PHYSIOLOGICAL ACTIVITY OF BACTERIA INDIGENOUS TO A LAGOON STABILIZING DOMESTIC WASTES

by

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GENERAL INTRODUCTION

The necessity of some method for the disposal of industrial and domestic wastes from congested urban centers is obvious. To be satisfactory any method of wastes disposal must result in the transformation of putrescible, organic wastes into stable compounds and at the same time eliminate the possibility of disease dissemination therefrom. The more efficiently and economically these ends can be achieved, the more satisfactory is the method.

Activated sludge and trickling filter units have been and still are extensively used for the treatment of domestic and industrial wastes. In recent years, lagoons have become very popular as a means of conditioning industrial and domestic wastes before any final innocuous disposal and their special features as a method for wastes disposal have already been discussed (25, 24, 76, 163, 200, 206). But lagoon design is presently being formulated on an empirical basis. While bacteria are the principal agents responsible for stabilizing the organic wastes introduced into lagoons, or into any wastes treatment facility for that matter, most reports on the function and efficiency of lagoons deal almost exclusively with engineering and technical aspects and little attention has been devoted to the microbial processes invoked by this method for wastes disposal. Because very little information is available on the biological activity of lagoon microflora,

this study was begun to elucidate some aspects of the ecology of the bacteria indigenous to a domestic wastes disposal unit (sewage lagoon) operating under climatic extremes, with emphasis upon the physical factors which control the biodegradation of various organic compounds that might be introduced into the lagoon and to correlate these results with the potential capacity of the lagoon to deal effectively with wastes disposal as a function of seasonal change.

HISTORICAL

Only within the past century have better methods been sought for the controlled disposal of man's domestic and industrial wastes. The most widely used processes include activated sludge, trickling filters, and stabilization ponds or lagoons.

The activated sludge process, developed around 1913 in England (132) is still a very popular wastewater treatment process. An activated sludge unit consists of two tanks in series; the first, an aeration tank, is followed by the second, a sedimentation tank. The wastes enter the aeration tank where diffused aeration provides dissolved oxygen and promotes mixing of the wastes. Here the microorganisms grow in large numbers and stabilize readily decomposable organic compounds. The wastes then flow into the sedimentation tank where micro-organisms degrade the resistant decomposable organic substances which have escaped from the aeration tank. Finally the organisms agglomerate with each other and with inert suspended solids forming aggregates which settle out in the sedimentation tank, producing a clear effluent. The process of agglomeration is called 'flocculation'. The suspended solids in the aeration tank, including organisms and suspended material from the wastes, are collectively known as 'activated sludge' (159). The settled material known as 'settled sludge' is biologically active, a part of which is returned to the aeration tank as

a seed inoculum and the remainder of which is discarded.

The capacity to purify wastes is usually estimated by comparing the Biochemical Oxygen Demand (BOD) of the influent and effluent. Biochemical Oxygen Demand is defined as the amount of oxygen required by bacteria while stabilizing decomposable organic compounds at 20 °C in 5 days. It is essential, therefore, that the sludge be separated from the mixed liquor before discharge because the organisms derived from the sludge would greatly increase the BOD of the effluent.

There are some problems associated with the activated sludge process. To function properly, the process is dependent upon an adequate supply of oxygen. The treatment efficiency is decreased by an inadequate amount of dissolved oxygen (70, 159) and oxygen is supplied to the activated sludge unit by air diffusion devices. The disposal of the excess microbial sludge may be troublesome. Often, it is disposed of by drying on sand beds and the dried residue is burned or used as a fertilizer. Many small communities utilize lagoons as a supplementary means for sludge disposal when drying beds are inadequate (156) but their use has been discouraged because of odor problems (219). The temperature and pH are important regulating factors for successful operation. Between pH 6.0 and 9.0, an activated sludge unit operates properly; above pH 9.0 or below pH 6.0 the efficiency is markedly reduced (94). Similarly, peak efficiency is achieved when the temperature

is between 25 °C and 30 °C (93). The temperature and pH, however, are not controlled by technical means and only rarely is their influence deleterious.

The main objection to the activated sludge process is its lack of stability. It is probably the hardest wastes treatment process to regulate especially if sudden changes occur in the concentration, composition, or flow rate of wastes. Activated sludge units are most suitable for large cities where only minor variations in the flow and the composition of wastes are experienced.

The activated sludge process has been largely ignored by the applied microbiologist and most research on this wastes treatment process has been conducted by either chemists or engineers. Wuhrmann (217) stated "There has been, and is still, too much engineering and too little microbiology in this field of environmental sanitation". Considerable research has been carried out to demonstrate the utilization by activated sludge of various organic compounds such as carbohydrates (49, 52, 53, 54, 81), amino acids (27), other nitrogen containing organic compounds (119, 120, 121, 197), and many aliphatic and aromatic compounds (30, 123). Investigators have tried to relate their data to the design and operation of activated sludge units but with little success (38, 62, 127, 128). A study of the ecological factors which control the microbial population should provide useful information in

improving the design and operation of activated sludge units but a great deal of work needs to be done before these improvements will be forthcoming.

Trickling filters are another type of widely used aerobic biological wastes treatment system. A trickling filter consists of a bed or tower of rough, hard particles approximately 1 1/2 to 2 1/2 in in diameter, normally encased in circular or rectangular units. Waste materials are uniformally distributed at the surface of the bed or tower by means of rotary or fixed distributors and percolate by gravity as a thin layer over the supporting structure. Organic substances such as carbohydrates, proteins, and fats are adsorbed and oxidized by aerobic bacteria coating the support. The amount of organic pollutant adsorbed and oxidized is measured as per cent BOD removed from the system.

For the oxidation process to be carried on, a continuous supply of oxygen is necessary. Oxygen is absorbed from the air and is transferred from the surface layer to the lower regions by percolation of oxygen rich solutions. To increase the efficiency of oxygen transfer, diffusion devices may also be employed. Underdrain channels may be interspaced in the trickling filter and are designed to carry a maximum quantity of air and waste materials for efficient operation (176). The microorganisms responsible for biodegradation are sensitive to changes in temperature and pH; the peak efficiency is

achieved around 28 °C (78) and at a pH of about 8.0 (207).

Not all the organic material contained in the waste is completely oxidized. Part of it is converted into new growth which tends to increase the thickness of the biological film. Normally, the excess growth is sloughed off and carried out in the filter effluent. If the portion retained in the trickling filter accumulates to an extent that it blocks the air flow, the condition is known as 'ponding'. The ponded part of the filter reduces the oxygen supply and the net result is a reduction in the efficiency of the filter proportional to the extent of ponding (176).

Initially, sand was used as the support but the need for greater loadings per unit volume prompted the use of gravel, small stones, coal, tile, and rocks. In recent years, preformed plastic media are being used (56, 142) to overcome the problems of clogging, high loading, high construction costs, and resistance to air flow commonly associated with other media.

A large number of modifications to trickling filters (42, 64, 184) have been proposed during the past few years but few have progressed beyond the experimental phase. Currently, the design and operation of trickling filters is based on field experience rather than on experimental data. To make further improvements in the design of trickling filters, research work pertaining to the biological aspects is needed.

The newest treatment process is the 'stabilization pond' or 'lagoon'. Some terms, such as oxidation pond, waste stabilization pond and lagoon are used interchangeably and will frequently be used. The "Glossary-Water and Sewage Control Engineering" (59) defines a waste stabilization pond as "a basin, natural or artificial, designed or used to treat organic wastes by natural, biological, biochemical and physical processes, commonly referred to as 'self purification'".

The wastes undergoing stabilization are conducted into the lagoon where settleable solids together with some suspended and colloidal particles settle to the bottom. Bacteria oxidize the settled organic matter and soluble organic compounds with the production of bacterial protoplasm, carbon dioxide, and water as the principal end products of metabolism.

Unlike other stabilization processes algae play an important role in the proper functioning of the majority of lagoons. Algae utilize the inorganic nutrients in wastes and carbon dioxide produced by the bacteria and, through the photosynthetic process, produce oxygen which is used by bacteria for the stabilization of remaining organic matter. Atmospheric oxygen also enters the lagoon as dissolved oxygen at the water-air interface but, without the aid of mechanical means, dissolution is a slow process. In a conventional lagoon, the most important method of oxygenation is through the photosynthetic activity of algae. A continuous cycle of

symbiotic bacterial-algae interaction takes place when sunlight furnishes the energy for photosynthesis and wind accomplishes the distribution of dissolved oxygen and essential nutrients.

In geographic regions which experience severe climatic conditions, a lagoon may be covered with ice and snow for several months of the year. Here, the ice-cover cuts off the supply of oxygen in the air and sunlight is reduced to the point where algal activity is completely inhibited. Under these conditions a lagoon quickly becomes anaerobic. When the ice disappears during late spring because of an increase in atmospheric temperature the transition from anaerobiosis to aerobiosis generally occurs in a matter of days.

Lagoons are usually operated at a depth of 3 to 5 ft and the retention period, i.e., the time required for wastes to pass through the lagoon, varies approximately from 3 weeks to 4 months depending upon the nature and volume of the wastes, climatic conditions, public health regulations, etc.

The concept of impoundment for raw sewage in lagoons was applied in Asia many centuries ago (45). During the early 1900's, lagooning of vegetable canning wastes was reported where lagoons were used primarily as seepage, settling, or holding basins (162). The first lagoon constructed specifically to treat domestic wastes was used in the United States, in California, in 1924 (25). The climatic conditions of this

geographic region are eminently suitable for a high efficiency of BOD removal throughout the year. In 1928, a lagoon was installed in Fesenden, North Dakota, and even though this mid-continental lagoon located at latitude 48° is susceptible to partial freezing for short periods of the year, after 40 years it is still operating successfully. Subsequently, the construction and operation of a properly but empirically designed lagoon in 1948 at Maddock, North Dakota, for the stabilization of raw sewage (200) led to the concept that lagooning as a method of treating domestic wastes could be applied successfully in spite of severe climatic conditions during portions of the year. The success of these early installations attracted much attention in the neighboring states and the use of lagoons spread rapidly throughout the mid-west area. Results of early installations in North Dakota were so encouraging that the North Dakota State Health Department recommended the use of lagoons for the disposal of domestic wastes for all small communities (205). A 10 acre lagoon with an effective depth of 3 to 5 ft and a retention time of approximately 120 days was recommended for cities of about 1,000 population. Successful operation of the lagoon at Maddock (200) was shown by 65 and 95% BOD removal respectively in winter and summer.

Following the lead of North Dakota, a large number of lagoons were installed in South Dakota. The first installation

serving a South Dakota community was placed into operation at Lemmon in 1951 and is still operating successfully (26). Extensive field studies of lagoons in both North and South Dakota were made by the Public Health Service, North Dakota Department of Health during 1955-1956. A summary of the report on the performance of five installations studied (177) contains the following statement: "Treatment obtained during both open water and ice cover is very good. Reduction in concentration of BOD ranged from 74 to 98 % during open water seasons and from 70 to 96 % under ice". The later figures, however, appear subject to scrutiny.

Although lagoons are becoming very common on the landscape, very little is known about the design criteria for the construction of efficient lagoons. Most lagoons are designed to dispose of an arbitrarily selected amount of BOD per acre per day. The recommended loading rate varies from 20 to 30 pounds per acre per day, with some lagoons reaching 50 pounds per acre per day (132). Many workers have proposed design criteria for the construction of lagoons (26, 72, 161) which include (a) land availability, (b) location, (c) quantity of waste, (d) type of waste, (e) loading per unit of surface area, (f) chemical quality of the water supply, (g) sunlight, (h) temperature, (i) depth, (j) inlet and outlet structures, and (k) cost. Currently, lagoons are constructed entirely on the basis of field experience. Much research is needed to understand the

biological changes invoked by the lagooning process before better design criteria can be established.

Lagoons may also be used in series. One or more cells, known as primary cells, are arranged in parallel and are integrated with one or more cells, called secondary cells, arranged in parallel; the primary and secondary cells are arranged in series. The wastes are first discharged into the primary cell where sedimentation of settleable solids and partial BOD removal is accomplished. The partially stabilized wastes materials are then conducted into the secondary cells for final stabilization of slowly decomposable organic compounds. Another purpose of lagoon operation in series is to effect better circulation and aeration of the wastes by successively flowing the effluent from one cell to the next.

The use of a 'series pond system' has been employed in many installations. On the basis of data collected from various lagoons treating domestic wastes in Texas,Meyers (141) recommended the use of lagoons in series at a depth of 3 to 6 ft and a BOD load of 40 pounds per acre per day. The purification of wastes by primary settling was stressed. Parker et al.(155) and Porter and Bishop (164) also advocated the use of dual cells in series. The disposal of industrial wastes in five cells in series was described by Neel et al (148). The cells were maintained at a depth of 2.5 ft and the BOD loads were 75, 60, 45, 30, and 15 pounds per acre per day.

Their studies showed that more than 80% of the BOD was removed throughout the year. The choice between the use of a single cell or multiple cells in series depends on local conditions, size of installation, type and volume of the waste to be treated, and other general conditions. The advantage of the series lagoons is realized only when the organic load is raised considerably above the recommended load. For small communities where no possibility of overloading exists, single unit lagoons have proven to be adequate.

Some workers have described the use of anaerobic lagoons. These are lagoons of considerable depth, up to 12 ft for example, only the top portion of which is oxygenated. Parker (152) and his associates (153, 154) claimed 70 to 87% BOD reduction in anaerobic lagoons treating domestic wastes in 2 to 3 days at about 20 °C. Stanley (187) claimed 70 to 75% BOD removal during both summer and winter in anaerobic lagoons having a retention time of 3 to 6 days. These high BOD removals in anaerobic lagoons are questionable. Bacteria rapidly oxidize readily utilizable organic materials with the production of bacterial protoplasm and the excess microbial growth and resistant organic materials are still retained in wastes under the conditions imposed for a BOD test. The wastes, therefore, become an unsuitable substrate for the BOD test and the efficiency of anaerobic lagoons described above should

be viewed with reservation. Parker (152) reported that few algae were found in anaerobic lagoons and stabilization of wastes was due to bacterial activity. The short term retention in anaerobic lagoons does not achieve a substantial reduction in bacterial numbers and it was recommended (152, 187) that anaerobic lagoons be followed by aerobic lagoons for bacterial removal.

Lagoons can be used following other treatment units to produce a more polished effluent. The use of a lagoon to treat the effluent from an activated sludge unit (44), a trickling filter unit (8, 61), and a septic tank (57) has been reported. McKinney (133) reported that many refineries utilize lagoons as polishing units.

The presence of coliforms in a water supply is used as an index of water pollution. Lagoons, however, have proven to be very effective in reducing the number of coliforms and several workers have reported the reduction of these organisms from 95 to 99% (25, 55, 58, 77, 107, 137, 138, 148, 200, 201). Caldwell (25) reported that the efficiency of stabilization ponds in removing coliforms was probably due to liberation of substances toxic to bacteria by the algae. However, Oswald and Gotaas (150) stated that no specific anticoliform activity can be credited to the algae. Higgins (75) reported that more likely coliforms and pathogenic bacteria cannot compete effectively for nutrient materials with the prodigious

flora and fauna of the lagoon.

In spite of the wide acceptance and effectiveness of lagoons, some workers have objected to their use. Smith and Leibee (179) made a survey of some lagoons used for the treatment of domestic wastes and concluded that there is a danger of the spread of diseases, particularly sewage-borne virus diseases, both by wind action or by birds. McAnulty (126) intimated that lagoons were responsible for an increased incidence of viral encephalitis diseases. Higgins (76) discussed the life history of this virus and proved that since it is not an enteric virus, its dissemination by lagoons was not possible. Kapee (90) reported that the discharge from lagoons contained abundant quantities of algae which accumulated and died in stream beds killing fish and aquatic life. Moreover, dead algae and weeds as well as other organic compounds produced putrescible toxic and odor producing compounds on decomposition. He also claimed that lagoons were effective only during sunlight hours.

Valid criticisms have been made concerning the lack of maintenance on lagoons (90, 126), and the general concept that lagoons require no attention once they have been put into operation is false. Babbitt (9) stated "The septic tank fell into disrepute because it was abused and neglected with resulting failure and nuisance. Let us not permit the stabilization pond to fall into the same category".

Unfavourable weather conditions reduce the purification capacity of lagoons and they may develop odors or other undesirable conditions. A review of stabilization pond practice with special attention to severe climatic conditions was given by Kalda (88). Ponds covered with ice restrain insoluble gases produced from the retarded anaerobic processes (26, 57, 72, 115, 134, 163) and lagoons may emit unpleasant odors for a period after spring breakup of ice in spite of proper maintenance (88, 134).

One promising solution to the odor problem is to aerate lagoons over the entire year. Aerated lagoons are created by introducing air into conventional lagoons by means of some mechanical or diffusion device. At present insufficient data are available to compare the operational characteristics of mechanical and diffused aeration systems. The primary purpose of aerating a lagoon is to increase the capacity of the unit. The first artificial aerated lagoon treating industrial wastes was described by Rice and Weston (167). An average of 55% BOD removal was obtained with a retention time of 3 days. Amberg reported that aeration for 4 days produced a BOD reduction in wastes in excess of 90% while the BOD reduction in unaerated wastes was reduced only 40% in 14 days (6). He also found that aeration increased the capacity of stabilization ponds 5 to 10 times (7). Bess and Conway (15) found that synthetic organic chemical wastes treated in an aerated lagoon

with a retention time of 2.5 days showed 75 to 85% BOD reduction at 25 °C and 50 to 60% near 15 °C. McKinney (135) described the use of two aerated lagoons treating domestic wastes to overcome the problem of overloading. Reid (166) described an aerated lagoon used in Alaska for the treatment of domestic wastes and during the severe winter the minimum BOD removal was reported to be 61% at 0.5 °C to 1.0 °C. With aeration much higher loadings and shorter retention times are possible but the optimum rate and duration of aeration is not known. Probably it will vary according to the type and volume of the wastes to be treated and the temperature of the lagoon. Because aeration prevents or reduces ice formation, the development of aeration systems appears to offer a solution to some of the problems of lagoon operation in cold climates.

The stabilization of wastes in all biological treatment systems is accomplished by the mutual interaction of a heterogeneous mixture of bacteria. However, the mechanics of handling wastes differs from one system to another and the choice of a specific waste treatment process depends on the feasibility of that system to meet a particular situation.

The present design of lagoons, as is the case in other waste treatment systems, is made entirely on an empirical basis and most investigators have ignored the biological aspects

of lagoon operation. No report has been seen which describes the biological characteristics of lagoons and considers ecological factors associated with lagoon operation. The construction of a lagoon serving the municipalities of St. James, Assiniboia and Tuxedo of metropolitan Winnipeg provided an opportunity to study some aspects of the microbial ecology of domestic wastes.

PART I

Physiological Activity of Lagoon Bacteria as a Function of Seasonal Change

INTRODUCTION

Industrial and domestic wastes contain both inorganic and organic compounds. Although inorganic compounds may pose biological problems in eutrophication (48, 106, 149), they generally tend to increase the rate of disposal of organic wastes (132). Further, the rate at which various organic compounds are utilized by living organisms is a distinguishing feature of each class of compound. Most soluble carbohydrates are metabolized easily and rapidly while proteins and fats, on the other hand, are degraded more slowly (41); some synthetic organic compounds are quite resistant to biodegradation (19, 31, 121).

Maximum efficiency in any microbiological waste treatment system is attained when the microorganisms are provided with an optimum environment. Temperature is an important factor for the regulation of all biological processes. Hurwitz et al. (81) reported that cellulose was degraded in an activated sludge unit at 25 °C but not at 12 °C. Ludzack et al. (122) found normal performance of an activated sludge process at 30 °C but at 5 °C the rate of BOD and COD removal was retarded and

inferior flocculation, accumulation of solids, and less nitrogen utilization occurred. Keefer (93) analyzed data from an activated sludge unit and found that the effluent had a lower BOD and contained smaller amounts of suspended solids at 25 °C than at 12 °C. Logan and Budd (117) reported that the loading rate had to be reduced when the temperature was decreased from 25 °C to 9 °C to produce normal sludge. Ling (115) found 87% BOD removal in the aerated lagoon at 20 °C in 4 days while in the same time 25% removal was accomplished at about 11 °C. McKinney (134) reported that the biological activity in aerated lagoons was reduced to almost zero as the temperature approached 0 °C. Since changes in climate alter the temperature of the lagoon, it was of interest to relate the impact of temperature to lagoon operation.

pH also influences significantly the efficiency of wastes treatment processes. The pH in lagoons treating domestic wastes varies from 6.5 to 11.2 (45, 73, 160). In an aerated lagoon treating chemical wastes, the pH varied from 3.4 to 7.5 (115). This pH variation is expected to influence the growth and metabolism of the microorganisms in a stabilization pond.

No study has been seen pertaining to the effect of temperature and pH on the bacterial activity in lagoons. The construction of a domestic wastes disposal unit serving the municipalities of St. James, Assiniboia and Tuxedo of metro-

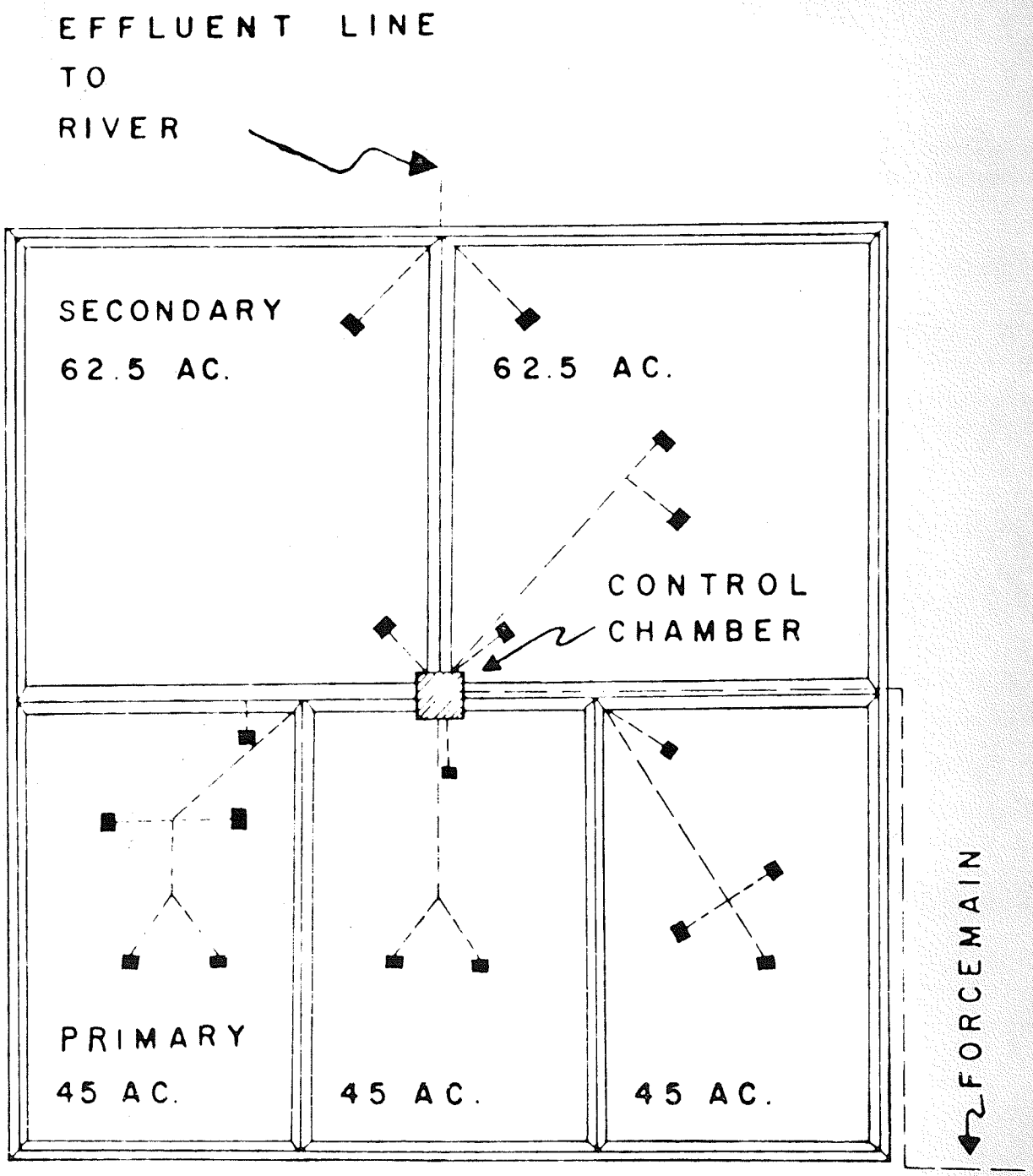
politan Winnipeg provided an opportunity to investigate the physiological activity of the microflora indigenous to a lagoon as a function of temperature and pH, and to relate these findings to lagoon operation as a function of seasonal change.

MATERIALS AND METHODS

Description of the Lagoon

The 'Charleswood Lagoon' was designed and constructed to serve portions of St. James, Assiniboia and Tuxedo which are adjacent municipalities of metropolitan Winnipeg. The lagoon was completed in May, 1965, and a scheme of this lagoon system is shown in Fig. 1. It is a dual cell unit which consists of three primary cells, arranged in parallel, integrated with two secondary cells arranged in parallel; the primary and secondary cells are arranged in series. The influent and effluent port positions have been varied in order that flow characteristics may be studied. Each primary cell has an effective area of 45 acres and each secondary cell has an area of 62.5 acres giving a total area of 260 acres. The design loading capacity is 80 lb BOD per acre per day with an operating depth of approximately 5 ft. The wastes, after pulverization and dilution with water derived from the adjacent Assiniboine River, are directed at will into one of the primary cells and after a short period of retention the partially stabilized material is conducted into one of the secondary cells for final polishing. The total retention period during the summer is about 40 days while the retention period during the winter is 120 days or greater as prescribed by the Manitoba Provincial Health

Fig. 1. Layout of the 'Charleswood Sewage Lagoon' showing the physical relationship of the primary and secondary cells. The modus operandi is provided in the text.



LAYOUT OF

CHARLESWOOD SEWAGE LAGOON

Department. Innocuous effluent is discharged into the Assiniboine River. The flow capacity of the lagoon is 5 million gallons per day but the operation during the summer is approximately 3 million gallons per day, which meets the 'on stream' flow of wastes from the community.

The areas served by this treatment facility are almost exclusively residential without any appreciable industrial development. This lagoon is under the operation and control of the Metropolitan Corporation of Greater Winnipeg, Waterworks and Waste Disposal Division.

Geographic Data

The lagoon is located at approximately 50°N latitude in a geographic region with warm summers and severe winters. The climate of the region is of the 'mid-continental' type. The average annual temperature range is approximately 68 °F; the average temperature for July, the warmest month, is 68.4 °F and the average temperature for January, the coldest month, is 0.1 °F (139). The climate creates lagoons that are ice-bound for approximately 5 months of the year. Winnipeg is noted for its bright, sunny days during summer, a condition promoting the introduction of oxygen through the photosynthetic activity of algae during this period of the year.

Sampling Procedure

Lagoon water was collected by a manually operated rotary hand pump mounted on the seat of a 16-ft aluminum boat equipped with an outboard motor. A calibrated hollow probe connected to the hand pump by high pressure hose was mounted onto the gunwale of the boat and could be lowered into the lagoon to any desired depth. The end of the probe was flared to a diameter of 6 in to prevent turbulence at the orifice; this ensured that the samples were derived from the desired locale. Samples were taken from both the surface and the bottom of either the Primary No. 1 or No. 2 cell equidistant from the inlet ports. The sampling positions were marked by permanent buoys. The pH and bacterial numbers were measured as a routine practice in each sample and the temperature of the lagoon was recorded at the time each sample was taken.

Determination of Bacterial Numbers in Lagoon Water

To determine the bacterial population as a function of seasonal change, a sample of lagoon water was filtered through Whatman No. 1 filter paper and then through a 10 μ Gelman membrane filter (Gelman Instrument Co., Ann Arbor, Mich.) to remove algae and protozoa. The bacterial cells in the filtrate were counted on a Coulter Counter Model A (Coulter Electronics,

Hialeah, Fla.) (191) as follows. The lagoon water was diluted 100-fold with physiological saline previously cleaned through a 0.45 μ Gelman membrane filter. The bacterial cell suspension was shaken thoroughly to achieve a uniform preparation and counts were then made on the suspension with the Coulter Counter using the 30 μ orifice at a threshold value of 10 with a current setting of 4. The volume counted was 0.05 ml and counts were recorded as the number of organisms per 1 ml of lagoon water. Corrections were made for background counts.

Preparation of Bacterial Resting Cell Suspensions

To study the physiological activity of lagoon bacteria as a function of seasonal change, the indigenous bacterial population was recovered from samples taken at about weekly intervals from the top 6 in of the lagoon whenever the weather was permissible from mid-May to the beginning of December, 1965. To prepare resting cell suspensions for manometric studies, the following procedure was developed. All organisms in a 20 liter sample of lagoon water were removed by centrifugation using a steam-driven high speed Sharples Super centrifuge (Sharples Centrifuges Ltd., Camberly, Surrey) with a standard clarifier rotor operating at 20 lb steam pressure (approximately 40,000 rpm). When the supernatant liquid was examined microscopically, no cells could be seen. The packed sediment consisting of algae, protozoa, and bacteria was washed from

the rotor with 0.05 M potassium phosphate buffer, pH 7.0, and thoroughly shaken on a mechanical shaker. Algae and protozoa were then separated from the bacteria by low speed centrifugation (365 x g) for 10 minutes using a Sorvall centrifuge Model RC-2 (Ivan Sorvall, Inc., Norwalk, Conn.). The sediment was washed in buffer once to recover trapped bacteria. The pooled supernatant liquid containing the bacteria was centrifuged at high speed (12,000 x g) for 15 minutes using the Sorvall centrifuge. The sedimented bacteria were resuspended in the same phosphate buffer and the final volume was made to 20 ml; thus, 1 ml of the cell suspension contained the equivalent of the total bacteria in 1 liter of lagoon water at the time of sampling. Microscopic examination of the bacterial suspensions showed only a very few small algae and no protozoa.

Manometric Studies

Since carbohydrates, proteins,and fats are the major constituents of the lagoon BOD load, the following representatives from these three classes of organic compounds were arbitrarily selected for study and were used either singly or in combination: acetate, palmitate, glucose, vitamin-free casamino acids (Difco Laboratories, Detroit, Michigan), egg albumin (Sigma Chemical Co., St. Louis, Missouri), and Liqui-nox (Alconox, Inc., New York, N.Y.), an alkylbenzene sulfonate

detergent marketed as 'biodegradable'. All soluble substrates were dissolved in 0.1 M potassium phosphate buffer, pH 7.0, to make 1% (w/v) solutions with the exception of Liqui-nox, which was dissolved in distilled water. To measure oxygen uptake a Precision Warburg Manometrican instrument equipped with both a heating coil and a cooling coil and having an operational temperature from 0 °C to 60 °C ± 0.2 °C was employed. Standard manometric techniques with air as the gas phase were used (204). Each flask contained 2.0 ml of bacterial cell suspension in the main compartment, 0.2 ml of 20% KOH and a fluted filter paper in the center well, and 0.4 ml of substrate in the side arm. The final volume was brought up to 3.2 ml with 0.05 M potassium phosphate buffer, pH 7.0. Palmitate, because of its poor solubility in water, necessitated a slight modification of the above procedure; 0.4 mg palmitate was weighed directly into the main compartment of a Warburg flask with double sidearm and the 2.0 ml of the cell suspension was distributed over the two arms. Manometric experiments were conducted at the same temperature as the lagoon at the time of sampling and flasks were shaken at a rate of 60 oscillations per minute. After an equilibration period of about 15 minutes, the oxygen uptake in μliters which occurred in 2 hours was recorded.

Stratification

Porges and MacKenthun (163) reported that practically all ponds have zones of aerobiosis and anaerobiosis, and it was thought that stratification might occur in the lagoon under study. To determine this, the rates of the physiological activity of equal numbers of bacteria derived from samples taken from both the surface and the bottom of the lagoon were compared. The indigenous population recovered from these samples was used in manometric experiments as described earlier. Glucose, Liqui-nox, and egg albumin were used as substrates and oxygen uptake was recorded over 8 hours at 23 °C. In addition, the utilization of glucose and Liqui-nox was measured colorimetrically (47, 186) using bacteria taken from the surface and the bottom of the lagoon. The procedure is described on page 58 and 84 for glucose and Liqui-nox utilization respectively.

Effect of pH on Substrate Oxidation

Acetate and vitamin-free casamino acids were used to study the effect of pH on the activity of the lagoon bacteria. In this investigation, the following buffers were used at a concentration of 0.05 M: potassium phosphate buffer, pH 4.0 to 9.0 at increments of one pH unit; borax-sodium hydroxide buffer, pH 10.0, and potassium phosphate-phosphoric acid buffer, pH 11.0 and 11.5. For these experiments, a resting cell

suspension was made in a buffer of the desired pH and the amount of oxygen consumed in 2 hours at 23 °C was measured manometrically according to the procedure described earlier.

RESULTS AND DISCUSSION

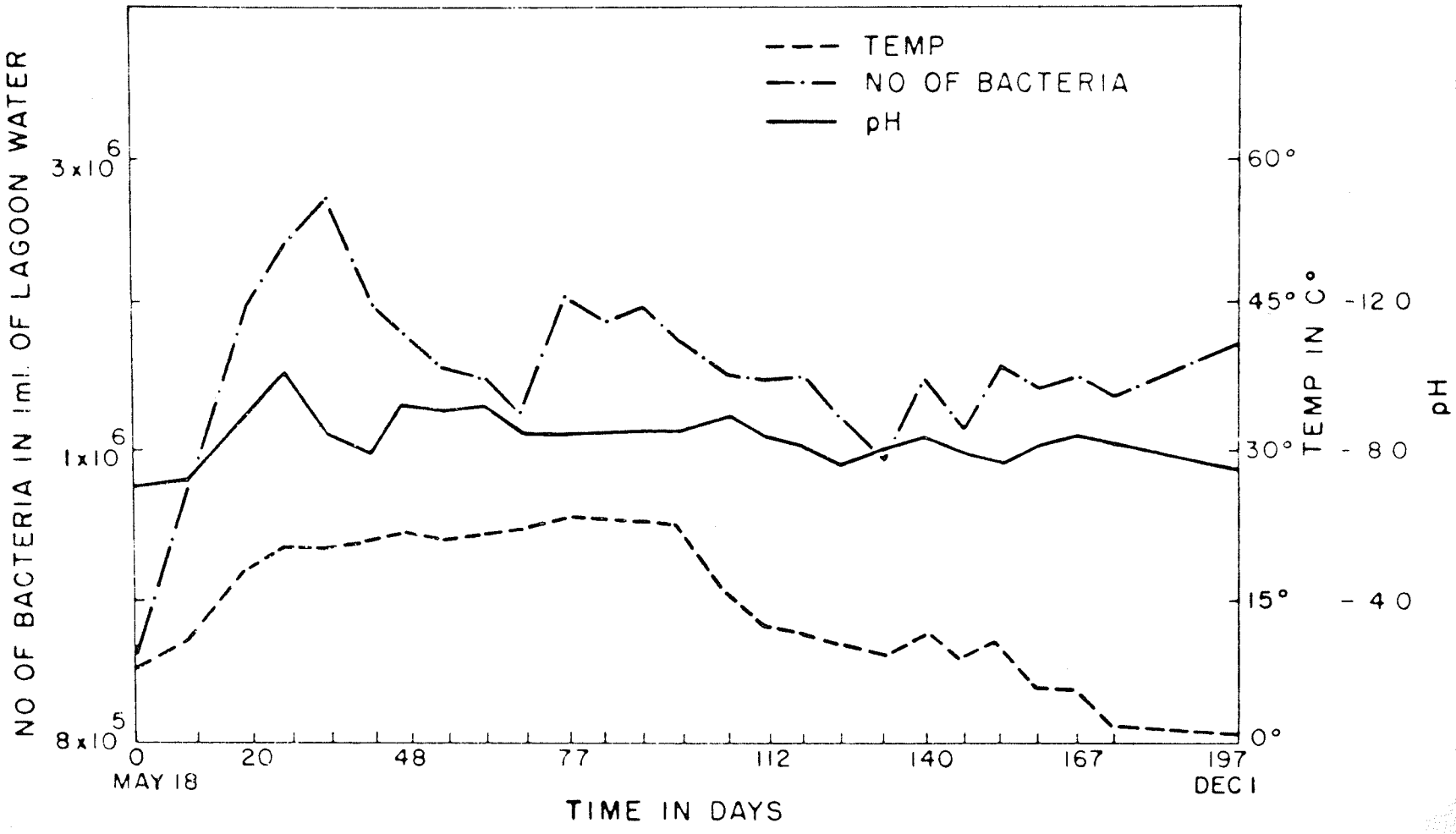
Variations in the Lagoon Temperature, pH, and Bacterial Numbers as a Function of Seasonal Change

A summary of the results obtained for the 26 week study showing the changes in temperature, pH, and bacterial numbers is presented graphically in Fig. 2.

The temperature in the lagoon ranged from a low of 2 °C to a high of 23 °C. The temperature was below 10 °C for approximately 54 of 197 ice-free days of operation. This proportion is of major importance because activity measured below 10 °C must be considered to be due principally to psychrophiles. Thus, in the stabilization process by the lagoon method, this group of organisms would be expected to play a significant role in some geographic regions.

Only minor fluctuations in the pH were experienced. The pH ranged from 7.1 to 9.2 and fluctuations occurred primarily in the early spring when the lagoon was undergoing equilization coincident with the spring thaw. The effect of pH upon substrate utilization over the naturally occurring range would be expected to be slight and no attempt was made to duplicate the pH of the lagoon in the manometric flasks. Small fluctuations in the pH encountered in the samples from the lagoon may be considered evidence for the constancy of the ecology of the lagoon microflora.

Fig. 2. Variations in the temperature, pH, and bacterial numbers in the Charleswood sewage lagoon as a function of seasonal change.


TEMP
NO OF BACTERIA
pH
NO OF BACTERIA IN 1ml. OF LAGOON WATER
3x10^6
1x10^6
8x10^5
60°
45°
30°
15°
0°
TEMP IN C°
12 0
8 0
4 0
pH
0
MAY 18
20
48
77
112
140
167
197
DEC 1
TIME IN DAYS

The bacterial population in the lagoon was low in May just after the ice cover had disappeared. Thereafter, there was a rapid increase in numbers with an increase in temperature after the spring warm-up. Vigorous bacterial growth is thought to be due to the metabolism of readily utilizable organic materials which had accumulated over the winter months. A sharp decline in numbers which followed the vigorous growth period is due probably to the exhaustion of metabolically active substrates. Mild fluctuations during the summer are due probably to changes in the operative BOD load demand made on the lagoon. Reciprocally, Burlington (21) reported that organic pollutants caused a major variation in the biota of a stream sampled at different stations and found that maximum numbers were present when there was maximum BOD load in the stream.

In the last week of August and onward there was again a gradual decrease in bacterial numbers concomitant with the decrease in temperature as a result of the onset of fall. This transition phase represents the shift from mesophilic to psychrophilic activity. A similar transition phase in the early spring is of less significance because an ice flotilla acts as a collant reservoir and once the ice-free condition is achieved the warm-up period to 10 °C is very short. A slight increase in numbers again appeared in the

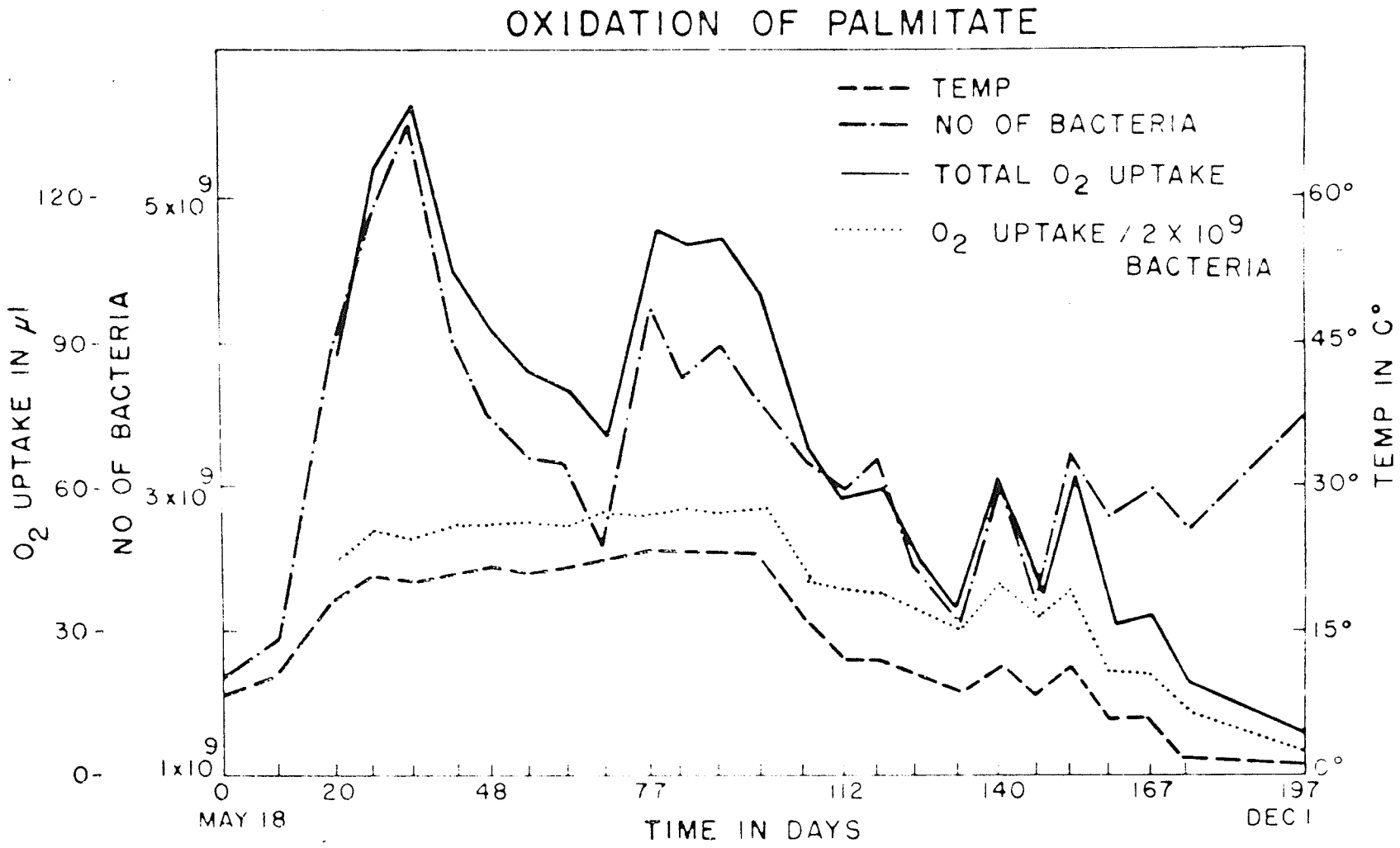
late fall when the entire surface of the lagoon was frozen solid but this apparent increase may be an artifact caused by a reduction of the liquid volume of the lagoon accompanying ice formation rather than an increase in numbers due to growth of psychrophiles.

The drop in the bacterial population late in the season is not unexpected. Ling (115) also reported that high numbers of bacteria and flagellates were found in a lagoon during the period from July to October when the temperature in the lagoon ranged from 11 °C to 25 °C but that the total population of bacteria and flagellates gradually fell when the weather became cold. But numbers increased again when the temperature increased.

Physiological Activity as a Function of Seasonal Change

The activity against palmitate, acetate, vitamin-free casamino acids, and glucose is shown in Figs. 3a, 3b, 3c, and 3d respectively. The results are expressed as the quantity of oxygen consumed in the Warburg vessel in 2 hours, a period arbitrarily selected as sufficient to demonstrate the comparative physiological activity of the lagoon bacteria, and are plotted on the basis of (a) the oxygen uptake by the total bacterial population recovered from a 2 liter sample of lagoon water and (b) the amount of oxygen consumed per population unit defined as 2×10^9 cells.

Fig. 3a. Oxidation of palmitate by lagoon bacteria as a function of seasonal change. Lagoon samples were taken roughly at weekly intervals. Each Warburg vessel contained: 0.2 ml of 20% KOH in the center well and 0.4 mgm of substrate, 0.05 M phosphate buffer, pH 7.0, to a final volume of 3.2 ml and the entire bacterial population recovered from a 2 litre sample of lagoon water in the main compartment. Oxidation was determined at a temperature identical to that of the lagoon at the time of sampling. The results are expressed as: (a) ——— total oxygen uptake in 2 hours by the recovered bacteria and (b) · · · · oxygen uptake in 2 hours by 2×10^9 cells.


OXIDATION OF PALMITATE
TEMP
NO OF BACTERIA
TOTAL O_2 UPTAKE
O_2 UPTAKE / 2 X 10^9 BACTERIA
O_2 UPTAKE IN µl
NO OF BACTERIA
TEMP IN C°
120
90
60
30
0
5×10^9
3×10^9
1×10^9
60°
45°
30°
15°
0°
0
20
48
77
112
140
167
197
MAY 18
DEC 1
TIME IN DAYS

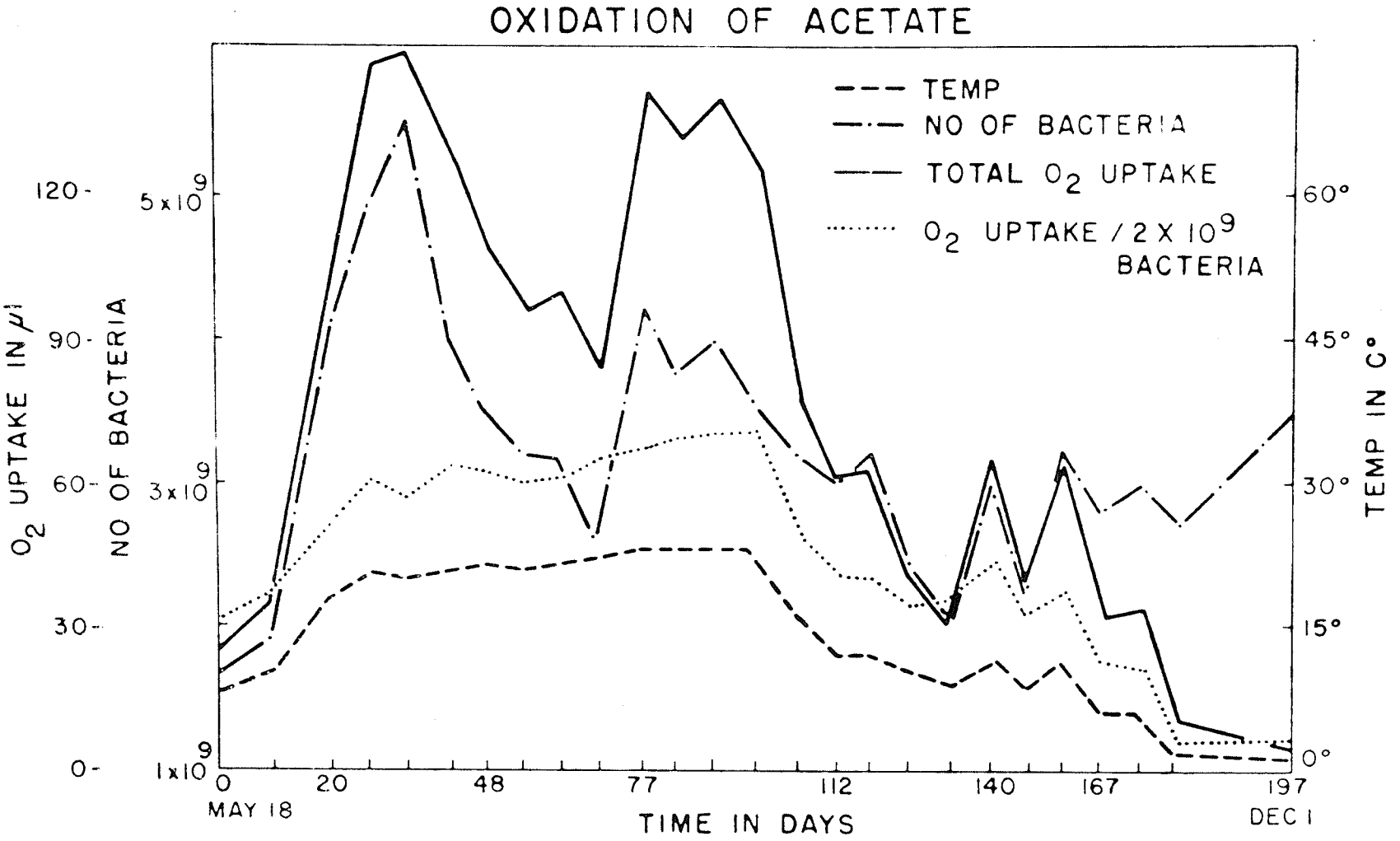

OXIDATION OF ACETATE
TEMP
NO OF BACTERIA
TOTAL O_2 UPTAKE
O_2 UPTAKE / 2 X 10^9 BACTERIA
O_2 UPTAKE IN μl
NO OF BACTERIA
TEMP IN C°
120
90
60
30
0
5×10^9
3×10^9
1×10^9
60°
45°
30°
15°
0°
0
20
48
77
112
140
167
197
MAY 18
DEC 1
TIME IN DAYS

Fig. 3c. Oxidation of casamino acids by lagoon bacteria as a function of seasonal change. Experimental as described in Fig. 3a.

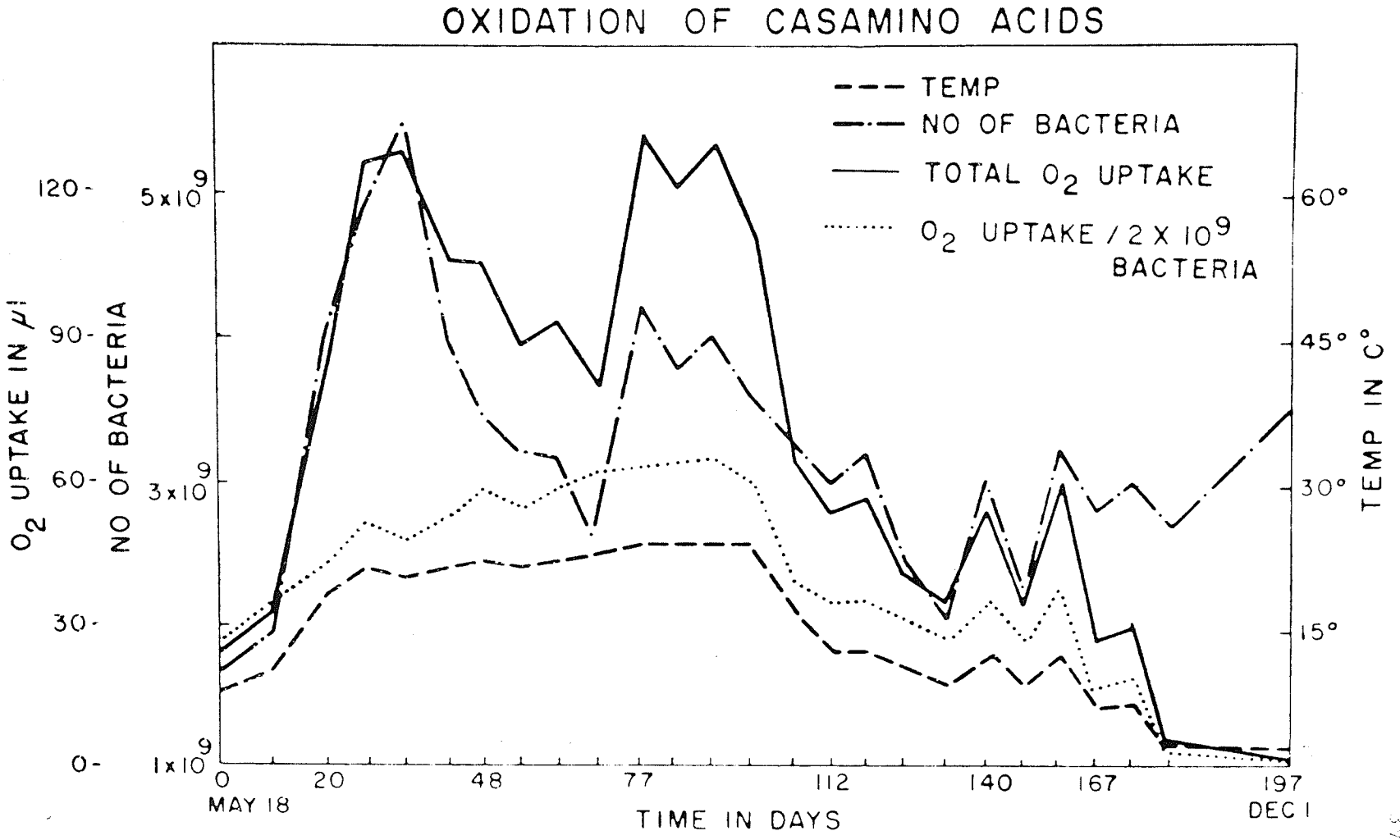
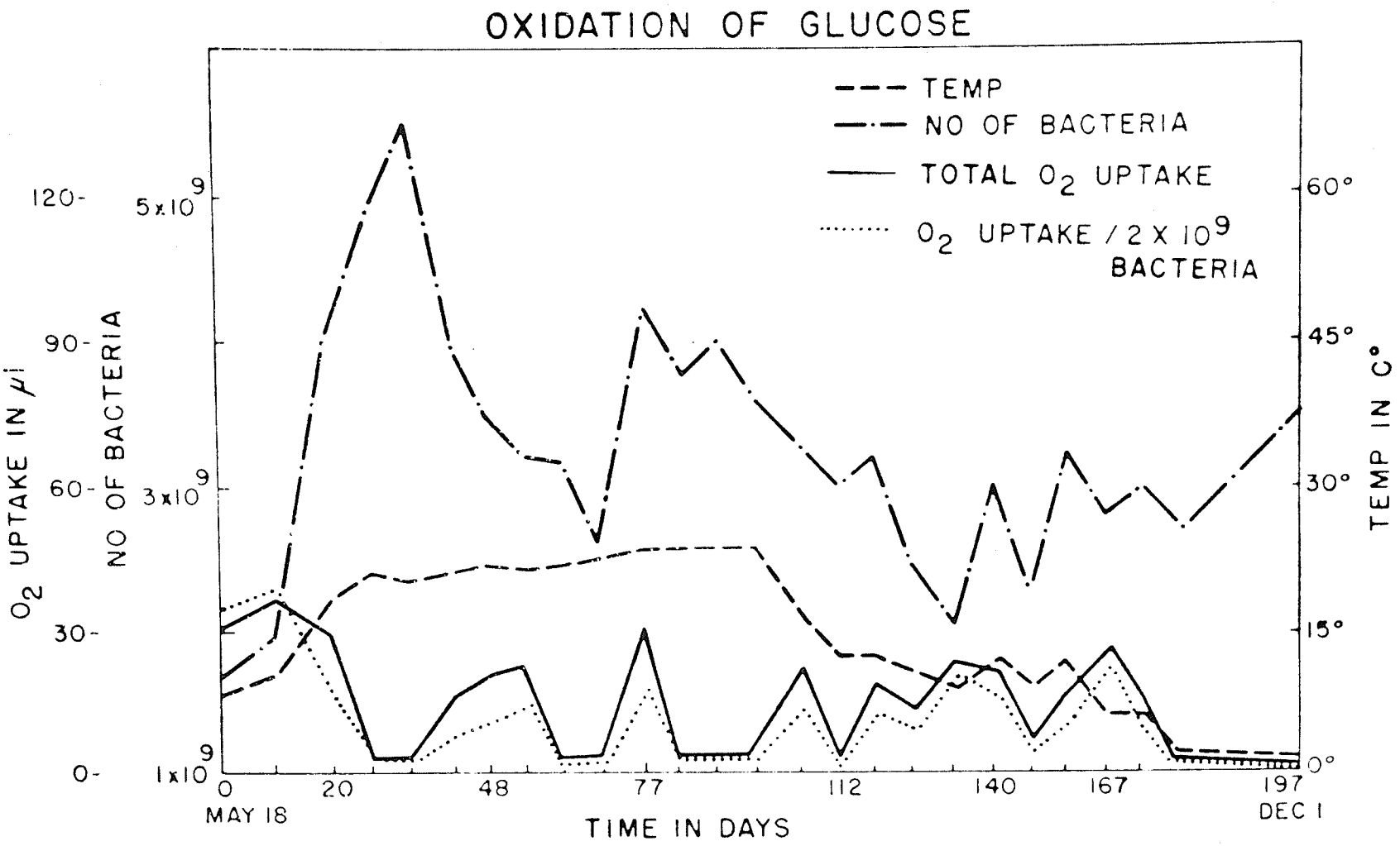

OXIDATION OF CASAMINO ACIDS
TEMP
NO OF BACTERIA
TOTAL O_2 UPTAKE
O_2 UPTAKE / 2 X 10^9 BACTERIA
O_2 UPTAKE IN μl
NO OF BACTERIA
TEMP IN C°
120
90
60
30
0
5×10^9
3×10^9
1×10^9
60°
45°
30°
15°
0
MAY 18
20
48
77
112
140
167
197
DEC 1
TIME IN DAYS

Fig. 3d. Oxidation of glucose by lagoon bacteria as a function of seasonal change. Experimental as described in Fig. 3a.


OXIDATION OF GLUCOSE
TEMP
NO OF BACTERIA
TOTAL O_2 UPTAKE
O_2 UPTAKE / 2 X 10^9 BACTERIA
O_2 UPTAKE IN μl
NO OF BACTERIA
TEMP IN C°
120-
90-
60-
30-
0-
5×10^9
3×10^9
1×10^9
60°
45°
30°
15°
0°
0
20
48
77
112
140
167
197
MAY 18
DEC 1
TIME IN DAYS

Let us consider the results of Figs. 3a-d expressed on the basis of the oxygen uptake by the total population. In the case of palmitate, acetate, or vitamin-free casamino acids, very little activity was found during the period of early spring when the lagoon temperature was below 10 °C. But activity increased markedly as the temperature increased to 18 °C and this high activity was increased further during the warm summer as a result of both rising temperatures and increased bacterial numbers. Maximum activity occurred at 23 °C, the highest temperature reached by the lagoon during summer. When the temperature of the lagoon was slowly reduced by the climate the activity subsided gradually until almost no activity could be demonstrated via manometry by bacteria collected from under the ice cover. In the case of glucose, oxygen uptake was sporadic as shown in Fig. 3d; clearly there are factors other than temperature regulating glucose oxidation.

Porges and MacKenthun (163) similarly found that in cold climates when the ice cover of stabilization ponds broke up, the rate of bacterial oxidation increased when the temperature increased.

Let us consider the results of Figs. 3a-d expressed on the basis of population units. That the temperature exerts an important regulating influence on the rate of metabolism is

evident because the activity shown by the population unit curve follows the temperature curve very closely.

When the temperature fell to 2 °C, activity could be demonstrated only against palmitate where 6% of the maximum activity (expressed on the basis of population units) was retained. No oxygen uptake in a 2 hour period could be shown with any other substrate. The results suggest that, because of the increased solubility of oxygen at low temperatures coupled with a markedly reduced requirement for molecular oxygen, it is unlikely the prevailing oxygen tension at low temperatures becomes a metabolic rate-limiting factor where air/water contact is possible. This concept merits study and could play an important role in aerated lagoon management in countries such as Europe, the U.S.S.R., the U.S.A., and Canada, regions of which experience rigorously cold climatic conditions during portions of the year.

The results obtained in this study compare favorably with those of Hawkes (71) and Ling (115). Hawkes found that in trickling filter sewage beds both the film fauna balance and the activity rate were a function of temperature with greater activity occurring at higher temperatures. Ling conducted his experiments on the basis of BOD removal from a lagoon through two winter seasons and found that BOD removal varied greatly with the temperature of the lagoon content. He observed that 87% of the BOD was removed during summer and,

in contrast, only 24% was accomplished in the same period of time during winter.

Independently of the temperature, the rate of metabolism of the substrates tested varied considerably. Some substrates were oxidized easily and rapidly while others were oxidized poorly or in a variable manner. Figures 3a, 3b, and 3c show that the rate of metabolism of palmitate, acetate, and vitamin-free casamino acids was fairly high. Glucose, as shown in Fig. 3d, appeared to be a poor substrate, for no or very little oxygen uptake was detected in a 2-hour period, even at the higher temperatures studied. Egg albumin, Liqui-nox, and a mixture of glucose and Liqui-nox behaved similarly to glucose and these results are not expressed graphically. However, all three substrates were oxidized to completion over an extended period and these results will be reported later.

Fair (41) reported that proteins and fats are metabolized very slowly. Similar observations on the behavior of some synthetic detergents have been reported by McGauhey and Klein (131).

The Warburg apparatus proved to be useful to assess the biodegradability potential of compounds which might be introduced into a lagoon. The ability of lagoon bacteria to oxidize any compound can be determined rapidly and the effect of other environmental conditions, such as temperature and pH,

may be measured quickly; oxygen uptake over the endogenous rate is indicative that decomposition is occurring.

Stratification

Bacteria recovered from samples of lagoon water taken from the surface and the bottom of the lagoon will be referred to as 'surface' and 'bottom' bacteria, respectively. The results of the Warburg studies are expressed on the basis of the amount of oxygen consumed by 2×10^9 cells while the results of glucose and Liqui-nox disappearance are expressed on the basis of utilization by 2×10^8 cells.

There was a lag of about 3 hours before the utilization of glucose by surface bacteria occurred (Fig. 4). However, the lag was reduced to about 2 hours by bottom bacteria. The unusual finding that glucose was metabolized after a lag demanded some explanation. The lag in the utilization of glucose by the lagoon bacteria could conceivably be due to two reasons. Most substrates are metabolized by constitutive enzymes, i.e., those formed only in response to stimulation by the presence of the substrate and the glucose catabolizing enzymes may not be constitutive and must be induced in the presence of glucose. Also plausible, the glucose permeation system is present in a repressed state. Since a mixed population indigenous to a lagoon would be expected to have

constitutive enzymes, the second explanation is more attractive but further experimentation is urgently needed to clarify this concept.

The relative rates of glucose oxidation varied and were higher by bottom bacteria. These results show that stratification among bacteria responsible for the utilization of glucose exists. This conclusion is further supported by evidence obtained on the quantitative utilization of glucose; Fig. 5 shows that glucose utilization was complete in 18 hours by bottom bacteria whereas the same number of surface bacteria utilized only 44% in the same time. Stratification in the lagoon of bacteria responsible for glucose oxidation may be the result of the decomposition of cellulose which is present in large quantities in domestic wastes. Glucose is the principal product in the breakdown of cellulose and the particulate nature of cellulose undoubtedly encourages its location at the bottom of the lagoon which is the principal site of glucose release and hence the site for the development of predominant bacterial species capable of utilizing glucose. When glucose is released from cellulose, it is readily degraded by bottom bacteria before it reaches the surface layers. These results might account for the fluctuations in the amount of oxygen consumed in the case of glucose as shown in Fig. 3d. It was noted that where no oxygen uptake occurred,

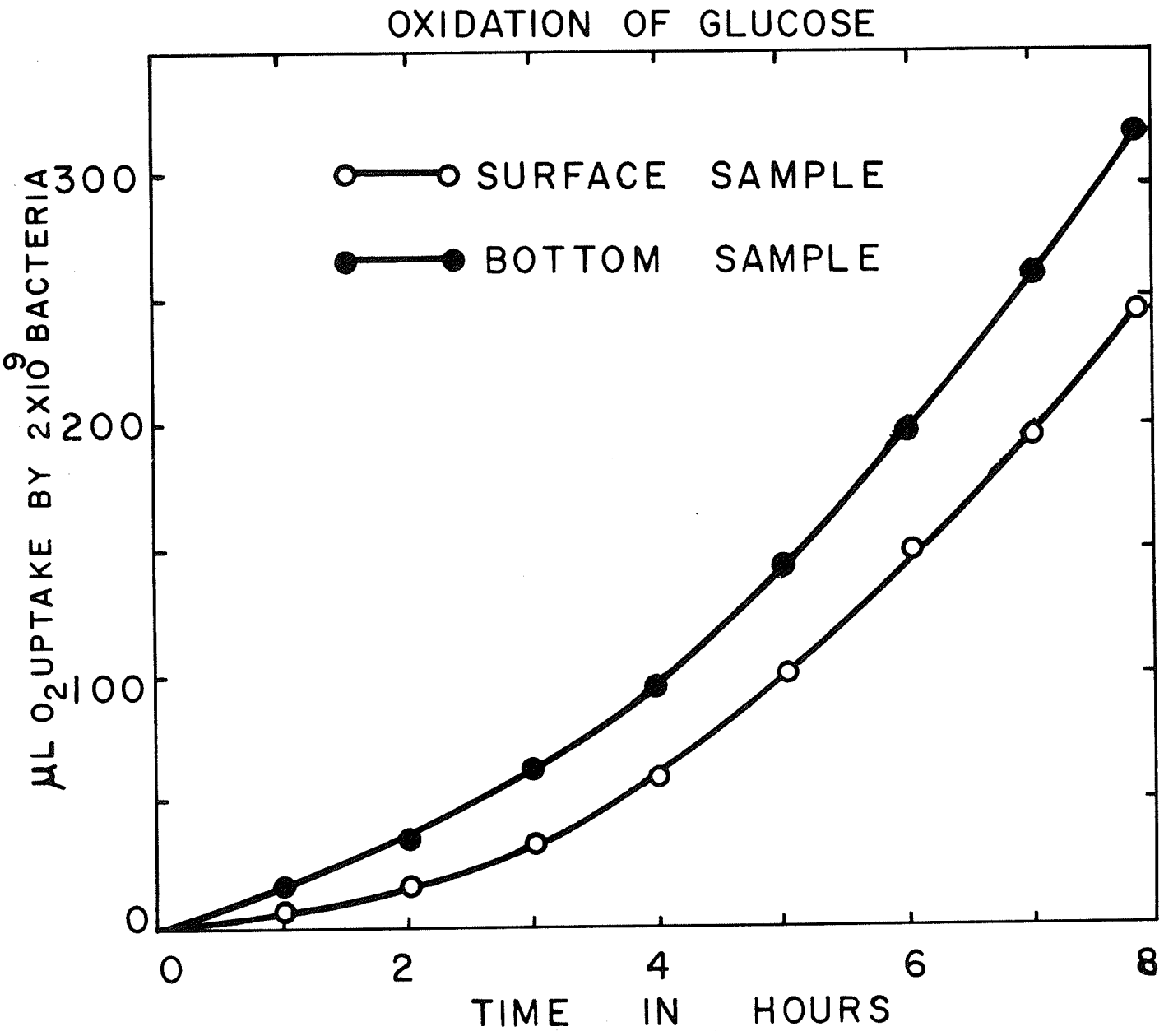
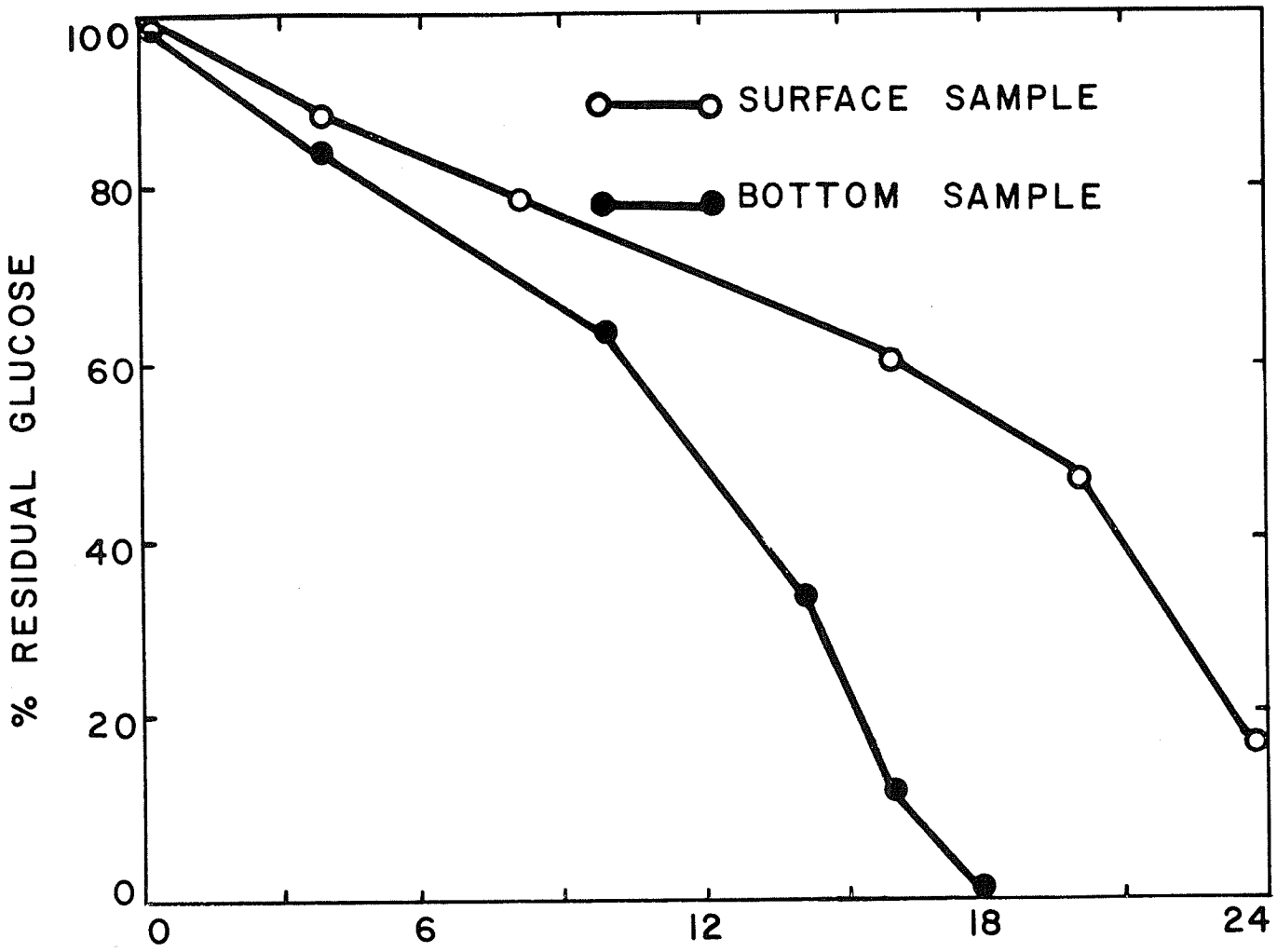

OXIDATION OF GLUCOSE
SURFACE SAMPLE
BOTTOM SAMPLE
μL O2 UPTAKE BY 2X10^9 BACTERIA
300
200
100
0
0
2
4
6
8
TIME IN HOURS

Fig. 5. Utilization of glucose by bacteria recovered from surface and bottom samples of lagoon water. Initial concentration of glucose was 92 mg/100 ml.

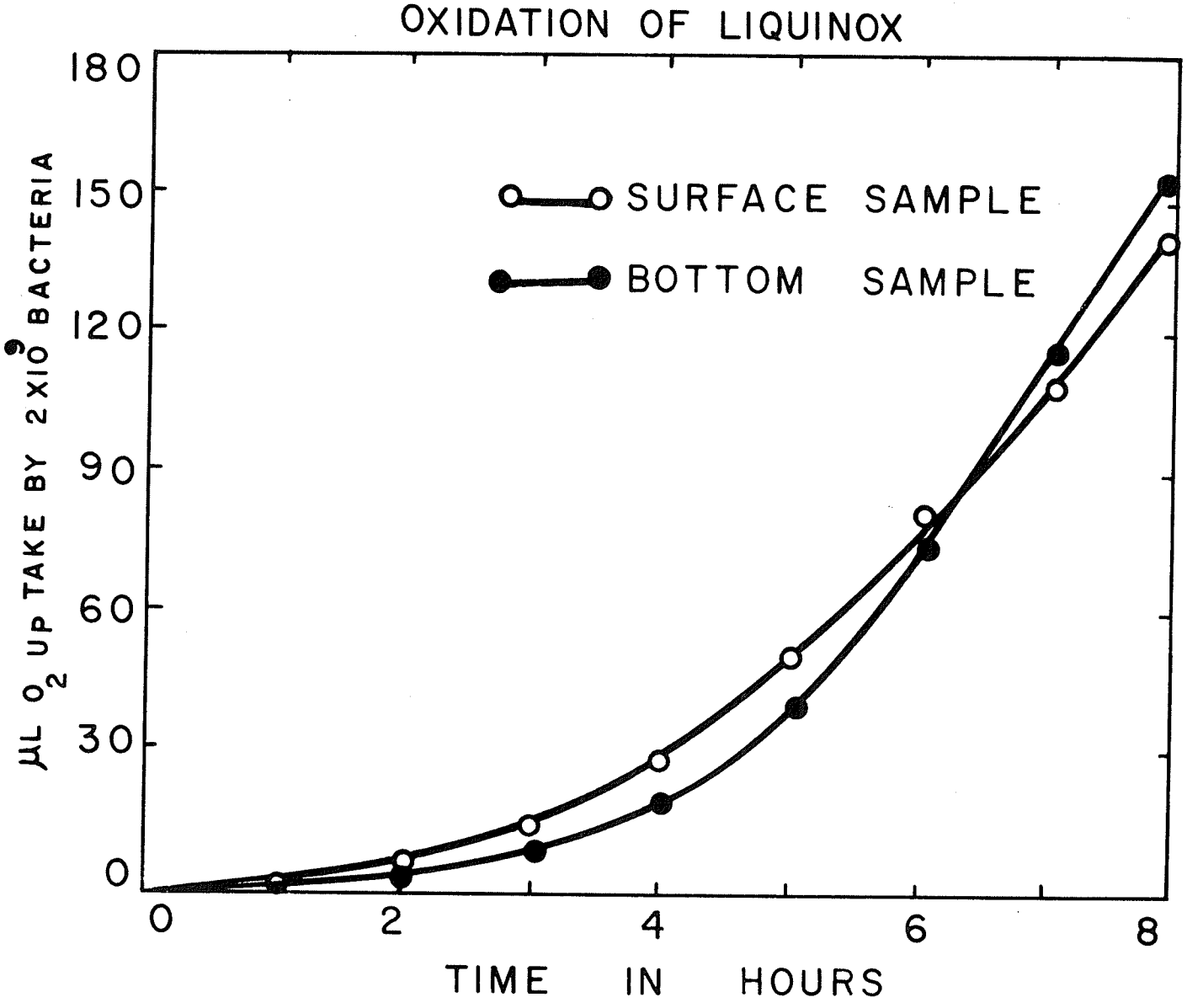


UTILIZATION OF GLUCOSE

the surface of the lagoon was calm and the bacterial population was free of organisms able to metabolize glucose. Where oxygen uptake occurred, the surface of the lagoon was turbulent, the wind velocity was in excess of 10 mph and it is proposed that bottom bacteria able to metabolize glucose were carried to the surface layer by wind action.

When Liqui-nox was used as a substrate, both surface and bottom bacteria required 3 hours before its oxidation began (Fig. 6). In all waste treatment systems different types of interaction between uncommon substrates and the heterogeneous population can be imagined. Slowly decomposable substances compete with the utilization of more readily utilizable compounds and the former group is only used when favorable substances are no longer available (50, 51, 52, 53, 97). This preferential utilization of one substrate over another is called 'diauxie phenomenon' (145). Liqui-nox in the lagoon is probably metabolized by the diauxic effect because of the availability of more easily oxidizable organic compounds. This preferential utilization of the carbon sources in a lagoon is a disadvantage for the biological degradation of substances such as detergents. A 3-hour period was possibly required for Liqui-nox acclimation by lagoon bacteria. The utilization of detergents after a lag

Fig. 6. Oxidation of Liqui-nox by bacteria recovered from surface and bottom samples of lagoon water. Curves are exogenous plot of oxygen uptake by resting cell suspensions. The Warburg vessel contents are identical to those of Fig. 3a.


OXIDATION OF LIQUINOX
180
150
120
90
60
30
0
µL O2 UP TAKE BY 2X10^9 BACTERIA
SURFACE SAMPLE
BOTTOM SAMPLE
0
2
4
6
8
TIME IN HOURS

by mixed cultures from activated sludge or river water has been demonstrated by Bogan and Sawyer (17) and Sawyer et al (174).

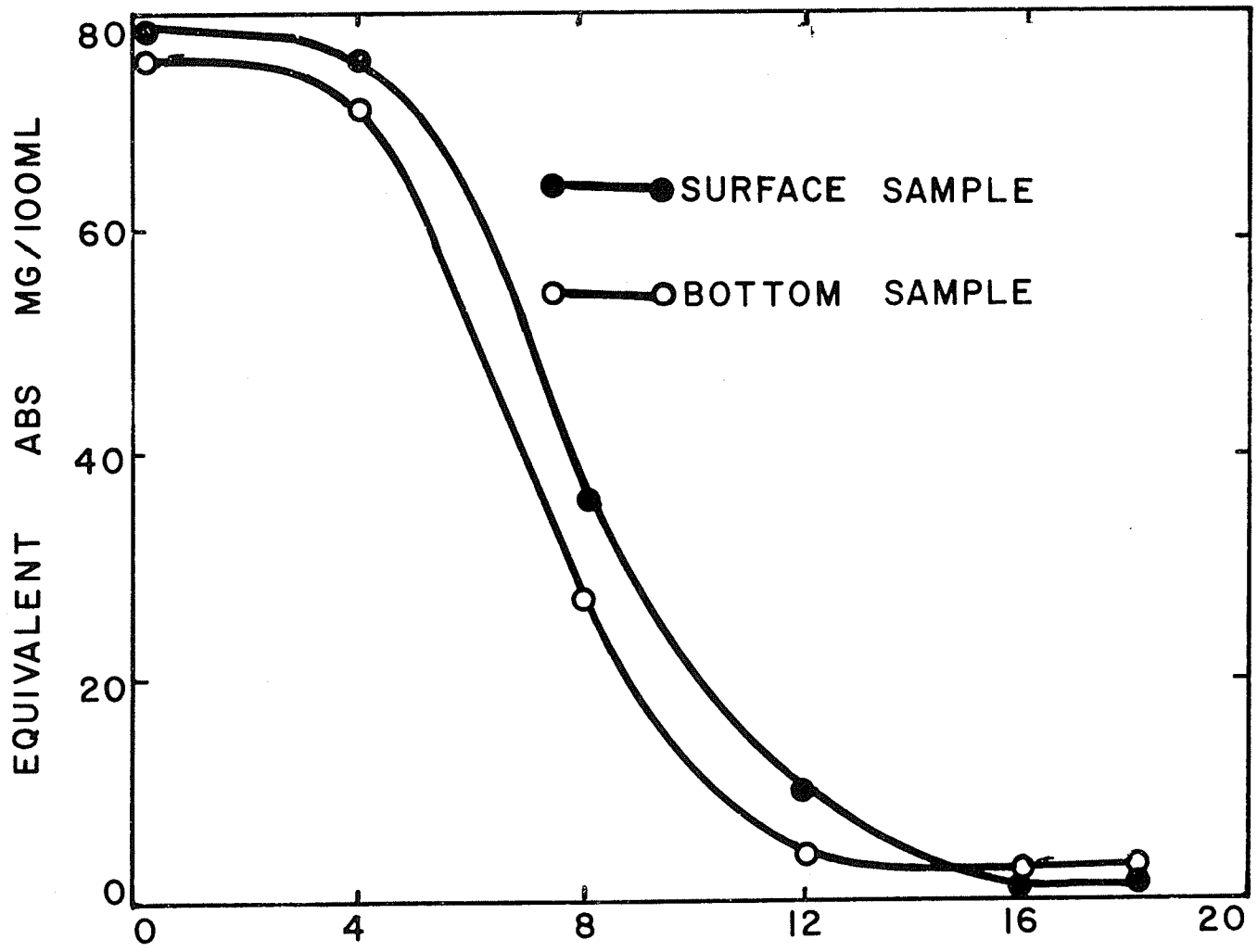
The comparative rates of oxidation of Liqui-nox by surface and bottom bacteria show that there was no difference in the total consumption of oxygen in 8 hours (Fig. 6). Further, no difference occurred in the rate of degradation of Liqui-nox by surface and bottom bacteria (Fig. 7). These results show that no stratification of bacteria responsible for Liqui-nox degradation exists in the lagoon.

Egg albumin proved to be a poor substrate, for no oxidation occurred over 8 hours at 23 °C by either surface or bottom bacteria. Egg albumin, however, was oxidized completely over an extended time and these results will be reported later.

Effect of pH

Fig. 8 shows the effect of pH on the oxidation of acetate and vitamin-free casamino acids by surface bacteria. The metabolism of both substrates changed markedly with a change in pH. Neither were oxidized at pH 4.0 and very little oxidation occurred at pH 4.5. Oxygen uptake of both substrates increased with an increase in pH and maximum activity was found at pH 7.0. A gradual decline in activity

Fig. 7. Utilization of Liqui-nox by bacteria recovered from surface and bottom samples of lagoon water. The results are expressed as the residual amount of Liqui-nox in terms of 'methylene blue active substance'.



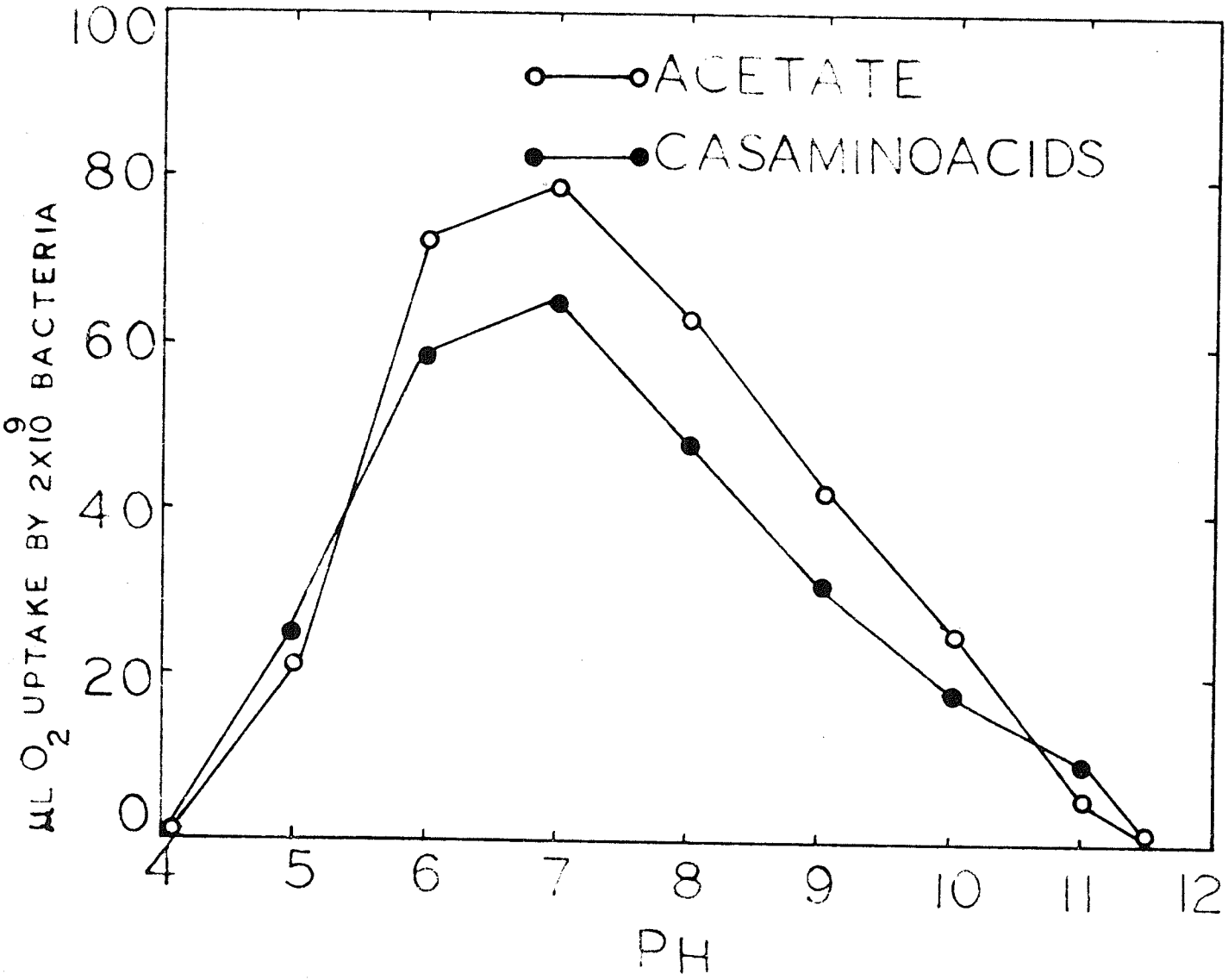
UTILIZATION OF LIQUINOX

occurred until pH 11.0 was reached and none occurred at pH 11.5. These results show that deviation from pH 7.0 would reduce the efficiency of lagoon operation. However, approximately 53% of the maximum activity against acetate was found at both pH 5.5 and 9.0, while about 50% of the maximum activity for casamino acids was retained at pH 5.3 and 9.0. The pH in the lagoon under study varied between pH 7.0 to 9.0 (see Fig. 2) which suggests that there is no need to control the pH in a lagoon treating domestic wastes.

Keefer and Meisel (94) found that an activated sludge unit could be acclimated for proper operation if the pH was within the range 6.0 to 9.0. However, when the pH of the influent domestic waste was above 9.0 the reduction of BOD was poor and above 10.0 reasonable operation was not possible. Beedham (14) reported that the activated sludge process was most effective when the pH was just below 7.0. Levine and Soopeland (110) found maximum oxidation of gelatin and milk proteins by the bacteria isolated from an activated sludge unit at a pH from 7.0 to 7.5 but at a pH of 5.0 to 5.5 decomposition stopped. Ling (115) reported that regardless of a favorable temperature the average BOD removal was only 25% in 6 days when the pH in a lagoon was 5.9, while in the same time 90% BOD removal was accomplished at a pH of 7.3. McDonald _et al_ (130) found that bacteria isolated from Arctic

Fig. 8. Effect of pH on the activity of bacteria recovered from surface samples of lagoon water. The results are expressed as the total oxygen uptake in μliters from acetate and vitamin-free casamino acids by 2×10^9 cells in 2 hours at 23 °C. Experimental as described in Fig. 3a.

Fig. 3b. Oxidation of acetate by lagoon bacteria as a function of seasonal change. Experimental as described in Fig. 3a.


ACETATE
CASAMINOACIDS
μL O_2 UPTAKE BY $2X10^9$ BACTERIA
100
80
60
40
20
0
4
5
6
7
8
9
10
11
12
PH

littoral and marine sediment samples oxidized casein most rapidly between pH 7.0 and 8.0. Below pH 7.0 and above pH 8.0 the activity was greatly reduced.

SUMMARY

1. To study the physiological activity of bacteria indigenous to a mid-continental domestic wastes disposal unit (lagoon) a method for the preparation of a bacterial cell suspension free of algae was developed.

2. The activity of the bacteria was found to be a function of (a) the number of bacteria, (b) the temperature of the lagoon, (c) the nature of the substrate, and (d) the site from which the bacteria were obtained.

3. The temperature in the lagoon ranged from 2 °C to 23 °C. The most rapid rates of oxidation occurred with casamino acids, acetate, and palmitate and maximum activity was found at 23 °C. The rates of oxidation decreased markedly when the temperature decreased as a result of the onset of fall.

4. Both glucose and Liqui-nox were metabolized after a lag of about 2 and 3 hours respectively.

5. Egg albumin was not oxidized even at 23 °C in 8 hours.

6. Stratification of bacteria in the lagoon was demonstrated; glucose was metabolized rapidly by bottom bacteria whereas it was degraded slowly by surface bacteria.

7. The pH in the lagoon ranged from 7.1 to 9.2. Maximum physiological activity occurred at pH 7.0; very little activity was found at pH 4.5 and 11.0, and none at pH 4.0 and 11.5.

PART II

A Comparative Study of the Mesophilic and Psychrophilic Activity of Lagoon Bacteria

INTRODUCTION

All activities of microorganisms are the result of an intricately co-ordinated series of metabolic reactions which are affected by the temperature of the environment. Most studies pertaining to the physiology of microorganisms associated with the disposal of domestic wastes have been conducted with mesophiles. Tischer et al (201) conducted a survey of the microbial population of stabilization ponds in Mississippi and found that ponds contained mostly mesophiles and few thermophiles. A large number of psychrophiles have been isolated from soil, mud, water, and marine sediments (34, 39, 74, 83, 108, 189, 214, 220) but none from lagoons.

Mesophiles grow in large numbers in lagoons during summer when the temperature of lagoon water varies from 18 °C to 25 °C. However, the temperature of lagoon water under an ice-cover hovers around 2 °C and would permit the growth of only psychrophiles. Microbial growth has been reported down to -5 °C (109) but little information about metabolic activities at low temperatures is available.

In geographic regions which experience severe winters during portions of the year, lagoons are covered with ice and

snow and the climate creates conditions which control the microbial population and their metabolic activities. It is expected that the activities of bacteria in such lagoons vary considerably during summer and winter and a study of the activity of lagoon bacteria as a function of seasonal change should provide useful information in improving the design and operation of lagoons. Therefore, this study was undertaken to compare the optimum activity of lagoon bacteria present during summer with that present during winter.

MATERIALS AND METHODS

Substrates

The following organic compounds which were considered representative of domestic wastes were used to test their susceptibility to degradation by lagoon bacteria: glucose, vitamin-free casamino acids, egg albumin, urea, creatinine, acetate, and palmitate. All these compounds were of the highest grade commercially available.

Preparation of Resting Cell Suspensions

Samples of lagoon water were collected from the surface 6 in of the lagoon during summer, 1966, and from under an ice-cover during winter, 1966-67. The quantitative determination of bacterial numbers in lagoon water samples and the preparation of resting cell suspensions of bacteria free of algae and protozoa have already been described (see page 25). Bacteria recovered from samples taken during summer will be referred to as "mesophiles" and the physiological activity of these organisms will be referred to as "mesophilic activity". Bacteria recovered from samples taken during winter will be referred to as "psychrophiles" and the physiological activity of these organisms will be referred to as "psychrophilic activity".

General Assay Method for the Quantitative Utilization of Substrates

A general procedure used for the quantitative utilization of all substrates, except palmitate to be described later, was as follows. For each experiment, a 40 liter sample of lagoon water was collected and a resting cell suspension was made to 80 ml in 0.05 M potassium phosphate buffer, pH 7.0. A suitable amount of the substrate being tested was added to the resting cell suspension and the total volume was made up to 100 ml with phosphate buffer. This cell suspension was subjected to controlled oxygen tension and temperature. Samples were removed at zero time and, at suitable time intervals thereafter, the cells were removed by centrifugation, and suitable aliquots of the supernatant were then analyzed for residual substrate.

Effect of Oxygen Tension on the Utilization of Substrates

To study the effect of oxygen tension on the oxidation of each substrate, studies were carried out under both aerobic and anaerobic conditions with both mesophiles and psychrophiles. For aerobic studies, 50 ml of the resting cell suspension containing the substrate under test was transferred to a 250 ml Erlenmeyer flask. The flask was then incubated at the desired temperature and was shaken continuously on a reciprocating shaker (New Brunswick Scientific Co., New

Brunswick, N.J.) to ensure an adequate supply of oxygen. For anaerobic studies, the remaining 50 ml of the resting cell suspension to which substrate had been added, was transferred to a 55 ml serum bottle. The bottle was sealed with a serum cap, flushed with nitrogen and kept in a static condition at the desired temperature. Samples from the serum bottle were removed by means of a hypodermic syringe and the bottle was flushed thoroughly with nitrogen after the withdrawal of each sample to ensure complete anaerobiosis. From both systems, samples were removed and analyzed according to the protocol already described (page 58).

Activity of Mesophiles and Psychrophiles

The physiological activity of mesophiles was investigated at 25 °C while the activity of psychrophiles was studied at 2 °C unless otherwise stated. The temperature was maintained by means of a water bath (Precision Warburg Manometrican) equipped with both a heating coil and a cooling coil and having an operational temperature from 0 °C to 60°C $\pm$ 0.2 °C.

Analytical Methods

Glucose was determined by the anthrone method (47). Vitamin-free casamino acids were measured by the biuret method (60) and egg albumin was measured by the method of

Lowry et al (118). Urea and creatinine were determined colorimetrically (140). Acetate was measured according to the procedure outlined in "Standard Methods for the Examination of Water and Waste Water" (186).

The procedure for the utilization of palmitate was modified from that of the general method used for other substrates because of its poor solubility in aqueous solutions. A resting cell suspension from 160 liters of lagoon water was made to 450 ml in 0.05 M potassium phosphate buffer, pH 7.0. This resting cell suspension was divided equally among nine 125 ml Erlenmeyer flasks and nine 55 ml serum bottles, 25 ml in each, and 10 mg of palmitate was added to each flask or bottle. The oxygen tension and temperature were maintained as described above for the other substrates. Palmitate was extracted from the entire contents of a flask or a serum bottle at zero time and appropriate time intervals thereafter using five 5 ml portions of redistilled petroleum ether (B.P. 58-60 °C). The ether extracts were filtered through a bed of anhydrous sodium sulfate, combined in a 50 ml ground glass round bottom flask, and concentrated to approximately 5 ml under a stream of nitrogen. The methyl derivative of palmitate was then prepared in the following way. To the concentrated extract, 5 ml of 2.5% methanol/HCl (w/v) was added and the resulting contents were refluxed for 2 hours

in a 60 °C water bath. After refluxing, the contents of the flask were extracted five to six times using 5 ml portions of redistilled petroleum ether each time. The combined ether extracts were dried over anhydrous sodium sulfate and reduced to exactly 1.0 ml under a stream of nitrogen. The amount of palmitate in the form of its methyl ester in the extract of each sample was determined by gas-liquid chromatography.

The instrument used for the quantitative analysis of palmitate was a Beckman GC-2 gas chromatograph (Beckman Instruments) fitted with a thermal conductivity detector. A 6' x 3/16" o.d. steel column packed with Butane diol succinate (BDS) on 70-80 mesh chromosorb W, was employed. Analyses were carried out at 220 °C with helium as the carrier gas at a pressure of 40 psi and a flow rate of 62.5 ml/min. An aliquot of 10 µl was injected into the gas chromatograph for analysis. The concentration of methyl palmitate in each sample was calculated by measuring the total area under the peak of each sample with the aid of a disc-type integrator (91) and comparing the area of the peak against methyl stearate used as an internal standard.

RESULTS

To compare mesophilic and psychrophilic activity on the utilization of each substrate, the results of all experiments in this section are expressed on the basis of the physiological activity of resting cell suspensions containing 4×10^8 cells/ml.

Glucose was metabolized by mesophiles in 7 hours both aerobically and anaerobically as shown in Fig. 9. Psychrophilic activity was comparatively slow; complete utilization of glucose occurred in 32 hours independent of molecular oxygen.

The degradation of vitamin-free casamino acids by mesophiles was complete in 12 hours under aerobic conditions while in the same time only 3.4% was utilized under anaerobic conditions (Fig. 10a). After 12 hours, the rate of degradation increased and it was metabolized completely in 60 hours. Casamino acids were not degraded by psychrophiles at 2 °C or 25 °C in 8 days either in the presence or absence of molecular oxygen (Fig. 10b).

Egg albumin was metabolized completely by mesophiles in 18 hours under aerobic conditions while under anaerobic conditions the rate of disappearance was considerably diminished and only 4% was utilized in 24 hours (Fig. 11a). However, mesophilic activity was appreciable after 24 hours

Fig. 9. Utilization of glucose by lagoon bacteria; 25 °C represents utilization by mesophiles recovered from samples taken during summer and 2 °C represents utilization by psychrophiles recovered from samples taken during winter.

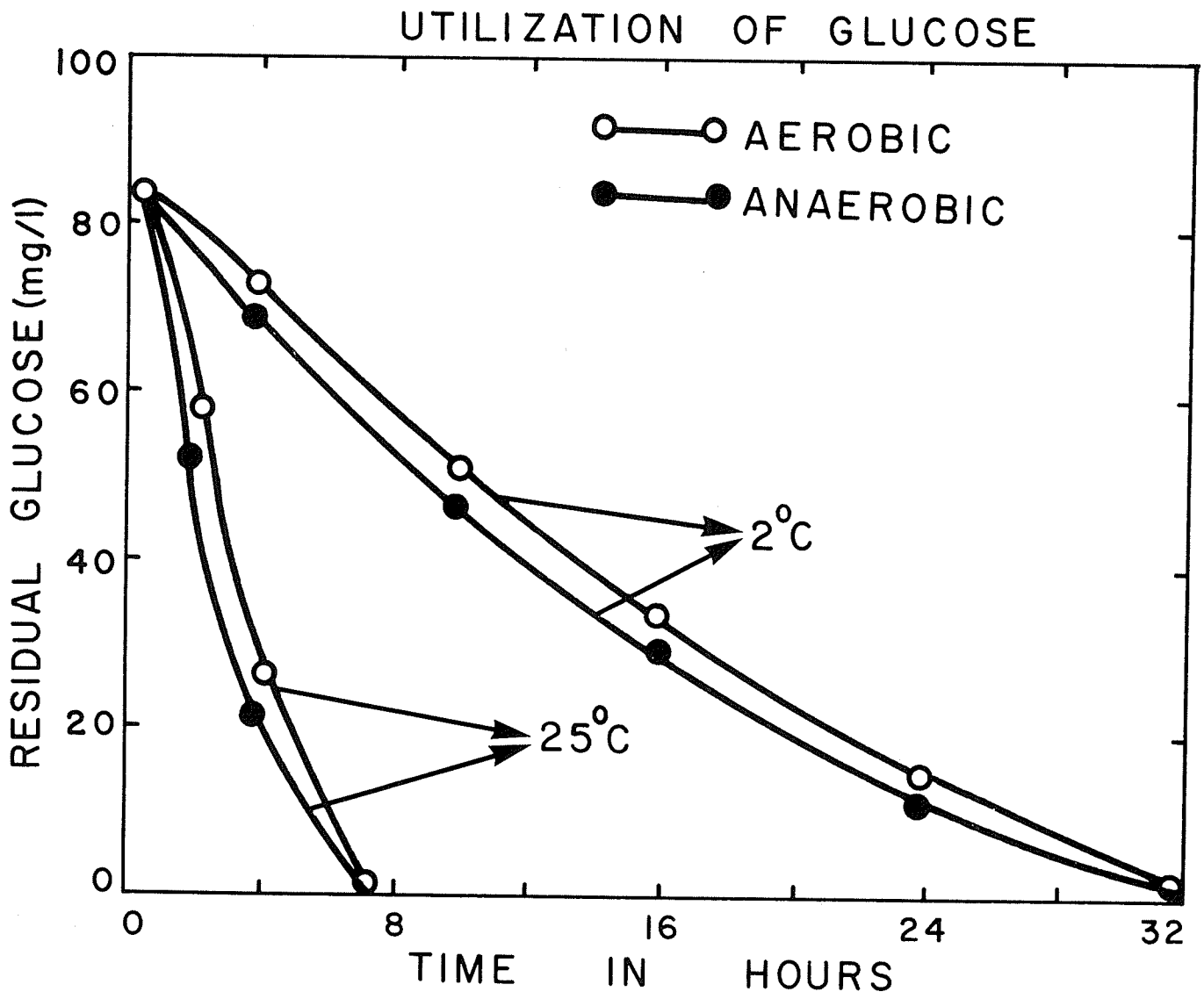

UTILIZATION OF GLUCOSE
AEROBIC
ANAEROBIC
2°C
25°C
RESIDUAL GLUCOSE (mg/l)
100
80
60
40
20
0
0
8
16
24
32
TIME IN HOURS

Fig. 10a. Utilization of vitamin-free casamino acids by mesophiles at 25 °C.

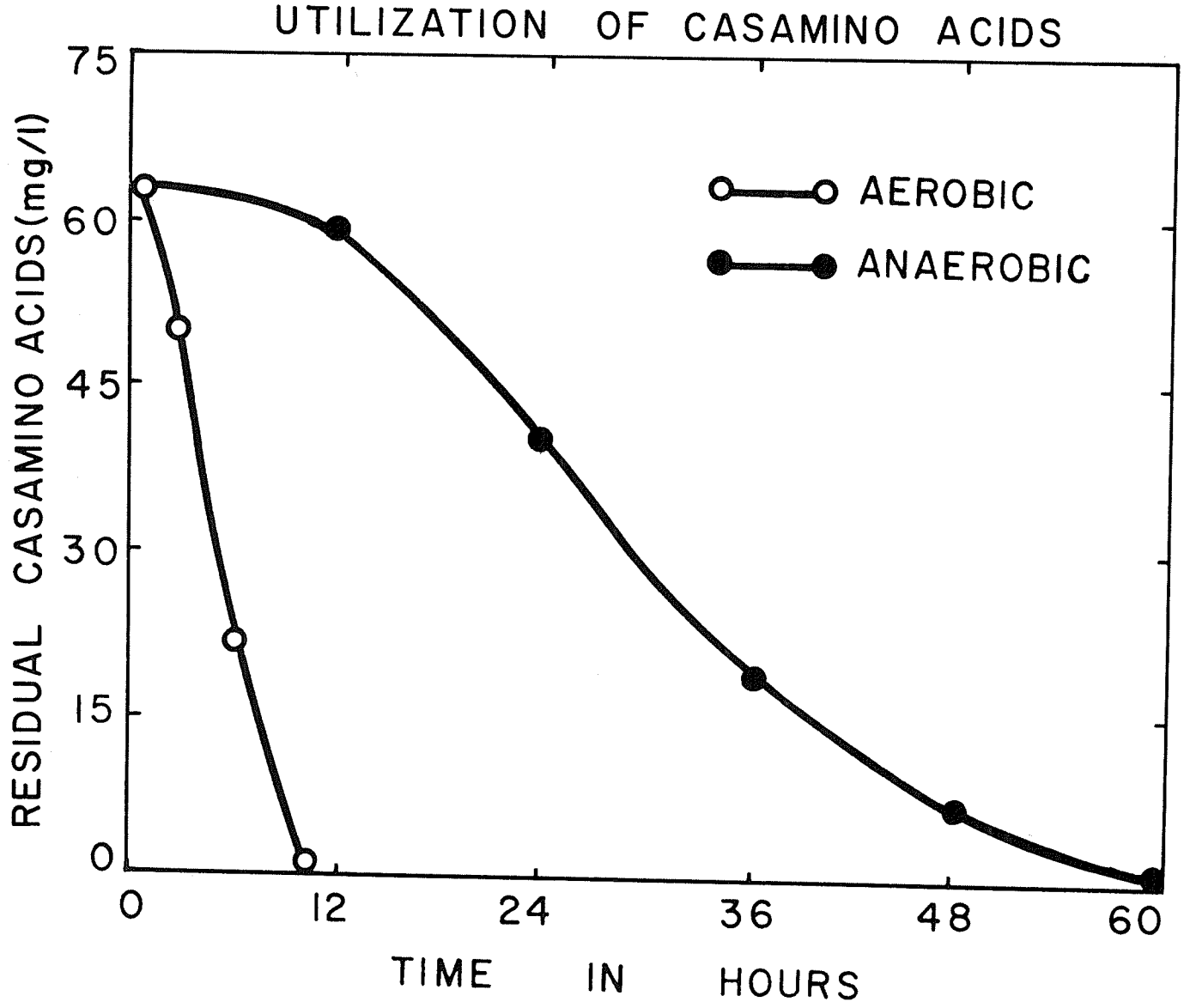

UTILIZATION OF CASAMINO ACIDS
RESIDUAL CASAMINO ACIDS(mg/l)
75
60
45
30
15
0
0
12
24
36
48
60
TIME IN HOURS
AEROBIC
ANAEROBIC

Fig. 10b. Utilization of vitamin-free casamino acids by psychrophiles at both 2 °C and 25 °C.

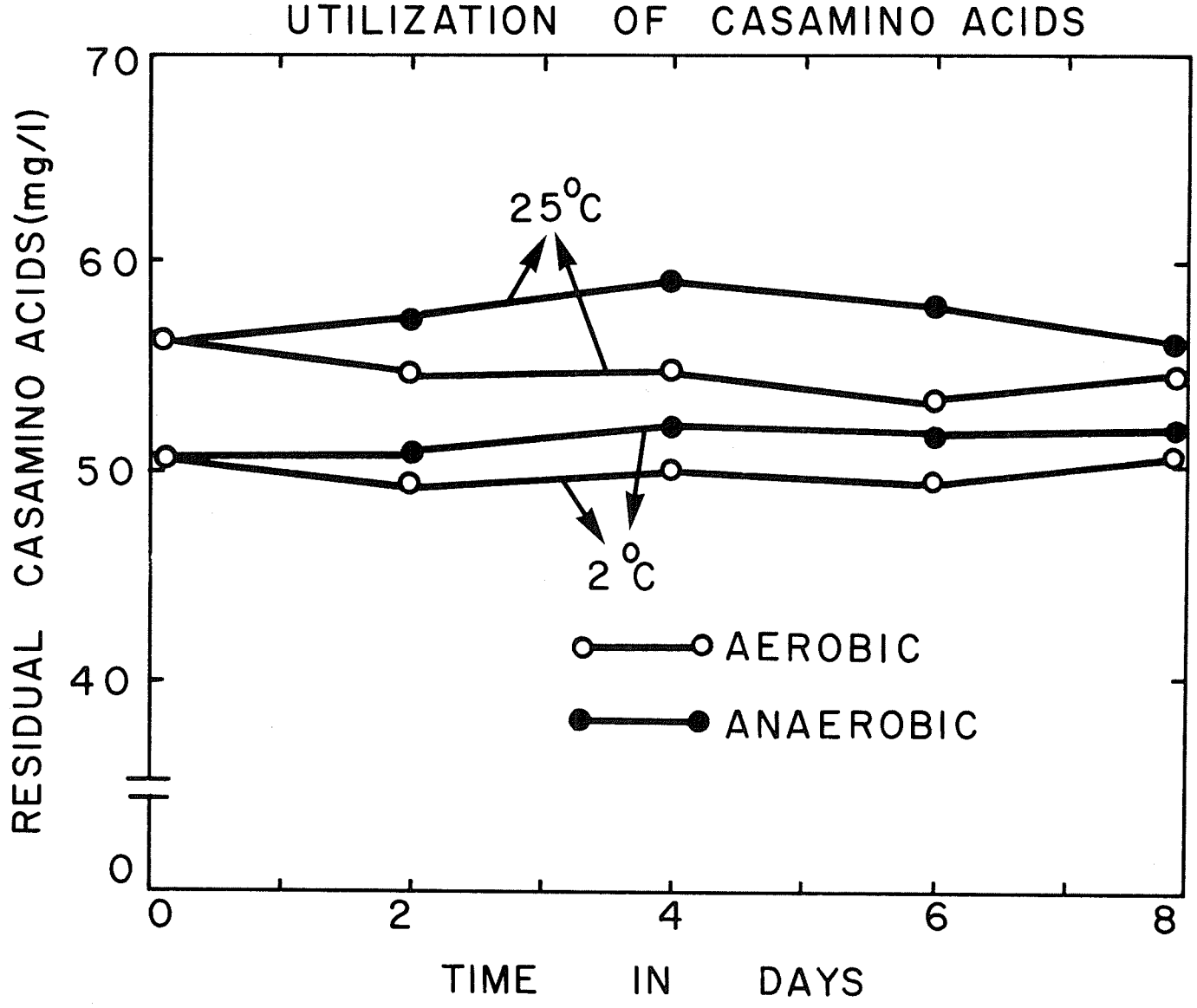

UTILIZATION OF CASAMINO ACIDS
RESIDUAL CASAMINO ACIDS(mg/l)
70
60
50
40
0
25°C
2°C
AEROBIC
ANAEROBIC
0
2
4
6
8
TIME IN DAYS

Fig. 11a. Utilization of egg albumin by mesophiles at 25 °C.

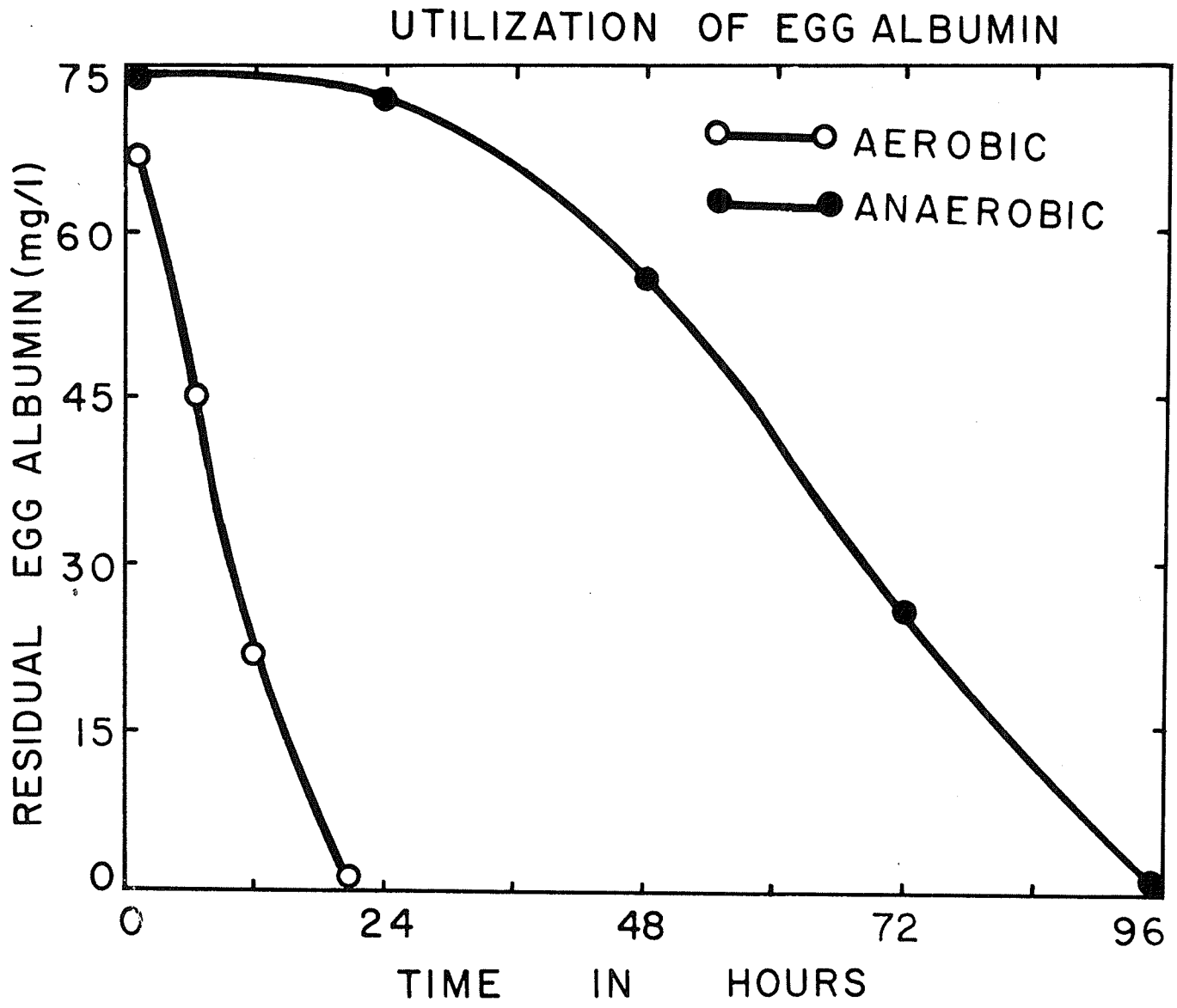

UTILIZATION OF EGG ALBUMIN
RESIDUAL EGG ALBUMIN (mg/l)
75
60
45
30
15
0
AEROBIC
ANAEROBIC
0
24
48
72
96
TIME IN HOURS

Fig. 11b. Utilization of egg albumin by psychrophiles at both 2 °C and 25 °C.

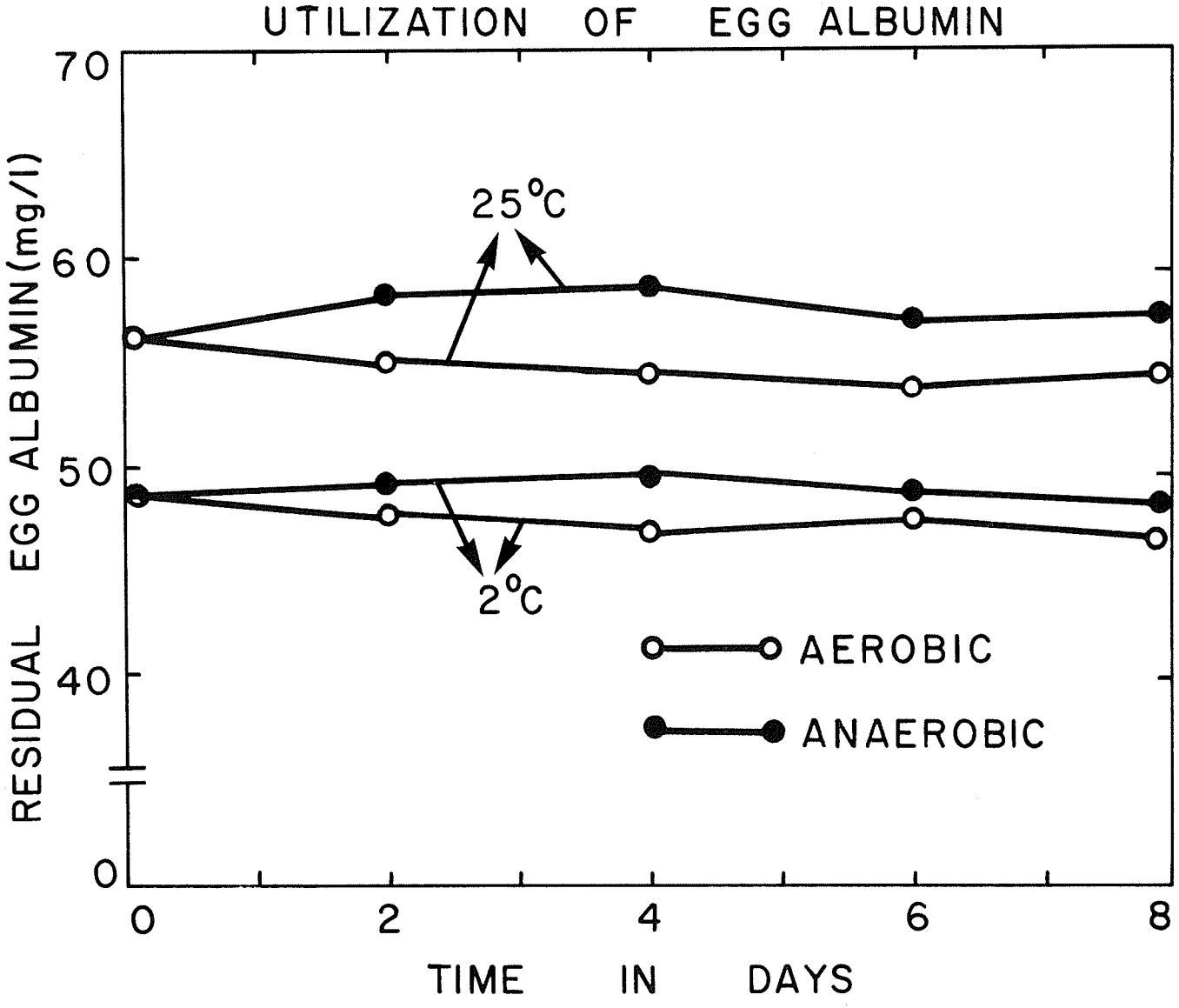

UTILIZATION OF EGG ALBUMIN
RESIDUAL EGG ALBUMIN (mg/l)
70
60
50
40
0
25°C
2°C
AEROBIC
ANAEROBIC
0
2
4
6
8
TIME IN DAYS

Fig. 4. Oxidation of glucose by bacteria recovered from surface and bottom samples of lagoon water. Curves are exogenous plot of oxygen uptake by resting cell suspensions. The Warburg vessel contents are identical to those of Fig. 3a.

and degradation was complete in 96 hours. No psychrophilic activity was found against egg albumin at 2 °C or 25 °C in 8 days either aerobically or anaerobically (Fig. 11b).

When urea was used as the substrate, mesophiles were able to use it completely in 8 hours independent of oxygen (Fig. 12a). Surprisingly, degradation of urea by psychrophiles was extremely rapid; utilization was complete in 2 hours (Fig. 12b). Psychrophilic activity was even more rapid at 25 °C; degradation was complete in 30 min.

Creatinine was metabolized by mesophiles in 16 hours both aerobically and anaerobically (Fig. 13); psychrophilic activity was comparatively slow but utilization of creatinine was complete in 56 hours.

Mesophilic and psychrophilic activity against acetate is shown in Fig. 14. It was metabolized completely by mesophiles and psychrophiles in 12 and 24 hours respectively under aerobic conditions. However, no degradation of acetate occurred under anaerobic conditions.

Palmitate was degraded completely by mesophiles in 30 hours while psychrophiles metabolized it in 108 hours in the presence of molecular oxygen and no degradation occurred in the absence of molecular oxygen (Fig. 15).

Fig. 12a. Utilization of urea by mesophiles at 25 °C.

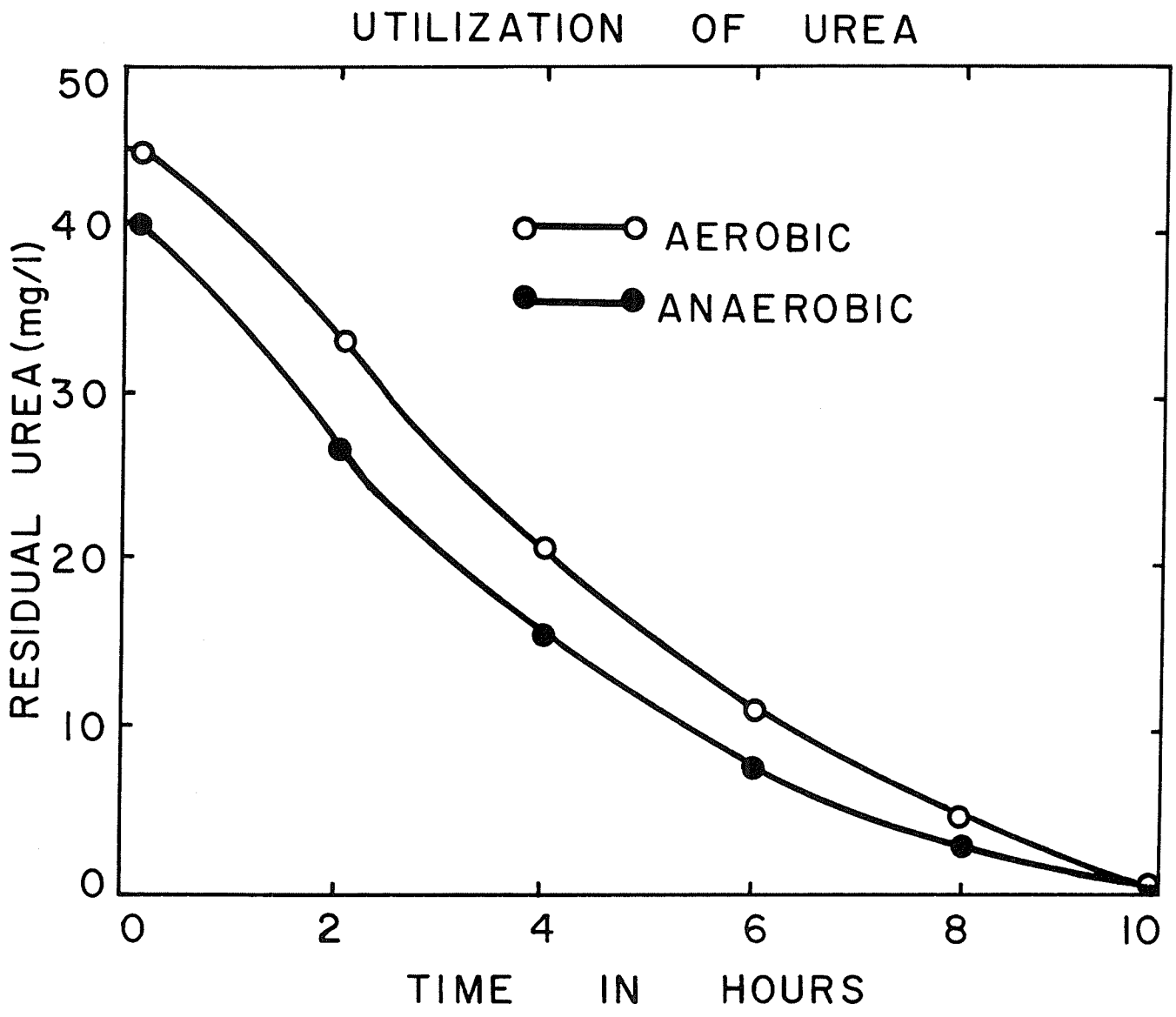

UTILIZATION OF UREA
RESIDUAL UREA (mg/l)
50
40
30
20
10
0
AEROBIC
ANAEROBIC
0
2
4
6
8
10
TIME IN HOURS

Fig. 12b. Utilization of urea by psychrophiles at both 2 °C and 25 °C.

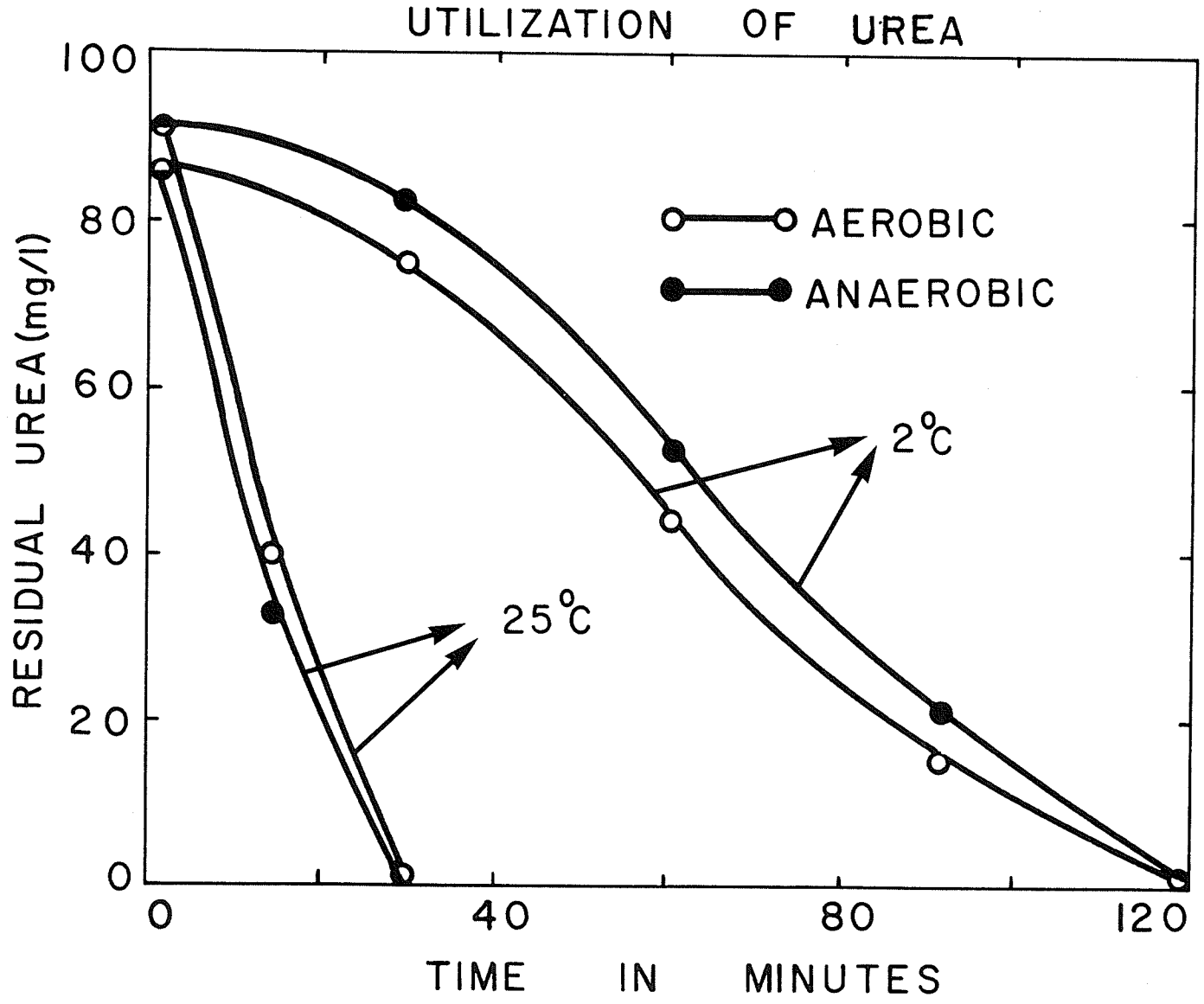

UTILIZATION OF UREA
RESIDUAL UREA (mg/l)
100
80
60
40
20
0
0
40
80
120
TIME IN MINUTES
AEROBIC
ANAEROBIC
2°C
25°C

Fig. 13. Utilization of creatinine by lagoon bacteria; 25 °C represents utilization by mesophiles recovered from samples taken during summer and 2 °C represents utilization by psychrophiles recovered from samples taken during winter.

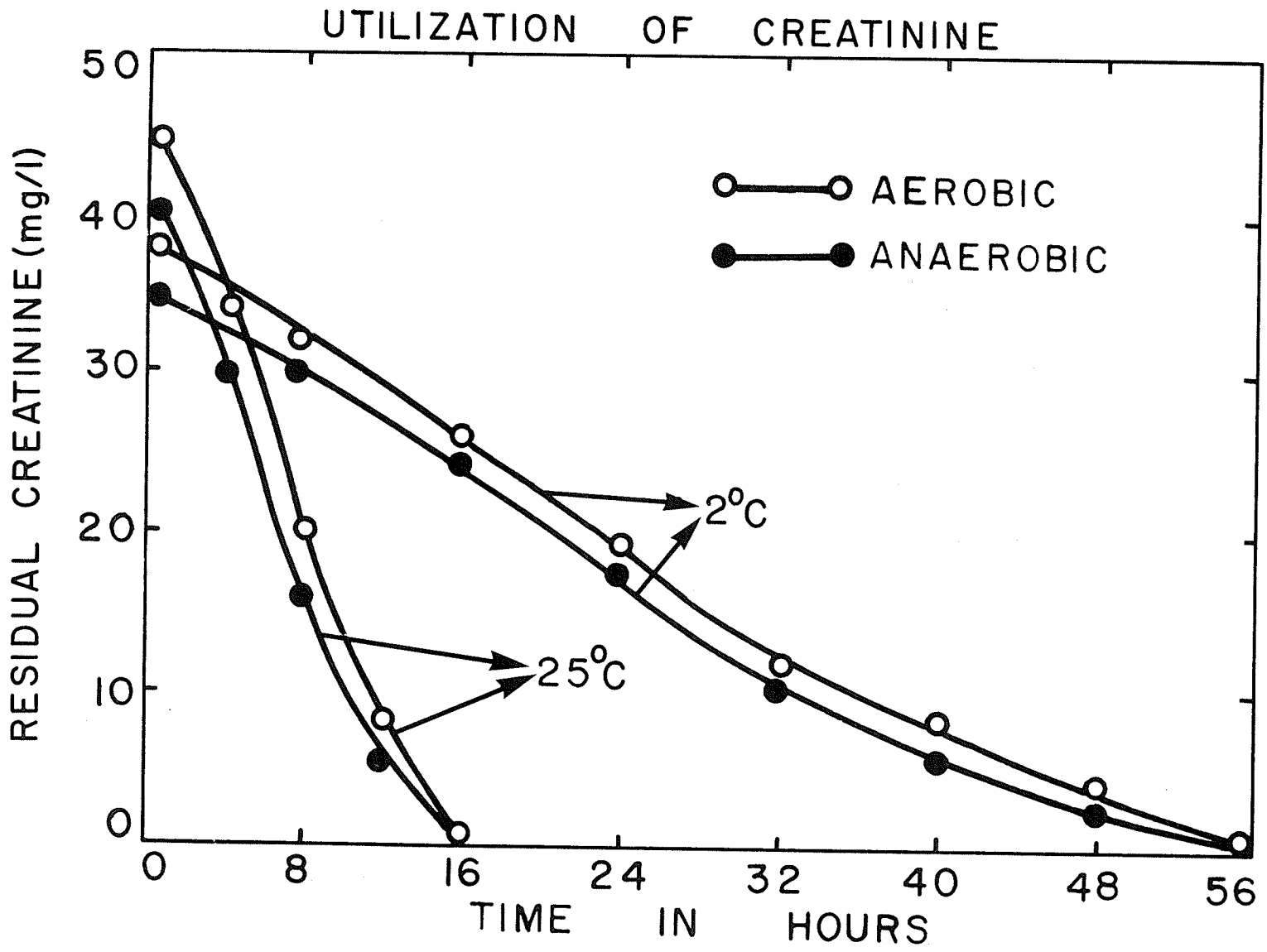

UTILIZATION OF CREATININE
RESIDUAL CREATININE (mg/l)
50
40
30
20
10
0
AEROBIC
ANAEROBIC
2°C
25°C
0
8
16
24
32
40
48
56
TIME IN HOURS

Fig. 14. Utilization of acetate by lagoon bacteria; 25 °C represents utilization by mesophiles recovered from samples taken during summer and 2 °C represents utilization by psychrophiles recovered from samples taken during winter.

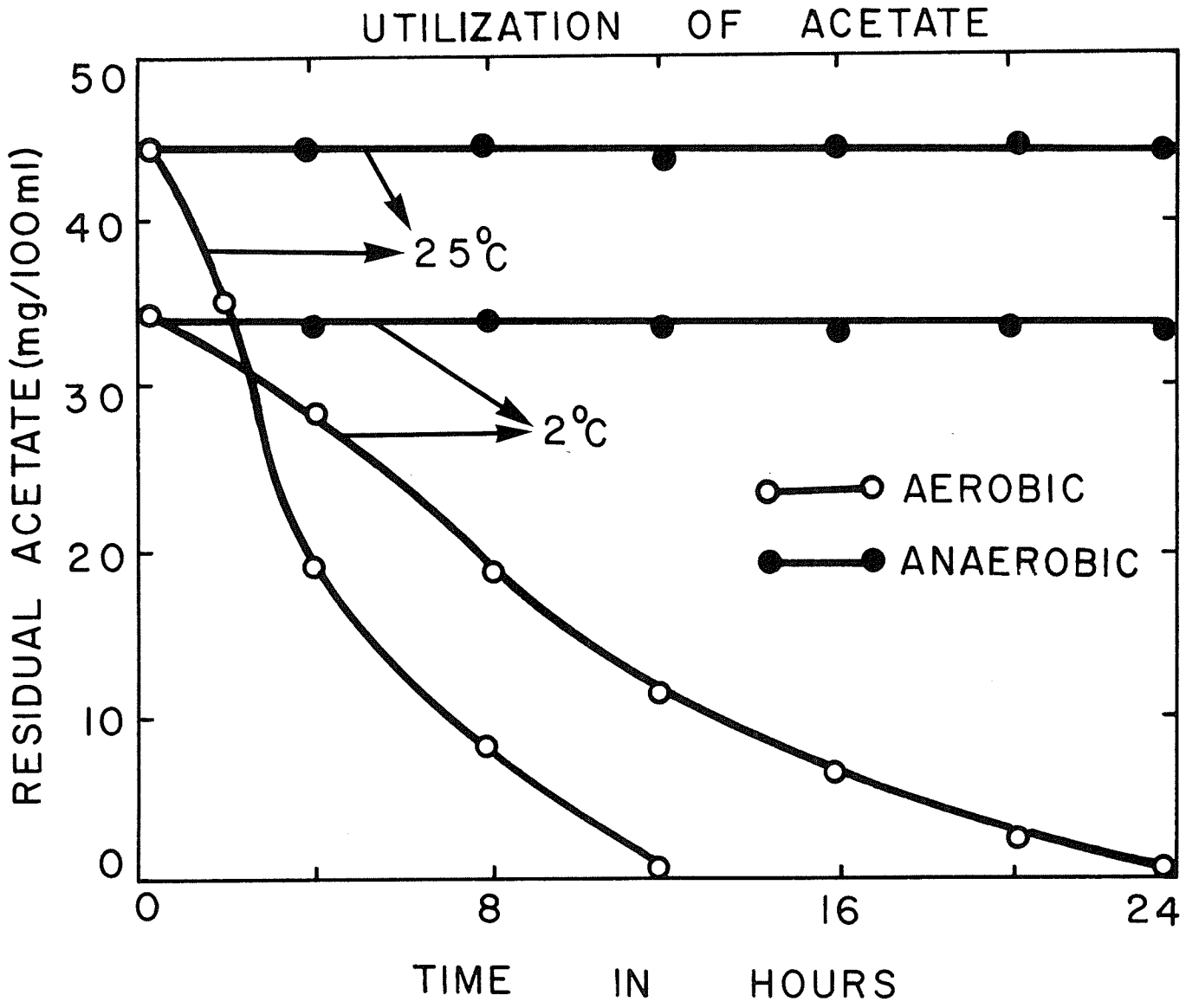
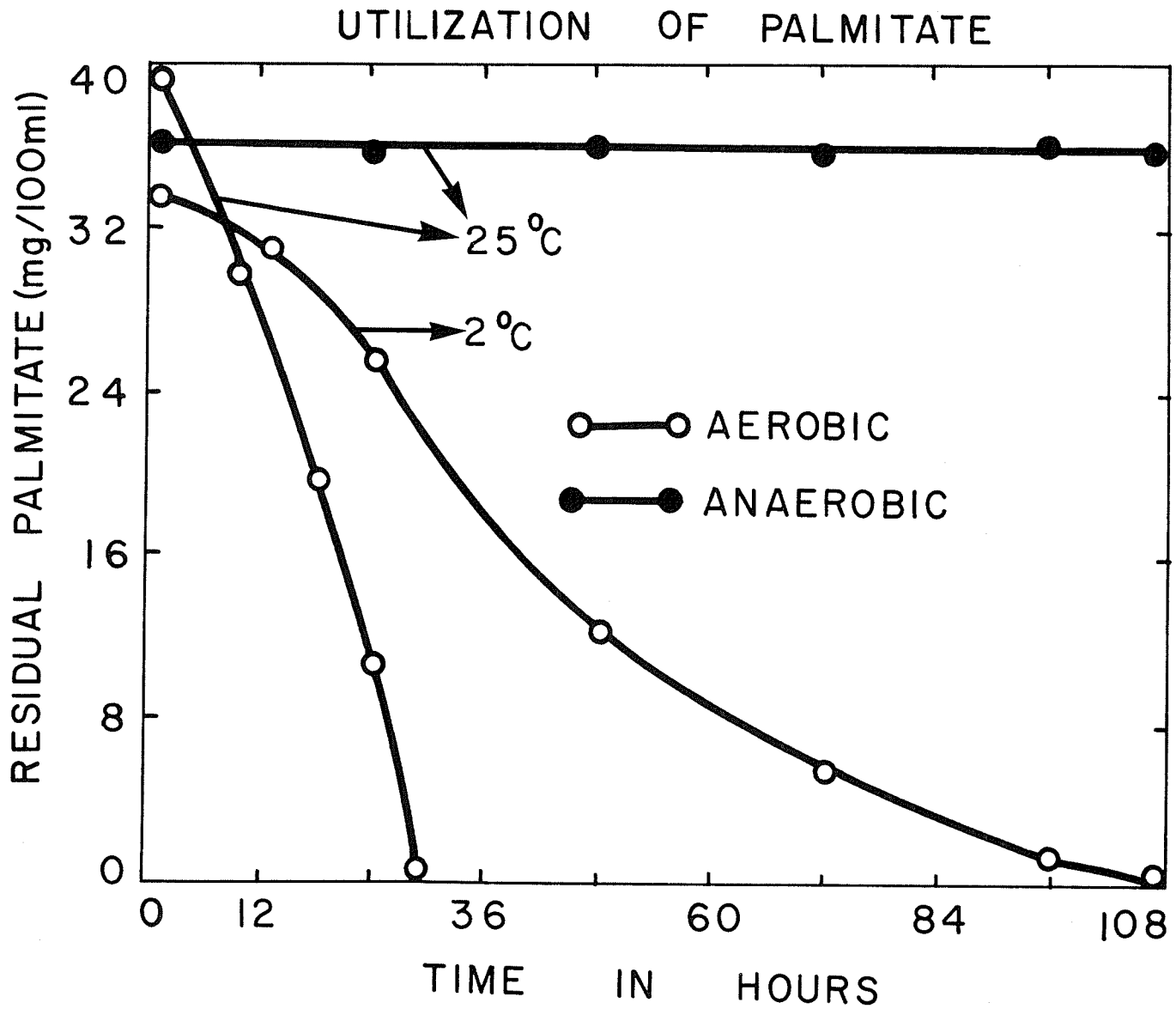

UTILIZATION OF ACETATE
RESIDUAL ACETATE (mg/100ml)
50
40
30
20
10
0
25°C
2°C
AEROBIC
ANAEROBIC
0
8
16
24
TIME IN HOURS

Fig. 15. Utilization of palmitate by lagoon bacteria; 25 °C represents utilization by mesophiles recovered from samples taken during summer and 2 °C represents utilization by psychrophiles recovered from samples taken during winter.


UTILIZATION OF PALMITATE
RESIDUAL PALMITATE (mg/100ml)
40
32
24
16
8
0
25 °C
2 °C
AEROBIC
ANAEROBIC
0
12
36
60
84
108
TIME IN HOURS

DISCUSSION

Carbohydrates, proteins, and fats encountered in biological waste treatment processes are the main energy sources for the bacteria in lagoons. The substrates tested were chosen as being representative of the various compounds of each category. Glucose was chosen to represent soluble carbohydrates. Vitamin-free casamino acids and egg albumin were chosen to represent proteins. Urea and creatinine were used because they are the major nitrogen ous compounds of domestic wastes. Acetate and palmitate were chosen to represent short and long chain fatty acids. When these compounds are degraded by a heterogeneous bacterial population, as is the case in lagoons, the mechanisms or pathways for their degradation must be diverse. The end products of metabolism of a substrate by one type of organism can serve as substrates for another type of organism.

Jeris and Cardenas (85) reported the utilization of glucose both aerobically and anaerobically at 25 °C and 35 °C respectively in a laboratory-scale digester unit seeded with microorganisms from a primary domestic waste treatment plant. Dawson and Jenkins (36) and Winzler (213) demonstrated glucose utilization at 28 °C by microorganisms taken from activated sludge or trickling filter units. No lag for

glucose utilization was detected in these studies. The results of the Warburg studies plotted in Fig. 4 show a lag of about 2 hours before glucose oxidation began; this may be attributed to acclimation by the lagoon bacteria. However, the results of the quantitative utilization of glucose (Fig. 9) do not support convincingly the interpretation given above and more work is needed to clarify this concept. Hess (74) detected the fermentation of glucose, sucrose, and maltose by strains of Pseudomonas, Flavobacterium, and Achromobacter at 0 °C in 22 days and at -3 °C in 65 to 120 days. The fermentation of glucose by psychrophiles at 0 °C and at lower temperatures has been demonstrated by Zobell (220), and Straka and Stokes (189). Ingraham and Bailey (82) found that the rate of growth and metabolic reactions of psychrophiles decreases more slowly with decreasing temperatures than that of mesophiles. The differences in rates of glucose oxidation between psychrophiles and mesophiles largely disappeared when cells were broken. They attributed that the difference in temperature response of mesophiles and psychrophiles is probably a result of some aspect of cellular organization rather than of enzymatic difference. The data obtained with lagoon bacteria show (Fig. 9) that glucose served as a good source of carbon and energy both for mesophiles and psychrophiles. The

transformations of glucose by bacteria are so diverse that it is not possible to predict the manner of its oxidation by lagoon bacteria.

Sperry and Rettger (182) demonstrated that some proteolytic bacteria were able to degrade albumin, serum proteins, egg albumin and crystalline edestin, but only in the presence of a small amount of peptone. They suggested that peptone was required for the synthesis of diffusible proteolytic enzymes. The failure of psychrophiles to decompose casamino acids and egg albumin (Figs. 11b, 12b) may be due to the inability of these proteins to enter the cells or a requirement for amendments to the amino acid pool required for the synthesis of proteolytic enzymes. Some amino acids are available to lagoon bacteria in domestic wastes (151), and proteins are expected to be degraded to simpler forms of nitrogen at the low temperatures found under natural conditions. Many thermophilic and mesophilic bacteria that produce proteolytic enzymes (16, 65, 146, 178, 203) have been isolated from natural sources but the present knowledge of the proteolytic activity of psychrophiles is limited (80, 158). Since the carbon moiety of amino acids and proteins on degradation yields either an intermediate of the TCA cycle or acetyl-coenzyme A, vitamin-free casamino acids and egg albumin are expected to be degraded by mesophiles in lagoons by means of the cycle.

Urea is readily hydrolyzed by urease into ammonia and carbon dioxide and the ammonia thus liberated may serve as a source of nitrogen for bacteria. The results (Fig. 12a) show that mesophiles are able to hydrolyze urea but the psychrophilic activity was extremely rapid (Fig. 12b). This rapid utilization of urea by psychrophiles provides evidence for seasonal fluctuation of organisms in the lagoon.

The data show (Fig. 13) that creatinine may also serve as a carbon and nitrogen source. Linneweh (116) reported the utilization of creatinine by some putrefactive bacteria isolated from soil. He identified ammonia and methyl-hydantoin as the end products of creatinine degradation. Dubos and Miller (37) using resting cell suspensions demonstrated the utilization of creatinine by two aerobic strains of bacteria isolated from soil. Urea and ammonia were identified as the end products of creatinine oxidation. Szulmajster (198) found that a resting cell suspension of a Clostridium was capable of metabolizing creatinine under anaerobic conditions.

Many microorganisms can derive all their carbon and energy from acetate (22, 99, 105). In this study, cell suspensions of mesophiles and psychrophiles were able to oxidize acetate under aerobic conditions. In a lagoon treating domestic wastes, all di- and tricarboxylic acids

necessary for the initiation of the TCA cycle are present and acetate in the form of acetyl-coenzyme A is probably completely oxidized under aerobic conditions by means of the cycle. Many workers have established that the TCA cycle is the only quantitatively important route for the complete oxidation of acetate (4, 100, 103). Further, studies with a variety of organisms have shown that, where the TCA cycle does not play a respiratory role, it still serves as the major source of precursors for cellular components (103, 101). McCarty and Vath (129) reported that acetate in an activated sludge digester was oxidized only in the presence of some unknown substances contained in the supernatant liquid of the domestic waste sludge digester. Speece and McCarty (181) reported that acetate utilization in an anaerobic digester unit was stimulated by the addition of iron filings and ferric chloride. Many workers have demonstrated that methane was an end product of acetate fermentation (23, 24, 86, 185). In these studies methane forming bacteria recovered from anaerobic systems were used. The lagoon bacteria were not able to ferment acetate probably because of a high redox potential near or at the top of the lagoon, the organisms capable of forming methane were absent or were not present in large numbers.

Palmitate may serve as a source of carbon for mesophiles and psychrophiles under aerobic conditions. Palmitate is most likely oxidized by the well established mechanism of β-oxidation; its utilization occurred only in the presence of molecular oxygen.

The results show that all the organic compounds used which are representative of domestic wastes were completely utilized by mesophiles and psychrophiles. The oxidation of fatty acids is oxygen dependent and proteins are also readily utilized aerobically, the aeration of ice-bond lagoons would make oxygen available at amounts required by the microorganisms. The comparative rates of mesophilic and psychrophilic activity on the utilization of organic substrates show that psychrophiles also play an important role in the stabilization of domestic wastes in lagoons. While the metabolic activities of psychrophiles are slow at 2 °C, the total contribution to stabilization by numerous processes which are taking place concurrently must be of vast significance.

SUMMARY

The results of a comparative study of mesophilic and psychrophilic activity on the utilization of several organic substrates have been presented. A rigorous climate induces a layer of ice over the lagoon for approximately 5 months of the year; this study was carried out using bacteria recovered from lagoon water during summer and winter. The results show the following:

1. Mesophiles were able to utilize glucose, vitamin-free casamino acids, egg albumin, urea, creatinine, acetate, and palmitate aerobically. However, under anaerobic conditions, acetate and palmitate were not metabolized.

2. Psychrophiles were able to utilize glucose, urea, creatinine, acetate, and palmitate in the presence of molecular oxygen, but no oxidation of vitamin-free casamino acids and egg albumin occurred at 2 °C and 25 °C. Moreover, acetate and palmitate were not degraded in the absence of molecular oxygen.

3. Urea was more rapidly metabolized by psychrophiles than by mesophiles.

4. With the exception of urea, psychrophilic activity was comparatively slow.

PART III

Degradation of Detergents by Lagoon Bacteria

INTRODUCTION

All synthetic detergents are not readily biodegradable and those which survive wastes treatment processes are ultimately discharged into surface or ground waters thereby creating deleterious effects such as foaming and bad taste. The anionic synthetic detergent, tetrapropylene alkyl benzene sulfonate (ABS), is one such noxious wetting agent. A plethora of reports has promulgated the serious problems arising from these undergraded 'syndets'.

Mixed bacterial populations indigenous to river water (67, 193, 194, 208), soil (168, 169), air (195), activated sludge (11, 17, 20, 68, 69, 96, 124, 165, 174, 175, 183, 192), trickling filters (96, 104, 183), septic tank percolation fields (95, 96, 190), and pilot scale oxidation ponds (95, 96) are known to degrade detergents of the linear alkylate sulfonate type (LAS) but the action of bacteria native to operational domestic wastes disposal units (lagoons) upon these detergents is not known. In spite of severe climatic conditions in some geographic regions, lagooning as a means of conditioning industrial and domestic wastes prior to any final innocuous disposal has become widely accepted. This

study was undertaken to investigate the effect of some physical parameters upon the degradation of several closely related anionic detergents by the microflora indigenous to a lagoon that experiences severe climatic conditions during portions of the year and to integrate these findings with the ecology of domestic wastes disposal by the lagooning method as a function of seasonal change.

MATERIALS AND METHODS

Detergents

The following detergents were used:

1. Tetrapropylene alkyl benzene sulfonate (ABS). A branched chain alkylate which is sulfonated to produce ABS.

2. Liqui-nox. Consists of anionic and non-ionic agents. The anionic agents are of the linear alkylate sulfonate type and the non-ionic portion consists of a linear chain octyl-phenol ethylene oxide condensate.

3. Commerical LAS Composite SDA 1-1. Prepared by sulfonation of a composite linear alkylate, chain length predominantly C_{11}, C_{12}, and C_{13}.

4. α-11 LAS. Prepared via alkylation of benzene with a narrow cut linear olifin derived from wax cracking, containing commercial type impurities.

5. α-14 LAS. Prepared from pure α-tetradecene.

6. $C_{11.3}$ LAS Commercial Type. Prepared by sulfonation of a linear alkylate having 11 carbon atoms and the phenyl group is attached to the 3 carbon.

7. $C_{13.3}$ LAS Commercial Type. Prepared by sulfonation of a linear alkylate having 13 carbon atoms and the phenyl group is attached to the 3 carbon.

ABS the only branched chain detergent used was kindly supplied by Dr. E.R. Blakley (Prairie Regional Laboratory, National Research Council, Saskatoon, Sask.). All other detergents were of the linear side chain type and were a generous gift of Dr. R.D. Swisher (Monsanto Chemical Co., St. Louis, Mo.) with the exception of Liqui-nox which is a commercial product (Alconox Inc., N.Y.).

Detergent Assay

The quantitative analysis of detergents was determined by the standard 'methylene blue' method (186). The method involves the solubilization by detergent action of methylene blue in chloroform and measuring spectrophotometrically the blue color of the chloroform solution. A colorimetric calibration curve prepared from ABS was used as the reference standard. The detergents measured in terms of ABS are more precisely reported as 'MBAS' (methylene blue active substance). Slight variations in the linear chain length of different detergents do not alter significantly the detergent action and no corrections were made for variations in chain length.

Detergent Utilization

To study the biodegradability of detergents, a mixed bacterial resting cell suspension prepared from the population indigenous to the surface layer of a domestic wastes disposal

unit (sewage lagoon) was used; the organisms from the primary cell were used. The sampling procedure, the quantitative determination of bacterial numbers in lagoon water and the preparation of resting cell suspensions of bacteria free of algae and protozoa have previously been described (see page 25).

To a 250 ml Erlenmeyer flask containing the resting cell suspension recovered from a 20 liter sample of lagoon water a suitable amount of the test detergent was added and the final volume was made up to 100 ml with distilled water. The flasks were then placed on a reciprocating shaker contained in a constant temperature water bath regulated to 25 °C $\pm$ 0.1 °C and were shaken constantly to ensure the oxygen supply was not limiting. Samples, usually 5 ml, were withdrawn at zero time and at appropriate time intervals thereafter, the cells were removed by centrifugation and the supernatant liquid was analyzed for residual detergent.

ABS, Liqui-nox, Commercial LAS, α-11 LAS, α-14 LAS, $C_{11.3}$ LAS and $C_{13.3}$ LAS were subjected to this treatment. Suitable controls were included in each case.

Effect of Temperature on the Utilization of Detergents

Liqui-nox and α-14 LAS were selected as representative detergents to study the effect of temperature on the utilization of detergents of the LAS type.

The activity of mesophiles was determined by utilizing the bacteria recovered from samples of lagoon water taken during mid-summer. Utilization was determined by the procedure already described with the temperature of the water bath regulated to 25 °C, 10 °C, and 2 °C $\pm$ 0.1 °C. Similarly, the activity of psychrophiles was determined by utilizing the bacteria recovered from samples of lagoon water taken from under an ice-cover during mid-winter. The utilization of each detergent was determined only at 2 °C $\pm$ 0.1 °C.

Effect of Oxygen Tension on the Utilization of Detergents

The detergents were not expected to be metabolized under anaerobic conditions, therefore, only Liqui-nox was selected as a representative compound to confirm the expected requirement of molecular oxygen for detergent degradation. In this experiment, utilization of Liqui-nox was studied both under aerobic and anaerobic conditions. For aerobic studies, 250 ml Erlenmeyer flasks containing resting cell suspensions to which Liqui-nox had been added were incubated at 25 °C on a reciprocating shaker. For anaerobic studies, serum bottles sealed with serum caps were kept static after combining the cell suspension and the substrate. To ensure completely anaerobic conditions in the serum bottles, samples were removed by means of a hypodermic syringe and the bottles were flushed thoroughly with nitrogen

after the withdrawal of each sample. In both systems, samples were removed at regular intervals and were analyzed for residual detergent as described previously.

Naturally Occurring 'MBAS' and its Biological Removal from Lagoon Water

From the beginning of June to mid-October, 1966, surface samples of lagoon water were collected at weekly intervals whenever the weather was permissible. In each sample, cells were removed by centrifugation and the supernatant liquid was analyzed for the presence of naturally occurring 'MBAS' by the standard method.

To determine the removal of naturally occurring 'MBAS' by the indigenous microflora of the lagoon, lagoon water was used both as the source of organisms and the source of detergent. The disappearance of a specific compound undergoing stabilization in a lagoon is difficult to measure because, under normal lagoon operating conditions, fresh material is being continually introduced to replace the effluent. To circumvent the problem of a continuous addition of detergent and to investigate the removal of naturally occurring 'MBAS', a surface sample of lagoon water (approximately 15 liters) was aerated in the laboratory at room temperature using moist sterile air.

Samples were withdrawn at zero time and appropriate time intervals thereafter and were analyzed for the presence of residual naturally occurring 'MBAS'.

RESULTS

To compare the rate of detergent utilization, the results are expressed on the basis of the physiological activity of resting cell suspensions containing 2×10^8 cells per ml. Table 1 compares the rate of removal of ABS, α-11 LAS, α-14 LAS, $C_{11.3}$ LAS, $C_{13.3}$ LAS and Commercial LAS. The resistance of ABS to biodegradation is readily seen; no utilization occurred at 25 °C over the entire 5 day test-period. In sharp contrast, all the LAS type detergents were utilized almost completely under these conditions. The slight difference in biodegradability of the linear chain detergents is not thought to be significant in terms of practical use.

Utilization of α-14 LAS and Liqui-nox as a Function of Temperature

The studies show that LAS type detergents were readily degraded by lagoon bacteria but the organisms responsible for this degradation are sensitive to temperature changes. The time-course degradation curves for α-14 LAS and Liqui-nox under aerobic conditions at 25 °C, 10 °C and 2 °C $\pm$ 0.1 °C are shown in Figs. 16 and 17. With an initial concentration of α-14 LAS and Liqui-nox of 44 and 48 ppm, both detergents

TABLE 1

Utilization of detergents by resting cell suspensions of lagoon bacteria

Time in hours	Detergent*					
	ABS	Commercial LAS	$C_{11.3}$LAS	$C_{13.3}$LAS	α-11 LAS	α-14 LAS
0	100.0‡	100.0	100.0	100.0	100.0	100.0
12	-	81.2	98.0	91.3	83.3	85.2
24	97.9	57.1	68.0	69.6	50.0	45.8
36	-	46.4	36.0	43.5	30.0	29.6
48	100.0	35.6	28.0	34.8	23.3	18.5
60	0	26.8	20.0	26.1	16.7	11.1
72	93.2	21.4	16.0	17.4	10.0	3.7
84	-	12.3	8.0	8.7	6.7	1.8
96	100.0	7.0	2.5	2.2	1.7	1.8
108	-	3.6	2.5	2.2	1.7	1.8
120	100.0	1.8	2.5	2.2	1.7	1.8

*Initial concentration

ABS; 29.3 ppm

Commercial LAS; 37.3 ppm

$C_{11.3}$ LAS; 40.0 ppm

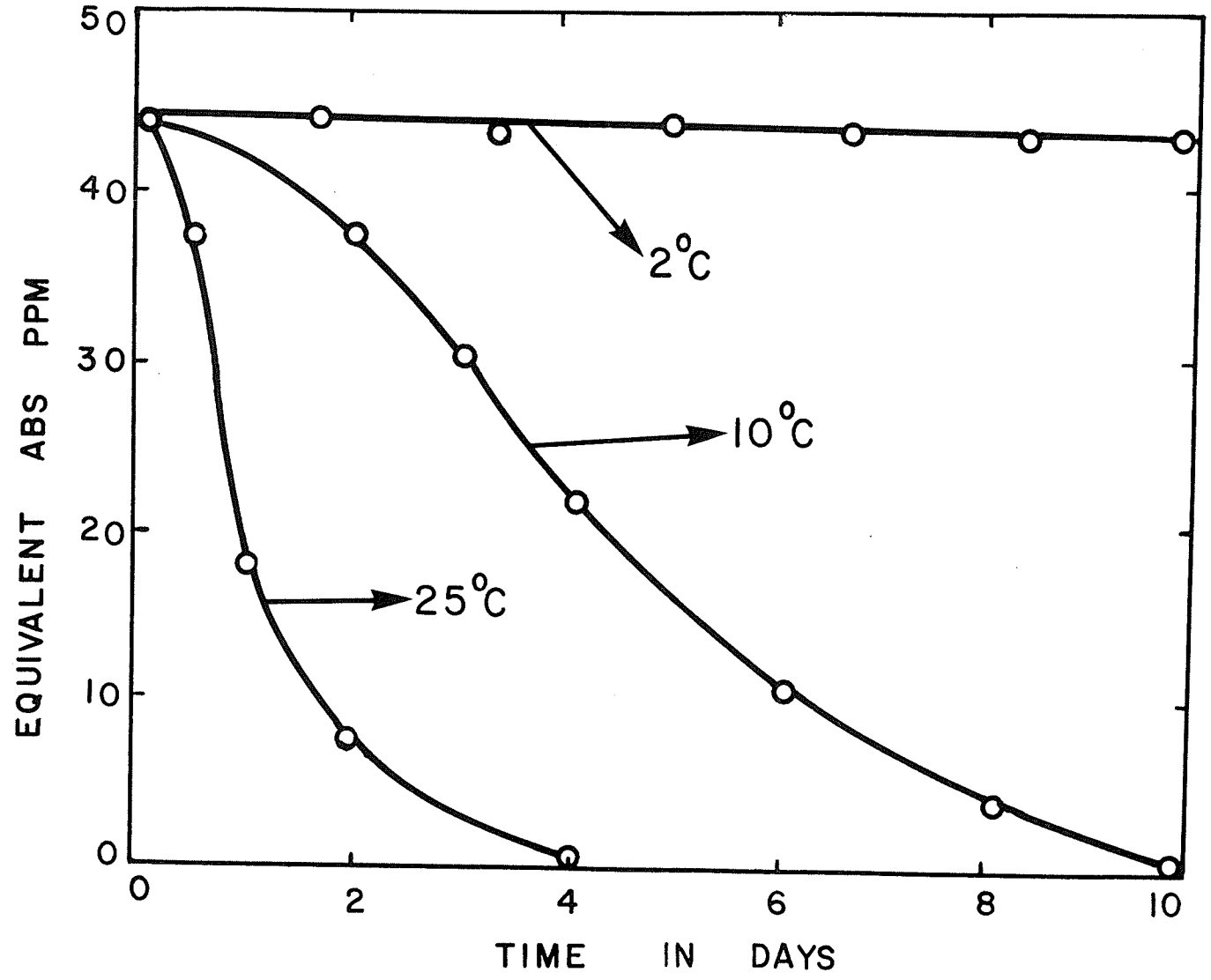
$C_{13.3}$ LAS; 36.8 ppm

α-11 LAS; 48.0 ppm

α-14 LAS; 43.2 ppm

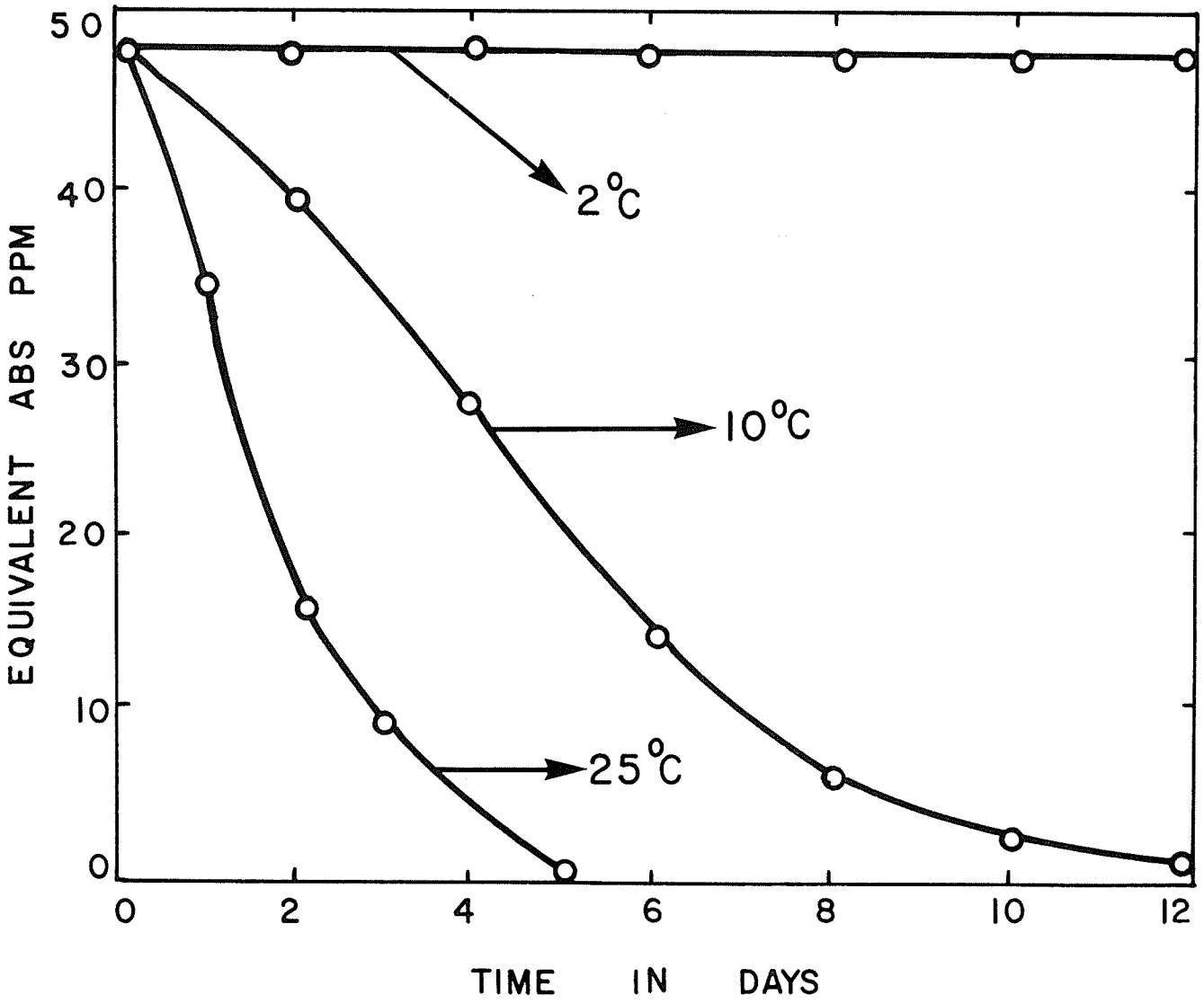
‡ Percent initial concentration.

Fig. 16. Utilization of α-14 LAS at various temperatures by a resting cell suspension of lagoon bacteria.



UTILIZATION OF α-14 LAS

Fig. 17. Utilization of Liqui-nox at various temperatures by a resting cell suspension of lagoon bacteria.



UTILIZATION OF LIQUINOX

were nearly completely utilized at 25 °C in a period of 4 and 5 days respectively whereas 10 and 12 days respectively were required to achieve the same result when the temperature was lowered to 10 °C. No utilization of either detergent occurred at 2 °C over the entire period studied. Bacteria recovered from under the ice-cover during mid-winter were not able to degrade any of the detergents used in this study at 2 °C.

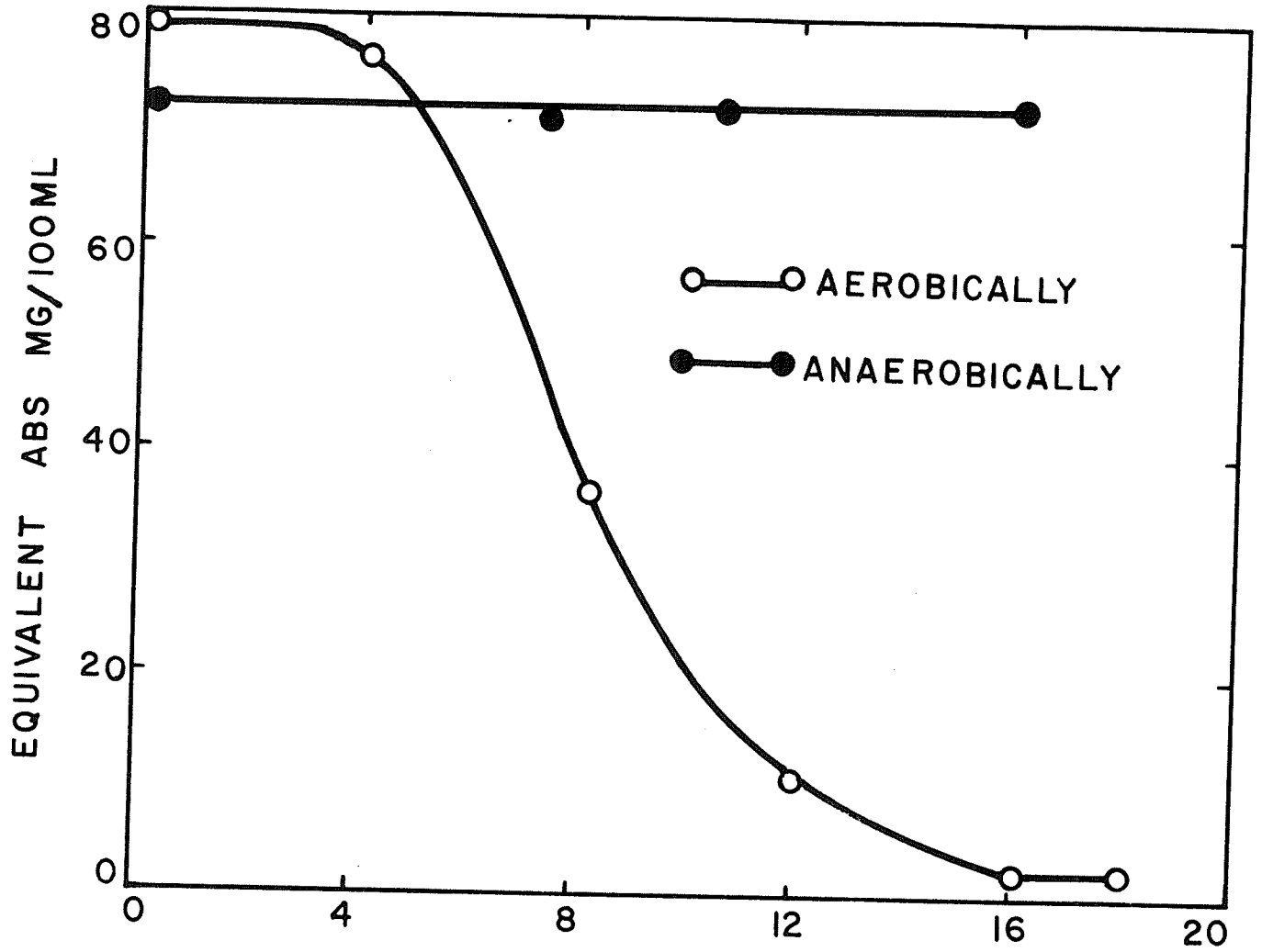
Utilization of Liqui-nox as a Function of Oxygen Tension

The disappearance curves for Liqui-nox under both aerobic and anaerobic conditions at 25 °C are shown in Fig. 18. When the initial concentration of Liqui-nox was 80 ppm, the utilization was preceeded by an acclimation period of approximately 4 days after which the detergent was almost completely degraded in 12 days. Metabolism did not occur in the absence of molecular oxygen.

Presence of Naturally Occurring 'MBAS' in Lagoon Water and its Utilization by Lagoon Bacteria.

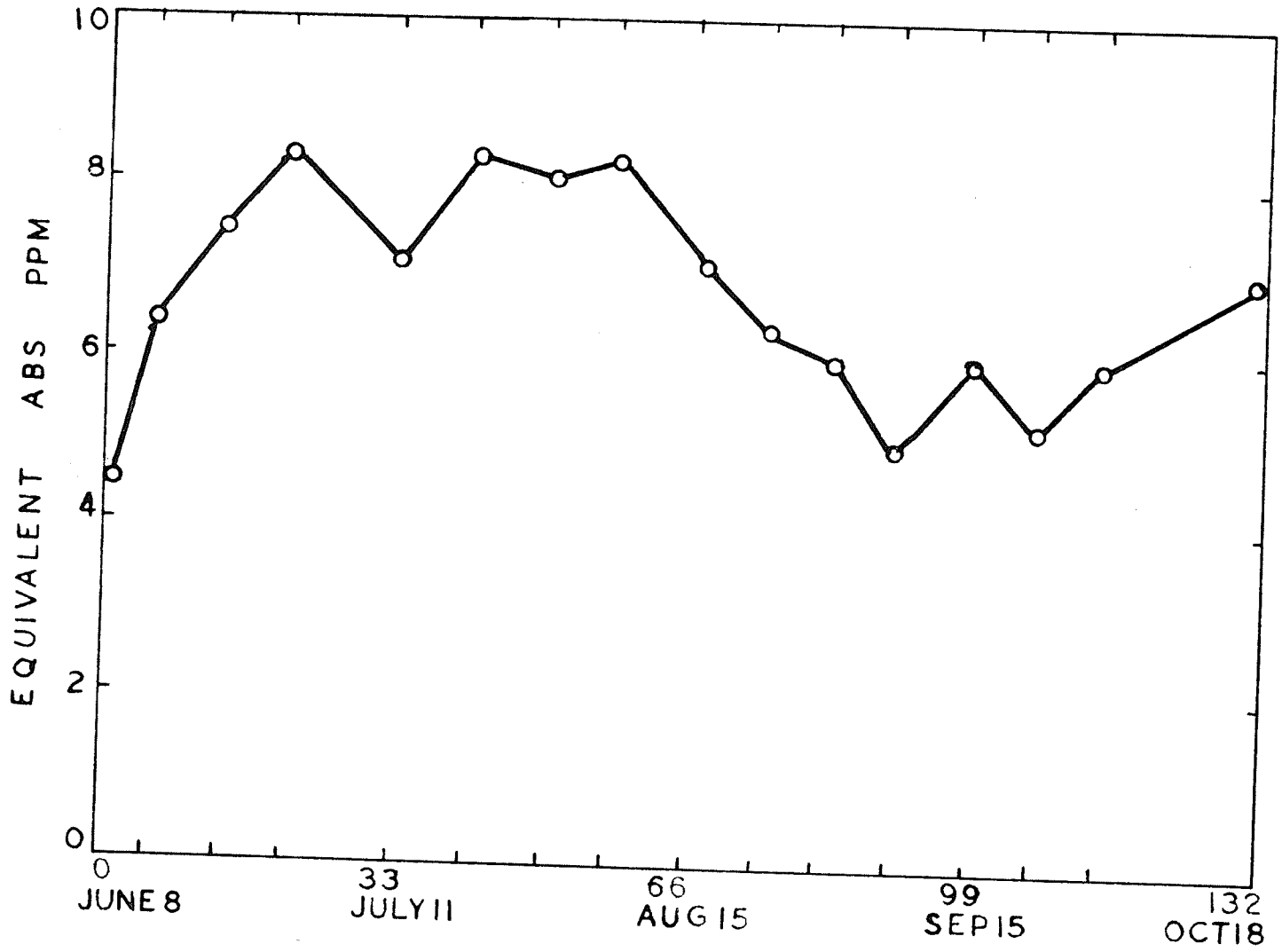
Fig. 19 shows the concentration of naturally occurring 'MBAS' found in the lagoon water from the beginning of June to the middle of October, 1966. The concentration varied from 4.2 to 8.2 'MBAS' ppm.

Fig. 18. Utilization of Liqui-nox by a resting cell suspension of lagoon bacteria under aerobic and anaerobic conditions.



UTILIZATION OF LIQUINOX

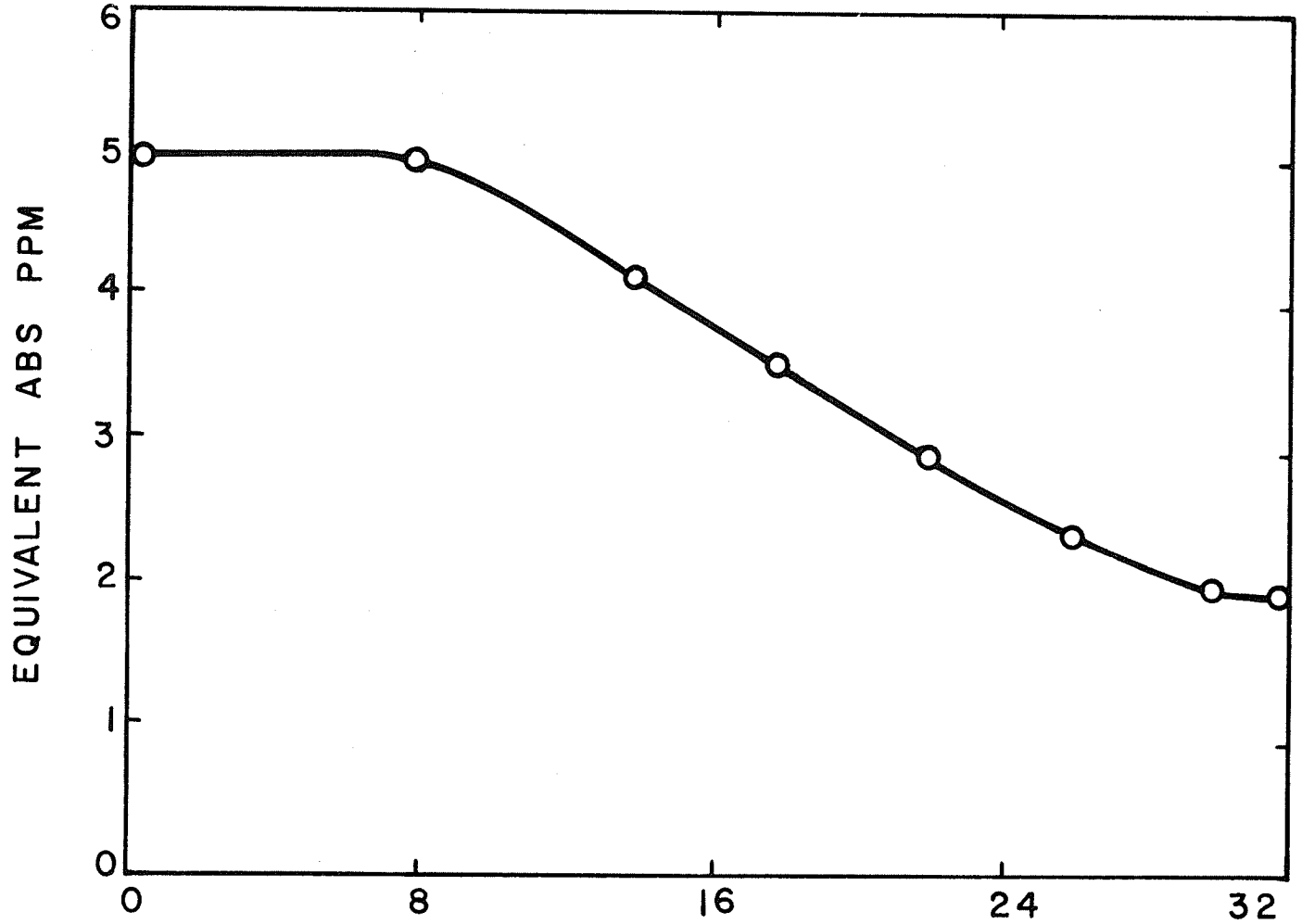
Fig. 19. Variation in the concentaration of 'MBAS' in the Charleswood lagoon as a function of seasonal change.



'MBAS' IN LAGOON WATER

The removal of naturally occurring 'MBAS' by indigenous lagoon microflora is shown in Fig. 20. The initial concentration remained unchanged at 5.0 'MBAS' ppm during an acclimation period of about 8 days after which degradation was initiated. In 30 days approximately 40% of the 'MBAS' was still present. The results show that significant amounts of the resistant branched chain ABS were still being used in the Winnipeg area in 1966.

Fig. 20. Utilization of naturally occurring 'MBAS' by lagoon bacteria.



REMOVAL OF 'MBAS' IN LAGOON WATER

DISCUSSION

The noxious nature of ABS, due to its resistance to biodegradation, is well documented (131, 136, 144, 174, 175, 193, 200). Currently, it accounts for the principal component in surfactant residues and it is not unexpected that lagoon microorganisms, like those flora associated with other sewage treatment processes, cannot degrade it. The detergent industry is now in the process of replacing resistant ABS with biodegradable LAS in its formulations, and since these anionic detergents account for approximately 75% of the detergent market, this transition when completed should reduce considerably problems associated with detergent residues.

Swisher (194), Sawyer and Ryckman (175), and Sweeney and Foote (192) have demonstrated 96 to 100 % removal of LAS detergent in river water in 6 to 40 days and in activated sludge units in 5 to 9 days. Similarly, Klein and McGauhey (96) reported 97% removal in septic tank percolation field systems, 93% in conventional type pilot stabilization ponds and 95% in standard rate activated sludge units. All these studies, however, were conducted in the mesophilic range and in the presence of molecular oxygen.

The failure of Liqui-nox to be metabolized anaerobically as shown in Fig. 18, is consistent with the molecular oxygen-

dependent β-oxidation mechanism for the degradation of long chained aliphatics and the oxygen-dependent mechanisms for the cleavage of aromatic ring (79, 196).

This is the first report of the utilization of LAS type detergents in full scale operational sewage lagoons. These studies show that linear alkylate sulfonates would be readily degraded by lagoon microflora during summer when favourable temperatures are induced by a warm climate and molecular oxygen is provided by the air/water interface and by oxygen release accompanying the photosynthetic action of algae. The lagoon under study is ice-bound for approximately five months of the year. Clearly, LAS detergents could not be degraded during this period of the year due to the absence of molecular oxygen.

It has never been established that psychrophiles are able to degrade LAS. These organisms, however, play a more important role in the stabilization process than has previously been recognized (66). During the ice-free period of 1965, the temperature of this lagoon remained below 10 °C for approximately 54 of 197 ice-free days (see Fig. 2). When the lagoon is ice-bound, the exclusion of molecular oxygen creates a habitat suitable only for the development of strict anaerobic and facultative anaerobic psychrophiles. Clearly, no organic transformations involving mechanisms

that are molecular oxygen-dependent would be expected to occur during this period.

A recent innovation in lagoon management employs aeration devices to provide a continuous supply of oxygen throughout the year. The indigenous population under these conditions might differ from the microflora of lagoons that are not aerated, particularly under climatic conditions that induce an ice cover. However, a heavy resting cell suspension prepared from the lagoon water samples collected from an aerated lagoon in the beginning of April, 1968, was not able to metabolize Liqui-nox and commercial LAS at 2 °C under both aerobic and anaerobic conditions.

The methylene blue test is a relatively specific test for the intact surfactant molecule. When the sulfate or sulfonate group is removed or the alkyl side chain is shortened to eight carbons in length or less, the surfactant property is lost. Thus, the test does not truly measure the extent of degradation, but loss of detergent action is concomitant with loss in the deleterious effects of the cleansing agents and the extent of decomposition need not be measured with greater precision.

Slight fluctuations in the 'MBAS' concentration during the summer months (Fig. 19) are due undoubtedly to variations in the bacterial populations (a function of the operative BOD

load), to changes in the concentration of fresh detergent supplied in the influent to the lagoon and to the dilution effect caused by fluctuations in the flow of river water associated with lagoon management. The increase in the naturally occurring 'MBAS' in lagoon water during June (Fig. 19) might be a 'diauxie effect' with biologically labile organic compounds which have accumulated over the winter months being preferentially utilized over the anionic detergent. This concept is strengthened by the detection of a pronounced lag period prior to the removal of naturally occurring 'MBAS' in lagoon water (Fig. 20) which corresponds to the time required to deplete the lagoon water of labile organic materials prior to detergent removal. Detergent which escapes decomposition in the primary cell is fed into the secondary cell where the lagoon water undergoes its final polishing. The physiological activity associated with the secondary cells of this dual cell stabilization unit has not been studied. Probably this is the site of the destruction of the least labile organic material in domestic wastes where readily utilizable organic materials are not abundant.

SUMMARY

The results of experiments investigating the degradation of several alkyl benzene sulfonate type detergents by the microflora indigenous to a lagoon that experience severe climatic conditions during portions of the year are presented.

1. α-11 LAS, α-14 LAS, $C_{11.3}$ LAS, $C_{13.3}$ LAS and commercial LAS were metabolized very readily by lagoon bacteria only during the summer months when favorable temperatures were induced by a warm climate.

2. ABS was not degraded even at 25 °C over the entire test period.

3. α-14 LAS and Liqui-nox were completely utilized by mesophiles at 25 °C and 10 °C but no utilization occurred at 2 °C.

4. Psychrophiles recovered from under the ice-cover during the mid-winter were unable to degrade any of the detergents at 2 °C, the only temperature studied.

5. Psychrophiles derived from ice-free aerated lagoon during the beginning of April, 1968, were unable to utilize commercial LAS and Liqui-nox at 2 °C.

6. Liqui-nox was not metabolized under anaerobic conditions.

PART IV

Degradation of Some Insecticides by Lagoon Bacteria

INTRODUCTION

The past two decades have seen an intensive increase in the production and use of synthetic organic pesticides (170) and the ecological hazards which pesticides and their residues create by contamination of soils, rivers, streams, and lakes are well known (84). These chemicals applied to control agricultural and forest pests enter surface or ground waters from many sources; run off from agricultural lands, drift from aerial and land applications, discharge of industrial wastes, and discharge of waste water from clean-up of equipment used for pesticide applications are some. The subject of pollution of the water environment by pesticides has recently been reviewed (43).

Chlorinated hydrocarbon insecticides are very widely used in spite of their resistance to degradation, and their persistence for substantial periods of time after application is well known (5, 46, 111, 112, 113, 114, 199, 211, 215, 216). DDT (18, 157, 171) and Dieldrin (156), for example, have been recovered from river water in concentrations of 1-20 ppb and 1 ppb respectively, the source of the pollutant being run off from the soil in juxtaposition to the river. More

than 50% of Dieldrin, BHC, and DDT have been recovered from soils 6 years after the initial application (170). Cole et al (33) were able to find DDT and Dieldrin in watershed soils and water samples taken from areas which had not previously been treated with these insecticides.

Contrary to chlorinated hydrocarbon insecticides, many organophosphorus insecticides are known to be metabolized very rapidly by bovine rumen fluid (3), soil bacteria (2, 125), fungi (125), yeasts and algae (2).

With the expanding use of insecticides, there is a great need for data pertaining to the direct and long term effects of chemicals used for pest control on soil and aquatic biota, on uptake by growing plants and on animals which ingest foods exposed to insecticides. Clearly, the replacement of resistant insecticides by those with a shorter 'half life' should be encouraged to avoid persistence and accumulation.

The examination of domestic wastes to be treated in the lagoon showed the presence of some organophosphorus and chlorinated insecticides. If these insecticides are not degraded by lagoon bacteria, the lagoon effluents when conducted into a river, will result in river water contamination. Therefore, this study was undertaken to determine the biodegradability by lagoon bacteria of some chlorinated hydrocarbon

insecticides with a known history of persistence and of some organophosphorus insecticides with a more acceptible performance of degradation.

MATERIALS AND METHODS

Insecticides

The following insecticides were selected for study: the organophosphates Malathion, Parathion and Diazinon and the chlorinated hydrocarbons Heptachlor, Dieldrin and DDT. These insecticides were of analytical grade and were used without further purification. All were obtained from City Chemical Corp., New York, except Malathion which was obtained from American Cyanamid Co., Agricultural Division, Princeton, N.J., N.Y.

Gas Chromatographic Analysis of Insecticides

A model GC-200 Micro-Tek (Baton Rouge, Louisiana) gas chromatograph equipped with an electron capture detector and a stacked sodium flame detector was used for the quantitative analysis of insecticides.

The following abbreviations are used:

Malathion: S-1,2 bis-(ethoxycarbonyl)-ethyl O,O-dimethyl phosphorodithioate.

Parathion: O-O-diethyl O-P-nitrophenyl phosphorothioate

Diazinon: O-O-diethyl O-(2 isopropyl-4-methyl-6 pyrimidinyl) phosphorothioate.

Heptachlor: 1,4,5,6,7,8,8-heptachloro-3α,4,7,7α-tetrahydro-4,7-methanoindene.

Dieldrin: 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4α,5,6,7,8,8α-octahydro-1,4,5,8-dimethanonaphthalene.

DDT: 1,1,1-trichloro-2,2-bis(P-chlorophenyl) ethane.

A pyrex column 2' x 1/4" packed with 5% silicone fluid QF1, FS1265 on 100-120 mesh chromosorb W acid-washed DMCS-treated (Chromatographic Specialties, Brockville, Ont.) was used.

For the analysis of chlorinated hydrocarbons, an electron capture detector was used with operating temperatures for the injection port, column, and detector at 200, 180, and 180 °C respectively. The carrier gas was a mixture of 5% methane and 95% argon at a flow rate of 40 ml per minute with a scavenger flow of 20 ml per minute. The pulsed power supply was operated at 50 v, 1-3 μsecs on and 50 μsecs off.

For the quantitative analysis of organophosphates, a stacked sodium flame detector was employed and the operating temperatures for the injection port, column, and detector were 310, 180, and 245 °C respectively. The carrier gas was nitrogen with a flow rate of 40 ml per min; other gases were hydrogen at a flow rate of 35 ml per minute for each flame and air at a flow rate of 200 ml per min for each flame. The upper flame ignitor coil was coated with sodium sulphate salt.

To increase the sensitivity of the instrument a special flash injector (180) was used which permits the injection of large aliquots. The integral power supply of the electrometer provided a 300 v negative D.C. accelerating voltage to

the upper and lower flame tips and coil assemblies of the detector. A series flame power supply (1) was employed to ignite the flames and to provide an additional means of heating the sodium sulphate-coated coil in the upper flame. A current of 1.2 amps was used to heat this coil to provide a background current of 3.2×10^{-10} amps.

Microorganisms

The organisms used were a mixed bacterial population indigenous to the primary cell of a dual cell domestic wastes disposal unit and were collected from the surface layer (top 6 in) of the lagoon during late summer, 1967. The quantitative determination of bacterial numbers in lagoon water, and the preparation of bacterial cell suspensions free of algae and protozoa were the same as previously described (page 25, 26).

Biodegradation of Organophosphates

The bacteria recovered from 40 liters of lagoon water were washed twice in 0.05 M potassium phosphate buffer, pH 7.0, and were resuspended in the same buffer to a volume of 180 ml. A standard insecticide solution containing 5,000 µgm per ml each of Malathion, Parathion, and Diazinon in ethanol was prepared, 2 ml of this solution was added to the resting cell suspension and the final volume was made up to 200 ml with

phosphate buffer. This gave a final concentration of approximately 50 ppm of each insecticide. Preliminary trials showed that the addition of several insecticides concurrently rather than singly did not affect the rate of insecticide utilization and accordingly the multiple addition procedure was adopted in order to reduce technical demands.

To investigate the effect of molecular oxygen on the degradation of insecticides by lagoon bacteria, the study was carried out under both aerobic and anaerobic conditions. For aerobic studies, 100 ml of the cell suspension to which the insecticides had been added was transferred to a 250 ml Erlenmeyer flask and the flask was shaken continuously on a wrist-action shaker (Burrell Corp., Pittsberg, Pa.) at a minimum speed in a constant temperature water bath at 25 °C. For anaerobic studies, the remaining 100 ml of cell suspension containing insecticides was transferred to a 120 ml serum bottle. The serum bottle was sealed with a serum cap, flushed with nitrogen for 30 secs, and kept static at 25 °C. At zero time and at appropriate intervals thereafter, 5 ml aliquots were withdrawn from both systems and transferred to specially constructed glass vials for extraction of residual insecticide (described later). To ensure complete anaerobiosis in the serum bottle, samples were removed by means of a hypodermic syringe and the bottle was flushed thoroughly with nitrogen after the withdrawal of each sample.

Biodegradation of Chlorinated Hydrocarbons

The procedure to determine the utilization of chlorinated hydrocarbons was slightly modified from that used for the organophosphates. A resting cell suspension was prepared from 20 liters of lagoon water to which were added the chlorinated hydrocarbons Heptachlor, Dieldrin, and DDT to a final concentration of 10 ppm for each insecticide. Immediately, 5 ml was pipetted into each of 16 glass vials. Magnetic stirring was employed during the pipetting to give as random samples as possible. This step was mediated by the poor solubility of these insecticides in water and was included to circumvent problems of sample heterogeneity due to possible binding of the insecticides to the bacterial cells and to the walls of the glass container. For aerobic studies, eight vials were shaken continuously on a wrist-action shaker at low speed at room temperature. One vial was removed periodically for analysis and the entire contents of each vial were extracted and analyzed for residual insecticides. For anaerobic studies, a parallel set of glass vials was prepared. These vials possessed constricted necks and could be capped with Teflon caps. They, too, were flushed with nitrogen for about 30 sec at zero time and remained static over the entire test period.

Extraction Procedure

A micro-extraction method was developed to recover insecticides from small samples; in this case as little as 5 ml. To each glass vial containing a 5 ml sample, an equal volume of hexane was added and the contents were mixed vigorously for 30 sec on a Vortex Jr. mixer (Scientific Industries Inc., Springfield, Mass.). The glass vials (25 ml capacity) possessed constricted necks to confine the extraction solvent. The emulsion which formed was broken by slow speed centrifugation and the hexane layer containing the insecticides was decanted and filtered through anhydrous sodium sulphate to remove dissolved and entrained water. The aqueous phase was extracted twice more and the hexane extracts from each sample were then combined.

For the analysis of organophosphates, the hexane extracts of each sample were concentrated to exactly 1 ml; for the analysis of chlorinated hydrocarbons, the hexane extracts were diluted to exactly 50 ml. Depending upon the concentration of the insecticides in the extract, 1-6 µl was injected into the gas chromatograph. No special clean-up procedure was required prior to analysis.

Extraction Efficiency

The efficiency of the extraction procedure and the percentage recovery of organophosphates and chlorinated hydrocarbons was determined. Both distilled water and resting cell suspensions were spiked with 50 ppm each of Malathion, Parathion and Diazinon or 10 ppm each of Heptachlor, Dieldrin and DDT. Immediately, 5 ml aliquots were extracted with hexane and analyzed as previously described.

RESULTS

Gas Chromatographic Analysis of Insecticides

Figs. 21 and 22 show standard curves for the quantitative determination of the chlorinated hydrocarbons and the organophosphates. At full scale recorder response, the electron capture detector can readily detect under operative conditions 0.1 ng of the chlorinated hydrocarbons and the stacked sodium flame detector can readily detect 100 ng of phosphorus containing compounds.

The retention time of insecticides used and some metabolites that were identified are given in Table II; all compounds are easily separated by the gas chromatography.

Extraction Efficiency

Table III shows that recoveries of 97-101% of the organophosphates added to either water or resting cell suspensions could be achieved by the micro-extraction procedure. Recoveries for the chlorinated hydrocarbons were considerably poorer and at best only 80% approximately of Heptachlor, Dieldrin, and 60% approximately of DDT could be recovered from resting cell suspensions.

Fig. 21. Linearity plot of peak height vs. sample size (in nanograms) of DDT, Dieldrin, and Heptachlor.

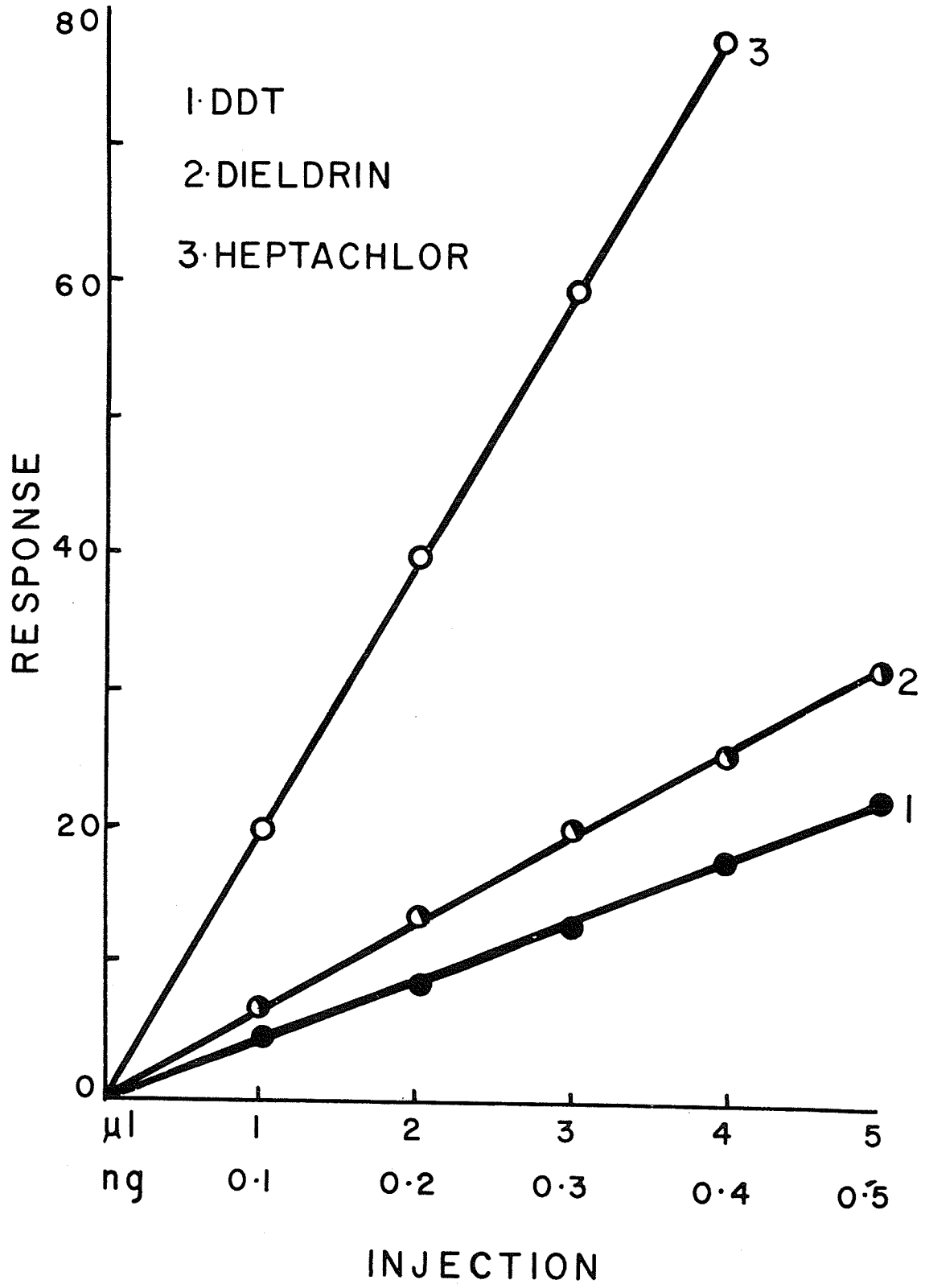

80
60
40
20
0
RESPONSE
1·DDT
2·DIELDRIN
3·HEPTACHLOR
3
2
1
µl
1
2
3
4
5
ng
0·1
0·2
0·3
0·4
0·5
INJECTION

Fig. 22. Linearity plot of peak height vs. sample size (in nanograms) of Parathion, Malathion, and Diazinon.

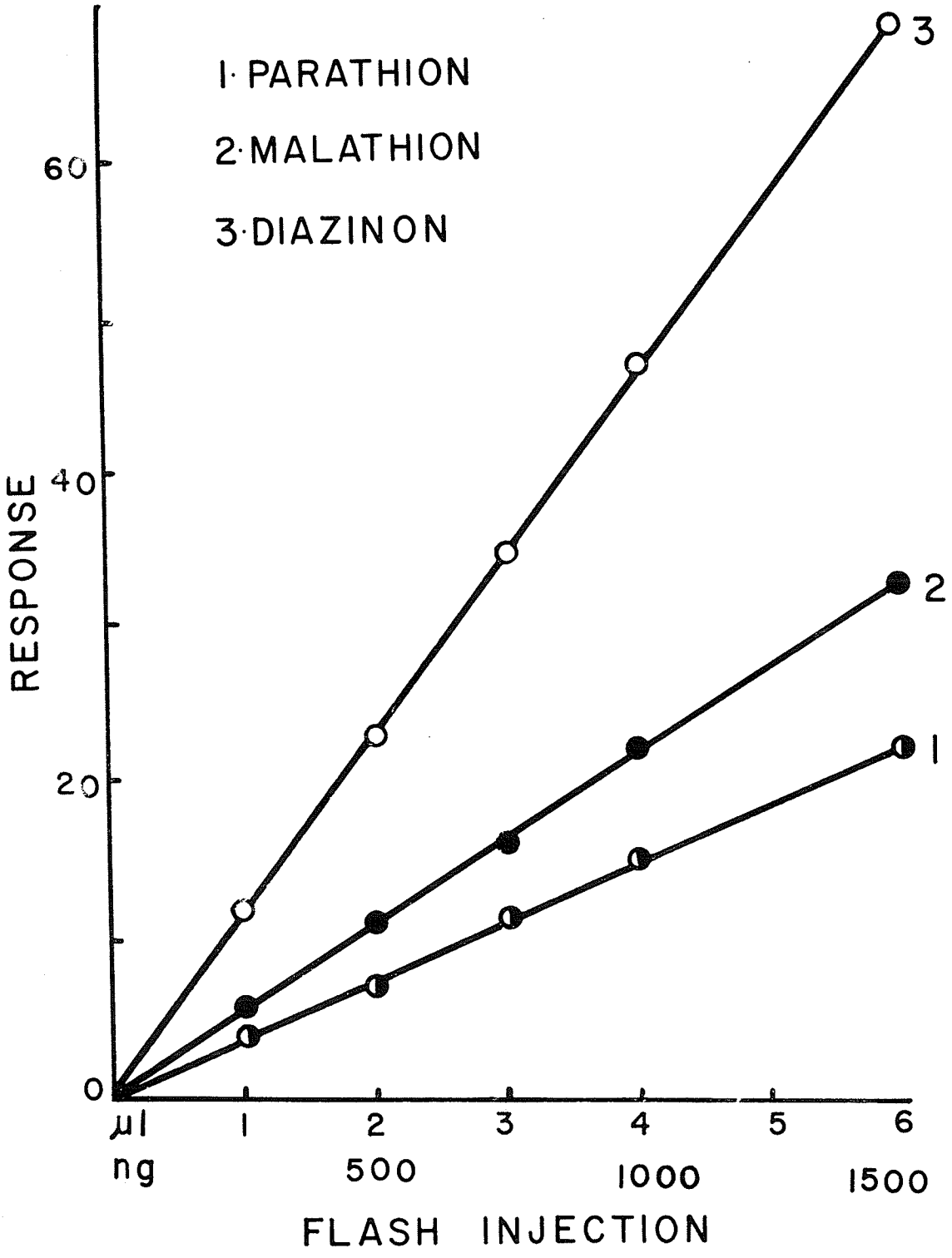

1·PARATHION
2·MALATHION
3·DIAZINON
RESPONSE
0
20
40
60
3
2
1
µl
ng
1
2
500
3
4
1000
5
6
1500
FLASH INJECTION

TABLE II

Gas chromatographic retention time of some organophosphate and chlorinated hydrocarbon insecticides, and some identified metabolites.

Insecticide	Detector	Retention time (min)
Diazinon	Stacked sodium flame	0.9
Malathion	Stacked sodium flame	2.2
Parathion	Stacked sodium flame	2.8
Heptachlor	Electron capture	1.8
Heptachlor epoxide	Electron capture	3.8
Dieldrin	Electron capture	6.0
DDD	Electron capture	7.4
DDT	Electron capture	8.2

TABLE III

Efficiency of extraction of insecticides.

Insecticide	Percent recovery	
	Distilled water	Resting cell suspension
Malathion	100.0	97.0
Parathion	101.0	100.0
Diazinon	98.8	98.0
Heptachlor	100.0	79.4
Dieldrin	86.1	79.0
DDT	88.0	60.4

All values are the average of four determinations.

Utilization of Malathion, Parathion, and Diazinon

The lagoon bacteria were able to utilize Malathion almost completely in 4 and 5 days under anaerobic and aerobic conditions respectively as shown in Fig. 23. Two small peaks appeared between Diazinon and Malathion in the sample taken after 3 days of incubation under aerobic conditions. One of these disappeared by day 4, while the concentration of the other (based on peak height) remained unchanged till day 6 but had disappeared by day 8. These metabolites were proven to be degradation products of Malathion but no attempt at identification was made.

The lagoon bacteria metabolized nearly all the Parathion in 5 days under both aerobic and anaerobic conditions (Fig. 24). One metabolite of Parathion was detected in the sample taken after 8 hours of incubation under anaerobic conditions. The concentration of this unknown metabolic product increased proportionally with a decrease in the concentration of Parathion reaching a maximum on day 6 after the parent compound had completely disappeared (see Fig. 24) and remained unaltered throughout the remaining test period.

Diazinon completely disappeared in 8 days under aerobic conditions while under anaerobic conditions its breakdown occurred slowly but steadily; only 56.6% was degraded in 10 days (Table IV).

Fig. 23. Degradation of Malathion by a resting cell suspension of lagoon bacteria under aerobic and anaerobic conditions.

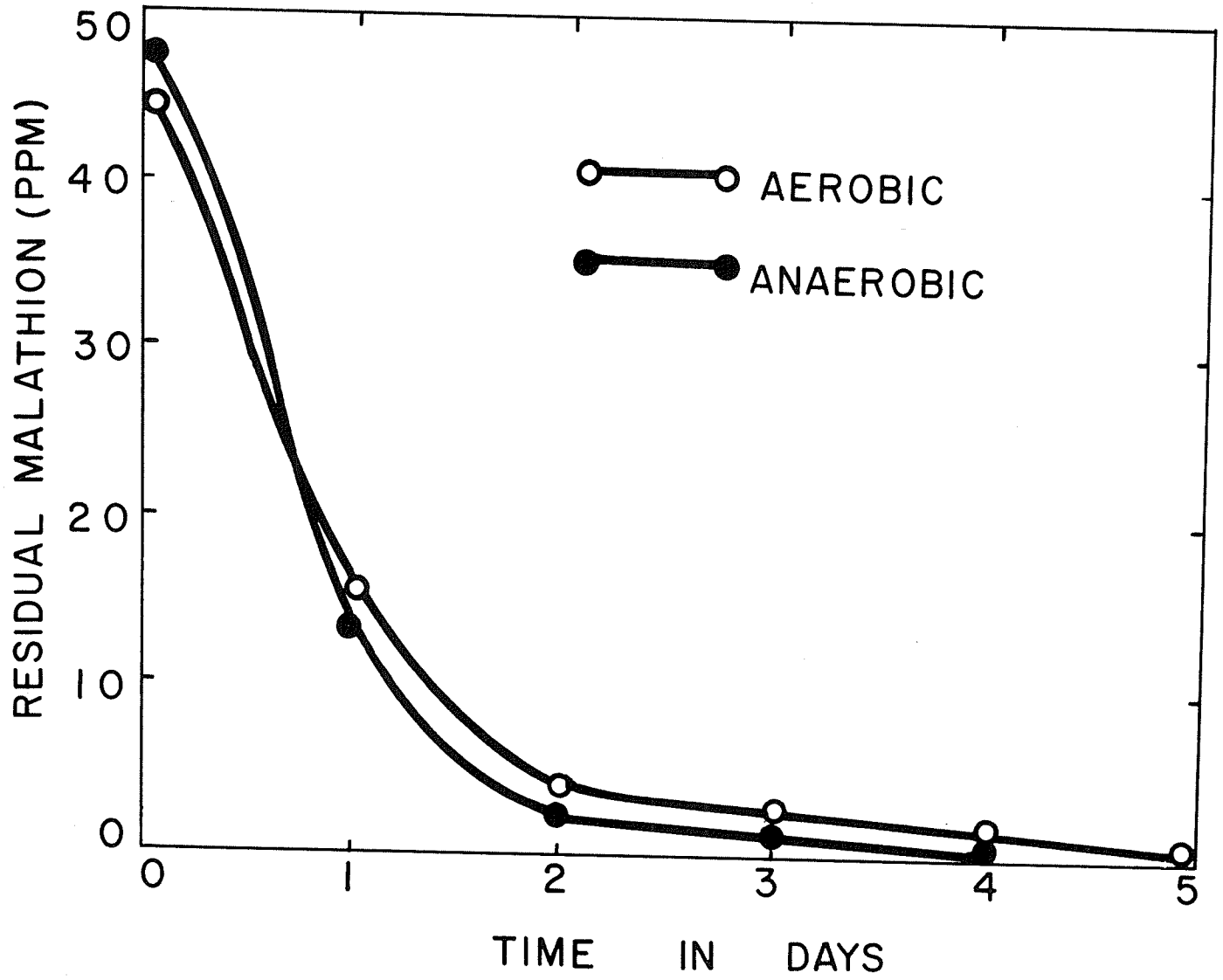

RESIDUAL MALATHION (PPM)
50
40
30
20
10
0
0
1
2
3
4
5
TIME IN DAYS
AEROBIC
ANAEROBIC

Fig. 24. Degradation of Parathion by a resting cell suspension of lagoon bacteria under aerobic and anaerobic conditions.

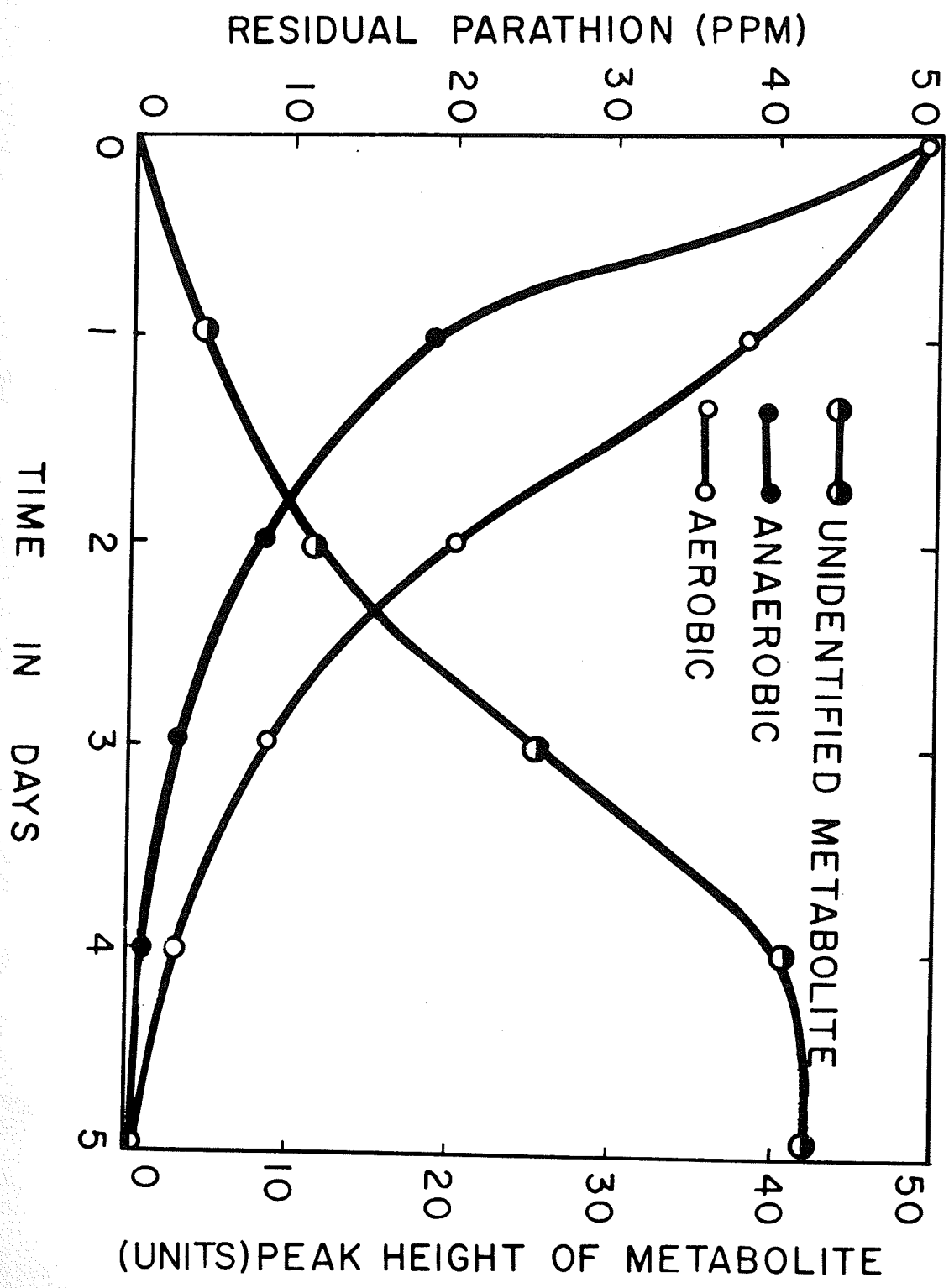

RESIDUAL PARATHION (PPM)
0
10
20
30
40
50
TIME IN DAYS
0
1
2
3
4
5
UNIDENTIFIED METABOLITE
ANAEROBIC
AEROBIC
(UNITS) PEAK HEIGHT OF METABOLITE

TABLE IV

Degradation of Diazinon by a resting cell suspension of lagoon bacteria.

Time in days	Residual insecticide (PPM)	
	Aerobically	Anaerobically
0	50.00	51.00
1	34.75	43.00
2	32.50	36.50
3	25.50	35.00
4	22.33	34.33
5	10.60	25.50
6	4.60	23.00
7	2.33	27.50
8	0.00	23.83
9	0.00	23.00
10	0.00	22.66

Utilization of Heptachlor, Dieldrin, and DDT

The lagoon bacteria degraded Heptachlor completely in 14 days under anaerobic conditions but no discernible metabolites were detected. Under aerobic conditions degradation proceeded consistantly but slowly and only 67.5% was metabolized in the 14 day period (Table V). A single small peak identified as Heptachlor epoxide appeared after 2 days of incubation. The concentration increased with time reaching a maximum of 1.6 ppm on day 5 (achieving 19% conversion) which remained unchanged throughout the course of this experiment.

The lagoon bacteria were not able to degrade Dieldrin anaerobically in 14 days while 23% was metabolized under aerobic conditions (Table V).

DDT appeared to be degraded aerobically but only to a slight extent; 27.6% loss was obtained and no DDD was detected. This result may be an artifact. Under anaerobic conditions, DDT was converted stoichiometrically to DDD quite rapidly. About 30% conversion had been achieved in 2 days but by day 4 conversion was complete. DDD was not further metabolized by the lagoon microflora.

TABLE V

Degradation of Heptachlor, Dieldrin, and DDT by a resting-cell suspension of lagoon bacteria

Time in days	Aerobically				Anaerobically			
	Heptachlor	Heptachlor epoxide	Dieldrin	DDT	Heptachlor	Dieldrin	DDT	DDD
0	8.6*	0.00	7.9	5.8	7.0	7.9	5.8	0.0
2	5.7	0.65	7.5	4.7	6.1	7.5	4.0	1.8
4	4.4	0.65	6.6	4.6	3.9	7.1	0.0	5.8
6	3.2	1.60	6.6	3.1	3.3	7.9	0.0	5.8
8	3.0	1.60	6.2	4.5	2.1	7.4	0.0	5.8
11	2.9	1.62	7.1	4.4	0.3	7.7	0.0	5.8
14	2.8	1.60	6.1	4.2	0.0	7.9	0.0	5.8

* All figures represent concentration in PPM.

DISCUSSION

The micro-extraction method described herein proved to be rapid, accurate, technically simple and modest in equipment requirements yet circumventing problems associated with extraction of large samples. The efficiency of extraction from water and resting cells spiked with insecticides was equally good or better than that achieved by most other workers in spite of the small sample size. Though the chlorinated hydrocarbons were poorly recovered (see Table III), other workers have experienced similar low recoveries with these insecticides (92).

In spite of the organic complexity of the mixed bacterial population, no clean-up pre-treatment was necessary. Wedemeyer (209) used essentially the same technique to study the dechlorination of DDT by sonicated suspensions of Aerobacter aerogenes and also found a clean-up step unnecessary.

The physiological mechanism of Malathion degradation by microorganisms is unknown. Coffin (32) showed that Malathion residues on lettuce decreased from 11.5 ppm to less than 0.1 ppm in 10 days when applied to field plots of lettuce. Malaoxon, an oxygen analogue of Malathion, and three unidentified metabolites were detected 2 days after application. Rowland (172) reported that when maize and wheat grains were

stored in sealed jars and analyzed after 6 months, dimethyl phosphorothiolate, Malathon mono-acid, and Malathion di-acid were identified. Other studies showed that Malathion applied to stored grains was degraded to dimethyl phosphate and Malaoxon (173).

Parathion is a thiophosphate compound which may form Paraoxon by oxidation or it may be isomerized to form the S-ethyl or S-phenyl isomer (32). In these studies, no metabolites of Parathion were detected under aerobic conditions; one which appeared under anaerobic conditions remained unidentified but it was not Paraoxon. Coffin (32) detected Paraoxon and two unidentified metabolites of Parathion on field plots of lettuce 2 days after application.

Lagoon bacteria degraded Diazinon to 56.6% under anaerobic conditions in 10 days and no metabolites were detected; accumulation of toxic products of metabolism may be responsible. Konrad et al (98) studied the degradation of Diazinon in soil, aqueous microbial systems and soil-free aqueous systems and found that no degradation occurred at pH 6.0 but it was hydrolyzed very readily at pH 2.0. They concluded that Diazinon degradation in soils is a chemical process and hydrolysis is a possible mechanism of degradation.

Heptachlor is metabolized to Heptachlor epoxide in soil (12, 13, 212, 218), by house flies (157), and by animals (35, 40). The metabolite is more toxic than the parent compound.

The lagoon bacteria degraded 48.8% Heptachlor in 4 days when only 0.35 ppm Heptachlor epoxide had accumulated. The level of Heptachlor epoxide increased to 1.6 ppm on day 6 and remained constant thereafter; an additional 17.7% Heptachlor was degraded in the subsequent 8 day period.

No reports on the microbial degradation of Dieldrin have been seen. Chacko et al (29) tested the ability of some actinomycetes and fungi to degrade Dieldrin and found that none were physiologically active. Kotre et al (102) reported that Dieldrin was not metabolized by molds.

The results reported here are in agreement with other workers who have shown the conversion of DDT to DDD under anaerobic conditions (63, 87, 143, 209). This mechanism is not confined to bacteria; it has been found to occur in other microorganisms (10, 28, 87, 89). Stenersen (188) and Wedemeyer (209) have both proposed that the efficiency of conversion of DDT to DDD by bacteria is inversely proportional to the availability of oxygen.

The data obtained with lagoon bacteria show that the chlorinated hydrocarbon insecticides are not susceptible to degradation while the organophosphorus insecticides are utilized rapidly. The lagoon effluents containing biologically resistant insecticides undoubtedly contribute to river water pollution due to accumulation.

SUMMARY

The results of studies investigating the biodegradability of some organophosphate and chlorinated hydrocarbon insecticides by a mixed bacterial population indigenous to a domestic wastes disposal unit are presented.

1. Malathion was degraded rapidly under both aerobic and anaerobic conditions. Two unidentified metabolites of Malathion were detected in the presence of molecular oxygen.

2. Degradation of Parathion was quite rapid independent of oxygen. One unknown metabolic product of Parathion degradation appeared under anaerobic conditions and its concentration increased proportionally with a decrease in the concentration of Parathion and remained unchanged after the parent compound had been completely utilized.

3. Diazinon was metabolized completely aerobically in 8 days while only 56.6% was degraded anaerobically.

4. Heptachlor degradation was complete in 14 days in the absence of oxygen. Under aerobic conditions, 67.5% was degraded in 14 days and Heptachlor epoxide was detected as a metabolic product of Heptachlor degradation.

5. Dieldrin was not degraded anaerobically whereas 23% was metabolized aerobically.

6. DDT was metabolized to 27.6% in the presence of molecular oxygen and no metabolite was detected. It was converted to DDD quite rapidly in the absence of oxygen.

A PROPOSAL FOR DETERMINING THE BIODEGRADABILITY POTENTIAL OR ORGANIC COMPOUNDS.

It is self-evident that stabilization in lagoon is achieved through the mutual interaction of a heterogeneous mixture of bacterial species. While the biotic types may vary (163), it is not so evident that the physiological activity of the indigenous population associated with a domestic wastes disposal unit remains reasonably constant. The gross homogeneity of the BOD load enriches the population for specific physiological types. Lagoons, therefore, afford an excellent and readily attainable source of a mixed bacterial population expressing a vast physiological spectrum which can be used to measure the biodegradability potential of both simple and complex natural products or products of the laboratory.

To determine the biodegradability potential of any organic compound, a simple method is proposed. A resting bacterial cell suspension is prepared from a sample of lagoon water and a desired amount of one or more substrates under test is added to the suspension. The preparation may be subjected to any desired environmental parameter. Samples are withdrawn at zero time and appropriate intervals thereafter and analyzed for residual substrate.

The proposed method is rapid, technically simple, and modest in equipment requirements. In addition to determining if the compound under study is biodegradable, it indicates a requirement for acclimation or adaptation. A distinct advantage of this method is that more than one substrate can be added to the same resting cell suspension reducing thereby the technical demands in determining the biodegradability potential of a multitude of substances. It is also possible by this method to establish whether under natural conditions the utilization of a specific compound in the presence of others is regulated by the 'diauxie' effect. Such information is invaluable, for example, in predicting the behaviour of a compound in wastes treatment plants.

The length of time over which the resting cell suspension can remain physiologically active has not as yet been established. Undoubtedly, some of the species are physiologically unstable and the net physiological activity of a preparation would be reduced with time. Further, the physiological stability will vary under different environmental conditions. A danger exists, where organic compounds are degraded slowly, that the resting cell suspension may become inactive before the complete utilization of the substance under test. In such cases the results must be interpreted with caution.

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