The Community Structure of Heterotrophic and Autotrophic Bacteria and Microflagellates in Four Lakes of the Precambrian Shield and the Possible Roles of Bacteria in Mercury Dynamics in Freshwater Systems.

by

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A Thesis Submitted to
The Faculty of Graduate Studies
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MICHAELA SAMEK

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ABSTRACT.

This study presents: 1/ the ecological significance of bacteria, flagellate and picoplankton in four lakes, 2/ development of methodology for the analyses of mercury (Hg) and methylmercury (MeHg) in freshwater particulate fractions, including the bacterial fraction using the AMICON® dewatering system, 3/ determination of the bacterial burden of Hg in one of the lakes examined.

Bacteria and flagellates collected during the ice-free season from water columns are described and quantified. The planktonic community was divided into three size fractions based on the longest linear measurements of each particulate fraction. The microbial fraction, measuring 0.1-3 µm, consisted mainly of bacteria and similar size organisms. The bacterial fraction was described and reported in terms of abundance, morphology, and biomass. The fraction measuring 3-20 µm consisted of a mixture of organisms, mainly flagellates, ciliates and algae. The largest fraction, >20 µm, consisted principally of zooplankton. A dewatering (AMICON®) system was used to separate and concentrate the microbial fraction from the other fractions. Methods for the analyses of Hg and MeHg were modified to assess these types of samples.

The microbial fraction is shown to be the main biotic compartment of Hg. The concentration of Hg in the microbial fraction ranged from 3.7 to 4.6 ng L^{-1} using the gas/liquid partition method (GLPM) and 5-8 ng L^{-1} by using the gold trap amalgamation method (GTAM) amalgamation method. Samples contained 0.09 to 0.45 ng L^{-1} Hg as MeHg. Other fractions and the filtrate contained less than 0.11 ng L^{-1} of Hg.

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1. Introduction.

Mercury widely occurs in the environment due to natural weathering processes and industrial pollution. Elevated Hg levels in fish reported in the 1970's from Lake St. Clair, North America, prompted investigation of the occurrence of Hg in aquatic organisms (Abernathy and Cumbie, 1977). Because of its chemical attributes and activity, Hg has the potential to cause enormous environmental and sociological problems. The extent of Hg uptake by humans and other animals through the aquatic ecosystem and its extreme toxicity became apparent after a chemical plant discharged effluents containing various Hg compounds, including MeHg (Fujiki, 1980a) into Minamata Bay, a port of the Sea of Japan. Due to this water contamination, fish were able to bioaccumulate MeHg (Rask and Verta, 1995; Cabana et al., 1994; Jackson, 1988a). Human consumption of the contaminated fish and shellfish resulted in "Minamata disease" (Fujiki, 1980a), which has been proved to cause severe disturbances of the central nervous system, leading to physical impairment and many deaths. Tracing the cause of human disorders led to investigations of the fate and toxic effects of Hg and methyl Hg (MeHg) in water bodies (Parks et al., 1989).

Similar discharges have been made into freshwater environments. In Canada, more research into these problems was sparked by increased Hg found in the English-Wabigoon River system (Parks et al., 1989). This waterway received Hg containing effluents from a chlorine-alkali plant located on the river near Dryden, Ontario. Discharges have been estimated to be between 9000-11000 kg of Hg between 1962-1969. Even though Hg discharges have been controlled, 12.5 kg is still added annually (Rudd, et al., 1983). Levels of Hg and MeHg in fish were still elevated in 1980 (Parks et al., 1989), some 10 years after major additions of Hg had ceased. The commercial fishery in Clay Lake, a part of the system where substantial amounts of Hg have been transported, was destroyed as amelioration procedures proved to be difficult (Rudd and Turner, 1983). Fish in the system can not be eaten because MeHg remains above the concentration levels permitted by local health authorities, e.g. Canadian Federal Food and Drug Directorate in Canada. Similar high levels of Hg in fish (Bodaly et al., 1984) were found in a newly inundated system in Southern Indian Lake located on the

Churchill River in northern Manitoba, (57°20'N, 98°20'W; Hecky, et al., 1984). Southern Indian Lake was impounded and rose by 3 m above the long term mean depth. Impoundment caused Hg to leach out of flooded soils and into the new water column. Dissolved organic carbon (DOC) was generated from decomposing organic material and oxygen became depleted. These responses are thought to be the major sources of stimulation of microbial production with subsequent high bacterially mediated Hg methylation (Hecky et al., 1984, Jackson, 1988a, Regnell and Tunlid, 1991, Hecky et al, 1991, St.Louis et al., 1994). When it was recognized that these processes led to the accumulation of MeHg in fish (Morrison and Therien, 1995; Boudou and Ribeyre, 1985; Richman et al., 1988), guidelines were established by the Canadian Federal Food and Drug Directorate. The first guidelines were established in the early 1990's, when concentrations of mercury in fish in many subarctic reservoirs were found to be frequently high (Canada-Manitoba Mercury Agreement, 1987). Fish with concentrations at or above 0.5 μg Hg g^{-1} wet weight muscle can not be marketed in Canada (Yingcharoen and Bodaly, 1993) and The World Health Organization imposed a maximum weekly intake of 200 μg Hg (as the methylated species) and 300 μg Hg (as total Hg) for a 70 kg human (Weber, 1993).

Bioaccumulated MeHg eventually ends up in fish and other biota (Fujiki, 1980b). Two non-exclusive theories suggest routes of bioaccumulation in fish either directly from water, due to passive diffusion of dissolved methylated Hg species across the gills of fish (Cossa et al. 1994; R.C. Playle, Wilfrid Laurier University, Waterloo, personal comm.) or through the food web, which is based on the microbial compartment in the water column (Allen-Gill et al., 1995; Becker and Bigham, 1995; Zillioux et al., 1993). Bioaccumulation through the food chain rests on the assumption that bacteria are not only Hg methylators, but also play an important role as Hg and MeHg carriers. This is the case of heavy metals such as cadmium (Kemp and Swartz, 1988), lead and other heavy metals (Sigg L. et al., 1987), which were reported to be associated with particulate matter. Particles consisting mainly of biologically generated material (Sigg L. et al., 1987; Kemp and Swartz, 1988) are suggested to be the main adsorption site for Hg by Allen-Gill et al. (1995) and Furutani and Rudd (1980).

Recent research efforts have addressed the amounts of Hg and MeHg occurring in water (Ahmed and Stoeppler, 1986; Bryan and Langston, 1992; Cossa et al., 1994; Regnell and Tunlid, 1991; Parks et al., 1989; Rodgers and Beamish, 1983), Hg methylation (Xun et al., 1987, Winfrey

and Rudd, 1990), and bioaccumulation (Rodgers and Beamish, 1983; Rudd et al., 1983; Hecky et al, 1991, Zillioux, et al., 1993). Prior to the present work there has been no reported research on Hg associated with planktonic bacteria in fresh waters.

This project was undertaken with three goals. First to describe the microbial compartments in two types of lakes in the ELA area. Biotic fractions in these lakes were separated by size, using the longest linear measurements of organisms. The smallest fraction, with linear measurements of 0.1 to 3 μm consisted mainly of bacteria, picoplankton (cyanobacteria and eucaryotic microalgae, size 0.2-2 μm) and very little detritus. The fraction which measured from 3 to 20 μm consisted of flagellates, ciliates and algae, and the fraction measuring >20 μm was mainly zooplankton. The second goal of the project was to develop methodologies for the analyses of Hg and MeHg in dilute fractionated microbial samples, as well as in unmanipulated lake water. Because of dilute concentrations of mercury in freshwater systems, methodological problems dealing with mercury analyses had to be addressed. Lastly, the distribution of Hg and MeHg within the microplankton of an inundated freshwater ecosystem was examined.

2. Ecological Relationships of Microplankton in Four Precambrian Lakes.

2.1 Literature Review.

2.1.1 The Microbial Compartments of the Water Column in Lakes: The Microbial Loop.

Bacteria have important roles as secondary producers in the regulation and flux of inorganic and organic materials in aquatic ecosystems (Tranvik, 1989, 1992) and in interactions with other members of the planktonic community (Bloem and Bar-Gilissen, 1989). Pomeroy (1979) first recognized that bacteria function not only as decomposers of organic material but as integral members of a food web likely to regulate energy transfer within that web. He questioned the commonly held theory that primary producers were the only major link in an almost linear food chain model leading from algae to fish. He comments that it is difficult to relate production of fish to unidentified primary consumers (flagellates) and to controversial primary producers (picoplankton). This approach led to the study of the role non-photosynthetic microbes play in energy transfer. As a part of a model of planktonic food chains, the term "microbial loop" was proposed by Azam et al. (1983) to describe the microbial input into other trophic levels. The microbial loop includes not only heterotrophic bacteria but picoplankton <2 μm, which in some systems contain 69% of chlorophyll a and are responsible for about 77% of primary production (Weisse, 1989). In the Red sea, these members of the microbial loop were responsible for as much as 90% of planktonic production (Weisse, 1989). Stockner (1988) and Sherr et al. (1986a,b) and Sherr and Sherr (1988) developed a model of the planktonic food web in which microplankton, including all prokaryotic and eucaryotic unicellular organisms, both autotrophic and heterotrophic, were separated into size categories: picoplankton (0.2-2.0 μm), nanoplankton (2.0-20 μm), and microplankton (20-200 μm) (Fig.1) based on the longest linear measurement of the organisms, and placed in trophic relationships. The microbial loop, consisting of bacteria,

flagellates, ciliates and small algae (Fig.1, red line) is now considered to be an important integral part of this larger microbial food web, which include the microbial food loop interactions with algae (Fig.1, blue line).

In this model allochtonous organic matter is degraded to dissolved organic carbon (DOC) by bacteria. DOC consists of exuded material from algae, dead organisms and zooplankton, and dissolved organic matter (DOM) of litoral, anthropogenic and terrestrial origin. DOC is identified as organic compounds of different molecular weights (M.W.) with distinct properties. High M.W. compounds (>10,000), for example humic acids, were found to be better substrates for bacterial growth than low M.W. (<1,000 M.W.) compounds, for example sugars and amino acids (Tranvik, 1990). However, low M.W. compounds, released by autotrophic organisms, can be involved in particle formation, where they "glue" DOC components together (Jensen and Sondergaard, 1982) or stick to surfaces of phytoplankton (Massalski and Leppard, 1979) creating particles. Based on these findings DOC is operationally defined and is generally accepted as material which passes through <1.2-0.45 µm pore sized filters. For example, Jensen and Sondergaard (1982) used 0.2-0.45 pore size filters and Miskimmin et al., (1992) used <0.7-1 µm pore size filters. According to this definition, DOC may contain particulate material as well as truly dissolved compounds. In the model presented, (Fig.1) DOC released from small algae can reach 50% of fixed carbon in the water column (Larson and Hagstrom, 1979), and may be subsequently ingested by larger algae measuring >5 µm, non-photosynthetic flagellates or ciliates. For example, heterotrophic flagellates are capable of ingesting and assimilating high molecular weight polysaccharides (Sherr, 1988). This primary production is available to metazoa only after assimilation, release and grazing through and within the microbial loop, a process usually referred to as "repackaging". Allochtonous material (terrestrial organic material of natural and anthropogenic origin) (Tranvik, 1990), is another source of dissolved organic matter, which can be utilized and taken up as DOC by bacterioplankton (Taylor and Joint, 1990; Hagstrom, 1988). The microbial loop is described both as a "source" of carbon to higher trophic levels and a "sink" of carbon unavailable to higher trophic levels. The loop is a "source" when

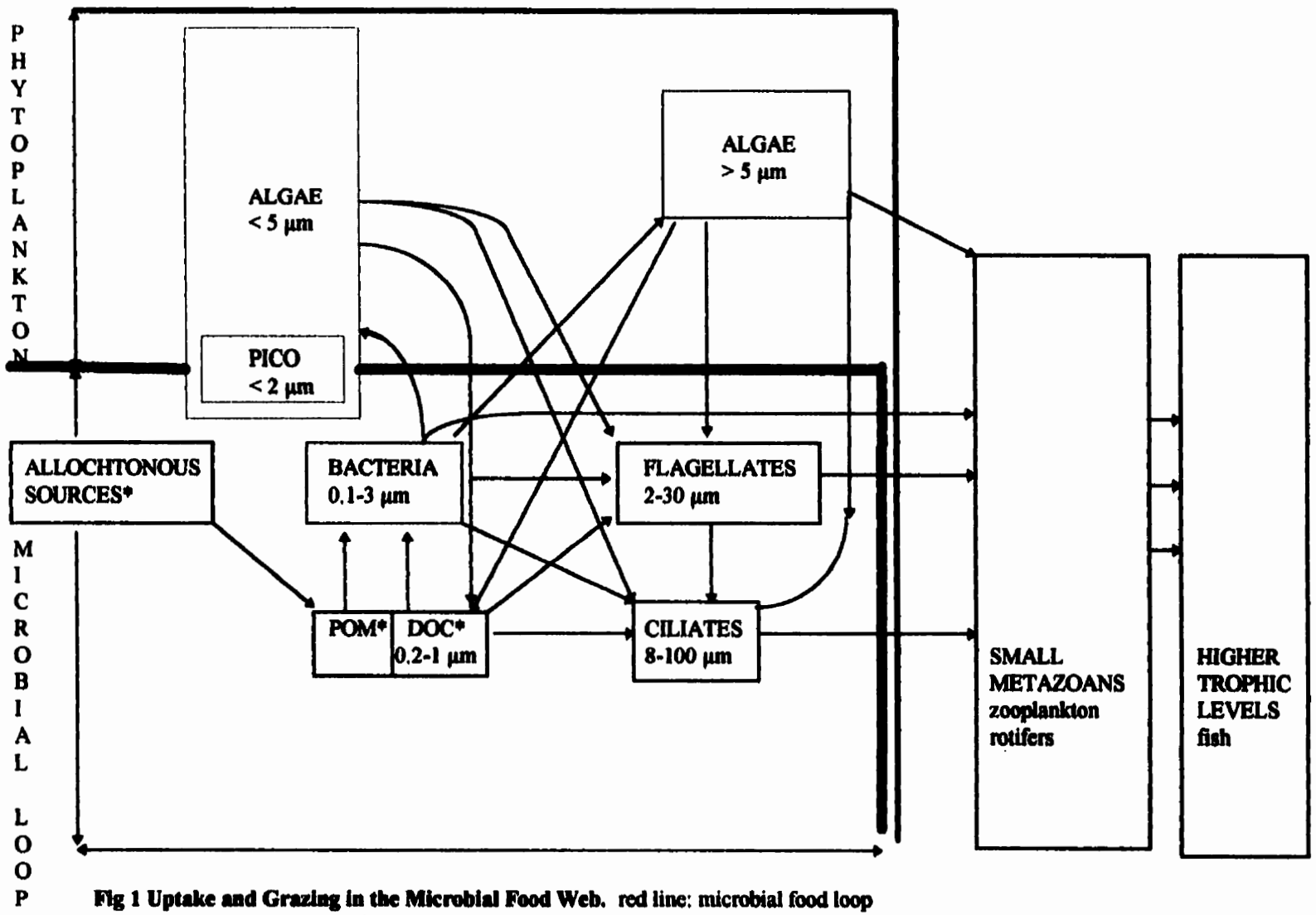


Fig 1 Uptake and Grazing in the Microbial Food Web. red line: microbial food loop
blue line: microbial food web (Redrawn and modified from Sherr and Sherr, 1988) * see text

bacteria assimilate and repackage DOM and make it available for bacterivores, algae and microzooplankton. For example, cyanobacteria and bacteria are grazed upon by heterotrophic nanoplankton and ciliates.

Small metazoans and rotifers also consume flagellate populations (Weisse, 1990). Flagellates and ciliates can ingest phytoplankton (Sherr and Sherr, 1988) and phytoflagellates ingest bacteria (Porter, 1988). As well, there are predator/prey interactions between flagellates and ciliates. The microbial loop may also be thought of as a carbon "sink" since only a small proportion of bacterially fixed carbon can be utilized by higher organisms, i.e., most of the carbon assimilated by bacteria is respired and lost as CO_2 at each new trophic level (Ducklow et al., 1986). The microbial food web, including microbial food loop and algae (Fig.1) is the ultimate food source for metazooplankton (Sherr and Sherr, 1988). Even if most of the primary production is respired within the microbial food web, the microbial based food web remains the only food source for metazoans. Within this process there is a need to take into account the interactions among bacteria, phytoplankton, and protozoa.

It is difficult to study the microbial control of DOC in water. DOC of autochtonous (extracellular organic carbon released by the phytoplankton) or allochtonous origin (organic material of terrestrial origin) can be involved in particle formation. Fuhrman and Azam (1980) used 0.45 µm filters to obtain a bacteria-free diluent for dilution experiments. They demonstrated that >90% of thymidine uptake occurred in the <1.0 µm particle size fraction. This suggests that the bulk of bacteria are within 0.45-1.0 µm size. These findings are the main reason why authors define particulate and dissolved fractions as >0.45 and <0.45 µm, respectively. It is then important to correctly define dissolved (<0.45 µm) and particulate (0.1-3 µm) fractions. Li and Dickie (1985) demonstrated that considerable number of bacteria will pass through the 0.45 µm pore size filters. They conducted dilution experiments and noted that bacteria were even able to pass through a 0.2 µm pore size filter. These small bacteria could grow at high rates (0.18 h^{-1}), increase in cell size and create colonies.

2.1.2 Spatial Distribution and Seasonal Succession of Bacteria, Picoplankton and Flagellated Protozoa.

The abundance of heterotrophic bacterial populations in the vertical columns of unpolluted lakes varies from 0.5 to 1.5 * 10^6 mL^{-1} depending on season, trophic status and depth. Those bacteria can be free living or attached to particulate matter. Attached bacteria are larger and can significantly contribute to biomass as described below. There are always more free living bacteria than occur attached to detritus in the water column (Clarke and Joint, 1986). The abundance of free living bacteria usually range from 5 * 10^5 in winter to 1 * 10^6 cells mL^{-1} during summer stratification, as found by Scavia and Laird (1987) in Lake Michigan. The density of attached bacteria varies: Pedros-Alio and Brock (1983) estimate that 1 and 30% of bacteria in Lake Mendota (Wisconsin), are associated with larger particles in winter and summer, respectively. Porter and Feigh (1980) estimate less than 10% of bacteria are attached in Lake Oglethorpe, an eutrophic manmade lake in Georgia. Bacterial abundance can be separated into several fractions to evaluate relative contribution of free and attached bacteria, e.g.: 0.1-1 µm, 1-3 µm and >3 µm fractions. The contribution of these three fractions to bacterial abundance in Lake Constance were on average 75, 19, and 4%, respectively. Even though the abundance of attached bacteria in >3 µm fraction could reach up to 100%, this represented always only about 3% of total bacterial counts. In polluted lakes, or those naturally carrying a high nutrient supply, bacterial numbers may be much higher. Within a thermally stratified lake there are differences in the vertical and spatial distribution of heterotrophic bacterial populations, picoplankton and other unicellular organisms of the microbial food web. Differences reflect vertical and horizontal variations in abiotic parameters such as light penetration, temperature, oxygen concentration and trophic status. Bacteria typically reach maximum abundance in two areas: within the thermocline and in the sediment/water interface, where the concentration of available organic matter is high (Rheinheimer, 1974).

Seasonal succession of planktonic bacteria has been described on several levels: as differences in abundance of morphological cell types and as changes in bacterial cell volume and carbon content. For example, the seasonal temperature pattern in Lake Constance was similar to other thermally stratified lakes in the northern hemisphere, with highest temperatures of 20°C from June to October. Seasonal trends for bacterial processes have been examined annually (Simon, 1987, 1988; Müller et al., 1991) and microbial abundance was found highest during spring diatom bloom ($>10^7$ mL^{-1}), while the lowest abundance recorded was in the hypolimnion and during winter. Above 20 m, the 0.2-1.0 µm fraction comprised 70-80% of total cell numbers (Simon, 1987, 1988).

Cyanobacteria (blue-green algae) form part of the picoplankton group. Cyanobacteria are found near lake surfaces where light penetration is adequate to support their phototrophic metabolism and where water temperatures are maximal (Caron et al., 1986). Their abundances vary from $1.8 * 10^2$ mL^{-1} in oligotrophic marine and freshwater systems (La Ferla, 1991) to 10^4 cells mL^{-1} in euphotic zones of lakes, and up to 10^6 cells mL^{-1} when temperatures are maximal, e.g., in Lake Ontario (Stockner and Antia, 1986).

Heterotrophic flagellates are found in a variety of habitats. Their occurrence depends on light penetration through the water column, water turbulence and flagellate feeding behavior (Porter et al., 1985). They can be free swimming or attached to particles or other substrata, which probably facilitates flagellate suspension feeding (Caron et al., 1982). During calm weather, flagellates attached to suspended material tend to sink, due to gravity. During windy periods turbulence tends to distribute flagellates through the entire water column. During calm weather the maximum abundance of free swimming flagellates occurs near the surface (2-3m). Weisse (1991), who studied the distribution of heterotrophic flagellates in Lake Constance, found flagellates were evenly distributed throughout the entire water column during the spring, but with the development of thermal stratification, their abundance was higher in the epilimnion than in deeper layers.

2.1.2.1 Assessment of Bacterial Production and Biomass.

Bacterial production may be expressed in terms of cell growth and division or in terms of dry weight biomass (carbon content per gram of dry bacterial weight). Methods for estimating bacterial biomass and production include measurements of ATP content (Karl et al., 1991, Jensen, 1989), frequency of dividing cells (FDC) (Hagstrom et al., 1979), tritiated nucleotides incorporation (Fuhrmann and Azam, 1982; Karl, 1981) and radioactively labeled bacteria (Hollibaugh et al., 1979). In order to obtain production estimates in terms of weight or carbon content, biomass must be measured. Many attempts have been made to obtain an universal conversion factor to convert microbial biovolume to bacterial carbon using various methodologies (Table 1). To obtain biovolume, bacteria stained with fluorochrome may be photographed and the volumes of different shapes of bacteria computed. Images can be taken by camera (Fuhrman and Azam, 1980), scanning electron microscope (SEM) (Fuhrman and Azam, 1980), transmission electron microscope (Borsheim, et al., 1990) or by image analyzer (present study). Fuhrman and Azam (1980) compared volumes and derived biomass estimates obtained by scanning electron microscopy, by camera and by visual estimation of size classes of bacteria using epifluorescent microscopy. They concluded that estimates made with epifluorescent microscopy were the closest to electron microscope measurements. Reliable estimations of bacterial volume cannot be done by an electronic particle analyzer, because other small organisms, organic debris, and inorganic particles cannot be distinguished from bacteria. Bacterial volumes may be converted to cell carbon content by application of factors experimentally ascertained or by use of values in the literature. Some authors have assumed that the carbon to biovolume ratio is constant. However, Lee and Fuhrman (1987) demonstrated that bigger cells tend to have lower concentrations of carbon than smaller cells. Their conversion factors average 0.38-0.5 pg C μm^{-3} (Table 1). Bratbak (1985) compared three methods for estimation of bacterial volume: SEM, epifluorescence microscopy (EFM), and electronic sizing (ES) and derived an estimate of average bacterial cell volume by combining the data from the three methods. He estimated bacterial production to be 0.56 pg C μm^{-3} using averaging methods. As well, derived two other conversion factors, 0.24 pg C μm^{-3} after corrections for shrinkage (see 2.1.4.2.),

and 0.22 pg C μm^{-3}, when using a dry weight/wet weight ratio of 0.4. Since these latter two methods take into consideration shrinkage and dry/wet weight supported in the literature, they seem to be better estimations than the much higher value of 0.56 pg C μm^{-3}, a calculated average for three quite different methods.

Table 1 Conversion Factors Used to Convert Biovolume of Bacteria to Carbon Content.

Conversion Factor [pg C μm^{-3}]	Conditions of Measurement	Source
0.12		Watson et al., 1977
0.24 and	correction-shrinkage	Bratbak, 1985
0.22	dry wt./wet wt. ratio 0.4	"
0.56	AVG of three method (SEM, EFM, ES)	"
0.35		Bjornsen, 1986
0.38-0.5		Lee and Fuhrman, 1987
0.154	POC/biovolume	Scavia and Laird, 1987
0.22		Bratbak and Dundas, 1984
0.4	[^{3}H]-leucine thymidine uptake	Bjornsen and Kuparinen,1991

The volume of the bacterial cell changes seasonally depending on the trophic status of the lake. Bacteria in oligotrophic lakes have smaller cell volumes (from 0.015 μm^3 during winter to 0.072 μm^3 during summer, Scavia and Laird, 1987), while cells in eutrophic lakes seem to have small volumes during summer (0.012 μm^3) and larger volumes during winter (0.10 μm^3)(Bjornsen et al., 1989). Gonzales et al. (1990) conclude that these differences in volumes are due to higher

growth rates of grazing flagellates close to the water surface. The higher abundance and growth rate of flagellates coincide with the maximum densities and growth rate of bacteria.

Bacterial biomass and cell numbers in Lake Constance showed the highest values during the spring and diatom bloom. Biomass ranged from 6.5 to 158.2 mg C m^{-3}, and cell volume from 0.029 to 0.313 μm^3 and from 0.048 to 0.353 μm^3 for free and attached bacteria, respectively (Simon, 1988). As attached bacteria are larger than free living bacteria, they can contribute significantly to bacterial biomass under special circumstances, e.g., when DOC concentrations rise during flooding of lakes (Simon, 1987).

2.1.3 Growth and Grazing Rates of Various Groups.

2.1.3.1 Growth of Bacteria.

Growth rates of bacteria in the environment have been measured in various ways including uptake of radio-labeled constituents, assessment of doubling cells and rates of increase under specific conditions. Dilution experiments are conducted in order to measure bacterial growth under reduced grazing pressure (Li and Dickie, 1985). In this type of experiment a small volume of unfiltered or partially filtered lake water is diluted with lake water from which all organisms have presumably been removed by filtration. In this way, bacteria are exposed to nutrients, but not predators. The number of bacteria often increases substantially over several hours and their apparent specific growth rate is in inverse proportion to the ratio of unfiltered to filtered water (Kirchman et al., 1982). The filtration method is used to estimate bacterial production without predators and/or bacterial grazing losses with different sizes of predators (Weisse and Scheffel-Moser, 1991). Tranvik (1989) used the dilution technique for growth rate estimation in Swedish oligotrophic lakes and found growth rates to range from 0.01 to 0.06 h^{-1} depending on the season.

Li and Dickie (1985) measured growth of bacteria using the

dilution technique combined with measurement of biomass by uptake of radio-labeled compounds. They measured growth rates of sea water bacteria of 0.18 h^{-1} with uptake of ^{3}H labeled amino acid mixture. Iturriaga and Mitchell (1986) reported growth rates of 0.67 h^{-1} for marine cyanobacteria using $H^{14}CO_3^-$.

2.1.3.2 Grazing Rate within the Microbial Loop.

The major sink for bacterial production is heterotrophic flagellates measuring <10 µm (Weisse, 1989). Their grazing rates range from 5-15 bacteria h^{-1} $flagellate^{-1}$ (Bjornsen, 1988) to 10-100 bacteria h^{-1} $flagellate^{-1}$ (Weisse, 1989, 1990). Larger bacteria are apparently selectively grazed by flagellates at rates up to three times higher than smaller forms (Gonzales et al., 1990). Grazing is also higher when attached flagellates are present. Nano- and micro-ciliates graze on bacteria, but also have been identified as major predators of flagellates (Weisse, 1990). If only flagellates are consumed the rate of ingestion is 3-40 flagellates h^{-1} $ciliate^{-1}$. Other protozoans and rotifers are of minor importance in controlling flagellate populations (Weisse, 1990). Both large and small cladocerans are important grazers of bacteria. When small cladocerans, e.g. *Bosmina*, are present, the flagellate grazing rate of bacteria is higher than when large cladocerans such as *Daphnia* are present (Vaque and Pace, 1992).

2.1.4 Methods in Microbial Ecology.

2.1.4.1 Principles of Fluorescent Microscopy.

The use of fluorescent dyes to visualize water bacteria was fundamental to the development of microbial ecology. Prior to the discovery of fluorescent dyes as bacterial staining agents, standard microscopical methods were insufficient to estimate bacterial abundance in water. Standard plate counts, which detect bacteria by growth of colonies, were unreliable as most natural water bacteria do not grow on

fortified agar (Coleman, 1980). The use of fluorescent stains improved visualization and counting of bacteria (Coleman, 1980; Porter and Feig, 1980) and flagellates (Haas, 1982) and at present remains the most reliable technique available.

Fluorescence microscopy of bacteria combines the use of fluorescent stains with epifluorescent microscopy in a system in which single and aggregated cells can be distinguished from other material. Specimens are dyed and illuminated with light of a short wavelength, e.g., ultraviolet or blue. In these techniques it is necessary to choose appropriate wavelengths and light filters in order to achieve high quality visualization. Fig.2,I. describes the general principle of the epifluorescent microscope (Bradbury, 1984) and Fig.2,II shows the specific function of Zeiss® filter set #02 used with 4',6-diamidino-2-phenylindole (DAPI) stain. Part of the excitation beam (Fig.2,Ia) from a light source (here a Hg lamp) is absorbed by the specimen (Fig.2,Ib) and part is reemited as fluorescence (Fig.2,Ic). Unnecessary wavelengths are filtered out by barrier filter (Fig.2,Id) placed between specimen and the eye. This filter should be fully opaque (Fig.2,IIb) up to the wavelength used for excitation (Fig.2,IIa) and fully transparent to longer wavelengths so as to transmit the fluorescence (Fig.2,IIc). The fluorescent object is then seen as a bright image against a dark background (Ploem and Tanke, 1987).

2.1.4.1.1 Fluorescence: Physics and Chemistry.

That part of the stain or fluorochrome which is responsible for fluorescence is the fluorophore. Molecules of fluorochrome can exist at any of several energy levels, each associated with a particular arrangement of the electronic orbitals. Molecules normally exist at the lowest available energy level, or the ground state. Molecules at higher energy levels are referred to as "in excited states". To proceed from the ground state the molecules must acquire energy, while the return to the ground state requires loss of energy. In the case of fluorescence this loss of energy is accomplished by the emission of a photon, i.e.,

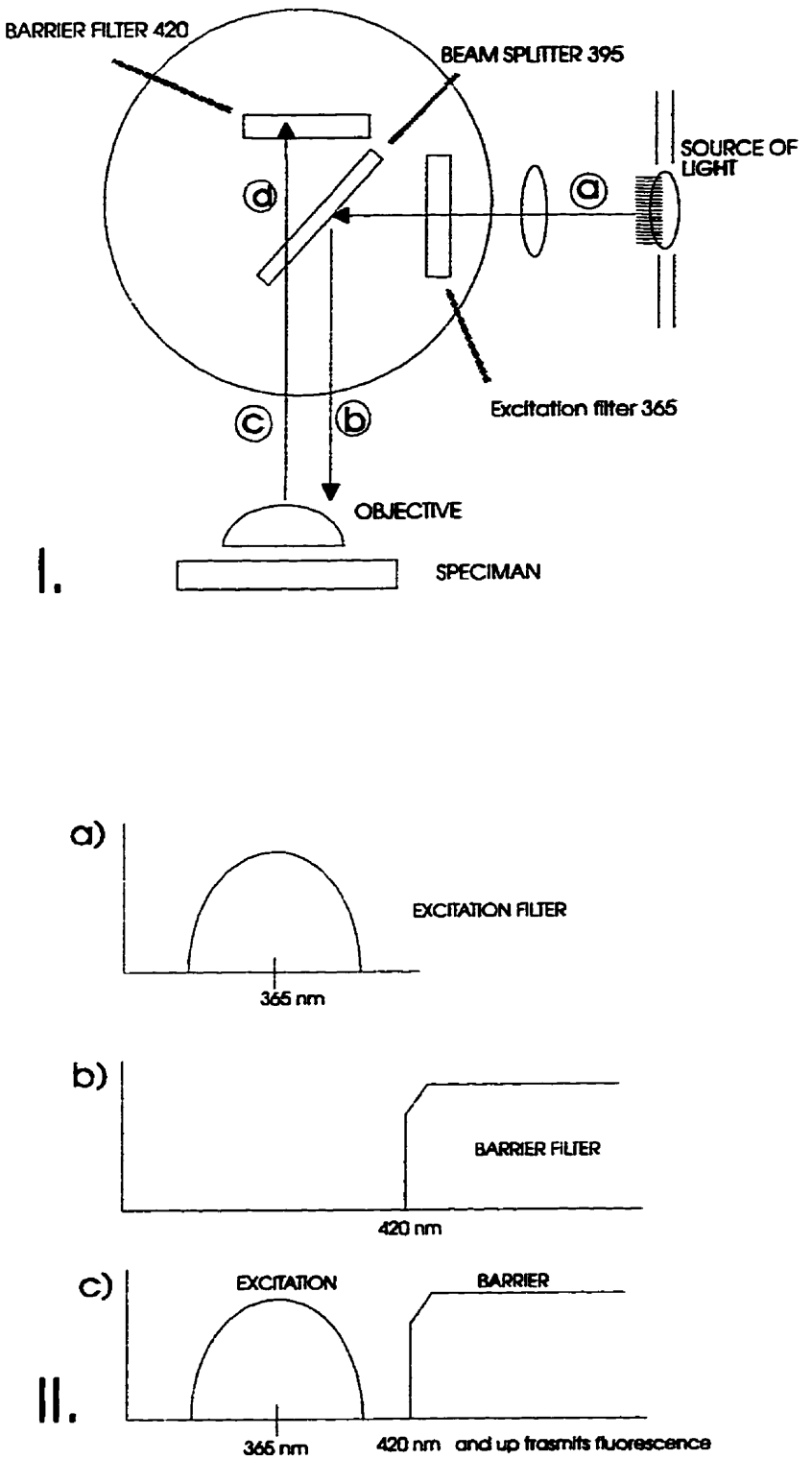


Figure 2 The Principles of Fluorescent Microscopy.

I. path of light from source to specimen and the eye II. Zeiss filter set 02 wavelengths used in conjunction with DAPI.

the production of light. Because the emitted photon has less energy than the excited photon, the wavelength of the emission is longer than that of excitation.

2.1.4.1.2 Photochemical Reactions and Fading.

It is characteristic of fluorescence preparations that they nearly always fade during irradiation. Fading varies from one specimen to the next and even within parts of the same specimen. Fading is due to photochemical reactions induced by the excitation light. The absorption of light raises the fluorophore to an excited state which is very reactive. A significant proportion of these excited molecules undergo photochemical reactions with the production of new molecules, which may be non-fluorescent or non-absorbent at the excitation wavelength. The rate of photobleaching depends on the nature of the fluorophore, its chemical environment and the intensity and quantum energy of the excitation. It is believed that fading is mainly due to a reaction between the excited fluorophore and oxygen (Ploem and Tanke, 1987), but fading can also be non-oxidative (Piccolo and Kaplan, 1984). Some fluorophores are particularly liable to rapid fading, while others are relatively stable. During prolonged irradiation, the rate of photobleaching varies. During the first few milliseconds of irradiation, fading does not occur, then there is a period of rapid bleaching lasting a few seconds and thereafter bleaching proceeds more slowly. Recovery for biological material is minimal. In order to minimize fading specimens are stored in the dark and at low temperatures.

2.1.4.2 Bacterial Fixation, Capture and Staining.

The most used bacterial fixatives are formaldehyde (HCHO, 1 or 2% final concentration, Bjornsen et al., 1989, Hobbie et al., 1977) or glutaraldehyde (1% final concentration, Bloem and Bar-Gilissen,

1989). Pomroy (1984) showed that bacteria preserved in Lugol's iodine solution, an algal preservative, did not vary in number after 10 years of storage.

Since bacteria occur in the 0.2-3 µm size range, polycarbonate Nucleopore® or Poretics® filters of pore size 0.2 µm or less are used to capture them. To eliminate autofluorescence, filters may be pre-stained in Irgalan black (Hobbie et al., 1977). To prevent hydrophobicity of the filters, a surfactant such as a 0.5% solution of Wayfos®, Phillip A Hunt Chemical Corp., East Providence, R.I., Hobbie et al., 1977), can be applied.

A variety of dyes are available for staining bacteria, but the most frequently used are DAPI (Porter and Feig, 1980) and acridine orange (AO) (Porter and Feig, 1980). DAPI is a highly specific stain for DNA under a wide range of conditions (Porter and Feig, 1980). It is used as a cytochemical probe for nuclear, mitochondrial and chloroplast DNA and for the detection of low levels of DNA. Fig.2,II shows the Zeiss filter set #2 used with this stain. When excited with light at a wavelength of 365 nm, the DNA-DAPI complex fluoresces a bright blue at or >390 nm. Material such as bacteria can be easily distinguished from other material. AO binds specifically with DNA and RNA and has to be excited at wavelengths of 346 or 490 nm. The DNA-AO complex fluoresces green, the RNA-AO complex red. With AO stain it is difficult to distinguish bacteria from nonliving particles (e.g. clays, detritus), which can also be stained or may autofluoresce. This is an important consideration when working with seston rich samples. DAPI, on the other hand, improves visualization of small (<1 µm) bacteria even in waters rich in suspended particulate matter (Porter and Feig, 1980).

2.1.4.3 Flagellate Fixation, Capture and Staining.

Since flagellated protozoa are very susceptible to rupture, bacterial fixation, filtration, and staining techniques are not suitable for them. During processing of photosynthetic flagellates, chlorophyll autofluorescence must be preserved so that they can be distinguished from heterotrophic flagellates. Many fixatives have been used to preserve flagellates, e.g., formaldehyde, glutaraldehyde, and Lugol's solution.

Bloem et al. (1986) investigated the quantitative fixation effects of formaldehyde, formaldehyde plus borax buffer, glutaraldehyde, and glutaraldehyde plus phosphate buffer. These fixatives were all used in combination with the fluorochrome primulin discussed below. By comparing counts of living flagellates before fixation with counts at several times after fixation, they showed that 5% formaldehyde (final concentration) offered the best compromise between preservation of cells and maintenance of fluorescence. Abundance of phototrophic flagellates in 5% formaldehyde fixed samples was constant for 1 week. In glutaraldehyde (0.3% - 1% final concentration), these cells tended to fade. When prepared slides were stored at 5°C, chlorophyll autofluorescence was preserved for 1 or 2 days, but storage at -30°C ensured that both heterotrophic and autotrophic flagellate counts were constant for at least 16 weeks. Filtration vacuum at or <3 kPa was recommended, since higher pressure showed 15-20% cell loss (Bloem et al., 1986). Flagellates occur in the 1-60 µm size range, therefore polycarbonate Nucleopore® or Poretics® filters with pore size 0.6 µm or more are used to capture them (Kuosa, 1988, Bloem and Bar-Gilissen, 1989). Choi and Stoecker (1989) studied the effect of fixation on cell volume of marine planktonic protozoa. Results of their work describe cell volume decrease with all fixatives used (Lugol's solution, 2% glutaraldehyde, 1% formaldehyde and a modified van der Veer fixative (2% glutaraldehyde and 2% tannic acid). Change in cell volume depends on the combination of the protozoan species present in the sample and the particular fixative used. The volume of fixed cells shrink by 20 to 55% of live cell volume. Even though the average volumes of live cells vary with growth stage, they still found significant differences between samples. Small changes in measured length and width of the cell result in large errors when volume is computed. As Lee and Fuhrmann (1987) showed, this can be corrected by calibration of cell images with fluorescent microspheres of known sizes. Bloem et al. (1986) reported no loss of flagella with 5% formaldehyde fixation and no distortion of cells. Cell volume distortion is often seen in electron micrographs of flagellates. Electron microscopy offers very high resolution and precise measurements, but artifacts caused by shrinkage and distortion of the cells have been found to cause considerable errors in volume estimations (Montesinos et al., 1983; Bratbak, 1985).

The fluorochromes primulin (Bloem et al., 1986; Kuosa (1988), proflavin (Kuosa, 1988), and Rose Bengal (Baldock, 1986) have been used to stain flagellates. Kuosa (1988) compared the fluorochromes

primulin and proflavine. Use of proflavine requires no rinsing during staining and filtration. However, in order to count picoplankton, an unstained slide has to be prepared, because proflavin-stained picoplankton cells are difficult to distinguish from other organisms. Primulin does not mask autofluorescence, so flagellates and picoplankton can be counted in one preparation (Table 2). Hass (1982) evaluated the storage time for proflavine stained flagellates and found that after 2 weeks of storage at -15°C, fading of both stained and autofluorescent cells was significant. A non-fluorescent stain, Rose Bengal (Baldock, 1986), can be used in samples with excess of organic material. Rose Bengal stains cytoplasmatic material pink, and specimens may be viewed under bright light for several hours.

Table 2 Conditions for Staining and Storage of Flagellate Samples.

Dye	Conc./Staining	Storage	Source
Primulin	63 mgL^{-1} 5 min.	16 weeks (-30°C)	Bloem et al.(1986)
Primulin	25 mgL^{-1} 3 min.	N/A	Kuosa (1988)
Proflavin	20 mgL^{-1} 1 min.	N/A	Kuosa (1988)
Proflavin	66.6mgL^{-1}2 min.	1-2 weeks (-15°C)	Hass (1982)
Rose Bengal	250 mgL^{-1}(70% MeOH)	hr. at room temp.	Baldock (1986)

N/A denote that storage test were not done.

2.2 Present Study: Structure of the Microbial Food Web in Four Precambrian Shield Lakes.

2.2.1 Introduction.

The naturally occurring microbial food webs of lakes in the Experimental Lakes Area (ELA) located in the Precambrian Shield of northwestern Ontario have not been studied prior to this present work. Work on the microbial compartments of ELA lakes has centered on methanogenesis processes of anaerobic bacteria in lake sediments (Rudd, at al., 1976; Kelly and Chynoweth, 1981; Kelly et al., 1988).

This present work was undertaken in order to understand the structure of the microbial food web, so that subsequent work on distribution of Hg in such systems could be done.

2.2.2 Methods and Materials.

2.2.2.1 Study Sites.

The Experimental Lakes Area (ELA) of northwestern Ontario, Canada (51°N, 94°W), contains a diversity of lakes (Cleugh and Hauser, 1971). Four ELA lakes, Lake 373 (L373, Fig.3) and Lake 375 (L375, Fig. 4.), were studied from May - October in 1992 and Lake 632 (L632, Fig.5) and Lake 979 (L979, Fig. 6,7) were studied from May - October in 1993. Those water bodies differ from each other in terms of hydrology, physiochemical characteristics and biology (Table 3). L373 and L375 are deep oligotrophic lakes, with maximum depths of 18 and 26 meters, respectively. These two lakes are fairly similar in terms of lake area, depths and volume, and chemical parameters such as DOC, alkalinity, pH and biota (Table 3). Lakes 632 and 979 are shallow ponds with mean depths of less than 1 meter. L632 is an unmanipulated reference basin. L979, a pond similar in many characteristics to L632, was inundated

between May and June 1993 in order to study the evolution of green house gases and the dynamics of Hg in the lake system and its biota.

Table 3 Selected Physical and Chemical Characteristics of the Four Study Lakes.

Lake	L373	L375	L632	L979 pre/post flooded
Lake area[a] [10^4 m^2]	27.3	18.73	0.86	2.38 /15.5
Max depth[a] [m]	18.0	26.0	1.5	1.45 /2.7
Mean depth[a] [m]	11.0	11.6	0.73	0.69 /0.79
Lake volume [10^4 m^3]	300.9[b]	217.4[b]	N/A	1.65-12.24[c]
DOC [µmol L^{-1}]	380-560[b]	380-530[b]	N/A	700-1200/700-1250[c]
POC [µgL^{-1}]	370-650[b]	350-620[b]	N/A	300-700/250-800[c]
Alkalinity [µeqL^{-1}]	337-403[b]	337-408[b]	N/A	N/A
Primary Production[d] [g$Cm^{-2}yr^{-1}$]	30.4	15.7	3.2	3.6/-

DOC: material <1.2 µm, POC: material >1.2 µm, N/A not available/not measured

[a] Greg McCullough, Freshwater Institute

[b] the ELA Database, Freshwater Institute

[c] M.J. Paterson et al., 1997.

[b] Eva Shindler, the ELA database

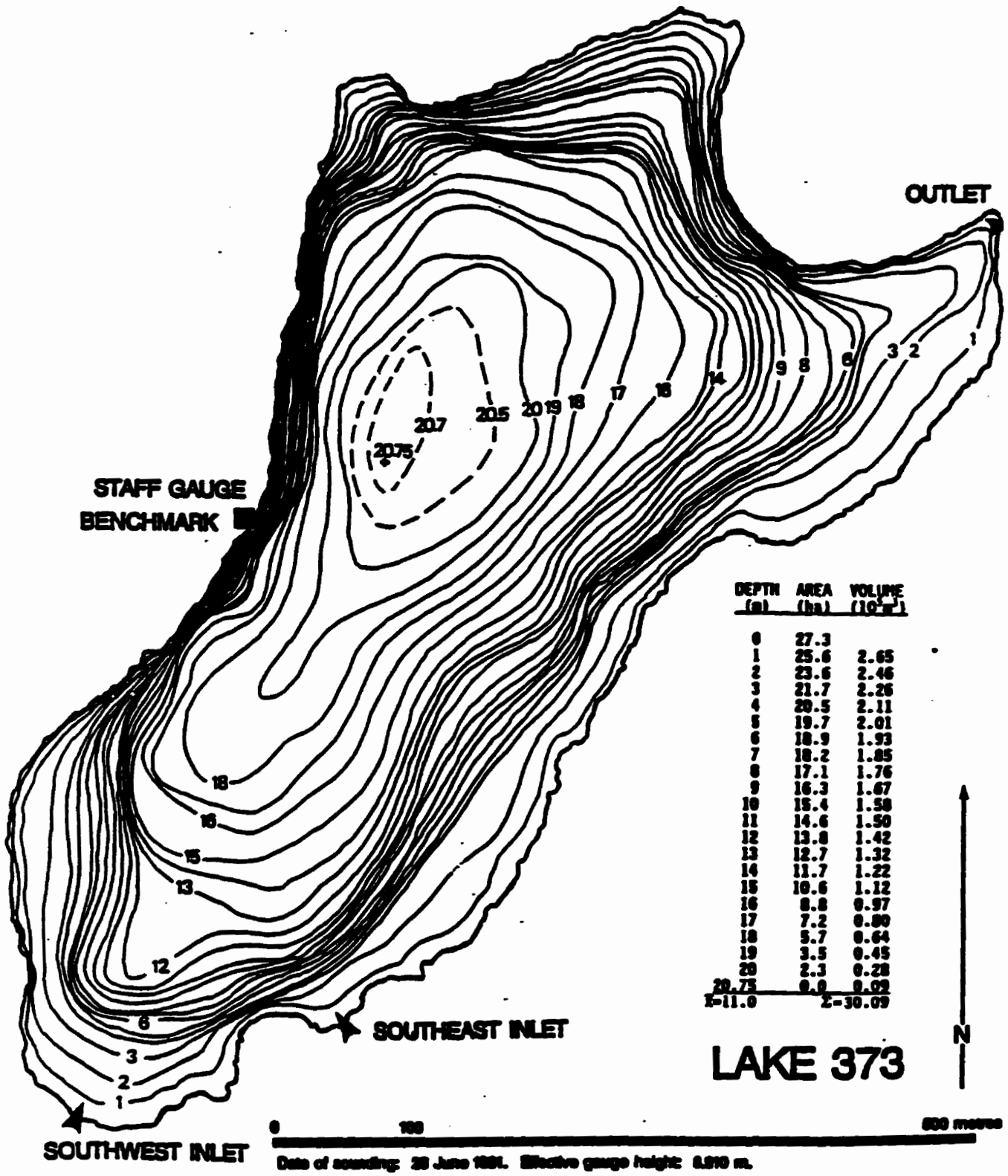
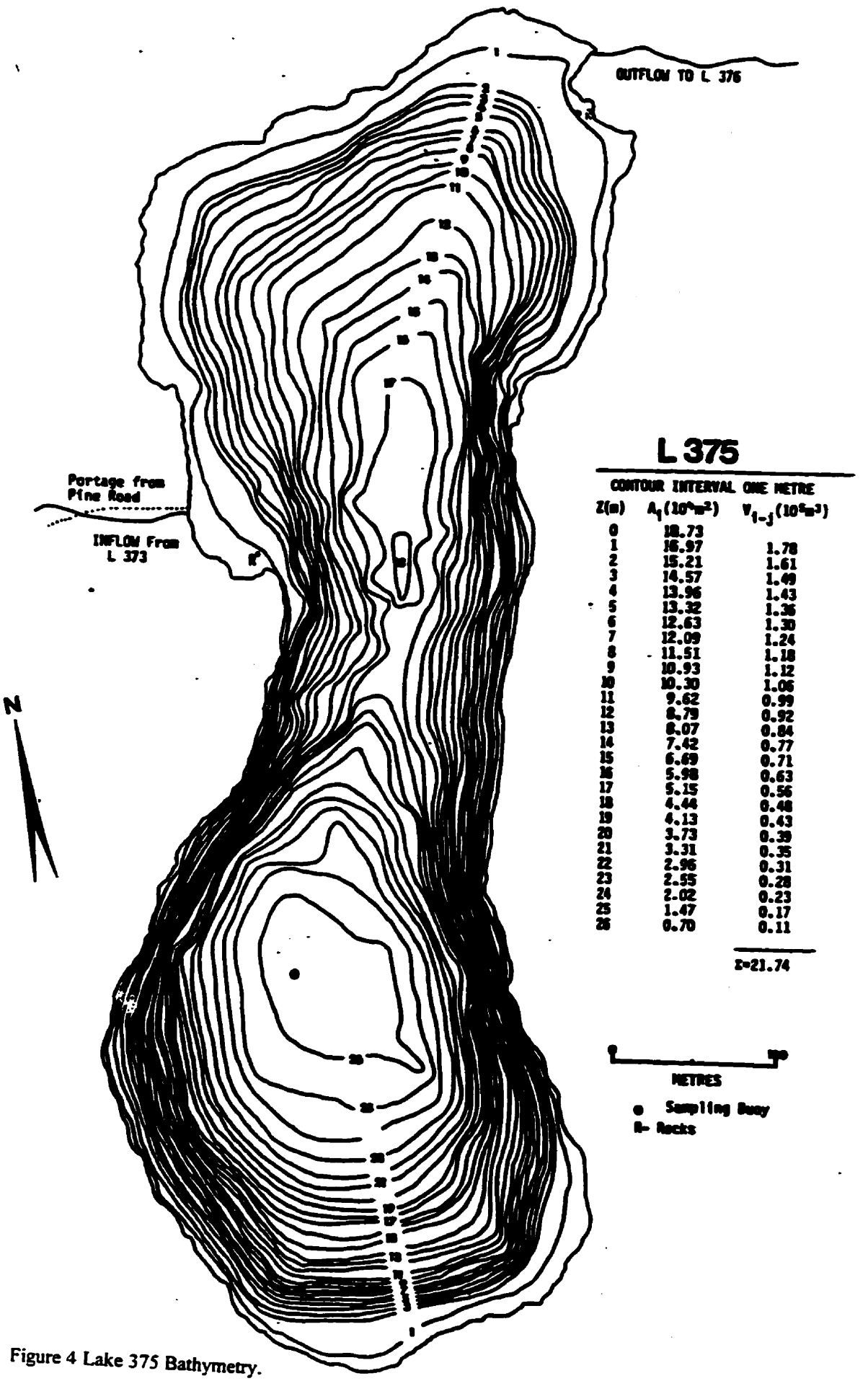


Figure 3 Lake 373 Bathymetry.



Z(m)	A_i ($10^4 m^2$)	V_{i-j} ($10^5 m^3$)
0	18.73	
1	16.97	1.78
2	15.21	1.61
3	14.57	1.49
4	13.96	1.43
5	13.32	1.36
6	12.63	1.30
7	12.09	1.24
8	11.51	1.18
9	10.93	1.12
10	10.30	1.06
11	9.62	0.99
12	8.79	0.92
13	8.07	0.84
14	7.42	0.77
15	6.69	0.71
16	5.98	0.63
17	5.15	0.56
18	4.44	0.48
19	4.13	0.43
20	3.73	0.39
21	3.31	0.35
22	2.96	0.31
23	2.55	0.28
24	2.02	0.23
25	1.47	0.17
26	0.70	0.11
		Σ=21.74

Figure 4 Lake 375 Bathymetry.

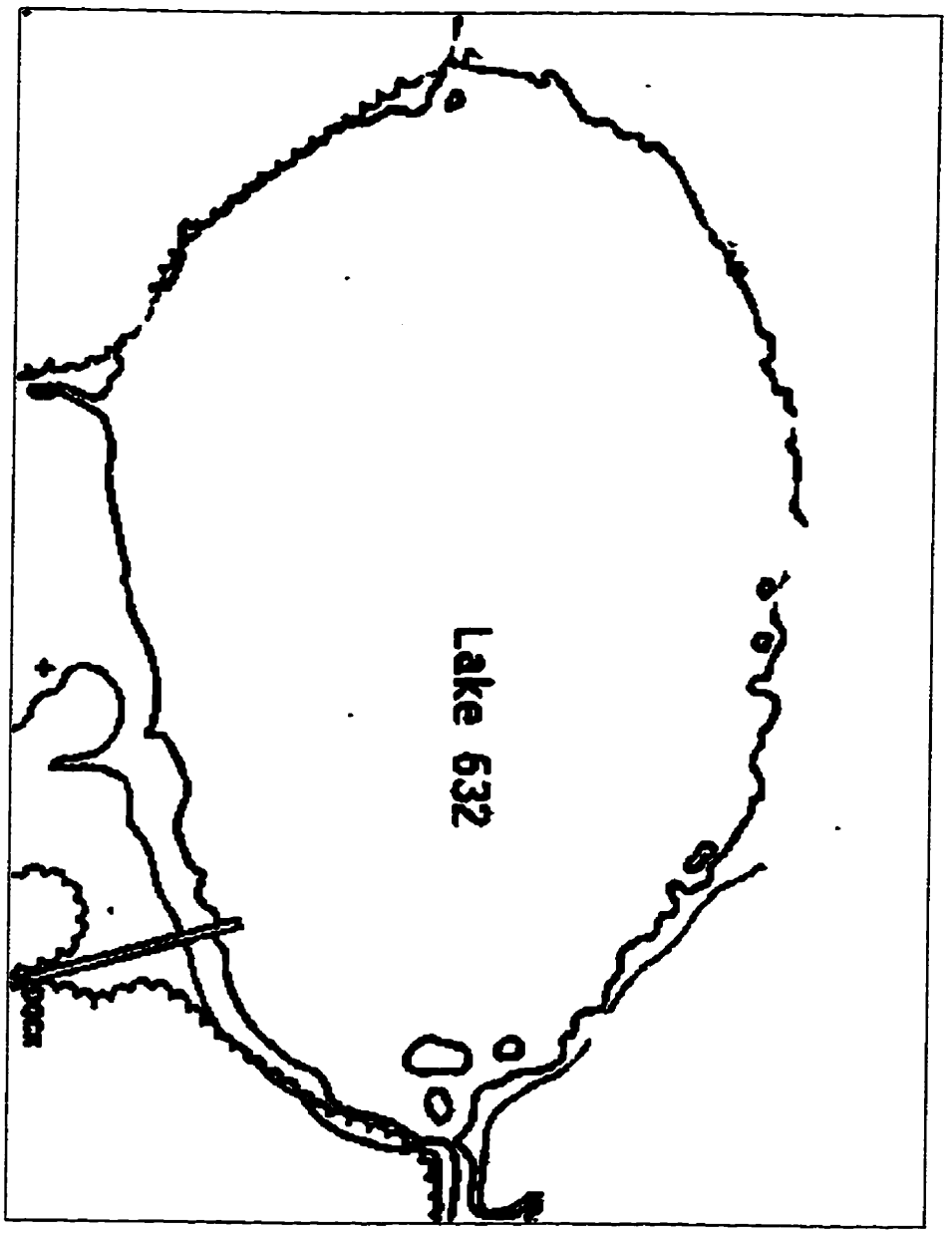


Figure 5 L632 Bathymetry.

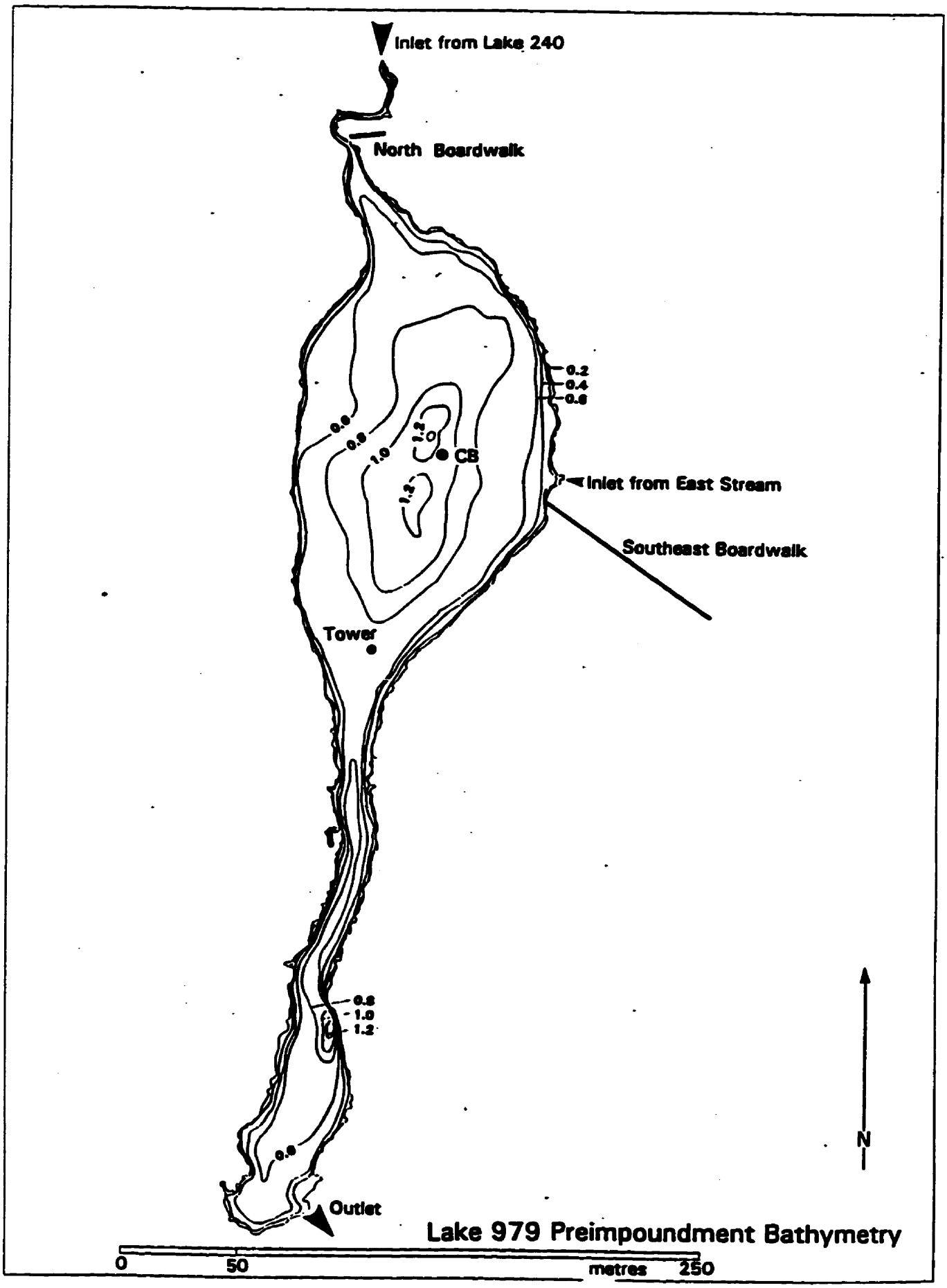


Figure 6 L979 Bathymetry Before Impoundment.

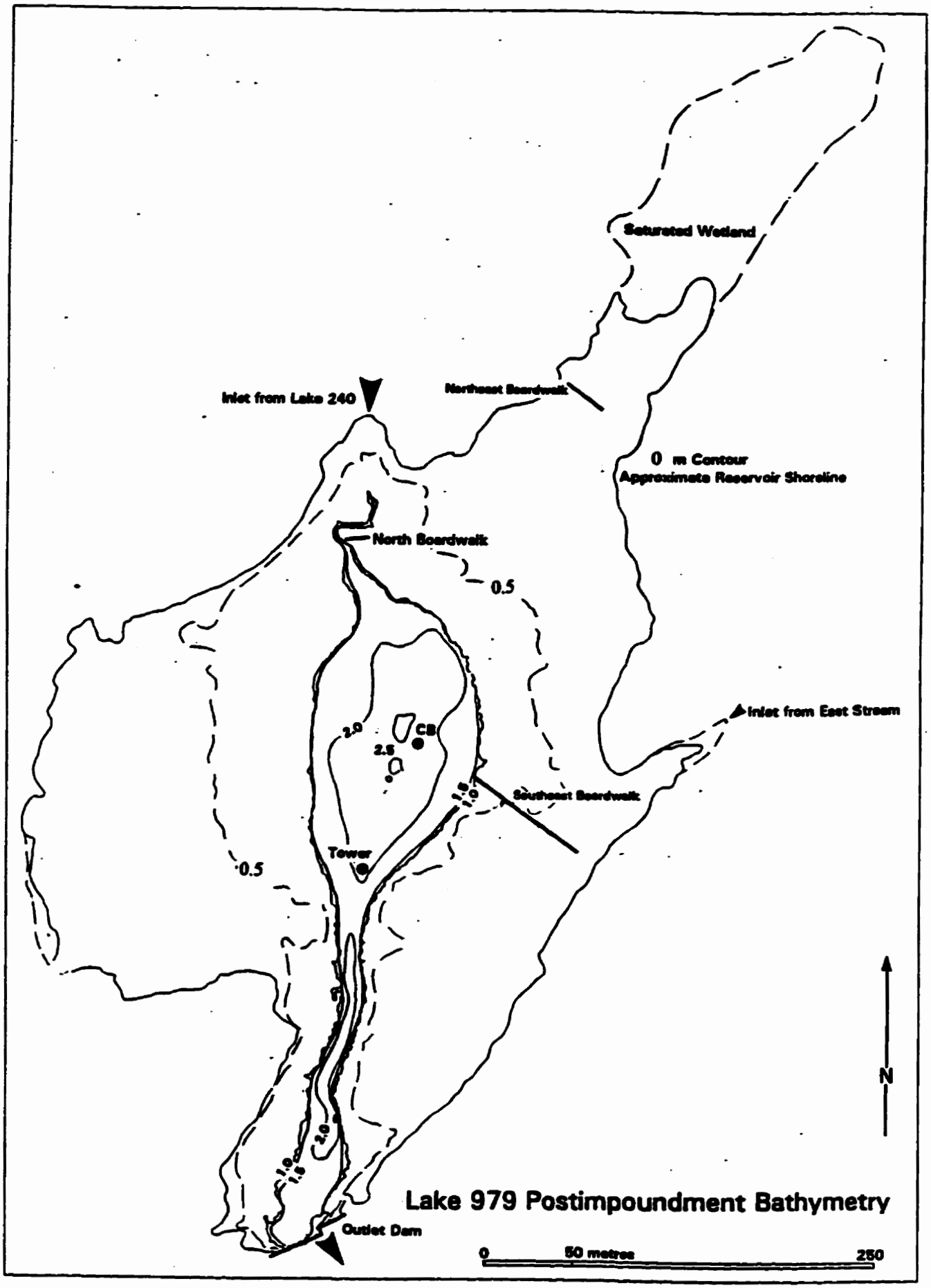


Figure 7 L979 Bathymetry After Impoundment.

2.2.2.2 Physical Measurements.

Temperature profiles were taken by temperature probe at the central buoy of each lake, located at the lake deepest point. Lakes were divided into strata based on temperature characteristics. The metalimnion is characterized by rapid decrease in temperature. The epilimnion is the water that lies above the metalimnion and the hypolimnion extends from the metalimnion to the bottom. Transparency of the water was measured by Secchi disk (a 30 cm metal plate circle, painted in black and white quadrants).

2.2.2.3 Collection of Microbial Samples.

Water to be assayed for bacteria and flagellates was sampled by means of a 4 L volume Van Dorn sampler at the central buoy of the lakes (Figs. 3 to 6). Samples, 20 mL volume for bacteria and 125 mL for flagellates, were preserved with undiluted, 0.2 µm filtered formaldehyde, 0.5% and 5% final concentrations, respectively. They were held at 4°C and processed within 4 days for bacteria and 1-2 days for flagellates. Water used in growth studies was passed through a 20 µm Nitex filter at the time of collection and stored at collection temperature until use in experiments.

2.2.2.4 Processing of Microbial Samples.

2.2.2.4.1 Epifluorescent Microscope.

For enumeration of bacteria and flagellates, an epifluorescent microscope (Carl Zeiss) equipped with 12.5x KPL eyepieces and Ph3 Neofluar 100x objective, was used. The Neofluar objective has a flat lens, necessary for good resolution of fluorescing organisms.

Bacteria, picoplankton and flagellates were processed separately in a dedicated specific filtration apparatus (Fig.8), to prevent cross contamination of dyes. Reagents and materials used are listed in Protocol 1. The support for filters was sintered glass or stainless steel as needed. If necessary, Nucleopore® filters were pre-stained in Irgalan black (2g L^{-1} in 2% acetic acid) for 2 to 24 hours. These filters were rinsed three times with distilled water, dried and stored at room temperature. Alternatively, pre-stained filters were obtained from Nucleopore® Inc. A 0.45 µm pore size polycarbonate Millipore® filter placed on the filtering tower and flushed with water was used as a backing filter to prevent the bacterial filter from coming into contact with the steel or glass screens and to distribute cells evenly. A Nucleopore® polycarbonate filter was placed on the Millipore filter, shiny side up. Filters and stains are described in Protocol 1. Detailed steps in slide preparation are shown in Protocols 2 and 3. Check slides to test for sterility of staining material were made with each preparation. Filtration was repeated as necessary.

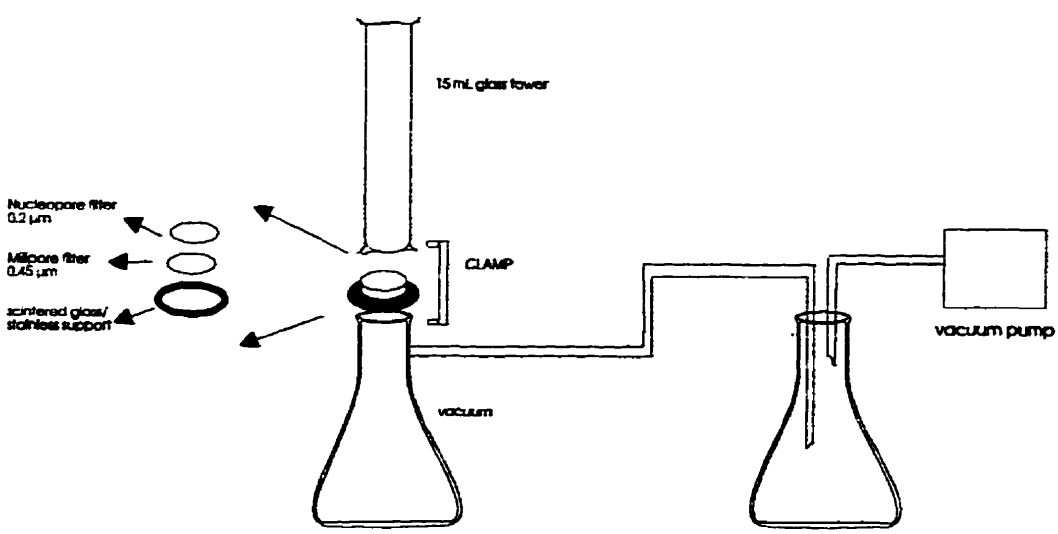


Figure 8 Filtration Apparatus for Filtration of Bacteria, Picoplankton and Flagellates.

Protocol 1 Filters and Stains Used in Bacterial, Picoplankton and Flagellate Slide Preparation.

Poretics® membrane filters: Poretic Corp., 25 mm diameter, pore sizes 0.2, 0.6, 0.8, 3 µm, stained with Irgalan Black.

Millipore® Filter: Millipore Filter Corp., pore size 0.45 µm, 25 mm diameter.

Formaldehyde: 37%, filtered through 0.2 µm sterile filters.

DAPI ($C_{16}H_{15}N_5 \cdot 2HCl$, 4',6-diamidino-2-phenylindole), Sigma Chem.Co.

stock #1: 5 mg, frozen resin

stock #2: weight 600 µg into 20 mL of distilled water to get 30 µg mL^{-1} final conc., filter through 0.2 µm sterile filter, distribute into 1.2 mL sterile plastic centrifuge tubes, (stored frozen indefinitely at -18°C).

final conc./staining: thaw stock #2 and use 100 µL (containing 30 µg DAPI ABS) into 3 mL of bacterial sample to get 1 µg mL^{-1} final conc.

Trizma - HCl: ($C_4H_{11}NO_3 \cdot HCl$, Tris[hydroxymethyl]aminomethane hydrochloride), Sigma Chem.Co., 0.1 M, pH 4. Stored at 4°C for several days. Filtered through 0.2 µm sterile filter.

Primulin: Direct Yellow 59, $C_{21}H_{14}N_3O_3S_3Na$, Sigma Chem.Co., 63 mg L^{-1} in 0.1 M trizma - HCl. Made up fresh with each use, kept at 4°C. Filtered through 0.2 µm sterile filter.

Irgalan black BGL, Pylam Products Company Inc.:2g L^{-1} in 2% acetic acid.

dH_2O: distilled water, 0.2 µm filtered.

Immersion oil: Zeiss West Company.

Protocol 2 Preparation and Viewing of Bacteria.

1. Place 3 mL of sample into a sterile vial, add 100 µl stock #2 DAPI, 1 µg mL^{-1} final conc., shake, place in dark for incubation period of 5 to 15 min.

2. Using vacuum pressure at water pressure of about 5 atm., filter sample onto Nucleopore filter, 0.2 µm pore size, treated with Irgalan Black, placed shiny side up on the filter apparatus. Wash the vial and walls with small amount of filtered (0.2 µm pore size filter) dH_2O and finish filtration. If the filtration takes longer than 15 min., prepare a new sample and start filtration immediately after adding the stain.

3. Sheet a thin layer immersion oil onto a microscope slide, mount the filter and place in the dark until the filter is transparent (about 2-3 min.).

4. Add drop of immersion oil onto the filter, cover with a coverslip and press firmly to get rid of air bubbles.

5. After each filtration wash the apparatus with 0.2 µm filtered dH_2O.

6. Bacteria slides were viewed using DAPI filter set 2; 420 nm barrier, 365 nm excitation filters.

7. Bacteria were counted at 1250x magnification using the 20 µm square grid (see below) in 32-40 fields, distributed in four rows.

Protocol 3 Preparation and Viewing of Flagellates.

1. To prevent flagellate damage use gentle vacuum (water pressure 1 atm.). Do not filter dry until the end of filtration.

2. Mount and wet 0.45 µm Millipore® filter ("backing filter" on the filtering tower. Place Nucleopore filter, 6 µm pore size on the Millipore filter, shiny side up. Filter required amount of sample to obtain reliable count of flagellates (1-10 per 64 µm square).

3. Rinse the filter with 2 mL aliquots of trizma-HCl, two times as the pump is running, leaving a small amount of the last rinse on the filter.

4. Stop the pump and flood the filter with 2 mL of primulin. Allow the stain to stand for 5 min. Restart the pump, but do not allow the filter to become dry. If the filtration takes longer than 15 min., repeat procedure, but start filtering immediately after addition of primulin.

5. Rinse the sample 4 times with 2 mL aliquots of trizma - HCl as the pump runs, allowing the filter to barely dry after the final rinse.

6. Allow the filter to air dry for about a minute, than mount on a slide sheeted with a thin layer of immersion oil. Place a drop of immersion oil on the coverslip, place it oil side down onto the filter, press slightly.

7. Flagellates were viewed using Carl Zeiss primulin filter set #7; 520 nm barrier and 436 nm excitation filters.

8. Flagellates were counted at 1250x magnification using a 63 µm grid square.

Steps in Preparation of Autotrophic Picoplankton Slides.

Autotrophic picoplankton were prepared as in Protocol 2 (bacteria) above except no stain was used. Samples were counted using the 64 µm field (Fig.9). Autotrophic cells were viewed using FITC filter set #9, which included 520 nm barrier and 450-490 nm excitation filters.

2.2.2.4.2 Calculations and Conversion Factors.

In order to obtain numbers of organisms per mL, each field grid and filtration tower was calibrated and assigned a conversion factor. Lengths of both small and large grids (Fig.9) in the micrometer eyepiece were measured by stage micrometer and the area was calculated as μm^2. The tower used for each dye was measured using an electronic caliper (N=10) and the average used for the area calculation (Protocol 4). This area was than divided by the area of the grid in order to obtain a conversion factor (Protocol 4). The conversion factor was used to obtain the number of cells per grid, and subsequently the number of cells per mL sample.

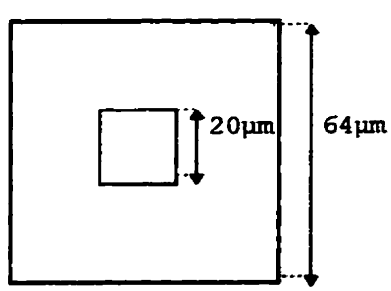


Figure 9 Eyepiece Grid for Cell Counts.

Protocol 4 Steps for Conversion Factor Calculation.

1. small grid area measurement:	20.5 μm	equals	420.25 [μm^2]
large grid area measurement:	64.0 μm		4096.00 [μm^2]
2. for DAPI filtration tower average diameter:			16511.67 [μm]
3. tower area calculation (Πr^2):	Π *(16511.67/2)^2		[μm^2]
4. conversion factor:	tower area/grid area		

Table 4 Conversion Factors for Cell Counts of Microbial Loop Organisms.

filtration tower	grid	d [μm]	conversion factor (tower/grid areas)
DAPI	small	16511.67	$5.095 \cdot 10^5$
(bacteria)	large		$5.228 * 10^4$
NO STAIN	small	16583.3	$5.139 * 10^5$
(picoplankton)	large		$5.273 * 10^4$
PRIMULIN	small	16548.33	$5.118 * 10^5$
(flagellates)	large		$5.251 \cdot 10^4$

2.2.2.4.3 Image Analyzer.

For measurements of bacterial volume, the epifluorescent microscope was inserted into an image analyzer system (Fig.10). The image analyzer for bacteria measurements consisted of a Carl Zeiss epifluorescent microscope, a low light level camera, monitor, computer and printer. The epifluorescent light from the sample was picked up by the low light level SIT TV camera and the image was transferred to the computer. The image was processed by the Bioscan Optimas® program using a specific calibration with configuration developed for measurement of bacteria. The image was brought up to the screen using 1250x magnification of the epifluorescent microscope and was further magnified 400x by the Optimas® program. The magnified image was calibrated to the microscope by stage micrometer with least dimensions of 10 µm. The precision of the system was 0.039 µm, which translated to about 3.8% and 1.3% of the measured length for small and large cells, respectively.

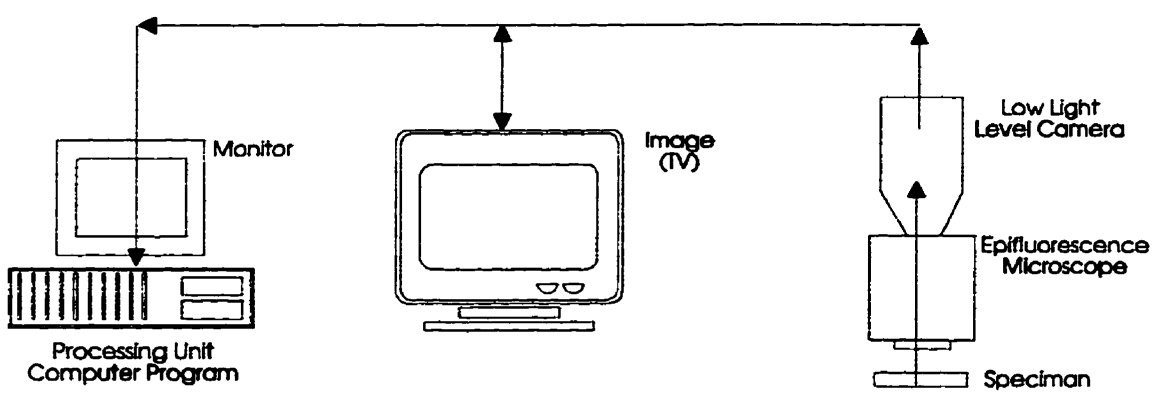


Figure 10 Image Analyzer System Used to Measure Microbial Loop Organisms.

2.2.2.4.4 Morphological Groups of Bacteria.

Bacteria were assigned to one of four morphological groups:

a) Coccus - small, slightly ellipsoid cells measuring 0.4 µm-0.6 µm
b) Rod - small cylinders, about 0.4 * 1.2 µm in length
c) Curved - curved, about 0.4-1.2 in diameter and 2.4 µm in length
d) Large - large cylindrical rods, about 0.6 * 3 µm

2.2.2.4.5 Bacterial Cell Volume.

Bacteria found in samples were coccoid, rod shaped or curved. I made an assumption about the cell shape and estimated the cell volume based on following equations. The smaller measurement [a] (width) was recalculated as area and the larger measurement [b] was used as length. Coccus bacteria were slightly elliptical in shape. Volumes of rod shaped bacteria were calculated as cylinders. Other types of bacteria such as vibrio or spirullum were not seen.

Calculation for volume of rod and ellipsoid bacteria (Fig.11):

$$\Pi * [a/2]^2 * b$$

width - a
length - b

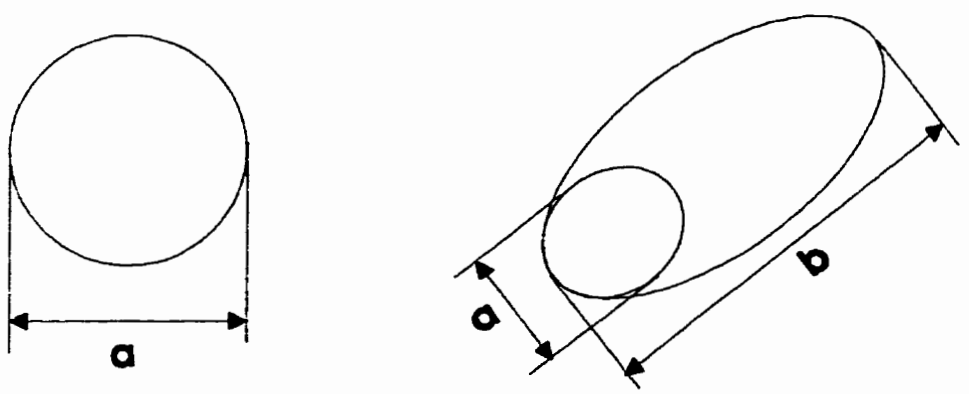


Figure 11 Volume of Ellipsoid Bacteria.

Calculation volume of curved bacteria (Fig. 12):

$$\Pi * [a/2]^2 * b * \Pi /2$$

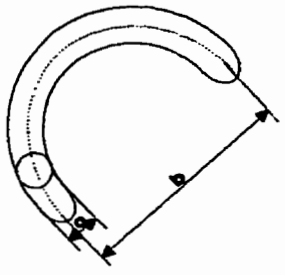


Figure 12 Volume of Curved Bacteria.

2.2.2.4.6 Morphological Groups of Flagellates.

Flagellates were assigned to four morphological groups according to size and color when stained.

a) Blue Small (BS) - small, blue cells with curved flagella, size 1-3 µm.
b) Blue Large (BL) - larger, blue cells of different morphology, size 5-10 µm.
c) Yellow Small (YS) - small cells, 4-5 µm.
d) Yellow Large (YL) - large cells, size 20-30 µm.

2.2.2.4.7 Bacterial Carbon Biomass and Production.

Cell volume of bacteria was converted into carbon biomass by using a conversion factor of 0.24 pg C μm^{-3} (Bratbak, 1985). This value

was chosen as the most conservative of the various values in the literature with correction for shrinkage. Calculations were done for each sampling day of the 1992 season in L373 and L375. Biomass estimates in L632 and L979 in 1993, where the cell volume was not measured, were calculated based on average cell volumes found in L373 and L375 bacteria.

2.2.2.4.8 Growth of Bacteria, Dilution Technique.

To get some insight in bacterial growth in these systems four dilution experiments were done using samples from L375. Samples were incubated for 6-22 h. at 20°C and specific growth rates assessed. Samples for growth assays were collected at the central buoy of L375 (Fig. 4) and water immediately filtered through the 20 µm filter to remove larger particles and plankton. The specific growth rates are calculated from the general exponential relation:

$$\ln(N_t/N_o) = rt \qquad \text{(Iturriaga and Mitchell, 1986)}$$

where N_t = abundance at time t; N_o = abundance at the time zero; r = specific rate coefficient; t = time. If the population is in steady state, the specific rate constants for growth, µ, grazing, g, and residual losses, l, satisfy the equation $r = \mu - g - l = 0$. Residual losses can represent losses otherwise not expressed in the equation, e.g., respiration and exudation or excretion by both prey and predator or grazers, which were not sampled. Each experiment consisted of mixtures in various proportions of the undiluted plankton sample and the diluent. The diluent was prepared by passing water through 0.2 µm Nucleopore® filters to remove particulate matter. Dilution was made from 100% to 20% of the original sample. The bacteria were allowed to grow for up to 22 hours at incubation temperature of 20°C. Subsamples were taken periodically and filtered onto Nucleopore® filters for abundance estimates.

2.3 Results and Discussion.

2.3.1 Water Parameters.

Transparency is a measure of the depth to which light penetrates in the water column. Penetration depends on the amount of particulate matter present and on water color. Secchi depth ranged from 2.5 to 5.5 m in L375 and from 4 to 8 m in L 373. Lake 375 was most transparent in May, while in L373, the highest Secchi depth, 8 m, was recorded in July. The most rapid decrease in Secchi depth occurred during August-September in both lakes. Secchi depth declined by 2 m in L375, but by 1 m in L373 (Fig.13). In comparison, values for Lake Constance, a deep cold lake, changed two to three meters within two days in May 1989. Transparency values in Lake Constance declined rapidly within one or two days with rapid increase of particulate matter content (Simon, 1994). Lake Constance is a very productive lake; low productivity is a feature of both lake 373 and 375.

The transparencies were consistently about 0.5 m in lakes 979 and 632, much lower than the much deeper lakes 373 and 375. This was the result of high humic coloration in these small peat ponds, specifically after the flooding of L979.

Seasonal changes in temperature profiles of lakes L373 and L375 follow the temperature/water density relationship. The highest temperature occurred during summer stratification near the surface and the lowest temperature near the bottom (Fig.14) of these temperate oligotrophic lakes. Temperatures ranged from 9 to 20°C in the epilimnion during May to October 1992 (Fig.14A, B). The metalimnion, the transition layer, showed rapid decrease of temperature from 16 to 12°C. Temperature in hypolimnion was uniform around 5°C from the metalimnion to the bottom of the lakes during sampling period from May to August 1992 (Fig. 14A, B). Similar relationship occurs in other lakes, e.g., in Lake Vechten (Bloem and Bar-Gilinssen, 1989).

Lakes 632 and 979 are shallow ponds and the entire water column varies slightly in temperature. Values measured at the peat surface ranged from 17°C in pre-flooded lake to 19°C in post-flooded lake during summer (Kelly et al., 1997).

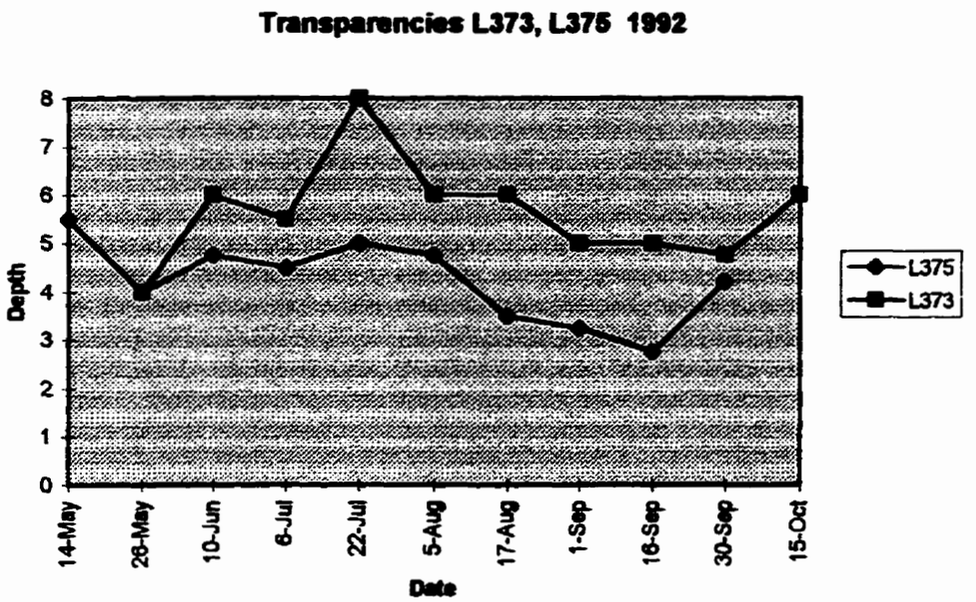
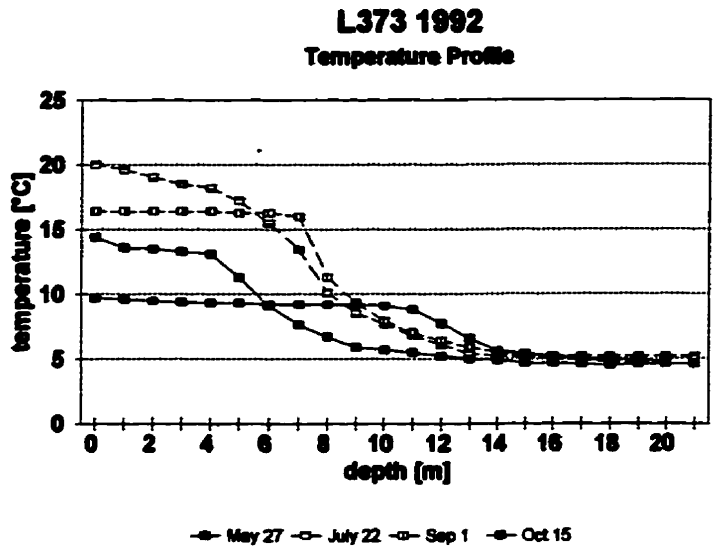


Figure 13 Transparencies L373 and L375, 1992 (Secchi disk depth).

a/



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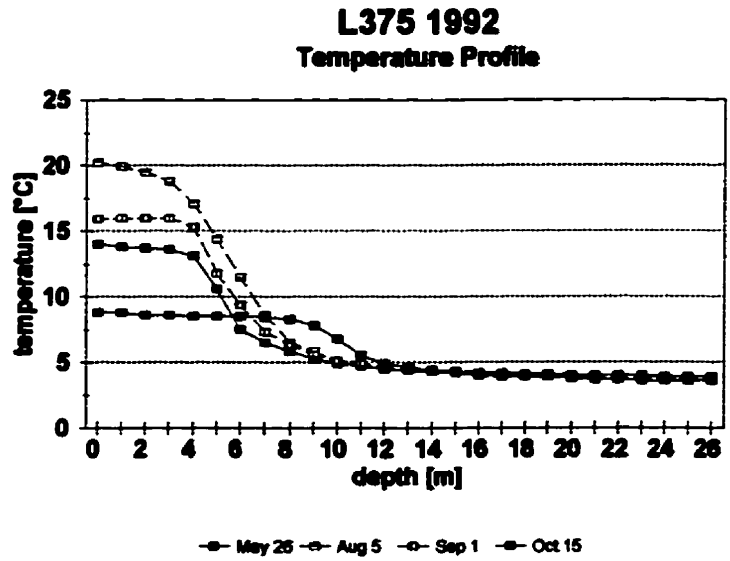


Fig.14 Temperature Profiles of Lakes 373 and 375, 1992.
a/ L373 , b/ L375

2.3.2 Autotrophic and Heterotrophic Bacteria.

2.3.2.1 Microscopy.

With proper use of filter sets and dyes, I was able to distinguish blue fluorescence in stained bacteria, yellow and blue fluorescence in protozoa, the orange autofluorescence of picoplankton and red fluorescence of algae. Blue material, which did not resemble bacteria either in shape or in size, probably detritus, occurred sporadically.

The estimation of growth by incubation in the laboratory actually changed the morphology in the bacterial assemblage. During experiments I encountered small, blue stained particles, viral or bacterial in nature. These cells, about 0.08 µm in diameter, were present in clouds of thousands, impossible to count. The use of temperature or ultrasound which can destroy all unwanted cells in the diluent may be warranted in future attempts to assess bacterial growth rates using this technique.

Unusual yellow autofluorescent particles encountered were round with a strong metallic yellow glare. They accounted for less than 1% of the bacteria present. These particles neither resembled bacteria by size or shape, nor increased in numbers, so were ignored as non-bacterial in nature. Caron et al. (1986) using a 450-490 µm excitation filter and 520 µm barrier filter enumerated glaring particles as cyanobacteria. They describe cells ranging in size from 0.7 to 1.3 µm in diameter, present in clumps or in tetrads and yellow in autofluorescence as indicative of the phycobiliprotein phycoerythrin.

2.3.2.2 Abundance of Bacteria.

Lakes 373, 375.

Bacterial and picoplankton numbers in the three thermal layers of L373 and L375 from May to October, 1992, are shown in Fig.15. Total abundance of bacteria varied from 0.5 to 3.5 * 10^6 mL^{-1}, similar to other oligotrophic environments (0.67-1.04 * 10^6 mL^{-1}, Scavia and Laird,

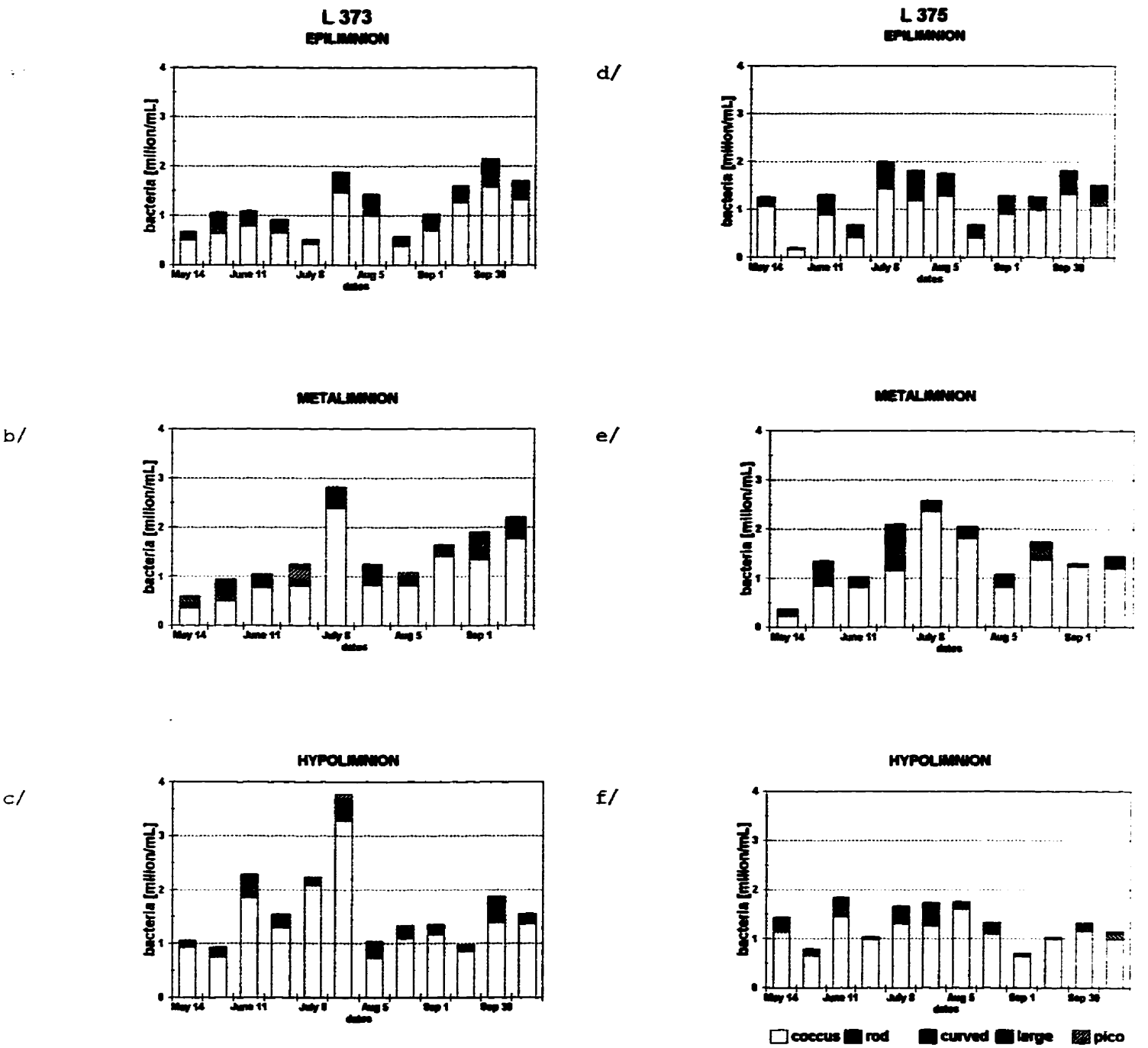


Fig. 15 Seasonal Bacterial Counts in Lakes 373 and 375, 1992.

1987). In both lakes, small coccoid bacteria were always in highest abundance. The second largest group was small rods.

Cyanobacteria occurred sporadically in all three layers of both lakes. Higher numbers of cyanobacteria occurred in the metalimnion of L373 than in L375. Munawar et al (1994) identified and enumerated the microbial food web organisms as bacteria, flagellates and picoplankton during microbial research in the Great Lakes. They found the highest bacterial numbers in contaminated areas and the lowest picoplankton counts in clean oligotrophic systems. They suggest that the abundance of picoplankton indicates good water quality. Picoplankton occurred in oligotrophic L373 and L375 (Fig.15) in numbers which point to their oligotrophic state and general good health.

Lakes 632, 979.

Fig.16 shows the seasonal bacterial abundance in L632 and L979 during the ice free season of 1993. The bacterial abundance of L632 and L979 show trends similar to those L375 and 373. The coccoid bacteria are in highest abundance, and total abundance is from 0.5 to 3.2 • 10^6 cells mL^{-1}. Before flooding L979 contained more (1.2-3.1 * 10^6 mL^{-1})of bacteria when compared with L362, which had much lower numbers (0.4- 1.4 • 10^6 mL^{-1}). During and after flooding of L979, (initiated June 23, 1993, fullest extent July 3, 1993), the abundance of bacteria in L979 doubled to 2-3 * 10^6 mL^{-1}. The counts of curved bacteria increased 600% and small rods 50%. The percent of dividing bacteria increased only slightly; values are not shown. Picoplankton did not occur in these small ponds.

2.3.2.3 Biomass and Carbon Content Estimate in the Concentrated Fraction (0.1-3 µm).

Lakes 375, 373.

The deep lakes L373 and L375 were confirmed oligotrophic at 0.015-0.052 g C m^{-3}. Bacterial carbon was the highest during the summer

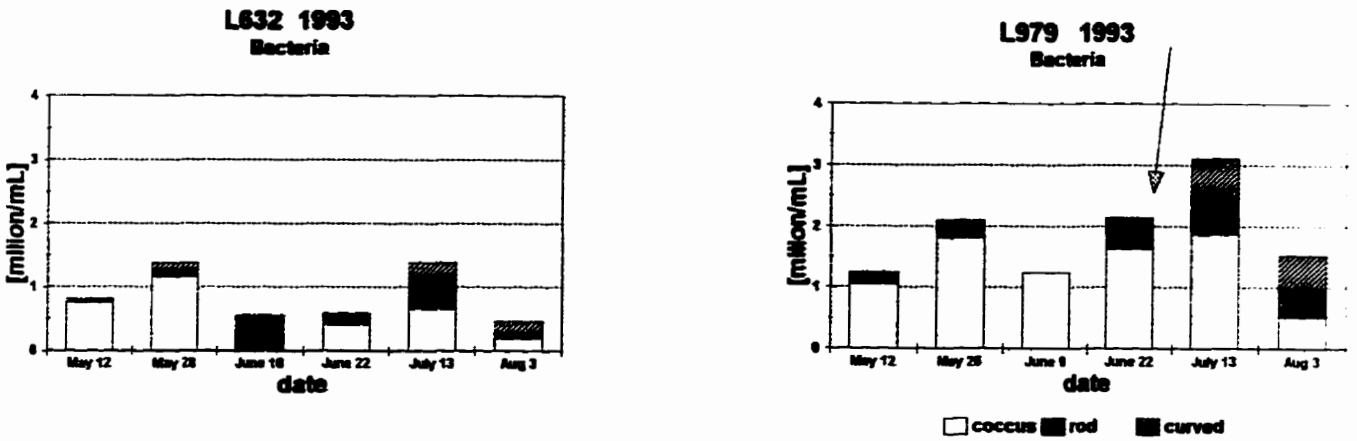


Fig. 16 Seasonal Bacterial Counts L632 and L979, 1993.
The arrow indicates initiation of flooding on June 23, 1993.

months, with the exception of a bloom in early spring in lake 373. The highest biomass in lake 373 occurred in the metalimnion (Fig.17b). The amount of bacterial carbon in the epilimnion of L373 was below 0.05 g C m^{-3} with only one exception, July 22 (Fig.17,a). The hypolimnion values ranged from 0.025 to 0.075 g C m^{-3} (Fig.17,c). The amount of bacterial carbon in the epilimnion of L375 was again below or around 0.05 g C m^{-3} with the exception of two dates, on July 22 and August 8 when carbon was found below 0.1 g C m^{-3} (Fig.17,d). The values in metalimnion varied from 0.01 to 0.075 g C m^{-3} (Fig.17,e). The values in hypolimnion of L375 were below 0.05 g C m^{-3} except on July 8, 1992, when bacterial biomass reached the highest level of 0.13 g C m^{-3} during spring and summer months in coccoid bacteria Fig.17,f). The largest biomass occurred in coccoid bacteria during spring and summer months in both lakes.

Lakes 632, 979.

Patterns similar to those in Lakes 373 and 375 can be seen in the small ponds where bacterial carbon concentrations were also below or around 0.05 g C m^{-3} (Fig.18a, b). The values estimated in present study ranged from 0.02 to 0.124 g C m^{-3}. After full flooding of L979 on July 13, 1993, the amount of carbon in the bacterial fraction rose to 0.124 g C m^{-3} (Fig.18b) indicating a significant increase in bacterial biomass.

Cho and Azam (1990) estimated particulate organic carbon to be up to 0.2 g C m^{-3} in the Pacific ocean. They found bacterial carbon to be 0.006 g C m^{-3}, based on bacterial volumes in the range 0.3-0.6 μm^3 and using conversion factor of 0.2 * 10^{-13} g C $cell^{-1}$. With this assumption they could underestimate bacterial carbon easily by 150%. Bjornsen and Kuparinen (1991) estimated an average value of 0.38 g C m^{-3} for bacteria in the northeast Atlantic ocean. In Lake Constance, bacterial carbon content averaged 0.0065-0.158 g C m^{-3} (Simon 1988). The amount of organic matter in L979 must have increased substantially after impoundment as the amount of bacterial carbon increased 2x from 0.065 before to 0.124 g C m^{-3} after impoundment. The values before impoundment are comparable to marine oligotrophic waters (0.006, after flooding (July 8 value of 0.124 g C m-3) L979 value was close to values

found in L. Constance, but still less than that reported for eutrophic waters (Bratbak, 1985, Simon and Azam, 1989).

Carbon Content Estimate in Four Lakes.

Since the ponds examined are small and thermally mixed, the carbon content of these water bodies (L632 and L979) can be most realistically compared to the epilimnion of the two thermally stratified lakes (L375 and L373). The amounts of bacterial carbon per m^3 in all lakes range from 0.014 to 0.124 g C m^{-3} (Table 5, Fig.17-18). A considerable increase of bacterial carbon production occurred immediately after flooding of L979 was initiated. The average values for summer (May-July) sampling period are considerably different between L979 and reference L632. The average bacterial carbon per m^3 is 2x higher (0.061 g C m^{-3}) in inundated L979 than in the reference (0.038 g C m^{-3}) L632 (Table 5). Two oligotrophic lakes L373 and L375 had average bacterial carbon content of 0.024 and 0.035 g C m^{-3}, respectively, comparable to L632 (Table 5). Summer is apparently the time when all four lakes reach peaks in particulate carbon: 0.066, 0.095 0.066, 0.124 g C m^{-3} for L373, L375, L632, and L979, respectively (Table 5).

The amount of bacterial carbon of 35 mg C m^{-3} should be included in POC of 250-800 mg C m^{-3} (Tab.3). The cut of for POC is 1.2 µm, so only smaller portion of bacteria retain on the filter. Even with differences in methodology one can speculate that about 10-40% of POC consists of bacteria. Because of the bacterial size, much larger portion is filtered through the filter to the DOC portion. The phytoplankton biomass in the size fraction 0-3 µm (D.Findlay, FWI, personal com.) was up to 5% of the total phytoplankton biomass. The algae in this fraction were small Chroococcoid algae (Chlorella sp.) and flagellates (<1%, Chrysophyceae) (D.Findlay, FWI, personal com.). Bacterial carbon in 0.1-3 µm fraction was major contributor to the material in this fraction (Table 6). The comparison of bacterial carbon to the total phytoplankton carbon also indicated bacterial population as major compartment of carbon in these lakes. The values range from 25 to 1800% of total phytoplankton (Table 6). It is obvious that even combined phytoplankton and bacterial biomass is only a fraction of particulate organic carbon. This POC also include zooplankton, ciliate, rotifers, crustaceans and other living creatures as well as detrital material.

Table 5 Bacterial Carbon per m^3 of Epilimnion of Studied Lakes.

DATE(1992)	L373	L375	DATE(1993)	L632	L979
May 14	0.015	0.032	May 12	0.02	0.035
May 27	0.027	0.014	May 28	0.04	0.056
June 11	0.021	0.016	June 10	0.044	0.027
June 24	0.028	0.059	June 22	0.021	0.065
AVG*	0.023	0.030		0.031	0.046
July 8	0.031	0.052	July 13	0.066	0.124
July 22	0.066	0.095			
Aug 5	0.047	0.093	Aug 3	0.023	0.078
Aug 17	0.01	0.026			
Sep 1	0.025	0.026			
Sep 16	0.03	0.007			
Sep 30	0.036	0.035			
Oct 16	0.021	0.018			

Values in g C m^{-3}. Average values are for dates which preceded flooding in L979.

Table 6 Comparison of Bacterial and Phytoplankton Carbon in the Size Fractions 0-3 and 0.1-3 µm, respectively, L979.

L979	A	B	C	C/A	D	C/D
DATE	PP carbon 0-3 µm	DATE	bacteria carbon 0.1-3 µm	B/PP	PP carbon total	B/TPP
12-May	0.125	12-May	35	280	103	0.34
		26-May	56			
9-June	5.707	9-June	27	4.7	108	0.25
		22-June	65		21.7	3
7-July	0.299	13-July	124	415	6.9	18
11-Aug	0.430	3-Aug	78	181	10.4	7.5

PP: phytoplankton, B/PP ratio: bacterial / phytoplankton (0-3 µm fraction) carbon ratio. B/TPP: bacterial / total phytoplankton carbon ratio. Units in mg C m^{-3}. Phytoplankton data supplied by Dave Findlay, FWI. Data in mg m^{-3}.

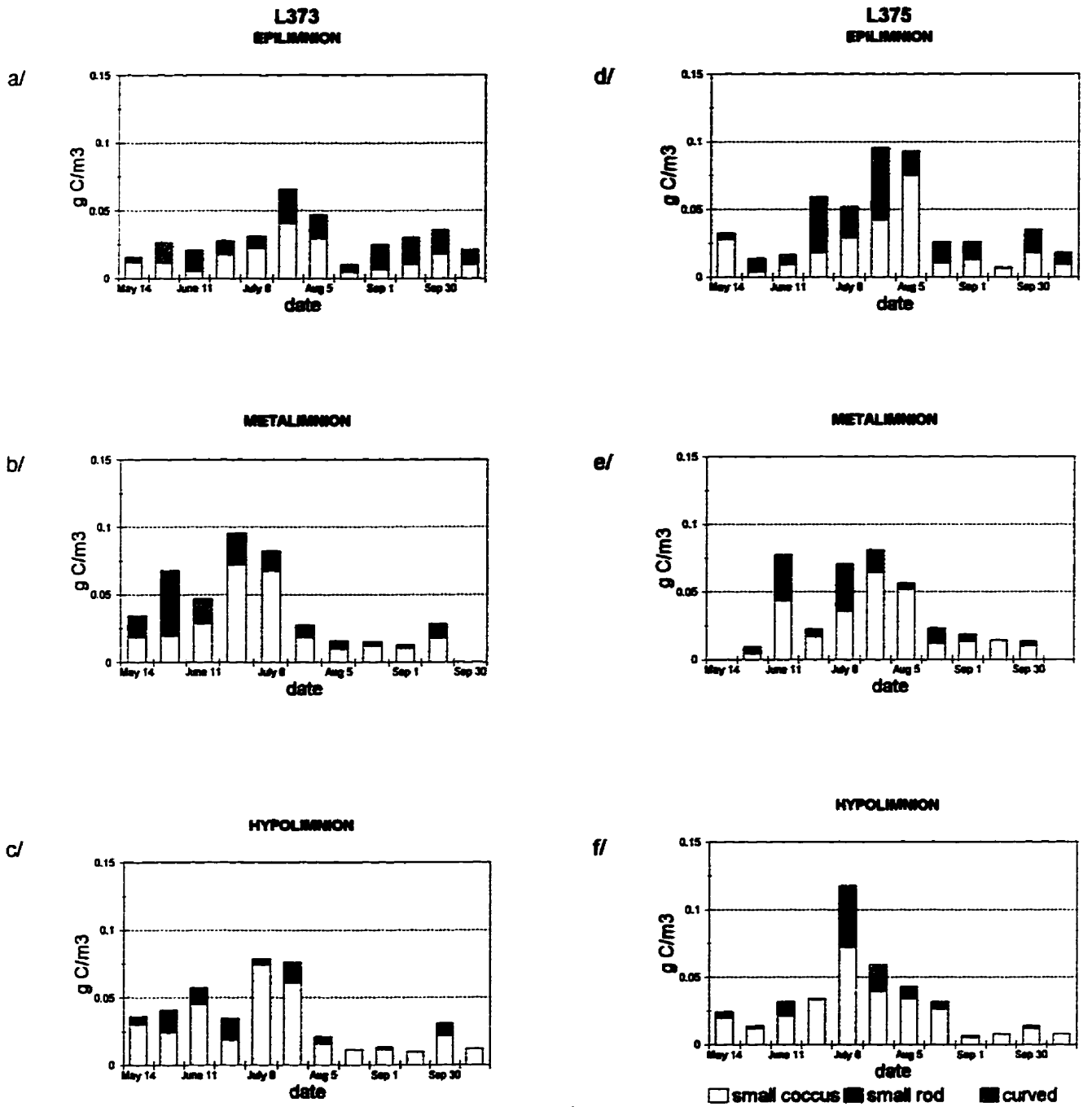


Fig. 17 Bacterial Carbon Content in Three Morphological Types in Lakes 375 and 373.

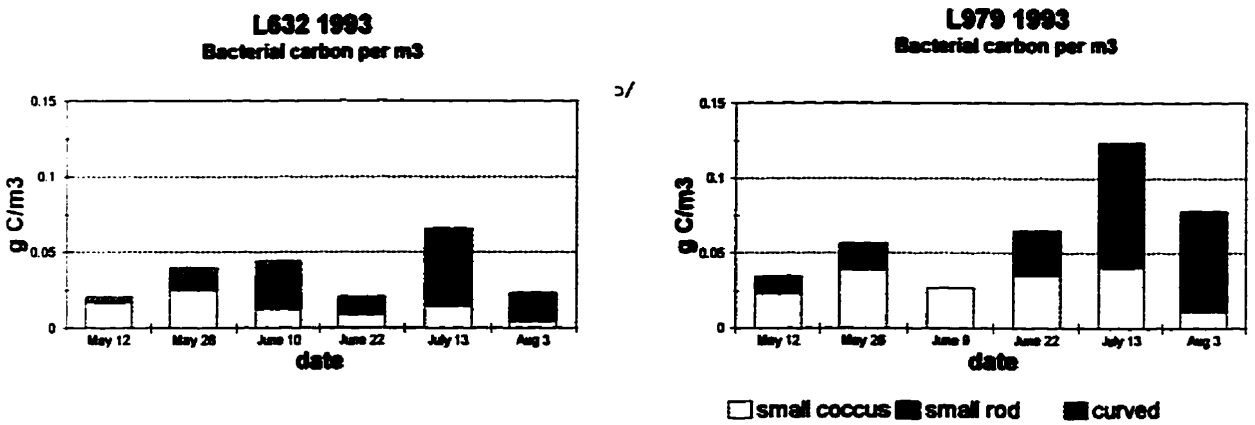
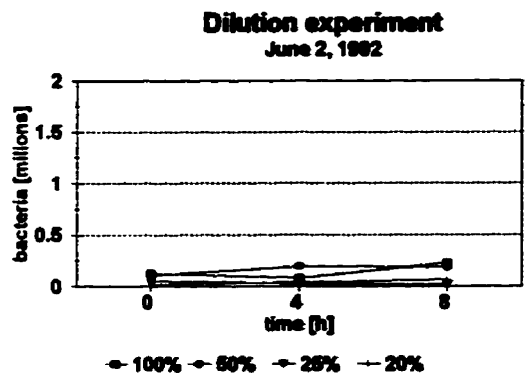


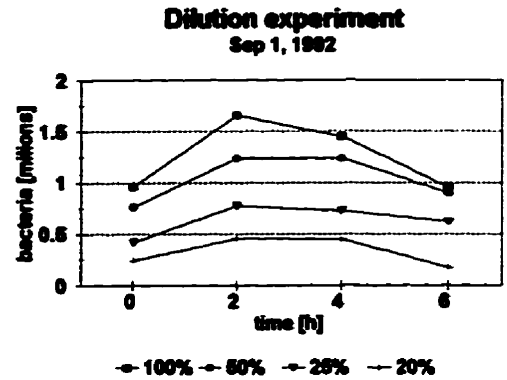
Fig. 18 Bacterial Carbon per m3 L632 and L979, 1993.

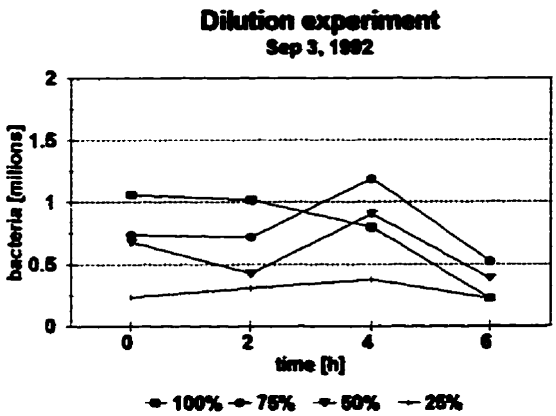
2.3.2.4 Bacterial Growth.

Bacterial growth in nature can fluctuate, e.g., intensive growth may be followed by long stationary periods. Bacterial growth did not occur during dilution experiments. The laboratory conditions and the possibility that substrates are added to the water from disrupted or damaged plankton organisms during filtration should have enhanced growth and changed the qualitative composition of the bacterial assemblage. No nutrients were added in these experiments so it is possible that bacteria used only the substrates available and than decreased in number. Another possibility exists: that actual doubling times of bacteria these oligotrophic lakes were much longer than the length of the longest running experiment (22 hours). Güde (1990) found that the mean bacteria doubling time in Lake Constance populations exceeded 10 days during the warm season and Cho and Azam (1990) report similar values in the euphotic zone of Pacific ocean. Water for my experiment was filtered through 54 and 2 µm filters in order to remove larger organisms, including zooplankton, ciliates and flagellates. These procedures may not be entirely successful as some protozoa can squeeze through 1 µm filters (Anderson and Fenchel, 1985; Cynar, et al., 1985). It is then possible that bacteria were grazed upon by flagellates and ciliates. To see coupled oscillations, experiments would have to be carried out for at least 100 h (Anderson and Fenchel, 1985).

The dilution experiments are shown in Fig.19. Bacteria in L375 were not fast growing and turn over slowly. First the bacterial abundance decreased, with one exception (Fig.19b), then showed an increase and then decrease in numbers. The overall specific growth rates showed little oscillation, staying at 0 or below zero at the end of the experiments, i.e., the bacterial populations were at steady state. The maximum specific growth rates were 0.09 h^{-1} and 0.042 h^{-1} day^{-1} for experiments B at 2h and D at 12h, respectively. The values were similar as found by Tranvik (1989) in oligotrophic lakes and Iturriaga and Mitchell, 1986. An indirect measure of the mean growth rate may be measured by the frequency of dividing cells (FDC, Hagstrom, 1970) within a specific sample. The relationship of the growth rate and







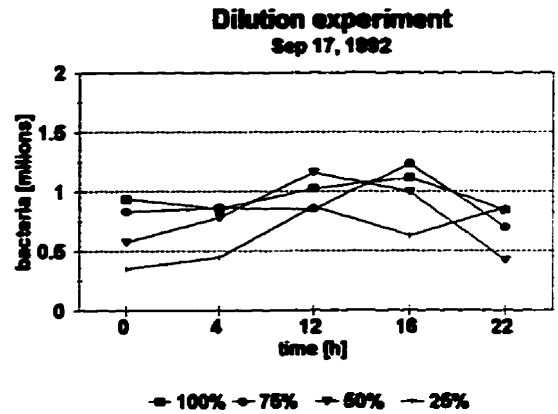


Fig. 19 Dilution Experiments, L375, 1992.

FDC of the natural population of bacteria in a particular environment is first established. As different temperatures do not significantly affect FDC, the growth rate can be estimated from the relationship at any time. The numbers of dividing cells ranged from 0 to 50,000 and 60,000 mL^{-1} in L373 and L375, respectively, always below 6% of the total number of bacteria.

DOC concentration in L979 ranged from 500 to 1200 µmol before flooding and were slightly higher, from 700 to 1250 µmol after flooding (Paterson et al., 1997). The DOC concentration was sampling site dependent; values up to 2000 µmol were obtained close to the shore (Paterson et al., 1997).

The concentration of DOC in deep oligotrophic lakes is extremely low (e.g., ELA lakes 239 and 302N have about 500-580 µmol, while bogs can have more than 2000 µmol Miskimmin et al, 1992). The DOC concentrations in L375 and L373 ranged about 380-560 µmol during 1993 (Table 5). Lack of suitable DOC substrate was probably the main reason for slow growth and slow turn over during experiments on L975 water samples. Both DOC and POC values have to be viewed with caution because the analytical method defines DOC as material passing through a GF/C filter (pore size 1.2 µm). This means a majority of unattached bacteria are passing through the filter into the filtrate which is defined as containing dissolved material (S.G. Lawrence, FWI, personal com.). This causes DOC values to be elevated and POC values to be depressed. The error with respect to DOC is small, but the error in POC tends to be large (see section 2.3.2.3.).

2.3.3 Flagellated Protozoa.

Lakes 373, L375.

Flagellates are important part of the microbial food web, but because they are difficult to study, they were usually ignored (Fenchel, 1986) or included as a part of algal biomass (e.g. Findlay et al., 1994, Paterson et al, 1997). Here, I present first insight into flagellate assemblage at ELA. Generally, numbers of flagellated protozoa in L373 and L375 were below 10^4 mL^{-1} with few higher values up to 4.5 * 10^4 mL^{-1} during the fall in L373 and hypolimnion in L375

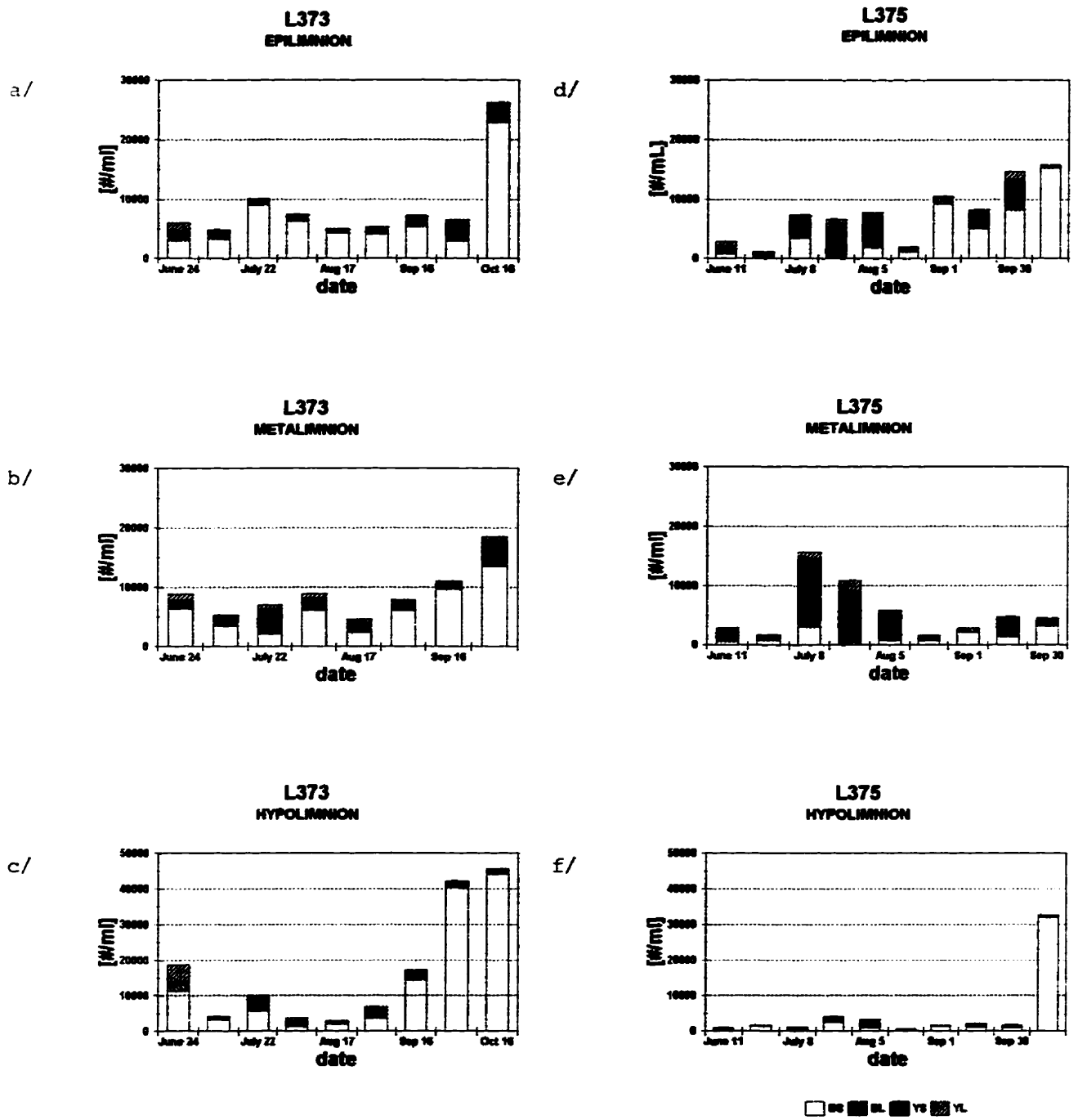


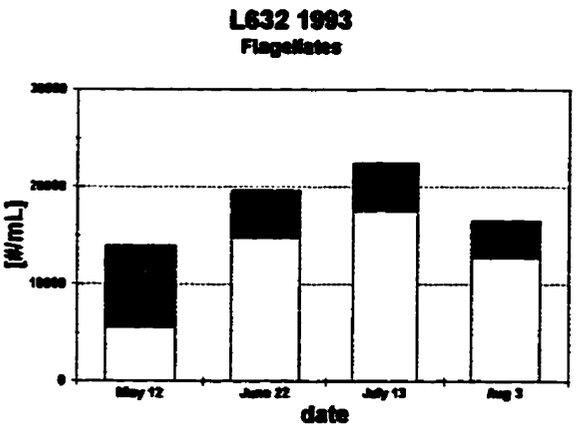
Fig.20 Seasonal Distribution of Flagellates in L373 and L375, 1992.
BS: blue small, BL: blue large, YS: yellow small, YL: yellow large

(Fig.20). These results are in agreement with published data for heterotrophic flagellates (HF). For example, Fenchel (1982), Sorokin (1979), and Davis and Sieburth (1982) report 200-3,000, 700-9,500 and 900-37,000 HF mL^{-1} for marine waters, respectively. Small blue flagellates were prominent in all three layers in L373 (Fig.20a, b, c). Flagellates were diverse in this lake and included from 50 to 90% of small blue, up to 30% of large blue, up to 30% of small yellow, and up to 20% of large yellow flagellates (Fig.20a, b, c). Large increases, up to $4.5 * 10^4$ mL^{-1}, of flagellates occurred in all three layers during the fall (October 16, 1992) (Fig.20a, b, c). The small yellow flagellates were prominent in L375 in both the epilimnion and metalimnion (Fig.20d, e). They constituted up to 70-80% of all flagellates on some dates. The numbers of flagellates in the hypolimnion of L375 were very low, except for one date in late fall (Fig.20f). The hypolimnion of L373 contained higher numbers of flagellates than L375. Small flagellates constituted about 1% of total algal biomass. The majority of large and colony forming flagellates were in the 3-20 and >20 µm fractions as discussed above.

L632 and L979.

In lakes 632 and 979 the abundance of flagellates was about $1.5-2.0 * 10^4$ organisms mL^{-1} (Fig.21b, d). The abundance was higher in small ponds than in the epilimnion of oligotrophic lakes, although the species diversity was lower. Small and large yellow stained flagellates occurred in all three layers in the oligotrophic lakes, but small yellow flagellates occurred only in L632 (Fig.21a). Large yellow flagellates did not occur in these ponds at all (Fig.21a, b).

a/



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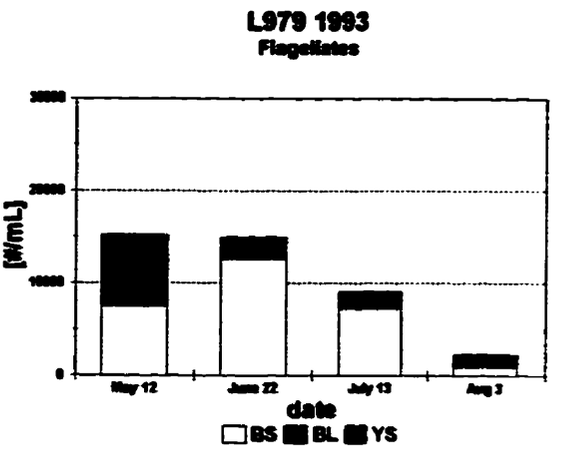


Fig. 21 Seasonal Flagellates Counts L632 and L979, 1993.
BS: blue small, BL: blue latge, YS: yellow small

2.4 Conclusions.

Abundance and biomass of bacteria and flagellates were estimated in two lakes 373 and 375 and two ponds 632 and 979, all located in the Experimental Lakes Area of Northwestern Ontario. The abundance of bacteria was similar in the two deep lakes and two shallow ponds, comparable to values found in the literature. The assessment of morphological types of bacteria revealed that coccoid bacteria were present in the highest proportion in all three layers of both lakes and ponds. However, when rod and curved bacteria were present, the amount of carbon in these types of bacteria contributed significantly to bacterial carbon, so that it was important to count curved and rod bacteria separately. Image analysis was chosen in estimation of volumes of the different types of bacteria.

Abundances of bacteria in lakes 373 and 375 ranged between 0.3-3.7 million mL^{-1}. The abundance of bacteria in L979 was higher (three times above 2 million mL^{-1}) than in L632 (always below 1.5 million mL^{-1}). The highest abundance of bacteria in L979 (above 3 million mL^{-1}) occurred after impoundment (July 13, 1993), indicating that flooding resulted in higher bacterial numbers.

The abundance of flagellates found in the two oligotrophic lakes was also similar to values found in literature. However, the species diversity was different in that the majority of flagellates in L373 consisted of small blue flagellates, while L375 had more small yellow flagellates. The types of flagellates in the small ponds differed from these in oligotrophic lakes. Small yellow flagellates were abundant only on one occasion, while big yellow flagellates did not occur at all. More study into species diversity and its implication within the microbial food web is needed. Ranges of bacterial carbon estimates in lake 373 and lake 375 were 0.02-0.09 and 0.01-0.12 g C m^{-3}, respectively, similar to values found in marine waters. The bacterial carbon estimates in lake 632 and lake 979 of 0.014-0.059 and 0.02-0.124 g C m^{-3} (the highest value after impoundment), respectively, are within the range found for oligotrophic waters (Cho and Azam, 1990). However, much higher values have been estimated elsewhere (Bjornsen and Kuparinen, 1984, Scavia and Laird, 1987). The major discrepancy between data is related to conversion factors, which must be applied to convert volume of bacteria into bacterial carbon. There is a need to evaluate

conversion factor for different types of bacteria in distinct ecosystems, and furthermore, to evaluate the analytical method for carbon estimation.

After impoundment of L979, higher bacterial carbon values suggested that organic carbon released from submersed vegetation supported the microbial population. However, the higher bacterial abundance was not followed by increased flagellate abundance, indicating that bacterial biomass was not controlled by flagellates at this time. Flagellates could not keep up with the bacterial population, which at this time reached over 3 million mL^{-1}, or other unknown factors contributed to low flagellate abundance.

Comparison to bacterial and phytoplankton biomass in the 0.1-3 μm fraction showed that bacterial biomass was far higher than algal biomass in this fraction. After the flooding, even when total phytoplankton carbon is considered, bacterial carbon was on average 13 times higher than phytoplankton carbon. Bacteria constitute a significant biological compartment in the freshwater trophic structure and must be therefore considered as important players in freshwater ecology.

3. Mercury: Chemistry, Analyses, Distribution and Toxicological Impact on the Aquatic System.

3.1 Literature Review.

3.1.1 Mercury Chemistry.

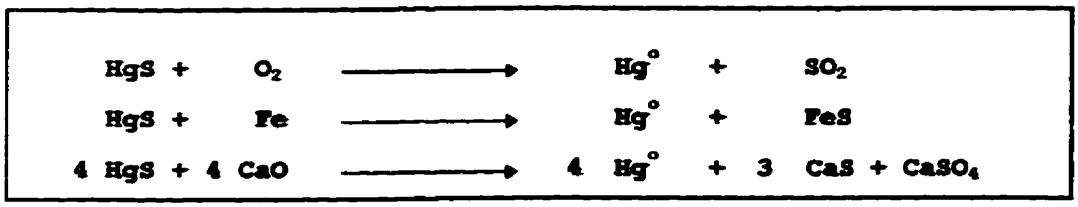
Mercury occurs mainly as HgS in nature, with traces of the pure element (Remy, 1972). Important ore bodies are found in the Almaden of Spain, Nikitov in Russia, the western U.S.A. and Mexico. Mercury in pure form was known and used in Greece and Rome. First known reports are from 300 BC. Ancient Rome exploited mines in the Almaden which are still being worked. Mercury was used as early as 600 AD to separate gold from its ore (Remy, 1972). The healing characteristics of Hg are known from antiquity, but wide spread use in medicine was prevented until recently because in very low concentration, Hg is extremely toxic to humans.

The basic chemical and physical characteristics of Hg are shown in Table 7. Mercury can be prepared by heating HgS in air (oxidation) or with iron and calcium as a catalyst (Protocol 5). As a metal, Hg is extensively used in technical fields, e.g., in thermometers, in production of ammunition and in dermatological ointments.

Table 7 Chemical and Physical Characteristics of Hg.

state at 20°C	**silvery, shiny liquid**
atomic number	**80**
molecular weight	**200.59**
boiling point	**356.95°C**
temperature at solidification	**-38.84°C**

Protocol 5 Industrial Preparation of Hg under Heat.



3.1.2 Distribution and Speciation of Hg in the Environment.

Mercury occurs naturally in the air, in the earth's crust, in water systems and in biota (Table 8). Bloom and Fitzgerald (1988) and Weber (1993), who developed methods for analyzing ng L^{-1} and pg L^{-1} concentrations of Hg in the environment, have been able to identify levels of contamination resulting from industrial activity. Concentrations of Hg found above natural (pristine) levels are considered to be anthropogenic pollution.

The primary form (>95%) of Hg in the atmosphere is elemental Hg (Hg°), with the reminder occurring as monomethyl Hg (MeHg). Total Hg in the air ranges from 1.43 to 5.52 pg L^{-1} (Bloom and Fitzgerald, 1988).

In oceanic waters, concentrations are typically from 2 to 100 pg L^{-1} (Weber, 1993). Values in freshwater range from 0.15 to 10 ng L^{-1} (Watras et al., 1995; Fitzgerald and Watras, 1989; J.Rudd, Freshwater Institute, personal comm.; this study).

The amounts of Hg in pristine soils are low, reaching 39 ng Hg g^{-1} of geologically defined upper layers of organic soil (Mills and Zwarich, 1975). The background measurements for cultivated and grasslands range from 29 to 39 ng Hg g^{-1} of soil (Mills and Zwarich, 1975). The occurrence of Hg in the sediment of lakes and rivers, has important implications for bioaccumulation of Hg into aquatic biota. Values for coastal waters, notably near the mouths of rivers receiving industrial waste, are high. For example, concentrations of Hg in United Kingdom estuarine water and sediments which are polluted can be up to 185 ng g^{-1} and 600 ng g^{-1}, respectively (Bryan and Langston, 1992). Mercury concentrations in surficial sediments in Ontario and Quebec lakes range from 3-267 ng g^{-1} dry sediment (Tremblay et al., 1995). Of

the organo-mercuric compounds, methylmercury [CH_3Hg^+, MeHg] is present in water in the highest concentration.

Mercury concentration in aquatic biota varies depending on differences in the lengths of pelagic food chains (Cabana et al., 1994). Concentration in plankton and benthic invertebrates seems to be controlled by suspended and sedimentary Hg-binding substances, with values ranging from 22 to 2272 ng Hg g^{-1} dry weight (Jackson, 1988b; Parkman and Meili, 1993). Mercury levels in fish range from 180 to 650 ng g^{-1}. Practically all such Hg is present as MeHg (Becker and Bigham, 1995; Cabana et al., 1994; Bodaly et al., 1993).

Table 8 Mercury Species with Levels of Concentrations in the Compartments within the Environment.

COMPARTMENT	SPECIES	LEVELS OF Hg	SOURCE
Air	Hg°	pgL^{-1}	Fitzgerald and Gill, 1979
	$(CH_3)_2Hg$	pgL^{-1}	Bloom and Fitzgerald, 1988
	$(CH_3CH_2)_2Hg$	$<pgL^{-1}$	"
	CH_3HgCl	$<pgL^{-1}$	"
	CH_3CH_2HgCl	$<pgL^{-1}$	"
Water	Hg°	pgL^{-1}	Fitzgerald and Gill, 1979
	Hg^{II}	ngL^{-1}	Weber, 1993, Winfrey and Rudd, 1990
	CH_3Hg^+	$<pgL^{-1}$	"
	CH_3HgCH_3	$<pgL^{-1}$	"
Soil	$Hg(OH)^+$	ngg^{-1}	Mills and Zwarich, 1995
	$Hg(OH)_2$	ngg^{-1}	"
Sediments	"total"	ngg^{-1}	Bryan and Langston, 1992
Biota	CH_3Hg^+	ngg^{-1}	Grieb et al., 1990

For specific concentrations under different conditions see text.

3.1.3 Mercury Speciation in the Aquatic Environment.

Due to weathering processes Hg may enter an aquatic system via several routes (Fig.22). The surrounding watershed of lakes may furnish substantive amounts to runoff, e.g., from cinnabar, HgS, in volcanically active areas and from exposure of Hg ore deposits during mining operations. Industrial processes may provide point source contamination, e.g., from industrial operations involving Hg electrodes or using inorganic Hg compounds as catalysts. The emanate input from pristine areas due to weathering processes is minute in comparison. Mercury in the atmosphere precipitates into terrestrial and aquatic systems as mercuric ion Hg^{II}. Inundation of terrestrial areas allows leaching of Hg from flooded soil into newly formed water bodies.

A primary factor controlling Hg fate and behavior in the aquatic environment is inorganic Hg speciation (Fig.22)(Nriagu, 1979). Once in the aquatic system, Hg^{II} may participate in a number of complex transformations (Weber, 1993). These include a) the bacterial process of reduction to Hg^{o} with subsequent volatilization to the atmosphere, (Mason et al., 1995), b) adsorption to particulate matter and dissolved organic carbon (DOC) (Bryan and Langston, 1992; Furutani and Rudd, 1980; Hintelmann et al., 1993), c) formation of humate complexes (Weber, 1993), d) precipitation as mercuric sulfide (HgS) in the presence of H_2S under anoxic conditions (Furutani and Rudd, 1980), and e) methylation to MeHg and other methylated species by bacteria (Ramlal et al., 1987).

In sediments, Hg may exist in the form of elemental Hg adsorbed to particles or be converted to Hg^{II} under both oxic and anoxic conditions. As Hg^{II}, Hg exists as mercuric humate complexes, e.g. $Hg(OH)^{+}$, $Hg(OH)_2$ at pH 7.5-8.0, and as well it may precipitate as HgS under anoxic conditions or be converted to MeHg and DiMeHg by abiotic (Weber, 1993) or biotic processes and with subsequent adsorption to particles (Fig.22). Furutani and Rudd (1980) considered the ability of organic and inorganic particulates in sediment to complex Hg. They found that Hg methylation associated with the sediment floc of a non-contaminated lake was higher than in the clay-floc of a contaminated lake. Mercury binds to the suspended particulate matter and bacteria

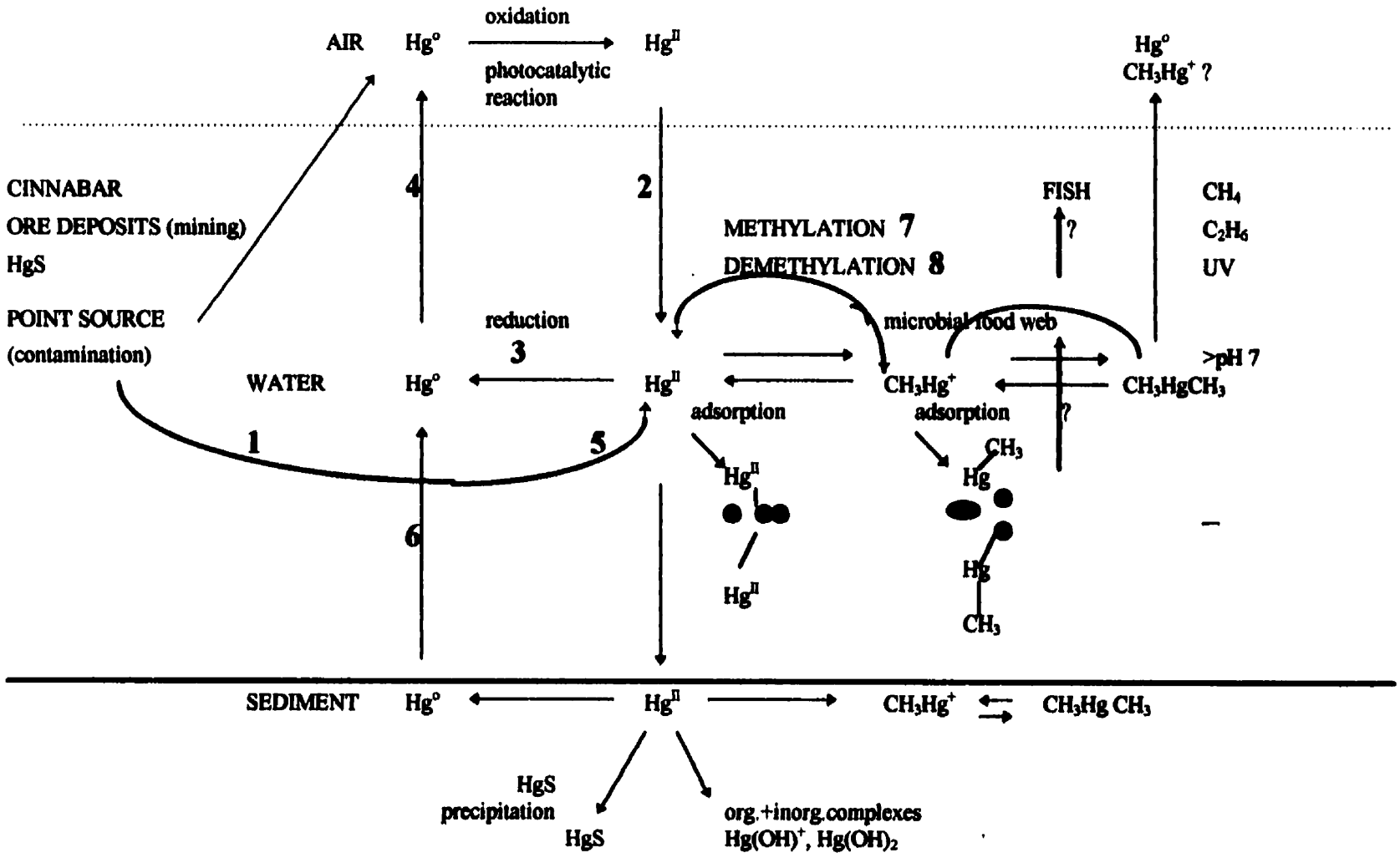


Fig. 22 Mercury Speciation in the Environment. Modified from Winfrey and Rudd, 1990. Particulate matter + DOC

which are attached to it. This binding of Hg is the main means of its transportation through the water column (Hintelman et al., 1993). Winfrey and Rudd (1990) mention Hg binding to particulates in water as a factor which increases Hg methylation. Suspended particulate material (Hintelman et al., 1993), settling particles (Sigg et al., 1987), particulate organic carbon (Cho and Azam, 1988), and particulates (Winfrey and Rudd, 1990) are cited as principal binding sites for Hg.

Methyl Hg is created within the water column mainly by bacterial processes as elaborated in chapter 4.1.1. (Parks et al., 1989; Regnell and Tunlid, 1991; Winfrey and Rudd, 1990). Methyl Hg is highly unstable (Ahmed and Stoeppler, 1986) and lipid soluble. The affinity for lipids allows MeHg to penetrate membranes (Boudou and Ribeyre, 1985) and become associated with proteins and amino acids of living organisms (Yamazaki et al., 1978). Methylmercury can be methylated or be converted abiotically to dimethylmercury (DiMeHg), which occurs primarily at pH <7 (Winfrey and Rudd, 1990). With exposure to ultraviolet light, dimethylmercury is readily dissociated into volatile Hg°, methane and ethane and passed into the atmosphere (Fig.22).

Methylmercury readily bioaccumulates in aquatic fauna, especially fish (Jackson, 1988b), but very little is known about pathways of uptake by biota and distribution of this and other forms of Hg (St. Louis et al., 1994).

3.1.4 Analytical Procedures for Mercury and Methylmercury in Water.

Methodologies available for Hg analyses were developed to assay relatively high Hg concentrations in tissue (Uthe et al, 1972) (Protocol 6,I). Mercury ordinarily occurs at very low concentrations (Parks et al, 1989) in water (ng L^{-1} and lower). This situation presents a methodological problem: either analytical methods of very high sensitivity are required for assay, or material containing low amounts of Hg must be concentrated in some way. Analytical methodologies are sequenced in three steps: preparation, separation and analyses.

I. Sample preparation. This determines what Hg species will be analyzed and how the samples will be taken and processed for assay. Sample preparation is a prerequisite to any separation system.

II. Separation Procedure. The selection of a separation procedure is based on the concentration of Hg and MeHg and on material from which Hg and MeHg must be separated. There are specific separation systems used in the analyses of both total Hg and organic Hg.

III. Instrumentation. The assessment instrumentation is chosen based on both sample preparation, separation procedures and the detection limit.

Protocol 6 Chemical Steps in Hg Analyses by Gas Liquid Partitioning Method and Gold Trap Amalgamation Method. See text for explanation.

I. Gas/Partition Method:

Methylmercury step:

$CH_3Hg - S - protein \longrightarrow H^+, CuSO_4, NaBr \longrightarrow CH_3HgBr$

Inorganic mercury step:

$CH_3HgBr + HNO_3 + H_2SO_4 \longrightarrow$ oxidize $\longrightarrow Hg^{2+}$ (mercuric ion)

CVAA monitor, reduction to elementary mercury:

$Hg^{2+} \longrightarrow$ reduced by $SnSO_4$, $(NH_2OH)_2$. H_2SO_4, $NaCl$ $\longrightarrow Hg^o$ (elementary Hg)

II. Gold Trap Method for Mercury:

$CH_3Hg - S - protein \longrightarrow NH_2OH \cdot HCl \longrightarrow CH_3HgCl$

$CH_3HgCl \longrightarrow BrCl \longrightarrow Hg^{2+}$

$Hg^{2+} \longrightarrow SnCl_2 \longrightarrow Hg^o$

3.1.4.1 Total Mercury.

3.1.4.1.1 Sample Preparation.

Sample preparation includes specification of sampling equipment and preservation techniques, the process of sampling itself and the preparation of samples for transportation to the site of analysis. In order to prevent contamination of samples taken in the field, all equipment including sampling bottles are double wrapped in clean plastic bags. Both teflon bottles (Ahmed and Stoeppler, 1986) and PVC bottles (Ahmed and Stockner, 1986) are used. Teflon bottles are deemed to be superior because of their inert surface and impermeability to gases, specifically MeHg (Ahmed and Stoeppler, 1986). Samples are taken against the wind to prevent entry of extraneous Hg (St. Louis et al, 1994). Freshly taken samples are preserved, usually with acid (Furutani and Rudd, 1980, Ahmed and Stoepler, 1986), to prevent microbial action and to ensure that Hg is preserved within material. Preservation techniques are specific with respect to material being assayed and to the analytical methods to be applied. To ensure that contamination does not occur during transport, samples are again double wrapped.

Preparation for Assay of Reactive Mercury.

Reactive Hg is defined as inorganic Hg species, labile organo-Hg associations, and Hg readily leached from particulate matter. Samples are acidified by high purity conc. HNO_3 (pH 1.2), 5% final concentration (Gill and Fitzgerald, 1985) or 0.5% HNO_3 (Bloom and Crecelius, 1983), followed by reduction with $SnCl_2$ (50% w/v). Analyses follows within 4-24 h (Bloom and Crecelius, 1983). This digestion does not release MeHg from protein sulfhydryl groups and therefore is limited.

Preparation of Samples to be Aged Prior to Assay.

This process is based on the assumption that after acidification the samples will be aged for several weeks so that particulate and organic forms of Hg will be gradually converted to ionic Hg (Bloom and Crecelius, 1983).

Preparation of Samples by Cold Oxidation.

In order to convert all forms of Hg to Hg^{II}, BrCl is added at the time of sampling (Bloom and Crecelius, 1983). Szakacss et al.(1980) showed that complete oxidation of organic Hg compounds to Hg^{II} took place immediately with such treatment. For effective digestion of bacterial tissue, a 20% solution of tetramethylammonium hydroxide can be used prior to analyses as an effective non-oxidizing solubilizer (Hintelman et al., 1993).

Preparation of Samples by Hot Oxidation.

Nitric acid (2% final concentration) is added to the sample which is then oxidized by hot $KMnO_4$ and $K_2S_2O_8$ to form MnO_2. The sample is then placed in a boiling water bath and heated for 1 hour. The sample is cooled and $NH_2OH{\cdot}HCl$ added to dissolve and reduce MnO_2 and Cl_2 (Bothner and Robertson, 1975). The permanganate-persulphate oxidizing reagent readily absorbs Hg and is often the source of high reagent blanks (Bloom and Crecelius, 1983).

3.1.4.1.2 Separation Systems.

3.1.4.1.2.1 Gas/Liquid Partitioning Method (GLPM).

Gas liquid partitioning method was developed by Armstrong and Uthe (1971) and is still widely used in analytical laboratories with minor improvements (Hendzel and Jamieson, 1976). This method is designed to liberate MeHg from protein and to convert all Hg species to the mercuric ion, Hg^{II} (Yamazaki et al, 1978)(Protocol 6,I). The oxidization is achieved by the action of nitric and sulfuric acids and application of heat. Mercuric ion is then reduced to elemental Hg (Hg°) with a solution of stannous sulfate, hydroxylamine sulfate and sodium chloride. The Hg° partitions between the solution and a measured volume of air. The GLPM (Hendzel and Jamieson, 1976) is adaptable for the analysis of many different substances. The method is usually set up to assay high levels of Hg, e.g., in waste water, effluents, discharges from mines and dental laboratories or biological materials such as contaminated blood and urine, and contaminated fish.

3.1.4.1.2.2 Two Stage Gold (Au) Amalgamation.

The analyses of Hg has been greatly improved by employment of the two stage amalgamation technique (Fitzgerald and Gill, 1979). Stannous chloride ($SnCl_2$) is added to the sample, Hg is released as Hg° and carried onto the system with Hg-free gas and amalgamated onto a gold trap (Protocol 6,II). Clean air (Fitzgerald and Gill, 1979), Ar (EnviroTest Labs.(ETL), Winnipeg) or N_2 (this study) can be used as the carrier gas. Since the gold trap may be deactivated by acidic or water vapor released in minute amounts from the reaction bottle, a trap containing distilled water and a small amount of $SnCl_2$ (Bloom and Crecelius, 1983) or $CaSO_4$ (this study) is placed between the reaction vessel and the gold trap. Mercury is transferred to a second gold trap in order to eliminate interference due to organic compounds or Cl_2

(Fitzgerald and Gill, 1979). This second transfer is done by sweeping with Hg-free gas while heating the gold trap to 400-500°C. Principal of this method is used by Flett Research Ltd.

3.1.4.2 Methylmercury.

3.1.4.2.1 Sample Preparation.

Samples for analyses of MeHg have to be preserved to prevent methylation/demethylation processes by microbial action. Furutani and Rudd (1980) used 0.13% HCl (final conc.) for termination of methylation and 1% HNO_3 for preservation of samples for total Hg. Precautions to prevent contamination of samples are taken as described in section 3.1.4.1.1.

3.1.4.2.2 Separation Systems.

3.1.4.2.2.1 Solvent Extraction.

Several techniques have been used to partition organic-Hg into solvents. Uthe et al. (1972) used acidic sodium bromide and copper sulfate to release organic-Hg from biotic material and then extracted it from solution with an organic solvent. Benzene, toluene, or hexane or a 3:2 mixture of methylene chloride (CH_2Cl_2) and hexane solution have been used (Uthe et al., 1972). The organic layer containing organic Hg species is then processed in the same way as inorganic Hg. Inorganic Hg is also released by reagents, but has been shown to reside in the aqueous phase (Hendzel and Jamieson, 1976; Uthe et al., 1972).

Other techniques include that of Bloom and Fitgerald (1988) who used chelating resins, with S- exchange sites to concentrate organomercury compounds on a carbotrap. Lee and Mowrer (1989)

synthesized an effective sulfhydryl cotton fiber (SCF) for MeHg concentration. SCF was packed into microlitre pipette tips and connected to the sample reservoir. The sample was then passed through the column by controlled gas pressure. Methylmercury, as CH_3HgCl, was eluted from the absorbent by acidification (HCl) and extracted into an organic solvent (benzene). These methods require large amounts of material and processing time (Lee and Mowler, 1989). Flett Research Ltd. used Bloom's (Bloom , 1989) aqueous ethylation method with distillation modifications (Horvat et al, 1993a, Horvat et al, 1993b) and GC separation improvement (Liang et al, 1994).

3.1.4.2.2.2 Aqueous Phase Ethylation.

Methylmercury can be stripped from aquatic samples by aqueous phase ethylation of ionic Hg species (Hg^+, CH_2Hg^+) at pH 4-6. In this process aqueous tetraethylborate anion reacts with Hg^{II} to form diethylmercury and with methylmercury species to form methylethylmercury. Elemental Hg and dimethylmercury, being non-ionic, do not react with the tetraethylborate anion, but can be specifically determined. Volatile Hg species are purged from the solution, trapped on a carbon trap and analyzed by cryogenic gas chromatography. Hg^0 present in the sample passes through the carbotrap and may be collected on a back up gold trap for subsequent analyses.

Excessive amounts of particulate matter inhibits the recovery of methylmercury. To dissolve particulates, KCl and HCl are added to samples. The samples are then extracted with CH_2Cl_2 and solvent evaporated (Bloom, 1989).

3.1.4.2.3 Instrumentation for Mercury Analyses.

Depending on sample preparation and separation, a cold vapor atomic absorption spectrophotometer (CVAA) (Bloom and Fitzgerald, 1988), cold vapor atomic fluorescence (CVAF) (Bloom, 1989) and/or gas

chromatograph (GC) instruments (usually electron capture detector with gas liquid chromatography (Uthe, et al, 1972) and cold vapor atomic fluorescence detector with gas chromatography) can be used for assessment of Hg and MeHg. In addition to this instrumentation, coupled GC-CVAF may be used (R.Flett, Flett Research Ltd.).

3.1.4.2.3.1 Atomic Absorption Spectrophotometry (AAS).

The general principle of atomic absorption lies in the absorption of radiation by atoms. Light is absorbed in a transparent absorption cell. The absorbance is measured within the linear working range of absorbance and concentration for a particular element. The AA spectrophotometer consists of a source of light (e.g., a Hg lamp), an atom reservoir (e.g., a source of elemental Hg), a device for isolation of atomic spectral lines, a detector for UV-visible radiation and a signal processor.

Types of atomic absorption include cold, flame, furnace and flame with a borohydride generator. Cold vapor AA was used in this study (The Mercury Monitor™ 3200, Elemental Mercury Detector, LDC Analytical).

Cold Vapor AA.

The hollow-cathode Hg lamp emits radiation characteristic of the cathode material. In the case of Hg, Hg° is produced after appropriate chemical treatment in the reservoir. Elemental Hg has an appreciable vapor pressure at room temperature. This allows for Hg absorption measurements to be made by sweeping Hg vapor into an unheated absorption cell (Loon, 1980). Ground state elements absorb radiation from the lamp thus reducing the intensity of the beam. The monochromator isolates the desired absorbing line. The radiation falls on the photomultiplier and the electrical signal is generated and processed.

3.1.4.2.3.2 Gas Chromatography (GC).

In this technique the components of a mixture are separated based on the rates at which they are carried through a stationary phase by a gaseous or liquid mobile phase (Skoog et al., 1992). The basic components of a gas chromatograph are a carrier gas, injection port, a separation column, a thermostatically controlled oven, a detector and a recorder.

The sample is injected onto the column and carried through the column by the carrier gas. Species travel through the column at different rates (retention times) so that they exit the column into the detector (AAS) sequentially (R. Flett, Flett Research Ltd.(FR), personal communication).

3.1.4.2.3.3 Cryogenic Gas Chromatography Coupled with Cold Atomic Absorption.

Organomercurials can be detected as Hg° by a CVAA spectrometer in line with the gas chromatograph (Baldi et al., 1995). Species are first collected on the gas chromatograph column at -196 °C, then eluted from the column by controlled heating from -196°C to +180°C over 15 minutes. The Hg species are eluted in order of increasing molecular weight in the following order: Hg°, $(CH_3)_2Hg$, $CH_3CH_2HgCH_3$, $(CH_3CH_2)_2Hg$. Column eluent is thermally decomposed to Hg° at 900°C and passed into the atomic absorption spectrophotometer for detection. Peaks are identified by comparison of retention time of known standards (Bloom, 1989).

3.2 Present Study

3.2.1 Development of Analytical Methodology for Assay of Mercury in Microbial Samples.

3.2.1.1 Introduction.

Because of the extremely low biomass of microbial loop material in freshwater, many analytical procedures for Hg usually applied to biological samples were not sensitive enough to assay for Hg in this material. Some procedures were possible to use in some treatments, but the sensitivity had to be improved. Procedures redeveloped for work with aquatic samples were the following: a Gas-Liquid Partitioning Method (GLPM)(3.2.1.3.) for total and organic Hg and the Gold Trap Amalgamation Method (GTAM)(3.2.1.4.) for total Hg.

Samples taken in the field consisted of whole (unmanipulated) lake water, and three biotic fractions separated into groups based on the linear measurements of the particles within the group. They are: the >20 µm (plankton) fraction, the 3-20 µm (protozoa) fraction, captured on a filter, the 0.1-3 µm (bacteria) fraction concentrated by means of a dewatering process, and the <0.1 µm (filtrate) fraction. These fractions were obtained and treated as follows:

Whole water

A subsample of 20 mL or less was digested by 1 mL conc. HNO_3 and 4 mL conc. H_2SO_4 in Hg clean glass tubes and analyzed by GLPM using CVAA (Flowchart 1, sample 1).

>20 µm fraction

Twenty (20) L of water was filtered through a 20 µm pore size agricultural Amiad 1.5" T filter (amiad filtration and irrigation systems, Amiad USA, Inc., Van Nuys, CA 91413, Berma and Kimor, 1983). The particulate fraction was rinsed into a Hg clean bottle by as little lake water as possible to keep sample in high concentration for

analyses. Replicate samples were taken and acidified with 1 mL of concentrated HNO_3 (Flowchart 1, sample 2).

3-20 µm fraction

After >20 µm organisms were removed from the 20 L aliquot of lake water, organisms were captured on a 3 µm pore size 147 mm diameter polycarbonate Nucleopore® filter. One quarter of the 3 µm pore size filter was used for total Hg analyses. The quarter section was washed twice with 2 mL of conc. HNO_3 to dissolve material on the filter. The resulting solution was pipetted into glass tubes. Sulfuric acid was added and the sample, containing both acids, was digested and then analyzed by GLPM using CVAA. The remaining ¾ of the filter was used for analyses of MeHg by either separatory funnel or teflon tube method.

0.1-3 µm fraction

After removal of 3-20 µm organisms, organisms measuring 0.1-3 µm were concentrated into 300-450 mL by water removal using an Amicon® system. Total Hg was assayed by the GLPM using CVAA (Flowchart 1, sample 4). MeHg was assayed using either separatory or teflon tube method, described below.

<0.1 µm

The filtrate of <0.1 µm was left after the dewatering process. A 20 mL sub-sample was taken and assayed by the GLPM and GTAM.

3.2.1.2 Sample Preparation.

Since the concentration of Hg in the aquatic system studied was very low, techniques which prevented contamination from handling, storing and analyses were adopted. All reagents and equipment were

blanked, e.g., checked for Hg contamination. Glassware and other material were soaked in 1% analytical grade HNO_3 overnight, then washed twice with 50% pure grade Baker HNO_3, followed by rinsing with tap water. FWI tap water was found to contain less Hg (~50 pg L^{-1}) than FWI distilled deionized water and so was used for final rinses. Pure grade Baker nitric acid was used for analysis, while other acids and reagents were found sufficient as analytical grades.

Two 20 L samples were taken on each sampling date and marked A or B. Fractions >20 µm and 3-20 µm were obtained and refrigerated on day 1. Samples 0.1-3 µm and filtrates A and B were processed chronologically since duplicate apparatus was not available. Therefore, sample A, collected on day 1, was transported on day 2, dewatered on day 3-6, and refrigerated; sample B was collected on day 1, but dewatered on day 7-10 and both A and B samples were then analyzed on day 11-12.

All equipment used in the sampling process was double wrapped in plastic bags. All craft used to reach sampling sites were oar or paddle driven to prevent general contamination which would ensue from use of gasoline and oil. The 20 L samples were taken against the wind to prevent entry of extraneous Hg. Samples were immediately preserved with 1% HNO_3 (final concentration) (pure grade Baker grade) and again double wrapped in plastic bags.

3.2.1.3 Inorganic Mercury Standard.

A Hg primary standard (10 µg mL^{-1}, Table 9, A) was prepared by adding 1 mL of Hg standard stock solution (1000 µg mL^{-1}) to acidified (1% HNO_3) tap water, final volume 100 mL. This solution was stabilized by addition of 100 µL of 5% potassium dichromate and stored in a sealed glass bottle for up to two weeks. The Hg working standard (100 ng mL^{-1}, Table 9, B) was 1 mL of primary standard diluted to 100 mL with 1% HNO_3 in tap water prepared fresh for immediate use. Required standard concentrations were prepared from the Hg working standards as in Table 9, I.C, II.C.

Table 9 Preparation of Hg Standard Solutions.

Amount	Conc.	Final Volume of Tap Water	Final Conc.
I. Gas/Liquid Partitioning Method.			
A. 1 mL of	1000 μg mL^{-1}	up to 100 mL	10 μg mL^{-1}
B. 1000 μL of	10 μg mL^{-1}	up to 100 mL	100 ng mL^{-1}
C. 100 μL of	10 ng mL^{-1}	up to 25 mL	10 ng mL^{-1}
300 μL of	10 ng mL^{-1}	up to 25 mL	3 ng (ABS)*
200 μL of	10 ng mL^{-1}	up to 25 mL	2 ng (ABS)*
100 μL of	10 ng mL^{-1}	up to 25 mL	1 ng (ABS)*
II. Gold Trap Amalgamation Method.			
A. 1 mL of	1000 μg mL^{-1}	up to 100 mL	10 μg mL^{-1}
B. 1000 μL of	10 μg mL^{-1}	up to 100 mL	100 ng mL^{-1}
C. 1000 μL of	100 ng mL^{-1}	up to 100 mL	1 ng (ABS)*

*ABS = absolute amount in final volume

3.2.1.4 Gas/Liquid Partitioning Method (GLPM).

Mercury and organic Hg in blanks, whole water, >20, 3-20, 0.1-3, and <0.1 μm fractionated samples were assayed using the GLPM (Fig.23). A sample or sub-sample was taken and acids added (Flowchart 1). The solution was heated to 40°C. Temperature was gradually increased to 180°C and samples digested for 8 hours. After cooling, samples were made up to 25 mL, if they were not already at this volume (as for example, the >20 μm fraction) and analyzed by CVAA (Fig.23). Mercuric ion (Hg^{II}) is reduced to elemental Hg (Hg°) with a solution of stannous sulphate ($SnSO_4$), hydroxylamine sulphate $(NH_2OH)_2$, H_2SO_4, and

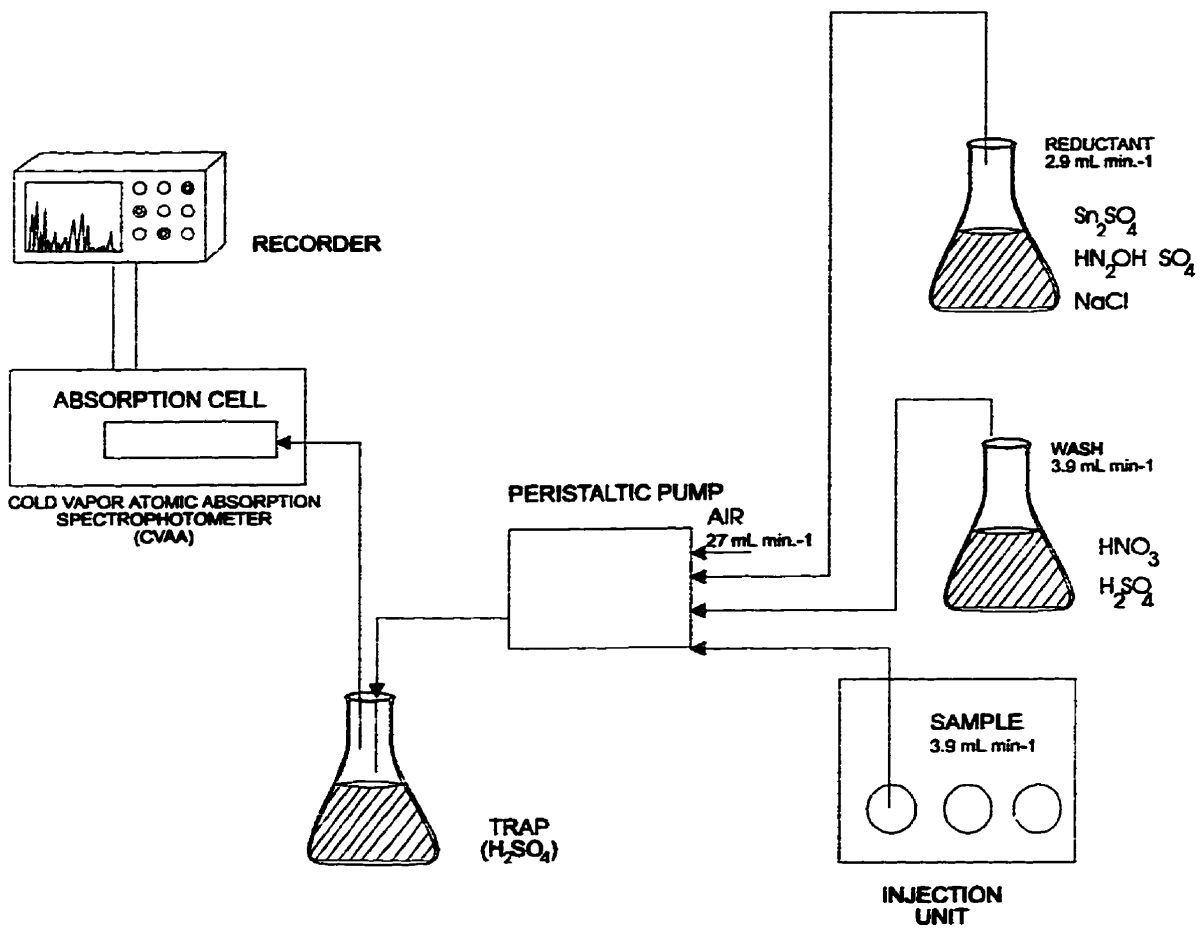


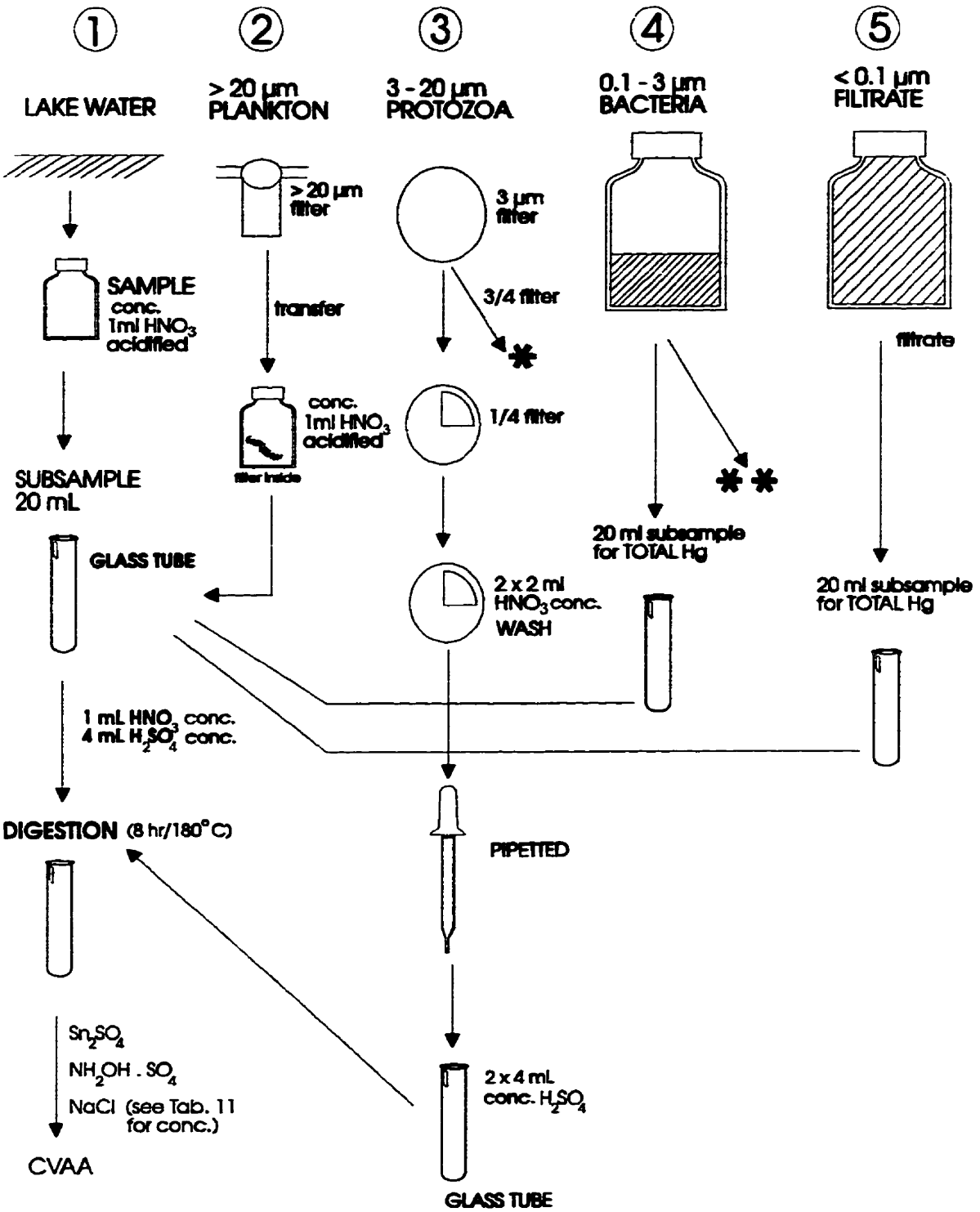
Figure 23 Gas/Liquid Partitioning Method (GLPM).

Protocol 7 Reagents Used in Gas/Liquid partitioning Method (GLPM).

Reagent:	Concentration:	Preparation: (in dH_2O)
I. **Reductant Solution:**		
H_2SO_4	10% v/v	100 mL conc. H_2SO_4/1000 mL
Sn_2SO_4	2.1% w/v	2.1 g Sn_2SO_4 / 100 mL
$(HN_2OH)_2$-SO_4	1% w/v	1.0 g $(HN_2OH)_2$-SO_4 / 100 mL
NaCl	0.05325% w/v	0.05325 g NaCl / 100 mL
II. **Wash Solution:**		
HNO_3:H_2SO_4	1%:4%	160 mL : 640 mL / 4000 mL
III. **Trap Solution:**		
H_2SO_4	100%	

sodium chloride (NaCl) (Protocol 7) and allowed to partition between the acid solution and a measured volume of air in a peristaltic pump system (Fig.23). Reagents are dissolved in dH_2O, H_2SO_4 to 10% (final concentration) is added, the mixture is cooled and stirred until clear and made up to the desired amount of reduction solution. The resulting Hg vapor (Hg^o) is determined by CVAA at 253.7 nm (Fig.23) (Hendzel and Jamieson, 1976; Uthe at al., 1972).

Pure acids were used for sample preservation and clean tubing for sample delivery and mixing. An acid trap was inserted between sample reduction and the analytical unit to minimize background noise. The spectrophotometer was set up for maximum sensitivity with the acid wash ratio changed to 1:4 from 3:2 at 10 mV. The method detection limit of 4 ng L^{-1} is discussed in section 3.3.5.1.



Flowchart 1 Preparing of Fractionated Samples for Assay by Gas/Liquid Partitioning Method .

*, ** see Flowchart 3.

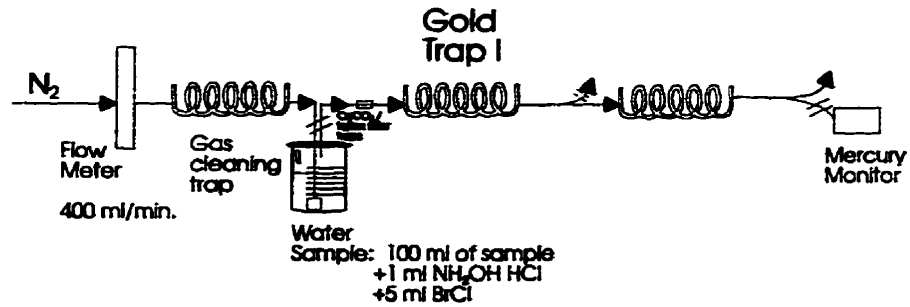
3.2.1.5 Gold Trap Amalgamation Method (GTAM).

Mercury was analyzed by the two-stage GTAM method using a technique similar to Fitzgerald and Gill (1979). The principal of the method as described in the literature review, section 3.1.4.1.2.2., was applied. Reagents and a schematic diagram of the two-stage gold trap system with specific modifications to this study is given in Protocol 6 and Flowchart 2, respectively. Vapor phase additions of 50 µL Hg-saturated air were used for system calibration. This Hg standard is prepared by allowing a small amount of Hg° to equilibrate with air at room temperature in a 250 mL glass container fitted with a rubber septum to allow syringe extraction. Mercury was than treated as an ideal gas. From the ideal gas law, Hg saturated air is calculated to contain 19.93 ng Hg cm^{-3} at 25°C and 1 atm. (Fitzgerald and Gill, 1979). A 100 mL aliquot of the sample was placed into a Hg clean 125 mL reaction bottle and 5 mL of BrCl solution added (Protocol 8). The oxidation reaction was allowed to proceed for 5 min. before 1 mL NH_2OH-HCl was added to remove residual Cl_2. The sample was inserted into the system and 5 mL of $SnCl_2$ (5%) was injected. The sample was stripped of Hg° with Hg clean N_2 flowing at 400 mL min^{-1} for 15 min. The free Hg° was collected on the first gold trap (Flowchart 2B). In the next step, Hg° was stripped from the first gold trap by controlled heating and transferred by N_2 (flow rate 70 mL $min.^{-1}$) onto a second gold trap (Flowchart 2C). In the last step, the second trap was heated and Hg° carried to the CVAA analyzer where absorption of Hg° was determined (Flowchart 2D). Mercury standards were prepared as in Table 11. The detection limit for this method was restricted by reagent blanks of about 30 pg for a 125 mL reaction bottle. Because of the low detection limit of this method, samples do not have to be concentrated.

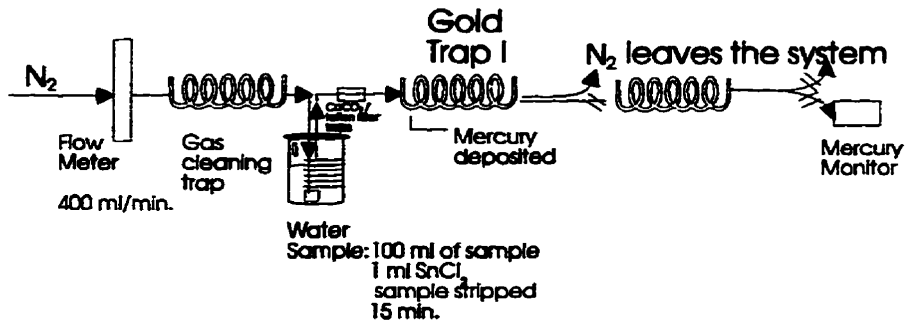
Protocol 8 Reagents Used in Gold Trap Amalgamation Method.

Reagent:	Concentration/Preparation:
N_2	pure grade, cleaned through the gold trap
$NH_2OH \cdot HCl$	12%
BrCl	11 g of $KBrO_3$, 15 g KBr in 200 mL of H_2O
$SnCl_2$	125 g of $SnCl_2 \cdot H_2O$ in 25 mL conc. HCl, dilute to 100 mL in dH_2O; purge the solution with N_2 overnight at a slow flow rate to remove Hg.

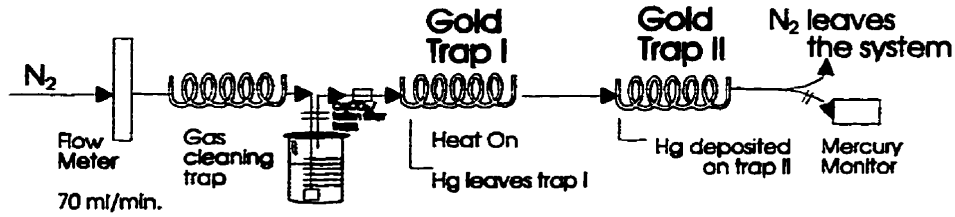
A) Gas flow established and system is cleaned of Hg



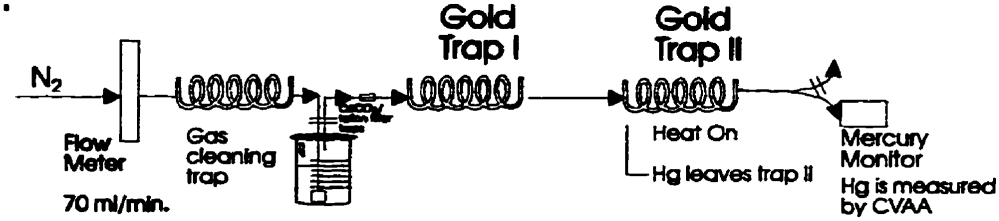
B) Sample Stripping.



C) Heat I.



D) Heat II.



Flowchart 2 Gold Trap Amalgamation Method.

3.2.2 Development of MeHg Analysis for Microbial Fractions (3-20 and 0.1-3 μm).

Methylmercury measurements were obtained for concentrated bacteria samples (0.1-3 μm) and for filtered 3-20 μm samples. Samples were preserved, transported and processed chronologically as described for total Hg in section 3.2.1.2., stored wrapped in a freezer to prevent contamination and analyzed for MeHg usually day 10-14 after sampling.

Methylmercury was released from protein with a solution of acidic NaBr and $CuSO_4$ and partitioned into the organic solvent methylene chloride. An organic layer aliquot was then analyzed as for inorganic Hg. This method was largely simplified and modified from Uthe et al (1972)

3.2.2.1 Methylmercury Standard.

The methylmercury primary standard (1 mg mL^{-1}) was prepared by dissolving 125.2 mg of CH_3HgCl in toluene and diluting to 100 mL (Table 10). The solution was stored in sealed glass bottle under refrigeration. The methylmercury working standard I., (1 μg mL^{-1}, Table 10), was 100 μL of primary standard in 100 mL of CH_2Cl_2. Methylmercury stock standard II, (1 ng mL^{-1}, Table 10) was 100 μl of stock standard I in 100 mL of CH_2Cl_2. One (1) mL of standard II gave 1 ng 25 mL^{-1} of working standard III in a digestion tube (using the GLPM) or 1 ng standard absolute in a 100 mL reaction bottle (using the gold trap method, Table 10). Except for the primary standard, all working standards were prepared fresh daily.

Table 10 Preparation of MeHg Standard Solutions.

Amount	Conc.	Final Volume	Final Conc.
Primary standard			
125.2 mg CH_3HgCl		100 mL toluene	1 mg mL^{-1}
Working standards			
I. 100 µL	1 mg mL^{-1}	100 mL	1 µg mL^{-1}
II. 100 µL	1 µg mL^{-1}	100 mL	1 ng mL^{-1} [a]
III. 1000 µL	1 ng mL^{-1}	25 mL	1 ng [b,c]

[a]used in GLPM

[b]used in gold trap amalgamation method

[c]absolute amount in final volume

3.2.2.2 Concentrated 0.1 - 3 µm Bacterial Samples.

3.2.2.2.1 Digestion of Material in 40 mL Teflon Tubes.

A 25 mL sub-sample of a 0.1-3 µm fraction was placed into a Hg clean 40 mL Teflon tube. Acidic copper sulphate (7.5 mL), sodium bromide (5 mL) and methylene chloride (2 mL) were added (Protocol 7A), and the teflon tube was shaken for 15 min. on a shaker. Samples were then centrifuged at 5,000 rpm for 5 min. A measured volume of the organic layer was withdrawn by air tight syringe, pre-cleaned by CH_2Cl_2. Centrifugation was repeated to allow maximum amounts of methylene chloride to be withdrawn. The organic layer was transferred to a digestion tube containing 5 mL of 4/1 H_2SO_4/HNO_3 concentrated acids. Digestion tubes were transferred to a warm (40°C) digestion block to evaporate solvents. After increasing the temperature to 180°C, the sample was digested overnight and analyzed by CVAA.

Protocol 9 Reagents Used for MeHg Analyses.

A) Reagents for MeHg 0.1-3 µm Concentrated Samples Teflon Tube Method

$CuSO_4$ 7.5 mL (5 g $CuSO_4$ + 75 mL of dH_2O, add 100 mL H_2SO_4 in 10 mL aliquots, cool in a water bath and make up to 200 mL with dH_2O)

NaBr 5 mL (30 g NaBr + 100 mL 4N H_2SO_4)

CH_2Cl_2,conc., 2 mL

HNO_3:H_2SO_4 conc., 1:4

B) Reagents for MeHg Analysis in 0.1-3 µm Concentrated Samples Separating Funnel Method

$CuSO_4$ For A mL of sample add 1.5 * A/100 g of $CuSO_4$

NaBr For A mL of sample add 12 * A/100 g of NaBr

H_2SO_4 For A mL of sample add A * 4/32 N (98%) H_2SO_4

CH_2Cl_2 3 x 10 mL

HNO_3 1 mL, conc.

H_2SO_4 4 mL, conc.

C) Reagents for MeHg Analysis 3-20 µm Filter Sample Methods

3/4 of the filter containing 3- 20 µm fraction

2 x 25 mL of 2.5% $CuSO_4$ (25 g in 1L 4N H_2SO_4), solutions added in ratio 7.5 mL $CuSO_4$: $5mL^{-1}$ NaBr.

B mL of $CuSO_4$ = B * 5/7.5.

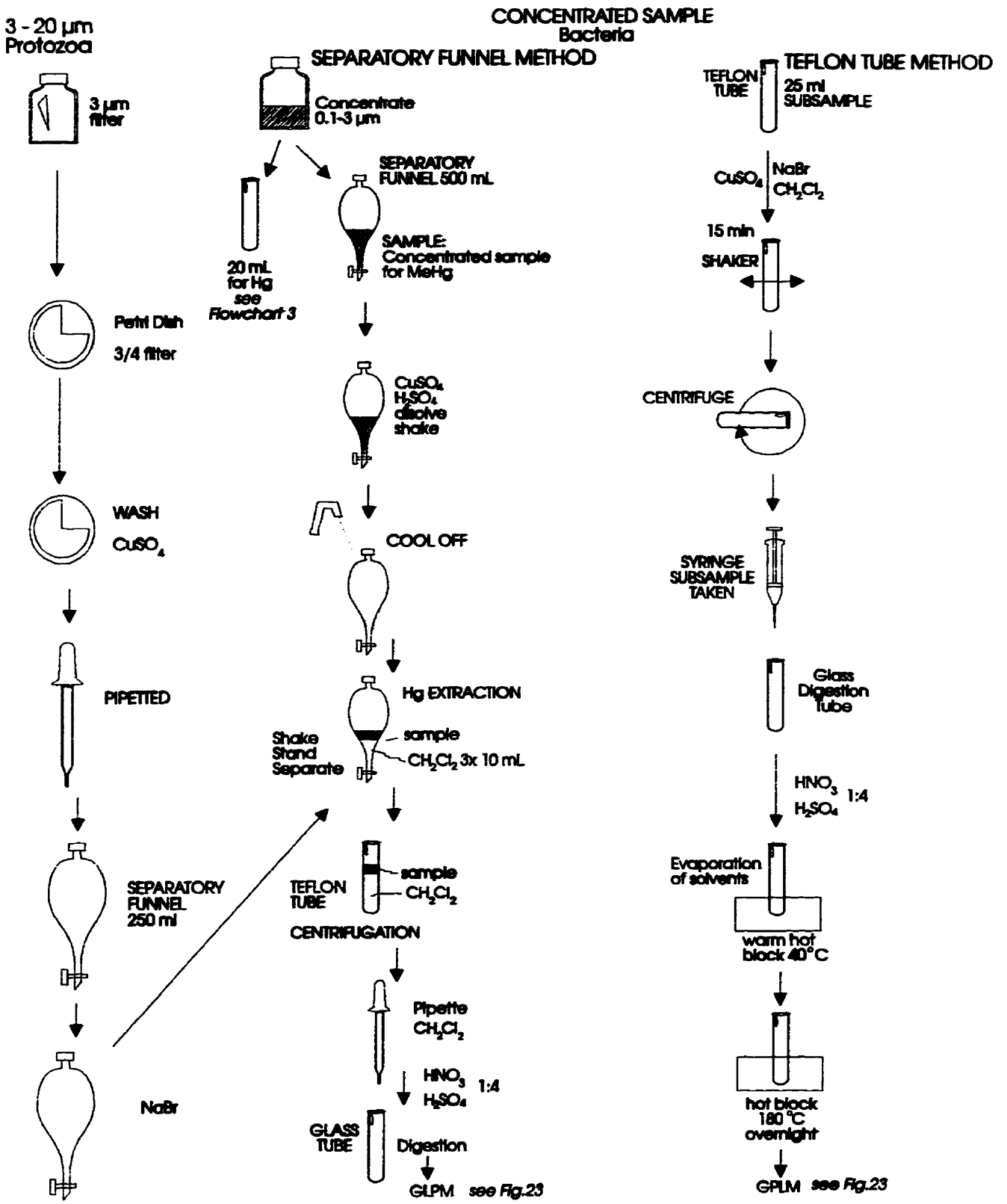
NaBr, 30% (30 g in 4N H_2SO_4 and dilute to 100 mL 4N H_2SO_4)

3.2.2.2.2 Digestion of Material in Separatory Funnels.

A measured, concentrated biotic sample was poured into a 250 or 500 mL separatory funnel. Copper sulphate ($CuSO_4$) and H_2SO_4 were added to the sample in proportions to its volume and dissolved (Protocol 7B). The solution was cooled in cold running water and Hg extracted in three steps by sequential additions of 3 x 10 mL aliquots of CH_2Cl_2. The funnels were vigorously shaken by hand between additions. The CH_2Cl_2 layer with partitioned Hg was separated into Teflon tubes after each addition by syringe. Teflon tubes were centrifuged at 5,000 rpm for 5 min. after which CH_2Cl_2 with extracted Hg was pipetted into a glass tube containing 4:1 sulfuric:nitric concentrated acids. Samples were analyzed by CVAA.

3.2.2.3 3-20 µm size Fraction Samples.

Three quarters (3/4) of the filter containing the 3-20 µm fraction was placed in a petri dish and washed 2x with 25 mL 2.5% $CuSO_4$ (Flowchart 3). The digesting solution was pipetted into a 250 mL separatory funnel. Acidic NaBr was added and dissolved. The amount of reagent added depended on the volume of sample, see Protocol 9. Subsequent extraction with CH_2Cl_2 and analysis for MeHg are as described at Flowchart 3.



Flowchart 3 Methylmercury Analyses of Concentrated (Bacterial) and Filter (Protozoa) Fractions.

3.3 Results and Discussion.

3.3.1 Sample Preparation.

Preparation of samples was as described in section 3.2.1.2. The amount of preservative used (1% HNO_3) was chosen based on literature research as the best preservative and stabilizer for Hg and MeHg in PVC bottles (Ahmed and Stoepler, 1986). Ahmed and Stoepler (1986) found the recovery of Hg to be 94.1% for polyethylene bottles. They found MeHgCl decomposes very rapidly in the absence of any reagents and recommended HNO_3 for short term preservation of samples. In contrast, with use of 1% HCl, only 53.8% of the Hg was recovered (Ahmed and Stoeppler, 1986). Furutani and Rudd (1980) used 1 ml of 4N HCl for 270 mL samples, (about 0.12% HCl) to stop the methylation process. For total Hg samples Furutani and Rudd (1980) used 0.05% chromate and 1% HNO_3 in preignited glass bottles. Similar sample preservation is practiced in laboratories which handle samples containing relatively high (method sensitivity >50 ng L^{-1}) Hg concentration. For example, at EnviroTest Labs. (Winnipeg) the technique calls for the use of both 1% of $K_2Cr_2O_8$ and 1% HNO_3 (Jan Zihrul, personal comm.). Since K_2MnO_4 causes high reagent blanks, these reagents were not used in present study.

3.3.2 Preparation of High Purity Water and Equipment.

The preparation of low Hg reagents and equipment blanks is a difficult but a crucial step. Gill and Fitzgerald (1987) cleaned water of Hg by using a multistage purification system. For laboratory ware they employed cleaning procedures including several steps of washing with pure water, HNO_3, chloroform and leaching in hot HNO_3 for several days. Similar cleaning procedures are being employed by Flett Research Ltd. (Winnipeg, MB). During this study all glassware and equipment were thoroughly cleaned by soaking in 1% HNO_3 overnight and than rinsing two times with 50% HNO_3, and finally rinse with tap water. The tap water in the FWI contained the least Hg of available water sources, with Hg

concentrations about 50 pg L^{-1}. The last rinse was checked for contamination. Alternate cleaning procedures were deemed to be unnecessary.

3.3.3 3-20 µm Fraction.

Attempts to solubilize the 3-20 µm material on the filter or to solubilize this material inside the separatory funnel by subsequent shaking caused the filter to adhere to the tube wall or remained folded, so that digestion of material was probably incomplete. As a solution, the filter was laid flat in a petri dish so that the material could be dissolved with conc. HNO_3 carefully applied to the filter by pipette. The digestion process was allowed to proceed for 15 minutes. The digested sample was then transferred into a digestion tube.

3.3.4 Whole, >20, <0.1 µm Fractions.

When subsamples of 20 mL of whole lake water, >20 µm fraction and <0.1 µm fraction were taken for analysis, the amount of Hg was too low to be detected by GLPM. Because these samples contained low concentrations of Hg, the maximum available volume of subsample (up to 65 mL) was placed into analytical vessel instead of the more usual 20 mL. Samples were acidified and placed on a hot block to evaporate 20 mL. Because of the small surface area presented, the time for the evaporation process was too long so this sample preparation technique was abandoned.

3.3.5 Hg Analysis: Separation System Improvements.

First attempts to assay Hg in fractionated samples using the GLPM with CVAA were unsuccessful (Fig.24) since samples apparently contained less Hg than the wash solution. Several steps were taken to improve the sensitivity of both GLPM and CVAA.

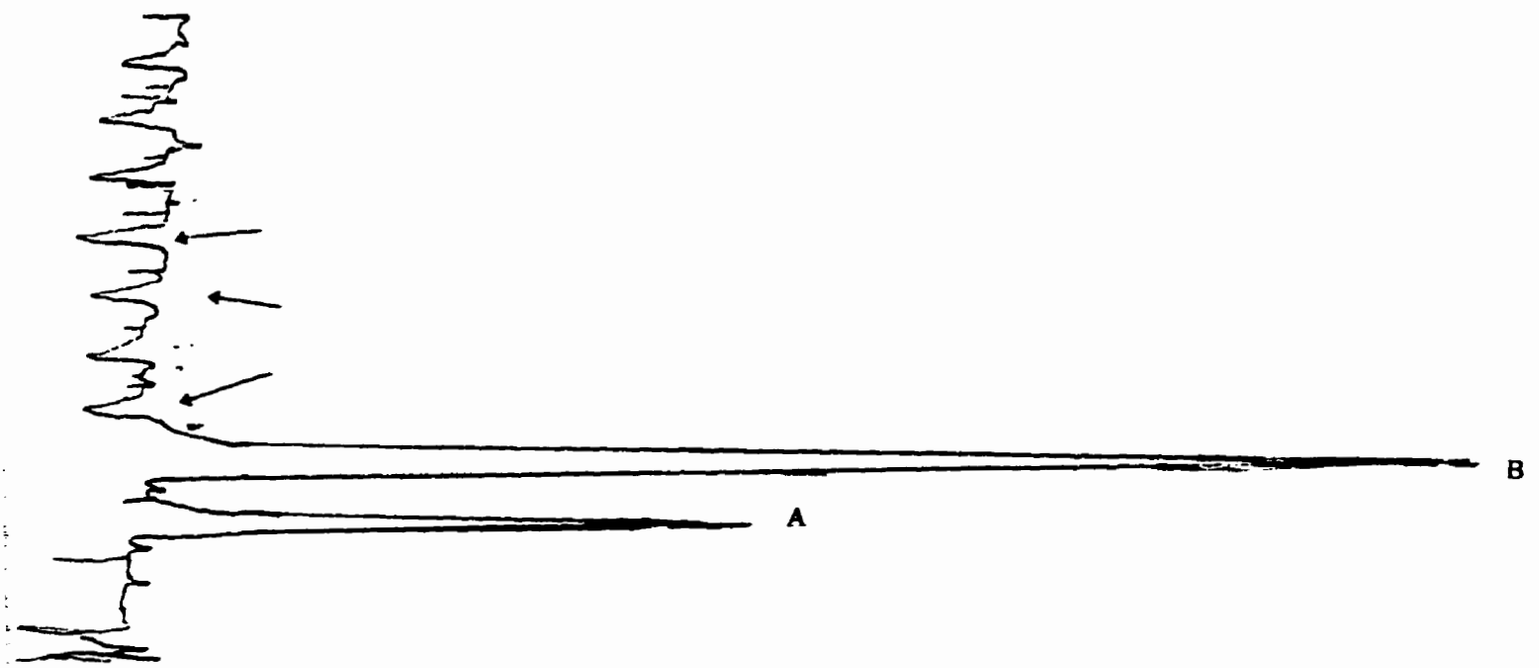


Figure 24 Example of Gas/Liquid Partitioning Method Chromatogram.

A: 1 ng STD, B: 2 ng STD The arrow indicates negative peaks.

3.3.5.1 Gas/Liquid Partitioning Method Improvement.

The GLPM for tissue samples, as used by different laboratories, is not sensitive to Hg concentrations lower than 40 ng Hg L^{-1}. Because the sensitivity of the method is not low enough, only samples in which biotic material has been concentrated, e.g., the 0.1-3 µm fraction, were supposed to be assayed by this method, as discussed below. The disadvantage of processing dewatered samples is that a large amount of sample (20 L) must be processed over several days giving rise to opportunity for contamination.

The CVAA spectrophotometer was specifically set up to detect low concentrations of Hg. In order to insure that samples were uncontaminated by the detection process, clean tubing, low Hg J.T. Baker acids and blank checks were analyzed throughout. Fig.24 shows the main features of the GLPM chromatogram and Appendix 1 the example of the GLPM chromatogram. The set of standards was run before and after each run. Blanks and one or two standards closest to the sample concentration were run every ten samples. The relationship between standards and number of unit of chromatogram was linear.

The following assumption was used in order to analyze Hg in the 0.1-3 µm fraction: If water contains from 1-10 ng L^{-1} Hg (Richman et al., 1988), and 20 L of such a sample is concentrated to about 300 mL, there will be 20-200 ng ABS or 67-670 ng L^{-1}. After modifications, the method gave reasonable results for all fractions except unmanipulated water samples and filtrates, where Hg was undetectable.

Interference may occur with high chloride (Cl^-) content due to liberation of free chlorine (Cl_2) during oxidation. Chlorine absorbs at 253.7 nm. As a solution to this problem, hydroxylamine was added to eliminate Cl_2.

Volatile organic compounds, especially aromatics can cause positive interference by absorbing at 253.7 nm. The interference can be confirmed by analyzing samples without $SnCl_2$. Care was exercised to prevent such interference.

3.3.5.1.1 The Detection Limit of the Gas Liquid Partitioning Method .

The instrument detection limit for the gas-liquid partition method applied to tissue samples is 0.2 ng mL^{-1} or 200 ng L^{-1}. The method detection limit is 10 ng Hg g^{-1} based on a 1 mL solvent aliquot of a 2 g sample extract.

After modifications the new improved CVAA instrument detection limit was based on 1 ng STD (Table 10) expanding to the full scale of the recorder. The linearity of the system was established near the samples concentration 0-3 ng L^{-1} ABS (Appendix 3). Maximum acceptable noise was established at about 5 mm of the baseline. The instrument detection limit was established as 1/10 of this 1 ng STD, i.e., 0.1 ng absolute. The new method detection limit was 4 ng L^{-1} based on 20 mL of concentrated and digested sample.

To prevent vapor from entering the instrument, a trap consisting of about 200 mL of reagent grade concentrated H_2SO_4, stirred constantly, was added between the tubing and the CVAA analyzer. At this stage the chromatogram showed negative peaks, meaning the samples were cleaner than the reagent used (Fig.24). To improve the sensitivity and minimize baseline noise of the chromatogram the wash had to be changed from 2%:3% of sulfuric and nitric acids to 1%:4% of HNO_3:H_2SO_4 which represented the same percentage of acids as in the sample. Reagent grade acids had sufficiently low blank to be used for washing. These procedures lessened system noise, so that the baseline held steady. Sensitivity, reagent blanks and baseline noise were improved substantially. The method was developed during spring and summer 1993.

3.3.5.1.2 Reproducibility and Accuracy of the Gas Liquid Partitioning Method.

The accuracy, the closeness of the measured value to the known value, was established by the linear regression to the known aqueous standards (Appendix 3). The reagents were prepared and machine set up slightly differently for each run, but the standards were always relative to samples. The standard deviation of standards was below 3% within run. There are two ways to establish the accuracy. First, a standard reference material of known concentration may be used. But

there is no reference material comparable to aqueous concentrated bacterial samples. Second, the same samples are analyzed by different laboratories (the minimum about twenty) and the mean value and standard deviation within laboratories calculated. Usually several concentrations are assessed. The procedure for such standard analyses are established at laboratories conducted by the certifying body of the Canadian Association for Environmental Analytical Laboratories (Inc.) (Suite 300, 265 Carling Ave., Ottawa, Ontario, K1S 2E1). Therefore no adequate measurement for accuracy was established and standard preparations were assumed to be correct.

3.3.5.2 Gold Trap Amalgamation Method Improvement.

Different techniques may be employed to protect the gold trap from deactivation. Deactivation is thought to be caused by moisture (Gill and Fitzgerald, 1987) or by acidic fumes (Bloom and Crecelius, 1983). Bloom and Crecelius (1983) trapped acid fumes in a solution of $SnCl_2$ placed between the reduction vessel and the gold trap. Gill and Fitzgerald (1987) placed a K_2CO_3 moisture/aerosol trap after the purging vessel and before the gold trap. For the present work, in order to trap moisture, a $CaCO_3$ trap was installed at the outlet of the purging bottle and before the gold trap (Flowchart 2). High quality non-contaminated Drierite ($CaSO_4$, W.A.Hammond drierite company, Xenig, Ohio) was obtained from R.Flett of Flett Research Ltd.(Winnipeg). As moisture was thought to be the main cause of problems, $CaSO_4$ was chosen as a trap because the color indicator $CoCl_2$ changes color from blue to pink when saturated by moisture. The trap worked well for several hours, but after that standard reproducibility became unsatisfactory. The trap had to be renewed and the system recalibrated. In the present study, water and acids did not present a problem while using the gold trap, but chlorine did. Therefore the $CaCO_3$ trap could be withdrawn. Addition of hydroxylamine sulfate was made to the sample after all other reagents had been added to prevent the gold trap being damaged by chlorine.

The method was put into working condition over a period of five weeks during summer, 1993. In comparison to GLPM, where the number

of injections could run up to 120 per day, the number of injections with GTAM Method was limited by the purging time (15 min.) for each injection. Conditioning of the equipment, standards preparation and running, blanks cleaning and running, and sample and reagent preparation limited time for sample analysis to a few per day.

3.3.5.2.1 Detection Limit of the Gold Trap Amalgamation Method.

The method detection limit was established as 1/10 of the standard expanding to the full scale of the recorder, e.g. 0.5 ng ABS. The established linearity of the system within the concentration range measured shows Appendix 4. The method detection limit was then estimated at 0.500 ng L^{-1} based on 100 mL of digested sample. This detection limit is comparable to that of Flett Research (30 pg per 70 mL sample or 0.428 ng L^{-1} , R.Flett, personal comm.). The reagent blank was estimated at 40 pg per sample or 0.4 ng L^{-1}. The single reagent analysis revealed that the highest contamination input is from the Drierite. The overall blank was low at 64 pg per 100 mL sample or 0.64 ng L^{-1}. Therefore, no further attempt at method improvement was made and the method blank was determined at the beginning and the end of analyses and subtracted from the samples. On several occasions the baseline noise could not be explained by sample variation or by contamination. The problem was recognized to be due to power unstability. Constant current appeared to be important in the maintenance of a low signal/noise ratio on the chart recorder. The precision calculated two times from two replicates on July 29, 93 indicated analytical error below ±2%. The frequency of data analyzes did not permit more replicates.

3.3.5.2.2 Reproducibility and Accuracy of Gold Trap Method.

Fig.25 shows the main features and accuracy and precision of the GTAM, Appendix 2 is an example of the original chromatogram. The standard of 0.5 ng ABS Hg had standard deviation of ±0.06 ng ABS. The gold traps were blanked after each sample, so that any carryover could be minimized and the gold trap cleaned. The method requires clean

reagents, e.g. UltraUltrex® J.T Baker reagents, and clean handling techniques. The blanks are minimal, always below 10 units, as shown on Fig.25. The method precision was assessed at 9% from five replicates of the same sample analyzed on the same day. The precision, the deviation of the number of replicates, was assessed for samples were the amount of material allowed measurement. For example, the precision of samples on Sep 15, 1993 was assessed at ±2.5 and ±6.3%.

3.3.6 Methylmercury Analysis: Extraction System Improvements.

Since MeHg occurs in natural waters in low concentrations (Parks et al, 1989), only concentrated 0.1-3 μm and 3-20 μm samples could initially be assayed for MeHg by GLPM. In the first attempt to analyze MeHg a tissue method (Uthe et al, 1972) was modified for use in teflon tubes. Ahmed and Stoeppler (1986) recommend teflon for its inert surfaces. The procedure involved extraction of methylmercury from the sample by acidic NaBr and $CuSO_4$ as methylmercury bromide (MeHgBr) and subsequent partition into CH_2Cl_2. The tubes were shaken lengthwise for 15 min. with two stainless steel balls, which functioned during extraction to fracture cell walls. Only one lot of CH_2Cl_2 was found pure enough for analysis. The teflon tube caps did not seal properly, so the liquid sample leaked. Furthermore, the teflon tubes available held only 25 mL of sample, which volume contained very low amounts of MeHg. For these reasons, this method had to be abandoned. Uthe et al. (1972) used stainless steel centrifuge tubes with stainless steel caps which prevented any leaking. They analyzed thicker and smaller (8 mL) tissue samples.

In order to obtain maximum amounts of MeHg for analysis, the entire volume of the whole concentrated 0.1-3 μm fraction (300-450 mL) and ¾ of 3-20 μm filter, less volume and ¼ filter for total Hg, respectively, were placed in separatory funnels. Concentrated samples contain water, so the amount of solid reagents to be added was recalculated and dissolved directly in the sample. The amount of

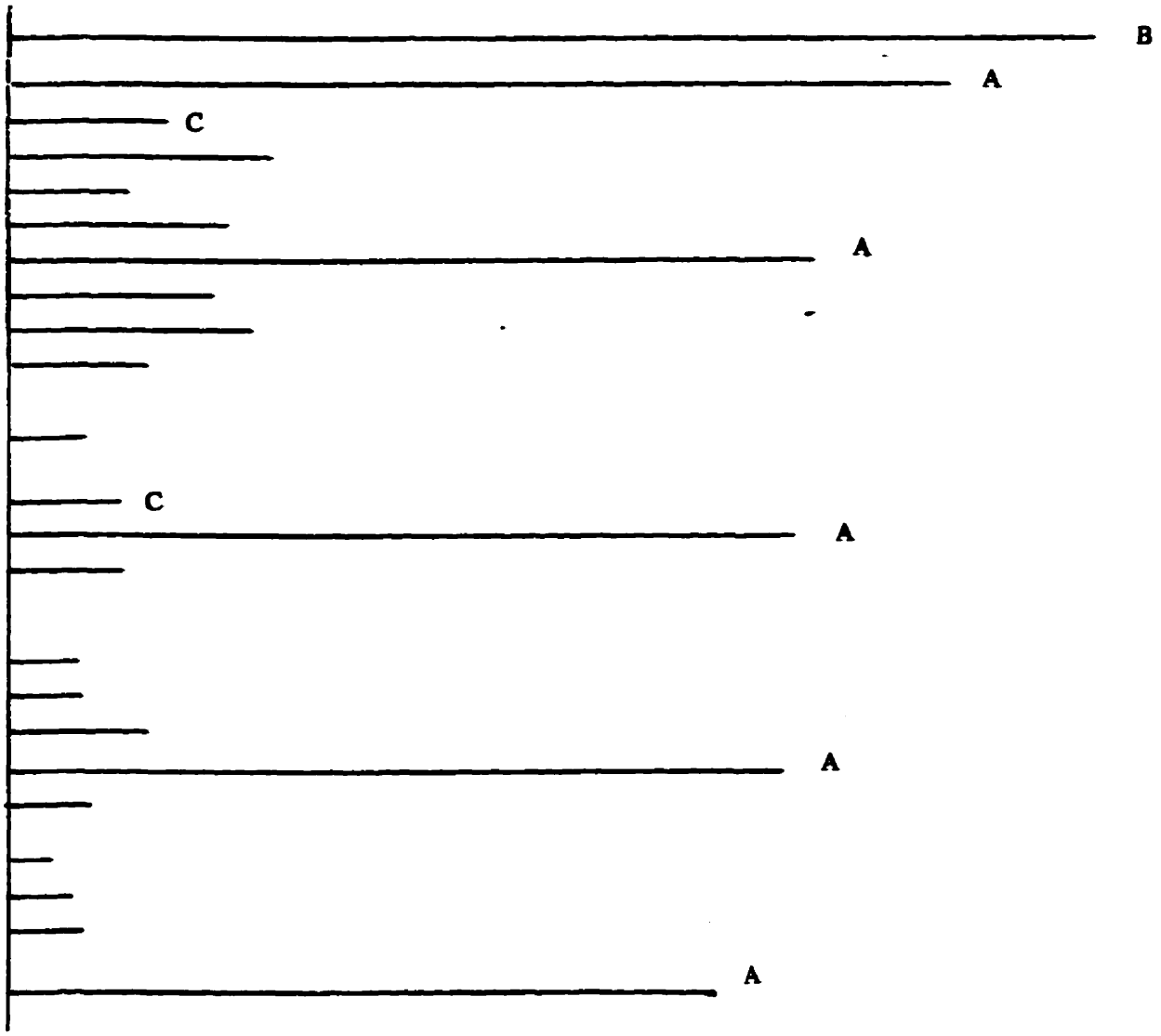


Figure 25 Example of Gold Trap Amalgamation Method Chromatogram.

High peaks: A: 0.5 ng STD absolute, B: samples

Low peaks: blanks, C: filtrate

reagents was in proportion to volume of sample and as that contributed to the reagent blanks. When a supply of high purity CH_2Cl_2 was obtained, the reagent blanks improved and preliminary data obtained (Tab.13).

3.4 Conclusions.

Both the gas liquid partitioning method (GLPM) and the gold trap amalgamation method (GTAM) are applicable to analyses of the low concentrations of Hg found in the freshwater systems. The GTAM requires specialized equipment and trained technicians. This method has several advantages: a) the sample can be small; b) no manipulation is required; and c) it is very sensitive (0.5 ng L^{-1}). The disadvantages are: a) it is relatively slow (about 4 samples h^{-1}); b) it requires ultra pure reagents, which are often costly; and c) it requires clean uncontaminated handling. The GTAM is very sensitive (500 pg L^{-1}) and applicable to unmanipulated samples containing natural background levels (pg L^{-1}) of Hg found in the environment. The GLPM also has many advantages: it is a) relatively simple and easy to use and well established in the literature; b) inexpensive; c) fast, as up to hundreds of analyses per week (depending on the matrix) can be performed; and d) not liable to contamination. Even though dewatering is labor and time intensive, this method can be used to furnish results in cases where maximum accuracy is not a priority, e.g., contaminated sites. The GLPM, commonly used in laboratories for analyzes of relatively high (µg g^{-1} Hg, detection limit 40 ng L^{-1}) tissue samples, but was adjusted to lower detection limit (4 ng L^{-1}) as described in this chapter and applied to concentrated freshwater samples containing naturally elevated concentrations (ng L^{-1}) of Hg.

Methylmercury analyses performed involved GLPM and extraction techniques. Measurements were difficult to obtain because extraction reagents blank were high. This method, as it stands would require Hg clean reagents for MeHg analyzes.

4. Microbial Interactions with Mercury.

4.1 Literature Review.

4.1.1 Bacteria as Mercury Methylators and Demethylators.

Environmental concentrations of Hg species are influenced by net methylation (methylation and demethylation) processes. The ability to methylate Hg has been found among anaerobes (Slotton et al., 1995), and facultative anaerobes and aerobes (Robinson and Tuovinen, 1984). Although bacterial methylation appears to be stimulated by anaerobic conditions (Parks et al., 1989), at least limited oxygen is required for these processes to occur in nature. Under anaerobic conditions, in the presence of hydrogen sulfide (H_2S), Hg will precipitate as HgS, which is considered unavailable for methylation (Winfrey, 1993). Biotic (microbial) methylation appears to produce most available methyl-mercury (MeHg), but abiotic methylation is known to occur (Weber, 1993). It is considered important in wetlands where metals act as catalysts in the methylation of organic matter (Zillioux et al., 1993). The amount of MeHg in lake water depends on ambient pH and the dissolved organic carbon (DOC) concentration. At lower pH (pH 5-7) MeHg is produced, while at higher pH (>7) more dimethylmercury (DiMeHg) is produced (Xun et al., 1987; Miskimmin et al., 1992). Methylation processes can be described as percentage of total mercury, e.g. the concentration of MeHg in the flooded L979 system can be up to 62% of the total mercury (Kelly et al., 1997). Methylation rates in water samples from eutrophic, natural, and experimentally acidified ELA lakes range from 3.8, 20-29, up to 132% L^{-1} h^{-1}, respectively (Xun et al., 1987).

The types of microorganisms responsible for methylation in freshwater ecosystems are not well known, but sulfate reducers group were linked to methylation by Berman et al (1990) and Gilmour et al (1992). Only a few species, such as *Desulfovibrio desulfuricans*, and compounds, as methylcobalamin, have been identified as Hg methylators

(Choi and Bartha, 1993). Positive net methylation produces the organo-Hg which is available for bioaccumulation. Mason et al. (1995) found that bacteria are also capable of reducing Hg^{2+} to Hg°, thus directly decreasing the amount of ionic Hg available for methylation. These microorganisms are <3 μm in size, heterotrophic bacteria in freshwater and cyanobacteria in marine waters (Mason et al., 1995). Methylmercury species can be converted by microbial processes to volatile Hg° in the process of demethylation. The main demethylation processes seem to occur in aquatic sediments under anaerobic conditions (Furutani and Rudd, 1980).

4.1.2 Bacteria as an Adsorption Site for Mercury.

It is recognized that Hg (Jackson, 1988b; Rudd and Turner, 1983; Rudd et al., 1983, Winfrey and Rudd, 1990) as well as other heavy metals (Sigg et al., 1987) present within the water column and sediment are controlled by adsorption to material, which can be described in several ways (Fig.22): a) as suspended and sedimentary Hg binding substances, such as FeOOH, MnOOH, organic matter, sulfides, and clay (Jackson, 1988b); b) as humic matter (Hessen, 1992), which can bind 50-90% of total Hg (Weber, 1993); c) as particulate matter, including bacteria (Winfrey and Rudd, 1990). Most bacteria fall into the size range of 0.1-2.0 μm and with their high surface/volume ratio are prime candidates for Hg adsorption; d) as DOC, which binds Hg^{II} (Miskimmin et al., 1992). The DOC is defined as "dissolved" within <0.2-0.45 μm size range by Jensen and Sondergaard (1982), but this size fraction actually contains bacteria and other particles such as small algae and detritus.

4.1.3 Bacteria, the Main Compartment for Hg in the Aquatic System.

Very little is known about the Hg burden of bacteria in fresh

waters. Links between methylation (Xun et al., 1987) or lake acidification with subsequent methylation and bioaccumulation of MeHg in fish have been suggested (Richman et al., 1988). Bioaccumulation of MeHg through the food chain is generally accepted as one possible route of uptake of MeHg into higher organisms (Richman et al., 1988; Riisgard and Hansen, 1990; Cabana et al., 1994). Recent work suggests that additional research on the role of bacteria in Hg transfer and bioaccumulation through the food chain is required (Zilloux et al., 1993; Bryan and Langston, 1992). If Hg is transferred via the food web, then a first step is to demonstrate that Hg is associated with biotic material. Bacteria not only produce, but can contain MeHg on or within their cells. Studies suggest that Hg can be bound onto the surfaces of particulate matter (Kuiper, 1981; Bryan and Langston, 1992) and, as well, that particles are responsible for the distribution of Hg in water bodies. Bacteria make up the bulk of particulate material in the size class (0.1-3.0 μm) studied [as discussed in section 4.2. citing work of Li and Dickie (1985) and Fuhrman and Azam (1980)]. Therefore it is likely that bacteria carry a high Hg-burden as well as contribute to transformation of Hg species.

4.2 Present Study.

4.2.1 Introduction.

Study Sites: L632 and Flooding of L979.

A full scale study of mercury (Hg) and green house gases, carbon dioxide (CO_2) and methane (CH_4), fluxes was conducted at L979 from 1991 to 1995 (Kelly et al, 1997). The pond was flooded two times during the summer months 1993 and 1994. The present study was conducted during 1993 to examine the Hg burden in the microbial fraction of the biota. The pond area had increased from 2.39 ha to 14.4 ha by flooding the peat land 1.3 m above the previous pond level (Kelly et al., 1997). L632 was chosen as a reference lake.

4.2.2 Methods and Materials.

4.2.2.1 Sample Collection.

Lake 979 was sampled before, during and after flooding at the same time as reference L632. Sampling was done from a canoe with all the precautions described as in section 3.2.1.2. All samples were preserved in 1% HNO_3, (final concentration), double wrapped, refrigerated and transported to Freshwater Institute. Lakes were sampled during the summer of 1993 at two weeks intervals. Two 20L samples were taken at each sampling interval and labeled as lake # A and lake # B, e.g. L979A and L979B.

4.2.2.2 Description of Fractionation Apparatus and Filtration Procedures.

4.2.2.2.1 The Whole Lake Water Samples.

The whole lake water samples (250 mL) were collected into Hg clean (blanked) polyethylene bottles at the arm depth at the central buoy, treated with HNO_3 to 1% (final concentration (Flowchart 4A, sample 1), refrigerated and transported for analysis.

4.2.2.2.2 >20 µm fraction.

Flowchart 4A shows how the >20 µm fraction was obtained. Two 20 L aliquots of lake water were filtered through a 20 µm pore Nitex® filter mounted in an Amiad 1.5" T filter holder (Amiad filtration and irrigation systems, Amiad USA, Inc., Van Nuys, CA 91413, Berma and Kimor, 1983) into stainless steel pressure cans, using a battery powered pump. The material retained on the filter was washed into separated 250 mL PVC bottles (Flowchart 4, sample 2).

4.2.2.2.3 3-20 µm fraction.

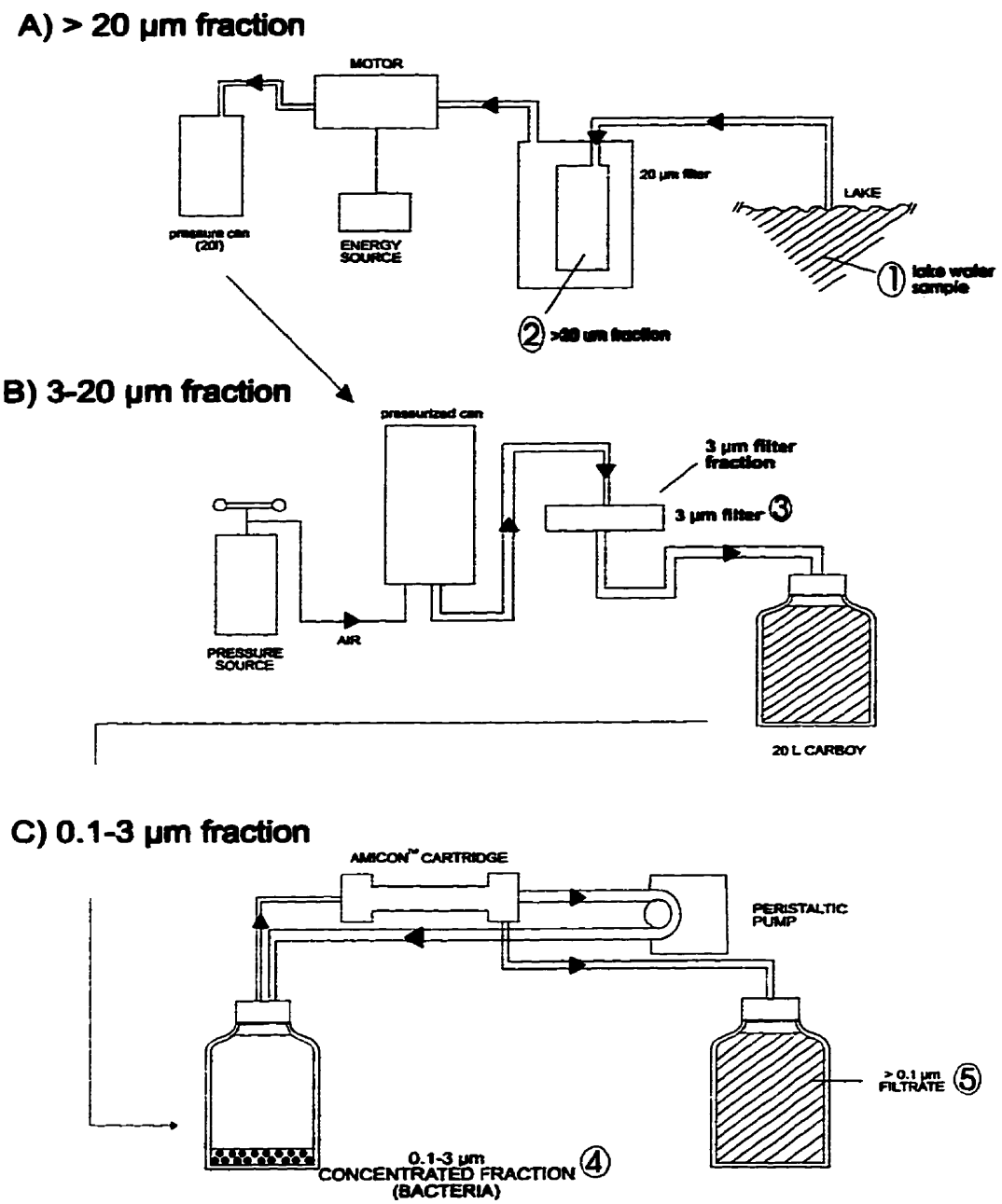
The two stainless steel cans containing the water filtered through >20 µm Nitex® were brought to the laboratory at the ELA camp and the water in each was immediately filtered under pressure through a 142 mm polycarbonate Nucleopore® diameter 3 µm pore size filter (Flowchart 4B). The filter was mounted on a stainless steel screen situated on a tripod, connected to a pressurized air cylinder which forced water at 50 pci (pound per cubic inch) through the filter. The filter retained material measuring 3-20 µm (Flowchart 4B, sample 3) and the filtrate, <3 µm particulate fraction, was collected into the 20L carboy (Flowchart 4B).

4.2.2.2.4 0.1-3 µm fraction.

Flowchart 4C shows how the concentrated 0.1-3 µm fraction (Flowchart 4C, sample 4) and the <0.1 µm filtrate (Flowchart 4C, sample 5) were obtained. The concentrating system consisted of an AMICON® cartridge (0.1 µm cut off pore size), the 20 L carboy containing the sample pre-filtered through a 3 µm filter, a pressure pump and a reservoir which received the filtrate. The unique principle of this system is based on dewatering instead of filtering the sample. Particulate material is pushed along the AMICON® cartridge, and is retained within the system (Flowchart 4C, sample 4), while water and colloids measuring <0.1 µm flow through the membrane and move into a collection vessel (Flowchart 4C, sample 5). Part of this concentrated sample has to be flushed out of the system because of the dead volume of the cartridge and tubing which serves to circulate the sample into the cartridge.

4.2.2.2.5 Mercury Assays.

Because leakage occurred during fractionation of Aug 3, 1993 samples, analytical results for concentrated and filtrate analytical results could not be obtained. September and October data were analyzed from fractions which contained the most Hg, e.g., in the concentrated (Table 11 and 12, column B) and filter (Table 11 and 12, column C) samples. The two methods were compared on September 15, 1993, as described in section 4.3.1.6. During routine chemical assays multiple analyses are not performed; the acceptable accuracy and reproducibility is supported by the methodology. In some cases the multiple analyses could not be even performed. For example fraction >3 μm is a filter which had to be divided for analyses of MeHg (3/4) and total Hg (1/4) on August 3, 1993. The same fraction was used four times (4 * 1/4 of a filter) by GTAM on Sep 15, 1993, giving four replicate results).



Flowchart 4 Whole Water Sample Fractionation Procedure.

A) <20 µm fraction processed on the lake, 1 lake water sample, 2 > 20 µm fraction; B) < 3 µm fraction processed at ELA lab, 3 3 µm fraction, C) >0.1 µm fraction processed at FWI, 4 concentrated fraction, 5 >0.1 µm filtrate

4.3 Results and Discussion.

4.3.1 Assays of Fractionated Samples for Total Hg and MeHg.

The analyses of total Hg were performed using gas/liquid partitioning method (GLPM) and gold trap amalgamation method (GTAM) as described in sections 3.3.5.1. and 3.3.5.2. The analyses of MeHg were performed by using GLPM method as described in section 3.3.5.1.

4.3.1.1 The Whole Lake Water Sample Total Hg Concentration Results.

The whole lake water sample (Flowchart 4A, sample 1) did not contain detectable amounts of total Hg as measured by GLPM. For this reason no data are shown for this fraction. The whole lake water was not analyzed by the GTAM.

4.3.1.2 > 20 µm Fraction.

The >20 µm fraction contained material bigger than 20 µm, mainly algae and zooplankton (Flowchart 4A, sample 2). Preliminary data from this fraction show low concentrations of Hg (Table 11 and 12, columns D). On August 3, 1993 the >20 µm fraction contained concentrations of Hg from 0.01 to 0.02 ng L^{-1} (Table 11, column D) or <1% of the total analyzable Hg by GLPM. On September 15, 1993 values obtained by GTAM were similar at 0.1 ng L^{-1} (Table 12, column D). I measured up to 110 pg L^{-1} of total analyzable Hg in this fraction, which represents mainly zooplankton and 6-70% of total biomass of algae (D.Findlay, personal com.). The analysis of single large zooplankters showed, that Hg concentration in single animal is very low and for some species, values were close to reagent blanks (M.Paterson, personal

com.). But the concentration of large numbers of certain size fractions of zooplankton by dewatering or other differentiated manipulation can gather enough biomass for assessment of Hg (Tremblay et al, 1995) in species known to be sensitive to heavy metal contamination (Lawrence and Holoka, 1991). Furthermore, nearly 100% of the analyzable Hg in large species of zooplankton and in some other biological compartments is MeHg (M.Paterson, unpublished data). The dewatering method than would be a prime candidate for MeHg analyzes in zooplankton. For example, during the peak period after flooding in October, 1993 I measured 6.7 ng L^{-1} of total Hg in the microbial fraction (Table 12, column B). During the same time the MeHg burden in zooplankton can be estimated from estimates of zooplankton carbon and MeHg burden to be 45 pg MeHg L^{-1} (M. Paterson, unpublished data). If this amount of MeHg is available on a daily basis to higher trophic levels, it has to be considered in bioaccumulation studies.

Table 11 Hg Concentration in Size Fractionated Biotic and Filtrate Samples by GLPM.

	S I Z E F R A C T I O N				Total Hg
Date/Lake Sample	A <0.1 µm	B 0.1–3 µm	C 3–20 µm	D >20 µm	A+B+C+D
3-Aug-93					
L632 A	a	3.69(94.6)	0.23(5.9)	0.009(0.23)	3.9(100)
L632 B	a	b	0.2	0.017	
L979 A	a	b	0.11	b	
L979 B	a	3.7(94.9)	0.21(5.4)	0.017(0.44)	3.9(100)
15-Sep-93					
L979A	d	4.02	c	d	
L979B	d	6.32	c	d	

Units in ng L^{-1}, % in parenthesis.

a not detectable

b sample lost during dewatering

c sample assayed using the GTAM

d not analyzed

Table 12 Mercury Concentration in Size Fractionated Biotic and Filtrate Samples by Gold Trap Amalgamation Method.

FRACTIONS OF TOTAL Hg BY GTAM						
DATE/COLUMN 15-Sep-93		A <0.1 µm	B 0.1-3 µm	C 3-20 µm	D >20 µm	A+B+C+D TOTAL Hg
	LAKE					
	L979 A	0.24(4.2)b	5.3(9.3)	0.12(2.1)	0.11(1.9)	5.7(100)
	L979 B	a	8.6	0.11	0.09	8.7
10 Oct-93	L979 A	a	6.7	0.11	a	
	L979 B	a	10.2	0.14	a	

Units in ng L^{-1}, % in parenthesis.

a not analyzed

b measured at the end of dewatering process

4.3.1.3 The Concentrated Filter Fraction, 3 - 20 µm.

Total Hg associated with the 3-20 µm fraction ranged from 0.11 to 0.23 ng L^{-1} as measured by GLPM on Aug 3, 1993 in both lakes 632 and 979 (Table 11, column C). Using GTAM, values of 0.11-0.12 and 0.11-0.14 ng L^{-1} were obtained on Sep. 15, 1993 and Oct. 6, 1993, respectively (Table 12, column C). These amounts represent from 2 to 6% of the total analyzable Hg in whole water samples. Most algae are present in this 3-20 µm (28-91%) and >20 µm (6-70%) fractions (D.Findlay, personal com.). Within these fractions the flagellates represented about 1% (D.Findlay, personal com.). According to my measurements fraction 3-20 µm contains 0.11-0.23 ng L^{-1}. The amounts of Hg (and MeHg) available in these fraction represents Hg which can be channeled through the food chain and accumulate in higher organisms.

4.3.1.4 The < 0.1 µm Filtrate.

Mercury was not detectable (<0.1 µm fraction, Flowchart 4C, sample 4) during the dewatering process. At the end of the dewatering process there was an increase (0.24 ng L^{-1}) in Hg concentration in the filtrate fraction as measured by GTAM on Sep. 15, 1993 (Table 12, column A). This amount represented about 4% of total Hg.

4.3.1.5 Bacterial Fraction 0.1 - 3 µm.

Samples were obtained from water taken from experimentally manipulated L979 and reference L632 as described in section 4.2.1. The fractionation is described at Flowchart 4C. In this study the cut off for living material was established at <0.1 µm. The dewatering process gently eliminated water from the sample leaving a microbial fraction sized from 0.1 to 3 µm behind in the concentrate. This method effectively retained all organisms larger than 0.1 µm and smaller than 3 µm in smaller dimension.

The microbial fraction 0.1-3 µm in linear diameter, was assayed by GLPM using CVAA as described in section 3.2.1.4. Bacteria were present in lake water in abundance of 0.5-3.2 * 10^6 mL^{-1} (Fig. 15, 16, section 2.3.2.2.), and flagellates at 2 * 10^3-2 * 10^4 mL^{-1} (Fig. 20, 21, section 2.3.3.). Most of both total Hg and MeHg was found to be present in this fraction (Tables 11-13). The amount of total Hg in the 0.1-3 µm fraction is an order of magnitude higher (3.7-8.5 ng L^{-1}, Table 11 and 12) than in the other fractions. From 92-98% of total analyzable Hg was contained in this fraction. Similar results (L632 of 1.46-6.73 and pre-flooded of 0.4-7.2 and post-flooded at 0.98-6.95 ng Hg L^{-1}, during whole year 1993) for total Hg show Kelly et al. (1997). The total Hg concentration in both flooded and reference lakes stays at the same level and the only major increase is in MeHg concentration after flooding. The values of MeHg measured on Aug 3, 1996 were 0.09 ng L^{-1} (2.4% of total Hg) at L632 and 0.45 ng L^{-1} (12.2% of total Hg) at L979 (Table 13). Kelly et al. (1997) report the concentration of MeHg before flooding to be on average 0.1 ng L^{-1} and after flooding on average 0.9

ng L^{-1} with maximum values over 2 ng L^{-1}. Kelly et al. (1997) determined MeHg concentration by aqueous phase ethylation as described in section 3.1.4.2.2.2. and total Hg by the GTAM method. Values were obtained by Flett Research Ltd., Winnipeg. The flooding did not have any effects on total analyzable Hg concentrations in L979. The total Hg concentrations include all organic, mainly MeHg, species, which amounts to an average of 5% of total Hg during the year. At maximum flooding of L979 MeHg concentrations rise several times to about 70% of total analyzable Hg (Kelly at al., 1997).

Table 13 Methylmercury Concentration in 0.1-3 µm Bacterial Samples on 3August, 1993.

Lake/Sample	conc. sample MeHg in 0.1-3 µm conc. sample	Percentage of Total Hg
L632 A	0.09	2.4
L632B	a	
L979A	a	
L979B	0.45	12.2

a lost sample during dewatering

This work was based on the assumption that dewatering using the AMICON® system (Flowchart 4C) would separate bacteria from other organisms and that Hg could be then measured in this concentrated fraction. Bacteria are usually <3 µm in their longest linear dimension, but other organisms fall into this size category as well. The system qualitatively and quantitatively separated bacteria and other unicellular organisms into the fraction size of 0.1-3 µm. Checks done by using epifluorescent microscopy showed that 99% of particulate material in the sample were bacteria. The fraction contained about 1% of detritus. Picoplankton did not occur in these samples. The concentrated sample become brown in color and likely contained high amounts of humic material (Tranvik and Hofle, 1987). Colloidal material does not stain with DAPI and therefore was not seen.

4.3.2 Interlaboratory Comparison.

The whole lake water sample contained all fractions added together, i.e., 3.9 ng L^{-1} (Table 11, column A+B+C+D) on August 3, 1993 and on average 7.2 ng L^{-1} on September 15, 1993 (Table 12, column A+B+C+D). An inter-laboratory comparison of data with Flett Research Ltd., (FR), Winnipeg was done on Sep. 15, 1993 (Table 14).

Table 14 Interlaboratory Analysis Between Flett Research (FR) and This Study.

Interlabolatory study	Date/Lake	A	B	C	B+C	D
	15-Sep-93	MeHg 0.1-3	Hg <0.1	Hg 0.1-3		TOTALHg
FR analyzes/gold trap St.Louis collection M.Samek collection	L979 B	0.52	3.84	2.8	6.64	2.5
This study analyzes GLPM	L979 A L979 B		a a	4.023 6.32		
GOLD TRAP	L979 A L979 B		0.24 a	5.26 8.55		

Analysis performed on independent samples taken from L979 on 15 September 1993. Units in ng L^{-1}. a not measured

Two samples were taken independently on 15 September, one for this study, the other by V.St. Louis. The St. Louis was analyzed at Flett Research as part of an ongoing research project contracted between FR and the FWI research group. My samples were processed as in section 3.2.1.2. and analyzed as in sections 3.2.1.5. for GTAM and 3.2.1.4. for

GLPM. Method used in this study was GLPM (3.1.4.1.2.1.) and GTAM method (3.1.4.1.2.2.) coupled with CVAA. As well, fractionated sample of this study was analyzed by FR. Results are shown in Table 14. The differences in these values are minimal considering that a) Hg values in L979 range between 0.72-7.2 ng L^{-1} of (Kelly et al., 1997), during 1993; b) different analytical methodologies were employed by FR and in the present study; and c) the time needed for dewatering was considerable, leading to possibility of contamination of the various fractions analyzed.

Table 15 gives a comparison of values obtained in this study and values obtained by FR collected close in time. The average of FR values is 5.57 ng L^{-1} and present study 5.08 ng L^{-1}. Results of this study are within the range of values found by FR during 1993.

Table 15 Values of Total Hg Measured by FR at L979 1993 on Dates Close to my Measurements.

DATE	FR	DATE	GLPM	GTAM
4-Aug-93	6.32[c]	3-Aug-93	3.9	
22-Sep-93	3.44[c]	15-Sep-93	4.02	5.26[a]
8-Oct-93	6.95[c]	10-Oct-93		6.7[b]

[a] measured week after first measurement [b] only 0.1-3 μm fraction
[c] values furnished courtesy J.Rudd (FWI), unpublished data

In the present study, sample manipulation was necessary during dewatering for use of the GLPM and subsequent dilution for assay by the GTAM method. It is then possible that some samples were contaminated, that is, Hg was added by some means to samples during processing. Such added Hg could be contained within or immediately adsorbed onto the microbial fraction. Bryan and Langton (1992), Miskimmin et al., (1992), and Kupier (1981) suggest that the distribution of Hg is dominated by adsorption onto suspended particles. This would mean that Hg can be stripped or attracted to the sample from the surrounding environment, the air, container walls or other equipment

in use. Laboratories analyzing for low levels of Hg use a Hg-clean environment, which means not only the use of ultra pure chemicals and Hg clean glassware, but also use of clean new rooms reserved for Hg analysis (V.St. Louis, personal comm.). Bothner and Robertson (1975) found that water samples stored in polyethylene bottles showed a 6-times increase in Hg concentration in 60 days. On the other hand, Yamazaki et al. (1978) in a study of inorganic and organic Hg compounds distribution within polyethylene and glass containers found losses of 30-50% Hg. Mercury was mainly vaporized (12%), or retained on side (30%) and bottom (52%) walls. In that study, mercury in solution amounted to only 6% of the initial amount added after 30 days.

Acidification of the sample prevents loss of Hg through adsorption to container walls or to methylation (Furutani and Rudd, 1980; Ahmed and Stoeppler, 1986). Ahmed and Stockner (1986) performed stability studies using ionic Hg and MeHg. They compared solutions in unmanipulated water, and in addition of 1% HNO_3, 1% HCl and 5% NaCl. The best results in PVC bottles were obtained with 5% NaCl or 1% HNO_3. Some authors (e.g. Cossa et al., 1994) recommend PTFE (teflon) bottles as the best containers for sampling, transportation and analyses of Hg, because their surfaces are inert and do not attract Hg. Teflon carboys of 20 L volume could not be found for the present work. However, carboys used in this study were double wrapped in black plastic bags and kept away from known sources of Hg. As well the black plastic bags prevent MeHg decomposition by UV light (Ahmed and Stoeppler, 1986).

Analyses are usually performed within about 24-48h of sampling to prevent changes in Hg due to contamination as well as biotic compartments. The concentration of samples was started immediately after arrival at the analytical laboratory, but the dewatering process took several days and could therefore be a source of either addition or loss of Hg. Several factors can cause Hg loss during manipulation are a) Hg is easily reduced by common reducing agents and lost as Hg°, b) Hg can adsorb to the surface of containers and penetrate through the walls, and c) Hg can undergo demethylation to Hg° due to microbial activities. Addition are through air or through non-Hg pure reagents.

4.3.3 Microbial Hg Burdens.

To evaluate the amount of Hg bound to microbial biomass,

carbon content of bacteria was estimated (Tab.5, section 2.3.2.3.). The amount of Hg carried by the 0.1-3 µm bacterial fraction on Aug 3, 1993 is estimated to be 47.4 µg Hg g C^{-1} in L979 and 160.7 µg Hg g C^{-1} in L632, 4x higher than in L979. The total Hg concentrations in the microbial fraction (0.1-3 µm) in both lakes are identical.

Because bacteria and similar size organisms create the basis of the food web, the amount of Hg each fraction holds provides information on Hg availability within the food web. Even if only a small portion of this Hg laden bacterial biomass is passed through the microbial loop to higher organisms, large amount of Hg may be transferred to organisms at higher trophic levels. For example, on Aug 3, 1993 there were 1.5 * 10^6 bacteria mL^{-1} (about 0.08 g C m^{-3}) and 3,000 flagellates mL^{-1} in L979. If we consider the grazing rate conservatively at 10 bacteria h^{-1} $flagellate^{-1}$, (grazing rate of 5-10 bacteria h^{-1} flagellate, Bjornsen, 1988, 10-100 bacteria h^{-1} $flagellate^{-1}$, Weisse, 1989, 1990) and no growth of bacteria or flagellates occurs, flagellates alone would consume almost 50% of the bacterial population in a single day. On Aug 3, 1993 there was a totally different dynamic in microbial populations in L632. Bacterial population counts reached only 0.5 * 10^6 bacteria mL^{-1} (0.02 g C m^{-3}), but flagellates numbered 17,000 flagellates mL^{-1}. This suggests that the bacterial population was controlled by flagellates as they can eat more than 8 x more bacteria than are available in a single day. Thus, the bacterial growth rate in L632 must have been high. Bacteria are consumed by flagellates and zooplankton, and flagellates are consumed by rotifers, zooplankton and planktivorous fish. Therefore, the microbial fraction of the microbial food web is an important factor in the estimation of whole water body MeHg production and in the potential of Hg for bioaccumulation within the food chain.

4.4 Conclusions.

It was found in this study that bacteria are associated with 94-98% of the Hg in the freshwater system examined. Since bacteria constitute the base of the food chain, they are likely to be the source of Hg to higher trophic levels. Algae and zooplankton fractions carry 1-6% and 1-2%, respectively, of the available Hg. Preliminary data indicates that bacteria in manipulated water bodies carry a higher

percentage of MeHg than in unmanipulated lakes. This is consistent with findings that MeHg represents high percentage of total Hg in biological compartments (e.g. Allen-Gill at al, 1995, Becker and Bigham, 1995) and flooded systems receiving input of organic matter (e.g. Hecky, 1991, Kelly et al, 1997).

Gold Trap Amalgamation Method is the analytical method of choice, because samples do not need to be manipulated for a long time before analysis. Gas Liquid Amalgamation Method is, however, a reasonable technique for some samples because it is available in most laboratories and is inexpensive.

Discussions on the validity of measurements and an inter-laboratory study are presented and values obtained compared with other available data.

5. Concluding Remarks and Speculation for Future Research.

This thesis focused on bacteria as an under-studied component of freshwater ecosystems. Microbes form a very active and important component of the trophic structure as they are involved in degradation of organic matter. As well, together with algae, they create the base of the food web in freshwater environments. The hypothesis, that bacteria are important compartment of Hg (MeHg), was confirmed. Mercury can be associated with dissolved compounds, colloids, DOC and humic substances or to particulate material: detritus, bacteria and algae. To evaluate the fate of Hg (and MeHg) within the food chain, the interactions of living organisms within this size fraction must be revealed. Specifically, the bacteria interactions with microbial food loop and microbial food web organisms and their Hg (MeHg) burden require more research.

The problem is not only to quantify bacteria in freshwater lakes by assessing their abundance and volume, but to evaluate bacterial carbon and distinguish between bacteria, DOC and other material of the same size. The microbial compartment contains populations of different entities, including living bacteria, flagellates, ciliates and algae, and non-living particles such as detritus, dead organisms and colloid material. From this point of view the fraction 0.1-3 μm contain so far by science indistinguishable amount of bacteria and DOC. Now, the question is: "Where does mercury and methylmercury reside within all this material?" Bacteria are known to be the principal methylators of Hg, but knowledge about the processes of bioaccumulation and biomagnification of Hg and MeHg within the food chain is limited. Since methylmercury is produced by bacteria, it should be found in highest concentrations in or on bacteria, thus making MeHg available for bioaccumulation in higher trophic levels. The identification of MeHg as associated with the bacterial fraction is only the first step in the study of Hg (MeHg) transfer through the food chain. Preliminary data indicate that most (up to 98%) of total analyzable Hg was found in the concentrated bacterial fraction.

Estimation of total Hg and MeHg by GLPM in unmanipulated samples was not possible due to low Hg concentration. Because of the complexity of the trophic structure, fractionation via a dewatering

process was used to separate and simplify the system. The application of the dewatering system together with the GLPM or GTAM allowed evaluation of naturally low Hg concentrations in freshwater samples.

The exact process and site of mercury methylation is not known at this time. Recent research focuses on rates of Hg methylation and MeHg fluxes within the ecosystem (T.Sellers, 1997). Methylation can occur abiotically or biotically, probably inside the cell (E.A.Henry, 1993). If MeHg does reside on or inside bacteria, this would shift views on MeHg transfer and accumulation. Future research could focus on simple food chain structures and assessment of concentrations of MeHg in discrete biological compartments such as bacteria, algae, zooplankton, benthos, and omnivorous and carnivorous fish. The fractionation and dewatering procedure is a promising method for analyzing low levels of Hg and MeHg in freshwater microbial samples.

Sulfate reducers are thought to be primary methylators of Hg in freshwater sediments (Gillmour et at, 1992) and in pure cultures (Choi and Bartha, 1993). Research should continue to identify other potentially important groups and species of bacteria in the methylation process. Research on such compounds as methylcobalamin (Choi and Bartha, 1993) indicate that these compounds are able to spontaneously transfer methyl groups to Hg, but more work is needed to explain why certain bacteria containing methylcorrinoids do or do not methylate Hg.

Genetic control of Hg transformation is promising for future research, since gene coding for organic mercury resistance has been found in some bacteria and fungi (Robinson and Tuovinen, 1984). Bacterial communities can adapt to Hg contamination by selection of specific genetic information (Barkay and Olson, 1986, Barkay et al, 1989). The *MER* operon, responsible for bacterial resistance to mercury (Barklay et al, 1990, 1991) could be used in future in mercury remediation. Methodologies involving those of genetic engineering (gene probes) could be applied (Nishio et al, 1997) to identify methylmercury producing and methylmercury resistant bacteria. Mercury resistant strains are able to turn on the genes in the presence of Hg and produce enzymes which volatilize Hg. This process is illustrated, for example, in experiments with *Thiobacillus ferrooxidants* (Baldi and Olson, 1987). Volatile Hg is lost to the atmosphere, becoming available for transport, deposition, methylation and bioaccumulation in other water bodies. This underlines the importance of studying freshwater bacterial populations and their involvement in the biogeochemical cycle of Hg (and MeHg).

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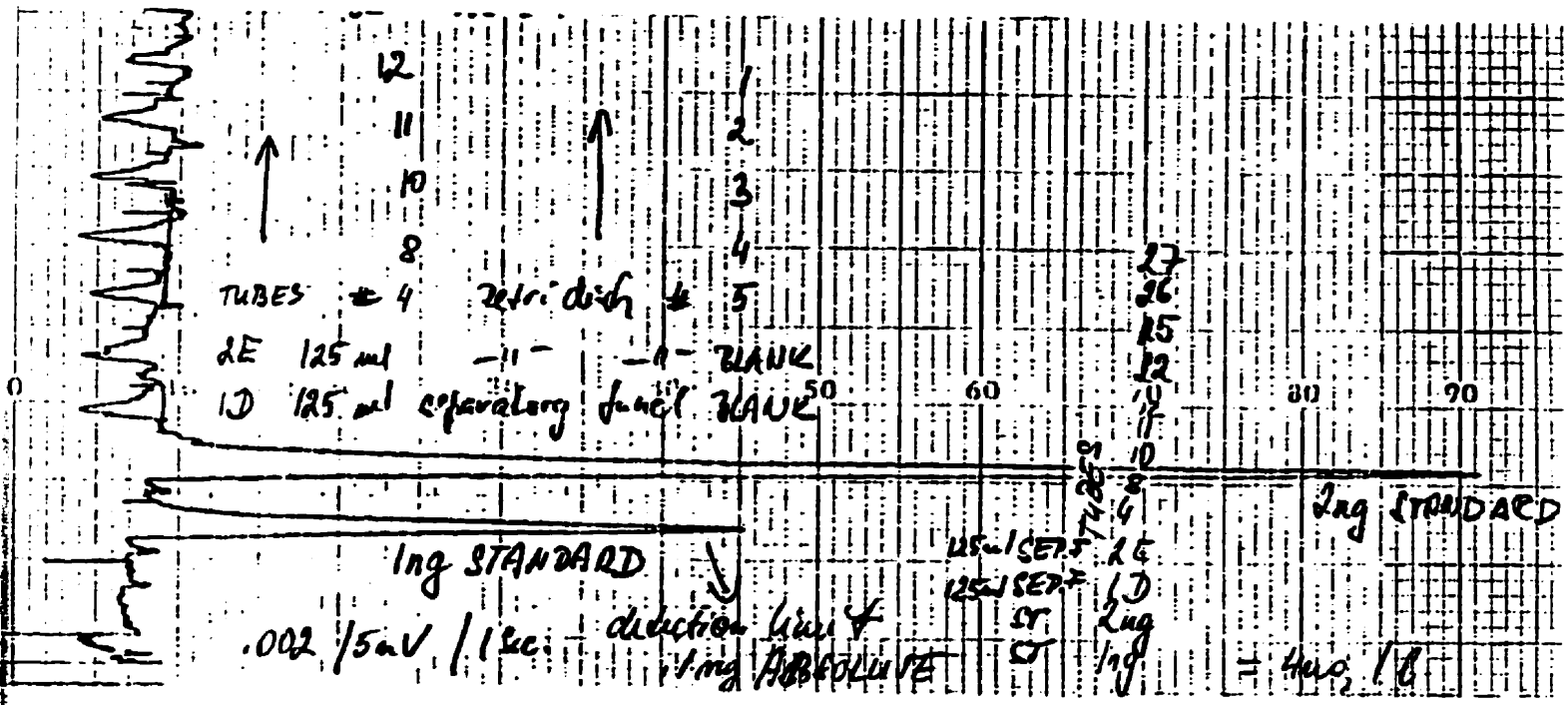
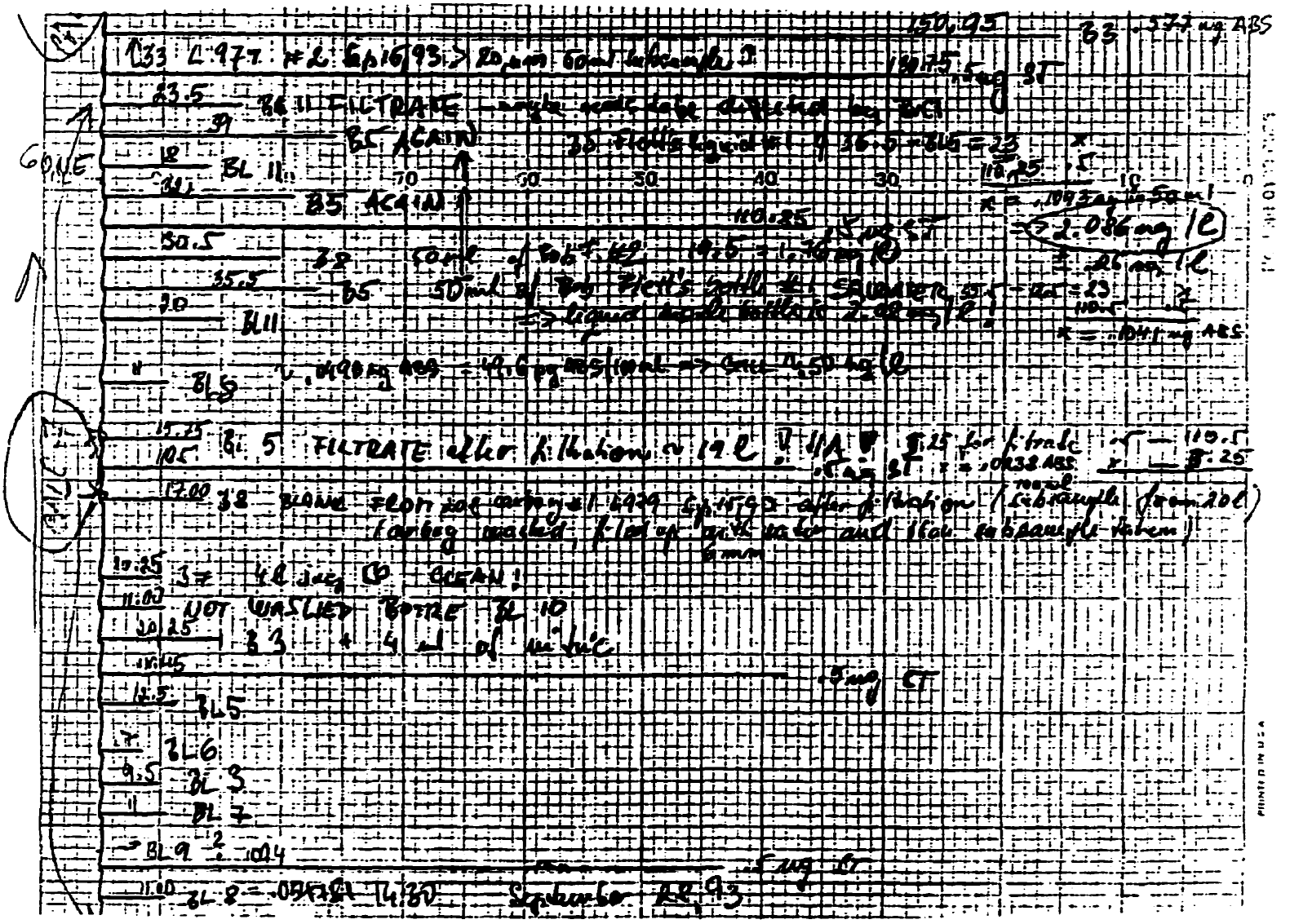
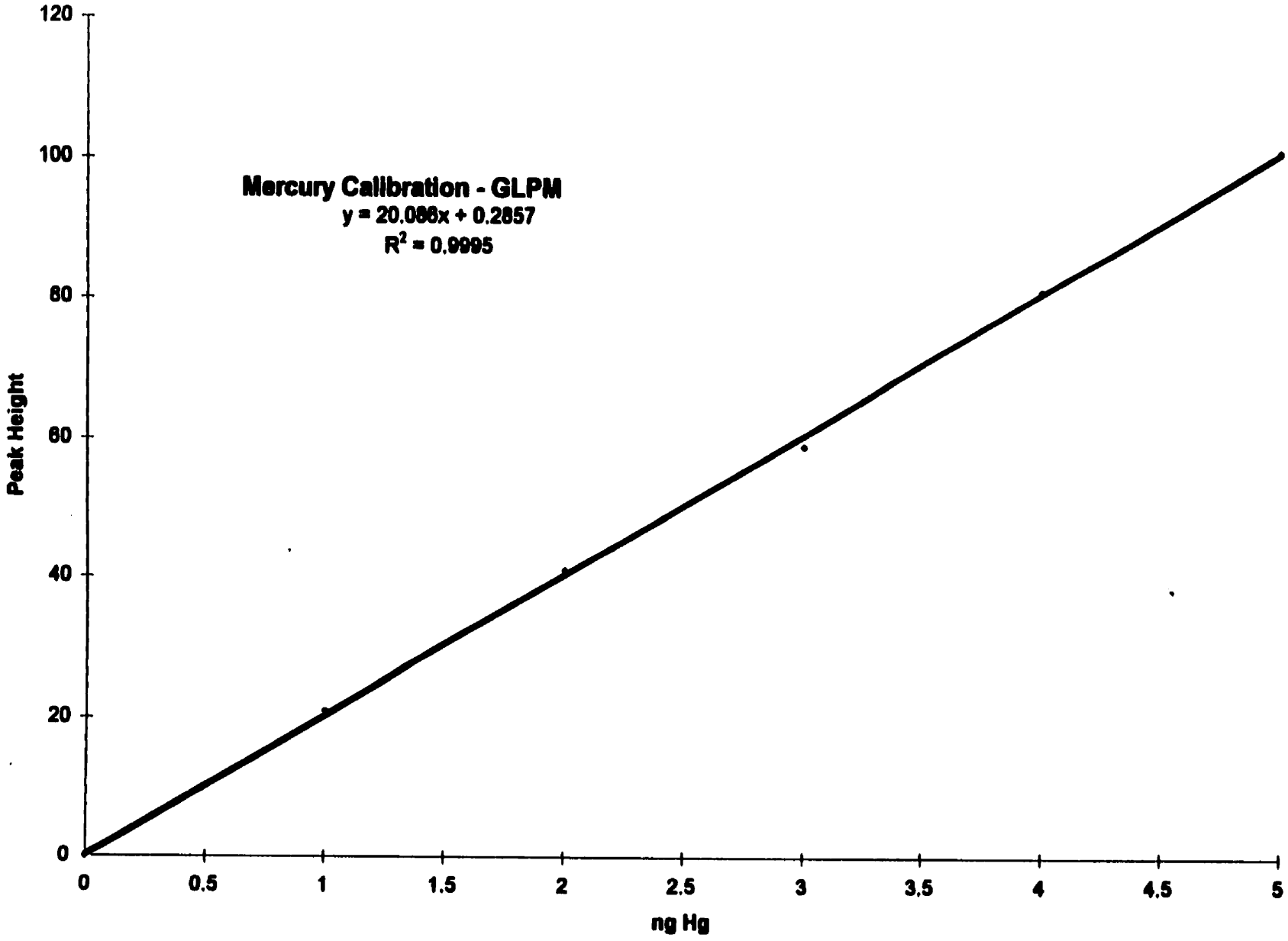


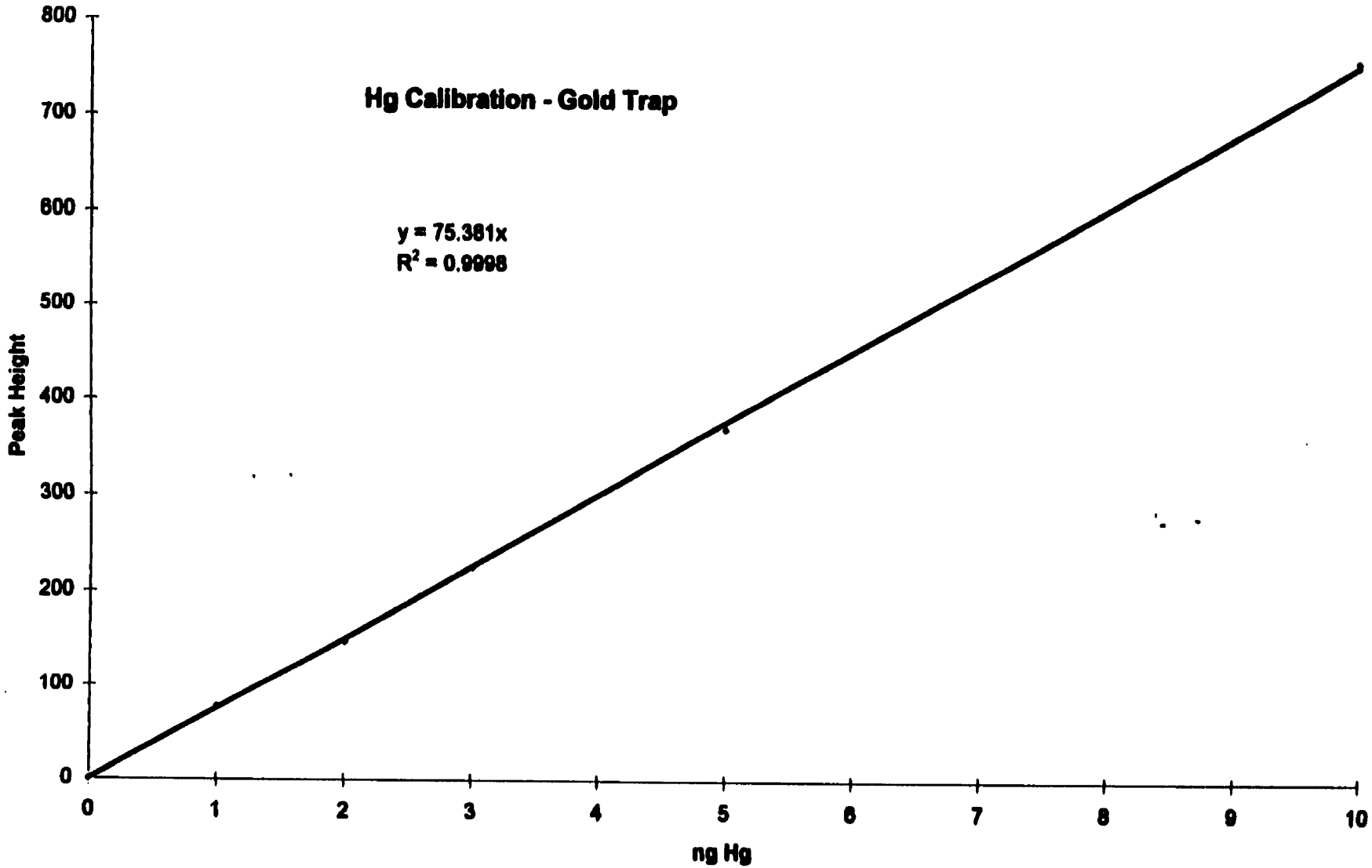
Appendix 1 Example of Gas Liquid Partitioning Method Chromatogram (original).



Appendix 2 Example of Gold Trap Amalgamation Method Chromatogram (original).



Appendix 3 Linearity of the GLPM.



Appendix 4 Linearity of the GTAM.