Microbial Study of Lake Winnipeg Sediment

By

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II Abstract

Eighteen sediment samples taken from selected Lake Winnipeg sites were analyzed using the Standard Plate Count Method to determine the average numbers of actinomycetes, bacteria, and fungi at 6°C and 23.5°C. Two-way analysis of variance, with temperature and location as fixed factors, were analyzed ($\alpha = 0.05$). Average actinomycete numbers varied with temperature ($p = 0.004$), but not location ($p = 0.762$). Average numbers of bacteria varied significantly with both ($p = 0.009, 0.045$), while those of fungi did not ($p = 0.156, 0.333$). The functional diversity of each sediment sample was assessed using Biolog© microplates. Substrate richness, the ability of organisms to metabolize 95 different carbon substrates and intensity, the rate at which these substrates were utilized, were determined. Substrate richness varied from 0 to 44.2% at 6°C and 0 to 78.9% at 23.5°C. Intensity varied from 0 to 2.77 at 6°C and 0 to 5.10 at 23.5°C. Richness and intensity varied significantly with temperature and location ($p = 0.000$) indicating communities in the South Basin are more functionally diverse. Both acid and alkaline phosphatase activity were analyzed with total activity being highest in the North Basin indicating microbial activity may contribute to phosphorus loading and eutrophication. Coliforms were isolated from 12 sediments, 9 in the South Basin and 3 in the North Basin. *Escherichia coli* were isolated from sites 3B, 60C, 7, 27S, 22 and 33 and fecal coliforms were detected at sites 3B, 60C, 7, 27S and 33. This indicates contamination of the lake and suggests sediment as a possible reservoir for coliforms.

III Acknowledgements

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1 Introduction

1.1 Indigenous microbial communities

In aquatic environments, one of the roles of microbial communities is the movement of matter, either in gaseous, in solid, or in dissolved phases (Prescott *et al.* 2002). The most prevalent cycle in aquatic environments is the microbial loop (Figure 1). From the water, photosynthetic phytoplanktons acquire nitrogen, phosphorus, carbon and other essential nutrients for growth. Some of the organic matter that phytoplankton synthesize during photosynthesis but not assimilate, enters the ecosystem as dissolved organic matter (DOM). When this plant exudate, or photosynthate, is metabolized by heterotrophic microorganisms and converted into microbial cell mass, it becomes part of the food chain as particulate organic matter (POM; Prescott *et al.* 2002). A portion of the POM is consumed by protozoa and zooplankton. After digestion, some of the nutrients from the bacteria and protozoa are released as carbon dioxide and constituent minerals (N, P, S, etc.) and cycled back to phytoplankton. Although this loop results in the rapid cycling of essential nutrients, it may limit the nutrients available for higher consumers (Prescott *et al.* 2002).

An important part of the loop is the cycling of phosphorus through the phosphorus cycle (Figure 2). Phosphorus is an essential, non-metallic, nutrient required by all life forms for the synthesis of nucleic acids, phospholipids and high energy adenosine triphosphate (ATP; Madigan *et al.* 1997). Phosphorus, unlike other cycled nutrients (N, S, and C) has no gaseous phase, and solely exists in dissolved organic and/or inorganic ionic forms. In the aquatic ecosystem, phosphorus is converted from the organic to the inorganic state (no change in oxidation state; Jones 2002). On a global scale, there are

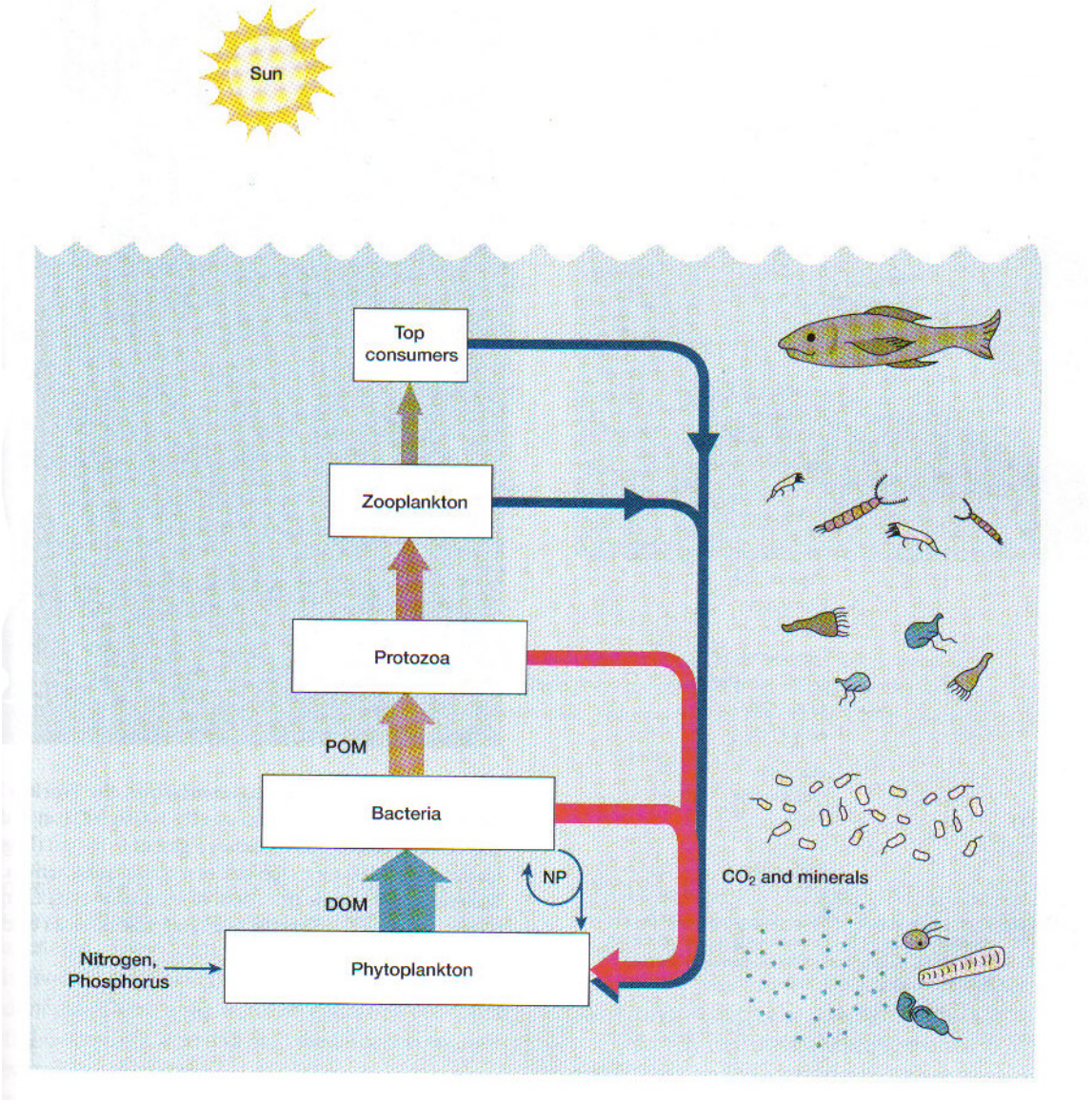

Figure 1: The microbial loop shown in red. Essentially nutrients contained in bacteria and protozoa are mineralized and looped back to phytoplankton. (Prescott *et al.* 2002).

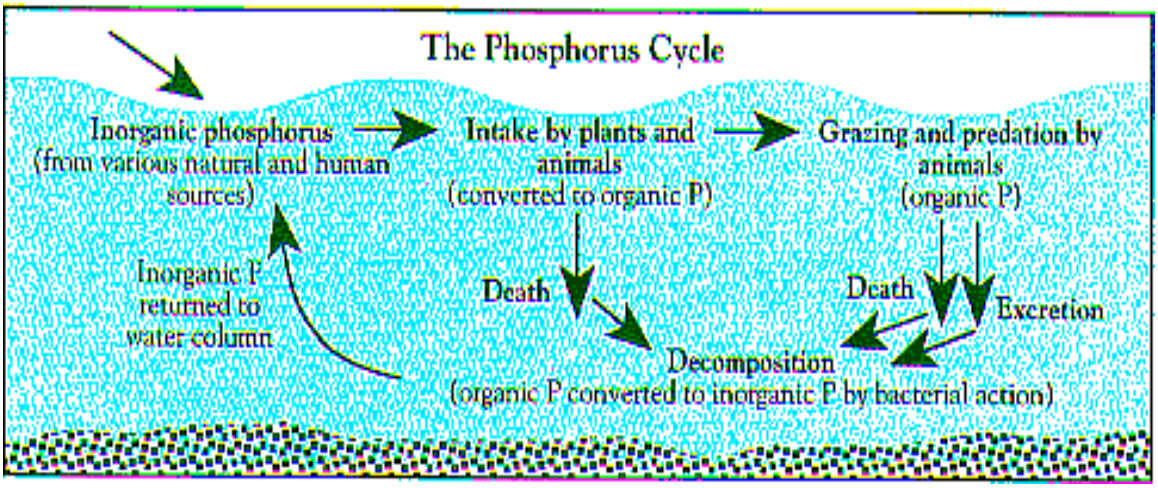

Figure 2: Phosphorus cycle occurring in a lake. In the aquatic phosphorus cycle, dissolved inorganic phosphorus is taken up by plants and animals and converted to organic phosphorus which then becomes a part of their tissue. As these organisms die or excrete the phosphorus, heterotrophic bacterial decomposition converts it back into inorganic phosphorus. During overturn of lake water, the phosphorus is once again released into the water column and the cycle repeats (EPA Volunteer Monitoring Publication 1994).

two pools of phosphorus: geologic phosphate found in the Earth's crust and sediments which is cycled slowly, and the biologically active pool, which although smaller is cycled more quickly (Jones 2002). In the geologic pool, phosphorus is typically found as salt-containing phosphate ions in terrestrial rock, such as apatite ($3Ca_3[PO_4]_2 \cdot Ca[FeCl]_2$). When released from the rock by weathering, it becomes dissolved in soil water which eventually enters the water column. This phosphorus pool is mined and used as inorganic fertilizers and detergents; however, this also leads to leaching of phosphorus from the soil into the water column (Miller 1998). It's the latter of the two pools in which microorganisms mediate the cycling of this nutrient through the microbial loop.

1.2 **Eutrophication**

Lake Winnipeg, similar to 1970s Lake Erie, has been labeled a "dead lake" as a result of its nutrient loading and algal blooms. Lake Winnipeg, although large with a relatively short water residence time (3 to 5 years), is shallow and has a high drainage to surface area which increases potential for nutrient loading (Lake Winnipeg Stewardship Board (LWSB 2005)). Over the past three decades, there has been a 10 per cent increase in phosphorus loading. Annually, 6600 tonnes of phosphorus are added into Lake Winnipeg by several sources. Approximately 41% originate in the United States, 6% from Alberta and Saskatchewan, 12% from Ontario, and the remaining 41% are from Manitoban sources (LWSB 2005). Phosphorus comes from anthropogenic sources, such as sewage, crop fertilizers, industrial discharges, livestock manure, and urban runoff; decaying vegetation and naturally rich soil; and the atmosphere (LWSB 2005).

Eutrophication is the acceleration of primary production in response to an increased nutrient supply; this process occurs naturally as a lake ages (Straskraba 1979,

Paerl 2002). However, cultural eutrophication results from nutrient loading, the addition of excessive levels of nitrogen and phosphorus into a lake, through non-natural processes. In either case, as inorganic matter content increases so too does the growth of aquatic plants, such as algae. During normal growth, algal cells absorb phosphates primarily for energy transformation reactions. Algal growth is actually limited by the amount of phosphates, usually in the form of condensed inorganic phosphates, present within the environment (Kuhl 1962). As levels of phosphates increase in the lake, excessive growth of algae creates "blooms", as seen in Lake Winnipeg (Figure 3).

There are several problems associated with algal blooms. (1) Blooms decrease the transparency of water blocking light from submerged plants. As light becomes more and more limited, the natural flora dies; eventually, this will lead to the destruction of the habitat of aquatic animals. (2) During winter, the dead and dying algae of the bloom settle to the bottom of the lake. As these masses of algae are decomposed by aerobic heterotrophic microorganisms in the sediment, oxygen is consumed reducing the total oxygen content in the benthic region of the lake. Oxygen depletion may result in the death of fishes and other aerobic organisms of the food web. (3) Also, as algal blooms are killed and are decomposed by heterotrophs, there is a release of phosphorus from the cells; this additional phosphorus will further support algal growth during the next growing season (Fitzgerald and Faust 1967). (4) Finally, many species of blue-green algae (cyanobacteria) produce and excrete toxins which may cause illness in those who consume the water.

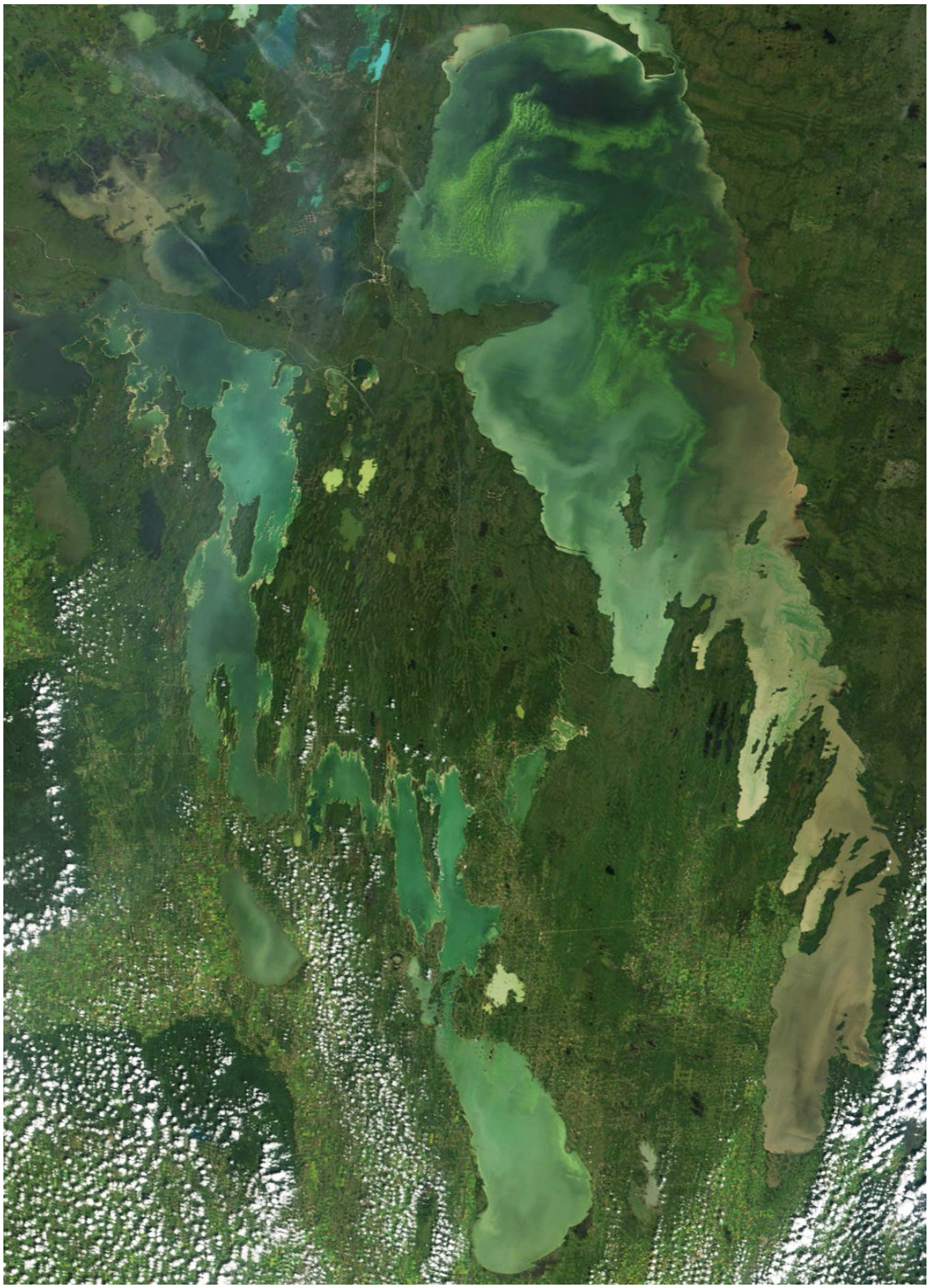

Figure 3: Satellite image recorded August 13, 2004 by NASA satellites using MODIS showing algal blooms (bright green areas) in the North Basin (Greg McCullough, University of Manitoba).

1.3 Transient microbial communities

Organisms associated with sewage and wastewaters that enter the aquatic ecosystem constitute "transient" or "migrant" organisms. Often the introduction of these organisms has a negative impact, since humans may acquire numerous diseases from microorganisms in water, either by consumption or contact with water itself (Hurst 2002). Some of the transient microorganisms are introduced into the aquatic habitat via the direct or indirect influx of animal and/or human feces into the water. The majority of these "fecal coliforms" are relatively harmless as they constitute normal inhabitants of the gastrointestinal tract; however, coliforms are often associated with intestinal pathogens (e.g. *Salmonella, Shigella,* etc.), so that the presence of feces always signals a potential "health risk". Thus levels of coliform bacteria have long been described as the best criterion for assessing the hygienic quality of water (Fujoka 2002).

1.4 Objectives

To elucidate the role to heterotrophic bacteria in nutrient cycling, the objectives of this study were to determine: (1) the number of culturable, heterotrophic actinomycetes, bacteria, and fungi; (2) the functional diversity of microbial communities of various sampling sites; (3) the phosphatase activity of microorganisms within the sediment; (4) the presence of total coliforms, *Escherichia coli*, and fecal coliforms in Lake Winnipeg sediment.

2 Materials and Methods

2.1 Sediment collection

Sediment samples were obtained from various sites across Lake Winnipeg by Dr. Adkins, LWRC scientists, and the crew aboard the Canadian Coast Guard Ship (C.C.G.S.) *Namao* during September and October, 2004 (Figure 4). Samples were collected using a spring-loaded Ekman dredge lowered to the bottom of the lake and closed using a messenger. The dredge was then brought to the surface and a 5 inch core was removed using a 10-mL syringe. Samples were placed in plastic bags and frozen (-20^{o}C) until examination.

Of the 65 samples collected during this period, 18 were chosen for study based on their (1) location relative to the shoreline, (2) location relative to each basin, and (3) the density of algal blooms within the lake (Figure 3).

2.2 Enumeration of microorganisms

The total numbers of culturable, heterotrophic actinomycetes, bacteria, and fungi in sediment were determined by serial dilution standard plate-count technique using the following selective culture media: Actinomycete Isolation Agar (AIA) amended with chlortetracycline (20 μg/mL), Nutrient Agar (NA), and Yeast D^{+} Glucose Chloramphenicol Agar (YGC) amended with Rose Bengal (0.05 g/L), respectively (Table 1). Media were autoclaved, cooled in a 50^{o}C water bath, amended with filtersterilized chlortetracycline or Rose Bengal and then poured. For each sample, an initial 10^{-1} dilution was prepared by mixing 1g of frozen sediment into 9 mL of sterile sodium pyrophosphate buffer (0.1% w/v) and 2 drops of Tween 80, in a 250-mL Erlenmyer flask. Sodium pyrophosphate buffer and Tween 80 were used to disperse sediment colloids.

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Figure 4: Map of Lake Winnipeg showing various sampling sites. South Basin: Sites 3B, 60C, 60, 60B, 7, 36S, 57, and 14. North Basin: Sites 48, 42S, 39, 23ES, 35S, 27S, 21, 22, 34S, and 33 (Map courtesy LWRC- Lake Winnipeg Research Consortium, Inc.).

Table 1: Composition of culture media used for the enumeration of actinomycetes, bacteria, and fungi and for the determination of the presence of coliforms, *Escherichia coli,* and fecal coliforms.

Isolate	Medium	Composition (g/L)	
Actinomycetes	Difco Actinomycete Isolation Agar	Sodium Caseinate	2
		Asparagine	0.1
		Sodium Propionate	4
		Dipotassium Phosphate	0.5
		Magnesium Sulfate	0.1
		Chlortetracyclin	0.02
		Ferrous Sulfate	0.001
		Bacto Agar	15
Bacteria	EM Science Nutrient Agar	Meat Extract	3.0
		Peptone from meat	5.0
		Agar-Agar	12.0
Fungi	EM Science Yeast Extract D^+ Glucose Chloramphenicol Agar	Yeast Extract	5.0
		D (+) Glucose	20.0
		Rose Bengal	0.5
		Chloramphenicol	0.1
		Agar-Agar	14.9
Coliforms	EM Science Brilliant Green 2%-Bile (BRILA) Broth	Peptone	10.0
		Lactose	10.0
		Ox bile, dried	20.0
		Brilliant Green	0.0133
Escherichia coli	EM Science Eosine Methylene-blue lactose agar	Peptone	10.0
		Lactose	10.0
		Di-Potassium hydrogen phosphate	2.0
		Eosin yellowish	0.4
		Methylene blue	0.065
		Agar-Agar	13.5
Fecal coliforms	Difco m FC Agar[a]	Pancreatic Digest of Casein	6.0
		Proteose Peptone No. 3	9.0
		Yeast Extract	3.0
		Lactose	12.5
		Bile Salts No.3	1.5
		Sodium Chloride	5.0
		Agar	15.0
		Aniline Blue	0.1

[a] Membrane Fecal Coliform agar, amended with 10 mL/L 1% Rosolic Acid in 0.2 M NaOH

The suspension was shaken on a rotary shaker at 170 rpm at 23.5°C, for an hour, and then allowed to settle for 15 minutes before final serial dilutions were performed. Using aseptic techniques, 4 mL of the 10^{-1} dilution were added to 36 mL of sterile saline (0.85%) and shaken to create a homogenous 10^{-2} suspension. Additional dilutions (10^{-3}-10^{-5}) were then prepared in 9 mL of sterile saline (0.85%). For each sample, replicate aliquots (0.1 mL) were removed for selected dilutions and spread onto the surface of the appropriate culture media. Plates to be incubated at 23.5°C (7 days) were inoculated with 0.1 mL from each of the dilutions (10^{-1}-10^{-5}). Plates to be incubated at 6°C (14 days) were inoculated as follows: NA: 0.1 mL of each dilution (10^{-1}-10^{-5}). AIA and YGC: 1.0 mL of 10^{-1} dilution and 0.1 mL from each of 10^{-1} to 10^{-4} dilution. After 7 days and 14 days of incubation at 23.5°C and 6°C, respectively, plates with between 30 to 300 colonies were recorded. The numbers of organisms per gram of wet sediment were calculated by multiplying the number of colonies by the dilution factor then dividing by the weight of sediment.

2.3 **Biolog© test for metabolic activity**

Biolog© (Biolog, Inc. 3938 Trust Way, Hayward, CA) microplates were initially designed by the manufacturer for the identification and characterization of pure cultures of bacteria; however, recently this method has been adopted by a number of researchers as a tool to examine biodiversity, functional potential, and overall heterotrophic activity of microbial communities in soil, sediments, and other natural habitats (Zak *et al*. 1994, Glimm *et al*. 1997).

Each GN2 Biolog© microplate consists of 95 wells each containing one of 95 individual, dehydrated carbon sources plus an indicator redox dye (tetrazolium violet;

Table 2). A 96th control well lacks a carbon substrate but contains tetrazolium violet. To each of the 96 wells, 100 μL of the 10^{-2} dilution prepared for enumeration, was added using an Eppendorf repeat pipette (Model 4780) with an 8-channel adaptor. For each sample, two plates were inoculated; one was incubated at 6^{o}C and the other at 23.5^{o}C. As a carbon source was metabolized by microorganisms, tetrazolium violet became reduced to formazan imparting a purple color to the well (Preston-Mafham *et al*. 2002). Wells containing a purple color were scored as positive. Plates incubated at 23.5^{o}C were assessed for the presence of purple every 12 hours beginning at 36 hours after inoculation and ending after 144 hours; plates incubated at 6^{o}C were scored for the presence of purple every 12 hours, beginning at 156 hours after inoculation and ending after 264 hours. Plates were scored from 1 to 10, with 10 showing purple color at 36 (23.5^{o}C) or 156 (6^{o}C) hours and a 1 for wells showing a residue at 144 (23.5^{o}C) or 264 (6^{o}C) hours (Table 3). A null value was used to score those wells that showed no purple residue after 144 and 264 hours.

2.4 **Phosphatase activity**

Phosphatase activity was determined following the procedures developed by Tabatabai and Bremner (1969), explained by Tabatabai (1982). Based on pH paper analysis of the sediment, the pH appeared to be only slightly acidic thus both acid and alkaline phosphatase activity was quantified. Phosphatase enzymes are generally described as enzymes that catalyze the hydrolysis of both esters and anhydrides of H_3PO_4 (Tabatabai 1982). Acid and alkaline phosphatases more specifically hydrolyze phosphomonoesters. This experiment was used to determine the amount of inorganic phosphorus released into the lake by the activity of sediment phosphatases. Acid

Table 2: List of substrates found in GN2 Biolog© microplates separated by substrate guilds (Biolog, Inc. 3938 Trust Way, Hayward, CA).

Carbohydrates	***Carboxylic Acids***	***Amino Acids***
Adonitol	Acetic Acid	D,L-Carnitine
D-Arabitol	cis-Aconitic Acid	D-Alanine
D-Cellobiose	Citric Acid	D-Serine
D-Fructose	D,L-Lactic Acid	Glycyl-L-Aspartic Acid
D-Galactose	D-Galactonic Acid Lactone	Glycyl-L-Glutamic Acid
D-Mannitol	D-Galacturonic Acid	Hydroxy-L-Proline
D-Mannose	D-Gluconic Acid	L-Alanine
D-Melibiose	D-Glucosaminic Acid	L-Alanyl-glycine
D-Psicose	D-Glucuronic Acid	L-Asparagine
D-Raffinose	D-Saccharic Acid	L-Aspartic Acid
D-Sorbitol	Formic Acid	L-Glutamic Acid
D-Trehalose	Itaconic Acid	L-Histidine
Gentiobiose	Malonic Acid	L-Leucine
i-Erythritol	p-Hydroxy-phenylacetic Acid	L-Ornithine
Lactulose	Propionic Acid	L-Phenylalanine
L-Arabinose	Quinic Acid	L-Proline
L-Fucose	Sebacic Acid	L-Pyroglutamic Acid
L-Rhamnose	Succinic Acid	L-Serine
Maltose	α-Hydroxybutyric Acid	L-Threonine
m-Inositol	α-Ketobutyric Acid	α-Aminobutyric Acid
N-Acetyl-D-Galactosamine	α-Ketoglutaric Acid	***Aromatic Chemicals***
N-Acetyl-D-Glucosamine	α-Ketovaleric Acid	Inosine
Sucrose	β-Hydroxybutyric Acid	Thymidine
Turanose	γ-Hydroxybutyric Acid	Uridine
Xylitol		Urocanic Acid
α-D-Glucose	***Amines***	***Phosphorylated Chemicals***
α-D-Lactose	2-Aminoethanol	D,L,α-Glycerol Phosphate
β-Methyl-D-Glucoside	Phenylethylamine	D-Glucose-1-Phosphate
	Putrescine	D-Glucose-6-Phosphate
Polymers	***Amides***	***Alcohols***
Dextrin	Glucuronamide	2,3-Butanediol
Glycogen	L-Alaninamide	Glycerol
Tween 40	Succinamic Acid	***Esters***
Tween 80	***Brominated Chemicals***	Pyruvic Acid Methyl Ester
α-Cyclodextrin	Bromosuccinic Acid	Succinic Acid Mono-Methyl Ester

Table 3: Intensity ratings for development of purple residue (formazan) in wells of Biolog© microplates.

Incubation Temperature	Time of presence of residue (hours)	Intensity rating
23.5°C	36	10
	48	9
	60	8
	72	7
	84	6
	96	5
	108	4
	120	3
	132	2
	144	1
	no reaction	null value
6°C	156	10
	168	9
	180	8
	192	7
	204	6
	216	5
	228	4
	240	3
	252	2
	264	1
	no reaction	null value

phosphatase is predominant in acidic sediment while alkaline phosphatase is predominantly found in basic sediment. In this experiment, p-nitrophenyl phosphate was hydrolyzed to p-nitrophenol; the quantity of p-nitrophenol was proportional to the amount of inorganic phosphorus released by enzymatic reaction.

One gram of sediment was placed into a 50-mL Erlenmeyer flask; to this we added: 0.2 mL of toluene, 4 mL of modified universal buffer (MUB; pH 6.5 for assay acid phosphatase or pH 11 for alkaline phosphatase), and 1 mL of p-nitrophenyl phosphate solution made in the same buffer. The flask was swirled for a few seconds, stoppered, and placed in a 37^{o}C incubator. After an hour, the stopper was removed and 1 mL of 0.5 M calcium chloride ($CaCl_2$) and 4 mL of 0.5 M sodium hydroxide (NaOH) added. The flask was swirled and contents were filtered using a Whatman No. 2 folded filter paper. The intensity of the filtrate was measured using a Spectronic 21 spectrophotometer set at a wavelength of 400 nm, the wavelength of highest p-nitrophenol absorbance.

Controls were performed to allow for color not derived from p-nitrophenol. Controls were performed similarly to above, that is 1 g of sediment was incubated with 0.2 mL of toluene, 4 mL of MUB (pH 6.5 or 11); however, 1 mL of p-nitrophenyl phosphate solution was added after the addition of 1 mL of 0.5 M calcium chloride and 4 mL of 0.5 M sodium hydroxide, after incubation but immediately before filtration. The control of each sample served as the blank.

The content of p-nitrophenol, the hydrolysis product of p-nitrophenyl phosphate, in the filtrate was determined by comparison to a calibration graph plotted from results obtained with standards containing 0, 10, 20, 30, 40, and 50 μg of p-nitrophenol. The

graph was prepared by diluting 1 mL of the standard p-nitrophenol solution to 100 mL in a volumetric flask and mixing thoroughly. Aliquots of 0-, 1-, 2-, 3-, 4-, and 5-mL were pipetted into a 50 mL Erlenmeyer flask and volume was adjusted to 5 mL with water; 1 mL of 0.5M $CaCl_2$ and 4 mL of NaOH were added then the solution was filtered as above. The absorbance was read at 400 nm and results were plotted against mass of p-nitrophenol. If the color intensity produced by a filtrate was not within the limits of the calibration graph, it was diluted with water until the reading fell within the limits of the graph.

To perform the analysis above, several additional solutions were required. Modified Universal Buffer (MUB) stock solution was prepared by dissolving 12.1 g of Tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, and 6.3 g of boric acid in 488 mL of 1 M sodium hydroxide (NaOH), and subsequently diluted to 1 Liter with double distilled water. Working solutions, pH 6.5-MUB and pH 11-MUB, were prepared as follows: 200 mL of MUB stock solutions were placed into two 500-mL beakers each containing a magnetic stirring bar, on a magnetic stir plate. The solutions were either titrated to the desired pH by the addition of 0.1 M hydrochloric acid (HCl) or 0.1 M sodium hydroxide (NaOH). The final volumes were adjusted to 1 Liter with double distilled water. The pH was measured using a Fisher Accumet® pH meter. The substrate p-nitrophenyl phosphate solution (0.025 M) was prepared by dissolving 0.420 g of disodium p-nitrophenyl phosphate tetrahydrate (Sigma 104, Sigma Chemical Co., St. Louis, Mo.) into approximately 40 mL of pH 6.5-MUB (for acid phosphatase assay) or pH 11-MUB (for alkaline phosphatase assay) and diluted to 50 mL using the same MUB solution in a volumetric flask. Calcium chloride ($CaCl_2$) 0.5 M was

prepared by dissolving 73.5 g of $CaCl_2 \cdot 2H_2O$ in 700 mL of water then diluted to 1 Liter with water in a volumetric flask. Sodium hydroxide (NaOH) 0.5 M was prepared by dissolving 20 g of NaOH in 700 mL of water then diluted to 1 Liter with water. The standard p-nitrophenol solution used to prepare the standard graph was prepared as follows: 1 g of p-nitrophenol was dissolved in 70 mL of water then diluted to 1 Liter with water. Solution was stored in the dark at 6^{o}C.

2.5 **Qualitative determination for presence of coliforms**

Coliforms are defined as facultatively anaerobic, Gram-negative, nonsporulating, rod-shaped bacteria that ferment lactose with acid and gas formation within 48 hours, at 35^{o}C (Prescott *et al*. 2002). All coliforms are members of the family *Enterobacteriaceae*.

To determine the presence of total coliforms (any or all members of the family *Enterobacteriaceae* irregardless of origin) in the sediment, approximately 0.5 g of sample was added to 20 mL of Brilliant Green 2%-Bile (BRILA) Broth with inverted Durham tubes and incubated at 37^{o}C for two days, monitored twice daily (Table 1). The presence of lactose-fermenting coliforms was determined based on the production of gas within the Durham tube. Tubes showing gas production were scored as positive (+).

Escherichia coli is a coliform which normally inhabits the intestine of man and other mammals (Pelczar and Reid 1972). This bacterium is considered an indicator organism for the potential presence of enteropathogenic organisms (Atlas and Bartha 1981). To determine if *Escherichia coli* was present in the lake sediment, Eosine Methylene-blue (EMB) Lactose Agar was inoculated from '+' BRILA tubes using the 4-way streaking technique. Plates were incubated at 37^{o}C for two days. When grown of the surface of EMB, *E. coli* produces blue-black colonies with a metallic green sheen.

This characteristic appearance is brought about by the large amount of acid produced during lactose fermentation causing precipitation of dye onto the growth's surface (Cappuccino and Sherman 2001). Due to this observation, *E. coli* can be differentiated from other coliforms, such as *Enterobacter aerogenes*. Plates with blue-black metallic green colonies were scored as positive.

Fecal coliforms are organisms associated with human or animal wastes which ferment lactose and produce gas at 44.5^{o}C with 24 hours while nonfecal coliforms are inhibited (Atlas and Bartha 1981). Bacteria from the genera *Escherichia, Klebsiella, Shigella,* and *Citrobacter* may be fecal coliforms and their presence within sediment and water indicates fecal contamination. To determine if the coliforms present in the sediment were human fecal coliforms, 0.1 mL aliquots of each BRILA broth (scored as positive) was aseptically spread onto Membrane Fecal Coliform (m FC) Agar plates amended with 1% Rosolic acid (10 mL/L) in 0.2 M NaOH. Plates were incubated in a sealed container in a hot water bath set at 44.5^{o}C for 4 days; observations were recorded at the end of the first and fourth day. At 44.5^{o}C, lactose is fermented by human fecal coliforms producing a blue color; all plates showing blue colonies were recorded as positive for human fecal coliforms.

2.6 **Statistical Analysis**

Two-way analysis of variance (ANOVA), with temperature and location as fixed factors, was used to assess the difference in average numbers of microorganisms, that is, actinomycetes, bacteria and fungi. For analysis, the dependent variable (colony forming units/ g wet wt. sediment) was square root transformed to stabilize variance since counts often follow a Poisson distribution where variance increases with the mean. To assess

the significance of temperature and location on substrate richness, the dependent variable of the data was converted to a fraction then arcsine square root transformed. Intensity was assessed with untransformed data also using two-way ANOVA and phosphatase activity was assessed using a singled tailed t-test with the level of significance equal to 5% ($\alpha = 0.05$).

3 Results

3.1 Assessment of microbial populations

Replicates were performed at 6^{o}C to determine the growth that would realistically be seen in the lake environment, a temperature similar to that of the lake during the fall and at 23.5^{o}C to determine the highest probable numbers within the sediment.

3.1.1 Actinomycetes

At 6^{o}C, growth was observed in only 7 sites; sites 60 and 7 in the South Basin, and sites 42S, 39, 27S, 34S, and 33 in the North Basin. At this temperature, the greatest amount of growth was observed at site 60 ($8.76x10^{1}$ Colony forming units (CFU)/g wet wt. sediment; Figure 5). At 23.5^{o}C, actinomycetes grew in all 18 sediment samples; site 35S has the greatest amount of growth ($1.67x10^{5}$ CFU/g wet wt.) and site 39 had the lowest amount of growth ($7.25x10^{1}$ CFU/g wet wt.).

3.1.2 Bacteria

Unlike actinomycetes, all sites had bacterial growth at 6^{o}C. Site 35S had the most growth ($8.20x10^{4}$ CFU/g wet wt. sediment), and site 34S had the least growth ($1.19x10^{3}$ CFU/g wet sediment; Figure 6). At 23.5^{o}C incubation, the numbers of CFUs ranged from $8.88x10^{5}$/g wet wt. (South Basin site 60B) to $1.81x10^{3}$ CFU/g wet wt. (North Basin site 42S).

3.1.3 Fungi

At 6^{o}C, site 48 had the greatest average growth ($1.46x10^{2}$ CFU/g wet wt. sediment) while several sites had no growth (Figure 7). At 23.5^{o}C, site 36S had the greatest amount of growth ($3.77x10^{5}$ CFU/g wet wt. sediment) and several sites incubated at this temperature had no growth. Overall the fungi have the least amount of growth.

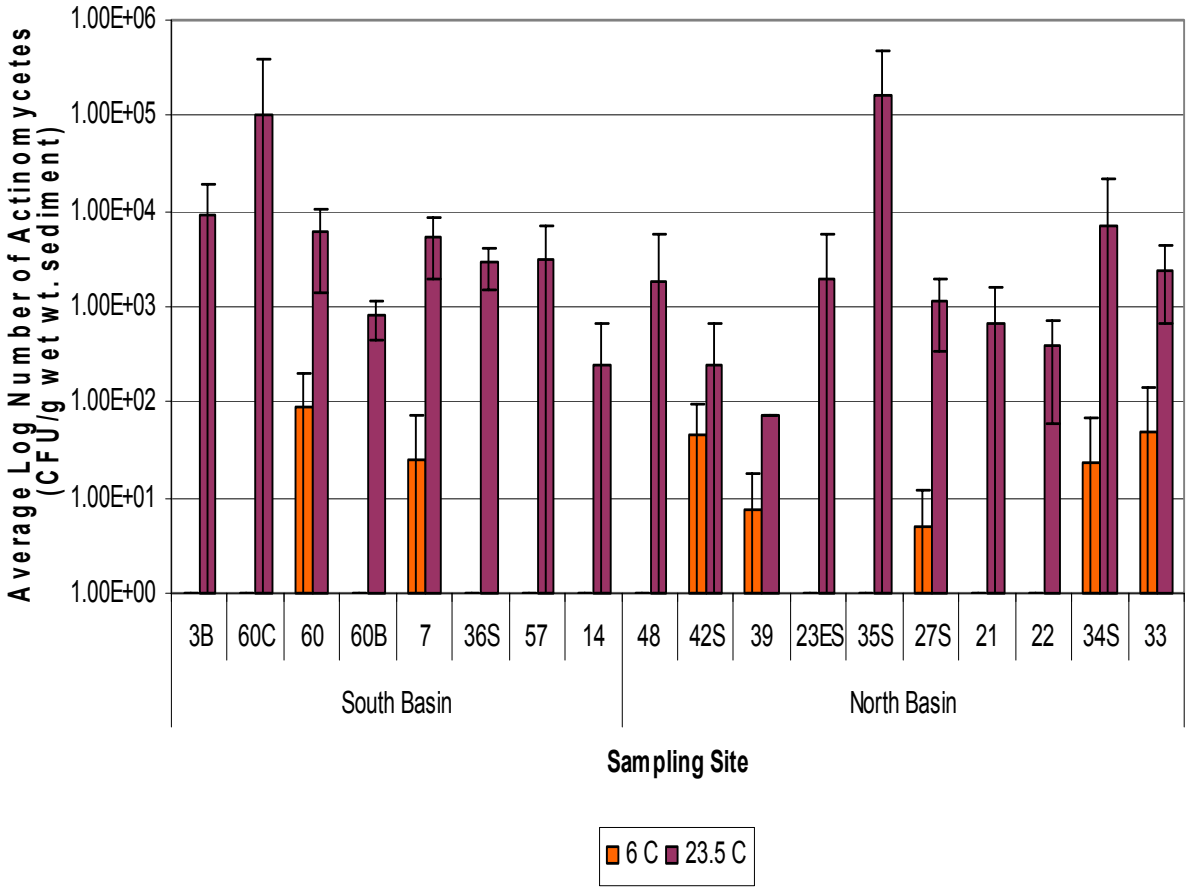

Figure 5: Average number of culturable, heterotrophic actinomycetes isolated from 18 sampling sites and incubated at 23.5°C (7 days) and 6°C (14 days; x ± standard deviation).

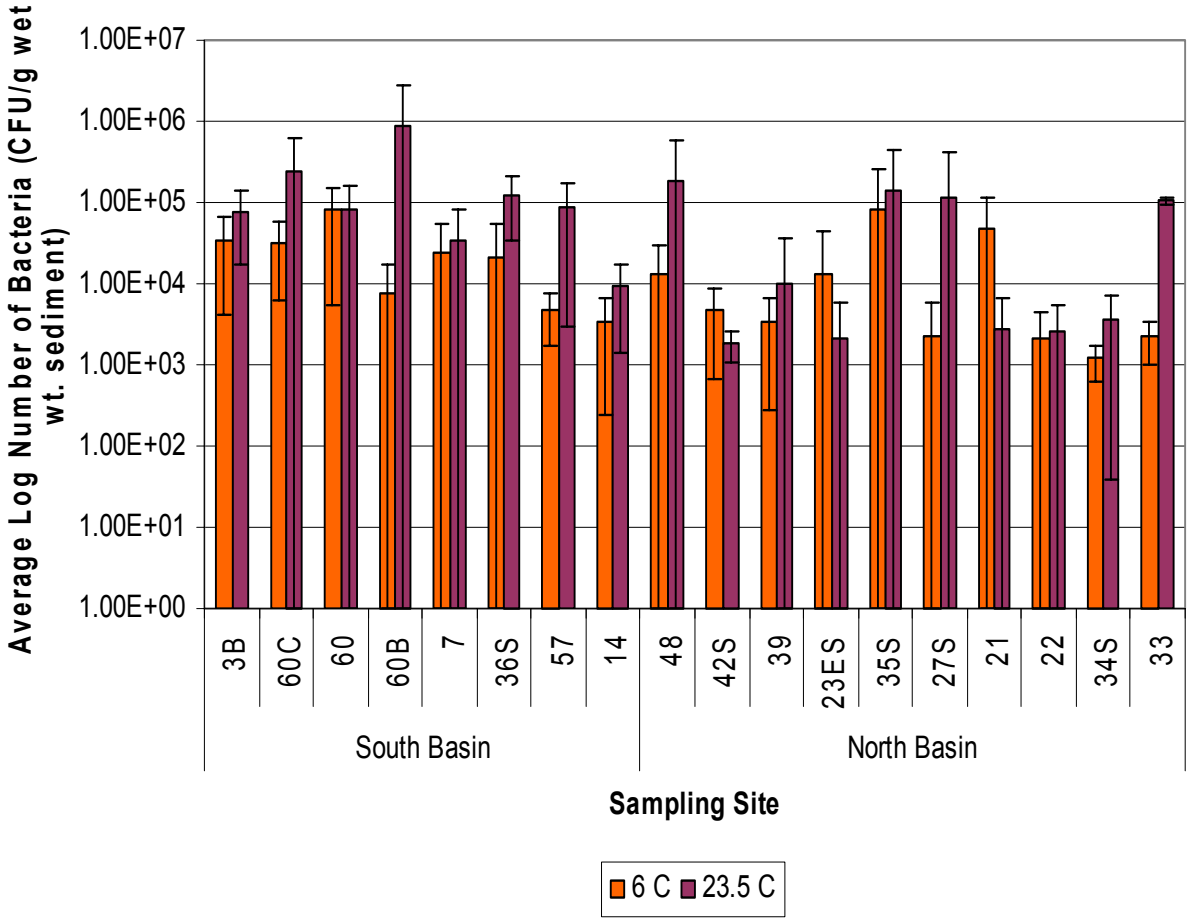

Figure 6: Average number of culturable, heterotrophic bacteria isolated from 18 sampling sites incubated at 23.5°C (7 days) and 6°C (14 days; x ± standard deviation).

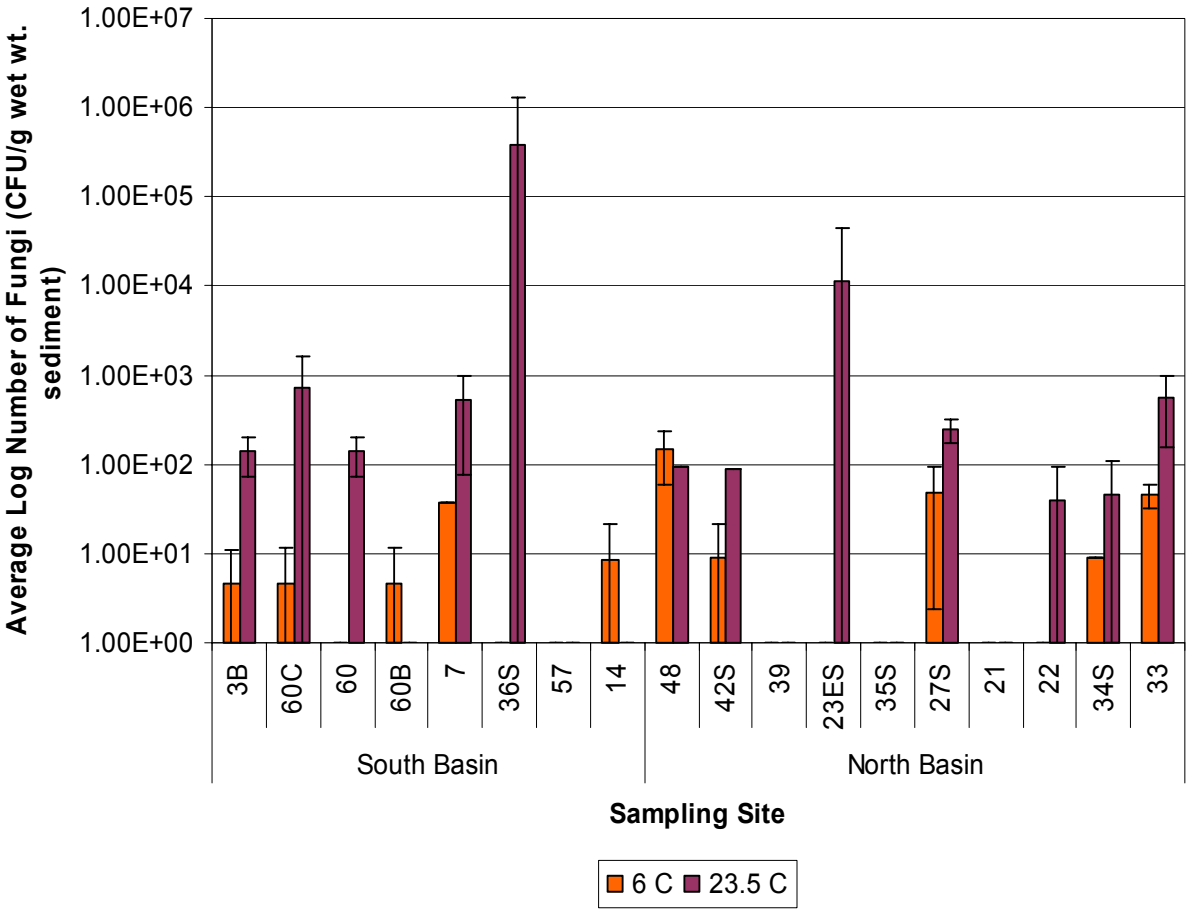

Figure 7: Average number of culturable, heterotrophic fungi isolated from 18 sampling sites and incubated at 23.5^{o}C (7 days) and 6^{o}C (14 days; x ± standard deviation).

3.1.4 **Statistics**

Two-way ANOVA indicated that average numbers of actinomycetes varied significantly with temperature ($p = 0.004$) but not location ($p = 0.762$; Table 4). Bacteria numbers varied significantly with temperature ($p = 0.009$) and location ($p = 0.045$) while fungi numbers did not vary with either ($p = 0.156$ and 0.333, respectively).

3.2 **Functional diversity of heterotrophic microbiota**

Functional diversity refers to the physiological characteristics, that is, biochemical and metabolic capabilities demonstrated by mixed heterotrophic microorganisms sharing a specific niche. Evaluation of functional diversity, rather than taxonomic diversity, provides greater insight to microbial roles in ecosystems and has proven to be an effective method to evaluate changes to community structure in response to natural and anthropogenic disturbances (Zak *et al*. 1994).

The Biolog© System is an easy to use system which produces data rich in information about functional diversity of bacteria and integrates the activities of a broad range of bacteria into assessments of functional diversity (Zak *et al*. 1994). Direct incubation of environmental samples in Biolog© plates produces patterns of metabolic response useful in classification and characterization of microbial communities and thus provides a sensitive and ecologically meaningful measure of heterotrophic microbial community structure (Garland and Mills 1991).

3.2.1 **Substrate richness**

Substrate richness or the number of carbon substrates metabolized by sediment of each sampling site is an indication of microbial enzymatic capability of the mineralzation of organic matter.

Table 4: Analysis of variance results showing calculated F-values and significance at a level of significance of 5% ($\alpha = 0.05$).

		F-value calculated	degrees of freedom	p-value	Significant (Y/N)
Actinomycete Counts[x]	Overall ANOVA	3.159	3,32	0.038	Y
	Temperature	9.384	1,32	0.003	Y
	Location	0.093	1,32	0.762	N
	Interaction	0.106	1,32	0.747	N
Bacterial counts[x]	Overall ANOVA	4.83	3,32	0.011	Y
	Temperature	7.78	1,32	0.009	Y
	Location	4.364	1,32	0.045	Y
	Interaction	1.707	1,32	0.201	N
Fungi counts[x]	Overall ANOVA	1.272	3,32	0.301	N
	Temperature	2.107	1,32	0.156	N
	Location	0.965	1,32	0.333	N
	Interaction	1.038	1,32	0.316	N
Substrate Richness[y]	Overall ANOVA	56.678	3,32	0.000	Y
	Temperature	19.805	1,32	0.000	Y
	Location	147.825	1,32	0.000	Y
	Interaction	4.144	1,32	0.050	Y
Intensity[z]	Overall ANOVA	47.861	3,32	0.000	Y
	Temperature	26.074	1,32	0.000	Y
	Location	107.541	1,32	0.000	Y
	Interaction	13.728	1,32	0.001	Y

x two-way ANOVA using square root transformed counts
y two-way ANOVA using fraction arcsine square root transformed data
z two-way ANOVA without transformed data
Interaction refers to Temperature x Location

The highest substrate richness was recorded for South Basin sites 14 (44.2%) and 60 (78.9%) at 6^{o}C and 23.5^{o}C, respectively. In the South Basin, substrate richness at 23.5^{o}C was higher than at 6^{o}C. At 23.5^{o}C, richness ranged from 48.4% to 78.9% while at 6^{o}C richness ranged from 11.6% to 44.2%. Also, the variation in richness observed for each sampling location varied; some sites had similar values at both temperatures (site 48) while other sites showed great variation (site 60). Overall, substrate richness was much lower in the North Basin with several sites showing no metabolic activity at either temperature (Figure 8).

Using two-way ANOVA, a significant difference was observed in average richness at 6^{o}C and 23.5^{o}C ($p = 0.000$) between the South and North Basin ($p = 0.000$; Table 4). Also, the interaction between temperature and location also proved significant ($p = 0.050$). This indicates richness was highest at 23.5^{o}C and in the South Basin.

3.2.2 **Intensity**

Intensity is a measure of the rate at which any of the 95 carbon substrates were utilized by microorganisms inoculated into each of the Biolog© microplate wells. This value gives an indication of metabolic activity and indirectly, density of organisms.

Overall intensity was greater in the South Basin than in the North Basin at both temperatures. In contrast to the South Basin, the intensity of substrate utilization in the North Basin samples was low with sites 23ES and 35S recording no intensity. Similar to substrate richness, intensity in the South Basin was higher at 23.5^{o}C with values ranging from 2.29 to 5.10 than 6^{o}C where values ranged from 0.48 to 2.77. Intensity for plates incubated at 6^{o}C was highest at site 14 (2.77) with several sites (23ES, 35S, 27S, and 33)

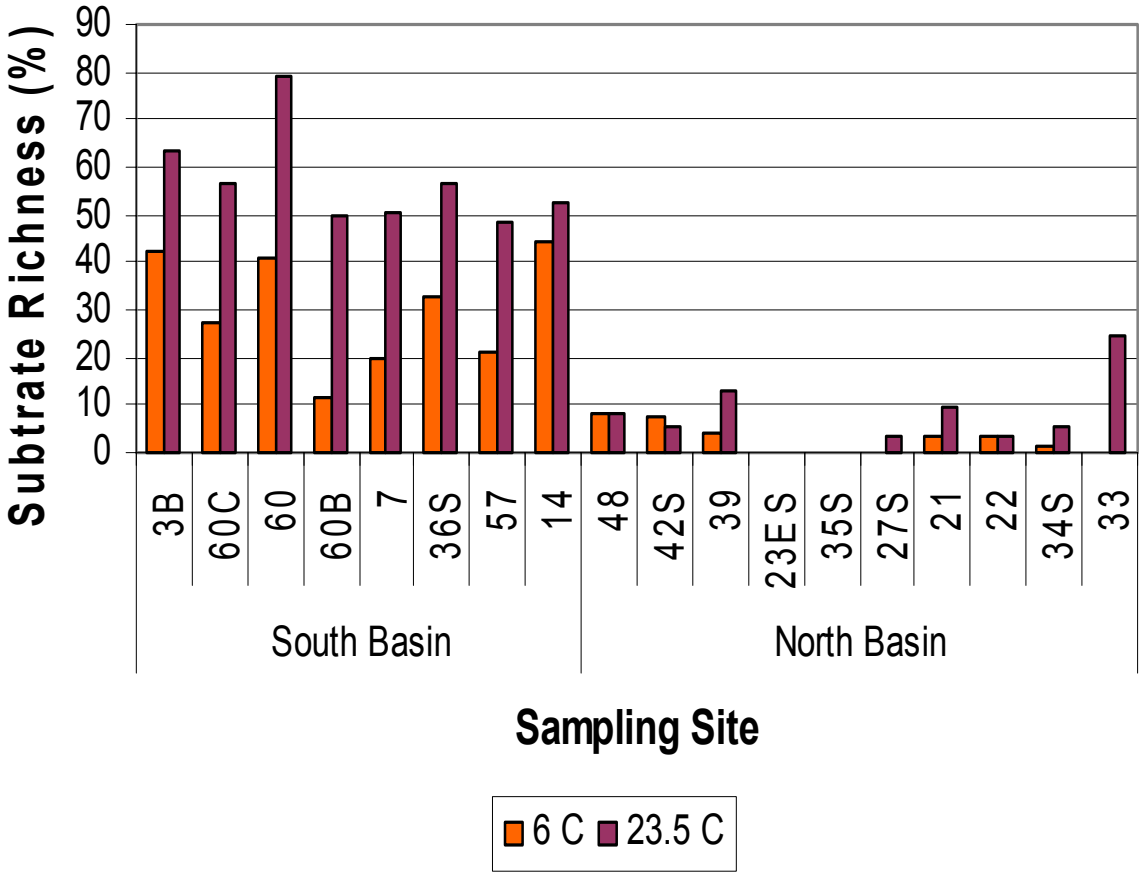

Figure 8: Substrate richness of 18 sediment samples as determined by GN2 Biolog plates.

showing no intensity (Figure 9). For plates incubated at 23.5^{o}C, intensity ranged from 0 to 5.10, with site 60 scoring the highest (5.10).

Using two-way ANOVA, both temperature ($p = 0.000$) and location ($p = 0.000$) proved to be significant variables for intensity of substrate utilization (Table 4). Interaction of temperature and location was also significant ($p = 0.001$). This indicates intensity was highest at 23.5^{o}C and in the South Basin.

3.3 Phosphatase Activity

Overall, phosphatase activity was higher in the North Basin compared to that in the South Basin (Figure 10). Acid phosphatase activity was highest at North Basin site 48 (151.3 ug p-np/g*hr) and lowest at South Basin site 3B (16.8 ug p-np/g*hr). The greatest amount of alkaline phosphatase activity was found at North Basin site 27S (109 ug p-np/g*hr). The slowest activity, that is, the least amount of p-nitrophenol released was observed at South Basin site 60B (11.4 ug p-np/g*hr).

Using a single-tail t-test, $\alpha = 0.05$, the activity difference between the South and North Basin for both acid and alkaline phosphatase was determined. At this level of significance, activity was statistically higher in the North Basin over the South Basin for both acid and alkaline phosphatases.

3.4 Qualitative determination for presence of coliforms

The qualitative assessment of the sediment using Brilliant Green 2%-Bile (BRILA) Broth indicated the presence of coliforms in 8 sediments in the South Basin and 4 in the North Basin (Table 5). In the South Basin, both *E. coli* and fecal coliforms were detected in three of the sediments whereas only two of the North Basin sediments tested positive for these organisms (Table 5).

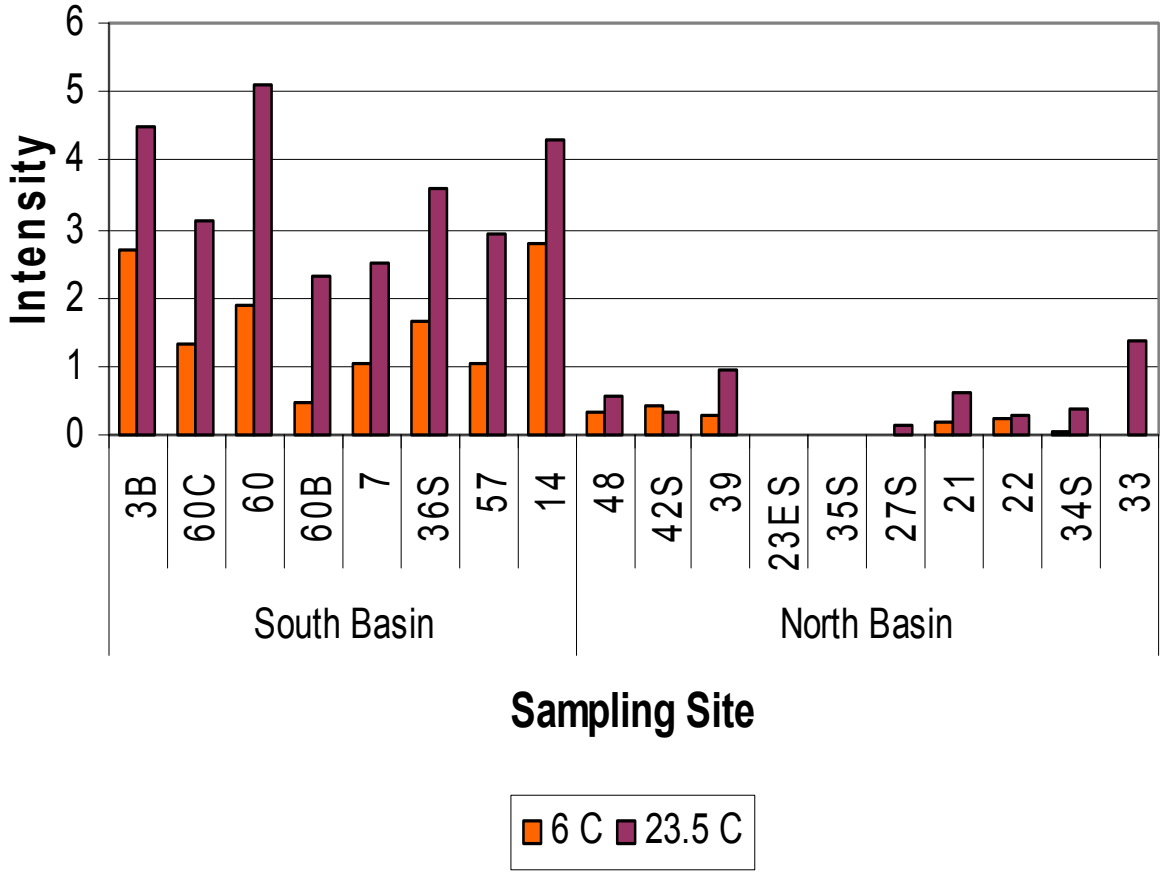

Figure 9: Intensity of 18 sediment samples determined by GN2 Biolog plates uses a 10-0 scoring system. Ten being given to substrates utilized after 36 and 156 hours (23.5^{o}C and 6^{o}C, respectively).

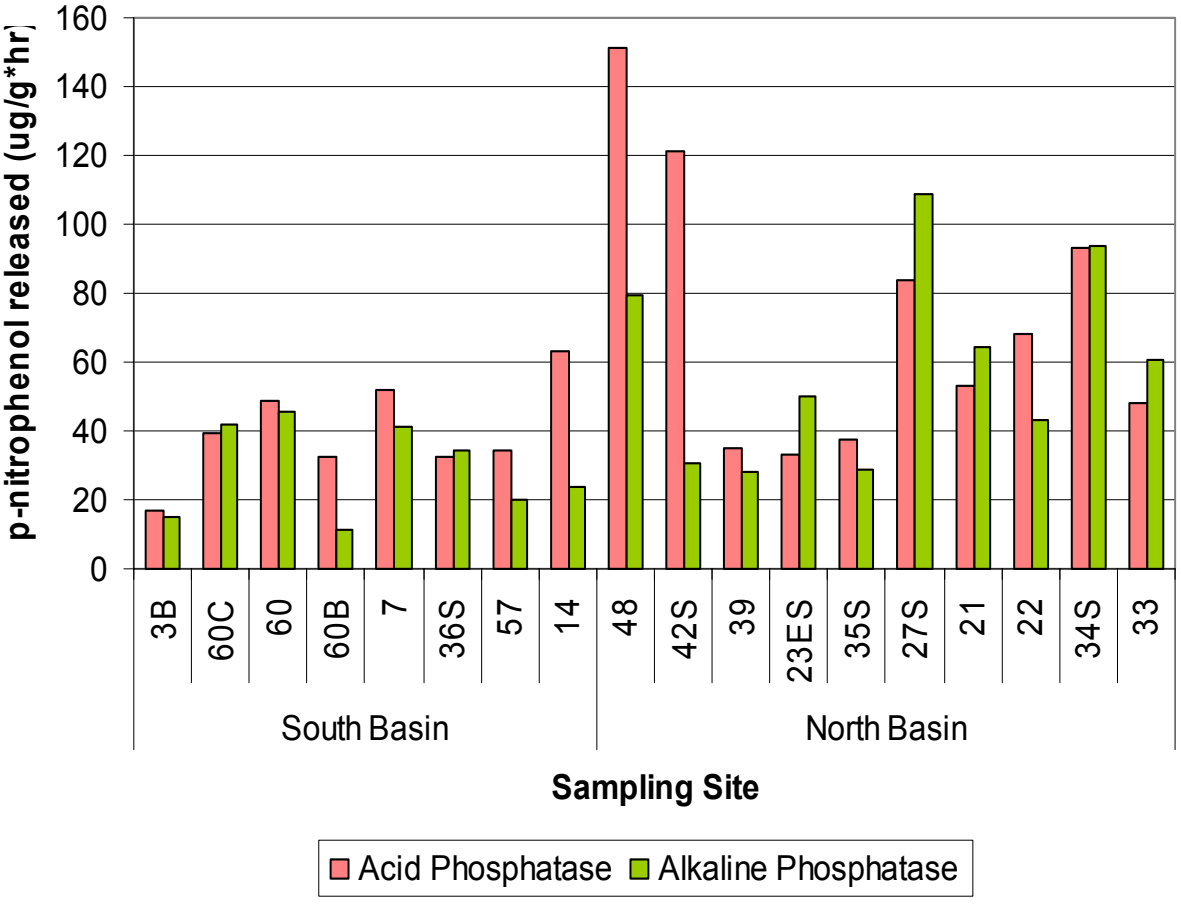

Figure 10: Content of p-nitrophenol released by hydrolysis of p-nitrophenyl phosphate by acid (pH 6.5) and alkaline (pH 11) phosphatase.

Table 5: Presence or absence of coliforms within selected sediment and water samples taken from Lake Winnipeg.

Location		Sediment			Water
		Total Coliforms[a]	*E. coli*[b]	Fecal Coliforms[c]	Fecal Coliforms[d]
South Basin	site 3B	+	+	+	+
	site 60C	+	+	+	\
	site 60	+	-	-	+
	site 60B	+	-	-	+
	site 7	+	+	+	+
	site 36S	+	-	-	+
	site 57	+	-	-	+
	site 14	+	-	-	-
North Basin	site 48	+	-	-	-
	site 42S	-	NA	NA	+
	site 39	-	NA	NA	\
	site 23ES	-	NA	NA	+
	site 35S	-	NA	NA	+
	site 27S	+	+	+	-
	site 21	-	NA	NA	+
	site 22	+	+	-	+
	site 34S	-	NA	NA	\
	site 33	+	+	+	-

+ indicates positive for coliforms
- indicates negative for coliforms
NA indicates test was not performed
\ indicates data not available
[a] performed using 20 mL BRILA broth inoculated with 0.5 g sediment. Formation of gas inside an inverted Durham tube was indication of lactose fermentation, a positive reaction.
[b] performed by streaking positively scored BRILA broth onto EMB agar and incubated at 37^{o}C. Presence of metallic green sheen on surface of colonies was an indication of *E. coli* growth, positive reaction.
[c] aliquots of 0.1 mL was taken from each BRILA broth scored as positive and plated onto a m FC agar plate which was then incubated at 44.5^{o}C. Presence of blue growth indicated a positive reaction, the presence of fecal colifoms.
[d] data obtained using a membrane filter method; 50 mL of water was filtered onto membrane filters incubated on m FC agar in a hot water bath at 44.5^{o}C.

Data obtained from the sediments were compared with water data obtained during the fall sampling off the C.C.G.S. *Namao* (Sept-Oct, 2004). Water column samples were treated on board and the number of fecal coliforms obtained. For the present study, only qualitative interpretations are presented. Whereas fecal coliforms had been isolated from the majority of water samples of the South Basin, only sediments from sites 3B, 60C, and 7 tested positive (Table 5). Although fecal coliforms were present in at least 50 per cent of the water samples from the North Basin, only sediments from sites 27S and 33 tested positive. Interestingly, no fecal coliforms had been detected in the corresponding water samples at these sites during the fall.

4 Discussion

Research has shown that the density of microorganisms (bacteria) in the aerobic layer of typical lake sediment is of the order of hundreds of millions of cells per gram moist silt (Kuznetsov 1970). Our enumeration data, on the other hand, indicate significantly lower numbers in Lake Winnipeg sediment. The reason for this requires further investigation; however, considering the present state of the lake, the suppression of heterotrophic microbial communities may be in response to inhibitory concentrations of pollutants in the benthic region of the lake.

Although all sampling sites supported growth of bacteria, at both incubation temperatures, this was not the case with actinomycetes and fungi. These results were not all together unexpected as these two groups of microorganisms, although adapted to environments subjected to variations of water and nutrient availability, have never been detected in significant abundance in any subsurface microbiological studies (Alexander 1971, Eugene and Ghiorse 1993). Since actinomycetes and fungi are strict aerobes, one explanation for their lack of growth may be the low oxygen diffusion (restricted oxygen availability) in an aquatic environment. The decrease in growth observed at $6^{o}C$, compared to that at $23.5^{o}C$, gives a good indication as to the relative amount of heterotrophic activity during various times of the year (seasonal activities).

It should be noted that the standard plate count method is imprecise and has been known to underestimate the numbers of organisms. In addition, the heterogeneity of the sediment adds to the difficulty of obtaining consistently comparative results (Kuznetsov 1970, DeBruyn *et al.* 2004). This leads to large standard deviations. However, it is a

useful technique when used in conjunction with other methods, such as the assessment of metabolic activities.

Average numbers of bacteria were statistically higher in the South Basin over the North Basin and it appears that microorganisms in the South Basin are more functionally diverse, having higher substrate richness and intensity. The greater degree of metabolic and biochemical activities suggests that South Basin sediment communities are distinctively different from those of the North Basin. An explanation for this may be related to the Red and Winnipeg Rivers which discharge approximately 56 per cent of the mean monthly flow of water entering the lake in the South Basin. Previous research by scientists from the Lake Winnipeg Research Consortium have shown that these sources carry significant amounts of fertilizers and nutrients from urban and industrial discharges (LWSB 2005) providing organisms in the South Basin with a diversity of substrates. As the water moves northward, it becomes diluted; thereby microorganisms in the North Basin sediment are exposed to a more restricted "diet".

Although microorganisms possess many enzymes for metabolic activity, phosphatases are specifically involved in the conversion of organic into inorganic phosphorus (Hurst 2002). Phosphatases are present in all organisms but only bacteria, fungi, and some algae are able to excrete these enzymes outside of their cells; as exoenzymes they participate in the dissolution and mineralization of organic phosphate compounds in the environment (Jones 2002). Without phosphatase enzymes, the presence of inorganic phosphorus would be limited to external sources, such as fertilizers, and productivity would be limited and dependent on these external sources. Phosphate would remain sequestered in cell matter and unavailable for primary producers. The

enzymatic activity of microbial communities is critical for the proper cycling of phosphorus within the lake.

Both acid and alkaline phosphatase activities are highest in the North Basin where the most profuse algal blooms have been observed over the past several years (LWSB 2005). It is possible that microbial phosphatase activity potentially contributes to these algal blooms by the release of phosphorus into the lake. An interesting feature of microbial phosphatases, especially alkaline phosphatase, is that they are not released in the presence of excess dissolved phosphate (Jones 2002). This may explain the lower phosphatase activity in sediments of the South Basin, which receives approximately 5100 tonnes of phosphorus-containing effluent (that is 77% of the total phosphorus load of Lake Winnipeg) annually by way of the Red and Winnipeg Rivers (LWSB 2005). As the water flows northward, dissolved nutrients become diluted and phosphorus concentrations decrease allowing phosphatase activity to resume. It is important to note, that activity at each site contributes to the total phosphorus being introduced into the lake; phosphorus being released by microbial activity from every site across the lake in the South and North Basins throughout the year may thus contribute to the total phosphorus introduced into the water. Although most of the inorganic phosphorus in the lake originates from anthropogenic sources, the results of this study indicate that microbial decomposition of organic matter in the sediment releases a significant amount of inorganic phosphorus into the ecosystem and as a result contributes toward primary production and eutrophication. Thus the biological role of microorganisms in the phosphorus cycle should not be ignored.

It is important to briefly comment on the protocol used to determine phosphatase activity in this study. Sediment samples were incubated at 37°C for an hour; this is a temperature microorganisms in the sediment will likely never be exposed to in their aquatic environment. Since enzymatic activity is known to double for every increase of 10°C (Tabatabai 1982), our results may in fact be an overestimation of the quantity of inorganic phosphorus released into the lake. If at the beginning of September the sediment temperature is approximately 21°C, and nearing the end of October, 5°C to 8°C (Pip 2004), the actual amount of phosphorus produced *in situ*, per hour, may in fact be a quarter to a half lower than that produced in the laboratory. However, in midsummer, sediment temperatures in areas of algal blooms have been recorded as high as 35°C (Pip 2004); therefore phosphatase activity in July may be as high as that shown in our data. In any case, microbial activity in the sediment likely contributes a sizeable amount of inorganic phosphorus into Lake Winnipeg, and thus toward the acceleration of eutrophication and algal growth.

In the past several years, occasional beach closures due to fecal contamination have prompted the provincial government to undertake a number of studies to determine the extent of fecal pollution in Lake Winnipeg. One such study seemed to indicate that shorebird populations (primarily gulls) were the primary cause of fecal pollution of Lake Winnipeg, or at least of its beaches (Williamson 2004). These findings were supported by a 2003 study conducted on Lake Michigan beach sand by Whitman and Nevers (2003). Because of their tendency to adhere to particulate matter, fecal bacteria are able to persist longer in the sand than in the water column. Thus beach sand serves as a fecal coliform reservoir. The presence of fecal coliforms in our samples indicates that

sediment, likewise, may serve as a reservoir for *E. coli* and other strains. Although the original source(s) of the sediment coliforms is/are unknown at this point, it is logical to assume (based on the mid-lake location of the sampling sites) that there are a number of contributing factors, including sewage from leaking septic tanks and wastewater treatment plants, livestock operations, etc.. Regardless of their origin, presence of fecal organisms in sediment presents a potential health hazard should they be released into the water. The World Health Organization (WHO) recommends a coliform count of 0 colonies per 100 mL for drinking water and the U.S. Environment Protection Agency (EPA) recommends a maximum level for swimming water of 200 colonies per 100 mL (Miller 1998). Although larger communities such as Victoria Beach and Berens River have water treatment plants, others, such as Loon Straits and Princess Harbour take water directly from Lake Winnipeg, increasing their vulnerability to water-borne diseases associated with fecal contamination (LWSB 2005).

4.1 **Future research**

Although this research project has illuminated several aspects of Lake Winnipeg sediment, future research needs to be conducted to determine the following:

(1) Nitrogen cycling of nutrients associated with microorganism activity. Nitrogen is said to be the limiting factor of bacteria growth; the role these bacteria play in cycling of this nutrient is important since nitrogen is found in excess amounts in the lake;

(2) Inorganic and organic content of sediment to determine the level of nutrients available to microorganisms of the sediment;

(3) Guild specific utilization of sole-carbon sources to determine which organic groups are preferentially metabolized by microbial communities within the sediment;

(4) Annual changes in microbial communities based on counts and functional diversity as changes in composition or activity of microbial communities might have immediate or lasting effects on ecosystem functioning since heterotrophic organisms are ecologically important in aquatic environments (Hobbie 1971, Zak *et al*. 1994);

(5) Quantification of total coliforms and fecal coliforms within the sediment and water to determine the level of contamination of the lake.

5 Conclusion

(1) In this study, the numbers of culturable, heterotrophic actinomycete, bacteria, and fungi were determined in 18 sediment samples taken from Lake Winnipeg. Statistically, actinomycete numbers varied with temperature (higher at 23.5^{o}C), bacteria numbers varied with temperature and location (highest at 23.5^{o}C and in the South Basin) while fungi numbers did not vary with either temperature or location.

(2) Substrate richness and intensity were highest at 23.5^{o}C and in the South Basin suggesting sediment microorganisms in the South Basin are more functionally diverse.

(3) Phosphatase activity, both acid and alkaline, was greatest in the North Basin indicating that microbial activity may contribute to eutrophication leading to algal blooms observed yearly in the North Basin of Lake Winnipeg.

(4) Coliforms were detected at 12 sites (primarily in the South Basin), while *E. coli* was located at 6 sites (in both the South and North Basin), and fecal coliforms were detected at 5 sites (3 in the South Basin). This suggests that sediment may be a reservoir of fecal coliforms.

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