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Techniques for enumerating protozoa in saturated subsurface sediments

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TECHNIQUES FOR ENUMERATING PROTOZOA IN SATURATED SUBSURFACE SEDIMENTS

BY

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DEDICATION

To Paul Gray,

Sills and Amoret Bunn.

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ABSTRACT

TECHNIQUES FOR ENUMERATING PROTOZOA
IN SATURATED SUBSURFACE SEDIMENTS

by

Amoret L. Bunn
University of New Hampshire, December, 1992

Three techniques were investigated for the enumeration of small (2-5 μm in diameter) flagellates and amoebae in sediments collected within and outside of a wastewater contaminated ground water plume at the U.S. Geological Survey Toxic Substances Hydrology Research site, located on Cape Cod, MA. An epifluorescent direct count technique was developed to enumerate DAPI stained protozoa on polycarbonate membrane filters. These estimates were compared to the those from the Darbyshire liquid media MPN and Singh solid media MPN techniques. In Fall 1991, sediment samples were collected to investigate the variability of the hold time of cores, total and encysted protozoan populations (MPN techniques only), and sites.

The population estimates changed significantly (with 95% confidence) from 1 to 28 days hold time: 1.22×10^4 to 7.71×10^3 protozoa/gdw for the epifluorescent technique; 2.94×10^4 to 3.82×10^4 total MPN/gdw for the Darbyshire MPN technique; and 6.85×10^2 to 1.74×10^5 total MPN/gdw for the Singh MPN technique. The epifluorescent technique had the

lowest variability of all techniques. The encysted population did not exceed 42% of the total population by either MPN technique.

Protozoan populations by all enumeration techniques were significantly higher from three cores at a contaminated site compared to those from three cores at an uncontaminated site. The largest source of variation for the protozoan estimates was the cores for the epifluorescent and Darbyshire MPN techniques and the subsamples within the cores for the Singh MPN technique. The maximum probable error calculated for each enumeration technique based on the components from the sampling of the contaminated site were: 6.07×10^3 protozoa/gdw for the epifluorescent technique; 5.56×10^4 total MPN/gdw for the Darbyshire MPN technique; and 5.86×10^4 total MPN/gdw for the Singh MPN technique. The changes over the hold time were within the detectable difference for the epifluorescent and Darbyshire MPN techniques. However, the significant increase in the Singh MPN estimates over time was not explained by the errors in the sampling technique and should be further investigated.

CHAPTER 1

INTRODUCTION

In 1986, experiments on the growth rate of indigenous microbial populations in closed bottle incubation studies were being conducted on contaminated ground water collected from a United States Geological Survey (U.S.G.S.) study site on Cape Cod, MA. Using a standard technique developed for marine samples, Dr. Ronald W. Harvey (unpublished data) determined that the apparent growth rate of free-living bacteria in unfiltered ground water samples was lower than that observed in prefiltered (3 μm pore size) samples. The difference in growth rate suggested that protozoan predation of the bacteria in the ground water was occurring. Further experiments on the transport of free-living bacteria in the aquifer at the organically-contaminated U.S.G.S. study site suggested that there was an additional sink for the bacteria other than that which could be attributed to adsorption and sediment straining. Again, subsurface protozoa were thought to explain the decrease in bacteria.

In 1987, weighted microscope slides were lowered into monitoring wells within the contaminant plume at the site. They were retrieved after several weeks and examined under the microscope. The biofilm that accumulated on the slides was the first direct evidence that small protozoa

(flagellates and amoebae) inhabited the saturated subsurface environment at the U.S.G.S. site (N.E. Kinner and R.W. Harvey, unpublished data). Research has continued since that time to characterize the protozoan population and its role in the saturated subsurface environment. This dissertation will discuss the techniques developed and applied to saturated subsurface sediments to enumerate the protozoan population.

I. RESEARCH STUDY SITE

The incidence of ground water pollution around the United States has led to the establishment of programs to investigate the chemical, physical and biological processes in the subsurface. One of the most comprehensive is the U.S.G.S.'s Toxic Substances Hydrology Program, with the intent: 1) to conduct research to understand the "movement and fate of hazardous substances" in the field and laboratory; and 2) to develop methods and techniques for sampling and analyzing contaminated subsurface material and ground water (Mallard, 1991). One of the study areas within the Toxic Substances program is the U.S.G.S. site in Cape Cod, MA (Figure 1), with the head of the plume originating at the Otis Air Base in Sandwich and extending into Falmouth county. At this site, the contaminant plume is a result of

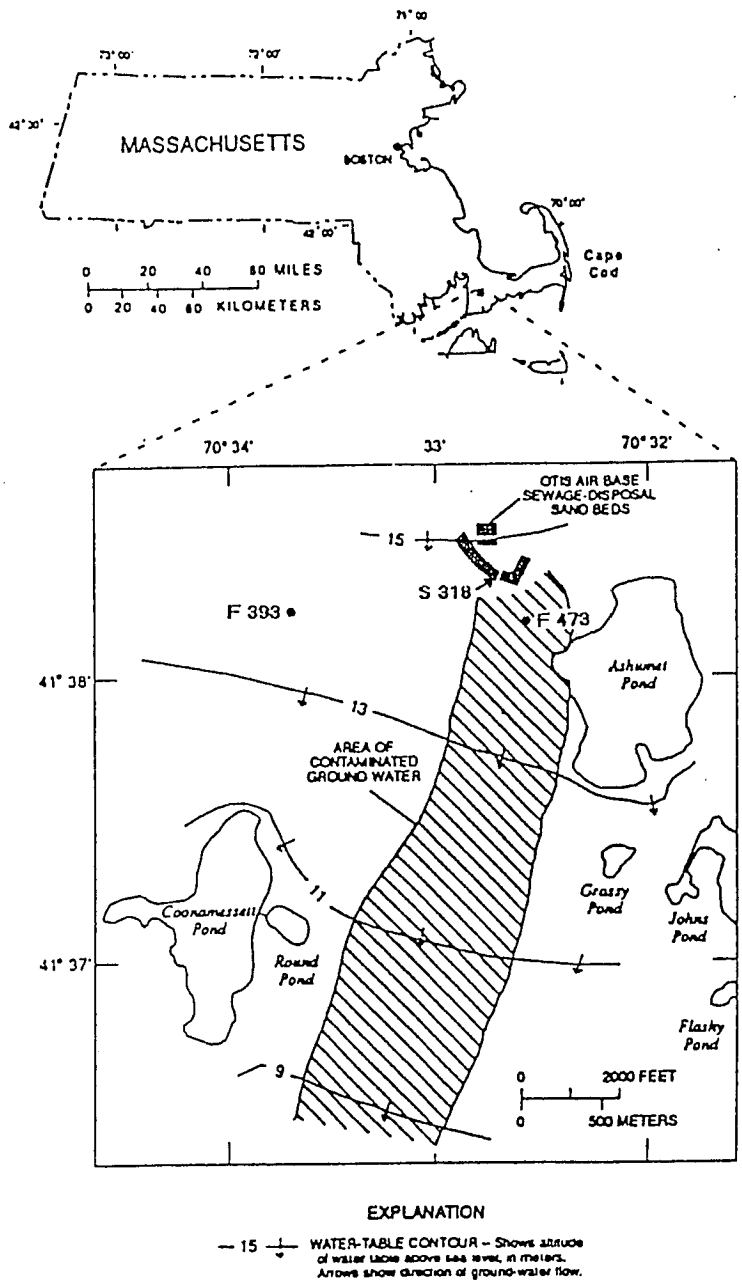


Figure 1: Location of U.S.G.S. site in Cape Cod, MA showing wastewater plume and sites where sediment samples were collected for protozoan enumeration. F 393 and S 318 were sites where sediment samples for protozoan enumeration during Fall 1991, and F 473 was the location of ground water samples for isolation of free-living bacteria used for one of the protozoan enumeration techniques. (Modified from Kinner et al. (1991).)

biologically treated wastewater being discharged onto rapid sand infiltration beds.

The U.S.G.S. site is located in a "broad sand and gravel outwash plain that was formed during the last Pleistocene glacial retreat" (Garabedian and LeBlanc, 1991). The slope of the outwash plain is to the south towards Nantucket Island. The plain has several valleys, with wetlands, and kettle holes, some of which contain ponds. A vertical profile of the site following the plume (Figure 2) shows that the top 30 to 50 m of sediment is composed of medium to coarse sand with some gravel overlying fine sand and silt to the north; and fine sand, silt and sandy till to the south. Below the unconsolidated sediments is a crystalline bedrock surface, sloping west to east throughout most of the study site.

The ground water table in the unconsolidated sediments of the site is unconfined. The annual fluctuation of the water table is 0.3 to 0.9 m, with the highest levels in the spring and the lowest in the fall. Recharge of the ground water primarily occurs from precipitation and underflow from upgradient areas. The horizontal ground water velocity in the sand and gravel ranges from 0.2 to 0.6 m/d with a porosity of 30 to 40%. The horizontal hydraulic conductivity of the sand and gravel is estimated to range from 60 to 90 m/d, and an aquifer test in 1984 measured

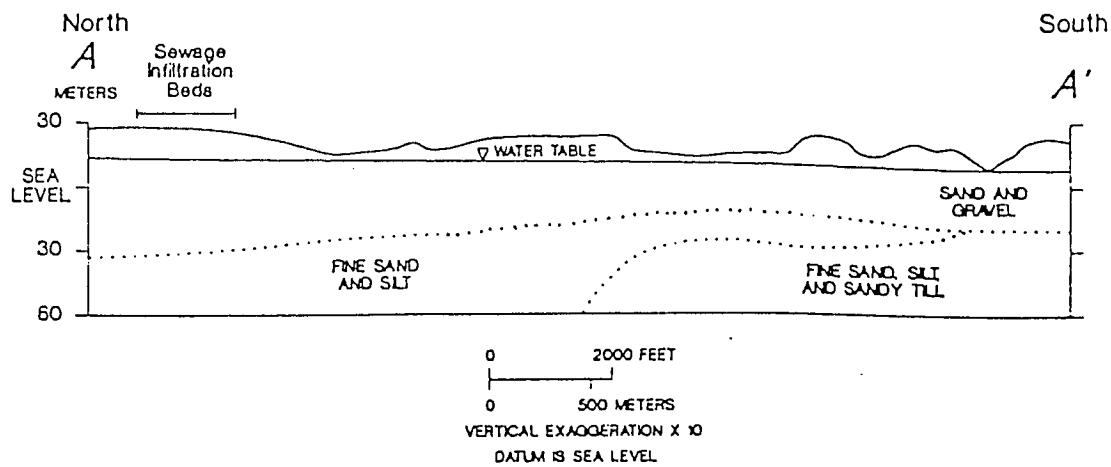


Figure 2: Cross-section of the length of the wastewater plume in Figure 1 and geohydrological features. (Modified from Garabedian and LeBlanc (1991).)

local values to be as high as 120 m/d. The underlying fine sand and sandy till is estimated to have only 10% of the horizontal hydraulic conductivity of the sand and gravel. The bedrock is considered to be the bottom of the aquifer (Garabedian and LeBlanc, 1991).

In 1936, a small treatment plant started discharging wastewater into 4 acres of sand beds. Then in 1941, the current trickling filter wastewater treatment plant at Otis Air Base was built and the effluent has been discharged into primarily two (out of 24 available) one-half acre sand beds (LeBlanc, 1984). Since the first treatment facility was established, more than 8 billion gallons of treated effluent has entered the sand and gravel aquifer (Garabedian and LeBlanc, 1991). As of 1988, the contaminant plume was 0.8 to 1.1 km wide, 23 m thick and 4.36 km long (D. LeBlanc, personal communication, 1992). The plume of sewage-contaminated ground water can be distinguished by elevated concentrations of dissolved solids, boron, chloride, phosphorus, ammonia, nitrate, detergents and in some locations volatile organic compounds (Garabedian and LeBlanc, 1991). Dissolved organic carbon concentrations $\geq$ 4 mg/L have been found near the head of the plume (Barber et al., 1988). The maximum NO_3-N concentration is 16 mg/L in the wastewater effluent yet the concentration in the plume immediately downgradient from the infiltration beds is below

detection due to microbial denitrification. Ammonia then becomes the predominant form of nitrogen in the plume within 1.5 km from the beds. The movement of ammonia in the aquifer is retarded by adsorption onto aquifer sediment. Beyond 1.8 km, nitrate again becomes the predominant nitrogen species, with concentrations rising to 3 to 4 mg/L NO_3-N. The highest detergent concentrations are found 0.9 to 3.0 km downgradient from the beds resulting from the disposal of non-biodegradable detergents (e.g., alkyl-benzenesulfonates, ABS) from 1946 to 1964 (Garabedian and LeBlanc, 1991).

Studies of the disposal of treated wastewater effluent into the subsurface by a joint team of the Massachusetts Division of Water Pollution Control and the U.S.G.S., New England Region began in 1978. In 1982, when the range of the sewage plume and the full extent of its impact on the ground water quality was realized, the U.S.G.S. Toxic Substances Hydrology Program became involved and investigations of the biological processes in the contaminated subsurface commenced (D. LeBlanc, personal communication, 1992). Sterile techniques developed at the site to take representative core samples of the sediment within the plume helped determine that greater bacterial populations occur in the sediment within the plume than in uncontaminated areas (Harvey et al, 1984). Research at the

site suggests that a diverse microbial community exists in the saturated subsurface.

A. Soil Characterizations

Soil profiles are generally divided into three major layers: the A,B and C horizons. The surface layer of soil, the A horizon, is the region with the most organic matter, the highest concentration of biological activity and the greatest amount of leaching (Alexander, 1977). This region commonly is called "topsoil". The B horizon underlies the A horizon, and is a region which usually has little organic matter and few plant roots. The C horizon is on the bottom and contains the parent material of the geological formation. The ground water table generally reaches into the C horizon.

Sediments below the ground water table are considered saturated; all voids between the particles are filled with water (Freeze and Cherry, 1979). The ground water table can be measured as the height of water in a monitoring well that is open to the atmosphere at the surface and fully screened in the saturated zone. Thus, the fluid pressure at the water table equals the atmospheric pressure, and the pressure increases with depth in the saturated zone. In porous material (including the sand and gravel found at the Otis Air Base), the capillary fringe is the region just

above the water table that is also saturated. However, in the capillary fringe there is negative water pressure compared to the water table itself. Above this region is the unsaturated or vadose zone (Freeze and Cherry, 1979).

At a pristine location, the C horizon usually has the lowest concentration of organic matter and the least amount of biological activity (Alexander, 1977). However, as more work has been conducted in the saturated subsurface, it appears there may be considerable biological activity in the C horizon (Ghiorse and Wilson, 1988).

B. Collection of Subsurface Sediments

Removal of aseptic, representative sediment samples for microbiological analyses is expensive, time consuming and often challenging (Ghiorse and Wilson, 1988; Phelps et al., 1989). Generally, the process has involved drilling a borehole into the subsurface with hollow-stem augers and then using a sampling device to collect the sediment at the desired depth. Until 1984, sediment samples were collected at the U.S.G.S. site with a split-spoon sampling device that was lowered into the hollow barrel of the augers and then driven hydraulically 0.3-0.6 m below the bottom of the augers. The sediment was then transferred into sterile 500 mL bottles and stored on ice until processed (Harvey et al., 1984). However, the recovery rate of the split-spoon

sampler was low due to the types of sediment at the U.S.G.S. site, adding to the time and expense of the sampling procedure.

Since 1984, sediment samples have been collected with the Waterloo corer which was designed specifically for the collection of the cohesionless sand and gravel sediments found in the aquifer at the U.S.G.S. site (Zapico et al., 1987). The corer consists of an exterior barrel, an interior aluminum core sleeve, a hardened steel drive shoe, a piston, the wireline, a drill-rod adapter and a drive head (Figure 3). Assembly involves attaching the drill rig's wireline to the piston which is placed in the opening of the aluminum core sleeve inside the corer. The corer is then lowered through the hollow-stem augers and driven into the sand and gravel. The wireline holds the piston in position while the corer is driven into the sediments (Figure 4). Suction develops in the saturated sediments as the core barrel passes the immobilized piston. The sediment and pore fluids are retained inside the core barrel (due to the suction and friction of the sand on the inner sleeve) while the corer is hoisted to the surface by the wireline. The aluminum core sleeve is removed from the corer, the outside wiped off with 95% ethanol, and plastic core caps (wiped with ethanol) are placed on the ends of the sleeve. A pipe cutter is used to further section the core sample. The top

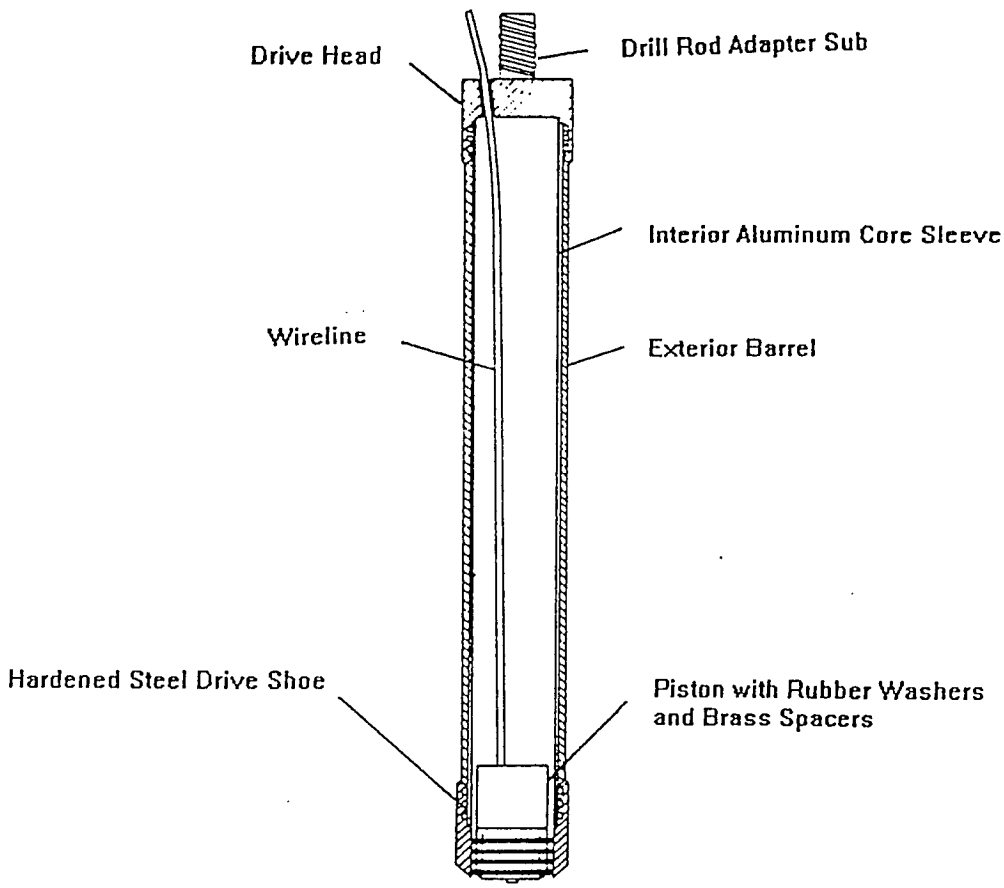


Figure 3: Waterloo corer used to collect saturated subsurface sediment samples from the U.S.G.S. site. (Modified from Zapico et al. (1987).)

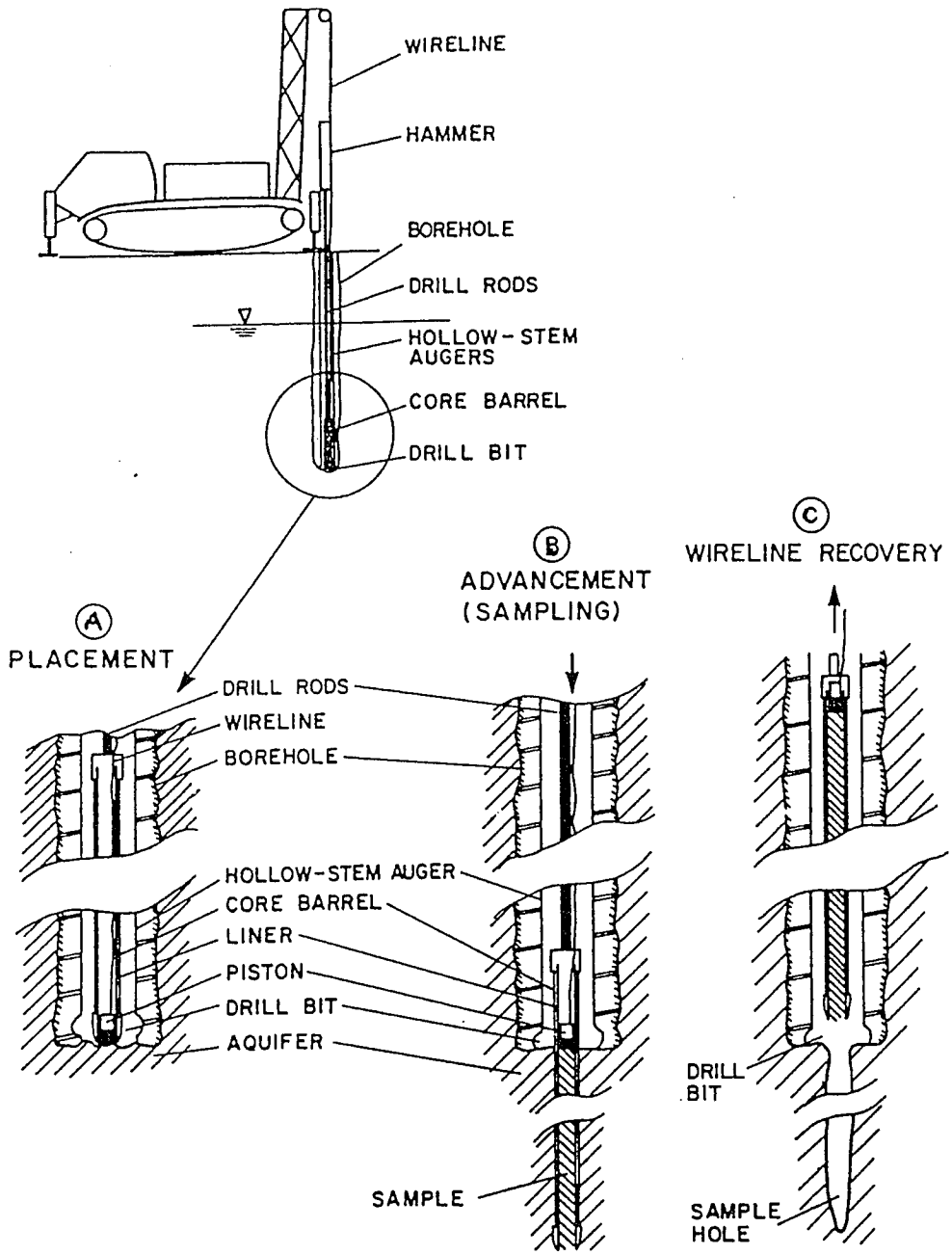


Figure 4: Collection of sediment samples by a drill rig with the Waterloo corer. The corer in the hollow-stem augers (A) is driven into the sediments (B) and then pulled to the surface (C). Note position of the piston in the corer during sediment sampling. (Modified from Zapico et al. (1987).)

and bottom 0.15 m of the sample are discarded since these regions have the greatest potential for contamination from topsoil that might have fallen down the borehole and/or from the unsterile piston. The core samples are sealed with electrical tape around the caps, labeled and stored on ice until processed.

The Waterloo corer has been used successfully for the recovery of sediment samples for protozoan enumeration at the U.S.G.S. site since 1987. Zapico et al. (1987) had a 97% recovery rate on tests with the corer at the U.S.G.S. site and found compaction of the sediments to be ~3%. The split-spoon sampler has not been used since representative sampling of the sediments was difficult and the necessity for transferring the aquifer material into a sterile bottle while in the field increased the chances for contamination. In Fall 1991, protozoan contamination of the core samples from non-sterile aluminum core sleeves was investigated, and the results will be discussed in this dissertation.

II. PROTOZOOLOGY

Protozoa are single-celled, eukaryotic organisms that range in size from approximately 2 μm to over 1 cm in length, however all terrestrial species are microscopic (Alexander, 1977). They differ from prokaryotic bacterial cells in that they have a nuclear membrane, mitochondria,

chromosomes and lack a cell wall (Nester et al., 1983). The shape of the protozoan cell is maintained by the cell membrane (Sleigh, 1989).

The word protozoa means "first animals", however, protozoa are now considered to be part of the Kingdom Protista (or Protoctista in Margulis et al., 1990) in the five kingdom system. Although the Protists still do not represent a group of organisms classified strictly on taxonomic characteristics, many were previously considered animals if they exhibited predatory behavior, or plants if they possessed chlorophyll. The 7 phyla of Protista are primarily classified according to cell morphology (structure) and locomotion, but only 2 phyla (Figure 5) are associated with aqueous ecosystems, Phylum Ciliophora and Phylum Sarcomastigophora (Curds and Warren, 1990).

Ciliophora (the ciliates) range in size from 10 μm to 80 μm in the terrestrial environment (Alexander, 1977). They are a distinct group of protozoa since they all: 1) posses cilia at some stage in their life cycle; 2) have two types of nuclei; and 3) exhibit a unique form of sexual reproduction, conjugation (Curds and Warren, 1990). Cilia are used for locomotion and/or feeding. Ciliates may be long and thin for moving within the interstices of porous media, individually stalked, arranged as colonies, or round with large mouth structures. The diversity of shapes found

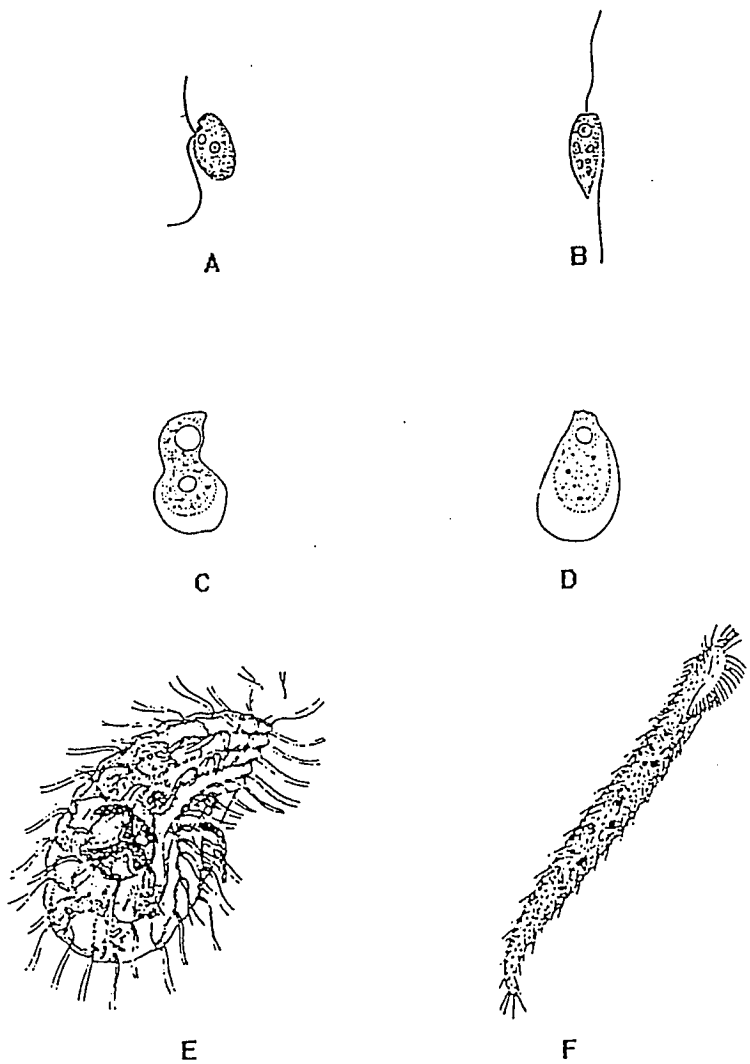


Figure 5: Examples of protozoa found in topsoils. Flagellates are the smallest protozoa, e.g., the 6-8 µm length *Bodo minimus* (A) and *Cercobodo radiatus* (B) (modified from Calaway and Lackey (1962). Naked amoebae vary greatly in size but the small (10 µm length) organisms are more common, e.g., *Vahlkampfia vahlkampfi* (C) and *Amoeba guttula* (D)(modified from Warren et al.(1990)). Ciliates have the most varied body forms, e.g., *Colpoda aspera* (30 µm length)(E) and *Engelmanniella mobilis* (122 µm length)(F)(modified from Foissner(1987)).

in this phylum exemplifies the ability of these organisms to populate a variety of ecosystems.

There are two subphyla in Sarcomastigophora, Mastigophora and Sarcodina. Mastigophora are the flagellated protozoa. They are divided into two classes depending on whether the flagellates are free-living, heterotrophs (Zoomastigophora) or they have chlorophyll and are capable of an autotrophic or heterotrophic existence (Phytomastigophora). These classes are for convenience and do not represent differences in taxonomy or evolutionary lineage. The organisms generally have 2 flagella, but up to 20 flagella have been observed in some species. Flagella are used for locomotion and capturing food (similar to the cilia on ciliates) or attachment to surfaces. The word "flagellate" will be used in this dissertation to refer to the heterotrophic flagellates that lack chlorophyll. These protozoa are also called nanoflagellates, microflagellates, zooflagellates and heterotrophic flagellates in the literature. "Heterotrophic" pertains to the nutrition of the flagellates which may include phagotrophic (feeding on bacteria and organic debris) or saprobic feeding (absorption of dissolved organic matter)(Sleigh, 1989).

Sarcodina are commonly known as amoebae (Class Rhizopoda) and are characterized by the presence of pseudopodia; appendages that the amoebae extend for

locomotion or feeding. The diversity of species ranges in size considerably: amoebae as small as 2 μm have been observed in ground water samples (Kinner et al., 1991) and those over 1 cm have been found in marine systems (Alexander, 1977). Amoebae may be naked or may possess a test (shell) that they produce or form from material found in their surroundings (Ogden and Hedley, 1980). The naked, non-spore forming, lobose (cylindrical and flattened in shape) amoebae are in the subclass Gymnamoebia (Page, 1988) and are the only type of amoebae that will be considered here. Some species have flagella during certain stages of their life cycle, making classification of these organisms confusing.

Soil protozoa have two discrete forms in their life cycle, a trophic (vegetative) stage and an encysted (resting) stage (Alexander, 1977). While some protozoa in other environments may be able to encyst, all soil protozoa appear to be able to produce "temporary and protective resting cysts" (Foissner, 1987). Cysts are not reproductive bodies like the spores formed by bacteria or fungi, but are produced by the organisms to withstand deleterious environments (e.g., desiccation, presence of toxic chemicals, or extremes in pH, temperature). Protozoa will excyst when there is enough moisture for physiological activity and locomotion, but the causes and mechanisms

associated with excystment are not completely understood. Feeding and reproduction are only possible during the trophic phase. Protozoa are generally considered to be phagotrophic, preying on bacteria and other protozoa or consuming particulate matter. However, small protozoa may be saprobic feeders ingesting soluble organic and inorganic substances across their cell wall. The contribution of saprobic nutrition to the protozoan community has not been well studied (Alexander, 1977).

The protozoa observed at the U.S.G.S. site during the examination of downwell samplers, ground water and sediment samples have generally been small (~2 to 5 μm) flagellates (Figure 6) and amoebae. Flagellates have been the dominant protozoa observed in all samples, with some amoebae detected in the downwell samplers and sediment samples using culturing enumeration techniques. Ciliates have been observed on a few downwell samplers, but are thought to represent contamination from the stagnant water in the monitoring well above the screened interval. Only flagellates have been successfully isolated and cultured from sediment samples (see Appendix C).

III. ENUMERATION OF SUBSURFACE PROTOZOA

The interest in subsurface microbial communities has coincided, not coincidentally, with growing concerns about

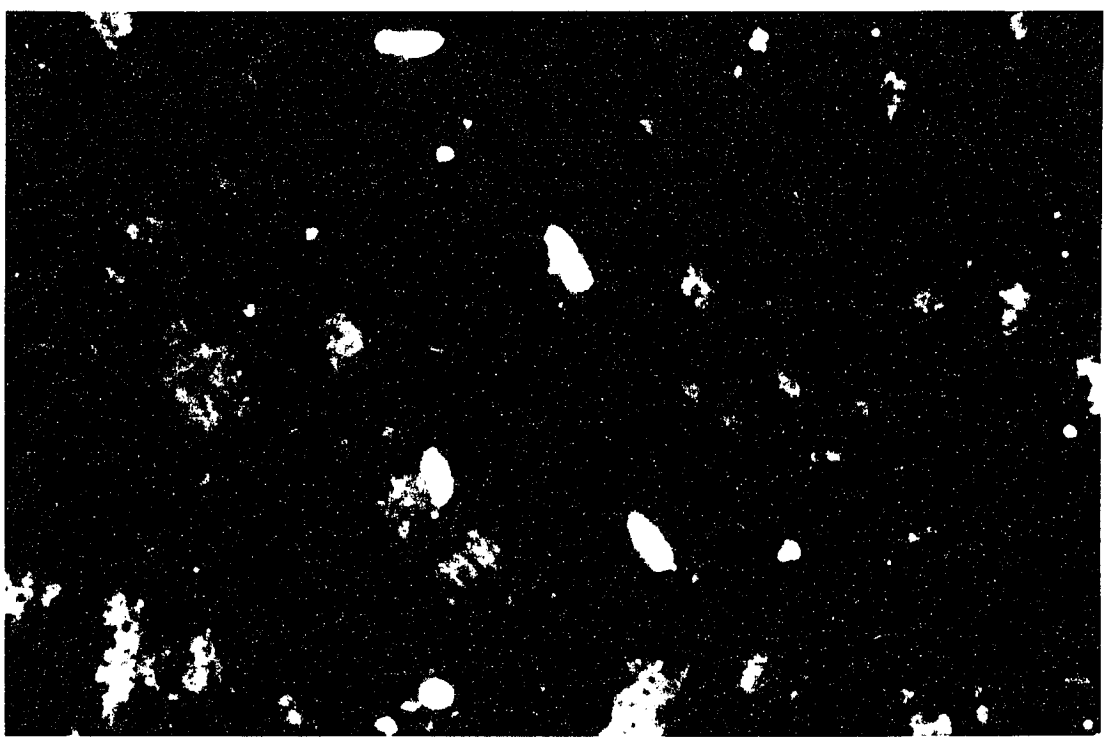


Figure 6: Epifluorescent photomicrograph of flagellates (~3 μm body length) in subsurface sediment samples stained with DAPI. Flagellate cells appear blue while non-DNA bound DAPI appears yellow (1250 X magnification).

ground water and subsurface contamination. Development of techniques for the estimation of microbial populations has been necessary to understand the role of microorganisms in the contaminated subsurface environment. Since much of the research on the subsurface microbial community has been devoted to the bacteria, methods for their enumeration have progressed considerably, while techniques for measuring the other constituents of the microbial community have lagged. Ironically, Severtzova noted the same discrepancy in methods for counting soil bacteria and protozoa in 1924.

Three different types of methods were identified by Severtzova (1924) for the enumeration of soil protozoa: direct counting and dilution methods with either liquid, or solid media. She felt that direct counting of soil infusions with a light microscope provided the best method for determining the activity and species of soil protozoa. Dilution techniques with liquid media involve the addition of a known amount of soil to the liquid for the inoculation of a series of dilution tubes, incubating the tubes and then examining the liquid for the presence or absence of protozoa. Similarly, solid media was also used for the quantification of soil protozoa. The problems Severtzova noted with these methods are still encountered with the enumeration of subsurface microorganisms. Only minute quantities of the soil can be examined with the microscope

and numerous samples must be observed. In some soils, the inability to distinguish the protozoa from the particles make microscopic examination ineffective and thus only soils rich in protozoa (e.g., 10^5 ciliates/gram dry weight (Foissner, 1987)) are practical for direct counting techniques. Separation of the protozoa from the soil also causes problems with dilution techniques, in which individual cells and soil aggregates should sequentially decrease for the procedure to precisely estimate populations. The media chosen for the dilution method often inhibits some protozoan species preventing their growth.

[N.B., the units associated with estimates by direct count and culture techniques are different and relate to the way in which the cells are detected. The protozoa are observed with a direct count technique and thus the population estimates are reported as *protozoa per volume*, or *ciliates per volume* in the case where the specific type of protozoa is quantified. However, the culture techniques generally consist of a dilution/extinction series and the populations are estimated by the *most probable number*, or MPN. Throughout this dissertation, MPN will refer **only** to the estimation of protozoa, otherwise the type of microorganism will be specified. Whenever possible, the population estimates for sediment samples will be reported *per gram dry weight*, or gdw.]

Some of these enumeration techniques have been applied recently to subsurface samples. In 1983, Hirsch and Rades-Rohkohl published the first paper in which ground water was examined for protozoa using enrichment techniques for topsoil bacteria. They used a number of different liquid and solid media to cultivate a variety of types of microorganisms in the ground water, but they did not quantify the protozoa. Federle et al. (1986) found polyenoic fatty acids (compounds unique to eukaryotic organisms) throughout subsurface samples, but they were not able to relate the concentration of the fatty acids to the populations present in the sediment. Sinclair and Ghiorse (1987) used an extinction-dilution technique with solid media, developed originally for soil amoebae, to enumerate the protozoa in sediment samples collected down to 8 m below land surface (the water table was 3 m below land surface). To date, no other techniques have been applied to enumerate subsurface protozoa that address the problems with quantitatively detecting these organisms. Quantitative and representative enumeration techniques for subsurface protozoan populations must consider the separation of the organisms from the sediment, concentration of the organisms for reliable detection, and elimination of bias due to media preferences.

IV. VARIABILITY OF ENUMERATION TECHNIQUES

In order to understand the role of protozoa in the subsurface environment, the variability associated with both *in situ* populations and enumeration techniques must be estimated before comparisons between sites can be made. Enumeration of the subsurface protozoan population involved drilling at the U.S.G.S. site, removing a sample within a 1.5 m aluminum core barrel, bringing a section of the core back to the laboratory and subsampling the core. Estimations of population size were made with direct count epifluorescent microscopy, the Darbyshire liquid media MPN method and the Singh solid media MPN method. Variability within each estimate of protozoa was delineated into that associated with the enumeration technique, core subsampling, intrasite location, holding time of the core, and the drilling process.

Each enumeration technique has a bias for the type of protozoa counted in the soil sample due to the method used to extract the organisms and the process of distinguishing the cells. Dilution techniques, such as the Darbyshire liquid media MPN and Singh solid media MPN methods, require the protozoa to grow in laboratory conditions which can be different from the soil environment. Detection of these organisms after incubation is based on the motion, size and shape of the cell, whereas the epifluorescent direct count

method only allows detection due to size, shape and type of fluorescence. The variability associated with the different enumeration techniques was estimated by comparing them with respect to the same subsurface sample.

Hierarchical experimental designs were used in the estimation of variability as a function of intrasite location and core subsampling for each enumeration technique. In Fall 1991, 3 cores were taken at a contaminated (S 318) and an uncontaminated (F 393) site. Each core was subsampled three times and analyzed using the epifluorescent direct count, Darbyshire liquid media MPN method and Singh solid media MPN method. The replication of the estimates of protozoa within a site and within each core allowed the application of the statistical design discussed by Gill (1978) and Box et al. (1978) to estimate the variability associated with the site, core and population estimate.

Analysis of variance (ANOVA) was used to investigate the variability of the enumeration techniques due to the hold time (between collection of the core samples and preparation of the enumeration methods in the laboratory) and contamination from the core barrels. The Fall 1991 samples collected at S 318 were enumerated 1, 5, 15 and 28 days after collection. Contamination from non-sterile core sleeves was also examined. Two additional cores at S 318

were taken with core sleeves that had been washed with 95% ethanol to determine if contamination from the sleeves affected population estimates compared to the untreated core sleeves.

Total and encysted populations were estimated with the Darbyshire liquid media MPN method and Singh solid media MPN method. The populations were distinguished by acidifying part of the sediment sample (pH~2) for 30 min and then neutralizing the solution before preparing the extinction/dilution series. The encysted population estimates were based on the assumption that the acid; 1) killed all the active cells; 2) all cysts survived; and 3) all cysts excysted during incubation of the samples. Trophic protozoan population was assumed to be equal to the encysted estimate subtracted from the total estimate. However, the indirect approach of estimating the active portion of the protozoan population was not discussed since the variability of each estimate was large.

V. ORGANIZATION OF CHAPTERS

In order to investigate the role of protozoa in the organically-contaminated, saturated subsurface environment, techniques for the study of the organisms had to be developed. The objectives of this dissertation are to discuss the enumeration techniques for protozoa found in the

sediment, and determine the variability associated with them and with the sampling techniques of the saturated subsurface.

Chapter 2 reviews the literature on techniques used for the estimation of protozoan populations from a variety of soil matrixes. Filtration, elutriation and centrifugation have been applied to physically separate the protozoa from sand grains and topsoil material. Other methods developed to remove motile protozoa from the sediments, e.g. geotaxism (organisms swim up to the surface of a sample) and seawater-ice extraction (organisms moving away from a descending salinity gradient), were also included. After separation of the protozoa from the abiotic particles in the sample, the techniques vary in the way protozoa are counted. Relevant literature investigating the variability associated with enumeration techniques and sampling the subsurface will also be reviewed.

Chapter 3 details the methods and materials used during the investigation of enumeration techniques for protozoa in sediments. Seawater-ice extraction, centrifugation and a shaking technique were evaluated for their ability to separate protozoa in marine sands. Then, the shaking method was used to extract protozoa from subsurface core material for enumeration by a filtration and epifluorescent staining technique, and two types of extinction-dilution.

Chapter 4 discusses the results of the studies of enumeration techniques. Details on the modifications of enumeration methods are also included. Three enumeration methods were applied to core samples collected during Fall 1991. Suggestions are also made for the number of cores to be collected at a new site, the number of replicates within each core and the enumeration technique considering these sources of variability.

Conclusions and recommendations are made in Chapter 5. The implications of the different enumeration techniques on saturated sediments are discussed with respect to the hold time and intrasite variability. Suggestions are made for future sampling programs to be conducted at the U.S.G.S. site to further investigate the enumeration techniques studied in this dissertation and their ramifications for determining the role of protozoa in an organically-contaminated subsurface environment.

CHAPTER 2

LITERATURE REVIEW

Enumeration procedures for soil protozoa all involve steps to enrich or concentrate the organisms since their number and size compared to the soil matrix generally precludes direct counting. Protozoa have been enumerated in agricultural soils since the early 1900's by culturing the organisms in a medium and determining their population size based on extinction-dilution series. The techniques used to enumerate protozoa have not changed with their more recent application to other environments such as lake sediments (Finlay et al., 1979), marine sands (Burnett, 1973; Uhlig et al., 1973), forest soils (Couteaux and Palka, 1988) and the subsurface (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990; Madsen et al., 1991). Procedures for direct observation of soil microorganisms have been developed based on the extraction of the organisms from the soil followed by a concentration process, but these techniques have not been widely used with protozoa.

This chapter will review the early work characterizing protozoa in soils, the qualitative and quantitative techniques of extracting and enumerating soil protozoa, and their application to subsurface samples. Table 1 is a schematic of the types of enumeration techniques discussed

Table 1: Protozoan enumeration techniques covered in the Literature Review, including references.

SEPARATION PROCESS	ENUMERATION TECHNIQUE	REFERENCES
None	Direct Counting	Bunt and Tchan, 1955; Finlay et al., 1979; Foissner, 1983; Alongi, 1986; Luftenegger et al., 1988
Dilution	Cultured in Liquid Media	Cunningham and Lönis, 1914; Cunningham, 1915; Severtzova, 1924, 1928; Darbyshire, 1973; Darbyshire et al., 1974; Anderson et al., 1978; Clarholm, 1981; Fenchel, 1982; Baldock, 1986; Couteaux and Palka, 1988; Kuikman et al., 1991
Dilution	Cultured in Solid Media	Cutler, 1920; Cutler et al., 1922; Severtzova, 1924, 1928; Singh, 1941, 1946, 1955; Darbyshire, 1973; Heal, 1971; Elliott and Coleman, 1977; Alabouvette et al., 1981; Vargas and Hatori, 1986; Sinclair and Ghiorse, 1987, 1989; Wiggins et al., 1987; Acea and Alexander, 1988; Beloin et al., 1988; Casida, 1989; Zaidi et al., 1989; Sinclair et al., 1990; Madsen et al., 1991
Filtration	Direct Counting of Filters	Couteaux, 1967; Uhlig et al., 1973; Dye, 1979; Louiser and Parkinson, 1981; Alongi, 1986; Baldock, 1986; Couteaux and Palka, 1988
Elutriation	Direct Counting or Filtration	Uhlig et al., 1973; Hopkins et al., 1991b
Centrifugation	Direct Counting or Filtration	Alongi, 1986; Griffiths and Ritz, 1988
Environmental Modifications	Direct Counting	Webb, 1956; Curds, 1963; Uhlig, 1964,1968; Fenchel,1967; Ruppert, 1972; Spoon, 1972; Arlt, 1973; Barnes, 1976; Groliere, 1977; Dye, 1979; Hartwig, 1980; Wright, 1982; Volkonitin, 1985; Agamaliev, 1986; Alongi, 1986; Armonies and Hellwig, 1986; Wilbert, 1986; Antes and Wilbert, 1987; Azovsky, 1988; Raikov and Volkonitin, 1989; Raikov et al., 1989

in the literature review. Also, this chapter will review the statistical techniques that have been used to estimate the variability of enumeration methods associated with the extraction, culturing conditions and detection of protozoan in sediment samples.

I. EARLY WORK ON SOIL PROTOZOOLOGY

The earliest work on enumeration of soil protozoa involved a dilution method commonly used for bacterial population estimates (Severtzova, 1928). This method has become the most common procedure for enumerating protozoa in forest litter and soil (Stout and Heal, 1967; Heal, 1970). A soil sample is mixed with a liquid and then serially diluted in liquid culture media. The dilutions are incubated and then examined for the presence of protozoa. Cunningham (1915) attributes the development of the dilution method to Rahn in 1914. Since then, numerous modifications have been made to account for trophic and encysted populations, the preferences of the protozoa for different types of bacteria (e.g., shape, motility or pigmentation), and the preference by different types of protozoa for liquid or solid media with varying concentrations of nutrients.

Much of the interest in protozoan enumeration in the early 1900's was motivated by the question of whether protozoa were harmful to a soil's agricultural productivity.

Cunningham and Löhnis (1914) reviewed the literature and found that most researchers believed that protozoa were harmful to soil productivity because the protozoa killed algae, fungi and bacteria, and were involved in the transport of "disease producers" to plants and transport of substances necessary for plant growth into deeper layers of the soil. They began to conduct experiments to determine if protozoa were actively feeding on other soil microorganisms and whether protozoa performed all these functions. They used an extinction-dilution method to evaluate broth media amended with different types of bacteria in order to determine which one resulted in the greatest protozoan growth. No single medium was found that supported flagellates, ciliates and amoebae, but they observed a succession of the protozoa growing over time. Cunningham and Löhnis speculated that the "protozoa live upon the bacteria" and were not involved in the "decomposition" of the medium.

Cunningham and Löhnis found that the dilution method could not distinguish between cysts and active (trophic) organisms since the media and the incubation time allowed both forms an "equal opportunity for development". They concluded that the function of soil protozoa could not be determined until the relative contributions of the encysted and active forms were understood. They expanded the work of

other colleagues on heat sterilization of soils, and found that trophic protozoa were killed when heated to 54°C and cysts were killed when heated to 72°C. They recommended using the dilution technique on untreated soil and heat-treated (55-60°C) soil to delineate total and encysted populations, respectively.

Cunningham (1915) continued to address these questions. He inoculated a phosphate buffer with bacteria and used this for dilution in the enumeration of total and encysted protozoa. The addition of bacteria decreased the excystment period and thus shortened the overall incubation time. Experiments with ammonifying solutions indicated that protozoa grazing on bacteria enhanced the ammonification process. Sterile soils were inoculated with bacteria and protozoa. Cultures with protozoa had a lower bacterial population than cultures without protozoa. Cunningham's experiments demonstrated some of the functions of soil protozoa not known by those who felt protozoa were detrimental to soil productivity.

Culter (1920) developed another method for estimating the number of trophic vs. encysted protozoa in a soil sample. He found that the heat treatment recommended by Cunningham killed an excessive number of cysts, so he compared the heating technique to treating the soil with 2% HCl "overnight" to eliminate trophic protozoa and hence

estimate the encysted population. Some cysts were killed by the HCl treatment, but many fewer than with heating. Culter speculated that the cysts killed were less resistant forms (e.g., those just beginning to encyst or those in the last stages of excystation, as well as reproductive cysts).

Cutler et al. (1922) conducted a one year, daily investigation of protozoan species and their abundance in soil. They used the dilution method to estimate protozoan populations with soil samples and dilution solutions inoculated onto the surface of agar media. The agar plates were examined after 28 days and flagellate, amoebae and ciliate species were identified. The medium used was thought to select against some species that were observed infrequently. Only two amoebae and four flagellate species were seen consistently throughout the study period and ranged from 10^3 to 10^5 per gram wet weight of soil.

Environmental conditions were also noted in conjunction with the sampling: soil moisture, rainfall, temperature, farm operations, and soil alkalinity. There were extreme daily fluctuations in these parameters as well as in the number of bacteria and protozoa. However, two week averages of the microbial enumerations showed that the maximum protozoan and bacterial populations occurred at the end of November and decreased to their minimum in December.

Severtzova (1924) compared the liquid media used by Cunningham and Löhnis to the solid media developed by Culter (1920) for estimating soil amoebae with the dilution method. Her interests were in developing a method for the isolation of soil amoebae and determining their role in agricultural soils. She found the solid agar surface provided a more precise estimate of the amoebae compared to the liquid medium. In 1928, she published another paper further developing the dilution method and applying the technique to investigations of soil amoebae and their preferences for different types of bacteria.

II. MODERN DILUTION METHODS

Two variations of the dilution method developed by the early protozoologists are commonly used today: the Singh solid media MPN method and Darbyshire liquid media MPN method. The Singh method refers to a technique developed by B.N. Singh in 1946 using subdivided agar plates that permit a number of replicates per dilution to be prepared on a single plate. The Darbyshire liquid media MPN method was a modification of Singh's technique by Darbyshire (1973) in which microtiter plates with 96 wells are used to grow the protozoa in liquid media.

A. Singh Solid Media MPN Method

Singh (1955) found the culture methods of Cunningham (1915), Cutler (1920), Cutler et al. (1922) and Severtzova (1924) to be unsatisfactory due to their choice of nutrient media. He believed that nutrient media encouraged the growth of inedible bacteria or bacteria and fungi that produced toxins. Hence, the presence of undesirable organisms on the agar surface led to the underestimation of soil protozoa. Singh stated that the work by Culter et al. (1922) was the first statistically acceptable procedure used in enumerating soil protozoa, yet he felt the research was inadequate due to insufficient replication, inappropriate source of bacterial food supply, and the large size of the petri dishes which meant that protozoa could be overlooked during examination.

Singh (1941, 1946, 1955) described a modification of the dilution culture method which overcame the problems encountered by Cutler et al. He used petri dishes which had been partitioned with glass rings in order to make replicates of the dilutions used to determine the population size. The modification also decreased the area to be examined for protozoa. Singh's plates were prepared by embedding eight glass rings in agar containing 0.5% (v/v) NaCl. Each ring was inoculated with a suspension of an edible bacterium (i.e., *Aerobacter* sp.) and a sterile

"fragment of soil" was placed in the center of each ring. Samples of soil (10 g wet weight) were shaken in 50 mL of water with 0.5% (v/v) NaCl for 5 min. Two-fold dilutions were prepared and 0.05 mL of the dilutions were inoculated into the rings of the petri dishes. The agar was kept moist during the two week incubation by adding sterile tap water or 0.5% NaCl solution to facilitate the growth and activity of flagellates or ciliates in the liquid on the agar surface. After the incubation period, the rings in which the bacterial lawn had disappeared were then observed on an inverted microscope to see if protozoa were present. To estimate the number of encysted protozoa in the soil, Singh (1946) compared heat-treated soil (Cunningham, 1915; Severtzova, 1924) to the acidification technique (Cutler, 1920). He found the heat treatment killed a number of cysts and therefore, underestimated the encysted population as compared to the acidification method.

Several modifications to Singh's glass rings have been proposed in the literature using materials that are readily available and thus easier to prepare. Darbyshire (1973) found that sterilizable polypropylene rings were suitable substitutions for glass rings. Casida (1989) used centrifuge caps to hold small samples of agar in place of rings in petri dishes. Sinclair uses plates with six 1 in.

diameter wells, available through many scientific equipment distributors (J.L. Sinclair, personal communication, 1992).

Singh (1946) appears to be the first to have applied the statistical technique developed by Fisher and Yates (1943) to determine the most probable number (MPN) of protozoa with the dilution technique. Prior to Singh's work, protozoologists reported the number of protozoa according to the dilution in which the organisms were no longer observed (i.e., the point of extinction). Since replicate samples often did not exhibit extinction at the same dilution, populations were often reported as ranges. Fisher (1922) developed the foundations of a statistical technique that considered the presence (positive) and absence (negative) of growth in a dilution series by an equation of maximum likelihood. Fisher and Yates (1943) provided convenient tables based on the equation for the determination of MPN depending on the dilution at which negative growth starts ("mean fertile level") and extinction occurs ("mean sterile level"). MPN calculations have been adapted to computer programs and are available for use with any dilution series (Hurley and Roscoe, 1983; Russek and Colwell, 1983)(see Appendix A).

Heal (1971) proposed a number of modifications to Singh's dilution culture method, stressing that the agar within the rings must be kept moist in order to prevent

restrictions on the movement of flagellates and ciliates. He also concluded that *Aerobacter* sp. was not a suitable substrate for all protozoa. Heal preferred the use of soil extract agar to encourage the indigenous bacteria in the sample to grow as the food source. Following Heal's method, soil bacteria would have to be added back into samples which had been treated with HCl for the enumeration of encysted populations.

Singh's technique and Heal's modifications were used by Elliott and Coleman (1977) to study protozoa in shortgrass prairie soils. They used cut up pieces of tygon tubing (1.27 cm diameter, 1.27 cm long) as rings in 3% soil extract agar (1:1 soil:water solution, autoclaved 30 min at 121^{o}C and then filtered). Distilled water was added to the soil dilution rings to maintain a thin liquid film during incubation. Naked amoebae appeared to grow well even if the water was not added, but more "free-swimming forms" (i.e., flagellates and ciliates) were observed if the rings were kept moist. Elliott and Coleman noted that this MPN modification may underestimate populations for two reasons: competition within a ring may result in the growth of only a few species from the soil community; and the culture conditions may not be suitable for the protozoa to grow.

In spite of these criticisms, Singh's method continues to be widely used in studies of soil protozoa. Alabouvette

et al. (1981) used Singh's method to study the density of *Thecamoeba granifera* sp. *minor* in soil samples, and Vargas and Hattori (1986) used the procedure to enumerate protozoa during studies on protozoan predation of bacteria in soil aggregates. Singh's technique has become the most commonly used enumeration method for subsurface protozoan populations (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990) and for assessing protozoan contributions to biodegradation (Wiggins et al., 1987; Acea and Alexander, 1988; Zaidi et al., 1989; Gurijala and Alexander, 1990; Madsen et al., 1991).

B. Darbyshire Liquid Media MPN Method

Instead of culturing on agar media, Darbyshire and his associates (1973, 1974) made dilution cultures in liquid media (soil extract) to avoid selection against ciliates and flagellates. His technique involved serial dilutions similar to those used by Singh (1946, 1955), but the samples were incubated in microwells (0.37 mL volume) for up to 14 days. The procedure used styrene microtiter plates with 96 wells per plate: 12 rows (for 12 dilutions) of 8 wells per row (for 8 replicates per dilution). Therefore, adequate replication of each dilution was possible within each plate. In addition, the wells were small enough to easily examine the sample thoroughly with an inverted microscope.

Darbyshire used soil extract as the diluent instead of Singh's saline solution. The soil extract was made by autoclaving (121^{o}C, 30 min) garden soil in water (1:1, w/v) and then filtering it (size No. 2 filter paper) to remove soil particles. No additional nutrients were available to the bacteria in soil extract as with Singh's saline solution, yet the conditions were similar to those encountered by the soil organisms. Darbyshire found that a variety of protozoa cultivated better in the soil extract as compared to the saline solution. He originally used microdiluters (capillary tubes that hold 25 μL) to transfer liquid from the wells, but no significant difference in the abundance of protozoa was detected in samples prepared with pipets and those prepared with microdiluters. Darbyshire and his co-authors reported great numbers of flagellates, amoebae and ciliates in their samples.

Anderson et al. (1978) used the extinction-dilution technique developed by Darbyshire et al. (1974) to estimate amoebae populations, while investigating the interactions of bacteria, amoebae and nematodes in soil microcosms. The authors modified the procedure to include the HCl treatment Singh used in estimating encysted amoebae.

Clarholm (1981) also used the microtiter plates described by Darbyshire et al. (1974) for enumeration of protozoa grazing on bacteria in soil experiments. Tryptone

soy broth in modified Neff's amoeba saline was used as the diluent, and the indigenous bacteria of the soil were allowed to grow and serve as a food source. She indicated that having the natural microflora available as a food source was a disadvantage at times when fungi grew in the microtiter wells and inhibited bacterial and protozoan growth. The plates were examined for ciliates after 3 days, flagellates after 3 and 5 days and naked amoebae after 7 and 10 days of incubation before the protozoa encysted due to lack of food.

Kuikman et al. (1991) used microtiter plates to enumerate protozoa in soils during experiments on the influence of moisture content, and predatory behavior on nitrogen mineralization in planted and fallow soils. They inoculated amoebae and flagellates into soils of various moisture content. After an incubation period, they extracted the protozoa into Neff's amoeba saline (similar to Singh's medium) using a shaking technique. Estimates of the populations demonstrated that flagellates were not as active at lower moisture contents as amoebae. All protozoan activity was limited at moisture contents of less than 11%. Nitrogen uptake by plants was greater in soils amended with protozoa than in soils without them.

III. DIRECT COUNTING

Direct counting of protozoa in soil samples using a microscope circumvents the problems of cultivating protozoa encountered in the dilution methods (Bunt and Tchan, 1955). The choice of media, bacterial food source, competition among species, and procedures for preparing dilutions have all been cited as reasons why the extinction-dilution method underestimates the protozoan population in soils. Severtzova (1928) stated that the direct observation of a drop of soil infusion (soil/water mixture) "is theoretically the best method of counting, but practically it may be adapted only to the soil especially rich in Protozoa".

Jones and Mollison (1948) described a procedure for direct observation of a known quantity of prepared soil in molten agar spread across a haemocytometer slide and then immersed the slide in acetic-aniline blue stain. They prepared the soil by sieving, grinding and washing it in distilled water. Molten agar (1.5%) was added to the soil suspension and the mixture was shaken to distribute the particles. The hot agar was then pipetted onto a haemocytometer for direct observation of the organisms with a microscope. They enumerated bacteria and fungi in the agar and speculated that the technique could be applied to the enumeration of other soil organisms.

Bunt and Tchan (1955) modified the Jones and Mollison procedure for use with soil protozoa in topsoil samples. They omitted the harsh sieving and grinding steps and simply suspended the material in a 1:4 ratio with agar, then stained the agar with erythrosin and methyl green or with Gram stains to differentiate the organisms from the soil particles. Attempts to use dark field illumination and phase contrast microscopy were unsuccessful since the presence of soil particles made the protozoa difficult to discriminate from algae and motile bacteria. They choose to develop this technique since Singh's (1946) method underestimated flagellates that "may not feed on bacteria". In addition to the stains mentioned above, Bunt and Tchan tried a fluorescent stain, acridine orange (AO), but they found it unacceptable since the soil also fluoresced and there was not enough contrast with the organisms. However, Hopkins et al. (1991a) successfully used the agar film technique with AO staining after extracting the microorganisms from similar sands with a centrifugation process.

Finlay et al.(1979) used direct observation of lake sediment (fine sand and silt) samples diluted 1:10 with membrane-filtered (0.45 μm) water to enumerate benthic ciliates. They examined fifty 5 μL samples of the suspension on microscope slides. Singh's method was not

used since the thin water film on the agar plates selected against diverse ciliate communities. In 1981, Finlay modified the direct observation technique by examining thirty 5 µL aliquots of pore water taken directly from the surface of sectioned cores. Due to the low densities of ciliates found in the sediments, this modification was necessary since dilution of the sediment would have made the enumeration process more difficult. While Finlay was satisfied with estimating benthic ciliate populations based on examinations of pore water, he felt attached forms of protozoa (i.e., amoebae) would probably not be recovered.

Foissner (1983) tested direct microscopic examinations by adding known amounts of ciliates, testate amoebae, naked amoebae, flagellates, green algae and diatoms to sterile topsoil. Soil suspensions were made by adding 0.05 g wet soil to 3 mL of tap water. Aliquots were dispensed on 10 slides and observed at 100 X magnification. He stated that "55%-100% of the ciliates, 30%-100% of the testacea, ~50% of the flagellates and diatoms, and about 2% of the *Chlorella* sp. and *Amoeba* sp. could be found again". The success that Foissner (1983, 1987) has had with direct observation would probably not be easily accomplished with different types of soil (e.g., large grain sizes would be difficult to fit under coverslip) or with a less experienced protozoologist.

Luftenegger et al. (1988) described a similar technique for enumerating ciliates, nematodes, rotatorians and testacea in topsoils. An amount of soil (i.e., 0.4 g of soil for ciliates, 0.1 g of soil for testate amoebae) was diluted with soil extract and stained with aniline blue. The sample was washed to remove excess stain, centrifuged, and then the soil pellet was "thoroughly macerated by a glass-stick". A suspension of the soil was made with 0.5 mL albumin-glycerin and then drops of the suspension were examined on a microscope slide (100 X magnification for ciliates and larger organisms; 400 X magnification for the amoebae). The recovery rates were similar to Foissner's (1983): "86% of the testacea, 85% of the nematodes, 72% of the ciliates and 47% of the rotatorians were recovered on average". Motile ciliates were difficult to observe during counts of random fields on the slide. They found that the recovery rate was dependent on the soil type, dilution and skill of the person performing the enumeration. In particular, the smaller ciliates had lower recovery rates presumably due to using a lower magnification.

Direct observation has also been used for the examination of large surfaces incubated in situ. This technique is selective for the organisms adapted to colonizing surfaces and does not include all species present. Removing leaves and rocks from bodies of water for

observation with a dissecting microscope is also common (Finlay et al., 1988). Objects which are more suitable to microscopic examination (e.g., petri dishes and microscope slides) can also facilitate direct counting of organisms in streams, rivers and ponds (Warren, 1983). Microscope slides have been attached to surfaces in wastewater treatment plants to examine biofilms (Kinner et al., 1990). Polyurethane foam sponges have also been introduced to water columns and the colonizing organisms squeezed from the pores and examined on microscope slides (Henebry and Cairns, 1980; Cairns, 1982; Finlay et al., 1988; Tremain and Mills, 1991). Results from contact slides (slides buried in the soil) are often found in school science fairs. This technique allows the investigation of microbial diversity and succession of organisms in the community. Quantitative estimations of colonizing populations are possible by observing a known area of the artificial matrix after it has reached steady state growth.

IV. SEPARATION AND CONCENTRATION METHODS

Often direct counting is tedious and unreliable because soil particles obscure the protozoa especially when present in low numbers. As a result, separation procedures have been developed which remove the organisms from the soil particles and dilute them in an excessive amount of

supernatant. After separation, the organisms are concentrated in another medium that facilitates enumeration. These methods exploit the different hydrodynamic behavior of the biotic and abiotic particles of a sample. The sinking rate and transport of the particles by water flow are functions of the size, specific weight, shape and surface structure of the particles (Uhlig et al., 1973). Filtration, elutriation, centrifugation and environmental modifications are commonly used as separation methods. They are designed to cause the organisms to be transferred from the soil matrix into a liquid medium that can subsequently be directly observed or cultured.

A. Filtration

Methods involving filtering soil suspensions onto a membrane and then observing them directly have been tested with a number of minor modifications. Dye (1979) described a procedure for separating protozoa from marine sands collected along the coast of South Africa. 50 cm^3 of sand was placed in a bottle and gently shaken for 1 min with 100 mL of filtered seawater. The sample was allowed to settle for 10 sec and then the supernatant was decanted into a beaker. The procedure was repeated twice generating 300 mL of supernatant. The supernatant was stained with AO and then filtered through a 5 μm membrane filter. The filter

was placed on top of a drop of immersion oil, covered with a coverslip and examined under a microscope. This procedure produced three times the recovery obtained using a seawater-ice extraction method (Fenchel, 1967).

Lousier and Parkinson (1981) adapted the membrane filter technique developed by Couteaux (1967) for extracting the tests of testate amoebae. A forest soil sample (1 g wet weight) was fixed with Bouin-Hollande solution, stained with xylidine de ponceau and then diluted up to 1 L with distilled water. The samples were suspended by manual agitation or maceration in a blender. Depending on the type of soil, 5 to 10 mL of suspension was removed and filtered through a 0.45 μm Millipore^{R} membrane. Maceration was found to yield higher numbers of testate amoebae than manual agitation, but the longer the duration of blending the more broken tests observed. Greater numbers and more species of testate amoebae were observed using this filtration technique compared to decanting and direct observation of agar films (Heal, 1967). Although the technique worked for testate amoebae, its usefulness for more fragile organisms has not been evaluated.

Baldock (1986) described an enumeration process for protozoa inhabiting fine grain, freshwater sediments. He fixed 5 μL samples with $HgCl_2$, suspended the sample in 0.5 M calcium chloride before filtering it through a 0.22 μm

Millipore^{R} membrane with vacuum pressure $\leq$ 25 mm Hg. The filters were stained with rose bengal, mounted on slides and observed with bright field illumination. He compared this technique to a direct counting method (Finlay et al., 1979) and the Darbyshire liquid media MPN method (Darbyshire et al., 1974). The flagellates were the most frequently observed protozoa in all enumeration techniques, yet they were not easily detected by the direct count method. Baldock noted that the large ciliates did not have a sufficient food source in the microtiter plates and as a result they were underestimated when compared with the filtration results. However, the Darbyshire MPN technique enumerated twice as many small flagellates (>5 μm) as his technique. Three problems were identified with the filtration procedure: 1) organisms could only be categorized by broad taxonomic groups (flagellates, amoebae and ciliates) based on size; 2) counting was time consuming, especially if there were numerous small flagellates; and 3) recognition and classification of the preserved material took some practice in order to be consistent.

Couteaux and Palka (1988) modified the filtration technique described by Couteaux (1967) and Lousier and Parkinson (1981) for enumerating soil ciliates, in particular *Colpoda aspera* (Kahl 1930-1935)(see Figure 5). *C. aspera* was inoculated into several microcosms consisting

of 1.5 g dry weight of humus soil held in syringes and maintained at a constant oxygen tension, moisture content and bulk density. Three days after inoculation, the samples were fixed, stored and diluted as outlined in Lousier and Parkinson (1981). Two more dilutions were made so that a 20 mL aliquot from the third dilution could be filtered through an 8 μm Millipore^{R} membrane. Then the filter was mounted on a slide and examined for *C. aspera* using a microscope. The filtration procedure was compared to a first dilution direct count (similar to Foissner, 1983) and a two-fold dilution method (adapted from Darbyshire et al., 1974), in which Volvic^{R} mineral water was used to make all dilutions. *C. aspera* cells were thought to have lysed in both dilution methods. Clumps of cysts were noticed on the filters, resulting in an underestimation of the ciliates present in the original sample. The greatest drawback of the filtration technique was that the dilution of the sample resulted in the equivalent of only 1 mg of soil (dry weight) being filtered, which limited the ability of the technique to detect low numbers of ciliates. Couteaux and Palka stated that the precision of the technique was improved by preparing a number of filters and observing numerous fields per filter.

To help differentiate the organisms from the particles on a filter, stains have often been added to increase the

contrast between the two. Soil bacteria have been enumerated by filtering samples and staining the cells with epifluorescent stains such as AO (Rades-Rohkohl et al., 1978) and 4'6'-diamidino-2-phenylindole (DAPI)(Huber et al., 1985). Only Dye (1979) has used filtration and epifluorescent stains for the enumeration of interstitial sediment protozoa. The advantage of fluorescent stains is their ability to make the organisms distinctly visible in contrast to soil particles. This occurs because the fluorescent stains combine with the DNA and RNA in the cells. Other fluorochrome stains such as fluorescein isothiocyanate (FITC; Caron, 1983), primulin (Caron, 1983; and Bloem et al., 1989) and proflavine (Kuosa and Marcussen, 1988) have been used with heterotrophic flagellates filtered from water samples.

Griffiths and Ritz (1988) tested combinations of 5 stains on protozoa extracted from centrifuged sediments. The stains (AO, DAPI and FITC) were used to enumerate the protozoa and measure the size of the cells. They also evaluated fluorescein diacetate (FDA) and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-pheyltetrazolium chloride (INT), which stain metabolically active protozoa. The AO-DAPI counterstaining provided the easiest procedure for the enumeration and sizing of the cells compared to other stains. INT was chosen as the stain for distinguishing

active cells since it stained fungal spores and did not interfere with the AO-DAPI staining.

Darbyshire (personal communication, 1990) found that 5-nitrosalicyl-aldehyde (NSA) and 4-methoxy-B-naphthylamine (MNA) stains, developed for use with tissue cells, also stained protozoa in a soil matrix. Some of the advantages of these stains included: the ease of application; the constant intensity of the stain during fluorescent illumination; and the limited indiscriminate staining of the soil particles.

B. Elutriation

Some separation techniques, like elutriation, exploit the different physical parameters of living organisms and abiotic soil particles after they are dispersed in a chelating agent or detergent solution. Elutriation devices generally consist of a sample receptacle where water can flow through the soil solution into a container and then out through a tube where the microorganisms are collected. Elutriation separates populations of particles (biotic and abiotic) from each other depending on their sedimentation velocity as described by Stokes' Law:

$$v = \frac{2r^2 g(\rho_p - \rho_m)}{9\eta} \qquad [1]$$

where **v** is the particle's sedimentation velocity; r is the radius of the particle; *g* is the gravitational constant; ρ_p is the particle's density; ρ_m is the liquid's density; and η is the liquid's kinematic viscosity (Macdonald, 1986; Hopkins et al., 1991b). The process involves flushing a sample of soil with a stream of water at the precise velocity to separate the less dense microorganisms (1.3 g/mL maximum density) from the heavier, faster settling mineral soil particles (2.6 g/mL particle density) and transporting the cells upward through the elutriator (Hopkins et al., 1991b). The enriched, washed out solution is then examined for the number and species of organisms present.

Uhlig et al. (1973) tested four modifications of elutriation on marine sand samples: 1) preserved and 2) unpreserved samples were concentrated on a 45 μm sieve in an open system; 3) narcotized organisms were collected on a 45 μm sieve in closed system (where the particles were circulated through the system many times); and 4) live organisms were caught on a sieve in a 55°C, open system. These techniques resulted in the collection of ciliates, tubellarians, gastrotrichs, nematodes, polychaetes, copepods, ostracods, and others. The greatest recovery of ciliates occurred with the preserved samples collected in the open system. Microorganisms smaller than the ciliates were not observed due to the large pore size of the sieves.

Hopkins et al. (1991b) calculated that using the water velocity recommended by Macdonald (1986), the elutriation device would theoretically have to be run for seven years to remove all the non-filamentous microorganisms from soil particles due to inconsistences in flow through a soil sample and drag forces exerted on the microorganisms as they were separated from the other particles. To limit the time required for processing a sample, Hopkins et al. had to increase the water velocity which resulted in larger volumes of collected supernatant and more soil particles than desired for direct observation. They had to further concentrate the sample to a point where it could be enumerated more easily. Filtration of the elutriated sample was a problem since too many soil particles remained. They chose to centrifuge the samples in order to further separate the less dense organisms from the soil particles. The supernatant above the soil was then filtered for enumeration with AO.

Hopkins (personal communication, 1990) felt that elutriation of subsurface sediments could be an effective process in extracting protozoa, although he had never worked with them. The efficiency of the elutriation procedure was dependent on: 1) the ability of the protozoa to separate from the other particles during the dispersion step with detergents or chelators; 2) the survival of the protozoa

during dispersion; and 3) the extent of the damage sustained during elutriation and a second concentration process, such as centrifugation. Hopkins felt these problems had to be addressed before elutriation could be used successfully in protozoan enumeration.

C. Centrifugation

Centrifugation has also been used to separate protozoa from soil suspensions. Alongi (1986) enumerated benthic protozoa from mangrove sediments with a density-gradient centrifugation method using a silica gel. He chose this technique based on its previous success for the collection of dinoflagellates and algae (Price et al., 1978), and nematodes, ostracods, and other organisms (Schwinghamer, 1981) from marine sediments. Sediment cores containing 2 cm^3 of sample were added to 30 mL round-bottomed centrifuge tubes containing 5 mL of the silica gel mixture. The tubes were vortexed for 1-2 min, allowed to settle for 1 hr and then centrifuged for 20 min at 490 x *g*. The supernatant was then poured into a gridded petri dish. The sediment pellet in the bottom was centrifuged with aliquots of silica gel three more times. The supernatants from each step were observed under a dissecting microscope to enumerate the ciliates and flagellates.

Schwinghamer (1981) worked with coarser sediments than Alongi. He did not mix the silica gel with the sand. Instead, the coarse sand sample was placed in the centrifuge tube, silica gel was added under the sand with a canula (pipet) and then the sample was centrifuged. He found that the speed and time of centrifugation was dependent on the type of sediment.

Alongi (1986) compared the centrifugation technique to methods consisting of decantation/preservation, cultivation and serial dilution, direct sampling of diluted mud with pipets, adhesion onto coverslips, seawater-ice extraction and sample flushing with $MgCl_2$. Ciliates and flagellates in 2 cm^3 samples of mangrove muds were observed with a dissecting microscope for all these procedures. The centrifugation technique yielded significantly ($P \leq 0.05$) more ciliates and flagellates than any of the other methods except for the cultivation and serial dilution technique, where the recovery was similar. The low estimates of the decanting/preservation method were attributed to the inability to extract attached protozoa and the inadequacy of formaldehyde as a preservative for the "soft-bodied" protozoa. The coverslips only recovered the "thigmotactic" (responding to contact with a solid surface) species. The "inherent sampling bias" of the direct sampling procedure and varying response of different species to the seawater-

ice and $MgCl_2$ extractions were thought to underestimate the protozoan population compared to the recovery of the centrifugation process.

Griffiths and Ritz (1988) described a procedure to separate protozoa from mineral soils with density gradient centrifugation for the enumeration of protozoa and biomass estimations. The procedure involved dispersing a soil sample in a buffer, centrifuging the sample, staining the supernatant and then passing it through a filter. They compared this procedure to the Darbyshire liquid media MPN method with acidification to determine the encysted population.

Griffiths and Ritz investigated different anionic, cationic and neutral detergents and buffers to determine the solution that achieved the greatest dispersion of the protozoa and soil particles in the smallest volume and also had the lowest toxicity. The detergents were not found to be as effective in dispersing the soil as the buffers. Although detergents could be used to disperse organisms that were "attached to the soil minerals via lipid-based or electrostatic mechanisms", the authors postulated that ciliates and flagellates were not attached by such mechanisms due to their "predatory" behavior, and that physical entrapment of protozoa within microaggregates was more likely responsible for their low recovery. Tris buffer

was chosen as the best dispersant since it extracted more cells compared to water, and was not toxic to the protozoa.

Griffiths and Ritz found no significant difference ($P<0.05$) in the number of protozoa observed in the samples treated by the centrifugation technique and Darbyshire liquid media MPN method. Estimates of active protozoa based on INT staining and the acidification process with the MPN method were also similar. Both techniques also required a similar amount of time to enumerate the protozoa. However, the INT staining was thought to give a more accurate estimate of the biomass of active protozoa than the MPN method since the measurements were made on the extracted rather than cultivated cells.

Though recovery of protozoa from soil samples with centrifugation was similar to the recovery with the extinction dilution methods (Alongi, 1986; Griffiths and Ritz, 1988), the efficiency of the separation technique depends on the ability of the organisms to be dispersed from the soil particles (Hopkins et al., 1991a). Indeed, all techniques reviewed may still underestimate the total protozoan population in the soil since they all depend on the sample to be dispersed so that individual organisms can be enumerated.

D. Environmental Modifications to the Soil

Techniques which concentrate the protozoa in another medium due to changes in the indigenous soil environment have been investigated for a number of years. Webb (1956) described a process in which mud samples from brackish tide pools were put in petri dishes and clean coverslips were placed on top of the moist mud. After an hour, the coverslips were removed and examined with a microscope to enumerate the attached protozoa. Presumably, the strongly thigmotactic protozoa swam up and attached to the coverslip, trying to escape the anaerobic conditions developing in the sediments.

Alongi (1986) compared Webb's technique to a centrifugation separation technique. He found that the diverse protozoan population in mangrove sediments were not well represented by thigmotactic species and the coverslip method underestimated the number of organisms.

Patterson (personal communication, 1990) qualitatively assessed flagellate populations in sediments with the coverslip technique. The coverslips often recovered some species that were not isolated easily by direct examination of fine sediment samples. He felt the process might not work with coarser sediments since the anaerobic conditions necessary to isolate the flagellates might not develop.

Spoon (1972) described a method for extracting and concentrating protozoa from sediments by forcing the organisms to swim to the surface of a test tube due to unfavorable conditions developing in the sediments. A sediment sample was placed in the bottom of a test tube (1.7 cm diameter, 14.5 cm long) and the tube was filled with water to a height of 4 cm below the top of the tube. Then an empty glass vial 2 mm smaller in diameter than the test tube was floated so that its mouth was just above the water's surface. After incubating the tubes for 16 hr, the glass vial was bobbed up and down in the test tube with a spring-wire retriever, so that the organisms in the upper layer of the water could be collected in the vial. Modifications such as applying an electric current, bubbling H_2S, N_2 or O_2 gas, or dispersing an irritant through the sediment did not improve the technique. Spoon found this method was especially successful in collecting hymenostome (e.g. *Paramecium*) and heterotrich (e.g. *Spirostomum*) ciliates. Curds (1963) describes a similar apparatus for collecting *Paramecium caudatum* from activated sludge by taking advantage of their negative geotaxis.

Uhlig (1964, 1968) separated protozoa from marine sands by using a salinity and temperature gradient to drive the organisms into a collection dish. The sediment was placed in a plastic cylinder (4.5 cm in diameter and 10 cm long)

that had one end covered tightly with nylon mesh (size of the mesh was dependent on the grain size of the sample). Cotton batting was placed on top of the sediment and the rest of the tube was filled with crushed seawater-ice. The tube was lowered into a culture dish, containing about 40 mL of filtered seawater, so that the nylon mesh just touched the water's surface. The interstitial organisms were forced out of the sediments and into the culture dish due to the melting seawater-ice forming a cold, salinity gradient that migrated down through the pores of the sediment. The culture dish was changed every 20 min until either enough sample for observation with a dissecting microscope had been collected, or the salinity in the dish equaled that of the seawater-ice mixture.

Further experiments demonstrated that the temperature change due to the melting ice had almost no effect on the extraction of the organisms from the sand (Uhlig, 1968). The interstitial fauna responded more to the salinity gradient than the flow of the seawater through the sample core. Uhlig (1968) found that the liquid remaining in the sediment usually did not rise above 50 parts per thousand (ppt) and that the salinity in the culture dish did not increase above 40 ppt. Also, the seawater-ice extraction was most successful with marine sediments of "capillary structure" (Uhlig, 1968). The technique was inadequate for

samples with a high mud content, presumably due to the low porosity inhibiting the migration of the salinity gradient and the motility of the organisms (Arlt, 1973; Armonies and Hellwig, 1986).

Many types of marine interstitial organisms have been extracted using Uhlig's technique. Uhlig (1968) found ciliates and flagellates as well as harpacticoides, ostracods, mystacocarids, nematodes, polychaetes, archiannelids, oligochaetes, turbellarians, gastrotrichs, and tardigrades. Higgins (1968) tried the seawater-ice extraction to collect kinorhynchs and Poizat (1975) collected opisthobranch gastropods. Armonies and Hellwig (1986) studied the movements of platyhelminthes in response to changes in moisture, salinity and temperature using the seawater-ice extraction.

The seawater-ice extraction has been used to study several ecological relationships of interstitial marine ciliates including their horizontal and vertical distribution (Ruppert, 1972; Barnes, 1976; Hartwig, 1980; Volkonitin, 1985; Agamaliev 1986) and succession in "new" sand (Groliere, 1977; Azovsky, 1988). It has also been used to discover new species (Wright, 1982; Wilbert, 1986; Raikov and Volkonitin, 1989); and observe of the fine structure of isolates difficult to culture (Antes and Wilbert, 1987; Raikov et al., 1989). Ruppert (1972) described a modified

coring device which allowed him to remove samples at different locations in the core for the seawater-ice extraction.

Uhlig's procedure has been compared to other quantitative sampling techniques of sandy soils. Alongi (1986) found that the seawater-ice method was not as efficient in extracting benthic protozoa as a centrifugation technique using a Percoll^{R}-sorbitol density gradient. Dye (1979) compared it to his filtration procedure and found three times the number of protozoa as compared to that of the seawater-ice extracted samples.

Uhlig et al. (1973) tested a number of quantitative extraction techniques (e.g. decanting and sorting, elutriation and seawater-ice extraction) on sand samples. Modifying the seawater-ice extraction by increasing the diameter of the tube did not improve the recovery of ciliates. Although the decanting and sorting procedure enumerated significantly more interstitial organisms, the seawater-ice extraction recovered the most ciliates.

No studies using the seawater-ice extraction technique on fresh water sediments have been found in the literature. The ability for fresh water organisms to respond to a salinity gradient would have to be evaluated. Changes in the osmotic concentration of the pore water might cause the cells to lyse. A possible modification of the technique

would be to allow fresh water ice to melt through the sediment sample. However, the success of the seawater-ice extraction was attributed to the advancement of the salinity gradient and not due to the change in temperature.

V. ENUMERATION OF SUBSURFACE MICROORGANISMS

In the past decade, the microbial communities in saturated subsurface sediments have been investigated in response to the growing concern for ground water quality and new bioremediation techniques for contaminated aquifers. Ghiorse and Wilson (1988) reviewed the recent literature regarding types, abundances and activities of microorganisms found in pristine and contaminated aquifers. They reported that protozoa were observed in samples from Segeberger Forest, northern Germany (Hirsch and Rades-Rohkohl, 1983), Lula, OK (Sinclair and Ghiorse, 1987; Beloin et al., 1988) and Nemaha, KS (Sinclair et al., 1990). Since that review was published, protozoan communities have been investigated in Aiken, SC (Sinclair and Ghiorse, 1989) and New York (Madsen et al., 1991).

Few investigations of the subsurface microflora have found protozoa, probably due to the difficulties associated with collection, sampling and the enumeration techniques. The first qualitative reports of protozoa from the saturated subsurface was by Hirsch and Rades-Rohkohl (1983). They

took ground water samples from four fully-screened (over 10 m), stainless steel wells and eight multilevel sampling wells (each with 18 sampling ports located 1 m apart from 5-23 m below the land surface). The ground water samples were inoculated into nutrient broth and basal mineral salts medium, and onto peptone yeast extract glucose vitamin agar, yeast malt extract agar, nutrient agar and solidified Baar's medium. Microscopic examination of the enrichments revelled flagellates, amoebae and ciliates growing in their cultures. They concluded that the organisms found in the ground water would probably only represent a small number of the entire population because many of the protozoa would probably be associated with the sediment and would not be collected in a ground water sample.

The most commonly used quantitative enumeration technique in the literature on subsurface protozoa populations was that developed by Singh (1946, 1955) using agar surfaces (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990). The Singh solid media MPN method has also been the preferred enumeration technique to count protozoa in microcosms for biodegradation experiments (Wiggins et al., 1987; Acea and Alexander, 1988; Zaidi et al., 1989; Madsen et al., 1991). The choice of the Singh solid media MPN method by all of these researchers probably is due to their affiliation with Cornell

University. Also, they have not investigated other enumeration techniques for protozoa.

Sinclair and Ghiorse (1987) modified the Singh solid media MPN method for the enumeration of subsurface protozoa. They poured 30 mL of 1.5% sterile, molten agar in phosphate buffer (2.2 mM KH_2PO_4, 4.02 mM K_2HPO_4, pH 7) into a petri dish and arranged five sterile glass rings (2 cm diameter, 1 cm tall) in the agar. Each ring was inoculated with *Enterobacter aerogenes* (harvested from Trypticase soy agar after one day of growth) as a food source for the protozoa. Undiluted samples consisted of 1 g of sediment from a core added to each ring with 1 mL of phosphate buffer. Dilutions were prepared by adding 10 g of sediment from a sample core to 90 mL of phosphate buffer. The sediment was shaken in the buffer for ~30 sec (J.L. Sinclair, personal communication, 1992) and then 1 mL of the buffer was pipetted into the five rings of a plate. At least three dilution levels were used for each core sample and one plate was used for each dilution (i.e., five replicates per dilution). Encysted protozoa were enumerated by decreasing the pH of the diluted samples to <2.0 (with 0.55 N HCl) for 15 min and then neutralizing the sample (with 1 N NaOH) before adding the sample to the plates. The plates were incubated at 20-23^{o}C for 14 days and the presence or absence of protozoa was determined by aseptically removing a sample

from each ring and examining it at 400 X magnification with phase-contrast microscopy. The MPN/gdw was determined with a computer program (Russek and Colwell, 1983) based on the same statistical technique as used by Singh (1946). They reported their detection limit to be 0.2 MPN/gdw.

Sinclair and Ghiorse (1987), and Beloin et al. (1988) used this modified Singh MPN technique to characterize the protozoa by depth at the Lula, OK site. Two boreholes were taken in January 1985 and then again in July 1985. The sediment samples were aseptically transferred into canning jars, transported to the laboratory where they were stored at 4°C. "Most" of the samples were analyzed for protozoa within 1 week after collection. The vertical profile at the Lula, OK site varied with depth below the surface: 0-3 m sandy loam; 3 m clay "confining layer" and the water table; >3 to 4 m sandy clay "interface zone"; 4-7 m sandy loam; 7-8 m sand and gravel; and 8-9 m "bedrock". [N.B., the regions of the vertical profile are an approximation of what Sinclair and Ghiorse (1987) illustrated and discussed.] When the confining layer was penetrated, ground water came up through the boreholes, indicating that a confined aquifer existed below 3 m.

At the surface, they reported small- and medium-sized flagellates, limax and filose amoebae (10^5-10^6 MPN/gdw) as well as ciliates (~2% of the total protozoan population).

No ciliates were found 0.5 m below the surface. Mostly flagellates were found in the unsaturated zone, decreasing in abundance with depth (from 10^3 to 10^0 MPN/gdw). At the interface zone, the number of flagellates and amoebae increased slightly (~10^2 MPN/gdw) before these populations dropped to zero where the clay content increased. No protozoa were found in the samples from 4-7 m. Flagellates and amoebae were found, however, in the gravel layer from 7-8 m (~10^1 MPN/gdw). Again, no protozoa were found in the bedrock layer.

Sinclair and Ghiorse (1987) concluded that there was a minimal pore space where the protozoa could no longer survive since no protozoa were found in the clay confining layer or bedrock regions. Two separate populations of protozoa existed at the site: the protozoa above the confining zone probably migrated from the surface; and the protozoa in the gravel layer probably were transported from a nearby river. They thought that the inconsistences between the population estimates at the same depth from samples taken in January and June were a function of changing hydrological or chemical parameters.

Finally, Sinclair and Ghiorse (1987) speculated on the role of the protozoa in the subsurface. In top soil environments where protozoa actively graze on bacteria, protozoa affect: 1) the regulation of bacterial densities;

2) bacterial species composition; and 3) bacterial mineralization processes, increasing their rate of degradation. However, the influence of protozoa on bacterial populations was thought to be minimal since the protozoan density was low and consisted mostly of cysts.

Beloin et al. (1988) found that generally regions where the protozoa densities were high other measures of microbial activity were also high (e.g., total and viable bacterial counts and ATP content). Sinclair and Ghiorse (1989) enumerated bacteria, protozoa, algae and fungi as well as recording soil texture, pore-water pH and metal concentrations with depth. They found the same trend in microbial abundance to the texture of the subsurface sediments in samples from Aiken, SC as in Lula, OK. Also, microbial populations correlated positively to pore water pH and negatively to pore water metal concentration. However, there was no apparent correlation between the types of microorganisms and depth. Similar results were also reported by Sinclair et al. (1990) for Nemaha, KS, but they did not detect any actinomycetes, fungi or algae.

Population estimates of protozoa with the Singh solid media MPN method often underestimate the total number of protozoa due to the selectivity of culturing conditions (Severtzova, 1924; Darbyshire et al., 1974; Elliott and Coleman, 1977; Sherr and Sherr, 1983). Also, the method may

be biased towards the enrichment of amoebae since the thin water film that has to be maintained on the agar may discourage the growth of ciliates and flagellates (Bunt and Tchan, 1955; C.R. Curds, personal communication, 1988). Foissner (1987) discourages reports of trophic protozoan populations based on indirect calculations from the total and encysted (i.e., acidified samples) MPN estimates because of the large variability of the technique.

The discrepancies between liquid media MPN and direct count estimations for protozoa have been more mixed. Caron et al. (1989) compared nanoflagellate (heterotrophic flagellates < 5 μm in size) population estimates by a direct count (DC) epifluorescent and Darbyshire liquid media MPN technique with seawater and marine snow collected throughout the North Atlantic and freshwater samples from three locations in Lake Ontario. In every type of sample, the MPN estimates "never constituted more than approximately 50% of the DC value". Neither of the techniques were consistent in enumerating nanoflagellates over an annual cycle at one of the sampling locations. However, ratios of MPN:DC enumeration estimates averaged for similar sampling environments "generally increased along an environmental gradient from oligotrophy to eutrophy". They found that the trend was positively correlated to the density of bacteria in the samples. The more bacteria present in the

environment the more bacterivorous nanoflagellates estimated by the DC vs. the MPN procedure.

Caron et al. compared the difference between the microtiter and direct count techniques to the "bacterial-count dilemma of marine bacteria reported by Jannasch and Jones" (1959). Underestimations of bacterial populations by the MPN technique were attributed to bacterial aggregates and bacteria associated with particulate matter, selectivity caused by the culture media and the presence of inactive or dead cells which could be enumerated with the DC method. These reasons were also found to be applicable to the bacterivorous nanoflagellates. MPN procedures were considered to be the lower limit estimate of the population since injured organisms would not culture and clumped organisms biased the results because they are not evenly distributed in dilution series. They found that slow steady growth of the natural biota of a sample resulted in higher MPN counts than with the addition of nutrient media or inoculations of bacteria. Direct counting may overestimate the protozoan population due to counting objects which are not alive or noneukaryotic. Despite the short comings of the MPN technique, the dilution method provided the only estimate of active vs. encysted forms due to acidification treatments. They concluded that the technique chosen for

enumeration was dependent on the organisms present in the samples and that more than one technique may be required.

In contrast, Fenchel (1982) found fairly close agreement between MPN (liquid media technique) and direct counting estimates of heterotrophic flagellates in estuarine and nearshore samples. Caron attributed the inconsistency between the enumeration techniques to the relative contribution of the flagellates to the heterotrophic nanoplankton community, and the trophic modes of the flagellate species affect the precision of the MPN estimates more than direct counting. Baldock (1986) found twice as many small flagellates in freshwater sediment samples with the Darbyshire liquid media MPN method than with counting cells on a filter. Darbyshire (1973) found significantly more soil protozoa (particularly flagellates) in samples prepared with the liquid media in his technique compared to those with the Singh solid media MPN.

Probably all techniques developed for studying soil microorganisms (bacteria or protozoa) detect only a portion of the entire community due to some discrimination of the procedure. Thus, Kieft and Rosacker (1991) recommended that more than one method should be used to characterize the protozoan population in the subsurface. In this dissertation, saturated subsurface sediment samples from the U.S.G.S. site have been enumerated for protozoa using

the epifluorescent, Darbyshire liquid media MPN and Singh solid media MPN techniques.

VI. STATISTICAL TECHNIQUES USED TO EVALUATE ENUMERATION TECHNIQUES

Many of the enumeration techniques discussed above have been evaluated to determine the variability associated with their estimate of protozoan populations as a function of their extraction method, culturing conditions and counting procedures. Some of the investigators used statistical techniques to consider the variability of the methods and to recommend procedures based on environmental conditions or microbial characteristics. These experiments will be discussed to demonstrate the application of methods and statistical techniques.

Results of field surveys conducted to elucidate causal relationships between the protozoan population and other physicochemical parameters will also be presented. These studies mention the conditions for the sterile sampling of the subsurface environment. Generally, the results have been summarized graphically (e.g., the number of organisms vs. depth of formation). Finally, this section will include a discussion of the statistical techniques that have been used in later chapters to determine the variability

associated with estimates of protozoan populations at the U.S.G.S. site.

A. Variability Within the Enumeration Techniques

In 1973, Uhlig et al. noted that the efficiency of the various enumeration methods for marine protozoa had only been evaluated qualitatively. Hence, they designed a study to compare 3 different separation techniques (decantation and sieving, elutriation, and seawater-ice extraction) conducted by eight groups of researchers on 4 different marine sands (fine, medium, coarse, and muddy sands). These samples were enumerated for ciliates and 7 taxa of metazoa. After they determined that the raw data had identical distributions (homogeneity of variance) and was normally distributed after log transformation, analysis of variance (ANOVA) was used to determine the variability associated with the concentration method and type of sediment.

Based on the results of the ANOVAs, Uhlig et al. (1973) concluded which separation methods were more suitable for certain sediment types and preservation techniques. They also qualitatively evaluated the techniques based on the time and expertise required to conduct the procedures. They found the technique that produced the largest quantity of ciliates with the least variability was the seawater-ice extraction. However, the technique was only efficient with

the medium sands (attributed to the "microporal structure") and was more time consuming than the elutriation technique.

Couteaux and Palka (1988) compared the Darbyshire liquid media MPN methods, direct counting of fixed material (in a Dolfuss dish) and a Millipore^{R} filtration method to enumerate ciliates in liquid cultures and humus forest soil microcosms. The results of enumerating trophozoites and cysts in liquid culture were assessed based on the 95% confidence intervals of the three methods: the Millipore^{R} filtration produced the highest number of ciliates/mL, followed by the direct count and finally, the liquid media MPN method. The change in osmotic pressure due to dilution with Volvic^{R} mineral water caused the trophozoites to lyse, and thus was considered to be the reason for the differentiation between the methods (especially since the organisms in the liquid media MPN method were diluted 12 to 15 times more than those observed by direct counting). However, there was no significant difference for the enumeration of cysts since there was an overlap of the 95% confidence intervals for all of the methods. Since cysts appeared to be resistant to the stress of dilution, their results were more consistent than those for the trophozoites.

ANOVA was used to determine the differences between the methods applied to the soil microcosms. There was no

mention of the determination of identical distributions nor power transformation of the data prior to statistical analysis. Highly significant differences (99% confidence interval) between the methods, microcosms and replicates of the microcosms were found for the enumeration of the trophozoites, but not the cysts. Again, the highest number of trophic ciliates were found with the Millipore^{R} technique, followed by the direct count and finally the liquid media MPN technique. Couteaux and Palka concluded that the Millipore^{R} filtration technique was "the best method tested for counting ciliates" considering the statistically significant difference between the methods and the number of ciliates enumerated by each method.

Caron et al. (1989) compared the Darbyshire liquid media MPN method (MPN/mL) to epifluorescent microscopy (protozoa/mL) for the enumeration of the populations of bacterivorous nanoflagellates (microflagellates) in seawater and freshwater samples. They used regression analysis to determine the population estimates of the epifluorescent counts and the log transformed MPN counts.

Although the densities of nanoflagellates spanned more than five orders of magnitude for the range of sample types examined, there was a significant positive relationship (r^2 = 0.79) apparent between the MPN and epifluorescent counts. A scatter plot of the log of MPN vs. epifluorescent counts

demonstrated that the regression line fell below a 1:1 correspondence between the two counting techniques. MPN counts never comprised more than 50% of the epifluorescent estimates. The slope of the regression line was 1.26, indicating that the MPN estimate constituted a larger fraction of the epifluorescent count at higher nanoflagellate densities than at lower densities.

B. Variability in Sampling the Subsurface

The Singh solid media MPN technique has been used by Sinclair and Ghiorse (1987 and 1989), Beloin et al. (1988), Sinclair et al. (1990) and Madsen et al. (1991) to enumerate protozoa in the subsurface. In all these investigations, protozoan populations were graphically compared to the subsurface depth from which the samples were taken. Sinclair and Ghiorse (1987 and 1989), and Beloin et al. (1988) were limited to only one sample per core due to the number of different analyses performed (e.g., enumeration of bacteria, adenosine triphosphate content and grain size analyses). In 1987 at the Lula, Oklahoma site, they found 10^2-10^5 MPN/gdw of protozoa in the first 0.5 m of sediment and less than 10 MPN/gdw of protozoa from 2 to 8 m below surface. The same decrease in protozoan population was found in the sites examined in Aiken, South Carolina (Sinclair and Ghiorse, 1989). Protozoa were only detected

at depths where the bacterial population was at least 10^4 CFU/gdw, but not all sites containing more than 10^4 CFU/gdw of bacteria had protozoa.

Estimates of the protozoan populations were also similar at sites in northeastern Kansas (Sinclair et al., 1990). Stepwise regression analysis was used to compare the population estimates to sediment type, number of types of protozoa, bacterial numbers (analyzed with plate counting and epifluorescent counts), number of bacterial colony types (plate counts only), ammonia, total organic carbon, and pH. The protozoan populations were found to be positively correlated only to the sediment type.

Madsen et al. (1991) investigated a site contaminated with buried coal tar and a nearby pristine site. The numbers of protozoa/gdw in the unsaturated and water table sediments were higher than those found at deeper locations at both sites. However, there were more than 400 MPN/gdw of protozoa in the plume's unsaturated zone and over 19,000 MPN/gdw of protozoa at the water table. Statistical analyses were not performed to compare these results with the bacterial populations or the concentrations of polyaromatic hydrocarbons. The authors felt the high protozoan population of the contaminated site compared with that observed in activated sewage sludges where predation by

protozoa on the bacteria simultaneously accelerates carbon cycling and increases biomass.

Federle et al. (1986) detected the presence of polyenoic fatty acids characteristic of eukaryotic microorganisms in samples in agricultural soils up to 3 m deep. The eukaryotic biomass was present throughout the cores in some of the sites tested. A series of stepwise multiple regression analyses was performed on the log transformed data to examine the influence of soil characteristics (e.g., % sand, % clay and cationic exchange capacity) on the classes of fatty acids derived from the phospholipids of the microbial community. The variability in the relative abundance of polyenoic fatty acids observed as a function of depth and site did not correlate with any combination of soil characteristics. The most predominant polyenoic fatty acid present in the cores was associated with fungi, although protozoa comprised a significant portion of the microbial community. The authors felt the technique of estimating the microbial community through the prevalence of fatty acids was less tedious, more independent of growth and more precise than other enumeration techniques. However, no work was done to directly correlate the quantity of fatty acids to the number of organisms present.

None of these studies included replicate core sampling, so no estimation of the variability associated with the

sites was possible. Correlations between protozoan populations and the whole microbial community or physicochemical parameters of the subsurface could be determined if the variability of the various enumeration techniques was better understood.

In order to obtain representative samples of subsurface sediments, contamination must be avoided during the drilling process. Experienced personnel operating the drilling equipment can prevent contamination from surface soils, water and drilling fluids (Ghiorse and Wilson, 1988). Drill rods and bits can be steam cleaned or autoclaved to prevent intrasite contamination from the drilling hardware (Phelps et al., 1989). However, drilling contamination within the site can only be prevented by the diligence of the drilling personnel to prevent sediment from falling back down the borehole. The use of drilling fluids depends on the depth of the borehole and the site's geological formation. Phelps et al. noted that contamination from the fluids is minimized by collecting a large sample below the level contaminated with the fluid and preventing the fluids from circulating through the core liner of the sampling device.

The sample variability can be reduced by collecting enough material to allow replication (Ghiorse and Wilson, 1988). Consequently, the region to be sampled must consist of similar sediments ~4 m thick (Phelps et al., 1989).

Sampling devices with core barrels can be used to preserve sediment sample integrity and facilitate disposal of potentially contaminated sections of the core. The top and sides of the core can be contaminated by particles and organisms from the overlying soil as the core barrel is pushed into the sediment. Sampling from the central portion of the core may reduce variability due to contamination (McNabb and Mallard, 1984). With the Waterloo corer, sectioning of the core in the field maintains the integrity of the sediment sample and reduces contamination from the transfer of the sample to another container (Zapico et al., 1987).

C. Statistical Methods for the Estimation of Variability of Subsurface Samples

In order to apply parametric statistics (e.g. regression analysis) to a sample population, the data sets must have equal variances and be normally distributed. The general linear regression model (ordinary least squares) takes the form:

$$E[Y_i] = \beta_o + \beta_1 X_{i1} + \beta_2 X_{i2} + \ldots + \beta_{K-1} X_{i,K-1}. \quad [2]$$

The $E[Y_i]$ denotes the expected value (population mean) of Y given the ith set of $X_1 \ldots X_{K-1}$ (independent estimates).

The number of parameters tested in the model is represented by "K". The coefficient on X_k, represented by β_K, equals the change in mean Y for each one unit increase in X_k as the other X variables remains the same. The actual Y value is the expected Y plus some random error (ε):

$$Y_i = E[Y_i] + \varepsilon_i. \qquad [3]$$

The assumptions about the ε include: 1) errors have identical distributions (i.e., mean=0 and equal variances), 2) errors are independent, and 3) errors are normally distributed (Hamilton, 1992).

An F-test can be used to determine if the variances are equal between two variables. Bartlett's test for homogeneity of variance can be applied to numerous variables (Rosner, 1982).

The distribution can be tested for normality by a variety of techniques. Graphical procedures such as histograms, box plots, quantile-normal and symmetry plots are fast methods of visually checking the distribution as well as the influence of outlying data points and checking the effect of power transformations on the data. Skewed data and the influence of outliers can generally be reduced with power transformations of the data. Distributions that are negatively skewed can approach a normal distribution

when the data is transformed with a power greater than one. Positively skewed data can be reduced to normal distributions using the logarithms or exponents less than zero. Once the data appears to be normally distributed, regression analyses and other parametric statistical techniques can be applied to the transformed data (Hamilton, 1992).

One technique for estimating the error associated with the components of a field test (components of variance) is the nested or hierarchical design (Gill, 1978; Box et al., 1978). The following discussion will use the terminology of Box et al., however Gill presents a more comprehensive review of the design. Assigning components of variance theory involves stages of an experiment where batches (e.g., the field site) are sampled (e.g., cores at a site) and tests are performed on the subsamples (e.g., replicates of the core). The deviation of an analytical result Y from the sample mean is called the analytical test error, ε_T; the deviation of the sample mean from the batch mean is called the sample error, ε_S; and the deviation of the batch mean from the process mean is called the batch error, ε_B. All errors are assumed to have zero means represented from independent tests with normal distributions having fixed variances of $\hat{\sigma}_T^2$, $\hat{\sigma}_S^2$, and $\hat{\sigma}_B^2$. The total number of analyses

would be the number of batches times the number of samples times the number of tests (B*S*T).

The components of variance are calculated through the sum of squares and mean squares of the different stages. If there are T replicated observations on each sample, then there are Y_{bs1}, Y_{bs2},..., Y_{bst} replicated Y analyses made on the sth sample of the bth batch. The estimate of the testing variance, $\hat{\sigma}_T^2$, is defined as:

$$V_T = \frac{\Sigma_b^B \Sigma_s^S \Sigma_t^T (Y_{bst} - Y_{bs})^2}{BS(T - 1)} \qquad [4]$$

where Y_{bs} is the mean of sample averages and V_T is an estimate of $\hat{\sigma}_T^2$ having BS(T - 1) degrees of freedom. The estimate of the sampling variance, $\hat{\sigma}_S^2$, is based on the S sample average, Y_{b1}, Y_{b2},...,Y_{bs}, of the bth batch:

$$V_S = \frac{\Sigma_b^B \Sigma_s^S (Y_{bs} - Y_b)^2}{B(S-1)} \qquad [5]$$

where Y_b is the mean of batch averages and V_S is an estimate of $\hat{\sigma}_S^2 + \hat{\sigma}_T^2/S$ having B(S - 1) degrees of freedom. Finally, in the three stage hierarchical design, the estimator of the batch variance is the B batch averages:

$$V_B = \frac{\Sigma_b^B (Y_b - Y)^2}{(B-1)} \qquad [6]$$

where Y is the average of all Y_{bst} observations and V_B is an estimate of $\hat{\sigma}_B^2 + \hat{\sigma}_S^2/S + \hat{\sigma}_T^2/ST$ having (B - 1) degrees of freedom. Therefore, the components of variance themselves are estimates of V_T, V_S and V_B:

$$\hat{\sigma}_B^2 = V_B - \left[\frac{\hat{\sigma}_S^2}{S}\right] - \left[\frac{\hat{\sigma}_T^2}{ST}\right] \qquad [7]$$

$$\hat{\sigma}_S^2 = V_S - \left[\frac{\hat{\sigma}_T^2}{T}\right] \qquad [8]$$

$$\hat{\sigma}_T^2 = V_T \qquad [9]$$

This model can be expanded to include more stages (Gill, 1978). Although hierarchical designs do not appear to have been used in microbial investigations of the subsurface, the design was used for the protozoan sampling program at the U.S.G.S. site.

VII. SUMMARY

As noted in this literature review, there have been a number of techniques developed to enumerate soil protozoa.

However, estimates of protozoan populations in the saturated subsurface environment have been limited to the Singh solid media MPN method. These studies have also not been designed to examine their variability within the subsurface environment, nor have they used more than one type of enumeration technique. The objectives of this dissertation research were to evaluate three different enumeration procedures for protozoa in sediment samples, and to determine the variability associated with the enumeration and sampling techniques.

CHAPTER 3

METHODS AND MATERIALS

The methods used to enumerate protozoa in sediments required the separation of the organisms from the soil particles followed by cultivation or direct counting. This section first describes the development of enumeration procedures for protozoa in saturated sediments that were tested on sands and subsurface material from 1990 until Summer 1991. In Fall 1991, a sampling program was designed to examine: 1) the variability associated with the enumeration techniques; 2) the effect of holding time between sampling and analysis; 3) the treatment of core sleeves for retrieving representative sediment samples; 4) the intrasite variability; and 5) the differentiation of trophic vs. encysted protozoa by the two MPN enumeration methods. Finally, the design of downwell samplers used to enumerate protozoa in ground water monitoring wells will be discussed.

I. DEVELOPMENT OF RELIABLE ENUMERATION TECHNIQUES FOR SEDIMENT SAMPLES

The methods and materials are presented here in the chronological sequence in which the research on sediment samples was conducted. Procedures to separate protozoa from

sediments were screened at the Natural History Museum (London, UK) during Winter 1990. The seawater-ice extraction (Uhlig, 1964, 1968), centrifugation (Hopkins et al., 1991a) and shaking (Dye, 1979) were evaluated and compared to determine the technique that caused the least harm to the organisms while removing them from the sediment particles. Subsequently, all sediment samples were extracted using the shaking technique.

Protozoa in sediment samples collected at the U.S.G.S. site were enumerated using three procedures: the Darbyshire liquid media MPN method, Singh solid media MPN method and a filtration technique with epifluorescent staining. The procedures discussed were used on cores collected during Fall 1990 and 1991.

A. Evaluation of Separation Techniques

Several different extraction techniques were tested at the Natural History Museum using thixotrophic sands (coarse, saturated sand easily dewatered when a load is applied). The sand was collected on February 8, 1990, as high tide retreated from the beaches at Castle Point, Canvey Island on the Thames River, UK. This site was chosen since the location was convenient and had previously been surveyed for protozoa (Carey, 1986). The salinity of the water was 25 ppt (measured using a temperature-compensated

refractometer; Optical Corp., Keene, NH). The sand was placed in a holding tank designed by Carey (1986) where artificial seawater (25 ppt salinity; Instant Ocean, Aquarium Systems, Sarrebourg, France) was continuously recirculated through it using an aquarium pump (420 L/h; Fluval 202, R.C. Hagen Corp., USA), an undergravel filter (Rena, Annecy, France; 180 L/h; Eheim 1007, G. Eheim Inc., Germany), and a counter-current protein skimmer (Sander WT250, E. Sander Elektronapparat., Germany). The sand was maintained in the holding tanks for two months and the protozoan species diversity remained constant throughout that time. [N.B., Carey found that ciliate communities survived for up to six months in the system.]

1. Seawater-Ice Extraction

The seawater-ice extraction procedure developed by Uhlig (1964, 1968) was used to remove protozoa from a core of sand taken from the holding tank (Figure 7). An artificial seawater solution of 32 ppt was prepared, frozen in an ice cube tray and then crushed prior to preparation of the sand sample. A 60 mL syringe (Becton-Dickinson & Comp., Rutherford, NJ), with its tip cut off and its plunger pulled 8 cm up into the barrel, was forced into the sand in the holding tank. A sample was removed by applying suction with the plunger and pulling the syringe from the tank. The sand

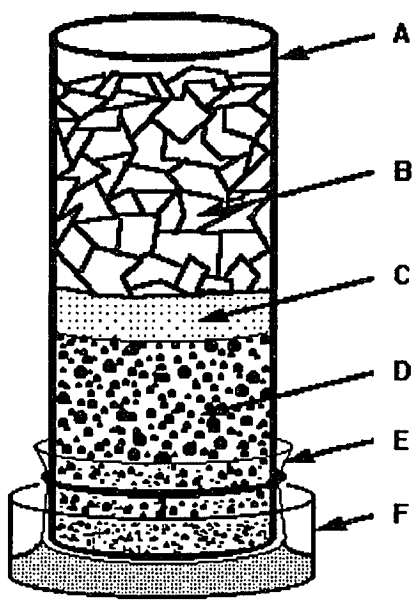


Figure 7: Apparatus for the separation of protozoa in sands from Canvey Island, U.K., using the Uhlig seawater-ice extraction technique. A plastic tube (A) holds crushed, melting seawater-ice (B) layered over cotton batting (C) and the sand sample (D). The salinity gradient established by the melting seawater-ice encourages the interstitial organisms to move downward, through the sand and nylon mesh (E), and into the liquid in the petri dish (F).

was immediately transferred to an acrylic plastic tube (3.5 cm I.D., 20 cm long). The bottom of the tube was covered with a piece of nylon mesh (140 μm mesh size; Lockertex, Warrington, Cheshire, UK), held in place with a rubber band. The end of the tube was then placed (~2 mm) above the bottom half of a small plastic petri dish (50 x 9 mm, sterile; Gelman Sciences Inc., Ann Arbor, MI). The tube was held upright by a ring stand. A piece of sterile cotton batting was placed on top of the sand (~2 cm deep in the plastic tube) and the tube was filled with ~10 cm of crushed seawater-ice.

As the ice melted, liquid collected in the petri dish. The height of the tube was adjusted so that the mesh remained in slight contact with the liquid in the dish, forming a meniscus between the liquid layer and the mesh. After ~3 mL of liquid was collected in the petri dish (enough to cover the bottom of the container), a new dish was placed under the sample. The salinity of the liquid eluting from the sand was checked with a refractometer before each dish was replaced. When the salinity became >25 ppt, the extraction was halted. The eluted liquid was examined for protozoa using a zoom dissecting microscope (0.7 to 45 X magnification; Olympus SZH Stereo Microscope; Olympus Optical Co., Ltd., Tokyo, Japan). The seawater-ice extraction produced a great amount of liquid (~20 mL) and

the examinations were tedious because of the volume collected, the low magnification (45 X) of the dissecting microscope and the small size of the protozoa. This was especially true for the flagellates (5-20 μm in size).

2. Centrifugation

The first test of centrifugation to concentrate protozoa in a small volume was tried on the liquid collected during a seawater-ice extraction. Approximately 5 mL of liquid was centrifuged (500 x *g*; GS-6 Beckman Centrifuge; Beckman Instruments, Scientific Division, Irvine, CA) in a glass, conical, 15 mL centrifuge tube (Corning Laboratory Sciences, Corning, NY) for 2 min. The liquid above the pellet was removed using a Pasteur pipet attached to the end of a tygon tube connected to an aspirator on a flowing, water faucet. In this manner, the liquid surface was continually vacuumed off as the tip of the pipet was lowered. The final 2 mL of sample was examined for protozoa under the dissecting microscope.

During a visit to the Department of Agricultural and Environmental Science, The University, Newcastle upon Tyne, UK, Dr. David W. Hopkins demonstrated his dispersal and differential centrifugation technique. The procedure was developed primarily for soil bacteria (Hopkins et al, 1991a). 5g of sand was shaken in a 10 mL solution of 0.1%

sodium cholate for ~30 sec, then transferred to a 50 mL centrifuge tube containing 10 mL sodium cholate solution, 10 mL of Na^+ form chelating resin and ~30 glass beads (3-4 mm diameter). This mixture was then shaken on a wrist-action shaker for 2 hr at 5°C. The sample was centrifuged at 500 x *g* for 2 min before the supernatant was decanted and observed under a dissecting microscope. Hopkins' procedure normally includes five more centrifugation steps on the pellet and resulting supernatants, but these steps were not tried on the sand sample since no protozoa were observed in the first fraction, which typically contains the most organisms.

3. Shaking Technique

Dye's (1979) shaking technique was also tested on the Canvey Island sand. 5 g of sand was added to a test tube containing 10 mL of 0.2 µm filtered, artificial seawater. The test tube was gently shaken up and down for 30 sec, so that the sand was continuously turned over in the liquid. The liquid was decanted into a petri dish and the process was repeated four more times. Each fraction was observed under the dissecting microscope for the presence of protozoa. The organisms could be maintained in the dishes for over a week at room temperature by daily addition of ~2-3 mL of filtered, artificial seawater (25 ppt; collected from the protein skimmer of the holding tanks). A

sterilized barley seed was added to some dishes to maintain some species of psammophilic (interstitial) ciliates and flagellates for observation and identification.

4. Comparison of Extraction Techniques

Uhlig's seawater-ice extraction, Hopkins' centrifugation procedure and Dye's shaking technique were compared using the Canvey Island sand. Three samples were removed from the holding tank with a modified syringe (as described for the seawater-ice extraction). Each sample was extracted using a different separation technique. The liquid fractions collected by all three methods were examined using the dissecting microscope. The three extraction procedures were evaluated based on: 1) the total number of organisms, 2) the type of protozoa (flagellates, amoebae or ciliates) collected, and 3) the organisms' physical condition after extraction (i.e., the number of broken or unrecognizable cells).

B. Enumeration Methods for Subsurface Core Samples

The Waterloo corer was used by the U.S.G.S. drill crews to collect the saturated subsurface sediments at the U.S.G.S. site in Cape Cod, MA. The methods for drilling and recovery of representative saturated, sediment samples are discussed in Chapter 1. All cores were stored vertically

(oriented in the same direction as they were in the subsurface) in a refrigerator (4°C) until they were sampled. [N.B., during transport, cores were kept in a cooler with frozen ice packs.] The holding time of the cores prior to sampling was investigated.

The procedure for sampling was developed to limit the removal of sediment from potentially contaminated areas within the core. The lower to middle sections of each core were sampled because the cores were stored vertically and these parts remained saturated during storage. The first 3 cm of the core was discarded since the end was exposed and potentially contaminated when the core was being sectioned in the field. A flame-sterilized, stainless steel spatula was used to remove only the central material from the core. Sediment touching the side of the aluminum core sleeve was not used since the sleeve was not sterilized prior to sampling the subsurface. Also, while collecting the samples at the U.S.G.S. site, sediment from depths closer to the land's surface could have been caught in the core and contaminated the sleeves. An experiment was performed to study contamination by comparing cores taken with untreated and ethyl alcohol-cleaned sleeves.

The shaking technique (Dye, 1979) was used for the extraction of microorganisms from cored material. 5 g of sediment from the central portion of a core was weighed

(Mettle P1200N Balance, Mettler Instrumentation Corp., St. Louis, MO) into a Whirlpak^{R} bag (114 mL). 25 mL of phosphate buffer (2.2 mM KH_2PO_4 and 4.02 mM K_2HPO_4 (Sinclair and Ghiorse, 1987)) was added immediately to the bag and the contents were shaken gently for 30 sec before the liquid fraction was decanted into a second bag. The process was repeated twice more until approximately 75 mL was collected in the second bag. The extracted samples were enumerated with epifluorescence microscopy and Darbyshire liquid media MPN method.

Dry weight conversions (i.e., moisture content) were calculated for all cores sampled to correct the counts of protozoa to gram dry weight (gdw). Empty beakers were labeled with the core location and depth, and their weights were recorded. Approximately 50 g of sediment was transferred to each beaker and the wet weight was recorded. The sediment was taken from the same region of the core that appeared to have the same moisture content as the sample for enumeration. The beakers were covered with foil and dried for 3 days in a drying oven set at 103^{o}C. The samples were removed from the oven and placed in a desiccator to cool before removing the foil and recording the dry weight of the samples. Calculations of dry weight conversions are discussed in Appendix A and were used with all population estimates for sediment samples.

1. Epifluorescent Enumeration of Sediment Samples

The epifluorescent enumeration procedure can be divided into: 1) types of epifluorescent stain; 2) filtration; 3) counting techniques; 4) preparation of sterile controls; and 5) calculation of protozoa/gdw. Three different epifluorescent stains have been used on samples from the U.S.G.S. site: acriflavin and DAPI have been used for enumerating protozoa from core samples; and Hydroethidine^{R} has been used to trace flagellates during transport experiments (both in laboratory column and field tests (Harvey et al., 1992))(see Appendix C). Preparation of filters are the same for all samples, but two different types of filtration apparati are described and their use depends on the number of samples to be filtered simultaneously. Filters were enumerated by counting fields or scanning. Either counting method could be used with any type of epifluorescent stain, although scanning was preferred for low concentrations of protozoa. An experiment to determine if the two methods were comparable will be discussed. Preparation of sterile controls is important for determining the presence of contaminants. Calculations for determining protozoa/gdw from the observation of filters will be discussed and examples given in Appendix A.

a. Epifluorescent stains. Two different epifluorescent stains were used during the enumeration of protozoa in cores from the U.S.G.S site. Acriflavin was the first stain applied to cores collected during Spring 1990. Based on the recommendation of Dr. David A. Caron (Woods Hole Oceanographic Institution, Woods Hole, MA), all subsequent samples were stained with DAPI.

i. Acriflavin staining. Acriflavin (Aldrich Chemicals, Milwaukee, WI) stock solution was prepared according to Bergström et al. (1986), by dissolving 0.022 g in 100 mL of 0.22 µm filtered, Milli-Q^R water (Milli-Q Standard Water Systems, Millipore Corp., Bedford, MA). The stain was stored in a dark (foil-covered), glass bottle in the refrigerator for $\leq$ 1 month.

The liquid collected from the shaking procedure was fixed for 30 min with 0.2 µm filtered formalin (37% formaldehyde, reagent grade; VWR, Boston, MA) to a final concentration of 0.1% (v/v). 5 mL of the formalin-fixed sample was transferred to the filtration apparatus with an adjustable pipettor (1-5 mL capacity; Fisher Scientific, Springfield, NJ). A vacuum <13 cm Hg (5 in. Hg or 13.30 Pa)(Caron, 1983) was applied with a vacuum pump (Gast Vacuum Rotary Pump, ¼ hp, model #0322, Emerson Motor Division, St. Louis, MO). As soon as the liquid was drawn through the

filter, the vacuum pump was disconnected from the apparatus. 2 mL of acriflavin stock was slowly added on top of the filter so that the particles collected on the filter were not disturbed. After 5 min of staining, the excess dye was removed with the vacuum pump.

Acriflavin stained filters were observed with a Nikon Optiphot^{R} light microscope equipped with epifluorescence (Nikon Episcopic-Fluorescence Attachment EF-D; B-2E combination filter with 510 nm dichroic mirror, 450-490 nm excitation filter and 520-560 nm barrier filter; 100 W high pressure Hg lamp; Osram, Germany). While the stain is suppose to be DNA-specific (Bergström et al., 1986), both DNA-acriflavin complexes and non-DNA material fluoresced green under epifluorescent light. Protozoan cells were distinguished from other fluorescing particles by their size, shape and/or the presence of internal organelles or flagella.

ii. DAPI staining. The DAPI (4',6-diamino-2-phenylindole; Sigma Corp., St.Louis, MO) stock solution was prepared using the concentration recommended by Rogerson (1988) for the examination of marine gymnamoebae (1 mg of DAPI in 20 mL of 0.22 µm filtered, Milli-Q^{R} water, or 2.5 µg DAPI/mL of sample). The stock was stored in a dark, freezer (0^{o}C) for up to 1 month.

Typically, 3 mL of the shaken extract of a sample was aseptically transferred to a test tube (18 mm O.D., 150 mm length, glass; VWR, Boston, MA) using an adjustable pipettor. However, if the shaking extract was turbid, the volume of sample was decreased and sterile water (121°C, 15 min) was added to adjust the total volume filtered to 3 mL. Samples were then fixed with an appropriate amount of filtered (0.22 µm, 45 mm, DM Metricel^{R} membrane filter; Gelman Sciences Inc., Ann Arbor, MI), 10% glutaraldehyde (stock = 25% glutaraldehyde, reagent grade; J.T. Baker Chemical, Phillipsburg, NJ) and 0.1 M cacodylic acid (sodium salt; Sigma Chemicals, St. Louis, MO) buffer (pH 7.0) so that the final concentration of glutaraldehyde was 1% (Caron, 1983). After a 20 min fixation time, 50 µL of DAPI stock was added for every 1 mL of sample in the test tube and the sample was stained for 10 min. Thus, for the above example of 3 mL sample volume, 0.300 mL of 10% glutaraldehyde solution and 0.150 mL of DAPI stock would be added to the sample.

The DAPI samples were observed at 400 X magnification on the Nikon Optiphot^{R} light microscope with the UV-1A or UV-2A combination filter (400 nm dichroic mirror, 365 nm excitation filter, 400 barrier filter; and 400 nm dichroic mirror, 330-380 nm excitation filter, 420 barrier filter, respectively). The intensity of the DAPI fluorescence was

observed to diminish with use of the Hg vapor lamp and Nikon^{R} recommends that the lamp be replaced after 200 hr (D. Yetman, personal communication, 1990). Color, size, shape and/or the presence of internal organelles or flagella distinguished the protozoa from other material. DAPI stained protozoa appeared blue and non-DNA, DAPI-bound material appeared yellow.

b. Filtration. Two different kinds of filtration apparatus were used in the preparation of the epifluorescent filters. A glass filter holder (16 mm I.D.; Nuclepore^{R} Corp., Pleasanton, CA) was used when only one filter needed to be prepared. For up to 12 simultaneous filtrations, the multiple port filtration apparatus was used (18 mm I.D.; Millipore, Bedford, MA). When fewer filtrations were required, the ports not being used were sealed with rubber stoppers.

Preparation of the filtration apparatus involved placing a backing filter on the support disk, then wetting it with a mild detergent (one drop of Triton X-100 (Alpkem Corp., Clackamas, OR) in 20 mL of deionized water). Two different kinds of backing filters were used. Metricel^{R} filters (0.45 µm, 25 mm; Gelman Sciences Inc., Ann Arbor, MI) were preferred to GF/C Whatman^{R} filters (25 mm; VWR, Boston, MA) since they did not disintegrate as quickly. Typically, the

Metricel^{R} backing filters can be used for up to 3 filtrations before being replaced.

A black, polycarbonate (PC) membrane filter (0.8 μm, 25 mm; Costar Products, Cambridge, MA) was placed on top of the wet backing filter with forceps. The sides of the PC filters are different and they are shipped with their dull side up. Typically, the dull side has been used as the filtration surface. However, Nuclepore^{R} recommends that the shiny side be used because some imperfections from the manufacturing process may be observed when the filters are used with the microscope (J. Saurer, personal communication, 1992). When prestained black filters were not available, the filters were stained with irgalan black (Porter and Feig, 1980). However, the background of these filters was not always as dark when examined microscopically as those prestained by Nuclepore^{R}.

The samples were drawn through the filters using a vacuum (≤13 cm Hg). As soon as all the liquid was drawn through the filter, the vacuum was stopped. In the case of the multiple filtrations, when the liquid disappeared, the port was covered tightly with a rubber stopper. For sample volumes ≤3 mL, all ports were usually finished within 1 min of each other.

The PC filter was separated from the backing filter with forceps, placed (sample side up) on a tissue paper and

allowed to air dry. Generally, fine-tipped forceps were easiest to use for this process. A drop of low fluorescence immersion oil (Cargille^{R} A; Fisher Scientific, Springfield, NJ) was placed in the center of a labeled microscope slide (25 mm x 75 mm, frosted end; VWR, Boston, MA). The dry PC filter was placed on the slide using a round-tipped forceps. The underside of the filter was covered with immersion oil by moving the filter over the surface of the oil droplet several times. Then the coverslip (25 mm x 25 mm, No.2, VWR, Boston, MA) was placed over the top of the filter (no additional oil was necessary). DAPI and acriflavin stained filters were stored in slide boxes in a refrigerator (4^{o}C) for up to 1 month before observation.

c. Methods of counting. Two different counting techniques were used to enumerate the epifluorescent filters: 1) counting fields; and 2) counting scans (with microscope stage brace). An experiment will be discussed that was designed to test: 1) the assumption of evenly distributed particles across the counting areas; and 2) significantly different enumerations by the two counting techniques. The experiment was conducted with fluorescing microspheres (MS).

i. Counting fields. Fields on the filter were examined for protozoan cells (or MS) at 400 X magnification. The fields were chosen by moving the microscope stage randomly (re: haphazardly) around to locate another field within 2 mm of the edge of the filtration area. The number of fields to be observed per filter was determined by the desired confidence interval for the estimate of protozoa/gdw. With sediment samples, fields were examined until $\geq$70 cells were observed (Cassell, 1965; Griffiths and Ritz, 1988). However, a minimum of 20 fields or 200 cells were examined per filter for samples with higher populations than those found in the sediment. [N.B., Fenchel (1982), Bergström et al. (1986) and Harvey (personal communication, 1990) have used this procedure for epifluorescent counts of bacteria.] Only protozoa (or MS) entirely within the field of vision were counted.

ii. Scanning. Filters were scanned at 400 X magnification, and only the protozoa (or MS) observed entirely within the field of vision were counted. Since the center of the filter was thought to have a more even distribution of particles than the edges, an aluminum brace was designed for the scanning technique that prevented the viewing of the outer 2 mm of an 18 mm filtration area (Figure 8).

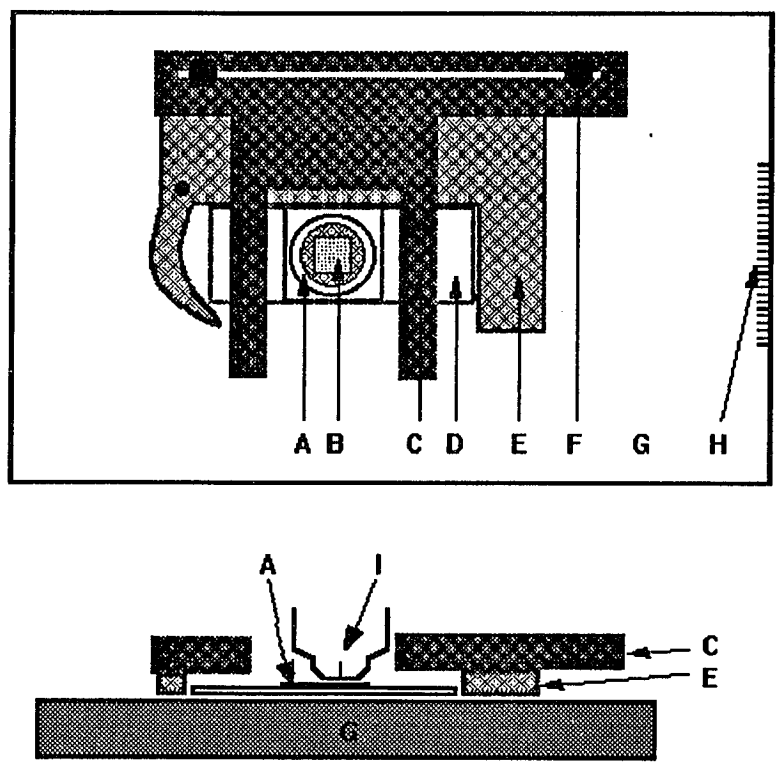


Figure 8: Brace (C) used on the microscope for the enumeration of epifluorescent samples using the scanning technique, in plan and cross-section. The epifluorescent filter (A) mounted on a slide (D) is placed in the slide holder (E). The brace is placed over the slide holder and attached to the microscope stage (G) with screws (F). The brace prevents the objective (I) from viewing the left, top and right edges of the filter. The starting point of the scan was chosen randomly within 10 mm from the back of the brace as measured by the micrometer (H) on the stage. Thus, only the central region of the filter (B) could be scanned.

The brace (Figure 8C) was attached to the top of the slide holder (Figure 8E) of the microscope's stage (Figure 8G) with the holder's screws (Figure 8F). The sample slide (Figure 8D) was placed into the slide holder. Then the brace was centered over the top of the filter and the screws were tightened to hold the brace and slide holder on the stage.

The dimensions of the brace prevented the objective (Figure 8I) from viewing the left, top and right edges of the filter. The length of the scan was the distance the objective traveled between the sides of the brace, which was 11.2 mm. Since the brace did not restrict the viewing of the bottom edge of the filter, the starting point for the scan was chosen to be within 10 mm from the back of the brace. A random numbers table (Koopmans, 1981) was used to determine a number between 0.0 and 9.9. That number was then measured on the stage's micrometer (Figure 8H) to locate the objective along the side of the brace for the starting point of a scan. Thus, only an area of the filter equivalent to a rectangle with the dimensions of 11.2 x 10 mm (Figure 8B) could be viewed. The scan's width depended on the diameter of the field in the microscope. Three scans were made per filter.

iii. Counting experiment. All solutions of MS were prepared by pipetting the stock solution into a VOA vial containing Milli-Q^R water. The theoretical concentration of MS/mL was calculated for 6.49 μm, Fluoresbrite^R yellow-green, plain MS (Polysciences^R, Warrington, PA) by:

$$\frac{MS}{mL} = \left[\frac{6W(10^{12})}{\rho\pi\phi^3}\right] D \qquad [10]$$

where W was 0.025 g for the 2.5% latex in the spheres; density, ρ, of the polymer was 1.05 g/mL; the diameter, ϕ, of the spheres was 6.49 μm; and the dilution factor, D, was dependent on the sample (Polysciences, Warrington, PA). The first dilution was prepared by shaking the stock bottle and then gravimetrically pipetting 0.102 g of MS stock into 40.026 g of water. Again, the solution was shaken and then 0.999 g of the first dilution was pipetted into 30.040 g of water. Four filters were prepared (according to the procedures outlined above) by filtering 5 mL of the second dilution. Theoretically, there were 1.45×10^4 MS/mL on the filter.

The filters were enumerated using the two types of counting methods. Counting fields was done by the same procedure described for sediment samples (i.e., enough fields to count >70 MS/filter). Scanning was accomplished by observing the area between two lines drawn on the

coverslip. The lines were produced with a fine tipped lab marker (VWR, Boston, MA) and were ~10 mm apart. The starting point of each scan was determined using the same technique as described for the brace. Four slides were counted per filter at 400 X magnification.

d. Sterile controls. The sterile controls were very important for the determination of contaminants and interferences with the epifluorescent procedure. At least one sterile control was prepared each sampling day. Sterile phosphate buffer was shaken in Whirlpak^{R} bags and then 5 mL of the liquid was fixed, stained and filtered following the procedures outlined above for the appropriate stain.

Contaminants and interferences observed on the filters determined if the integrity of the epifluorescent samples had been compromised. Although protozoa have never actually been observed on the sterile controls, objects which were similar in size, shape or color have been found. Such objects have been attributed to the agglutinization of glutaraldehyde, and residues in the sterile bags (used during the shaking technique) and on slides and coverslips. Background fluorescence from moisture in the immersion oil can be reduced with longer exposures to the UV light. If contaminants were observed, new samples were prepared when possible.

e. Calculation of protozoa/gdw. Calculations of protozoa/gdw differed depending on the procedure used to make the filter and how the filter was enumerated. Protozoa/filter were calculated from either the number of protozoa observed per field or per scan. The size of the filtration area on the filter and the diameter of the field of vision also had to be known. The volume filtered and the dilution due to the shaking technique were used to calculate protozoa/mL. Finally, the moisture content of the sediment was used to convert to protozoa/gdw. Examples of calculations from the various parameters are in Appendix A.

2. MPN Enumeration of Sediment Samples

Two types of extinction-dilution MPN techniques were tested on the subsurface sediment: Darbyshire liquid media MPN and Singh solid media MPN methods. Core sediments were extracted using different shaking techniques. The extracted liquid was sampled to estimate the total protozoan (trophic + encysted) population, and then acid treated to estimate the encysted protozoa. The population estimates were the most probable number (MPN) based on the statistical method developed by Fisher (1922).

a. Darbyshire Liquid Media MPN Method. The Darbyshire liquid media MPN method can be divided into: 1) preparation

of microtiter plates; 2) sample preparations; 3) incubation of plates; 4) analysis of plates; and 5) calculation of MPN/gdw. The general procedures were described by Darbyshire (1973) and his associates (1974) with some modifications for the use of a computer program to calculate MPN/gdw. An experiment to determine if the enumeration technique required a supplemental source of bacteria will also be discussed.

i. Preparation of microtiter plates. The Darbyshire method involves the dilution of the extracted liquid through a series of wells in a microtiter plate (6.4 mm bottom diameter, 10.8 mm depth, 96 well, flat bottom, tissue culture treated, sterile; Corning Laboratory Science, Wexford, PA). The diluent for the Darbyshire liquid media MPN method was the same phosphate buffer used during the extraction of the sediments with the shaking technique. Since the concentration of protozoa in the sediments was not known, one plate was used per sample for the maximum number of 12 dilutions (i.e., for a projection of ~10^7 protozoa/mL in wells in the first row). Preparation of the microtiter plates involved filling 11 of the 12 rows of wells with sterile buffer using a 200 µL Octapette^R (Costar, Cambridge, MA) equipped with 200 µL pipet tips (yellow, 96 per box, sterile; VWR, Boston, MA). The first row of wells was left

empty. The lid of the plate was opened as little as possible while filling the wells to help prevent any particles in the air from contaminating the samples. Since the plates were sterile, they could be filled and kept cool for hours in advance of sampling as long as the diluent volume did not change (e.g., through evaporation). Pipet tips were reused after they were soapy water washed, reorganized in their boxes, sterilized (121^{o}C, 15 psig, 15 min), and dried in a drying oven (60^{o}C for 12 hr). A box of 200 µL pipets (96 pipet tips per box) was required to dilute all the wells of a microtiter plate.

ii. Sample preparation. On each sampling day, three types of samples were prepared for incubation: 1) plates for the estimation of total MPN/gdw; 2) plates with diluted acidified samples for the estimation of encysted MPN/gdw; and 3) sterile controls. All the microtiter plates were prepared as described above.

Just prior to subsampling, the bag containing the extracted liquid from the core was shaken to resuspend all particles. Approximately 10 mL of the liquid was poured into a sterile petri dish (100 x 15 mm, plastic, sterile; VWR, Boston, MA) (i.e., enough liquid to cover the bottom of the dish). The bag was then resealed until it was used for the acidification process. [N.B., the bag was stored at

room temperature since the next sample was started in ≤10 min.] The box of pipet tips was opened, the first row of pipets was attached to the 200 µL Octapette^{R} and withdrawn from the box, which was then closed. Sample was removed from the petri dish by putting the pipet tips into the liquid, sucking and then discharging the liquid three times to mix the sample, before transferring 200 µL into each well in the first row of the microtiter plate. A 50 µL Octapette^{R} was used to transfer 50 µL of the sample from the first to the second row of wells. Again, this was done by pulling the liquid into the pipet and discharging three times to mix the buffer and sample, before transferring into the next set of wells. The process was continued with the 50 µL pipettor until all wells in the plate were serially diluted. Great care was taken to draw liquid slowly into the pipet tips to prevent the Octapette^{R} from becoming contaminated. [N.B., pipet tips with acetate filters are now available that would prevent liquid from entering the Octapette^{R}.]

The extracted liquid remaining in the bag was acidified with 10 mL of 0.5 N HCl, lowering the pH to ~2. After 30 min, the pH was neutralized with 0.5 mL of 1 N NaOH. Then, the same procedures were followed to prepare a second microtiter plate with the acid treated sample.

On every sampling day, a sterile control was prepared. Sterile phosphate buffer was treated the same as sediment samples. The microtiter plate with the sterile buffer was incubated the same amount of time as other samples prepared on the same day. At least one well per row in the microtiter plate (for a total of 12 wells) was examined for the presence of protozoa. No acid treated sterile controls were evaluated.

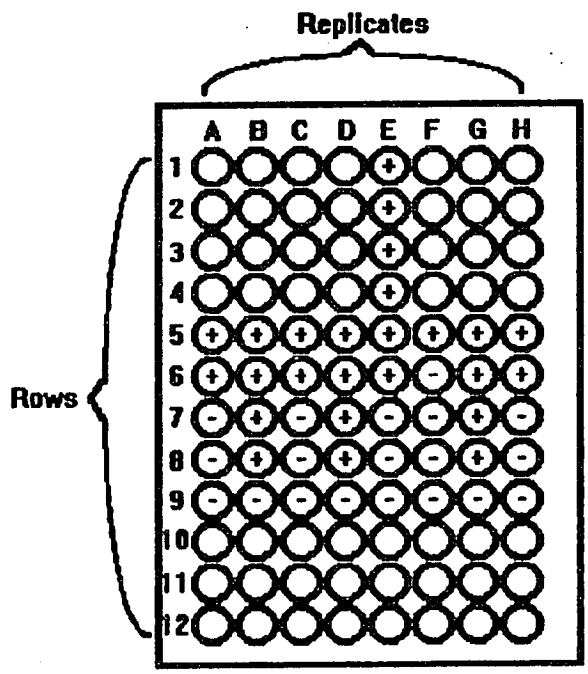
iii. Incubation of plates. All of the microtiter plates were incubated for 12 days in a 12°C (~temperature of the ground water when sample was collected) refrigerator (Precision Scientific, GCA Corp.,). A beaker of water was kept inside the refrigerator to provide humidity and prevent excessive evaporation during incubation. The plates were removed from the refrigerator and kept in the dark until they reached room temperature. Then they were examined at 300 X magnification using a Nikon Diaphot-TDM^R inverted microscope (Nikon Kogaku K., Garden City, NJ) equipped with Hoffman modulation contrast optics (Hoffman Modulation Contrast System, Modulation Optics Inc., Greenvale, NY).

iv. Analyses of plates and calculation of MPN/gdw. Positive protozoan activity in a well was determined by looking for characteristic motion, size and shape of

protozoa. Typically, the first rows of wells had protozoa in each replicate (i.e., all 8 wells in a row) and the last rows had no signs of activity (Figure 9).

A search pattern was designed to minimize the number of observations required for the MPN calculation. Instead of observing all 96 wells in a plate, a replicate well in the first row (dilution factor = $1.00x10^0$ in Figure 9) was oriented on the inverted scope so that the whole width of the well could be scanned (replicate E in Figure 9). A well was scanned from one edge to the other at least three times to determine if protozoa were present. Once positive activity in a well was confirmed, the same replicate well in the second row was searched (row 2, replicate E in Figure 9). The comparable well in each row was examined until a well that had no protozoa was found (row 9 in Figure 9). Then all 8 replicate wells in that row were observed for the presence of protozoa. The search pattern was expanded until the series of rows where all the replicates had positive (+) activity and all the replicates had negative (-) activity were completely observed (rows 5-9 in Figure 9).

A computer program was used to calculate the MPN/mL of sample (Hurley and Roscoe, 1983; Arnold, 1992). The record of + and - protozoa for a plate, dilution factors, and the volume per row were required by the program for each sample (see Appendix A for discussion of program). Then the MPN/mL



Dilution Factor	Volume per Row (mL)	# Positives/ # Subsamples
1.00×10^{0}	0.15	8/8
2.00×10^{-1}	0.20	8/8
4.00×10^{-2}	0.20	8/8
8.00×10^{-3}	0.20	8/8
1.60×10^{-3}	0.20	8/8
3.20×10^{-4}	0.20	7/8
6.40×10^{-5}	0.20	3/8
1.28×10^{-5}	0.20	3/8
2.56×10^{-6}	0.20	0/8
5.12×10^{-7}	0.20	0/8
1.02×10^{-7}	0.20	0/8
2.05×10^{-8}	0.25	0/8

Figure 9: Microtiter plate used for enumerating sediment protozoa with the Darbyshire liquid media MPN technique. The extinction dilution procedure uses 96 wells (i.e., 8 replicates/dilution and 12 rows of dilutions) for the liquid media. A search pattern was designed to provide the information necessary for the MPN determination and minimize the number of wells that had to be examined for the presence (+) or absence (-) of protozoa in the wells. The protozoan population estimate shown in the microtiter plate is 4.38×10^{4} MPN/mL of sediment sample.

was converted to MPN/gdw by the dilution factor from the volume of phosphate buffer used, the quantity of sediment sampled and the moisture content of the core (see Appendix A for calculations).

v. Experiment on bacterial supplement. An experiment was conducted with a core sample to determine if the enumeration of protozoa by the Darbyshire liquid media MPN method required the addition of bacteria. The experiment was conducted as a comparison to one of the requirements of the Singh solid media MPN technique.

Microtiter plates were prepared with 1) unseeded phosphate buffer, 2) buffer inoculated with *Escherichia coli* sp. (courtesy of R. Mooney, Department of Microbiology, University of New Hampshire, Durham, NH, 1990), and 3) buffer inoculated with heat-killed *E. coli*. *E. coli* was chosen since *Enterobacter aerogenes*, the species used by Sinclair and Ghiorse (1987), was unavailable. The seeded phosphate buffer was prepared using an inoculating loop (4 mm loop; VWR, Boston, MA) to remove a small amount of culture from a slant of standard plate count agar (Difco Laboratories, Detroit, MI). The inoculum was transferred into 10 mL of sterile phosphate buffer. The water became turbid from the addition of the bacteria, but the concentration of bacteria in the liquid was not determined.

A second solution of bacteria prepared in the same manner was heat-killed by flaming the liquid over a Bunsen burner until the liquid began to boil (A. Warren, personal communication, 1990). These solutions were used as the diluent for the microtiter plates. In this experiment, the plates were incubated and examined for protozoa as stated above.

b. Singh solid media MPN method. Subsurface samples from the U.S.G.S. site were enumerated by the Singh solid media MPN method according to the procedures of Sinclair and Ghiorse (1987). One slight modification to their procedure was the use of two different kinds of dilution plates: 1) petri dishes with plastic rings in agar; and 2) 6 well polystyrene macrotiter plates. Details of their procedure are discussed regarding: 1) preparation of dilution plates; 2) sample preparation; 3) incubation of plates; and 4) analyses of plates and calculation of MPN/gdw.

i. Preparation of dilution plates. Sinclair and Ghiorse (1987) used 2 cm diameter glass rings to divide petri dishes into 5 replicate wells for their dilution plates. Since cutting the glass tubing proved difficult, plastic rings were cut from clear polyvinyl tubing as an alternative (1" O.D., 0.75" I.D.; Plumbmaster, Inc., Chicago, IL). Non-

nutrient agar (1.5% Bacto agar; Difco Laboratories, Detroit, MI) was poured into sterile petri dishes (100 x 15 mm, plastic, sterile) and 4 sterilized plastic rings (121°C, 15 psig, 15 min) were arranged in the molten agar with sterilized forceps (Figure 10A).

Samples collected in Fall 1991 were processed using a different kind of dilution plate. "Macrotiter" plates (Figure 10B), similar to microtiter plates, but with only 6 "rings" (34.6 mm ϕ, 17.6 mm depth, flat bottom wells, tissue culture treated, sterile; Corning Laboratory Science, Wexford, PA), were used because they were more convenient than preparing the plates with the plastic rings. A thin agar layer (~3 mm thick) was poured into each well which was subsequently inoculated with the bacterial suspension. After the agar hardened in the plates, each ring was inoculated with a bacterium indigenous to the subsurface samples. The bacterium came from a ground water sample collected from F 473 at a depth within the plume. A sample from the culture was spread onto standard plate count agar (Difco Laboratories, Detroit, MI) and incubated at room temperature for three days. Only two bacterial colony morphologies were apparent and both were comprised of 0.5 µm cocci. One was a white, opaque colony and it was streaked on a second plate to check the purity of the isolate and maintain a stock available for the Singh MPN enumeration

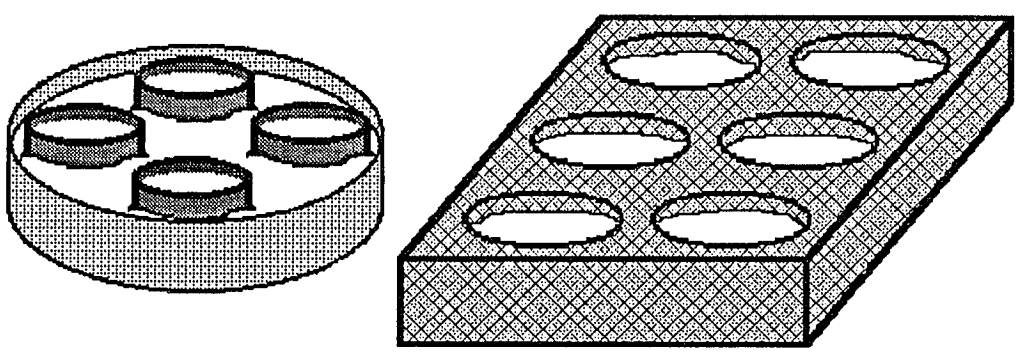


Figure 10: Plates used for the Singh solid media MPN technique for the enumeration of protozoa in sediment samples. Plastic rings are arranged in non-nutrient agar (A) or agar is poured into wells of macrotiter plates (B) to serve as media for the extinction dilution procedure.

technique. Since the colonies on the plates did not appear to be clearing, it was assumed that no protozoa were present. [N.B., some of the inoculum should be inspected on a light microscope to ensure that it is not contaminated with protozoa.] A small amount of bacteria from the plate was transferred to 10 mL of sterile water with an inoculating loop and the dilution was mixed with a Maxi-mixer (Type 16700 Mixer, Thermolyne Corp., Dubuque, IA). The inoculum was assumed to have at least 10^6 bacteria/mL when the solution appeared to be slightly turbid (A. Warren, personal communication, 1990). Each ring in the dilution plates was seeded with an inoculating loop of bacterial suspension. The plates were used shortly after the suspension was applied or left to incubate at room temperature overnight.

ii. Sample preparation. The procedures for sampling cores was a modification of those used by Sinclair and Ghiorse (1987). Processing of the dilution plates occurred simultaneously with the opening of a core sample.

Two macrotiter plates (6 wells each) were needed to analyze each sample (i.e., 4 dilutions and 3 replicates per dilution). To prepare the undiluted replicates, a plate was placed on the analytical balance and 1.0 g of sediment from the core was placed in three separate wells. An automatic

pipettor (100-1000 µL; Eppendorf, Brinkmann Instruments, Inc., Westbury, NY) was used to flood the sediment in each ring with 1 mL of sterile phosphate buffer (same as that used for the shaking technique). A 10^{-1} dilution of the sediment was prepared by weighing 1.0 g of core material into a sterile test tube containing 10 mL of phosphate buffer. The test tube was mixed for 30 sec, rested for 30 sec and mixed again for 30 sec on a Maxi-mixer before 1 mL of the dilution was transferred to each of 3 wells on a plate. The next dilution, 10^{-2} was prepared by adding 1 mL of 10^{-1} solution into 9 mL of sterile phosphate buffer. Representative samples of the dilutions were collected by submerging the pipet tip ~1 cm below the surface of the liquid. All subsequent dilutions were prepared in the same manner (including the mixing, resting and mixing procedure).

The diluted sediment samples were treated with acid for the enumeration of encysted protozoa. To each of the dilutions, 1.2 mL of 0.5 N HCl was added, mixed and after 30 min the acid was neutralized with 300 µL of 0.1 N NaOH (as determined with pH paper). These samples were then processed as outline above. The undiluted sample (1 g of sediment on the plate, or 10^{0} dilution) was not used with the acidified samples, and the wells used for the undiluted sediment were instead filled with 2.5 mL of sterile phosphate buffer and served as sterile controls.

iii. Incubation of plates. All plates for the Singh method were incubated in a 12°C refrigerator for up to 12 days. The incubation period varied depending on the time required for the bacteria to decrease enough to view the protozoa. The humidity was maintained in the refrigerator using an open beaker of water. However, every week the plates were opened and 2.5 mL sterile phosphate buffer was added to each well to ensure >1 mm of liquid covered the agar throughout the incubation period.

iv. Analyses of plates and calculation of MPN/gdw. To observe the plates, a sterile inoculating loop was used to swipe the area of the agar in a well and transfer ~60 µL to a microscope slide (60 µL was enough to float the coverslip on top of the sample without any air pockets or excess liquid beyond the edges of the 25 x 25 mm coverslip). The liquid was covered with a coverslip and observed at 400 X magnification (Nomarski interference optics) with the Nikon Optiphot^{R} light microscope. The slides were scanned from one edge of the coverslip to the other for the presence of amoebae or flagellates. The contents of all rings were examined for presence (+) or absence (-) of protozoan activity.

A computer program was used to calculate the MPN/mL of sample (Hurley and Roscoe, 1983; Arnold, 1992). The record

of + and - protozoan activity for each set of dilution plates and their respective dilution factors were required by the program (see Appendix A for discussion of program). Then the MPN/mL was converted to MPN/gdw using the moisture content of the core (see Appendix A for calculations).

II. DESIGN OF SAMPLING PROGRAM FOR VARIABILITY STUDY

The epifluorescent (DAPI), Darbyshire liquid media MPN and Singh solid media MPN enumeration techniques were used to analyze samples collected in Fall 1991 from two sites at the U.S.G.S. site. The objectives of this study were to determine: 1) the intrasite variability; 2) the variability associated with the enumeration techniques; 3) the effect of hold time on core samples; 4) the effect of treatment of core sleeves for retrieving representative sediment samples; and 5) the differences in estimates of trophic vs. encysted protozoa obtained from the two MPN enumeration methods. The cores were processed at the University of New Hampshire. Sieve analyses were also performed on representative cores from the two sites.

A. Location of Sites

The sites (Figure 1) chosen for the variability study were: 1) a contaminated site, S 318, 10.5-11.1 m below land's surface, 0.05 km from the head of the plume and

located within an infiltration bed used for disposal of wastewater effluent; and 2) an uncontaminated site, F 393, 11.4-11.7 m below land's surface, 2.06 km southwest of the head of the contaminant plume. These sites represented two extremes in the bacterial population (i.e., attached and free-living bacteria) and chemical composition (e.g., dissolved organic concentration) of the ground water, but they were physically (e.g., grain size of sediment) very similar.

At each site, 3 core samples were removed with the Waterloo coring device using the drilling rig owned and operated by the U.S.G.S. Connecticut office (John O'Brian and Roger Freeman, operators). The same drilling protocol was performed at each site. The drill rig collected a core and was moved forward ~1 m before beginning the next borehole. All cores were taken within 6 m of a monitoring well and at a depth similar to the screened interval of the well. If a core had less than 1 m of sediment recovery in the sleeve, then the sample was rejected since too little material was available for all of the analyses. The core barrel with the recovered material was sectioned in the field using a pipe cutter. Plastic caps (wiped with 95% ethanol) were placed on each end of the core and secured with several wraps of electrical tape. The top and bottom 0.15 m of the sediment in a core was removed and examined by

a geologist for soil characterization. The rest of the core was divided into 3, ~0.3 m sections: the top section for sieve analysis; the middle section for bacterial enumeration; and the bottom section for protozoan enumeration.

B. Enumeration Techniques

The intrasite variability and the variability associated with the enumeration techniques was estimated by components of variation analysis (Gill, 1978). The cores were sampled according to a hierarchical design which allowed the variability associated with each site, the number of cores within a site and the replicates within a core for each enumeration technique to be calculated (equations 7-9, Chapter 2 and Appendix B). [N.B., Site = Batch, Core = Sample and Replicate = Test in the terminology used by Box et al. (1978).]

Due to the time requirements to perform the enumeration techniques, different numbers of replicates were used for the epifluorescent and the two MPN methods. To prevent contamination from the non-sterile core sleeve, sediment material was removed from the central portion of the core after the first 3 cm of the bottom material was removed. Three 5 g replicate samples were taken from the core and subjected to the shaking technique for use in epifluorescent

analysis. Two of these replicates were also diluted for the Darbyshire method. For the Singh method, 1 g of sediment from the core was weighed into each of the wells in the macrotiter plate to serve as undiluted sample. Also, two 1 g samples were weighed into test tubes to prepare the other dilutions for the macrotiter plates. The core was resealed with the plastic cap and stored at 4°C. Plates were also prepared for the "acid" treated samples to determine the fraction of the protozoan population that was encysted at the time of sampling.

Often the drill rig can collect more core material in a day than the laboratory can process. To estimate the variability of the enumeration techniques due to the holding time of the core sample before processing, the 3 cores collected at S 318 were enumerated at 1, 5, 15 and 28 days after the sediment was removed from the site. The cores were always sampled from the bottom. After sediment was removed from a core on a sampling day, the core was resealed and returned to the 4°C incubator.

In addition to the 3 replicate cores at S 318, 2 more cores were drilled with core barrels that were washed with 95% ethyl alcohol (using nylon brushes (5.5 cm diameter, 12 cm length) attached to 1.83 m sections of fiberglass, chimney sweep rods) and then rinsed with distilled water (Poland Spring^{R}, Poland Spring, ME). The outside of the

core sleeve and plastic caps for the core were cleaned with 95% ethyl alcohol. All of the other cores were only rinsed with water from the drill rig (collected each morning at a fire hydrant) and the end caps wiped with tissue. Core samples with cleaned barrels were only processed using epifluorescent enumeration.

C. Sieve Analyses

The core sections for sieve analyses were emptied into an aluminum pan and dried for three days at 103°C. Subsequently, 500-1000 g of the dried sediment was weighed and placed in the top of a stack of sieves: 3/8"; No.4; No.8; No.16; No.30; No.50; No.100; and No.200 sieves (brass, U.S. Standard Sieves; W.S. Tyler Comp., Cleveland, OH) followed by a pan. Each sieve and the pan was weighed prior to the introduction of the sample. The sediments were shaken in the sieves with a mechanical sieve shaker for 10 min, and then each sieve and the pan was reweighed. Cumulative frequency curves were developed (Holtz and Kovacs, 1981) for the contaminated (S 318) and uncontaminated (F 393) sites.

III. STATISTICAL ANALYSES

The statistical computer package STATA^{R} (Computing Resource Center, Los Angeles, CA) was used to determine the

distribution of the data and for all other statistical techniques unless mentioned otherwise. Appendix B contains: 1) the commands used in STATA^{R} for the various tests used in this dissertation; 2) procedure for untransforming the data after statistical analysis; and 3) the components of variance analyses.

CHAPTER 4

RESULTS AND DISCUSSION

The results of the procedures outlined in the previous chapter can be divided into four parts: 1) separation techniques tested at the Natural History Museum; 2) methods used for enumerating protozoa in subsurface sediments; 3) variability associated with the subsurface sediment enumeration techniques; and 4) enumeration of ground water protozoa on downwell samplers. This chapter will include qualitative discussions of all procedures (e.g., information learned during the development of the methods and the advantages and disadvantages associated with the techniques applied to the subsurface sediments) and quantitative results from the enumeration of core samples (e.g., variability associated with the enumeration techniques, sites in and out of the plume, and hold time of the core samples). Finally, the results of enumerating ground water protozoa on slides and sponges from downwell samplers will be discussed.

I. ASSESSMENT OF SEPARATION TECHNIQUES

Three separation procedures--the seawater-ice (Uhlig, 1964, 1968), centrifugation (Hopkins et al., 1991a) and shaking (Dye, 1979)--were evaluated for their ability to

remove protozoa from sediment. All procedures were tested with the sand collected at Canvey Island and maintained in the laboratories at the Natural History Museum. The evaluation was qualitative since the actual number of protozoa in the sand samples was not known. These techniques were chosen after a search of relevant literature, and considering the equipment available and the fact that the seawater-ice method had been used by others in the laboratory. The criteria for evaluating the suitability of the techniques included: 1) the number of protozoa recovered; 2) the types of protozoa recovered (e.g., ciliates, amoebae, flagellates); and 3) the physical condition of the organisms found. No dry weight analysis was performed on the sand, however, comparisons were valid because all techniques were evaluated using the same sample (Table 2).

Table 2: Evaluation of separation techniques on the Canvey Island samples. Each technique involved observation of sample fractions collected during the procedure; "-" indicates that the fraction was not sampled; ">1" means that more than 1 organism was present, but the total number could not be distinguished.

Protozoa per Fraction	Seawater-Ice	Centrifuge	Shaking
1	15	>1	29
2	9	-	19
3	5	-	8
4	0	-	0
5	-	-	0
Total Protozoa	29	>1	56
Weight of Sample (g)	30.2	5.0	5.0
Protozoa/ g wet weight	<1	?	11

A. Seawater-Ice Technique

The Uhlig seawater-ice method recovered <1 protozoa/g wet weight (Table 2). A number of psammophilic ciliates and one type of flagellate were observed as well as metazoa, such as nematodes, copepods and rotifers. No amoebae were found. The first fraction of eluant had 15 organisms and small sand particles were scattered across the bottom of the petri dish. The number of organisms and sand particles decreased with each successive fraction until no organisms were found in the fourth fraction.

Ciliates (typically 50-200 μm) were the most common type of protozoa extracted. Only 3 flagellates (15-20 μm) were found and they were all observed in the second fraction. Observation of the flagellates was difficult with the dissecting microscope (45 X maximum magnification) through 3 mL of eluant, and some cells could have been overlooked.

The eluant evaporated quickly from the petri dishes, so they had to be covered and kept in a cool location until they were observed. The organisms did not appear to be physically damaged by the extraction technique (i.e., cells were not crushed, tangled or missing appendages). They could be removed (e.g., with a micropipettor) for further identification under higher magnification. However, after several hours of incubation the organisms stopped moving, and thus became more difficult to distinguish from the sand particles.

The organisms might have died due to the exposure to the high salinity gradient created by the separation technique. The melting seawater-ice often had a salinity of 40-50 ppt. Since the Canvey Island samples came from a tidal region of the Thames estuary, these organisms were acclimated to daily fluctuations in salinity ranging from 0-32 ppt but, they might not have the capability of withstanding the higher salinity (40-50 ppt). The organisms might have survived longer if seawater from their holding tanks had been added

to the petri dishes, but this dilution would have only increased the difficulty in searching for small protozoa.

The seawater-ice technique required the motile cells to detect the advancing salinity gradient and move downward with the flowing pore water. Uhlig et al. (1973) found that the seawater-ice technique was better at extracting large ciliates compared to other procedures, however, a decanting method extracted more small ciliates (<100 μm) and flagellates. Thus, the technique was biased toward the recovery of large motile organisms such as ciliates. Amoebae, if they were present in the sample, would probably not be recovered since they move slowly. Burnett (1973) reported observing amoebae in scanning electron micrographs of deep-sea benthic sediment samples, but was unable to separate them with the seawater-ice technique. These observations indicated that this separation technique might not be suitable for the kind of small flagellates and amoebae found in the saturated sediments at the U.S.G.S. site.

No work has been reported in the literature using the seawater-ice on freshwater sediments. However, in July and October 1988 a modified Uhlig technique, using only a temperature gradient, was used to extract protozoa from cores collected in the contaminant plume (S 318, S 314,

F 230 and S 316) and upgradient from the plume (S 315) (see Figure 1 for locations). Sediment (~5 g) from the cores was placed in sterile cheese cloth in a funnel, covered with distilled water ice (0 ppt salinity), and ~50 mL of pore water and melted ice was collected in a 125 mL bottle below the funnel. A slide with 60 µL of the eluant was prepared (the same method as described for the examination of the pore water from the downwell samplers) and was scanned for protozoa until it began to dry out. Generally, only 1-2 flagellates were observed in the eluant.

One problem with the separation procedure was dilution of the sample. If the modified Uhlig technique were able to recover 100% of the protozoan cells in the sediment compared to the population estimates of other techniques, the detection of the protozoa would be difficult considering the dilution of the sediment. For example, 1.44×10^4 flagellates and amoebae/mL were observed in the sponge on the downwell sampler incubated in the adjacent monitoring well at site F 230. Since the moisture content of the sediment at the site was 15%, there was 0.75 g of pore water (0.75 mL) in the 5 g of sediment extracted by the 50 mL of melted ice. Considering the concentration if all 1.44×10^4 protozoa/mL were removed from the pore water into the eluant, then there would be 13 protozoa under the coverslip of the slide prepared for examination:

$$\frac{1.44\times10^4 \text{ protozoa}}{\text{mL}} \times 0.75 \text{ mL} = 1.08\times10^4 \text{ protozoa in sediment;}$$

$$\frac{1.08\times10^4 \text{ protozoa}}{\text{mL}} = \frac{216 \text{ protozoa}}{\text{mL}} \text{ final conc. in diluent;}$$

$$\frac{216 \text{ protozoa}}{\text{mL}} \times 0.06 \text{ mL on slide} \sim 13 \text{ protozoa.}$$

At 400 X magnification, there were 88 scans/coverslip (2.5 cm width of the coverslip and 0.0285 cm diameter of the field). On average, 7 scans would have to be made in order to find 1 protozoon on the slide, but the technique only examines 3 scans/slide. [N.B., Fall 1990 sediment samples were estimated to have populations of 3×10^4 MPN/gdw (Darbyshire liquid media MPN technique) and 1×10^5 protozoa/gdw (epifluorescent technique). However, the Uhlig separation probably estimated the population of protozoa more likely to be found in the sponge of the downwell sampler than in the sediments because those recovered would be biased toward more motile species.]

The abundance of protozoa separated by the modified Uhlig technique had to exceed 3.26×10^4 protozoa/mL of eluant in order to detect 1 protozoon in 3 scans of the slide (assuming the protozoa were evenly distributed on the slide). Thus, without considering the efficiency of the

separation technique, the theoretical detection limit of the procedure was too high to be used with these sediments.

Another problem with the technique was that it relied on a temperature gradient to induce protozoan movement out of the sediments from the U.S.G.S. site. Since the ground water temperature ranges from 9-13^{o}C in the plume (Ceazan et al., 1984; Lee, 1991), the temperature gradient from the melting ice was probably not drastically different from the ambient conditions. Uhlig (1968) found in laboratory tests that the temperature in the sediments did not fall below 9^{o}C. He also stated that the advancing of the salinity gradient due to the melting seawater-ice was more effective in recovering the interstitial protozoa than cold, flowing seawater or mechanical separation. The modified Uhlig procedure did not use seawater-ice since it would have exposed the organisms to a high salinity and they might have lysed due to their inability to compensate for changes in osmotic pressure.

Only flagellates and amoebae have been observed in sediments from the U.S.G.S. site. These protozoa have also been reported as the dominant types found at other saturated subsurface sites (Hirsch and Rades-Rohkohl, 1983; Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990). The data in comparison to the theoretical limit and other enumeration methods indicated the modified Uhlig

technique did not efficiently remove these organisms. Hence, no further attempts were made to use the modified Uhlig technique on core material after 1987.

B. Centrifugation Extraction

Centrifugation was used to: 1) separate protozoa from sediment; or 2) concentrate protozoa in liquid samples to decrease the volume of liquid necessary for examination. The centrifugation technique developed by Hopkins et al. (1991a) was found to be ineffective for extracting protozoa from the Canvey Island sand (Table 2). The method did recover between 1 and 10 protozoa in the first liquid fraction removed from above the sediment after centrifugation in the centrifuge tube, however, the cells could not be counted since they were tangled together. This problem was also reported by Sherr and Sherr (1983). No further fractions were tested because of the damage to the cells.

Hopkins' technique involved dispersing the sediment and organisms in sodium cholate and chelating solutions, then separating the organisms by centrifugation at 500 *g* for 2 min. The method tried to optimize dispersion of the microorganisms and soil aggregates since Hopkins felt that entrapment of the organisms in the aggregates accounted for the limitations of the enumeration technique. Sodium

cholate, an anionic detergent (Griffiths and Ritz, 1988), is used to dissociate the organisms from the particles by creating negative charges on all surfaces, which then repel other like-charged surfaces (Solomans, 1980). However, Hopkins did not comment on how well the sodium cholate dissociated bacterial cells attached by stronger mechanisms (e.g., a glycocalyx). The chelator reduces the electrolyte concentration and further increases the repulsive interaction between the like-charged surfaces of the cells and particles in solution (Hopkins et al, 1991a).

Hopkins and his associates (1991a) found the technique to be effective for enumerating bacterial cells in clay loam, sandy loam and peat. They repeatedly suspended the pellet in solutions to disperse the soil aggregates and then separate the bacteria into the supernatant by centrifugation up to five times. The soils they tested all had a grain size of <0.1 mm which appeared to be smaller then the average grain size of the Canvey Island samples. Although the cholate and chelating resin separate the protozoa from the sand, the recovery of protozoa in this method is probably also a function of the interstitial organisms being pushed up into the liquid fraction due to their lighter density and the concurrent compression of the sediment particles by the forces of the centrifuge. The Canvey Island sand did not compress into a "pellet" as was observed

with the soil that Hopkins' used during his demonstration, so any organisms in the interstitial liquid would have only been separated based on their lower density. The use of centrifugation to recover the entrapped organisms was not effective with the large grain size of the sand.

Separation by density centrifugation was also not effective as a means of concentrating the protozoa in the eluant of the seawater-ice technique. The density of the protozoa (1.12 g/mL; Griffiths and Ritz, 1988) was slightly greater than the seawater (1.02 g/mL at 20^{o}C and 32 ppt; Bishop, 1983), so centrifugation should have concentrated the organisms in the bottom of the centrifuge tube. However, the protozoa found in the bottom of the centrifuge tube became tangled together in an unrecognizable mass and could not be enumerated.

Griffiths and Ritz (1988) used Tris buffer instead of sodium cholate to dissociate protozoa and mineral soil particles before centrifuging the sample in a linear density gradient. The gradient was prepared by adding Percoll^{R} sol and Sörensen's phosphate buffer mixtures into a centrifuge tube in layers and then allowing the dispersed soil solution to sediment through the liquid gradient before centrifugation. The density of the layers ranged from 1.00 g/mL at the top of the centrifuge tube to 1.12 g/mL above the soil pellet after centrifuging. Since the protozoa have

a density of <1.12 g/mL, they are typically recovered in the layers above the soil pellet after centrifugation. Griffiths and Ritz did not report problems with distinguishing ciliates (e.g., *Colpoda steinii* and *Engelmanniella halseyi*) and flagellates (e.g., *Heteromita* sp.) since the shapes of these soil protozoa were not as elaborate nor as likely to tangle as those found in the Canvey Island sand.

Centrifugation has not been tried on the sediments collected at the U.S.G.S. site. The linear density gradient procedure used by Griffiths and Ritz (1988) might be effective with these samples since they reported 10^4-10^5 MPN/gdw of ciliates, flagellates and amoebae in their mineral top soils (as estimated by the Darbyshire liquid media technique), which is in the range of the flagellates and amoebae in the subsurface sediments at the U.S.G.S. site. However, the time involved in preparation of the linear gradients (~5 hr) and centrifugation (2 hr) prior to enumeration was considered impractical for the subsurface samples.

C. Shaking Technique

The shaking technique (Dye, 1979) recovered more organisms than the other two techniques, 11 protozoa/g wet weight (Table 2). More than 50% of the protozoa were found

in the first fraction, and no protozoa were observed in the fourth and fifth fractions. Ciliates and flagellates were the most numerous types of protozoa present in the decanted liquid. One amoebae was observed. No attempt was made to quantify the ciliates and flagellates by type. Although more sediment particles were found in the decanted fractions than with the other two techniques, the protozoa could be distinguished and did not appear to be damaged by the procedure. Several species of ciliates and one specie of flagellate extracted by the shaking technique were cultured in the laboratory for several weeks.

The results of the seawater-ice and shaking techniques were similar to those reported by Dye (1979): three times more protozoa were enumerated in marine sediments separated by the shaking technique than with seawater-ice. Uhlig et al. (1973) compared a decanting technique (similar to the shaking procedure) to the seawater-ice method, and found more flagellates and small ciliates in the decanted fractions. However, all of the protozoa recovered were still larger than the 2-5 μm flagellates and amoebae observed at the U.S.G.S. site.

D. Conclusions and Recommendations for Subsurface Sediment Samples

The shaking technique was chosen as the extraction procedure to be used for the enumeration of protozoa in sediment collected from the U.S.G.S. site since it recovered the greatest numbers and diversity of protozoa without damaging the cells. Only mechanical separation was involved with the shaking technique (e.g., the Canvey Island sand was shaken in additional seawater and the liquid fraction was decanted for examination). No gradients were established to encourage the organisms to separate from the sand. Also, no chemicals were added to break covalent, electrostatic or hydrogen bonds between the protozoa and sand. The process essentially "washed" the organisms out of the sand and decanted them off repetitively.

Separation procedures for protozoa differ from bacteria since the organisms' association with the soil matrix is different. Some bacteria attach to surfaces with extracellular glycocalyxes or charge interactions (Atlas and Bartha, 1987). However, soil protozoa are unlikely to attach by such mechanisms since they use their motility to search for food sources within the soil matrix (Griffiths and Ritz, 1988). Flagellates have been observed temporarily attaching their flagella to surfaces while feeding (Clarholm, 1984). Amoebae are more closely associated with

surfaces and glide over them in search of food (Clarholm, 1984). There is no mention in the literature about strong interactions (e.g., permanent attachment) between flagellates and amoebae and soil particles similar to the formation of glycocalyxes by soil bacteria. Since the recovery of protozoa from soil is thus related to the entrapment of the cells in the aggregate, the separation procedure must be effective in dispersing the cells from the soil particles to facilitate enumeration.

Dye (1979) used seawater for the shaking technique on his marine sediments. Since seawater has a higher ionic strength and buffering capacity than the ground water at the U.S.G.S. site, Dye's shaking technique was modified for application with the saturated, fresh water sediments. The potassium phosphate buffer (2.2 mM KH_2PO_4 and 4.02 mM K_2HPO_4) that Sinclair and Ghiorse (1987) used with saturated sediments for the modified Singh MPN technique was chosen as the diluent/decanting liquid. The buffer was used to help prevent cells from lysing due to osmotic shock (Griffiths and Ritz, 1988; Hopkins et al., 1991a). The dissociation of the salt and protonation (and/or deprotonation) of the phosphate in water helped to maintain a stable pH:

KH_2PO_4 ---> $K^+ + H_2PO_4^-$ $pK_a=7.2$

H_2PO^- ---> $H^+ + HPO_4^{-2}$ $pK_a=6.8$

HPO_4 ---> $H^+ + PO_4^{-3}$ $pK_a=1.7$.

Also, the buffer decreased changes in osmotic pressure by increasing the ionic strength of the solution (Christian, 1980).

Buffers have been used with other enumeration procedures for soil protozoa. Cunningham (1915) used a 0.05% K_2HPO_4 solution for the enumeration of soil protozoa by his dilution method. Griffiths and Ritz (1988) used Tris buffer (tris(hydroxymethyl) aminomethane or $(HOCH_2)_3CNH_2$). Tris is frequently used with biological samples since it has a greater buffering capacity than phosphate solutions and does not inhibit as many enzyme systems (Christian, 1980).

Griffiths and Ritz (1988) evaluated anionic, cationic and neutral detergents, in addition to Tris buffer, because the detergents were thought to increase the recovery of cells that were attached to particles by a lipid-based chemical bond or electrostatic mechanisms. The charges on the detergents and their hydrophilic/hydrophobic configuration were thought to assist in disassociating the cells and the particles. These particular detergents had been used to recover bacteria from soil samples. However, the concentrations of the detergents had to be low to

prevent inhibition of the protozoa, and at those concentrations the detergents were not as effective in dispersing the soil particles as Tris buffer. Whether Tris is better at dispersing particles than phosphate buffers is unknown.

The shaking technique by Dye (1979) was further modified for the convenience of sampling the sediments from the U.S.G.S. site. During Spring 1990, Ziploc^{R} bags were used for sediment separations because they were readily available and assumed to be sterile since they are used to store food. However, blue fluorescing threads (~5-60 μm long) were observed on a number of epifluorescent slides, especially the sterile controls, and they were thought to be from these bags. Whirlpak^{R} bags were used for all subsequent samples since they were sterilized with ethylene oxide, and microbial contamination was not a concern. The blue threads were rarely found on the sterile controls and other slides prepared in the Whirlpak^{R} bags. The metal ties on the Whirlpak^{R} bags made them easier to hold and to decant liquid than the Ziploc^{R} bags.

The phosphate buffer was added immediately to each sediment sample to keep it saturated. Aliquots of sterile buffer were prepared in three 25 mL volumetric flasks prior to weighing the sediment, to facilitate the rapid transfer of the liquid to a bag. Three fractions were chosen based

on the results of the Canvey Island sand samples. This agreed with the findings by Dye (1979) that the efficiency of the technique did not increase after 3 shakings. A total volume of 75 mL was found (through trial and error) to dilute the decanted sediment particles enough to be able to filter a sufficient quantity (2-3 mL) of sample and distinguish the organisms from the abiotic particles on the filter . Adding the buffer to the sample and keeping the sediment samples saturated was important since protozoan cells have been observed to lyse upon exposure to air. Amoebae, in particular, will attach to surfaces, so the shaking process must be performed quickly (i.e., within seconds according to S. Brown, personal communication, 1990) to limit the loss of organisms due to adhesion to the bags.

II. ENUMERATION METHODS FOR SUBSURFACE CORE SAMPLES

The techniques applied to the subsurface sediment samples included epifluorescence microscopy and two extinction-dilution MPN methods--the Darbyshire liquid media MPN technique and the Singh solid media MPN technique. Modifications of the shaking technique by Dye (1979) were used to separate the organisms from the soil for the epifluorescent and Darbyshire MPN enumerations, and a similar process was used for the Singh MPN enumeration. The

samples processed with these techniques were collected in Spring and Fall 1990, and Summer and Fall 1991.

A. Epifluorescent Enumeration

There has not been any published work on the epifluorescent enumeration of subsurface protozoa to date. However, epifluorescent enumeration of subsurface bacteria has been widely used (Wilson et al., 1983; Harvey et al., 1984; Balkwill and Ghiorse, 1985; Beloin et al.,1988; Federle et al., 1990; Pederson and Ekendahl, 1990; Sinclair et al., 1990; Hazen et al., 1991). None of these researchers has reported observing protozoa on the filters used to enumerate bacteria probably because they used filters with small pore size and higher vacuum that damaged the protozoan cells.

The following categories were considered during the development of the epifluorescent procedure described here: 1) cell integrity; 2) enhancement of visualization; 3) distribution of particles on the filters; 4) enumeration procedure; 5) time management; and 6) cost of sample analysis. The use of PC filters to concentrate the protozoa was based on the criteria established by Hobbie and his associates (1977) for a successful direct counting technique: 1) the filter must retain all cells; 2) all cells must be visible on the filter surface; and 3) the

highest contrast between the cells and the background must be achieved with the stain and optical conditions. The work of Caron (1983; and personal communications, 1990), Rogerson (1988), Griffiths and Ritz (1988), and Cowling (personal communication, 1990) influenced the development the epifluorescent staining method for protozoa.

1. Cell Integrity

The procedures for enumerating bacteria with epifluorescent stains had to be modified to consider the differences compared to the ultrastructure of protozoa. Protozoa have different ultrastructure than bacteria. The cell membrane of most trophic protozoa lacks the composition of a bacterium's cell wall that imparts rigidity and maintains the integrity of the cell when exposed to changes in osmotic pressure. Few protozoan species have the means to withstand drastic changes in osmotic pressure, and those that do generally are found in environments such as salt (Sleigh, 1989) or soda lime lakes (D. McL. Roberts, personal communication, 1990). However, the wall produced by the encysted protozoa protects the organisms from a variety of environmental changes (e.g., desiccation).

All of the steps of the procedure had to maintain the integrity of the cells (both trophozites and cysts). The phosphate buffer used during the shaking procedure was ~pH 7

and had an ionic strength designed to limit osmotic shock. The addition of sodium cacodylate to the glutaraldehyde solution was important for lowering the pH ~7 and increasing the buffering capacity of the fixative (Caron, 1983).

Fixation of the samples was important to the preservation of the cells when they were exposed to air during vacuum filtration because the glutaraldehyde added rigidity to the cell membrane (D.A. Caron, personal communication, 1990). Caron (1983) emphasized that the vacuum used during filtration of protozoa must $\leq$ 13 cm Hg, and the time of exposure to the vacuum must be as short as possible (the vacuum was terminated as soon as all the liquid passed through the filter) to prevent lysing of the cells. Since the protozoa are larger than bacteria, filters with 0.8 µm pores were used to retain the cells. The larger pore size allowed the sample to filter faster than with the standard 0.2 µm pore size filters used with bacterial enumeration. Thus, the cells were further protected by decreasing the exposure time to the vacuum.

2. Enhancement of Visualization

Enumeration by the epifluorescent technique was based on observation of fixed material. The enhancement of the particles on the filters was related to the type and application of the epifluorescent stain. Procedures were

developed and modified in order to improve the detection of the protozoa. These efforts reduced the systematic errors in the calculation of protozoa per sample.

a. Epifluorescent stains. Epifluorescent stains cause objects to fluoresce when they are excited by a narrow band of light. Some dyes, such as AO, acriflavin and DAPI, will only fluoresce when bonded to the DNA and RNA of viable cells. The fluorescing stain was used to distinguish biotic from abiotic particles. AO has never been used to enumerate protozoa for sediment samples at the U.S.G.S. site because Sherr and Sherr (1983) found AO was too specific for staining the nucleotides in heterotrophic flagellates and the stain did not always fluoresce the outline of the cell or presence of flagella, important features for distinguishing flagellates from other particles.

Acriflavin dye was chosen as the first epifluorescent stain to test for enumerating the protozoa in core samples from the U.S.G.S. site since the procedure was successful with counting bacteria in other sediments (Bergström et al., 1986; Bunn, 1989). Also, Robertson (1929) used this stain to detect the internal structures of the free-living flagellate *Bodo caudatus*. However, acriflavin was not as specific to the protozoa as desired since many abiotic particles also fluoresced green. As a result, in the

U.S.G.S. samples it was very difficult and time consuming to distinguish the sediment particles from the objects that appeared to be the size and shape of flagellates and amoebae.

Caron (personal communication, 1990) recommended that DAPI be used as the epifluorescent stain for the subsurface protozoa since it was more specific for DNA than acriflavin. When excited by UV-light (365 nm), the DNA-DAPI complex appears blue while unbound DAPI and non-DNA material appears yellow (Porter and Feig, 1980). With this stain, protozoa could not only be distinguished by their size and shape, but also by color, an important advantage when the filter was congested with sediment particles.

The concentration of the stain and the exposure time prior to filtration of DAPI samples may also affect the ability to differentiate the protozoa from the sediment. The DAPI concentration for the sediment samples, 2.5 μg DAPI/mL, was used by Rogerson (1988) for the enumeration of a variety of cultured amoebae, and was recommended by Cowling (personal communication, 1990) for use with soil flagellates. These concentrations were higher than the ones recommended by Porter and Feig (1980) and Huber et al. (1985) for staining bacteria (0.01 μg/mL). Rogerson did not specify why he chose such a high DAPI concentration, but perhaps more DAPI is needed to penetrate to the membrane-

bound DNA of the amoebae and through the wall of cysts. Experiments with active flagellates (cultured from the sediments collected at the U.S.G.S. site) showed that 0.3 µg DAPI/mL was sufficient for producing adequate fluorescence and keeping the cells viable for feeding and transport studies. However, the higher 2.5 µg DAPI/mL concentration was routinely used for the enumeration of sediment samples in order to assure sufficient staining of all protozoa.

All samples from the U.S.G.S. site were stained with DAPI for 10 min. Rogerson (1988) used 15 min for all species of gymnamoebae, but Cowling found that 10 min was sufficient for soil samples containing flagellates and amoebae. Staining time has been variable for bacterial enumerations stained with 0.01 µg DAPI/mL: Porter and Feig (1980) stained aquatic bacteria with DAPI for "5 min and longer"; >12 hr staining times have been used with saturated subsurface bacteria (R.W. Harvey, personal communication, 1991). For the U.S.G.S. subsurface sediment samples stained with 2.5 µg DAPI/mL for 10 min, there has always been sufficient fluorescence to distinguish cells from other particles.

The intensity of the fluorescence observed will also be affected by the conditions of the episcopic attachment to the microscope. The Hg vapor lamp's ability to provide sufficient UV light for the fluorescent observation

diminishes with time and the lamp should be replaced frequently. Based on personal observations, the length of time before changing the lamp depends on the epifluorescent stain being observed: ~200 hr for DAPI; and >200 hr for acriflavin. Also, fingerprints on the Hg vapor lamp, dirt on the collector lens or objective, or changes in the excitation filter cube (with excessive use) may all contribute to the loss of the cells' fluorescence (D. Yetman, personal communication, 1990).

b. Observation of filters. Counting particles on a polycarbonate (PC) filter is difficult if there are interferences due to improper preparation of the filter. Materials used to prepare the samples may cause background fluorescence, which makes the blue color of the DAPI stained protozoa difficult to distinguish. Problems with mounting the filter on the slide may also prevent the enumeration of the sample.

Black stained filters provide a dark background that improves the visibility of particles (Hobbie et al., 1977; Porter and Feig, 1980). Now that manufacturers of PC filters are selling black stained filters, the process of soaking them in Irgalan black prior to preparing a sample is no longer necessary. Also, the pre-stained, black

Nuclepore^{R} filters appear to be more evenly pigmented than those stained in the laboratory.

When PC filters were first used for enumeration of samples collected in Spring 1990, several steps were taken to limit interferences due to the irregularities in the Nuclepore^{R} membranes, including the orientation of the filter and wetting the filter with a surfactant. The filters were always used with the shinny side down because at one time samples on filters oriented in the opposite direction were obscured by background fluorescence (Eighmy, personal communication, 1987). Hobbie et al. (1977) and Porter and Feig (1980) do not mention what side of the PC filters they used. The use of the filters with their shiny side down contradicts the instructions provided with each package of Nuclepore^{R} filters, which recommends the use of the filter for filtration with the shinny side up. Saurer (personal communication, 1992) explained that the different sides are due to the manufacturing process of the PC membranes. The dull side, which was in contact with the belts during production, may have irregularities that can be observed under the microscope. However, Saurer noted that as long as no problems with the filters were observed either side could be used, but to avoid problems one side should be used consistently.

Hobbie et al. (1977) and Porter and Feig (1980) observed wet filters within an hour of preparation and did not mount them with immersion oil. Bergström et al. (1986) reported that the fluorescence of the samples diminished as the filters dried, and they tested different immersion oils to preserve them and increase storage time. The best results (i.e., the least amount of background fluorescence) were obtained when the filters were dried prior to mounting them in immersion oil. They added a second drop of oil to the top of the filter prior to adding a coverslip.

With the samples from the U.S.G.S. site, the immersion oil was found to cause other problems associated with the observation of the fields. Particles were observed moving around on the filter when a second drop of oil was added. Also, the excess oil caused the filters to be uneven, which made them difficult to observe under the microscope. One drop of oil under the filter seemed sufficient for mounting the samples since the oil moved easily through the 0.8 μm pores.

Another problem encountered was background fluorescence (i.e., "blue haze") which resulted from excitation of the immersion oil by the epifluorescent light. Bergström et al. (1986) found Cargille^{R} A immersion oil to have the least amount of background fluorescence compared to other brands investigated. The shelf life for an open bottle of

immersion oil is ~3 months since background fluorescence increases due to the oil absorbing moisture from the air (D.A. Caron, personal communication, 1990).

3. Distribution of Particles on a Filter

The calculation of protozoa per sample was based on the observation of protozoa on a portion of the filter. The distribution of all the particles on the filter was assumed to be uniform (i.e., the particles were scattered evenly across the filtration area). Careful preparation of the filters contributed to the validity of the assumption.

The distribution of particles on the filters was influenced by the filtration of the samples. The combination of the backing (e.g., the Metricel^{R} filters used with the DAPI procedure) and PC filters formed a tight seal on the filtration apparatus so that an even vacuum pressure was applied across the filtration area. Wetting the backing filter on the filter holder assisted in placing the PC filter flat and preventing air bubbles between the filters. Dilute Triton X-100, a surfactant, was used as the wetting solution to decrease the hydrophobicity of the PC filters (Hobbie et al., 1977; R.W. Harvey, personal communication, 1990). Hydroscopic regions appeared to filter more slowly compared to other areas. All of these procedures assisted

in the equal distribution of particles across the PC filter (Porter and Feig, 1980; Bergström et al., 1986).

The edges of the filter area had more particles than the center of the filter despite efforts to maintain an even vacuum. The edge effects were probably due to particles settling while the liquid portion of the sample was being pulled through the filter. Particles that were attracted to the funnel were swept down to the edge of the filter area by the meniscus of the sample. Near the end of the filtration, the liquid cleared through the center of the filter before the edges, which pulled some particles to the side of the filtration area. Generally, the funnel was not rinsed when 3 mL or less of sample was filtered in the multiport filtration apparatus because the depth of the liquid was so shallow that only ~5 mm along the sides of the port was exposed to the sample. When the filter was rinsed, the distilled water was added carefully so as not to disturb the particles already on the filter. In the acriflavin procedure, the stain was applied after the sample was filtered. If the stain was added too forcefully into the funnel, the particles were pushed to the edges.

4. Enumeration Procedure

The procedures for examining the filters were also important to the assumption of randomly distributed

particles across the filters. Since edge effects occurred during filtration, these areas had to be excluded from the enumeration of the sample. Two different procedures were used to count the protozoa on the filters: counting individual fields and scanning. An experiment with microspheres (MS) was conducted to examine the validity of the assumption of the equal distribution of particles across the filters (excluding the edges) and to compare the two counting procedures.

a. Counting Fields and Scanning Procedures. "Random" field observations were used to enumerate the acriflavin samples collected in Spring 1990. For this method, fields were chosen around the filter until $\geq$70 organisms had been counted. However, no standard procedure for ensuring random sampling (e.g., the use of random number tables) was used, so the procedure would technically be considered "haphazard" (Miller and Miller, 1984). All papers that discussed how to enumerate epifluorescent filters stated that they observed "fields", but made no mention of how they were chosen to be random (Hobbie et al., 1977, Porter and Feig, 1980, Caron, 1983, and Bergström et al., 1986). For the examination of samples from the U.S.G.S. site, the process of finding fields by moving the microscope stage to specific areas

randomly chosen (i.e., using a random number generator) on the filter was considered too time consuming.

Fields on the acriflavin stained filters were enumerated according to the benchmark of 70 organisms per filter recommended by Griffiths and Ritz (1988). They cited the work of Cassell (1965) as their choice of this benchmark. Cassell used a graphical method to estimate the precision of direct microscopic counting data by assuming a confidence interval and the average count of cells per field, assuming that the protozoa were uniformly distributed across the filter. Three graphs were developed by Cassell for 90%, 95% and 99% confidence, respectively. Griffiths and Ritz assumed 70 organisms per filter (which corresponded to 0.5 cells/field) represented a sufficient sample size "to give 95% confidence limits of 20% of the mean abundance", but this value actually came from Cassell's graph for the 90% confidence level. Thus, by counting fields until 70 organisms were observed the significant differences between estimated populations would be detected when their averages were $\pm$20% with 90% confidence.

Fenchel (1982) prepared filters from marine, nearshore water samples and tried to count at least 200 flagellates per filter, the same number as he was counting for enumerating bacteria. However, the concentration of flagellates was so low that the number of fields to be

examined was excessive. He therefore compromised on the counting procedure for the flagellates and examined the filters for 20 to 50 cells per sample. Using Cassell's (1965) technique, Fenchel would have detected significant differences between the mean population estimates of his samples only if they differed by >37% of the mean abundance using a 90% confidence interval.

Often over 100 fields had to be observed per filter for the U.S.G.S. site samples in order to find 70 protozoa when 5 mL was filtered. As the number of fields that had to be observed increased (100 fields is ~3% of the filter area), the chance of viewing the same field more than once grew. The process of locating fields, even haphazardly, and discriminating protozoa from particles was time consuming and difficult. Filtering more sample so that there were more protozoa on the filter was not possible since more particles also became trapped on the filter and obscured the view of the protozoa.

An alternative approach, the scanning procedure, was developed to enumerate the DAPI stained filters. The scanning procedure was not a random sampling technique. For the procedure to be considered random, all members of a population must have an equal chance of being included (Miller and Miller, 1984). Since scanning only uses one

axis of the filter, the procedure looses an element of randomness.

Scanning was originally achieved using a coverslip (with two lines drawn 10 mm apart) centered over the filter; these lines defined the left and right boundaries for the counting area of the filter. Only the central 10 mm square of the filter was considered for the initiation of a scan; this defined the top and bottom boundaries for enumeration. A random number table was used to determine the starting point for a scan, so at least this element of the procedure could be considered as random. Drawing lines exactly 10 mm apart on the coverslips was almost impossible and very tedious. To improve the scanning technique, a brace (see Figure 8) was developed for the microscope stage that only allowed the objective to travel a known distance across a filter. The width of the brace prevented counting of the edges of the filter. The process for choosing the starting point for a scan was also based on a random number table.

b. Counting experiment. An experiment was designed using microspheres (MS) to: 1) determine if particles were distributed randomly across the filter, and 2) compare the two counting procedures. Table 3 shows the estimate of the concentration of MS used in the experiments and the results.

Table 3: Results of counting experiment with microspheres (MS) using the counting fields and scanning methods of enumeration.

Filter	Fields (MS/mL)	Scanning (MS/mL)
1	3812	3719
2	3191	3442
3	4299	3513
4	3634	3446
Mean ± Standard Deviation	3734 ± 458	3530 ± 130
Relative Standard Deviation	12%	4%
% Recovery*	26%	24%
Coefficient of Skewness	0.56	0.09
Coefficient of Kurtosis	2.82	2.48

*Theoretically, 1.45×10^4 MS/mL were added to each filter.

If the spheres were scattered evenly across the filtration areas examined, then the MS counted "randomly" by both techniques should be described by a normal (Gaussian) distribution (Hamilton, 1990). The coefficients of skewness and Kurtosis for both counting techniques (Table 3) provided a means of predicting the distribution of the MS on the filters. Skewness represents the symmetry of the distribution and equals 0 when the median is symmetrical about the mean; values >0 indicate the distribution is skewed to the right, positively. Kurtosis represents the "peakedness" of the distribution and equals 3 when the data is normally distributed; values <3 indicates a flatter than

normal distribution (STATA^{R}, 1988). The distribution for the counting fields procedure was more positively skewed than the scanning procedure, however, the opposite was true considering the peakedness of the data. Since neither counting technique had coefficients equal to that for a normal distribution, graphical analysis of the distribution was necessary.

The distribution of the MS on the filters was also checked with quantile-normal, or normal probability, plots. Figures 11A and 12A illustrate the MS/mL determined by counting fields (n = 72 fields) and scanning (n = 16 scans), respectively, on all the filters versus the quantiles of the normal distribution with the same mean and standard deviation (Hamilton, 1992). Neither technique demonstrates serious problems with the distribution of the data (e.g., skewness or outliers). The granularity (or the "stair step" effect) in the quantile-normal plot was due to "rounding off" measurements by the counting technique and does not affect the interpretation of the distribution of MS on the filter (Hamilton, 1992). The "rounding" occurred because a MS was only counted when the entire sphere was visible, and a "fraction" of a MS was not included. Since all points lay close to the line, the quantile-normal plots suggest the data is similar to the normal distribution.

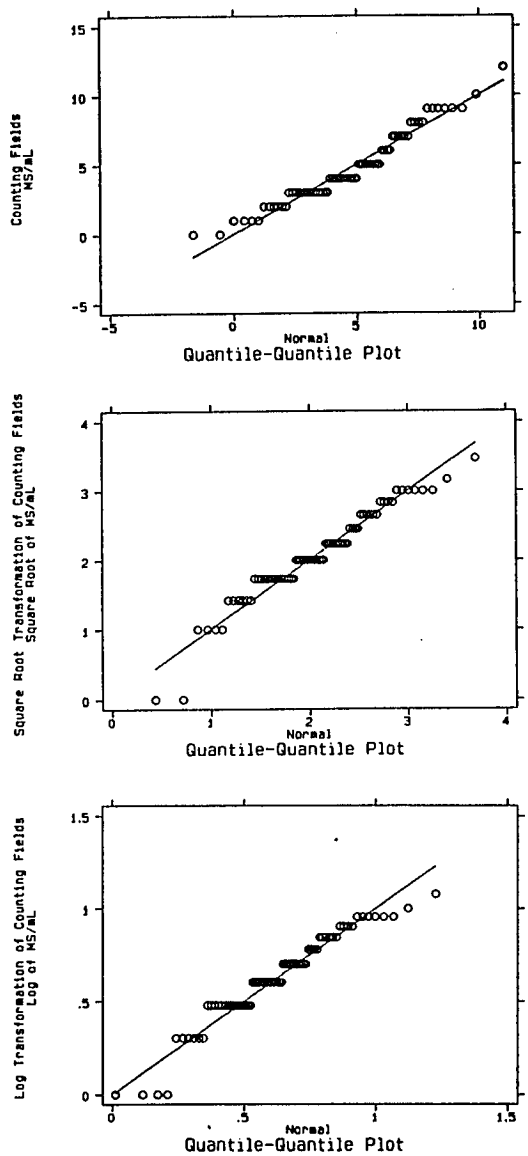


Figure 11: Quantile-normal plots of microspheres/mL (MS/mL) enumerated with the counting fields technique verses the corresponding quantile for a normal distribution with the same mean and standard deviation. Untransformed (A), square root transformed (B) and log transformed (C) data are shown to demonstrate that the untransformed data has the closest fit to the normal distribution.

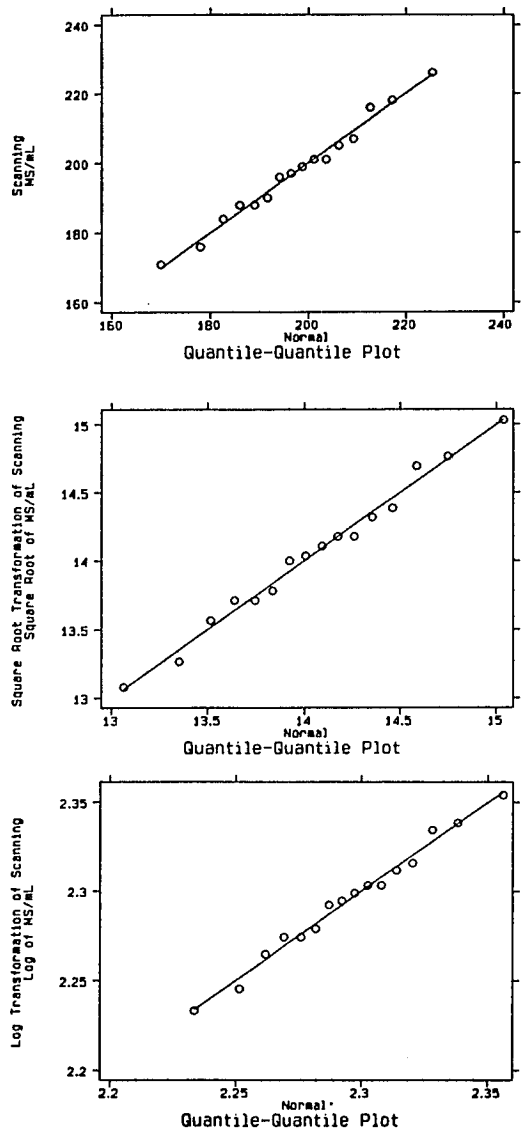


Figure 12: Quantile-normal plots of microspheres/mL (MS/mL) enumerated with the scanning technique verses the corresponding quantile for a normal distribution with the same mean and standard deviation. Untransformed (A), square root transformed (B) and log transformed (C) data are shown to demonstrate that the untransformed data has the closest fit to the normal distribution.

Even though the coefficients of skewness and Kurtosis were not equal to the exact values corresponding to a normal distribution, the quantile-normal plots did not indicate that the data deviated seriously from a normal distribution. The coefficient of skewness indicated that the data were positively skewed, but the quantile-normal plots showed that the skew was minimal. Positively skewed data can approximate the normal distribution when a square root or log transformation of the data is considered (Hamilton, 1992). However, neither transformation improved the distribution of the data. Counting fields became negatively skewed when its values were square root or log transformed (Table 4) which was apparent in the quantile-normal plots (Figures 11B and C). Although the skew of the scanning technique decreased with the square root and log transformations, the Kurtosis (Table 4) and quantile-normal plots (Figures 12B and C) remained relatively unchanged. Hence, these indicators of normal distribution showed that the MS were deposited fairly evenly across the area of the filters (excluding the edges), but they do not demonstrate if the 2 counting techniques sampled the same MS population.

Table 4: Coefficients of skewness and Kurtosis for transformation of the counting fields and scanning techniques for the MS experiment.

Counting Technique	Transformation	Coefficient of Skewness	Coefficient of Kurtosis
Fields	Untransformed	0.56	2.82
	Square Root	-0.60	4.10
	Log	-0.60	3.23
Scanning	Untransformed	0.09	2.48
	Square Root	-0.07	2.48
	Log	0.01	2.47

The average concentrations of MS (MS/mL) by the two procedures were not significantly different ($P>F=0.42$) based on one way analysis of variance (ANOVA). Bartlett's test indicated that the variances for the estimates of MS/mL by the counting techniques were homogeneous ($P>chi^2=0.07$). The lower relative standard deviation of the scanning technique compared to the counting fields technique was probably due to the greater amount of the filter observed. The counting fields technique examined only 18 fields or 0.45% of the filter, whereas the scanning technique observed 1.25% of the filter when 4 scans were conducted. However, the ANOVA demonstrated that the population estimates of the counting techniques was not significantly different from that of the scanning method.

One problem with the MS experiment was the discrepancy between the number of MS enumerated on the filters and the theoretical quantity in the filtered solution, as illustrated in the low percent recovery in Table 3 (26% for

counting fields and 24% for scanning). One possibility for the low recovery of MS was that a majority of the spheres were deposited on the edges of the filtration area which was excluded in both of the counting procedures. If a majority of the MS were deposited around the filtration edges, the mass of spheres would have been readily apparent because of the intensity of the fluorescence. However, when the edges of some filters were observed no excessive deposition of MS was noticed.

Another, more likely, possibility was that the procedures for preparing the dilution of the MS stock was responsible for the large systematic error. The stock of MS was only shaken for ~1 min before an aliquot was pipetted. Two serial dilutions were made for the preparation of the solution filtered. Further experience with microspheres has shown that shaking does not disperse the spheres homogeneously in solution; the stock should be sonicated at least 1 hr before removing a sample. Serial dilutions are very difficult to make since the solutions are generally not homogeneous and the spheres tend to clump when exposed to air during pipetting or when vigorously shaken, or stick to surfaces. Although the diluted solution of MS was filtered immediately after it was prepared, storage of MS solutions has been a problem since the MS settle and clump readily in solution.

Despite the low percent recovery of the experiment and the non-random sampling for the two counting techniques, the results indicated that the procedures for preparing filters evenly distributed the MS across the filtration area, which approximates the normal distribution. Also, the two methods for enumerating the filters were not significantly different.

Caron (1983) also found that estimations of primulin-stained flagellates in marine samples from counting fields and scanning were comparable. His scanning technique involved measuring the length of each scan with the microscope stage's micrometer. [N.B., he did include the edges of the filtration area in his enumerations.] Caron reported that the relative standard deviation (coefficient of variation) for both methods together was 10.8% for 80 population counts. Caron's results were similar to the MS experiment where the relative standard deviation was 9.1% with both methods.

5. Time Considerations

Some of the procedures used for the epifluorescent sediment samples decreased the amount of time required for analysis. No short cuts were possible in the preparation of the filters themselves, since this would have jeopardized the integrity of the sample analysis, but the filtration

apparatus could be assembled while the samples were being fixed. DAPI staining the samples was possible during the last 10 min of fixation. This was never found to be a problem with any sediment samples from the U.S.G.S. site. The length of the staining time for DAPI did not appear to be important. Anywhere from 5 min to 24 hr has been reported for bacteria (Porter and Feig, 1980).

The MS experiment was used to demonstrate the effect of the number of scans per field on the confidence in the estimation of MS/mL. The maximum probable error, E, was calculated at the 95% confidence interval for MS/mL estimates based on an increasing number of scans per filter:

$$E = z_p \left[\frac{\hat{\sigma}^2_{sc}}{sc}\right]^{\frac{1}{2}} \qquad [11]$$

where, Z_p equals the t-value (n=16) for the chosen confidence level; $\hat{\sigma}^2_{sc}$ is the variance of the estimate for all the scans; and "sc" equals the number of scans (Provost, 1984). The maximum probable error can be considered as the detectable difference (i.e., in order to determine two estimates to be significantly different they must differ by their average $\pm$ the detectable difference at a given confidence level). Figure 13 shows the detectable differences for the enumeration of MS depending on the

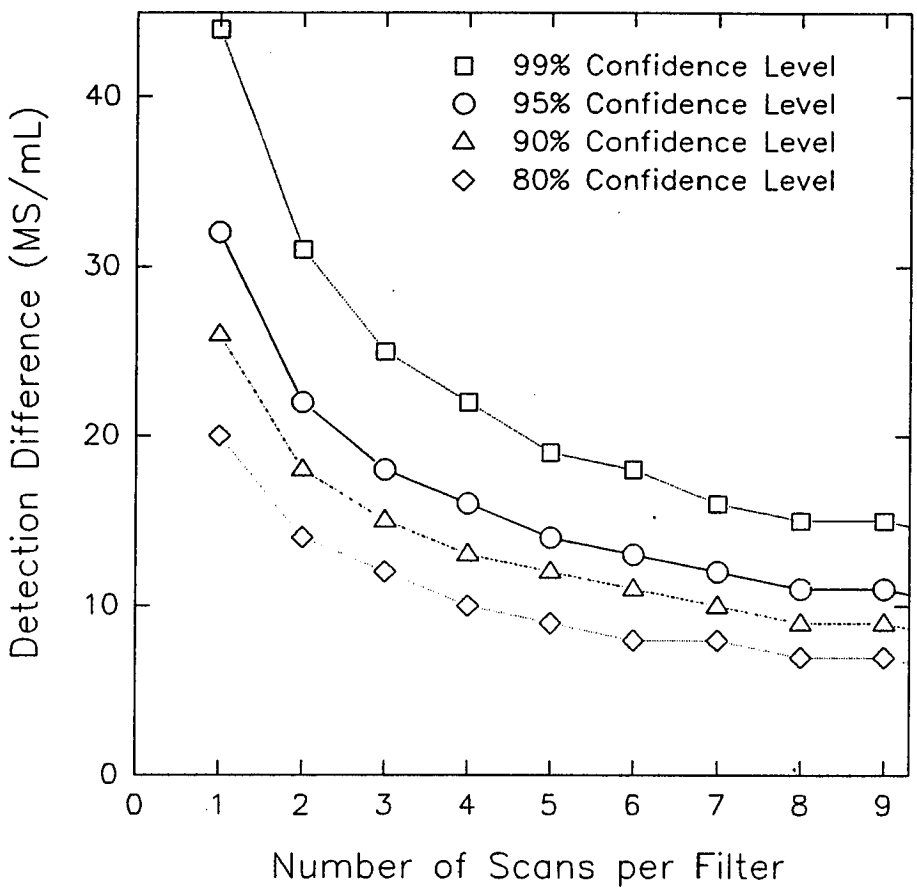


Figure 13: Detectable differences for the enumeration of microspheres (MS) on filters using the scanning method with a given confidence level.

number of scans per filter for the 80, 90, 95 and 99% confidence level. The greatest change in the detectable difference occurred when the MS were enumerated over 2 scans compared to only 1 scan. After 3 scans the amount of information gained by each additional scan decreased further, and there was no relative change in the detectable difference after 8 scans per filter at any confidence interval. A lower confidence level could be accepted if a lower detectable difference was desired. For example, the detectable difference was 16 MS/mL for 4 scans with 95% confidence which is greater than the difference of 14 MS/mL for 2 scans with 80% confidence.

The detectable differences calculated for the observation of MS on a filter cannot be applied directly to the enumeration of protozoa from sediments collected at the U.S.G.S. site (see Section III.C. for the errors of the epifluorescent enumeration of sediment samples) since the population of MS/mL ($\sim 3 \times 10^3$ enumerated on the filter, Table 3) was more then 6 times the average population of protozoa/mL ($\sim 5 \times 10^2$ enumerated on filters from F 393 and S 318 samples, see Table 13). Cassell (1965) showed that the average number of cells per field affects the detectable difference of such low populations as are found in the sediments. However, the maximum probable error due to the number of scans per filter calculated for the MS does show

that a minimum number of scans can be conducted without changing the detectable difference.

Three scans/filter were chosen as the optimum counting technique for the enumeration of DAPI stained protozoa in sediment samples based on the results of the MS experiment and considering the time necessary to count the filters. For the same area of the filter to be observed by both counting techniques, 150 fields would have to be examined in order to equal the area of 3 scans (3.76% of the filter). The amount of time required to enumerate this many fields was 1.5 hr compared to only 45 min for three scans. Since the examination of filters was tedious, the technician found that observation on the epifluorescent microscope was not possible for more than 5 hr and thus only by using the scanning technique one core (i.e., 6 slides) could be enumerated in a day (4.5 hr for scanning vs. 9 hr for counting fields).

6. Cost Considerations

The type of filters and stains used during the development of the epifluorescent enumeration technique did affect the cost of the analysis. Black Nuclepore^{R} filters cost ~$0.12 more per filter than unstained ones. Since the Irgalan black stain is so concentrated, the same number of filters could probably be prepared for less money. However,

the quality of the filters stained in the laboratory was lower than for the black Nuclepore^{R} filters. The darkness of the background and occasional problems with background fluorescence with the laboratory stained filters affected the integrity of the sample's enumeration.

PC black filters are now available from Poretics^{R} (Livermore, CA) at a lower cost (~$0.10/filter). The filters appeared to perform similarly during vacuum filtration and no difference was apparent during microscopic examination.

DAPI was a more expensive stain than acriflavin ($0.06/DAPI sample vs. >$0.01/acriflavin sample). All of the aspects related to increased contrast between the cells and abiotic particles on the filter justify the additional cost for DAPI.

7. Summary for Epifluorescent Enumerations

Counting protozoa on filters can be reliable if the cells are evenly distributed across the filter and easily distinguished from abiotic particles. The integrity of the cells must be protected by using buffered solutions for the shaking procedure and fixation of the organisms, and also a low vacuum (<13 mm Hg) applied during filtration. It is important to ensure an even distribution on the filters by using a wet backing filter under the PC filter and careful

handling of the filter after filtration to prevent redistribution of the particles. Interferences with the observation of the filters can be limited by placing the PC filter as flat as possible on a drop of Cargille^{R} A immersion oil. The epifluorescent stain, DAPI, created the greatest contrast between the protozoa and abiotic particles on the filters. The choice of counting method, fields or scans, was dependent on the population of cells on a filter. Scanning the filter was faster than counting fields using the epifluorescent enumeration for protozoa at the U.S.G.S. site.

B. Darbyshire Liquid Media MPN Enumeration

Fall 1990 and 1991 core samples from the U.S.G.S. site were enumerated with the Darbyshire liquid media MPN method using the modified procedures of Darbyshire and his associates (1973, 1974). Handling of the core material through the shaking technique was not unlike their processing of topsoils in soil extract. Other modifications to the procedure concerned changes in laboratory equipment since the 1970's. This section discusses the modifications of the procedures for use with aquifer material and the potential effect on observation of the microtiter plates. The results of an experiment to determine the effect of using a supplemental bacterial source are also discussed.

1. Observation of Microtiter Plates

The microtiter plates prepared for the Darbyshire liquid media MPN enumerations were incubated at 12°C for at least 12 days. After incubation, they were removed and examined on an inverted microscope. The incubation temperature was similar to the temperature found in the ground water at the U.S.G.S. site. The first samples prepared from cores collected during Spring 1990 were observed after 5, 10, 12 and 18 days incubation. Protozoa were not observed in any of the microtiter wells after 5 days of incubation. Protozoa were observed in the same wells after 10, 12 and 18 days of incubation. Thus, 12 days was chosen as a convenient time to analyze microtiter plates for the presence of protozoa. In some cases, protozoa were not observed after 12 days of incubation in even the first row of wells or there was an excessive number of bacteria obstructing the view of protozoa. In these cases, the plates were incubated a few more days and then reexamined (e.g., 14 or 16 days of incubation). The lids on the plates remained in place throughout incubation and examination to prevent evaporation and contamination.

Detecting the presence or absence of protozoa in the microtiter plates involved looking for moving particles that were 2-5 μm and shaped like flagellates and amoebae. Plates that were below room temperature could not be observed for

~10-15 min because temperature gradients in the wells caused all particles present to move. Protozoan activity was distinguished from Brownian motion by searching for particles that were moving from one location to another rather than those quivering in place. Once the plate had adjusted to room temperature, extraneous particle motion was minimized. The best way to become familiar with how to find protozoa was by observing cultures of subsurface protozoa (prepared by adding a few grams of sediment from a core to a liquid medium (e.g., Cerophyl-Prescott's infusion; Page, 1988)).

The flagellates typically present in the plates were about 2-3 μm diameter and their flagella were almost impossible to discern using the inverted microscope at 300-600 X magnification, even using Hoffman modulation contrast optics. The resolution of the protozoa could not be improved since the plastic plates were not as optically clear as glass slides and the light was refracted around the edges of the wells. The Hoffman modulation contrast optics was advertised as more suitable for observation through plastic material than Nomarski interference optics since the refracted light of Hoffman optics does not distort the polarized light as much as the Nomarski optics (Hoffman Modulation Contrast System, Modulation Optics Inc., Greenvale, NY). 300 X magnification (20 X objective in

conjunction with 15 X oculars) was often better for scanning the wells than 600 X magnification (40 X objective in conjunction with 15 X oculars). However, the higher magnification was used to assist in identifying particles suspected of being protozoa. This was particularly critical for identifying the slow moving amoebae, though they were rarely observed in the microtiter plates.

2. Modifications for the Darbyshire Liquid Media MPN Method

Relatively few modifications to the original procedures in the literature were necessary for the use of the Darbyshire liquid media MPN technique with the core material. Darbyshire and his associates (1973, 1974) applied the technique to several types of topsoils. All had higher organic content and smaller grain size than the sediments at the U.S.G.S. site. They excavated their samples, sieved the material and then added 10 g of soil to 50 mL of sterile soil extract. The "original dilution" was then shaken for 5 min on an orbital shaker before using the liquid fraction for the dilution series in microtiter plates.

The shaking technique (Dye, 1979) used for the core material differed primarily in the dilution factor, type and length of shaking time. 5 g of core material was diluted in 75 mL of phosphate buffer. This dilution factor was chosen

primarily to be compatible with the epifluorescent technique, where excessive sediment particles were detrimental. In this manner, the same diluted sediment sample could be used to minimize sample preparation and assist in the comparison of both enumeration techniques.

The shaking technique essentially washed the core sediments with three 25 mL aliquots of phosphate buffer rather than simply suspending the material in the diluent. Dye (1979) called for the diluted sediments to be "gently shaken by hand for 60 sec" before decanting the liquid portion. The length of time for each shaken aliquot was shortened to 30 sec for the Canvey Island sands, similar to the mixing times used by Balkwill and Ghiorse (1985) for the characterization of subsurface bacteria. The delicate interstitial ciliates found in the Canvey Island sands were thought to be incapable of surviving the longer and stronger shear forces created by mechanical shakers. However, the flagellates and amoebae found in the core sediments are very different from ciliates. No tests were performed to optimize the shaking procedure for the core material.

Octapettes^{R} have become available since Darbyshire et al. (1974) was published. They compared use of wide-mouth glass pipets to microdiluters, and found the enumeration results similar. Like the microdiluters, the Octapettes^{R} do eight dilutions simultaneously. Darbyshire et al. were

concerned that the rinsing process of the micordiluters' vanes between dilutions was not sufficient to prevent cross-contamination of the wells. Since the pipet tips could easily be replaced on the Octapette^{R} between dilutions, cross-contamination was a function of how well the instrument was used and not a function of tip cleaning. The liquid had to be pipetted slowly to prevent any of the sample from contaminating the Octapette^{R}. Recently, disposable pipet tips with acetate filters have become available that would help to prevent this kind of contamination in the microtiter plates.

The other modification for the U.S.G.S. site material was the use of a computer program to calculate MPN values. Darbyshire (1973) used Fisher's (1922) "method for negative plates" and the tables in Fisher and Yates (1943) for the calculation of MPN values. Each table was specific for the number of replicates within a dilution series (e.g., 8 replicates for the Darbyshire MPN enumerations). The computer program (see Appendix A) used for all MPN calculations on the U.S.G.S. site samples was based on a similar equation to that used by Fisher and Yates, and the results were comparable to within 10 MPN/gdw.

The advantages of determining MPN values with the computer program are its flexibility and convenience for the number of replicates and different dilution schemes. For

example, the dilution series in the microtiter plates resulted in different volumes for the 12 rows (see Figure 9). MPN tables for this procedure are not available, but there are equations available to modify the tables. The volumes could have been equalized throughout the dilution series, but this would have required additional pipet tips and increased the chances of contamination. The computer program allowed entry of different volumes and dilution factors for every dilution level necessary to determine extinction of protozoan activity (see Appendix A). It could also be modified to assume the volumes of a standardized procedure as used with the enumeration of core samples.

The search pattern for the analysis of a microtiter plate was based on the least amount of information necessary for the MPN calculation to minimize the time required to observe the plates. That is, the program first asks for the number of rows to extinction (negative activity for all wells within a row) and the starting row where extinction begins (the first row with $\geq$ 1 negative well). Only the number of wells, sample volume (in mL) and dilution factor are then entered for each row prior to the initiation of extinction because all wells in these rows are assumed to have positive protozoan activity. Information on the number of wells, volume, dilution factor and number of positive replicate wells is required for all rows throughout the

dilutions to extinction. The program calculated the MPN/mL corresponding to the population estimate in 1 mL of the same dilution as in row 1 and did not consider the dilution due to the phosphate buffer, amount of sediment or moisture content.

Considering the example seen in Figure 9, the number of rows to extinction is 9 and the starting row where extinction begins is 6. Therefore, the computer only asks for the dilution factor, sample volume and number of wells for rows 1 through 5; the number of positive replicates in these rows is assumed to be 8. At row 6, the computer starts to prompt for the number of positive wells (row 6 = 7; row 7 = 3; row 8 = 3; row 9 = 0). The MPN/mL is calculated ("for a sample with dilution factor 1 and volume 1 mL"). Thus, if the program coding is modified for the Darbyshire liquid media procedure (i.e., 0.15 mL in the first row, 0.20 mL in rows 2-11, etc.) then after the initial questions the program only asks for information at the row where extinction started.

The search pattern provides <100% of the information available from a microtiter plate (i.e., not all wells are observed). The disadvantage with the pattern is that the presence of protozoa in the wells after extinction or any irregularities in the wells at low dilution would not be observed and yet these cases would affect the MPN value.

The computer program includes a "deviance" value in the output for each MPN calculation which is an indicator for the validity of the MPN calculation. When the "deviance" exceeds the 5% Chi^2 value, as under the circumstances of contamination, the MPN value would be rejected (see Appendix A). None of the Darbyshire MPN estimates for the cores collected in Fall 1991 were rejected as indicated by the "deviance". Although the search pattern does not provide a complete data set for the MPN estimation, the time saved by minimizing the number of wells observed could allow for more subsampling of the core material which would decrease the maximum probable error of the estimation (see Section III.C.). Sterile controls also indicate contamination.

3. Experiment on Supplemental Bacterial Source

The Darbyshire liquid media MPN enumeration is an extinction-dilution technique where the organisms are serially diluted to a level in which there are no more protozoa present. Generally, MPN techniques are based on the assumption that organisms grow and reproduce at the different dilutions. Often MPN techniques use gas production or turbidity as an indicator of the presence of the test organism. It is not clear whether the protozoa in the Darbyshire plates actually grow and multiply during incubation.

One core sample was used to determine if the protozoa needed a source of bacteria in addition to the indigenous population in the sample. Three replicate plates were prepared with one of the following dilution solutions in rows 2-12: (1) phosphate buffer (unseeded); (2) buffer inoculated with *Escherichia coli* sp.; (3) buffer inoculated with heat-killed *E. coli*. Protozoa are known to be selective in the types of bacteria they will consume (Severtzova, 1928; Singh, 1941; Clarholm, 1981; Fenchel, 1982; Foissner, 1987). *E. coli* was chosen since the species is commonly used as a food source for a variety of flagellate and amoebae cultures (Thompson et al., 1988) and was readily available at the time of the experiment. Protozoan cultures are often given heat-killed bacteria to limit the quantity of food present and prevent excessive bacterial growth (S. Brown, personal communication, 1990).

The plates inoculated with the live *E. coli* were difficult to examine since several of the replicates had an overgrowth of bacteria in the bottom of the wells even after 20 days of incubation. The patchiness of growth in the samples was a problem since the abundance of bacteria could have been due to the absence of protozoa in the well or the lack of protozoan grazing (possibly due to bacterial species preference or use of an alternative source of organic material).

There was no significant difference (P>0.05 based on a t-test) in the MPN/gdw calculated from the results of the plates seeded with heat-killed *E. coli* (1.97 x 10^5 MPN/gdw) and the unseeded plates (1.56 x 10^5 MPN/gdw). Protozoa became diluted out after 6 rows (dilution factor=3.2 x 10^{-4}) in both cases. Darbyshire (1973), Darbyshire et al. (1974), and Couteaux and Palka (1988) also found that the natural biota in the diluted samples was sufficient for the enumeration of protozoa in microtiter plates. Baldock (1986) concluded that the addition of bacteria to the dilutions resulted in a reduction in the total protozoan population estimate from fresh water sediments. Since the natural populations present in the samples from the U.S.G.S. site appeared to suffice for the needs of the incubating subsurface protozoa, all further samples were prepared without the addition of bacteria, eliminating the time required to prepare the amended dilution solutions and problems of bacterial overgrowth.

C. Singh Solid Media MPN Enumeration

The Singh solid media MPN technique was also used for enumerating core samples from the U.S.G.S. site. Solid agar was the surface available for culturing the protozoa in serial dilutions, similar to the process with the Darbyshire liquid media MPN enumerations. This section will discuss

the different preparations of the agar plates used over several sampling periods, and problems with incubation and detection of the protozoa on the solid surfaces.

1. Plates Used with the Singh Solid Media MPN Enumeration

Singh (1946) used glass rings to divide the agar surfaces in petri dishes into smaller sections that could be more easily examined for the presence or absence of protozoa. Cutting the numerous glass rings needed for all the replicates and dilutions was time consuming, expensive and difficult. Plastic tubing was substituted for glass (Elliott and Coleman, 1977) in the core samples analyzed in Spring 1990 and Summer 1991 since the plastic was less expensive and much easier to cut into rings. The plastic was not difficult to cut with a shear (designed to cut slotted angle framing), however, many of the rings had to be discarded because they were not a consistent height or did not fit inside the petri dishes. After the plates were examined for protozoa, the rings were removed from the agar, washed in soapy water, packed into glass beakers and sterilized before reuse. Each plate took ~45 min to prepare including cutting of the rings and cleaning them for reuse.

For the samples analyzed in Fall 1991, the solid agar was poured into the 6 wells of the "macrotiter" plates. The protozoa observed in the wells of the macrotiter plates or

plastic rings did not appear to be different, although the same samples were never compared using the different plates for Singh enumerations. Other researchers have used the macrotiter plates without problems (M.L. Krumme, personal communication, 1990; J.L.Sinclair, personal communication, 1992).

There were several advantages to the macrotiter plates: they were easier to prepare (~15 min/plate including inoculation of bacteria into each ring); contamination due to opening the plates to arrange the rings in the molten agar was eliminated; and all of the wells were the same depth, thus the chance for cross contamination between wells was decreased. However, the macrotiter plates were not reusable and they were twice as expensive as the plastic rings in disposable petri dishes ($1.50 vs. ~$0.70 per plate).

2. Incubation of the Singh MPN Samples

The greatest difficulty with incubating the plates for the Singh solid media MPN enumerations was evaporation of the liquid. Evaporation was not a problem during incubation of the microtiter plates (Darbyshire enumeration) because those wells had a smaller surface area to volume ratio compared to the macrotiter plates (1.60cm^2/mL vs. 9.40cm^2/mL). Singh (1946, 1955) mentions that he added

extra saline solution to the rings to enhance growth of ciliates and flagellates on the agar surfaces. Based on experience with both types of plates, a liquid layer above the agar could be maintained by adding 2.5 mL of sterile phosphate buffer to each ring once a week during incubation. However, the process of transferring buffer was both time consuming and increased the chances for contamination.

Bacteria were inoculated into each ring of the plates for protozoan grazing in the Singh solid media MPN enumerations. Sinclair and Ghiorse (1987) tested the preference of the subsurface protozoa for *Enterobacter aerogenes* vs. indigenous bacteria with their enumerations and found equivalent MPN values with both food sources. The bacterium used in the Singh solid media enumerations for Summer and Fall 1991 samples was isolated (on standard plate count agar) from ground water collected from within the plume at the U.S.G.S. site (well F 473, near F 383). The inoculum was prepared by adding enough bacterial culture to 10 mL of phosphate buffer to turn the buffer turbid (10^6 bacteria/mL; A. Warren, personal communication, 1990). The bacteria were not heat-killed. Each ring was inoculated with a loop of bacterial solution and the plates were allowed to sit for at least 1 hr prior to diluting them with sediment extract. No experiments were performed to compare

the Singh enumeration procedure for plates incubated without a bacterial source or with heat-killed bacterial inoculum.

The incubation time of the agar plates was dependent on the decrease of bacteria with time. If there were too many bacteria after 12 days of incubation, then the samples were reexamined several days later. Protozoan grazing on the bacteria was not observed and the flagellates and amoebae appeared to be the same size as in the wells of the microtiter plates for the Darbyshire enumerations where bacterial numbers were much lower. The sterile wells in the acid-treated macrotiter plates were examined for contamination. Sinclair (personal communication, 1992) has found that cross-contamination of wells occurred occasionally in samples incubated for ~30 days.

3. Detection of Protozoa in the Singh MPN

Sinclair and Ghiorse (1987) used a similar procedure to that described in the Methods and Materials for detecting protozoa. The examination of the wells required ~1.5 hr, about the same amount of time necessary for the observation of the Darbyshire liquid media MPN samples, but fewer were observed for each dilution (i.e., 3 replicates/dilution in the Singh MPN and 8 replicates/dilution in the Darbyshire MPN).

Flagellates and amoebae from the Singh plates were not difficult to detect with the use of Nomarski interference optics because flagella and internal organelles were easier to distinguish making positive identifications possible. In these samples, the numbers of each type of protozoa were not recorded, but should be included in future studies. MPN calculations with computerized programs were not as difficult as with the Darbyshire liquid media technique since the volume of the dilutions for the Singh solid media plates were all equal.

Flagellates were the most dominant type of protozoan present, however, amoebae were found more frequently on the Singh solid media (~5 % of the total protozoa observed) compared to the Darbyshire liquid media (<1%). Solid media is more commonly used to culture amoebae than other types of protozoa (S. Brown, personal communication, 1990) and many ecological studies have used solid media for characterizing soil amoebae (Severtzova 1924, 1828; Singh 1941, 1946, and 1955; Elliott and Coleman, 1977; Alabouvette et al., 1981). Also, Sinclair and Ghiorse (1987, 1989) reported finding greater proportions of amoebae in some layers of the subsurface when the protozoa were enumerated on Singh MPN plates. Whether amoebae prefer solid to liquid media has not been established, however, the observed differences in the diversity of the protozoa by both MPN techniques does

support the use of more than one type of enumeration technique.

D. Qualitative Comparison of Enumeration Techniques

The epifluorescent direct count, Darbyshire liquid media and Singh solid media enumeration techniques have never been compared analyzing a common sediment sample. Some basic differences between the techniques probably influence the counts derived including: 1) separation of the cells and sediment particles; 2) direct examination vs. culturing; 3) methods of detecting and distinguishing organisms from soil particles; 4) liquid vs. agar media; 5) and variability of the estimate due to the replication within the procedure. Finally, the analyses also differ in the amount of time required to enumerate a core sediment, though this does not directly affect the population estimates.

1. Separation Techniques

The shaking technique used for the epifluorescent and Darbyshire MPN methods was based on Dye (1979), whereas the separation procedure for the Singh MPN method was based on Sinclair and Ghiorse (1987). The Singh MPN samples included an undiluted sample as well as serial dilutions which was not possible to prepare from the shaking procedure.

The amount of sediment used from the core in the Singh technique also differed. The shaking technique for the epifluorescent and Darbyshire samples used 5 g of sediment in 75 mL of phosphate buffer. Several samples were removed from the core for a Singh MPN estimate: each 10^0 well received 1 g of sediment (for the total estimate); and the dilution series was prepared from 1 g of sediment in 10 mL of phosphate buffer mixed on the Maxi-mixer^R (for the total and encysted estimates). Thus, eight 1 g samples of sediment were removed from the core for duplicate Singh enumerations and only 5 g of sediment was removed for the epifluorescent and Darbyshire MPN enumerations.

Since all three techniques are not prepared from the same sediment aliquot from the core there is the potential that the estimates might differ from the heterogeneity in the sediment instead of the enumeration technique itself. There was no separation of the cells and sediment in the undiluted (10^0) replicates used in the Singh samples. All of the MPN calculations for the Singh samples from Fall 1991 were equally affected since all of the undiluted and 10^{-1} diluted replicates were positive for the presence of protozoa. There was some selection involved with removing an aliquot of sediment from the core. The sediment around the edges of the core were avoided to limit contamination from the core sleeve. Also, the aliquot removed from the

core had to have several sand grains; a piece of gravel that weighed ≥1 g was rejected as a sample. The heterogeneity in the sediment samples was included in the analytical variability of the slide component of variance for the epifluorescent technique and the subsample component for the MPN techniques for the hierarchical design (see Section III.C.3.)

2. Direct Counting vs. Extinction/Dilution Techniques

The major difference between the enumeration techniques is the examination of live vs. fixed sample material. Direct examination of the sediment itself was not possible due to the small size and abundance of the protozoa compared to the sediment particles. Hence, the organisms had to be separated from the sediment. Epifluorescent enumeration is a direct counting technique, however, it only includes the protozoa separated from the sediment by the shaking method. Unfortunately, soil particles which were carried into the shaking extract, occasionally obscured the view of protozoa and thus affected the enumeration of the sample. Trophic and encysted protozoa both contain DNA which can be stained by the DAPI and appear blue when exposed to UV light. Trophic flagellates could be differentiated from amoebae and cysts if their flagella were still present. However, differentiating between cysts and amoebae was not possible

because the fixed organisms were all similar in size, shape and fluorescence. Only flagellates, whose flagella remained intact through the preparation of the filters, could be discerned from other types of cells.

Live organisms were observed in the Singh and Darbyshire extinction/dilution methods. Both of these enumeration techniques had the advantage of differentiating between trophic and encysted protozoa by acidification. The motion of the live organisms offered another means for characterizing the protozoa in addition to their size and shape. Flagellates and amoebae could be distinguished in the MPN methods since the former are typically far more mobile than the latter. However, enumeration of the MPN techniques can be seriously affected by the selectivity of the medium or availability of an appropriate food source for the protozoa.

3. Detection and Distinction of Protozoa

The Singh solid and Darbyshire liquid media techniques differed in the type of microscope and optics used to examine the samples. In the Singh method, the protozoa were easy to detect with the Nomarski optics since the polarized light emphasized the organelles and flagella (if present). Since the amoebae moved, they could be distinguished from cysts. The cell wall of the cysts was also apparent under

the polarized light which distinguished them from the soil particles. However, during the transfer of the contents from the agar wells to the slides some protozoa could have been missed or contamination might have occurred which could have affected the enumeration of the sample.

Protozoa were more difficult to find in the Darbyshire liquid media samples. The inverted scope was used and the organisms were viewed through plastic which affects the resolution of all objects. Slow moving amoebae and cysts were not easy to find and could easily be overlooked, which could have affected the enumeration of the sample. However, the samples were examined in situ, unlike the Singh solid media, and thorough scans could be made in each well to confirm the absence of protozoa.

4. Liquid vs. Solid Media

Different species of protozoa are known to prefer either liquid or solid media. Facilities that culture protozoan species for research and for sale generally grow small amoebae on agar plates and small flagellates in liquid media (Thompson et al., 1988; S. Brown, personal communication, 1990). Since amoebae glide on surfaces and flagellates generally swim or attach and wave their bodies in the passing fluid, these types of protozoa probably prefer the medium that best facilitates their type of motility.

Sterile phosphate buffer was added frequently to maintain a liquid layer above the agar in the Singh solid media enumerations and this probably contributed to the observation of both amoebae and far more flagellates than have been reported in other subsurface sediment samples.

Sinclair and Ghiorse (1987) looked at subsurface sediments in Lula, OK, and found 95% of the protozoa (enumerated with the Singh solid media MPN technique) were flagellates and the rest were amoebae in the sand and gravel layer. The same proportion of flagellates to amoebae were found with the Singh technique in the sand and gravel sediments at the U.S.G.S. site. Sinclair and Ghiorse did report the discovery of a new species of filose amoebae. Some species of amoebae have flagellated stages during their life cycle. However, there has not been any extensive work conducted of the types of flagellates and amoebae present in the subsurface (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990; Kinner et al., 1991; Madsen et al., 1991), and the proportion of flagellates that represent amoebic species is not known.

The dilutions of both MPN techniques required ~12 days of incubation before protozoa could be analyzed, but there was no evidence of growth of the organisms with time. The organisms could have encysted due to the stresses during preparation of the MPN techniques and not been apparent

during the early days of incubation. Cysts are difficult to detect in the Singh plates if high bacterial populations are present. In addition, the Hoffman optics used were not sufficient to distinguish them from other particles in the microtiter plates. If the protozoa had grown due to consumption of bacteria on the Singh plates, they probably would have been larger in size than those in the Darbyshire enumerations. Also, the cells were never observed consuming bacteria or dividing into daughter cells in either MPN technique. Instead, the incubation period seemed to be required for the trophozoites to reappear after they had acclimated to the conditions of the enumeration procedure.

5. Comparison of Space Requirements for the Enumeration Techniques

Since the epifluorescent slides took less time to prepare and observe, 6 slides were generally fixed for each core sample: 3 replicate samples from each core (i.e., each replicate was 5 g of sediment processed with the shaking technique) and 2 slides per replicate. Also, each slide was enumerated by 3 scans, which covered more area on the slide compared to procedures generally used for enumeration of direct counting procedures (Cassell, 1965) and thus should have improved the precision of the analysis.

Only 2 replicates per core were prepared for the MPN methods, since they were more difficult to prepare and

analyze and enumeration of the encysted protozoa was also performed. However, the two MPN methods contrasted in the number of replicates per dilution and the dilution series used. The microtiter plates for the Darbyshire liquid media MPN method had 96 wells, which were convenient for the preparation of 8 replicates per dilution of sample. However, the macrotiter plates of the Singh solid media MPN method had 6 wells. Hence, only 3 replicates/dilution and 2 dilutions/plate were used for the Singh MPN estimates due to space limitations in the incubator and the time required to examine subsamples from each well.

The dilution series for the MPN methods also differed. The Darbyshire samples were prepared with one-fifth dilution series (due to the types of Octapettes^{R} available). Thus, with 12 rows available in each microtiter plate, 10^{-9} dilutions were possible. However, the Singh samples were prepared with a one-tenth dilution series (for convenience). In this case, the number of dilutions prepared depended on the number of macrotiter plates that were used, generally only three for a maximum 10^{-5} dilution. If the most diluted replicates in the Singh samples were not all negative, the MPN/gdw was not definitive (MPN/gdw could only be given as greater than a certain value).

The replication with each enumeration technique also affected the amount of space required to store the samples.

The epifluorescent slides were stored in microscope slide boxes and could be kept for up to one month prior to observation. The microtiter plates for the liquid media were compact considering each plate contained 8 replicates/dilution and 12 dilutions/plate, and 4 plates (2 for the acid-treated samples) were required per sediment sample. Unfortunately, a large amount of incubator space was required for the macrotiter plates since each had only 2 dilutions/plate. Therefore 3 plates were necessary for the dilution series of the total and acid-treated samples, a total of 12 plates per core.

6. Time Requirements for the Enumeration Techniques

Each type of enumeration technique required different amounts of time for general preparation, processing of samples and examination (Table 5).

The epifluorescent samples required the least amount of time for the enumeration of the sediment samples. General preparation time consisted of making the DAPI stain and setting up the filtration apparatus with filters. The shaking technique was the same for the epifluorescent and Darbyshire MPN techniques. The time required to fix, stain, filter and mount the 6 filters prepared per core was included as time for the processing of the sample. Only the

Table 5: Estimated time required for each step in the processing of the epifluorescent, Darbyshire liquid media MPN and Singh solid media MPN enumeration techniques.

Time Per Core Sample*	Epifluorescent Enumeration (min/sample)	Darbyshire Liquid Media MPN Method (min/sample)	Singh Solid Media MPN Method (min/sample)
General Preparation	10	30	40
Shaking Technique	15	15	15
Processing of Sample	50	80	70
Observation	270	360	360
Total	345 (5.75 hr)	485 (8.08 hr)	485 (8.08 hr)

*Assuming:
6 epifluorescent slides/core (2 slides/subsample and 3 subsamples/core);
4 Darbyshire liquid media MPN estimates/core (2 subsamples/ core for total MPN and 2 subsamples/core for encysted MPN);
4 Singh solid media MPN estimates/core (2 subsamples/core for total MPN and 2 subsamples/core for encysted MPN).

scanning technique was considered for determining the observation time since counting fields was too difficult for the enumeration of the low populations of protozoa in the sediment (see Section II.A.5).

The MPN methods involved essentially the same amount of time. Preparation prior to sampling for the Darbyshire method involved sterilizing the equipment, and filling and labeling the microtiter plates. However, the Singh method

was more involved since the procedure called for a bacterial inoculum which had to be maintained, even between collection of core samples. Since there were less wells in the macrotiter plates to be diluted, the Singh technique required less time to process the samples than Darbyshire. Observation time was the same for both MPN techniques, but longer than for the epifluorescent technique.

The discrepancy between the epifluorescent technique and MPN methods in total time required to enumerate a core was due to the shorter time for observation of the samples. A lower proportion of the epifluorescent filter was enumerated compared to the amount of area that had to be examined to determine if protozoa were absent from an MPN dilution. To a lesser extent, the time required for general preparation of the epifluorescent technique was shorter than that for the two MPN methods, which also contributed to the shorter total time necessary to process a core.

III. QUANTITATIVE COMPARISON OF ENUMERATION TECHNIQUES

In the Fall 1991, 8 cores were taken at two locations at the U.S.G.S. site and used to compare the protozoan enumeration techniques and the variation associated with the sites, the hold time of the cores, estimations of the total population and encysted protozoa (MPN techniques), and the use of sterile and non-sterile core sleeves (Table 6).

S 318 and F 393 were considered the contaminated and uncontaminated sites, respectively (see Figure 1). A hierarchical design was used for the sampling program at the two sites to determine the variation associated with the site, cores and subsamples. This was then used to estimate the minimum sampling regime necessary for an acceptable detectable difference. From previous sampling events, the microbial population was known to be greater at S 318 than F 393. Therefore, more extensive analyses were performed on the S 318 cores since the higher population estimates increased the chances to detect differences in the protozoan enumerations that could be attributed to the hold time or contamination from the core sleeves.

A. Data Analyses

To use parametric statistics, the enumeration data had to be evaluated to determine if the assumptions of 1) random sampling, 2) normal distribution of the data, and 3) homoscedasticity or homogeneity of variances were met (Hamilton, 1992). Random sampling of the cores and observation of the enumeration techniques are discussed in Chapter 3.

Table 6: Cores collected at the uncontaminated F 393 and contaminated S 318 sites at the U.S.G.S. site.

Site	Core #*	Depth (m)	Core Sleeves	Hold Time (days)	Enumeration Techniques+
F 393	C 15	11.4-11.7	unsterile	14	E D S
F 393	C 16	11.4-11.7	unsterile	14	E D S
F 393	C 17	11.4-11.7	unsterile	14	E D S
S 318	C 15	10.5-10.8	sterile	1	E
S 318	C 17	10.5-10.8	sterile	1	E
S 318	C 18	10.8-11.1	unsterile	1,5,15,28	E D S
S 318	C 19	10.8-11.1	unsterile	1,5,15,28	E D S
S 318	C 21	10.5-10.8	unsterile	1,5,15,28	E D S

*Core numbers were assigned according to how many boreholes had been taken from the site since 1983.

+Enumeration techniques are abbreviated: E = epifluorescent direct counts; D = Darbyshire liquid media MPN; and S = Singh solid media MPN.

All data from the sediment enumeration techniques were found to be positively skewed around the mean (Figures 14-16 A). Power transformations of less than 1 were tested to reduce the skewness of the distributions (i.e., pull in the upper tail) and reduce the influence of outliers (Hamilton, 1992): square root transformations (Figures 14-16 B); log transformations (Figures 14-16 C); and inverse square root transformations (Figures 14-16 D). The transformed data were used in all parametric statistical tests.

As in the MS experiment (see Section II.A.4.b), normal distribution of the data was judged based on the calculation of skewness and Kurtosis of the data (Table 7). Graphical

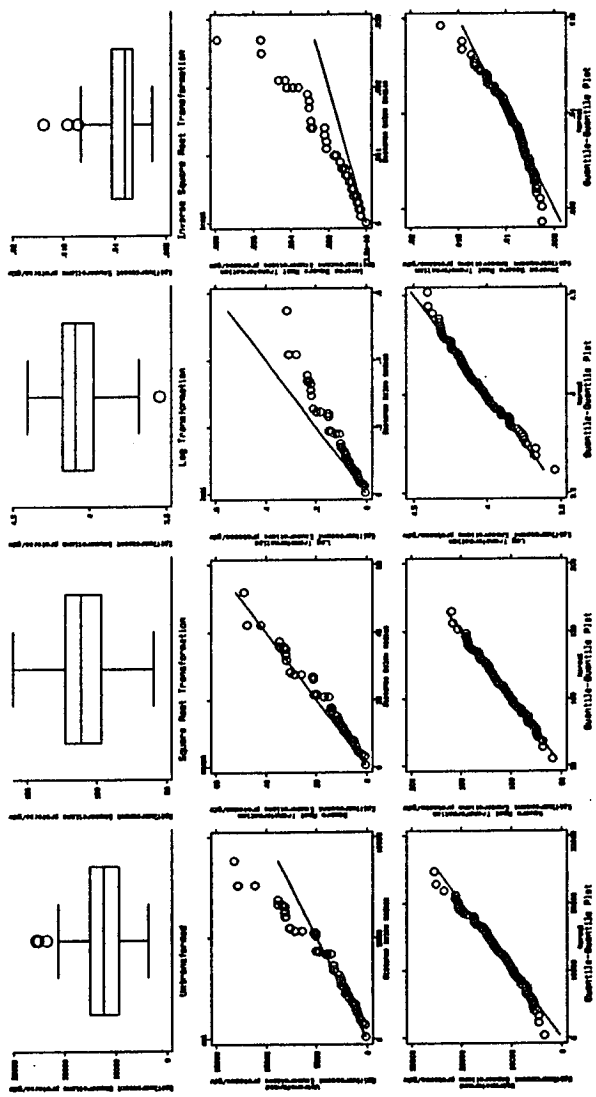


Figure 14: Power transformations for protozoan estimates with the epifluorescent technique: untransformed (A), square root transformed (B), log transformed (C) and inverse square root transformed (D). Box, symmetry and quantile-normal plots are shown to demonstrate that the square root transformed epifluorescent data approximates the normal distribution the best.

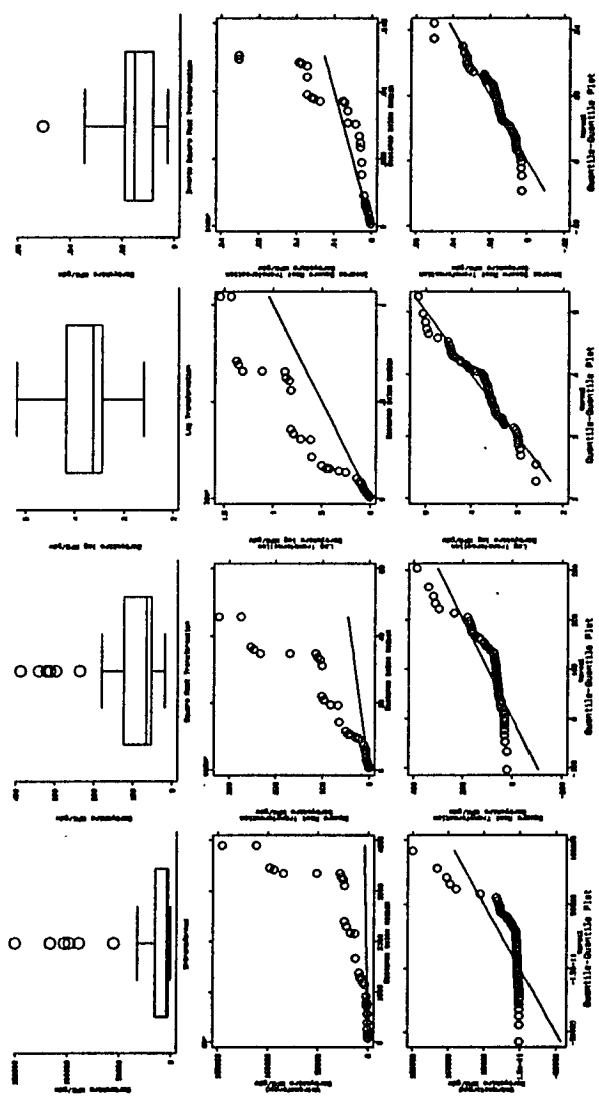


Figure 15: Power transformations for protozoan estimates with the Darbyshire liquid media MPN technique: untransformed (A), square root transformed (B), log transformed (C) and inverse square root transformed (D). Box, symmetry and quantile-normal plots are shown to demonstrate that the log transformed Darbyshire MPN data approximates the normal distribution the best.

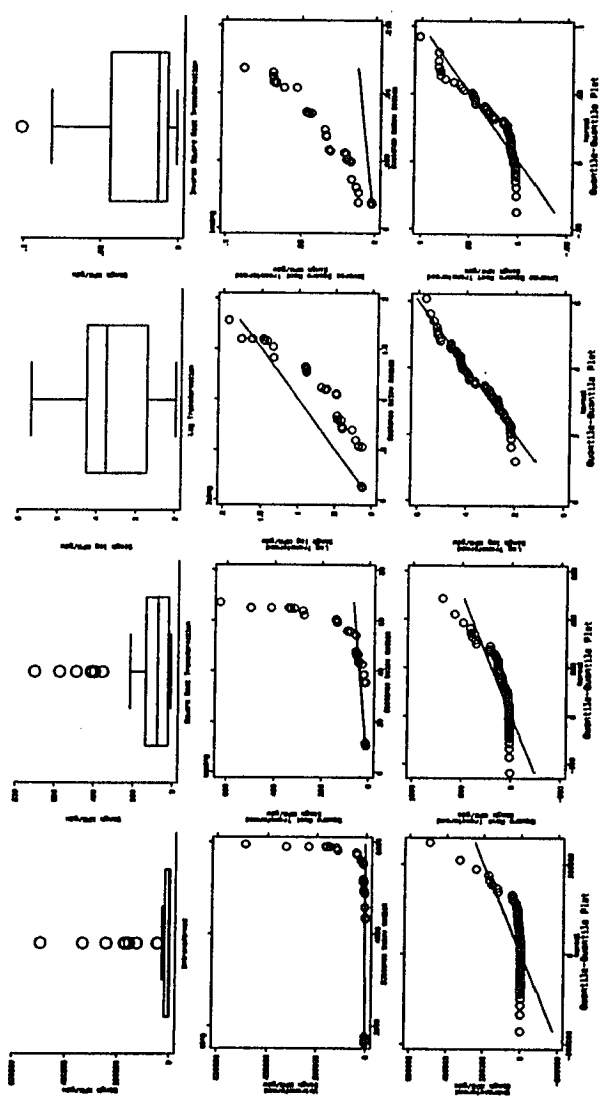


Figure 16: Power transformations for protozoan estimates with the Singh solid media MPN technique: untransformed (A), square root transformed (B), log transformed (C) and inverse square root transformed (D). Box, symmetry and quantile-normal plots are shown to demonstrate that the log transformed Singh MPN data approximates the normal distribution the best.

representation of the data was also used to determine normal distribution. Box plots can show the presence of outliers (circles on the box plots in Figures 14-16) and how they skew the data. The box represents the median (middle line) enclosed by the first and third quantile. The error bars around the box are one standard deviation unit from the sample mean. Symmetry plots show the data's distance from the median (the line shown on the symmetry plots in Figures 14-16). Quantile-normal plots show the data's quantiles around the theoretical normal distribution (the line shown on the quantile-normal plots in Figures 14-16) with the same mean and standard deviation (Hamilton, 1992).

The square root transformation was the best transformation for the epifluorescent technique based on all the measures of the normal distribution. Figure 14 illustrates that the square root transformation of all epifluorescent estimates for the Fall 1991 cores collected at the U.S.G.S. site was closest to the normal distribution. There were no outliers and the median was centered in the box plot for the square root transformation. The square root of the estimates was closest to the median in the symmetry plot and to the normal distribution in the quantile-normal plot. Also, the coefficients of skewness and Kurtosis combined (Table 7) were both closest to the normal distribution for the square root transformation of

Table 7: Coefficients of skewness and Kurtosis for the untransformed, square root, log and inverse square root transformations of the epifluorescent, Darbyshire MPN and Singh MPN estimates from the cores collected in the Fall 1991 at the U.S.G.S. site. Sample size (n) is also given for each enumeration technique.

Enumeration Technique	Transformation	Coefficient of Skewness	Coefficient of Kurtosis
Epifluorescent (protozoa/gdw) (n=102)	Untransformed	0.50	2.92
	Square Root	0.04	2.64
	Log	-0.46	2.97
	Inverse Square Root	1.03	4.20
Darbyshire (MPN/gdw) (n=60)	Untransformed	2.74	9.91
	Square Root	1.83	5.67
	Log	0.48	2.74
	Inverse Square Root	1.22	4.66
Singh (MPN/gdw) (n=60)	Untransformed	3.43	15.68
	Square Root	1.99	6.62
	Log	0.17	1.94
	Inverse Square Root	1.04	2.95

the epifluorescent estimates compared to the others (i.e., skewness ~0 and Kurtosis ~3). The process of counting cells in fields (or by scanning areas), as with the microscopic examination of the epifluorescent filters, follows a Poisson distribution (Cassell, 1965) that can be normalized by taking the square root of the data (Parkinson et al., 1971).

The Darbyshire and Singh MPN counts were found to be normally distributed when the data was log-transformed based on all measures of the normal distribution. Both of the log transformed MPN estimates had coefficients of skewness and Kurtosis combined (Table 7) closest to the normal

distribution. This is best illustrated graphically by the box and quantile-normal plots in Figures 15C and 16C. There are no outliers and the data falls closest to the normal distribution in the quantile-normal plots for both the Darbyshire and Singh MPN estimates. Log transformations for enumeration techniques in which exponential growth of the organisms occurs over time are common (Atlas and Bartha, 1987; Caron et al., 1989; N.E. Kinner, personal communication, 1992).

The third assumption, homoscedasticity, was estimated with Bartlett's test for homogeneity of variance before choosing a parametric test to make comparisons of the data. This estimate involves an F-test to determine if all variances of the groups are equal. When the probability of F is less than the α-level chosen, then the variances are considered equal (Rosner, 1982). All data were tested with the appropriate transformation to yield a normal distribution for any statistical comparisons.

Oneway analysis of variance (ANOVA) was used to determine significant differences in the data if their variances were equal (i.e., homogeneous variances as determined by Bartlett's test). The probability of the F-statistic calculated by the ANOVA was used to determine if a significant difference existed between the variances. If the F-statistic calculated by the ANOVA was greater than the

probability (P<F) of the significance level, then the comparison was considered to be significantly different. STATA^R calculates P-values to the fourth decimal place. In the case of highly significant differences, STATA^R indicates "P>F=0.0000". However, P>F cannot be zero. In this dissertation, such cases of highly significant differences will be reported as "P>F<0.001".

The significance level (α) was considered as the probability of making a Type 1 error (i.e., the probability of rejecting the null hypothesis (no difference between observations) when it should have been accepted). Although α=0.05 is commonly used, for biological sampling an α-level as high as 0.20 (i.e., 80% confidence level) is often acceptable for making a correct decision (Hamilton, 1990). Thus, the risks of a Type I error increase but protects against a Type II error, β, (i.e., the probability of accepting the null hypothesis when the alternative hypothesis (significant difference between observations) should have been accepted). For example, when determining the difference between the protozoan estimates by two techniques, using a larger α (i.e., increasing Type I error) would mean reducing the chance of accepting that the protozoan estimates are significantly different when they are **not** significantly different (i.e., decreasing Type II error).

Although the significance level can be as low as 0.20 (80% confidence), a statistical test that is significantly different at an α=0.05 does not have to be tested at a lower level. In this dissertation, the significance level will be α=0.20 unless all comparisons of a set of statistical tests meets higher α-level (e.g., multiple comparison tests) and the probability value of the statistical tests will be included.

The nonparametric, Kruskal-Wallis test was used to determine significant differences if the variances were inhomogeneous. In the Kruskal-Wallis procedure, all data were assigned a rank and then the ranks were used for the analysis of variance. If the Kruskal-Wallis statistic exceeded the probability of the desired α-level (P>K-W), then the comparison was considered significantly different (Wall, 1986).

Scheffé's test was used to make multiple comparisons of the counting means while holding the "experimentwise" error rate (α_{ER}) the same for all possible comparisons of the samples (Wall, 1986). In this case, the Type 1 error rate describes the probability of rejecting the difference in at least one comparison (null hypothesis) when in fact there was a significant difference (Gill, 1978). The experimentwise error rate, α_{ER}, was calculated with the following equation:

$$\alpha_{ER} = 1 - (1 - \alpha)^k \qquad [12]$$

where α is the significance level for the comparisons, and k is the number of comparisons (Wall, 1986).

The data had to be "untransformed" (i.e., converted back to its original form) to interpret and compare the results of statistical tests with transformed data. The "log of the mean" (geometric mean) was converted by taking the antilog of the transformed mean; the "square root of the mean" was converted by taking the square of the transformed mean. However, the conversion of the transformed standard deviation (or variance) was not as easy to calculate because these estimates were unequal about the mean in the original form. Thus, on a graph the vertical error bars that represent the standard deviation(s) about the geometric mean on a log scale appear to be equal, but on a linear scale the lower bar is shorter than the upper bar. For example, to calculate the lower standard deviation (s_L) from log transformed data: the standard deviation of the log transformed data is subtracted from the mean of the log transformed data (i.e., geometric mean), and then the mean of the untransformed data (i.e., arithmetic mean) is subtracted from the antilog of the difference. The upper standard deviation (s_U) would be calculated similarly, but with the addition of the standard deviation of the log

transformed data and the geometric mean. The same procedure would also be used for square root transformed data except that the value would be squared in place of the antilog (see Appendix B for examples of the calculation). Therefore, a range of the standard deviations for each untransformed mean will be reported.

B. Variability Associated with the Hold Time of the Cores

Cores were often collected faster at the U.S.G.S. site than they could be processed in the laboratory with the enumeration techniques. Since the MPN techniques had to be examined after a specific incubation period, the number of cores processed in a single day depended on the time available to examine the MPN samples after their incubation period. Therefore, the effect of the hold time on the estimation of protozoan populations in the sediments was investigated with three cores from S 318. These cores were enumerated by all 3 techniques 1, 5, 15 and 28 days after they were collected. For the purposes of this dissertation, graphical and statistical comparisons of the protozoan enumerations over the storage period will be presented before their implications are discussed.

1. Graphical and Statistical Comparisons

Figures 17 and 18 show the results of the three enumeration techniques as a function of hold time. Geometric means were used for all protozoan estimates. This was appropriate for the MPN counts since the data approached the normal distribution when log transformed. The vertical error bars represent one standard deviation unit around the means, and are symmetrical about the geometric mean on the log scale. However, there was no such formal plotting procedure available for square root transformed epifluorescent estimates. Hence, the geometric means of the epifluorescent data are presented on the log scale of Figure 17 for ease of comparison. Regression lines were used to show the relationship between the protozoan estimates over the hold period for each enumeration technique (Figure 17, r^2= 0.10, 0.02 and 0.56 for epifluorescent, Darbyshire total, and Singh total enumerations; Figure 18, r^2= <0.00 and 0.29 for Darbyshire encysted, and Singh encysted enumerations, respectively). Table 8 contains all means, and upper and lower standard deviations, and 95% minimum and maximum confidence intervals in their original units for the epifluorescent, Darbyshire and Singh protozoan estimates (total and encysted). That is, the transformed estimates were used in the statistical analyses and then untransformed to calculated the means, lower and upper standard

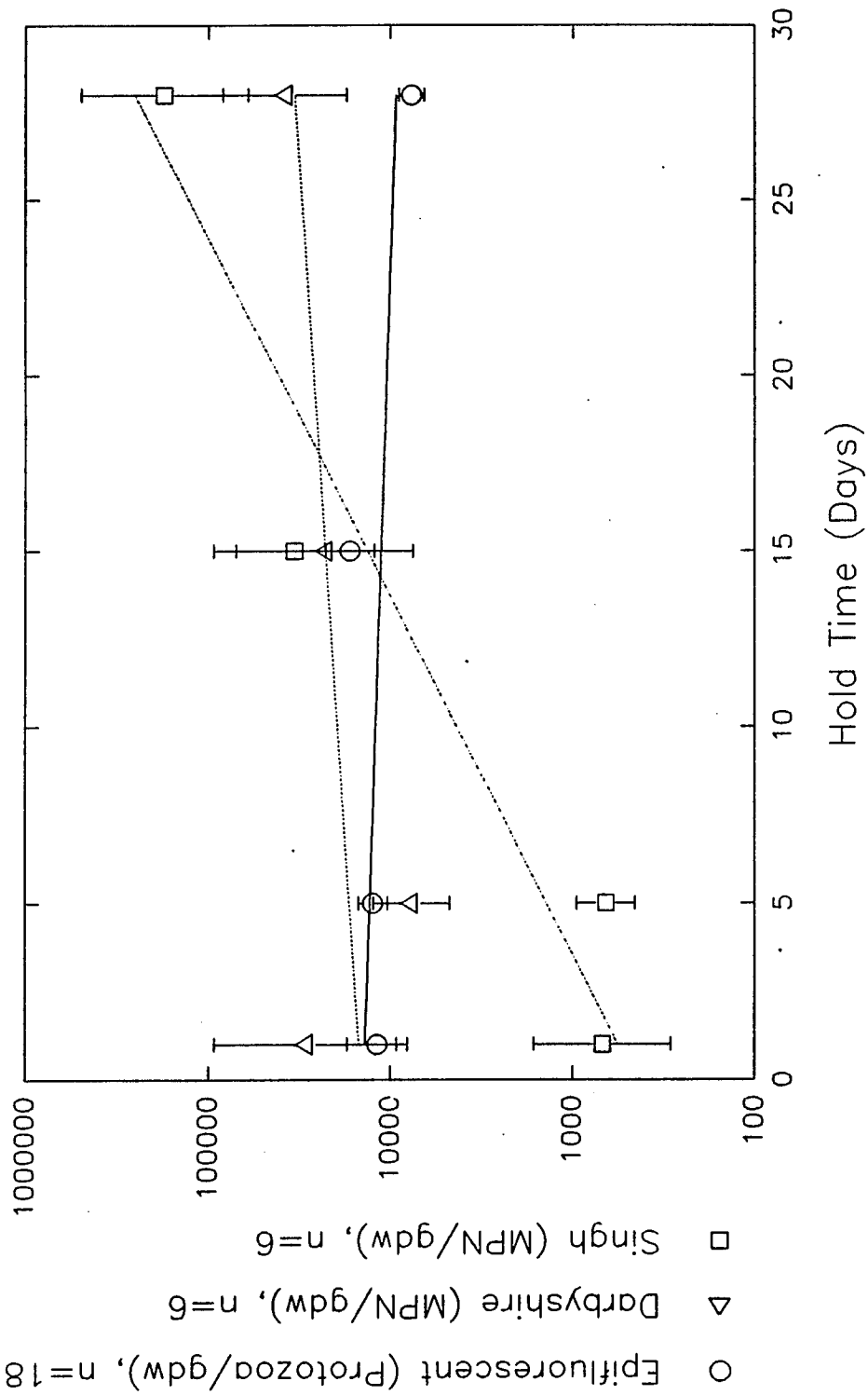


Figure 17: Effects of hold time on protozoan estimates using the epifluorescent, Darbyshire liquid media MPN and Singh solid media MPN techniques. Sediment samples were taken from three cores collected at S 318 at the U.S.G.S. site. Geometric means and standard deviations are shown.

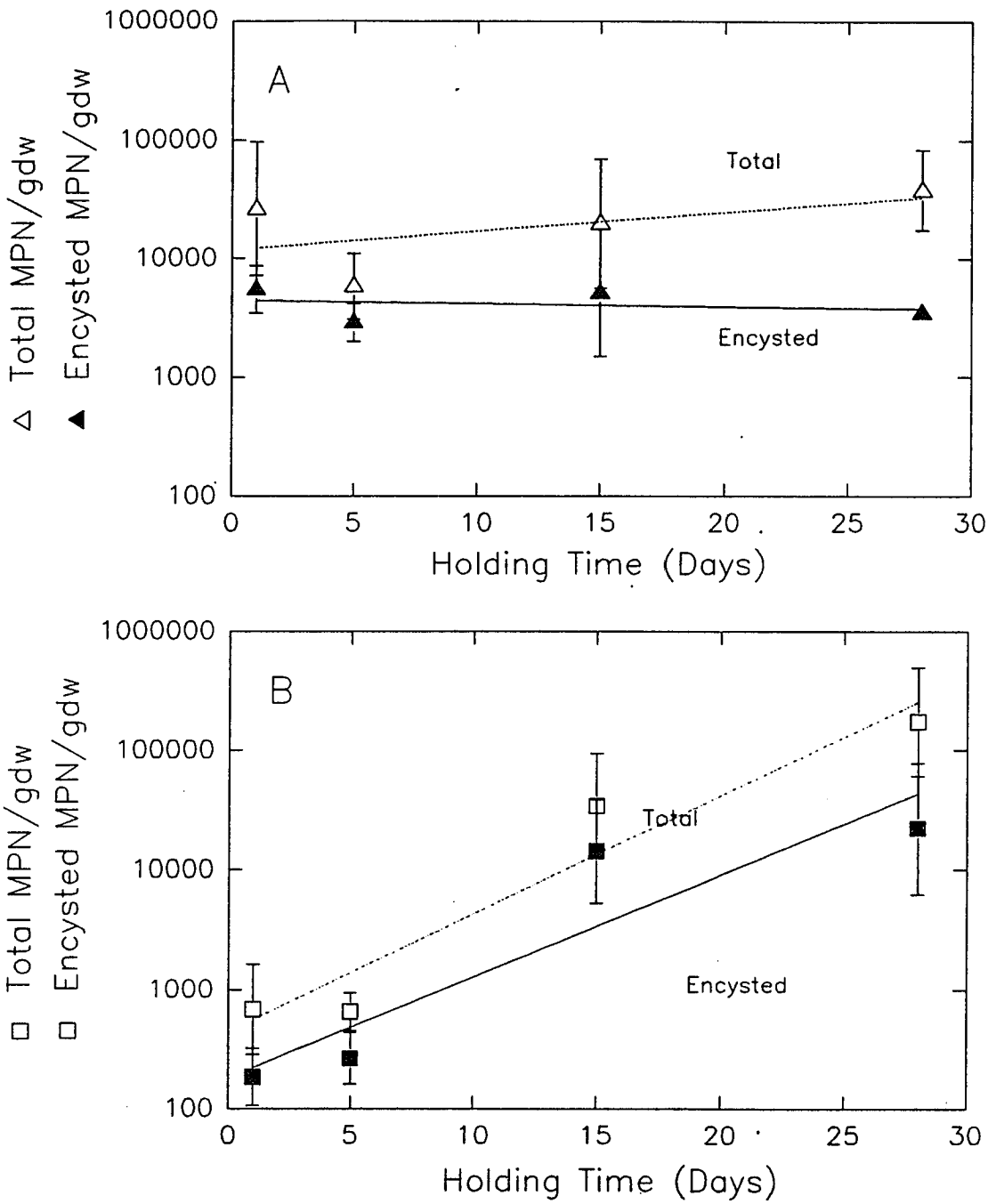


Figure 18: Effects of hold time on total and encysted protozoan populations using the Darbyshire liquid media MPN technique (A) and Singh solid media MPN technique (B). Sediment samples were taken from three cores collected from S 318 at the U.S.G.S. site. Geometric means and standard deviations are shown.

Table 8: Summary of mean, and upper and lower standard deviation (s_L and s_U), and 95% minimum and maximum confidence intervals for the epifluorescent, Darbyshire liquid media MPN and Singh solid media MPN protozoan estimates for 1, 5, 15 and 28 days hold time. Results based on three cores collected in Fall 1991 from S 318 at the U.S.G.S. site.

Enumeration Technique		Day 1	Day 5	Day 15	Day 28
Epifluorescent (protozoa/gdw)	Mean	1.22×10^{4}	1.26×10^{4}	1.71×10^{4}	7.71×10^{3}
	s_L	3.69×10^{3}	2.34×10^{3}	4.76×10^{3}	1.19×10^{3}
	s_U	4.35×10^{3}	2.58×10^{3}	5.54×10^{3}	1.29×10^{3}
	95%min	1.14×10^{4}	1.21×10^{4}	1.60×10^{4}	7.43×10^{3}
	95%max	1.32×10^{4}	1.32×10^{4}	1.84×10^{4}	8.02×10^{3}
Darbyshire Total (MPN/gdw)	Mean	2.94×10^{4}	7.68×10^{3}	2.30×10^{4}	3.82×10^{4}
	s_L	2.01×10^{4}	2.94×10^{3}	1.55×10^{4}	2.07×10^{4}
	s_U	6.40×10^{4}	4.76×10^{3}	4.73×10^{4}	4.53×10^{4}
	95%min	2.12×10^{4}	6.48×10^{3}	1.67×10^{4}	2.97×10^{4}
	95%max	5.55×10^{4}	9.62×10^{3}	4.23×10^{4}	5.67×10^{4}
Darbyshire Encysted (MPN/gdw)	Mean	4.88×10^{3}	2.20×10^{3}	4.42×10^{3}	3.47×10^{3}
	s_L	2.30×10^{3}	1.02×10^{3}	3.21×10^{3}	3.64×10^{2}
	s_U	4.35×10^{3}	1.89×10^{3}	1.18×10^{4}	4.06×10^{2}
	95%min	3.94×10^{3}	1.79×10^{3}	3.10×10^{3}	3.33×10^{3}
	95%max	6.65×10^{3}	2.97×10^{3}	9.23×10^{3}	3.64×10^{3}
Singh Total (MPN/gdw)	Mean	6.85×10^{2}	6.56×10^{2}	3.40×10^{4}	1.74×10^{5}
	s_L	3.98×10^{2}	2.02×10^{2}	2.17×10^{4}	1.13×10^{5}
	s_U	9.48×10^{2}	2.92×10^{2}	5.99×10^{4}	3.25×10^{5}
	95%min	5.23×10^{2}	5.73×10^{2}	2.52×10^{4}	1.28×10^{5}
	95%max	1.07×10^{4}	7.75×10^{2}	5.85×10^{4}	3.07×10^{5}
Singh Encysted (MPN/gdw)	Mean	1.84×10^{2}	2.65×10^{2}	1.43×10^{4}	2.22×10^{4}
	s_L	7.80×10^{1}	1.04×10^{2}	9.00×10^{3}	1.59×10^{4}
	s_U	1.35×10^{2}	1.71×10^{2}	2.42×10^{4}	5.61×10^{4}
	95%min	1.53×10^{2}	2.22×10^{2}	1.07×10^{4}	1.57×10^{4}
	95%max	2.40×10^{2}	3.35×10^{2}	2.42×10^{4}	4.51×10^{4}

deviations, and 95% minimum and maximum confidence intervals that are shown in Table 8.

Storage of the cores did have an effect on protozoan estimates since none of the slopes of the regression lines in Figure 17 were zero. The slopes were: -145 protozoa/gdw per day for the epifluorescent technique (i.e., there was a decrease of 145 protozoa/gdw in the epifluorescent technique for every day of storage); +556 MPN/gdw per day for the Darbyshire total MPN technique; and +8,970 MPN/gdw per day for the Singh total MPN technique. The variability of the epifluorescent technique was lower than that for the Darbyshire total MPN and Singh total MPN methods. The epifluorescent and Darbyshire total MPN estimates also appeared to be more consistent throughout the hold time (these techniques were prepared from the same core samples). The Singh total estimates were affected the most by storing the cores and increased more than two order of magnitude over the 28 day hold time.

The difference between the total and encysted estimates represents the trophic protozoan population. The slope for the Darbyshire encysted MPN estimates (Figure 18 A) was only +10 MPN/gdw per day, which was even lower than the slope for the epifluorescent technique on Figure 17. The slope for the Singh encysted MPN estimates (Figure 18 B) was +1,680 MPN/gdw per day, which was not as steep as the slope for the

total MPN estimates. The estimates for the total population were always greater than the encysted population on each day and for both techniques. The percentage of encysted protozoa never exceeded more than 42% of the total population (Table 9). However, estimates of the encysted protozoan population for the hold time of the core were variable depending on the enumeration technique.

Table 9: Darbyshire MPN and Singh MPN estimates of encysted protozoa as a percentage of the total population.

Enumeration Technique	Day 1	Day 5	Day 15	Day 28
Darbyshire MPN	17%	29%	19%	9%
Singh MPN	27%	40%	42%	13%

a. Statistical Comparisons between Epifluorescent, Darbyshire Total MPN and Singh Total MPN Enumerations. The trends in Figure 17 were analyzed statistically to test for significant differences: 1) due to hold time for each enumeration technique; and 2) among the enumeration techniques for each day. The Darbyshire total MPN and Singh total MPN estimates for each day of the hold time were found to have homogeneous variances ($P>chi^2 = 0.29$ and 0.19, respectively) and a oneway ANOVA was performed. Each of the total MPN techniques were found to be significantly

different over the storage period (P>F=0.03 and 0.00 for Darbyshire and Singh, respectively). The epifluorescent technique had inhomogeneous variances (P>chi^2 = 0.00) and the Kruskal-Wallis test found the estimates also to be significantly different over the entire hold time (P>K-W = 0.00). Therefore, the slope of the regression lines in Figure 17 changed significantly for each enumeration technique from days 1 to 28.

Scheffé's multiple comparisons test was used to specifically determine where significant differences occurred among the days for each enumeration technique. The experimentwise error rate (α_{ER}) for 6 comparisons (k=6) was 0.26 for α=0.05 (equation 12). Where P>F exceeded α_{ER} in Table 10, there was a significant difference between the protozoan estimates for the hold times being compared.

There was no significant difference between days 1 and 5 for either the epifluorescent or Singh total estimates (Table 10). The Darbyshire total MPN counts were not significantly different on days 1 and 15, 1 and 28, 5 and 15, and 15 and 28. All other comparisons of hold times were significantly different for these techniques, with at least α_{ER}=0.43 (based on α=0.10). The significant differences within the enumeration techniques over the hold time are illustrated on Figure 18, especially the differences for the

Singh total technique where the protozoan estimates rise sharply after day 5.

Table 10: Multiple comparisons using Scheffé's test to determine when significant differences occurred due to hold time of the cores from S 318 at the U.S.G.S. site for the epifluorescent, Darbyshire total MPN and Singh total MPN techniques. Values in the table represent the probability of a greater F-statistic.

Comparison of Hold Time (Days)	Epifluorescent Technique (P>F)	Darbyshire Total MPN (P>F)	Singh Total MPN (P>F)
1 & 5	0.988	0.132	1.000
1 & 15	<0.001	0.975	<0.001
1 & 28	<0.001	0.970	<0.001
5 & 15	0.005	0.272	<0.001
5 & 28	<0.001	0.055	<0.001
15 & 28	<0.001	0.824	0.034

Figure 17 supports the findings of the multiple comparison tests for all enumeration techniques: the standard deviations of one day usually encompass the range of the second day's standard deviations in order for the days to be considered not significantly different. For example, the upper and lower vertical error bars (i.e., the standard deviations) for the day 5 Singh total MPN estimate fell within the range of the vertical error bars for the day 1 Singh estimate, and Scheffé's multiple comparison found these two days to be not significantly different. The epifluorescent estimates of days 1 and 5 were also not

significantly different. However, the Darbyshire total MPN estimate of day 5 fell below that of day 1. This comparison was found to be significantly different by Scheffé's test with an α_{ER}=0.26 (for 95% confidence). The significant difference for the comparison of the day 5 and 15 estimates by the Darbyshire technique was also apparent on Figure 17, where only the upper vertical error bar of day 5 overlapped the lower vertical error bar of day 15.

The protozoan estimates among the techniques for each sampling day were investigated. The Kruskal-Wallis test was used to determine if all three enumeration techniques differed significantly on each day of hold time since all were found to have inhomogeneous variances ($P>chi^2>0.001$). On days 1, 5 and 28, all enumeration techniques were found to have significantly different estimates of protozoa (P>K-W>0.001). However, all estimates of protozoa by the three enumeration techniques were not significantly different on day 15 (P>K-W=0.33). In Figure 17, all enumeration estimates converge on day 15.

Multiple comparisons were made to determine which of the techniques differed on each day (Table 11). For three comparisons, the probability had to exceed α_{ER}=0.14 for α=0.05. Scheffé's test agreed with the ANOVA that all protozoan estimates by the enumeration techniques were

significantly different on days 1, 5 and 28, and not significantly different of day 15.

Table 11: Multiple comparisons using Scheffé's test to determine if there were significant differences among the epifluorescent, Darbyshire total MPN and Singh total MPN enumerations due to hold time of the cores from S 318 at the U.S.G.S. site. Values in the table represent the probability of a greater F-statistic.

Comparison among Enumeration Techniques	Day 1 (P>F)	Day 5 (P>F)	Day 15 (P>F)	Day 28 (P>F)
Epifluorescent & Darbyshire	0.091	0.002	0.893	<0.001
Epifluorescent & Singh	<0.001	<0.001	0.153	<0.001
Darbyshire & Singh	<0.001	0.004	0.452	<0.001

b. Statistical Comparisons Between Total and Encysted MPN. The total and encysted populations estimated by the Darbyshire and Singh MPN methods (Figure 18) were found to be significantly different on days 1, 5, 15 and 28 (in each case, the oneway ANOVA had a P>F<0.17). The encysted population never exceeded more than 42% of the total population (Table 9), which indicated that a majority of the protozoa were remaining active in the sediments throughout the storage period.

Figure 18 shows that the techniques enumerated the encysted proportion of the total population differently. Scheffé's test compared MPN estimates of encysted protozoa

made on different days (Table 12). There were no significant differences found for the Darbyshire encysted MPN due to hold time (α_{ER}=0.26 for 95% confidence). This agrees with the flat slope for the Darbyshire encysted MPN estimates (Figure 18A). The Singh encysted MPN estimates were not significantly different on days 1 and 5, and 15 and 28, which was apparent on Figure 18B. These results are not very different from the differences detected with Scheffé's test for the Darbyshire encysted MPN and Singh encysted MPN estimates (Table 10).

Table 12: Multiple comparisons using Scheffé's test to determine when significant differences occurred due to hold time of the cores from S 318 at the U.S.G.S. site for the Darbyshire encysted MPN and Singh encysted MPN techniques. Values in the table represent the probability of a greater F-statistic.

Comparison of Hold Time (Days)	Darbyshire Encysted MPN (P>F)	Singh Encysted MPN (P>F)
1 & 5	0.406	0.917
1 & 15	0.997	<0.001
1 & 28	0.905	<0.001
5 & 15	0.515	<0.001
5 & 28	0.801	<0.001
15 & 28	0.962	0.862

2. Discussion of the Hold Time Experimental Results

All interpretations of the trends found in the hold time experiment were based on the results of analyzing three cores from one site in Fall 1991. In particular, the similarity of all estimates on day 15 may have been a coincidence and should be further investigated. Until the results of the hold time experiment can be confirmed, the results illustrated in Figures 17 and 18 can only be used to speculate on the ability of the enumeration techniques to estimate the protozoa at S 318, or any other site.

The conditions in the core samples may have changed the association between the protozoa and the sediment particles during storage and may be responsible for: 1) the negative slope of the epifluorescent enumerations over the hold time; 2) the similarities between the epifluorescent and Darbyshire estimates; and 3) the increasing population estimates for the Darbyshire and Singh techniques. During the storage of the cores, the temperature and saturation of the sediments remained constant. Temperature was maintained at 12^{o}C, about the ground water temperature at F 393 and ~6^{o}C cooler than the ground water at S 318 at the time the cores were collected. The cores remained saturated with pore water. No additional source of carbon or nutrients was added. Harvey (personal communication, 1991) has found that cores from the U.S.G.S. site remain aerobic in the aluminum

core sleeves for at least one month if they were stored in a refrigerator (4°C).

The low slope of the epifluorescent enumerations with time (-145 protozoa/gdw per day) suggests that any increased association between the protozoa and sediment particles over the storage period was not irreversible. The efficiency of the epifluorescent technique to estimate the protozoan population depends only on the separation of cells from the particles during the shaking procedure. Thus, any type of strong attachment of the protozoa on the sediments would have affected the precision and accuracy of the technique. Wilson et al. (1983) observed stability in epifluorescent bacterial estimates of saturated subsurface core samples stored over 28 days.

Figure 17 and Table 8 demonstrated several similarities between the epifluorescent and Darbyshire protozoan estimates over the hold time. Both of these techniques had relatively flat regression lines, indicating storage had little effect on the population estimates. The Darbyshire estimates had a greater variability than the epifluorescent estimates on each sampling day, as illustrated by the heights of the vertical error bars around each estimate in Figure 17 and the 95% minimum and maximum confidence intervals around the mean in Table 8. Thus, no significant differences were found between the days for the Darbyshire

estimates (if comparisons with day 5 were excluded). Since the epifluorescent estimates were more precise, they were more significantly different almost throughout the storage period (Table 10), despite the low slope of the regression line. However, the epifluorescent and Darbyshire population estimates were approximately the same order of magnitude throughout the hold time, even though they were found to be significantly different on days 1, 5 and 28. It was expected that these techniques might produce similar estimates since they were prepared from the same sediment sample shaken in three 25 mL aliquots of phosphate buffer. The relative similarities of the two techniques have implications for the interpretation of results from sites sampled throughout the contaminant plume during Fall 1990 at the U.S.G.S. site, where the hold times varied among the sites (from 1 to 24 days) and the protozoan population estimates by the epifluorescent and Darbyshire total MPN techniques for a site differed by as much as two orders of magnitude (Kinner et al., 1991; Bunn et al., 1992)..

The significant increase in the Singh MPN population estimates contrasted with the epifluorescent and Darbyshire techniques. The obvious difference between the methods was that the dilutions for the Singh MPN samples were prepared by the procedures of Sinclair and Ghiorse (1987) instead of using the shaken phosphate buffer extract. If the

separation technique for the Singh samples was more efficient than the shaking technique, then the Singh technique should have overestimated the epifluorescent and Darbyshire samples throughout the hold time. However, the relatively consistent population estimates by the epifluorescent technique indicate that the efficiency of the separation procedure remained the same despite storage of the sediments. Instead, the significant increase in Singh MPN populations suggested that the protozoa were responding differently to the media conditions of the MPN techniques as a function of the hold time before processing.

The conditions in the stored cores did not change sufficiently to cause a majority of protozoa to encyst (Table 9). Soil protozoa encyst when conditions limit the maintenance of the cells (Foissner, 1987). It appears the sediment environment in the core remained favorable for trophic protozoa since the percent encysted of the total population did not increase with storage time. In fact, there was a lower percentage of encysted protozoa on day 28 compared to day 1 as estimated by both MPN techniques. Since the Singh MPN technique estimated a higher percentage of encysted protozoa with time compared to the Darbyshire MPN technique, some species with greater preference for the solid media might have been responding to changes in the sediment conditions with hold time. This hypothesis may be

tested if the diversity of the MPN samples is observed in future analyses.

The most apparent change in the core was that the sediments were no longer exposed to ground water flow while they were stored over the 28 day hold time. The average flow of the ground water at the U.S.G.S. site is 0.5 m/yr (Garabedian and LeBlanc, 1991) through relatively wide pore spaces (70 μm critical pore openings; Harvey, personal communication, 1992). The cells may become more oriented to surfaces and adapted to a static flow environment (i.e., no ground water flow) during extended holding periods.

The Darbyshire MPN estimates of the protozoan population were relatively consistent over the hold time compared to the Singh MPN estimates. The environment within the microtiter plates of the Darbyshire MPN technique may have simulated the sediments and the protozoa responded consistently throughout the storage period (i.e., the protozoa were use to an environment where there was sufficient spaces filled with liquid in which to move freely about and the conditions in the Darbyshire samples were similar). The slight increase in time observed for the Darbyshire estimates on Figure 17 may have been due to the protozoa adapting to the static flow conditions of the cores.

The Singh MPN technique had a more limited liquid environment than the Darbyshire MPN technique. The low initial estimates of the Singh samples may have been a response by the protozoa to the more surface oriented environment on the agar plates. The overestimation of the Singh samples on day 28 suggests that the protozoa adapted to the static core conditions might have been related to the addition of bacteria or the agar to the dilutions. The protozoa were never observed consuming the bacteria in the Singh samples and they were similar in size to those in the Darbyshire samples. However, the bacterial inoculum transferred from nutrient agar for the Singh technique may have increased the nutrient content of the dilutions compared to the phosphate buffer in the microtiter plates. The adaptation of copiotrophic bacteria (i.e., growing in high nutrient conditions) to be associated with surfaces for nutrient uptake in oligotrophic environments is well documented (Atlas and Bartha, 1987; Balkwill and Ghiorse, 1988; Ghiorse and Wilson, 1988; Hazen et al., 1991). In the cores, the bacteria and nutrients may become more surface oriented vs. pore water associated with time. Hence, over the hold time the protozoa may have become more affiliated with the surfaces in the cores and responded better to the Singh enumeration technique which has higher nutrient concentrations associated with its agar and bacterial film.

Darbyshire (1973) appears to be the only researcher who has compared the Singh MPN technique to his method. He recorded the types of protozoa in topsoil (holding time <24 hr) located in Aberdeen Scotland (the same region investigated by Cutler et al., 1922). There were significantly more flagellates found in the Darbyshire MPN samples than in the Singh MPN samples ($P>0.05$), and no significant difference was found for the enumeration of amoebae ($P<0.05$). Darbyshire concluded that liquid media with indigenous bacteria to support protozoan growth was better for enumerating a range of protozoa compared to solid media with bacterial supplement. This agrees with the protozoa estimates of the subsurface sediments from the U.S.G.S. site that were enumerated within 5 days.

All published accounts of protozoan populations in subsurface sediments have been enumerated with the Singh solid media MPN technique (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990). The highest protozoan estimates were in sand and gravel layers in the subsurface: 67 MPN/gdw in Lula, OK; 1.19×10^3 MPN/gdw in Aiken, SC, and 30 MPN/gdw northeastern Kansas. All sediment samples had a hold time of ≤ 7 days. Hence, their generally low estimates of protozoa agree with the significantly lower Singh MPN estimates observed with short hold times for the S 318 samples from the U.S.G.S. site.

Researchers have generally concluded that "MPN techniques underestimate the total number of protozoa by failing to measure those species which do not grow under the culture conditions" (Baldock, 1986). The soil amoebae that Severtzova (1924) was studying depleted the bacterial populations on agar plates within days and then encysted. Caron et al. (1989) found that epifluorescent direct counts estimated a higher density of nanoflagellates than the Darbyshire MPN technique. They also demonstrated that the nanoflagellates had a preference for the food source and that the growth phase of the bacteria affected the estimates by the Darbyshire MPN technique. Baldock found generally twice as many large ciliates (15-120 μm) in freshwater sediments by direct counting compared to Darbyshire liquid media MPN estimates and attributed the difference to the lack of food in the dilutions for these large predatory protozoa. He chose not to amend the dilutions since he thought the addition of bacteria would result in a reduction in the total population estimates. However, Baldock also found that the Darbyshire estimates were twice as high for flagellates (>5 μm in size).

Other researchers have found estimations of small flagellates by direct counting and dilution/extinction techniques similar to those reported for sediments from the U.S.G.S. site. Fenchel (1982) found nanoflagellates

populations were comparable when enumerated with a direct count technique or liquid media MPN in marine nearshore water samples. Also, Griffiths and Ritz (1988) found no significant difference in the means (with t-tests and an α=0.05) of trophic protozoan abundances in mineral soils as estimated by an epifluorescent method (with FDA or INT staining) and the Darbyshire MPN technique.

The significant relationships illustrated in Figures 17 and 18 suggest that storage of the cores does have an effect on the enumeration of protozoa. The results of the three cores analyzed during the hold time experiment indicated that storage of the core material for several days would result in similar estimates by all three techniques. The epifluorescent technique has the lowest variability and the most consistent estimates with time and therefore can be used as a reliable enumeration method for subsurface sediment protozoa. If only one type of MPN enumeration were possible, then the Darbyshire technique should be chosen since the effects of hold time appears to be less influential on the total and encysted protozoan estimates. However, the hold time experiment should be repeated to demonstrate the convergence of the estimates on day 15 and the significant increase in the Singh MPN samples throughout storage. Recording the diversity of the protozoa in the sediment samples and using the same separation procedure for

all three techniques might provide more information on the processes influencing the protozoa/sediment association with time.

C. Intrasite Variability

The variability associated with the two sites, F 393 and S 318 was assessed for each enumeration technique using: 1) oneway ANOVA, and 2) components of variance analyses. The ANOVA tested the null hypothesis that the mean population estimates of both sites were equal whereas, the components of variance examined the variances associated with the design of the sampling program at the sites and their influence in the confidence limits around the means of the population estimates. The two types of analyses reached similar conclusions, and improvements in the sampling procedures were suggested by the components of variance. Grain size was also examined to determine if the two sites were physically different.

1. Grain Size Analyses for F 393 and S 318

F 393 and S 318 were known to have different chemical constituents in the ground water because of their location with respect to the contaminant plume. Grain size distributions for the sediment at a site affect the porosity and subsequently, the transport of chemical constituents and

the mobility of the microorganisms. Sinclair and Ghiorse (1987, 1989), Beloin et al. (1988), and Sinclair et al. (1990) have found that protozoan populations have been positively correlated to the soil texture of the sampling site. Their highest population estimates were in layers of sand and gravel, where the porosity was the greatest. Sinclair and Ghiorse (1987) speculated that the protozoa found in sediments above the clay confining layer (3 m below surface) were transported down from the top soils above. The gravelly, loamy sand layer 7.5 m below surface had from 4-10 protozoa (MPN)/gdw, with >80% of the population being flagellates. They thought that the protozoa in the subsurface were transported from a nearby river along with nutrients and organic substances.

Grain size analyses were conducted on all cores collected at F 393 and S 318, except for S 318 C 15 (the sterile sleeve core)(Figure 19). The grain size distributions of the samples from F 393 and S 314 were not significantly different (P>F=0.87). Greater than 50% of all the particles at both sites had an average grain size of 0.7 mm, which would be classified as a medium sand (Alexander, 1977). Harvey has found that the 0.5-1.0 mm size fraction predominates in the sediments at the U.S.G.S. site. Therefore, the comparison of the protozoan populations from F 393 and S 318 was probably not biased due to the grain

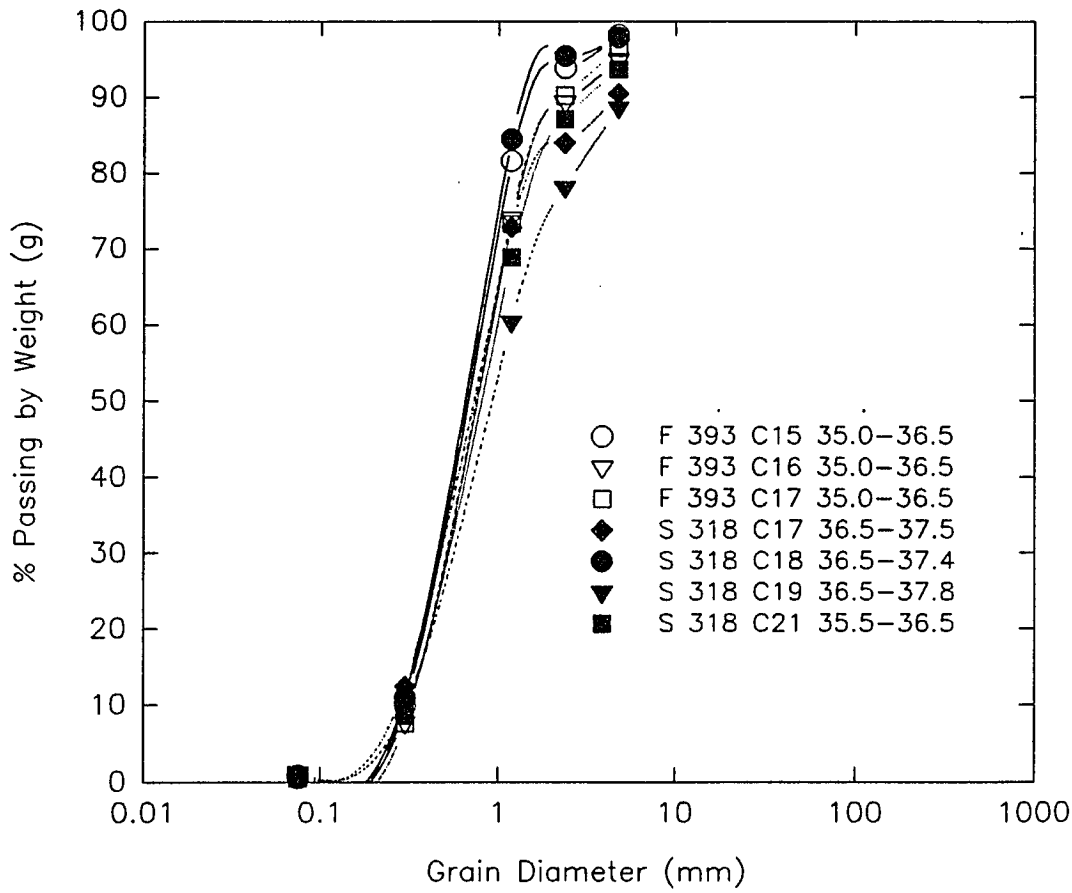


Figure 19: Grain size distribution for sediments collected at the U.S.G.S. site.

size. Based on the literature, large populations of protozoa should be detected in the sediments with this grain size distribution.

The performance of the Waterloo corer in collecting sediment samples was dependent on the grain size distribution (Zapico et al., 1987). The corer was designed to remove "cohesionless" sand and gravel samples with their pore fluids and without excessive compaction. Zapico et al. tested the corer in the medium sand and gravel sediments at the U.S.G.S. site and found compaction to be <3% with a recovery rate of 97% (continuous recovery over 20 m in length). However, they found the recovery rate of the corer decreased and necessitated the use of drilling muds (which are undesirable for microbiological samples due to increased chances of contamination) as the percent fines increased for other sites tested.

2. Oneway ANOVA for Determining Intrasite Variability

The F 393 cores were sampled 14 days after they were collected. The protozoan estimates by the epifluorescent, Darbyshire and Singh techniques from this uncontaminated site were compared to those collected at S 318 (cores stored for 15 days only). Based on the results of the hold time experiment, comparisons of protozoan estimates should be made on cores held the same amount of time, and similar

estimates by the various techniques occurred at ~15 days of storage. The variances of all estimates from both sites were found to be homogeneous ($P<0.05$). Significant differences between the sites were determined with a oneway ANOVA for each technique (Table 13). The population estimates at F 393 and S 318 were found to be significantly different based on all three enumeration techniques with a 90% confidence level. The greater variability in the Singh

Table 13: The mean, 95% minimum and maximum confidence intervals, and results of the oneway ANOVA for the enumeration techniques and sites. All values are based on the square root transformed epifluorescent estimates and log transformed MPN estimates.

Enumeration Techniques	Site	Mean	95% C.I. Minimum	95% C.I. Maximum	P>F
Epifluorescent* (protozoa/gdw)	F 393	1.06×10^4	9.52×10^3	1.19×10^4	<0.001
	S 318	1.71×10^4	1.60×10^4	1.84×10^4	
Darbyshire** (Total MPN/gdw)	F 393	1.38×10^3	1.09×10^3	1.97×10^3	<0.001
	S 318	2.30×10^4	1.67×10^4	4.23×10^4	
(Encysted MPN/gdw)	F 393	8.73×10^2	6.66×10^2	1.36×10^3	0.029
	S 318	4.43×10^3	3.12×10^3	9.24×10^3	
Singh** (Total MPN/gdw)	F 393	1.04×10^4	7.80×10^3	1.74×10^4	0.066
	S 318	3.40×10^4	2.52×10^4	5.85×10^4	
(Encysted MPN/gdw)	F 393	4.21×10^3	3.08×10^3	7.52×10^3	0.067
	S 318	1.43×10^4	1.07×10^4	2.42×10^4	

*Epifluorescent enumerations were based on 18 slides: 2 slides/subsample, 3 subsamples/core and 3 cores/site.

**Darbyshire and Singh enumerations were based on 6 dilution series: 2 subsamples/core and 3 cores/site.

MPN enumerations required a higher significance level (i.e., α=0.10) in order for there to be a significant difference between the sites, which for biological samples is not uncommon (see Section III.A.). No more information about the sampling regime could be ascertained from the ANOVA.

The means (square root transformed for the epifluorescent samples and geometric for the MPN samples) for each estimate at S318 were greater than those at F 393. Figure 20 shows the addition of F 393 14 day estimates along with the hold time estimates of S318 (Figure 17) to illustrate the differences between the sites (not including the encysted MPN samples). The Darbyshire total MPN estimate for F 393 (1.38×10^3 MPN/gdw) was significantly different (i.e., lower) than the estimates of the other two enumeration techniques. It appears that both of the non-acidified microtiter plates used for estimating the Darbyshire total MPN population for F 393 may have underestimated the protozoa. This is supported by the fact that the total Singh and epifluorescent estimates for F 393 were significantly higher than the Darbyshire total MPN counts. In addition, the acidified plates for the Darbyshire encysted MPN estimates for F 393 represented 63% of the total MPN estimate, while in the other cases (i.e., at S 318) the Darbyshire estimates of percent encysted were much lower (9-29%) and more consistent (Table 9). The Singh

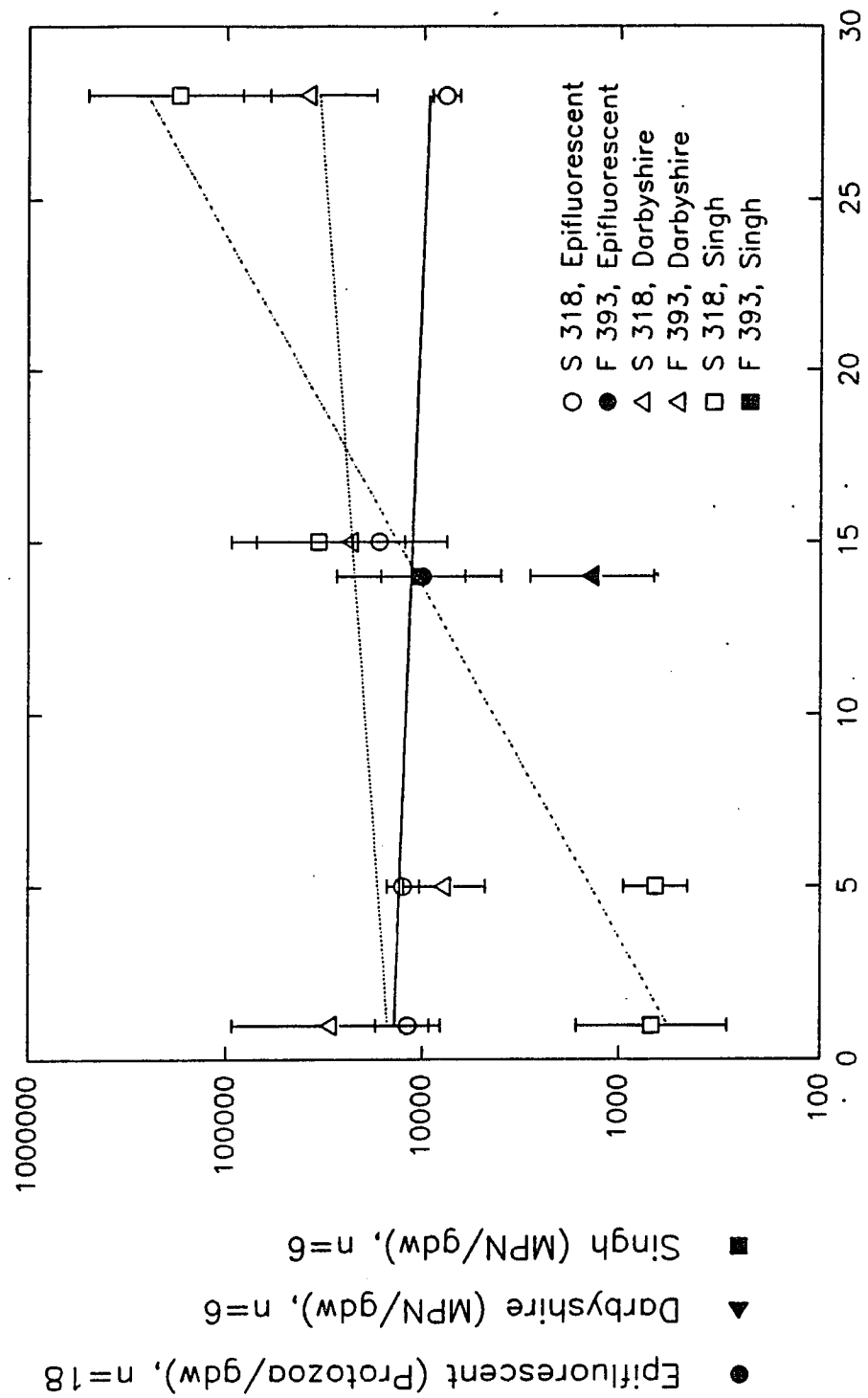


Figure 20: Intrasite variability and the effects of hold time on protozoan estimates using the epifluorescent, Darbyshire liquid media MPN and Singh solid media MPN techniques. Sediment samples were taken from three cores collected at S 318 (Hollow symbols) and F 393 (filled symbols) at the U.S.G.S. site. Geometric means and standard deviations are shown.

estimate of percent encysted for F 393 was 40% which was comparable to the 42% estimated for S 318.

3. Components of Variance Analyses for Determining Intrasite Variability

The sampling scheme used for Fall 1991 at sites F 393 and S 318 was a hierarchical design and allowed the results of each enumeration technique to be analyzed for components of variance (i.e., replication of samples allowed for the calculation of the variances associated with the sites, cores and subsamples from the cores). The analyses did not determine the significant difference between the sites, as with the ANOVA, but did illustrate the influence of each sampling component within the sites.

Previous studies devoted to the characterization of protozoa in the subsurface have based their estimates on only one core taken at a site, which is typical because of time and cost constraints (Sinclair and Ghiorse, 1987, 1989; Beloin et al., 1988; Sinclair et al., 1990). For the hierarchical design, three cores were taken at each site (i.e., one core more than would practically be taken for any other type of subsurface investigation).

The hierarchical design for the epifluorescent direct counts included 3 cores from each site, subsampling each core three times and then preparing duplicate slides for each subsample (Figure 21A). The number of subsamples and

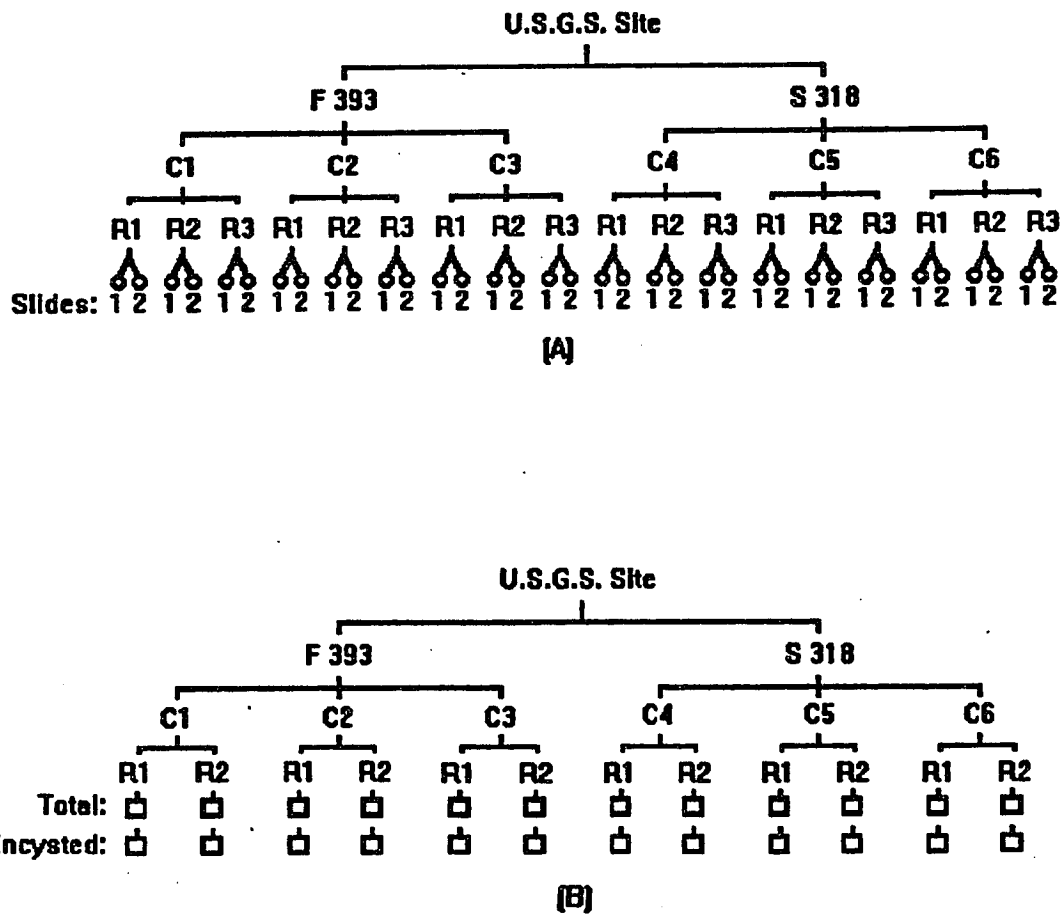


Figure 21: Hierarchical design for the epifluorescent (A), Darbyshire MPN and Singh MPN (B) enumerations of core material collected in Fall 1991 at the U.S.G.S. site. At sites F 393 and S 318, three cores (C) were taken and subsampled (R). Two slides were prepared from each subsample for the epifluorescent enumeration. Total and encysted population estimates were made for each subsample enumerated by the Darbyshire liquid media MPN and Singh solid media MPN technique.

slides were chosen based on the practical limit of preparing and enumerating the samples (see Section II.D.5). Thus, a total of 18 slides was counted for each site.

The Darbyshire liquid media MPN and Singh solid media MPN techniques were only performed on two subsamples from each core since each MPN estimate required substantially more time to examine compared to the epifluorescent slides (Figure 21B). Only six plates were prepared per site to estimate the total (trophic + encysted) population of protozoa by the Darbyshire and Singh MPN techniques. Six additional plates were prepared on these subsamples after they were acidified for 30 min in order to estimate the encysted protozoan population by the MPN techniques. Thus, a total of 12 MPN estimates were prepared for each site. Since there were 8 replicate wells for Darbyshire technique and only 3 replicate wells for Singh, the Darbyshire estimates had a higher confidence in the MPN calculation (see Appendix A for values of the standard error).

The components of variance were calculated using equations 7-9 (see Chapter 2) for each enumeration technique. The individual estimates were transformed to a normal distribution for the calculation of the components (i.e., in the components of variance data sheets in Appendix B): square root transformation for the epifluorescent counts; and log-transformation for the MPN results. Then

the variances were untransformed to give actual concentrations for use as detectable differences. Since all the data in its original form was positively skewed (Figure 14-16), the upper standard deviation, variance or confidence interval was always larger than the component below the mean. Hence, to be conservative, the upper standard deviations were used to return the components of variance back to their original form (see Appendix B). Thus, the components presented represent the worst case scenario (i.e., the greatest error).

The maximum probable error, E, for the hierarchical designs used in the Fall 1991 was calculated as (Tables 14-16):

$$E = z_p \left(\left[\frac{\hat{\sigma}_S^2}{S} \right] + \left[\frac{\hat{\sigma}_C^2}{C} \right] + \left[\frac{\hat{\sigma}_R^2}{R} \right] + \left[\frac{\hat{\sigma}_{sl}^2}{sl} \right] \right)^{\frac{1}{2}} \quad [13],$$

where z_p is the percentile of the standard normal distribution; S is the number of sites; C is the number of cores from a site; R is the number of subsamples per core; and sl is the number of slides per subsample (for the epifluorescent enumerations only). If the maximum probable error is desired with 95% confidence, then z_p is 1.96, the $t_{infinity}$-value at the 95% confidence interval (Provost, 1984). Changing the confidence in the error would thus only

Table 14: Components of variance and maximum probable error for epifluorescent enumerations of core samples collected at F 393 and S 318. All the components were calculated with the most conservative variances from the square root transformation of the estimates.

Component of Variance	Comparison of F 393 & S 318 (protozoa/gdw)	F 393 (protozoa/gdw)	S 318 (protozoa/gdw)
$\hat{\sigma}^2_S$, site	1.38×10^7 (24%)*	-	-
$\hat{\sigma}^2_C$, core	3.23×10^7 (55%)	3.43×10^7 (77%)	2.63×10^7 (64%)
$\hat{\sigma}^2_R$, subsample	0	0	0
$\hat{\sigma}^2_{sl}$, slide	1.23×10^7 (21%)	1.01×10^7 (23%)	1.46×10^7 (36%)
$\hat{\sigma}^2_T$, total	5.84×10^7	4.44×10^7	4.09×10^7
95% maximum probable error	6.96×10^3	6.79×10^3	6.07×10^3
80% maximum probable error	4.56×10^3	4.44×10^3	3.97×10^3

* Each component was expressed as the percentage of the total variance, $\hat{\sigma}^2_T$, which was the sum of all components of variance.

Table 15: Components of variance and maximum probable error for Darbyshire total MPN enumerations of core samples collected at F 393 and S 318. All the components were calculated with the most conservative variances from the log transformation of the estimates.

Component of Variance	Comparison of F 393 & S 318 (total MPN/gdw)	F 393 (total MPN/gdw)	S 318 (total MPN/gdw)
$\hat{\sigma}^2_S$, site	6.75×10^8 (89%)*	-	-
$\hat{\sigma}^2_C$, core	5.39×10^7 (7%)	2.76×10^5 (14%)	2.10×10^9 (77%)
$\hat{\sigma}^2_R$, subsample	3.30×10^7 (4%)	1.65×10^6 (86%)	6.24×10^8 (23%)
$\hat{\sigma}^2_T$, total	7.62×10^8	1.96×10^6	2.72×10^9
95% maximum probable error	3.67×10^4	1.19×10^3	5.56×10^4
80% maximum probable error	2.40×10^4	7.77×10^2	3.64×10^4

* Each component was expressed as the percentage of the total variance, $\hat{\sigma}^2_T$, which was the sum of all components of variance.

Table 16: Components of variance and maximum probable error for Singh total MPN enumerations of core samples collected at F 393 and S 318. All the components were calculated with the most conservative variances from the log transformation of the estimates.

Component of Variance	Comparison of F 393 & S 318 (total MPN/gdw)	F 393 (total MPN/gdw)	S 318 (total MPN/gdw)
$\hat{\sigma}^2_S$, site	4.28×10^8 (28%)*	-	-
$\hat{\sigma}^2_C$, core	0	3.98×10^7 (16%)	0
$\hat{\sigma}^2_R$, subsample	1.11×10^9 (72%)	2.10×10^8 (84%)	5.37×10^9 (100%)
$\hat{\sigma}^2_T$, total	1.54×10^9	2.50×10^8	5.37×10^9
95% maximum probable error	3.43×10^4	1.71×10^4	5.86×10^4
80% maximum probable error	2.24×10^4	1.12×10^4	3.84×10^4

* Each component was expressed as the percentage of the total variance, $\hat{\sigma}^2_T$, which was the sum of all components of variance.

be a matter of changing the value of z_p (e.g., 1.28 for an 80% confidence). As an example, to calculate the maximum probable error for the comparison of F 393 and S 318 with the Darbyshire liquid media MPN estimates S = 2 sites, C = 3 cores, R = 2 subsamples, and the sl component was dropped from the calculation since slides were not used for this analysis. Only the relevant components were used to calculate the desired maximum probable error (e.g., for the Darbyshire F 393 enumerations only $\hat{\sigma}_C^2$ and $\hat{\sigma}_R^2$ were used). A low maximum probable error increases the ability to determine more subtle differences in the population estimates between sites.

In the epifluorescent technique, the largest source of variation was for the cores, $\hat{\sigma}_C^2$, ranging from 55-77% of the total variance (Table 14). This means that the confidence limits for the epifluorescent enumerations would be most improved if more cores were analyzed at each site. The variance of the slides was not as influential on the error rate as the cores and the variance of the subsamples was not important at all. [N.B., all components were negative for the subsamples and were reported as zero. These values probably represent negatively correlated experimental errors within their respective groups and may be considered

negligible in their contribution to the overall error (Gill, 1978)]. Therefore, changing the number of subsamples per core would not affect the detectable difference. When the components for the sites were calculated independently, the variance due to the comparison of sites, $\hat{\sigma}^2_S$, became part of the core and slide component.

The low component of variance due to the enumeration of the slides compared to the total variance agrees with the low variability observed with the hold time experiment of the epifluorescent technique (Figure 17). The variance of the slides includes the error due to the preparation and enumeration of the filters. Thus improvements in the enumeration would decrease the variance of the slides and improve the precision of the analysis (e.g., counting more area of the filter). However, the percentage of the total variance for the slides was about half that for the cores. The increased cost and drilling time incurred when taking another core would need to be balanced against the benefit of the decreased error in the population estimate.

For the Singh total MPN enumerations, the greatest component was for the subsamples taken from the cores (Table 16). In the case of all MPN techniques, the subsample component was the measure of analytical variability (like the slide component for the epifluorescent technique) and was related to the way in which the Singh dilutions were

prepared. It is possible that the subsample component was more important in the Singh technique because of the heterogeneity in the numerous sediment samples used for the estimates (see Section II.D.1). Also, the relatively large standard error calculated for the Singh total MPN estimates with only three replicates per dilution could have increased the variance for the subsamples compared to the Darbyshire total MPN estimates (see Appendix A).

The relative contribution of the components for the Darbyshire total MPN technique (Table 16) was not as readily apparent as with the other techniques. With the Darbyshire total MPN estimates, the importance of the core and subsample components of variance changed for the site sampled. The subsample component for the Darbyshire MPN estimates at F 393 was the largest source of the total variance (84%) compared to the core component (16%). As mentioned previously, the Darbyshire total MPN estimate for F 393 appeared to be lower than expected considering the percent encysted and the estimates by the other techniques (see Section III.C.2.). The contrast between the subsample and core components for the each site was the reason why the site component for the comparison of F 393 and S 318 was so large for the Darbyshire MPN technique (89%).

The core was the largest component of variance for the Darbyshire total MPN technique at S 318, similar to the

importance of the core to the variance of the epifluorescent technique. Since both of the techniques were prepared from the same core sediment sample, they were expected to have relatively the same core components of variance. However, the greater variances for the Darbyshire MPN estimates compared epifluorescent estimates at S 318 reflect the larger analytical variability of the MPN technique.

The equation for the maximum probable error, E, was also used to determine the detectable differences with different sample sizes for 95% and 80% confidence interval (equation 13). Table 17 shows the detectable differences for hierarchical designs with up to 4 cores/site, 4 subsamples/core and 3 epifluorescent slides/subsample. Tables 18 and 19 illustrates the detectable differences for the total protozoan population estimates by the Darbyshire and Singh MPN techniques, respectively, for a total up to 4 cores and 3 subsamples/core. The untransformed components of variance for the contaminated site, S 318, were used for the calculations (Tables 14-16) since there were no apparent problems with these estimates. Since untransformed variances from transformed estimates have a greater range above the mean then below, the upper variance was used in the calculations to represent the worst case scenario.

The maximum probable error, E, varies according to the importance of the variance components (see equation 13).

Table 17: The maximum probable error for the enumeration of protozoa in sediment samples using the epifluorescent technique. Based on the upper range of the untransformed components determined for S 318.

Core	Subsample per Core	Slide per Subsample	Total # of Slides per Core	Maximum Probable Error with 95%C.I. (P/gdw)	Maximum Probable Error with 80%C.I. (P/gdw)
1	1	1	1	1.25E+04	8.20E+03
1	1	2	2	1.14E+04	7.43E+03
1	1	3	3	1.09E+04	7.16E+03
1	2	1	2	1.14E+04	7.43E+03
1	2	2	4	1.07E+04	7.02E+03
1	2	3	6	1.05E+04	6.87E+03
1	3	1	3	1.09E+04	7.16E+03
1	3	2	6	1.05E+04	6.87E+03
1	3	3	9	1.04E+04	6.77E+03
1	4	1	4	1.07E+04	7.02E+03
1	4	2	8	1.04E+04	6.80E+03
1	4	3	12	1.03E+04	6.72E+03
2	1	1	2	8.86E+03	5.80E+03
2	1	2	4	8.03E+03	5.25E+03
2	1	3	6	7.74E+03	5.06E+03
2	2	1	4	8.03E+03	5.25E+03
2	2	2	8	7.58E+03	4.96E+03
2	2	3	12	7.43E+03	4.86E+03
2	3	1	6	7.74E+03	5.06E+03
2	3	2	12	7.43E+03	4.86E+03
2	3	3	18	7.32E+03	4.79E+03
2	4	1	8	7.58E+03	4.96E+03
2	4	2	16	7.35E+03	4.81E+03
2	4	3	24	7.27E+03	4.76E+03
3	1	1	3	7.24E+03	4.73E+03
3	1	2	6	6.56E+03	4.29E+03
3	1	3	9	6.32E+03	4.13E+03
3	2	1	6	6.56E+03	4.29E+03
3	2	2	12	6.19E+03	4.05E+03
3	2	3	18	6.07E+03	3.97E+03
3	3	1	9	6.32E+03	4.13E+03
3	3	2	18	6.07E+03	3.97E+03
3	3	3	27	5.98E+03	3.91E+03
3	4	1	12	6.19E+03	4.05E+03
3	4	2	24	6.00E+03	3.93E+03
3	4	3	36	5.94E+03	3.88E+03
4	1	1	4	6.27E+03	4.10E+03
4	1	2	8	5.68E+03	3.72E+03
4	1	3	12	5.47E+03	3.58E+03
4	2	1	8	5.68E+03	3.72E+03
4	2	2	16	5.36E+03	3.51E+03
4	2	3	24	5.25E+03	3.44E+03
4	3	1	12	5.47E+03	3.58E+03
4	3	2	24	5.25E+03	3.44E+03
4	3	3	36	5.18E+03	3.39E+03
4	4	1	16	5.36E+03	3.51E+03
4	4	2	32	5.20E+03	3.40E+03
4	4	3	48	5.14E+03	3.36E+03

Table 18: The maximum probable error for the enumeration of protozoa in sediment samples using the Darbyshire liquid media MPN technique. Calculations based on the upper range of the untransformed components of variance determined for S 318.

Core	Subsample per Core	Total # of Subsamples per Core	Maximum Probable Error with 95% C.I. (MPN/gdw)	with 80% C.I. (MPN/gdw)
1	1	1	1.02E+05	6.69E+04
1	2	2	9.63E+04	6.30E+04
1	3	3	9.42E+04	6.16E+04
2	1	2	7.23E+04	4.73E+04
2	2	4	6.81E+04	4.45E+04
2	3	6	6.66E+04	4.36E+04
3	1	3	5.91E+04	3.86E+04
3	2	6	5.56E+04	3.64E+04
3	3	9	5.44E+04	3.56E+04
4	1	4	5.11E+04	3.35E+04
4	2	8	4.81E+04	3.15E+04
4	3	12	4.71E+04	3.08E+04

Table 19: The maximum probable error for the enumeration of protozoa in sediment samples using the Singh solid media MPN technique. Calculations based on the upper range of the untransformed components of variance determined for S 318.

Core	Subsample per Core	Total # of Subsamples per Core	Maximum Probable Error with 95% C.I. (MPN/gdw)	Maximum Probable Error with 80% C.I. (MPN/gdw)
1	1	1	1.44E+05	9.39E+04
1	2	2	1.02E+05	6.64E+04
1	3	3	8.29E+04	5.42E+04
2	1	2	1.02E+05	6.64E+04
2	2	4	7.18E+04	4.70E+04
2	3	6	5.86E+04	3.84E+04
3	1	3	8.29E+04	5.42E+04
3	2	6	5.86E+04	3.84E+04
3	3	9	4.79E+04	3.13E+04
4	1	4	7.18E+04	4.70E+04
4	2	8	5.08E+04	3.32E+04
4	3	12	4.15E+04	2.71E+04

For example with the epifluorescent technique, the largest source of variance was the core component and therefore, E decreased the most with each additional core analyzed. The lowest E for the enumeration of one core involved the examination of 12 slides (1.03×10^4 protozoa/gdw) was still greater than the E for only 2 slides enumerated from 2 cores (8.86×10^3 protozoa/gdw). Since the subsample component for the epifluorescent samples was negligible, the E depended only on the number of slides enumerated for the core sample. For example, the same E was calculated whether the analysis of one core included 1 subsample and 2 slides/subsample, or 2 subsamples and 1 slide/subsample (i.e., 1.14×10^4 protozoa/gdw).

In the case of the Darbyshire total MPN estimates the greatest component of variance at S 318 was that due to the analyses of the cores (Table 18). The influence of the cores was apparent from the 95% confidence intervals for the error for one core and 2 or 3 subsamples/core--they differed by only 0.21×10^4 total MPN/gdw due to the limited importance of the subsample component and the importance of the subsample component to the total variance. In the Singh MPN enumerations (Table 19), the variance due to the cores was negligible, and the same error was calculated when the total number of subsamples/core was the same (e.g., the maximum probable error was 1.02×10^5 MPN/gdw when 1 core and 2

subsamples/core or 2 cores and 1 subsample/core were analyzed).

Since significant differences were found between F 393 and S 318 for all of the enumeration estimates (with the oneway ANOVA, Table 13), the detectable differences calculated using equation 13 should be approximately the same or less than the differences between the means. The detectable difference (E) for 3 cores, 3 subsamples/core and 2 slides/subsample was 6.07×10^3 protozoa/gdw with 95% confidence which was $<6.50 \times 10^3$ protozoa/gdw for the difference between the means of the epifluorescent technique for F 393 and S 318. Therefore, the detectable difference would have found the sites significantly different without the use of a statistical test. However, because conservative calculations for the errors (i.e., the largest value of the standard deviation was used) were made often the values of E (even with 80% confidence) were slightly greater than the differences in the means for the MPN techniques: 3.64×10^4 MPN/gdw vs. 2.16×10^4 MPN/gdw for the Darbyshire technique; and 3.84×10^4 MPN/gdw vs. 2.36×10^4 MPN/gdw for the Singh technique, respectively.

Figure 17 illustrated that the differences due to hold time were relatively small for the epifluorescent and Darbyshire MPN technique compared to the Singh MPN technique. The detectable differences weighed the

importance of the analyses to the components of variance. The change in protozoa per day of hold time was -145 protozoa/gdw for the epifluorescent estimates, so over 28 days the overall change was 4.06×10^3 protozoa/gdw, which was less than the detectable difference with 95% confidence (6.07×10^3 protozoa/gdw). Based on the same approach, the change in Darbyshire MPN estimates (1.56×10^4 MPN/gdw) was also less than the detectable difference (5.56×10^4 MPN/gdw). However, the Singh MPN estimates dramatic increase over the hold time (2.51×10^5 MPN/gdw) exceeded even the detectable difference (5.86×10^4 MPN/gdw). Whereas, the statistical analyses were too sensitive (i.e., oneway ANOVA, Kruskal-Wallis and Scheffé's test) the detectable differences indicated that the epifluorescent and Darbyshire MPN estimates were independent of the 28 day hold time compared to the sources of variance inherent to the field, laboratory and analytical procedures. Also, the detectable differences suggest that the increase in the Singh MPN estimates over time should be further investigated.

The detectable differences can be used in establishing criteria for future sampling programs. There was no benefit in the analysis of a fourth core by any of the techniques since the decrease in the maximum probable error was not significant. In the case of the epifluorescent estimates, the error for 3 cores, 2 subsamples/core and 2

slides/subsample ($6.19x10^3$ protozoa/gdw) was less than that for 4 cores, 1 subsample/core and 1 slide/subsample ($6.27x10^3$ protozoa/gdw). The cost of collecting core samples greatly outweighs the scenarios presented in Tables 17-20, especially if more than one site is to be considered in the study. The time to prepare slides and subsamples for the enumeration techniques was minimal compared to the time required to examine the samples (Table 5). Therefore, if only one core were to be taken at a site, then the lowest detectable difference would be with the analysis of 2 subsamples/core and 3 slides/subsample ($1.05x10^4$ protozoa/gdw) since the difference between this scenario and the lowest error for 1 core ($1.03x10^4$ protozoa/gdw) was only 200 protozoa/gdw with 95% confidence (which could be exceeded by the differences due to hold time). The best sampling program for the Darbyshire MPN technique would be 2 subsamples/core ($9.63x10^4$ MPN/gdw) since the variance due to the core was negligible. At least 3 subsamples/core ($8.29x10^4$ MPN/gdw) should be taken for the Singh MPN technique. However, the effect of hold time of the Singh estimates was so great that the detectable differences should be further investigated, perhaps with more replicates per dilution.

D. Sterile vs. Non-sterile Core Sleeves

Five cores were collected at S 318 to investigate the use of sterile sleeves vs. the normal procedure for handling the cores: two sleeves (C 15 and C 17) were washed with 95% ethanol in the field prior to coring; and the other three sleeves were left untreated (Table 6). All cores were enumerated within 1 day after the samples were collected (to limit variability due to hold time) with epifluorescent direct counts (previous sampling events had indicated that the epifluorescent technique had the lowest variability and thus the greatest ability to differentiate between estimates). The counts of the cores with sterile sleeves were compared to those with non-sterile sleeves using oneway ANOVA (Table 20) and no significant difference ($P>F=0.18$) was found between the protozoan enumerations. The variability of the sterile estimates was greater than that for the non-sterile (the standard deviations were smaller), which indicates that the epifluorescent technique could have detected contamination.

The protocol for sampling the cores in the laboratory (sediment only from the center of the sleeve was removed) appears to have been sufficient for limiting the effects of contamination from the core sleeves. Although cleaning core sleeves with 95% ethanol was not as rigorous of sterilization process as autoclaving, the procedure was the

Table 20: Comparison between the epifluorescent estimates on the cores taken with sterile core sleeves and non-sterile core sleeves. The mean, lower and upper standard deviations, and 95% minimum and maximum confidence intervals calculated from the square root transformed data. All estimates made after 1 day hold time.

Epifluorescent Estimates	Sterile Core Sleeves (protozoa/gdw)	Non-sterile Core Sleeves (protozoa/gdw)
Mean	1.41×10^4	1.22×10^4
s_L	3.69×10^3	2.32×10^3
s_U	4.35×10^3	2.53×10^3
95% Minimum Confidence Interval	1.34×10^4	1.14×10^4
95% Minimum Confidence Interval	1.48×10^4	1.32×10^4

only option available at the time and there was no way to maintain a sterile environment in the field. For example, loading the piston of the Waterloo corer into the sleeve required a lot of effort and could not have been done very easily using sterile techniques. Therefore, prevention of contamination is most important by sampling the sediments in the cores carefully and analyzing sterile controls for the enumeration techniques. Occasional use of sterile core sleeves should be included in the sampling programs to monitor the core sleeves' potential for contamination.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

Microbial communities in the saturated subsurface environment have been investigated over the last two decades, primarily to determine their influence on the degradation of organic contaminants in ground water. The focus of a majority of the research has been on the bacterial populations. The diversity of the microbial community was revealed by Hirsch and Rades-Rohkohl (1983) when they reported bacteria, fungi and protozoa in cultured samples of ground water. However, the importance of protozoa in the subsurface environment has only been recently studied since the techniques for detecting them have not been as intensively developed as the bacterial enumeration methods. This dissertation has reviewed available enumeration techniques and discussed the development of an epifluorescent direct count technique and modifications to two types of MPN techniques for subsurface sediment samples.

Core samples collected at the U.S.G.S. site in Cape Cod, MA since Summer 1988 have been enumerated for protozoa with several techniques. A modified Uhlig ice extraction technique was unsuccessful since the procedure diluted the samples below the detection limit for the protozoa. Cores

collected in Spring 1990, Fall 1990, Summer 1991, and Fall 1991 were analyzed with either the epifluorescent direct counts, Darbyshire liquid media MPN or Singh solid media MPN techniques. The protozoa were separated from the sediment particles by a shaking procedure (modification of Dye (1979)) for the epifluorescent and Darbyshire MPN techniques. The dilutions for the Singh MPN technique (Sinclair and Ghiorse, 1987) were prepared using a mechanical mixer. Each time sediment samples were enumerated valuable experience was gained that reduced the variability of the population estimates. Improvements were made with the preparation for the various techniques (e.g., glass and plastic rings vs. macrotiter plates for Singh MPN) and detection of the protozoa in the samples (e.g., acriflavin vs. DAPI stain). Modifications of the procedures also reduced the amount of time required to analyze the samples without compromising the integrity of the technique (e.g., random field vs. scanning of epifluorescent slides).

The sampling program conducted in Fall 1991 investigated the variability associated with the hold time of the cores, the total and encysted population estimates (for the MPN techniques), and each site. All analyses were based on three cores collected from an uncontaminated (F 393) and contaminated (S 318) site. Three subsamples/core and two slides/subsample were enumerated for the epifluorescent

technique. Two subsamples/core were enumerated for the total and encysted protozoan estimates by the Darbyshire and Singh MPN techniques. Also, two additional sediment samples were collected at S 318 with sterile core sleeves and analyzed with the epifluorescent technique to determine if there was any significant contamination due to the preparation of the core sleeves. The following conclusions were made:

- There were significant differences in the protozoan estimates as a function of the hold time of the cores for each enumeration technique (95% confidence). The epifluorescent technique had the lowest variability for each sampling day and throughout the 28 days of storage. The Darbyshire total MPN estimates did not change more than one order of magnitude over the storage period. The Singh total MPN estimates increased approximately two orders of magnitude during the period between 5 and 28 days. However, there was no significant difference among the three enumeration estimates at 15 days after the core samples were collected. The similarities of the epifluorescent and Darbyshire total MPN estimates indicated that the shaking separation procedure was efficient despite any changes with the protozoa population over the storage period. The Singh technique provided an additional nutrient source that may

have been responsible for the apparent increase in population estimates with time.

- The encysted population never exceeded more than 42% of the total population estimated by either MPN technique over the 28 day hold time of the S 318 sediments. The highest proportion of encysted protozoa estimated by the Darbyshire MPN technique was 29% on day 5 and decreased to 9% by day 28. The highest proportion of encysted protozoa by the Singh MPN technique was 42% on day 15 and decreased to 13% by day 28. Therefore, the conditions in the cores during storage remained favorable for trophic protozoan populations.

- There was a significant difference between the protozoan estimates by each of the enumeration techniques for the uncontaminated site (F 393, hold time = 14 days) and the contaminated site (S 318, hold time = 15 days), with 90% confidence. However, there were some discrepancies with the F 393 Darbyshire total MPN estimates since they appeared to be underestimations based on the results of the other techniques and the encysted protozoan estimates. The largest source of variation for the S 318 samples with the epifluorescent and Darbyshire total MPN estimates was due to the variability of the cores since the subsample component (analytical variability) was reduced by increasing the replication within the sample estimates (i.e., 2

slides/subsample for the epifluorescent estimate and 8 replicates/dilution for the Darbyshire MPN estimate). The subsample component was the largest source of variability for the Singh total MPN estimates since they were based on smaller samples from the cores which were more influenced by the heterogeneity of the sediments. The variability observed during the 28 day hold time was less than the maximum probable error (E) for the epifluorescent and Darbyshire total MPN techniques, therefore there was no detectable difference between these estimates due to storage. However, E was less than the increase in the Singh total MPN estimates with storage of the cores. This suggests that the lower estimates of protozoan population reported by researchers using the Singh total MPN technique (on samples analyzed within 1 week of collection) may be due to the observed effect of storage time on the analysis of the sediment.

- Epifluorescent estimates of protozoa in sediments collected in non-sterile core sleeves were not significantly different from those collected in sterile core sleeves with 95% confidence (hold time = 1 day). Therefore, contamination due to the treatment of the core sleeves was not significant using the procedures described for sampling in the field and analyses in the laboratory.

From the conclusions, several recommendations can be made for future sampling programs for protozoan estimates of sediments collected at the U.S.G.S. site:

- The researcher will need to be experienced with each enumeration technique in order to detect the small (2-3 μm) protozoa found in the sediments from the U.S.G.S. site. Appendix C discusses culturing techniques that can be used to cultivate the sediment protozoa for observation in epifluorescent preparations and wet mounts examined with Nomarski interference and Hoffman modulation optics. Appendix D describes a sampler that can be incubated in monitoring wells at the site and then examined for protozoa found in the ground water.
- The diversity and relative cell size of the protozoa observed should be recorded for the MPN enumerations since flagellates and amoebae may respond differently to the conditions of the techniques and storage time of the cores.
- The significant difference in the Singh total MPN estimates compared to the epifluorescent and Darbyshire total MPN estimates as a function of hold time of the cores should be further investigated. The focus of additional research should be to determine if the association of the protozoa to the sediment particles is responsible for the differences in the Singh estimates compared to the other

techniques. The results of the Fall 1991 samples suggested that the additional source of nutrients in the Singh analysis was responsible for the underestimation of protozoa in fresh samples and then overestimation after 28 days of storage. An experiment could be conducted to compare estimates from macrotiter plates (with and without bacteria inoculated into the wells of the Singh MPN technique) to the Darbyshire liquid media MPN technique. Additional macrotiter plates should also be prepared using the same shaking extract used for the epifluorescent and Darbyshire estimates and thus, all three enumeration techniques could be directly compared. The first dilution for these Singh estimates should be the shaking extract and successive 1/5 dilutions should be prepared with mechanical mixing. These results will demonstrate the efficiency of removing the protozoa with the more vigorous mixing procedure. Also, the variability of the Singh technique may be reduced compared to the other techniques if the number of replicates per dilution is increased. Each macrotiter plate could be devoted to a single dilution. However, the examination of more wells would increase the time and space required for the analysis and therefore make the Singh technique the most expensive and time consuming of the enumeration techniques.

- Analysis of more cores could demonstrate if the convergence of estimates around 15 days after collection was

coincidental or a phenomenon of the hold time. The sample program should include the acid treated samples of the MPN techniques to determine if the active population continues to be the major proportion of protozoa in the cores with storage time.

- Sterile core sleeves should be used occasionally during a sampling program to monitor contamination, especially in cores that must be stored for a considerable length of time.

- If only one core can be collected at each site and only one subsample/core can be analyzed in a sampling program, then for the protozoan estimates to be considered significantly different with 80% confidence they must differ by more than: 8.20×10^3 protozoa/gdw for the epifluorescent technique (with 1 slide/subsample); 6.69×10^4 MPN/gdw for the Darbyshire total MPN technique; and 9.39×10^4 MPN/gdw for the Singh total MPN technique. The relative improvement of the estimates by increasing the number of subsample depends on the enumeration technique. Therefore, in a limited sampling program, the epifluorescent technique would be the best to chose since the time and space requirements are less compared to the other techniques, and thus more slides/core could be prepared to further reduce the variability of the estimates.

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APPENDIX A

Appendix A contains the computer program to calculate MPN, the formulas used to calculate the protozoan populations and the raw data for the Fall 1991 samples.

I. MPN CALCULATIONS

The computer program used to calculate the MPN values for the Darbyshire and Singh estimates was based on the work of Hurley and Roscoe (1983) as modified by Arnold (1992). The terminology to be used is shown in Figure 9 and discussed in Chapter 4, Section II.B.2. The method of maximum likelihood is the basis of the MPN calculation:

$$\Sigma_{i=1}^{k} \left(\frac{v_i d_i p_i}{1-e^{-v_i d_i x}} \right) = \Sigma_{i=1}^{k} v_i d_i n_i \qquad [14]$$

where k is the number of levels of dilution to extinction; v_i is the volume of each subsample (in mL); d_i is the dilution factor at level i; p_i is the number of positive subsamples at level i; and n_i is the number of subsamples at level i. The above equation is an iterative numerical technique which is more difficult to solve than the method of moment estimation used by Fisher (1922). However, the corresponding algorithm is not difficult to solve with a computer and the method of maximum likelihood does not restrict the type of dilution series chosen by the researcher (Hurley and Roscoe, 1983).

The program written by Hurley and Roscoe (1983) includes the calculation of the MPN/mL, standard error ($\log_{10}$), 95% confidence interval and deviance. Some experience with BASIC computer language is required to use the program. Arnold (1992) modified the BASIC code to current conventions and customized the program to the dilution series used for the Darbyshire and Singh protozoan estimates as well as a procedure for printing the output to a printer or an ASCII file. The following section includes the general program (the operator must enter v_i, d_i and n_i for each dilution level) and a description of how to use the program.

PROGRAM: MPNFIL
GENERAL PURPOSE MICROORGANISM ENUMERATION PROGRAM
MODIFIED BY KURT E. ARNOLD

THIS PROGRAM SENDS OUTPUT TO THE FILE: MPNFIL.RES

```
1 CLS
10 REM ******************************************
20 REM GENERAL PURPOSE PROGRAM FOR MPN, ITS S.E,
30 REM C.I. AND HOMOGENEITY TEST STATISTIC.
40 REM ******************************************
50 REM
60 DIM A(10,6),X2(3,9)
70 COMMON YUP
80 COMMON TYPE$
90 REM SET PROGRAM LIMITS
100 D9=10
110 U9=50
120 SPCASE = 0
130 L9=0
140 A1=.0005
150 E1=85
160 GOSUB 1000
170 GOSUB 2000
180 GOSUB 3000
190 GOSUB 4000
200 GOSUB 5000
210 GOSUB 6000
220 END
1000 REM SET CHI-SQUARED SIGNIFICANCE LEVELS
1010 FOR I=1 TO 3
1020 FOR J=1 TO 9
1030 READ X2(I,J)
1040 NEXT J
1050 NEXT I
1060 REM PERCENT LEVELS DF=1...9
1070 DATA 3.84,5.99,7.81,9.49,11.07
1080 DATA 12.59,14.07,15.51,16.92
1090 REM 1 PERCENT LEVELS
1100 DATA 6.63,9.21,11.34,13.28,15.09
1110 DATA 16.81,18.48,20.09,21.67
1120 REM .1 PERCENT LEVELS
1130 DATA 10.83,13.81,16.27,18.47,20.52
1140 DATA 22.46,24.32,26.12,27.88
1150 RETURN
2000 REM READ IN RESULTS OF A DILUTION SERIES
2010 PRINT "MPN GENERAL PURPOSE PROGRAM"
2020 PRINT "**************************"
2030 PRINT " "
2040 PRINT "M.A.HURLEY AND M.E.ROSCOE"
2050 PRINT "MODIFIED BY KURT E. ARNOLD"
2060 PRINT " "
2070 PRINT "NUMBER OF DILUTION LEVELS.....K=";
```

```
2080 INPUT N
2090 IF N>10 THEN GOTO 8000
2100 IF N<1 THEN GOTO 2070
2110 IF N<=09 THEN GOTO 2140
2120 PRINT "ERROR *** LEVELS EXCEED MAXIMUM"
2130 STOP
2140 S1=0
2150 FOR I=1 TO N
2160 PRINT " "
2170 PRINT "LEVEL NUMBER.....I=";I
2180 PRINT "DILUTION FACTOR.....D=";
2190 INPUT A(I,2)
2200 PRINT "SUBSAMPLE VOLUMN.....V=";
2210 INPUT A(I,1)
2220 PRINT "NUMBER OF SUBSAMPLES.....N=";
2230 INPUT A(I,3)
2240 PRINT "NUMBER OF POSITIVE SUBSAMPLES...P=";
2250 INPUT A(I,4)
2260 PRINT "IS THE DATA CORRECT FOR LEVEL ";I;"(Y OR N)";
2270 INPUT R$
2280 IF R$="Y" OR R$="y" THEN GOTO 2300
2290 GOTO 2160
2300 A(I,5)=A(I,1)*A(I,2)
2310 A(I,6)=A(I,5)*A(I,4)
2320 S1=S1+A(I,5)*A(I,3)
2330 NEXT I
2340 RETURN
3000 REM CALCULATES AND PRINTS MPN
3010 B1=0
3020 S3=0
3030 FOR J=1 TO N
3040 E2=A(J,5)*U9
3050 IF E2<E1 GOTO 3080
3060 E2 = 0
3070 GOTO 3090
3080 E2=EXP(-E2)
3090 S3=S3+A(J,6)/(1-E2)
3100 NEXT J
3110 IF S3-S1>=0 THEN GOTO 3130
3120 GOTO 3200
3130 FOR I=1 TO N
3140 A(I,5)=A(I,5)*2
3150 A(I,6)= A(I,6)*2
3160 NEXT I
3170 S1 = S1*2
3180 B1=B1+1
3190 GOTO 3020
3200 X3=L9
3210 X4=U9
3220 X=(X3+X4)/2
3230 S=0
3240 FOR I = 1 TO N
3250 E2=A(I,5)*X
3260 IF E2<E1 GOTO 3290
3270 E2=0
3280 GOTO 3300
3290 E2 = EXP(-E2)
3300 S=S+A(I,6)/(1-E2)
3310 NEXT I
3320 IF ABS(S-S1)<A1 THEN GOTO 3380
3330 IF S-S1>0 THEN GOTO 3360
3340 X4=X
3350 GOTO 3220
3360 X3=X
3370 GOTO 3220
3380 X5=X*(2^B1)
3390 PRINT "IS THE SOLUTION ACIDIFIED?"
3400 INPUT O$
3410 PRINT "WHAT IS THE SITE NAME ?";
```

```
3420 INPUT SITE$
3430 PRINT "WHAT TYPE OF TEST IS THIS? (e.g. MICROTITER, SINGH, ect)";
3440 INPUT TEST$
3445 OPEN "MPN.RES" FOR APPEND AS 1
3450 IF SPCASE = 0 THEN GOTO 3490
3460 PRINT#1, "**************************************"
3470 PRINT#1, "**          SPECIAL CASE            **"
3480 PRINT#1, "**************************************"
3490 PRINT#1, "------------------------------------------"
3500 PRINT#1, TEST$ " TEST RESULTS AT" N " DILUTIONS"
3510 PRINT#1, "AT SITE: "SITE$
3520 IF O$ = "N" OR O$ = "n" GOTO 3540
3530 PRINT#1, "ACIDIFIED"
3540 PRINT#1, " "
3550 PRINT#1, "MPN=";X5
3560 PRINT#1, "FOR A SAMPLE WITH DILUTION FACTOR 1"
3570 PRINT#1, "                        AND VOLUMN 1"
3580 RETURN
4000 REM CALCS AND PRINTS S.E. OF LOG10(MPN)
4010 S2 =0
4020 FOR I=1 TO N
4030 X3=A(I,5)
4040 E2 = X3*X
4050 IF E2<E1 GOTO 4080
4060 X4 = 0
4070 GOTO 4090
4080 X4=EXP(-E2)
4090 S3=X3*X3*A(I,3)*X4
4100 S3=S3/(1-X4)
4110 S2=S2+S3
4120 NEXT I
4130 V = 1/(X*X*S2)
4140 S1=SQR(V)/LOG(10)
4150 PRINT#1, " "
4160 PRINT#1, "S.E.OF LOG10(MPN)=";S1
4170 RETURN
5000 REM CALCS 95 PERCENT C.I. FOR MPN
5010 X3=LOG(X) + B1*LOG(2)
5020 S2=SQR(V)
5030 U=EXP(X3+1.96*S2)
5040 L=EXP(X3-1.96*S2)
5050 PRINT#1, "95 PERCENT C.I. =";L;"TO";U
5060 RETURN
6000 REM CALCS. AND PRINTS DEVIANCE
6010 S3=0
6020 FOR I = 1 TO N
6030 S4=0
6040 IF A(I,4)<=0 GOTO 6110
6050 E2 = A(I,5)*X
6060 IF E2<E1 GOTO 6090
6070 E2=0
6080 GOTO 6100
6090 E2=EXP(-E2)
6100 S4=A(I,4)*LOG(A(I,4)/(A(I,3)*(1-E2)))
6110 S3=S3+S4
6120 S4=0
6130 IF A(I,4)>=A(I,3) GOTO 6160
6140 S4=A(I,3)-A(I,4)
6150 S4=S4*(LOG(S4/A(I,3))+A(I,5)*X)
6160 S3=S3+S4
6170 NEXT I
6180 D=2*S3
6190 REM CHI-SQUARED TEST OF DEVIANCE
6200 V=N-1
6210 PRINT#1, "DEVIANCE =";D;" ON";V;" D.F."
6220 PRINT#1, " "
6230 PRINT#1, "CHI-SQUARED SIGNIFICANCE LEVELS FOR";V;" D.F."
6240 PRINT#1, "     5  PERCENT   ";X2(1,V)
6250 PRINT#1, "     1  PERCENT   ";X2(2,V)
```

```
6260 PRINT#1, "     .1 PERCENT   ";X2(3,V)
6270 PRINT#1, "----------------------------------------"
6275 CLOSE #1
6280 CHAIN "MAIN",300
6290 END
8000 REM WHAT ROW WOULD YOU LIKE TO BEGIN WITH
8010 Z2 = N-9
8020 REM WHAT ROW WOULD YOU LIKE TO FINISH WITH
8030 Z3 = N
8040 Z5 = Z3 - Z2
8050 IF Z3 < Z2 THEN GOTO 8000
8060 IF Z2<1 THEN GOTO 8000
8070 IF Z5<=D9 THEN GOTO 8100
8080 PRINT "ERROR *** LEVELS EXCEED MAXIMUM"
8090 STOP
8100 S1=0
8110 FOR I=1 TO Z5+1
8120 PRINT " "
8130 PRINT "LEVEL NUMBER.....................I=";I + Z2 -1
8140 PRINT "DILUTION FACTOR.................D=";
8150 INPUT A(I,2)
8160 PRINT "SUBSAMPLE VOLUMN................V=";
8170 INPUT A(I,1)
8180 PRINT "NUMBER OF SUBSAMPLES............N=";
8190 INPUT A(I,3)
8200 PRINT "NUMBER OF POSITIVE SUBSAMPLES...P=";
8210 INPUT A(I,4)
8220 PRINT "IS THE DATA CORRECT FOR LEVEL ";I+Z2-1;"(Y OR N)";
8230 INPUT R$
8240 IF R$="Y" OR R$ = "y" THEN GOTO 8260
8250 GOTO 8120
8260 A(I,5)=A(I,1)*A(I,2)
8270 A(I,6)=A(I,5)*A(I,4)
8280 S1=S1+A(I,5)*A(I,3)
8290 NEXT I
8300 N=Z5
8310 SPCASE = 1
8320 GOTO 2340
```

The NSF-MPN subdirectory is accessed by executing the following commands (CAPITALS indicate keyboard entry):

1) type GWBASIC, return
2) hit the "F3" button for the LOAD" prompt
3) type MAIN, return
4) type RUN, return

MAKE SURE THE CAPITAL LOCK BUTTON ON YOUR KEYBOARD IS ON! YOU WANT TO INPUT ALL CAPITAL LETTERS TO MAIN. MAIN IS THE ONLY PROGRAM OF THE SET THAT IS CAPITAL SENSITIVE.!!!!!

The menu screen will appear with the program options for the subdirectory. You MUST follow the instructions indicated concerning the printer in order for the program to work with the printing option. The printer must not only be turned on but online as well. Please also adjust the printer to the top of the page.

Eight programs are available within MAIN. MAIN also displays the preset criteria within each of the programs. Three of the eight programs obtain results for the cases where laboratory testing followed preset standards. These three laboratory tests are: the Darbyshire Test, the Singh test for 3 subsamples per dilution and the Singh Test for 4 subsamples per dilution. An addition program is provided for the case where the laboratory procedure did not follow any of the fore mentioned testing procedures. Each of these programs can supply output to a preset file or to the printer. In total four programs push output to the printer and four push output to a printer. In the case that an output file does not exist one will be created, in the case that the output file already exists the output will be appended to the end of the current file.

In the case that the user wishes to send results to a results file. The following table shows the name of the program, as selected from MAIN, and the output file that will either be created or appended too.

NOTE: In the case that the results file already exists: the latest results sent to that file will be tacked on after the last resultant set sent to it (RESULTS ARE ADDED TO THE BOTTOM OF THE OUTPUT FILES).

NOTE: To save your self some confusion you should copy the resultant data file to another directory with a appropriate name after your done with your session. You should then delete these files as you finish your computations for that daily session. It is recommended that the user copy these files to a directory that contains just resultant files with an extension that represents the day of computation (the date the data was processed by the computer).

EXAMPLE: The resultant file created is named SIGN3D.RES
The day the data was processed was April 21.

COPY THIS FILE TO A DIRECTORY ENTITLED RESULTS: (BY THE COMMANDS)

COPY SIGN3D.RES A:\results\SIGN3D.A21

Where the .A21 is the extension and in this case represents the first letter of the month the data processed and the day of that month. "A" could represent April and the 21 being the 21st day of April.

The user should then delete the original data file SIGN3D.RES as to avoid appending future results to this file.

TABLE OF PROGRAM SELECTED FROM MAIN	OUTPUT FILE CREATED OR APPENDED TO
DF	DARFILE.RES
S3F	SIGNFIL.RES
S4F	SIGNH4DF.RES
GF	MPNFIL.RES

Four Major program types are available from MAIN. The addition of either a P or an F after the test name specified in MAIN will either send the output to a printer or a file. "P" for Printer and "F" for a File. The file that the results will be sent to depends on which test you select in MAIN and the name of the created or appended file can be found in the Table above.

The major programs available from MAIN are as follows:

"D" --- calculates MPN values for the Darbyshire microtiter plates where there are 12 levels (a.k.a rows) of 8 subsamples (a.k.a. replicates) per dilution and the volume of the first level=.15 mL, twelfth level = .25 mL and all other levels = .20 mL.

"S3" -- calculates MPN values for the Singh plates with 3 subsamples per dilution, ten-fold dilutions and the volume per subsample = 1 mL.

"S4" -- is similar to "S3", but with 4 subsamples per dilution.

"G" --- is a generic program for calculating MPN values. You must enter the subsample volume, dilution factor, number of subsamples and number of positive subsamples at every level. This program is available for any calculations that do not fit the descriptions given with the other programs.

EXAMPLE: The user wishes to obtain MPN results for Darbyshire Test Data. The user also wishes to send that data to a output file so that they can organize the results using a spreadsheet or word processor.

SOLUTION: Execute MAIN as described previously. From the menu in MAIN, type:

DF

The Darbyshire results program is then activated and results will be sent to the output file DARFILE.RES. The user can then load these results into a spreadsheet or word processor by specifying to that program that DARFILE.RES is a ASCII type file.

QUESTIONS ASKED BY PROGRAMS AND THEIR MEANINGS:

"Is this a special case?" -- Are there more than 10 dilution levels in the microtiter plate. If there are, hit "Y" (for yes); if there are not, type "n" (for no). SEE HINTS below for instructions with special cases.

"How many dilution levels?"- The total number of dilutions needed for extinction (the first level at which all replicates are negative).

"Starting Row?" ---------- The first row in which not all replicates are positive.

"Is the solution Acidified?"-The output will indicate ACIDIFIED if you answer "Y" to this question.

"What is the site name?"---- The output will contain the message you enter after the question. You should try to include as much information pertinent information as you can (e.g. test date, sample drawn from, lab personnel that did testing).

NOTE: Make sure the printer is ON and ONLINE, because after you hit return to the last question, the program will start sending results to the printer if you selected that output option in MAIN. The following message will result if the printer is not ON and ONLINE if you selected the print option in MAIN: "DEVICE ERROR".

NOTE: The MAIN program will automatically advance the printer to the next page after every third output. You must start the program with the top of page adjusted correctly on the printer to avoid results ending up on page perforations.

"Do you want more calculations from this program?"-- If "Y" the MAIN program will assume the user wants the same type of output and for the same type of test. If "N", the program will end and allow the user to enter another program from MAIN's menu.

NOTE: To end the program (in MAIN) type: END

NOTE: If you end out of the program you'll still be in GWBASIC, to get out of GWBASIC and back to DOS (the "C:\" prompt) type: SYSTEM.

HINTS:

If you make a mistake, hit "CTRL C" at any time to hault program execution. Type LOAD"MAIN, return, RUN, return. This will kick you back to MAIN where you can reselect the appropriate program and enter your data again.

II. CALCULATION OF PROTOZOZOAN POPULATIONS

The following formulas were used to calculate the protozoan estimates expressed in units of "gdw". Moisture content was determined for all sediment samples (see Chapter 3, Section I.B.) and the results were used to calculate a dry weight conversion (DW):

$$DW = \frac{(B_o - B_{ws}) - (B_o - B_{ds})}{(B_o - B_{ws})} \qquad [15]$$

where B_o is the beaker tare (in g); B_{ws} is the beaker weight when containing wet sediment; and B_{ds} is the beaker weight when containing dried sediment.

The formula for the epifluorescent technique depends on which counting technique is used: fields or scanning. Counting fields relates the number of protozoa/field to the total number of fields available on the filter:

$$\frac{\text{protozoa}}{\text{gdw}} = \left[\frac{P_F}{F}\right]\left[\frac{\frac{\pi}{4}(d_F)^2}{\frac{\pi}{4}(d_f)^2}\right]\left[\frac{1}{V_F}\right]\left[\frac{V_d}{x}\right] DW \qquad [16]$$

where P_F is the total number of protozoa observed in all fields counted; F is the number of fields observed; d_F is the diameter of the area of the filter covered with filtrate; d_f is the diameter of the field; V_F is the volume of the sample filtered; V_d is the volume of the diluent; and x is the wet weight of the sediment extracted with the diluent.

Scanning the filter to enumerate the epifluorescent protozoa relates the number of protozoa/scan to the area of the filter observed:

$$\frac{\text{protozoa}}{\text{gdw}} = \left[\frac{P_S}{S}\right]\left[\frac{\frac{\pi}{4}(d_F)^2}{L\ d_f}\right]\left[\frac{1}{V_F}\right]\left[\frac{V_d}{x}\right] DW \qquad [17]$$

where P_S is the total number of protozoa observed in all scans; S is the number of scans; and L is a length of the scan.

The MPN estimations calculated by the computer program are expressed "per 1 mL in the first dilution". All the estimates by the Darbyshire and Singh, total and encysted, can be converted to "MPN/gdw" with the following equation:

$$\frac{MPN}{gdw} = \left(\frac{MPN}{mL}\right)\left(\frac{Vd}{x}\right) DW \qquad [18]$$

where MPN/mL is the value calculated with the MPN computer program.

III. RAW DATA

The following pages contain the data used to calculate the protozoan estimates for each enumeration technique using the Fall 1991 cores.

Epifluorescent Enumerations for Fall 1991 Sampling Program, U.S.G.S. Si

Site: F 393; Hold Time: 14 days; Core Sleeve: Non-sterile
C = Core C = 1 = F 393 C15 11.4-11.7 m
R = Core subsample 2 = F 393 C16 11.4-11.7 m
sl = slide 3 = F 393 C17 11.4-11.7 m

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
1	1	1	1	8	20	1.1834	4717
1	1	1	2	6			
1	1	1	3	6			
1	1	2	1	9	23	1.1834	5425
1	1	2	2	7			
1	1	2	3	7			
1	2	1	1	7	24	1.1834	5661
1	2	1	2	8			
1	2	1	3	9			
1	2	2	1	10	29	1.1834	6840
1	2	2	2	9			
1	2	2	3	10			
1	3	1	1	7	20	1.1834	4717
1	3	1	2	6			
1	3	1	3	7			
1	3	2	1	7	24	1.1834	5661
1	3	2	2	7			
1	3	2	3	10			
2	1	1	1	19	60	1.1767	14072
2	1	1	2	19			
2	1	1	3	22			
2	1	2	1	13	41	1.1767	9616
2	1	2	2	10			
2	1	2	3	18			
2	2	1	1	10	49	1.1767	11492
2	2	1	2	19			
2	2	1	3	20			
2	2	2	1	24	80	1.1767	18762
2	2	2	2	30			
2	2	2	3	26			
2	3	1	1	14	45	1.1767	10554
2	3	1	2	19			
2	3	1	3	12			
2	3	2	1	14	43	1.1767	10085
2	3	2	2	14			
2	3	2	3	15			
3	1	1	1	26	89	1.1511	20419
3	1	1	2	38			
3	1	1	3	25			
3	1	2	1	11	43	1.1511	9865
3	1	2	2	16			
3	1	2	3	16			
3	2	1	1	20	76	1.1511	17436
3	2	1	2	37			
3	2	1	3	19			
3	2	2	1	15	57	1.1511	13077
3	2	2	2	17			
3	2	2	3	25			
3	3	1	1	20	65	1.1511	14913
3	3	1	2	25			
3	3	1	3	20			
3	3	2	1	23	76	1.1511	17436
3	3	2	2	19			
3	3	2	3	34			

Epifluorescent Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 1 day; Core Sleeve: Non-sterile
C = Core
R = Core subsample
sl = slide

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
4	1	1	1	26	72	1.2098	17360
4	1	1	2	19			
4	1	1	3	27			
4	1	2	1	23	73	1.2098	17601
4	1	2	2	30			
4	1	2	3	20			
4	2	1	1	19	65	1.2098	15672
4	2	1	2	28			
4	2	1	3	18			
4	2	2	1	14	62	1.2098	14949
4	2	2	2	17			
4	2	2	3	31			
4	3	1	1	22	62	1.2098	14949
4	3	1	2	18			
4	3	1	3	22			
4	3	2	1	20	68	1.2098	16396
4	3	2	2	26			
4	3	2	3	22			
5	1	1	1	22	58	1.1577	13382
5	1	1	2	15			
5	1	1	3	21			
5	1	2	1	14	50	1.1577	11536
5	1	2	2	17			
5	1	2	3	19			
5	2	1	1	21	63	1.1577	14536
5	2	1	2	19			
5	2	1	3	23			
5	2	2	1	21	58	1.1577	13382
5	2	2	2	17			
5	2	2	3	20			
5	3	1	1	10	46	1.1577	10613
5	3	1	2	15			
5	3	1	3	21			
5	3	2	1	13	42	1.1577	9691
5	3	2	2	15			
5	3	2	3	14			
6	1	1	1	5	15	1.1655	3484
6	1	1	2	6			
6	1	1	3	4			
6	1	2	1	10	35	1.1655	8130
6	1	2	2	13			
6	1	2	3	12			
6	2	1	1	13	35	1.1655	8130
6	2	1	2	10			
6	2	1	3	12			
6	2	2	1	16	55	1.1655	12776
6	2	2	2	19			
6	2	2	3	20			
6	3	1	1	13	49	1.1655	11382
6	3	1	2	17			
6	3	1	3	19			
6	3	2	1	15	50	1.1655	11614
6	3	2	2	14			
6	3	2	3	21			

Site: S 318; Hold Time: 5 days; Core Sleeve: Non-sterile

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
4	1	1	1	17	53	1.2093	12774
4	1	1	2	17			
4	1	1	3	19			
4	1	2	1	15	44	1.2093	10605
4	1	2	2	15			
4	1	2	3	14			
4	2	1	1	22	58	1.2093	13979
4	2	1	2	17			
4	2	1	3	19			
4	2	2	1	31	86	1.2093	20728
4	2	2	2	27			
4	2	2	3	28			
4	3	1	1	20	61	1.2093	14702
4	3	1	2	19			
4	3	1	3	22			
4	3	2	1	17	53	1.2093	12774
4	3	2	2	17			
4	3	2	3	19			
5	1	1	1	7	38	1.2366	9365
5	1	1	2	18			
5	1	1	3	13			
5	1	2	1	15	46	1.2366	11337
5	1	2	2	14			
5	1	2	3	17			
5	2	1	1	15	45	1.2366	11090
5	2	1	2	13			
5	2	1	3	17			
5	2	2	1	13	42	1.2366	10351
5	2	2	2	15			
5	2	2	3	14			
5	3	1	1	11	39	1.2366	9611
5	3	1	2	13			
5	3	1	3	15			
5	3	2	1	13	44	1.2366	10844
5	3	2	2	16			
5	3	2	3	15			
6	1	1	1	20	54	1.2545	13502
6	1	1	2	15			
6	1	1	3	19			
6	1	2	1	15	50	1.2545	12502
6	1	2	2	17			
6	1	2	3	18			
6	2	1	1	21	58	1.2545	14502
6	2	1	2	17			
6	2	1	3	20			
6	2	2	1	15	54	1.2545	13502
6	2	2	2	20			
6	2	2	3	19			
6	3	1	1	17	53	1.2545	13252
6	3	1	2	19			
6	3	1	3	17			
6	3	2	1	17	55	1.2545	13752
6	3	2	2	20			
6	3	2	3	18			

Epifluorescent Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 15 days; Core Sleeve: Non-sterile
C = Core
R = Core subsample
sl = slide
C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
4	1	1	1	26	83	1.2089	19998
4	1	1	2	22			
4	1	1	3	35			
4	1	2	1	28	85	1.2089	20480
4	1	2	2	36			
4	1	2	3	21			
4	2	1	1	38	106	1.2089	25539
4	2	1	2	33			
4	2	1	3	35			
4	2	2	1	20	65	1.2089	15661
4	2	2	2	24			
4	2	2	3	21			
4	3	1	1	28	81	1.2089	19516
4	3	1	2	20			
4	3	1	3	33			
4	3	2	1	26	85	1.2089	20480
4	3	2	2	31			
4	3	2	3	28			
5	1	1	1	26	91	1.1671	21168
5	1	1	2	36			
5	1	1	3	29			
5	1	2	1	34	108	1.1671	25122
5	1	2	2	40			
5	1	2	3	34			
5	2	1	1	34	91	1.1671	21168
5	2	1	2	29			
5	2	1	3	28			
5	2	2	1	23	74	1.1671	17213
5	2	2	2	27			
5	2	2	3	24			
5	3	1	1	19	56	1.1671	13026
5	3	1	2	16			
5	3	1	3	21			
5	3	2	1	38	101	1.1671	23494
5	3	2	2	34			
5	3	2	3	29			
6	1	1	1	19	55	1.1346	12437
6	1	1	2	19			
6	1	1	3	17			
6	1	2	1	26	69	1.1346	15602
6	1	2	2	22			
6	1	2	3	21			
6	2	1	1	16	43	1.1346	9723
6	2	1	2	15			
6	2	1	3	12			
6	2	2	1	20	51	1.1346	11532
6	2	2	2	14			
6	2	2	3	17			
6	3	1	1	13	54	1.1346	12210
6	3	1	2	22			
6	3	1	3	19			
6	3	2	1	15	46	1.1346	10402
6	3	2	2	14			
6	3	2	3	17			

Site: S 318; Hold Time: 28 days; Core Sleeve: Non-sterile
C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
4	1	1	1	8	29	1.2070	6976
4	1	1	2	10			
4	1	1	3	11			
4	1	2	1	9	26	1.2070	6255
4	1	2	2	10			
4	1	2	3	7			
4	2	1	1	8	30	1.2070	7217
4	2	1	2	11			
4	2	1	3	11			
4	2	2	1	7	24	1.2070	5773
4	2	2	2	9			
4	2	2	3	8			
4	3	1	1	8	25	1.2070	6014
4	3	1	2	8			
4	3	1	3	9			
4	3	2	1	10	29	1.2070	6976
4	3	2	2	9			
4	3	2	3	10			
5	1	1	1	12	38	1.1806	8942
5	1	1	2	15			
5	1	1	3	11			
5	1	2	1	12	34	1.1806	8000
5	1	2	2	10			
5	1	2	3	12			
5	2	1	1	14	39	1.1806	9177
5	2	1	2	15			
5	2	1	3	10			
5	2	2	1	13	40	1.1806	9412
5	2	2	2	13			
5	2	2	3	14			
5	3	1	1	15	37	1.1806	8706
5	3	1	2	10			
5	3	1	3	12			
5	3	2	1	13	43	1.1806	10118
5	3	2	2	15			
5	3	2	3	15			
6	1	1	1	14	35	1.1451	7988
6	1	1	2	11			
6	1	1	3	10			
6	1	2	1	9	31	1.1451	7075
6	1	2	2	11			
6	1	2	3	11			
6	2	1	1	11	35	1.1451	7988
6	2	1	2	13			
6	2	1	3	11			
6	2	2	1	12	31	1.1451	7075
6	2	2	2	10			
6	2	2	3	9			
6	3	1	1	15	39	1.1451	8900
6	3	1	2	11			
6	3	1	3	13			
6	3	2	1	10	31	1.1451	7075
6	3	2	2	10			
6	3	2	3	11			

Epifluorescent Enumerations for Fall 1991 Sampling Program, U.S.G.S. Si

Site: S 318; Hold Time: 1 day; Core Sleeve: Sterile
C = Core C = 7 = S 318 C15 10.5-10.8 m
R = Core subsample 8 = S 318 C17 10.5-10.8 m
sl = slide

C	R	sl	scan	Protozoa	Ps	DW	Protozoa/gdw
7	1	1	1	21	75	1.1751	17565
7	1	1	2	27			
7	1	1	3	27			
7	1	2	1	22	59	1.1751	13818
7	1	2	2	16			
7	1	2	3	21			
7	2	1	1	21	64	1.1751	14989
7	2	1	2	17			
7	2	1	3	26			
7	2	2	1	28	68	1.1751	15925
7	2	2	2	20			
7	2	2	3	20			
7	3	1	1	25	61	1.1751	14286
7	3	1	2	18			
7	3	1	3	18			
7	3	2	1	13	47	1.1751	11007
7	3	2	2	19			
7	3	2	3	15			
8	1	1	1	16	43	1.1874	10176
8	1	1	2	14			
8	1	1	3	13			
8	1	2	1	17	46	1.1874	10886
8	1	2	2	15			
8	1	2	3	14			
8	2	1	1	19	72	1.1874	17038
8	2	1	2	30			
8	2	1	3	23			
8	2	2	1	23	64	1.1874	15145
8	2	2	2	22			
8	2	2	3	19			
8	3	1	1	19	60	1.1874	14199
8	3	1	2	24			
8	3	1	3	17			
8	3	2	1	23	63	1.1874	14909
8	3	2	2	21			
8	3	2	3	19			

Darbyshire Liquid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: F 393; Hold Time: 14 days; Core Sleeve: Non-sterile
C = Core
R = Core subsample

C = 1 = F 393 C15 11.4-11.7 m
2 = F 393 C16 11.4-11.7 m
3 = F 393 C17 11.4-11.7 m

Darbyshire Total MPN Estimates

C	R	MPN/mL	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
1	1	54	0.1492	27	105	0.9322	17	6	1.1834	950
1	2	60	0.1498	30	118	2.2912	17	6	1.1834	1063
2	1	58	0.1495	29	113	8.0423	17	6	1.1767	1019
2	2	54	0.1492	27	105	0.9322	17	6	1.1767	945
3	1	343	0.1511	174	679	3.1171	18	7	1.1511	5928
3	2	69	0.1511	35	136	3.1171	17	6	1.1511	1186

Darbyshie Encysted MPN Estimates

1	1	43	0.1515	21	84	3.8827	15	5	1.1834	870
1	2	41	0.1511	21	82	3.117	16	5	1.1834	834
2	1	20	0.1527	10	39	3.7351	15	5	1.1767	392
2	2	20	0.1527	10	39	3.7351	15	5	1.1767	392
3	1	213	0.1515	107	421	3.8827	17	6	1.1511	4193
3	2	48	0.1529	24	95	4.6456	15	5	1.1511	945

Darbyshire Liquid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 1 day; Core Sleeve: Non-sterile
C = Core
R = Core subsample

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Darbyshire Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	1460	0.1496	743	2868	4.9075	20	8	1.2098	26494
4	2	740	0.1538	370	1482	8.3094	20	8	1.2098	13429
5	1	755	0.1491	385	1480	7.2367	18	7	1.1577	13111
5	2	543	0.1514	274	1074	6.1364	18	7	1.1577	9421
6	1	5600	0.1494	2853	10993	11.5884	22	9	1.1655	97902
6	2	8587	0.1511	4342	16981	3.1171	22	9	1.1655	150117

Darbyshie Encysted MPN Estimates

4	1	543	0.1514	274	1074	6.1364	18	7	1.2098	11223
4	2	200	0.1508	101	395	4.7970	17	6	1.2098	4137
5	1	203	0.1501	103	400	8.2558	18	7	1.1577	4025
5	2	88	0.1542	44	176	6.5594	17	6	1.1577	1741
6	1	271	0.1545	135	545	1.4061	17	6	1.1655	5407
6	2	384	0.1559	190	775	2.0860	17	6	1.1655	7649

Site: S 318; Hold Time: 5 days; Core Sleeve: Non-sterile

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Darbyshire Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance Chi-sq	1%	D.F. Conv.	DW	MPN/gdw
4	1	350	0.1554	174	706	3.3775	18	7	1.2093	6349
4	2	481	0.1538	24	96	1.5121	17	6	1.2093	8730
5	1	306	0.1550	152	617	2.0552	18	7	1.2366	5681
5	2	228	0.1494	116	446	4.1411	18	7	1.2366	4220
6	1	907	0.1514	458	1795	6.1363	20	8	1.2545	17062
6	2	480	0.1546	239	964	7.2973	20	8	1.2545	9033

Darbyshie Encysted MPN Estimates

4	1	49	0.1489	25	95	15.5561	18	7	1.2093	1006
4	2	75	0.1517	38	148	15.3463	18	7	1.2093	1544
5	1	69	0.1511	35	136	3.1171	17	6	1.2366	1454
5	2	185	0.1500	94	363	2.0532	17	6	1.2366	3905
6	1	123	0.1501	62	241	2.2097	17	6	1.2545	2628
6	2	229	0.1524	115	456	5.3308	17	6	1.2545	4921

Darbyshire Liquid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 15 days; Core Sleeve: Non-sterile

C = Core
R = Core subsample

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Darbyshire Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	5653	0.1494	2880	11095	2.6664	22	9	1.2089	102515
4	2	1720	0.1511	87	340	3.1170	20	8	1.2089	31190
5	1	204	0.1501	104	402	2.2097	18	7	1.1671	3574
5	2	770	0.1546	393	1509	7.1507	17	6	1.1671	13480
6	1	1913	0.1524	962	3804	5.3308	20	8	1.1346	32562
6	2	1720	0.1511	870	3402	3.1170	20	8	1.1346	29272

Darbyshie Encysted MPN Estimates

4	1	2685	0.1515	1355	5320	1.0900	20	8	1.2089	55505
4	2	96	0.1529	48	192	1.0900	17	6	1.2089	1985
5	1	169	0.1494	86	332	8.5484	17	6	1.1671	3381
5	2	319	0.1491	145	650	5.7018	18	7	1.1671	1862
6	1	110	0.1512	56	218	6.1016	17	6	1.1346	2134
6	2	264	0.1542	131	529	6.5594	17	6	1.1346	5122

Site: S 318; Hold Time: 28 days; Core Sleeve: Non-sterile

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Darbyshire Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	1540	0.1500	783	3030	2.0532	20	8	1.2070	27882
4	2	1540	0.1500	783	3030	2.0532	20	8	1.2070	27882
5	1	6560	0.1492	3346	12861	6.6219	22	9	1.1806	116175
5	2	5013	0.1503	2544	9878	9.7038	22	9	1.1806	88784
6	1	1020	0.1501	5185	2008	2.2097	20	8	1.1451	17520
6	2	1283	0.1491	655	2516	5.7018	20	8	1.1451	22043

Darbyshie Encysted MPN Estimates

4	1	169	0.1494	86	332	8.5484	17	6	1.2070	3496
4	2	173	0.1495	88	340	8.0423	17	6	1.2070	3573
5	1	154	0.1491	78	301	5.7018	17	6	1.1806	3105
5	2	185	0.1500	94	363	2.0532	17	6	1.1806	3729
6	1	206	0.1511	104	408	3.1171	17	6	1.1451	4034
6	2	154	0.1491	78	301	5.7018	17	6	1.1451	3012

Singh Solid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: F 393; Hold Time: 14 days; Core Sleeve: Non-sterile

C = Core
R = Core subsample

C = 1 = F 393 C15 11.4-11.7 m
2 = F 393 C16 11.4-11.7 m
3 = F 393 C17 11.4-11.7 m

Singh Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
1	1	15000	0.2829	4183	53784	0.9141	15	5	1.1834	17751
1	2	15000	0.2829	4183	53784	0.9141	15	5	1.1834	17751
2	1	21600	0.2800	6103	76441	4.1581	15	5	1.1767	25418
2	2	7500	0.3219	1755	32054	2.1989	15	5	1.1767	8826
3	1	1475	0.2809	415	5239	1.0030	15	5	1.1511	1698
3	2	9400	0.3079	2343	37715	0.6067	15	4	1.1511	10821

Singh Encysted MPN Estimates

1	1	7500	0.3218	1755	32054	2.1989	15	5	1.1834	7718
1	2	4300	0.3133	1046	17681	0.3434	15	5	1.1834	4425
2	1	15000	0.2829	4183	53784	0.9141	15	5	1.1767	15349
2	2	7500	0.3218	1755	32054	2.1989	15	5	1.1767	7674
3	1	938	0.3080	233	3764	0.6067	13	5	1.1511	939
3	2	1475	0.2809	415	5239	1.0030	15	5	1.1511	1476

Singh Solid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 1 day; Core Sleeve: Non-sterile
C = Core
R = Core subsample

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Singh Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	938	0.3080	233	3765	0.6066	13	4	1.2098	1135
4	2	147	0.2810	41	522	1.0029	13	4	1.2098	178
5	1	275	0.2830	77	986	7.8994	15	5	1.1577	318
5	2	738	0.3207	173	3136	2.2436	15	5	1.1577	854
6	1	938	0.3080	233	3765	0.6066	13	4	1.1655	1093
6	2	1475	0.2809	415	5239	1.0030	15	5	1.1655	1719

Singh Encysted MPN Estimates

4	1	425	0.3124	104	1741	0.3434	13	4	1.2098	447
4	2	92	0.3070	23	369	0.6622	13	4	1.2098	97
5	1	147	0.2810	41	522	1.0029	13	4	1.1577	148
5	2	147	0.2810	41	522	1.0029	13	4	1.1577	148
6	1	275	0.2830	77	986	7.8994	15	5	1.1655	279
6	2	147	0.2810	41	522	1.0029	13	4	1.1655	149

Site: S 318; Hold Time: 5 days; Core Sleeve: Non-sterile

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Singh Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance Chi-sq	1%	D.F. Conv.	DW	MPN/gdw
4	1	425	0.3124	104	1741	0.3434	13	4	1.2093	514
4	2	425	0.3124	104	1741	0.3434	13	4	1.2093	514
5	1	425	0.3124	104	1741	0.3434	13	4	1.2366	526
5	2	425	0.3124	104	1741	0.3434	13	4	1.2366	526
6	1	938	0.3080	233	3764	0.6066	13	4	1.2545	1177
6	2	738	0.3207	173	3136	2.2436	15	5	1.2545	926

Singh Encysted MPN Estimates

4	1	147	0.2810	41	522	1.0029	13	4	1.2093	155
4	2	147	0.2810	41	522	1.0029	13	4	1.2093	155
5	1	211	0.2761	61	733	4.2856	13	4	1.2366	227
5	2	275	0.2830	77	986	7.8994	15	5	1.2366	296
6	1	425	0.3124	104	1741	0.3434	13	4	1.2545	464
6	2	425	0.3124	104	1741	0.3434	13	4	1.2545	464

Singh Solid Media MPN Enumerations for Fall 1991 Sampling Program, U.S.G.S. Site

Site: S 318; Hold Time: 15 days; Core Sleeve: Non-sterile
C = Core
R = Core subsample

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Singh Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	15000	0.2829	4183	53784	0.9141	15	5	1.2089	18133
4	2	15000	0.2829	4183	53784	0.9141	15	5	1.2089	18133
5	1	15000	0.2829	4183	53784	0.9141	15	5	1.1671	17507
5	2	109600	0.3223	25586	469476	0.0001	15	5	1.1671	127918
6	1	15000	0.2829	4183	53784	0.9141	15	5	1.1346	17018
6	2	109600	0.3223	25586	469476	0.0001	15	5	1.1346	124348

Singh Encysted MPN Estimates

4	1	7500	0.3219	1755	32054	2.1989	15	5	1.2089	7884
4	2	7500	0.3219	1755	32054	2.1989	15	5	1.2089	7884
5	1	4300	0.3132	1046	17681	0.3434	15	5	1.1671	4364
5	2	46400	0.3308	10428	206466	0.0077	15	5	1.1671	47092
6	1	15000	0.2829	4183	53784	0.9141	15	5	1.1346	14799
6	2	46400	0.3308	10428	206466	0.0077	15	5	1.1346	45777

Site: S 318; Hold Time: 28 days; Core Sleeve: Non-sterile

C = 4 = S 318 C18 10.8-11.1 m
5 = S 318 C19 10.8-11.1 m
6 = S 318 C21 10.5-10.8 m

Singh Total MPN Estimates

C	R	MPN	S.E. log	95% L.C.I.	95% U.C.I.	Deviance	1% Chi-sq	D.F.	DW	MPN/gdw
4	1	275200	0.2834	76563	989183	7.8829	18	7	1.2070	332167
4	2	409600	0.3097	101233	1657284	0.3465	18	7	1.2070	494387
5	1	147200	0.2809	41427	523041	1.0030	18	7	1.1806	173790
5	2	204800	0.2759	58963	711343	5.2621	18	7	1.1806	241795
6	1	21000	0.2760	6041	73000	4.2856	17	6	1.1451	24047
6	2	147200	0.2809	41426	523041	1.0030	18	7	1.1451	168556

Singh Encysted MPN Estimates

4	1	148000	0.2834	41197	531693	0.9143	17	6	1.2070	155337
4	2	43200	0.3136	10490	177899	0.3436	17	6	1.2070	45341
5	1	27600	0.2836	7674	99266	7.8828	17	6	1.1806	28335
5	2	9400	0.3078	2343	37715	0.6067	15	5	1.1806	9650
6	1	4300	0.3133	1046	17681	0.3434	15	5	1.1451	4282
6	2	14700	0.2810	4136	52244	1.0029	17	6	1.1451	14637

APPENDIX B

This section will discuss the commands used in STATA^R for the statistical tests evaluated, and calculation of standard deviations from power transformed data. The components of variance data sheets are also included.

I. STATA^R COMMANDS

All of these commands are given with more detail in the STATA^R Reference Manual, Release 2 (1988). This list is intended as a reference for experienced users. The following format will be used: the command line (always initiated by a ".") will be followed by a discussion of the command; *italicized* words indicate the use of variables ("*var*") or file names.

.infile *var list* using *file*
Used to import ASCII file into STATA^R. Each column of data will be considered a variable that must be named.

.save *file*
For creating a file (.dta) of imported data in STATA^R.

.use *file.dta*
For accessing files saved in STATA^R.

.drop_all
For clearing STATA^R in order to use another file.

.exit, clear
To leave STATA^R.

.log using prn:
To record STATA^R output.

.log off
To stop printing STATA^R output.

.summarize *var*, detail
Lists percentiles, maximum and minimum values, mean, standard deviation, variance, skewness and Kurtosis.

.graph *var*, normal bin(9) xlab ylab border b1(*label y-axis*) saving(*file*)
Histogram with 9 divisions on the x-axis.

.graph *var*, box ylab b1(*labels y-axis*) l1(*label x-axis*) saving(*file*)
Box plot (more than one variable can be shown).

.symplot *var*, xlab ylab border l1(*label x-axis*) saving(*file*)
Symmetry plot (must open Graph.kit).

.qnorm *var*, xlab ylab border l1(*label x-axis*) saving(*file*)
Quantile normal plot (must open Graph.kit).

.oneway *varlist*
Oneway ANOVA table with Bartlett's test for equal variances.

.oneway *varlist*, noanova scheffe
Scheffé multiple comparison table (not including the ANOVA table).

.kwallis var_1, by(var_2)
Kruskal-Wallis non-parametric test.

Printing graphs requires leaving the program. For a dot-matrix or laser printer, type at the c-prompt "gphdot *file.gph*".

II. CALCULATIONS OF UNTRANSFORMED STANDARD DEVIATIONS

The square root of the epifluorescent estimates and logarithms of the Darbyshire and Singh MPN estimates were used for all statistical analyses. The mean and standard deviations were untransformed and reported in their original units. The process is not straight forward for the standard deviations since they are no longer symmetrical about the mean when untransformed (Taylor, 1990). An example will be used to demonstrate the procedure using the raw data in Appendix A.

A. Epifluorescent Estimates for F 393

Summary of Data:

mean, $x = 10574$ protozoa/gdw
square root transformed mean, $x_t = 102.8331$
square root transformed standard deviation, $s_t = 24.7384$

An upper standard deviation value was calculated by adding x_t and s_t, then squaring the value. The upper standard deviation, s_u, was then the difference between the standard deviation value and x.

$$(x_t + s_t)^2 = (102.8331 + 24.7384)^2 = 16274$$

$$s_u = 16274 - 10574 = 5700 \ \frac{\text{protozoa}}{\text{gdw}}$$

The lower standard deviation, s_l, was calculated similarly.

$$(x_t - s_t)^2 = (102.8331 - 24.7384)^2 = 6099$$

$$s_l = 10574 - 6099 = 4475 \ \frac{\text{protozoa}}{\text{gdw}}$$

B. Darbyshire Total MPN Estimates for F 393

Summary of Data:

mean, $x = 1378$ MPN/gdw
log transformed mean, $x_t = 3.1391$
log transformed standard deviation, $s_t = 0.3126$

An upper standard deviation value was calculated by adding x_t and s_t, then taking the antilog of the value. The upper standard deviation, s_u, was then the difference between the standard deviation value and x:

$$10^{(x_t + s_t)} = 10^{(3.1391 + 0.3126)} = 2829$$

$$s_u = 2829 - 1378 = 1451 \frac{\text{MPN}}{\text{gdw}}.$$

The lower standard deviation, s_l, was calculated similarly:

$$10^{(x_t - s_t)} = 10^{(3.1391 - 0.3126)} = 671$$

$$s_l = 1378 - 671 = 707 \frac{\text{MPN}}{\text{gdw}}.$$

The same procedure was used for all log transformed data, including the Singh MPN estimates.

III. COMPONENTS OF VARIANCE DATA SHEETS

The calculations for the components of variance were discussed in Chapter 2, Section VI.C. All estimates were normalized using the appropriate power transformation. The components were calculated in the following tables using procedures recommended by Box et al. (1978). Then the components were untransformed and the upper component value (larger value of the two and hence, most conservative) was listed in Tables 14, 15 and 16.

			Hierarchical Design											
			Fall '91 Epifluorescent Enumeration (DAPI Stain)											
			All cores held for 14-15 days prior to processing											
S	C	R	sl	Sqrt of P/gdw (A)	Avg. Reps. (B)	Avg. Cores (C)	Avg. Sites (D)	Avg. All Est. (E)	A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2	[D-E]^2
1	1	1	1	68.68	71.17	74.03	102.83	116.84	-2.49	6.19	-2.87	8.21	-28.80	829.45	196.23
1	1	1	2	73.65					2.49	6.19					
1	1	2	1	75.24	78.97				-3.73	13.93	4.94	24.39			
1	1	2	2	82.70					3.73	13.93					
1	1	3	1	68.68	71.96				-3.28	10.76	-2.07	4.30			
1	1	3	2	75.24					3.28	10.76					
1	2	1	1	118.63	108.34	110.67			10.28	105.72	-2.33	5.41	7.84	61.41	
1	2	1	2	98.06					-10.28	105.72					
1	2	2	1	107.20	122.09				-14.89	221.62	11.42	130.37			
1	2	2	2	136.97					14.89	221.62					
1	2	3	1	102.73	101.58				1.15	1.33	-9.09	82.65			
1	2	3	2	100.42					-1.15	1.33					
1	3	1	1	142.90	121.11	123.80			21.79	474.64	-2.69	7.23	20.96	439.48	
1	3	1	2	99.32					-21.79	474.64					
1	3	2	1	132.05	123.20				8.85	78.24	-0.60	0.36			
1	3	2	2	114.35					-8.85	78.24					
1	3	3	1	122.12	127.08				-4.96	24.63	3.29	10.79			
1	3	3	2	132.05					4.96	24.63					
2	1	1	1	141.41	142.26	142.05	130.85		-0.85	0.72	0.21	0.05	11.20	125.38	196.23
2	1	1	2	143.11					0.85	0.72					
2	1	2	1	159.81	142.48				17.34	300.50	0.43	0.18			
2	1	2	2	125.14					-17.34	300.50					
2	1	3	1	139.70	141.41				-1.71	2.91	-0.64	0.41			
2	1	3	2	143.11					1.71	2.91					
2	2	1	1	145.49	152.00	141.35			-6.51	42.32	10.65	113.35	10.50	110.23	
2	2	1	2	158.50					6.51	42.32					
2	2	2	1	145.49	138.35				7.15	51.05	-3.00	9.02			
2	2	2	2	131.20					-7.15	51.05					
2	2	3	1	114.13	133.71				-19.58	383.18	-7.64	58.42			
2	2	3	2	153.28					19.58	383.18					
2	3	1	1	111.52	118.22	109.15			-6.70	44.82	9.06	82.11	-21.70	470.72	
2	3	1	2	124.91					6.69	44.82					
2	3	2	1	98.61	103.00				-4.39	19.27	-6.15	37.86			
2	3	2	2	107.39					4.39	19.27					
2	3	3	1	110.50	106.25				4.26	18.11	-2.91	8.46			
2	3	3	2	101.99					-4.26	18.11					
								Sum of squares =		3599.87		583.58		2036.66	392.45
									Vsl =	199.99	Vr =	48.63	Vc =	509.17	392.45
									Sig^2 =	199.99		-51.37		492.96	222.73

			Hierarchical Design for F 393											
			Fall '91 Epifluorescent Enumeration (DAPI Stain) - F 393											
			All cores held for 14-15 days prior to processing											
S	C	R	sl	Sqrt of	Avg.	Avg.	Avg.		A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
				P/gdw	Reps.	Cores	F 393							
				(A)	(B)	(C)	(D)							
1	1	1	1	68.68	71.17	74.03	102.83		-2.49	6.19	-2.87	8.21	-28.80	829.45
1	1	1	2	73.65					2.49	6.19				
1	1	2	1	75.24	78.97				-3.73	13.93	4.94	24.39		
1	1	2	2	82.70					3.73	13.93				
1	1	3	1	68.68	71.96				-3.28	10.76	-2.07	4.30		
1	1	3	2	75.24					3.28	10.76				
1	2	1	1	118.63	108.34	110.67			10.28	105.72	-2.33	5.41	7.84	61.41
1	2	1	2	98.06					-10.28	105.72				
1	2	2	1	107.20	122.09				-14.89	221.62	11.42	130.37		
1	2	2	2	136.97					14.89	221.62				
1	2	3	1	102.73	101.58				1.15	1.33	-9.09	82.65		
1	2	3	2	100.42					-1.15	1.33				
1	3	1	1	142.90	121.11	123.80			21.79	474.64	-2.69	7.23	20.96	439.48
1	3	1	2	99.32					-21.79	474.64				
1	3	2	1	132.05	123.20				8.85	78.24	-0.60	0.36		
1	3	2	2	114.35					-8.85	78.24				
1	3	3	1	122.12	127.08				-4.96	24.63	3.29	10.79		
1	3	3	2	132.05					4.96	24.63				
							Sum of squares =			1874.11		273.71		1330.34
									Vsl =	208.23	Vr =	45.62	Vc =	665.17
									Sig^2 =	208.23		-58.50		649.96
			Hierarchical Design for S 318											
			Fall '91 Epifluorescent Enumeration (DAPI Stain) - S 318											
			All cores held for 14-15 days prior to processing											
S	C	R	sl	Sqrt of	Avg.	Avg.	Avg.		A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
				P/gdw	Reps.	Cores	S 318							
				(A)	(B)	(C)	(D)							
2	1	1	1	141.41	142.26	142.05	130.85		-0.85	0.72	0.21	0.05	11.20	125.38
2	1	1	2	143.11					0.85	0.72				
2	1	2	1	159.81	142.48				17.34	300.50	0.43	0.18		
2	1	2	2	125.14					-17.34	300.50				
2	1	3	1	139.70	141.41				-1.71	2.91	-0.64	0.41		
2	1	3	2	143.11					1.71	2.91				
2	2	1	1	145.49	152.00	141.35			-6.51	42.32	10.65	113.35	10.50	110.23
2	2	1	2	158.50					6.51	42.32				
2	2	2	1	145.49	138.35				7.15	51.05	-3.00	9.02		
2	2	2	2	131.20					-7.15	51.05				
2	2	3	1	114.13	133.71				-19.58	383.18	-7.64	58.42		
2	2	3	2	153.28					19.58	383.18				
2	3	1	1	111.52	118.22	109.15			-6.70	44.82	9.06	82.11	-21.70	470.72
2	3	1	2	124.91					6.69	44.82				
2	3	2	1	98.61	103.00				-4.39	19.27	-6.15	37.86		
2	3	2	2	107.39					4.39	19.27				
2	3	3	1	110.50	106.25				4.26	18.11	-2.91	8.46		
2	3	3	2	101.99					-4.26	18.11				
							Sum of squares =			1725.76		309.87		706.33
									Vsl =	191.75	Vr =	51.64	Vc =	353.16
									Sig^2 =	191.75		-44.23		335.95

		Hierarchical Design									
		Fall '91 Log of Darbyshire - Total Population									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of Sites (C)	Avg. of All Est. (D)	A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
1	1	1	2.9779	3.0023	3.1391	3.7175	-0.0244	0.0006	-0.1368	0.0187	-0.5784	0.3345
1	1	2	3.0267				0.0244	0.0006				
1	2	1	3.0080	2.9917			0.0163	0.0003	-0.1474	0.0217		
1	2	2	2.9754				-0.0163	0.0003				
1	3	1	3.7729	3.4235			0.3494	0.1221	0.2844	0.0809		
1	3	2	3.0740				-0.3495	0.1222				
2	1	1	5.0108	4.7524	4.2958		0.2584	0.0668	0.4566	0.2085	0.5783	0.3344
2	1	2	4.4940				-0.2584	0.0668				
2	2	1	3.5532	3.6454			-0.0922	0.0085	-0.6504	0.4230		
2	2	2	3.7376				0.0922	0.0085				
2	3	1	4.5127	4.4896			0.0231	0.0005	0.1938	0.0376		
2	3	2	4.4664				-0.0232	0.0005				
						Sum of squares =		0.3976		0.7904		0.6690
							Vr =	0.0663	Vc =	0.1976	Vs =	0.6690
							Sig^2 r =	0.0663	Sig^2 c =	0.1645	Sig^2 s =	0.6031
		Hierarchical Design for either F 393 and S 318									
		Fall '91 Log of Darbyshire - Total Population - F 393									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. at F 393 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
1	1	1	2.9779	3.0023	3.1391		-0.0244	0.0006	-0.1368	0.0187		
1	1	2	3.0267				0.0244	0.0006				
1	2	1	3.0080	2.9917			0.0163	0.0003	-0.1474	0.0217		
1	2	2	2.9754				-0.0163	0.0003				
1	3	1	3.7729	3.4235			0.3494	0.1221	0.2844	0.0809		
1	3	2	3.0740				-0.3495	0.1222				
						Sum of squares =		0.2460		0.1213		
							Vr =	0.0820	Vc =	0.0607		
							Sig^2 r =	0.0820	Sig^2 c =	0.0197		
		Fall '91 Log of Darbyshire - Total Population - S 318										
		All cores held 14-15 days before processing										
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. at S 318 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
2	1	1	5.0108	4.7524	4.2958		0.2584	0.0668	0.4566	0.2085		
2	1	2	4.4940				-0.2584	0.0668				
2	2	1	3.5532	3.6454			-0.0922	0.0085	-0.6504	0.4230		
2	2	2	3.7376				0.0922	0.0085				
2	3	1	4.5127	4.4896			0.0231	0.0005	0.1938	0.0376		
2	3	2	4.4664				-0.0232	0.0005				
						Sum of squares =		0.1516		0.6691		
							Vr =	0.0505	Vc =	0.3345		
							Sig^2 r =	0.0505	Sig^2 c =	0.3093		

		Hierarchical Design									
		Fall '91 Log of Darbyshire - Encysted Population									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of Sites (C)	Avg. of All Est. (D)	A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
1	1	1	2.9396	2.9303	2.9410	3.3257	0.0093	0.0001	-0.0107	0.0001	-0.3847	0.1480
1	1	2	2.9210				-0.0093	0.0001				
1	2	1	2.5937	2.5937			0.0000	0.0000	-0.3473	0.1206		
1	2	2	2.5937				0.0000	0.0000				
1	3	1	3.6225	3.2990			0.3235	0.1047	0.3580	0.1282		
1	3	2	2.9754				-0.3236	0.1047				
2	1	1	4.7443	4.0210	3.7104		0.7233	0.5232	0.3106	0.0965	0.3847	0.1480
2	1	2	3.2977				-0.7233	0.5232				
2	2	1	3.5290	3.5908			-0.0618	0.0038	-0.1196	0.0143		
2	2	2	3.6526				0.0618	0.0038				
2	3	1	3.3292	3.5193			-0.1901	0.0361	-0.1911	0.0365		
2	3	2	3.7094				0.1901	0.0361				
						Sum of squares =		1.3358		0.3962		0.2960
							Vr =	0.2226	Vc =	0.0990	Vs =	0.2960
							Sig^2 r =	0.2226	Sig^2 c =	-0.0123	Sig^2 s =	0.2630
		Hierarchical Design for either F 393 and S 318									
		Fall '91 Log of Darbyshire - Encysted Population - F 393									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. at F 393 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
1	1	1	2.9396	2.9303	2.9410		0.0093	0.0001	-0.0107	0.0001		
1	1	2	2.9210				-0.0093	0.0001				
1	2	1	2.5937	2.5937			0.0000	0.0000	-0.3473	0.1206		
1	2	2	2.5937				0.0000	0.0000				
1	3	1	3.6225	3.2990			0.3235	0.1047	0.3580	0.1282		
1	3	2	2.9754				-0.3236	0.1047				
						Sum of squares =		0.2095		0.2489		
							Vr =	0.0698	Vc =	0.1244		
							Sig^2 r =	0.0698	Sig^2 c =	0.0895		
		Fall '91 Log of Darbyshire - Encysted Population - S 318										
		All cores held 14-15 days before processing										
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. at S 318 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
2	1	1	4.7443	4.0210	3.7104		0.7233	0.5232	0.3106	0.0965		
2	1	2	3.2977				-0.7233	0.5232				
2	2	1	3.5290	3.5908			-0.0618	0.0038	-0.1196	0.0143		
2	2	2	3.6526				0.0618	0.0038				
2	3	1	3.3292	3.5193			-0.1901	0.0361	-0.1911	0.0365		
2	3	2	3.7094				0.1901	0.0361				
						Sum of squares =		1.1262		0.1473		
							Vr =	0.3754	Vc =	0.0736		
							Sig^2 r =	0.3754	Sig^2 c =	-0.1141		

		Hierarchical Design									
		Fall '91 Log of Singh Plates - Total Population									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of Sites (C)	Avg. of All Est. (D)	A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
1	1	1	4.2492	4.2492	4.0189	4.2755	0.0000	0.0000	0.2303	0.0530	-0.2566	0.0658
1	1	2	4.2492				0.0000	0.0000				
1	2	1	4.4051	4.1754			0.2297	0.0528	0.1565	0.0245		
1	2	2	3.9457				-0.2297	0.0528				
1	3	1	3.2299	3.6321			-0.4022	0.1618	-0.3868	0.1496		
1	3	2	4.0343				0.4022	0.1618				
2	1	1	4.2585	4.2585	4.5321		0.0000	0.0000	-0.2736	0.0749	0.2566	0.0658
2	1	2	4.2585				0.0000	0.0000				
2	2	1	4.2432	4.6751			-0.4319	0.1865	0.1430	0.0204		
2	2	2	5.1069				0.4319	0.1865				
2	3	1	4.2309	4.6628			-0.4319	0.1865	0.1307	0.0171		
2	3	2	5.0946				0.4319	0.1865				
						Sum of squares =		1.1750		0.3395		0.1317
							Vr =	0.1958	Vc =	0.0849	Vs =	0.1317
							Sig^2 r	0.1958	Sig^2 c =	-0.0130	Sig^2 s =	0.1034
		Hierarchical Design for either F 393 or S 318									
		Fall '91 Log of Singh Plates - Total Population - F 393									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of F 393 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
1	1	1	4.2492	4.2492	4.0189		0.0000	0.0000	0.2303	0.0530		
1	1	2	4.2492				0.0000	0.0000				
1	2	1	4.4051	4.1754			0.2297	0.0528	0.1565	0.0245		
1	2	2	3.9457				-0.2297	0.0528				
1	3	1	3.2299	3.6321			-0.4022	0.1618	-0.3868	0.1496		
1	3	2	4.0343				0.4022	0.1618				
						Sum of squares =		0.4291		0.2271		
							Vr =	0.1430	Vc =	0.1136		
							Sig^2 r	0.1430	Sig^2 c =	0.0421		
		Fall '91 Log of Singh Plates - Total Population - S 318										
		All cores held 14-15 days before processing										
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of S 318 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
2	1	1	4.2585	4.2585	4.5321		0.0000	0.0000	-0.2736	0.0749		
2	1	2	4.2585				0.0000	0.0000				
2	2	1	4.2432	4.6751			-0.4319	0.1865	0.1430	0.0204		
2	2	2	5.1069				0.4319	0.1865				
2	3	1	4.2309	4.6628			-0.4319	0.1865	0.1307	0.0171		
2	3	2	5.0946				0.4319	0.1865				
						Sum of squares =		0.7460		0.1124		
							Vr =	0.2487	Vc =	0.0562		
							Sig^2 r	0.2487	Sig^2 c =	-0.0681		

		Hierarchical Design									
		Fall '91 Log of Singh Plates - Encysted Population									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of Sites (C)	Avg. of All Est. (D)	A-B	[A-B]^2	B-C	[B-C]^2	C-D	[C-D]^2
1	1	1	3.8875	3.7667	3.6244	3.8903	0.1208	0.0146	0.1423	0.0203	-0.2659	0.0707
1	1	2	3.6459				-0.1208	0.0146				
1	2	1	4.1861	4.0356			0.1506	0.0227	0.4112	0.1691		
1	2	2	3.8850				-0.1506	0.0227				
1	3	1	2.9726	3.0709			-0.0983	0.0097	-0.5535	0.3063		
1	3	2	3.1692				0.0983	0.0097				
2	1	1	3.8968	3.8968	4.1562		0.0000	0.0000	-0.2594	0.0673	0.2659	0.0707
2	1	2	3.8968				0.0000	0.0000				
2	2	1	3.6399	4.1564			-0.5165	0.2668	0.0002	0.0000		
2	2	2	4.6729				0.5165	0.2668				
2	3	1	4.1702	4.4154			-0.2452	0.0601	0.2592	0.0672		
2	3	2	4.6606				0.2452	0.0601				
						Sum of squares =		0.7476		0.6301		0.1414
							Vr =	0.1246	Vc =	0.1575	Vs =	0.1414
							Sig^2 r =	0.1246	Sig^2 c =	0.0952	Sig^2 s =	0.0889
		Hierarchical Design fro either F 393 or S 318									
		Fall '91 Log of Singh Plates - Encysted Population - F 393									
		All cores held 14-15 days before processing									
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of F 393 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
1	1	1	3.8875	3.7667	3.6244		0.1208	0.0146	0.1423	0.0203		
1	1	2	3.6459				-0.1208	0.0146				
1	2	1	4.1861	4.0356			0.1506	0.0227	0.4112	0.1691		
1	2	2	3.8850				-0.1506	0.0227				
1	3	1	2.9726	3.0709			-0.0983	0.0097	-0.5535	0.3063		
1	3	2	3.1692				0.0983	0.0097				
						Sum of squares =		0.0938		0.4957		
							Vr =	0.0313	Vc =	0.2478		
							Sig^2 r =	0.0313	Sig^2 c =	0.2322		
		Fall '91 Log of Singh Plates - Encysted Population - S 318										
		All cores held 14-15 days before processing										
S	C	R	Log of MPN/gdw (A)	Avg. of Cores (B)	Avg. of S 318 (C)		A-B	[A-B]^2	B-C	[B-C]^2		
2	1	1	3.8968	3.8968	4.1562		0.0000	0.0000	-0.2594	0.0673		
2	1	2	3.8968				0.0000	0.0000				
2	2	1	3.6399	4.1564			-0.5165	0.2668	0.0002	0.0000		
2	2	2	4.6729				0.5165	0.2668				
2	3	1	4.1702	4.4154			-0.2452	0.0601	0.2592	0.0672		
2	3	2	4.6606				0.2452	0.0601				
						Sum of squares =		0.6538		0.1345		
							Vr =	0.2179	Vc =	0.0672		
							Sig^2 r =	0.2179	Sig^2 c =	-0.0417		

APPENDIX C

This section will discuss the culturing techniques and another epifluorescent stain, Hydroethidine^R, used on subsurface sediments collected at the U.S.G.S. site. Cultures of flagellates from the sediments were used to train other researchers to enumerate the microorganisms as well as for transport studies (Harvey et al., 1992). No amoebae have been successfully cultured in the laboratory. Hydroethidine^R is a vital stain that has been used to trace protozoa during transport studies.

I. CULTURING TECHNIQUES

A variety of media has been used to culture subsurface sediment protozoa, including: Bold's basal medium, *Euglena gracilis* medium, proteose peptone yeast extract medium, soil extract medium with added salts, soil/water biphasic medium, and dilute secondary wastewater effluent (Finlay et al., 1988; Thompson et al., 1988; Cowling, 1992). However, the most successful medium used for culturing the subsurface sediment protozoa has been the Cerophyl-Prescott medium. The following stock solutions are required:

1. 0.433 g $CaCl_2 \cdot 2H_2O$,
   0.162 g KCl;
   Dilute to 100 mL with distilled water.

2. 0.512 g K_2HPO_4;
   Dilute to 100 mL with distilled water.

3. 0.280 g $MgSO_4 \cdot 7H_2O$;
   Dilute to 100 mL with distilled water.

Add 1 g of Cerophyl^R rye or wheat grass (Sigma, St. Louis, MO) to 1 L of distilled water. Autoclave (15 psig, 121^oC) the solution for 10 min, cool and then filter the solution through GF/C filter paper (VWR, Boston, MA) to remove the particulate material. Add 1 mL of each of the stock solutions to the 1 L of Cerophyl^R infusion and pH adjust to 7.0 (or 7.4 if the cells are to be stained with Hydroethidine^R). Autoclave the solution for 15-20 min.

The strength of the medium can be varied (0.1% to full strength) depending on the desired culturing conditions. The Cerophyl-Prescott medium must be stored under sterile conditions. Storage periods of more than 1 month are not recommended since the solution can easily become contaminated with bacteria and fungi.

The greatest variety of flagellates have been observed in petri dishes with 1-2 g of sediment in ~10 mL of Cerophyl-Prescott medium. When the flagellates are cultured in flasks, biofilm growth can become a problem. The flagellates also grow to 5-7 μm in length in these liquid cultures. Size selection has been achieved by adding cultures to sterile (15 psig, 121°C for 90 min), sieved sediments (0.5-1 mm diameter). The flagellates growing in the liquid film on top of the sediments tend to be 5-7 μm in length, but the cells in the pore water tend to be 2-4 μm in length. To maintain active populations of protozoa, the media is replaced (draw and fill) approximately every 5 days using sterile technique.

Not all of the protozoa observed on the downwell samplers (Kinner et al., 1991) have been seen in cultures prepared in the laboratory. Cowling (1992) describes several other procedures for isolating free-living flagellates that might be successful with the subsurface protozoa.

II. HYDROETHIDINE^R STAINING

The procedure for staining cells with Hydroethidine^R was similar to the instructions given by the manufacturer (Data Sheet #351; Polysciences, Inc., Warrington, PA) including the modification used with the ciliate, *Colpidium campylum*, by Graham (1990). The stock solution consisted of 7 mg of Hydroethidine^R ($C_{21}H_{21}N_3$; 315.5 molecular weight) in 1 mL of N,N-dimethylacetamide. This was stored in a 2.2 mL VOA vial fitted with a teflon-coated septum (Wheaton Glass, Millville, NJ). The stock solution apparently oxidizes with time and Polysciences recommends that the solution be stored in a nitrogen atmosphere. Since nitrogen gas was not readily available, the stock was stored in the freezer (0°C) and discarded if not used within 1 week.

Only 200 μL of stock solution was necessary to stain 100 mL of sample. The organisms were allowed to stain for 15 min and then were diluted 1:10 with the solution that the protozoa were in prior to staining (e.g., Cerophyl-Prescott medium). Flagellates exposed for longer periods of time to Hydroethidine^R appeared to quiver irradically. The cells

were red when observed with UV light (UV-2A combination filter, 400 nm dichroic mirror, 330-380 nm excitation filter, 420 barrier filter) from a 100W Hg vapor lamp with <100 hr of use. [N.B., the manufacturer notes that Hydroethidine^{R} stains the cytoplasm blue and the chromatin red.] Fluorescing cells were observed up to 1 month after staining.

Hydroethidine^{R} is a vital stain and the organisms must be alive in order to absorb the compound and fluoresce under UV light. Hence, unlike acriflavin and DAPI, the stain must be applied **before** fixation. If active cells are not to be examined, then the sample can be fixed (**after** the protozoa are stained) with 1% glutaraldehyde/cacodylic acid solution (final concentration). They can then be filtered onto a 0.8 µm PC black filter and mounted onto a microscope slide, as described above for DAPI staining. Slides prepared of Hydroethidine^{R} stained cells were stored in slide boxes at 4^{o}C for up to 1 month (however, the best results were obtained when the slides were viewed the same day they were prepared).