A LABORATORY STUDY OF REDUCTION OF THE BIOCHEMICAL OXYGEN DEMAND OF SYNTHETIC SEWAGE BY ZOOGLOEA RAMIGERA

by

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INTRODUCTION

In order to improve the design and to control satisfactorily the operation of the modern sewage disposal and industrial waste treatment plants, a great deal of work remains to be done by competent sanitary engineers, biologists, and chemists. It is the general belief that the stabilization of organic wastes is a biological phenomena. This indicates that a concentrated study of the organisms that are responsible for such stabilization should be undertaken and the part that each organism contributes should be evaluated. The lack of specific knowledge has resulted in "rule of thumb" methods of design and control. A considerable amount of work on the biology of sewage disposal has been done by the New Jersey Experiment Station (4)(5)(8); by C. T. Butterfield and Elsie Wattie of the U. S. Public Health Service (10)(13) and by others, but a concentrated effort is still needed.

James B. Lackey (1), has done an excellent job in summing up the work accomplished to date in the field of sanitary biology in his article "Sewage Treatment Biology". In this article, Dr. Lackey lists four things that must be known before a clear picture can be presented on the subject of sewage and waste treatment. These points are as follows: (1) More precise information on the species of each group of organisms working in treatment plants. (2) The relative abundance of each so that no important (numerically or volumetrically) organism is neglected. (3) The range - not optimum - of environmental conditions under which the organism works. (4) The work accomplished by the organisms - whether a small segment of the stabilizing process, or a large one.

Gerald A. Rohlick (2), in discussing Lackey's article, places special emphasis on additional experimental work that should be done on the activities of protozoa and higher forms of life.

The editors of Sewage Works Journal (3) have summed up the problem in the following statement, "When we can answer completely the what, how, who, when and why of the organisms that populate our digestors, aerators and trickling filters, we shall simultaneously solve the problems of treatment plant design and operation that are of present concern".

In an effort to contribute something to the fund of knowledge of sanitary biology, the author undertook an investigation based on the four needs suggested by Lackey.

The investigation is divided into three major parts: (1) A preliminary investigation; (2) the construction of a pure culture testing apparatus; and (3) the testing of a pure culture of organisms. The complete investigation is confined to studies of the organisms found in the aerobic phase of treatment, the precise source being the trickling filter at the Virginia Polytechnic Institute sewage disposal plant. This confinement of purpose was to allow the investigator to conduct a concentrated study of a single phase of treatment.

The preliminary investigation consisted of taking frequent samples from the trickling filter and examining them under the microscope. The predominate organisms were noted and the development of pure cultures of each was attempted.

The construction of a pure culture filter consisted of an attempt to duplicate in the laboratory as nearly as conditions would allow, the

actual conditions that exist in the trickling filter at the plant, while at the same time observing pure culture requirements and techniques.

The testing of the organisms consisted of measuring the amount of purification exerted by the organism on a synthetic sewage as it passed through the filter.

The results of this investigation should answer two questions: First, can a pure culture apparatus be constructed and operated with such success that it can be used as a standard device for determining in the laboratory the degree of purification exhibited by organisms in pure culture, and Second, is it possible for a pure culture of organism to carry on the purification process. The answer to the second question will, of course, depend on a positive answer to the first question.

REVIEW OF LITERATURE

Summarizing work that has been done on the biological aspects of sewage treatment is ably performed by Dr. James B. Lackey (1).

The earliest and most complete investigations on the biology of sewage disposal were performed at the New Jersey Agricultural Experiment Station (4). Studies of the biological populations of sewage treatment plants have been performed by Gaub (5), Hotchkiss (6)(7), Lackey (8), and others (9).

Work in this field which pertains particularly to the nature of the present investigation is that accomplished by Butterfield and Wattie (10), and Pillai and Subrahmanyan (11). The results reported by these two groups of investigators are conflicting, therefore the writer deems it important to review at this time the works of each.

Butterfield and Wattie (10) believed the predominating bacteria of trickling filter units and the predominate bacteria of activated sludge units to be essentially the same organism. These investigators isolated the predominate bacteria from these two units and identified them as Zoogloea ramigera. A pure culture experimental trickling filter was then constructed with which the degree of purification of the organism could be determined. The bacteria were tested separately and in combination in the pure culture filter and also under activated sludge aeration conditions. From the results of these tests the investigators concluded that the organisms were similar and were able under pure culture conditions to definitely purify sewage. The investigators were able to obtain varying degrees of reduction in the standard 5-day biochemical oxygen demand of a

synthetic sewage (composition included under Operation of Filter) as it passed through the filter, depending on the rate of flow. A maximum reduction of 72% was proclaimed at a flow of less than 1 million gallons per acre per day, with an average of 50% reduction at an average flow rate of 1 million gallons per acre per day.

The work of Pillai and Subrahmanyan (11) in India, however, places special emphasis on the role of protozoa. The ciliate Epistylis sp was used in their studies, and they concluded that "the isolated protozoa can bring about practically all the changes associated with the purification". The part played by the bacteria is claimed to be almost negligible. They state that further evidence is available to show that the conditions affecting the life and activity of the protozoa also affect the efficiency of the purification. In examining sludges from activated sludge and other aerobic systems of treatment from various parts of India they concluded that the presence and active functioning of protozoa is evident wherever the purification is proceeding satisfactorily, and that if the protozoa are absent or found dead or encysted, there is no purification. The investigators presented the dilution method as a means of isolating the protozoa but did not include the method used in testing the degree of purification nor the values determined.

Cramer (12) and Wagner (original not seen) (2) have demonstrated the regulatory function of protozoa on bacteria, which reinforces somewhat the work done by Pillai and Subrahmanyan (11). Wagner using a small model sewage purification tank added inhibitors to destroy the protozoa and allow the bacteria to grow. The bacteria increased greatly in numbers

but the degree of purification of the unit was adversely effected. On the renewal of the protozoan fauna the number of bacteria again decreased and the original degree of purification was established.

A review of these works indicates a definite need for further work on the role of the organisms in sewage purification and on the relationship between these organisms as well.

Some basic work has been accomplished at Virginia Polytechnic Institute on the respiration rates of bacteria in decomposing waste materials. The rate of respiration of the bacteria in decomposing waste materials is a reflection of the rate of purification which is taking place. P. H. Watkins*, of the Chemical Engineering Department, has made a study of the respiration rates of some common water bacteria using a Warburg apparatus. L. G. Rich*, Sanitary Engineering Department, is completing a similar study of Zoogloea ramigera using a Sargent-Heyrovsky Polarograph apparatus.

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* Information obtained from personal contact.

PRELIMINARY INVESTIGATION

The preliminary investigation was carried on jointly with Professor L. G. Rich. Part of this investigation consisted of a quest and examination of all publications containing information on the fauna and flora of trickling filter units, isolating and culture methods of microorganisms, and pure culture technic and methods. This intense reading and probing program was carried out to orientate ourselves in this field of investigation and to obtain a speaking knowledge of the work that has been carried out thus far.

The second part of the preliminary investigation consisted of the examination of the microflora and fauna of the trickling filter unit at the Virginia Polytechnic Institute sewage disposal plant. Samples of the slime from the stones at the surface to a depth of 6 inches were collected for a period of one month. These samples were examined under a microscope to determine the flora and fauna of this unit. Relative density of the organisms were studied and the predominate organisms were especially noted.

At this point in the work, the investigators felt that a division in the investigation and a concentrated effort on a small segment would yield a more successful result in a shorter period of time. It was thus decided that Rich would concentrate on isolating the predominate bacteria found in the samples in an effort to obtain a pure culture for testing and the writer would concentrate on the protozoa and higher forms of life for the same purpose. The results of Rich's investigation can be found in his Ph. D. dissertation (Virginia Polytechnic Institute, 1951). The results of the writer of this part of the investigation are discussed in the paragraphs

that follow.

From the microscopic examination of the samples it was determined that members of the Phylum Rotifera, commonly known as Rotifers, were predominate according to their numbers, and that members of the Phylum Nematoda, commonly called Nematodes, Epistyles sp., and Arcella sp. followed in that order.

A concentrated effort was made to get all of these organisms in a healthy, reproductive culture. A fair degree of success is claimed for the Rotifers but complete failure resulted from the work on the Nematodes, Arcella sp., and Epistyles sp.

Several methods were tried in an attempt to separate the organisms in large numbers in order that culture methods and media could be attempted. Filtration through different materials, filtration through a Sedgwick-Rafter apparatus, and centrifuging all proved unsatisfactory for this separation. By using a finely drawn eye dropper under the x45 lens power of a stereoscopic microscope the organisms could be removed from the sample relatively free from other organisms. The organism was then placed in distilled water for washing purposes and further removed by the same process to a watch glass containing 2 ml. of media for incubation. This procedure was slow but excellent results were obtained, especially with the Rotifers.

Four types of media were used in an attempt to obtain a healthy reproducing culture; (1) raw trickling filter influent, (2) sterile trickling filter influent (sterilized in an autoclave), (3) synthetic sewage (sterilized in an autoclave), and (4) concentrated trickling filter influent (concentrated by evaporating to one-half the original volume). All of

these media supported the organisms but only the synthetic sewage media materially effected the growth and reproductive ability of any of the organisms. This effect was more pronounced in the Rotifer cultures. The Epistyles sp. would multiply rapidly but would die off very quickly. No success was obtained in keeping this organism in a healthy reproductive state.

The writer was able to obtain the Rotifers in excellent cultures and believes it to be well worth while to bring to the reader's attention the observations, methods used, and results with these cultures.

The particular organism under observation is the Philodina roseola and is easily distinguished by a slender rose-colored body. The head is provided with two ciliated trochal discs which aid in locomotion and draw food into the mouth. The tail or foot is bifurcated and adheres to objects by means of a secretion from a cement gland. The body is covered by a shell-like cuticle. A thick milky like substance is excreted in large quantities at intervals from the anus. The egg is carried inside the body of the organism and is easily distinguished by its large size. The average length of the Philodina roseola is 0.5 mm. and the egg is about one-fourth the size of the organism.

Two different species were observed. More likely they were the male and female of the same species. The organisms were similar in appearance except for the size and predominate method of motility. The small organism was about half the size of the larger and the method of motility was usually free swimming using a slow rotating motion. Seldom did this organism move in "snail-like" fashion across the surface of the container. The larger

organisms were different in that they moved about almost entirely in "snail-like" fashion and were seldom seen free swimming.

The living organism is easily distinguished by the movement of the chitinous jaws which are constantly at work, breaking up the food. The dead organism is much harder to distinguish because most of the familiar characteristics are concealed. The easiest way to recognize the dead organism is the bifurcated foot which becomes very outstanding.

Using the eye dropper technic the Rotifers were removed from the sample and washed in three watch glasses containing distilled water. The organism was then transferred to a watch glass containing 2 ml. of sterile synthetic sewage and incubated at room temperature. Incubation at 30° C and 37° C has no accelerating effect on the development of the culture. After a period of 1 to 3 days of incubation numerous Rotifers were observed containing one egg in their bodies and also numerous eggs could be found in the free state. The actual separation of the egg from the Rotifer was never observed. No movement could be observed in the egg for a period of 12 to 24 hours but movement of the integral parts could be observed after this time and several hours later the Rotifer would evolve into a full grown organism. No residue from the egg could be observed.

One of the most important developments which resulted from this phase of the investigation is the unique washing technic that can be applied to these organisms. This technic may play an important part in the development of a pure culture of the organism in some later work. The method is unique in that it utilizes a special property of the organism to accomplish its purpose. The Rotifers secrete a sticky substance which enables them to adhere firmly to the bottom of the watch glass. Under such

conditions one may pour off the media and wash the watch glass thoroughly with distilled water from an eye dropper or pressure water bottle with the loss of a minimum number of the organisms. The media can then be applied and the culture again incubated.

Using this technic, the writer was able to obtain cultures completely free from any other type of flora or fauna except possibly bacteria. No attempt was made to free the culture of bacteria.

In the meantime Rich had succeeded in obtaining in pure culture the predominate bacterium that was found in the trickling filter. This bacterium was identified as Zoogloea ramigera. Due to a limiting time factor, it was recommended that the writer begin concentrating on the testing in the pure culture filter the degree of purification exhibited by Zoogloea ramigera in pure culture.

MAJOR INVESTIGATION

Construction of Filter

The development of an apparatus, duplicating the trickling filter, to be used to explore the degree of purification exhibited by a pure culture of organisms was accomplished with a certain degree of success only after considerable time had been spent and numerous failures had been experienced. The basic principals of the apparatus were taken from the work done by Butterfield and Wattie (10). A number of improvements are believed to be incorporated in the apparatus. In order that the testing apparatus and testing conditions might be reproduced by others, the investigator has included a detailed description of the apparatus.

The apparatus employed is shown diagrammatically in Figure 1. Twenty liters of sterile synthetic sewage (b) is placed in a 5-gallon carboy (a) which must be of pyrex glass to withstand the heat and pressure of the autoclave during the sterilizing of the media. Non-absorbent cotton packing (c) is used to protect against vacuum leaks around the supply bottle stopper. A one-hole tight-fitting stopper (d) is held in place by Scotch tape. The entire weight of the supply bottle rests on this stopper when the apparatus is in operation. Hard glass tubing (e) is permanently fixed in stopper (d) and should be at least 9 mm in outside diameter to prevent air locks between the supply bottle and the constant head bottle (o). This tubing extends through the stopper into the supply bottle for a distance of 1/4 inch to prevent the insoluble particles from passing into and fouling the influent tube. Heavy rubber tubing (f) is attached

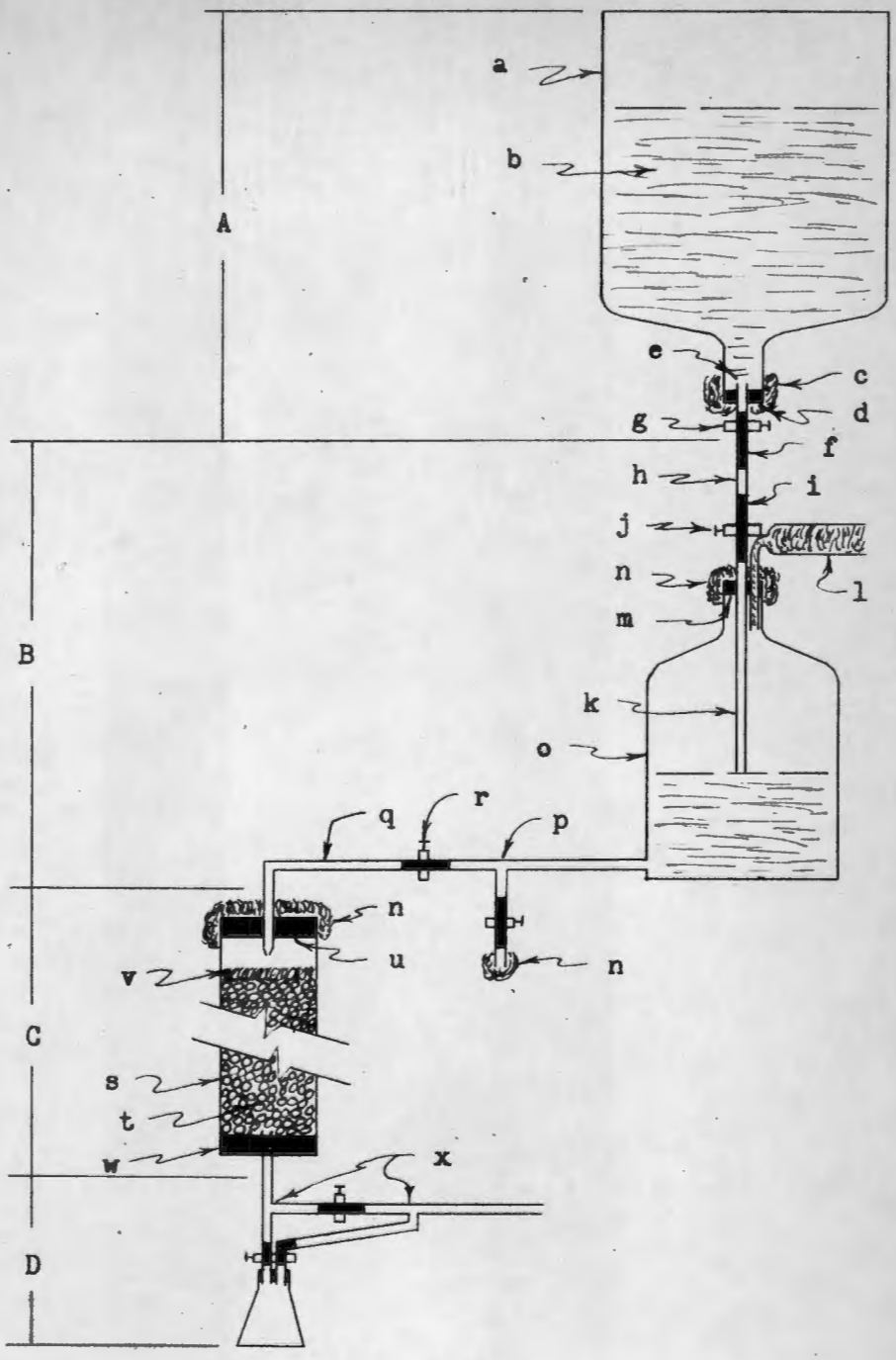

Figure 1 - Component Parts of the Pure Culture Experimental Trickling Filter

permanently to tubing (e) which can be clamped, sealing the supply bottle during assembly and dismantling of the apparatus. Adjustable clamp (g) is used to seal the supply bottle and also to allow a controlled equilibrium of pressure in the supply bottle and constant head bottle after assembly. The 9 mm. O. D. hard glass tubing (h) serves only to connect the supply bottle to the constant head bottle but must be of hard glass since it has to be sterilized by flaming during assembly of the apparatus. Heavy rubber tubing (i) is permanently attached to tubing (k). This tubing serves the purpose of sealing the constant head bottle during a change of supply bottles. An adjustable clamp (j) is used on tubing (i) only during a change of supply bottles. The 9 mm. O. D. glass tubing (k) serves the purpose of regulating the head in the constant head bottle. The desired head is obtained by moving the tube (k) up or down. The air filter (l) consists of 6 inches of non-absorbent cotton. Two-hole tight fitting rubber stopper (m) is held firmly in place with Scotch tape. Non-absorbent cotton packing (n) is used as protection from leaks around stopper (m). A one gallon aspirator bottle (o) is used to maintain a constant head in the system providing a constant rate of flow. A 6 mm. O. D. glass "T" (p) provides for a means of sampling the media before it passes on to the filter. Another 6 mm. O. D. hard glass tube (q) is used as the influent tube. The end of this tube which fits in the filter is drawn to aid in regulating the rate of flow and also to decrease capillary action. Several trials are necessary before the desired result of drawing is obtained. An adjustable clamp (r) is used to control the rate of application of media to the filter (s). The pyrex glass cylinder (s) 2-7/8 inches in

diameter and 25 inches in length is used as the filter proper. In the author's investigation this cylinder was necessarily limited in length by the size of the autoclave. Smooth pebbles (t) which pass through a 3/8 inch mesh screen and are retained on 1/4 inch mesh screen, were arbitrarily chosen, as to size, to furnish the largest possible area of contact and yet to remain free from clogging. A three-hole rubber stopper (u) is used to seal the top of the filter. Two of the holes were plugged with non-absorbent cotton to provide filtered air for circulation in the filter. The third hole is for the placement of the influent tube. A layer of absorbent cotton (v) is placed on top of the stones to provide equal distribution of flow over the entire surface of the filter. One-hole rubber stopper (w) furnishes a permanent air-tight seal to the filter bottom. Glass "T" (x) permits the sampling of the filter effluent.

The size of the assembled apparatus was too large to be sterilized intact. This necessitated a division into four sections for sterilization purposes. These sections, unit A - stock supply of synthetic sewage; unit B - the constant head reservoir and the influent tubing; unit C - the filter proper; and unit D - the effluent tubing, are also shown in Figure 1.

A timing device was constructed in the laboratory to provide for intermittent flow to the filter. This was later considered an unnecessary refinement and was not used in the operation.

Operation of Filter

The operation of the filter consists of the preparation of the media, sterilization of the apparatus, seeding of the filter, and the assembly and operation of the apparatus.

The synthetic sewage used was the same used by Butterfield and Wattie (10) which was developed and used by Butterfield, Ruchhoft and McNamee (13). The composition is as follows:

Peptone, Difco, Bacto grade	gram	0.3
Meat extract, Liebigs	do	0.2
Urea, C. P. grade	do	0.05
Disodium hydrogen phosphate, C. P.	do	0.05
Sodium chloride, C. P.	do	0.015
Potassium chloride, C. P.	do	0.007
Calcium chloride, C. P.	do	0.007
Magnesium chloride, C. P.	do	0.005
Water, distilled	milliliters	1000

The disodium hydrogen phosphate, and the chloride salts were weighed in stock quantities and dissolved in an amount of distilled water such that 500 milliliters in 20 liters of medium made up the correct quantities. This saved a tremendous amount of time in preparing the medium. The peptone, meat extract, and urea were weighed in the correct proportions for each 20 liter supply of medium and then dissolved in distilled water. The medium was sterilized in the five gallon pyrex carboy, properly protected from subsequent contamination, in an autoclave for 20 minutes at 15 pounds pressure. It was allowed to cool overnight in the autoclave and sealed before removal. If the carboy is sealed before the media is cooled, a vacuum is produced in the bottle. Upon installing the carboy, the equalizing of the difference in pressure in the constant head bottle

and the carboy takes place so rapidly that contamination is freely invited.

Contamination of the supply medium was one of the most serious problems encountered in the operation of the filter, and was never completely solved. The longest continuous period of operation without contamination was 22 days. The source of contamination is believed to be in the technic of changing the supply bottles. For this reason the writer should like to include the procedure for the changing of the supply which provided the highest degree of success. The following is the recommended procedure, with designations referring to Figure 1: Fasten clamps (g) and (j) securely in place. Slip rubber tube (f) from the connecting glass tube (h) thus freeing the supply bottle. Replace the empty carboy with a full one. The glass connecting tube (h) will be filled with medium which is exposed to the atmosphere. Do not attempt to remove this medium as the surface of the medium will serve to catch the contaminating bacteria in the air. It is much easier and a more thorough sterilization is provided by flaming the tube until the medium boils. The rubber tubing (f) is also flamed and the connection again made. Clamp (j) can then be loosened and removed. The pressure difference in the carboy and constant head bottle will cause air to be sucked into the carboy at such a rate that the air filter will be overloaded. This rate of equalization of pressure can be controlled by carefully loosening clamp (g).

One other point to be observed in the prevention of contamination of the supply is to close clamp (g) before an influent sample is taken. This proved to be a very important point in operation.

Sterilization of the apparatus intact could not be accomplished as stated previously. The filter, effluent tubing, constant head bottle and

influent tubing were sterilized at the same time but not assembled. Sterilization was accomplished in the autoclave at 20 pounds steam pressure for 30 minutes. All openings were covered with cotton packing for protection against subsequent contamination.

The assembly of the apparatus followed a definite order. The supply carboy and the constant head bottle were connected first. The difference in pressure between the two containers was allowed to equalize and the medium in the constant head bottle was allowed to seek its level. During this operation the flow rate control clamp was closed. Next, the effluent tube was connected to the filter, however the vacuum was not applied at this time. The connection of the influent tube to the filter was not made until the synthetic sewage was ready to be applied. There are several good reasons for this. It allows an access to the filter for seeding purposes. Also the atmosphere of the filter will contaminate the influent tube and thus the supply if a flow is not maintained in the tube after the connection is made.

Aseptic technic was observed at all times during the seeding operation. For the method of isolation and the development and maintenance of a stock culture of Zoogloea ramigera, see L. G. Rich's Ph. D. dissertation (Virginia Polytechnic Institute, 1951). One loop of the stock culture was used to inoculate 10 milliliters of synthetic sewage fortified by 1% yeast extract. This medium was then incubated at 25° C. for 24 hours at which time a very turbid growth could be observed. The culture was then transferred by means of a sterile pipette to a flask containing one liter of sterile synthetic sewage. This flask was incubated at 25° C.

for 24 to 48 hours. The culture was then transferred to the filter where it was allowed to stand for two hours in order that the bacteria might become well attached to the stones in the filter. The seeding culture was then carefully withdrawn through the effluent tube connected to a suction pump and the filter was allowed to stand idle for another period of two hours. The influent tube was then connected to the filter and the synthetic sewage applied at a very slow rate for 24 hours. This was done to prevent excessive washing away of the seeded organisms. The flow of synthetic sewage was adjusted to the desired rate and the apparatus was ready for testing.

Slides were made of the seeding material after each seeding to determine the presence of contamination. The slides were prepared by placing several loops of seed material on a clean slide and allowing them to dry in air. The slide was then stained with crystal violet for two minutes, washed with a copper sulfate solution, and examined under oil immersion.

The writer feels that several definite improvements over a similar apparatus and methods used by earlier investigators, have been incorporated in this set-up, the principal improvement being the method of effluent sampling and effluent disposal. A diagram of the method used is shown on Figure 2. During normal operation clamp (B) is closed, sealing off the two tubes leading to the sampling flask. Clamp (A) is open, allowing the effluent to be deposited in the flask and the air drawn off by the suction pump. The degree of suction is not changed during sampling, which is important since the rate of flow of synthetic sewage through the filter is also measured during the sampling period.

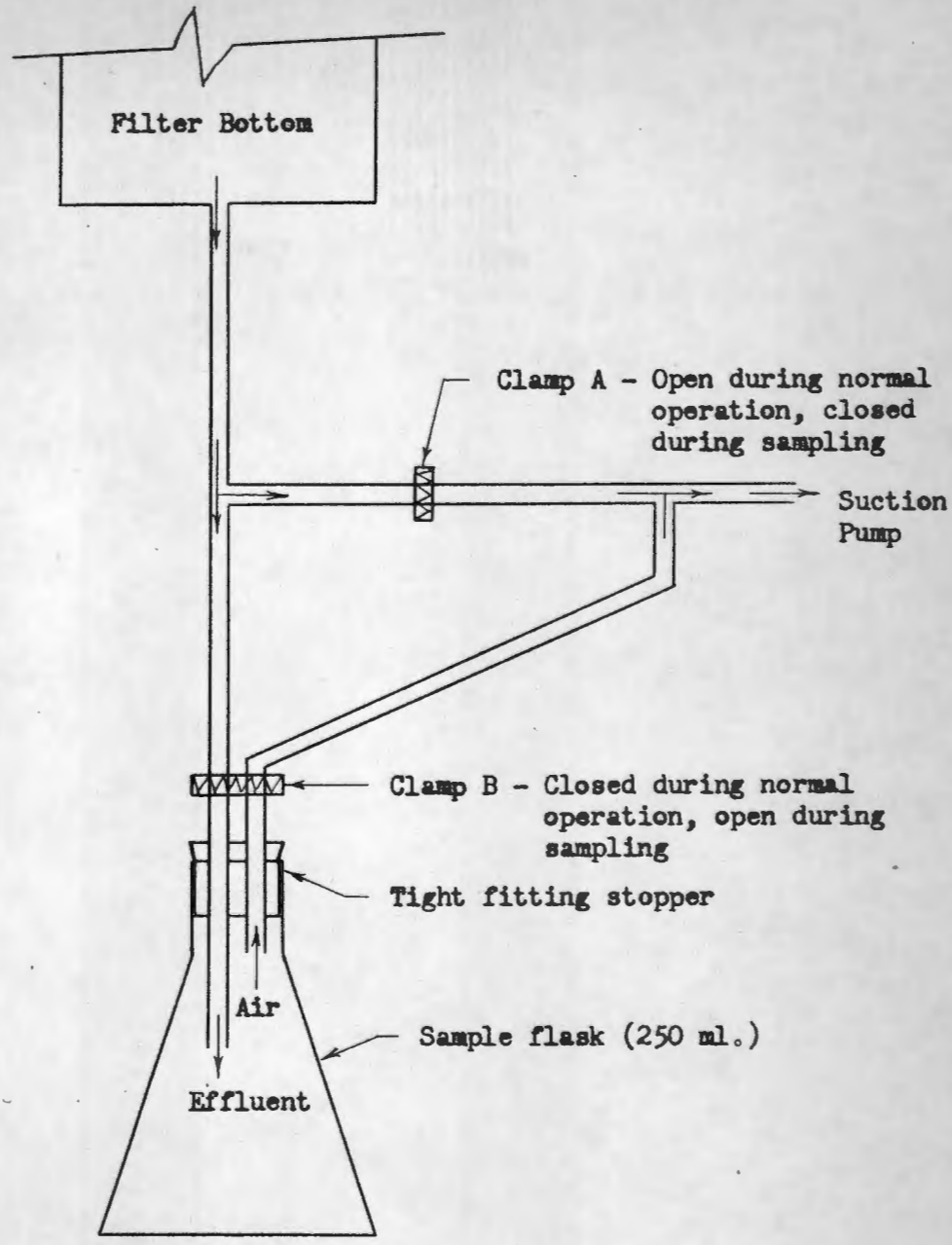

Figure 2 - Component Parts of the Filter Effluent Sampling and Disposal Set-up

Another improvement is the forced air ventilation. This is not a duplication of the equipment at the Virginia Polytechnic Institute sewage disposal plant but it is felt to be a close approximation of the existing conditions. Without forced air the ventilation in the experimental filter would be only slight, if any, while under actual conditions at the plant the exposed area of the filter offers ample ventilation.

Testing Procedure

The biochemical oxygen demand or B.O.D. is that demand of oxygen by the organisms which are active in breaking down, decomposing, and therefore stabilizing the putresible material present in a water. The biochemical oxygen demand is the sanitary engineer's best single criterion for the measure of organic material present in sewage and water. A reduction in this demand is indicative of the extent of purification that has taken place.

The extent of purification of the synthetic sewage accomplished as it passed through this pure culture trickling filter was measured by comparing the 5-day biochemical oxygen demand of the influent with the corresponding 5-day biochemical oxygen demand of the effluent. These B.O.D. determinations were made in accordance with standard procedure (14) except as noted in the dissolved oxygen determinations. Each set of samples (influent, effluent and diluting water) was put up for this determination in appropriate dilution and seeded. The initial dissolved oxygen content was determined from one bottle of each of the samples. Duplicates of each sample were incubated for 5 days at 20° C. and the final dissolved oxygen content was determined after this period of incubation. The average

values of the duplicates were used in the calculations of B.O.D. reported in the results. The use of the average value of the duplicates provides the investigator with a more reliable value since the variation in this part of the test is biochemical in nature and is not controllable by the investigator.

The diluting water used was distilled water buffered with sodium bicarbonate (13). One milliliter of seed was added to each of the 300 milliliter B.O.D. bottles both for the initial and final dissolved oxygen determination. The seed consisted of primary settling basin effluent from the Virginia Polytechnic Institute sewage disposal plant which had been aerated for 24 hours and allowed to stand for a period of two weeks.

The rates of flow of the synthetic sewage through the filter were based on the loading criterion, million gallons per acre per day. The investigator attempted to provide a flow rate of one million gallons per acre per day for testing purposes but some of the tests vary considerably from this rate. In all cases when the rate of flow was changed, the filter was allowed to run overnight at the new rate to allow for the adjustment of conditions in the filter before a test was made.

Dissolved Oxygen Test

The dissolved oxygen test used was the Alsterberg or sodium azide modification of the Winkler method except as noted herein. The writer feels that the complete procedure should be included since the volume of the samples varied from that given in "Standard Methods" (14) which required a change in the amount of reagents. The procedure used for the

dissolved oxygen determination is as follows. The water was exhausted from the water seal of the standard B.O.D. bottle (308 milliliters average volume) containing the sample. Two milliliters of manganous sulfate solution and two milliliters of alkaline - potassium iodide with azide solution were then added to the sample. The sample was shaken immediately for 20 to 25 seconds by inverting the bottle 50 times. The floc was allowed to settle for several minutes, three milliliters of concentrated sulfuric acid were added and the sample shaken immediately by inverting the bottle 15 times. The sample was allowed to stand for at least 5 minutes before a determination was made.

The next step as given in the standard methods is to titrate a certain volume of the sample with sodium thiosulfate until the iodine color, produced by the addition of the sulfuric acid, disappears using a starch solution as an indicator toward the end of the titration. The investigator found a number of limitations in this step. First, the sodium thiosulfate solution has to be standardized and restandardized at very frequent intervals to prevent errors from the loss of strength of the sodium thiosulfate. Secondly, the starch solution has to be made up at very frequent intervals. The third and most important restriction is the element of time which was consumed in the titration of the number of samples handled by the investigator.

These restrictions were approached by the investigator from the view point that the intensity of iodine color produced by the addition of the sulfuric acid revealed the dissolved oxygen content of the sample. A measure of the color intensity could be obtained on the electrophotometer. The investigator, therefore, determined the dissolved oxygen content of

samples containing various quantities of dissolved oxygen by titrating with standard sodium thiosulfate and then recording the reading of the electrophotometer for each sample. From the results obtained a curve was plotted as shown in Figure 3. The electrophotometer was calibrated several times during the investigation to compensate for variations in the instrument itself. This method was used for all dissolved oxygen determinations in this investigation. The sodium thiosulfate had to be standardized to calibrate the electrophotometer and no starch solution was required. The actual time of testing was divided in half.

Observing the curve on Figure 3, it will be noticed that the curve is fairly steep in the range of higher dissolved oxygen content. An error or variation in the electrophotometer reading would therefore result in a larger error in the dissolved oxygen content value. It therefore seemed advisable to determine the error which would be introduced by variations in the electrophotometer readings. Dissolved oxygen determinations were made on 16 bottles of a sample containing the same dissolved oxygen content and the electrophotometer readings were recorded for each bottle. The sample contained a high dissolved oxygen content such that the investigation was carried on at the steep slope of the curve given a value of the maximum range of error. The readings are treated by statistical methods (15) to determine this range of error in Table 1 and the following calculations:

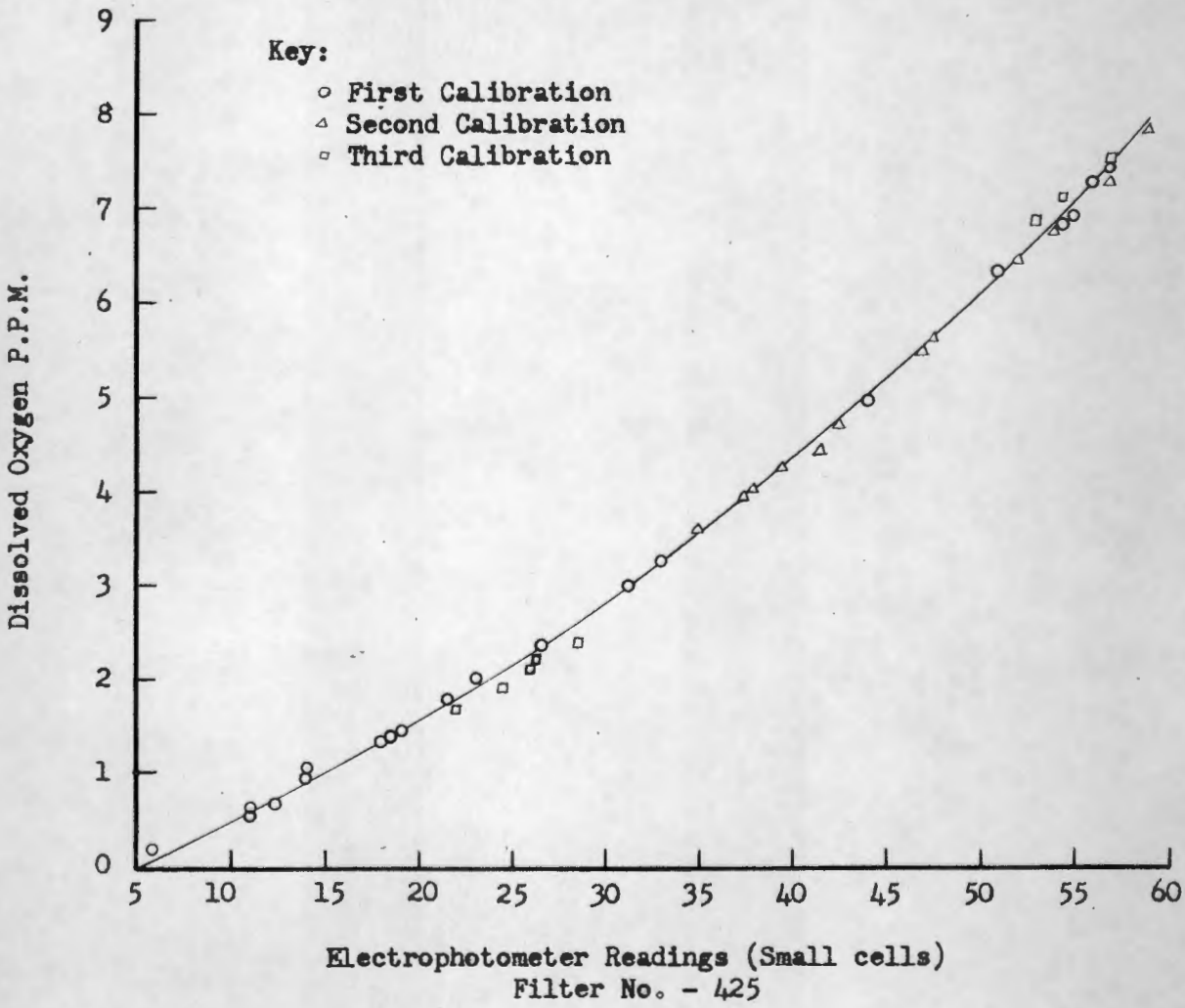

Figure 3 - Calibration Curve for Dissolved Oxygen Determinations by Electrophotometer Methods

Table 1

Dissolved Oxygen Calibration of Electrophotometer Treated by Statistical Methods

Sample No. n	Photometer Reading	Dissolved Oxygen Content from Curve	Deviation from Mean $(x - \bar{x})$	$(x - \bar{x})^2$
1	57.1	7.35	0.02	.0004
2	57.8	7.50	0.17	.0289
3	57.3	7.40	0.07	.0049
4	57.1	7.35	0.02	.0004
5	56.8	7.25	0.08	.0064
6	56.9	7.30	0.03	.0009
7	57.2	7.35	0.02	.0004
8	56.9	7.30	0.03	.0009
9	56.9	7.30	0.03	.0009
10	56.8	7.25	0.08	.0064
11	56.8	7.25	0.08	.0064
12	57.8	7.50	0.17	.0289
13	56.9	7.30	0.03	.0009
14	56.8	7.25	0.08	.0064
15	56.9	7.30	0.03	.0009
16	56.9	7.30	0.03	.0009
		Ave. = 7.33		Sum = .0949

$$\text{Standard deviation} = \sqrt{\frac{\text{Sum } (x - \bar{x})^2}{n}} = \sqrt{\frac{.0949}{16}} = .077$$

Range of error 90% of the time = $\bar{x} \pm a$ (Standard deviation)

$= 7.33 \pm .453\ (.077)$

$= 7.33 \pm .03$

Therefore, for 90% of the time the value of the dissolved oxygen content determined by electrophotometer readings are within the range of ± 0.05 parts per million of the true value.

It is very probable that this method of determining the dissolved oxygen content has been used before but no record of its use was found recorded in literature by this investigator.

B.O.D. Investigation

The variation in the actual 5-day B.O.D. values obtained for the sterile synthetic sewage caused the investigator a great deal of concern. The limitations of the B.O.D. test are well understood by the investigator but it seemed that much closer results should be obtained under the controlled conditions provided. For this reason the writer conducted an investigation of the characteristic B.O.D. curve for synthetic sewage. This investigation consisted of setting up and incubating a number of samples, properly diluted and seeded, and determining the B.O.D. of one sample each day over a period of twenty days. The plotting of the results of time versus B.O.D. is shown in Figure 4. The data was further analysed by using the slope method (16) to evaluate the constants of the first stage B.O.D. This analysis consists of Table 2 and the following

Note: The reduction of dissolved oxygen in the diluting water was not considered.

Key:

▫ Test 1 - 1.5% synthetic sewage; 1 ml. seed per bottle
○ Test 2 - 1.0% synthetic sewage; 1 ml. seed per bottle

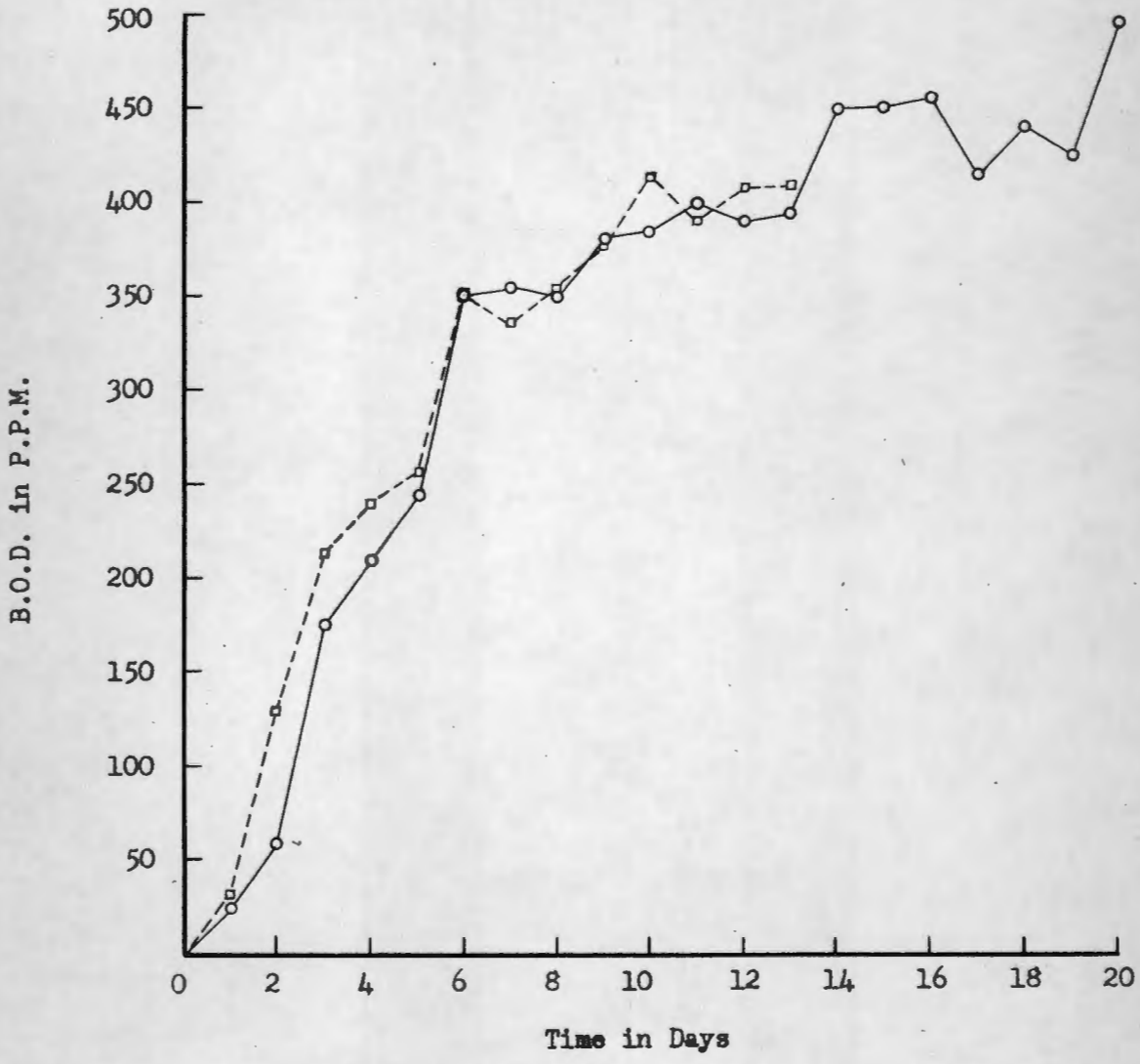

Figure 4 - Characteristic B.O.D. Curve for Synthetic Sewage

Table 2

Evaluation of B.O.D. Constants by the Slope Method
for a Twenty Day Testing Period

Time in days	B.O.D. observed y	Rate of change of B.O.D. y'	Product y'y	Product y^2
0	0	0	0	0
1	29	47	1363	841
2	95	83	7885	9025
3	195	65	12675	38025
4	225	28	6300	50625
5	251	62	15562	63001
6	350	48	16800	122500
7	346	0	0	119716
8	351	16	5616	123201
9	378	25	9450	142884
10	400	8	3200	160000
11	395	0	0	156025
12	400	3	1200	160000
13	401	25	10025	160801
14	450	25	11250	202500
15	450	3	1350	202500
16	455	-17	-7735	207025
17	415	-8	-3320	172225
18	440	5	2200	193600
19	425	27	11475	180625
20	495			
	= 6946	= 445	= 105296	= 2465110

I $na + b\Sigma y - \Sigma y' = 0$

$19a + 6946b - 445 = 0$

II $a\Sigma y + b\Sigma y^2 - \Sigma yy' = 0$

$6946a + 2,465,110b - 105,296 = 0$

$11 \times \frac{19}{6946} = 111$

III $19a + 6743b - 288 = 0$

Subtracting III from I

$$\begin{array}{r} 19a + 6946b - 445 = 0 \\ -(19a + 6743b - 228 = 0) \\ \hline 203b - 217 = 0 \end{array}$$

$$b = 1.07$$

$$K = \frac{b}{2.3026} = \frac{1.07}{2.3026} = .4647$$

The value of K (the reaction velocity constant) obtained by this analysis is abnormal in that it is considerably higher than the 0.10 value which is normally assumed for 20° C. first stage B.O.D. calculations. This analysis was conducted under the assumption that all the values of B.O.D. were part of the first stage B.O.D. curve.

It is well known that the normal B.O.D. curve consists of two separate stages, the carbonaceous stage and the nitrification stage, but many times the two stages are not clearly defined, as is the case with this set of data. However, knowing that the two stages do exist, the investigator examined the curve for factors which would characterise the division of the two stages. A definite leveling off of the curves can be noticed along the third, fourth, and fifth day, and a sharp break and increase in B.O.D. on the sixth day is very apparent. This is characteristic of the

break in the first stage B.O.D. curve and the point where the nitrification stage becomes evident.

It was considered advisable to analyze the data concerned with the first five days on the curve and evaluate the constants of the first stage B.O.D. The analysis of the data and the evaluation of the constants by the Slope Method is included in Table 3 and the following calculations:

I $na + b\Sigma y - \Sigma y' = 0$

$4a + 795b - 223 = 0$

II $a\Sigma y + b\Sigma y^2 - \Sigma yy' = 0$

$795a + 98516b - 28223 = 0$

$11 \times \frac{4}{795} = 111$

III $4a + 496b - 142 = 0$

Subtracting III from I

$$\begin{array}{r} 4a + 795b - 223 = 0 \\ -(4a + 496b - 142 = 0) \\ \hline 299b - 81 = 0 \end{array}$$

$b = .2709$

$K = \frac{b}{2.3026} = \frac{0.2709}{2.3026} = .1176$

$a = \frac{1}{4}(795(.2709)\ 223) = \frac{438}{4} = 109.5$

$L = \frac{a}{b} = \frac{109.5}{.2709} = 404$ ppm

The value of the reaction velocity constant obtained in this evaluation is very close to the value of 0.10 normally assumed for the first stage 20° C. B.O.D. calculations. This was conclusive proof to the investigator that the first five days were part of the first stage curve

Table 3

Evaluation of First Stage B.O.D. Constants by the Slope Method for Five Day Testing Period

Time in Days t	B.O.D. observed y	Rate of change of B.O.D. y'	Product y'y	Product y^2
0	0	0	0	0
1	29	47	1363	841
2	95	83	7885	9025
3	195	65	12675	38025
4	225	28	6300	50625
5	251			
	= 795	= 223	= 28223	= 98516

and that nitrification was interferring around the fifty day. For these reasons considerable variation would be expected in the results of the 5-day B.O.D. tests performed.

Results

The results of measuring the degree of purification of a synthetic sewage as it passes through an experimental trickling filter seeded with a pure culture of Zoogloea ramigera, are tabulated and presented in Table 4. The filter reached its normal operating conditions around the tenth day after seeding. The reduction in the five day B.O.D. after this time with varying rates of flow is presented in Table 5 and also shown graphically in Figure 5.

Table 4

Tabulation of Results of the Removal of 5-Day B.O.D. of Synthetic Sewage by Zoogloea ramigera in an Experimental Trickling Filter

Test number	Time elapsed since seeding in days	Rate of flow million gallons per acre per day	5-day B.O.D. filter influent	Average 5-day B.O.D. filter effluent	% of 5-day B.O.D. removal
1	1	2.20	222	212	4.5
2	2	0.65	242	198	18
3	4	1.23	210	154	27
4	5	1.32	258	195	24.5
5	6	1.20	302	200	32.5
6	7	1.5	292	225	23
7	8	0.5	234	146	38
8	9	0.5	248	150	39.5
9	10	0.35	200	67	66.5
10	11	0.40	190	69	64
11	12	0.60	200	82	59
12	13	0.60	216	100	54
13	14	0.60	203	82	60
14	15	1.40	193	106	45
15	16	1.30	198	128	35
16	17	0.85	206	88	57
17	18	0.95	258	134	48
18	19	0.95	234	109	53.5
19	20	0.82	213	91	57
20	21	0.82	212	58	77
21	22	0.70	215	76	65

Table 5

Average Reduction in the 5-Day B.O.D. of Synthetic Sewage by Zoogloea ramigera with Varying Rates of Flow

Range of flow in million gallons per acre per day	Average flow for period	Number of tests included in average	Percent of 5-Day B.O.D. removal
0.0 - 0.49	0.37	2	65.2
0.5 - 0.74	0.63	4	59.5
0.74 - 0.99	0.88	5	58.5
1.00 - 1.24	—	-	—
1.25 - 1.49	1.35	2	40.0

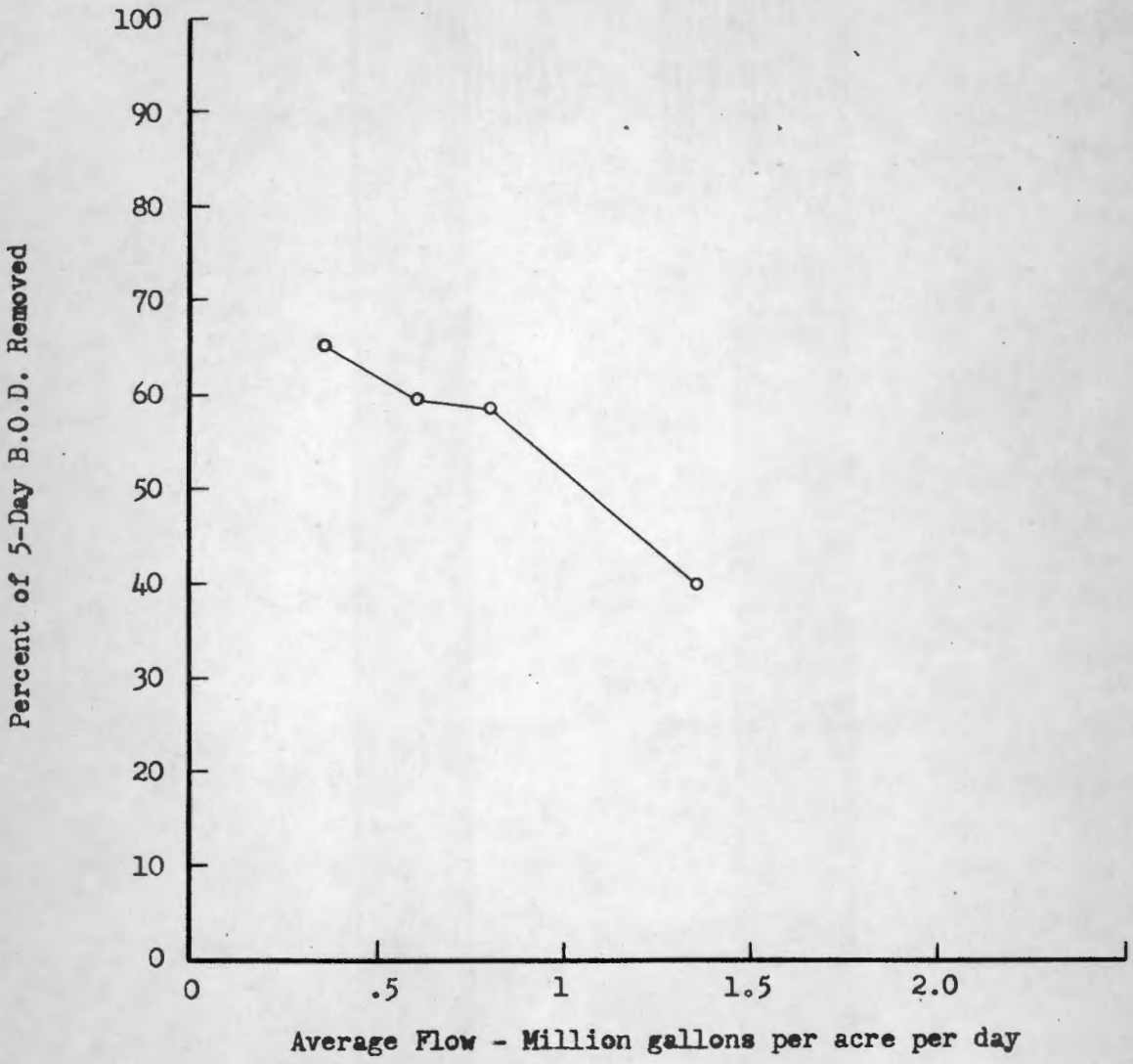

Figure 5 - Average Reduction in 5-Day B.O.D. of Synthetic Sewage by Zoogloea ramigera with Varying Rates of Flow

DISCUSSION OF RESULTS

Two observations may be made regarding the results presented. First, there is a marked degree of purification of the synthetic sewage as it passes through the filter. Second, the degree of purification is directly related to the rate of flow of the synthetic sewage through the filter. The results obtained compare very favorably with the results of Butterfield and Wattie (10).

No doubt the reader has questioned the term pure culture filter and rightly so. The investigator had no idea how long a pure culture could be maintained in the filter or whether the results obtained would be significant if contamination was present. A number of stained slides were made of the growth present in the filter after the testing was completed. These slides were critically examined under oil immersion to determine if contamination was present and the degree of contamination. This examination revealed the presence of contaminating bacteria but the writer was extremely surprised at the minor degree of contamination. Rather than try to explain to the reader the extent of contamination, the writer has included a photograph of a typical field showing the extent of contamination. This photograph is presented in Plate 1. Many fields revealed no visual sign of contamination.

For this reason the investigator believes that the results are significant in that the degree of purification of the synthetic sewage is a sole product of the Zoogloea ramigera.

Due to the depth difference between the experimental filter and the plant filter and also the difference in time required for the sewage to

flow through the two units, no correlation can be made at this time.

Plate 1

Typical Microscopic View Showing the Extent of Contamination in the Filter Growth

Note: The contaminating organisms are circled on the photograph.

SUMMARY AND CONCLUSIONS

The construction, method of inoculation, and operation of an experimental trickling filter unit under pure culture conditions is described in detail. The apparatus is not believed to be refined or perfected to such an extent that it could be used as a standard method of pure culture studies in a laboratory at this time. More time was spent in maintaining the condition of the apparatus than was applied to the actual testing procedures. It is believed, however, that the use of sterile lamps in the vicinity of the supply bottle and the constant head bottle would eliminate the contamination of the supply which would be a major improvement in the apparatus.

Although no direct correlation can be made between the experimental and actual trickling filter conditions and results at this time, this method of study affords an excellent means of evaluating the purification properties of a pure culture of organisms. By testing enough of the organisms isolated from a specific unit in a treatment plant in both pure cultures and mixed pure cultures, an excellent picture can be presented of the organisms and conditions responsible for the purification that takes place in the unit.

The investigator is not satisfied with the present method of determining and reporting B.O.D. It is felt that a reported 5-day B.O.D. is absolutely worthless unless the characteristic B.O.D. curve is known. The data and discussion under B.O.D. Determination is a perfect example of the value of the characteristic curve. The 5-day B.O.D. of the synthetic sewage would be reported as 250 ppm. By using the standard

formulas for B.O.D. calculations (17), the sixth day B.O.D. could be calculated as follows:

$$X_t = (L_a)_t \quad (1-10^{-kt})$$

$$(L_a) = \frac{X_t}{(1-10^{-kt})} = \frac{250}{(1-10^{2.5})} = \frac{250}{.684} = 366 \text{ ppm} = \text{1st Stage}$$

$$X_t = (366)(1-10^{-.6}) = 366 \times .749 = 274 \text{ ppm} = \text{6th day}$$

The sixth day B.O.D. would then be reported as 274 ppm but the sixth day B.O.D. had been determined experimentally to be 350 ppm. By not knowing the characteristic B.O.D. curve of the waste, the reported B.O.D. would be in error by 28%. Therefore, by determining the characteristic B.O.D. curve of a particular waste, the investigator has an excellent picture of the rate of biological action which is taking place.

The electrophotometer method of dissolved oxygen determination affords the investigator a speedy and reliable tool in the field of sanitary engineering. This method of determining the dissolved oxygen content required only half the time required by the conventional method. It was shown that the results obtained are reliable and that no significant sacrifice of accuracy is required by the method.

The investigation revealed that pure cultures of Zoogloea ramigera were able to carry on the process of purification of a synthetic sewage. The degree of purification is dependent on the rate of flow of the sewage through the experimental filter. An average reduction, in the 5-day B.O.D. of the sewage, of 52 percent at a flow of one million gallons per acre per day, is shown by the results.

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