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THE MICROBIOLOGY OF CHITIN DECOMPOSITION
IN LAKE ERIE SEDIMENTS

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
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* * * *

The Ohio State University
1974

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FIELD OF STUDY

Microbial Ecology
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INTRODUCTION

The understanding of the cycling of nutrients within an environment can lead to constructive work towards maintaining or preserving that environment. Within an aquatic ecosystem, the wide variety of sources of energy present at any one time makes study of the system as a whole complex and difficult to interpret. One means of studying nutrient cycling within an environment is to enrich for those organisms and factors within the environment that are involved in the cycling of the particular substance.

One nutrient associated with the aquatic environment is chitin. Chitin is a macromolecule composed of N-acetylglucosamine subunits with $\beta-1,4$ linkage. Chitin is present in shells of microcrustaceans and arthropods all of which are found in abundance in aquatic systems. None of these substances accumulate substantially within this environment. Because of the large quantity of chitin within the aquatic environment, and since it does not accumulate substantially, its cycling is a significant and integral part of the food chain.

This study has been conducted in hopes of further elucidating the role chitin may play in the cycling of nutrients within the aquatic environment. Chitin, because of
its macromolecular nature, is not a utilizable source of energy for most species found within this environment. However, N-acetylglucosamine or its breakdown products, such as, glucose, acetate, and ammonia, are readily utilized by a much more diverse group of microorganisms. The intent of this research is to examine more closely the mechanism by which microorganisms utilize chitin within the aquatic environment.

The study of this process has been broken down into three levels of diversity: 1) experimentation dealing with mud samples upon enrichment for chitinolytic organisms, 2) studies with chitinolytic isolates from the aquatic environment, and 3) studies dealing with the extracellular enzymes of chitinolytic organisms.
LITERATURE REVIEW

Various parameters of sediment samples of Lake Erie as well as the other Great Lakes have been studied in great detail over the years. Before any biological study of sediment cores from a lake was undertaken, an examination of their physical characteristics was advisable. The western basin of Lake Erie, where the present study was conducted, was very shallow averaging 20 ft in depth and characterized by turbidity and mixing of the water column. The surficial sediments were generally considered aerobic with anaerobic zones below 2 cm, a depth at which significant mechanical mixing did not occur.

A detailed study of physical characteristics of lake cores was carried out by a number of investigators, including Kemp (25,26), Konrad, et al. (27), and Vanderpost (52). On cores collected from Lake Ontario, Vanderpost (52) noted a significant drop in organic carbon found within the depths of the sediment column from surface to approximately 6 cm. At this depth the organic carbon continued to decrease but at a much slower rate. The Eh as well as the water content also showed significant decreases in value after the first 6 cm of sediment. An Eh change of approximately 200 mv from 0-6 cm was found
regardless of core or date of sampling. Water content dropped from over 70% to 50% in the same zone. Determination of pH within the column showed little change, remaining fairly constant at a pH of 7.1-7.6.

Kemp (25,26) studied sediments from Lakes Erie, Ontario, and Huron. He examined the sediments for changes in organic carbon and nitrogen as well as Eh with increasing depth. Kemp (26) found organic carbon and nitrogen decreased sharply from the surface sediments down to 10 cm with a leveling off at that point. The organic carbon in Lake Erie sediments was lower than those values obtained in the other lakes studied, especially in the western basin where he attributed microbial mineralization processes as the cause of the phenomenon. The Eh values dropped with depth as did the values reported by Vanderpost (52).

Konrad, et al. (27) compared the organic carbon and nitrogen content of various cores taken from oligotrophic, mesotrophic, and eutrophic Wisconsin lakes. Much higher values of organic carbon were found at all depths in all lakes tested when compared to work done with sediments from the Great Lakes. The results showed no significant change of organic carbon within the depths of the sediments between the mesotrophic, oligotrophic, and eutrophic lakes.

The physical factors associated with sediment cores have been correlated with bacterial numbers and nutritional types by Vanderpost (52) as well as by Zobell and Anderson (56). The
latter investigators first recognized the correlation in 1936. They examined marine cores up to 68 cm in depth. Their data showed that "utilizable organic matter is the most important single factor which influences the distribution of bacteria in marine sediments" (56). However, it was also recognized that animals feeding on bacteria may influence bacterial numbers (33). The change in oxidation-reduction potential was likewise correlated with bacterial populations. Zobell and Anderson (56) attributed the decrease in O-R potential within the core to biological activity. The ability of the bacteria recovered from the marine sediments to undergo biochemical processes, such as, ammonification, proteolysis, nitrate reduction, nitrification, macromolecular digestion, and fermentation were reported with percentage values given of the total population obtained. The ability of bacteria to concentrate in colloidal clay preferentially over large particle size suspensions, such as, sand and silt, was also noted. This was one more correlation between organics, which are preferentially concentrated in clays, and bacterial numbers.

Vanderpost (52) also noted the correlation that existed between bacterial numbers, organics, and Eh within sediments. Cores collected from Lake Ontario over a period of several months were used for this study. A direct relationship existed between bacterial numbers and organic carbon. Highest values of each were noted in surficial sediments with decreasing values with increasing depths.
Various nutritional types were enumerated by Vanderpost (52). Included were anaerobic and aerobic heterotrophs, sulfate reducing bacteria, sulfur oxidizing bacteria, and nitrifying bacteria to name a few. Numbers ranged from $4.2 \times 10^7$ heterotrophic aerobes in surficial sediments to $1.8 \times 10^6$ aerobes/g dry wt at 18-21 cm in the column. Heterotrophic anaerobes numbered $1.6 \times 10^5$ to $2.4 \times 10^4$/g dry wt in surficial and 18-21 cm sediments, respectively. No significant seasonal variation was noted. Numbers of bacteria of the various nutritional types were noted to decrease with increasing depths.

Further work on Ontario sediments has been conducted by Bell and Dutka (3,4). Their work was limited to enumeration of anaerobic and aerobic heterotrophs. They used a spread plate method as did Vanderpost (52) but also compared their results with membrane filter technique results conducted on the same core and found little differences in numbers due to methods employed (4). Values of $10^7$ aerobes/g dry wt in surficial sediments to $10^4$/g dry wt at a depth of 12-15 cm were encountered. Anaerobes varied from $10^6$ to $10^5$/g dry wt at surficial and 12-15 cm, respectively. As with the previously mentioned authors (56,52), the correlation between numbers within the column and Eh was drawn by Bell and Dutka (3).

Weeks (55) examined heterotrophic bacterial populations in sediments of western Lake Erie as in the present study. Surface sediment counts gave values of $6-18 \times 10^6$ aerobes and $1.2-2.9 \times 10^5$ anaerobes/g dry wt which were noted to be subject to
phytoplankton blooms. Higher values were obtained following periods of phytoplankton blooms and increased turbidity. Sediments from 2 cm depth showed values of $2.6-7.7 \times 10^6$ aerobes and $1.8-3.3 \times 10^5$ anaerobes/g dry sediment.

Other methods have been used to quantitate bacteria in lake sediments. Lee, et al. (31) correlated ATP content with cell numbers in sediments. These results were easily correlated with bacterial numbers in sterilized sediments inoculated with indigenous bacteria. However, results with unsterilized sediments did not correlate well with plate count values due to protozoa within the sample. The method consequently relied on the accuracy of the investigator to microscopically enumerate the protozoa within the sediments.

Pamatmat and Bhagwat (42) correlated anaerobic bacterial numbers with dehydrogenase activity within sediments. This process did not, however, account for dehydrogenase activity attributed to aerobic metabolism, and was, therefore, not considered any more valid than plate or membrane filter methods of enumeration.

A nutritional type of bacteria not mentioned thus far was the chitinolytic bacteria present within the sediments. Using a minimum dilution technique, Zobell and Rittenberg (57) attempted enumeration of chitinoclastic organisms from marine sediments. They found a very uneven distribution of chitinoclasts between cores taken within a few cm of each other. The numbers varied by a factor of 100 from each other. This was
attributed to uneven distribution of nutrients in the sediments. An interesting finding was their observation that high numbers of chitinoclasts were usually associated with coarse sedimentary materials such as sand. The chitinous particles were concentrated by a sorting action in the larger particles of the sediments, thereby having an enriching effect for chitinolytic organisms.

The chitinolytic organisms of marine origin were not limited to sediments. Campbell and Williams (8) conducted a study isolating and identifying a number of chitin digesting microorganisms from the water column.

Attempts to enumerate chitinolytic organisms within aquatic sediments have not been carried out. However, numerous chitinolytic isolates have been selected from lake environments (5,14,36,54). Enumeration of chitinolytic organisms was conducted on surficial sediments of Lake Erie by Warnes (54) who found values of approximately $10^4$/ml surficial water.

Quantitation of chitinolytic microorganisms has been conducted in other environments. Skinner and Davis (47) enumerated chitinolytic microorganisms found in a variety of soils by the MPN method and found from $288/g$ dry soil of sand to $10^6/g$ dry soil in rich black loam. Veldkamp (53) worked with sandy soils and showed $3.5 \times 10^5$ chitinoclasts/g dry soil. The values obtained by the two authors differed because of techniques. Veldkamp (53) used a dilution plate technique while an MPN method was used by Skinner and Davis (47).
In addition to enumeration of chitinolytic microorganisms in various environments, characterization and identification of numerous strains have been conducted by many investigators (24, 8, 53, 10, 36, 54). The habitats of these organisms are very diverse. They have been found in the intestines of many fish, reptiles, and mammals as well as in the soils and waters of various locations (5).

One of the best studied habitats was that of sea water. Benecke, as reported in Benton (5), was the first individual to isolate a bacterium capable of digesting chitin. He isolated the organism from a marine environment and chose the name Bacillus chitinovorous for the Gram negative, aerobic, motile organism. Zobell and Rittenberg (57) isolated and characterized numerous chitin digesting strains from marine muds, water, and marine crustacea. All were Gram negative, nonmotile rods. Their ability to degrade starch varied with none being able to utilize cellulose. Their ability to ferment sugars varied.

Campbell and Williams (8) isolated and identified a number of species of chitinolytic organisms from sea water. Species of the genera Achromobacter, Pseudomonas, Flavobacterium, and Micrococcus were identified.

In 1932 Johnson (24) published work on chitinolytic microorganisms found on the exoskeleton of hard shell crabs. All organisms isolated showed pigmentation on chitin agar. She classified a number of the isolates as being in the genus Myxococcus. Fruiting bodies were observed with the isolates.
Cook and Lofton (11) isolated organisms from diseased shrimp off the coast of Mississippi. The genera Beneckea, Vibrio, and Pseudomonas were found in association with the diseased portion of the crustacean shell. All three were found to have chitinolytic capabilities. The type suspected to be the causative agent was Beneckea partially due to 100% incidence with the disease.

Less work has been done with characterization and identification of chitinolytic organisms within the lake sediment. Ericson (14) studied strains of Micromonospora chalcae isolated from Lake Mendota in Wisconsin. A number of strains were studied and noted to be strongly chitinolytic with no need for addition of nitrogen in the form of nitrate for digestion to occur.

Studies with Lake Erie isolates by McCabe (36) showed four strains of Micromonospora and one strain of Streptomyces demonstrating chitinolytic abilities. Using surficial sediments of Lake Erie, Warnes (54) isolated 2 strains of chitinolytic Pseudomonas, one of Aeromonas, one of a eubacterium, possibly Beneckea, and 2 strains of Streptomyces.

Many isolates were examined by Benton (5) from water and sediments from Lake Alexander in Minnesota. By morphological and biochemical means she identified chitinolytic organisms which most closely resembled bacteria of the genera Pseudomonas, Cellvibrio, and Cellulomonas. Of strains isolated from the aquatic environment but not identified to genus were a peritrichous, fermentative, Gram negative organism, and a number of plump, polarly flagellated rods with or without fermentative
abilities. A pleomorphic form was also found which was described as resembling coryneform bacteria.

Probably the most extensively studied environment in which chitinolytic microorganisms were found was the soil. Benton (5) was one of the first to investigate this environment. She characterized a number of chitinolytic vibriods but did not identify them.

Of the 50 chitin decomposing bacterial strains isolated by Veldkamp (53), representatives of the following genera were identified: Achromobacter, Flavobacterium, Chromobacterium, Bacillus, Cytophaga, and Pseudomonas. In addition, a Gram positive nonsporeforming rod, possibly a coryneform bacteria, was isolated. A number of actinomycetes were also identified in his study. Twelve strains of Streptomyces, 2 of Nocardia, and 9 of Micromonospora were identified as chitinolytic.

Using a buried slide approach, Okafor (38,39,40) studied the ecology of chitin decomposition in temperate and tropical soils at different temperatures. Isolates of the probable genera of Sarcina, Streptomyces, Actinoplanes, and Micromonospora as well as an isolate identified to the order Myxobacterales were noted (38). The predominant type of bacteria, a coccal form of 1.3 um in diameter, was not identified.

Temperature effect was examined by Okafor (40). He concluded that actinomycetes were only active in chitin decomposition when soil temperature reached 20 C or more. He speculated that the importance of actinomycetes in chitin decomposition in
soil may not be as great as concluded by Skinner and Davis (47) and Veldkamp (53).

Working with chitin from deproteinized and dewaxed insect wings, Okafor (39) isolated 2 strains of Streptomyces, a Pseudomonas, and a Bacillus capable of hydrolyzing chitin. Of the various classes used by Okafor (fungi, bacteria, and actinomycetes), the bacteria always predominated, although the fungi and actinomycetes were also present.

The importance of chitinolytic bacteria to the ecology of soils was exemplified by the work of Hocking and Cook (21). A fungal strain containing chitin in its cell wall was known to be responsible for pathogenicity in roots of pine seedlings. They inoculated potted seedlings with strains of chitinolytic Cytophaga and Sorangium. A decrease in the degree of pathogenicity was observed after this treatment when compared to controls.

A number of papers have been published on chitin decomposition in soils by workers from the University of Liverpool. Baxby and Gray (2) identified chitinolytic strains of the genera Micromonospora, Streptomyces, Chromobacterium, and Bacillus from soils. A number of chitinolytic fungi were also identified. In addition to the above mentioned isolates, Gray and Baxby (17) identified Aeromonas, Vibrio, and Pseudomonas species as chitinase positive organisms. Gray, et al. (18) compared decomposition of chitin in sea water, marine mud, and forest soil. Bacillus circulans was identified as present in the forest soil by fluorescent antibody technique. Gray and Bell (16)
isolated a number of fungi and one bacterium growing on buried chitin in soil. The bacterium resembled *Pseudomonas chitinovorans* as described by Veldkamp (53).

In addition to the above mentioned isolates, others have taken type cultures and tested them for the ability to hydrolyze chitin. This information was necessary because work done in the various habitats mentioned have been nearly all aerobic studies. Clarke and Tracey (10) identified strains of *Clostridium*, *Klebsiella*, *Vibrio*, and *Erwinea* possessing chitinase systems.

Monreal and Reese (34) found strains of *Serratia marcescens*, *Enterobacter liquefaciens*, *Cellvibrio vulgaris*, and *Bacterium* sp. that could hydrolyze chitin preparations.

The sources of chitin within the water environment and other habitats are varied. Tracey (50) detected chitin in the green alga *Cladophora* and *Spirogyra* and in fungi. Chitin was not detected in vascular plants or bacteria.

The microcrustacea were the major source of chitin in an aquatic environment. Many authors (9,12,13,7,22) have enumerated these organisms in the water column. Frey (15) and Harmsworth and Whiteside (20) have enumerated and/or discussed cladoceran remains within aquatic sediments.

Arthropod exoskeletons were another source of chitinous substrates within the aquatic environment. Numerous insect populations use the lake sediment for maturation of larval and pupal stages.
Studies conducted to enumerate or isolate chitinolytic organisms from a given environment usually attributed a positive result either by the organism's ability to produce a ring of hydrolysis on chitin agar or ability to grow in a chitin mineral salts media as for MPN determinations. A number of authors, however, have tried to detect chitinolytic activity by measurement of end products. Jeuniaux (23) discussed various means of assaying for chitinase activity. He outlined the method of Morgan and Elson (35) for the colorimetric determination of N-acetylglucosamine (NAGA), the subunit of chitin. Veldkamp (53) suggested that the high C:N ratio of chitin (6.8:1) might suggest ammonia release from degradation of the subunit or possibly hydrolysis of the amine portion of the chitin molecule. Tracey (50) suggested tests for glucosamine and acetic acid, also possible products of chitin digestion.

A very qualitative means of determining chitinolytic activity has been employed by Okafor (38) and Gray and Bell (16). This method, adapted from Tribe (51), employed the burial of a slide with an adhering piece of chitin in soils for varying incubation periods. The morphological types associated with the slide were observed and photographed. A control slide with no chitin was employed for comparison of normal versus enriched populations due to the chitin.

An even more direct approach to the study of chitinolytic activity of isolates was the study of the chitinase enzyme system. The turbidimetric method described by
Jeuniaux (23) has been applied to enzyme preparation from numerous isolates. A decrease in OD values at 420 nm in a colloidal chitin, buffer, enzyme system was indicative of chitinase activity.

It should be noted that the chitinase system was composed of 2 separate enzymes. The chitinase enzyme was active on the chitin macromolecule down to chains of two and three subunits. The chitobiase enzyme must be present for production of NAGA. Chitobiase was active in hydrolyzing the disaccharides and trisaccharides of chitin (23). The system was usually considered to be extracellular in nature but this was not without exception (49).

Ability to hydrolyze NAGA appeared to be widespread. Ortiz, et al. (41) reported that exo-N-acetylglucosaminidase was common in Gram positive and negative bacteria. It has also been found in known chitinolytic microorganisms by Sundarraj, et al. (49) and Nord and Wadstrom (37). N-acetylglucosamine hydrolysis was not specific to chitinolytic organisms although some possessed the ability.

Most articles published on the chitinase system have dealt with preparations of strains of Streptomyces. Reynolds (45) used 10 ml of a cell-free system from a culture of Streptomyces sp. and 50 mg of chitin in 50 ml Erlenmeyer flasks which were incubated on a rotary shaker for 48 hr. Net production of reducing sugars over that of control flasks were tested for by the method of Sumner (48). Chitinase activity was found to be maximal at pH 6.5-7.0. Optimal activity was observed at 37 C
incubation. No attempt was made to fractionate the culture supernatant to concentrate the enzyme system.

Further work with crude supernatants was conducted by Clarke and Tracey (10). A positive chitinase test was determined by production of NAGA after incubation of a colloidal chitin, buffer, and enzyme solution. The method of Morgan and Elson (35) was used to determine NAGA.

Berger and Reynolds (6) were among the first to attempt purification of the enzyme system. They used a strain of *Streptomyces griseus*. Assays were conducted for NAGA, total reducing sugars, and protein content of the culture fluid. Chitinase activity was determined by noting a decrease in OD at 420 nm in a buffer, colloidal chitin, enzyme solution after incubation at 35°C. A unit of chitinase activity was defined as the amount of enzyme which brought about a decrease of 0.001 OD unit per min. Specific activity was described as units per mg of protein. Using ammonium sulfate fractionation, the enzyme was concentrated using 70% saturation. The maximum activity of the enzyme system was found to be at pH 6.5. Further separation of the chitinase system into chitinase and chitobiase was conducted using zone electrophoresis on starch beds.

The chitinase system of *Serratia marcescens* has been investigated by Monreal and Reese (34). Differences in chitin substrate preparation, pH, and inducibility were studied. Maximum activity occurred on colloidal chitin as opposed to particulate chitin at a pH optimum of 6.5. The inducible nature of the
enzyme was also noted. Ammonium sulfate fractionation showed maximum activity in the 40-80% fraction. Chitinase activity was determined by production of reducing sugar.

Sundarraj, et al. (49) have studied the chitinase system of strains of Cytophaga johnsonii. The test for chitinase activity was identical to the turbidometric method used by Berger and Reynolds (6). No activity could be found with culture filtrates of one strain at any stage of growth, therefore, it was assumed that the particular strain produced no extracellular chitinase enzyme. Cell free extracts, however, did show activity in the supernatant and pellet after spinning for 30 min at 15,000 x g.
MATERIALS AND METHODS

Collection of cores

Sediment cores were taken using a brass corer into which a 3 ft butyrate tube was inserted. The tubing had dimensions of 1-3/8" ID x 1-1/2" OD. Samples were collected manually using 5 ft sections of aluminum pipe with appropriate couplers. Removal of the sample from the corer was done as rapidly as possible but could not involve aseptic technique. Samples were capped and stored in an ice chest until return to Columbus. Cores were stored at -20 C upon arrival until needed for experimentation.

Samples were taken approximately 30 m north of Rattlesnake Island. No other site was sampled because of inclement weather. Collection occurred on October 25, 1973.

Preparation of chitin substrates

Chitin fragments were obtained from Calbiochem (Los Angeles, California). All subsequent work involving chitin as a substrate was done using this substance.

Commercial chitin, being of a particulate nature, necessitated the preparation of a colloidal form for both plating
experiments and enzyme analysis. The further purification of the commercial form for studies involving particulate chitin was also done.

The particulate chitin was purified by a modification of the method of Skerman (46). Ten g particulate chitin were first soaked in 1 liter of 1% HCl to remove Ca²⁺ salts. Occasional stirring aided in the process. Following this procedure, the chitin was washed until neutral to Hydrion paper and resuspended in 2% KOH to remove pigments. During this treatment, the solution was heated to boiling with subsequent removal and replacement of the KOH solution after which the heating was repeated until no further pigment was extracted. The chitin was then washed with tap water until neutral to pH Hydrion paper. After drying, the chitin was washed in 100% ethanol and again dried. The above preparation produced a chitin sufficiently cleaned to be used as a substrate in liquid media.

The particulate chitin could further be treated to form a reprecipitated chitin conventionally referred to as colloidal chitin in the literature. The term "colloidal" chitin will be used in this work to refer to reprecipitated chitin. The colloidal chitin was necessary for use in agar, enzyme assays, and liquid cultures. Skerman's (46) method was employed in its preparation. Approximately 10 g of the treated particulate chitin were dissolved in 100 ml of 50% H₂SO₄ which had previously been cooled to room temp. With continual stirring of the mixture, the chitin was dissolved within 15 min, at
which time at least 2 liter of cold ice water was added to the solution. The chitin precipitated out as a white precipitate at that point. This chitin suspension was filtered through a small mesh screen and spun down using an RC2B centrifuge at $5860 \times g$ for 10 min. The chitin was resuspended in tap water until the pH reached neutrality, sterilized, and stored at 4°C until used.

Media used during course of experimentation

The following media were used during the course of this study:

1. Skerman's Mineral Salts Medium (46)

\[
\begin{align*}
K_2HPO_4 & \quad \text{1.0 g} \\
MgSO_4 & \quad \text{0.5 g} \\
NaCl & \quad \text{0.5 g} \\
CaCl_2 \cdot 2H_2O & \quad \text{0.1 g} \\
FeCl_3 \cdot 6H_2O & \quad \text{0.001 g} \\
\text{Distilled H}_2\text{O} & \quad \text{1030 ml}
\end{align*}
\]

FeCl_3 \cdot 6H_2O was used instead of the FePO_4 \cdot 2H_2O as suggested by Skerman (46). The above medium containing 2% agar was used for all chitin agar plates.

Chitin agar plates were prepared by the overlay technique. Approximately 10 ml of mineral salts agar were poured into 50 ml petri dishes and allowed to harden. Five ml of mineral salts and colloidal chitin agar were overlayed on the above hardened agar.

Enough colloidal chitin was added to give an opalescent
quality to the plates, thus, allowing for visualization of hydrolysis.

2. Difco Plate Count Agar (PCA) was used for bacterial enumeration of the mud column.

3. Difco Nutrient Broth (NB) and Nutrient Agar (NA) were used for growth of isolates.

**Enumeration of microorganisms within the sediment column**

The core was sawed while frozen into 2 cm sections and placed in sterile beakers. After thawing, 1 ml of sediment was pipetted into sterile 9 ml water blanks and diluted for plating. Duplicate 1 ml samples were pipetted into preweighed pans for dry weight determination. Appropriate dilutions of the mud from the 7-2 cm segments were plated on PCA and chitin agar. A volume of 0.25 ml was used per plate. Duplicate plates were incubated at room temperature both anaerobically and aerobically.

**Collection of crustacean remains**

A readily available source of chitin within the sediment samples would be crustacean shells. To determine if fragments of crustacea are present at depths up to 14 cm within the cores, the method of Frey (14) was employed. One ml of mud was suspended in 10% HCl, heated, and stirred until no bubbles were noticed. The solution was centrifuged and washed twice with distilled H₂O. The sediment was next suspended in 10% KOH with heat and stirring. Again the solution was centrifuged and washed twice with distilled
water. The sediment was then suspended in 10 ml distilled water. Examination of 0.05 ml of solution mounted in glycerin should have allowed a quantitative count of crustacean fragments, although it was unsuccessful, due to nonreproducibility of results. This method did allow, however, for the observation of insect parts as well as crustacean remains within the sediments.

**Chitinolytic isolates used**

All isolates used in this investigation were isolated from surficial sediments of Lake Erie during the summer of 1972. Partial characterization of the isolates has been conducted and described by Warnes (54) but attempts at speciation were not made. Isolates designated 2 and 8 were considered to be of the genus *Pseudomonas*. Isolate 6 was characterized as an *Aeromonas*, while isolates 7 and 9 were considered to be *Streptomyces* species. Isolate 5 was a peritrichous microorganism tentatively designated *Beneckea* because of its chitinolytic ability. The isolates were all selected because of their ability to produce rings of hydrolysis on chitin agar.

**Density gradient fractionation of sediment and isolates**

The banding of bacteria from water and sediments by Pfister, *et al.* (43) and Hammers (28,29,30) led to the use of this technique to attempt to concentrate bacteria from bottom sediments.

Organic debris and bacterial cells have a density range of 1-1.2 (29,30). Using a 25% sucrose (w/v) as the light fluid
and 75% sucrose (w/v) as the heavy fluid a linear gradient was obtained having a density range of 1.06 to 1.26. The gradient was formed by an ISCO Model of 570 Gradient Former at a delivery speed of 10 (maximum). The gradient was collected in cellulose nitrate tubes 5/8" x 4" to a volume of 15 ml. Either one or 2 ml of the sample was overlaid on the gradient in preparation for centrifugation.

The gradient was centrifuged on a Beckman LC-2B Ultracentrifuge using the SW27 swinging bucket rotor head. Various g forces were tried with 12,800 x g for 30 min forming a narrow band.

Although suspensions of bacterial cells did show banding, little or no banding could be observed with mud suspensions. Therefore, no further work was carried out with this technique.

End product analysis of chitin degradation within the sediments

The absence of large quantities of chitinous remains in the water column or sediments was indicative of biological activity effecting its disappearance. To further define this activity, an enrichment technique was employed to favor the growth and activity of chitinolytic microorganisms anaerobically and aerobically within the surficial water and sediments up to 14 cm in depth.

The core was cut, as previously described, into 7-2 cm sections while frozen and allowed to thaw. Test tubes containing
8 ml of sterile mineral salts medium and 1 ml colloidal chitin (4.57 mg) were inoculated with 1 ml of mud from the core segment being tested. The appearance of the tube following inoculation with mud showed 3 layers; mud sediments in the bottom, colloidal chitin appearing as a fluffy precipitate over the mud, and clear media above the chitin. Triplicate tubes were incubated aerobically and anaerobically at 25 C for 4 or 14 days. Controls containing 9 ml mineral salts and 1 ml mud were used to account for activity due to growth on substrates in the mud inocula. Aerobic cultures were placed on a rotary shaker while anaerobic cultures were placed in an anaerobic incubator flushed with 95:5 mixture of N₂ and CO₂ gas. After 4 and 14 days, the tubes were removed and analyzed for production of NH₃, NAGA, and bacterial growth.

NH₃ was detected by Nesslerization as described in Standard Methods for the Examination of Water and Wastewater (1) with slight modifications. The following reagents were required: 100 g ZnSO₄·7H₂O diluted to 1 liter; 6N NaOH; stock NH₄Cl, 1.22 mg/ml; and Nessler reagent. The Nessler reagent was prepared by first dissolving 25 g KI in a small volume of distilled water. To this solution, HgCl₂ was added to the solution with constant stirring until precipitate remained (approximately 12.2 g added). Next 200 ml of 50% NaOH was added and the solution was diluted to 500 ml and allowed to settle.

The samples were treated in the following manner for NH₃ determination. To 10 ml sample 0.1 ml of ZnSO₄ solution was
added and mixed in the tube. One drop of 6N NaOH brought the solution to the correct pH (10.5). A white precipitate formed. The contents of the tube were centrifuged and 1 or 2 ml of the supernatant were diluted to a total volume of 25 ml. To this solution 1 ml of Nessler's reagent was added. The addition of EDTA or Rochelle salt interfered with the determination and was not used as suggested. The solution was mixed by inversion and allowed to stand at room temperature. The solutions were read at 425 nm or 450 nm after 10, 20, and 30 min. Maximum readings were recorded after 20 min. All readings were taken on a Bausch and Lomb (B and L) Spectronic 20 Spectrophotometer.

Different cores were used for the 4 and 14 day tests. Four days after incubation both sets of tubes showed little or no hydrolysis, therefore, a longer incubation time was desirable.

Detection of another possible end product of chitinolytic activity was NAGA. The method of choice for its detection was that reported by Morgan and Elson (35). Required for this experiment were 0.5N Na₂CO₃, glacial acetic acid (Dupont reagent grade), 2% dimethylaminobenzaldehyde (DMAB) (Baker) in 95:5 glacial acetic acid to HCl (Dupont reagent grade), and standard NAGA.

The procedure called for mixing 0.1 ml of 0.5N Na₂CO₃ in a test with 1 ml of supernatant being tested. The mixture was placed in a boiling water bath for 5 min, removed, and cooled to room temp. To this solution was added 8 ml of glacial
acetic acid and 1 ml DMAB solution. The solution was mixed by inversion and read after 45 min at 540 nm on a B and L Spectronic 20 Spectrophotometer.

**End product analysis of chitin degradation with selected isolates**

The inoculum of isolates 2, 5, 6, 7, 8, and 9 was a loopful of logarithmically growing cells. Growth curves were run on each isolate to determine the time of logarithmic growth. All isolates were known to be chitinolytic.

The procedure and tests used were analogous to those used on the sediments. The difference in procedure was that 9 ml of sterile mineral salts media and 1 ml of colloidal chitin were used per tube to obtain the final volume of 10 ml. Again cultures were grown anaerobically and aerobically as previously described. Isolate 2 was not run because of its failure to grow on nutrient broth for determination of exponential growth.

Incubation time used was 14 days after which most, if not all, colloidal chitin had been hydrolyzed.

**Buried slide procedure**

The buried slide procedure was employed to obtain a profile of morphologic types which may be associated with chitin degradation in sediments. This procedure has been used by soil microbiologists when dealing with microhabitats (16, 51, 38).

Colloidal chitin was used as an enrichment substance for chitinolytic microorganisms. Various methods of making the chitin
adhere to the acid and alcohol cleaned slide were tried. The colloidal chitin was air dried, heat fixed, or mixed with 1% agar and poured on the slide. Of these methods, only the heat fixed chitin could withstand the mechanical maneuvering required of a buried slide and remain on the slide. Subsequently, all slides were prepared for use with heat fixed colloidal chitin adhering to them.

Slides were buried in a mud jar showing distinct aerobic and anaerobic zones. The sediment was obtained from Lake Erie at the same site coring was done. The jar was stored at 4°C until use. Approximately 2 cm of the slide remained in the water covering the mud. About 5 cm of the slide was immersed in sediments. Slides were removed after 2, 4, 6, 7, 10, 13, and 20 days of incubation. Slides with no chitin adhering were likewise buried and removed after 2 and 7 days incubation.

Upon removal from the jar, the mud was removed from the uncoated side. The slide was then heat fixed to help adhesion of cells to the slide. The slide was then washed with tap water which removed nearly all the particulate sediment. Safranin was used to stain the slide with heat again being applied. The excess stain was washed off and dried. The slide was first examined under a light microscope for gross changes in the chitin layer before examination under oil immersion. Fields of vision were photographed on the Carl Zeiss 16010 Microscope at a magnification of 400X using phase contrast. Kodak panatomic-X film was used for all photomicrographs. Observations of morphological
types and hydrolysis of chitin at various depths and periods of incubation were recorded.

**Enzyme assay for chitinase with various isolates**

In conducting the enzyme assay for chitinase activity, a method proposed by Berger and Reynolds (6) was used. Their method was based on a decrease in OD at 420 nm in an enzyme, buffer, colloidal chitin system. This method was applied to all isolates worked with during the course of this study.

Both PCA and chitin agar grown cells were used as inocula. All isolates except isolate 2 grew well on PCA. Preliminary studies showed that inocula grown on chitin agar gave better production of the chitinase enzyme system. The inducibility of the system would support this conclusion (54,34). Plates showing good growth after 48 hr incubation were used as the inocula using PCA as the culture medium. Isolates grown on chitin agar usually required three days for good growth to appear at which time they were used as inocula. Two ml of sterile distilled water were pipetted onto each plate after which a spreader was used to suspend the cells. One ml of this fluid was used as inoculum for all culture flasks.

The growth of the cultures was conducted as described by Reese (44). Cells were grown in 250 ml Erlenmeyer flasks containing 25 ml of mineral salts medium and either 1% particulate chitin (44) or approximately 0.2% colloidal chitin as used by Sandarraj, et al. (49). Cultures were incubated for various times to determine
when maximum chitinase activity was obtained. Isolate 2 produced maximum activity after 2 days incubation while isolates 5, 6, 7, 8, and 9 showed best results after 5 days incubation. All cultures were grown on a rotary shaker.

The supernatants of the cultures were harvested by centrifugation on a Sorvall SS-1 centrifuge at 14,350 x g at 4°C for 15 min. The supernatant collected was considered the crude enzyme preparation. Ten ml of this fluid was used for protein fractionation with NH₄SO₄. Fractionation of 60% and 75% saturation was carried out. To 10 ml of fluid 3.56 g of NH₄SO₄ was added with stirring to give 60% saturation (19). The solution was allowed to stand at 4°C for 10 minutes before spinning at 20,228 x g for 15 min. The supernatant was saved and the precipitate redissolved with the proper buffer. To the supernatant was added 0.97 g to give 75% saturation, mixed, and spun at 20,280 x g for 15 min. The supernatant was thrown out and the precipitate redissolved in 5 ml of the appropriate buffer. This gave 3 solutions to be tested for chitinase activity and protein estimation: crude enzyme, 0-60% fraction, and 60-75% fraction.

These three solutions were tested for chitinase activity as described by Berger and Reynolds (6). The following reagents were prepared for this and related experiments: 0.2M phosphate buffers of pH 6.0, 6.2, 6.7, and 7.0; 0.2M acetate buffers of pH 5.0 and 5.3; and colloidal chitin. The assay was conducted using 2 ml of the enzyme preparation. The 5 ml solution was such that OD values at 420 nm were between 0.28 and 0.5 at 0 time. The
solutions were read every 10 min for 30 min in matched cuvetts at 420 nm. The tubes were incubated in a 37°C water bath during the 30 min. A B and L Spectronic 20 Spectrophotometer was used for all readings.

In addition to the determination of activity by a decrease in turbidity, the crude preparations and fractions were tested for the production of NAGA after the 30 min incubation period. The method was that described earlier by Morgan and Elson (35).

A unit of chitinase activity was expressed as units of activity per mg of protein. Total activity was merely determined by multiplying the units of activity/ml by the total ml in the preparation, this being 10 ml for the crude enzyme and 5 ml for each of the fractions. Percent recovery was expressed by dividing the total activity in the fraction by the total activity in the crude enzyme preparation and multiplying by 100.

Before conducting the assay on all the fractions, the optimum pH of the chitinase enzyme systems of the various isolates was determined using the crude enzyme supernatant. The range of pH tested was from 5.0 to 7.0. Once the optimum value was determined, the assay was run on each of the fractions.

The effect of substrate preparation was also tested. Cells were grown on a particulate as well as colloidal chitin substrate. Chitinase production was more rapid in cultures grown on colloidal chitin, which was subsequently used in the remaining tests.
Protein determination was carried out by the method of Lowry, et al. (32). Required for this test were 2% Na$_2$CO$_3$ in 0.1N NaOH, 0.5% CuSO$_4$·5H$_2$O in 1% Na$_2$Ktartrate, phenol reagent (1N) and protein standard of human serum albumin. The Na$_2$CO$_3$ solution and CuSO$_4$ solutions were mixed just prior to using in a ratio of 50:1. One ml of the substance being tested was mixed with 5 ml of the above 50:1 mixture and allowed to stand 10 min. To this was then added 0.5 ml of the phenol reagent, mixed, and allowed to react for 30 min. The solution was read at 540 nm on a B and L Spectronic 20 Spectrophotometer. Protein standards were run with every sample of fractions being tested.
Determination of viable cells in sediments

To determine viable numbers of bacteria present within the sediments, plate counts were taken at depths from 0 to 14 cm with 2 cm sections. Figure 1 shows the number of bacteria/g dry wt of sediment having the ability to grow on PCA after storage at -20 C. These numbers represent the heterotrophic population at the various depths. Values of Fig. 1 were plotted as the average depth of the 2 cm segment, therefore, the 0-2 cm section was plotted at 1 cm, the 2-4 section at 3 cm, etc. The number of anaerobic microorganisms obtained was usually less than the number of aerobic organisms obtained regardless of depth. Many Gram positive spore formers were noted, both anaerobically and aerobically, at all depths tested. Highest values on both anaerobically and aerobically incubated plates were obtained within the top 2 cm with numbers decreasing to 4-6 cm and leveling off at approximately \(2.5 \times 10^5\) bacteria/g dry wt of sediment at that depth. At the lower depths the number of bacteria growing anaerobically and aerobically were similar.

Of the heterotrophic microorganisms found within the core, the percentage displaying chitinolytic qualities was

RESULTS
Figure 1. Heterotrophic population within sediments after 48 hr incubation at 25 C on PCA in core 1.
BACTERIA/G DRY SEDIMENT

SEDIMENT DEPTH (CM)

BACTERIA/G DRY SEDIMENT

AEROBIC

ANAEROBIC
ascertained using chitin agar plates and counting those colonies showing rings of hydrolysis. Counts were taken when maximal numbers were obtained (approximately 12 days). Again, as on those samples plated out on PCA, the greatest number of bacteria was obtained in the upper layers. Figure 2 contains the number of chitinolytic microorganisms obtained up to 14 cm in depth. Again values were plotted as the average depth of the 2 cm section. The inocula for the plates were taken from the same core as used for counts shown in Fig. 1. The numbers differed from the PCA grown samples by the factor of approximately 100. The numbers of bacteria varied by a factor of approximately 10 when grown aerobically and anaerobically on chitin agar. Many Gram positive spore formers were found growing on the anaerotic plates. Their growth on chitin agar was often spreading and feathery in appearance. The species was more than likely of the genus *Clostridium*. The chitinolytic abilities of strains of the genus have been reported elsewhere (10).

A second core was plated for chitinolytic microorganisms to determine if large differences existed between cores. Figure 3 shows the numbers of chitinolytic organisms found in the second core. Numbers were usually higher within the second core, but similar trends were displayed. The highest counts were seen in the uppermost sediments with numbers decreasing in the lower depth. Chitinolytic bacterial numbers were generally between $10^3$ and $10^4$ bacteria/g dry wt in each core.
Figure 2. Chitinolytic population within core 1 obtained after 12 days incubation at 25°C on chitin agar.
AEROBIC

ANAEROBIC

BACTERIA/G DRY SEDIMENT
Figure 3. Chitinolytic population within core 2 obtained after 12 days incubation at 25°C on chitin agar.
Demonstration of chitinous substrates within the sediment core

That potentially chitinolytic organisms existed within depths up to 14 cm of sediment within Lake Erie was not proof that this potential was displayed. Further evidence supporting the contention that chitinolytic organisms were active within the sediments was the demonstration that substrate existed for this metabolic process. Using a method of Frey (15), sediments were tested for the presence of particulates of chitinous origin. Among such particles were remains of crustaceans and arthropod exoskeletons. Although the method as explained by Frey (15) was quantitative, quantitation was not found to be possible in this study.

Numerous samples examined from the same depth contained counts that varied drastically from each other. However, as a means of preparing mud samples for a qualitative identification of crustacean remains, the system served a useful function. Chitinous remains from microcrustaceans and arthropods were found in all sediments tested.

Separation of bacteria from sediments

Density gradient fractionation of sediments was conducted to determine if this method could serve as a means of separating bacteria from the inorganic debris found in the sediments. At best the method could give a mixed culture from which chitinolytic microorganisms could be identified. The osmotic pressure, being
different from bacterial cells, should influence the counts from such a collection.

Linear gradients were formed with various concentrations of sucrose proved to give the appropriate density range when tested by the refractive indices of the various fractions. Density values of 1.06 to 1.26 were obtained with these solutions.

The examination of gradients with no overlaid suspension showed some error in the system. When density values of the fraction approached 1.2, the linearity of the gradient no longer held. Density values became erratic. This density value approached the maximum of sucrose gradients.

To test the banding of bacteria of the gradient two isolates were chosen. Mixtures of a pseudomonad (isolate 6) and a streptomycete with vegetative cells and spores (isolate 7) were overlaid on the gradient. A peak was observed at a density of 1.2035.

When the gradient was overlaid with suspended sediment, a pellet was formed but no definite banding occurred. Ultraviolet analysis concurred with the visual assessment. A slight peak in absorbance occurred in one mud sample at a density range of 1.1930-1.2055. This value approached the limits of the sucrose gradient. An even smaller peak was observed in the same range with a second sample. Upon plating out of the fractions on PCA two types of bacterial colonies were identified. The first was a large, mucoid, cream colored colony and the second was a small yellow colony. The former was a Gram positive rod while the latter was a Gram negative rod. When every other fraction
collected was plated out, very few if any colonies were formed. The method as described did not serve the purpose for which it was initially intended, although the method has worked well for others (28,29,30,43) using lake water. The bacteria in the sediment were either not released by the suspension process, or they simply reaggregated with the organic and inorganic debris pelleted out in the gradient. Possibly a discontinuous gradient, as used by McCabe (36), would separate the bacteria from the organic and inorganic debris in the sediments.

**Demonstration of chitin digestion within the sediments**

The enumeration of potentially chitinolytic microorganisms from the sediment column of Lake Erie and the presence of chitinous remains within the sediment suggested a possible interaction between the two. To test this hypothesis, probable end products of chitin digestion were assayed in chitin enriched cultures as well as noting visible changes in bacterial growth and chitin disappearance.

After 4 days incubation, only a few tubes showed visible hydrolysis of the chitin or observable turbidity due to bacterial growth under anaerobic and aerobic conditions. Tests for NAGA and NH$_3$ showed little accumulation of either product after 4 days. A general decrease of free NH$_3$ with depth was noted, however, in both anaerobically and aerobically incubated tubes. Figure 4 shows the amount of free NH$_3$ detected after 4 days incubation in the 10 ml culture medium. Values of NH$_3$ found in the control tubes were subtracted before the values were graphed. Subtraction of control values gave a net reduction of free NH$_3$ in some tubes.
after 4 day incubation with colloidal chitin in mineral salts medium.

Figure 4. Net production of NH$_3$ within the sediment column after 4 day incubation with colloidal chitin in mineral salts medium.
over those enriched with chitin and were displayed as a negative value on Fig. 4. One would expect more NH$_3$ release in an anaerobic environment since anaerobically growing organisms would be expected to assimilate less of the ammonia because of dependence on less efficient anaerobic energy yielding processes, but this did not seem to be evident in this system. A decrease in NH$_3$ was actually observed in certain sediment inocula. Ammonia release from chitin may possibly have allowed use of other substrates within the inocula.

No NAGA was detected in tubes incubated for 4 days although all tubes were tested. This correlated with the visual assessment and the low value of NH$_3$ release.

Tubes incubated for 14 days showed visible signs of chitin hydrolysis. Aerobically incubated tubes were usually very turbid from bacterial growth. The sediment within the tubes was usually light in appearance and very little, if any, visible colloidal chitin could be seen.

The reducing conditions in those tubes incubated in 95:5 mixtures of N$_2$:CO$_2$ was evidenced by the darkening of the mud sediments to a nearly black appearance as one would expect from cultures in which sulfide had been produced and combined with reduced iron to form FeS.

No turbidity due to bacterial growth was observed in the anaerobically grown cultures. Biological activity had occurred, though, as evidenced by the lack of visible colloidal chitin and reducing conditions of the mud.
After incubation, a loopful of a number of the cultures were streaked on PCA and chitin agar and incubated anaerobically or aerobically in accordance with past incubation of the tube. A diversity of morphological types was not observed. Either Gram positive or negative rods grew on the plates. The Gram positive organisms were usually spore formers and were isolated from both anaerobically and aerobically grown plates on both media.

The amount of free NH$_3$ found within the cultures after 14 days incubation was considerably more evident than after 4 days. All cultures enriched with colloidal chitin produced more NH$_3$ than in unenriched control tubes containing mineral salts and the inoculum of sediments. Figure 5 shows the amount of free NH$_3$ determined in culture with inocula from the various depths. Again aerobically incubated samples showed higher values of free NH$_3$ than did anaerobically incubated tubes with inocula from the same depth with the exception of the 4-6 and 10-12 cm sections. All values plotted on Fig. 5 are net values with the NH$_3$ produced in the control tubes having been subtracted.

Attempts to demonstrate NAGA with the fluid showed no positive results. The enzymatic breakdown of chitin by all chitinase systems studied to date showed the release of the amine sugar as a product of chitin hydrolysis. However, the sugar is very biodegradable and, therefore, may not show up at any given time.
Figure 5. Net production of \( \text{NH}_3 \) within the sediment column after 14 day incubation at 25 C with colloidal chitin in mineral salts medium.
Demonstration of chitin digestion with various isolates

The same procedure used for sediments was applied to selected isolates. Isolate 1, originally observed as being chitinolytic, had lost the ability to show hydrolysis on chitin agar plates. However, it did grow anaerobically and aerobically in tubes supplemented with colloidal chitin.

Table 1 shows the type of growth and presence or absence of chitin after 14 days of incubation. On occasion little, if any, decrease in colloidal chitin was observed although growth was evident. The anaerobically incubated cultures of the actinomycetes, isolates 7 and 9, showed only minimal, if any, growth although end products were determined in the supernatant. The controls were inoculated tubes of mineral salts media without colloidal chitin addition.

Figures 6 and 7 show the amounts of NH$_3$ and NAGA, respectively, found within the cell free culture media of isolates after 14 day incubation. The aerobic nature of the actinomycetes was readily seen in Fig. 6 by the difference in NH$_3$ values when cultures were incubated anaerobically and aerobically. Isolate 1 produced significant amounts of NH$_3$ both anaerobically and aerobically as well as did isolate 8. Isolate 8 did not show visible growth in the culture tube but was associated with the colloidal chitin.

Figure 7 shows the production of NAGA by the various isolates. This product was produced by all chitinolytic
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Incubation</th>
<th>Type of Growth</th>
<th>Degree of Chitin Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>An</td>
<td>slight growth on chitin surface</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>Aer</td>
<td>turbid</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>An</td>
<td>growth at chitin-water interface</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Aer</td>
<td>turbid</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>An</td>
<td>slight turbidity</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Aer</td>
<td>media clear, no visible growth</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>An</td>
<td>pellicle growth</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Aer</td>
<td>chitin clumped together</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>An</td>
<td>media clear, no visible growth</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Aer</td>
<td>media clear, no visible growth</td>
<td>+</td>
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<tr>
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<td>An</td>
<td>pellicle growth</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Aer</td>
<td>chitin clumped, growth on sides of tube</td>
<td>++</td>
</tr>
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</table>
Figure 6. Production of $\text{NH}_3$ after 14 day incubation of isolates in colloidal chitin mineral salts medium.
Figure 7. Production of NAGA after 14 day incubation of isolates in colloidal chitin mineral salts medium.
microorganisms, but it may also act as a substrate for the organ­isms. Of the 6 isolates tested only 3 showed the presence of NAGA within the supernatant. Of these 3 only 2 produced signifi­cant amounts both anaerobically and aerobically. Isolate 8, although showing no visible signs of growth, such as turbidity, produced the most NAGA of any of the isolates treated. Isolate 9, which produced the highest amount of NH₃ of the isolates tested, showed no detectible NAGA. Possibly this was because the actino­mycete has an enzyme system capable of hydrolyzing the amine portion off the amino sugar moiety to give a sugar, NH₃, and acetate.

Examination of buried slides

These above described studies showed that, under labora­tory conditions, microorganisms within sediments can and do hydrolyze chitin both anaerobically and aerobically. The study did not, however, provide a good look at the morphological types of organisms that may be involved in the hydrolysis of chitin. To examine this aspect, photomicrographs were taken of slides coated with chitin and buried within the mud column up to a depth of approximately 5 cm. A mud jar composed of mud that had not been frozen but stored at 4 C was used. Therefore, a greater variety of morphological types should be present. It also more closely resembled the ecology of lake sediments.

Gross changes in bacterial populations and in the colloidal chitin substrate were noted as a function of depth within
the column and length of incubation on Table 2. The intact chitin formed a pink film after staining. Areas of hydrolysis were noted by absence of the film and bacterial growth at the edges of the film. It appeared as if hydrolysis of colloidal chitin was slow within the sediment column but fairly rapid within the water column. Both the water-air and water-mud interfaces were the first to show substantial bacterial populations adhering to the slide.

Figure 8 shows bacteria on the control slide with no chitin at the water surface (8a) and within the water (8b) after 7 days incubation. Refractile bacterial spores were present in large numbers in both photomicrographs. An actinomycete hypha can be seen on 8b along with many spores and sediment debris. An algal filament and more bacterial spores may be seen in Fig. 9. These photomicrographs were taken in the aerobic zone of the mud column (above 2 cm). In 9b the slime of a fungal or algal filament was embedded with bacterial cells probably depending on secretions for nutrients. The bacteria seen in these photomicrographs (Figs. 8 and 9) were considered part of the normal flora of the water column and sediments.

After 2 days incubation of a chitin coated slide, large masses of bacteria were observed at the water surface. The mass of cells may be seen in Fig. 10a. Large numbers of cells were also observed in the water column including a *Hyphomicrobium* species (10b). It is doubtful that this organism has any
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*Does not apply.*
Figure 8. Bacteria and debris found at water surface (a), and within the water column (b) on control slide (no chitin) after 7 day incubation in mud jar. Refractile bacterial spores (s) are noted. Bar indicates 5 µm in both photomicrographs.
Figure 9. Bacteria associated with the control slide at the water-mud interface (a) and within the aerobic zone of the mud column (b) after 7 day incubation. An algal filament is noted by an arrow in (a) while vestiges of a fungal filament (arrows) may be seen in (b). Bar indicates 5 μm in both photomicrographs.
Figure 10. Bacteria associated with chitin coated slides after 2 day incubation. Typical mass of bacteria at the water-air interface (a), rod shaped bacteria of the water column (b), and bacteria of the water-mud interface (c) are seen. Note Hyphomicrobium species (H) in (b). Bar indicates 5 μm in all photomicrographs.
function in chitin hydrolysis because of its known metabolic processes. Its low incidence would also eliminate it. The apparent growth of the rod shaped organisms into the colloidal chitin would be indicative of this organism's possible role in chitin degradation. Microorganisms similar to those found within the water column are seen in surficial sediments in Fig. 10c. A diatom frustule is apparent in the center of the print.

After 4 days incubation, the majority of cells at the water's surface was still rods of 3 μm in length (Fig. 11a). In addition to the rods, a filamentous organism displaying curved growth may be seen. It was never again noted and probably lacked significance in chitinolytic processes. A smaller (1-2 μm) more dense cell, as seen in 11b, was found to be quite prevalent in surficial sediments. A few vibrioid cells were also noted. In 11c a lawn of colloidal chitin appeared to be slightly hydrolyzed by bacteria multiplying within the cleared area. The growth of bacteria within the sediment column was beginning after 4 days although little, if any, hydrolysis was noted.

After 6 days, bacterial numbers were very high within the water column and surface sediments. The bacteria literally coated the adhering chitin. The rod shaped organisms tended to predominate but more coccoid to ovoid organisms appeared. Figure 12a and b showed a type of organism unique to this incubation period. It was not found on other slides at other depths,
Figure 11. Bacterial cells associated with water-air interface (a), mud-water interface (b), and aerobic mud zone (c) after 4 day incubation on chitin coated slide. Note area of hydrolysis (arrow) in (c). Bar indicates 5 μm in all photomicrographs.
Figure 12. Bacterial cells associated with chitin coated slide after 6 day incubation at the water-surface (a and b) and within the water column (c). Large granulated organisms (arrows) are seen in (a) and (b) while rods predominate in (c). Bar indicates 5 µm in all photomicrographs.
but only at the water surface. The organisms appeared to be flexing, highly granulated bacteria having dimensions of 1-2 μm x 3-7 μm. This organism was probably not chitinolytic because of its low incidence. Figure 12c shows typical rod shaped organisms approximately 2 x 0.5 μm found on numerous occasions within the water column. The refractile spores and filamentous bacteria also appeared in increasing numbers after 6 days.

Growth in the aerobic zone of the sediments increased after 6 days of incubation as shown in Fig. 13a and b. Both show areas of hydrolysis of the colloidal chitin layer with the bacteria within the cleared area and more specifically as in 13a apparently attached to the circumference of the hydrolyzed area. This may be indicative of hydrolytic qualities of the vibriod organism that predominated in the area. Figure 13b shows another "ring" of hydrolysis by a colony of rod shaped organisms.

A more diverse population of bacteria, morphologically, was found after 7 days on incubation. The rod shaped organisms were still present in large numbers (14a), but bacterial spores (14b), smaller, more ovoid rods (14c), and rods with capsular material (14d) were present also. The encapsulated microorganisms may be zooglael in nature. All these photomicrographs were taken at or near the air-water interface.

The anaerobic zone after 7 days had begun to show areas of hydrolysis. Spiral organisms appeared to be active in chitin.
Figure 13. Bacterial growth within the aerobic zone of mud sediments associated with a chitin coated slide after 6 day incubation. Rings of hydrolysis may be seen in both (a) and (b). Bar indicates 10 μm in both photomicrographs.
Figure 14. Bacteria associated with the water surface of a chitin coated slide after 7 day incubation. Large, pseudomonad-type rods may be seen in (a), bacterial spores in (b), ovoid cells in (c), and encapsulated rods in (d). Bar indicates 5 μm in all photomicrographs.
breakdown or metabolism of its by-products. Figure 15 shows a heterogenous population of spirilla as evidenced by their varying wavelengths. They appeared to be from 5 to 30 μm in length with a width less than 0.4 μm. Also associated with these organisms were rods but in smaller numbers than the spiral forms.

Slides removed after a 10 day incubation period showed a decrease in the bacterial population found within the aerobic zone. Hydrolysis was nearly complete in all the aerobic zones, possibly accounting for the decrease in numbers. Figure 16 shows the bacterial population growing within the water column and on the water surface. Very small bacterial spores were noted by their refractile qualities in both Fig. 16a and b. In addition to the spores, very slender (0.4 μm) filaments, showing no branching but a flexuous nature, can be seen. These filaments presumably are chains of bacilli. Shorter filaments with similar width dimensions were seen in 16b showing the bacillary nature of the filament.

The anaerobic zone of the mud column showed increasing bacterial numbers and populations after 10 days. Figures 17 and 18 show a few types found within the anaerobic zone. Figure 17a shows a mass of vibriod cells approximately 2 μm in length covering the chitin coat. The vibriod microorganisms seemed to become increasingly important in chitin hydrolysis with increasing time of incubation. Large rods of 5 μm in length were seen attached to colloidal chitin at a depth of approximately 3 cm (Fig. 17b).
Figure 15. Bacteria adhering to chitin coated slide after 7 day incubation. Photomicrograph taken at depth of 4 cm in the mud column. Note spirilla (arrows).
Figure 16. Bacterial populations associated with chitin coated slide at the water-air interface (a) and water column (b) after 10 day incubation. Note bacterial spores (S) and the bacillary filaments (arrows). Bar indicates 5 μm in both photomicrographs.
Figure 17. Vibriod (a) and rod shaped bacteria (b) associated with chitin coated slide after 10 day incubation. Photomicrographs are of the anaerobic zone within the mud column. Bar indicates 6 μm in both photomicrographs.
Figure 18. Bacteria associated with chitin coated slide after 10 day incubation at anaerobic depths within the mud column (a and b). Note spiral (S), vibrioid (V), and Spirochaetal-like (P) forms. Bar indicates 7 μm in both photomicrographs.
At the maximum depths of the slide, spiral, and vibriod cells predominated after 10 days. Figure 18 shows a number of these morphologic types. The vibriod microorganisms observed in 18a may be similar to cellulose oxidizing vibrios, such as, Cellfacicula sp. The dimensions were similar (1 x 2 µm) and a dense central body, possibly a metachromatic granule, appeared present. The larger spiral organism in 18a may possibly be an anaerobic spirochaete or a Spirillum sp. In Fig. 18b the majority of cells present are probably of the genus Spirillum.

After 13 days, anaerobic hydrolysis was still active as seen in Fig. 19. Zones of hydrolysis can be seen in 19a surrounded by spiral and vibriod cells. The vibriod cells appeared to be the major morphologic type present. Figure 19b shows an organism displaying what may be volutin granules. The organism appeared to display morphology similar to that found in coryneform bacteria.

The aerobic zone including the water column and aerobic sediments displayed very little change from 10 through 20 days. The slide incubated for 17 days again displayed the predominant vibriods and also a large spiral organism approximately 0.5 x 20 µm. These forms may be seen in Fig. 20.

Those photomicrographs of slides incubated for 10-17 days showed similar bacteria as found at 20 days. However, a very narrow spirillum was observed in the anaerobic zone and is
Figure 19. Bacterial forms found on chitin coated slide after 13 day incubation under anaerobic conditions. Various spirilla (S) and vibriods (V) may be seen in (a). Definite clearing is also noted. Coryneform-like bacteria are seen in (b). Bar indicates 8 µm in both photomicrographs.
Figure 20. Bacterial forms found on chitin coated slide after 17 day incubation at 2 cm depth within the mud column. Vibriod (V) and spiral (S) forms may be noted.
seen in Fig. 21. This was only half as wide as those spirilla seen in Fig. 20 although their length was similar.

**Enzyme assay for chitinase system**

The last phase of the examination of chitin cycling within the environment was the study of the biological mechanisms by which the substrate was degraded to a more readily utilizable nutrient. The method of selection was a study of the extracellular chitinase enzyme systems of a number of microorganisms previously identified as chitinolytic. The isolates used, although not isolated at the time of sediment collection, were recovered from surficial water using chitin agar and appear to represent morphologically and, to some extent, biochemically organisms attached to the chitin coated slides.

The optimum pH values for each isolate were determined. Using the crude supernatants, the assay was carried out as described earlier and readings were taken at 0 and 30 min. The results are seen on Fig. 22. Except for isolate 5, the enzymes appeared relatively active at pH values from 5.0 to 7.0. This may be a definite advantage for an extracellular enzyme. As a result, work with enzyme preparations of isolates 5, 7, and 9 were carried out at pH of 6.5, isolates 8 and 6 at pH 7.0, and isolate 2 at pH 6.0.

The effect of using colloidal versus particulate chitin in the culture flask was also investigated. Results using isolate 2 showed an increase of nearly 2-3 times the specific
Figure 21. Spiral (S) forms found on chitin coated slide after 20 day incubation in the anaerobic zone of the mud column.
Figure 22. Effect of pH on the activity of crude enzyme preparations in a colloidal chitin, buffer, enzyme system.
activity when grown on the colloidal substrate. Isolate 9 also showed significant activity increase when grown on colloidal chitin. These preliminary studies showed the advantage of use of colloidal chitin and it was subsequently used in all assays.

The data collected by the method of Berger and Reynolds (6) is listed in Table 3. The inocula for all flasks were taken from chitin agar and the media contained colloidal chitin as a substrate.

Purification of the chitinase enzyme system by ammonium sulfate fractionation showed that the enzyme was concentrated in the 0-60% fraction. On occasion activity was observed in the 60-75% solution, but it did not show any increase in specific activity over that obtained in the crude supernatant.

Of the isolates tested, the highest specific activity was obtained in the 60% fraction of isolate 9. This finding might be expected in view of the well-documented ability of actinomycetes to hydrolyze macromolecules, such as cellulose, chitin, and starch.

The 60% fractions of cultures of isolate 2 also showed high specific activity values. The values were 2/3 that found for isolate 9.

The specific activity of the crude supernatants did not vary significantly from isolate to isolate. The relatively minor change due to pH and the concentration of the enzyme in the 0-60% fraction from one isolate to another supported the
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contention that the enzyme system is the same from organism to organism. The organism's ability to degrade chitin did, however, vary from one isolate to another.

An additional test for NAGA for enzyme activity was conducted on the supernatants after 30 min incubation period. Only isolate 2 showed any significant production of NAGA within the time. Therefore, the test was not considered as valid a test for the enzyme as that of Berger and Reynolds (6).
DISCUSSION

The various physical factors associated with aquatic sediments are most favorable for bacterial metabolism at the surface and decrease with increasing column depth. Organic carbon content within the sediments decreases with increasing depths (55,51,24,25) and is the most important determining factor influencing bacterial growth within the sediments (55) due to the heterotrophic nature of most bacteria. Increasingly reduced conditions and drops in nitrogen content with increasing depths also limit the numbers of bacteria metabolizing at the lower depths. The pH effect of sediments seems only to vary slightly from neutrality and, therefore, is not a major factor to be considered in sediments.

The correlation existing between these physical factors and bacterial numbers appears to apply to the sediments used within this study. Although no data were taken on the physical parameters of the sediments, reducing conditions were observable upon collection and presumably the other parameters followed work done on similar Great Lakes cores (52,55,3,25,26). The lower organic carbon content found in Lake Erie by Kemp (25) was attributable to higher rates of bacterial mineralization and also may apply to the present study.
The bacterial numbers obtained by Weeks (55) of $6 \times 10^6$ within the top 2 cm is near the value obtained in the present study ($2 \times 10^6$). Slightly higher values ($10^7$) were obtained by Bell and Dutka (3) with Lake Ontario sediments. At lower depths a significantly larger drop in numbers of aerobic heterotrophs was noted by both Bell and Dutka (3) and Vanderpost (52) than was noted in the present study with Lake Erie sediments.

The number of anaerobic heterotrophs within sediment cores showed less variance with depth than did aerobic heterotrophs. Bell and Dutka's (3) findings of $10^7$ to $10^6$ bacteria/g dry wt of sediment at surficial and 12-15 cm depths, respectively, and Vanderpost's (52) findings of $10^5$ and $10^4$ bacteria/g dry sediment from surficial to 12-15 cm depths, respectively, corresponds favorably with values of $10^6$ to $10^5$/g dry sediment found in the present study at surface and 12-14 cm depths.

The differences, however slight, between values in this study and those of others may be accounted for by differences in media used, differences in core collection and most obviously between the cores themselves. The means of storage, if stored, must also be considered.

Although various nutritional types have been enumerated within the sediments of aquatic environments, no other work besides the present has attempted to quantitate chitinolytic organisms within the sediments. However, Skinner and Davis (47) and Veldkamp (53) enumerated chitinolytic microorganisms
within soils. Approximately the same number of chitinolytic organisms were found in the soils as found growing aerobically in surface sediment.

The aquatic environment seemed to be fairly active in chitin degradation when compared with other environments. Gray, et al. (18) noted this phenomenon with work on sea water, marine mud, and forest soils. The most rapid hydrolysis took place in the water column followed by the marine sediments and finally the soils. Enumeration of chitinoclasts in the aquatic environment also showed significant numbers that may be active in chitin digestion (54).

Variation in bacterial numbers obtained occurs between cores, as noted by Zobell and Anderson (56), as well as in the present study. The total numbers varied but the trend of fewer bacteria found with increasing depths was still observable.

Separation of bacteria from sediments by linear density gradient fractionation with sucrose failed probably because of the ability of the bacteria to adhere to the particulates of the sediments as shown by Zobell (58) and McCabe (36). The slight peak seen at a density value of approximately 1.2 was probably bacterial cells that had been separated from the particulate matter. The addition of a chemical to reduce surface tension may have helped in separation of the cells and the particulate matter.

The demonstration of chitin digestion with lake sediments by the visual as well as chemical means of detection used in
this work may be compared to work done by Veldkamp (53) with saturated soils. Because of the low C:N ratio of chitin, he theorized that $\text{NH}_3$ may be released and possibly converted to nitrate. The present work showed $\text{NH}_3$ production to be low while Veldkamp (53) showed $\text{NH}_3$ production to be absent in the tube although nitrate production increased. He attributed this to nitrification occurring after release of the $\text{NH}_3$. Vanderpost (52) demonstrated the presence of nitrifying bacteria in Great Lakes sediment; therefore, this explanation might hold in sediment studies. The activities of the nitrifiers within the sediments could also explain the occasionally lower values of $\text{NH}_3$ found within aerobically grown cultures when compared with anaerobically grown cultures.

The most demonstrative result of chitin digestion within the sediments was the occurrence of turbidity in the media with accompanying disappearance of the colloidal chitin upon incubation of 14 days. Since a particulate chitin was used in Veldkamp's (53) work, an analogous description of results was not given. The appearance of turbidity in the suspending water may have been noticed, but it was not reported.

The results obtained from filtrates of the mud inoculated cultures showed no NAGA produced after 4 or 14 days. No similar work has been reported. However, production of NAGA in the supernatants of isolates incubated for varying times has been reported by a number of authors (53,6,34). Veldkamp (53) showed production
in *Pseudomonas chitinovorans* and *Cytophaga johnsonii*; Monreal and Reese (34) in *Serratia marcescens* and *Enterobacter liquefaciens*; and Berger and Reynolds (6) and Reynolds (45) in strains of *Streptomyces*. Similar results were shown in the work on two pseudomonads and a *Streptomyces* sp. Ammonia production was also detected in isolates used. Higher values were obtained with the isolates than in the mud inoculated tubes possibly due to the lack of nitrifiers that appeared active in mud sediments. The production of $\text{NH}_3$ and NAGA showed an inverse relationship with the isolates. This would be expected since $\text{NH}_3$ release would negate results for NAGA production.

Examination of the photomicrographs taken of the chitin coated slides led to a number of general conclusions that may be made concerning chitinolytic processes within the water column and sediments. Hydrolysis of chitin was more rapid within the water column and the aerobic sediments. These results substantiated work done by Gray and Baxby (17) in the marine environment. The microorganisms responsible for this phenomenon appeared to be bacillary or pseudomonad type microorganisms. Okafor's (38) findings pointed to a predominance of coccoid microorganisms within chitin amended soil using a similar technique as applied in this study. The numbers of bacteria increased significantly when the environment had been enriched with colloidal chitin.

The anaerobic process was slower for two probable reasons: the particulate nature of the sediments and the lower
rate of metabolism of anaerobes in general. Anerobic degredation appeared to be initiated by rod shaped organisms but these appeared to lose ground to the vibriod and spiral microorganisms as time progressed. Such types were commonly found in aquatic sediment by Benton (5). Actinomycetes, normally considered to have chitinolytic properties, were not observed in large quantities by this adaptation of the buried slide procedure. This did not, however, exclude their activity within this environment but merely the possible failure of the technique to allow the actinomycetes to effectively compete with the other organisms or their inability to attach to the slide.

The present findings on the nature of the extracellular chitinase enzyme substantiated previous work and further elucidated the nature of the enzymes involved. Previous work on numerous isolates has shown an optimum pH of 5.0 (10), 6.5 (34), and 7.0 (6). The present study showed pH values with the isolates from 6.0 to 7.0. The use of a colloidal chitin to obtain maximum activity was shown in the study by Monreal and Reese (34) and in the present study. The inducibility of the system has been documented by Warnes (54) and Monreal and Reese (34). The lack of activity of cells grown on NA in the present study supported this contention. Clarke and Tracey (10), however, stated that a constituitive chitinase enzyme system existed in many bacteria.

The concentration of the enzyme in a 60% ammonium sulfate fraction found in this work substantiates the work of Berger
and Reynolds (6). Their findings indicated concentration in the 70% fraction. The finding of the concentration of a chitinase system in the 40-80% ammonium sulfate fraction by Monreal and Reese (34) further defines the enzyme system. The 40-60% fraction must, therefore, contain the enzymes of the chitinase system, chitinase and chitobiase.
SUMMARY

1. Aerobic and anaerobic heterotrophic populations decrease within the sediments with increasing depths.

2. At any given depth, from 1 to 10% of the anaerobic or aerobic heterotrophic populations possessed chitinolytic abilities.

3. A direct correlation exists between various physical factors of sediments, such as, Eh, organic carbon, and nitrogen and the numbers of bacteria found within the sediments.

4. Chitinous remains were found within Lake Erie sediments up to 14 cm in depth.

5. Bacteria failed to separate from sediments when layered on linear sucrose gradients of appropriate densities.

6. Chitinase activity was present in anaerobic and aerobic sediments from depths up to 14 cm when incubated with chitin, as determined by visual observation and chemical analyses.

7. Selected chitinolytic isolates produced NH₃ and/or NAGA from chitin.

8. Aerobic chitin digestion was rapid (6 days) in Lake Erie sediments and water with rod shaped organisms primarily responsible.
9. Anaerobic chitin digestion was slow (10-20 days) in Lake Erie sediments showing a succession from a predominance of ovoid to vibriod to spiral organisms.

10. The nature of the chitinase system was studied noting its inducibility, optimum pH of 6-7, preference of colloidal chitin, and ability to fractionate in a 60% \( \text{NH}_4\text{SO}_4 \) solution.
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