Glucose Mineralization and Chitin Hydrolysis by Bacteria Associated with the Sediment in Four Lakes in the Lake Washington Drainage Basin

by

Marleen Marie Baker Wekell

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We have carefully read the dissertation entitled Glucose mineralization and chitin hydrolysis by bacteria associated with the sediment in four lakes in the Lake Washington drainage basin submitted by Marleen Marie Baker Wekell in partial fulfillment of the requirements of the degree of Doctor of Philosophy and recommend its acceptance. In support of this recommendation we present the following joint statement of evaluation to be filed with the dissertation.

The study of bacteria from lake sediments has been carried out in many lakes of the United States and other countries; however, the Lake Washington drainage basin has received very little effort. With the Coniferous Biome Study (International Biological Program), an opportunity to study lakes of this drainage basin in the Seattle watershed was available.

Many of the techniques perfected in the early part of this study were used for the measurement of glucose mineralization. These techniques were used to determine the capabilities of bacteria isolated from the sediment of four lakes. Of more importance, these techniques were also used to measure several parameters of growth of bacteria from littoral and benthic zone sediments to help explain the mineralization functions of bacteria in the lake sediments.

During the latter parts of the study, bacteria capable of digesting chitin were isolated and studied. Ms. Wekell developed a technique for the nondestructive measurement of chitin hydrolysis carried out in test tubes. This method is also applicable to studies with other substrates and bacteria. Special emphases were placed on the measurement of optimum temperatures for chitin hydrolysis by cells, cell-free extracts, and enzyme preparations. These data were also correlated with optimum growth temperatures. The enzyme systems of chitinase and chitobiase were studied and characterized. These studies have significantly contributed to the body of knowledge of the mineralization of organic compounds in lake sediments and show the importance of bacteria in preventing the build-up of litter on the earth's surface. These studies fulfill the academic requirements for fundamental research and interpretation.

We recommend that this thesis be accepted as partial fulfillment of the requirements for a Ph.D.

DISSERTATION READING COMMITTEE: Jack R. Matches

Frieda B. Taub

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INTRODUCTION AND LITERATURE REVIEW

HISTORICAL ASPECTS OF AQUATIC MICROBIOLOGY

Because of the difficulties involved, aquatic microbiology and especially the study of heterotrophic activities developed slowly. Although early in the twentieth century, bacteria were recognized as a necessity for aquatic ecosystems in the recycling of materials, biologists at that time felt that water bacteria belonged to no special class but were merely "...germs from the air, from the soil, from decomposing animal and plant substances and from the healthy and diseased tissues of animals and plants [that] may at times find their way into water..." (Jordan, 1918). Most of the initial work with freshwater bacteria was from a standpoint of sanitation and not ecology (Henrici, 1933).

Early studies in aquatic microbiology, not related to sanitation, were basically descriptive and involved the enumeration of aquatic microorganisms and the effects of various parameters on the numbers, distribution, and species composition. One of the earliest studies of bacteria in lakes was made by Kleiber (1894) who examined the fate of bacteria washed into Lake Zurich by inflowing rivers. Pfenniger (1902) studied fluctuations and the distribution of bacteria in Lake Zurich; and in 1920, a similar study in this lake was conducted (Minder, 1920). In 1901, Jordan (1903) studied bacteria in river waters and found bacteria in polluted waters were different from those in nonpolluted areas. In the late 1920s and early 1930s, Henrici (1933) noted the beginning of a movement directed towards the study of bacteria in their environment.

In the United States, much of the early work was done on numbers of bacteria in lakes as affected by water depth, season, influx of storm-sewer

water (Fred et al., 1924) and the presence of chromagenic bacteria (Snow and Fred, 1926). Snow and Fred (1926) compared bacterial plate counts on a variety of culture media including those used for soil bacteria, and recommended the use of sodium caseinate for culturing water bacteria, a component of media still in use today for these organisms.

In the early 1930s, studies of aquatic bacteria included vertical distribution and seasonal fluctuations of lake bacteria (Dᾶgelli, 1939; Graham and Young, 1934; Ruttner, 1932; Stark and McCoy, 1938) and the horizontal distribution of bacteria in lakes (Graham and Young, 1934; Stark and McCoy, 1938). During this time, Henrici (1933) found that water bacteria are not free floating organisms, but grow attached to surfaces. New techniques were developed for enumerating bacteria, such as the counting of bacteria colonizing submerged glass slides (Henrici, 1933, 1936) and direct counting of the total number of bacteria in water (Bere, 1933; Cholodny, 1929; Kusnetzow and Karzinkin, 1931). Direct counting methods necessitated the concentration of bacteria in the water sample by flocculation, filtration, or evaporation under reduced pressure (Collins, 1963). Total counts using these methods yielded bacterial numbers between ten and ten thousand times the numbers obtained by the plate count (Collins, 1963; Ruttner, 1972). Direct counting methods have since been improved and are still widely in use. In the 1930s, stalked bacteria were discovered (Henrici and Johnson, 1935), and comparative microbiological studies of lakes of varying trophic status were made (Henrici, 1940).

Aquatic microbiology proceeded along these lines until the 1950s when the function and activities of aquatic bacteria were questioned as the problem of eutrophication of water bodies emerged. The availability of radionuclides made these studies of aquatic bacterial activities possible.

Aquatic bacteria developing in sediments (Rodina, 1972) and the water column (Hobbie and Wright, 1968; Zeikus and Brock, 1972) are now considered to be vital indicators of contamination of water masses.

Aquatic microbiologists now tend to focus not on descriptive studies, but rather on the activities of aquatic heterotrophic bacteria. During the past 10 years, there has been a growing awareness of the importance and role of these organisms as transformers of carbon and subsequent availability of bacteria as food to higher organisms, bacterial production and mineralization, and the complex relationships between bacteria and organisms at higher trophic levels.

The measurement of in situ bacterial activities still remains one of the most important problems in microbial ecology (Overbeck, 1974) due to the difficulties involved in taking direct measurements (Fjerdingstad, 1971). The statement of Hobbie and Crawford (1969b) that "...knowledge is still limited on what bacteria are doing and the rates involved...." is still true.

Recent findings regarding the role of aquatic microorganisms will be discussed on the following pages, and will include: (1) the role of bacteria in aquatic ecosystems; (2) temperature and bacterial activities; (3) methods of studying bacterial activities; (4) kinetic measurement of uptake and respiration of ^{14}C labeled substrates; (5) glucose and aquatic bacteria; (6) cellulose and aquatic bacteria; (7) chitin; (8) chitin decomposition; (9) the chitinase system; and (10) enzymes in the equatic environment.

The final pages of this discussion concern the 4 lakes that were part of this study.

ROLE OF BACTERIA IN AQUATIC ECOSYSTEMS

Aquatic bacteria are important as a source of nourishment for other members of the food chain and in the production, mineralization, and

regeneration of nutrients.

Bacteria as Food

Bacteria are one of the first links joining the biotic with the abiotic world (Rodina, 1972). Bacterial biosynthesis is one of the most important large-scale processes in the transformation of organic matter in aquatic ecosystems, and is important in the formation of basic food resources in freshwater lakes (Sorokin, 1971). Dissolved organic matter is a basic energy source for aquatic bacteria and represents 90% of the total organic matter (Sorokin, 1971). Bacteria can concentrate dissolved organic matter, such as extracellular products of algal photosynthesis and allochthonous material, inside cells during biosynthesis and serve as food for some species of invertebrates (Overbeck, 1974; Sorokin, 1971). Bacteria are thought to be the main food for zooplankton by some (Romanenko, 1969) and somewhat less important by others (Saunders, 1969). Bacteria are food for filter feeders and detritivores (Sorokin, 1968), e.g., chironmid larvae (Jónasson, 1969), protozoa, crustacea, and molluscs (Brock, 1966, p. 277), so that the energy is eventually dissipated through other members of the food chain. Aquatic bacteria are capable of producing the main vitamins, and when consumed, provide protein and vitamins (Sorokin, 1971). Algal associations in small ponds may be determined partially by vitamin B_{12} that is produced by microorganisms in the mud (Hutchinson, 1957, p. 827). It has been suggested that bacteria adhering to phytoplankton modify these cells either by partially digesting them or producing vitamins, so that they are more palatable to zooplankton (Parsons, 1963). Algae are fairly indestructible by mammalian digestive systems. However, bacterial actions will render them digestible; bacterial synthesis probably compensates for the nutritional inadequacies of the algae (Golueke and Oswald, 1964).

Bacterial action on sedimented material is necessary for the conversion of organic detritus into utilizable food for a number of benthic organisms (Seki et al., 1968). In the marine environment, bacteria on aggregates are consumed by copepods and fish larvae (Sorokin, 1971), and are an important food source in the pelagic zone (Seki et al., 1968). In marsh dominated systems, conversion of low protein marsh grass to high protein microbial biomass is an important link in the food chain (Gosselink and Kirby, 1974).

Bacterial Production

Microbial primary production has been defined as microbial production at the expense of allochthonous organic matter; microbial production at the expense of autochthonous energy sources is secondary microbial production (Sorokin and Kadota, 1972). In microbial secondary production, substrates, not utilized by animals, are attacked by bacteria. Bacterial production is compensated by grazing zooplankton (Sorokin and Kadota, 1972). Bacterial secondary production cannot be separated from decomposition (Sorokin and Kadota, 1972), and both primary and secondary bacterial production are difficult to separate from mineralization.

Some authors feel that bacterial production should be added to phytoplankton primary production, for in some lakes, allochthonous sources are greater than autochthonous organic matter (Vinberg, 1971a). Kuznetsov (1968) found that bacterial production in Russian reservoirs was significantly higher than phytoplankton production. In the marine environment, intensive bacterial production in the upper layer of the warm waters of the ocean occurs, and bacterial production often exceeds photosynthesis of phytoplankton (Sorokin, 1971). Not all authors share Sorokin's view, however (Taub, personal communication).

Methods for measuring microbial production are given by Sorokin and Kadota (1972) and Vinberg (1971b).

Mineralization

The most recognized role of heterotrophic microorganisms in the seas is the respiration of organic compounds and the consequent regeneration of inorganic nutrients (Williams and Askew, 1968). In lakes, heterotrophic bacteria are important in CO_2 cycling (Allen, 1971), by mineralizing proteins of dead algae (Mikhaylenko and Kulikova, 1973), and utilizing the products of phytoplankton production and allochthonous organic matter (Kuznetsov, 1968). Heterotrophic bacteria play an essential role in replenishing the relatively small supply of CO_2 available for photosynthesis (Alexander, 1971b, p. 407); and without microorganisms as decomposers of organic remains, the living world would eventually run out of CO_2 (Brock, 1966, p. 260). At the sediment-water interface, bacteria control organic exchange (Hayes, 1964); and in this region, bacterial CO_2 production may exceed CO_2 production by the macrobenthic community (Brinkhurst et al., 1972).

Activities of microorganisms have a direct or indirect influence on the production of aquatic organisms of various trophic levels (Sorokin and Kadota, 1972). Bacterial mineralization and algal primary productivity are closely related (Kadota et al., 1966) with the rate of bacterial mineralization for nutrient regeneration perhaps the main factor controlling algal primary productivity (Golterman, 1972). Every increase in bacterial activity shortens the regeneration of nutrients within the aquatic ecosystem, and delivers more growth promoting metabolites (Rodhe, 1969).

Bacteria can effectively remove substrates present at low in situ concentrations, keeping these substrates at low levels and preventing heterotrophic growth of algae in nature (Wright and Hobbie, 1966).

TEMPERATURE AND BACTERIAL ACTIVITIES

The lowest bacterial growth temperature has been reported at -10° C (Larkin and Stokes, 1968), and the highest at over 90° C in hot springs (Brock, 1967; Bott and Brock, 1969). The majority of bacteria, however, are adapted to live in the moderate range of temperatures around the mean temperature of the earth, at 12° C (Blum, 1961).

Temperature is one of the most important variables affecting the growth of bacteria (Ingraham, 1962; Rose, 1968). It is also one of the most important environmental conditions determining how rapidly substrates are metabolized by bacteria (Rose, 1968). Direct correlations have been found in environmental microbiological studies between environmental temperature and bacterial biomass (Henrici, 1938; Mataruyeva, 1971), and bacterial activities such as mineralization (Alexander, 1961; Inniss, 1975; Kadota et al., 1966; Sorokin and Kadota, 1972), respiration (Hargrave, 1969), and uptake of organic substrates (Wetzel et al., 1972; Wright, 1973). Direct correlations have been found specifically between environmental temperature and maximal bacterial uptake velocities for amino acids (Burnison and Morita, 1974), and acetate and glucose (Allen, 1969, 1971). Not all workers have found temperature correlations to bacterial activities however (Hall et al., 1972). Turnover times for bacterial uptake of glucose and acetate have been found to be inversely proportional to water temperature with increasing turnover times throughout the water column of lakes in the fall and early spring with decreasing temperatures (Allen, 1969). Some investigators claim that temperature effects on bacterial activities and biomass are greater than substrate availability during parts of the year (Bott, 1975; Wetzel et al., 1972); while others claim availability of substrate is important (Okutani et al., 1972) and perhaps more so than temperature

(Straskrabovka-Prokesova, 1966). Probably both temperature and substrate availability are important, but interactions between the two factors, related to bacterial biomass and activities, are extremely difficult to separate.

Bacterial biomass and activities have been found, not surprisingly, to be related as well. The maximal velocity of uptake of organic substrates tends to increase with total numbers of bacteria in pure culture (Hobbie and Wright, 1968; Wright, 1973), and in the waters of subarctic (Morgan and Kalff, 1972) and eutrophic (Allen, 1969) lakes. Static measurements of mineralization have also demonstrated correlations of mineralization to bacterial numbers in the sea (Okutani et al., 1972). The proportionality of the maximum velocity of uptake or V_{max} to increases in bacterial biomass is not always uniform (Overbeck, 1974) and V_{max} per cell can vary for different species of bacteria (Wright, 1973); therefore, changes in V_{max} with temperature and bacterial biomass may indicate qualitative as well as quantitative changes in bacterial populations. Different species of bacteria can also have varying optimum temperatures for V_{max}. Allen (1971) reports a Caulobacter sp. with an optimum temperature for the maximal velocity of glucose uptake at low temperatures, indicative of a higher enzyme efficiency at lower temperatures. Organisms in the aquatic environment have also been found with multiple temperature optima for growth (Sieburth, 1967) and for the V_{max} of glucose and acetate uptake (Allen, 1971; Bott, 1975). These interesting findings suggest that bacteria can possess enzyme systems capable of functioning at temperatures different from the apparent optimal temperature of the organism.

METHODS OF STUDYING HETEROTROPHIC BACTERIAL ACTIVITIES

Heterotrophic bacteria are capable of utilizing a wide variety of substrates, for there are only a few nonbiodegradable substances identified; it has

been suggested that failure to demonstrate bacterial degradation of "recalcitrant molecules" may be due not to microbial fallibility, but to the fallibility of microbiologists! (Horvath, 1972). There exists no indicator substrate, for which in situ or laboratory measurements of uptake or mineralization by bacteria would indicate all of the activities of the heterotrophic bacteria in aquatic ecosystems. Approximations of heterotrophic bacterial activities have been determined by the measurements of: (1) oxygen consumption; (2) CO_2 production; (3) heterotrophic CO_2 fixation; (4) measurement of growth rates; and (5) kinetic measurement of uptake and mineralization of labeled organic substrates. Other methods used to determine biological activities that can be adapted to measurement of heterotrophic bacteria include dehydrogenase (Curl and Sandberg, 1961; Pamatmat and Bhagwhat, 1973) and electron transport activities (Packard et al., 1971). All of these methods have certain limitations.

1. Oxygen uptake. The biological uptake of oxygen in water has been found to be statistically correlated to total numbers of bacteria (Straskrabova, 1968), and has been used as a basis for comparing microbial activity in different types of sediment and detrital material (Hargrave, 1972).

2. CO_2 production. Nonkinetic measurements of the production of CO_2 by the bacterial degradation of ^{14}C-labeled or unlabeled compounds has been used by some workers as an indicator of heterotrophic activity (Kadota et al., 1966; Boylen and Brock, 1973). Ohle (1956) preferred this method for biological activity measurements rather than use of the oxygen deficit. This technique is not a kinetic measurement and this is considered by some to be a serious limitation (Wright, 1973).

3. Heterotrophic CO_2 fixation. This measurement is based on the indications that 6% of the external CO_2 carbon is involved in cell synthesis in the $^{14}CO_2$ dark fixation by natural populations of aquatic heterotrophic microflora.

The heterotrophic production is calculated from the measurements of the dark $^{14}CO_2$ fixation and compared to primary production (Overbeck, 1974); this method is limited by the fact that the % CO_2 in bacterial biomass is subject to strong variations and CO_2 is used by all kinds of cells in basic metabolic processes not restricted to heterotrophic organisms.

4. Microbial growth rate. Microbial growth rates have been studied as an indication of microbial activities. Various methods used are summarized by Brock (1971). Growth studies utilizing radioautography (Brock, 1967a; Ramsay, 1974) appear to be promising for in situ studies of bacterial activities.

5. Kinetic measurement of uptake and mineralization of ^{14}C-labeled substrates. Aquatic heterotrophic bacterial activities have been measured using ^{14}C-labeled compounds, based on a method first described by Parsons and Strickland (1962), modified to give kinetic data by Wright and Hobbie (1965) and later corrected for respiratory $^{14}CO_2$ loss (Hobbie and Crawford, 1969b). Recently methods have been developed to measure uptake and mineralization kinetics of organic ^{14}C compounds by heterotrophic bacteria associated with sediment in the marine (Wood, 1973) and freshwater environment (Hall et al., 1972; Harrison et al., 1971). Some difficulties involved with this method are outlined by Wright (1973) and Williams (1973), although both authors recommend the use of kinetic measurements rather than static ones. This method of kinetic measurement was described recently in the Russian literature by Fursenko (1972).

KINETIC MEASUREMENT OF UPTAKE AND RESPIRATION OF ^{14}C LABELED SUBSTRATES

The kinetic analysis of the mineralization of uniformly labeled ^{14}C glucose was used in this study of 4 lakes, and so will be presented in greater

detail than the other methods for measuring bacterial activities.

The kinetic method has been used to measure the uptake of labeled substrates by bacteria in marine (Hamilton and Austin, 1967; Hamilton et al., 1966; Hamilton and Preslan, 1970; Vaccaro, 1969; Vaccaro and Jannasch, 1966, 1967), and in freshwaters (Allen, 1969, 1971; Burnison and Morita, 1973, 1974; Morgan and Kalff, 1972; Thompson and Hamilton, 1973; Wright and Hobbie, 1966).

Parsons and Strickland (1962) in studies of the uptake of ^{14}C-labeled organics by marine planktonic microorganisms in inshore Pacific waters, found that the velocity of uptake rapidly approached a maximum uptake velocity with increasing substrate concentrations, and that such uptake could be analyzed by Michaelis-Menten enzyme kinetics. Wright and Hobbie (1965) reported two distinct uptake mechanisms operating simultaneously in natural populations. Uptake at low substrate levels was due to bacteria and was mediated by active transport. Uptake at higher concentrations was due to diffusion and was considered by these authors to be associated with algae. From the original Michaelis-Menten equation of Parsons and Strickland (1962) for uptake at a given substrate concentration, Wright and Hobbie (1965) derived the equation:

$$\frac{C\ \mu t}{c} = \frac{(K + S_N)}{V} + \frac{A}{V}$$

Where:

C = counts per minute (CPM) for 1 μCi of ^{14}C

c = CPM of filtered organisms

μ = number of microcuries added to the sample

t = incubation time in hours

S_N = natural substrate concentration (μgm/l)

K_t = a constant similar to the Micahelis Menten constant

A = added substrate concentration (µgm/1)

V = maximum uptake velocity in µgm/1/hr

The method involves incubating water samples with varying amounts of labeled substrate, fixing the sample after a suitable incubation time, filtering the sample on membrane filters, and determining the radioactivity of the filtered organisms. The velocities of uptake at a number of substrate concentrations can then be plotted linearly in the form of a modified Lineweaver-Burk plot as Cµt/c against A. The inverse of the slope gives the maximum velocity of uptake (V_{max}) and the y intercept, the turnover time of the substrate. The V_{max} is the theoretical maximum rate of the bacterial population at the time of measurement and at the sample temperature for a given substrate (Hobbie and Crawford, 1969a; Wright, 1973), and is a measure of what the existing population is capable of doing. The V_{max} varies with the trophic state of a lake (Hobbie and Wright, 1968), gives information about the size and function of the bacterial population (Wright and Hobbie, 1965), and can be a sensitive measure of pollution (Hobbie and Wright, 1968).

The turnover time or time required for the uptake or mineralization of all of the substrate at the in situ concentration is also an important parameter. A short turnover time indicates rapid utilization of the substrate by the bacterial population at the time and temperature of sampling.

It is now known that a significant percentage of the substrate added is respired as $^{14}CO_2$ during the incubation (Hamilton and Austin, 1967; Hobbie and Crawford, 1969b), and respiration corrections were made by Hobbie and Crawford (1969b). Harrison et al. (1971) pointed out that the respiration fraction of uptake when considered independently of assimilation, is actually mineralization and so extended the method for use in measuring mineralization

by lake sediment bacteria.

For this method to give valid results, the investigator must assume that: (1) there is no appreciable reproduction or death during the analysis; (2) there is no removal of substrate by plankton during the analysis; (3) there is no induction; (4) there is little change of substrate concentration during the incubation period; (5) there must be linearity of response with time; and (6) the concentration of added substrate, temperature, and time of reaction must be specified and carefully controlled (Harrison et al., 1971; Wright, 1973; Wright and Hobbie, 1965).

If the substrate concentration added is too high, induction of additional transport and mineralization enzymes can occur in a short period of time as shown by Harrison et al. (1971). The uptake and assimilation of a solute by microorganisms shows first order kinetics only if bacterial growth and/or induction of enzymes does not occur; therefore, the time of incubation should be at a minimum needed to obtain significant radioactivity at the lowest substrate level used (Wright, 1973).

GLUCOSE AND AQUATIC BACTERIA

Glucose was used in this study and has been used by many aquatic microbiologists in the study of heterotrophic bacterial activities. The use of glucose by bacteria has been assumed to be typical of other organic compounds being used by bacteria, and is therefore considered to be a general measure of metabolic activities of the bacterial population. Most bacteria are permeable to glucose and have enzymes to utilize it (Ramsay, 1974). Uptake of glucose at low concentrations in aquatic bacteria is mediated by specific transport systems or permeases (Cohen and Monod, 1957). Glucose is one of the most easily decomposible substrates for most aquatic microorganisms (Kadota et al., 1966;

Sorokin and Kadota, 1972) and is used more rapidly than most other carbon sources by the majority of bacteria (Pardee, 1961). It has been suggested that glucose is one of the main factors responsible for the development of aquatic microorganisms (Yenaki, 1969) and for the development of particle aggregations mediated by aquatic bacteria (Paerl, 1974).

Dissolved free glucose has been found in lakes, and levels are higher in sediments than in the water column (Wood and Chua, 1973b). Of carbohydrates found in lake sediments, glucose is present in the largest amounts up to 1.0 gm/kg sediment organic matter (Vallentyne, 1957). Chemical determinations of glucose concentrations in the water column of lakes have revealed levels of 5 µgm/L (Vallentyne and Whittaker, 1956) and from 3.6 to 10 µgm/L (Hicks and Carey, 1968). Similar values are reported for sea water (Vaccaro et al., 1968) and it has been reported as one of the components present in particulate matter in the sea (Handa and Tominaga, 1969).

Fluctuations in glucose levels have been reported in Russian reservoirs with high levels in spring and summer, due to leaching from phytoplankton, and low levels in the winter (Yenaki, 1969). Other workers have found no seasonal fluctuations of glucose levels in lakes, with levels remaining constant at less than 10 µgm/L (Allen, 1969; Hobbie and Wright, 1968).

Uniformly labeled ^{14}C-glucose has been used extensively in studies of heterotrophic activities because it is available at a high specific activity (320 µc/µM, or greater than 80% ^{14}C), higher than many other substrates, and hence lesser amounts of glucose, approximating the <u>in situ</u> concentration can be used.

Glucose is also the end product of the decomposition of the more refractory carbon compounds normally present in lakes, cellulose, and chitin.

CELLULOSE AND AQUATIC BACTERIA

Cellulose, one of the compounds identified in the sediment of lakes (Vallentyne, 1957), is one of the most abundant naturally occurring organic compounds (Hofsten and Edberg, 1972). It is the principal cell wall constituent of higher plants, provides their main structural element, and is found as a constituent of various algae. Cellulose is a linear chain of 1-4, β -D gluco-pyranose residues, with intra- and inter-molecular hydrogen bonds; the antiparallel arrangement of adjacent chains may accommodate intermolecular hydrogen bonding (Aspinall, 1970).

Cellulose is highly crystalline and occurs in various states of purity in all higher plants and algae; wood fibers are about 45% cellulose and it is often associated with various other polysaccharides, hemicellulose, and lignin (Berg et al., 1972b). The structure of the cellulose fiber influences the rate of its decomposition by cellulolytic organisms (Berg et al., 1972b) and its biodegradation is generally considered as a prerequisite for the functioning of most ecosystems (Hofsten and Edberg, 1972).

Bacterial Decomposition of Algae

The capacity for microorganisms to utilize cellulose is responsible for the rapid destruction of algae in nature (Gunnison and Alexander, 1975b). Algae differ in susceptibility to biodegradation by microorganisms (Kudryautsev, 1972) with the cell wall the major determinant of resistance of some algal species (Gunnison and Alexander, 1975a). It has been demonstrated that in species of algae susceptible to microbial attack, cellulose is a major component of the algal surface and is attacked by bacterial cellulases (Gunnison and Alexander, 1975b). Cytophaga species capable of lysing blue-green algae have been found to digest cellulose, with lysis caused by the dissolution of algae

after digestion of the cell walls (Stewart and Brown, 1969). Differences in further decomposition of algal cells can be mediated by the absence or presence of oxygen, with the production of dissolved organic carbon increased under anaerobic conditions (Otsuki and Hanya, 1972a, 1972b).

Occurrence of Cellulolytic Activities

The cellulase system (E.C. 3.2.1.4) is a multienzymic system and hydrolyzes only β-D (1--4) glucosidic bonds (Barman, 1969; Reese and Mandels, 1963). Cellobiase or β-glucosidase (E.C. 3.2.1.21) is often associated with the cellulase system and acts synergistically with it (Berghem and Pettersson, 1974; Boretti et al., 1973). The cellulases are inhibited by mercuric acetate, but not p-chloromercuribenzoate, or EDTA, indicating that divalent metal ions are not involved in the catalytic reaction and no free SH groups are present (Berg et al., 1972a).

The end products of cellulose hydrolysis are usually consumed during the growth of cellulolytic organisms (Fusee and Leatherwood, 1972; Reese and Mandels, 1963). Cellulase is produced by a variety of microbial forms, including protozoans, fungi, bacteria and actinomycetes (Stutzenberger, 1972b). Cellulose is degraded by only a few groups of bacteria (Hofsten et al., 1971) and fungi (Berg et al., 1972a). Other organisms cannot catabolize cellulose and depend on prior conversion by microorganisms (Gosselink and Kirby, 1974). Cellulase activity appears to be widespread in marine invertebrates (Elyakova, 1972; Hultin and Wanntorp, 1966; Koningsor et al., 1972), although many workers do not rule out the possibility of microbial cellulase activities in the samples analyzed.

Studies suggest that the spore forming Clostridia are the dominant cellulolytic organisms in anaerobic environments; under aerobic conditions, flexibacteria or myxobacteria of the Cytophaga group and various pseudomonads

with simple growth requirements, are the most important bacterial cellulose decomposers (Hofsten and Edberg, 1972). Electron micrographs suggest that aerobic bacteria degrade cellulose through direct contact between cells and fibers, and anaerobes may use a similar mechanism (Hofsten and Edberg, 1972). Reactivity of cellulose is enhanced by increasing the available surface by grinding, acid-swelling, or with the use of water soluble derivatives (Reese and Mandels, 1963). The cellulase system is complex and consists of the C_1 and C_x factors.

C_1 Factor of Cellulase Complex

Only a few microorganisms can rapidly degrade solid or native cellulose due to the lack of the C_1 cellulase factor (Stutzenberger, 1972b) which can attack highly organized crystalline native cellulose (Li et al., 1965), but may not catalyze the actual hydrolytic event (King and Vessal, 1969). The mode of action of the C_1 component is not completely understood, but it is thought to activate the resistant substrate to enzyme hydrolysis (Stutzenberger, 1972a) by cleaving the inter-molecular hydrogen bonding system (King and Vessal, 1969).

C_x Cellulase System

The C_x system hydrolyzes cellulose to soluble cellulose derivatives (Stutzenberger, 1972a), and through the action of cellobiose glucose is the end product. C_x activity cannot be used as a criterion to evaluate cellulolytic ability of microorganisms in nature because it is active against soluble substituted celluloses such as carboxy methyl cellulose, but shows no detectable activity against native cellulose (e.g., cotton fibers) without the C_1 enzyme (Stutzenberger, 1972b).

The extracellular cellulase system of bacteria consists of from 3 to 4 physically and enzymically distinct C_x enzymes (King and Vessal, 1969; Lee

and Blackburn, 1975; Suzuki et al., 1969) and in many organisms the enzyme system is constitutive (Suzuki et al., 1969). Formation of one or more of the enzymes is enhanced by growth in cellulose (Suzuki et al., 1969) and is stimulated by yeast extract (Lee and Blackburn, 1975). Enzyme production (but not activity) is repressed by growth in high concentrations of glucose or cellobiose (King and Vessal, 1969; Lee and Blackburn, 1975). It has been suggested that the cellulase system is controlled by a catabolite repression mechanism similar to that described by Jacob and Monod (1961).

The cellulase C_x system of fungi also consists of several physically and enzymically distinct components and cellobiase is also present (Boretti et al., 1973; Berghem and Pettersson, 1974; Fusee and Leatherwood, 1972; Li et al., 1965; Okada et al., 1968). Cellulase is inducible in fungi with cellobiose postulated as the inducer (Fusee and Leatherwood, 1972). There is decreased cellulase activity when cellulolytic fungi are grown in glucose (Fusee and Leatherwood, 1972).

Production of Cellulase

The cellulase enzyme system is found in the cell-free medium during the stationary phase when bacteria and actinomycetes are grown in pure culture (Berg et al., 1972a). The released cellulase is often bound to cellulose fibers. Enzyme activity varies with incubation time, carbon source, and is increased by shaking (Stutzenberger, 1972a) and by sonication (Berg et al., 1972a) of the cultures. Differences in the crystal structure of cellulose can affect induction. Enzymically and physically distinct cellulase systems are induced with fungal cultures in the presence of different crystal forms of cellulose (Rautella and King, 1968).

Most microbial cellulases, originally found in viable cells, usually occur as typical extracellular enzymes and are not the result of autolysis

(Suzuki et al., 1969). Most cell-bound cellulase resides in the cell wall region and/or on the surface of the cytoplasmic membrane fraction; some cellulolytic bacteria, Cellvibrio gilvus, Clostridium thermocellum, and Ruminococcus flavefaciens have cell-bound cellobiase phosphorylase, and can more efficiently utilize the glucosyl bond energy (Suzuki et al., 1969).

The location of cellulase in Cellvibrio fulvus depends upon the carbon source for growth and the age of the culture. When cells are grown on glucose or cellobiose, all of the C_x cellulases are cell bound, with only part of the activity located on the cell surface. Thus, the enzyme is not unnecessarily "lost" to the organism; cellobiase is also bound to the membrane fraction and located in the periplasm (Berg, 1975). Cultures of this organism grown on cellulose contain cell-free C_x cellulases (Berg, 1975).

Significance of Cellulose Decomposition

Without cellulose decomposition, a tremendous amount of carbon would be "tied up" and unavailable to members of the aquatic ecosystem. The decomposition of cellulose in nature is mediated primarily by microorganisms. The production of the cellulase complex is finely tuned to the needs of the microorganisms and is either inducible or its production is enhanced by proximity to the substrate and/or its soluble oligomers. Different forms of cellulose can elicit the production of physically distinct cellulase systems and even the location of these systems within the bacterial cell. The production of cellulase is repressed by glucose, the end product of cellulose decomposition, so that cellulolytic organisms will produce the enzyme and liberate it as an exoenzyme only when the represser is absent. In this way, cellulose can be seen as a store of "reserve nutrient" that is decomposed only by microorganisms, and only when other more labile substrates have been decomposed or utilized.

Rates of cellulose breakdown in the aquatic environment are difficult to measure. Measurements are either conducted in situ by suspending cellulose fibers in inert nylon bags in the sediments and waters of lakes and measuring weight loss (Hofsten and Edberg, 1972), or by experiments with pure or mixed cultures in the laboratory under conditions approximating those prevailing in the environment.

Chitin, another refractory carbon compound, and an analogue of cellulose, can also be viewed as a "reserve nutrient" in lakes and will be discussed on the following pages.

CHITIN

Next to cellulose, chitin is probably the most abundant of naturally occurring organic compounds (White et al., 1968, p. 51). Like cellulose, chitin is decomposed to glucose. Chitin is a structural component of the exoskeletons of crustaceans, insects, coelenterates, annelids, molluscs, brachiopods, fungi, and yeasts (Hood and Meyers, 1973; Rudall and Kenchington, 1973). For more extensive information on the distribution of chitin in animal phyla, see Jeuniaux (1963).

The first attempt to define the chemical nature of a material which contained chitin are found in the earlier works of Braconnot (1781-1855, cited by Rudall and Kenchington, 1973). In 1811, Braconnot proposed the name "fungine" for the insoluble residue remaining after boiling mushroom species in weak alkali. The name "chitine," derived from the Greek word for tunic, was proposed by Odier in 1823 (Rudall and Kenchington, 1973) for the material of insect cuticles which remained unchanged in external form, after several treatments with a hot solution of caustic potash. In the 1870s Ledderhose found an amino sugar, which he called glucosamine, and acetic acid in the acid hydrolysate of

arthropod chitin; it was later shown that these products were formed in equimolar proportions (Rudall and Kenchington, 1973). It is now accepted that chitin is a β-1-4 linked polysaccharide composed of N-acetyl D-glucosamine units. Chitin is similar to cellulose, another structural linear polysaccharide, except that in chitin, the hydroxyl group at carbon 2 is replaced by the acetamido group ($-NHCOCH_3-$). Chitin is insoluble in aqueous solvents, other than concentrated acids, and is de-acetylated to chitosan, after treatment with hot concentrated alkali (Aspinall, 1970).

Structure of Chitin

On the basis of x-ray diffraction patterns, chitin is separated into 3 main types, alpha, beta, and gamma, which differ in arrangement (Hood and Meyers, 1973). The intermolecular arrangement of chitin has been studied by x-ray diffraction and polarized infrared spectroscopy (Aspinall, 1970). Dweltz (1960) reported that chitin is composed of two polysaccharide chains running in opposite directions with four asymmetric N-acetyl glucosamine units. The chains are separated by a distance of 4.69 Å perpendicular to the "plane" of the pyranose rings. The NH and CO groups in the neighboring aminoacetyl side chains are hydrogen bonded. The hydroxyl methyl side chains, containing a hydroxyl group, are also hydrogen bonded, the OH of one linked to the oxygen of a similar group from a neighboring chain running in the opposite direction. The short hydroxyl group is intrahydrogen bonded to the oxygen of the amide group in the same asymmetric unit. The straight chain structure was questioned by Carlström (1962), who suggested that a bent chain is the only spatial configuration of the polysaccharide chain of chitin which is sterically satisfactory and fits x-ray diffraction data.

Alpha chitin is found in insects and crustaceans. The beta type of chitin, found in coelenterates, annelids, molluscs, and brachiopods, and the

gamma, from cuttlefish shell, are both associated with an elastin (Rudall, 1967). For a more detailed explanation of the structure of chitin, see: Carlström, 1963; Dweltz, 1960; Ramakrishnan and Prasad, 1972; Rudall and Kenchington, 1973).

Pure chitin is rarely found in nature but occurs as a native glycoprotein complex combined with calcium and trace amounts of other inorganic salts (Hood and Meyers, 1973). The protein is linked to chitin by covalent bonds (Hackman, 1960) through aspartyl and histidyl residues (Hackmann, 1964), with the degree of bonding varying with the type of chitinous material (Jeauloz, 1963). Weak bonding occurs between chitin and protein in soft cuticle, and strong bonding is present in hard sclerotized cuticles (Jeauloz, 1963). Chitin-protein complexes form a variety of states of aggregation ranging from micelles to microfibers, and in cuticle, to microscopically visible fibers ("Balken") (Kent, 1964). The calcification of cuticle resembles that of bone in higher animals (Kent, 1964). Calcium carbonate, the most common crystalline deposit, is associated with the chitinous exoskeleton of crustaceans and ranges from trace quantities to 99% of the cuticle (Kent, 1964). The percent calcium carbonate varies with the species (Hood and Meyers, 1973). Other inorganic salts are present in trace amounts and include: $MgCO_3$, $Ca_3(PO_4)_2$, SiO_2, $(Al, Fe)_2O_3$, MgO, CaO, and $CaSO_4$ (Richards, 1951). Although it appears that chitin probably always exists in complex with proteins, inorganic salts and pigments, a pure homogeneous crystalline 1-4' linked 2-acetamido-2-deoxy-β-glucan has been isolated from fibers from certain diatoms. The name chitan has been proposed for this material. It has a different macrostructure from the chitin derived from crustacean sources, but it may be converted irreversibly into a form indistinguishable from that of chitin by treatment with hot aqueous lithium thiocynate (Aspinall, 1970).

Abundance of Chitin

In 1957, no data was available on the abundance of chitin in aquatic sediments (Vallentyne, 1957). Even today little work has been reported on the abundance and degradation of chitin in fresh waters and sediments. Most of the studies of chitin and its degradation have been conducted in the marine environment. Zobell and Rittenberg (1938) calculated an annual chitin production of several billion tons from marine copepods. Copepods have been stressed as a chitin source because they are usually considered to be the most abundant marine crustacean, and chitin makes up about 5% of the dry weight of copepods (Seki and Taga, 1963a). It has been calculated that euphausidd (krill) shells average 66% chitin by weight (Richards, 1951), which can also add several billion metric tons per year to the world's oceans (Chan, 1970). When chitin produced by other crustaceans is considered, the annual chitin production in the oceans must be enormous. The fact that chitin does not accumulate in the sea in amounts equal to its production is indirect evidence for its decomposition. It has been assumed that chitin in the sea is degraded by microorganisms (Zobell and Rittenberg, 1938). Simulated in situ studies (Chan, 1970; Seki and Taga, 1963c, Seki, 1965a, b) have demonstrated that chitin is decomposed by microbial activity in marine sediments and waters.

CHITIN DECOMPOSITION

Chitin is degraded by microorganisms, primarily the bacteria, actinomycetes, and fungi, although chitinases have been found in invertebrates and vertebrates (including some mammals). Of these, microorganisms provide the most convenient source of chitinase and consequently these systems have been extensively studied.

Chitinoclastic Bacteria and Actinomycetes

Benecke (1905) was the first investigator to describe a chitin digesting bacteria, isolated from enrichment of sea water with chitin and mineral broth. The organism, Bacillus chitinovorous, was aerobic, gram negative, rod shaped and peritrichously flagellated. The first isolation of chitinoclastic nonfruiting myxobacteria of the Sporocytophaga and Cytophaga types was by Johnson (1932) from the shell of the Dungeness crab, Cancer magister. Stanier (1947) isolated similar strains of myxobacteria from chitin enrichment cultures inoculated with soils. These bacteria closely resembled those described by Johnson (1932) and together they were grouped under the name Cytophaga johnsonae. Zobell and Rittenberg (1938) found a total of 31 strains of chitinoclastic bacteria in water and bottom sediment samples along the coast of California. A total of 31 strains, primarily asporogenous gram negative rods, were isolated using an enrichment of chitin and mineral salts. Clarke and Tracey (1956) found that chitinase was produced by a number of soil and water organisms including a number of human enteric pathogenic bacteria. Another human pathogen, Vibrio parahaemolyticus is able to degrade chitin (kaneko and Colwell, 1973) and is found in the marine environment (Baross and Liston, 1970). Chitinoclastic bacteria have been suggested as the causitive agent of "brown spot" disease in shrimp (Seki, 1965b; Seki and Taga, 1963d; Sindermann, 1971). Recently chitinoclastic Clostridia have been isolated from marine mud (Timmis et al., 1974).

Most of the marine chitinoclastic bacteria belong to the genera: Vibrio, Pseudomonas, Cytophaga, Aeromonas, Beneckea, and Photobacterium (Hood and Meyers, 1973). Chitinoclastic actinomycetes have also been found and belong to the genera: Micromonospora, Streptomyces, and Nocardia (Hood and Meyers, 1973).

Chitinoclastic bacteria have been found in the digestive tract of marine fishes (Chan, 1970; Okutani, 1966), whales (Seki and Taga, 1965a) and a number of invertebrates (Chan, 1970; Seki and Taga, 1963d). The function and possible symbiotic and nutritional role of these bacteria in the digestive tract of marine animals is still unknown. Seki and Taga (1963d) calculated that the amount of chitin decomposed by these bacteria to utilizable products in the host was a negligible proportion of the hosts' total diet. These authors did not dismiss the possible symbiotic role of these organisms in providing vitamins and other products to the host.

Chitinoclastic bacteria and actinomycetes have been isolated from non-marine sources including soils (Benton, 1935; Gray and Baxby, 1968; Okafor, 1966; Skinner and Dravis, 1937; Veldkamp, 1955), the rhizosphere of winter wheat and sugar beets (Mihaly, 1960), dead insects, compost materials, the intestinal tract of birds, bats, and fish (Benton, 1935), and in the mud and waters of lakes (Chan, 1970; Erickson, 1941; Kinkel, 1936; Steiner, 1931).

Chitinoclastic Fungi

A number of chitinoclastic fungi have been isolated, mostly from non-marine sources (Gray and Bell, 1963; Leopold and Seichevtova, 1967; Skinner and Dravis, 1937; Veldkamp, 1955). It has been postulated that in Phycomyces blackesleeanus, chitinase may be involved in the growth response system by loosening the rigid framework of chitin at specific and defined points, thus rendering it more extensible for growth (Cohen, 1974).

Nonmicrobial Chitin Degradation

Nonmicrobial chitinases have been found in seeds, bean, wheat, cabbage and almond (Powning and Irzykievicz, 1965) and may act as fungistatic agents. A number of invetebrates and vertebrates (including mammals) are

able to degrade chitin. Chitinase has been found in the hypodermis of the shore crab, Carcinus maenas (Lunt and Kent, 1960), and it has been suggested to play a role in partial resorption of chitin during molting. Over one-half of the N-acetyl glucosamine was recently found to be reabsorbed and incorporated into new pre-molt chitin in crayfish (Gwinn and Stevenson, 1973), although the presence of chitinase was not investigaged by these authors. Chitinases have been found in the snail (Kent, 1964; Jeuniaux, 1961), marine fishes (Okutani, 1966), a number of marine invertebrates (Elyakova, 1972), protozoans, nematodes, earthworms, arthropods, polychaete worms (Jeuniaux, 1961) and in the gastric mucosa of a number of insectivorous birds and mammals (Jeuniaux, 1961). Chitinase has recently been found in the serum of the goat, cow, hen, sheep, and pig (Lundblad et al., 1974). However, chitinase has not been found in omnivorous animals, including man (Jeuniaux, 1963). Whether the chitinase systems found in various animals examined are similar to those of microbial origin remains to be demonstrated. Of those studied, the goat serum chitinase appears to have a different isoelectric point and twice the molecular weight of the chitinase from Streptomyces antibioticus (Lundblad et al., 1974). The chitinase from beans and other seeds also appears to differ from the microbial enzyme (Powning and Irzykievicz, 1965). In many of these studies the possibility of microbial chitinoclastic activity was not ruled out.

Jeuniaux (1971) considered all chitinase systems examples of "regressive evolution." Chitinases and chitobiases are widespread in primitive unicellular organisms. In the more advanced phyla, however, chitinase systems are less common and characterized in many cases by the loss of one of the chitinolytic enzymes. Enzymes are retained only in those organisms that consume chitinous foods. Animals consuming foods devoid of chitin have subsequently lost the ability to produce chitinases.

THE CHITINASE SYSTEM

The chitinase system (E.C. 3.2.1.14) is very similar to the cellulase system; and like the cellulase system, chitinase is multienzymic and exocellular. Chitinase is specific for linear polymers of N-acetyl D-glucosamine; carboxymethyl chitin, glycol chitin, and chitin sulfates are hydrolyzed in addition to chitin (Barman, 1969). The enzyme does not attack chitin nitrates, cellulose, mucin, hyaluronate, and alginate (Barman, 1969). Chitobiase (E.C. 3.2.1.29) or β -glucosidase is often associated with the chitinase system.

Microbial Chitinases

The majority of investigations concerning microbial chitinases have involved preparations from chitinoclastic Streptomyces sp. Chitinases from these organisms have been purified sufficiently and in large enough quantities to obtain physical and chemical data on the properties of the enzyme system (Skujins et al., 1970). Chitinase from Streptomyces antibioticus is composed of 3 enzymes (Jeuniaux, 1959). With Streptomyces griseus, two chitinases with similar enzyme activities and a chitobiase have been separated (Berger and Reynolds, 1958). The two described chitinases differed in electrophoretic mobility and thermal stability. They showed no difference in substrate specificity or in reaction rate (chitosan was not hydrolyzed). Only N-acetyl glucosamine and chitobiose were detected in the hydrolysate (Berger and Reynolds, 1958; Reynolds, 1954). Berger and Reynolds (1958) proposed that with chitin, only the ends of the chitin chains would be exposed to chitinase due to limited hydration of the insoluble molecule, so that chitodextrins (soluble oligomers) would not be found as an intermediate. The chitobiose would then be hydrolyzed by chitobiase to give N-acetyl glucosamine.

Chitinase from a Streptomyces sp. yielded two chitinase enzymes with a pH optimum of 5.0 and molecular weight determined at 29,000 daltons (Skujins et al., 1970). Four calcium atoms were found to be associated with a molecule of chitinase and calcium was found to be necessary in trace amounts to maintain enzyme stability with increasing degrees of purification (Skujins et al., 1970).

The pH optimum for the Streptomyces griseus chitinase has been determined at 6.3 (Berger and Reynolds, 1958) which is similar to that from Serratia marcescens (Monreal and Reese, 1969). Partially purified chitinases of the marine chitinoclastic bacteria, Aeromonas chitinophthora and Vibrio gerris had pH optima of 5.5-6.0 and 7.0, respectively (Okutani, 1966).

There is some evidence for a prehydrolytic factor (CH_1) analogous to the C_1 factor of the cellulase complex. Monreal and Reese (1969) found greater activity on swollen chitin compared to crystalline chitin with the chitinase of Serratia marcescens. This result could be due to the presence of a CH_1 factor capable of modifying the crystalline chitin to a form susceptible to chitinase. Further evidence for a CH_1 factor was found by Jeuniaux (1957a, 1957b, 1959) who observed a synergistic effect when purified chitinases from Streptomyces sp. were recombined.

Chitinase may be synthesized in the absence (constitutive) or in the presence (induced) of its substrate (Jeuniaux, 1966). Clark and Tracey (1956) found that chitinase was constitutive in many species of bacteria, and chitinase has been reported to be inducible in Clostridia (Timmis et al., 1974), Serratia marcescens (Monreal and Reese, 1969) and in Streptomyces sp. (Reynolds, 1954). Soluble oligosaccharides have been postulated to act as inducers (Monreal and Reese, 1969). Like cellulase, chitinase production is subject to catabolite repression by glucose in Serratia marcescens (Monreal and Reese, 1969) and by glucose and N-acetyl glucosamine in Streptomyces sp. (Reynolds,

1954). The fact that chitinase is repressed by glucose and N-acetyl glucosamine makes it difficult to determine if the enzyme is indeed constitutive, for if glucose or N-acetyl glucosamine are present in a medium, no extracellular chitinase would be expected and thus it may appear that the enzyme is inducible.

The source of chitin can affect the degradation rate and the production of chitinase by microorganisms. Chitin from cuttlefish has been found more easily degraded by a Streptomyces sp. than chitin prepared from crab and lobster shells (Nord and Wadström, 1972). In another study, shrimp chitin elicited higher yields of chitinase from Serratia marcescens than did mushroom chitin (Monreal and Reese, 1969). Particle size can also influence chitin degradation rates, with the rate inversely related to particle size (Chan, 1970; Seki, 1965a, b; Seki and Taga, 1963c).

Chitinase from Streptomyces sp. is a relatively stable enzyme with respect to drying and heating (Skujins et al., 1970), and both crude and purified chitinases are stable when frozen for at least two years (Jeuniaux, 1966). The enzyme is inhibited by divalent cations in the following order of decreasing inhibition: Zn^{2+}, Co^{2+}, Mg^{2+}; high concentrations of Ca^{2+} and Al^{3+} were also found to be inhibitory; although at concentrations less than 0.04 M, $CaCl_2$ exhibited some stimulatory effect (Skujins et al., 1970). Copper ions are inhibitory, but p-chloromercuribenzoate and iodobenzoic acid have no effect (Jeuniaux, 1966).

Mode of Chitinase Action

Chitin is an insoluble colloidal substrate and the enzymic reaction takes place after adsorption of the enzyme onto the surface of the substrate (Skujins et al., 1970). The affinity of chitinase for the substrate is so great that the enzyme can be purified by selective adsorption onto chitin. After several hours, the chitin is hydrolyzed by the adsorbed enzyme (Jeuniaux, 1956, 1966).

Removal of protein (Hood and Meyers, 1973) and melanin (Bull, 1970) is necessary before chitin is susceptible to enzymic attack. Microbial decomposition of chitin in shrimp exoskeletons was found by Liston et al. (1965) to be preceded by decalcification and proteolysis. It has been suggested (Chan, 1970) that the passage of chitinous material through the intestinal tract of fishes may render the chitin more susceptible to attack, partly by freeing it from calcium and protein. Because passage is rapid through the guts of most fishes, the "conditioned" chitin is probably ultimately decomposed by the action of chitinoclastic bacteria adsorbed from the gut and from the immediate surroundings into which the chitin is excreted (Chan, 1970).

The terminal steps in chitin breakdown have not been established. No evidence has been found to indicate the presence of an exocellular deacetylase or deaminase produced by a chitinoclastic microorganism (Hood and Meyers, 1973), although workers have detected ammonia, acetic acid, and reducing sugars in media when marine chitinoclastic bacteria were grown with chitin (Campbell and Williams, 1951; Zobell and Rittenberg, 1938). Bacteria capable of decomposing N-acetyl glucosamine have been found in sea water and bottom sediments (Okutani and Kitada, 1970), and a deacetylase has been found in E. coli (Faulkner and Quastel, 1956).

An enzyme catalyzing the deacetylation of glycol chitin, chitin, and oligomers of chitin has been found in fungi and a number of bacteria including: Bacillus cereus, B. megaterium, B. subtilis, E. coli, Streptococcus faecalis, and Staphylococcus aureus (Araki and Ito, 1975). This enzyme released about 30% of the acetyl groups of glycol chitin giving a product with decreased sensitivity to lysozyme. The presence of this enzyme accounts for the occurrence of chitosan in fungi and unusual peptidoglycan containing N-deacetylated residues in cell walls of bacteria and probably protects both bacteria and fungi from

enzymatic degradation (Araki and Ito, 1975).

ENZYMES IN THE AQUATIC ENVIRONMENT

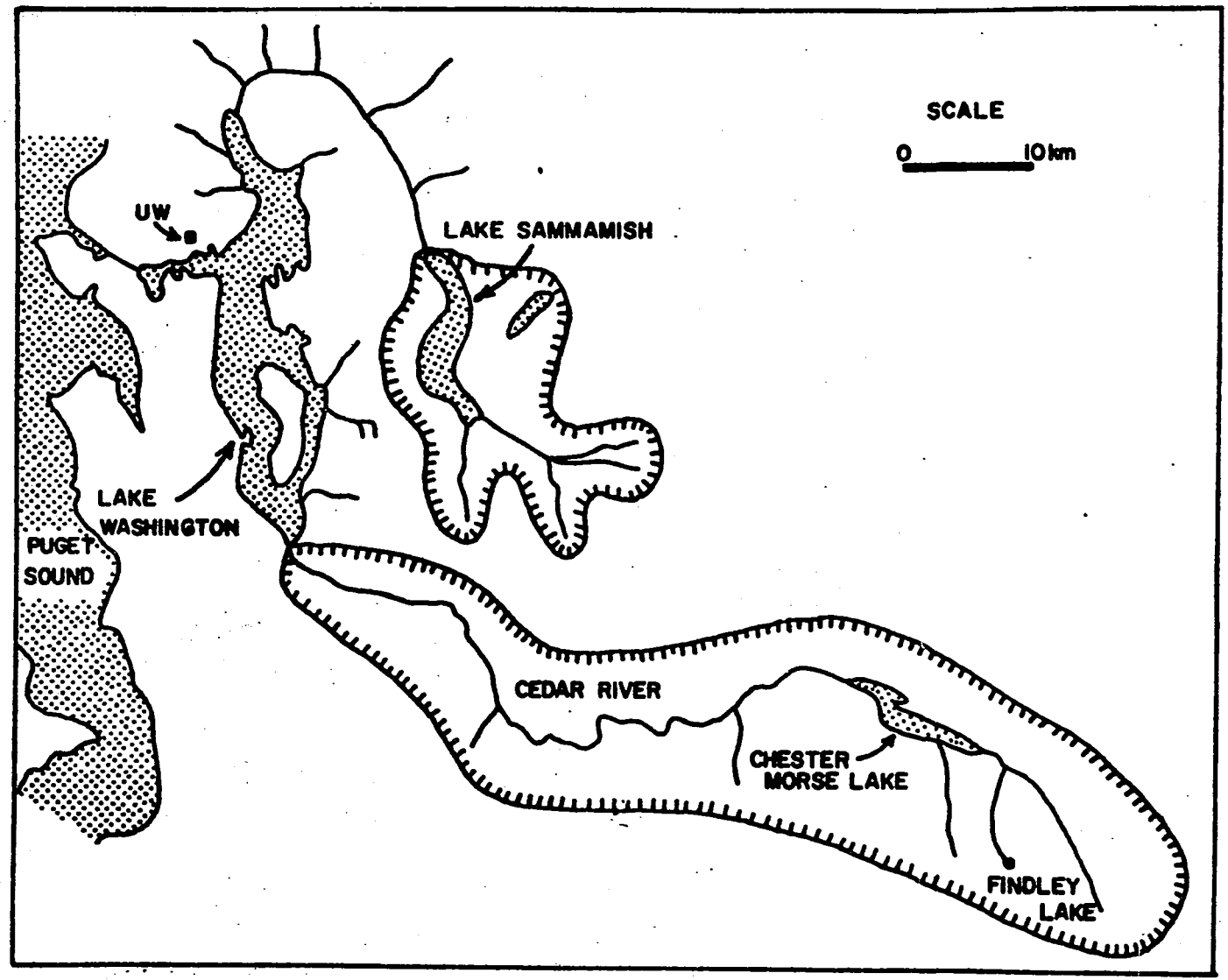
The stability of chitinase may allow this enzyme to remain active for some time in lake sediments. It has been suggested by many workers that bacterial enzymes may be concentrated in marine (Zobell, 1946a) and lake sediments (Vallentyne, 1957) and function long after the death of the bacteria that produced them. Consequently, estimations of live microbial populations cannot always be relied upon to give an accurate picture of the biochemical activity in a given area (Wood, 1965, p. 38). Free enzymes may be important in chemical transformations in both lakes and oceans (Vallentyne, 1957). In fact, these enzymes may be extremely important in the biogeochemistry of lake sediments (Doyle, 1968). Free enzymes such as phosphatases (Overbeck, 1974; Reihardt, 1973), catalases, peroxidases, proteolytic enzymes (Vallentyne, 1957) and esterases (Doyle, 1968) have been found in sediments. The concentrations of many of these enzymes decrease with depth below the surface of the sediment (Vallentyne, 1957). Oxygen may also play a role for the availability of oxygen is a most important factor controlling release of extracellular bacterial proteolytic enzymes (Sizemore and Stevenson, 1974) and bacterial growth and release of chitin (Chan, 1970) and cellulose (Hofsten and Edberg, 1972) digesting enzymes.

THE FOUR LAKES

The four lakes in this study represent lakes of differing trophic status and meteorological conditions. These four lakes are part of the Lake Washington drainage system.

The Lake Washington drainage system (Fig. 1) contains the Lake Sammamish and Cedar River basins. Lake Sammamish drains into the north

Figure 1. The Lake Washington Drainage Basin


SCALE
0 10km
UW
LAKE SAMMAMISH
LAKE WASHINGTON
PUGET SOUND
CEDAR RIVER
CHESTER MORSE LAKE
FINDLEY LAKE

end of Lake Washington via the Sammamish River. The Cedar River picks up drainage from Findley Lake, passes through Lake Chester Morse, and empties into the south end of Lake Washington.

Lake Washington

Both Lakes Washington and Sammamish were formed 14,000 years ago at the end of the Wisconsin Glaciation when the Vashon Glacier receded leaving terminal moraine blocking a preexisting valley (Liesch et al., 1963). After the ice cleared from the Strait of Juan de Fuca, Lake Washington was connected to Puget Sound and was later converted to a freshwater lake by the damming of the basin at its southern end by delta building (Gould and Budinger, 1958). The deposit of limnic peat (gyttja), 17 meters deep in some places in the lake, is interrupted only by a volcanic ash layer at 6,700 years and a layer of varved material having an estimated age of 5,000 years (Gould and Budinger, 1958). Submerged forests, masses of drowned trees, exist in some areas of the lake (Stockner and Benson, 1967). Settlement of Seattle began before 1860 and reached the shores of Lake Washington before the turn of the century (Stockner and Benson, 1967).

The first published record of work done on Lake Washington was an exploratory study in 1913 by Kemmerer et al. (1923), which consisted of a single series of chemical and plankton observations. In 1933, Scheffer and Robinson made a more detailed study on plankton (Scheffer and Robinson, 1939).

The Lake Washington ship canal, constructed in 1916, was formally opened on July 4, 1917. Before the canal was built, Lake Washington and Lake Union were separate lakes. The outlet of Lake Washington was at the south end of the lake through the Black River and into the Duwamish River (Gould and Budinger, 1958). The Cedar River was diverted into Lake

Washington and the Black River was left dry (Gould and Budinger, 1958). Lake Washington was lowered by 2.4 m after the opening of the ship canal (Shapiro et al., 1971). With the lowering of Lake Washington, the Sammamish River had a stronger current and it was dredged to make it navigable to small craft (Ajwani, 1956).

Between 1910 and 1920 there were 30 sewage outfalls discharging raw sewage into the lake along the west side (Stockner and Benson, 1967). Three primary sewage treatment plants were constructed in 1924. Diversion of sewage effluents began between 1926 and 1930 and was completed by 1936 (Stockner and Benson, 1967). From 1936 to 1941, the lake received only storm sewage overflow from the city of Seattle; urbanization and extensive development of the east side of the lake occurred from 1941 to 1955 (Stockner and Benson, 1967). Between 1941 and 1959, eleven secondary sewage treatment plants were built on both sides of the lake, on Mercer Island, south and on the Sammamish River (Stockner and Benson, 1967).

By 1955, Edmondson reported the presence of a large population of the nuisance blue-green alga, Oscillatoria rubescens (Edmondson et al., 1955). There was no record of this organism before 1955. The mean summer concentration of dissolved phosphate tended to increase in the hypolimnion of Lake Washington from 1933 to 1955; the 1955 oxygen deficit in the lake had increased to 2.7 times the rate observed in 1933 and 1.8 times greater than that of 1952, due to the increase in treated sewage added to the lake as a result of extensive urbanization (Edmondson et al., 1955). The mean summer chlorophyll in the epilimnion increased from 2 µgm/L to 10 µg/L from 1950 to 1964, related to the greater abundance of phytoplankton; the mean summer secchi disc transparency decreased from 3.5 m in 1950 to 1.1 m in 1964 (Edmondson, 1966). In 1955, Lake Washington was receiving effluent from 10 secondary treatment

plants amounting to 24,200 m^3/day and by 1957, this sewage effluent was contributing 56% of the phosphate and 12% of the nitrogen income of the lake (Edmondson, 1970). A program for diversion of all sewage effluents emptying into the lake was voted by public action and began in 1963 (Edmondson, 1970). By 1965, the volume of sewage influents were reduced to 55% of the original; almost 99% of sewage was diverted in March 1967, and by February 1968, sewage diversion was completed (Edmondson, 1970, 1972). After the first diversion, the condition of the lake began to improve steadily; the winter concentrations of phosphates and nitrate decreased, but at different rates (Edmondson, 1967, 1970). Nitrogen values are still high, but the water supply to the lake is higher in nitrogen than in phosphorus, and phosphorus is the dominating limiting element (Edmondson, 1970). The high phosphorus content of the sediment laid down in the years of maximum enrichment will continue to be indicated by a maximum in the sediments, while nitrogen does not show a corresponding older maximum (Shapiro et al., 1971). Examination of the diatom remains in recent Lake Washington sediment also reveals a correlation with the pattern of sewage enrichment over the past 80 years; in deeper sediment deposited prior to cultural enrichment, the relative composition of diatoms is constant but with increasing levels of sewage discharge into the lake, many indicator species changed in proportion (Stockner and Benson, 1967).

Presently, there are two major sources of inflow to Lake Washington, the Sammamish River, flowing from the north and the Cedar River, flowing from the south. All discharge from the lake is controlled through the ship canal and government lock system which drain west to Puget Sound (Gould and Budinger, 1958). Lake Washington has been classified as oligotrophic-mesotrophic before settlement of the area and can be considered a oligotrophic by morphometric factors (Stockner and Benson, 1967). Today the lake is classified as mesotrophic

(Hendry, 1973). The lake is presently heavily settled and trunk and sewer lines surround it; no sewage presently flows into the lake (Hendry, 1973).

Lake Washington is used intensely for recreation, including sports fishing, water skiing, recreational boating, etc., and the shores of the lake are heavily urbanized. The dominant fish in the lake is the andramous sockeye salmon (Oncorhynchus nerka) which was introduced about 30 years ago (Taub et al., 1972). Other anadromous and resident fish populations also are present.

Lake Washington is a monomictic lake and has a distinct temperature stratification period from May to October. In late fall, hypolimnetic oxygen levels decrease but these levels do not go as low as those in Lake Sammamish. Lake Washington is a deep narrow trough with a maximum depth of 65 m (Table 1). Convective flow takes place in the lake during the winter overturn on all steep marginal slopes eroding the sediment from the trough at the base of the slopes and redepositing it to form the medial ridge of the basin floor (Gould and Budinger, 1958).

Lake Sammamish

Lake Sammamish is 16 km southeast of Seattle and lies in a north-south direction. The lake, classified as mesotrophic (Emery et al., 1973a, b), is at an elevation of 12 m above sea level, and has a maximum depth of 31 m (Table 1). The lake has two principal inflowing streams: Issaquah Creek and Tibbets Creek, both entering at the southern end (Horton, 1972). Issaquah Creek supplies 70% of the surface inflow (Isaac et al., 1966). There are over 40 other sources of surface inflows to the lake. Ground water augmentation may also be high for much of the lake is underlain with stratified glacial sands and gravel (Liesch et al., 1963). The only major outlet is the Sammamish River which flows from the north end of the lake and eventually enters the northern end of Lake Washington.

Table 1. Physical Features of Lakes Washington, Sammamish, Chester Morse, and Findley*

	Lake Washington	Lake Sammamish	Lake Chester Morse	Lake Findley
Max. Depth, m	65	31	35	28
Mean Depth, m	33	17	19	8.2
Area, km^2	87.60	19.80	6.81	0.158
Volume, km^3	2.884	0.328	0.128	0.0013
Length, km	35.1	12.9	8.1	-
Elevation, m	4.3	8.5	473	1131
Av. H_2O Exch. time, yr.	2.5[2]	Appr. 2-4	0.27	Appr. 0.20-0.25

*Data courtesy of Peter Birch (1974).

The Cedar River basin was once dominated by virgin coniferous forests. Exploitation of these began in the late nineteenth century when logging became an important industry; Lake Sammamish served sawmill operations until 1924 (Emery, 1972). Remnants of sawmill pilings and debris can still be found in the lake bottom and around the perimeter of the lake. Early mining activity in the Lake Sammamish watershed was probably important in inducing sedimentary layers of silt on the bottom of the lake (Emery, 1972). The Lake Sammamish watershed is in the process of rapid residential development. Much of the western and southern sides of the lake are developed into suburban housing tracts. The east side is rural, and the town of Issaquah is on the southern end. To the north is the city of Redmond and Bellevue is on the west. Freeways run along the south and west ends of the lake. The west and south parts of the east shore

watershed are provided with sewer lines and most residences are connected (Hendry, 1973).

Wastes entering Issaquah Creek were the object of sewage interception and diversion began in 1968 at a cost of over $3 million. Very little information is available regarding Lake Sammamish before nutrient diversion (Edmondson, 1969). The lake was closely monitored for biological and nutrient changes after sewage diversion; however, the response of Lake Sammamish was less dramatic than that of Lake Washington (Emery, 1972; Moon, 1973). The diversion of about 40% of the external phosphorus inflow to the lake had little or no effect on either the P levels, which remained constant at 25–40 µgm/L, or the algae concentrations (Emery et al., 1973a). The P and N concentrations by 1972 had not changed significantly except the total P concentration immediately following the fall overturn in post-diversion years has been reduced by about the same percentage of P diverted in 1968; the P levels are not reduced in other seasons of the year (Emery, 1972). The seasonal levels of phytoplankton production and biomass are not decreased significantly since nutrient diversion, but a change in the composition of phytoplankton populations has occurred (Emery, 1972). Emery's results (1972) with unialgal cultures in vitro as well as in situ with natural populations of phytoplankton and periphyton indicate that urban runoff is not now a significant source of nutrient enrichment for Lake Sammamish.

The first major limnological investigation of Lake Sammamish was made by the Municipality of Metropolitan Seattle (METRO) from June 1964 to December 1965 (Isaac et al., 1966). Recent studies have shown that the levels of oxygen, iron, and phosphate are related in Lake Sammamish. The lake sediments have a great capacity for phosphorus sorption under aerobic conditions, and release phosphorus under anaerobic conditions (Horton, 1972). The phosphorus levels in Lake Sammamish are controlled by the presence and form of

iron which is in turn controlled by the redox conditions of the system. As thermal stratification proceeds and the oxygen is reduced in bottom water, iron is reduced and the associated phosphorus is released, with subsequent re-oxidation of iron in the upper more oxygenated waters. At the time of the fall overturn in Lake Sammamish, the solubilized phosphorus and iron forms are mixed upward into the aerobic surface waters. The ferrous ion oxidizes, forming hydrated colloidal ferric oxides, phosphorus is sorbed by ferric oxides, and is precipitated from the surface (Horten, 1972). As much as 75% of the phosphorus released into the hypolimnion of Lake Sammamish is supplied by the sediments (Monahan, 1974). It has been suggested that the lack of response in trophic status of Lake Sammamish following nutrient diversion in 1968 could be due to the ability of the lake sediments to supply large amounts of nutrients to the lake water column (Monahan, 1974).

Lake Sammamish is monomictic with thermal stratification beginning in May. Maximal water column stability occurs by late August. Downward erosion of the thermocline begins in late September and vertical thermal mixing is complete by late November (Emery, 1972). Light is the principal temporal control of primary production in Lake Sammamish and phosphorus is growth limiting to phytoplankton (Hendry, 1973). Copepods are the most abundant component of zooplankton in Lake Sammamish (Pederson, 1974). Some of the fish species in Lake Sammamish are the same as in Lake Washington, including the sockeye salmon, although in Lake Sammamish, the numbers are not as great as in Lake Washington (Taub et al., 1972).

Lake Chester Morse

Both Lakes Chester Morse and Findley are in the Seattle watershed. Use of the watershed is closely regulated and residential and recreational uses have been prohibited by Seattle City Ordinance No. 19061 since 1908 (Lamb,

1914). Timber rights are owned by both governmental and private organizations which have been operating road building and clear cut timber operations for many years (Hendry, 1973). Lake Chester Morse lies 10.6 km southeast of the city of North Bend, Washington, and has served as a supply of much of the water for the city of Seattle since 1911 (Horten, 1972).

The glaciation of this region is responsible for the alpine valleys and pronounced morrains. Lobes of the Vashon glaciation moved into this area during the period of continental glaciation between 21,000 and 14,000 B.C. with the maximum period of glaciation at a later date, after the end of the period when local glaciation had scoured river valleys; the Vashon ice damned these valleys, causing formation of glacial lakes (Mackin, 1941). Glacial debris from morrains, till and outwash from this continental glaciation formed the morrainal impoundment at the northwest end of Lake Chester Morse (Mackin, 1941). In 1904, a crib dam was built to raise the level of then called Cedar Lake from 466.3 m to 471.2 m to improve its use as a water reservoir for the city of Seattle (Hendry, 1973). This dam is still in place and marks the end of Lake Chester Morse. A second masonry dam was built in 1914, 2.3 km downstream from the crib dam; severe flooding occurred 2 km from the new dam due to permeable morrain, consequently the reservoir is now allowed to rise 474 m, although the new dam was intended to provide a maximum of 484.6 m (Hendry, 1973).

Oligotrophic Lake Chester Morse is a dimictic lake, with pronounced thermal stratification in summer (May to October), with a shorter less stable stratification in winter. The bed of the lake is a uniform trough, and the maximum depth is 35 m (Table 1). Meterological data are collected at Lake Chester Morse by the National Oceanic and Atmospheric Administration (NOAA) (Cedar Lake Station). The average mean annual temperature is 8.7^{o} C; the average

annual rainfall is 266 cm.

The phytoplankton growing season was found by Hendry (1973) to begin in early April with the maximum production by mid-May in 1972. The greatest effect on light penetration is by autochthonous biomass and suspended allochthonous material with secchi disc readings varying from 3.3 to 9.7 m with a reading of 8.8 m, mean value during the growing season (Hendry, 1973). Primary production is limited by phosphorus during the April to October thermal stratification; but during the rest of the year, high turbidity reduces light due to mixing deep in the water column and light becomes growth limiting (Hendry, 1973).

Lake Chester Morse has a resident fish population of rainbow trout (Salmo gairdneri), Dolly Varden (Salvelinus malma), whitefish (Prosopium sp.), and sculpins (Cottus sp.) (Wyman, 1975). There is a low average numerical abundance and biomass of zooplankton, with rotifers and cladocerans of approximate equal importance; maximal total biomass occurred in June of 1972 and July of 1973 (Pederson, 1974).

Findley Lake

Findley Lake is a subalpine cirque lake in the western Cascade Mountains, 18 km southwest of Snoqualmie Pass. The land area around Findley Lake consists of undisturbed mature coniferous forest, with most of the area timbered by relatively homogenous old-growth Pacific silver fir (Abies amabilis) (Olson et al., 1972). Widespread talus accounts for 16.2% of the total basin and soils of mixed materials, divided as forested, semiforested, and nonforested, account for 56.2, 4.3 and 1.6%, respectively, of the total basin; soil profiles show evidence of two relatively recent fires as well as three volcanic ash depositions (Olson et al., 1972). Access to Findley Lake is by a 14.5 km logging road above Cedar Falls, then by foot over a trail of 2 km to a ridge at

152 m above the lake. The trail descends in steep switchbacks to the lake level at 1128 m (Hendry, 1973). The trail to the lake was made in the summer of 1971 under guidance of IBP research workers.

The watershed of the Findley Lake glacial cirque was carved out at least 15,000 years ago and has an area of 2.6 km^2; the surrounding ridge crests attain an elevation of over 1447 m, the cirque walls are steep, with prominent talus slopes and avalanche scars (Olson et al., 1972). The lake has a maximum depth of 28 m and this basin is a continuation of a talus slope beneath cliffs on the western side of the lake (Table 1). Numerous temporary inflow streams from snow melt supply water to the lake. The outlet is through a single stream, Findley Creek at the northwest corner of the lake. Findley cirque opens to the north ridge crest around the rest of the lake causing considerable shading in early morning and late afternoon.

Findley Lake is the only lake of the study group that freezes in the winter months, with the snow pack reaching 4 m. Melt usually occurs in June-July, and the ice-free period usually lasts four months until ice over which begins in October-November.

Findley Lake is dimictic with pronounced thermal stratification in summer and in winter. After melt, complete turnover and restratification occur in a few weeks. The lake remains stratified in summer. Complete occlusion of light from the lake because of snow cover prevents winter phytoplankton production, which peaks immediately following the clearing of snow from the surface, followed by a rapid decline in the production rate (Hendry, 1973). Biomass is primarily responsible in controlling light penetration during the snow-free period (Hendry, 1973). Most phytoplankton in the 27.5 m water column takes place below the thermocline due to surface inhibition of primary productivity thought to be caused by UV light; high surface primary productivity was found

only on a dark cloudy day (Hendry, 1973).

The relation between production and nutrient concentration in oligotrophic Findley Lake is not clear. Ortho phosphate is low and approaches the lower detection limit, ranging from 0.5 to 8.3 µgm/1. The mean N concentration fluctuates toward the end of the growing season, total N was at a minimum level of 12 µgm/1 in September 1971 and increased to 76 µgm/1 in late March 1972, declining after ice out. Nitrogen and phosphorus were found to be most limiting to phytoplankton growth (Hendry, 1973). In Findley Lake, seasonal changes in phytoplankton production are regulated primarily by light; after ice out, the nutrient levels are high, with ortho phosphate 4.5 µgm/1, and available nitrogen, 44.9 µgm/1 (Hendry, 1973). In Findley Lake, Pederson (1974) found low average numerical abundance and biomass values for zooplankton, with Cladocerans and Copepods of equal importance in biomass, with maximal numbers occurring in September of 1972 and August 1973. Findley Lake has no resident fish population but has three species of amphibians in abundance in the lake and lower pond: the rough-skinned newt (Taricha granulosa), western toad (Buffo boreas), and Cascade frog (Rana cascadae); less common are the northwestern salamander (Ambystoma gracile) and the Pacific treefrog (Hyla regilla) (Olson et al., 1972).

SUMMARY OF THE LITERATURE REVIEW AND APPROACH TO THE PROBLEM

Aquatic heterotrophic bacteria, present in water and sediments, are important in the breakdown of complex organic compounds to simpler molecules that can be readily assimilated by higher organisms. Heterotrophic bacteria are also responsible for the removal of some of these dissolved organic substrates and, as a result, keep the net balance of these compounds relatively low in the aquatic environment (Allen, 1969; McCoy and Sarles, 1969; Wright and Hobbie, 1966). Many of the earlier studies of aquatic bacteria in freshwater were from the standpoint of sanitation and not ecology. Later ecological studies of these organisms were primarily descriptive. In recent times, as the problem of eutrophication of water bodies emerged, the function and activities of aquatic bacteria were questioned. The availability of radionuclides made these studies of aquatic bacterial activities possible. The role of these bacteria has been studied using isotopes, in marine waters (Hamilton and Austin, 1967; Hamilton et al., 1966; Hamilton and Preslan, 1970; Vaccaro, 1969; Vaccaro and Jannasch, 1966, 1967), and freshwaters (Allen, 1971; Burnison and Morita, 1973, 1974; Morgan and Kalff, 1972; Thompson and Hamilton, 1973; Wright and Hobbie, 1966). Aquatic heterotrophic bacterial activities have been measured using ^{14}C-labeled compounds based on a method first described by Parsons and Strickland (1962), modified to give kinetic data by Wright and Hobbie (1965), and later corrected for respiratory $^{14}CO_2$ loss (Hobbie and Crawford, 1969b). Most of the studies of aquatic bacteria concerned those found in the water column. Recently methods have been developed

to measure uptake and mineralization kinetics of organic ^{14}C compounds by heterotrophic bacteria associated with sediment in the marine (Wood, 1973) and freshwater environment (Hall et al., 1972; Harrison et al., 1971). Although numerous studies have been carried out in the aquatic environment, very little is known of the activities of heterotrophic bacteria associated with the sediment, a site of intense microbial activity, in lakes of the Pacific Northwest.

This study was begun in 1972 as part of the Coniferous Forest Biome (International Biological Program) to determine the activities of sediment heterotrophic bacteria and to compare these activities in lakes of differing trophic status and meterological conditions in the Lake Washington drainage basin. Glucose mineralization rates in undiluted sediment, using uniformly labeled ^{14}C glucose were determined to approximate the activities of heterotrophic bacteria in situ in Lakes Washington, Sammamish, Chester Morse, and Findley. Various parameters affecting the bacterial glucose mineralization rate were investigated and included:

1. Seasonal fluctuations of the glucose mineralization rate and bacterial biomass.
2. Relation of trophic state of each lake to the bacterial biomass and glucose mineralization rate.
3. Relation between temperature and glucose mineralization rate and bacterial biomass.

Although the rate of glucose mineralization is a measure of the potential capabilities of bacteria present in the environment, glucose, as such, is present in only very low concentrations as a result of bacterial activity, and for only very short times. Glucose is a building block of a number of complex materials formed by photosynthesis and is present in structural materials of a number of aquatic animals. These materials or refractory carbon compounds probably supply the major portions of glucose found in aquatic environments. To be available as a source of carbon and energy to many aquatic organisms,

these refractory compounds must be hydrolyzed to their component building blocks which are in turn mineralized further. The microbial degradation of refractory carbon compounds provides an input of carbon in aquatic environments. Therefore, bacteria degrading these compounds were studied. Although much information exists regarding microbial decomposition of the refractory carbon compound, chitin in the marine environment, very little is known about chitinoclastic bacteria in fresh waters. Except for cellulose, chitin is probably the most abundant of naturally occurring organic compounds (White et al., 1968). Chitin is a β-1-4 linked polysaccharide composed of N-acetyl glucosamine units, and like cellulose is decomposed to glucose. Chitin is a structural component of the exoskeletons of crustaceans, insects, coelecterates, annelids, molluscs, brachiopods, fungi, and yeasts (Hood and Meyers, 1973; Rudall and Kenchington, 1973). Chitin is degraded by microorganisms, primarily the bacteria, actinomycetes, and fungi (Hood and Meyers, 1973). The chitinase system is very similar to the cellulase system, and like the cellulase system is multi-enzymic (Berger and Reynolds, 1958; Jeuniaux, 1959, 1966; Skujins et al., 1970). Chitobiase or β-glucosidase is often associated with it (Jeuniaux, 1966).

The second part of this study involved the degradation of chitin by chitinoclastic bacteria isolated from the 4 lakes and included:

1. The occurrence and seasonal fluctuations of chitinoclastic bacteria in sediments of the 4 lakes.

2. Effects of environmental parameters (oxygen, nutrient, temperature) on the development and activity of chitinoclastic bacteria.

3. The effect of nutrient and temperature on growth of chitinoclastic bacteria, production of chitinase, and activity of chitinase, using representative isolated pure cultures of chitinoclastic bacteria isolated from the 4 lakes, and cell-free extracts prepared from these organisms.

4. Characterization of the chitinase enzyme system using cell-free extracts from the isolates:

   a. determination of pH optimum for activity

   b. determination of optimum temperature for activity and range

   c. purification of enzyme using ammonium sulfate fractionation

   d. determination of the stability of the enzyme.

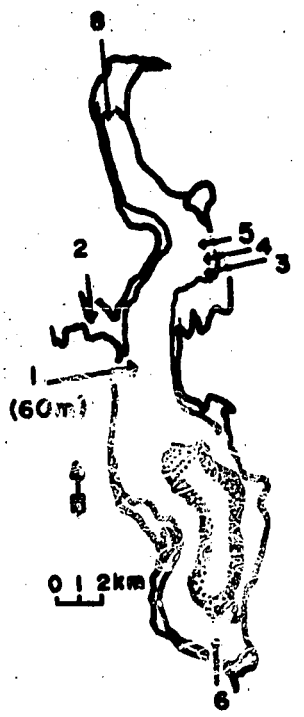
MATERIALS AND METHODS

Sampling Stations

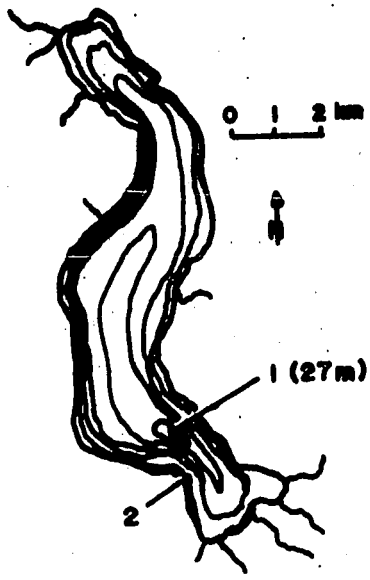
Sampling stations used in this study were representative areas of the 4 lakes chosen by Coniferous Forest Biome researchers.

Samples from the sediment-water interface were collected in each of the 4 lakes at two main stations in the littoral and benthic zones as shown in Figure 2. The benthic stations were located at the maximum depth of each lake. Occasionally, samples were taken from stations other than the main ones and are also shown in Figure 2. Station No. 1 at Findley Lake was sometimes difficult to locate in the winter months due to a thick ice cover. Consequently, some of the sediments collected from this site, although close to the area of maximum depth of 27 m, were from a lesser depth. During the ice cover period at Findley Lake, samples were obtained by cutting a hole in the ice with an auger. Maximum ice thickness averaged 40 cm in the winter. After ice out, the 27 m station was marked by a buoy and samples were taken from a boat. The 33 m station at Lake Chester Morse was also marked by a buoy. Benthic stations in Lakes Sammamish and Washington were found by compass and visual sightings. Temperature profiles of the water column and sediment were routinely taken at the time of sampling with a Tri-R electronic thermometer (Tri-R Instrument Co., Rockeville Centre, New York). Three sample temperature profiles for Findley Lake in 1972 are shown in Figure 3. In August, the lake exhibited a typical summer stratification. In October, the thermocline had descended to 15 m, and by November, the lake had turned over with a uniform temperature of 5^{o} C, from the top to the bottom of the lake.

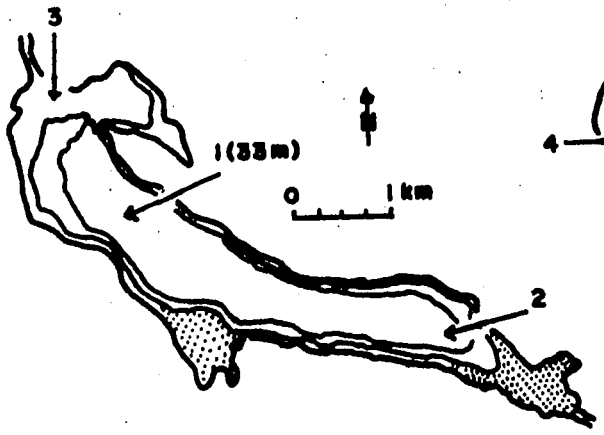
Figure 2. Sampling Stations with Recorded Depths at Each of the Four Lakes within the Lake Washington Drainage Basin



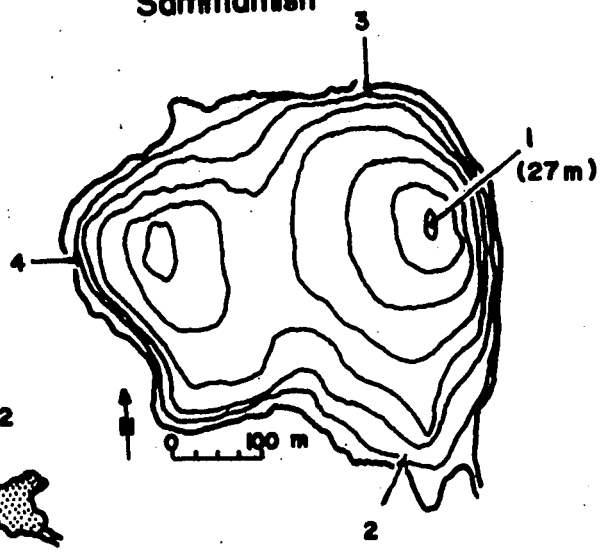
Washington



Sammamish

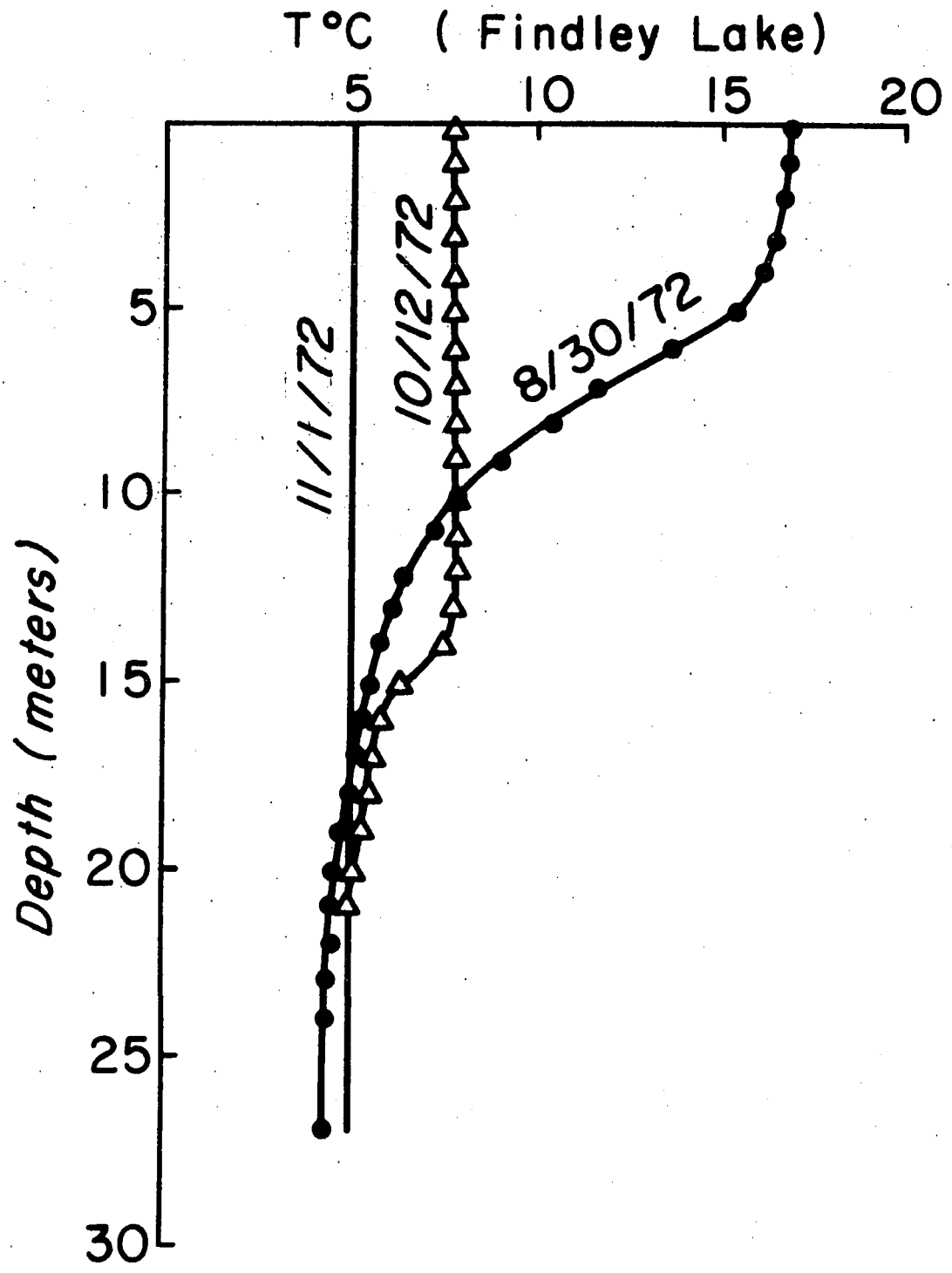


Chester Morse



Findley

Figure 3. Temperature Profiles for Findley Lake, 1972


T°C (Findley Lake)
5
10
15
20
5
10
15
20
25
30
Depth (meters)
11/1/72
10/12/72
8/30/72

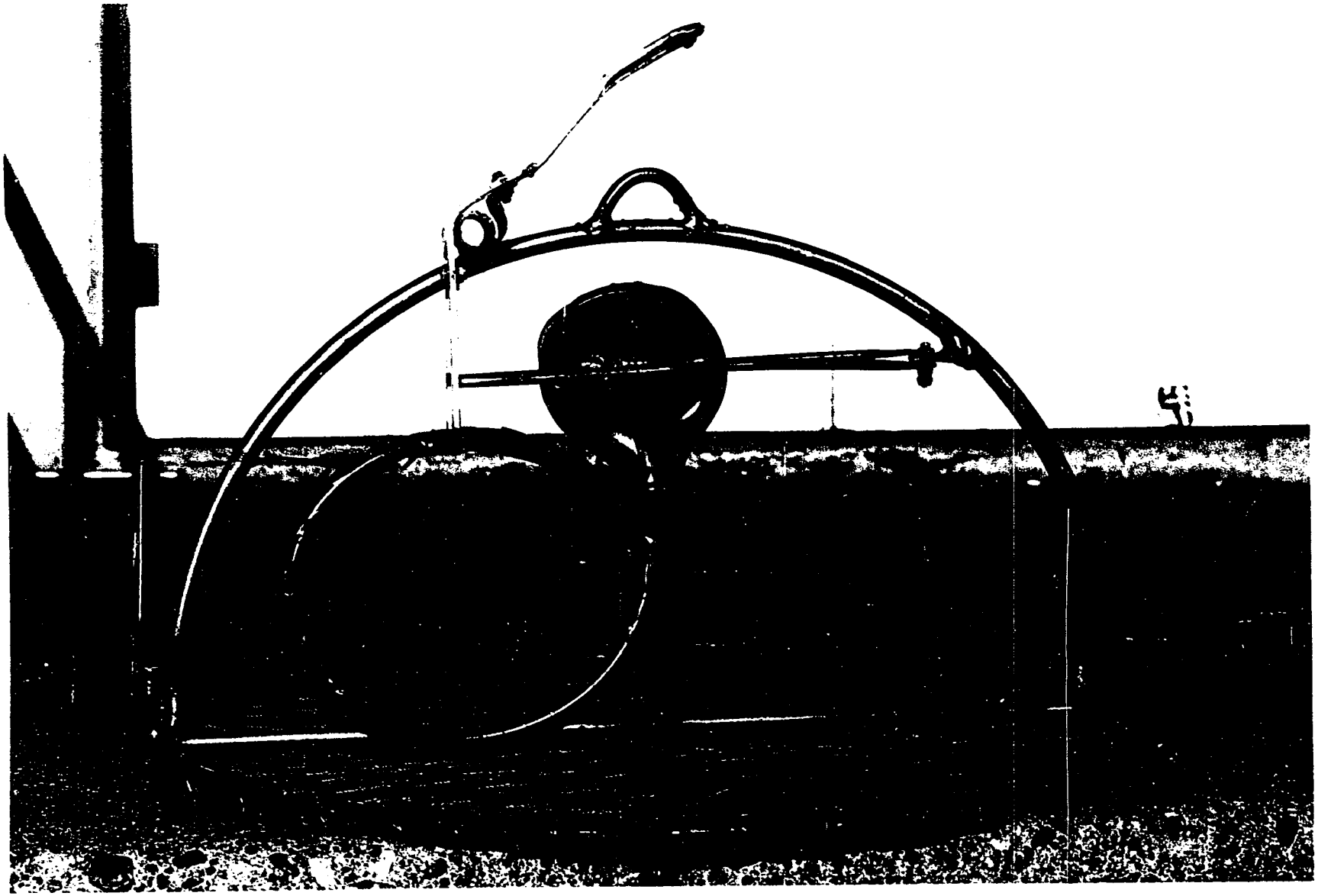
Temperature profiles for the 4 lakes are included in the appendix (Table 50).

Sampling Devices

Sediment samples were taken from the benthic areas of the lakes with a suction device patterned after the "ooze sucker" described by Welch (1948). This device (Plate 1), consisted of a loop made from a 60 cm length of extruded acrylic plastic tubing (ID 5/16 in., 1/16 in. wall thickness) connected to a 2 oz. rubber bulb and wrapped with plastic tape. The lower end of the plastic tubing extended approximately 0.5 cm from a bottom support ring which rested on the bottom of the lake when in use. To load the device, the rubber bulb was clamped shut, with the ends of the clamps extended through a tripping mechanism attached to wire cable. When properly positioned on the lake bottom, a messenger was dropped down the cable, releasing the clamps. The sample, containing the top 2 cm of sediment plus interstitial water in a volume of approximately 50 ml, was aspirated into the bulb and attached plastic tubing. This device was observed to cause very little disturbance of the sediment-water interface before the sample was collected when positioned on the lake bottom. Before use, the suction device was washed with 95% ethanol, followed by 4 rinsings with sterile distilled water to reduce the possibility of terrestrial contamination.

Sediment and water samples from littoral areas of Lakes Washington and Findley, were collected with a sterile plastic 10 ml pipet, with the tip removed, attached to a sterile 2 oz. rubber bulb. The top 1-2 cm of sediment plus interstitial water was aspirated. The pipet was easier to use for littoral areas than the suction device, due to the presence of rocks and other submerged material in littoral areas of these two lakes, preventing the use of the suction device. No differences in results were found for samples collected with the pipet compared to those taken with the suction device.

Plate 1. Suction Sampling Device in the Loaded Position. Sampler Patterned After the "Ooze Sucker" Described by Welch (1948).



Core samples were taken using a 13 x 34 cm plastic hollow tube attached to a messenger tripped geological gravity coring device. The plastic tube was rinsed with 95% ethanol, immediately followed by 4 rinsings with sterile distilled water. After sample collection, the core was plugged at both ends with sterile rubber stoppers, and maintained on ice during transport to the laboratory.

Sampling

Sediment samples of the fine sediment-water interface were collected seasonally in Lakes Washington, Sammamish, Chester Morse, and Findley. All samples were collected between 1000 and 1300 hours. The sediment samples were transferred to sterile, screw capped glass containers, and maintained on ice until analysis. All samples were analyzed within 3 to 4 hours of collection.

Samples collected for the isolation of cellulolytic bacteria in Lake Sammamish (littoral station, sunken forest) were collected with a sterile plastic 10 ml pipet, attached to a sterile rubber bulb. Samples were obtained at a depth of 2 to 3 meters by skin diving. The top 1 to 2 cm of sediment around sunken tree stumps was aspirated. Upon return to the laboratory, these samples were serially diluted, plated out on freshly prepared cellulose agar plates, and incubated aerobically and anaerobically at the approximate *in situ* temperature.

Glucose Mineralization Rate Determinations

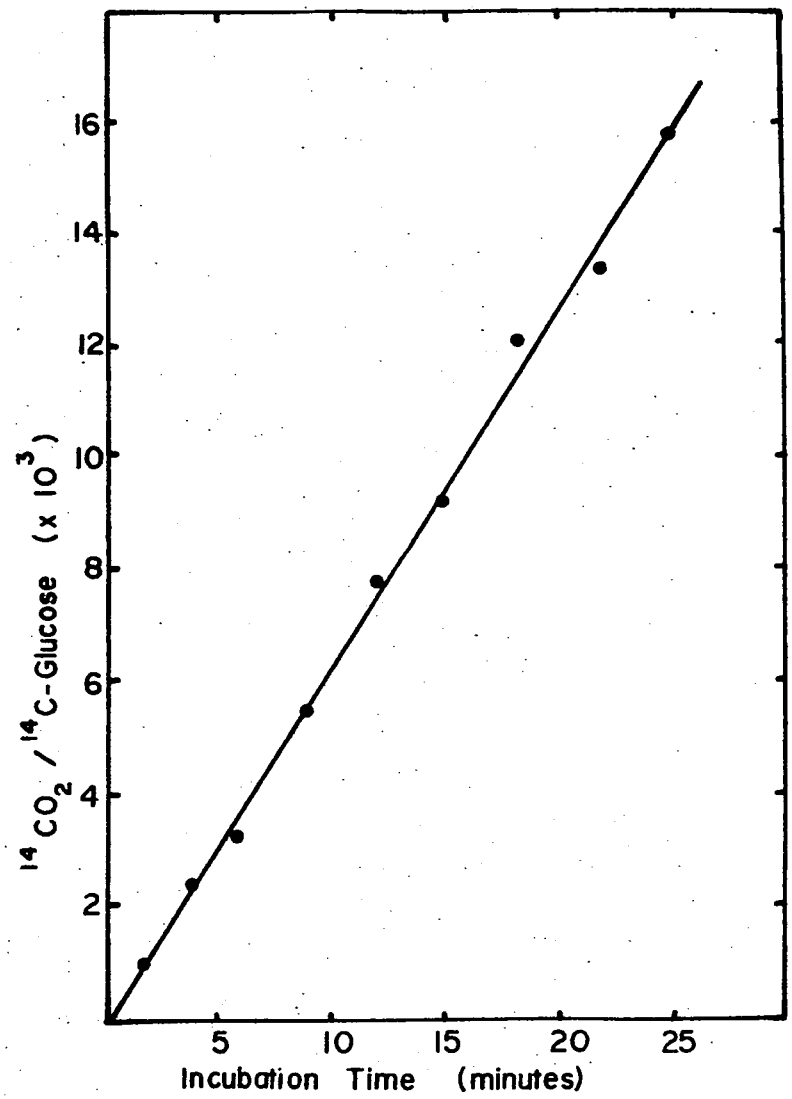
Glucose mineralization rate determinations were patterned after the method of Harrison et al. (1971). All data are reported on the basis of dry weight of the sediment. Dry weight was determined by taking the mean of 5 replicate samples, containing 5 ml sediment/sample in tared aluminum cups,

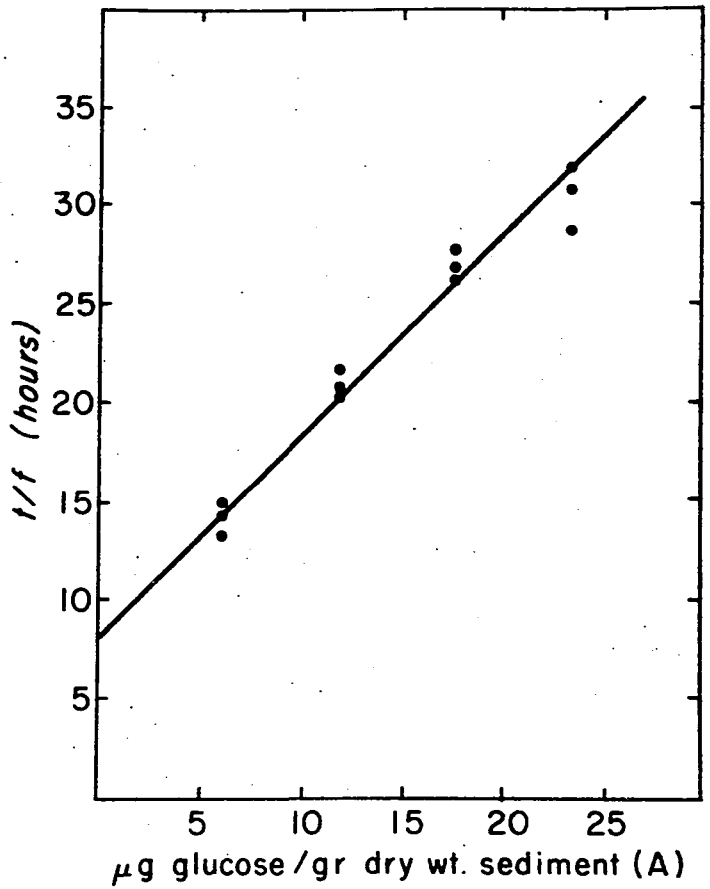
and dried at 90° C for 24 hours. A sterile magnetic stirring bar was placed in the sediment sample. The sample was slowly stirred on a magnetic mixer (Mag Mix, heavy duty) just sufficiently to ensure a homogenous slurry and 5 ml portions containing approximately 200 mg dry weight, were dispensed into sterile 50 ml serum vials. A sterile plastic pipet with the tip removed by a hot (flame sterilized) scalpel blade was used to measure the sediment slurry. The vials were capped with sterile aluminum foil and equilibrated at the prevailing in situ temperature for one hour in a thermostatically controlled refrigerated water bath. After temperature equilibration, sterile unlabeled D-glucose (Merck) contained in 1.0 ml was added to 4 replicates in concentrations of 0.2, 0.4, 0.6, and 0.8 µgm, for a total of 16 samples. The unlabeled glucose was at the in situ temperature to minimize temperature shock. One of each replicate served as a blank and contained 1 ml of 2 N H_2SO_4. This was immediately followed by addition of 0.06 µmole, in 0.1 ml, of sterile ^{14}C uniformly labeled glucose (Amersham Searl, 250 mCi/mmole). The ^{14}C glucose was kept frozen until use. A 100 ul Hamilton syringe sterilized with 95% ethanol, followed by a sterile distilled water rinse was used to dispense the labeled glucose. Immediately after the addition of ^{14}C glucose, the vials were carefully closed with a serum cap pierced by a plastic cup (Kontes Glass Co., Vineland, N.J.: K-882320) containing a 2 x 5 cm accordian pleated filter paper (Whatman No. 1), taking care not to splash the liquid in the serum vial on the filter paper. The samples were incubated at the in situ temperature for 10 minutes. Samples collected during the winter months were incubated for 15 to 20 mimutes due to slower reaction rates at the lower temperatures. The reaction was linear within these time periods and temperatures (Fig. 4).

Samples analyzed to determine the temperature optimum for glucose mineralization were temperature equilibrated for one hour and analyzed at

Figure 4. Fraction of ^{14}C-UL glucose converted to $^{14}CO_2$ as a function of time in minutes for Lake Washington littoral zone sediment sample incubated at 6°C. Values represent mean of duplicate samples of 5 ml sediment, incubated with 0.6 µgm unlabeled glucose, +0.1 µCi (0.06 ugm) UL-C-14 glucose.

Figure 5. Plot of t/f versus A where t = time of incubation in hours, f = fraction C-14 glucose mineralized to C-14 CO_2 and A = µgm total glucose (labeled + unlabeled) per gram dry weight of sediment. Data from Lake Chester Morse littoral station sediment.


$^{14}CO_2$ / ^{14}C-Glucose (x 10^3)
2
4
6
8
10
12
14
16
5
10
15
20
25
Incubation Time (minutes)


t/f (hours)
5
10
15
20
25
30
35
5
10
15
20
25
μg glucose/gr dry wt. sediment (A)

various temperatures other than the in situ temperature.

All samples were incubated unshaken under normal laboratory lighting conditions.

The reaction was terminated with 1 ml of 2 N H_2SO_4, added with a 10 ml plastic syringe through the serum cap to kill the microorganisms and to drive off the dissolved CO_2. After one hour, 0.2 ml of scintillation grade phenylethylamine (Eastman Kodak) was added with a 1 ml syringe through the serum cap to the filter paper. After 2 hours, the paper was removed with forceps and placed in a glass scintillation vial containing 10 ml of Instagel™ (Packard Instrument Co., Downers Grove, Ill.). Samples were counted (10 minutes/sample) in a Packard Tri Carb Scintillation Counter. Corrections for quenching were made by running a series of similarly quenched ^{14}C glucose and $^{14}CO_2$ standards. Recovery of $^{14}CO_2$ from sediment samples was complete. When 0.3 μCi of $Na_2\,^{14}CO_3$ was added to each of 5 aliquots of sediment slurry and acidified as described, all of the label was recovered. Data were corrected for blank activity.

Calculation of Glucose Mineralization Rates

The adapted Lineweaver-Burk equation derived by Wright and Hobbie (1965) was used to give the glucose mineralization turnover time and the maximal velocity or V_{max}. This equation is:

$$t/f = \frac{(K + S)}{V_{max}} + \frac{A}{V_{max}}$$

Where:

t = incubation time in hours

f = fraction of available substrate mineralized

K = constant related to uptake

V_{max} = maximal velocity for mineralization (which would apply when transport is at maximal velocity)

S = natural substrate concentration (unknown)

A = concentration of added substrate (labeled + unlabeled) per gram dry weight sediment

The glucose turnover times and V_{max} values were calculated by plotting t/f versus A (Fig. 5). Extrapolation to the y intercept of the line, where A = O, gives the turnover time for mineralization at the natural substrate concentration. The inverse of the slope of the line is a measure of V_{max}. Computer analysis, using a linear regression program (Hammerly and Knopf, 1972) was used to calculate the glucose turnover time and V_{max} values.

Incubation of Sediment with ^{14}C Cellulose

Uniformly labeled ^{14}C cellulose with a specific activity of 50 μC/mg, was obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). The dried powder was suspended in sterile distilled water to give 0.1 μCi/ml. The labeled cellulose suspension was added in 0.1 ml amounts to each of 10 ml aliquots of lake sediment suspension that had been temperature equilibrated for one hour. Sediment and labeled cellulose were incubated in 50 ml serum bottles, closed with sterile aluminum foil, at specified temperatures. At timed intervals, samples were centrifuged for 20 minutes at 12,000 x G in a refrigerated centrifuge. Two ml aliquots of the supernatant, containing labeled soluble oligomers and glucose released from the cellulose, were counted in Instagel™ in a scintillation counter. Labeled cellulose incubated with 10 ml of sterile water served as a control. Triplicate samples were run for each time period.

Preparation of Reprecipitated Cellulose

Reprecipitated cellulose was prepared by dissolving 5 gm of cellulose powder (Whatman CF 11) in a solution of 100 ml of concentrated sulfuric acid and 60 ml of distilled water at 50° C. After 20 seconds, 6 liters of cold tap water was added to precipitate the cellulose. The mixture was allowed to stand for a few hours or overnight. The liquid was removed by siphon. The cellulose was washed, centrifuged at 4,000 x G for 15 minutes and the pH adjusted to 7.0 with 2 N NaOH. The cellulose was centrifuged and washed with distilled water 5 times. The neutral cellulose slurry was sterilized at 121° C for 15 minutes to prevent microbial growth. Unsterilized cellulose was found to be contaminated with heavy fungal growth after a few days. The dry weight of the cellulose slurry was determined, and the sterilized cellulose slurry was stored at 4° C until use.

Preparation of Reprecipitated Chitin

Reprecipitated chitin was prepared according to a method patterned after Chan (1970). The same chitin preparation was used throughout this study. Practical grade chitin (Eastman Kodak, P-2061) was pulverized with an electric mill and sifted through a 0.2 mm screen. Fifty grams of pulverized chitin was slowly added with stirring to 2000 ml of 18 N sulfuric acid, prechilled to 4° C in a 6000 ml Erlenmeyer flask. The viscous mixture was constantly stirred at 4° C for 10 hours, and was filtered through several layers of spun glass wool into 19 liters of chilled tap water in a 20 liter carboy. The carboy was shaken to facilitate dilution and reprecipitation. After standing for 24 hours at room temperature, the reprecipitated chitin separated as a mass and floated to the top. The acid layer was removed from the bottom by siphon. The chitin was centrifuged at 4,000 x G for 15 minutes, the supernatant decanted, and the

remaining chitin neutralized with 5 N NaOH to pH 7.0. The neutralized chitin was centrifuged at 4,000 x G for 15 minutes and the supernatant decanted. The chitin was placed in 6.4 cm, SSDC type cellophane tubing (Union Carbide, Chicago, Ill.) and dialyzed in cold running tap water for 48 hours. After dialysis, the chitin was centrifuged at 4,000 x G for 15 minutes, supernatant decanted, and chitin washed with distilled water, centrifuged and placed in glass jars. The chitin was steamed at 100° C for 15 minutes to prevent microbial growth. The dry weight of the chitin was determined and chitin was stored at 4° C until use.

Media

Media used in this study included freshwater (FW), freshwater chitin (FWCh), freshwater cellulose (FWC), freshwater glucose (FWG) and minimal media (MM), all at a pH of 7.1. All pH adjustments were made with 1.0 N H_2SO_4 or NaOH. The composition of FW and MM media are given in Tables 2 and 3. The FW medium consisted of the basic medium described by Taylor (1940), modified by adding yeast extract (YE) (0.2 gm/liter). YE was added to Taylor's medium because higher counts and better growth was achieved with YE added, especially at low incubation temperatures. Data in Table 4 demonstrate plate counts of sediment from Lake Washington littoral station collected April 25, 1975 of 5 replicate plates each of FW and Taylor's agar, incubated at 4, 12, and 22° C. Plates incubated at 4 and 12° C had significantly higher counts at the 5% and 0.1% confidence interval (Student T Test) on FW agar than on Taylor's agar, respectively. The plate count on FW agar was higher at 22° C than the corresponding plate count at 22° C on Taylor's agar but the difference was not statistically significant.

The FWCh medium was prepared by mixing 2.0 gm of reprecipitated chitin with 300 ml of distilled water in a Waring Blender at top speed for one minute. The remaining water and ingredients for FW medium were added.

Table 2. Composition of Freshwater Medium[a] (FW)

Peptone	0.5 gm	
Yeast extract	0.2 gm	
Sodium Caseinate	0.5 gm	
Soluble starch	0.5 gm	pH adjusted to 7.1
Glycerol	1.0 gm	
K_2HPO_4	0.2 gm	Sterilized at 121° C for 15 minutes
$MgSO_4$	0.05 gm	
$FeCl_3$	trace	
Distilled water	1,000 ml	

[a]patterned after the media described by Taylor (1940); for solid media, 15 gm agar added/liter.

Table 3. Composition of Minimal Medium[b] (MM)

K_2HPO_4	0.2 gm	pH adjusted to 7.1
$MgSO_4$	0.05 gm	
$FeCl_3$	trace	Sterilized at 121° C for 15 minutes
Distilled water	1,000 ml	

[b]for solid media, 15 gm agar added/liter.

Table 4. Plate Counts of Lake Washington Littoral Zone Sediment on Taylor's and FW (Taylor's + yeast) Media. Data are from 5 replicate plates at a serial dilution of 10^{-2}.

Temperature	Parameter	Taylor's Medium	FW Medium	Significance (Student T Test)
4	mean plate count ± std. dev.	83 ± 9.5	116.0 ± 24.0	5.0%
	variance	90.8	576	
12	mean plate count ± std. dev.	79.8 ± 25.8	145.0 ± 26.7	0.1%
	variance	667.7	717.8	
22	mean plate count ± std. dev.	161.0 ± 35.7	174.0 ± 25.0	NS
	variance	1276	632	

The FWC medium was prepared similarly except that 1.0 gm of reprecipitated cellulose was added in place of chitin. The FWG medium was composed of FW medium and 5.0 gm D-glucose (Merck) added per liter. For solid FWG agar, 0.015 gm of bromocreso purple (Difco) was added per liter as an indicator of acid production.

All agar plates were dried, inverted for 2 hours at 43° C or 24 hours at 37° C and were stored at 4° C until use.

The FWCh agar plates were poured as double layer plates. The first layer of FW agar of approximately 15 ml was poured, allowed to solidify, dried inverted for 2 hours at 43° C, and chilled to 4° C. An overlay of 5 ml of FWCh agar was poured over the prechilled FW agar. Prechilling the plates ensured rapid solidification of the chitin agar, thus preventing settling out of the suspended chitin.

The FWC plates were prepared similarly as overlay plates, but were dried and used immediately.

Dilution blanks were composed of 0.1% peptone in distilled water, dispensed in 9 ml aliquots in screw capped culture tubes and sterilized at 121° C for 15 minutes.

Enumeration of Bacteria

All plate count results are reported for dry weight of the sediment.

Samples of sediment or water were serially diluted using sterile 0.1% peptone blanks and plated in 0.1 ml aliquots onto duplicate agar plates. An L-shaped glass rod, sterilized by dipping in 95% ethanol, followed by flaming, was used to spread the inoculum evenly over the agar plate. Inoculated plates were incubated at temperatures approximating the in situ temperature. Samples collected at temperatures from 0 to 10° C, 11 to 17° C, and 18 to 25° C were incubated at 4, 15, and 22° C, respectively. Samples incubated aerobically at

4, 15, and 22° C were counted after incubation times of 42, 20, and 12 days, respectively. Samples incubated anaerobically at these temperatures were incubated for 52, 30, and 22 days, respectively. No further increases in bacterial numbers occurred after these incubation times at these temperatures.

Replicate plates for anaerobic counts were incubated in anaerobic jars with Gas Paks™ (BBL) at the approximate in situ temperature.

Sediments from core samples were counted from samples extruded from the core into a sterile pan. The extruded core was divided into sections with a sterile scalpel. Sediment from the interior of each section was mixed with a minimum of sterile distilled water, serially diluted and inoculated onto FW agar plates. The dry weight of each section was determined. All plate counts reported are from plates supporting the growth of from 50 to 180 colonies.

Enumeration of Facultative Anaerobes

Isolated colonies from spread plates on FW agar were picked using a flame sterilized loop. All of these plates had been initially incubated under anaerobic conditions. These colonies, 24/test, were immediately streaked onto FW agar plates after removal of the plates from the anaerobic jar, and incubated aerobically at the initial temperature for the appropriate time. Samples incubated anaerobically served as controls. If samples had visible growth after specified times, the organisms were classified as facultative anaerobes and the numbers calculated.

Determination of Temperature Effect on Growth of Heterotrophic Bacteria

Isolated colonies from FW agar spread plates of highest dilution were picked using a flame sterilized loop. All of these plates had been initially incubated under aerobic conditions at 4 or 22° C. These cultures, 24/test, were

streaked onto FW agar and incubated aerobically at a different temperature; for example, isolates from plates initially grown at 4° C were incubated at 22° C, and those initially grown at 22° C were incubated at 4° C. Cultures, streaked and incubated on FW agar at the original temperature served as controls. Positive values were assigned to cultures exhibiting visible growth after 42 and 12 days at 4° C and 22° C, respectively.

Enumeration of Chitinoclastic and Cellulolytic Bacteria

Numbers of bacteria capable of digesting chitin were estimated after incubation of duplicate spread plates of sediment on FWCh agar, incubated at approximate in situ temperatures aerobically and anaerobically. Chitinoclastic activity could easily be detected on this medium by the presence of a clear zone which appeared around the colonies of bacteria capable of hydrolyzing chitin. Cellulolytic bacteria were detected in the same manner after growth on FWC agar.

Purification and Maintenance of Cultures

Isolated colonies of chitinoclastic bacteria were picked from plates of highest dilution and streaked on FWCh agar and incubated at 15 and 22° C until visible growth had occurred. Isolated colonies were restreaked and visually examined for purity. Gram stains and wet mounts of 24-48 hour cultures were examined using both light and phase contrast microscopy. Pure cultures were transferred to slants of FWCh agar, incubated at 22° C until growth had appeared and stored at 4° C. Pure cultures were restreaked on FWCh agar, examined for purity and transferred to fresh FWCh slants every 4 to 6 months. Some of the isolates were grown in FWCh broth, distributed in 2 ml aliquots in 4 dram sterile vials, and frozen at -30° C. Most of these cultures were not

viable after one month; therefore, this method of storage was discontinued.

All pure cultures were coded in a two part designation which included the experiment number and the specific isolate number; for example, 62-1 was isolate number 1 from the experiment numbered 62 (Lake Chester Morse, Cedar River inlet). The specific details of the lake, station, and initial incubation temperature of the original plate were recorded in laboratory notebooks and appear in the appendix (Table 51).

Biochemical Tests

To test glucose utilization, the medium of Hugh and Leifson (1953) was used. The medium was adjusted to pH 7.1, steamed at 100° C for 25 minutes, dispensed in 10 ml aliquots in screw capped culture tubes and sterilized for 10 minutes at 121° C. The tubes, after inoculation, were covered with 3 cm of sterile mineral oil to test for glucose fermentation. Oxidation of glucose was also tested using this medium.

Activity of Chitinoclastic Bacteria on FWCh Agar

Rates of chitin hydrolysis by chitinoclastic isolates were measured using the zone-ratio method of Chan (1970). A 0.01 ml aliquot of a 24-hour culture grown in FWCh broth at 22° C was applied in duplicate to FWCh plates. After absorption of the inoculum, the plates were inverted and sealed with masking tape to prevent drying and microbial contamination, and incubated at specified temperatures. The colony diameter and cleared zone diameter were measured in mm periodically. Activity was expressed as the ratio of the colony diameter to the cleared zone diameter.

Activity of Chitinoclastic Bacteria in FWCh Broth

Chitinoclastic bacteria were observed to grow in FWCh broth at the chitin-liquid interface. As the bacteria hydrolyzed the chitin, the amount of suspended chitin visibly decreased until no chitin remained in the tube. It was possible to use this decrease in chitin, relative to the amount initially present in inoculated screw capped culture tubes as a rate measurement of chitin decomposition analogous to the solid agar zone-colony ratio described by Chan (1970). The amount of chitin measured in mm in the tubes can be correlated with the weight of the chitin. In tubes containing FWCh broth inoculated with bacteria incapable of chitin hydrolysis, the suspended chitin did not settle due to packing by the cells. In uninoculated control tubes, the chitin suspension did not settle appreciably after holding times as long as 90 days.

This method has the further advantage over gravimetric analyses because samples are not disturbed during the measurement, thus a single culture can be studied over a long period of time. Chitin decomposition rates in liquid medium were measured by this ratio method. Samples were inoculated in 16 x 125 mm screw capped culture tubes, and incubated at specified temperatures without shaking. After 24 hours, the distance from the bottom of the tube to the chitin-liquid interface was carefully measured in mm and is designated the initial measurement. On successive days, as the chitin was hydrolyzed, the distance in mm from the bottom of the tube to the chitin-liquid interface was measured. This value was divided into the initial measurement to give the ratio. Measurements were discontinued when the chitin occupied only the curved bottom part of the test tube.

Growth and Activity of Chitinoclastic Bacteria at Various Temperatures

A temperature gradient incubator, described by Matches and Liston (1973) was used to study the effects of temperature on the growth and activity of chitinoclastic isolates. This incubator can be adjusted over the entire temperature range for psychrophilic, mesophilic, and thermophilic bacteria so that organisms can be grown and biochemical responses measured over a wide temperature range at one time.

Some of the test organisms grew more slowly than others, therefore, requiring longer incubation times for the inoculum. A 48, 96, or 144 hour culture from a FWCh agar plate incubated at 22, 15, or 4° C, respectively, was used to inoculate 10 ml of FWCh broth in a screw capped culture tube. The tube was incubated at the same temperature as the plate, without shaking for either 24, 48, or 96 hours at temperatures of 22, 15, or 22° C, respectively. This is designated the primary inoculum. All cultures were inoculated into medium at the temperature of incubation to prevent temperature shock. After the specified incubation time, 5.0 ml of the primary inoculum was mixed with a Vortex mixer and transferred to 45 ml of FWCh broth and grown under the same conditions prevailing for the growth of primary inoculum. The entire 50 ml of inoculum was transferred to 450 ml of FWCh broth and 10 ml aliquots were transferred with a sterile 10 ml pipet into sterile 16 x 125 mm culture tubes. The inoculum was slowly stirred during inoculation to prevent the chitin from settling out. The flask was flamed after every other removal to prevent contamination. Tubes inoculated in this manner were rarely found to be contaminated. For samples grown at 4° C, the inoculating flask and tubes were packed in ice during inoculation of tubes. The inoculated tubes were immediately placed in the temperature gradient incubator and incubated for specified times. At the end of every experiment, all tubes were checked for purity and viability by streaking onto FW, FWCh,

and FWG agar plates. Samples of each culture were also serially diluted and plated out on these agars to test for purity and biomass.

The optimum temperature for chitin decomposition by bacteria in this study, is defined as the temperature at which 10 ml of inoculated FWCh broth in a 16 x 125 mm culture tube first reaches a ratio of 3.0 or over. The temperature range for chitin decomposition is defined as the temperature range in these tubes where the ratio is 1.5 or over after 50-60 days of incubation.

To determine the optimum growth and growth temperature range, chitinoclastic isolates were grown without chitin. The primary inoculum, containing 5.0 ml in FWCh broth, mixed by a Vortex mixer, was transferred to 45 ml of FWG broth and treated as previously described, except FWG broth was used instead of FWCh broth. The cultures were grown while shaking at 22^{o} C, prior to inoculation in the culture tubes. Bacterial growth was determined by turbidity measurements of optical density at 660 nm in a Bausch and Lomb Spectronic 20. After 24 hours at 22^{o} C, cells reached a level of 10^{8} organisms/ml and were inoculated at a 10:1 dilution into tubes and incubated on the temperature gradient incubator. The optimum growth temperature is defined as that temperature where maximum growth first occurs within 10 to 31 hours of inoculation. The growth temperature range is defined as the lower and upper limits for growth detectable by turbidity measurements at 660 nm after 60 days incubation. After incubation for 60 days, tubes were checked for purity by plating out on FWCh and FWG agars.

Effect of End Products on Chitin Degradation

To determine the effects of end products on chitin degradation by chitinoclastic bacteria in liquid medium, a primary inoculum was prepared by picking an isolated colony from a FWCh plate and inoculated into 10 ml of FWCh

broth. The cells were grown unshaken at 22° C for 48 hours, mixed by a Vortex mixer and transferred in 1 ml aliquots into tubes containing 9 ml of FWCh broth plus varying amounts of N-acetyl glucosamine (NAG) or glucose. Tubes were incubated unshaken at 22° C. The rate of chitin degradation was measured by the ratio method in these tubes. Controls consisted of isolates inoculated and incubated under the same conditions in FWCh broth.

Chitin degradation was examined on solid medium using plates prepared with a 0.4% gradient of NAG. Plates were prepared by pouring 10 ml of FW agar containing 0.4% NAG. The plates were allowed to solidify in a slanted position so that one side contained no medium. A second layer of FWCh agar (10 ml) was poured over the prechilled FWCh-NAG gradient plates and was allowed to solidify in a horizontal position. The plates were dried and inoculated with 0.01 ml of a 24-hour culture grown in FWCh broth at 22° C. The inoculations were on four positions on the gradient plates, so that NAG levels by diffusion upwards from the first layer to the second were at 0, 0.15, 0.3, and 0.4%. The inoculated plates were incubated inverted at 22° C, and chitin hydrolysis measured by the zone ratio method.

Enrichment for Chitinoclastic Bacteria

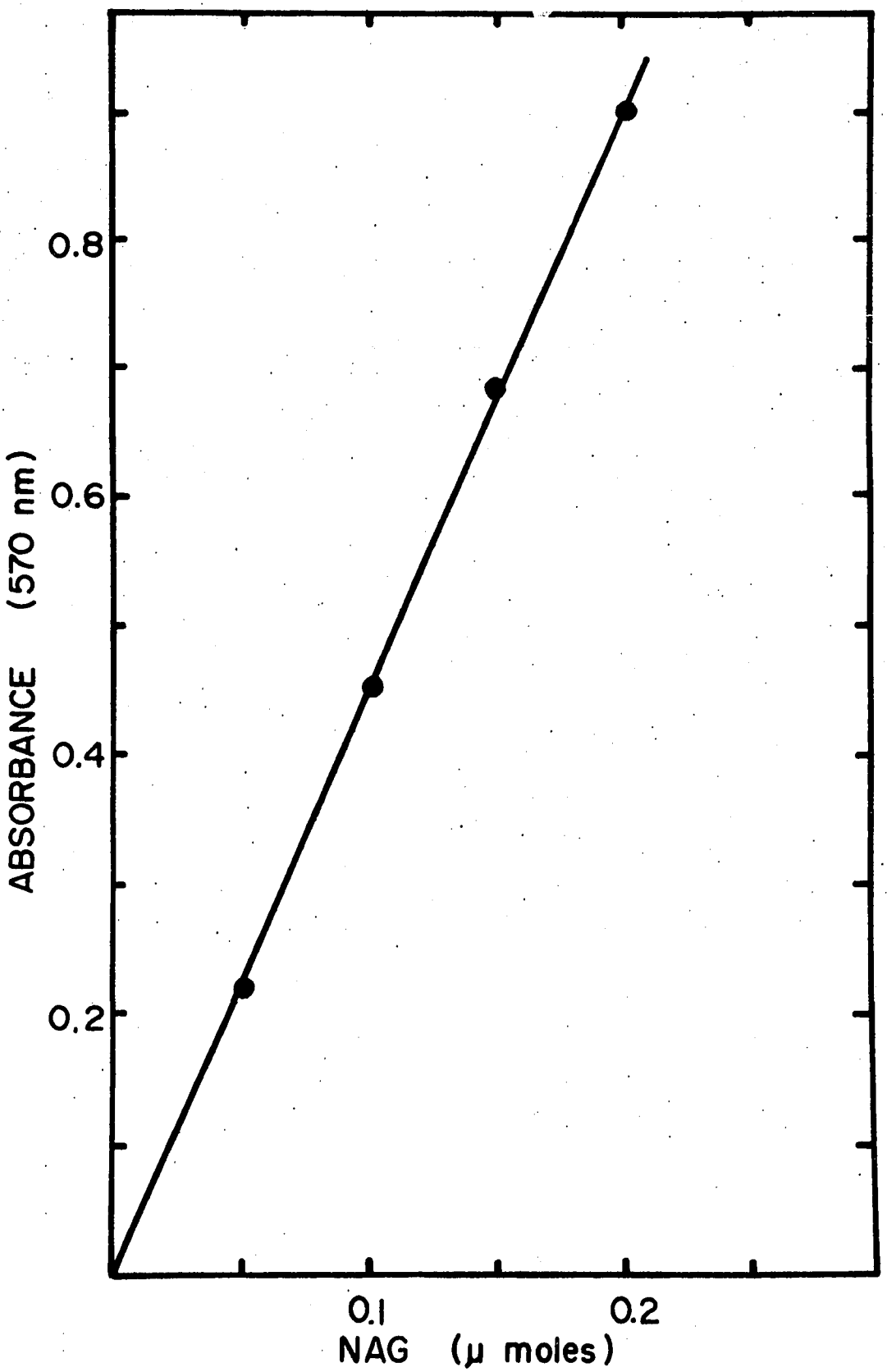
Sediment samples for enrichment experiments were distributed in 9 ml aliquots by a sterile plastic 10 ml pipet with the tip removed, into sterile 25 x 125 screw capped culture tubes. Substrates were added to the sediment in 1 ml volumes, and included peptone, glucose, NAG, glucosamine to final concentrations of 0.2%. Sterile FWCh broth, $CaCO_3$, cellulose, and chitin were added to final concentrations of 10, 0.4, 0.01, and 0.01%, respectively. Glucose, glucosamine, and NAG were filter sterilized prior to addition. The remaining substrates were sterilized before addition by heating at 121° C for

15 minutes. A 1 ml volume of sterile distilled water added to 9 ml of sediment served as a control. Periodically, these samples were mixed by a Vortex mixer, serially diluted and plated out on FWG, FW, and FWCh agars. Enrichment samples and plates were all incubated at the in situ temperature.

Determination of N-Acetyl Glucosamine (NAG)

The method of Reissig et al. (1955) was used to determine NAG. Culture tubes (10 x 100 mm) containing 0.5 ml sample were mixed with 0.1 ml of potassium tetraborate (0.8 M H_3BO_3 titrated to pH 9.1 with 0.8 M KOH). Distilled water, 0.5 ml, served as a blank. The tubes were loosely capped and heated in a boiling water bath for exactly 3 minutes and cooled in tap water. After cooling, 3 ml of p-dimethylaminobenzaldehyde (DMAB) reagent was added, the tubes were mixed with a Vortex mixer, and placed in a water bath at 38° C. After precisely 20 minutes, the tubes were cooled in tap water and read immediately in a Bausch and Lomb Spectronic 20 at 570 nm. Tubes had to be read immediately because the color faded at a rate of 1.5% every 5 minutes after cooling to room temperature. Standards from 0.05, 0.1, 0.15, and 0.2 μmoles of NAG (Calbiochem, San Diego, Calif.) were run with each assay. A typical standard curve is shown in Figure 6. Samples containing more than 0.2 μmole NAG, were diluted with sterile distilled water. The DMAB reagent was prepared by dissolving 10 gm of p-dimethylaminobenzaldehyde (Eastman Kodak, Rochester, N.Y.) in 100 ml of analytical reagent grade glacial acetic acid which contained 12.5% by volume 10 N HCL (analytical reagent grade). This DMAB reagent was stable for one month when stored at 4° C. Shortly before use, DMAB reagent was diluted with 9 volumes of reagent grade glacial acetic acid.

Figure 6. Standard Curve for the Determination of N-Acetyl Glucosamine (NAG) by the Method of Reissig et al. (1955)


0.8
0.6
0.4
0.2
ABSORBANCE (570 nm)
0.1
0.2
NAG (μ moles)

Determination of Glucose

Glucose was determined by a colorimetric method utilizing a Calbiochem STAT-Pack™ (Calbiochem, San Diego, Calif.). To prepare the activated reagent solution, 15.5 ml of distilled water was added to a vial containing NADP. The contents were gently swirled to dissolve the NADP; when dissolved, the contents were added to a vial containing excess ATP, hexokinase, and glucose-6-phosphate dehydrogenase. When all of the material was dissolved, the vial was placed in an ice bath until use. For each analysis, 3 ml of reagent was dispensed into a clean dry cuvette with a 1 cm light path. The cuvette was placed in a spectrophotometer set at 340 nm and the absorbance adjusted to zero, using 3 ml of distilled water and 20 µl of culture as a blank in the cuvette. The bacterial culture (20 µl) was added to the cuvette using a 100 µl Hamilton syringe and contents were gently mixed by inversion. The absorbance changes occurring as the glucose was utilized thereby generating NADPH were recorded on a Gilford-2000 multiple sample absorbance recorder attached to a Beckman DU spectrophotometer. After 5 minutes, when absorbance changes had stopped, the stabilized absorbance was read and recorded. Absorbance readings were converted to mg % glucose using a standard curve prepared from glucose standards ranging from 10 to 350 mg %.

Protein Determination

Proteins were determined using the method of Lowry et al. (1951) with bovine serum albumin (Sigma, St. Louis, Mo.) as a standard.

Preparation of Cell-Free Extract

A primary inoculum of a chitinoclastic bacterium isolate was prepared by taking a loop of a 48-96 hour culture from a FWCh agar plate incubated at 4, 10, or 22° C, and inoculated into 10 ml of FWCh broth in a screw capped

culture tube. The organisms were grown without shaking. After 24, 48, or 96 hours at temperatures of 22, 10, or 4° C, respectively, the entire contents of the tube were mixed with a Vortex mixer and transferred to 40 ml of FWCh broth in a 125 ml Erlenmeyer flask (stoppered with sterile cotton in cheesecloth) and incubated with stirring under the same temperature and time period used for the primary inoculum. To prevent heating of the inoculated flask by the magnetic mixer, a 3 cm thick section of styrofoam was placed between the flask and the mixer. After the specified time period, the entire 50 ml were transferred to 300 ml of FWCh broth in a 1 liter Erlenmeyer flask to give a total count of 10^6 cells/ml, and grown under the same conditions as before. After 5, 7, or 9 days for cells grown at 22, 10, or 4° C, respectively, when a significant proportion of the chitin had visibly decreased, the cells were checked for purity by plating out on FWCh agar. The cell suspension was sonicated for 2 minutes using a Biosonic sonicator (Bronwill Scientific, Rochester, N.Y.) in a 250 ml beaker packed in ice to prevent heating. The sonicated cells were centrifuged at 27,000 x G for 20 minutes in a refrigerated centrifuge (Sorvall, RC IIB). After centrifugation, the cells were discarded, and the supernatant was filter sterilized using a 0.22 μ Millipore filter. Cell-free extracts prepared in this manner were stored at 4° C.

Chitinase Assay of Cell-Free Extracts

Cell-free extracts were assayed for chitinase activity by incubating these extracts with reprecipitated chitin. The NAG released from chitin by the extract was measured by the colorimetric method of Rossig et al. (1955). This method can be applied to chitinase estimation only if the hydrolysis proceeds in the presence of chitobiase. Chitin hydrolysis was also followed by measuring the decrease in turbidity at 420 nm of the assay mixture as the chitin

was hydrolyzed. All chitinase assays were conducted under aseptic conditions. Nonsterile samples incubated longer than 24 hours were frequently found to be contaminated and gave spurious results.

Cell-free extracts were assayed with varying amounts of sodium phosphate-citric acid buffer, pH 7.0 at 28° C. With additions of 0.01 M buffer, enzyme activity was 81% greater than with sterile distilled water, and remained at this level up to 1.0 M buffer concentration. All enzyme assays in this study were therefore conducted with the addition of 0.5 M sodium phosphate-citrate buffer, pH 7.0, with final concentration of 0.07 M buffer in the assay mixture. Some assays were also run using 0.5 M sodium phosphate-acetate buffer, pH 7.0. There was no difference in activity between sodium phosphate-acetate buffer and sodium phosphate-citrate buffer.

All chitinase assays were run with a standard assay mixture containing 5 ml cell-free extract, 1 ml reprecipitated chitin (1 mg/ml), and 2 ml of 0.5 M solution of $NaHPO_4$ buffered to pH 7.0 with 0.5 M citric acid. Assays were conducted in screw capped culture tubes. A tube containing the assay mixture with sterile distilled water in place of cell-free extract served as a control. No change in turbidity measurement or production of NAG was ever observed in control samples. Initial OD measurements of turbidity were taken. After a specified time period (including a 0 time) of incubation at 28° C, turbidity measurements were taken, and the tubes were boiled for 10 minutes and centrifuged. The supernatant was used for NAG determinations. No NAG was ever detected in the 0 time samples. Since the colorimetric method of Reissig et al. (1955) for NAG determination requires a basic pH for the formation of an intermediate and a final acidic pH for full color development, a volume of not more than 0.25 ml of the centrifuged and boiled enzyme assay mixture was used per NAG assay. No interference with color development occurred with chitinase assay samples

incubated at pH ranges from 4.0 to 9.0 with this volume. However, considerable interference, presumably from the buffer in the assay mixture, occurred when sample volumes greater than 0.25 ml were used.

The enzyme reaction rate as assayed by NAG release, was linear for the isolates when tested for 2-3 hours of incubation at 28° C, pH 7.0 and continued for several hours (Fig. 7). After the first 2-3 hours, the reaction had a slower rate, but could still be detected after 4 days. The reaction on the basis of turbidity measurement was linear for the first hour of incubation, and then decreased (Fig. 8). To calculate the units of enzyme per ml of cell-free extract, assays were conducted for 1 hour at 28° C, pH 7.0 in sodium phosphate-citrate buffer, 0.5 M. A unit is defined as the amount of enzyme catalyzing the liberation of 1 μmole of NAG per hour, or the decrease in turbidity by 1.0 OD unit per hour. Specific activity is defined as units/mg protein.

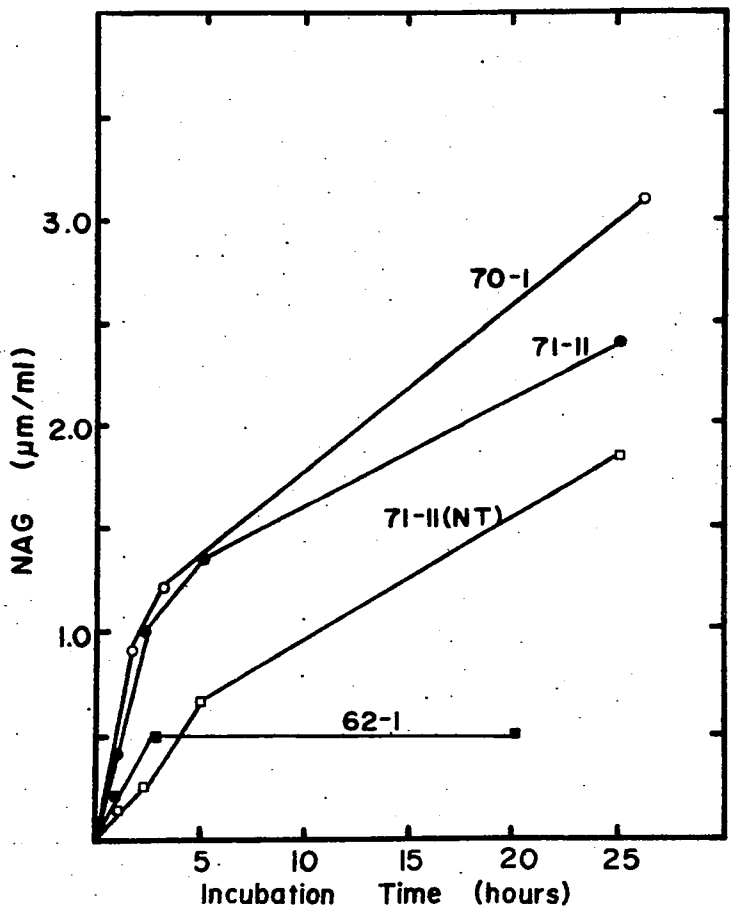
There was no difference in activity of cell-free extracts prepared by sonication and centrifugation and sonication without centrifugation, when incubated with chitin and assayed for NAG release. Thus, it appeared that the chitinase-chitobiase enzymes were present almost entirely in the supernatant after sonication and were not attached to the bacterial cells or to the chitin.

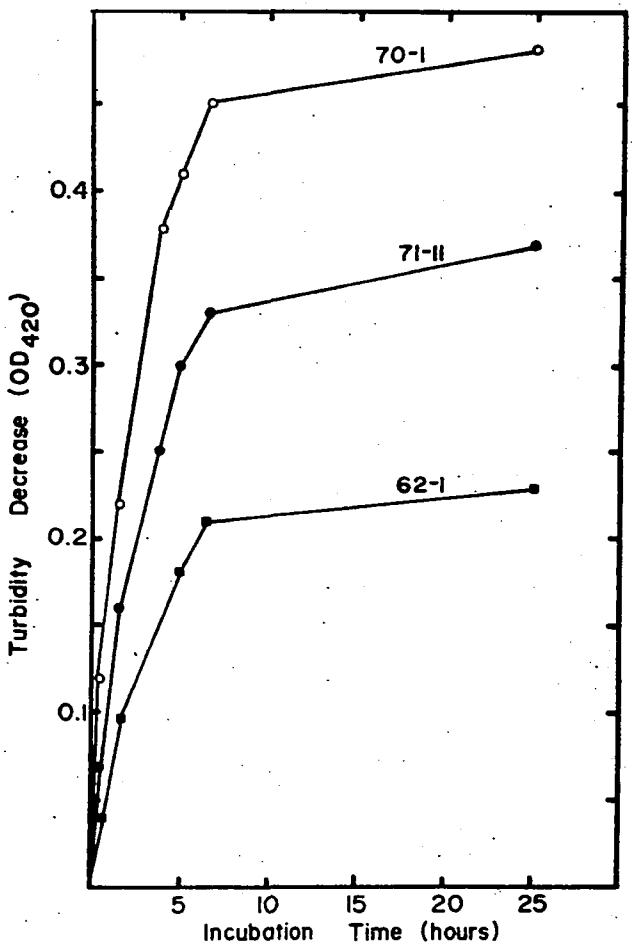
Incubation of Cell-Free Extracts with End Products of Chitin Hydrolysis and Potential Inhibitors of Enzyme Activity

To the standard assay mixture was added 0.2 ml of a sterile solution of either EDTA, glucose, glucosamine, NAG, $CaCO_3$, or $CuSO_4$. Controls consisted of the standard assay mixture plus 0.2 ml of sterile distilled water. The assay mixture was incubated for 1 hour at 28° C, and enzyme activity calculated.

Figure 7. Chitin Hydrolysis as a Function of Time as Determined by Assay for N-Acetyl Glucosamine (NAG). All samples sonicated except 71-11 not treated (NT), and all grown in FWCh at 22° C. Assays conducted under standard conditions.

Figure 8. Chitin Hydrolysis as a Function of Time as Determined by Turbidity Decrease at 420 nm; cell-free extracts prepared from 3 isolates grown at 22° C in FWCh broth. Assays conducted under standard conditions.


NAG (μm/ml)
3.0
2.0
1.0
70-1
71-II
71-II(NT)
62-1
5
10
15
20
25
Incubation Time (hours)


Turbidity Decrease (OD420)
0.4
0.3
0.2
0.1
70-1
71-II
62-1
5
10
15
20
25
Incubation Time (hours)

Chitinase Activity of Cell-Free Extracts at Various Temperatures

To study the effect of temperature on chitinase activity, cell-free extracts were incubated under aseptic conditions with chitin in the temperature gradient incubator over a range of -0.8° C to 33.0° C. Samples incubated at 37, 45, and 50° C were held in thermostatically controlled water baths. The assay mixture consisted of 100 ml cell-free extract, 20 ml reprecipitated chitin (1 mg/ml) and 40 ml 0.5 M $NaHPO_4$ buffer adjusted to pH 7.0 with 0.5 M citric acid, dispensed in 4 ml aliquots into 16 x 125 mm screw capped culture tubes. Initial turbidity measurements were taken for each tube at 420 nm. At specified times, turbidity measurements were taken and subtracted from the initial reading to give the change in optical density (ΔOD_{420}). NAG levels were also measured. A sample of the assay mixture containing sterile water in place of cell-free extract served as a control.

pH Optimum Determination

The optimum pH for chitinase and chitobiase activity of cell-free extracts was determined by adjusting the standard assay mixture (5 ml cell-free extract, 1 mg reprecipitated chitin, 2 ml $NaHPO_4$-citrate, 0.5 M, at specified pH) to the desired pH by either 1.0 M citric acid or 1.0 M NaOH in a volume less than 0.2 ml. The pH was measured with a Corning pH meter (Model 7) equipped with a single electrode. After a specified time of incubation at 28° C, the pH was again measured. The final pH is the one reported.

Ammonium Sulfate Fractionation

Solid ammonium sulfate was added to 100 ml of filter sterilized cell-free extract to give an initial 35% of saturation. The mixture, held in an ice bath, was stirred for 1 hour and then centrifuged for 20 minutes at 12,000 x G in a refrigerated centrifuge. The resulting precipitate was dissolved in 10 ml

of chilled sterile 0.01 M $NaHPO_4$-acetate buffer, pH 7.0). Solid ammonium sulfate was added to the supernatant and the procedure repeated. The dissolved precipitates (35, 64, and 78% of saturation fractions) were tested for enzyme activity. The percent of saturation was calculated using a nomogram (Dixon and Webb, 1964, p. 40).

RESULTS

The first part of this section deals with the mineralization of a labile organic carbon compound, glucose, by heterotrophic bacteria associated with lake sediments in four lakes. Glucose mineralization rates of undiluted sediment, using uniformly labeled ^{14}C glucose were determined to approximate the activities of heterotrophic bacteria in situ in Lakes Washington, Sammamish, Chester Morse, and Findley. A number of aspects of the glucose mineralization rate method of Harrison et al. (1971) used in this study were initially investigated including reproducibility of the method; effect of sampling device, light, storage, shaking during analysis; and the effects of added substrates on the glucose mineralization rate of lake sediment bacteria. All of the glucose mineralization rate and plate count data are presented for each lake separately, followed by studies of: temperature effects on glucose mineralization rate, bacterial plate counts, and cellulolytic bacteria.

The second part of this section deals with the degradation of a more refractory organic carbon compound, chitin, by chitinoclastic bacteria from lake sediment, in pure culture, and by cell-free extracts. Included in Part II are: the seasonal abundance of chitinoclastic bacteria in the 4 lakes; substrate and temperature enrichment for chitinoclastic bacteria from lake sediment; isolates of chitinoclastic bacteria; effects of bacterial growth temperature, incubation temperature, and end products on chitin hydrolysis rates of viable whole cells and cell-free extracts; optimal pH and temperature for chitin hydrolysis by cell-free extracts; purification and stability of cell-free extracts.

PART I

GLUCOSE MINERALIZATION BY HETEROTROPHIC BACTERIA ASSOCIATED WITH SEDIMENTS IN FOUR LAKES

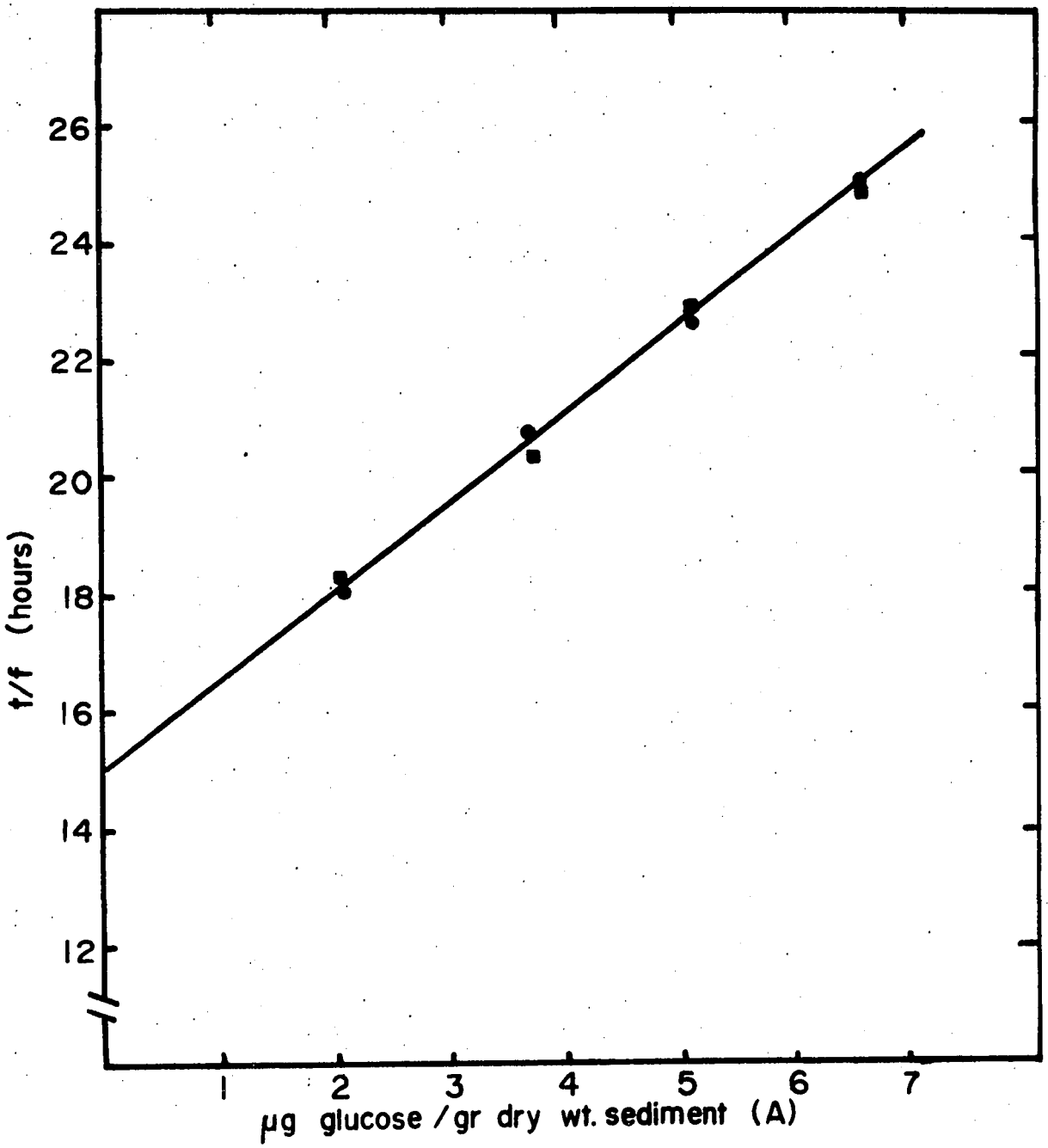
Aspects of the Glucose Mineralization Rate Determination

A number of parameters of the glucose mineralization rate method of Harrison et al. (1971) were initially investigated to determine the sensitivity of the method and to attempt to duplicate in situ conditions as much as possible. These parameters included the effects of: sampling device, light, sample storage, shaking, and effect of added substrates on the glucose mineralization rate of bacteria associated with the sediment. The reproducibility of the method was also determined.

Reproducibility. Glucose mineralization rates were measured in duplicate analyses conducted 2 hours apart at 7.0° C with a sediment sample collected at the Lake Washington littoral station. Both analyses yielded the same turnover time of 15 hours and V_{max} values of 0.66 µgm glu/gm/hr (Fig. 9), showing that the method yields reproducible results when duplicate samples are analyzed under the same conditions.

Effect of sampling device. The effect of the sampling device on data outcome was investigated for sediment collected at both shore and benthic stations at Lake Chester Morse. Sediment samples were collected with the suction device and the geological gravity corer. The suction device was estimated to collect approximately the top 1-2 cm of sediment and interstitial water. With the coring device, the top 3 cm of the core and interstitial water were transferred by pouring into a sterile glass jar. Glucose mineralization rates were determined on sediments collected by the two sampling methods, and those collected with

Figure 9. Results of Duplicate Analyses of Glucose Mineralization Rate for Sediment Collected at the Lake Washington Littoral Station and Incubated at 7.0° C. Points on the line represented by circles represent mean of 3 replicates. Points on the line represented by squares represent mean of 3 replicates for a separate analysis.


26
24
22
20
18
16
14
12
t/f (hours)
1
2
3
4
5
6
7
µg glucose / gr dry wt. sediment (A)

the suction device showed the greatest bacterial activity. The glucose turnover times were shorter and the V_{max} values were highest in both littoral and benthic sediments collected by this device (Table 5).

Effect of light. The effect of lighting conditions during analysis on data outcome was investigated for samples collected from both the littoral and benthic zones in Lake Washington. No differences were found between the results of benthic samples incubated in a well-lighted and a darkened room (Table 6); however, with littoral samples, the V_{max} in µgm glucose/gm/hr decreased from 1.47 in the well-lighted room to 0.49 in the darkened room.

Effect of sample storage. Glucose mineralization rates changed markedly in samples stored from 1 to 7 days (Table 7); however, the changes were not consistent. In some samples, rates increased with storage time; for example, with Lake Washington littoral zone sediment, the V_{max} of 2.58 measured initially after collection, increased to 7.2 after storage at 4^{o} C for 24 hours. In other instances, the rates decreased; for example, with Lake Washington littoral sediment, the rates decreased from an initial V_{max} of 1.46 to 0.43 after storage at 4^{o} C for 24 hours. The glucose mineralization rate as measured by V_{max} was most affected by storage, while the glucose turnover time changed very little for Lake Washington littoral zone sediment stored at 4^{o} C up to 24 hours. Variable results were obtained for sediment samples collected at Lakes Chester Morse and Findley for samples stored for longer periods up to 8 days. Although the changes in rates were not consistently increased or decreased, these data show the necessity of processing samples as soon as possible after collection.

Table 5. Effect of Sampling Device on Glucose Mineralization Rate Determination. Samples taken from Lake Chester Morse, 5/22/73.

Station	In Situ T°C	Sampling Device	V_{max}* µgm/gm/hr	Turnover* Time (hr)
littoral	12.0	corer	0.47 ± 0.1	13.5 ± 0.7
	12.0	suction device	0.66 ± 0.1	5.6 ± 0.7
benthic	5.5	corer	0.12 ± 0.1	18.8 ± 5.9
	5.5	suction device	0.22 ± 0.05	8.5 ± 1.4

*Values in this and following tables represent mean ± standard deviation of the curve generated by plotting t/f versus A.

Table 6. Effect of Lighting Conditions on Glucose Mineralization Rates of Lake Washington Sediment Bacteria. Samples analyzed in darkened room and in well lit room, incubated for 15 minutes at the in situ temperature.

In situ T°C	Station	Illumination	V_{max} µmole/glu/ hr/gm	Glucose Turnover Time (hr)
9.8	littoral	light	1.47 ± 0.17	4.7 ± 0.26
		dark	0.49 ± 0.16	4.8 ± 0.71
7.5	#1 60 m	light	0.39 ± 0.09	12.1 ± 1.6
		dark	0.21 ± 0.07	8.8 ± 2.0

Table 7. Effect of Storage on Sediment Samples on Glucose Mineralization Rate. Samples were stored at indicated time periods at 4^{o} C and analyzed for glucose mineralization rate.

Lake	Station	T of Analysis	Storage Time	V_{max} µgm/glu/ gm/hr	Glucose Turnover Time
Washington	littoral	10.2	0	2.58 ±	3.2
		10.2	24 hrs	7.2 ± 0.2	2.7 ± 0.1
Washington	littoral	9.8	0	1.46 ± 0.17	4.7 ± 0.26
		9.8	24 hrs	0.43 ± 0.07	3.9 ± 0.46
Washington	60m, #1	8.7	0	0.82 ± 0.06	6.4 ± 0.37
		8.7	24 hrs	0.63 ± 0.05	8.4 ± 0.41
Findley	27m, #1	5.0	0	0.66 ± 0.3	35.8 ± 19.0
		5.0	6 days	0.16 ± 0.3	45.8 ± 12.5
Findley	23m, #1	4.0	0	0.09	32.0
		4.0	7 days	0.09 ± 0.07	13.3 ± 2.7
Chester Morse	littoral	5.0	0	0.18 ± 0.18	3.6 ± 1.2
		8.0	8 days	1.05	7.6

Effect of sample shaking during analysis. Glucose mineralization rates were increased when samples were shaken during analysis resulting in higher V_{max} values and shorter turnover times (Table 8). In every analysis of shaken samples, the counts per minute (CPM) or amount of $^{14}CO_2$ evolved was up to two times greater than corresponding samples incubated without shaking. All analyses in this study were conducted on unshaken samples to duplicate in situ conditions as much as possible.

Effect of added substrates on glucose mineralization rate. A variety of substrates were added to Lake Washington sediment samples to determine the sensitivity of the method of measuring glucose mineralization, especially to determine if perturbations in the environment, such as oil spills, or sudden increases in nutrients could be detected by the glucose mineralization rate method. Antibiotics were investigated in a preliminary study to determine if the influence of organisms not sensitive to antibiotics (fungi, zooplankton, etc.) could be contributing towards the mineralization of glucose in addition to the bacteria. The substrates tested included the following in final concentrations: No. 2 diesel oil, 1.0%; streptomycin, 6 mg %; PO_4, 0.1 M; and freeze-dried plankton, 2 mg %. Substrates were added to the sediment samples and the same volume of sterile distilled water was added to controls. These samples were incubated for given time periods and glucose mineralization rates were measured (Table 9).

The No. 2 diesel oil caused a decrease in the V_{max} from 7.20 in the control to 1.23 in the littoral zone sediment sample incubated for 17 hours in the oil; the turnover time and aerobic plate count were not significantly affected.

Streptomycin added to inhibit microbial growth, had little effect on the V_{max} value or the plate count, for sediment from the littoral zone, although the turnover time appeared to increase from 17.7 in the control to 25.4 hours in the

Table 8. Effect of Shaking of Sample During Analysis on Glucose Mineralization Rate. Shaken samples were shaken 1 hour prior to analysis conducted while sample shaken in water bath.

Lake	Station	T of Analysis	Conditions of Analysis	V_{max} µgm/glu/ gm/hr	Glucose Turnover Time
Sammamish	26m, #1	21.0	shaken	5.7 ± 0.35	4.1 ± 1.4
		21.0	not shaken	1.32 ± 0.34	10.6 ± 0.45
Washington	littoral	21.9	shaken	6.65	2.34
		21.9	not shaken	3.07	4.4
Chester Morse	33m, #1	25.2	shaken	0.43	100.0
		25.2	not shaken	0.21	110.0
Chester Morse	littoral	19.5	shaken	22.4 ± 0.37	1.6 ± 0.05
		19.5	not shaken	12.6 ± 0.29	3.4 ± 0.07

Table 9. Effects of Added Substrates on the Glucose Mineralization Rate Measured for Sediment Collected in Lake Washington

Station	Substrate, Final Concentration	Pre-Incubation Time (hrs)	V_{max} (µgm glu/gm hr)	Turnover Time (hrs)	Aerobic Plate Count x 10^6
littoral	#2 diesel oil, 1.0%	17	1.24 ± 0.05	2.30 ± 0.16	130
	control		7.20 ± 0.20	2.70 ± 0.10	150
littoral	streptomycin, 6.3 mg %	2.5	0.65 ± 0.26	25.40 ± 5.90	25
	control		0.63 ± 0.12	17.7 ± 2.80	97
littoral	potassium phosphate, 0.1 M	19.5	0.05 ± 0.15	39.20 ± 8.20	160
	control		0.04 ± 0.07	3.90 ± 0.46	200
60 m	freeze-dried plankton, 2 mg %	2.0	0.98 ± 0.11	16.40 ± 0.52	28
	control		0.82 ± 0.06	6.40 ± 0.37	32
	freeze-dried plankton, 2 mg %	24.0	1.90 ± 0.38	20.40 ± 0.97	33
	control		0.63 ± 0.05	8.40 ± 0.41	29

sample incubated with streptomycin for 2.5 hours at 4° C. A longer turnover time indicates a slower rate.

Littoral zone sediment samples were incubated with 0.1 M phosphate buffer at pH 6.5, the same pH as that measured for the sediment sample. Phosphate had a dramatic inhibitory effect on the glucose mineralization rate after incubation at 4° C for 19.5 hours. Control samples had a V_{max} and turnover time of 0.43 and 3.9, respectively, compared to corresponding values of 0.047 and 39.2 for the phosphate treated samples.

When sediment samples from the benthic station were incubated with freeze-dried plankton for 2 hours at 4° C, the glucose turnover time was increased from 6.4 hours for the control to 16.4 hours in the treated sample. The V_{max} was not affected. After 24 hours, the glucose turnover time of the control remained lower in the treated sample compared to the control; however, the V_{max} of the treated sample (1.0) was significantly higher than that of the control (0.63). The plankton was collected with a 40 mesh net from the area of the benthic station and immediately lyophilized, 1 week prior to taking the sediment sample from this station.

Glucose Mineralization Rate Measurements in the Four Lakes

Glucose mineralization rates were determined for sediments collected from the four lakes between July 1972 and April 1974 (Table 10), at the two main sampling stations. All data are given in Tables 52-55 in the Appendix.

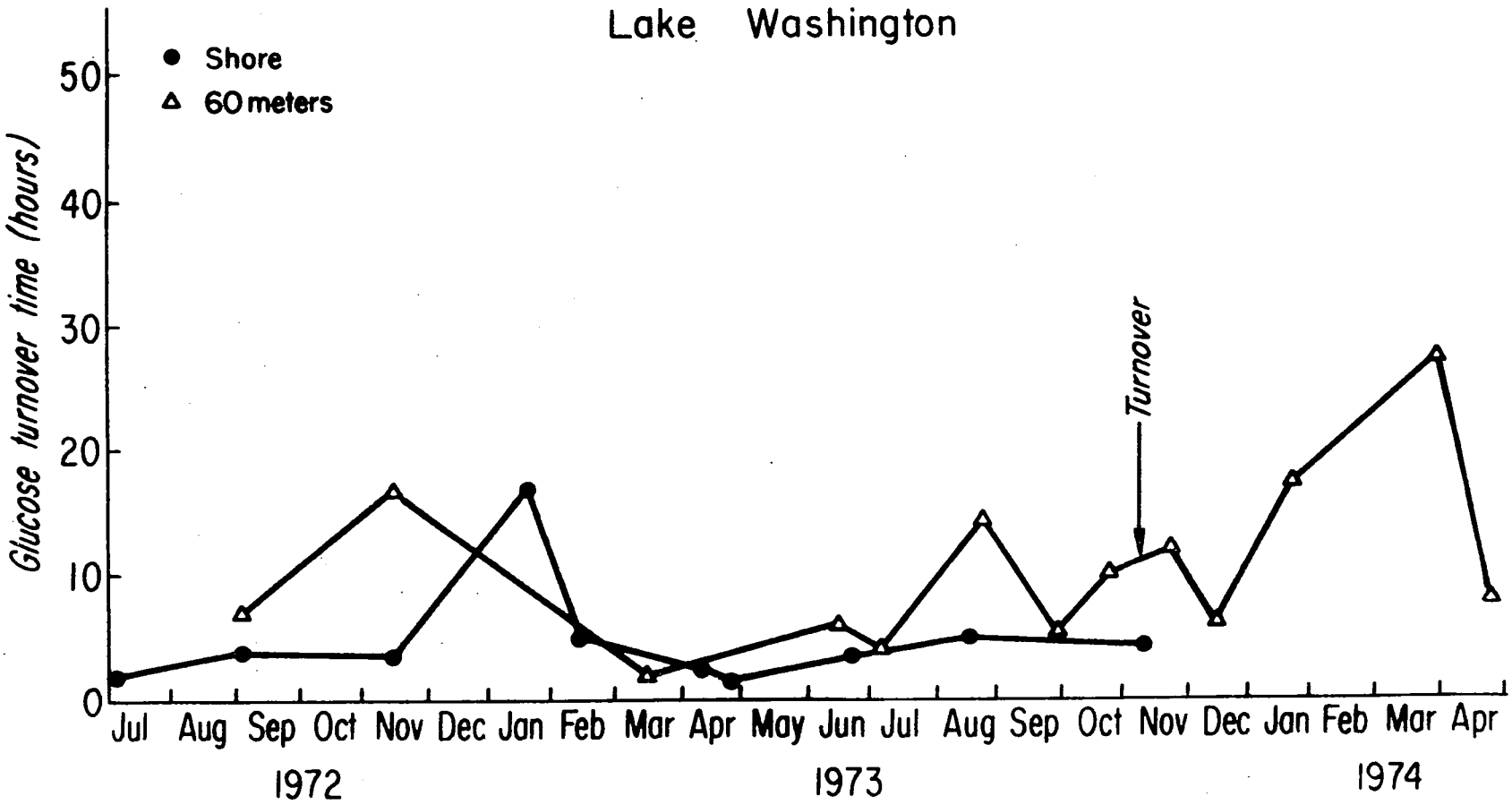
Lake Washington. Sediments obtained from Lake Washington were analyzed for glucose mineralization activity from September 1972 to October 1974, and included a total of 12 littoral zone samples, 13 samples from the benthic station at 60 m, and 8 samples from stations other than the main sampling areas.

Table 10. Sediment Samples Taken in Four Lakes of Lake Washington Drainage Basin and Analyzed for Glucose Mineralization Rate

Lake	Station	Depth in Meters	Number of Samples	Sampling Period
Washington	1	60.0	9	9/ 8/72 - 4/25/74
	2	0.5	13	9/ 8/72 - 11/ 8/73
Sammamish	1	25.0	14	9/20/72 - 4/ 3/74
	2	2.0	11	9/20/72 - 12/ 5/73
Chester Morse	1	33.0	16	8/22/72 - 1/30/74
	2	4.0	12	8/22/74 - 2/12/74
Findley	1	27.0	16	7/ 7/72 - 2/28/74
	2	0.5	14	7/ 7/72 - 2/28/74

Samples collected from the littoral area usually exhibited faster glucose mineralization rates than did those from the benthic station. Glucose turnover times (TT) in both stations were shortest in the late spring to summer months when the bacteria were most active and reached much slower rates in the winter months (Fig. 10). Glucose turnover times are inversely related to the rate; for example, when glucose turnover times are low, it is indicative of a faster rate for it takes less time for the bacteria to mineralize the glucose to CO_2. In the littoral zone, shortest TT of 2.4 hours, and 1.8 hours occurred in July 1972 and April 1973, respectively. The highest TT of 17.7 hours for this station occurred in January 1973. In the benthic station, shortest TT of 7.5, 2.3, and 8.0 hours occurred in September 1973, March 1973, and late April 1974, respectively. The V_{max} values in μgm glu/gm sediment/hr, for the benthic

Figure 10. Glucose Turnover Times for Lake Washington Sediments from July 1972 to April 1974. Data from Table 52 in the Appendix.


Lake Washington
Shore
60 meters
Glucose turnover time (hours)
0
10
20
30
40
50
Turnover
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
1972
1973
1974

area varied with the season (Fig. 11) and reached highest levels in the summer (0.75 and 0.53 in September 1974 and July 1973, respectively). Lowest levels were obtained in the winter-early spring (0.15, 0.15, and 0.13 in March 1973, January 1974, and April 1974, respectively). For littoral samples, highest V_{max} values of 1.03 and 9.3 occurred in September 1972 and April 1973, respectively, and lowest V_{max} value of 1.03 was found for the February sample of 1973. A high V_{max} of 8.3 was observed in November 1972.

The highest numbers of bacteria growing aerobically on Lake Washington benthic sediments were located in the top 2 cm of sediment. Total aerobic plate counts on FW agar decreased from 10^7 organisms/gm at the sediment-water inferface, 10^6/gm at 3 cm, 10^4/gm at 11 cm, to 10^3/gm at 18 cm below the sediment surface (Fig. 12). The numbers of bacteria in sediments collected from the littoral area were also higher than numbers in sediments collected from the benthic zone. Aerobic plate counts on FW agar ranged from 3.8 to 970 x 10^6 organisms/gm for littoral and 2.1 to 75 x 10^6 organisms/gm for littoral and 2.1 to 75 x 10^6 organisms/gm for benthic samples (Table 11, Fig. 13). The numbers fluctuated only slightly with season with higher counts for benthic samples in the summer than in winter months. Plate counts also increased in benthic samples after the 1973 fall turnover in November. Pigmented bacteria in littoral samples ranged from 8 to 38% and 24 to 70% in benthic samples (Table 11).

Other stations were sampled (Table 12). Lowest glucose mineralization rates were found in May and September of 1973 at the Cedar River inlet (No. 6) near the city of Renton, Washington, when compared to other samples collected and analyzed on the same day. In March 1973, an area (Station No. 4) on the eastern side of the lake near the city of Kirkland had a very low rate when compared to littoral and benthic (Nos. 1, 3, 5) samples collected and analyzed

Figure 11. V_{max} Values for Lake Washington Sediment Samples from July 1972 to April 1974. Data from Table 52 in the Appendix.

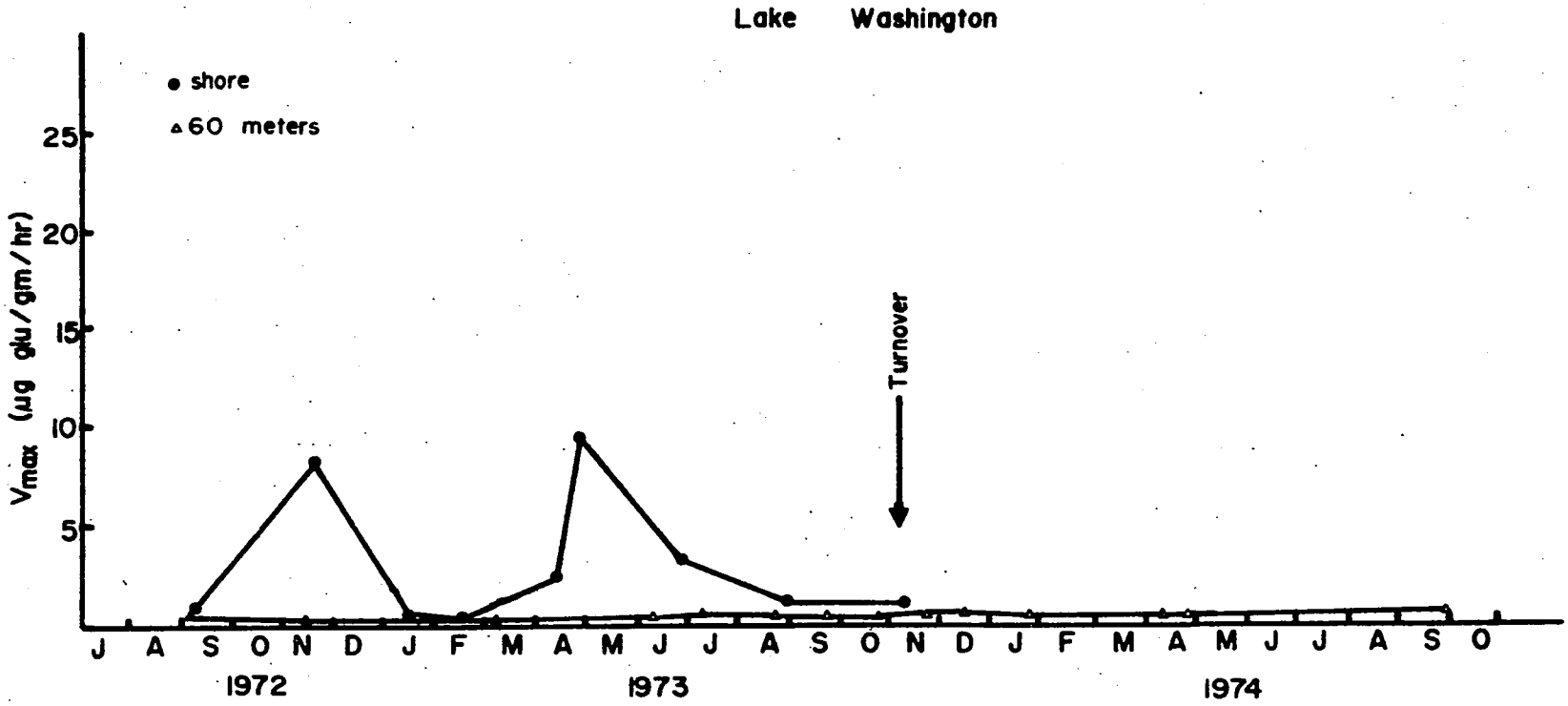

Lake Washington
• shore
▲60 meters
V_{max} (µg glu/gm/hr)
25
20
15
10
5
Turnover
J A S O N D J F M A M J J A S O N D J F M A M J J A S O
1972
1973
1974

Figure 12. Log Bacterial Plate Count with Depth into the Sediment for Lake Washington Benthic Station. Values represent the mean counts from replicate counts on FW agar.

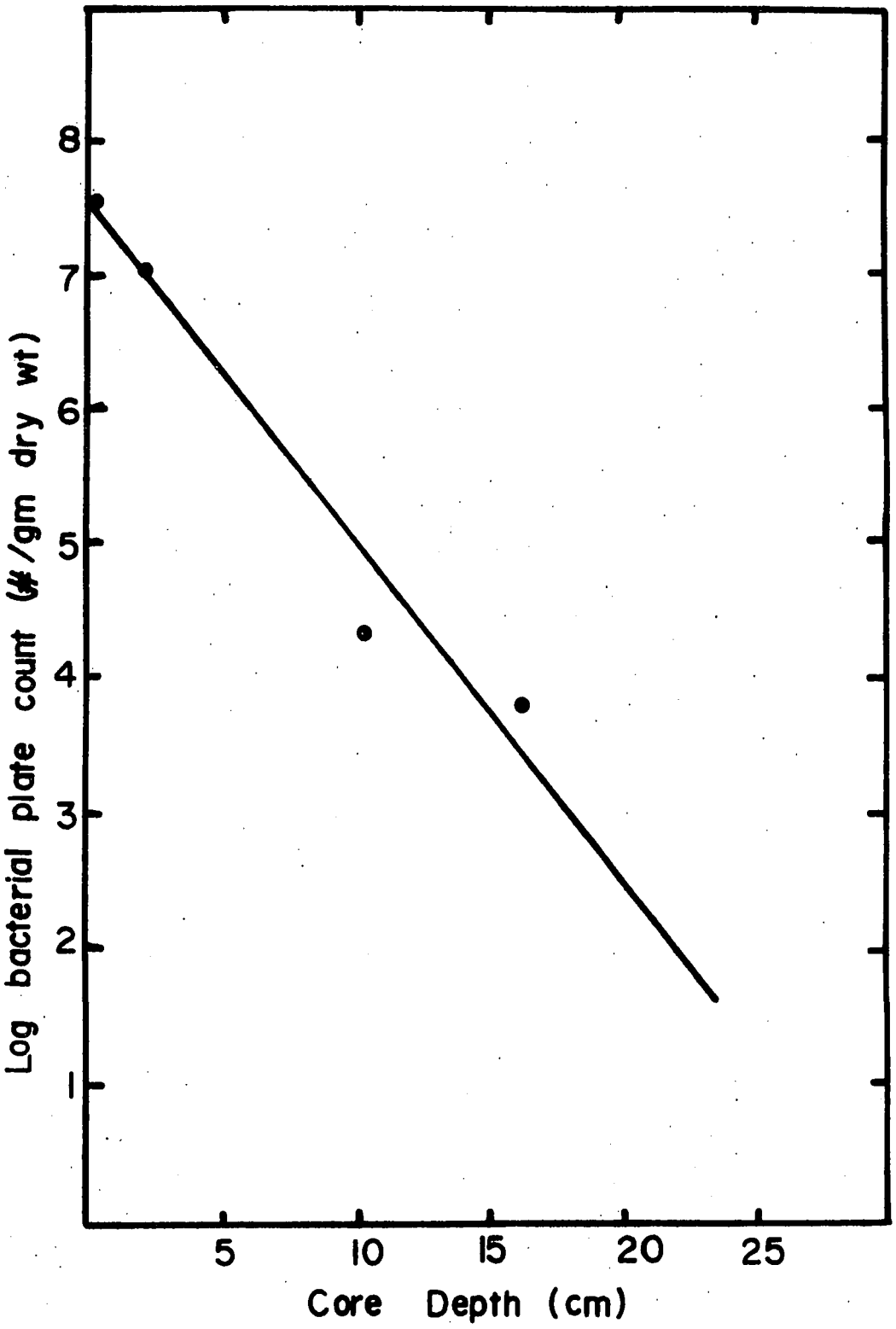

Log bacterial plate count (#/gm dry wt)
8
7
6
5
4
3
2
1
5
10
15
20
25
Core Depth (cm)

Table 11. Total Aerobic Plate Counts of Sediment from Lake Washington

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6	Percent Pigmented Bacteria
#1, 60 meters	9/ 8/72	7.6	6.7	40
	11/15/72	7.0	3.6	-
	3/ 6/73	6.9	6.3	-
	4/20/73	6.7	9.8	-
	6/19/73	7.2	24.0	-
	7/31/73	8.0	52.0	-
	8/23/73	8.0	9.2	-
	9/25/73	8.1	2.1	-
	10/25/73	7.9	2.8	56
	11/20/73	8.5	75.0	24
	12/18/73	8.7	32.0	32
	1/25/73	6.5	4.6	57
	4/ 4/74	6.6	30.0	-
	4/25/74	7.0	47.0	-
	9/24/74	6.6	3.8	-
#2, 0.5 meters	6/19/72	20.0	78.0	8
	6/28/72	20.0	750.0	-
	9/ 8/72	19.0	76.0	-
	11/15/72	10.0	120.0	30
	1/23/73	6.0	97.0	-
	2/18/73	9.0	170.0	-
	4/10/73	10.5	150.0	-
	4/26/73	13.0	970.0	-
	6/26/73	15.5	630.0	-
	8/20/73	19.0	170.0	-
	11/ 8/73	9.8	380.0	-
	8/22/74	20.0	950.0	-
	10/ 1/74	15.0	730.0	-
	11/ 5/74	13.5	40.0	-
	1/ 2/75	7.0	3.8	-
	2/ 4/75	7.0	61.0	-
	3/25/75	9.0	57.0	-
	4/25/75	10.5	270.0	-
	5/15/75	15.0	250.0	-

*Values represent mean counts of replicate plates incubated at the approximate in situ temperature.

Figure 13. Log Bacterial Plate Count on FW Agar from July 1972 to February 1974 for Sediment Collected from Lake Washington. Values represent mean of replicate plate counts incubated at the approximate in situ temperature. Data from Table 11.

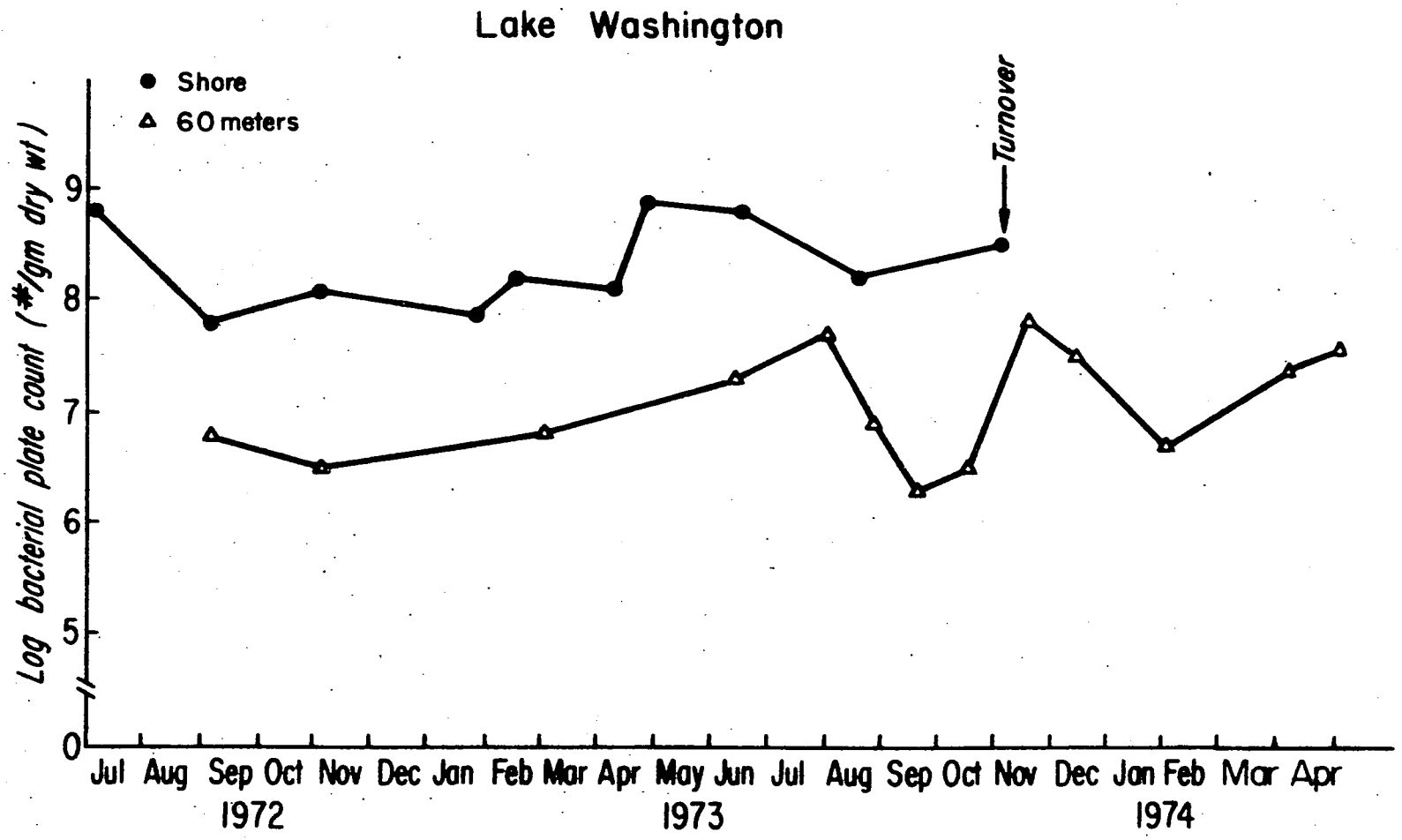

Lake Washington
Shore
60 meters
Turnover
Log bacterial plate count (*/gm dry wt)
9
8
7
6
5
0
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
1972
1973
1974

Table 12. Glucose Mineralization Rates for Stations Other than Main Sampling Stations in Lake Washington. Location of stations given in Figure 2.

Date	In Situ T°C	Station	TT (hrs)	V_{max} (µgm glu/ gm/hr)	Plate Count x 10^6/gm* Aerobic	Anaerobic
9/ 8/72	18.5	2 (H_2O)	100	0.006	0.0016/ml	-
3/ 7/73	7.0	1	2.30 ± 1.40	0.15 ± 0.04	6.3	0.88
	7.0	3	7.00 ± 0.23	9.20 ± 0.60	8.3	6.2
	7.0	5	6.20 ± 3.70	0.21 ± 0.15	3.1	7.1
	7.0	8	8.00 ± 2.80	0.35 ± 0.13	9.4	13.0
	7.0	4	23.20 ± 6.00	0.41 ± 0.45	2.1	5.7
5/10/73	7.0	6	32.00 ± 5.80	0.02 ± 0.02	12.0	-
	7.0	5	9.80 ± 2.00	0.14 ± 0.08	13.0	-
	13.0	8	7.20 ± 0.11	0.13 ± 0.09	9.6	-
9/25/73	8.2	1	4.10 ± 0.77	0.16 ± 0.03	2.1	5.3
	11.0	6	9.00 ± 0.45	0.85 ± 0.13	56.0	0.59
	17.5	8	6.20 ± 0.28	2.05 ± 0.15	44.0	1.2
	7.5	5	11.10 ± 2.80	0.16 ± 0.09	2.8	2.8

*Plate counts mean of replicate FW agar plates incubated at the approximate in situ temperature.

that day.

In September 1972, glucose mineralization rates were measured for a water sample taken 10 cm below the lake surface with a sterile pipet, at the littoral station (depth, 0.5 M). The glucose turnover time was 100 hours and V_{max} value of 6.7×10^{-3} µgm glu/gm/hr. The comparable glucose TT and V_{max} values measured for littoral zone sediment collected on that day was 7.5 hr and 1.0 µgm glu/gm/hr, respectively.

Anaerobic plate counts of sediments were approximately the same or slightly lower than corresponding aerobic counts and ranged from 8.1 to 130 x 10^6 organisms/gm and 0.88 to 23 x 10^6 organisms/gm for littoral and benthic zones, respectively (Table 13). Facultative anaerobes made up 87 to 100% of the population growing anaerobically in benthic zone sediments.

Table 13. Anaerobic Plate Counts on FW Agar for Sediment Samples from Lake Washington

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6
#1, 60 meters	3/ 6/73	6.9	0.88
	7/31/73	8.0	0.77
	9/25/73	8.1	5.3
	11/20/73	8.5	2.5
	1/25/74	6.5	3.7
	4/ 4/74	6.6	23.0
#2, 0.5 meters	1/23/73	6.0	8.1
	4/26/73	13.0	38.0
	11/ 8/73	9.8	130.0
	8/22/74	20.0	9.8

*Values represent mean counts of replicate plates incubated at the approximate in situ temperature.

Lake Sammamish. Lake Sammamish sediments were analyzed for glucose mineralization activity from September 1972 to April 1974. Samples obtained from the littoral station usually had faster glucose mineralization rates than did those from the benthic station; however, in the fall months of 1972 and 1973, benthic sediment exhibited shorter turnover times than did littoral samples (Fig. 14). In the littoral zone, shortest glucose TT of 8.1 and 5.3 hr were observed in November 1972 and July-August 1973, respectively. The longest TT of 20.6 hr occurred at this station in December 1973. For the benthic station, shortest TT of 6.1 and 6.7 hr occurred in September 1972 and August 1973, respectively; longest TT of 19.5 and 32.5 hr were found in April 1973 and February 1974, respectively. The V_{max} values determined for Lake Sammamish sediment samples from both stations were the lowest of all lakes sampled (Fig. 15). The V_{max} values for littoral zone sediment were highest at 2.2 from August to October 1973; the lowest V_{max} value of 0.17 for this station was found in April 1973. For the benthic station, highest V_{max} values of 1.0 and 1.3 occurred in September 1972 and August 1973, respectively; the lowest V_{max} value of 0.06 occurred in April 1973.

Aerobic plate counts on FW agar for both stations ranged from 5 to 380 x 10^6 organisms/gm sediment (Table 14, Fig. 16), with highest counts for both stations occurring after the fall turnovers in 1972 and 1973 and lowest counts in April 1973. Pigmented bacteria ranged from 18 to 25% of the total count for littoral and 23 to 32% for benthic samples (Table 14).

Anaerobic plate counts (Table 15) ranging from 0.4 to 4.0 x 10^6 organisms/gm for littoral and 2.1 to 31.0 x 10^6 organisms/gm for benthic stations were lower than corresponding aerobic plate counts with the exception of the anaerobic count of October 1973. On this date, the anaerobic count for the benthic station sediment was 28 x 10^6 organisms/gm as compared to the

Figure 14. Glucose Turnover Times for Lake Sammamish Sediment from September 1972 to April 1974. Data given in Table 53 in the Appendix.

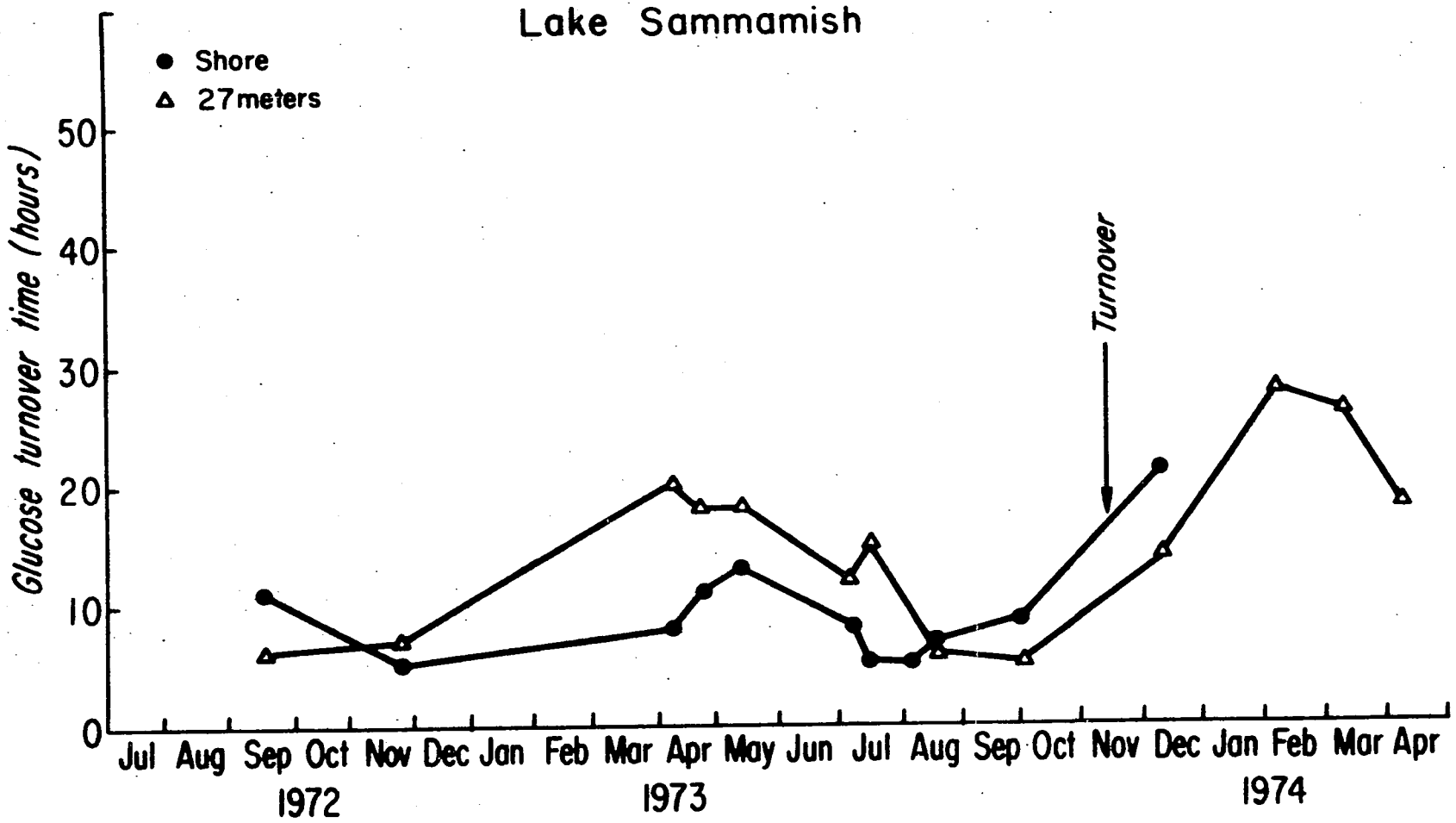

Lake Sammamish
Shore
27meters
Glucose turnover time (hours)
0
10
20
30
40
50
Turnover
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr
1972
1973
1974

Figure 15. V_{max} Values for Lake Sammamish Sediment from September 1972 to April 1974. Data given in Table 53 in the Appendix.

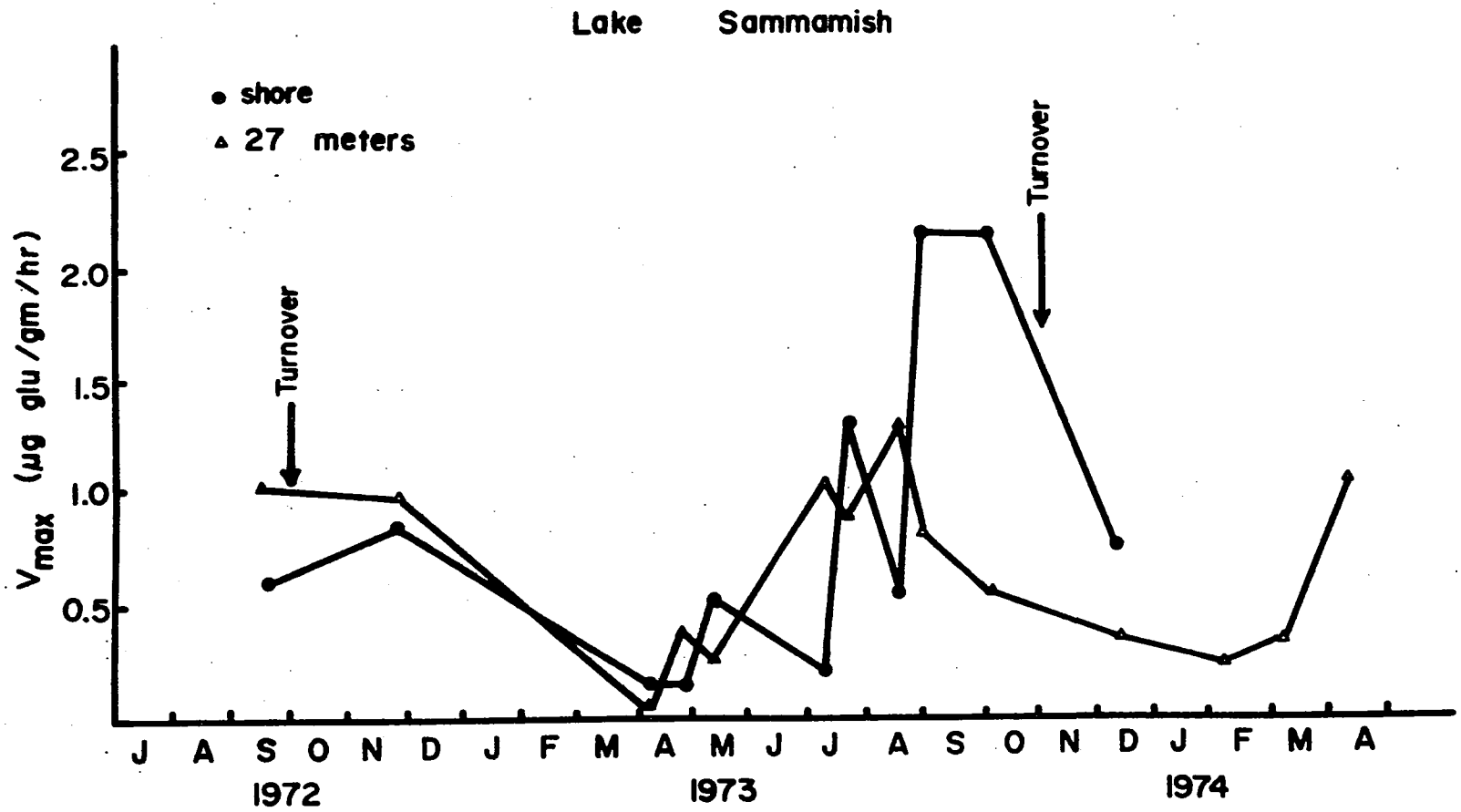

Lake Sammamish
shore
27 meters
Turnover
Turnover
V_{max} (µg glu/gm/hr)
2.5
2.0
1.5
1.0
0.5
J A S O N D J F M A M J J A S O N D J F M A
1972
1973
1974

Table 14. Total Aerobic Plate Counts of Sediment from Lake Sammamish

Station	Date	In Situ $T^{o}C$	Organisms/gm dry wt* Sediment x 10^6	Percent Pigmented Bacteria
#1, 27 meters	9/20/72	8.0	19.0	-
	11/29/72	7.8	34.0	-
	4/13/73	6.2	3.8	-
	4/27/73	7.0	15.0	-
	5/11/73	6.9	21.0	-
	7/13/73	7.9	10.0	-
	7/17/73	7.9	13.0	-
	8/ 2/73	7.9	12.0	-
	8/21/73	7.8	41.0	-
	10/ 2/73	7.9	6.7	-
	12/ 5/73	8.9	380.0	26
	2/ 5/74	5.8	15.0	25
	3/ 6/74	6.0	22.0	32
	4/ 3/74	6.0	23.0	-
#2, 2 meters	9/20/72	15.5	84.0	-
	11/29/72	9.0	280.0	-
	4/13/73	11.2	5.4	-
	4/27/73	10.3	23.0	-
	5/11/73	11.4	13.0	-
	7/13/73	23.0	6.3	18
	7/17/73	24.0	16.0	-
	8/ 2/73	23.0	32.0	-
	8/21/73	20.5	26.0	-
	10/ 2/73	18.5	30.0	-
	12/ 5/73	8.9	170.0	25

*Values represent mean of replicate plates incubated at the approximate in situ temperature.

Figure 16. Log Bacterial Plate Count on FW Agar from September 1972 to April 1974 for Sediment Collected from Lake Sammamish. Values represent mean of replicate plate counts incubated at the approximate in situ temperature. Data from Table 14.

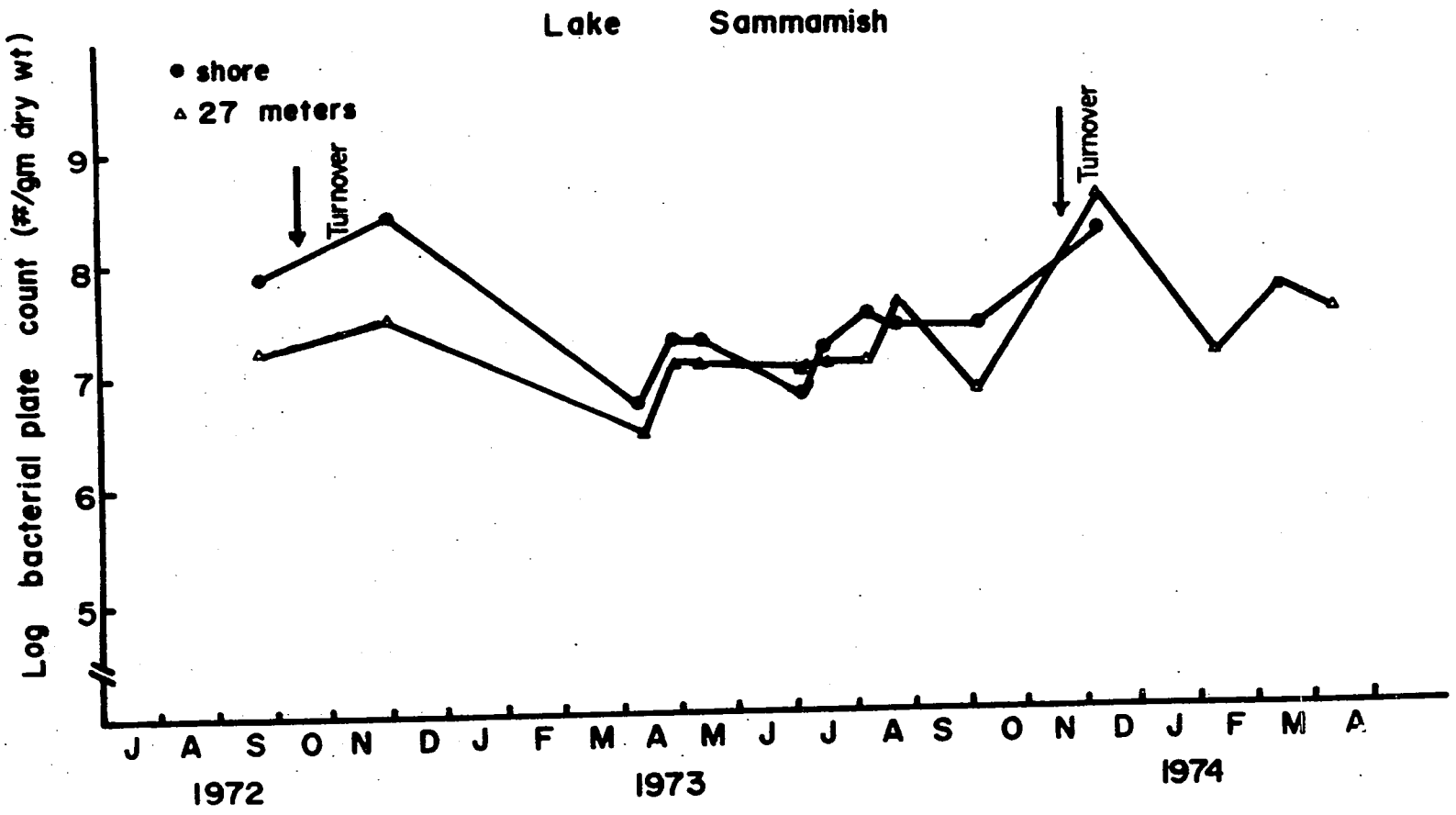

Lake Sammamish
• shore
▵ 27 meters
Turnover
Turnover
Log bacterial plate count (#/gm dry wt)
9
8
7
6
5
J A S O N D J F M A M J J A S O N D J F M A
1972
1973
1974

Table 15. Anaerobic Plate Counts on FW Agar for Sediment Samples from Lake Sammamish

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6
#1, 27 meters	7/13/73	7.9	2.1
	8/21/73	7.8	3.1
	10/ 2/73	7.9	28.0
	12/ 5/73	8.9	4.7
	2/ 5/74	5.8	4.6
	4/ 3/74	6.0	31.0
#2, 2 meters	7/13/73	23.0	0.45
	8/21/73	20.5	3.1
	10/ 2/73	18.5	3.5
	12/ 5/73	8.9	4.3

*Values represent mean of duplicate plates incubated at the approximate in situ temperature.

aerobic plate count of 6.7×10^6. After the fall turnover, the anaerobic plate count for benthic samples decreased to 4.7×10^6 and corresponding aerobic plate counts increased to 380×10^6 organisms/gm. Facultative anaerobes made up 96 to 100% of the population growing anaerobically in benthic zone sediments.

Lake Chester Morse. Sediments from Lake Chester Morse were analyzed for glucose mineralization activity from August 1972 to February 1974. Sediment samples obtained from the littoral area at the inlet of the Cedar River (No. 2) had faster glucose mineralization rates with shorter turnover times (Fig. 17) and higher V_{max} values (Fig. 18) than benthic samples. In the littoral zone samples, shortest TT of 1.0 and 1.9 hr were found for samples collected in August 1972 and July 1973, respectively; longest TT of 33 hr was observed in April 1973. For benthic zone sediment, the longest TT of 51 hr occurred in

Figure 17. Glucose Turnover Times for Sediment from Lake Chester Morse from August 1972 to February 1974. Data given in Table 54 in the Appendix.

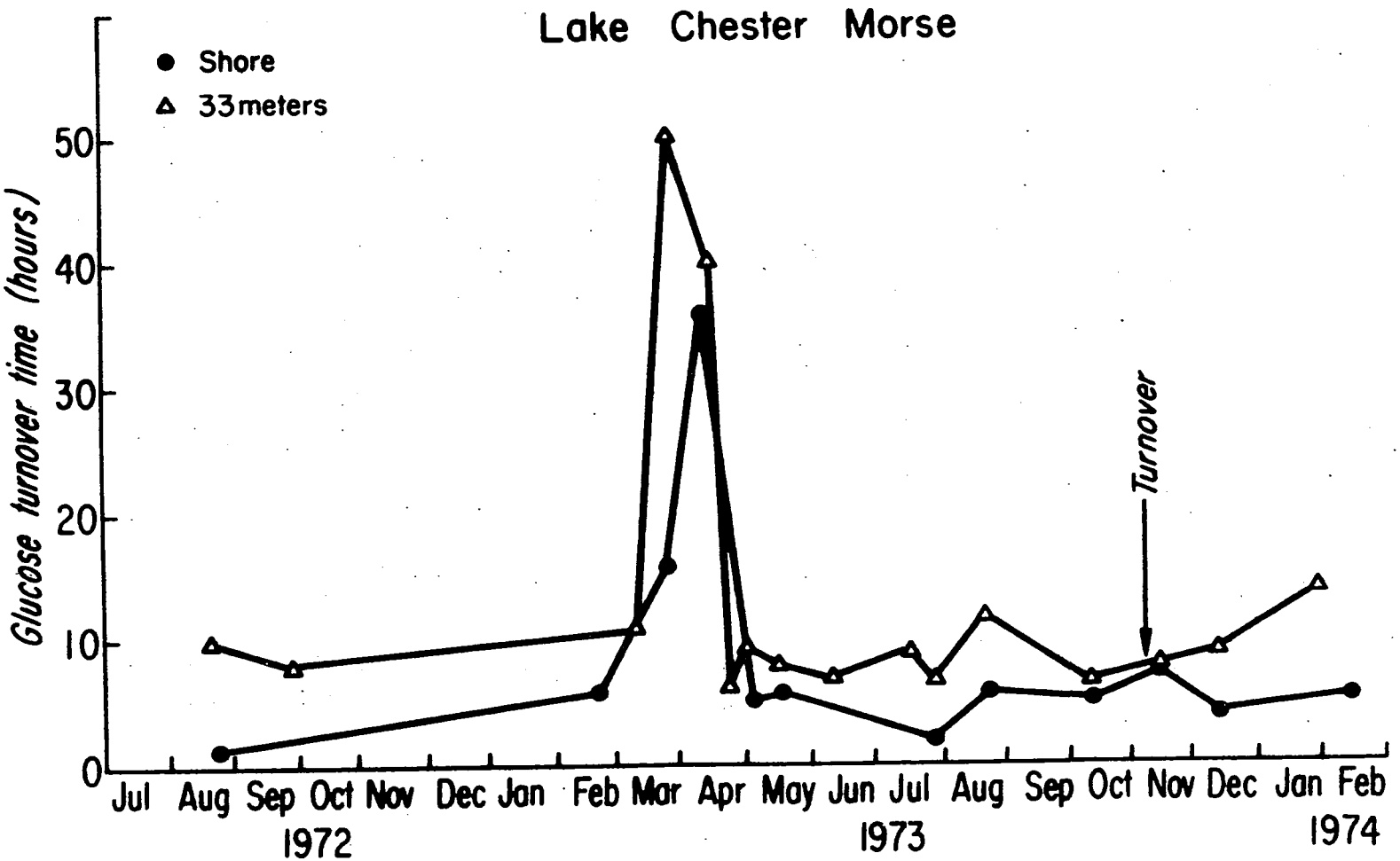
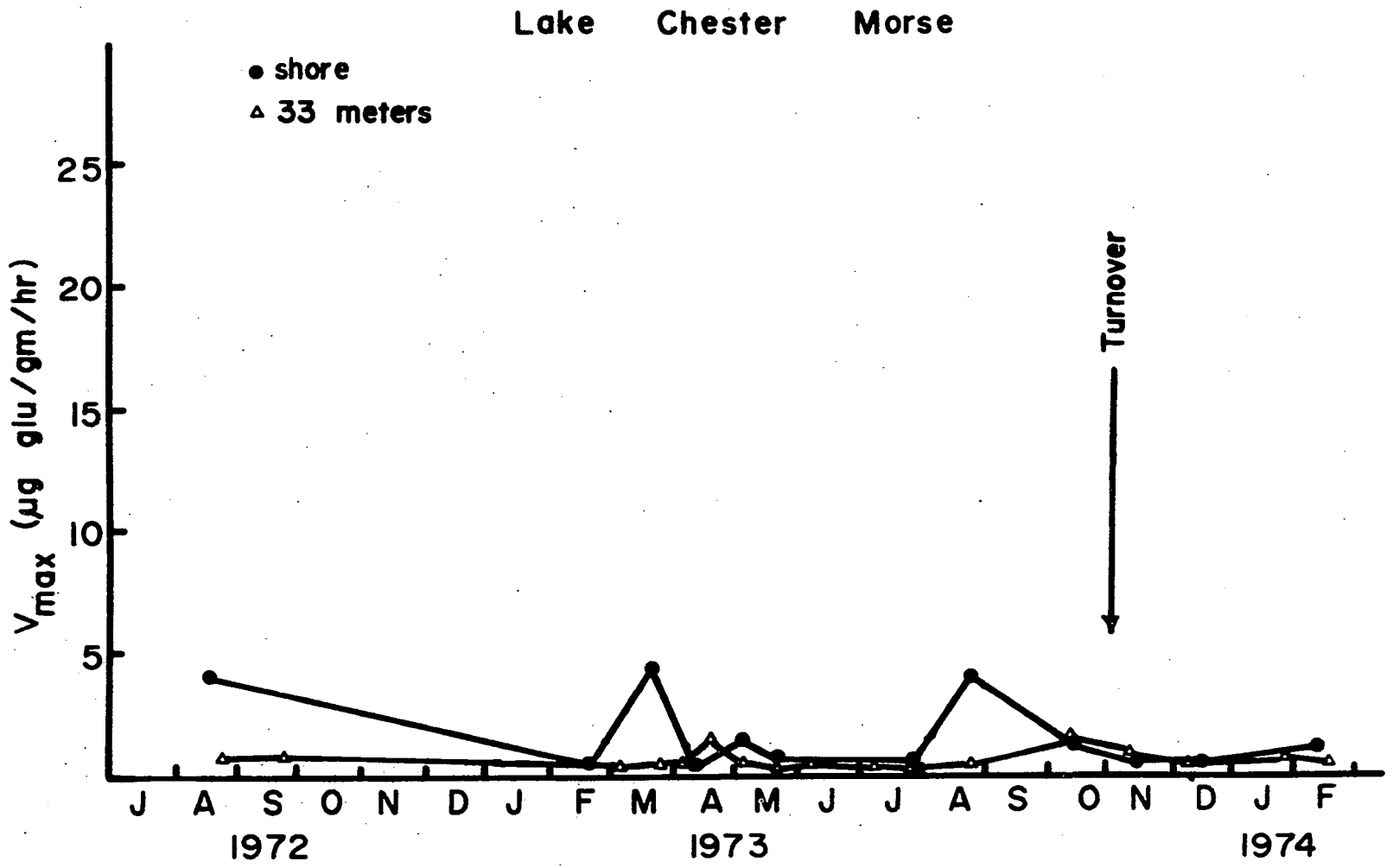

Lake Chester Morse
Shore
33 meters
Glucose turnover time (hours)
0
10
20
30
40
50
Turnover
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb
1972
1973
1974

Figure 18. V_{max} Values for Sediment Collected from Lake Chester Morse from August 1972 to February 1974. Data given in Table 54 in the Appendix.


Lake Chester Morse
● shore
△ 33 meters
25
20
15
10
5
V_{max} (µg glu/gm/hr)
Turnover
J A S O N D J F M A M J J A S O N D J F
1972
1973
1974

March 1973, which decreased to 8 hr by May 1973 and remained relatively constant until November with the exception of a higher turnover time in August of 11 hr. The V_{max} values of benthic zone sediments reached highest levels of 0.94, 1.3, and 1.3 in August-September 1972, March 1973, and October 1973, respectively; the lowest V_{max} values of 0.1 and 0.09 were found in April 1973 and July 1973, respectively. For littoral zone sediment, the highest V_{max} of 5.4 and 4.4 occurred in March 1973 and August 1973; the lowest of 0.04 was obtained in April 1973.

Aerobic plate counts on FW agar varied from 0.53 to 340 x 10^6 organisms/gm for benthic and 1.5 to 1100 x 10^6 organisms/gm for littoral area sediment (Table 16, Fig. 19). Littoral zone plate counts were usually higher than corresponding benthic area plate counts. Highest counts were found for samples collected in early spring and fall for both stations with lower counts during summer and winter months. Pigmented bacteria comprised 4 to 20% for littoral and 10 to 26% of the total count for benthic sediment samples (Table 16).

Anaerobic plate counts (Table 17) ranging from 0.006 to 12.0 x 10^6 organisms/gm for benthic and 2.1 to 31.0 x 10^6 organisms/gm for littoral zones were the same or lower than corresponding aerobic plate counts. Facultative anaerobes constituted 96% of the population growing anaerobically in benthic zone sediments.

Other stations were sampled in March 1973 and included the Cedar River outlet compared to the inlet (Table 18). Rates of glucose mineralization were higher on the basis of TT at 2.3 hrs for the inlet than the outlet at 8.9 hrs; there was no significant difference in V_{max} values between the two stations. Aerobic plate counts for the inlet of the Cedar River were an order of magnitude higher than those for the outlet, while there was no difference in the anaerobic plate counts between the two stations. In June 1973, samples were collected

Table 16. Total Aerobic Plate Counts of Sediment from Lake Chester Morse

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6	Percent Pigmented Bacteria
#1, 33 meters	8/22/72	7.0	1.8	-
	3/ 8/73	2.2	340.0	-
	3/20/73	3.4	1.6	-
	4/ 3/73	3.8	0.53	-
	4/18/73	4.8	6.1	-
	5/ 1/73	4.7	10.0	-
	5/22/73	5.9	6.1	-
	6/ 5/73	6.9	31.0	-
	7/10/73	6.9	7.3	-
	7/24/73	7.1	4.6	-
	8/28/73	6.9	180.0	-
	10/16/73	6.9	1.5	26
	11/13/73	7.8	11.0	10
	12/11/73	5.0	6.9	24
	1/30/74	4.0	6.6	25
	9/ 5/74	7.6	1.5	-
#2, 4 meters	8/22/72	19.0	140.0	-
	2/28/73	3.0	21.0	-
	3/ 8/73	2.2	26.0	-
	3/20/73	3.5	1100.0	-
	4/ 3/73	3.2	3.1	20
	5/ 1/73	7.5	13.0	-
	5/22/73	11.3	47.0	-
	7/24/73	18.4	21.0	-
	8/28/73	16.0	290.0	-
	10/16/73	8.5	1.5	4
	11/13/73	7.8	8.7	3
	12/11/73	5.0	4.7	14
	2/12/74	3.2	24.0	-
	9/ 5/74	13.5	11.0	-

*Values represent mean counts of replicate plates incubated at the approximate in situ temperature.

Figure 19. Log Bacterial Plate Count on FW Agar from August 1972 to February 1974 for Sediment Collected from Lake Chester Morse. Values represent mean of replicate plate counts incubated at the approximate in situ temperature. Data from Table 16.

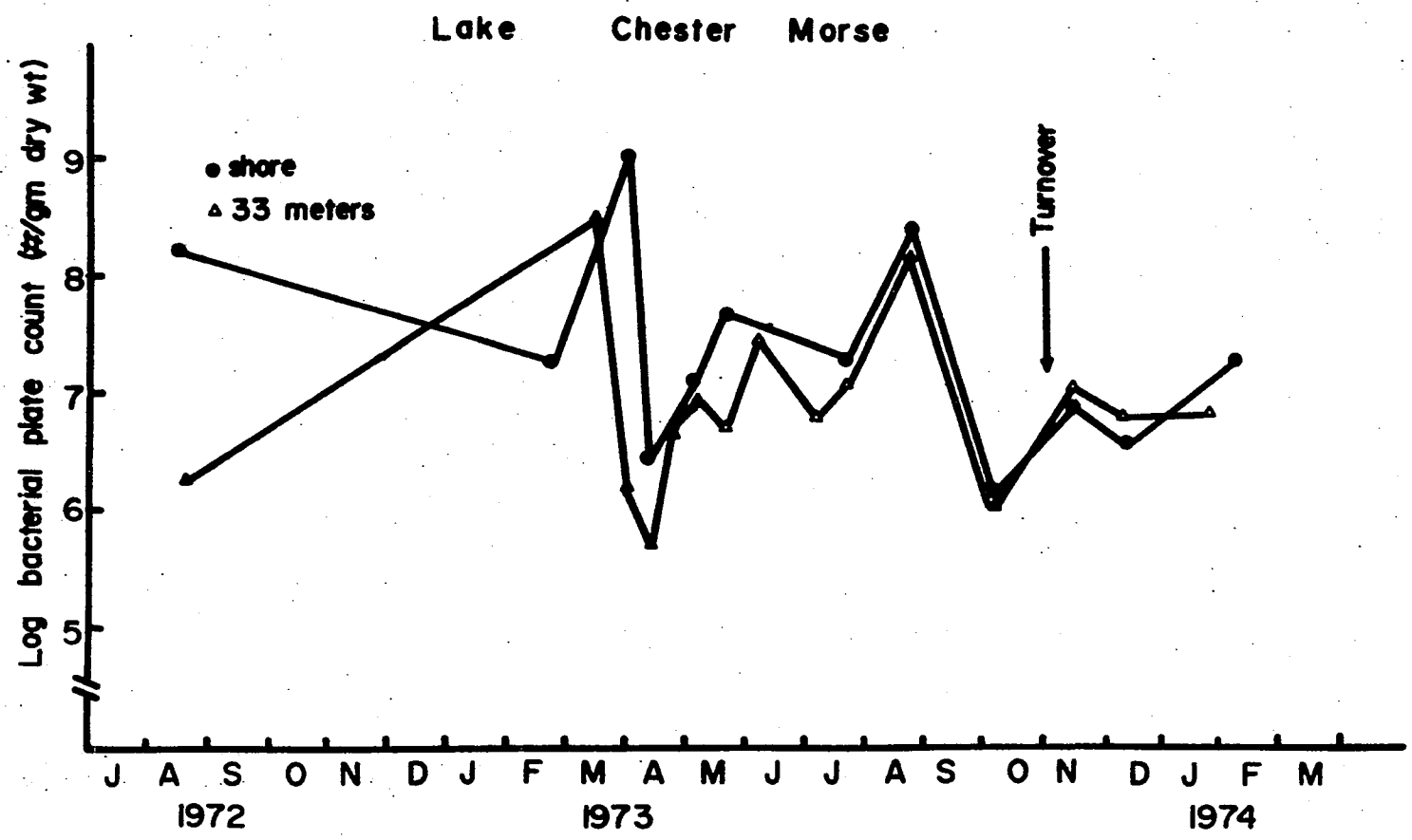

Lake Chester Morse
Log bacterial plate count (#/gm dry wt)
• shore
△ 33 meters
Turnover
9
8
7
6
5
J A S O N D J F M A M J J A S O N D J F M
1972
1973
1974

Table 17. Anaerobic Plate Counts on FW Agar for Sediment Samples from Lake Chester Morse

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6
#1, 33 meters	3/ 8/73	2.2	0.35
	4/18/73	4.8	6.0
	7/10/73	6.9	4.8
	8/28/73	6.9	0.32
	10/16/73	6.9	0.73
	11/13/73	7.8	12.0
	1/30/74	4.0	0.006
	9/ 5/74	7.6	0.6
#2, 4 meters	3/ 8/73	2.1	0.27
	8/28/73	16.0	0.81
	10/16/73	8.5	2.0
	11/13/73	7.8	7.0
	9/ 5/74	13.5	0.91

*Values represent mean of replicate plates incubated at the approximate in situ temperature.

Table 18. Glucose Mineralization Rates and Bacterial Plate Counts of Sediment from Stations Other than Those Routinely Sampled in Lake Chester Morse

Date	In Situ T°C	Station	TT (hrs)*	V_{max}* (µgm glu/ gm/hr)	Plate Count x 10^6/gm Aerobic	Plate Count x 10^6/gm Anaerobic
3/8/73	2.2	2 (inlet)	2.30 ± 2.10	0.09 ± 0.09	26.0	0.27
	2.2	4 (outlet)	8.90 ± 2.80	0.11 ± 0.16	2.6	0.2
6/5/73	6.8	1	7.40 ± 0.40	0.68 ± 0.10	31.0	-
	6.8	1 (100 m to west)	8.80 ± 0.60	0.51 ± 0.06	23.0	-

*Values represent mean ± standard deviation.

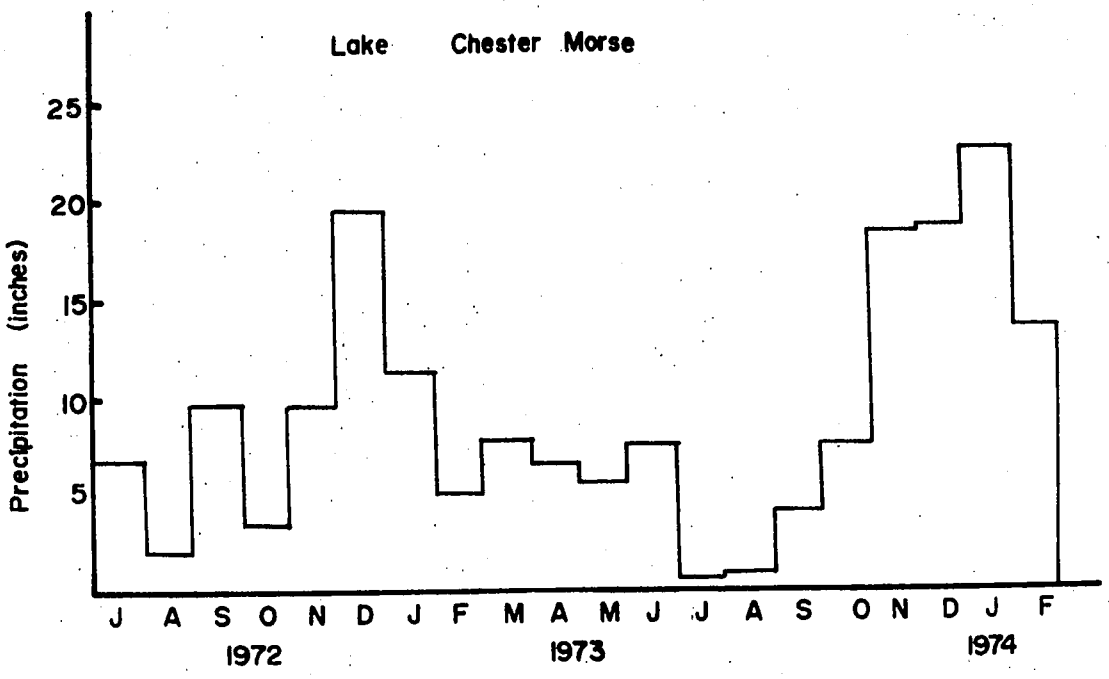
within 100 m at the benthic station and analyzed. There was no statistical difference in rate between the two samples, or in the aerobic plate count (Table 18).

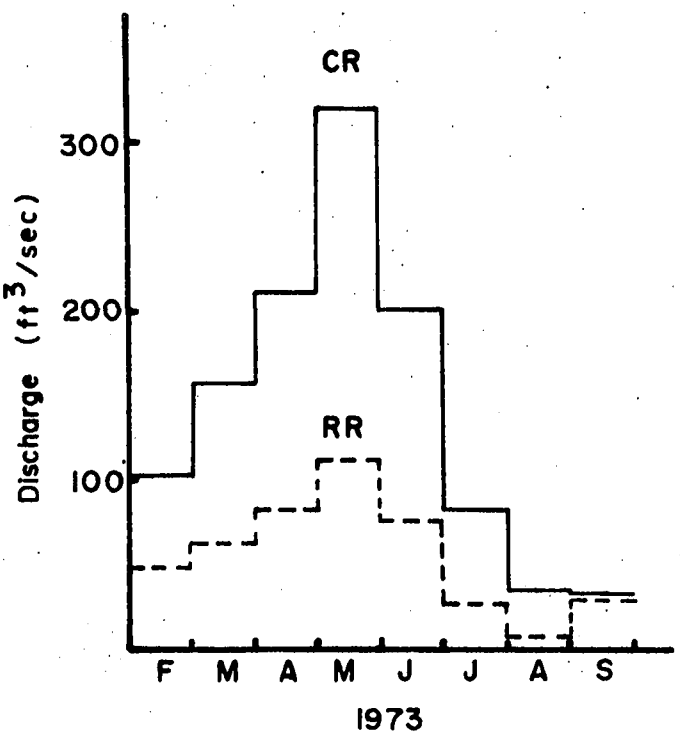
Precipitation in inches at Lake Chester Morse from July 1972 to February 1974 is illustrated in Figure 20a. The months of greatest precipitation for 1972 to 1973 occurred from November to January, and in 1974 from November to February. The driest months of the year occurred in August and October in 1972, and July and August in 1974. There was little correlation between plate count and glucose mineralization with precipitation, although plate counts tended to be higher, V_{max} higher and TT lower in the drier summer months.

Discharge to Lake Chester Morse in 1973 is shown in Figure 20b. Highest river inflow rates for both the Cedar and Rex Rivers occurred during the month of May. Preceding this high inflow rate in April, bacterial plate counts for both benthic and littoral stations decreased from 340 and 1100 x 10^6 cells/gm to 0.53 and 3.1 x 10^6 cells/gm, respectively, in the month of May (Table 16, Fig. 19). In May, a slight increase in littoral V_{max} was observed, while benthic V_{max} values decreased in May (Fig. 18). The glucose turnover times sharply decreased in April and May for both littoral and benthic zone sediments (Fig. 17).

Figure 20a. Precipitation in Inches at Lake Chester Morse from July 1972 to February 1974. Data courtesy of the Seattle City Water Department.

Figure 20b. Cedar River (CR) and Rex River (RR) Discharge to Lake Chester Morse, 1973. Data courtesy of Peter Birch.


Lake Chester Morse
Precipitation (inches)
25
20
15
10
5
J A S O N D J F M A M J J A S O N D J F
1972
1973
1974


CR
RR
Discharge (ft3/sec)
300
200
100
F M A M J J A S
1973

Findley Lake. Sediments from Findley Lake were analyzed for glucose mineralization activity from July 1972 to February 1974. Samples collected from the littoral area usually exhibited faster glucose mineralization rates than benthic samples. In the littoral zone samples, shortest TT (Fig. 21) of 1.0 and 2.0 hrs. were found in August 1972 and June 1973; the longest TT of 22.4 and 28.5 hrs. were found in May 1973 and February 1974, respectively. For benthic zone sediment, the longest TT of 64.5 hrs. occurred in May 1973. Shortest TT of 6.7 and 4.9 hrs. occurred in October 1972 and July 1973, respectively. The V_{max} values (Fig. 22) of benthic zone sediment were highest at 2.0 in July 1972, decreased to a minimum of 0.09 in March 1973, then increased to a constant value of 0.4 during the summer of 1973, followed by an increase to 1.3 and 1.6 in November 1973 and February 1974, respectively. For the littoral zone samples, highest V_{max} values of 30.0 and 20.0 occurred after melt during the month of July 1972 and 1973, respectively. The maximum V_{max} values for Findley Lake after ice out were the highest of all lakes in this study. The V_{max} values were lower during the winter months remaining constant at 0.4, with a decrease to 0.27 in May 1973 just prior to ice out. Sediment samples collected from Findley Lake had greater seasonal variation in glucose mineralization rates than samples collected from the other three lakes. In August 1972, glucose mineralization rates were measured on a water sample taken at littoral station No. 2. The glucose turnover time was 250 hrs. and V_{max} value 0.0011, compared to the rates measured at the sediment water interface for TT and V_{max} of 5.5 hrs. and 9.8 (Table 19). Other stations in the littoral area (Nos. 3, 4) and the lower lake (No. 5) were sampled in the summer of 1973 (Table 19). All of the other stations consistently exhibited more rapid glucose mineralization rates than did the 27 m benthic station. In June, highest V_{max} values were found at the littoral station No. 4; July, highest values were found at the lower lake; late July highest at littoral

Figure 21. Glucose Turnover Times for Sediment from Findley Lake from July 1972 to February 1974. Data given in Table 55 in the Appendix.

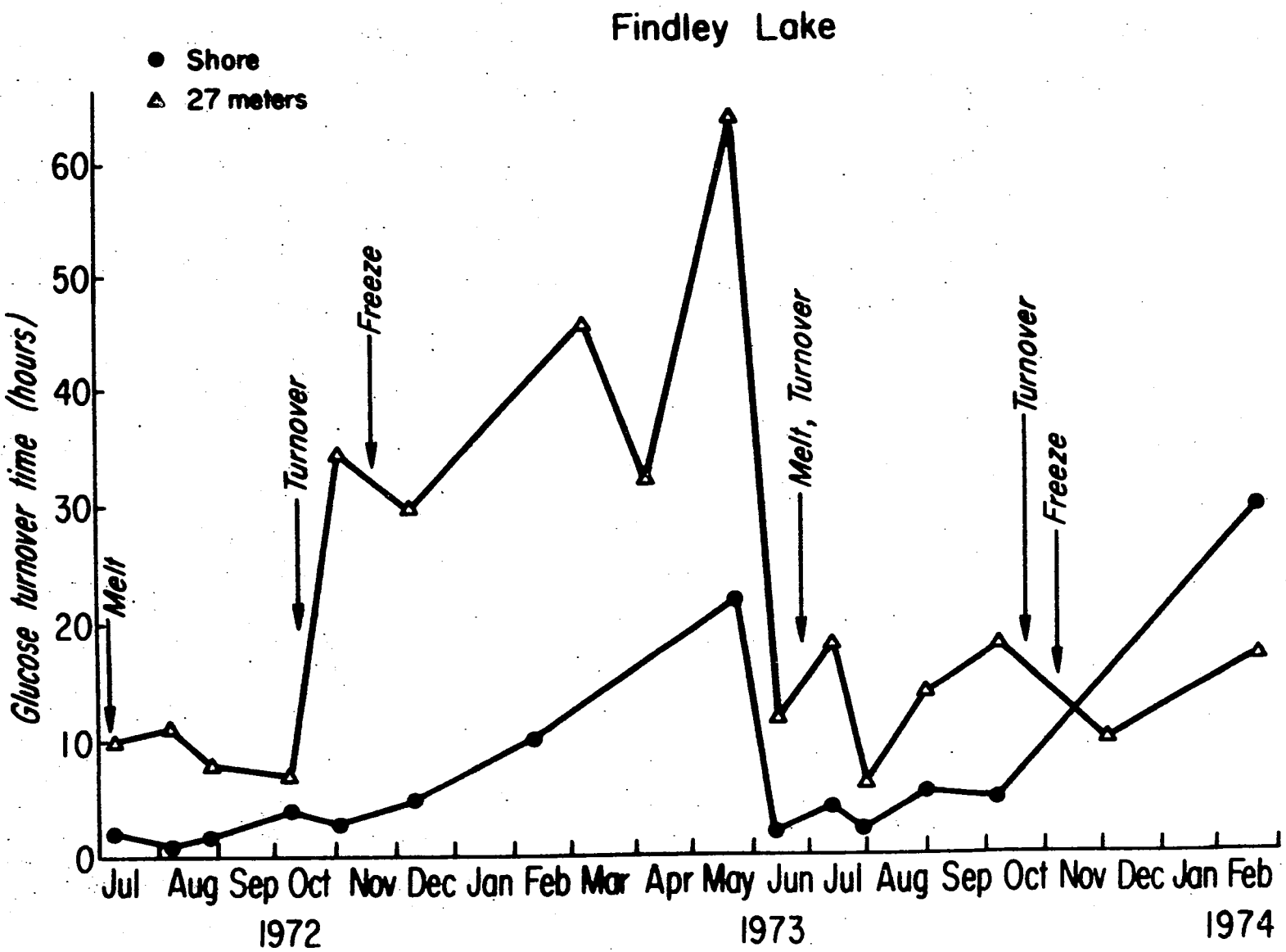

Findley Lake
Shore
27 meters
Glucose turnover time (hours)
0
10
20
30
40
50
60
Melt
Turnover
Freeze
Melt, Turnover
Turnover
Freeze
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb
1972
1973
1974

Figure 22. V_{max} Values for Sediment Samples Collected from Findley Lake from July 1972 to February 1974. Data given in Table 55 in the Appendix.

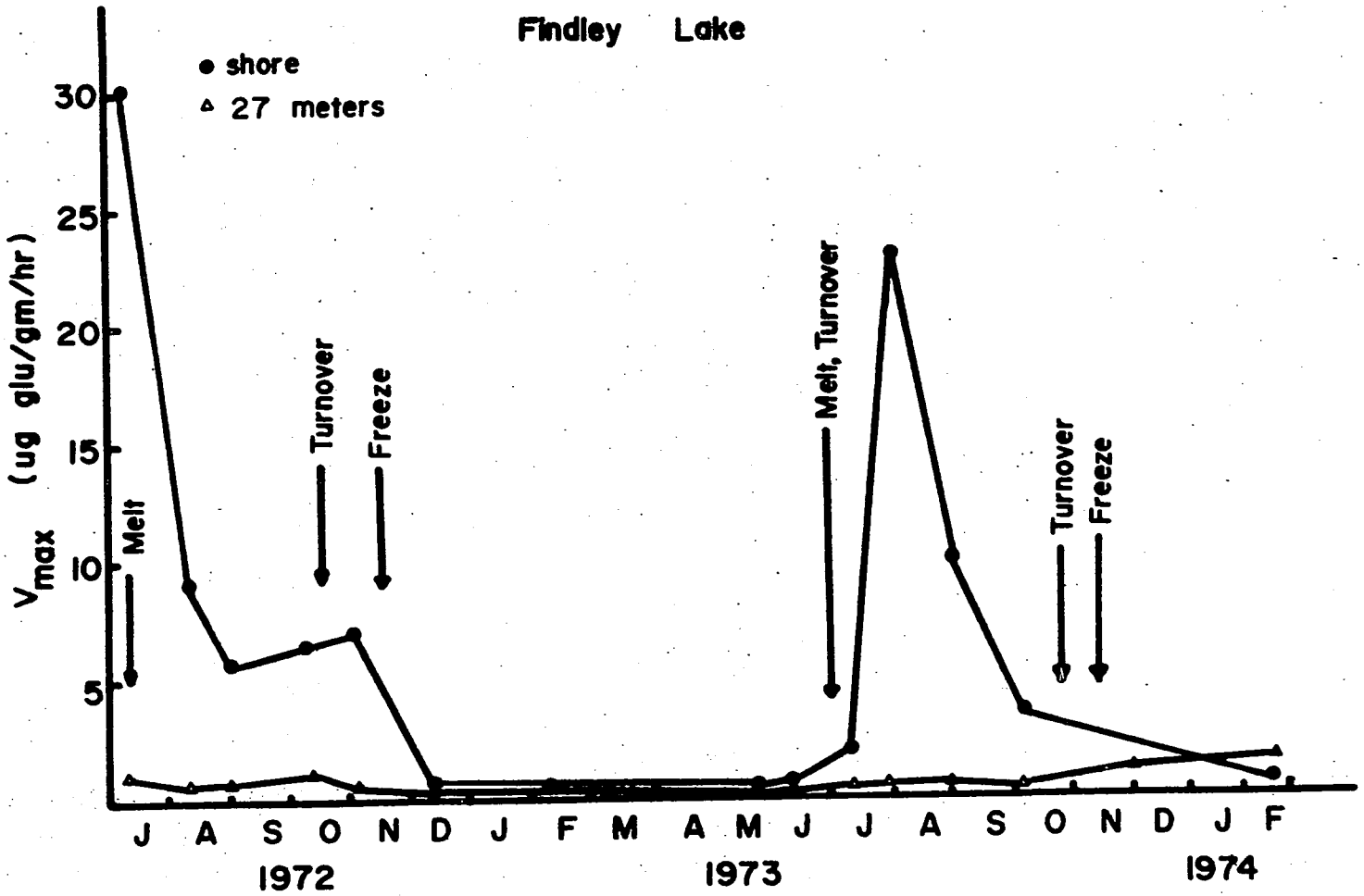

Findley Lake
• shore
▵ 27 meters
V_{max} (ug glu/gm/hr)
30
25
20
15
10
5
Melt
Turnover
Freeze
Melt, Turnover
Turnover
Freeze
J A S O N D J F M A M J J A S O N D J F
1972
1973
1974

Table 19. Glucose Mineralization Rates and Bacterial Plate Counts for Other Stations Sampled at Findley Lake. Stations shown in Figure 2.

Date	Station[1]	T°C	Depth Meters	Glucose[2] Turnover Time (hrs)	V_{max}[2] (µgm glu/ gm/hr)	Plate Count x 10^6/gm Aerobic	Anaerobic
8/30/72	2 (water)	19.0	0.1	250	1.1×10^{-3}	.001 (/ml)	
6/ 7/73	1	4.4	27.0	12.8 ± 2.0	0.3 ± 0.1	7.2	--
	4	4.5	0.5	5.3 ± 0.3	8.6 ± 1.0	39.0	--
	5*	5.0	0.5	6.2 ± 0.7	4.5 ± 0.2	150.0	--
	2	5.0	0.5	2.0 ± 0.4	0.6 ± 0.1	18.0	--
7/ 5/73	1	4.8	27.0	17.5 ± 3.0	0.38 ± 0.2	11.0	3.4
	5*	11.5	0.5	4.5 ± 0.3	0.68 ± 0.2	140.0	31.0
	3	13.0	0.5	3.9 ± 0.3	2.1 ± 0.1	110.0	45.0
	2	13.0	0.5	3.9 ± 0.3	1.7 ± 0.1	160.0	21.0
8/30/73	2	13.5	0.5	5.5 ± 0.2	9.8 ± 0.2	64.0	.74
	1	6.0	27.0	13.6 ± 0.9	0.37 ± 0.06	4.3	.50
	3	14.5	0.5	4.6 ± 0.2	14.2 ± 0.2	1000.0	2.9
	5*	13.5	0.5	3.7 ± 0.2	5.2 ± 0.1	38.0	.43
7/26/73	3	23.0	0.5	2.4 ± 0.1	4.4 ± 0.15	20.0	2.0
	2	23.0	0.5	2.1 ± 0.4	23.6 ± 0.4	24.0	.11
	1	4.5	27.0	4.9 ± 2.0	0.34 ± 0.17	8.0	1.7

[1]Station 5* is lower Findley Lake.

[2]Values are mean ± standard deviation.

No. 2; August, highest values at No. 3; and in October, littoral station No. 2 had the highest V_{max}.

Aerobic plate counts on FW agar for littoral samples ranged from 2.0 to 1100 x 10^6 organisms/gm (Table 20, Fig. 23), with the highest counts occurring after ice out in 1972 and 1973. Aerobic plate counts of benthic zone sediment were usually lower than littoral samples with counts ranging from 0.16 to 60.0 x 10^6 organisms/gm. Pigmented bacteria comprised 7 to 55% for littoral and 15 to 44% of the total count for benthic sediment samples (Table 20).

Anaerobic plate counts (Table 21), ranging from 0.15 to 6.5 x 10^6 for benthic and 0.11 to 21.0 x 10^6 organisms/gm for littoral zone sediments were lower than corresponding aerobic plate counts. Highest anaerobic counts occurred with sediment samples collected in October 1973. Facultative anaerobes from both stations made up 100% of the population growing anaerobically.

Table 20. Total Aerobic Plate Counts of Sediment from Findley Lake

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6	Percent Pigmented Bacteria
#1, 27 meters	7/ 7/72	4.0	0.34	-
	8/ 8/72	4.0	10.0	-
	8/30/72	4.3	1.1	-
	10/12/72	4.9	0.16	-
	11/ 1/72	5.0	0.76	-
	3/ 5/73	4.0	60.0	-
	3/27/73	4.0	2.9	-
	5/24/73	4.0	0.27	-
	6/ 7/73	4.0	7.2	-
	7/ 5/73	4.6	11.0	38
	7/26/73	4.1	8.0	-
	8/30/73	5.9	4.3	44
	10/ 4/73	5.0	3.1	15
	11/30/73	4.0	2.5	17
	2/28/74	4.0	1.3	18
	9/19/74	4.5	1.0	-
#2, 0.5 meters	7/ 7/72	10.5	10.0	-
	8/ 8/72	20.5	1100.0	-
	8/30/72	18.8	24.0	-
	10/12/72	7.8	3.3	-
	11/ 1/72	5.0	3.9	7
	2/ 9/73	0.5	2.3	-
	5/24/73	3.5	9.7	-
	6/ 7/73	5.9	18.0	-
	7/ 5/73	13.5	160.0	-
	7/26/73	17.4	24.0	-
	8/16/73	16.0	310.0	-
	8/30/73	13.5	64.0	-
	10/ 4/73	12.5	48.0	-
	2/28/74	1.0	2.0	55
	9/19/74	12.9	93.0	-

*Values represent mean of replicate plates incubated at approximate in situ temperature.

Figure 23. Log Bacterial Plate Count on FW Agar from July 1972 to February 1974 for Sediment Collected from Findley Lake. Values represent mean of replicate plate counts of plates incubated at the approximate in situ temperature. Data from Table 20.

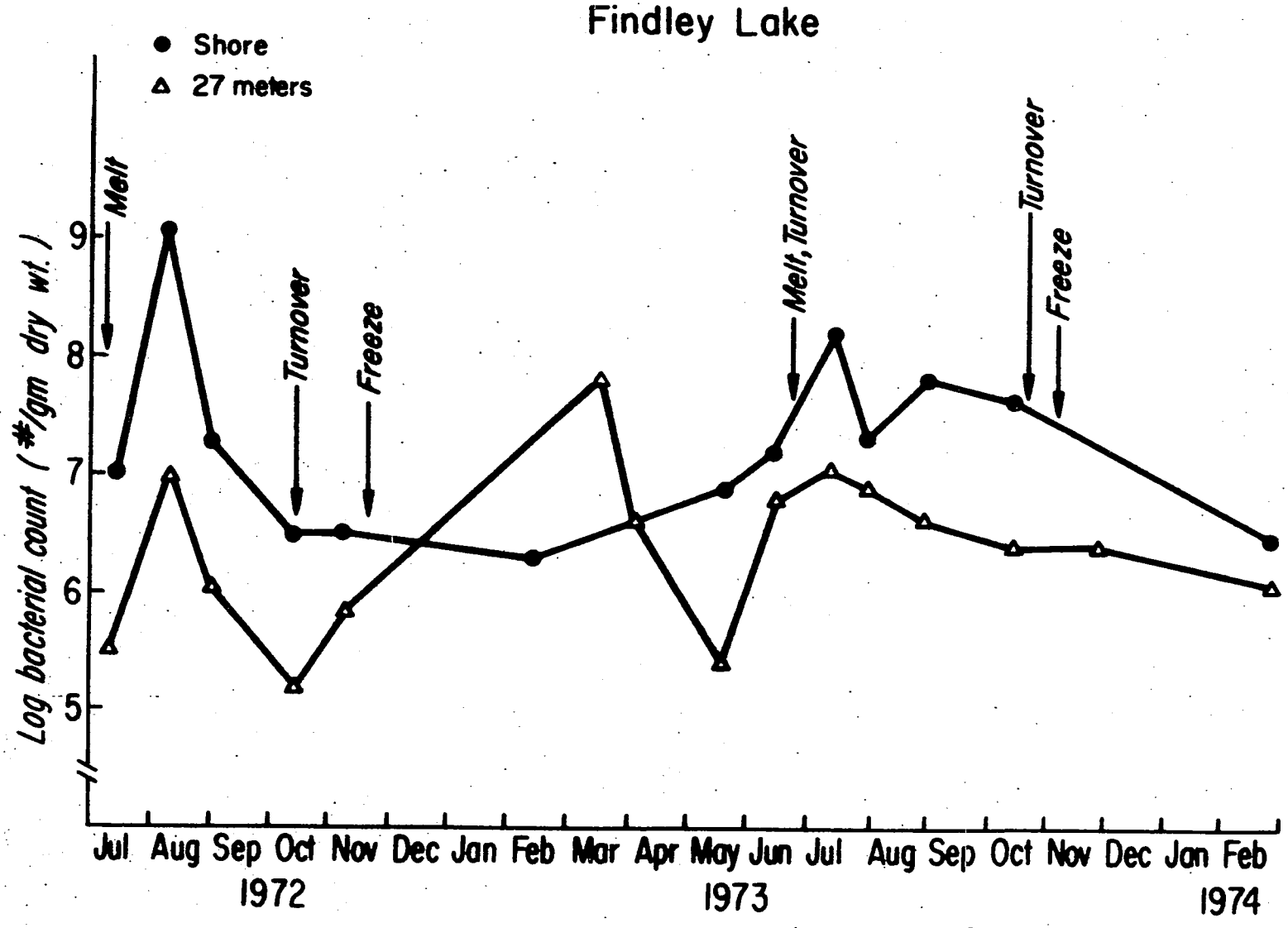

Findley Lake
Shore
27 meters
Log bacterial count (*/gm dry wt.)
9
8
7
6
5
Melt
Turnover
Freeze
Melt, Turnover
Turnover
Freeze
Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb
1972
1973
1974

Table 21. Anaerobic Plate Counts on FW Agar for Sediment Samples from Findley Lake

Station	Date	In Situ T°C	Organisms/gm dry wt* Sediment x 10^6
#1, 27 meters	3/ 5/73	4.0	0.15
	3/27/73	4.0	3.3
	7/ 5/73	4.6	3.4
	7/26/73	4.1	1.7
	8/30/73	5.9	0.5
	10/ 4/73	5.0	6.5
	11/30/73	4.0	4.9
	2/28/74	4.0	0.29
#2, 0.5 meters	2/ 9/73	0.5	0.5
	7/ 5/73	13.5	21.0
	7/26/73	17.4	0.11
	8/16/73	16.0	3.6
	8/30/73	13.5	0.74
	10/ 4/73	12.5	11.0
	2/28/74	1.0	1.5

*Values represent mean counts of replicate plates incubated at the approximate in situ temperature.

Four Lakes

A total of 50 littoral, 55 benthic, and 18 sediment samples, collected at other than the main stations, were analyzed for glucose mineralization activity from July 1972 to April 1974 in Lakes Washington, Sammamish, Chester Morse, and Findley. Higher glucose mineralization rates occurred at the sediment-water interface than in the water column in the 4 lakes. Bacteria associated with the littoral zone sediment were usually more numerous and exhibited higher glucose mineralization rates than did those from benthic stations with the exception of Lake Sammamish. In Lake Sammamish sediments, glucose mineralization rates were higher in benthic sediment in September 1972 and from August to December in 1973 (Fig. 14).

Seasonal variation in glucose mineralization rate occurred in all lake sediments with higher rates in summer and lowest in the late winter to early spring months. Greatest seasonal variation in glucose mineralization rate was found in sediments from Findley Lake. Lowest V_{max} values for all lakes sampled were found in sediments from Lake Sammamish.

Aerobic heterotrophic bacteria were found to be most numerous in the top 2 cm of sediment at the Lake Washington benthic station (60 m), and numbers decreased logarithmically with depth into the sediment. There was a tendency for seasonal variation in bacterial biomass in the lakes, with higher numbers occurring in the summer and lower numbers in the winter months. Increases in biomass occurred in Lakes Washington and Sammamish after the fall turnover. Greatest seasonal variation in bacterial numbers was observed with Findley Lake sediments. The bacterial numbers varied considerably in Lake Chester Morse sediments with little correlation to season.

Anaerobic plate counts for all stations in the 4 lakes were the same or lower than corresponding aerobic counts, with one exception. Anaerobic plate

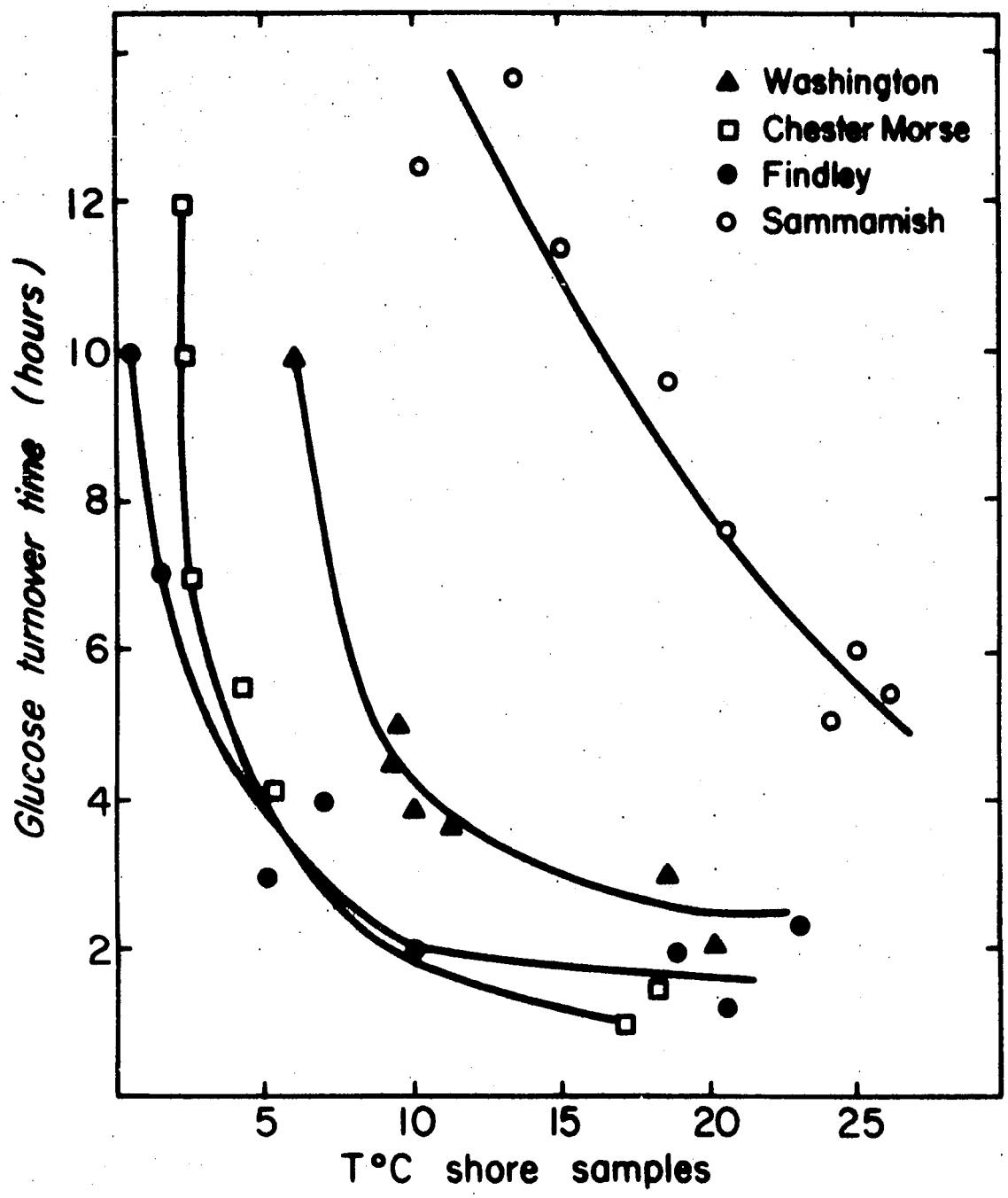
counts for benthic zone sediment collected from Lake Sammamish in October 1973 were an order of magnitude higher than the corresponding aerobic count. The majority of anaerobes tested, from 87 to 100%, were facultative anaerobes. A high proportion of the bacteria found associated with the sediments in the 4 lakes were gram-negative motile rods. Pigmented bacteria ranged from 8 to 70% with a mean value of 30%. The majority of the pigmented bacteria were yellow, although smaller numbers of purple, orange, and red pigmented bacteria were also noted.

Effect of Temperature on Glucose Mineralization Rate

Glucose mineralization rates, on the basis of turnover times measured for littoral zone sediment bacteria, were indirectly related to the in situ temperature (Fig. 24). Although this relationship later proved not to be statistically significant for Findley Lake, there was a tendency for longer glucose turnover times at lower temperatures. Data for this lake are included in Figure 24 for comparison to the other 3 lakes. It is apparent from Figure 24 that sediment from Lake Chester Morse exhibits higher rates, with shorter turnover times at lower temperatures, than do the lakes lower in altitude, Washington and Sammamish. Bacteria associated with the littoral zone of sediment from Lake Sammamish were least responsive to temperature fluctuations.

A number of relationships were tested for significance by linear regression analysis and calculation of the correlation coefficient with a Wang computer Model 700 c (Wang Lab., Inc., Twekesbury, Mass.) using a linear regression analysis program. Many of these relationships have been claimed to exist by other workers and data generated in this study suggested possible significance. These relationships included: (1) log turnover time versus 1/T absolute, (2) log V_{max} versus 1/T absolute, (3) log bacterial plate count versus temperature

Figure 24. Relationship Between Glucose Turnover Time to In Situ Temperature (Celsius) for Bacteria Associated with Sediment Collected from the Littoral Zone for the 4 Lakes


Washington
Chester Morse
Findley
Sammamish
Glucose turnover time (hours)
12
10
8
6
4
2
5
10
15
20
25
T°C shore samples

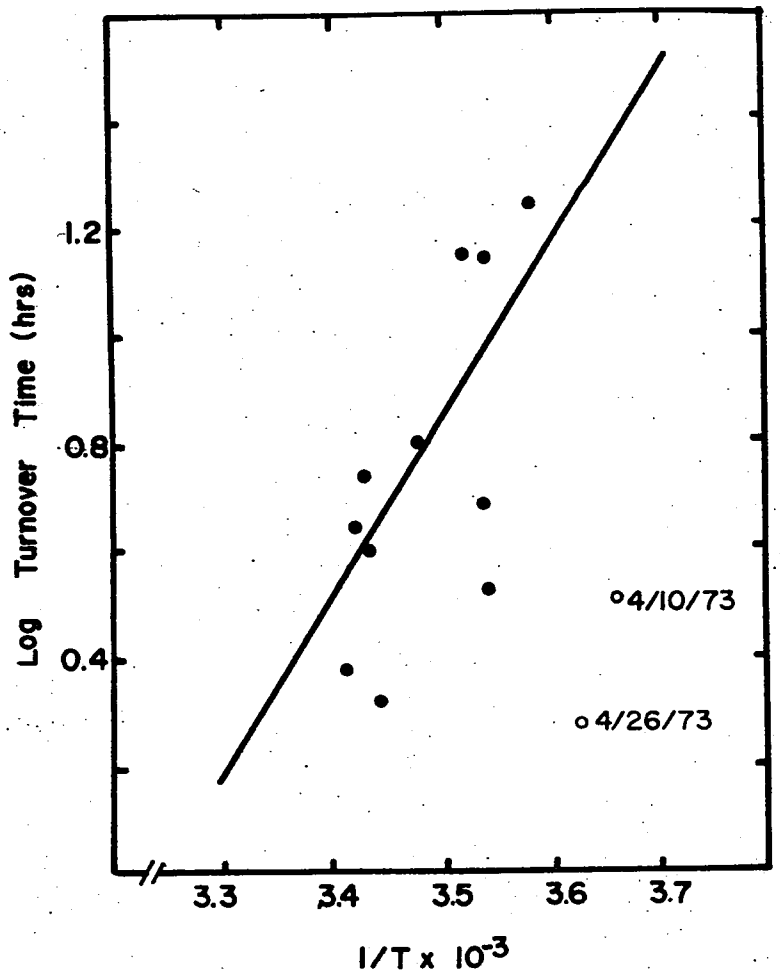
(Celsius), (4) log bacterial plate count versus glucose turnover time, (5) log bacterial plate count versus log V_{max}. In a few cases, data yielded a cluster of points which fell along a line with 1 or 2 points strikingly out of the range of the majority of the data points. Only when these points were excluded from the calculations was a statistically significant relationship obtained. These points were deleted only if some other governing factor beside temperature could be suggested. The seasonal glucose turnover times for littoral zone sediment were drawn as Arrhenius plots (log glucose turnover time versus 1/T absolute). Best fit to the resulting line was calculated by linear regression analysis, and the correlation coefficient calculated. Significance at the 5% level occurred for littoral sediment from Lake Washington (Fig. 25), Lake Sammamish (Fig. 26), and Lake Chester Morse (Fig. 27); however, with values for Lake Washington, the relationship was significant only if the two values for April 1973 were deleted. All data were included in the analysis of the other 3 lakes. Data from Findley Lake were not statistically significant.

The temperature coefficient, defined as the slope of the line generated by plotting either log glucose turnover time or log V_{max} as a function of 1/T absolute (Arrhenius plot) was calculated. The temperature coefficient calculated from Arrhenius plots for littoral zone sediment glucose turnover times for Lake Washington (6-20°C), Lake Sammamish (9-24°C), and Lake Chester Morse (2.2-19.0°C), were 3.34×10^3, 0.31×10^3, and 4.06×10^3, respectively. Values for Lake Washington and Lake Chester Morse were similar; however, the temperature coefficient for Lake Sammamish was lower, indicating less responsiveness to temperature.

For benthic samples, a similar turnover time to temperature relationship earlier in the course of this study appeared to exist; but as more data were collected, only Lake Sammamish data from sediments of the benthic zone, when

Figure 25. Arrhenius Plot for Sediments from the Lake Washington Littoral Zone (Log Glucose Turnover Time Versus 1/T abs). Values for 4/10/73 and 4/26/73 were not included in the linear regression analysis. Sediment collected from June 1972 to Jan. 1973 and analyzed at the in situ temperature.

Figure 26. Arrhenius Plot for Sediments from the Lake Sammamish Littoral Zone (Log Glucose Turnover Time Versus 1/T abs). Sediment collected from Sept. 1972 to Dec. 1973 and analyzed at the in situ temperature.


Log Turnover Time (hrs)
1.2
0.8
0.4
3.3
3.4
3.5
3.6
3.7
1/T x 10⁻³
o 4/10/73
o 4/26/73

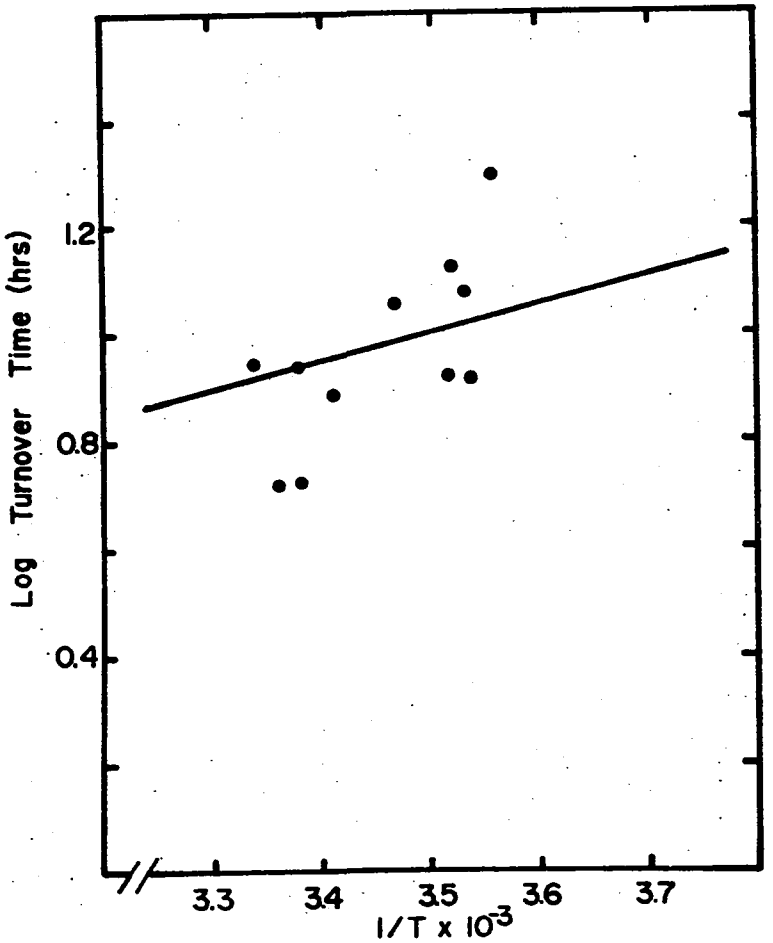
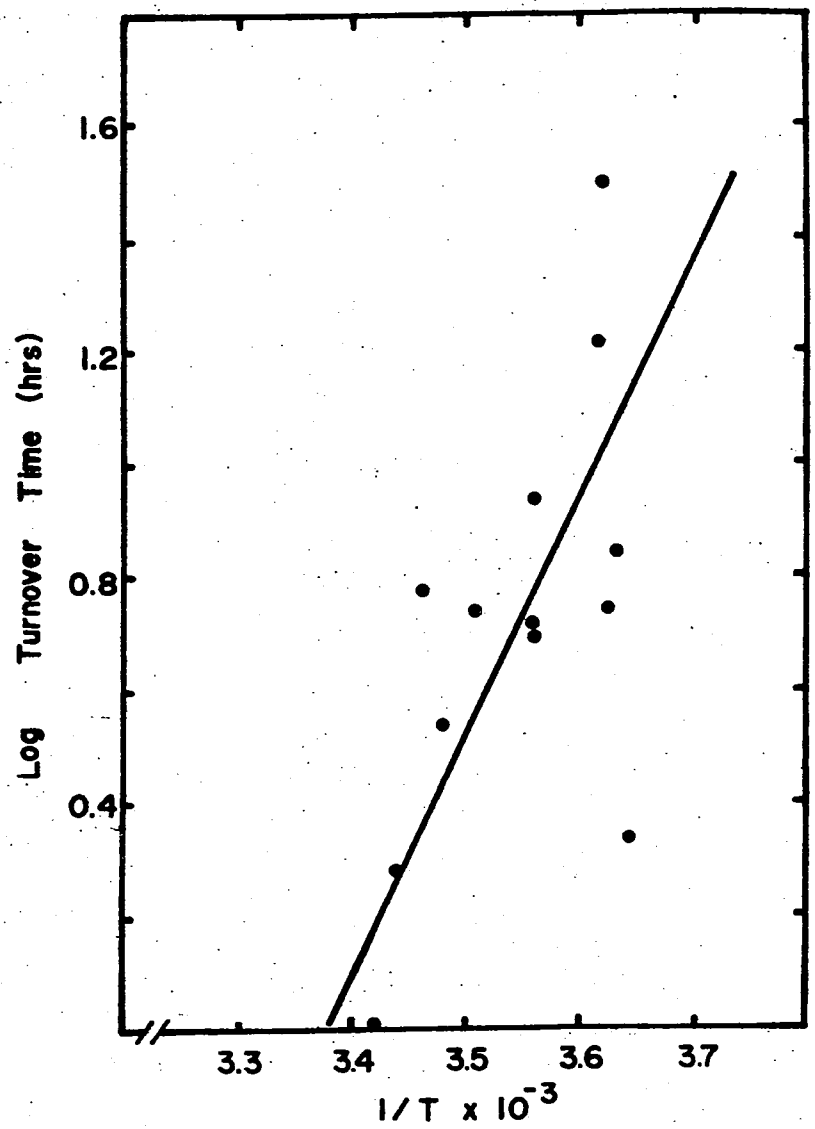
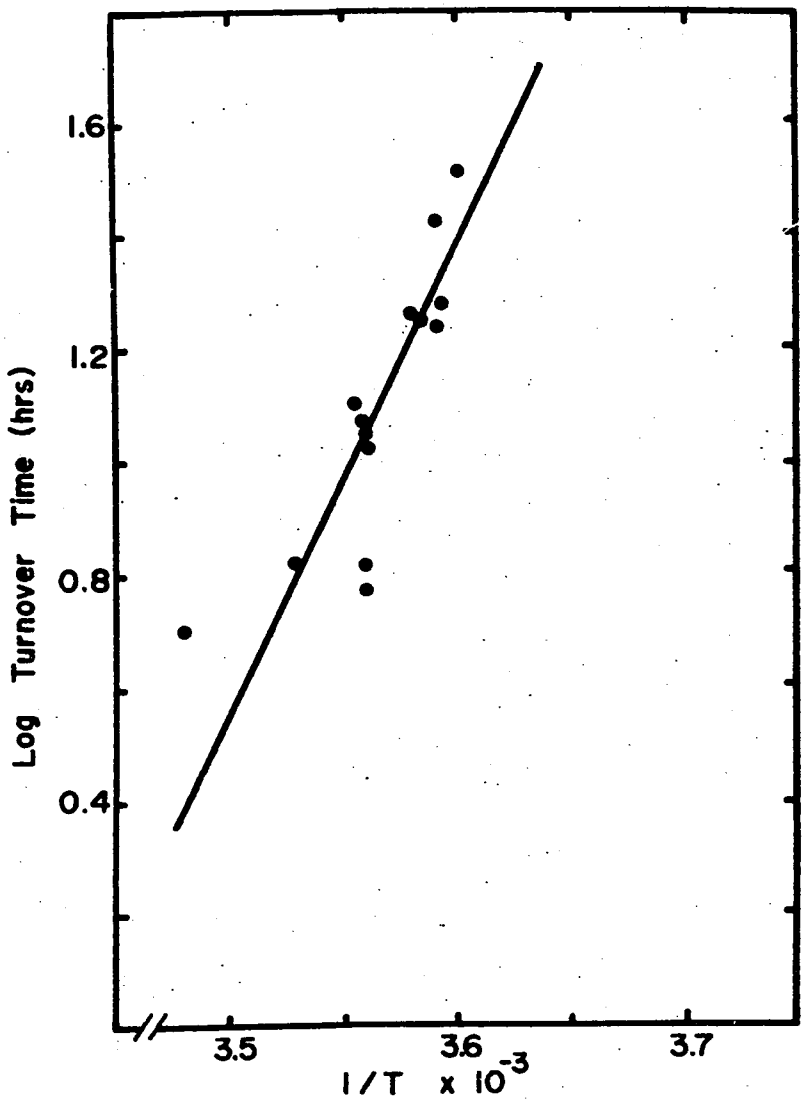

Log Turnover Time (hrs)
1.2
0.8
0.4
3.3
3.4
3.5
3.6
3.7
1/T x 10⁻³

Figure 27. Arrhenius Plot for Sediments from Lake Chester Morse Littoral Zone (Log Glucose Turnover Time Versus 1/T abs). Sediment collected from Aug. 1972 to Feb. 1974 and analyzed at the in situ temperature.

Figure 28. Arrhenius Plot for Sediments from Lake Sammamish Benthic Zone (Log Glucose Turnover Time Versus 1/T abs). Sediment collected from Sept. 1972 to Dec. 1973 and analyzed at the in situ temperature.


Log Turnover Time (hrs)
1.6
1.2
0.8
0.4
3.3
3.4
3.5
3.6
3.7
1/T x 10^-3


Log Turnover Time (hrs)
1.6
1.2
0.8
0.4
3.5
3.6
3.7
1/T x 10^-3

analyzed statistically, demonstrated a significant relationship (0.1%) with all data points included (Fig. 28). The temperature coefficient was 8.25×10^3 (6.0 - 8.0°C), greater than that found for the littoral zone sediment from this lake (0.31×10^3).

Arrhenius plots for log V_{max} versus 1/T absolute, when analyzed statistically were significant for littoral samples only from Lake Sammamish (Fig. 29) (5%), and Findley Lake (Fig. 30) (1%). For Findley Lake data, all values were used; however, for Lake Sammamish, April and July 1973 data points were excluded and are shown in Figure 29. The temperature coefficients for Lake Sammamish (9-24°C) and Findley Lake 1.0-20.5°C) littoral zone were -2.05×10^3 and -6.4×10^3, respectively.

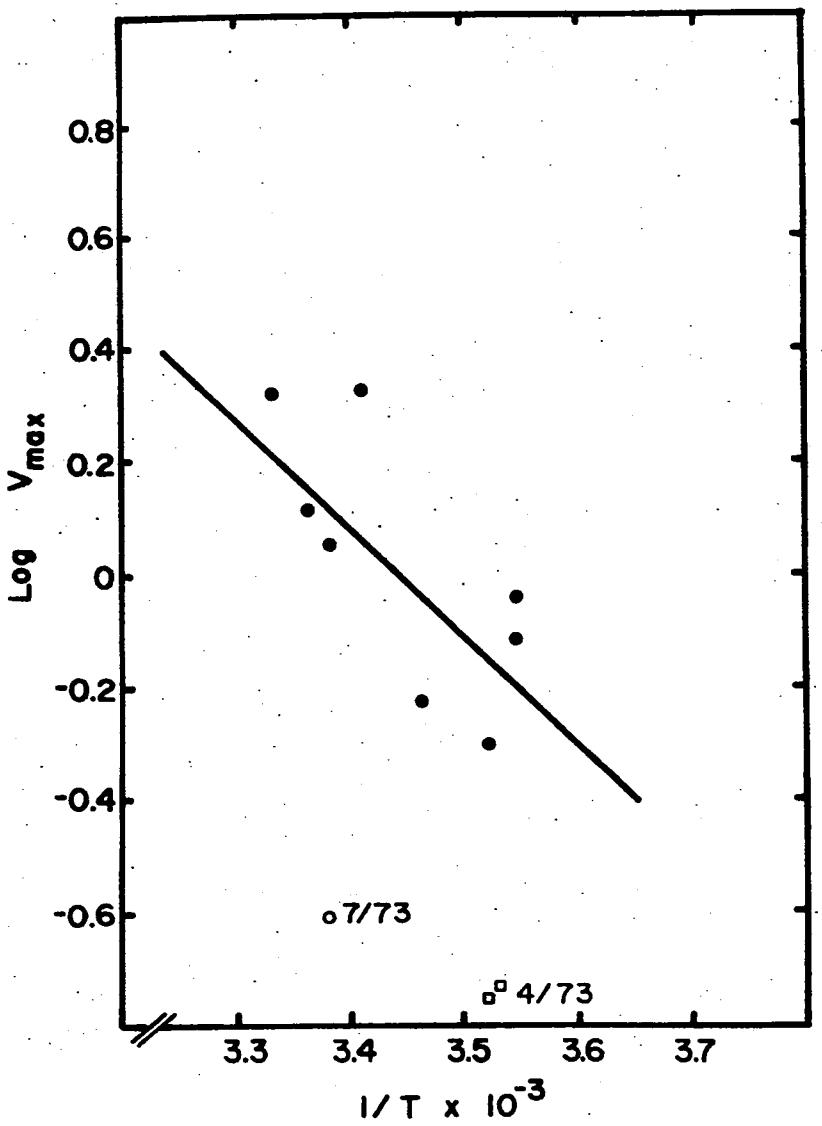
For benthic zone samples, the relationship between log V_{max} and 1/T absolute was significant only for Lake Washington sediment (1%) with all measured values included (Fig. 31). The temperature coefficient was -8.57×10^3 (6.5-8.7°C).

Of the other relationships tested, only log V_{max} and temperature were significant for Lake Chester Morse littoral zone (5%), and the log plate count and temperature (Celsius) was significant for sediments from the littoral zone of Findley Lake (0.1%) with all measured values included (Fig. 32). For every 10 degree rise in temperature (Celsius), the bacterial plate count increased by one log unit.

A summary of the linear regression analysis is given in Table 22. More of the relationships were significant for data from sediments collected in the littoral zone than corresponding data from benthic zone sediments. Only data from the benthic zone of Lakes Washington and Sammamish were significant. Data points excluded from the analysis for both Lake Washington littoral (log turnover time versus 1/T absolute) and Lake Sammamish littoral (log V_{max}

Figure 29. Arrhenius Plot for Sediments from Lake Sammamish Littoral Zone (Log V_{max} Versus 1/T abs). Values for 4/10/73, 4/26/73 and 7/13/73 were not included in the linear regression analysis. Sediment collected from Sept. 1972 to Dec. 1973 and analyzed at the in situ temperature.

Figure 30. Arrhenius Plot for Sediments from Findley Lake Littoral Zone (Log V_{max} Versus 1/T abs). Sediments collected from July 1972 to Feb. 1974 and analyzed at the in situ temperature.


0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
Log V_{max}
o 7/73
□ 4/73
3.3
3.4
3.5
3.6
3.7
1/T x 10^{-3}

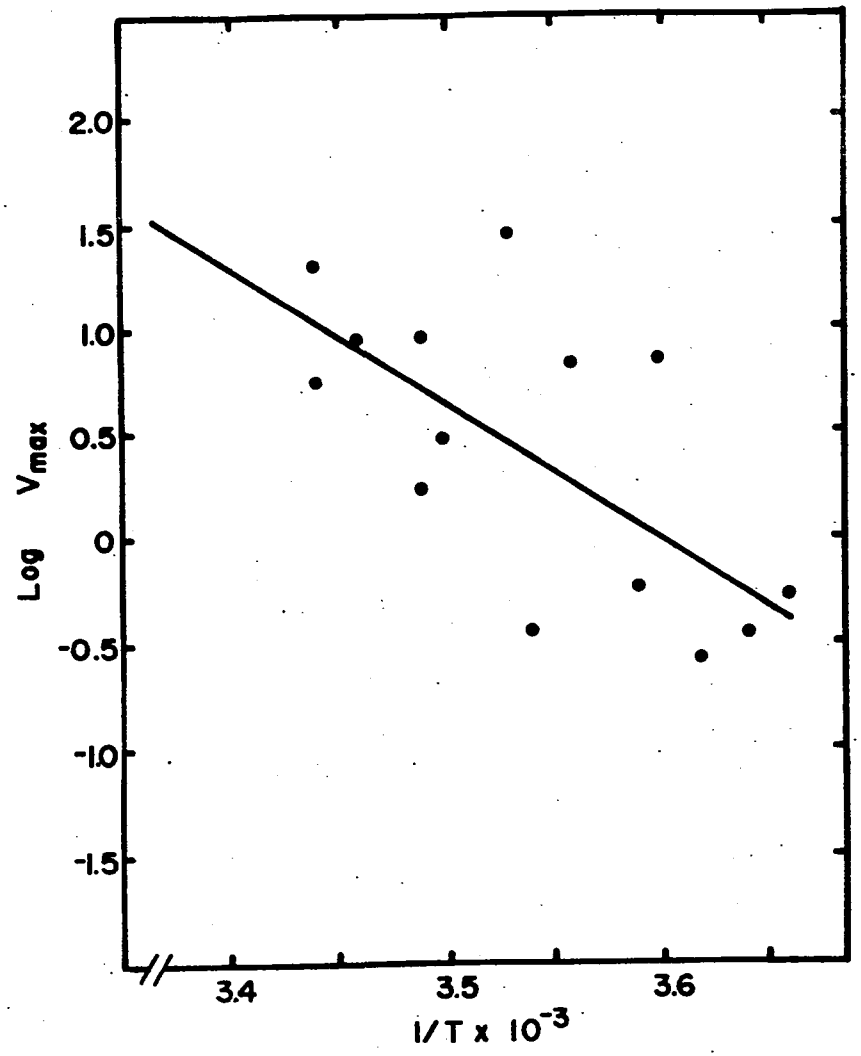
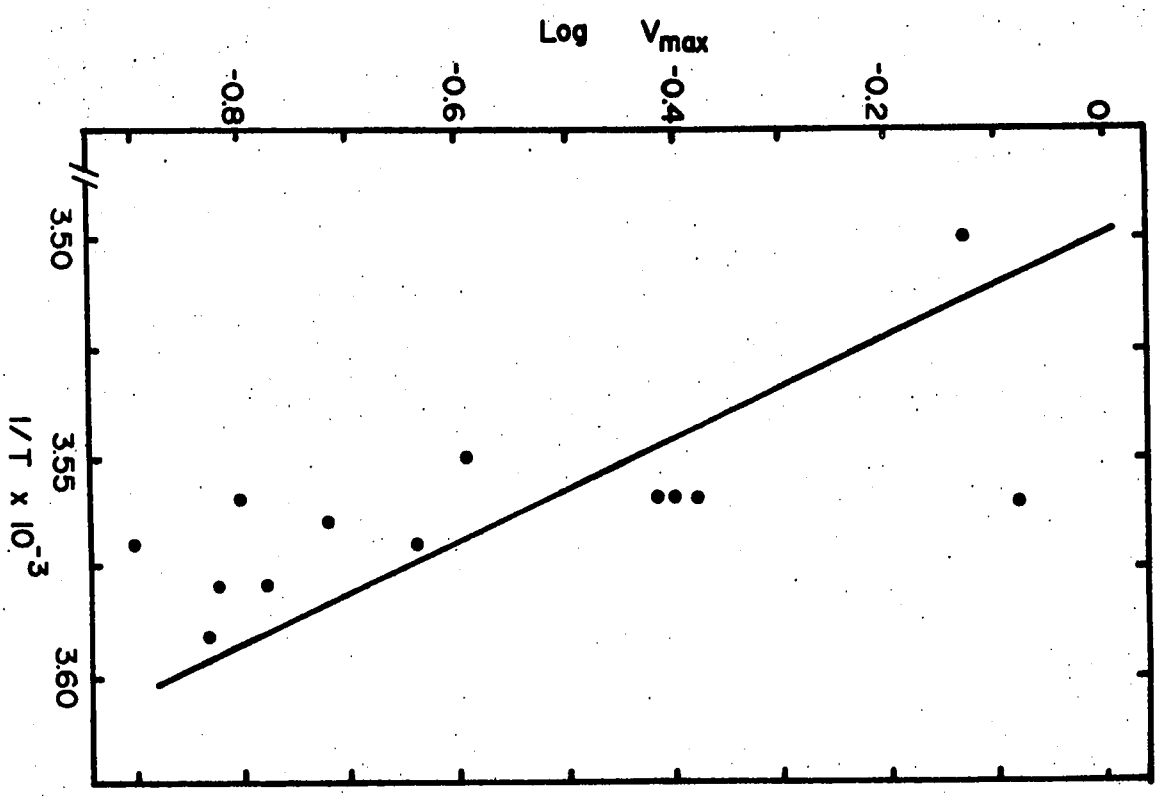

2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
Log V_{max}
3.4
3.5
3.6
1/T x 10^{-3}

Figure 31. Arrhenius Plot for Sediment from Lake Washington Benthic Zone (Log V_{max} Versus 1/T abs). Sediment collected from Sept. 1972 to April 1974 and analyzed at the in situ temperature.

Figure 32. Log Bacterial Plate Count (Organisms/gm dry weight sediment) as a Function of Temperature (Celsius) for Findley Lake Littoral Zone Sediment. Sediment samples collected from July 1972 to Sept. 1974, inoculated on FW agar and incubated at the approximate in situ temperature.


Log V_{max}
-0.8
-0.6
-0.4
-0.2
0
3.50
3.55
3.60
1/T x 10^{-3}

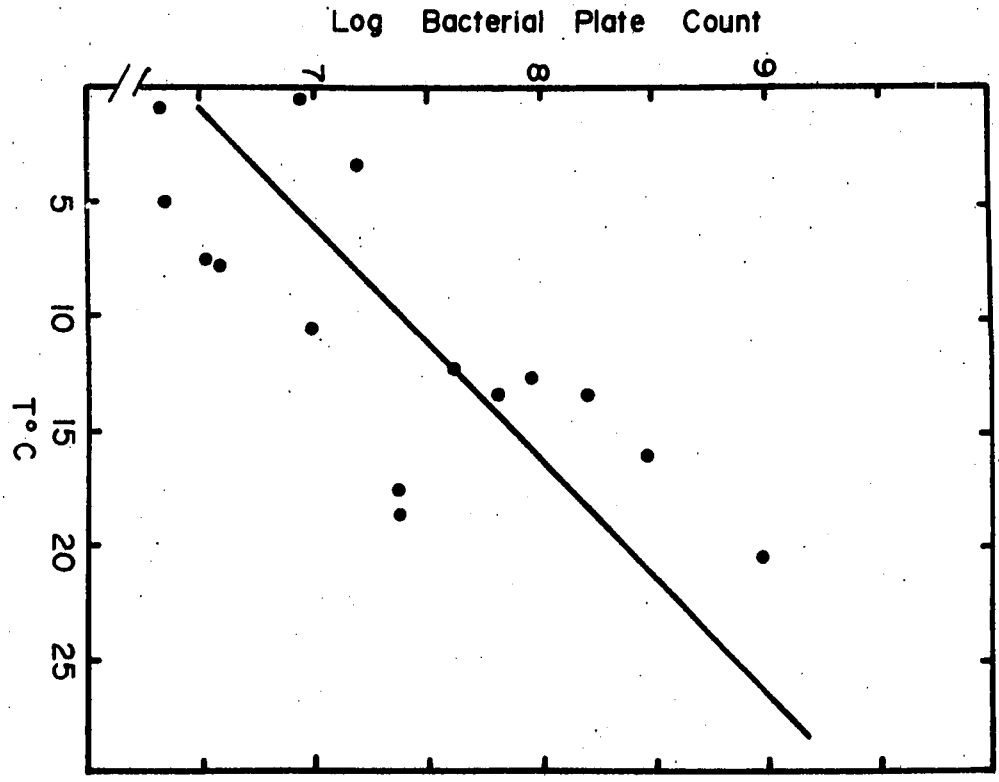

Log Bacterial Plate Count
7
8
9
5
10
15
20
25
T°C

Table 22. Relationships for Glucose Mineralization Rate Data, Temperature, and Log Bacterial Plate Count for Sediments Collected and Analyzed at the In Situ Temperature for the Four Lakes in the Study Group

Parameter	Station	Lake[1]	r^2	No. of Samples	$a_{(1)}$[3]	Significance[4]
Log turnover-time versus 1/T absolute	littoral	W	0.7028	11	3.334×10^3	5.0
		S	0.6574	11	0.310×10^3	5.0
		CM	0.6163	13	4.400×10^3	5.0
		F	0.4344	14	--	NS
	benthic	W	0.3776	12	--	
		S	0.7967	14	8.250×10^3	0.1
		CM	0.3955	14	--	NS
		F	--	16	--	NR
Log V_{max} versus 1/T absolute	littoral	W	0.0643	12	--	NS
		S	-0.7445	8	-2.05	5.0
		CM	-0.4033	13	--	NS
		F	-0.6699	14	-6.4	1.0
	benthic	W	-0.6817	13	-8.57	1.0
		S	-0.5449	13	--	NS
		CM	-0.1357	16	--	NS
		F	--	16	--	NR
Log bacterial plate count versus T, C	littoral	W	0.4522	19	--	NS
		S	--	11	--	NR
		CM	0.3957	12	--	NS
		F	0.8131	15	0.102	0.1
Log bacterial plate count versus T, C	benthic	W	--	14	--	NR
		S	0.3325	14	--	NS
		CM	0.4495	12	--	NS
		F	--	14	--	NR

(Continued)

Table 22 (continued)

Parameter	Station	Lake[1]	r^2[2]	No. of Samples	$a_{(1)}$[3]	Significance[4]
Log bacterial plate count versus turnover time	littoral	W	-0.5399	13	--	NS
		S	--	11	--	NR
		CM	--	13	--	NR
		F	--	14	--	NR
	benthic	W	0.0394	13	--	NS
		S	--	14	--	NR
		CM	-0.080	14	--	NS
		F	--	14	--	NR
Log bacterial plate count versus log V_{max}	littoral	W	--	13	--	NR
		S	--	11	--	NR
		CM	0.6571	12	0.524	5.0
		F	0.3331	13	--	NS
	benthic	W	0.3010	13	--	NS
		S	-0.2074	14	--	NS
		CM	-0.2583	15	--	NS
		F	--	16	--	NR

[1]Lake Washington (W), Lake Sammamish (S), Lake Chester Morse (CM), Lake Findley (F).

[2]Correlation coefficient.

[3]Slope of the line.

[4]Not significant (NS); not a relationship and no statistical analysis made (NR).

versus 1/T absolute) were those collected in April. An additional data point from July 1973 was also deleted for Lake Sammamish. In April, the light intensity is increased and vernal algal outbursts occur. These data suggest that perhaps factors other than temperature, such as nutrient from algal blooms may be influencing the glucose mineralization. (This will be discussed at greater length in the Discussion section.)

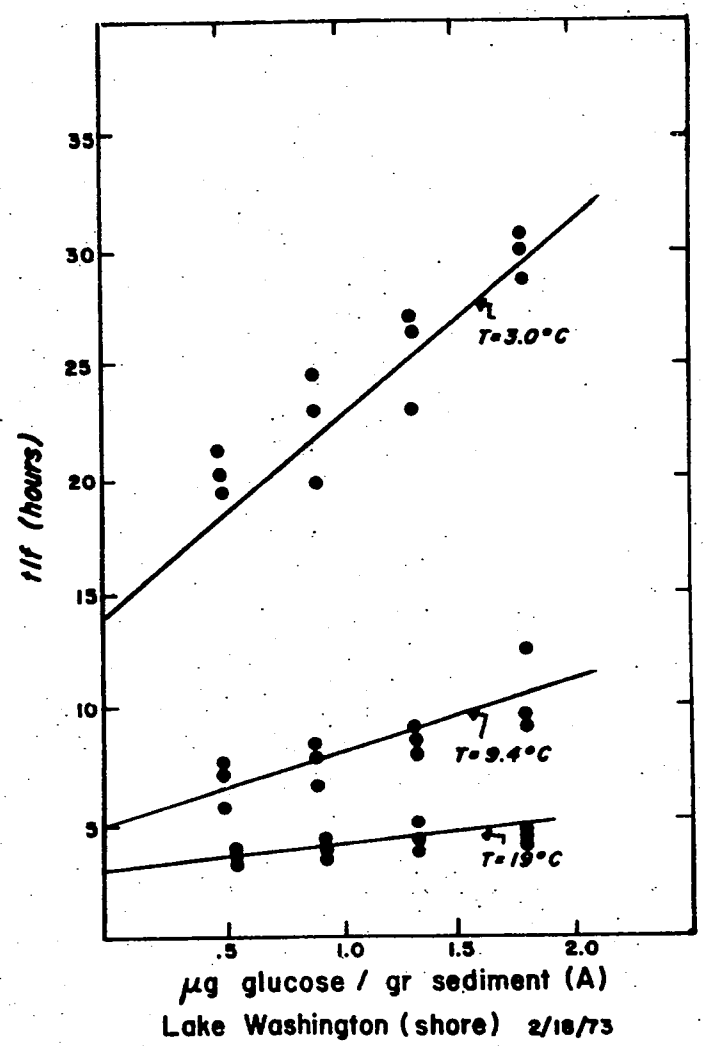
Glucose turnover times were found to be indirectly related to the in situ temperature for the littoral zone sediment of all the lakes but Findley. The response to temperature fluctuations on the basis of temperature coefficients was similar for sediment data from Lakes Washington and Chester Morse; however, Lake Sammamish had a lower temperature coefficient than the other 2 lakes. The inverse relationship between turnover time and in situ temperature was significant for benthic sediment only for Lake Sammamish and the response to temperature was greater than that calculated for the littoral zone of this lake.

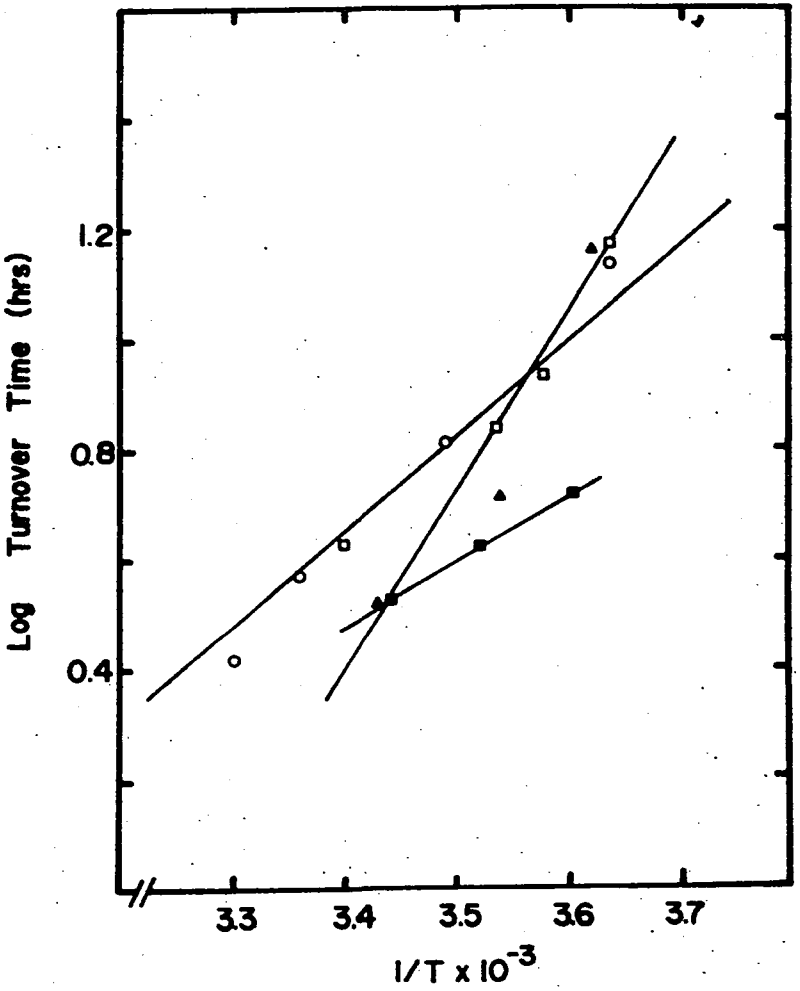
Temperature and Glucose Mineralization Rate-Experimental

When sediment samples were incubated at increasing temperatures, the reaction was faster as demonstrated by Figure 33 for sediment collected from the Lake Washington littoral zone. This increase in V_{max} and decrease in glucose turnover time with increasing temperature of incubation occurred with all of the sediment samples analyzed at increased temperatures. Temperature coefficients were calculated from Arrhenius plots for glucose turnover times measured for the Lake Washington littoral zone at various temperatures with sediment samples collected in winter, summer and fall (Fig. 34). In February 1973, the temperature coefficient of 3.33×10^3 was similar to that measured for seasonal samples analyzed at the in situ temperature (3.34×10^3). The temperature coefficient calculated for samples collected and analyzed in June 1973

Figure 33. t/f Versus A for Sediment from the Littoral Zone of Lake Washington in Feb. 1973 and Analyzed at 3.0, 9.4, and 19.0°C.

Figure 34. Arrhenius Plots for Sediments from Lake Washington Littoral Zone in Feb. 1973 ▲ , June 1973 ■ , Aug. 1974 □ , and Oct. 1974 ○ , and Analyzed at Various Temperatures (Log Glucose Turnover Time Versus 1/T abs).


1/f (hours)
35
30
25
20
15
10
5
T=3.0°C
T=9.4°C
T=13°C
.5
1.0
1.5
2.0
μg glucose / gr sediment (A)
Lake Washington (shore) 2/18/73


Log Turnover Time (hrs)
1.2
0.8
0.4
3.3
3.4
3.5
3.6
3.7
1/T x 10^-3

was 1.15×10^3, and for August and October 1974 samples the value was 1.81×10^3.

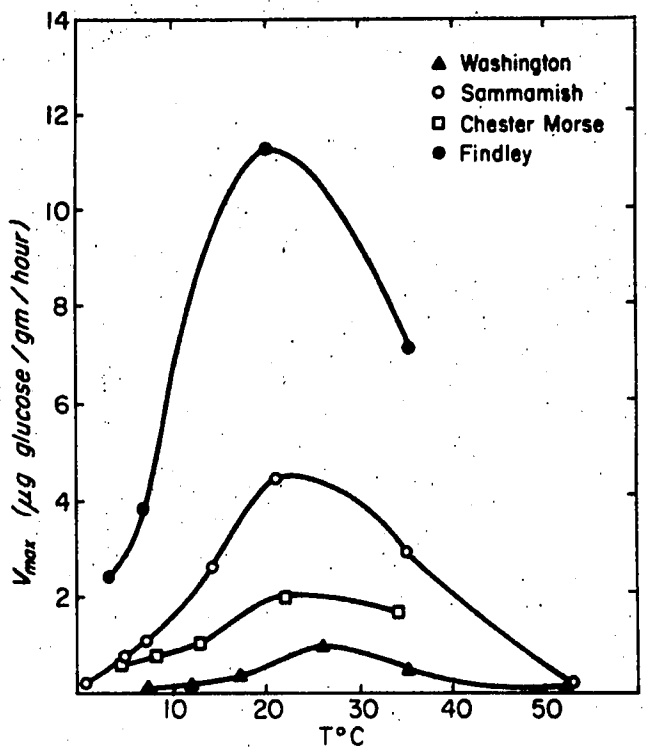
Sediment from the benthic stations from the 4 lakes were collected in February 1973 and analyzed at temperatures up to 50°C, temperatures never encountered in this region. When V_{max} values were plotted against temperature of incubation, a bell-shaped curve was obtained (Fig. 35). The maximum rate or optimum temperature for glucose mineralization occurred between 20 and 30°C, temperatures higher than normally encountered at these depths. The V_{max} values obtained for the Findley Lake samples were unusually high for the month of February, when compared to the other 3 lakes. Bacteria associated with the sediment from Findley Lake were most responsive to temperature, followed by Lakes Sammamish, Chester Morse, and Washington. When glucose turnover time was plotted against temperature of incubation, the lakes showed a similar relationship as found for littoral samples, analyzed at the in situ temperature (Fig. 36). Below 10°C, Findley Lake sediments had more rapid rates or shorter glucose turnover times, followed by Chester Morse, Washington, and Sammamish.

Benthic samples from Lakes Washington, Chester Morse, and Findley were analyzed in the same manner in September 1974. Findley Lake samples were not as sensitive to temperature as in February and the temperature optima for this lake appeared slightly shifted from 20 to 23°C of February to 25 to 30°C in September (Fig. 37). No shift in temperature optimum was observed for sediment from the other two lakes.

Lake Washington littoral zone samples were collected in August and October in 1974 and analyzed at varying temperatures (Fig. 38). The optimum temperature for glucose mineralization using V_{max} values, occurred at 30°C for both August and October samples.

Figure 35. V_{max} Values as a Function of Temperature for Samples from Benthic Stations in Feb. 1973. Lake Sammamish sample collected in April 1973.

Figure 36. Glucose Turnover Times as a Function of Temperature for Samples from Benthic Stations in Feb. 1973. Lake Sammamish sample collected in April 1973.


Washington
Sammamish
Chester Morse
Findley
V_{max} (µg glucose / gm / hour)
T°C

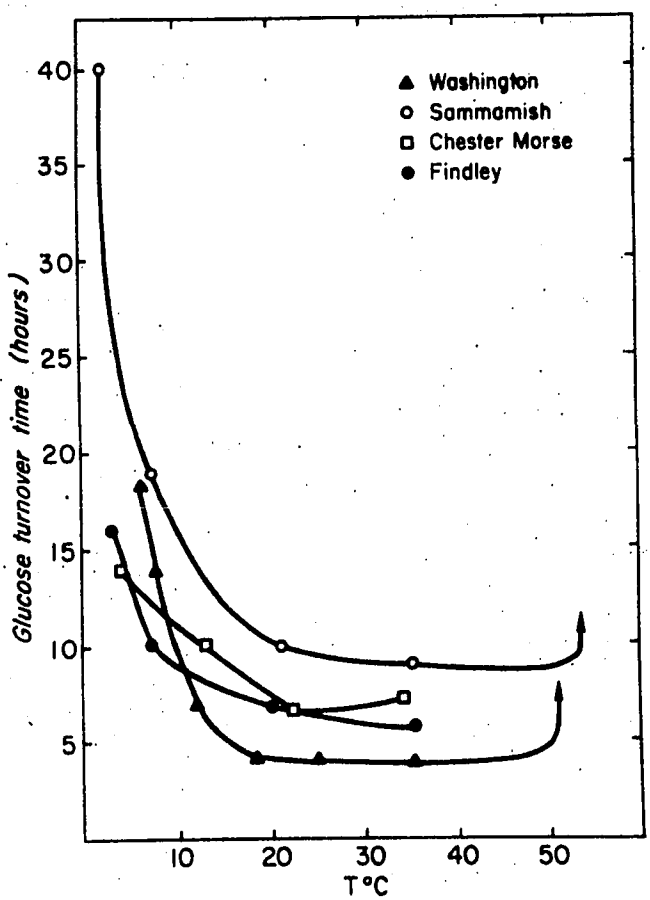
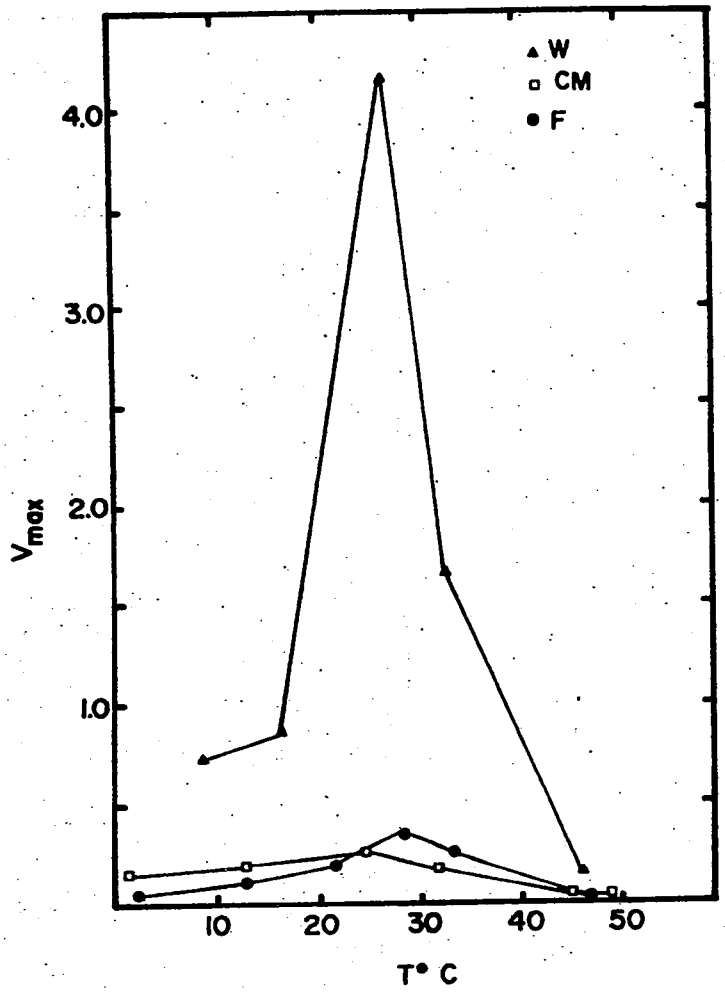
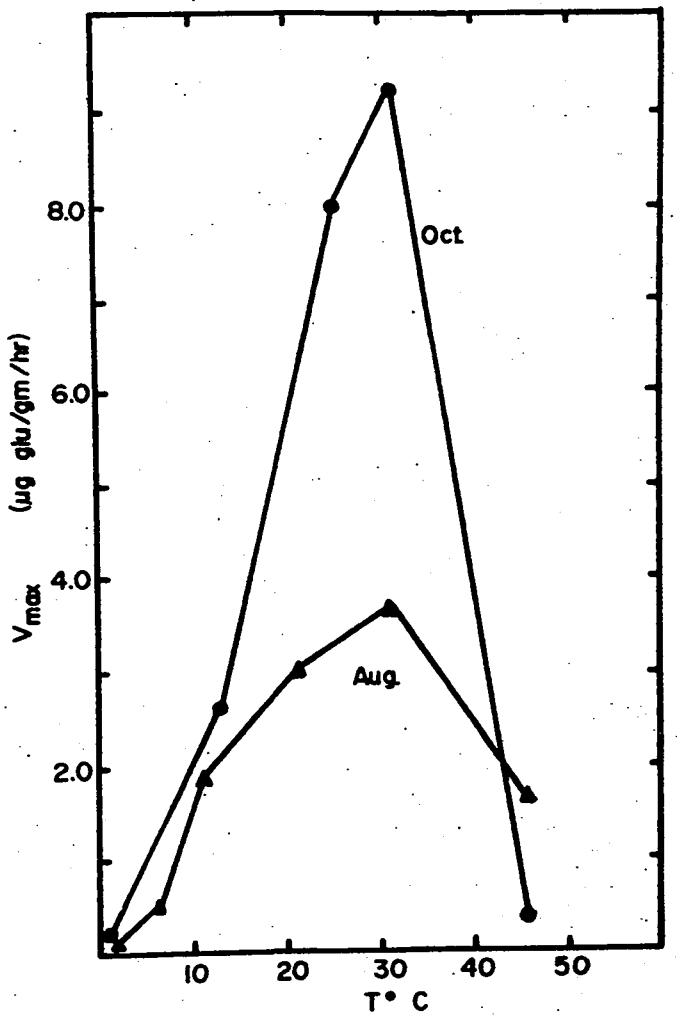

Washington
Sammamish
Chester Morse
Findley
Glucose turnover time (hours)
T°C

Figure 37. V_{max} Values as a Function of Temperature for Samples from Benthic Stations in Sept. 1974. (▲ Lake Washington (W), □ Lake Chester Morse (CM), and ● Findley Lake (F).)

Figure 38. V_{max} Values as a Function of Temperature for Samples from the Littoral Zone in Lake Washington in Aug. and Oct. 1974.


▲ W
□ CM
● F
4.0
3.0
2.0
1.0
V_{max}
10
20
30
40
50
T° C


8.0
6.0
4.0
2.0
V_{max} (µg glu/gm/hr)
Oct
Aug.
10
20
30
40
50
T° C

Temperature and Bacterial Culture Studies

Sediment samples were collected from the 4 lakes; duplicate FW agar spread plates were inoculated and incubated under aerobic conditions at various temperatures. Bacterial plate counts were usually higher when plates were incubated at temperatures higher than 4°C. Decreases in bacterial plate counts occurred for plates incubated at 37°C. Colonies always grew much faster at 22°C than at 15 or 4°C. Colonies appearing on plates initially incubated at 4°C, yielded 87 to 100% organisms able to grow at 22°C. Of the colonies appearing on plates initially incubated at 22°C, 29 to 71% of the organisms did not grow at 4°C. All organisms isolated from sediments and initially cultivated at 4°C, grew better on subsequent incubation at 22°C.

A total of 8 sediment samples were taken from the littoral, 4 from the benthic zone in Lake Washington, and inoculated spread plates were incubated at the 3 temperatures (Table 23). For benthic samples, counts were the same or slightly increased with increasing temperature of incubation. Increases in plate counts with temperature were observed with the littoral samples collected from August 1974 to January 1975. For example, the October sediment plate count for 4, 15 and 22°C was 1.7, 730, and 1300 x 10^6 organisms/gm, respectively. Little or no increase in plate counts occurred for littoral samples tested monthly from February to May 1975. Decreases in plate count occurred when samples were incubated at 37°C. For benthic zone samples, 91% of the samples initially grown at 4°C and cultured at 22°C grew; however, only 29% of the bacteria initially grown at 22°C grew at 4°C (Table 24). For littoral samples tested monthly from February to May 1974, 100% of the organisms initially grown at 4°C grew on subsequent incubation at 22°C; 56 to 80% of the organisms initially grown at 22°C grew at 4°C (Table 24).

Table 23. Bacterial Plate Counts of Sediment from the Four Lakes and Incubated at Various Temperatures. Incubation times: 4°C, 42 days; 15°C, 20 days; 22°C, 12 days; 37°C, 3 days.

Lake/Station	Date	In Situ T°C	Plate Count x 10^6 at* Designated Temperatures 4°C	15°C	22°C	37°C
Washington, benthic	1/25/74	6.5	4.6	14.0	13.0	-
	4/ 4/74	6.6	30.0	11.0	-	-
	4/25/74	7.0	-	47.0	220.0	-
	9/24/74	6.6	3.8	24.0	44.0	-
Washington, littoral #2	8/22/74	20.0	7.6	78.0	950.0	-
	10/ 1/74	15.0	1.7	730.0	1300.0	-
	11/ 5/74	13.5	4.4	40.0	47.0	-
	1/ 2/75	7.0	3.8	21.0	19.0	-
	2/ 4/75	7.0	61.0	66.0	63.0	-
	3/25/75	9.0	57.0	58.0	72.0	-
	4/25/75	10.5	380.0	470.0	560.0	63.0
	5/15/74	15.0	140.0	250.0	700.0	17.0
Sammamish, benthic	2/ 5/74	5.8	15.0	52.0	53.0	-
	3/ 6/74	6.0	22.0	49.0	-	-
	4/ 3/74	6.0	23.0	-	99.0	5.1
Chester Morse, benthic	1/30/74	4.0	66.0	23.0	24.0	-
	9/ 5/74	7.6	1.5	2.4	2.6	-
Chester Morse, littoral	2/28/73	3.0	21.0	-	37.0	-
	2/12/74	3.2	24.0	120.0	140.0	-
	9/ 5/74	13.2	1.3	11.0	6.7	-
Findley, benthic	2/28/74	4.0	1.3	4.2	6.3	-
	9/19/74	4.5	1.0	1.9	2.2	-
Findley, littoral #2	2/28/74	1.0	2.0	16.0	14.0	-
	9/19/74	12.9	45.0	13.0	120.0	-

*Values are counts per gram dry weight sediment and represent mean counts from replicate plates (FW agar) incubated aerobically.

Table 24. Aerobic Growth of Cultures of Bacteria Isolated from Lake Sediments. Incubation times: 4°C, 48 days; 22°C, 12 days.

Lake/Station	Date	In Situ T°C	Initial Growth Temperature °C	Experimental Growth Temperature °C	Growth at Experimental Temperature %*
Washington, benthic	1/25/74	6.5	4	22	91
			22	4	29
Washington, littoral	2/ 4/75	7.0	4	22	100
			22	4	75
	3/25/75	9.0	4	22	100
			22	4	75
	4/25/75	10.5	4	22	100
			22	4	56
	5/25/75	15.0	4	22	100
			22	4	80
Sammamish, benthic	2/ 6/74	5.8	4	22	100
			22	4	50
	3/ 6/74	6.0	4	22	100
	4/ 3/74	7.0	4	22	100
Chester Morse, benthic	1/30/74	4.0	4	22	100
			22	4	54
Chester Morse, littoral #2	2/12/74	3.2	4	22	88
			22	4	38
Findley, benthic	2/28/74	4.0	4	22	100
			22	4	69
Findley, littoral #2	2/28/74	3.0	4	22	92
			22	4	61

*Values in % represent 25 subcultures isolated from inoculated plates incubated at one temperature and subcultured at the other. Subcultures grown at the initial incubation temperature served as controls.

A total of 3 sediment samples were taken from the benthic zone of Lake Sammamish for this study. Little or no difference in bacterial numbers occurred with plates incubated at the 3 temperatures for the months of February, March, and April of 1974 (Table 23). Bacterial numbers decreased from 10^7 organisms/gm at 22°C to 10^6 organisms/gm at 37°C. All colonies tested appearing on plates initially grown at 4°C, yielded organisms able to grow at 22°C; 50% of the cultures originally grown at 22°C, grew at 4°C (Table 24).

From Lake Chester Morse, 2 samples from the benthic zone and 3 from the littoral zone were tested. Benthic zone samples collected in January 1974 showed an increase in plate count from 10^6 organisms/gm at 4°C to 10^7 organisms/gm at 15°C with no further increases in numbers at 22°C (Table 23). Benthic samples collected in September 1974 showed no increase in bacterial biomass with increasing temperature of incubation and remained at 10^6 organisms/gm. Of the three samples collected from the littoral zone, two increased at 15°C, one of these decreased and one remained constant at 22°C. The other sample, collected February 1973, showed no change in plate count with increasing incubation temperature. For benthic zone sediment, all of the colonies tested originally grown at 4°C, grew at 22°C; 54% of the colonies grown at 22°C also grew at 4°C (Table 24). For littoral zone sediment, 87% of colonies originally grown at 4°C grew at 22°C; 37% of colonies initially grown at 22°C grew at 4°C.

For Findley Lake, 2 samples from the 27 m station and 2 from the littoral (No. 2) station were tested (Table 23). Samples from the benthic zone showed no increases in bacterial plate count with increasing temperature of incubation. Samples from the littoral zone collected in February 1974, increased from 10^6 organisms/gm at 4°C, to 10^7 organisms/gm at 15°C and remained at that level at 22°C. Littoral samples collected in September 1974 were 10^7

organisms/gm at 4°C and 15°C, and increased to 10^8 organisms/gm at 22°C. Of colonies originally grown at 4°C, 92% and 100% grew at 22°C for littoral and benthic samples, respectively; of colonies initially grown at 22°C, 61% and 68% grew at 4°C for littoral and benthic samples, respectively (Table 24).

Cellulolytic Bacteria

The microbial decomposition of cellulose can represent a further source of glucose and eventually carbon in the aquatic environment. Therefore, a search for cellulolytic bacteria in the sediments of the 4 lakes was instigated.

Cellulolytic bacteria in lake sediments. Sediments from the 2 main sampling stations in each of the 4 lakes were inoculated on FWC and MM + cellulose agars and incubated aerobically and anaerobically at the approximate in situ temperature. Cellulolytic bacteria were found on inoculated plates from sediment collected from the littoral zone of Lake Washington, and at both stations in Lake Sammamish.

Cellulolytic bacteria isolated in April and August of 1973 from Lake Washington littoral zone sediment comprised 1.3 and 2.4%, respectively, of the total anaerobes counted; the organisms numbered 10^4 organisms/gm sediment. Samples from Lake Sammamish collected in July 1973 were 2.5 and 1.2% of the total anaerobes in the littoral and benthic stations, respectively, with a total number of 10^3 organisms/gm for both stations. All of the cellulolytic organisms detected were gram positive rods, and were obligate anaerobes. No aerobic cellulolytic organisms were found using the cellulose-agar zone clearing detection method.

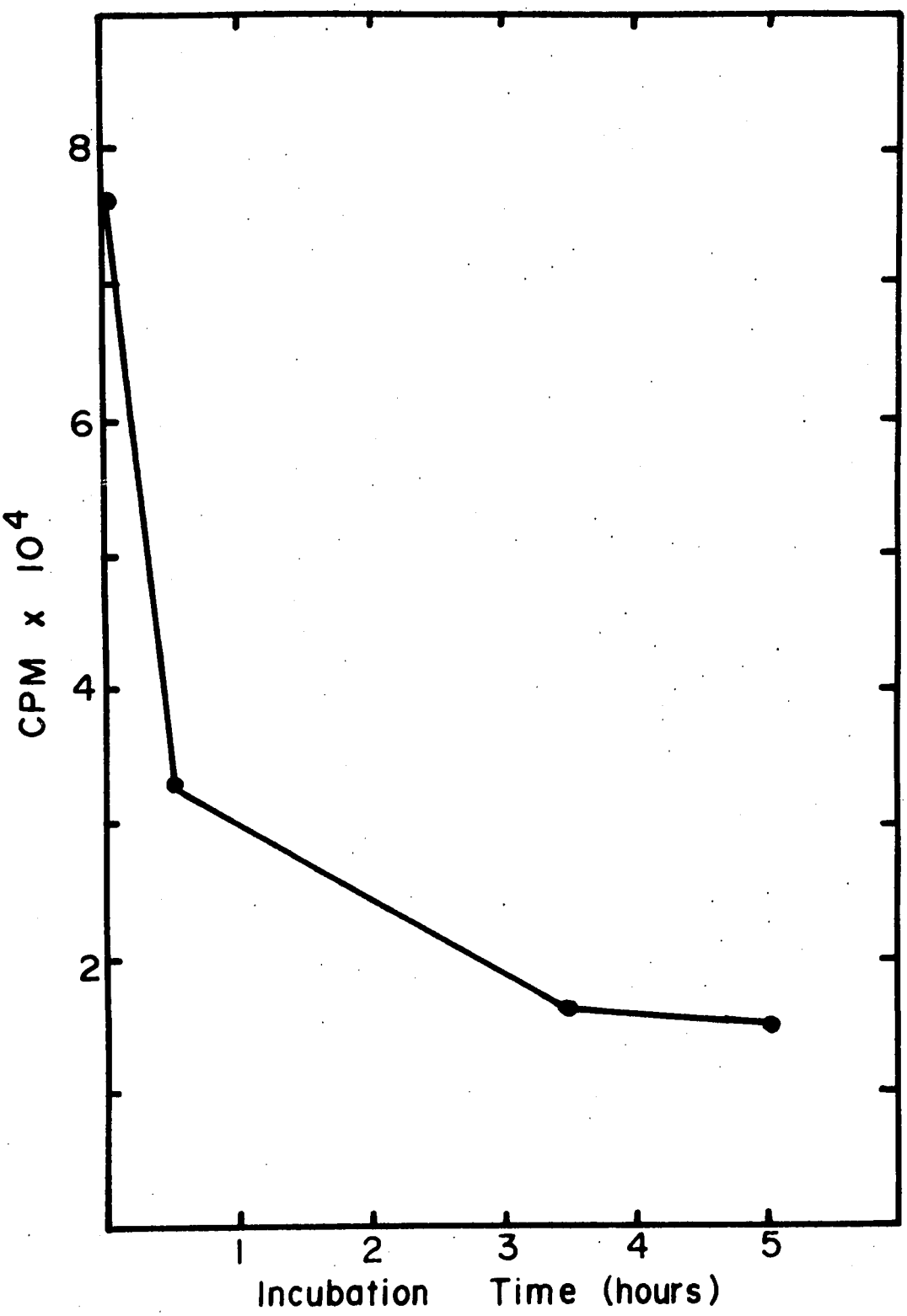
Attempts were made to grow the cellulolytic bacteria in pure culture; however, this proved to be extremely difficult. In many cases, the obligate

anaerobes were growing with a gram negative facultative organism, and the two were difficult to separate. The facultative organism was unable to digest the cellulose, when isolated, and diminished cellulolytic activity occurred when the obligate anaerobe was tested in pure culture.

When sediment samples from the Lake Washington littoral station were incubated at 15°C with sterile strips of cigarette papers, the papers grew noticeably thinner and eventually disappeared after 6 months; however, no aerobic or anaerobic cellulolytic organisms were ever successfully isolated from these tubes.

Incubation of sediment with ^{14}C cellulose. Sediment samples from the Lake Washington littoral zone were incubated at 13°C (unshaken) and 29°C (shaken) with 0.1 μCi/10 ml sediment slurry for incubation periods up to 5 hours. After timed intervals the supernatant was counted in a scintillation counter to determine if sediment bacteria had hydrolyzed the cellulose to soluble labeled products. Instead of the anticipated increase in soluble ^{14}C products of cellulose hydrolysis, the counts decreased (Fig. 39) in all samples, reaching equilibrium (15.7×10^3 CPM) after 5 hours. Samples incubated at 29°C (with shaking) had even greater decreases after 5 hours (4.4×10^3 CPM). The ^{14}C stock suspension of cellulose when centrifuged and supernatant counted had 760×10^3 CPM. Apparently, microorganisms in the sediment preferentially metabolized the soluble material present in the ^{14}C labeled cellulose suspension. The labeled cellulose was washed with sterile water and centrifuged repeatedly with little decrease in the supernatant radioactivity. Perhaps if sediment samples had been incubated for longer time periods, after all of the soluble labeled material was metabolized, microorganisms would have hydrolyzed the cellulose, and the supernatant would have increased in activity. The study of cellulolytic bacteria in lake sediments was discontinued at this point and greater effort was spent on the isolation and study of chitinoclastic bacteria.

Figure 39. Counts per Minute (CPM) with Time of Incubation of Lake Washington Littoral Zone Sediment Incubated with ^{14}C-labeled Cellulose at 13°C. Counts are those of supernatant after centrifugation and represent the mean of 3 replicate samples.


8
6
4
2
CPM x 10^4
1
2
3
4
5
Incubation Time (hours)

PART II

CHITINOCLASTIC BACTERIA

Chitin from the exoskeletons of fungi, crustaceans, and insects can represent a considerable amount of carbon and nitrogen in the aquatic ecosystem. The breakdown of this refractory material is thought to be mediated chiefly by microorganisms. Like cellulose, chitin is hydrolyzed to glucose and therefore represents a further source of glucose to the aquatic environment. Although the microbial breakdown of chitin has been thoroughly investigated in the marine environment, very little is known about the microorganisms responsible for chitin degradation in fresh water lakes. Therefore, a search for chitinoclastic bacteria in the sediments of the 4 lakes was conducted. The effects of substrate enrichment and temperature on lake sediment samples was determined. Several representative chitinoclastic organisms were isolated and experiments were conducted in the laboratory with these cultures to determine the effects of temperature and presence of end products on microbial chitin decomposition. Finally, several aspects of chitin decomposition using cell-free extracts prepared from pure cultures of chitinoclastic bacteria was studied.

Seasonal Abundance of Chitinoclastic Bacteria in Four Lakes

Chitinoclastic bacteria are defined in this study as those bacteria demonstrating hydrolysis of reprecipitated chitin. Hydrolysis, degradation, digestion, and activity are terms used interchangeably here to describe the action of these chitinoclastic bacteria.

Chitinoclastic bacteria were found in both littoral and benthic sediments in all 4 lakes in the study group. In Lake Washington littoral sediment, chitin digesting bacteria varied from 10^4 to 10^6 organisms/gm and comprised from

0.6 to 7.0% of the total heterotrophic population (Table 25). In benthic samples, these organisms varied from 10^4 to 10^6 organisms/gm and comprised from 0.2 to 26.0% of the bacterial population. Little seasonal variation in abundance of chitinoclastic bacteria was observed for Lake Washington, although a tendency existed for slightly higher numbers of these organisms in the warmer summer months.

In Lake Sammamish, the numbers of chitinoclastic bacteria ranged from 10^5 to 10^6 organisms/gm and these represented from 0.7 to 6.0% of the total heterotrophs counted for littoral and 1.5 to 4.6% for benthic samples (Table 26). There was little seasonal variation in abundance of these organisms in Lake Sammamish.

Higher percentages and numbers of chitinoclastic bacteria were found at the Lake Chester Morse littoral station than at any other station of the lakes sampled. The numbers ranged from 10^5 to 10^7 organisms/gm and comprised from 3.0 to 80.0% of the population (Table 27). In the benthic zone, chitinoclasts ranged from 10^4 to 10^5 organisms/gm and represented from 0.8 to 14.5% of the total heterotrophic population counted. Highest numbers and percentages of chitinoclastic bacteria occurred in the winter months at the littoral station, and in July and November at the benthic station. The exoskeletons of living crayfish collected in Lake Chester Morse were found to contain large populations of chitinoclastic bacteria. Quantitative evaluations of their numbers were not made.

In Findley Lake, the numbers of chitinoclastic bacteria at littoral station No. 2 ranged from 10^5 to 10^6 organisms/gm and represented from 0.4 to 15.0% of the population counted (Table 28). For the benthic station, the numbers ranged from 10^4 to 10^5 organisms/gm when present and represented from 1.2 to 40.0% of the total heterotrophic population, a higher percentage than that found

Table 25. Chitinoclastic Bacteria in Lake Washington Sediments

Station	Date	In Situ T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
Benthic	7/31/73	8.0	1.2	0.67
	9/25/73	8.1	ND	-
	11/20/73	8.5	1.7	1.70
	1/25/73	6.5	2.3	0.22
	2/ 4/75	6.6	0.3	0.06
	3/25/75	7.0	1.6	4.00
	9/24/74	6.6	26.0	2.20
Littoral	1/23/73	6.0	1.5	0.01
	2/18/73	9.0	3.1	5.20
	4/10/73	10.5	2.8	3.70
	6/26/73	18.5	1.5	1.60
	8/20/73	19.0	7.1	18.00
	11/ 8/73	9.8	0.6	1.90
	8/22/74	20.0	3.0	1.00
	10/ 1/74	15.0	7.0	3.30
	11/ 5/74	13.5	2.0	0.73
	1/ 2/75	7.0	2.0	0.29
	2/ 4/75	7.0	1.2	0.80
	3/25/75	9.0	4.2	0.25
	4/25/75	10.5	3.1	3.20
	5/15/75	15.0	1.9	6.30

*ND, chitinoclastic bacteria not detected.

**Values are counts per gram dry weight sediment and represent mean of duplicate samples inoculated on FWCh agar and incubated at the approximate in situ temperature.

Table 26. Chitinoclastic Bacteria in Lake Sammamish Sediments

Station	Date	In Situ T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
Benthic	4/13/73	6.2	ND	-
	8/ 2/73	7.9	1.5	0.20
	8/21/73	7.8	ND	-
	2/ 5/73	5.8	1.6	0.48
	3/ 6/74	6.0	4.6	2.20
	4/ 3/74	6.0	2.3	2.50
Littoral	4/13/73	11.2	2.6	0.15
	8/ 2/73	23.0	6.0	1.50
	8/21/73	20.5	0.7	2.60

*ND, chitinoclastic bacteria not detected.

**Values are counts per gram dry weight sediment and represent mean of duplicate samples inoculated on FWCh agar and incubated at the approximate in situ temperature.

Table 27. Chitinoclastic Bacteria in Lake Chester Morse Sediments

Station	Date	In Situ T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
Benthic	4/ 3/73	6.9	14.5	0.95
	7/10/73	7.8	4.6	0.63
	11/13/73	4.0	0.8	0.03
	1/30/73	7.6	ND	-
	9/ 5/73	3.2	ND	-
Littoral	2/28/73	3.0	80.0	17.00
	4/ 3/73	3.2	10.5	0.57
	11/13/73	7.8	72.5	6.80
	2/12/74	3.2	20.6	15.00
	9/ 5/74	13.5	3.1	0.28

*ND, chitinoclastic bacteria not detected.

**Values are counts per gram dry weight sediment and represent mean of duplicate samples inoculated on FWCh agar and incubated at the approximate in situ temperature.

Table 28. Chitinoclastic Bacteria in Findley Lake Sediments

Station	Date	In Situ T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
Benthic	10/12/72	4.9	1.8	0.39
	11/ 1/72	5.0	ND	-
	3/ 5/73	4.0	40.0	4.50
	3/27/73	4.0	33.0	0.43
	7/ 5/73	4.6	1.2	0.06
	8/16/73	4.6	ND	-
	10/ 4/73	5.0	ND	-
	11/30/73	4.0	24.5	0.58
	2/28/74	4.0	16.0	0.17
	9/19/74	4.5	ND	-
Littoral #2	10/12/72	7.8	2.4	2.20
	11/ 1/72	5.0	ND	-
	2/ 9/73	0.5	ND	-
	7/ 5/73	13.5	0.4	0.77
	7/26/73	17.4	ND	-
	8/16/73	16.0	3.1	3.50
	10/ 4/73	12.5	ND	-
	2/28/74	1.0	15.0	0.21
	9/19/74	12.9	8.2	8.00
Littoral #3	7/ 5/73	13.0	4.0	3.60
	8/16/73		8.6	0.75
	7/26/73	23.0	ND	-
	10/ 4/73	12.5	1.8	0.17
Lower Lake #5	7/ 5/73	11.5	2.4	1.70
Littoral #2, H_2O	11/ 1/72	5.0	4.4	20/ml
	2/ 9/73	0.5	10.5	1300/ml

*ND, chitinoclastic bacteria not detected.

**Values are counts per gram dry weight sediment and represent mean of duplicate samples inoculated on FWCh agar and incubated at the approximate in situ temperature.

for littoral zone sediment from this lake. Chitinoclastic bacteria were also found in samples from the lower lake, from littoral station No. 3, and from water samples from littoral station No. 2. Higher numbers of chitinoclastic bacteria and higher percentages occurred in the winter months for the benthic station. The highest percentage (15.0%) of chitinoclastic bacteria found for the littoral zone was in sediment collected in winter (February 1974).

Substrate Enrichment for Chitinoclastic Bacteria

Enrichments for chitinoclastic bacteria were conducted using Lake Washington littoral zone sediment to determine the effects of a number of substrates on the development of these organisms. Various substrates were added to the sediment collected in November 1974, and incubated without shaking at the in situ temperature (10°C). These substrates were end products of chitin hydrolysis, chitin alone, or in combination with organic materials, or other substrates that may have a potential effect upon the hydrolysis of chitin as suggested by data generated in this study and others. Periodically, samples were removed and the effects of the added substrates on the numbers of chitinoclasts, total heterotrophic bacteria, and percentage of chitinoclastic bacteria of the heterotrophic population were determined using duplicate spread plates on FW and FWCh agars. An increase in the percentage of chitinoclastic bacteria in a sample would indicate preferential growth of chitinoclastic bacteria in relation to the total heterotrophic population. For samples collected in November 1974 (Fig. 40), the highest percentage of chitinoclastic bacteria was elicited by FWCh broth and chitin + peptone, with 80% of the total population composed of chitinoclasts after 9 days of incubation with both additions; 40% of the population was composed of chitinoclasts after 9 days in the sample enriched by chitin. In sediment enriched with peptone, 33% of the population were chitinoclastic bacteria after 4 days. No enrichment

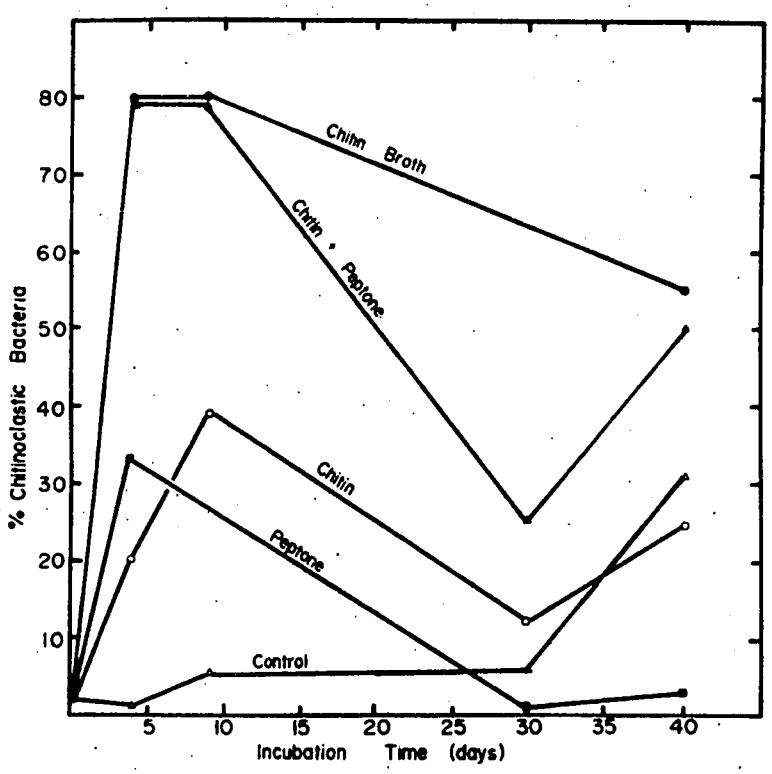
occurred with glucose, glucose + $CaCO_3$, cellulose, $CaCO_3$, and N-acetyl glucosamine (NAG), for up to 30 days of incubation. In all samples the highest percentage of chitinoclasts were reached after 9 days incubation with decreases up to 30 days. A slight increase was evident after 38 days incubation.

On the basis of total numbers of chitinoclastic bacteria (Fig. 41), the most rapid increase occurred during the first 9 days of incubation with a slight increase up to 38 days. The sample enriched with chitin + peptone had the highest count of 3.7×10^{12} organisms/gm followed in order of decreasing number: chitin, glucose + $CaCO_3$, FWCh broth, peptone, NAG, and glucose. Cellulose and $CaCO_3$ did not enrich for chitinoclasts, and in fact exerted a slight inhibitory effect.

Results from a second enrichment on a sediment sample collected from the Lake Washington littoral station in January 1975 were similar to the first experiment. The highest percentage of chitinoclastic bacteria occurred at 14 days with: chitin + peptone, 75%; chitin, 45%, and FWCh broth, 22% (Fig. 42). Little effect was observed for samples incubated with glucosamine. The sample enriched with peptone had the highest percentage of chitinoclasts of all substrates tested after 6 days at 64%, but a decrease to 25% occurred after 14 days. On the basis of total numbers of chitinoclasts after 14 days incubation at $10^{o}C$, numbers in order of decreases were: peptone and FWCh broth, 10^{12} organisms/gm; peptone, 10^{11} organisms/gm; chitin, 10^{9} organisms/gm; and glucosamine, 10^{7} organisms/gm (Fig. 43). The control remained at a constant level of 10^{5} organisms/gm up to 14 days and increased to 10^{6} organisms/gm after 30 days of incubation. After 30 days of incubation, both percentages and numbers of chitinoclasts had decreased in all samples when compared to values obtained after 14 days. In the second experiment, chitin broth did not elicit as high a percentage of chitinoclasts as in the first experiment, although total numbers were comparable.

Figure 40. Percentage of Chitinoclastic Bacteria on FWCh Agar After Substrate Enrichment of Lake Washington Littoral Zone Sediment. Substrates included in final concentration: FWCh broth, 10%; chitin, 1 mg % + peptone, 0.2%; chitin, 1 mg %; and control. No enrichment with glucose, glucose + $CaCO_3$, $CaCO_3$, cellulose, and NAG.

Figure 41. Log Plate Count Chitinoclastic Bacteria on FWCh Agar After Substrate Enrichment of Lake Washington Littoral Zone Sediment. Substrates included in final concentration: chitin, 1 mg % + peptone, 0.2%; FWCh broth, 10%; chitin, 1 mg %; glucose, 0.2%; and control. No enrichment with $CaCO_3$, 0.4% or NAG, 0.2%.


% Chitinoclastic Bacteria
80
70
60
50
40
30
20
10
Chitin Broth
Chitin + Peptone
Chitin
Peptone
Control
5
10
15
20
25
30
35
40
Incubation Time (days)

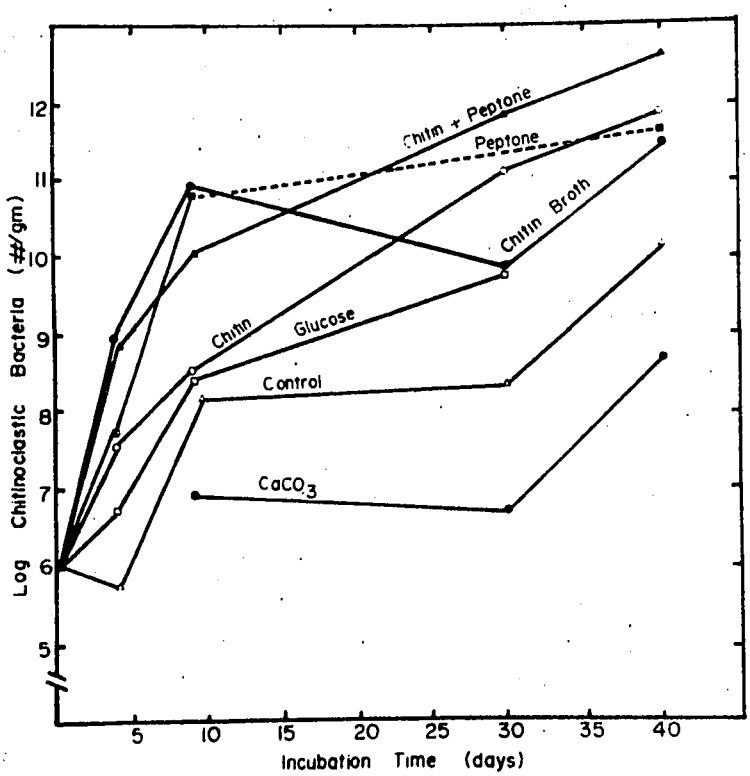
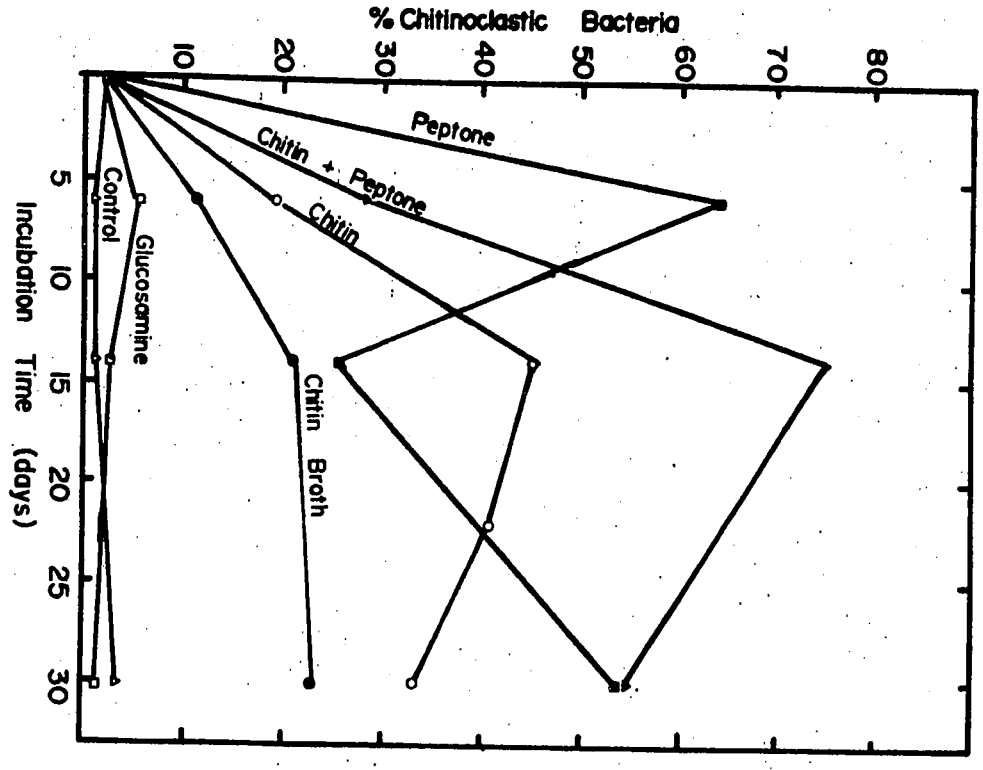
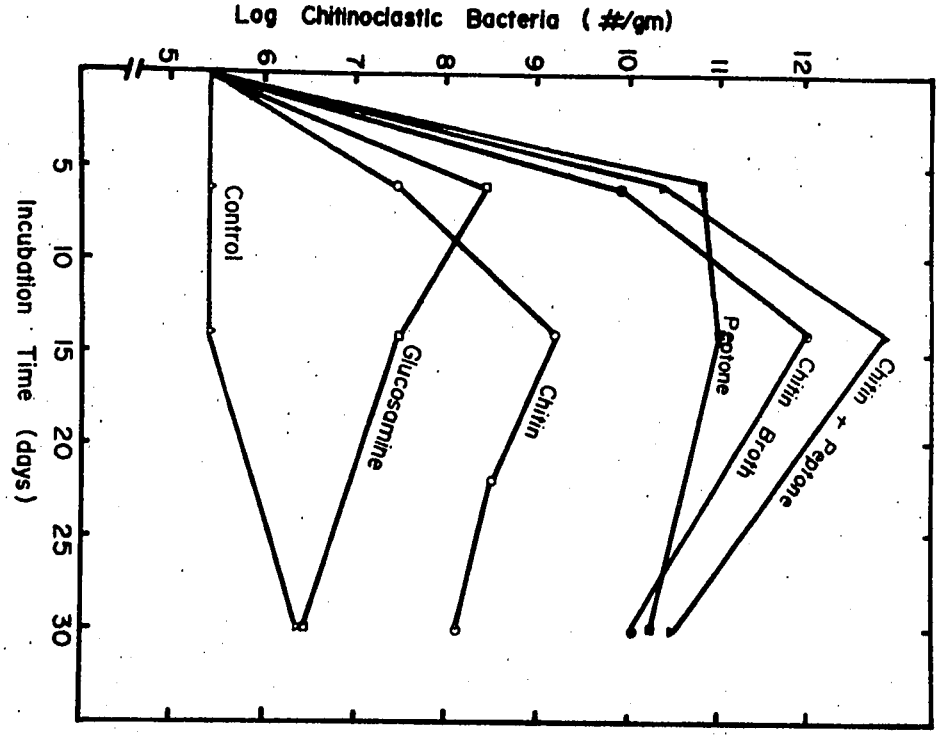

Log Chitinoclastic Bacteria (#/gm)
12
11
10
9
8
7
6
5
Chitin + Peptone
Peptone
Chitin Broth
Chitin Glucose
Control
CaCO3
5
10
15
20
25
30
35
40
Incubation Time (days)

Figure 42. Percentage of Chitinoclastic Bacteria on FWCh Agar After Substrate Enrichment of Lake Washington Littoral Zone Sediment. Substrates included in final concentration: peptone, 0.2%; chitin, 1 mg % + peptone, 0.2%; chitin, 1 mg %; FWCh broth, 10%; glucosamine, 0.2%; and control.

Figure 43. Log Plate Count Chitinoclastic Bacteria on FWCh Agar After Substrate Enrichment of Lake Washington Littoral Zone Sediment. Substrates included in final concentration: chitin, 1 mg % + peptone, 0.2%; FWCh broth, 10%; peptone, 0.2%; chitin, 1 mg %; glucosamine, 0.2%; and control.


% Chitinoclastic Bacteria
10
20
30
40
50
60
70
80
Peptone
Chitin + Peptone
Chitin
Control
Glucosamine
Chitin Broth
Incubation Time (days)
5
10
15
20
25
30


Log Chitinoclastic Bacteria (#/gm)
5
6
7
8
9
10
11
12
Control
Glucosamine
Chitin
Peptone
Chitin Broth
Chitin + Peptone
Incubation Time (days)
5
10
15
20
25
30

In both experiments, higher numbers of chitinoclastic bacteria were obtained by enrichment in chitin + peptone and FWCh broth than by chitin alone. Glucose, glucosamine, and NAG had little effect on the percentage of chitinoclasts, because total numbers of both heterotrophic bacteria and chitinoclasts increased proportionately in these samples. Greatest increases in percentage of chitinoclastic bacteria occurred after 9 to 14 days of incubation with the enriching substrates used in this study.

Effect of Temperature on Chitinoclastic Bacteria

Spread plates were inoculated with serial dilutions of sediment from the 4 lakes and incubated at 4, 15, and 22°C to determine the effect of incubation temperature on the percentage and total numbers of chitinoclasts. With most of the samples, results were inconclusive. The total numbers of chitinoclasts either did not change or increased with temperature increase. Variable results were obtained when data were analyzed for percentage of chitinoclasts. The variable changes in both percentage and numbers of chitinoclastic bacteria are perhaps reflective of different thermal types of these organisms during the seasons of the year.

Variable temperature effects were found for Lake Washington benthic and littoral zone sediment (Table 29). For benthic samples, collected in January, the total numbers and percentage of chitinoclasts increased from 2.3% and 10^5 organisms/gm at 4°C, to 39% and 10^6 organisms/gm at 15°C. The percent decreased to 8.6% at 22°C, with little change in total numbers. With benthic samples, tested in April and September, either decreases or little change occurred in percent chitinoclastic bacteria and total numbers with increasing temperature. For littoral zone sediment samples, little change in percent chitinoclasts occurred with temperature increase, except for the sample

Table 29. Chitinoclastic Bacterial Plate Counts of Lake Washington Sediments on FWCh Agar and Incubated at Various Temperatures. Incubation times: 4°C, 42 days; 15°C, 20 days; 22°C, 12 days.

Date	Station	In Situ T°C	Temperature T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
1/25/74	benthic	6.8	4.0	2.3	0.22
			15.0	39.0	4.70
			22.0	8.6	1.10
4/ 4/74		7.0	4.0	0.3	0.06
			15.0	ND	-
4/25/74		7.0	15.0	1.6	4.00
			22.0	2.9	0.28
9/25/74		6.6	4.0	26.0	2.20
			15.0	8.9	2.20
			22.0	13.5	3.90
8/22/74	littoral	20.0	4.0	9.3	0.86
			15.0	6.1	2.40
			22.0	3.0	1.00
10/ 1/74		15.0	4.0	1.9	0.23
			15.0	7.0	3.30
			22.0	21.6	12.00
11/ 5/74		13.5	4.0	6.0	0.17
			12.0	2.0	0.73
			22.0	8.0	3.20
1/ 2/75		7.0	4.0	3.8	0.14
			10.0	1.9	0.29
			22.0	2.1	0.20
2/ 4/75		7.0	4.0	2.5	1.60
			10.0	1.2	0.80
			22.0	4.1	2.80
3/25/75	littoral	9.0	4.0	1.1	1.80
			10.0	4.2	0.25
			22.0	3.3	0.57
4/25/75		10.5	4.0	4.5	4.90
			10.0	3.1	3.20
			22.0	2.3	13.00
5/15/75		15.0	4.0	0.7	2.50
			10.0	1.9	6.30
			22.0	0.7	2.50

*ND, chitinoclastic bacteria not detected.

**Values representing mean of duplicate samples are counts per gram dry weight sediment, incubated aerobically.

collected in October which increased in percent at 22°C compared to 15°C. For total numbers of chitinoclastic bacteria, little change occurred for most samples, except a decrease from 10^6 at 4°C, to 10^5 organisms/gm at 10°C for the sample collected in March.

Sediment from the Lake Sammamish benthic station showed little change in percentage chitinoclasts with temperature change, but total numbers of chitinoclastic bacteria increased slightly with increasing incubation temperature (Table 30).

With littoral zone sediment from Lake Chester Morse, decreases in the percent chitinoclasts and total numbers occurred with increasing temperature for sediment collected in the winter (Table 31); however, for sediment collected in summer, the percentage chitinoclasts decreased with increasing temperature of incubation, but not to the extent observed for the winter sample. No chitinoclastic bacteria were detected at any of the 3 incubation temperatures for benthic sediment collected in September; however, for the sample collected in winter (January), incubation temperature had little effect on percent, but numbers increased from 10^4 organisms/gm at 4°C to 10^5 organisms/gm at 15°C. Chitinoclastic bacteria were not detected in benthic sediments incubated at 22°C.

With Findley Lake benthic sediments, the percent chitinoclasts increased with increasing incubation temperature (Table 32). With littoral zone sediments, the percentage and total numbers of chitinoclasts changed little with increasing temperature up to 15°C, and decreased in percent at 22°C.

For littoral stations from both of the lakes of higher elevation--Chester Morse and Findley--decreases in percentage chitinoclasts with temperature increase occurred. This decrease may be due to the presence of chitinoclastic bacteria adapted to colder temperatures in these lakes, and demonstrates the necessity of maintaining samples on ice before analysis. Inoculated plates

Table 30. Chitinoclastic Bacterial Plate Counts of Lake Sammamish Sediments on FWCh Agar and Incubated at Various Temperatures. Incubation times: 4°C, 42 days; 15°C, 20 days; 22°C, 12 days.

Date	Station	In Situ T°C	Incubation Temperature T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
2/ 6/74	benthic	5.8	4.0	1.6	0.47
			15.0	ND	-
			22.0	2.7	1.40
3/ 6/74		6.0	4.0	ND	-
			15.0	4.6	2.20
4/ 3/74		7.0	4.0	2.3	0.25
			22.0	2.5	2.50

*ND, chitinoclastic bacteria not detected.

**Values representing mean of duplicate samples are counts per gram dry weight sediment, incubated aerobically.

Table 31. Chitinoclastic Bacterial Plate Counts of Lake Chester Morse Sediments on FWCh Agar and Incubated at Various Temperatures. Incubation times: 4°C, 42 days; 15°C, 20 days; 22°C, 12 days.

Date	Station	In Situ T°C	Incubation Temperature T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
1/30/74	benthic	4.0	4.0	0.8	0.03
			15.0	1.5	0.31
			22.0	ND	-
9/ 5/74		7.9	4.0	ND	-
			15.0	ND	-
			22.0	ND	-
2/12/74	littoral	3.2	4.0	20.6	15.00
			15.0	9.6	4.40
			22.0	1.0	2.20
9/ 5/75		13.5	4.0	8.4	0.09
			15.0	3.1	0.28
			22.0	2.5	0.19

*ND, chitinoclastic bacteria not detected.

**Values representing mean of duplicate samples are counts per gram dry weight sediment, incubated aerobically.

Table 32. Chitinoclastic Bacterial Plate Counts of Findley Lake Sediments on FWCh Agar and Incubated at Various Temperatures. Incubation times: 4°C, 42 days; 15°C, 20 days; 22°C, 12 days.

Date	Station	In Situ T°C	Incubation Temperature T°C	Percent Chitinoclastic Bacteria*	Numbers Chitinoclastic Bacteria x 10^6**
2/28/74	benthic	4.0	4.0	16.0	0.17
			15.0	28.5	8.40
			22.0	ND	-
9/ 9/74		5.0	4.0	ND	-
			15.0	ND	-
			22.0	5.6	0.59
2/28/74	littoral	3.0	4.0	15.0	0.21
			15.0	18.2	5.70
			22.0	3.3	0.72
9/ 9/74		13.0	4.0	13.2	1.50
			15.0	8.2	8.00
			22.0	6.3	8.00

*ND, chitinoclastic bacteria not detected.

**Values representing mean of duplicate samples are counts per gram dry weight sediment, incubated aerobically.

should also be incubated at temperatures as close to the prevailing in situ temperature as possible.

Isolates of Chitinoclastic Bacteria

Several representative isolates of chitinoclastic bacteria were selected and picked from spread plates of sediment from the 4 lakes. The cultures were checked for purity and maintained in pure culture. All of the isolates were gram negative, oxidase positive, motile rods. Most were capable of fermenting glucose to acid without gas production in Hugh Leifson medium (1953); thus these bacteria were classified as members of the Vibrio or Aeromonas group. These isolates represented the most abundant species of chitinoclastic bacteria observed in the lake sediment samples, although a small percentage of chitinoclastic Streptomyces, Pseudomonas, and gliding bacteria, Cytophaga sp, were sometimes present.

The pure cultures were stored on slants in FWCh agar at 4°C. Some samples, early in the course of this investigation, stored on media without chitin exhibited subsequently decreased rates and in one case no chitin hydrolysis when restreaked on FWCh agar. Although the isolates were facultative anaerobes, they were not capable of hydrolyzing chitin anaerobically, or did so very slowly.

The chitinoclastic bacterial isolates were capable of hydrolyzing not only reprecipitated chitin but also "crude" chitin as well. Of cultures tested, all were capable of hydrolyzing an overlay of 0.5% chitin in FWCh agar on FW agar. However, the rate of hydrolysis was slower on plates prepared from "crude" chitin than with reprecipitated chitin. The "crude" chitin was prepared from pulverized lobster shells ground in an electric mill and sieved through a 0.2 mm screen.

All data collected on the chitinoclastic bacterial isolates are given in Table 51 in the Appendix.

Chitin Degradation by Viable Cells

The hydrolysis of chitin by viable cells was studied in the laboratory to gain a better understanding of microbial chitin breakdown in the environment. Chitin hydrolysis was studied using the isolates of chitinoclastic bacteria growing in both solid and liquid media containing reprecipitated chitin.

Chitin decomposition on FWCh agar. A clear zone of hydrolysis surrounding colonies of chitinoclastic bacteria growing on FWCh agar usually appeared within 1 to 7 days of inoculation. Both the time of zone appearance and the ratio of the cleared zone diameter to colony diameter were temperature dependent (Table 33). Higher initial zone to colony ratios occurred with cultures grown at 22°C and 15°C than at 4°C. At 4°C hydrolysis was usually initially slower, and with some cultures a cleared zone did not appear until after 25 days of incubation as shown in Figure 44 for isolate 61-2.

The initial rate of chitin hydrolysis was usually linear during the first 5 days after the appearance of a zone. However, with some cultures after additional time, the colony and zone sizes increased at equal rates resulting in no net increase in the ratio (Fig. 45). With some cultures, for example isolate 61-2 (Fig. 44), colony and zone sizes increased in a stepwise fashion resulting in ratio fluctuations. For this reason, measurement of hydrolysis on FWCh agar was terminated after 30 days of incubation.

The rate of chitin hydrolysis varied with the test organisms. For example, isolate 19-5 (Fig. 46) had a slower rate than did isolate 71-11 (Fig. 45). After 15 days of incubation, the ratios for 19-5 incubated at 4, 15, and 22°C were 1.03, 1.47, and 1.45, respectively. For isolate 71-11 at 4, 15, and 22°C, ratios were 1.6, 1.90, and 1.85, respectively, after 15 days of incubation. While no hydrolysis occurred with isolate 19-5 incubated at 4°C, until after 15 days, the highest ratio of 2.3 occurred for this isolate after 35 days at 4°C,

Table 33. Rate of Chitin Decomposition of Isolates on Solid FWCh Agar at Given Temperatures

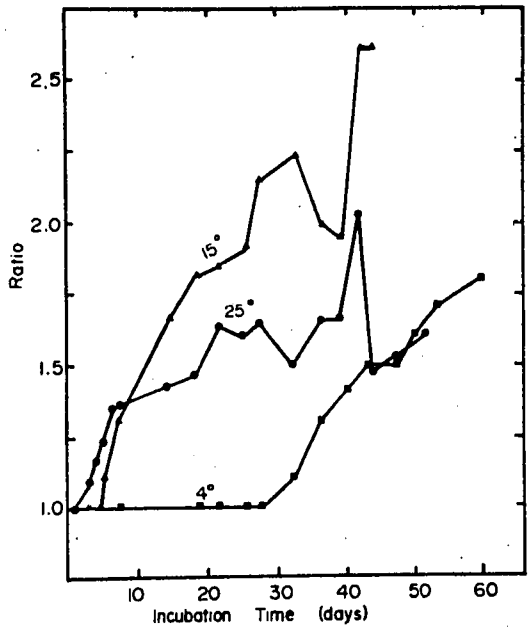
Isolate	Lake	Days to Reach Ratio of 1.5 at Temperature* 22°C	15°C	4°C
61-2	Washington	19	11	43
70-1	Sammamish	4	6	22
74-1		24	12	9
62-1	Chester Morse	10	16	23
62-2		6	11	33
62-3		28	-	37
71-7		6	6	11
71-11		5,5	7,5	10,10
64-3	Findley	-	25	21
19-5		17	17	25
72-3		6	8	18
72-21		5	-	16

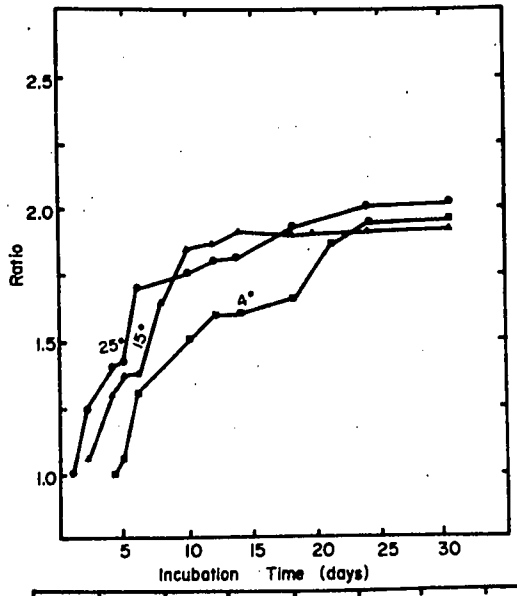
*Values are days required to reach a ratio (zone of hydrolysis/ diameter of colony) of 1.5.

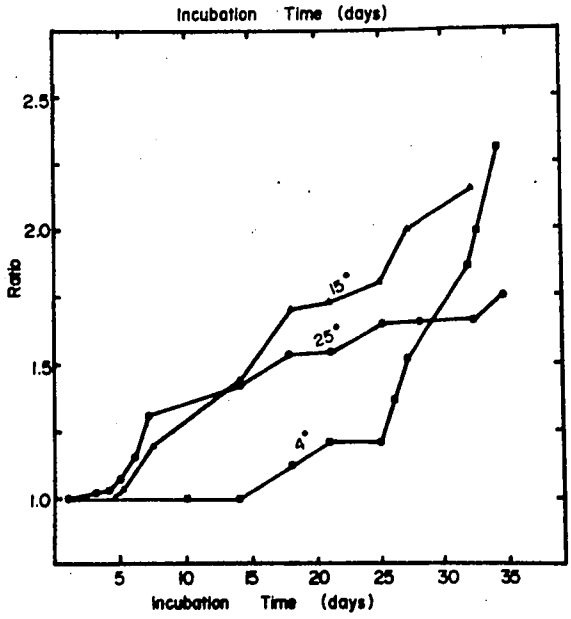
Figure 44. Activity of Isolate 61-2 on FWCh Agar at 4, 15, and 22°C as Measured by the Ratio Method.

Figure 45. Activity of Isolate 71-11 on FWCh Agar at 4, 15, and 22°C as Measured by the Ratio Method.

Figure 46. Activity of Isolate 19-5 on FWCh Agar at 4, 15, and 22°C as Measured by the Ratio Method.


2.5
2.0
1.5
1.0
Ratio
15°
25°
4°
10
20
30
40
50
60
Incubation Time (days)


2.5
2.0
1.5
1.0
Ratio
25°
15°
4°
5
10
15
20
25
30
Incubation Time (days)


2.5
2.0
1.5
1.0
Ratio
15°
25°
4°
5
10
15
20
25
30
35
Incubation Time (days)

compared to ratios of 2.15 and 1.75 at 15 and 22°C, respectively (Fig. 46).

The zone-ratio method of measuring chitin hydrolysis rates on FWCh agar could not be used to study some of the isolates due to extensive spreading of the colonies. These cultures were often very rapid chitin digesters, often with the culture extending over the clearing an entire FWCh agar plate after 30 days. Therefore, the zone-colony ratio method on FWCh agar was not a suitable method to describe the activity of these bacteria.

Chitin decomposition in liquid media. Pure cultures of chitinoclastic bacteria would grow in FW broth only with the addition of chitin, glucose or NAG. These bacteria always grew at the chitin-liquid interface when chitin was present in the media. In FWG (0.5% glucose) broth and FW broth containing 0.5% NAG, the organisms initially grew as a suspension in the broth and after one to two days, settled out on the bottom of the tube. All isolates tested were capable of growth in minimal media + chitin, with chitin the sole carbon and nitrogen source.

Chitin decomposition rates were measured by the ratio method in liquid broth for several of the isolates. Hydrolysis rates were usually linear for the first 30 days. Supernatants from several of the inoculated FWCh broth tubes were assayed for NAG, glucose, protein, and chitinase activity (by NAG release when the supernatant was incubated with chitin) (Table 34). Chitinase activity was not detected in the supernatant in any of these tubes during the course of the incubation for up to 62 days. Neither NAG nor glucose was detected in the supernatant as well. Protein concentrations decreased in the supernatant with increasing incubation times, indicating utilization of protein by these organisms. The FWCh medium initially contained 740 µgm/ml protein and 0.34 µmole/ml NAG. The MM + chitin initially contained no detectable NAG and less than 20 µgm protein/ml. After 15 days of incubation, NAG and protein

Table 34. Protein and N-acetyl glucosamine Levels in FWCh Broth Inoculated with Specified Isolate at Specified Times of Incubation. No chitinase or glucose detected in the supernatant of all samples tested. Samples designated MM are minimal media broth with 0.6% reprecipitated chitin.

Isolate	Incubation Time, Days	N-acetyl glucosamine µmole/ml	Protein µgm/ml	Ratio
Blank	FWCh broth	0.34	740	1.0
Blank	MM + chitin	0.0	0-20	1.0
64-1	1	0.14	0	1.0
	2	0.14	0	1.15
	3	0.14	300	1.19
	4	0.16	150	1.26
	15	0.10	0	1.47
	15, MM	0.0	500	1.35
	62	0.14	-	2.3
	62, MM	0	-	gone
64-2	1	0.2	500	1.0
	2	0.2	500	1.14
	3	0.2	350	1.28
	4	0.16	500	1.36
	15	0.16	250	1.86
	15, MM	0.0	-	1.80
	62	0.22	-	6.85
	62, MM	0.04	-	5.0
70-1	1	0.2	500	1.0
	2	0.2	500	1.13
	3	0.2	350	1.27
	4	0.16	500	1.35
	15	0.16	250	1.9
	15, MM	0	-	2.36
	62	0.22	-	10.5
	62, MM	0.04	-	gone
71-7	1	0.16	600	1.0
	2	0.16	620	1.15
	3	0.2	500	1.22
	4	0.16	400	1.27
	15	0.16	450	1.65
	15, MM	0.66	-	1.74
	62	0.38	-	9.5
	62, MM	0.96	-	4.26

(Continued)

Table 34 (continued)

Isolate	Incubation Time, Days	N-acetyl glucosamine µmole/ml	Protein µgm/ml	Ratio
71-11	1	0.2	650	1.0
	2	0.2	500	1.21
	3	0.2	300	1.32
	4	0.16	350	1.4
	15	0.16	-	1.84
	15, MM	0.26	500	1.67
	62	0.96	-	4.6
	62, MM	0.58	-	3.46
71-11B*	1	0.2	650	1.0
	2	0.16	550	1.19
	3	0.16	350	1.27
	4	0.2	350	1.31
	15	0.42	150	1.80
	15, MM	0.26	-	1.59
	62	0.96	-	9.5
	62, MM	0.58	-	3.06
72-21	1	0.16	670	1.0
	2	0.14	600	1.14
	3	0.16	500	1.21
	4	0.14	-	1.25
	15	0.1	450	1.67
	15, MM	0	-	2.0
	62	0.24	-	5.0
	62, MM	0.84	-	4.15
74-1	1	0.2	-	1.0
	2	0.16	650	1.17
	3	0.16	100	1.32
	4	0.16	-	1.36
	15	0.16	200	2.05
	15, MM	0	-	2.2
	62	> 1.1	-	13.7
	62, MM	> 1.1	-	gone
74-2	1	0.24	750	1.0
	2	0.2	700	1.16
	3	0.16	450	1.28
	4	0.2	450	1.33
	15	0.32	150	1.9
	15, MM	0.28	640	2.5
	62	1.0	-	gone
	62, MM	0.8	-	gone

*71-11B, duplicate of 71-11, grown on FWCh agar from slant, separately from 71-11, to check reproducibility of method.

levels had decreased for 11 of the isolates tested, except 71-11B and 74-2; in these two samples, increases were observed. The organisms were still viable in all of the tubes after 15 days of incubation. After 62 days, NAG levels had increased substantially over initial measured values in many of the samples. However, no viable cells were cultured from the tubes with high NAG levels, indicating perhaps that NAG was released by residual enzyme, and no organisms were present to further degrade this carbohydrate.

Isolates varied in rates of chitin hydrolysis in liquid media as in solid, with some organisms capable of more rapid rates than others (Table 35). Rates of chitin hydrolysis in liquid media were temperature dependent, with higher rates at higher temperatures up to 28°C.

Effect of growth temperature on optimal temperature for chitin hydrolysis. The results of this study and of others suggest that temperature is one of the most important environmental variables affecting the breakdown of chitin. Therefore, the effect of temperature on various aspects of growth and chitin breakdown by chitinoclastic bacteria was investigated.

Four chitinoclastic bacterial isolates, 62-1 (Lake Chester Morse, littoral), 70-1 (Lake Sammamish, benthic), 71-11 (Lake Chester Morse, littoral), and 74-1 (Lake Sammamish, benthic) were grown as inoculums at 4, 15, and 22°C. These organisms were inoculated into FWCh medium and incubated in the temperature gradient incubator from -0.8 to 32.9°C. Most of the organisms were capable of chitin decomposition over the entire temperature range studied and had temperature optima for chitin decomposition from 20.5 to 25.7°C (Figs. 47-50). An example of chitin decomposition by isolate 71-11 (inoculum grown at 4°C) from incubation times of 7 to 60 days is shown in Plate 2.

The inoculum growth temperature had little effect on the temperature optimum and range for chitin decomposition (Table 36), for these organisms

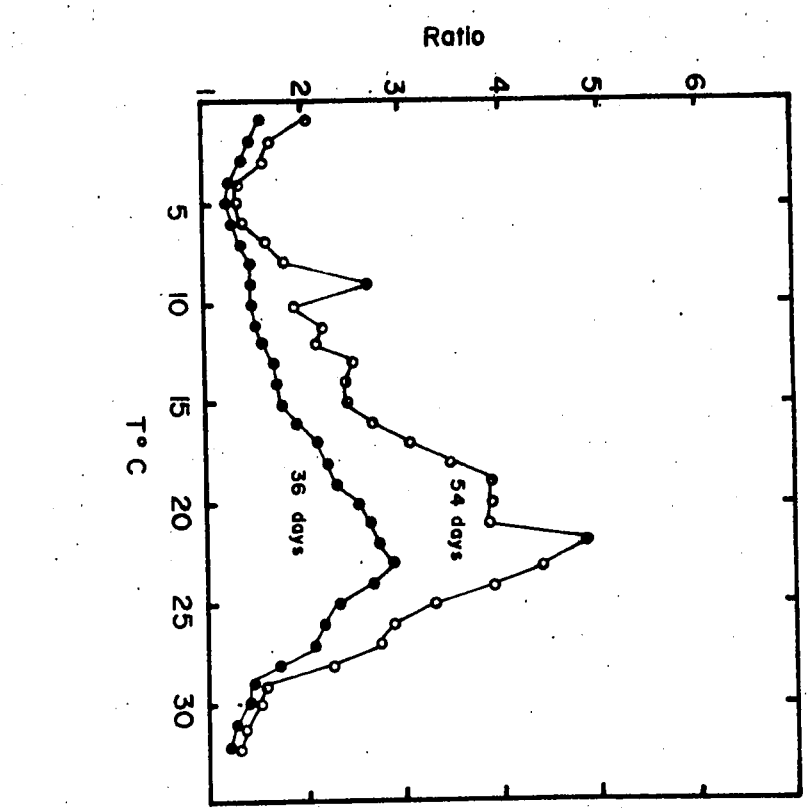
Table 35. Chitin Decomposition Rates of Isolates in FWCh Broth Incubated at 22°C

Isolate	Lake	Days to Reach Ratio of 1.5 at 22°C*
61-2	Washington	9, 9, 11, 10, 9
77-1		12, 11
77-2		10, 10
77-3		16, 15
77-4		18
70-1	Sammamish	10, 8, 9
70-2		27
74-1		6, 6
74-2		6, 7, 6
62-1	Chester Morse	9, 9, 11, 10, 9
62-2		11, 15
62-3		18, 19, 15
71-1		> 50
71-2		12
71-7		10, 12, 13
71-11		8, 8, 10, 10
19-5	Findley	31
64-1		10, 9
64-2		8, 12, 12, 12
64-3		38, 36
72-4		36
72-3		24, 21
72-21		10, 12, 12

*Values are days required to reach a ratio of 1.5 (ratio = length in mm of chitin liquid interface to bottom of 26 mm diameter tube/length in mm of chitin-liquid interface to bottom of tube at specified time). Multiple values are separate measurements.

Figure 47. Chitin Hydrolysis by Isolate 62-1 in FWCh Broth after 36 ● , and 54 o , days incubation in the temperature gradient incubator. (Inoculum grown at 15°C.)

Figure 48. Chitin Hydrolysis by Isolate 70-1 in FWCh Broth after 37 ● , and 68 o , days incubation in the temperature gradient incubator. (Inoculum grown at 22°C.)


Ratio
1
2
3
4
5
6
5
10
15
20
25
30
T° C
36 days
54 days

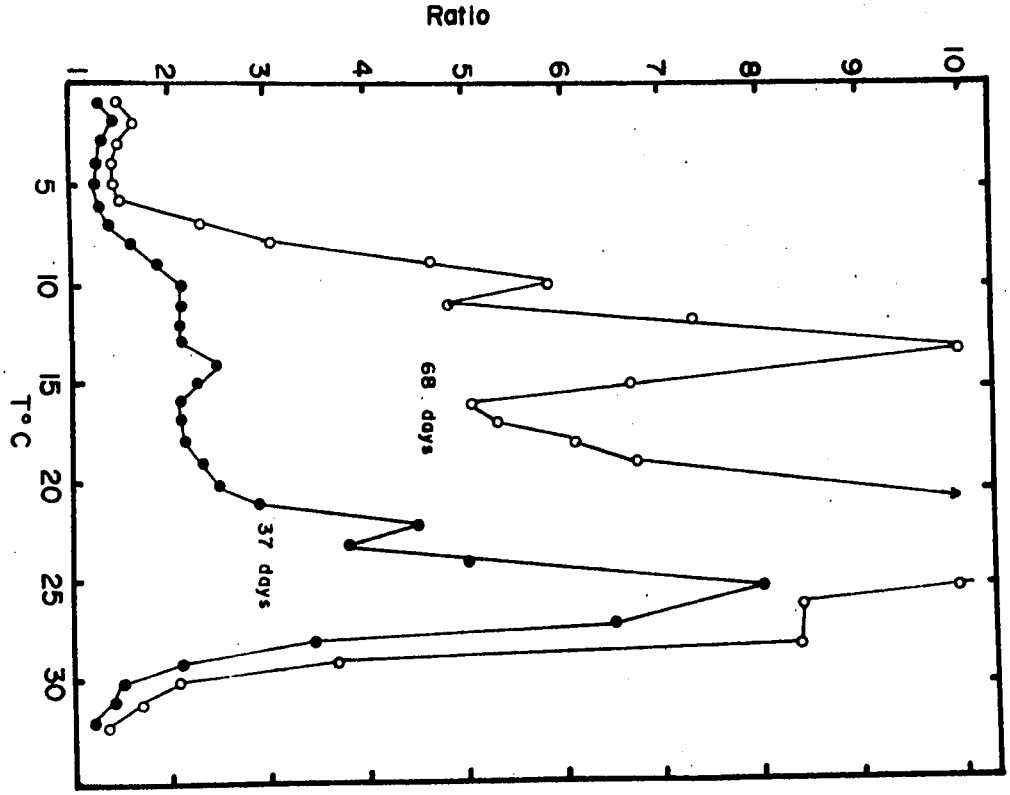
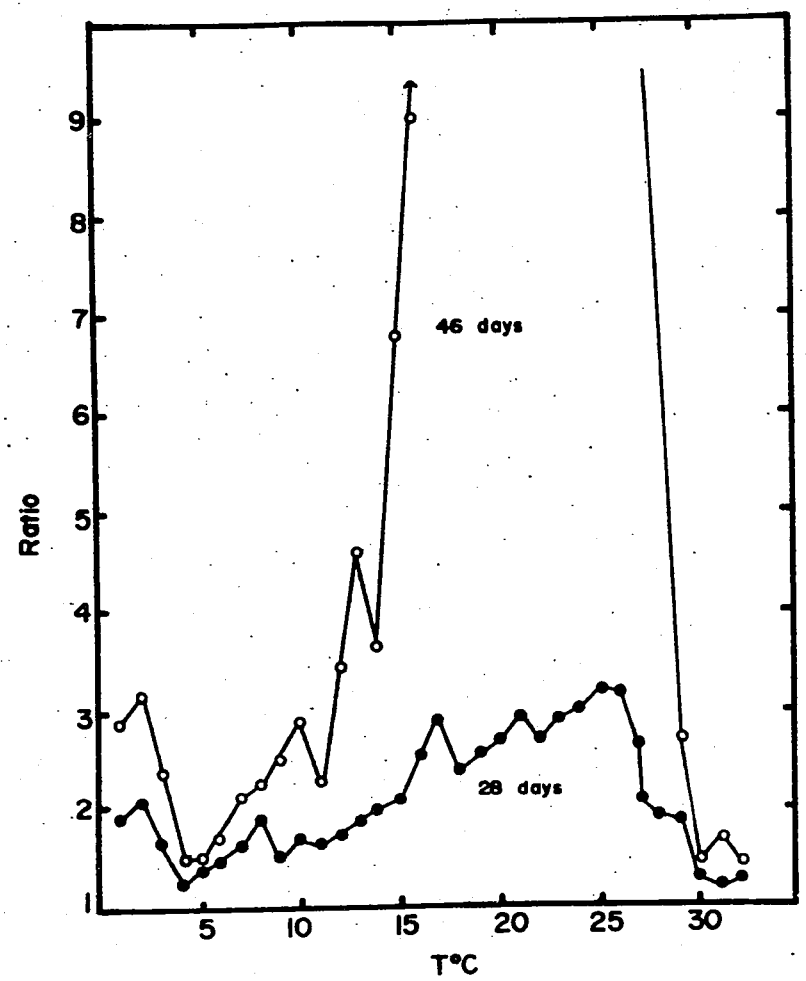

Ratio
1
2
3
4
5
6
7
8
9
10
5
10
15
20
25
30
T° C
68 days
37 days

Figure 49. Chitin Hydrolysis by Isolate 71-11 in FWCh Broth after 28 ● , and 46 o , days in the Temperature Gradient Incubator. (Inoculum grown at 4°C.)

Figure 50. Chitin Hydrolysis by Isolate 74-1 in FWCh Broth after 37 ● , and 68 o , days in the Temperature Gradient Incubator. (Inoculum grown at 22°C.)


Ratio
T°C
46 days
28 days

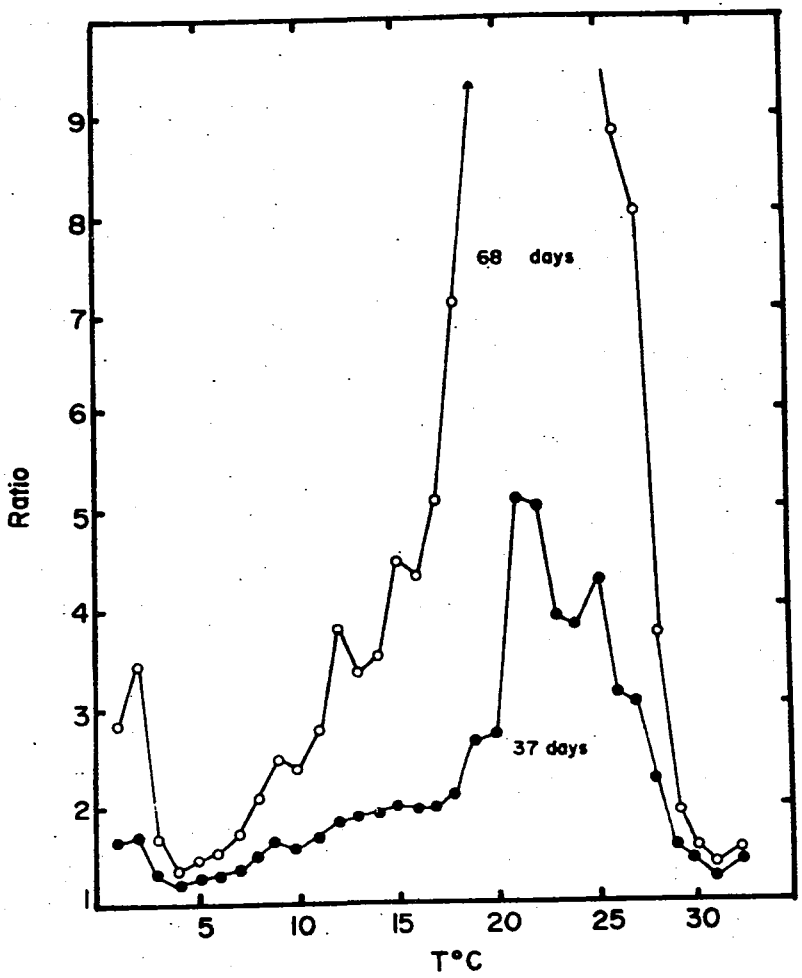
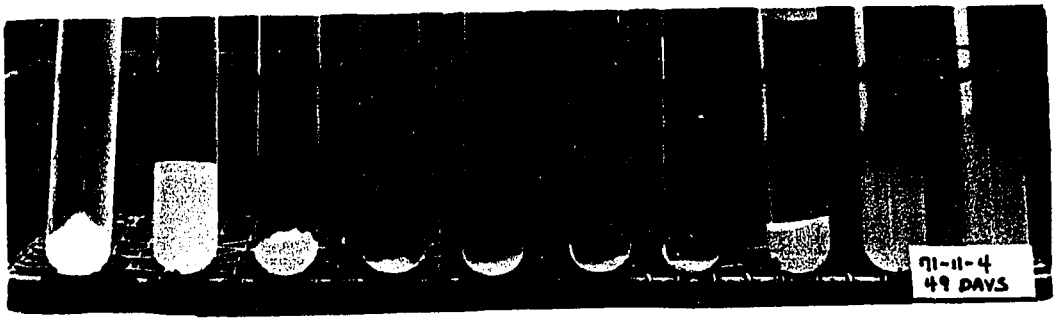

Ratio
T°C
68 days
37 days

Plate 2. Chitin Degradation by Viable Cells of Isolate 71-11 in FWCh Broth after 7, 15, 49, and 60 days incubation in the temperature gradient incubator. Inoculum was grown at 4°C. Tubes shown from left to right are from positions 1, 5, 10, 15, 20, 23, 25, 27, 30, and 32 on the temperature gradient incubator and correspond to these temperatures (Celsius). The black line indicates the initial level of chitin in the tubes.


71-11-4
7 DAYS


71-11-4
15 DAYS


71-11-4
49 DAYS

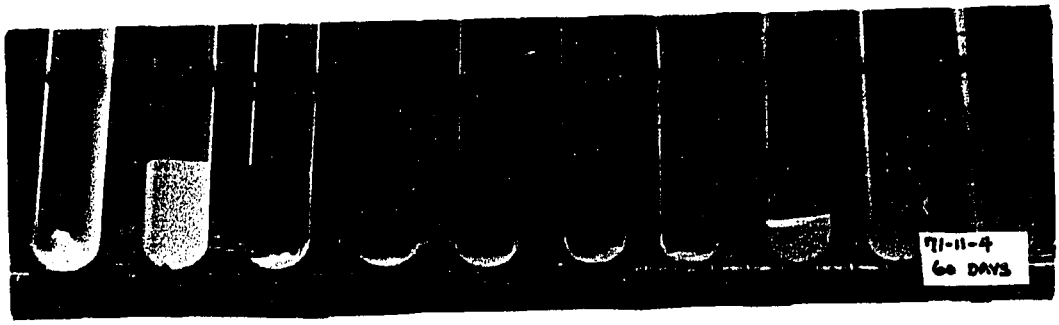

71-11-4
60 DAYS

Table 36. Optimal Temperature and Temperature Optimum Range for Bacterial Chitin Decomposition. Optimal temperature, where ratio reaches a value over 3.0. Optimum temperature range where ratio is 1.5 or over after 54 days incubation.

Isolate	Temperature of Inoculum	Optimum Temperature	Optimal Temperature Range	
62-1	4.0	24.7	-0.8	+30.9
	15.0	23.6	-0.8	+29.8
	20.0	20.5	-0.8	+29.8
70-1	4.0	23.6	-0.8	+32.0
	15.0	24.0-26.8	+0.4	+32.0
	20.0	25.7	+0.4	+32.0
71-11	4.0	25.7	-0.8	+32.0
	15.0	25.7	-0.8	+32.0
	20.0	25.7	-0.8	+32.9
74-1	4.0	21.6	-0.8	+32.9
	15.0	23.6	-0.8	+30.9
	20.0	21.6-22.7	-0.8	+30.9

tested with the exception of 62-1. With this isolate, the optimum temperature for chitin decomposition decreased slightly with increasing inoculum growth temperature. The optimum temperature for chitin hydrolysis was 24.7, 23.6, and 20.5°C for inoculums grown at 4, 15, and 22°C, respectively.

Some of the isolates exhibited smaller peaks of chitin decomposition at 1-2°C and at 15°C, as shown for isolate 71-11, inoculum grown at 22°C and incubated 39 days (Fig. 51).

After 68 to 100 days of incubation, the tubes were removed from the temperature gradient incubator and tested for viability. Some of these samples were assayed for NAG levels (Table 37). In most of these tubes, viable organisms were found in tubes incubated at temperatures approaching the optimum for chitin digestion. At temperatures near the optimum, both above and below, viable cells were not present. The NAG levels were consistently at 0.2 µmoles/ml or lower in tubes containing viable cells and higher in tubes containing non-viable cells. This was also mentioned in the previous section on "Bacterial decomposition in liquid media." For example, with isolate 74-1, inoculum grown at 22°C, no viable organisms were found at temperatures from 23 to 32°C, and NAG levels were 1.6 µmoles/ml or lower in these tubes. The temperature optimum for chitin digestion for this isolate was 21-22°C. Similar results were obtained for the other isolates tested.

While the inoculum growth temperature had relatively little effect on the temperature optimum and range for chitin digestion, there was a pronounced effect on the rate of chitin degradation for the four isolates as measured by the ratio method. For example, Figure 52 demonstrates the rate of chitin degradation at position 2 (+0.5°C) on the temperature gradient incubator for isolate 71-11 over a period of 60 days. The inoculum grown at 4°C had a faster rate of chitin degradation at 0.5°C, with more chitin hydrolyzed than did the inocula grown at

Figure 51. Chitin Hydrolysis by Isolate 71-11 in FWCh Broth after 39 Days Incubation in the Temperature Gradient Incubator. (Inoculum grown at 22°C.)

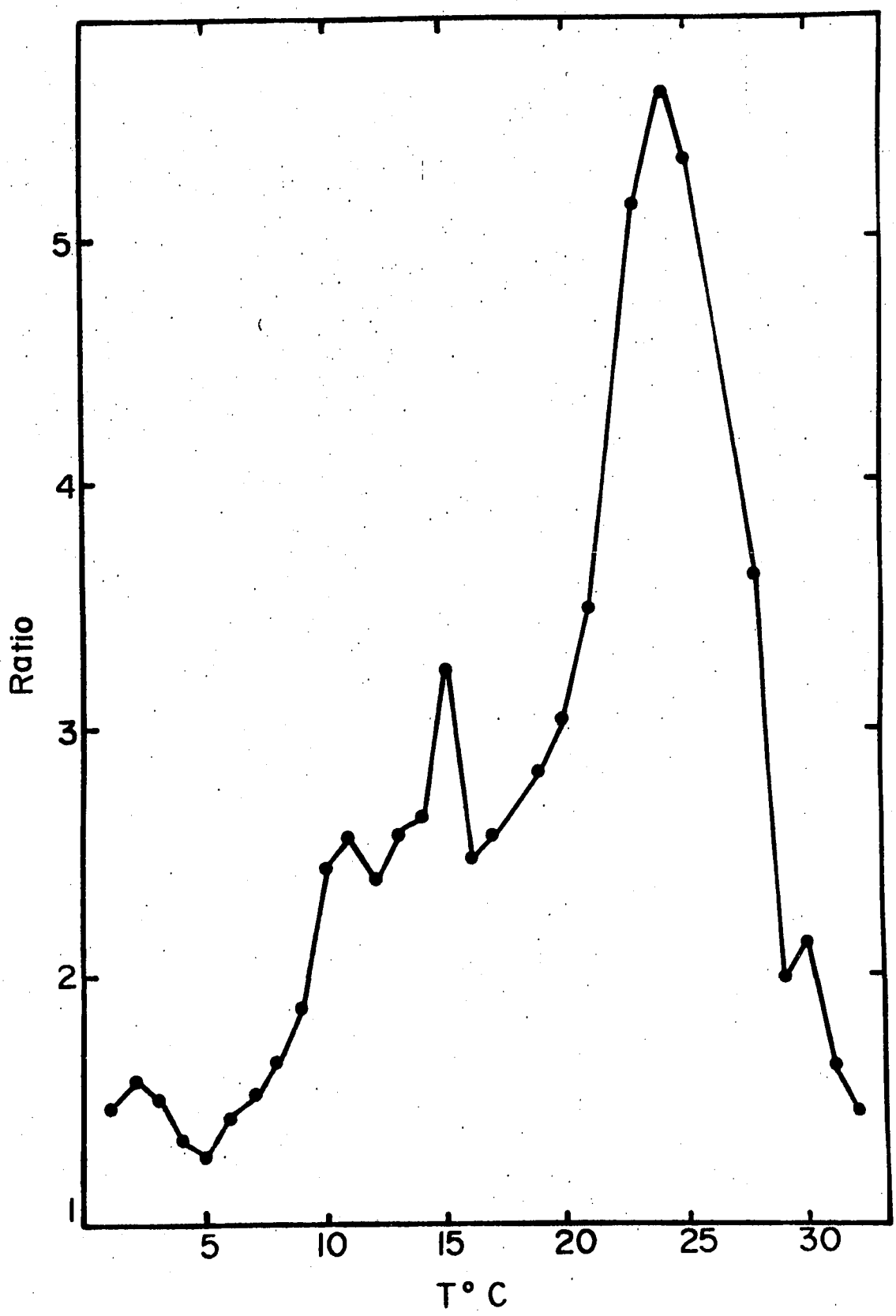

Ratio
1
2
3
4
5
5
10
15
20
25
30
T° C

Table 37. Viability of Organisms and N-acetyl glucosamine Levels in Tubes Incubated for Specified Times in the Temperature Gradient Incubator. All tubes containing viable organisms consistently had N-acetyl glucosamine levels at 0.22 μmoles/ml. Organisms grown in FWCh broth.

Isolate	Growth Temperature of Inoculum	Optimum Temperature Chitin Digestion	Days	Temperature of Nonviable Samples	N-acetyl glucosamine in Nonviable Tubes μmole/ml
62-1	22	20	89	22-32	ND
	4	24	100	23	1.0
				24	0.6
				27	1.5
				28	0.9
				29	0.8
				30	0.6
				31	0.6
				32	0.4
70-1	22	25	68	25	0.45
				26	0.52
				32	0.35
	15	24-26	90	22-28	ND
				32	
	4	23	90	31-32	ND
71-11	22	25	80	25-28	0.23
				30	0.23
				32	0.37
	15	25	90	19-20	ND
				22	
				24-32	
	4	25	100	28	0.5
				32	0.4
74-1	22	21-22	72	23	1.5
				24	1.5
				25	1.6
				26	1.6
				27	1.1
				28	0.8
				29	0.4
				30	0.3
				31	0.3
				32	0.3

(Continued)

Table 37 (continued)

Isolate	Growth Temperature of Inoculum	Optimum Temperature Chitin Digestion	Days	Temperature of Nonviable Samples	N-acetyl glucosamine in Nonviable Tubes µmole/ml
74-1	15	23	90	22-28	ND
				32	
	4	21	100	26	0.8
				27	0.5
				28	0.7
				29	0.5
				30	0.5
				31	0.3
				32	0.2

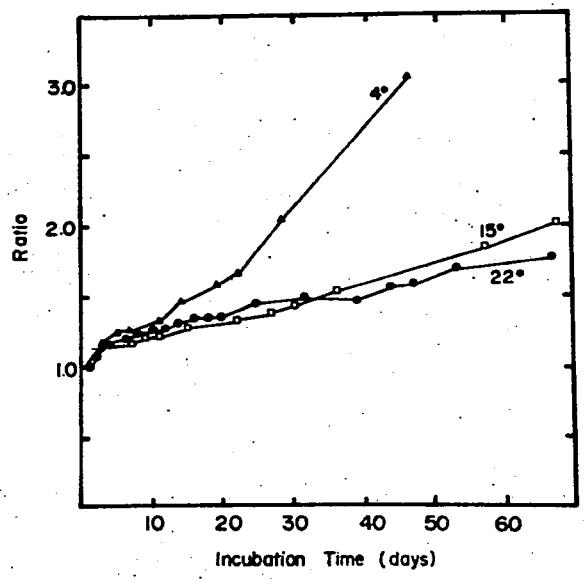
15 and 22°C. Very little difference in chitin degradation occurred for the latter two inocula. When tubes at position 15 (15.0°C) were similarly analyzed (Fig. 53), the inocula grown at 22 and 4°C had equal rates of chitin hydrolysis, although chitin was completely digested in the inoculum grown at 4°C in 59 days, while a small amount was still present in the inoculum grown at 22°C after 71 days. The inoculum grown at 15°C had a significantly slower rate and hence less chitin was hydrolyzed than the other 2 inocula. When position 27 (27.8°C) was analyzed in the same manner (Fig. 54), the situation was reversed and the inoculum grown at 22°C exhibited the highest rate of chitin hydrolysis followed by the inoculum grown at 4°C. The inoculum grown at 15°C had the slowest rate of chitin digestion at all 3 temperatures analyzed. The other 3 isolates analyzed in this manner exhibited the same phenomena (Table 38) with the exception of isolate 74-1. With this isolate, the inoculum grown at 4°C had the fastest rate of chitin hydrolysis at all 3 inocula growth temperatures. Surprisingly, the inoculum for all isolates grown at 15°C did not exhibit as rapid a rate of chitin degradation as the other 2 inocula growth temperatures. The reason for this is not clear.

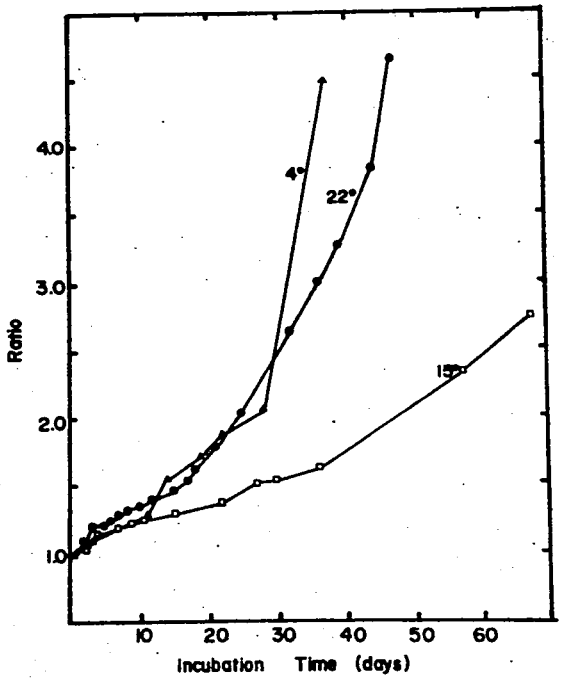
Growth temperature optima and range. To separate the interaction between bacterial growth and chitin hydrolysis with temperature, the temperature optimum and growth temperature range were determined for a number of isolated (Table 39). Typical turbidity measurements after 8 and 12 hours of incubation in the temperature gradient incubator, for isolate 71-11, are shown in Figure 55. This organism had an optimum growth temperature of 27°C and was able to grow over a temperature range of -0.8 to 37.0°C after 60 days. All of the organisms tested exhibited an optimum growth temperature between 19 and 27°C and were capable of growth from -0.8 to 32.9°C. The optimal temperatures for growth and chitin digestion for the 4 isolates were very close (Table 39).

Figure 52. Rate of Chitin Degradation in FWCh Broth by Isolate 71-11 at 0.5°C Position in the Temperature Gradient Incubator (inoculums grown at 4 ▲ , 15 □ , 22°C ●).

Figure 53. Rate of Chitin Degradation in FWCh Broth by Isolate 71-11 at 15.0°C Position in the Temperature Gradient Incubator (inoculums grown at 4 ▲ , 15 □ , and 22°C ●).

Figure 54. Rate of Chitin Degradation in FWCh Broth by Isolate 71-11 at 27.8°C Position in the Temperature Gradient Incubator (inoculums grown at 4 ▲ , 15 □ , and 22°C ●).


Ratio
3.0
2.0
1.0
4°
15°
22°
10
20
30
40
50
60
Incubation Time (days)


Ratio
4.0
3.0
2.0
1.0
4°
22°
15°
10
20
30
40
50
60
Incubation Time (days)

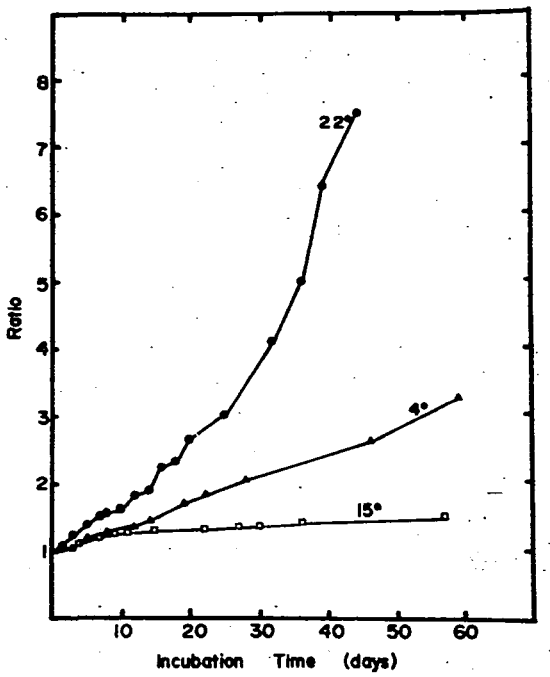

Ratio
8
7
6
5
4
3
2
1
22°
4°
15°
10
20
30
40
50
60
Incubation Time (days)

Table 38. Chitin Digestion as Measured by the Ratio Method for Isolates of Chitinoclastic Bacteria after Incubation in FWCh Broth in the Temperature Gradient Incubator for 40 Days

Isolate	Temperatures in Gradient Incubator	Ratio for Inoculum Grown at Temperatures			Inoculum Growth Temperature with Highest Ratio at Temperature in Gradient Incubator
		4°C	15°C	22°C	
62-1	0.5	2.98	1.59	1.70	4
	15.0	2.75	1.90	2.45	15
	25.7	2.60	2.60	3.90	22
70-1	0.5	1.83	1.40	1.48	4
	15.0	3.45	2.25	2.50	4
	27.8	9.00	3.66	9.00	4,22
71-11	0.5	2.70	1.60	1.50	4
	15.0	5.20	1.60	3.40	4
	27.8	2.40	1.40	6.60	22
74-1	0.5	2.55	1.55	1.90	4
	15.0	6.00	2.22	2.20	4
	26.8	8.50	3.42	3.40	4

Figure 55. Growth of Isolate 71-11 in FWG Broth as Measured by Turbidity at 600 nm after 8 and 12 hours Incubation in the Temperature Gradient Incubator.

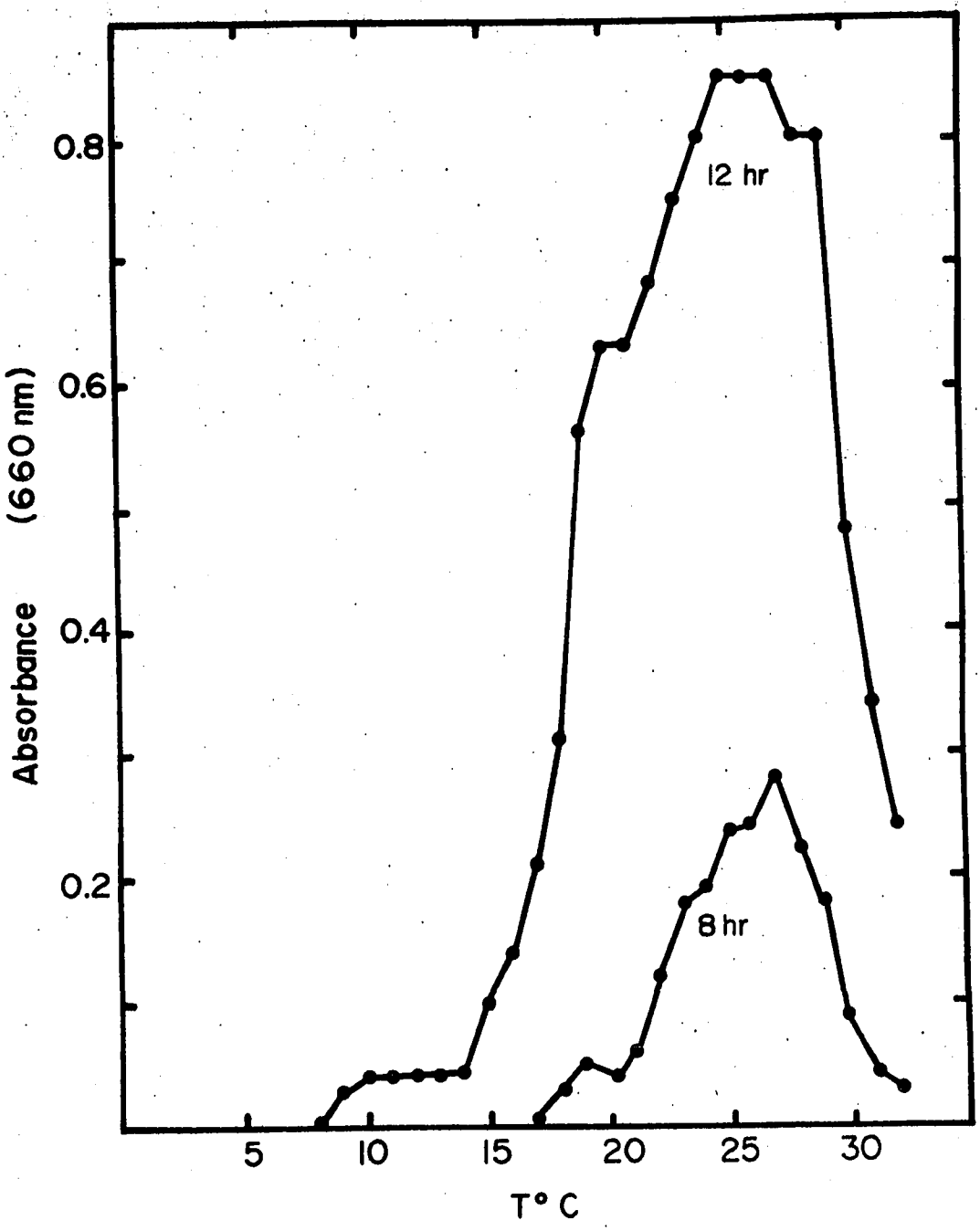

Absorbance (660 nm)
0.8
0.6
0.4
0.2
12 hr
8 hr
5
10
15
20
25
30
T° C

Table 39. Growth Temperature and Range of Chitinoclastic Bacteria. Isolates were grown as inoculums at 22°C.[1]

Isolate	Optimum Growth[2] Temperature	Growth Temperature[3] Range
62-1	19.5-25.7	-0.8 + 32.9
70-1	24.7-27.8	-0.8 + 30.9
71-11	27.8	-0.8 + 37.0
72-3	24.7	-0.8 + 26.8
72-21	24.7	-0.8 + 27.8
74-1	26.8	-0.8 + 32.9
77-4	27.8	+1.6 + 30.9

[1]Optimum temperature for chitin digestion and range is given in Table 36.

[2]Optimum growth temperature is defined as that temperature where maximum growth first occurs within 10-31 hours of inoculation. Growth determined by turbidity measurements of optical density at 660 nm.

[3]Growth temperature range is defined as the lower and upper limits for growth detectable by turbidity measurements at 660 nm after 60 days of incubation.

Effect of end products on chitin decomposition. Isolates were inoculated into culture tubes containing FWCh broth and varying amounts of either glucose or NAG, and grown at 22°C to determine the effects of these end products on the chitin hydrolysis rate. Cultures inoculated into FWCh broth served as controls. Rates of chitin decomposition were calculated by the ratio method. Decreased rates of chitin hydrolysis and lower ratios occurred in tubes containing glucose and NAG compared to control tubes for all isolates tested as demonstrated for isolate 64-1 (Figs. 56 and 57). This decrease in chitin hydrolysis by end products is further shown in Plate 3 for isolate 62-1 after 42 days of incubation. Very little chitin remained in the control tube compared to those containing glucose and NAG at varying concentrations. The decreases in rate of chitin decomposition were directly related to the concentration of the end product added. Ratios remained constant after 55 days of incubation in tubes containing over 0.1% glucose or NAG (Table 40).

Similar decreased rates of chitin hydrolysis occurred on a solid medium with a NAG gradient of from 0 to 0.4% NAG with an overlay of FWCh agar as shown for isolate 71-7 (Fig. 58).

Chitin Degradation by Cell-free Extracts

Chitin hydrolysis by cell-free extracts was studied to gain a better understanding of the microbial breakdown of chitin in the environment. There are many complex interacting factors in the environment (e.g., temperature, nutrient, predation, competition) affecting bacteria and their responses. It is extremely difficult to separate these factors in situ. Therefore, it is sometimes enlightening to remove bacteria from the environment and study their activities in the laboratory as described in the previous section. Further understanding of microbial activities can be gained by studying microbial enzymes responsible

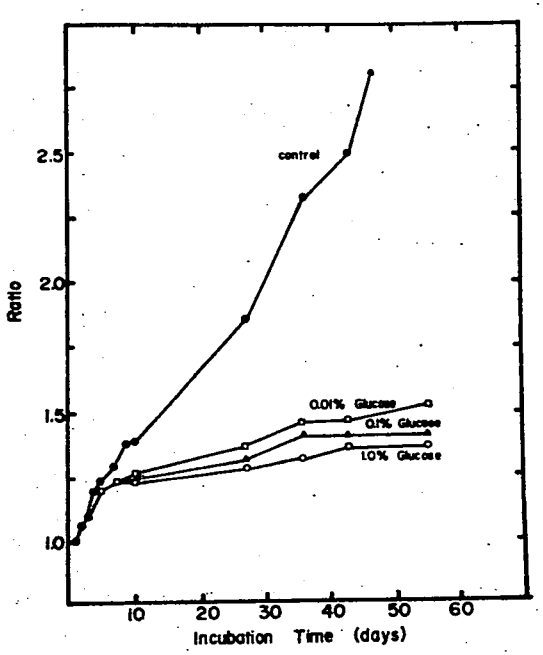
Plate 3. Chitin Degradation by Viable Cells of Isolate 62-1 in FWCh Broth after 42 days of incubation at 22°C. Tubes from left to right contain: control, 0.01% glucose, 0.1% glucose, 1.0% glucose, 0.01% NAG, 0.1% NAG, and 0.4% NAG. Black line indicates the initial level of chitin in the tubes.

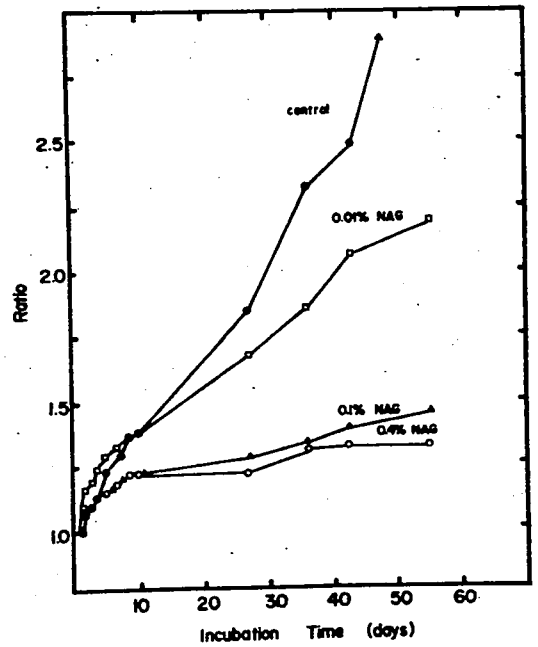
62-1
42 DAYS

Figure 56. Rate of Chitin Degradation for Isolate 64-1 in FWCh Broth Containing Varying Amounts of Glucose Incubated at 22°C.

Figure 57. Rate of Chitin Degradation for Isolate 64-1 in FWCh Broth Containing Varying Amounts of N-acetyl glucosamine (NAG) incubated at 22°C.

Figure 58. Rate of Chitin Degradation by Isolate 71-7 at 22°C on FWCh Agar over a Gradient of N-acetyl glucosamine (NAG) from 0 to 0.4% in FW Agar.


Ratio
2.5
2.0
1.5
1.0
control
0.01% Glucose
0.1% Glucose
1.0% Glucose
10
20
30
40
50
60
Incubation Time (days)


Ratio
2.5
2.0
1.5
1.0
control
0.01% NAG
0.1% NAG
0.4% NAG
10
20
30
40
50
60
Incubation Time (days)

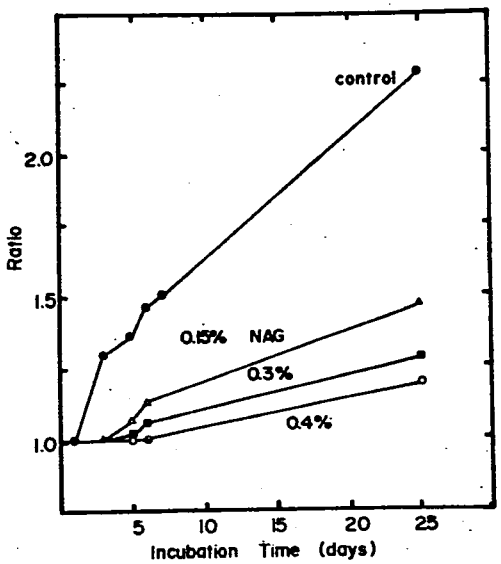

Ratio
2.0
1.5
1.0
control
0.15% NAG
0.3%
0.4%
5
10
15
20
25
Incubation Time (days)

Table 40. Chitin Digestion by Isolates in FWCh Broth Containing Varying Concentrations of Glucose and N-acetyl glucosamine. Incubated at 22°C for 55 days.

		Glucose			N-acetyl glucosamine		
Isolate	Control	0.01%	0.1%	1.0%	0.01%	0.1%	0.4%
62-1	*	2.50	1.65	1.46	4.12	2.00	1.65
62-2	5.70	1.94	1.42	1.35	3.18	1.46	1.36
62-3	3.18	2.67	1.35	1.39	2.32	1.56	1.48
64-1	3.70	1.52	1.40	1.35	2.18	1.44	1.32
64-2	4.85	2.00	1.70	1.36	2.18	1.48	1.41
70-1	2.31	1.84	1.43	1.42	2.64	1.36	1.55
71-7	4.25	2.06	1.50	1.45	3.10	1.68	1.52
71-11	2.67	1.80	1.32	1.27	2.27	1.50	1.40
72-3	2.20	1.74	1.44	1.40	2.31	1.42	1.48
72-4	1.56	1.45	1.36	1.35	1.61	1.46	1.35
72-21	6.00	2.54	1.43	1.33	2.85	1.55	1.46
74-1	*	3.10	1.76	1.47	12.00	2.20	1.72
74-2	2.19	1.68	1.43	1.38	1.85	1.38	1.42
77-1	6.60	3.40	1.38	1.45	5.50	1.54	1.46
77-2	8.25	1.79	1.33	1.40	4.55	1.45	1.45
77-3	6.60	1.88	1.39	1.43	3.88	1.48	1.35
77-4	16.00	2.54	1.59	1.39	7.00	1.57	1.39

*No chitin remaining.

Values are the ratio index (amount of chitin 24 hours after inoculation with designated chitinoclastic isolate compared to the amount of chitin remaining after 55 days of incubation).

for specialized activities (e.g., chitinase) and thus relate all of these data back to the environment. This final section includes studies of chitin degradation by cell-free extracts prepared from the isolates used in the study of chitin hydrolysis by viable cells. To maximize enzyme yields, the effects of sonication and incubation time of cells were investigated. The optimal pH and temperature for chitin hydrolysis, stability of the enzyme, and the effects of end products on enzyme activity were all determined. The enzyme was also partially purified by ammonium sulfate fractionation.

Effect of sonication on enzyme release. Sonication of cell suspensions usually resulted in greater enzyme release compared to untreated samples (Table 41). Enzyme activity, expressed as units of enzyme per ml of cell-free extract were from 7 to 85% higher (with a mean of 55% for 10 preparations) in sonicated samples than with untreated ones. Specific activity, units/mg protein, increased from 26 to 83% (mean of 68% for 8 samples), in 8 sonicated samples; in two sonicated preparations, no difference occurred in one and a decrease in specific activity in the other occurred due to increased protein levels as a result of sonication.

The effect of length of sonication time was tested for a suspension of isolate 71-11 grown 5 days in FWCh broth (Table 42). The enzyme levels, as measured by assay for NAG production from chitin, increased with increasing time up to 2 minutes with 71-11 (Fig. 59). With isolate 71-11, used extensively in this study, the greatest increases in enzyme level were obtained after 2 minutes of sonication; therefore, this treatment was used throughout the study. Greater increases in enzyme levels as measured by turbidity decrease also occurred in sonicated samples. Protein levels in the cell-free extracts were also directly related to sonication time. Total protein concentrations increased to a greater extent with increasing time of sonication (Fig. 60) than did enzyme

Table 41. Effect of Sonication on Enzyme Levels in Cell-free Extracts. Activity determined by NAG release from chitin incubated with extracts. All assays run at 27°C. Cells grown in media shown in parentheses at 22°C unless otherwise noted. All sonicated cell-free extracts sonicated for 2 minutes.

Organism	Units/ml[1] S	Units/ml[1] NT	Percent Increase Units/ml by Sonication	Protein µgm/ml S	Protein µgm/ml NT	Specific Activity[2] Units/ng Protein S	Specific Activity[2] Units/ng Protein NT	Percent Increase Specific Activity by Sonication
74-1 (MM)	0.08	0.05	40	210	100	0.38	0.48	--
74-1 (FWCh)	0.84	0.79	7	1230	1150	0.68	0.68	--
71-11 (MM)	0.67	0.21	69	174	130	3.86	1.60	60
71-11 (FWCh)	1.12	0.23	80	760	600	1.47	0.38	74
62-1 (FWCh)	0.42	0.09	78	590	436	0.71	0.21	70
70-1 (FWCh)	0.75	0.12	84	760	760	0.98	0.16	83
71-11 (FWCh)	0.71	0.11	85	780	566	0.91	0.19	79
71-11-30 (FWCh)	0.37	0.11	71	640	600	0.59	0.18	70
71-11-22 (FWCh)	0.213	0.16	25	693	693	0.31	0.23	26
71-11-12 (FWCh)	0.77	0.13	83	700	640	1.1	0.2	82

[1]Units defined as amount of NAG (µM) produced per ml cell-free extract per hour when incubated with reprecipitated chitin at 27°C. S-sonicated cell-free extract; NT-nontreated cell-free extract.

[2]Specific activity defined as units/mg protein.

Table 42. Effect of Sonication Time on Enzyme Levels in Cell-free Extracts (determined by NAG assay)

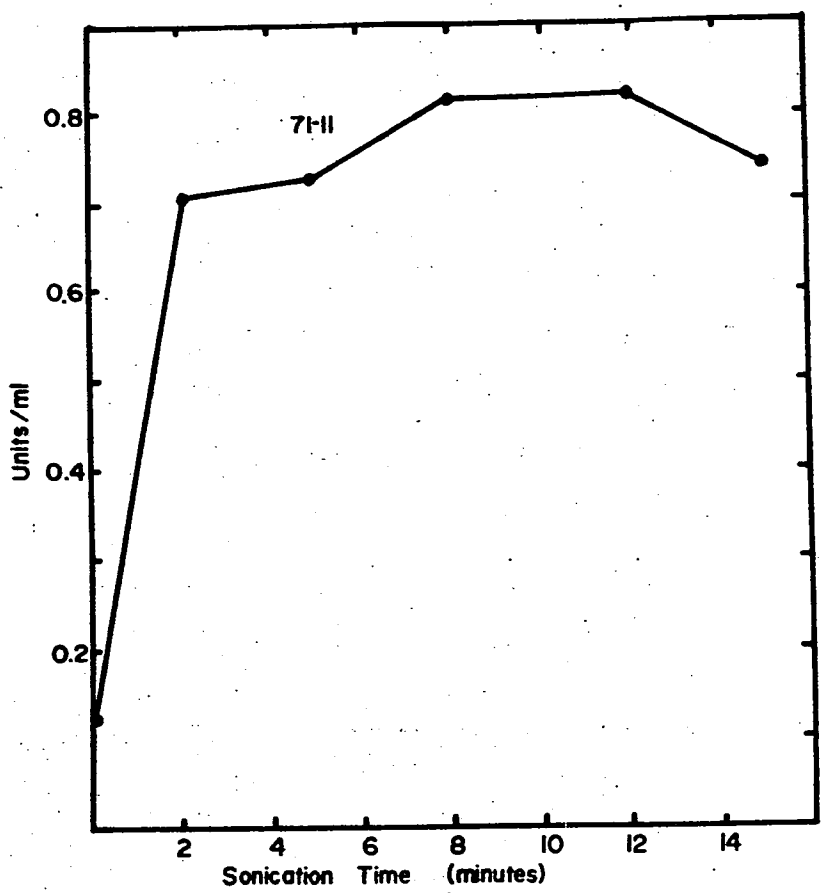
Isolate	Sonication Time (Minutes)	Units/ml[1] Cell-free Extract	Protein µgm/ml	Specific Activity[2] Units/ml Protein
70-1 (MM + Ch)	0	0.10	156	0.66
	4	0.13	220	0.58
	7	0.16	260	0.62
	14	0.19	390	0.49
71-11 (FWCh)	0	0.11	566	0.19
	2	0.71	780	0.91
	5	0.73	780	0.94
	8	0.82	786	1.05
	12	0.83	833	0.99
	15	0.75	833	0.90

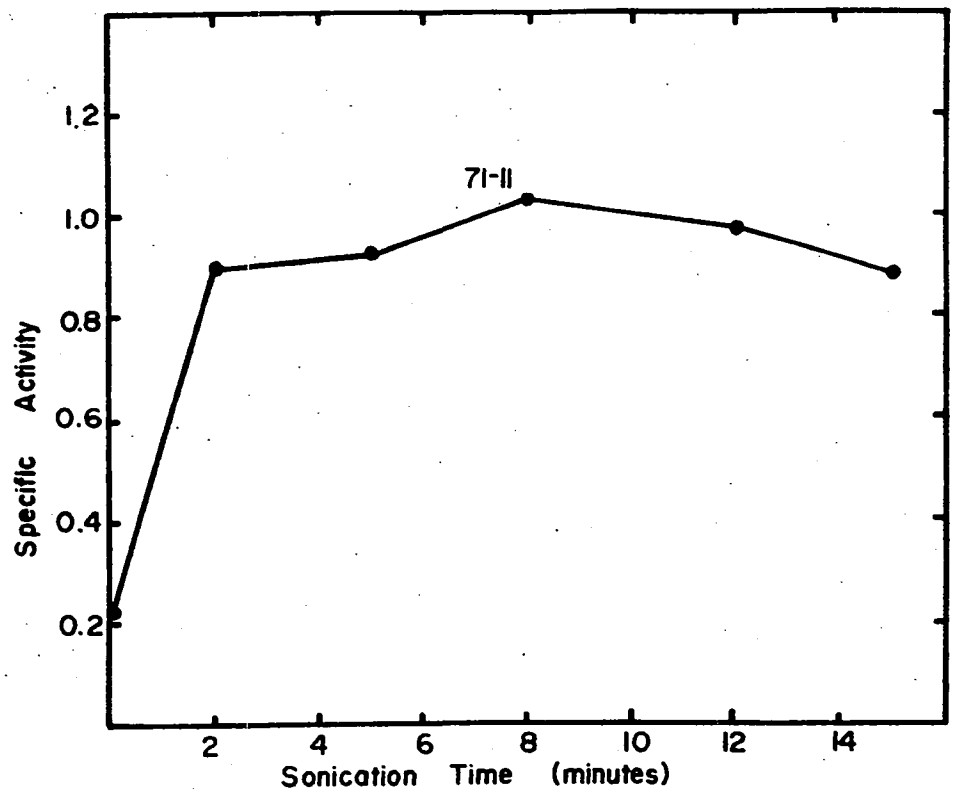
[1]Units defined as amount of NAG (µM) produced per ml cell-free extract per hour when incubated with reprecipitated chitin at 27°C.

[2]Specific activity defined as units/mg protein.

Figure 59. Enzyme Activity in Units/ml (μmoles NAG released/hr) as a Function of Sonication Time for Isolate 71-11 Grown in FWCh Broth.

Figure 60. Specific Activity in Units/mg Protein (μmoles NAG released/hr/mg protein) as a Function of Sonication Time for Isolate 71-11 Grown in FWCh Broth.


71-11
0.8
0.6
0.4
0.2
Units/ml
2
4
6
8
10
12
14
Sonication Time (minutes)


71-11
1.2
1.0
0.8
0.6
0.4
0.2
Specific Activity
2
4
6
8
10
12
14
Sonication Time (minutes)

protein levels, resulting in specific activity decreases.

Effect of incubation time on enzyme production. A 48-hour culture of isolate 71-11, grown at 22°C in 50 ml of FWCh broth, was inoculated into 350 ml of FWCh broth in a 1 liter Erlenmeyer flask and incubated on a rotary shaker at 22°C. Both sonicated and untreated cell-free extracts were prepared from aliquots taken from the culture daily during the 6 days of incubation. These extracts were tested for chitinase activity by measuring NAG release from chitin (Table 43). Bacterial numbers were determined by plate counts on FWCh agar (Fig. 61). Initially, the inoculated flask contained 5.4×10^6 organisms/ml; after 1 day, counts increased to 1.6×10^{10} cells/ml, and reached a maximum number of 5.0×10^{11} cells/ml on day 2. After 3 days, the plate count decreased to 9.1×10^8 cells/ml and remained at 10^8 cells/ml up to 6 days.

There was a lag of chitinase activity following the cell counts by 2 days. No chitinase activity was detected in the cell-free extract after 1 day of incubation (Table 43, Fig. 62). Activity increased to 0.09, 0.64, and 0.72 units/ml after 2, 3, and 4 days, respectively, and then decreased to 0.49 and 0.032 units/ml by days 5 and 6, respectively. Protein levels were measured in the cell-free extracts and decreased from an initial concentration of 866 µgm/ml to 476 µgm/ml in sonicated preparations and to 3.6 µgm/ml in untreated preparations by day 6. Enzyme levels and protein concentrations were higher in sonicated preparations than in untreated ones. The decreased protein levels with increasing time of incubation was due presumably to protein consumption by the test organisms during growth.

Maximum specific activity (Table 43) was present on the fifth day of incubation, and was slightly higher for the untreated sample, which contained less protein than the sonicated preparation. From these data, it can be seen that chitinase appears in the culture medium of isolate 71-11 during the

Table 43. Effect of Incubation Time of 71-11 Isolate in FWCh Broth on Plate Count, Enzyme Produced, and Protein Concentration. Plate counts are the mean of duplicate counts on FWCh agar. All incubations at 22°C.

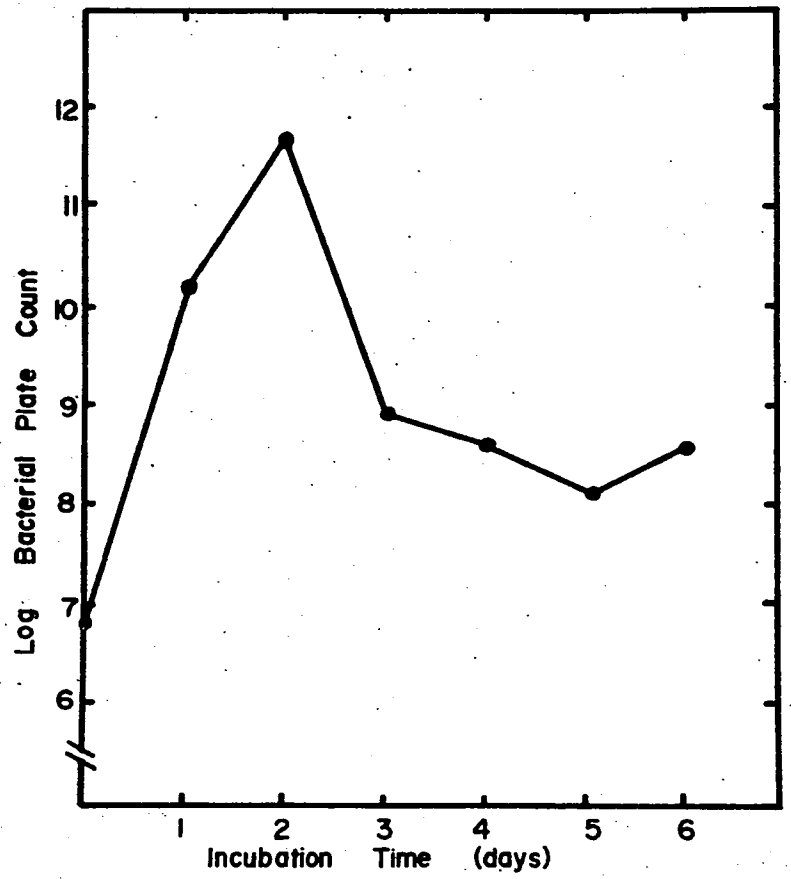
Incubation Time/Days	Plate Count cells/ml	Units/ml[1] S	Units/ml[1] NT	Protein µgm/ml S	Protein µgm/ml NT	Specific Activity[2] Units/mg Protein S	Specific Activity[2] Units/mg Protein NT
0	5.4×10^6	0	0	866	866	0	0
1	1.6×10^{10}	0	0	777	633	0	0
2	5.0×10^{11}	0.09	0.06	853	656	0.11	0.09
3	9.1×10^8	0.64	0.40	753	650	0.85	0.61
4	4.3×10^8	0.72	0.49	790	700	0.91	0.71
5	1.3×10^8	0.57	0.49	433	333	1.30	1.51
6	4.0×10^8	0.19	0.03	476	316	0.41	0.1

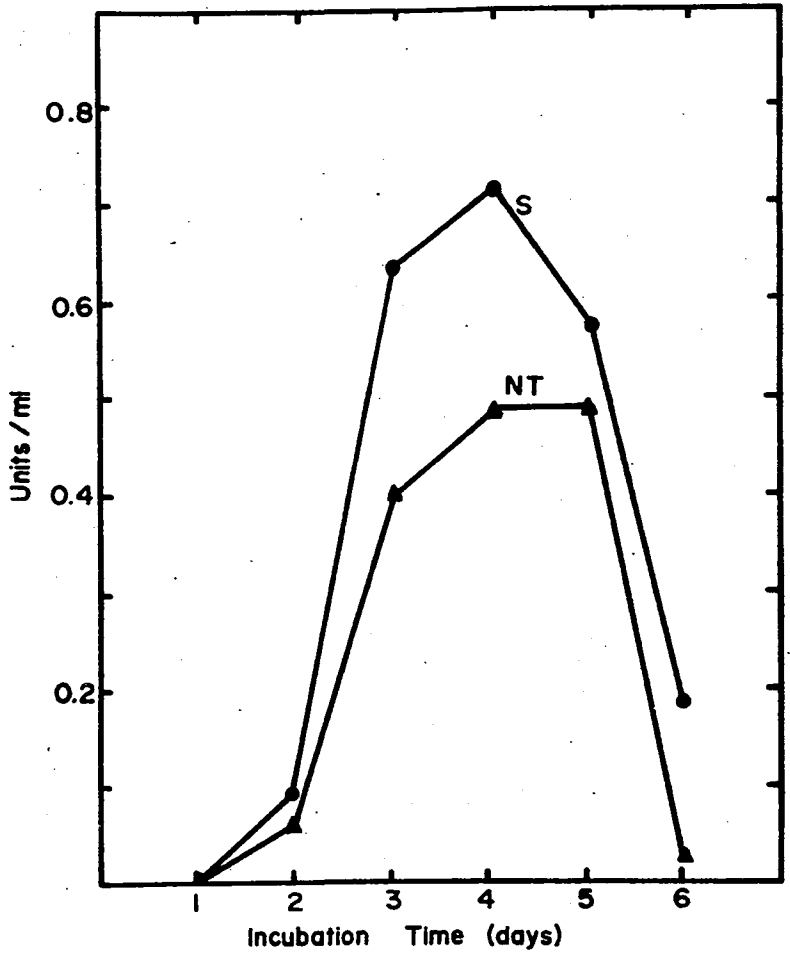
[1]Units are defined as amount of NAG produced per ml cell-free extract per hour when incubated with reprecipitated chitin at 27°C. S-sonicated cell-free extract; NT-nontreated cell-free extract.

[2]Specific activity is defined as units/mg protein.

Figure 61. Log Bacterial Plate Count/ml for Isolate 71-11 Grown in FWCh Broth at 22°C from 0 to 6 days.

Figure 62. Enzyme Activity in Units/ml (μmoles NAG released/hr/ml) of Cell-free Extract Prepared from Isolate 71-11 Incubated at 22°C in FWCh Broth from 0 to 6 days (sonicated (S) and nontreated (NT) cell-free extract).


Log Bacterial Plate Count
12
11
10
9
8
7
6
1
2
3
4
5
6
Incubation Time (days)


Units / ml
0.8
0.6
0.4
0.2
S
NT
1
2
3
4
5
6
Incubation Time (days)

stationary or late log phase. Accurate plate counts were extremely difficult to obtain after 3 days, presumably due to attachment of the bacteria to the chitin particles known to occur with many chitinoclastic bacteria (Kaneko and Colwell, 1975).

Optimal pH for chitin hydrolysis. The optimal pH of chitinase activity was determined for cell-free extracts prepared from cultures grown in FWCh broth and MM + chitin broth (Table 44). These optima, as determined by NAG release from chitin, in FWCh broth were pH 7.0 for isolates 74-1, 71-11, and 62-1, and pH 6.6 for isolate 70-1 (Fig. 63). The optimum pH when using either minimal medium or FWCh broth remained unchanged for isolate 71-11, but decreased to pH 6.3 for isolate 74-1. The release of NAG from chitin by cell-free extract was very pH dependent (Fig. 64) with maximum activity at pH 7.0. Activity rapidly decreased both above or below pH 7.0. The effect of pH on turbidity change was much less pronounced (Figs. 63, 65) with a wider optimum.

Optimal temperature of chitin hydrolysis. Cell-free extracts prepared from the 4 isolates, grown in FWCh broth at 22°C were assayed over a temperature range extending from -0.8 to 50°C. Chitinase was active throughout the entire temperature range, although activity was diminished at both extremes, as shown for isolate 62-1 (Fig. 66). Optimal temperature for NAG release for isolates 70-1, 62-1, 74-1, and 71-11 were 28, 26, 24, and 30°C, respectively. The temperature optimum for chitin degradation by cell-free extracts reported here is the same or slightly higher than the optimum temperature observed for chitin degradation by whole viable cells (ratio method in Table 45) and the optimum growth temperature for the test organisms (Table 39).

Cell-free extracts of isolate 71-11, grown in FWCh broth and MM broth + chitin at various temperatures, were prepared and incubated with chitin over

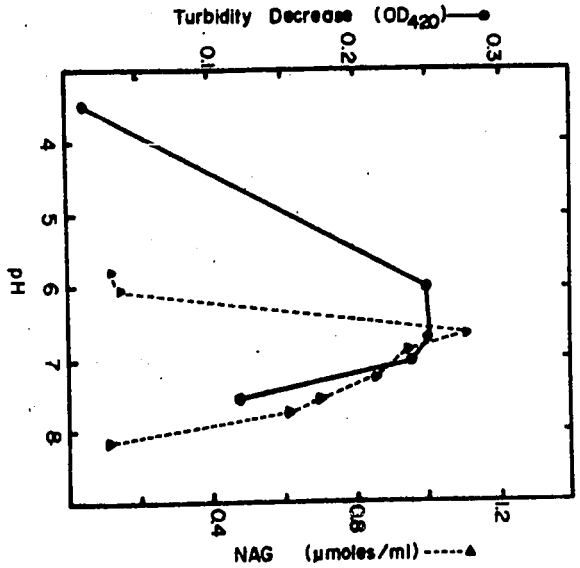
Table 44. Optimal pH of Cell-free Extracts Prepared from Cultures Grown in Two Media at 22°C

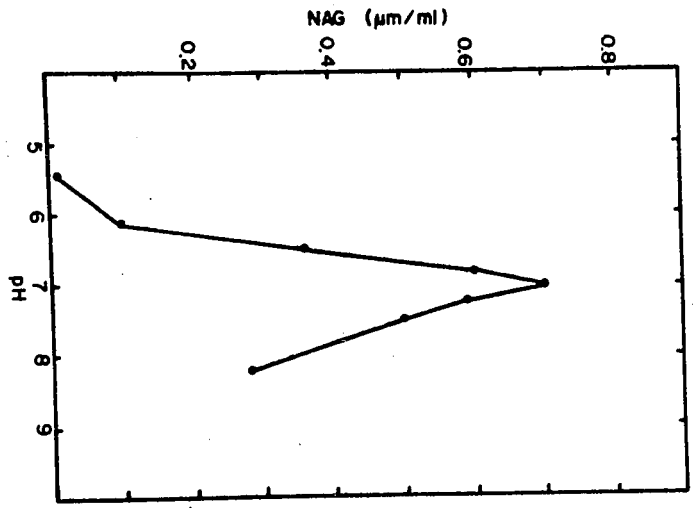
Isolate	Medium (broth)	Optimal pH NAG Assay	Optimal pH Turbidity
74-1	FWCh	7.0	
74-1	MM + Ch	6.3	
71-11	FWCh	7.0	6.1-6.9
71-11	MM + Ch	7.0	
62-1	FWCh	7.0-7.2	
70-1	FWCh	6.6	6.0-6.7

Figure 63. Enzyme Activity as Measured by Turbidity Decrease and NAG Release as a Function of pH for Cell-free Extract Prepared from Isolate 70-1 Grown in FWCh Broth at 22°C (enzyme assay conducted in 0.5 M $NaHPO_4$-citrate buffer at 28°C).

Figure 64. Enzyme Activity as Measured by NAG Release as a Function of pH for Cell-free Extract Prepared from Isolate 71-11 Grown in MM + Chitin Broth at 22°C (enzyme assay conducted in 0.5 M $NaHPO_4$-citrate buffer at 28°C).

Figure 65. Enzyme Activity as Measured by Turbidity Decrease as a Function of pH for Cell-free Extract Prepared from Isolate 71-11 Grown in FWCh Broth at 30°C (enzyme assay conducted in 0.5 M $NaHPO_4$-citrate buffer at 28°C).


Turbidity Decrease (OD420)
0.1
0.2
0.3
4
5
6
7
8
pH
0.4
0.8
1.2
NAG (μmoles/ml)


NAG (μm/ml)
0.2
0.4
0.6
0.8
5
6
7
8
9
pH

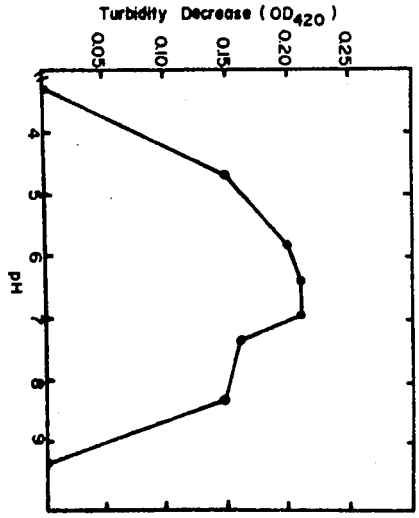

Turbidity Decrease (OD420)
0.05
0.10
0.15
0.20
0.25
4
5
6
7
8
9
pH

Table 45. Optimum Growth Temperature, Optimum Temperature of Chitin Digestion by Viable Cells, and Temperature Optimum Chitin Degradation by Cell-free Extracts for Isolates Grown at 22°C in FWCh Broth. For optimum growth temperature measurements, cells were grown in FWG broth.

Isolate	Optimum Growth[1] Temperature	Optimum Temperature[2] Chitin Hydrolysis Whole Viable Cells	Optimum Temperature Chitinase Cell-free Extract
62-1	19.5-25.7	20.5	26.8
70-1	24.7-27.8	25.7	28.8
71-11	27.8	27.8	27.8-30.9
74-1	26.8	21.6	24.7

[1]Data from Table 39.

[2]Data from Table 36.

the temperature range from -0.8 to 50°C. Chitinase activity was assayed by turbidity decrease and NAG production (Table 46). For many of the cell-free extracts, as with viable whole cells, multiple temperature optima were observed as demonstrated for 71-11 (Fig. 67). Maximal activity occurred at 29°C, with smaller activity peaks at 4 and 12°C. Similar multiple optima, although less pronounced, were found for the same organism with turbidity measurements (Fig. 68).

The growth medium had little effect on the optimal temperature for chitin hydrolysis by cell-free extracts of isolate 71-11 grown in MM + chitin broth or FWCh broth at 4°C. For cells grown at 22°C, however, chitinase activity had a lower temperature optimum of 28°C for cells grown in MM + chitin and 32°C for cells grown in FWCh broth (Table 46).

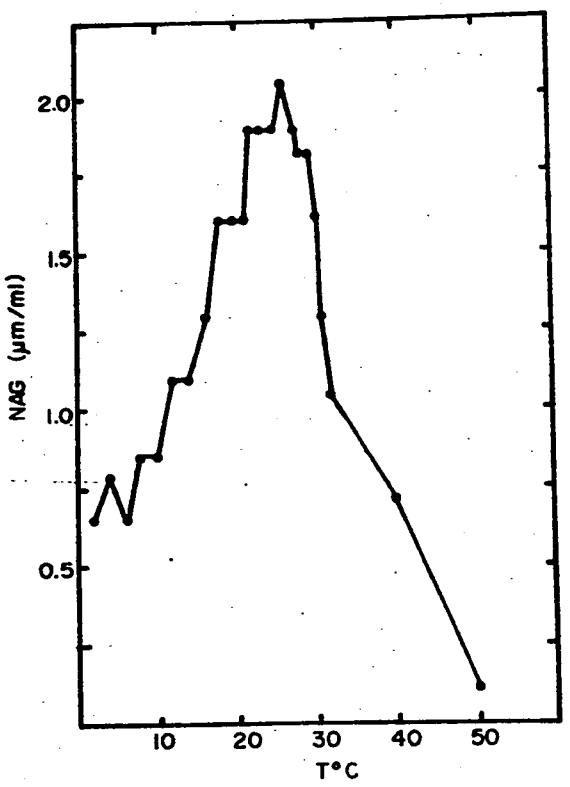
The growth temperature exerted a slight effect on the temperature optimum as determined by both assays. Cells grown at 22°C produced a chitinase with a slightly higher temperature optimum compared to those grown at 4°C (Table 46). Cells grown at 30°C in FWCh broth produced a chitinase with a pronounced increase in temperature optimum compared to those grown at 4°C. Cells grown at 12°C in FWCh broth had enzyme activity equal to and greater than those grown at 22°C. The reason for this is not clear.

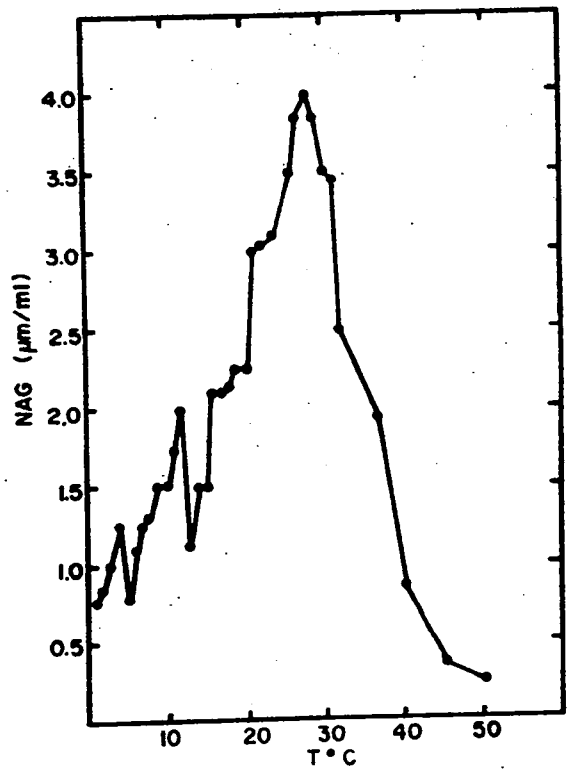
Chitinase also appeared to have increased heat stability at 40°C with increasing growth temperature of the organism (Table 46). When the activity at 40°C was compared to that measured at the temperature optimum, cells grown in MM + chitin at 4°C had 62.5% and 16.7% activity at 40°C for turbidity and NAG release, respectively, as compared to that measured at the optimum temperature. Cells grown at 22°C, on the other hand, had 89% and 20.7% activity at 40°C for turbidity and NAG release, respectively, as compared to that measured at the optimum. Cells grown in FWCh broth at 30°C compared to those grown at 4°C also showed the same increased heat stability.

Figure 66. Enzyme Activity as Measured by NAG Release as a Function of Temperature for Cell-free Extract Prepared from Isolate 62-1 Grown in FWCh Broth at 22°C.

Figure 67. Enzyme Activity as Measured by NAG Release as a Function of Temperature for Cell-free Extract Prepared from Isolate 71-11 Grown in MM + Chitin Broth at 22°C.

Figure 68. Enzyme Activity as Measured by Turbidity Decrease at 420 nm as a Function of Temperature for Cell-free Extract Prepared from Isolate 71-11 Grown in FWCh Broth at 4°C.


NAG (µm/ml)
2.0
1.5
1.0
0.5
10
20
30
40
50
T°C


NAG (µm/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
T°C

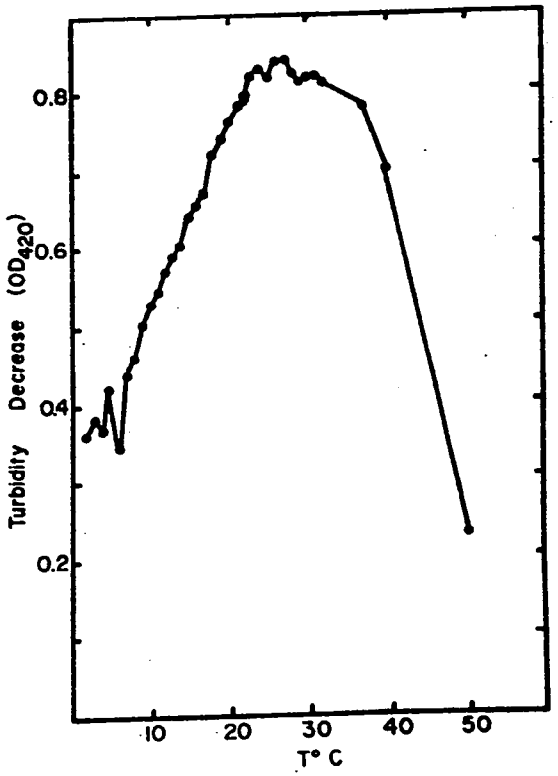

Turbidity Decrease (OD_{420})
0.8
0.6
0.4
0.2
10
20
30
40
50
T° C

Table 46. Effect of Growth Temperature of Inoculum on Temperature Optimum for Chitinase Activity for Isolate 71-11

Media	Inoculum Growth Temperature	Temperature Optima Chitinase Turbidity	Chitobiase NAG Release	Activity at 40°C / Activity at Optimum T. Chitinase Turbidity	Chitobiase NAG
MM + ch	4	28	26	62.5	16.7
MM + ch	22	31	28	89.0	20.7
FWCh	4	26-27	25-26	83.0	26.6
FWCh	12	27-40	31-32	100.0	28.3
FWCh	22	30	32	96.0	25.5
FWCh	30	32-40	32-37	100.0	45.5

It appears that chitinase (turbidity measurement) was more heat stable than chitobiase (NAG release).

When cell-free extracts or organism 71-11 grown in FWCh and MM + chitin broth were held at 45°C for 23 hours, enzyme activity was lost or severely reduced (Table 47). Cells grown at 4 and 12°C on both media lost all enzyme activity for NAG release after the heat treatment. Cells grown at 22°C in MM + chitin and FWCh broth and cells grown at 30°C in FWCh broth retained only 9, 14, and 3% activity, respectively. On the basis of turbidity measurements, all the cell-free extracts retained 16% of the activity shown by control samples. The chitinase enzyme appeared to be more heat stable when heated at 45°C in the presence of chitin and some activity was usually observed after heating cell-free extracts at 45°C in the presence of chitin. The chitin offered some protection to the enzyme, however, this was not pursued further.

Table 47. Effect of Growth Media and Temperature on Heat Stability of Cell-free Extracts, after Incubation at 45°C for 23 hours, for Isolate 71-11. Nontreated aliquot served as a control.

Medium	Growth Temperature	Percent Activity Remaining Compared to Control	
		Percent Activity NAG	Percent Activity Turbidity
MM	4	0	16
MM	22	9	16
FWCh	4	0	16
FWCh	12	0	16
FWCh	22	14	16
FWCh	30	3	16

Stability of the enzyme. Enzymes prepared from cell-free extracts could not always be tested immediately after preparation. Therefore, the stability of the enzymes after storage at 4°C was investigated. Stability of the enzyme could also indicate whether this enzyme could persist in the environment. No change in enzyme activity occurred after storage at 4°C for 24 hours. Storage of cell-free extracts for several days at 4°C had little effect on activity. Cell-free extracts prepared from isolates 62-1, 71-11, and 70-1 (grown in FWCh broth), retained 84, 96, and 87% activity after storage for 16, 18, and 8 days, respectively, at 4°C.

Effect of end products and potential inhibitors on enzyme activity. To determine if the enzyme from the test organisms were similar to those enzymes from other chitinoclastic bacteria, a number of end products and potential inhibitors were added to assay mixtures.

The activity of cell-free extracts from isolate 71-11 grown in MM + chitin broth was tested in the presence of glucose, glucosamine, and NAG compared to untreated controls. The end products of chitin hydrolysis had no effect on the enzyme activity at concentrations up to 0.5% in the assay mixture, as tested by both assays showing that a feedback inhibition was not operating.

To test for the possibility of metal cofactors, EDTA (0.01 M) was incubated with cell-free extracts with no effect on activity.

Calcium ($CaCO_3$, 0.8%) and copper ($CuSO_4$, 0.2%) were also tested. The $CaCO_3$ at the concentration tested had no effect on enzyme activity; however, a 78% reduction in activity occurred with the $CuSO_4$. Calcium (Skujins et al., 1970) and copper (Jeauniaux, 1966) are known to inhibit microbial chitinases and so were tested here.

Amonium sulfate fractionation. Cell-free extracts were treated with ammonium sulfate to precipitate extraneous protein. Initially before testing, reconstituted precipitates were dialyzed overnight at 4°C against 6 liters of 0.01 M $NaPO_4$-citrate buffer, pH 7.0. In every case, all enzyme activity was lost after dialysis, even when traces of $CaCl_2$ (several drops of 0.01 M $CaCL_2$ added per liter) were added to the dialysis buffer, as found necessary for stabilization of the enzyme by Skujins et al. (1970). When two separate preparations of sonicated cell-free extract from 71-11, grown in FWCh broth, were dialyzed under the same conditions (with and without traces of $CaCl_2$), 44% of all activity was lost. This decrease in activity was also accompanied by a 60% and 80% loss in protein in each sample. Because of this loss of both protein and enzyme activity, subsequent samples were not dialyzed.

Solutions of cell-free extracts were initially brought to 35% of saturation with ammonium sulfate. At 35% of saturation, enzyme activity was present; however, at 64% of saturation, greatest increases in amount of enzyme

precipitated occurred (Table 48). With cell-free extracts prepared from 71-11 grown at 4°C in MM + chitin broth, the untreated sample contained 0.5 units/ml; the 35% of saturation fraction contained 0.36 units/ml; and the 64% fraction 2.24 units/ml, all on the basis of NAG release. The specific activity for these preparations on the basis of NAG release, increased from 3.0 units/mg protein for untreated samples, to 36.4 and 224.0 units/mg protein for the 35% and 64% of saturation fractions, respectively. Similar increases occurred for activity and specific activity on the basis of turbidity measurements (Table 48). No activity was detected in the 78% of saturation fraction. The semipurified enzymes were extremely active with less than 0.2 ml capable of hydrolyzing all of the chitin in the standard assay mixture within 4 hours of incubation at 28°C.

Precipitated protein from the 35% and 64% of saturation fractions were reconstituted in sterile distilled water, 0.01 M $NaPO_4$-acetate buffer pH 7.0, and 0.001 M $CaCl_2$ + 0.01 M $NaPO_4$-acetate buffer, pH 7.0, and tested for activity (Table 49). In some cases, highest activity occurred with the precipitates reconstituted with 0.01 M $NaPO_4$-acetate + 0.001 M $CaCl_2$, although differences between the three reconstituting liquids was somewhat minimal.

The pH optimum for the enzyme from isolate 71-11, obtained by ammonium sulfate fractionation was identical to that observed for the sonicated cell-free extract.

Table 48. Purification of Chitinase by Ammonium Sulfate Fractionation of Cell-free Extracts from Isolate 71-11

Media	Percent $(NH_4)_2SO_4$ of Saturation	NAG[1] Units/ml	Turbidity[2] Units/ml	Protein µgm/ml	Specific Activity Units/mg Protein NAG	Specific Activity Units/mg Protein Turbidity
MM + chitin (4°C)	0	0.5	0.14	168	3.0	0.82
	35	0.36	0.17	10	36.4	16.8
	64	2.24	0.52	10	224.0	52.4
	78	0	0	0	0	0
FWCh (22°C)	0	0.05	0.07	770	0.06	0.09
	35	0.11	0.07	10	11.0	6.8
	64	0.70	1.68	160	1.27	12.0

[1]NAG unit defined as amount of enzyme catalyzing the formation of 1 µmole NAG/hr under standard conditions.

[2]Turbidity unit defined as amount of enzyme catalyzing the hydrolysis of chitin to produce a change in turbidity of 1.0 OD unit/hr under standard conditions, at 420 nm.

Table 49. Activity of Reconstituted Precipitates from Ammonium Sulfate Fractionation of Cell-free Extracts Prepared from Isolate 71-11.

Growth Medium Temperature	Percent Fraction of Saturation $(NH_4)_2SO_4$	H_2O		$NaPO_4$-acetate (0.01 M) + 0.001 M $CaCl_2$		$NaPO_4$-acetate (0.01 M)	
		NAG µm/ml	OD_{420}	NAG µm/ml	OD_{420}	NAG µm/ml	OD_{420}
MM + chitin 4^oC	35	0.6	0.21	0.8	0.37	0.66	0.2
	64	3.86	0.51	4.1	0.51	4.3	0.55
FWCh 22^oC	35	0.2	0.8	0.24	0.15	0.24	0.1
	64	1.56	0.46	1.53	0.5	1.53	0.34

Values are results after 4 hour incubation of reconstituted precipitate with chitin at 28^oC.

DISCUSSION

This study was initiated in 1972 as part of the Coniferous Forest Biome (International Biological Program) to determine the activities of sediment heterotrophic bacteria and to compare these activities in lakes of differing trophic state and meterological conditions in the Lake Washington drainage basin. Bacteria associated with the sediment-water interface were selected for study because this area is known to be a site of intense microbiological activity in lakes. Bacterial activities were determined by taking kinetic measurements of mineralization of ^{14}C-labeled glucose to $^{14}CO_2$ based on a method described by Harrison et al. (1971). Glucose was used in this study as a "representative" organic compound. It is readily utilized by the majority of aquatic bacteria and is one of the carbohydrates found in lake waters and sediments. Glucose is also an end product of the decomposition of chitin, one of the more refractory carbon compounds normally present in lakes. Therefore, chitinoclastic bacteria were also enumerated in the 4 lakes. The degradation of chitin under laboratory conditions by chitinoclastic bacteria isolated from the sediment of the 4 lakes and by cell-free extracts prepared from these isolates was also studied.

ASPECTS OF THE GLUCOSE MINERALIZATION RATE DETERMINATION

Before extensive sampling of the 4 lakes was begun, a number of parameters known to affect the bacterial glucose mineralization rate were investigated. These parameters included: (1) reproducibility of the method of Harrison et al. (1971); (2) effect of sample storage; (3) differences between stations in the lakes;

(4) effect of sampling device; (5) effect of lighting conditions during analysis; (6) effect of shaking the sediment sample during analysis; and (7) the effects of additions of substrates or inhibitors on the glucose mineralization rate.

Glucose mineralization rates measured for bacteria associated with sediment from the 4 lakes were reproducible if samples were analyzed within 2 to 4 hours of collection; however, results were variable from samples analyzed after storage in glass sample jars at 4°C from 1 to 8 days. Glucose turnover times usually remained unchanged after storage for 24 hours. The V_{max} value was most affected in the majority of stored samples, either increasing or decreasing. This change in rate is most likely due to the "wall" effect, or the increase in microbial numbers for (sea) water samples stored for long periods in glass bottles, noted by Zobell and Anderson (1936). The magnitude of the change in microbial numbers is primarily a function of the organic content of the water, and the rate of change is a function of temperature (Zobell, 1946a). Storage at low temperatures minimizes changes in microbial populations. Zobell (1946a) found mud samples more stable during storage than water samples.

This altered V_{max} for glucose mineralization after storage is caused probably by depletion of nutrients in the sample jar, and the establishment of dominant bacterial species with different rates of glucose mineralization with the accompanied death of bacteria not able to compete in a closed system. Other workers have noted that storage of samples in closed containers results in a more uniform metabolism due to general population increases attendant on a reduced number of species (Vaccaro, 1960; Zobell, 1946a). Vaccaro et al. (1968) found that storage of sea water samples with or without glucose, resulted in increased maximum uptake velocities of ^{14}C-labeled glucose.

In general, differences in glucose mineralization rate occurred in samples taken from widely separated stations in lakes, but not in areas in close

proximity (within 100 m). In Lake Chester Morse sediment samples, higher glucose mineralization and aerobic plate counts were found for sediment from the inlet of the Cedar River, than sediment collected at the outlet. In Lake Washington, slower glucose mineralization rates occurred in sediments collected at the Cedar River inlet near an industrial area, and on the eastern side of the lake near the city of Kirkland, than in sediments collected at the mouth of the Sammamish River, a shallow silty area. In Findley Lake, differences between various littoral sampling areas were observed with high rates at station No. 3 an area located at the base of recent avalanches. Differences between littoral zone stations in Findley Lake were not as great as differences observed within the other 3 lakes, suggesting that differences between stations are not as great in small lakes compared to larger ones. Hall et al. (1972) found differences in uptake of ^{14}C-labeled substrates in samples taken from various stations at Marion Lake. These differences between bacterial activities and biomass from different stations in lakes, have been ascribed to different sources of in situ organic compounds, availability of bacterial attachment surfaces, such as silt (Paerl and Goldman, 1972), and physiological differences in resident bacterial species (Allen, 1971). Observations from these data indicate that the choice of sampling sites should be carefully considered and should be as "representative" of a lake as possible. Also more sampling sites may be necessary in larger lakes than in small ones.

The method of collecting samples will affect the glucose mineralization rate and this is most likely due to the amount of the "fluffy layer" collected at the sediment-water interface, and the depth to which the sampler penetrates. Higher glucose mineralization rates occurred in sediment samples collected with the suction device, or modified "ooze sucker," which collected the "fluffy layer" and the top two cm of sediment, than sediment collected with a geological

gravity coring device, which collected the top 3 cm of sediment. Hall et al. (1972) found a decrease in ^{14}C-labeled acetate and glucose uptake with increasing depth into the sediment with cores taken from Marion Lake. This decrease in bacterial activity with increasing depth into the sediment is most likely due to higher concentrations of bacteria at the sediment-water interface (Hargrave, 1969; Henrici and McCoy, 1938; Innis, 1975; Ocevski, 1966; Zobell, 1946a, 1946b); populations decrease logarithmically with depth into the sediment. It is advisable, therefore, in comparative studies, to use the same or at least similar sampling devices to reduce variation. The same modified "ooze sucker" was used throughout the 4 lakes for sample collection. Sampling devices such as the Ekman dredge are not advisable for use in microbiological studies of the sediment-water interface. When the dredge hits the bottom, clouds of sediment, including the "fluffy layer," are pushed away by the pressure wave created ahead of the sampler, as shown in the photographs of Aarefjord (1972). Many of these aspects of bottom sampling are discussed further by Lackey (1961).

Previous workers (Hall et al., 1972) have reported increases in the V_{max} of uptake and mineralization for glucose and acetate measured with sediment cores incubated in the dark. This was tested for sediments from Lake Washington but the reports could not be substantiated. In fact, rates were lower when sediment samples were incubated in the dark than in a well-lighted room. The reason for lower rates in the dark is not apparent. It is relevant to point out that this occurred in sediments collected from the littoral zone which is subject to light fluctuations, but not in sediments from the benthic zone at 60 m which is below the photic zone.

Some investigators incubate sediment or water samples under static conditions, while others subject samples to shaking during the analysis for substrate uptake, mineralization of organic solutes, or oxygen consumption. The

effect of shaking sediment samples during the analysis for glucose mineralization has not been reported by other workers and so was examined here. Shaking the sediment samples during incubation with ^{14}C glucose always resulted in up to twice the amount of $^{14}CO_2$ evolved as compared to unshaken samples, resulting in a higher V_{max} and shortened turnover time. Since most sediments in lakes are not subjected to shaking, with the exception of wave action near the shore and convective currents occurring after temperature destratification, shaking samples during analysis is not to be recommended to duplicate in situ conditions. However, this variable should be considered in comparing data obtained by other investigators measuring aquatic microbial activities. The effect of shaking on rate may be due to dislodging bacteria attached to sediment or detrital particles. Bacteria in aquatic habitats probably live primarily attached to sediment or detrital particles, or in colonial masses rather than as free cells (Bott and Brock, 1970; Floodgate, 1972; Hunding, 1973; Mikhaylenko and Kulikova, 1973; Paerl, 1974, 1975; Rodina, 1963, 1966). The mixing that occurs during shaking can also cause increases in V_{max} by increasing the probability of bacteria coming into contact with the substrate. In statically held samples, microzones of substrate depletion can occur around bacterial cells, and shaking would reduce this. Wood and Chua (1973b) found differences in substrate concentrations in sediments where thorough mixing was physically inhibited. Allen (1971) found that shaking epiphytic samples resulted in increased uptake rates of ^{14}C-labeled glucose and ascribed it to dispersion of clumped bacteria. Stirring sediment also increases oxygen uptake. Statically held samples may have oxygen depletion around settling particles, with lower oxygen uptake resulting (Hargrave, 1972).

Various substrates known to affect bacteria were added to sediment samples to determine if influences of these substrates on sediment bacteria could be detected by the glucose mineralization rate method of Harrison et al.

(1971). These substrates or additions included: phosphate; diesel oil; and freeze-dried plankton. Inhibition occurred when high concentrations of phosphate and No. 2 diesel oil were used. Inhibition appeared to be noncompetitive (decreased V_{max} and prolonged turnover time) with diesel oil, a potential pollutant in aquatic ecosystems. With potassium phosphate, the inhibition was competitive (V_{max} unchanged, turnover time prolonged). The high levels of phosphate may have competed for bacterial uptake sites, or exercised intracellular inhibition. It would be interesting to test the effects of lower concentrations of phosphate on the glucose mineralization rate, because it is thought to be stimulatory to bacteria in situ (Paerl and Goldman, 1972; Thomas, 1969).

Since glucose mineralization rates of sediment bacteria were affected by these additions, the method of Harrison et al. (1971) may be of use in determining the effects of substrates or potential inhibitors on aquatic bacteria. These data indicate the glucose mineralization rate method could be of value in pollution studies provided controls or baseline data were available.

The addition of freeze-dried plankton caused an initial competitive inhibition of glucose mineralization rate by sediment bacteria after 2 hours of preincubation. This suggests that the sediment bacteria were using labile materials from the plankton as well as the labeled glucose, thus increasing the turnover time, but with no effect on V_{max}. After 24 hours, the turnover time of the sample incubated with plankton was still longer than the control, but the V_{max} had increased 3 times over that observed for the control. Perhaps an induction of uptake systems and mineralization enzymes had occurred similar to that observed by Harrison et al. (1971) for sediment samples incubated with high levels of glucose. The presence of other carbohydrates is also known to affect glucose uptake by microorganisms. Lactose has been found to be stimulatory to glucose uptake, whereas cellobiose, galactose, sucrose and maltose decreased net uptake in one

study (Wood, 1973). Algae contain several carbohydrates such as structural polysaccharides (Shnyukova and Pirozhenko, 1972) and several monosaccharides (Kvasnikov et al., 1973), thus it is not surprising that incubating sediment samples with freeze-dried plankton affected the glucose mineralization rate and may be of use in future studies.

SEDIMENT BACTERIAL GLUCOSE MINERALIZATION RATES IN THE FOUR LAKES

A total of 50 littoral, 55 benthic and 18 sediment samples collected at stations other than the main sampling stations were analyzed for glucose mineralization activity from July 1972 to April 1974 in the 4 lakes.

Littoral Zone

In the 4 lakes studied, higher glucose mineralization rates and bacterial plate counts were found in sediment from the littoral than benthic zones, with the exception of Lake Sammamish. This is understandable because the littoral zone of a lake is an active area of the lake ecosystem. A significant portion of the total biomass is algae and bacteria attached to aquatic plants. The bacteria in the littoral zone may possess a greater biomass than the phytoplankton in this region (Allen, 1971). The sediment-water interface, under shallow water, appears to be dominated by bacteria (Hayes, 1964) which are more prevalent in shallow areas than in deep water (Goman, 1973) due to the high concentration of organic matter in the water of this region (Collins, 1963). The littoral zone plays an important role in the lake ecosystem as a source of dissolved organic material (Allen, 1971). Epiphytic bacteria and sediment forms are responsible for the removal of much labile organic carbon from this area. Wave activity contributes to the replacement of littoral water, thus increasing nutrient availability (Allen, 1971). The littoral zone contributes to

autochthonous production with indirect effects on pelagic and benthic carbon metabolism; in the majority of lakes, the littoral zone contributes a major component of the organic carbon flux (Wetzel and Rich, 1973).

Increased bacterial activities and numbers found in the littoral zone compared to the benthic zone, may be related to particle size. There is an inverse relationship between mean grain size of sediment and microbial activity (Fenchel, 1970; Gosselink and Kirby, 1974; Hargrave, 1972) carbon: nitrogen ratio of detritus (Newell, 1965), bacterial biomass (Dale, 1974; Fenchel, 1970) and bacterial growth (Gosselink and Kirby, 1974).

In the marine environment, there are similar findings of greater biological activity and bacterial biomass in inshore waters and estuaries than in areas remote from land (Okutani et al., 1972; Sorokin, 1971; Vaccaro, 1969). In shallow marine water, bacterial respiration accounts for 64% of the total community respiration, while in deep sea waters, bacterial activity is less important (Smith, 1974; Smith and Teal, 1973).

Lake Sammamish

Glucose mineralization rates in Lake Sammamish were higher for benthic than for littoral sediments in September 1972 and from August to December 1973. The increased benthic rates coincided with the development of anoxic conditions in the hypolimnion in late summer of both years. Increased bacterial glucose mineralization rates under anaerobic conditions in Lake Sammamish, may be caused by the decreased efficiency of bacterial metabolism under anaerobic conditions. Most bacteria under aerobic conditions are approximately 50% efficient in substrate utilization, while under anaerobic conditions, the efficiency is lowered to 5 to 10% (Brock, 1966). The increased rate may also be partially responsible for the oxygen depletion and should be studied further. Sediment bacteria, under conditions of low water flow in lakes, can completely consume oxygen

in the hypolimnion due to heterotrophic activities (Konstantinova, 1971). Also, the interaction of the sediment bacteria and phosphate released from the sediments under increasing anaerobic conditions is of interest. Phosphorus is the dominant limiting element to phytoplankton in Lake Washington (Edmondson, 1970) and the Cedar River Watershed lakes (Welch et al., 1975). The chemical basis for phosphorus recycling in Lake Sammamish has been well documented. Horton (1972) found that sediments of this lake act as a "sink" for phosphate, and the phosphorus levels are controlled by iron and the redox conditions of the lake. After the fall overturn, there is usually a sharp rise in total phosphorus (Horton, 1972). Monahan (1974) found as much as 75% of the phosphorus released into the hypolimnion of Lake Sammamish was supplied by the sediments. It is surprising that up to this time, no one has investigated the microbial role in the phosphorus cycle in Lake Sammamish, for bacteria are known to be important in promoting phosphorus exchange at the sediment-water interface (Harrison et al., 1972; Hayes, 1953), and accelerate the rate of phosphorus return from sediment to the water (Hayes and Phillips, 1958). The decomposing organisms, chiefly bacteria, are responsible for most of the recycling of organic phosphorus (Nazarkin, 1960). Bacteria can be the primary cause of rapid turnover of phosphate in lakes (Rigler, 1956), and bacteria can compete effectively with algae for the available phosphate (Rhee, 1972; Rigler, 1956).

Bacteria that can solubilize a number of phosphates ($FePO_4$, $Ca_3(PO_4)_2$, $CaHPO_4$, $Al_2(PO_4)_2$ and $Mg_3(PO_4)_2$) have been isolated from sediment (Harrison et al., 1972). Solubilization is associated with carbohydrate metabolism and aerobic growth, with solubilization attributed to the formation of organic acids which function as chelating agents, releasing free phosphate that can be utilized by algae (Harrison et al., 1972).

Bacteria can also transform organic phosphate to inorganic phosphate through the action of alkaline phosphatase, and bacteria possessing this enzyme are an essential constituent of the bacterioplankton in lakes (Reihardt, 1973). Alkaline phosphatase activity is easily detected by adding phenolphthaleinphosphate to a CPS medium. A clear zone around bacterial colonies, indicates hydrolysis due to alkaline phosphatase activity (Reihardt, 1973). It would be most interesting to study the role of bacteria in the phosphorus cycle in Lake Sammamish, as this area has received very little attention.

The Sediment-water Interface

Higher glucose mineralization rates occurred at the sediment-water interface than in the water column in the 4 lakes. Higher glucose mineralization activity was found at or near the sediment-water interface than in the water column in other lakes as well (Allen, 1971; Harrison et al., 1971; Wood and Chua, 1973a) and has been attributed to the microbial community rather than macrobenthic organisms (Brinkhurst et al., 1972). The higher rates of microbial activity and greater bacterial biomass (Stokes and Redmond, 1966) at the sediment-water interface may be due to higher concentrations of dissolved free labile organic solutes in sediments than in the water column (Wood and Chua, 1973b), and in the availability of solid surfaces in the sediment to which bacteria can attach (Bott and Brock, 1970; Floodgate, 1972). Attachment is considered advantageous to microorganisms, may be necessary for survival in oligotrophic waters, and may involve some interaction between the organism and the settlement surface (Floodgate, 1972; Jannasch and Pritchard, 1972).

Seasonal Variation in Glucose Mineralization Rates in the Four Lakes

Glucose mineralization rates varied seasonally in all of the 4 lakes, with higher rates in summer than in winter. On the basis of V_{max} values,

summer rates in Findley Lake, the lake of highest altitude, exceeded the summer maximum values observed for the two mesotrophic lakes, Washington and Sammamish. Increased uptake rates in summer and lower rates in winter have been found by other workers for bacteria in freshwater lakes for amino acids (Burnison and Morita, 1974; Crawford et al., 1973), glucose (Allen, 1969, 1971; Boylen and Brock, 1973; Crawford et al., 1973; Morgan and Kalff, 1972; Wetzel et al., 1972), sucrose (Thompson and Hamilton, 1973), and acetate (Paerl and Goldman, 1972) in the water column and for uptake of glucose and acetate in lake sediments (Hall et al., 1972). The maximum uptake of these solutes by aquatic bacteria in summer has been attributed to higher water temperatures, increased dissolved organic material resulting from phytoplankton blooms (Allen, 1969; Crawford et al., 1973) and to metabolic differences between bacterial species predominating in lakes during the summer as compared to winter months (Allen, 1971; Burnison and Morita, 1974).

BACTERIA IN THE FOUR LAKES

The majority of the bacteria in the 4 lakes were aerobic or facultative anaerobic gram negative rods, with pigmented (usually yellow) species making up 30% of the population. Most of the organisms were motile. Other workers have reported that sediment bacteria were composed of aerobic and facultative anaerobic species (Hargrave, 1969) with the facultative anaerobes capable of more luxurient growth under aerobic conditions (Taylor, 1940). Most bacteria in lakes and oceans, where the concentration of organic matter is low, are filamentous or rod-shaped (Bowers and Bishop, 1966; Collins, 1963; Henrici, 1933; Ocevski, 1966; Mataruyeva, 1971; McCoy and Sarles, 1969; Zobell, 1946a). The numbers of gram negative cells exceed gram positive organisms in the marine and freshwater environment (Baig and Hopton, 1969; Bere, 1933;

Bowers and Bishop, 1966; Collins, 1963; Henrici, 1933; Ocevski, 1966; Sorokin and Kadota, 1972; Taylor, 1940), but the reverse is true in soil (Taylor, 1942). The prevalence of gram negative organisms in the sea and freshwaters is not completely understood; however, one hypothesis attributes this to antibacterial substances secreted by certain species of phytoplankton (Pratt et al., 1944; Say et al., 1963; Sieburth, 1959) which are more inhibitive to gram positive than gram negative bacteria. Costerton et al. (1974) suggest that gram negative bacteria are more adapted for live in the dilute aqueous environment of lakes for unlike gram positive, gram negative bacteria retain degradative enzymes in a highly protective association with the cell wall so that products of enzymic digestion are immediately available to the transport system of the cell.

Most aquatic microorganisms are motile (John and Boull, 1965) which is probably a means of distribution of bacteria and enables microorganisms to respond quickly to changes in the environment (Brock, 1966).

Pigmented bacteria are found in the aquatic environment (Taylor, 1942). Hunding (1973) reported 40% of the bacteria in lakes to be pigmented with mostly yellow and orange chromagens. The pigments have been suggested to play a protective role, shielding bacteria against photoinactivation (Alexander, 1971; McCoy and Sarles, 1969).

Bacteria were more numerous in littoral than in benthic sediments, with maximal numbers at the sediment-water interface. Bacterial numbers at the sediment-water interface were also 3 orders of magnitude higher than in the overlying water. High numbers of aerobic bacteria were present in the top 1 to 2 cm of sediment at the benthic station in Lake Washington and the numbers decreased with depth into the sediment. This is in agreement with the findings of other workers (Henrici and McCoy, 1938; Ocevski, 1966; Zobell, 1946b), although Hayes and Anthony (1959) found that bacterial numbers did not decrease

with depth into the sediment until beyond the first 5 cm of sediment. The logarithmic decline of bacteria with depth into the sediment is probably a function of the "actual layer," or that layer in the sediment subjected to mixing by the bottom fauna (Kleckner, 1967; Nichols, 1974) and is in contact with the water, as compared to the historical layer. Pamatmat and Bhagwat (1973) found that the actual layer for Lake Washington sediment is thinner than the 10 cm suggested for northern European lakes, so that it is not surprising that bacterial numbers decrease after 1 to 2 cm of depth into the sediment in this lake.

Bacterial Plate Count

Bacterial numbers in this study were determined by the plate count method using a modified medium based on that described by Taylor (1940). While plate count data are open to criticism on the basis of the selectivity of media, attempts to count bacterial populations by direct microscopic examination have fared little better (Bell et al., 1974). It is extremely difficult to count sediment bacteria by direct counting methods due to the interference from sediment and detrital particles, bacterial clumping and attachment to detritus particles, and presence of bacterial cells inside detritus particles (Olah, 1972; Rodina, 1963, 1966). The main source of error of the plate count is thought to be due to this clumping effect (Jannasch, 1958). Increases in plate counts have been achieved by treating samples with nonionic detergents at low concentrations (Jones and Jannasch, 1959), although this technique was not used in this study. While the plate count underestimates substantially the numbers of bacteria compared to direct counts (Collins, 1963; Jannasch and Jones, 1959; Sorokin and Kadota, 1972), it is valuable in microbiological studies of the aquatic environment. Different metabolic types of bacteria can be determined quantitatively and qualitatively by counting on agar plates.

The spread plate is the most suitable method for estimating numbers of viable bacteria (Jones, 1970), and is preferable to the pour plate. A significant decrease in bacterial numbers can occur with pour plates after the transient stress of warmed agar (Klein and Wu, 1974; Zobell and Conn, 1940). Starvation of bacteria that can occur in low nutrient aquatic environments leads also to an increasing susceptibility to secondary temperature stress (Klein and Wu, 1974).

The medium used by many workers in aquatic microbiology, and used in this study, was first suggested by Snow and Fred (1926), and later modified by Taylor (1940). This medium has been found best for counting aquatic bacteria in both unpolluted (Collins and Willoughby, 1962; Jones, 1970), and polluted rivers and streams (Staples and Fry, 1973). Of all the media tested by Jones (1970), the medium of Taylor (1940) supported the best growth.

The addition of yeast extract to media has been found stimulatory to marine bacteria (Carlucci and Pramer, 1957) and also significantly increased plate counts of lake sediment samples incubated at 4 and 12°C. Therefore, yeast extract (0.5 gm/L) was added to Taylor's medium and used throughout these studies.

Seasonal Variation in Bacterial Numbers

Bacterial numbers in the lakes tended to vary with season, with higher counts in the warmer summer months than in winter which was also reported by Bere (1933). Greatest seasonal variation in bacterial numbers occurred in Findley Lake, with lowest counts occurring during the winter ice cover. One exception was a high benthic zone count in March 1973. In Lake Chester Morse, there were fluctuations in bacterial biomass, but with no apparent correlation to season.

Increases in bacterial plate counts occurred after the autumnal circulation or turnover in the 4 lakes and have been observed in other lakes as well (Collins, 1963; Taylor, 1940). As early as 1902, Pfenniger (1902) found fluctuations of bacterial populations in Lake Zurich corresponding to periods of circulation and stratification of the water. After turnover, there are changes in species composition (Collins, 1963), with complete mixing of water column bacteria from the bottom of the lake to the top (Henrici, 1938) and dispersion of microbiological zones formed during thermal stratification (Collins, 1963). Autumnal circulation also causes homogenous distribution of algae and nutrients in the water column (Pechlaner, 1971), and increases in bacterial numbers at this time may be due to increased availability of nutrients.

There are many interacting factors responsible for the seasonal fluctuations of bacteria in aquatic ecosystems. Some workers reported fluctuations in bacterial populations as random and not seasonal (Weeks, 1944) and with no correlation to the concentration of dissolved substances in the water (e.g., NH_2, NO_3, silicates, PO_4, and O_2) (Taylor, 1940). Other workers have ascribed seasonal changes in bacterial biomass to nutrients from dying plankton (Goman, 1973), hydrobiological characteristics of the lake (Maturuyeva, 1971), and to meteorological conditions (Taylor, 1940). Bacterial plate counts have also been correlated with other measurements thought to be related to productivity such as alkalinity, conductivity, and oxygen consumption in clear water lakes (Hayes and Anthony, 1959).

Trophic Status and Bacterial Plate Count

For bacterial plate counts of sediment collected from the benthic zone, highest mean counts in 1973 were found for Lake Sammamish, a mesotrophic lake, and lowest for Findley, an oligotrophic lake. For littoral zone sediment,

highest mean values in 1973 were found for Lake Washington and lowest for Lakes Sammamish and Findley. Greater numbers of bacteria in lake water and sediments have been observed in eutrophic than in oligotrophic lakes (Bere, 1933; Henrici and McCoy, 1938; McCoy and Sarles, 1969; Ocevski, 1966; Romanenko, 1969; Straskraba and Straskrabova, 1969). In these 4 lakes, plate counts of sediment did not completely reflect the trophic state of the lake. Bacterial plate counts for the two oligotrophic lakes, Chester Morse, and Findley, were higher in the summer months than counts from the mesotrophic lakes, Washington and Sammamish, during this time; however, during the colder winter months, the plate counts of sediment from the two oligotrophic lakes were lower than winter counts from Lakes Washington and Sammamish. If a comparison had been made between oligotrophic and eutrophic lakes, greater differences would have been expected.

TROPHIC STATUS AND GLUCOSE MINERALIZATION RATE OF SEDIMENT BACTERIA

The kinetic uptake of glucose by bacteria has been measured in aquatic environments of different trophic status (Allen, 1969; Hamilton and Preslan, 1970; Hobbie and Wright, 1968; Vaccaro and Jannasch, 1966, 1967; Vaccaro et al., 1968; Wright and Hobbie, 1965). Glucose uptake rates seem to be related to trophic state as indicated by phytoplankton primary production (Overbeck, 1974), with increased rates of uptake measured for eutrophic (Hobbie and Wright, 1968; Overbeck, 1974; Romanenko, 1973; Wright and Hobbie, 1965, 1967) and mesotrophic (Morgan and Kalff, 1972) compared to oligotrophic lakes. Increases in V_{max} of uptake have also been found to be indicators of pollution with higher V_{max} values found in rivers and streams at discharge points of sewage effluent and wastes from a phosphate mine (Hobbie and Crawford, 1969).

Trophic status and glucose mineralization rates were related on the basis of turnover times but not V_{max} values. The turnover times for the two mesotrophic lakes, Washington and Sammamish, were shortest of all lakes sampled, with the lowest turnover time found for Lake Washington. Summer rates for all lakes were about the same. Winter glucose turnover times were longest for the two oligotrophic lakes, Chester Morse and Findley, with the longest turnover time measured for Findley Lake, just prior to melting of the ice.

On the basis of V_{max} values, the trophic state and glucose mineralization rate were related only during the winter months with highest values found for mesotrophic Lake Washington. The V_{max} values for Lake Sammamish, however, were the lowest of all lakes sampled. The consistently low V_{max} values determined for this lake may indicate that:

1. The bacterial population in this lake is not respiring as much glucose to CO_2 as in the other lakes. The percentage of glucose utilized by bacteria has been found higher in eutrophic lakes (Romanenko, 1973) and perhaps may be higher in mesotrophic than oligotrophic lakes as well.

2. Perhaps glucose levels are lower in this lake for the V_{max} is related to substrate level (Wright, 1973).

3. The bacteria of the sediment in Lake Sammamish contain a smaller percentage of organisms able to use glucose, for V_{max} is the maximum rate at which a population can use a given substrate.

4. The lower V_{max} in the littoral zone of this lake may be due to lower numbers of bacteria in this zone of Lake Sammamish than in the other lakes for V_{max} is also directly related to bacterial biomass (Morgan and Kalff, 1972) in some lakes.

The V_{max} values for Findley Lake after melting of the ice were the highest of all lakes sampled probably due to the influx of organic materials from the melting ice and snow. Greatest seasonal variation in rate of bacterial glucose mineralization and greatest fluctuations in bacterial numbers occurred in the two oligotrophic than in the mesotrophic lakes.

FINDLEY LAKE

Sediments collected from Findley Lake showed the greatest seasonal variations in both bacterial glucose mineralization rates and plate counts, as well as secondary production reported by Pederson (1974). In Findley Lake, as in other high mountain lakes (Rodhe et al., 1966), the greatest temperature variations and extremes in photic conditions occur. The UV light penetration, thought to be inhibitory to algae, is increased due to the optical purity of the water, and hence algal production occurs at lower depths with peak values at 15 m (Hendry and Welch, 1974). Inhibition of algal production by UV light has been called into question by Pechlaner (1971). This author finds UV light inhibition unlikely, and suggests inhibition is due to the fact that nutrients used by algae are in the metalimnion and hypolimnion. The intensities of radiant energy are optimal for algae under an adequate supply of nutrients, but detrimental when nutrients are lacking. Therefore, the depth of algae in these lakes is a nutrient-dependent light effect because in the ice-free period the meta- and hypolimnion are the only receivers of colder inflow nutrient-rich surface waters. Whatever the reason for algal production at lower depths, more plankton detritus settles to the bottom in Findley Lake for mineralization by benthic bacteria than in the other 3 lakes. This may explain the higher rates observed in this lake than in the other lakes during the summer. In Findley Lake, the highest sediment bacterial glucose mineralization rate, maximum phytoplankton primary productivity (Hendry and Welch, 1974) and emergence of Chironomid larvae (Bissonnette, 1974) occurred immediately after ice out in July 1972. The peak in secondary productivity in 1972 followed one month later (Pederson, 1974). The peak of bacterial glucose mineralization also occurred after ice out in June 1973. The increases in biological activity and biomass in Findley Lake, following ice melt,

may be due to the input of forest litter which comprises the major part of the tripton in the lake and which enters the lake mainly on completion of snow and ice melt (Birch, 1974). Also, the temperature and light intensity increase at this time. In the winter, bacterial glucose mineralization rates were at minimal levels, especially just prior to ice melt. Uptake of ^{14}C glucose and acetate (Allen, 1971) and primary production (Wetzel et al., 1972) has been found to occur at minimal rates under ice cover in other lakes as well. The complete occlusion of light from the lake by snow and ice cover also prevented phytoplankton production in Findley Lake (Hendry and Welch, 1974). In some high mountain lakes, during winter ice cover, a maximum of phytoplankton occurs just under the ice (Pechlaner, 1971). This phenomenon and attendant microbiological interactions would be interesting to investigate in Findley Lake.

RELATION BETWEEN ALGAL PRODUCTION AND BACTERIAL ACTIVITY

Algal primary productivity and heterotrophic bacterial glucose mineralization could be correlated in both Lakes Sammamish and Findley. Primary productivity data were not available for Lakes Washington and Chester Morse to make similar comparisons.

In Lake Sammamish, bacterial glucose mineralization rates increased one month after the maximum in phytoplankton primary production. In Findley Lake, both bacterial biomass and heterotrophic activity, and phytoplankton primary production reached maximal values at the same time, immediately following the melting of the ice in 1972. The bacteria, stimulated by increased nutrient after ice melt may have supplied growth factors for the phytoplankton (Allen, 1971) or the phytoplankton may have supplied dissolved nutrients known to be utilized (Kuznetsov, 1968) and stimulatory to bacteria (Bell et al., 1974). Morgan and Kalff (1972) found a similar sharp peak in bacterial numbers and glucose uptake

rates in the water column of high arctic lakes after spring melt, but they could not relate bacterial cycles to phytoplankton cycles due to the low rates involved. In high mountain lakes there is considerable temporal parallelism between bacterial biomass and phytoplankton production rates (Pechlaner, 1971); a high population of bacteria develops in high mountain lakes in summer and decreases parallel to that of phytoplankton in the ice covered lake. Many workers have found a direct correlation between bacterial biomass and activity (respiration, mineralization, uptake of ^{14}C-labeled organic substrates) and phytoplankton biomass and production (Allen, 1971; Burnison and Morita, 1974; Goman, 1973; Hargrave, 1969; Henrici, 1938; Hobbie and Wright, 1968; Holm-Hansen and Paerl, 1972; Kadota et al., 1966; Kuznetsov, 1968; Mikhaylenko and Kulikova, 1973; Overbeck, 1974; Paerl and Goldman, 1972; Taylor, 1940). The coupling of bacteria and phytoplankton is not simple. The carbon flow in aquatic ecosystems is a set of oscillating systems with heterotrophic bacteria involved in short time oscillations (Overbeck, 1974). With this rapid recycling of primary productivity products, there is little net loss to the system (Hargrave, 1969). It has been suggested (Golterman, 1972) that the rate of bacterial mineralization is the main factor controlling primary productivity and without this mineralization or recycling of products from algal cycles, algal growth in most lakes would come to an end. Bacteria grown with algae in nonanexic (Overbeck, 1974) and anexic cultures separated by membrane filters, permitting cross feeding (Scott and Ball, 1975) instantly mineralize extracellular metabolites of algae and influence algal growth rates via the CO_2 system and subsequently enlarge algal growth ranges. Blue-green algae in all stages of development are good substrates, energy sources, and are growth stimulating to bacteria (Mikhaylenko and Kulikova, 1973). Many strains of nitrogen fixing blue-green algae liberate from 5 to 50% of the total carbohydrates formed during photosynthesis (Kosenko,

1973), including monosaccharides and all the components needed for growing heterotrophic microorganisms (Kvasnikov et al., 1973). Bacteria capable of lysing blue-green (Daft and Stewart, 1971; Granhall and Berg, 1972; Gromov et al., 1972; Shilo, 1970) and green algae (Stewart and Brown, 1969) have been isolated. The products of algal lysis, which include carbohydrates and amino acids, may induce bacterial attack on algal cells (Golterman, 1972). The use of these bacteria capable of lysing algae have been suggested as a possible biological control of algal blooms (Stewart and Brown, 1969).

The amounts of carbohydrates liberated by algae are greater under poor growing conditions and increase with the age of the culture (Kosenko, 1973). Batch culture data indicate that bacteria are stimulated only during algal senescence (Bell et al., 1974). It is not surprising then to find that in many studies, bacterial numbers and activities lagged behind the pulses of phytoplankton biomass and productivity as occurred with Lake Sammamish. After phosphate depletion in the epilimnion, growth of phytoplankton stops, and the breakdown products lead to an increase in bacterial numbers (Overbeck, 1974).

Some investigators have found considerable diel variations in bacterial activities. Changing light and excretion patterns can vary throughout the day (Overbeck, 1974). This can affect bacterial mineralization due to organic substrates leaching out of algal cells from shortly before noon through the afternoon, with a peak of 1700 to 1900 hours as found by Hunding (1973) in the littoral zone of an oligotrophic lake. The material leaching out of algal cells supported the bacterial requirement for organic material, resulting in a logarithmic increasing rate of bacterial respiration during the afternoon. Respiration rates of sediment cores incubated at night have been found to be two to three times higher than during the day (Hargrave, 1969). Diel variations in sediment bacterial glucose mineralization rate were not investigated, but may be of interest for future work.

All sediment samples were collected in the 4 lakes from 1000 to 1300 hours.

TEMPERATURE

Lake sediment bacterial glucose mineralization rates and bacterial plate counts were affected by temperature. A number of relationships were tested for significance using linear regression analysis on data obtained from the littoral and benthic zones in each of the lakes.

1. Log glucose turnover time and 1/T absolute. Turnover times for bacterial uptake of glucose and acetate have been found to be inversely related in the water column of lakes (Allen, 1969). Turnover times for glucose mineralization were indirectly related to temperature for the littoral zone of Lakes Washington, Sammamish, and Chester Morse and significant at the 5% level. Bacteria associated with sediments from Lake Chester Morse had higher rates at lower temperatures with shorter turnover times than did those from a lake of lower altitude, Lake Sammamish. Sediment bacteria from Lake Sammamish littoral zone were the least responsive to temperature. Data from Findley Lake littoral zone suggest a similar relationship between glucose turnover times and temperature, but the data were not statistically significant. For Lake Washington, most of the data points fell along a straight line; however, data collected twice in April in 1973, fell outside the majority of the points, and the relationship between temperature and turnover time was significant only if these 2 points were excluded. During April, light intensities were increasing, causing vernal outbursts of algae; this algal activity could have affected bacterial activity in addition to temperature, rather than temperature alone. For benthic samples, only Lake Sammamish data were significant when analyzed statistically; benthic bacteria were more responsive to temperature than those from sediment of the littoral zone of this lake. Perhaps this is indicative of a different species

composition in the 2 regions of this lake.

2. Log V_{max} and 1/T absolute. The maximal velocity of uptake of substrates and temperature have been correlated by other workers (Allen, 1969, 1971; Burnison and Morita, 1974). The relationship between log V_{max} for glucose mineralization and 1/T absolute was significant for littoral zone sediment samples from Lake Sammamish (5%) and Findley Lake (1%). For Lake Sammamish, the relationship was significant only if data collected in April and July 1973 were excluded. In 1973, the maximum peak of algal production occurred March 17, at 1581 mgC/m^2/day, followed by a second pulse, July 13, at 859 mg C/m^2/day (unpublished data courtesy of Dr. Eugene Welch, Dept. Civil Engineering, Univ. Washington). It is possible that these peaks of primary productivity, and attendant organic material produced by the phytoplankton, were affecting the sediment bacteria at this time to a greater extent than temperature. For benthic samples, the relationship was significant at 1% only for sediments from Lake Washington.

3. Log bacterial plate count and temperature. Numerous studies indicate that temperature and bacterial biomass are related. The log of the plate count was significantly related to temperature only for sediments from the littoral zone of Findley Lake (0.1%). For every 10 degree rise in temperature (Celsius), the bacterial plate count increased by one log unit.

4. Log bacterial plate count and log V_{max}. Morgan and Kalff (1972) found a significant correlation between total bacterial numbers and V_{max} of glucose uptake for a high arctic lake. However, Innis (1975) could not find a relationship between respiration of $^{14}CO_2$ from labeled glucose and bacterial numbers in lake sediments using cores. Innis suggested that this may be due to the lag in response of dominant bacterial populations to temperature changes in natural water environments found by Sieburth (1967). The relationship of

plate count to V_{max} of glucose mineralization was significant at the 5% level only for data from the littoral zone of Lake Chester Morse. Perhaps if inoculated spread plates had been incubated at temperatures closer to the in situ temperature, a better correlation between bacterial biomass with temperature and bacterial activities would have emerged. Inoculated plates unfortunately were incubated only at an approximate in situ temperature (4, 15 and 22°C) due to a lack of thermostatically controlled low temperature incubators. Also, it is possible that plate counts, known to underestimate bacterial biomass, are not sufficiently sensitive to show relationships between temperature and bacterial numbers. If glucose had been incorporated into the medium used in this study, perhaps a correlation would have been found between plate count and glucose mineralization activity. Correlations have been found between plate counts on media supplemented with various single organic acids and uptake of these respective acids by bacteria in the water column of lakes (Robinson et al., 1973). The same relationship could be expected using glucose.

There were more interactions between temperature and bacterial activities for sediment samples from littoral than from the benthic areas of the 4 lakes. This finding suggests that temperature in the benthic zone, which fluctuates seasonally by only $\pm$ 2°C, is not a variable influencing sediment bacterial biomass and activities. Other factors such as meteorological, nutritional, and hydrobiological are probably involved.

Zobell et al. (1953) found that bacterial activities in Lake Mead sediment raised the temperature of sediment near the surface by as much as 5°C over that of the overlying water. Such a temperature increase was sought in this study and was found only once with sediments of lower Findley Lake in late summer. The temperature at the sediment-water interface was 5.3°C, while that of the overlying water was 5.0°C. The lower lake at this time had

very little water flowing through it, had very high sediment bacterial glucose mineralization rates, and relatively high bacterial plate counts. Therefore, the increased temperature in the sediment may have been due to bacterial activities.

When sediments were incubated at temperatures up to 20 or 30°C above the prevailing in situ temperature, there was a linear increase in the maximum velocity of glucose mineralization for bacteria associated with the sediment in all 4 lakes. At temperatures above 30°C, inhibition occurred and the V_{max} decreased with temperature increase. Similarly, the glucose turnover times were inversely related to temperature up to 30°C; above this temperature, the turnover time increased with increasing temperature. The temperature optimum for glucose mineralization of benthic zone samples collected in winter ranged from 20°C for Findley to 30°C for Lake Washington. This optimum temperature was considerably higher than the in situ temperature of 4 to 6°C prevailing in the lakes at this time. The temperature optimum also shifted with season with the optimum of winter isolates less than 20°C, while the summer temperature optimum was 28°C. This shift in optimum temperature for the maximum velocity of glucose mineralization occurred with sediments from the venthic station of only Findley Lake; however, since the temperature optimum appears to vary with prevailing water temperatures in this lake, similar findings might result with further studies of sediment from the other 3 lakes. The populations of sediment bacteria apparently are not closely adapted to the prevailing temperature of the sediments, but to temperatures several degrees above as determined by the V_{max} for glucose mineralization. This agrees with the findings of Bott (1975) for uptake of ^{14}C-labeled glucose by bacteria in streams and with Boylen and Brock (1973) for mineralization of ^{14}C-labeled glucose by bacteria in lakes. At suboptimum temperatures, the growth rate and production of bacteria are lower than at the optimum temperature (Brock, 1970). With most organisms, the

temperature optimum is near the maximum and lethal temperature (Brock, 1970; Zeikus and Brock, 1972). Therefore, it is dangerous for organisms in unstable thermal habitats to have a temperature optimum for growth or activities exactly at the environmental temperature. Organisms adapted to a higher optimum than the prevailing temperature will survive if increases in temperature occur.

Although the temperature optimum for ^{14}C-labeled substrate incorporation by benthic bacteria increases in parallel with water temperature, increased water temperature does not always result in increased activity of the adapted bacterial population (Zeikus and Brock, 1972), as predicted by the Arrhenius equation (Bott and Brock, 1969). Instead there is a seasonal selection of thermal types by water temperature (Allen and Brock, 1968). The optimum growth temperature of organisms is genetically determined (Brock, 1970), is unaffected by growth temperature (Jennison, 1935; Reihardt, 1973), but can be influenced by the nature of the medium (O'Donnovan and Ingrahan, 1965; Sorokin and Kadota, 1972). Recent studies indicate that the membrane structure may be a key factor determining the growth temperature ranges of psychrophilic (Finne, 1974) and thermophilic (Chan et al., 1973) bacteria.

Seasonal differences in temperature response occurred in the lakes. Sediment bacteria were more responsive to temperature increases during the winter months than in the summer. These data indicate that the bacterial species composition changes with seasonal temperature changes in the sediment of the lakes, with bacteria capable of mineralizing glucose at lower temperatures predominant in the winter months. During the warmer summer months, the predominant bacteria in the sediments are not as responsive to temperature and mineralize glucose at a slower rate at lower temperatures. In aquatic ecosystems, the seasonal selection of thermal types by water temperature, occurs in all taxonomic groups with no apparent suppression or enhancement of any

taxonomic group (Sieburth, 1967). Changes in temperature will alter the species composition of the bacteria and adaption to altered temperature is likely to occur by changes in species composition rather than through physiological or genetic changes in the species already present (Alexander, 1961). Psychrophilic bacteria in lakes (Reihardt, 1973) and in estuaries (Pierce et al., 1975) decrease in number as water temperatures increase. Similarly, the temperature optimum of bacteria that develop on cover slips in termally polluted sections of a river is higher than in nonpolluted sections (Bott and Brock, 1970).

Bacteria from the sediment of the 4 lakes grew faster at temperatures higher than the in situ temperature. Of the bacteria tested in pure culture, the temperature optimum for growth was usually from 20 to 28°C, similar to the optimum temperature for glucose mineralization. Very few of the organisms from the 4 lakes were capable of growth in either solid or liquid media above 32°C. These data are in accord with the reports that most aquatic microorganisms have optimum growth temperatures much below 37°C (Sorokin and Kadota, 1972). Psychrophilic bacteria, organisms able to grow at 0°C but not at 22°C were found in sediments from the Lake Washington benthic zone and the littoral zones of the higher altitude lakes, Chester Morse and Findley.

CELLULOSE AND CELLULOLYTIC BACTERIA

Cellulolytic bacteria were present in sediments from the littoral zone of Lake Washington and at both stations at Lake Sammamish. All of the cellulolytic bacteria isolated were gram positive obligately anaerobic rods, comprised from 1.3 to 2.4% of the total anaerobes counted, and numbered 10^4 organisms/gm dry weight sediment. The cellulolytic bacteria were difficult to purify and to maintain in pure culture. Other workers encountered the same difficulties and found cellulolytic Clostridia hard to isolate and characterize

(Skinner, 1960). In many cases, cellulose digesters grew symbiotically with a gram negative facultative organism unable to digest cellulose. Diminished cellulolytic activity occurred when the obligate anaerobe was tested in pure culture. Other workers have also found mixed cultures more effective in degrading cellulose than pure cultures with cellulolytic Clostridia (Enebo, 1949) and with aerobic Cellulomonas sp (Hofsten et al., 1971). It has been suggested that the noncellulolytic bacteria which can grow on glucose and cellobiose, but not cellulose are living on the cellulose decomposition products and may stimulate the cellulolytic organisms by removal of end products or may secrete growth factors (Hofsten et al., 1971).

SEASONAL ABUNDANCE OF CHITINOCLASTIC BACTERIA IN THE FOUR LAKES

There have been very few studies of chitinoclastic bacteria in freshwater lake sediments, although in the marine environment, these bacteria have been studied for many years.

Bacteria capable of hydrolyzing chitin appear to be widely distributed in the sediments of lakes in the Lake Washington drainage basin. Chitinoclastic bacteria were present in sediment at both littoral and benthic zone stations in all 4 lakes. The total numbers and proportion chitinoclasts to the total heterotrophic population changed very little with season in the two lakes of lower altitude, Washington and Sammamish, and comprised from 1 to 7% of the aerobic population. There was a slight increase in chitinoclastic bacteria in Lake Washington in the summer months. This has been reported by other authors for marine chitinoclastic bacteria. In the marine environment, the abundance of chitinoclastic bacteria is related to temperature, with higher numbers in the warmer summer months (Chan, 1970; Kaneko and Colwell, 1973; Seki, 1965a, 1965b; Seki and Taga, 1963a, 1965b). The observed increases may be related

to high nutrient content in the form of dissolved organic matter and dead plankton in the summer in addition to temperature (Seki and Taga, 1963a). Quite different seasonal data were obtained for the two oligotrophic lakes, Chester Morse and Findley. Higher numbers and percentages, up to 80% chitinoclastic bacteria, were found in these lakes with largest populations occurring in the winter months. Perhaps in these two lakes, which experience the most extreme meteorological and nutrient conditions of all the lakes sampled, chitin represents a valuable food reserve for the chitinoclastic bacteria during winter after the more labile materials from summer algal blooms have been consumed, and thus these bacteria proliferate in the winter.

Numbers of chitinoclastic bacteria varied from 10^4 to 10^7 organisms per gram dry weight of sediment in the 4 lakes and are within the range of values reported for the marine environment. Numbers of chitinoclastic bacteria have been reported at levels from 10^3 cells/gm, 10^4 cells/gm, and 10^3 cells/ml in sediments off the California coast (Zobell and Rittenberg, 1938), sediments of the Mediterranean Sea (Bianchi, 1971) and surface waters in Sagami Bay (Seki and Taga, 1965c), respectively. In Puget Sound, Chan (1970) found 10^4 cells of chitinoclastic bacteria per gram and 10^3 cells/gm in freshwaters. The distribution of chitinoclastic bacteria in a salt marsh appears to be related to organic matter, chitin deposition and temperature as reported by Hood and Meyers (1973). Highest concentrations of chitnoclasts (10^6 cells/gm sediment) occurred in areas of high organic content while the peak of bacterial biomass coincided with optimum numbers of chitin-producing animals in the water column (Hood and Meyers, 1973).

The abundance of chitinoclastic bacteria varied somewhat between stations in Lakes Chester Morse and Findley. In Lake Chester Morse chitinoclastic bacteria were more abundant in the littoral zone. In Findley Lake the total

numbers of bacteria were about the same at both stations, but a higher percentage of chitinoclastic bacteria was present in the benthic zone. In Lakes Washington and Sammamish, the abundance of chitinoclastic bacteria was about the same in both littoral and benthic sediment. It has been suggested that the abundance of chitinoclastic bacteria is related to the availability of chitin or crustacean zooplankton (Buck and Barbaree, 1971; Lear, 1961; Seki and Taga, 1965c). Chitinoclastic bacteria have been found adsorbed onto living copepods (Kaneko and Colwell, 1975; Seki, 1965a) for example. Chitinoclastic bacteria were found on exoskeletons of living crayfish from Lake Chester Morse and it may be logical to assume the chitinoclastic bacteria may be associated with chitinous organisms in the other 3 lakes as well.

ENRICHMENT

Increases in both numbers and percentages of chitinoclastic bacteria were achieved by enriching sediment samples with a variety of substrates. In most cases, numbers of both chitinoclastic bacteria and total heterotrophs increased steadily with incubation time on many of the substrates. The most significant parameter to measure, therefore, is the proportion of chitinoclastic bacteria in relation to the total biomass of the heterotrophic bacterial population. An increase in the percentage of chitinoclastic bacteria thus signifies preferential or more rapid growth of these organisms in relation to the other heterotrophic bacteria in the sample. Maximum percentages of chitinoclastic bacteria were elicited by organic substrates within 9 to 14 days of incubation at the in situ temperature. After 15 days or more, the percentage of chitinoclastic bacteria decreased to low levels at 30 days. Slight increases occurred after this time up to 40 days. This decrease followed by an increase may have been due to auxotrophic growth or to the succession of different species of bacteria as nutrients were consumed.

While media with chitin gave an increased proportion of chitinoclastic bacteria, the maximum increase occurred when organic substrates such as peptone or FW broth were added in addition to chitin. Chan (1970) also found that organic substrates in addition to chitin enriched for the greatest numbers of chitinoclastic bacteria in marine sediment samples. A number of chitinoclastic bacteria from a variety of sources degrade chitin more readily (Clarke and Tracey, 1956; Johnson, 1932; Reynolds, 1954; Zobell and Rittenberg, 1938) and exhibit increased chitinase production (Monreal and Reese, 1969) when protein is added to the medium. Furthermore, shell disease in brine shrimp, thought to be caused by chitinoclastic bacteria seldom occurs in sea water deficient in organic nutrients (Seki, 1965a), while the richest source of marine chitinoclastic bacteria are the remains of decomposing crustaceans (Zobell and Rittenberg, 1938). The exoskeletons and residual tissues of these crustaceans contain proteins which substantiates the reports on the importance of proteins to the development and activity of chitinoclastic bacteria.

The fact that chitinoclastic bacteria were enriched by organic substrates other than chitin indicates that chitinoclastic bacteria are able to increase in numbers when other substrates are available. The rapid establishment of a chitinoclastic flora would be conducive to <u>in situ</u> chitin decomposition. Labile organic material such as proteins and amino acids accompanying chitinous materials thus serve as the initial substrate used by the developing chitinoclastic flora (Chan, 1970), and when labile compounds are consumed, microbial chitin decomposition proceeds.

Glucose, NAG, glucose + $CaCO_3$, and cellulose did not enrich for chitinoclastic bacteria. Chan (1970), however, found a substantial enrichment with NAG in marine sediments. The differences in enrichment by end products of chitin decomposition in this study and that of Chan (1970), may be due to

differences in the flora of fresh versus marine waters or to other factors.

Cellulose and $CaCO_3$ exerted a slight inhibitory effect. The inhibitory effect of $CaCO_3$ may have been due to inhibition of bacterial adsorption to chitin by residual Ca^{2+} ions when sediment samples were inoculated onto FWCh agar. Divalent cations have been found to inhibit adsorption and possibly the ability of Vibrio parahaemolyticus to degrade chitin (Kaneko and Colwell, 1975). Cellulose may have "inhibited" enrichment or the detection of chitinoclastic bacteria by acting as a surface to which chitinoclasts could adsorb, and because of this adsorption of chitinoclasts to cellulose, these organisms could have been underestimated by the plate count.

The effects of incubation temperature on chitinoclastic plate counts of sediment collected seasonally in the 4 lakes were inconclusive. Incubation temperature had variable effects on both the percentages and numbers of chitinoclastic bacteria. Highest percentages of chitinoclastic bacteria generally appeared on plates incubated at 15°C or below, thus it appears that many of the chitinoclastic bacteria in these lakes are adapted to growth at lower temperatures.

ISOLATES OF CHITINOCLASTIC BACTERIA

All of the chitinoclastic bacteria isolated from the lakes and selected for study were gram negative, oxidase positive, motile rods. Most of these were capable of fermenting glucose to acid without gas production and can be classified as belonging to the genera Vibrio or Aeromonas. These bacteria represented the most abundant chitinoclastic in the sediments of the 4 lakes. These two genera of chitinoclastic bacteria have been found in the marine environment by a number of workers (Chan, 1970; Kaneko and Colwell, 1973, 1975; Okutani, 1966; Zobell and Rittenberg, 1938). Little difference exists between these two genera (Bain and Shewan, 1968; Shewan and Veron, 1974),

although the two organisms can be distinguished from one another by sensitivity to the Vibriostatic agent, Pteridine (0/129) (Shewan et al., 1954).

A few chitinoclastic Streptomyces, Pseudomonas and Cytophaga sp were also found in the lake sediments. Both Streptomyces sp (Berger and Reynolds, 1958; Jeuniaux, 1966; Skujins et al., 1970), Cytophaga sp (Johnson, 1932; Jeuniaux, 1966; Stanier, 1947) and Pseudonomas sp (Jeuniaux, 1966) from a variety of environments have been known for many years to possess chitinoclastic activity.

Most of the chitinoclastic bacteria were motile. Many types of marine chitinoclastic bacteria are also motile (Seki, 1965a), and motility may be important for contact between the organisms and chitin. Chitinoclastic organisms showed activity on both reprecipitated and crude chitin. Hydrolysis rates were higher on the reprecipitated chitin, which has smaller particle sizes. The rate of microbial chitin hydrolysis has been found by other workers to be inversely related to the chitin particle size (Chan, 1970; Seki and Taga, 1963c).

It was necessary to maintain chitinoclastic isolates in a medium containing chitin, otherwise either decreased rates or no chitin hydrolysis occurred when retested. Johnson (1932) similarly found with Cytophaga johnsonae that cultures lost their ability to hydrolyze chitin when cultivated in media containing no chitin. Zobell and Rittenberg (1938) found symbiotic relationships between members of mixed cultures, with 2 or more different species of bacteria capable of attacking chitin when together but no hydrolysis occurred when bacteria were in pure culture. Occasionally higher rates of chitin hydrolysis occurred with mixed cultures than with pure cultures, although this was not investigated. All of the chitinoclastic bacteria were isolated with greater ease than were cellulolytic organisms from the 4 lakes which did demonstrate symbiotic relationships.

Sediment samples were incubated both aerobically and anaerobically on FWCh agar. All of the chitinoclastic bacteria were found to be aerobic species by the zone clearing method. No obligately anaerobic chitinoclastic bacteria were found, although on one occasion a chitinoclastic bacterial colony appeared on a plate incubated anaerobically. This organism proved to be facultative, and when grown aerobically on FWCh agar, the rate of chitin hydrolysis was significantly higher than under anaerobic conditions. Chan (1970) also found increased rates of chitin hydrolysis with a facultatively anaerobic chitinoclastic marine Vibrio sp. when grown aerobically. In the pelagic region of the oceans, microbial mineralization of chitin is considered to occur aerobically in sediments (Seki, 1965b; Zobell, 1946), although recently obligate anaerobic chitinoclastic Clostridia were isolated from marine sediments (Timmis et al., 1974). The chitin agar zone technique is unsatisfactory for detection of anaerobes; therefore, these organisms must be isolated by anaerobic enrichment techniques (Timmis et al., 1974). All of the isolates tested were unable to digest chitin anaerobically or did so at decreased rates. Although anaerobic chitinoclasts may be present in the sediment of the 4 lakes, in this study they would not have been detected by the zone clearing method used.

Seki and Taga (1965c) found two types of marine chitinoclastic bacteria based on nutritional requirements. These were classified as: (1) chitin cannot serve as a sole organic nutrient source for the bacteria, but is utilized with other available organic nitrogenous matter and (2) chitin can serve as the sole organic nutrient source without additional organic materials. Most of the isolates from the sediment of the 4 lakes were able to grow in a minimal medium containing chitin as the sole source of carbon and nitrogen. The isolates were capable of growth in a medium without chitin only if either glucose or NAG was added; however, growth was usually more luxuriant when chitin was added to

the medium. Many species of marine chitinoclastic bacteria are capable of utilizing chitin as a sole carbon and nitrogen source (Campbell and Williams, 1951); but these organisms can use other carbon and nitrogen sources if chitin is absent.

CHITIN DEGRADATION BY VIABLE CELLS

Chitin digestion by viable cells was studied by measuring the relative hydrolysis of chitin on chitin agar plates and in FWCh broth. The optimum growth temperatures and ranges were determined for these isolates as well as the effects of end products on chitin degradation by viable cells. The effects of growth temperature on the optimum temperature for chitin degradation by viable cells was also investigated.

Chitin Decomposition on FWCh Agar

The rate of chitin hydrolysis on FWCh agar varied with the test organisms and rates were directly related to temperature. At lower temperatures ($4^{\circ}C$), chitin hydrolysis did not proceed until after several days of incubation. In some cases, although the initial hydrolysis rate was lower, more chitin was hydrolyzed at lower temperatures compared to that at a higher temperature ($25^{\circ}C$) after prolonged incubation up to 30 to 60 days. The zone ratio method of measuring chitin hydrolysis was used by Chan (1970) who also found that chitin hydrolysis rates were directly related to temperature up to the optimum, and varied with the species of marine chitinoclastic bacteria.

Chitin Decomposition in FWCh Broth

The zone ratio method was unsatisfactory for a number of isolates due to extensive spreading of the colonies; therefore, a method was devised to measure rates of chitin hydrolysis by viable cells in liquid media. This method

was patterned after that used on agar medium. Pure cultures of chitinoclastic bacteria always grew at the chitin liquid interface on the surface of chitin particles, when chitin was present in the medium. Bacteria in the marine environment (Kaneko and Colwell, 1975; Zobell, 1946; Zobell and Rittenberg, 1938) and in freshwater (Erikson, 1941) are known to grow attached to chitin. The efficiency of adsorption of chitinoclastic bacteria to chitin is dependent on the pH of the medium and on the strain of bacteria; divalent cations inhibit adsorption of the chitinoclast, Vibrio parahaemolyticus onto chitin (Kaneko and Colwell, 1975). The mechanism of adsorption is not clear, but it is postulated to involve the electrokinetic potential between the positively charged chitin particle and the negatively charged bacteria (Kaneko and Colwell, 1975). If the adsorption is related to the levels of divalent cations present, then one may expect to find higher adsorption efficiencies of chitinoclastic bacteria to chitin in freshwater containing fewer divalent cations than in saltwater.

Results obtained with chitinoclastic bacteria grown in a liquid medium containing chitin were similar to results using a solid medium. Chitin hydrolysis rates in the liquid medium were a function of temperature up to the optimum and varied with the species. Several pure cultures of chitinoclastic bacteria were grown in FWCh broth and assayed for glucose and NAG. Neither glucose nor NAG were detected in the supernatant of these cultures, but protein levels in the medium decreased with incubation time, indicative of utilization of these substrates by the bacteria. Also enzymes were not found in the supernatant indicating that they were either bound to the chitin or to the cells. NAG was detected in the medium only after extended incubation when the cells were no longer viable. Hence, it appears that the enzymes released from the cells continued to degrade chitin after the death of the cells. In many cases, these enzymes continued to degrade chitin until all of the chitin in the tube had

disappeared. This observation supports the view expressed by others (Doyle, 1968; Overbeck, 1974; Reihardt, 1973; Vallentyne, 1957; Zobell, 1946a) that free microbial enzymes may persist in the environment, e.g., lake sediments, and continue to attack substrates after the bacteria have died. Cell-free extracts in this study were also found to retain activity after prolonged storage times at 4°C for several days. The stability of the chitinase system found in this study and in others (Clarke and Tracey, 1956; Skujins et al., 1970) makes it likely that this enzyme can occur in lake sediments. Also chitinases have a great adsorptive affinity for the substrate (Jeuniaux, 1956, 1966; Skujins et al., 1970) and the enzymes are even more stable (e.g., to heating) when adsorbed onto chitin (Jeuniaux, 1966), which should allow this enzyme to persist for some time when attached to chitin in lake sediments.

Effect of Growth Temperature on Optimum Temperature for Chitin Hydrolysis

Four of the chitinoclastic isolates were grown at temperatures of 4, 15, and 22°C and incubated in the temperature gradient incubator. The inoculum growth temperature had little effect on the temperature optimum and range for chitin decomposition. Most of the isolates tested were capable of digesting chitin at temperatures from -0.8 to 33°C. Many psychrophilic bacteria of the Vibrio sp are known to be very efficient chitin digesters at 0°C (Morita, 1975). Some of the isolates in this study exhibited multiple optimum temperatures for chitin digestion as measured by the ratio method. The single largest peak occurred from 20 to 25°C, depending on the isolate, with smaller peaks at 1 to 2°C, and 15°C. Organisms in the aquatic environment have been found with multiple temperature optima for growth (Sieburth, 1967) and for the uptake of glucose and acetate (Allen, 1971; Bott, 1975). These findings suggest that bacteria can possess enzyme systems capable of functioning at temperatures different from

the apparent optimum temperature for growth.

For many of the cell-free extracts, as with viable cells, multiple temperature optima for chitinase activity were observed. Multiple temperature optima could be partially due to different temperature optima for the three to four enzymes known to make up the chitinase and chitobiase system. The microbial chitinase system contains enzymes identical in activity, but different in electrophoretic mobility (Jeuniaux, 1957b, 1959; Skujins et al., 1970), and heat stability (Berger and Reynolds, 1958), so it is not surprising that these chitinases may also exhibit different optimum temperatures for chitin digestion. These different temperatures for activity would ensure chitin digestion within a wide range of temperatures and provide the organism with greater chances of survival in the aquatic environment.

The maximum peak of chitin digestion for viable cells, from 20 to 25°C coincided very closely with the optimum temperature for growth of these organisms. It has been reported that psychrophilic bacteria, having a maximum growth temperature at 13°C, produce chitinase enzymes with an optimum temperature for activity much higher, at 27°C (Morita, 1975). This optimum is similar to the optimum temperature for chitin digestion of lake sediment chitinoclastic bacteria.

Although the inoculum growth temperature had no effect on the optimum temperature for chitin hydrolysis, it did have a pronounced effect on the rate of chitin degradation at different temperatures for the 4 isolates. Cultures grown for a period of several weeks at 4°C had higher rates of chitin hydrolysis at lower temperatures (+0.5°C) than at 15 or 27.8°C. The situation was reversed for cultures grown at higher temperatures. These same cultures grown at 22°C for several weeks, with one exception, had higher rates of chitin hydrolysis at 27.8°C than at the other two temperatures. Results of studies with cell-free

extracts also demonstrated a tendency for increasing optimum temperatures for activity and increased thermal stability for chitinase and chitobiase with increasing growth temperature of viable cells used to prepare these cell-free extracts. Cells grown at higher temperatures of 30°C produced enzymes with higher optima for activity (32-40°C) than those grown at the lower temperature of 4°C (26-27°C). Bacteria are known to respond to environmental stresses by altering their metabolic patterns; these response mechanisms include increases in enzyme synthesis and changes in enzyme activity (Pardee, 1961). The adaption to temperature demonstrated by the chitinase data may not merely be a result of growth alone. It is known that during the lag phase of growth, at any temperature, microorganisms carry out metabolic processes enabling that organism to start growing at a rate characteristic for that temperature (Farrell and Rose, 1967). If cultures grown at one temperature are reinoculated into fresh medium, and incubated at the same temperature, the lag phase disappears or is of very short duration (Farrell and Rose, 1967). The data from the chitinase study with both viable cells and cell-free extracts suggest that this adaption to temperature by microorganisms may be due to the synthesis of enzymes having increased activity near the incubation temperature. It also appears that the growth temperature of the bacteria has an influence on the thermal stability of the enzymes produced. The possibility of different temperature optimums for the three to four enzymes known to make up the chitinase system has already been discussed. Perhaps the growth temperature selects for the production of one or more of the chitinase enzymes having a temperature optimum for activity close to the growth temperature. In the aquatic environment this ability to produce enzymes or isoenzymes in different amounts in response to the prevailing temperature would provide great versatility to the organisms. Bacteria would have increased ability to function at the prevailing temperatures and subsequently have greater

chances of survival when temperature fluctuations occur. This would also ensure that even at lower temperatures, refractory materials such as chitin would be broken down and the carbon and nitrogen consequently would be recycled back into the environment. The chitinase system may provide a useful tool for the further study of the interactions of both temperature and nutrient and their influence on aquatic bacteria.

Effects of End Products on Chitin Degradation

The hydrolysis of chitin by isolates was inhibited by the addition of the end products of chitin hydrolysis, glucose and NAG. This inhibition was directly related to the concentration of these end products added to the medium. Even after prolonged incubation of up to 90 days, decreased hydrolysis of chitin occurred in samples containing as little as 0.01% glucose or NAG. Since neither glucose nor NAG inhibited chitin hydrolysis by crude or partially purified cell-free extracts, this decreased rate may be due to catabolite repression of the synthesis or inhibition of the release of the exocellular chitinases. Microbial chitinase production has been found to be subject to catabolite repression by other workers (Clarke and Tracey, 1956; Monreal and Reese, 1969; Reynolds, 1954). Recently Berg (1975) found with the cellulase system of Cellvibrio fulvus, that cultures grown in glucose or cellobiose, contained cellulase bound to the membrane with only part of the activity located on the cell surface. Growth in the presence of cellulose caused a release of cellulase from the cells and possibly further stimulated enzyme formation. While glucose was thought to repress enzyme formation, some cellulase was found in the periplasm of the cells grown in glucose and cellobiose and thus glucose and cellobiose may function additionally as inhibitors of cellulase release. Because the cellulase and chitinase enzyme systems are very similar, this same phenomenon may occur with the

chitinase complex; i.e., perhaps the end products of chitin hydrolysis may not only repress the synthesis of chitinase, but may inhibit "pre-formed" chitinase from being released by the cells. This would be advantageous to the organism for the enzyme would be present in the cell, but would not be released until needed; i.e., when chitin is present and represents the only carbon source.

CELL-FREE EXTRACTS

Cell-free extracts were prepared from 4 of the chitinoclastic bacterial isolates to separate the complex effects of metabolizing viable cells from the action of the enzymes alone on the degradation of chitin.

Cell-free extracts contained active chitinase only if the cells grown in chitin were shaken during incubation. Other workers have also found that shaking increased growth (Okutani, 1966) and chitin breakdown (Reynolds, 1954) when cultures of chitinoclastic bacteria are grown in the presence of chitin. Shaking may increase the availability of the substrate to the bacterial enzymes, and hence stimulate chitinase production or increased aeration may stimulate the growth and utilization of end products, which if not removed from the medium, may act as repressors of enzyme synthesis.

Enzyme activity did not appear in the supernatant of cells growing in the presence of chitin until either the late log or the stationary phase, and then enzyme levels decreased. Decreases may be due either to irreversible adsorption of the enzyme to the substrate or, perhaps, to the action of proteolytic enzymes also produced by some chitinoclastic bacteria (Skujins et al., 1970). Plate counts of chitinoclastic bacteria grown in chitin usually gave spurious results, presumably due to clumping of the cells or attachment of the cells to chitin as reported by Kaneko and Colwell (1975). Many exocellular enzymes appear to be liberated at the end of the log phase (Polluck, 1962) or the stationary phase

(Lampen, 1965). Most investigators harvest cells to prepare chitinase after 4 to 6 days of incubation at room temperature (Berger and Reynolds, 1958; Reynolds, 1954; Skujins et al., 1970). Enzyme yields were increased by sonicating the cell suspensions. Sonication probably increased enzyme yields by disrupting cells and releasing any enzymes located within the cells and not yet liberated. Also, most enzymes which can normally be found in culture supernatants can also be detected attached to the cells themselves (Pollock, 1962). Chitinase also adsorbs strongly to chitin (Jeuniaux, 1956, 1966); sonication, therefore, would release the attached chitinases.

Enzyme activity was measured in two ways. The action of chitinase was determined by turbidity measurements; the action of chitinase and chitobiase were measured by assay for release of NAG from chitin.

Optimum pH and Temperature for Chitin Hydrolysis

The pH optima of chitinase and chitobiase activity as measured by NAG release for cell-free extracts prepared from the isolates tested were from 6.3 to 7.0. This is higher than the pH optimum of 5.0 measured for Streptomyces sp chitinase (Skujins et al., 1970), but is within the range of those reported for Serratia marcescens (Monreal and Reese, 1969) and Vibrio gerris (Okutani, 1966). The release of NAG from chitin by cell-free extracts was extremely pH dependent with rapid decreases above and below the optimum. The effect of pH on turbidity change (chitinase) by the cell-free extracts was much less pronounced with a wider optimum. The results of pH effects on activity of cell-free enzymes from the test organisms show that these enzymes should be operable in the pH ranges reported for lake water.

The optimum temperature for chitin hydrolysis by chitinase was slightly higher than for chitobiase. However, chitobiase was more heat stable

than the chitinase. Both enzymes lost almost all of the activity after heating at 45°C for 23 hours. The optimal temperatures for chitinase and chitobiase were the same or slightly higher than the optimal temperatures observed for chitin degradation by viable cells and the optimal growth temperatures for the test organisms. Many microorganisms have enzymes stable at the optimum temperature of growth for the organism, but not at temperatures much higher (Brock, 1967b). This seems to be the case for the chitinase system of bacteria isolated from the sediments of the 4 lakes. In most cases the optimal temperatures for the cell-free extracts were 20 to 25 degrees higher than the maximum recorded temperatures at the sampling stations, and especially for the benthic stations. Therefore, these microbial enzymes would not be subjected to heat stress in the environment. The optimum temperatures for chitinase activity of cell-free extracts varied from 24 to 32°C, depending on the isolate and the growth temperature of the organism. This is below the 40°C optimum reported for marine chitinoclastic Aeromonas and Vibrio species (Okutani, 1966) and the 50°C optimum reported for Serratia marcescens (Monreal and Reese, 1969). However, for the bacteria found in sediments from the 4 lakes, this enzyme would not be subjected to temperatures higher than 26°C in the littoral zone or 8°C in the benthic zone.

Ammonium Sulfate Fractionation

Ammonium sulfate fractionation was used to purify and concentrate the enzymes from cell-free extracts.

The specific enzyme activity was increased by 70-fold after treatment with ammonium sulfate at 64% of saturation. These preparations were so active that extremely small quantities (10-50 µgm protein) added to a standard assay mixture was sufficient to digest all of the chitin within a few hours. Jeuniaux (1956, 1957a, 1957b, 1959) has achieved similar purification from 25- to 130-fold

of Streptomyces sp chitinase with selective adsorbtion of chitinase to chitin followed by ammonium sulfate fractionation at 55% of saturation. Calcium has been found to be necessary to stabilize chitinase from Streptomyces sp (Skujins et al., 1970) with increasing degrees of purification. The addition of calcium had little effect in this study, and since no enzyme inhibition occurred when EDTA was added, it appears that divalent metal cofactors are not involved in the reaction for the isolates tested. The enzyme was inhibited by copper which is an inhibitor of many enzymes including the chitinase prepared from Streptomyces sp (Jeuniaux, 1966).

The enzyme from Streptomyces sp (Jeuniaux, 1966) is dialyzable. In this study, dialysis was accompanied by both a loss of enzyme activity and protein. Fungal chitinases also lose activity after dialysis (Cohen, 1974) and this is thought to be due to the loss of a dialyzable activator. The loss in this study may also be due to a dialyzable activator, although as demonstrated by lack of effect with EDTA, a metallic cofactor does not seem to be involved. Perhaps, the enzyme adsorbed to the sides of the dialysis tubing.

SUMMARY

Bacteria in lakes play an important role in the cycling of nutrients. These bacteria are capable of degrading refractory carbon compounds into particulate forms available to other organisms in the food chain. Bacteria are also capable of rapidly degrading carbon compounds such as glucose, present in water and sediments and hence, keep these labile compounds at low levels. One of the methods used to study aquatic bacterial activities has been the uptake and respiration of ^{14}C-labeled glucose. Recently a method has been described to measure respiration or mineralization of ^{14}C-labeled glucose in lake sediments (Harrison et al., 1971). The sediment-water interface is a site of intense microbial activity and until recently, the bacteria associated with sediments have not received much attention, especially in lakes in the Pacific Northwest. Consequently, this study of glucose mineralization by lake sediment bacteria in 4 lakes of differing trophic status in the Lake Washington drainage basin was initiated. To gain a further understanding of sediment bacteria, the degradation of chitin by chitinoclastic bacteria associated with the sediment in the 4 lakes was also studied. Chitin is a relatively refractory carbon compound, degraded primarily by bacteria ultimately to glucose.

This work was divided into two parts. Part I deals with glucose mineralization in the 4 lakes by sediment bacteria. The effects of temperature on glucose mineralization rates was investigated as well as seasonal variation in rate, relationship of glucose mineralization rate and bacterial plate count to trophic status. The second part of this study concerns chitinoclastic bacteria in the sediment of the 4 lakes, seasonal variation in numbers of chitinoclastic

bacteria, and effects of temperature and substrate enrichment on the development of chitinoclastic bacteria. Laboratory studies of chitin hydrolysis and the effects of temperature were conducted with pure cultures of chitinoclastic bacteria and finally with cell-free extracts prepared from these isolates.

A total of 50 littoral, 55 benthic, and 18 sediment samples collected at stations other than the main sampling stations were analyzed for glucose mineralization activity from July 1972 to April 1974 in 4 lakes of the Lake Washington drainage basin. These 4 lakes (Washington, Sammamish, Chester Morse, and Findley) are of varied trophic state and experience different meteorological conditions. Lakes Washington and Sammamish are in an urbanized environment and have recently undergone a limited eutrophication and subsequent sewage diversion. Lake Washington has recovered remarkably; however, Lake Sammamish, for reasons still not entirely understood, has not. Both Lakes Chester Morse and Findley are in the Seattle watershed, an area closed to the public, and represent lakes in a more pristine environment. Both lakes are oligotrophic. Findley Lake, the lake of highest altitude, is the only lake of the study group that completely freezes over during the winter months.

The littoral zone proved to be an extremely microbiologically active area of these lakes. Bacteria associated with the littoral zone were more numerous and exhibited higher glucose mineralization rates than did those from the benthic stations with the exception of Lake Sammamish. Increased glucose mineralization rates at the littoral zone in these lakes may be due to increases in allochthonous nutrient supply in this region, presence of submerged vegetation providing increased bacterial attachment sites and nutrients, increased temperatures, and the predominance of particles in this zone with greater surface areas. All of these factors would be favorable conditions for the activities and, consequently, the growth of bacteria in the littoral zone of lakes.

In Lake Sammamish sediment, glucose mineralization rates were higher in benthic than littoral sediment during the time of hypolimnetic oxygen depletion. This increased rate may be partially responsible for the oxygen depletion and should be studied further. Increased benthic rates could also be due to decreased efficiencies of bacterial metabolism under anaerobic conditions as well. Lake Sammamish has not responded to sewage diversion to the same extent as Lake Washington. This lack of response has been partially attributed to the capacity of the sediments to adsorb phosphorus during aerobic conditions and to release it during anaerobic conditions. The chemical basis for phosphorus recycling in this lake has been extensively studied; however, the role of sediment bacteria in the phosphorus cycle of this lake warrants further attention.

The glucose mineralization rate varied seasonally in all 4 lakes. The two oligotrophic lakes had the greatest seasonal variation. Higher rates occurred in all lakes in the summer while lowest rates were observed in the late winter to early spring months. Higher rates in the summer are probably due to higher water temperatures, increased dissolved organic material from phytoplankton blooms, and to metabolic differences between bacterial species predominating in the lakes during the summer as compared to winter months.

It has been suggested that uptake rates of ^{14}C-labeled substrates be used as a measure of trophic state analogous to the use of phytoplankton primary productivity. Bacteria in lakes which are higher in nutrients or eutrophic should exhibit higher uptake rates than those in lakes less enriched or oligotrophic. Data from this study suggest that trophic states of each of the 4 lakes and the glucose mineralization rate were related, but only by comparing data generated during the winter months. The glucose turnover times for the two mesotrophic lakes, Washington and Sammamish, were the most rapid of all lakes sampled

in the winter. Summer turnover times for all lakes were about the same. Winter glucose turnover times were longest for the two oligotrophic lakes, Chester Morse and Findley. The relationship between trophic status and V_{max} was not as anticipated, especially during the summer months. The summer V_{max} values for oligotrophic Findley Lake were the highest of all the lakes sampled. Maximal velocities for glucose mineralization occurred after melting of the ice and was most likely due to the influx of organic materials from the melting ice and snow. Furthermore, the lowest V_{max} of all lakes sampled were observed for mesotrophic Lake Sammamish. These data suggest that the glucose mineralization rate method for the determination of trophic state of lakes in comparative studies be used with care. Trophic state as determined by this method should not be assigned on the basis of a single measurement but rather after a series of determinations extending over at least a year.

The glucose mineralization rate method may be useful in pollution studies and perhaps may be used to generate baseline data. The method was useful in detecting the effects of diesel oil, which could be anticipated to be a pollutant, on the glucose mineralization rate of sediment bacteria. Bacteria respond very quickly to changes in the environment, and could be useful indicators of pollution.

Algal primary productivity and heterotrophic bacterial glucose mineralization rates appeared to be related in Lakes Sammamish and Findley. Phytoplankton production data collected by other investigators were not available for the other two lakes. In Lake Sammamish, the glucose mineralization rate increased one month after the vernal peak of algal primary productivity. In Findley Lake, both bacterial peak activities and primary production peaks of the phytoplankton occurred simultaneously after the melting of the ice and snow cover. It is difficult to determine if the favorable increases in light and

temperature occurring at these times stimulated activities of the bacteria and phytoplankton independently or if there was indeed interaction between them. In Findley Lake, bacteria stimulated by the increased nutrient after ice melt may have supplied growth factors for the phytoplankton or the phytoplankton may have provided dissolved nutrients known to be utilized and stimulatory to bacteria. Results of laboratory experiments with additions of freeze-dried plankton to sediment samples suggested that the bacteria were stimulated by additions of these organisms and were able to utilize them.

During the early phases of this investigation, it appeared that temperature was an important parameter affecting the glucose mineralization rates of sediment bacteria. As more data were collected, it became apparent that glucose mineralization rates were indeed directly affected by the in situ temperature, and bacteria in each lake differed in their temperature response. A number of relationships involving temperature were tested for significance using linear regression. The glucose turnover time measured seasonally at the in situ temperature was indirectly related to the temperature for the littoral zones of Lakes Washington, Sammamish, and Chester Morse (5% level of significance). Bacteria in sediment from Lake Chester Morse, higher in elevation, exhibited higher rates at lower temperatures than bacteria in the other two lakes, while bacteria associated with sediment of Lake Sammamish were least responsive to temperature fluctuations.

For benthic samples, only those from Lake Sammamish demonstrated a significant (0.1%) relationship of temperature to bacterial activities. The bacteria of the benthic zone of this lake were far more responsive to temperature than the bacteria from the littoral zone.

The maximum velocity for glucose mineralization was also related to temperature for the littoral zones of Lake Sammamish (5%), Findley (1%) and

the benthic zone of Lake Washington (1%). The V_{max} value was also correlated to bacterial plate count for sediment from the littoral zone of Lake Chester Morse (5%).

There were more significant interactions between temperature and bacterial activities for the littoral than for the benthic areas. This suggests that temperature, which fluctuates very little in the benthic zone of these lakes, is not an important variable affecting bacterial activities in this region.

For Lakes Washington and Sammamish littoral zone sediments, significant temperature relationships were obtained by linear regression analysis only if data obtained during the times of spring and summer peaks of phytoplankton primary production were excluded. It appears that nutrients from algal production at these times had greater influence on bacterial activities than temperature. The influence of temperature and nutritional factors on aquatic bacteria are extremely difficult to separate; however, it appears that statistical analysis of the relationship of temperature to bacterial glucose mineralization rates can indicate which of these two variables is predominant at a given time, provided base line data are available. The coupling of glucose mineralization rate of lake sediment bacteria to temperature may prove to be a useful tool in future studies of aquatic bacteria. It may also prove especially helpful in the mathematical modeling of lakes.

The optimum temperature under experimental conditions for bacterial glucose mineralization in the benthic zone was considerably higher than the temperatures normally encountered in this region. The optimum temperatures determined for sediment collected during the winter months from this region were 20°C, 25-30°C, 25-30°C, and 27-30°C for Lakes Findley, Sammamish, Chester Morse, and Washington, respectively. Findley Lake benthic sediments which experienced the lowest <u>in situ</u> temperatures also had the lowest temperature

optimum. The temperature optimum for glucose mineralization in Findley Lake also changed with the season. Samples collected in summer had a temperature optimum of 28°C, higher than the 20°C optimum measured in the winter, and is probably due to the seasonal selection of bacterial types. Shifts in temperature optimum were not observed in the other lakes. These data indicate that the bacteria in these lakes are not optimally adapted to the prevailing temperatures of the sediment, but to temperatures several degrees higher. The bacteria are capable of operating over a wide range of temperatures and if temperatures were increased (e.g., thermal pollution) and utilizable substrates were available, increased bacterial metabolic rates and subsequent hypolimnetic oxygen depletion could be expected. Furthermore, temperature studies demonstrated seasonal differences in bacterial temperature responses. Bacteria predominating in the sediments during the winter were more responsive to temperature fluctuations than those in the summer. In the winter, bacteria were capable of mineralizing glucose at a higher rate at lower temperatures than those predominating in the summer. This difference is probably a reflection of the changes in species composition during different times of the year.

The glucose mineralization method of Harrison et al. (1971) was reproducible when samples from the same station in a given lake were analyzed within 2 to 4 hours of collection (littoral zone). There was also little difference in rate observed with benthic samples taken in close proximity (within 100 m). Samples from widely separated sites exhibited vastly different rates with lower rates near the sites of industrial areas compared to higher rates near river inlets. These variations in rates at different sampling sites are most likely due to differences in nutrients, availability of attachment sites, and physiological responses of different bacterial species within specific areas of a lake.

Perhaps the type of sampling device used is one of the most important and often overlooked factors considered when undertaking studies of aquatic bacteria. There is a great need for the development of a sediment sampling device that will permit aseptic sampling, without exposure to terrestrial and water column bacteria, and still take a representative sample with minimum disruption of the sediment-water interface or the "fluffy" layer. The amount of the "fluffy" layer obtained by a sampler can have a direct influence on the rates of bacterial activities measured. The majority of the bacteria and hence bacterial activities are associated with this "fluffy" layer. If the sampler disturbs this layer and causes it to disperse, then measured rates and bacterial numbers may be found to be lower than would be the case if a truly "representative" sample had been obtained. Also if possible, to reduce variation, the same or at least similar devices should be used throughout a comparative study. The modified "ooze sucker" used in this study appeared to obtain a representative sediment sample without disturbing the sediment-water interface on descent and contact with the lake bottom.

Bacteria were enumerated by the plate count method on Taylor's medium, used by many investigators in freshwater aquatic microbiology. The addition of yeast extract significantly increased plate counts of sediment and was, therefore, used throughout this study. The majority of the bacteria in the 4 lakes were aerobic or facultative anaerobic gram negative, motile rods, with pigmented species making up 30% of the population. Bacteria associated with littoral sediments were more numerous than those of benthic sediments. In the lakes studied, bacterial numbers at the sediment-water interface were three orders of magnitude higher than in the overlying water. Furthermore, numbers of aerobic bacteria were highest in the top 1 to 2 cm of sediment and decreased with depth into the sediment.

Bacterial numbers in the lakes tended to vary with season with higher counts in the warmer summer months than in the winter. The greatest seasonal variation in bacterial numbers occurred in Findley Lake, with lowest counts occurring during the winter ice cover. Increases in bacterial plate counts occurred after the fall overturn in the 4 lakes. This increase is most likely due to the mixing of bacteria and nutrients that occurs at this time.

The results of the relationship between plate count and trophic status of the lakes were similar to those found for the glucose mineralization rate and trophic state. Plate counts of sediment did not completely reflect the trophic state of each lake in the summer. However, in the winter, plate counts for sediment from the two oligotrophic lakes were lower than corresponding counts from the mesotrophic lakes.

The effects of in situ temperature on bacterial biomass were studied and were related for sediment only from Findley Lake. For every 10 degree rise in temperature (Celsius) the bacterial plate increased by one log unit. Bacteria from the sediment of the 4 lakes were capable of growing faster at temperatures higher than the in situ temperature. Of the bacteria tested in pure culture, the temperature optimum for growth was usually from 20 to 28°C, similar to the optimum for glucose mineralization. Very few organisms from the 4 lakes were capable of growth above 32°C. Psychrophilic bacteria, bacteria capable of growth at 0°C, but not at 22°C, were found in benthic zone sediment in Lake Washington, and the littoral zones of the higher altitude lakes, Chester Morse and Findley. These organisms comprised from 8 to 12% of the total heterotrophic population.

Cellulolytic bacteria were present in sediment collected at the littoral zone of Lake Washington and at both stations in Lake Sammamish. All of the cellulolytic organisms were gram positive, obligately anaerobic rods. These

organisms comprised from 1.3 to 2.4% of the total anaerobes counted and numbered 10^4 organisms/gm sediment. Many of these cellulolytic bacteria degraded cellulose more rapidly when grown in mixed culture than when isolated.

Chitinoclastic bacteria were present in the sediments of the 4 lakes. The total numbers and percent chitinoclasts in the sediment of Lakes Washington and Sammamish changed very little with the seasons. These organisms numbered from 10^4 to 10^6 organisms/gm and comprised from 1 to 7% of the total heterotrophic population. In the two oligotrophic lakes, Chester Morse and Findley, chitinoclastic bacteria varied with the season with highest numbers occurring during the winter months. Higher percentages and numbers of chitinoclastic bacteria were found at the Lake Chester Morse littoral station than at any other station of the lakes sampled. The numbers ranged from 10^5 to 10^7 organisms/gm and comprised from 3 to 80% of the population. It appears that chitin in oligotrophic lakes may represent a food reserve for bacteria able to hydrolyze it, during the winter months after the more labile substrates have been consumed.

The development of chitinoclastic bacteria was enhanced by the addition of organic substrates. While chitin alone enriched for chitinoclastic bacteria, greatest numbers of these organisms developed if peptone was added in addition to chitin. Additions of only peptone also enriched for chitinoclastic bacteria. Chitinous material from dead animals, e.g., crustaceans, in the environment would be expected to be accompanied by organic substrates thus favoring the development of chitinoclastic bacteria. Glucose, NAG, $CaCO_3$, and cellulose did not enrich for chitinoclastic bacteria. Cellulose and $CaCO_3$ in fact exerted a slight inhibitory effect. Besides substrate, temperature was also found to be an important factor for the development of chitinoclastic bacteria. Most of the chitinoclastic bacteria in these lakes were adapted to growth at lower temperatures.

A small percentage of chitinoclastic Pseudomonas, Streptomyces, and Cytophaga were found in the lake sediments. However, most of the chitinoclastic bacteria in the lakes were gram negative, oxidase positive, motile rods, capable of fermenting glucose to acid without gas production. These organisms appeared to belong to the genera Vibrio or Aeromonas. A number of these organisms were isolated and maintained in pure culture for laboratory studies of chitin degradation. It was necessary to maintain pure cultures of these organisms in a medium containing chitin, otherwise decreased chitin hydrolysis rates occurred when retested.

Chitin hydrolysis rates were related to the particle size of chitin. All of the isolates were capable of hydrolyzing larger sized chitin particles (up to 2 mm), but rates were highest with reprecipitated chitin.

The hydrolysis of chitin by these organisms was favored by aerobic conditions. Thus in well-oxygenated oligotrophic lakes, chitin hydrolysis should occur. Chitin hydrolysis was also increased by shaking which may be due to the increased aeration as a result of agitation. Most of these organisms did not hydrolyze chitin or did so very slowly under anaerobic conditions. Since these organisms were facultative, they should be able to successfully compete in environments where levels of organic nutrients are high and as a consequence, oxygen levels are reduced. While sediment chitinoclastic bacteria could survive anaerobic conditions in the hypolimnion, they would not hydrolyze chitin during this time, or would do so at diminished rates. All of the isolates tested were able to utilize chitin as the sole carbon and nitrogen source. However, these organisms grew more luxuriantly if organic substrates were added to the medium. Isolates were capable of growth without chitin only if glucose or NAG was added to the medium.

The formation of chitinase and perhaps its release by the cells appears to be subject to catabolite repression. Both glucose and NAG inhibited chitin hydrolysis by viable cells but did not inhibit the enzyme system in cell-free extracts. Bacteria in lake sediment would therefore not synthesize the enzyme or release it until needed. Furthermore, in the environment, catabolite repression would play an important role in governing the rate of chitin degradation. One would expect chitin to be broken down readily in oligotrophic lakes. However, in eutrophic lakes, rich in nutrients, chitin degradation may proceed at a slower rate.

Chitin degradation was studied using viable cells and cell-free extracts. Chitinase activity was measured in cell-free extracts by measuring either NAG release or turbidity decreases after incubation of extracts with chitin. Chitin hydrolysis by pure cultures of viable chitinoclasts was studied in liquid and solid media. In FWCh agar, chitin hydrolysis was assayed by taking the ratio of the colony diameter to the cleared zone diameter. This ratio method was not a suitable method for measuring chitin hydrolysis with some of the isolates due to extensive spreading of some colonies. Therefore, a new method was devised to measure chitin degradation in liquid media. This method consisted of measuring the depth of a reprecipitated chitin layer in a culture tube 24 hours after inoculation. This initial measurement was compared to measurements taken on successive days of incubation as the chitin decreased. This method is better to use than gravimetric analyses because samples are not disturbed during the measurement, and a single culture can be studied over a long period of time. Culture tubes containing uninoculated FWCh broth served as controls.

Chitin hydrolysis by viable cells varied with the test organisms in both liquid and solid media and rates were directly related to temperature. Glucose and NAG were not detected in the FWCh broth containing viable cells, and protein

levels decreased with time in these tubes, indicating utilization of these substrates by the bacteria. Free enzymes were not detected in these tubes. With cell-free extracts sonication resulted in increased enzyme yields, therefore, it appears that the chitinase may have been bound to the cells or to the chitin. After cell death, NAG levels proceeded to increase with a concomitant decrease in chitin. In many cases, residual enzymes continued to degrade chitin until all of the chitin in the tubes had disappeared. Later in this study, it was found that cell-free extracts remained active after prolonged storage times of several weeks at 4°C. The stability of the microbial chitinase system suggests that free chitinases may persist and remain active in lake sediments long after the bacteria that produced them have died.

Because temperature appeared to be an important variable affecting the growth and glucose mineralization rate of sediment bacteria, the effects of temperature on chitin degradation by chitinoclasts was also studied. All of the isolates were able to grow and to digest chitin over a temperature range extending from -0.8°C to 32.0°C. The optimal temperatures for growth and chitin hydrolysis were the same or slightly lower than the optimum temperature for glucose mineralization. Two smaller temperature optimal peaks for chitin digestion by viable cells and cell-free extracts were found at 1 to 2°C, and at 15°C. These multiple temperature optima may be due to differences in temperature optima between the three to four enzymes known to make up the chitinase enzyme system. Also the chitinase system as measured by turbidity decrease had a slightly higher temperature optimum than did the chitobiase as measured by NAG release.

The inoculum growth temperature did not affect the temperature optimum for chitin hydrolysis by viable cells but did affect the optimum temperature of cell-free extracts. Cell-free extracts prepared from cells grown at higher

temperatures (30°C) had a higher temperature optimum for chitin hydrolysis and greater thermal stability than did extracts from cells grown at lower temperatures (4°C). Enzymes synthesized by these bacteria were also adjusted to the temperature of subculture. Isolates subcultured at low temperatures for several weeks had higher chitin hydrolysis rates using viable cells at low temperatures than did those subcultured at high temperatures and retested at low temperatures. The same was true for isolates subcultured at high temperatures. This may indicate a selection by temperature for either the production of different amounts of enzyme comprising the chitinase system or the production of enzymes with different temperature optima for activity. Chitinase synthesized by bacteria would have maximum activity near the growth temperature of the organism. In the environment, this phenomenon would ensure that chitin would be hydrolyzed at the in situ temperature at an optimum rate even though that temperature may be several degrees below the temperature optimum for growth of that organism.

The optimum pH for chitin hydrolysis by cell-free extracts for most of the test organisms was 7.0. The relationship between pH and activity for chitobiase generated a steep curve with rapid decreases in activity above and below the optimum. The effect of pH on chitinase was much less pronounced with a wider optimum. The chitinase does not appear to require divalent metal cofactors for activity, and is inhibited by copper. Losses of both activity and protein occurred after dialysis and may be due to adsorption of the enzyme to the dialysis tubing or perhaps to the loss of a small molecular weight cofactor. The enzyme was purified 70-fold by ammonium sulfate fractionation (64% of saturation). This semipurified enzyme was extremely active; small quantities (10-50 µgm protein) were sufficient to hydrolyze all of the chitin in the standard assay mixture within a few hours.

The results of this study indicate that temperature is an important environmental factor affecting the growth and activities of lake sediment bacteria. The temperature optima for microbial growth, glucose mineralization, and chitin degradation by viable cells and cell-free extracts were similar. These temperature optima were considerably higher by several degrees (20) than the temperatures encountered by these organisms in the environment. Furthermore, temperature selects for different bacterial species in lake sediments. Bacteria found in the sediments during the winter months were more responsive to temperature than those predominating during the summer. Also bacteria in the sediments of the higher altitude lakes which experience the most extreme meteorological conditions were more responsive to temperature than those from the sediments of lakes at elevations near sea level. Glucose mineralization rates and bacterial plate counts were indicative of the trophic state of the lakes when winter but not summer data were compared.

The microbial chitinase enzyme system may be a useful tool for the study of bacteria and effects of environmental temperature fluctuations. The heat stability and optimum temperature for chitin hydrolysis by cell-free extracts were affected by the subculture temperature of isolates of chitinoclastic bacteria. Cultures held for several weeks at low temperatures ($4^{o}C$) were capable of hydrolyzing chitin at these low temperatures at higher rates than the same cultures maintained for several weeks at higher temperatures ($22^{o}C$). The stability of the microbial chitinases also suggests that free chitinases may persist and remain active in lake sediments.

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APPENDIX

Table 50. Temperature Profile of Four Lakes.
Lake Washington

Depth (Meters)	9/8/72 T°C	11/15/72 T°C	3/6/73 T°C	5/10/73 T°C	6/19/73 T°C	7/31/73 T°C	9/25/73 T°C
Surface	20.0	10.5	7.45	10.9	16.5	22.5	17.6
1	20.0	10.5	7.45		15.8		17.6
2	20.0	10.5	7.45	10.8	15.5	22.3	
3	20.0	10.5	7.25		15.5		17.5
4	20.0	10.5	7.1		15.4	22.2	
5	20.0	10.5	7.0	10.8	15.4		17.6
6	20.0	10.5	7.3			21.5	
7	20.0	10.5			15.3		
8	20.0	10.5	7.25			19.0	
9	20.0	10.5		10.7			
10	20.1	10.5	7.0	10.6	15.1	18.5	17.6
11	20.2	10.5		10.8			
12	20.1	10.5	6.95	10.6		17.2	
13	20.1	10.5		10.6	15.0		17.4
14	16.0	10.5		10.6		16.0	
15	13.6	10.5	6.9	10.6			14.0
16	12.6	10.5		10.5	14.9	15.0	
17	12.2	10.5	6.85	10.5	12.5		11.7
18	11.5	10.5		10.5	10.1	12.5	
19	11.0	10.5	6.8	10.5	9.7		
20	10.4	10.5	6.8	10.4	9.4	11.0	10.7
21	9.8	10.5	6.8	10.2	9.0		
22	9.5	10.5	6.8	10.1	9.0	10.0	
23	9.4	10.5	6.8	10.0	8.7		
24	9.2	10.4	6.8	10.0	8.5	9.4	
25			6.8	10.0			9.8
26				9.9		9.2	
27	8.8	8.8		9.8	8.1		
28				9.5		9.0	
29				9.5			
30	8.4	8.0	6.75	9.5	8.0	8.8	9.1

Table 50. Lake Washington (continued)

Depth (Meters)	9/8/72 T°C	11/15/72 T°C	3/6/73 T°C	5/10/73 T°C	6/19/73 T°C	7/31/73 T°C	9/25/73 T°C
31				9.3			
32				9.2			
33	8.3	8.8	6.75	9.2	7.8		
34				9.2		8.5	
35			6.8	9.0			8.9
36	8.1	7.7		9.0	7.9	8.5	
37				8.6			
38				8.9		8.5	
39	8.0	7.5					
40				8.2		8.4	8.6
41				8.0			
42	8.0	7.4		7.5	7.5	8.4	
43				7.4			
44				7.3		8.3	
45	7.8	7.5		7.2	7.4		
46				7.1		8.2	
47							
48	7.8	7.4		7.0	7.3	8.2	
49							
50						8.1	8.7
51	7.8	7.4			7.2		
52					7.2		
53					7.2		
54	7.6	7.3			7.2		
55			6.8		7.2		
56					7.2		8.1
57	7.6	7.2			7.2		
58			6.85		7.2	8.1	8.1
59					7.2		
60	7.6	7.0	6.9	6.7	7.2	8.0	

Table 50. Lake Washington (continued)

Depth (Meters)	10/25/73 T°C	11/20/73 T°C	12/17/73 T°C	1/25/74 T°C	4/4/74 T°C	4/25/74 T°C	9/24/74 T°C
Surface	13.6	10.0	8.7	6.4	6.8	8.4	19.3
1				6.5	6.5	8.0	19.4
2						8.0	19.1
3							19.1
4							19.0
5							18.9
6							18.9
7							18.8
8							18.7
9							18.6
10			8.7	6.5			18.6
11							18.5
12							16.6
13							15.5
14							15.0
15							13.0
16	13.6						11.8
17							10.8
18	13.5				6.5		9.9
19							9.6
20	13.2	10.0	8.7	6.5			9.3
21							9.3
22	13.0						9.0
23							8.8
24	12.5						8.4
25	11.0	9.8					
26	10.5						
27	9.8						8.1
28	9.5						
29							
30	9.5	9.5	8.7	6.5	6.3		7.8

Table 50. Lake Washington (continued)

Depth (Meters)	10/25/73 T°C	11/20/73 T°C	12/17/73 T°C	1/25/74 T°C	4/4/74 T°C	4/25/74 T°C	9/24/74 T°C
31							
32	9.1						
33							7.5
34	9.1						7.3
35		9.3					
36							
37	8.7						
38					6.1		7.2
39	8.5						
40	8.0	9.3	8.7	6.5			
41							
42	7.9						7.0
43							
44							7.0
45		9.0			6.0		
46							
47							
48							6.9
49							
50		8.8	8.7	6.5			
51							
52							
53							
54							
55		8.7					
56							
57							
58							
59							
60	7.9	8.5	8.7	6.5	6.0	6.6	6.6

Table 50. Lake Sammamish

Depth (Meters)	9/20/72 T°C	11/29/72 T°C	4/13/73 T°C	4/27/73 T°C	5/11/73 T°C
Surface	17.6	8.9	13.3	12.2	13.3
1	17.6	8.9	13.0	12.1	12.8
2	17.8	9.0	12.4	12.1	12.5
3	17.8	9.0	11.2	12.1	11.9
4	17.7	9.0	10.4	12.1	11.3
5	17.7	9.0	9.7	12.1	11.0
6	17.8	9.0	9.2	12.0	10.9
7	17.8	8.9	8.8	10.6	
8	17.7	8.9	8.6	10.2	10.8
9	17.0	8.9	8.4	9.8	10.6
9.5	16.5	8.9			
10	15.4	8.9	8.3	9.4	10.2
11	13.6	8.9		8.9	9.9
12	12.0	8.9	8.2	8.8	9.7
13	10.5	8.9	7.8	8.6	9.6
14	9.6	8.9	7.6	8.4	9.4
15	9.3	8.9	7.4	8.0	8.7
16	9.0	8.9	7.0	7.8	8.4
17	8.6	8.9	6.7	7.6	
18	8.5	8.9	6.6		
19	8.4	8.9			
20	8.2	8.3	6.4	7.6	8.1
21	8.0	8.2	6.4	7.3	7.8
22	8.0	8.1	6.4	7.2	7.5
23	8.0	8.0	6.3	7.0	7.4
24	8.0	7.9			7.3
25	8.0	7.8			
26			6.2	7.0	7.0
27	8.0	7.8			6.9

Table 50. Lake Sammamish (continued)

Depth (Meters)	7/13/73 T°C	7/17/73 T°C	8/10/73 T°C	8/21/73 T°C	10/2/73 T°C
Surface	21.5	24.0	23.0	20.4	18.5
1	21.0	23.5	23.1	20.6	18.2
2		23.3	23.2	20.8	18.0
3		23.2	23.5	20.9	17.8
4	20.8	23.0	23.4	20.7	17.8
5	20.8	21.4	23.4	20.7	17.7
6	19.5	20.4	22.6	20.7	17.7
7	17.9	19.0	22.6	20.7	17.6
8	14.5	17.0	15.7	20.7	17.6
9	14.2	15.0	14.9	17.4	17.6
10	11.7	13.6	13.1	14.6	16.6
11	10.5	11.7	11.6	13.3	14.1
12	10.0	10.2	11.0	11.6	12.4
13	9.5	9.5	10.4	10.2	11.0
14	9.3	9.3	9.8	9.6	10.4
15	9.0	9.1	9.4	9.4	9.7
16	8.8	8.9	9.1	9.0	9.3
17	8.6	8.7	9.0	8.6	9.0
18	8.5	8.6	8.9	8.5	8.8
19	8.4	8.5	8.6	8.2	8.6
20	8.3	8.3	8.5	8.1	8.5
21	8.1	8.0	8.4	8.0	8.3
22	8.0	7.9	8.3	7.9	
23			8.2	7.7	
24	7.9		8.0	7.8	8.0
25		7.9	8.0	7.8	
26			8.0		7.9
27	7.9		7.9		

Table 50. Lake Sammamish (continued)

Depth (Meters)	10/12/73 T°C	12/5/73 T°C	2/5/74 T°C	3/6/74 T°C	4/3/74 T°C
Surface	18.5	8.9	5.8	6.0	6.0
1	18.2				
2					
3	18.0				
4	17.8				
5	17.7				
6	17.7				
7	17.6				
8					
9	17.6				
10	16.6	8.9	5.8	6.0	
11	14.1				
12	12.4				
13	11.0				
14	10.4				6.0
15	9.7				5.9
16	9.3				5.9
17	9.0				5.9
18	8.8				5.8
19	8.6	8.9	5.8	6.0	
20	8.5				5.6
21	8.3				5.6
22					
23					
24	8.0				5.4
25					
26	7.9				
27	7.9	8.9	5.8	6.0	5.3

Table 50. Lake Chester Morse

Depth (Meters	8/22/72 T°C	9/27/72 T°C	3/20/73 T°C	4/3/73 T°C	4/19/73 T°C	5/1/73 T°C
Surface	18.0	11.4	0	4.8	6.7	7.9
1	17.8	11.3		4.5	6.8	7.7
2	17.8	11.2	3.5	4.4	6.7	7.4
3	17.8	11.1		4.3		7.2
4	17.7	11.1				7.1
5	17.6	11.1			6.6	7.0
6	17.0	11.1		4.3	6.4	7.0
7	16.2	11.1			6.2	
8	14.2	11.1	3.45		6.0	6.9
9	12.5	10.7		4.3	5.9	
10	10.9	10.5	3.45			6.8
11	10.0	10.2			5.9	
12	9.4	10.0		4.3		6.5
13	8.9	9.8			5.8	
14	8.6	9.4				6.3
15	8.4	9.3		4.2	5.6	
16	8.2	8.8				6.2
17	8.2	8.7			5.5	
18	8.0	8.3		4.2		5.8
19	8.0	8.0			5.3	
20	8.0	7.9				5.6
21	7.8	7.9		4.2	5.2	
22	7.8	7.9				5.5
23	7.6	7.8			5.1	
24	7.6	7.5		4.2	5.0	5.4
26						
27	7.4	7.2		4.2	4.9	5.1
28			3.5			
29						
30	7.0	6.8	3.4	4.2	4.8	4.8
31						
32			3.4	4.2	4.8	4.7
33	7.0	6.8				

Table 50. Lake Chester Morse (continued)

Depth (Meters)	5/22/73 T°C	6/6/73 T°C	7/10/73 T°C	7/24/73 T°C	8/28/73 T°C	10/16/73 T°C
Surface	11.5	12.5	17.6	18.0	16.1	
1	11.4	12.0	17.2	18.5	16.4	
2			17.1	18.3	16.6	
3	11.3	11.9	16.9		16.7	
4	11.2		16.9	18.2	16.8	
5			16.9	18.1	16.8	
6		11.8	16.0		16.8	
7	10.9	11.5	14.8	18.0	16.8	
8	10.7		14.4	16.1	16.8	
9	9.9		13.5	13.0	15.1	
10		11.1	11.9	11.6	11.7	
11	9.7	10.9	11.0	10.7	10.6	
12	9.0	10.6	10.3	10.1	9.6	
13	8.9	10.1	9.7	9.6	8.9	
14	8.7	10.0	9.5	9.4	8.4	
15	8.5	9.5	9.0	9.1	8.1	
16	8.3	9.1	8.8	8.9	7.9	
17	8.0	8.5	8.5	8.5	7.8	
18	7.8	8.4	8.3	8.4	7.6	
19		8.0	8.1	8.3	7.5	
20	7.7	8.0	8.0	8.2	7.5	
21		7.9	7.9	8.1	7.4	
22	7.3	7.7		7.9	7.3	
23		7.7		7.7	7.3	
24	7.1	7.5	7.5	7.4	7.2	
25		7.3				
26		7.2				
27	6.7	7.0	7.3		6.9	
28		6.8				
29		6.8				
30	6.4	6.9	7.0	7.3	6.9	
31					6.9	
32						
33	5.9		6.9			

Not Measured

Table 50. Lake Chester Morse (continued)

Depth (Meters)	11/13/73 T°C	12/11/73 T°C	1/30/74 T°C	2/12/74 T°C	9/5/74 T°C
Surface	7.6	5.0	4.0	4.0	17.4
1					17.3
2					17.3
3					17.0
4					16.9
5					16.6
6					16.2
7					15.0
8					14.2
9					13.2
10	7.6	5.0	4.0	4.0	11.9
11					10.9
12					10.4
13					9.5
14					9.2
15					8.9
16					8.8
17					8.6
18					8.6
19					8.6
20	7.6	5.0	4.0	4.0	8.5
21					8.4
22					8.4
23					8.3
24					8.3
25					8.2
26					8.2
27					8.2
28					8.0
29	7.6				7.9
30		5.0	4.0	4.0	
31					
32					
33	7.8	5.0	4.0	4.0	7.9

Table 50. Findley Lake

Depth (Meters)	8/8/72 T°C	8/30/72 T°C	10/12/72 T°C	11/1/72 T°C	12/13/72 T°C
Surface	19.5	16.9	7.8	5	0
1	19.5	16.9	7.8	5	
2	18.5	16.6	7.8	5	
3	14.0	16.5	7.8	5	
4	12.2	16.2	7.8	5	
5	10.6	15.5	7.8	5	
6	9.5	13.7	7.8	5	
7	8.8	11.7	7.8	5	
8	7.7	10.5	7.8	5	
9	6.8	9.0	7.8	5	
10	6.0	7.9	7.8	5	
11	5.6	7.2	7.8	5	
12	5.2	6.5	7.8	5	
13	5.0	[illegible].1	7.8	5	
14	4.8	[illegible].8	7.5	5	
15	4.5	5.5	6.2	5	
16		5.3	5.9	5	
17		5.2	5.7	5	
18	4.2	5.0	5.5	5	
19		4.8	5.1	5	
20		4.6	5.0	5	
21	4.0	4.5	4.9	5	
22		4.5		5	
23		4.4		5	
24	4.0	4.4		5	
25				5	
26				5	
27	4.0	4.3		5	

Not Measured

Table 50. Findley Lake (continued)

Depth (Meters)	3/27/73 T°C	5/24/73 T°C	6/7/73 T°C	7/6/73 T°C	7/26/73 T°C
Surface	0.4	0.5	4.5	13.4	17.4
1	2.1	1.6			17.0
2	3.3	3.0		13.2	16.5
3	3.5	3.5		11.0	16.0
4	3.7	3.5		9.5	15.5
5	3.7	3.6		8.5	13.5
6	3.8	3.7		8.2	12.6
7		3.7	4.5	7.9	11.5
8		3.8	4.2	7.4	10.2
9		3.8	4.1	6.6	7.4
10		3.8	4.0	6.2	7.3
11			4.0	5.5	
12		3.9	4.05	5.5	6.5
13			4.0	5.4	6.2
14				5.2	6.8
15		3.9			6.5
16				5.0	6.4
17		4.0			6.3
18					6.0
19					5.9
20		4.0			5.6
21					4.5
22		4.0		4.9	
23					4.4
24		4.0			4.2
25					4.2
26					
27				4.6	4.1

Table 50. Findley Lake (continued)

Depth (Meters)	8/30/73 T°C	10/4/73 T°C	11/30/73 T°C	2/28/74 T°C	9/19/74 T°C
Surface	15.0		5.1		12.9
1	15.1		6.1		
2	15.2		6.6		
3	15.2		6.7		
4			6.8		
5			6.9		12.5
6			6.9		
7			6.9		
8	15.0		7.0		
9	12.1		7.0		
10	11.0		7.1		8.5
11	10.5		7.2		
12	9.5		7.4		
13	8.7	Not Measured	7.5	Not Measured	
14	8.3		7.6		
15	7.8		7.7		
16	7.5				
17	7.2		7.7		
18	6.9		7.8		
19	6.6		7.9		
20	6.4				Not Measured
21	6.2				
22					
23	6.0				
24			7.9		
25					
26	5.9		8.0		
27	5.9		8.1		

Table 51. Pure Cultures of Chitinoclastic Bacteria Isolated from the Four Lakes, Characteristics[1]

Isolate	Lake	Station[2]	Date	In Situ T°C	Isolate[3] T°C	Hugh Leifson[4]
19-5	F	(H_2O) #2	2/ 9/73	0.5	4.0	+
61-2	W	#2	11/ 8/73	9.8	15.0	+
62-1	CM	#2	11/13/73	7.8	4.0	+
62-2					4.0	+
62-3					4.0	+
64-1	F	#1	11/30/73	4.0	4.0	+
64-2					4.0	+
64-3					4.0	+
70-1	S	#1	2/ 6/74	5.8	25.0	+
70-2					25.0	-
71-1	CM	#2	2/12/74	4.0	4.0	+
71-2					4.0	+
71-7					4.0	+
71-11					4.0	+
71-15					4.0	+
72-1	F	#2	2/28/74	1.0	15.0	-
72-2					15.0	+
72-3					15.0	+
72-4					15.0	+
72-12					4.0	+
72-21					4.0	+
72-23					4.0	+
72-24					4.0	+
74-1	S	#1	4/ 3/74	6.0	4.0	+
74-2					4.0	+
77-1	W	#2	8/22/74	20.0	4.0	+
77-2					4.0	+
77-3					4.0	+
77-4					4.0	+

[1]All chitinoclastic bacteria isolated from the sediment, except 19-5; all isolates were gram negative, oxidase positive motile rods.

[2]W - Lake Washington; S - Lake Sammamish; CM - Lake Chester Morse; F - Findley Lake.

[3]Indicates initial incubation temperature of inoculated plate from which culture isolated.

[4]Positive response indicates capable of fermenting glucose to acid (without production of gas); all isolates were capable of oxidizing glucose to acid.

Table 52. Glucose Mineralization Data for Sediments from Lake Washington.[1] Plate count data in Tables 11 and 13.

Station	Date	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/gm/hr)
#1, 60 meters	9/ 8/72	7.6	7.5 ± 1.0	0.75 ± 0.12
	11/15/72	7.0	17.8 ± 3.5	0.23 ± 0.15
	3/ 6/72	6.9	2.3 ± 1.4	0.15 ± 0.04
	6/19/73	7.2	7.1 ± 1.3	0.26 ± 0.06
		17.0*	4.6 ± 1.1	4.60 ± 1.10
	7/31/73	8.0	4.0 ± 1.3	0.53 ± 0.09
	8/23/73	8.0	14.8 ± 2.0	0.40 ± 0.10
		12.5*	5.0 ± 2.0	0.20 ± 0.08
		26.0*	4.7 ± 0.9	0.90 ± 0.10
	9/25/73	8.1	4.1 ± 0.7	0.16 ± 0.03
	10/25/73	7.9	7.2 ± 1.0	0.19 ± 0.04
	11/20/73	8.5	12.1 ± 1.6	0.39 ± 0.09
	12/18/73	8.7	6.4 ± 0.3	0.82 ± 0.06
	1/25/73	6.5	15.8 ± 3.7	0.16 ± 0.13
		12.8*	3.8 ± 2.6	0.14 ± 0.07
		35.0*	3.9 ± 0.7	0.47 ± 0.06
	4/ 4/73	6.6	23.5 ± 5.2	0.21 ± 0.10
		51.0*	353.0 ± 29.2	0.02 ± 0.06
	4/25/73	7.0	8.0	0.13
	9/24/74	6.6	3.4	0.75
		17.0*	3.8	0.85
		26.8*	2.6	4.15
		32.3*	4.0	2.86
		45.6*	57.0	0.15
#2, 0.5 meters	6/28/72	20.0	2.4	23.00
	9/28/72	19.0	7.5 ± 4.0	1.03 ± 0.36
	11/15/72	10.0	3.4 ± 0.2	8.30 ± 0.23
	1/23/73	6.0	17.7 ± 2.8	0.63 ± 0.12

Table 52 (continued)

Station	Date	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/gm/hr)
#2, 0.5 meters	2/18/73	9.0	5.2 ± 0.8	0.41 ± 0.20
		3.0*	15.0 ± 1.3	0.12 ± 0.12
		19.0*	3.4 ± 0.3	2.80 ± 0.79
	4/10/73	10.5	3.2	2.58
	4/26/73	13.0	1.9 ± 0.2	9.30 ± 0.30
	6/26/73	18.5	3.4 ± 0.2	3.31 ± 0.25
		5.0*	5.2 ± 0.5	0.88 ± 0.14
		11.0*	3.6 ± 0.5	1.10 ± 0.19
	8/20/73	19.0	5.5 ± 0.8	1.10 ± 0.40
	11/ 8/73	9.8	4.7 ± 0.2	1.46 ± 0.17
	8/22/73	20.0	4.4	3.07
		1.8*	15.0	0.18
		6.3*	9.0	0.56
		10.8*	7.0	1.92
		30.9*	3.1	3.72
		44.8*	3.3	1.65
	10/ 1/74	15.0	6.5	2.68
		1.5*	13.0	0.40
		25.0*	3.8	8.00
		30.5*	2.7	9.20
		35.0*	2.9	9.50
		43.5*	22.0	0.35

[1]Values are mean ± standard deviation.

*Denotes temperature of analysis.

Table 53. Glucose Mineralization Data for Sediments from Lake Sammamish.[1] Plate count data in Tables 14 and 15.

Station	Data	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/gm/hr)
#1, 27 meters	9/20/72	8.0	6.1	1.00
	11/29/72	7.8	5.1 ± 0.5	0.89 ± 0.06
	4/ 3/73	6.2	19.5 ± 2.0	0.06 ± 0.08
	4/27/73	7.0	18.2 ± 0.9	0.42 ± 0.10
	5/11/73	6.9	17.6 ± 1.7	0.20 ± 0.07
	7/13/73	7.9	11.3 ± 0.7	0.96 ± 0.10
	7/17/73	7.9	15.0 ± 1.7	0.89 ± 0.10
	8/ 2/73	7.9	10.6 ± 1.0	1.30 ± 0.25
	8/21/73	7.8	6.7 ± 0.6	0.81 ± 0.10
	10/ 2/73	7.9	6.4 ± 0.7	0.55 ± 0.10
	12/ 5/73	8.9	12.7 ± 1.5	0.35 ± 0.08
	2/ 6/74	5.8	32.5 ± 5.3	0.24 ± 0.22
	3/ 6/74	6.0	26.4 ± 3.0	0.33 ± 0.23
	4/ 3/74	6.0	17.6 ± 1.0	1.00 ± 0.17
		1.0*	49.5 ± 5.1	0.27 ± 0.20
		21.0*	10.6 ± 0.4	5.70 ± 0.35
		35.0*	8.0 ± 0.6	2.70 ± 0.20
		53.0*	308.0 ± 4.8	0.19 ± 0.15
#2, 2.0 meters	9/20/72	15.5	11.5	0.60
	11/29/72	9.0	8.1 ± 1.3	0.85 ± 0.10
	4/13/73	11.2	8.3 ± 1.0	0.17 ± 0.06
	4/27/73	10.3	12.2 ± 1.6	0.18 ± 0.10
	5/11/73	11.4	13.4 ± 0.7	0.50 ± 0.10
	7/13/73	23.4	8.5 ± 0.2	0.25 ± 0.20
	7/17/73	24.0	5.3 ± 0.2	1.30 ± 0.10
	8/ 2/73	23.0	5.3 ± 1.9	1.16 ± 1.12
	8/21/73	20.5	7.6 ± 0.2	2.15 ± 0.20
	10/ 2/73	18.5	9.7 ± 0.5	2.14 ± 0.42
	12/ 5/73	8.9	20.6 ± 1.5	0.77 ± 0.29

[1]Values are mean ± standard deviation. *Denotes temperature of analysis.

Table 54. Glucose Mineralization Data for Sediments from Lake Chester Morse.[1] Plate count data in Tables 16 and 17.

Station	Date	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/gm/hr)
#1, 33 meters	8/22/72	6.5	10.0	0.92
	9/27/72	7.0	8.1 ± 2.3	0.94 ± 0.14
	3/ 8/73	2.2	11.6 ± 8.0	0.16 ± 0.06
	3/20/73	3.4	51.0 ± 9.4	1.30 ± 0.60
	4/ 3/73	3.8	38.9 ± 10.2	0.10 ± 0.40
	4/18/73	4.8	6.2 ± 0.5	1.07 ± 0.23
	5/ 1/73	4.7	9.2 ± 1.6	0.42 ± 0.15
	5/22/73	5.9	8.5 ± 1.4	0.22 ± 0.05
	6/ 5/73	6.9	7.4 ± 0.4	0.68 ± 0.10
	7/10/73	6.9	9.5 ± 1.0	0.55 ± 0.10
	7/24/73	7.1	7.1 ± 2.8	0.09 ± 0.05
	8/28/73	6.9	6.0 ± 0.8	4.40 ± 0.70
	10/16/73	6.9	5.8 ± 0.1	1.25 ± 0.06
	11/13/73	7.8	8.0 ± 0.7	0.81 ± 0.28
	12/11/73	5.0	9.1 ± 0.5	0.68 ± 0.11
	1/30/74	4.0	11.9 ± 1.0	0.66 ± 0.16
		13.0*	10.5 ± 2.6	1.00 ± 0.64
		22.0*	6.8 ± 0.7	2.15 ± 0.35
		34.0*	7.2 ± 0.5	1.76 ± 0.20
	9/ 5/74	1.0*	41.0	0.10
		13.5*	24.0	0.18
		25.2*	10.5	0.20
		32.0*	5.6	0.21
		45.2*	14.0	0.13
		50.0*	41.5	0.07
#2, 4.0 meters	8/22/72	19.0	1.0 ± 0.7	4.09 ± 0.20
	2/28/73	3.0	6.7 ± 1.4	0.27 ± 0.14
	3/20/73	3.5	16.5 ± 1.8	5.40 ± 0.68
	4/ 3/73	3.2	33.2 ± 15.5	0.04 ± 0.02

Table 54 (continued)

Station	Date	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/ gm/hr)
#2, 4.0 meters	5/ 1/73	7.5	5.0 ± 0.6	1.50 ± 0.19
	5/22/73	11.3	5.6 ± 0.7	0.66 ± 0.10
	7/24/73	18.4	1.9 ± 0.3	0.73 ± 0.10
	8/28/73	16.0	6.0 ± 0.8	4.40 ± 0.70
	10/16/73	8.5	5.0 ± 0.3	1.00 ± 0.13
	11/13/73	7.8	8.7 ± 1.2	0.29 ± 0.27
	12/ 1/73	5.0	3.6 ± 1.2	0.18 ± 0.18
	12/12/74	4.0	5.5 ± 0.5	1.33 ± 0.30
		6.5*	4.0 ± 0.2	3.40 ± 0.27
		19.9*	3.4 ± 0.1	12.60 ± 0.20
		29.0*	2.8 ± 0.2	3.00 ± 0.20

[1]Values are mean ± standard deviation.

*Denotes temperature of analysis.

Table 55. Glucose Mineralization Data for Sediments from Findley Lake.[1] Plate count data in Tables 20 and 21.

Station	Date	In Situ T°C	Glucose Turnover Time (Hrs)	V_{max} (µgm/glu/gm/hr)
#1, 27 meters	7/ 7/72	4.0	10.0	2.00
	8/ 8/72	4.0	11.5 ± 2.5	0.75 ± 0.13
	8/30/72	4.3	7.9 ± 1.8	0.84 ± 0.13
	10/12/72	4.9	6.7 ± 4.0	1.18 ± 0.16
	11/ 1/72	5.0	35.8 ± 19.0	0.66 ± 0.30
	12/13/72	4.0	30.3 ± 4.0	0.40 ± 0.10
	3/ 5/72	4.0	46.0 ± 8.0	0.18 ± 0.30
	3/27/73	4.0	32.0	0.09
	5/24/73	4.0	64.5 ± 20.0	0.07 ± 0.11
	6/ 7/73	4.4	12.8 ± 2.0	0.30 ± 0.10
	7/ 5/73	4.6	17.5 ± 3.0	0.38 ± 0.20
	7/26/73	4.1	4.9 ± 2.0	0.34 ± 0.17
	8/30/73	5.9	13.6 ± 0.9	0.37 ± 0.06
	10/ 4/73	5.0	17.8 ± 3.3	0.25 ± 0.10
	11/30/73	4.0	9.3 ± 0.5	1.27 ± 0.16
	2/28/74	4.0	16.1 ± 0.8	3.20 ± 0.70
		7.0*	10.7 ± 3.1	7.00 ± 0.40
		20.0*	6.5 ± 0.2	11.50 ± 0.27
		35.0*	5.6 ± 0.3	6.50 ± 0.40
	9/19/74	2.9*	150.0	0.005
		13.5*	60.0	0.094
		23.4*	22.0	0.18
		29.6*	22.0	0.23
		34.4*	40.0	0.25
		46.0*	550.0	0.003
#2, 0.5 meters	7/ 7/72	10.5	2.0	30.0
	8/ 8/72	20.5	1.0 ± 0.1	8.70 ± 0.10
	8/30/72	18.8	1.9 ± 0.5	5.50 ± 0.30
	10/12/72	7.8	4.0	6.50

Table 55 (continued)

Station	Data	In Situ $T^{o}C$	Glucose Turnover Time (Hrs)	V_{max} (ugm/glu/ gm/hr)
#2, 0.5 meters	11/ 1/72	5.0	3.0 ± 0.5	7.50 ± 0.09
	12/13/72	2.0	3.2 ± 2.4	0.34 ± 0.10
	2/ 9/73	0.5	10.2	0.42 ± 0.10
	5/24/73	3.5	22.4 ± 2.0	0.27 ± 0.10
	6/ 7/73	5.9	2.0 ± 0.4	0.60 ± 0.10
	7/ 5/73	13.5	3.9 ± 0.3	1.70 ± 0.10
	7/26/73	17.4	2.2 ± 0.4	23.60 ± 0.40
	8/30/73	13.5	5.5 ± 0.2	9.80 ± 0.20
	10/ 4/73	12.5	4.6 ± 0.1	3.10 ± 0.07
	2/28/74	1.0	28.5 ± 2.1	0.36 ± 0.09
		15.0*	12.5 ± 1.2	3.00 ± 0.37

[1]Values are mean ± standard deviation.

*Denotes temperature of analysis.

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