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DNA CONCENTRATION AS AN ESTIMATE OF SLUDGE BIOMASS

ENVIRONMENTAL PROTECTION AGENCY WATER QUALITY OFFICE

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DNA CONCENTRATION AS AN ESTIMATE OF SLUDGE BIOMASS

by

Southwest Missouri State College
Springfield, Missouri 65802

for the

WATER QUALITY OFFICE

ENVIRONMENTAL PROTECTION AGENCY

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ABSTRACT

The objective of this project was to determine the feasibility of using DNA concentration as an estimate of sludge biomass. Such an estimate would be valid provided the DNA present in the sample represented only viable cells. This assumption was satisfied by experimentation. Since DNA constitutes about four percent of the organic matter of bacterial cells, DNA expressed as percent of volatile solids was used to estimate the amount of organic matter represented by viable cells in a sludge sample.

Sludge population in terms of cells per ml was estimated by assuming the weight of one cell to be 1×10^{-9} mg. The population size as based on DNA analyses was then compared with that of a cell count obtained from the most probable number (MPN) method. Population estimates of this type were performed on activated sludge.

This report was submitted in fulfillment of Project Number 17070DHO, under the partial sponsorship of the Water Quality Office, Environmental Protection Agency.

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This investigation has shown that DNA released from dead cells is rapidly degraded by activated sludge. The DNA concentration of this sludge may therefore be used as an estimate of the viable population, or the amount of organic matter represented by viable cells.

It was found that viable cells may represent from 75 to 100 percent of the organic matter of activated sludge. The average of 20 representative analyses during the months of July and August, 1970, were 98 percent.

INTRODUCTION AND BACKGROUND INFORMATION

The purpose of this project was to determine whether the biomass of activated sludge could be estimated by a DNA analysis of that sludge.

It is well known that a cell count of activated sludge cannot be obtained by the conventional plate count as used for uniform suspensions of cells. The main difficulties that prevent the use of the plate count are the unknown nutritional requirements of the many species present in the sludge and the incorporation of the organisms in a gelatinous matrix. DNA is a unique constituent of living protoplasm, and since the DNA content of bacterial cells is fairly constant, it was proposed that the quantity of bacterial protoplasm present in the sludge could be estimated from the DNA content of that sludge.

DNA constitutes about four percent of the volatile matter of bacterial cells. In order to make use of this relationship, one must assume that the DNA becomes degraded when the organism dies. This DNA degradation could possibly be catalyzed by enzymes that normally function in the synthesis of DNA in the viable cell, or the DNA may be degraded by other organisms present in the environment.

In order to use DNA as an estimate of viable cells, it must first be demonstrated that DNA from dead cells does not contribute significantly to the total content of sludge DNA. This was done by adding DNA extracted from sludge to activated sludge. DNA degradation was determined by periodic sampling.

Once the DNA content of the sludge has been determined, the percent DNA of volatile solids is easily obtained:

(1) $\frac{\text{ug DNA} \times 100}{\text{ug volatile solids}}$

The percent of sludge organic matter that is represented by viable bacterial protoplasm may be calculated as shown below:

(2) $\frac{\text{\% DNA} \times 100}{4.0}$

The biomass may be obtained as follows:

(3) $\frac{\text{mg DNA/ml} \times 100}{4.0} = \text{mg biomass/ml}$

For example, if the sludge was found to contain 160 ug DNA/ml, the biomass would be 4000 ug or 4.0 mg per ml.

An estimate of the number of cells per ml of sludge or per gram of dry weight may be obtained by assuming the weight of one cell to be approximately 1×10^{-9} mg. The number of cells/ml would be represented by the expression:

(4) $\frac{\text{mg biomass/ml}}{1 \times 10^{-9}} = \text{mg biomass/ml} \times 10^{9} \text{ cells/mg} =$

$$\frac{\text{mg DNA/ml} \times 100}{4.0} \times 10^{9} \text{ cells/mg} = \text{cells/ml}$$

MATERIALS AND METHODS

Preparation of Sample.

The sludge used in this research was obtained from the Southwest Springfield Waste Treatment Plant. The samples were stored in ice during the transport from the plant to the laboratory.

The procedure for extraction of DNA is based on that of Agardy and Shephard (1965).

1. Filter chilled sludge through cheese cloth.

2. Pipette 1.0 ml of chilled and filtered sludge into 4.0 ml of 12.5% trichloroacetic acid, (TCA), or 2.0 ml of sludge into 3.0 ml of 17.0% TCA. In either case the final concentration of TCA is 10%. Mix sludge and TCA by pipetting.

3. Centrifuge in the cold at 8.2 x 1000 G for 10 minutes.

4. Discard supernatant.

5. Add 5.0 ml of 95% ethanol to pellet. Mix well.

6. Centrifuge at 8.2 x 1000 G for 10 minutes. Discard supernatant.

   Steps 5 and 6 may need to be repeated if the sample contains a large amount of lipid material.

7. Add 4.0 ml of 0.5 N perchloric acid, (PCA), to pellet. Heat at 90°C for 15 minutes. Mix 2 or 3 times during this period.

8. Centrifuge at 8.2 x 1000 G for 10 minutes. Save supernatant. This is the first DNA extract.

9. Add 2.0 ml of 0.5 N PCA. Heat at 90°C for 20 minutes. Mix 2 or 3 times during this period.

10. Centrifuge at 8.2 x 1000 G for 10 minutes.

11. Combine supernatant with extract from step 8.

12. Repeat steps 9-11.

13. Perform DNA analysis of combined extracts.

The DNA analysis of supernatants was performed in the following manner:

1. Dilute supernatant with PCA to make the final concentration 0.5 N PCA, e.g. 9.0 ml of supernatant may be mixed with 1.0 ml of 5.0 N PCA, or 5.0 ml of supernatant may be mixed with 5.0 ml of 1.0 N PCA.

2. Heat the acidified supernatant for 15 minutes at 70°C.

3. Do DNA analysis.

Colorimetric Analysis of DNA

This analysis is based upon that of Burton (1965).

Reagents:

1. Aqueous acetaldehyde, 1.6 g per 100 ml.

2. Diphenylamine reagent: dissolve 1.5 g of diphenylamine in 100 ml of acetic acid. Add 1.5 ml of concentrated H_2SO_4.

   Store in the dark. Just before use add 0.5 ml of aqueous acetaldehyde for each 100 ml of reagent.

3. 1.0 N perchloric acid, (PCA).

4. 0.5 N PCA.

5. Stock standard DNA: prepare by dissolving 40 mg highly polymerized calf thymus DNA in 100 ml of 5 mM NaOH.

   Standard DNA solutions are obtained by diluting the stock solution with 5 mM NaOH. The standard solutions must be heated at 70°C for 15 minutes with equal volumes of 1 N PCA.

   The standard curve is prepared by mixing 2.0 ml of standard DNA with 4.0 ml of diphenylamine reagent containing acetaldehyde. The tubes are incubated for 16-20 hours at 25-30°C. The OD is read at 600 nonemeters.

The range of the analysis is from about 5 to 80 micrograms DNA/ml.

A typical DNA standard curve is shown in Figure 1.

Extraction of Crude DNA from Activated Sludge.

1. Strain sludge through cheese cloth and allow to settle. Pour off a volume of supernatant equal to one half of the original volume.

2. Resuspend the solids and sonicate to rupture cells and release DNA. The time required for maximum release of DNA will depend upon the size and concentration of the sample. See Figure 2.

3. Centrifuge the sonicated sludge at 12.8 x 1000 G for 10 minutes.

4. Heat the supernatant from step 3 for 15-30 minutes at 70°C in order to pasteurize the sample and coagulate protein.

5. Centrifuge at 21.6 x 1000 G for 10 minutes. The supernatant contains crude DNA.

Media.

The composition of the medium used in estimating the cell popula-

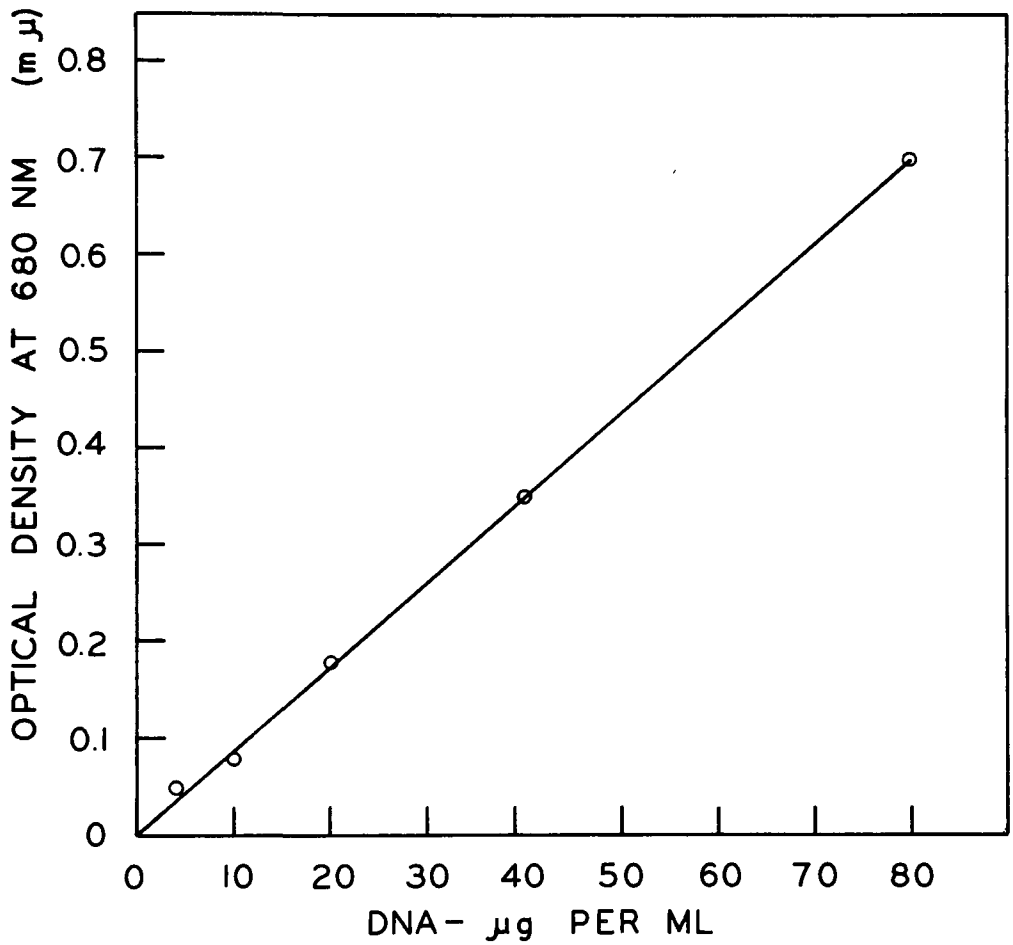

FIGURE I. TYPICAL STANDARD DNA CURVE

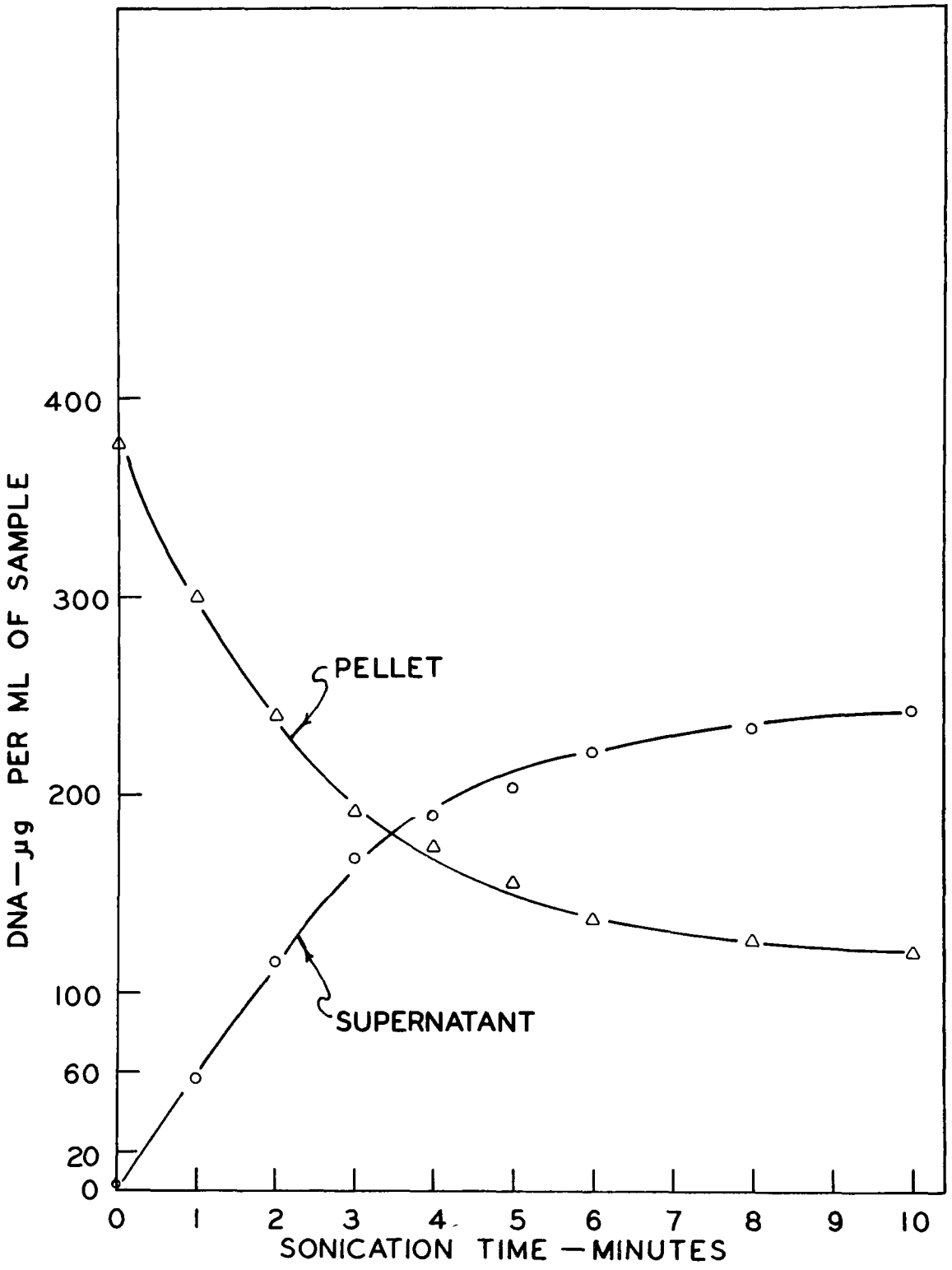

FIGURE 2. THE EFFECT OF SONICATION ON THE RELEASE OF DNA FROM ACTIVATED SLUDGE

tion of the sludge samples by the MPN method is given in Table 1.

Table 1. Composition of Medium Used in Estimating Sludge Population.

Component	g/liter
Glucose	5.0
Yeast extract	10.0
Nutrient broth powder	4.0
K_2HPO_4	1.0

EXPERIMENTAL PHASE AND DISCUSSION

Effect of Sonication on MPN Count.

In order to determine the sonication time that would give the maximum number of cells as determined by the most probable number (MPN) method, 10 ml of 5 times concentrated sludge was sonicated for various periods of time at 80 watts.

The results are shown in Table 2.

Table 2. Effect of Sonication Time on Most Probable Number Count.

Time minutes	MPN cells/ml
0	6.9×10^8
1	4.8×10^9
2	3.2×10^{10}
4	3.4×10^9
8	1.6×10^9
10	1.8×10^8

The results indicate that the floc particles are broken apart and the individual cells of the floc are released within the two first min-

utes of sonication. Further sonication causes destruction of the cells.

It was found that in general, the largest population of cells was obtained after two minutes of sonication. However, this was not always true. The time required appears to be determined by the characteristics of each individual sludge sample.

It must be made clear that even though the largest population was obtained after two minutes of sonication, this number does not represent the total population since many cells would have been destroyed by this time. Other experiments, such as the one shown in Figure 2, indicate that the number of cells killed during the two first minutes of sonication approximates 10 percent of the total population.

Effect of Sonication on Quantity of DNA Released.

In order to determine the effect of sonication time on the quantity of DNA released from sludge floc, the following experiment was performed. Activated sludge from the nitrification tank was strained through cheese cloth and centrifuged. The solids were concentrated 2.5 times by resuspending in part of the supernatant. Ten ml samples were sonicated for 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10 minutes. The sonicated sludge was centrifuged and a DNA analysis was performed on the pellet and the supernatant. The results are shown in Figure 2.

It is evident from the graph that the DNA is released very rapidly from the floc during the first three minutes. After 10 minutes of sonication there is a leveling off where about 24/360 x 100 = 67% of the total DNA has been released, i.e. at this time about 67% of the population has been killed. Supernatant DNA obtained in this manner was

partially purified by heating at 70°C for 30 minutes in order to coagulate proteins which were removed by centrifugation. The partially purified DNA was added to activated sludge to check for DNA degradation.

Effect of Ultra Violet Light.

In order to demonstrate the degradation of DNA of dead cells, several experimental techniques were employed. Ultra violet light was employed as the lethal agent in a series of experiments. The idea behind these experiments was to destroy 99 percent of the population by exposure to ultra violet light. The DNA of the non-viable cells would either be destroyed by the enzymes released from the dead cells or by the remaining viable cells. However, any time a microbial population is exposed to a pasteurizing agent such as ultra violet light, heat, or chemicals one must consider the possible growth of the remaining viable cells provided the conditions following exposure are favorable for growth.

In our experiments where we destroyed cells by exposure to ultra violet light, heat, or chemicals we were unable to demonstrate a degradation of DNA corresponding to the initial reduction in population. In one experiment using ultra violet light as the pasteurizing agent, the population was reduced initially from 4.4×10^7 cells per ml to less than 8.0×10^5 cells per ml. During four hours of incubation following the exposure, the population increased to 1×10^7 cells per ml. Consequently, in experiments of this type, where there is a simultaneous degradation and synthesis of DNA, one cannot demonstrate the degradation of the DNA of the non-viable cells.

The experiments using ultra violet light as a pasteurizing agent

were performed as described below.

1. Sonicate 10 ml of strained and concentrated sludge for one minute at 80 watts.

2. Expose 5.0 ml of sonicated sludge to UV light for 7 minutes. Use 60 mm petri dishes and keep the sludge stirring during the exposure.

3. Incubate the UV treated sludge on rotary shaker. Sample periodically for DNA analysis.

4. Determine viable population at zero time, and periodically thereafter.

Table 3. Killing Effect of UV Exposure on Population Size of Sludge.

Exposure time minutes	Population Size cells/ml	ug DNA/ml
0	6.4×10^9	192
5	6.8×10^6	194
7	2.8×10^6	195

This shows that the population may be reduced by 99 percent by exposure to ultra violet light for five minutes. Further exposure has little effect. It is evident that the DNA as determined in our analysis is not effected by the ultra violet light, since the variation between the zero time and seven minute determinations are insignificant.

Table 4. The Effect of UV on the Rate of Degradation of DNA.

	Time hours	MPN cells/ml	DNA ug/ml	DNA Degraded ug/ml
Control No UV	0	4.4×10^7	156	0
	1		136	20
	2		130	26
	3		113	43
	4		108	48
UV Treatment 7 Minutes	0		136	0
	1		131	5
	2		121	15
	3		117	19
	4		113	23

As may be seen from Table 4 there is a decrease in the DNA of the control population as well as in the UV treated population. This suggests that the DNA degraded is that which was released from the one minute sonication of the sample in order to break apart the floc.

Since the control sample has a considerably larger population than the UV treated sample, it is expected that the DNA is degraded more rapidly in this population. Unfortunately, separate analyses of the DNA of the supernatant and of the solids were not performed in this experiment. Such analyses would have indicated whether the DNA broken down was that released from the initial sonication.

A repeat of the above experiment gave very similar results.

Degradation of DNA by Activated Sludge.

The rapid degradation of DNA by activated sludge was finally demonstrated in the experiments described below.

The DNA used in this experiment was prepared as described on page 4 in this report.

The experimental procedure was performed as follows:

1. Filter activated sludge.
2. Allow to settle.
3. Pour off a volume of supernatant equal to half of the original volume. Save.
4. Add DNA extract.
5. Add supernatant to obtain original concentration of sludge.
6. Incubate on rotary shaker and sample periodically. Do separate analyses on supernatant and solids.
7. As control use DNA extract diluted with sterile water.
8. A second control consisting of the activated sludge without the added DNA may also be included.

The results of the above experiment are shown in Figure 3.

The data for the two controls are not shown on the graph. DNA extract diluted with water to a final concentration of 175 ug DNA per ml was not effected by four hours of shaking. The concentration after four hours of shaking was the same as that of zero time.

Activated sludge without the addition of DNA maintained about three micrograms of DNA per ml of supernatant. The DNA of the pellet varied between 105 and 110 micrograms per ml.

From Figure 3 it may be seen that there is a slight increase in the DNA of the pellet at the end of the first half hour of incubation following the addition of DNA. This increase is probably due to absorption of DNA. There is, however, no increase in the DNA of the pellet corresponding to the DNA decrease of the supernatant. This indicates that under the conditions of this experiment the DNA is degraded and used mainly as a source of energy and cell material other than DNA. Otherwise there would have been an increase in the DNA of the pellet.

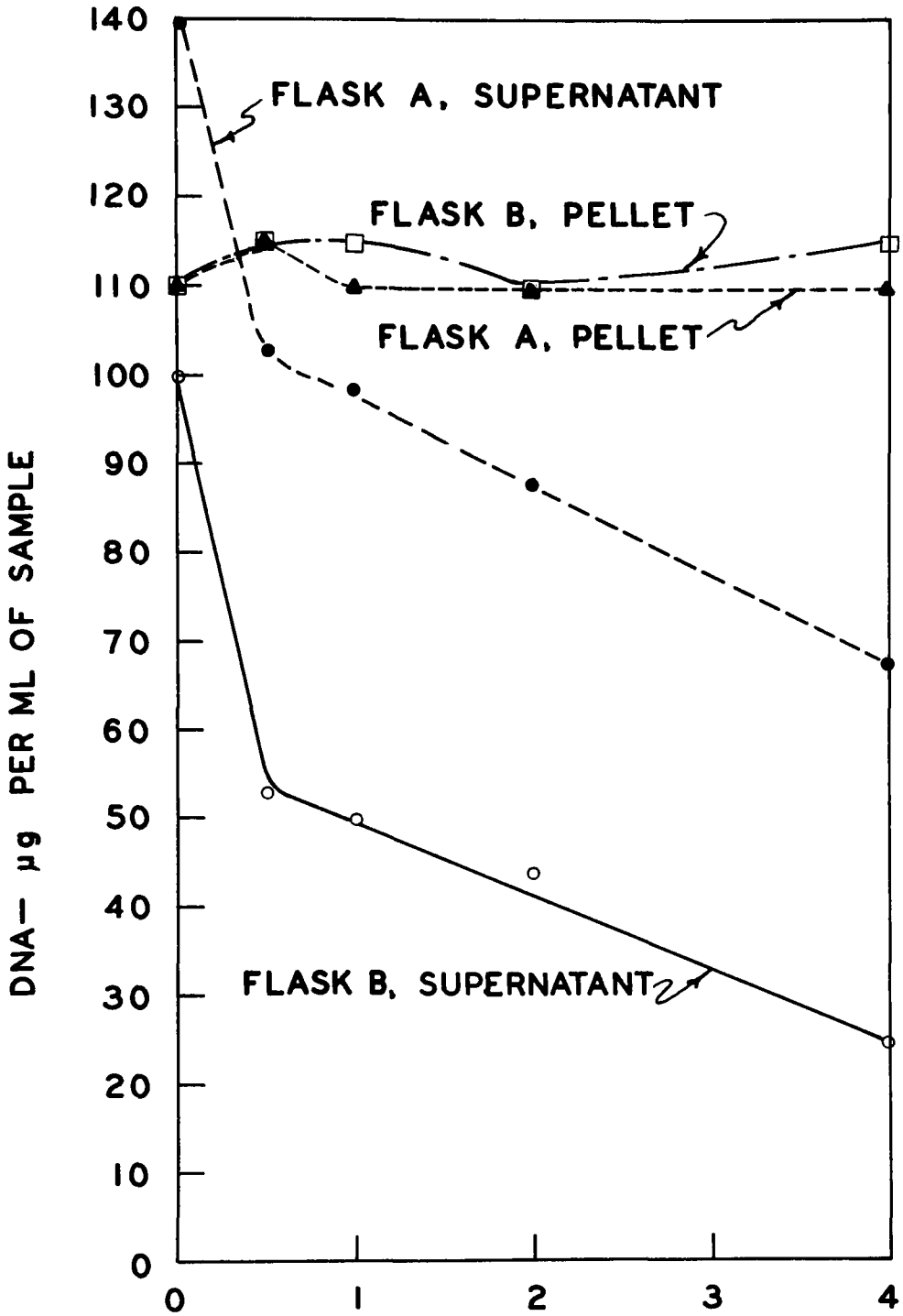

FIGURE 3. DEGRADATION OF DNA BY ACTIVATED SLUDGE

From the data shown in Figure 3 it may be calculated that 27.5 percent of the DNA added to flask A was degraded in the first half hour of contact, and 48.5 percent was degraded after four hours of contact.

In flask B, 47.0 percent of the DNA was degraded at the end of one half hour of contact and 76.0 percent was degraded at the end of four hours.

It is quite evident from the graph that the initial absorption and degradation occurs very rapidly. Then, as the cells become saturated, the rate at which the DNA is being degraded decreases from 1.57 ug per minute to 0.14 ug per minute. That is, the final rate is less than one tenth of the initial rate.

The above results do not take into account the amount of degradation that must have taken place during the initial mixing before the first sampling. In the case of flask B, the zero time concentration of DNA in the supernatant should have been 117 ug per ml. The graph shows 100 ug per ml. However, if the graph is extrapolated to take into account four minutes of sample preparation, the initial concentration becomes 117 ug DNA per ml of supernatant.

Figure 4 shows the results of an experiment very similar to that mentioned above. In this case the DNA extract was clarified by centrifugation, but was not heated. The results are similar to those of the previous experiment. Twenty percent of the DNA added was degraded in the first 15 minutes of the experiment, 28.0 percent in the first 30 minutes and 80.0 percent in the first four hours. At the end of 24 hours, 94.0 percent of the DNA had been degraded.

In this experiment the DNA of the pellet increased from 140 ug per

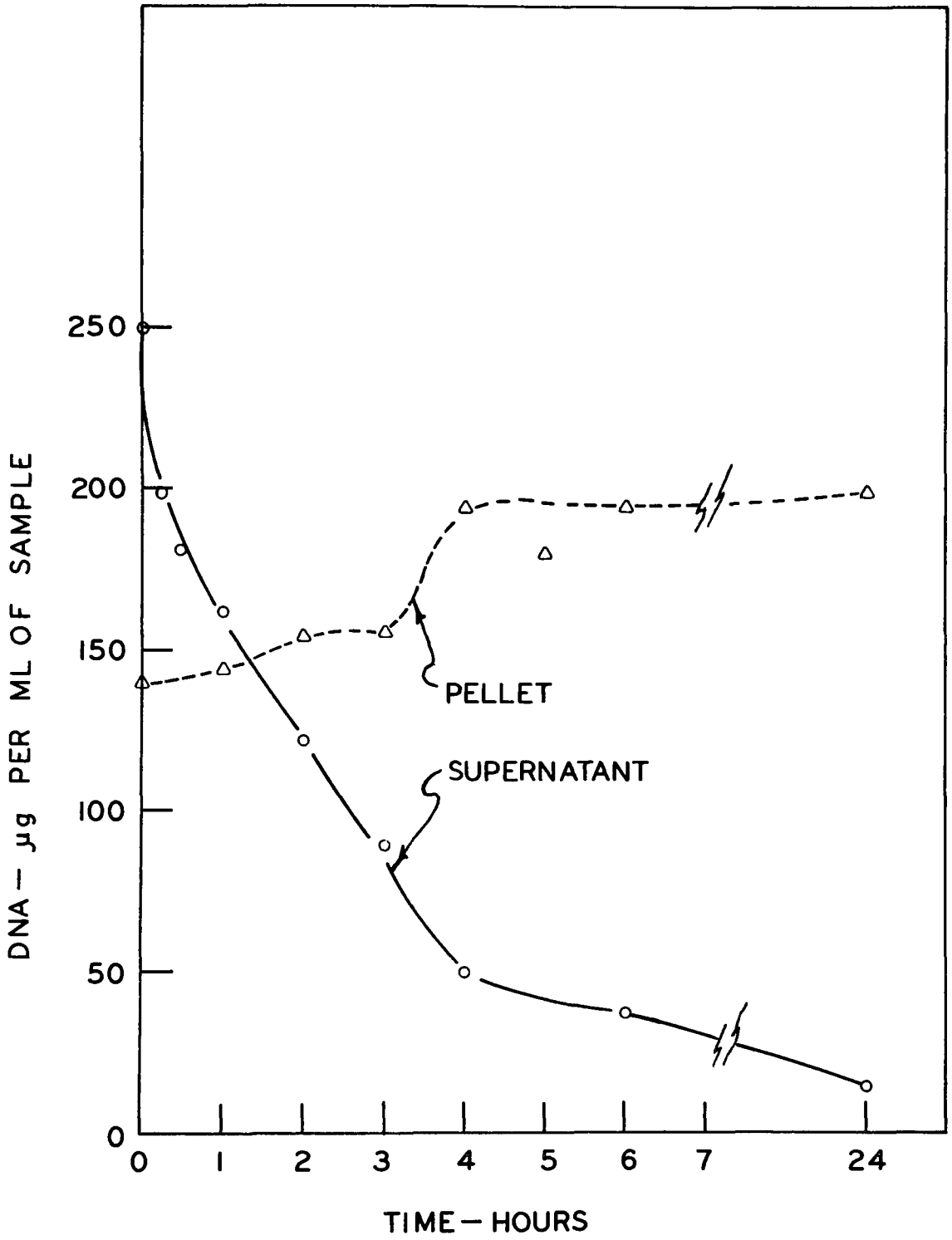

FIGURE 4. DEGRADATION OF DNA BY ACTIVATED SLUDGE

ml at zero time to 195 ug per ml at the end of four hours. This corresponds to a population increase of 27 percent.

The results of these two experiments seem to indicate that the manner in which the DNA is utilized by the cells depends upon the condition of the floc, that is, the phase of growth that the cells are in.

These experiments were repeated using calf thymus DNA rather than sludge DNA. The results were very similar. In one experiment the DNA concentration of the supernatant dropped from 58 ug per ml to 17 ug per ml in three hours, that is, a reduction of 41 ug per ml or 71.0 percent. At the same time, the DNA of the solids increased from 138 to 161 ug per ml, an increase of 23 ug per ml. This corresponds to a population increase of 17 percent.

From these experiments and from the fact that there is only a trace of DNA present in the supernatant of activated sludge, it is apparent that any DNA present in the sewage or released from dead cells is rapidly degraded by the sludge floc. The DNA of the sludge therefore represents viable cells.

Population of Activated Sludge.

If one assumes the weight of one bacterial cell to be 1×10^{-9} mg, the population size as based on DNA analyses can be compared with that of a cell count obtained from the most probable number method.

Population estimates of this type were performed on activated sludge, return sludge and nitrification tank sludge. The latter tank contains return sludge mixed with supernatant from the anaerobic digester. The results are shown in Table 5.

When comparing the population estimates obtained from the two

methods mentioned above, it was found that the population based on the DNA content of the sludge was greater in 70 percent of the cases. This is as would be expected due to the flocculant characteristic of the sludge inoculum and the inability of many bacteria to grow in the counting medium.

From the data in Table 5 it may be shown that in many cases the viable bacteria represent nearly 100 percent of the organic matter in activated sludge.

Table 5. DNA Content of Various Sludges, October - December, 1969.

Date	Type of Sludge	ug DNA/ml	% DNA	Cells/ml x 10^{-9} MPN	Cells/ml x 10^{-9} Based on DNA
Oct. 22	A	112	2.6	0.4	2.8
24	A	47	1.7	0.7	1.2
27	A	72	3.1	2.6	1.8
29	A	54	2.1	32.0	1.4
Nov. 3	A	61	2.4	4.8	1.5
5	A	60	2.5	2.6	1.5
	N	126	2.1	3.7	3.2
10	A	71	2.5	5.6	1.8
12	N	109	2.3	4.4	2.7
14	N	196	2.9	6.4	4.9
17	N	192	2.4	2.2	4.8
19	N	151	2.4	2.2	3.8
24	N	156	2.5	0.1	3.9
26	A	70	2.5	0.3	1.7
	R	65	2.4	0.5	1.6
	N	144	2.4	1.0	3.6
Dec. 3	N	124	2.1	0.2	3.1
12	A	53	2.1	0.3	1.3
	R	58	2.3	0.7	1.5
	N	132	1.8	1.0	3.3
15	N	139	1.9	2.2	3.5
17	N	134	2.1	2.2	3.4

A indicates aeration tank
N indicates nitrification tank
R indicates return sludge

Table 6. DNA Content of Various Sludges, January - August, 1970.

Date	Type of Sludge	ug DNA/ml	% DNA	Cells/ml x 10^{-9} MPN	Cells/ml x 10^{-9} Based on DNA
Jan. 5	A	66	2.5	1.1	1.7
	R	52	2.2	0.2	1.3
	N	142	2.2	1.1	3.6
23	A	63	2.6	0.3	1.6
	R	56	2.4	0.5	1.4
	N	142	2.2	0.5	3.6
Feb. 20	A	86	3.5	0.7	2.2
	R	183	3.0	1.4	4.6
	N	212	2.8	0.2	5.3
April 6	A	55	2.5	1.1	1.4
	R	57	2.4	0.1	1.4
	N	65	2.3	0.5	1.6
22	A	58	2.6	6.4	1.5
	R	42	2.4	3.7	1.1
	N	156	2.1	6.4	4.4
May 4	A	34	2.3	0.2	0.9
	R	37	2.3	0.5	0.9
	N	132	2.0	1.4	3.3
18	A	60	2.6	0.7	1.5
	R	44	2.0	3.2	1.1
	N	175	2.4	1.0	4.4
June 24	A	79	3.3		2.0
	R	208	2.7		5.2
	N	170	2.3		4.5
29	N	242	3.9		6.1
July 4	N	243	4.2		6.1
8	N	300	4.2		7.5
15	A	108	4.4		2.7
	R	200	5.6		5.0
	N	240	3.4		6.0
20	A	88	3.0		2.2
	R	175	3.7		4.4
	N	200	3.0		5.0
27	A	93	3.6		2.3
	R	250	4.1		6.3
	N	250	4.1		6.3
Aug. 4	A	88	4.0		2.2
	R	295	3.9		7.4
	N	280	3.9		7.0
17	A	100	3.6		2.5
	R	185	4.0		4.6
	N	255	3.7		6.4
24	A	100	3.5		2.5
	R	185	4.0		4.6
	N	255	4.0		6.4

Bacterial Population of Sewage as Based on DNA Analysis.

To obtain an estimate of the bacterial population of sewage, 40 ml of strained sewage was centrifuged at 8.2 x 1000 G for 10 minutes. The resulting pellet was analyzed for DNA. The sewage was found to contain 1.63 ug DNA per ml and 0.38 percent DNA. By the use of Equation (2) on page 2 it may be calculated that viable bacteria represent only 9.5 percent of the organic matter in sewage. The population may be estimated by use of Equation (4) on page 2.

$$\frac{1.63 \text{ ug/ml} \times 10^{-3} \text{ mg/ug}}{4.0 \times 10^{-9} \text{ mg/cell}} = 4.1 \times 10^{6} \text{ cells/ml}$$

This value is in accordance with most estimates of bacterial population of sewage.

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27 Abstract

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Abstractor: Roar L. Irgens

Institution: Southwest Missouri State College

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