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Microbially mediated redox transformations of manganese (II) along with some other trace elements: A study from Antarctic lakes

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Abstract:

The significance of fresh water systems in global manganese cycles is well appreciated. Yet, the polar systems which encompass the largest fresh water repository in the world have been least studied for their role in manganese cycling. Here, we present results from a study that was conducted in the brackish water lakes in the Larsemann Hills region (East Antarctica). The rate of *in situ* manganese oxidation ranged from 0.04 to 3.96 ppb/day. These lakes harbor numerous manganese oxidizing bacteria (10^{5-6} CFUL⁻¹), predominantly belonging to genera *Shewanella*, *Pseudomonas* and an unclassified genus in the family Oxalobacteriaceae. Experiments were conducted with representatives of predominant genera to understand their contribution to Mn cycling and also to assess their metabolic capabilities in the presence of this metal. In general, the total and respiring cell counts were stimulated to a maximum when the growth medium was amended with 10mM manganese. The addition of manganese promoted the use of D-Mannitol, Maltose etc, but inhibited the use of Maltotriose, L-Serine and Glycyl L-Glutamic acid. The bacterial isolates were able to catalyze both the redox reactions in manganese cycling. *In vitro* manganese oxidation rates ranged from 3-147 ppb/day while manganese reduction rates ranged from 35-213 ppb/day. It was also observed that the maximum stimulation of manganese oxidation occurred in the presence of cobalt (81±57 ppb/day), rather than iron (37±16 ppb/day) and nickel (40±47 ppb/day). Our studies suggest that cobalt could have a more profound role in manganese oxidation while nickel promoted manganese reduction in polar aquatic systems.

Keywords: Antarctica, Lakes, Manganese, Oxidation, Bacteria

Introduction:

Manganese (Mn) is an important trace element essential in microbial, plant, and animal nutrition. It is required as an activator by a number of crucial enzymes such as isocitric dehydrogenase, malic enzyme etc. It is also required in oxygenic photosynthesis, where it functions in the production of oxygen from water by Photosystem II (e.g., Klimov 1984). Hulth et al. (1999) speculated on the biochemical coupling of anaerobic lithotrophic nitrification to MnO₂ reduction. According to Luther et al. (1997), denitrification due to oxidation of NH₄⁺ and organic nitrogen by MnO₂ can predominate over nitrification and may account for 90% of the N₂ formed in the continental margins. Mn (II) could also serve as an energy source in mixotrophs (Lieske 1919; Sartory and Meyer 1947) while Mn(III) and Mn(IV) function as terminal electron acceptors in anaerobic respiration. Wide spread occurrence of Mn oxidizing and reducing microbes in terrestrial, freshwater, and marine environments signify the important role they play in the geochemical cycle of Mn. Jackson (1901a, 1901b) and Beijerinck (1913) were the first to describe the existence of Mn-oxidizing bacteria. Some of the Mn oxidizers deposit oxidized Mn on their cells, sheath, or spore (Okazaki et al. 1997; Mulder 1972; Rosson and Neelson; 1982). In addition, the relatively higher resistance of Mn(II) to auto oxidation as compared to Fe(II) at pH values below 8 increase the importance of Mn oxidizers in the environment (Crerar and Barnes 1974). Also, when Mn²⁺ concentrations are below 10mgL⁻¹, auto oxidation is not considered likely to happen (Crerar and Barnes 1974). The most important geomicrobial interactions with Mn are those that lead to precipitation of Mn²⁺ in an insoluble phase (immobilization) or solubilization of insoluble forms of Mn (mobilization). Further, the suggested central role for manganese oxides is due to their remarkable surface area and charge distribution, which make them a potentially rich reservoir of adsorbed metals (Bratina et al. 1998). Linking Mn(II) oxidation to cellular function, although still not well understood, continues to drive efforts to characterize Mn biomineralization. Diem and Stumm (1984) and Tipping et al. (1984) found that Mn(IV) oxide was formed microbiologically as the first recognizable oxidation product in Mn-containing water samples. Tipping et al. (1984, 1985) found that the oxide they recovered resembled MnO₂. Environmentally it is possible to associate rapidly formed, fresh Mn(IV) oxide deposits with biological catalysis and Mn(III) oxide deposits with abiotic reactions (Ehrlich 1996). Recently, complexed-Mn(III) has been shown to be a transient intermediate in Mn(II) oxidation to Mn(IV), suggesting that the reaction might involve a unique multicopper oxidase system capable of a two-electron oxidation of the substrate (Tebo et al. 2005).

Average concentration of Mn in freshwater has been reported to be 8µg kg⁻¹, while in sea water the average concentration is reported to be 0.2µg kg⁻¹ (Bowen 1979). Some of the earlier studies in cold regions have been observed in a glacial, oligotrophic, thermally stratified lake; Punnus-Yarvi on the Karelian Peninsula in the former U.S.S.R. (Sokolova-Dubinina and Deryugina 1967a, 1967b). *Metallogenium sp.*, which was found in all parts of the lake, was responsible for Mn oxidation while *Bacillus circulans*, *B. polymyxa*, and an unidentified non-spore forming rod were thought to be involved in the formation of rhodochrosite (MnCO₃) from bacteriogenic Mn oxide. Davison et al. (1982) reported that microbial Mn reduction in the deeper sediments of Esthwaite Water (U.K.) was intense during the winter months when Mn oxides accumulated transiently in the sediment. In the summer months, Mn oxides generated in the water column were reduced in the hypolimnion before reaching the sediment.

In spite of its global significance, the studies on the biogeochemical aspects of Mn in soil and aquatic environments are still few. An approximately forty-fold rise in the levels of Mn in the freshwater systems when compared to marine systems is a testimony to the role played by such systems in the global cycling of Mn. Even though the polar regions, especially the Antarctic, have the single largest repository of fresh water in the world, the study of Mn biogeochemical cycling or metal-microbe interactions in general have been sparse (e.g. Bratina et al. 1998) and to date there has been no attempt to study the microbially mediated Mn oxidation under *in situ* conditions and understand the microbial flora catalyzing these crucial reactions.

The Larsemann Hills are located along the Ingrid Christensen Coast of Princess Elizabeth Land, East Antarctica (69°30' S, 76°20' E), and comprise a series of N–S oriented, ice-free rocky peninsulas, which extend from beneath the Antarctic ice-cap towards the ocean. One of the features of the Larsemann Hills is the presence of more than 150 freshwater lakes. Most of these lakes are small (5000–30,000 m²) and shallow (less than 5 m), and their water chemistry is primarily influenced by natural processes such as snow melt-water and sea-spray influx, erosion and weathering of the country rock, biological activity, and evaporation (Burgess et al., 1988; Burgess and Kaup, 1997; Gasparon and Burgess, 2000; Gasparon et al., 2002; Gasparon and Matschullat, 2006; Gillieson et al., 1990). All the lakes are ultra-oligotrophic and ice covered for 9–11 months each year.

Microbial manganese oxidation in the fresh water lakes of Larsemann Hills was assessed during the austral summer. *In situ* incubation experiments were carried out for a period of 7 days to access the net dynamics of Mn²⁺. Mn oxidizing bacteria were enumerated, representative strains isolated, identified and tested *in vitro* for their ability to grow and immobilize Mn by itself and also in the presence of Fe, Co and Ni. BIOLOG studies were also employed to have some basic understanding on the influence of Mn on the metabolic profiles of bacterial strains.

Materials and Methods:

Study site, water sampling and physiochemical parameters:

Water samples were collected from the twelve fresh/brackish water lakes in the Larsemann Hills region of the East Antarctica during the XXVI Indian Scientific Expedition to Antarctica (March, 2007). Figure 1 shows the lakes in the Larsemann Hills region and the locations of sampling. Since the surface of the lakes was frozen, a hole was drilled in the ice through which we could lower the water sampler (Go-Flow bottles). The thickness of the ice cover was fairly thin which could be easily dislodged with a sterile chisel with a polypropylene sleeve. Sub surface samples (<0.5m) were collected into polypropylene bottles cleaned with trace element grade HCl and rinsed thrice with the lake water prior to collection of each sample. The samples were stored in near to ambient conditions (~4°C) in a refrigerator for about 2 days prior to microbial analysis.

Salinity was measured as conductivity using a handheld conductivity meter. While temperature and pH were measured using a hand held portable pH meter with pH and temperature probe (Thermo Orion).

Direct counts (DC) and culture of Heterotrophs (THB) and Mn oxidizing bacteria (MnOB):

Microbes (DC) in the surface water samples (10 ml) were stained with both acridine orange (AO) (Hobbie et al. 1977) and/or 4',6-diamidino-2-phenylindole (DAPI) (Hicks et al. 1992) before enumeration. DAPI was used when cells (from lakes L4, L6 and L7A) were not discernible on 0.2 μ black track etched membrane filters when stained with AO due to high background fluorescence. Cells stained with DAPI were first fixed with formaldehyde (final concentration, 3.7%) to preserve the cell morphology and improve the staining efficiency. Stained cells were filtered onto black polycarbonate membranes (Nucleopore, Whatman) and counted by using an Olympus epifluorescence microscope (BX 51). The filters used for DAPI was Olympus U-MWU2 (Excitation 330-385 and Emission 420) and Acridine Orange was Olympus U-MWB2 (Excitation 460-490 and Emission 520IF). Counts were done using a Whipple grid and the objective used was 60X (Olympus UPLNFLN).

An inoculum of 100 μ l was used for the enumeration of culturable MnOB by surface plating. The lake waters were also checked for total culturable heterotrophic bacterial population on both marine agar (THB-SAL) and nutrient agar (THB-FW). Media were prepared using diluted nutrient agar in lake water [dilNA = 2 gm l⁻¹ nutrient broth (HiMedia) in lake water+2% agar agar (Difco)] for THB-FW and dilNA supplemented with 1 and 10 mM MnCl₂.4H₂O for MnOB (1 mM Mn l⁻¹ has 54.9 mg Mn l⁻¹ and hereafter referred as 1 mM Mn). The THB-SAL was enumerated on diluted Difco Marine Agar 2216 [dilMA = 2 gm l⁻¹ Difco Marine Agar in lake water+2% agar (Difco)]. Culturable counts in the form of colony forming units (CFU) were recorded after incubating the plates at 2°C (\pm 0.1°C) for 30 days. Representative isolation was done from Mn amended plates based on the variability in colony morphology. Each isolate represented a percentage of colonies having similar morphologies recorded in the plate counts. Well-isolated colonies were checked for purity by microscopic examination after Gram staining. Further subculture and maintenance was done on 1mM Mn supplemented nutrient media as described above.

Effect of Mn on growth and respiration:

An assay was performed to understand the growth of three representative isolates (LH – Larsemann Hills) LH-5, LH-8 and LH-11 in the presence of Mn. They were selected based on their abundance and colony characteristics. Tests were done at 1, 10 and 100 μ M MnCl₂.4H₂O; as well as 1, 10 and 100 mM MnCl₂.4H₂O in 25% nutrient broth. An unamended (25% nutrient broth without added Mn) combination was used as the control. Exponentially growing cells were harvested from Mn supplemented dilNA plates in physiological saline. The suspension was centrifuged at 5000 rpm for 10 min. Cell pellets were washed twice with sterile saline and resuspended by vortexing. The inoculum size was calculated by direct cell counts in a cell counting chamber. Triphenyl tetrazolium chloride (TTC; 0.025%) was added to every tube prior to incubation. The experiments were done in triplicate at pH 7.6–7.8 and incubated at 2°C (\pm 0.1°C) in dark for a period of 20 days. Over the incubation period, respiring cells convert TTC to triphenyl formazan (Lenhard 1956) that gives a deep pinkish appearance. As measurements were carried out spectrophotometrically, a longer incubation period was necessary to facilitate formazan production by the slow growing bacteria. Cell counts (respiring and total) were carried out in a cell counting chamber and observed with a bright field (Olympus BX 51). The amount of formazan produced corresponded to the respiring

activity of cells. Hence to measure the amount of TTC reduced to formazan, samples were centrifuged at room temperature at 8000 rpm for 10 min. Three milliliters of methanol was added to the cell pellet and the tubes were vortexed. The tubes were kept overnight for complete extraction of formazan by methanol and centrifuged again. The supernatant was measured spectrophotometrically at 520 nm to estimate the formazan formed.

Effect of Mn on single-C substrate utilization:

Isolates were examined for their ability to oxidize various compounds in presence and absence of Mn by using GN2 (Biolog, Inc. USA). A Biolog assay involves inoculating microbial cultures into microplates that contain 95 sole C-source in addition to a “tetrazolium violet dye”. The GN (gram negative) plates are made up of 95 sole carbon sources such as carbohydrates (26), carboxylic acids (24), amino acids (20), amines/amides (8), polymers (5) and miscellaneous (12). The utilization of any C-source by the bacteria results in the respiration-dependent reduction of the dye and purple-coloured formation that can be quantified and monitored over time. Negative wells remain colorless, as does the reference well with no carbon source. Young cultures [three days old at 2°C ($\pm 0.1^\circ\text{C}$)] were harvested from 1mM Mn supplemented dilNA plates with sterile physiological saline (0.9%). Suspension was washed 5-6 times by centrifuging at 6000rpm for 10 minutes at 10°C with subsequent vortexing each time. This was necessary to exclude any nutrients coming from the agar plates which could otherwise result in a false positive test. Almost same turbidity (0.8 ± 0.05 at OD_{600}) was maintained for all three isolates. This cell suspension was then divided in two separate sets of test tubes. $100\mu\text{M}$ of Mn was added in one of the tubes. All wells of the microplates were inoculated with precisely 150 μl of cell suspension. Separate plates were used for inoculation of sample from Mn amended and unamended suspensions. Incubation was done at $2(\pm 0.5)^\circ\text{C}$. Absorbance was measured at 595nm in a microplate reader (BMG Optima) for every 24 hour interval. Measurements were carried out for five days till the OD measurements plateaued for each well. Average Well Color Development (AWCD) and further normalized values were computed for each substrate (Garland and Mills 1991). Wells which was showing the highest variability in normalized value (compared to that of the previous day reading) was considered to have the substrate that was preferentially utilized during that particular period.

Identification and characteristics of Mn oxidizing bacteria:

The manganese oxidizing isolates were classified based on a phylogenetic analysis of their 16S rRNA gene (16S rDNA) sequences. The 16S rDNA was amplified by PCR from single colonies (Louws et al. 1996) by using oligodeoxynucleotide primers designed to anneal to conserved regions of the bacterial 16S rDNA. The forward primer EUB f 5'-GCACAAGCGGTGGAGCATGTGG-3' corresponded to positions 933 to 955 of V6 ,V7,V8 of the 16S rDNA from *Escherichia coli*, and the reverse primer EUB r 5'- CCCGGGAACGTATTCACCG -3' corresponded to positions 1387 to 1368 of V6,V7,V8 region. The amplified product was purified on QIAquick PCR Purification Kit, QIAGEN recommended by the manufacturer. Cycle sequencing was performed on Eppendorf Mastercycler, Hamburg, Germany and the products were analyzed on a DNA sequencer. A partial sequence of 16S rDNA (ca. 400 to 500 nucleotides) was obtained for each of the 3 isolates from primers EUB f and EUB r. Each sequence was compared with sequence of strains belonging to the same phylogenetic group obtained from the Genbank database and from a BLAST search of the National Centre for Biotechnology Information (NCBI). Phylogenetic analysis was performed using the BioEdit and Mega 4.1 software package.

For fatty acid methyl ester analysis, cell mass of the isolates were harvested from Soyabean Casein Digest Agar plates after incubation for one day at 28°C. The fatty acid methyl esters were extracted and prepared according to standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser 1990).

Culture specimen for electron microscopy and EDS analysis was prepared as follows. Suspensions of Mn oxidizing colonies of all the isolates cultured on 1 mM Mn amended diINA medium were prepared in 0.9% sterile saline. Clean glass pieces were immersed in the suspensions and left for a week for the cells to form a biofilm on the glass surface. The glass pieces were retrieved and the biofilm was then subjected to dehydration by running it through a series of increasing acetone concentrations from 10, 30, 50, 70, 90 and 100%. The samples were then air dried, mounted on a stub and sputter coated (JEOL JFC- 100) with platinum. The specimens were then visualized with a JEOL JSM-6360 Scanning electron microscope (SEM). The presence of Mn-oxide deposits in the culture was corroborated using an energy dispersive X-ray spectrometer (EDS) in conjunction with the SEM.

Mn oxidation assay:

(i)Field studies

Three large lakes (L7, L2 and L3; Figure 1) were selected for logistic reasons and close proximity to the Indian research base to monitor the changes in dissolved metal ions (Mn, Fe, Co & Ni). Immediately after collection the water samples were sub sampled (100ml) into seven sterile poly propylene, wide mouth square bottles (Nalgene). After fixing the 0 day samples with 2% ultra pure HNO₃, the rest of the sample-containing bottles were closed airtight and incubated under ambient conditions just below (<0.5m) the frozen lake surface. Individual incubated bottles were retrieved at 24-hour intervals over a 6-day period and their content was filtered through a 0.015 μ filter (Whatmann) and fixed with 2% ultra pure HNO₃. The prevailing temperature of incubation ranged from 0.1-0.5°C. The concentration of metal ions in the samples was analyzed by X series Inductive Coupled Plasma Mass Spectrometer (ThermoElemental). The trace element standard used in the present study (Multimetal Standard 6) was procured from Merck. The instrument was tuned to achieve a detection limit of 0.087, 0.052 and 0.02 ppb for Fe, Mn and Co respectively.

(ii)Isolate studies

All the three isolates were examined for their multi-metal immobilization/mobilization capability in 0.1% nutrient broth. The cultures were prepared as was done for Biolog based studies. Eight different combinations of four metals Fe, Mn, Co and Ni viz Mn; Mn&Fe; Mn&Co; Mn&Ni; Mn,Fe,&Co; Mn, Fe,&Ni; Mn,Co&Ni; Mn,Fe,Co&Ni were used to study the effect of similar transition metal ions on the immobilization/mobilization of Mn. Uninoculated controls for each combination were incorporated to check the levels of auto oxidation. The concentrations of all metals at the zero hour were kept at 1 μ M. Each batch was inoculated in triplicate with 100 μ l of cell suspension and incubations were done at 2(\pm 0.1) $^{\circ}$ C. A 0-day batch was kept as control and preserved by adding 2% ultra pure HNO₃. Remaining batches were analyzed on the 2nd, 4th, 7th, 9th and 13th day for their metal concentrations. Respective samples were spun at 6000 rpm for 15 minutes at 10°C. The supernatant was collected and preserved by adding 2% ultra pure HNO₃. All preserved samples were analyzed by

ICPMS after 1:1 dilution with milliQ. No extraneous source of oxides has been added and mobilization was measured only in terms of the reversed reaction where immobilized ions become re-mobilized.

Results:

Cultivability, Identity and culture characteristics:

Figure 2 shows that culturable counts on THB-FW and THB-SAL ranged from non-detectable (ND) to 10^6 CFU L⁻¹, while MnOB was in the range of 10^{5-6} CFUL⁻¹. The magnitude of DC (10^{7-8} cells L⁻¹) suggests that the majority of the culturable counts were less by two orders of magnitude than the DC. A total of 100 Mn oxidizing isolated from various lakes were biochemically characterized and checked for the precipitation of oxides of Mn. Ninety eight percent of the colonies showed brown precipitation on prolonged incubation. There were 6 morphologically different colony types. All the isolates tested were gram-negative, short rods ($\sim 0.6 \times 0.4 \mu\text{m}$). Of these isolates, 60% showed taxonomic affinities to *Shewanella sp.* and 30% comprised *Pseudomonas sp.* The rest belonged to an unclassified genus in the family Oxalobacteriaceae. The three isolates used in the study LH-5(FJ476127), LH-8 (FJ357794.1) and LH-11(FJ595995) resembled the three different genus viz. *Pseudomonas*, the unclassified genera in the family Oxalobacteriaceae and *Shewanella* respectively. The colony morphotype LH-5 represents 48% of the total MnOB CFU from Lake 2 (9×10^5 CFU L⁻¹), while LH-11 represented 72% of the total MnOB CFU from Lake 3 (6.2×10^5 CFU L⁻¹). The strain LH-8 was isolated from lake L-7, which is the largest lake in this region (Figure 1) and represented only 26% of the total MnOB CFU (8.3×10^6 CFUL⁻¹). Intense brown color observed possibly due to deposition of oxide by the bacterial colonies of the three strains was the major criterion for the selection of strains LH-5, 8 and 11 for a detailed study. SEM-EDS could not detect Mn oxide on individual bacterial cells since the oxides were only found to be present within the bacterial exopolymeric secretions (45.14% MnO). The oxide is assumed to be of Mn(IV) since it is most likely deposited on the bacterial surface on prolonged incubation (Ehrlich 1999).

All the isolates have a dominant fraction of cis-7-Hexadecenoic acid, 2 hydroxy-iso-pentadecanoic acid and hexadecenoic acid. In addition to this, cis-7-octadecenoic acid, Cyclo-heptadecanoic acid and Iso-pentadecanoic acid were dominant in LH-5, 8 and 11 respectively. LH-5 resembled *Pseudomonas syringae syringae* with a similarity index of 0.887 (Library - TSBA40 4.10), while LH-8 had a very low similarity index. MIS-FAME identity of LH-11 showed a partial concurrence with the 16SrDNA results indicating that the strain belongs to the genus *Shewanella*. LH-11 had the closest 16S rDNA similarity to *S. baltica* while MIS-FAME analysis shows it to be *S. putrefaciens* with a similarity index of 0.368.

Growth and respiration:

Figures 3A, B and C illustrate growth, respiring cell counts and respiration rates for isolates LH-5, 8 and 11. Growth and respiration experiments with three representative isolates showed similar results. At the end of a 20-day incubation period, the respiring cell counts in control tubes of unamended incubations (-Mn) for strains LH-5, 8 and 11 were 2.8×10^8 , 8×10^7 and 1.3×10^8 cells ml⁻¹ respectively. In LH-5, the respiring cell counts were observed to be stimulated to a maximum of 6.9×10^8 cells ml⁻¹ in 10mM Mn supplement (Figure 3A), while for LH-8 and 11 it was 8.3×10^8 and 1.0×10^9 cells ml⁻¹ respectively in 10mM Mn supplement (Figure 3B and C). Maximum stimulation in growth of LH-5 (8.1×10^8 cells ml⁻¹)

occurred at 1mM, while for both LH-8 (8.3×10^8 cells ml^{-1}) and 11 (1.0×10^9 cells ml^{-1}) it was observed in 10mM amendment. A marginal decrease in both growth and respiring counts was observed at 100mM supplement irrespective of the isolate. Respiration rate in the unamended medium was 2.9, 2.6 and 1.7×10^{-17} $\mu\text{g O}_2 \text{ cell}^{-1} \text{ day}^{-1}$ for LH-5,8 and 11 respectively while the peak was observed in 100mM Mn amendment in LH-5 (20.1×10^{-17} $\mu\text{g O}_2 \text{ cell}^{-1} \text{ day}^{-1}$), LH-8 (21×10^{-17} $\mu\text{g O}_2 \text{ cell}^{-1} \text{ day}^{-1}$) and LH-11 (21.5×10^{-17} $\mu\text{g O}_2 \text{ cell}^{-1} \text{ day}^{-1}$) indicating enhanced metabolism. An increase in respiration rates with increased initial Mn concentration could indicate stimulation of metabolism to utilize available manganese optimally

Effect of Mn on C utilization:

Table 2 gives an account of the major carbon compounds (carbohydrates and amino acids) utilized by the three isolates. Considerable difference in the utilization of carbohydrates was observed by the isolates in the presence and absence of added Mn. In the absence of added Mn (Figure 4 A), Maltotriose was the most preferred sugar followed by α -D-Glucose and D-Trehalose. In the presence of Mn (Figure 4B), α -D-Glucose was utilized with high levels of preference followed by D-Mannitol. It is also important to note that Maltotriose was not used in the presence of Mn, while addition of Mn triggered the use of Maltose, D-Galactose, D-Sorbitol and D-Mannitol. D-Melibiose and D-Melezitose were the other sugars that were not utilized in the presence of Mn. In respect to amino acid utilization, L-asparagine was the most preferred followed by L-Glutamic acid, L-Serine and Glycyl L-glutamic acid in the absence of added Mn (Figure 4A). In the presence of added Mn (Figure 4B), L-Serine and Glycyl L-glutamic acid were not preferred. L-asparagine was used preferentially in the presence and absence of added Mn during the first 24 h except by LH-8 in the absence of added manganese. During the next 24 h, LH-5 continued to use asparagine preferentially in the presence and absence of Mn, whereas LH-8 and LH-11 used it preferentially only in the presence of added manganese. At the end of third day L-Glutamic acid was preferred by all the isolates with and without added manganese except for LH-11 (+Mn) which still maintained a preference for L-Asparagine. The preference for L-Glutamic acid by LH-5(+Mn), LH-8(+Mn) and LH-11 (- Mn) continued till the end of the experiment. L-serine was used preferentially in the absence of added manganese by LH-5 and LH-8, whereas L-glutamic acid was used preferentially by LH-11 in the presence of added manganese. L-Glutamic acid dominated the preference list at the end of incubation for all the three isolates in the presence and absence of added manganese, except for LH-8 in the absence of added manganese, which preferred glycyl L-glutamic acid.

Mn oxidation assay:

(i) Field studies

Figure 5 illustrates the changes in manganese concentration during in-situ incubation of lake water samples for 6 days. In general, the rate of net Mn (II) oxidation in these lakes ranged from 0.04 to 3.96 ppb/day. Whereas a continuous phase of oxidation of Mn was noted in L7, manganese oxidation in L2 and L3 was intermittent and showed phases of (MnIII/MnIV) reduction. The Mn (III/IV) reductions observed in the other lakes ranged from 0.5 to 1.1 ppb/day. It was observed during the end 3rd and 6th day for Lake L3, while for L2 it was at the end of 3rd and 5th day.

(ii) Isolate studies

Figure 6, 7 and 8 illustrates the changes (average value of the triplicates) of added Mn(II) and other ions added in incubation experiments with LH-5, LH-8 and LH-11 respectively. All the standard deviations were <5 ppb for Mn and <0.5 ppb for Fe, Co and Ni. In all the incubation experiments, the first four days were marked by a net rise in the level of Mn (II). In all the incubations of LH-5 the initial level of Mn increased more than ~700ppb at the end of fourth day while for LH-8 and 11 it was >~500 and >~300ppb respectively. In most of the cases, except for combinations of Mn, Mn-Fe and Mn-Fe-Co of LH-11 reduction was succeeded by oxidation. However, the period of oxidation varied with culture and incubation conditions. LH-5 (Figure 6) showed enhanced oxidation activity compared to the other isolates. LH-5 was able to immobilize about 40-98% of Mn²⁺ available in the medium after the initial reduction phase, while that for LH-8 and 11 ranged between 17-62% and 14-45% respectively. For all the isolates, Fe in the Mn-Fe combination showed net reduction (Figures 6b, 7b and 8b) while in combinations of Mn-Fe-Co (Figures 6e, 7e and 8e), Mn-Fe-Ni (Figures 6f, 7f and 8f) and Mn-Fe-Co-Ni (Figures 6h, 7h and 8h), the net effect was oxidation. The net changes in Co and Ni in all the incubation experiments of LH-5 (Figures 6 c to h), LH-8 (Figures 7 c to h) and LH-11 (Figures 8 c to h) indicated net oxidation. The auto oxidation of Mn in the control tubes was <1ppb/day.

Discussion:

The significance of fresh water systems in global Mn cycles is well appreciated. Though polar regions especially the Antarctic being the single largest repository of fresh water in the world, to date there have been no attempt to study the microbially mediated Mn oxidation and understand the microbial flora catalyzing these crucial reactions. Hence, in order to probe into these issues, the Larsemann Hills region in the East Antarctica (Figure 1) which represents one of the numerous coastal oases that carry large suites of lakes, most of them shallow, small and ultra-oligotrophic covered with ice for 9-11 months each year was selected as a representative geographic region to study these reactions.

Bratina et al. (1998) have reported that there was no significant difference between counts of cells stained with DAPI or AO. However, the detritus load in the sample is extremely critical for accurate counting of microbial cells on AO stained specimens. In DAPI stained specimens the presence of microbes is only indicative. In the present study DC obtained by both or either the methodologies has not been discriminated for discussion as there was no significant difference. The culturable counts were lower by more than two orders of magnitude than the direct counts (10^{7-8} cells⁻¹). It was interesting to note that in spite of having the highest DC; Lake L7 showed poor cultivability of THB-FW and THB-SAL. However on addition of Mn the cultivability was improved by almost 6 orders of magnitude. This probably shows that the bulk fraction was viable but non-culturable and may indicate the relevance of Mn in governing the viability of microbial cells under psychrophilic conditions. In addition it was observed that the lakes which were showing higher DC (eg.L7, L7A and L5) also harbored higher levels of MnOB and very low levels of THB-SAL. This strengthens our argument on the role of fresh water systems in governing Mn cycling. The occurrence of THB-SAL in these fresh water lakes strengthens their ecological history which is that these lakes evolved from oligotrophic, proglacial lagoons to brackish lakes affected by periodic influxes of salt water from sea spray and surges produced by glacial calving (Gillieson 1991).

The three isolates used in the study LH-5, LH-8 and LH-11 resembled three different genera viz. *Pseudomonas*, the unclassified genera in the family Oxalobacteriaceae and *Shewanella* respectively. The involvement of *Shewanella* and *Pseudomonas* in the Mn cycle has been widely reported (eg. Bratina et al.1998; Fernandes et al.2005; Schweisfurth et al.1978). Colony abundance estimates show that in general the major culturable taxa involved in Mn oxidation could be *Pseudomonas* and *Shewanella* as they represented 48% and 72% respectively of the total MnOB CFU in these regions. Although, LH-8 represented only 26% of the total MnOB CFU it could have a very profound ecological significance for a couple of reasons. The most important aspect being that they belong to an unclassified genus in the family Oxalobacteriaceae and the lake (L7) from which these bacterial strains were isolated harbors the greatest number of MnOB in the region.

A universal response to low temperature is a change in the lipid composition of membranes. Phospholipids with unsaturated fatty acids have lower melting points and a greater degree of flexibility than phospholipids with saturated fatty acids. This, homeoviscous adaptation was first shown in *E.coli* (Sinensky 1974). The occurrence of *cis*-7-Hexadecanoic acid in all these strains is unique feature considered to be a signature fatty acid for autotrophic bacteria isolated from mining lakes (Knief et al. 2003). This probably indicates their mixotrophic nature (Mn based) in these ultra-oligotrophic systems. In addition unsaturated fatty acids like *cis*-7-octadecenoic acid which are present in all the isolates indicate their ability to live in perennial, extremely cold conditions. The occurrence of elevated levels of hexadecanoic, 2 hydroxy-iso-pentadecanoic, cyclo-heptadecanoic, iso-pentadecanoic acids etc in these organisms exhibits the metabolic plasticity of these isolates to withstand a wide range of temperature.

Growth and respiration (Figures 3A-C) experiments conducted on the three representative strains on varying amendments of Mn (1µM–100 mM) revealed the stimulatory effect at even lower concentrations. At the end of a 20-day incubation period, the maximum stimulation in respiration was found in amendments of 10mM Mn. Except for LH-5 where growth was stimulated at a maximum of 1mM, the growth peaked for the other isolates at 10mM. A marginal decrease in both growth and respiring counts observed at 100mM probably indicate the level of tolerance. Comparatively higher rates of respiration coupled to lower respiring/total counts observed at 100mM could be considered as an index of stress. Fernandes et al. (2005) had shown that manganese oxidation could be linked to lithotrophic mode of nutrition. In the present study since there was significant differences between the amended and unamended (-Mn) incubations, the oxidation of Mn could be coupled to the growth of the bacteria in a mixotrophic mode.

Though probably not obligately coupled to carbon metabolism, the presence of Mn had a considerable impact on the preference for utilization of carbohydrates and amino acids in these isolates. In general though Maltotriose was the most favored sugar followed by α D-Glucose and D-Trehalose, the addition of Mn inhibited the use of Maltotriose, D-Melibiose and D-Melezitose. The addition of Mn was also stimulated the metabolism of carbohydrates such as D-Mannitol, D-Sorbitol etc. Addition of Mn was found to prevent the uptake of amino acids like L-Serine and Glycyl L-Glutamic acid indicating their role in influencing protein synthesis.

Lake L7 is characterized by a comparatively high level of Mn²⁺ (18.23 ppb) which probably sustained continuous Mn (II) oxidations. This could also be achieved by very short turn over times or continuous Mn inputs from the sediment bed.

The periodic shifts from Mn oxidation to reduction or vice versa in the much shallower lakes L3 and L2 could be due to Mn limitation. ANOVA of Mn oxidation rates from the three lakes show that there is significant variability ($df = 2$, $p < 0.02$) between the lakes. These observations indicate that the largest lake L7 could have different environmental conditions compared to the others where a Mn redox cycle was observed to change phases in about two days time.

Green et al. (1986a) observed that the transition metals Mn, Fe and Co are the most reactive elements in the upper lake and are removed most rapidly, while Ni has longer residence times. Further they speculated that, in contrast to the major elements, there appeared to be effective removal processes regulating the concentrations of trace metals probably scavenging of these elements by clay particles, sorption onto hydrous manganese and iron oxide surfaces etc. Our studies emphasize the involvement of microbes in governing the fate of transition elements in these lakes. All the isolates showed well defined phases for oxidation and/or reduction (Table 3). During incubation of our cultures, the first four days were marked by a net rise in the level of Mn (II) suggesting the expulsion of Mn from the cells to the media or probably the reduction of cell surface bound Mn oxides. The net Mn reduction for LH-5 peaked at 213 ppb/day while for LH-8 and LH-11 it was 126 and 59 ppb/day respectively. It was also observed that the maximum stimulation of Mn reduction in LH-5 occurred in the combination Mn and Mn-Fe (>200 ppb/day). For LH-8 it peaked in the combination of Mn-Ni and Mn-Fe-Ni (>125 ppb/day) while for LH-11 it was in the combinations of Mn-Ni, Mn-Fe-Ni and Mn-Fe-Co-Ni (>50 ppb/day) suggesting the critical role of Ni in Mn reduction. Recently, Konhauser et al (2009) had reported that Ni played a significant role in governing the redox state of the earth before the great oxidation event. Further, they showed that Ni was extremely critical for catalyzing reductive pathways and the famine of nickel resulted in the oxidation event.

In all the isolates Mn oxidation was found to peak in the second half of the incubation period with LH-5 and LH-8 showed Mn oxidation maxima of 147 and 55 ppb/day respectively between 7-13 days. LH-11 showed the maxima (41 ppb/day) during the period 9-13 days. In, LH-5 the lowest rate of Mn oxidation (55 ppb/day) was encountered in both Mn-Ni and Mn-Fe-Co-Ni combinations. In LH-8 the same was observed at a rate of 24 ppb/day (Mn combination) while in LH-11 there was no net Mn oxidation in Mn-Fe combination. In general it was observed that the Mn oxidation rates of LH-5 in all the combinations were higher than LH-8 and LH-11. In most of the incubations the additional ion(s) added in addition to Mn showed little variability ($\sim 1-3$ ppb/day) however they significantly influenced the redox kinetics of Mn (Table 3). It was also observed that the maximum stimulation of Mn oxidation occurred in the Mn-Co combination (81 ± 57 ppb/day), rather than combinations of Fe (37 ± 16 ppb/day) and Ni (40 ± 47 ppb/day) probably suggesting the role of Co in Mn oxidation. The contrasting behavior of Fe and Mn was previously observed in Lake Joyce, Antarctica (Schat et al. 2004). Green et al. (1986b) and Canfield et al. (1995) reported that water column separations (between Fe and Mn) of 10 m was observed in highly stratified systems of Lake Vanda. Additionally, our studies showed that all of our bacterial isolates oxidized Mn at a faster rate in the presence of Co than in its absence irrespective of other trace metal combinations (Table 3). Though Fe is thought to be having a more pronounced role in the biological reactions involving trace metals, our studies indicate that probably inert oxides of Co on primary Mn oxides could serve as a catalyst in governing the trace metal cycles. Ni probably retarded Mn oxidation by LH-5 and Fe had a negative influence on Mn oxidation by LH-8 and LH-11 (Table 3).

Conclusion:

The fresh water lakes in the Larsemann Hills region (East Antarctica) harbor numerous MnOB (10^{5-6} CFUL⁻¹) and the occurrence of THB-SAL indicate their past maritime history. The presence of Mn in the bacterial culture media enhanced their growth by six orders of magnitude. The bulk fraction of the culturable MnOB belonged to *Shewanella*, *Pseudomonas* and an unclassified genus in the family Oxalobacteriaceae. The addition of Mn promoted the use of D-Mannitol, Maltose etc but inhibited the use of Maltotriose, L-Serine and Glycyl L-Glutamic acid. The rate of Mn oxidation in the lakes ranged from 0.04 to 3.96 ppb/day while under *in vitro* the isolates oxidized Mn from 10 to 100 times faster. Our studies suggest that cobalt could have a more profound role in manganese oxidation while nickel promoted manganese reduction in polar aquatic systems.

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Legend to figures:

- Figure 1: Fresh water lakes in the Larsemann Hills region. The labels indicate Lake ID and area of sampling.
- Figure 2: Abundance (median values) of culturable fractions of Heterotrophic Bacteria (Fresh Water and Saline) and Manganese Oxidizing Bacteria and Total microbial counts from various lakes. Error bar represent the statistical range. [TC – Total Counts; THB-FW – Total Heterotrophic Bacteria enumerated on nutrient agar; THB-SAL - Total Heterotrophic Bacteria enumerated on marine agar; MnOB – Manganese Oxidizing Bacteria].
- Figure 3: Median values for growth and respiration of isolates LH-5 (A), LH-8 (B) and LH-11 (C) in different combinations of Mn amendments. Legends for (B) and (C) are the same as given in (A). Growth (●) and Respiring counts (○) are given on the primary Y axis. Respiration rates (▲) for the isolates are given on the secondary Y axis (axis on the right hand side). The temperature of incubation was 2°C (±0.1°C). Error bar represent the statistical range.
- Figure 4: Highest recorded normalized value for sugars and amino acids that showed maximum variability in –Mn (A) and +Mn (B) amended incubations for LH-5, 8 and 11.
- Figure 5: Variation (median values) in the level of Mn in the in situ incubations conducted in Lake L2, 3 and 7. Error bar represent the statistical range.
- Figure 6: Variations in the level of Mn (●), Fe (□), Co (Δ) and Ni (◇) in various combinations of Mn (A), Mn-Fe (B), Mn-Co (C), Mn-Ni (D), Mn-Fe-Co (E), Mn-Fe-Ni (F), Mn-Co-Ni (G), Mn-Fe-Co-Ni (H) in incubation experiments of Isolate LH-5.
- Figure 7: Variations in the level of Mn (●), Fe (□), Co (Δ) and Ni (◇) in various combinations of Mn (A), Mn-Fe (B), Mn-Co (C), Mn-Ni (D), Mn-Fe-Co (E), Mn-Fe-Ni (F), Mn-Co-Ni (G), Mn-Fe-Co-Ni (H) in incubation experiments of Isolate LH-8.
- Figure 8: Variations in the level of Mn (●), Fe (□), Co (Δ) and Ni (◇) in various combinations of Mn (A), Mn-Fe (B), Mn-Co (C), Mn-Ni (D), Mn-Fe-Co (E), Mn-Fe-Ni (F), Mn-Co-Ni (G), Mn-Fe-Co-Ni (H) in incubation experiments of Isolate LH-11.

Figures:

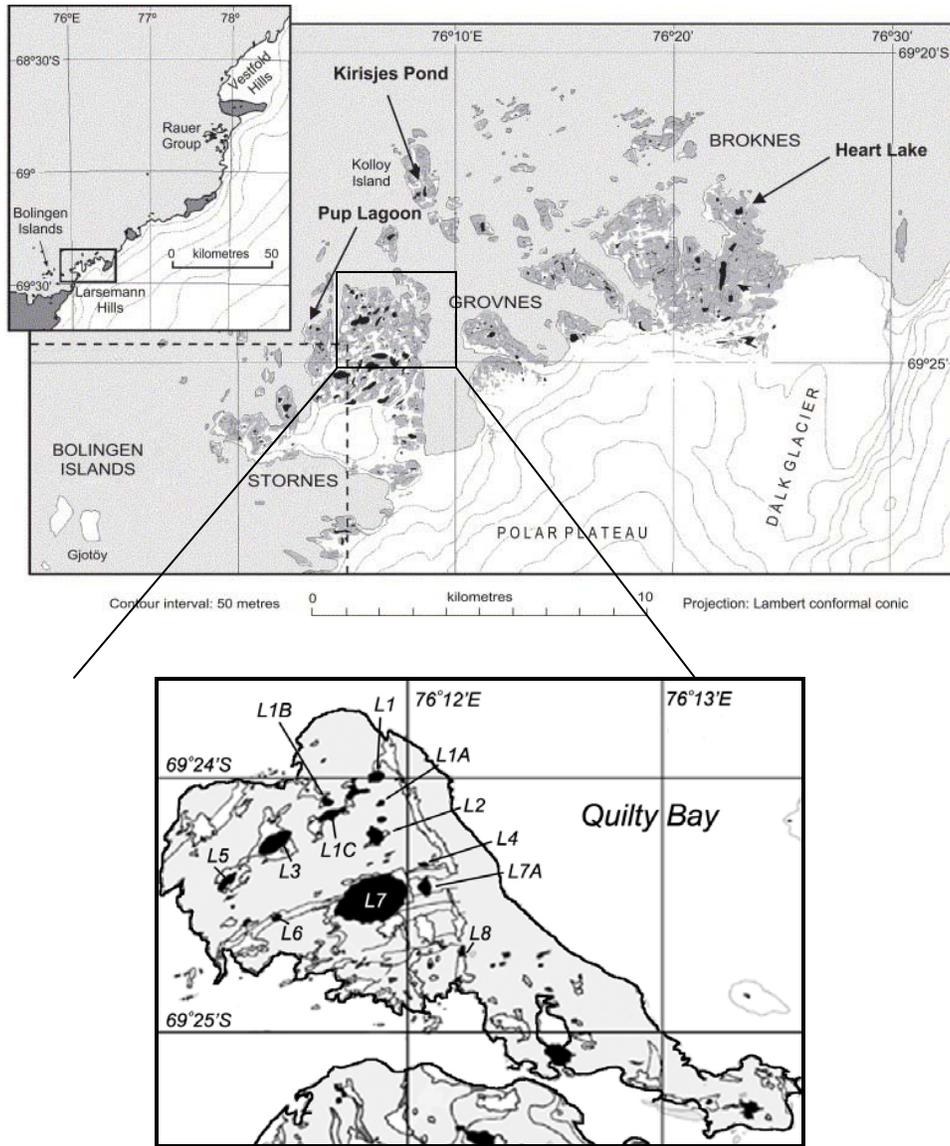


Figure 1

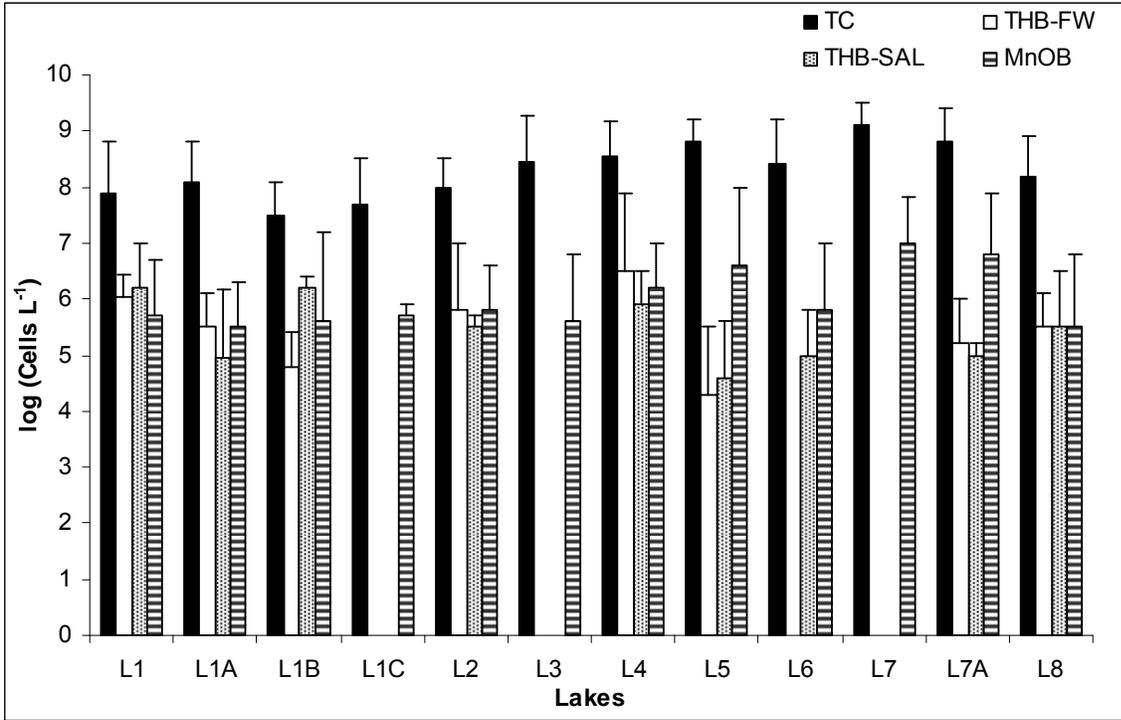


Figure 2

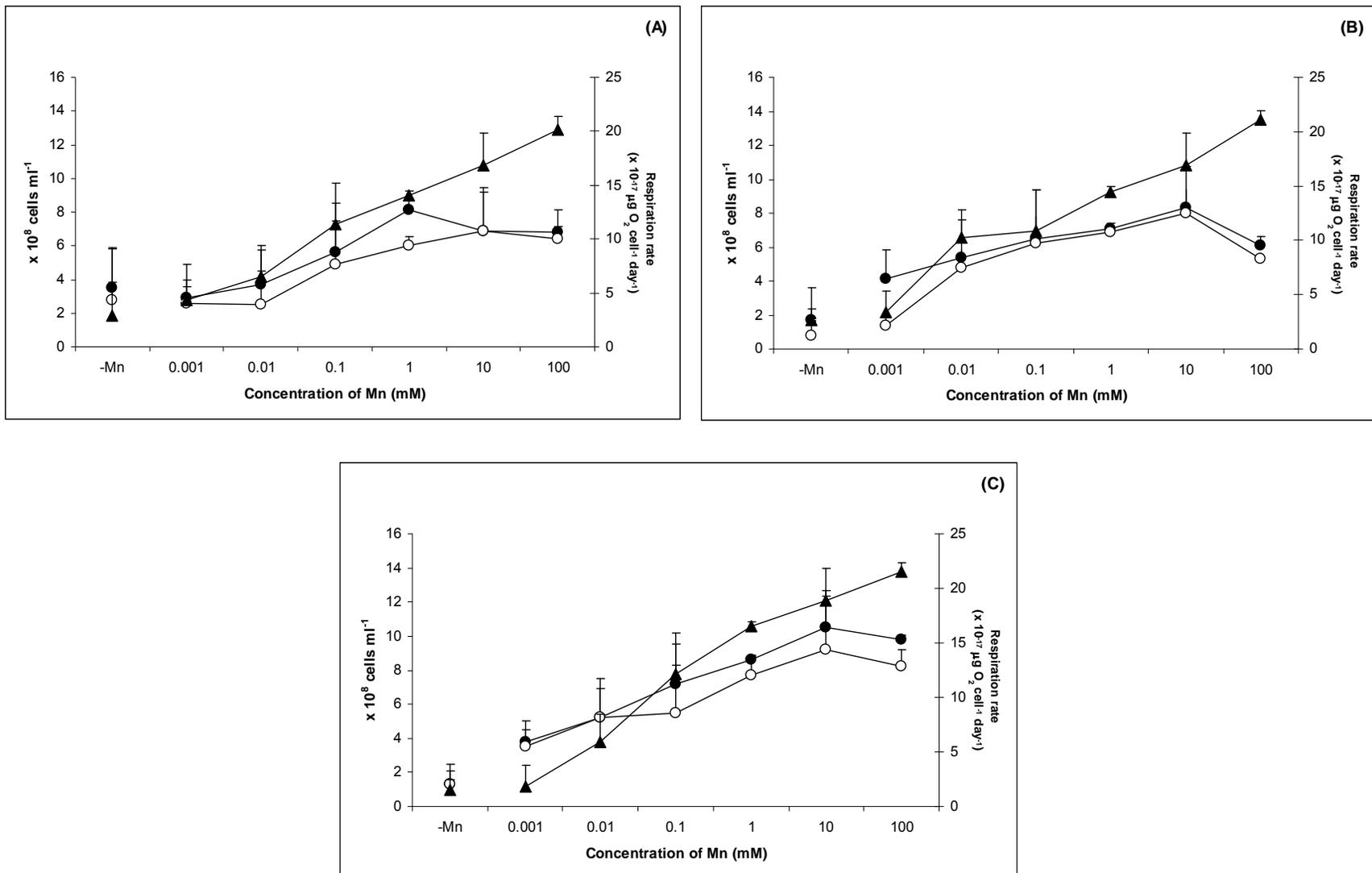


Figure 3

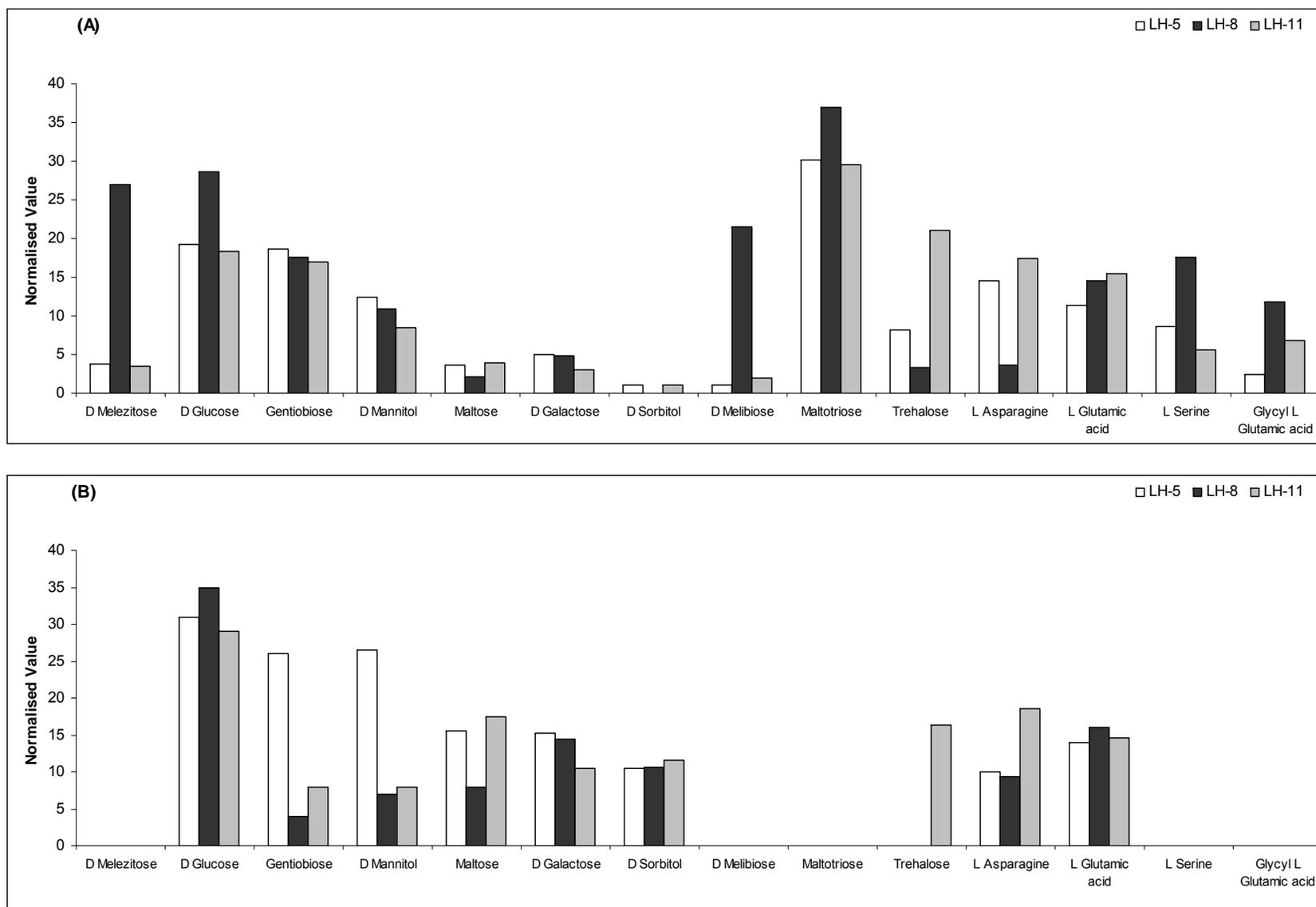


Figure 4

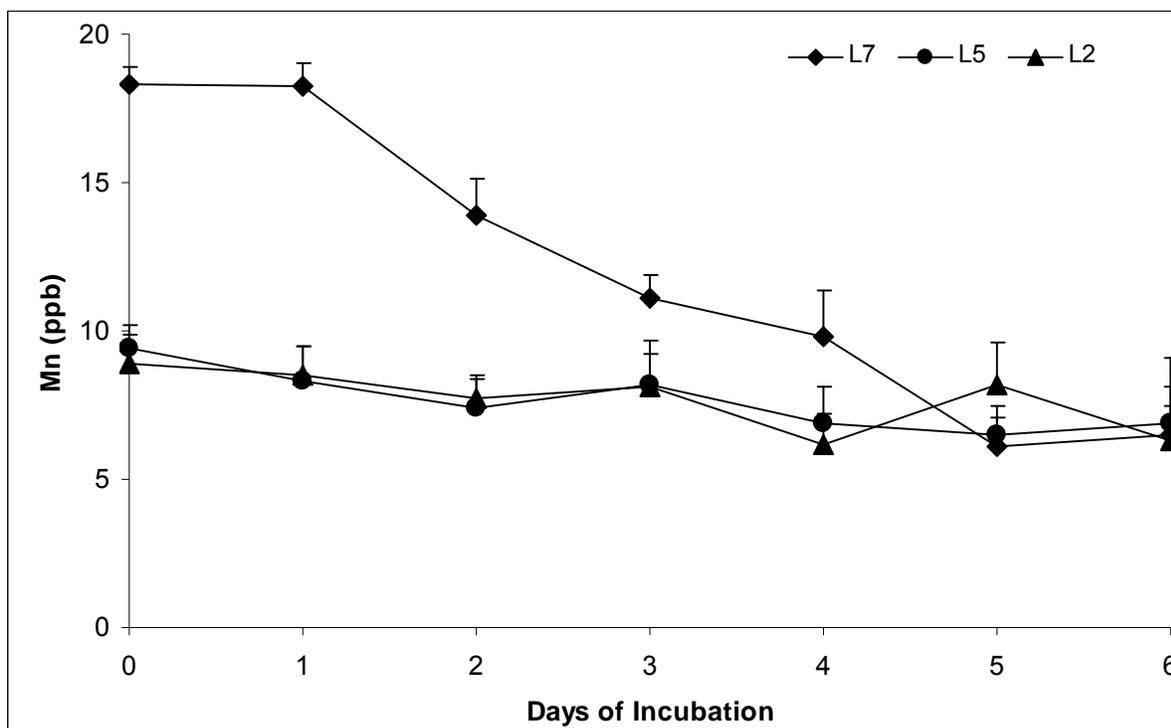


Figure 5

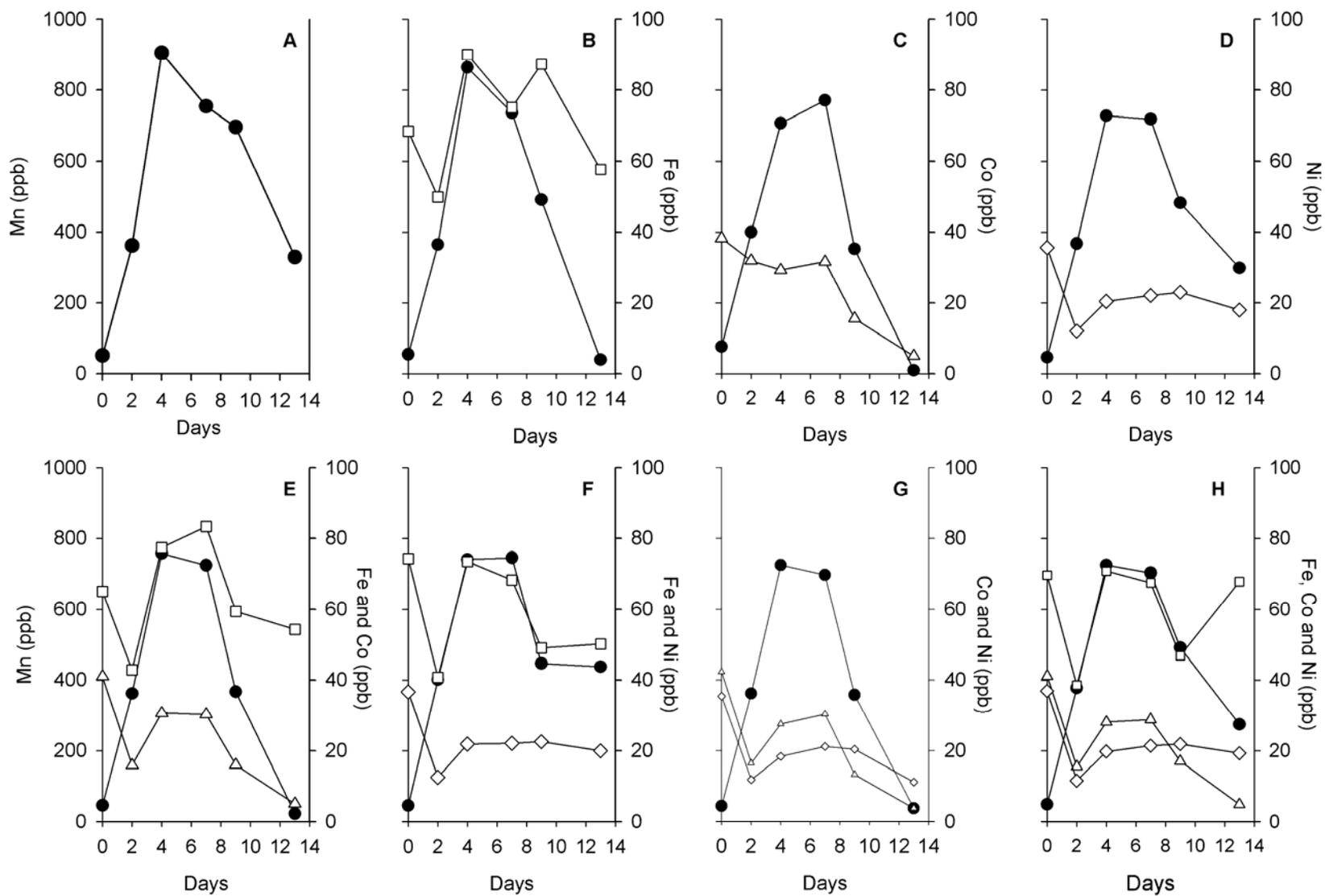


Figure 6

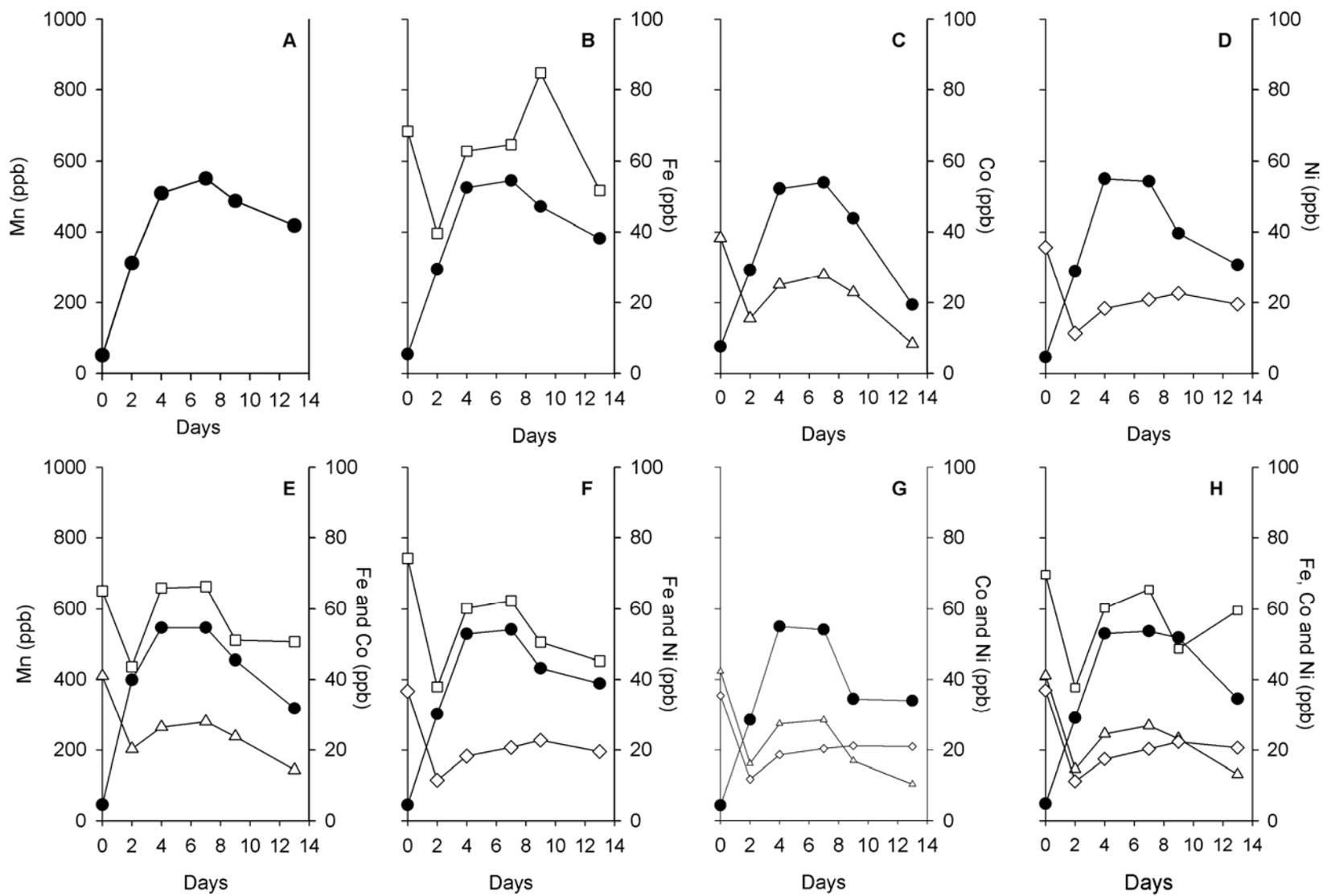


Figure 7

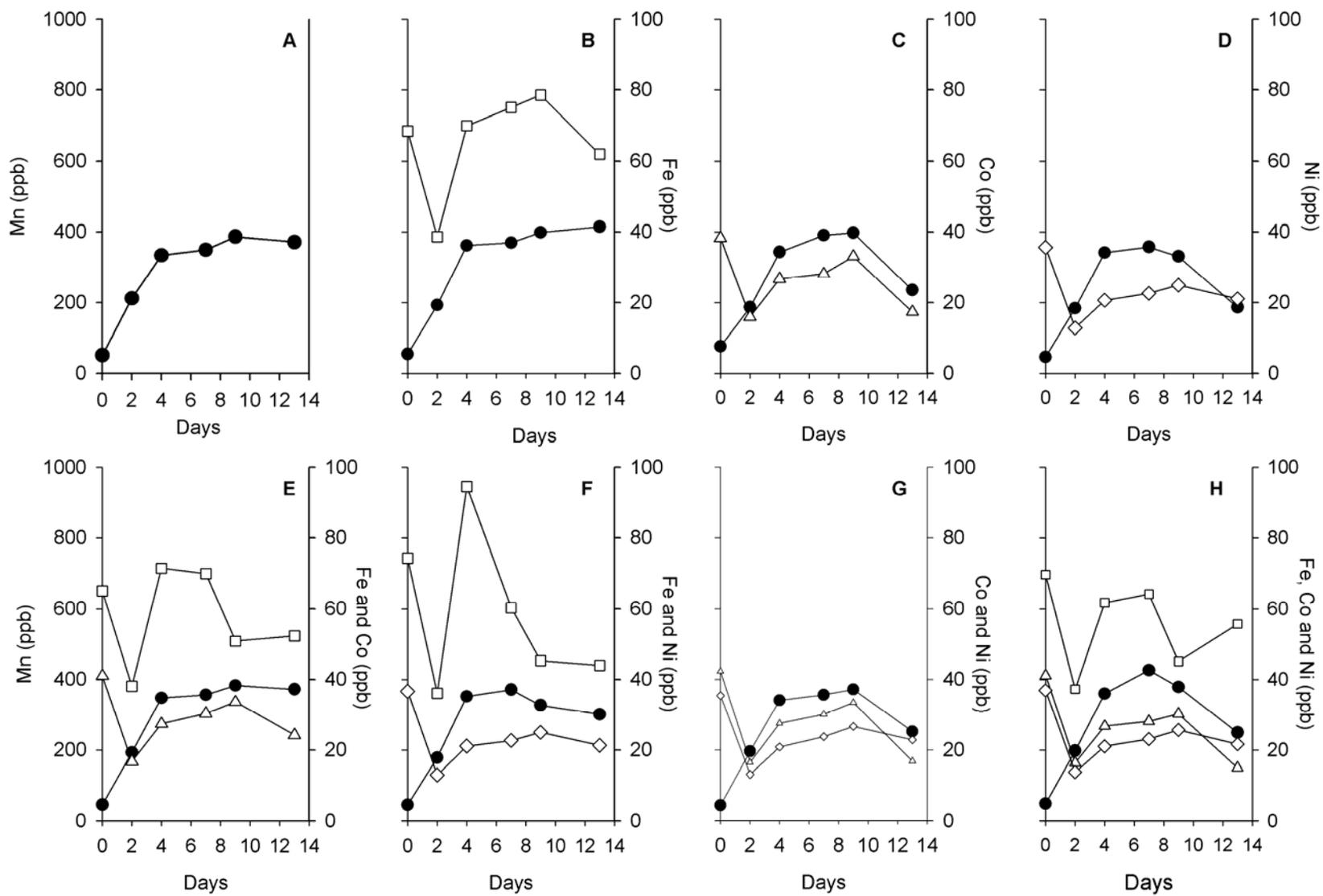


Figure 8

Table 1: Physiochemical parameters of the lakes at the time of sampling

Lakes	Latitude	Longitude	Salinity (psu)	Temperature (°C)	pH	Depth at the point of sampling (m)	Spatial extent (Km ²)
L1	69°24.427`S	76°11.293`E	<1	-0.3	7.1	0.5	0.0045
L1A	69°24.335`S	76°11.500`E	<1	2.6	7.5	0.7	0.0007
L1B	69°24.310`S	76°11.662`E	<1	1.5	7.5	0.2	0.0012
L1C	69°24.365`S	76°11.365`E	<1	0.4	7.8	0.2	0.0047
L2	69°24.434`S	76°11.709`E	<1	2.7	7.8	1.5	0.0057
L3	69°24.458`S	76°11.124`E	<1	0.4	7.4	0.2	0.0275
L4	69°25.084`S	76°12.838`E	<1	0.5	6.8	1.6	0.0005
L5	69°24.512`S	76°10.823`E	<1	1.5	8.1	1.8	0.0093
L6	69°24.613`S	76°11.042`E	<1	-0.1	8.2	0.9	0.0019
L7	69°24.578`S	76°11.593`E	1	-0.4	7.1	13.0	0.1082
L7A	69°24.546`S	76°11.917`E	3	0.7	7.8	1.5	0.0071
L8	69°24.898`S	76°12.733`E	8	0	7.9	1.1	0.0008

Table 2: Most preferred carbohydrates and amino acids by isolates LH-5, 8 and 11 in the presence and absence of added Mn. "-" means same as above

	Day	LH-5		LH-8		LH-11	
		-Mn	+Mn	-Mn	+Mn	-Mn	+Mn
Carbohydrate	1	α -D-Glucose	D-Mannitol	D-Melibiose	D-Sorbitol	D-Trehalose	α -D-Glucose
	2	-"	-"	α -D-Glucose	α -D-Glucose	Maltotriose	-"
	3	Gentiobiose	Gentiobiose	D-Melezitose	D-Galactose	-"	-"
	4	Maltotriose	α -D-Glucose	Maltotriose	α -D-Glucose	D-Trehalose	D-Trehalose
	5	-"	-"	-"	-"	-"	Maltose
Amino acid	1	L-Asparagine	L-Asparagine	-	L-Asparagine	L-Asparagine	L-Asparagine
	2	-"	-"	L-Serine	-"	L-Glutamic acid	-"
	3	L-Glutamic acid	L-Glutamic acid	L-Glutamic acid	L-Glutamic acid	-"	-"
	4	L-Serine	-"	L-Serine	-"	-"	L-Glutamic acid
	5	L-Glutamic acid	-"	Glycyl L-glutamic acid	-"	-"	-"

Table 3: *In vitro* incubation experiments with LH-5, LH-8 and LH-11. 'O' and 'R' indicates net oxidation and net reduction respectively. Entries under the column heading 'Period' refer to Fig.5.

	LH- 5		LH- 8		LH-11	
	Mn Redox Kinetics		Mn Redox Kinetics		Mn Redox Kinetics	
Combination	Period (days)-Phase ('O'/'R')	Rate ppb/day	Period (days)-Phase ('O'/'R')	Rate ppb/day	Period (days)-Phase ('O'/'R')	Rate ppb/day
Mn	(0-4)'R'	213	(0-7)'R'	80	(0-9)'R'	41
	(4-13)'O'	56	(7-13)'O'	24	(9-13)'O'	4
Mn-Fe	(0-4)'R'	202	(0-7)'R'	80	(0-13)'R'	35
	(4-13)'O'	92.6	(7-13)'O'	29		
Mn-Co	(0-7)'R'	112	(0-7)'R'	76	(0-9)'R'	38
	(7-13)'O'	147	(7-13)'O'	55	(9-13)'O'	41
Mn-Ni	(0-4)'R'	170	(0-4)'R'	125	(0-7)'R'	51
	(4-13)'O'	55	(4-13)'O'	32	(7-13)'O'	24
Mn-Fe-Co	(0-4)'R'	177	(0-7)'R'	83	(0-9)'R'	41
	(4-13)'O'	91	(7-13)'O'	40	(9-13)'O'	3
Mn-Fe-Ni	(0-7)'R'	116	(0-7)'R'	81	(0-7)'R'	53
	(7-13)'O'	75	(7-13)'O'	32	(7-13)'O'	14
Mn-Co-Ni	0-4'R'	170	0-4'R'	126	0-4'R'	38
	4-13'O'	86	4-13'O'	34	4-13'O'	30
Mn-Fe-Co-Ni	(0-4)'R'	168	(0-7)'R'	80	(0-7)'R'	59
	(4-13)'O'	55	(7-13)'O'	26	(7-13)'O'	28