A STUDY OF THE DISTRIBUTION OF NAEGLERIA SP. IN SOIL

By

Ndu Umeche

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ABSTRACT

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A study was conducted at Rose Lake Wildlife Research Area in Michigan to ascertain the distribution of Naegleria sp. in different types of organic and mineral soils, to estimate their populations and to characterize some factors influencing their distribution in soils. Core samples taken throughout the year did not reveal the presence of high temperature strains of N. gruberi or pathogenic strains of N. fowleri. Four non-pathogenic N. gruberi isolates were obtained: 3 from mineral soils (Ottokee loamy sand, Fox loam and Miami loam) and 1 from organic soil (Rifle peat). Percentage moisture, pH, bulk density and porosity of soils were determined. The isolates were obtained from sites with litter and soil layers containing 17 and 35% moisture levels, whereas other sites with higher moisture levels up to 177% contained predominantly Colpoda sp. and other protozoa. The number of Naegleria and Naegleria-like amoebae (amoebae that resemble Naegleria in cyst and trophozoite morphologies but did not transform into the flagellate stage) per gram of litter and soil were estimated using a dilution method by Singh (1946). The populations which ranged from

159 to 16,000 per gm varied from soil to soil, layer to layer and time to time, with more cells in the soil than in the litter layers. Using autoclaved soils, inoculation of Naegleria isolates per gm of organic and mineral soils in excess of population range in nature with bacterial food added resulted in death of cells and lack of growth. Inoculation of cells within natural population range with bacterial food added resulted in growth of cells, although the increase in number was not significant. These results suggest limited growth of the amoebae in soil environments, whereas they grew rapidly in pure laboratory cultures. Using unautoclaved soils, inoculation of Naegleria isolates per gm of organic and mineral soils with no bacterial food added resulted in significant growth in samples from positive sites and poor growth or death of cells in samples from negative sites. An inference was made that the positive sites might contain edible bacteria or other edible materials that favored growth, while negative sites might contain inedible bacteria or other toxic materials that inhibited Naegleria development at those sites.

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INTRODUCTION

Protozoa have been isolated and described from soil cultures since the later part of the nineteenth century (Muller, 1887). In experiments with partially sterilized soils, protozoa were found to be one of the factors limiting the development of bacteria in soils and this limitation affected soil fertility (Russell and Hutchinson, 1909). Most protozoa cultured from soils developed from resting cysts (Goodey, 1911). Examination of freshly fixed soil films showed that protozoa could also exist in an active trophic state in normal soils (Martin and Lewin, 1915).

Although a few protozoa in soil samples could be observed directly under the microscope by staining (Heal, 1964), most of them were observed by incubating soil samples in various media at various temperatures for several days. Nutrient agar, soil extract agar and hay infusion liquid media were commonly used depending on the organism. In some studies, the soil samples were incubated on the media and the protozoa obtained their food from the indigenous bacterial flora of the soil (Cutler, 1919; Crump, 1920). In other studies, the soil samples were incubated with pre-grown bacterial cultures (Cunningham, 1915; Cutler, 1923). Some methods had been developed to estimate the protozoa populations in the soil. A method of enumeration similar to that used in estimating bacterial numbers was described by Cunningham (1915). In this method, bacteria were inoculated into soil extract medium and incubated for two days. Then a series of soil dilutions was made in test

tubes and inoculated into the pre-grown bacterial cultures and incubated for several days before counting the protozoa. To obtain counts of cysts only, the soil samples were heated to 58°C to kill the vegetative cells. However, it was found that heating also killed a considerable number of cysts. A more reliable method of eliminating the vegetatives without affecting the cysts consisted of treating the soil samples with 1.5 to 2.0% hydrochloric acid for 24 hr. (Cutler, 1920). The dilution method was modified further by Singh (1946) using Fisher and Yates (1943) Statistical Table $VIII_2$ for estimating the densities of microorganisms. Singh's method involved a twofold dilution series of soil samples ranging from 1/5 to 1/81,920. Each dilution series was inoculated into 8 glass rings embedded in agar plates containing edible bacteria. After incubation, sterile and fertile rings were counted and the number of protozoa per gram of soil was estimated with the Table from the count of negative cultures. Two estimates would differ significantly at the 5% level when their numbers of negative cultures differed by 8. Excellent estimates of protozoa numbers have been obtained by various workers using the dilution method with appropriate modifications.

Soil protozoa feed mainly holozoically, although saprozoic nutrition occurs in some species. Holozoic feeders like amoebae and ciliates ingest bacteria and other small organic materials, while saprozoic feeders like some flagellates obtain their nutrients by absorption. Protozoa play a dominant role in regulating the bacterial population in the soil. This was confirmed by studies in which known numbers of protozoa and bacteria were inoculated into sterilized soils: as the

numbers of protozoa increased, the bacterial numbers decreased; in soils without protozoa, a high bacterial density was maintained (Cutler, 1923; Danso and Alexander, 1975; Habte and Alexander, 1977). Some bacteria are eaten by soil protozoa, but others are not. Both Gram-positive and Gram-negative bacteria are eaten (Singh, 1941). Many pigmented species like Chromobacterium violaceum and toxin-producing species like Serratia marcescens are not eaten. Pseudomonas pyocyanea produces pyocyanin pigment and other metabolic products that are toxic to soil amoebae, flagellates and ciliates (Singh, 1942; Singh, 1945). When food is lacking, encystment or death takes place; in the presence of edible bacteria, bacterial extracts or amino acids, excystation occurs and growth takes place (Singh et al., 1971).

Studies of the rhizosphere of plants using the buried slide technique revealed the presence of large numbers of cysts and vegetative protozoa (Starkey, 1938). The protozoa numbers and diversity were higher in the rhizosphere than in the surrounding soil. The most abundant populations occurred around decaying roots where rapid bacterial multiplication was favored, although the abundance of particular genera varied with plant species and age (Geltzer, 1963; Darbyshire and Greaves, 1967). The protozoa were probably attracted to the rhizosphere by the abundant bacterial population there or as a consequence of the nutrients diffusing out of the root cells, or both (Linford, 1942).

Most protozoa are found between the upper one inch and twelve inches of soil. This region contains most of the organic materials and nutrients that favor development of bacteria on which protozoa subsist. It is not unusual to find practically no protozoa below twelve inches

in some orchard and garden soils (Waksman, 1916). Protozoa have also been obtained from excavation depths of over twenty feet in ranch soils, but their maximum numbers occurred at a depth of four inches (Kofoid, 1915). A study of protozoa populations in a shortgrass prairie using core samples indicated that the numbers of cysts did not differ with depth, but the numbers of vegetatives decreased significantly with depth (Elliott and Coleman, 1977).

The presence of protozoa increased the amount of atmospheric nitrogen fixed by Azotobacter chrococcum and A. vinelandii in soil cultures. Cultivation of various species of protozoa with Azotobacter in artificial culture media or in sand cultures and estimation of the nitrogen by Kjeldahl method indicated fixation of 36% or more over control plots. The factors inducing these large fixations were not completely known, but it was believed that a symbiotic relationship was involved between the protozoa and the bacteria. The protozoa might be reducing the acidity of the media and such reduction would lead to increased growth and fixation by Azotobacter. The protozoa might also be removing some of the metabolic products or wastes formed by Azotobacter thereby increasing their nitrogen-fixing efficiency (Nasir, 1923; Cutler and Bal, 1926; Hirai and Hino, 1928).

Soil is a complex environment consisting of inorganic (mineral) and organic components. Soils with predominantly mineral constituents and about 1 to 10% of organic matter are called mineral soils. The mineral particles, namely clay, silt and sand are bound into aggregates by organic matter, adsorbed cations and slimy surfaces of microorganisms. The structure of the soil is determined by the size, shape and

arrangement of the aggregates. The aggregates also determine the amount of pore spaces available for moisture and aeration (Griffiths, 1965). Soils that contain a high amount of organic matter, about 80 to 95% are referred to as organic soils. The two major types are peat and muck. Peat consists of partially decomposed identifiable organic matter, while muck consists of organic matter in advanced stages of decomposition such that the organic matter could not be identified (Dawson, 1956). The principal sources of organic matter are fallen leaves, animal remains and excreta. Organic matter is the major site of activities of soil microorganisms (Robinson, 1949).

The activities of protozoa in the soil are influenced by some physical and chemical factors. Moisture is considered to be the most important factor; protozoan populations increase significantly when water is added, and die or encyst during periods of water stress (Band and Umeche, 1976; Elliott and Coleman, 1977). The moisture holding capacity of organic soils is about two times or more greater than that of mineral soils (Feustel and Byers, 1936). Since protozoa are aerobic, oxygen from the atmosphere diffuses through the soil and is used for metabolism, while carbon dioxide diffuses out in a similar way (Griffin, 1963). Most protozoa are found in environments around pH 6 to 8, although a few species survive at lower or higher pH values. The temperature of the soil varies from season to season, but greater fluctuations occur at the upper layers than at the deeper layers (Buckman and Brady, 1969). High temperatures tend to increase the metabolic rate while low temperatures slow it down, for example, Colpoda inflata multiplied three times faster at 27°C than at 10°C (Lackey, 1938).

Four major types of protozoa are found in soils: the flagellates, the ciliates, the testate amoebae and the naked amoebae. The flagellates possess one or more flagella and swim or creep in moisture films. They feed holozoically or saprophytically. The common soil genera are Cercomonas, Oikomonas and Heteromita. All form resistant cysts in adverse environments. Some species such as Heteromita globosa form resting structures that resemble cysts by losing the flagella and rounding up. These structures are less refractile and less resistant than cysts (Sandon, 1927). Flagellates number from 100 to 10,000 per gm in field soils (Crump, 1920).

Colpoda is the most common and most predominant of all ciliate genera. Other genera reported in the literature are Blepharisma, Metopus, Halteria and Dileptus. They feed mainly on bacteria, but some feed on amoebae, algae and smaller ciliates (Bick and Buitkamp, 1976). Species of Colpoda are widespread in many types of soil because they can tolerate a wide range of environmental conditions and also form cysts. Examples of Colpoda resistance were shown by studies in which dry soil and hay samples stored for several decades contained viable Colpoda cysts (Goodey, 1915; Dawson and Hewitt, 1931). Some ciliate species such as Blepharisma are more specialized in their environmental requirements, for example, they prefer places with low oxygen tension (Stout, 1958; Noland, 1925). Ciliates number from 100 to 5,000 per gm in forest soils (Bamforth, 1971).

Testate amoebae are amoebae that possess shells or tests. The common soil genera are Euglypha, Difflugia, Arcella, Assulina, Nebela and Centropyxis. They construct their tests with silica, sand and

other organic and mineral particles in the soil. Availability of test materials influence their distribution in soils. Stump (1936) showed that in cultures lacking test materials, Pontigulasia vas failed to reproduce, but when test materials such as powdered sand and glass were added, reproduction started to occur. Extensive surveys of soil testacea have been made by Heal (1961), Heal (1962), Heal (1964) and Heal (1965). Their habitats ranged from bog areas (pH 3.2 to 4.6) to marble soil (pH 6.0 to 8.5). Their classification is based on test morphology, for example, Centropyxis and Plagiopyxis have flattened tests while Difflugia and Nebela have pyriform tests. Most testaceans respond to unfavorable conditions by encysting. Encysted forms are rounded with closed tests. These forms could be distinguished by staining the animal with phenolic aniline blue on agar film slides. Total numbers of testacea recorded from grassland soils ranged from 40,000 to 69,000 per gm dry soil, while woodland soils ranged from 4,000 to 31,000 per gm dry soil.

Naked amoebae are the most numerous protozoa in the soil, where they feed mainly on bacteria. Common soil genera are Naegleria, Hartmanella, Vahlkampfia, Acanthamoeba and some unidentified Amoeba sp. Some genera such as Naegleria possess a flagellate stage and are called amoebo-flagellates, but most lack a flagellate stage. The naked amoebae are considered to be the most important of all soil protozoa because they play a major role in controlling bacterial population in the soil. Their number may reach as high as 230,000 per gm in soil (Cutler, 1923; Sandon, 1927).

Naegleria gruberi is an amoebo-flagellate which has been described

as one of the most common soil amoebae. It was first isolated from diarrhoeic faeces by Schardinger (1889). Since then, various strains have been isolated from the soil and other environments world-wide (Rafalko, 1947; Singh, 1952; Chang, 1958; Schuster, 1963a, b; Kingston and Warhurst, 1969). A pathogenic strain of Naegleria designated as N. fowleri was isolated recently from the soil by Anderson and Jamieson (1972). From soils, they contaminate freshwater pools and lakes where humans swim and become infected. N. fowleri cysts and vegetative cells were recovered from the cerebrospinal fluid of patients who died from the amoebic meningoencephalitis (Culbertson et al., 1968; Butt et al., 1968; Callicott et al., 1968; Carter, 1968; Cerva and Novak, 1968; Symmers, 1969; Dos Santos, 1970).

N. gruberi has a triphasic life cycle: the amoeboid, the flagellate and cystic phases. The vegetative organism ranges in size from 8 u to 35 u. The cyst has a double wall with one or more pores and ranges in size from 8 u to 16 u. The amoeba moves with broad pseudopodia, feeds by engulfing bacteria and reproduces by fission. The flagellate is a non-feeding stage which lasts from one to several hours. Transformation from the amoeboid to the flagellate stage occurs when the bacterial nutrients are washed off and the amoebae are suspended in distilled water or buffer solutions at room temperature. The flagellate stage may change back to the amoeboid stage or may encyst (Rafalko, 1947).

The objectives of the present study were: 1) to isolate pathogenic and non-pathogenic strains of Naegleria from different types of soil, 2) to enumerate Naegleria populations in these soils and 3) to characterize some factors influencing Naegleria distribution in these soils.

MATERIALS AND METHODS

SAMPLING SITES: All soil samples were obtained from Rose Lake Wildlife Research Area. The Area consists of 3,334 acres of forests, grasslands, marshes and lakes located 12 miles northeast of Lansing, Michigan. Twelve sampling sites were selected and identified with the aid of a Michigan Department of Natural Resources (1969) map of Rose Lake Wildlife Research Area, Johnsgard et al., (1942) Clinton County Soil Survey, and Threlkeld and Feenstra (1974) Shiawassee County Soil Survey. The sites spread out over the entire area and are interspersed by hard-surfaced roads, gravel roads and trails which are easily accessible. Tags were placed on trees at sites for easy identification. Samples were taken monthly throughout the year.

SAMPLING METHODS: Soil samples were taken with modified core samplers made of metal pipes 18" long and 2" diameter. An opening 1/2" wide was cut in each pipe for observation of the profile. To take a sample, the vegetation cover and litter were carefully removed, the sampler was held vertically and driven with a hammer into the ground down to a depth of 12". It was then pulled out gently, wrapped with polyethylene sheets and placed into a bucket. The litter was collected and placed on top of the profile or in separate container. The vegetation cover was identified using methods described by Symonds (1958). Samples were transported to the laboratory where they were analyzed the same day or stored in a cold room at 8°C for later analysis. During the months of January, February and March, 1977, deeper layers of the soils were frozen;

only the litter and the 1" soil layers were sampled.

CULTURE METHODS:

Culture media: All soil samples and the protozoa isolates were incubated at 23°C and 37°C on Dilute Stock Agar Glucose - DSAG (Balamuth, W. Personal Communication) which was composed of $MgCl.6H_2O$ 2.13 g, KH_2PO_4 0.136 g, Na_2HPO_4 0.568 g, trypticase 1 g, yeast extract 1 g, glucose 1 g, agar 15 g, and distilled water 1L. The DSAG was enriched with Escherichia coli K12 suspension in Low Salt - L.S. (Band and Mohrlok, 1969) composed of NaCl 2.92 g, $MgSO_4$ 0.65 g, $CaCl_2$ 0.04 g, and distilled water 1L. The DSAG was autoclaved in a flask and poured into sterilized culture plates. E. coli was grown on sterilized Stock culture agar (Difco) slants in test tubes at 37°C for 24 hr. The bacterial cells were harvested with a loop, placed into L.S. and shaken with a Vortex mixer.

Soil Sample Preparation: Soil samples were prepared for incubation by the following modification of the dilution method described by Singh (1946): warm DSAG was pipetted into wells of Falcon Multiwell Tissue Culture plates just enough to cover the bottoms. When the agar solidified, the wells were labelled in groups of 8, since 8 replicates of each dilution series would be made. Then 0.3 ml of E. coli suspension was added into each well.

Some soil samples were taken out through the openings in the core samplers at the 1" and the 8" markings and placed into watchglasses. Big rocks and stones were removed from the samples. One gram of each soil sample was weighed out on a balance and placed into sterilized test tubes containing 5 ml of L.S. The soil in L.S. was mixed on a Vortex

mixer and then centrifuged.

Each litter sample was immersed in L.S. on a 1:5 ratio and mixed by grinding inside a Waring blendor. Five ml of the ground litter was placed into a test tube.

Fifteen, twofold dilution series were made from the soil and litter 1:5 dilution, i.e. series ranging from 1/5 to 1/81,920. Then 0.05 ml of each homogenate was pipetted out and added into each of the 8 appropriately labelled Falcon wells containing E. coli suspension. The plates were shaken slightly and incubated at 23°C and 37°C for 48 hr. After incubation, any excess fluid was drained off and the plates were examined directly under a phase contrast microscope. Naegleria and Naegleria-like cysts were transferred to glass slides and identified under high power.

Naegleria Isolation: To isolate Naegleria, amoebae or cysts were transferred with a loop from positive Falcon wells to DSAG Petri dishes. A few drops of E. coli suspension were spread on the cells and the dishes were incubated for 24 hr. Serial subculturing continued on fresh DSAG Petri dishes until pure isolates were obtained. The amoebae were washed three times in L.S. by centrifugation and transformed into flagellates in a rotary shaker maintained at 28°C. Stock cultures of all isolates were stored on DSAG Petri dishes inside a refrigerator at 8°C and subcultured monthly.

Protozoa Count: The number of Naegleria and Naegleria-like amoebae present in 1 g of soil or litter from positive sites was determined by application of Singh's (1946) method, and Fisher and Yates (1943) Statistical Table $VIII_2$ for estimating the densities of microorganisms.

Positive and negative wells of the 8 replicate cultures per dilution series were counted. Total numbers of both cysts and vegetatives were determined and corrected to number per gm dry soil. To obtain cysts only, each sample was treated with 1% solution of sodium dodecyl sulfate (SDS) in order to kill the vegetative cells and leave the cysts unharmed. Treated samples were washed three times with L.S. by centrifugation and serially diluted as described above.

Growth in Autoclaved and Unautoclaved Soils: Soil samples were autoclaved for 3 hr. at 121°C and 15 lbs/sq. in. pressure. Naegleria isolates from #8 Peat (Org) and #7 Loamy sand (Min) were grown, washed and counted by using a hemacytometer. Various numbers of cells of #8 Peat (Org) isolate were inoculated into each gram of autoclaved and unautoclaved organic soils from different layers, while cells of #7 Loamy sand (Min) isolate were similarly inoculated into mineral soils. The bacterial food was added to the autoclaved soils but none was added to the unautoclaved soils. The inoculated samples were incubated at 23°C for 48 hr. and then serially diluted as described.

Hemacytometer Count and Dilution Method Count: To test the reliability of the counts obtained by the dilution method, a "recovery test" was conducted. This consisted of counting cells of the two isolates on a hemacytometer and inoculating known numbers of the cells into each gram of autoclaved soils in order to see how many cells that would be recovered. The inoculated soils were serially diluted immediately and incubated. After incubation, the actual numbers of negative cultures observed were compared with the numbers of negative cultures that would be obtained by calculations. The result outlined in Table 16 shows the

hemacytometer count inoculated, the expected and the observed negative cultures by the dilution method count, and the differences in negative cultures. It can be seen that the differences in negative cultures were significant in only two samples: #8 Rifle Peat (Org) at 1" and #7 Ottokee Loamy Sand (Min) at 8" layers. The remaining six samples did not differ significantly. Two estimates would differ significantly at the 5% level when their numbers of negative cultures differed by 8. Since the differences were not significant in most instances, it was concluded that reliable estimates of the amoeba numbers could be obtained with the soil samples.

PERCENTAGE MOISTURE DETERMINATIONS: Soil and litter samples obtained as described earlier were transported to the laboratory in metal containers to prevent moisture loss. Each wet sample was weighed and placed into a pre-weighed watchglass. The weighed samples were dried for 24 hr. inside an oven set at 110°C. After drying, the weights of dried samples were determined and subtracted from the weights of wet samples. The percentage moisture was then calculated.

pH DETERMINATION: Soil samples taken out from the 1" and 8" layers of the core were mixed thoroughly on a 1:1 ratio with double distilled water in small beakers. Litter samples were mixed by a similar ratio but ground up in a Waring blendor. The pH of each homogenate was read from a pH meter.

BULK DENSITY AND POROSITY DETERMINATION: Bulk density and porosity were determined by a modification of the Keen and Raczkowski (1921) box method. Cardboard boxes were used instead of brass boxes. Each box was made as follows: A square 8 cm x 8 cm was cut out from the cardboard

sheet. Four rectangular pieces 8 cm x 4 cm were also cut out and glued to all sides of the square with a masking tape. The volume of the box was 256 cc. To compare the volumes of all boxes, each box was filled with water which was then poured into a graduated cylinder and read. All volumes were approximately the same. The boxes were weighed, labelled and taken to the sampling sites.

Samples were obtained by cutting out approximately 8 cm x 8 cm x 4 cm pieces of soil with a shovel, lifting and trimming the samples with a spatula and placing them into the boxes. The samples were placed into metal containers with lids for transportation to the laboratory where they were weighed immediately. Small holes were punctured through the boxes at all corners to ensure even distribution of heat throughout the soil. The samples were dried at 110°C for 24 hr. The oven-dried soils were cooled briefly at room temperature and weighed again. The bulk density and porosity were then calculated by methods of Richards (1969).

RESULTS

SAMPLING SITES AND SOIL TYPES: The twelve sampling sites comprised different types of organic soils (peat and muck) and mineral soils (loamy sand, sandy loam and loam). The locations of the sites and detailed descriptions of the soil types are outlined on the map of Rose Lake Wildlife Research Area (Figure 1 and Table 1). The photographs of some representative sites and their corresponding profiles are presented in Figures 2 through 13.

DISTRIBUTION OF LIFE BY STRATA: The layers of all organic and mineral soils studied throughout the year did not reveal the presence of high temperature strains of N. gruberi, or N. fowleri. However, low temperature, free-living strains of N. gruberi (Figures 14 through 16), Naegleria-like amoebae and other protozoa were obtained at different times. Naegleria-like amoebae refer to two species of amoebae that resemble N. gruberi in both cyst and trophozoite morphologies, but did not transform into the flagellate stage. One of the two species designated as (N-1k) has the same size as N. gruberi, while the other species designated as (N-1k sml) is smaller than N. gruberi.

From September to December 1976, N. gruberi was isolated from only three sites, namely #7 Ottokee loamy sand, #8 Rifle peat and #9 Fox loam; numerous Naegleria-like amoebae, Acanthamoeba and the ciliate Colpoda were present at almost all sites in various layers (Table 2)

From January to March 1977, N. gruberi was still present at the

three positive sites and absent at all other sites; all sites showed dominance of Colpoda and Acanthamoeba , while Naegleria-like amoebae were present at some sites (Table 3).

From April to June 1977, N. gruberi was isolatable from only one of the three positive sites, namely #7 Ottokee loamy sand; all sites contained predominantly Colpoda; Naegleria-like amoebae were present at some sites; the ciliate Blepharisma was seen in the litter layers of #15 Sebewa loam (Table 4)

From July to August 1977, N. gruberi was isolated from the #10 Miami loam site; it was also present at all three sites of initial isolation; Naegleria-like amoebae, Acanthamoeba and Colpoda were present. During this period, Blepharisma was still present at site #15 Sebewa loam (Table 5). Therefore, the months of July to August appeared to be the growing season for all the protozoa observed.

CHARCTERISTICS OF THE FOUR NAEGLERIA ISOLATES: Three of the N. gruberi isolates were obtained from mineral soils (#7 Ottokee loamy sand, #9 Fox loam and #10 Miami loam), while one came from organic soil (#8 Rifle peat). In both mineral and organic soils, Naegleria could be obtained from the 1" and 8" layers at different times. However, in the mineral soil (#10 Miami loam), it was found only in the litter layers (Table 6).

The time of transformation from amoebae to flagellates was as expected in all cases. The dilution series from which the isolates originated were as follows: 1/80 for #7 Ottokee loamy sand, 1/5 for #8 Rifle peat, 1/40 for #9 Fox loam and 1/2,560 for #10 Miami loam.

VEGETATION COVER: The vegetation cover of sampling sites was composed of grass, leaves or a mixture of grass and leaves (Table 7). The following types of leaves were observed at the indicated sites: White poplar (Soil #5 Houghton muck), Elm (#7 Ottokee loamy sand), Ash (#11 and #14 Bellefontaine sandy loam), Oak (#12 Bellefontaine S.L. and #13 Hillsdale S.L.), and Cherry (#15 Sebewa loam and #16 Sloan loam).

For all positive sites, three out of four had grass cover; i.e. two for loam (Min) and one for peat (Org). Only one site had leaf cover; i.e. loamy sand (Min). None had a mixture of grass and leaves.

For all negative sites, one had grass cover; i.e. muck (Org). Two had leaf cover; i.e. sandy loam (Min) and five had a mixture of grass and leaves; i.e. sandy loam, loam (Min) and muck (Org).

PERCENTAGE MOISTURE: The percentage moisture in different layers of all organic and mineral soils determined during the months of October and November 1977 showed that the greatest amount of moisture was present at the litter layers of all sites, while lesser amounts were found at the 1" and 8" layers (Table 8).

The highest percentage moisture recorded was 177% in the litter layers of #9 Fox loam (October) and #15 Sebewa loam (November). The lowest was 8% in the 8" layer of #15 Sebewa loam (November). The difference between percentage moisture from October to November ranged from 17 to 71% in the litter layers, 4 to 25% in the 1" layers and 2 to 9% in the 8" layers. This indicates that moisture in a given soil sample fluctuates widely within a short period of time (Table 9). All N. gruberi cysts and vegetatives isolates were obtained from sites with moisture levels between 17 and 35%.

BULK DENSITY AND POROSITY: The bulk density measurements for the organic and mineral soils in some layers were found to be slightly higher than values obtained for these types of soils in other studies. This was due to many pieces of stones and rocks which had to be left intact within the samples. The percentage pore space for organic soils ranged from 68% in muck to 77% in peat, while mineral soils ranged from 39% in loamy sand to 50% in loam (Table 10). No major differences from normal range of porosity were observed.

pH DETERMINATION: The pH of selected samples varied from 5.6 to 6.3 for organic soils (peat and muck) and 6.2 to 6.5 for mineral soils (loamy sand and loam). Therefore, both the organic and mineral soils tested were slightly acidic at all layers (Table 11).

NAEGLERIA GROWTH IN THE SOIL:

Numbers Per Gram Dry Soil: The numbers of Naegleria and Naegleria-like cysts and vegetative cells per gm of dry soil in different layers of positive sites during the months of August and September 1977 are shown in Table 12. Generally, more organisms were present in August than in September. There were more vegetative cells than cysts at most layers and more cells in soil layers than in litter layers.

In litter layers, the highest number of cells per gm recorded were 6,111 (August, #7 Ottokee loamy sand) of which 1,524 were cysts; the lowest number was 159 (September, #10 Miami Loam) of which 98 were cysts. At the 1" layers, the highest number was 6,021 (August, #10 Miami loam) with 2,532 cysts; the lowest was 225 (September, #9 Fox loam) with 205 cysts. Finally, at the 8" layers, the highest number recorded was 16,288 (August, #9 Fox loam) with 2,618 cysts, while the

lowest number was 714 (September, #9 Fox loam) containing 326 cysts.

Thus the numbers of organisms per gm in different layers varied from soil to soil, from layer to layer and from month to month. Both significant and non-significant differences were observed in both months at different layers (Appendix A and B).

Growth in Autoclaved Soils: Inoculation of the #8 Peat (Org) and the #7 Loamy sand (Min) Naegleria isolates into 1 g of autoclaved soils from different layers of organic and mineral soils respectively in excess of population range in nature resulted in death of cells and lack of growth (Table 13). About 51,000 to 145,000 amoebae were inoculated per gm of soil. The percentage survival ranged from 1.04% in #15 Miami loam (Min) at the 8" layer to 29.72% in #8 Rifle peat (Org) at the 1" layer. The organisms did not exhibit preferential growth in positive sites over negative sites in autoclaved samples.

Inoculation of these Naegleria isolates within population range in nature resulted in growth and increase in number of cells, although the increases were not significant (Table 14). About 4,000 amoebae per gm were inoculated. Growth percentages ranged from 7% in #8 Rifle peat (Org) at 8" layer to 80.25% in #15 Miami loam (Min) also at the 8" layer. Positive sites #8 Rifle peat (Org) and #7 Ottokee loamy sand (Min) were again not preferred over negative sites #6 Carlisle muck (Org) and #15 Miami loam (Min) in autoclaved samples.

Growth in Unautoclaved Soils: Inoculation of #8 Peat (Org) and #7 Loamy sand (Min) Naegleria isolates into 1 g of unautoclaved soils from different layers of organic and mineral soils respectively with no bacterial food added resulted in significant growth in all layers

from positive sites and poor growth or death of cells in all layers from negative sites (Table 15).

The highest growth percentages which were 187.68% and 245.18% occurred at the 1" layers of positive sites #8 Rifle peat (Org) and #7 Ottokee loamy sand (Min) respectively; the lowest growth percentages were -64.83% and -54.43% at the 8" layers of negative sites #6 Carlisle muck (Org) and #15 Miami loam (min) respectively.

For all positive sites, the total number of cells after growth in unautoclaved soils far exceeded the total number in autoclaved soils by a wide margin. For all negative sites, growth was greatly inhibited in unautoclaved soils (Table 14 and 15).

Table 1. Soil samples and soil descriptions.*

Soil #	Soil Type	Soil descriptions
5	Houghton muck (Organic soil)	Slightly acid black fibrous organic material 6 to 8" deep. More fibrous organic material with increasing depth. Around lakes and filled-in basins. Drain with difficulty; therefore, wet in character. Natural vegetation: grass, trees.
6	Carlisle muck (Organic soil)	Slightly acid to alkaline black granular organic material 10 to 12" deep. Dark-brown organic material with increasing depth. In lake basins and drainage valleys. Nat. vegetation: trees.
7	Ottokee loamy sand (Mineral soil)	Dark grayish brown loamy sand 9" thick. Below is pale-brown sand. Soil is formed in sand. Permeability rapid; therefore, available water is low. Nat. vegetation: trees.
8	Rifle Peat (Organic soil)	Acidic granular fibrous organic material 8" to 3 ft. deep. Fibrous brown organic material with increasing depth. In lake basins. Natural vegetation: grass, shrub.
9	Fox Loam (Mineral soil)	High content of silt, clay and plant nutrients 15" deep. Great moisture-retaining capacity. Well-drained. Nat. vegetation: crop plants.
10	Miami loam (Mineral soil)	High clay content. Medium to low in organic matter. Slightly acid. Good moisture retention. Well-drained. Natural vegetation: trees.

Table 1 (cont'd.)

11	Bellefontaine sandy loam (Mineral soil)	Strongly acid sandy loam containing small quantity of finely divided organic matter 8 to 15" thick. Free percolation; therefore, low moisture retention. Gravel and stone throughout and on surface. Natural vegetation: trees, shrub.
12	Bellefontaine sandy loam (Mineral soil)	See #11
13	Hillsdale sandy loam (Mineral soil)	See #11
14	Bellefontaine sandy loam (Mineral soil)	See #11
15	Sebewa loam (Mineral soil)	Dark brown loam 11" deep. Below is brownish gray coarse sand. Poorly drained. Found on flood plains of rivers and streams. Nat. veg: trees.
16	Sloan loam (Mineral soil)	See #15

* Summarized from Johnsgard et al., (1942) Soil Survey of Clinton County, Michigan; and Threlkeld and Feenstra (1974) Soil Survey of Shiawassee County, Michigan.

Table 2. Distribution of life in the soils by strata (Sept-Dec) 1976.

	Organic soils				Mineral soils								
Soil layers	#5	#6	#8	Soil layers	#7	#9	#10	#11	#12	#13	#14	#15	#16
Litter	N-lk Acan	N-lk Acan	N-lk Colp	Litter	N-lk Colp	N-lk Colp	N-lk Colp	Colp Acan	N-lk Acan	Colp Acan	Colp Acan	N-lk Colp	Colp Acan
1"	N-lk Acan	N-lk Acan	Naeg N-lk Acan	1"	Naeg N-lk Acan	Naeg Acan	Colp Acan	N-lk Acan	N-lk Acan	N-lk Acan	N-lk Acan	Colp Acan	Colp Acan
8"	N-lk Acan	N-lk Acan	N-lk Acan Colp	8"	N-lk Acan	Naeg Acan	N-lk Acan			N-lk Acan			N-lk Acan

Naeg - Naegleria gruberi
N-lk - Naegleria-like amoebae
Colp - Colpoda
Acan - Acanthamoeba

Table 3. Distribution of life in the soils by strata (Jan-Mar) 1977.

	Organic soils				Mineral soils								
Soil layers	#5	#6	#8	Soil layers	#7	#9	#10	#11	#12	#13	#14	#15	#16
Litter	Colp Acan	Colp Acan	Colp Acan	Litter	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan
1"	Colp	Colp	Naeg	1"	Naeg	Naeg	N-lk	N-lk (sml)	N-lk (sml)	Colp	Colp	N-lk (sml)	Colp
	Acan	Acan	Colp			Colp	Colp Acan	Colp Acan		Acan	Acan	Acan Colp	Acan
8"	Froz	Froz	Froz	8"	Froz	Froz	Froz	Froz	Froz	Froz	Froz	Froz	Froz

N-lk (sml) - small Naegleria-like amoebae
Froz - frozen, no samples taken

Table 4. Distribution of life in the soils by strata (Apr-Jun) 1977).

	Organic soils				Mineral soils								
Soil layers	#5	#6	#8	Soil layers	#7	#9	#10	#11	#12	#13	#14	#15	#16
Litter	Colp	Colp	Colp	Litter	Colp	Colp	Colp	Colp	Colp	Colp	Colp	Colp Blep	Colp
1"	N-lk (sml) Colp	Colp	Colp	1"	Colp	Colp	Colp	N-lk Colp	Colp	Colp	Colp	Colp	Colp
8"	Colp	Colp	Colp	8"	Naeg N-lk (sml) Colp	Colp	N-lk Colp	Colp	Colp	Colp	Colp	Colp	Colp

Blep - Blepharisma

Table 5. Distribution of life in the soils by strata (Jul-Aug) 1977.

	Organic soils				Mineral soils								
Soil layers	#5	#6	#8	Soil layers	#7	#9	#10	#11	#12	#13	#14	#15	#16
Litter	Colp Acan	Colp Acan	Colp N-lk	Litter	N-lk Colp	N-lk Colp	Naeg N-lk	Colp Acan	Clop Acan	Colp Acan	Colp Acan	Colp Blep	Colp Acan
1"	Colp Acan	Colp Acan	Naeg N-lk Colp	1"	Naeg Colp	Naeg Colp	N-lk Colp	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan
8"	Colp Acan	Colp Acan	Naeg Colp	8"	Naeg Colp	Naeg Colp	N-lk Colp	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan	Colp Acan

Table 6. Characteristics of the four Naegleria isolates.

Soil #	Soil Type	Isolation date	Strata	Transformation time
7	Ottokee loamy sand (Mineral soil)	(Oct. 1976)	1", 8"	62 mins
9	Fox loam (Mineral soil)	(Nov. 1976)	1", 8"	60 mins
10	Miami loam (Mineral soil)	(Jul. 1977)	Litter	60 mins
8	Rifle peat (Organic soil)	(Nov. 1976)	1", 8"	60 mins

Table 7. Characteristics of vegetation cover of organic and mineral soils (Nov. 1977).

Soil #	Soil type	Vegetation cover	*Naegleria* sp.
5	Houghton muck (Org)	Grass and White poplar leaves	-
6	Carlisle muck (Org)	Grass	-
7	Ottokee loamy sand (Min)	Elm leaves	+
8	Rifle peat (Org)	Grass	+
9	Fox loam (Min)	Grass	+
10	Miami loam (Min)	Grass	+
11	Bellefontaine sandy loam (Min)	Grass and Ash leaves	-
12	Bellefontaine sandy loam (Min)	Grass and Oak leaves	-
13	Hillsdale sandy loam (Min)	Oak leaves	-
14	Bellefontaine sandy loam (Min)	Ash leaves	-
15	Sebewa loam (Min)	Grass and Cherry leaves	-
16	Sloan loam (Min)	Grass and Cherry leaves	-

(+) - Positive sites
(-) - Negative sites

Table 8. Percentage moisture in different strata of organic and mineral soils (Oct. 1977).

Organic soils				Mineral soils								
Soil layers	#5	#6	#8	Soil layers	#7	#9	#10	#11	#12	#13	#14	#15	#16
Litter	51	132	29	Litter	69	177	35	49	66	163	31	150	29
1"	29	39	26	1"	29	28	31	31	40	53	26	35	26
8"	25	13	17	8"	20	23	21	21	21	11	13	16	17

Table 9. Comparison of percentage moisture fluctuation in organic and mineral soils (Oct/Nov. 1977).

Soil layers		Organic soils #8 (Peat)	Organic soils #6 (Muck)	Mineral soils #7 (Loamy sand)	Mineral soils #15 (Loam)
Litter	October	29	132	69	150
	November	100	115	120	177
	Difference	71	17	51	27
1"	October	26	39	29	35
	November	22	19	12	10
	Difference	4	20	17	25
8"	October	17	13	20	16
	November	15	10	11	8
	Difference	2	3	9	8

Table 10. Bulk density and porosity of organic and mineral soils (Nov. 1977).

Soil layers		Organic soils #8 (Peat)	Organic soils #6 (Muck)	Mineral soils #7 (Loamy sand)	Mineral soils #15 (Loam)
1"	Bulk density (g/cc)	0.27	0.29	1.40	1.30
	Particle density (g/cc)	1.20	1.20	2.65	2.65
	Porosity (% pore space)	77	75	47	50
8"	Bulk density (g/cc)	0.34	0.38	1.60	1.50
	Particle density (g/cc)	1.20	1.20	2.65	2.65
	Porosity (% pore space)	71	68	39	43

Table 11. pH of selected organic and mineral soils (Nov. 1977).

	Organic soils		Mineral soils	
Soil layers	#8 (Peat)	#6 (Muck)	#7 (Loamy sand)	#15 (Loam)
Litter	6.0	5.6	6.2	6.3
1"	6.2	6.0	6.3	6.2
8"	6.3	6.1	6.5	6.4

1:1 double distilled water and soil
1:1 double distilled water and litter ground up in a blendor

Table 12. Number of Naegleria and Naegleria-like cysts and vegetatives corrected to number per gm dry soil in different layers of positive samples. E. coli added. (Aug/Sept 1977)

		Cysts					Total (Cysts vegetatives)			
Soil layers		#7 (L. sand)	#9 (Loam)	#10 (Loam)	#8 (Peat)	Soil layers	#7 L. sand	#9 Loam	#10 Loam	#8 Peat
Litter	AUG	1524	44	446	1924	Litter	AUG 6111	179	3014	2500
	SEPT	259	179	98	136		SEPT 907	237	159	809
1"	AUG	1572	838	2532	4454	1"	AUG 4864	2000	6021	4863
	SEPT	468	205	315	253		SEPT 510	225	376	787
8"	AUG	1701	2618	3402	5707	8"	AUG 8103	16288	6226	6224
	SEPT	550	326	425	600		SEPT 1432	714	1432	1100

Table 13. Estimation of growth of inoculated Naegleria isolates into different layers of autoclaved organic and mineral soils. Cells inoculated in excess of population range in nature. E. coli added. (Oct. 1977).

Soil layers		Organic soils		Mineral soils	
		#8 (Peat)	#6 (Muck)	#7 (L. sand)	#15 (Loam)
1"	Total # inoculated/g soil (Hemacytometer count)	69,000	61,000	51,000	70,000
	Total # after growth, 48 hrs. (Dilution method count)	20,000	17,000	4,280	5,540
	Survival %	29.71	28.36	8.39	7.91
8"	Total # inoculated/g soil (Hemacytometer count)	61,000	69,000	145,000	145,000
	Total # after growth, 48 hrs. (Dilution method count)	5,080	5,540	3,020	1,510
	Survival %	8.32	8.02	2.08	1.04

Table 14. Estimation of growth of inoculated Naegleria isolates into different layers of autoclaved organic and mineral soils. Cells inoculated within population range in nature. E. coli added. (Oct. 1977)

Soil layers		Organic soils #8 (Peat)	Organic soils #6 (Muck)	Mineral soils #7 (L. sand)	Mineral soils #15 (Loam)
1"	Total # inoculated/g soil (Hemacytometer count)	4,000	4,000	4,000	4,000
	Total # after growth, 48 hrs. (Dilution method count)	6,040	4,670	4,670	5,540
	Growth %	51.00	19.25	19.25	38.50
8"	Total # inoculated/g soil (Hemacytometer count)	4,000	4,000	4,000	4,000
	Total # after growth, 48 hrs. (Dilution method count)	4,280	5,080	6,600	7,210
	Growth %	7.00	27.00	65.00	80.25

Table 15. Estimation of growth of inoculated Naegleria isolates into different layers of unautoclaved organic and mineral soils. No bacterial food added. (Dec. 1977).

Soil layers		Organic soils #8 (Peat)	Organic soils #6 (Muck)	Mineral soils #7 (L. sand)	Mineral soils #15 (Loam)
1"	Total # inoculated/g soil (No E. coli added)	4,000	4,000	4,000	4,000
	Total # after growth, 48 hrs.	12,200	4,280	14,500	5,540
	Total # after growth, 48 hrs. (CONTROL, No Naeg or E. coli added)	693	265	693	223
	Total corrected #	11,507	4,015	13,807	5,317
	Growth %	187.68	0.38	245.18	32.93
8"	Total # inoculated/g soil	4,000	4,000	4,000	4,000
	Total # after growth, 48 hrs.	9,380	1,650	8,570	2,140
	Total # after growth, 48 hrs. (CONTROL)	126	243	154	317
	Total corrected #	9,254	1,407	8,416	1,823
	Growth %	131.35	-64.83	110.40	-54.43

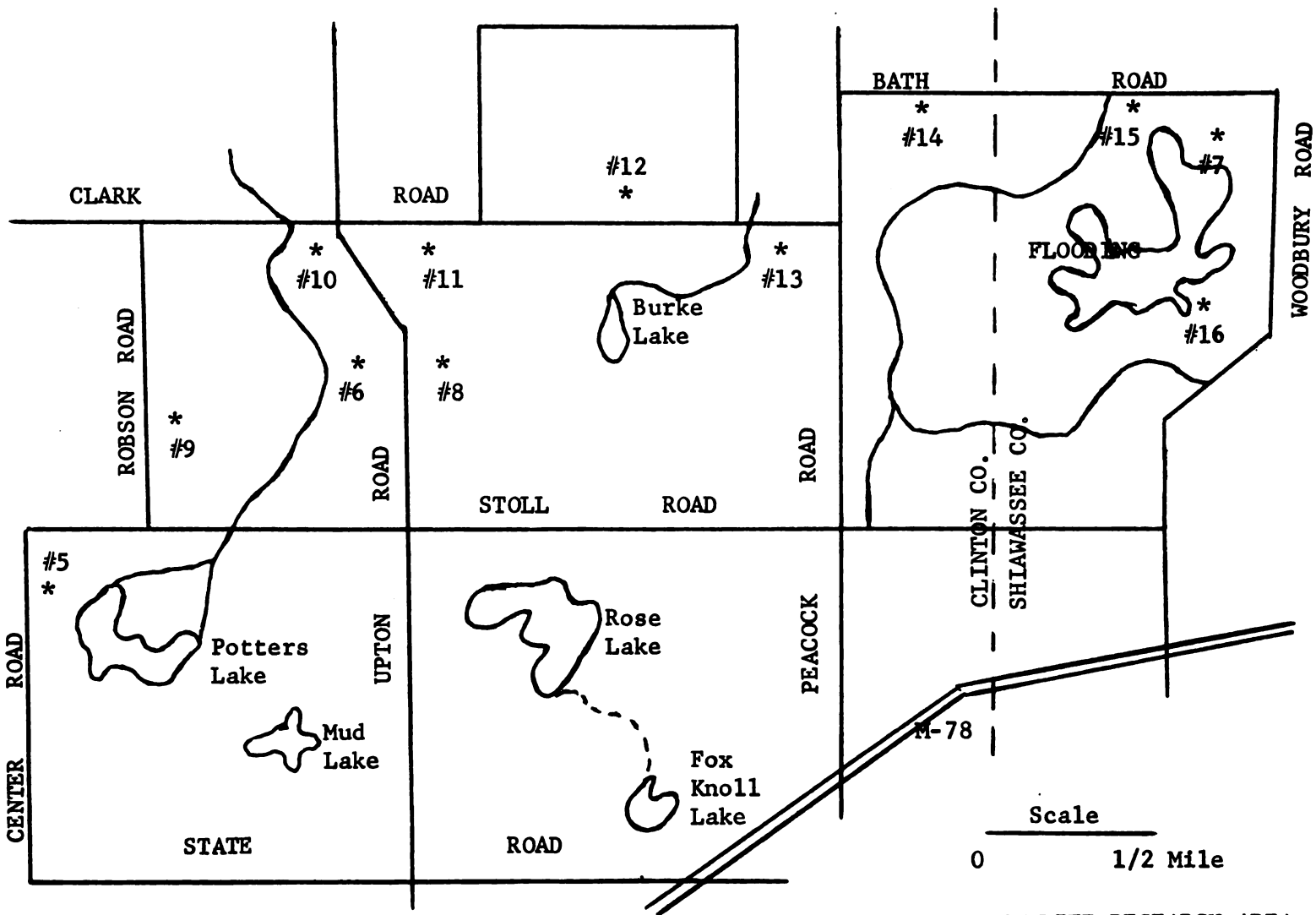
Total # in CONTROL refers to Naegleria or Naegleria-like amoebae.

Table 16. Comparison of hemacytometer count and dilution method count (Oct. 1977).

Soil layers		Organic soils #8 (Peat)	Organic soils #6 (Muck)	Mineral soils #7 (L. sand)	Mineral soils #15 (Loam)
1"	Total # inoculated/g soil (Hemacytometer count)	800	800	800	800
	Expected negative cultures (Dilution method count)	85	85	85	85
	Total # after growth, 48 hrs. (Dilution method count)	1,800	1,270	1,390	1,070
	Observed negative cultures	76	80	79	82
	Difference in negative cultures	9	5	6	3
8"	Total # inoculated/g soil	800	800	800	800
	Expected negative cultures	85	85	85	85
	Total # after growth, 48 hrs.	1,510	979	1,650	1,170
	Observed negative cultures	78	83	77	81
	Difference in negative cultures	7	2	8	4

2 estimates differ signif. at 5% level when their #'s of neg. cultures differ by 8.

Figure 1. A map of Rose Lake Wildlife Research Area with numbers indicating sampling sites.



ROSE LAKE WILDLIFE RESEARCH AREA

Figure 1

Figure 2. A photograph of site #7 Ottokee loamy sand (Mineral soil).

Figure 3. A profile of site #7 Ottokee loamy sand (Mineral soil).

Figure 2

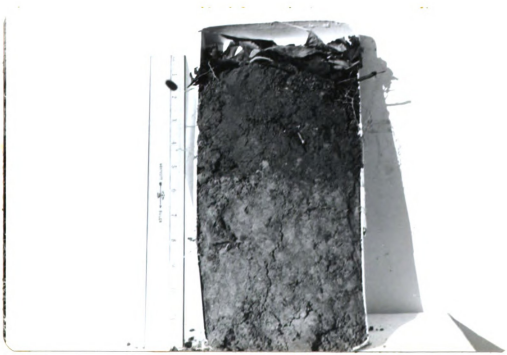


Figure 3

Figure 4. A photograph of site #6 Carlisle muck (Organic soil).

Figure 5. A profile of site #6 Carlisle muck (Organic soil).

Figure 4

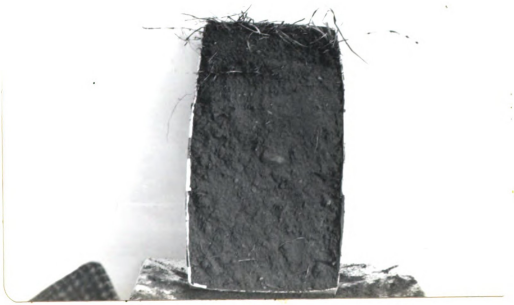


Figure 5

Figure 6. A photograph of site #8 Rifle peat (Organic soil).

Figure 7. A profile of site #8 Rifle peat (Organic soil).

Figure 6

Figure 7

Figure 8. A photograph of site #10 Miami loam (Mineral soil).

Figure 9. A profile of site #10 Miami loam (Organic soil).

Figure 8

Figure 9

Figure 10. A photograph of site #13 Hillsdale sandy loam (Mineral soil).

Figure 11. A profile of site #13 Hillsdale sandy loam (Mineral soil).

Figure 10

Figure 11

Figure 12. A photograph of site #15 Sebewa loam (Mineral soil).

Figure 13. A profile of site #15 Sebewa loam (Mineral soil).

Figure 12

Figure 13

Figure 14. A diagram of amoeba cell of Naegleria gruberi.

Figure 15. A diagram of flagellate cell of N. gruberi.

Figure 16. A diagram of cyst of N. gruberi.

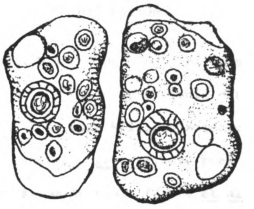


Figure 14

Figure 15

Figure 16

DISCUSSION

NAEGLERIA DISTRIBUTION AND ISOLATION: Failure to isolate high temperature strains of N. gruberi or pathogenic strains of N. fowleri from soil samples of the Rose Lake Wildlife Research Area was not surprising. High temperature N. gruberi have been isolated from water samples of heated swimming pools (Cerva et al., 1969), while N. fowleri was isolated from thermally polluted lakes (De Jonckheere and Von de Voorde, 1977) and from spinal fluids of patients who became infected by swimming in such environments. So far, only a single report from Australia (Anderson and Jamieson, 1972) confirmed isolation of N. fowleri from the soil. This supported the possibility that soil was the source from where the pathogen contaminated the freshwater pools, ponds and lakes. Since these strains thrive at high temperatures, the cool climate of Michigan might be a factor in explaining their absence from the soils sampled.

At the onset of the present study, during the months of June to September 1975, soil samples were taken from a total of 50 locations in the Canal Zone, Panama City and adjacent districts in the Republic of Panama in order to isolate N. fowleri and high temperature N. gruberi. The soils ranged from slightly acidic to slightly alkaline (pH 6.1 to 8.3) Of the 50 sites sampled, only 5 sites contained high temperature N. gruberi; no N. fowleri was isolated. The hot tropical climate of Panama appeared to favor growth of high temperature N. gruberi which

were lacking in Michigan soils sampled.

In a recent study, Wellings et al., (1975) showed that there was a direct relationship between growth of Naegleria and the amount of supplements added to Chang's calf serum-yeast extract-casein (CYSEC) medium. The two supplements were: 1) bacterial homogenate (BH) obtained by sonically disrupting heat-killed cells of Enterobacter aerogenes and 2) cell-free lysates (CFL) obtained by centrifuging and filtering the bacterial cells. Pathogenic Naegleria grew axenically in unsupplemented medium, but non-pathogenic Naegleria had limited growth in this medium. When CYSEC medium was supplemented with BH or CFL, the non-pathogenic Naegleria grew as much as the pathogenic Naegleria. This implies that growth requirements may play a role in their isolation. Isolation of pathogenic and non-pathogenic Naegleria in the present study was done on Dilute Stock Agar Glucose (DSAG) enriched with live E. coli suspension in Low Salt (L.S.). This meant that all isolates obtained were capable of growing on DSAG and subsisting on E. coli. It is not known whether N. fowleri or more N. gruberi isolates could have been obtained from the areas sampled if a different medium or a different bacterial species was used.

In the literature, N. gruberi was generally called one of the most common soil amoeba. Kofoid (1915) inferred that the flagellated stage might enable N. gruberi to be widely distributed in the soil. Allison (1924) using both solid agar medium and liquid hay infusion medium for isolation, found Dimastigamoeba (=Naegleria gruberi) in most soil samples he studied from various parts of the U.S.A., except two soils from Ohio and Wisconsin. In the present study, N. gruberi did not

appear to be the most common or the most widely distributed amoeba in the areas sampled, since only four isolates were obtained during more than a year of continuous sampling. Acanthamoeba sp. was clearly the most common amoeba encountered (Tables 2 to 5).

NAEGLERIA SPECIES PER GRAM OF SOIL: To isolate and identify Naegleria sp. two major criteria were used, namely the cyst morphology and transformation of the amoeba into flagellate in buffer solutions. Using these criteria, three species were encountered: N. gruberi (Naeg), Naegleria-like amoeba (N-lk) and small Naegleria-like amoeba (N-lk sml) as shown in Tables 2 to 5. Only N. gruberi could transform into the flagellate, while the other two did not. These could be isolated from 1 g of soil from various layers in association with themselves or with other protozoa.

In earlier works, Sandon (1928) studying the protozoa of some American soils found N. gruberi and an unidentified limax amoeba which he designated as "species D". "Species D" could not transform into flagellate but had a smooth, thin-walled cyst like that of Naegleria. Cutler et al., (1922) investigating the protozoa fauna of the soil also encountered N. gruberi and an unidentified small limax amoeba which they designated as "species ✕". "Species ✕" was smaller than N. gruberi and could not transform into flagellate. The Naegleria-like amoeba (N-lk) encountered in the present study corresponded to "species D", while the small Naegleria-like amoeba corresponded to "species ✕". Since these two species resemble N. gruberi in all but name, they might be mutant strains of N. gruberi that have lost their ability to transform into the flagellate.

PHYSICOCHEMICAL FACTORS: The influence of moisture on protozoa activity was well-established. All N. gruberi cyst and vegetative isolates were obtained from samples with moisture levels between 17 and 35% (Table 8). Other protozoa especially the ciliate Colpoda sp. thrived in the soil at higher moisture levels up to 177%, but not N. gruberi. This preference for low moisture levels can be further ascertained by the fact that in solid media cultures, Naegleria isolates grew better under thin moisture films than under flooded conditions. Since N. gruberi is an obligate aerobe, the concentration of oxygen might be affected by the presence of excess moisture within the pore system because oxygen diffusion is slower in water than in air (Griffin, 1963). Singh et al., (1971) studying the effect of E. coli extract on excystment of amoebae, obtained 94 to 98% excystment at 1/50 extract dilution, but only 0 to 30% excystment at 1/25,600 extract dilution. In soil environments, dilution of nutrients due to excess moisture might prevent excystation so that growth might not occur. The present study supports the findings of Losina-Losinsky and Martinov (1930) who observed more activities of the amoeba Vahlkampfia sp. than the ciliate Colpoda sp. at small moisture content of the soil, but more Colpoda sp. activity at greater moisture content of the soil.

In a recent study, Chang (1978) determined the resistance of pathogenic Naegleria trophozoites and cysts to some physical and chemical agents. He found that drying the cells on slides killed the trophozoites instantly while the cysts were killed in less than 5 minutes; on freezing, the trophozoites degenerated in minutes while the cysts became nonviable in hours. The death of cells was attributed to dena-

turation of proteins resulting from loss of cytoplasmic water when cells were dried or crystallization of cytoplasmic water when cells were frozen. Although Chang's observations were made with axenic cultures of pathogenic Naegleria, it is not known whether similar results could be obtained with soil cultures. All the fluid on a glass slide could disappear by simple evaporation at room temperature, but it takes overnight drying at 110°C to remove all the moisture in a soil sample. In the present study, air-dried soil samples from positive sites were kept for several months and still contained viable Naegleria cysts. During the cold winter months, frozen soil samples obtained from positive sites were thawed and found to contain many viable Naegleria cysts. Therefore, lethality of Naegleria due to drying or freezing may not be an important factor in explaining their presence or absence in the soil samples studied.

NUMBERS PER GRAM SOIL: The estimates of native Naegleria sp. populations showed that the number of organisms per gm of litter or soil varied at different times from one site to the other and from layer to layer. The total cell populations ranged from 179 per gm in grass litter to 16,288 per gm in deeper layers of Fox loam soil; the cysts numbered from 259 per gm in Elm litter to 1,701 per gm in deeper layers of Ottokee loamy sand soil (Table 12).

In similar studies using Singh's dilution method, Stout (1962) counted 94 to 7,000 rhizopod amoebae per gm in Beech litter and 22 to 50 per gm in Clay loam and Silt loam soils. Bamforth (1971) obtained 400 to 24,000 testate amoebae per gm in litters and 400 to 7,800 per gm in loam, peat and loamy sand soils. Hence, these investigators observed

more cells in the litter layers than in soil layers. In the present study, generally more cells were obtained in the soil layers than in the litter layers.

Studies on rhizosphere soils exhibited similar population variations. Darbyshire and Greaves (1967) observed 12,500 to 80,000 total amoebae in the rhizosphere of the perennial ryegrass Lolium perenne and 5,900 to 32,430 cells per gm in unplanted soil. The present study did not separate the rhizosphere soil from the unplanted soil, so that the rhizosphere was included as a part of the general soil environment.

GROWTH IN AUTOCLAVED SOILS: Some data on the number of cells that existed per gm of dry soil were presented on Table 12. Using autoclaved organic and mineral soils, inoculation of Naegleria isolates in excess of this population range per gm resulted in death of cells (Table 13). One possible explanation would be that the density of cells was so high that accumulation of their waste products exerted depressing effects on their numbers. Cutler et al., (1922) called this phenomenon autointoxication. The experimental conditions lacked means of removal of wastes and renewal of nutrients so that once wastes began to accumulate, the organisms died or encysted. When cells were inoculated within population range in nature, growth occurred, although the increase in numbers was not significant (Table 14). This illustrates the fact that these organisms have very limited growth in the soil, whereas they grow rapidly in pure laboratory cultures.

GROWTH IN UNAUTOCLAVED SOILS: Inoculation of Naegleria isolates per gm of unautoclaved soils with no bacterial food added resulted in significant growth in all layers from positive sites, while negative

sites exhibited poor growth and death of cells (Table 15). Since no bacterial food was added, it could be inferred that the amoebae utilized native bacterial flora present in the soil for growth. Other factors that favor Naegleria growth such as amino acids and amino sugars (Singh et al., 1971) have been isolated from organic and mineral soils using paper partition chromatography (Bremner, 1952). Isolation from organic soils of diaminopimelic acid which is unique to bacteria provided evidence that some of these amino acids and amino sugars have bacterial origin (Dawson, 1956).

Experimental determinations have shown that some species of the common plant pathogen Phytomonas were not only inedible to the amoebae, but also produced exotoxins that were harmful to the amoebae. Pseudomonas pyocyanea and Chromobacterium violaceum produced toxic pigments; Serratia marcescens produced diffusible exotoxin that could prevent amoebae from eating other edible bacteria (Singh, 1942). These toxic secretions caused the amoebae or flagellates to round up and finally burst (Singh, 1945). Such studies support the possibility that inedible bacteria or toxic extracts might be present at the negative sites tested. Isolation of these bacteria and their extracts might help to explain whether they played any role in the absence of Naegleria in the negative sites sampled.

SUMMARY

In this study, twelve sites comprising different types of organic soils (peat and muck) and mineral soils (loamy sand, sandy loam and loam) at Rose Lake Wildlife Research Area were studied. Attempts to isolate high temperature strains of N. gruberi or pathogenic strains of N. fowleri from litter and soil samples taken monthly throughout the year were not successful. However, four strains of non-pathogenic N. gruberi and other protozoa were isolated from litter and soil layers.

Of the four N. gruberi isolates, three were obtained from mineral soils (Ottokee loamy sand, Fox loam and Miami loam), while one came from organic soil (Rifle peat). The isolate from Miami loam was found only in the litter layers, while the other isolates were found only in the soil layers.

Of all the physicochemical factors studied, moisture was found to have more direct correlation to the Naegleria presence. The isolates were obtained from litter and soil environments with moisture levels between 17 and 35%. Other sites with higher moisture levels up to 177% contained predominantly Colpoda sp. and other protozoa.

The number of Naegleria and Naegleria-like amoebae per gm of litter and soil was estimated. The populations were found to vary from soil to soil, from layer to layer and from time to time. Generally, more cells were observed in the soil layers than in the

litter layers.

Using autoclaved soils, inoculation of Naegleria isolates per gm of organic and mineral soils in excess of population range in nature with bacterial food added resulted in death of cells and lack of growth. Inoculation of cells within population range in nature with bacterial food added resulted in growth of cells, although the increase in number was not significant. This suggested limited growth of the amoebae in soil environments, whereas the same organisms grew rapidly in pure laboratory cultures.

Using unautoclaved soils, inoculation of Naegleria isolates per gm of organic and mineral soils with no bacterial food added resulted in significant growth in samples from positive sites and poor growth or death of cells in samples from negative sites. Therefore, an inference was made that the positive sites contained edible bacteria or other edible materials that favored growth, while the negative sites contained inedible bacteria or other toxic materials that inhibited Naegleria development at those sites.

LIST OF REFERENCES

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Allison, R. V. 1924. A note on the protozoan fauna of the soils of the United States. Soil Sci. 18:339-352.

Anderson, K. and A. Jamieson. 1972. Primary amoebic meningoencephalitis. Lancet 2:379.

Bamforth, S. S. 1971. The numbers and proportions of Testacea and ciliates in litters and soils. J. Protozool. 18:24-28.

Band, R. N. and S. Mohrlok. 1969. The respiratory metabolism of Acanthamoeba rhysodes during encystation. J. Gen. Microbiol. 59:351-358.

———— and N. Umeche. 1976. The moisture requirement of vegetative amoebae in the soil. J. Protozool. 23:23A.

Bick, H. and U. Buitkamp. 1976. Ciliated protozoa from Canadian grassland soils. Trans. Amer. Micros. Soc. 95:490-491.

Bremner, J. M. 1952. The nature of soil-nitrogen complexes. J. Sci. Food Agric. 3:497-500.

Buckman, H. O. and C. Brady. 1969. The nature and properties of soils. The Macmillan Co. Toronto. 653 pp.

Butt, C. G., C. Baro and R. W. Knorr. 1968. Naegleria sp. identified in amoebic encephalitis. Amer. J. Clin. Path. 50:568-574.

Callicott, J. H., E. C. Nelson, M. M. Jones, J. G. dos Santos, J. P. Utz, R. J. Duma and J. V. Morrison. 1968. Meningoencephalitis due to pathogenic free-living amoebae. Report of two cases. J. Amer. Med. Assoc. 206:579-582.

Carter, R. F. 1968. Primary amoebic meningoencephalitis: Clinical and pathological epidemiological features of six fatal cases. J. Path. Bact. 96:1-25.

Cerva, L. and K. Novak. 1968. Amoebic meningoencephalitis: sixteen fatalities. Science 160: 92.

————, V. Novak and K. Novak. 1969. Amoebic meningoencephalitis. A new amoeba isolate. Science 163:575-576.

Chang, S. L. 1958. Cytological and ecological observations on

flagellate transformation of N. gruberi. J. Gen. Microbiol. 18:579-585.

_____. 1978. Resistance of pathogenic Naegleria to some common physical and chemical agents. Appl. Microbiol. 35:368-375.

Crump, L. M. 1920. Numbers of protozoa in certain Rothamsted soils. J. Agric. Sci. 10:182-198.

Culbertson, C. G., P. W. Ensminger and W. M. Overton. 1968. Pathogenic Naegleria species. Study of a strain isolated from human cerebrospinal fluid. J. Protozool. 15:353-363.

Cunningham, A. 1915. Studies on soil protozoa. J. Agric. Sci. 7:49-74.

Cutler, D. W. 1919. Observations on soil protozoa. J. Agric. Sci. 9:430-444.

_____. 1920. A method for estimating the number of active protozoa in the soil. J. Agric. Sci. 10:135-143.

_____. 1923. The action of protozoa when inoculated into sterile soil. Ann. Appl. Biol. 10:137-141.

_____ and D. V. Bal. 1926. Influence of protozoa on the process of nitrogen fixation by Azotobacter chrococcum. Ann. Appl. Biol. 13:516-534.

_____, L. M. Crump and H. Sandon. 1922. A quantitative investigation of the bacterial and protozoan population of the soil, with an account of the protozoan fauna. Phil. Trans. Roy. Soc. B. 211:317-350.

Danso, S. K. and M. Alexander. 1975. Regulation of predation by prey density: the Protozoan-Rhizobium relationship. Appl. Microbiol. 29:515-521.

Darbyshire, J. F. and M. P. Greaves. 1967. Protozoa and bacteria in the rhizosphere of Sinapsis alba L., Trifolium repens L., and Lolium perenne L. Can. J. Microbiol. 13:1057-1068.

Dawson, J. E. 1956. Organic soils, in Advances in Agronomy VIII:377-401.

Dawson, J. A. and D. C. Hewitt. 1931. The longevity of encysted Colpodas. Am. Nat. 65:181-186.

De Jonckheere, J. and H. Von de Voorde. 1977. The distribution of N. fowleri in man-made thermal waters. Am. J. Trop. Med. Hyg. 26:10-15.

Dos Santos, J. G. 1970. Fatal primary amoebic meningoencephalitis: A retrospective study in Richmond, Virginia. Amer. J. Clin. Path. 54:737-742.

Elliott, E. T and D. C. Coleman. 1977. Soil protozoan dynamics in a shortgrass Prairie. Soil Biol. Biochem. 9:113-118.

Feustel, I. C. and H. G. Byers. 1936. The comparative moisture-adsorbing and moisture-retaining capacities of peat and soil mixtures. U.S.D.A. Tech. Bull. 532:1-25.

Fisher, R. A. and F. Yates. 1943. Statistical Tables for biological, agricultural and medical research. 2nd. ed. London: Oliver & Boyd.

Geltzer, J. G. 1963. On the behavior of soil amoebae in the rhizospheres of plants. Pedobiologia 2:249-251.

Goodey, T. 1911. A contribution to our knowledge of the protozoa of the soil. Roy. Soc. Proc. B. 84:165-180.

_____. 1915. Note on the remarkable retention of vitality by protozoa from old stored soils. Ann. Appl. Biol. 1:395-399.

Griffin, D. M. 1963. Soil moisture and the ecology of soil fungi. Biol. Rev. 38:141-166.

Griffiths, E. 1965. Microorganisms and soil structure. Biol. Rev. 40:129-142.

Habte, M. and M. Alexander. 1977. Further evidence for the regulation of bacterial populations in soil by protozoa. Arch. Microbiol. 113:181-183.

Heal, O. W. 1961. The distribution of testate amoebae (Rhizopoda: Testacea) in some fens and bogs in Northern England. J. Linn. Soc. (Zool.) 44:369-382.

_____. 1962. The abundance and micro-distribution of testate amoebae (Rhizopoda: Testacea) in Sphagnum. Oikos 13:35-47.

_____. 1964. The use of cultures for studying Testacea (Protozoa: Rhizopoda) in soil. Pedobiologia 4:1-7.

_____. 1965. Observations on testate amoebae (Protozoa: Rhizopoda) from Signy Island, South Orkney Islands. Brit. Antarct. Surv. Bull. 6:43-47.

Hirai, K. and I. Hino. 1928. Influence of soil protozoa on nitrogen fixation by Azotobacter. Proc. 1st Int. Congr. Soil Sc. Wash. 3:160-165.

Johnsgard, G. A., T. E. Nivison, H. T. Stone, G. M. Welles,

M. M. Striker, J. W. Moon and Z. C. Foster. 1942. Soil Survey of Clinton County, Michigan. U.S. Dept. Agric. Series 1936, No. 12. 71 pp.

Keen, B. A. and H. Raczkowski. 1921. The relation between the clay content and certain physical properties of a soil. J. Agric. Sci. 11:441-449.

Kingston, D. and D. C. Warhurst. 1969. Isolation of amoebae from the air. J. Med. Microbiol. 2:27-36.

Kofoid, C. A. 1915. On the relative numbers of rhizopod and flagellates in the fauna of soils. Science 42:937-940.

Lackey, J. B. 1938. A study of some ecological factors affecting the distribution of protozoa. Ecol. Monogr. 8:501-527.

Linford, M. B. 1942. Methods of observing soil flora and fauna associated with roots. Soil Sci. 53:93-103.

Losina-Losinsky, L. and P. F. Martinov. 1930. A method of studying the activity and rate of diffusion of protozoa and bacteria in the soil. Soil Sci. 29:349-362.

Martin, C. H. and K. R. Lewin. 1915. Notes on some methods for the examination of soil protozoa. J. Agric. Sci. 7:106-119.

Michigan Dept. Nat. Resources. 1969. Rose Lake Wildlife Research Area map.

Muller, P. E. 1887. Studien uber die naturlichen humusformen. Berlin: Julius Springer.

Nasir, S. M. 1923. Some preliminary investigations on the relationship of protozoa to soil fertility with special reference to nitrogen fixation. Ann. Appl. Biol. 10:122-133.

Noland, L. E. 1925. Factors influencing the distribution of freshwater ciliates. Ecology 6:437-452.

Rafalko, J. S. 1947. Cytological observations on the amoebo-flagellate N. gruberi. Jour. Morphol. 81:1-44.

Richards, L. A. 1969. Diagnosis and improvement of saline and alkali soils. U.S. Dept. Agric. Handbook No. 60. 160 pp.

Robinson, G. W. 1949. Soils. Their origin, constitution and classification. An introduction to pedology. John Wiley & Sons Inc. N. Y. p. 233.

Russell, E. J. and H. B. Hutchinson. 1909. The effect of partial

sterilization of soil on the production of plant food. J. Agric. Sci. 3:111-144.

Sandon, H. 1927. The composition and distribution of the protozoan fauna of the soil. Oliver & Boyd, Edinburgh. 237 pp.

_____. 1928. A study of the protozoa of some American soils. Soil Sci. 25:107-121.

Schardinger, F. 1899. Entwicklungskreis einer Amoeba lobosa (Gymnamoeba): Amoeba gruberi. Sitzber. Akad. Wiss. (Wien) Math. Natur. Abt. 1. 108:713-734.

Schuster, F. 1963a. An electron microscope study of the amoeboflagellate N. gruberi. The amoeboid and flagellate stages. J. Protozool. 10:297-313.

_____. 1963b. An electron microscope study of the amoeboflagellate N. gruberi. The cyst stage. J. Protozool. 10:313-320.

Singh, B. N. 1941. Selectivity of bacterial food by soil amoebae in pure mixed culture and in sterilized soil. Ann. Appl. Biol. 28:52-64.

_____. 1942. Selection of bacterial food by soil flagellates and amoebae. Ann. Appl. Biol. 29:18-22.

_____. 1945. The selection of bacterial food by soil amoebae and the toxic effects of bacterial pigments and other products on soil protozoa. Brit. J. Expt. Path. 26:316-325.

_____. 1946. A method of estimating the numbers of soil protozoa especially amoebae, based on their differential feeding on bacteria. Ann. Appl. Biol. 33:112-119.

_____. 1952. Nuclear division in 9 species of small free-living amoebae and its bearing on the classification of the Order Amoebida. Phil. Trans. Roy. Soc. Lond. Ser. B. 236:405-461.

_____, D. Datti and G. P. Dutta. 1971. Factors inducing excystation of free-living amoebae. Indian J. Expt. Biol. 9:350-357.

Starkey, R. L. 1938. Some influences of the development of higher plants upon the microorganisms in the soil: VI. Microscopic examination of the rhizosphere. Soil Sci. 45:207-249.

Stout, J. D. 1958. Biological studies of some Tussock grassland soils. N. Z. J. Agric. Res. 1:974-984.

_____. 1962. An estimation of microfaunal populations in soils and forest litter. J. Soil Sci. 13:314-320.

Stump, A. B. 1936. The influence of test materials on reproduction in Pontigulasia vas (Leidy) Shouteden. Biol Bull. 70:142-147.

Symmers, W. St. C. 1969. Primary meningoencephalitis in Britain. Brit. Med. J. 4:449-454.

Symonds, G. W. D. 1958. The tree identification book. A new method for the practical identification and recognition of trees. William Morrow & Co. N. Y. 272 pp.

Threlkeld, G. W. and J. E. Feenstra. 1974. Soil survey of Shiawassee county, Michigan. U.S. Dept. Agric. Soil Conserv. Ser. 113 pp.

Waksman, S. A. 1916. Studies on soil protozoa. Soil Sci. 1:135-152.

Wellings, F. M., P. T. Amuso, S. L. Chang and A. L. Lewis. 1977. Isolation and identification of pathogenic Naegleria from Florida lakes. Appl. Microbiol. 34:661-667.

APPENDICES

APPENDIX A

A TABLE FOR ESTIMATING PROTOZOAL NUMBERS

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A TABLE FOR ESTIMATING PROTOZOAL NUMBERS

The following table taken from Singh (1946) shows estimates of protozoal numbers per gm of soil from numbers of negative cultures by dilution method. Fifteen, twofold dilution series (1/5 to 1/81,920) with 8 replicates per series and 0.05 ml per inoculum were used. Two estimates would differ significantly at 5% level when their numbers of negative cultures differ by 8. A difference of 8 cultures corresponds to approximately 100% difference in population estimates between two individual counts. This table was computed from Fisher & Yates (1943) Statistical Table $VIII_2$ for estimating the densities of microorganisms.

No. of negative cultures	Organisms per gm	No. of negative cultures	Organisms per gm	No. of negative cultures	Organisms per gm
4	1,690,000	21	232,000	38	49,400
5	1,430,000	22	211,000	39	45,200
6	1,230,000	23	192,000	40	41,000
7	1,060,000	24	175,000	41	37,900
8	931,000	25	159,000	42	34,700
9	824,000	26	145,000	43	31,800
10	729,000	27	132,000	44	29,200
11	650,000	28	121,000	45	26,700
12	581,000	29	110,000	46	24,500
13	520,000	30	101,000	47	22,400
14	467,000	31	92,000	48	20,500
15	421,000	32	84,000	49	18,800
16	380,000	33	77,000	50	17,300
17	344,000	34	70,000	51	15,800
18	311,000	35	64,000	52	14,500
19	282,000	36	59,000	53	13,300
20	256,000	37	54,000	54	12,200

No. of negative cultures	Organisms per gm	No. of negative cultures	Organisms per gm
55	11,100	88	635
56	10,200	89	582
57	9,380	90	534
58	8,570	91	490
59	7,860	92	450
60	7,210	93	412
61	6,600	94	377
62	6,040	95	346
63	5,540	96	317
64	5,080	97	290
65	4,670	98	265
66	4,280	99	243
67	3,920	100	223
68	3,600	101	203
69	3,300	102	185
70	3,020	103	169
71	2,770	104	154
72	2,540	105	140
73	2,330	106	126
74	2,140	107	113
75	1,960	108	101
76	1,800	109	90.2
77	1.650	110	79.4
78	1,510	111	69.2
79	1,390	112	60.2
80	1,270	113	51.3
81	1,170	114	42.9
82	1,070	115	34.8
83	979	116	27.4
84	898		
85	823		
86	755		
87	693		

APPENDIX B

ACTUAL PROTOZOA COUNT

APPENDIX B

ACTUAL PROTOZOA COUNT

Examples of actual Protozoa count by the dilution method with soil samples from #7 Ottokee loamy sand (Mineral soil).

1 gm soil from 1" layer

Dilutions	Amoebae +	Amoebae −
1/5	2	6
1/10	4	4
1/20	0	8
1/40	2	6
1/80	2	6
1/160	3	5
1/320	3	5
1/640	3	5
1/1,280	6	2
1/2,560	2	6
1/5,120	2	6
1/10,240	6	2
1/20,480	6	2
1/40,960	6	2
1/81,920	7	1
TOTALS	54	66

Positive cultures 54
Negative cultures 66
No. per gm soil 4,280

1 gm soil from 8" layer

Dilutions	Amoebae +	Amoebae −
1/5	2	6
1/10	1	7
1/20	2	6
1/40	1	7
1/80	4	4
1/160	2	6
1/320	4	4
1/640	4	4
1/1,280	6	2
1/2,560	7	1
1/5,120	4	4
1/10,240	5	3
1/20,480	7	1
1/40,960	6	2
1/81,920	4	4
TOTALS	59	61

Positive cultures 59
Negative cultures 61
No. per gm soil 6,600