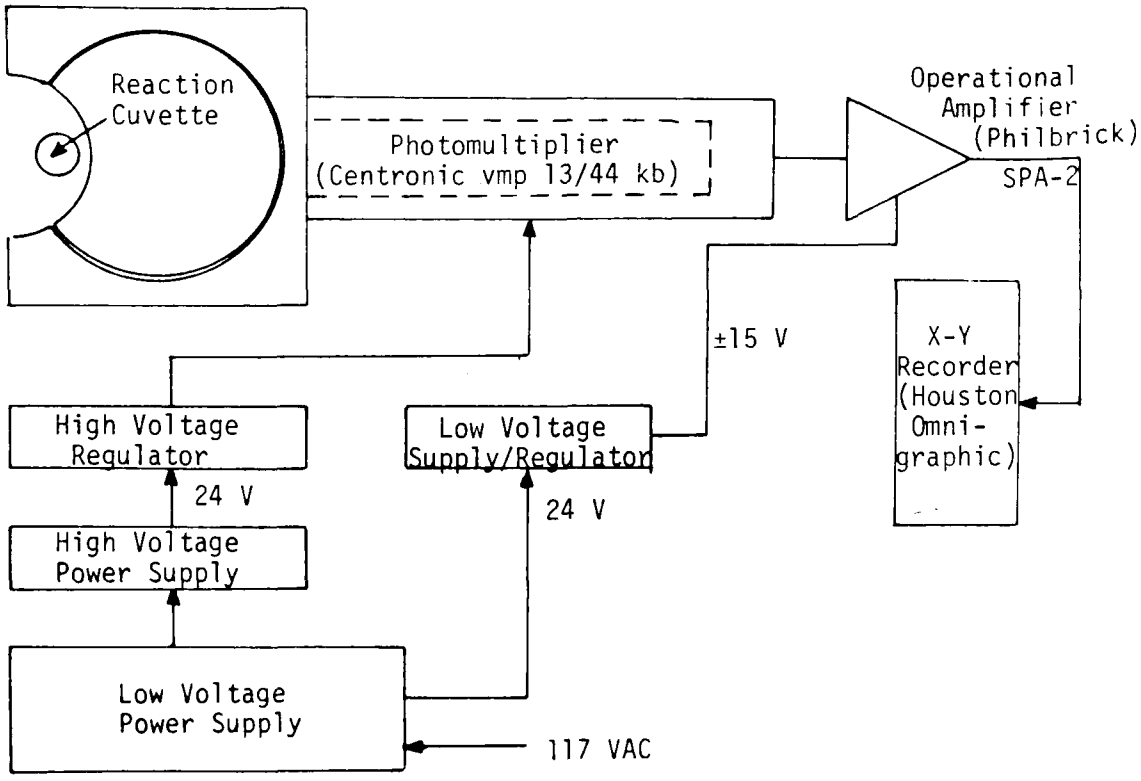


FIGURE 4

Block Diagram of Electronics Circuitry

This extraction technique was used to determine the quantitative relationship between ATP and numbers of cells. Measurement of an unidentified pure culture isolated from mixed liquor, as shown in Figure 5, verified the linear relationship between ATP and cell numbers as determined by bacterial plate counts. This, and several similar experiments, also showed that ATP measurements could be performed on pure bacterial cultures with an average coefficient of variation of 10%

The extraction of mixed liquor from the District of Columbia Sewage Treatment Plant was also investigated to determine if a linear relationship between measured ATP and concentration of mixed liquor could be obtained. Representative results are shown in Figure 6.

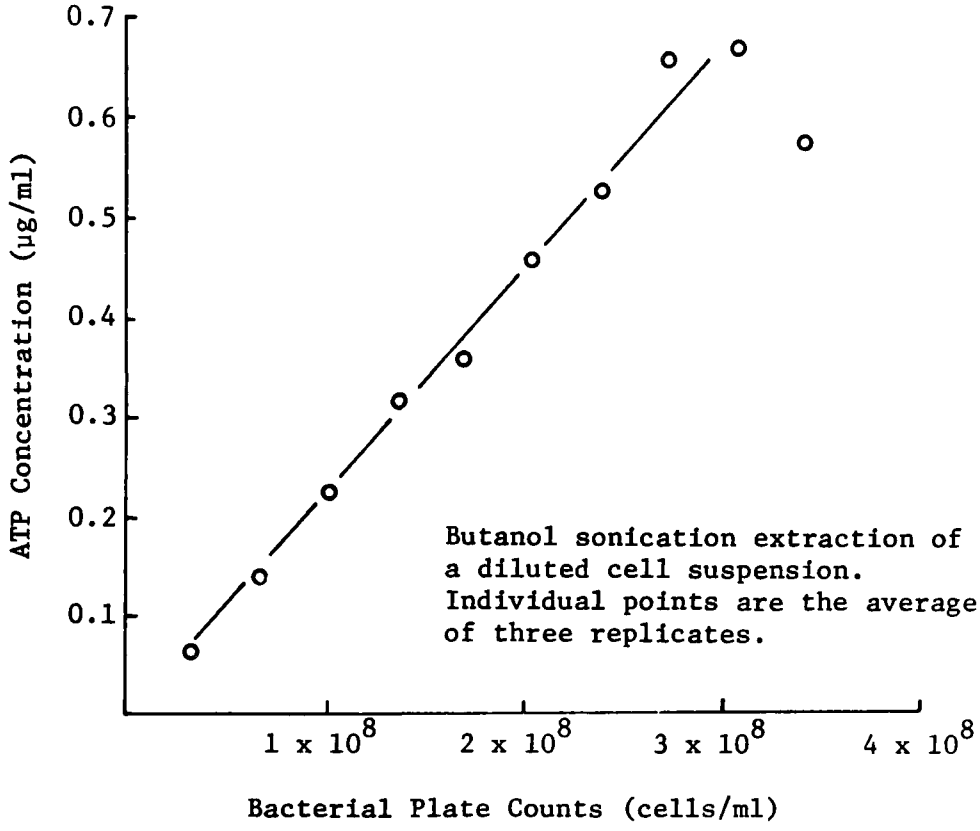


FIGURE 5

Relationship of Measured ATP Concentration
to Bacterial Cell Concentrations of Pure Cultures

The precision in performing the ATP assays on mixed liquor was achieved with a coefficient of variation of approximately 10%. Measurements of ATP and bacterial plate counts of mixed liquor were also compared. As shown in Figure 7, a linear relationship with some scatter was found.

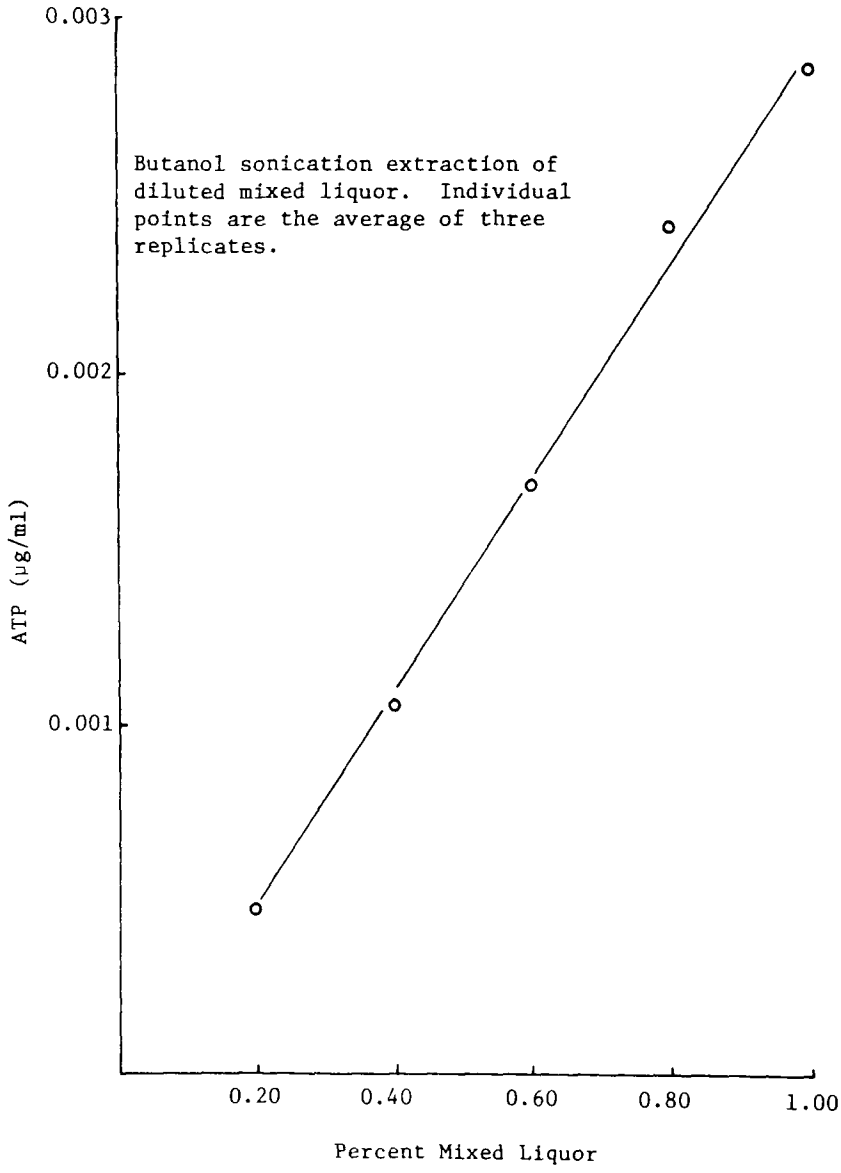


FIGURE 6

Relationship of Measured ATP Concentration to Mixed Liquor Concentrations

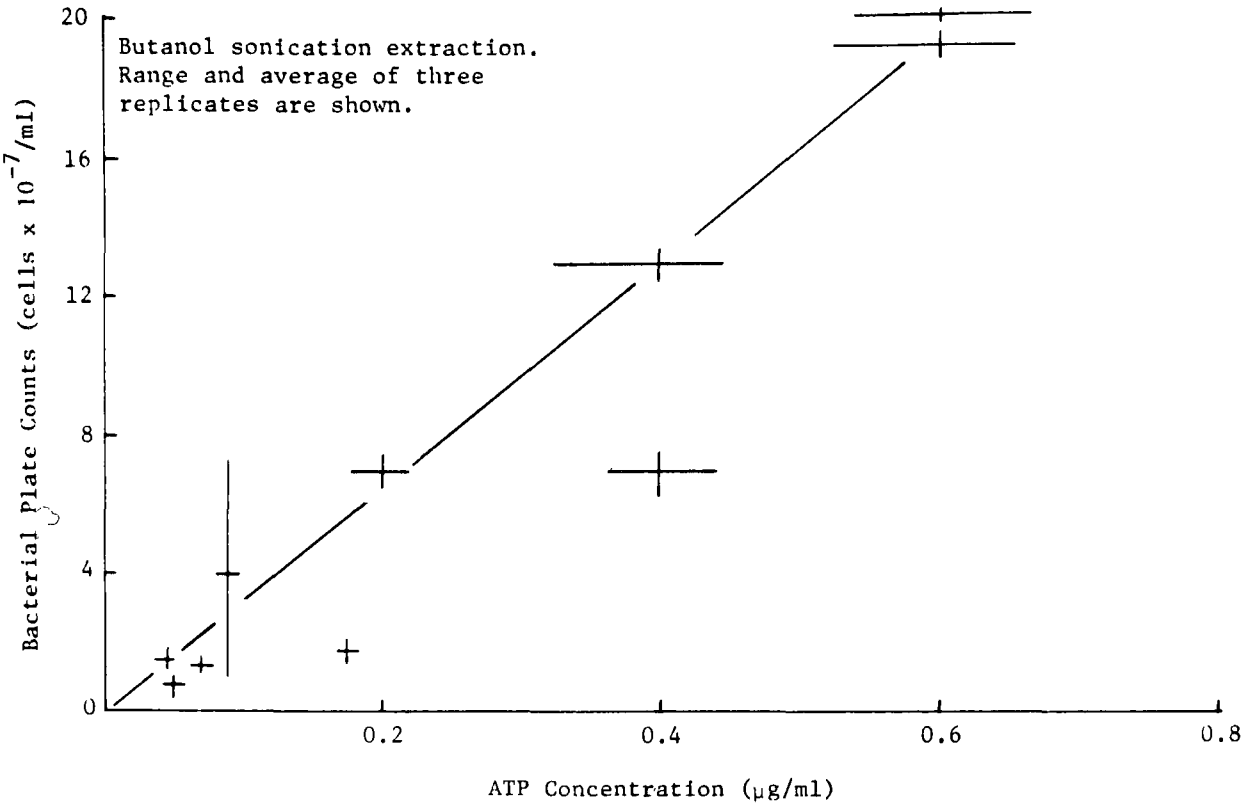


FIGURE 7

Relationship Between Measured ATP
Concentration and Number of Bacteria
in Mixed Liquor

The question concerning the presence of extracellular ATP (that not contained in microorganism which could enter mixed liquor with primary effluent or result from death and lysis of sludge microorganisms) was raised. A series of experiments was, therefore, conducted on mixed liquor and mixed liquor filtrate to determine the percentage of ATP which might be expected to occur outside viable organisms. Figure 8 shows the results of a typical experiment which was conducted. Extracellular concentrations of ATP were found to be relatively insignificant in proportion to the levels present in the particulate fraction of sludge.

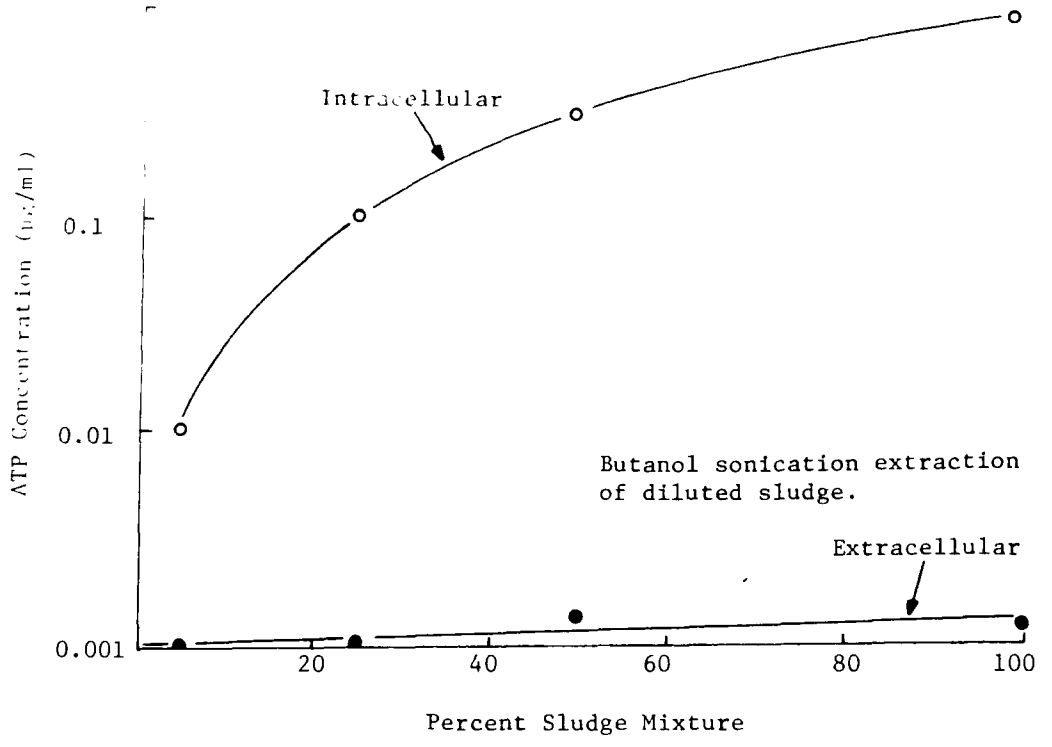


FIGURE 8

Concentration of Intracellular and Extracellular ATP

Correlation between ATP concentration and suspended solids in return sludge was investigated in the activated sludge component of the Baltimore Back River Sewage Treatment Plant. Measurements of ATP, SVS and SS levels in return sludge were made on separate days. As shown in Figure 9, there was no correlation between ATP and SVS. However, many of the changes in ATP level could be explained in terms of operational changes made at the plant.

A number of analyses were also performed on samples which were withdrawn from various stages in the aeration basin. Figure 10 shows the typical correlation between SVS, ATP, and BOD which were found. As shown, an increase in ATP throughout the aeration basin appeared to correlate with decreases in BOD. The SVS and SS (not shown) remained relatively constant.

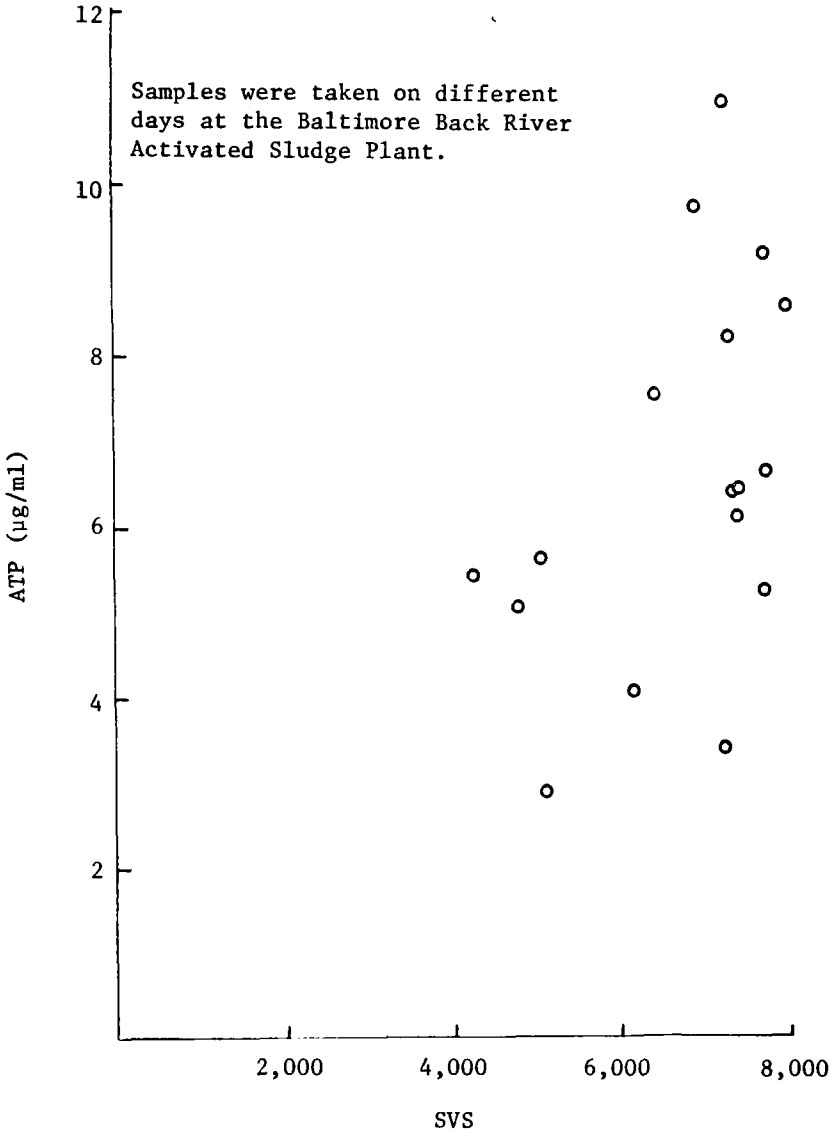


FIGURE 9

Relationship of ATP to SVS in Return Sludge

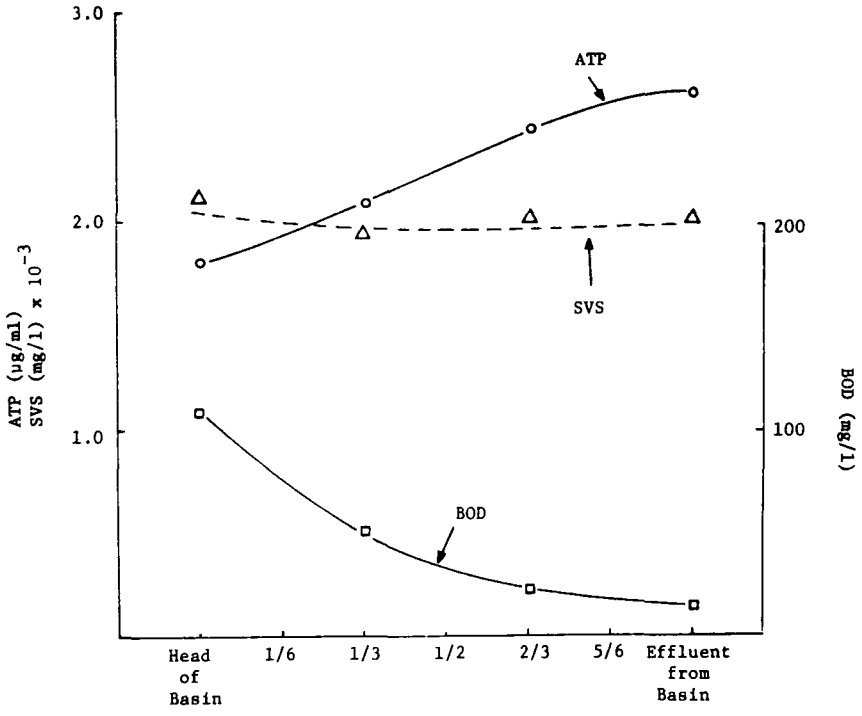


FIGURE 10

ATI , BOD (settled) and SVS at Various Points in the Aeration Basin at Baltimore Back River Treatment Plant

A series of flask studies were performed to determine the effect of ATP concentration on BOD removal. Utilizing return sludge and primary effluent from the Back River Plant in Baltimore, nine mixed liquors were prepared for each of a series of laboratory experiments. Return sludge for the nine mixed liquors ranged from zero percent to 40%, in increments of 5%, as measured by volume. The initial ATP concentration of each mixed liquor was prepared by calculation based on ATP measurements of the return sludge and primary effluent. Flasks containing these mixed liquors were then aerated for eight hours. Typical results are shown in Figure 11. An ATP concentration of 1-2 µg/ml was found to be the minimum concentration which could effect 95% BOD reduction during the eight-hour aeration period.

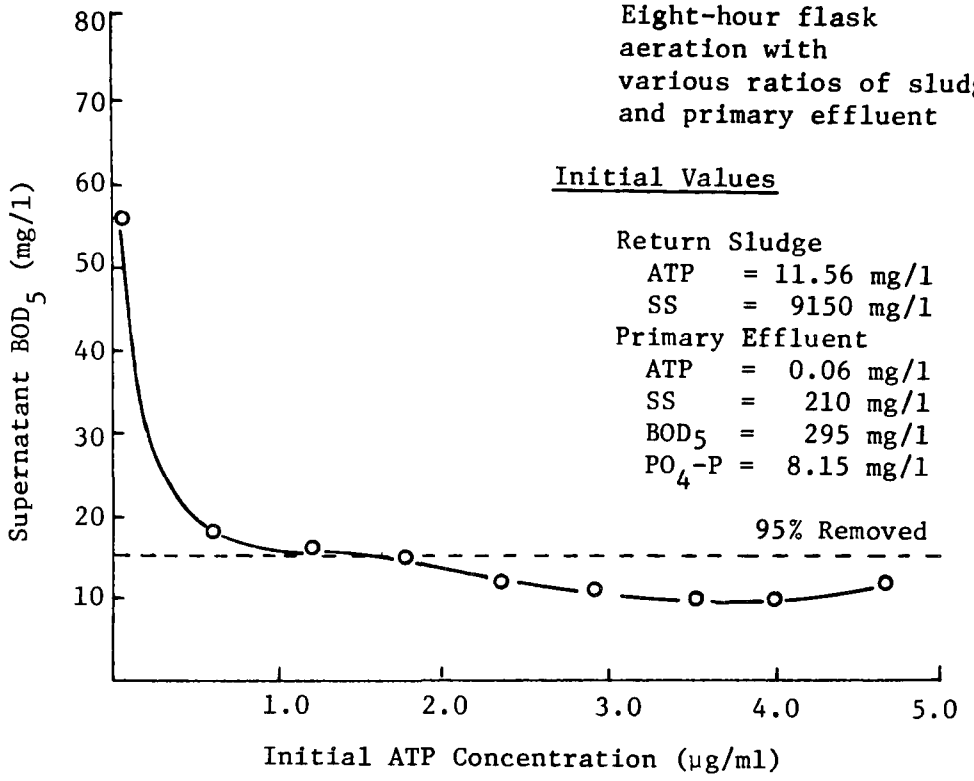


FIGURE 11

BOD_5 Reduction vs. ATP Concentration

Increased initial ATP concentration did not significantly increase BOD reduction. However, no experiments with Baltimore sludge were performed using shorter aeration periods to determine whether the higher ATP concentrations might shorten the time required for 95% BOD removal.

Similar experiments were run on sludge and primary effluent from a Washington, D. C. plant. A two and one-half hour aeration period was used. Concentrations of ATP (below 1 µg/ml) appeared to be most effective, again indicating that heavier than needed (on the basis of BOD_5 reduction) MLSS were being carried at the plant.

Flask studies were undertaken to measure the metabolic activity of mixed liquor in relation to the measured biomass level. Mixed

liquor from the Baltimore Back River Treatment Plant was compared with mixed liquor from the Washington, D. C. plant. Oxygen uptake rate and evolution of $^{14}CO_2$ from media containing ^{14}C-glucose by sludge were the methods of determining metabolic activity. As shown in Table 1, the ATP concentration of both systems was similar; however, the Washington sludge was far more active than the Baltimore sludge when placed in either Washington or Baltimore primary effluent. The difference was thought to be caused by the large BOD concentration introduced by the Washington sludge as the result of digester supernatant being introduced into the return sludge line.

TABLE 1

Comparison of BOD Concentration, ATP Concentration, $^{14}CO_2$ Evolution Rate and O_2 Uptake Rate at Various Intervals During the Aeration of Mixed liquor from the Baltimore and Washington, D. C. Sewage Treatment Plants

	WASHINGTON				BALTIMORE			
	BOD_5 mg/l	O_2 Uptake Rate ppm/ min.	$^{14}CO_2$ Evolution CPM	ATP mg/l	BOD_5 mg/l	O_2 Uptake Rate ppm/ min.	$^{14}CO_2$ Evolution CPM	ATP mg/l
Primary Effluent	209	-	-	-	185	-	-	-
Return Sludge	-	10.1	5,762	7.75	-	2.5	1,652	7.74
Aero Time Aeration	323	-	800	1.94	143	-	200	2.00
0.5 Hrs. Aeration	-	1.90	-	2.29	-	0.88	-	2.29
1.0	-	1.90	547	2.62	-	0.89	156	2.61
2.0	-	1.37	1,583	2.83	-	0.56	548	2.99
3.0	-	1.70	1,057	2.79	-	0.49	466	2.79
4.0	-	1.38	1,063	3.19	-	0.37	393	2.99
5.0	-	1.11	-	2.38	-	-	-	2.43
Final Effluent	108	-	-	-	41	-	-	-

SECTION V

TECHNIQUE IMPROVEMENT

The ATP assay procedure was further developed to provide greater accuracy and precision. The earlier technique had been to dispense 0.1 ml of enzyme solution into a cuvette, place the cuvette in the bioluminescence instrument and inject 0.01 ml of ATP solution into the assay cuvette. The ATP solution was quantitatively measured with a 0.05 ml Hamilton syringe. A rubber septum in the top of the instrument was used as the injector port.

A careful step by step analysis of this procedure revealed several sources of error which *in toto* reduced both the accuracy and precision of the assay.

ATP was found to adhere to the inside walls of the Hamilton syringe. A "memory" of prior solutions was thus carried over to subsequent solutions. Even with extensive rinsing procedures using several different wash solutions, contamination with ATP could not be overcome. The problem was most critical when a solution containing a low level of ATP was assayed after assay of a solution which had relatively high levels of ATP. The injection of the ATP solution was also critical. A rapid, smooth injection directly into the reaction mixture was necessary; however, the injection itself was not visible; therefore, one could not be sure that the entire sample had quantitatively entered the reaction mixture. Aerosol formation and splash during injection could easily result in an undetermined amount of sample not reaching the bottom of the cuvette. Careful scrutiny of the injection needle after injection, but prior to withdrawal from the injection port, did reveal the presence of minute droplets and a liquid film. It was subsequently found that the septum wiped the needle during withdrawal, and continuous injections caused a large droplet to form on the bottom of the septum. This meant that the needle could easily become contaminated during insertion.

Several changes in the procedure were made to minimize these difficulties. The injection port was changed in such a way as to eliminate the septum. An adaptor containing O-ring seals was fixed to the barrel of the injection syringe. This adapted syringe therefore formed the light seal when fitted into the recess in the top of the instrument. The injection procedure was also reversed. Instead of ATP solution being injected into reaction mixture, the reaction mixture was injected into the ATP solution. This eliminated the necessity of rinsing the injection syringe since that syringe was used only for reaction mixture and therefore did not become contaminated. The volume of reaction mixture was also found to be noncritical, therefore, a precise quantitative delivery into the

cuvette was not necessary. Losses due to spray or splash were essentially not important. It was also determined that a plateau in response occurred with an injection of 0.04 ml or more of reaction mixture. A 0.05 ml injection volume was therefore adopted. This procedural change represented a saving since costs for the reagents were greatly reduced.

Solutions containing ATP were placed in cuvettes by the following procedure: 26 gauge teflon spaghetti tubing was cut into sections approximately 10 cm in length. A section of this tubing was then placed over the tip of a 50 µl Hamilton syringe, and a 10 µl sample drawn up into the tubing and measured according to calibrations on the syringe. However, no sample was allowed to come in contact with the syringe needle or body. Excess sample was tapped or touched-off from the tip of the tubing and the measured quantity extruded slowly and carefully into the bottom of the cuvette. Since the aqueous sample does not wet the teflon, the entire sample is quantitatively removed from the tubing with no remaining droplet. Placement of the entire 0.01 ml sample in the bottom of the cuvette may be easily observed. The teflon tubing is disposable and was discarded after each sample; hence, the problem of carryover was completely eliminated.

In attempting to simplify the extraction procedure, a number of extractants were tried, including dimethylsulfoxide (DMSO), boiling arsenate buffer, boiling Tris buffer, perchloric and trichloroacetic acids.

A number of experiments were run using DMSO at concentrations varying from 20 to 90% on sludge to extract ATP and also on standard ATP solutions. Conditions included the use of hot DMSO solutions and solutions chilled in an ice bath and sonication. When compared with the butyl alcohol procedure, the DMSO procedures were all unsatisfactory. While high concentrations of DMSO appeared to extract ATP from sludge, this result was offset by the fact that similar concentrations of DMSO inhibited the reaction of the ATP standards.

Knowles and Smith (3) used perchloric acid extraction to measure the ATP content of Azotobacter vinelandii. This procedure was adapted for the extraction of ATP from sludge. Perchloric acid inhibits the action of the enzyme luciferase on ATP at the concentrations required for the extraction of ATP from cells. Consequently, two steps must be taken to eliminate the inhibition. The first step is to use a concentrated potassium hydroxide solution to precipitate potassium perchlorate, which has a low solubility, and then to filter off the insoluble salt. The second step requires further dilution to remove completely the inhibitory effect of the remaining perchlorate ions. The neutralization of perchloric acid generates considerable heat, and the mixture must be chilled at this step. The subsequent filtration and dilution provide a solution that gives satisfactory values for both the sludge and the standards. Objections to this procedure are the time consuming manipulations involved and the large dilution factor imposed.

Patterson, et al. (2) used boiling Tris buffer to extract ATP from sludge. Holm-Hansen and Booth (4) had also used this extractant in the assay of ATP in ocean water. The Tris buffer method has a number of advantages. The use of a buffer at the same pH as the enzyme reaction mixture subsequently employed in the assay eliminates neutralization and filtration. The final dilution can be controlled to provide an ATP concentration range that is suitable for the ATP assay. The contact time of the diluted sludge with the boiling Tris buffer is short, and the whole extraction procedure can be performed speedily by an operator, or it could be readily automated. The dilution factor is also small in comparison with the other procedures.

The use of boiling arsenate buffer for extraction was also investigated since enzyme preparations used during the early portion of the study (Contract No. 14-12-419) were made up in this buffer (0.02 M, pH 7.4). Arsenate buffer had also been used for preparation of ATP standard solutions. During the extraction studies ATP standards were found to be deactivated by freezing in arsenate buffer, but at least partially reactivated by boiling.

This unusual finding prompted a study on quantitation of standard ATP solutions, which is presented in Section VI of this report. As a result, the use of arsenate buffer was eliminated from all phases of the extraction and assay procedures.

The butanol sonication extraction procedure, employed the use of arsenate buffer. After the problems with this buffer were discovered, attempts were made to substitute Tris buffer. These attempts were not successful. Since an alternate procedure to butanol sonication was being sought, emphasis was placed on other extraction techniques and the use of butanol sonication was abandoned.

The use of trichloroacetic acid in relatively dilute solutions, 5 to 10%, for the extraction of cell components and the precipitation of protein has been a standard method for many years. The extraction and precipitation can be done without the use of heat and, because of the low concentration of extractant, the pH adjustment of this solution to that required for the ATP assay could be readily accomplished by dilution with Tris buffer. The method is simple to use and can be accomplished rapidly, either manually or by automation.

A series of comparisons were made using Tris buffer or perchloric or trichloroacetic acids as the extractants of ATP from sludge. The validity of these methods was also established on ATP standard solutions of known concentration. The determination of the purity of the ATP employed, the replication of standard solution assays, and the extraction of ATP by the three methods are given in detail in order to establish the validity of the assays subsequently presented.

SECTION VI

QUANTITATION

The ATP used was adenosine-5-triphosphate disodium salt 4.5 H_2O, molecular weight 632.2, purchased from Calbiochem, Los Angeles, California. Two methods were used to determine purity. The first was based upon the ultraviolet absorption measured at wave length 257 nm. The second was based upon paper chromatrography and visualization of the spot under ultraviolet light.

For the absorption method, 15 mg of the ATP were dissolved in 500 ml H_2O. Absorbance average of five measurements was 761. Molecular weight was calculated as follows:

$$\frac{15.4 \times 30}{761} = 607.9$$

In this equation, 15.4 is the molar extinction coefficient as given in the literature, 30 is the concentration of ATP in mg/l and 761 is the observed absorbance. The experimental molecular weight was 607.9, the calculated molecular weight is 632.2; the sample is, therefore, 96.2% of the theoretical value.

Paper chromatography was done using the following solvent system: isobutyric acid, ammonium hydroxide, water, ratio 66:1:33. The spot can be seen only under ultraviolet light. The four different spots in the chromatograph (Figure 12) represent areas of four different concentrations circled with a pencil. Under ultraviolet light, only one migration spot is visible for each concentration. If ADP were present a spot would have shown on a different site. None was observed. It is apparent that the purchased sample has a high degree of purity.

Five separate samples of ATP were weighed and serially diluted to provide concentrations of 1.0×10^{-3}, 1.0×10^{-4}, and 1.0×10^{-5} mg/ml. Each dilution was assayed in quadruplicate. The assay was conducted on 10 µl of the standard solution contained in disposable cuvettes. The enzyme reaction mixture, 50 µl, was injected into the ATP solution. The average of the responses for the three dilutions was 569 mv/µg/ml with a range of ±2%.

The precision of the four replicates is illustrated by the typical examples in Table 3. It is apparent that the replication range is not more than 3% and the average responses over a dilution range of one hundred fold is of the same magnitude.

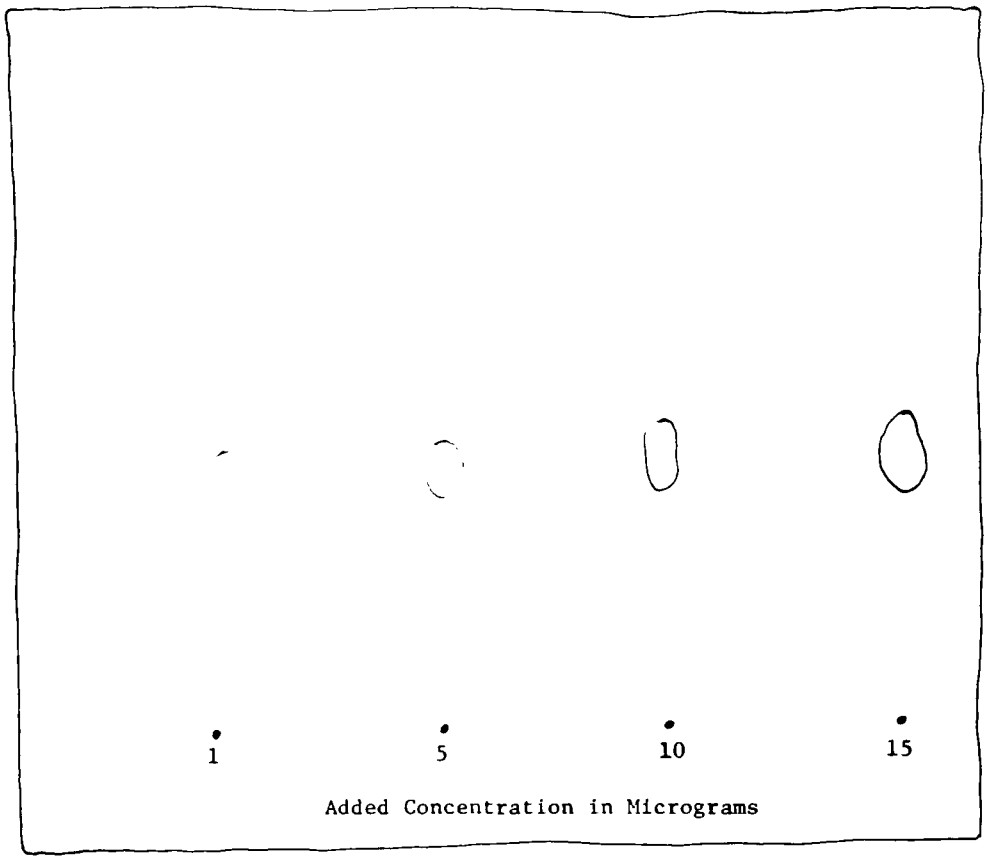


FIGURE 12

Chromatogram of ATP Standard

A series of assays on ATP dilutions ranging from 1 x 10^{-0} µg/ml through 1 x 10^{-5} µg/ml using 10 µl assay solution was run. Figure 13 is a logarithmic plot of the mv responses for each sample. A straight line is achieved between 1.0 x 10^{-0} µg/ml and 1.0 x 10^{-4} mg/ml. At the highest dilution (10^{-5} µg/ml), there is deviation.

Five separate stock solutions of ATP were made and dilutions of 1.0 x 10^{-3} mg/ml, 1.0 x 10^{-4} mg/ml, and 1.0 x 10^{-5} mg/ml were prepared from each. These were assayed using freshly prepared enzyme solutions and the assays were repeated four days later with fresh enzyme solutions prepared from the same batch. The ATP solutions were stored in the refrigerator during the interval. The results are given in Table 4.

TABLE 2

Analysis of ATP Standard Solutions

Sample No.	ATP (mg/ml)	Millivolts	Millivolts*/(ug/ml)
1	1.0×10^{-3}	557	557
2	1.0×10^{-3}	546	546
3	1.0×10^{-3}	581	581
4	1.0×10^{-3}	554	554
5	1.0×10^{-3}	563	563
		Avg. 560	Avg. 560 range ±3%
1	1.0×10^{-4}	56.5	565
2	1.0×10^{-4}	58.6	586
3	1.0×10^{-4}	56.4	564
4	1.0×10^{-4}	55.1	551
5	1.0×10^{-4}	56.2	562
		Avg. 57.4	Avg. 574 range ±2%
1	1.0×10^{-5}	5.76	576
2	1.0×10^{-5}	6.05	605
3	1.0×10^{-5}	5.80	580
4	1.0×10^{-5}	5.76	576
5	1.0×10^{-5}	5.68	568
		Avg. 5.81	Avg. 581 range ±4%

Average of all values and standard error 569 ±4%

*Sample Nos. 1-5 indicate separate weighings of ATP standard.

The responses after the four-day storage were slightly higher, 8.4, 6.1, and 0.8%, respectively, for the three dilutions. These small differences could be due to small differences in the activities of the freshly prepared enzyme solutions.

Table 2 and Table 4 give the results of the assays on ten separate weighings of ATP. There were five weighings in each table, and the preparation of 15 separate dilutions for each group produced a total of 30 solutions that were assayed in quadruplicate. The standard error for the means of all values was calculated in Table 2, and the average and its standard error is 569 ± 4%; in Table 4, the proper comparison is column 1 with an average of 590 ± 20.5%. The effect of storage is shown in column 2 of Table 4, and the average is 618 ± 7.4%. Comparison of these three means by student's t test showed that there were no significant differences between the means. The

TABLE 3

Replicate Assays of ATP Solutions

ATP(mg/l)	Millivolts*	
1.0×10^{-3}	545	
	554	
	579	
	550	
	Avg. 557	range ±3%
1.0×10^{-3}	569	
	559	
	538	
	550	
	Avg. 554	range ±3%
1.0×10^{-4}	57.0	
	55.8	
	55.7	
	56.9	
	Avg. 56.4	range ±1%
1.0×10^{-4}	56.0	
	56.9	
	55.0	
	57.0	
	Avg. 56.2	range ±2%
1.0×10^{-5}	5.79	
	5.80	
	5.80	
	5.82	
	Avg. 5.80	range ±1%
1.0×10^{-5}	5.89	
	5.64	
	5.83	
	5.68	
	Avg. 5.76	range ±2%

*Four responses are result of four assays of single solution.

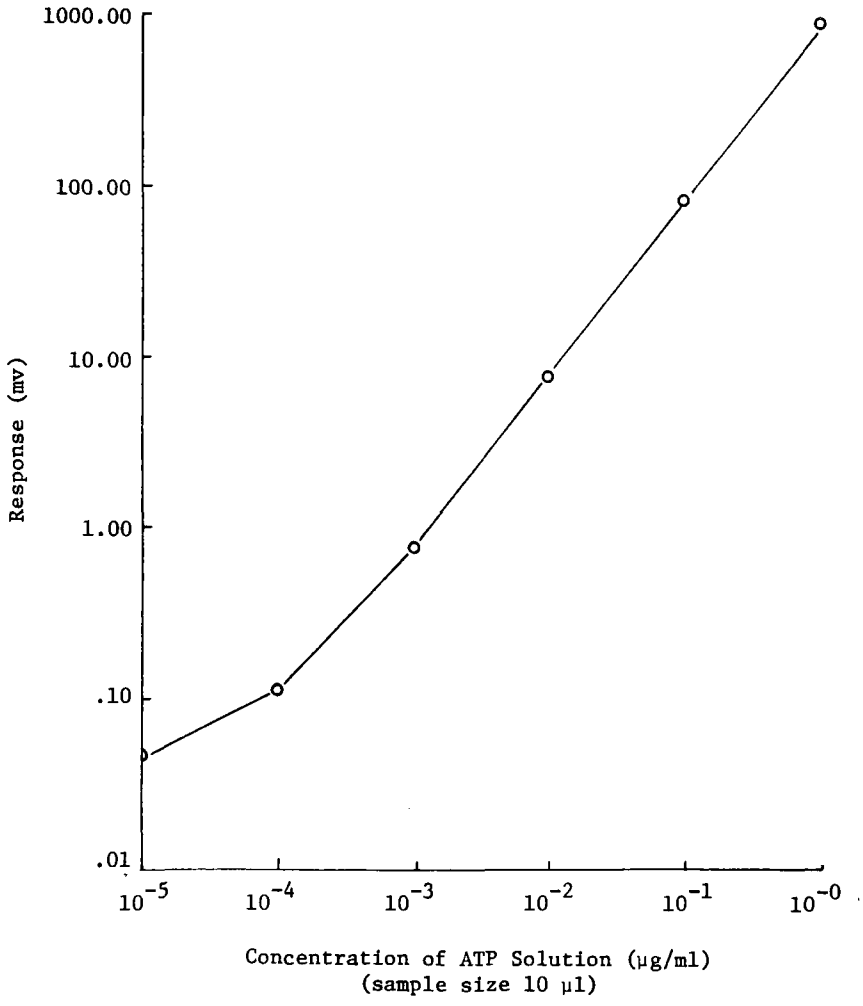


FIGURE 13

Instrument Response as Function of ATP Concentration

TABLE 4

Stability of ATP Solutions - Four-Day Storage

Sample No.	ATP(mg/l)	Initial Response Millivolts*	Four-Day Response Millivolts*
1	1.0×10^{-3}	555	567
2	1.0×10^{-3}	540	576
3	1.0×10^{-3}	568	600
4	1.0×10^{-3}	525	579
5	1.0×10^{-3}	480	571
		Avg. 534	Avg. 579
1	1.0×10^{-4}	553	588
2	1.0×10^{-4}	570	594
3	1.0×10^{-4}	581	616
4	1.0×10^{-4}	556	584
5	1.0×10^{-4}	531	578
		Avg. 558	Avg. 592
1	1.0×10^{-5}	650	620
2	1.0×10^{-5}	606	606
3	1.0×10^{-5}	767	787
4	1.0×10^{-5}	759	782
5	1.0×10^{-5}	612	623
		Avg. 679	Avg. 684
Average of all values and standard error		590 ±20.5	618 ±7.4

*Response expressed as mv per 1 µg ATP in 1 ml.

value for the difference of 21 between 659 of Table 2 and 590, first column, Table 4, was 1.0; the t value for the difference of 28 between the means in Table 4 was 1.3; and both values were well below the t value of 1.7 for significance at the 10% level. It is apparent that the storage for four days had not significantly affected the assay results.

In order to determine the stability of enzyme solutions, the standard solutions shown in Table 2 were assayed using enzyme solutions prepared one and four days previously and stored in the refrigerator. The results are shown in Table 5.

The assays with the day-old enzyme solution showed a slightly higher response, but it was well within the variation of the method. The assays with the four-day-old enzyme solution showed an average decrease in activity of 13%.

TABLE 5

Comparison of Assay Values With One-Day and Four-Day Old Enzyme Solutions

ATP (mg/l)	Millivolts* Original Value from Table 1	One-Day-Old Enzyme		Four-Day-Old Enzyme	
1.0×10^{-3}	557	594		516	
1.0×10^{-3}	581	587		503	
1.0×10^{-3}	563	575		493	
	Avg. 567	Avg. 585	+3%	Avg. 504	-11%
1.0×10^{-4}	565	581		497	
1.0×10^{-4}	564	588		495	
1.0×10^{-4}	562	574		489	
	Avg. 564	Avg. 581	+3%	Avg. 494	-12%
1.0×10^{-5}	576	566		486	
1.0×10^{-5}	580	599		493	
1.0×10^{-5}	568	568		490	
	Avg. 575	Avg. 578	+.5%	Avg. 490	-15%

*Response expressed as mv per 1 μg ATP in 1 ml.

The concentration of ATP in mixed liquor and in the return sludge is such that the samples must be diluted in order to reach a final concentration range (after extraction) between 2×10^{-5} and 2×10^{-6} mg/ml, which produces maximum extraction and falls within the range of linear responses.

Table 6 shows the effect of sample size on the extraction efficiency of the boiling Tris buffer extraction. A 3 ml sample gave results that were 25% lower than when a 1.5 ml sample was used. Note also that the precision of assay was reduced considerably by the larger sample. An experiment in which sludge was diluted in increasing amounts prior to extraction with boiling Tris buffer is shown in Table 7. The higher concentrations of sludge which were extracted yielded lower results than those which were diluted further prior to extraction. An average of 4.4 μg/ml was found in dilutions 2 to 10. Assuming this value is correct, the sludge which was not diluted prior to extraction showed an assay value which was 40% low. It was decided that sludge with an ATP concentration greater than 1 μg/ml (1×10^{-3} mg/ml) should be diluted prior to extraction.

TABLE 6

Effect of Sample Size on Boiling Tris Buffer Extraction

Boiling Tris Buffer Extraction and Assay of Five 1.5 ml Mixed Liquor Aliquots

0.99 µg ATP/ml sludge
1.05 µg ATP/ml sludge
1.00 µg ATP/ml sludge
1.00 µg ATP/ml sludge
1.04 µg ATP/ml sludge
Avg. = 1.02 µg ATP/ml sludge

Coefficient of Variation 2.75%

Boiling Tris Buffer Extraction and Assay of Five 3 ml Mixed Liquor Aliquots

0.78 µg ATP/ml sludge
0.87 µg ATP/ml sludge
0.72 µg ATP/ml sludge
0.70 µg ATP/ml sludge
0.85 µg ATP/ml sludge
Avg. = 0.78 µg ATP/ml sludge

Coefficient of Variation 9.7%

On the other hand, the procedure should not produce a dilution so great that the multiplication of the response times the dilution results in a large magnification of the error in precision. The perchloric acid procedures applied to return sludge produce a high dilution factor; the trichloroacetic acid and Tris buffer procedures give lower dilution factors.

The three procedures are described in detail so that the times required to perform each can be compared and also the dilution factors.

Perchloric Acid Procedure

Dilute 1 ml of sludge to 20 ml with distilled water. Add 1 ml of the diluted sludge to 4 ml chilled water in an ice bath, then add 1 ml of 30% perchloric acid solution. Neutralize carefully with 1.3 ml of a solution prepared by mixing 67.5 ml of saturated potassium hydroxide solution with 180 ml of Tris buffer pH 7.75. Filter and dilute 2 ml of the filtrate to 50 ml with Tris buffer pH 7.75. This solution, which represents a dilution of 3650, is

TABLE 7

Effect of Sludge Concentration on the Efficiency of Boiling Tris Buffer Extraction

Dilution Factor Before Extraction	Dilution Factor Due to Extraction	Concentration of ATP (ug/ml) of Nondiluted Sludge	Calculated* Concentration of ATP Actually Measured (mg/ml)	Calculated* Concentration of ATP Prior to Extraction (mg/ml)
1	50	2.59	8.8×10^{-5}	4.4×10^{-3}
2	50	3.93	4.4×10^{-5}	2.2×10^{-3}
4	50	4.44	2.2×10^{-5}	1.1×10^{-3}
6	50	4.05	1.5×10^{-5}	7.5×10^{-4}
8	50	4.36	1.1×10^{-5}	5.5×10^{-4}
10	50	4.74	8.8×10^{-6}	4.4×10^{-4}

*Based upon average value obtained in dilutions 2 to 10.

assayed. The elapsed time for preparation and assay is approximately 20 minutes.

Tris Buffer Procedure

Dilute 1 ml of sludge to 10 ml with distilled water. To approximately 35 ml of Tris buffer pH 7.75 in a 50-ml volumetric flask that has reached a temperature of at least 98°C in a boiling water bath add 1 ml of the diluted sludge. Mix thoroughly and immediately chill in an ice bath, dilute to 50 ml with Tris buffer, filter, and assay. The dilution factor is 500. The elapsed time for preparation and assay is approximately ten minutes.

Trichloroacetic Acid Procedure

Dilute 1 ml of sludge to 10 ml with distilled water. To 4 ml of 5% trichloroacetic acid solution, chilled in an ice bath, add 1 ml of the diluted sludge. Mix thoroughly, then add 1 ml of the mixture to approximately 35 ml of chilled Tris buffer pH 7.75 in a 50-ml volumetric flask. Make to volume with Tris buffer and assay.

The dilution factor is 2500. The trichloroacetic acid method can be used at a dilution factor of 1250 by bringing 1 ml of the mixture to 25 ml with Tris buffer. The values reported hence were obtained with the 2500 dilution factor. The elapsed time for preparation and assay is approximately five minutes.

Tables 8 and 9 compare results obtained by the three procedures using the Tris buffer as the standard. As shown in Table 8, the Tris buffer method gave a higher ATP concentration than the perchloric acid extraction. The perchloric acid procedure requires more manipulation than does either the Tris buffer or the trichloroacetic acid procedure, and the high dilution which results is a source of error that must be considered. The Tris buffer and trichloroacetic acid procedures, Table 9, gave close results. Either of these methods will provide reproducible results.

The effect of boiling time on ATP extraction from sludge in the Tris buffer procedure was examined. As shown in Figure 14, maximum extraction occurs very soon after immersion of the sample into boiling Tris buffer. A gradual decrease in activity resulted as boiling time was increased. Maximum extraction was achieved within one minute and this time was selected for the procedure.

The use of a blending technique to aid sampling was investigated. Table 10 shows that a lower ATP value was always obtained for blended samples. It is likely that blending resulted in the rupture of larger cells (protozoa, algae, and fungi) and that the released ATP was rapidly metabolized by other organisms. Differences in the percent decrease due to blending may be due to differences in the populations present. Although precision of sampling was improved by blending the procedure was considered detrimental; investigation into the lysis of various microbial forms by blending might provide useful information.

Recovery experiments of ATP added to sludge presented a problem. When ATP was added directly to sludge, the ATP began to disappear immediately as the viable organisms incorporated it into their metabolic cycle. To stop the action of the organisms in the instances of perchloric acid and trichloroacetic acid extractions, the ATP solution was added immediately after the mixture of the diluted sludge and the acid. In the boiling Tris buffer procedures, the ATP solution was added immediately after the diluted sludge was introduced into the boiling Tris buffer. These procedures effectively stopped the action of the viable cells, and the recoveries, as shown in Table 11, were considered satisfactory. Each value shown in the table is the average of three individual determinations.

TABLE 8

Comparison of Tris Buffer and
Perchloric Acid Extractions

Sample	ATP (µg/ml sludge) Tris Buffer	Perchloric Acid	Perchloric/Tris %
H 1	4.03	3.04	
H 2 duplicate	4.03	3.42	
	Avg. 4.03	Avg. 3.22	80
E 1	3.71	3.23	
E 2 duplicate	3.77	3.42	
	Avg. 3.74	Avg. 3.33	88
M 1	3.18	2.70	
M 2 duplicate	3.82	2.66	
	Avg. 3.50	Avg. 2.68	77

TABLE 9

Comparison of Tris Buffer and
Trichloroacetic Acid Extractions

Sample	ATP (µg/ml sludge) Tris Buffer	Trichloroacetic Acid (TCA)	TCA/ Tris %
a	3.39	3.56	100
b	3.88	3.26	84
c	3.20	3.34	104
d	3.28	3.16	97
e	3.08	3.42	111
	Avg. 3.39	Avg. 3.35	99.2

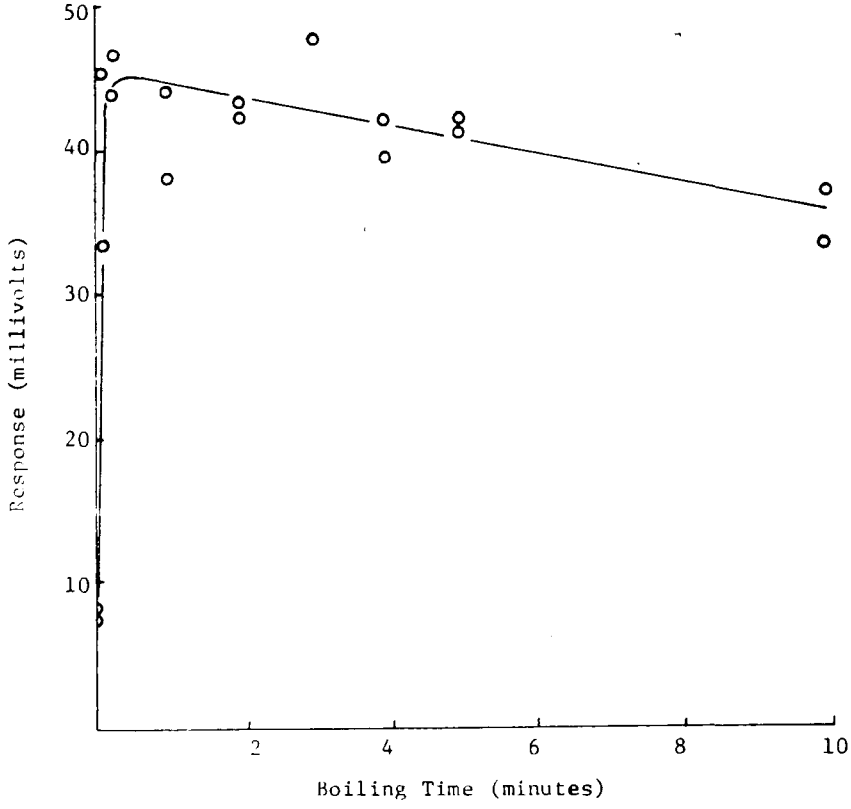


FIGURE 14

Efficiency of Extraction by Variation of
Boiling Time in Tris Buffer

Inspection of the results shows that, essentially, the recovery of ATP added during the extraction procedure is the same for all three methods. This type of experiment provides no absolute information relative to the efficiency with which ATP is extracted from the cells. If one procedure is significantly more effective than another in the extraction of ATP, then higher results would be obtained by that procedure. The values by the Tris buffer procedure and by extraction with trichloroacetic acid are practically identical, Table 9, whereas the perchloric acid procedure gave extractions ranging from 10 to 25% less, in the three cases listed in Table 8.

TABLE 10

Effect of Blending on the
Extraction of ATP from Sludge

**Sludge Samples	Blended*	Non-Blended	Decrease Due to Blending
1	2.00	2.70	28%
	1.98	2.85	
2	1.62	2.56	25%
	1.92	2.14	
3	1.31	1.41	10%
	1.20	1.36	
4	0.76	1.05	10%
	0.80	0.67	

* Blending was conducted for two minutes using the high speed setting on a Sears Eight Speed Blender.

**Samples were obtained on separate days from Biospherics' Pilot Plant.

TABLE 11

Recovery of ATP in the Tris Buffer, Perchloric,
and Trichloroacetic Acid Extraction Procedures

	ATP in Sludge (µg/ml)	ATP Added (µg/ml)	Total ATP Measured (µg/ml)	Added ATP Recovery (µg/ml)
Tris Buffer	3.63	2.0	5.63	2.08
	2.93	2.0	4.93	1.78
	3.05	2.0	5.05	1.82
				Avg. 1.89 (94.5%)
Perchloric Acid	4.70	5.0	9.70	5.75
	4.02	5.0	9.02	4.80
	4.30	5.0	9.30	5.22
				Avg. 5.25 (105%)
Trichloroacetic Acid	2.15	2.0	4.15	1.82
	2.15	2.0	4.15	2.03
	2.30	2.0	4.30	1.84
				Avg. 1.90 (95%)

N.B. These recoveries were performed on different sludge samples.

SECTION VII

CORRELATION BETWEEN ATP AND OTHER PARAMETERS

In the experiments on pure cultures, the ATP content of the microorganisms was determined and the following parameters were also measured: the tyrosine content of the cells (to provide an identication of total mass based upon protein concentration), the oxygen uptake rate (to measure the metabolic state of the organisms), turbidity, and microscopic cell counts.

Oxygen uptake rates were determined using a Beckman Model 77 oxygen analyzer and a polarographic sensor. Samples were placed in a stirred 125-ml Erlenmeyer flask. The O_2 uptake rate was measured either immediately or after a one-minute aeration period to provide an initial O_2 concentration. Calibration of the instrument and O_2 uptake determinations were performed according to instructions provided with the instrument.

Tyrosine determination was made according to the Folin-Ciocalteu method (5). One ml of test solution was placed in a 50-ml volumetric flask, 2 ml of 5N NaOH were added, and the mixture was heated in a boiling water bath for five minutes. The flask was then removed from the bath, 25 ml of H_2O were added, and the solution was cooled on ice. Next, 3 ml of phenol reagent were added, and the solution was brought to the mark with water. After a five-minute color development, the absorbance was measured in a B & L Spectronic 20 spectrophotometer at 640 nm. Tyrosine concentration was determined from a standard curve prepared from standard solutions of tyrosine. In the case of pure cultures, a sample of culture was filtered through a 2 cm glass fiber filter, washed with 0.25 M of pH 7.75 Tris buffer, and the entire filter was subjected to treatment as described above.

Microscopic counts were made in a Levy ultra-plane counting chamber, using a Zeiss phase contrast microscope. Cell suspensions were diluted 1:10 immediately after withdrawal from the culture flask with 0.1 N KI_3. This solution stopped all further cell replication and imparted a light stain on the cells.

Pure cultures of several typical organisms found in wastewater were grown in a 2-liter flask equipped as shown in Figure 15. Oxygen uptake rate was monitored periodically with a Beckman Oxygen Analyzer connected to a Hewlett-Packard recorder. Both influent and effluent air passed through a sterile glass wool filter. An air flow rate of approximately 150 cc/min. was used to maintain a disolved oxygen level of at least 4 mg/l in the growing culture. Oxygen uptake readings were made by stopping the inflowing air and recording the decrease in oxygen concentration at a known chart speed. Air was

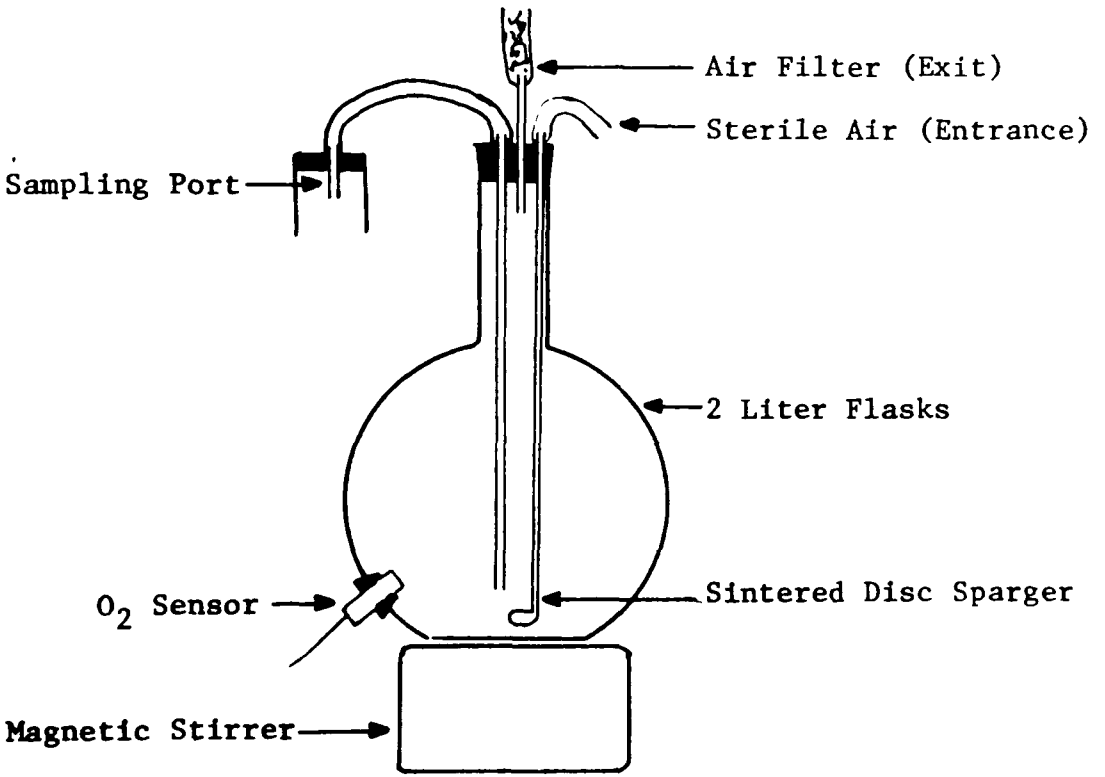


FIGURE 15

Apparatus Used for Pure Culture Studies

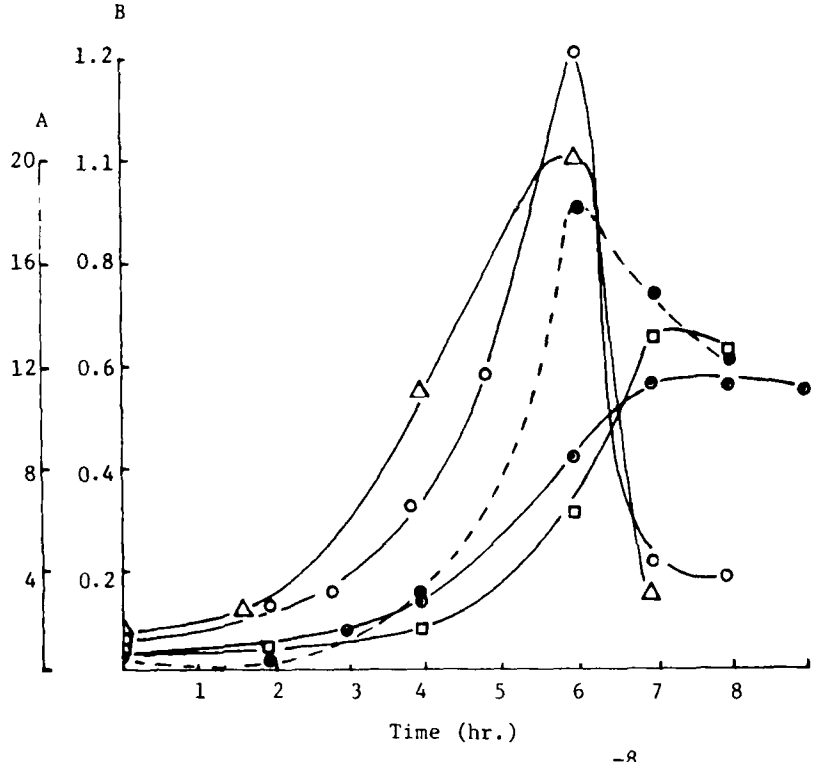
shut off during these readings for a maximum of four minutes. Sampling of the culture was performed by clamping the exit air tube and collecting the sample which was pressure siphoned up and out of the sampling port. Samples were analyzed immediately after collection.

Figure 16 illustrates the results obtained upon a culture of E. coli. Oxygen uptake rate and tyrosine and ATP contents reached a maximum at six hours of growth and then declined, whereas turbidity remained at a high level and total count declined slightly. Similar results, shown in Figure 17, were obtained with a culture of Z. ramigera; in the case of the latter organism, the total count declined rapidly. Since total microscopic count measures both viable and nonviable organisms, it is possible that death rate and cellular decay are greater for Z. ramigera than they are for E. coli. It is difficult to estimate the total counts for Z. ramigera because the organism forms clumps. Assays were also performed on a gram-positive spore forming rod designated Bacillus sp. A typical run with this organism is shown in Figure 18. Concentration of ATP per unit volume of culture reached a maximum before either turbidity or total microscopic counts attained maximum values, then decreased rapidly.

In all cultures studied, the ATP concentration showed a peak which preceded stationary growth as determined by turbidity. As the cultures aged, the ATP concentration fell to a low level. Of all parameters measured, the ATP levels followed oxygen uptake rates most consistently.

Tables 12, 13, and 14 show the concentration of ATP per cell, based upon the total microscopic count and the ratio of the ATP concentration to tyrosine (ATP µg:tyrosine µg). The Bacillus sp. cultures provided the most reliable ATP/cell data because these large rods could be counted more accurately than the smaller Zooglea and E. coli cells. Data from three separate Bacillus sp. cultures yielded an average value of 7.4×10^{-9} µg ATP/cell with a coefficient of variation of 15%. Values of the same order of magnitude had been reported by Chappelle and Levin (6). Several cultures of Sphaerotilus natans were assayed. The results could not be evaluated in terms of total counts since the organisms formed trichomes. The ratio of ATP/tyrosine for the large cells of Bacillus sp. and of Sphaerotilus fell within the same range of values that were obtained for the smaller cells of E. coli and Z. ramigera.

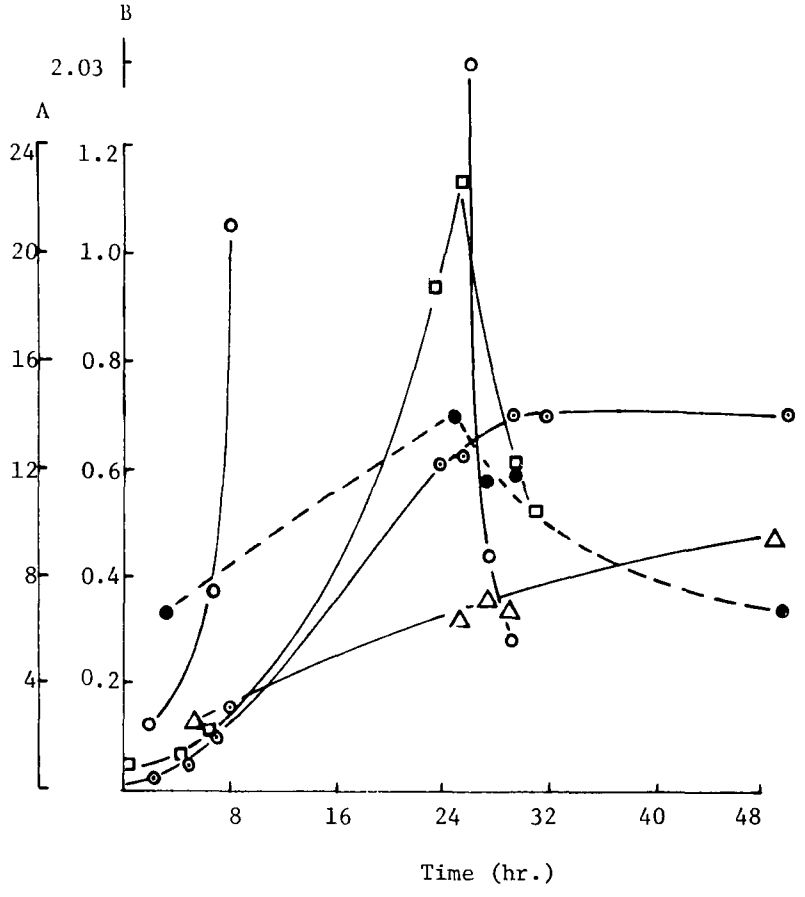
Some cultures showed a slightly elevated ATP/cell or ATP/tyrosine concentration during logarithmic growth. However, a generalization could not be made. Z. ramigera (Table 13) showed the highest levels during the lag phase of growth. Some cultures after reaching stationary growth showed a decrease in ATP/cell or ATP/tyrosine concentration, but others failed to show a decrease after more than



A = □ - Total microscopic counts ($x10^{-8}$)
△ - Tyrosine (µg/ml)
B = ○ - Oxygen uptake rate (mg/l/min)
● - Turbidity (optical density)
● ATP extracted with perchloric acid (µg/ml)
Nutrient broth, 25°C

FIGURE 16

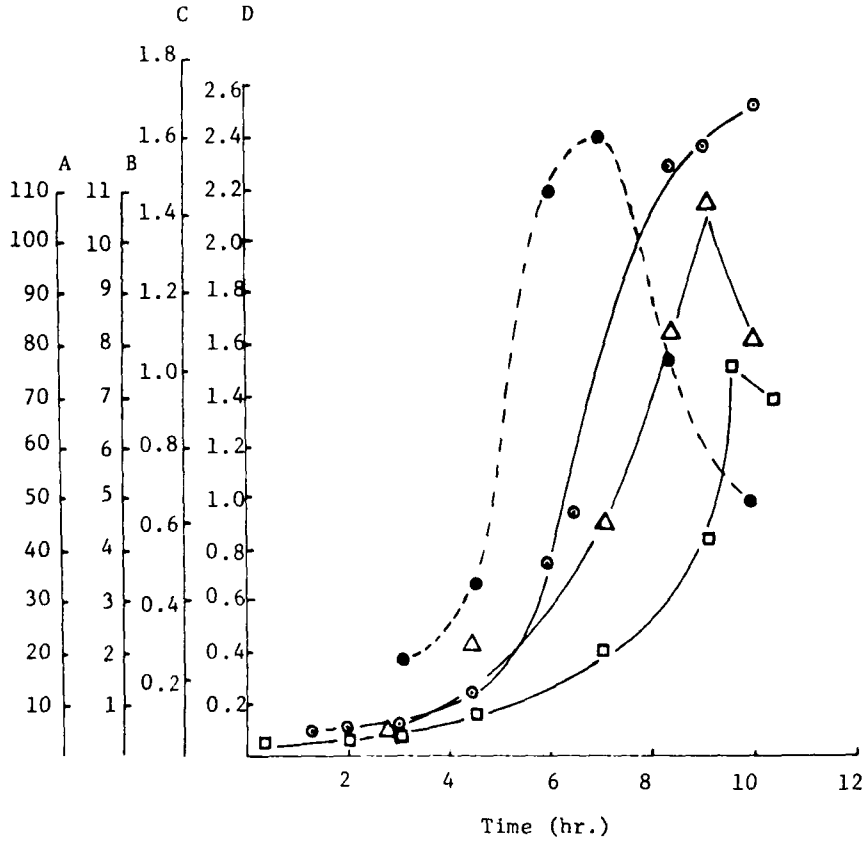
ATP, Tyrosine, Oxygen Uptake Rate,
Total Count and Turbidity of a Growing E. coli Culture



A = □ - Total microscopic bacterial counts ($x10^{-8}$)
△ - Tyrosine (µg/ml)
B = ○ - Oxygen uptake rate (mg/l/min)
⊙ - Turbidity (optical density)
● - ATP extracted with perchloric acid (µg/ml)

FIGURE 17

ATP, Tyrosine, Oxygen Uptake Rate,
Total Count and Turbidity of a Growing Z. ramigera Culture



A = △ - Tyrosine (µg/ml)
B = □ - Total microscopic counts (x 10^{-8} cell/ml)
C = ● - ATP extracted with perchloric acid (µg/ml)
D = ⊙ - Turbidity (optical density)

FIGURE 18

ATP, Tyrosine, Total Microscopic Counts
and Turbidity of a Growing Bacillus sp. Culture

TABLE 12

Concentrations of ATP Per Cell, Based on Total Count and Amount Per µg of Tyrosine Found During the Growth of the E. coli Culture

Time (hr.)	ATP (µg/cell)	Ratio ATP (µg) to Tyrosine (µg)
0	3.9×10^{-10}	0.09
2	6.3×10^{-10}	0.12
4	17.0×10^{-10}	0.13
6	6.3×10^{-10}	0.10
8	6.3×10^{-10}	0.11

TABLE 13

Concentration of ATP Per Cell, Based on Total Count and Amount Per µg of Tyrosine Found During the Growth of Z. ramigera Culture

Incubation Time (hr.)	ATP (µg/cell)	Ratio ATP (µg) to Tyrosine (µg)
5.5	30.8×10^{-10}	0.56
25.5	3.1×10^{-10}	0.54
29.5	5.0×10^{-10}	0.45
50.0		0.17

TABLE 14

Concentration of ATP Per Cell, Based on Total Count and Amount Per µg of Tyrosine Found During the Growth of the Bacillus sp. Culture

Incubation Time (hr.)	ATP (µg/cell)	Ratio ATP (µg) to Tyrosine (µg)
3	7.2×10^{-9}	0.19
4.5	5.3×10^{-9}	0.10
6	9.7×10^{-9}	0.32
7	7.6×10^{-9}	0.17
21	7.5×10^{-9}	0.23
24	7.1×10^{-9}	0.18

12 hours of stationary growth. Analysis of all pure culture data failed to show a definite pattern in maximal or minimal ATP concentrations relative to growth phase. Very old cultures would very likely show decreased ATP levels; however, they were not studied.

Differences in ATP concentration per cell, due either to cell size or growth conditions may not be important if the ratio of ATP to dry weight of viable biomass is constant. Attempts to measure the ATP to viable biomass ratio are difficult.

Viable plate counts do not give cell mass; microscopic counts do not give viability or cell mass; tyrosine and dry weight determinations do not distinguish viable cells. The use of a chemostat to achieve steady state culture conditions would probably provide the best source of material from which to study the ATP to dry weight ratio. However, this latter approach was not undertaken in the current study.

A number of dry weight determinations were made on the E. coli culture. The relationship of ATP content to dry weight is shown in Table 15. The range of values, 0.82 to 2.00 µg ATP/mg dry weight, is in agreement with the average value of 2.0 µg ATP/mg dry weight cited by Patterson, et al. (2).

TABLE 15

Concentration of ATP Per mg Dry Weight of E. coli

Time (hr.)	µg ATP/ mg dry wt.
0	1.0
2	.82
3	1.76
5	2.00
6	1.64
8	.85

This study showed apparent differences in the concentration of ATP per cell of approximately ten fold depending upon the growth phase of the culture. However, these differences do not necessarily demonstrate a difference in the ATP/viable biomass ratio. Cell size, and viability must be considered. Bacterial cultures show a variation in cell size which occurs during the various growth phases. Figure 19 shows the generalized variation in cell mass (g/cell) which occurs during the normal growth curve. Cells are found to increase in size during logarithmic growth and to decrease as the culture becomes older. Herbert (7) has found that the average cell mass of Aerobacter aerogenes may vary by as much as four fold, depending upon the growth rate (See Figure 20).

On the other hand, as shown in Figure 20, the content of protein per unit of dry weight varies only slightly over the range of growth rates studied. The percent tyrosine in proteins from various sources, as shown below, varies only slightly. The selection of the tyrosine assay as a determinant of cellular protein was, therefore, made to provide the same type information as dry weight.

	Tyrosine Content Moles Percent	Reference
E. coli	2.1	(8) p. 20
Mycobacteria (11 strains)	2.0 - 2.5	(8) p. 20
Plant Proteins	3.2 - 6.3	(9) p. 131
Blood Proteins	3.0 - 6.8	(9) p. 132
Egg Albumin	3.7	(9) p. 132

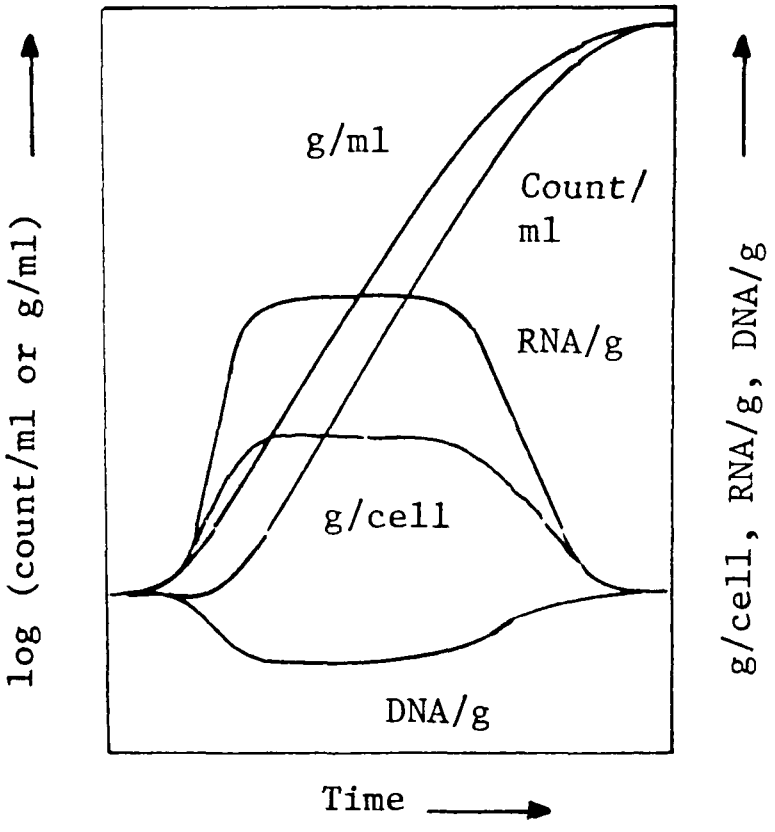
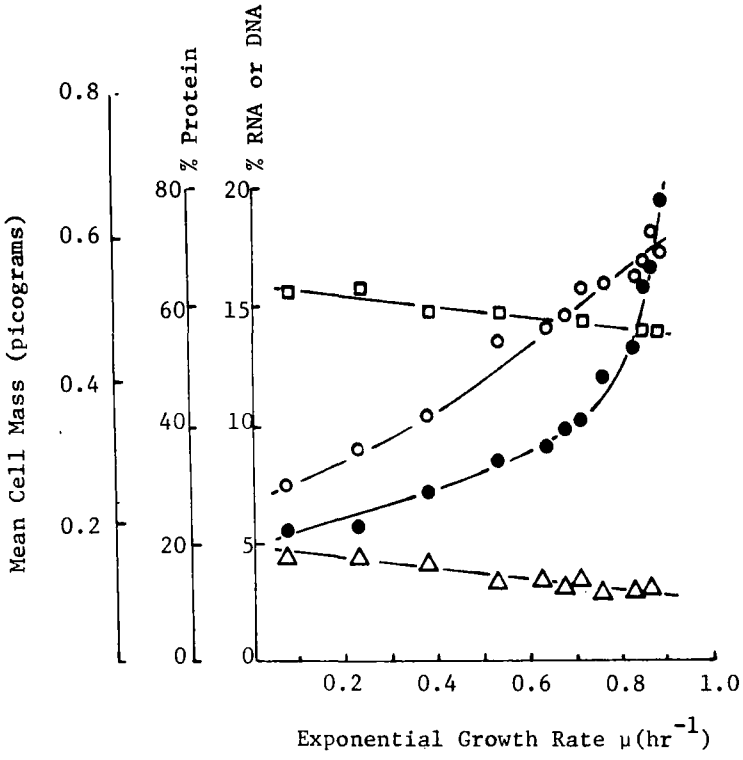


FIGURE 19

Changes in Population (count/ml), Dry Weight (g/ml), Cell Weight (g/cell), RNA/g of Cells and DNA/g of Cells in an Idealized Bacterial Culture Following Inoculation with Cells in Lag Phase (Mallette 1970) (10)



Content of proteins (□), and nucleic acids RNA (○) and DNA (△) as % of dry weight and mean cell mass (●) (dry weight/ml divided by total cell count/ml) as a function of growth rate μ. Cultivation medium: glycerol - NH_3 - salts; glycerol is the limiting factor.

FIGURE 20

Growth of Aerobacter aerogenes in Continuous Culture (Herbert 1961) (7)

Tyrosine would be present in dead cell structure, however, unless the dead cell lysed, it would contribute falsely to a tyrosine based determination of biomass.

Nonetheless, since the concentration of tyrosine would be greater for larger cells, the measurement of ATP biomass based upon tyrosine was expected to be more constant than ATP biomass based upon cell numbers. Table 12 showed that, while the concentration of ATP/cell varied four fold during the various growth phases, the concentration of ATP per unit tyrosine varied only 18%. As shown in Table 13, the ATP/cell of Z. ramigera was ten fold greater for 5.5 hours than it was after 22.5 hours. However, the ATP/unit tyrosine showed less than 2% variation during this time. This latter culture did show a three fold decrease in 50 hours which was probably indicative of a loss in viability in which cells remained intact thereby maintaining the tyrosine level. Table 14 showed three fold differences in concentration of ATP/unit tyrosine; however, these values and those from all other cultures fell within a range of approximately 0.06 to 0.5 μg ATP/μg tyrosine. This means that the variation in ATP/biomass on the basis of all cultures measured did not exceed ten fold regardless of genus or physiological state.

It can be assumed that a certain percentage of tyrosine measured at some growth phases was due to non-viable cells; therefore, the range in ATP/biomass variation was probably less than ten fold. The range in ATP/dry weight which was found in this study falls within the same range of values determined by other workers (see Table 16).

Work concerning growth measurements and calculated energy yields supports the theory of a constant ATP pool. It has been found, Gunsalas and Shuster, that the dry weight of bacterial cells produced by the utilization of a given substrate is directly proportional to the moles of ATP produced during the metabolism of that substrate by a known pathway (11). A remarkably constant value of 10.5 g dry weight of cells per mole of ATP produced has been found for many different organisms and substrates. For example, if Streptococcus faecalis is fed one mole of glucose (which it utilizes via the Embden-Meyerhof pathway, producing 2 moles of ATP), the yield in dry weight of cells is 21 g.

This same organism degrades arginine via citrulline for a yield of one mole of ATP per mole of arginine. The molar growth yield for arginine grown S. faecalis has been found to be 10.5 g dry weight of cells per mole of arginine utilized. Many organisms including yeasts have been shown to yield an approximate value of 10.5, although more recent studies involving continuous culture have shown a variation in yield which is dependent upon growth rate. Decreased yields are explained by Tempest in terms of energy of maintenance requirements (12).

TABLE 16

Range of Values for ATP/mg Dry Weight Determined by This and Other Studies are as Follows:

Organism	µg ATP/mg Dry Wt. Cells	Reference
Based on Tyrosine - All Growth Phases		
Escherichia coli	0.92 - 1.3	This Study
Zooglea ramigera	1.1 - 6.0	This Study
Bacillus sp.	0.6 - 3.3	This Study
Mixed E. coli & *Bacillus* sp.	0.6 - 1.3	This Study
Based on Dry Weight		
E. coli	1.0	Chappelle and Levin (1968) (6)
Saccharomyces cerevisiae	1.1	Chappelle and Levin (1968) (6)
Streptococcus faecalis	2 - 12	Patterson (1970) (2)
Pseudomonas sp.	0.7 - 2.2	Patterson (1970) (2)
Mammalian Cells	3.7	West & Todd (1962) (13)

This evidence, although not concerned directly with an ATP pool as measured during this study, shows that many different organisms utilize different substrates and conduct a flow of energy in the form of ATP from the degradation of substrates to the construction of cellular structures. If large variations in an ATP pool existed, a constant relationship between ATP and cell production would not be expected. Energy in the form of ATP is rapidly utilized in energy requiring synthesis. No storage of ATP occurs and the means by which cells uncouple energy production and growth in a non-energy limited medium have been studied. Gunsalus and Shuster (11) have discussed three mechanisms of energy dissimilation other than the production of protoplasm which occur: (1) accumulation of polymeric products, either in storage form or as unusual waste; (2) dissipation as heat by ATP use mechanisms, and (3) activation of shunt mechanisms bypassing energy yielding reactions or requiring a greater expenditure of energy for priming.

The location and state of transfer of ATP which is extracted from cells and measured by the bioluminescent reaction is not known. However, the close coupling of ATP production and utilization argues for the constancy of its concentration in living cells.

A ten-fold variation in a method of microbial quantitation of mixed populations is not great. Patterson (2) has calculated that only

15% to 20% of the MLVSS at the University of Florida plant was viable biomass. McKinney (1) has estimated that the ratio may range from 25 - 50%. Lesperance (14) suggests that 80% of the MLVSS in normal sludge is biological material, but cautions that this figure does not hold true for certain industrial wastes. These estimates indicate that the range in percent viable biomass of normal sludge may be in the neighborhood of five fold. Toxic materials could easily reduce the viable biomass.

Thus, the variation in biomass determination by the ATP method is about as great as that of the more conventional techniques. However, it is quite possible that additional work will narrow the ATP range. It might be shown, for example, that treatment correlates more closely with the mass of living cells than with the numbers of cells. ATP appears more closely tied to mass than cell numbers which relationship, if further verified, will reduce the range of ATP determination as applied to sewage treatment. Furthermore, the variations in ATP associated with various physiological stages of the cell may be effectively reduced. The extremes of this range are between young logarithmic cells and very old stationary cells. For the various respective sampling points in a sewage treatment plant, at known levels of loading and sludge age, the physiological state of the cells will be sufficiently well known to reduce the possible range of ATP/ unit biomass. Further, since ATP comparison for treatment control purposes will be made at respective sampling points at which the physiological state of the cells would be the same from sampling time to sampling time (except perhaps for periods of upset detected by ATP assays at other plants), the measurements should serve adequately.

The possibility that organism differences caused by specific wastes and geographical locations may give rise to slightly different ATP/ biomass ratios has not been excluded. An empirical determination of the optimum biomass concentration may be necessary for individual treatment plants.

In further work aimed at this problem, a prime difficulty exists. This is the possibility that the ATP assay may be more precise a measure of biomass than any of the techniques against which the researchers may attempt to calibrate it. Colony forming units may be the result of single cells of clumps. Many living cells do not survive to produce colonies because of culturing deficiencies. Direct microscopic examinations cannot distinguish living from dead cells. Fluorescent staining suffers from these problems plus one of background discrimination. DNA, protein, or enzyme determinations vary with cell types and physiological state.

Jannasch and Jones found discrepancies as high as 13 to 9,700 fold among standard culturing and enumeration procedures (15).

SECTION VIII

PILOT PLANT STUDIES

The Biospherics Incorporated pilot sewage treatment plant was operated for a month to test the feasibility of the control of plant operation through the determination of ATP at various selected points in the plant (Figure 21).

The plant has a 500-gallon capacity reservoir tank to contain the synthetic sewage. Table 17 gives the composition of the synthetic sewage, as recommended by Eberhardt and Nesbitt (16). The mixture was prepared daily in 360-gallon batches. The synthetic waste flowed into two identical treatment systems. Each system was composed of three 15-gallon aeration tanks, connected in series. The flow rate was 168 gallons per day into each system; retention time in the aeration tanks was 6.5 hours. The effluent from the third aeration tank entered a final clarifier, volume 30 gallons, from which the final effluent was sampled. The conical shape of the final clarifier permitted the sludge to settle and be returned to the first aeration tank or be wasted. The entire system was controlled by valves and pumps, which were activated by timers programmed for a desired return sludge flow and wasting schedule. Aeration which was at the rate of 3 l/min. produced a level of 4 mg/l dissolved oxygen in the first aeration tank and 8 mg/l in the third aeration tank.

The systems were primed with several gallons of sludge from a local sewage treatment plant; both systems were run in the same manner for one week. The following measurements were then made on each system: ATP, oxygen uptake rate, tyrosine, optical density, suspended solids, BOD, and TOC. Table 18 lists the tests employed and the sampling points. The numbers of the sampling points refer to the numbers on Figure 21.

The BOD of the synthetic sewage was determined daily and had an average value of 244 mg/l with a standard deviation of 18, and the TOC had an average value of 151 mg/l with a standard deviation of 3.4. The ratio of TOC to BOD had an average value of 1.63, with a standard deviation of 0.15 and a coefficient of variation of 2.1%.

After one week of operation, when both sections of the pilot plant showed similar values for the parameters measured, one section was continued in the same manner and the second section was altered to provide a test section. Prior to changes in the test section, the ATP content of the first aeration tank was 4 µg/ml (18 January 1971). Based upon previous bench studies in this laboratory, it was decided to reduce the ATP content of the first aeration tank to 2 µg/ml. Accordingly, on 15 January and again on 18 January, the flow of

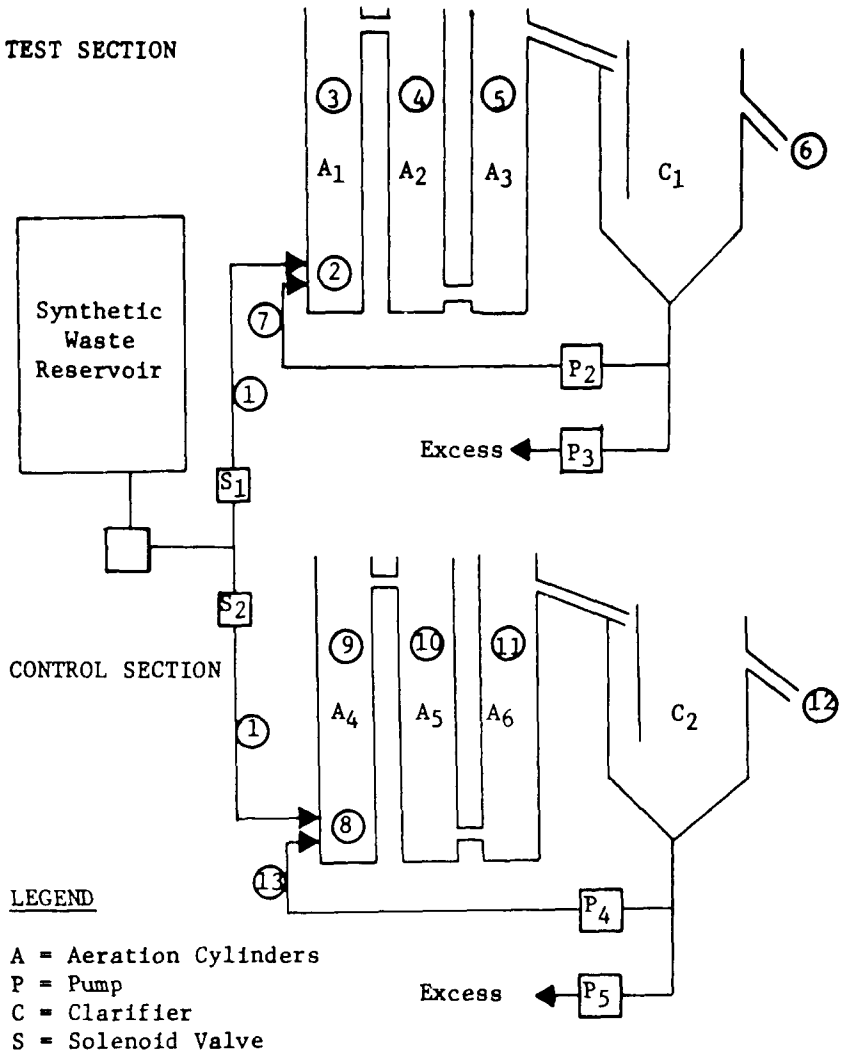


FIGURE 21

Diagram of Identical Pilot Plants Which Were Used for Test and Control Experimentation

TABLE 17

Composition of Synthetic Sewage (16)

Nutrient Broth	496 g
Urea	49 g
KH_2PO_4	39 g
add 360 gallons water	
BOD	244 ±18 mg/1
TOC	151 ±3.4 mg/1

return sludge in the test system was reduced to achieve a return flow of one-fourth that of the control system. The ATP content of the first aeration tank responded to the reduction by declining 50% to an average value of 2 μg/ml over the seven-day test period, 18 January to 25 January 1971. The BOD_5 reduction averaged almost 95% and did not suffer with respect to the control despite the drastic reduction in the amount of sludge returned. Figures 22 through 26 provide information on the parameters determined and their relation to the first aeration tank. These charts were selected from all those prepared on other sampling points because they represent most clearly the differences between the test and control systems. Figure 27 shows that the reduction in BOD_5 and TOC, as determined by comparison of the analyses of the primary effluent and the final effluent. Figure 28 describes the changes in pumping rates of the returned sludge for both systems and also the changes in the waste pumping. Suspended solids during the period of 20 January to 22 January 1971 were decreased by a factor of three in the test system (Figure 24). However, ATP and O_2 uptake rate (Figures 22 and 23) were decreased much less, therefore, indicating an increase in viability and/or activity of the mixed liquor. The tyrosine concentration (Figure 26) showed that levels averaged 80% less in the test than in the control system.

The reductions in BOD in the two systems, as shown in Figure 27, were similar.

MLSS of the control section were allowed to increase (no wasting as shown in Figure 28) in an attempt to reach a food/microorganism ratio of approximately 0.35, which is recommended by Lesperance for conventional treatment (17). At these higher MLSS levels sludge tends to accumulate in the clarifier; therefore, the return sludge pumping rate was accelerated as shown in Figure 28.

TABLE 18

Tests and Sample Points Employed During the Pilot Plant Study

Sample* Points	BOD	TOC	ATP	SS (OD)	O_2 Uptake	Tyrosine	SS (Dry Wt.)
Test							
1	X	X					
2		X	X	X	X		
3		X	X	X	X		
4		X	X	X	X		
5		X	X	X	X		
6	X	X		X			
7			X	X	X	X	X
Control							
8		X	X	X	X		
9		X	X	X	X		X
10		X	X	X	X		
11		X	X	X	X		
12	X	X		X			
13			X	X	X	X	X

*Sample points correspond to those shown in Figure 21.

Over the weekend starting 25 January 1971, a technical difficulty caused the settled sludge in the final clarifier of the test system to form a rather solid mass and become anaerobic. The mass broke apart and was partially pumped to the aeration tanks before the problem was discovered and the remaining sludge wasted. This effect was evidenced by an interruption in the pattern of results shown in Figures 22-27 between 26 January and 28 January 1971. The system

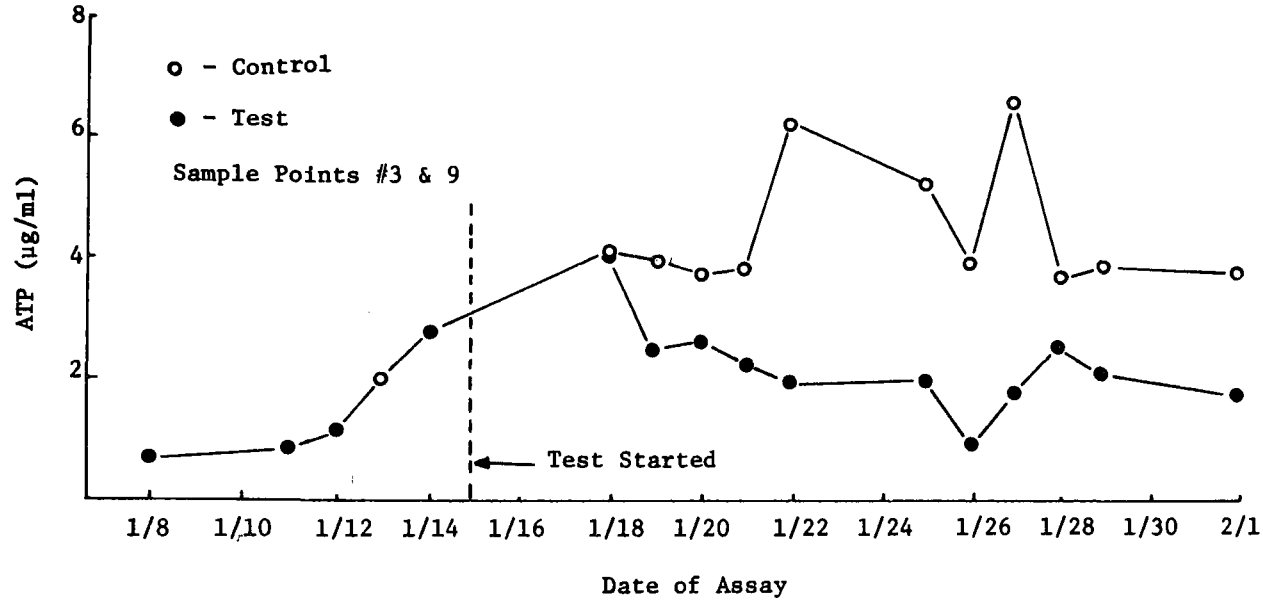


FIGURE 22

Concentration of ATP (µg/ml) in the Mixed Liquor of the First Aeration Cylinder

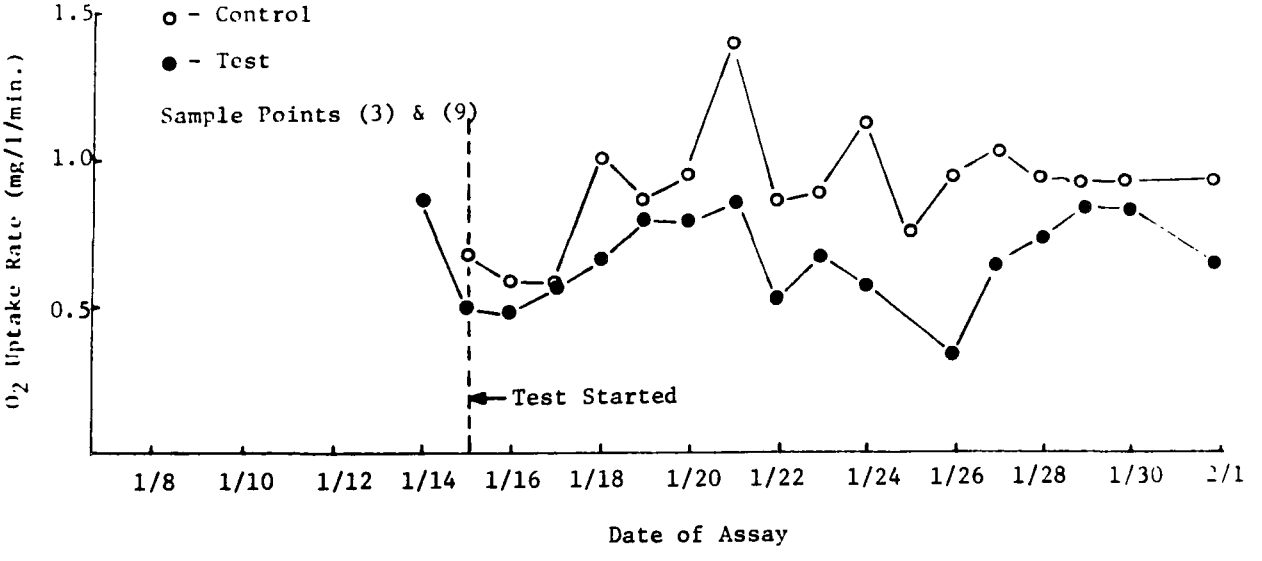


FIGURE 23

Oxygen Uptake Rate of Mixed Liquor in the First Aeration Cylinder

corrected itself in approximately three days and was functioning as before.

The operation of the Biospherics Incorporated pilot sewage treatment plant was discontinued when it was felt that the feasibility of the ATP control system had been demonstrated. The remainder of the contract period was devoted to the transfer of knowledge gained from laboratory conduct and pilot plant studies, to the control of full-scale municipal sewage treatment plants.

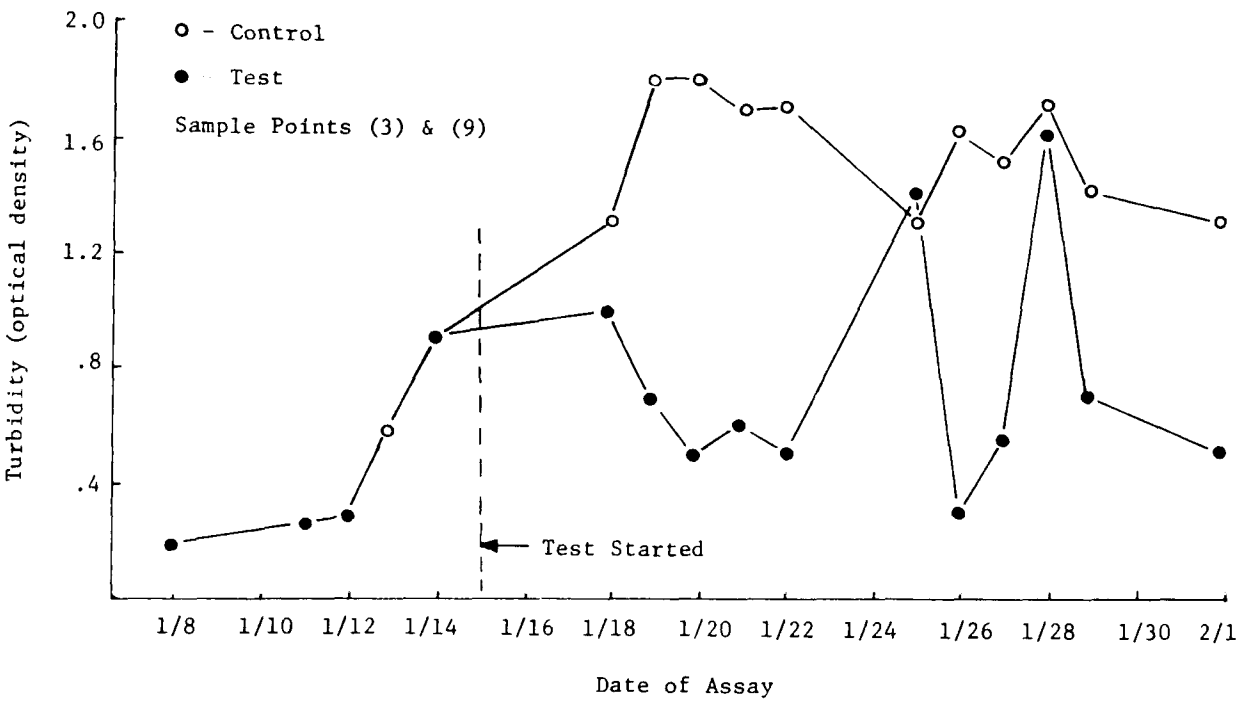


FIGURE 24

Suspended Solids of Mixed Liquor as Determined by Optical Density in the First Aeration Cylinder

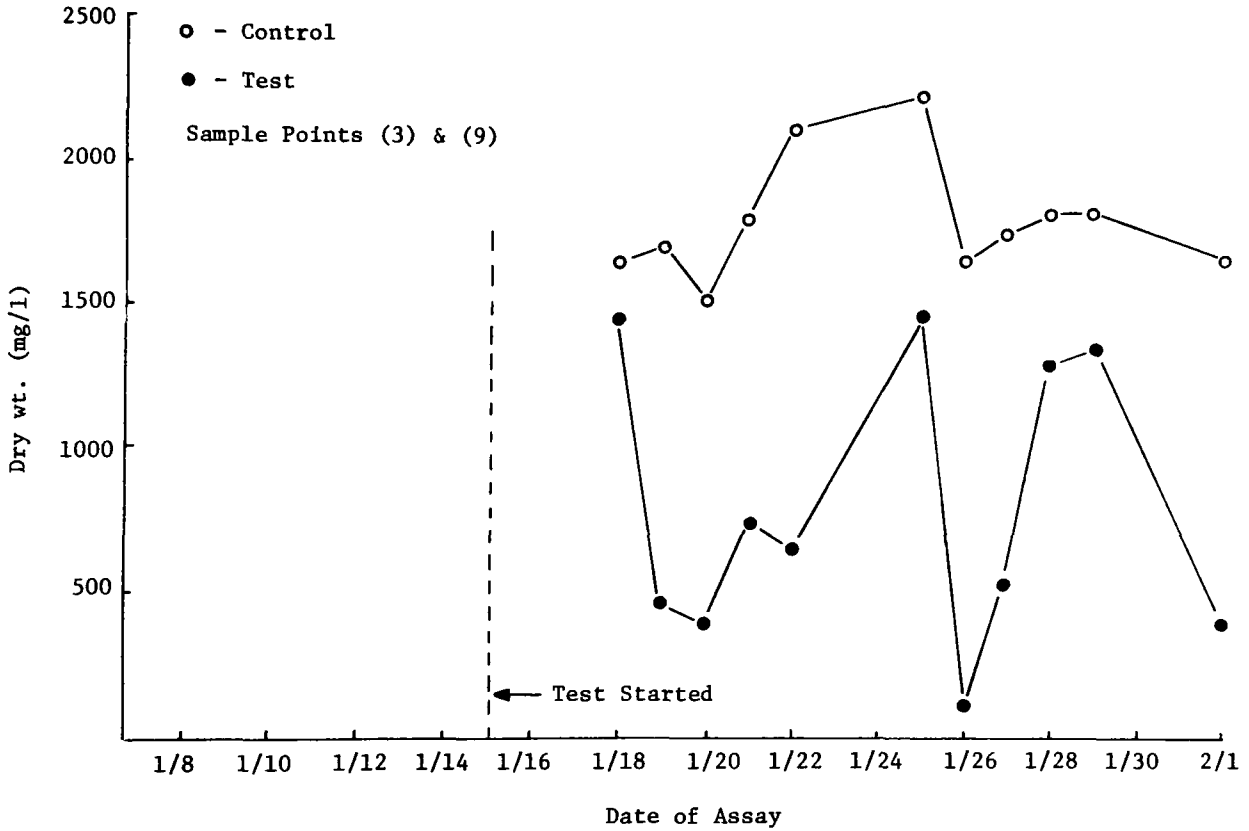


FIGURE 25

Suspended Solids of Mixed Liquor as Determined
By Dry Weight (105°C) in the First Aeration Cylinder

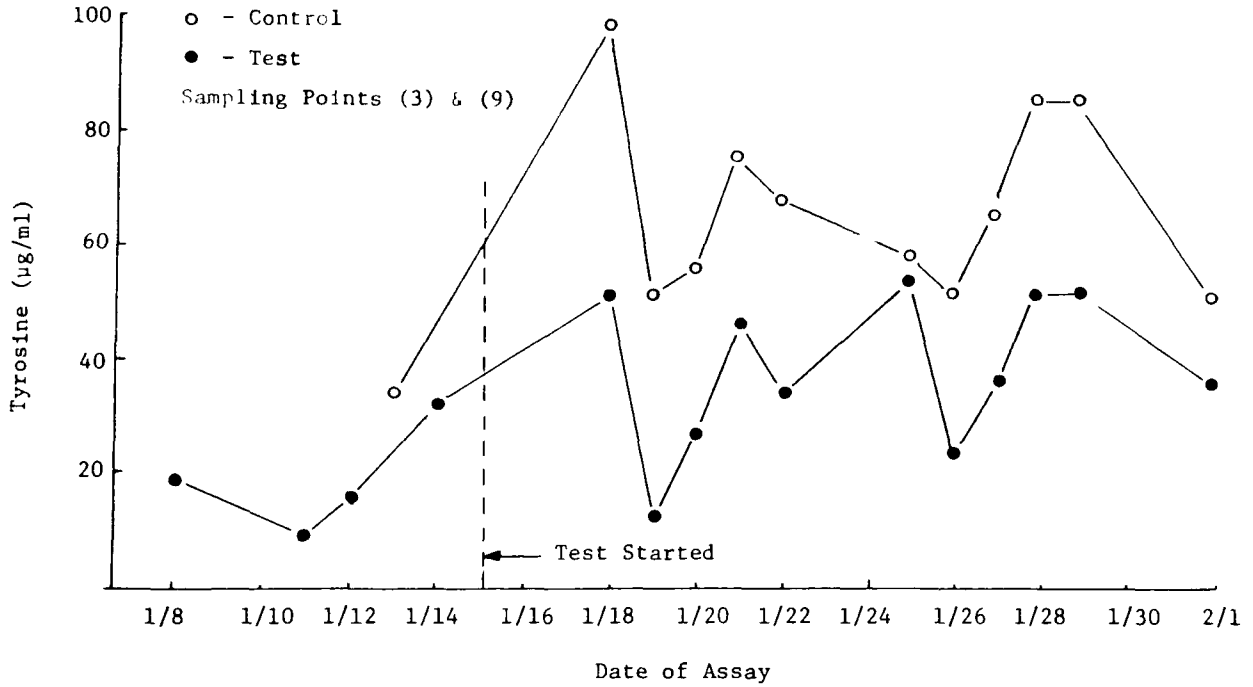


FIGURE 26

Tyrosine Content of Mixed Liquor
in the First Aeration Cylinder

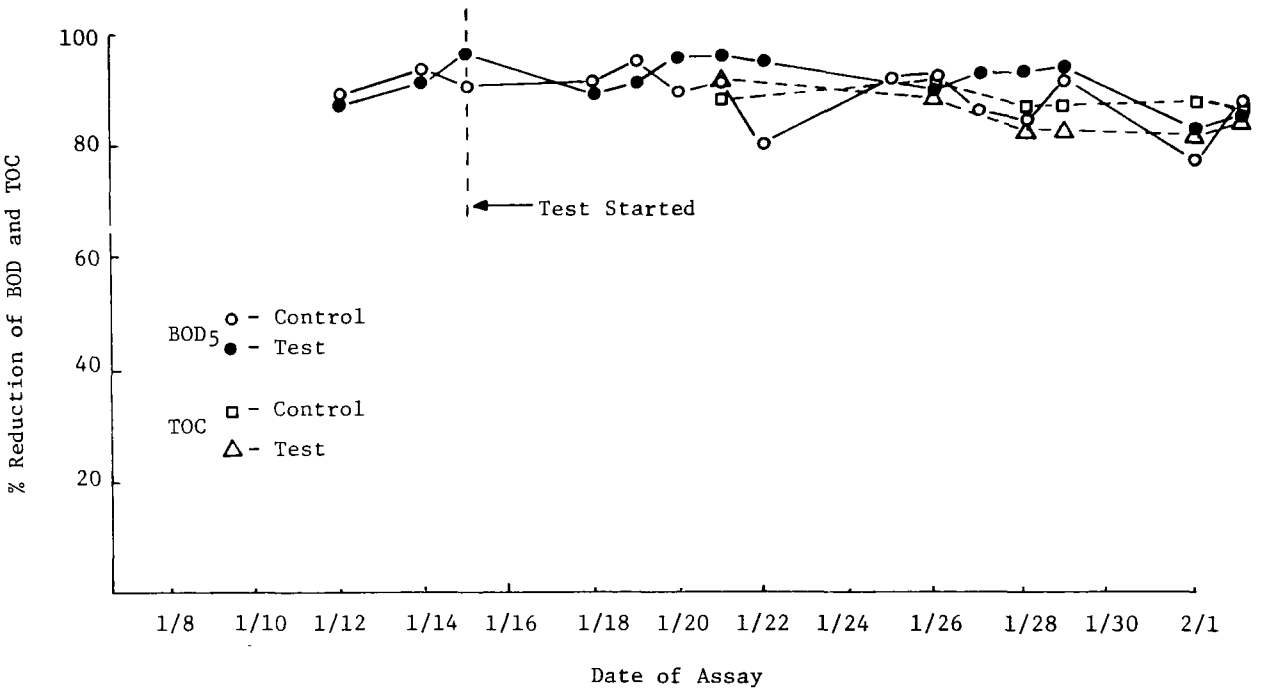


FIGURE 27

Efficiency of Treatment Expressed as Percent Reduction in Biochemical Oxygen Demand (BOD_5) and Total Organic Carbon (TOC)

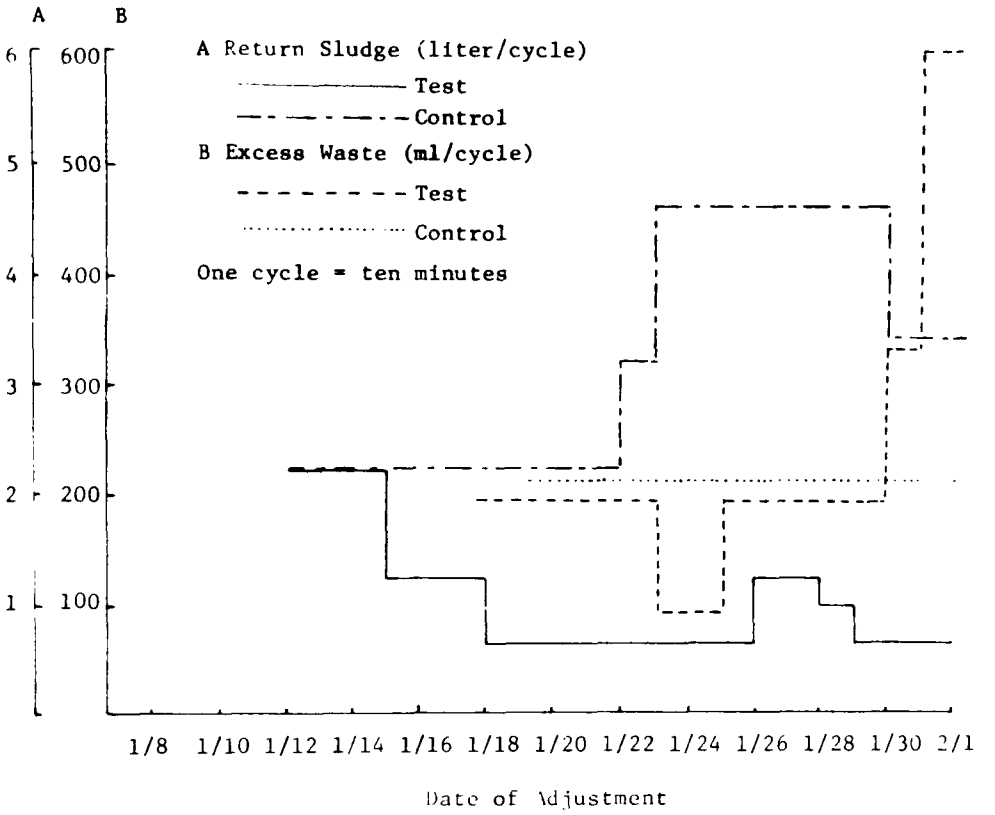


FIGURE 28

Schedule of Return Sludge and Excess Waste Pumping

SECTION IX

OPERATION OF THE BALTIMORE CITY BACK RIVER ACTIVATED SLUDGE SEWAGE TREATMENT PLANT

Control by ATP Assay

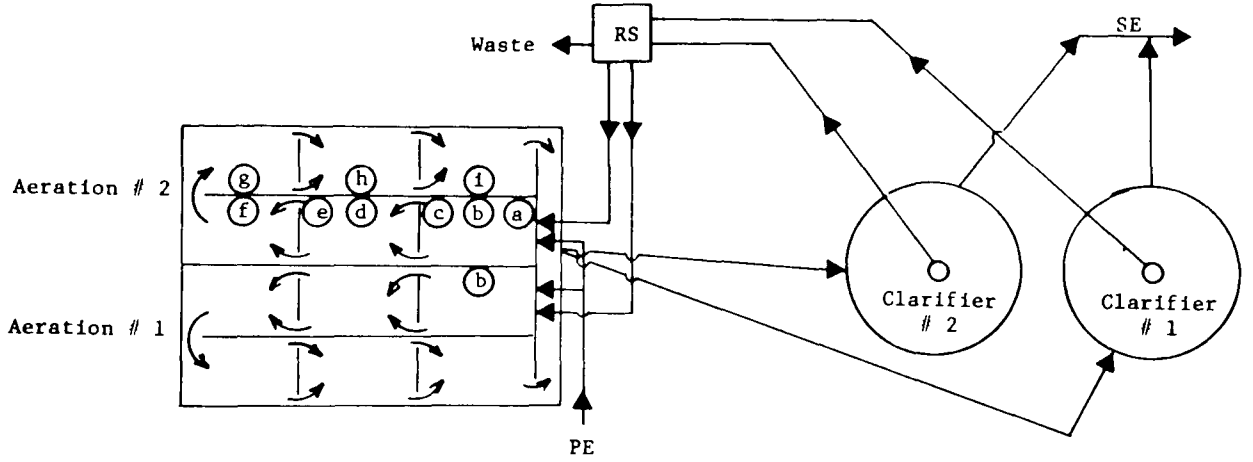
The Baltimore Back River Treatment Facility maintains and operates a conventional activated sludge system which was built with a design capacity of 20 MGD. Although the Plant was at one time divided into two separate sections to accommodate phosphate removal study (18), more recent changes have integrated the two sections.

As shown in Figure 29, return sludge is collected from the two clarifiers and mixed by continuous pumping from a sump well to an overflow head basin. Return flow of sludge is metered from this basin to the heads of the two aeration basins. Mixed liquor, which moves as a plug, flows through the aeration systems, exits into a common sluice and goes to the secondary clarifiers.

Primary effluent at the Baltimore Plant is treated by both activated sludge and trickling filters. Design is such that the activated sludge receives a constant flow (20 MGD) regardless of raw sewage influent flow variation. Normal operating procedures at the plant prescribe a mixed liquor suspended solids level of 1500 to 2000 mg/l, which is generally achieved with a 25% return sludge rate. Aeration time is approximately five hours. Two pumps are used for excess sludge wasting (capacities of 0.40 MGD + 0.96 MGD). The smaller one is generally operated continuously, whereas the larger pump is used only when the sludge blanket in the clarifier exceeds the 2 ft. level.

This field study was undertaken to observe the ATP content and O_2 uptake rate of sludge and of mixed liquor and to attempt to correlate these parameters with other characteristics of plant operation. The use of these measurements in providing more effective control was tested by actual operation of the plant, using ATP as the criterion of the return sludge rate. BOD and TOC reductions were used as measures of effectiveness.

Approximately one week was spent in observation of the plant, during which no changes were made in the method of operation. After this baseline period of operation, sludge return was regulated on the basis of ATP measurement. Sludge wasting was also geared to maintaining this parameter. Based on pilot plant studies using waste of similar BOD strength to that found in Baltimore, a concentration of approximately 2 µg/ml was selected as the test MLATP concentration. It was also felt that this concentration would not be an overly drastic change that could impair treatment for some time until solid levels



LEGEND

SE - Secondary Effluent
PE - Primary Effluent
RS - Return Sludge Pumping System

Circled letters in aeration basin indicate sampling points.

FIGURE 29

Simplified Diagram of the Activated Sludge Portion of the Baltimore Back River Sewage Treatment Plant

were reestablished. Adjustments were made to attempt maintenance of 2.0 μg ATP/ml in the mixed liquor.

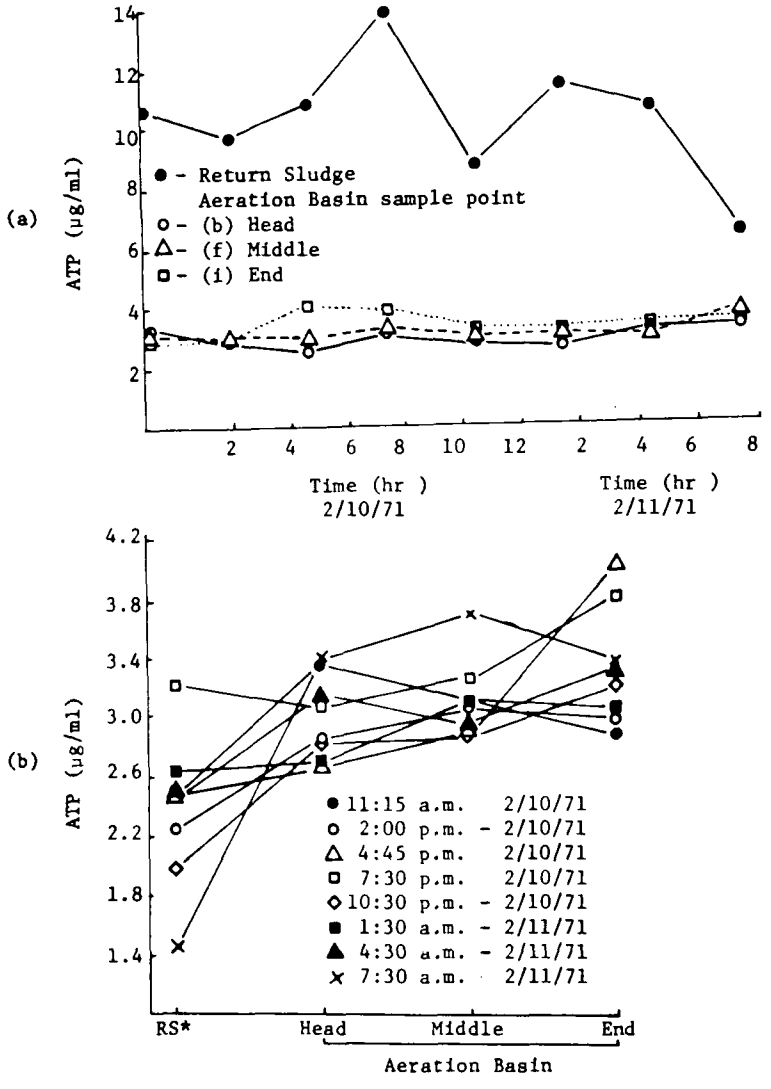
The activated sludge system was initially found to be operating excellently. Information supplied by the City of Baltimore showed that BOD reduction during the preceding month averaged 97% and the sludge volume index, which had declined steadily over the past months, was approximately 100. The MLSS were being held at 1600 to 1700 mg/l.

An experiment was conducted to determine (a) the difference in ATP concentration that might be expected in return sludge and in the mixed liquor at the head, middle, and end of the aeration basin, and (b) the daily ATP fluctuation in mixed liquor and return sludge. Results of an experiment performed on 10 February 1971 through 11 February 1971, before ATP control was initiated, are shown in Figure 30 and Table 19.

Of interest was the fact that MLATP levels were relatively stable throughout the course of a 24-hour period. Measurements of the RSATP, however, showed a wider variation. The reason for this variation is not known; however, ATP assays on return sludge from the laboratory pilot plant showed similar fluctuations. Although the flow of primary effluent to the plant is constant, the biological loading fluctuates widely throughout a diurnal cycle. Figure 31 shows the daily fluctuations in TOC and phosphate concentrations which were routinely measured. These fluctuations correspond quite well to raw waste flow data supplied by the Back River Wastewater Treatment Plant. The approximately two hour detention time required for primary treatment is probably responsible for the fact that maxima and minima of PO_4-P and TOC of the primary effluent are slightly out of phase with the flow of raw waste.

In exploring the best location for sampling to control the return sludge by ATP content, it was decided to use the head of the aeration basin. The ATP fluctuations are dampened at this location with respect to those observed in the return sludge. Inasmuch as the ATP content of the raw sewage is relatively insignificant compared to that in the return sludge, the former can be ignored. Therefore, percent changes observed in mixed liquor ATP can be compensated by equal percent changes in the rate of return sludge. This assumes no radical change in ATP content of the return sludge such as might be caused by an overdraught on it. However, such changes in sludge composition will be detected by the ATP-monitoring of the mixed liquor. Accordingly, sample point (b), Figure 29, was selected for use throughout the control period.

As shown in Table 19, the MLATP increased by 3.6% in the first half of the aeration basin and by 7% in the second half. However, Figure 32 shows that the O_2 uptake rate was much greater at the head of the aeration basin than in the middle or end. Profiles of O_2 uptake and



*Values were calculated by multiplying measured RSATP times percent sludge return (.122).

FIGURE 30

ATP Levels in Mixed Liquor (2/10/71 - 2/11/71) at the Baltimore Back River Activated Sludge Plant Prior to ATP Regulation

TABLE 19

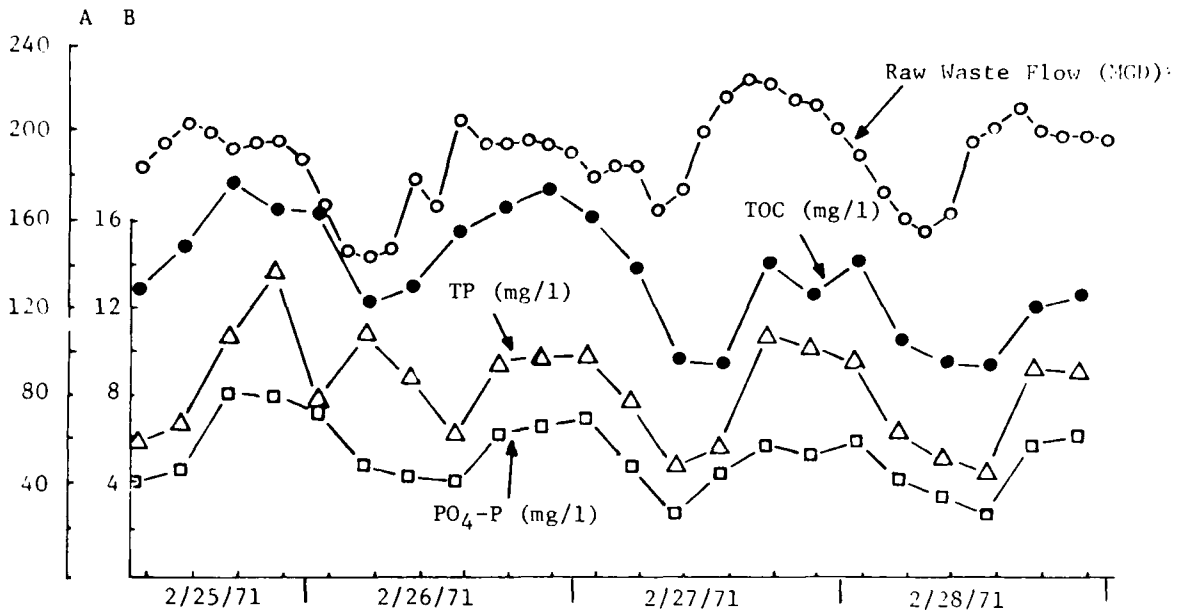
ATP Concentrations in Mixed Liquor and Return Sludge (2/10/71 to 2/11/71) at the Baltimore Back River Activated Sludge Plant Prior to Onset of ATP Regulation

	ATP(μg/ml)			
	Aeration Basin			
Time	Head (b)	Middle (f)	End (i)	Return Sludge
2/10/71				
11:15 a.m.	3.37	3.10	2.89	10.58
2:00 p.m.	2.85	3.03	3.00	9.69
4:45 p.m.	2.66	2.90	4.05	10.78
7:30 p.m.	3.07	3.26	3.82	13.90
10:30 p.m.	2.84	2.87	3.24	8.65
2/11/71				
1:30 a.m.	2.68	3.09	3.07	11.40
4:30 a.m.	3.16	2.93	3.33	10.65
7:30 a.m.	3.39	3.70	3.39	6.39
Average ±s. d.	3.00 ±.29	3.11 ±.27	3.35 ±.41	10.25 ±2.30
	→ 3.6% Increase		→ 7% Increase	

dissolved oxygen in the aeration basin are shown in Figures 33 and 34. These data suggest that although a rapid respiration rate occurred primarily in the first one-sixth of the aeration basin, ATP production occurred throughout the aeration basin and predominated in the second half.

From Table 19, the measured average RSATP concentration was 10.25 μg/ml. Since it is diluted to approximately 23% with primary effluent, the expected average MLATP concentration would be 2.36 μg/ml. This value is 21.4% lower than the 3.00 μg/ml which was actually measured; therefore, an increase in biomass occurred during contact of return sludge with primary effluent. Since the end MLATP value was 3.35 μg/ml, the total increase in ATP during an aeration cycle was 0.99 μg/ml or 29.5%.

An experiment was performed to determine the profile of soluble TOC in the aeration basin. Samples of mixed liquors were collected and a portion was measured for O_2 uptake rate. Results of this experiment which are shown in Figure 34 show that the decrease in soluble TOC parallels the decrease in O_2 uptake rate.



*Data supplied from treatment plant records.

FIGURE 31

Fluctuation in the Concentration of TOC, Orthophosphate (PO_4-P) and Total Phosphate (TP) of Primary Effluent with Daily Variation in Raw Waste Flow At the Baltimore Back River Sewage Treatment Plant

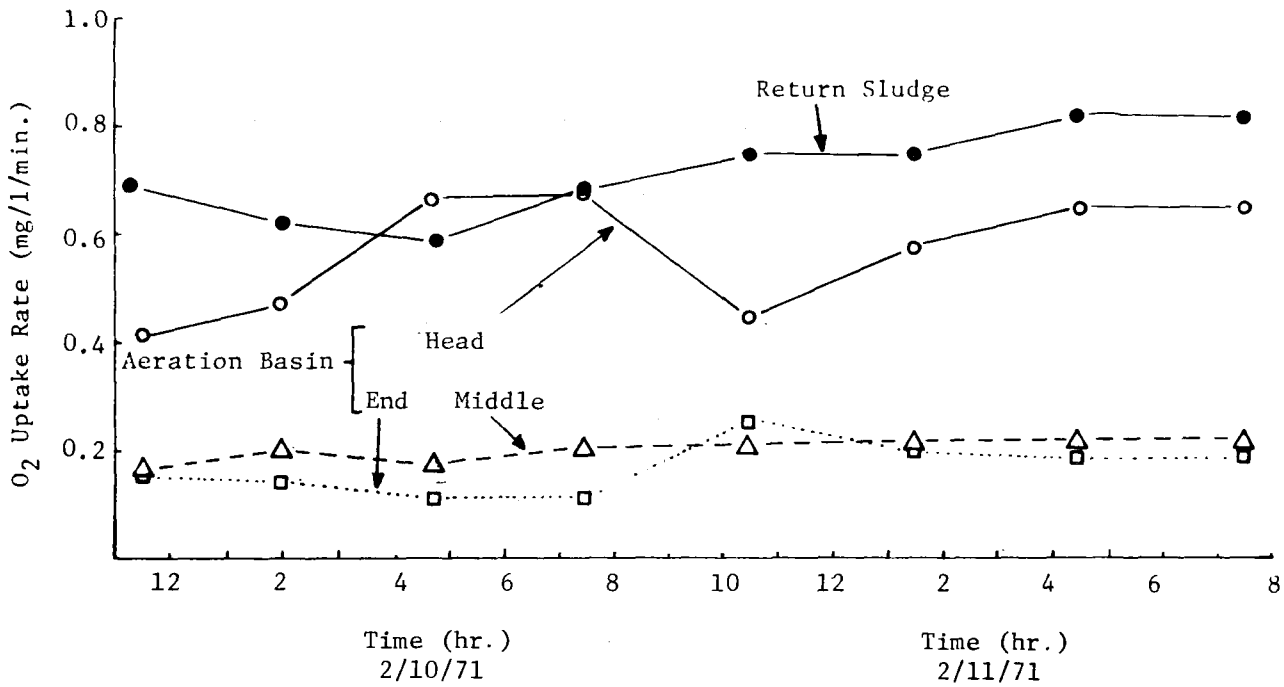


FIGURE 32

Oxygen Uptake Rate in Mixed Liquor and Return Sludge (2/10/71 - 2/11/71) at the Baltimore Back River Activated Sludge Plant Prior to ATP Regulation

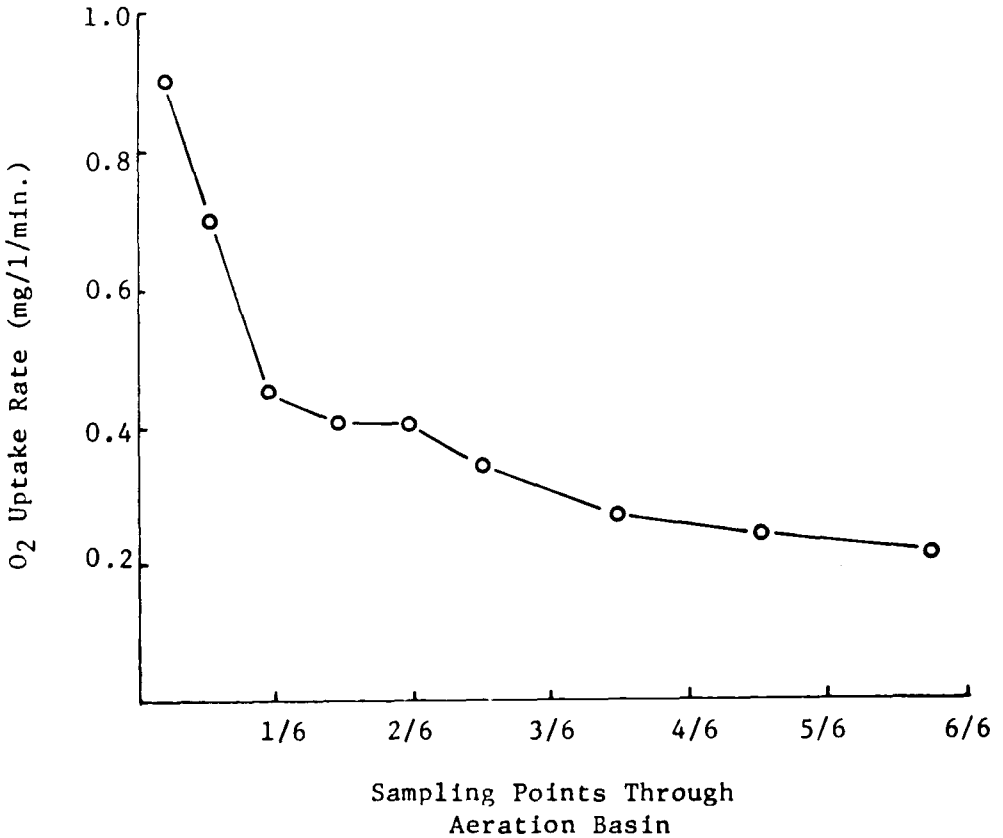


FIGURE 33

Profile of O_2 Uptake Rate Throughout the Aeration Basin at the Baltimore Back River Sewage Treatment Activated Sludge Plant on 2/12/71 Prior to ATP Regulation

As shown in Figure 36, the treatment plant was initially found to contain a MLATP concentration greater than 3 µg/ml. This was considerably more than the desired concentration. A large backlog of sludge was also found in the clarifiers and a sludge retention of approximately four hours was calculated. On February 14, 1971, a large slug of sludge was wasted. This resulted in a sharp reduction of the MLSS and MLATP. MLATP concentration was now close to the 2 µg/ml level believed desirable. Adjustments in the rate of return sludge and wasting which were made during the following weeks, were made in order to attempt to maintain this 2 µg/ml level. Except where indicated on Figure 36, the small waste pump was operated continuously. Rate of aeration throughout the experiment was controlled so that the dissolved oxygen level at the end of the mixed

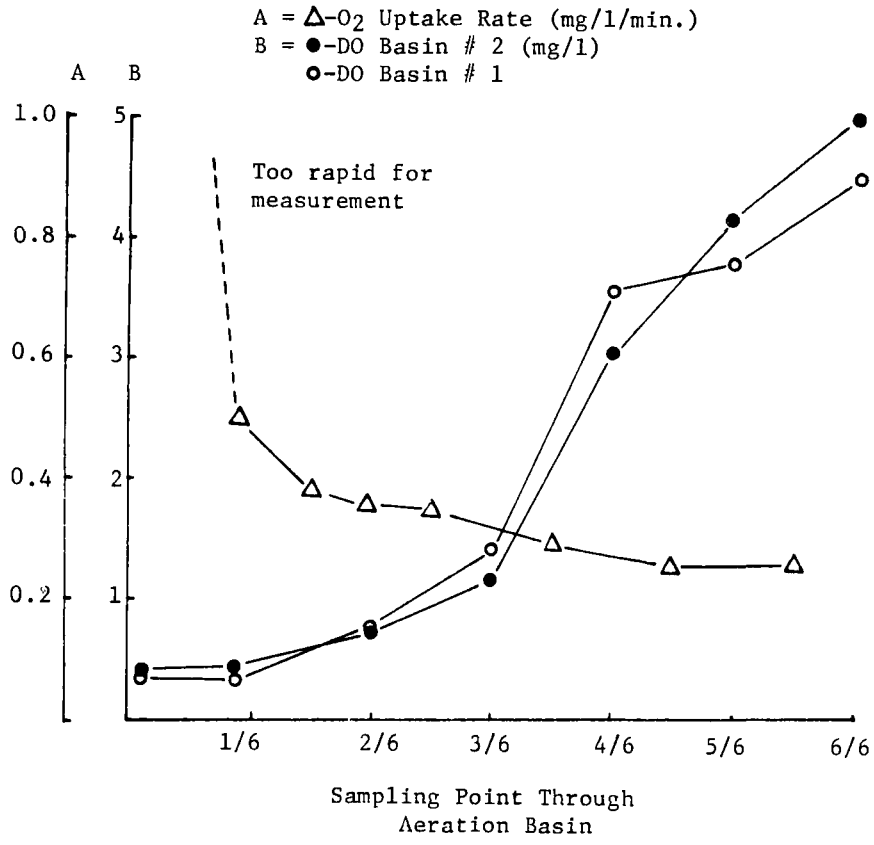


FIGURE 34

Profile of O_2 Uptake Rate and Dissolved Oxygen (DO) in the Aeration Basin at the Baltimore Back River Sewage Treatment Activated Sludge Plant During ATP Regulation Period 3/10/71

liquor basin never fell below 5 ppm. With 23% sludge return rate, the MLATP level climbed back above the 2 µg/ml level; therefore, beginning on 18 February 1971, the return sludge rate was decreased in an attempt to reduce the MLATP to a lower stable level. However, after decreasing the RS rate to 8%, the MLATP decreased slowly to a concentration of 1.2 g/ml. It appeared that the 8% return rate was too low to maintain a stable level of MLATP. On 27 February 1971, the return sludge level was increased to 12.3%. This caused an ATP increase. On 2 March 1971, the ATP level fell sharply; however, it was observed that the percent of solids in the sludge was also decreased, and a very low accumulation of sludge was measured in both clarifiers. It was concluded that the rate of wasting was in excess of sludge production; therefore, the small waste pump was shut off for 24 hours. This caused the MLATP level to

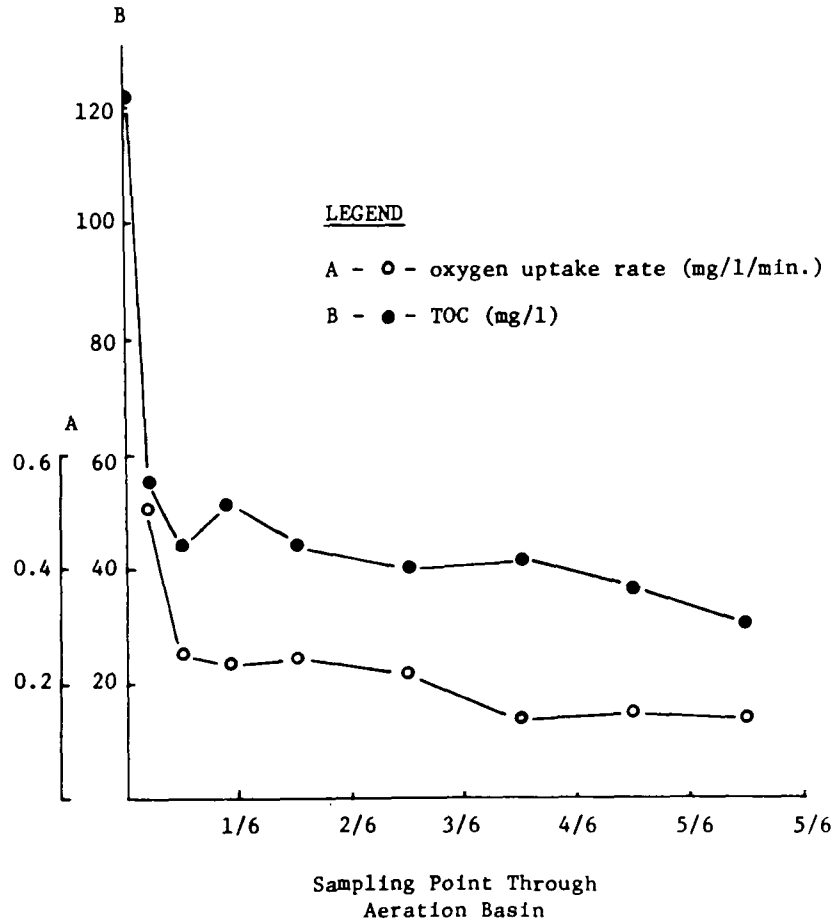
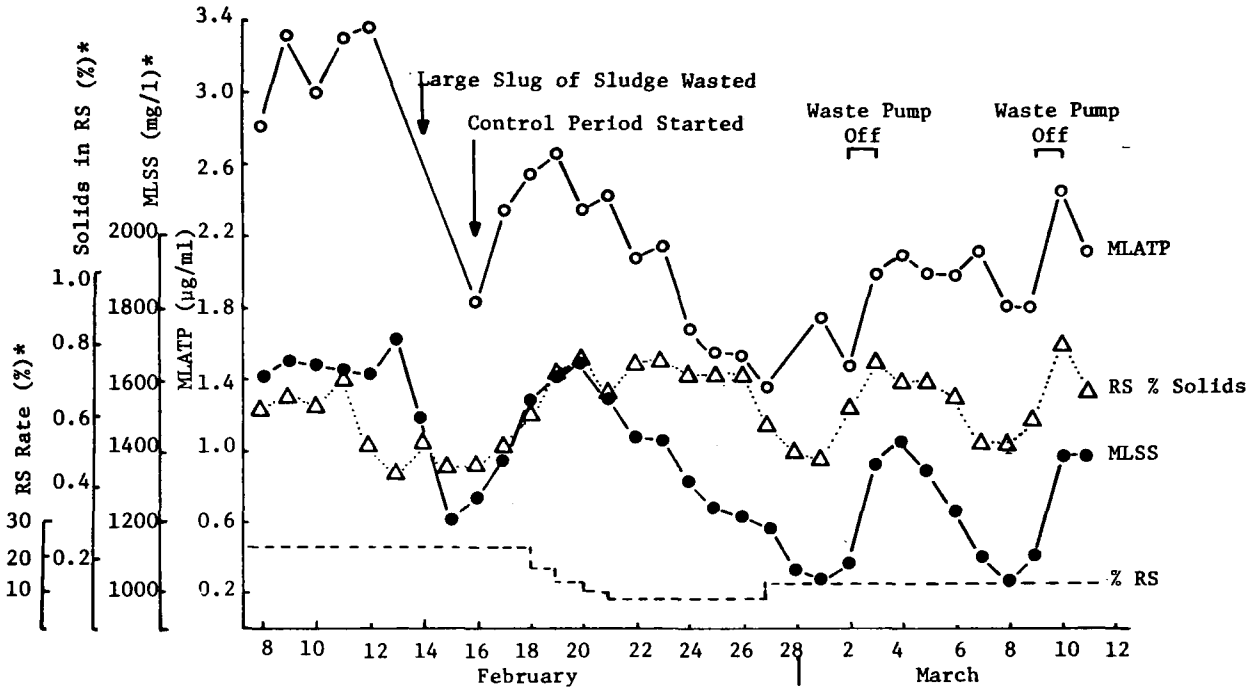


FIGURE 35

Profile of O_2 Uptake Rate and Soluble TOC in the Aeration Basin at the Baltimore Back River Sewage Treatment Activated Sludge Plant on 2/12/71 Prior to ATP Regulation



*Data supplied from treatment plant records.
N.B. MLVSS = 0.718 ± 0.024 x MLSS

FIGURE 36

Parameters Used for Control of the Baltimore
Back River Activated Sludge Plant From 2/8/71 to 3/11/71

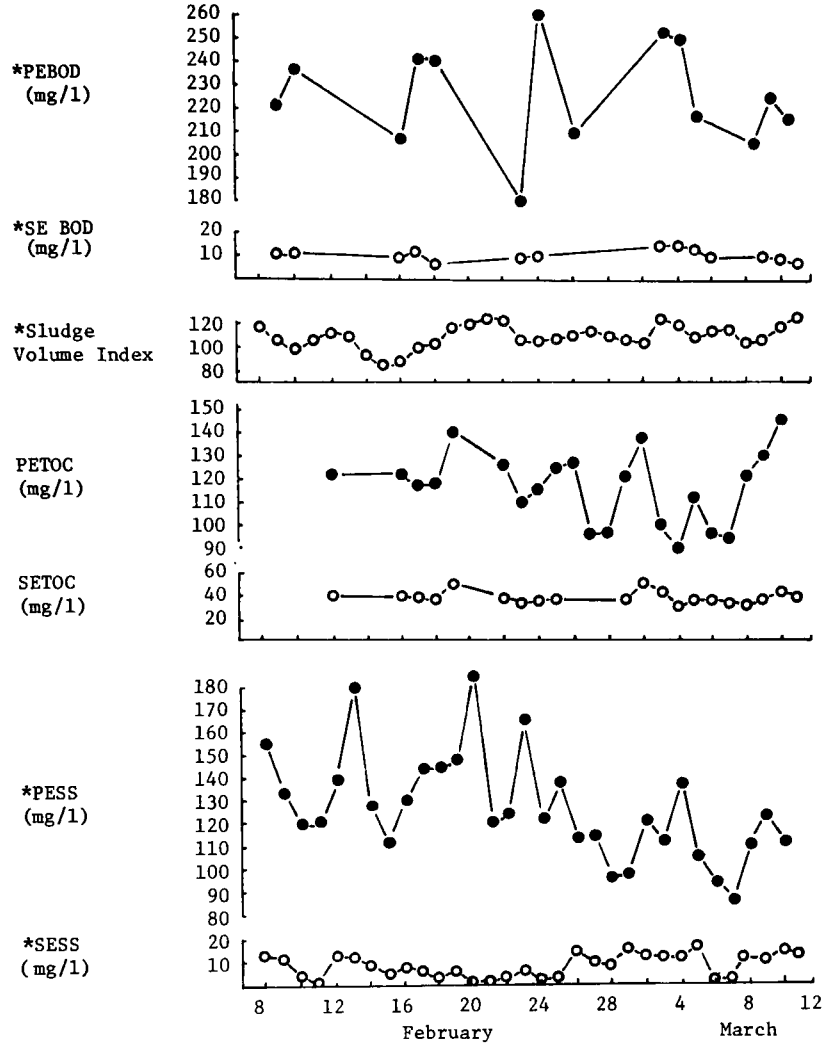
increase to a value near 2 μg/ml, where it remained fairly stable for five days. On 8 March 1971, the ATP level again declined; however, a condition similar to that experience on 2 March 1971 was found and the waste pump was again shut off for 24 hours. Following this action, the ATP level rose again.

A comparison of MLATP and MLSS (Figure 36) shows that these two parameters followed somewhat similar patterns with one significant difference. Changes in the return sludge rate caused a change in the MLATP level within 24 hours. However, a change in the MLSS was not observed until the second day. The decrease in return sludge rate which was initiated on 18 February 1971 and 19 February 1971 was followed by a MLATP decrease on 20 February 1971, but no decrease in MLSS until 21 February 1971. On 27 February 1971, the RS rate was increased and an increasing trend in MLATP was observed on 28 February 1971, but did not occur in the MLSS until a day later.

Prior knowledge of an impending trend in the MLSS level, hours or days before that effect occurs, would be an important advantage for the plant operator.

Figure 37 shows the effect of ATP-based control on the efficiency of waste treatment. The BOD reduction of composite samples collected and assayed by plant personnel averaged above 95%; TOC reduction performed every four hours averaged 70%, and suspended solids reduction calculated from plant records averaged 93% during the period of experimentation. Thus, the high efficiency of the Baltimore plant was maintained, despite the fact that considerably less sludge than normal was returned. The experiment demonstrated that ATP may be used effectively to control a full-scale treatment plant. Although the MLSS level was changed drastically, no decrease in plant efficiency or problems associated with settling occurred. Records showed that the sludge volume index was lower during the period of experimentation, including the first week of baseline observation, than for the previous six months of operation. During the period of ATP-based control, a dense, rapidly settling sludge was characteristic. The secondary effluent TOC (SETOC) averaged approximately 35 mg/l despite broadly fluctuating concentrations in the primary effluent. The City of Baltimore phosphate removal study (18) included much data on the concentration of SETOC; however, the TOC level which they measured rarely fell below 50 mg/l.

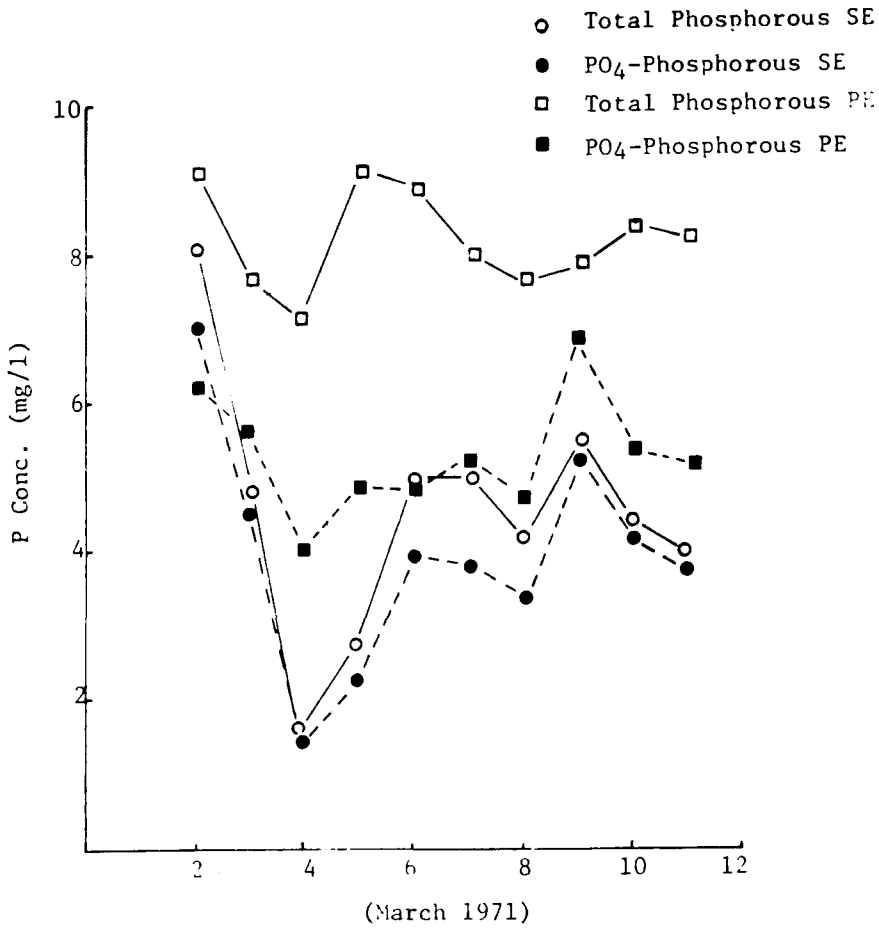
Total and orthophosphate analyses were performed during the last ten days of the current study; however, good phosphate removal was observed for only one brief period. As shown in Figure 38, approximately 80% phosphate removal was observed on 4 March 1971. However, the efficiency of removal was much poorer at other times. Comparison of Figure 38 with Figure 36 shows that the occurrence of phosphate removal corresponded to a peak on the MLSS. It has been demonstrated (18)



*Data supplied from treatment plant records.

FIGURE 37

Parameters of Plant Effectiveness at Baltimore Back River Activated Sludge Plant from 2/8/71 to 3/11/71



Points represent the daily average of assays run every 4 hours.

FIGURE 38

Phosphate Removal During ATP Control
On Activated Sludge

that effective phosphate removal in the Baltimore plant will occur at low solids levels, but is seriously interrupted by a prior rapid decrease in the suspended solids level. Although fluctuations in the solids level did occur during the period when phosphate analysis was made, the rate of decrease never exceeded 150 mg/l/day.

Daily oxygen rates were determined on mixed liquor samples at points a, b, c, and i (Figure 29). The rate of O_2 uptake appeared to be due to both the activity and/or viability of the sludge organisms and to the concentration of nutrients in the incoming waste. As shown in Figure 39, variations of O_2 uptake rate (sample point a) correlated most clearly with PETOC. But, it appeared that a sudden

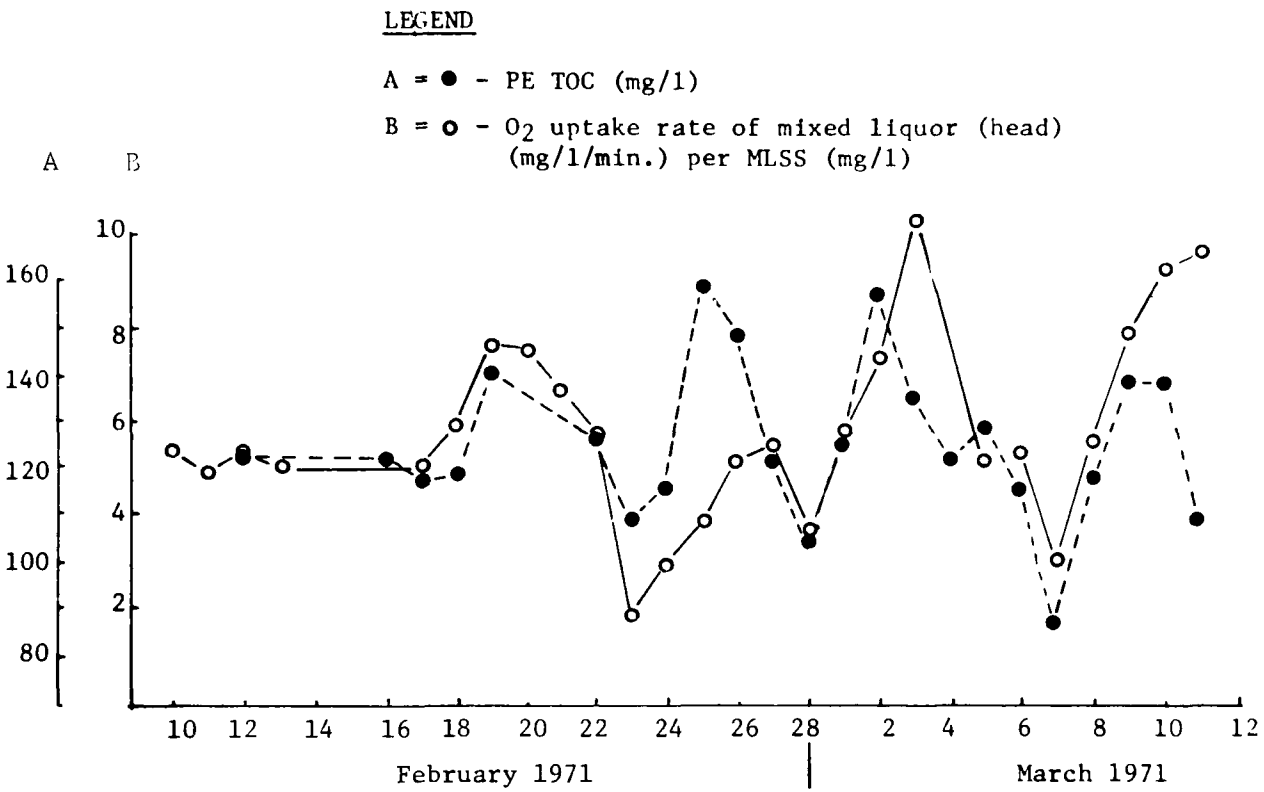


FIGURE 39

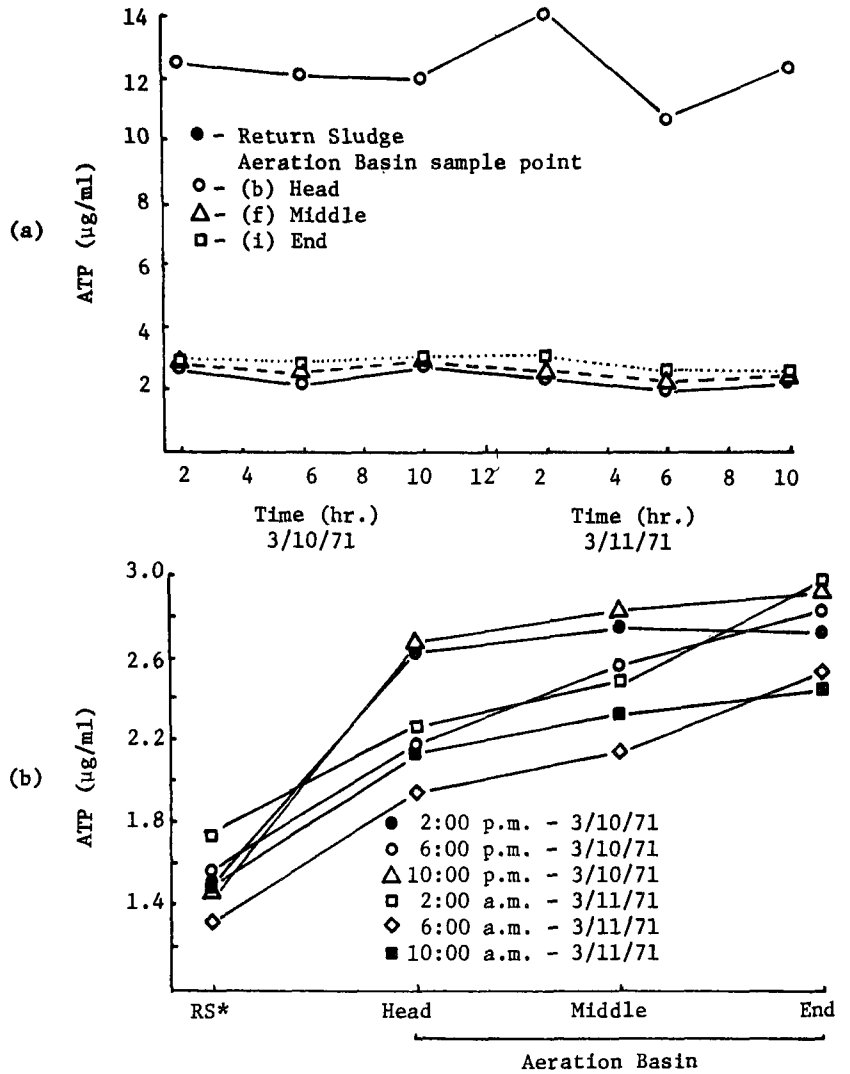
Correlation Between O_2 Uptake Rate/mg MLSS at the Head of the Aeration Basin and TOC of the Primary Effluent at the Baltimore Back River Activated Sludge Plant

drop in TOC did not result in an immediate O_2 uptake rate decrease; and after a lag of approximately 24 hours, the O_2 uptake rate would exhibit the effects of the earlier decreased nutrient concentration and fall. This might mean that cellular activity could remain at a potentially high level for perhaps only one day after experiencing a sudden drop in organic loading. This decrease in activity was not reflected by changes in the ATP level and, therefore, cannot be interpreted as a loss in viability.

A repeat of the 24-hour MLATP experiment, as presented in Figure 30 and Table 19, was conducted on 10 March 1971 to check the earlier findings. Results of this experiment, given in Figure 40 and Table 20, confirmed the earlier findings. A greater average increase in ATP throughout the mixed liquor may have been observed in this second experiment. A comparison of data from Table 19 and Table 20 was made as follows:

From Table 19	From Table 20	
2.36 µg/ml (0.23 x 10.25)	1.42 µg/ml (0.11 x 12.31 µg/ml)	= Concentration of ATP in return sludge relative to its dilution in mixed liquor
3.00 µg/ml	2.31 µg/ml	= Average concentration of MLATP at the head of the aeration basin
3.35 µg/ml	2.73 µg/ml	= Average concentration of MLATP at the end of the aeration basin
Therefore:		
0.64 µg/ml	0.89 µg/ml	= ATP produced upon contact of return sludge and primary effluent
0.35 µg/ml	0.42 µg/ml	= ATP produced throughout aeration basin
0.99 µg/ml	1.31 µg/ml	= Total increase in ATP during one aeration cycle

The BOD loading at the time of the earlier experiment (before ATP control) was approximately 5% higher than at the time of the latter experiment. However, as shown, the production of ATP, i.e., viable biomass, was 23% greater after the period of ATP based control. This may mean that after the period of ATP based control, return sludge was better able to act upon available substrate and convert it into viable material.



*Values were calculated by multiplying measured RSATP times percent sludge return (.122).

FIGURE 40

ATP in Mixed Liquor and Return Sludge at the Baltimore Back River Activated Sludge Plant During the ATP Regulation Period (3/10/71 - 3/11/71)

TABLE 20

ATP Concentrations in Mixed Liquor and Return Sludge at Baltimore Back River Activated Sludge Plant, During ATP Regulation Period (3/10/71 - 3/11/71)

	ATP(μg/ml)			
	Aeration Basin			
	Head (b)	Middle (f)	End (i)	Return Sludge
3/10/71				
2:00 p.m.	2.47	2.77	2.82	12.5
	2.84	2.80	2.69	12.6
3:30 p.m.	2.23	2.37	2.32	
6:00 p.m.	2.18	2.47	2.72	12.19
	2.20	2.71	2.97	12.35
10:00 p.m.	2.64	2.76	2.95	12.00
	2.73	2.95	2.95	12.02
3/11/71				
2:00 a.m.	2.27	2.53	2.96	14.10
	2.27	2.48	3.03	14.10
6:00 a.m.	1.94	2.17	2.62	11.2
	1.95	2.15	2.51	10.2
10:00 a.m.	2.23	2.25	2.46	12.3
	2.98	2.43	2.49	12.3
Average ±s. d.	2.31 ±.35	2.52 ±.24	2.73 ±.25	12.31 ±.79
	→ 8.4% Increase		→ 7.6% Increase	

N.B. Statistical analysis of the percent deviation between duplicate assays yielded: Std. Dev. 2.67, Std. Error of the Mean 0.39, and Coefficient of Variation 2.67.

Also, it might be noted that approximately equal quantities of ATP were produced in both halves of the aeration basin. In the earlier run ATP production predominated in the second half. This would mean that, in the second run, cells were in better condition for biomass production earlier after the start of aeration.

Although the average values shown in Table 20 were used to compute the percent increases shown, a statistical comparison of these averages fails to show significance if only the mean values are compared. A better comparison is shown in Figure 40b. For each sampling time, the MLATP was higher at the middle than the head

12/13 times. It was also higher at the end than at the middle 11/13 times. The probabilities that the MLATP increased between the head and middle and middle and end of the aeration basin were, therefore, 0.92 and 0.85, respectively. The probabilities of increase during the earlier experiment were somewhat less, being 0.75 and 0.5, respectively.

The possibility that the composition of the incoming waste had changed sufficiently to provide a more usable substrate during the second experiment cannot be ruled out. No specific analyses of the primary effluent were made. Additional experiments of this type are needed to substantiate these preliminary findings.

Of additional interest in Table 20 is the degree of reproducibility with which the ATP extraction and assay are routinely performed. Each set of duplicate numbers represent two separate dilutions, extractions, and ATP assays of nonblended return sludge or mixed liquor sample. These data are typical of those obtained during the one-month study at Baltimore.

SECTION X

OPERATION OF THE ARLINGTON COUNTY ACTIVATED SLUDGE SEWAGE TREATMENT PLANT

The Arlington County Secondary Treatment Facility is a step aeration, activated sludge plant with a design capability of 24 MGD. As shown in Figure 41, there are two aeration tanks, each having four passes. Settled sludge is returned to the A pass (gates s_1 and s_2) and is aerated throughout its length. A variable flow of primary effluent enters at the ends of the first three passes (gates b, c, and d). Mixed liquor from both aeration tanks (gates e_1 and e_2) mixes in a common sluice before being distributed to the three secondary clarifiers. Settled sludge moves by gravity to a sump well; a portion of it is returned to the aeration basin, and the excess is wasted.

Gate heights controlling inflowing primary effluent are adjusted according to changes in the sludge density index (SDI). In practice, openings of the gates are regulated to shift the waste load gradually forward in the aeration tank to improve treatment and then back to improve settling qualities of the sludge.

Figure 42 shows the diurnal fluctuations in flow rate, waste concentration measured by TOC, and DO level in the aeration tank. Although there is some question concerning accuracy of meters, these flow data show a daily average of approximately 22 MG of settled sewage.

A peculiarity encountered in the Arlington plant was the occurrence of a heavy, dark foam, which, at times, covered the surface of the aeration tanks and spread onto walkways. It accumulated most heavily at the head of the aeration tank, but some escaped with the final effluent, reducing its quality. Microscopic examination showed the predominance of branching, filamentous organisms.

The BOD of primary effluent at Baltimore had averaged 215 mg/l. Effective treatment had been maintained when the concentration of ATP in the mixed liquor at the head of the aeration basin was maintained at 2 µg/ml. The BOD of Arlington's primary effluent averaged approximately one-half the Baltimore value. Therefore, a lower level of MLATP was expected to be effective. But contact time in the Baltimore plant approximated five hours in contrast to that in the Arlington aeration tank, which varied from several hours to approximately 30 minutes, depending upon sewage flow rate, return sludge flow rate, and gate openings.

Measurement of ATP levels was made on samples collected at the points indicated in Figure 43. Essentially, the same parameters were measured at the Arlington plant as in the Baltimore study. In addition to the

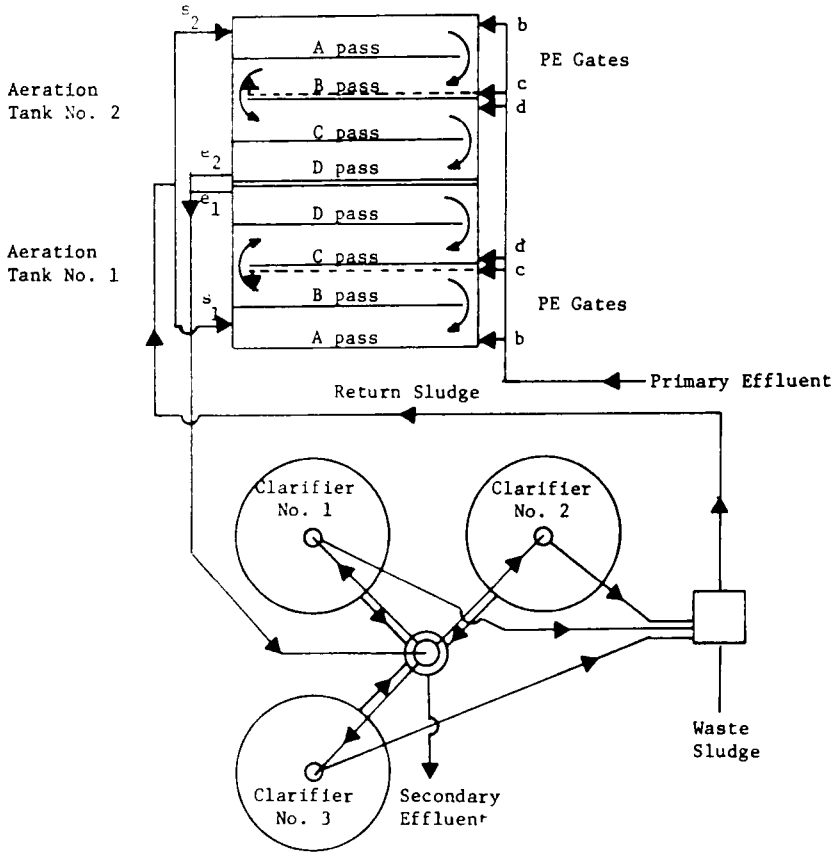


FIGURE 41

Simplified Diagram of the Activated Sludge
Portion of the Arlington County Secondary Treatment Plant

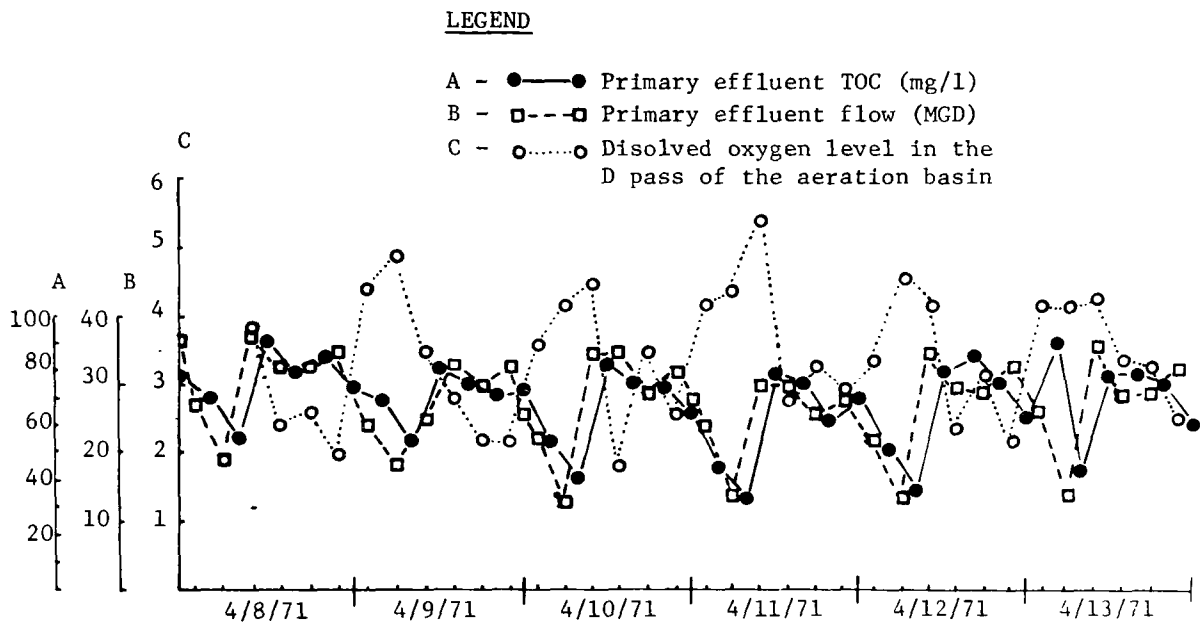


FIGURE 42

Daily Fluctuations at the Arlington Sewage Treatment Plant

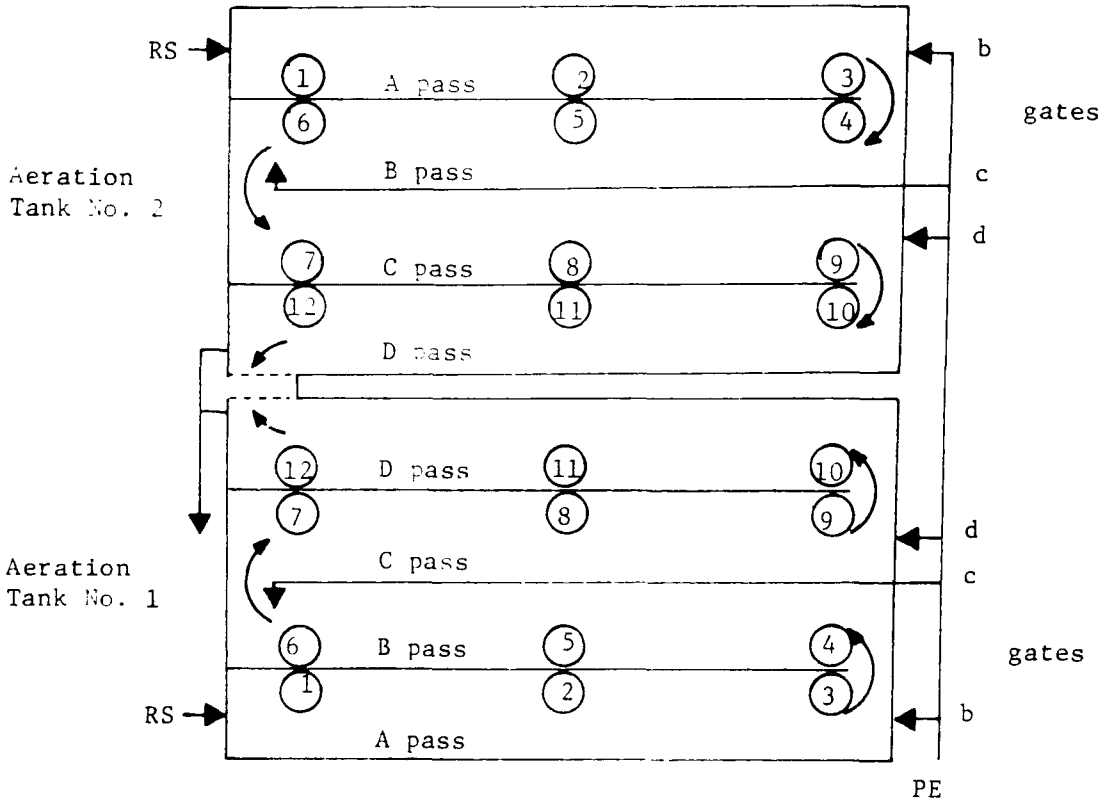


FIGURE 43

Diagram of Aeration Tanks Showing Sampling Points (Circled Numbers) Initially Used for Study

determination of ATP, these included: oxygen uptake rate, TOC, suspended solids (turbidity), phosphate, ammonia, nitrate, nitrite, and total nitrogen.

When work started on 18 March 1971, a return sludge rate of 16 MGD was in effect. The mixed liquor suspended solids level was 3350 mg/l in the A pass and 1000 mg/l at the end of the D pass. An average of 82% reduction in BOD and 55% reduction of suspended solids had been achieved during the first three weeks of March. Determination of ATP levels were made on samples collected at the points shown in Figure 43. The results of the determinations of ATP and of oxygen uptake rates made on several days are shown in Figures 44 and 45.

At the Baltimore plant, ATP levels had increased slightly and progressively throughout the plug flow aeration basin. At Arlington, ATP levels in each of the passes were expected to reflect the dilutions which occurred at each point of waste entrance. However, stepwise decreasing levels of ATP were not found in the aeration basin. As shown in Figure 44, there was a decrease, but it was not characterized by sharp drops at the waste entrance points. Very small differences in ATP levels of the C and D passes were observed. However, the d gate was set to admit approximately 50% of the total flow. The ATP values indicated that an increase in concentration occurred concomitant with dilution. This might result from poor mixing. The fact that sample points near the waste inlets showed more scatter in values than others supports this explanation. Sample points were selected (Figure 46) that showed minimum scatter and would most probably provide representative values for the individual passes.

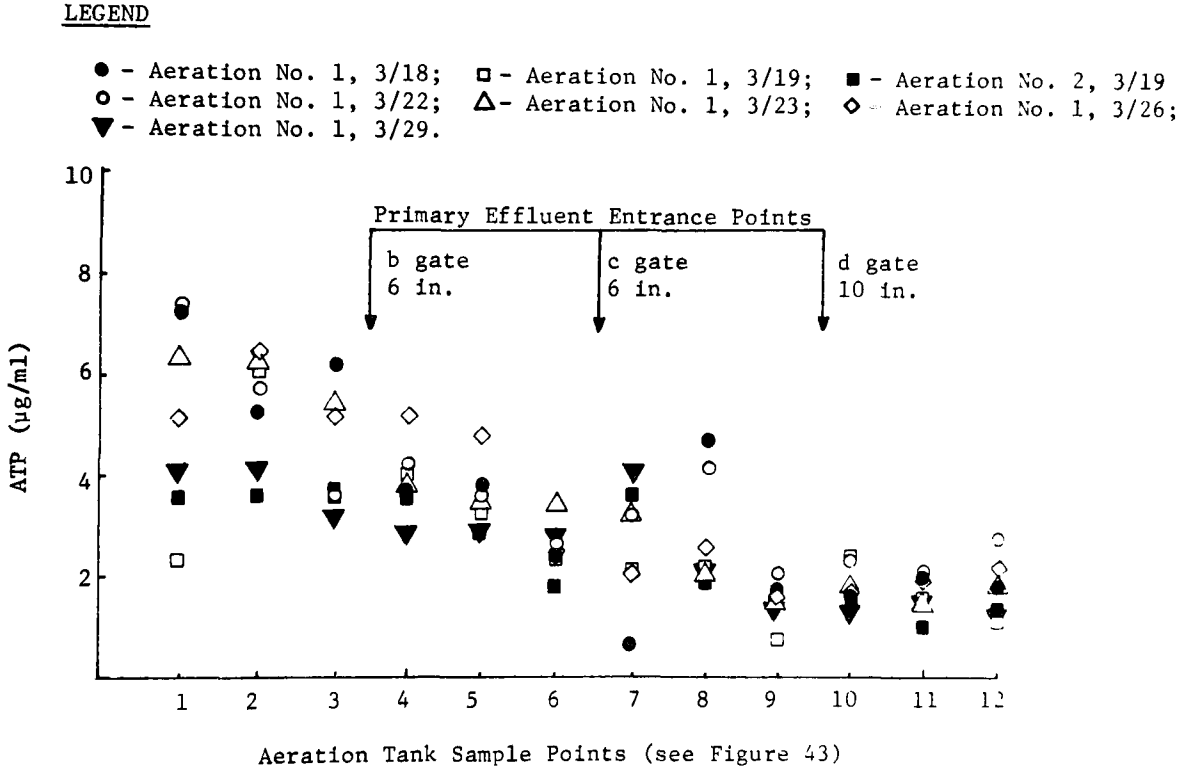


FIGURE 44

Concentration of ATP in the Aeration Tank at the Arlington Sewage Treatment Plant on Several Days

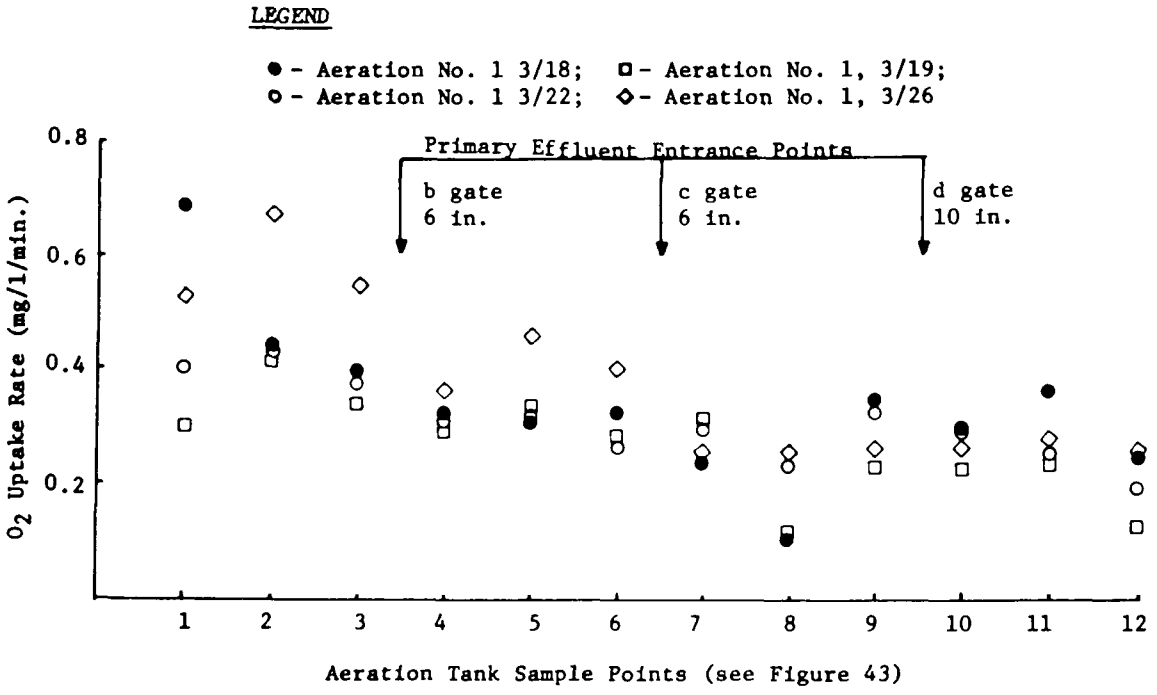


FIGURE 45

Oxygen Uptake Rate in the Aeration Tank at the Arlington Sewage Treatment Plant

Oxygen uptake rate (Figure 45) showed results similar to those for ATP. Contact of sludge and waste at the three feed points was expected to produce a spike in the oxygen uptake rate, but instead, a gradual decrease was observed throughout the aeration basin. The individual waste gates were not metered. Since the flow rate of the incoming waste changed constantly, it was not possible to determine flow rates for each of the three gates. A definite pattern of dilutions relative to the gate openings at the three feed points could not be established.

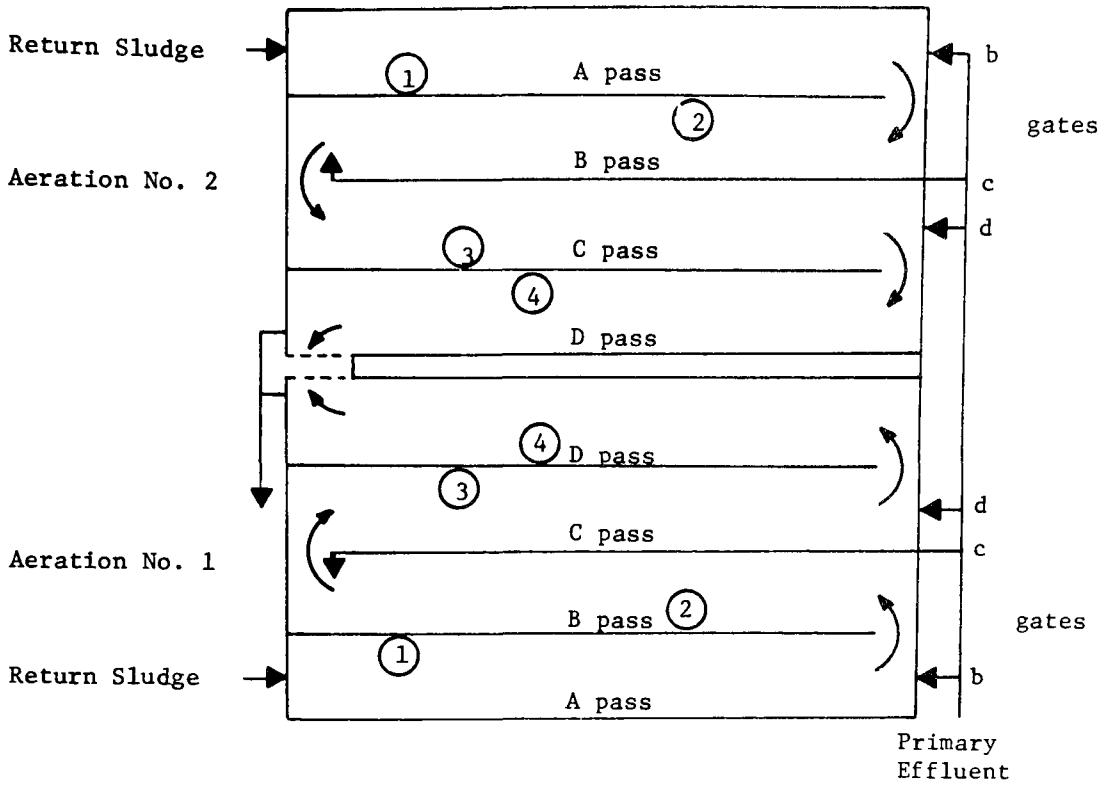


FIGURE 46

Diagram of Aeration Tanks Showing Sampling Points Used for Study After 3/29/71

An experiment was performed to determine the fluctuations in ATP concentration and oxygen uptake rate which might be expected in a 24-hour period during which the waste flow rate varied widely. The results are shown in Figures 47 and 48. As expected, the ATP concentration and the oxygen uptake rate vary with the flow rate. However, these variations were not great in comparison with the variations in flow rate.

Total phosphate analyses were run on a series of grab samples. The phosphate reductions during a week of observations, Table 21, averaged 15%.

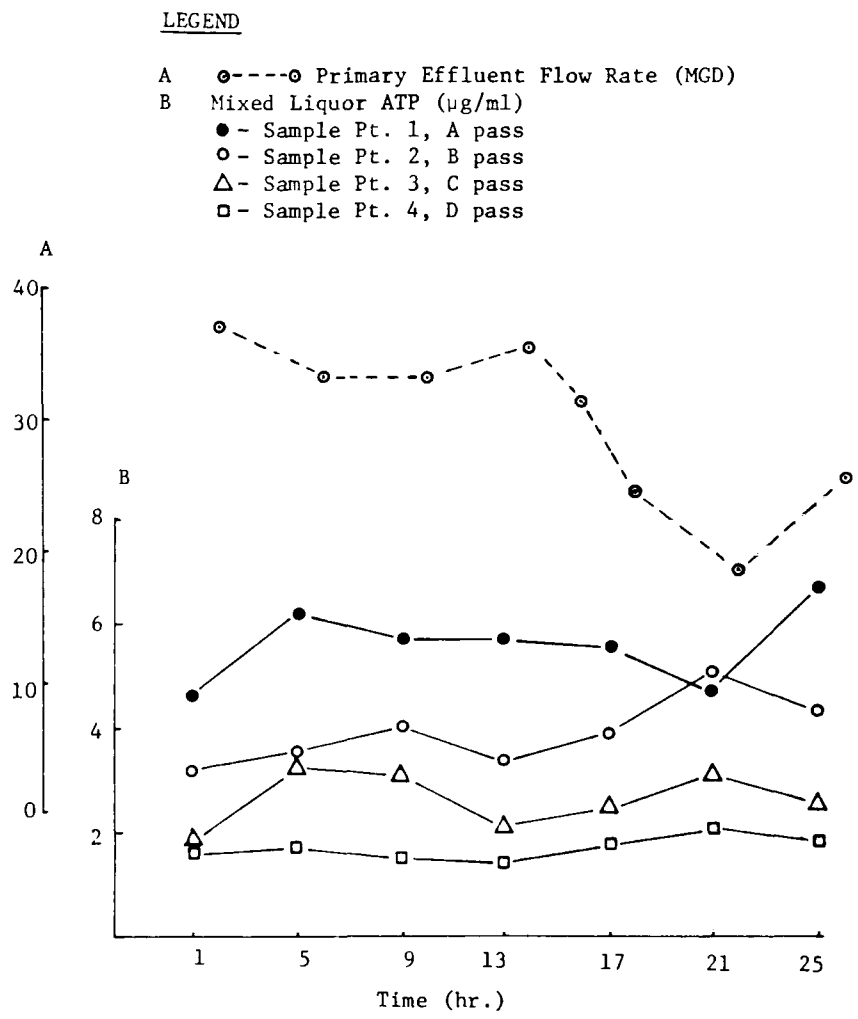


FIGURE 47

ATP Concentration of Mixed Liquor From the Aeration Tank and Flow Rate of Primary Effluent At the Arlington Secondary Treatment Plant

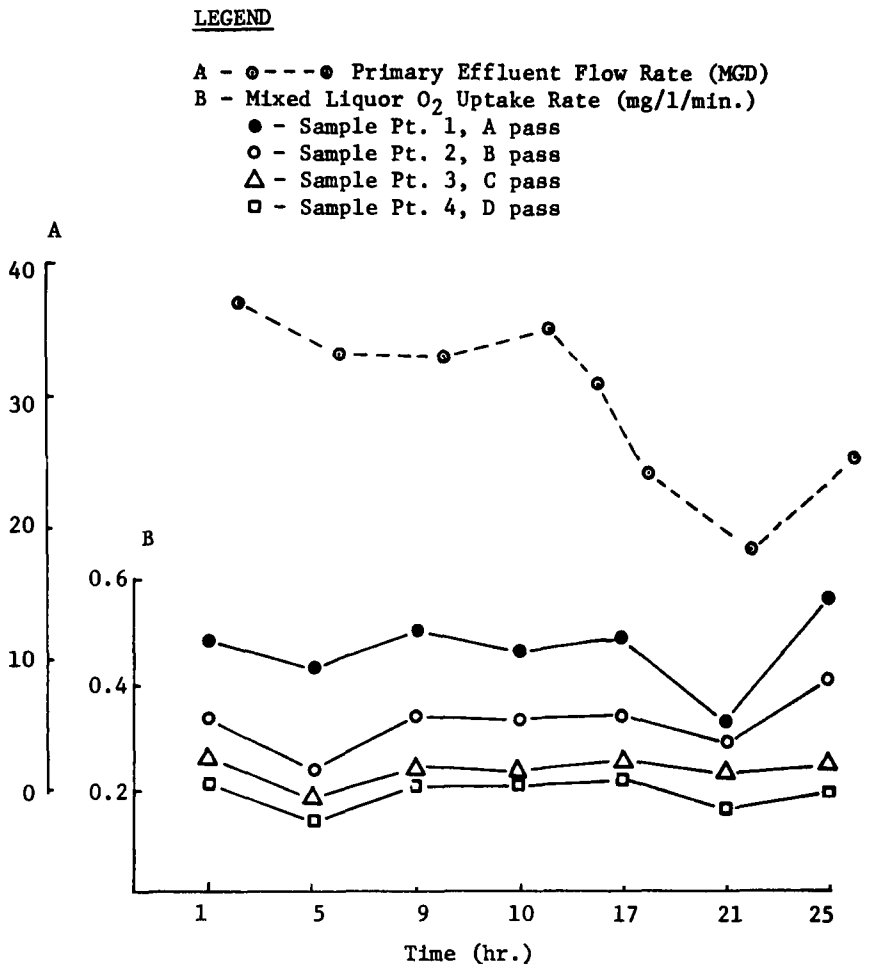


FIGURE 48

Oxygen Uptake Rate of Mixed Liquor From the Aeration Tank and Flow Rate of Primary Effluent at Arlington Secondary Treatment Plant

TABLE 21

Phosphate Analysis Made at the Arlington County Sewage Treatment Plant Prior to ATP-Based Control

Date*	Total Phosphate, PO_4-P (mg/l) Primary Effluent	Secondary Effluent
3/18/71	7.4	7.3
3/19/71	7.0	6.1
3/22/71	6.9	6.1
3/23/71	7.7	5.7
3/24/71	7.4	5.6
Average	7.3	6.2

Reduction = 15%

*Six 2-hour samples were collected between 8:00 a.m. and 6:00 p.m. on each day. Values expressed are an average of these six assays.

The most critical biomass concentration was believed to exist in the D pass. The effect of return sludge rate change on ATP measured biomass level in the D pass was, therefore, used for control. Control measures reduced the return sludge rate to decrease the BOD-to-ATP ratio and extend the reaeration period of return sludge in the A pass. An attempt was also made to shift the waste loading from predominance in the D pass to a slight predominance in the B pass. These changes produced a decrease in the sludge density index. In an attempt to counter the drop in index, changes in gate openings were made starting on 20 April 1971 to shift the PE load back to the D pass. Figure 49 shows the levels of ATP measured in the four passes of the aeration tank prior to and during the ATP based control period.

The ATP level in all passes rose during the period of decreased return rate. This rise was parallel, but not proportional to, changes in the suspended solids (Figure 50). Return sludge suspended solids showed, at most, a doubling during the period of decreased return rate, but ATP levels increased more than three fold. The initial change in return rate, made on 5 April 1971, first produced a decrease in the ATP level in the D pass. However, after two days, the ATP concentration in this pass increased and remained at a level 25% above that observed prior to 5 April 1971. The suspended solids

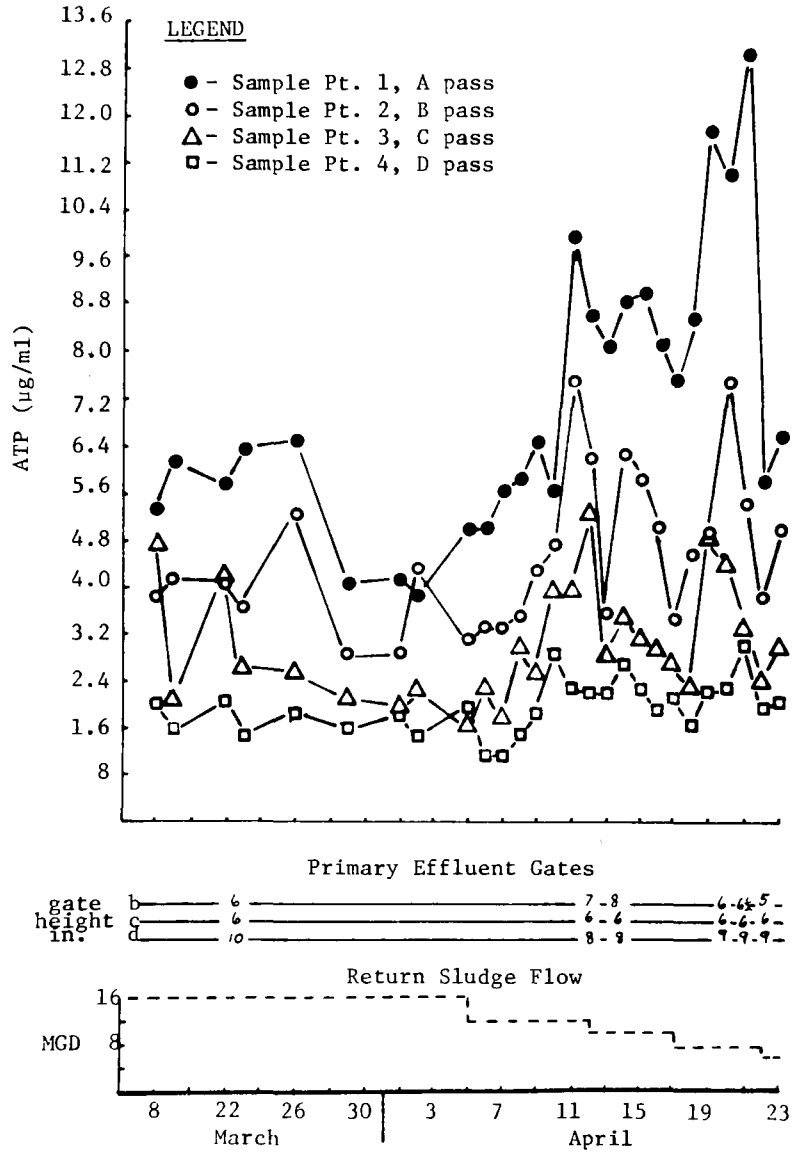
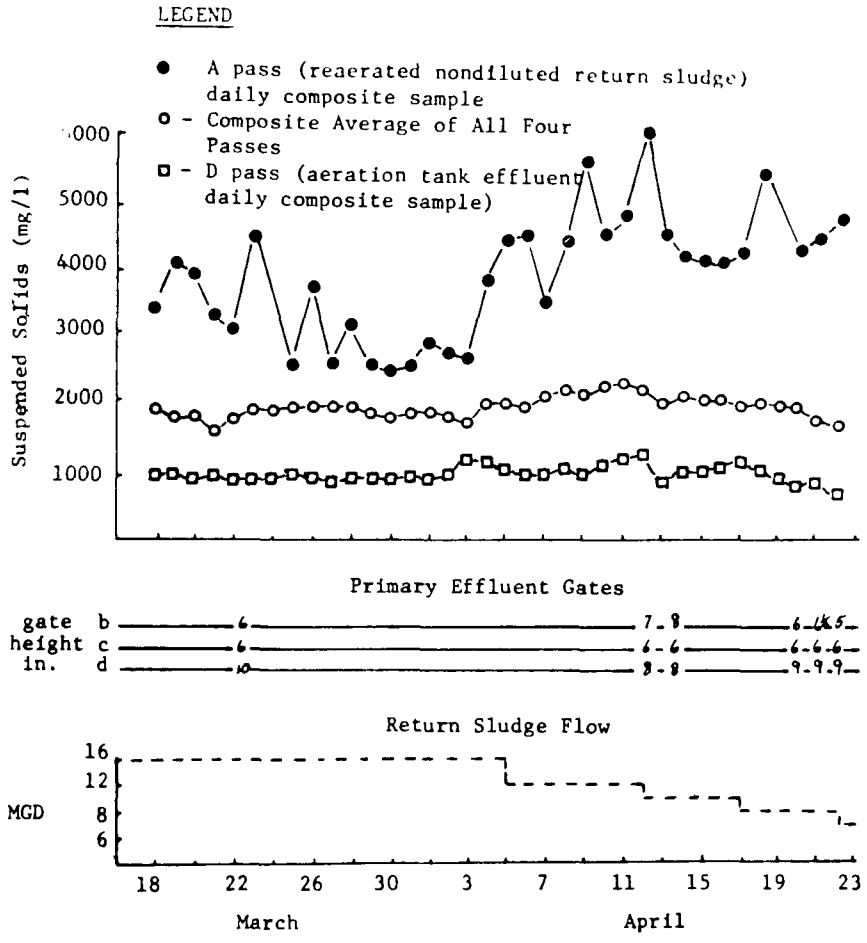


FIGURE 49

Concentration of ATP Found in the Aeration Tank of the Arlington Sewage Treatment Plant Between 3/18/71 and 4/23/71



Values supplied by plant personnel.
N.B. MLVSS = 0.80 ± 0.017 x MLSS

FIGURE 50

Suspended Solids Found in the Aeration Tank of the Arlington Sewage Treatment Plant Between 3/18/71 and 4/23/71

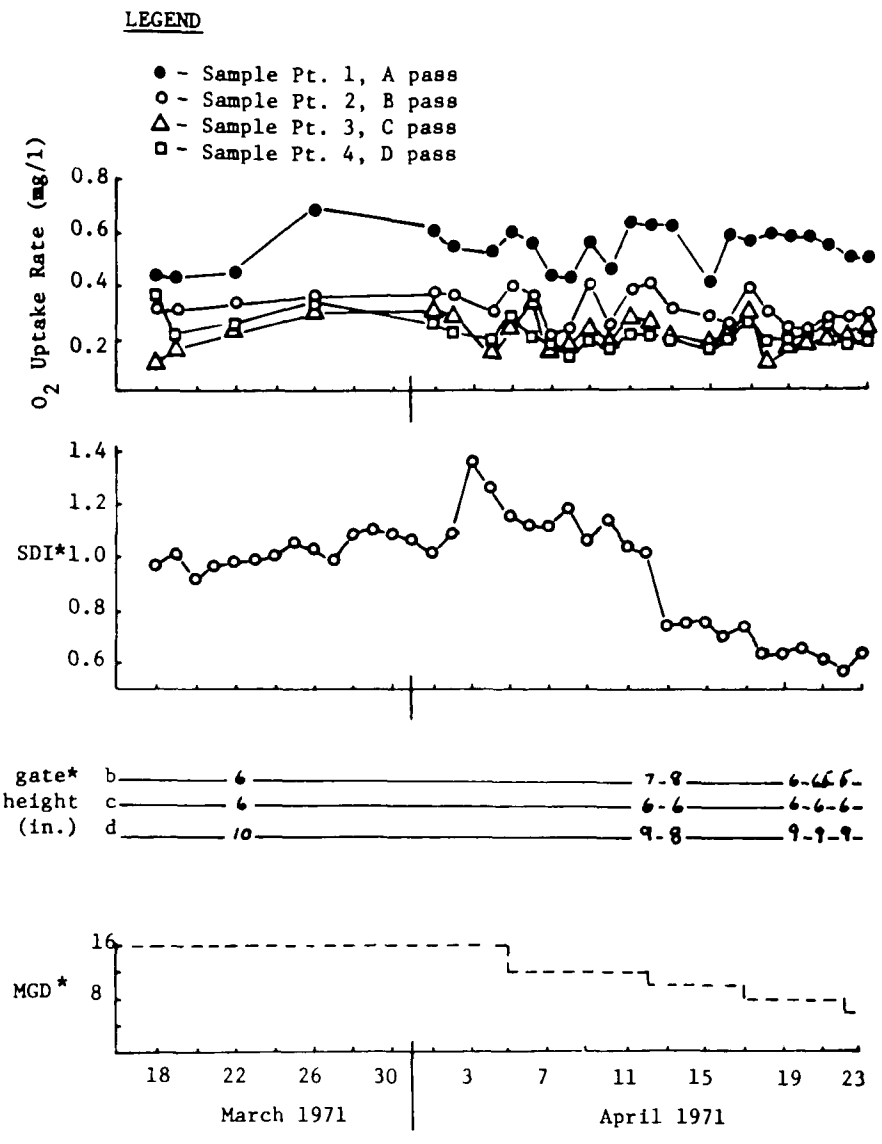
level in D pass did not reflect the increase in ATP. The sharp drop in ATP in the A pass which occurred on 22 April 1971, was indicative of cell death and it was most likely a response to the gate changes which were made in an attempt to halt a decreasing trend in the sludge density index (SDI). As shown in Figure 51, the SDI fell sharply after the 12 April 1971 changes and continued to decrease gradually thereafter. Although the elevated ATP levels were observed during the period of decreasing SDI, the decrease in settling ability is considered to have been associated with the points and ratio of waste entrance into the aeration basin. Prior to the gate changes made on 12 April 1971, the SDI had remained above 1.0. The oxygen uptake rate, Figure 51, remained relatively constant throughout the entire period of testing. The relatively low level of activity, in comparison to that observed in Baltimore, may be indicative of the lower BOD concentration of the Arlington waste.

Twenty-four hour composite samples of primary and secondary effluents were assayed for ammonia, nitrite, and nitrate. The results are presented in Figure 52. Ammonia was reduced by an average of 27%, while nitrate and nitrite concentrations increased 5.9 and 7.0 times between primary and secondary effluents. There was some tendency for nitrate levels to decrease during the test period, but there were no indications of major changes in ammonia or nitrite concentrations.

The efficiency of waste treatment in terms of BOD, TOC, and SS removal during the period 18 March 1971 to 23 April 1971 is shown in Figure 53. All indices showed improved treatment results during the test period. However, further studies will be required to specifically determine the factors which contributed to this improvement.

A summary of the effects of ATP based control at both the Baltimore and ARlington plants are shown in Table 22. Data obtained during normal plant operation immediately prior to testing and over a two-month period prior to testing are shown.

The advantages of the ATP based biomass control are: (1) real time control which makes possible the measurement of active biomass in time to adjust the process to the waste being accepted. In this way, the ATP measurement could provide information for the optimum mixing ratio of return sludge and primary effluent. (2) better control since the assay measures the active fraction of sludge or mixed liquor. Factors which effect cell viability or the degree of inert accumulation in sludge would not affect the control system. (3) rapidness of response when changes are made in the system. It is possible that changes in the population structure during the initial stages of bulking may be observed as a change in biomass. Proper counter measures to prevent an upset may become feasible with this early



*Values obtained from plant records

FIGURE 51

Sludge Density Index (SDI) and O_2 Uptake Rates Measured in the Aeration Tank of the Arlington Sewage Treatment Plant

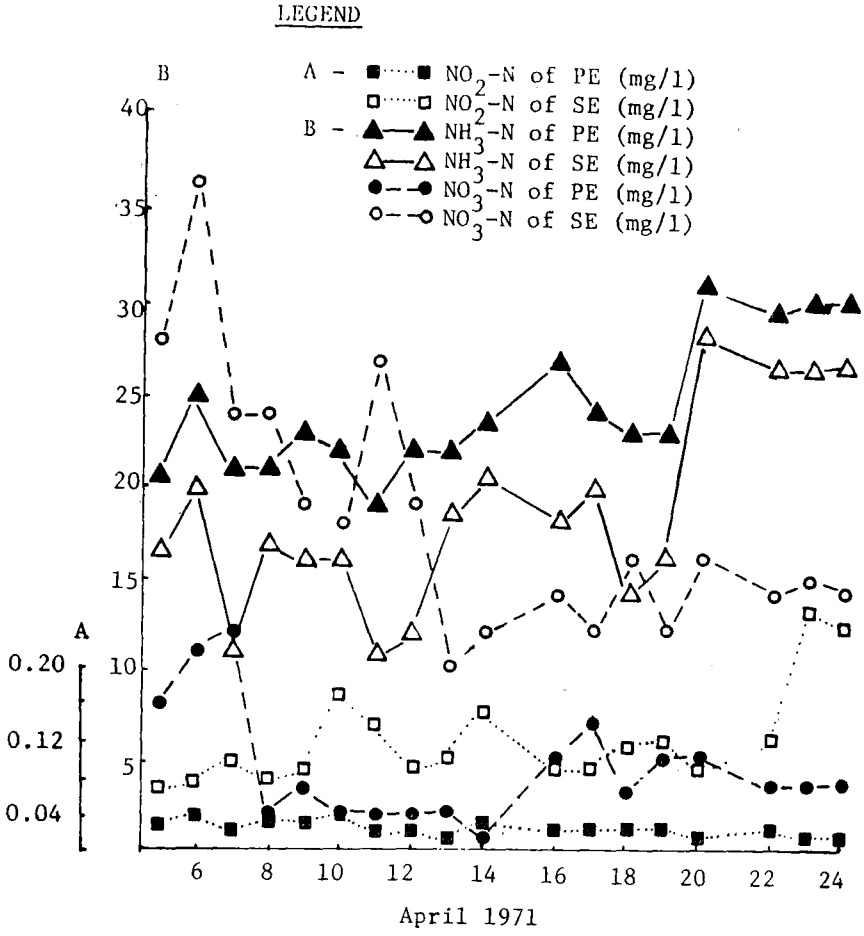


FIGURE 52

Ammonia Nitrogen (NH_3-N), Nitrite Nitrogen (NO_2-N)
and Nitrate Nitrogen (NO_3-N)
Measured in the Primary and Secondary Effluent
At the Arlington Treatment Plant

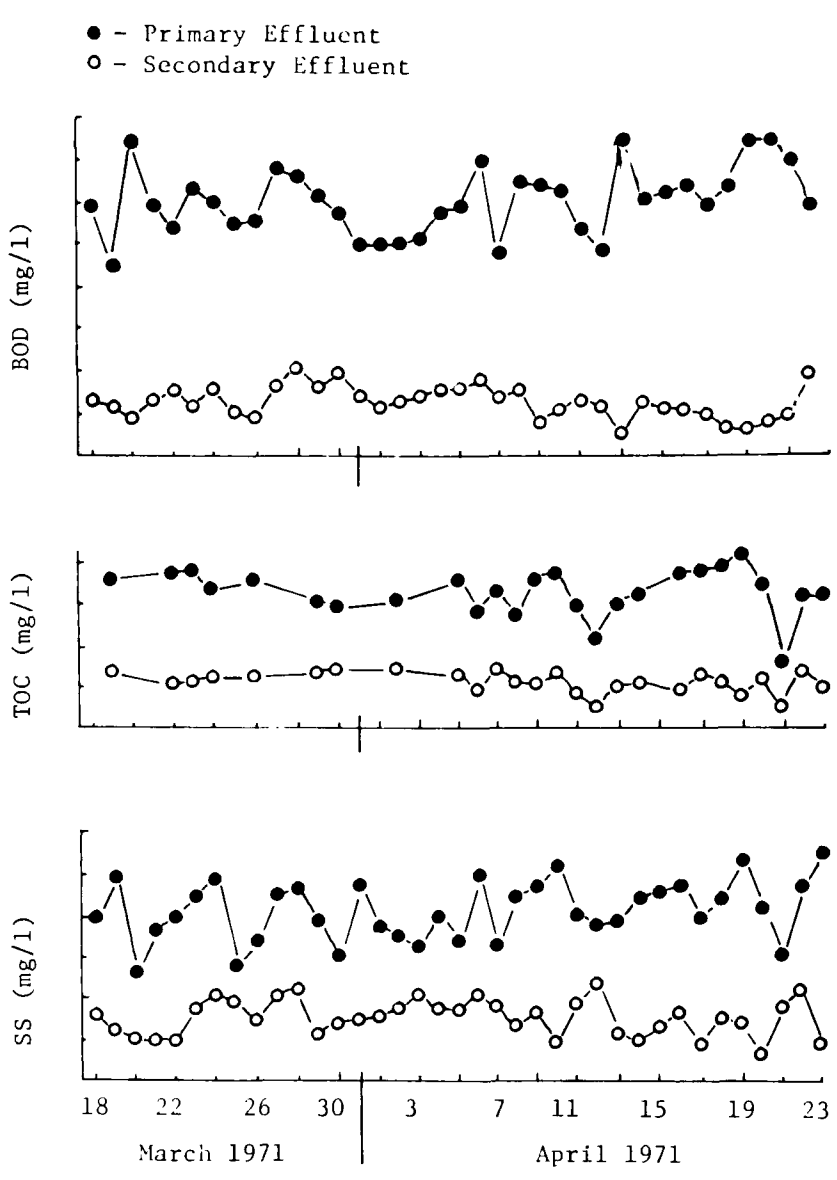


FIGURE 53

BOD, TOC and SS of Primary and Secondary Effluent (Composite Samples) at the Arlington Sewage Treatment Plant

TABLE 22

Effects of Using ATP Based Control on Full-Scale Treatment

	Baltimore		Arlington	
	Prior to Test	During Test	Prior to Test	During Test
Return Sludge (MGD)	4.5	2.6	16	8
BOD Reduction (%)	96 (95)	95	76 (77)	82
TOC Reduction (%)	69	71	62	68
SS Reduction (%)	94 (91)	93	50 (48)	66
Waste Sludge Produced (lbs./day)	(18,900) 20,300	18,600	-	-
Sludge Density Index	0.88 (0.45)	0.89	1.0	1.2 - 0.6

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 approximately two-week period immediately preceding the reduction in return sludge.

Sludge production at Baltimore rose to 22,300 lbs./day during the first week following the reduction in return sludge rate. It then fell to the average shown during the final two weeks of testing.

warning. (4) Much can be learned about the functioning of a treatment plant from the assay. The response of organisms to various conditions, sites of metabolic activity, changes in the percent active fraction of sludge and the effect of aeration and settling time in various processes could be observed and serve as important control and engineering design criteria. (5) Additional work may demonstrate that ATP is the best parameter for measuring the living biomass responsible for sewage treatment.

SECTION XI

SUMMARY

The extraction procedures used included the use of butyl alcohol and sonication, chilled perchloric acid, boiling Tris buffer, dimethylsulfoxide, and trichloroacetic acid. Several of these procedures, such as butyl alcohol extraction with sonication and chilled perchloric acid, gave satisfactory results; however, they were cumbersome to perform, time-consuming, and would be difficult to automate (an important consideration for future development). Dimethylsulfoxide inhibited the reaction. Tris buffer and trichloroacetic acid extraction procedures were satisfactory, could be conducted rapidly, and can readily be automated. The procedures were validated on pure ATP solutions, pure cultures of several organisms, and return sludge.

A technique for ATP assay which eliminated syringe contamination by ATP, allowed assays of some sludge and mixed liquor to be performed with a coefficient of variation of approximately 3%. However, variations in the sludge did have some effect since some sludges could not be as reproducibly assayed. Recovery of ATP added to sludge was found to be approximately 100% provided the sludge organisms were first killed. Viable sludge attacks exogenous ATP at a very rapid rate.

The efficiency of extraction was found to be maximum between certain limits of sludge concentration. Dilution of sludge prior to extraction greatly improved the extraction efficiency. However, the dilution needed to be held to a minimum since large dilution factors greatly magnified errors in assay and sampling.

Methods of handling standard ATP solutions and enzyme reaction mixtures were established. The reagents were found to be stable and of consistent quality.

ATP assays were conducted on pure cultures of organisms to determine the constancy of ATP concentration per cell and cell mass during various phases of growth. Tyrosine content was measured as an indicator of total protein of both viable and nonviable organisms. Oxygen uptake rate of the cultures was taken as a measure of the metabolic activity of the cells. Turbidity measurements, total cell counts, and dry weights were used as parameters to provide indicators related to mass. The organisms studied included *Escherichia coli*, *Zooglea ramigera*, *Sphaeratilus natans*, and *Bacillus sp.*

Concentrations of ATP in some cultures were found to vary by an order of magnitude on a per cell basis, but concentrations of ATP per µg of tyrosine showed much less deviation. *Bacillus* cells which were much larger than *Z. ramigera* and *E. coli*, had an average of 7×10^{-9} µg ATP/cell, while the latter organisms had an average of 5×10^{-10} µg ATP/cell. However, concentration of ATP per µg of tyrosine for all species tested showed similar ranges of values. The largest differences

occurred in very young and very old cultures where cell viability may be questioned. The data tend to indicate that the ratio of ATP to viable cell mass is relatively constant.

Growing bacterial cultures exhibited a peak in ATP concentration which coincided with a peak in O_2 utilization. The peak occurred in the latter portion of the logarithmic growth phase as determined by turbidity measurements and total microscopic counts. ATP concentration and O_2 uptake rate of the culture decreased rapidly during stationary growth.

The correlation of these two parameters would seem to be significant, since they indicate the presence of living organisms undergoing metabolic respiration - the specific basis of biological waste treatment.

The concept of using the ATP content as a measurement of viable biomass was used to control the operation of the Biospherics Incorporated pilot sewage treatment plants. Two completely independent, but identical pilot plants were employed, one serving as control and the other, as the test system. They were operated simultaneously on a synthetic sewage (average BOD 244 mg/1). In addition to the determination of ATP content, measurements were made of the oxygen uptake rate, optical density, tyrosine, suspended solids, biochemical oxygen demand (BOD), and total organic carbon (TOC). It was found that reductions in sludge recycling did not produce proportionate reductions in mixed liquor ATP levels. A 75% decrease in return rate was necessary to effect a 50% decrease in the mixed liquor ATP level. Mixed liquor of 2 µg ATP/ml in the test section exhibited respiration rates that were nearly as high as mixed liquor containing 4 µg ATP/ml. Effectiveness of treatment in terms of BOD and TOC reduction, was enhanced slightly by the mixed liquor ATP concentration of 2 µg/ml.

The feasibility of using the ATP content of the mixed liquor as a means of controlling full-scale plant operation was tested at the Baltimore Back River activated sludge sewage treatment plant. This is a conventional plug flow activated sludge secondary treatment plant with an influent constant flow of 20 MGD. Average strength of primary effluent was 240 mg/1 BOD. Control changes were initiated to reduce the ATP content of the mixed liquor at the head of the aeration basin from approximately 3 µg/ml to a level of 2 µg of ATP per ml, which had been effective in pilot studies. To effect this reduction, the return rate of the sludge was decreased to 12%. The BOD reduction, as determined by the Baltimore plant staff, averaged more than 95% during the baseline and test periods. The reduction in TOC also remained constant with an average reduction of 70%. Changes in return sludge rate caused a change in the mixed liquor ATP concentration within 24 hours. However, a change in the mixed liquor

suspended solids was not observed until the second day. The early and prompt response of the ATP level to changes in the system is viewed as an advantage to plant operation.

The changes in oxygen uptake rate appeared to correlate most closely with the concentration of the nutrients in the primary effluent as measured by its TOC. However, a sudden drop in TOC did not produce an immediate decrease in the oxygen uptake rate; a lag of approximately 24 hours was required to notice the effect. This decrease in rate was not reflected by changes in the ATP level and, therefore, cannot be interpreted as a loss in viability.

In order to continue testing the feasibility of ATP control on full-scale plant operations, the methodology was next applied to the step aeration secondary treatment plant located in Arlington County, Virginia. At this plant, each aeration tank consists of four passes. Return sludge alone enters at the head of the first pass and is aerated throughout the length of the pass. Settled waste enters at the end of the first, second, and third passes. The volume of waste entering each pass is under gate control. The BOD of the primary effluent averaged 116 mg/l daily, approximately half that of the Baltimore plant. In contrast to the five-hour contact time in Baltimore, contact time in the Arlington plant varied from several hours to as little as 30 minutes.

Measurements of the ATP content and oxygen uptake rates of the mixed liquor at a number of points through the four passes were conducted to determine the points at which samples would be most representative. During the test period, the ratio of ATP to BOD was started slightly above that found effective in Baltimore. The rate of return sludge was reduced gradually from 16 MGD to 8 MGD in four steps.

During the test period, the ATP level rose in all passes. Return sludge suspended solids concentration doubled, whereas ATP levels increased three fold. The ATP level in the final pass increased, and remained at a level 25% above that found in the pretest period where the suspended solids level of that pass remained constant, therefore, indicating an increase in sludge viability. The oxygen uptake rate remained low and relatively constant throughout the entire period of testing. This low level of activity in comparison to that found in Baltimore may be indicative of the lower BOD concentration of the Arlington waste.

During the test period, the reduction of BOD, TOC, and SS showed improvement by 8, 10 and 10%, respectively. Although the factors contributing to these improvements were not determined, the effective reduction of BOD, TOC and SS loading on the receiving stream would have been reduced by 25, 16, and 17%, respectively.

The results of field testing are encouraging, but not conclusive. The test periods were relatively short and parallel controls could not be run.

After establishment of procedural techniques in the laboratory, no technical difficulties with the ATP assay were encountered during the latter phase of study. The control method appears promising and is now ready for more extended comparative, full-scale trials.

This first preliminary attempt at full-scale control was concerned with the most obvious parameters of operation. ATP levels were adjusted on the basis of laboratory studies and some trial and error. The optimum concentration of ATP in mixed liquor would depend upon several factors. The strength of incoming waste would be of prime importance. TOC measurements, which can be performed rapidly enough to be of control significance, may be needed to monitor the influent stream and so determine the rate at which sludge of a certain ATP content should be recycled. Also, the recycling of sludge based upon ATP content of sludge alone, may very well not be sufficient. The state of microbial activity, in addition to viability, may be important. Oxygen uptake rates were approached in this program as a possible measure of this activity.

Another factor of considerable importance is the contact time between waste and biomass. A greater concentration of viable organisms would be expected to be necessary when contact time is decreased. It would also be important to test processes like contact stabilization, to determine the optimum concentration for maximum absorption.

Temperature and dissolved oxygen level are two additional parameters which would affect the optimum biomass level. Increased temperature and/or dissolved oxygen would increase the rate of cellular metabolism, therefore, within a given time frame the required concentration of viable cells should be less.

In overview of continuous monitor of TOC, DO, waste flow, temperature and O_2 uptake rate, in addition to ATP measurements would be integrated into a signal for sludge recycling.

SECTION XII

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The laboratory research and field studies reported herein were performed by Biospherics Incorporated, Rockville, Maryland. The research team which directed the project and prepared the report consisted of Drs. Gilbert V. Levin, Principal Investigator, J. Rudolph Schrot, Research Microbiologist, and Walter C. Hess, Senior Biochemist. Mr. George Alvarez, Chemist, and Mrs. Vivian Brooks, Technician, performed most of the analytical work. Messrs. Donald G. Shaheen, Senior Chemist, George Topol, Chemist Engineer and Barry Forster, Technician, assisted in various capacities. Dr. Patricia Straat reproduced the figures included in this report.

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SECTION XIII

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SECTION XIV

ABBREVIATIONS USED IN TEXT AND FIGURES

ATP	Adenosine Triphosphate
BOD	Biochemical Oxygen Demand
DO	Dissolved Oxygen
MGD	Million Gallons per Day
MLATP	Mixed Liquor Adenosine Triphosphate
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
PE	Primary Effluent
PETOC	Primary Effluent Total Organic Carbon
RSATP	Return Sludge Adenosine Triphosphate
SDI	Sludge Density Index
SE	Secondary Effluent
SEBOD	Secondary Effluent Biochemical Oxygen Demand
SESS	Secondary Effluent Suspended Solids
SETOC	Secondary Effluent Total Organic Carbon
SS	Suspended Solids
SVS	Suspended Volatile Solids
TOC	Total Organic Carbon
TP	Total Phosphate

SECTION XV

APPENDIX

Description of Analytical Methods

Solutions Required for ATP Assay

Tris Buffer

6.075 g Tris dissolved in 2 l of sterile, distilled, deionized ATP-free water. Adjust to pH 7.75 with hydrochloric acid (equal parts concentrated HCl and ATP-free water). It is important that the water is ATP free. Ordinary distilled water run through a deionizing column should be autoclaved (two hours) to hydrolyze ATP. The buffer should be tested for ATP activity. The response of 10 µl of buffer should be less than 0.1 millivolts and is normally approximately 0.03 millivolts. After preparation Tris buffer should be refrigerated.

Enzyme Solution

A commercial preparation (Dupont) was employed. The buffer is supplied in tablet form. One tablet is dissolved in 3.0 ml of ATP-free water. To this solution is added the entire contents of one vial of enzyme powder. Allow the resulting solution to stand 15 to 30 minutes at room temperature before using.

ATP Standard Solutions

Adenosine-5-phosphate, disodium salt, 4.5 H_2O molecular weight 632. 62.5 mg equivalent to 50 mg of ATP, are dissolved in 50 ml of Tris buffer (1 mg/ml concentrated). Serial dilutions using Tris buffer are made to provide the concentrations desired to prepare a standard curve. The dilutions should run from 1.0×10^{-2} to 1.0×10^{-6} mg/ml ATP (10-0.001 µg/ml). Dilutions of 1, 0.1 and 0.01 µg/ml were routinely run with each assay.

Trichloroacetic Acid

Dissolve 5 g of TCA in 100 ml of ATP-free H_2O.

Methods of Extraction

Place 35 ml of Tris buffer in a 50-ml volumetric flask. Bring to 100°C in a boiling water bath and add 1 ml of the diluted solution to be assayed. Mix rapidly and transfer the flask to an ice bath and chill.

After the temperature has been reduced to approximately that of the ice bath, make to volume with Tris buffer, filter, and assay. In lieu of filtration, a brief settling period may be used.

TCA Extraction

Place 4.0 ml of the 5% TCA solution in a test tube maintained in an ice bath, add 1 ml of the diluted solution to be assayed, and mix well. To 35 ml of Tris buffer in a 50-ml volumetric flask, add 1 ml of the TCA mixture, dilute to volume with Tris buffer, and assay. If desired, the 1 ml of TCA mixture can be diluted to 25 ml with Tris buffer.

Method of Assay

Aliquots (10 µl) of the solution to be assayed are placed in cuvettes. The transfer is effected by placing a 10 cm length of 26 gauge Teflon tubing on the tip of a microsyringe. The plastic tubing is replaced for each solution to be assayed. The cuvette is inserted in the analyzer and 50 µl of the enzyme solution contained in a microsyringe are injected into the assay solution. Quantitation is based upon peak height of the energy output.

Determination of NH_3

Place 10 ml of the solution to be assayed (filtered primary or secondary effluent) in a 50-ml volumetric flask, add NH_3-free, distilled water to make approximately 35 ml. The flask, together with a blank solution similarly treated, is kept in an ice bath. Add, with shaking, 1.0 ml of Nessler's solution. Wait ten minutes after diluting to volume and read in a colorimeter at wavelength 520 nm. The blank is set at 100% transmittance.

Solutions Required

Nessler's Reagent

Dissolve 10 g mercuric iodide and 7 g potassium iodide in approximately 70 ml distilled water. Add the resulting solution, slowly with stirring, into 15 ml of water containing 10 g sodium hydroxide, dilute to 100 ml.

Nitrogen Standard

Dissolve 0.3819 g of ammonium chloride in 100 ml of distilled water (1.0 ml of this solution diluted to 100 ml contains 0.01 mg N per ml).

Determination of NO_2

Solutions Required

Sulfanilic Acid

Dissolve 0.6 g of sulfanilic acid in 70 ml of hot H_2O. Cool. Add 20 ml of concentrated HCl and dilute to 100 ml with water.

Naphthylamine HCl

Dissolve either 16 g anhydrous sodium acetate or 27.2 g sodium acetate trihydrate to yield 100 ml of an aqueous solution.

Sodium Nitrite Standard

Dissolve 49.3 mg sodium nitrite to yield 100 ml of an aqueous solution. This is the stock solution. Dilute 1 ml of the stock solution to 200 ml with water; 1.0 ml contains 0.5 µg nitrogen.

Procedure

Place 10 ml of the solution to be assayed (filtered primary or secondary effluent) in a 50-ml volumetric flask. Add 1.0 ml sulfanilic acid solution and wait three minutes. Add 1.0 ml naphythylamine solution and 1.0 ml sodium acetate solution. Dilute to volume, wait ten minutes, and read in a colorimeter at 520 nm. A water blank is run through the procedure and set at 100% transmittance.

Determination of NO_3

Solutions Required

Brucine-Sulfanilic Acid

Dissolve 1 g brucine sulfate and 0.1 g sulfanilic acid in approximately 70 ml of hot, distilled water. Add 3 ml concentrated HCl, cool, and dilute to 100 ml.

Sulfuric Acid Solution

Carefully add 500 ml of a concentrated sulfuric acid to 75 ml of distilled water. Cool to room temperature before using.

Nitrate Standard

Dissolve 72.18 mg anhydrous potassium nitrate in 100 ml water. This solution contains 0.1 mg N per ml.

Procedure

Place 2.0 ml of the solution to be assayed (filtered primary or secondary effluent) in a 50 ml beaker. Add 1.0 ml of the brucine reagent. Into a second 50 ml beaker, place 10 ml of the sulfuric acid reagent. Mix the contents of the two beakers. Allow the treated sample to remain in the dark for ten minutes. During the interval, add 10 ml distilled water to the second beaker. After ten minutes, add the water to the sample and mix. Allow to cool in the dark for approximately 20 minutes and read in a colorimeter at 410 nm. A water blank run through the procedure is used to set the instrument at 100% transmittance.

Determination of Total Nitrogen

Solutions Required

Digestion Mixture

To 30 ml phosphoric acid, add 5 ml of a 5% cupric sulfate solution and 10 ml of concentrated sulfuric acid.

Standard Solution

The same standards can be used as described under the Determination of Ammonia.

Procedure

It should be noted that the digestion method does not reduce nitrate to ammonia and, therefore, the nitrate-nitrogen values should be added to the total nitrogen values that are determined by digestion. To 25 ml of the solution to be assayed (filtered primary or secondary effluent), add 2 ml of the digestion mixture. A 50 ml Kjeldahl flask can be used. Digestion is continued until the solution becomes colorless. Then cool in an ice bath, add 35 ml of water and 15 ml of Nessler's reagent, all contained in a 50-ml volumetric flask. After ten minutes, dilute to volume and read at 520 nm. Standards and readings are the same as for ammonia determination.

Tyrosine Determination

Solutions Required

Phenol Reagent

In a 1 l flask equipped with a reflux condenser, place 50 g sodium tungstate, 12.5 g sodium molybdate, 350 ml water, 25 ml, 85% phosphoric acid, and 50 ml concentrated hydrochloric acid. Reflux gently for ten

hours. Add 75 g lithium sulfate, 25 ml water, and several drops of bromine. Boil the mixture for 15 minutes, without the condenser, to remove excess bromine. Cool, dilute to 500 ml, and filter. Before use, the reagent should be diluted with an equal volume of water.

Tyrosine Standard

Dissolve 20 mg tyrosine in 100 ml of 0.1 N hydrochloric acid; this is the stock solution. Dilute 10 ml of the stock solution to 100 ml with distilled water (1 ml contains 0.02 mg tyrosine/ml).

Micromethod Procedure

To 5 ml of the solution to be assayed (filtered primary or secondary effluent) add 0.2 ml 5N sodium hydroxide. Place the test tube in a boiling water bath for 15 minutes. Cool, add 0.2 ml of the phenol reagent, dilute to 10 ml, and read at 690 nm. A blank is similarly treated. Standard curve is prepared from 1.0 - 5.0 ml aliquots of the tyrosine standard solution.

Orthophosphate Determination

Solution Required

Ammonium Molybdate Solution

Dissolve 25 g of ammonium molybdate in 175 ml distilled water. Cautiously add 280 ml concentrated sulfuric acid to 400 ml distilled water. Cool, add the molybdate solution, and dilute to 1 l. This is the stock solution. To 1 ml of the stock solution, add 3 ml 50% sulfuric acid and 36 ml water. This is the reagent used for the micromethod.

Stannous Chloride Solution

Dissolve 10 mg stannous chloride in 2.5 ml 10% hydrochloric acid; add 22.5 ml distilled water.

Phosphate Standard

Dissolve 0.4393 potassium dihydrogen phosphate in 1 l distilled water. This is the stock solution. Dilute 10 ml to 200 ml with distilled water. This solution contains 0.005 mg phosphorus in 1 ml (5 ppm).

Procedure

To 5 ml of the solution to be assayed (filtered primary or secondary effluent) add 2 ml molybdate solution and 0.3 ml stannous chloride solution. After ten minutes, read in a colorimeter at 690 nm. A blank and a series of phosphate standards are run at the same time.

Total Dissolved Phosphate

Acid Hydrolyzing Solution

Slowly add 30 ml concentrated sulfuric acid to approximately 60 ml distilled water. When cool, add 0.4 ml concentrated nitric acid and dilute to 100 ml.

Procedure

To 5 ml of the solution to be assayed (filtered primary or secondary effluent), add 0.1 ml of the acid hydrolyzing solution. Boil gently for 90 minutes. Cool and neutralize to a faint pink color (phenolphthalein indicator) with 5N sodium hydroxide solution. Restore volume to 5 ml and determine the orthophosphate content as described above.

Oxygen Uptake Rate Measurement

Principle

The sample (mixed liquor or return sludge) is sparged with air to create a measurable DO level and introduced into a vessel containing the oxygen sensor, Figure 1. The vessel is closed to exclude air, and the rate at which organisms consume the dissolved oxygen is measured and recorded. Rate is calculated from the slope of the recorded curve.

Apparatus

Beckman O_2 Analyzer, Model No. 777 with polarographic oxygen sensor, and a Hewlett-Packard recorder.

Procedure

A 600 ml aliquot of the sample is aerated for about one minute and immediately introduced into the flask. The solution is allowed to overflow to prevent trapping of air bubbles in the neck of the flask. The flask is stoppered and stirred with a magnetic stirrer bar. Both analyzer and recorder are turned on and measurement is continued for three minutes. From the resulting linear graph, the decrease in the dissolved oxygen level is divided by the time required. Results are expressed as the oxygen uptake rate in mg/l of oxygen per minute.

Total Organic Carbon (TOC)

TOC measurements were performed on a Beckman Model No. 915 Total Organic Carbon Analyzer. Samples were acidified with concentrated HCl to approximately pH 2 at the time of collection, and then stored under refrigeration (no longer than three days), until assay.

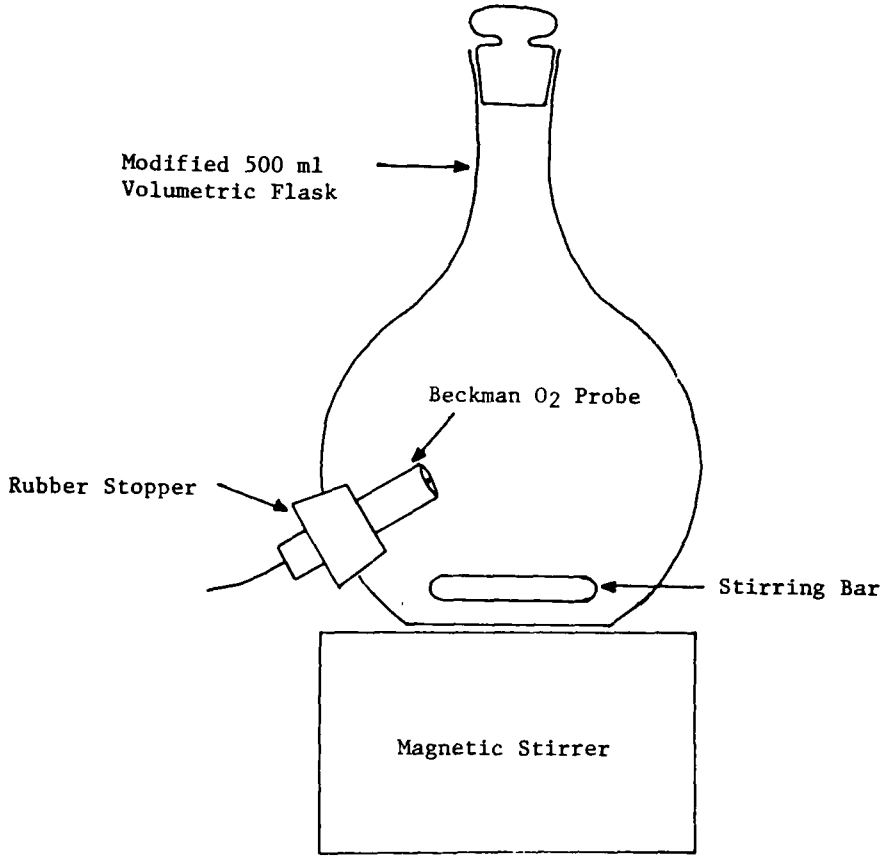

FIGURE 1

Glass Stoppered Vessel Used for
Measuring O_2 Uptake Rates

Biochemical Oxygen Demand (BOD)

All BOD values presented are for the five-day BOD test which is described in Standard Methods for the Examination of Water and Wastewater, American Public Health Association, New York, p 592 (1967).

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16. Abstract Research was conducted to determine the feasibility of using ATP as a measure of viable biomass in activated sludge. Methods were developed for the extraction of ATP from sludge and mixed liquor, and for the determination of ATP using the firefly bioluminescent procedure. Measurements of ATP were conducted on various pure cultures, pilot plant and full-scale activated sludge treatment plants. Additional parameters including BOD, TOC, oxygen uptake rate, and suspended solids were measured to provide comparative and supportive information. Preliminary tests in which ATP measurements of biomass were used to control the percent sludge return were conducted at two full-scale municipal sewage treatment plants. Lowered return sludge rates were found to produce effective treatment and increase the biological activity of the sludge. Changes in the rate of return sludge resulted in changes in ATP concentration of mixed liquor which preceded changes in suspended solids by as much as 24 hours. The assay was found to be reproducible and rapid. Results can be obtained within approximately ten minutes.

17a. Descriptors
Wastewater Treatment, Sewage, BOD Removal

17b. Identifiers
Biomass, ATP, Activated Sludge

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