Determining temporal trends of metal exposure in Lake Trout (*Salvelinus namaycush*) otoliths using microchemical analysis

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ABSTRACT

Otoliths are calcified structures located in the inner ear of teleost fish. They are formed by the crystallisation of calcium carbonate in the form of aragonite onto a protein matrix. Otoliths grow continuously during the lifespan of the fish by the deposition of concentric layers of aragonite and protein. During the formation of otoliths, trace elements are potentially incorporated into the otolith either by substitution for Ca or through co-precipitation with other carbonates. Since it has been suggested that otolith composition is reflective of the external environment (i.e. food and/or water) otoliths have been used as a temporal record to address many fisheries questions.

Generally, toxicological studies focus on soft tissues (e.g. liver, kidneys, muscle) to determine metal exposure in fish. However, interpretation of the metal concentrations can be challenging due to the labile nature of metals in these tissues. A more recent approach to investigate temporal trends of metal exposure involves the use of otoliths. Otoliths are metabolically inert so that, once metals are incorporated they are not subject to remobilization.

Red Lake, located in the Canadian Shield in Northwestern Ontario is recognized as a popular fishing area. However, over the last decade lake trout have experienced a drastic decline, with concurrent increase in the age distribution. One hypothesis for the recruitment failure has focused on the metals present in the water as a result of past mining activities. A retrospective

analysis of the elemental signatures in the lake trout otoliths was performed to determine whether trace element concentrations have changed in Red Lake over the past three decades. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was used to determine trace elements in otoliths recovered from lake trout from 1960 to 2008. Mn was incorporated into the otoliths and there was a suggestion that concentrations peaked between 1980 and 1989 in Red Lake. Concentrations of Mn in lake trout otoliths ranged from 0 mg/g to 10.1 mg/g. Potential contributors to the elevated concentrations could have been associated to the local geology. In addition to geology further uncertainties concerning the water and diet to otolith relationships could also contributed to the Mn concentrations. Augmented diets failed to increase metal concentrations in otoliths. Further research is required to further investigate the relationship between Mn in the environment and the incorporation into the otolith.

PREFACE

This thesis is organized as a "sandwich style" thesis where chapters 2 and 3 are organized as manuscripts to be submitted for publication. Therefore, each chapter contains an abstract, introduction, results, and discussion. Each chapter and associated manuscripts were written and presented by Leslie Carroll, as indicated as primary authorship listed below.

Each author played an important role in the research presented in each manuscript. Nadine Thébeau communicated past and present research that was conducted on Red Lake; Norm Halden communicated appropriate otolith analyses and oversaw the operations of the LA-ICP-MS laboratory; Vince Palace was my primary supervisor who offered guidance and support throughout my research project.

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Carroll L.C, Thébeau N, Halden NM, Palace VP. 2010. Incorporation of Cu, Cd, Ni, Pb and Zn in otoliths of rainbow trout (*Oncorhynchus mykiss)* fed experimental diets. *Bulletin of Environmental Contamination & Toxicology.* To be submitted January 2011.

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1 General Introduction

1.1 Introduction

1.1.1) Background

Over the last decade a drastic population decline has occurred in the once renowned lake trout (*Salvelinus namaycush* Walbaum 1792) in Red Lake, Ontario (Thébeau, 2008). In 2001, the Ministry of Natural Resources (MNR) in Red Lake conducted a spring littoral index netting (SLIN) survey. This was the first population survey conducted since 1992, and the first indication that there was a problem. The smallest lake trout caught was 60 cm and the youngest was 11 years old. These results indicated an ageing lake trout population with a serious recruitment problem. Since then the MNR have been conducting a variety of spawning assessments, surveys and bioassays in an attempt to identify a causal agent for the ongoing recruitment failure.

Prior to 2001, only two population assessments were ever conducted. In 1989, MNR conducted the first survey of the shoals within Red Lake. The shoals were mapped and their size and location were recorded for future studies (Figure 1.1) (Spicer and Barnes, 1990; Thébeau, 2008). Following the survey, MNR conducted the first spawning assessment of these spawning shoals. Results suggested that the lake trout were using the shoals for spawning, and in particular the spawning shoals within Pipestone Bay were being utilized. Among the 187 lake trout collected in the 1989, only 7 lake trout were caught outside of Pipestone Bay. Results from the assessment directed the main focus for further investigations on Pipestone Bay (Thébeau, 2008). In 1992, a second spawning assessment was conducted in Pipestone Bay. This assessment included measuring, tagging, weighing and recording the sex

of the lake trout. A total of 289 lake trout were captured. The data collected in 1989 and 1992 showed a healthy lake trout population with a reasonably well age distribution. However, in 2001 the SLIN results suggested that the lake trout population was unevenly distributed with a higher number of older fish and few young fish. Results showed that seventy seven fish were between the ages 10 and 20, sixty two fish caught were over the age of 20, and two fish caught were under the age of 10 (unpublished data, Thébeau). These results suggested that lake trout population was an aging population.

In 2002, the MNR conducted a spawning assessment, similar to that conducted in 1992. Results showed a shift in the age distribution of the lake trout population compared to the 1989 and 1992 results (Figure 1.2). Of the lake trout collected (n = 176), 77 fish were between the ages 10 and 20 (77 fish), 62 fish were over the age of 20, and 2 fish were under the age of ten

The 2002 assessment established that the state of the lake trout population had changed since the last assessment in 1992, and that there was a decrease in the number of young fish in the population. In order to identify a causal agent for the recruitment failure further studies were initiated.

In October, 2003, lake trout eggs from Pipestone Bay were collected and taken to the Ministry of Natural Resources' Fish Culture Station, in Chatsworth ON where they were incubated and monitored to verify egg fertility, viability, alevin and fry survival, and thiamine deficiency. The incubation established that eggs developed normally, fertility and viability, and survival rates among alevin and fry were adequate, and there were low concentrations of metals (Thébeau, 2004). In addition, an *in-situ* study was conducted to further investigate egg survival. Incubation trays were placed at four spawning shoals within Pipestone Bay, and one

at reference site (Figure 1.2). The latter was used to follow developmental stages. Eggs retrieved at the reference site developed and survived. However, eggs from Pipestone Bay exhibited 0.03% survival. Given that the lake trout eggs had developed in the hatchery and at the reference site, the low survival rate in Pipestone Bay suggested a localized problem that could potentially be associated to the recruitment failure. To further investigate a potential localized problem MNR felt it was important to repeat the incubation study. Therefore, in October 2004, incubator trays were placed at five sites within Pipestone Bay and at thirteen sites throughout Red Lake (Figure1.3). The number of sites were increased to confirm the high mortality in the 2003 study in Pipestone was reflective of the environment, and to verify the geographical extent of the problem. Results showed that the reference site had 90% survival while eggs incubated in Pipestone Bay exhibited 10% survival after one week and 0% by the following week. One exception was a site in the southwest end of the bay that exhibited 90% survival. These results suggested that potential factors associated with the Pipestone Bay substrate could have been the source of the observed mortality. Factors could include contamination derived from mining activity over several years. Unfortunately MNR had no empirical evidence to show if this was the cause of the mortality in the eggs. Therefore, the past mining activity (and metals associated with mining) in the area were suspected to be a possible source.

1.1.2) Mining History

The Red Lake district has a history of gold mining that dates back to the early 1930's. Historical records show that over the last eighty years seventeen mines have operated in this

region (CIMMP, 1998). More specifically, around Pipestone Bay there were five gold mines that were once in operation, Cole Gold Mine, Mount Jamie Mine, Miles (Red Lake) Mine, May-Spiers and West Red Lake Mine (CIMMP 1998). The Cole Gold Mine and Mount Jamie mines were the major mines around Pipestone Bay (CIMMP, 1998). The Cole Gold Mine was comprised of 58 original claims that covered approximately 2333 acres. Mining activity began in 1926, with prospecting, stripping and trenching a 20 ft shaft was sunk onto a promising vein. The area encompassed three parallel gold bearing quartz veins. In 1933, a company was formed and began operation. However, in April, 1938 all underground work was suspended until electric power could be secured. Cole Gold Mine never continued operation due to delays of securing electrical power and falling gold prices. The mine never produced any gold (Parrott and Cole, 1968). The Mount Jamie Mine was comprised of two shafts. Shaft # 1 was located 1.6 km away from Pipestone Bay and was the only shaft that was fully operational. The total mining activity was minimal. In 1976, shaft #1 recovery rate of gold was 78.8%, producing 209 oz. of gold and Shaft #2, located 750 m away from Pipestone only operated for 1 year (1940-1941). In 1984, work was suspended and the mine never reopened (CIMMP, 1998).

Today, there is one major mining company, Gold Corp Inc. in the Red Lake area. It is Canada's largest gold mine, and one of the world's richest. Gold Corp Inc. operates two mining complexes within the Red Lake District, Red Lake Complex and the Campbell Complex. Both complexes release treated discharge into Balmer Lake which discharges to Balmer Creek and then downstream to the Chukuni River, which is 3 km downstream to Red Lake and 31 km downstream to Pipestone Bay (Figure 1.4). Generally mine discharge consists of water treatment chemicals (i.e. sulphate, chloride, ammonia and cyanide), and

metals (e.g. Ni, Cu, Co, Pb, Se and As) which could potentially impact fish, and fish habitat (Ribey et al. 2002; Driedger et al., 2010).

1.1.3) Metals as a Potential Driver for Lake Trout Recruitment Failure

Many metals occur in the natural environment as trace metals (e.g. Mn, Fe, Zn, and Cd) (Moore, 1991). However, in high concentrations these metals can become toxic and impact the aquatic ecosystem (Moore, 1991; Schmitt et al. 2007). Metal mining operations (e.g. lead, zinc, and copper mining) have been shown to negatively influence aquatic environments as the result of continuous release of metal containing effluents into aquatic systems (Woody et al., 2010). Metals that exist in the aquatic system as trace metals become elevated causing impacts on the local biota (Brumbaugh et al. 2005; Woody et al., 2010). Research has shown that metal toxicity, as a result of mining activity has impacted aquatic environments. Studies have found that metal toxicity can reduce species diversity, impact the flora and fauna (Amisah and Cowx 2000;Gemici 2004; Brumbaugh et al. 2005; Concas et al. 2006), cause changes in various fish behaviours, for instance changes associated to forging, predator avoidance, reproduction and social hierarchies (Scott and Sloman, 2004). Additional research on the impacts of contamination in fish has also observed reduced reproductive capacity, retarded maturation, and impaired larval survival (de Lafontaine et al., 2002).

Gold mining operations have also been shown to contribute to metal toxicity in aquatic environments. In gold mining, gold is recovered by two different processes. One method is from dug up soils and hard rock; the second is from dredged bottom sediments. Neither processes is superior to other environmentally, both release industrial waste into the aquatic environment and the atmosphere (Salomons, 1995). During the mining process a cyanide

solution or liquid mercury are used to separate the fine gold particles, following this procedure waste is discharged into the natural environment. However, the amount of waste that is discharged is dependent on the mining site, the separation process, the waste, and its chemical concentration (Salomons, 1995). Cadmium, lead and zinc are common metals found elevated in aquatic systems as a result of gold mining (Nimmo *et al*., 1998; Carola *et al.,* 2009). However, other metals such as, manganese have also been found to be elevated.

A review by Woody (2010) discussed a study conducted involving the Summitville Mine, a gold mine located in south western Colorado. The study determined that the increased concentrations of manganese, aluminum, copper, iron and zinc observed 29 km downstream in the Alamosa River was the result of contamination from Summitville Mine. Betancourt et al. (2005) found the Puyango Basin contaminated with manganese, lead and mercury due to local gold mining. The manganese and lead concentrations reached levels that surpassed the limits allowed for safe human consumption, 50 μg Mn/L and 10 μg Pb/L (WHO, 1995; Drinking Water Services, 2005). Veado et al (2006) analyzed a suite of metals including manganese in water, sediment, forage, and fish samples collected from gold mine polluted sites and reference sites within the Minas Gerais State in Brazil. Results found elevated manganese concentrations in water (860 ± 80 μg/g) and sediment (1460 ± 140 μg/g), and above average manganese concentrations in the forage (roots 620 μg/g, stems and leaves 153 μg/g) were high. These studies suggest that gold mining could influence the concentrations of naturally occurring trace elements, such as manganese in the aquatic systems which could potentially influence the health of the local biota in the aquatic system.

Research has shown that metal toxicity in aquatic environments can influence the water quality and potentially have negative impacts on different facet of reproduction in fish

populations. Therefore, metals present in Red Lake could potentially be a driver for the reproductive failure observed in the lake trout population.

1.2 Metals and Their Traditional Measurement in Soft Tissues

1.2.1) Metals of Concern in Red Lake

The results from the 2004 incubation study conducted by MNR suggested that the shoals within Pipestone Bay could be contaminated and potentially the reason for the high egg mortality. Therefore, in 2005 the Ministry of Environment (MOE) carried out an embryology toxicity study involving substrate collected from Pipestone Bay. The study was a simple static test whereby 100 newly fertilized rainbow trout *Oncorhynchus mykiss* eggs (Rainbow Springs Trout Farm - Thamesford, ON) were exposed either to Pipestone Bay substrate or reference substrate (washed, coarse aquarium gravel) for 30 days. After 2 weeks 100% of the eggs exposed to Pipestone Bay substrate became black and died. The acceptable mortality rate for the Red Lake lake trout was 25%. The colour change in the eggs prior to death suggested the presence of manganese either being released from the substrate or within the pore water. At concentrations >0.15 mg/L manganese is known to discolour pipes, and at concentrations <0.05 mg/L manganese can form coatings as black precipitates (CCREM, 1987). Manganese tends to occur in higher concentrations in ground water compared to lakes and reservoirs (Health Canada, 1987). MOE analyzed pore water samples collected from eight sites in Red Lake, that showed elevated concentrations of manganese (0.89 - 12.6 mg/L), copper (up to 15 ug/L), aluminum (2 - 3.4 mg/L), and iron (1 - 5.5 mg/L). MOE suggested manganese as a potential source for the mortality since concentrations were elevated however further research

would be required. Generally, manganese concentrations in natural waters (free from anthropogenic influence) range from <0.01mg/L to >10 mg/L). However, manganese concentrations in surface waters rarely exceed 1.0 mg/L and are typically < 0.2 mg/L (McNeely et al., 1979). Manganese data collected by BC Environment seldom observed Mn concentrations in surface waters that exceeded 1.0 mg/L (Reimer, 1999).

Manganese is an element that occurs in surface waters as a dissolved or suspended matter generally in concentrations below 0.05 mg/L (CCREM, 1987; NRCC, 1988). Concentrations of manganese tend to be higher in ground water compared to lakes and rivers due to the reducing conditions of the underground environment CCREM, 1987). Manganese toxicity in the lakes and rivers is uncommon. Acute toxicity studies have shown that certain fish species however are sensitive to manganese. Rainbow trout have been shown to be most sensitive to manganese. The NRCC (1988) conducted a 96 hour toxicity study on the early life stages of rainbow trout; results showed fingerlings to be more sensitive while fry and eggs were more tolerant. Lethal concentrations ranged between 0.37 mg/L and 11.6 mg/L among the fingerlings, fry and eggs.

Since Mn was elevated, it was suggested that manganese could be associated with the death of eggs in MOE's embryology study. However, it is important to note that additional metals could also be involved because several metals associated with mining can be elevated.

1.2.2) Metal exposure

The impacts of metal exposure due to anthropogenic sources are of great concern and have been the focus of many toxicological studies. To better understand the impacts, studies

have been conducted to examine the chronic and acute effects of metal exposure in the laboratory or field or both combined. The effects of single and/or multiple metal exposure have been investigated in many different fish species (e.g. rainbow trout (*Oncorhynchus mykiss*) and invertebrates (*Daphnia*). Heavy metals such as zinc (Zn), copper (Cu), cadmium (Cd), manganese (Mn) and lead (Pb) have been included in many studies because of their toxicity, and biological availability (Hansen et al., 1999; Scott and Sloman, 2004; Svobodova et al., 2004; Uysal et al., 2009). Each of theses metals possess unique characteristics in terms of their impacts and at elevated concentrations can influence biological processes. Elevated concentrations of Cu and Zn in Mantiouwadge Lake, ON impaired reproductive performance and growth rates in white sucker (*Catostomus commersoni*) populations (Munkittrick and Dixon, 1988). At high concentrations, Zn and Cd reduced spawning success in white sucker in Hamel Lake, SK (MacFarlane and Franzin, 1978). In addition to impaired reproduction performance and spawning success, metal exposure can influence fish development, growth, and behaviour (MacFarlane and Franzin, 1978; Munkittrick and Dixon, 1988; Hanen et al., 1999; Scott and Sloman, 2004; Svobodova et al. 2004; Johnston et al. 2008). Spinal damage was observed in the minnow *Phoxinus* at 7.5 mg/L of Cd (Bengtsson et al., 1975). Reduced growth rates were observed in white suckers exposed to high concentrations of copper and zinc while growth rates in white sucker at reference sites were not affected (Munkittrick and Dixon, 1988). Hansen (1996) reported that when rainbow trout exposed to low metal mixture concentrations of Cu, Cd, Pb, and Zn (1.2 µg/L, 0.11 µg/L, 0.32 µg/L, and 5.0 µg/L) avoidance behaviour was exhibited suggesting that rainbow trout have a low tolerance to these metals. Research has shown that impacts are observed in fish as a result of metal exposure. However, the observed impacts are varying due to the test species, the specific

metal or metal mixture being tested, concentration of metal mixture, and duration of exposure. Therefore continuous efforts are required to continue research to understand and be able to make links between the impacts observed in fish and the metal exposure.

1.2.3) Labile nature of soft tissues for metal analysis

In fishes, soft tissues such as the liver, kidney, muscle and gill are commonly used to investigate metal exposure. These tissues are most likely to concentrate metals from the environment in which the fish have been exposed (Cooley and Klaverkamp, 2000; Ranaldi and Gagnon, 2008). However, interpreting concentrations of metals over time poses difficulties due to certain biological processes. The liver, kidney, muscle and gill have the ability to eliminate toxic metals and repair damage tissues (Dove and Kingsford, 1998; Ranaldi and Gagnon, 2008). Metals are segregated by metal binding compounds and can be transported to specific organs. Once at the organ the metal can be excreted or retained at sites within the cell (Dove and Kingsford, 1998). Metals present in soft tissues are likely to have been taken up from the surrounding aquatic system (Ranaldi and Gagnon, 2008), however, because of the labile nature of soft tissues it can be difficult, to identify when they were assimilated (Dove and Kingsford, 1998; Ranaldi and Gagnon, 2008). Therefore, continuous biological sampling (costly and timely) would be required to assess the status of the environment, and to relate this to temporal trends of accumulation in biota (Palace et al., 2007; Ranaldi and Gagnon, 2008). Another approach to investigate temporal trends of accumulation is to use calcified tissues, such as otoliths which are known to incorporate metals over time. The annular structure of the otolith can be a useful tool when investigating temporal metal exposure in fish.

1.3 Otoliths

1.3.1) Otolith Description

The inner ear of fish is a paired structure that is located within the cranium on either side of the head near the midbrain (Figure 1.4a). Each individual ear is comprised of many canals, sacs and ducts that are filled with a fluid (endolymph) with a unique viscosity (Popper and Lu, 2000; Wright et al., 2002; Duffin, 2007). Among teleosts, the inner ear includes three semi-circular canals (anterior, posterior and horizontal) all at right angles to each other (Figure 1.4b). These canals are connected to three endolymphatic sacs (otic sacs), the utriculus, sacculus and lagena. Each sac is unique in size and shape, and each contains their own distinctive pair of calcareous structures called otoliths. These are called sagitta in the sacculus, the lapillus in the utriculus and the asteriscus in the lagena (Popper and Lu, 2000; Murayama et al., 2002; Wright et al 2002; Popper et al., 2005; Lenaz et al., 2006; Duffin, 2007) . Generally, the sagitta otoliths are the largest otoliths among most species. Otoliths are formed by the crystallisation of calcium carbonate in the form of aragonite onto a protein matrix known as otolin (Wright et al., 2002). Otoliths grow continuously by the deposition of concentric layers of aragonite and protein (Halden et al., 2000; Romanek and Gauldie, 1996). The annually deposited layers are recognized by alternating wide translucent bands (rapid summer growth), composed mainly of inorganic material, and thin opaque bands (slowed winter growth) composed of mainly organic material (Figure 1.5) (Panella, 1971; Campana,

1999; Halden et al., 2000; Melancon et al., 2005). It has been shown that the different bands have different structural characteristics, density, and different production rates (Panella, 1971; Campana and Thorrold, 2001). Otoliths are commonly used for age estimations and otolith formations studies (Panella, 1971; Wright et al., 2002; Duffin, 2007).

1.3.2) Otolith Function in fish and as aging structure

The otolith is very important for the survival of teleost fish. The otolith is associated with balance and hearing in all teleost fish species (Campana, 1999; Popper et al., 2005; Melancon et al., 2008). In the inner ear of the fish there are two systems; the auditory system, whereby the sacculus and lagena are associated with the detection of sound waves, and the vestibular system, whereby the utriculus is involved with detection of linear and angular movements facilitating the fish's ability to maintain balance within its environment (Radtke and Shafer, 1992; Popper and Lu, 2000; Murayama et al., 2002; Wright et al, 2002; Duffin, 2007).

Otoliths are involved with the mechanoreception. The otic sacs also contain a sensory tissue, the macula, which allows fish to detect linear accelerations and sound (Secor et al., 1992; Takagi, 2002; Wright et al., 2002; Duffin, 2007). Inside the otic sacs each otolith is positioned over the macula by an otolithic membrane. It operates as a sound and displacement transducer which converts forces of energy into electrical impulses by displacing the "hair" cells (kinocilia) of the macula (Radtke and Shafer, 1992; Popper and Lu, 2000; Takagi, 2002; Wright et al., 2002; Duffin, 2007). Relative motion between the macula and the otolith bends

the ciliary bundles located on the apical surface of the hair cells, which generates a receptor potential in the cells and excites the eighth cranial nerve (Popper and Lu, 2000; Wright et al., 2002).

In addition to functioning as a sensory organ, otoliths can serve as aging structures (Campana and Neilson, 1985). Age estimations are carried out on the assumption that the annual structures (deposited layers of calcium carbonate) of the otolith are produced at a steady rate and that the distance between each annulus is relative to fish growth (Campana and Neilson, 1985; Friedrich and Halden, 2008; Melancon et al., 2008; Ranaldi and Gagnon, 2008). Reibisch (1899) first described the utility of otoliths for aging fish. The annual periodicity of the growth increments in the otolith provides fisheries biologists with a technique to gather precise age estimations for fish populations (Panella, 1971; Campana and Neilson, 1985). The first publication on daily increments in otoliths was published by Panella (1971), and since then the use of otoliths for age estimation in teleost fish has increased considerably (Campana, 1999; Pontual et al., 2001; Campana and Thorrold, 2001; Jackson, 2007). In 1999, a global survey of thirty fisheries laboratories was conducted. The survey established that 800, 000 otoliths were aged globally, and in 2000 nearly 2 million age estimations were made by means of otoliths (Campana and Thorrold, 2001).

1.3.3) Otolith Structure

Biomineralisation is an acellular process that forms minerals within living tissues. Organisms such as fish, algae and mammals have the ability to form biominerals. In fish, the otolith is an example of a biomineral (Weiner and Dove, 2003). The biomineralization of

otoliths is dependent on the surrounding endolymph. Thus, the composition of the endolymph plays an important role during otolith formation (Borelli et al., 2003). The endolymphatic fluid is supersaturated with calcium and bicarbonate ions, and also contains proteins. The proteins, in addition to the precise concentrations of calcium and bicarbonate ions, are essential to the biomineralisation process (Pontual (de) and Geffen, 2001; Murayama et al., 2002; Borelli et al., 2003; Popper et al., 2005; Melancon et al., 2008). The biomineralization of otoliths is unique compared to other biominerals such as the vertebrate bone and molluscan shell (Asano and Mugiya, 1993; Campana, 1999; Pontual (de) and Geffen, 2001; Borelli et al., 2003). It is unique because the otolith forms within the endolymphatic fluid without contacting other cells and away from the region of calcification (Takagi, 2002). Therefore, the calcification is dependent upon the composition of the endolymph (Campana, 1999). Several elements, ions and ionic complexes can be incorporated into otoliths, including carbonates, phosphates, sulfates, and silicates.

It was first discovered in the 1980's that otoliths could incorporate elements other than calcium from the surrounding environment into the otolith microstructure (Pontual et al., 2001). Campana (1999) reported that 31 elements could be detected in otoliths with the majority of elements occurring in minor and trace quantities. Once an element has been incorporated into the otolith it is generally not altered, or removed (Campana, 1999; Campana and Thorrold, 2001; Melancon et al., 2008). However, Mugiya and Uchimura (1989) reported that under anaerobic stress, the rate of calcium retention in otoliths and other elements was significantly reduced in goldfish (*Carassius auratus).* Elements that are incorporated into the otolith can provide historical information about a fish's behaviour, diet, and local environment as well as the surrounding geology (Friedrich and Halden, 2008; Ranaldi and

Gagnon, 2008). However, the understanding of the environmental influence associated with otolith composition can be a complicated process since the transfer of elements must pass through several biological barriers (gill, blood –endolymph, and endolymph-otolith) before being incorporated into the otolith (Kalish, 1991; Campana, 1999; Melancon et al., 2008).

Trace metals from the aquatic environment are transported, first into the fish through the gills or intestine, then to the blood plasma, followed by the endolymph, and finally into the crystallizing structure of the otolith. The uptake of metals in aquatic animals is very different compared to uptake in terrestrial animals. Fish are continuously submerged in water allowing for continuous exposure to various metal ions. There are two major pathways for metal uptake. In freshwater fish, this is primarily across the gill epithelium, while in marine fish metals cross the intestinal wall (Olsson et al., 1998; Campana, 1999). In freshwater fish, approximately 20 L (Olsson et al., 1998) of water move across the gills at one time which allows for a high chance of metal uptake. The metals diffuse across the gill epithelium and into the blood (McKim, 1994). In marine fish, metals are taken up from food to the gastrointestinal tract, and from the water. Metals enter the fish in direct amounts, relative to their surrounding by assimilation from the intestine, and by "drinking" the sea water. Marine fish drink water extensively. Therefore, marine fish have two pathways that could increase their chance for metal uptake, compared to one pathway thought to occur in freshwater fish. In addition, metal uptake has been shown to increase with increasing salinity and water hardness. These factors do not play a major role in metal uptake in freshwater fish.

Once metals are transported into the fish, their transport is regulated by proteins, and distributed throughout the body system to different tissues (Olsson et al., 1998). Deposition of

metals into otoliths has been suggested to be more regulated compared to the incorporation into soft tissues and it includes a multi-stage process that encompasses independent barriers (Campana, 1999),). It is important to note that at each barrier along the pathway trace metal concentrations are reduced therefore, limiting the amount of ions available for uptake in the otolith. The rate of loss is unpredictable with each barrier (Campana, 1999).

In freshwater fishes, the primary source of trace metals is at the gill-water interface, where the metal uptake pathway begins, and where the largest hindrance for metal uptake occurs (Olsson et al., 1998; Campana, 1999). This should be expected since the gill-water interface is an active site for several life functions (gas exchange, osmoregulation, and excretion) (McKim, 1994; Olsson, 1996; Campana, 1999). Osmoregulation regulates the flux of metals from the freshwater to the fish (McKim, 1994; Campana, 1999). The surface of the gills includes chloride channels that are specialized for ion-transport. The chloride cells are covered by pavement cells. The pavement cells regulate the flux of ions across the gills by modifying the area where the chloride cell is in contact with the water. The apical membrane of the chloride cell contains a sensitive cation transporter which is permeable to divalent ions (McKim, 1994; Olsson, 1998). Divalent ions such as Zn^{2+} can be transported through the chloride cells (Olsson, 1998). Divalent Mn^{2+} could potentially be transported via these chloride cells as well. However, no studies have been carried out for Mn specifically. It has been suggested that water hardness, as $CaCO_3$, is one the largest influences on the metal uptake at the gill-water interface (Meyer et al., 1994; McKim, 1994) such as hard water that results in the decrease of uptake of other elements.

The next barrier is at the blood-endolymph interface. Unfortunately there is little research on the relationship between blood plasma and endolymph composition. At this interface the ion composition of the endolymph is generally low, and generally similar to the otolith composition compared to the composition of water or blood plasma (Campana, 1999). Factors influencing the endolymph composition have not been studied as much as well as the mechanisms for metal ion transport (Campana, 1999; Pontual (de) and Geffen, 2002). It has been suggested that the transfer of metal ions at this interface occurs through the saccular epithelium since there are active and regulated ionic transports that occur at the epithelium interface (Pontual (de) and Geffen, 2002). It has been suggested that Sr and Ca maybe transported into the endolymph via a paracellular pathway (Kalish, 1991). However, Mugiya and Yoshida (1995) suggested that Sr and Ca were transported via a transcellular pathway. It is clear that further research is needed to understand the transport of metal ions at the blood-endolymph interface.

The final barrier of the pathway is between the endolymph and the crystallizing otolith. Studies have shown that there is a strong relationship between the endolymph composition and the otolith composition (Campana, 1999; Takagi, 2002). However, literature on the extent of the relationship between the varying concentrations that occur within the endolymph and the otolith composition are still limited (Pontual (de) and Geffen, 1994). The otolith is mostly composed of calcium carbonate but also contains an organic matrix of proteins, carbohydrates and lipids (Degens et al., 1969). The organic matrix is created from cells of the membranous wall of the otolith organ, which are secreted into the endolymph, which forms the framework for otolith crystallization (Takagi and Takahashi 1999; Takagi 2000; Takagi et al., 2000). This process suggests that the otolith composition is

reliant on the composition of the endolymph (Campana, 1999; Takagi, 2002). Physical factors (i.e. pH and temperature) regulate the endolymph and play an important role in the rate of calcification in otoliths. However, recently it has been suggested that the water-soluble proteins within the organic matrix are most important in the calcification rate of otoliths (Campana, 1999). During the calcification process, trace metals from the surrounding environment can enter the otolith matrix at three locations; within the crystal lattice, adsorbed to the otolith surface or in association with the protein matrix. Within the crystal lattice, metals are incorporated into the otolith by substitution or co-precipitation (Campana, 1999; Pontual (de) Geffen, 2002). During calcification, metals ions can be eliminated that can implies chemical reduction leading to lower concentrations present in the otolith.

Incorporation of elements into crystal structures can occur differently for individual elements and/or groups of elements. It is important to mention *Goldschmidt's rules of substitution* when discussing the internal structure of a crystal. The fundamental principle of these rules is that an ion of one element can replace the ion of another of similar size and charge (Faure, 1991). That basic principle can explain how elements are incorporated into otoliths. Element incorporation can occur by three processes. Substitution or co-precipitation occurs when cations of similar size and charge are substituted for Ca^{2+} or co-precipitated for another carbonate. Inclusion occurs when elements are included within the interstitial spaces of the crystal lattice. Finally, association refers to elemental adherence to the proteinaceous matrix of otoliths (Dove and Kingsford, 1998; Campana, 1999; Ranaldi and Gagnon, 2008). Substitution and co-precipitation process are the most studied and, therefore, more fully understood. For example studies have reported that Sr can replace Ca^{2+} via substitution.

Barium and Pb are also thought to substitute or co-precipitate (Degens et al., 1969; Campana, 1999; Ranaldi and Gagnon, 2008).

Otoliths can exist as one of three crystal polymorphs of calcium carbonate; aragonite, vaterite or calcite. Sagittal and lapilli otoliths are mainly composed of polycrystalline aragonite, while asterisci otoliths are generally vaterite. Generally, otoliths are composed primarily of aragonite however, under certain conditions one example being stress vaterite can replace the aragonite partially or completely (Halden and Friedrich, 2008; Lenaz et al., 2006). In circumstances when vaterite has replaced aragonite the otolith is identified as "crystalline" (Gauldie, 1986; Gauldie, 1993; Melancon et al., 2005). Studies have not yet determined the mechanisms behind the switch between aragonite and vaterite. However studies have suggested that vateritic otoliths could be associated to genetics or amino acid biochemistry (Gauldie, 1986).

1.3.4) Otolith Microchemistry

In the early 1970's researchers began using a new approach for the analysis of calcified structures. The approach focused on the chemical composition (Thresher, 1999; Pontual (de) and Geffen, 2002), using chemical analysis on a micro-scale. Data collected from this type of analysis can be useful in addressing fisheries questions related to environmental exposure, population structure, and fish migrations (Radtke and Shafer, 1992; Campana, 1999; Thresher, 1999; Pontual (de) and Geffen, 2002; Takagi, 2002). With the discovery of daily increments in the otolith microstructure, promoting age estimations and the continuous improvements with the chemical analysis technique the use of microchemical analysis on

otoliths has grown popular. The microchemical analysis can provide data on the concentrations of metals that occur in the fish's surrounding environments during its lifespan (Campana, 1999; Kalish, 1992; Kalish, 1989). The data paired with age estimations can provide a chronological record of the environmental history of the fish (Campana, 1999; Ranaldi et al., 2008).

1.4 Objectives

The purpose of this thesis was to:

1) To determine temporal trends in Mn in otoliths of lake trout captured from the Red Lake area of Ontario.
2) To determine if the timing of lake trout recruitment failure in Pipestone Bay, Red Lake is associated with an increase in Mn concentrations in otoliths of lake trout from Red Lake.
3) To link otolith microchemical analyses to environmental exposure of Mn using sediment chemistry as a proxy.

1.5 Hypotheses to be tested

1) Concentrations of Mn in otoliths from lake trout captured in Pipestone Bay are elevated compared to otoliths of lake trout collected from a reference watershed, not

receiving mine inputs; 2) Elevated Mn concentrations in otoliths of lake trout from Pipestone Bay will coincide with the timing of lake trout reproductive failure; 3) That the temporal history measured in the sediment cores will provide us link between the environmental exposure of manganese to concentrations in lake trout otoliths.

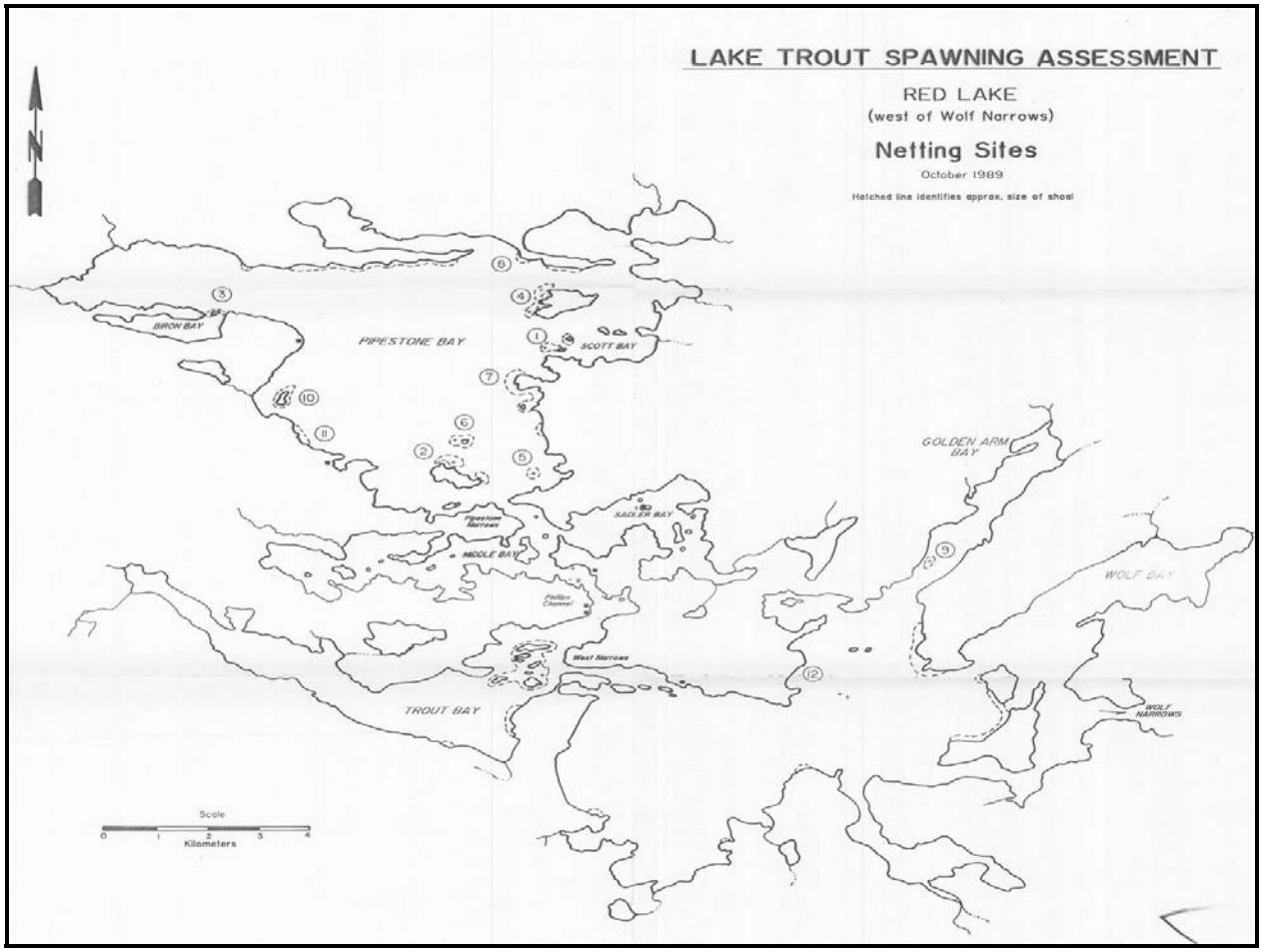

Figure 1.1 Map of the first survey of the spawning shoals within Red Lake in 1989.

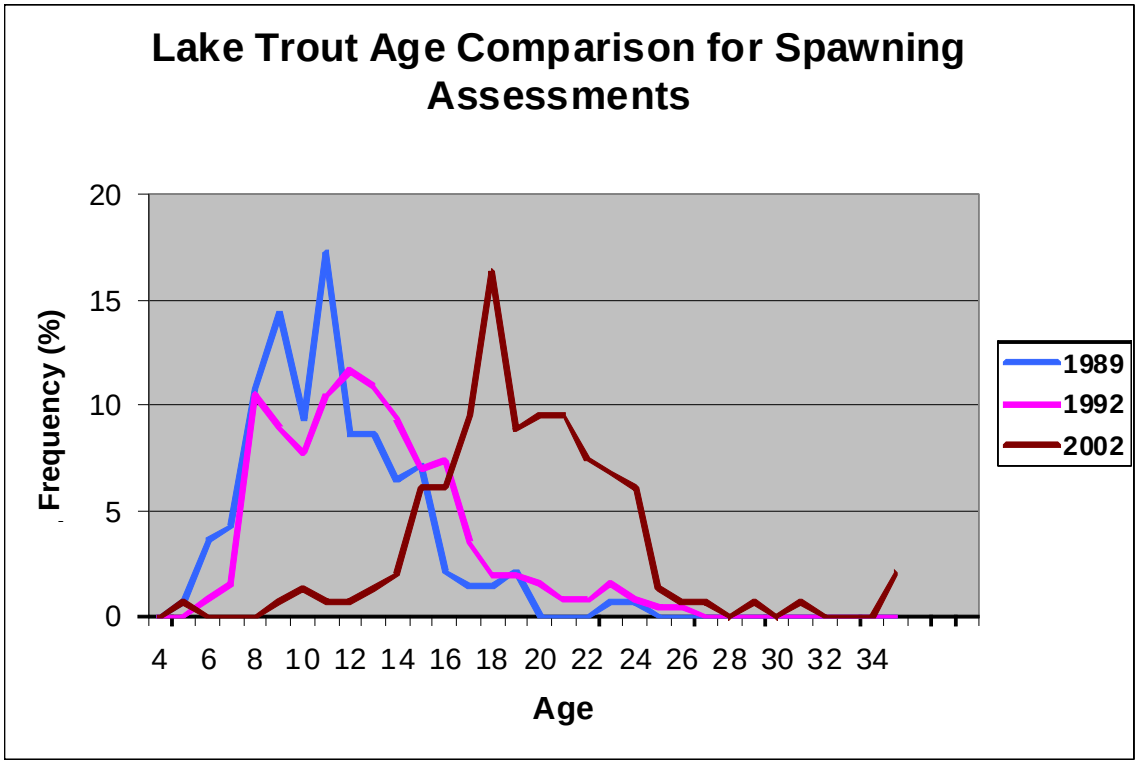

Figure 1.2 Trout age comparison for spawning assessments conducted in 1989, 1992 and 2002 on Red Lake (Thébeau, 2008).

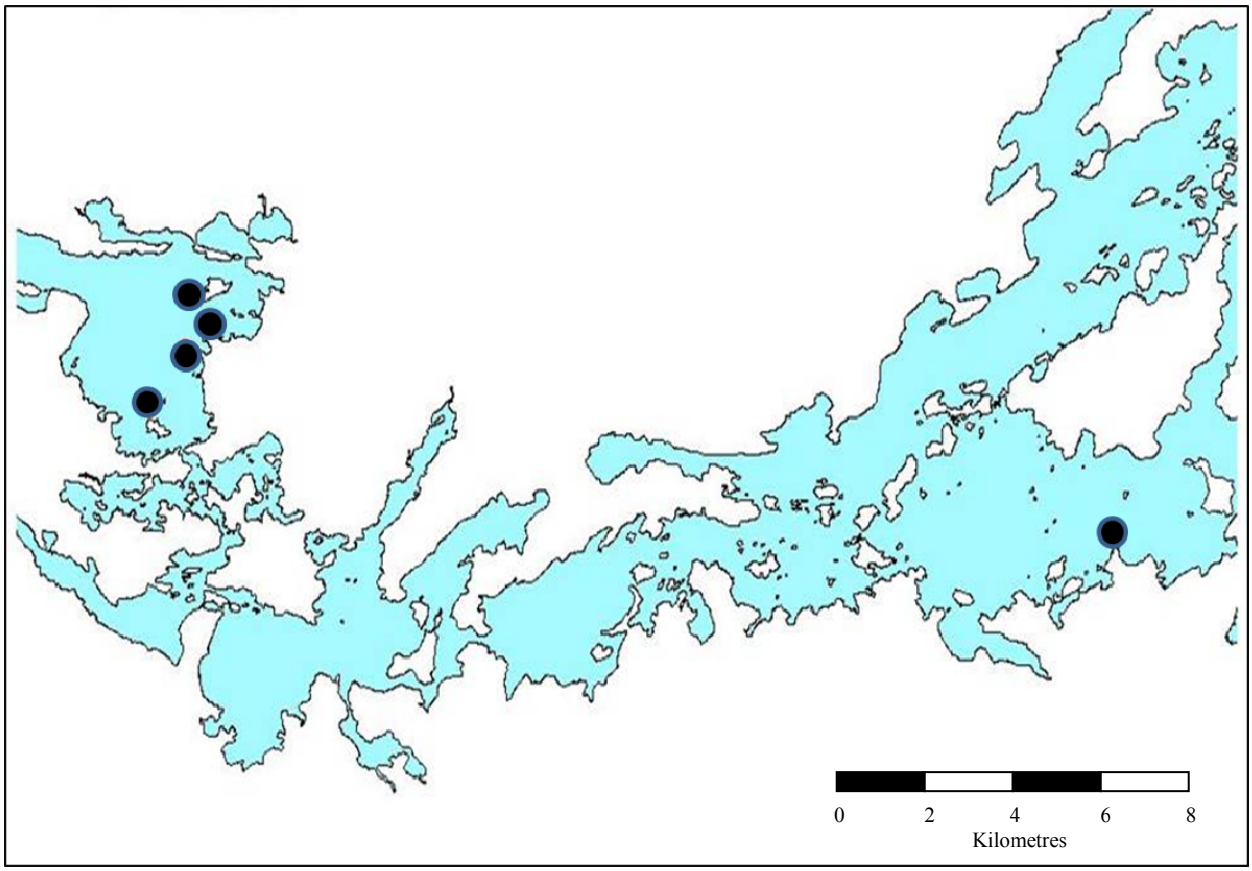

Figure 1.3 A map of Red Lake in Northwestern Ontario (51° 01' 59.81" N 93°49' 59.73" W) indicating the four sites where incubation trays were placed (●) in the 2003 *in situ* survival study.

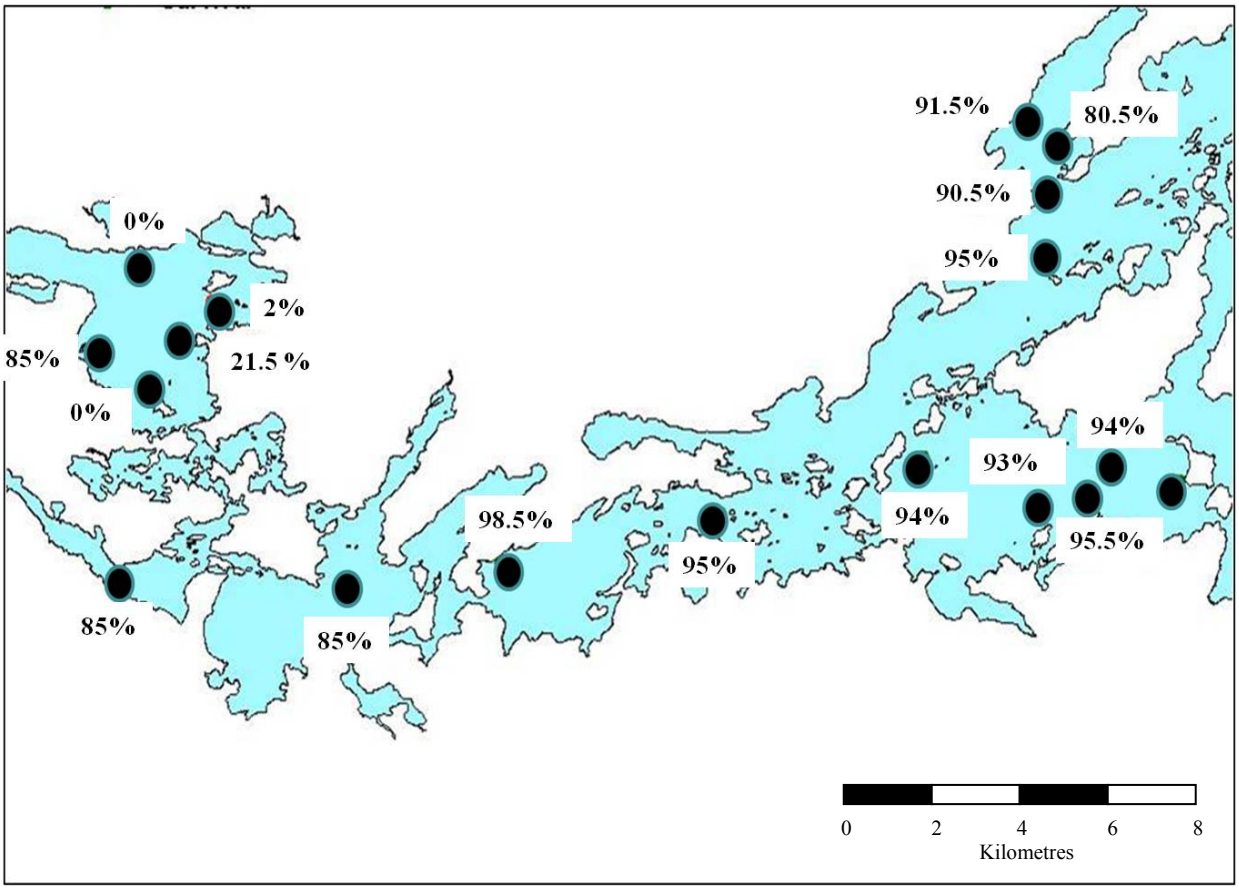

Figure 1.4 A map of Red Lake in Northwestern Ontario (51° 01' 59.81" N 93°49' 59.73" W) indicating the eighteen sites where incubation trays were placed (●) in the 2004 *in situ* survival study and the survival rate after 20 days.

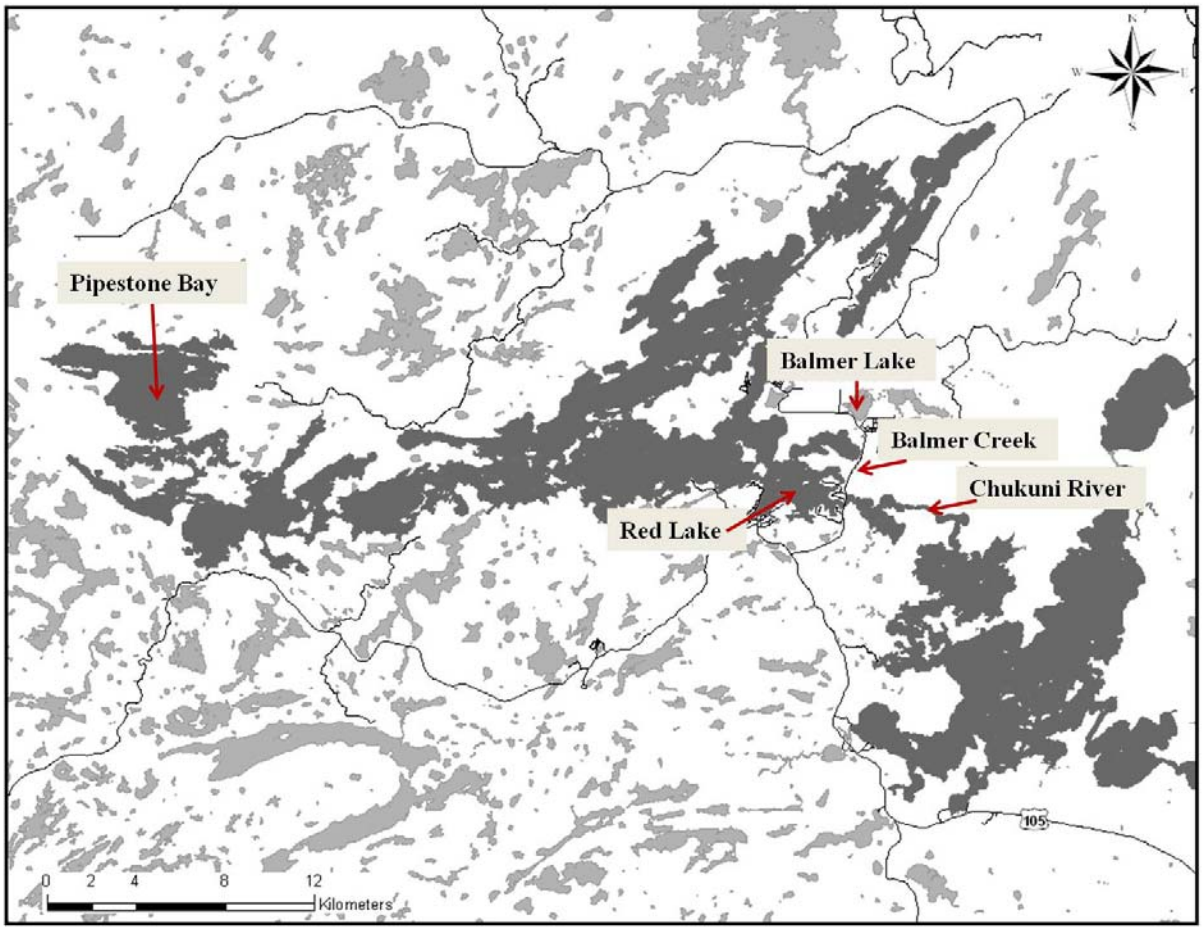

Figure 1.5 A map of the creeks and rivers downstream of Gold Corp Inc.

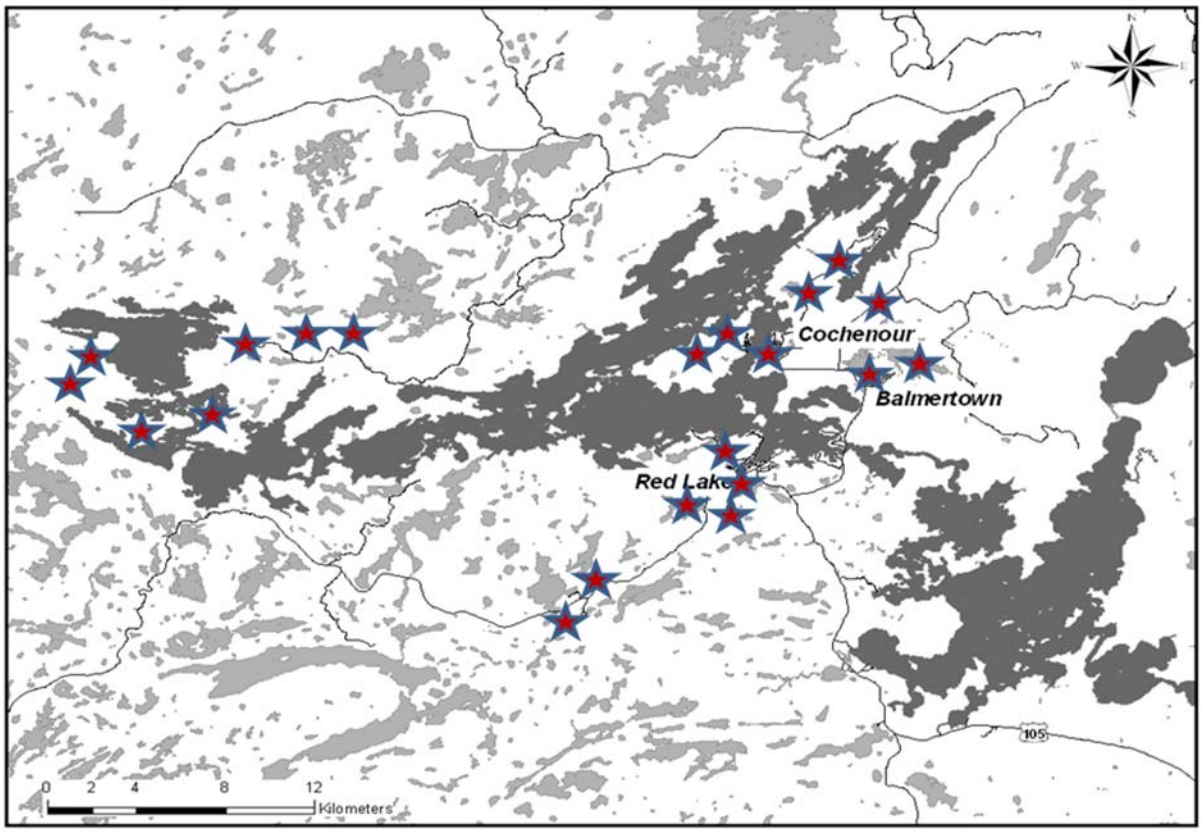

Figure 1.6 A map of Red Lake with (★) indicating gold mines that operated in the Red Lake Region.

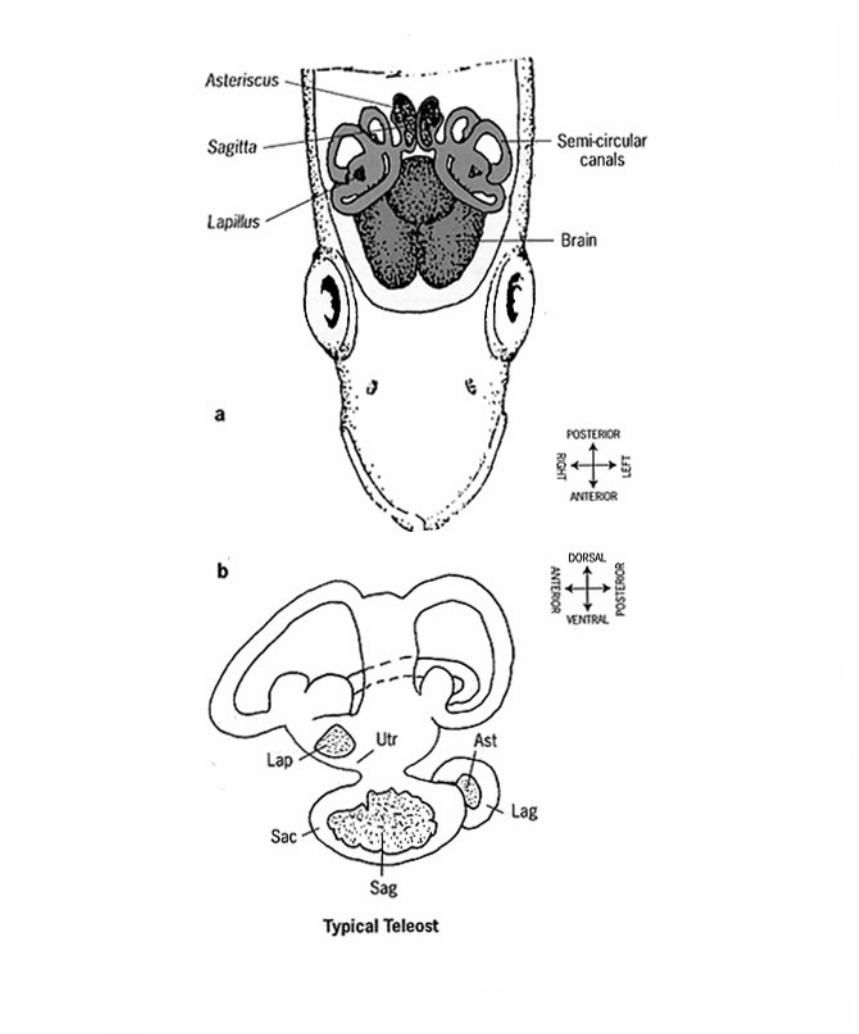

Figure 1.7a) Diagram of a head of the fish containing the midbrain identifying the location of the three pairs of otoliths, semi-circular canals. b) Diagram of one of the semi-circular canals that is found within the ear of a teleost fish (image was modified from Secor *et al*., 1992 with permission).

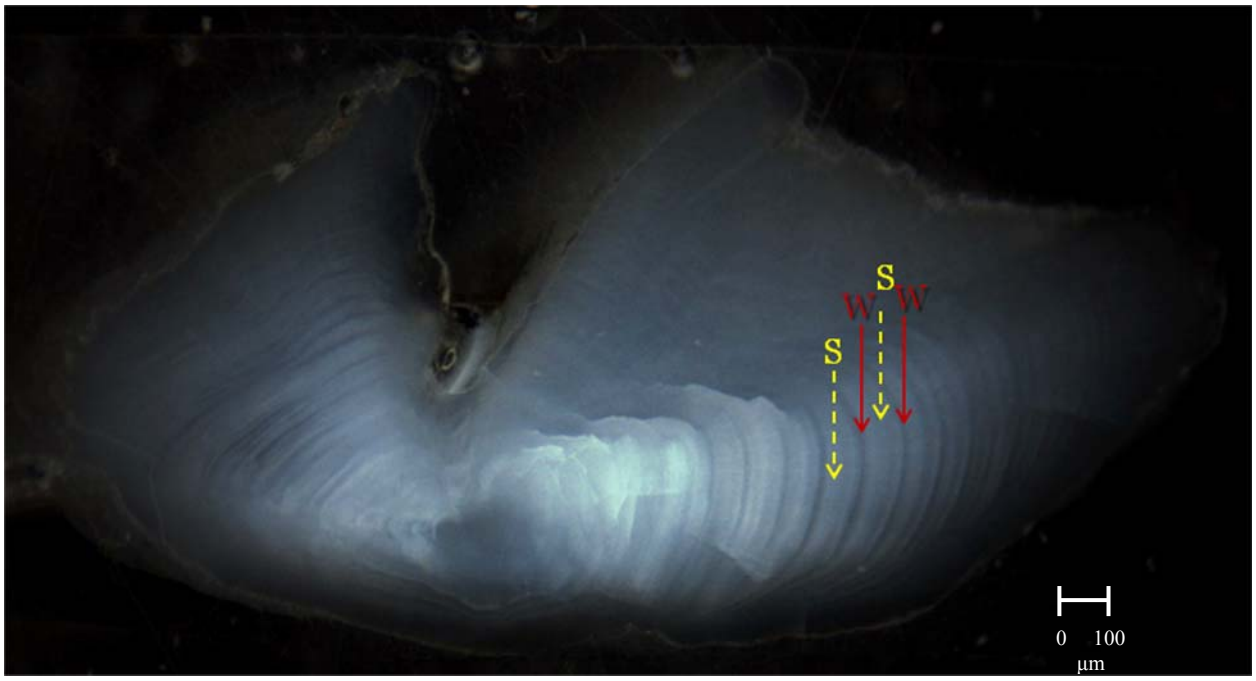

Figure 1.8 Otolith image illustrating the visible summer (S) and winter (W) annual growth bands.

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Chapter 2:

Retrospective analysis of Manganese in otoliths from lake trout (*Salvelinus namaycush*) exhibiting reproductive failure

ABSTRACT

Over the last decade a decline in abundance of the lake trout (*Salvelinus namaycush*) population of Red Lake, Ontario has been documented, coincident with apparent recruitment failure. Since 2001 spawning assessments, surveys, and bioassays focussed on the trout's primary breeding shoals in Red Lake's Pipestone Bay have been conducted to attempt to discover the causal agent for the ongoing recruitment failure. The Red Lake area has a history of gold mining. Metals associated with gold mining warrant special attention as the driver of low recruitment. Manganese (Mn) is a metal associated with mining, and previous water quality data from Red Lake show elevated Mn concentrations more mortality, was associated with Mn in an acute toxicity study. It was suggested that Mn could be a driver for the ongoing recruitment failure in the lake trout population. A retrospective analysis of Mn in archived lake trout otoliths (1963-2008) was performed using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). The aim of the study was to compare Mn concentrations in otoliths from Red Lake and two reference sites; Trout Lake and Confederation Lake. The aim of the study was to determine if temporal concentrations of Mn in the otoliths support its role in recruitment failure. Over a ten year period Mn concentrations in otoliths obtained from the three lakes were: Pipestone bay: 0.00 – 6.61μg/g; Trout Lake: 0.00 – 4.24 μg/g; Confederation Lake: 0.00 – 10.19 μg/g. There were significant differences ($P < 0.001$) in yearly Mn concentration in all three lakes. It appears that Mn is incorporated into otoliths at low concentrations. Mn concentrations were highest in otoliths from Confederation Lake compared to the other two lakes. Even though Mn concentrations were low, peaks associated with certain time periods could be discerned. There was no mining activity on Red Lake and

Trout Lake however elevated manganese concentrations were measured in otolith sections corresponding to the period between 1980 and 1990. The aim of the study was to determine whether manganese was a causal agent of recruitment failure, it should not be ruled out as a contributing factor to Lake trout recruitment failure.

2.1 Introduction

A more recent approach to investigate temporal trends of metal exposure in fish has included the use of otoliths, calcified structures composed of annually deposited altering layers of protein and aragonite (a morph of calcium carbonate). Otoliths are used for balance and hearing in teleost fish (Degens et al., 1969; Romanek and Gauldie, 1996; Campana, 1999; Halden *et al.,* 2000; Popper *et al*., 2005; Palace et al., 2007; Melancon et al., 2008; Ranaldi and Gagnon, 2008) that begin to form before time of hatch. They continue to grow throughout the lifespan of the fish (Campana and Neilson, 1985). It was first discovered in the 1970's that the otolith structure was comprised of daily increments which form an annular structure in otoliths. This breakthrough facilitated a better understanding of the otolith microstructure, and lead to a new approach for age estimation with enhanced capabilities and precision (Panella, 1971; Campana and Neilson, 1985). A decade later, studies revealed that otoliths had the capability to incorporate metals (e.g. Mn and Zn) into the annular structure (Campana, 1999). Since it was assumed that otolith composition was reflective of the external environment (Ranaldi et al., 2008), this discovery lead to several studies examining chemical make up of otoliths. These have address different fisheries questions associated with stock discrimination (Thresher et al., 1999). Because otoliths are acellular and metabolically inert (metals from the surrounding environment are not available for reabsorption or alteration)

they act as permanent recorder of a fish's exposure history (Campana, 1999; Halden and Friedrich, 2008). Otolith microchemistry has since been used as a tool to reconstruct exposure histories, specifically during particular periods of growth and development (Radtke and Shafer, 1992; Olsson et al., 1998; Campana, 1999; Halden *et al.,* 2000; De Pontual (de) and Geffen, 2002; Palace et al., 2003; Ranaldi and Gagnon, 2008).

Red Lake, located in the Canadian Shield in Northwestern Ontario is recognized as a popular fishing area. In the past the lake supported three essential sport fisheries, walleye *(Sander vitreus*), northern pike *(Esox lucius)* and lake trout (*Salvelinus namaycush*) (Thébeau, 2008). However, over the last decade the once renowned Red Lake lake trout has experienced a drastic decline, with a concurrent shift in the age distribution towards older fish (Figure 2.1). The Ministry of Natural Resources (MNR) have prioritized the lake trout population as a management concern; and are taking great efforts to restore the population and to identify a causal agent for the recruitment failure (Thébeau, 2008).

The MNR have established that the recruitment problem is isolated to the main spawning basin, Pipestone Bay. This area has been the main focus of research over the last nine years. One hypothesis for the failure has focused on the metals associated with gold mining. Historical records showed that over the last eighty years seventeen mines have operated in the Red Lake area, dating back as early as the 1930's. To further investigate the hypothesis, MNR carried several surveys, assessments and bioassays. A static test included rainbow trout *Oncorhynchus mykiss* eggs exposed to either Pipestone Bay or reference substrate for 30 days. Eggs exposed to Pipestone Bay substrate turned black and died after 14 days, while 50% of the eggs on the reference substrate died after 30 days (Thébeau, 2008).

The colour change in the eggs prior to death suggested the presence of manganese, either released from the substrate or within the pore water (CCREM, 2004). In addition to these results, water quality results from Red Lake found elevated manganese concentration (0.89 - 12.6 mg/L). Concentrations were considered high however, in Canada there is no guideline for the Protection of Aquatic life for manganese. Because Mn is associated with mining, it was measured at high concentrations in pore water samples, and because of the mortality of eggs in the embryology toxicity study, it was suggested that manganese could be a potential driver for the ongoing recruitment failure in the lake trout population.

The objectives of this study were 1) to analyze otoliths using laser ablation inductively coupled mass spectrometry (LA-ICP-MS) for Mn in the otolithic structure to reconstruct exposure history of the lake trout; 2) compare and contrast metal concentrations in otoliths from Pipestone Bay and reference sites, Trout Lake and Confederation Lake; 3) to link otolith Mn microchemical analyses to environmental exposure using sediment chemistry; 4) to link Mn signatures in the Pipestone Bay otoliths to identify possible causal agent(s) for the recruitment failure.

2.2 Material and Methods

2.2.1) Study Site

Red Lake is located in the Canadian Shield of Northwestern Ontario (51° 01’ 59.81” N 93°49’ 59.73” W) (Figure 2.2). It provides water for communities within the Township of Red Lake and is a popular fishing and hunting area for both local residents and visitors. The lake is 15, 591 ha in area and is comprised of various basins with depths up to 43 m. The lake typically stratifies in late June or early July at an estimated depth of 18m, and has an average

pH of 7.8 during the summer months (Thébeau, 2008). The geology of Red Lake is an important site characteristic to highlight. It is underlain by ultramafic rocks and a greenstone belt which consists of bedrock, till, mafic metavolcanic, a suite of gneissic and follated tonalite, and felsic to intermediate metavolcanic rocks

The primary focus of this study was Pipestone Bay, located in the western end of Red Lake (Figure 2.2). This is the main spawning basin for the lake trout of Red Lake and the location of recruitment failure. Pipestone Bay has an estimated mean depth of 40 m with an average summer temperature of 10.9 °C. In 2004, the estimated mean pH was 8.11, with a mean concentration of dissolved oxygen (DO) 9.1 mg/L measured at 30 m (Unpublished data, 2002).

Trout Lake and Confederation Lake were used as reference sites since they were similar in size, dominated by similar fish species and were most similar to Pipestone Bay geologically. Trout Lake is located in the Canadian Shield of Northwestern Ontario, 30 km northeast of Red Lake (Figure 2.2). The lake is 34,807 ha with a maximum depth of 47.3 m. It is an oligotrophic lake supporting fishes such as Lake Trout, Walleye, Lake Whitefish, Northern Pike, White Sucker, Red, and Ling. Geologically, Trout Lake is surrounded and underlain by granite (Stott and Corfu 1991). Confederation Lake is also located in Canadian Shield of Northwestern Ontario, 150 km from Red Lake (Figure 2.2). The lake is 4174 ha with a maximum depth of 32 m. The geological formation of Confederation Lake consists of a greenstone belt around it but, without ultramafic rock.

2.2.2) Otoliths

The Natural Ministry of Resources in Red Lake (MNR) have been collecting and archiving lake trout otoliths from the Red Lake area for several years. For this study, MNR provided 42 Pipestone Bay lake trout otoliths which were collected in 1987, 1988, 1989, 1990, 1991 and 2007. An additional 10 otoliths were collected as part of this thesis research in 2008 (n = 52). MNR also supplied otoliths from Trout Lake (n = 32) and Confederation Lake (n = 24) collected in 1989, 1993, 1997, and 1998.

2.2.3) Otolith Preparation

All otoliths used in this study were sagittal otoliths. Prior to metal analysis each otolith was rinsed in deionized distilled water (DDW), dried, embedded into Buherler® Epoxicure Resin to reinforce the otolith during sectioning. After the resin hardened (3- 5 days), the nucleus of each embedded otolith was marked and sectioned (Buehler® Isomet low speed saw) transversely to produce a dorso-ventral cross section through the nucleus and to expose all the annuli (Figure 2.3). The posterior half of the cut otolith was then re-embedded into a 25- mm acrylic microprobe ring (generally 4-7 otoliths per ring). Each ring was sequentially ground with three grades (30, 9 and 0.3 μm) of wetted 3M Imperial® lapping film to smooth the surface of the ring. Once the surface was free of abrasions, the rings were polished using Buheler® Masterprep Polishing Suspension (0.05 μm) and Buherler ® MeaServ 2000 Variable Speed Grinder-Polisher. Finally, the rings were ultrasonically cleaned with Ultrapure water for 2-3 minutes with a Branson® 1510 Ultrasonic cleaner.

2.2.4) Laser ablation- inductively coupled plasma mass spectrometry (LA- ICP-MS)

Elemental concentrations in lake trout otoliths were determined by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS). The analysis was performed on a Thermo Electron Finnigan Element 2 ICP-MS coupled to Merchantek LUV 213 Nd-YAG laser. Table 2.1 describes the general instrument conditions used during the analysis. For this study, the beam size diameter was 30 μm moving at speed of 2 μms^{-1} across the surface of the otolith. Standardization and calibration are essential for each analysis. Calcium (as 56 wt. % CaO) was used an internal standard (known concentration of one element in the unknown) while a glass reference NIST 610 (known concentrations of elements) was used for external calibration. After one sample (1 microprobe ring) was analyzed, the glass reference was analyzed to quantify and correct for instrumental drift. The concentrations of elements were determined by measuring the following isotopes: ^{55}Mn ^{60}Ni ^{65}Cu ^{66}Zn ^{111}Cd ^{208}Pb. The concentrations, standard deviations and detection limits of each element were processed using GLITTER software and exported to Microsoft Excel® and CorelDraw® for final presentation.

2.2.4) Aging

All otoliths collected by MNR in 1987, 1988, 1989, 1990, 1991 and 2007 from Pipestone Bay were aged at time of collection by MNR staff (See Appendix V). All 10 Pipestone Bay lake trout otoliths collected in 2008, 13 archived otoliths collected in 1998 and 1991, and 19 otoliths collected from the reference lakes collected between 1989 and 1997 were aged at the Freshwater Institute, Winnipeg, as described in Chilton and Beamish (1982).

2.2.5) Sediment Core Collection

On March 11, 2009, three intact sediment cores (inside diameter: 5 cm, length 13-25 cm) were collected at two different sites within Pipestone Bay. Two were obtained from the deepest part of Pipestone Bay (40 m), *Pipestone Bay deep 1* and *2* N 51° 03'42.6" W 094°12'40.8"and one core was from a recognized spawning shoal along the eastern shore of Pipestone Bay (30 m), Shoal 2 N 51° 03' 59.1" W0 94° 11' 29.9". Cores were retrieved with a Kajak-Brinkhurst (K-B) Corer as previously described (Murdoch and Mac Knight, 1991). At the surface, the core tube was immediately capped, to prevent cores from freezing, and transported to be processed (1 – 2 hr) (upright and covered). The *Pipestone Bay deep* core 1 was 18 cm, *Pipestone Bay deep* core 2 was 13 cm and *shoal 2* was 20 cm). Cores were sectioned at 1-cm intervals, which were stored in whirl packs and placed in a cooler for transport back to the laboratory at the Freshwater Institute, Winnipeg. In the laboratory, wet weights were measured. Prior to analysis, the samples were freeze-dried using a Labconco Freeze Dry/ Shell Freeze System. For each section total dry weights were obtained.

2.2.6) Sediment digestion

For each core, *Pipestone Bay* (20 samples) and *Shoal 2* (18 samples), a 0.1 g subsample of homogenized sediment was added to a Teflon sample vessel. In a well vented fumehood, 5 mL of concentrated nitric acid (HNO_3) was pipette into to each sample vessel capped with a single-ported cap equipped with a pressure release valve, then placed into a MARS 3051 Microwave Digestion System XP-1500™. In addition, 0.1g of two Certified

Reference Materials (CRMs), Mess-3 and Buffalo River Sediment were also digested for recoveries and blank digests were also included. The microwave was programmed to run samples under maximum power of 1600 W, maximum temperature of 175°C, and maximum pressure of 300 psi. The ramp time was 3 minutes; once maximum temperature was reached the temperature was held for 5 minutes followed by a cooling period of 3 minutes. After cooling, each sediment sample was emptied into a vial, 1.5 µL of sediment solution was measured into a 15mL vial then diluted with Ultrapure water, to a total volume of 15 mL.

2.2.7) Trace metal analysis

Digested sediment samples were analyzed by the Ultra Clean Trace Elements Laboratory (UCTEL) at the University of Manitoba, Winnipeg for manganese and iron. Samples were analyzed using inductive coupled mass spectrometry (ICP-MS) using a ELAN® DRC II Instrument from Perkin Elmer. The samples were analyzed under 1100 W plasma power with a sample flow rate of ~1 mL/min in standard mode. To acquire optimal sensitivity the ELAN DRC II instrument was optimized daily for nebulizer gas flow, lens voltage and auto lens. Certified reference materials NIST-SRM 1643e, and TM 26.3 and TM 28.3 were used to assure quality performance of the instrument during the run.

2.3 Results

2.3.1) Otolith Microanalysis

Manganese was suspected as a source for the mortality in the lake trout eggs, therefore, only manganese results, as well as zinc will be discussed. Zinc had shown to

exhibits a systemic distribution associated to the annular structure in otoliths, and could potentially offer some information on the habitat (Halden et al. 2000). See Appendix I, II, and II for the results for the remaining metals from all three lakes.

Prior to processing the data, isolated spikes caused by instrument noise were removed from the dataset. This was carried out to reduce instrument noise, and therefore identify the signals associated to the otolith chemistry. To illustrate, unmanipulated Mn is presented in Figure 2.4a. Figure 2.4b is the same data but with isolated spikes caused by instrument removed.

The peak Mn concentrations detected by the LA-ICP-MS in the lake trout otoliths collected from Pipestone Bay (n= 38), Trout Lake (n = 28) and Confederation Lake (n =17) were compiled into three illustrations (Figure 2.4, 2.5, 2.6). Otoliths from all three lakes were collected over a similar 48 year time period (1960 - 2008) to determine whether Mn concentrations between the three lakes were different and, whether peak Mn concentrations occurred within the same time period. In Pipestone bay, Mn concentrations ranged between 0.00 - 6.61 μg/g with the majority of the Mn concentrations falling between 0.25 - 4.07 μg/g during 1977 and 1990 (Figure 2.4). The highest concentration occurred in 1980. In Trout Lake, Mn concentrations ranged between 0.00 - 4.24 μg/g, with the majority of the Mn concentrations falling between 0.46 - 3.55 μg/g during 1978 and 1988 (Figure 2.5). The highest concentration occurred in 1983. In Confederation Lake, Mn concentrations ranged between 0.00 - 10.19 μg/g, with the majority of the Mn concentrations falling between 0.49 - 5.82 μg/g during 1983 and 1996 (Figure 2.6). The highest concentration occurred in 1991. The Mn concentrations measured in Confederation Lake were higher than those in Trout Lake

(4.24 μg/g) and Pipestone Bay (6.61 μg/g). To determine manganese exposure during the early life stages (egg and fry) Mn concentrations were measured in the first year of the lake trout from each lake. In Pipestone Bay, peak Mn concentrations ranged between 0.00 - 6.61 μg/g during 1963 and 1985 (Figure 2.7). In Trout Lake, peak concentrations ranged between 1.59 – 4.24 μg/g during 1972 and 1983 (Figure 2.8). In Confederation Lake, peak Mn concentrations ranged between 0.00 – 10.05 μg/g during 1981 and 1992 (Figure 2.9)

A one-way ANOVA (SigmaPlot 11.10) was performed to determine whether there were significant differences in Mn concentration among all three lakes. This was carried out by analyzing mean Mn concentrations from all three lakes from 1980 to 1989 (the period where otolith data overlapped) from all three lakes. There was a significant difference ($P < 0.001$) in the Mn concentration between all three lakes for that time period (Figure 2.11, Table 2.3). A Tukey's Studentized Range (HSD) showed that Mn concentrations were higher in Confederation Lake than both Red Lake and Trout Lake (Table 2.3). The HSD showed no significant differences between Red Lake and Trout Lake.

Zinc was included in the microanalysis because of its documented natural periodicity in otoliths. A representative illustration of the Zn results is given in Figure 2.12. Zinc concentrations in all the otoliths ranged between 33 – 304.00 μg/g, with the majority of the data falling between 6.07 -103.00 μg/g. Zinc concentrations were highest in the core region of the otolith (otolith development begins), with an annual oscillatory pattern that continued throughout the majority of the fish's life until the latter years where the oscillatory pattern gradually decreases to an almost flat profile (Figure 2.12). The oscillatory pattern corresponds to the annual growth zones in each otolith (Figure 2.12). As fish age, the annual growth zones compress, and it becomes harder to assign a corresponding year.

An oscillatory pattern was also observed in the Mn signature (Figure 2.13). However, the periodic pattern of Mn decreased at earlier annual growth zones compared to the Zn profile. The Mn signatures were also highest in the core and corresponded to the early life stages of the fish (Figure 2.10).

2.3.2) Vertical Distribution of Iron and Manganese

Mn concentrations in the sediment cores collected from Pipestone Bay deep peaked at 1cm with a peak concentration of 4987.9 µg/g (dry weight) (Figure 2.14). The Mn concentrations in the sediment cores collected from Shoal 2 were much lower with peak concentration at 566.0 µg/g (dry weight) at 1 cm (Figure 2.15).

Fe concentrations in the deep sediment cores collected from Pipestone Bay were highest at 2 cm with a peak concentration of 74.6 mg/g (dry weight) (Figure 2.16). The Fe concentration in the sediment cores collected from Shoal 2 was highest at 1 cm with a peak concentration of 31.3 mg/g (dry weight) (Figure 2.17).

2.4 Discussion

2.4.1) Otolith Microchemistry

Mn is a ubiquitous element found throughout the water and can be found in elevated concentrations near metal mining operations (Stubblefield et al., 1997; Dubé et al., 2005). Analyses of otoliths from Red Lake, Trout Lake and Confederation Lake indicated that Mn concentrations were greatest in Confederation Lake compared to the other two study sites. It was unexpected that mean Mn concentrations in the Confederation Lake would be significantly higher than those obtained in Red Lake's Pipestone Bay and Trout Lake. While not known to us at the outset of this study, it was determined that between 1971 and 1980 a copper-zinc-mine (South Bay Mine) operated on a shoreline of Confederation Lake (Andreas Lichtblau, Ministry of Northern Development (MND), pers. commun. May. 19, 2010). While the mine was in operation it produced 1.6 million tons of ore with the average content of 11.06% Zn/ton, 1.8% Cu/ton and 2.12 ounces Ag/ton. Statistics regarding Mn releases are not known for this mine, but it is well known that one of the key anthropogenic inputs of Mn to the aquatic environment is through wastewater and/or emissions from mining processes (Moore, 1991; WHO, 2004). Environment Canada estimated that the total Mn emissions from these sources were 1225 tonnes on a global scale during the 1980's. During this time aquatic environments received Mn inputs from wastewater (58-171 x 10^3 tonnes/yr), non-ferrous metal refining (2-15 x10^3 tonnes/yr), metals manufacturing (2.5-20 x 10^3 tonnes/yr), and base metal mining/dressing (0.8-12 x10^3 tonnes/yr) (Nriagu et al., 1988). The lower concentrations observed in Pipestone Bay and Trout Lake otoliths may be because of the absence of significant mining activity near these lakes. A review of archived data indicted that Pipestone Bay had one mine located ~1 km away from the shore but it operated for two years (1986-1988) and only produced 13, 023 tons of gold ore. Trout Lake has no record of mining activity in or around the region (Andreas Lichtblau, (MND), pers. commun. May 19, 2010).

Even though historical mining data showed no significant activity during 1980-1989, and Mn concentrations were lower compared to Confederation Lake, it is important to draw attention to the fact that Mn concentrations in Pipestone Bay peaked during 1980-1989. Coincidently, when lake trout recruitment failure was thought to have commenced, suggesting another source of Mn exposure other than mining activity such as natural deposits, industrial wastes, or pesticides. The Mn concentrations measured in the first year of the lake trout in all three lakes show similar trends with Pipestone Bay with elevated concentrations during 1980's supporting the potential for another Mn source.

Mn concentrations observed in the lake trout otoliths from Red Lake's Pipestone Bay could potentially be reflective of the dissolved manganese with the water column. At each barrier along the elemental pathway there is a decline in metal ions. The decline of ions could account for the low Mn concentrations observed in the otoliths from all three study sites. There have been only a small number of studies that have investigated the influence of Mn concentrations present in aquatic environments on the otolith microchemistry. Among the little research that is been conducted, most of the focus has been on marine and estuarine fish species and their environments (Dove and Kingsford, 1998). Elsdon and Gillanders (2003) conducted a study on the relationship between water and metal uptake in the marine species, Black Bream (*Acanthopagrus butcheri).* Even though Mn concentrations were variable, the element was still incorporated into the otolith at detectable concentrations. However, because Mn is involved in metabolic processes (enzyme co-factor) (CCME, 1987; Barceloux, 1999), and at high concentrations Mn can be toxic, manganese may be physiologically maintained at consistent concentrations.

The LA-ICP-MS analysis showed that Mn was incorporated into lake trout otoliths at low concentrations. This suggests that dissolved Mn concentrations were low in Pipestone Bay. However, there were a higher concentration between 1980 and 1988, suggesting an increase in the environmental Mn concentrations during that time. The total manganese present in freshwater systems can be quite variable (Moore, 1991). In natural waters, free from anthropogenic influence the Mn concentration can occur in low concentrations that range between <0.01mg/L to >10mg/L (McNeely et al., 1979) or 0.002 to >4 mg/L (Moore, 1991). Since it occurs at low concentrations, Mn is considered to be a non toxic element (Moore, 1991). Fish and invertebrates tend to bioconcentrate Mn at low concentration (<0.1 mg/kg wet weight) (Kwasnik et al., 1978; Brush et al., 1979; Moore, 1991) and there is no usually no major concern that Mn will bioconcentrate and cause any effects. However, it is important to note that the rate of bioconcentration is different for each species, organ, and environment. Dubé et al. (2005), measured Mn concentration in water, and whole body tissue of Atlantic salmon (*S. solar*) exposed to different concentrations of metal mine effluents. The study found that Mn demonstrated consistent increases in both water and whole body tissues. However, the body burden of Mn only increased between 1 to 2 fold. Since the Mn concentration in the 80% treatment group was 0.3 mg/L, way below the reported IC_{25} by Stubblefield et al. (1997) the study concluded that Mn was not source for biological impacts (i.e. survival, growth) on *S. solar*.

Wiener and Giesy (1979) examined Mn concentrations in axial muscle, liver and whole body of eight species in an acidic pond in south-western USA. Lake Chubsucker (*Erimyzon sucetta*) had the highest Mn concentration among all resident species, Mn 75.1 μg/g (dry weight), the Chain pickerel (*Esox niger*) had the highest Mn concentration in axial muscle

with a Mn concentration of1.21 μg/g (dry weight), and bluegill had the highest Mn concentration in the liver with concentrations of 7.18 μg/g (dry weight). This study concluded suggested that Mn concentrations accumulated in tissues however only at low concentrations.

Halden and Friedrich (2008) examined the influence of exposure to tailings effluent on otolith chemistry. The manganese concentrations in otoliths of lake trout (*Salvelinus namaycush*) collected from two mine impacted lakes were low, 0.1- 2 μg/g. Manganese had an oscillatory pattern that corresponded to the annuli, suggesting that Mn availability and/or uptake is periodic and potentially seasonal. The Mn concentrations measured between 1980 and 1988 in lake trout otoliths in the present differ considerably when compared to Mn concentrations observed in lake trout otoliths in Halden and Friedrich (2008).

Thorrold et al. (1998) attempted to use elemental signatures as an indicator of American shad (*Alosa sapidissima*) natal rivers. Unfortunately the discrepancies observed in the Mn concentrations in the otoliths could not explain the variation in the metal concentrations between all three study sites. They concluded that genetics or diet could have influenced the incorporation of Mn in the otoliths.

There have been several studies that have investigated diet as a source of metal uptake (Hoff and Fuiman, 1995; Geffen et al., 1998; Milton and Chenery, 2001; Buckel et al., 2004). However, studies have suggested that otolith uptake of certain trace metals from food was minimal. For example, for Ca and Sr the majority of the otolith composition is probably derived from the water (80-90%) in freshwater fish (Hoff and Fuiman, 1995).

There is still much to learn about environmental Mn concentrations and their relationship with fish otolith composition. Variations in Mn concentrations may be attributed

to metal regulation by fish, dietary influences, and genetics. All these remain to be fully investigated. Additional research regarding the controls and mechanisms of barriers and pathways of metal uptake into the fish and otolith are also needed (McKim, 1994; Campana, 1999).

2.4.2) Geology of Red Lake

Another explanation for the differences in otolith Mn concentrations could be the underlying geology of each of the three lakes. Manganese occurs naturally in the environment and enters the environment through physical and chemical weathering (CCME, 1987). Confederation Lake and Red Lake are both surrounded and underlain by felsic and mafic rock. In terms of Mn, felsic rocks are known to contain 1000 μg/g Mn while mafic rocks can have Mn concentrations near 2000 μg/g. Samples of rock collected from Confederation Lake by the Ministry of Northern Development (MND) (Red Lake District) showed concentrations as high as 2000 μg/g Mn (Andreas Lichtblau, (MND), pers. commun. May. 19, 2010). Red Lake's Pipestone Bay, also has a large component of ultramafic rock which tends to have concentrations around 1300 μg/g of Mn. However, ultramafic rocks collected from Pipestone Bay by MND contained only 774 μg/g of Mn (0.10% MnO) (Andreas Lichtblau, (MND), pers. commun. May. 19, 2010). In contrast, Trout Lake is surrounded and underlain by granitic rocks. Samples collected from the western section of Trout Lake by MND contained Mn concentrations between 450-500 μg/g (Lichtblau, (MND), pers. commun. May. 19, 2010). The amount of Mn being released into the aquatic environment is dependent not only on concentrations but also the susceptibility of each rock type to weathering. Ultramafic rocks are more susceptible to weathering than felsic rocks because felsic minerals are

comparatively more stable at surface conditions than ultramafic minerals (Strahler and Strahler, 1973; Halden, University of Manitoba, pers. Commun. May. 26, 2010). The availability of Mn to the environment is also reliant on the minerals contained in each rock type. Unfortunately there is limited research on the influence of Mn concentrations in substrate and its relationship to otolith concentrations. Hanson and Zdanowicz (1999) conducted a study on the influence of 13 elements (Ag, As, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Se, Sn and Zn) on the otolith composition of Atlantic croaker (*Micropogonias undulates)* in Galveston Bay, Texas. Manganese, Cr, and Ni concentrations were greater than natural background levels. The study found no correlations between the trace metal concentrations in sediment, liver and otoliths. This suggests that biological, not geochemical factors influenced the elemental composition of the otoliths (Hanson and Zdanowicz, 1999).

Manganese comprises 0.1% of the earth's crust and is a component of at least 100 minerals including sulfides, oxides, carbonates, silicates, phosphates, and borates (Campell and LaZerte, 1988; Moore, 1991; Barceloux, 1999). Manganese generally occurs in trace to minor amounts in the form of ferrous oxides as well as spinels. Manganese can exist in three different oxidation states, Mn (II), Mn (III) and Mn (IV). Mn (II) is most important in aquatic environments since it is the form that is reduced, soluble and geochemically mobile compared to Mn (III) and Mn (IV) which are the insoluble forms of Mn (Wetzel, 1975; Moore, 199; Davison, 1993). The Mn present in the ultramafic rock is typically Mn^{2+} while oxide minerals are Mn^{3+} and Mn^{4+}. However, if the minerals are exposed to the atmosphere they are oxidized to Mn^{2+} and more available to the aquatic environment. If minerals consisted of particularly high levels of Mn and weathering occurred, then elevated concentrations of Mn might result from changing redox conditions. At the redox boundary of lake sediment, oxidized particulate

matter is reduced to a soluble form that can diffuse upwards or downwards. If diffusion is upwards, the particulate matter re-enters oxic conditions where it is re-oxidized back into the insoluble form and redeposit's at the sediment surface. The heavier particulate matter settles downward through the redox boundary completing the redox cycle (Davison et al., 1980; Sundby et al., 1981; Belzile et al., 1989). The closer the redox boundary is to the sediment water interface the more likely that elements are to escape the oxic tarp and enter the water column.

2.4.3) Sediment Chemistry

Sediment cores were collected to attempt to link chemical signatures present in the sediments to chemical signatures in the otoliths. Manganese and iron are key constituents in the aquatic redox processes, and are both associated with the cycling and bioavailability of metals in aquatic environments (Wetzel, 1975; Förstner and Wittman, 1979; Belzile et al., 1989; Ochieng et al., 2007). Sediment cores collected from Pipestone Bay showed Mn concentrations as high as 4987.9 μg/g (dry weight) approximately 1 cm under the sediment-water interface (Figure 2.11). Redox boundaries can extend over many meters within the water column or occur in less then a centimetre in the sediments (Davison, 1980; Sundby et al., 1981). Our results suggest that the redox boundary is very close to the sediment-water interface which would promote reducing conditions supporting the reduction of Mn^{4+} to soluble Mn^{2+} (Moore, 1991). The thin 1 cm layer of oxidized sediment suggests that Mn near the sediment-water interface had become dissociated from oxyhydroxides in anoxic deep sediments, and actively diffused upward. This would allow Mn a better opportunity to escape into the overlying water column more than if the redox boundary was 10 cm deep. This

suggests that Mn is more mobile in Red Lake sediments, and not as a precipitate from the water column.

There are several mechanisms that influence redox reactions at the sediment-water interface that favour the release of Mn from the sediments to the overlying water. The mobility of Mn could have been influenced by pH and electron activity (pE) (Wetzel, 1975). The pH can decrease the oxidation rate of Mn promoting the formation of manganese oxyhydroxides near the sediment-water interface (Belzile et al., 1989). Belzile et al., (1989) found that in conditions where pH < 6, no manganese oxyhydroxides formed, suggesting slow kinetics of Mn oxidation. In conditions where pH > 6 (i.e. conditions that occur at or near the sediment-water interface) manganese oxyhydroxides were formed. There were no pH values recorded (at bottom sediments) during this study. However, data collected by MNR in 1980 showed pH conditions at 41 m ranged between 6.47 and 7.35. If pH conditions have not changed greatly over time, oxyhydroxides were favoured that would increase precipitation and lower manganese concentrations in the overlying water column.

The electron activity (pE) is also known to influence the mobility of Mn in sediments. In the sediments, areas with reducing conditions generally have large pE values. Thus, a high pE suggests a high affinity for oxidation (Förstner and Wittmann, 1979; Stumm and Morgan, 1981). In well oxygenated conditions, where pE is high (pE > 11) Mn occurs as Mn (IV). However, when reduction occurs this depletes oxygen and the pE decreases. As a result, Mn (II) in the sediments increases with depth (Förstner and Wittmann, 1979). Belzile et al., (1989) conducted an *in situ* study on the mobility of iron and manganese oxyhydroxides in natural sediments. Results found that the oxidation of Mn (II) occurred at a much higher pE value, and required a higher pE value for oxidation compared to iron. Even though the pE

was not measured in this study Mn results followed a similar pattern to what has been described. Mn concentrations were observed higher in the upper sediments (2 cm), near the sediment- water interface where oxidation most likely occurred and decreased with depth as oxygen declined. Previous research indicates that pE influences metal mobility however, we can only assume that pE was high since Mn concentrations followed a similar pattern that has observed in previous studies.

Fe concentrations were more variable than Mn concentrations in sediments (Figure 2.13, 2.11). Iron can occur as Fe (II) or Fe (III) in aquatic environments. Both forms are electroactive in the aquatic environment (Wetzel, 1975). In natural waters, Fe^{2+} is the more soluble form while Fe^{3+} is insoluble. Similar to Mn, at the redox boundary the oxidation of Fe^{2+} to Fe^{3+} is influenced by pH, redox potential (Eh) and temperature (Wetzel, 1975; Belzile et al., 1989; Amatya and Mika, 2008). The redox status of lakes is always changing (Davison et al., 1980). The variability in the Fe concentrations could have been associated with changes in the physico-chemical properties promoting reduction of Fe^{3+} to Fe^{2+}, allowing the diffusion of Fe^{2+} up into the upper sediments (Davison et al., 1980; Belzile et al., 1989). As mentioned previously redox processes are greatly influenced by pH, redox potential (Eh), electron activity (pE) and temperature (Wetzel, 1975). The pE decreases with depth due to the decreasing oxygen in the sediment. Fe does not require a high pE value therefore the oxidation rate would be faster compared to Mn (Kawashima et al., 1988; Belzile et al., 1989; Amatya and Mika, 2008). In addition, the shape of the concentration curve of iron in lakes has been found to have an amorphous shape that causes it to be more labile and readily reduced in anoxic conditions (Tipping et al., 1981; Tipping et al., 1982). Manganese and iron act similar at the redox boundary. Due to relative redox similarities it was important to

examine iron concentrations along with Mn because Fe can indicate the depth at which the redox boundary was located which was important for this study.

To establish whether Mn concentrations in Pipestone Bay were a causal agent for recruitment posed a challenge since Canada has no guideline for manganese included in the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 2007). In the United States, a concentration of 1000 μg Mn/L has been used as a standard exclusively on the suggestion by McKee and Wolf (1963). This proposed concentration is not supported by much toxicity data and does not consider other influential factors (i.e. water hardness) (Stubblefield et al., 1997). Unfortunately, the proposed Mn standard for water quality can not be applied to this study to establish a relationship between Mn concentrations present Pipestone Bay and lake trout otoliths. As previously mentioned, trace metal uptake in fish occurs over a multi-barrier pathway. Metals are discriminated at each barrier at varying extents, and discrimination occurs differently for each trace metal (Campana, 1999). This prevents us from identifying a relationship between Mn concentrations in Pipestone Bay and the Mn concentrations observed in the lake trout otoliths. Further research on how manganese is taken up, transferred across the barriers, and incorporated into the otolith will help better understand the relationship between Mn concentrations in otoliths and water concentrations.

Manganese generally exerts a low toxicity, and therefore relatively minimal effects on aquatic organisms (Hanna and Spryer, 1988). Several acute and chronic studies have been carried out with salmonid species to investigate the impacts of Mn toxicity. Stubblefield (1997) investigated the relationship between Mn toxicity, and the influence of water hardness

and pH by conducting a 62 day chronic toxicity study on the early life stages of brown trout (*Salmo trutta*). The study was carried out at three different levels of water hardness 30, 150 450 mg/L as $CaCO_3$. Mn concentrations ranged from 0.51up to 15.5 mg Mn/L for brown trout exposed to 30 hardness (mg/L as $CaCO_3$), from 2.78 up to 74.90 mg Mn/L for brown trout exposed to 150 hardness (mg/L as $CaCO_3$), and from 2.54 up to100.82 mg Mn/L for brown trout exposed to 450 hardness (mg/L as $CaCO_3$). Brown trout embryos were found to be tolerant to Mn exposure. The hatching success was not affected by any tested concentrations. The success rate for embryos was between 86% and 98.2-%. In contrast, larvae were not so tolerant. The rate of larval survival decreased with increasing Mn concentration. The mean percent survival for the control groups exposed to each water hardness was 86.7, 86.7, and 43.8% while survival at 2.54, 4.55, and 8.68 mg Mn/L exposed to 450 hardness (mg/L as $CaCO_3$) was 90.0, 90.8, and 83.3%. In addition, mortality was observed sooner in the higher treatment groups compared to lower treatment groups. The time interval was 15-20 d post hatch in higher treatment groups while the time interval was 35-40 d post hatch in lower treatment groups. The post hatch life stage appeared more sensitive to metal exposure compared to the embryo stage. Stubblefield et al. (1997) concluded that there was important relationship between water hardness and Mn toxicity in brown trout, and results could be useful towards establishing an estimate for potential effects of chronic Mn exposure in salmonid species.

Water hardness was not a focus in the present study therefore not recorded. However, MNR collected water hardness measurements for all three study sites in 2004 and 2008. In Pipestone Bay, the mean water hardness was 30.4 mg/L $CaCO_3$ measured 1.5 m below the surface; in Confederation Lake, mean water hardness was 25.1 mg/L $CaCO_3$ 1 m below the

surface; and Trout Lake the hardness (calc) in 1985 was 10.7 mg/L $CaCO_3$. Water that contains <60 mg/L of $CaCO_3$ is considered soft water (IPCS, 1996). From water hardness data collected from Pipestone Bay, data suggests that Pipestone Bay is a relatively soft water lake. Therefore, suggesting water hardness was not a factor since both Mn, and water hardness were low. Trout Lake would be considered a very soft lake, suggesting that water hardness would not have been an influence on the Mn concentrations. Confederation Lake had the highest Mn concentrations measured in the otoliths. Since it had the highest Mn concentrations it would be suspected that the water hardness may be elevated however, that wasn't the case. The water hardness in Confederation Lake was lower compared to Pipestone. Suggesting that water hardness was not a factor in the Mn concentrations observed in the lake trout otoliths. In the present study, water hardness did not appear to play an important role in the Mn concentrations observed in the lake trout otoliths collected from all three study sites.

2.4.4) Summary and Conclusions

There were two hypotheses being tested in this study, 1) concentrations of Mn in lake trout otoliths collected from Pipestone Bay were elevated compared to otoliths of lake trout collected from a reference watershed, not receiving mine inputs; 2) elevated Mn concentrations in lake trout otoliths from Pipestone Bay would coincide with the timing of lake trout reproductive failure.

Research on manganese toxicity in freshwater systems and the influence on otolith microchemistry are limited. However, the available data, suggest that manganese has a low toxicity in the aquatic environments resulting in low impacts on fish populations. The LA-

ICP-MS results show that Mn was incorporated into the otoliths; however, only at low concentrations. Even though Mn concentrations were low, differences were found to be temporally associated to past mining activity on Confederation Lake. There was no mining activity on Red Lake and Trout Lake between 1980 and 1990. We concluded that the manganese was incorporated into the lake trout otoliths and peak manganese concentrations were observed in Pipestone Bay, Trout Lake and Confederation Lake. The peak Mn concentrations in Pipestone Bay correspond to the time period when the recruitment failure was thought to have begun however since Mn is redox active the temporal history from sediment cores was uncertain. Therefore, no link could be made between the chemical signatures in the sediments and the chemical signatures to indicate manganese as causal agent for recruitment failure in Red lake. Further investigation is required.

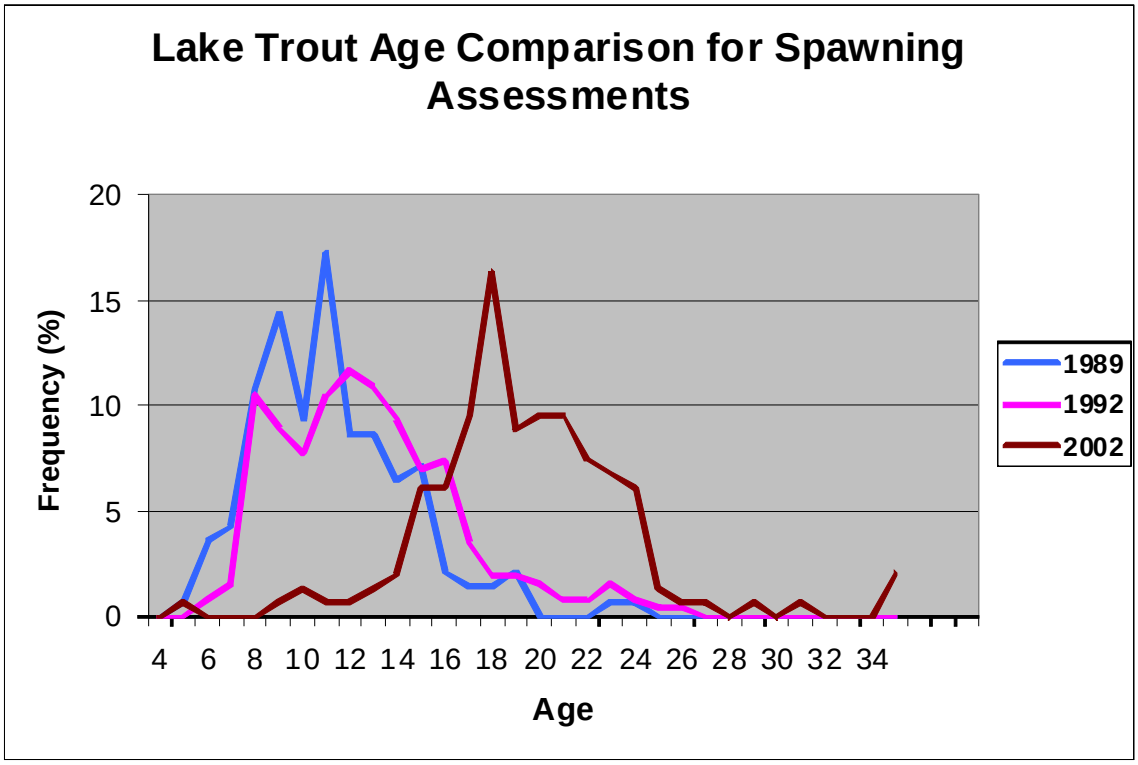


Figure 2.9 Trout age comparison for spawning assessments conducted in 1989, 1992 and 2002 on Red Lake (Thébeau, 2008).

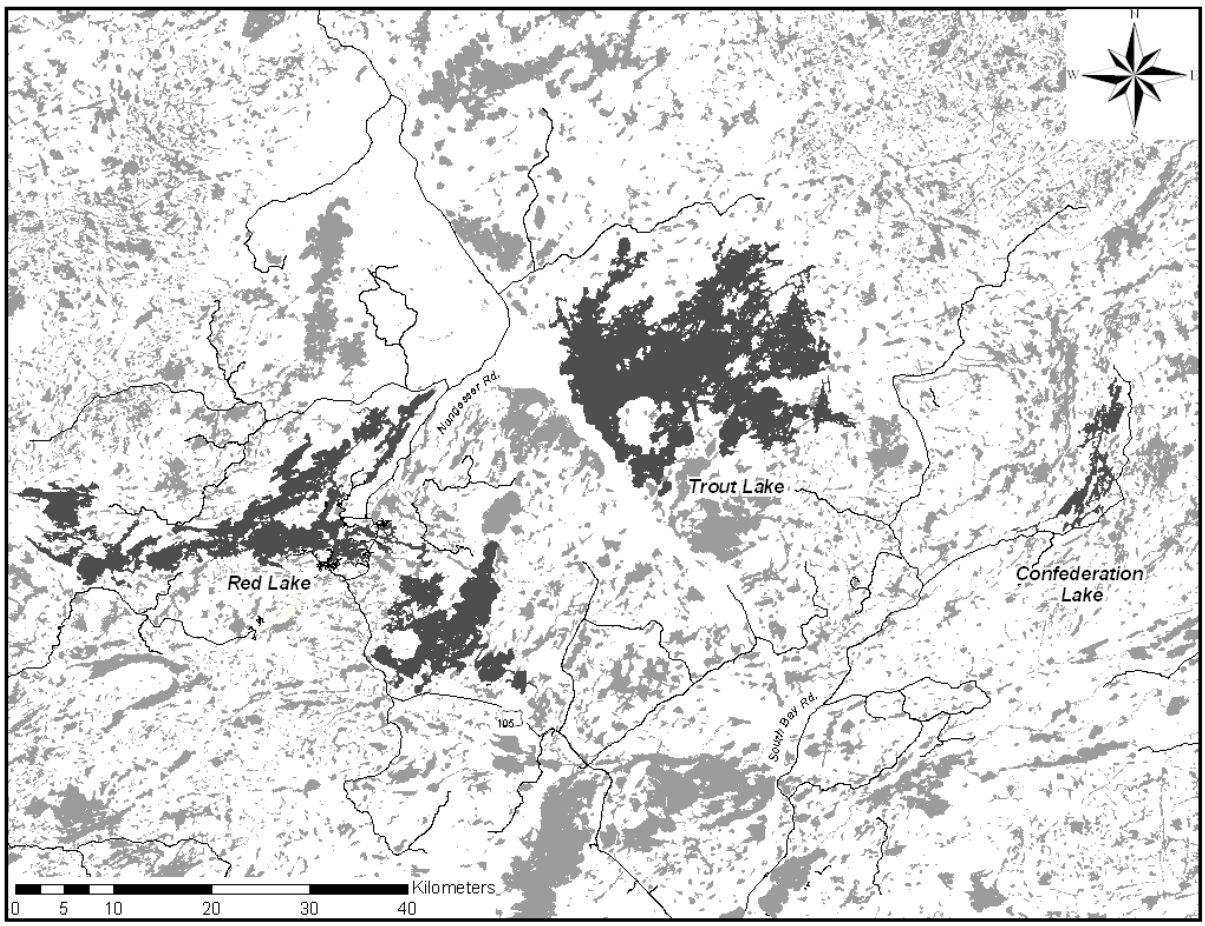


Figure 10.2 Map of Red Lake, Trout Lake and Confederation Lake.

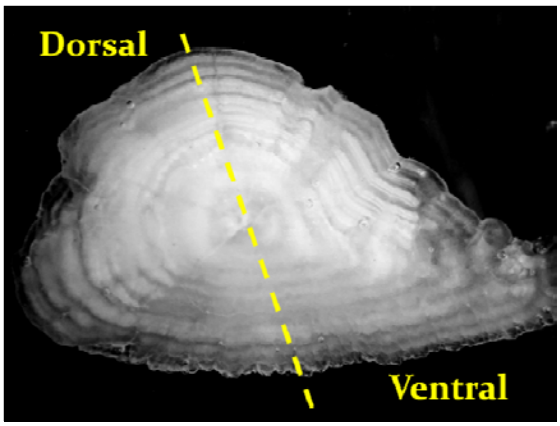


Figure 2.11 A dorso-ventral cross section of an otolith

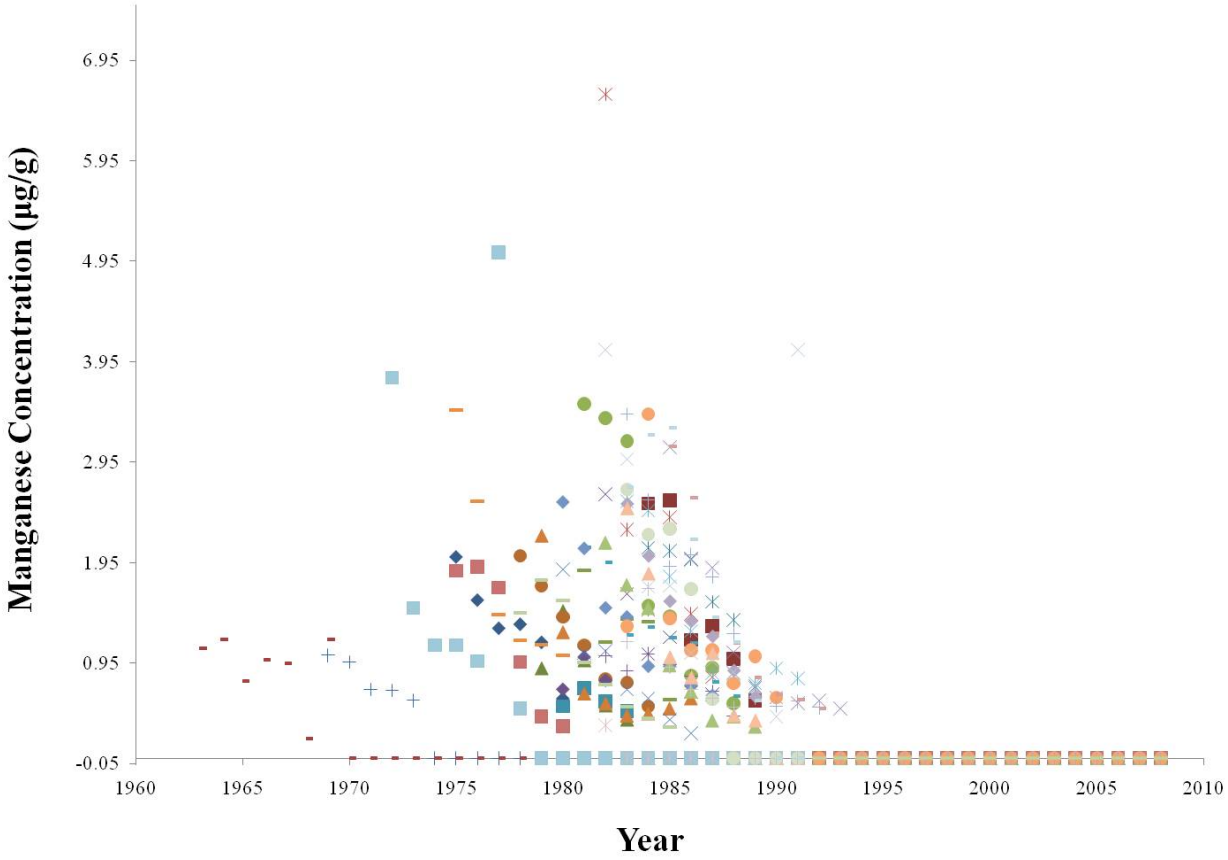


Figure 2.12 Peak concentrations of manganese in all the otoliths collected from Pipestone Bay between 1960 and 2008. Each symbol represents an individual fish and their Mn concentration measured in the given year.

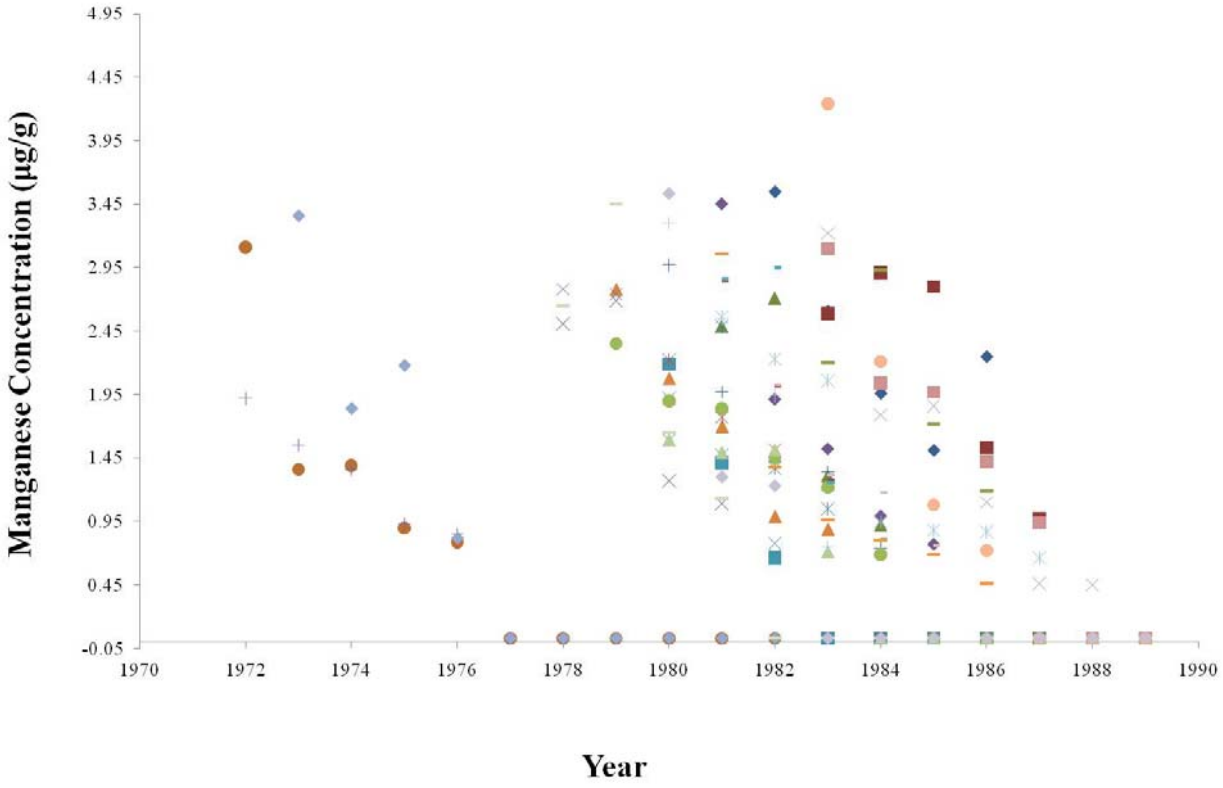


Figure 2.13 Peak concentrations of manganese in all the otoliths collected from Trout Lake between 1970 and 1989. Each symbol represents an individual fish and their Mn concentration measured in the given year.

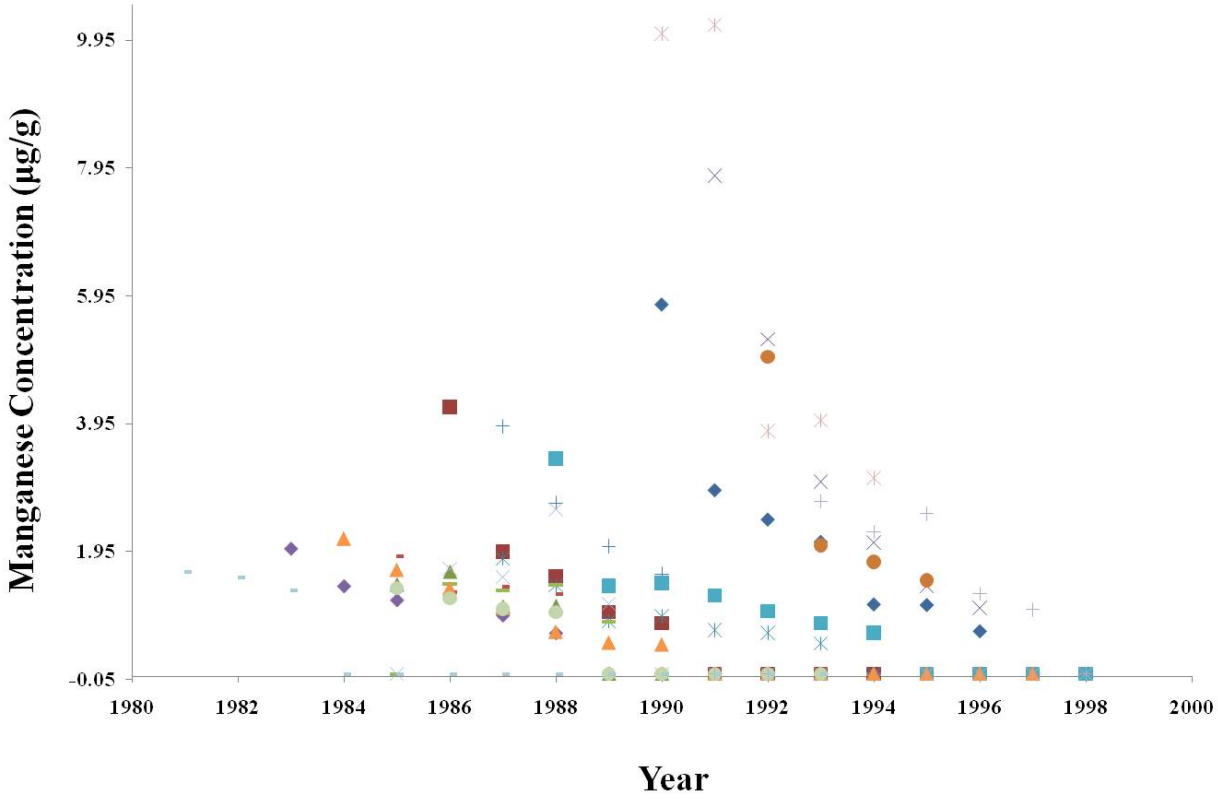


Figure 2.14 Peak concentrations of manganese in all the otoliths collected from Confederation Lake between 1980 and 1998. Each symbol represents an individual fish and their Mn concentration measured in the given year.

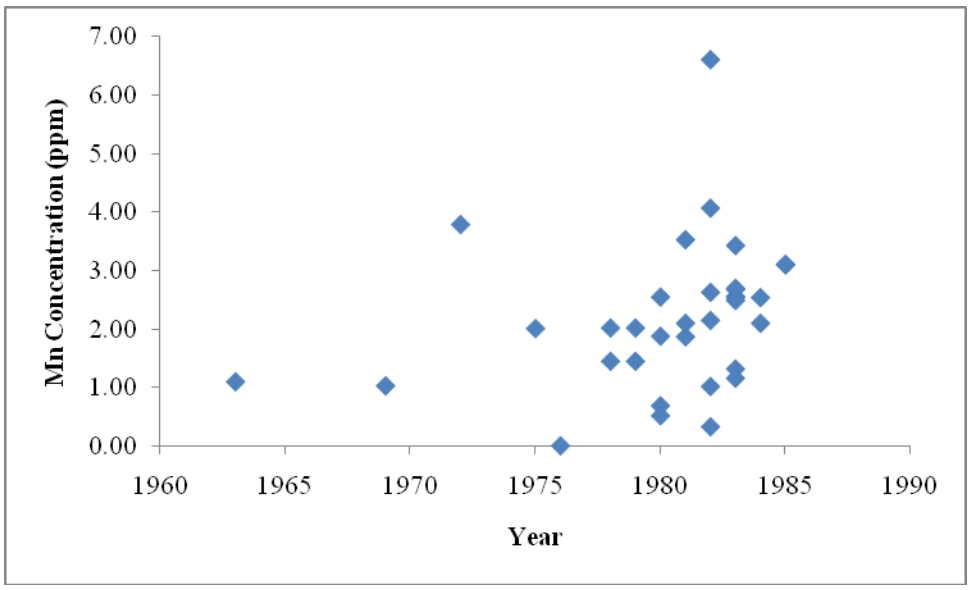


Figure 2.15 Peak manganese concentrations measured in the first year of lake trout otoliths collected from Pipestone Bay

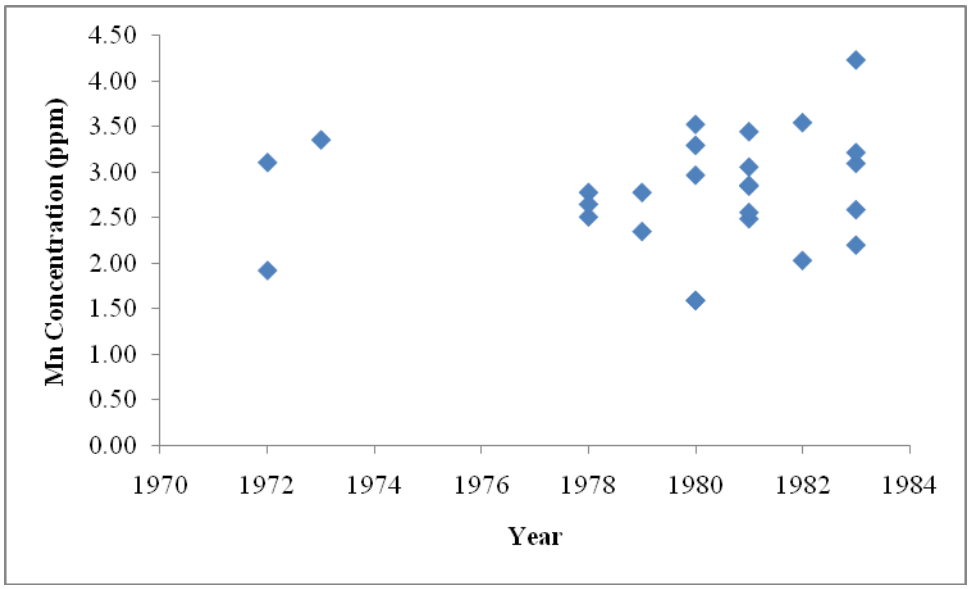


Figure 2.16 Peak manganese concentrations measured in the first year of lake trout otoliths collected from Trout Lake.

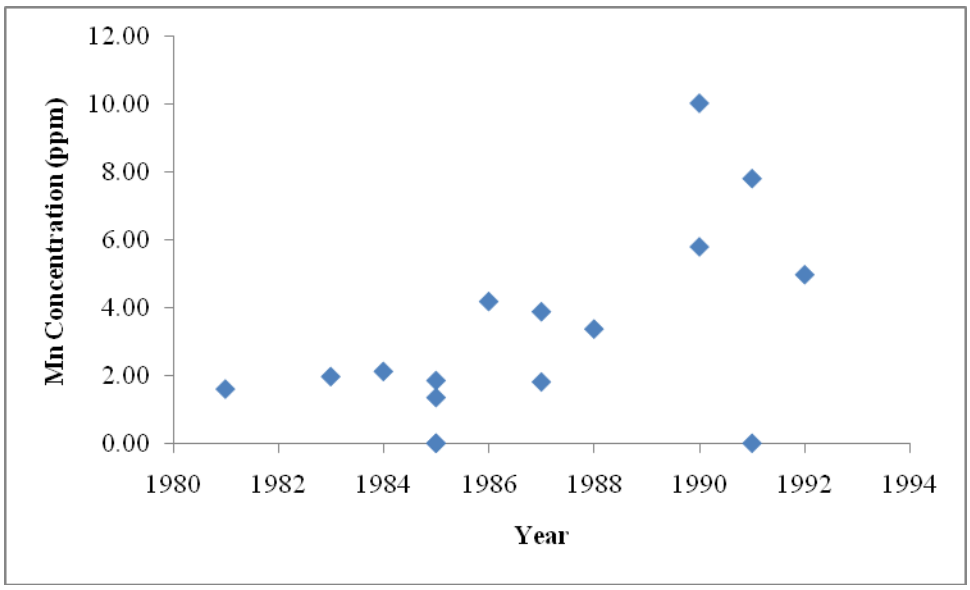
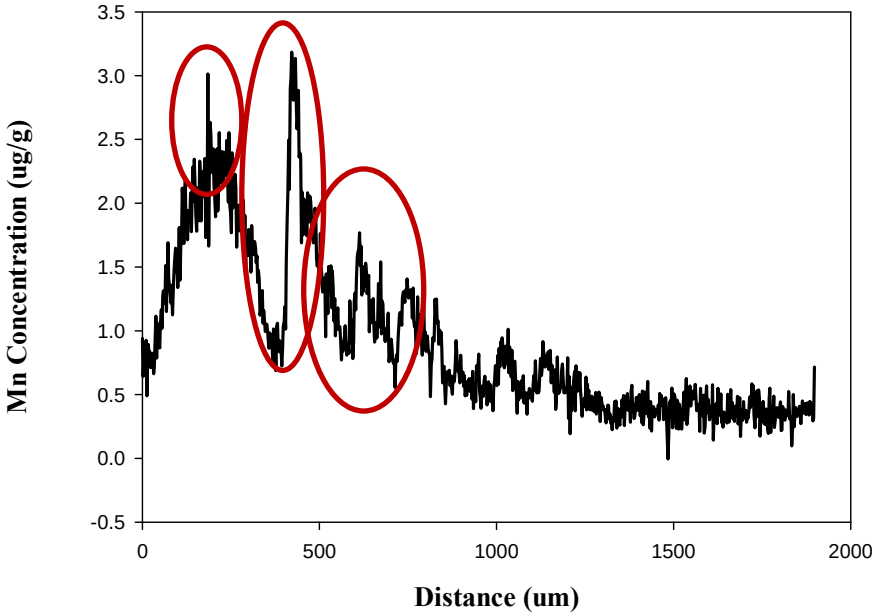
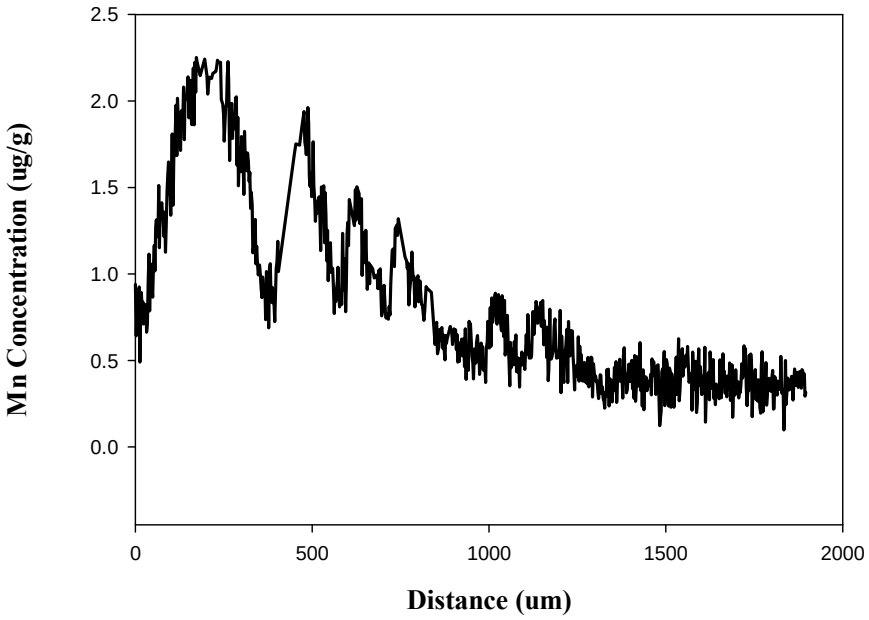


Figure 2.17 Peak manganese concentrations measured in the first year of lake trout otoliths collected from Confederation Lake.

A)





B)

Figure 2.18 (A) Mn profile of a lake trout otolith from Pipestone Bay

including isolated peaks that were thought to be a result of machine error. The red circles identify the single isolated peaks (B) Mn profile of a lake trout otolith from Pipestone Bay with single data points removed.

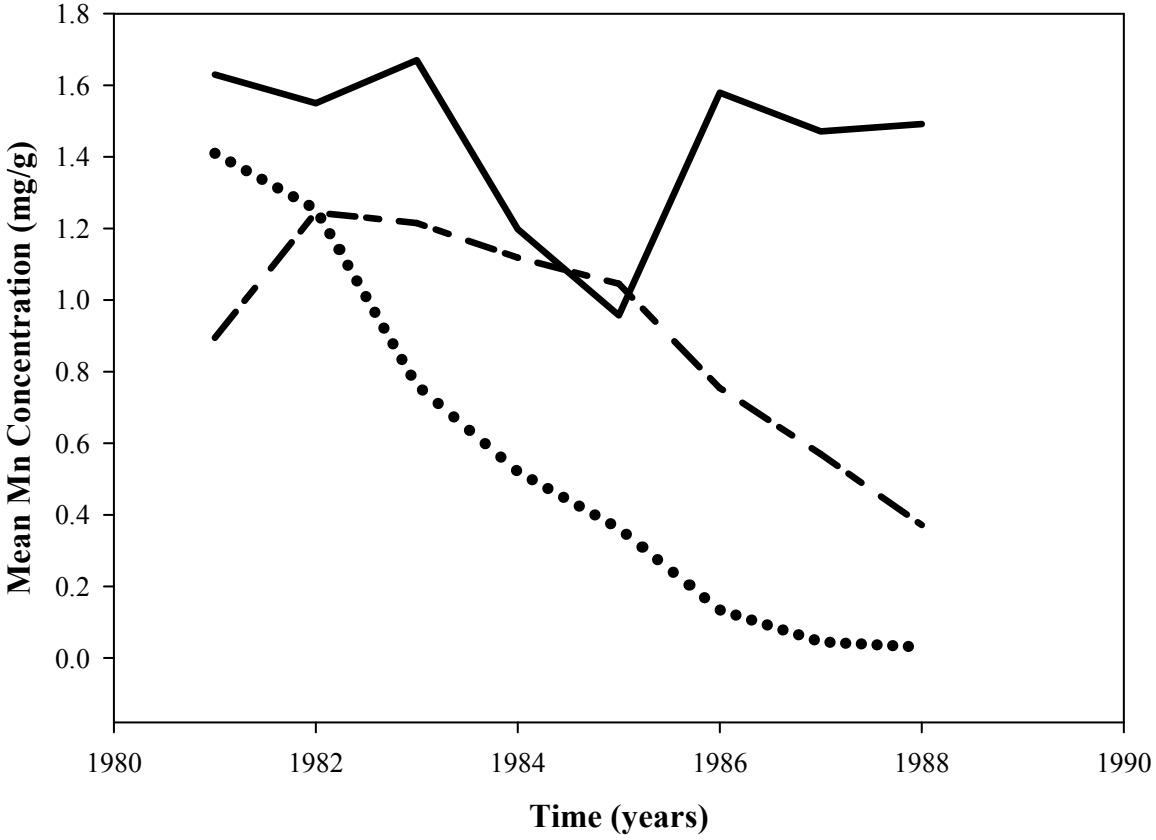


Figure 2.19 Mean Mn concentrations for otoliths collected from Red Lake (•••), Trout Lake (▬ ▪), and Confederation Lake (▬) between 1980 and 1988.

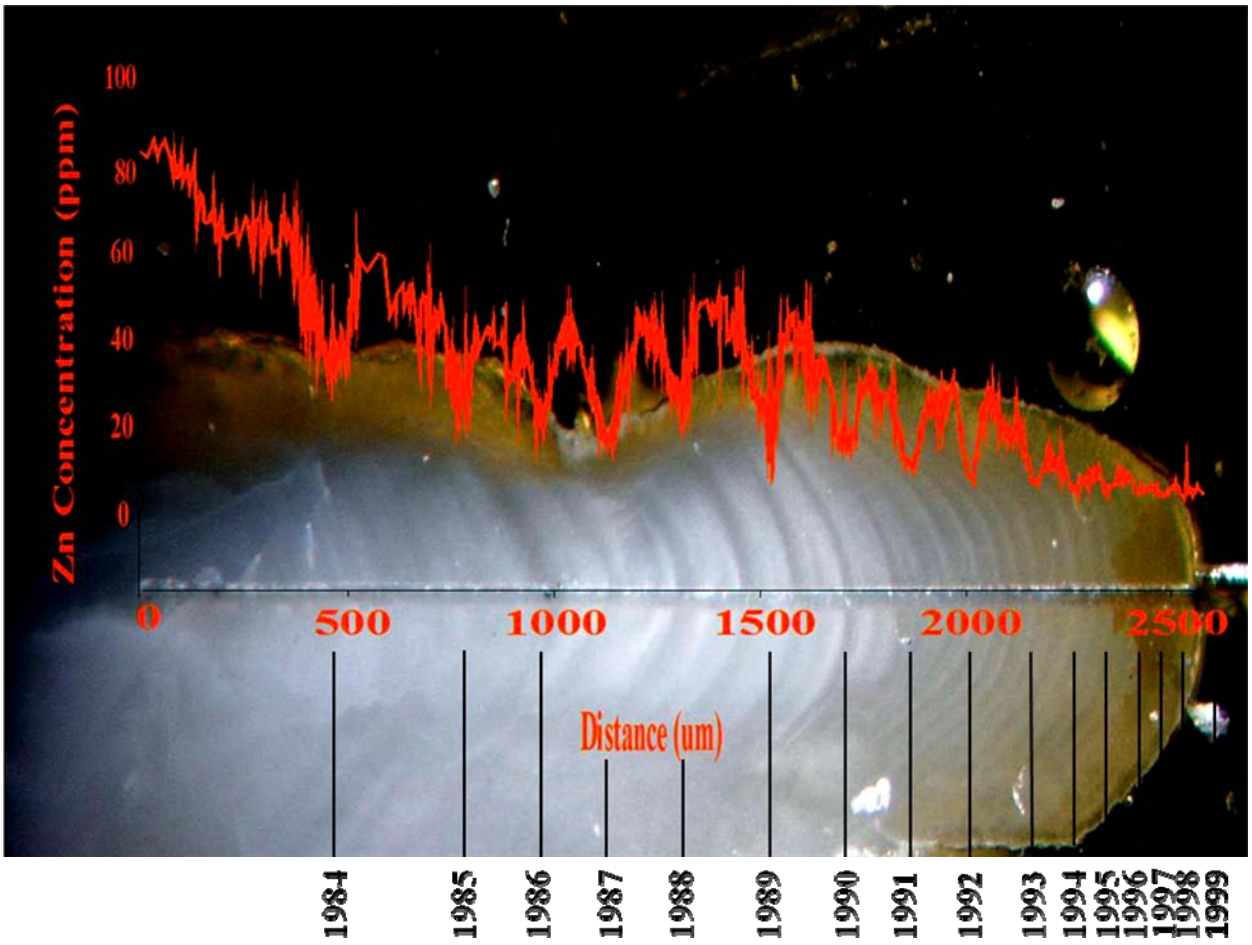


Figure 2.20 Overlay of LA-ICP-MS Zn profile on an image of a lake trout otolith from Pipestone Bay. Years correspond to the oscillatory pattern of the annual growth zones.

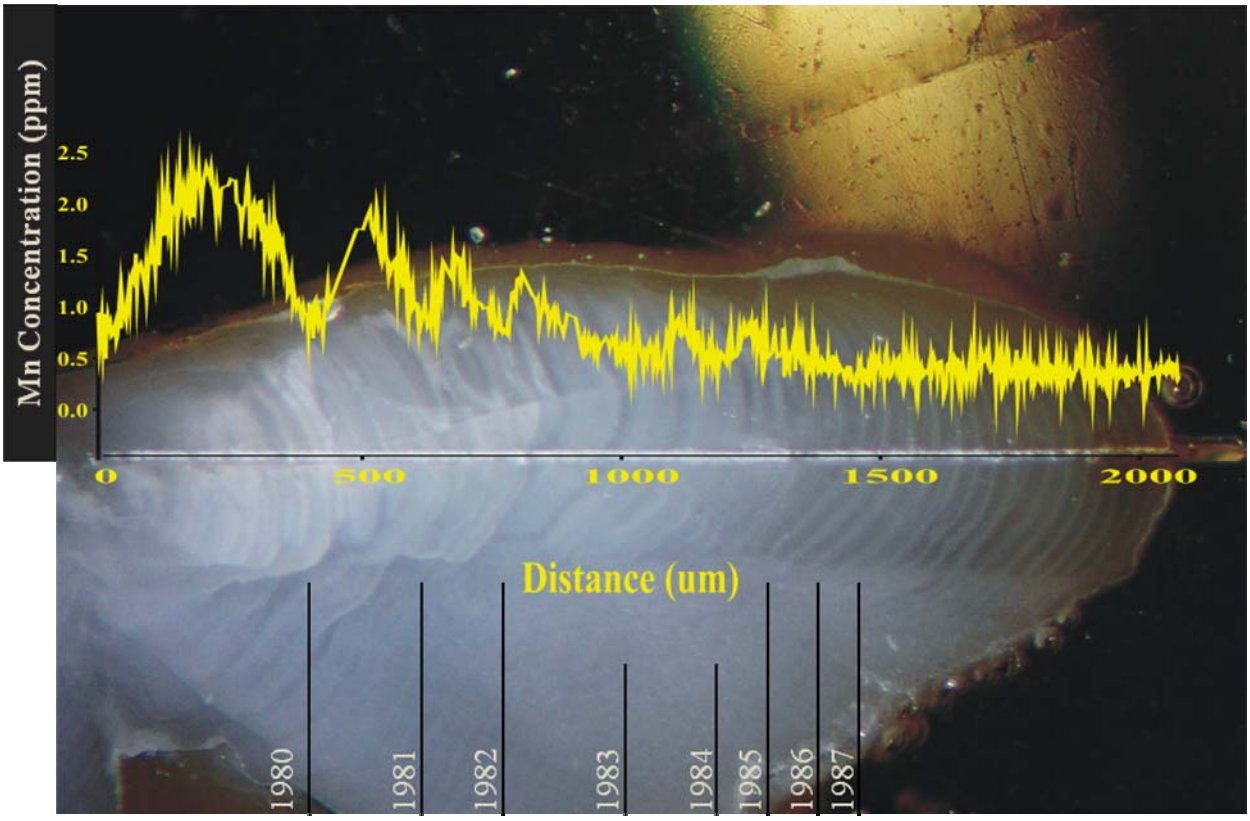


Figure 2.21 Overlay of LA-ICP-MS Zn profile on an image of a lake trout otolith from Pipestone Bay. Years correspond to the oscillatory pattern of the annual growth zones.

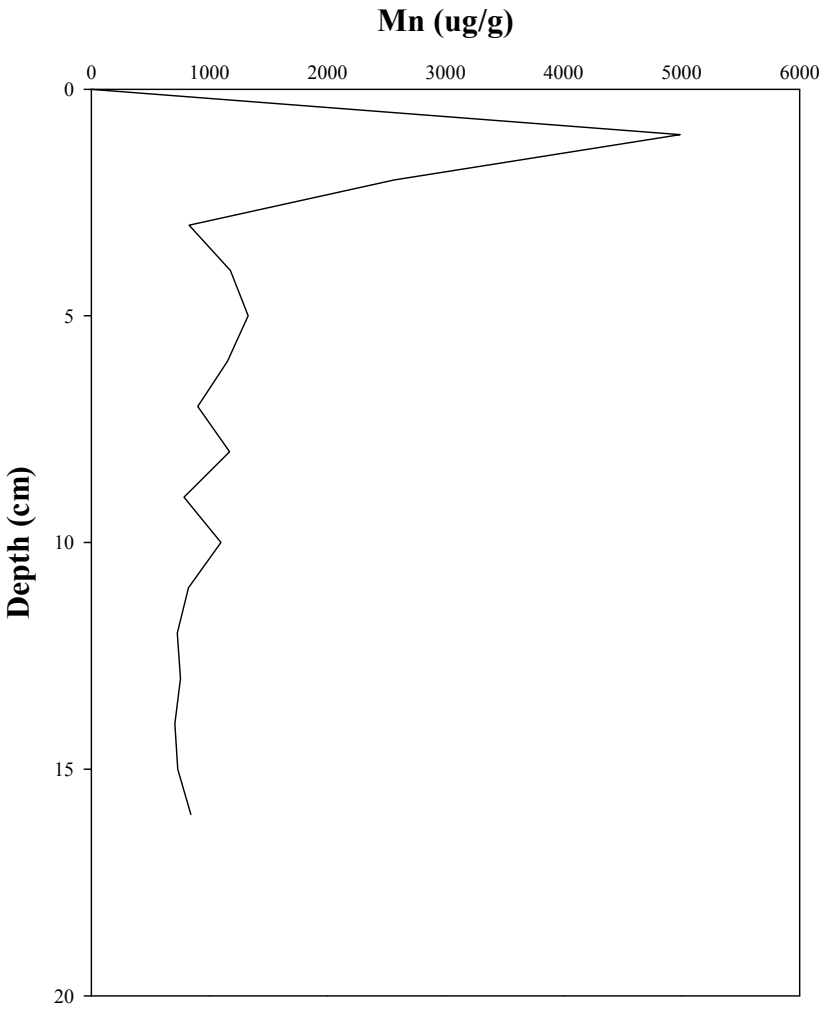


Figure 2.22 Profile of the Mn concentration in a sediment core collected from Pipestone Bay deep.

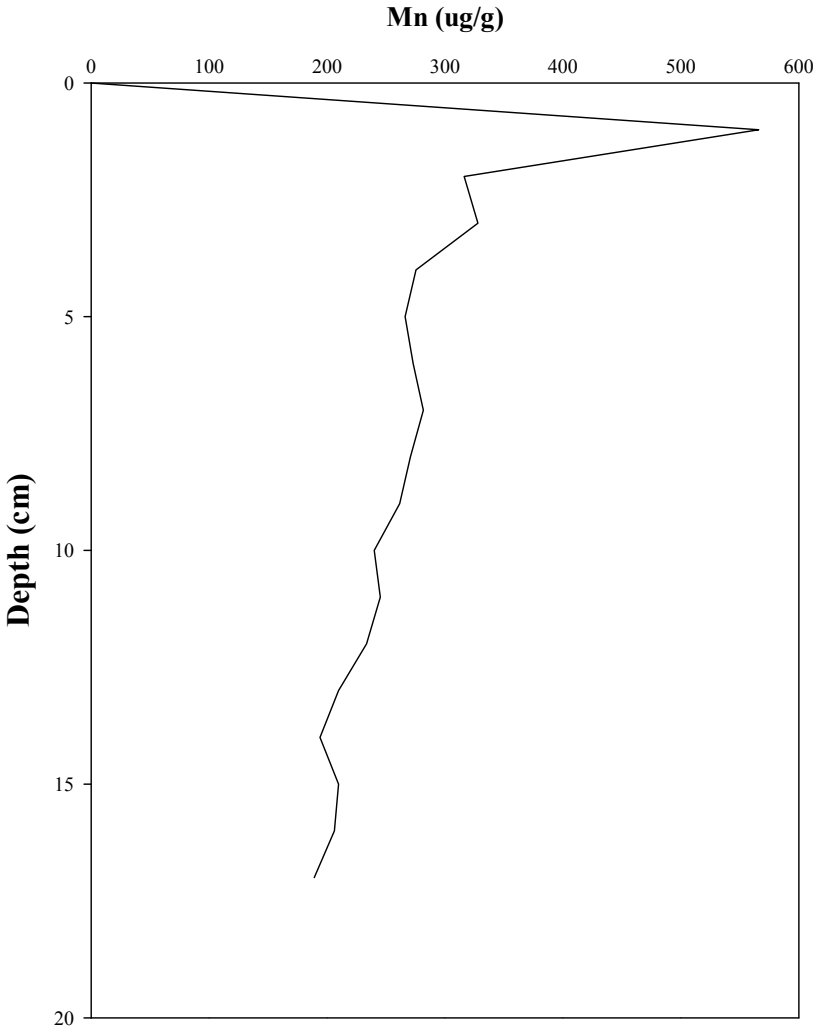


Figure 2.23 Profile of the Mn concentration in a sediment core collected from Shoal 2.

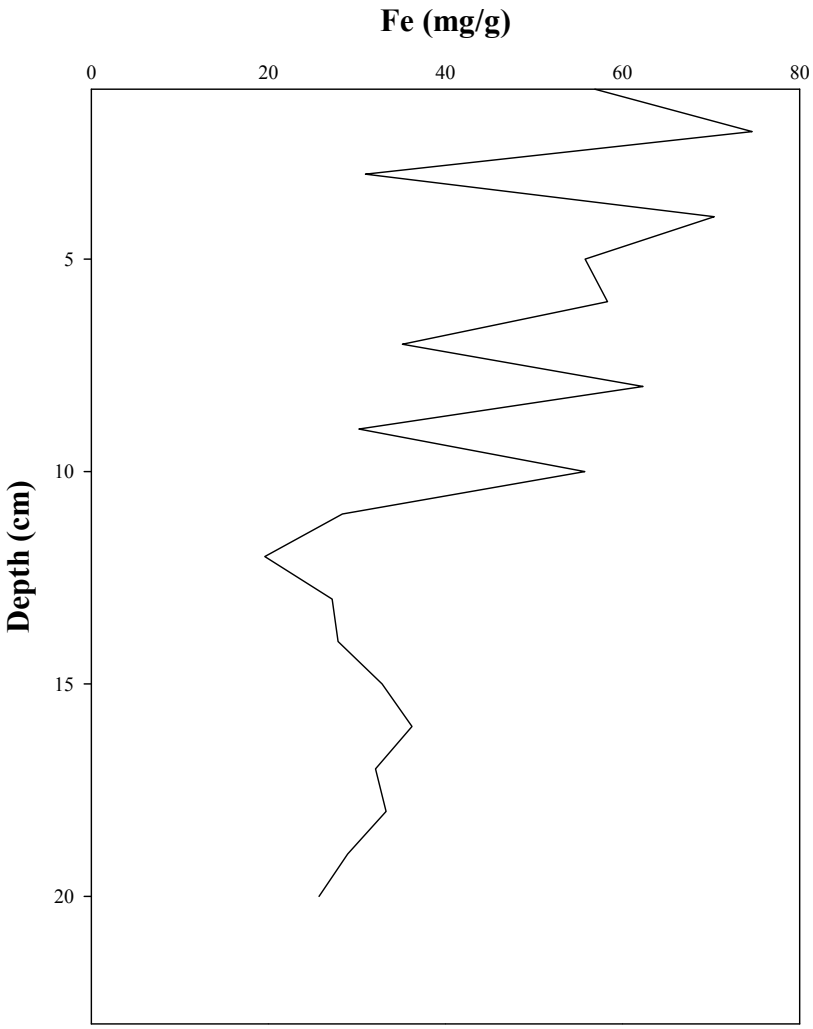


Figure 2.24 Profile of the Fe concentration in a sediment core collected from Pipestone Bay deep.

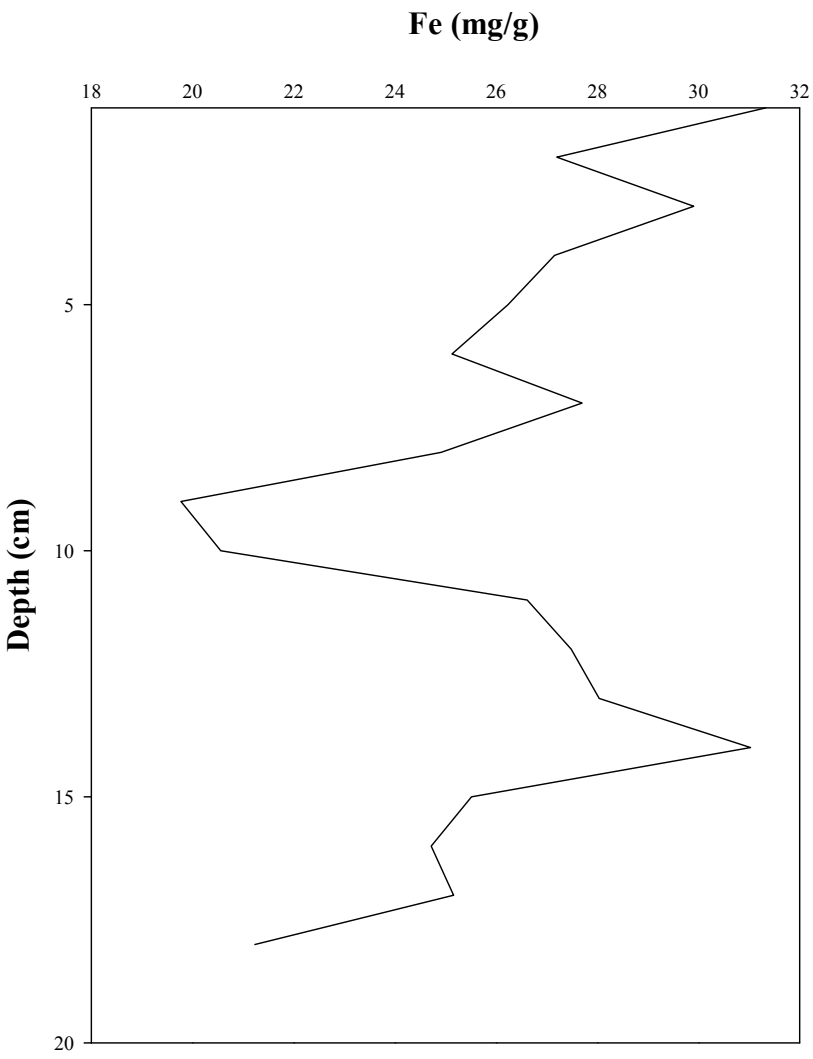


Figure 2.25 Profile of the Fe concentration in a sediment core collected from Shoal 2.

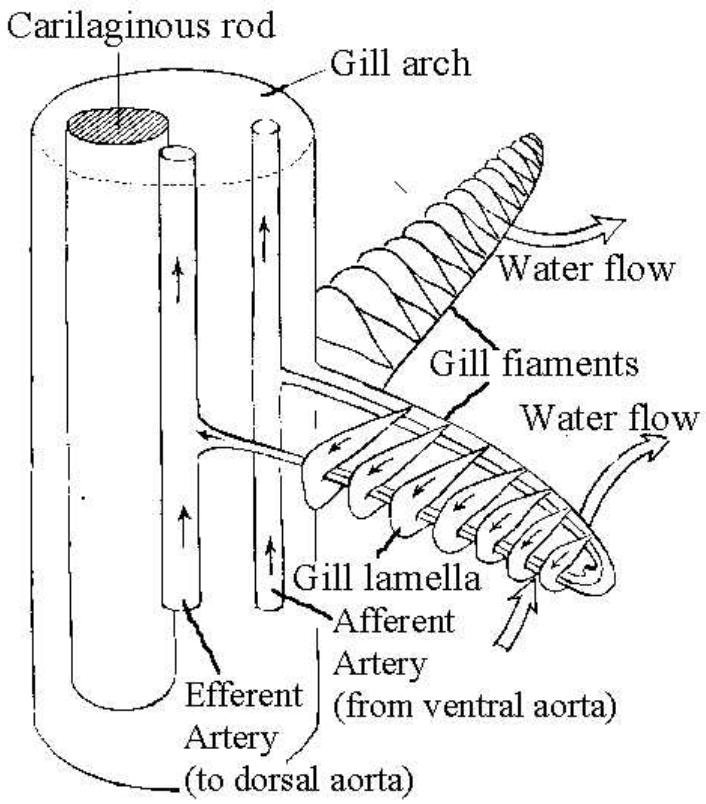


Figure 2.26 Diagram of the gill (from Wedemeyer, G.A., Meyer, F.P., and Smith, L. (1976). *Diseases of fish: Environmental stress and fish diseases*. TFH. Publications, Inc. Ltd, New York. With permission).

Table 2.1 Analytical operating conditions for otolith analysis.

ICP-MS	
Forward power	1238 W
Gas flows	
Plasmas (Ar)	15.50 L/min
Auxiliary (Ar)	1.0 L/min
Sample (Ar/He)	0.66 L/min
Shield electrode	Used for analysis
LA	
Repition rate	20Hz
Beam size	30 μm
Wavelength	213 nm
Energy on sample	8 J/cm^2
Incident pulse energy	
Pulse duration	5ns
Laser scan speed	2μm/sec
Pre-ablation warm-up	~60 sec
Data acquisition	
Data acquisition protocol	Time resolved analysis
Scanning mode	BScan and EScan
Detector mode	Analog and counting
Isotopes determined	^{43}Ca, ^{55} Mn, ^{60} Ni, ^{65} Cu, ^{66}Zn, ^{111}Cd, ^{208}Pb
Dwell time (segment duration)	0.1 sec
Magnet settling time	0.001-0.3 ms
Runs/Passes[a]	109*1

[a] Pass is a measurement cycle through the mass spectrum; 1 pass collected 1000 times producing 1000 blocks of data per sample

Table 2.2 Biological measurements for all the lake trout used for this study.

Lake	n	Mean Age (years)	Mean Body Weight (g)	Mean Total Length (cm)	Mean Condition Factor
Pipestone Bay	38	17	2646.4	70.8	0.87*
Trout Lake	28	9	2175.9	62.0	0.89
Confederation Lake	17	10	1703.1	57.3	0.90

*Mean condition was calculated for 16 fish since weights were not available for 2007 and 2008 fish.

Table 2.3 Mean Mn concentration in lake trout otoliths with SE for the three lakes.

Time (years)	**Confederation Lake**[a]	**Trout Lake**[b]	**Red Lake**[b]
1981	1.63 ± 0.00	1.41 ± 0.20	0.89 ± 0.23
1982	1.55 ± 0.00	1.25 ± 0.21	1.24 ± 0.33
1983	1.67 ± 0.33	0.76 ± 0.18	1.21 ± 0.20
1984	1.20 ± 0.62	0.52 ± 0.15	1.12 ± 0.18
1985	0.96 ± 0.28	0.36 ± 0.11	1.05 ± 0.17
1986	1.58 ± 0.37	0.13 ± 0.05	0.75 ± 0.13
1987	1.47 ± 0.29	0.05 ± 0.02	0.57 ± 0.10
1988	1.49 ± 0.28	0.03 ± 0.00	0.37 ± 0.07

[a,b] indicates significant differences

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Chapter 3:

Examination of Cu, Cd, Ni, Pb and Zn in otoliths of rainbow trout (*Oncorhynchus mykiss*) fed experimental diets

Abstract

A laboratory feeding experiment was conducted to determine how concentrations of five dietary trace metals correspond to deposition in the otoliths of juvenile rainbow trout (*Oncorhynchus mykiss)*. Fish were kept in holding tanks in freshwater for 364 days and were fed a diet enriched with Cu, Ni, Pb and Zn. The three experimental diets (low, medium and high dose) were fed at 8, 10 and 12 week intervals, and between each interval, fish were fed an unfortified diet for a minimum of 6 weeks. A control group, receiving food not enriched with metals was also included in the experiment. At the end of the feeding periods, otoliths were removed and processed by Laser Ablation Inductively Coupled Plasma Mass Spectrometery (LA-ICP-MS) for analysis of all 5 metals. Pb was below detection limits < 0.12 μg/g. Copper and nickel were present in the otolith of exposed and control fish, very low concentrations (mean concentrations for each experimental diet and control; Cu 0.17 μg/g, Ni 0.41 μg/g). There were no observed differences in Cu and Ni concentrations between the three experimental diets and control. This suggests that either Cu, Ni were not deposited in the otolith via the diet, (ii) concentration of these metals used in the experimental diet were not sufficiently high enough to be observed in the otolith, or not enough time elapsed for the metals to be deposited on the otolith. In contrast, Zn was observed at relatively high concentrations in all groups but again not significant between dose groups (reference 87.78 μg/g, low dose 108.78 μg/g, medium dose 92.06 μg/g, high dose 106.78 μg/g). This suggests that Zn is taken up to a greater extent from the diet compared to the other metals. One unexpected observation was a high proportion (70%) of "crystalline" otoliths. This could be due to stress associated to the hatchery conditions. Dietary zinc is most distinctly deposited in

otoliths, however it may be regulate, or differences in diet concentrations may not have been large enough to differentiate via the otolith concentrations. In regards to the other metals, their incorporations appear to be less distinct via the diet.

3.1 Introduction

To investigate metals present in the environment and their impacts on fish populations it is important to conduct field and laboratory studies. However, interpretation of field studies can be limited by the uncertainty of exposure. Field studies have little control over exposure to the contaminant of interest because test organisms are capable of migrating into and out of the study area (Palace et al., 2007). In addition, chronic impacts and the early onset of environmental deterioration are difficult to measure in a field setting (Sprague et al., 1984; NRCC, 1985). Recently, new developments in analytical techniques are making it easier to reconstruct exposure histories of fish collected from a field studies. In particular, laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS); a microbeam technique used to analyze metal incorporation in calcified structures (e.g. otoliths) has improved sensitivity and capability to analyze a larger suite of metals to reconstruct exposure histories (Halden and Friedrich, 2008). Currently there is insufficient data regarding the rates of incorporation of metals into different calcified structures, such as otoliths, and additional information is required. To better understand the incorporation rates of metals, results are better derived from laboratory studies where metal exposures can be closely regulated.

The microchemical composition of otoliths can be reflective of the past environments that fish have inhabited. The annular structure of otoliths acts as an "information storage structure" which records the life history including events such as environmental change or metal exposure (Friedrich and Halden, 2009). Archived otoliths can offer historical insight to the environmental conditions experienced over long periods of time. A growing amount of

research is being conducted using the analytical microbeam analysis of otoliths in order to better understand the elemental composition in otoliths and therefore, the chronological exposure to ambient elements. Strontium (Sr) has been used in many fishery studies investigating temperature and salinity, migration patterns, and population structure (Kalish, 1989; Hoff and Fuiman, 1995; Campana, 2000). One of the most popular analytical microbeam techniques is laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). It has the ability to detect a large suite of elements with detection limits in the low μg/g range.

Changes in the metal concentrations in either food or water could influence the amount of metals available for uptake by the otolith. Only a few studies have been carried out to investigate the relationship between metal concentrations in otoliths and metals present in the diet or water (Hoff and Fuiman, 1995; Limburg, 1995; Milton and Chenery, 2001; Elsdon and Gillanders, 2003). For example, recent laboratory studies have shown that elevated concentrations of Zn in the otoliths of pink snapper were the result of dietary exposure (Ranaldi and Gagnon, 2008, 2009). In addition, laboratory studies have shown that Sr: Ca and Ba: Ca concentration ratios in the otoliths of Black Bream (*Acanthopagrus butcheri*) were associated to concentrations present in the surrounding aquatic environment (Elsdon and Gillanders, 2003). However these studies have not definitively identified diet or water as the confounding factors to metal concentrations in otoliths.

The primary objective of the studies detailed in this chapter was to investigate the relationship between metal concentrations in the diet. Specifically, how the concentrations of copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were augmented in the diet to examine

how they would influence concentrations deposited in the otolith of juvenile rainbow trout (*Oncorhynchus mykiss)*.

3.2 Material and Methods

3.2.1) Experimental diets

Four experimental diets containing either low, med, or high concentrations of metals, as well as a reference diet not supplemented with metals (Table 3.1), were prepared. The levels of metals in the augmented diets were determined based on a previous study (Klaverkamp et al. 2002). The low treatment group diet was enriched with metals that were reflective of slightly above background levels in the environment (Zn = 22.24µg/g, Cu = 2.82µg/g, Ni = 0.06µg/g, Pb = 0µg/g); the "medium" treatment group diet was enriched with metals reflective of moderately contaminated mining sites (Zn = 15µg/g, Cu = 3.5µg/g, Ni = 0.25µg/g, Pb = 5 µg/g; and the "high" treatment group diet was enriched with metals that were reflective of highly contaminated mining sites (Zn = 29.5µg/g, Cu = 7.62µg/g, Ni = 0.565µg/g, Pb = 0.10µg/g) (Klaverkamp et al. 2002).

Diets were prepared by homogenizing two parts of crumbled trout feed (Martin Mills Inc., Silver Cup Crumble; comprised of 52% crude protein, 14% crude fat, 3% crude fibre, and 12% ash) with one part deionized distilled water (DDW) containing Xg per litre of 60 bloom gelatine (Sigma Chemical Co. St Louis, MO) in a Hobart Food Grade mixer. After homogenizing (>20 minutes) the food was allowed to air dry for 1 hr until a thick paste

consistency was achieved. The food was then extruded into noodles, air dried in the dark at 25 °C for 48 hrs, crushed into powder and stored frozen at - 20 °C. Daily rations were removed from the freezer, weighed and thawed for 20 minutes prior to feeding. Initial experimental diets were analyzed for metal content.

3.2.2) Fish

On July 11, 2007 approximately two hundred juvenile rainbow trout (*Oncorhynchus mykiss)* were received from Stevenson's Rainbow Trout Hatchery, Thamesford, ON. For seven weeks prior to the feeding experiment, fish were fed the reference diet (1.4 g/day) of Martin Mills Inc. Silver cup crumble and acclimated to experimental conditions.

3.2.3) Tanks

Fish were randomly distributed (40 fish in the low, 25 fish in med, 29 fish in high, and 40 fish in the reference tank) among the four fibreglass tanks (200 L). Each tank received dechlorinated Winnipeg City tap water at a flow rate of 1L/min. Water is filtered through activated carbon filters, ozonated, UV sterilized, sock filtered, then proceeds through multimedia beds, gas balancers and finally aerated. Chlorine content was monitored weekly to maintain Cl_2 at or below 10 ppb (free chlorine). Throughout the experiment dissolved oxygen (DO) was maintained between 80-95% saturation, water temperature ranged between 12.7°C and 17.1°C, pH was maintained between 7.11 and 8.24, and conductivity was maintained between 150 – 165 uS/cm. The photoperiod was static at 12 hours on 12 hours off

3.2.4) Exposure Procedures

Commencing on August 29, 2007, rainbow trout were fed the experimental diets six days a week for 364 days at 8, 10 and 12 week intervals (Figure 3.1). Fish were fed at the different intervals to observe depositional differences in the otoliths. Between each interval fish were fed the reference diet for a minimum of 6 weeks (Figure 3.1). A reference group, receiving food not enriched with metals was also included in the experiment. Twenty fish from each treatment group were weighed on days 06, 39, 56, 78, 149, 234, 293 to adjust the feed ration to a rate of 2 % body weight per day.

3.2.5) Sampling Procedures

On August 28, 2008 each experimental group was anaesthetized with buffered solution of tricaine methane sulfonate (MS-222; SIGMA Chemical Co., St. Louis MO) at a concentration of 0.6 g MS-222/ 4L until fin movement ceased. For each fish, the total body weight (g) and fork length (cm) were measured and recorded. Livers, otoliths, scales, pectoral fins, eyes and muscle were dissected from the carcass. The livers and otoliths were analyzed for trace metals (Cu, Ni, Pb, and Zn) to determine a relationship between metal concentrations in the otoliths the livers. Scales, pectoral fins, eye and muscle were stored for future studies. Livers, eyes and muscle were stored at - 90 °C, while scales, pectoral fins and otoliths were stored at room temperature (20 °C) until time of analysis.

3.2.6) Tissue analysis for trace metals

A subsample (n = 39), of the livers collected from the feeding experiment (10 from the low, medium and high groups, and 9 from the control group) were sent to CANTEST Professional Analytical Services, Winnipeg MB for trace metal analysis following their methodologies described below.

Samples were weighed into 50 mL plastic digestion tubes. Weights were recorded to 0.0001g. For each batch, 1 duplicate for every 10 samples was included in addition of 2 matrix blank samples and 1 sample including certified reference material NIST 1577b. For each sample, 7.5 mL of concentrated OmniTrace nitric acid was added and samples were left overnight. The following day, samples were placed in a hot block at a temperature between 102 -108 °C until 2-3 mL of the sample remained (approximately 2 hours). Afterward, samples were cooled to room temperature, 3 mL of hydrogen peroxide was added and samples were left for 2 hours at room temperature. An additional 7.5 mL of concentrated OmniTrace nitric acid was added to the samples and left overnight. Finally, samples were diluted with Ultrapure water to a bulk volume and analyzed by inductively coupled mass spectrometry (ICP-MS).

3.2.7) Otolith Preparation

As described for Chapter 2 Material and Methods, Section 2.2.3.

3.2.8) Laser Ablation ICP-MS

As described for Chapter 2 Material and Methods, Section 2.2.4.

3.2.9) Cathodoluminescence (CL)

Cathodoluminescence microscopy is a non-destructive procedure that is able to detect any structural defects and/or elemental substitutes within the crystal lattice structure of otoliths (Marshal, 1988). CL has the ability to detect trace metals in otoliths in extremely low levels (ppb range) (Halden *et al.*, 2004; Marshall, 1988). For the purpose of this study CL was conducted to asses the chemical variation in the otoliths after exposure to the experimental diets. Five rainbow trout otoliths from the high treatment group were analyzed using cathodoluminescence (CL) microscopy as described in Halden et al. (2004).

3.5 Results

3.5.1) Morphological indices

The consumption of the metal enriched diets for 364-days did not significantly (ANOVA, $P = 0.480$) alter weight, length or condition factor (K) of the rainbow trout (Table 3.2) relative to the fish fed the reference diet.

3.5.2) Metal accumulation in otoliths

Analyses of the otoliths from juvenile rainbow trout by LA-ICP-MS indicated that concentrations of Cu, Ni and Pb in otoliths from all four experimental groups were below or near the detection limit (DL) (Table 3.3). Therefore, no significant differences in the

concentration of any of the metals were evident between the treatments or within regions of the otoliths from fish fed pulses of the metal laden diets. In contrast, Zn concentrations were detectable in all four experimental groups (Table 3.4). Zn concentrations were much higher in treatment groups compared to other metals. There were observed differences in the Zn concentrations within regions of the otolith, however, there was no oscillatory pattern in the Zn concentrations that could not be linked to the pulses of the metal laden diet. Zinc typically exhibits an oscillatory pattern of peaks and troughs which correspond to high and low concentrations. These peaks and troughs, along with age estimations can be used to link high and low Zn exposure in the fish. Statistical analysis showed no significant differences in Zn concentrations between treatment groups.

3.5.3) Metal accumulation in tissue

Liver tissues were analyzed to assess metal uptake in another tissue from the rainbow trout and to compare to metal concentrations determined in the otoliths. The comparisons were conducted to determine patterns in the metal accumulation in otoliths and tissues on an individual fish level. Exposure to the metal enriched diets resulted in concentrations of Ni, and Pb near the detection level in rainbow trout from all four treatment groups (Table 3.5). Cu concentrations were the highest among all six metals analyzed (Table 3.5).

3.5.4) Microchemistry of aragonite and vaterite

One unexpected observation from the experiment was a high proportion of what can be characterized as “crystalline” otoliths (70%). An otolith is considered crystalline when the

aragonite form of $CaCO_3$ is partially or completely replaced by vaterite, a polymorph of $CaCO_3$. A vateritic otolith has a different crystalline structure, abnormal shape and is visibly translucent. To distinguish whether an otolith is crystalline (vaterite polymorph) versus non-crystalline (aragonite polymorph), strontium (Sr) was included in the suite of isotopes analyzed on the LA-ICP-MS. Sr concentrations are in vateritic otoliths (Figure 3.2) compared to aragonite otoliths (Figure 3.3). Vaterite possess a different crystal structures and spacing compared to aragonite otoliths, which can lead to different trace metal composition (Tomás and Geffen, 2003). The change in the crystal structure alters the molecular arrangement and limits the space for any inclusion of other trace metals. Since Sr has a larger ionic radius compared to Ca, Sr concentrations were lower in vaterite otoliths.

There was a higher proportion of crystalline otoliths compared to non-crystalline otoliths, from this study but there were no significant differences in the proportion of crystalline otoliths among all four treatment groups ($P = 0.0886$). However, there were statistically significant differences ($p < 0.001$) among the Sr and Zn concentrations present in the aragonite and vaterite.

3.5.5) Cathodoluminescence microscopy

All five rainbow trout otoliths exhibited two patterns of luminescence. Otoliths showed concentric zones of luminescence, corresponding to the annular structure and asymmetrical patches of luminescence. An example of the luminescence observed in one of the five otoliths is shown in Figure 3.4. Otoliths typically emit a yellow-green or alternating green with dark blue colours in CL images. No otoliths from this study observed this colour

variation. Each polymorph of calcium carbonate emits different luminescence. The emitted colour from true aragonite otoliths have been described to have a green and yellowish-green colour (Marshall, 1988; Sommer, 1972), calcite has been described with an orange-red, yellow-orange, and orange (Smith and Stenstrom, 1965; Martin and Zeepers, 1969; Sippel and Glover, 1965), and since vaterite has a similar CL spectrum as calcite it emits a similar luminescence (Sommer, 1972). The colours observed in all five rainbow trout otoliths were a red-orange and purplish-blue.

3.6 Discussion

3.6.1) Metal accumulation in otoliths

The aim of this study was to determine if the concentrations of Cu, Ni, Pb and Zn in a fish's diet correlated with the deposition of those elements in the otoliths of juvenile rainbow trout (*Oncorhynchus mykiss)*. However, no differences were detected in the metal concentrations in otoliths among fish from the experimental diet treatments and the reference group. Cadmium, Ni, and Pb were below detection limits. Analysis of the liver tissue (Table 3.5) showed Ni, and Pb were below detection limits in fish fed the metal enriched diet. Copper and Zn concentrations were higher compared to the other metals. Previous research has shown conflicting results for the relationship between diet and metal uptake in otoliths. Ranaldi and Gagnon (2008) have shown diet as a primary source of Zn in pink snapper while Milton and Chenery (2001) observed no influence of metal uptake in barramundi as result of diet exposure.

Literature on the influence of metal concentration from a dietary source, and otolith composition is limited. Milton and Chenery (2001) found that the when barramundi (*Lates calcarifer*) were exposed to Sr, Pb, Cu, Li, and Ba via the diet and water exposure, that Sr, Pb and Cu showed significant increases in otoliths of fish exposed to these metals via water exposure while no increases in the metal concentrations in otoliths from fish fed the enriched diets were observed. Results supported the argument that water exposure is a major source for metal uptake (Farrell and Campana, 1996) while diet contributes very little similarly to what has been observed in the wild (Hoff and Fuiman, 1995).

Trace metals from the aquatic environment or diet are transported, first into the fish through the gills (freshwater) or intestine (marine), then to the blood plasma, into the endolymph, and finally into the crystallizing structure. Along this pathway, there are certain barriers where ion concentrations decline, resulting in decreased concentrations available for uptake into the otolith. Barriers occur between the gill-water and intestine - water interface, blood - endolymph, and the endolymph- crystallizing otolith interface (Campana, 1999). The gill - water and intestine - water are the two major barriers where metal ions are lessened (Campana, 1999; Olsson et al., 1998). In Milton and Chenery (2001) decreased concentrations of Cu and Pb in the otoliths were attributed to the blood - endolymph barrier. It was suggested that Cu and Pb were reduced so much that only small concentrations reached the blood, and transported onward to the endolymph (Wright et al., 1992). In this study, it is thought that the metal concentrations in the experimental diets may not have been sufficient to elevate otolith concentrations.

Another study by Hoff and Fuiman (1995) examined the relationship between diet, and the elemental composition of Mg, Sr, Na, and K in the otoliths of red drum (*Sciaenops ocellatus*). There were no significant differences in the otolith chemistry between fish receiving the dietary supplements and the reference fish which suggested that diet was not an important determinant or metal contributor to otolith metals. In contrast, temperature had an influence on metal incorporation in otoliths. The concentrations of Ca, Sr and K in the otolith were all correlated with five constant temperatures. A linear relationship was observed between 27 and 34 °C with little change in the otolith composition below this temperature range. The lower temperatures (21 and 23°C) exhibited no changes in the trace metal composition of the otoliths suggesting that physiological mechanisms could have influenced the incorporation of elements in the otoliths. The temperatures in the present study ranged between 12.7°C and 17.1°C, which were similar to temperatures rainbow trout experience in the natural environment. During the summer rainbow trout generally inhabit waters around 12°C. Since temperatures were within the ideal range for rainbow trout, results suggest that temperature was not a factor in the influencing the elevation of metal composition in the otoliths.

In contrast to the previous studies, others have provided evidence that diet does influence the otolith composition for certain elements. Farrell and Campana (1996) examined the contribution of Cu, Sr and Pb through the diet in order to better understand whether the otolith microchemistry of Nile tilapia (*Oreochromis niloticus)* reflected the elemental concentrations in the surrounding aquatic environment. Diet had a small influence (20%) on the Sr and Ca concentrations in the otolith. Similar to the results from this study, Farrell and

Campana (1996) suggested that the concentrations of Cu, Sr and Pb used in their experimental diet were not sufficiently high to contribute to the otolith composition.

In contrast to the Cu, Ni, and Pb, Zn occurred in relatively high concentrations in the otoliths compared to the other metals (Table 3.3, 3.4). Liver also had higher Zn concentrations in the tissues of fish exposed to elevated concentrations compared to the reference diet fed fish. Even though Zn concentrations were high in the otoliths there were no differences in concentrations among experimental groups (Table 3.5). The difference between Zn concentrations and the concentrations of the remaining metals suggests that Zn was more available for uptake from the diet. In contrast to our study, Ranaldi and Gagnon (2008) observed significant differences in the Zn concentrations in otoliths of pink snapper that were fed Zn enriched diets. Waterborne Zn exposure did not significantly alter otolith Zn. They concluded that diet played an important role in the incorporation of Zn into the otoliths of *P. auratus* compared to water. The lack of disparity of Zn among the experimental groups from this study could be linked to the Zn concentrations used in the experimental diets. It may have been that the Zn concentrations used in the experimental diet were not sufficiently high enough to observe differences among the experimental groups. The metal enriched diets contained Zn concentrations of 22.24mg/kg (low dose), 15mg/kg (med dose), and 29.5mg/kg (high dose) to replicate varying levels in the natural environment from background to highly contaminated concentrations. These concentrations were much lower compared to concentrations (6000-9000 mg kg, or approximately 8-17 fold higher than our exposures) used by Ranaldi and Gagnon (2008). The pathway of Zn from environment to otolith is thought to be different for each species of fish. Moreover, many of those the pathways are not clearly understood. However, diet does seem to be the primary source for Zn

incorporation into the otolith (Halden and Friedrich, 2008). Ranaldi and Gagnon (2008) suggest that Zn uptake in the otolith was through the increased Zn absorption in the intestine. The intestine - water interface is the major barrier in the elemental pathway, and it is the most important route for Zn uptake in marine fishes (Campana, 1999; Powell et al., 1999). In freshwater systems Zn uptake seems to be greatly influenced by water chemistry (i.e. pH and salinity). Gill - water interface is the most important barrier in the elemental pathway in freshwater fish since it controls the ion movement into the fish (Campana, 1999).

Studies examining how freshwater species absorb Zn from dietary sources and how it is incorporated in otoliths are limited. No other studies have been conducted on Zn accumulation in otoliths of freshwater species.

3.6.2) Microchemistry of aragonite and vaterite

There are three polymorphs of calcium carbonate in the otoliths of teleost fish; aragonite, vaterite, and calcite. Aragonite is the most common polymorph. However, in certain circumstances the aragonite can be replaced by vaterite, creating what is referred to as a crystalline otolith (Carlstrom, 1963). Crystalline otoliths are considered abnormal since they are transparent, have an irregular shape, and the surface is typically uneven. These features are opposite to the aragonite otolith (Carlstrom, 1963; Bowen et al., 1999). Many studies have attempted to identify the cause of vaterite crystallization in otoliths (Gauldie, 1986, 1996; Gauldie et al., 1997; Bowen II et al., 1999; Tomas and Geffen et al., 2003; Sweeting et al., 2004; Ma et al., 2008). It has been suggested that crystalline otoliths occur more frequently in salmonids species (Bowen II et al., 1999), and are more common in hatchery reared fish

compared to wild fish (Sweeting et al. 2004; Bowen et al. 1999; David et al. 1994; Gauldie 1986). Sweeting et al., 2004 compared the occurrence of crystalline otoliths in hatchery reared and wild juvenile coho salmon (*Oncorhynchus kitsutch*). Results for a 100 pairs of otoliths showed a higher percentage of crystalline otoliths in hatchery- reared (20-36%) coho salmon compared to wild (2-10%) coho salmon. Bowen II et al. (1999) examined the occurrence of crystalline otoliths between stocked and wild lake trout (*Salvelinus namaycush*). The study found that 77% of the stocked lake trout had crystalline otoliths. Bowen et al. (1999) suggested that the changes in the otolith structure occurred mostly in the early life stages, and further examined otoliths from wild and stocked lake trout fingerlings (3, 4, 5 month old). Results found vateritic otoliths in both wild and stocked fish. However, greater numbers of crystalline otoliths were observed in the stocked lake trout, suggesting that prior to stocking the hatchery conditions could have lead to changes in the calcium carbonate structure. Gauldie et al. (1986) investigated the occurrence of crystalline otoliths in hatchery reared Chinook salmon (*Oncorhynchus tshawytscha*), and found that 34 % of the otoliths were crystalline.

The rainbow trout used in the present study were reared in hatchery conditions prior to the experiment. There were 60 rainbow trout otoliths analyzed for this study, 70% were crystalline. The hatchery environment prior the study or perhaps the practices to maintain a stable hatchery conditions throughout the study could have been responsible for the vaterite crystallization in the rainbow trout. It has been suggested that holding and rearing density stress, weighing often, and temperature stress associated with hatchery practices could influence the occurrence of crystalline otoliths in hatchery reared fish.

In addition, results also support the statement that salmonids typically have higher proportion of crystalline otoliths. Salmonid species were used in all the previous studies (Gauldie, 1986; Bowen II et al., 1999; Sweeting et al., 2004). Strong et al. (1986) investigated the occurrence of crystalline otoliths in the Scotian Shelf pollock (*Elachius viren.).* Pollock otoliths were completely comprised of the calcite polymorph of calcium carbonate rather than vaterite or aragonite polymorph. Blacker et al. (1974) investigated the occurrence of crystalline otoliths in Atlantic cod (*Gadus morhua)* and haddock (*Melanogrammus aeglefinus*). Only 1% of the Atlantic cod otoliths were vaterite, and 5% of haddock otoliths were vaterite. These studies clearly support that statement that vateritic otoliths occurs in much higher proportions in salmonid species compared to other fish species.

In addition to hatchery practices, the genetics and endolymph composition (chemical or protein) have been suggested as potential sources for vateritic otoliths (Gauldie, 1993; Campana, 1999; Payan et al., 1999; Sweeting et al., 2004). Malfunction of a gene could potentially influence the formation of aragonite versus a vaterite otolith (Gauldie, 1986). Söllner et al. (2003) found a gene, referred to as the starmaker gene that was thought to be associated to be otolith formation in zebrafish (*Danio rerio).* Söllner et al. (2003) observed that when the starmaker gene activity was limited, the otolith morphology and crystal lattice structure was altered. Different expression levels of the starmaker gene could influence the morphology of otoliths. Unfortunately we did not examine gene expression in this study. However further studies are important to gain a better understanding of the association between gene expression and crystalline otoliths.

There are three are pairs of otoliths found in teleost fish, sagitta, lapillus and astericus (Carlstrom, 1963). Sagitta and lapillus otoliths typically exist in the aragonite form while the

astericus otoliths can occur in many fish in the vaterite form suggesting that the composition of the endolymph must change (Carlstrom, 1963; Lowenstein and Weiner, 1989; Oliviera et al., 1996; Sweeting et al., 2004). The calcification process relies highly on the composition of the endolymph (Ranaldi and Gagnon, 2009). Studies have examined the relationship between the endolymph composition and otolith crystallisation, and have found that when changes occur within the endolymph it promotes the vaterite or calcite to form rather than aragonite. These studies suggest that the formation of vateritic otoliths could be associated to the proteins and solubility of the endolymphatic fluid surrounding the otolith (Falini et al. 1996; Payan et al., 1997; Campana, 1999; Tomás et al. 2004). Payan et al. (2004) carried out a study that examined the effects of environmental stress on endolymph composition, blood plasma, and otolith growth in rainbow trout (*Oncorhynchus mykiss*). Within the endolymph the protein concentration increased, ion composition decreased, as otolith growth decreased. The study concluded that stress promoted changes in the protein concentrations and ionic compositions in the endolymph. This is important since the composition of the endolymph has been shown to be essential in otolith composition (Campana, 1999). There are many factors that could be the lead to the formation of vateritic otoliths.

The elemental composition of vateritic otoliths varies greatly compared to aragonite otoliths (Tomás and Geffen 2003; Melancon et al. 2005). In toxicological studies the elemental composition of the otolith is important in order to determine chemical signatures and environmental exposure. It has been suggested that metals are incorporated into vateritic otoliths differently compared to aragonitic otoliths. One theory suggests that the elemental differences between the two polymorphs are associated with the geometry of the crystals in aragonite and vaterite (Melancon et al., 2005; Gauldie et al., 1997). The molecular

arrangements of the crystals of the calcium carbonate polymorphs could leave little space for the inclusions of other elements (Curti, 1999) resulting in different element concentrations (Tomás and Geffen, 2003; Melancon et al. 2005). For instance, elements with larger ionic radiuses (i.e. Sr and K) compared to Ca would be less available for inclusion in the $CaCO_3$ structures, and elements with a smaller ionic radius (i.e. Na) would be less influenced by either $CaCO_3$ polymorph. Aragonite generally incorporates the larger cations (Sr^{2+}) while smaller cations (Mn^{2+}) are incorporated into vaterite. This is due to the different crystal structure of the polymorphs. Vaterite is incapable of incorporating larger cations without significantly distorting its structure (Casanova et al., 2004). To distinguish vaterite and aragonite otoliths in the present study, Sr was included in the suite of metals analysed on the LA-ICP-MS. Strontium is an indicator of true aragonite (Melancon et al., 2005). Significant differences in the Sr ($P = <0.001$) concentrations in aragonite otoliths compared to vateritic otoliths were found. Sr concentrations were much higher in aragonite portions. Once the laser transect entered the vateritic portion of the otolith Sr concentrations decreased dramatically (Figure 3.2). Results provide supporting data that Sr cations are incorporated into the aragonite crystalline structure and not in the vaterite structure. In addition, results showed significant differences in Zn ($P <0.001$) concentrations between aragonite and vateritic otoliths. Melancon et al., 2008 examine the concentrations of Sr, Ba, Mg, Mn, Li, Rb, and Zn between aragonite and vaterite otoliths in coho salmon. Results found major differences in the metal concentrations between the two polymorphs. In the aragonite portions of the otolith Sr and Ba were high while Mg and Mn were low. Results for Li, Rb and Zn, concentrations were almost equal for both CaCO3 polymorphs. This suggests that Zn uptake in coho and rainbow trout otoliths was not influenced by the forming $CaCO_3$ polymorph. Consideration should be

taken when applying otolith chemistry in comparative studies among species that could potentially be comprised of both aragonite and vaterite polymorphs (Melancon et al., 2008).

3.6.3) Cathodoluminescence microscopy

Cathodoluminescence microscopy is a non-destructive procedure that is able to detect any structural defects and/or elemental substitutes within the crystal lattice structure of otoliths (Marshal, 1988). CL has the ability to measure the chemical variation, and detect the presence of trace metals in otoliths at extremely low levels (in the ppb range) (Halden *et al*, 2004; Marshall, 1988). CL is the light given off by a substance that is bombarded by electrons. The light is due to the change in the electrons between the conducting band and the valence band of the material, where the wavelength of the light is proportional to the energy caused by the change in electrons. The intensity of the light is relative to the number of emission sites. The emission sites could be imperfections of the crystal lattice framework or trace metal substitutions in the crystal lattice. Results from the present study showed that the rainbow trout otoliths exhibited both concentric zones and asymmetrical patches of luminescence (Figure 3.4). Visible luminescence is generally observed in non pure, distorted insulators such as carbonates. Luminescence intensity is dependent activators, sensitizers, and quenchers. Each are impurities within a carbonate however each act differently when excited. Activators are ion impurities that emit by releasing absorbed energy as photons. Sensitizers are impurities that absorb energy, and later transfer the absorbed energy to an activator. Once all the absorbed energy is transferred to activator ions, the sensitizer is spectroscopically unrecognizable. Activators intensity increases through this process known as sensitization. Quenchers are impurities that abate activator luminescence by "trapping" some or all of the

absorbed energy (Machel et al., 1991; MacRae and Wilson, 2008). The visual CL colours and intensities are typically the result of the ratio of Mn^{2+} and Fe^{2+} constituents in carbonates. Mn^{2+} is an activator while Fe^{2+} is a quencher. The interaction of Mn^{2+} and Fe^{2+} potentially influence the strength of the luminescence.

All five otoliths exhibited two patterns of luminescence; concentric zoning and asymmetrical patches. The concentric zoning corresponds to the annular structure of the otolith suggesting that elemental uptake follows an annual pattern. The asymmetrical patches suggest that elemental uptake differs between aragonite and vaterite. The colour emitted from otoliths was a red-orange and purplish- blue luminescence colour. Red-orange luminescence is most commonly observed in calcite, a polymorph of calcium carbonate not typical of sagittal otoliths. In sagittal otoliths polycrystalline aragonite is the predominant polymorph and therefore a greenish-yellow colour would have been expected by CL. Although, vaterite, a common polymorph of calcium carbonate in sagittal otoliths shares the similar spectra as calcite, suggesting the red-orange luminescence was due to the presence of the vaterite polymorph rather then the calcite (Sommer, 1972). Typically, Mn^{2+} appears to be the most abundant and important activator in calcite, replacing calcium and emitting an orange-red colour. In vaterite otoliths, larger ions can not be incorporated into the crystal structure, therefore it too favours the incorporation of Mn^{2+} (Melancon et al., 2005). The red-orange luminescence observed in the rainbow trout otoliths suggests it was due to the presence of the polymorph vaterite which appears to share some similar properties as calcite. The blue colour is typical of intrinsic luminescence in carbonates and is common in calcite. The blue luminescence indicates regions where lattice defects could have occurred (Machel et al., 1991). Vaterite has a different cell structure compared to aragonite. As the otolith grows, the

vaterite replaces the aragonite leading to alterations in the crystal structure of the otolith. The blue could be an indicator of the alterations of the crystal structure as the vaterite was replacing the aragonite.

Halden et al. (2004) conducted CL microscopy on cisco, walleye (*Stizostedion vitreum*) northern pike (*Esox lucius*), lake white fish (*Coregonus clupeaformis*), white sucker (*Catostomus commersoni*), cisco (*Corgeonus artedii*) and yellow perch (*Perca flavescens).* Results from the CL microscopy showed that green and yellow-green luminescence was the most common observed in the study. However, there were otoliths that exhibited a red-orange and purplish-red luminescence. However, it was suggested by Halden et al. (2004) that the presence of Mn and Sr could have promoted the red-orange colour in the otoliths. The results from the present study are not supported by the results found by Halden et al. (2004) since portions of the otoliths that exhibited red luminescence where vaterite was present and consisted of low Sr concentrations (Figure 3.4). Vateritic otoliths do not incorporate Sr into the crystalline structure since Sr is a larger ion and cannot be incorporate into the crystal structure.

3.6.4) Summary and Conclusions

Metal concentrations in the experimental diets may not have been sufficiently high to be observed in concentrations in the otoliths. It may also have been that not enough time elapsed for the metals to be deposited on the otolith. While diet is the primary source for Zn incorporation into the otolith, no dose response relationships was evident in otoliths from the

four dietary groups. The high occurrence of crystalline otoliths in the study could be associated to the rearing practices either at the hatchery prior to the study, or perhaps during the study. The two polymorphs have different partition coefficients which precludes the use of otoliths with multiple polymorphs in environmental reconstructions (Reeder, 1983). Further research is needed in defining a cause for the switch between aragonite and vaterite and why it occurs in different species.

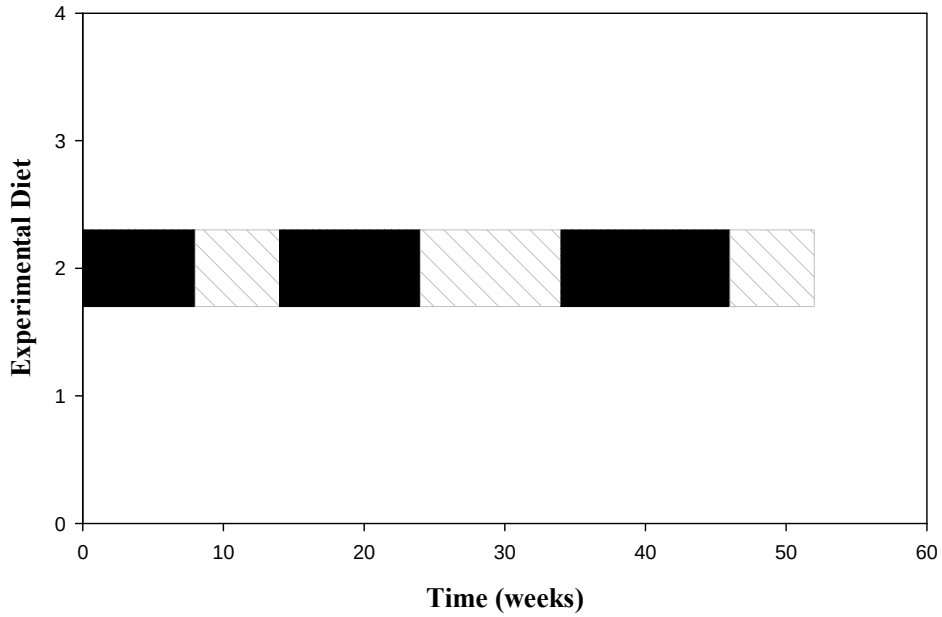

Figure 3.1 The intervals at which the four experimental diets were fed.

■ Indicates the interval in which fish were fed the metal enriched diet.

▨ Indicates the interval in which fish were fed the reference diet.

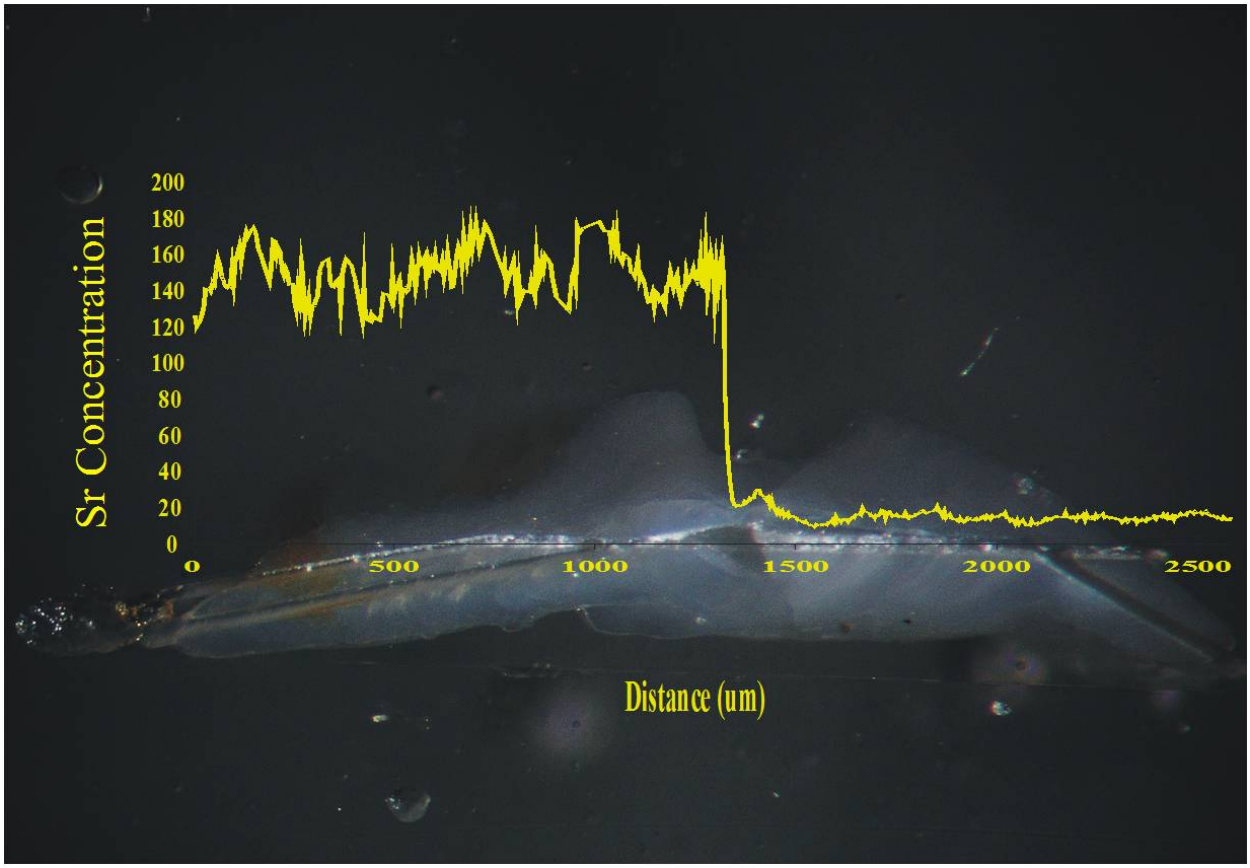

Figure 3.2 LA-ICP-MS line-scan data overlaid on a crystalline rainbow trout otolith from the high treatment group. The drastic change in Sr concentration indicates the change between aragonite and vaterite portions in the otolith.

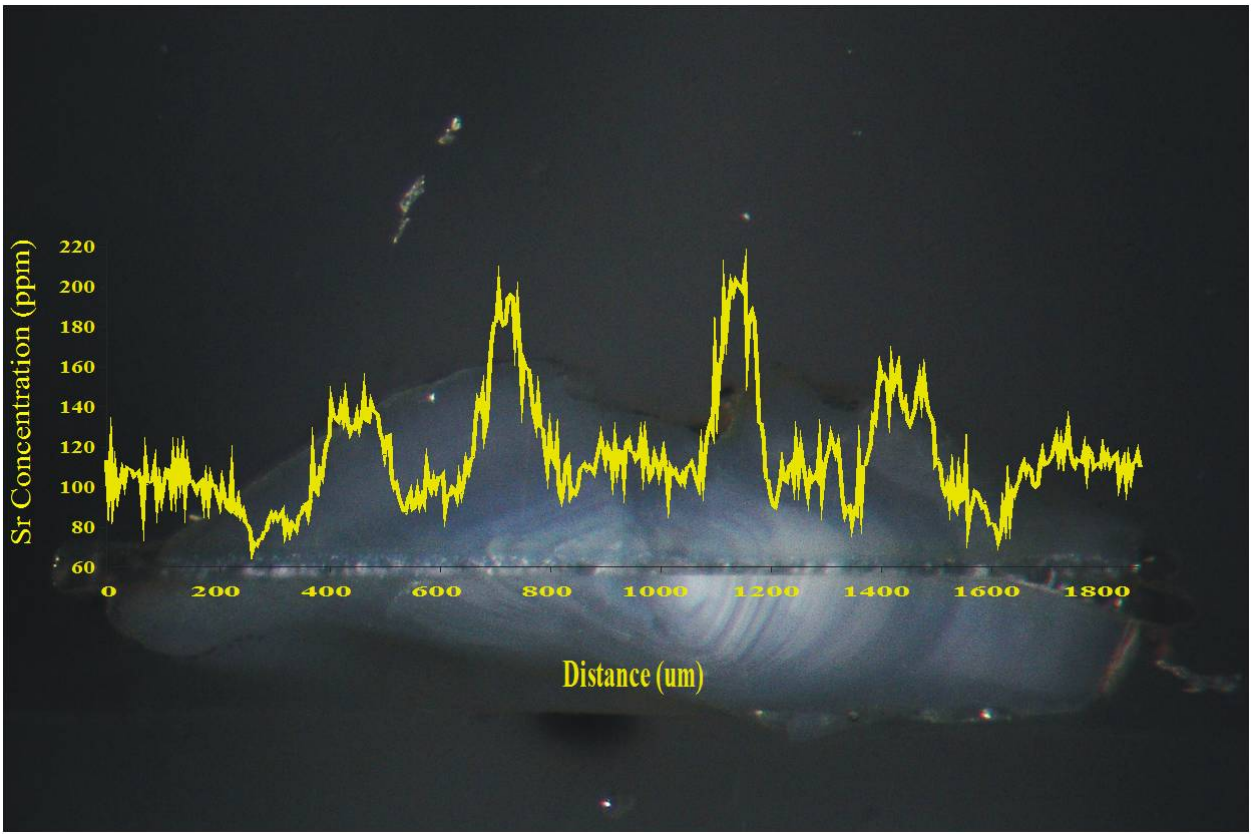

Figure 3.3 LA-ICP-MS line-scan data overlaid on a non crystalline rainbow trout crystalline from the reference group. The continued Sr concentration indicates a true aragonite otolith.

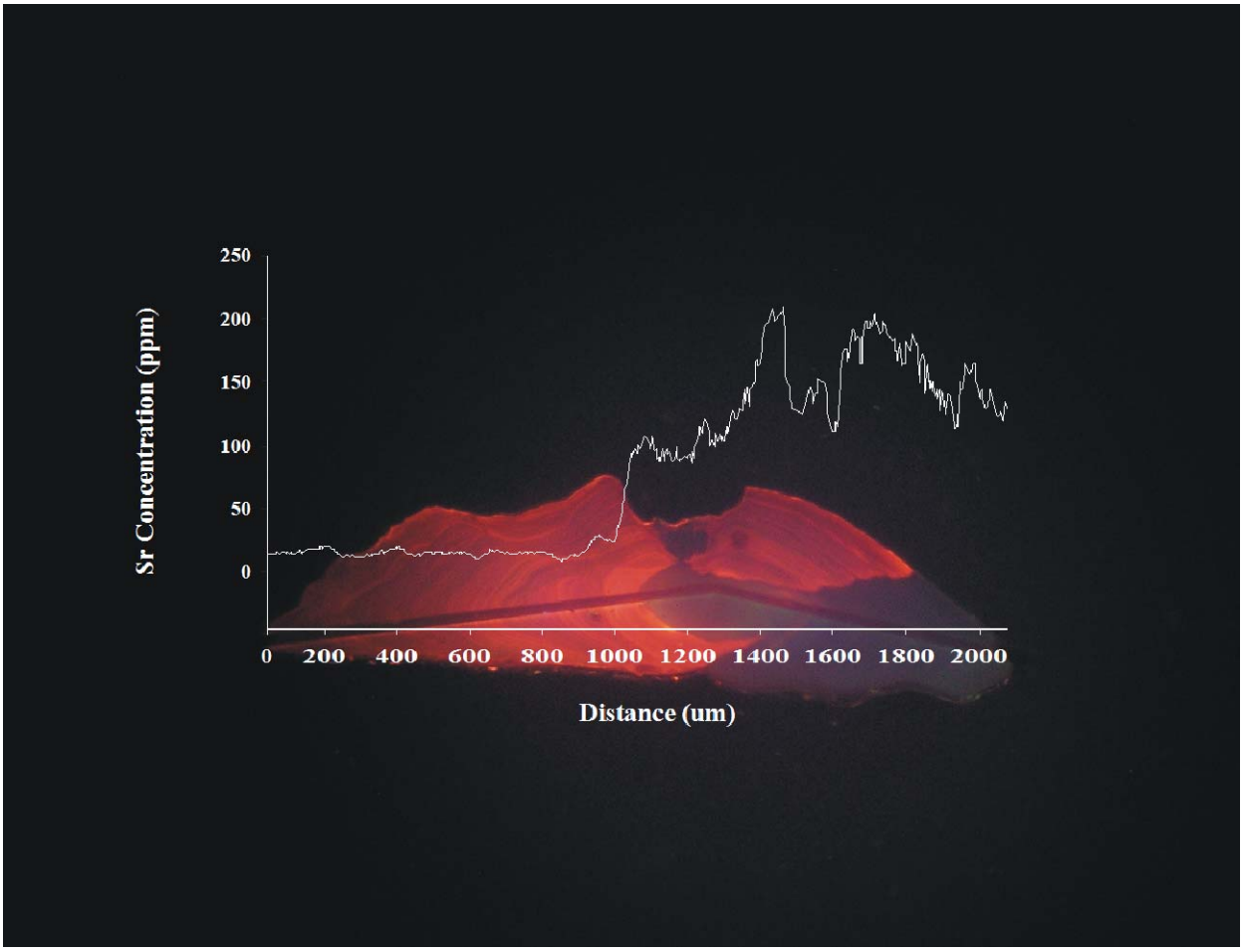

Figure 3.4 LA-ICP-MS line-scan data overlaid on an image of a luminescent otolith from the high treatment group. The change in Sr concentration indicates the change between vaterite and aragonite in the otolith

Table 3.4 Metal concentrations in the three augmented diets

Metal	Low dose (µg/g)	Med Dose (µg/g)	High Dose (µg/g)
Zn	22.24	15	29.5
Cu	2.82	3.5	7.62
Ni	0.06	0.25	0.57
Pb	0.00	0.05	0.10

Table 3.5 Mean body weight, fork length and condition factor for all fish used for the LA-ICP-MS analysis.

Treatment	*n*	Weight (g)	Fork Length (cm)	Condition Factor
Reference	16	28.2 ± 10.35	13.5 ± 1.55	1.15 ± 0.08
Low Dose	14	30.6 ± 8.57	13.7 ± 1.47	1.18 ± 0.09
Medium Dose	12	31.5 ± 7.70	13.9 ± 1.44	1.17 ± 0.20
High Dose	18	23.2 ± 10.97	12.2 ± 1.76	1.21 ± 0.06

Table 6.3 Mean metal concentrations and DL (µg/g) values present in rainbow trout otoliths.

Treatment Group	n	Ni (µg/g)	Ni DL	Cu (µg/g)	Cu DL	Pb (µg/g)	Pb DL	Zn (µg/g)	Zn DL
Reference	16	0.28	0.74	0.15	0.15	0.01	0.02	87.78	0.23
Low Dose	14	0.34	0.63	0.14	0.17	0.02	0.13	108.78	0.23
Medium Dose	12	0.71	0.45	0.25	0.15	0.1	0.01	92.06	0.21
High Dose	18	0.31	0.46	0.15	0.14	0.02	0.01	106.78	0.19

Table 3.7 Mean metal concentrations present in liver tissue.

Treatment	Cu (µg/g)	Ni (µg/g)	Pb (µg/g)	Zn (µg/g)	Se (µg/g)
Reference	44.7 ± 9.31	ND	ND	20.8 ± 1.24	8.07 ± 10.1
Low	39.4 ± 14.30	0.06 ± 0.02	ND	20.7 ± 1.39	1.89 ± 0.82
Medium	50.6 ± 24.50	0.04± 0.03	ND	21.2 ± 1.97	3.24 ± 0.92
High	43.3 ± 25.80	0.04 ± 0.00	0.08 ± 0.01	20.7 ± 2.08	5.67 ± 2.43

3.7 References

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4: General Conclusions

4.1 General Conclusions

4.1.2) Summary

This study had two key objectives, to determine temporal trends in Mn in otoliths of lake trout captured from the Red Lake area of Ontario, and to determine if the timing of lake trout recruitment failure in Red Lake's Pipestone Bay was associated with an increase in Mn concentrations in otoliths of lake trout from Red Lake.

Chapter 2 examined manganese concentrations present in lake trout otoliths collected from Pipestone Bay and two reference sites, Trout Lake and Confederation Lake, to compare temporal trends in lake trout otoliths. Peak manganese concentrations were observed in otoliths collected from all three sites. Significant differences in manganese otolith content between Pipestone Bay, Trout Lake, and Confederation Lake were determined and were likely the result of past mining activity on Confederation Lake. Red Lake and Trout Lake did not have any significant mining activity during study period (1980 to 1989). However, peak manganese concentrations occur during 1980 and 1989 which coincides with the time period when the lake trout recruitment failure was thought to have begun suggesting another potential source of Mn exposure rather than mining activity.

Chapter 2 also examined the sediment chemistry of sediment cores collected from Pipestone Bay to link otolith microchemical analyses to environmental exposure of manganese. Concentrations in sediment cores were measured highest at 4987.9 μg/g (dry weight), approximately 1 cm under the sediment-water interface. The thin 1 cm layer of oxidized sediment suggests that Mn near the sediment-water interface had become dissociated from oxyhydroxides in anoxic deep sediments, and actively diffused upward.

This allowed a better opportunity for Mn to escape into the overlying water column, and available for uptake by lake trout. The otolith composition is thought to be reflective of the fish's surrounding environment (i.e. water or diet exposure). However, establishing a relationship between the otolith composition and environment was difficult. In fish, trace metals, including manganese present in the environment are taken up and across a multi-barrier pathway where metals are discriminated (fractionation) against at varying degrees. The discrimination (fractionation) of manganese at the different barriers is not well understood and prevented us from identifying a relationship between Mn concentrations in Pipestone Bay and Mn concentrations in otoliths. There is a lack research on the role of manganese in the environment, and how it, as well as other metals is incorporated into the otolith via exposure through diet or water. In Chapter 3, the relationship between metal exposure through diet, and how the metal is incorporated into the otolith was investigated.

In addition to the fractionation of trace metals another factor that influences otolith chemistry is the regulation of metals. Manganese is used for metabolic processes, and when Mn concentrations are high (above ambient concentrations) Mn can become toxic to fish (Rainbow, 1997). Consequently, fish are required to maintain Mn at steady levels to ensure metabolic processes function, and to avoid death if concentrations become intolerable. Thus, influencing the Mn concentrations available for otolith uptake (Gillanders & Kingsford 1996, 2000; Sanchez-Jerez et al. 2002).

Chapter 3 determined how the concentrations of Cu, Ni, Pb, Se and Zn present in a fish's diet corresponded to the concentrations deposited in the otolith of juvenile rainbow trout (*Oncorhynchus mykiss)*. These four metals were selected since concentrations have

been found to have elevated concentrations at mining sites. Metal exposure through the diet had no influence on the concentrations of Cu, Ni, and Pb in the rainbow trout otoliths from all three experimental diets and reference group. However, diet did have an influence on the concentrations of Zn in the otoliths collected from the three experimental diets. Diet appeared to be the primary source for Zn incorporation into the otolith. The otoliths collected from the feeding experiment had an unexpected high percentage (70%) of crystalline otoliths. Metals were incorporated differently between the two otolith structures and lead to difficultly in determining chemical signatures.

The measurement of metal concentrations in the rainbow trout otoliths were influenced by the high percentage of crystalline otoliths in the study. The change in the aragonite structure (non crystalline otoliths) to the vaterite structure (crystalline otoliths) prevents the comprehension of how the otolith incorporates metals from the environment. The composition of vateritic otoliths can vary greatly compared to aragonite and cause difficulty in determining chemical signatures. Metals are incorporated differently in vateritic otoliths compared to aragonitic otoliths due to the geometry of the crystal lattice structure. Aragonite generally incorporates the larger cations (e.g. Sr^{2+}) while smaller cations (e.g. Mn^{2+}) are incorporated into vaterite. The vaterite structure is incapable of incorporating larger cations without significantly distorting its structure (Casanova et al., 2004). Based on this laboratory study, the crystalline structure in otoliths can differ. The discrepancy in the crystalline structure can cause variations in the elemental composition of otoliths. From the Sr and Zn results, aragonite and vaterite demonstrate no relationship between their elemental chemistry and definitely incorporate these elements differently from diet. Previous research has shown that aragonite otoliths can reflect metal exposure from the diet (Farrell and

Campana, 1996; Ranaldi and Gagnon, 2009). If this is accurate, vaterite otoliths likely not reflect the diet chemistry equally. Therefore, the use of vateritic otoliths would not be ideal for examining elemental composition. Caution should be taken when working with crystalline otoliths.

4.1.2) Recommendation for Future Research

Manganese moves along an elemental pathway where significant discriminations can occur resulting in varying concentrations between the environment and otolith composition. The reasons for the discrimination of Mn at each barrier are not clearly understood. Further investigation on the mechanisms associated with discrimination (fractionation) of Mn between water and the otolith is required. Laboratory experiments to investigate the influence of Mn and the otolith chemistry through exposure via diet and water exposure could be utilized. In addition to laboratory experiments, partition coefficients could be calculated to conduct comparisons between trace metal compositions.

Research including toxicity testing using manganese and freshwater species should be carried out since current research on freshwater species is quite limited. These types of studies would supply additional information on the effects of manganese toxicity on freshwater species. In addition to these toxicity tests, studies should also focus on the effects that manganese toxicity has the early life stages (embryo, fry, and larvae) of freshwater species in order to better understand the sensitivity to manganese exposure. Variations in Mn concentrations may be attributed to metal regulation by fish, dietary influences, and genetics. Each of these influential factors have been investigated slightly but remained to be fully investigated combining both laboratory and field approaches should be included to better

understand the controls and mechanisms of barriers and pathways of metal uptake into the fish and otolith. Investigations on the relationships between manganese concentrations in water and otolith need to be conducted. It should also be noted that future studies should try to include the use of wild fish rather then hatchery fish to investigate temporal trends in metal exposure to avoid the occurrence of vaterite otoliths.

The recruitment failure in the lake trout population in Red Lake's Pipestone Bay requires further research. A better understanding of the uptake of manganese will promote a better understanding of manganese exposure in the aquatic environment.

The continued recruitment failure requires action and leadership. MNR has begun taking action by implementing a stocking and monitoring program for the lake trout. Lake trout yearlings are released in deep basins outside of Pipestone Bay every spring. To monitor the lake trout population, a SLIN (spring littoral index netting) survey and SPIN (summer profundal index netting) are conducted annually to assess the success rate of the stocked fish and potentially provide information the lake trout population and the habitat of the juvenile lake trout (Thébeau, 2008).

4.1.3 Conclusions

Peak manganese concentrations were observed in otoliths from Pipestone Bay, Trout Lake and Confederation Lake. The peak manganese concentrations observed in Pipestone Bay corresponded to the time period (1980-1989) that could be related to environmental exposure.

However, since manganese is redox active the temporal history gathered from the sediment cores was uncertain.

Diet had no influence on the concentrations of Cu, Ni, and Pb in the rainbow trout otoliths. These metals were (i) not deposited in the otolith through the diet, (ii) concentration of these metals used in the experimental diet were not sufficiently high enough to be observed in the otolith, or not enough time elapsed for the metals to be deposited on the otolith. In contrast, diet was identified as the primary source of the Zn concentrations in the rainbow trout otoliths. Crystalline otoliths prevent examining the elemental composition and caution should be taken when using them to examine temporal trends in metal exposure.

4.2 References

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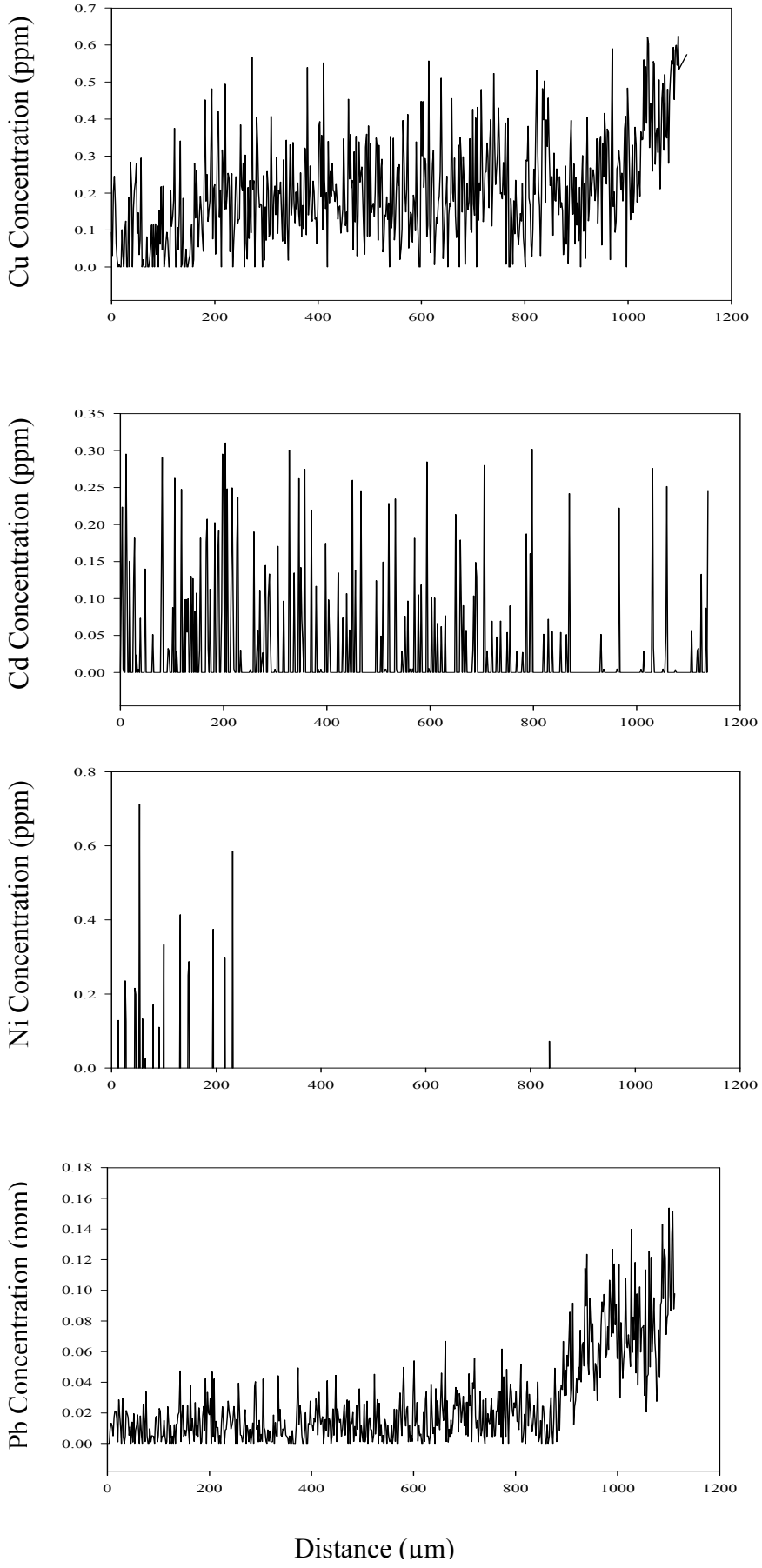
APPENDIX I

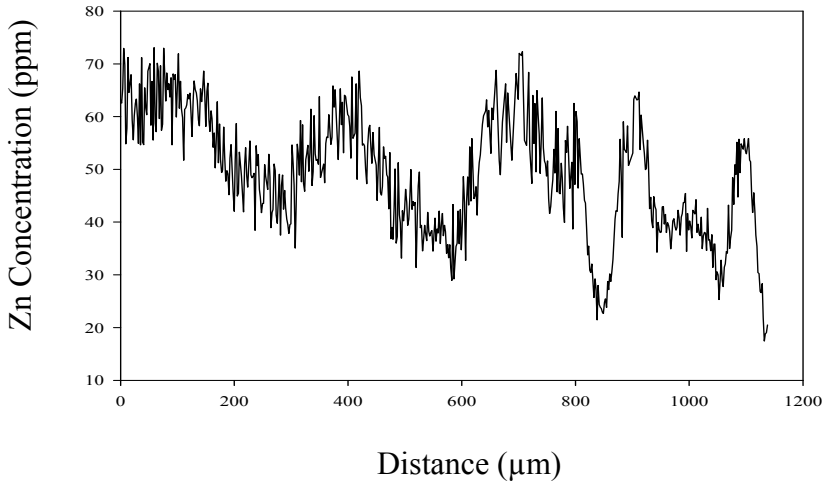
Metal Results for Pipestone Bay

RL-V-63

Age: 9+

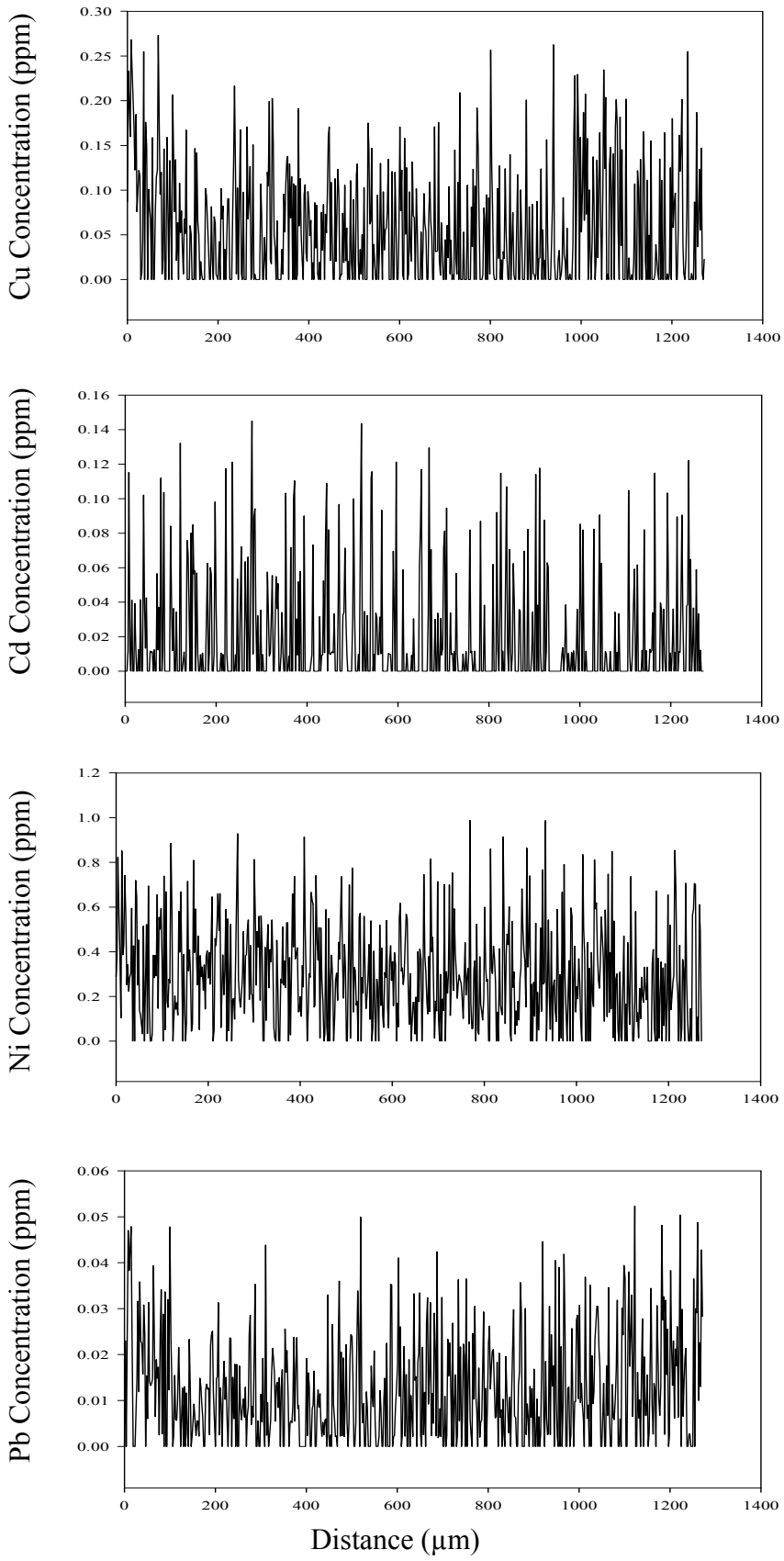

Cu Concentration (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
Cd Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Distance (µm)

RL-V-63

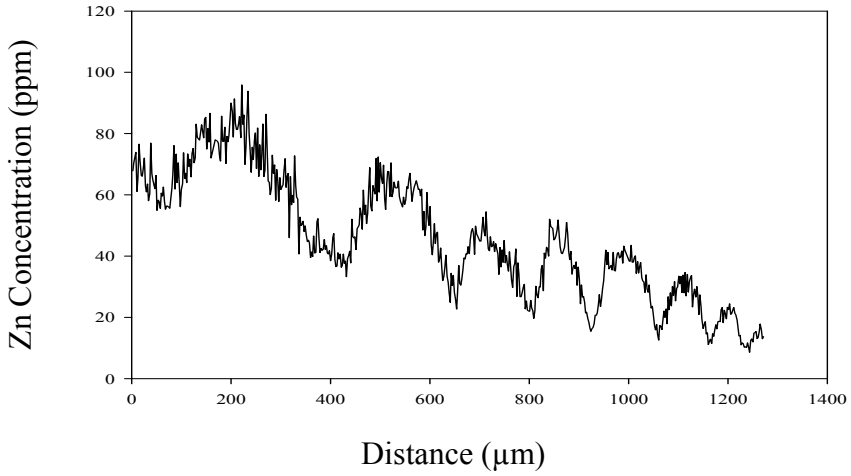

Zn Concentration (ppm)
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
Distance (μm)

RL-v-64

Age: 8+

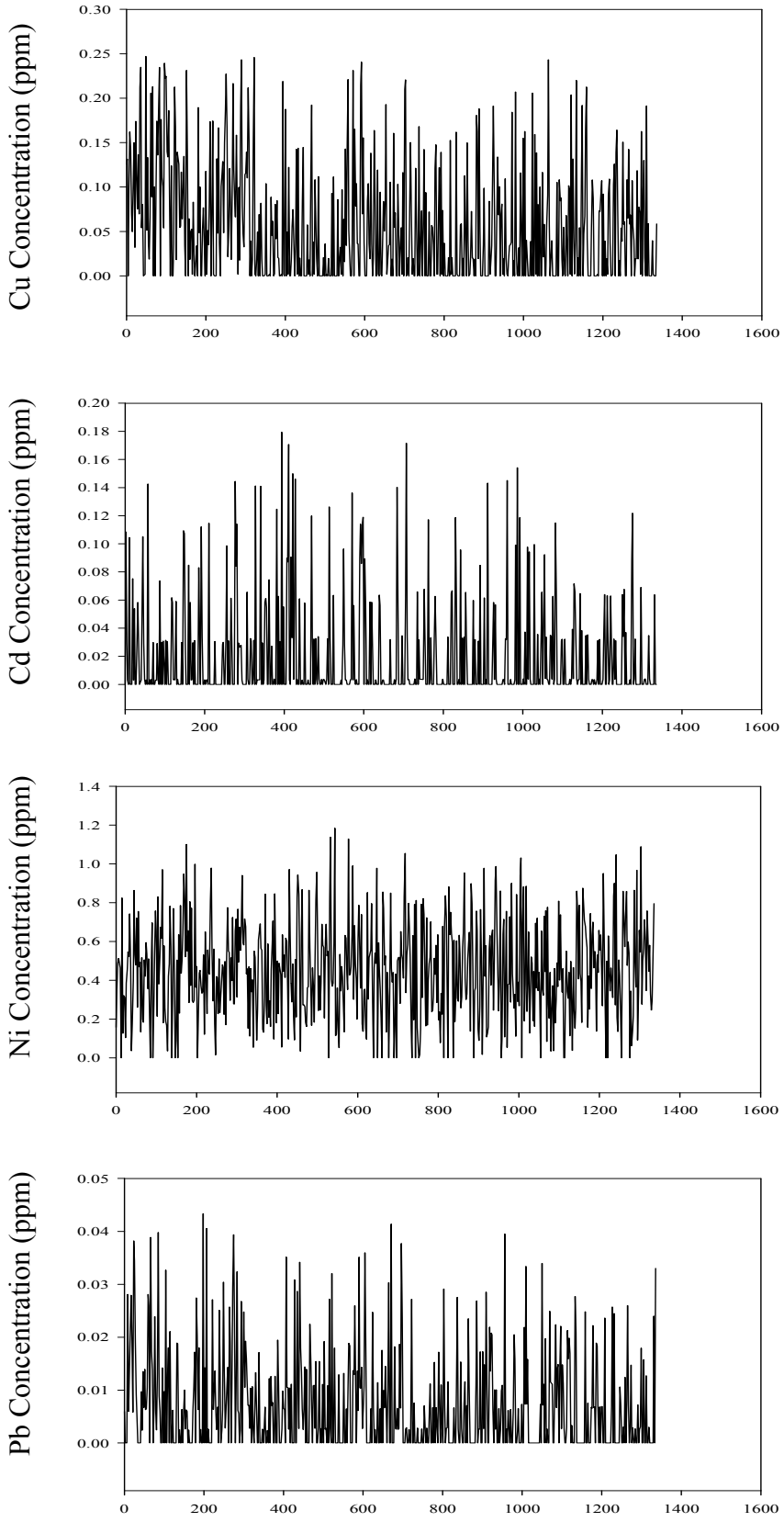

Cu Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (µm)

RL-v-64

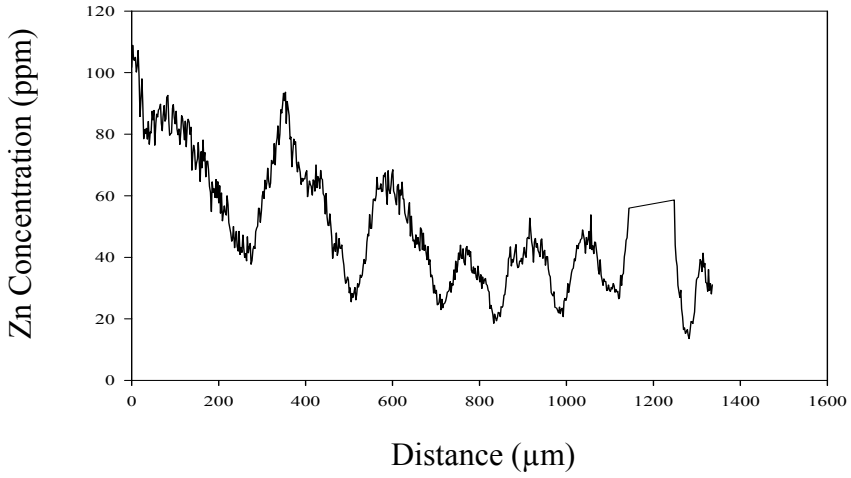

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

RL-V-65

Age: 8

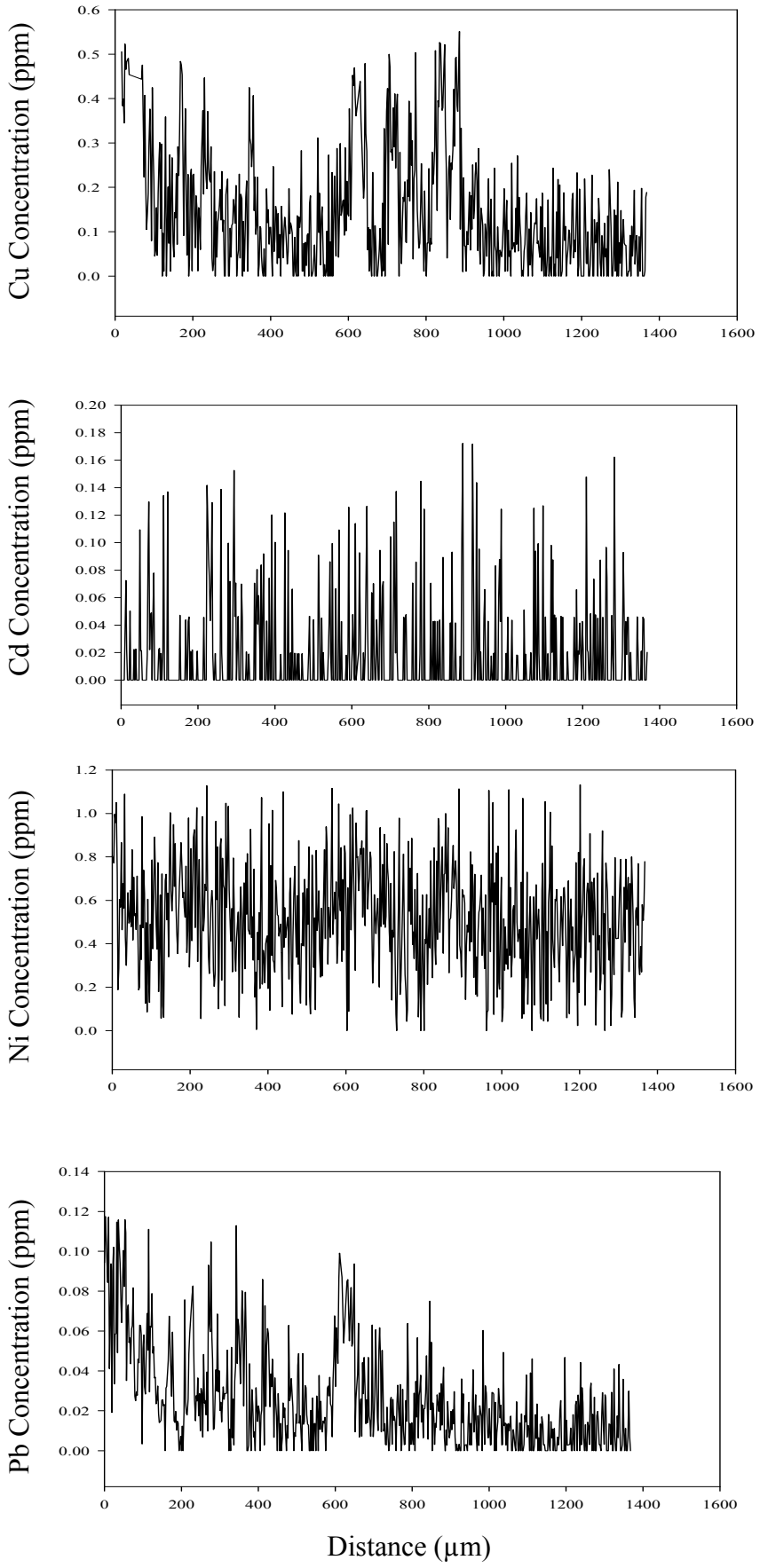

Cu Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Cd Concentration (ppm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ni Concentration (ppm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.05
0.04
0.03
0.02
0.01
0.00
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

RL-v-65

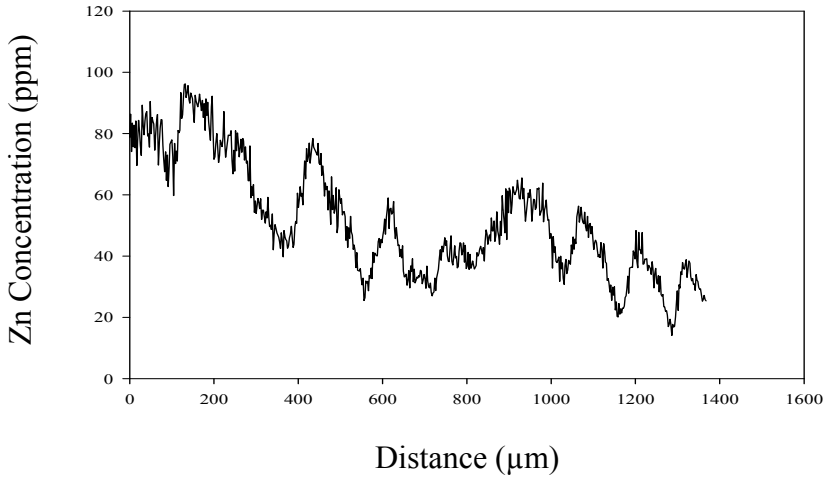

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

RL-V-72

Age: 8

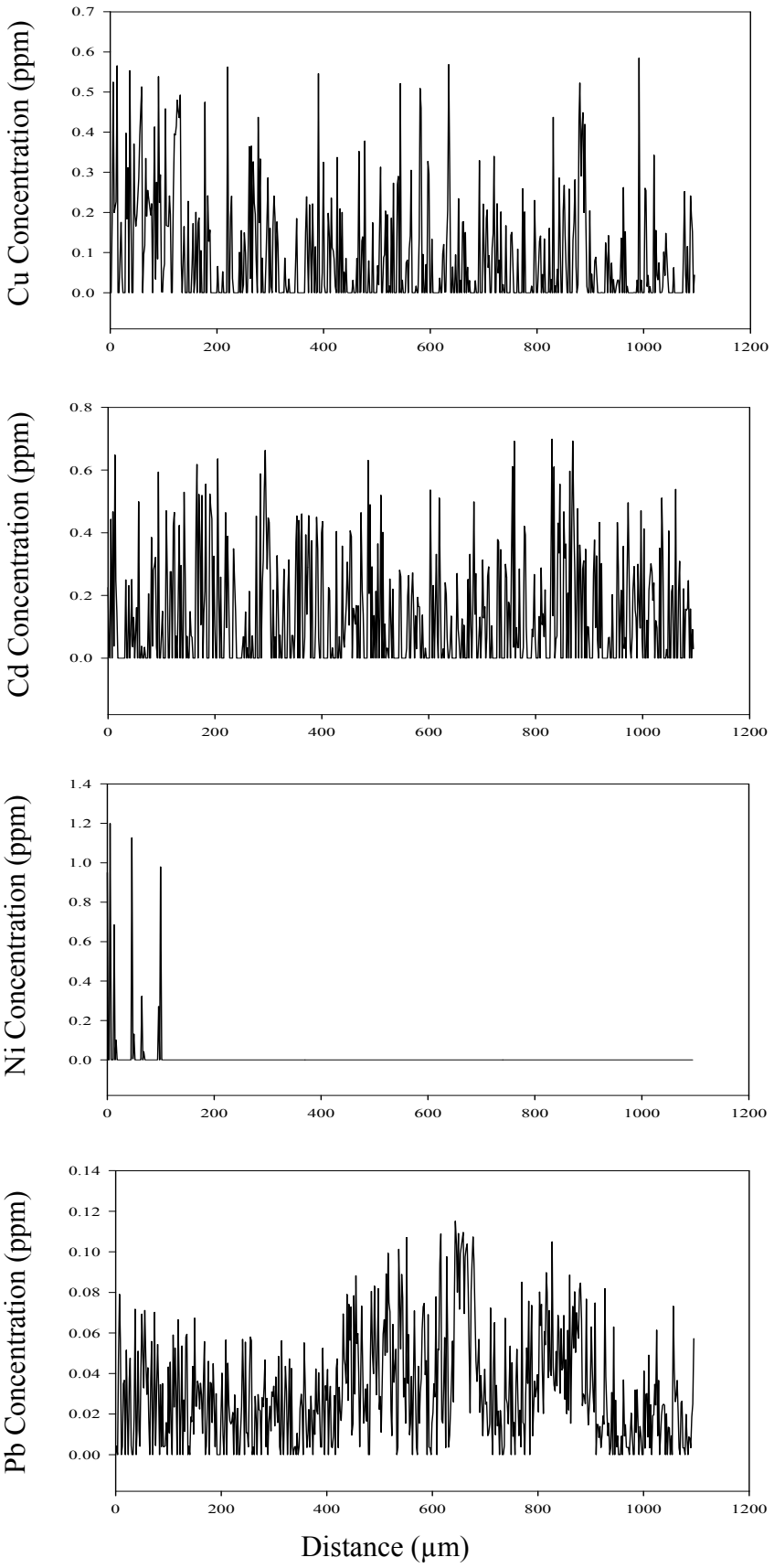

Cu Concentration (ppm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Distance (μm)

RL-v-72

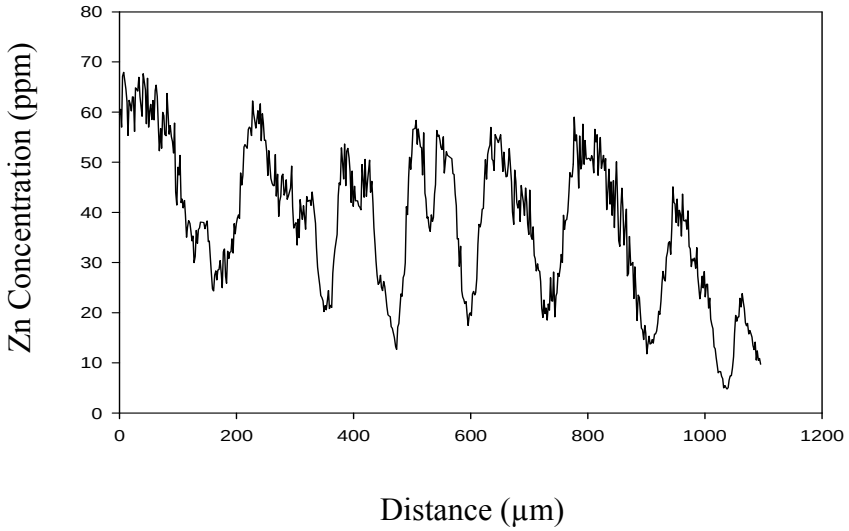

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-v11-48

Age: 9

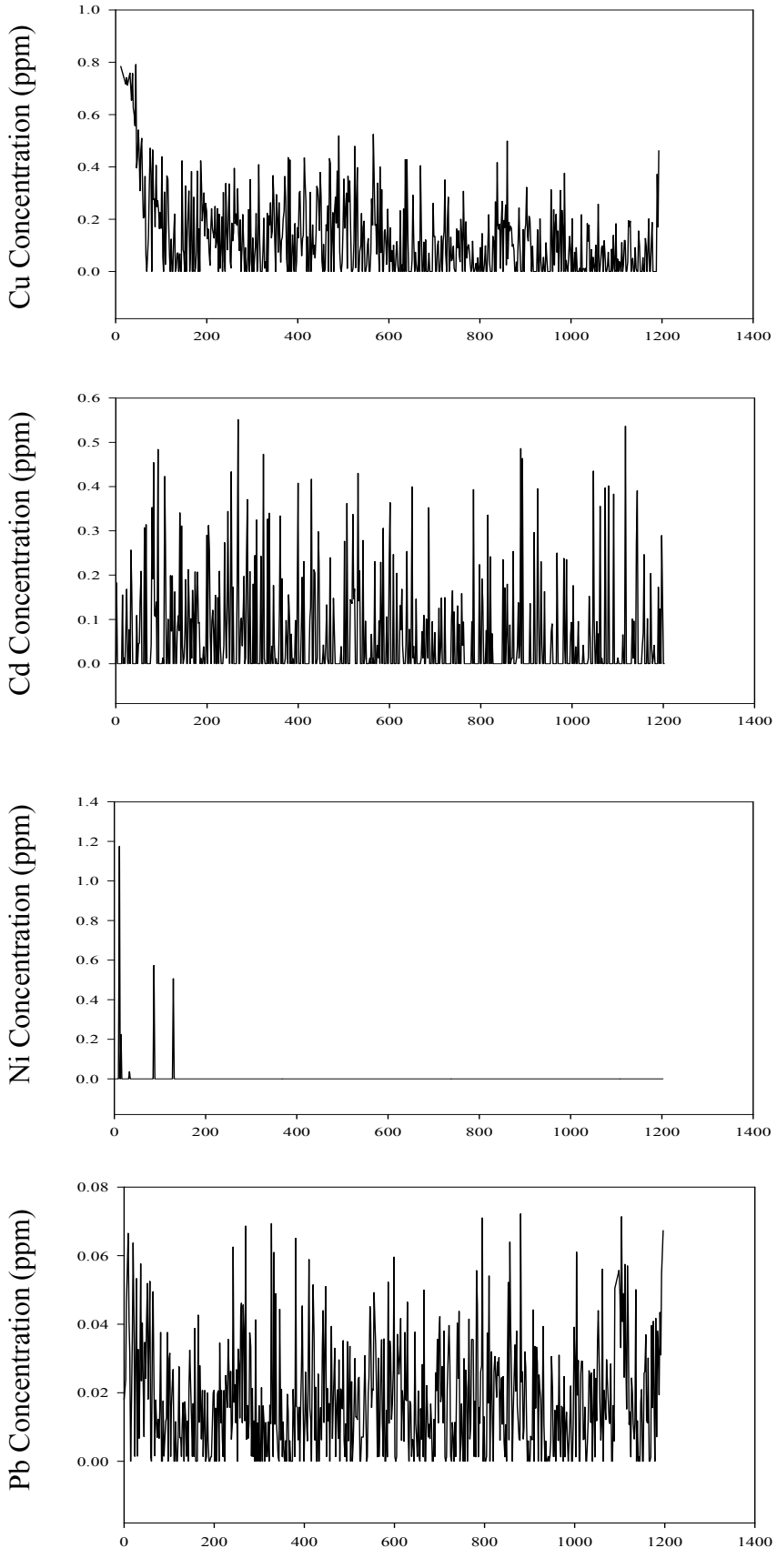


RL-v11-48


Zn Concentration (ppm)
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
Distance (µm)

RL-v11-49

Age: 10



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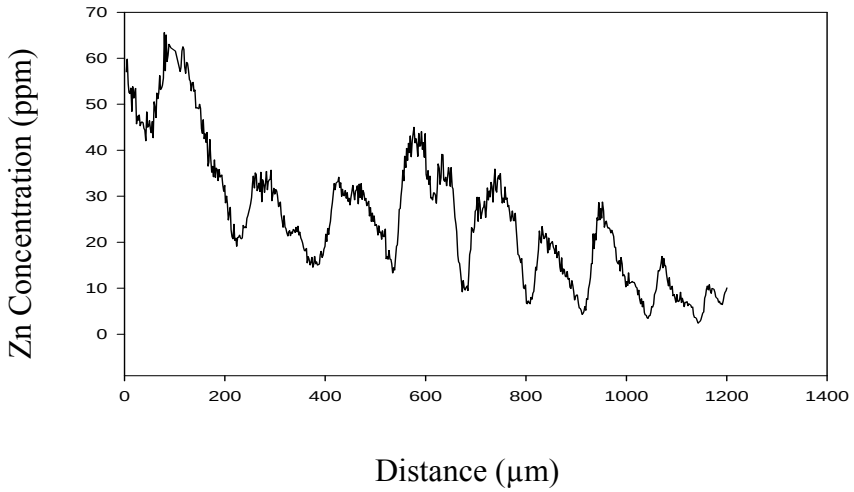
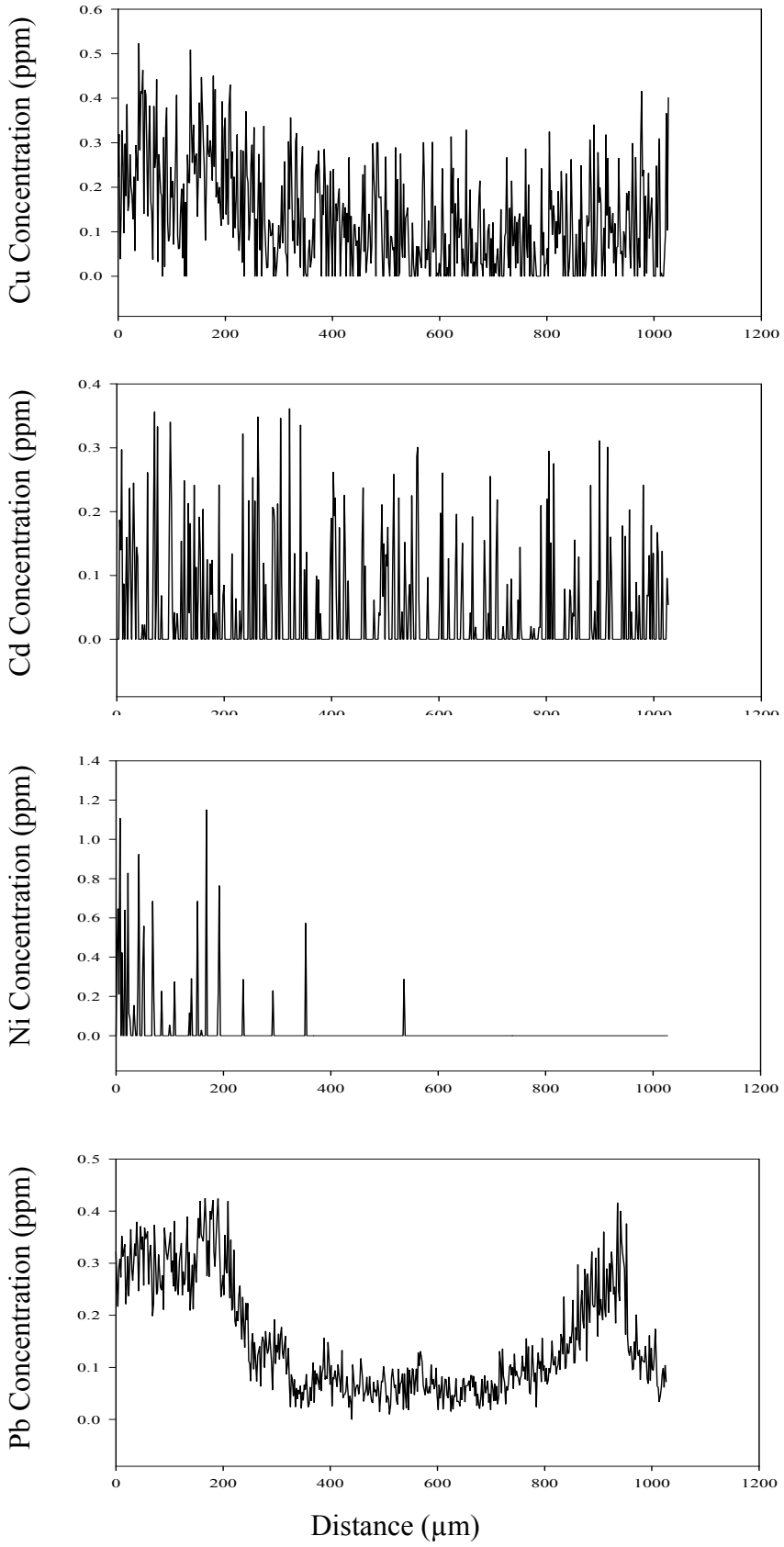

Zn Concentration (ppm)
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

RL-v11-50

Age: 10+



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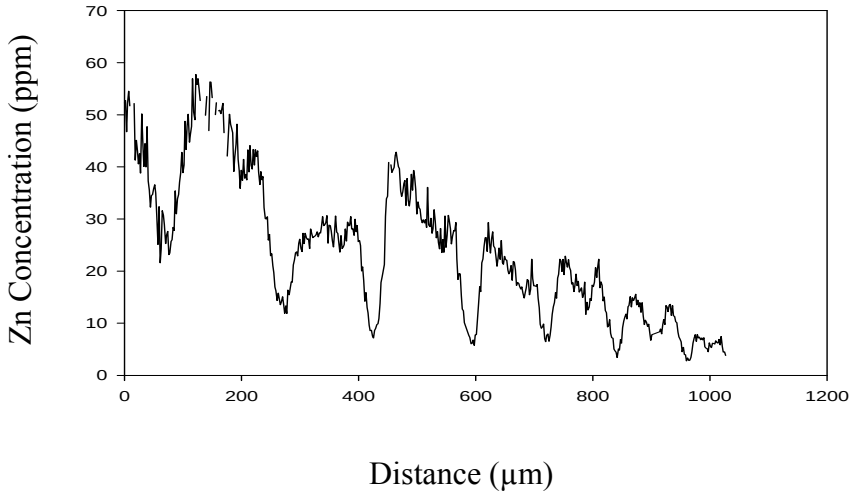
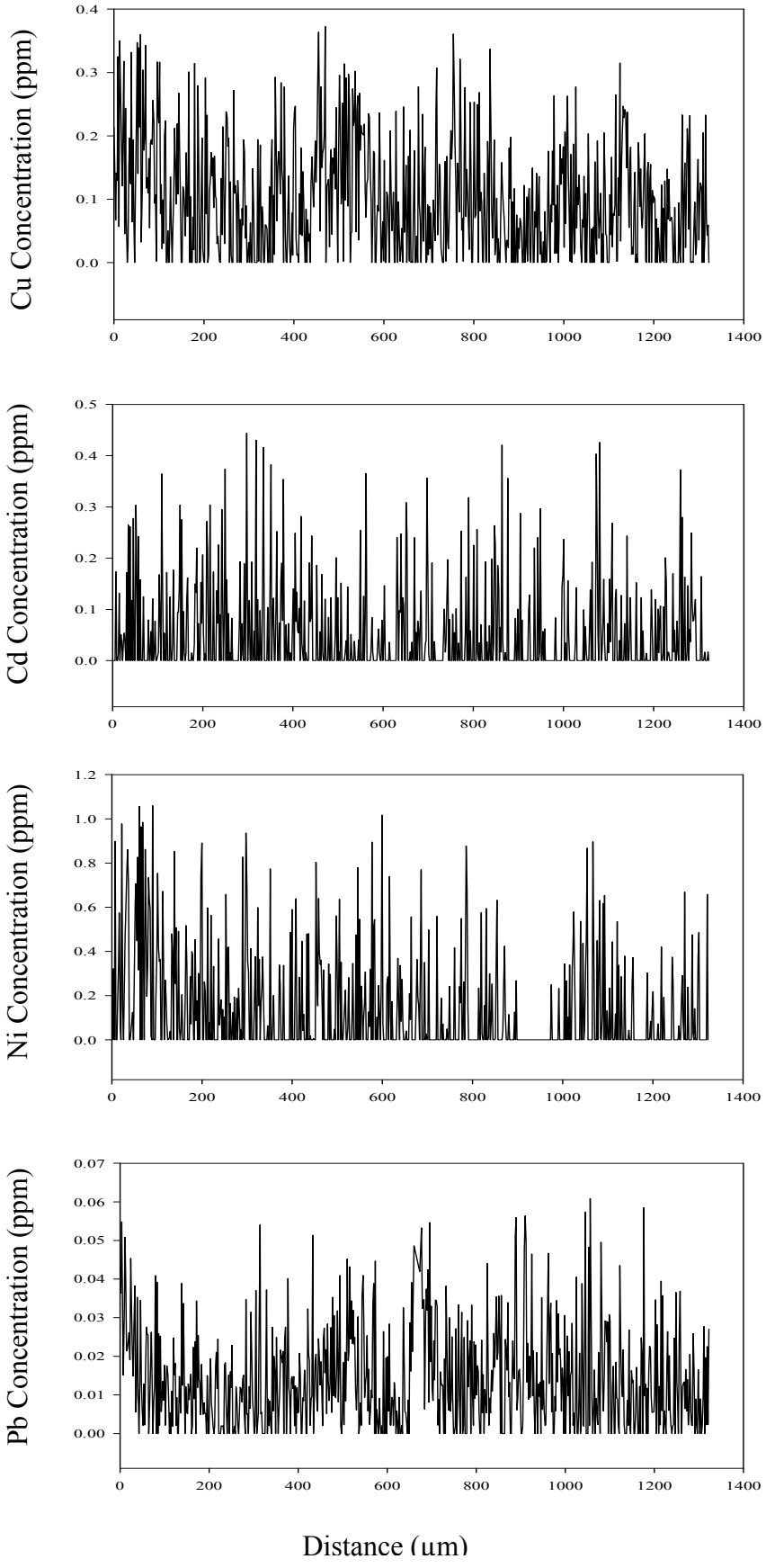

Zn Concentration (ppm)
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
Distance (μm)

RL-v11-51

Age: 11+



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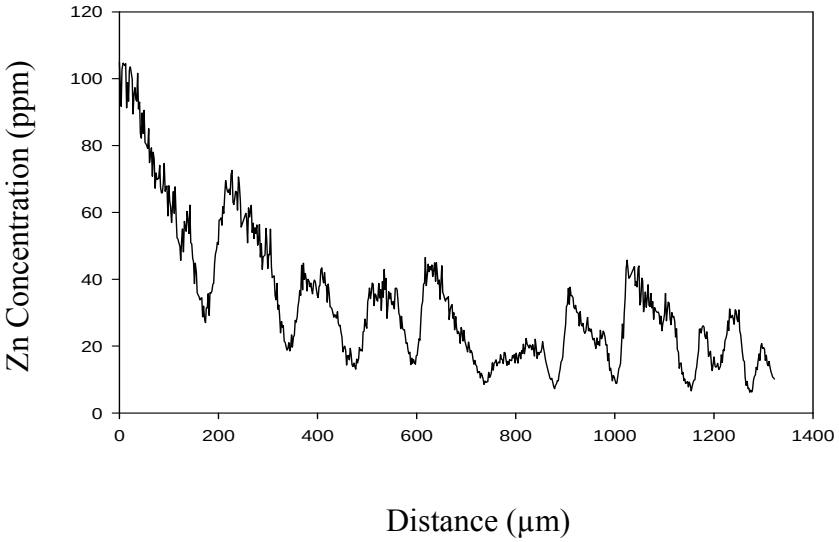
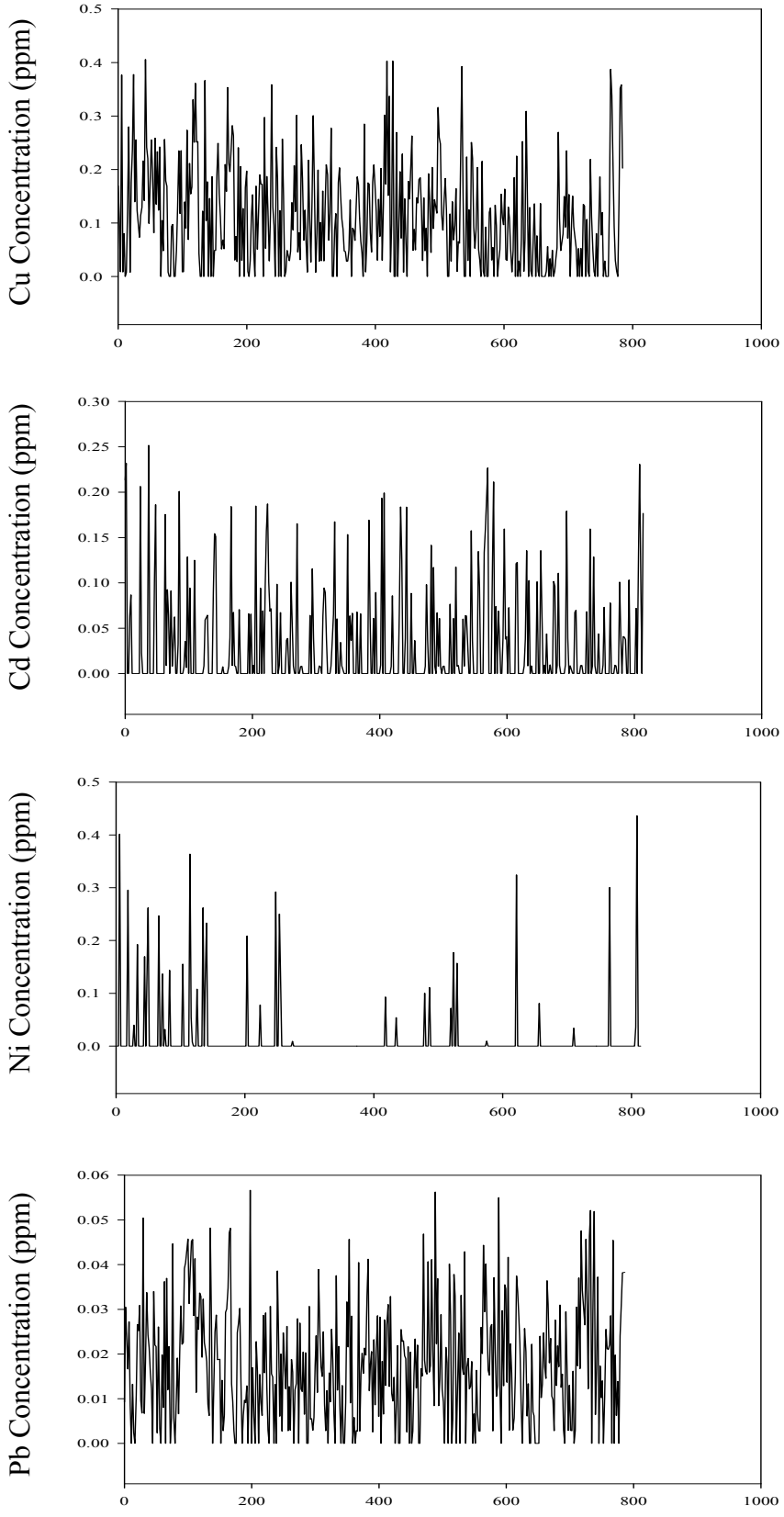

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (µm)

RL-v11-53

Age: 9+



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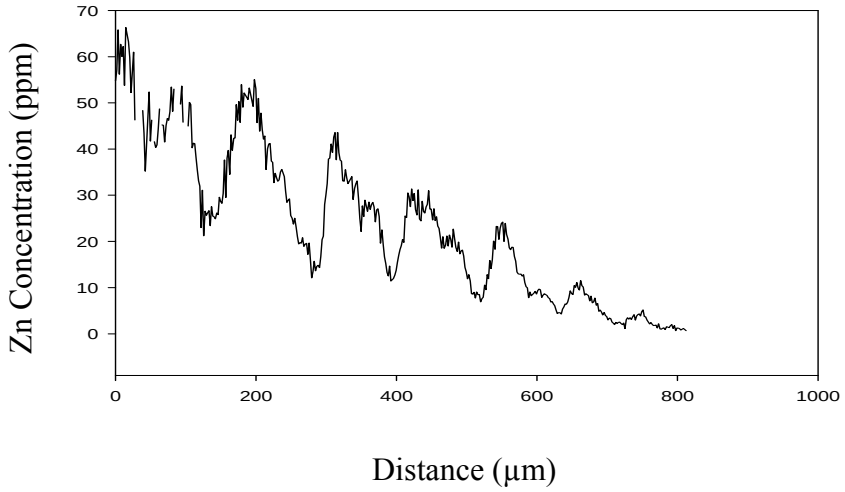
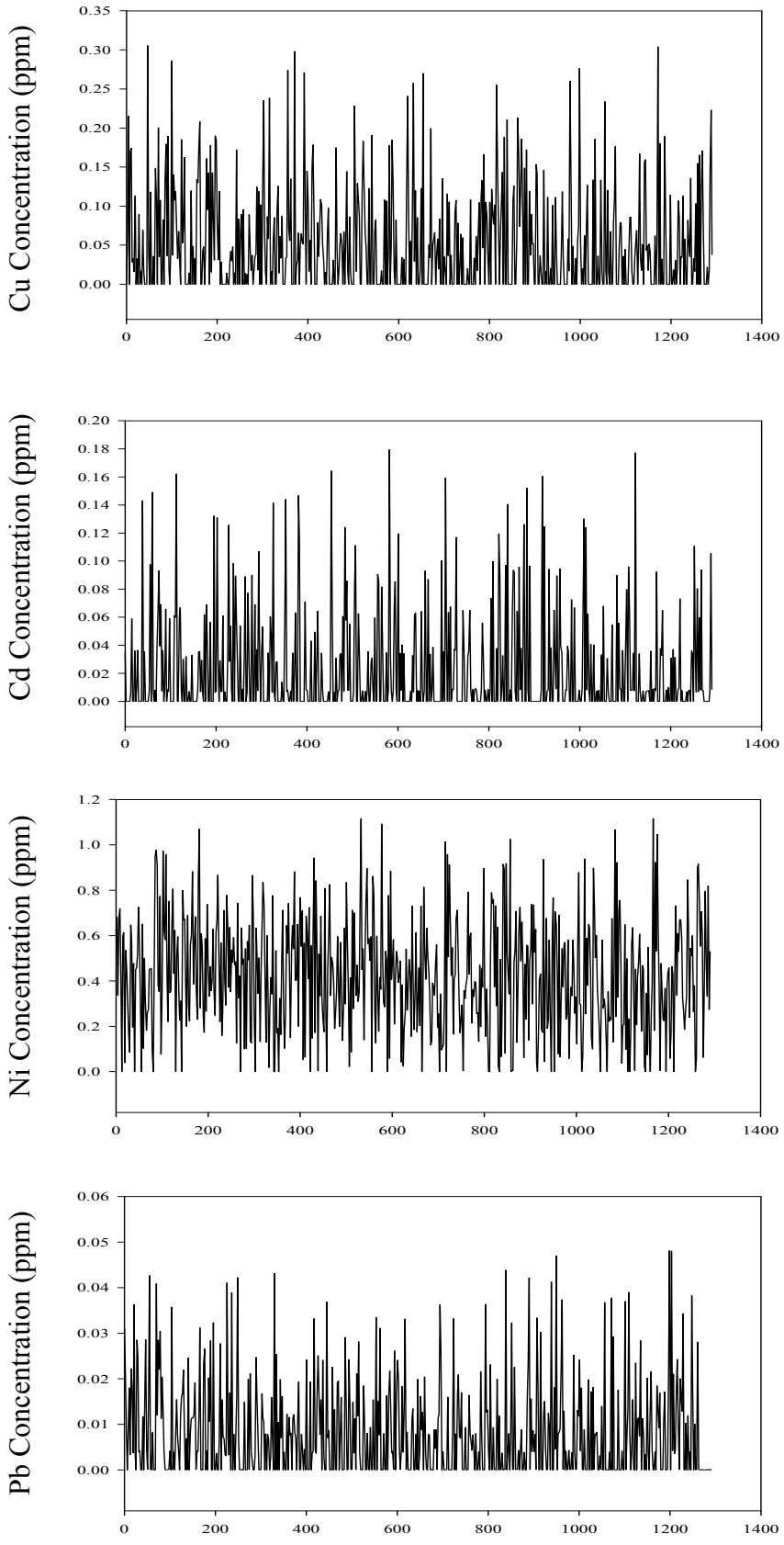
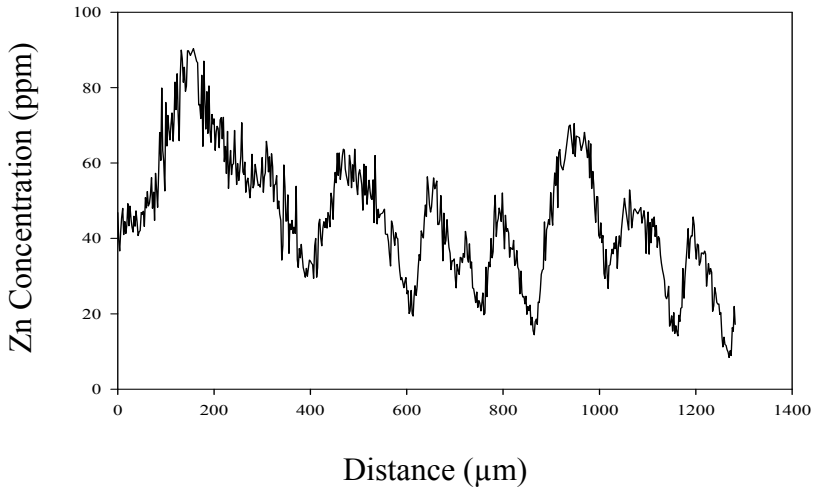

Zn Concentration (ppm)
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
Distance (μm)

RL-4-61

Age: 7+

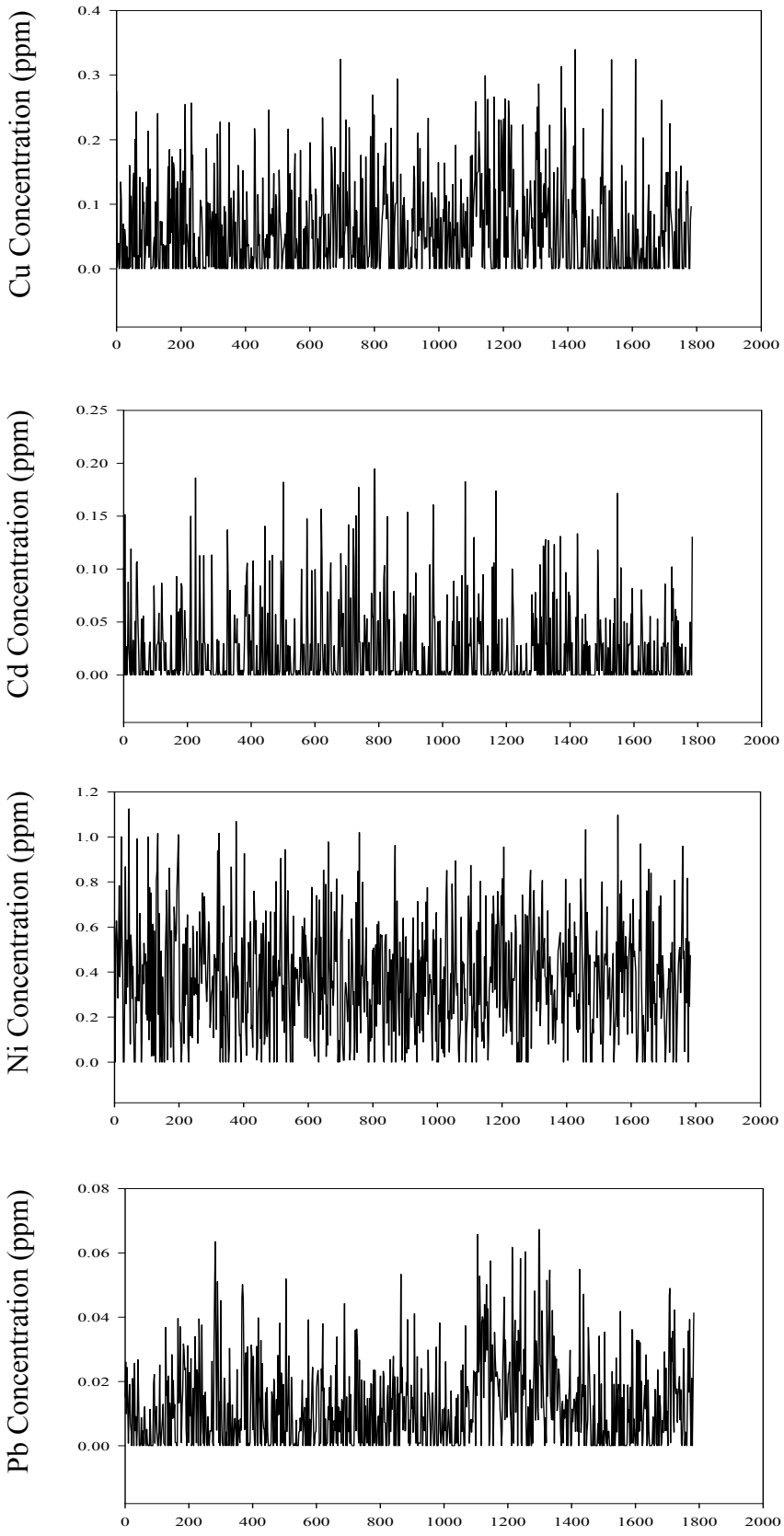

Cu Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (μm)

RL-4-61

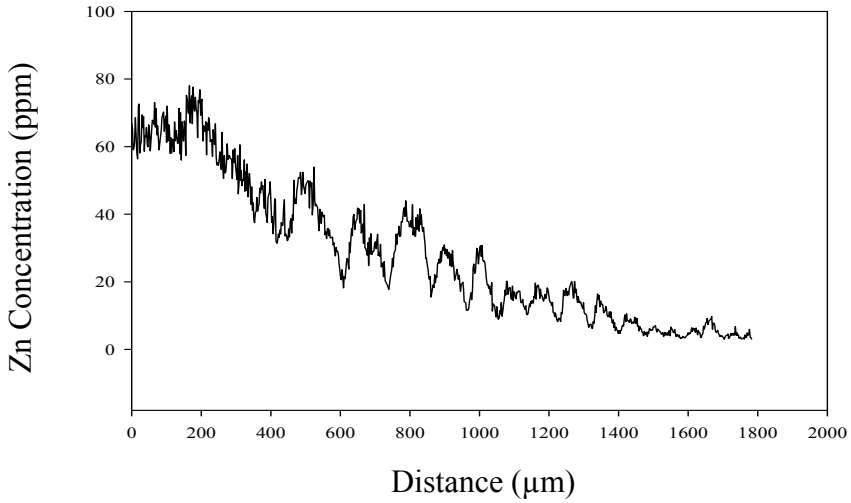

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

RL-4-66

Age: 19

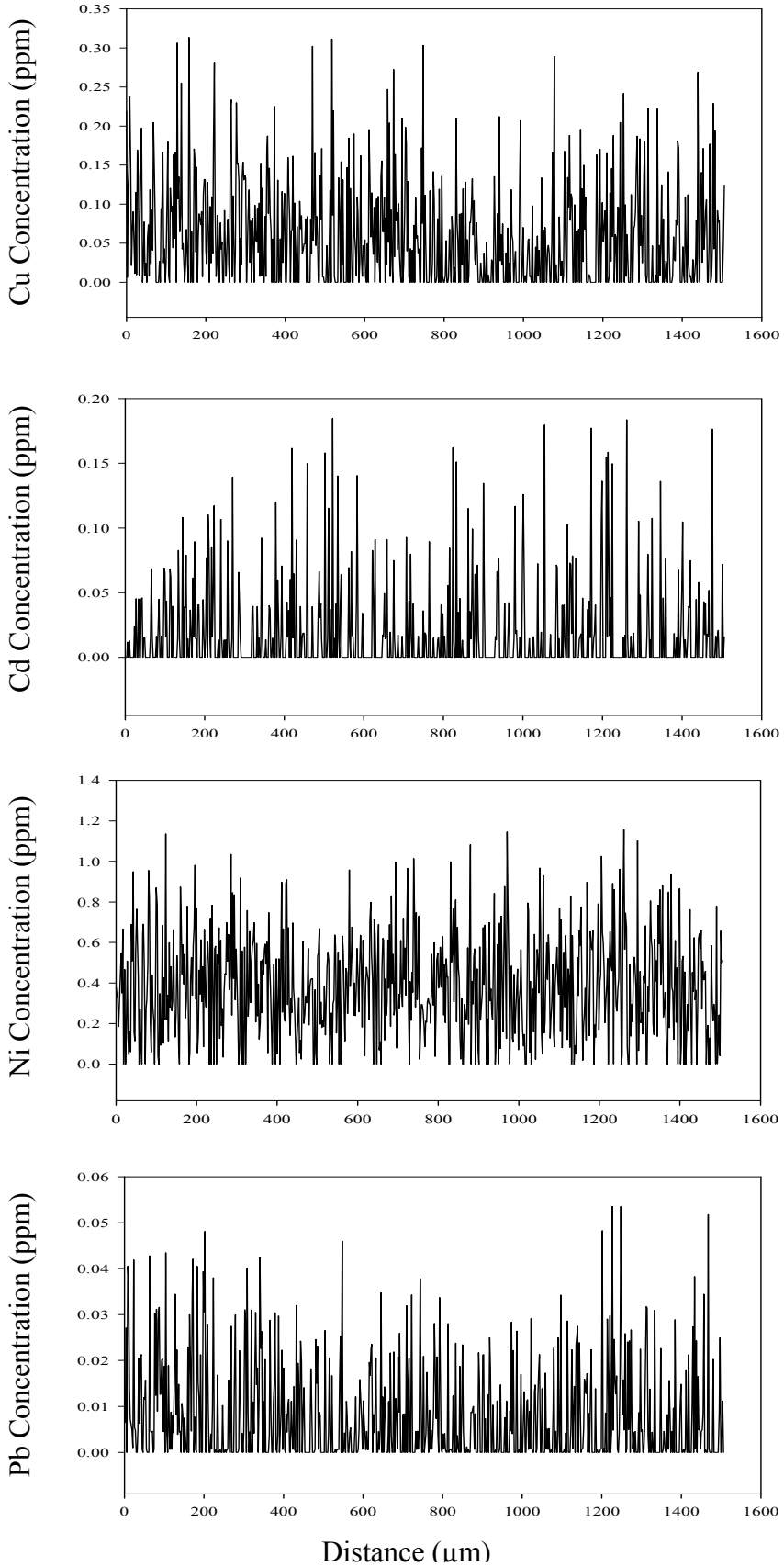

Cu Concentration (ppm)
0.4
0.3
0.2
0.1
0.0
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (µm)

RL-4-66

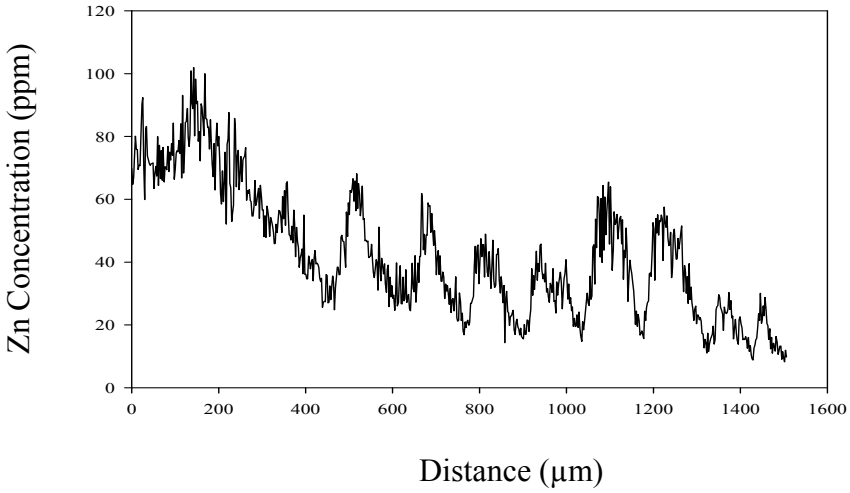

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL-4-67

Age: 9

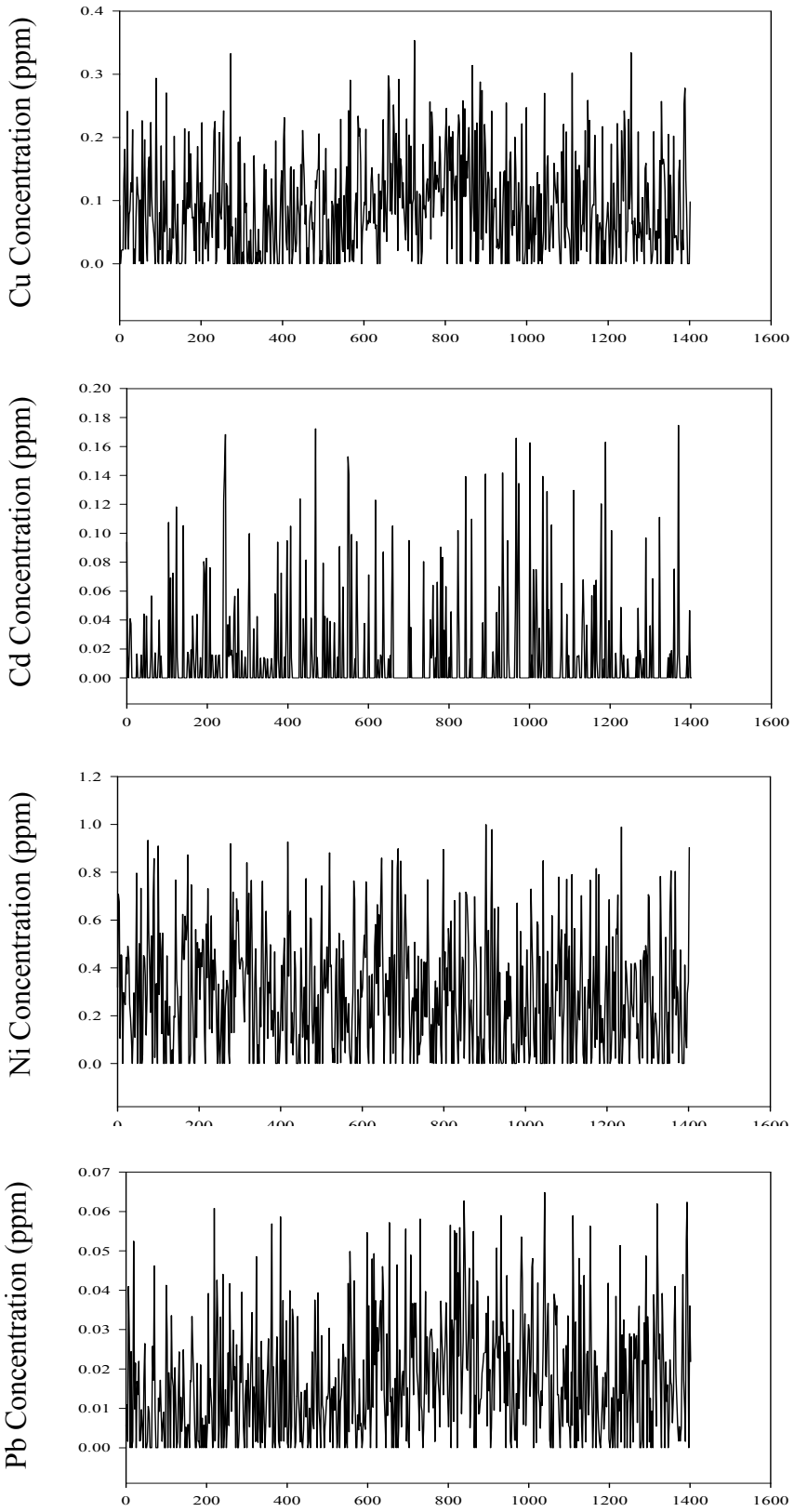

Cu Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (µm)

RL-4-67

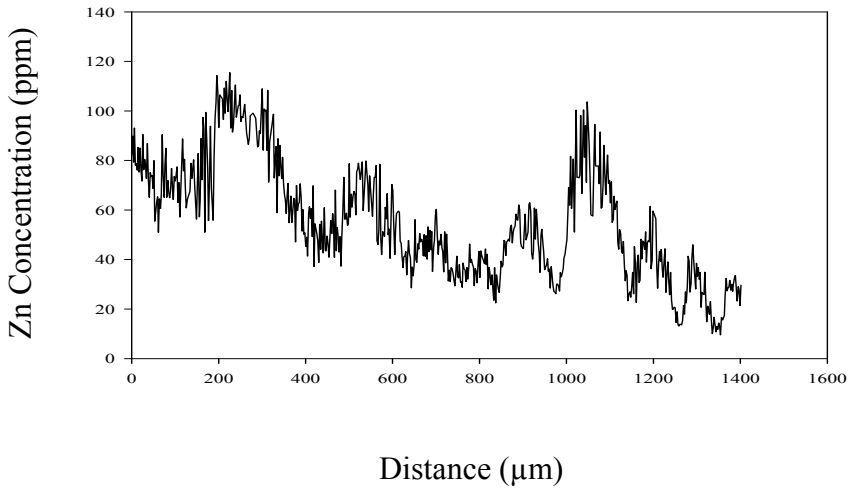

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-4-69

Age: 8

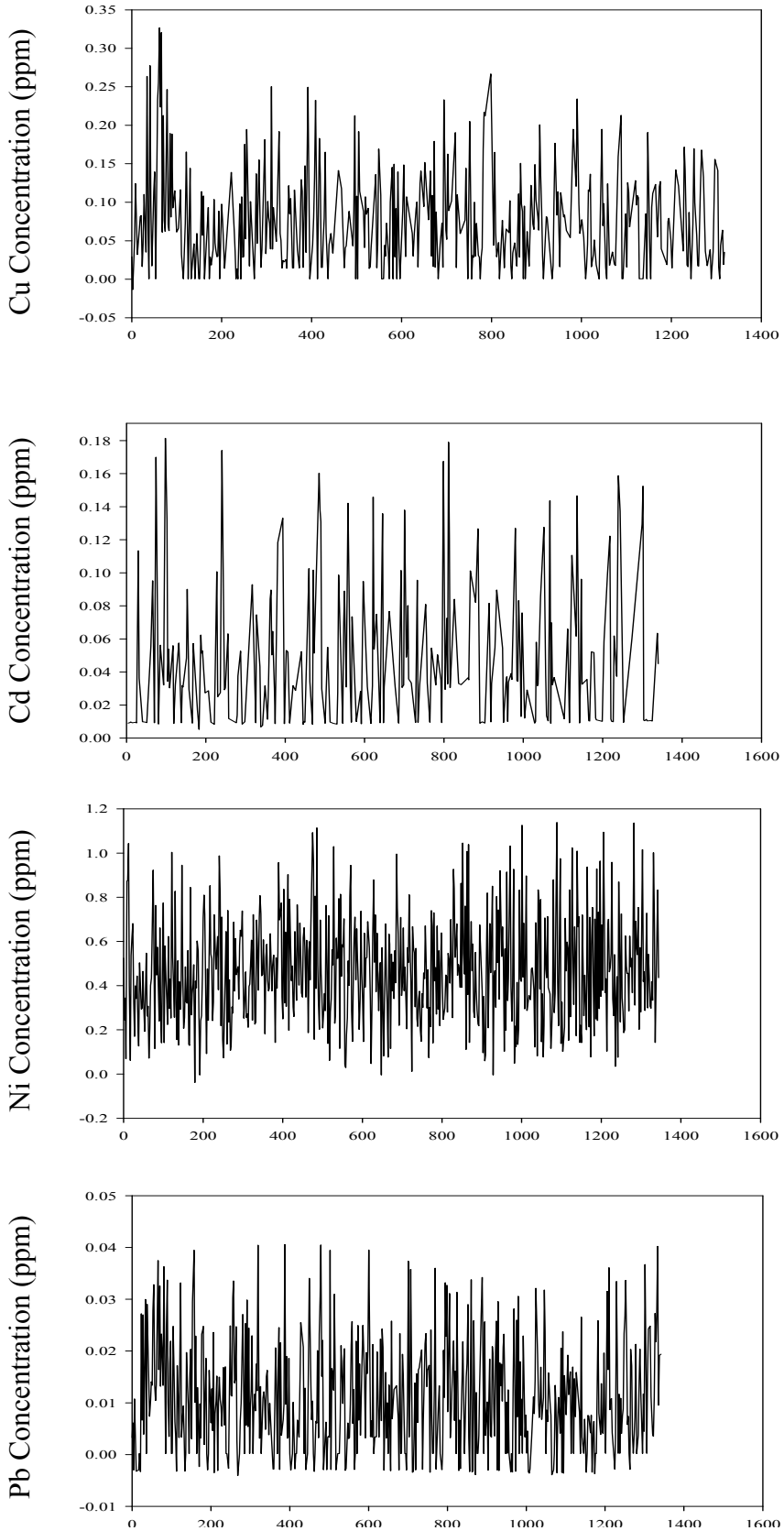

Cu Concentration (ppm)
0.4
0.3
0.2
0.1
0.0
Cd Concentration (ppm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

RL-4-69

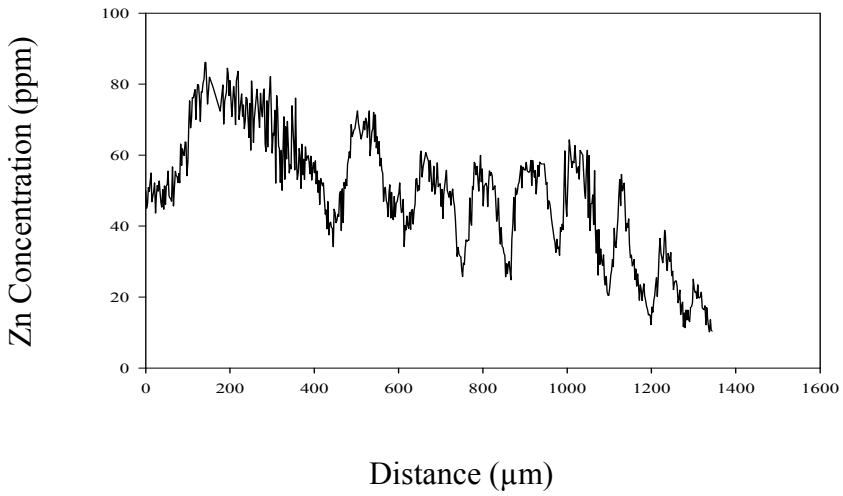

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-3-59

Age: 9+

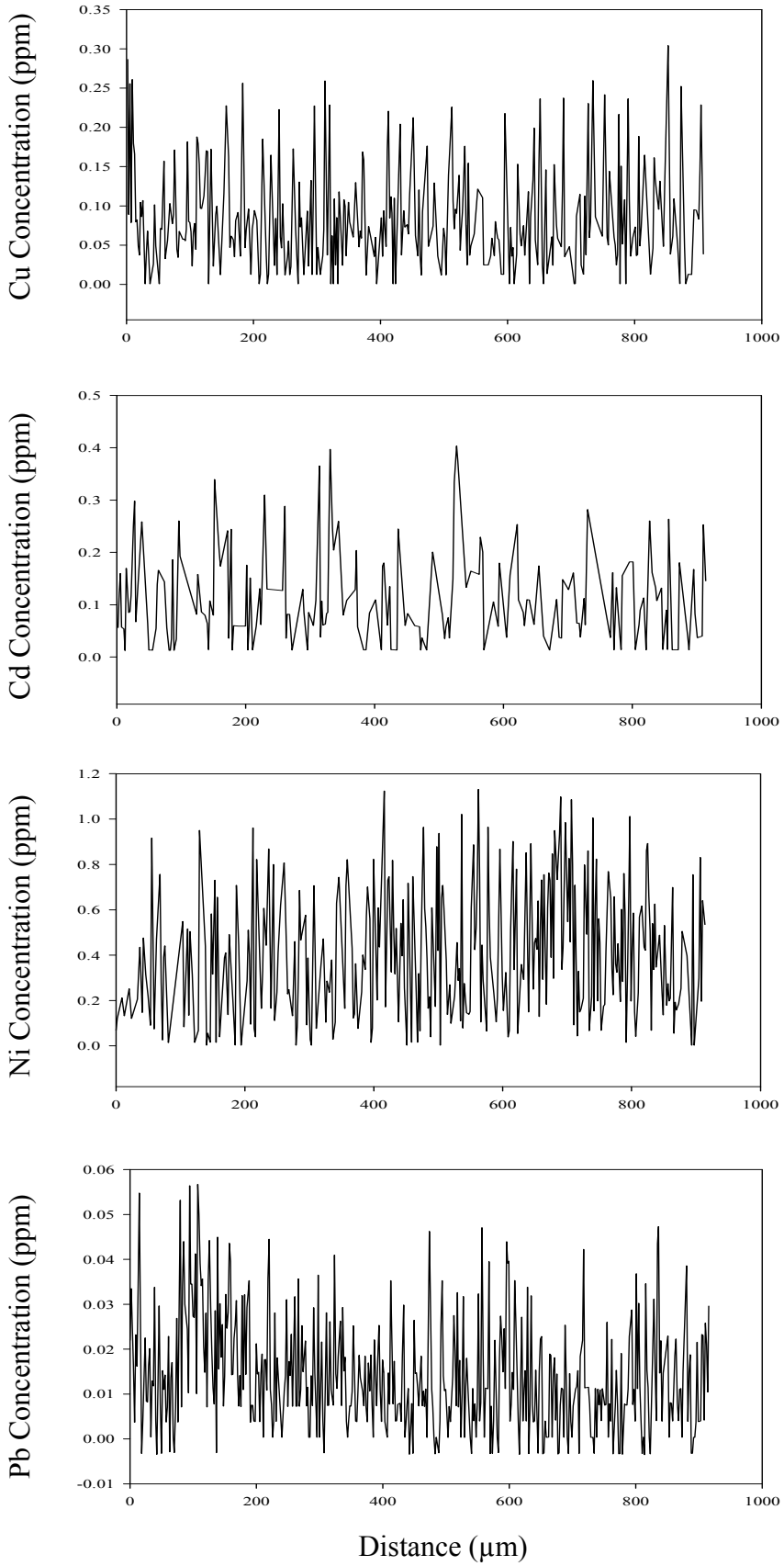

Cu Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
200
400
600
800
1000
1200
1400
1600
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
200
400
600
800
1000
1200
1400
1600
Pb Concentration (ppm)
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

RL-3-59

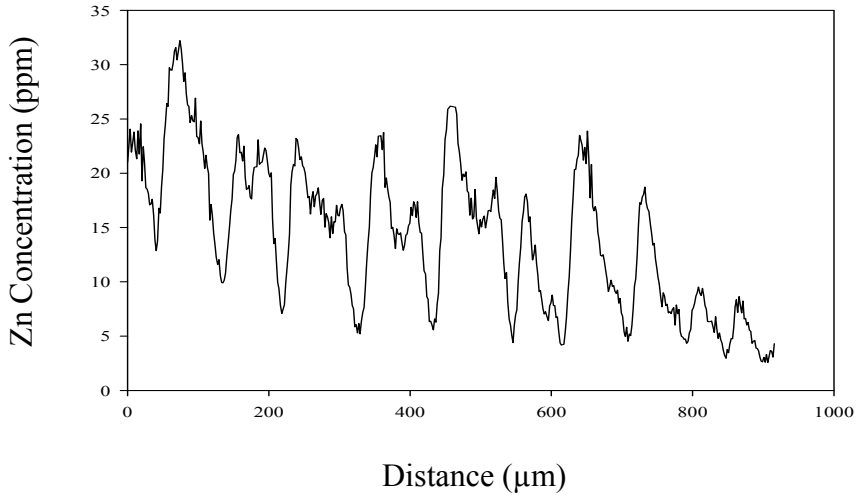

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-3-68

Age: 15+

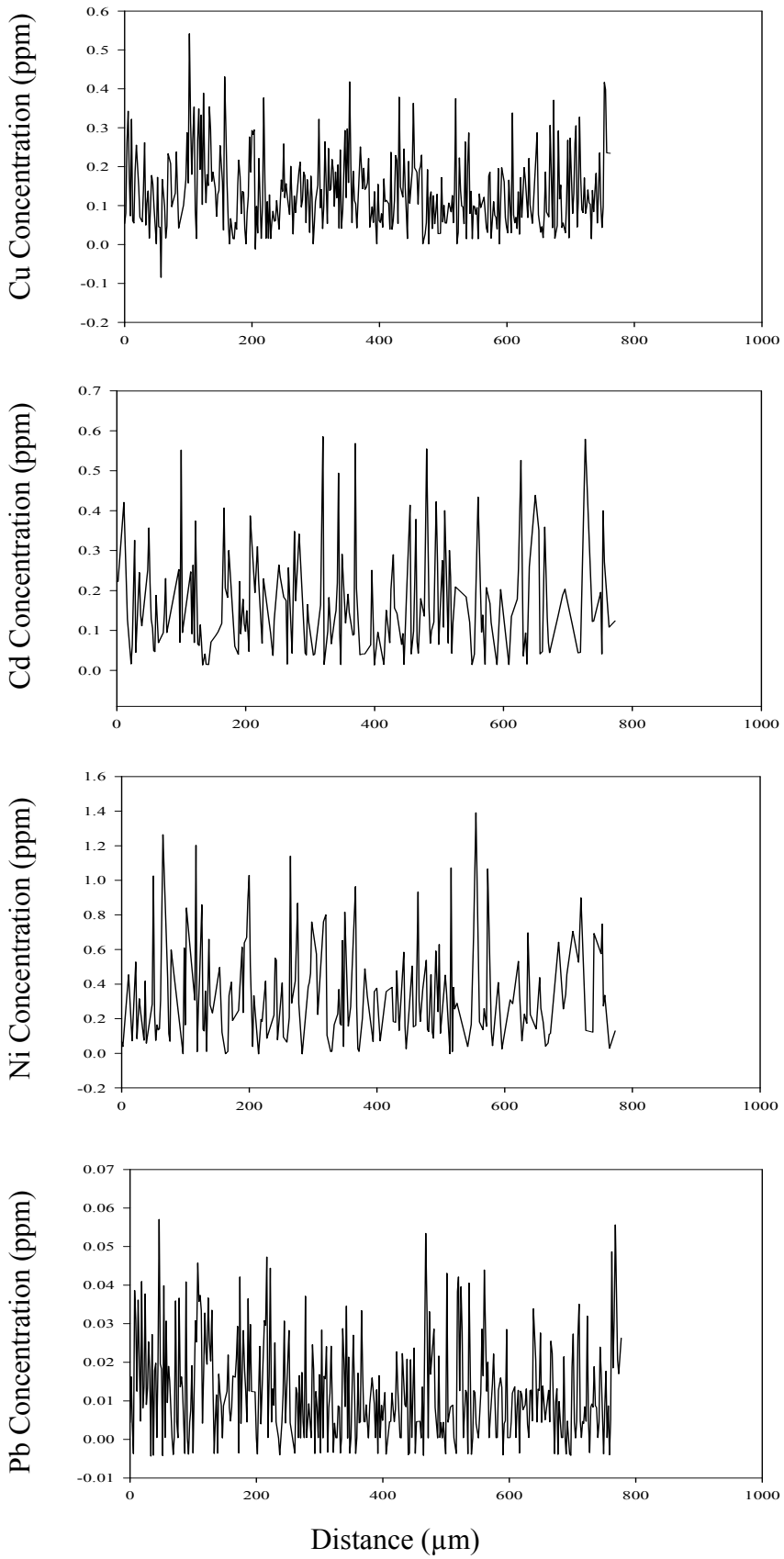

Cu Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
200
400
600
800
1000
Cd Concentration (ppm)
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
0
200
400
600
800
1000
Distance (μm)

RL-3-68

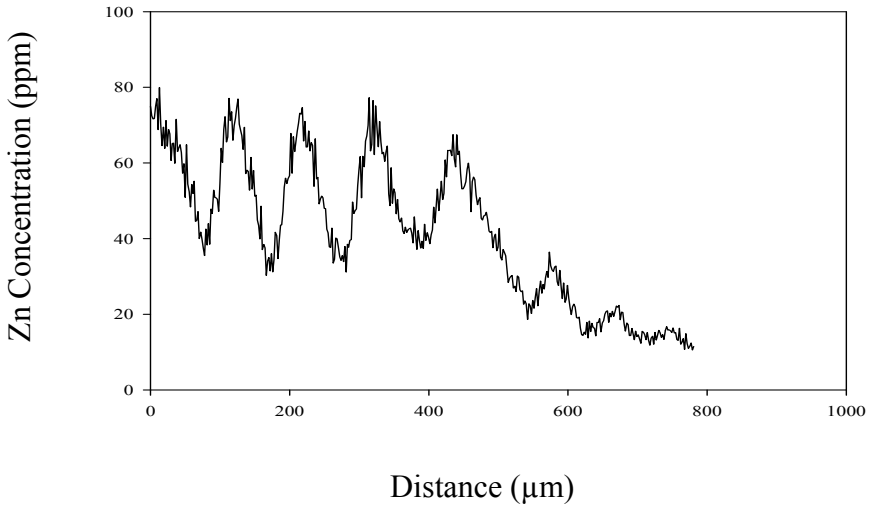

Zn Concentration (ppm)
35
30
25
20
15
10
5
0
0
200
400
600
800
1000
Distance (µm)

RL-3-75

Age: 9+

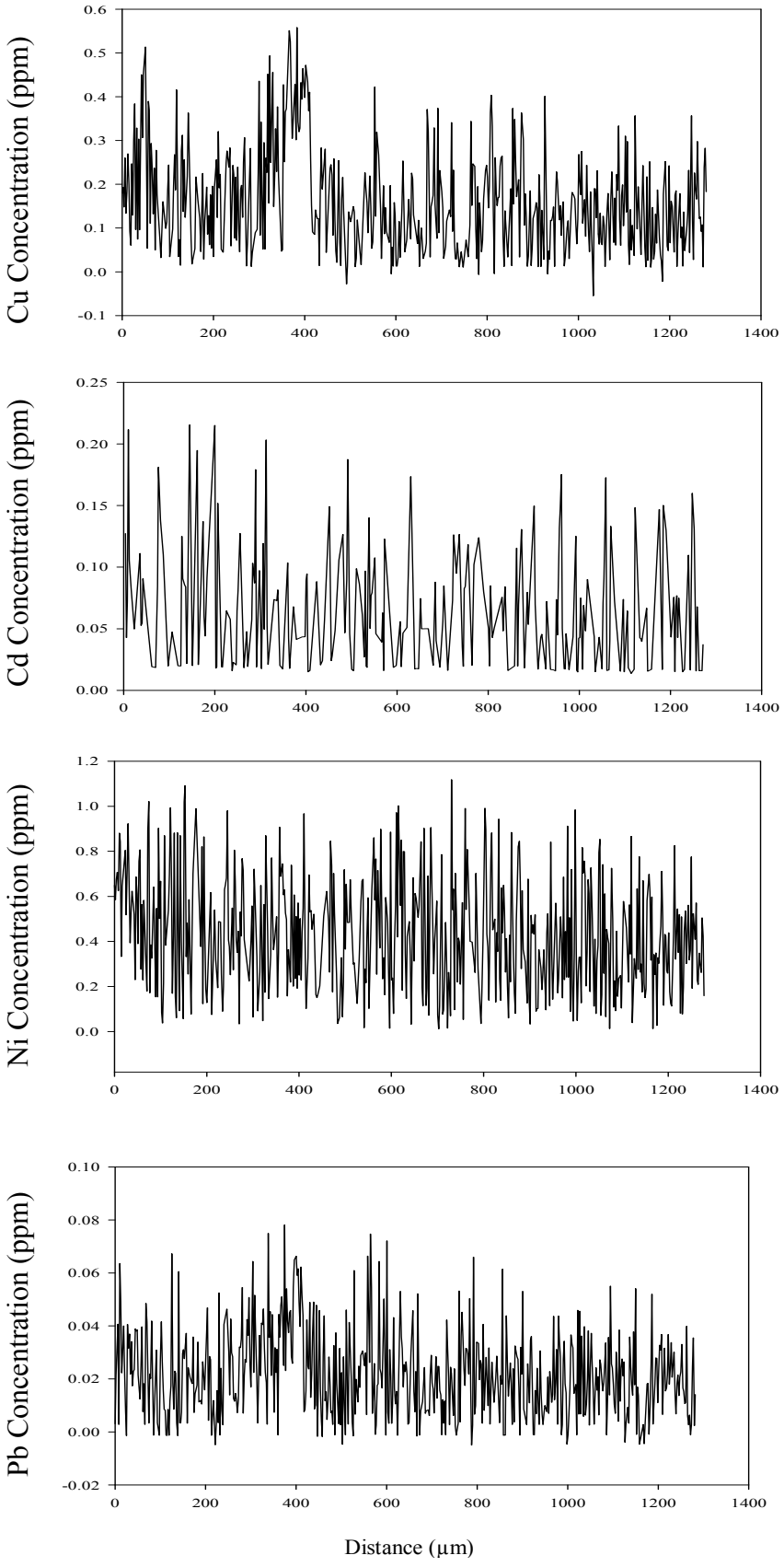

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

RL-3-75

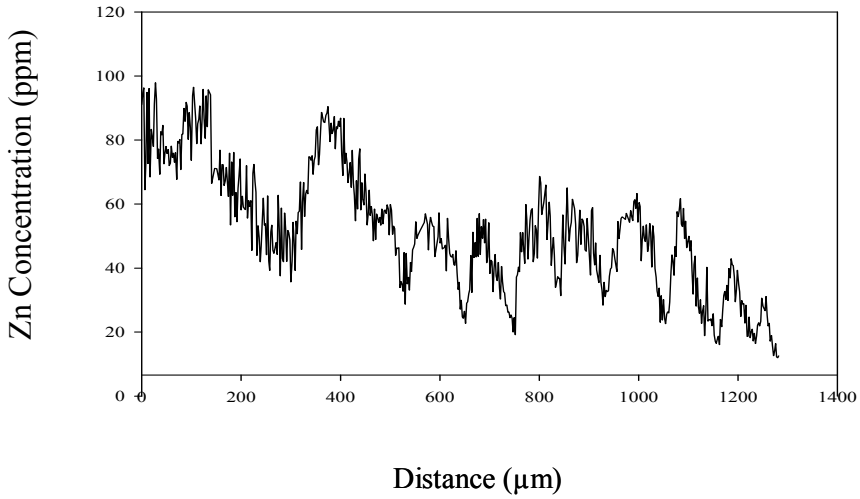

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
Distance (μm)

RL-3-76

Age: 10

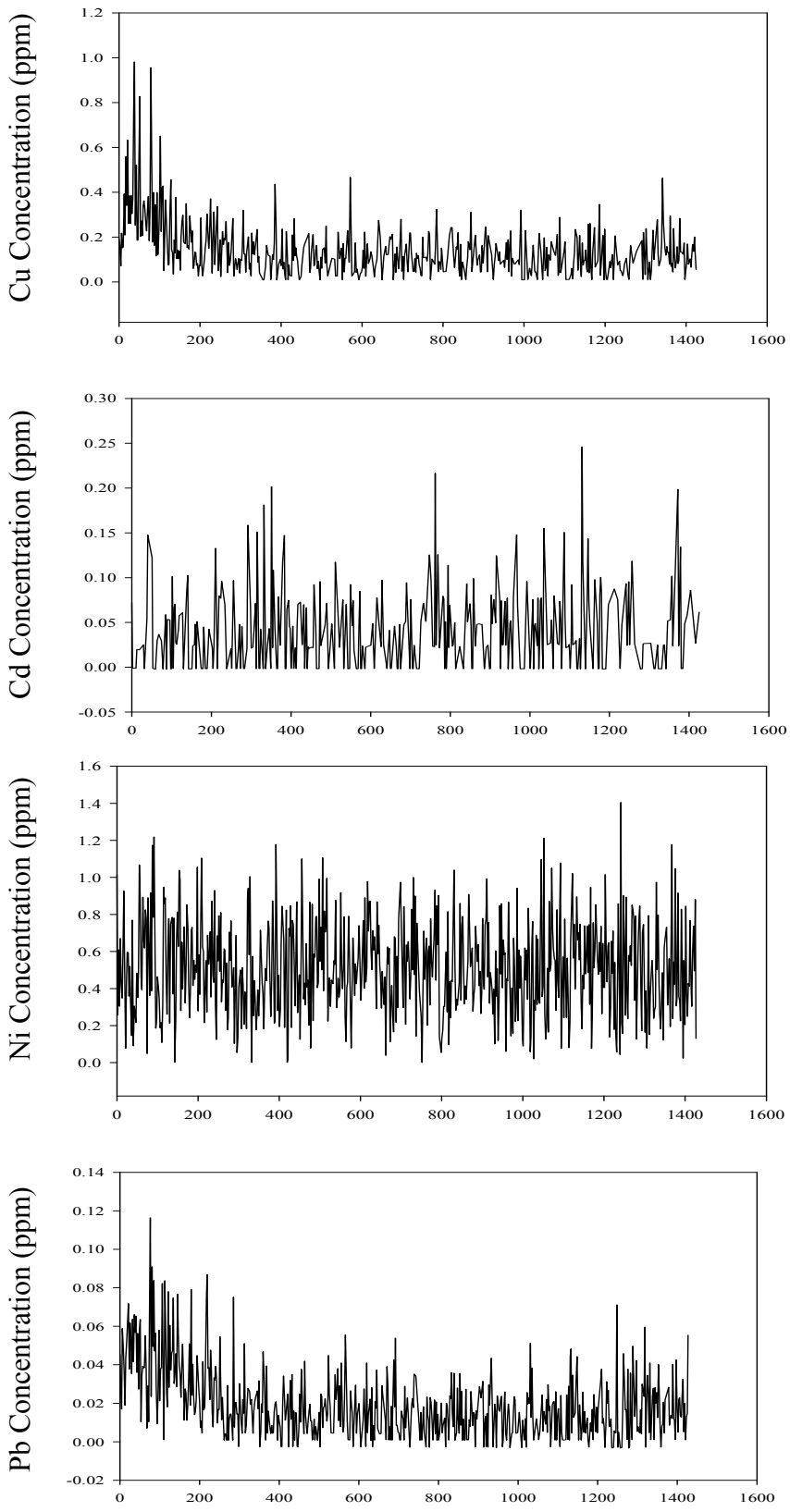

Cu Concentration (ppm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
Distance (μm)

RL-3-76

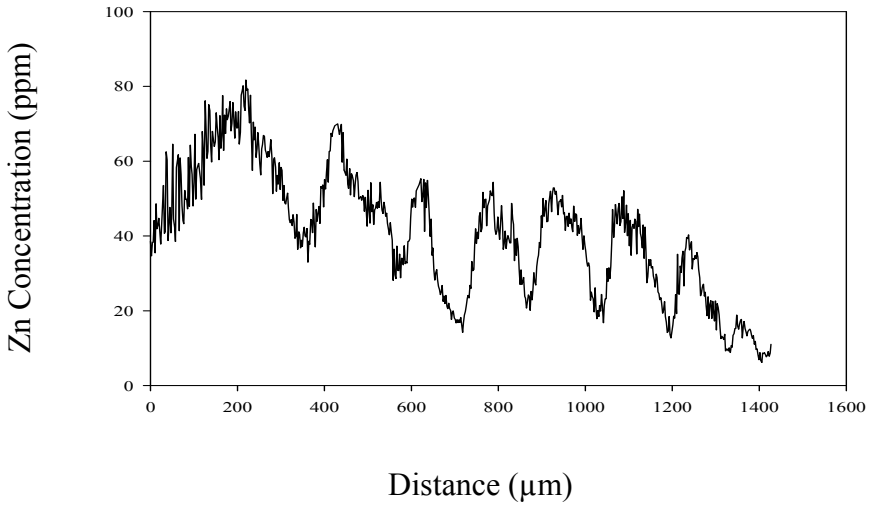

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (µm)

RL-3-77

Age: 10

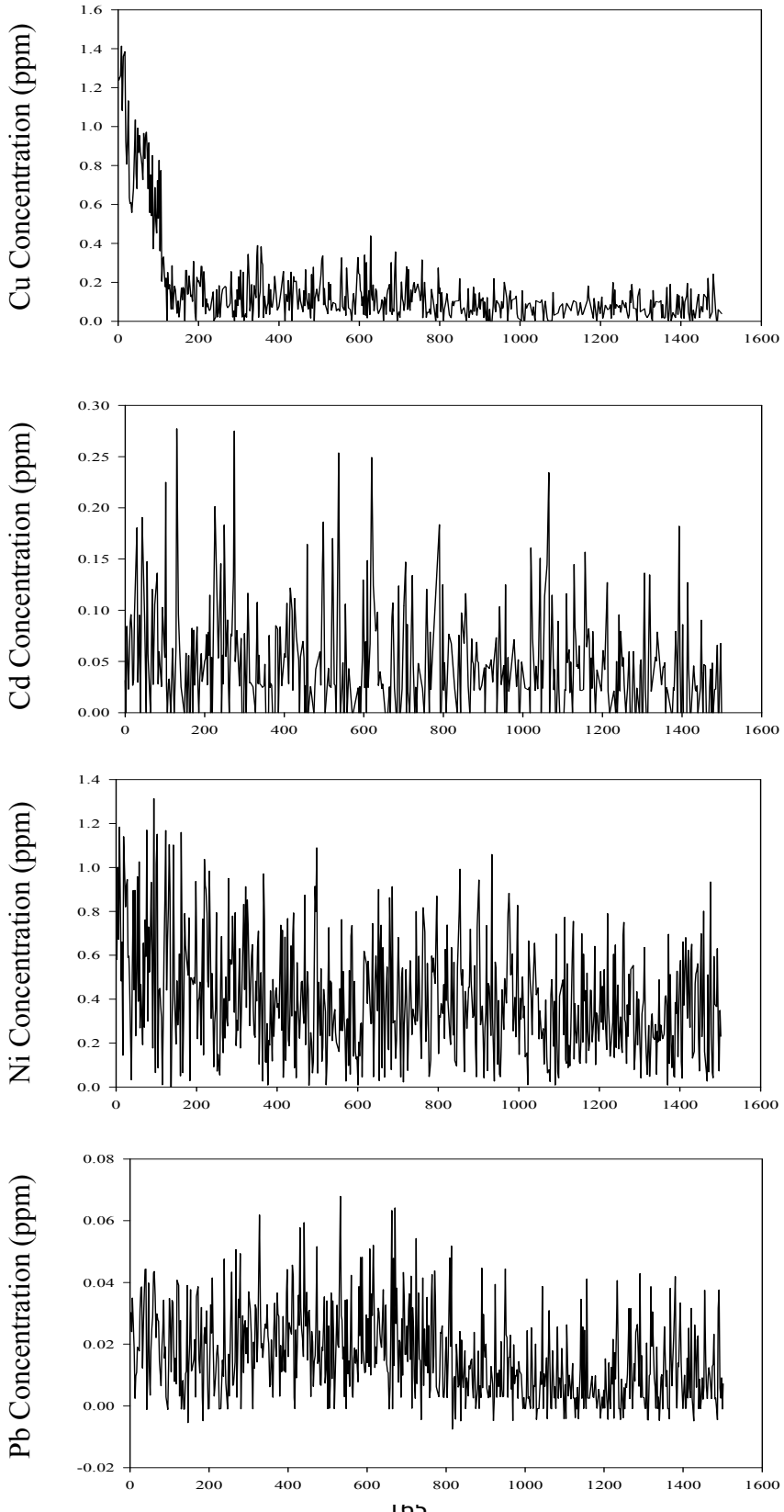

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

RL-3-77

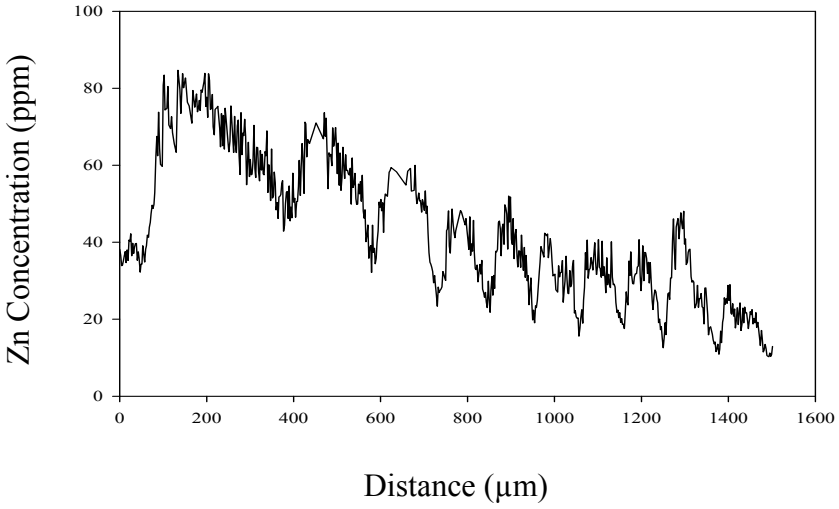

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-3-78

Age: 16+

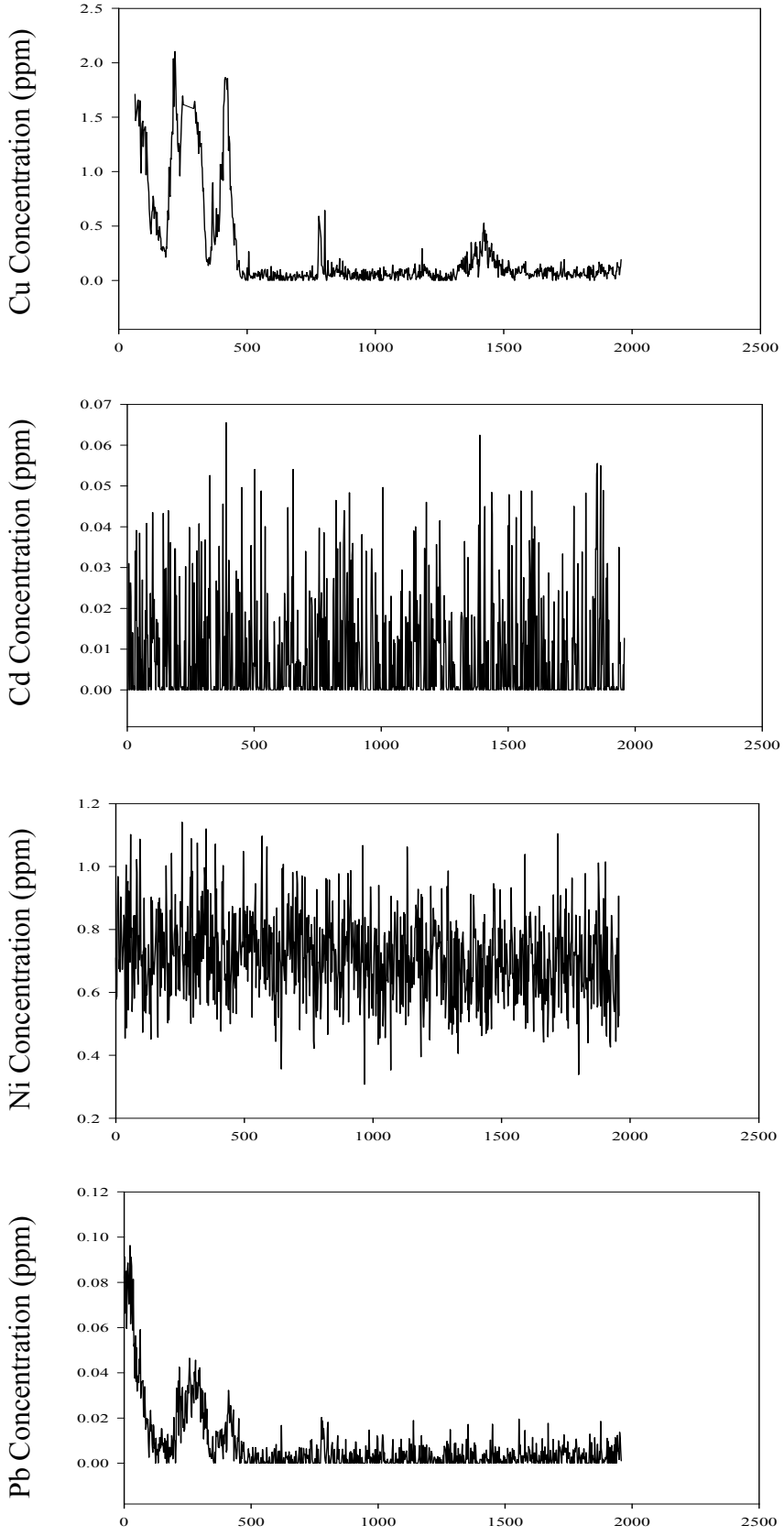

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

RL-3-78

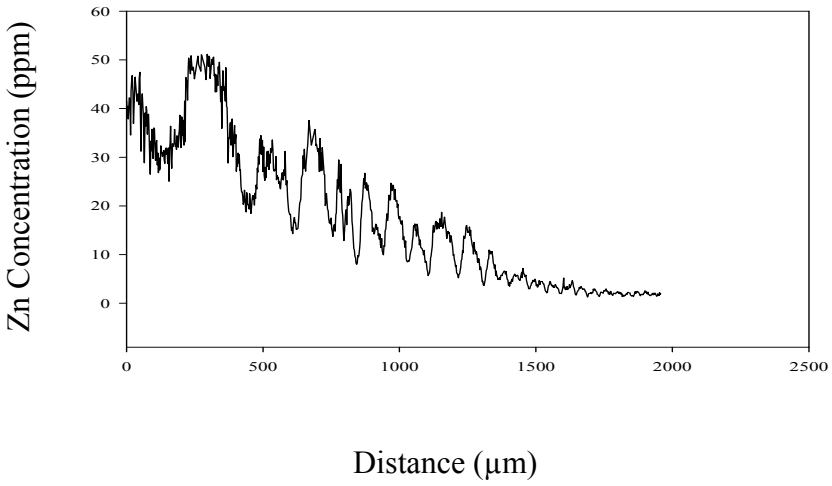

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

RL-1x-7

Age: 14+

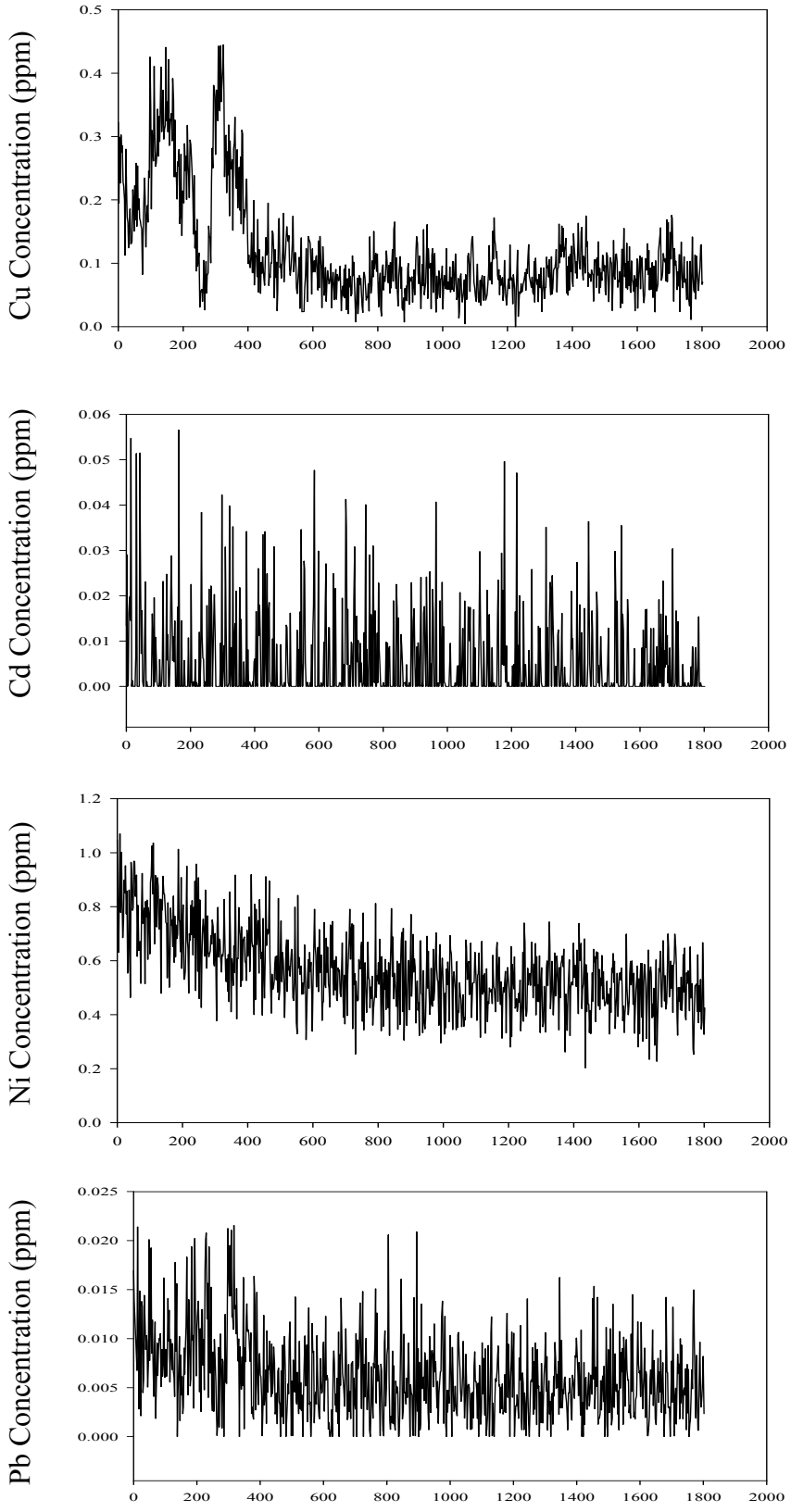

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

RL-1x-7

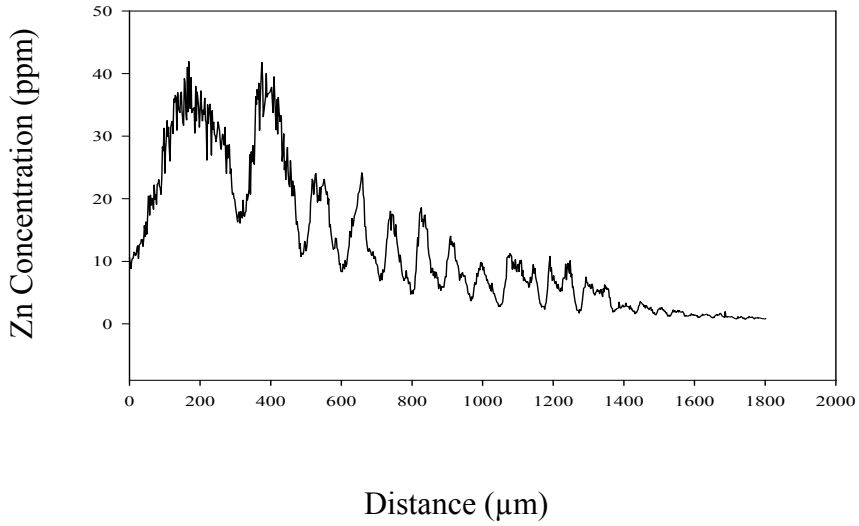

Zn Concentration (ppm)
60
50
40
30
20
10
0
0
500
1000
1500
2000
2500
Distance (μm)

RL-1x-40

Age: 28+

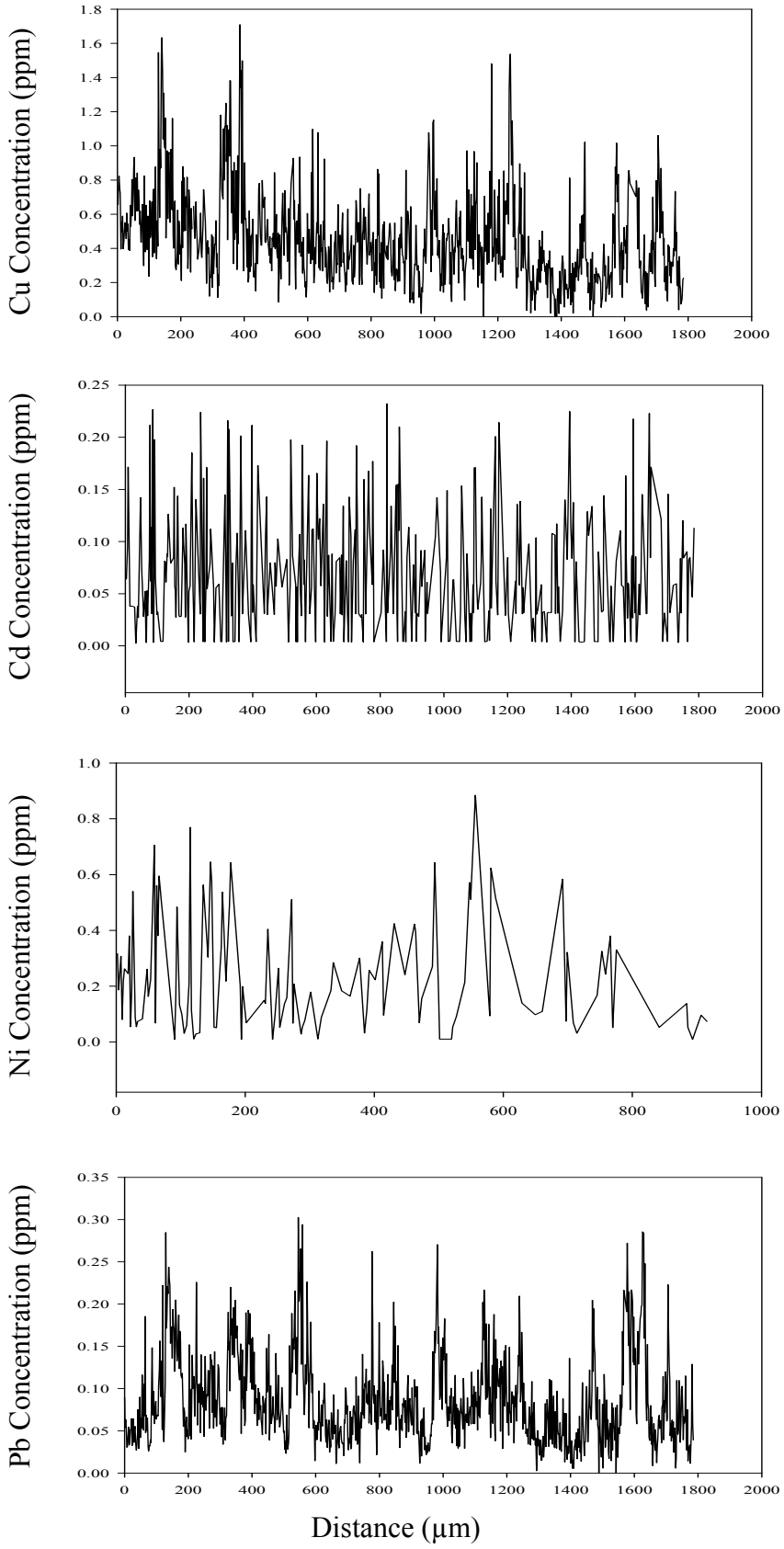


RL-1x-40

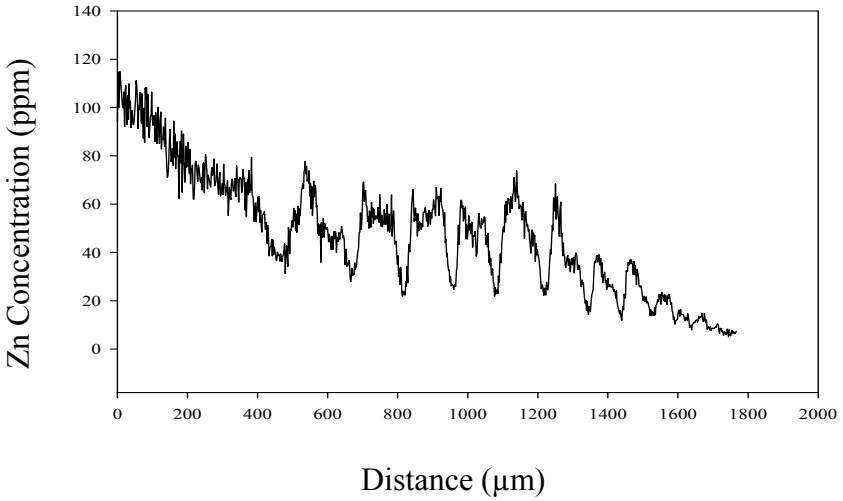

Zn Concentration (ppm)
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (µm)

RL-1-01

Age: 32+

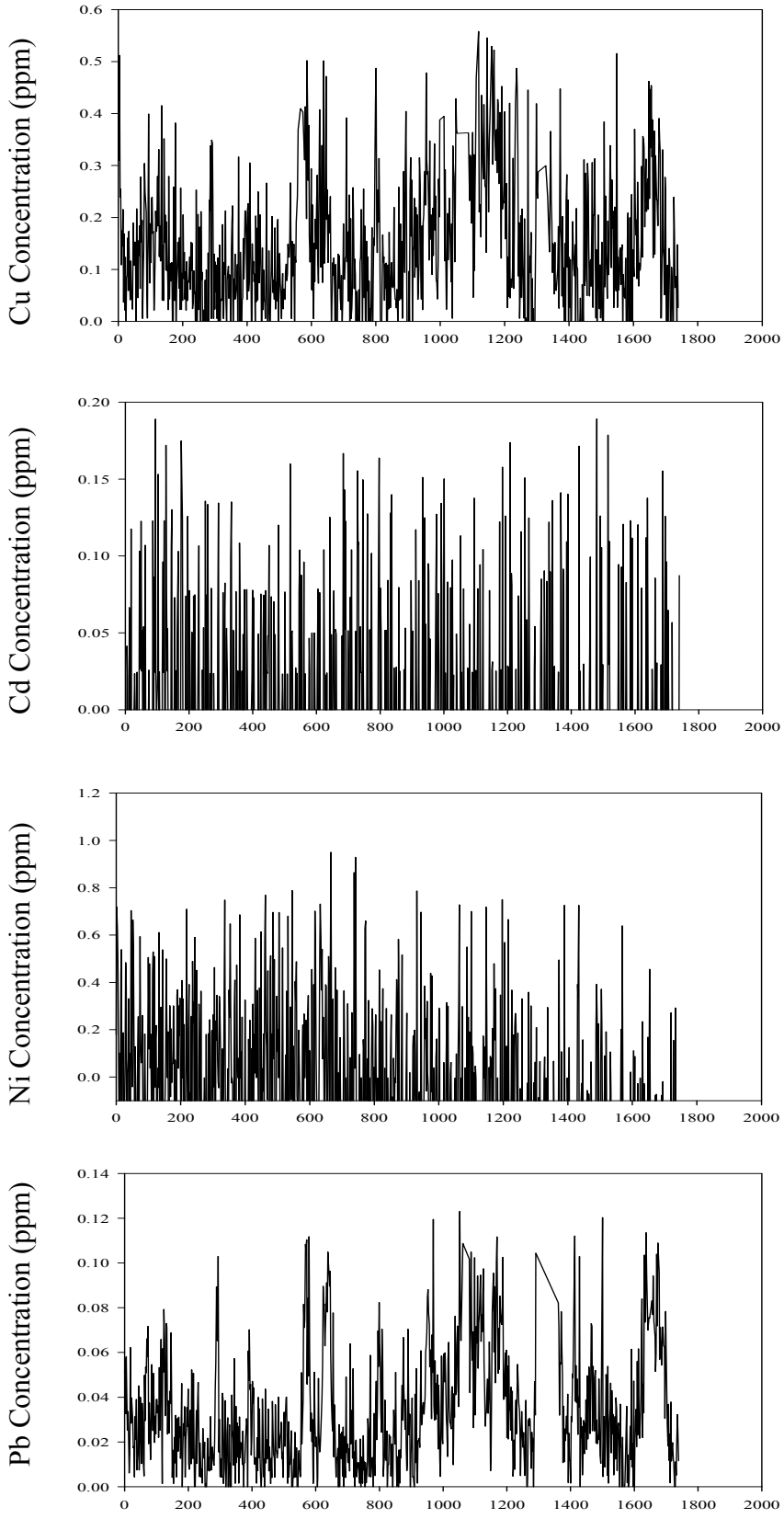

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

RL-1-01

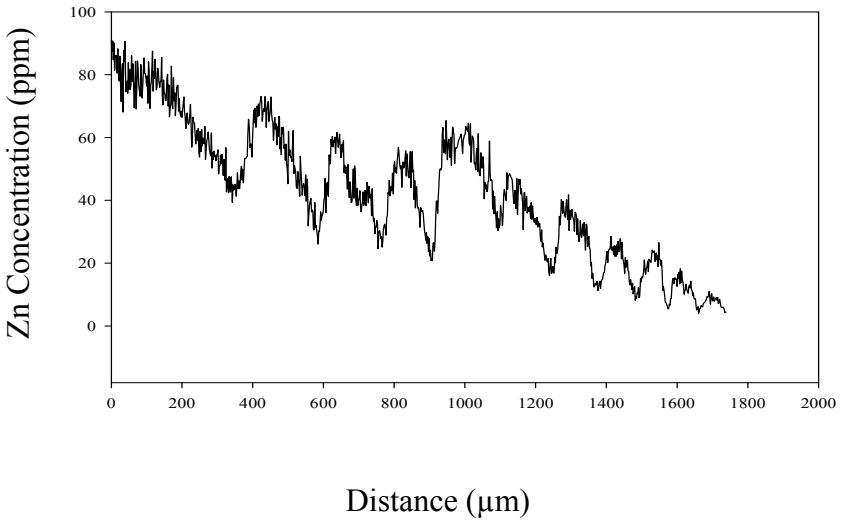

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL-1-02

Age: 23+

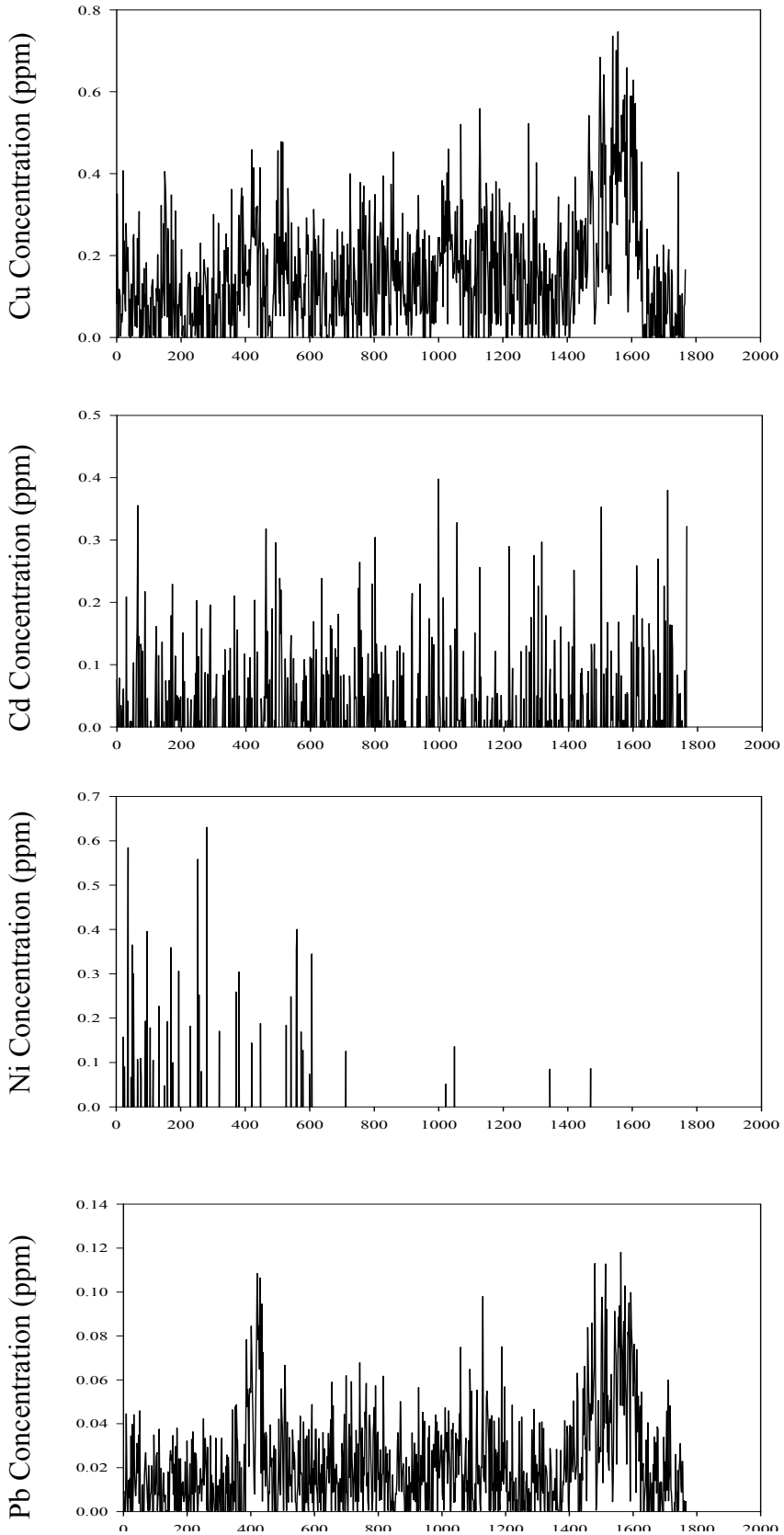

Cu Concentration (ppm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Cd Concentration (ppm)
0.00
0.05
0.10
0.15
0.20
Ni Concentration (ppm)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Pb Concentration (ppm)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL-1-02

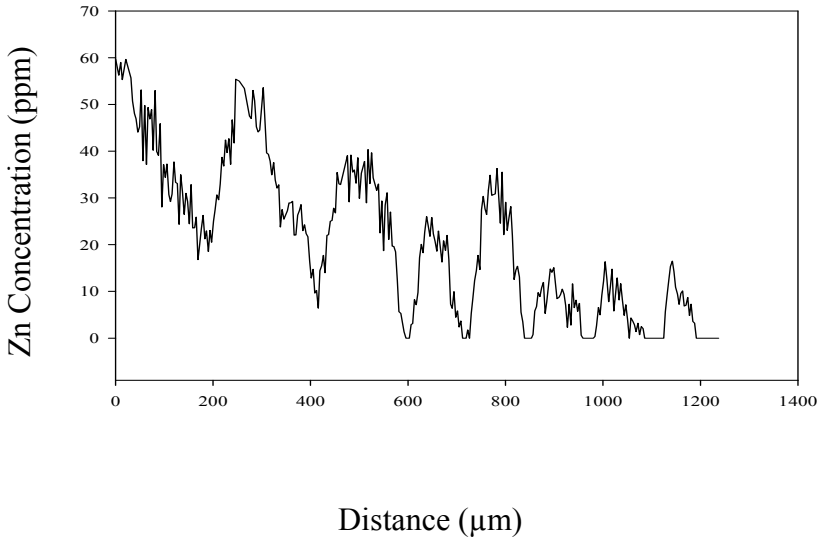

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL-1-03

Age: 28+

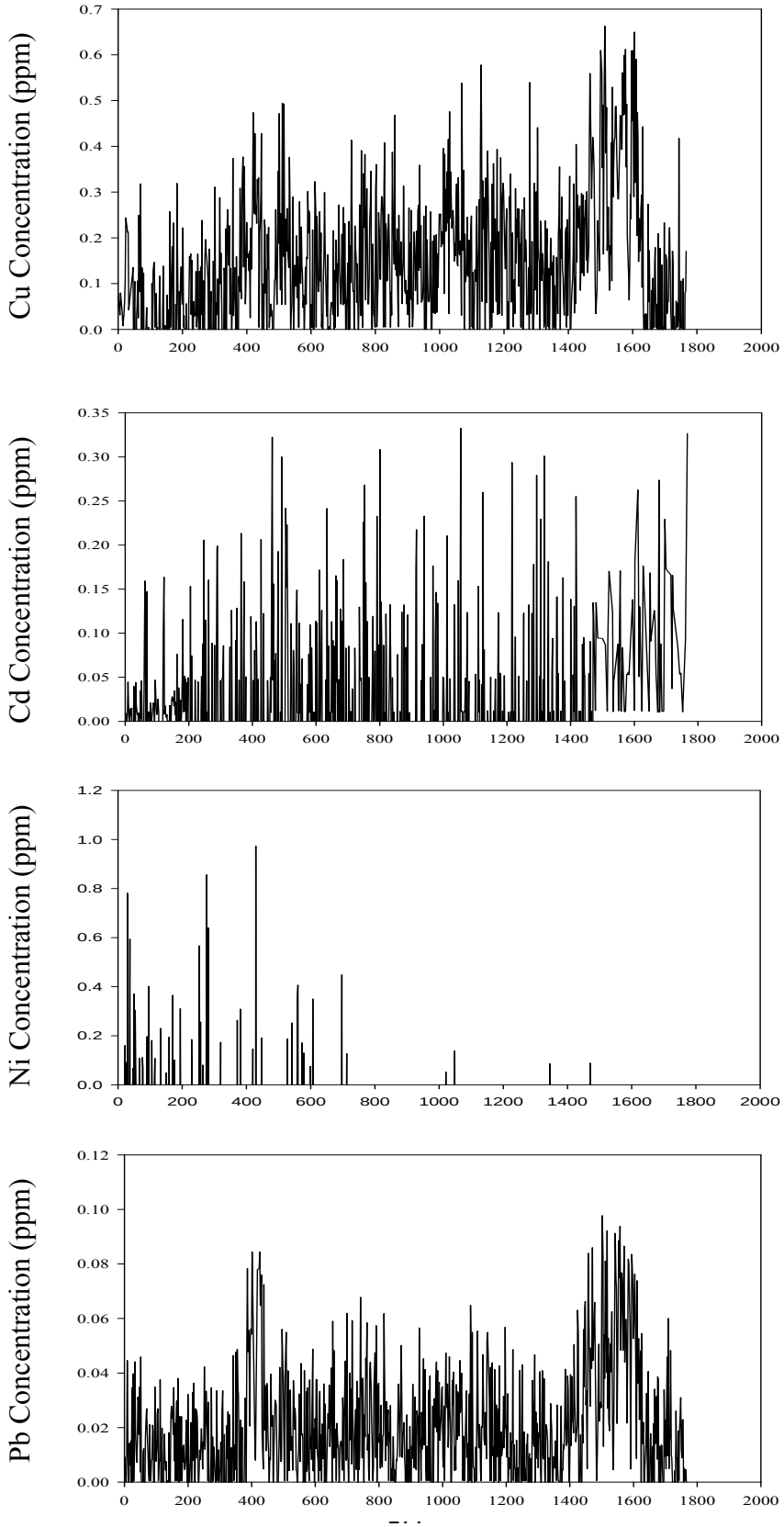

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

RL-1-03

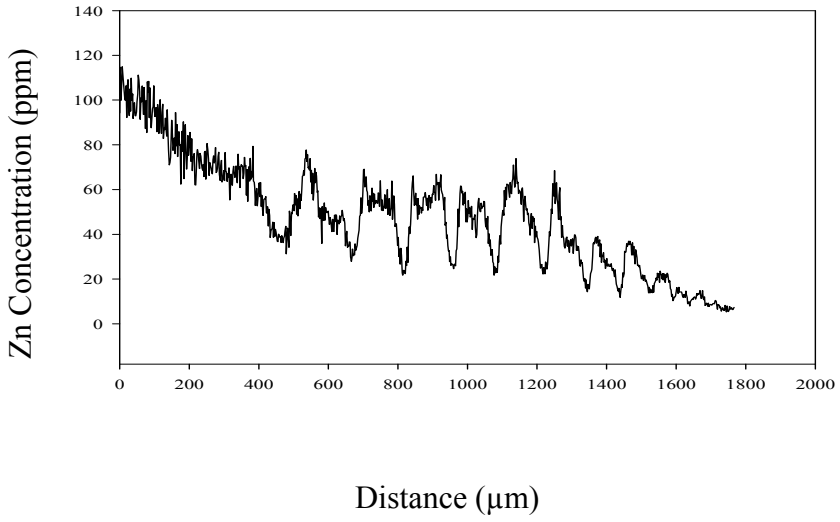

Zn Concentration (ppm)
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

RL-1-04

Age: 25+

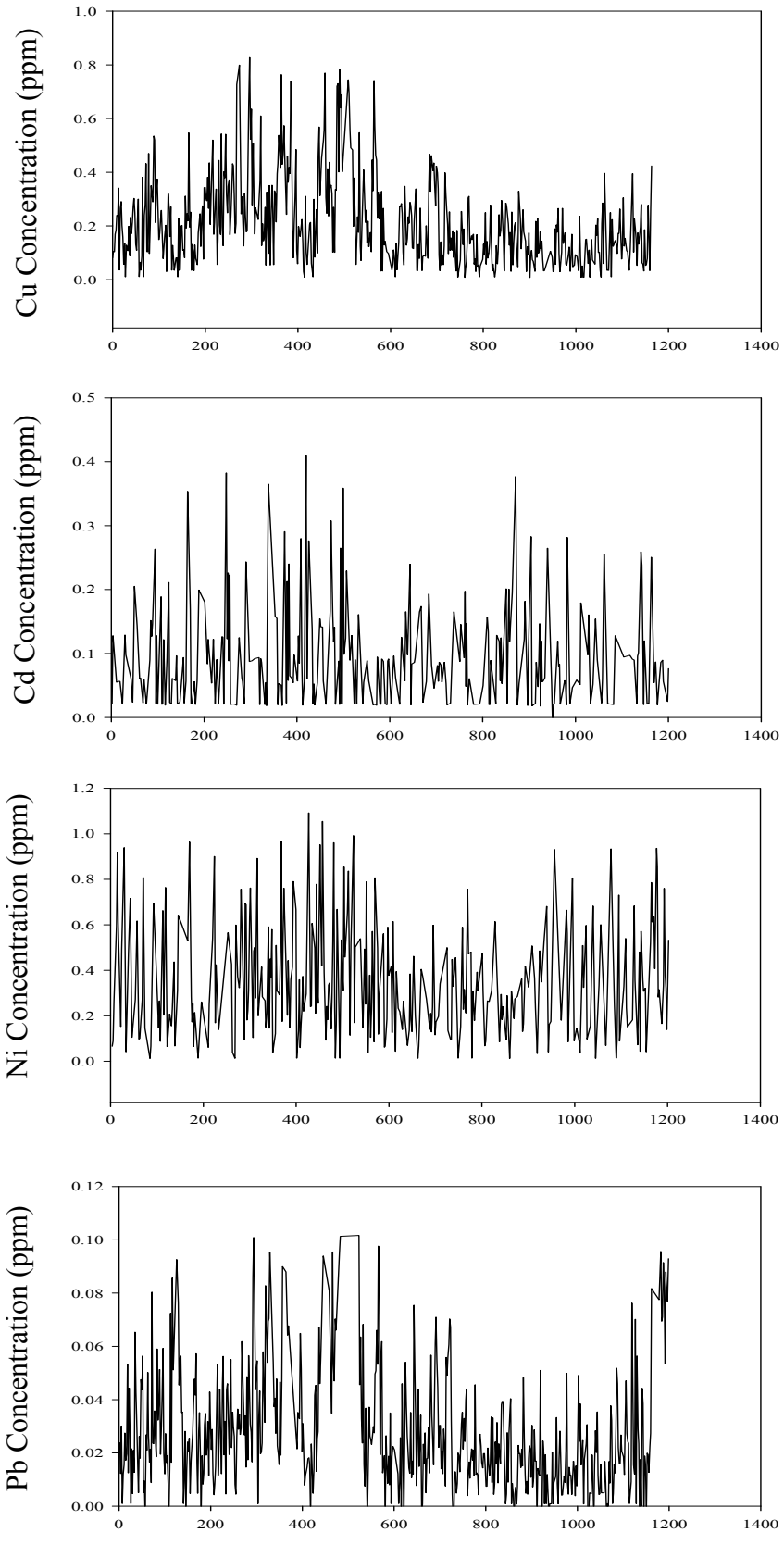

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

RL-1-04

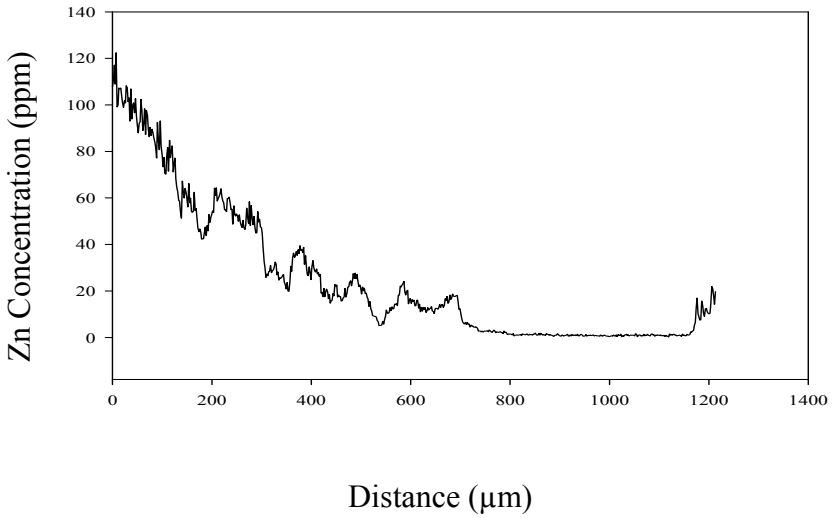

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL-1-06

Age: 23+

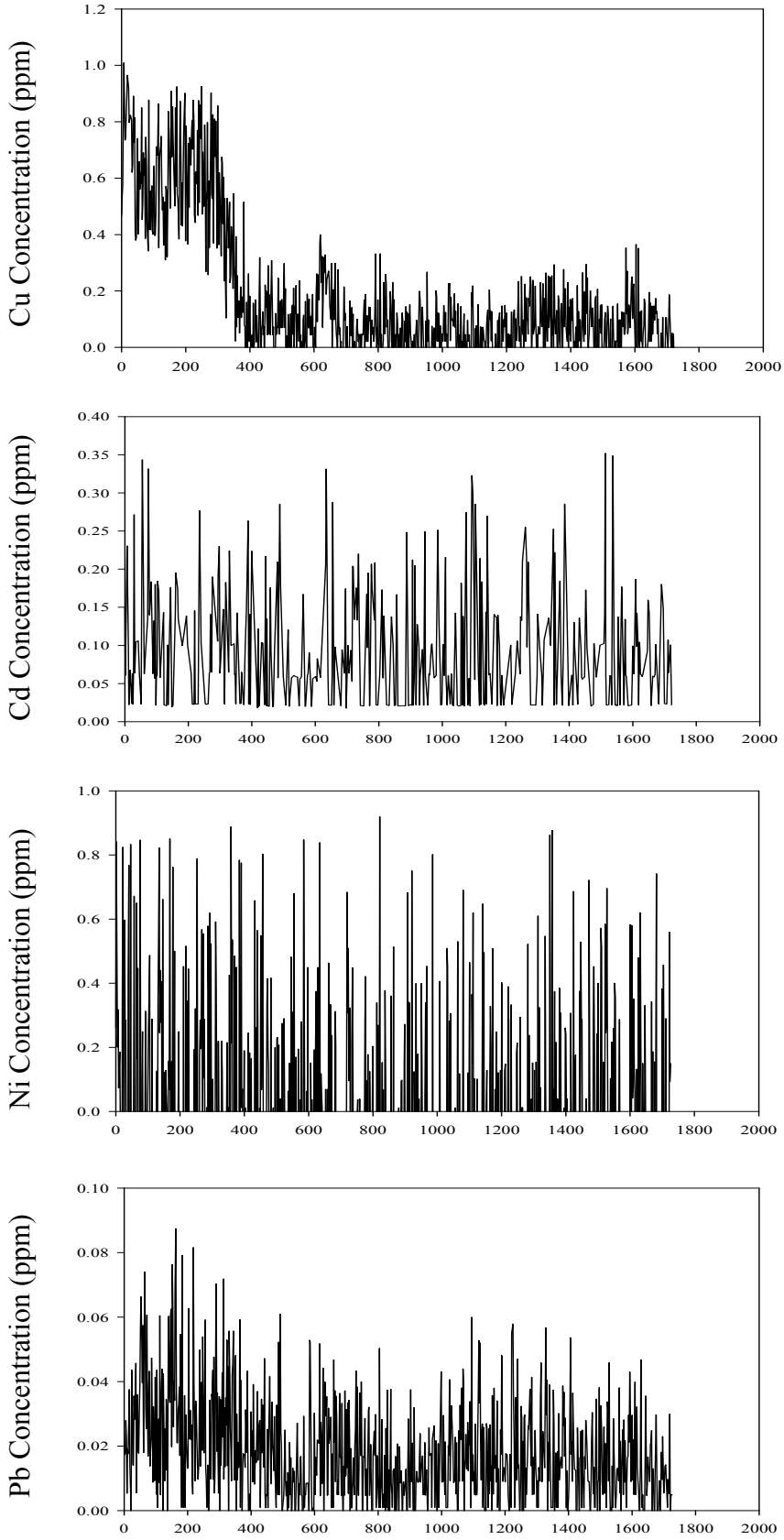

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

RL-1-06

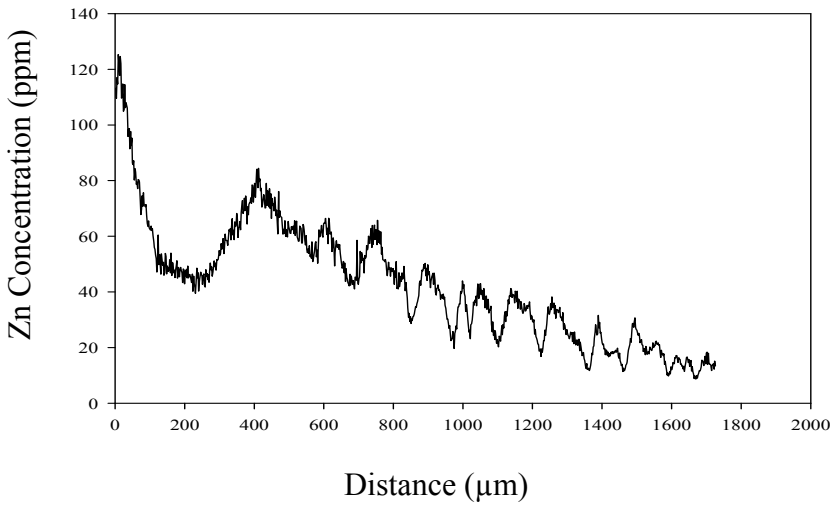

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

RL-1-09

Age: 29+

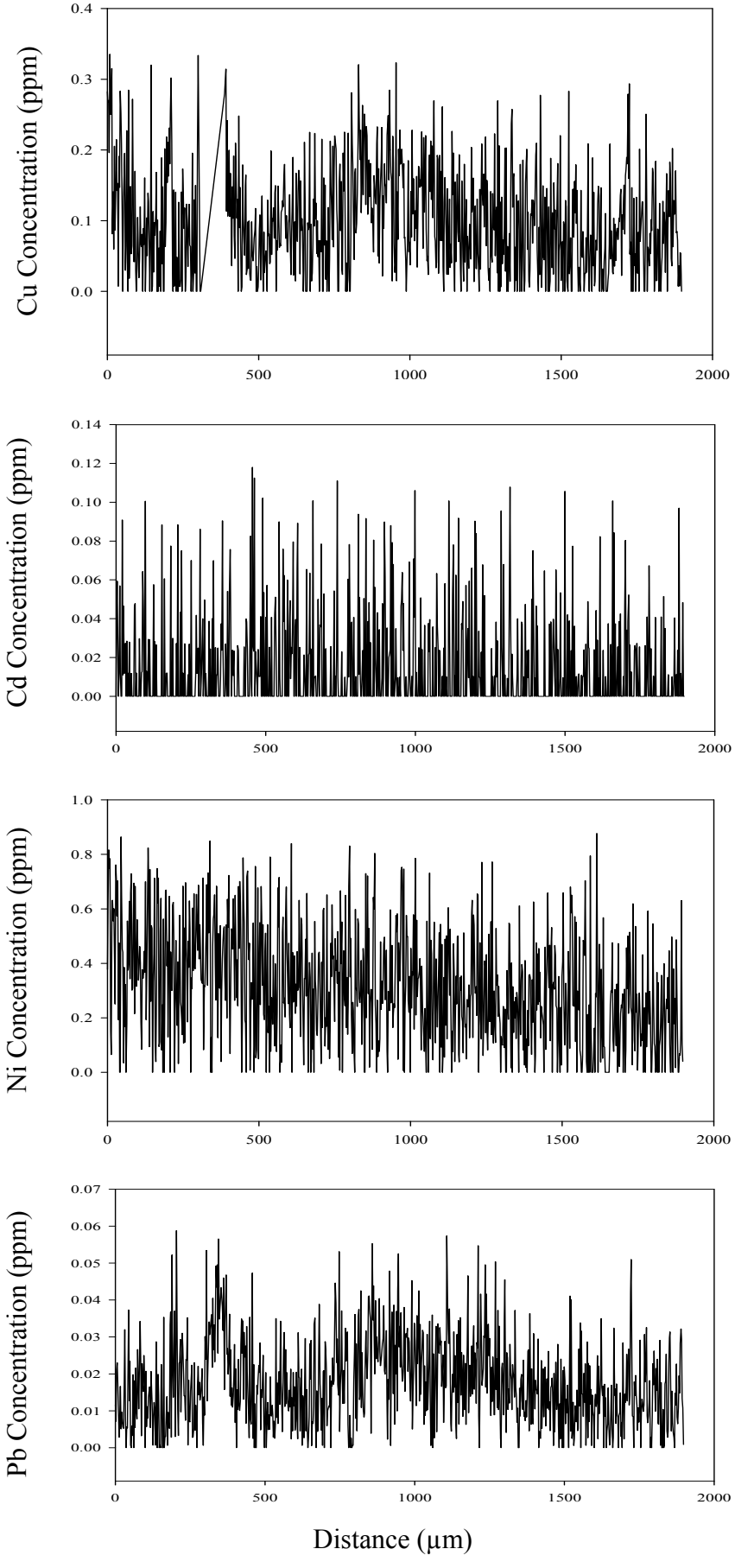

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

RL-1-09

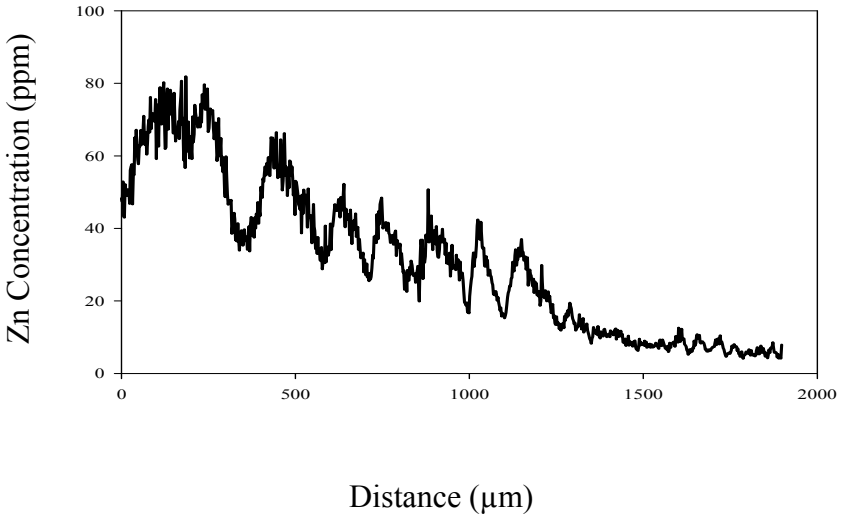

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL08-0134

Age: 28+

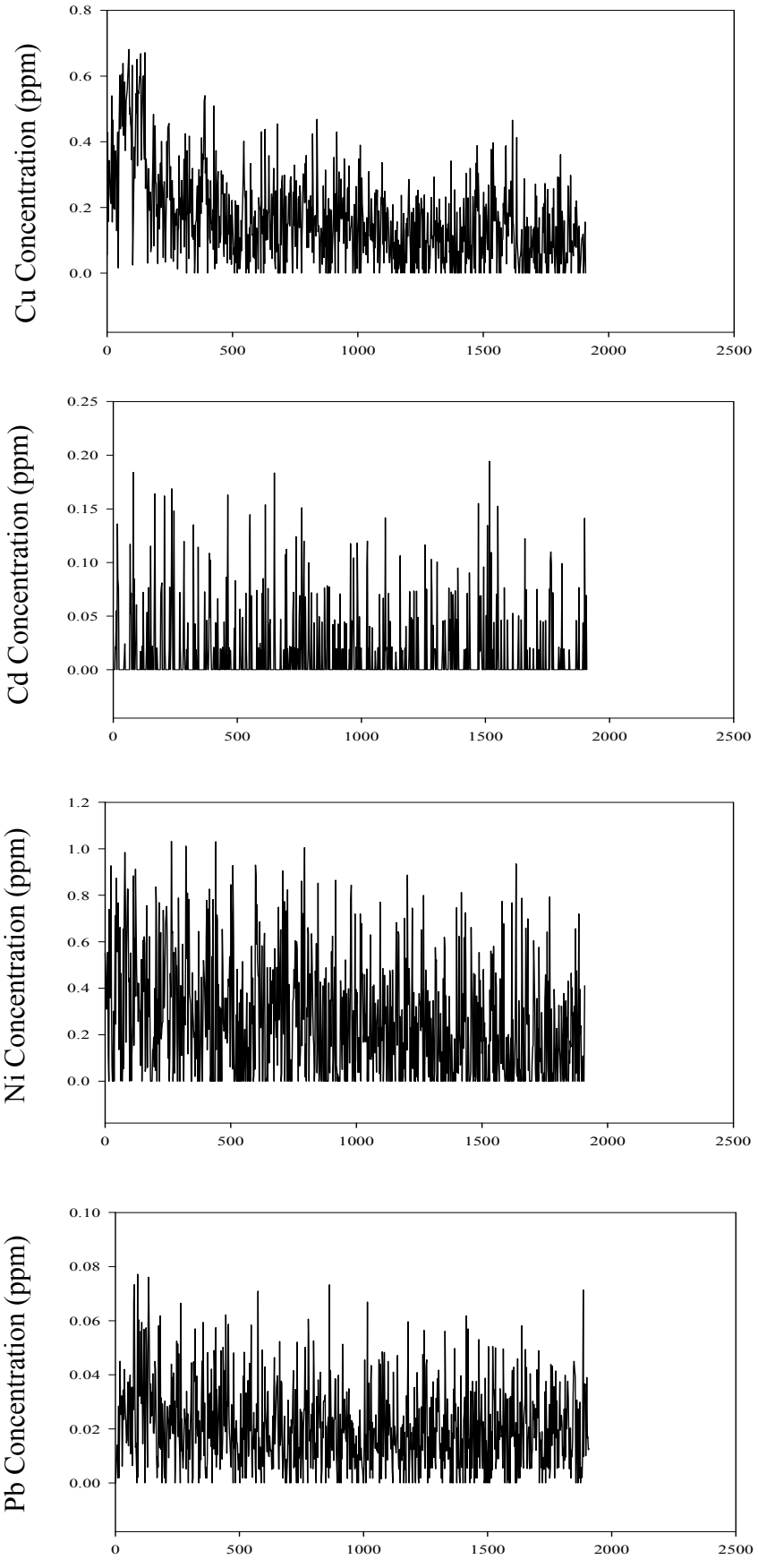


RL08-0134

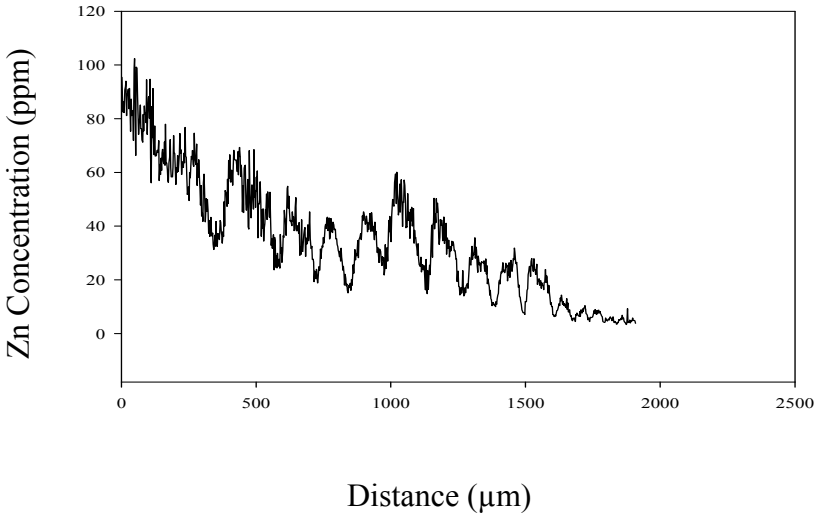

Zn Concentration (ppm)
100
80
60
40
20
0
0
500
1000
1500
2000
Distance (μm)

RL08-4022

Age: 25+

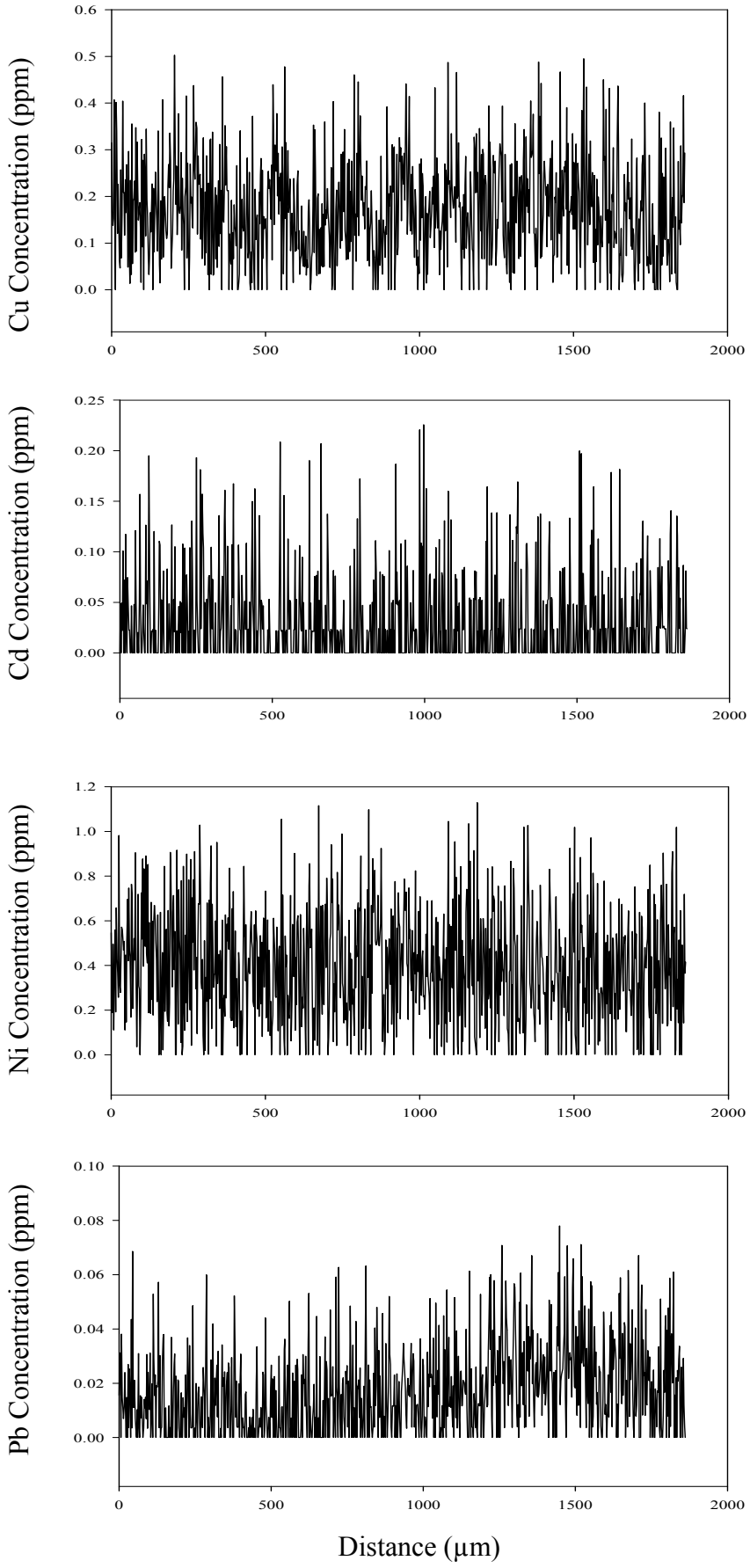


RL08-4022

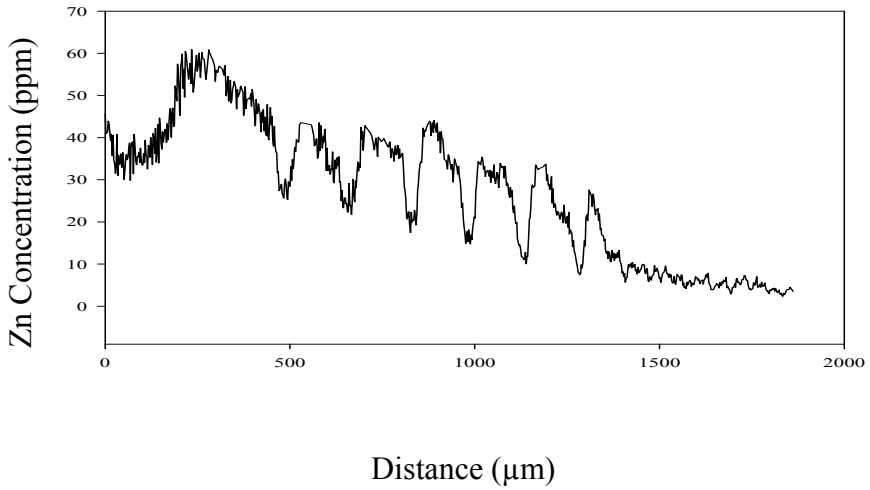

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

RL08-4029

Age: 25+

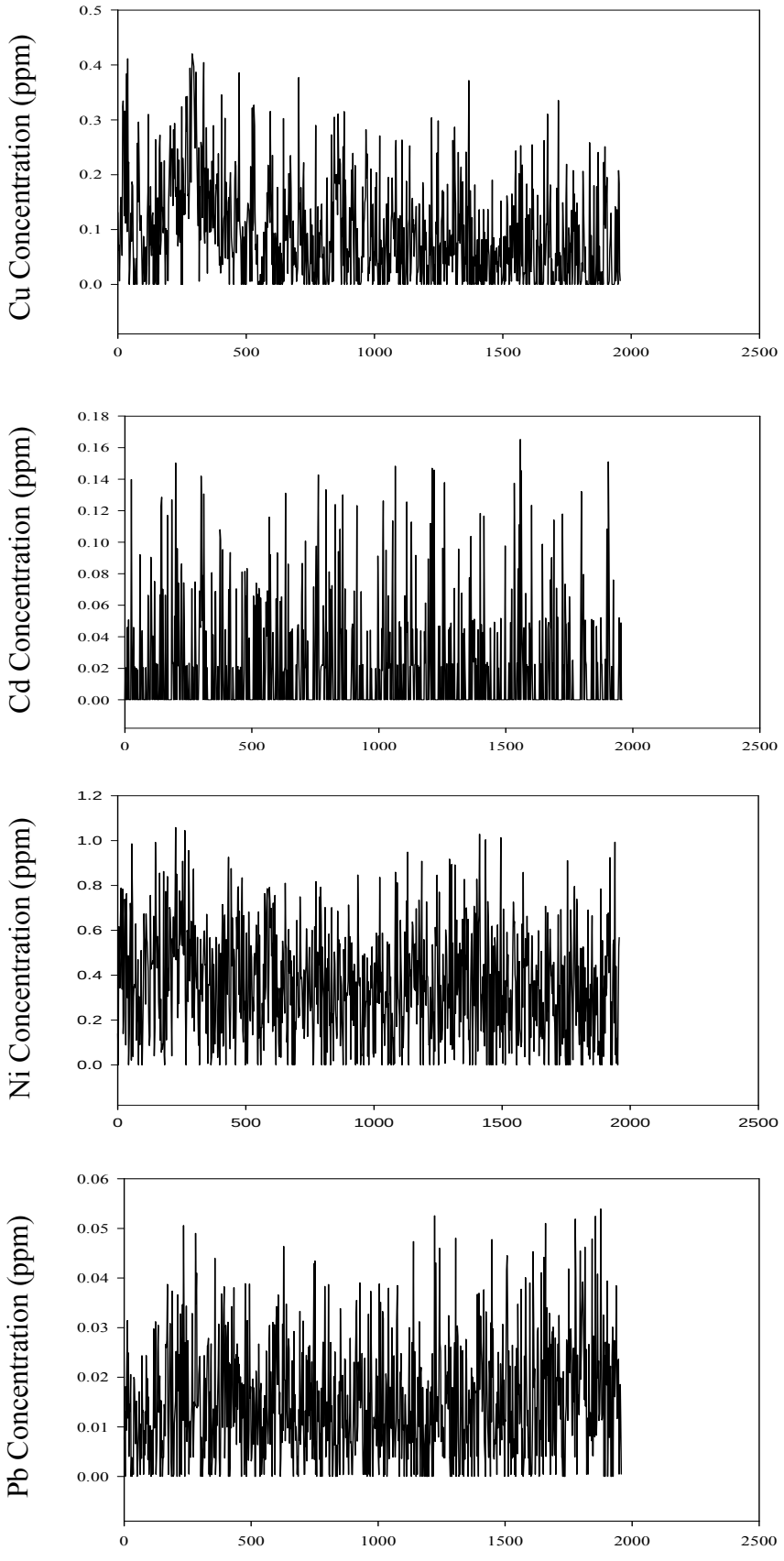


RL08-4029

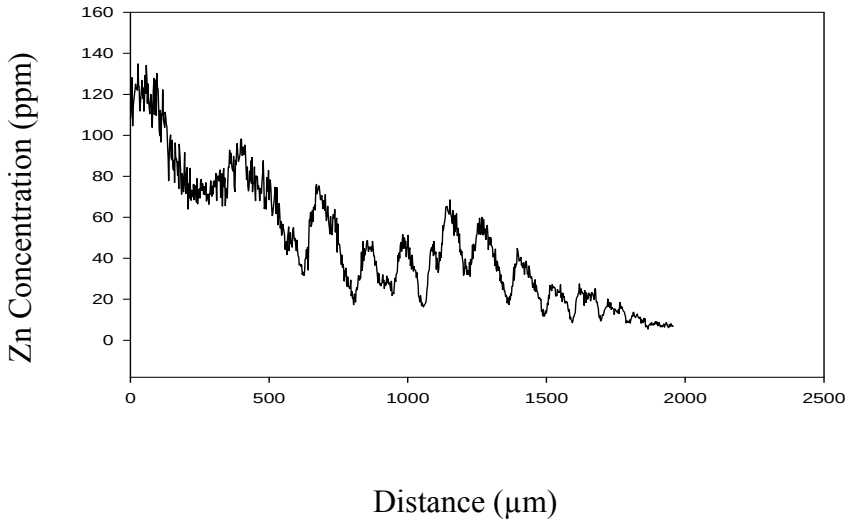

Zn Concentration (ppm)
70
60
50
40
30
20
10
0
0
500
1000
1500
2000
Distance (μm)

RL08-4068

Age: 25+

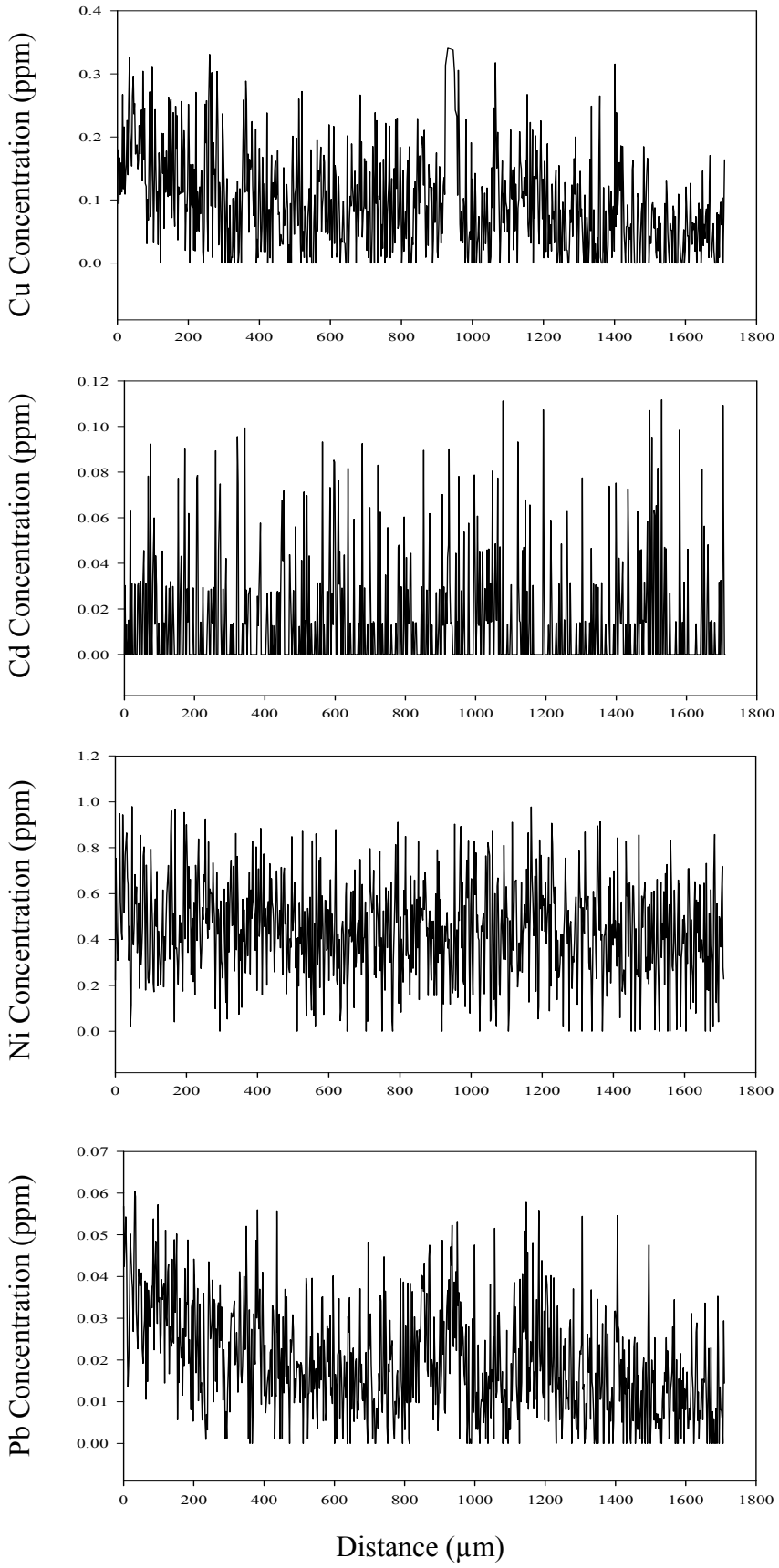


RL08-4068

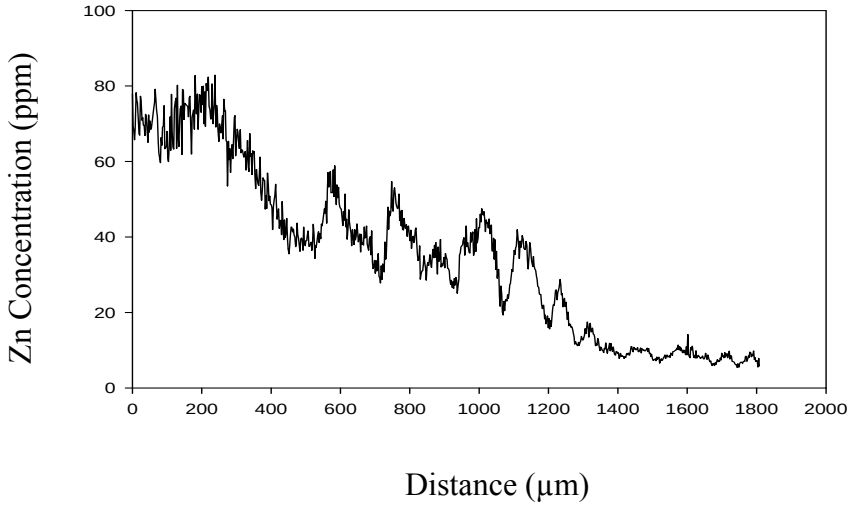

Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (µm)

RL08-4079

Age: 33+

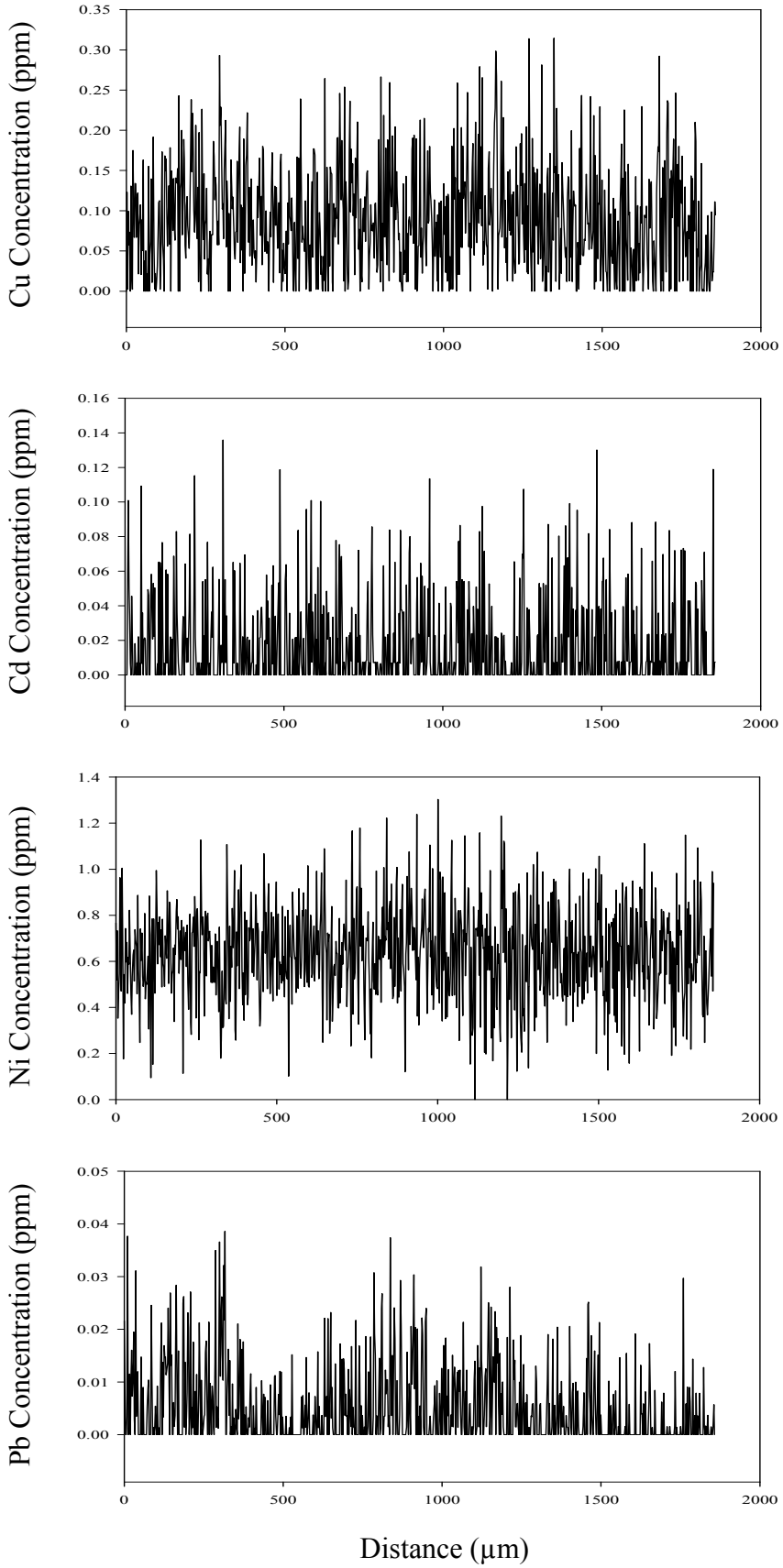


RL08-4079

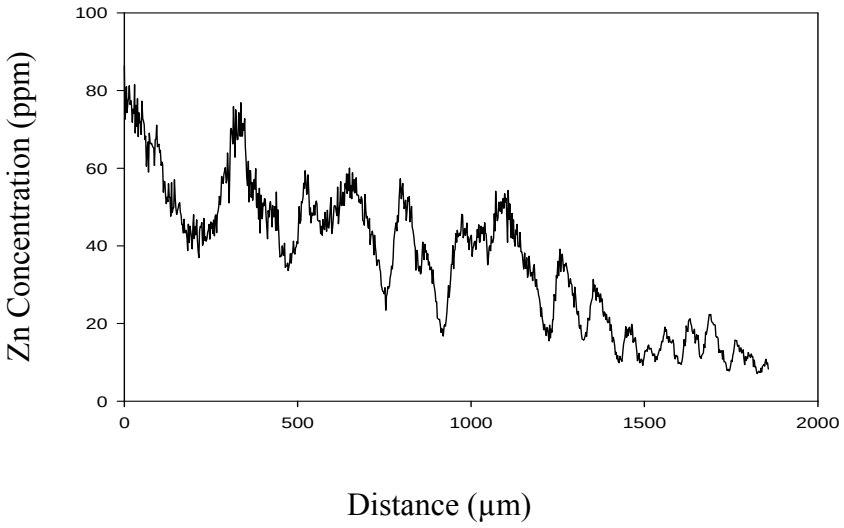

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

RL08-4081

Age: 26+

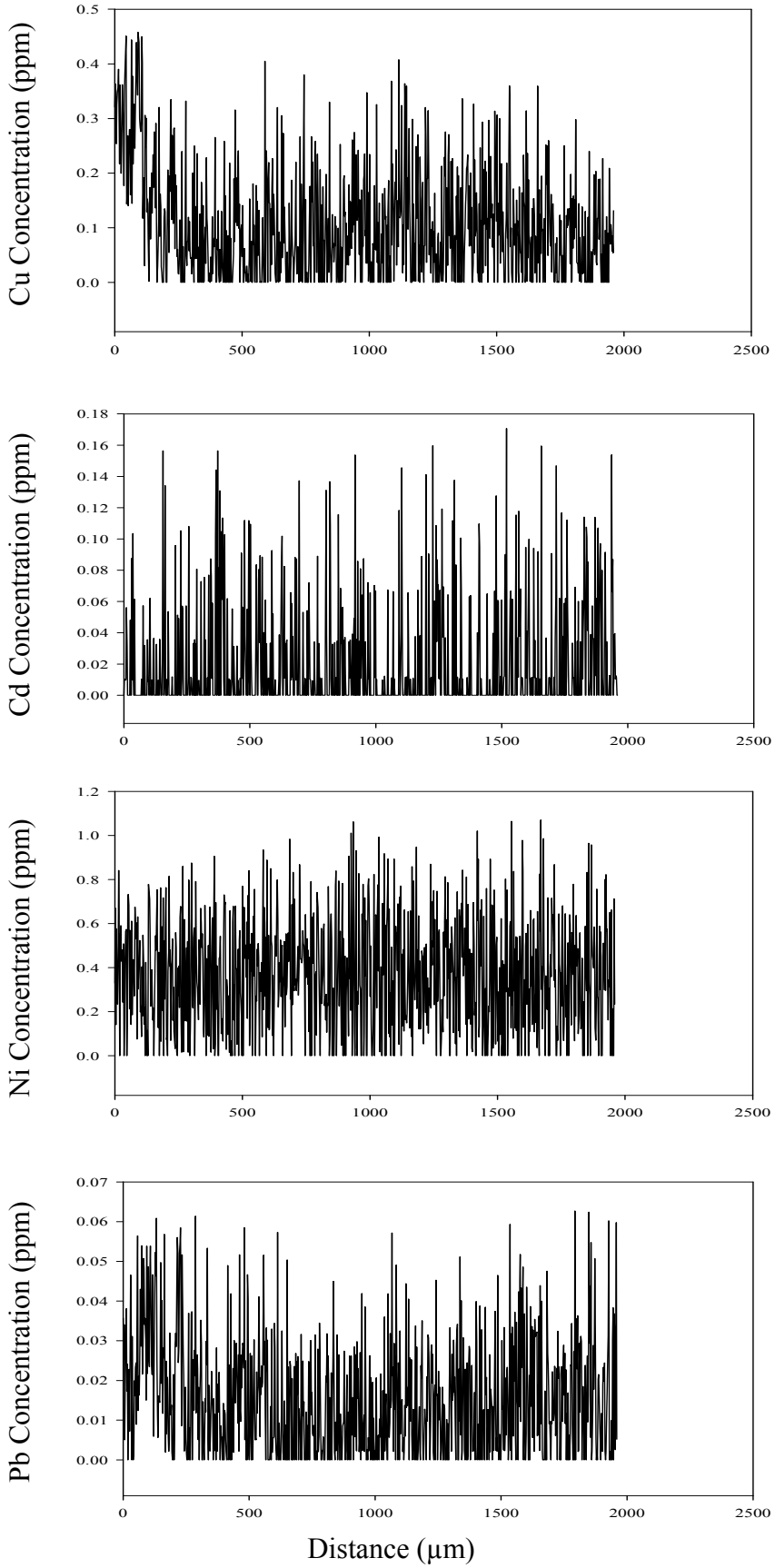


RL08-4081

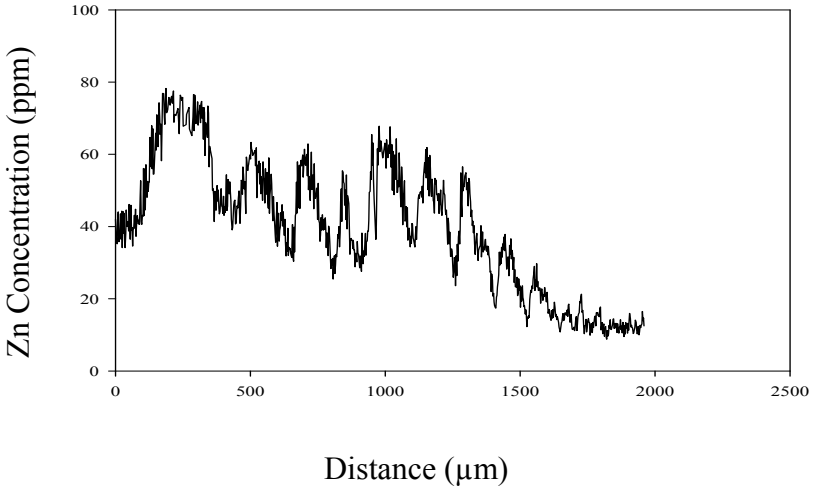

Zn Concentration (ppm)
100
80
60
40
20
0
0
500
1000
1500
2000
Distance (µm)

RL08-4100

Age: 23+

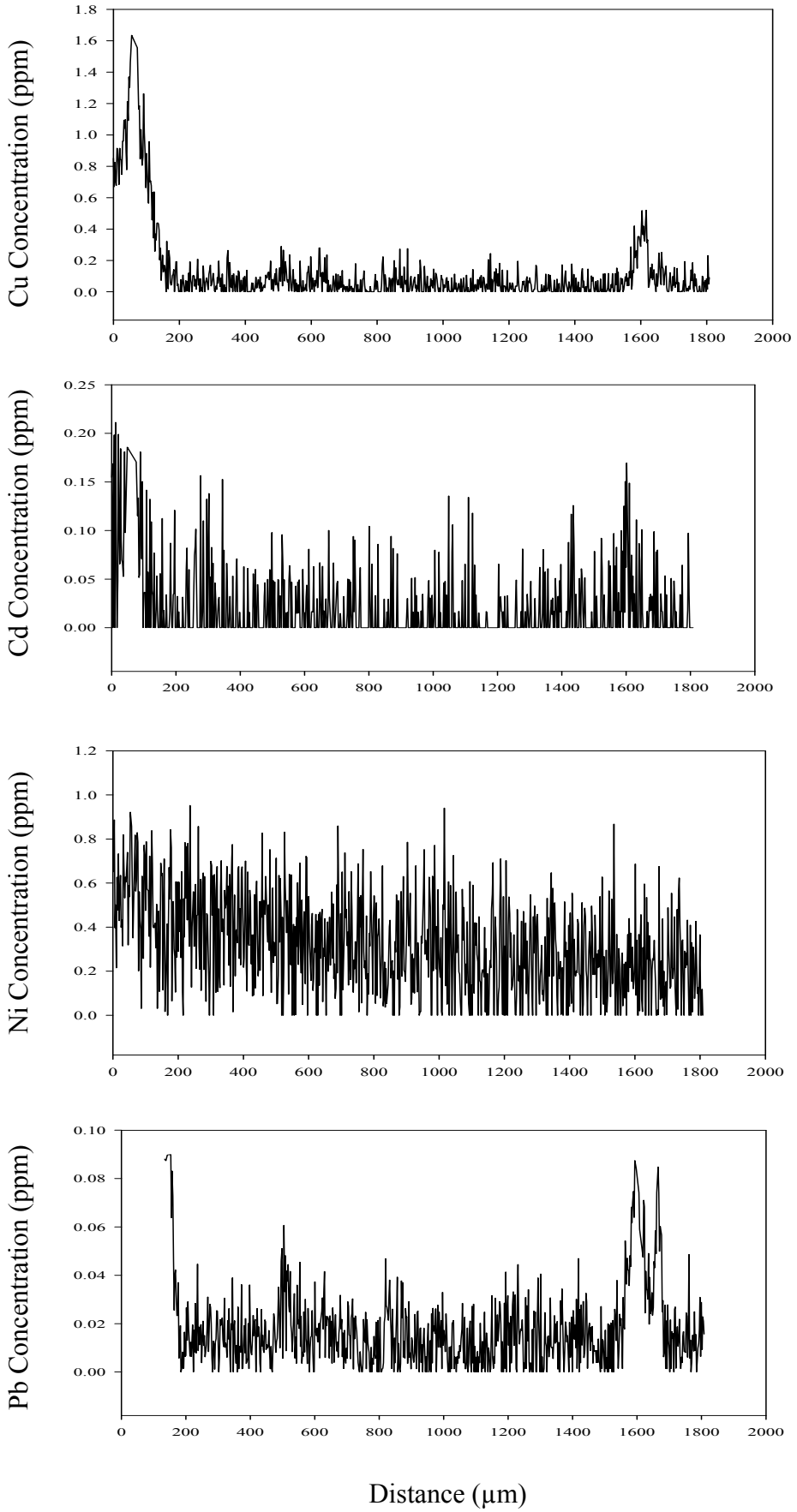


RL08-4100

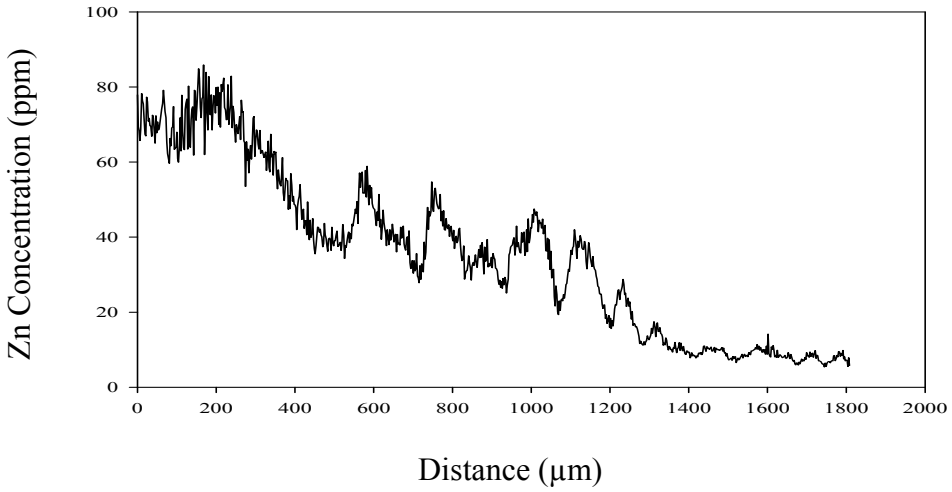

Zn Concentration (ppm)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

RL08-4101

Age: 23+

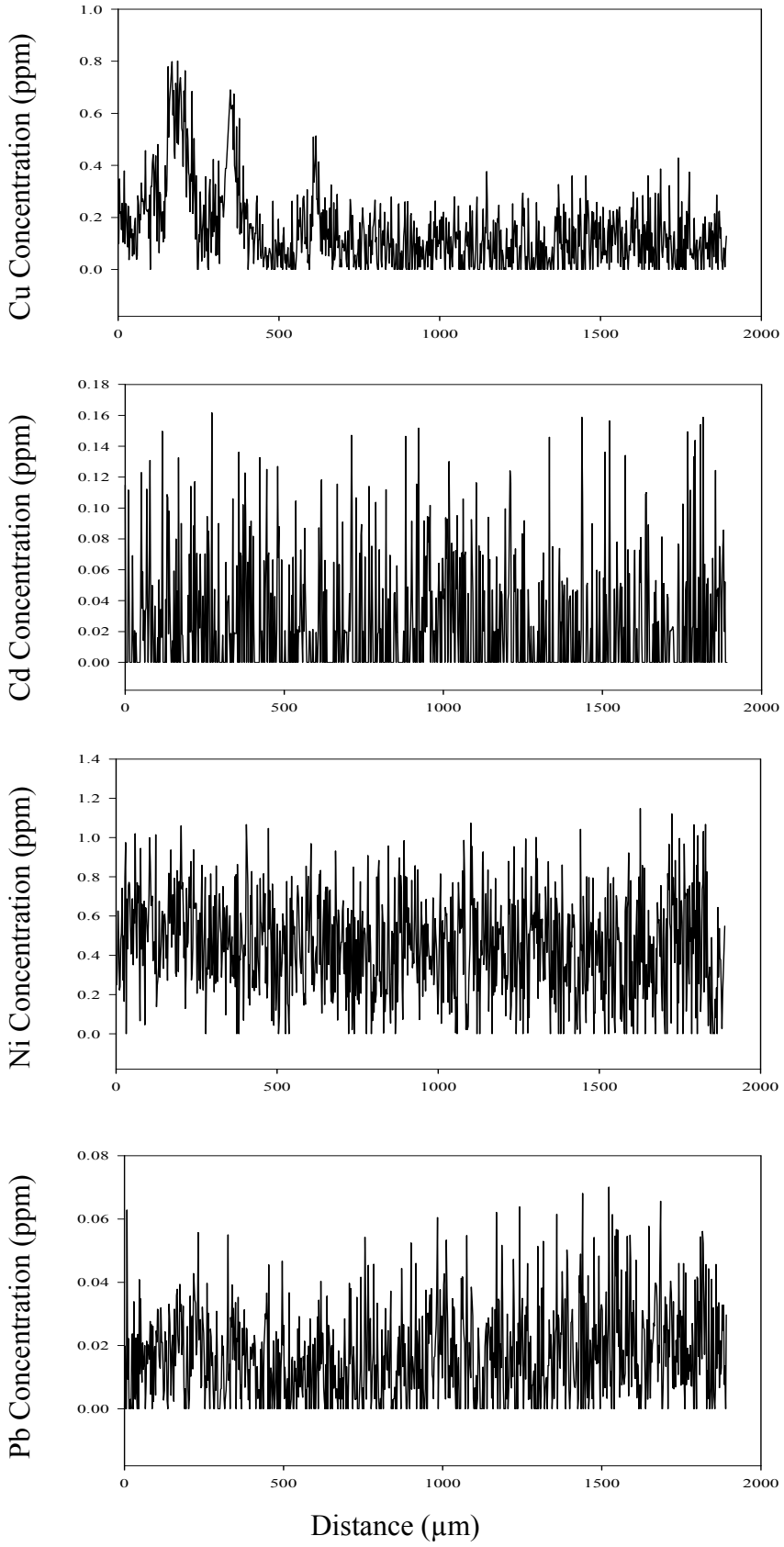


RL08-4101

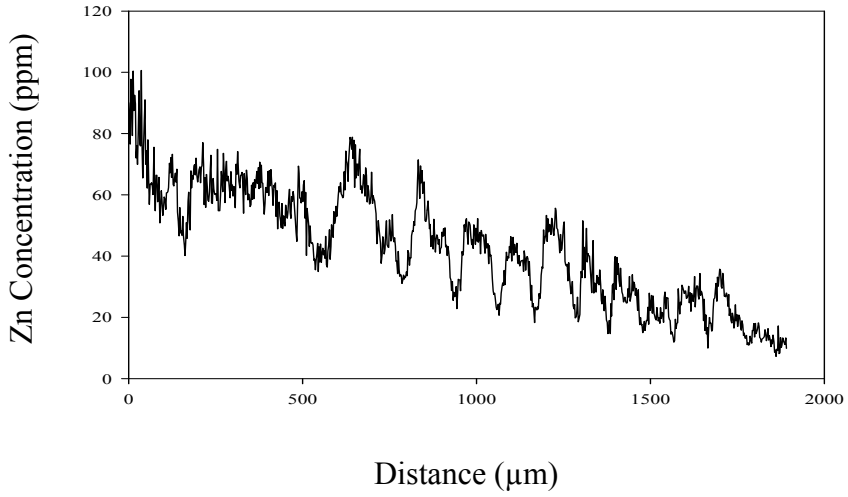

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (µm)

RL08-F39

Age: 31+



RL08-F39

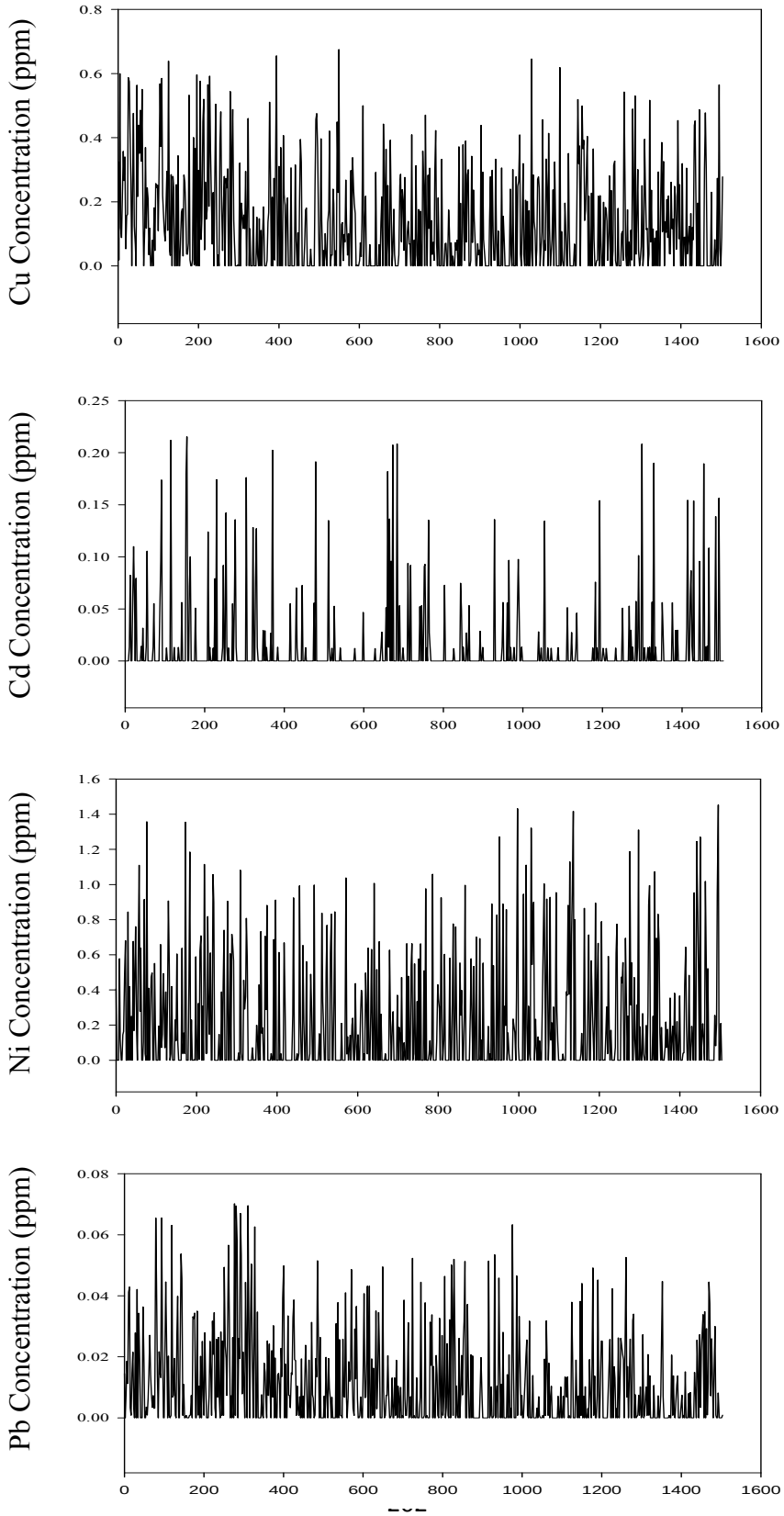

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
500
1000
1500
2000
Distance (μm)

APPENDIX II

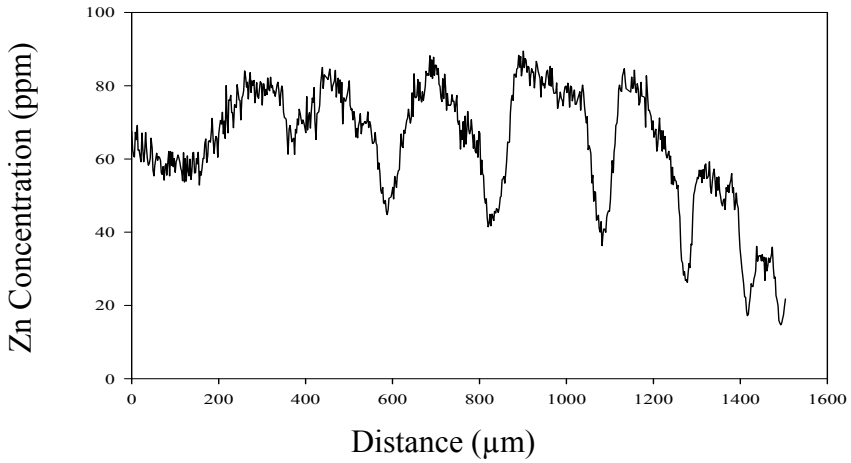
Metal Results for Trout Lake

TL01-02

Age: 7+

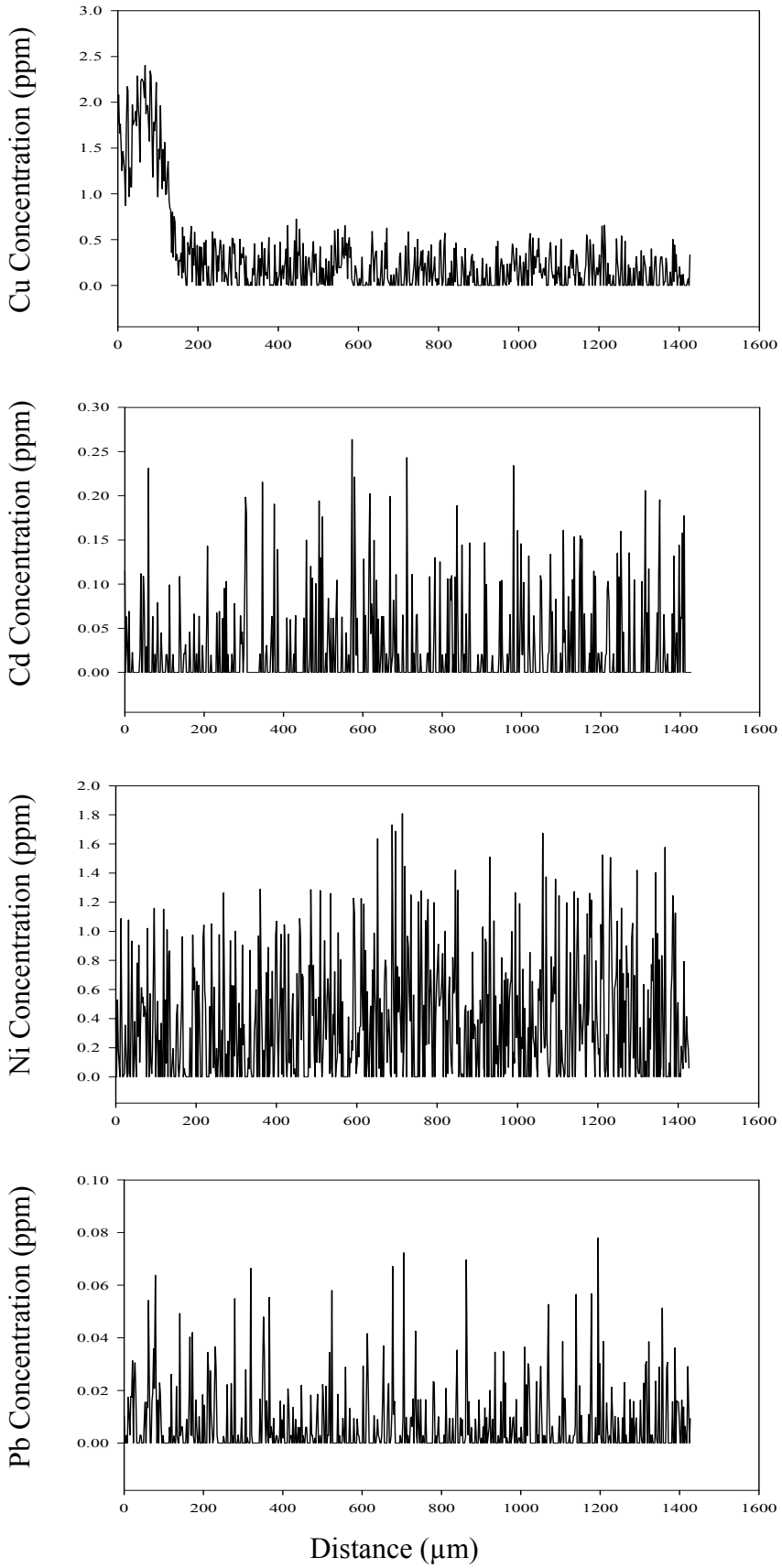

Cu Concentration (ppm)
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (µm)

TL01-02

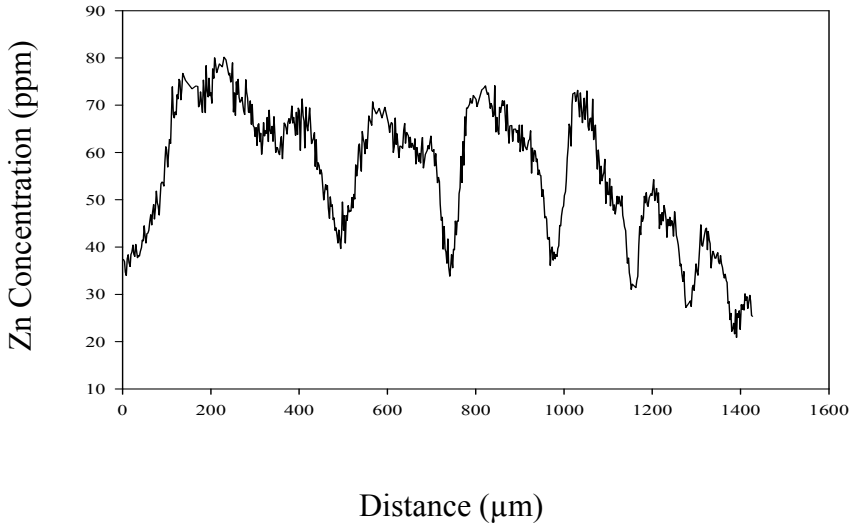

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL01-12

Age: 6+

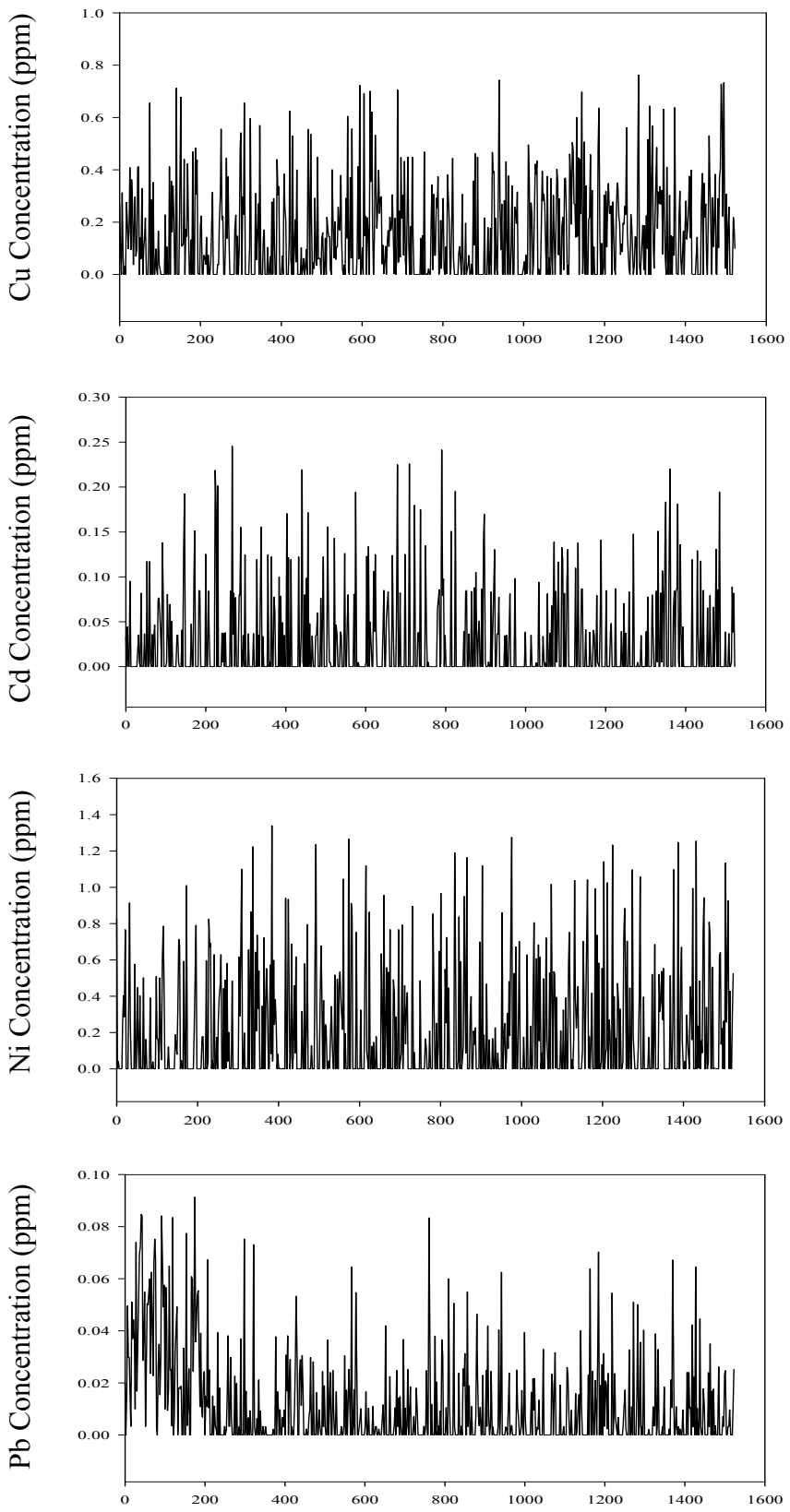

Cu Concentration (ppm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.10
0.08
0.06
0.04
0.02
0.00
Distance (µm)

TL01-12

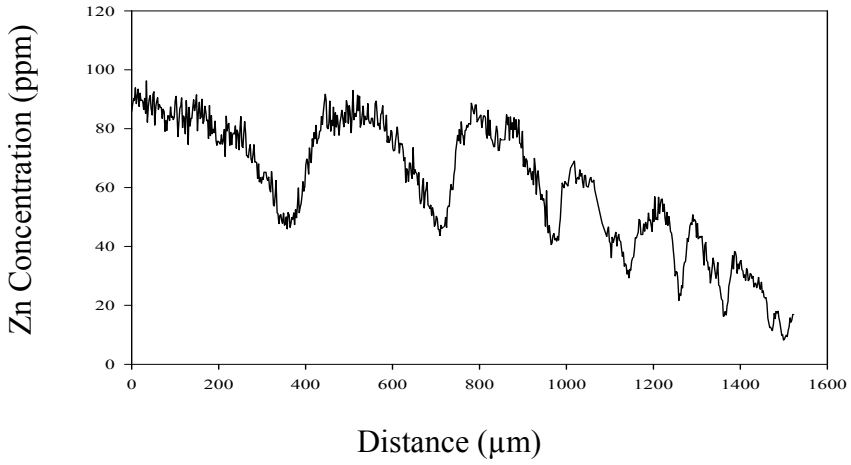

Zn Concentration (ppm)
90
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

TL01-13

Age: 8+

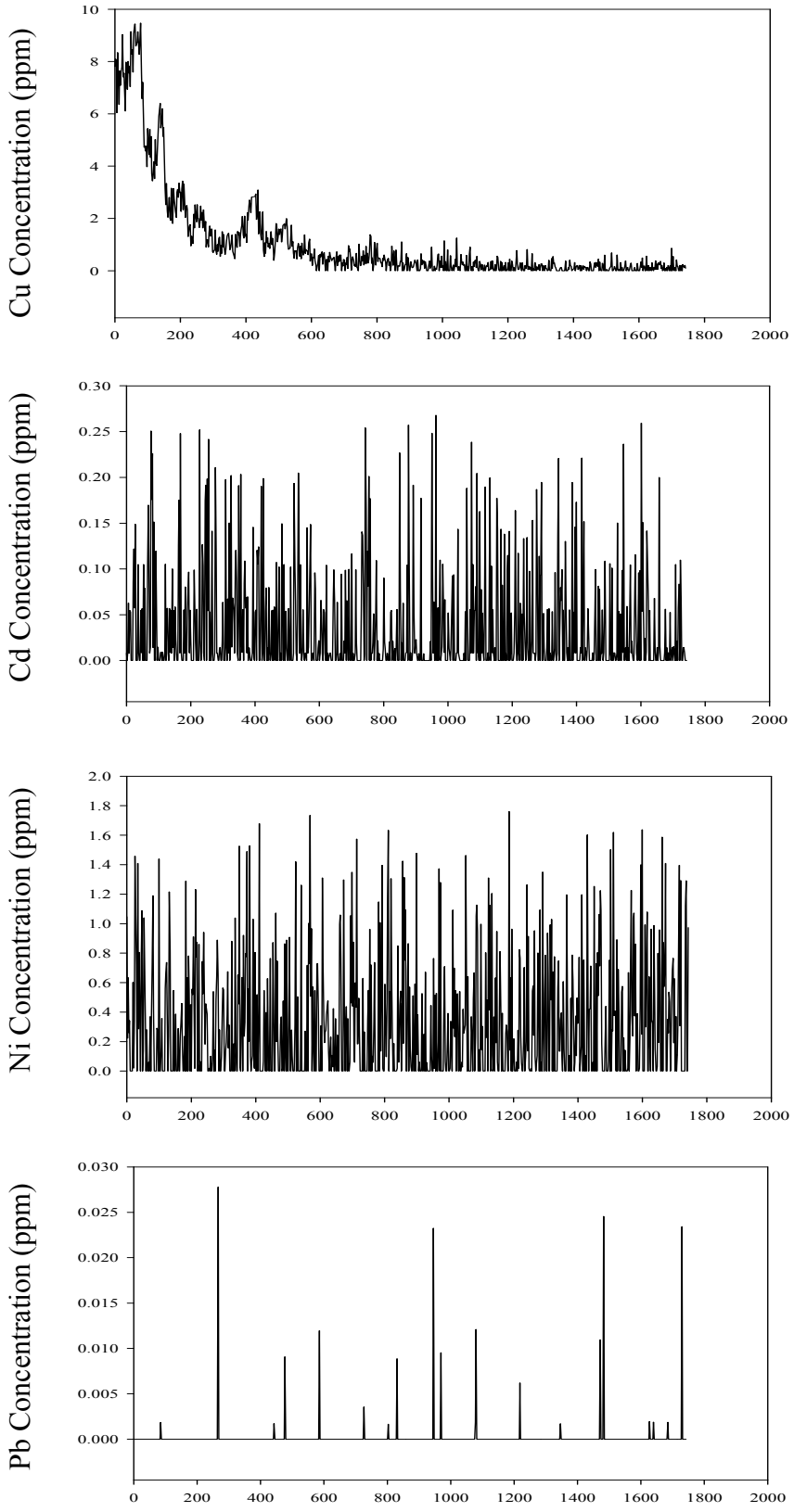

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

TL01-13

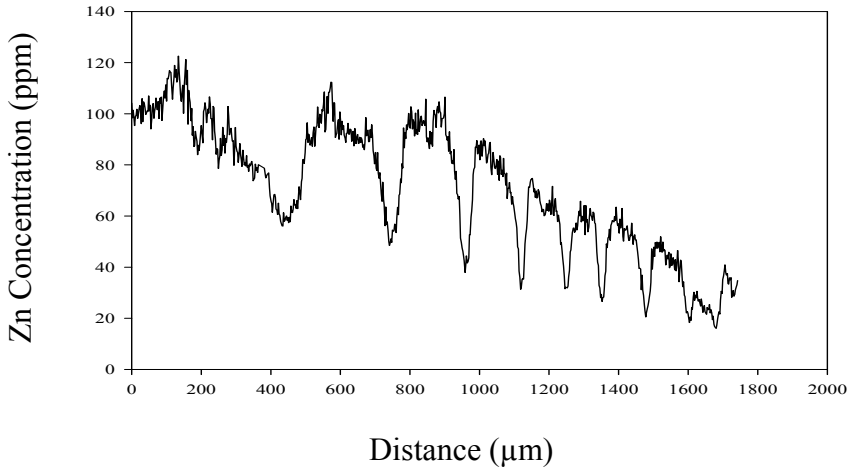

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

TL01-14

Age: 11+

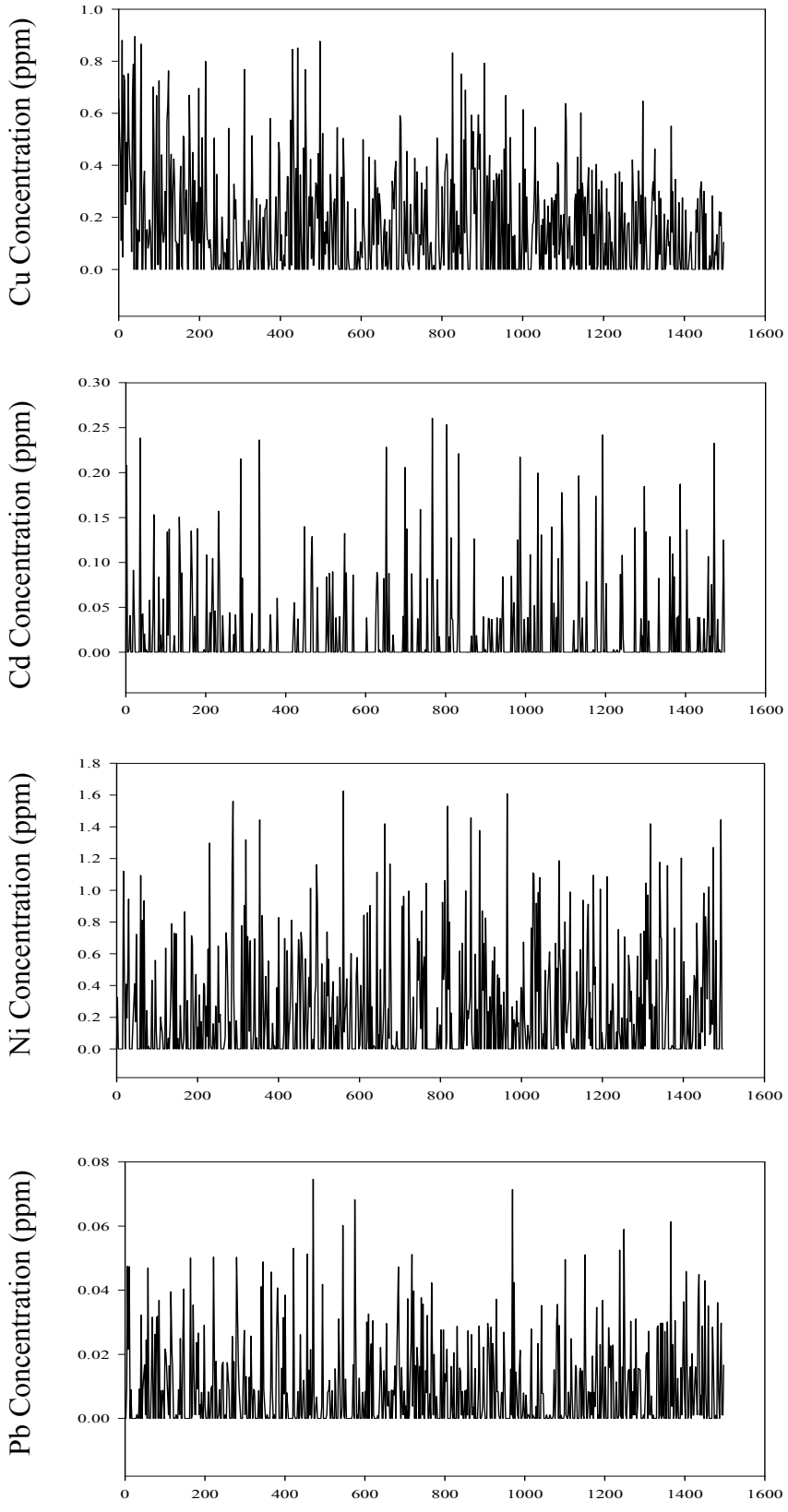


TL01-14

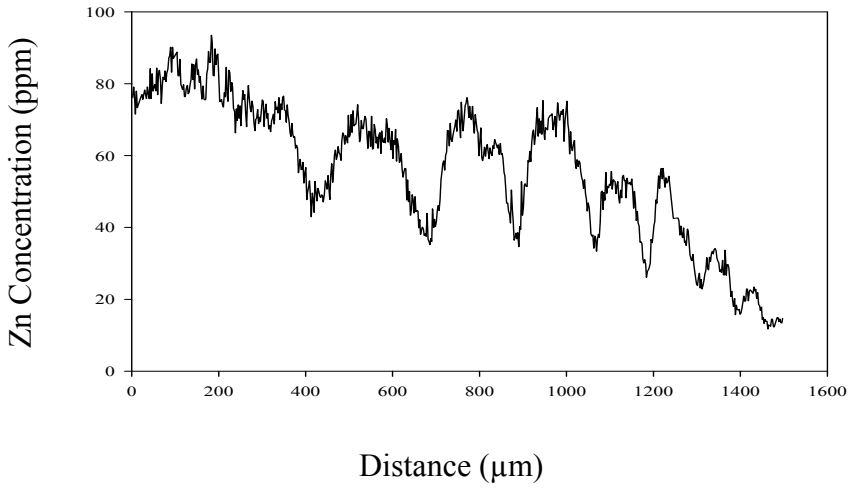

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

TL01-15

Age: 19+

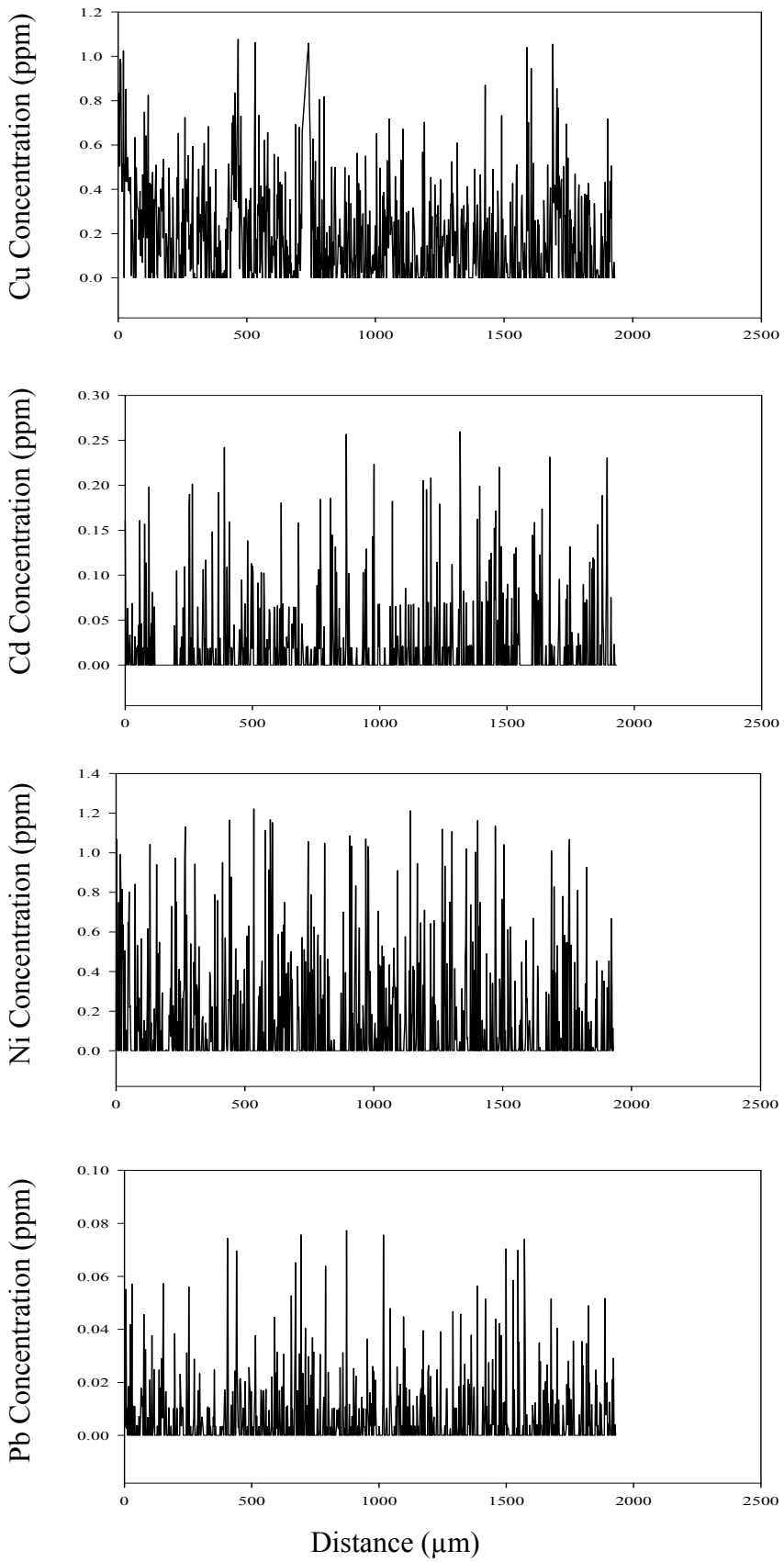


TL01-15

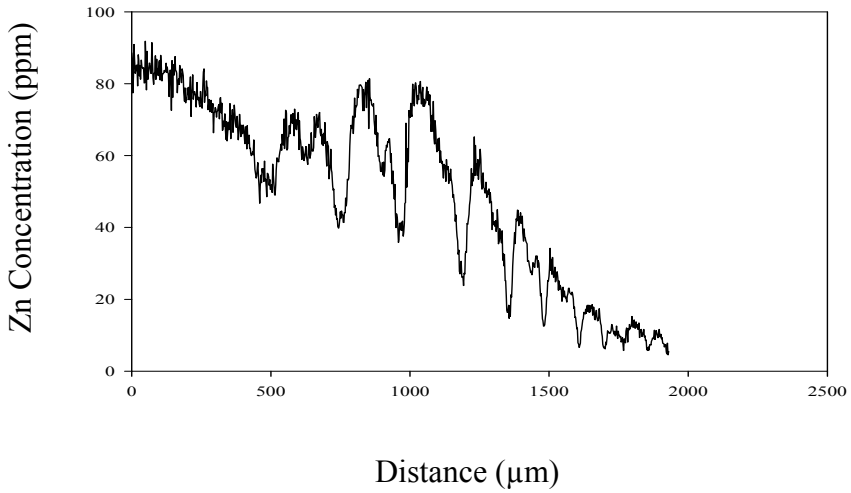

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL01-26

Age: 17+

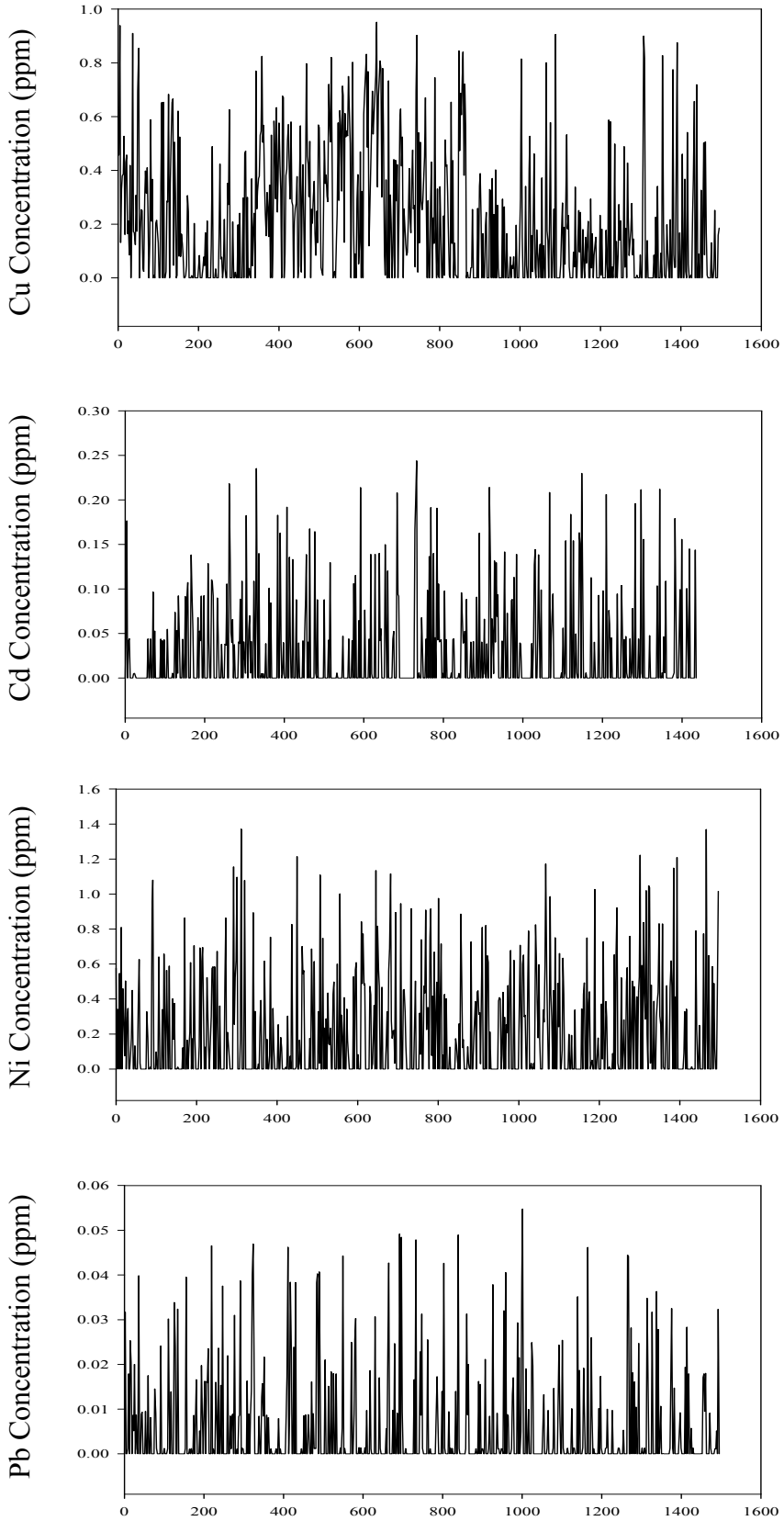


TL01-26

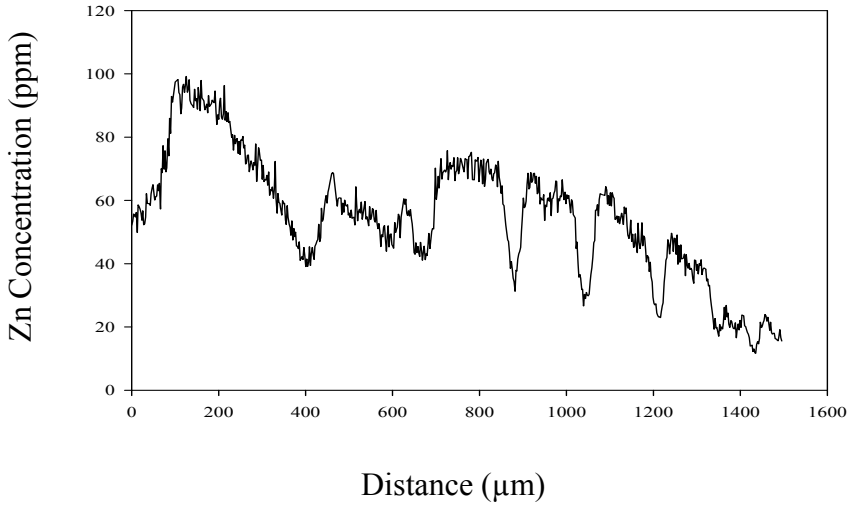

Zn Concentration (ppm)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

TL01-27

Age: 9+

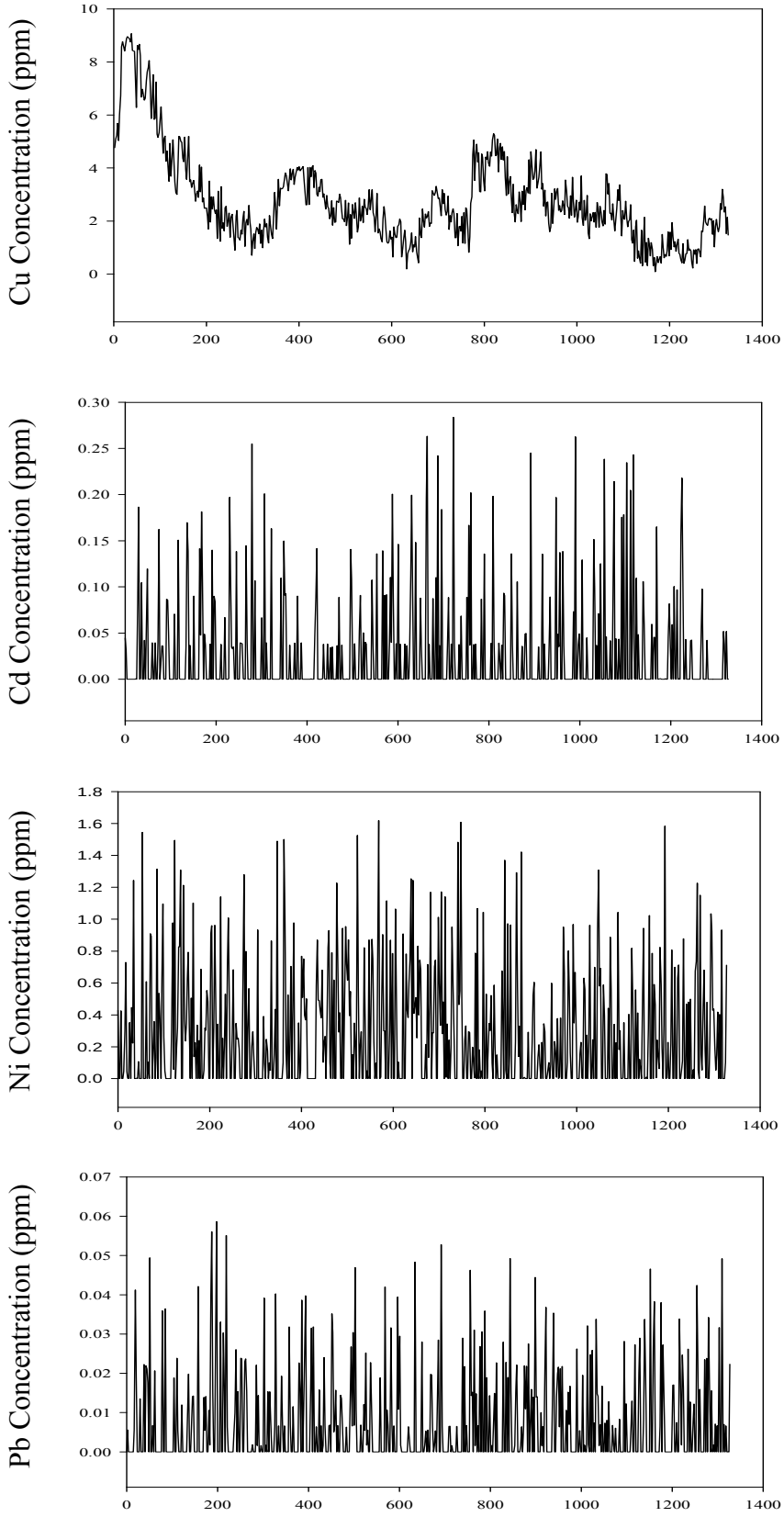

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

TL01-27

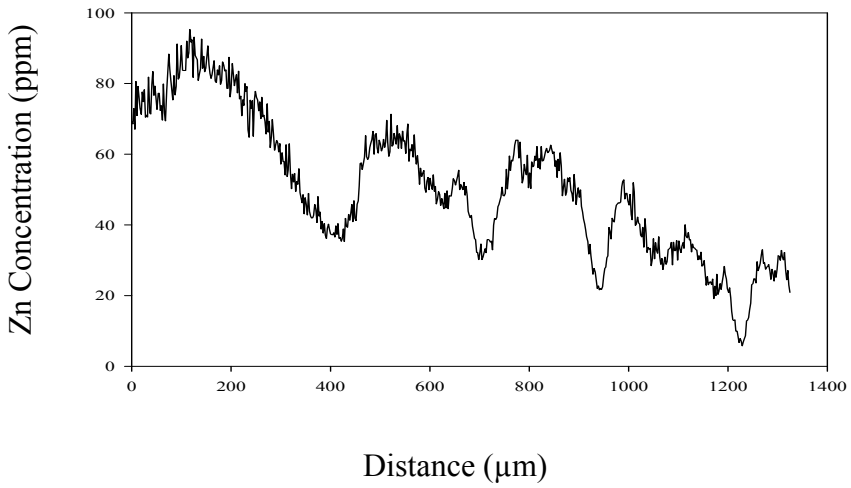

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL01-28

Age: 8+

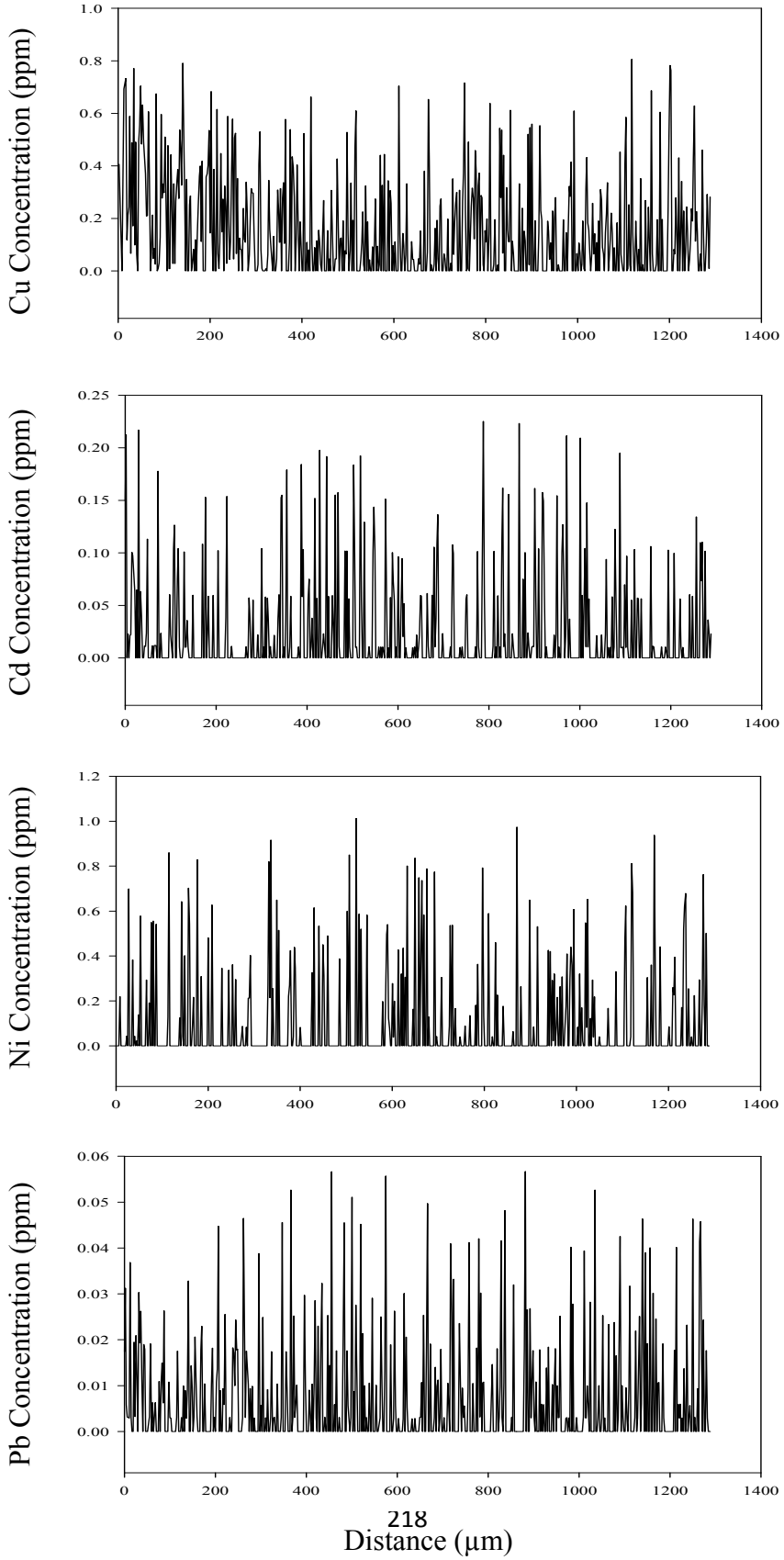

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

TL01-28

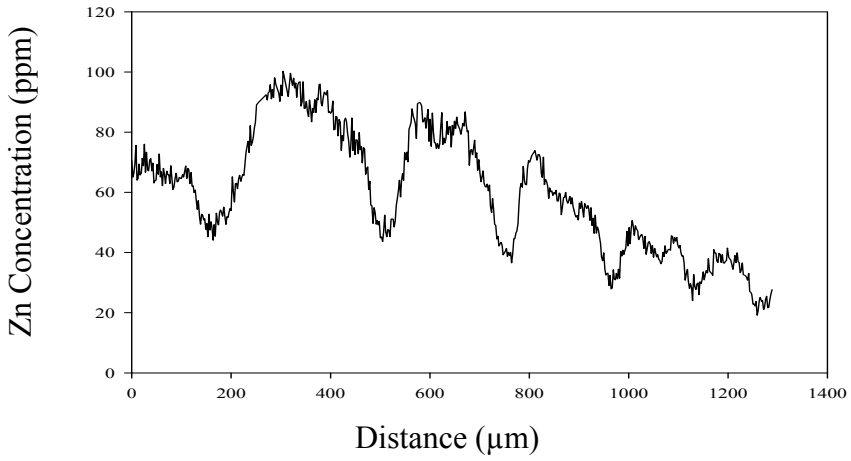

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

TL02-31

Age: 6+

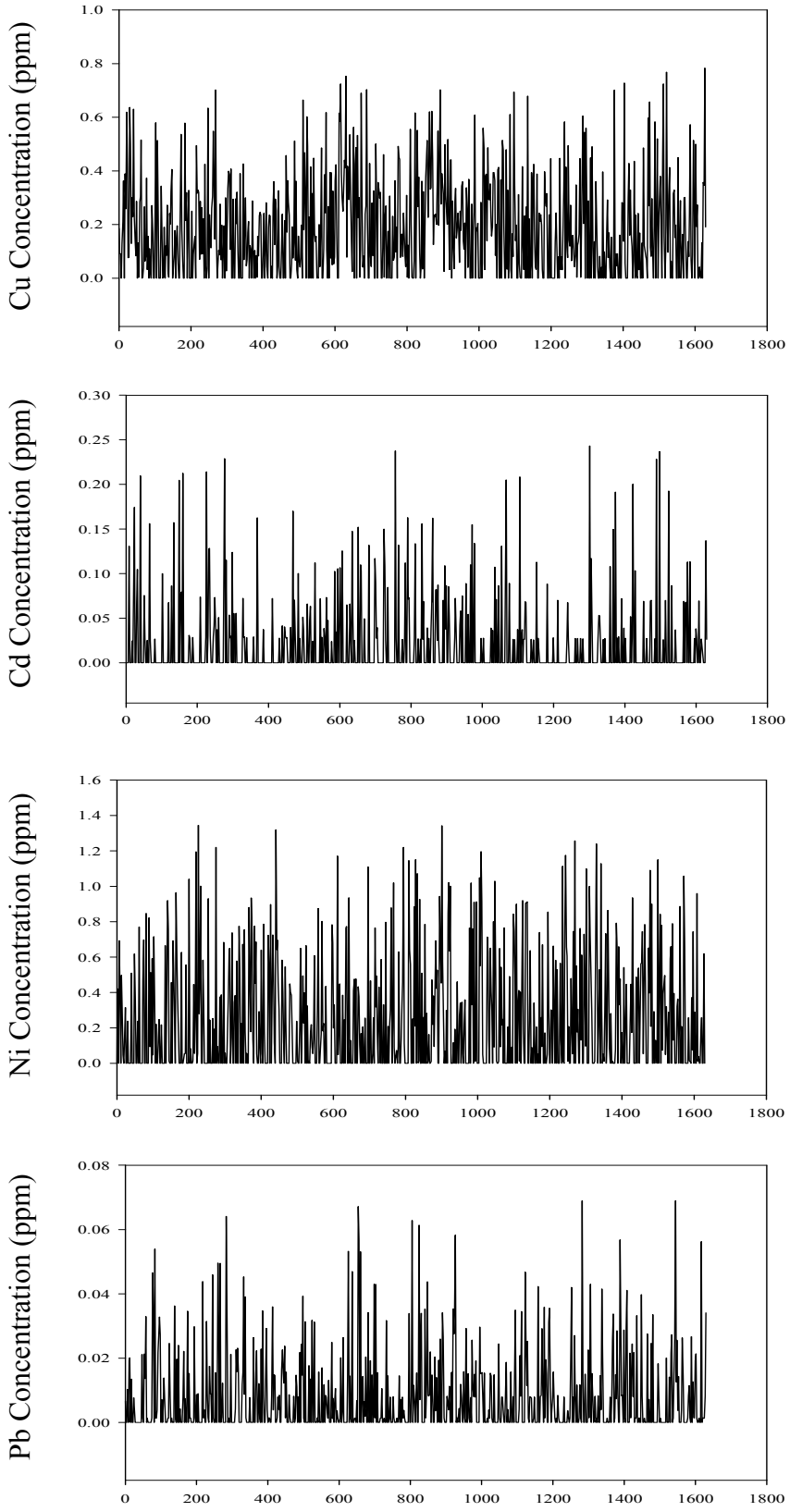

Cu Concentration (ppm)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (μm)

TL02-31

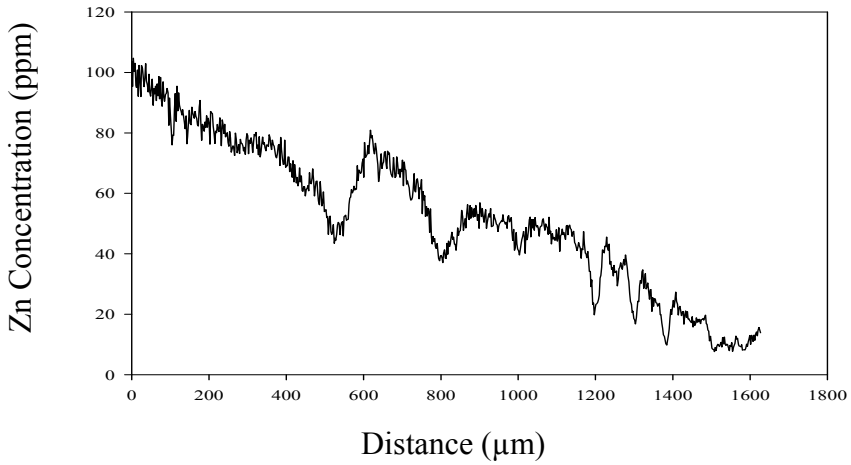

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

TL02-33

Age: 9+

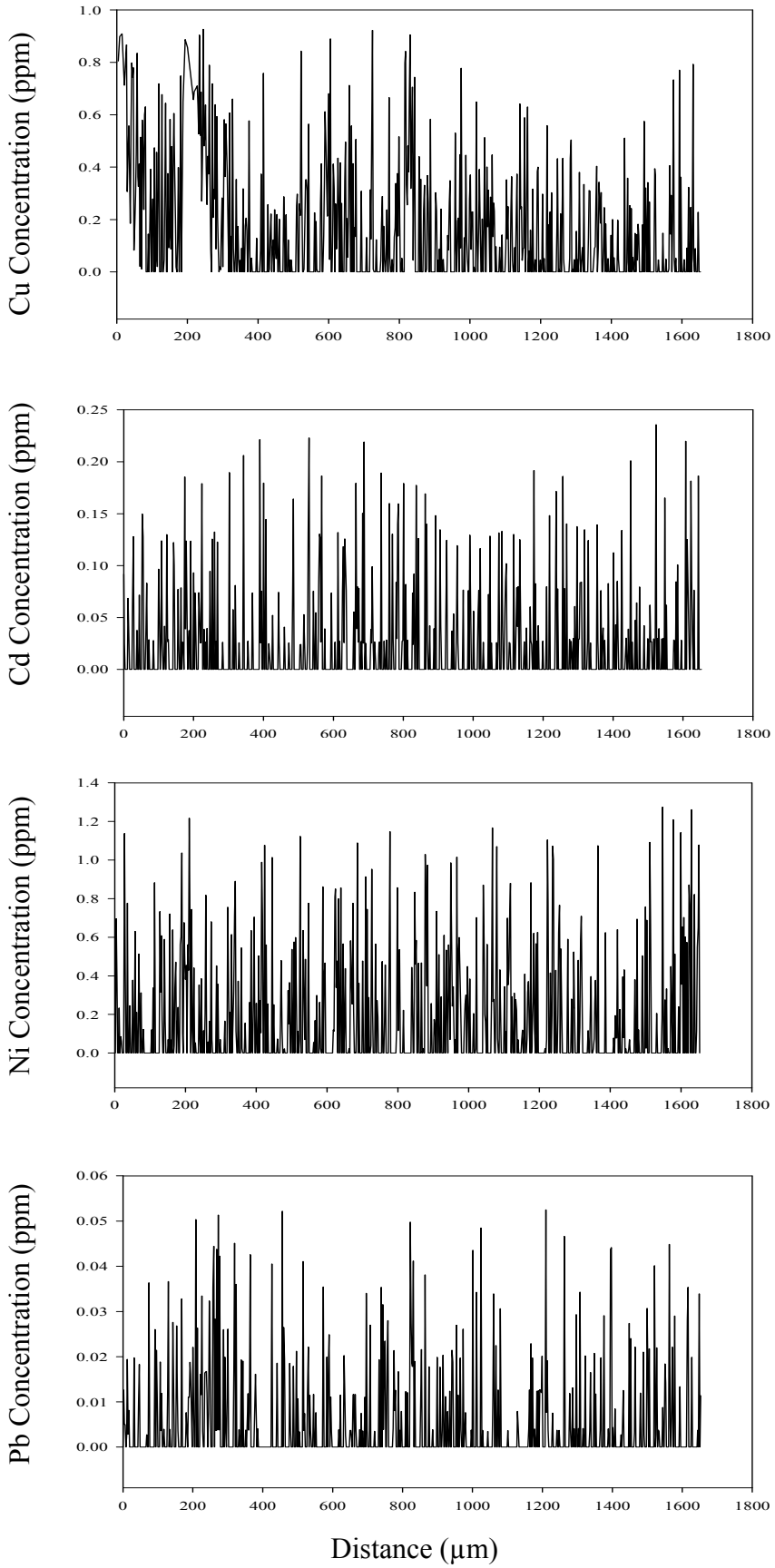

Cu Concentration (ppm)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
1600
1800
Cd Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
220
Distance (μm)

TL02-33

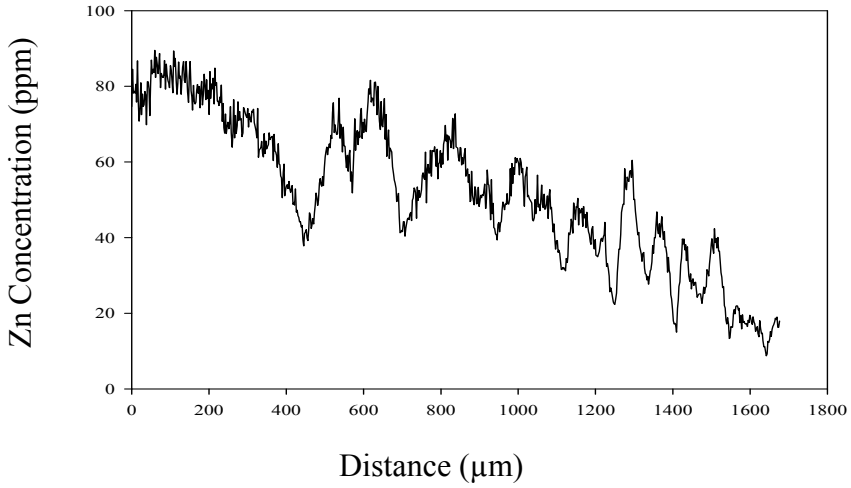

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL02-34

Age: 10+

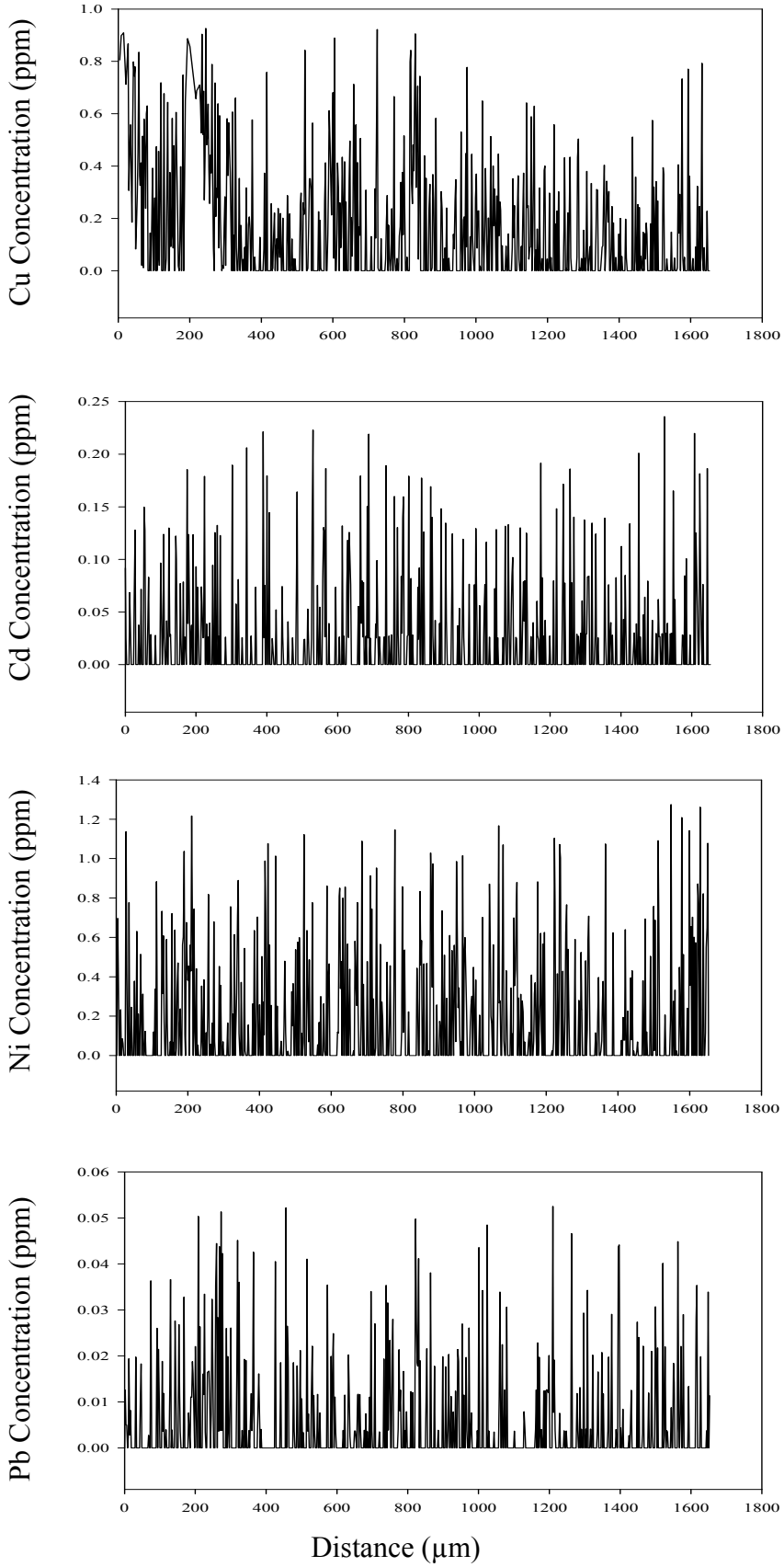


TL02-34

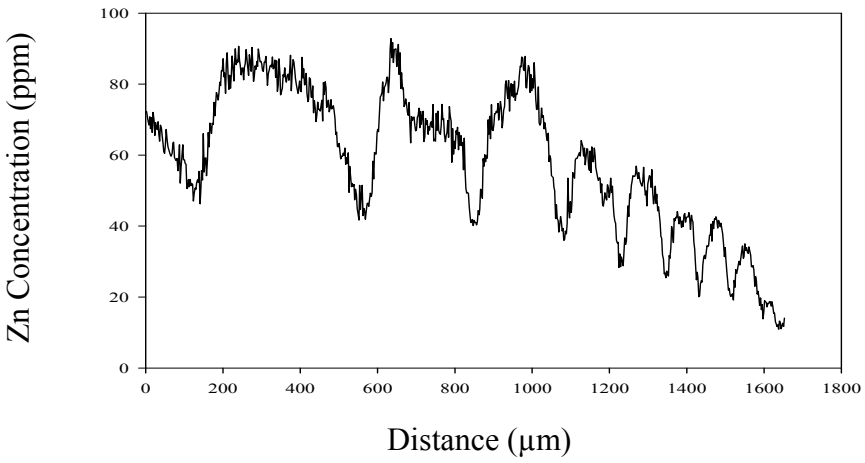

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL02-35

Age: 11+

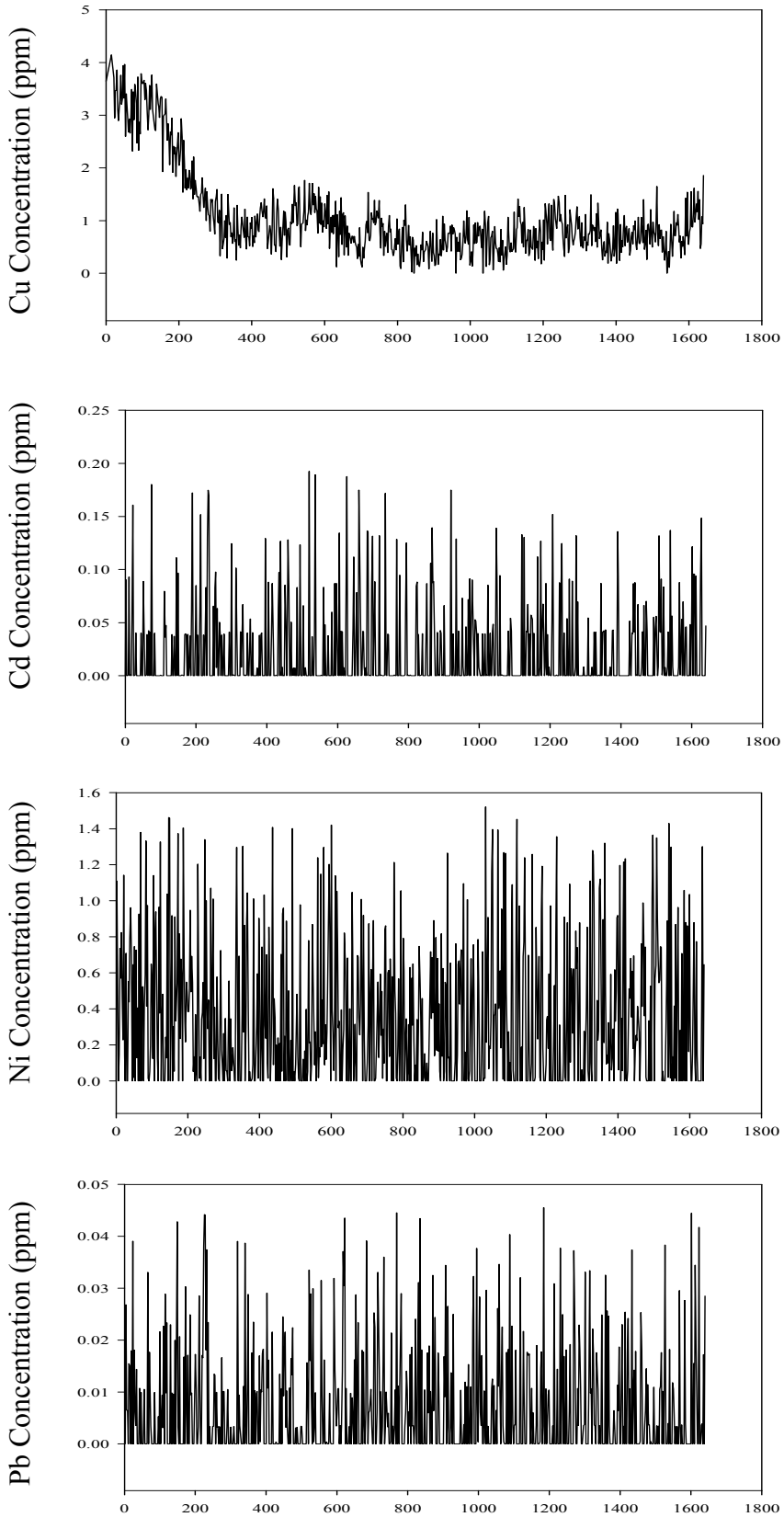


TL02-35

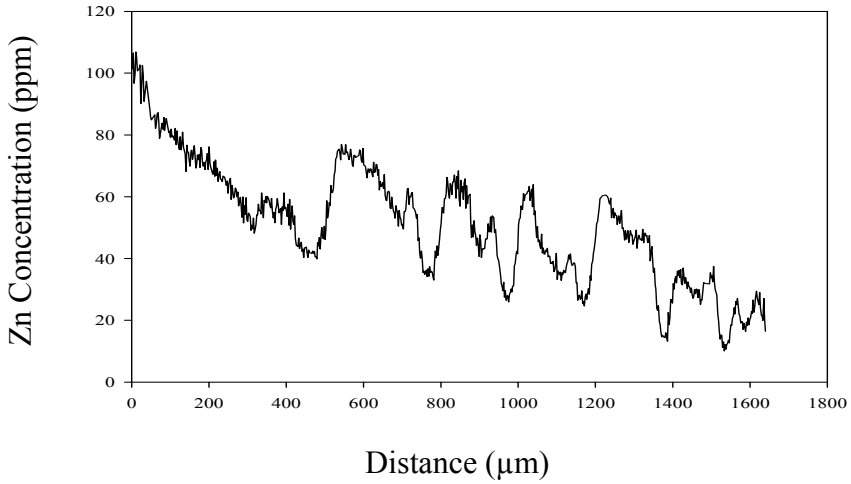

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (µm)

TL02-36

Age: 9+

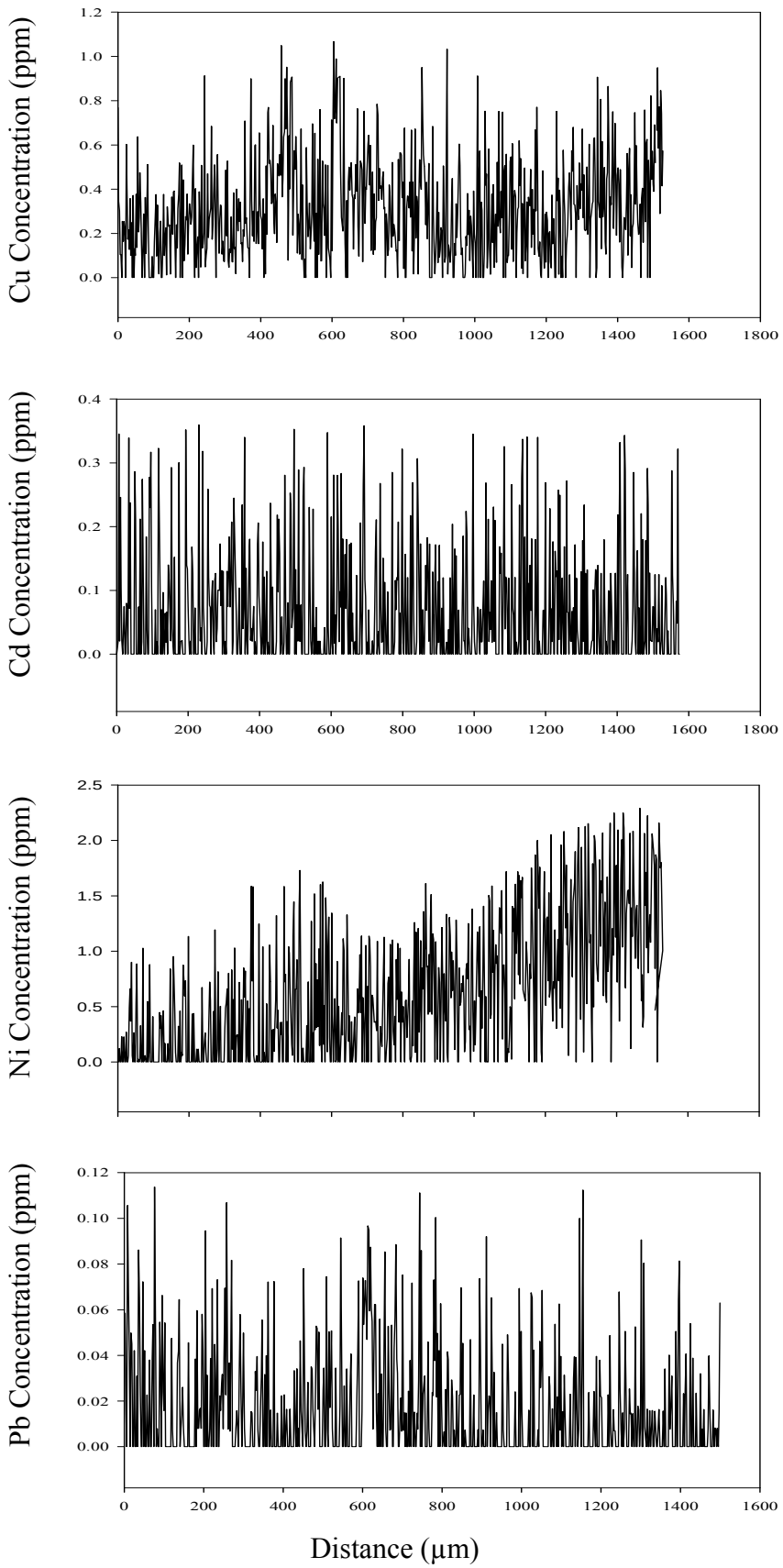

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

TL02-36

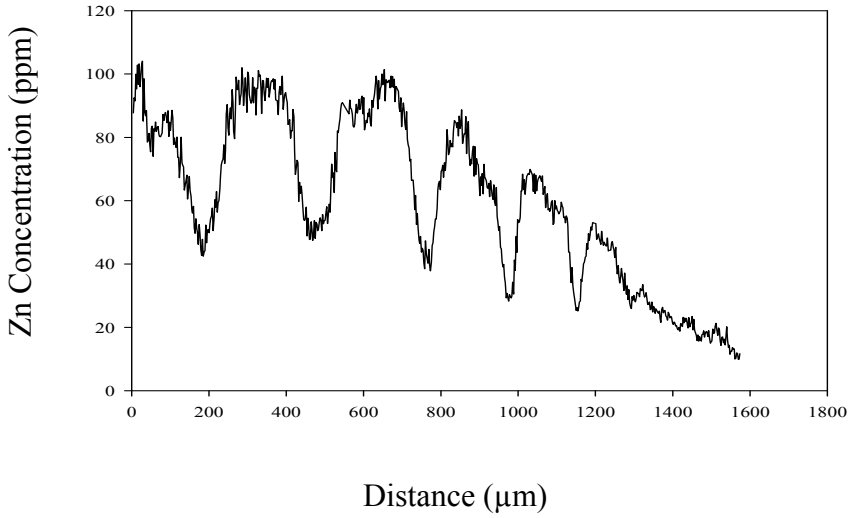

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL03-04

Age: 10+

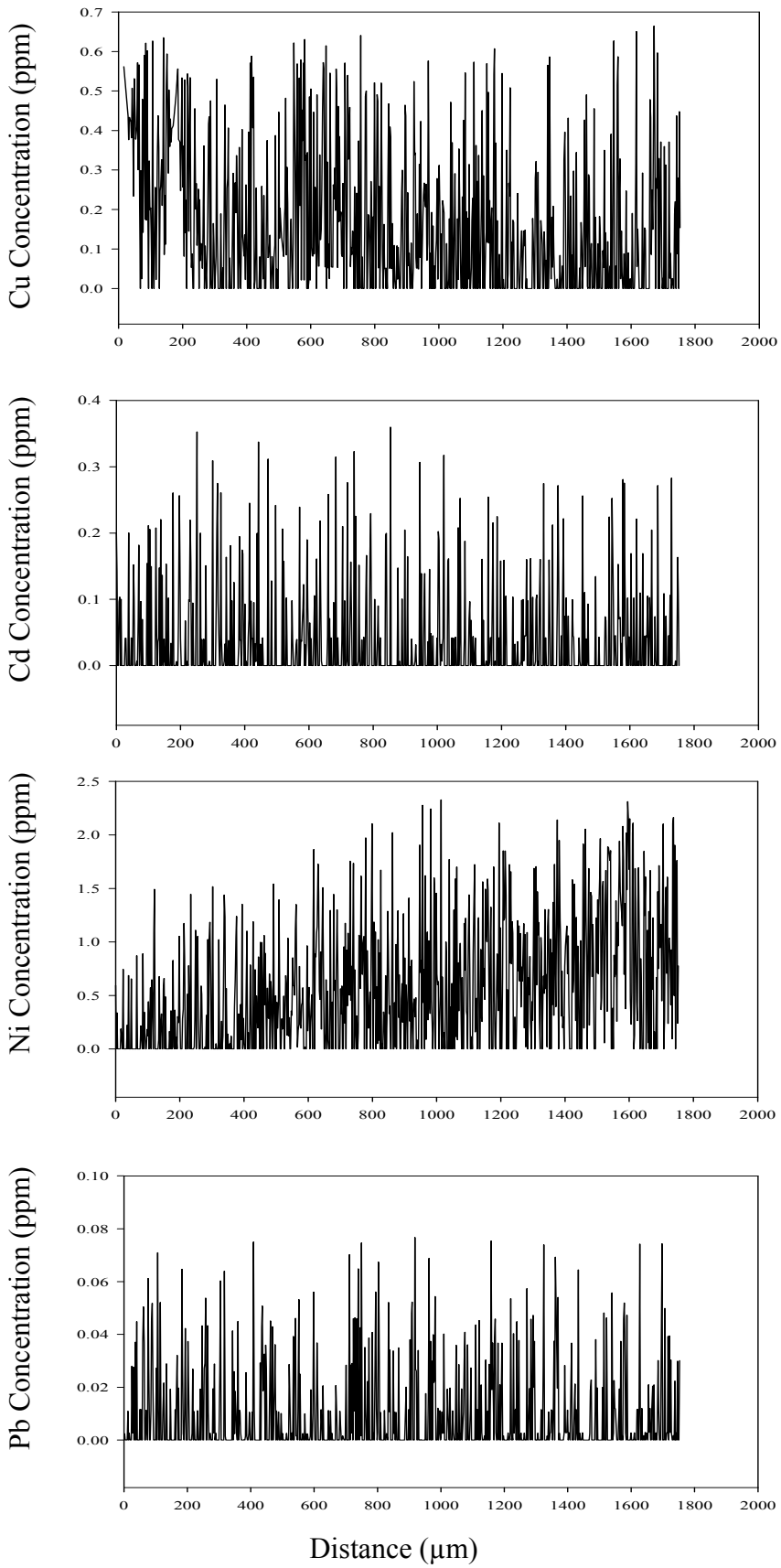


TL03-04

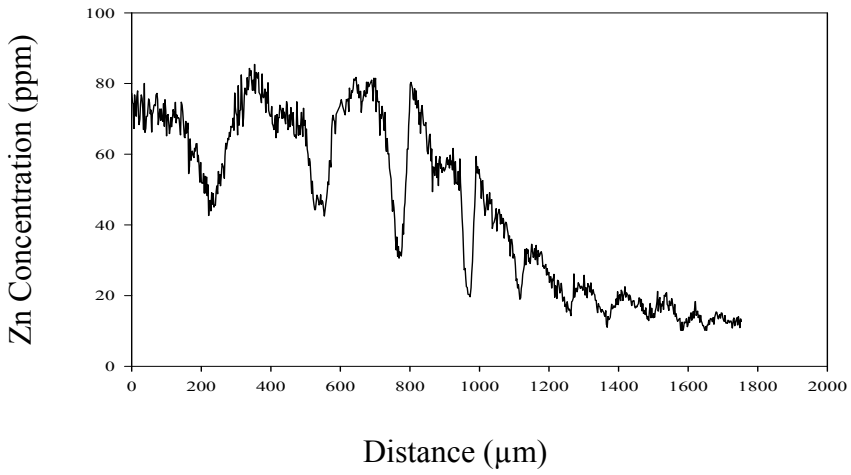

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL03-07

Age: 17+

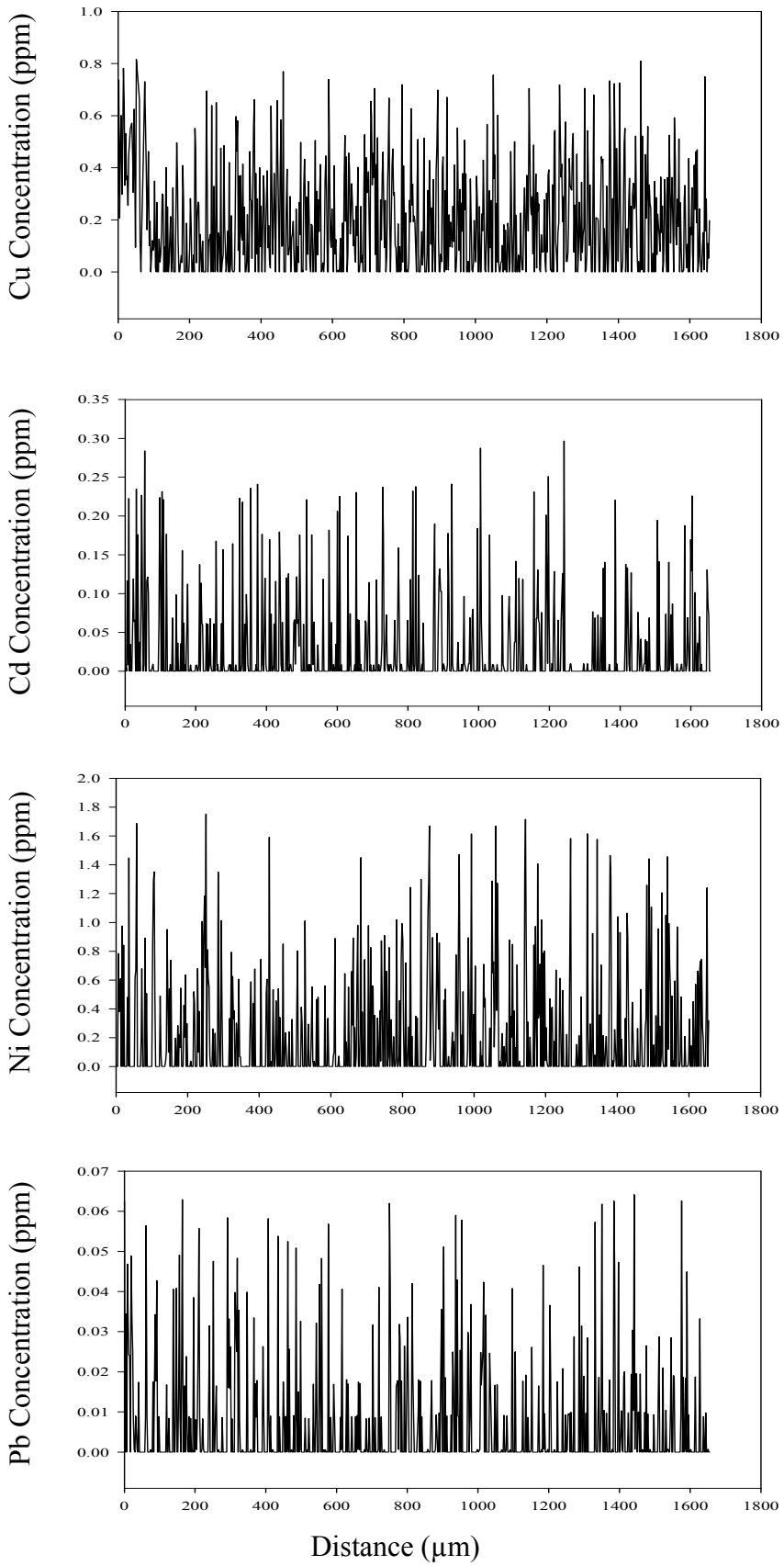


TL03-07

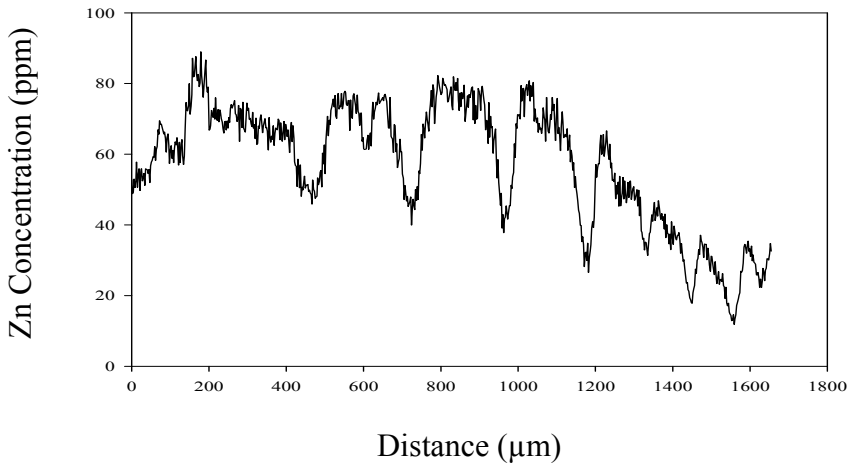

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

TL03-08

Age: 8+

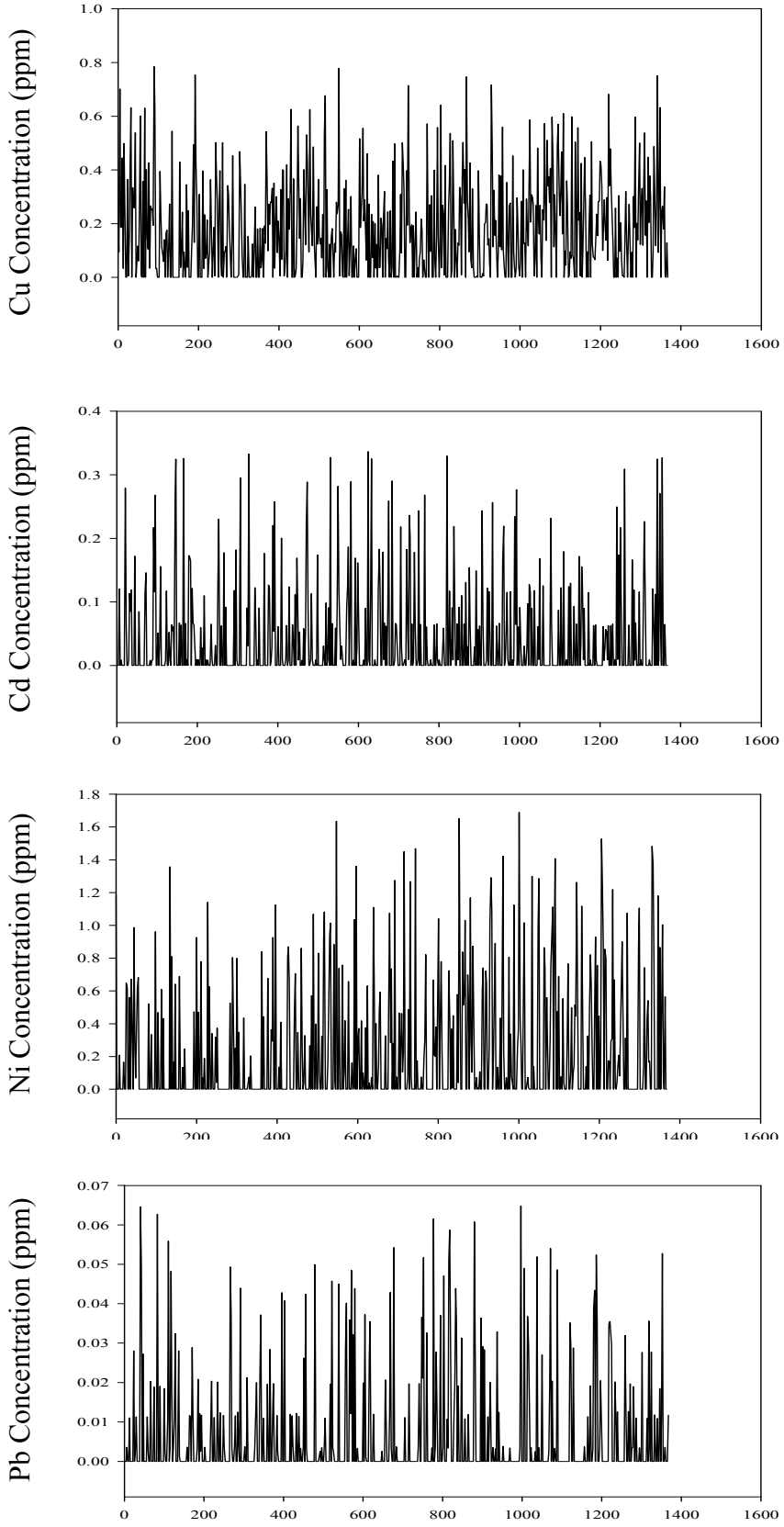

Cu Concentration (ppm)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
1600
1800
Cd Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (μm)

TL03-08

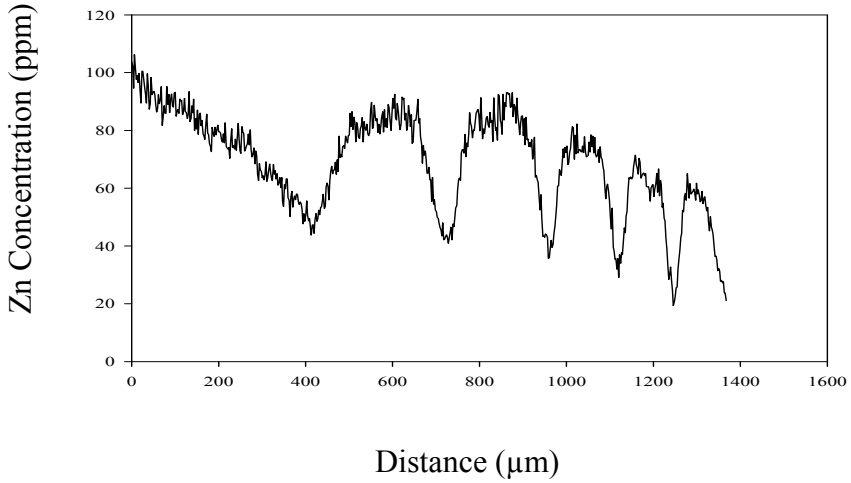

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL03-09

Age: 8+

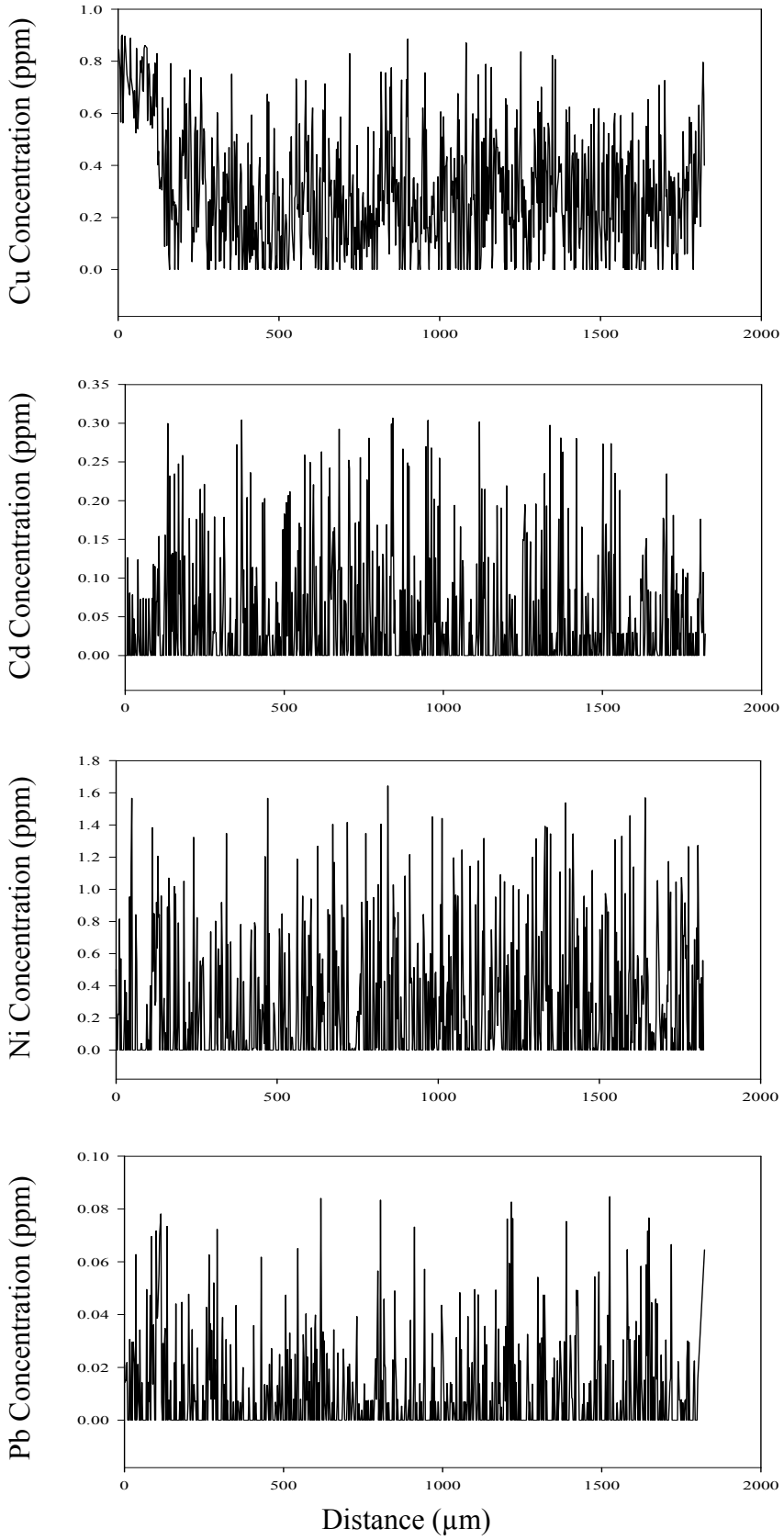

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (μm)

TL03-09

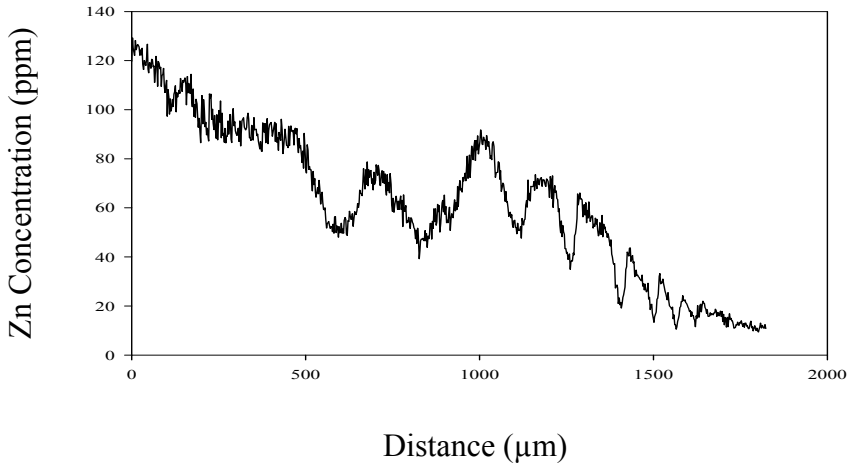

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL03-10

Age: 16+

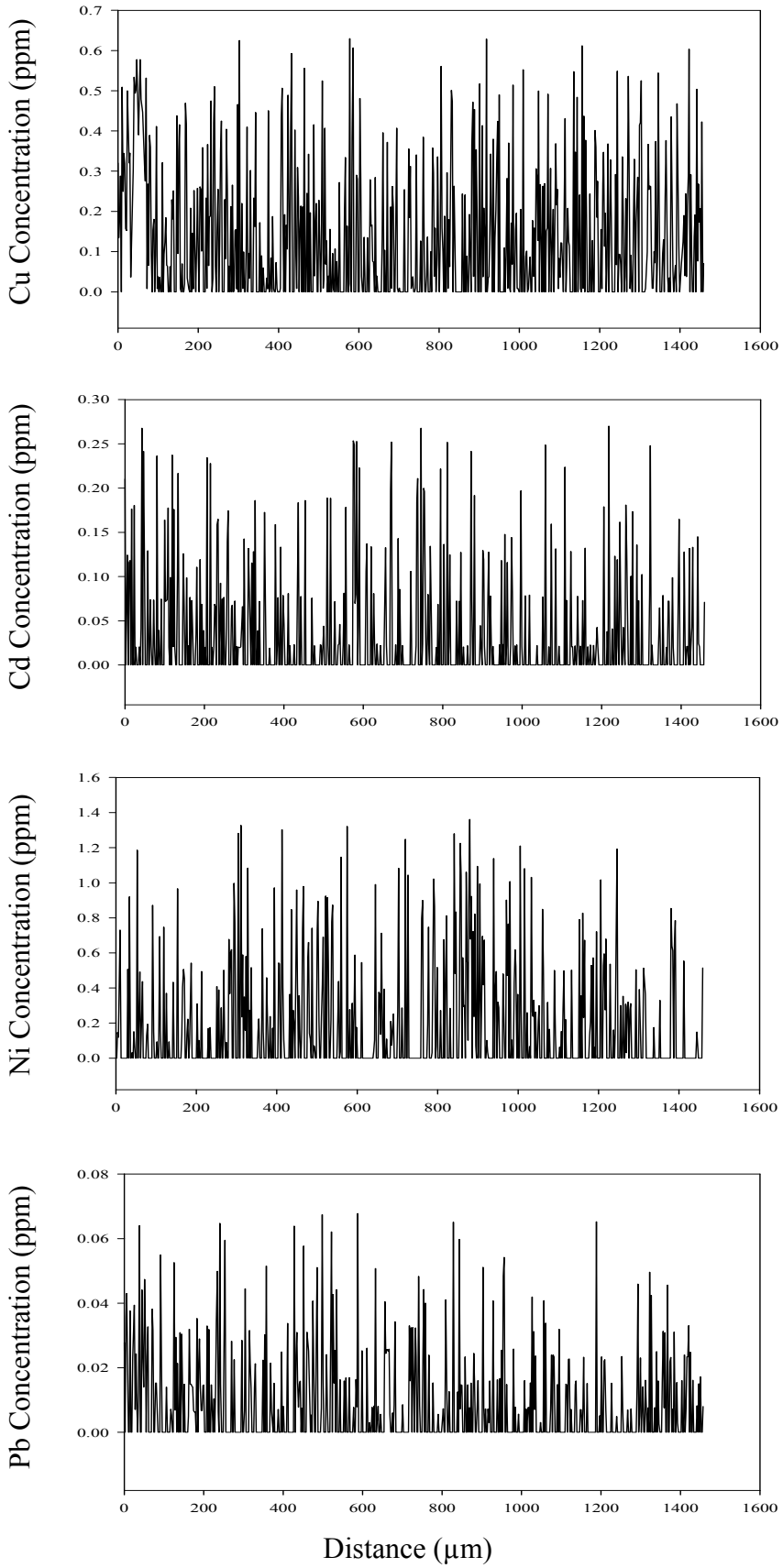


TL03-10

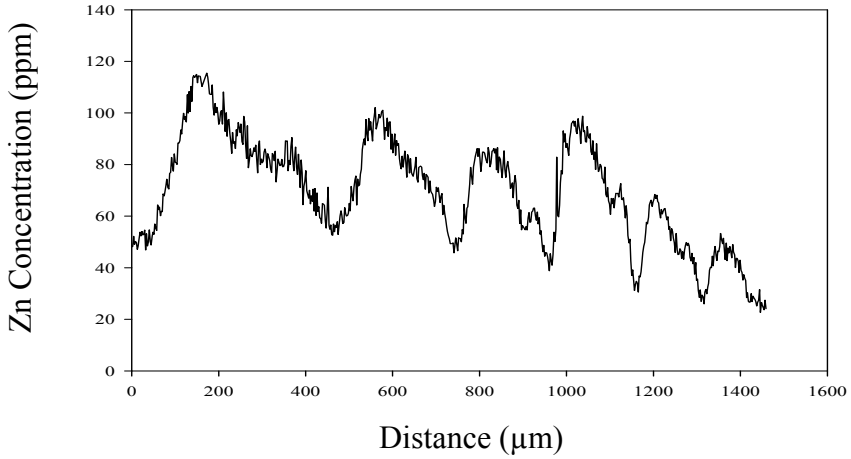

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
Distance (μm)

TL03-11

Age: 6^{+}

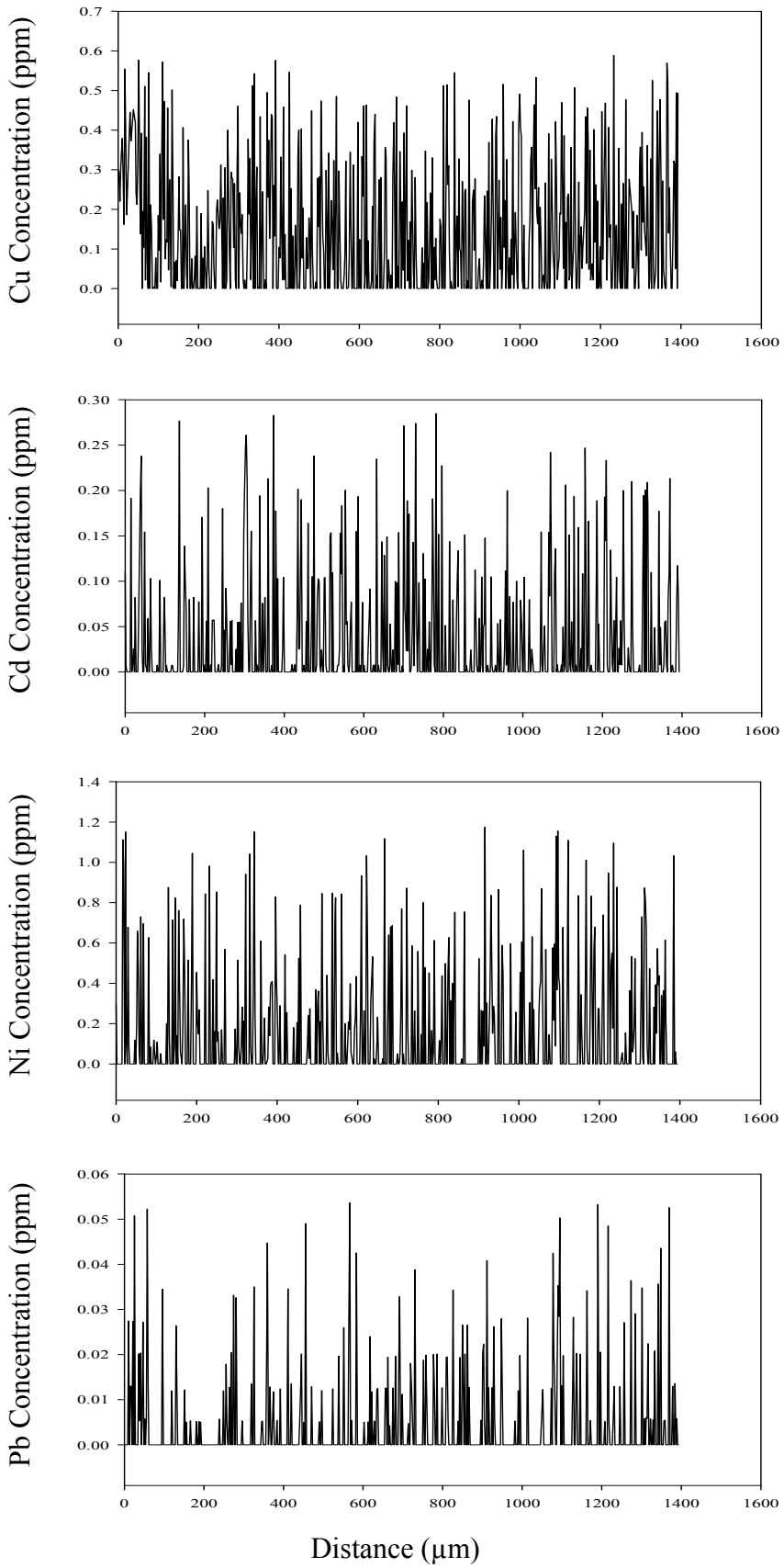

Cu Concentration (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (μm)

TL03-11

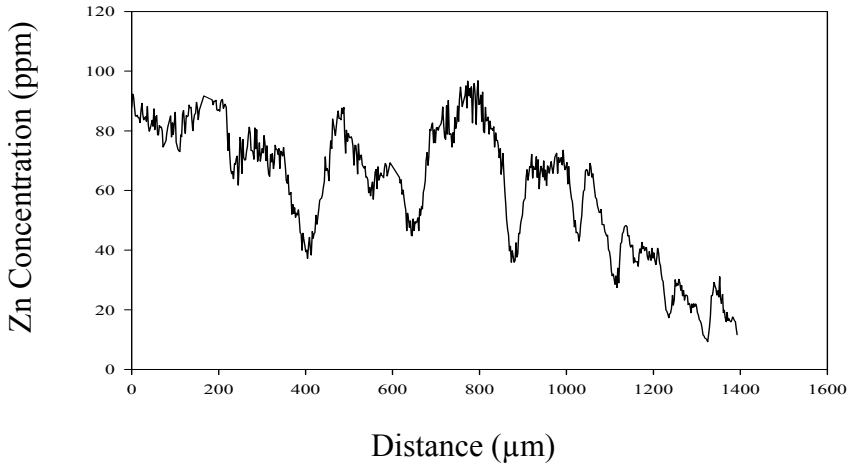

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL04-16

Age: 9+

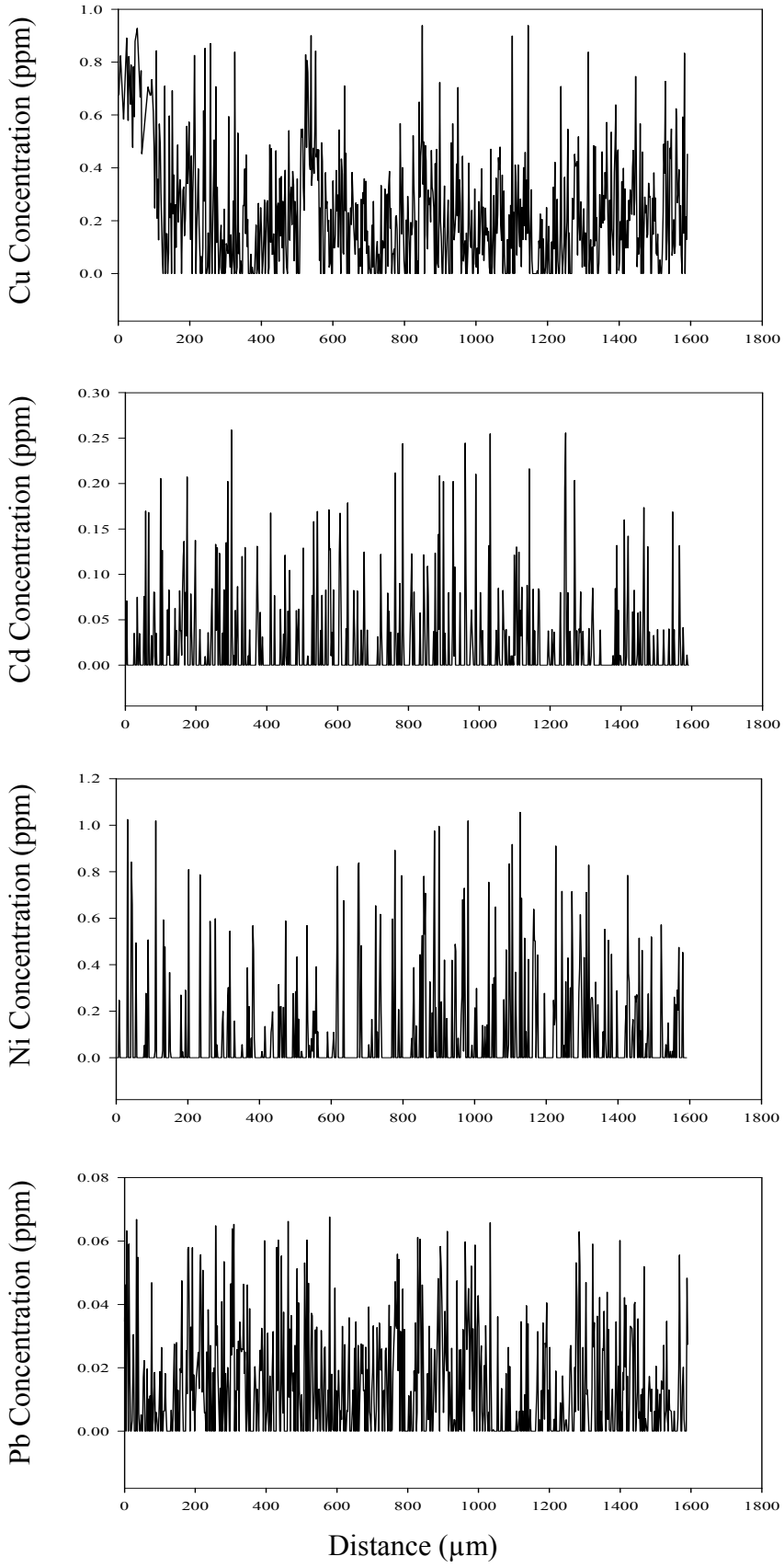

Cu Concentration (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Distance (μm)

TL04-16

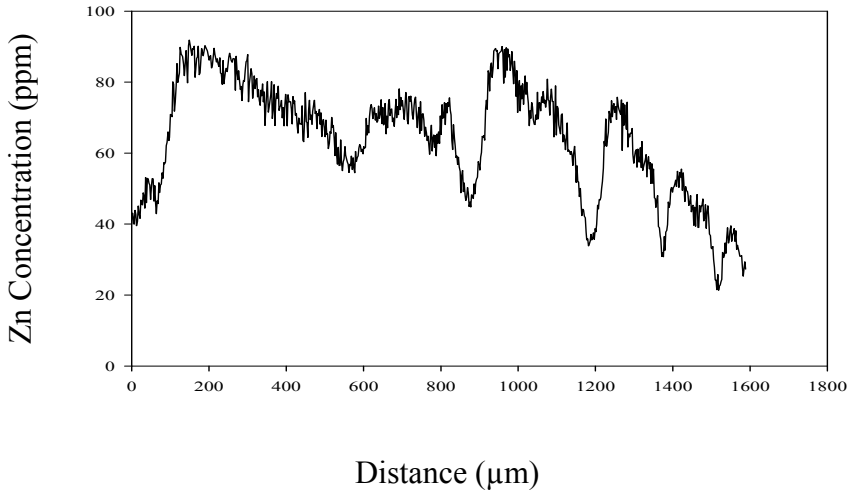

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL04-17

Age: 6+

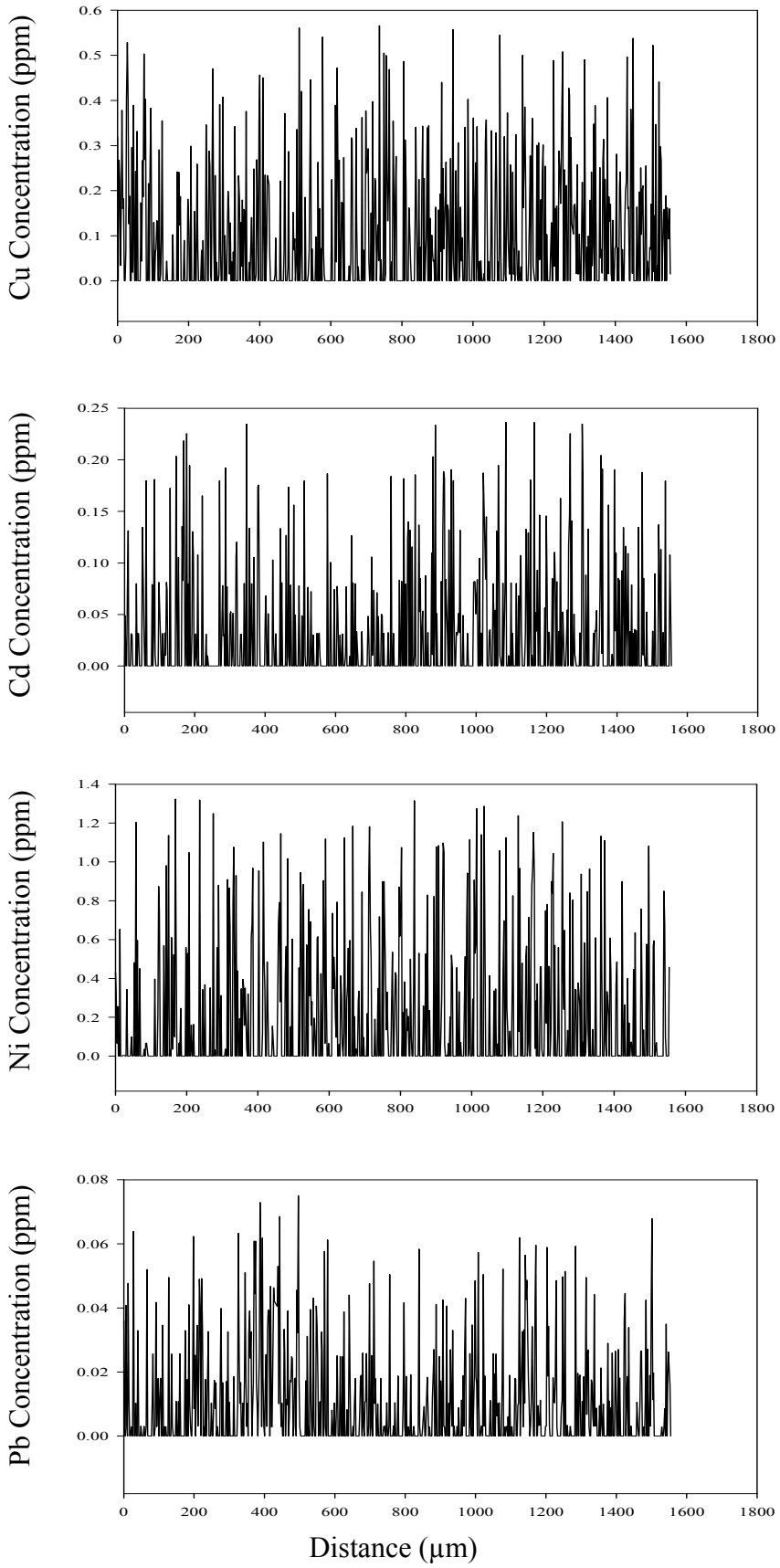

Cu Concentration (ppm)
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
1600
1800
Cd Concentration (ppm)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (μm)

TL04-17

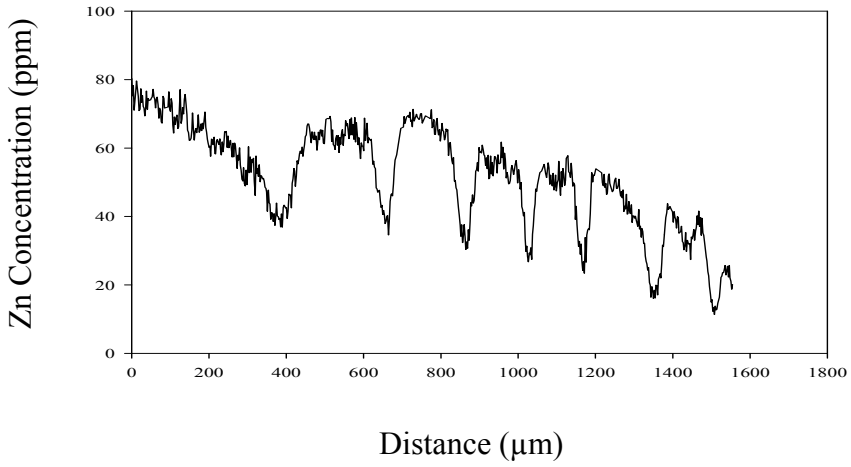

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL04-18

Age: 8+

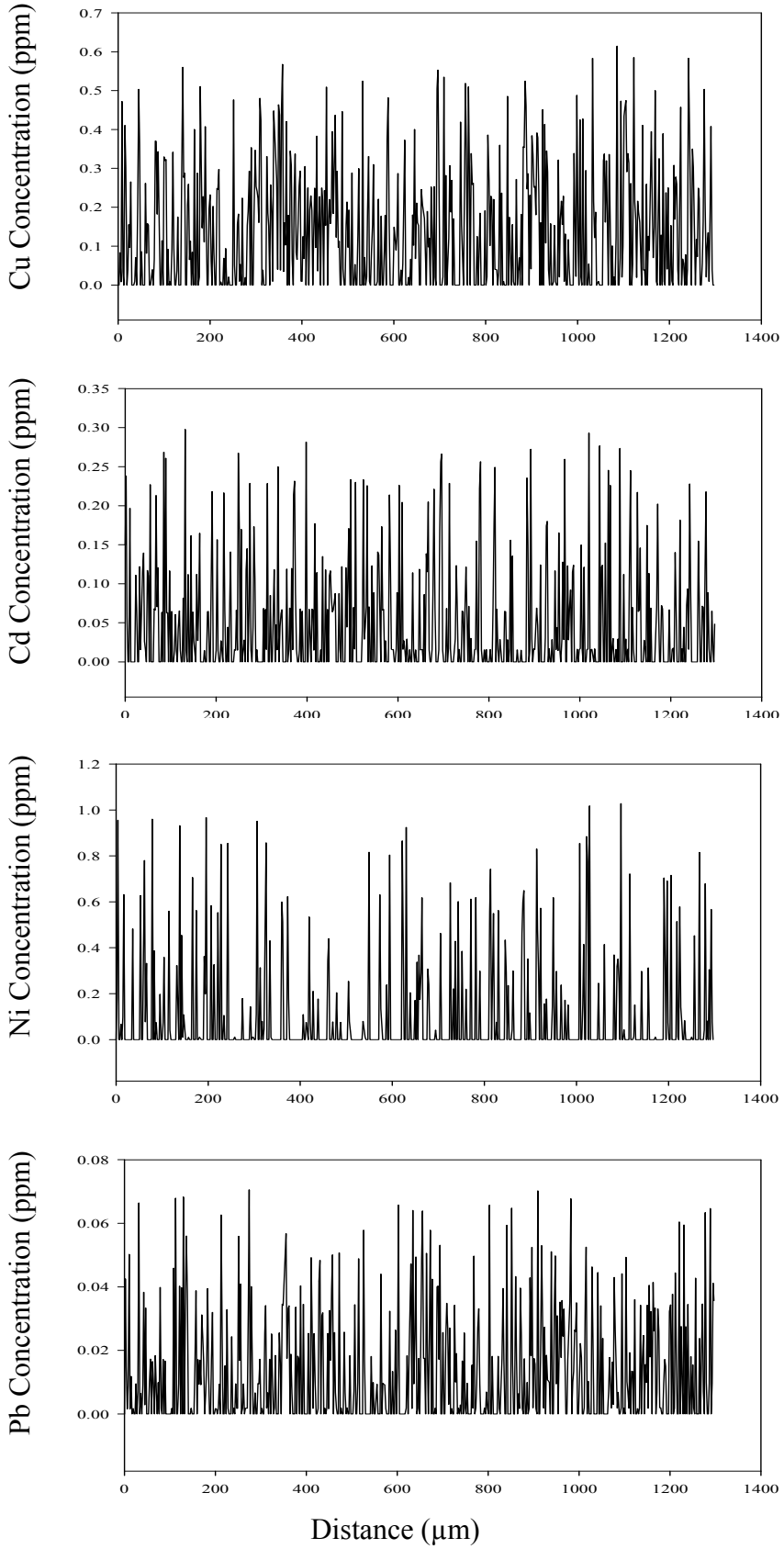

Cu Concentration (ppm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
1800
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (μm)

TL04-18

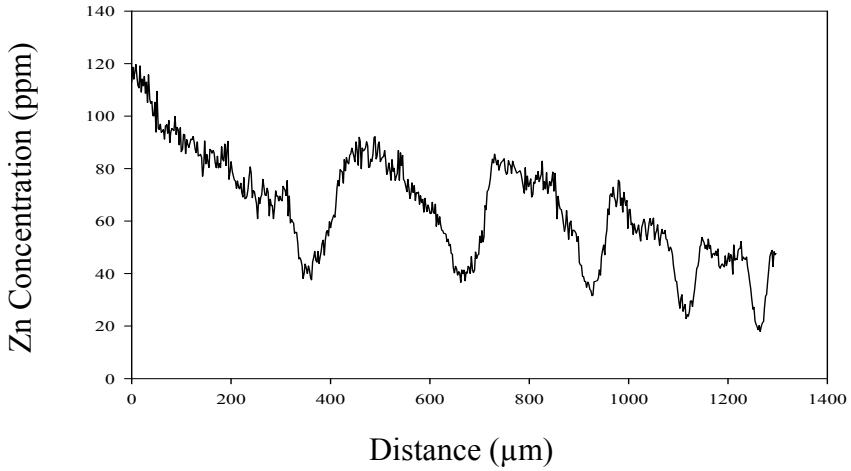

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL04-19

Age: 6+

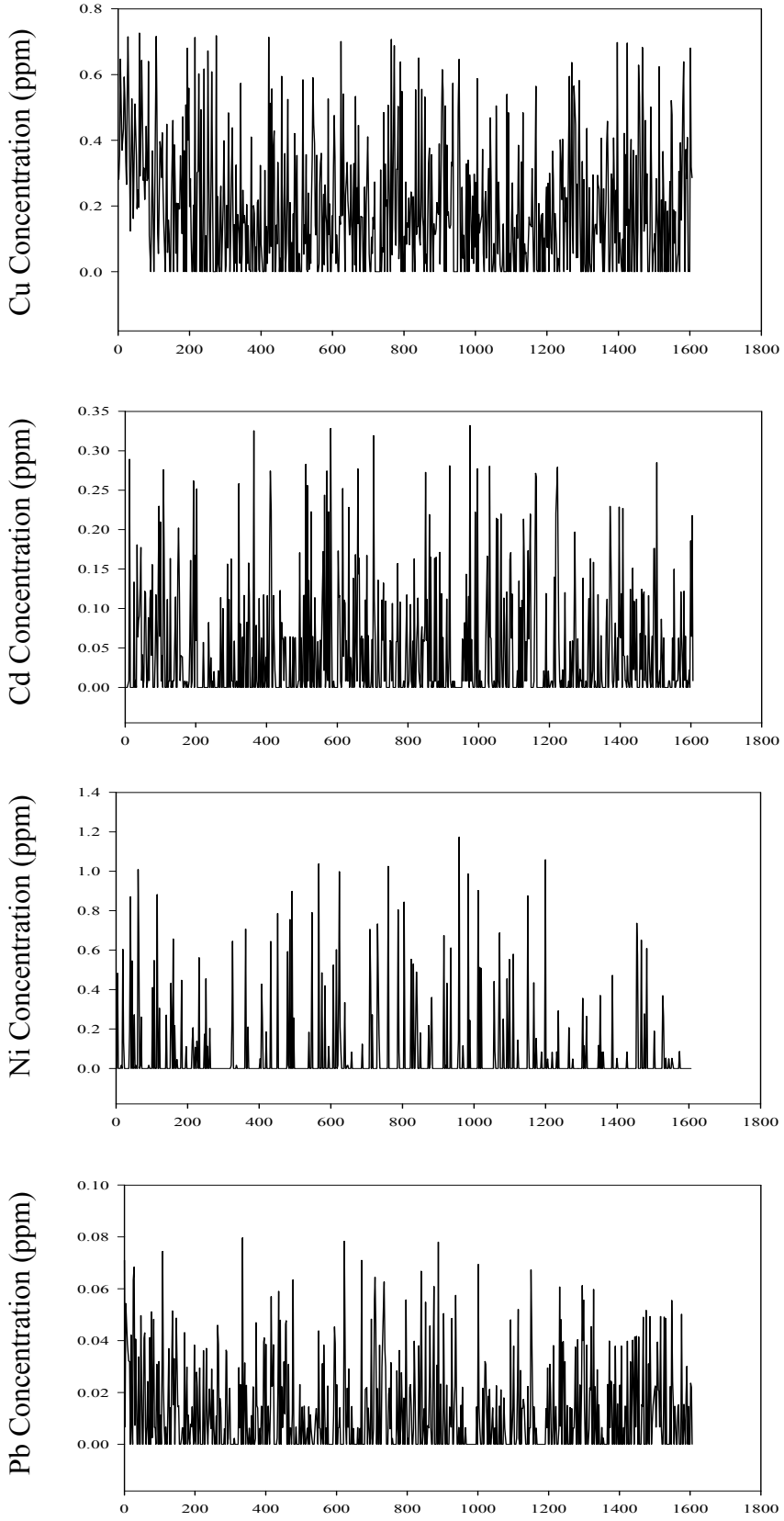

Cu Concentration (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
Cd Concentration (ppm)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (µm)

TL04-19

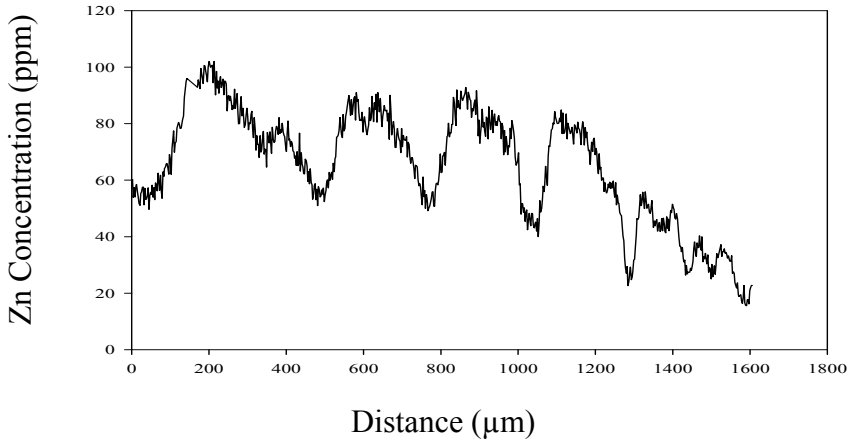

Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
Distance (μm)

TL04-20

Age: 10+

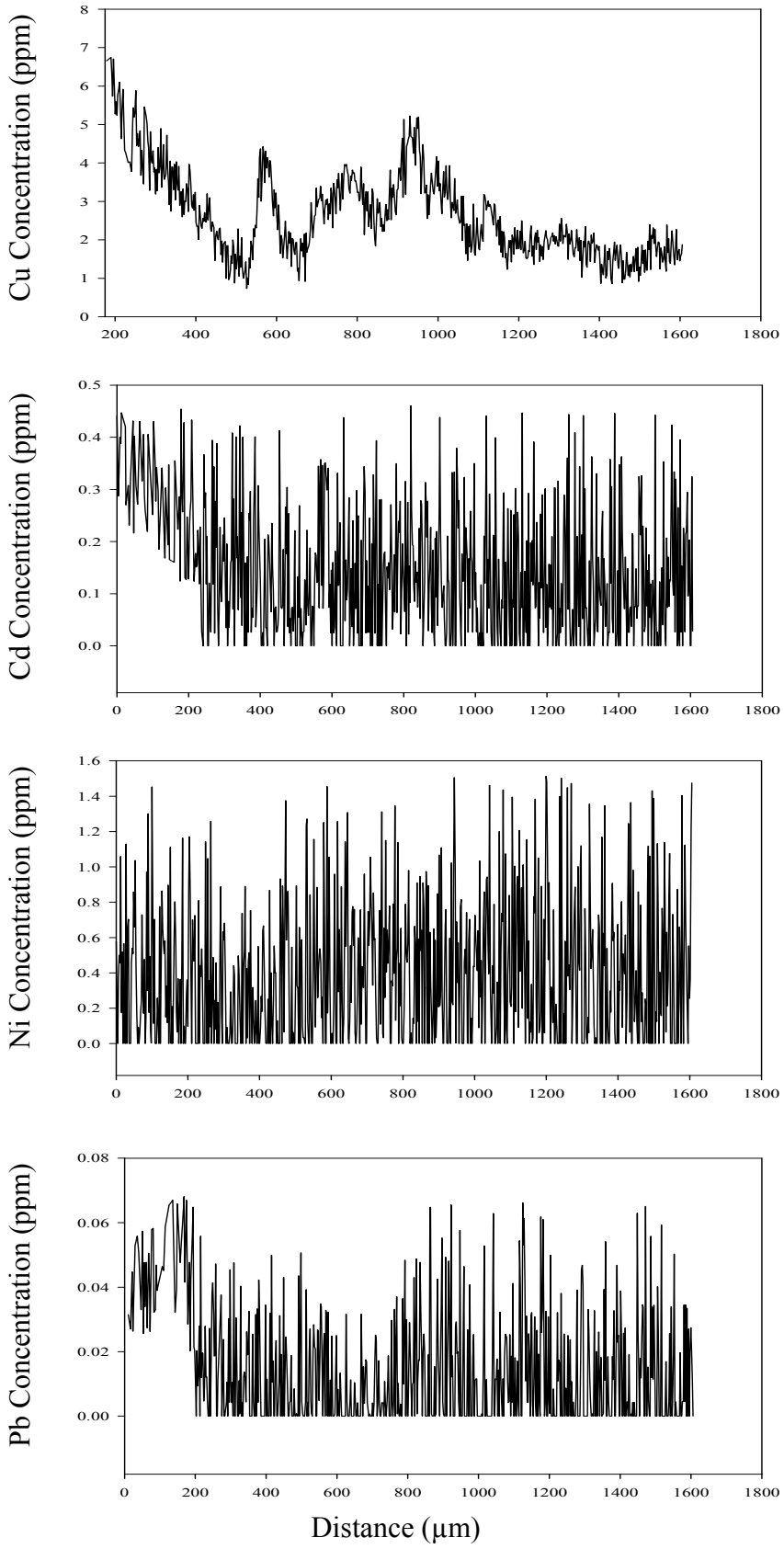


TL04-20

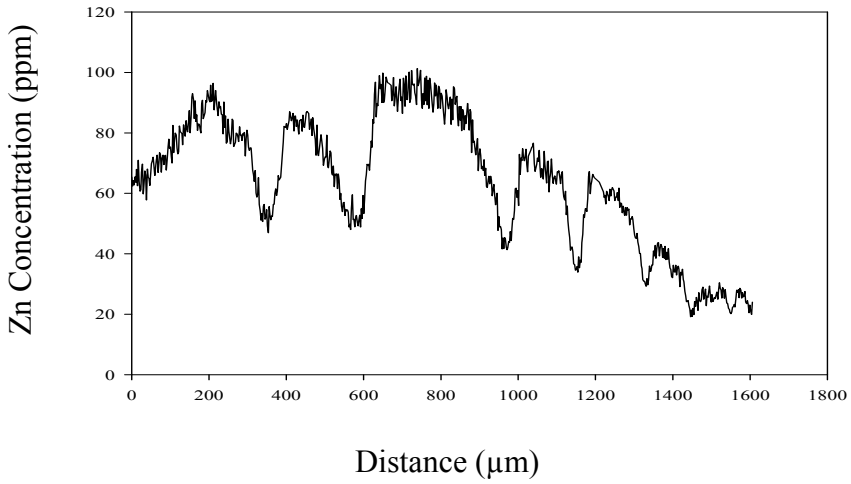

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL04-21

Age: 7+

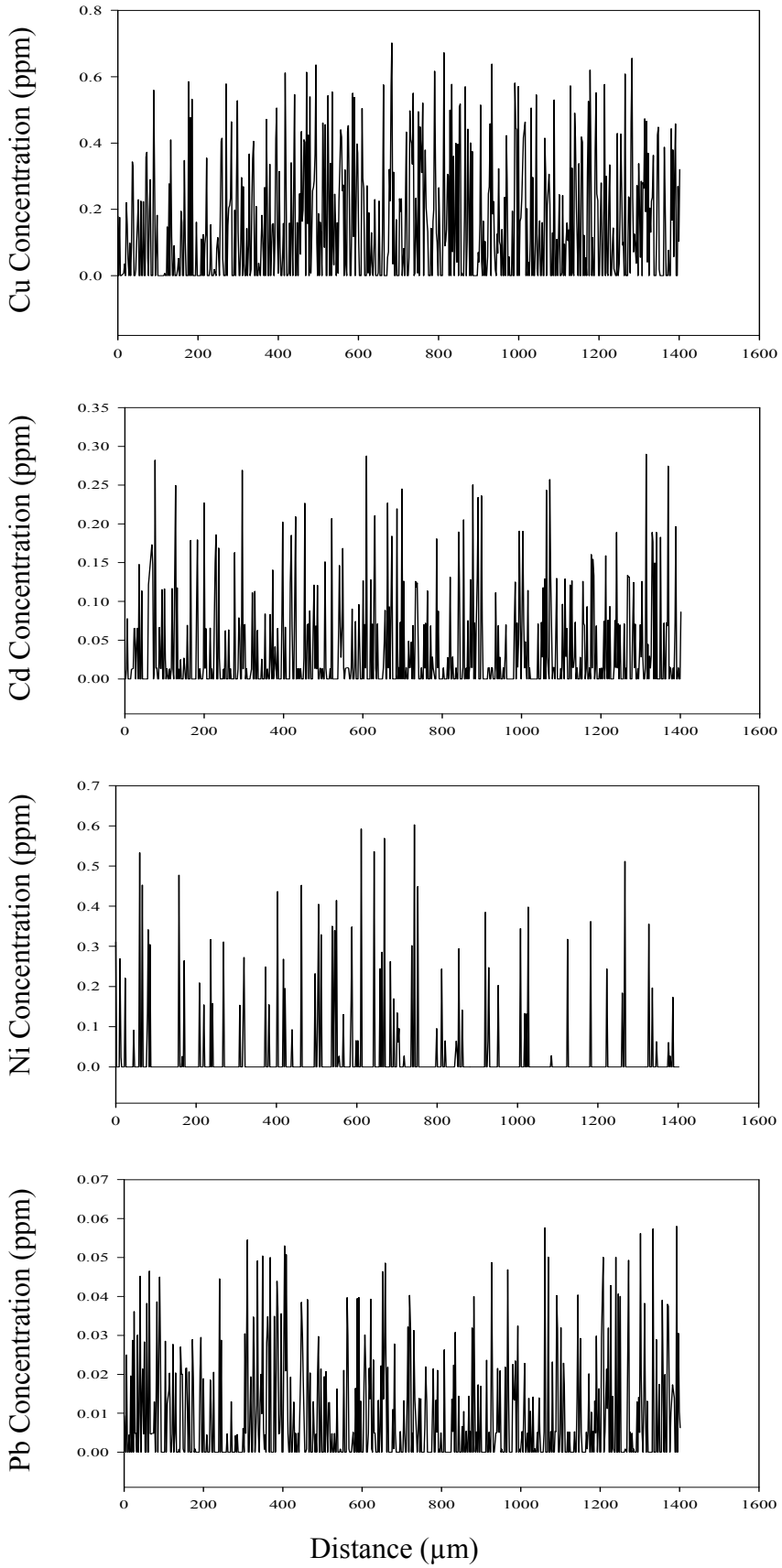

Cu Concentration (ppm)
Cd Concentration (ppm)
Ni Concentration (ppm)
Pb Concentration (ppm)
Distance (µm)

TL04-21

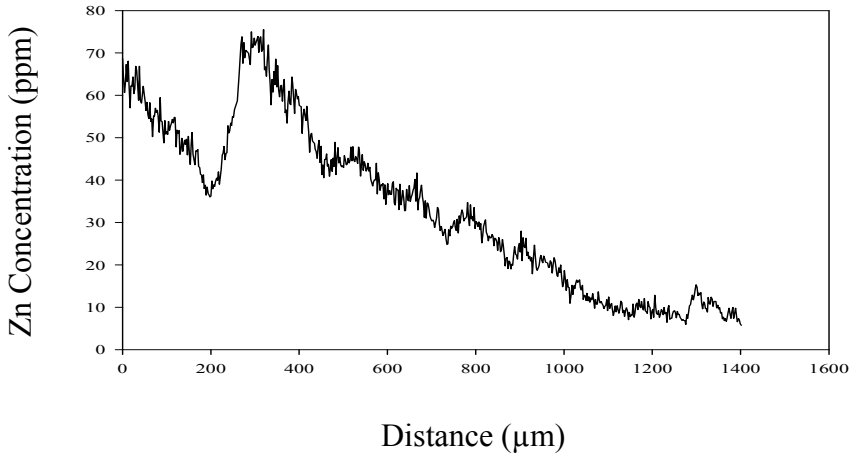

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

TL04-23

Age: 11+

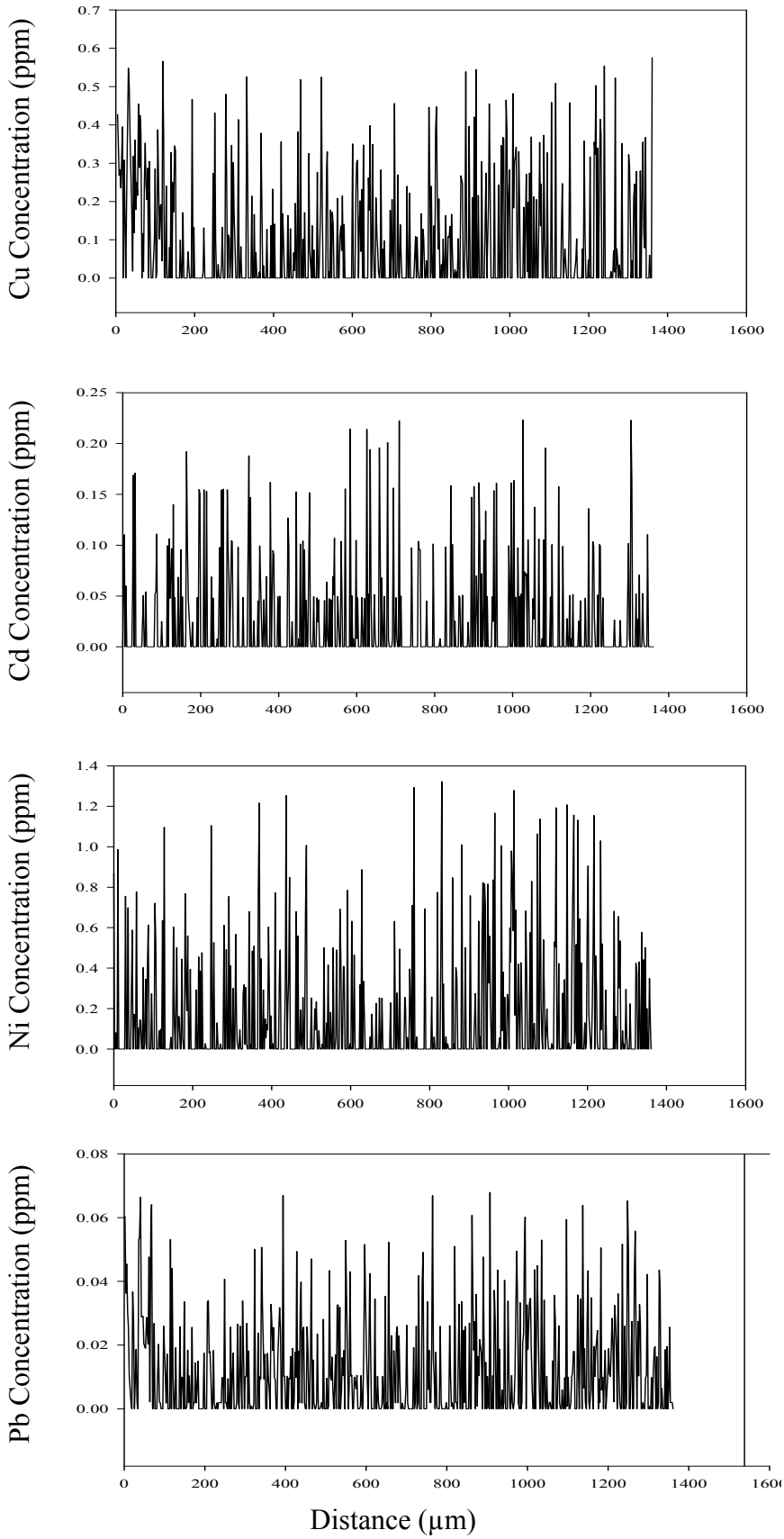


TL04-23

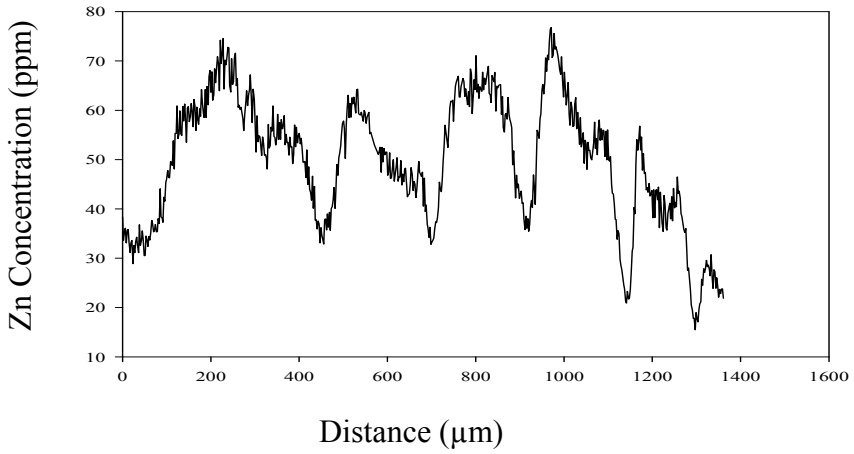

Zn Concentration (ppm)
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

TL-04-24

Age: 9+


Cu Concentration (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
600
800
1000
1200
1400
1600
Cd Concentration (ppm)
0.25
0.20
0.15
0.10
0.05
0.00
Ni Concentration (ppm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pb Concentration (ppm)
0.08
0.06
0.04
0.02
0.00
Distance (μm)

TL04-24

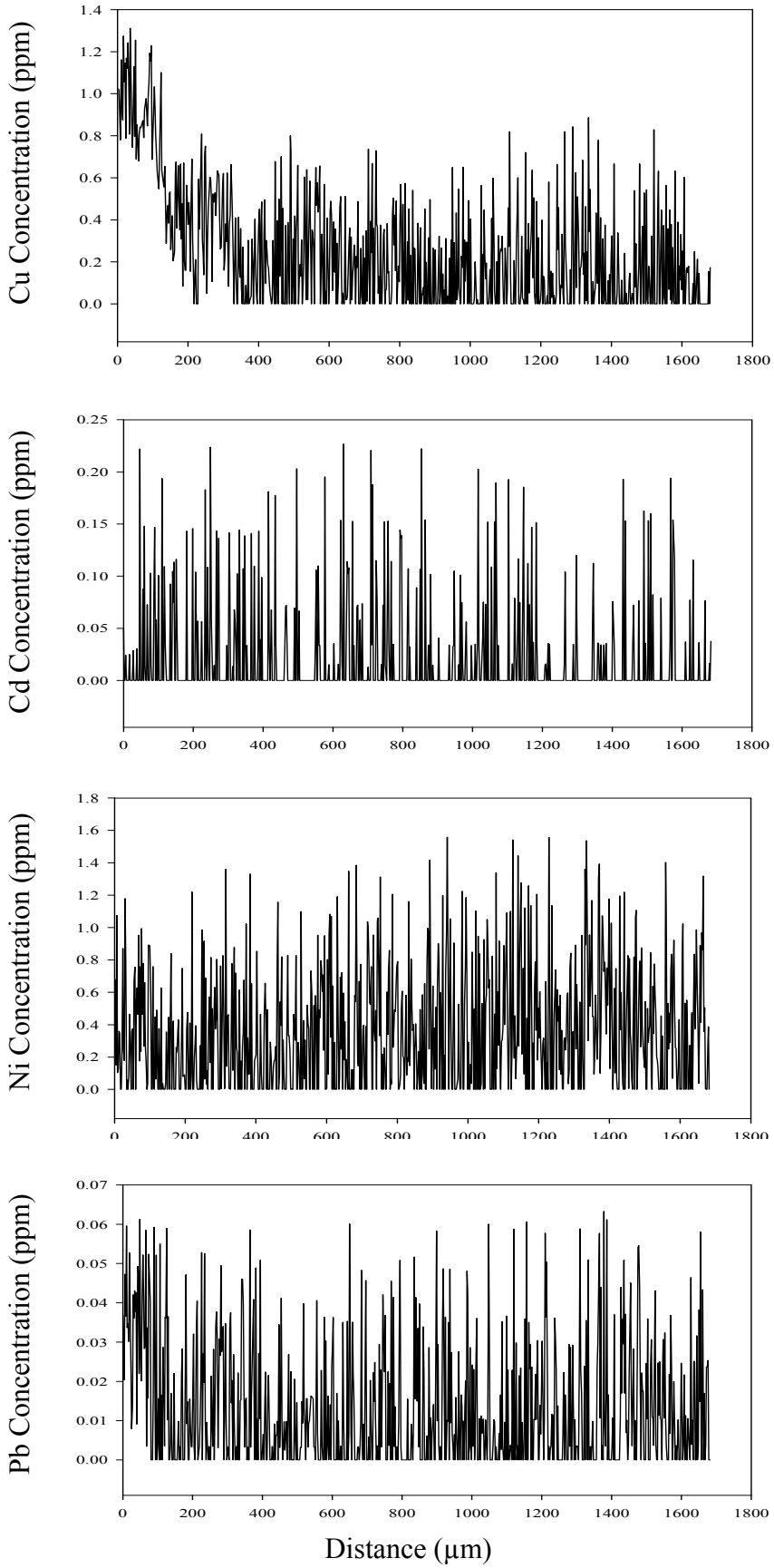

Zn Concentration (ppm)
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

APPENDIX III

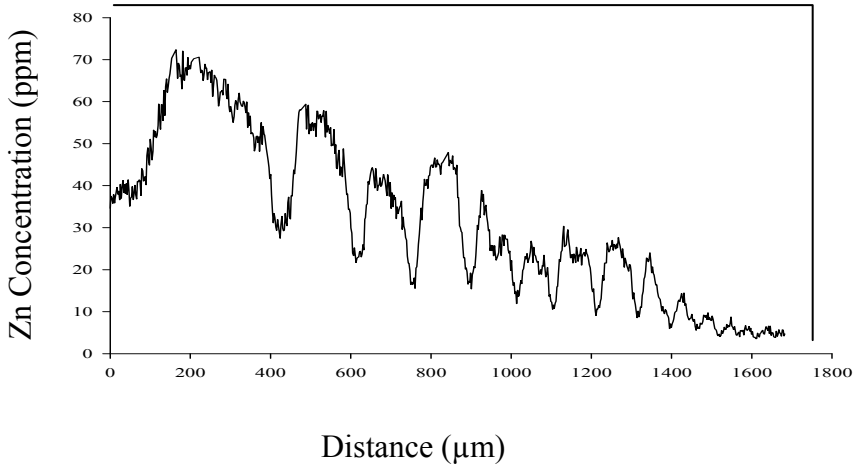
Metal Results for Confederation Lake

CFDL01-01

Age: 14

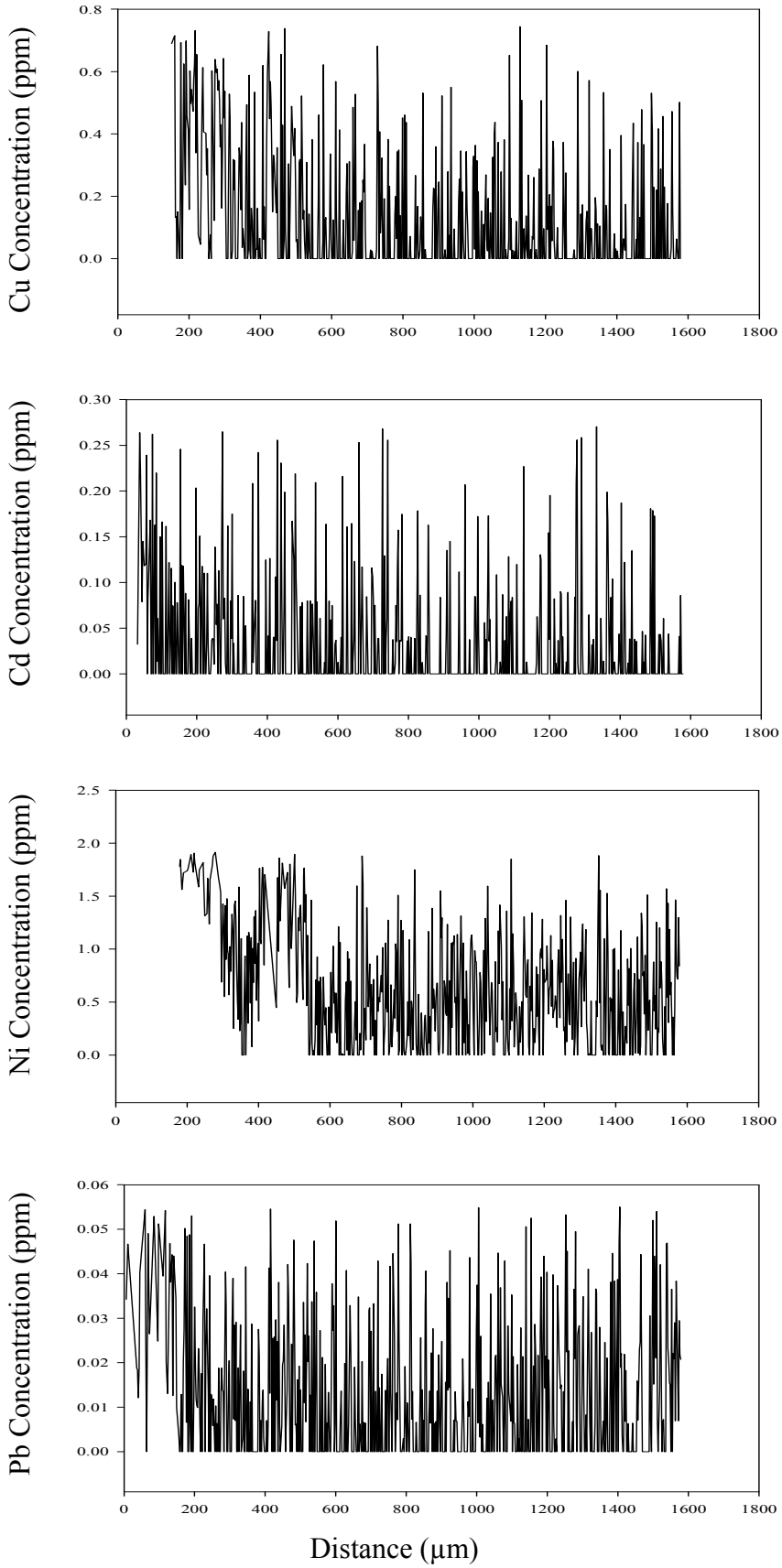


CFDL01-01

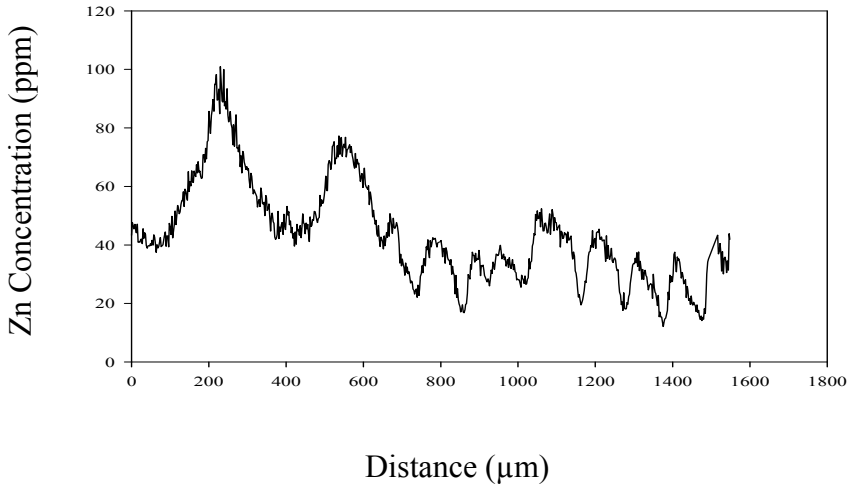

Zn Concentration (ppm)
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

CFDL01-02a

Age: 9

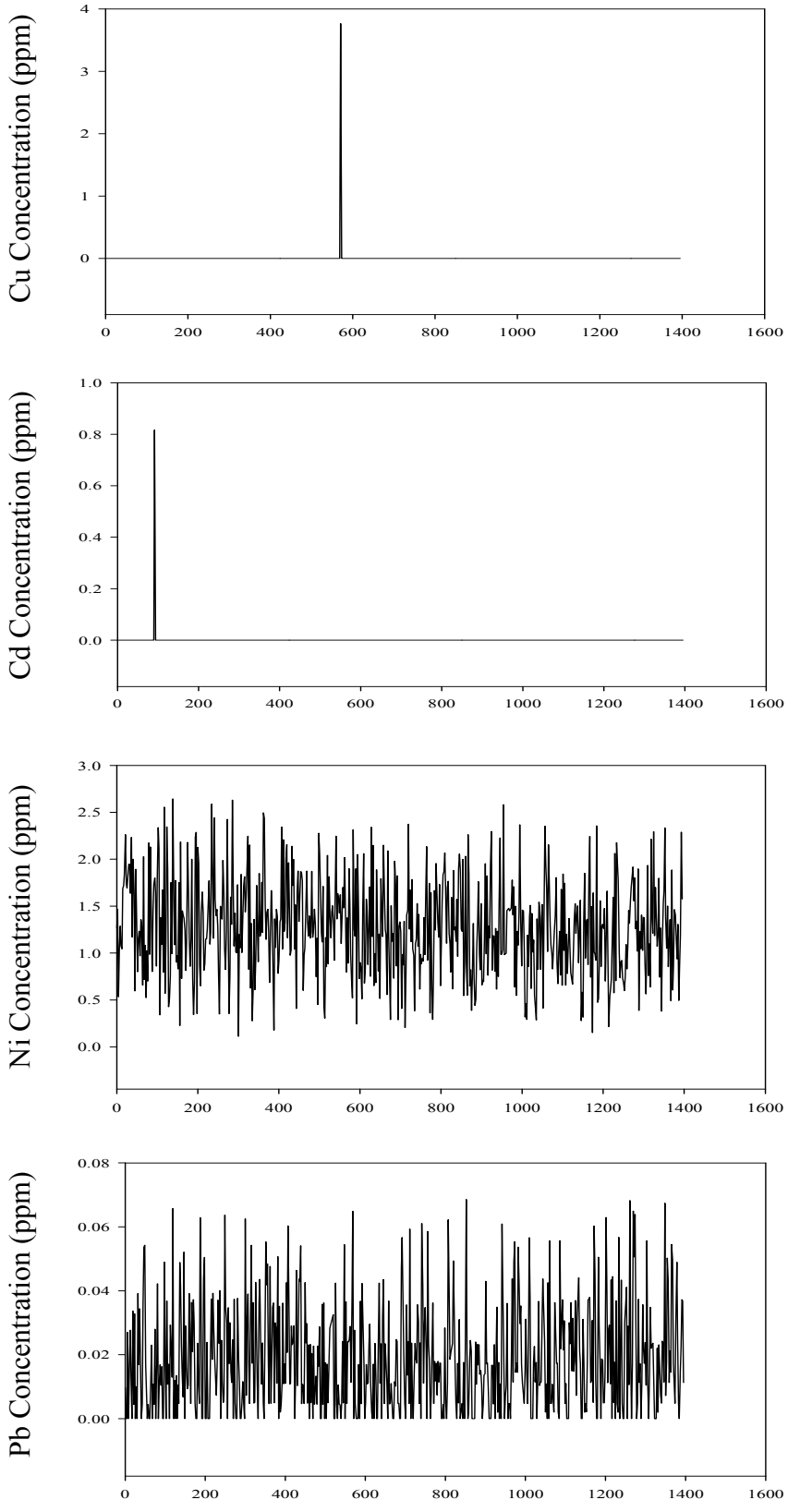


CFDL01-2a

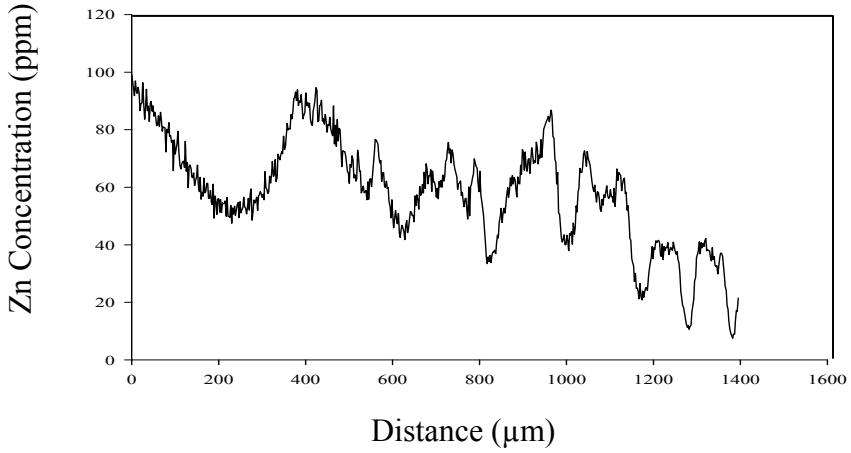

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

CFDL01-03

Age: 8+

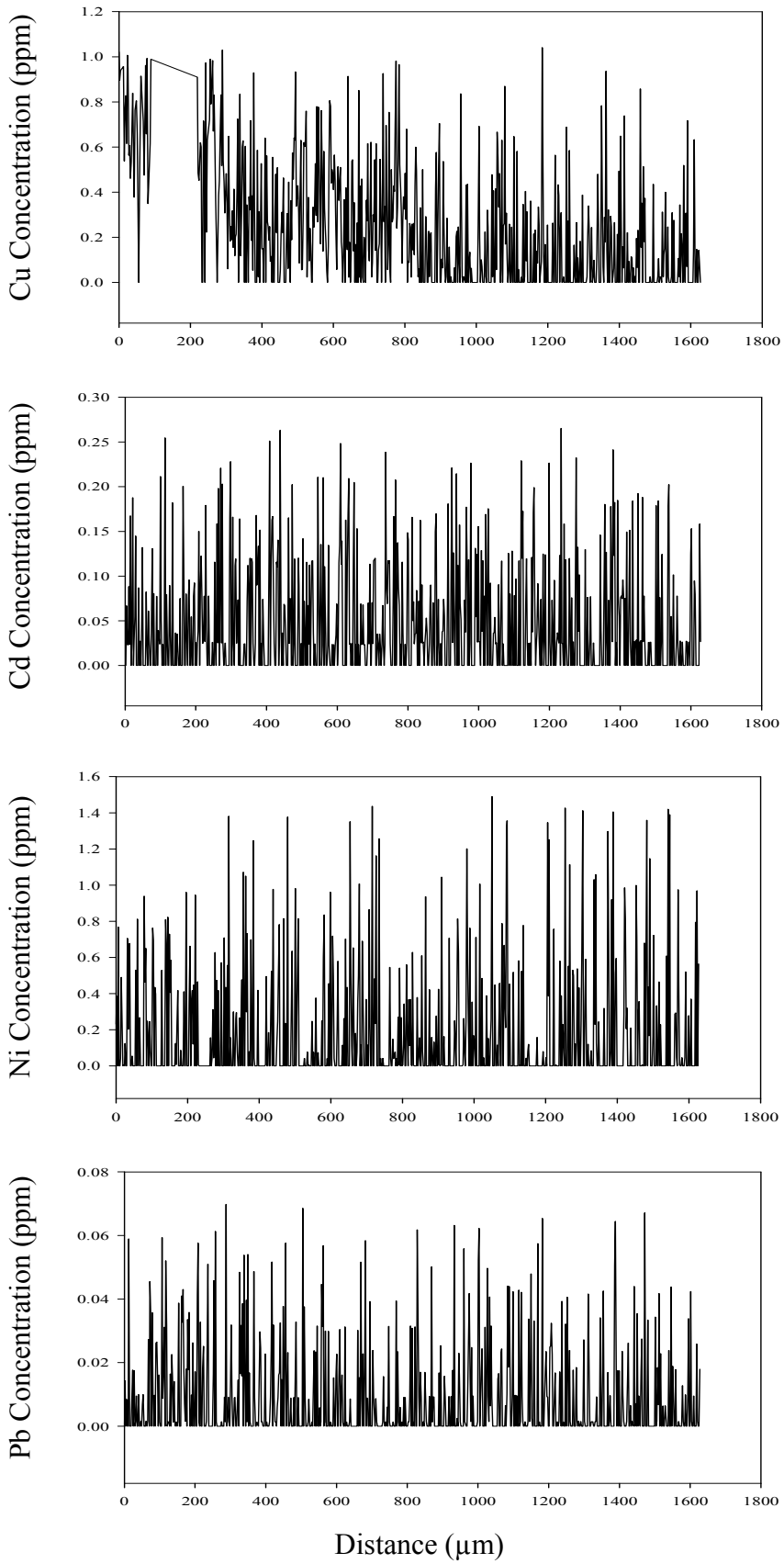


CFDL01-03

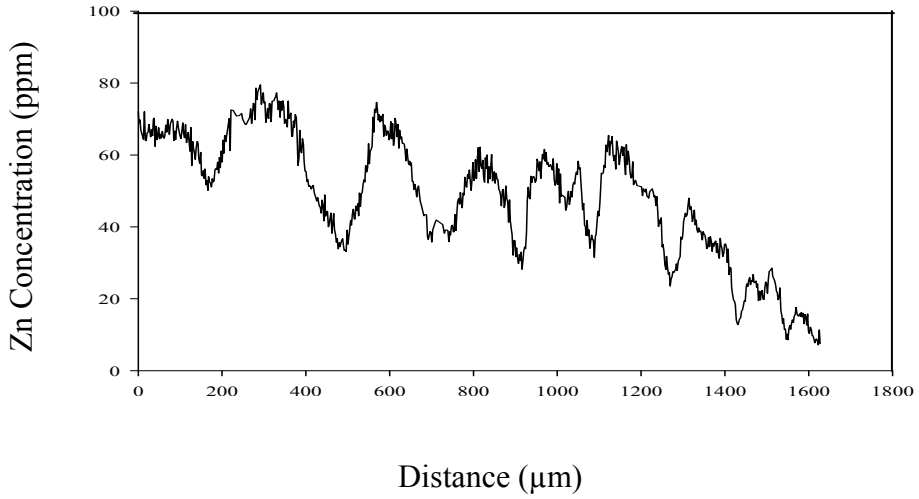

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL01-04

Age: 9+

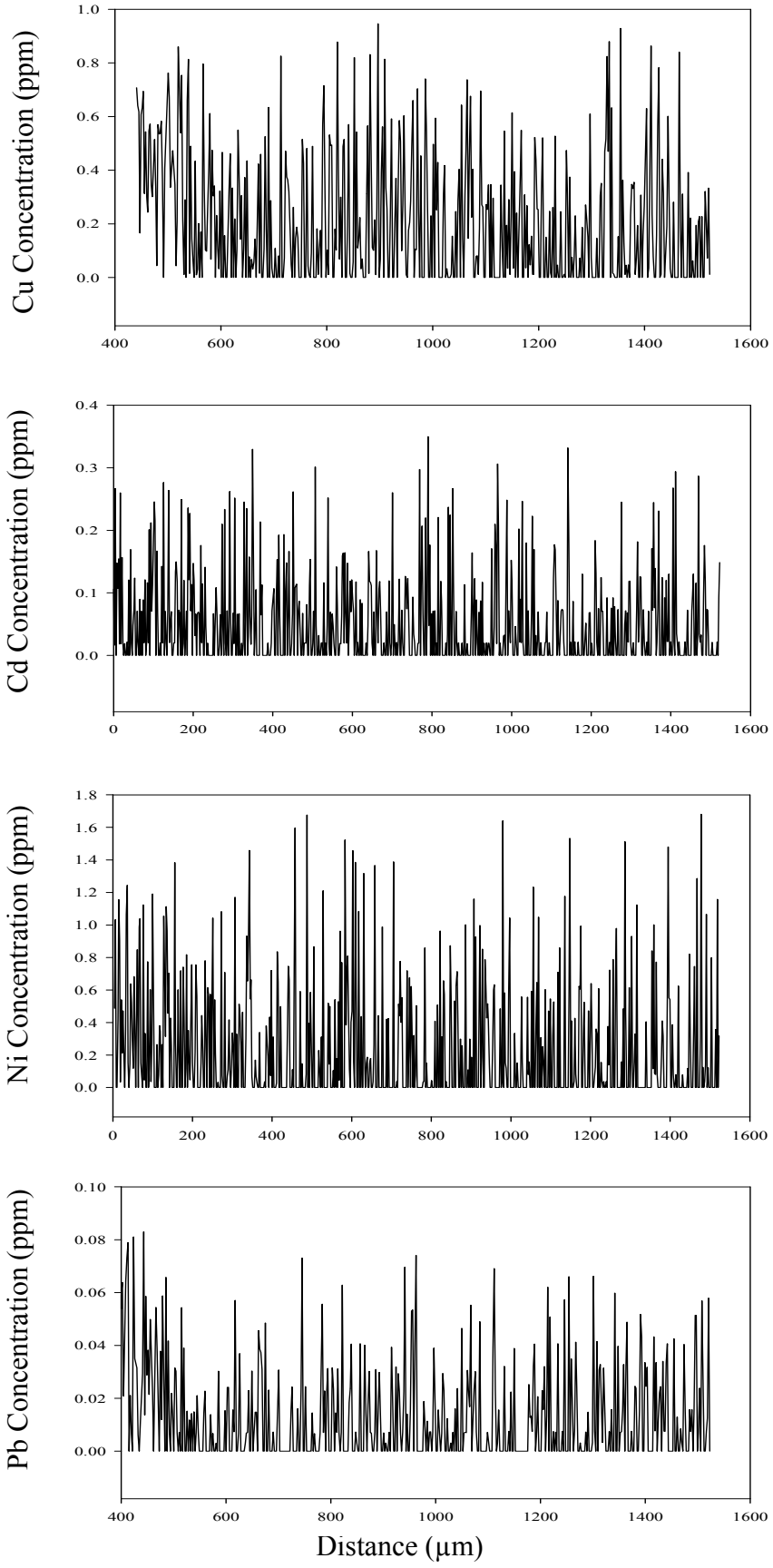


CFDL01-04

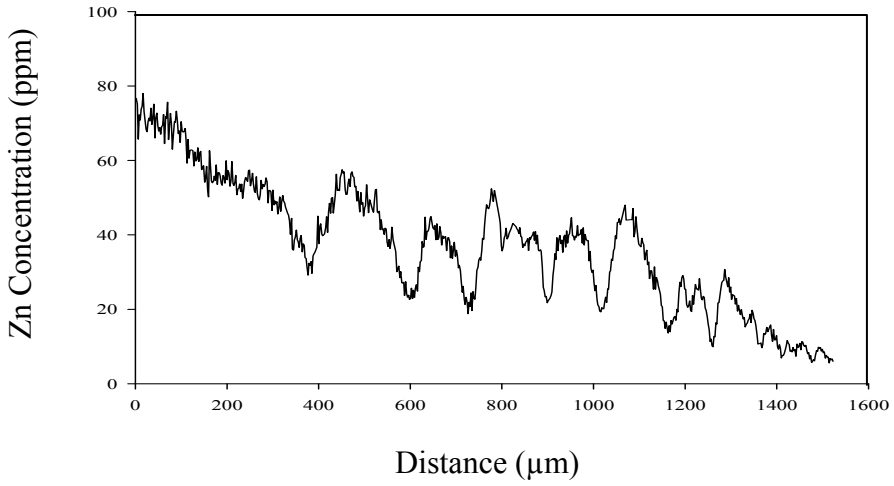

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

CFDL01-5a

Age: 13

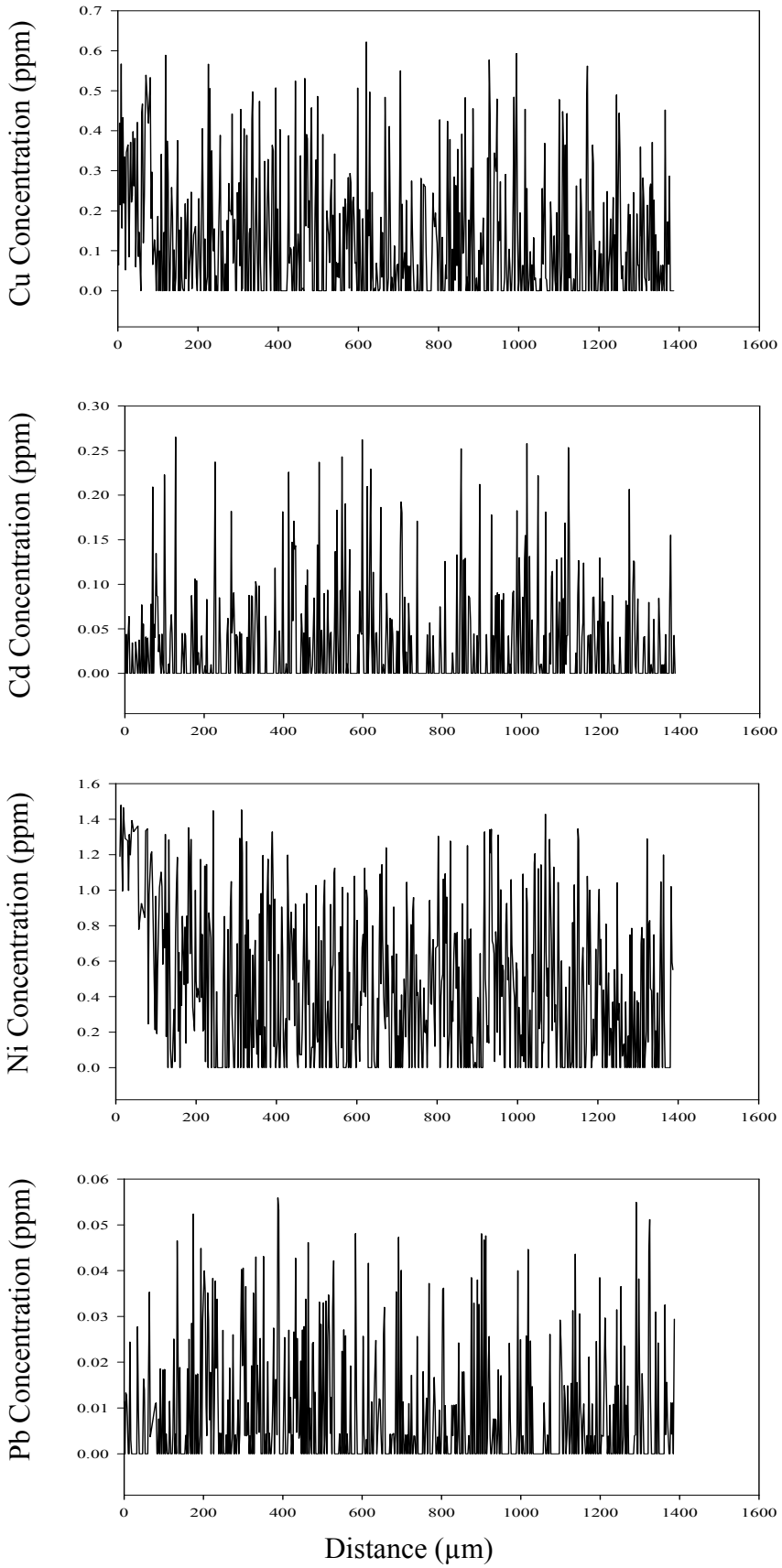


CFDL01-5a

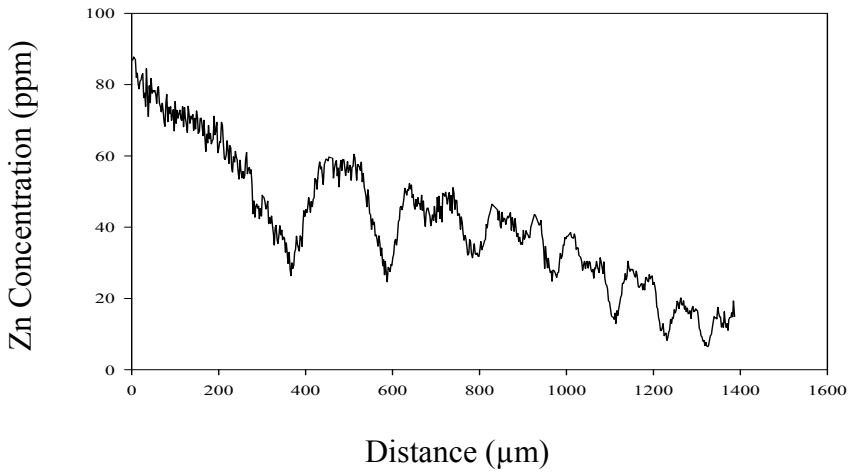

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL02-05

Age: 8

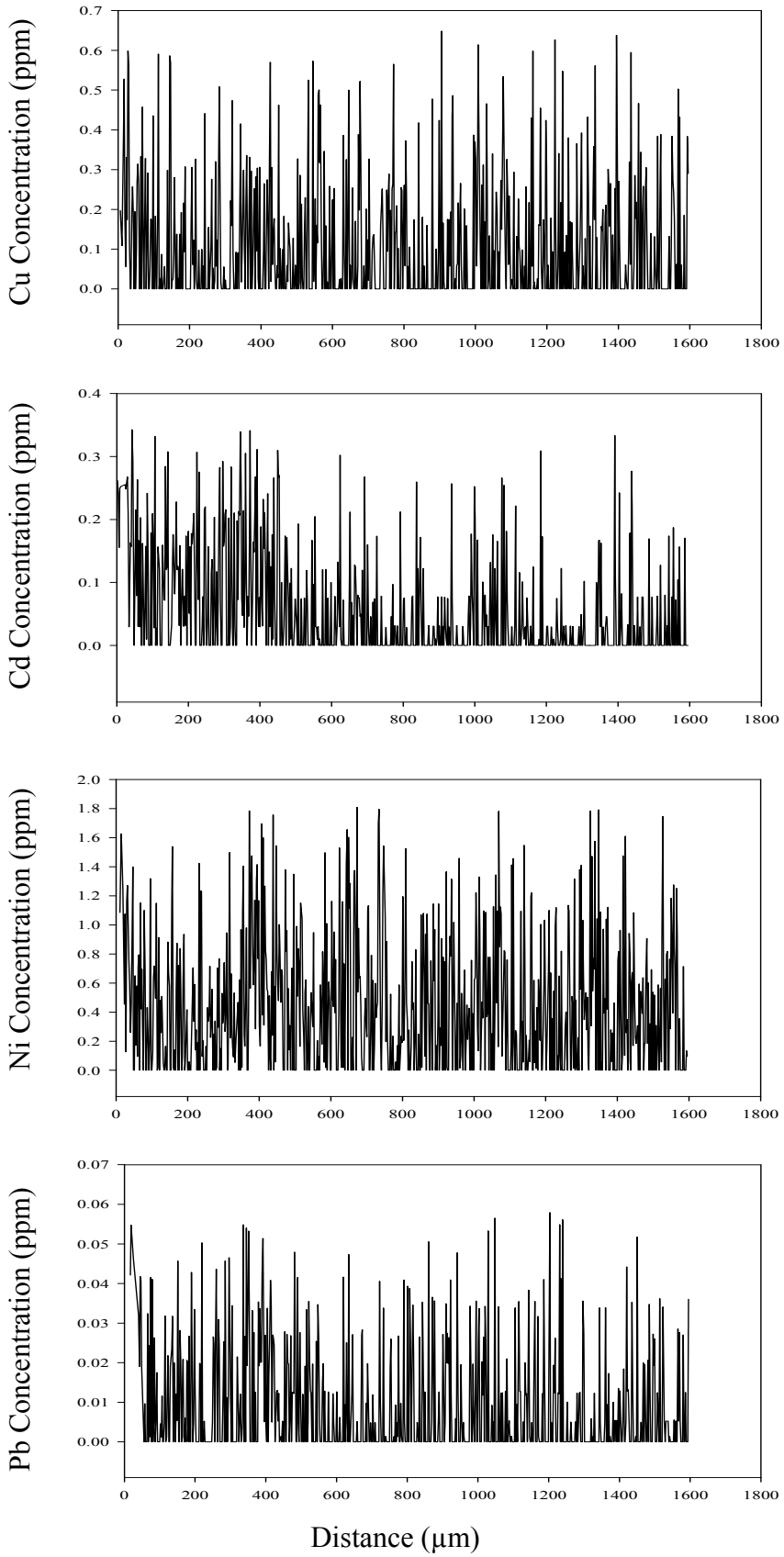


CFDL02-05

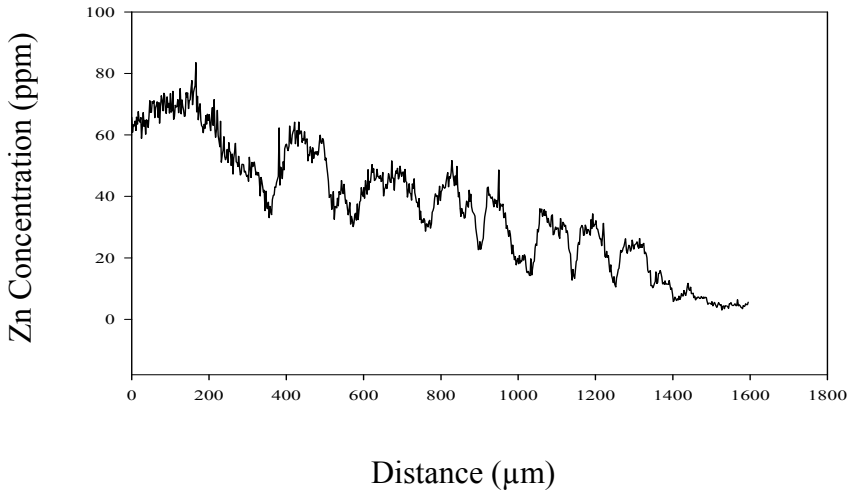

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL02-06

Age: 12+

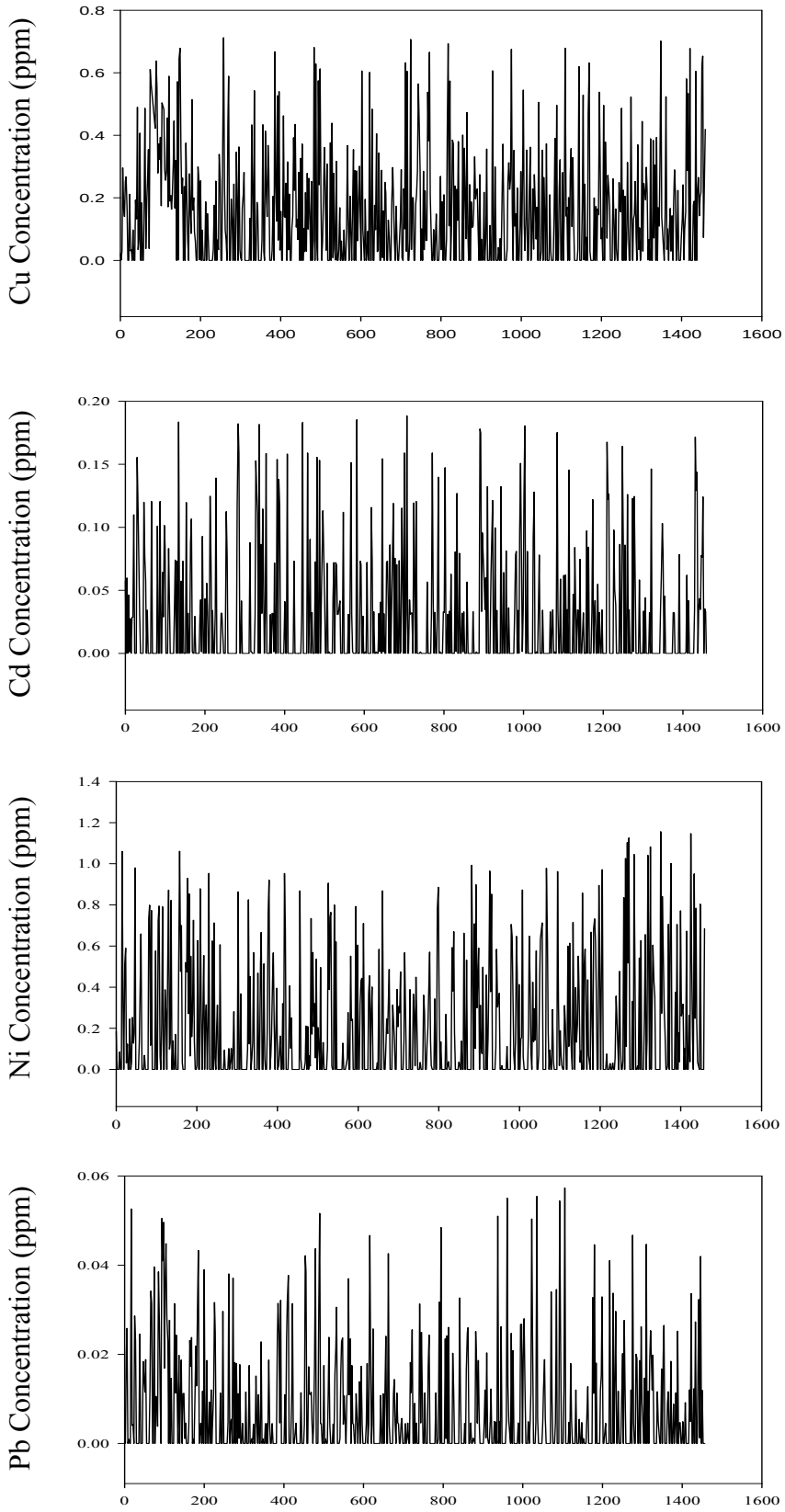


CFDL02-06

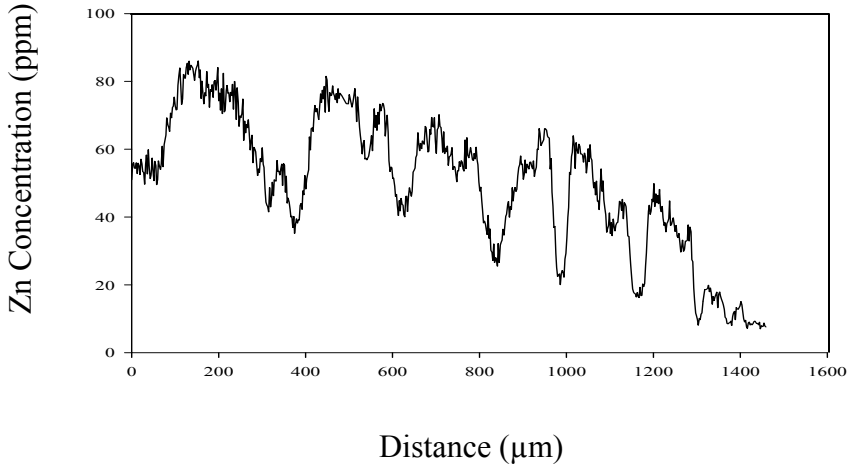

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

CFDL02-07

Age: 8

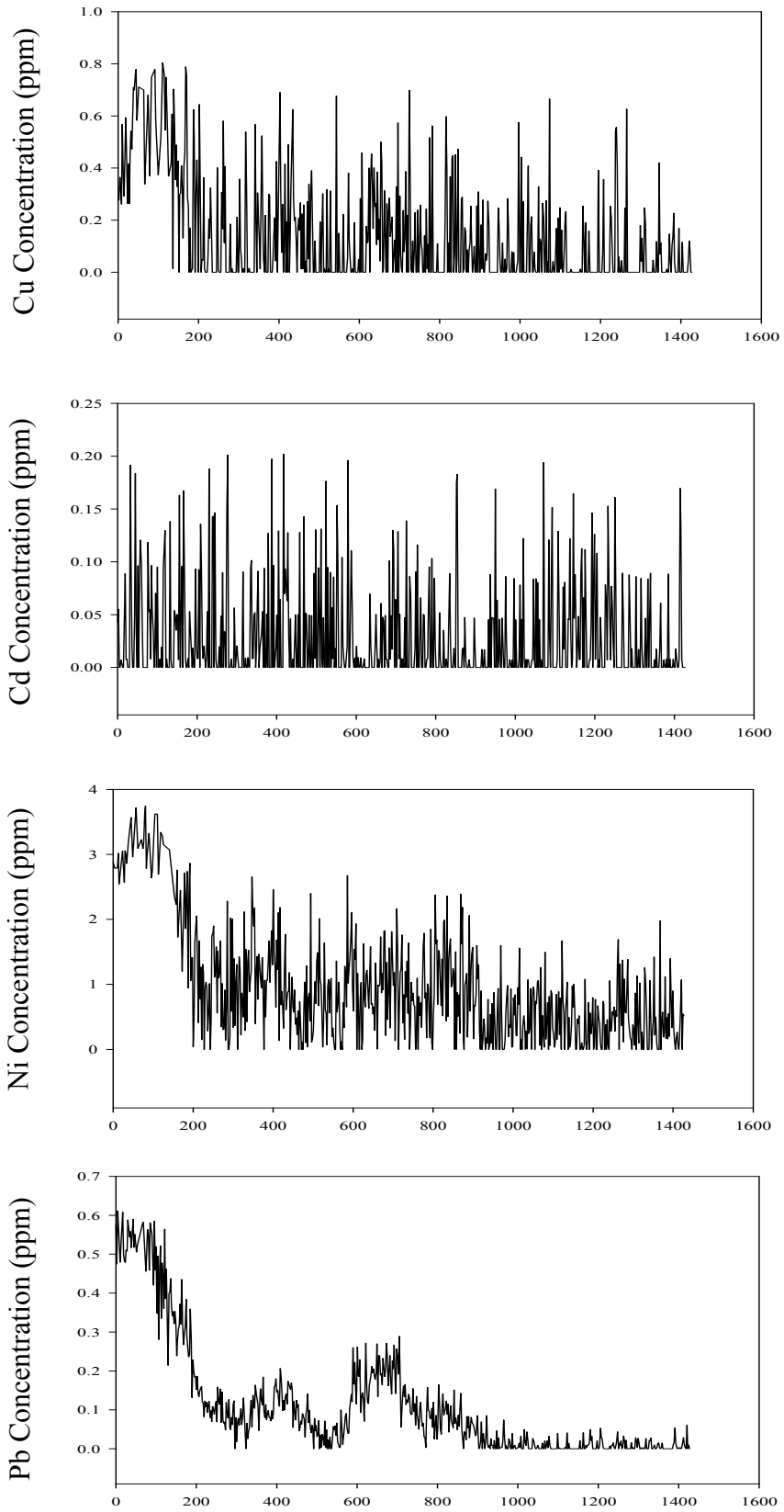


CFDL02-07

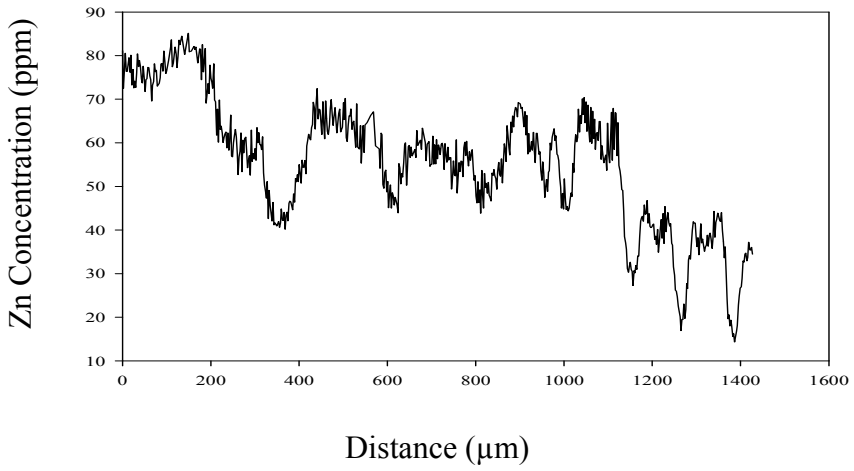

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL02-09

Age: 7+

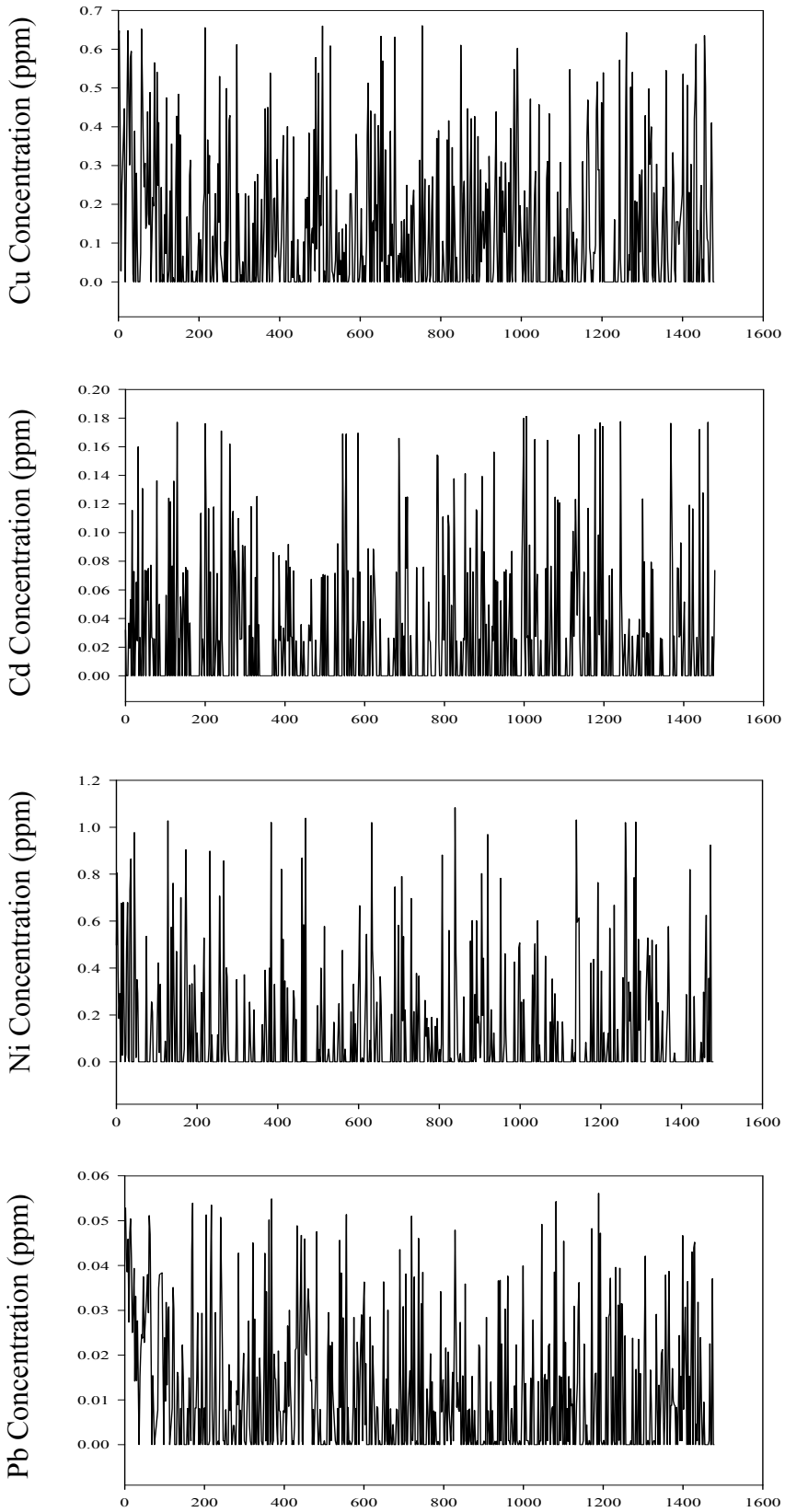


CFDL02-09

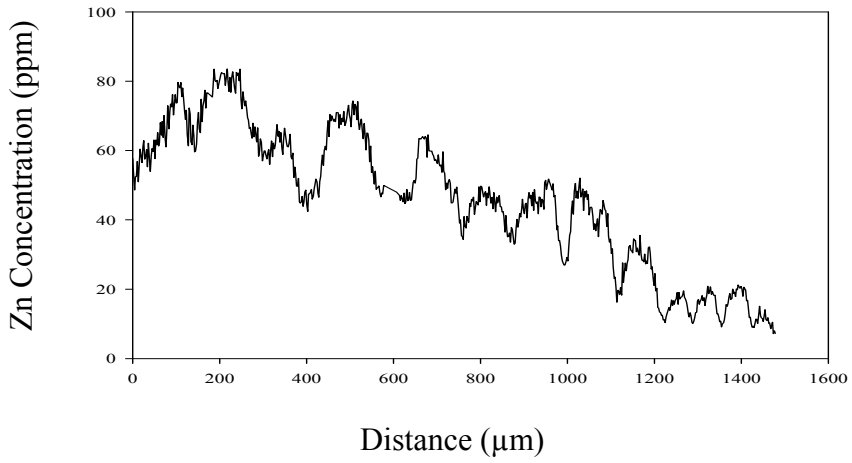

Zn Concentration (ppm)
90
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL02-10

Age: 11+

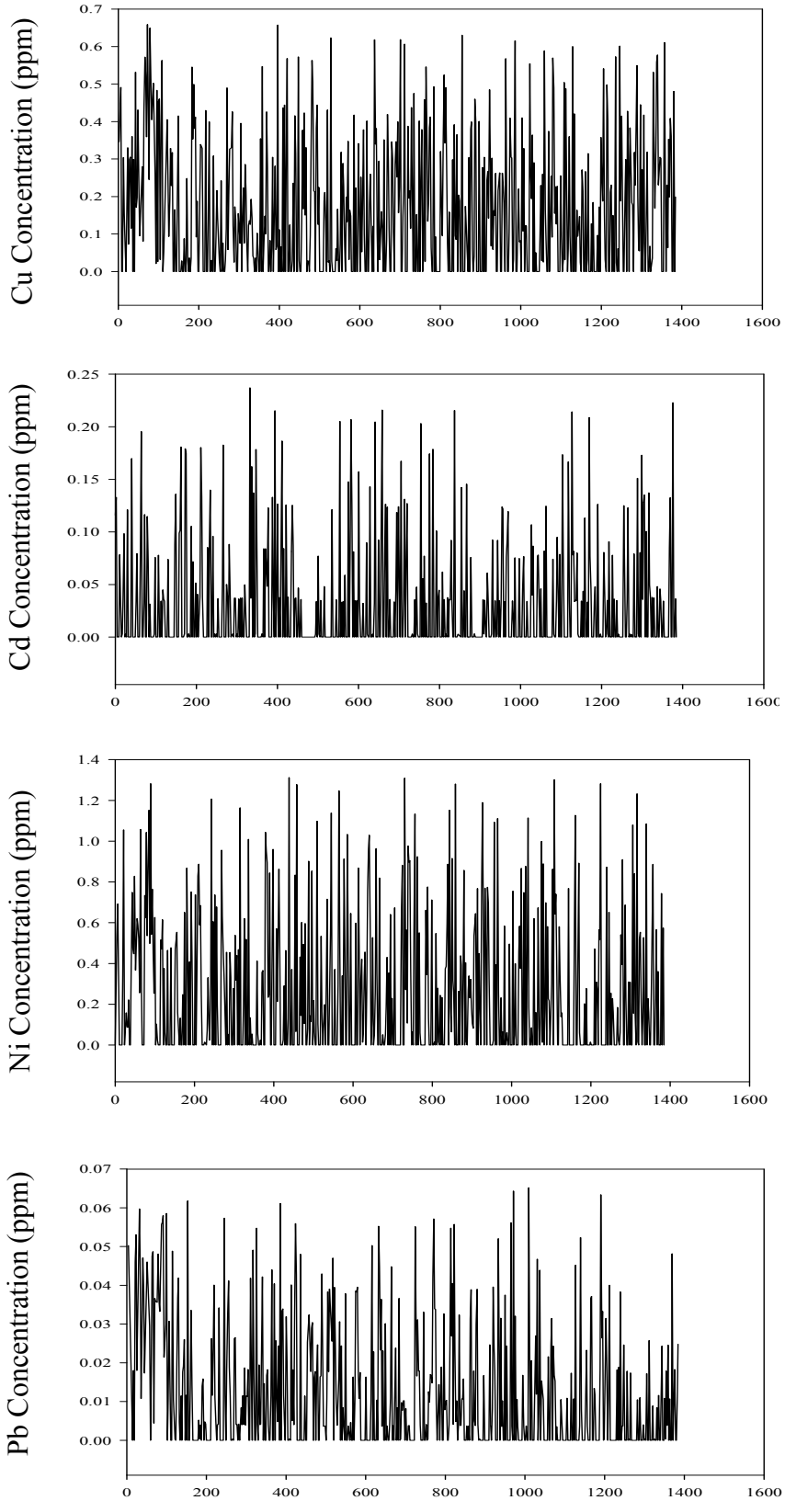


CFDL02-10

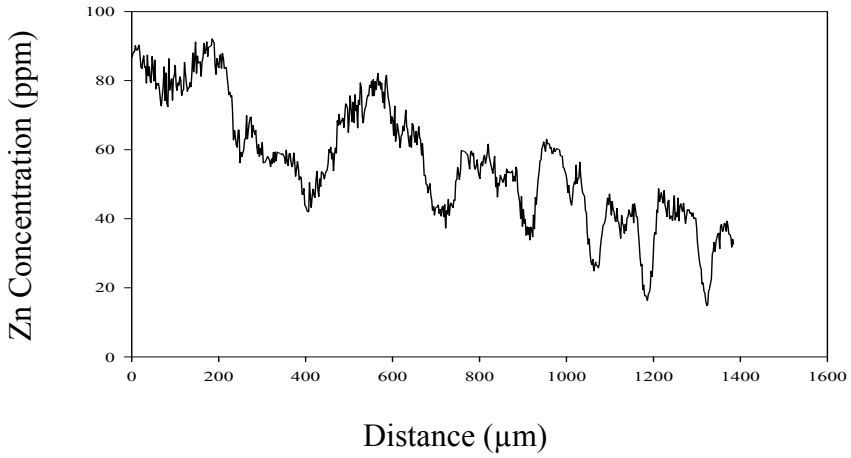

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (µm)

CFDL02-12

Age: 12

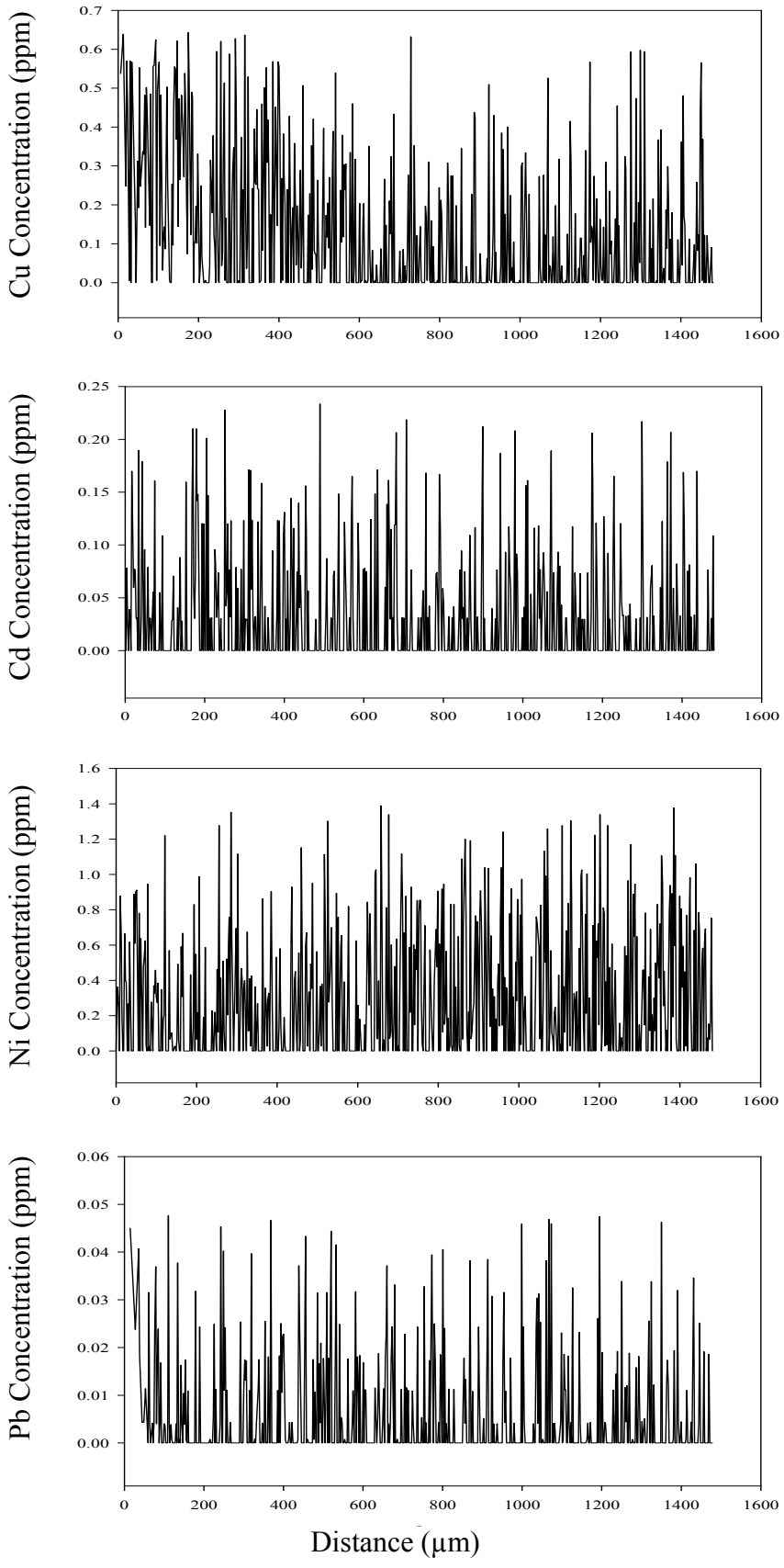


CFDL02-12

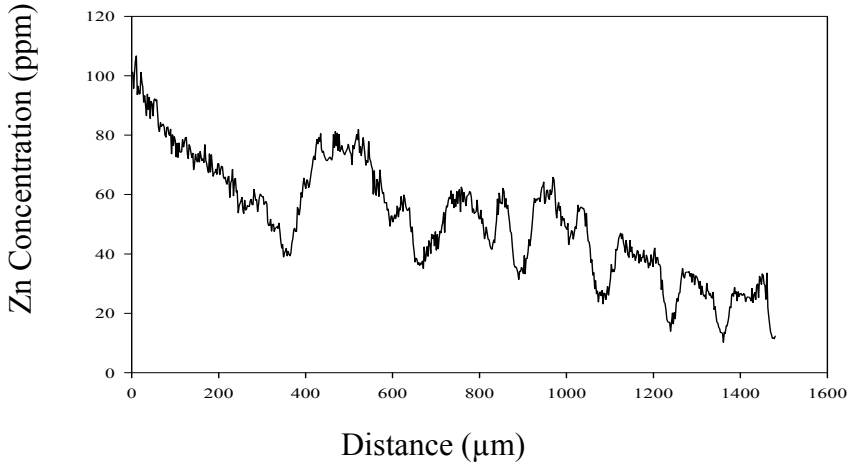

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL03-12b

Age: 7+

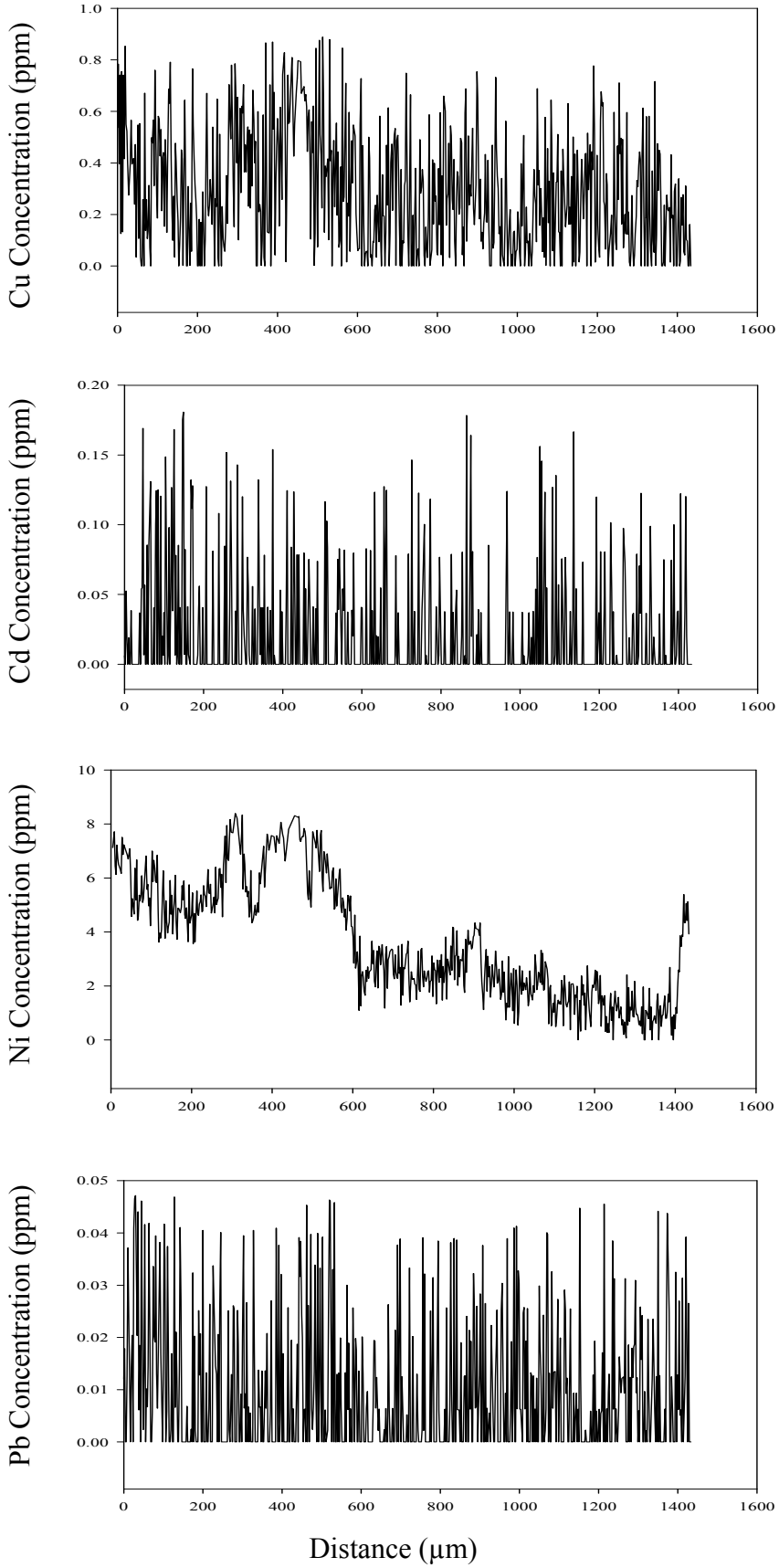


CFDL03-12b

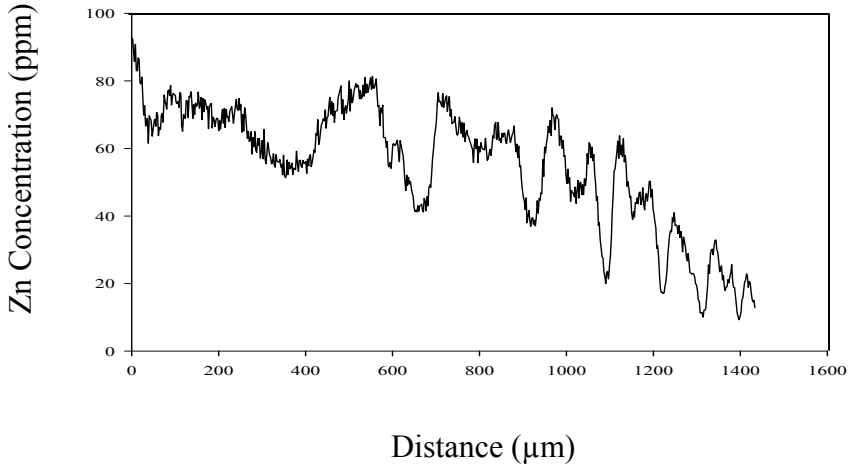

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL03-14

Age: 9+

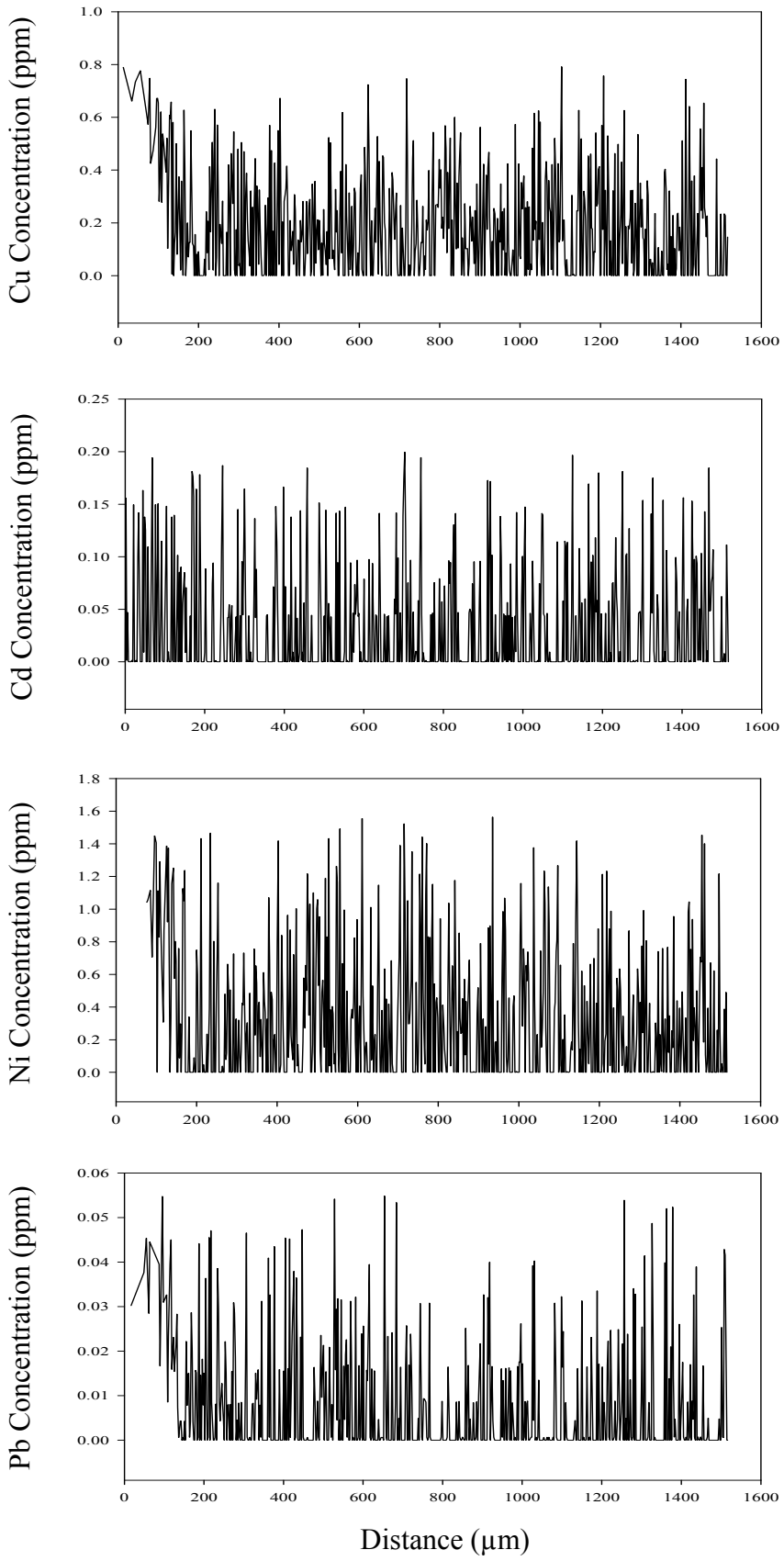


CFDL03-14

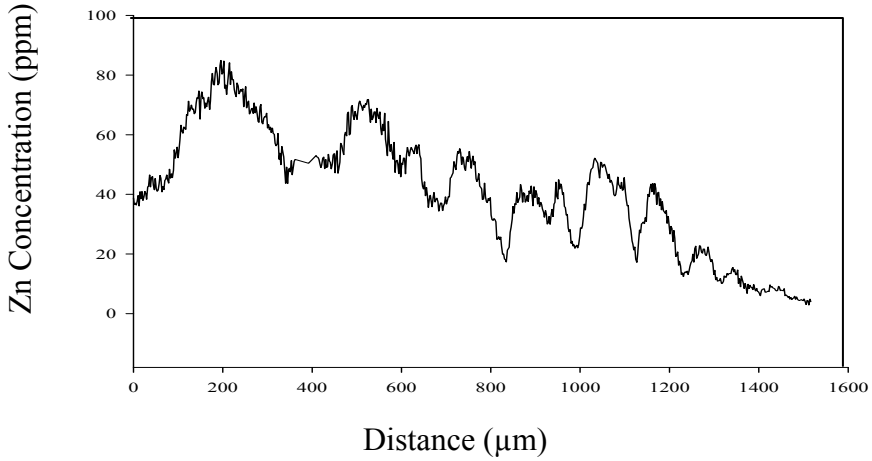

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL03-15

Age: 14+

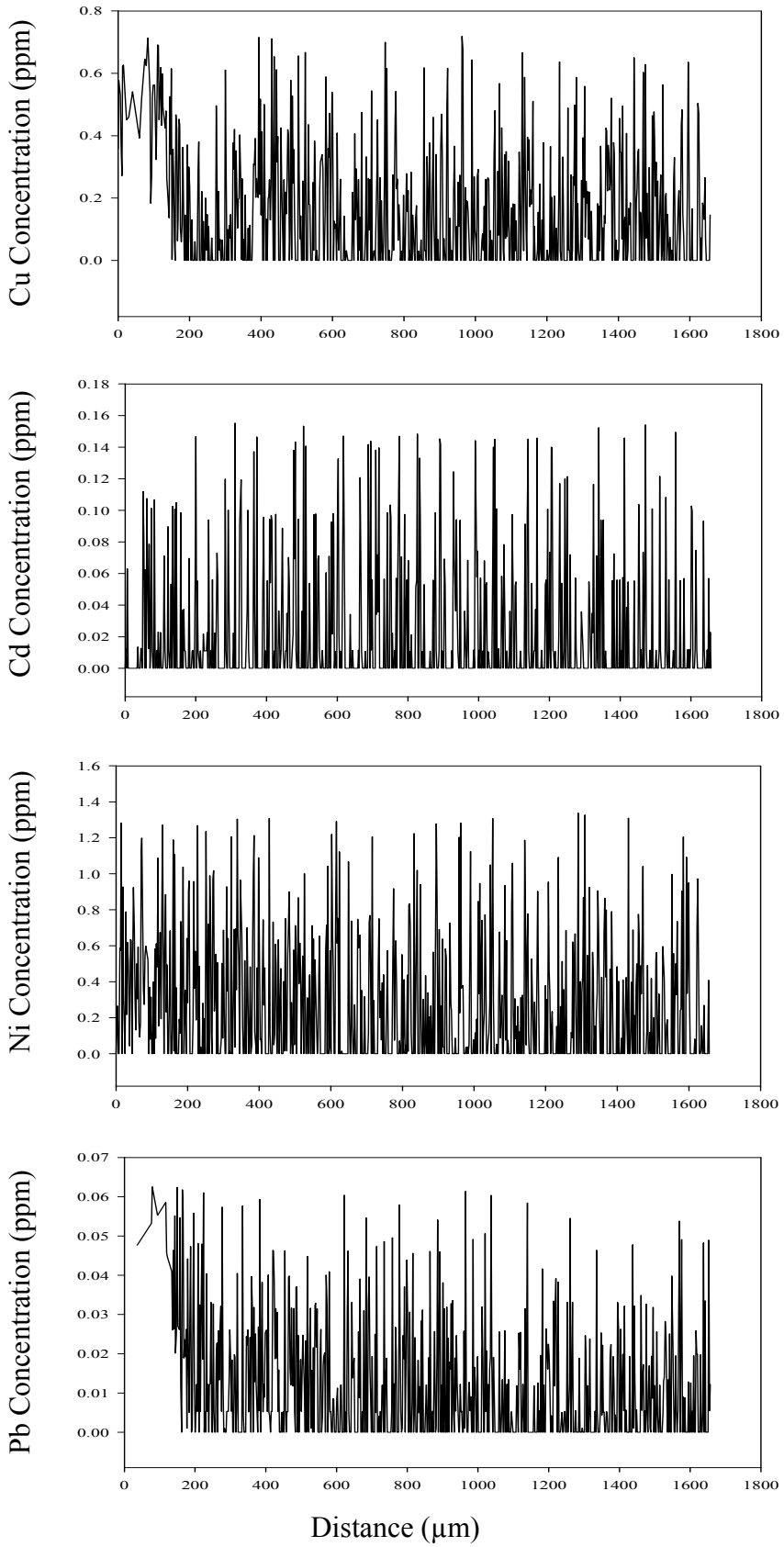


CFDL03-15

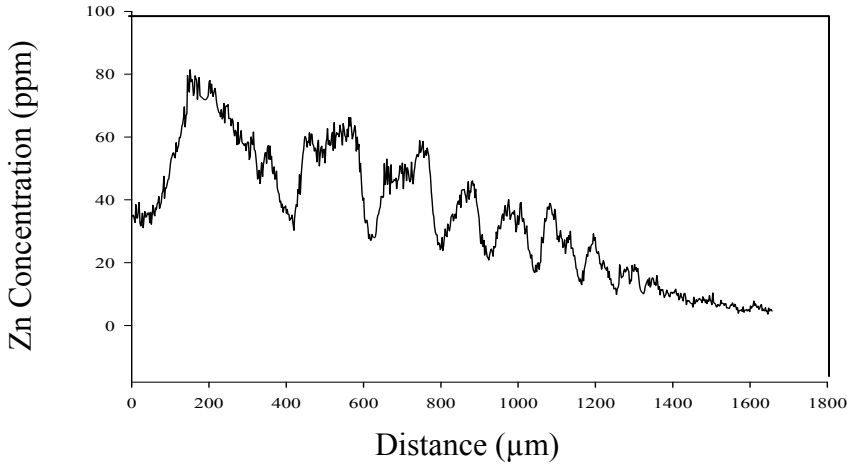

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

CFDL03-19

Age: 15+

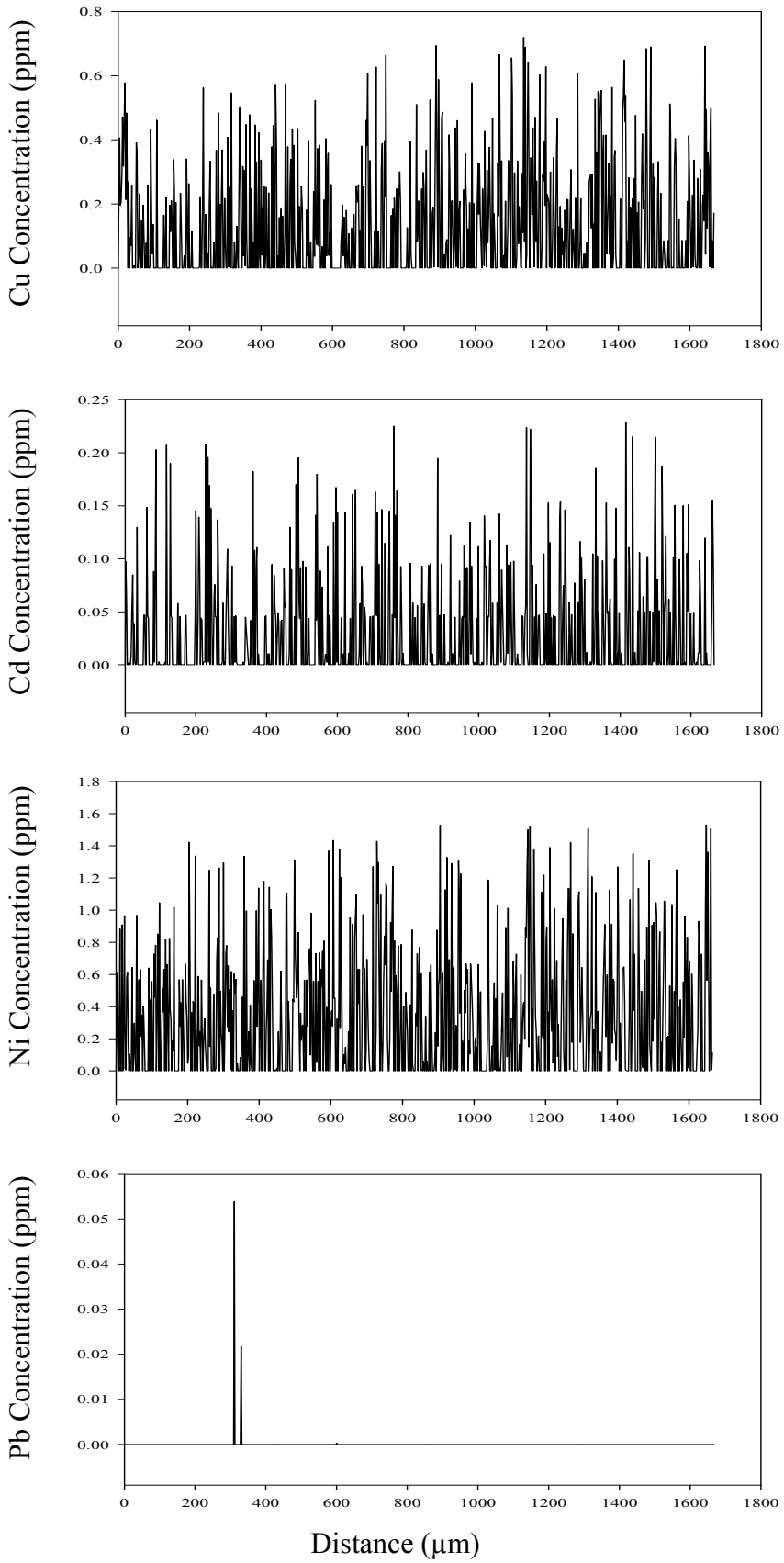


CFLD03-19

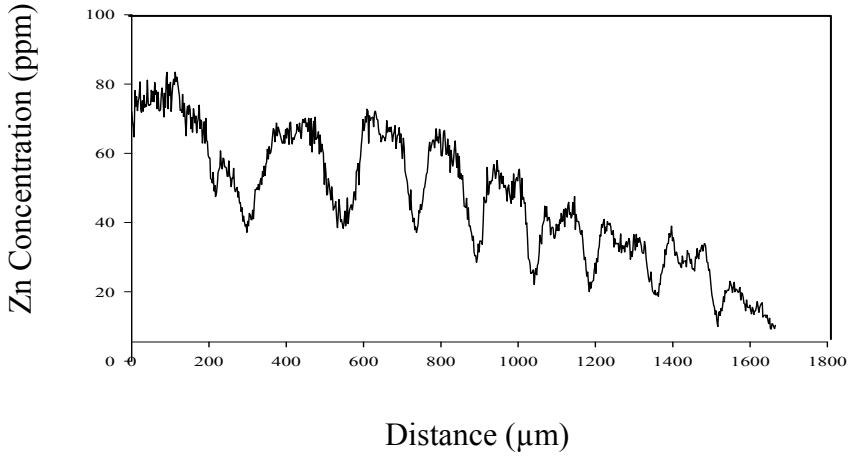

Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

CFDL03-20

Age: 10+



CFDL03-20


Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (µm)

APPENDIX IV

Metal Results for the Feeding Experiment

IV.I Reference Group

IV.II Low Dose Group

IV.III Medium Dose Group

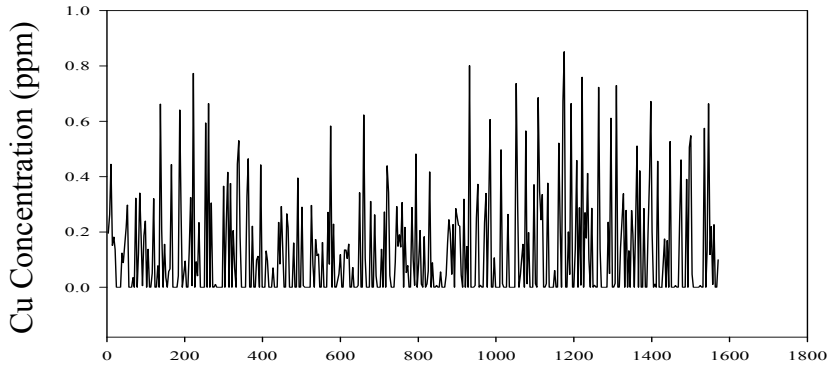
IV.IV High Dose Group

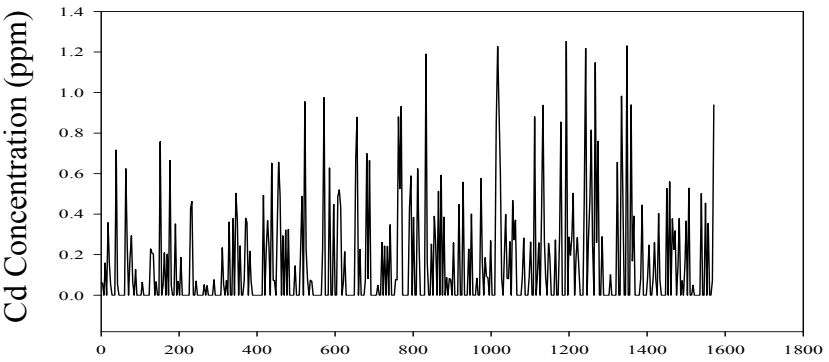
IV.I Reference Group

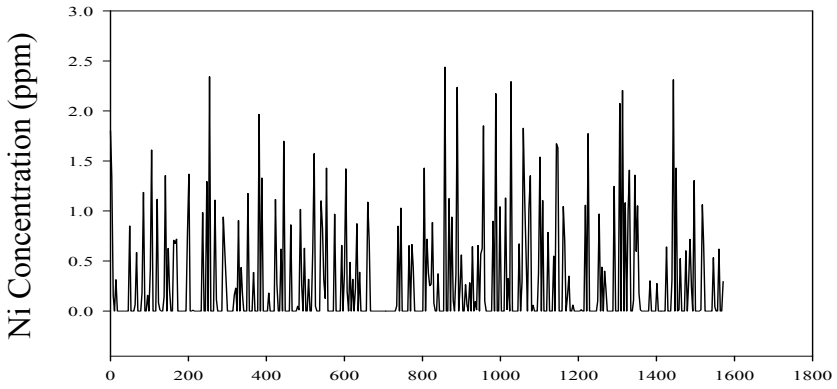
C1-C08

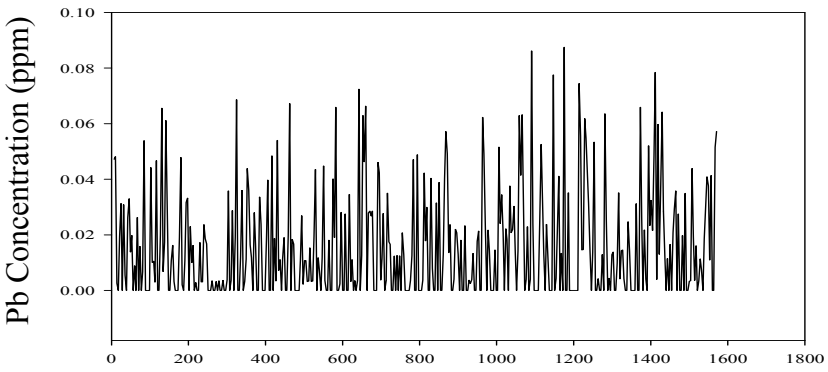
Reference

Non-Crystalline



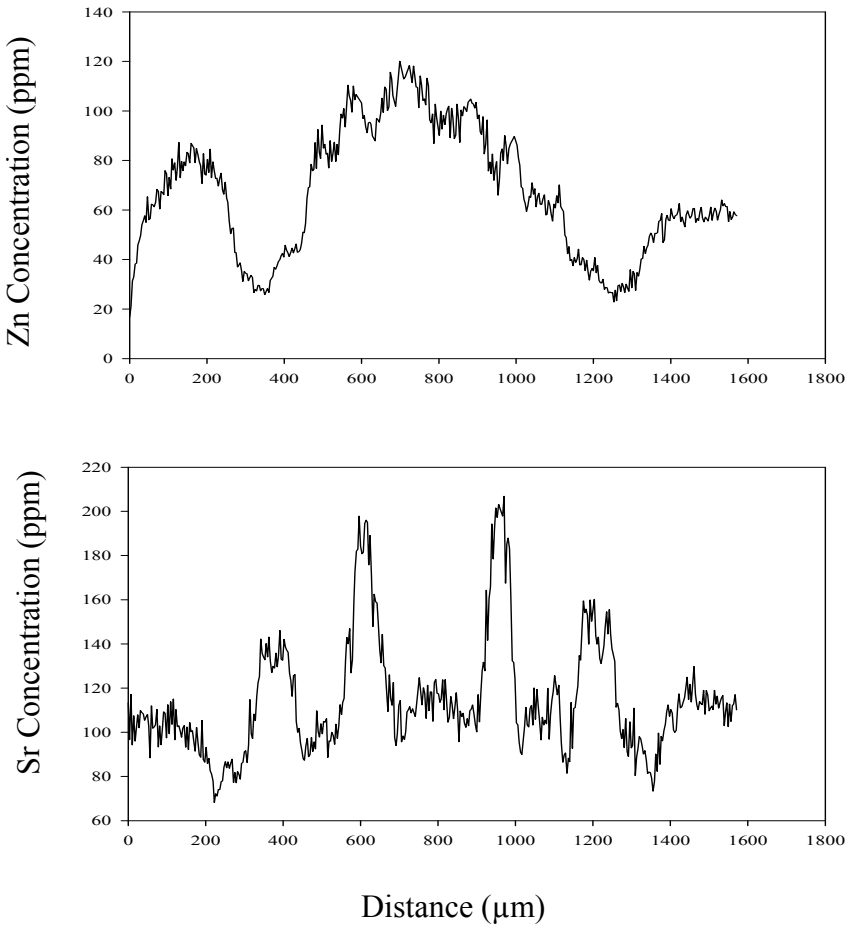






Distance (μm)

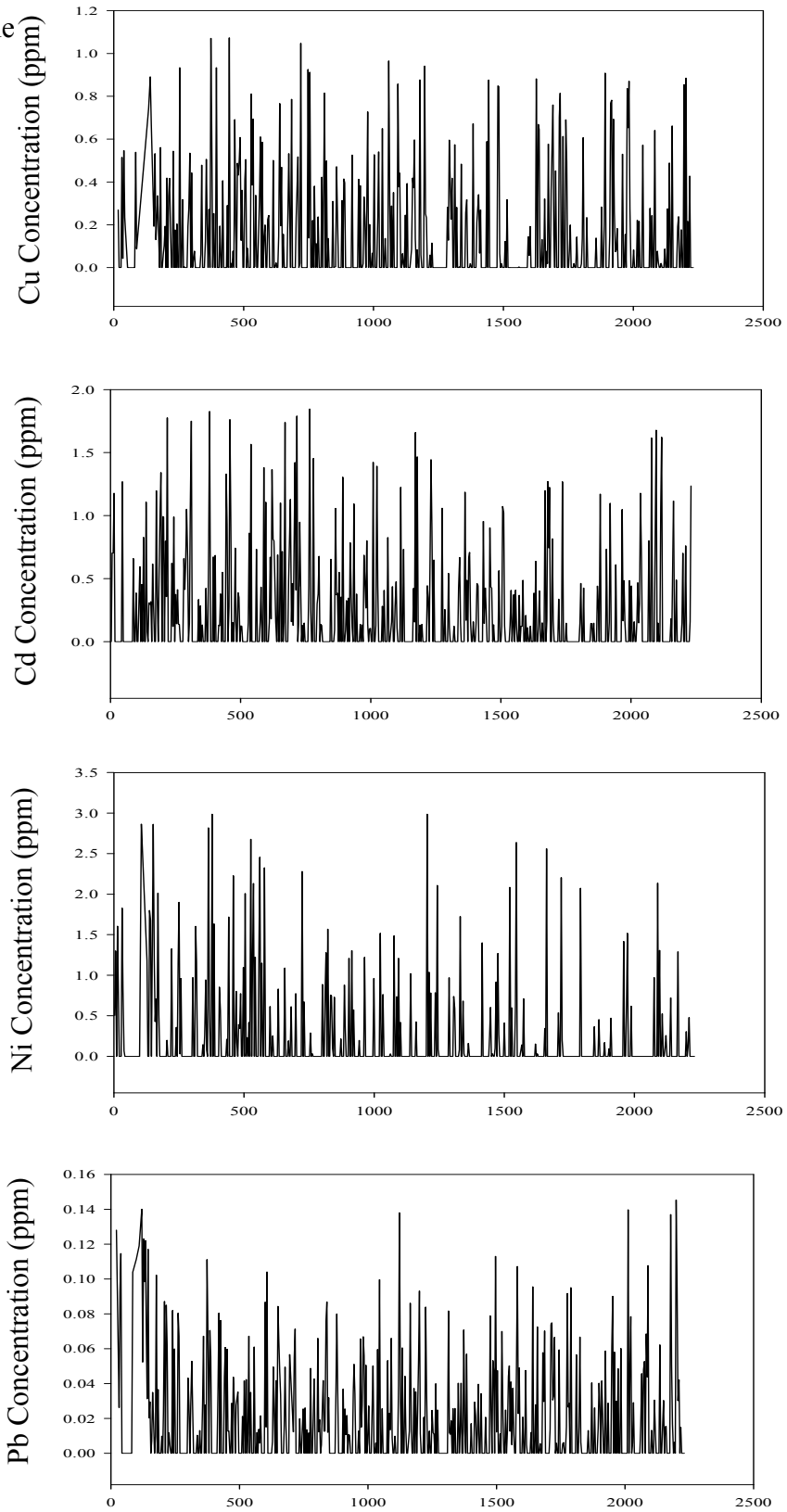
C1-C08



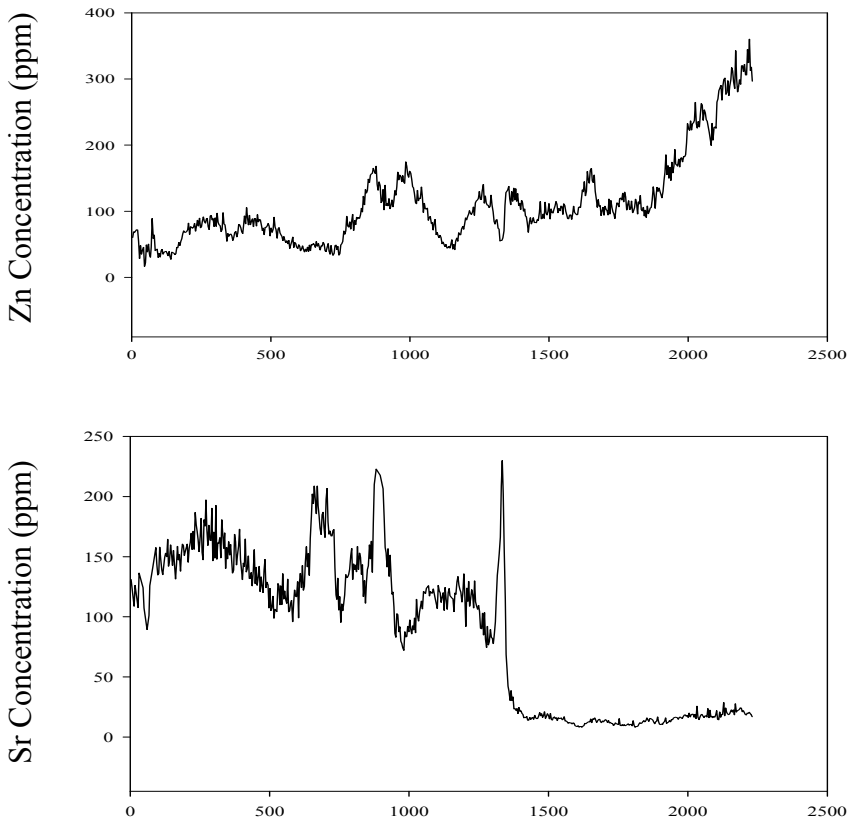
C1-C13

Reference

Non-crystalline



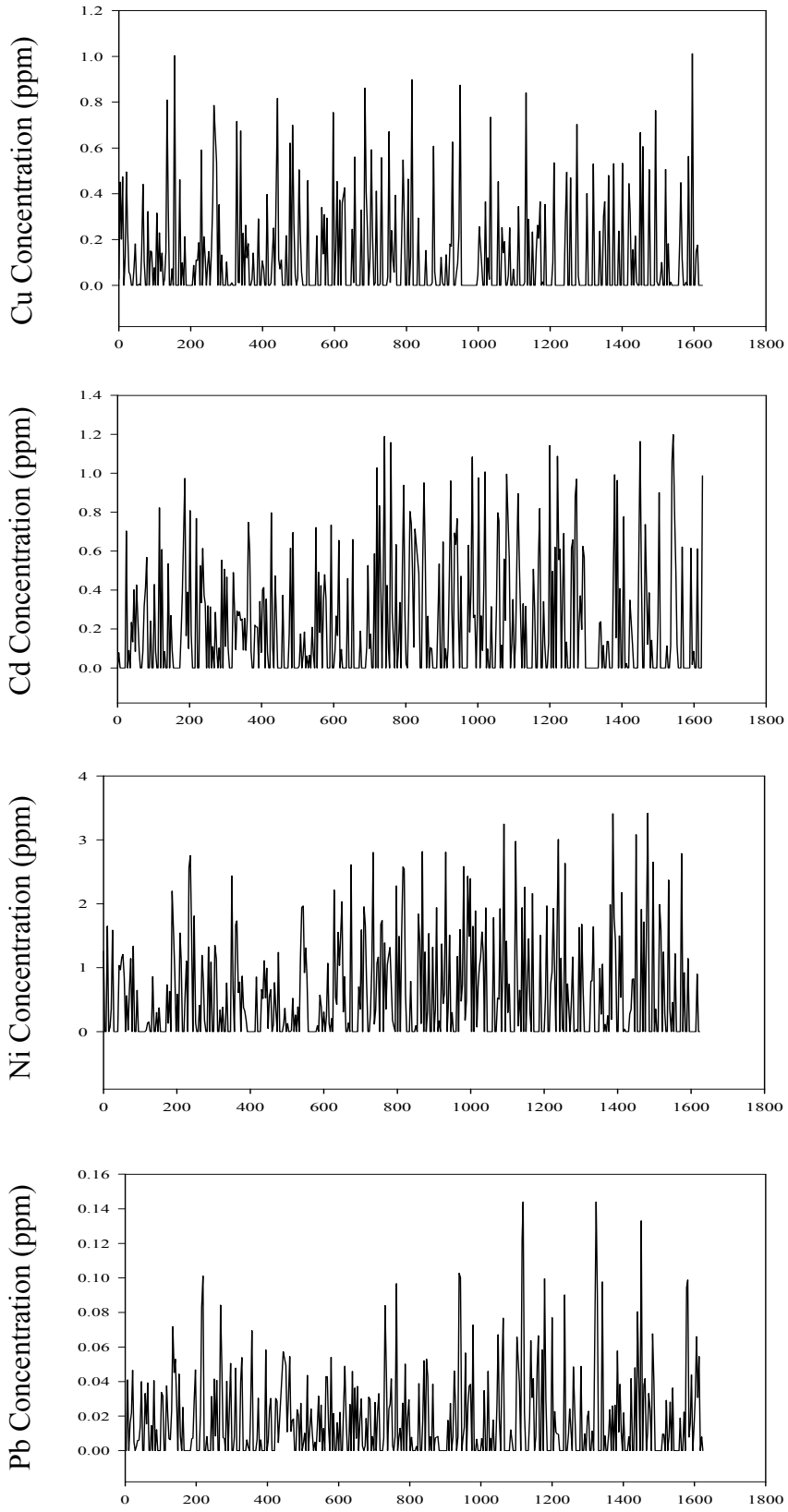
C1-C13


Zn Concentration (ppm)
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Distance (μm)

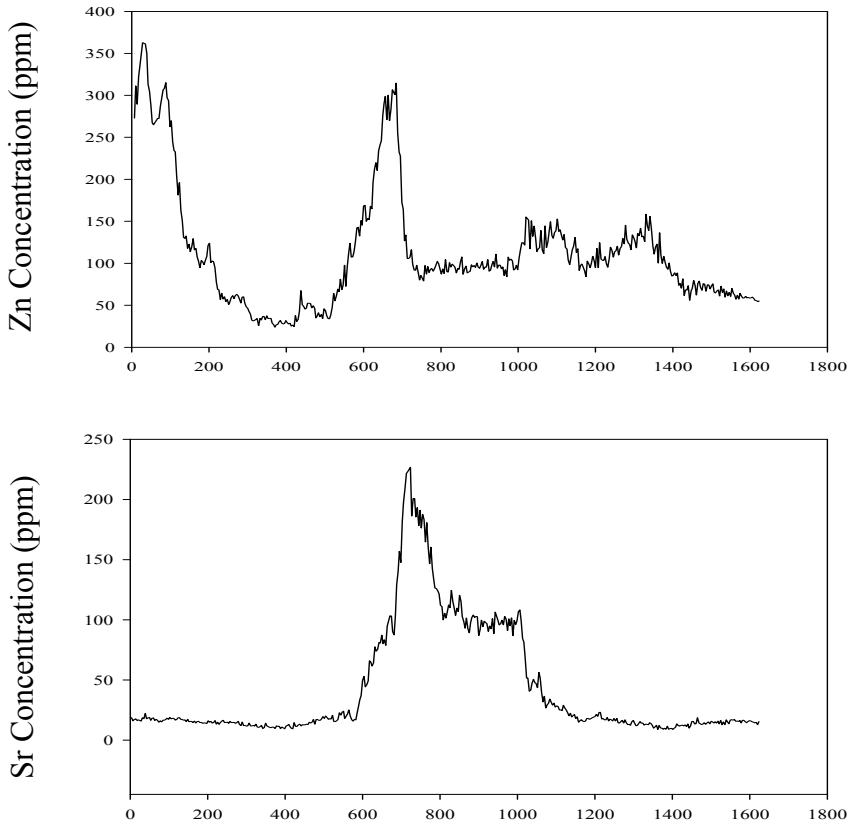
C1-C15

Reference

Crystalline



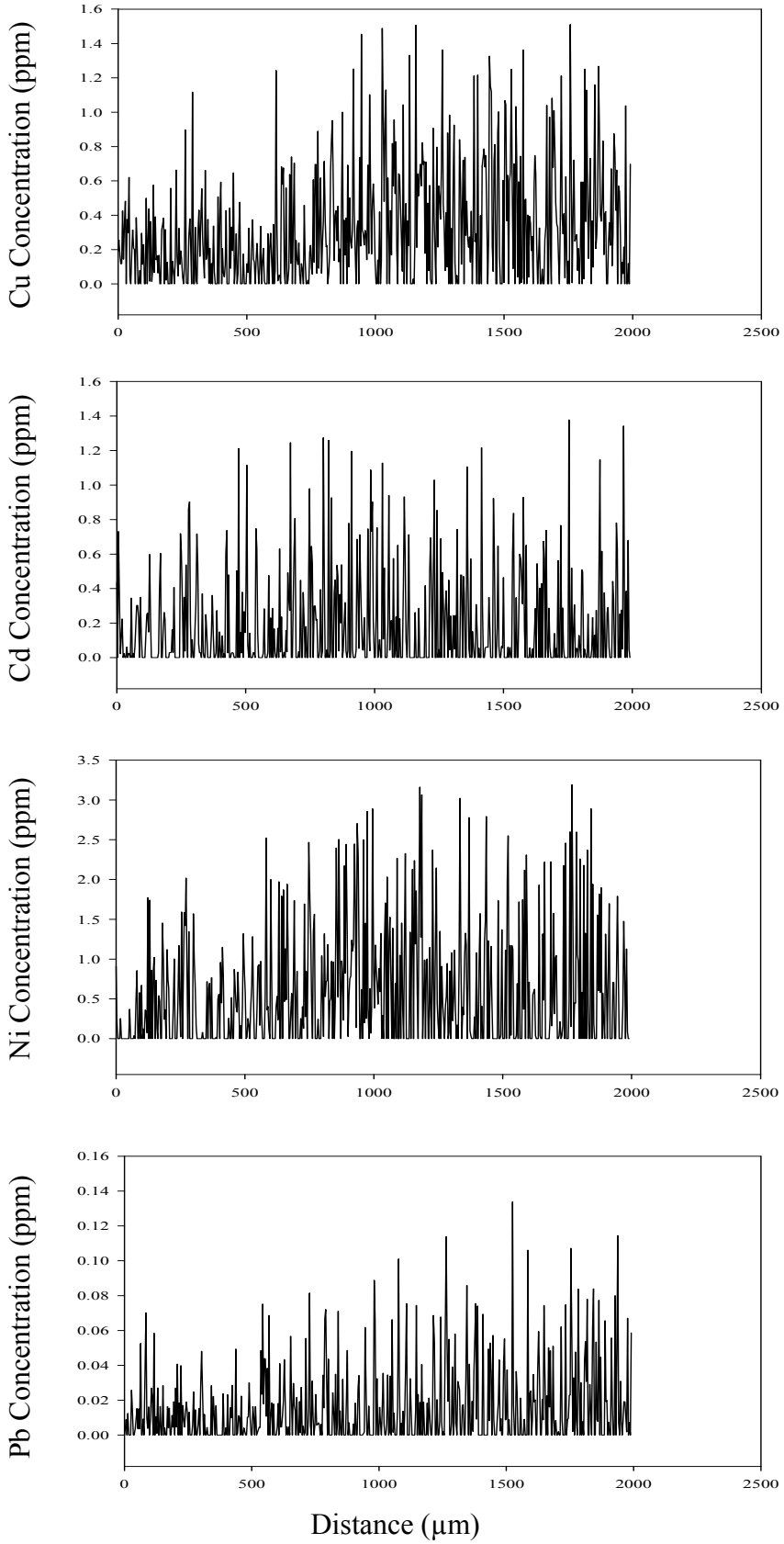
C1-C15


Zn Concentration (ppm)
400
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

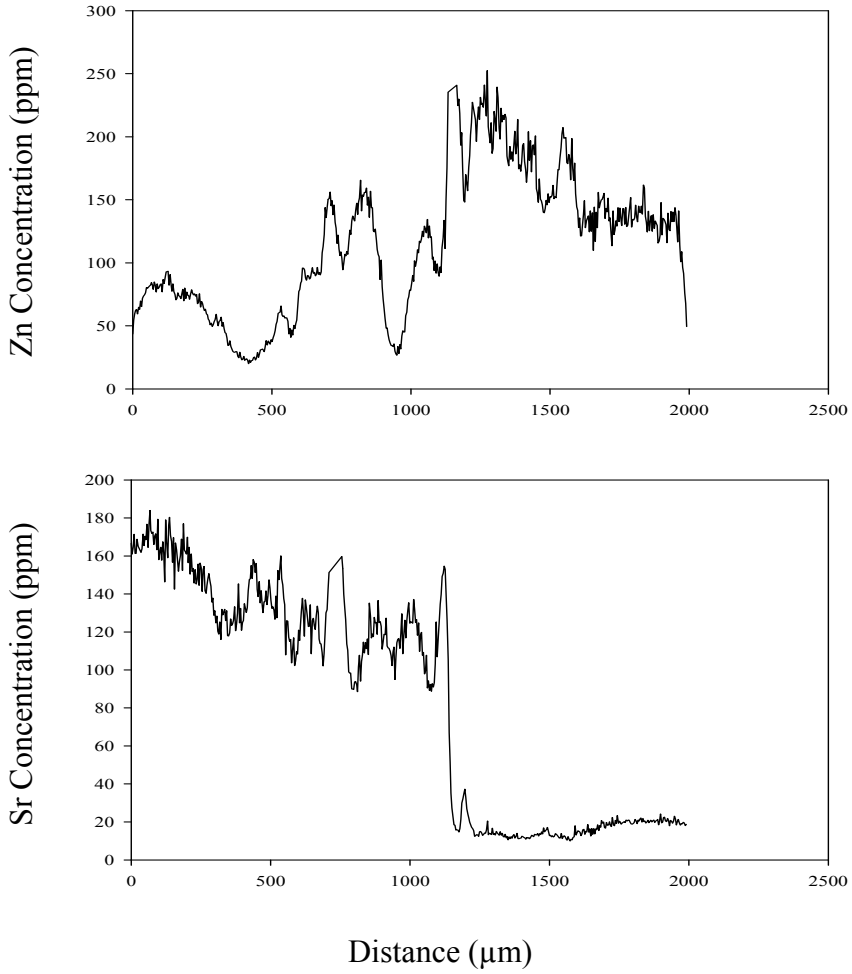
C1-C19

Reference

Crystalline



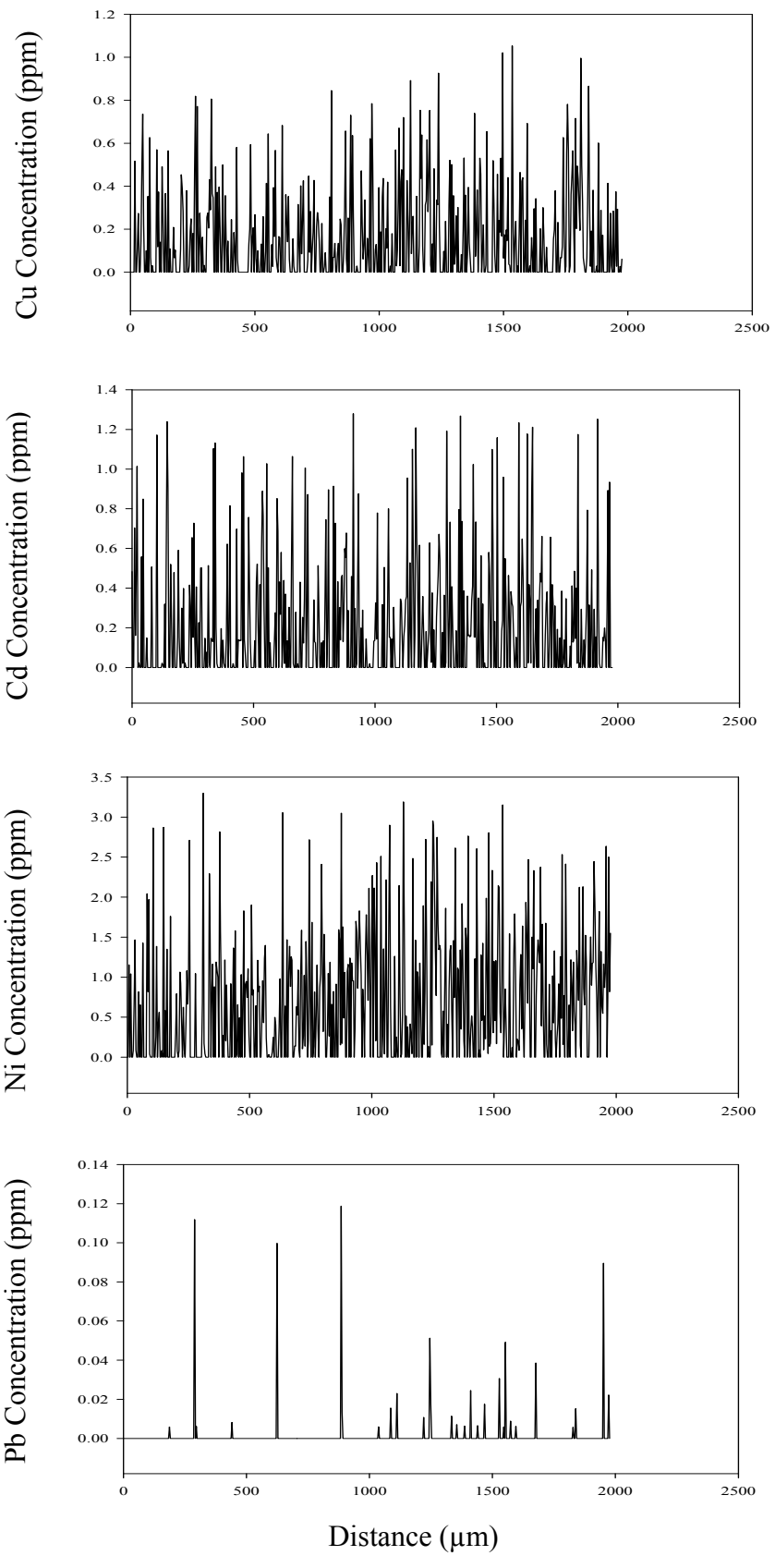
C1-C19


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
200
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

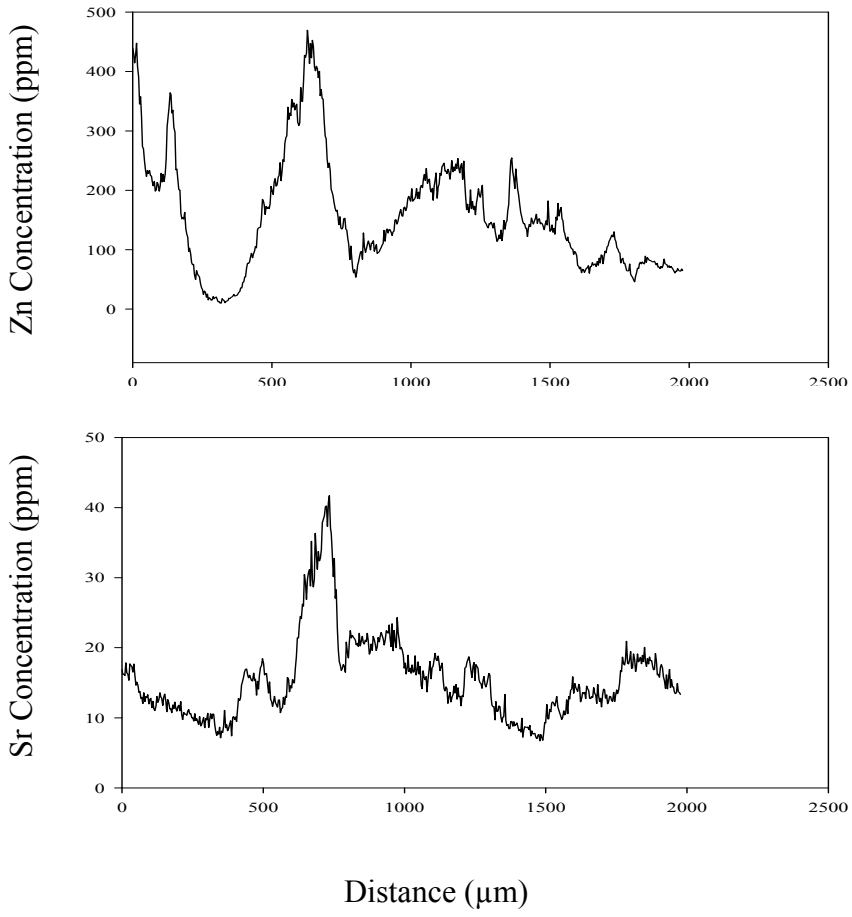
C1-C20

Reference

Non-crystalline



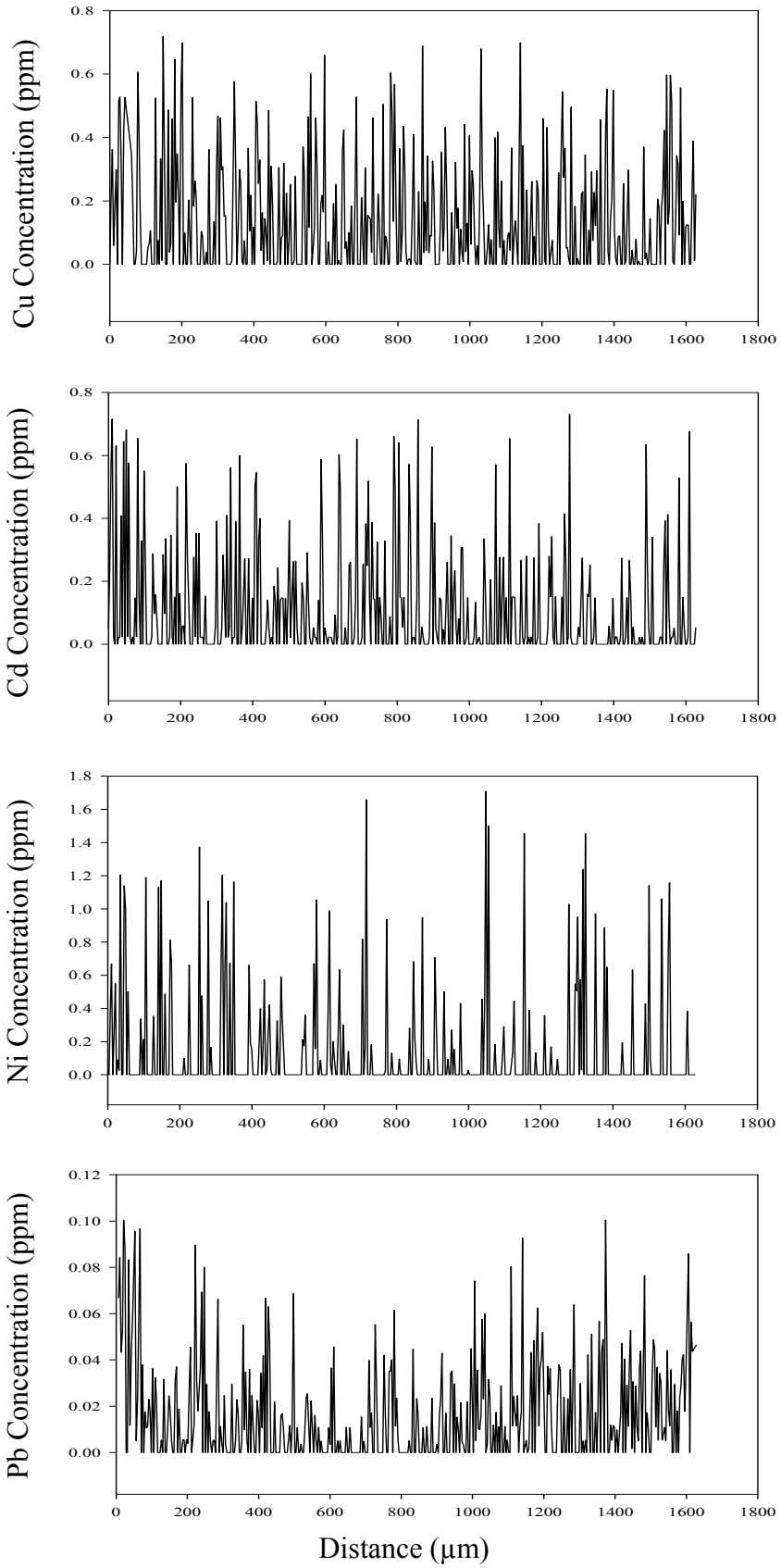
C1-C20


Zn Concentration (ppm)
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
50
40
30
20
10
0
0
500
1000
1500
2000
2500
Distance (µm)

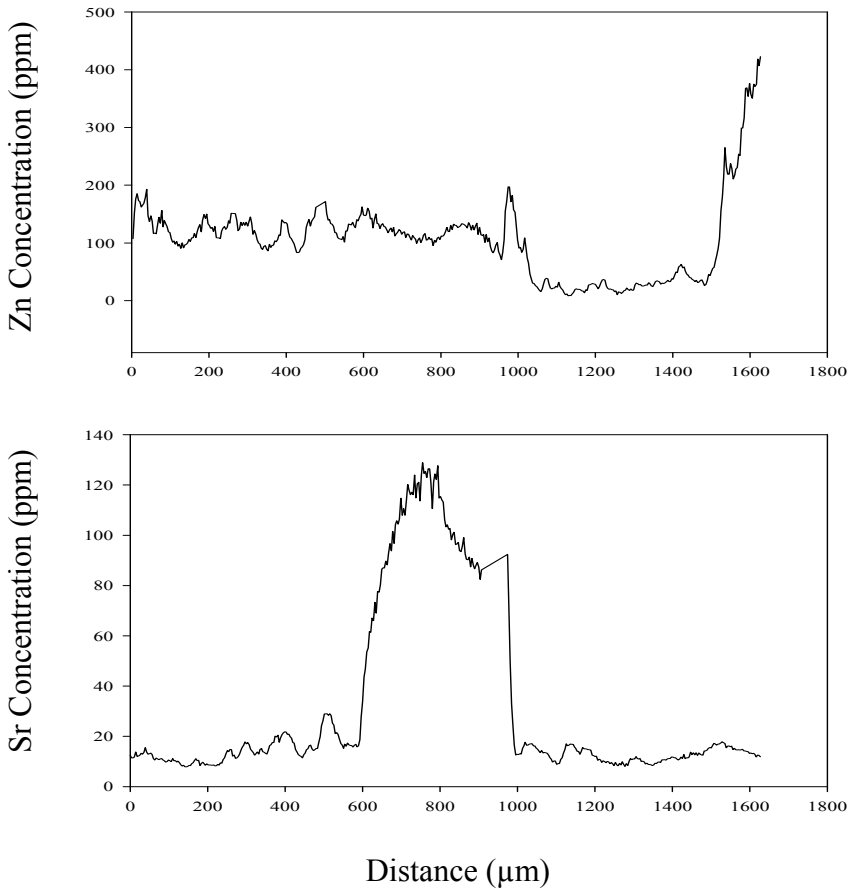
C1-C23

Reference

Crystalline



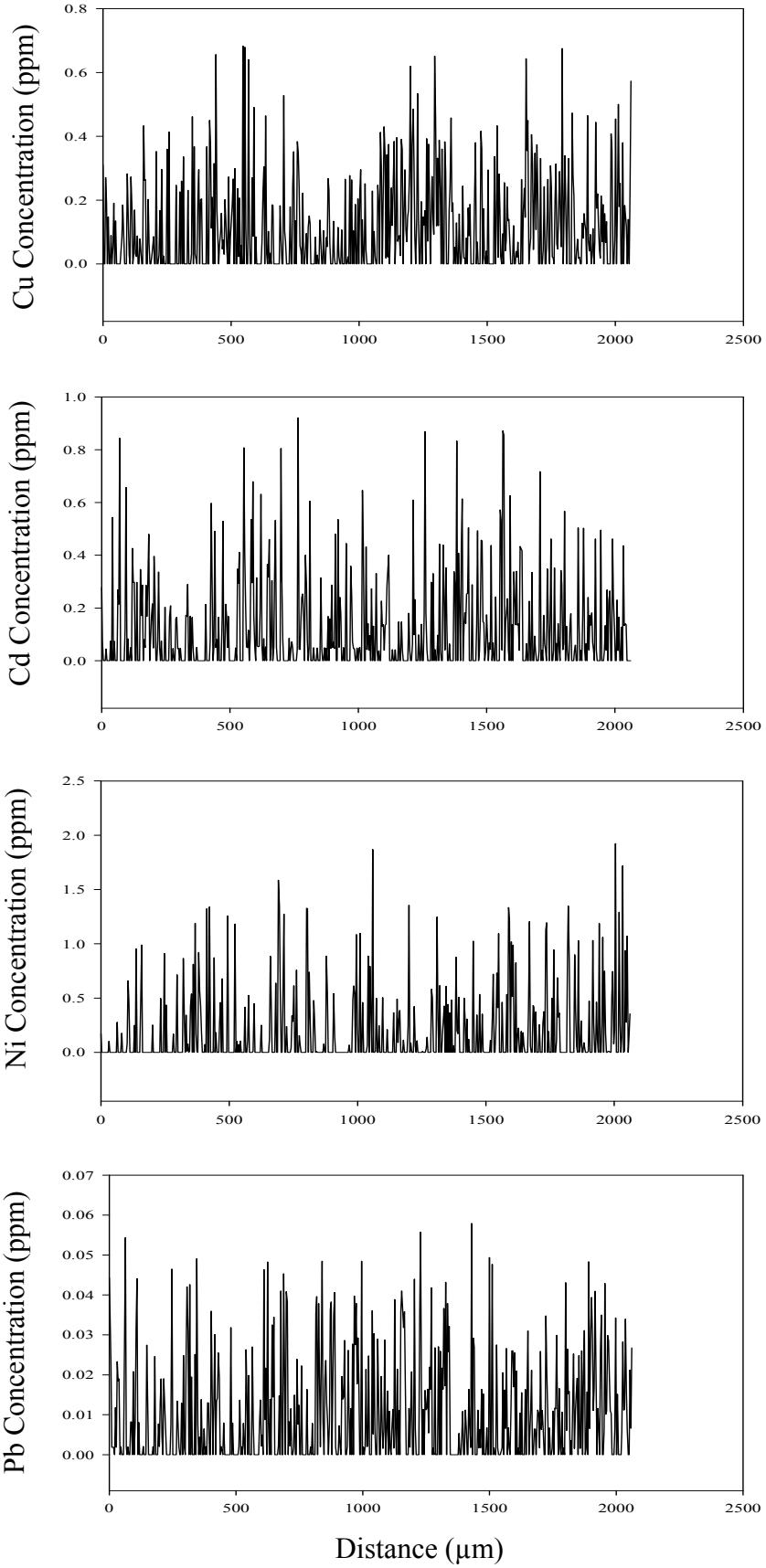
C1-C23


Zn Concentration (ppm)
500
400
300
200
100
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (µm)

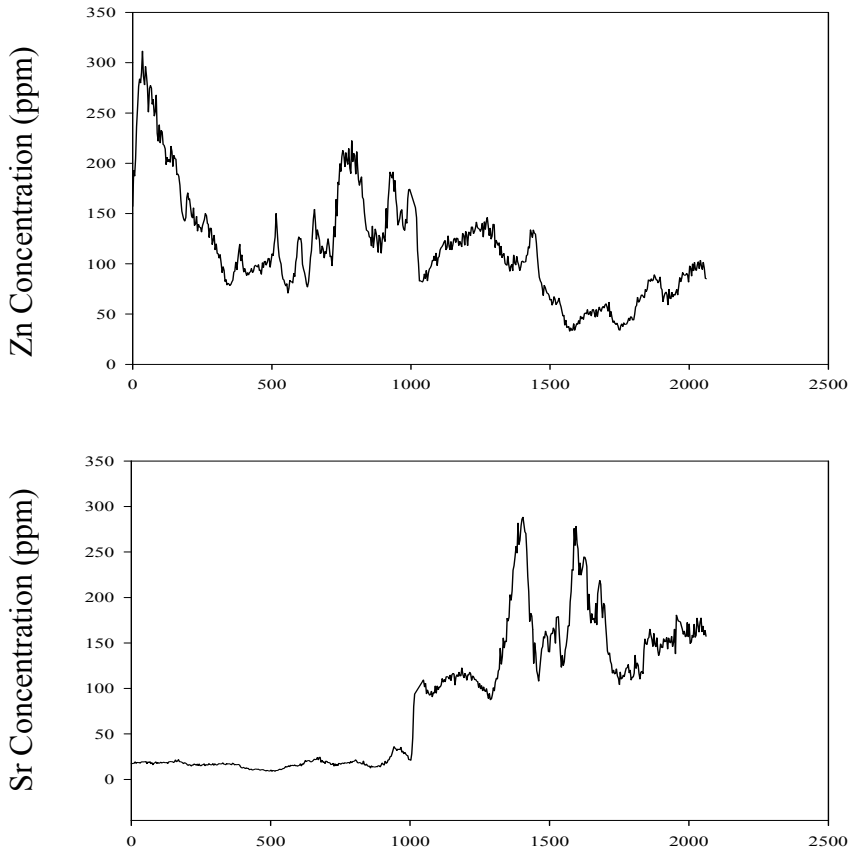
C02-C24

Reference

Crystalline



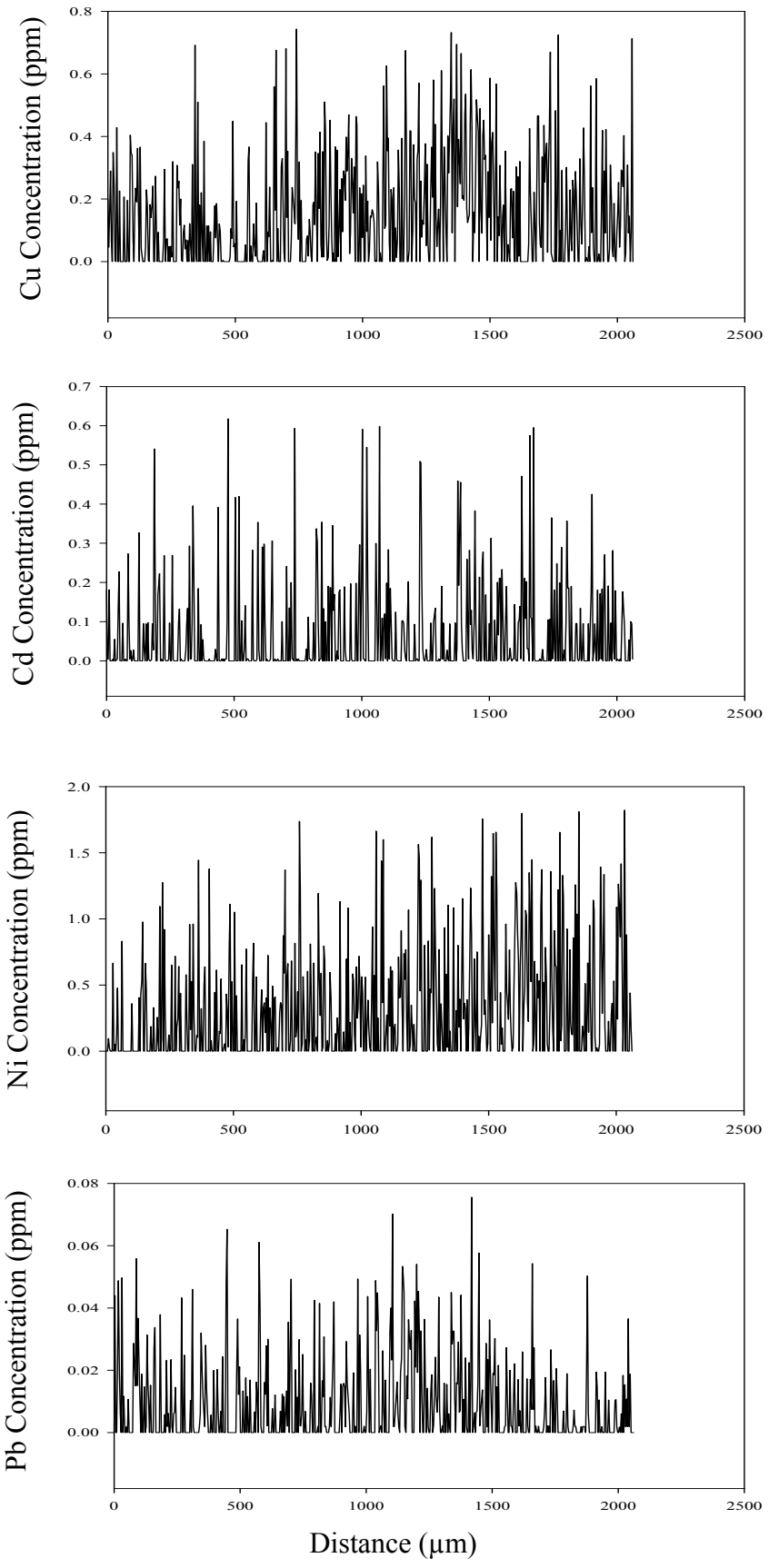
C02-C24


Zn Concentration (ppm)
Sr Concentration (ppm)
0
50
100
150
200
250
300
350
0
500
1000
1500
2000
2500
Distance (μm)

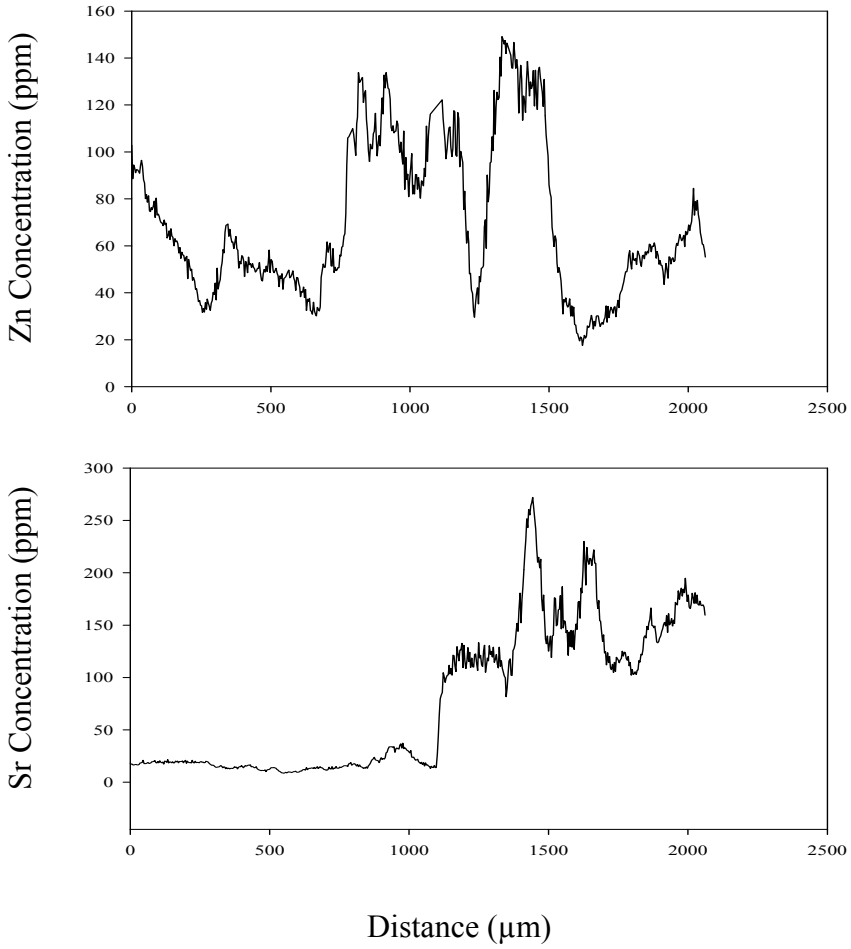
C02-C25

Reference

Crystalline



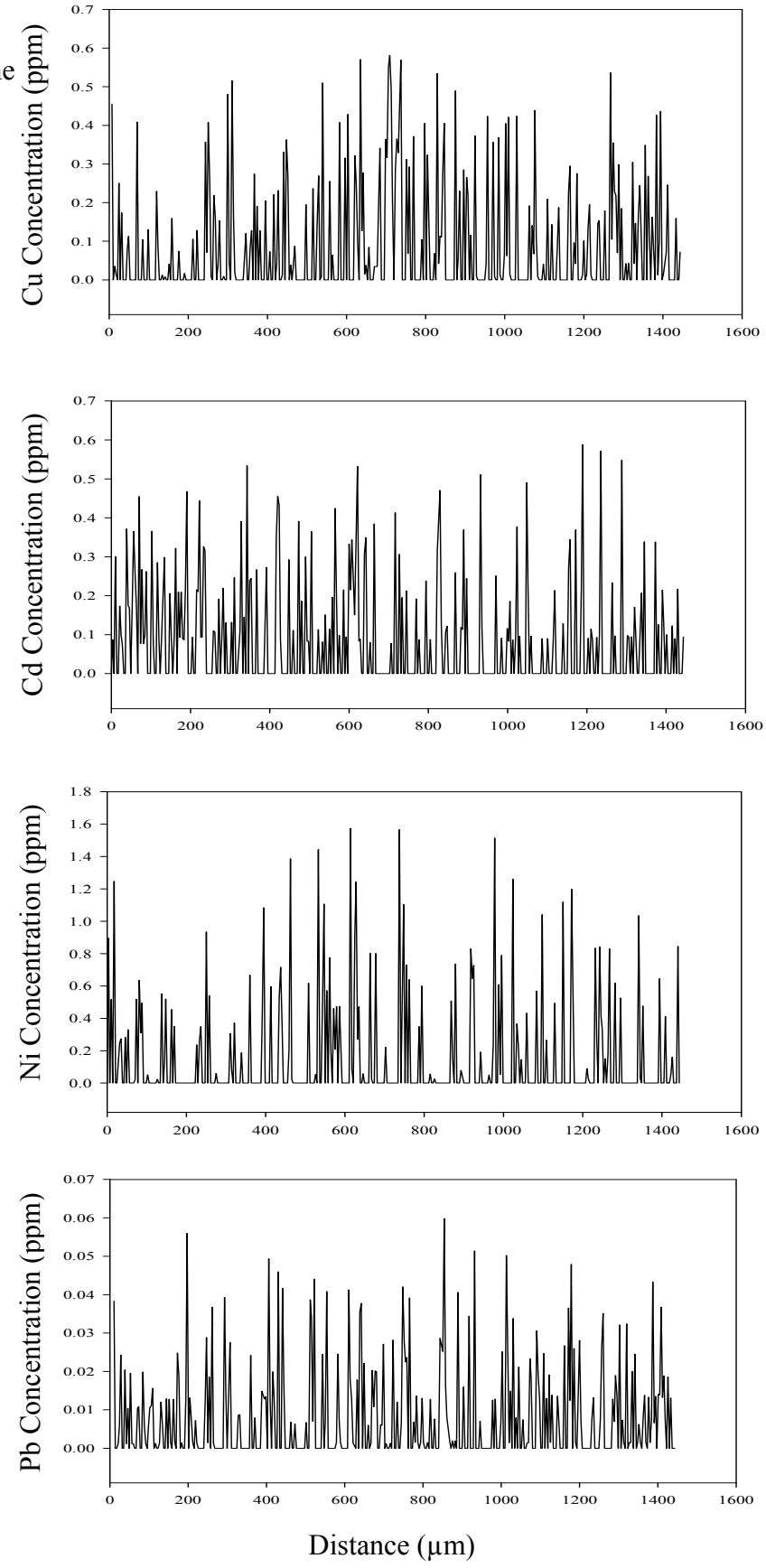
C02 C25


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Distance (μm)

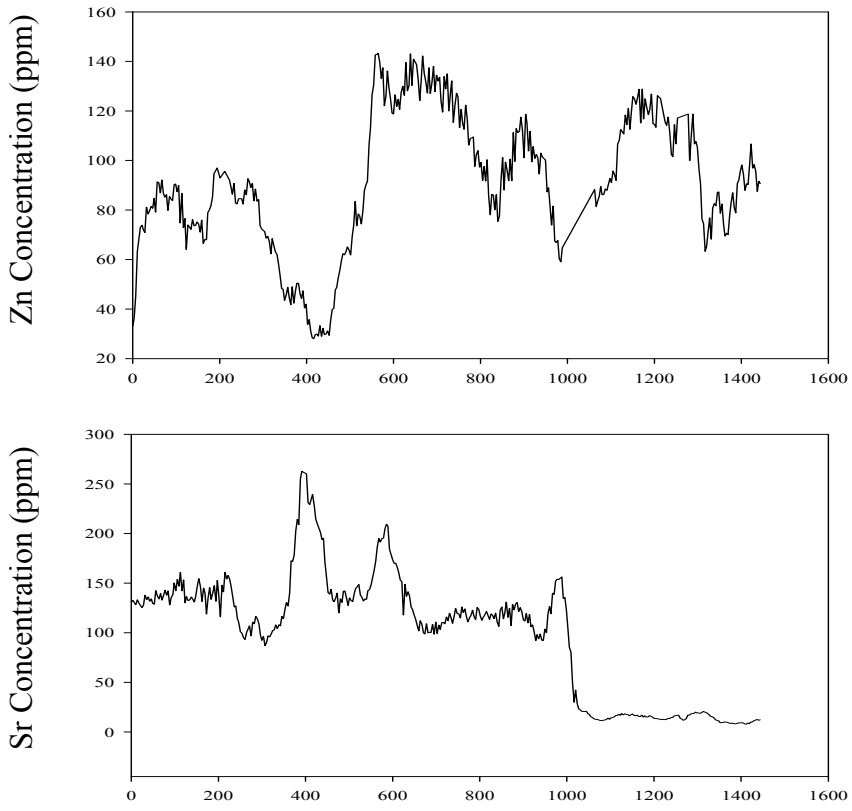
C2-C26

Reference

Partially Crystalline



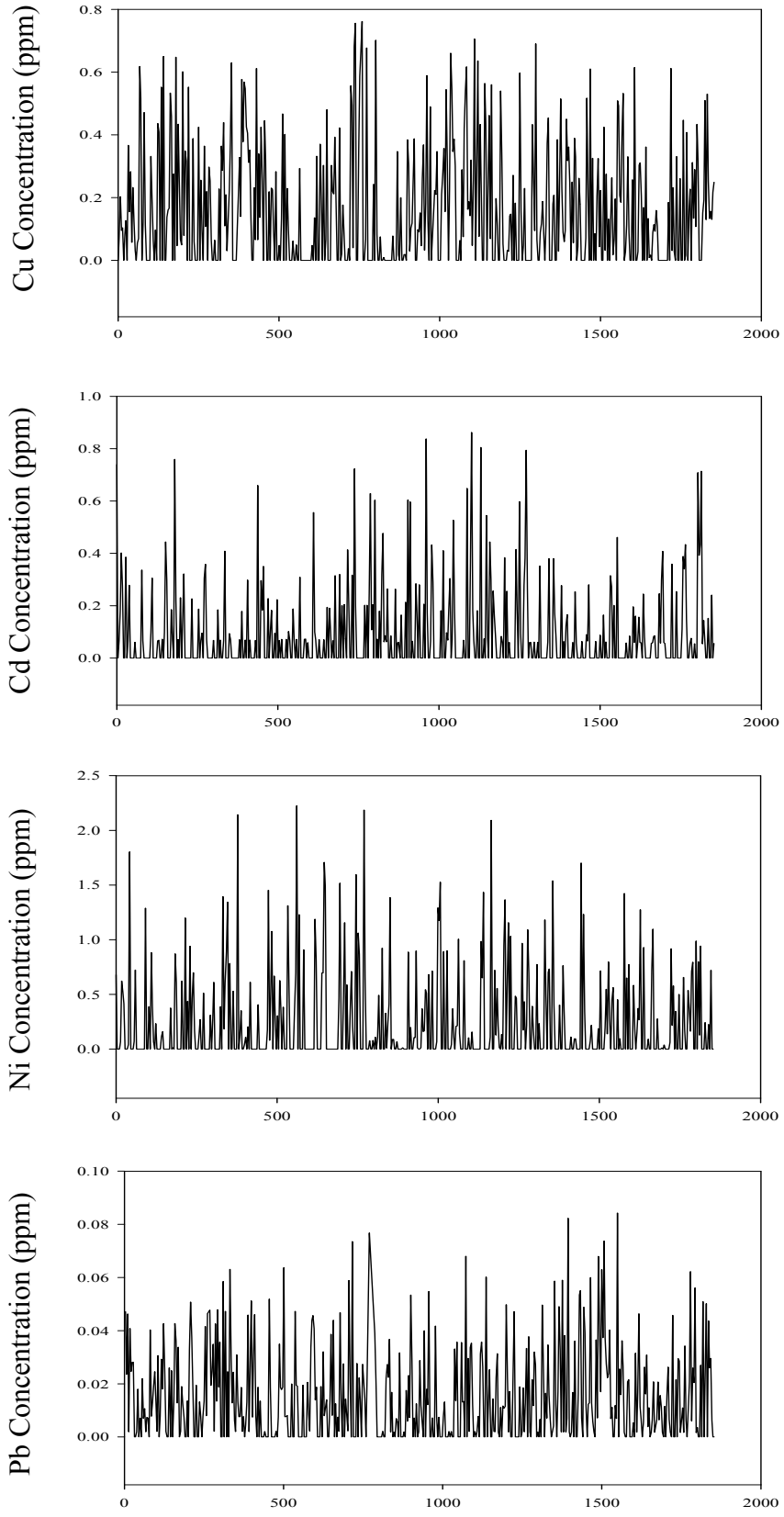
C2-C26


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
Sr Concentration (ppm)
300
250
200
150
100
50
0
Distance (μm)

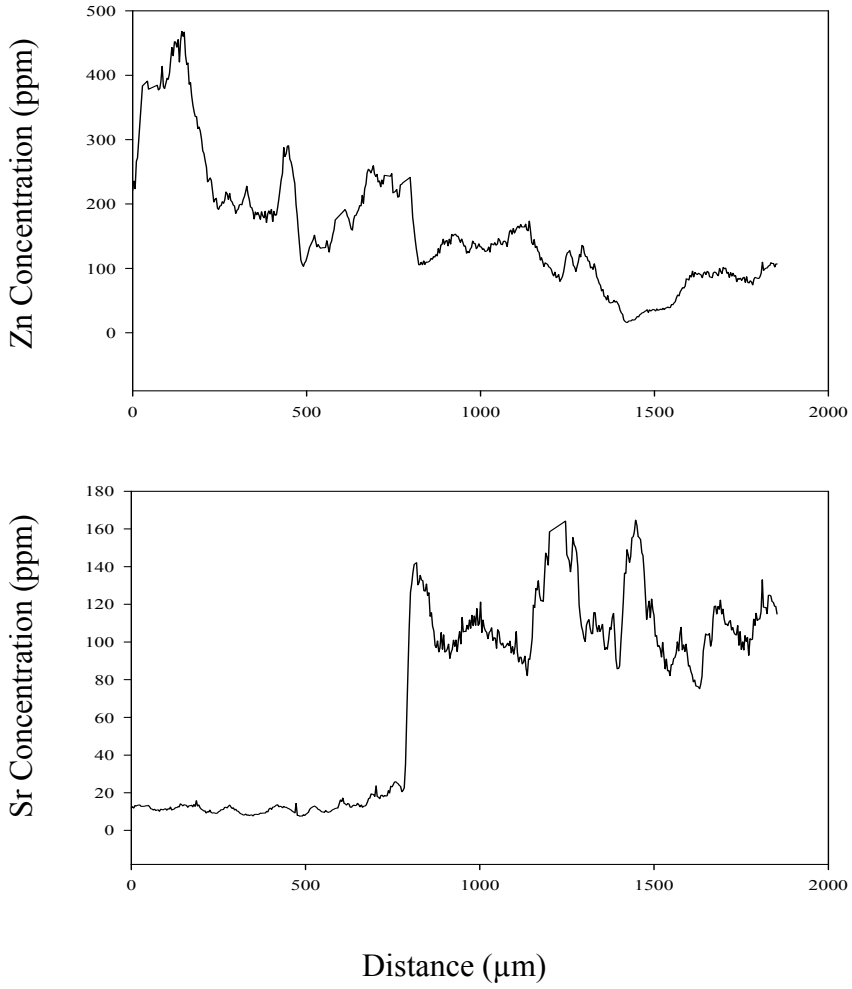
C02-C27

Reference

Crystalline



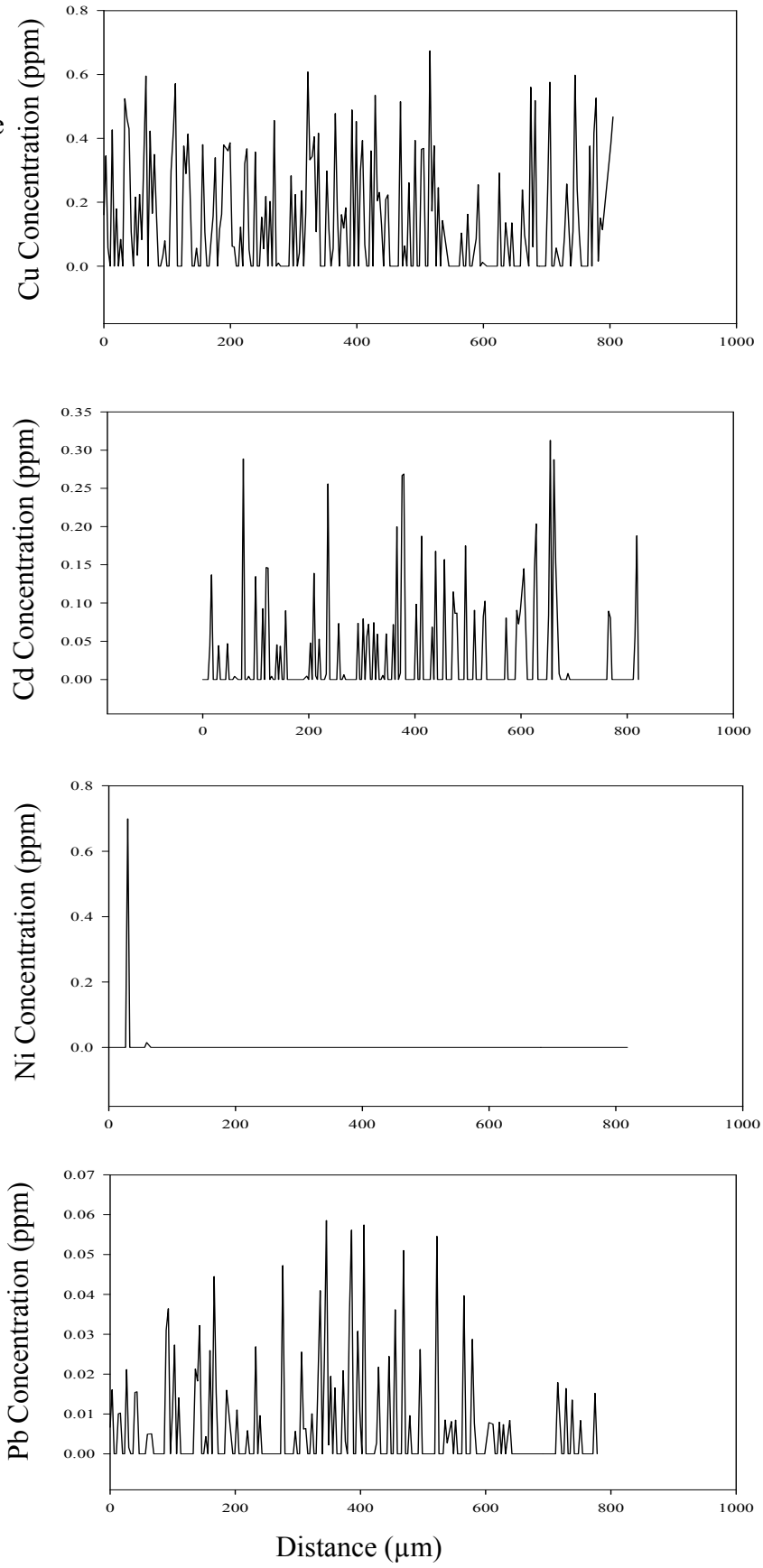
C02-C27


Zn Concentration (ppm)
500
400
300
200
100
0
0
500
1000
1500
2000
Sr Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
Distance (μm)

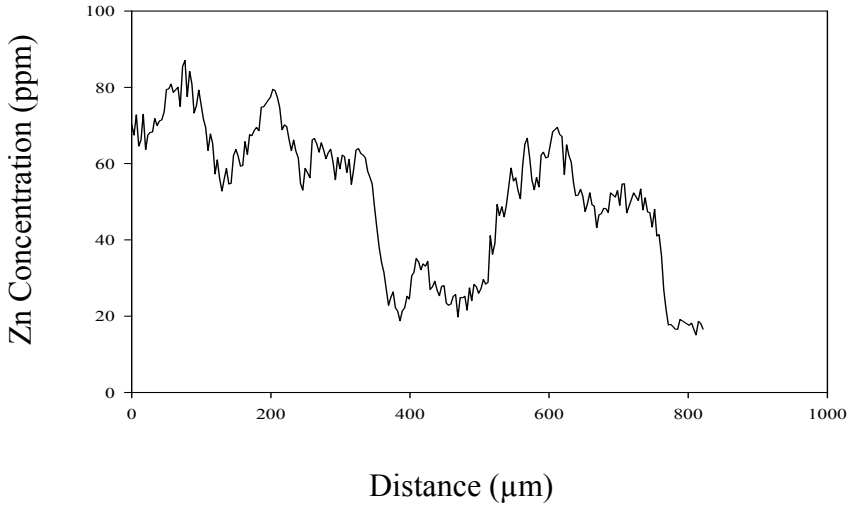
FD03 C14

Reference

Non-crystalline



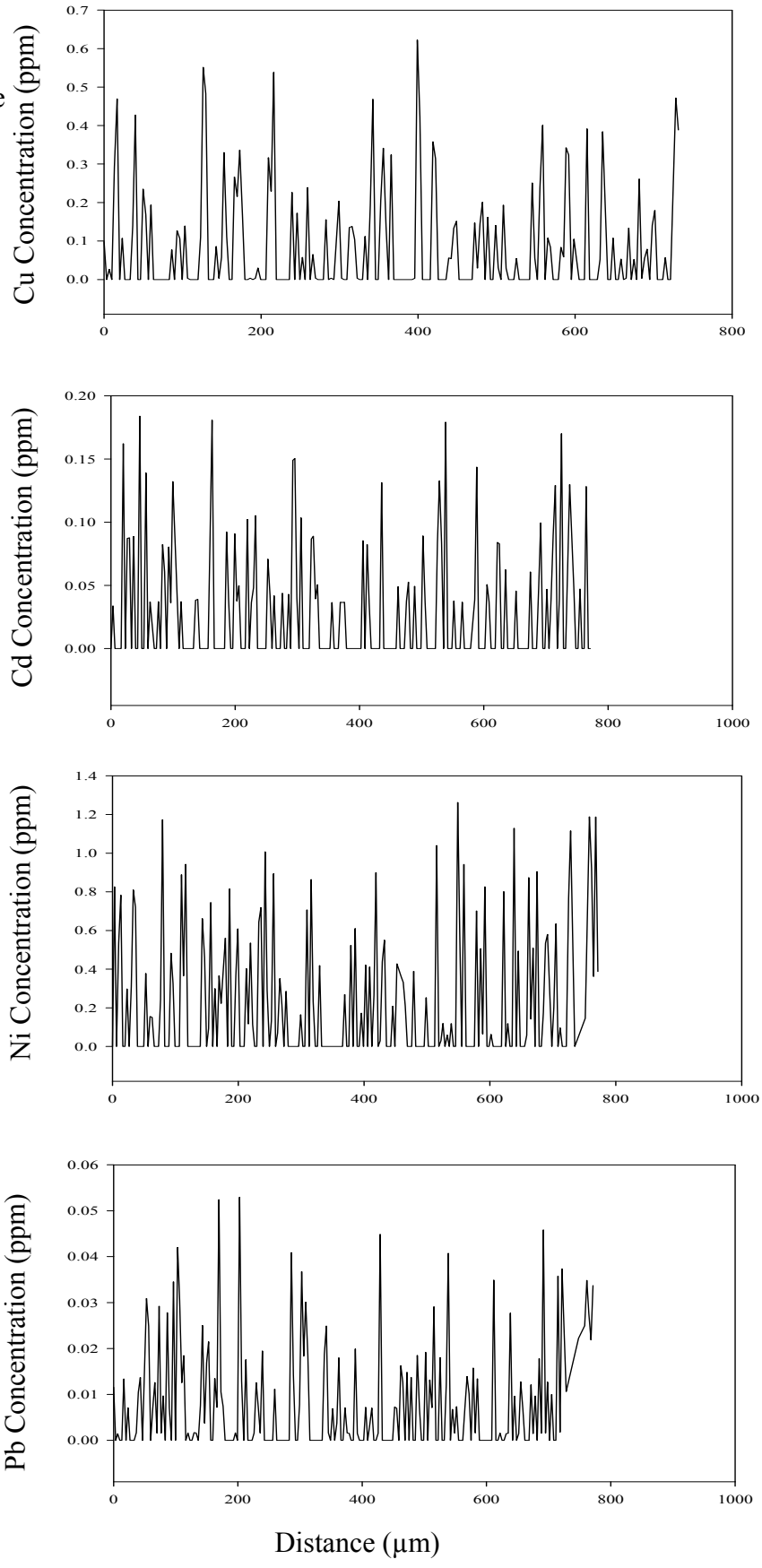
FD03-C14


Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
Distance (μm)

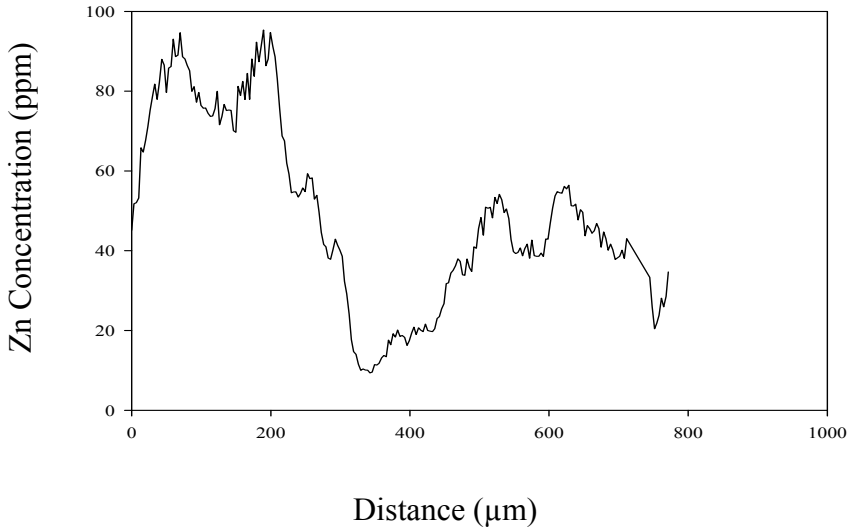
FD03 C21

Reference

Non-crystalline



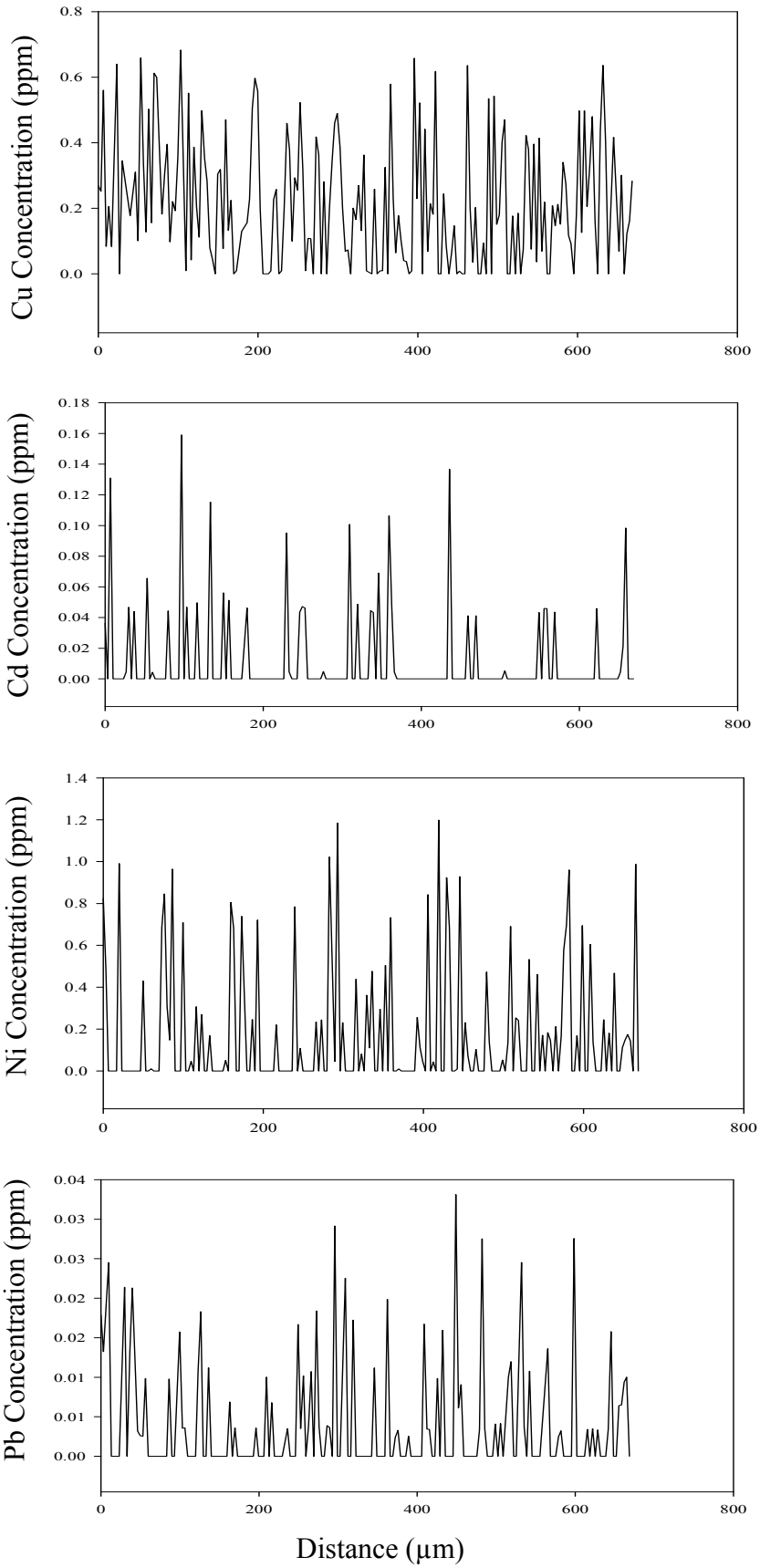
FD03 C21


Zn Concentration (ppm)
100
80
60
40
20
0
0
200
400
600
800
1000
Distance (μm)

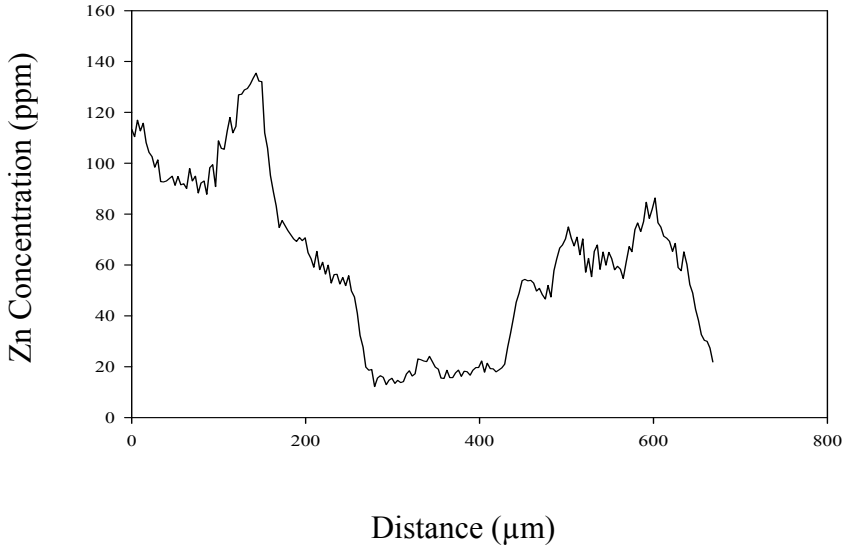
FD03 C28

Reference

Non-crystalline



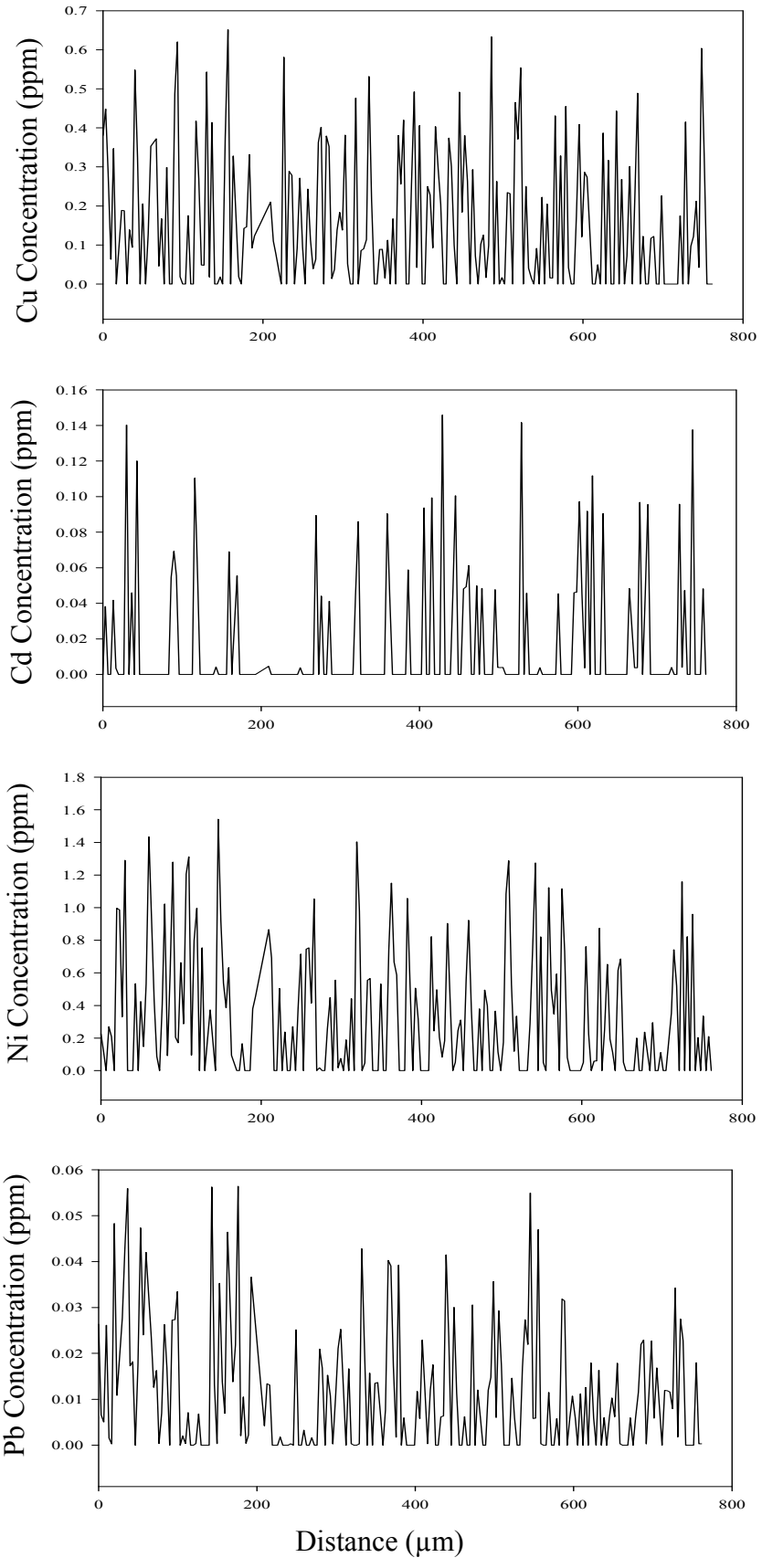
FD03 C28


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
0
200
400
600
800
Distance (µm)

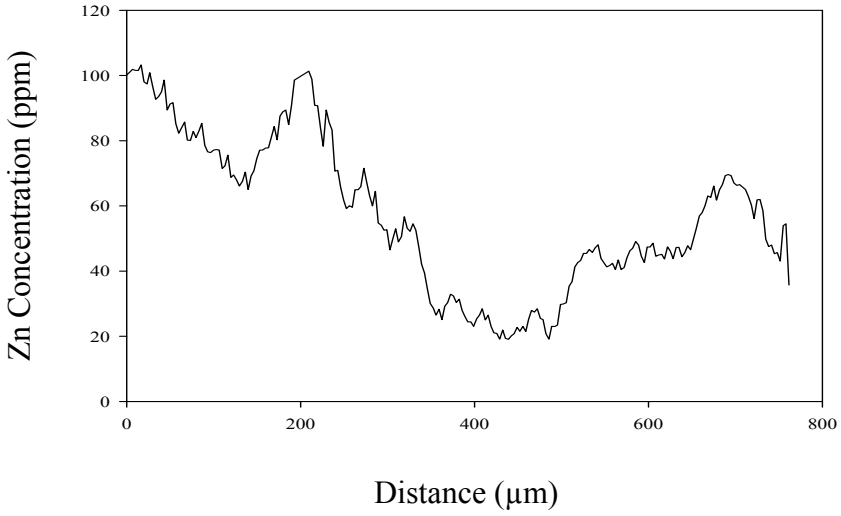
FD03 C29

Reference

Non-crystalline



FD03 C29

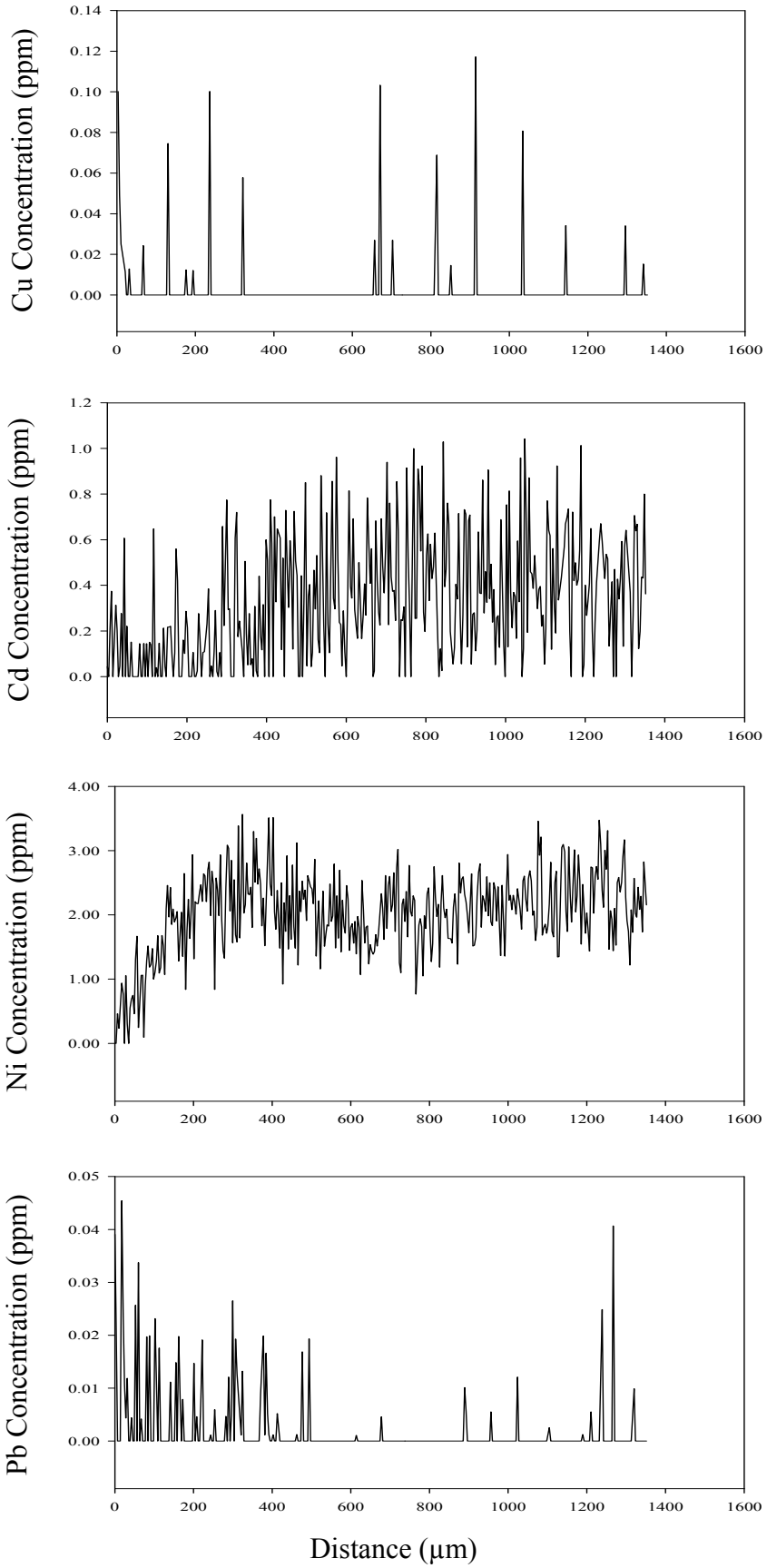

Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
Distance (µm)

IV.II Low Dose Group

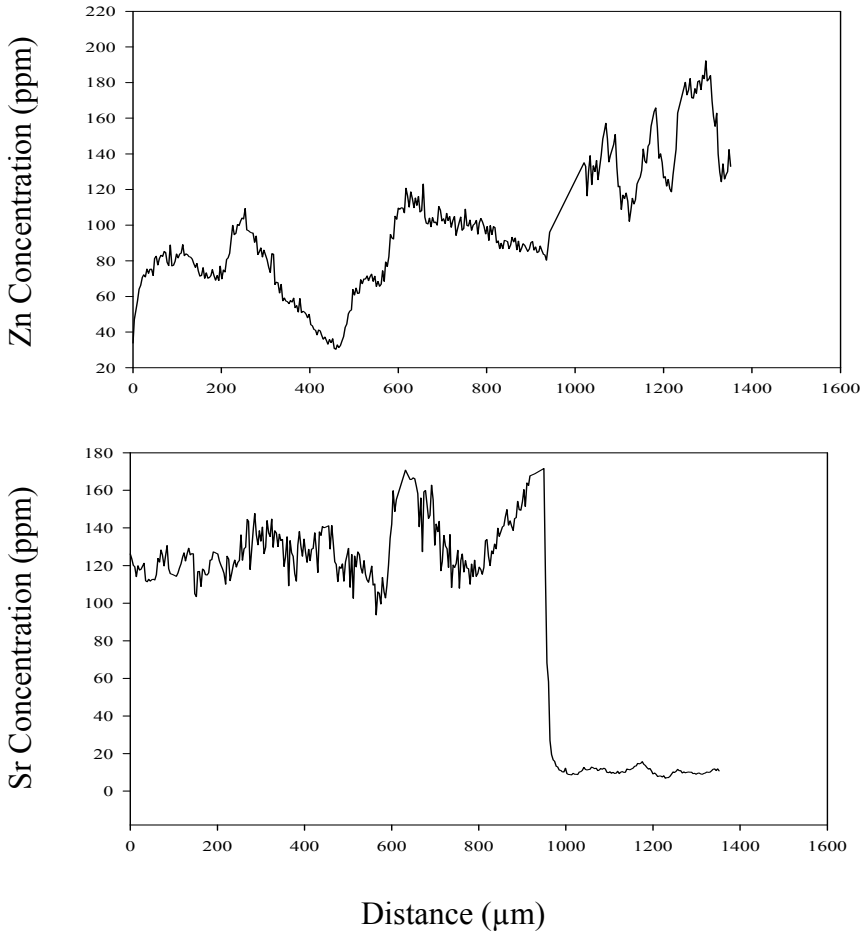
LD01-01

Low Dose

Crystalline



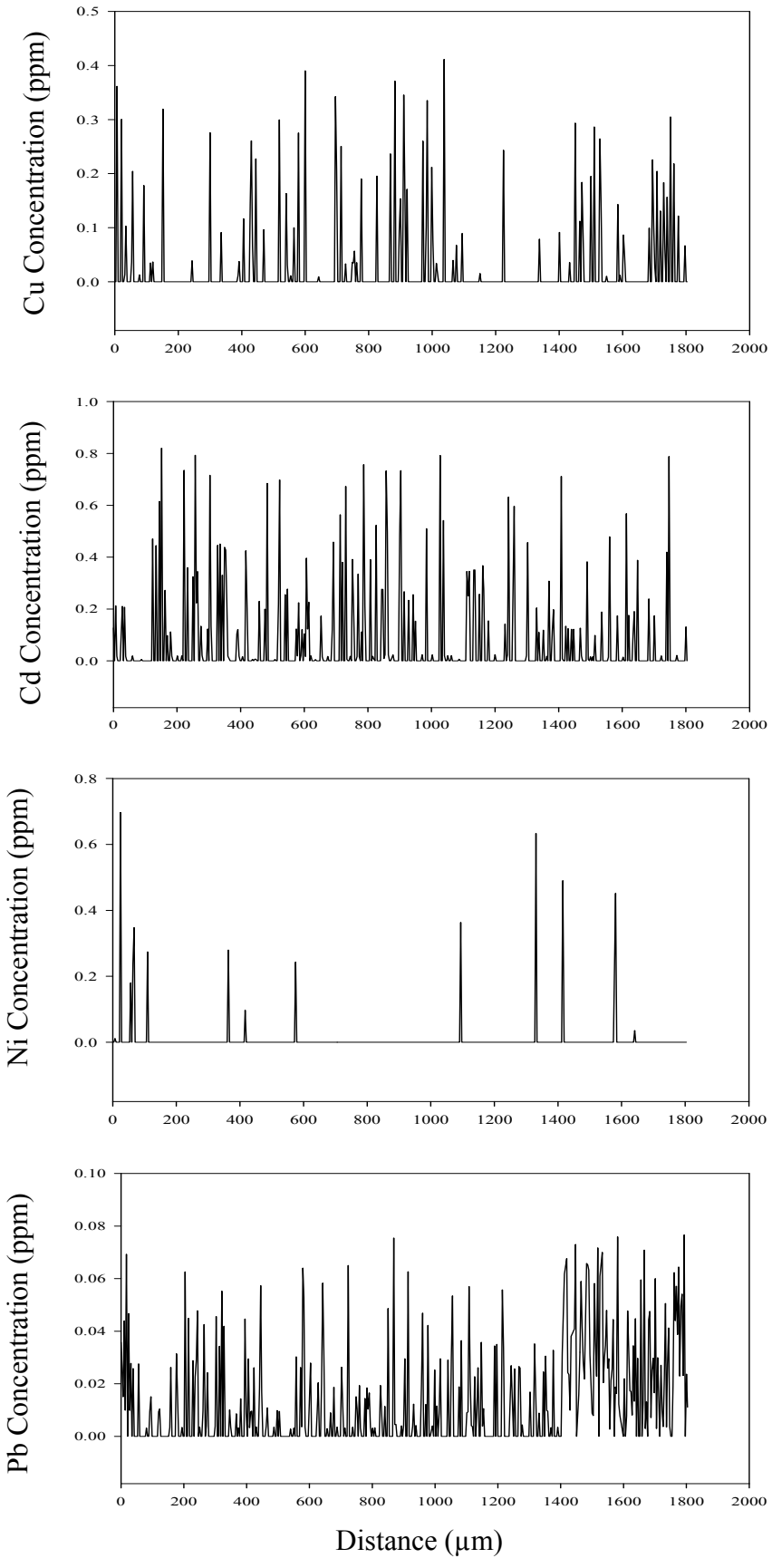
LD01-01


Zn Concentration (ppm)
220
200
180
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
Sr Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

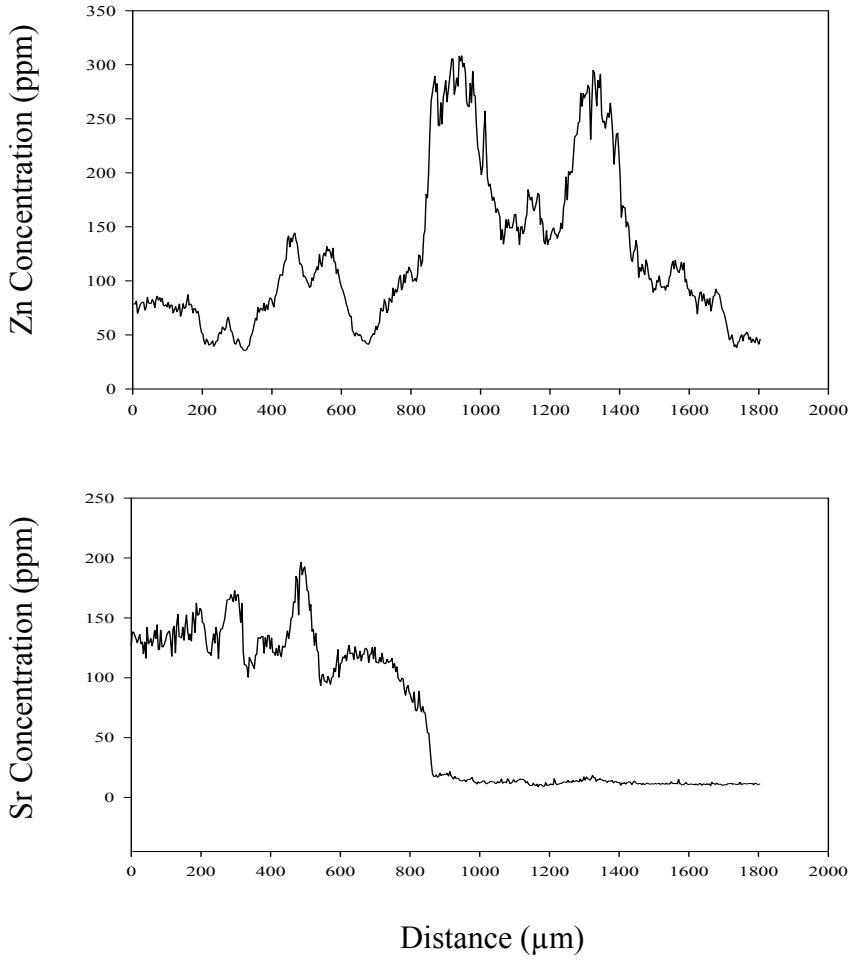
LD01-L04

Low dose

Crystalline



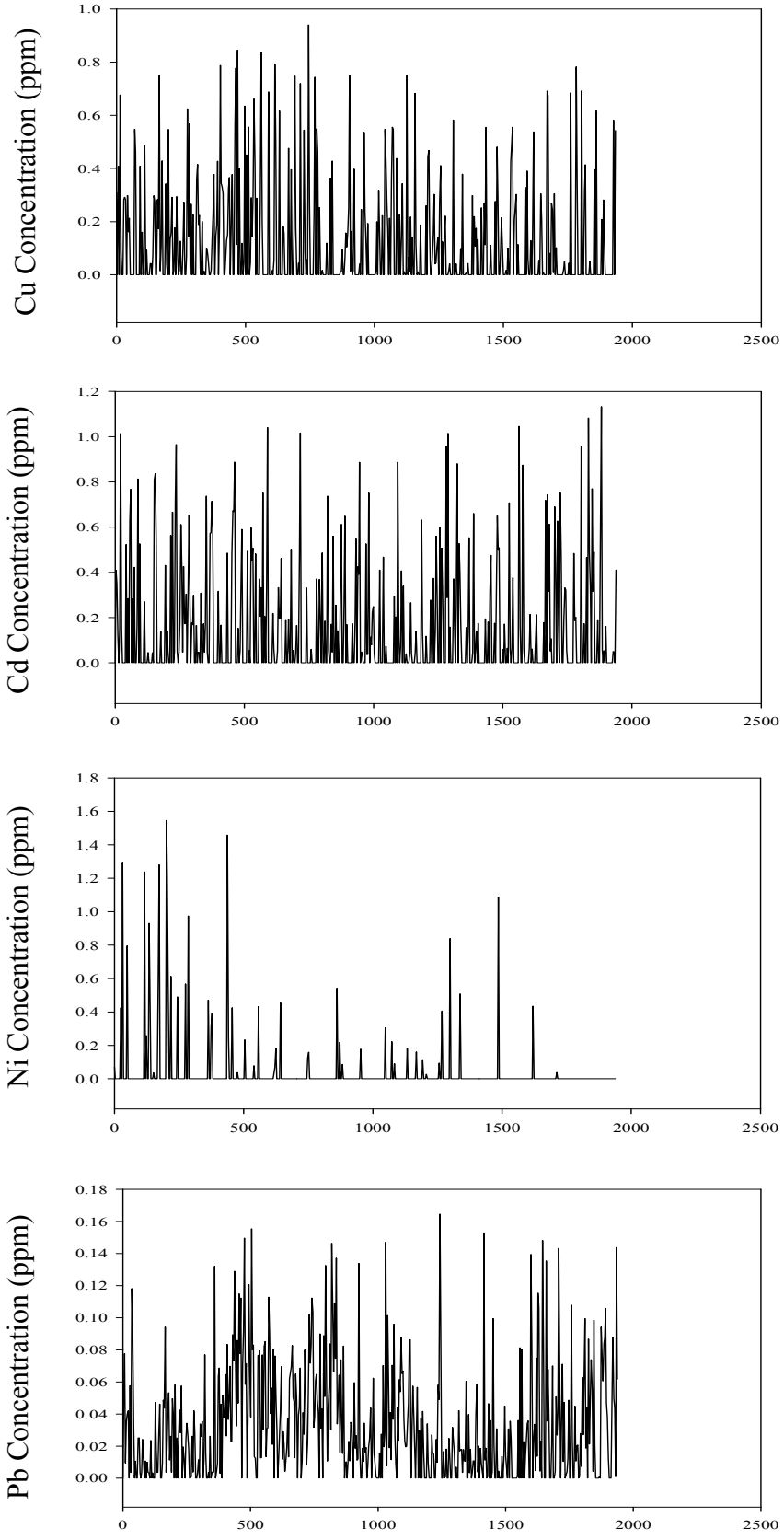
LD01-L04


Zn Concentration (ppm)
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (µm)

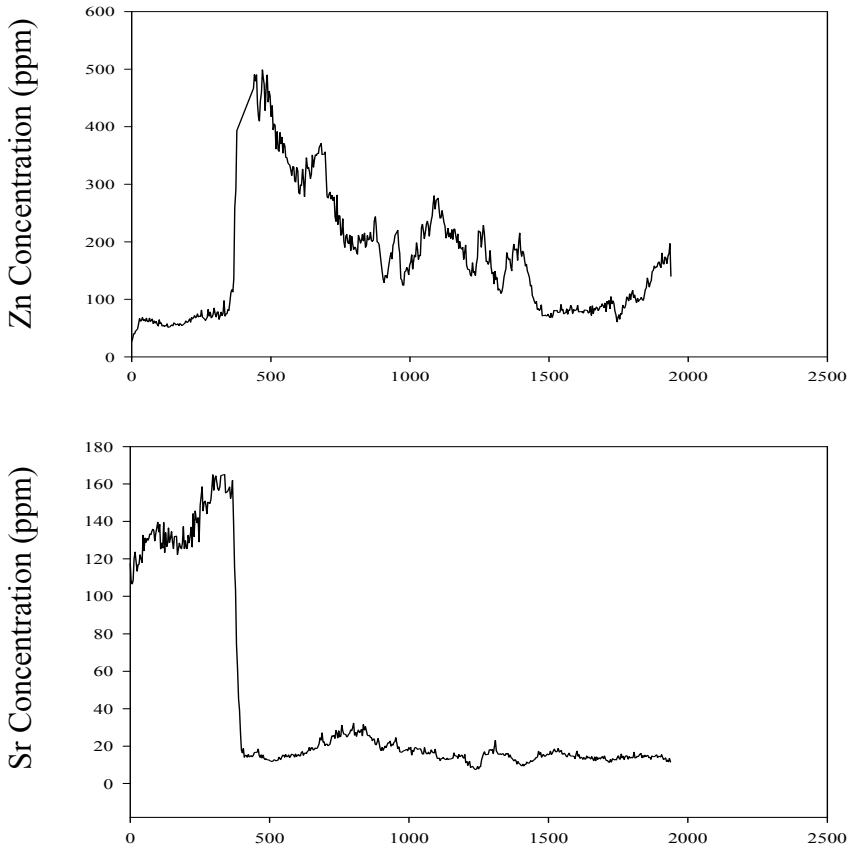
LD01-05

Low Dose

Crystalline



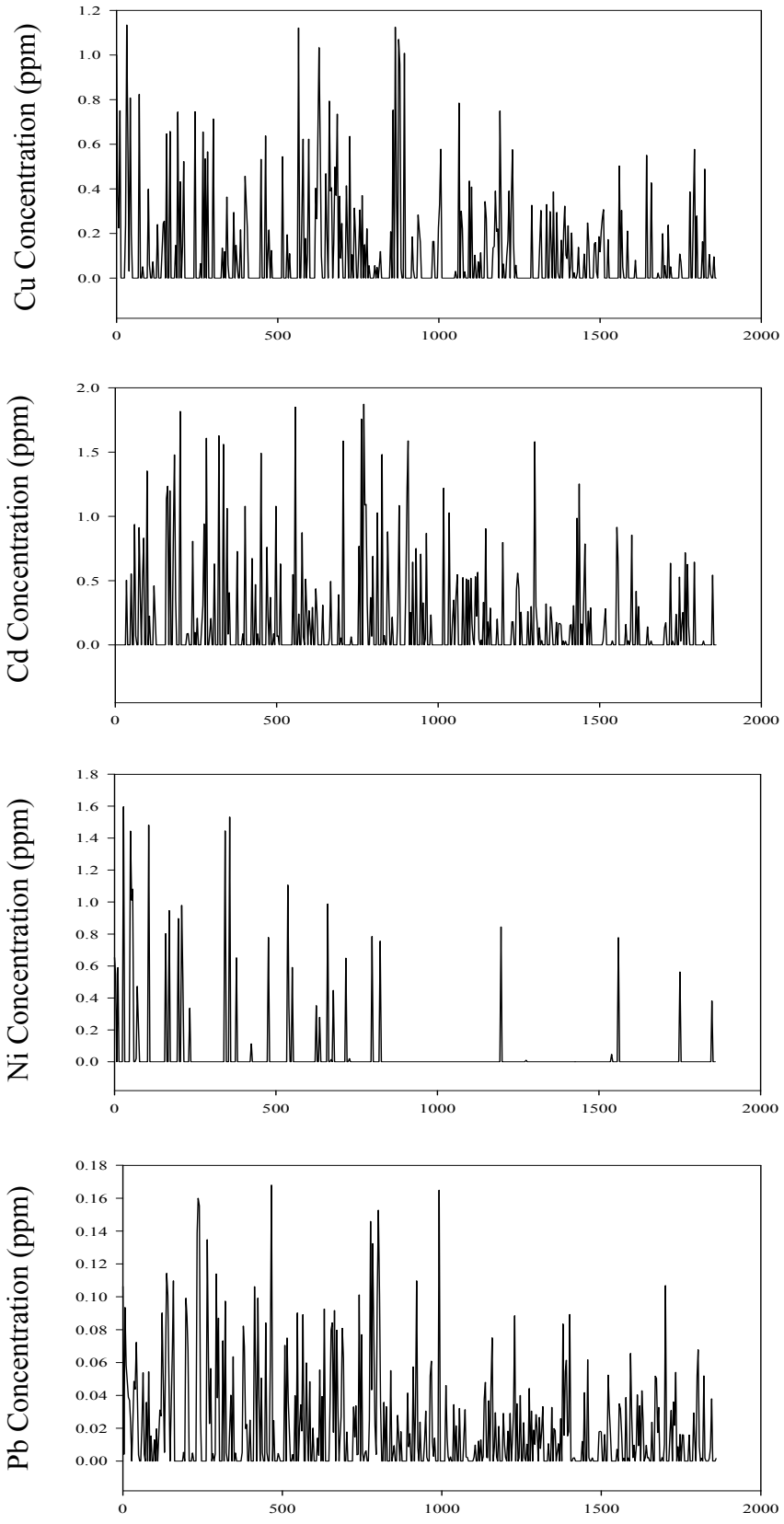
LD01-05


Zn Concentration (ppm)
600
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

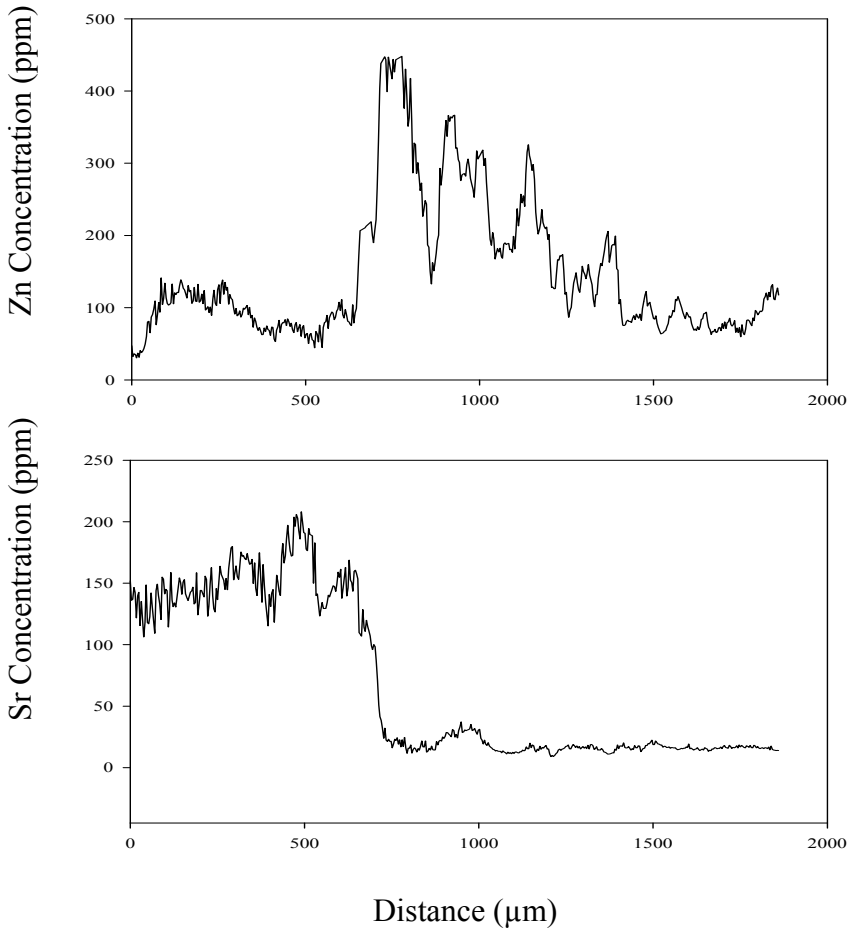
LD01-06

Low dose

Crystalline



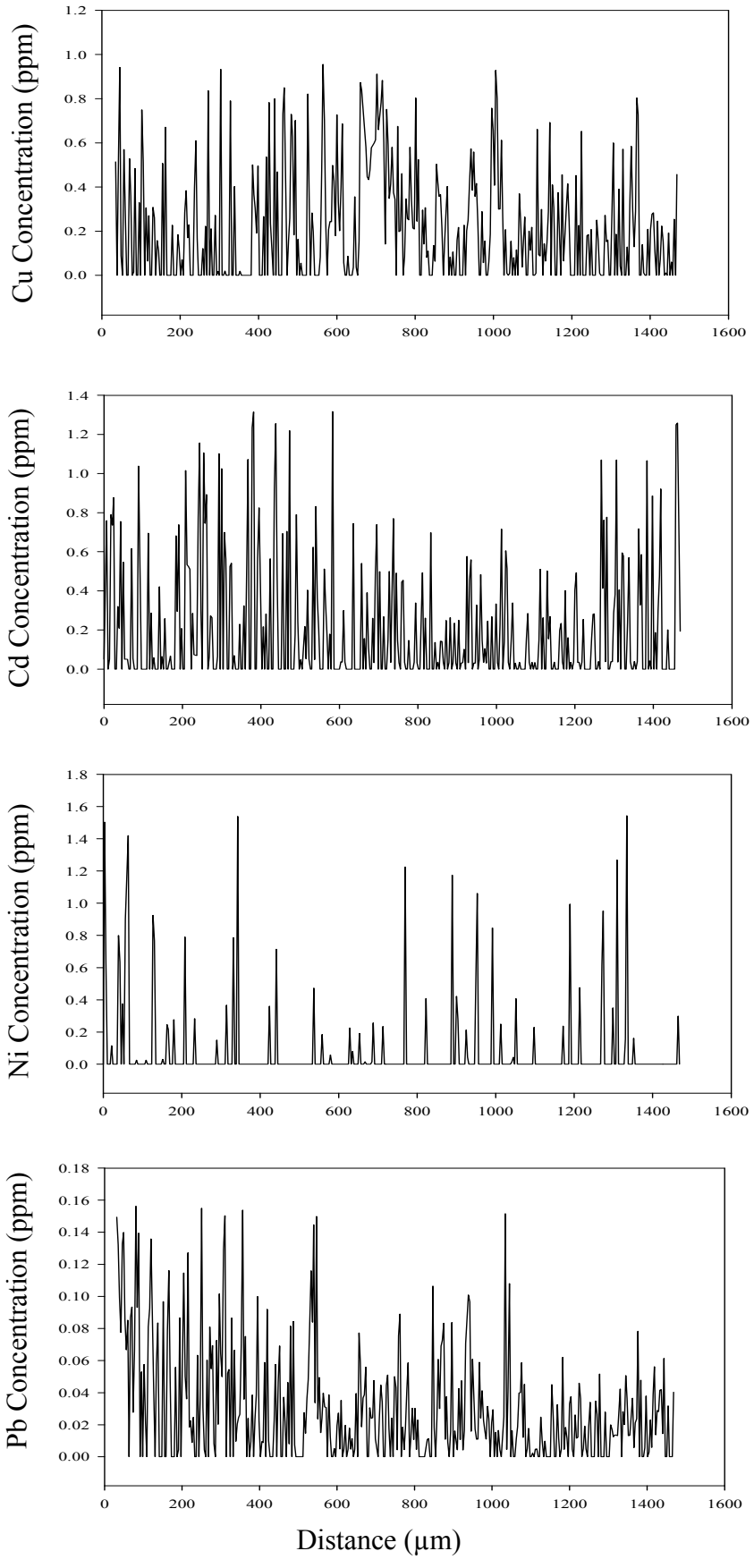
LD01-06


Zn Concentration (ppm)
500
400
300
200
100
0
0
500
1000
1500
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Distance (µm)

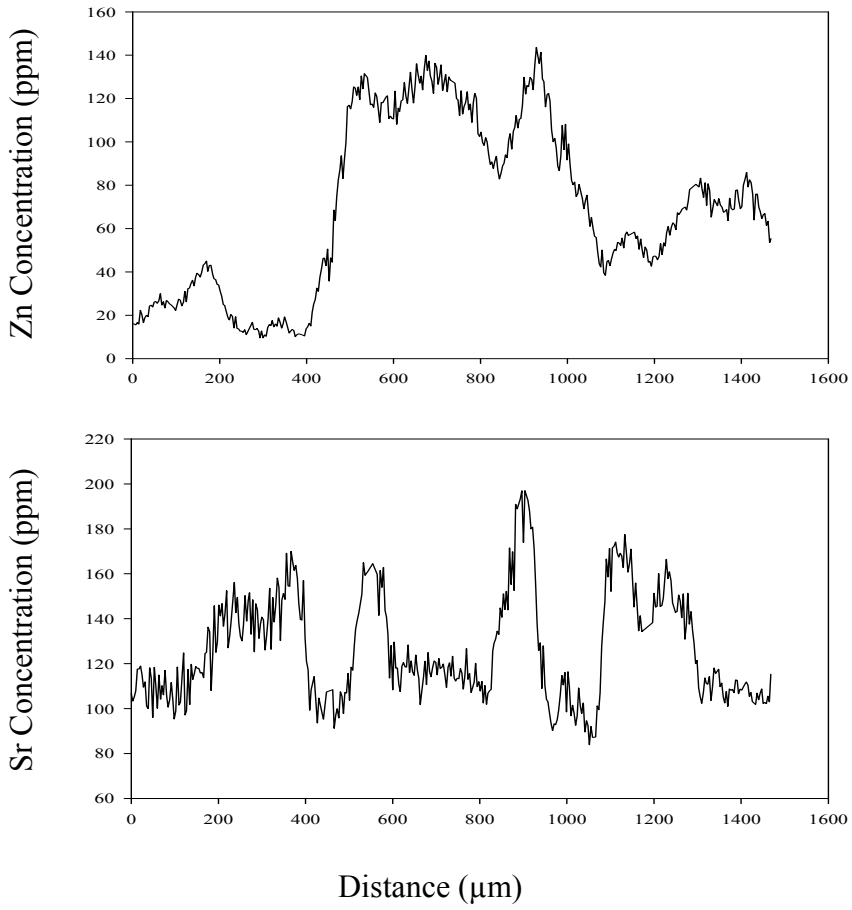
LD01-L07

Low Dose

Non-crystalline



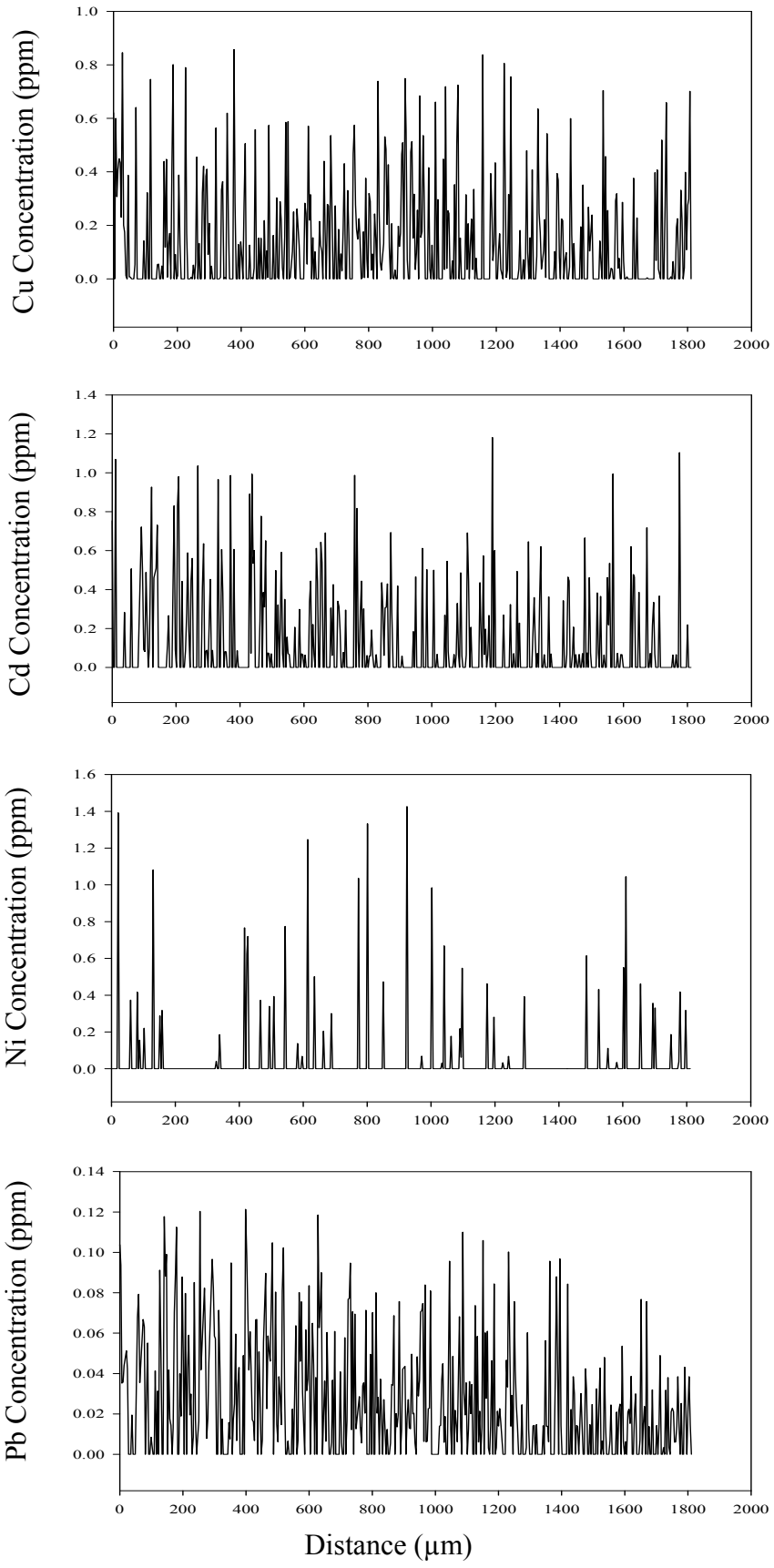
LD01-L07


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Sr Concentration (ppm)
220
200
180
160
140
120
100
80
60
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

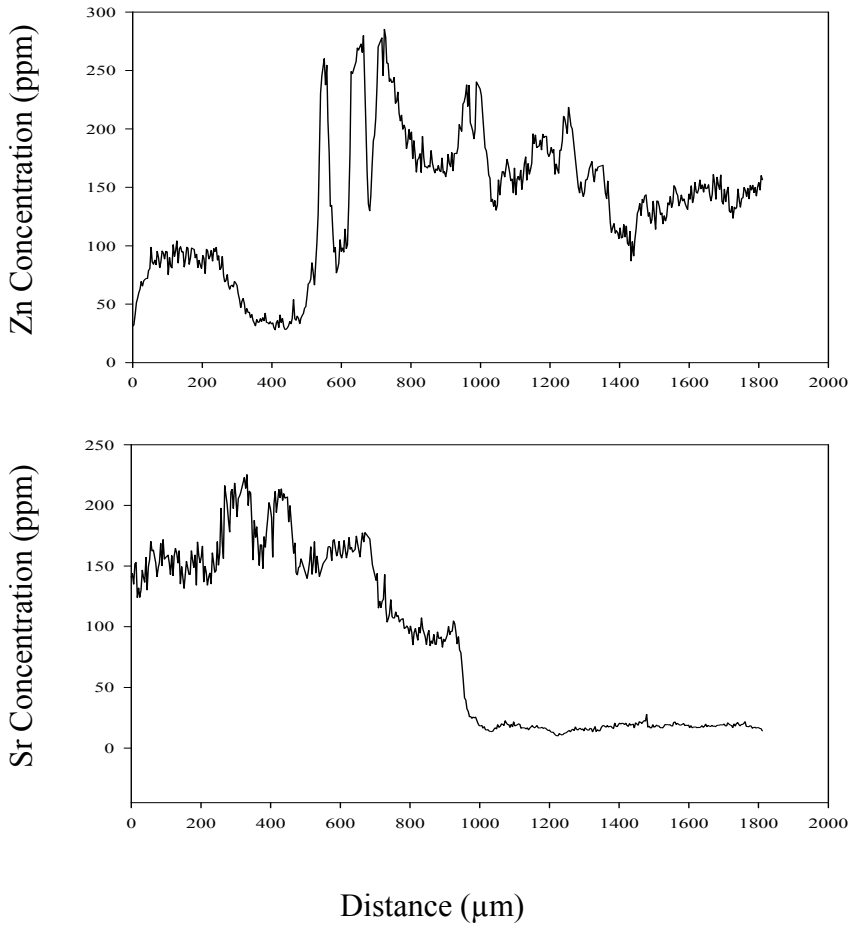
LD01-L08

Low dose

Crystalline



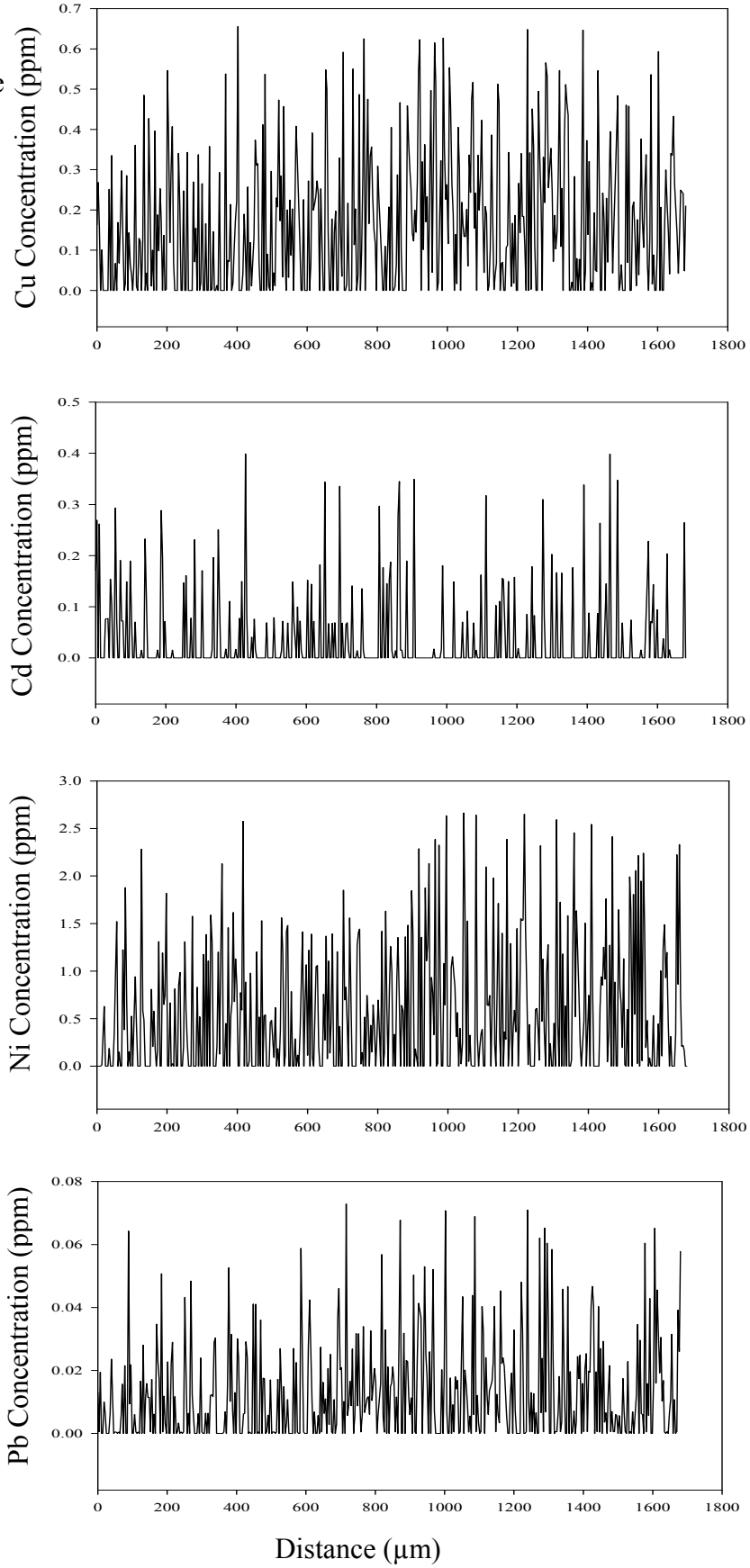
LD01-L08


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

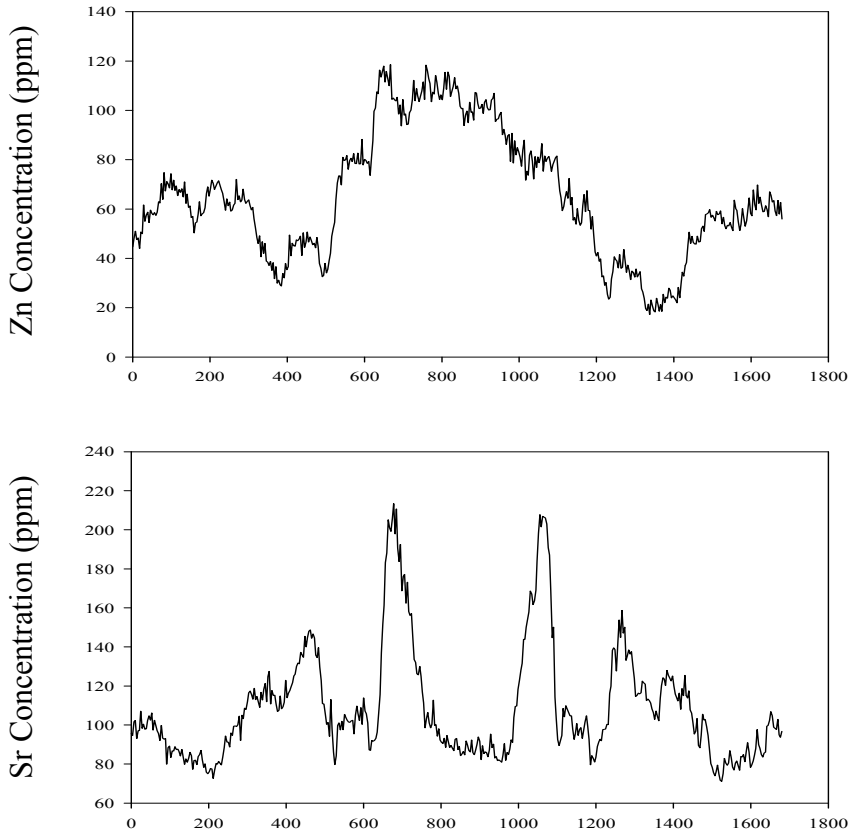
LD02-L15

Low dose

Non-crystalline



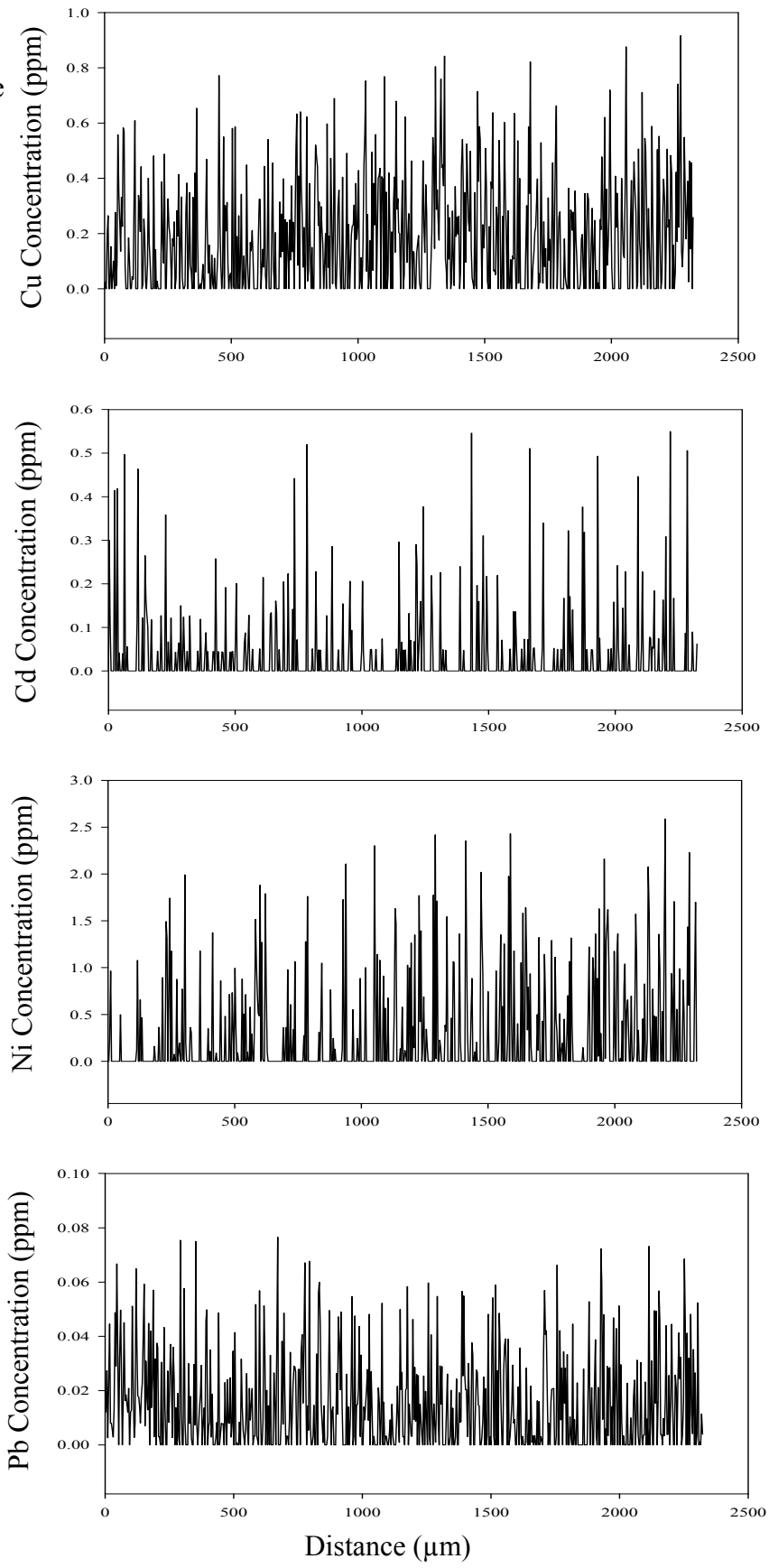
LD02-L15


Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
240
220
200
180
160
140
120
100
80
60
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

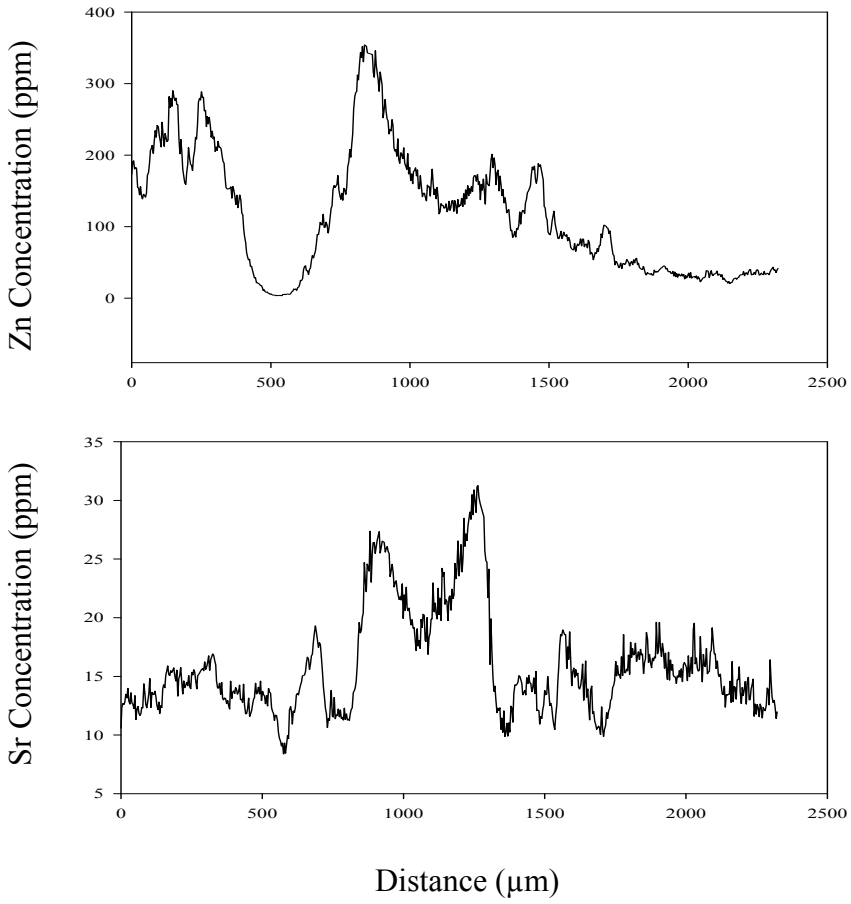
LD02-L16

Low dose

Non-crystalline



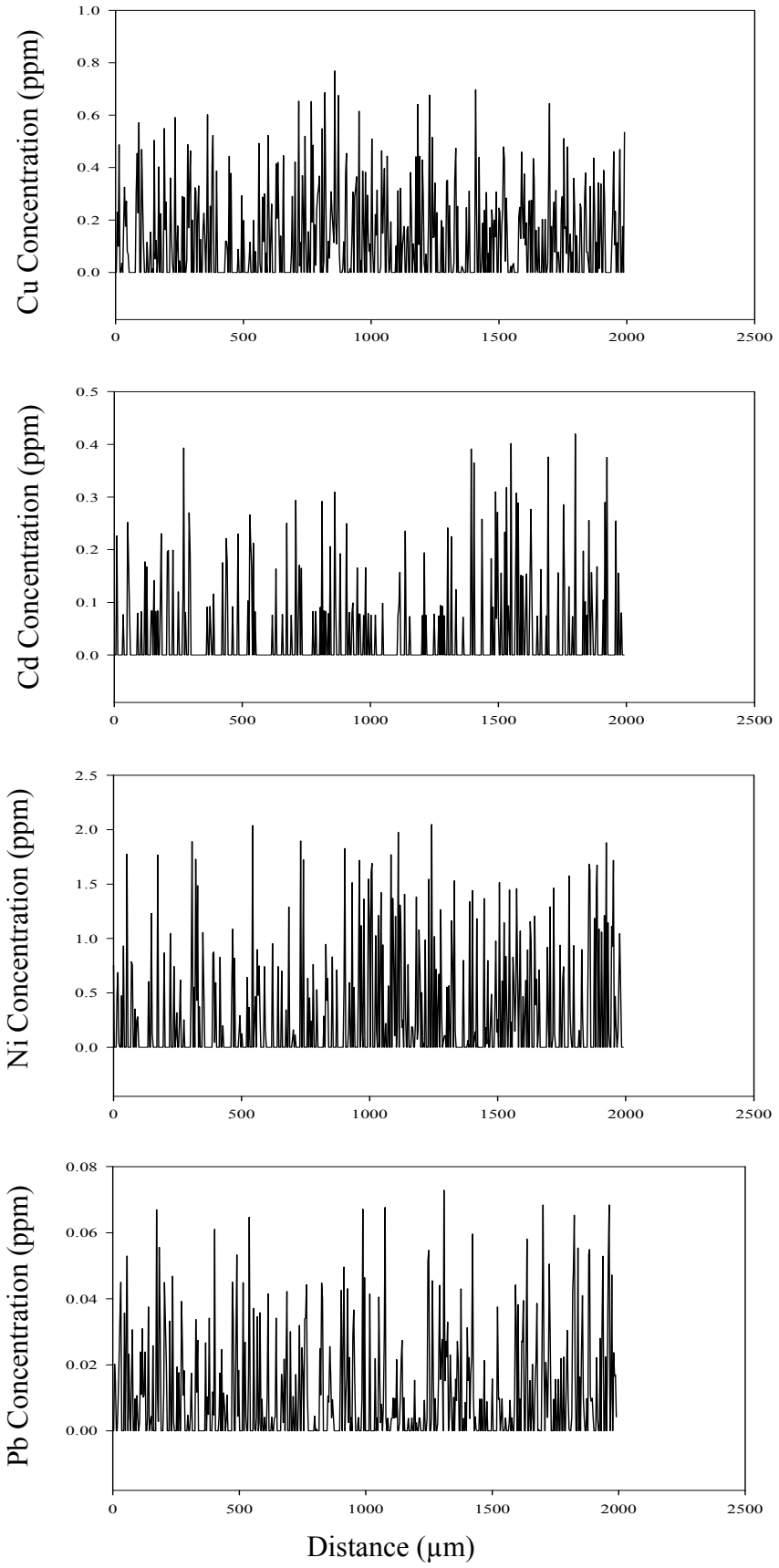
LD02-L16


Zn Concentration (ppm)
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
35
30
25
20
15
10
5
0
500
1000
1500
2000
2500
Distance (µm)

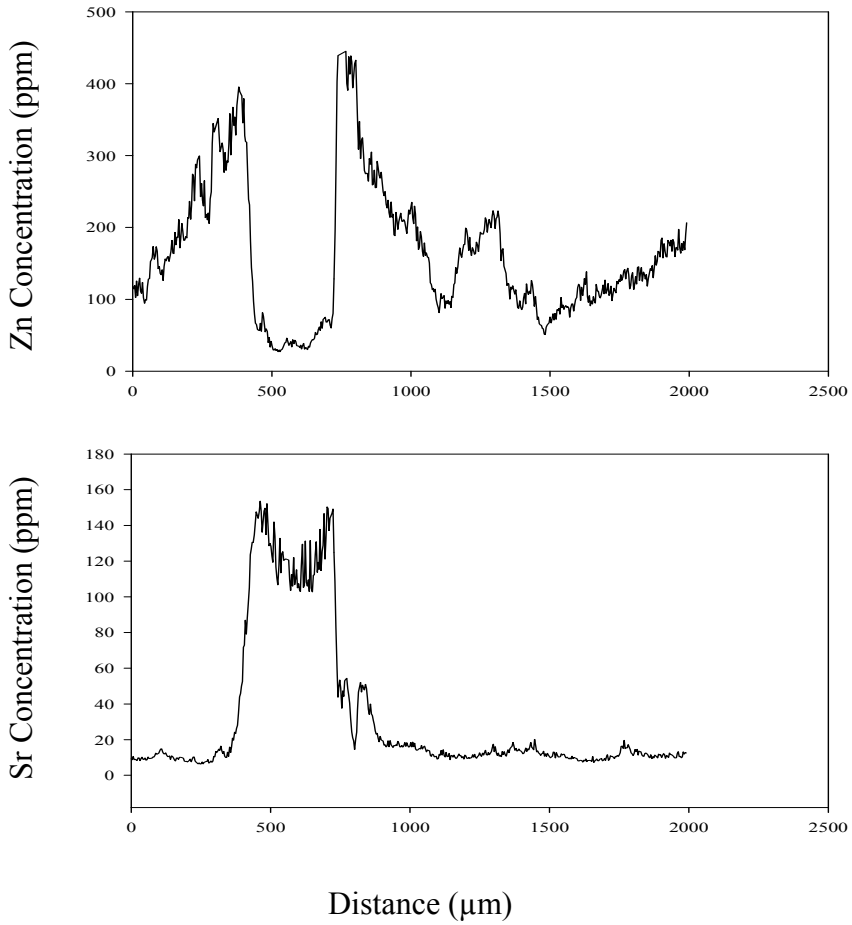
LD02-L18

Low dose

Crystalline



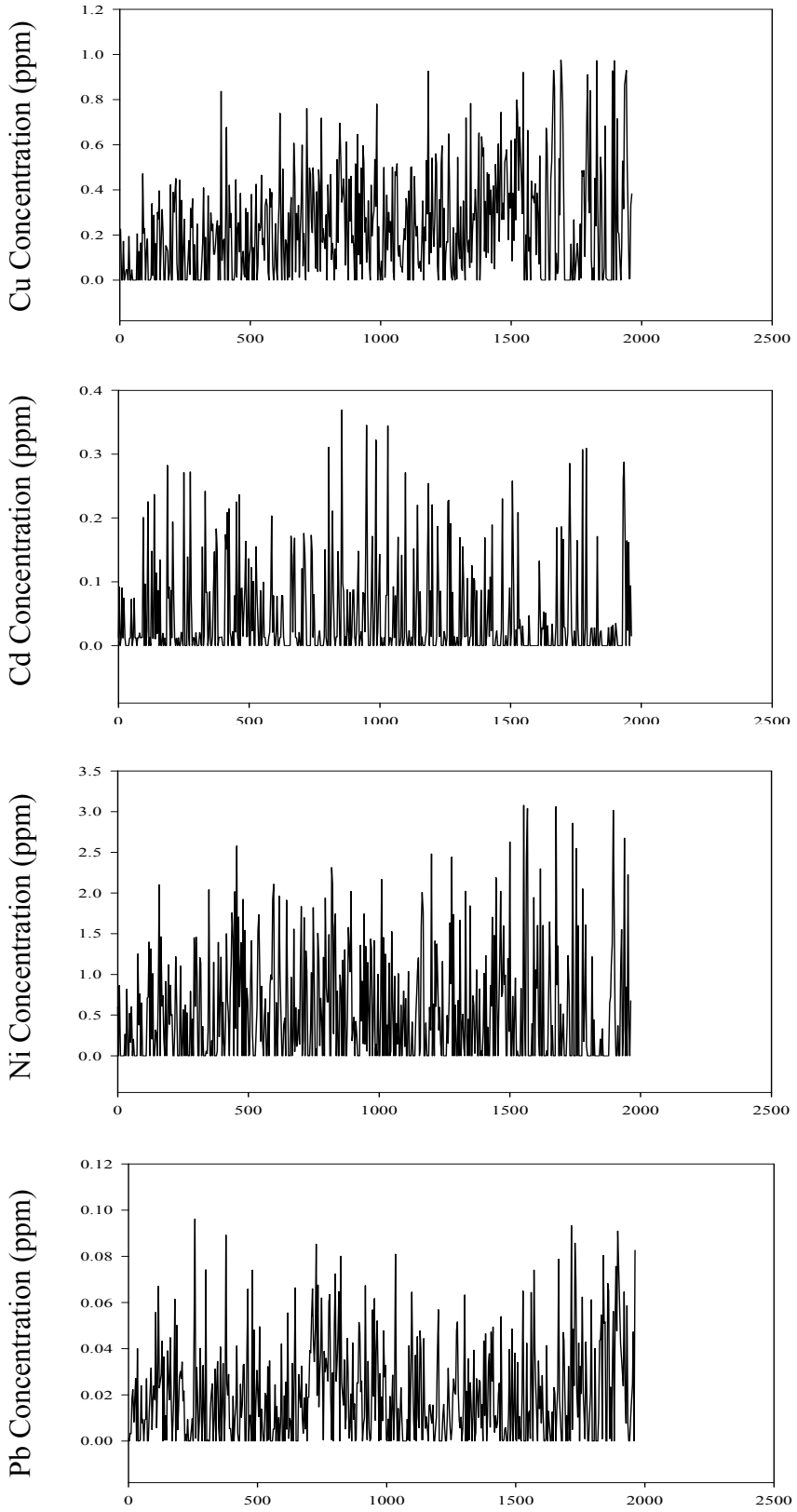
LD01-L18


Zn Concentration (ppm)
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (μm)

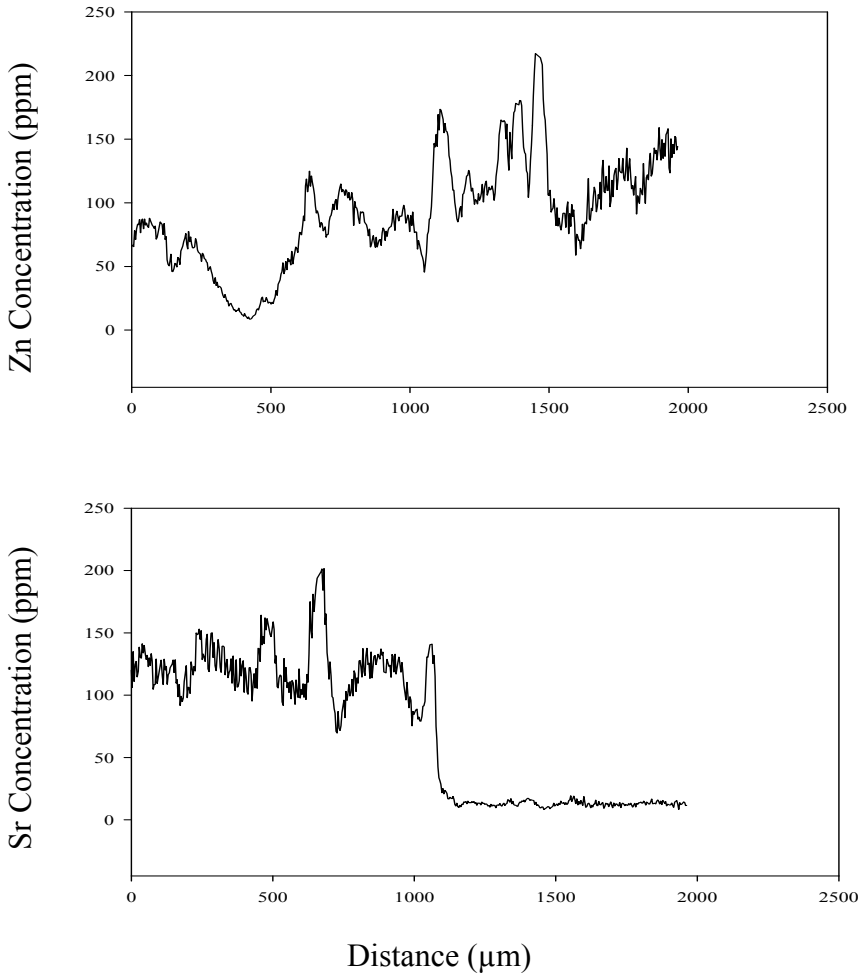
LD02-L19

Low dose

Crystalline



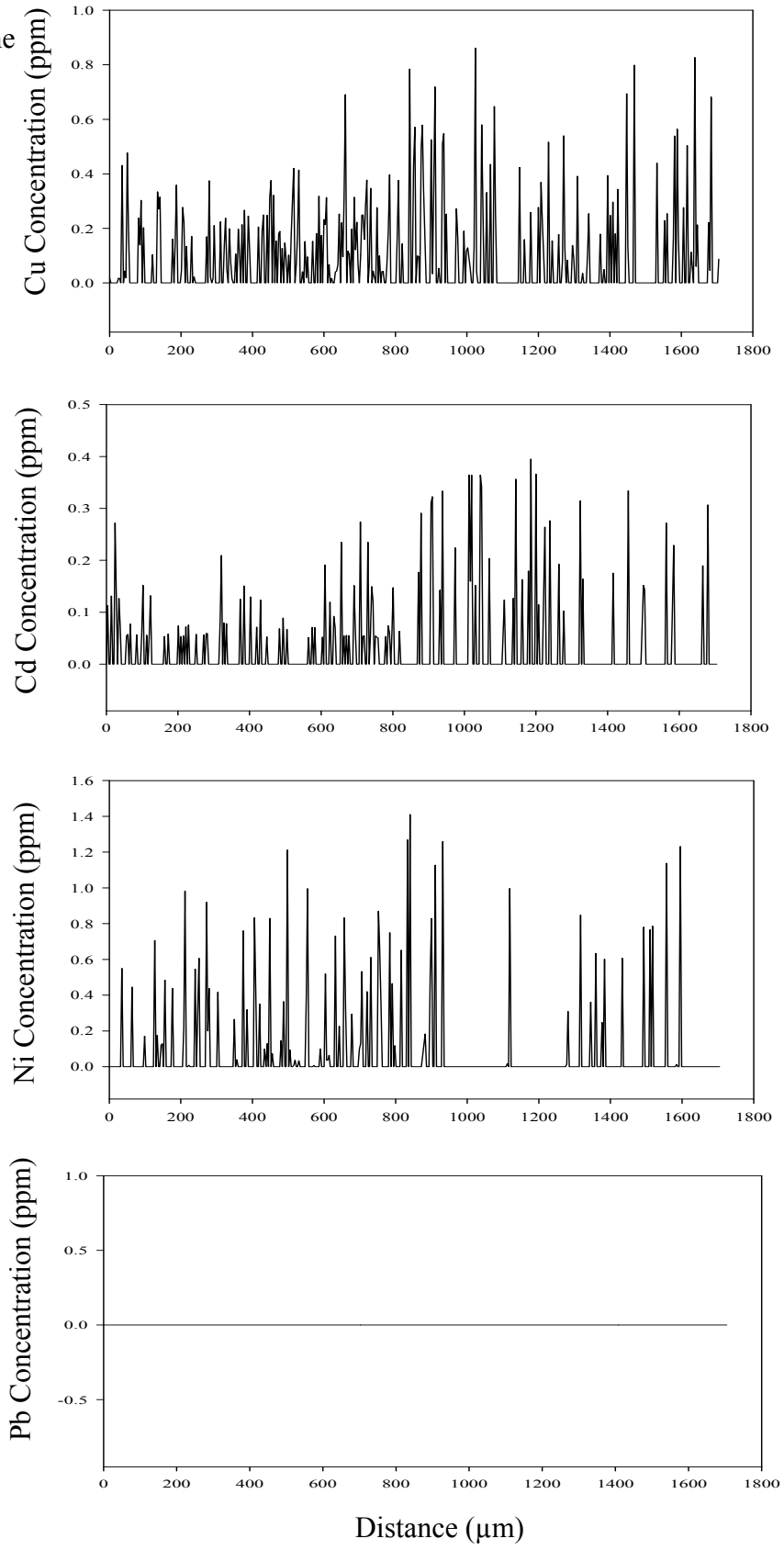
LD02-L19


Zn Concentration (ppm)
Sr Concentration (ppm)
Distance (μm)
0
50
100
150
200
250
500
1000
1500
2000
2500

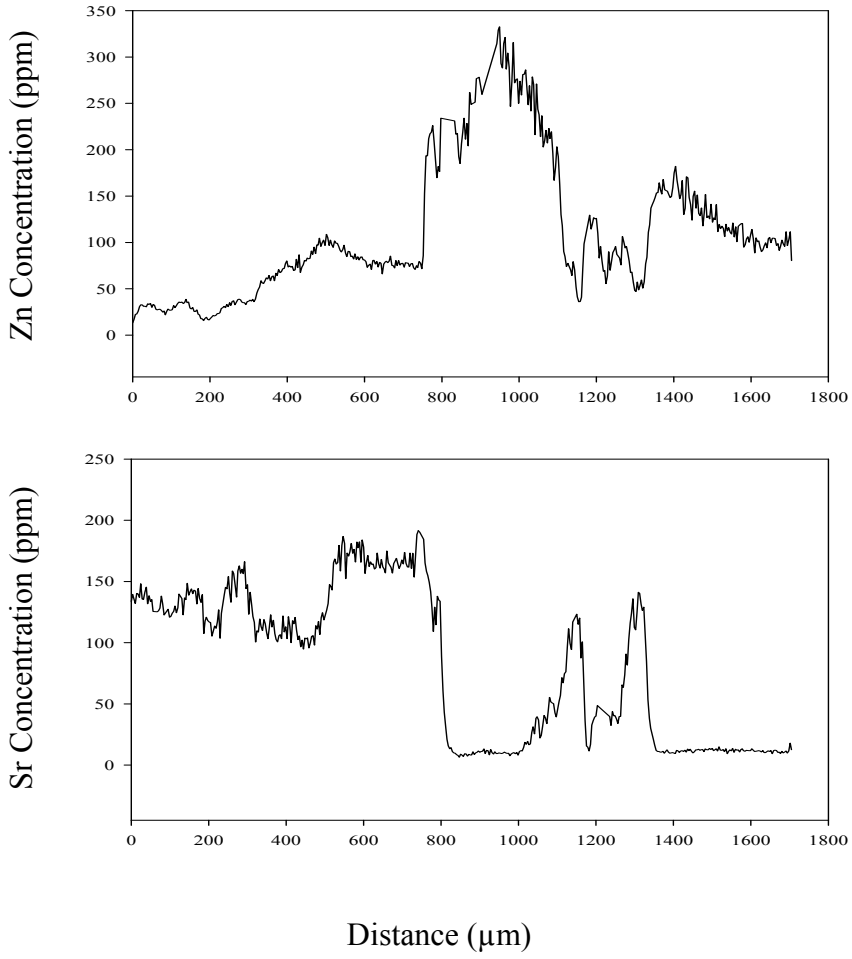
LD02-L20

Low dose

Partially crystalline



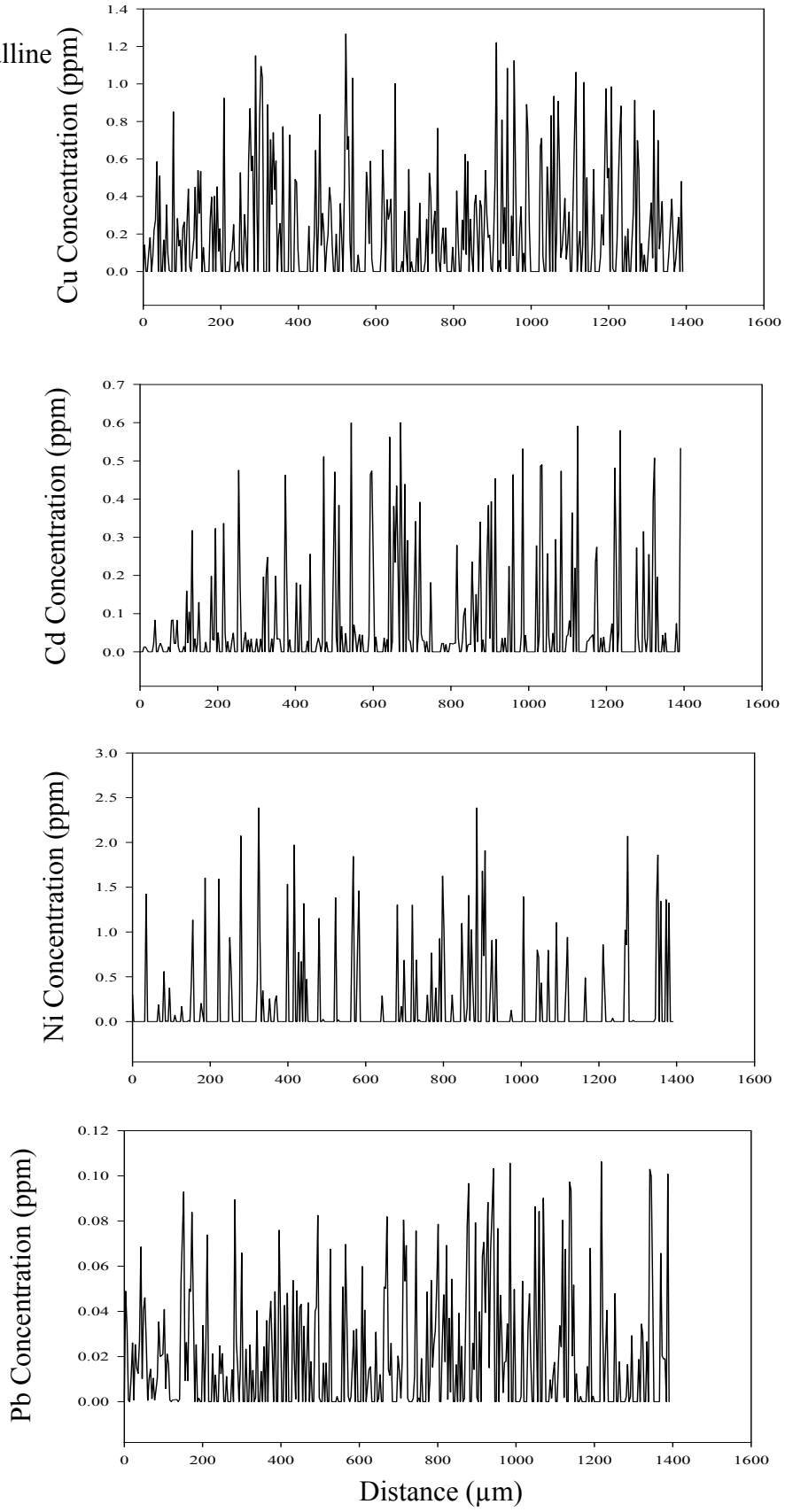
LD02-20


Zn Concentration (ppm)
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

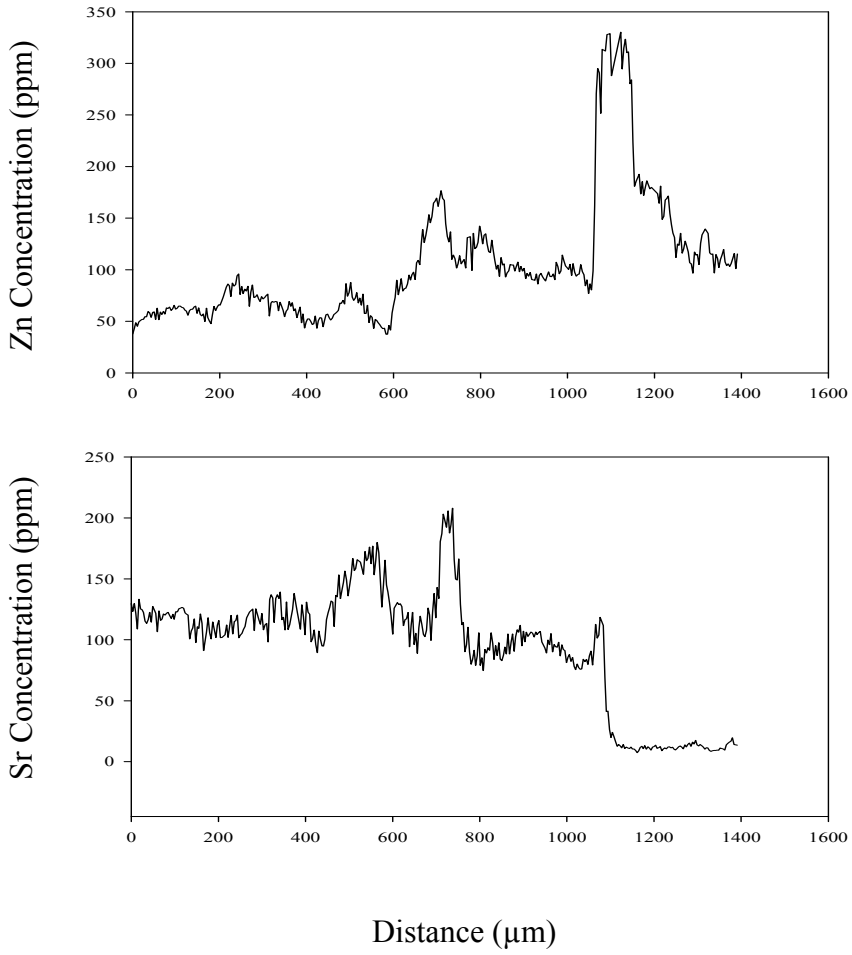
LD02-L21

Low dose

Partially crystalline



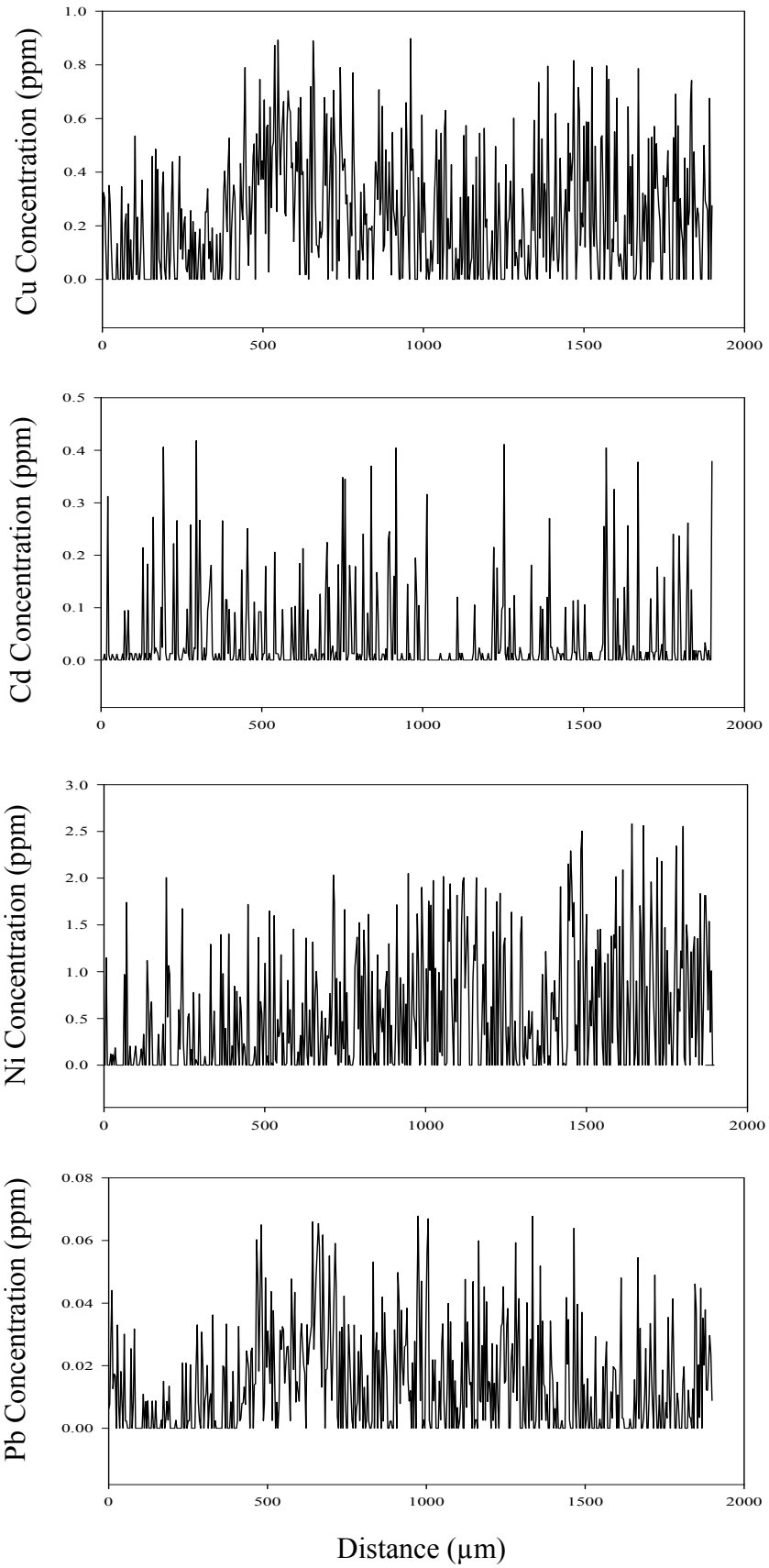
LD02-L21


Zn Concentration (ppm)
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)

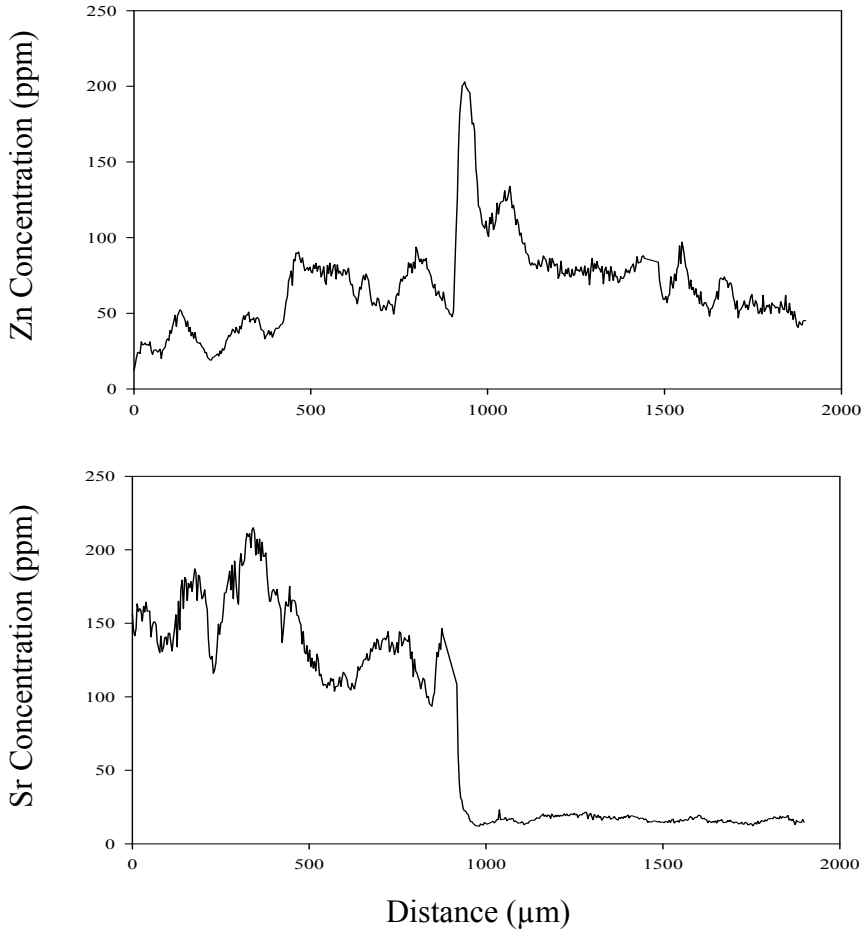
LD02-L25

Low dose

Crystalline



LD02-L25

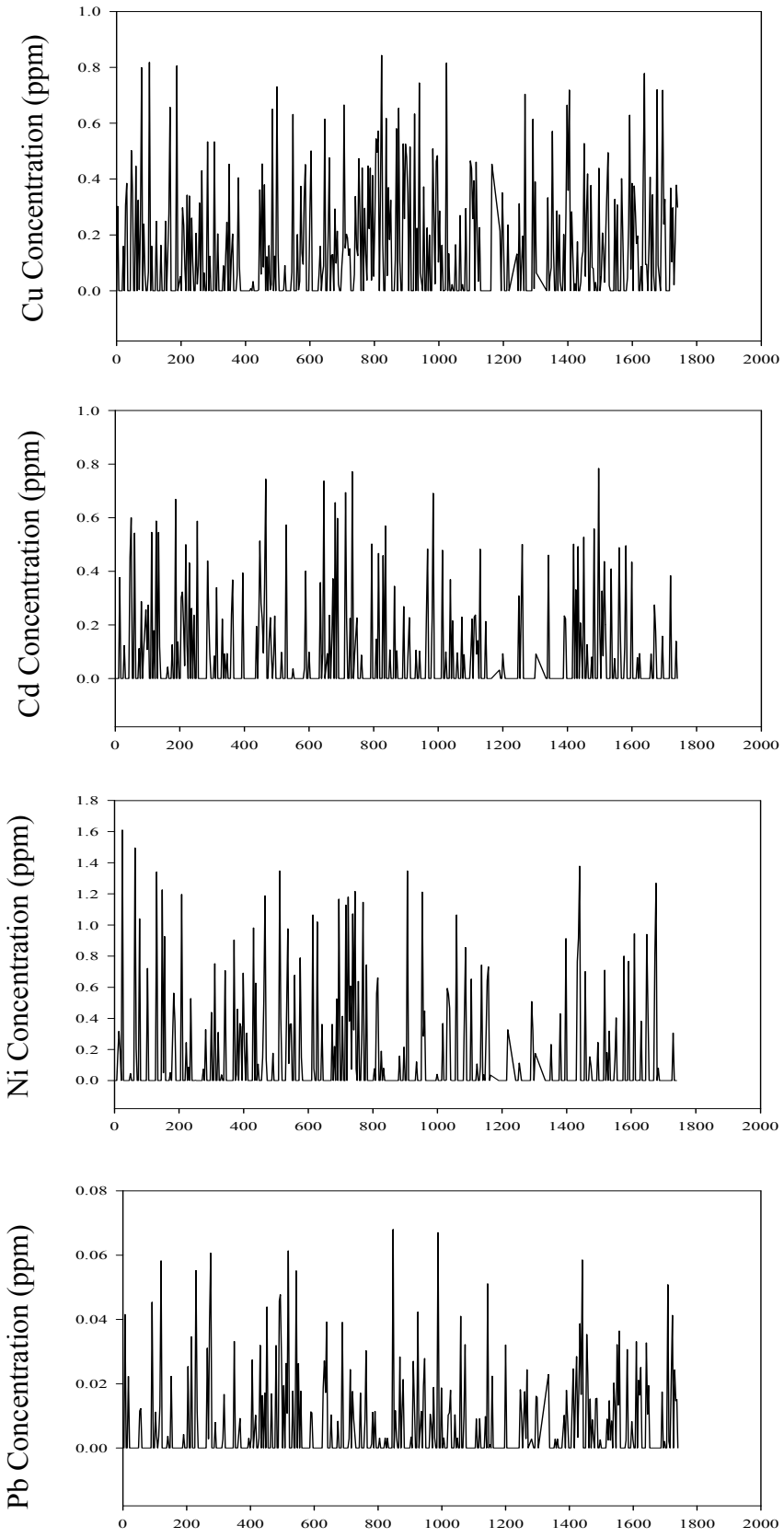

Zn Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Distance (µm)

IV.III Medium Dose Group

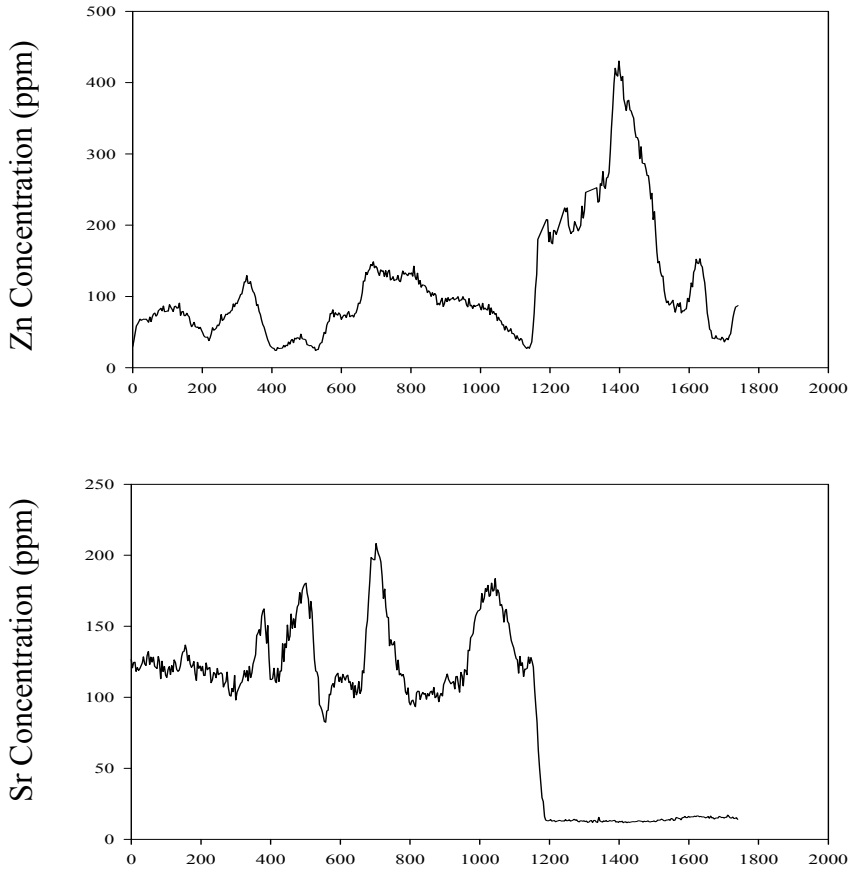
MD01 M02

Med dose

Crystalline



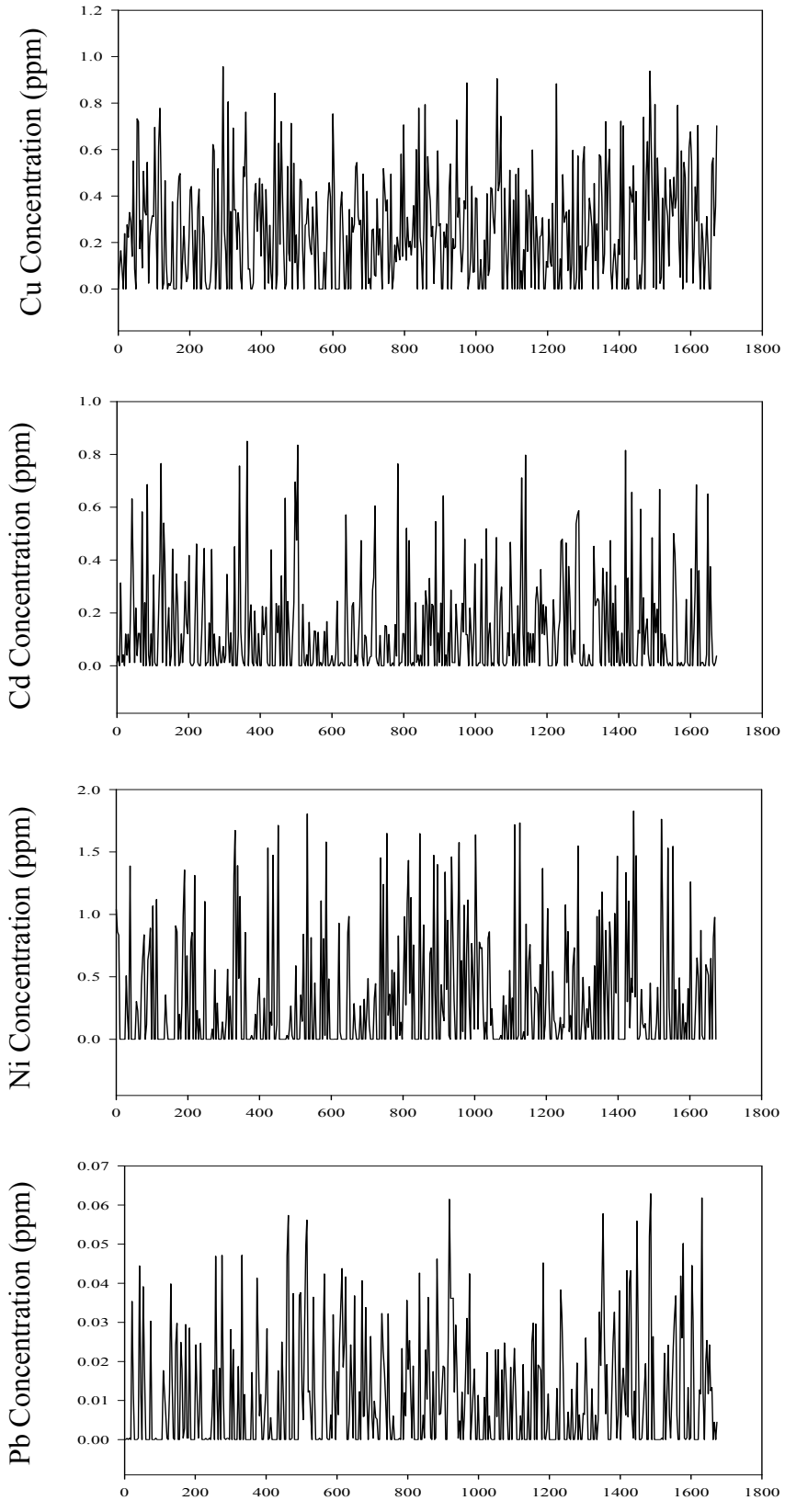
MD01 M02


Zn Concentration (ppm)
500
400
300
200
100
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (µm)

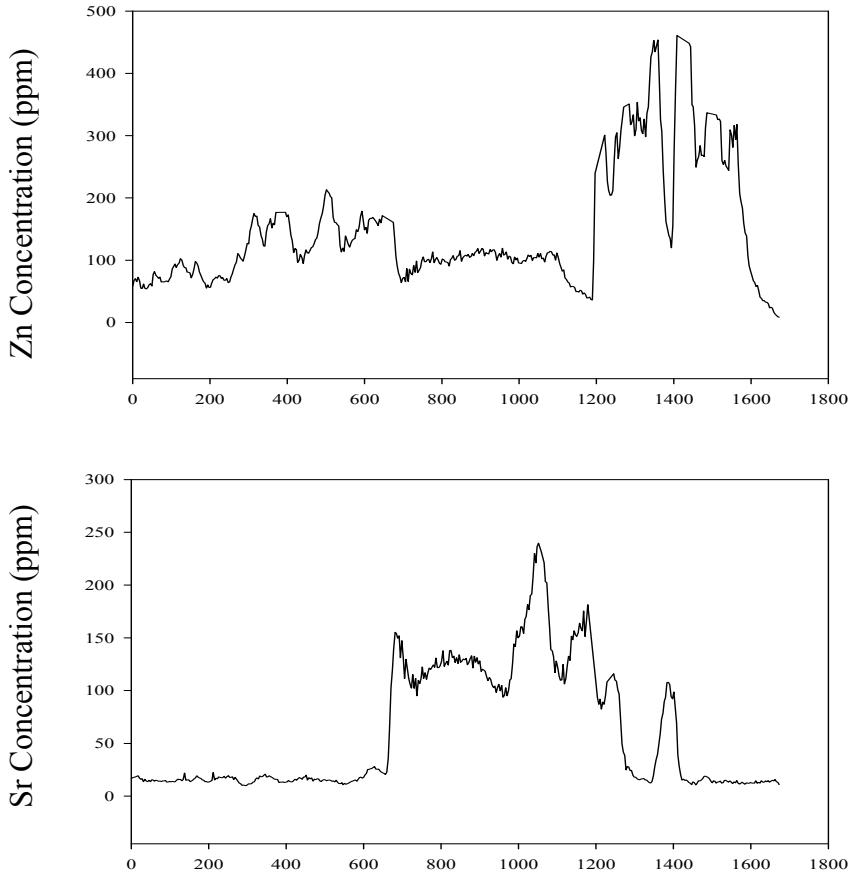
MD01 M03

Med dose

Crystalline



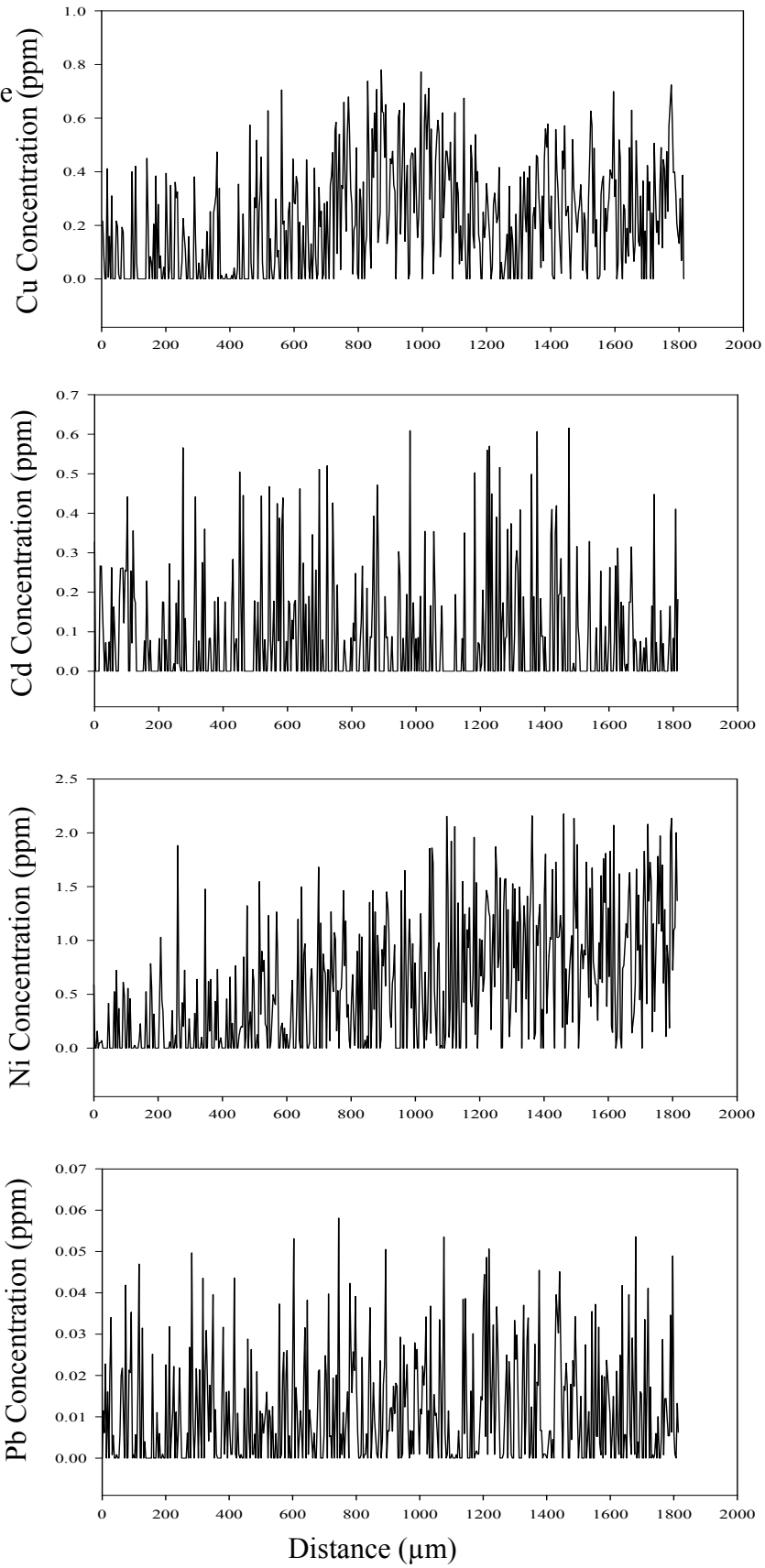
MD01 M03


Zn Concentration (ppm)
500
400
300
200
100
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

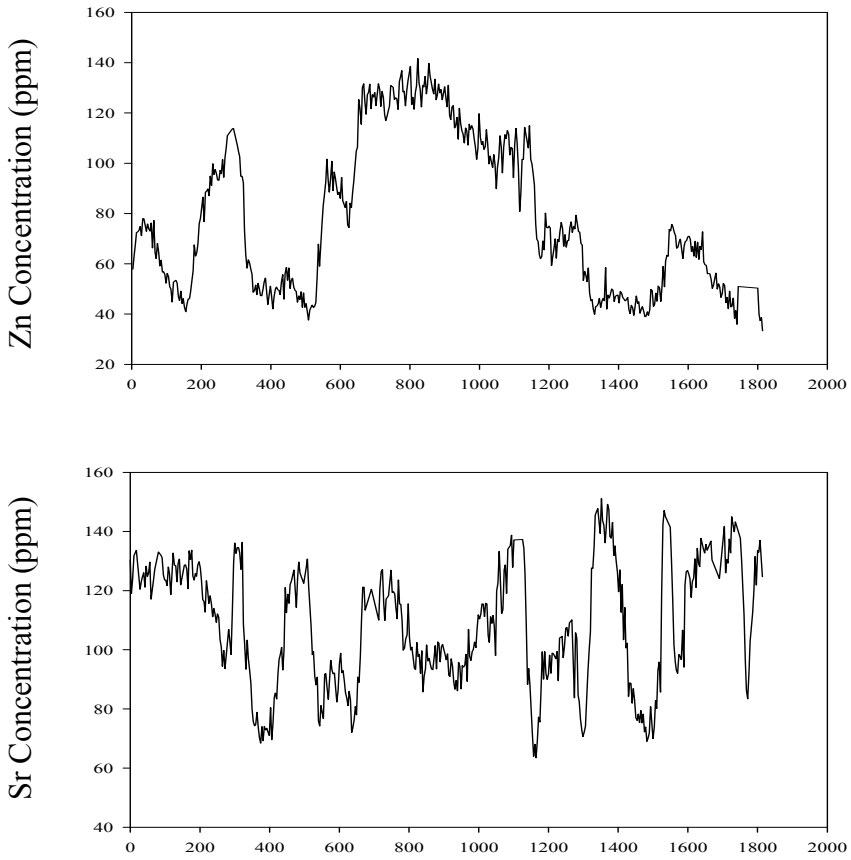
MD01 M04

Med dose

Non-crystalline



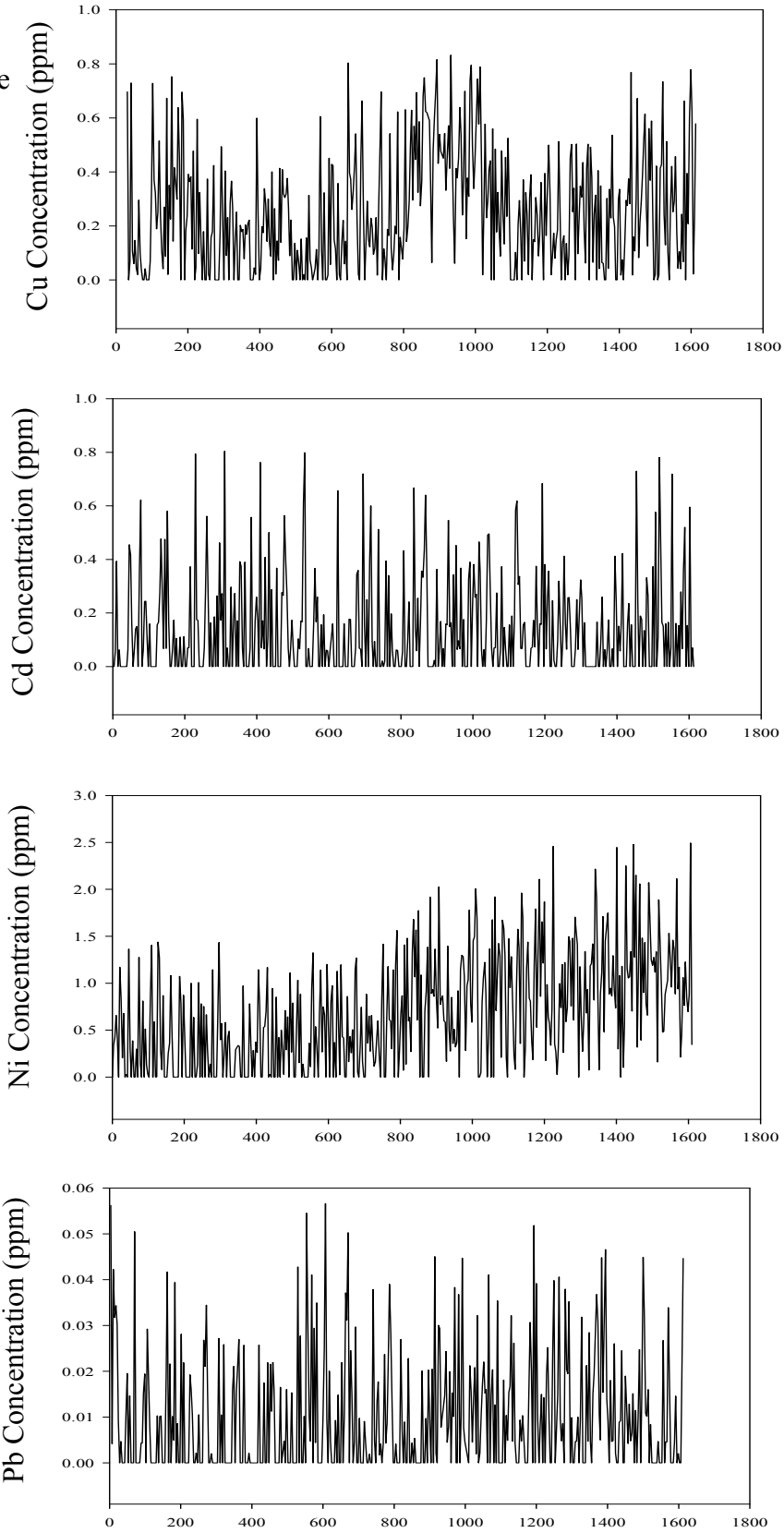
MD01 M04


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
160
140
120
100
80
60
40
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

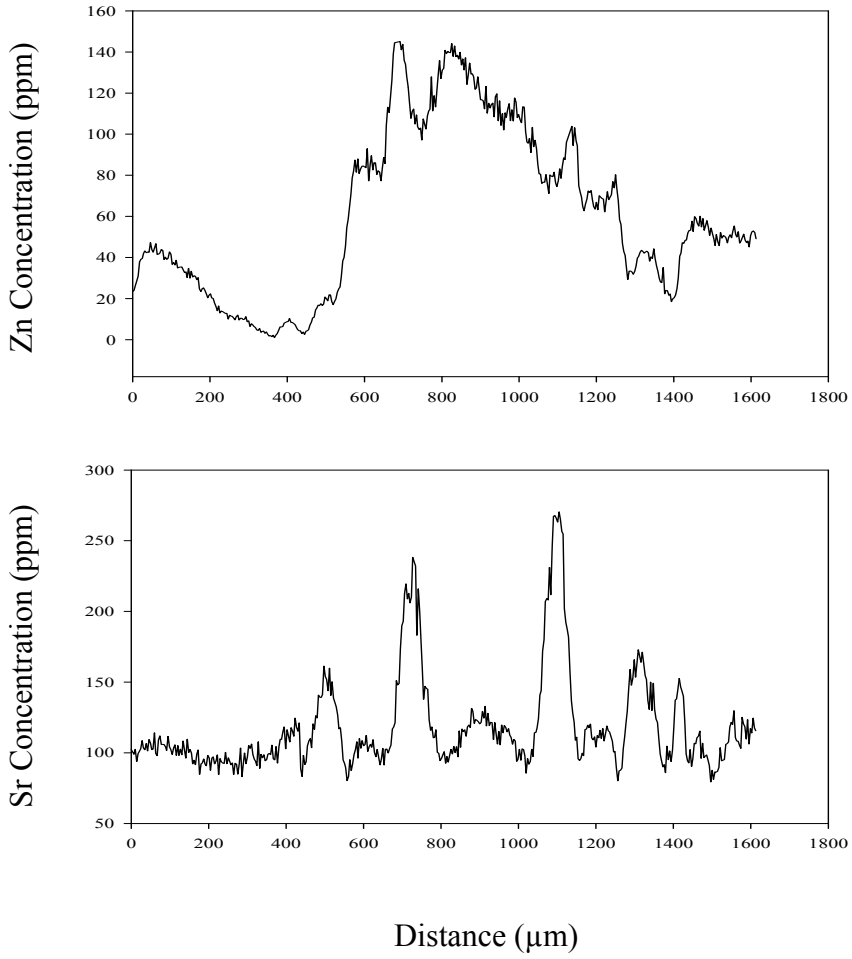
MD01 M05

Med dose

Non-crystalline



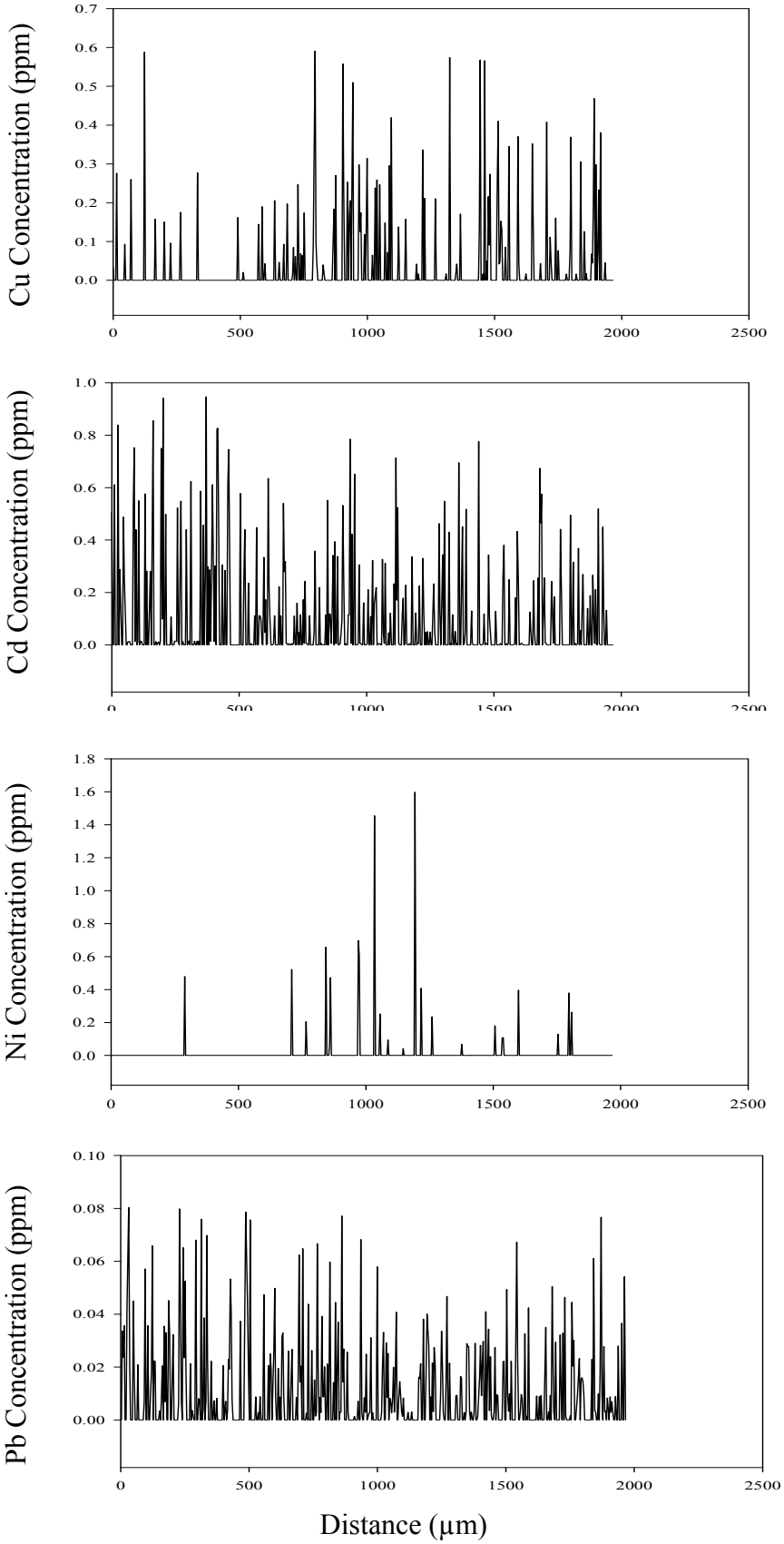
MD01 M05


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
300
250
200
150
100
50
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

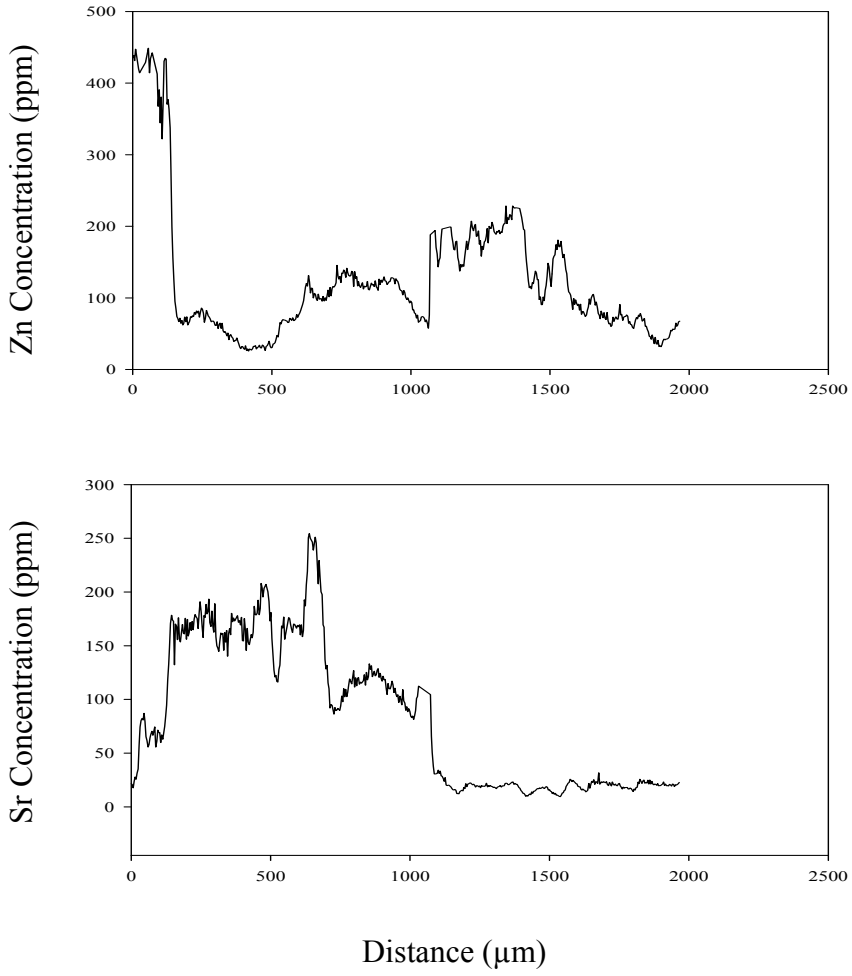
MD01 M07

Med dose

Crystalline



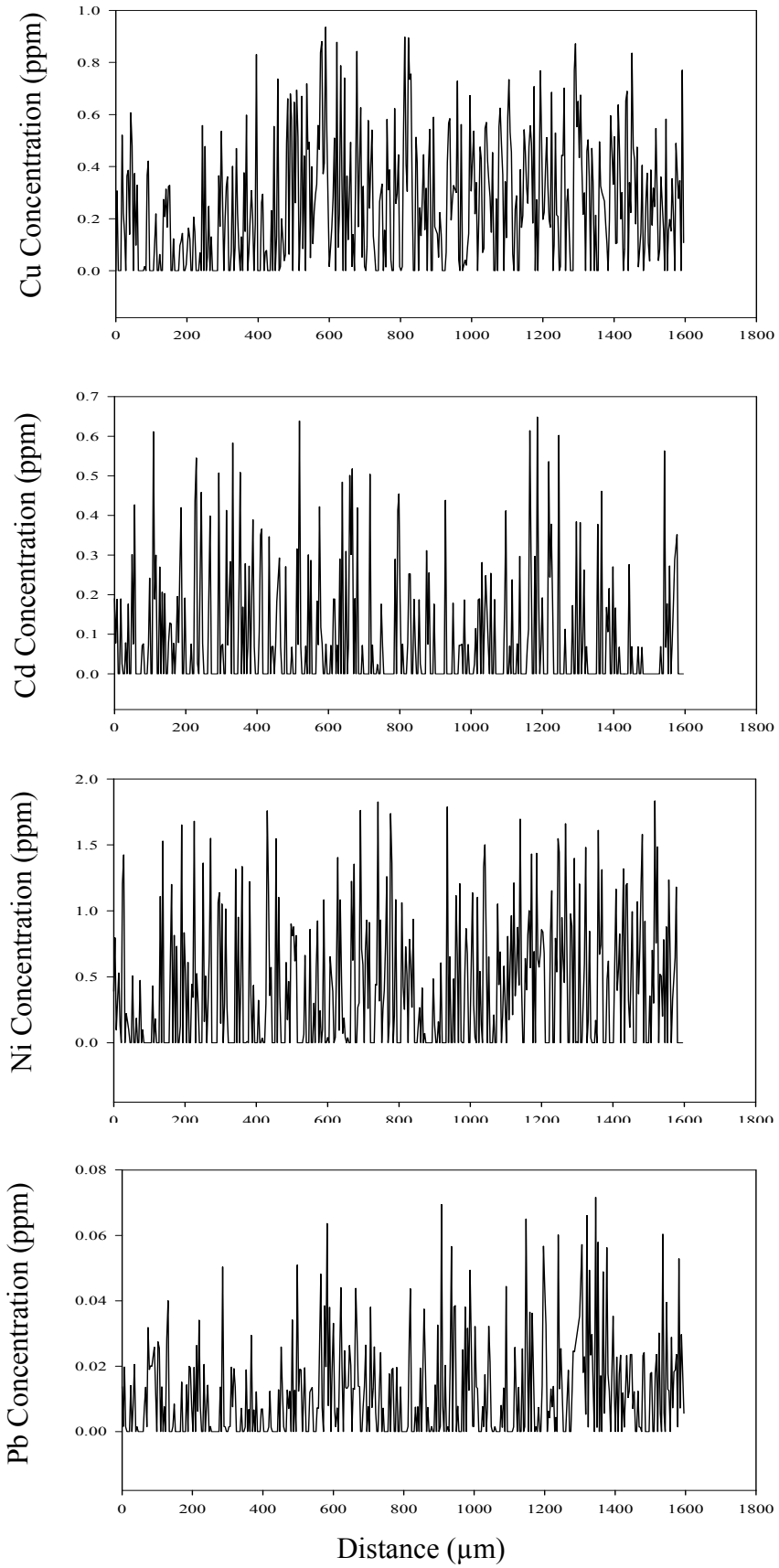
MD01 M07


Zn Concentration (ppm)
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Distance (μm)

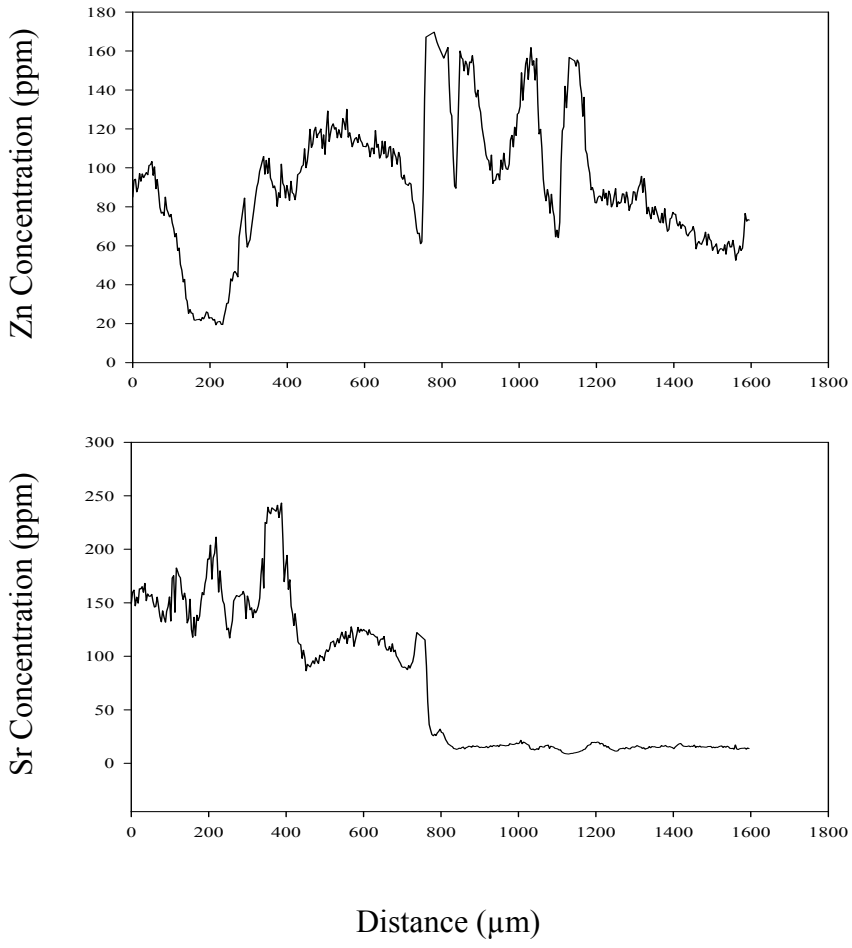
MD01 M08

Med dose

Crystalline



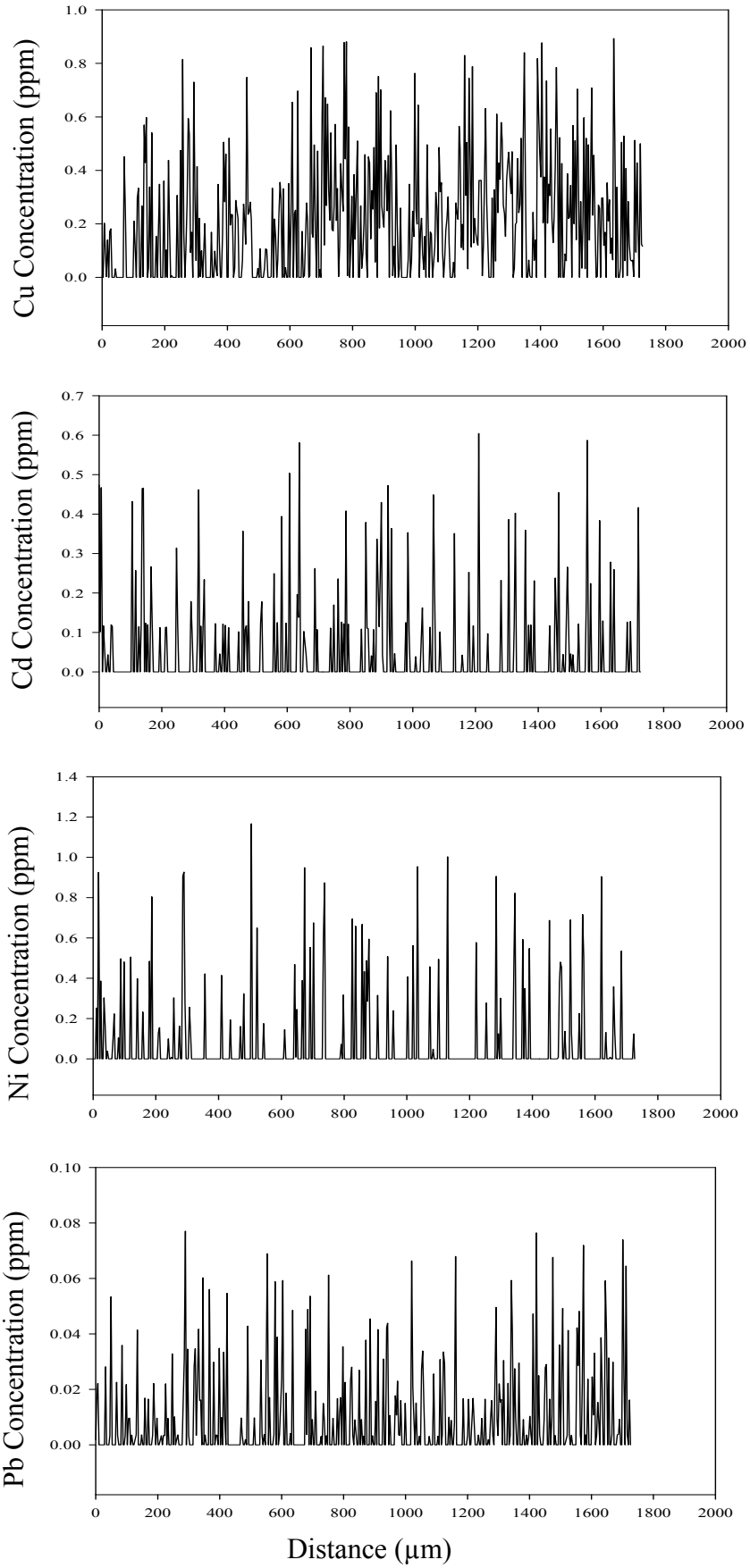
MD01 M08


Zn Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

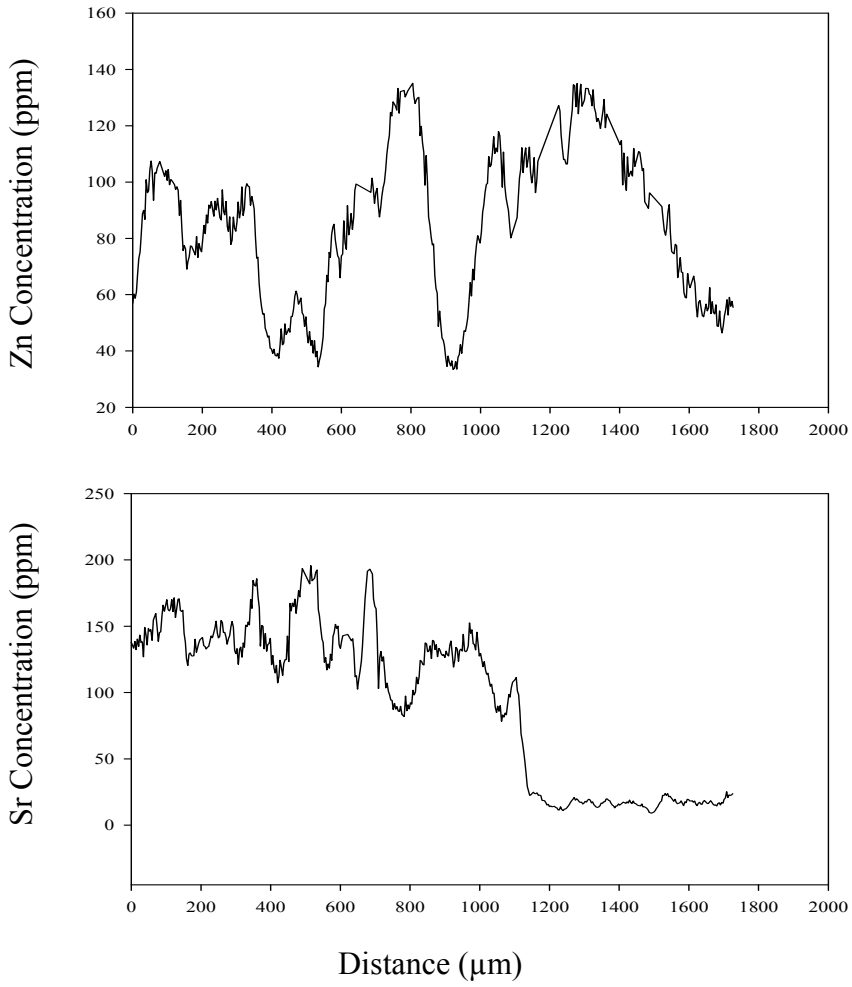
MD01 M11

Med dose

Partially crystalline



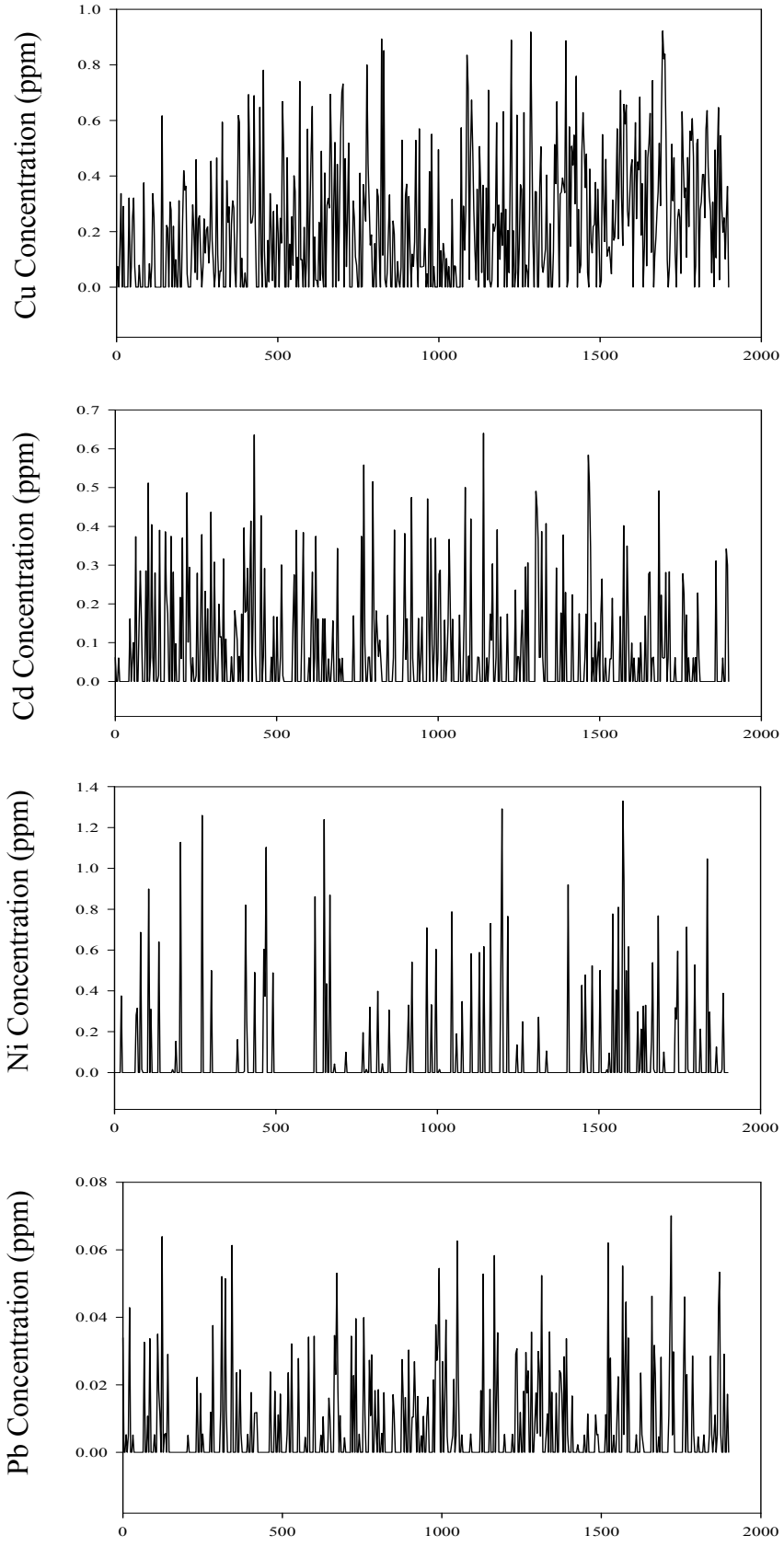
MD01 M11


Zn Concentration (ppm)
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
250
200
150
100
50
0
Distance (μm)

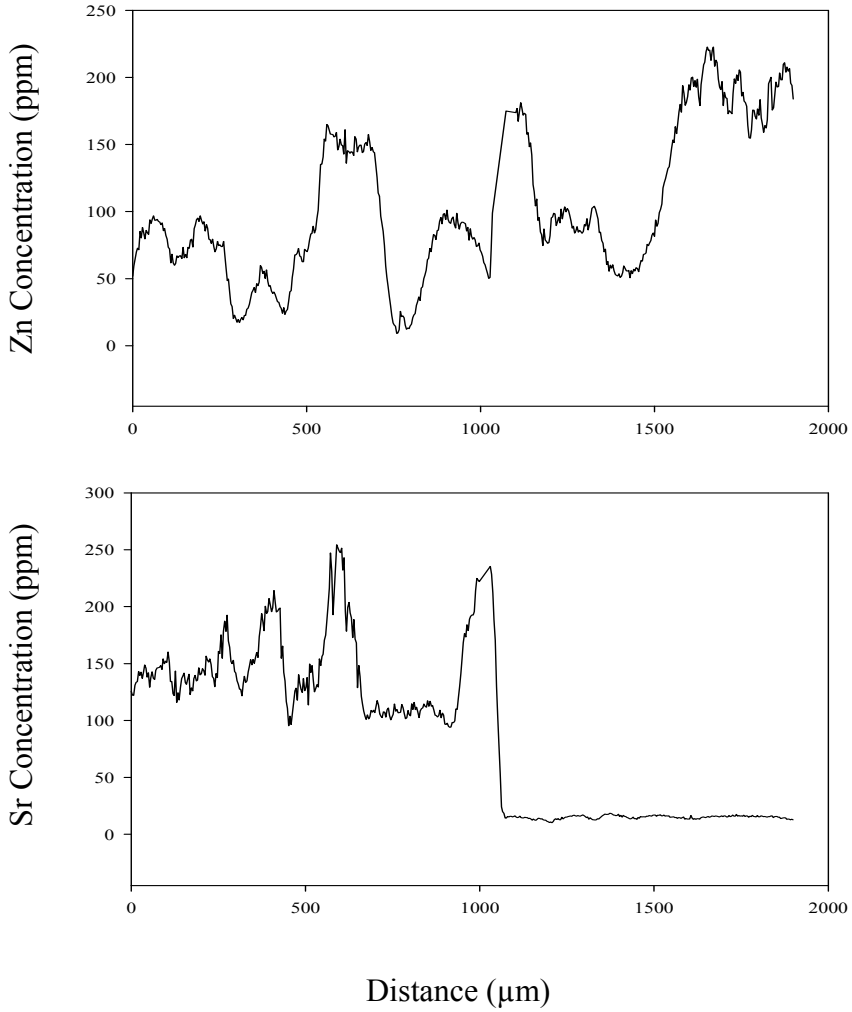
MD01 M13

Med dose

Crystalline



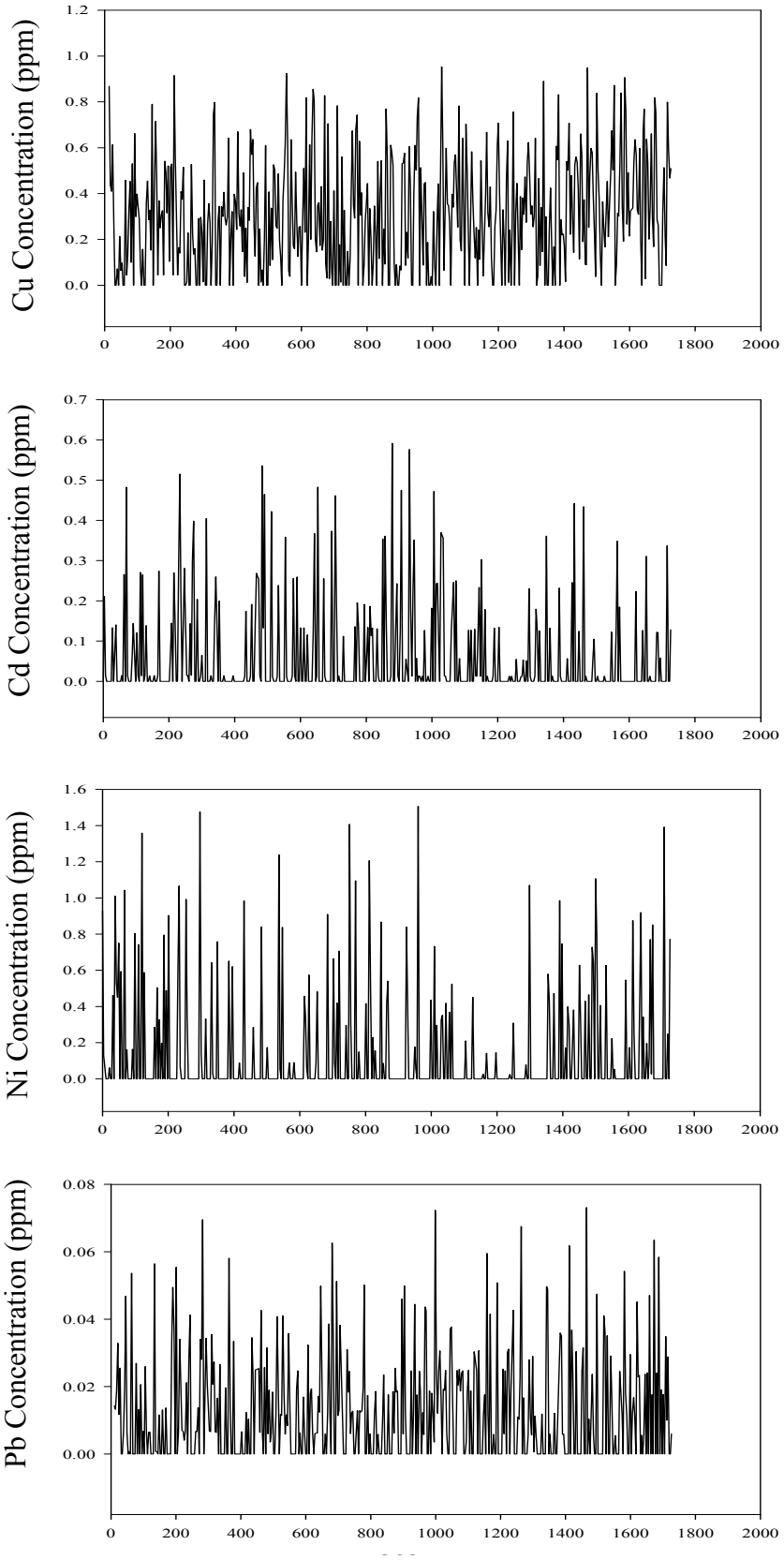
MD01 M13


Zn Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Sr Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
Distance (μm)

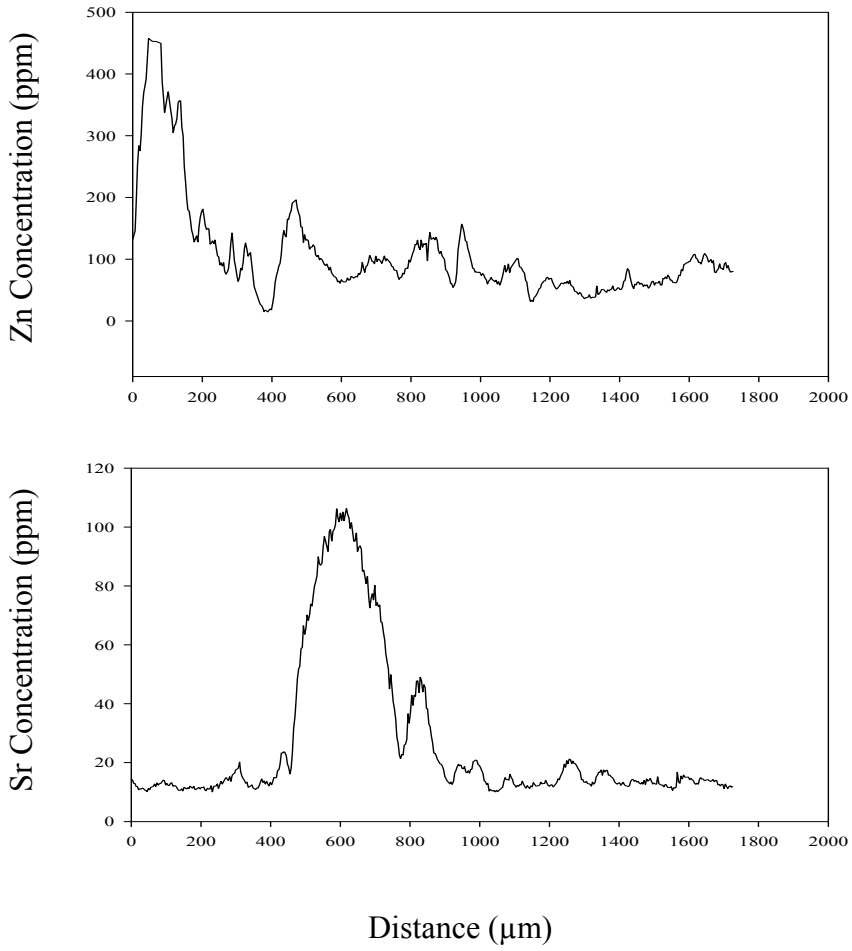
MD01 M15

Med dose

Crystalline



MD01 M15

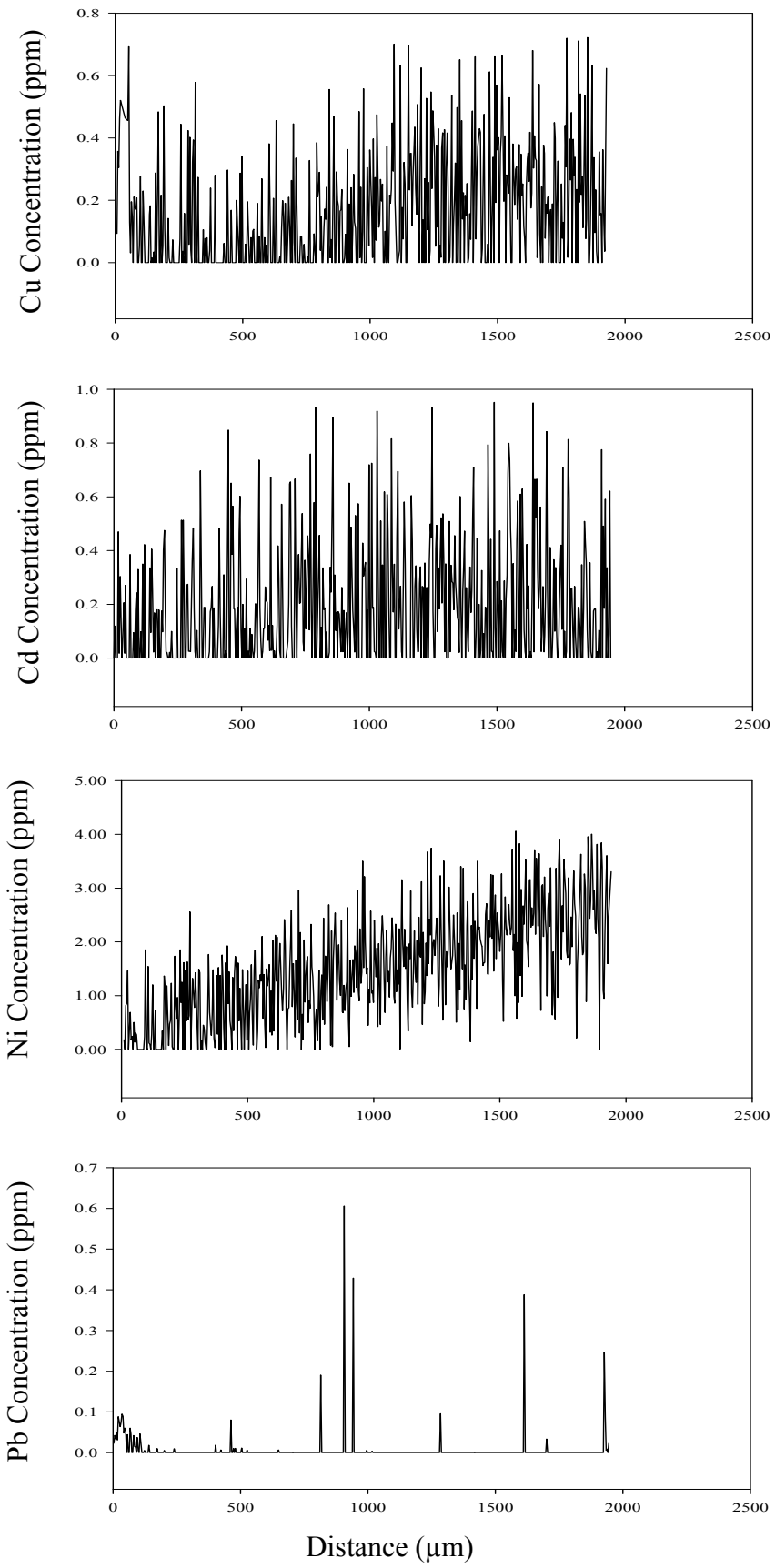

Zn Concentration (ppm)
500
400
300
200
100
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

IV.IV High Dose Group

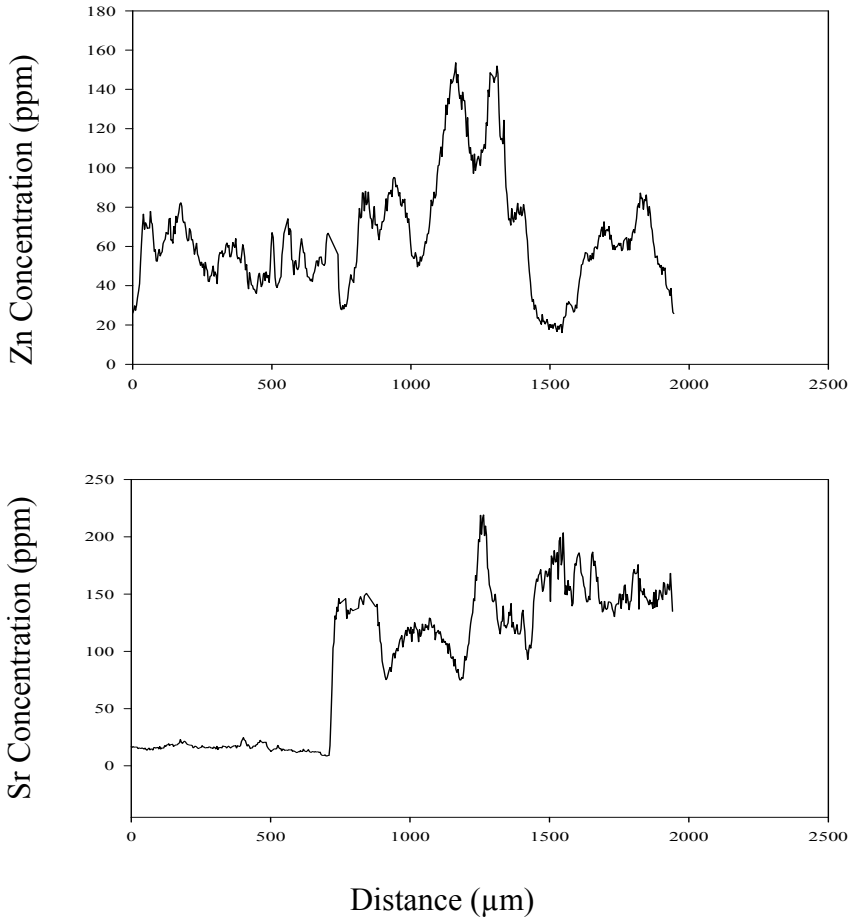
H01 H01

High dose

Crystalline



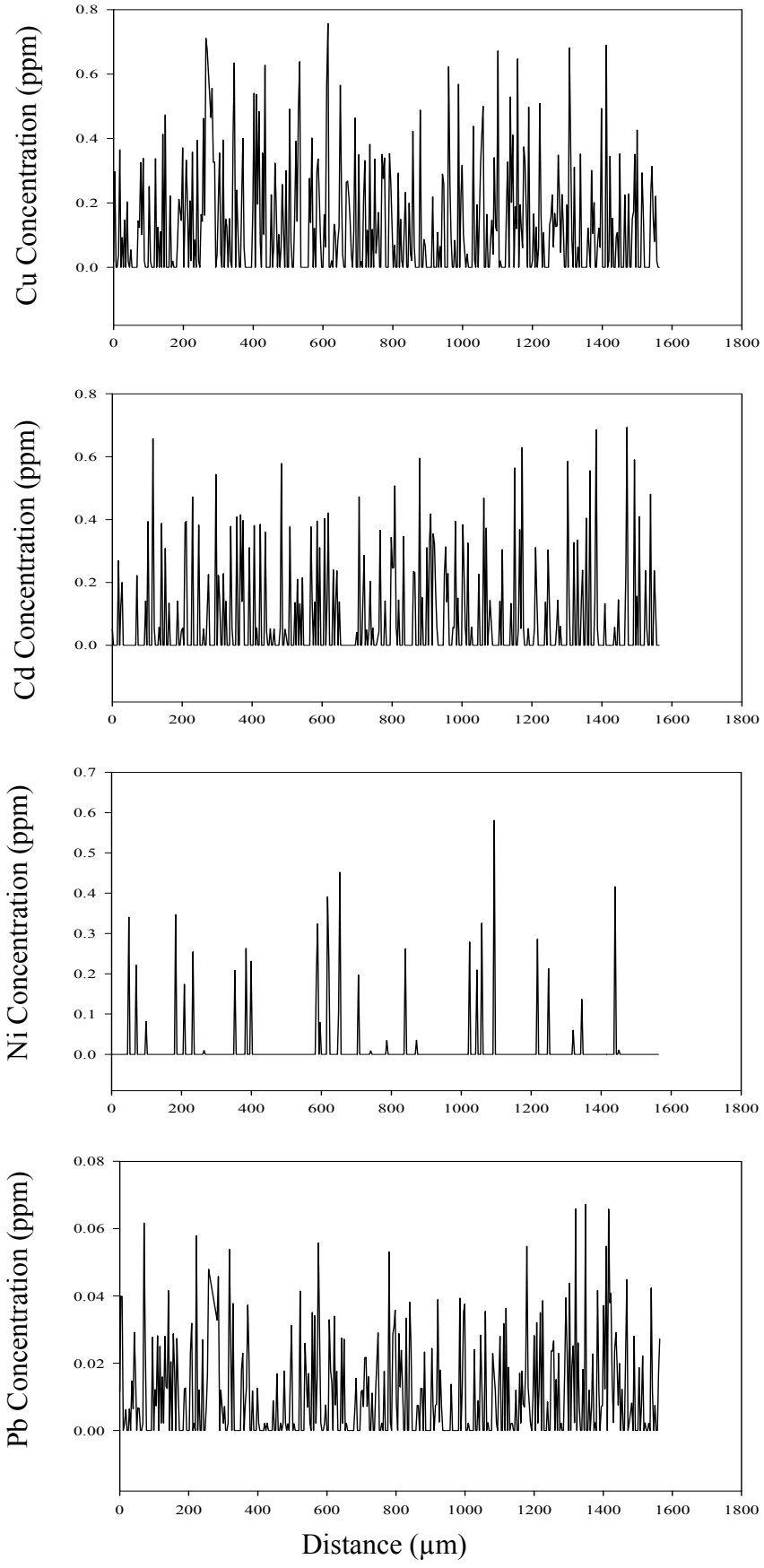
H01H01


Zn Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Distance (μm)

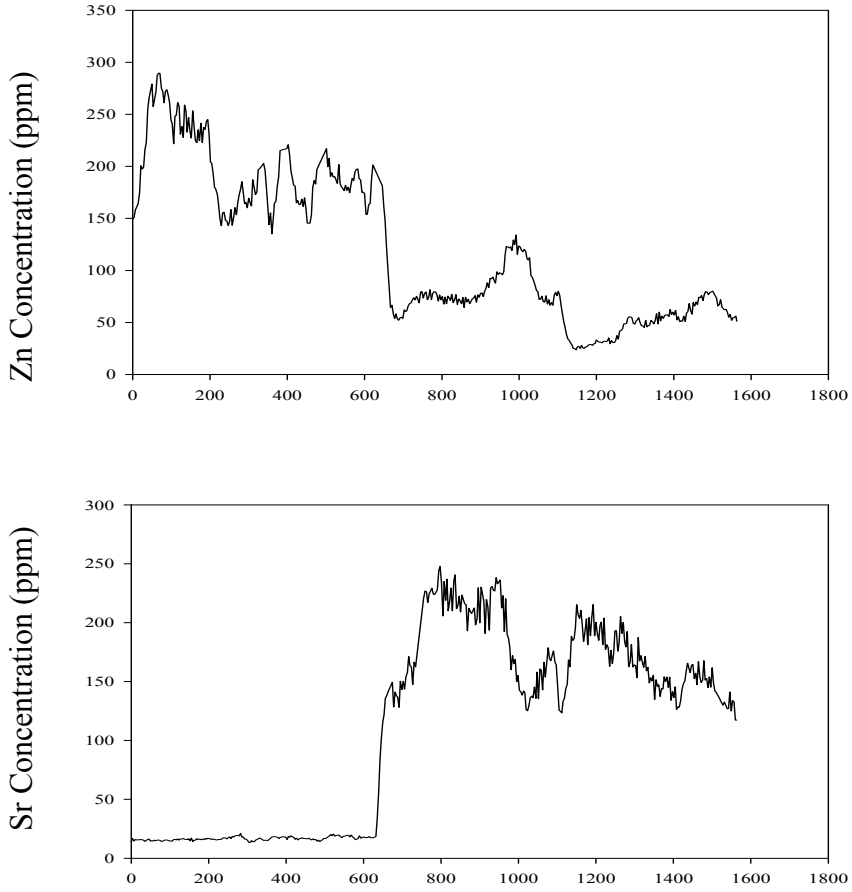
H01 H02

High dose

Crystalline



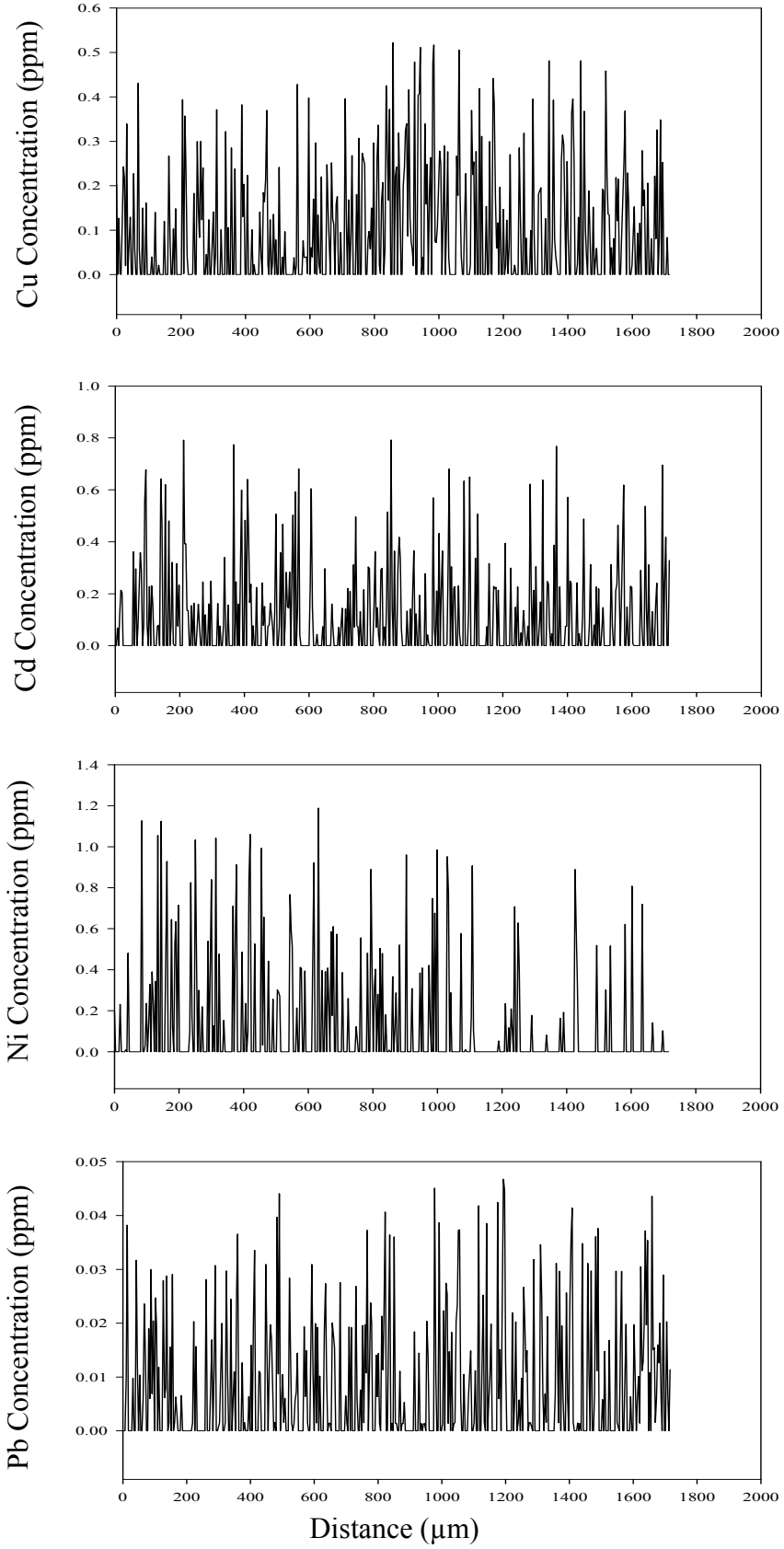
H01 H02



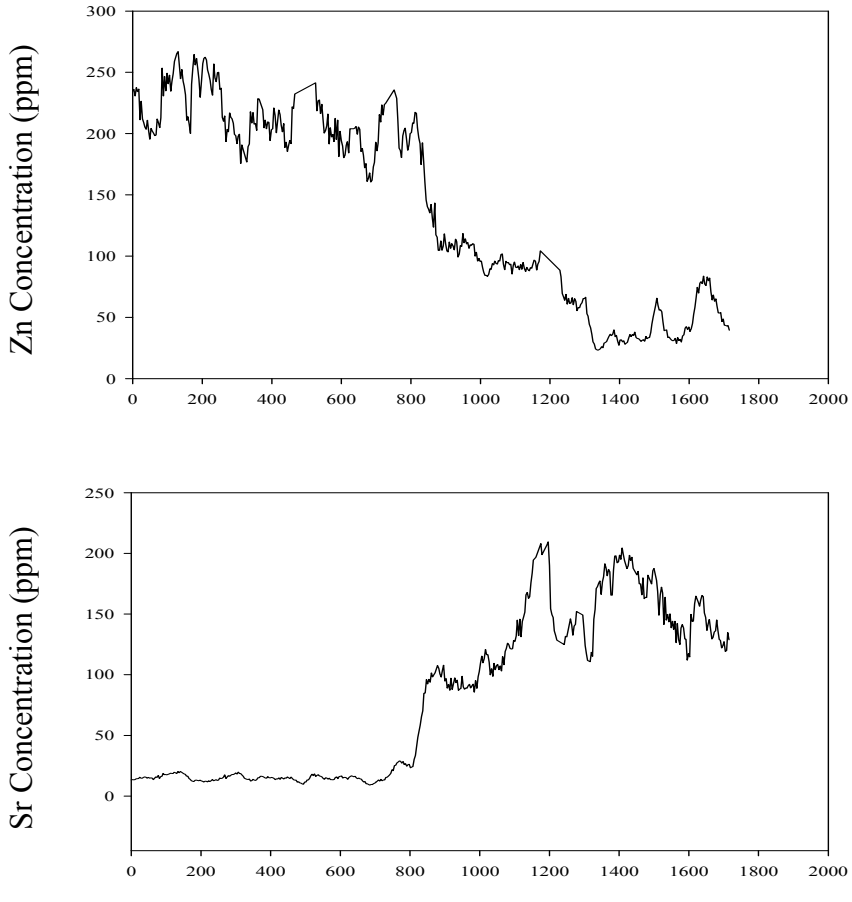
H01 H03

High dose

Crystalline



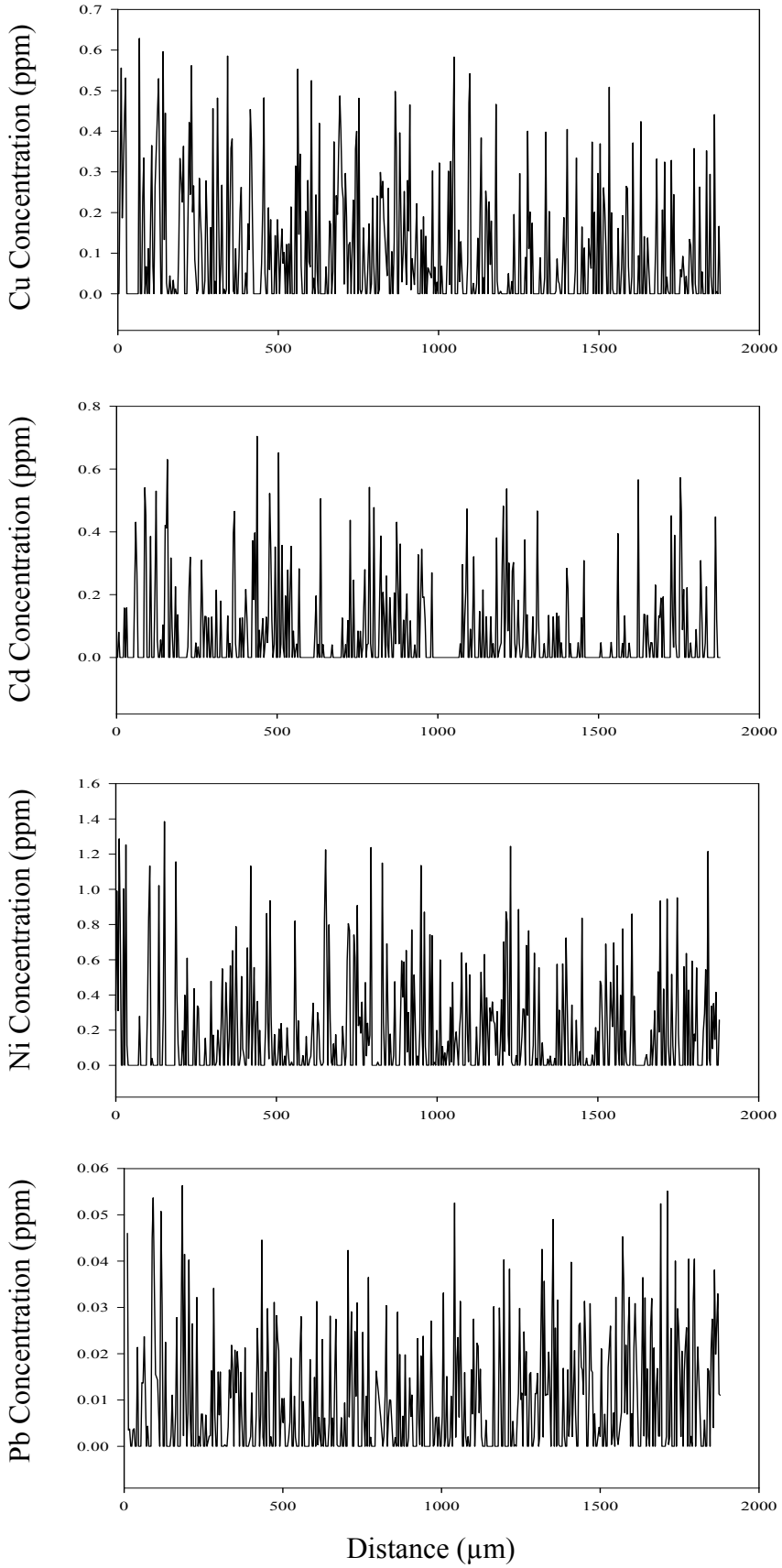
H01 H03


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Distance (μm)

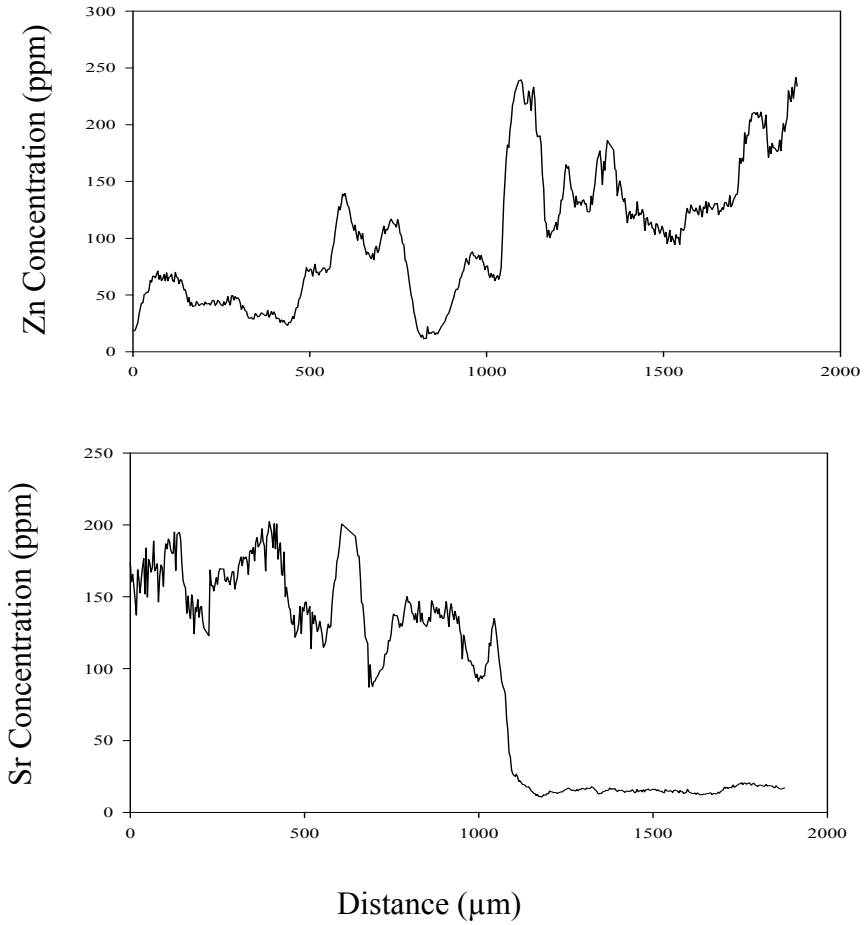
TL05-H04

High dose

Crystalline



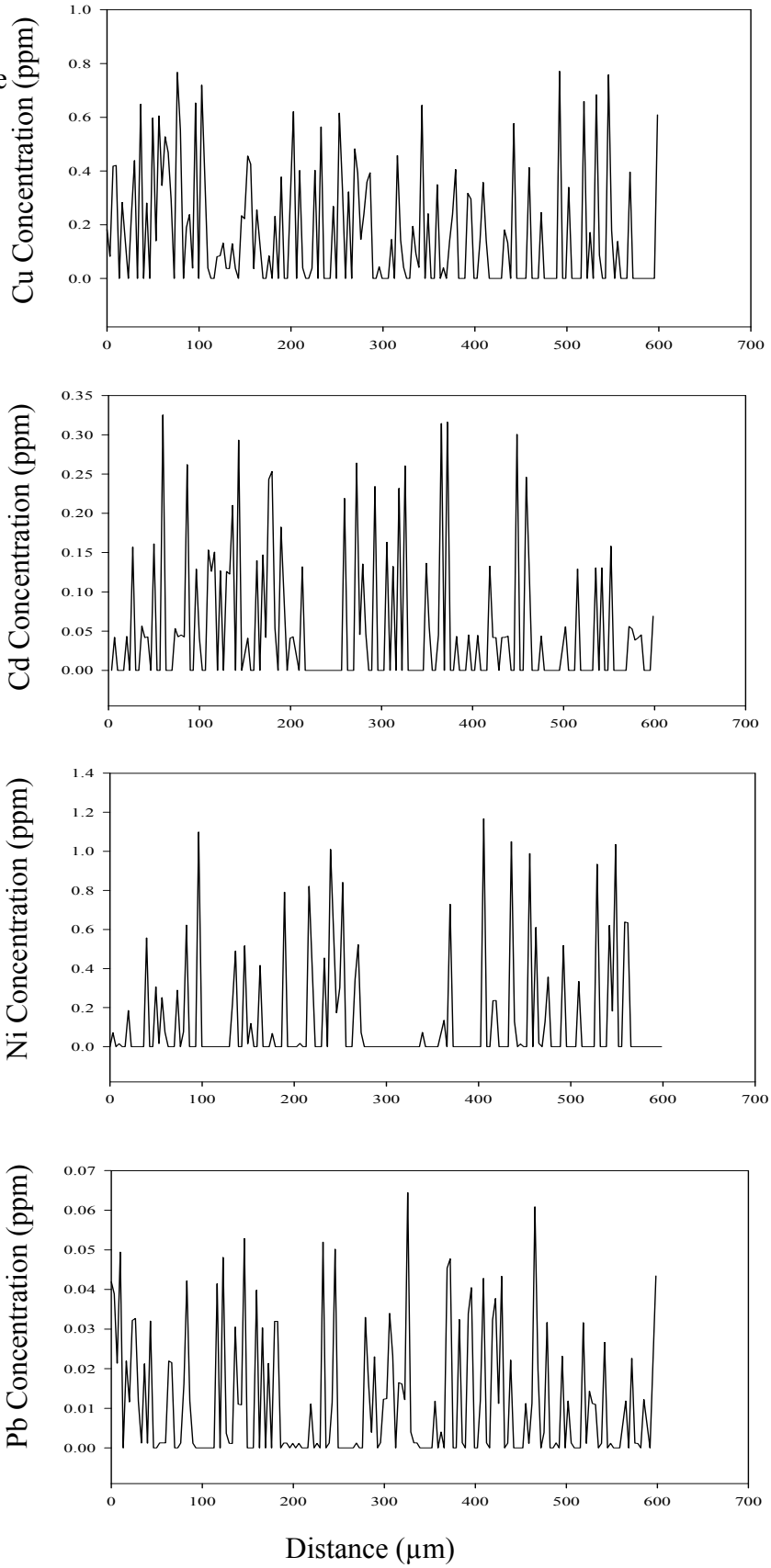
TL05 H04


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Distance (μm)

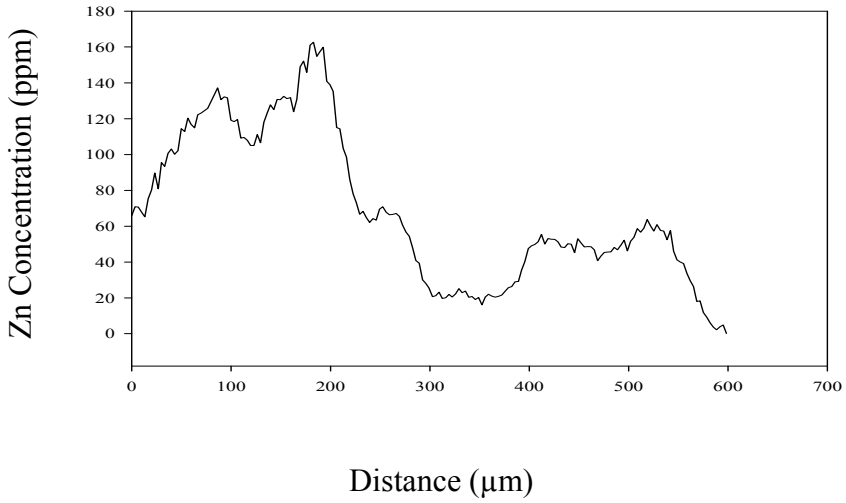
FD01-H05

High dose

Non-crystalline



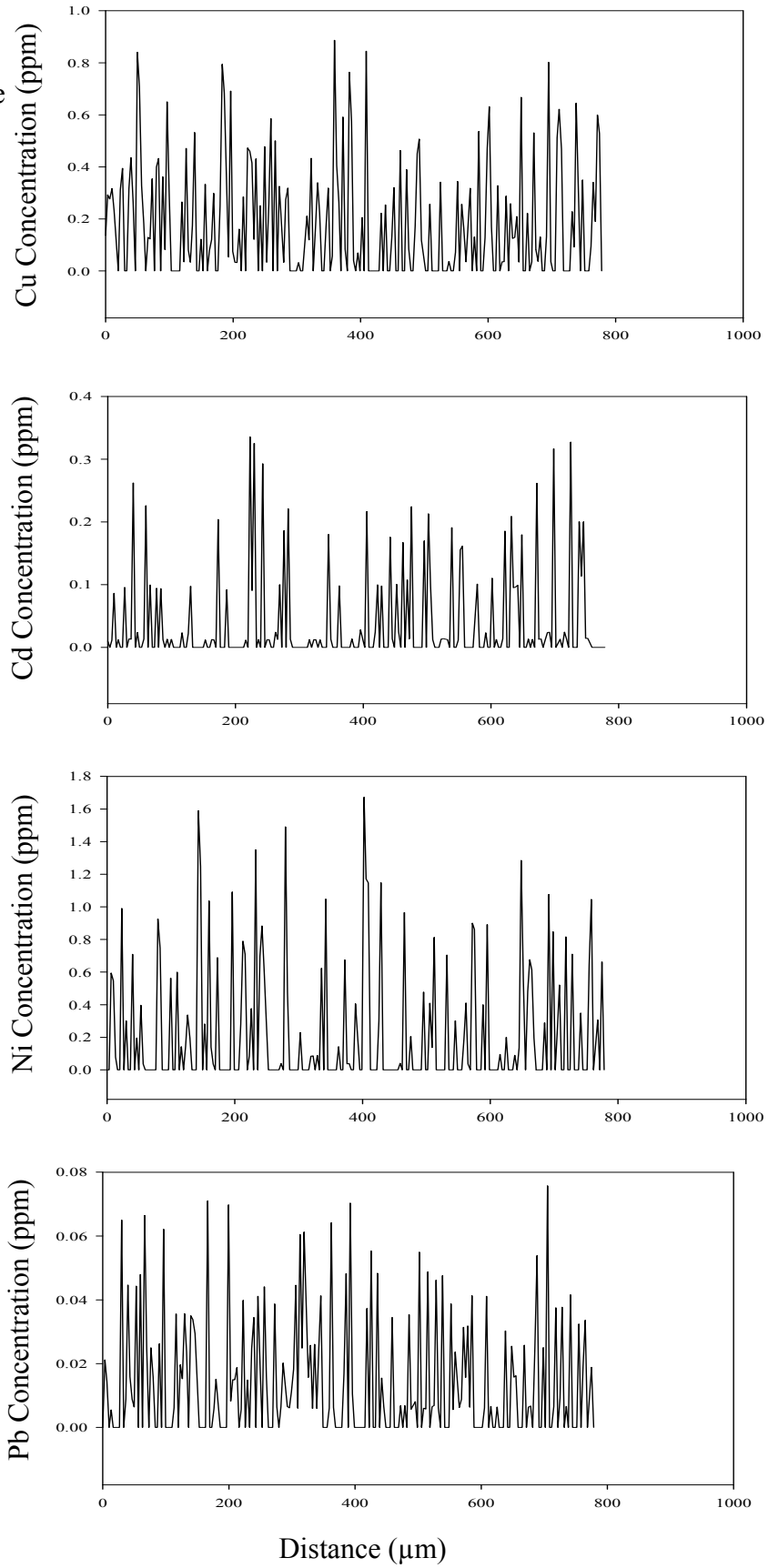
FD01-H05


Zn Concentration (ppm)
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Distance (μm)

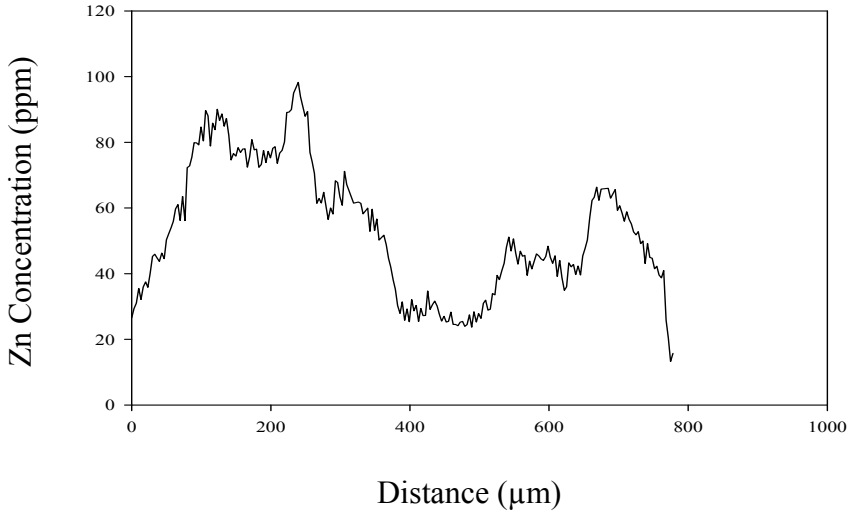
FD01-H06

High dose

Non-crystalline



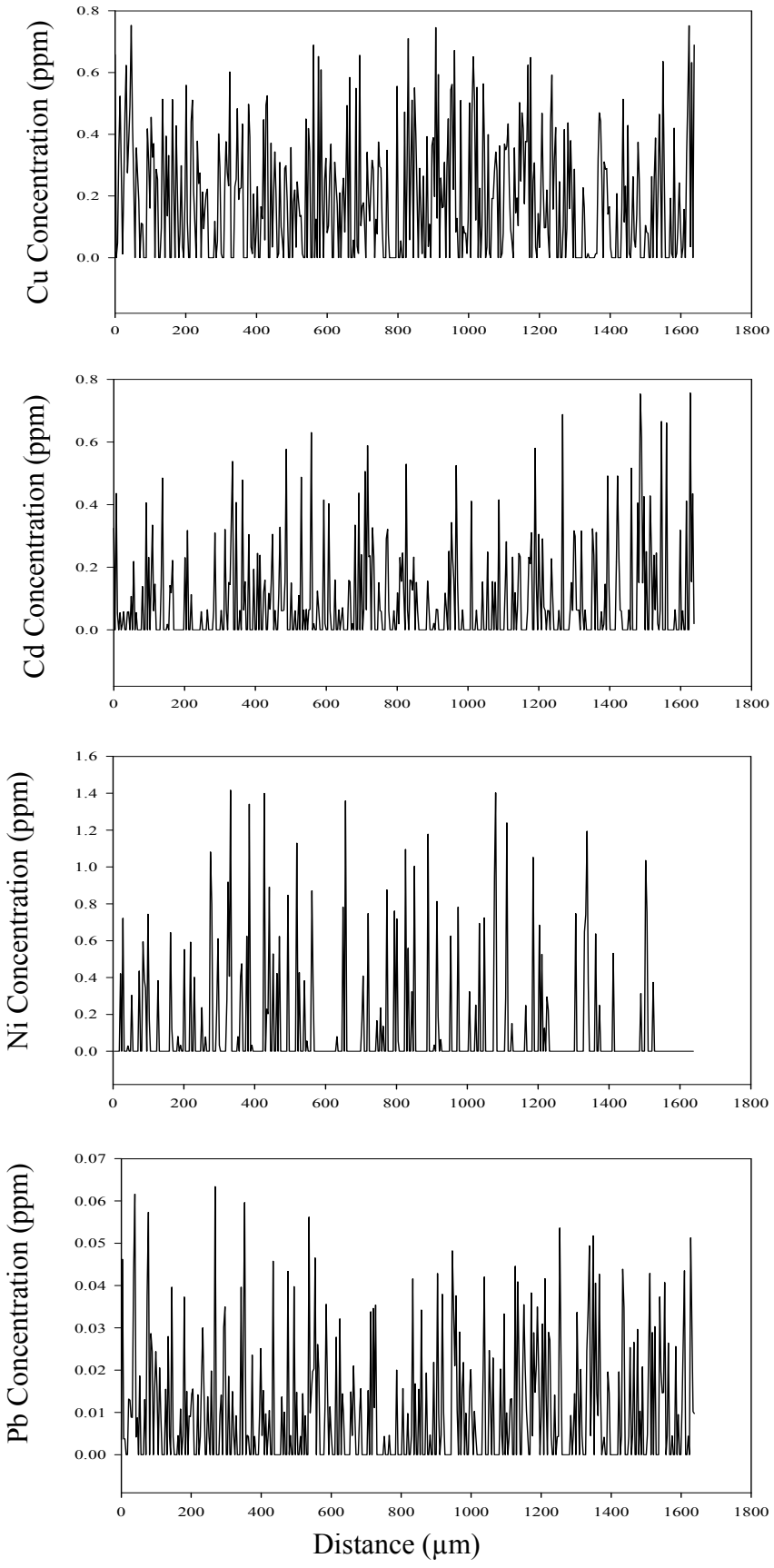
FD01-H06


Zn Concentration (ppm)
120
100
80
60
40
20
0
0
200
400
600
800
1000
Distance (μm)

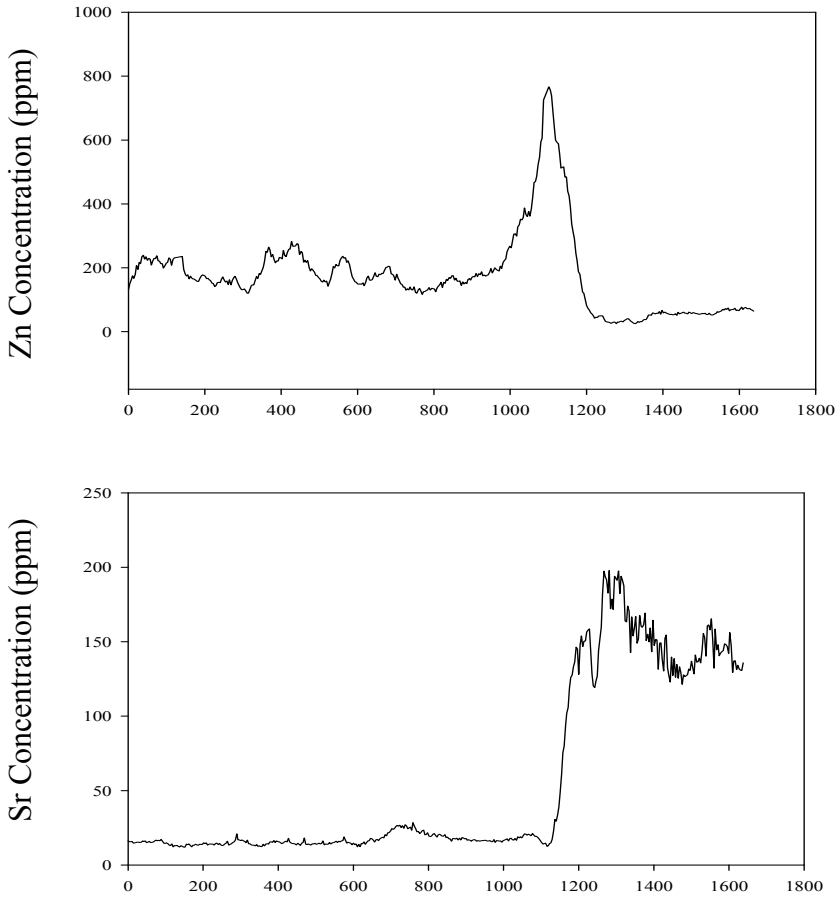
H01 H10

High dose

Crystalline



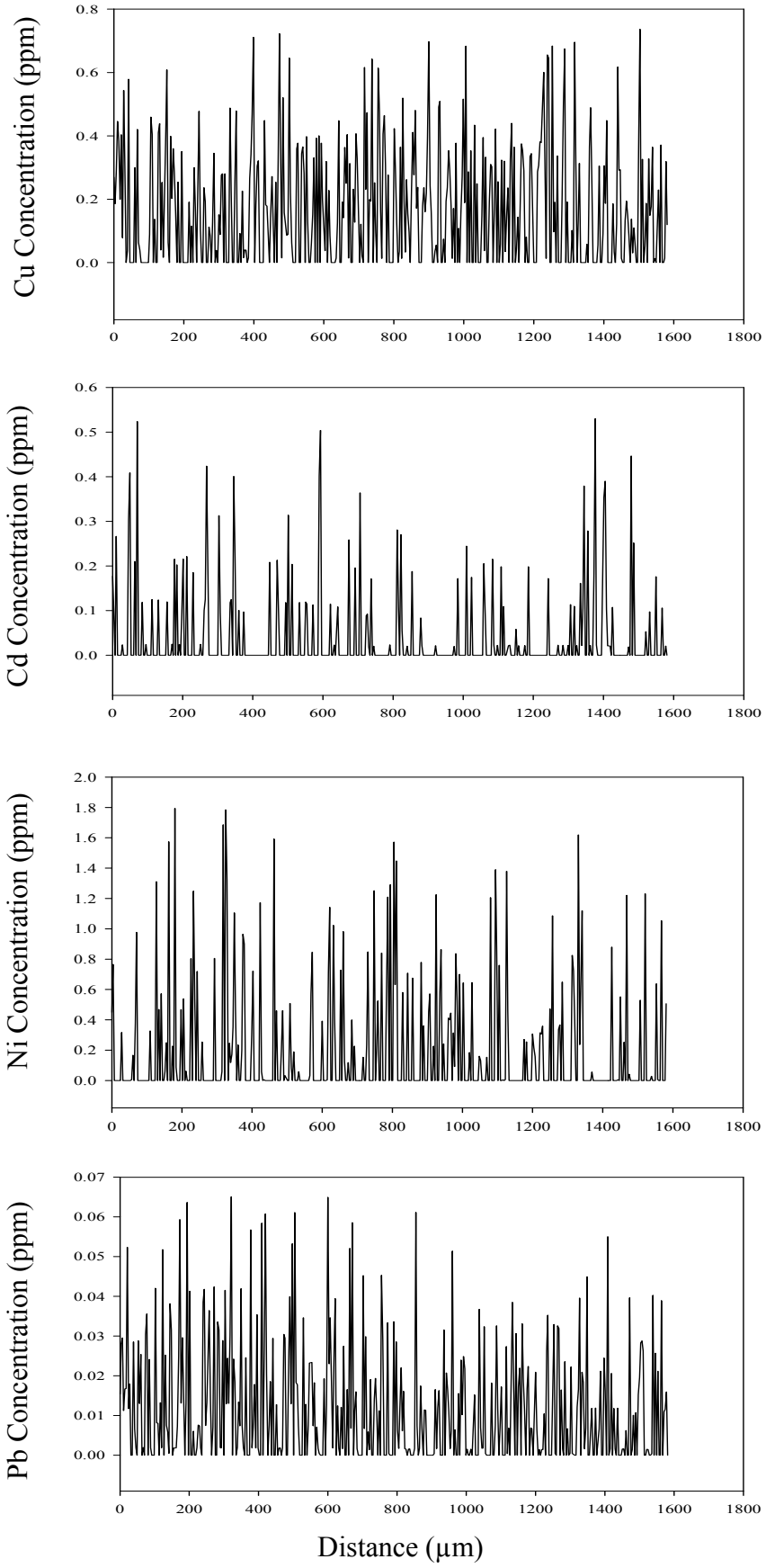
H01 H10


Zn Concentration (ppm)
1000
800
600
400
200
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (μm)

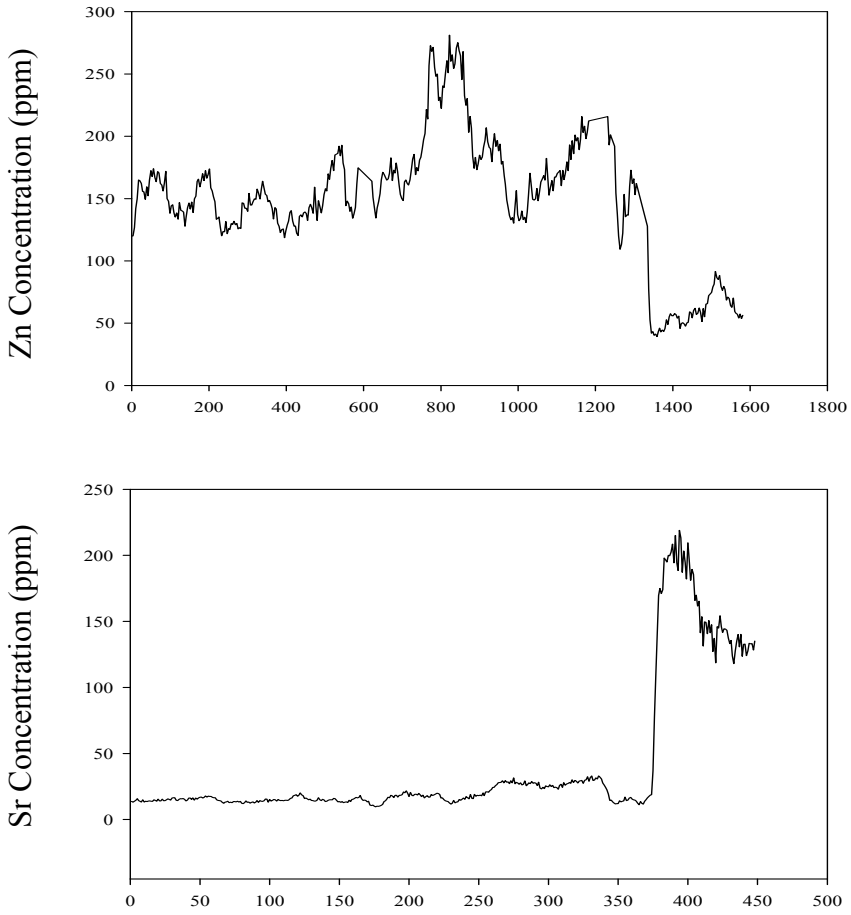
H01 H11

High dose

Crystalline



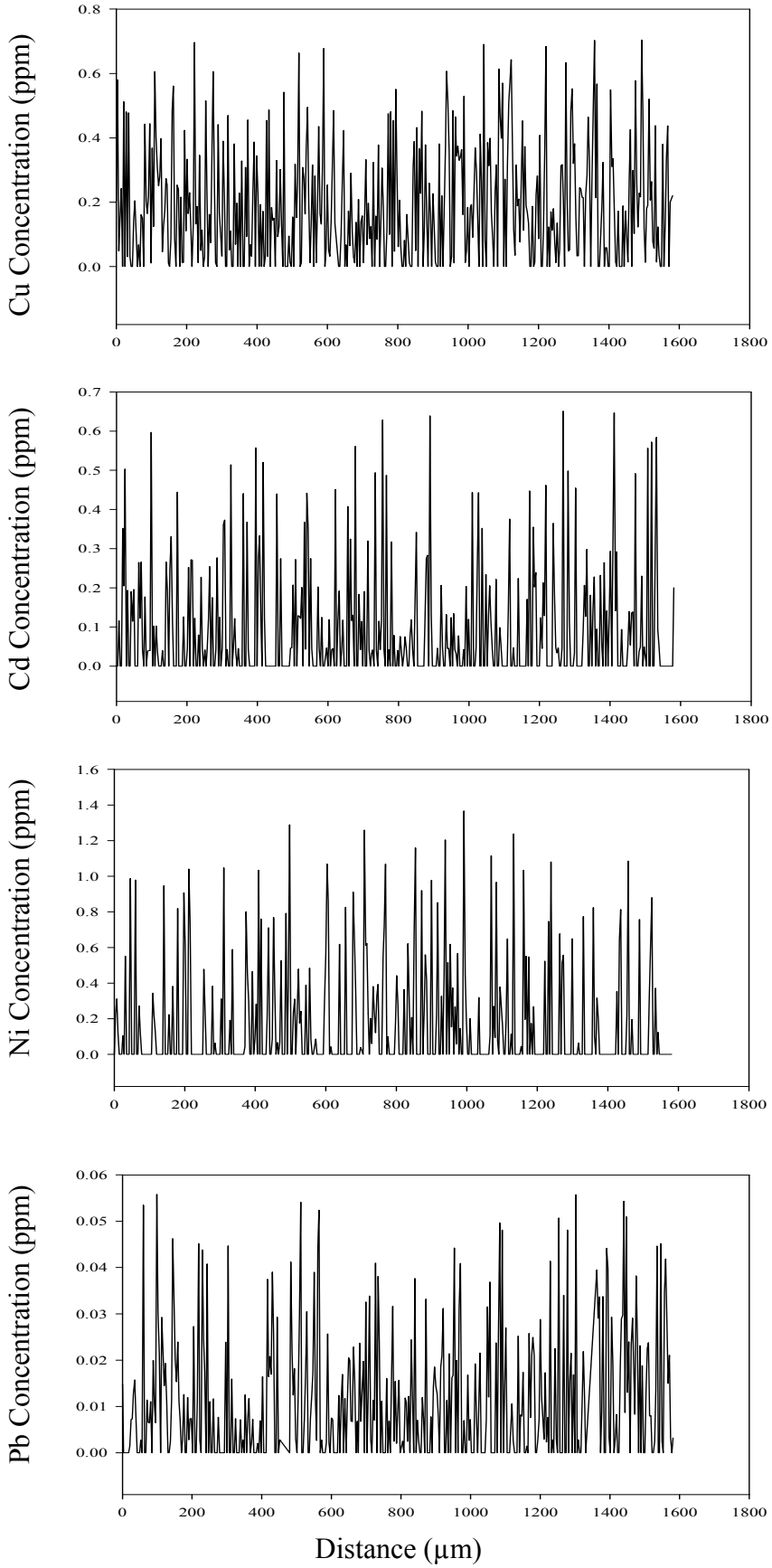
H01 H11


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
450
500
Distance (μm)

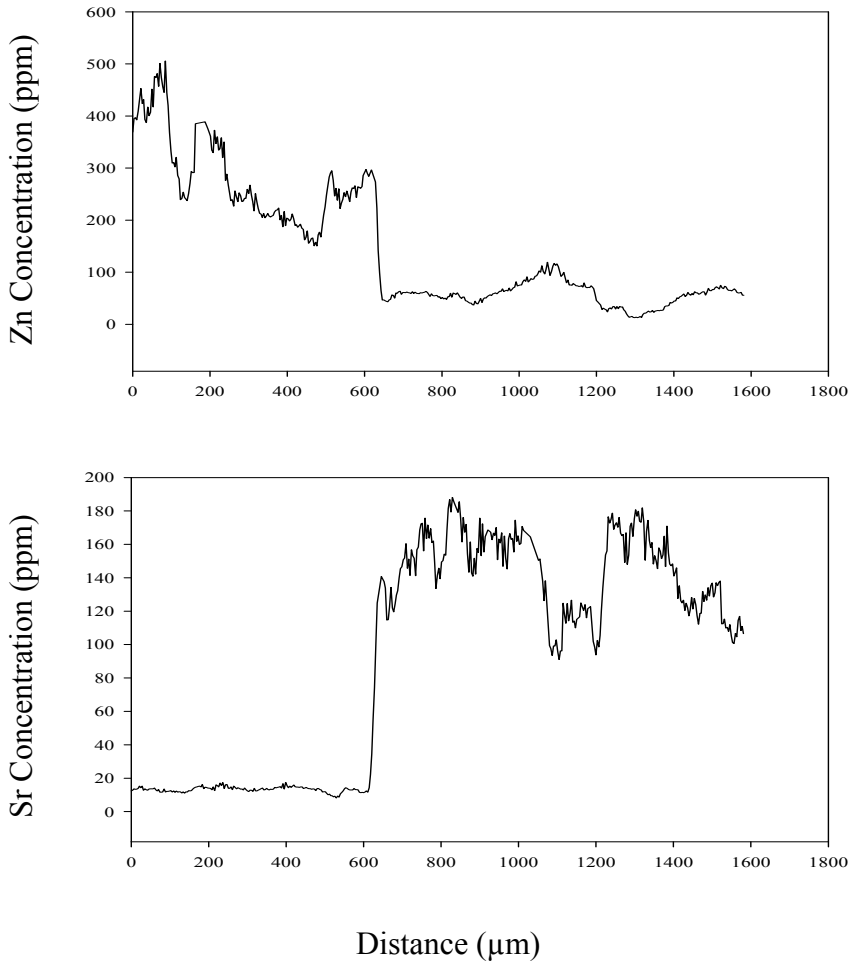
H01 H12

High dose

Crystalline



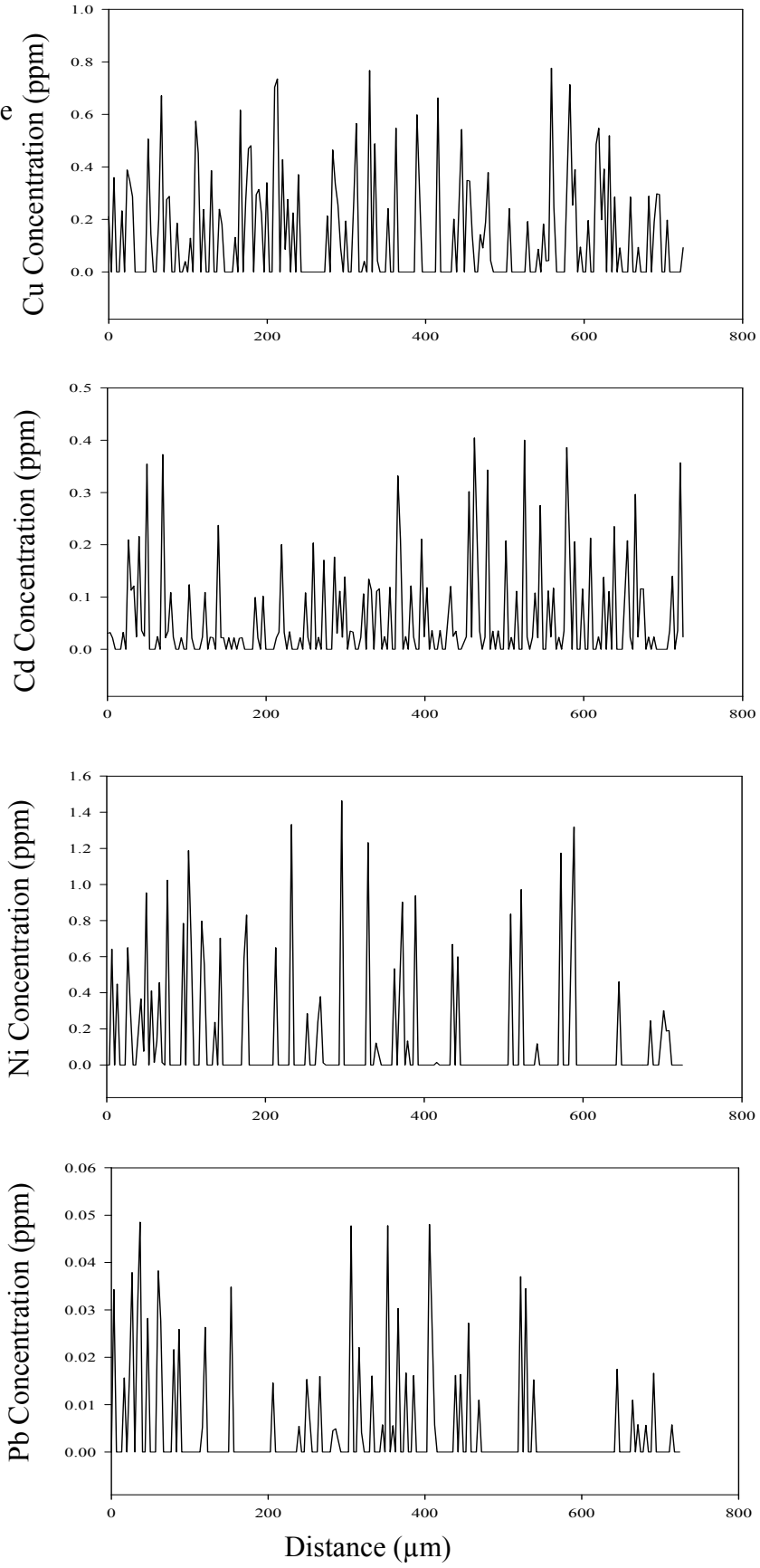
H01 H12


Zn Concentration (ppm)
600
500
400
300
200
100
0
0
200
400
600
800
1000
1200
1400
1600
1800
Sr Concentration (ppm)
200
180
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
Distance (µm)

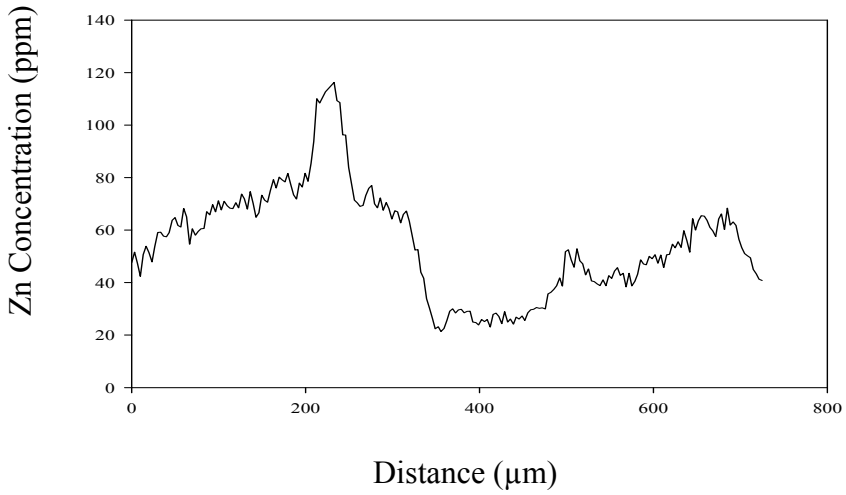
FD01-H13

High dose

Non-crystalline



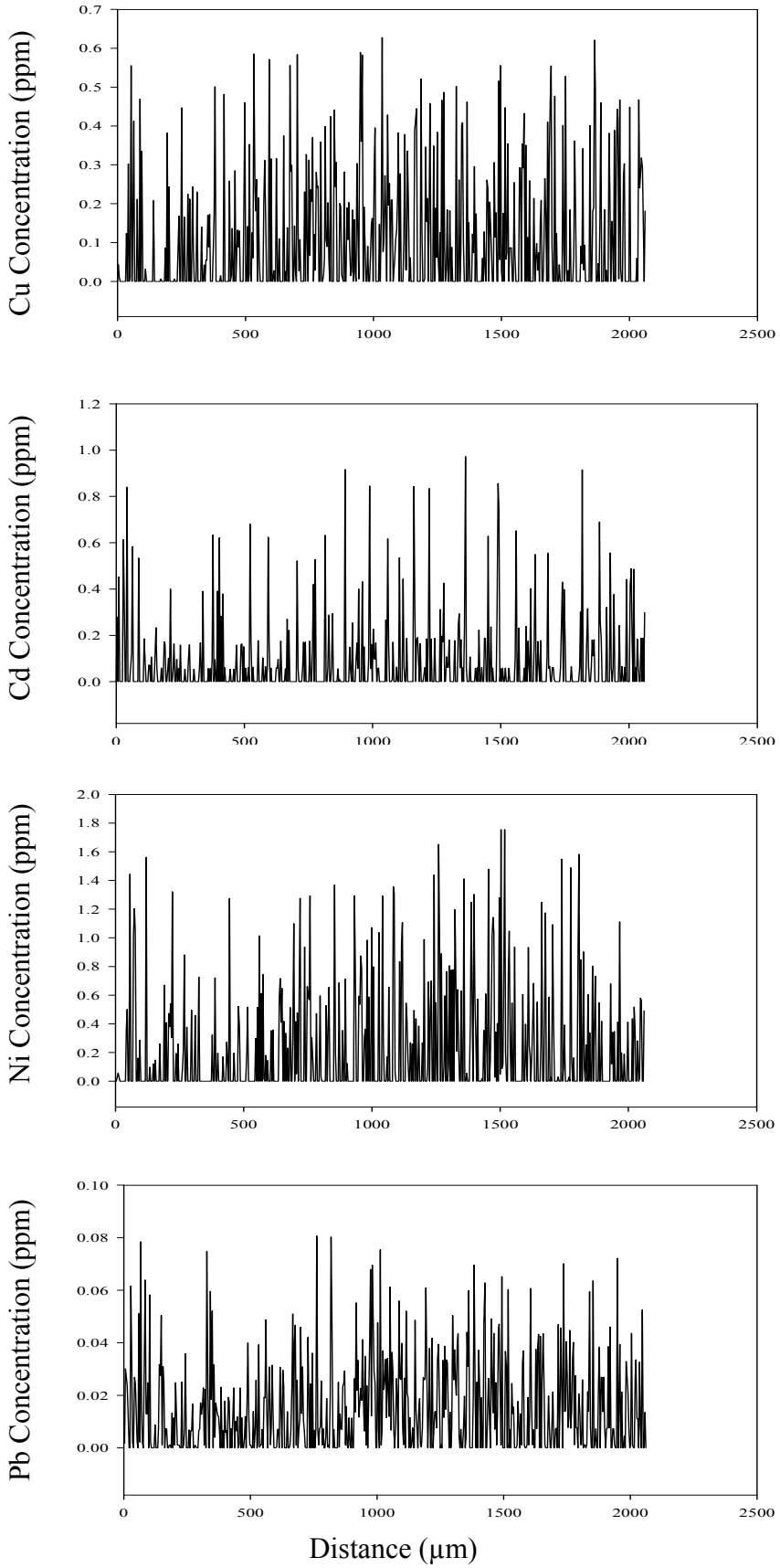
FD01-H13


Zn Concentration (ppm)
140
120
100
80
60
40
20
0
0
200
400
600
800
Distance (μm)

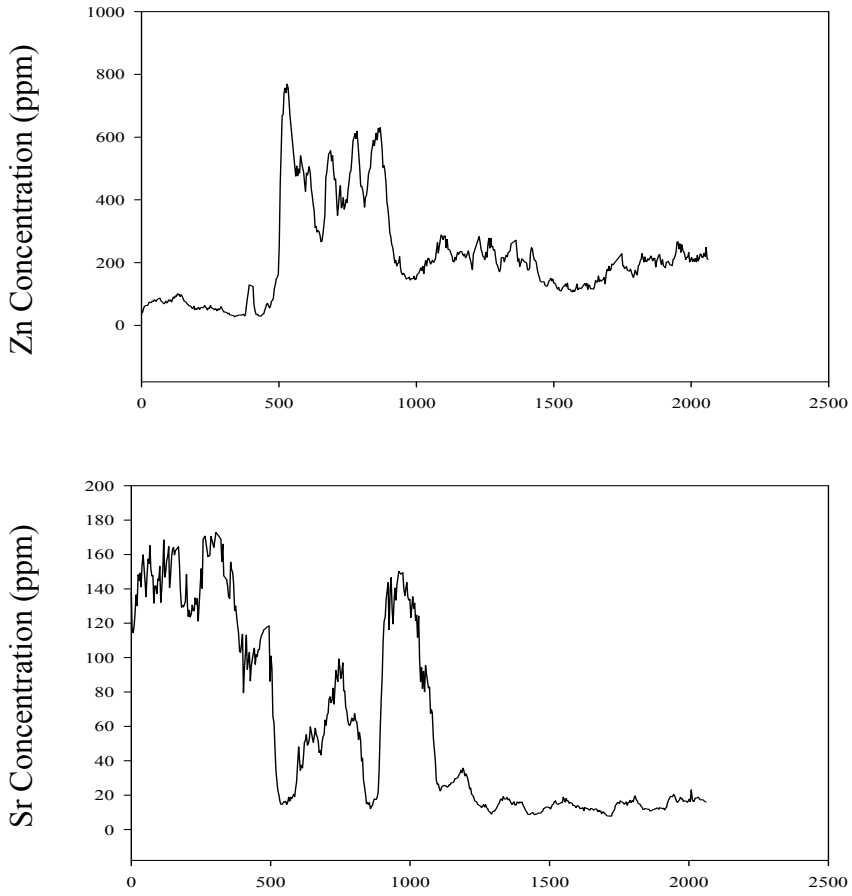
H02 H15

High dose

Crystalline



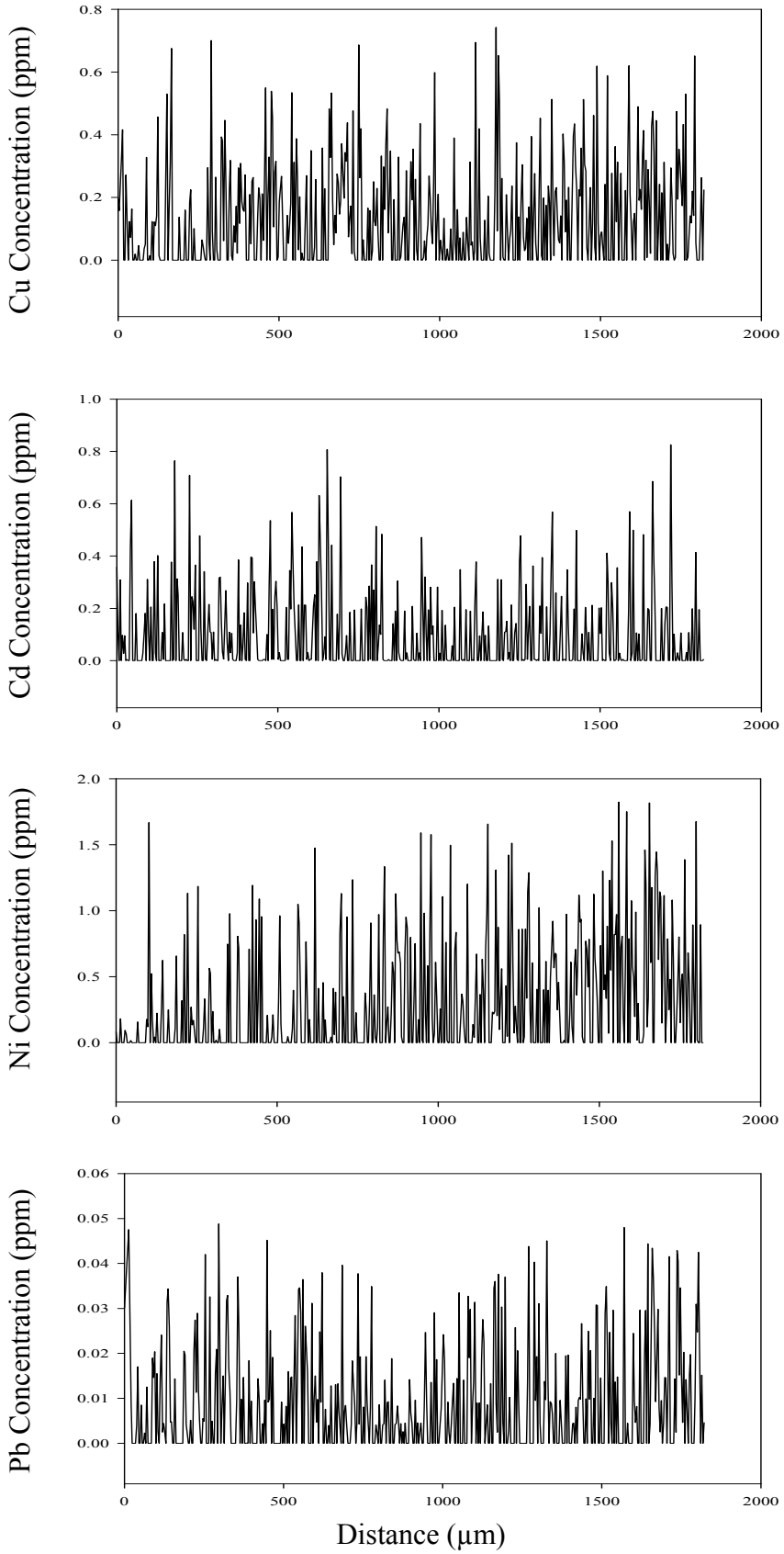
H02 H15


Zn Concentration (ppm)
1000
800
600
400
200
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
200
180
160
140
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
Distance (µm)

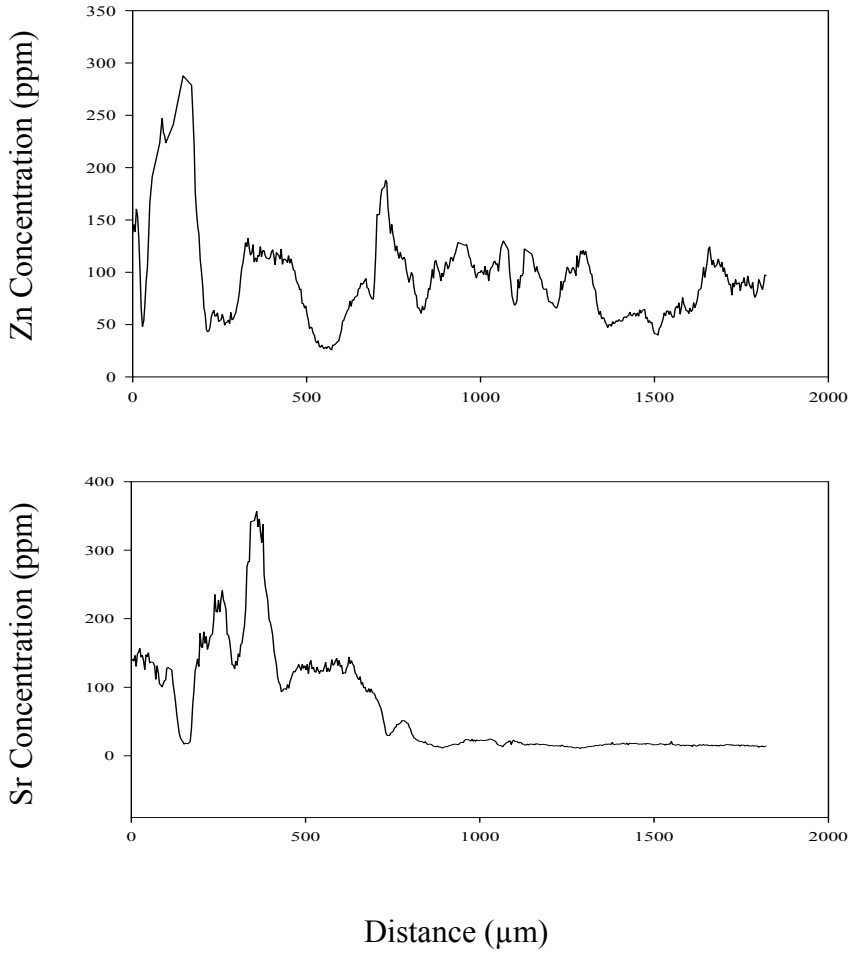
H02 H16

High dose

Crystalline



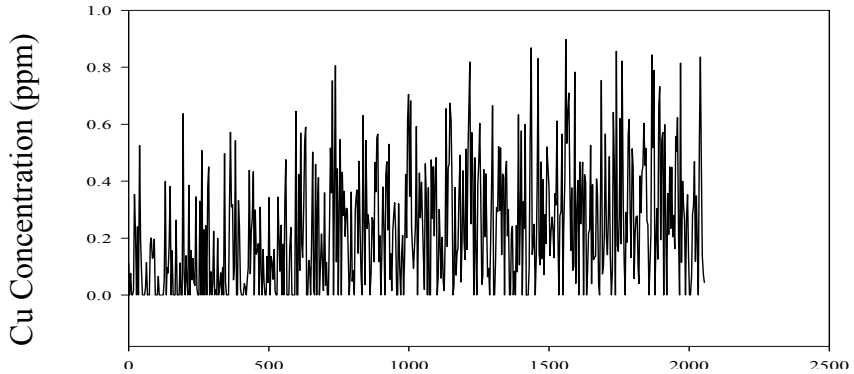
H02 H16

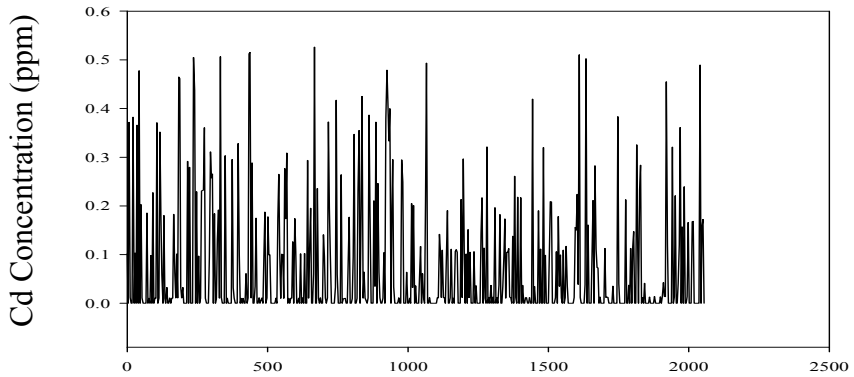

Zn Concentration (ppm)
350
300
250
200
150
100
50
0
0
500
1000
1500
2000
Sr Concentration (ppm)
400
300
200
100
0
0
500
1000
1500
2000
Distance (μm)

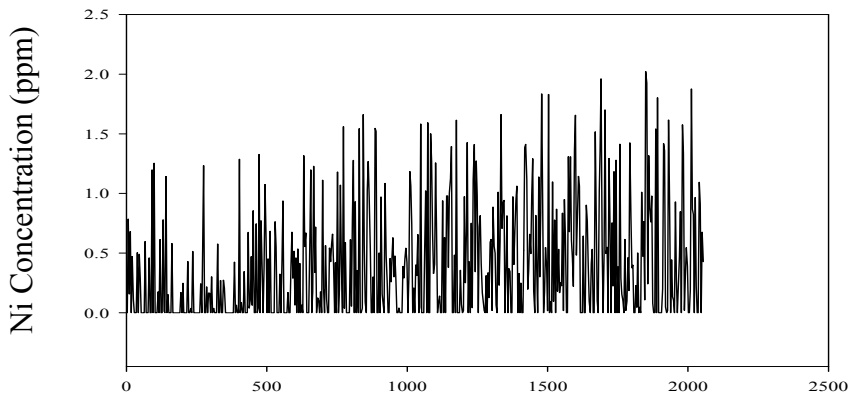
H02 H17

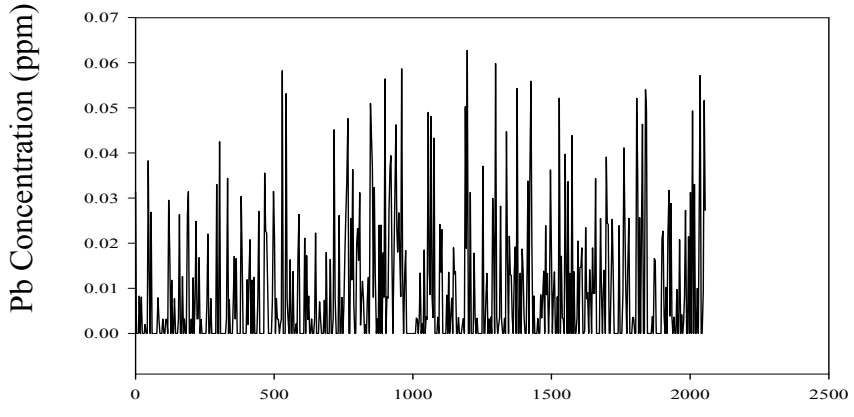
High dose

Crystalline

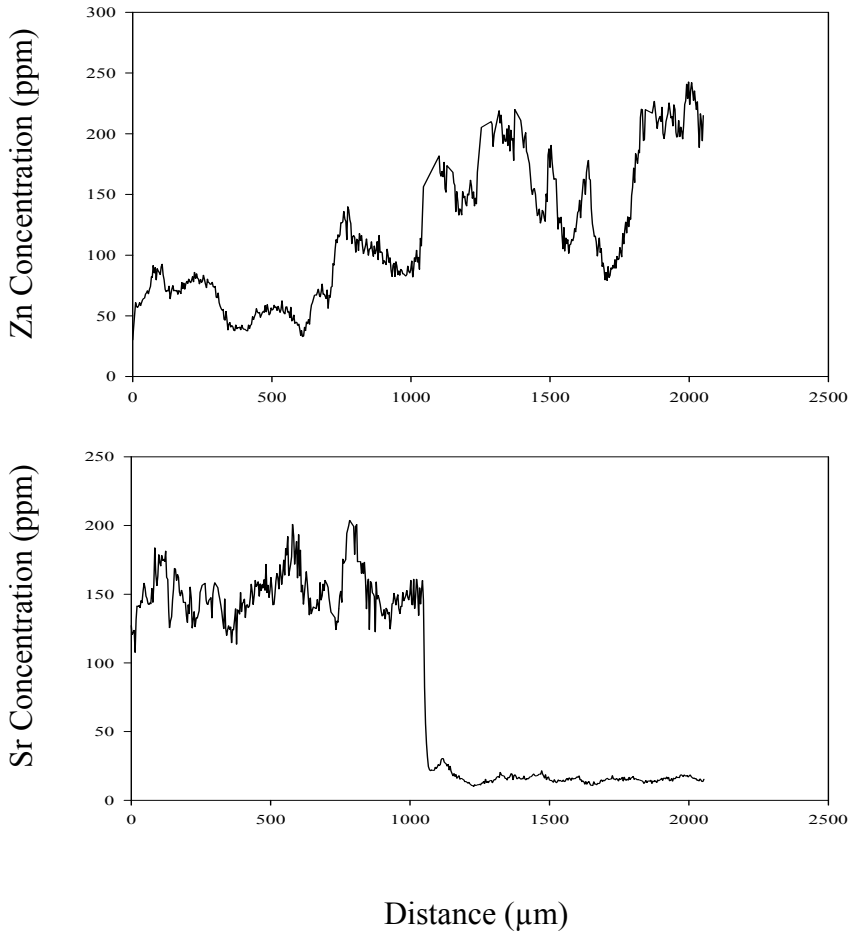








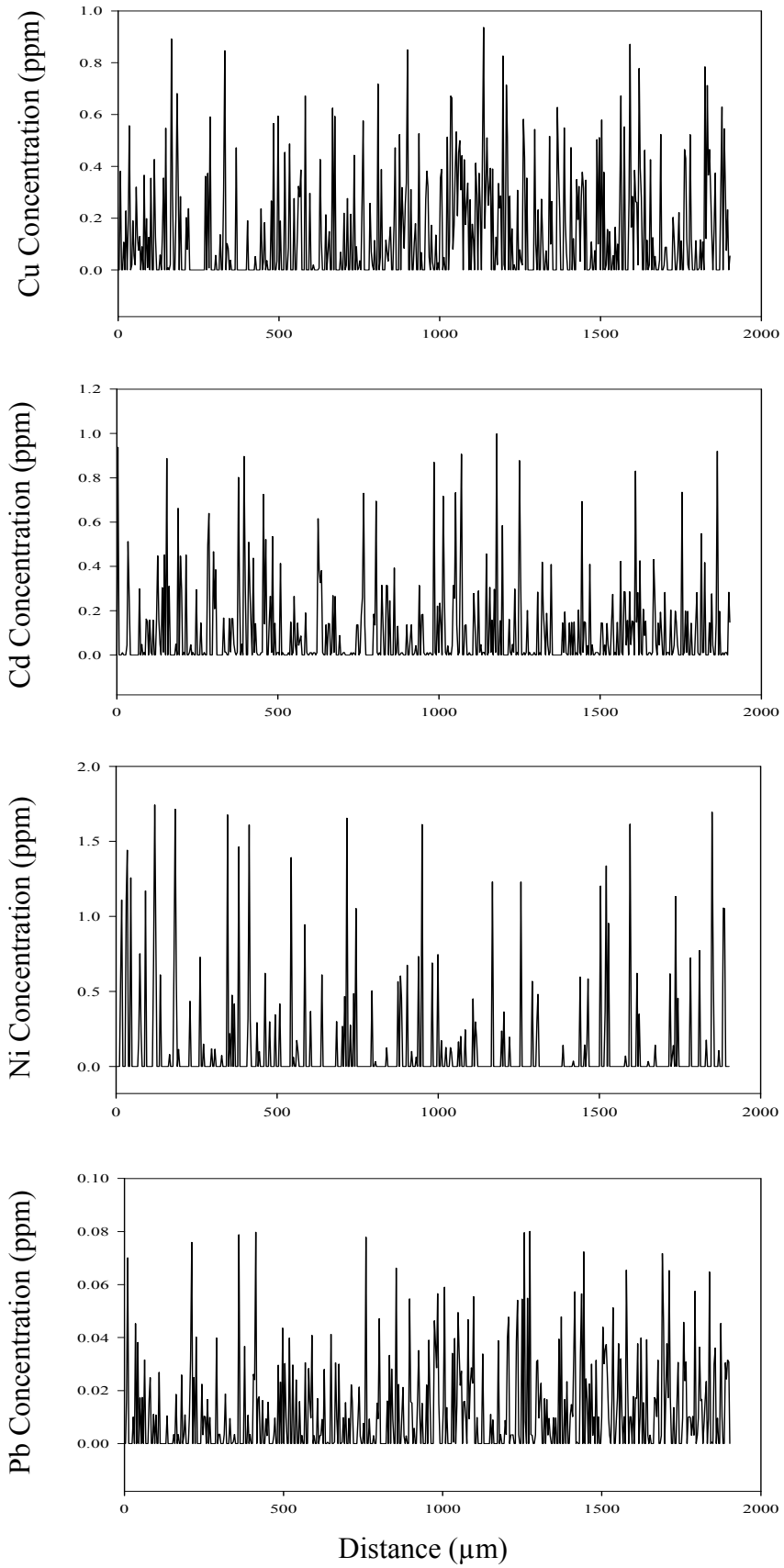
H02 H17


Zn Concentration (ppm)
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
2500
Distance (μm)

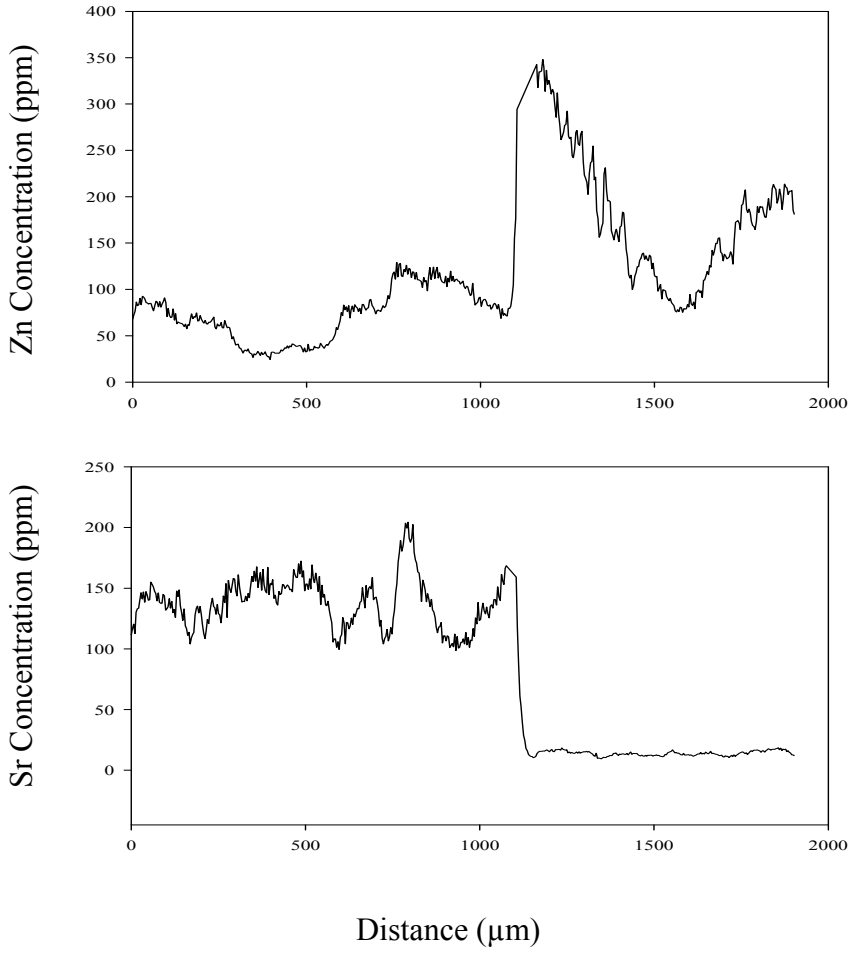
H02 H18

High dose

Crystalline



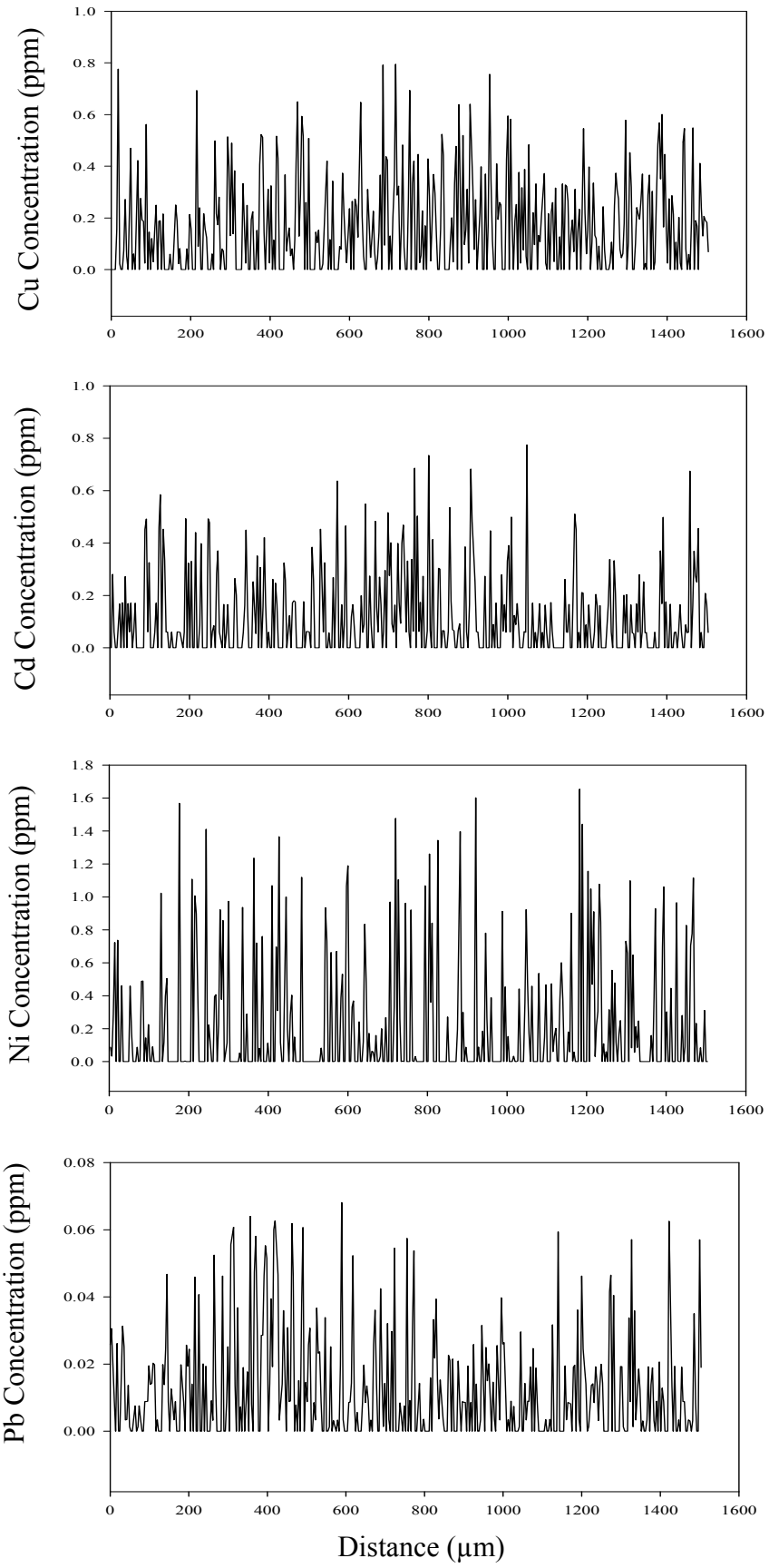
H02 H18


Zn Concentration (ppm)
400
350
300
250
200
150
100
50
0
0
500
1000
1500
2000
Sr Concentration (ppm)
250
200
150
100
50
0
0
500
1000
1500
2000
Distance (μm)

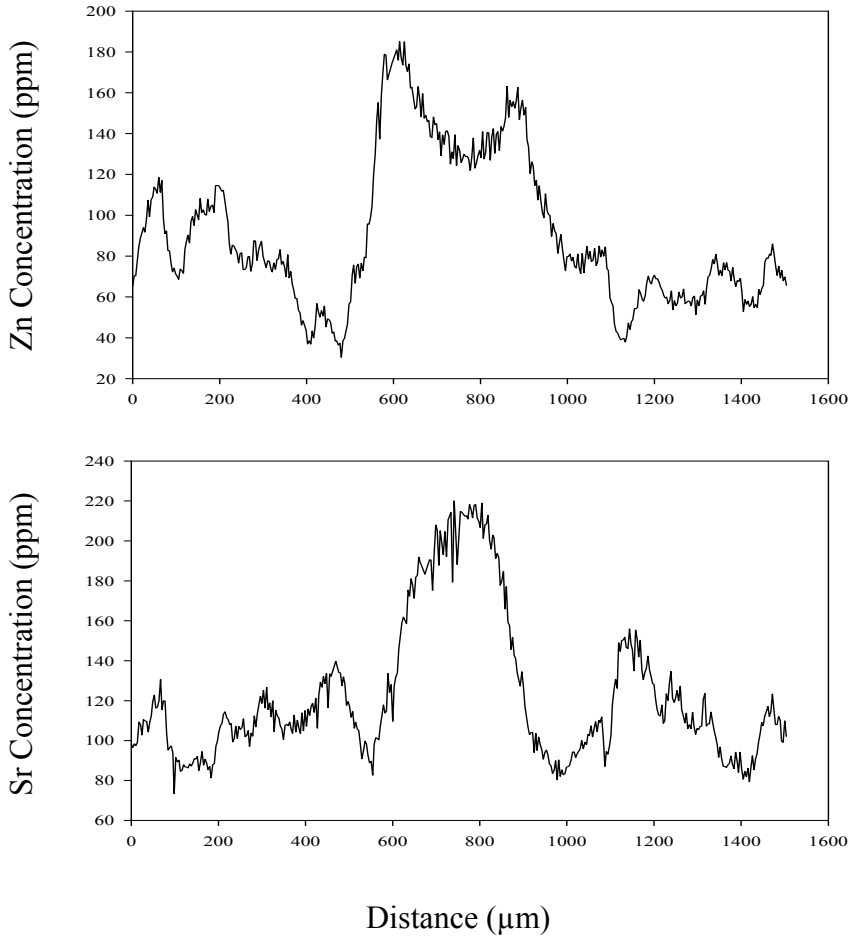
H02 H20

High dose

Crystalline



H02 H20


Zn Concentration (ppm)
200
180
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
1400
1600
Sr Concentration (ppm)
240
220
200
180
160
140
120
100
80
60
0
200
400
600
800
1000
1200
1400
1600
Distance (μm)