RAPID, SMALL-VOLUME RESPIROMETRY AS A SUBSTITUTE FOR THE FIVE DAY BOD ASSAY

By

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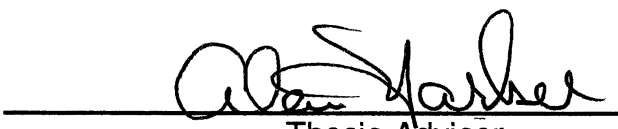

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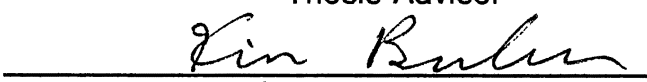

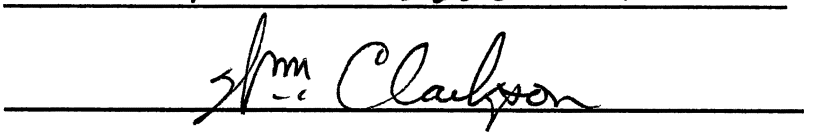

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CHAPTER I

INTRODUCTION

As our society progresses towards a more environmentally conscious people, the role of wastewater management becomes increasingly important. The need to know how much and what we are releasing into our water reservoirs has brought about the need for more accurate and rapid devices to determine wastewater parameters. The biochemical oxygen demand (BOD) test is one of the oldest assays used to evaluate wastewater organic strength. The BOD test measures the pollutional strength of domestic and industrial wastes in terms of the amount of oxygen required by bacteria to oxidize the organic matter under aerobic conditions. The efficiency of a treatment facility as a whole or individual plant components is evaluated based on their BOD reduction. Sewer surcharges are based on the BOD of the effluent. There are, however, several limitations to the standard five day BOD (BOD_5) due to the biological nature of the test. The world has experienced a surge of technological advances since the introduction of the BOD test at the beginning of the twentieth century. However, since its introduction very little has been accomplished to update and improve the currently recognized standard five day BOD test. The most significant shortcoming of the standard BOD_5 is the amount of time involved. Five days is acceptable for obtaining a historical perspective of a treatment system or to determine sewer surcharges, but the requirement imposed on the BOD has shifted to the monitoring and control of wastewater treatment systems. Process engineers who must evaluate daily fluctuations in their effluent must have access

to a rapid estimation of the BOD in order to respond to these fluctuations and thereby maximize the efficiency of their systems.

A rapid method for determining BOD results is proposed which provides results within fifteen minutes after sample acquisition. The short-term assay measures substrate dependent oxygen consumption and relates this value to the BOD_5. The objective of this study was to test a broad range of synthetic sample substrates falling into three general classes of compounds- carbohydrates, proteins and fatty acids, and to compare the BOD values obtained using the short-term assay to those obtained using the standard BOD_5. In addition, industrial samples containing particulate materials were tested in order to determine problems associated with the short-term assay when used in an actual process control situation. The proposed assay may not prove to be a substitute for the standard, EPA approved BOD_5. However, it certainly has potential to become a valuable process control tool which would provide real-time BOD results.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

The concept of biochemical oxidation and its relationship to wastewater treatment was recognized in 1898 when the British Royal Commission on Sewage Disposal was appointed to report on methods of waste treatment and measurement, and to establish a set of guidelines governing river pollution. In their investigations, they discovered that a significant relationship existed between the dissolved oxygen content of river waters and the degree of pollution of these waters. This resulted in their proposal that the weight of oxygen required for the biochemical oxidation of the organic material present in a water sample after a five day incubation at 65 °F (18.3 °C) be representative of the degree of pollution in the sample. This temperature was chosen because 65 °F was the maximum expected stream temperature in Britain during the summer months. A five day incubation period was chosen to allow a statistically significant amount of sample oxidation while decreasing the likelihood of nitrification. In addition, all the rivers in Britain required less than five days to reach the sea; after five days, it was assumed that oxygen demand would no longer be significant as the river water would be diluted with oxygen-saturated sea water (LeBlanc, 1974). The BOD procedure used today was adopted in 1936 by the American Public Health Association Standard Methods Committee. Although various other analytical methods now exist to characterize wastewater organic strength, the BOD test still remains one of the most important parameters used to assess the concentration of biodegradable substances in a wastewater.

The standard procedure consists of placing a sample in an airtight bottle which also contains oxygen-saturated dilution water and a seed culture. The bottle is incubated for five days at 20 °C; the BOD is calculated from the difference between the initial and final dissolved oxygen content. The dilution water provides essential nutrients and ensures that the pH of the incubated sample remains in a range suitable for bacterial growth. Another purpose of the dilution water is to bring the oxygen demand and supply into balance, since most wastewaters contain more oxygen requiring substrates than the amount of dissolved oxygen available in a 300 mL bottle of air-saturated water. By diluting the sample and ensuring that a certain amount of oxygen remains at the end of the five day incubation period, it is the organic carbon that is the limiting growth factor in the bottle, not the oxygen. A dilution water blank and a seed control are used as a check on the quality of the dilution water and as a measure of oxygen consumption due to endogenous respiration, respectively (APHA et al., 1985).

The five day BOD test has one advantage in that if the seed culture is not acclimated to the substrates present in the sample, five days is usually enough time to activate any inducible enzyme systems. The standard BOD_5 test, however, has a number of disadvantages including poor reproducibility, long incubation period, limited information, and interferences due to dilution water and seed culture. A myriad of papers has been published concerning problems associated with the five day BOD, as well as methods to improve the reliability of the BOD_5 (Stover and McCartney, 1984; Kostina et al., 1988; Woodring and Clifford, 1988; Heddle, 1984; Stack, 1984). As previously stated the most obvious disadvantage related to the standard BOD_5 is the time between sample acquisition and results. Smaller industries which must contract outside sources for their BOD determinations can expect turnaround times of up to ten days for their BOD results. On a more practical note, the test can only be initiated on

certain days of the week (Wednesday, Thursday, Friday) when laboratory facilities are closed for weekends (similar problems arise for holidays). Also since an oxygen concentration is taken only at the beginning and end of a five day incubation period, the standard procedure does not give any information concerning the rate of sample degradation by the seed culture or whether a particular concentration of sample would maximize microbial oxidation rates. In fact, one does not even know if sample stabilization is complete at the end of five days. In addition, for samples containing toxic substances that inhibit bacterial respiration, dangerous conclusions can be drawn when relying on the BOD_5. For example, if the bacterial seed culture used in the BOD_5 is inhibited by the sample substrate being tested, this could lead to a conclusion that the sample had a low BOD, since oxygen consumption during the five day incubation would be decreased.

One of the requirements placed on the dilution water quality is that the oxygen demand must be below 0.2 mg/L as stated in Standard Methods. This requirement is often difficult to meet. Since the oxygen demand of the dilution water is not determined until after the fact, and is also not accounted for in the calculation of BOD_5, it is likely that the quality of the dilution water is often ignored. In addition, a high degree of variation of BOD results has been shown when different dilutions are used for the same sample (Stack, 1984; Woodring and Clifford, 1988). There is also evidence that the activity and acclimation state of the seed culture used can significantly effect the resulting BOD values (Stover and McCartney, 1984; Kostina et al.,1988). In summary, the BOD has outlived its usefulness; its main use today is to provide a historical perspective and to meet specific EPA composite sample effluent data requirements. Most of the current research efforts in improving the standard five day BOD test are focused on reducing the time involved in performing the assay while obtaining values

comparable to the five day test. Another goal of an improved BOD assay is to enhance the accuracy of the test compared to the five day method. The following sections will review present and past research efforts involved in the development of a short-term BOD assay.

Attempts to shorten the standard five day BOD test fall into three general categories: manometrically measuring short-term oxygen consumption, electrochemically measuring short-term oxygen consumption, and shortening standard five day methodologies with extrapolation of the data to predict the BOD_5. In performing most of the short-term BOD assays, several guidelines must be noted. First, the bacterial seed culture must be adapted to the sample substrate being examined and the ratio of bacteria to sample substrate must be relatively large. In addition, the assay must account for the oxygen requirement which does not enter into substrate oxidation (i.e. oxygen depletion due to endogenous respiration)(Riegler, 1987).

The determination of BOD using a continuous recording of oxygen uptake, manometrically or electrochemically, is a common procedure, and several types of these exist. The automatic respirometer made by Tech-Line Instruments, Arthur Technology, Inc. is an automated system which manometrically measures oxygen consumption in the presence of the sample and converts it to a BOD_5 value. The system consists of a closed air recirculation system with an air pump, a fine-bubble air diffuser, and a 1- to 4-Liter aeration column containing a CO_2 scrubber. Once filled with the sample to be analyzed, the aeration column is closed and measurement of the dissolved oxygen uptake rate begins (Therien and Ilham, 1982). Since CO_2 is produced in equimolar amounts to oxygen consumed during sample oxidation, and this CO_2 is scrubbed out of the system, a net decrease in total pressure results from the utilization of oxygen. This change is sensed by a transducer which converts the information to an electrical

signal which can be calibrated to oxygen consumption and recorded (Arthur and Hursta, 1968).

Therien and Ilhan (1982) conducted a study to compare BOD results obtained from analyzing wastewater using the Tech-Line automatic respirometer to BOD values derived from the standard BOD_5. Domestic sewage and industrial wastewater of prescribed concentrations were used to compare the two BOD assays. This procedure seems to correlate with BOD_5 values, but it is also extremely expensive (making it inaccessible to smaller companies), large samples must be used (1-4 L), and it is not portable.

Many of the attempts to shorten the standard BOD_5 are similar to the automatic respirometer described above, but differ in the way that oxygen depletion is measured. One of the first electrochemical methods to determine BOD was developed in 1960 by Clark. As in the Tech-Line system, as oxygen is consumed in the presence of the sample substrate and CO_2 is produced in equimolar amounts, this CO_2 is scrubbed out of a closed system. This results in a drop in the gas phase pressure inside the reaction chamber. The initial pressure in the system is reestablished by the electrolytic generation of oxygen. The BOD calculation is based on the amount of oxygen generated to maintain the initial oxygen concentration (Clark, 1960). This first system had problems related to temperature and atmospheric pressure fluctuations. Since then, several modifications have been made to make the system independent of atmospheric pressure and external temperature fluctuations but have increased its mass (Hickey and Nagel, 1985; Cadena et al, 1989).

Another rapid method for estimating BOD involves the use of a biofilm electrode. First developed by Karube (1977), this procedure uses a microbial cell paste on top of an oxygen electrode. When the biofilm electrode is placed in a solution containing the sample substrate and oxygen consumption by the seed

culture begins, less oxygen diffuses across the biofilm to the electrode, which then leads to a drop in the current of the system. Several variations to this method exist, primarily differing in the type of seed culture utilized. One group chose a heterogeneous seed culture (Strand and Carlson, 1984), but most chose a single species of yeast to comprise the biofilm (Riedel et al., 1990; Harita et al., 1985). This method did correlate well with BOD_5 for the limited sample substrates tested. However, the biofilms were not stable for longer than three to four weeks. Most treatment plants do not have the facilities to produce and maintain these biofilms, making this method inaccessible to them.

The following two methods most closely resemble the proposed short-term BOD assay. The first one, the BOD-M3 developed by Dr. Gunther Riegler, (Darmstadt, West Germany) is an on-line device which requires about 3 minutes between sample acquisition and display of the measured BOD value. This unit consists of a bioreactor containing bacteria growing on the surface of many plastic rings and two oxygen electrodes to measure the oxygen content of diluted sample entering and leaving the reaction vessel, which is operated at a prescribed liquid retention time. The system is designed to keep the oxygen demand of the bioreactor at a certain level, so that if the oxygen difference between the inlet and outlet falls below that level, the dilution ratio is lowered; on the other hand if the oxygen difference rises, less effluent is pumped in and more dilution water is used. This mixing ratio determines the nutrient concentration in the sample and ultimately serves as the basis for the BOD determination. Results are plotted on a paper tape by a graphic printer. Limited published data from the BOD-M3 seems to correlate with the five day method (Koehne, 1985). Like the Tech-Line respirometer, the BOD-M3 is an on-line system with a cumbersome amount of equipment involved. The cost of this system has not

been evaluated but it seems likely that it will be less costly than the Tech-Line system.

Another similar rapid BOD assay, on a much smaller scale, consists of a transformer, recorder, water bath (30 °C), magnetic stirrer, flask, and oxygen electrode. The flask contains 10 g immobilized cell beads containing a facultative bacterium, *Bacillus polymyxa* D-21. In this method, 90 mL of a diluted sample is saturated with oxygen by vigorously stirring and is then placed in the reaction vessel flask. Air is excluded from the flask and consumption of oxygen by the immobilized cells causes a current decrease which is displayed on the recorder. The slope of the response curve correlates to the drop in dissolved oxygen. The total assay time is not more than 15 minutes. This short-term assay seemed to correlate with the BOD_5 very well in a study of a sugar company waste, although the authors did not go into their methods of calculating the short-term BOD value (Su, Huang, and Lu, 1986). This method provides a simple, inexpensive means of obtaining BOD values, but limits the sample range to substrates degraded by *B. polymyxa*. It is unlikely that a single microorganism could use all the organic compounds present in a sample of wastewater, therefore this assay could probably be improved by using a mixed culture of bacteria in the cell beads. Furthermore, various types of sample substrates need to be tested for a better comparison to the BOD_5. By using one species of microorganism and one type of sample wastewater for their study, the authors severely limited the applicability of their assay to other wastewater situations.

The final category of short-term BOD assays involves no specialized equipment. This procedure follows the same methodology as the five day BOD, but over shorter time frames. Ademoroti (1984) conducted a study to establish a relationship between 5-day BOD values and those of 4-, 3- and 2- day BOD values in the form of a simple equation: $BOD_5 = K * BOD_n$ (n=2, 3, 4; where K is

determined experimentally), so that the BOD test could be conducted in fewer than five days and the results could be accurately converted to 5 day BOD values using the K_n value. He compared the 2, 3, 4 and 5 day values using two sample wastewaters: one from a hospital, the other from a brewery. K values were obtained by taking the ratio of BOD_5/BOD_n for the two types of wastewater. The K values obtained for the two types of wastewater agreed within one percent. The K values were obtained by taking the mean of these two sets of data and were tested with several other water/wastewater samples to see if the BOD_5 could be predicted. This proved to be successful over the range of BOD_5 values tested. The major disadvantage to this procedure is that 2, 3 and 4 days is still much too long for process control requirements, although it does alleviate some of the time involved in the five day method. Raviv and Ben-Yaakov (1984) have developed an algorithm that can predict the BOD_5 within 15% using the BOD taken after a 36 hour incubation period. As stated above, this is an improvement over the five day method, but 36 hours is still too long.

Various other analytical procedures have been developed in an effort to overcome some of the problems associated with the BOD assay. The chemical oxygen demand test is a rapid procedure which uses potassium dichromate to completely oxidize most organics in a sample. The amount of dichromate reduced is measured colorimetrically and is related to the oxygen demand of substances in the sample. The COD test is more precise and requires less time (2-3 hours) than the BOD_5, however the COD test does not distinguish between biodegradable and nonbiodegradable compounds. The COD may be used as a substitute for BOD only for samples that are consistent in the amount of biodegradable substances present and if standard BOD_5 assays are conducted periodically as a check to ensure that the established empirical relationship of the COD to BOD remains consistent (Ademoroti, 1986; Gil et al, 1989). In addition,

the Total Oxygen Demand (TOD), Total Carbonaceous Biochemical Oxygen Demand (T_cBOD), and Total Organic Carbon (TOC) methods have all been correlated to the BOD_5. Like the COD, these methods determine oxygen demand based on nonbiological procedures and therefore include oxygen demand for nonbiodegradable compounds in the wastewaters (Basei, 1984; Jones, 1969; Heddle and Tavener, 1981; Lueck, Dishman, and Thayer, 1981).

The proposed short-term BOD assay provides rapid BOD results that are proportional to the BOD_5, as did most of the methods described above. In addition, some of the problems associated with the other short-term assays such as cost, amount of equipment involved, portability, and substrate range are addressed in this study.

CHAPTER III

MATERIALS AND METHODOLOGY

Seed Culture Selection

Two heterogeneous cultures and one pure culture were used in the short-term biochemical oxygen demand (BOD) assay to determine which culture utilized the most substrates without any acclimation period prior to the test. A fecal sample and a sewage sample were used to obtain the two heterogeneous cultures. *Alcaligenes eutrophus*, which is known to utilize a broad spectra of substrates (A. Harker, personal communication), was used as the pure culture. Numerous carbohydrates, amino acids and hydroxy acids were tested. Following these data, it was determined that the heterogeneous culture collected from the Stillwater Municipal Treatment Facility provided a broad substrate utilization as well as being the most convenient for obtaining consistent samples for inoculations. Every two to three months following, a fresh sample was collected, grown up overnight in Total Nutrient Broth (TNB) (Table I), and used as the inoculum for the seed cultures in both the short-term and five day assays.

TABLE I

TOTAL NUTRIENT BROTH RECIPE

Distilled Water	500 mL
Tryptone	2.50 g
Yeast Extract	1.25 g
Dextrose	0.50 g
NaCl	2.50 g
KNO_3	2.00 g

Growth and Acclimation Conditions of Seed Culture

In order to obtain rapid results using the short-term BOD assay, a culture in log phase growth was needed. Since many of the substrates to be tested did not utilize constitutive enzymes in their degradation, the culture was allowed to grow on the sample substrate prior to the test, to induce the necessary enzyme systems. The seed culture was grown in minimum mineral medium (MMO) plus 0.1%(w/v) of the sample to be tested. Table II presents the composition of the minimum mineral medium which provides buffering capacity as well as supplying the elementary trace metals required for bacterial growth. The seed culture was maintained in TNB during the course of this study. Twenty microliters was used to inoculate 50 mL of TNB. This culture was then incubated at room temperature on a bench-top shaker (Labline, #3520). This culture was used as the inoculum for the acclimation growth media containing MMO and 0.1% of the sample substrate.

TABLE II

COMPOSITION OF MINIMUM MINERAL MEDIUM

Distilled Water	930 mL
Solution A	40 mL
Solution B	20 mL
Solution C	10 mL

Solution A: 1 M Phosphate Buffer

Sodium Phosphate Dibasic (1 M)	220 mL
Potassium Phosphate Monobasic (1 M)	190 mL

Solution B:

Nitrilotriacetate	10.00 g
Magnesium Sulfate	14.45 g
Calcium Chloride (Dihydrate)	3.34 g
Ammonium Molybdate	9.25 mg
Ferrous Sulfate (Heptahydrate)	0.10 g
Metal "44"	50.00 mL
Potassium Hydroxide	7.40 g

Dissolve above chemicals in distilled water to make 1000 mL total of solution.

Solution C: 10% Ammonium Sulfate

Distilled Water	100 mL
Ammonium Sulfate	10 g

Metal "44"

Distilled Water	100 mL
EDTA	0.250 g
Ferrous Sulfate (Heptahydrate)	0.500 g
Manganous Sulfate (Monohydrate)	0.154 g
Cupric Sulfate (Pentahydrate)	0.039 g
Cobalt Nitrate	0.025 g
Zinc Sulfate	0.110 mg
Sodium Tetraborate	0.018 mg

A few drops of sulfuric acid should be added to retard precipitation.

Early in this study it was determined that in order to have a log phase seed culture ready for a short-term assay, a continuous culture system needed to be implemented. The stock feed solution was MMO plus 0.1% (w/v) of the sample to be tested. Figure 1 illustrates the physical assembly of the feed bottles. Since solutions A, B and C must be autoclaved separately, a special cap was placed on a feed flask containing the appropriate amount of distilled water. Following sterilization, the three solutions were added by a syringe through a rubber septum placed on a glass pasteur pipette. The opening of the flask was sealed with cotton to prevent contamination during the acclimation period of the seed culture. One liter of the feed solution was made and slowly added to another flask containing the seed culture using a Masterflex Pump Controller. Nutrient was supplied at a rate of 0.2 mL/min using Tygon Microbore Tubing (0.03 cm ID). The continuous culture apparatus is shown in Figure 2. Flasks were designed with a side arm to allow the continuous removal of old culture.

For all of the sample substrates tested, the seed culture was grown up in the continuous culture for one to three days depending on the acclimation period that was necessary to turn on the metabolic pathways used in the degradation of that substrate. A turbid broth indicated that acclimation to the sample substrate had been achieved.

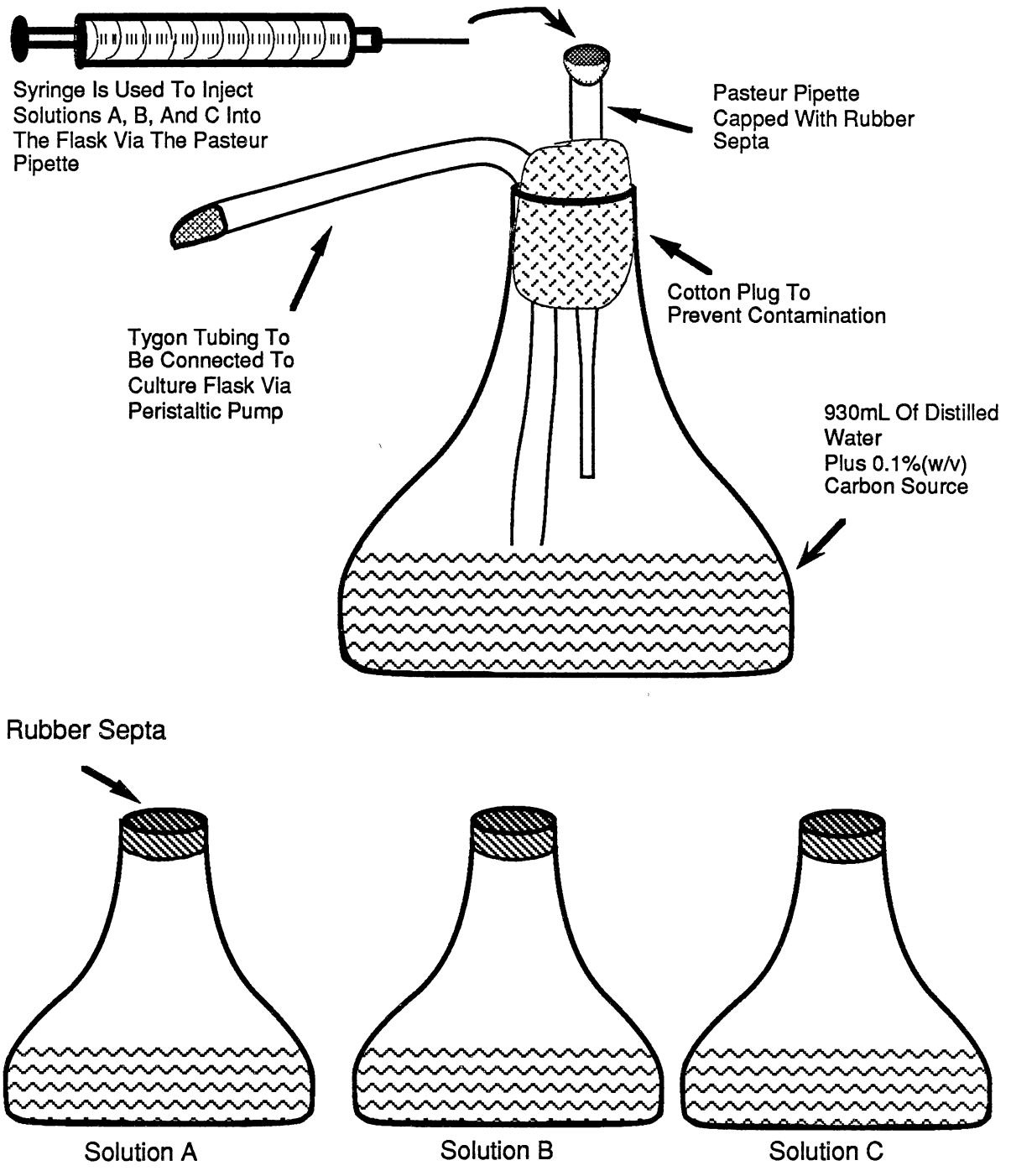

Figure 1. Physical Assembly of the Stock Feed Solutions.

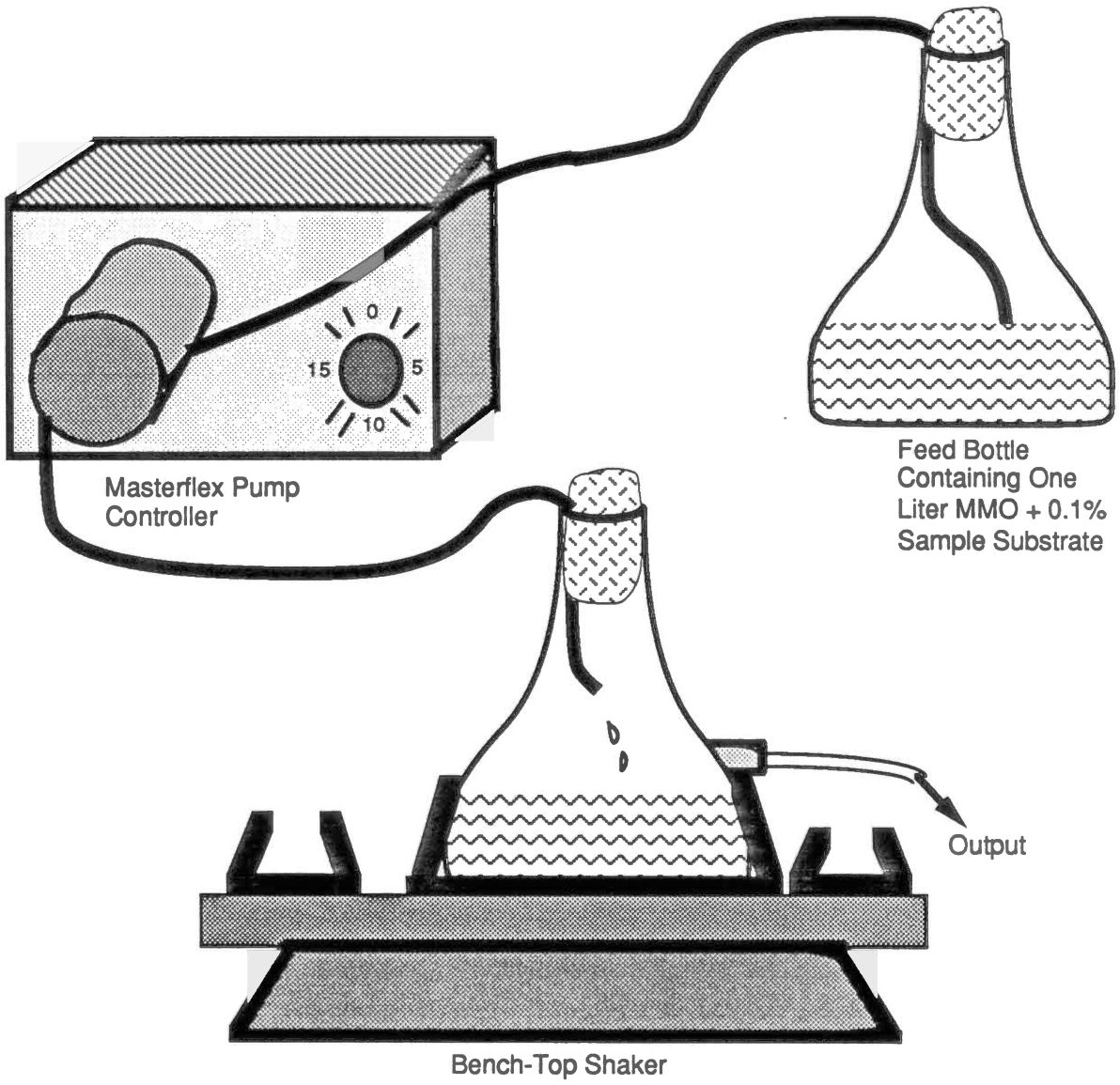

Figure 2. Continuous Culture Apparatus.

Short-Term Assay

Apparatus

The equipment involved in the short-term BOD assay is shown in Figure 3. A Clark-type oxygen electrode (Yellow Springs Instruments) is placed in the side of a water-jacketed reaction vessel (Gilson Medical Electronics) which contains a chamber used to hold 2 mL of seed culture. The reaction chamber is fitted with a glass stopper to prevent the exchange of oxygen between the atmosphere and the seed culture during an assay. The glass stopper contains a small opening through which the sample is injected. The oxygen electrode is connected to an interface box which allows the system to be calibrated using air-saturated water. Water contains 8.9 ppm of oxygen at 22 °C; this value was used to calibrate the oxygen electrode. As an additional check to ensure that the electrode was functioning properly, the oxygen concentration of a solution of Na_2SO_3 (12.5 g in 250 mL distilled water) was measured. A reading of 0.1 ppm O_2 or less indicated that the oxygen electrode was functioning properly. The oxygen permeable membrane covering the electrode was replaced every two months, or more frequently if needed. Unstable readings on the stripchart recorder usually indicated a need to change the membrane.

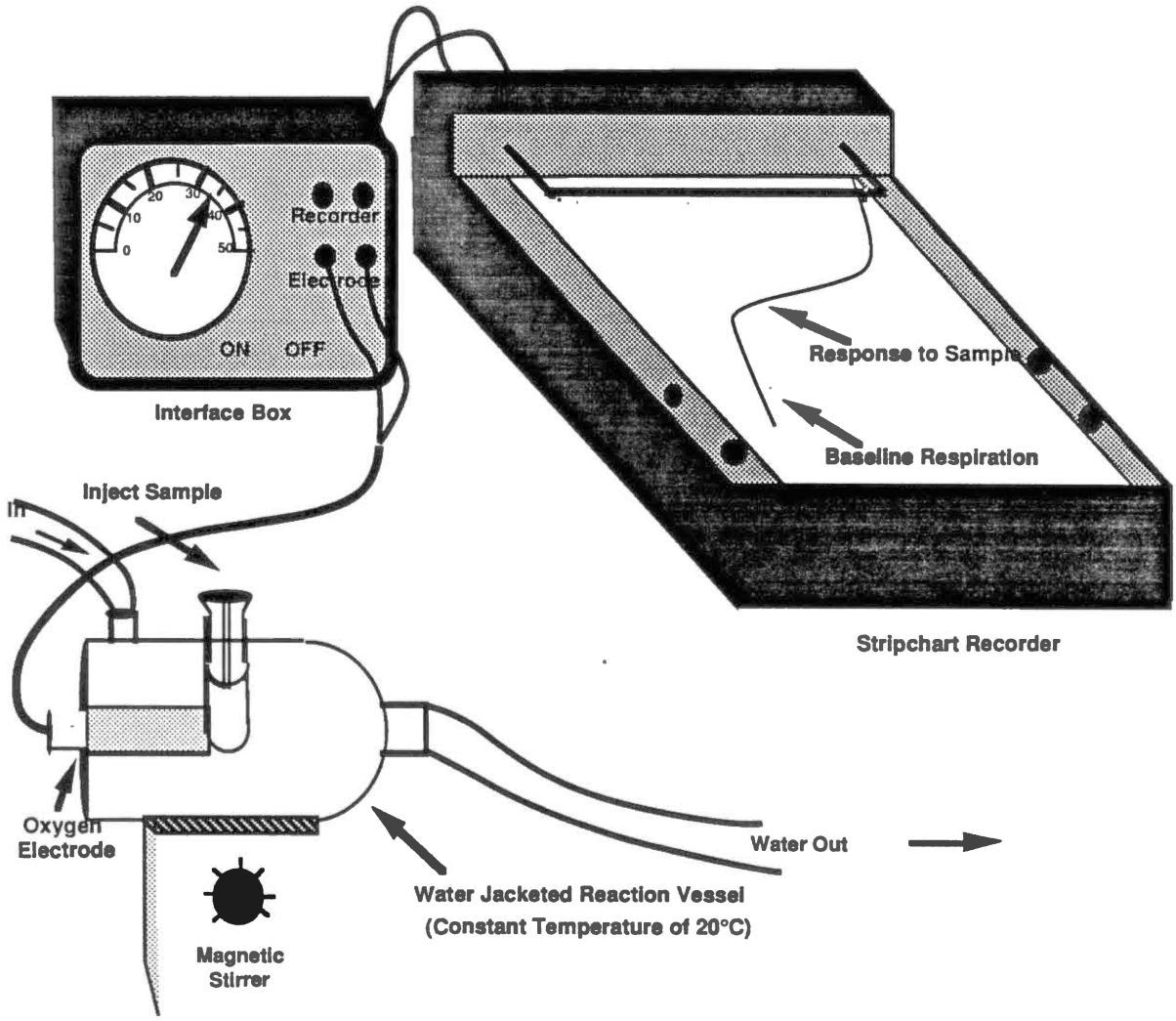

Figure 3. Short-Term BOD Apparatus

Procedure

As previously stated, prior to performing a short-term assay, a 0.1% (w/v) solution of the sample substrate in MMO was inoculated using the TNB-maintained seed culture. This culture was grown at room temperature in the continuous culture apparatus for at least twenty-four hours (some samples required several days of acclimation to the sample substrate). Two hours prior to running a short-term assay, 50 mL of the acclimated culture was centrifuged

at 10,000 rpm for fifteen minutes. The pelleted cells were then brought up in 20 mL of MMO without a carbon source, and centrifuged a second time at 10,000 rpm for fifteen minutes. After the second centrifugation, the cells were brought up in MMO to an optical density of approximately 1.0 at 425 nm. This seed culture was then placed in a sterile flask and incubated at room temperature on the bench top shaker for approximately one hour. The purpose of the incubation period prior to BOD testing was to allow the cells to reduce the amount of endogenous substrates which would cause a high endogenous respiration rate. After incubation, 1800 µL of the cell suspension was placed in the reaction chamber. The temperature was maintained at 20 °C by using a Lauda Refrigerating Circulator (Brinkman, #RM6). Using a glass pasteur pipette, the cell suspension was vigorously aerated to saturate the system with oxygen. The glass stopper was placed on top on the reaction chamber, and the BOD determination began after the sample was injected through the top of the glass stopper. The chart speed of the stripchart recorder (Houston Instruments, #D5000) was 0.5 cm/min; input current range of the recorder was 10 mv.

Calculation of the Short-Term BOD

To calculate the BOD_{ST}, the following formula is used:

$$BOD_{ST} = [(DO_1 - DO_2) - R\,(T_2 - T_1)](1800\ \mu L/S)(X)$$

Where:

DO_1 = Initial oxygen concentration (ppm) at T_1

DO_2 = Final oxygen concentration (ppm) at T_2

T_1 and T_2 = Initial and final times

R = Stable rate of endogenous oxygen removal at T_1 and T_2

Volume of reaction vessel = 1800 µL

S = Volume of sample (µL)

X = Dilution factor prior to sample injection

A shortcut calculation of the BOD_{ST} was accomplished by extrapolating the initial endogenous respiration rate line to a point above the final endogenous respiration rate line. This accounted for the $R(T_2 - T_1)$ term in the above equation and then the difference in the oxygen concentration between the two parallel lines could simply be multiplied by the (1800 µL/S) term to calculate the BOD (Figure 4).

Five Day BOD Analysis

Procedure

The determination of the standard five day BOD was performed according to the procedure given in Standard Methods (APHA et al., 1985). At least triplicate tests of the five day BOD were done for each sample tested. Dilution water was made according to the recipe given in Standard Methods. The dilution water was stored in a carboy and aerated overnight prior to five day BOD testing. An Orion oxygen electrode (Model #97-08-00) in conjunction with an Orion Digital Ionalyzer (Model #501) was used to measure the oxygen concentration at the beginning and end of the five day incubation at 20 °C. The electrode was calibrated using water-saturated air according to manufacturers instructions each time the electrode was used. As a further check on the proper calibration of the electrode, the oxygen concentration of a solution of Na_2SO_3 (12.5 g/250 mL distilled water) was measured. An oxygen reading of less than 0.1 ppm indicated that the electrode was properly calibrated. This second

check was done every three months or when the electrode had been stored for more than several weeks without use.

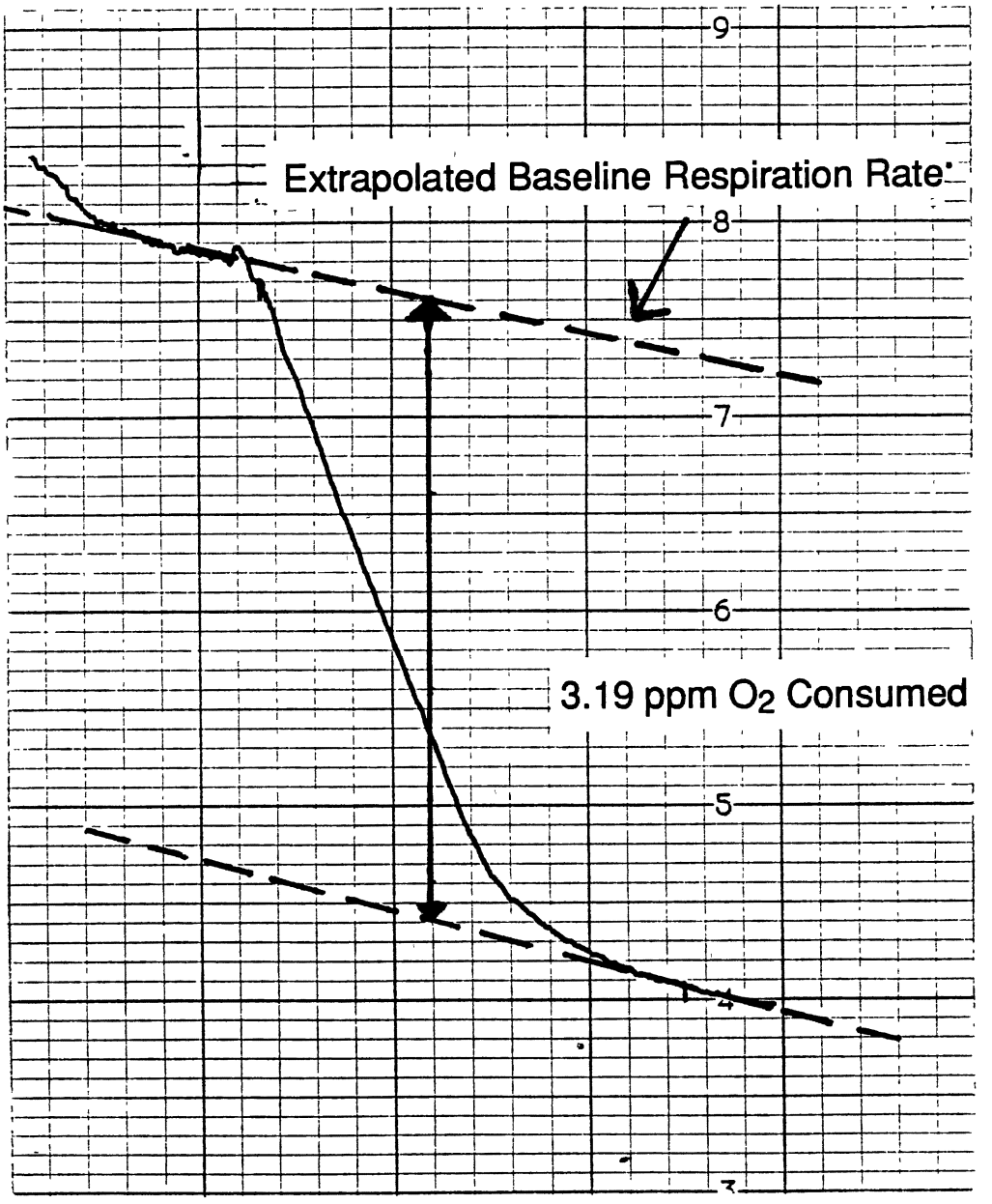

Figure 4. Example Short-Term BOD Graph.

Calculation of the Five Day BOD

To calculate the five day BOD, the following formula is used:

$$BOD\ (mg/L) = \frac{(D_1-D_2)-(B_1-B_2)}{P}$$

Where:

D_1 = Dissolved oxygen of diluted sample immediately after preparation, mg/L.

D_2 = Dissolved oxygen of diluted sample after five days incubation at 20 °C, mg/L.

P = Decimal volumetric fraction of sample used.

B_1 = Dissolved oxygen of seed control before incubation, mg/L.

B_2 = Dissolved oxygen of seed control after incubation, mg/L.

The seed control is used as a check on the quality of the dilution water as well as a measure of part of the oxygen consumption due to endogenous respiration. The BOD of a dilution water blank is also determined to ensure that the dilution water is free of organics. Dilution water was considered acceptable according to Standard Methods when the BOD was below 0.2 mg/L. In addition, a standard glucose/glutamic acid solution (0.3 mg/mL) was used as a check on the five day test. This standard sample should have a BOD of 200 mg/L (+/- 37 mg/L). Triplicate BOD determinations were done on this sample each time the five day test was used. If the BOD of this solution was outside the acceptable range, all of the data from the other samples assayed at this time were considered to be invalid and the tests were repeated until all criteria were met.

Sample Preparation

Synthetic Samples

The synthetic carbohydrate and protein samples were made by dissolving 0.03 g and 0.10 g respectively in 100 mL of distilled water (nanopure by reverse osmosis). The samples were then autoclaved to allow long-term storage of the samples without contamination. Even though fatty acids are not soluble in water, in order to remain consistent with the way that previous samples were prepared, as well as attempting to represent a real sample containing fatty materials, 10 µL (for low melting point fatty acids) of the fatty acid was added to 10 mL of distilled water in a sterile test tube. For the fatty acids with a higher melting point, 10 mg was added to 10 ml of distilled water in a test tube. These samples were heated prior to use in order to produce a liquid fatty acid sample. Prior to sample removal for BOD determinations, the test tube containing the fatty acid sample was vigorously vortexed in an attempt to evenly distribute the fatty acid micelles so that when a portion was removed for BOD testing, the sample was a representative one.

To test the effects of varying temperature, optical density of seed culture, and dilution of samples on the short-term test, several experiments were done using a 0.75 mg/mL maltose solution. The sample solution was made as described above for the synthetic carbohydrate samples. Also, five different concentrations of a maltose solution were made and tested using the short-term assay in order to show a linear increase in the short-term BOD with increasing sample concentrations. The following concentrations of maltose solutions were used: 0.25, 0.50, 0.75, 1.0, and 1.25 mg/mL.

Industrial Samples

To further test the short-term BOD assay and to identify problems related to sample preparation for wastewater samples containing particulate materials, the BOD of three industrial effluents was determined using both the short-term and the five day methods. These samples would provide a more accurate representation of the types of sample substrates that would be used in later field studies. The samples were collected from the following companies located in Oklahoma:

1. Moore Business Forms: this effluent contained mostly organic solvents as well as large clumps of a white sponge-like material dispersed throughout the sample.
2. Farm Fresh Dairy: soluble dairy by-products.
3. Ralph's Meat Packing Company: slaughter house run-off. Diluted blood with solid materials (?) settled on the bottom of the collection bag and layer of fat floating on top.

After collection from the sample site, these samples were kept on ice during transport back to the laboratory. A 10% growth medium was made using MMO plus the sample. This seed culture was allowed to grow up overnight on a bench-top shaker at room temperature. The following morning, the seed culture was washed and incubated as described above for the synthetic samples. For the two samples that contained particulate materials (Moore Business Forms and Ralph's Meat Packing), a portion of the sample collected was filtered to remove all solids and the BOD was determined for both the filtered and unfiltered portions.

Short-Term BOD Analysis of ^{14}C-Glucose

In an attempt to delineate the fate of a sample substrate after completion of the short-term BOD assay, a ^{14}C-Glucose sample solution was used in the short-term assay. The ^{14}C-Glucose was purchased from NEN Research Products as a crystalline solid. All six carbons of the ^{14}C-Glucose were labeled. The total amounnt purchased was 50 μCi, having a specific activity of 2.3 mCi/mmole. The total amount purchased was brought up in 1000 μL of distilled water and then aliquotted into ten 100 μL fractions. Each of these fractions was then brought up to a total volume of 1000 μL with distilled water. These samples were then referred to as the stock solution; the activity of the stock solutions was 0.23 mCi/mmole. The concentration of the stock solutions was calculated to be 0.392 mg/mL (2.174×10^{-3} mM) based on the following:

Purchased: 50 μCi glucose Activity: 2300 μCi/mmole

Then: 50 μCi/2300 μCi $mmole^{-1}$ = 2.174×10^{-2} mmole

Converting this to gram weight:

(2.174×10^{-2} mmole)(180.2 mg/mmole) = 3.92 mg

Therefore the 3.92 mg of ^{14}C-Glucose was added to 1 mL of water and then diluted 1/10 to obtain the stock solutions having a final concentration of 0.392 mg/mL (2.174×10^{-3} mM).

A standard curve was generated relating concentration of the stock solution to counts per minute on a Beckman scintillation counter (LS 5000 CE). Dilutions of the stock solution were 1/10, 1/25, 1/50, 1/100, 1/250, 1/1000, 1/5000, and 1/10,000. Triplicate samples were counted and values reported were averages. Scintillation vials (7 mL) were purchased from Fisher Scientific. A 100 μL portion of the dilution sample was added to 400 μL of

Ready Safe Cocktail (Beckman). The eight dilution samples above were converted to molar concentrations so that the resulting standard curve was reported as counts per minute (CPM) vs Molarity.

The short-term assay was performed in the usual manner using the ^{14}C-Glucose stock solution as sample substrate, except that a side-arm was added to the glass stopper to allow the removal of CO_2 during and after the test. Also, the top of the glass stopper was sealed with a rubber septum to retain the evolved CO_2 within the system. Three traps were placed in succession, the first was connected directly to the side arm of the glass stopper and used as an overflow container. The second and third trap contained 1 mL of Solvable (NEN) which was used to trap the evolved CO_2. Tygon microbore tubing was used to connect the reaction vessel, the overflow container and the two CO_2 traps. The experiment, run at 20 and 30 °C, was repeated one week later to evaluate the reproducibility of the assay and the resulting scintillation counts. After completion of the short-term assay, 100 µL of a 40% (w/v) sodium azide solution was added into the reaction vessel to stop any further sample oxidation by the seed culture. After inhibiting bacterial respiration, approximately 30 cc of air was bubbled through the reaction vessel using a 5 cc syringe, in an attempt to displace CO_2 retained in the reaction vessel. After the gas displacement phase, two 400 µL portions of the buffer/cell solution in the reaction vessel were removed and placed into two 1 mL eppendorf tubes. These were then centrifuged for three minutes on a tabletop centrifuge to separate the seed culture from the supernatant. Following centrifugation, the supernatant was removed from each tube and placed in another eppendorf tube. Four drops of concentrated hydrochloric acid was added to one of the duplicate tubes to release any residual CO_2. The cell pellets were washed twice with MMO and brought up in 400 µL of MMO after the final washing. Five samples were used

to determine the activity, and therefore the fraction of the injected glucose in each after completion of the short-term assay: supernatant, acidified supernatant, seed culture suspension, and the two CO_2 traps. From each sample, 100 µL was added to 400 µL of cocktail before determining the activity on the scintillation counter. The concentration of the glucose in the reaction vessel was calculated and using the standard curve, the total expected CPM was determined for the solution in the reaction vessel. This amount was used to determine the percentage of the total for each of the three fractions (supernatant, cells, and CO_2).

CHAPTER IV

RESULTS AND DISCUSSION

Carbohydrate and Protein Samples

The results of the BOD values obtained using both the five day and short-term BOD assays are shown graphically in figures 5-7 for the synthetic carbohydrate and protein samples. The BOD values shown are averages taken from tests performed on at least triplicate samples (see Appendix A). These data show that the short-term assay consistently underestimates the five day values by approximately one-half. There are several possibilities that might explain the lower BOD attained when using the fifteen minute method. The first and most obvious explanation is based on evidence that the BOD in the five day method is augmented by cellular turnover and autodigestion of newly generated cell mass (Chapter II). That is, during the first one to two days of the five day test, organics in the sample are rapidly metabolized and incorporated into cellular constituents. This leads to an increase in cell mass, which eventually will die and serves as a food source for other cells in the BOD bottle (Busch, 1958). At the end of the five day incubation period, the oxygen depletion that has occurred is a result of degradation of the sample substrate, as well as the degradation of newly formed cell mass.

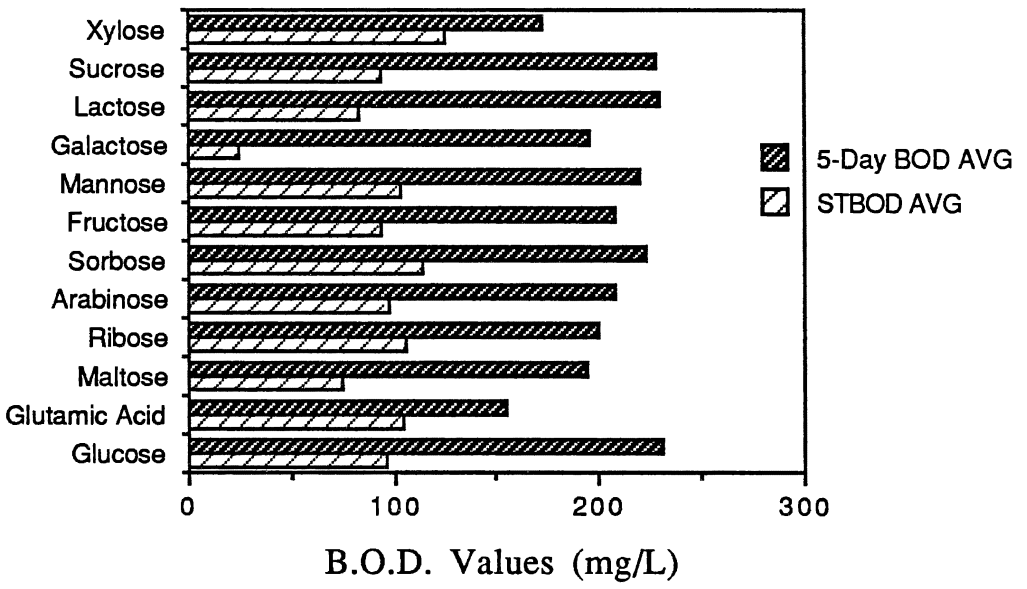

Figure 5. Comparison of Short-Term BOD Values to Standard BOD_5 Using Synthetic, One-Part Carbohydrate Samples (0.3 mg/mL).

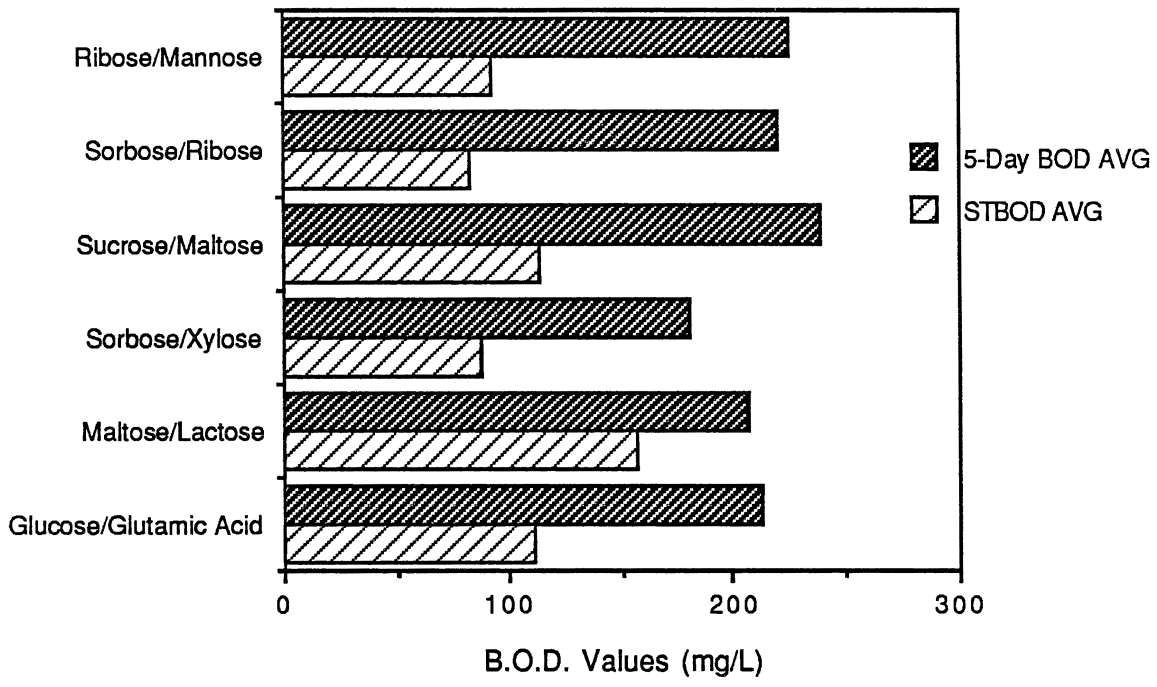

Figure 6. Comparison of Short-Term BOD Values to Standard BOD_5 Values Using Synthetic, Two-Part Carbohydrate Samples(0.3 mg/mL).

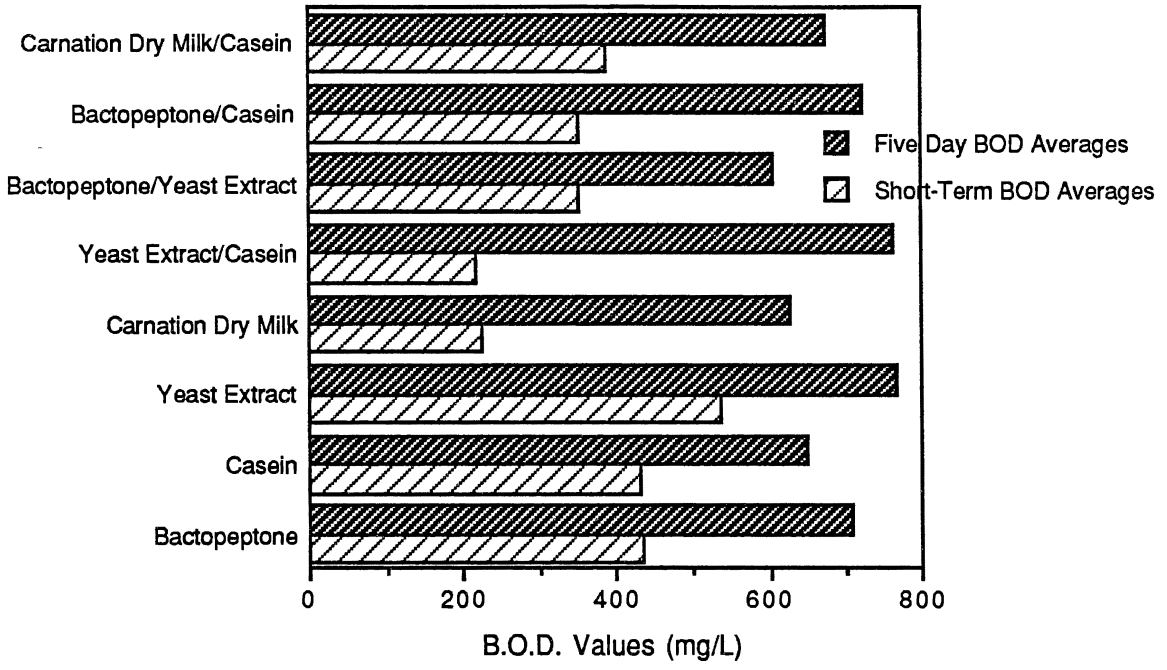

Figure 7. Comparison of Short-Term BOD Values to Standard BOD_5 Values Using Synthetic, Heterogeneous Protein Samples (1.0 mg/ml).

Some of this autodigestion can be accounted for by determining the oxygen consumption in a seed control (Chapter III), but due to the formation of new cells using the sample substrate, all of it cannot be totally accounted for. During a fifteen minute period (short-term assay), autodigestion and cellular turnover probably does not occur. Over this time frame, oxygen consumption will primarily be due to sample oxidation alone. An alternate explanation for the lower BOD obtained using the short-term assay may be that part of the sample is converted into cellular constituents rather than being entirely oxidized. During the first few days of the five day test, part of the sample is converted into new cellular constituents while the rest is used in energy producing reactions

required for cellular growth, and hence are fully oxidized to CO_2 and water. However, the portion of the sample that was converted into cell mass will eventually be oxidized, as these cells die and are used as a source of nutrients and energy by other cells in the bottle. Again, during a fifteen minute assay, sample that is converted into cellular constituents will probably remain in that state throughout the assay, as turnover of these products is not likely in this short period (The doubling time for *E. coli* under optimal nutrient conditions, and at a temperature of 37°C, is about 17 minutes. *Pseudomonas*, which would likely be one of the predominant organisms in a sewage sample, has a much greater doubling time, especially at a temperature of 20°C) (Pelczar, Chan, and Krieg, 1986). Finally, the bacterial cells may encounter some problems with substrate transport. The concentration of the sample after injection into the reaction chamber may be below that needed for efficient uptake into the cytosol. Therefore, a portion of the sample (up to 50%) may be left out in the supernatant. It is likely that several of these factors play a part in the resulting lower BOD values obtained using the short-term method. These questions will be addressed in a later study using ^{14}C-glucose as a sample substrate in the short-term assay to track the reaction products throughout the test..

Synthetic Fatty Acid Samples

The fatty acid samples must be addressed separately from the synthetic carbohydrate and protein samples, as no consistency was observed when the results from the short-term assay were compared to the five day values. Several of the short-term BOD values were actually higher than the five day values. Figure 8 graphically compares the BOD of the fatty acid samples using both methods. As in the previous graphs, the BOD values shown are averages

of at least triplicate assays for each sample (See Appendix A).

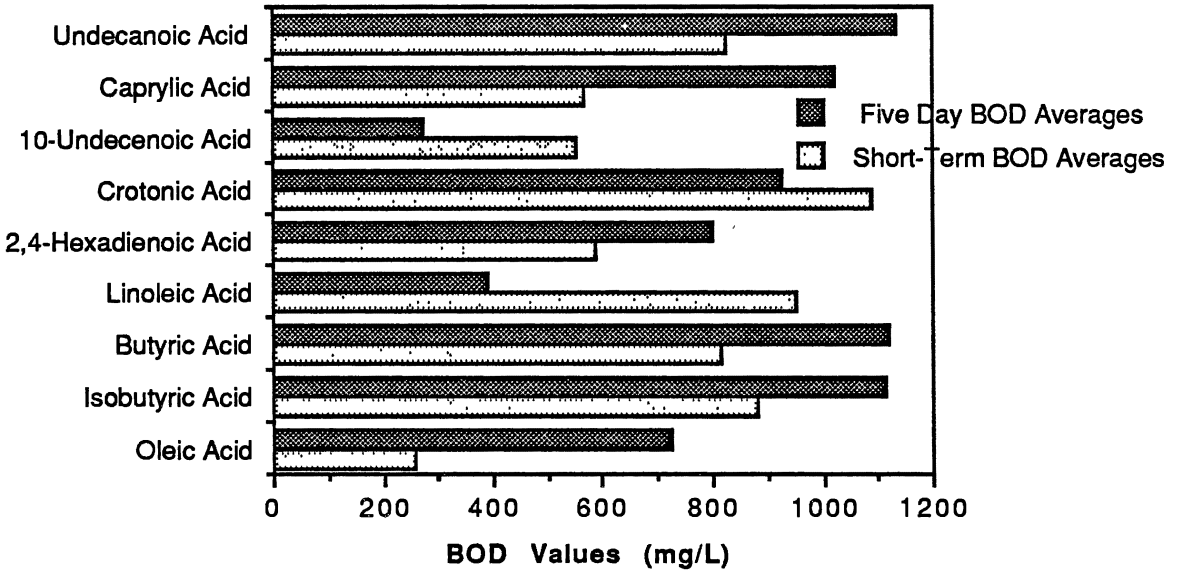

Figure 8 Comparison of Short-Term BOD Values to Standard BOD_5 Values Using Synthetic Fatty Acid Samples (1.0 mg/mL or 1.0 mL/mL).

The percent difference of the short-term assay compared to the five day method ranged from 64% less to 59% greater than the BOD_5. This inconsistency is probably related to the chemical nature of the sample substrate, *i.e.* its insolubility in water. As described in the materials and methods section, the fatty acid samples were prepared in a manner similar to that of the carbohydrate and protein samples, that is, a small amount of the fatty acid was added to water in order to dilute the substrate. When a portion was

withdrawn for both BOD assays, the sample was vigorously vortexed in an attempt to evenly distribute the fatty acid. Immediately after the vortexing was stopped, the fatty acid began to migrate to the top of the water. This created the possibility of withdrawing unrepresentative samples for both methods of analysis, since both required a high dilution. The standard deviation for both assays was unusually high, especially for the five day method, indicating that indeed, unrepresentative samples probably were the cause of the inconsistent BOD results.

Industrial Samples

In order to obtain a more realistic perspective on how the short-term assay would compare to the five day method when testing complex, real effluent samples, several types of wastewaters were collected from companies located in Oklahoma. The BOD values obtained using both the five day and the short-term assay for each of the industrial samples are shown in Figure 9. Short-term values are the averages of duplicate or triplicate tests, while five day values are averages of at least triplicate samples. Even more consistently than the synthetic sample results, the short-term BOD again underestimated the five day value by approximately one half. All of the percent differences except for one sample were within five percent of being exactly one half of the five day value.

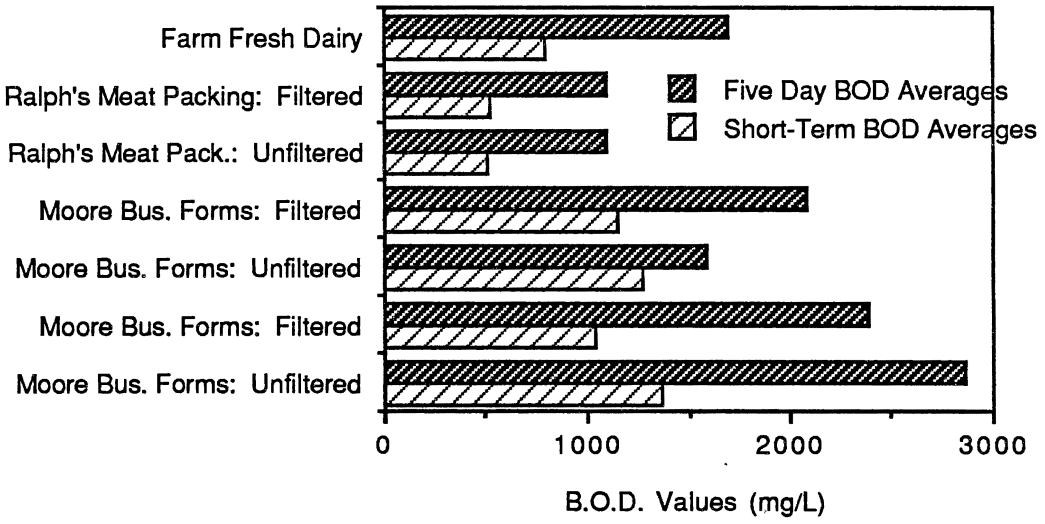

Figure 9. Comparison of Short-Term BOD Values to Standard BOD_5 Values Using Various Industrial Samples (see text for a complete description of samples).

Another objective of using the industrial samples was to determine the effects of particulate wastewater on the rapid assay . Both the Moore Business Forms sample and Ralph's Meat Packing contained particulate matter (see materials and methods for a description). For this reason, part of the samples were filtered to compare the BOD values of filtered versus unfiltered samples using both assays. The BOD of these samples was high, requiring a large dilution for the short-term assay as well as the five day method. This involved withdrawing a 5-10 µL portion for the short-term method and approximately 500 µL sample for the five day method to be diluted in 1800 µL and 300 mL respectively. Therefore, both methods were actually only measuring the soluble BOD since particulate materials were not withdrawn for either method. Referring to Figure 9 again, it can be seen that that there is no observable

difference between the BOD values of the filtered and nonfiltered samples. Most of the rapid automated and nonautomated BOD methods discussed in Chapter II also only measure the soluble BOD, since screens and filters are used to separate the effluent samples prior to the BOD analysis. In fact, the five day method is usually a measure of the soluble BOD for effluents requiring a high dilution since when withdrawing a small portion of sample to be tested, larger solid particles will not be removed. Therefore effluent samples containing particulates do not pose a problem for the short-term procedure.

Effects of Temperature and Optical Density of Seed Culture

The short-term assay was used to determine the BOD of a synthetic maltose sample at 20, 25 and 30 °C. Before this experiment, it was thought that increasing the temperature would shorten response time and increase slope of the oxygen uptake line, but not affect the BOD value. On some of the short-term graphs, return to original endogenous respiration did not occur, that is, the final baseline respiration rate was slightly higher than the original rate. In these instances, some estimation of the point of return to initial endogenous respiration rate had to be made. It was believed that an increase in temperature would eliminate the final baseline respiration rate tapering. However, as the temperature was increased, the apparent BOD increased proportionally (Figure 10). These preliminary results seem to indicate that between 20 and 30 °C a linear increase in the BOD occurs. Therefore in future studies using the short-term assay, it may be beneficial to increase the temperature of the short-term assay in order to obtain BOD values that are closer to the five day values.

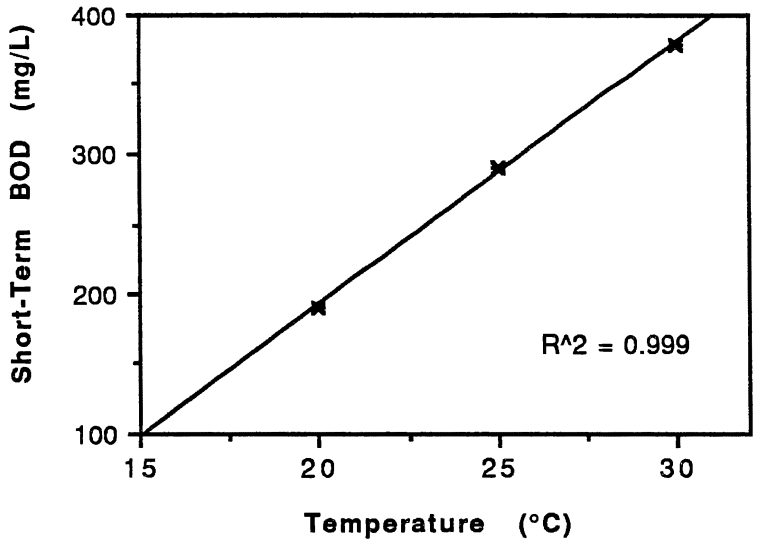

Figure 10. Temperature Versus Short-Term BOD Using a Synthetic Maltose Sample (0.75 mg/mL).

Three different densities of seed cultures were also used in a short-term BOD analysis of the synthetic maltose sample (0.75 mg/mL) to determine their effects on the short-term results (Figure 11). As the optical density is increased from 0.5 to 1.5 (425 nm) there is an increase in the short-term BOD (from approximately 170 to 240 mg/L). This suggests that in order to obtain maximum short-term BOD results, high density seed cultures should be used. The limited data suggest that BOD as a function of optical density will reach a maximum. The experiment could be extended over a broader range to determine the optimum inoculum density to maximize short-term BOD.

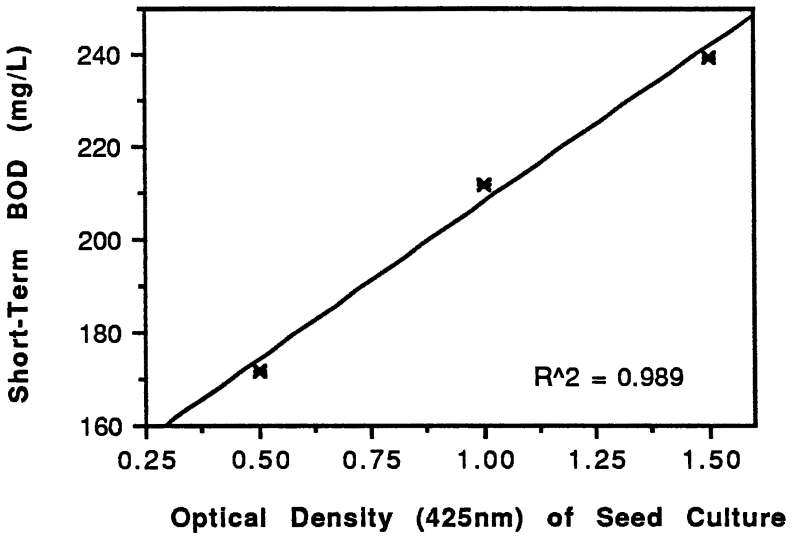

Figure 11. Short-Term BOD Versus Optical Density of Seed Culture Using a Synthetic Maltose Sample (0.75 mg/mL).

Sample Concentration and Dilution Variation

The next two experiments were done to show that the short-term assay was consistent when varying dilution factors and concentration of the same synthetic sample. In the former experiment, sample volumes of 10, 20, 30, 40 and 50 µL were used, representing dilutions ranging from approximately one-half to three percent. For the latter experiment, five synthetic maltose samples were made using concentrations of 0.25, 0.50, 0.75, 0.10, and 1.25 mg/mL of maltose to show that as the concentration of a sample increased, the BOD obtained using the short-term assay increased proportionally. The results shown in Figures 12 and 13 provide evidence that the short-term assay is consistent over a large dilution and concentration range for the same sample.

This is significant as there is ample evidence that the standard BOD_5 is not consistent when the BOD is determined using varying dilutions of the same sample (Chapter II).

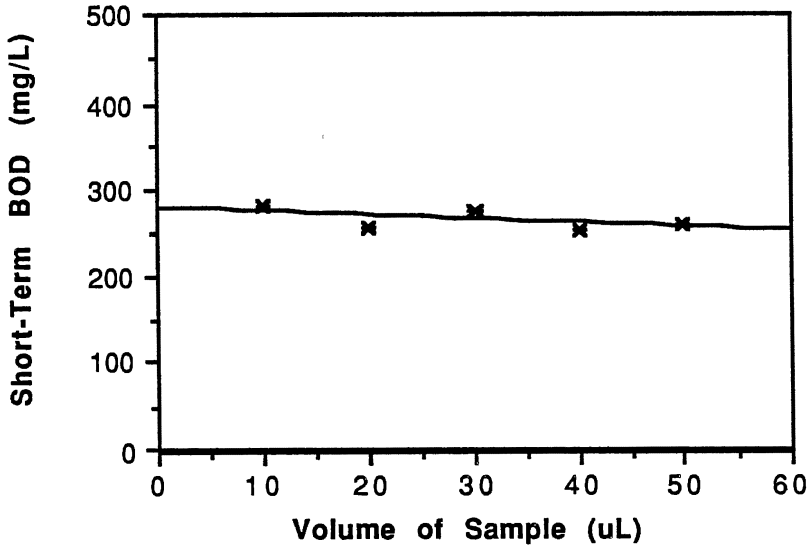

Figure 12. Short-Term BOD Versus Varying Sample Volumes (Dilutions) Using The Same Synthetic Maltose Sample (0.75 mg/mL).

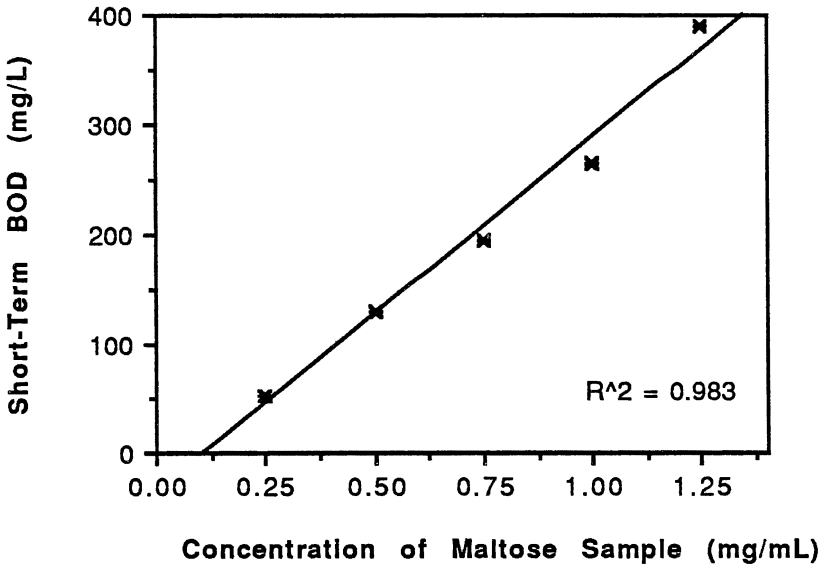

Figure 13. Short-Term BOD Versus Concentration of Sample Using a Synthetic Maltose Sample.

Short-Term BOD Analysis of ^{14}C-Glucose

Figure 14 shows the standard curve generated after converting the dilution series to molar concentrations and graphically comparing them to counts per minute (CPM) obtained using a scintillation counter. The total initial concentration of the glucose solution inside the reaction vessel was calculated based on the addition of a 75 μL sample of stock solution into a 1800 μL volume of seed culture. Therefore the concentration was 825.6 X 10^{-4} μmole/mL. Based on the standard curve, the activity at this concentration should be approximately 39,800 CPM. The counts for each of the three fractions were divided by the total possible activity and multiplied by 100 to calculate the percentage of the total for each fraction (TABLE III).

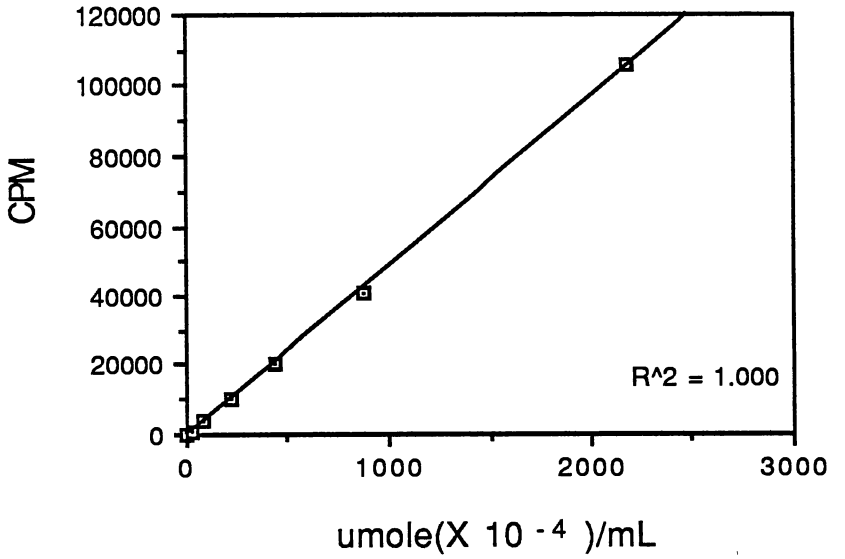

Figure 14. Standard Curve Relating CPM to Concentration For a ^{14}C-Glucose Stock Solution.

TABLE III

PERCENTAGE OF TOTAL GLUCOSE IN EACH FRACTION

	Trial #1			Trial #2	
	20 °C	30 °C		20 °C	30 °C
Supernatant	23%	24%		30%	47%
Cells	19%	31%		23%	12%
Carbon Dioxide*	58%	45%		47%	41%

* The total CO_2 activity was taken from the counts obtained from both of the CO_2 traps, from the difference between counts of the supernatant sample minus the acidified supernatant sample, and the inferred CO_2. The inferred CO_2 was calculated based on all unaccounted CO_2 (Total possible activity minus supernatant, cell, and CO_2 trap activities equals the inferred CO_2).

Based on the data given above, roughly one-half of the organic carbon is being converted into CO_2, while the other half is left within the cells or in the supernatant (Specific CPM data are given in Appendix B). The data suggest, as expected, that the low BOD values obtained using the proposed short-term assay are due to several factors. First, it seems that the cells cannot transport all of the substrate into the cytosol. Secondly, approximately one-eighth to one-fourth remains within the cells as storage products or cellular constituents. The data obtained on glucose at 30 °C, compared to the normal 20 °C temperature, did not support the data presented in the previous section using a synthetic maltose solution. In the previous section it was shown that the BOD_{ST} was almost double for several assays run at 30 °C compared to those run at 20 °C. Based on this evidence, it would be expected that the percentage of total carbon in the CO_2 fraction would increase accordingly. The results in Table III do not show an increase in the CO_2 fraction. In fact, it is slightly lower for the assays performed at the higher temperature even though the resulting BOD values were approximately 25% higher for both of the 30 °C assays. However, in Trial #2 at 30 °C there was a slight increase of total carbon in the cellular fraction. The data indicate that all of the sample substrate is not being metabolized, that some is left within the cells and a portion is left in the supernatant. The data for the temperature variations is somewhat contradictory. The resulting BOD values are higher when run at a higher temperature. Supposedly the higher BOD should result from more complete sample oxidation, but this is not accounted for in the scintillation results. Therefore, before definitive conclusions can be drawn for temperature variations, a more in depth study is necessary.

CHAPTER V

CONCLUDING REMARKS

The purpose of this study was to compare the proposed short-term BOD assay to the standard, EPA approved five day method, in terms of its precision and accuracy. A broad range of samples was tested, including several industrial samples, using both methods. The BOD values obtained when using the short-term assay were on the average about 50% lower than the five day BOD values. However, fatty acid samples did not show this type of consistency, with the short-term values ranging from 60% lower to 60% higher than the BOD_5. This was probably due to the immiscibility of these substrates. Difficulty in obtaining representative samples contributed to inconsistent results for both fatty acid BOD assays.

Table IV gives the results of the percent standard deviations calculated for each of the sample types, for both methods. The percent standard deviation was higher for the five day method in 70% of the samples tested (although 5 of the 28 samples that were higher for the five day method were within two percent of the short-term percent standard deviation). Based on this limited statistical analysis, the short-term assay appears to be slightly more reproducible compared to the five day method, if not at least as reproducible as the five day method. There have been numerous papers published concerning the lack of precision related to the five day method (CHAPTER II). One reason for this is the seed culture. Standard Methods does not include any specifications for the type or amount of seed culture that should be used for the five day method.

TABLE IV

COMPARISON OF PERCENT STANDARD DEVIATIONS FOR THE STANDARD BOD-5 AND SHORT-TERM ASSAYS

Sample	Short-Term Assay	Five Day Assay
Maltose	16	17
Ribose	6	12
Arabinose	9	10
Sorbose	11	18
Fructose	19	16
Mannose	5	9
Galactose	12	14
Sucrose	7	16
Lactose	5	18
Xylose	7	16
Glucose	1	9
Glutamic Acid	10	13
Glucose/Glutamate	7	10
Maltose/Lactose	15	15
Sorbose/Xylose	14	39
Sucrose/Maltose	3	14
Sorbose/Ribose	22	27
Ribose/Mannose	16	12
Galactose	20	14
Bactopeptone	3	12
Casein	7	9
Yeast Extract	7	10
Carnation Dry Milk	13	5
Yeast Extract/Casein	28	6
Bactopeptone/Y. Extract	10	7
Bactopeptone/Casein	9	7
Casein/Carn. Dry Milk	6	3
Undecanoic Acid	9	7
Caprylic Acid	7	36
10-Undecenoic Acid	13	29
Crotonic Acid	4	23
2,4-Hexadienoic Acid	18	25
Linoleic Acid	7	45
Butyric Acid	12	21
Isobutyric Acid	10	12
Oleic Acid	32	40
MBF-Unfiltered	*	*
MBF-Filtered	34	11
MBF-Unfiltered	*	*
MBF-Filtered	*	*
Ralph's Meat-Unfiltered	14	9
Ralph's Meat-Filtered	7	23
Farm Fresh Dairy	7	34

*Too few data points were available to calculate standard deviation.

Since it has been shown that varying seed cultures can significantly alter the results of the BOD_5, this is one probable explanation for the higher standard deviations obtained during this study. Since the proposed short-term BOD assay eliminates interferences due to variation in the types of seed cultures by accounting for the endogenous respiration, different types or activities of seed cultures should not affect the BOD_{ST} results.

Limited data are available for short-term assays performed on separate days using different seed cultures. If Figure 13 is used as a standard curve for any concentration of a maltose sample solution, it can then be predicted that the BOD_{ST} of a 0.3 mg/mL maltose solution should be approximately 70 mg/L. The BOD_{ST} that was determined for a 0.3 mg/mL maltose solution during the earlier work on carbohydrate samples was 62 mg/mL. These assays were done almost a year apart using different seed cultures. Based on this example, it seems that the reproducibility of the proposed short-term assay is unaffected by using different seed cultures. Again, however, more data is needed as the results shown in Figures 12 and 13 are not as similar. The BOD reported for a 0.75 mg/mL maltose sample is 250 mg/L in Figure 12, and aproximately 200 mg/L in Figure 13.

As a basis of comparison for the short-term results, as well as the five day BOD results, the theoretical Chemical Oxygen Demand (COD_{th}) was calculated for the synthetic monosaccharide samples. The COD_{th} for these samples (all of the single substrate carbohydrate samples except maltose, lactose and sucrose) is 320 mg/L. The short-term BOD values for these samples ranged from 94 - 124 mg/L, while the five day values ranged from 173 - 231 mg/L BOD. Therefore, just as the short-term method underestimates the five day assay, the BOD_5 is also an underestimate of the oxygen required for the complete oxidation of a sample.

The proposed short-term assay gives BOD results almost instantaneously after sample acquisition compared to a minimum of five days for the standard method. Consistent BOD_{ST} results were shown when varying dilutions and sample concentrations. It is known within a few minutes whether a sample dilution was done correctly, which results in fewer wasted tests than the standard method. The short-term method is not affected by improper dilution water, nitrification, or seed culture interferences, which have all been shown to alter BOD_5 results. The BOD_{ST} test can be done on any day of the week regardless of whether laboratory facilities are open on the weekend or holidays. The proposed short-term assay gives the operator some indication of the acclimation state of the seed culture. The slope of the oxygen consumption line in the presence of sample substrate gives an operator much more useable information than is routinely obtained from the five day method.

There are several questions that should be addressed in future studies. First, the effect of temperature on the short-term procedure needs to be examined in greater depth. Some of the data obtained in this study was contradictory concerning the effects of increasing temperature on the short-term results. Secondly, more comparisons need to be made on the short-term assays on separate days, using the same sample but varying the seed culture type, since this seems to be one of the main problems related to the precision of the BOD_5 method. Because the proposed short-term assay is proportional to the standard BOD_5, as well as repeatable for a particular sample, the short-term BOD assay should have practical applicability to real-time process control.

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APPENDICES

APPENDIX A

SHORT-TERM AND FIVE DAY BOD VALUES

Maltose

Date: 10/30/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
1.90	50.00	68.40		177.50
2.56	50.00	92.16		177.00
2.11	50.00	75.96		156.50
2.14	50.00	77.04		228.50
1.66	50.00	59.76		233.00
	Mean	74.66		194.50
	Std. Deviation	11.99		34.20

% Difference
61.61

Ribose

Date: 11/1/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.85	50.00	102.60		184.00
3.21	50.00	115.56		180.00
2.93	50.00	105.48		184.50
2.83	50.00	101.88		221.50
2.85	50.00	102.60		226.00
	Mean	105.62		199.20
	Std. Deviation	5.72		22.53

% Difference
46.98

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Arabinose

Date: 11/2/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.06	50.00	110.16		191.00
2.77	50.00	99.72		232.00
2.50	50.00	90.00		189.00
2.80	50.00	100.80		188.50
2.40	50.00	86.40		218.00
				224.00
	Mean	97.42		207.08
	Std. Deviation	9.43		19.78

% Difference
52.96

Sorbose

Date: 11/2/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.10	50.00	111.60		210.00
3.22	50.00	115.92		221.00
2.50	50.00	90.00		161.00
3.50	50.00	126.00		250.50
3.30	50.00	118.80		267.50
3.38	50.00	121.68		
	Mean	114.00		222.00
	Std. Deviation	12.74		41.07

% Difference
48.65

Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Fructose

Date: 11/14/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
1.95	50.00	70.20		234.50
3.20	50.00	115.20		169.00
2.30	50.00	82.80		171.50
2.68	50.00	96.48		227.00
2.88	50.00	103.68		235.00
	Mean	93.67		207.40
	Std. Deviation	17.61		34.07

%Difference
54.84

Mannose

Date: 11/15/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.98	50.00	107.28		210.50
2.85	50.00	102.60		221.50
2.63	50.00	94.68		191.00
2.94	50.00	105.84		236.50
2.97	50.00	106.92		241.00
	Mean	103.46		220.10
	Std. Deviation	5.25		20.29

%Difference
52.99

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Glucose

Date: 3/31/91	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.68	50.00	96.48		210.00
4.00	75.00	96.00		227.00
3.97	75.00	95.28		216.00
				237.50
				263.50
	Mean	95.92		230.80
	Std. Deviation	0.60		21.10

%Difference
58.44

Galactose

Date: 12/1/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
1.60	100.00	28.80		182.00
1.50	100.00	27.00		213.50
2.28	200.00	20.52		165.00
2.66	200.00	23.94		162.00
2.72	200.00	24.48		223.50
				227.00
	Mean	24.95		195.50
	Std. Deviation	3.16		26.88

%Difference
87.24

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Lactose

Date: 12/1/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.50	75.00	84.00		221.00
2.40	50.00	86.40		226.00
2.20	50.00	79.20		167.00
3.30	75.00	79.20		258.50
3.60	75.00	86.40		273.50
	Mean	83.04		229.20
	Std. Deviation	3.64		41.13

%Difference
63.77

Lyxose

Date: 11/27/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.63	75.00	87.12		No 5 Day Data
5.22	75.00	125.28		
5.10	75.00	122.40		
	Mean	111.60		
	Std. Deviation	21.25		

Not Available

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Sucrose

Date: 11/29/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.00	50.00	108.00		216.00
3.84	75.00	92.16		201.00
5.07	100.00	91.26		212.00
5.24	100.00	94.32		283.50
5.02	100.00	90.36		
4.97	100.00	89.46		
	Mean	94.26		228.13
	Std. Deviation	6.93		37.46

%Difference
58.68

Xylose

Date: 11/17/89	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.21	50.00	115.56		176.50
3.29	50.00	118.44		161.50
3.75	50.00	135.00		139.50
3.32	50.00	119.52		188.50
				201.50
	Mean	124.32		172.75
	Std. Deviation	9.26		27.73

%Difference
28.03

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Glucose/Glutamic Acid

Date: 1/12/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.84	75.00	116.16		203.50
4.76	75.00	114.24		200.00
4.79	75.00	114.96		245.00
4.44	75.00	106.49		201.00
2.98	50.00	107.10		
3.47	50.00	124.85		
4.34	75.00	104.16		
4.16	75.00	99.84		
	Mean	110.97		212.38
	Std. Deviation	8.03		21.80

%Difference
47.75

Maltose/Lactose

Date: 1/4/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.66	50.00	167.76		185.50
3.51	50.00	126.36		221.50
3.80	50.00	136.80		168.00
4.80	50.00	172.80		184.50
4.18	50.00	150.48		238.50
5.16	50.00	185.76		245.50
	Mean	156.66		207.25
	Std. Deviation	22.72		32.17

%Difference
24.41

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Sorbose/Xylose

Date: 1/4/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.70	100.00	84.60		177.50
5.51	100.00	99.18		207.50
2.96	75.00	71.04		83.50
5.16	100.00	92.88		113.00
				267.50
				234.50
	Mean	86.93		180.58
	Std. Deviation	12.16		70.98

%Difference
51.86

Sucrose/Maltose

Date: 1/8/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.82	75.00	115.68		217.50
4.77	75.00	114.48		233.00
4.50	75.00	108.00		198.00
4.80	75.00	115.20		270.00
3.11	50.00	111.96		275.50
	Mean	113.06		238.80
	Std. Deviation	3.17		33.44

%Difference
52.65

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Sorbose/Ribose

Date: 1/11/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.91	60.00	87.30		174.50
3.53	75.00	84.72		175.00
3.91	75.00	93.84		186.50
2.36	50.00	84.96		262.50
1.87	50.00	67.32		303.00
4.84	75.00	116.16		
2.51	75.00	60.24		
2.85	75.00	68.40		
	Mean	82.87		220.30
	Std. Deviation	17.81		58.98

%Difference
62.38

Ribose/Mannose

Date: 1/14/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.42	100.00	97.56		193.50
2.04	50.00	73.44		234.00
5.14	100.00	92.52		201.00
4.53	75.00	108.72		207.50
				252.50
				257.50
	Mean	93.06		224.33
	Std. Deviation	14.73		27.44

%Difference
58.52

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Glutamic Acid

Date: 1/12/90	Concentration of Sample: 0.3 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.18	75.00	100.37		139.00
2.65	50.00	95.40		143.50
4.28	75.00	102.60		150.00
4.09	75.00	98.16		184.00
3.38	50.00	121.68		
3.58	50.00	128.88		
	Mean	103.64		154.13
	Std. Deviation	10.43		20.42

% Difference
32.75

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Bactopeptone

Date: 5/24/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.90	20.00	441.00		622.50
5.08	20.00	457.20		657.00
3.58	15.00	429.60		672.00
3.48	15.00	417.60		796.50
4.80	20.00	432.00		795.00
	Mean	435.48		708.60
	Std. Deviation	14.74		81.56

% Difference
38.54

Casein

Date: 5/29/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.11	20.00	459.90		681.00
3.82	15.00	458.40		681.00
2.18	10.00	392.40		714.00
4.63	20.00	416.70		582.00
4.70	20.00	423.00		594.00
	Mean	430.08		650.40
	Std. Deviation	28.90		58.69

% Difference
33.87

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Yeast Extract

Date: 5/23/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.98	10.00	536.40		654.00
5.74	20.00	516.60		792.00
5.50	20.00	495.00		790.00
4.90	15.00	588.00		816.00
	Mean	534.00		763.00
	Std. Deviation	39.77		73.62

% Difference
30.01

Carnation Dry Milk

Date: 6/1/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.93	20.00	263.70		648.00
3.18	25.00	228.96		622.50
2.43	20.00	218.70		595.50
2.68	25.00	192.96		667.50
				604.50
	Mean	226.08		627.60
	Std. Deviation	29.30		29.99

% Difference
63.98

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Yeast Extract/Casein

Date: 6/18/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.27	50.00	189.72		730.50
5.26	50.00	189.36		814.50
4.20	35.00	216.00		715.50
3.78	20.00	340.20		727.50
5.26	50.00	189.36		808.50
5.17	50.00	186.12		
	Mean	218.46		759.30
	Std. Deviation	60.65		48.03

% Difference
71.23

Bactopeptone/Yeast Extract

Date: 6/20/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.10	25.00	367.20		660.00
4.36	25.00	313.92		634.50
5.47	30.00	328.20		543.00
4.48	20.00	403.20		606.00
4.81	25.00	346.32		594.00
	Mean	351.77		607.50
	Std. Deviation	35.00		44.24

% Difference
42.10

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Bactopeptone/Casein

Date: 6/19/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.22	25.00	375.84		735.00
4.80	25.00	345.60		738.00
4.45	25.00	320.40		631.50
4.32	25.00	311.04		763.50
6.20	30.00	372.00		744.00
5.32	25.00	383.04		
	Mean	351.32		722.40
	Std. Deviation	30.48		52.01

% Difference
51.37

Casein/Carnation Dry Milk

Date: 6/19/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.98	25.00	430.56		688.50
4.20	20.00	378.00		666.00
5.38	25.00	387.36		675.00
3.98	20.00	358.20		693.00
5.35	25.00	385.20		651.00
5.10	25.00	367.20		
4.50	20.00	405.00		
	Mean	387.36		674.70
	Std. Deviation	24.24		17.05

% Difference
42.59

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Undecanoic Acid

Date:6/13/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.43	10.00	797.40		1122.00
5.25	10.00	945.00		1242.00
4.90	10.00	882.00		1173.00
4.26	10.00	766.80		1092.00
4.50	10.00	810.00		1041.00
4.20	10.00	756.00		
	Mean	826.20		1134.00
	Std. Deviation	72.22		77.04

% Difference
27.14

Caprylic Acid

Date: 5/10/90	Concentration of Sample: 1.0 ul/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.51	10.00	631.80		627.00
4.81	15.00	577.20		610.50
6.10	20.00	549.00		1020.00
6.10	20.00	549.00		1012.50
5.96	20.00	536.40		1521.00
				1350.00
	Mean	568.68		1023.50
	Std. Deviation	38.32		369.33

% Difference
44.44

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

10-Undecenoic Acid

Date: 6/14/90	Concentration of Sample: 1.0 ml/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.38	20.00	484.20		381.00
6.60	20.00	594.00		277.50
5.50	20.00	495.00		202.00
3.78	10.00	680.40		233.00
5.53	20.00	497.70		
5.90	20.00	531.00		
6.32	20.00	568.80		
	Mean	550.16		273.38
	Std. Deviation	70.41		78.16

%Difference
-50.31

Crotonic Acid

Date: 6/5/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.08	5.00	1108.80		981.00
5.97	10.00	1074.60		979.50
5.52	10.00	993.60		687.00
6.20	10.00	1116.00		675.00
6.30	10.00	1134.00		980.00
5.97	10.00	1074.60		1248.00
6.25	10.00	1125.00		
	Mean	1089.51		925.08
	Std. Deviation	48.23		215.69

%Difference
-15.09

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

2,4-Hexadienoic Acid

Date: 5/30/90	Concentration of Sample: 1.0 mg/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.18	10.00	752.40		798.00
3.43	10.00	617.40		718.50
2.80	10.00	504.00		633.00
3.10	10.00	558.00		586.50
2.52	10.00	453.60		1071.00
5.40	15.00	648.00		1006.00
	Mean	588.90		802.17
	Std. Deviation	107.24		197.97

% Difference
26.59

Linoleic Acid

Date: 5/21/90	Concentration of Sample: 1.0 ul/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.22	6.00	966.00		564.00
4.53	8.00	1019.25		417.00
3.80	8.00	855.00		628.50
5.60	10.00	1008.00		282.00
5.13	10.00	923.40		255.00
				201.00
	Mean	954.33		391.25
	Std. Deviation	67.18		175.21

%Difference
-59.00

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Butyric Acid

Date: 6/18/90	Concentration of Sample: 1.0 ul/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.11	10.00	919.80		948.00
4.92	10.00	885.60		826.50
4.73	10.00	851.40		1352.00
4.20	10.00	756.00		1348.00
3.75	10.00	675.00		1269.00
				960.00
	Mean	817.56		1117.25
	Std. Deviation	100.42		232.07

% Difference
26.82

Isobutyric Acid

Date: 5/31/90	Concentration of Sample: 1.0 ul/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.15	10.00	747.00		1017.00
4.98	10.00	896.40		922.50
5.23	10.00	941.40		1302.00
5.22	10.00	939.60		1210.00
				1122.00
				1101.00
	Mean	881.10		1112.42
	Std. Deviation	91.79		134.80

% Difference
20.79

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Oleic Acid

Date: 6/18/90	Concentration of Sample: 1.0 ul/mL			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.05	30.00	183.00		1173.00
3.93	40.00	176.85		492.00
4.70	40.00	211.50		444.00
4.38	40.00	197.10		718.00
1.18	10.00	212.40		790.00
4.85	40.00	218.25		
6.06	40.00	272.70		
4.78	40.00	215.10		
5.20	40.00	234.00		
2.52	20.00	226.80		
5.81	40.00	261.45		
6.36	30.00	381.60		
6.00	25.00	432.00		
4.42	20.00	397.80		
	Mean	258.61		723.40
	Std. Deviation	83.41		290.78

% Difference
64.25

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Moore Business Forms

Date:8/1/90	Sample: Unfiltered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.92	7.00	1522.29		3193.30
3.36	5.00	1209.60		2801.20
				2576.00
	Mean	1365.94		2856.83
	Std. Deviation	221.10		312.39

% Difference
52.19

Moore Business Forms

Date: 8/1/90	Sample: Filtered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
4.02	5.00	1447.20		2665.00
2.15	5.00	774.00		2319.30
5.00	10.00	900.00		2164.00
	Mean	1040.40		2382.77
	Std. Deviation	357.89		256.46

% Difference
56.34

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Moore Business Forms

Date: 8/23/90	Sample: Unfiltered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.74	5.00	1346.40		1596.00
3.34	5.00	1202.40		1566.00
				1612.00
	Mean	1274.40		1591.33
	Std. Deviation	101.82		23.35

% Difference
19.92

Moore Business Forms

Date: 8/23/90	Sample: Filtered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
2.87	5.00	1033.20		2196.00
3.54	5.00	1274.40		1740.00
				2304.00
	Mean	1153.80		2080.00
	Std. Deviation	170.55		299.36

% Difference
44.53

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Ralph's Meat Packing

Date: 8/22/90	Sample: Unfiltered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
5.95	25.00	428.40		996.00
4.40	15.00	528.00		1194.00
6.28	20.00	565.20		1084.00
	Mean	507.20		1091.33
	Std. Deviation	70.73		99.20

% Difference
53.52

Ralph's Meat Packing

Date: 8/22/90	Sample: Filtered			
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
6.10	20.00	549.00		900.00
2.65	10.00	477.00		1014.00
2.87	10.00	516.60		1376.00
	Mean	514.20		1096.67
	Std. Deviation	36.06		248.53

% Difference
53.11

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

Farm Fresh Dairy

Date: 9/19/90				
Oxygen Consumed*	Sample Volume*	BOD-ST*		Five Day BOD*
3.73	8.00	839.25		2172.00
3.30	8.00	742.50		756.00
3.32	8.00	747.00		1800.00
3.76	8.00	846.00		1260.00
				2168.00
				2060.00
	Mean	793.69		1702.67
	Std. Deviation	56.61		577.25

% Difference
53.39

*Oxygen concentrations and BOD values are given in ppm, sample volumes are in microliters.

APPENDIX B

^{14}C-GLUCOSE EXPERIMENT

^{14}C- Glucose Standard Curve Data

Dilution^{-1}	Concentration (μCi X 10^{-4})	Concentration (μmole X 10^{-4})	Average CPM
10	5000	2174.00	105964
25	2000	870.00	40891
50	1000	435.00	20140
100	500	217 40	10476
250	200	87.00	4129
1000	50	21.74	1010
5000	10	4.35	207
10000	5	2.17	125

^{14}C-Glucose Scintillation Data for Short-Term BOD Assay

Fraction	Trial #1: Average CPM @ 20°C	Average CPM @ 30°C
Supernatant	12983	13868
Acidified Supernatant	9131	9386
Cells	7593	12505
CO2 #1	4150	2433
CO2 #2	835	285

Fraction	Trial #2: Average CPM @ 20°C	Average CPM @ 30°C
Supernatant	14923	25672
Acidified Supernatant	12019	18710
Cells	9218	4616
CO2 #1	1735	6902
CO2 #2	34	22

VITA

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Master of Science

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